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# **ENDOCRINE ASPECTS OF DISEASE PROCESSES**

*Proceedings of the Conference held in honor of*

**HANS SELYE**

*Mont Tremblant, Quebec*

*Edited by*

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\* Not present at the meeting.

## Preface

THE Hans Selye Conference was held amidst the picturesque scenery of our Laurentian Mountains, near Montreal, Quebec. It was a great moment long anticipated by Selye's colleagues and former graduates from l'Institut de Médecine et de Chirurgie expérimentales de l'Université de Montréal, who wished to pay him a tribute on the occasion of his 60th birthday. Needless to say, a cordial atmosphere prevailed and all the graduates coming from different parts of the world were most eager to communicate their personal feelings and to review their scientific achievements at this very exceptional gathering.

As has been our custom over the years, both French and English were used at the reunion; but the proceedings, except for part of the voluntary discussion, have been edited entirely in English. They will provide the reader with a good insight into "the productive ideas" instilled by Selye into his trainees during the past 25 years. The presentation of the papers follows the same sequence as was observed during the Conference. Their number had to be restricted because of time limitations. But original contributions and pertinent illustrations were incorporated within the discussions, to give us an opportunity to hear from alumni and colleagues engaged in active research.

Great advances have been made during these past years in the concept of endocrine factors in disease processes and, in agreement with the participants, I can only say that this Conference illustrates the multiple pathways opened by Selye to his disciples as to all categories of workers in experimental medicine. In many of his writings, Professor Selye refers to the *triad* as the starting point of a series of discoveries on the non-specific reactions in biology. Similarly, the tripartite theory of disease production that he postulated proved to be highly productive in the design of experiments for the study and understanding of pathological processes. The essential components are: 1) the stimulus; 2) the sensitizer; and 3) the target tissue. Many of the experiments reported here were planned in accordance with this scheme.

My editorial task was facilitated by the full collaboration of all

the contributors and the invaluable assistance of Mrs. Bridget Sacra and Miss Francesca Pozzy in the preparation of the manuscripts. I also wish to express my thanks to Warren H. Green, Inc., who contributed by simplifying our editorial task. The Conference would not have been possible without the collaboration of the very efficient members of the Organizing Committee: Drs. Eugène Robillard, Vice-Dean, M. Cantin, P. Jean and G. Gabbiani from the Faculty of Medicine, Université de Montréal. As the president of this Committee I wish to express my gratitude to the Medical Research Council of Canada, le Conseil de la Recherche Médicale du Québec, and to all the pharmaceutical firms\* who, by their kind financial assistance, contributed to the success of this reunion.

Université de Montréal  
January 1968

GAËTAN JASMIN

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## Hypothalamic Hormones Regulating the Secretions of the Anterior Pituitary

ROGER GUILLEMIN

THE generation to which I belong was taught that the anterior pituitary was the "master" of all other peripheral endocrine glands with, admittedly, some debate upon its control of parathyroids and the pancreas; also that a subtle and exquisite reciprocal equilibrium between concentrations of circulating "peripheral" hormones and quantities of the corresponding pituitary hormones maintained endocrine homeostasis. This *feedback* or *push-pull theory*, which had been suspected as early as 1931 by Aron and his collaborators and which was later expanded by Selye, Hoskins, Evans, and Sayers, satisfactorily explained the experimental compensatory hypertrophy of peripheral endocrines following unilateral ablation, the development of goiter, the compensatory atrophy of the contralateral adrenal in the case of a unilateral adrenal tumor, the atrophy of the adrenal cortex following administration of crude extracts of the adrenals, etc. These were also the days when the first extensive purification of anterior pituitary hormones was reported from the laboratories of Collip and of Evans, from Kamm and Du Vigneaud's laboratory for the posterior pituitary hormones, reaching in 1952, the isolation, determination of the molecular structure and total synthesis of the two principles of the posterior lobe of the pituitary by Du Vigneaud. Then followed a twilight period in which numerous aspects of the elegant physiological construction of the previous fifteen years lost their appealing clarity and heuristic value, as it was realized that there were circumstances in which the simple pituitary-target-organ relationship was not exclusively operative. Thus, the simple feedback theory could not explain the original observation by J. S. L. Browne and Venning of the prolonged excretion in the urine of large quantities of corticoids during

prolonged stress or exercise or the hypertrophy of the adrenal cortex during chronic stress as reported by Selye as early as 1936; nor could it explain the extreme rapidity of the changes in ACTH secretion induced by stress, with no evidence of a preceding fall in plasma concentration of the peripheral corticoids that could have triggered the feedback system; finally came the demonstration by Sayers himself that increased secretion of ACTH upon exposure to stress could take place in adrenalectomized animals maintained on constant high levels of corticoids. Meanwhile, clinicians and experimenters, with Cushing, Roussy and Mos-singer, Ranson, Magoun, Markee, Benoit, and Harris, had been reporting more and more evidence that lesions in certain areas of the base of the brain (in the hypothalamus), which anatomically did not come in contact with the pituitary, could produce various syndromes of pituitary dysfunction: inhibition of the stress-induced release of ACTH, permanent diestrus, permanent estrus with an ovarian picture reminiscent of that of the Stein-Leventhal syndrome, testicular atrophy, obesity, etc. Today, we teach our students that for the most part the center of control of the adenohypophysial functions is to be found in the hypothalamus, that the feedback relationships between peripheral hormone levels and adenohypophysial secretions are mainly transhypothalamic, and that the hypothalamic control over the pituitary functions is exerted through the secretion of hypothalamic substances, the *hypothalamic hypophysiotropic hormones or releasing factors*, which reach the anterior pituitary through the hypothalamo-hypophysial portal system.

It is interesting that our information about the endocrinology of the hypothalamus was acquired through the simple intellectual and experimental processes that have led to all the basic knowledge in endocrinology: the deficiency syndrome produced by the classical surgical ablation of the suspected endocrine tissue was achieved here by localized destruction of various parts of the hypothalamus through stereotactically placed electro-coagulations or by subtracting the hypothalamic influence from the pituitary by isolating the gland in peripheral grafts or by *in vitro* explantation or incubation; replacement therapy was achieved by injecting hypothalamic extracts into the animals with hypothalamic lesions

and studying the function of the adenohypophysis or, more simply, by adding these extracts to the pituitary isolated *in vitro* and studying its release of hormones. A unique feature of this *neuroendocrinology* has been the possibility of electrically stimulating hypothalamic areas to specifically induce the release of the pituitary hormone as well as recording the electrical activity of hypothalamic nuclei as modified by the levels of circulating hormones and also of correlating this electrical activity with pituitary secretion. Precise localization of the origin of a given releasing factor cannot be ascertained in terms of specific hypothalamic nuclei as they have been described by the neuroanatomists. Rather, the neuroendocrine areas in the hypothalamus appear to be somewhat diffuse if we consider the overlapping of hypophysiotropic activities related to one area or another. This question of the exact hypothalamic origin of specific releasing factors requires further investigation. In its present and perhaps final state, the question is reminiscent of the conclusions reached by the neurophysiologists not many years ago regarding the absence of specific localization in the hypothalamus for sympathetic and parasympathetic integration, for instance.

With this important restriction in mind we can say, however, that stimulation of the posterior or ventral hypothalamus is followed by secretion of ACTH, whereas stimulation of a more anterior area will trigger secretion of TSH. Somewhere in between is an area that upon electric stimulation will produce ovulation in suitably prepared animals and, hence, may be considered to be related to secretion of gonadotropins (LH and FSH). It appears also that this mid-hypothalamus area is under the command of a somewhat more anterior (supra-chiasmatic) area, the interplay between the two regulating "basal" secretions of gonadotropins and "spurt" secretions as in the triggering of ovulation. Conversely, minute lesions as made by electro-coagulation of the same areas of the hypothalamus will reduce or inhibit secretions of ACTH, TSH, FSH and LH. After lesion of the ventral hypothalamus, particularly of the area known as median eminence, the acute release of ACTH that usually follows exposure to any stressful stimulus is completely inhibited. Lesions of the anterior hypothalamic region can prevent the acute secretion of TSH that

takes place when animals are exposed to a cold environment. Similarly, lesions in somewhat different areas of the hypothalamus will render female animals anovulatory in a syndrome of permanent diestrus or permanent estrus. Rather massive destruction of the ventral hypothalamus will also impair growth, possibly by reducing the secretion of growth hormone. Some of these lesions lead, on the other hand, to an increased and prolonged secretion of prolactin, with signs of mammary development, lactation, persistence of corpora lutea, as if the normal influence of the hypothalamus were a tonic *inhibition* of the secretion of prolactin—a conclusion also supported by the fact that the pituitary when separated from the hypothalamus as by peripheral grafting or *in vitro* transplantation will produce unusually large quantities of prolactin. Lesions of the ventral hypothalamus will lead to an increased secretion of the melanophoretic hormone (MSH) in amphibians and perhaps also in mammals as in a mechanism reminiscent of the hypothalamic inhibitory control over prolactin secretion.

Section of the pituitary stalk as an experimental means of severing hypothalamo-pituitary connections has only temporary effects, reestablishment of adenohypophysial functions being regularly correlated with regeneration of the cut hypothalamo-pituitary portal vessels. The explantation of pituitary fragments *in vitro* for short-term incubation or long-term tissue or organ cultures, the transplantation of the anterior lobe to some location remote from median eminence, as auto- or homo-transplants in hypophysectomized animals, will produce lasting and profound changes in the pituitary secretions: in brief, there is considerable reduction of the secretion of all pituitary hormones with the exception of prolactin, which in all these experimental situations appears to be released from an active inhibitory control that normally tends to restrain its secretion. Actually, peripheral pituitary transplants seem to secrete minute quantities of pituitary hormones (other than prolactin), but as in the case of the paralytic secretion of the adrenal medulla following splanchnicectomy, no increases or decreases in this minimal basal secretory activity of the transplant seem to be possible. It is as if the hypothalamic connections were necessary to assure some sort of an amplitude modulation of what may be considered a steady carrier secretory ability of the pituitary

tissue itself. Indeed, when such peripheral pituitary transplants are replaced in contact with the median eminence or when in the case of *in vitro* explants crude or purified extracts of hypothalamic tissues are added to the incubation system, the pituitary transplants rapidly resume normal secretory activity quantitatively and qualitatively. At the same time, reestablishment of the hypothalamic proximity or the injection of hypothalamic extracts in the case of the peripheral transplants will restore to normal the cytological appearance of the transplants, which while disconnected from the hypothalamus assume a classical dedifferentiated histology.

All these data from *in vivo* and *in vitro* experiments constitute the solid basis on which the concept of the hypothalamic control of the secretion of the anterior pituitary is now well established.

What is known about the *hypothalamic hypophysiotropic hormones* or *hypothalamic releasing factors*, the existence of which was postulated from some of the early observations summarized above? On the basis of results obtained with various bioassay techniques, there is now good evidence that extracts of hypothalamic tissues of several mammalian sources contain several substances that can specifically stimulate or inhibit the secretion of several pituitary hormones. Hypothalamic extracts contain a TSH-releasing factor (TRF), an LH-releasing factor (LRF), an FSH-releasing factor (FRF), a corticotropin-releasing factor (CRF), possibly a growth hormone (somatotropin)-releasing factor (SRF); also, it appears that the tonic inhibition of the secretion of prolactin exerted by the hypothalamus and which we mentioned above may well be due to a substance of hypothalamic origin, prolactin-inhibiting factor (PIF); there is also preliminary evidence for both an MSH-releasing factor (MRF) and MSH-inhibiting factor (MIF). Hypothalamic TRF, LRF and FRF have been obtained in high degree of purity. Purification of a hypothalamic SRF has also been reported, on the basis, however, of a somewhat questionable bioassay; this important conclusion will require confirmation. Hypothalamic CRF and PIF have been purified, but their isolation has not been approached. MRF and MIF as independent and/or novel entities are the subject of some controversy as this review is being written.

Several studies have recently led to claims of observations of

physiological or experimental variations in the hypothalamic content of releasing factors, CRF, LRF, FRF, TRF, SRF and PIF, in rapport with concomitant dynamic variations of the corresponding pituitary secretions. Even though the conclusions presented are quite attractive and well in keeping with theoretical expectations, I personally think that they will need confirmation with a methodology more secure than the one presently available and employed so far. The technical problems involved are considerable and revolve primarily, as I see them, about the specificity and even more so about the precision of the bioassays currently involved as the end-points to assess biological activities. Of considerable interest are recent reports from the laboratory of G. W. Harris, which claim the presence of LRF and TRF in blood coming from the primary plexus of the hypothalamo-hypophysial vessels—an observation reminiscent of the earlier conclusions by John Porter who was the first investigator to report, several years ago, the presence of CRF activity in portal blood. If we are eventually capable of measuring hypothalamic content and portal blood concentration of releasing factor, while at the same time measuring pituitary content and blood concentration of the corresponding hypophysial hormones, as well as the peripheral (target) secretions, we will then be in possession of all the elements necessary to understand the physiological relationships involved in the control and regulation of adenohypophysial secretions. The technical problems involved are still considerable but not insuperable.

In spite of the important progress that the information reviewed above unquestionably represents, we still have no definite knowledge of the chemical nature of the hypothalamic hormones as of the time of writing this manuscript. This is a rather humbling statement, after almost ten years of effort to isolate the hypothalamic hormones and establish their chemical nature and molecular structure. The first hypothalamic hormone purified to any degree several years ago was CRF. It was purified by simple techniques of paper chromatography and electrophoresis from acid extracts of dog, beef, pig, sheep hypothalamus and was shown to concentrate along with basic polypeptides but to be different from vasopressin; this was in keeping with the hypothesis proposing

that if substances of hypothalamic origin existed at all as hypophysiotropic hormones, they would most probably be of polypeptidic nature as were the other two products of neurosecretion, oxytocin and vasopressin. From crude extracts of the posterior pituitary, two substances were isolated ( $\alpha$ -CRF,  $\beta$ -CRF) that specifically stimulate release of ACTH. The polypeptidic structures of these two substances have been established. At this time it is not clear what relationship, if any, these two polypeptides bear to the nature of hypothalamic CRF, which has not been isolated as yet in homogeneous form. For the next ten years, the hypothesis of the polypeptidic nature of hypothalamic hormones was to prove fruitful. With the use of methods classically designed to purify polypeptides, the existence as chemical entities and the purification of LRF, TRF, FRF were reported. These three releasing factors have been now obtained in states of high purity and actively release pituitary hormones at millimicrogram doses when injected in a peripheral vein in several assay preparations. Recently, we have questioned the concept of a simple polypeptidic nature for the hypothalamic hormones, more particularly in the case of TRF. Approximately 2.0 mg of a preparation of TRF extracted and purified from more than half a million hypothalamic fragments of sheep brains have been obtained and are currently being used in an effort to establish the molecular structure of this hypothalamic hormone. It has been observed that the biological activity of the material is totally resistant to incubation with a series of proteolytic enzymes, and that following HCl hydrolysis, amino acids account for only a small fraction of the dry weight of aliquots of this preparation of highly purified material. Thus, the hypothalamic hormones may not be the simple polypeptides that they were considered to be originally. Recent studies of high resolution nuclear magnetic resonance spectra of TRF suggest that we are dealing with a highly saturated alicyclic or heterocyclic structure with peripheral CH<sub>3</sub> groups, without ruling out completely a polyamide structure. So far, our attempts to probe the structure of TRF by mass spectrography have met with disappointment, because the natural product is totally non-volatile as have been all the derivatives classically made under such circumstances. With the minute quantities of purified material

available and the considerable cost involved in procuring the fragments of brain that constitute the starting material, efforts are being made to devise a new micro or even submicro methodology where only millimicrogram or microgram quantities of material can be meaningfully used to obtain information about the elemental composition and chemical structure.

Recent observations may have considerable significance in regard to the mode of action of hypothalamic releasing factors in their mechanisms to stimulate secretion of pituitary hormones. The effects of TRF and LRF in releasing TSH and LH are not inhibited by pretreatment with the drugs puromycin, cycloheximide or actinomycin, under conditions in which these antibiotics are known to interfere with protein synthesis. Thyroxine inhibits at the pituitary level the action of TRF to stimulate release of TSH. TRF and LRF appear to require the presence of  $\text{Ca}^{++}$  to exert their activity. As this review is being written, a series of experiments still in progress have led us to wonder whether the releasing factor may not work by specifically altering the membrane permeability of specific pituitary cells, thus triggering the release of the pertinent pituitary hormones; the effects of the releasing factors appear to be duplicated *in vitro* for the release of ACTH, TSH, and LH by increasing the concentration of  $\text{K}^+$  in the medium of pituitary incubation experiments. Several new working hypotheses about the mechanisms of the hypothalamic control of adenohypophysial functions are suggested by these novel observations.

Thus, we can conclude that in spite of many points that are still obscure, controversial or difficult to approach, the concept of a neurohumoral control of hypothalamic origin for the secretions of the adenohypophysis is now well established and recognized. Hypothalamic hormones are presently being studied and discussed as were, several years ago, the hormones of the adenohypophysis and the neurohypophysis. We can reasonably envision that, in the not too distant future, their chemical structure will be finally established, their synthesis achieved; the hypothalamic hormones will then be available for clinical purposes, as there is considerable interest in their diagnostic and therapeutic significance. The efforts of many groups of investigators throughout the world will have led to this achievement in what will have been a gallant

competition. For those who were among the early protagonists on this vast scene, the rewards have already been realized in their always pleasant and so often humbling association with many enthusiastic collaborators and colleagues and in seeing old hypotheses take shape, become common knowledge, and engender new theories more subtle than ever in the facts and observations that they are seeking.

### Abstract

A brief review is presented of the development over the last twenty years regarding the concept of a hypothalamic neurohumoral control of the secretions of the anterior pituitary. The recent evidence for the existence of specific hypothalamic hypophysiotropic hormones or releasing factors is summarized. These are releasing factors for corticotropin (CRF), thyrotropin (TRF), luteinizing hormone (LRF), follicle stimulating hormone (FRF), with the possible existence of a somatotropin releasing factor (SRF) and a prolactin inhibiting factor (PIF). Hypothalamic TRF, LRF, and FRF have been obtained in highly purified form and are active in various *in vivo* or *in vitro* preparations at millimicrogram doses. Recent evidence questioning a simple polypeptidic nature for TRF is discussed, along with our current information on the nature of TRF. Regarding the mode of action of the hypothalamic hormones in their release of pituitary hormones, neither cycloheximide nor actinomycin D inhibits or prevents TSH-release due to TRF, in conditions under which the drugs are shown to inhibit incorporation of H<sub>3</sub>-leucine or H<sub>3</sub>-uridine in pituitary proteins and RNA, respectively. Small doses of cycloheximide or actinomycin D can prevent the thyroxine-induced inhibition of TSH-release due to TRF. Increasing K<sup>+</sup> content of the medium in which pituitary tissues are incubated releases TSH, ACTH, and LH. K<sup>+</sup>-induced release of TSH is prevented by pretreatment with thyroxine or preincubation in Ca<sup>++</sup>-free medium. Similarly, TRF requires the presence of Ca<sup>++</sup> to stimulate release of TSH. These results suggest several hypotheses according to which the hypothalamic hormones may affect the membrane potential of the pertinent adenohypophyseal cells in their stimulating release of pituitary hormones.

The concept that some years ago proposed the existence of specific hypothalamic hypophysiotropic factors for the control of the secretion of anterior pituitary hormones now seems solidly established.

### Abbrégé

Une revue rapide est présentée du développement pendant les vingt années passées, de l'hypothèse d'un contrôle neurohumoral hypothalamique sur les sécrétions de l'hypophyse antérieure.

Les résultats récents confirmant l'existence d'hormones hypothalamiques

spécifiques sont résumés. Il existe, en effet, un facteur hypothalamique de décharge de l'hormone adrénocorticotrope (CRF), de l'hormone thyréotrope (TRF), de l'hormone de lutéinisation (LRF), de l'hormone folliculo-stimulante (FRF); probablement, il existe aussi un facteur hypothalamique stimulant la sécrétion de l'hormone de croissance (SRF) et un facteur inhibant la sécrétion de prolactine (PIF). Les hormones hypothalamiques TRF, LRF, FRF ont été dernièrement préparées à un degré de purification considérable et sont actives *in vivo* ou *in vitro* à des doses de l'ordre du millimicrogramme. Nous discutons la possibilité que l'hormone hypothalamique TRF ne soit pas un polypeptide simple comme on l'avait considéré pendant longtemps; nous rapportons aussi nos observations récentes sur la structure moléculaire de TRF.

En ce qui concerne le mode d'action des hormones hypothalamiques, il a été établi que ni cycloheximide ni actinomycine ne modifient la stimulation de la sécrétion de TSH due à TRF, dans des conditions où l'on a démontré que ces drogues inhibent l'incorporation d'un acide aminé marqué ( $H_3$ -leucine) et  $H_3$ -uridine respectivement dans les protéines et les ARN hypophysaires. Par contre de faibles doses des mêmes drogues peuvent empêcher l'inhibition par la thyroxine de la sécrétion de TSH produite normalement par TRF. De plus, l'augmentation du contenu en  $K^+$  du milieu dans lequel sont incubés des fragments hypophysaires stimule la sécrétion de TSH, ACTH, LH. La sécrétion de TSH stimulée *in vitro* par  $K^+$  est inhibée par élévation de la concentration du milieu en thyroxine ou par incubation en absence de l'ion  $Ca^{++}$ . Il en est de même de la sécrétion de TSH stimulée par TRF. Ces résultats suggèrent plusieurs hypothèses dans lesquelles une dépolarisation de la membrane cellulaire des cellules hypophysaires serait impliquée pour expliquer l'action des hormones hypothalamiques.

Le concept qui proposait, il y a plusieurs années, l'existence d'hormones hypothalamiques hypophysiotropes contrôlant la sécrétion des hormones antéhypophysaires semble donc bien établi.

### Acknowledgements

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Over the years, I have been privileged to work in the laboratory on the physiology and chemistry of the hypothalamic hormones with many students, collaborators and associates. The names of most of them appear in the list of references quoted at the end of this short review.

### References

The titles below refer to a few recent reviews in which the interested reader could find keys to the literature on the hypothalamic control of the anterior pitui-

tary. In keeping with the spirit of this Conference, these titles will also include references of a few publications from this laboratory, which will show how our own contributions to this field evolved over the years.

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### **Contributions from our Laboratory**

These are listed according to the year in which they were published.

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**J. C. Beck:** Roger Guillemin has already reported on the evidence obtained by various workers including McCann and Schally and their associates supporting the concept of a Somatotropin-Releasing Factor (SRF). Our own laboratory's interest in the factors regulating the bio-synthesis, storage and release of growth hormone (GH) led us to join hands with Roger Guillemin's group and I should like to summarize our findings, since I believe they clearly dictate the development of more refined methods for study of these problems. We will report our experience in the bio-assay for SRF, both in the side fractions derived from the Sephadex-G25 filtration of ovine hypothalamus as performed in Dr. Guillemin's main program of purification of the thyrotropin releasing factor, and in the complete effluent of similar

gel filtrations devoted entirely to the search for growth-hormone-releasing activity.

Fractions dissolved in saline were injected into the external jugular vein of ether-anaesthetized male or female rats weighing 150 to 200 g. In other experiments, we have slowly infused the carotid artery under pentobarbital anaesthesia. Fifteen to thirty minutes later the rats were decapitated and the anterior pituitary glands from the two to six rats in the various treatment groups were extracted in saline to the concentration required for injection in the tibia test. These extracts and plasma from each treatment group were frozen for later assay. In the tibia test for growth hormone, extracts of control pituitary from rats injected with saline were assayed at two dilutions. Pituitary extracts from rats injected with hypothalamic fractions were assayed at a dose equivalent by weight to the higher dose of the control, or bracketed between the two doses of the control pituitary extract. Treatment groups in a tibia test comprised four to eight female rats hypophysectomized at 28 days of age. In the following week, injections of L-thyroxine, 0.5  $\mu\text{g}/\text{day}$  were begun. After four daily injections of pituitary extracts, the proximal tibial epiphyseal cartilage width was measured as described by Greenspan. A bovine preparation of growth hormone (NIH-GH-B7) has been used as growth hormone standard. The significance of differences between responses in each assay was determined using either Duncan's multiple range test, or a three- or four-point bioassay analysis. The validity of the growth hormone assay has been tested by repeated assays of anterior pituitary extracts from rats decapitated following ether anaesthesia with the appropriate bovine growth standards in the multiple four-point bioassay system. These assays have had a composite lambda of 0.142. The potency ratios had mean 95% confidence limits that were equivalent to 25 to 41  $\mu\text{g}$  of standard per mg of wet adenohypophysis. I wish to emphasize that, in all assays, we employed statistical analyses whose criteria of significance are similar to this.

Figure 1 shows the responses obtained when eleven aliquots from the effluent of a gel filtration of 10,000 fragments of sheep hypothalamus were injected in a single experiment. The narrow black line referring to the ordinate on the left shows the optical density in the Goa reaction. The shaded area refers to the right hand ordinate and depicts the range of tibia cartilage width where no difference from the control response exists by the multiple range test. In order to infer a depleting effect, a decrease from the response of the control

## BIOASSAY OF EFFLUENT FROM SINGLE CHROMATOGRAM

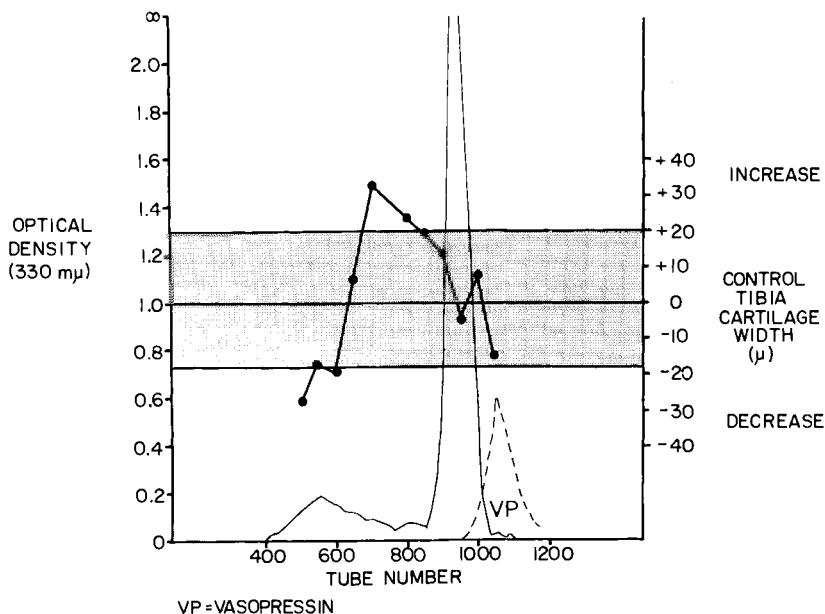


FIGURE 1

pituitaries of over 18 micra is necessary. This is seen in responses to 150 to 200  $\mu$ g fractions from the first eluted peak. These are equivalent to depletions of 45 to 60% of control potency. The increase of potency prior to the second peak possible corresponds to the activity recently described in ovine hypothalamus by Krulich and his associates, which inhibits the release *in vitro* of pituitary substances active in the tibia test. Although evidence of a SRF has been associated with fractions from the first eluted peak in four of the seven experiments manifesting reduction of potency, one such fraction subjected to repeated bioassay at doses of 50 and 100  $\mu$ g per rat has shown notable variability of response. On some occasions, a depleting effect was demonstrated, where, with reference to the responses of the control extract, decreases of 24 and 30 micra occurred. On other occasions, the same preparation induced an increase of potency at 100  $\mu$ g but was inactive at 50  $\mu$ g. In this latter experiment, the assay system was capable of detecting a depletion of 47%, exactly comparable to the one previously described. Repeated bioassays of

the retarded fraction, which in Figure 1 showed that it was capable of increasing the potency of the pituitary extracts, has shown equally variable effects. No consistency of response has been achieved with the additions of albumin to the hypothalamic material, the administration of glucose to rats prior to intracarotid injection or with the intracarotid infusion of hypothalamic extracts. In some studies, the response to an alkaline extract of porcine hypothalamus infused for 20 minutes into the carotid artery of rats under pentobarbital anaesthesia suggested a depletion whose 95% confidence limits were from 20 to 100%. Extracts of ovine hypothalamus and cortex were inactive.

Table I summarizes 30 assays. In 15 of these, considered valid in that we were at least 95% certain of difference between the two dilutions of controlled pituitary extracts, the medium detectable reduction of tibia cartilage width was 22 micra, constituting a median depletion of 62% of control potency. In six, depletion consistent with the effects of a SRF was produced. Augmentation occurred in three, and no effects were observed in six experiments. In the remaining fifteen experiments, where sensitivity was so low as to detect not less than a 90% reduction of control potency, two instances of depletion have been observed. In four experiments, ovine cerebral cortex has been inactive. If the infrequent positive responses are interpreted as random events, we have not demonstrated any modification of growth hormone secretion. However, if differences from control tibia cartilage width were due to more than chance, their magnitude was frequently compatible with the effects of variable quantities of, not only somatotropin, but other hormones in these crude pituitary extracts.

To permit more specific interpretation of these responses, radioimmunoassay of growth hormone in plasma and pituitary extracts

TABLE I  
INCIDENCE OF POSITIVE RESPONSES

Preparation	"Valid" Experiment*			Invalid Experiment*		
	$P < 0.05$			$P > 0.05$		
	Decrease $P < 0.05$	Increase $P < 0.05$	No Effect	Decrease $P < 0.05$	Increase $P < 0.05$	No Effect
Ovine Hypothalamus .....	5	3	5	2	1	9
Porcine Hypothalamus .....	1	—	1	—	—	3
Ovine Cortex .....	—	—	2	—	—	2

\* Based on 2 doses of control pituitary extracts.

## RADIOIMMUNOASSAY OF EFFLUENT FROM SINGLE CHROMATOGRAM

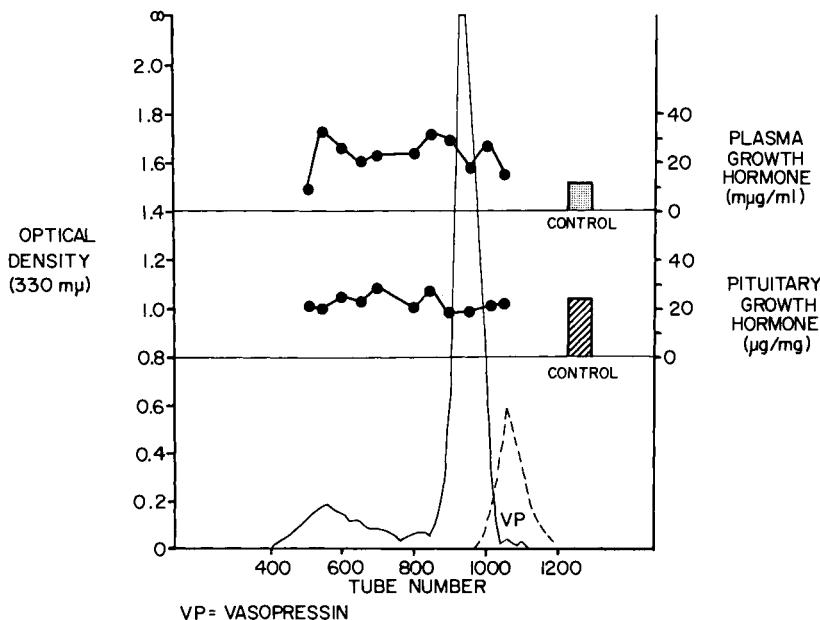


FIGURE 2

from rats that have received ovine and porcine hypothalamus in these bioassays have been performed by Dr. Donald Schalch and Dr. Seymour Reichlin, using methods previously described. Figure 2 shows their results in the radioimmunoassay of the previously shown chromatogram, wherein fractions from the non-retarded peak elicited in the bioassay depletions of potency of 45 to 60% that were consistent with the effects of a SRF. The top line referring to the ordinate on the right shows the absence of the increments of plasma growth hormone that one would expect following the release of such large amounts of pituitary growth hormone. Below, the radioimmunoassay of the pituitary extracts from the same rats shows no indication of the decreases or the increases of potency compared to control that were suggested by responses in the tibia test. Correlations with eleven of our bioassays including eight showing statistically valid positive effects provide no convincing evidence of growth hormone release. Whatever the interpretation of these results, positive effects in this relatively non-specific, two-step bioassay have been presented in a

minority of experiments. A dose response relation was absent. Extreme variability of response has been observed upon repeated testing of the same preparation, when strict statistical criteria were applied. Plasma and pituitary extracts from these experiments did not show radioimmunological changes, which should have been easily detectable had the bioassay responses corresponded to the release of growth hormone. While we firmly believe in the existence of factors facilitating the release of growth hormone, our experience with this two-step bioassay would suggest it to be so insensitive as to preclude its use in a study of the factors controlling the growth hormone content of the pituitary. Other possible explanations that deserve consideration are: 1) Ineptitude of our laboratories—I believe this to be unlikely. 2) Species specificity of the releasing factor comparable to that which exists for the growth hormones. 3) Radioimmunoassay of rat growth hormone may fail to measure biologically active growth hormone. Schalch, Reichlin and their associates feel they have verified the validity of the immunoassay. 4) Presence of factors in the hypothalamic extracts that either inhibit, release, or facilitate synthesis, as well as those stimulating release "cancelling themselves out" and thus giving variability in the bioassay. These data have been presented to point out some limitations of this bioassay for hypothalamic substances affecting growth hormone secretion.

I wish to acknowledge my collaborators in the studies that have been reported here. These are Doctors R. Burgos and Roger Guillemin of Baylor University, and Dr. Wilson Rodger, recently of my own Department but now a new Faculty member in the Department of Medicine at the University of Western Ontario, London, Ontario. Dr. Rodger carried out this work while a Research Trainee in my Department.

## **Corticosterone-binding by Transcortin and Pituitary-Thyroid-adrenocortical Interactions in the Rat**

F. LABRIE, J. P. RAYNAUD, G. PELLETIER,  
P. DUCOMMUN and C. FORTIER

THE simultaneous occurrence of high total plasma corticosteroid levels and increased adrenal cortical activity in rats treated with high doses of thyroxine, and the opposite phenomena observed as a result of thyroidectomy, were difficult to reconcile with the concept of a negative feedback mechanism, whereby pituitary adrenocorticotrophic activity adjusts to changing levels of adrenal cortical hormones until secretory equilibrium is restored.

Assuming feedback involvement, the following two possibilities could account for the observed discrepancy:

1) Reset of the closed-loop controller by thyroid hormone. By a modification of the set-point or reference value, the controlled variable, in terms of total plasma corticosterone concentration, would be maintained at higher levels in hyperthyroidism and at lower levels in hypothyroidism.

2) Enhancing effect of thyroid hormone on the binding affinity of transcortin for corticosterone. It should be recalled, in this connection, that corticosterone is bound by at least two plasma proteins, i.e., transcortin, a specific corticosteroid-binding globulin, and an albumin of lesser binding affinity, and that it exists, therefore, in equilibrium distribution among three forms in plasma: the native or unbound steroid and two steroid-protein associations. Assuming that unbound, as opposed to total, corticosterone concentration is the controlled variable, an enhancing effect of thyroxine on the binding capacity of transcortin could conceivably account for a relative hypocorticoidism, notwithstanding the high total corticosterone concentration induced by thyroxine, and for the opposite phenomenon in hypothyroidism.

Studies aimed at testing the latter hypothesis yielded the following conclusions:

- 1) Thyroid hormone, administered to the intact rat, markedly enhances corticosterone-binding by transcortin, through an increase in the number of transcortin-binding sites.
- 2) Alteration of the number of transcortin-binding sites observed as a result of thyroxine administration represents the algebraic summation of two opposite effects: enhancement by thyroxine and depression by corticosterone, with predominance of the stimulating effect of thyroxine.
- 3) Chronic administration of graded doses of thyroxine results in step-wise increases of total plasma corticosterone concentration, without significant alteration of the absolute level of the unbound fraction. This strongly suggests that the unbound, as opposed to the total, corticosterone concentration is the variable under feedback control and that the binding capacity of transcortin is responsible, in the rat, for the adjustment of the secretion rate of corticosterone to changes in thyroid activity. The increase in the number of transcortin-binding sites resulting from thyroxine administration would tend to decrease free corticosterone, the constancy of which could only be insured by a feedback-mediated increase in pituitary-adrenocortical activity resulting, in view of the unaltered metabolic clearance rate of corticosterone, in a proportional elevation of the total plasma concentration of this steroid. A new dynamic equilibrium determined by the increase in the number of transcortin-binding sites, and characterized by a virtual depression of the free corticosterone level compensated by an accelerated rate of corticosterone secretion and a resulting increase in the total plasma concentration of corticosterone, thus provides a satisfactory explanation for the simultaneous occurrence of high total plasma corticosteroid levels and increased adrenal cortical activity in the hyperthyroid rat, and for the opposite changes observed as a result of thyroidectomy.
- 4) From a comparative assessment of the effects of adenohypophyseal, adrenal cortical, and gonadal hormones, it is inferred, furthermore, that thyroxine alone has a direct enhancing effect on the binding capacity of transcortin and that the stimulating

effect of estrogens and progesterone is exerted through the pituitary-thyroid axis.

### **Abstract**

The chronic administration of graded doses of thyroxine to intact rats resulted in progressive increases of adrenal weight, evidencing a corresponding enhancement of ACTH secretion. Graded doses of thyroxine concurrently resulted in step-wise increases of total plasma corticosterone concentration, without significant alterations in the absolute level of the unbound fraction. This strongly suggests that the unbound, as opposed to the total, corticosterone concentration is the variable under feedback control and that the binding capacity of transcortin is responsible, in the rat, for the adjustment of the secretion rate of corticosterone to changes in thyroid activity. From a comparative assessment of the effects of adenohypophysial, adrenal cortical, and gonadal hormones, it is inferred, furthermore, that thyroxine alone has a direct enhancing effect on the binding capacity of transcortin and that the stimulating effect of estrogen and progesterone is exerted through the pituitary-thyroid axis.

### **Abbrégé**

L'administration de thyroxine entraîne, chez l'animal intact, une hyperactivité cortico-surrénaliennes accompagnée d'une augmentation de la capacité de liaison de la transcortine pour la corticostérone, alors que des effets inverses sont observés après thyroïdectomie. L'administration de doses croissantes de thyroxine cause une élévation progressive de la corticostérone plasmatique totale, sans modification décelable de la concentration absolue de la fraction libre du stéroïde. Il en découle que la fraction libre de la corticostérone, par opposition à la concentration totale du stéroïde, est la variable sous contrôle hypothalamique et que la capacité de liaison de la transcortine est responsable, chez le rat, de l'ajustement du taux de sécrétion de la corticostérone à l'activité thyroïdiennes. De l'évaluation des effets des hormones adénohypophysaires, cortico-surrénaliennes et sexuelles, il ressort, en outre, que la thyroxine seule a un effet stimulateur direct sur la capacité de liaison de la transcortine et que l'effet stimulateur de l'estradiol et de la progestérone est médié par l'axe hypophyso-thyroïdien.

### **Acknowledgements**

The studies summarized in this paper were supported by grants from the Medical Research Council of Canada (MT-1205) and the U.S. Air Force Office of Scientific Research (AF-AFOSR-511-64, 511-65 and 511-67). The findings were the subject of several reports (1, 3-8) and a Ph.D. thesis (2).

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**P. Ducommun:** Comme introduction à la discussion de la très brillante conférence du Dr. Fortier, j'ai choisi de vous exposer quelques expériences que nous avons faites tant à l'Université Laval à Québec qu'à Baylor University à Houston. Le Dr. Selye nous a appris, il y a quelques années, que le stress augmentait la sécrétion d'ACTH et, dans le textbook qu'il a écrit en 1949, il a montré que l'activité de la thyroïde diminuait durant le stress aigu. C'était, je pense, la première base des expériences qui nous a conduit de Québec à Houston à étudier les interrelations existant entre l'ACTH et la TSH.

Nous avons étudié deux situations différentes: Les interrelations entre l'ACTH et la TSH en cas de *stress aigu* d'une part, et les interrelations de ces mêmes hormones au cours d'un *stress aigu*, mais chez des animaux qui auraient été durant plusieurs jours ou plusieurs mois soumis à des petits stress répétés tels que manipulations quotidiennes

des rats, injections sous-cutanées de solution saline et anesthésies à l'éther.

La première chose que nous observons, c'est qu'un stress aigu augmente brusquement l'ACTH dans le sang, alors que la TSH disparaît du plasma. Cette notion avait conduit à l'idée qu'il y avait une relation réciproque entre ces deux hormones, c'est-à-dire que toutes les fois que l'ACTH montait, obligatoirement la TSH descendait. En reprenant cette étude, chez des animaux bloqués par l'association déexaméthazone-nembutal, il a été possible de dissocier cette relation inverse, c'est-à-dire que le stress aigu ne modifiait plus le taux d'ACTH (étant donné le blocage par la déexaméthazone-nembutal), mais diminuait néanmoins le taux de la TSH plasmatique.

Le deuxième point était de savoir si nous pouvions avoir une augmentation de l'ACTH et une augmentation de la TSH simultanément. En reprenant ces expériences avec le froid, il a été possible de montrer que l'ACTH augmentait sous l'influence du froid, de même qu'augmentait la TSH plasmatique. De plus, si nous soumettions ces animaux au froid dans les mêmes conditions que précédemment, c'est-à-dire que nous les bloquions avec la déexaméthazone-nembutal, nous pouvions supprimer l'augmentation d'ACTH plasmatique et de plus augmenter significativement le taux de la TSH plasmatique. Une autre expérience nous a permis de voir que ce mécanisme d'action se passait probablement au niveau hypophysaire, puisque nous avions les mêmes résultats si, au lieu de les soumettre au froid, nous injections le TRF: les rats bloqués par la déexaméthazone-nembutal étaient capables de sécréter beaucoup plus de TSH que les animaux non bloqués. Ces résultats démontrent que le stress aigu et unique détermine une chute de la TSH plasmatique indépendante des variations de l'ACTH sanguine.

Si les rats sont manipulés ou injectés chaque jour, que va-t-il se passer lorsqu'ils seront soumis à un stress aigu? Nous avons pu voir que dans les 10 ou 15 premiers jours de stress répétés, la réponse à un stress aigu reste la même, c'est-à-dire que la TSH plasmatique chutera à des valeurs qui ne sont plus mesurables avec la méthode de McKenzie. A partir du 20e jour, il n'y aura plus de chute de la TSH plasmatique; par la suite et jusqu'à 5 mois de stress quotidiens, le taux de base de la TSH plasmatique va s'élever progressivement. Après 5 mois de manipulations quotidiennes, nous observons une réponse différente au stress aigu: les animaux de contrôle chutent leur TSH plasmatique, alors que les animaux stressés chroniquement ne la modifient pas significativement. De plus, dans ce dernier groupe, le

*taux de base de la TSH est de l'ordre de 50 mU/100 ml, donc nettement plus élevé que les taux normaux (env. 25 mU/100 ml). Ainsi nous voyons que la variation de la TSH secondaire au stress aigu est influencée par des stress chroniques. Au contraire, la sécrétion d'ACTH n'est pas modifiée dans les deux groupes expérimentaux: en effet, il y a parallélisme entre la courbe de la corticostérone plasmatique des animaux de contrôle non stressés pendant 5 mois et la courbe de la corticostérone plasmatique des animaux ayant eu 5 mois de stress chroniques.*

Vous voyez, Dr. Selye, vous avez influencé notre travail pendant de nombreuses années. J'aimerais vous dire combien je suis heureux de participer à cette réunion d'anniversaire et, au nom de mes camarades suisses, je vous souhaite de pouvoir continuer vos travaux de recherche.

**H. Selye:** First I wish to congratulate Dr. Fortier on this remarkable piece of work. We have been interested in pituitary-adrenal interrelations for many years and some of you will remember the animated controversy we had with George Sayers about the importance of the hormonal feedback mechanism during stress. There was never any doubt about the existence of this mechanism, since partial adrenalectomy results in compensatory hypertrophy of the residue, while excess corticoid administration causes compensatory atrophy of the adrenal cortex. However, what struck us most was that it was precisely during periods of stress that this mechanism did not seem to work well. Even in rats whose adrenals became atrophic as a consequence of prolonged treatment with cortisone, exposure to stress caused adrenal enlargement and hypersecretion; the same was true even in animals acutely overdosed with the highest tolerable amounts of cortisone. It is on the basis of these observations that we expressed some doubts about the importance of the feedback mechanism during the alarm reaction and postulated that, in acute stress, a great excess of circulating glucocorticoids is compatible with continued hypersecretion of ACTH. Indeed, if the feedback mechanism were perfect, it would be impossible to obtain such an intense cortical hyperactivity as is observed during a severe alarm reaction. I wonder whether a change in transcartin formation could explain the occurrence of intense and often long-maintained hypercorticoidism during stress.

My second remark is directed to Dr. Ducommun. If I understood him right, he has shown that the "anterior pituitary shift mechanism," as we called it, does not actually exist. I think he did it very elegantly, because somehow he managed to prove that we were wrong, and yet put it in the form of a compliment. Certainly an unsurpassed feat!

However, perhaps we were not totally wrong and I would like to know what he thinks of the shift concept as a whole. You see, it is not only the interrelationship between ACTH and TSH that must be considered, because the indirect evidence that served as a basis for our hypothesis suggested that ACTH-secretion is greatly augmented during stress at the cost of almost every other pituitary hormone. Animals under stress have large adrenals, owing to increased ACTH-secretion; but they do not grow well, perhaps as a consequence of diminished STH-secretion. The gonads undergo atrophy both in males and in females during stress, suggesting diminished LSH- and FSH-secretion. Milk production is greatly diminished when lactating animals are exposed to stress, which was ascribed to a decreased prolactin secretion. Of course, in all these cases, decreased end-organ sensitivity to the trophic hormones may also be at work. Yet in some cases, this possibility could be excluded; for example, the atrophic gonads of stressed female rats continue to respond well to exogenous gonadotrophic hormone.

I wonder whether Dr. Ducommun could summarize the present status of our knowledge concerning the "pituitary shift" during stress for those of us who have not followed developments in this field lately. Apparently, there is no necessary antagonistic interrelationship between ACTH- and TSH-secretion; but it seems difficult for me to accept that the anterior lobe can simultaneously secrete all of its hormones in excessive amounts, maximal production of one principle having no influence on the concurrent elaboration of others. Still, it must be admitted that the problem can be solved only by direct hormone-determination techniques as used by the speaker.

## **Calcitonin—Hormone of the Ultimobranchials**

D. HAROLD COPP

### **Introduction**

CALCIUM is one of the most precisely controlled constituents of body fluids, which is not surprising, in view of the important role that ionic calcium plays in many vital processes. These include its effects on muscle contraction, membrane permeability and neuromuscular excitability. Carruthers *et al.* (10) observed that the diurnal fluctuation in plasma calcium in normal human subjects was less than  $\pm 3\%$ . Sanderson *et al.* (43) found that in healthy young dogs, the initial calcium levels were restored within 2-4 hours after the plasma calcium level had been elevated by iv infusion of calcium gluconate (15 mg Ca/Kg) or lowered by infusion of the calcium complexing agent EDTA (ethylene-diaminetetraacetate—50 mg/Kg). However, they found that this efficient homeostatic control was absent in thyroparathyroidectomized dogs.

Until recently, it was thought that this precise regulation depended on the function of the parathyroid glands, which secreted a hormone (parathormone) that mobilized calcium from the vast reservoir present in the skeleton. McLean (35) proposed a sensitive negative feedback, whereby a fall in plasma calcium stimulated output of parathyroid hormone, and increased calcium mobilization; control of hypercalcemia was thought to be due to suppression of parathormone production.

However, it is now apparent that such passive control of hypercalcemia cannot explain the precise control that exists. Because of the prolonged effect of parathormone on bone, Rasmussen (42) pointed out that such a simple feedback system would tend to oscillate. It also does not account for the impaired control of hypercalcemia observed by Sanderson *et al.* (43) in thyroparathyroidectomized dogs. We first obtained evidence for a more active

control of hypercalcemia in experiments carried out in 1958 and reported some years later (13). Parathyroid extract (1 unit/Kg/hr) was infused iv for 8 hours into anesthetized dogs. Plasma calcium rose from 10 to 11 mg % and remained at that level for several hours after the infusion was stopped. However, if the thyroid and parathyroid glands were removed at the end of the infusion, the plasma calcium promptly rose to 13-14 mg %, suggesting release from an active hypercalcemic control.

The significance of these observations did not become apparent until 3 years later, when we were carrying out experiments to test the McLean feedback hypothesis directly. Copp and Davidson (16) developed a technique for perfusing the isolated thyroid-parathyroid gland complex in the dog, using blood in which the blood calcium had been elevated or lowered 20% by addition of suitable amounts of calcium salts or EDTA. With this preparation, it was clearly demonstrated that output of parathormone was controlled directly by the level of calcium in the perfusing blood, and did not require mediation by the nervous system or other endocrines. We were surprised by the rapid fall in systemic plasma calcium that occurred when the glands were perfused with high calcium blood, and decided to determine whether this could be explained by the McLean hypothesis (i.e., that control of hypercalcemia depends on suppression of parathormone release). In the experiment illustrated in Figure 1, we perfused the glands for successive 2-hour periods with alternatively high- and low-calcium blood. At the end of the second EDTA infusion, we removed the thyroid and parathyroids, and anticipated the fall in systemic plasma calcium predicted by the McLean hypothesis. Instead, the plasma calcium continued to rise for the next 10 hours. It was evident that the rapid fall observed with high calcium perfusion of the glands was not due to suppressed parathyroid function, but must have been due to a humoral factor released from the glands. We found that there was indeed a hypocalcemic substance in the high calcium perfusates, which would lower the calcium when injected into a second dog. We concluded that this must be a previously unrecognized hormone, and named it *Calcitonin*, since it was apparently involved in controlling the level or "tone" of calcium in body fluids. A preliminary report of this work (17)

### Effect of Perfusion of the Parathyroid & Thyroid.

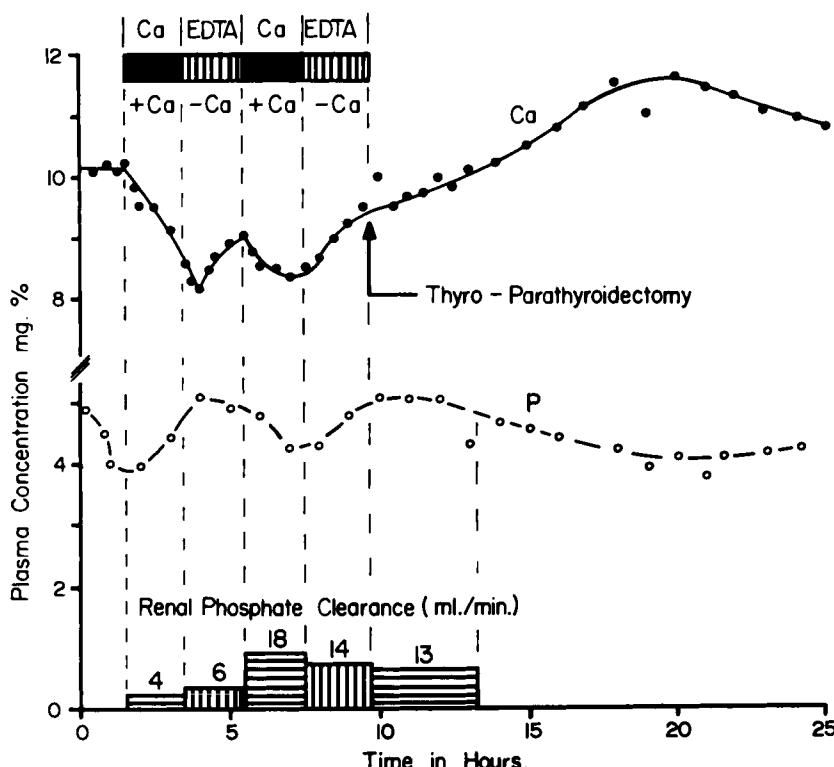


FIGURE 1. Effect of successive perfusions of the thyroid and parathyroid glands with alternatively high- and low-calcium blood. The glands were removed at the end of the last EDTA perfusion (14). (From Copp *et al.*: *Endocrinology*, 70:638, 1962. Courtesy J. B. Lippincott Co., Philadelphia.)

was presented at the meeting of the Canadian Federation of Biological Societies in June 1961, and a full report was presented the following year (14). The observations were subsequently confirmed by Kumar *et al.* (32), using a slightly different technique for altering the calcium level in the perfusates. Originally thought to come from the parathyroids (18), it soon became apparent that calcitonin was derived from cells present in the mammalian thyroid. Hirsch *et al.* (27) observed that removal of the parathyroids by hot-wire cautery caused a much more rapid and pro-

found fall in plasma calcium than did simple surgical removal of the glands, and they suggested that this might be the result of release of a hypocalcemic substance from the cauterized thyroid. They found that a potent hypocalcemic and hypophosphatemic substance could be extracted from thyroid with N/10 HCl, and suggested that it might be identical with calcitonin. They proposed the name "*Thyrocalcitonin*" to indicate this possible relationship and the thyroid origin of the hormone. The thyroid origin of calcitonin was confirmed by high-Ca perfusion experiments in the goat (23) and in the pig (8). In the latter, there is no parathyroid tissue present in the thyroid. There is now no doubt that calcitonin and thyrocalcitonin are identical.

### **Chemistry and Assay**

Hirsch *et al.* (29) described a simple method of bioassay, which involved injecting the extracts subcutaneously into intact young male rats weighing 150-200 g. Approximately 1 hour later, the rats were bled by heart puncture and the plasma calcium was determined by the method of Copp (12). The fall in plasma calcium was directly related to the logarithm of the dose, and the unknown could be compared with a standard preparation. A Hirsch unit was defined as the dose that would lower the plasma calcium 1 mg % in the one-hour period. A somewhat similar biological assay has been described by Kumar *et al.* (33) in which the test sample is infused iv for 50 minutes into male rats that have been fasted overnight. A third method described by Copp and Kuczerpa (20) involves ip injection of the test sample into rats that have been maintained on a low calcium diet for at least 24 hours. Blood samples are collected and analyzed for plasma calcium at 0, 1, 3, 6 and 9 hours in each rat, and the plasma curve drawn. The response to hormone is taken as the area (in mg %-hrs) between this curve and the control plasma calcium curve in animals that received only saline. Both age and the level of dietary Ca and P were found to have a profound effect on this response (19). For example, the response in a 9-month-old rat was only 1/20 that in a 30-day-old weanling.

Recently, the Division of Biological Standards of the National Institute for Medical Research, Mill Hill, London, England, has

TABLE I

## COMPARISON OF THE AMINO ACID COMPOSITION OF PARATHORMONE AND CALCITONIN

<i>Amino Acid Residues</i>	<i>Parathormone (5)</i>	<i>Calcitonin (41)</i>
Alanine . . . . .	7	3
Glycine . . . . .	4	5
Leucine . . . . .	7	4
<i>Isoleucine</i> . . . . .	3	0
Serine . . . . .	5	6
Threonine . . . . .	1	3
Valine . . . . .	6	2
Aspartic acid . . . . .	7	2
Glutamic acid . . . . .	9	2
Arginine . . . . .	5	3
Lysine . . . . .	8	1
Histidine . . . . .	4	2
<i>Tryptophane</i> . . . . .	1	—
Proline . . . . .	3	4
Tyrosine . . . . .	1	1
Phenylalanine . . . . .	2	4
<i>Cystine</i> . . . . .	0	0
Methionine . . . . .	2	1
<hr/>		<hr/>
Residues . . . . .	75	43
Molecular weight . . . . .	9,500	4,500

made available to investigators a lyophilized, partially purified standard preparation of pork thyroid calcitonin, which is referred to as "Research Standard A." It has provided a very useful standard of reference. One MRC unit is defined as the content of 4 ampoules, and weighs approximately 40 mg. This unit is estimated to equal 100 Hirsch units as defined above.

The original method of purification described by Hirsch *et al.* (29) consisted in homogenizing fresh minced hog thyroid glands with 10 volumes of N/10 HCl, and then ultracentrifuging at 100,000 g for 24 hours. The yield was 2.6-4.3 MRC units/g hog thyroid tissue. After lyophilization, further purification was accomplished by column chromatography with carboxymethyl-Sephadex G-25. The method of Baghdiantz *et al.* (6) consisted in extracting acetone-dried pig thyroid tissue with 0.2 N HCl for 5 minutes at 60°-70° C and subsequent dialysis and salt fractionation. However, the most efficient method appears to be that described by Tenenhouse *et al.* (46) and is very similar to that used for the preliminary purification of parathormone. These authors

originally thought that they had obtained the pure homogeneous hormone, but it was soon shown that the material was largely inert protein with only a small hormone content. Recently, Potts *et al.* (41) have presented evidence for the isolation of what appears to be a very potent and homogeneous peptide that may well be pure hormone. It is a straight chain peptide with 43 amino acid residues and a molecular weight of 4,500 (it is similar in size to ACTH). The activity of this peptide is reported to be 200 MRC units/mg. A comparison of the amino acid composition of parathormone and calcitonin is shown in Table I.

### **Ultimobranchial Origin of Calcitonin**

For some time, there was controversy as to whether calcitonin was reproduced by parathyroid or thyroid tissue. However, there is now substantial evidence that it is neither a thyroid nor a parathyroid hormone, but is, in fact, a hormone of the ultimobranchial glands (15). As the name suggests, these arise from the ventral floor of the last ("ultimo") branchial pouch just behind the

### Derivatives of the Branchial Pouches of the Frog

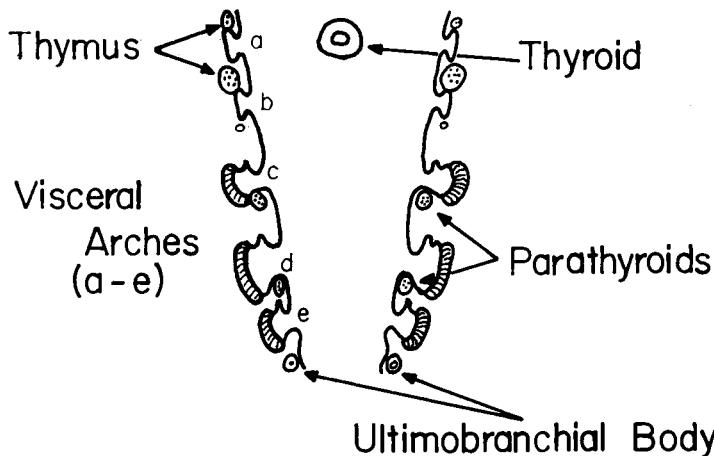


FIGURE 2. Glandular derivatives of the branchial pouches of the frog. (Based on Greil's modifications of Maurer's drawings. *Anat. Hefte*, 1905.)

parathyroids, which arise from a similar position in the third and fourth branchial pouches. The relationship in the early stages of development of the frog is shown in Figure 2. In fish, amphibia, reptiles and birds, the ultimobranchials exist as discrete glands, but in mammals the ultimobranchial cells become intimately associated with the thyroid gland, and the internal parathyroid (40). Here, they constitute the so-called parafollicular cells, or "light" cells, which can be distinguished from the regular thyroid cells by the absence of colloid and radioiodine uptake, and by certain specific staining reactions (19). Their possible role in the production of calcitonin was first proposed by Foster *et al.* in 1964 (24). Pearse (39) has suggested that these be referred to as "C" cells because of their role in calcitonin production. Bussolati and Pearse (7), using guinea pigs, prepared antibodies against purified pig calcitonin. By standard immunofluorescent staining techniques, they clearly demonstrated that the antibodies were localized in the C cells, but were absent from the regular colloid-containing thyroid cells. The conflicting report of Hargis *et al.* (26) must be discounted, because the preparation that they used for immunization was impure and contained very little hormone.

To test the hypothesis that calcitonin might be an ultimobranchial hormone, we prepared extracts of the thyroid and ultimobranchial glands in chickens, where the two are completely separate (15). Glands were collected from young 1-2-month-old chickens, and extracts were prepared by the method of Hirsch *et al.* (29). The ultimobranchials weighed 6-10 mg, the thyroids 70-100 mg. Bioassay was carried out by the method of Copp and Kuczerpa (20) using 7-week-old rats of the Long Evans strain. Extracts were injected ip, and tail blood samples were taken at 0, 1, 3, 6 and 9 hours. Typical plasma calcium curves are shown in Figure 3. The relative calcitonin content of the glands is shown in Table II. No calcitonin activity could be detected in extracts from as many as 5-6 thyroid glands, confirming the observations of Kraintz and Puil (31). It is evident that calcitonin is not a thyroid hormone in the chick. However, the concentration in ultimobranchial tissue was enormous. Extract from as little as 0.1 mg fresh gland tissue caused a significant fall in plasma calcium. The content per gram was over 100 times that of fresh hog thyroid.

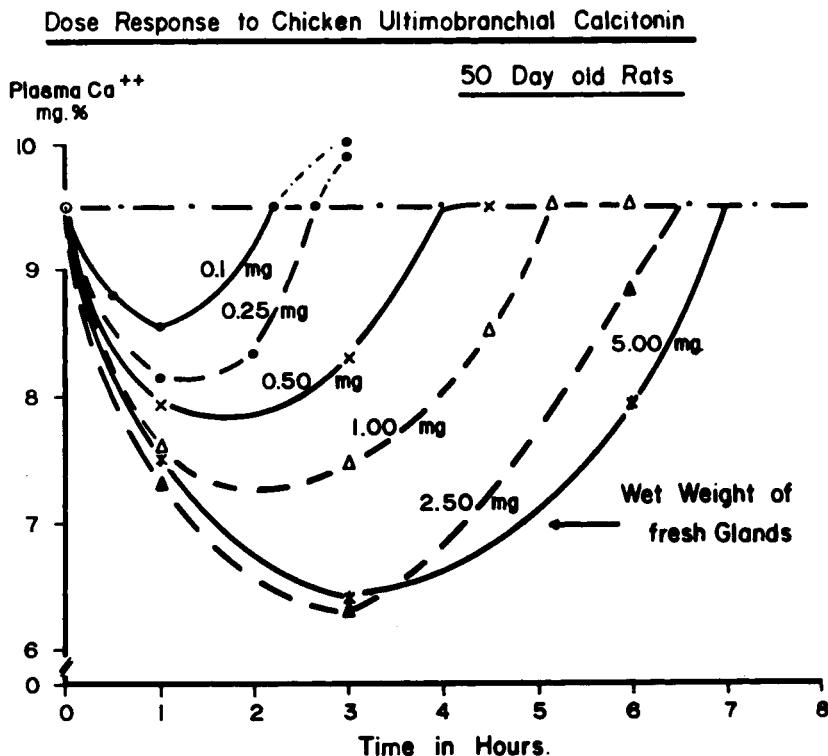


FIGURE 3. Plasma calcium curves showing response to injection of extracts of chicken ultimobranchial glands into 7-week-old rats. Dose is expressed in terms of mg fresh gland extracted. Curves represent average for 6 rats at each dose level.

This is not surprising, since the C cells make up only 1-2% of mass of mammalian thyroids. Protein present in the extracts was determined by the method of Lowry, and gave a specific activity of 25-35 MRC units/mg protein. Assuming that the preparation of Potts *et al.* (41) with an activity of 200 MRC units/mg represents essentially pure calcitonin, this would indicate that our preparation contained 12-15% pure hormone.

We have also used the same procedure to extract calcitonin from the ultimobranchial gland of the Pacific dogfish (*Squalus suckleyi*)—a small shark found in large numbers off the west coast. The gland is found on the left side beneath the floor of

TABLE II  
SPECIFIC CALCITONIN CONTENT  
MRC UNITS/mg

	<i>Hog Thyroid</i>	<i>Source</i>	<i>Chicken</i>	<i>Ultimobranchials</i>
Fresh glands .....	0.003-0.005	(38)	0.4	(15)
TCA powder .....	0.1-0.7	(41)		
Acid extract (ultracentrifuged) .....	1	(38)	25-35	(15)
G-25 Sephadex .....	10-16		100-150	
"Pure" peptide .....	200	(41)		

the posterior end of the pharynx in the triangle formed by the basibranchial and ceratobranchial cartilages and the coraco-branchial muscle. The activity was approximately  $\frac{1}{10}$ th of that found in chicken ultimobranchials but 10 times the activity of fresh pork thyroid tissue. The relationship between response and log dose for calcitonin extracted from chicken and dogfish ultimobranchial glands and from beef thyroid is shown in

Area Response  
mg.%-hrs.

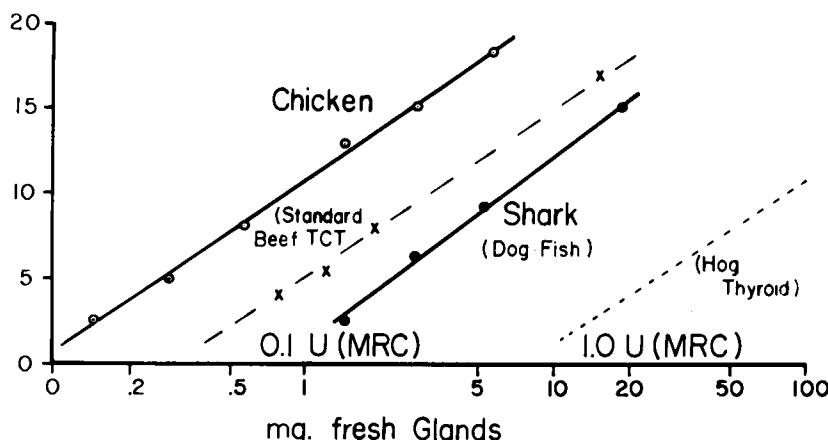


FIGURE 4. Relationship between response and log dose of acid extracts of ultimobranchial glands of chickens and dogfish. Response to a standard partially purified preparation from beef thyroid is shown for comparison.

Figure 4. The slopes are essentially parallel, suggesting similar biological activity for calcitonin prepared from these various sources. It is particularly interesting to find calcitonin present in an elasmobranch, which has no parathyroid or boney skeleton.

These results clearly indicate that calcitonin is an ultimobranchial hormone that can be extracted from mammalian thyroid and parathyroid tissue only because of the ultimobranchial cells, which have become associated with these organs. They also provide strong support for the view that the C cells in mammalian thyroids are of ultimobranchial origin. Calcitonin can scarcely be considered a true thyroid hormone, since the presence of ultimobranchial cells in mammalian thyroid is no different from that of parathyroid tissue in this gland. Further, in all vertebrates other than mammals, the glands are distinct.

Under these circumstances, the name "thyrocalcitonin" seems inappropriate. The terms ultimobranchiocalcitonin (UBC) or ultimobranchial hormone (UBH) appear cumbersome, and in our opinion, the simplest solution would be to drop the suffix "thyro" and retain the original name "calcitonin" (which, incidentally, is the official name for the hormone in the Index Medicus). It is particularly interesting that the glands responsible for calcium regulation develop from similar regions in adjacent branchial pouches.

#### Action of Calcitonin

The action of calcitonin (thyrocalcitonin) has been discussed in a number of excellent recent reviews (4, 37, 38). The principal effect of the hormone is to lower the level of plasma calcium and phosphate, and this occurs in nephrectomized animals (29), in eviscerate rats, and in the absence of the parathyroids (2). Kenny and Heiskell (30) failed to observe any significant changes in the calcium and phosphate in soft tissues. It seems logical that the primary target of the hormone is bone.

Aliopoulos *et al.* (1) showed that calcitonin (thyrocalcitonin) inhibited osteolysis in cultures of calvaria from young mice, and was particularly effective in counteracting the osteolytic effect of adding parathyroid extract to the cultures. Friedman and Raisz (25) also observed that the hormone inhibited bone resorption as

indicated by reduced release of  $\text{Ca}^{45}$  from previously labelled radii and ulnae from 19-day-old rat embryos. Administration of the hormone also reduced the urinary excretion of hydroxyproline in intact animals (34). This would fit in with the suggestion that the hormone inhibits bone resorption. Foster *et al.* (21) made a study of the effect of chronic administration of calcitonin to young (40-60 g) parathyroidectomized rats. They found that there was a significant reduction in the osteoclast count in the treated animals, as well as an accumulation of trabecular bone and increased bone density, which could be explained on the basis of reduced bone resorption and remodelling. In view of this, it seems possible that the hormone may be of practical value in osteolytic bone disease. It is particularly significant that Selye *et al.* (44) found that chronic administration of calcitonin inhibited cutaneous calciphylaxis.

### Calcitonin in Man

Milhaud *et al.* (36) first reported successful extraction of calcitonin from human thyroid tissue—testing the extracts in rats and monkeys. They also observed that the hormone from pork thyroid produced a small (0.4 mg %) but significant reduction in plasma calcium when injected into human subjects.

Aliopoulos *et al.* (3) also succeeded in extracting calcitonin from human thyroids, but found that the average concentration of hormone was only 0.4 MRC units/g. This is 1/10th the activity found in hog thyroids, and 1/1000th the activity reported in chicken ultimobranchials. No hypocalcemic activity could be demonstrated in two thyroid adenomas. In one case of long-standing pseudo-hypoparathyroidism, the concentration was reported to be 100 times higher than that in the average human thyroid, which suggests the intriguing possibility that this condition may be due to excess calcitonin.

Foster *et al.* (22) studied the effect of iv injection of hog calcitonin in human subjects. Very little response was obtained in a normal subject, but significant decreases in plasma calcium (1.5-2.0 mg %) were observed in three patients with secondary bone metastases and hypercalcemia. The doses given ranged from 1-22 MRC units.

### Homeostatic Role of Calcitonin

There is growing evidence that removal of the thyroid glands impairs control of hypercalcemia. Copp (13) observed a sudden increase in plasma calcium when dogs were thyroparathyroidectomized after a prolonged infusion of parathyroid extract. Hirsch and Munson (28) have recently reported significantly higher plasma calcium values when parathyroid extract (85 U/100 g) was administered to thyroparathyroidectomized rats than when the same dose was given to intact or parathyroidectomized rats. Talmage *et al.* (45) clearly demonstrated the importance of the thyroid in lowering the plasma calcium after it had been artificially raised by iv infusion of calcium salts. This was confirmed by Care *et al.* (9), who infused calcium iv in pigs. Plasma calcium rose, and then levelled off, even though the perfusion was continued. However, when the thyroid gland was removed, there was a prompt increase in plasma calcium, similar to that observed after parathyroid extract infusion (13). These results can all be explained on the basis of increased calcitonin release by the ultimobranchial cells present in mammalian thyroids. In a preliminary report, Care (personal communication) measured the calcitonin

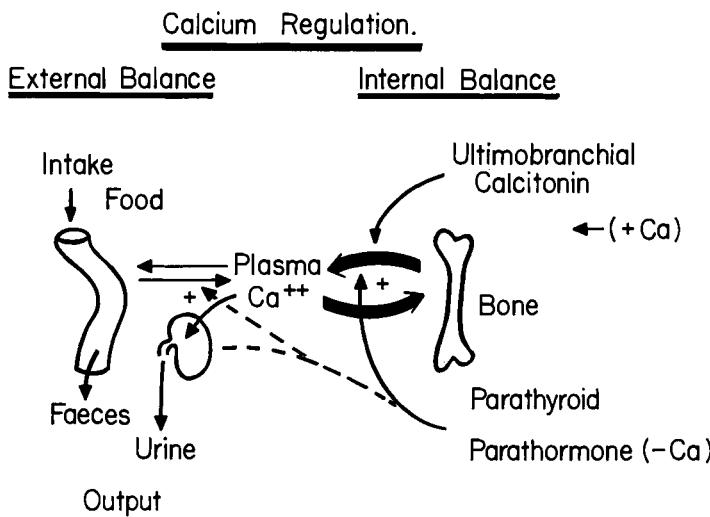


FIGURE 5. Model of factors in regulation of blood calcium.

level in blood collected from the veins of pig thyroid perfused with blood containing added calcium. The increase in hormone secretion paralleled the increase in plasma calcium, indicating direct humoral control and an excellent feedback system.

As illustrated by the model in Figure 5, the precise control of plasma calcium depends on two endocrine glands that arise from neighbouring branchial pouches. The parathyroids are stimulated by hypocalcemia to produce parathormone, which increases osteolysis and so restores the plasma calcium to normal. Ultimobranchial cells, whether in thyroid or in a discrete gland, respond to hypercalcemia by releasing calcitonin, which suppresses osteolysis and lowers plasma calcium. These two negative feedback mechanisms provide one of the most efficient regulatory systems in the body.

### Abstract

The precise control of plasma calcium depends on two endocrine glands that arise from branchial pouches—the parathyroids, which produce parathormone and control hypocalcemia, and the ultimobranchials, which produce calcitonin and control hypercalcemia. Calcitonin is a recently discovered peptide hormone with a molecular weight of 4,500. It apparently lowers plasma calcium by suppressing osteolysis. In mammals, ultimobranchial cells may be recognized by special staining techniques, and are found in the thyroid and internal parathyroids. In birds, the ultimobranchials are distinct glands with a very high calcitonin content. Chicken thyroid contains no calcitonin, while chicken ultimobranchials contain over 200 MRC units/g (compared with 3·4 units/g in hog thyroid) and dogfish gland contains 50.

### Abrégé

Le contrôle précis du niveau du calcium du sang dépend de deux glandes endocrines qui proviennent des sacs branchiaux: la parathyroïde qui produit la parathormone et combat l'hypocalcémie et les corps ultimobranchiaux qui produisent la calcitonine et combattent l'hypercalcémie. La calcitonine est une nouvelle hormone peptidique découverte récemment ayant un poids moléculaire de 4,500. Chez les mammifères on identifie les cellules ultimobranchiales par des techniques histochimiques; elles se trouvent dans la thyroïde et la parathyroïde interne. Chez les oiseaux, les glandes ultimobranchiales sont distinctes et séparées de la thyroïde: elles ont un grand taux de calcitonine. Chez la poule, la thyroïde ne contient pas de calcitonine, mais la glande ultimobranchiale contient plus de 200 MRC u/g (en comparaison de 3·4 MRC u/g de la thyroïde du porc) et la glande du requin en contient 50.

### Acknowledgements

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**F. C. McLean:** Harold Copp has made a very significant contribution to the physiology of calcium metabolism. It is now clear that, in many species, there is a hormone that is capable of controlling hypercalcemia, and that it is elaborated by the ultimobranchial bodies, or the ultimobranchial glands as they now deserve to be called. The physical interrelationships between the thyroid, the parathyroids, and the ultimobranchial glands are different in different species, and the parathyroid glands are not found at all in fishes and in still lower aquatic forms. Because they are buried in thyroid tissue in many species, the parathyroids remained undiscovered until relatively recently, even though they are discrete aggregations of cells quite different morphologically from those of the thyroid. The ultimobranchial cells, on the other hand, are in many higher vertebrates randomly distributed in thyroid tissue; and since they closely resemble cells of the thyroid, they have escaped detection and identification until even more recently. As Copp has pointed out, it is particularly interesting that the glands contributing to calcium homeostasis develop from similar regions in adjacent branchial pouches, and it is this that has made it possible for cells with quite different functions to be intermingled during embryonic development.

Some years ago it was suggested that the ultimobranchial bodies in certain fishes elaborate a hormone with parathyroid-like activity, but

this has not been confirmed and the idea appears to have been lost sight of. Copp's demonstration of high calcitonin activity in the ultimobranchial glands of chickens leaves little room for the possibility that they may also exhibit parathyroid activity. The situation now demands that similar studies be made in other species, and particularly in fish.

It is tempting and indeed logical to assume, as Copp does, that precise calcium homeostasis depends upon the opposing actions of two hormones, i.e., that of the parathyroid glands and that of the ultimobranchial bodies. The situation is thus similar to that of homeostasis of the blood glucose concentration. Two hormones, insulin and glucagon, with opposing effects on the concentration of glucose in the blood, are produced by the islands of Langerhans in the pancreas; the physiologic significance of glucagon is still under investigation.

Copp quotes Rasmussen as stating that a simple feedback mechanism, involving the parathyroid hormone alone, would tend to oscillate. It may be pointed out that all regulatory mechanisms that depend upon negative feedback require oscillation to be effective. When we refer to homeostasis as maintenance of constant concentrations, we actually mean that oscillation is held to a minimum; by definition, the function of negative feedback is to relay the information that the concentration is changing. The same reasoning applies to the control of the concentration of glucose in the blood. In cybernetic terms, the organism has proportional, derivative, and integral controls at its disposal in its many self-regulating mechanisms, and these tend to reduce the range of oscillation. It is not safe to assume that glucagon is essential for glucose homeostasis, or that calcitonin is required for regulation of the calcium level in the blood. This statement in no way detracts from the importance of its discovery; it rather points the way to further investigations. Finally, as to the name of the calcium-lowering principle, it would now seem that its association with the thyroid gland is an accident in embryonic development in certain species, and that there is no reason to perpetuate this accidental association in the name of the principle. I agree with Copp that a return to the original name—*calcitonin*—is desirable.

**L. F. Bélanger:** I would first like to thank my good friend Harold Copp for the renewed interest that he has introduced into embryology at a time when the "advanced thinkers," and I say that with quotations, are in the process of removing the subject from the medical

course. So, thanks to Harold, I am assured of a job for a few more months; but on the other hand, I have some problems with this tremendously rapid increase in the field of calcitonin origin, calcitonin function. Like Dr. McLean, I should like to say that it seems that the original confusion as to the origin of this hormone might very well come from the fact that some of this ultimobranchial material becomes incorporated in an aberrant fashion into the parathyroid; thus, the original work of Harold Copp in finding the calcium-lowering principle from this gland and then in the thyroid gland. But nobody speaks about that other enormous derivative of the area, the thymus. I am wondering, Sir, whether it is known that there could be some calcitonin activity from the thymus? Now, when I had the pleasure of working in your laboratory, I saw, on looking at the thymus of sheep (which you so graciously provided), that there were islands of cells that, at the time, looked to me like parathyroid cells. I frequently saw them, but they could just as well have been C cells, as I did not know any better at the time and looked at them only under the light microscope. I am also greatly surprised to hear of the most recent work in the dogfish because, as McLean has stressed, it is only the most advanced teleosts that we consider to have calcium homeostasis. Now, if the dogfish developed such large amounts of calcitonin, is it because he was already prepared for his grandson to have bone?

**D. H. Copp:** Thank you for those kind remarks, Dr. Bélanger. Naturally, the Pacific coast dogfish are superior. A number of years ago we tested thymus extracts for hypocalcemic activity, but were unable to detect any. Of course, as with the rabbit thyroid, we may have taken our tissue from a place that contained no C cells. It would be interesting to know whether these cells can be found in thymus tissue. There does seem to be a kind of endocrine gregariousness—especially in mammals—so that parathyroid and ultimobranchial tissues become imbedded in thyroid.

The point you raised concerning the cartilaginous nature of the dogfish skeleton is a fascinating one. The ultimobranchial gland is highly vascular, and certainly looks as if it were functioning as an endocrine organ. It also contains almost 10 times the calcitonin concentration present in hog thyroid. The question is, how does it act in an animal without a bony skeleton? This should provide an interesting problem for investigation by a comparative endocrinologist. It is also interesting that the parathyroids and ultimobranchials, which regulate calcium, and the aortic and carotid bodies, which

have a role in regulating oxygen levels and pH, all arise from similar tissue in the branchial pouches.

**C. H. Li:** I should like to congratulate Dr. Copp for the beautiful demonstration of the discovery of a new hormone, including its origin, property and chemical identification. I would like to ask him though: "Have you done any hypophysectomy in the fish or in a dog to find out what happened to the ultimum ultrabranchial cells?" The second comment is: I am not as pessimistic as Dr. McLean that this dogfish that he is working with has high concentrations of this hormone and yet has nothing to do with calcium. You may recall that prolactin is chiefly concerned with the development and lactation of mammary glands and yet G. Pickford (*Science*, 130:454, 1959) found that prolactin exerts biological effects in the fish. I should now like to comment on the presentations by Dr. Guillemin and by Dr. Beck. I think that Dr. Guillemin gave a very thorough summary on releasing factors, except that he did not commit himself that there is a growth-hormone-releasing factor. Dr. Beck somehow very gently said that he agreed with him, although his data seem to indicate the absence of a growth-hormone-releasing factor. I think everyone writing a paper on growth-hormone-releasing factor should take note of the short paper of R. Hertz (*Endocrinology*, 65:926, 1959), who has transplanted the pituitary gland on the capsule of the kidney of the rat; the rat continues to grow, but the adrenal is atrophied and so is the thyroid and the ovary. This experiment of Hertz was independently carried out in 1957 and confirmed by I. I. Geschwind in our laboratory.

**D. H. Copp:** These are interesting and important questions. However, we began our studies on the dogfish last week, and haven't got around to determining the effect of hypophysectomy . . . perhaps next week. Seriously, there is no evidence that removal of the anterior pituitary affects either of the two calcium regulating glands, while our original gland perfusion studies in the dog indicate that an altered plasma calcium level provides adequate stimulus for the release of parathyroid hormone or calcitonin.

**R. Guillemin:** In answer to Dr. Li's question, I would like to say this. In the current literature, as many of you know, a considerable number of publications have recently come out concluding, on the basis of the bioassay summarized by Dr. Beck, to the existence of a growth hormone releasing factor. I think we have no reason to doubt the results, but I think we have reason to question the conclusions derived from them. I think that Dr. Beck has put it quite clearly

that other possible interpretations of the results of this complicated bioassay could be involved. We are dealing with a multi-step bioassay system from which these other groups have obtained observations that they consider to mean secretion of pituitary growth hormone. It could be that entirely different mechanisms are at play; for instance, it could be that stimulation of the lysosomes will locally destroy the adenohypophysial content of growth hormone. I think that one of the most striking observations as reported by Dr. Beck in his summary is the fact that no group, to my knowledge, has ever been able up to now to correlate levels of circulating growth hormone as measured by bioassay (which by the way is a rather difficult endeavour) or by immunoassay, with any of the changes observed at the level of the anterior pituitaries. I think that this is a striking observation that still points to the necessity of further investigation. In other words, the pituitary "depletions" do not appear to be necessarily correlated with unequivocal evidence of secretion. I was glad that Dr. Li reminded us of this paper by Hertz and further work by Geschwind that there is also good evidence from their investigations that pituitary tissue can indeed secrete growth hormone for weeks and months in the complete absence of hypothalamic control or hypothalamic connection—even though some hypothalamic modulation of growth hormone secretion may also be operative in the normal individual.

# **Brain Monoamines and Thyroid Hormones on the Emotional Stress Induced by Sympathomimetic Agents in Aggregated Animals\***

BERNARD HALPERN, CAROLA DRUDI-BARACCO, \*\*  
DENISE BESSIRARD and FRANÇOISE MARTINEAU

**A** NUMBER of sympathomimetic amines (3, 6, 8) and MAO inhibitors (9) can display two types of toxicity: 1) the absolute toxicity that results when the drug is administered in isolated animals; 2) the "group toxicity" (G.T.) observed when the animals are aggregated in groups.

The G.T. is reflected, not only by a reduction in the lethal dose, but also by a characteristic pattern of emotional behaviour resulting from the aggregation of the animals in a restricted space. Ambient temperature is another important factor.

The emotional disturbance that may produce a lethal paroxysm results from the sensory stimuli due to the presence of other animals. G.T. is an attribute of various chemical agents that, although belonging to different pharmacological groups, possess central nervous stimulating properties in common. However, this effect is far from being shared by all centrally stimulating drugs. Moreover, Halpern *et al.* (10, 11) have recently shown that thyroid hormones play a prominent role in the severity of the disorders and in the mortality caused in aggregated as well as isolated animals by certain adrenergic amines and their precursors. Finally, the emotional stress involves peculiar changes in brain monoamines, the patterns and the significance of which have been investigated.

In this study, the following points will be discussed: 1) The relationship between the pharmacological properties of various drugs and their ability to induce G.T.; 2) The mechanism of the protection afforded by reserpine and chlorpromazine; 3) The

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\* This paper was presented in French.

\*\* Deceased.

reversal of this protection by MAO inhibitors; 4) The potentiation of the toxicity of sympathomimetic amines by MAO inhibitors and thyroid hormones; 5) The correlation between the emotional paroxysm as expressed by the phenomenon of G.T. and the brain and myocardial monoamines.

### **Materials and Methods**

All the experiments were performed on male albino Swiss mice originating from the same breeding colony and weighing between 18 and 22g. Care was taken to use animals shortly after their arrival in the laboratory, as it has been observed that mice kept for prolonged periods in smaller groups are less susceptible to G.T.

Experiments were carried out at a temperature of  $27^{\circ} \pm 0.5^{\circ}\text{C}$ . The animals were kept in glass containers, 21cm in diameter and 10cm high and placed in a ventilated, thermostatically regulated incubator. The temperature is of great importance for obtaining constant results with a high mortality rate in G.T. experiments. The intensity of the reactions decreases abruptly with a lowering of the environmental temperature.

The experimental procedures were initiated in the morning after 4 hours of fasting. The substances used were dissolved in saline and all the injections were given intraperitoneally in a volume not exceeding 0.5ml/20g.

To study the G.T., groups of ten animals were placed, immediately after injection, in the previously-warmed glass containers, the only exception being the experiments carried out with DL-Dopa, where groups of 20 were used. The behaviour of the animals was carefully observed, and the mortality noted during the following 24 hours.

In the studies dealing with absolute toxicity, the injected animals were also kept at an ambient temperature of  $27^{\circ}\text{C}$ , but they were placed in isolated glass containers.

The brain and myocardial monoamines [noradrenaline (N.A.) and 5-hydroxytryptamine (5-HT)] were determined according to Shore and Olin (17) with some minor modifications. The tissues were homogenized in H Cl 0.01N and the monoamines extracted with rectified butanol. Extraction was found to be facilitated by

saturation of the aqueous phase with NaCl. Another extraction in an aqueous phase (H Cl 0.01N) was then carried out by the addition of heptane, which decreases the solubility of the monoamines in the butanol phase. The monoamines were transformed by oxidation with iodine into fluorescent trihydroxyindoles. The solutions were activated by exposure during 30 minutes to U.V. and the fluorescence was measured with the "Amino" spectrophotofluorometer. In the case of N.A., activation was carried out at 405 m $\mu$  and the fluorescence measured at 500 m $\mu$ , while for 5-HT, activation occurred at 300 m $\mu$  and the fluorescence measured at 540 m $\mu$ . The quantities of the tissue monoamines were extrapolated from standard curves prepared with known amounts of N.A. or 5-HT and treated in the same way. The "blank" was obtained from extracts of the same tissues, similarly treated except that no oxidation with iodine was carried out.

## Results

### I) The Ability of Various Substances to Induce G.T.

In Table I are grouped the various substances that have been studied for their ability to induce G.T. They can be divided into three pharmacological classes: 1) sympathomimetic amines, 2) catecholamines and their precursors, and 3) MAO inhibitors (MAOI).

It is evident that the G.T. phenomenon cannot be correlated

TABLE I  
ABILITY OF VARIOUS SUBSTANCES TO INDUCE G.T.

<i>Pharmacological Classes</i>	<i>Positive G.T.</i>	<i>Negative G.T.</i>	<i>G.T. after Treatment with MAOI</i>
Sympathomimetic amines	DL-Amphetamine	L-Ephedrine Tyramine	L-Ephedrine Tyramine
Catecholamines and their precursors	DL-Dopa DL-Metatyrosine	Adrenaline L-Noradrenaline	Dopamine
MAOI	Pheniprazine Tranylcypromine Nialamide	Iproniazid Isocarboxazide Hydrazine-2-Octane Pargyline	

with either a particular chemical structure or a definite pharmacological class. In each of the three classes, there are substances possessing the ability of inducing G.T., while others lack this property. It should be emphasised, however, that the number of compounds tested is insufficient to allow any valid hypothesis concerning the chemical structure or the fundamental pharmacological property with which G.T. is correlated.

## **II) The Protective Effect of Reserpine and Chlorpromazine Against G.T.**

Burn and Hobbs (3) found that G.T. can be abolished by previous administration of reserpine or chlorpromazine. We have confirmed this observation (8) and our studies have provided new information on this subject. There are fundamental differences in the patterns of action of reserpine and of chlorpromazine. In mice given amphetamine, the action of reserpine was established slowly and it required about 1 to 2 hours (after intraperi-

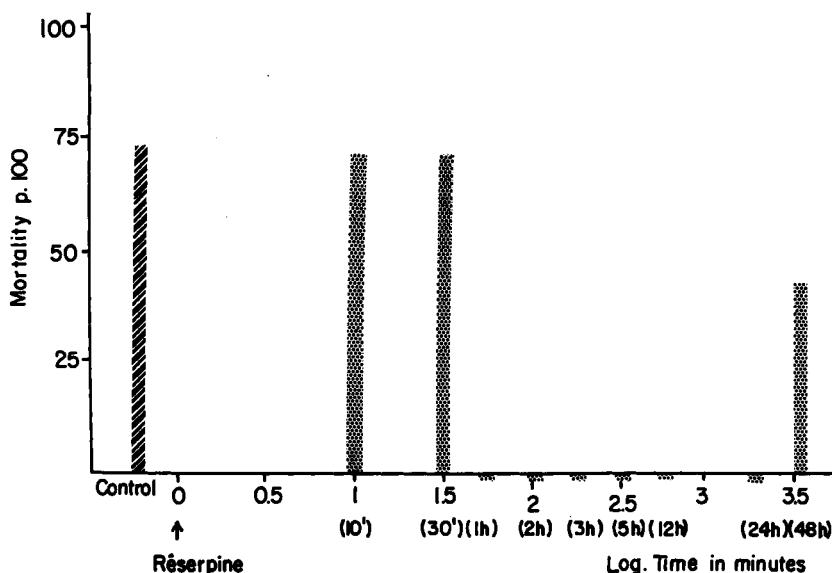


FIGURE 1. Inhibition by reserpine (0.5 mg/kg administered at 0 time) of G.T. induced by subsequent injection of amphetamine (14 mg/kg). *Ordinate: Mortality %.* *Abscissa: Time in minutes (logarithmic scale).* Note the slow onset and prolonged duration of action of reserpine.

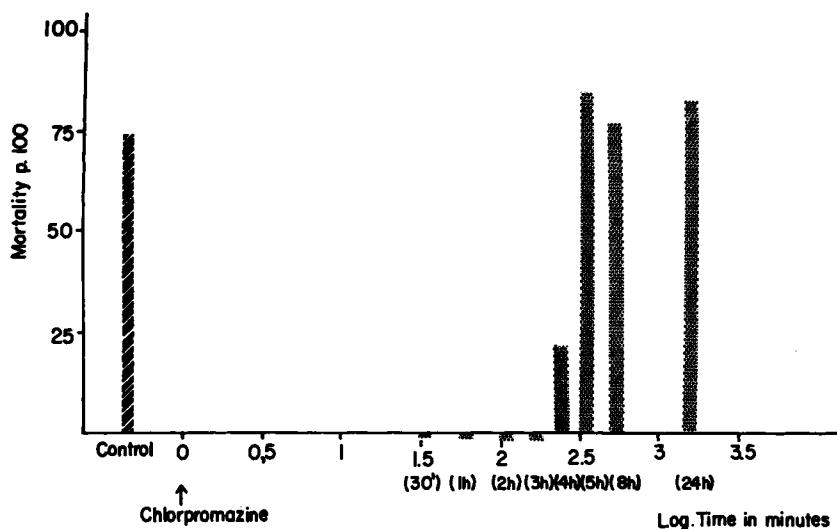


FIGURE 2. Inhibition by chlorpromazine (1 mg/kg given at 0 time) of G.T. induced by subsequent injection of amphetamine (14 mg/kg). Note the rapid onset and the short duration of the protective action of chlorpromazine.

toneal administration of 0.5mg/kg) to attain its maximum; the protective action persisted for more than 24 hours. The action of chlorpromazine given at about the same dose was maximal in less than 30 minutes, but the protective action subsided after 5 to 6 hours.

TABLE II

MODALITIES OF THE PROTECTIVE ACTION AFFORDED BY RESERPINE  
AND CHLORPROMAZINE AGAINST THE G.T. INDUCED BY AMPHETAMINE,  
TRANYLCPROMINE AND DL-DOPA IN MICE

	Dose mg/kg	Reserpine					Chlorpromazine				
		0.5 %	1 %	5 %	10 %	20 %	1 %	5 %	10 %	20 %	
DL-Amphetamine	14	0					0				
	28	80	67	12			85	0			
	42	100	70	35	17			0			
	80				45	37		5	0		
Tranylcypromine	60		65	30	13	20	70	55	58	53	
	90				35	40	80	68			
	140					65				80	
DL-Dopa	700		50	83	68	70	80	65	55	20	

Other data concerning the protection afforded by reserpine and chlorpromazine against G.T. induced by amphetamine, MAO inhibitors (tranylcypromine) and DL-Dopa, respectively, are summarized in Table II.

The differences observed in the modalities of action of reserpine and chlorpromazine cannot be attributed to a simple difference in absorption or elimination of the two drugs. Rather, they suggest that the mechanism by which the two substances produce protection against mortality in aggregated animals may be fundamentally different.

### *III) Reversal of the Protection Conferred by Reserpine and Chlorpromazine Against G.T.*

Halpern *et al.* (8) provided evidence that the protective effect of reserpine against G.T. can be reversed by iproniazid. In Table III is shown the reversal by various substances of the protection afforded by reserpine or chlorpromazine against G.T. The following conclusions may be drawn. All MAO inhibitors reversed the

TABLE III  
REVERSAL BY VARIOUS SUBSTANCES OF THE PROTECTION AFFORDED  
BY RESERPINE\* AND CHLORPROMAZINE† AGAINST G.T.

<i>Substances Tested for Their Reversing Properties (mg/kg)</i>		<i>Substances Inducing G.T. (mg/kg)</i>		<i>Reversal of Action of Reserpine</i>	<i>Reversal of Action of Chlorpromazine</i>
DL-Ampetamine ●	5 mg	Pheniprazine 100 mg Amphetamine 14 mg		+	-
Iproniazid ○	100 mg	Amphetamine 14 mg		+	-
Pheniprazine ●	1 mg	Amphetamine 14 mg Pheniprazine 100 mg		+	-
Hydrazine-2-Octane ○	10 mg	Amphetamine 14 mg Pheniprazine 100 mg		+	-
Imipramine ○	80 mg	Amphetamine 14 mg		+	-
Tranylcypromine ●	5 mg	Amphetamine 14 mg		+	-
Isocarboxazide ○	10 mg	Amphetamine 14 mg		+	-
Nialamide ●	7 mg	Amphetamine 14 mg		+	-
Pargyline ○	20 mg	Amphetamine 14 mg		+	-
Thyroxine ○ (‡)	0.2 mg	Amphetamine 14 mg		+	-

\* Dose of reserpine 1 mg/kg i.p.

† Dose of chlorpromazine 1 mg/kg i.p.

‡ Thyroxine 0.2 mg/kg daily during 3 preceding days.

● Full circle = substance capable of inducing G.T.

○ Open circle = substance unable to induce G.T.

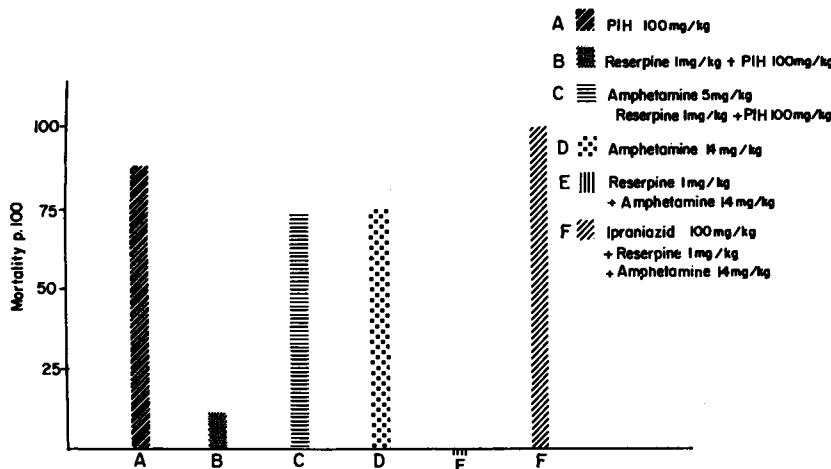


FIGURE 3. Reversal by iproniazid or amphetamine of the protection afforded by reserpine against the G.T. induced by a MAO inhibitor: pheniprazine. *A* = mortality caused by 100 mg/kg pheniprazine (PIH) in aggregated mice. *B* = mortality caused by the same dose of pheniprazine in animals pretreated, 4 hours earlier, with reserpine (1 mg/kg). *C* = mortality in aggregated mice receiving:—amphetamine (5 mg/kg).—2 hours later, reserpine (1 mg/kg).—1 hour later, pheniprazine (100 mg/kg). Note the reversal by amphetamine of the protective action usually afforded by reserpine against the G.T. normally caused by this dose of pheniprazine.

protective action of reserpine against G.T., the effective doses depending on their chemical structure. There is no relationship between the capacity of a substance to reverse the action of reserpine and its ability to induce G.T. In no case can the antagonistic action of chlorpromazine be reversed. Amphetamine reverses the protective action of reserpine towards the G.T. induced by a MAO inhibitor, in very much the same way as a MAO inhibitor reverses the protective action of reserpine against amphetamine (Fig. 3). It is, consequently, suggested that amphetamine acts in this respect like a MAO inhibitor.

#### *IV) Potentiation by MAO Inhibitors of the Toxicity of Sympathomimetic Amines and Catecholamine Precursors*

Previous single or repeated injections of various MAO inhibitors resulted in a considerable increase of the toxicity of various sympathomimetic amines and of DL-Dopa. This increase in tox-

TABLE IV

POTENTIATION BY IPRONIAZID OF THE ABSOLUTE TOXICITY AND G.T.  
OF DL-AMPHETAMINE, L-EPHEDRINE AND DL-DOPA

Substances	Control Animals			MAOI*-Treated Animals		
	Isolated Animals		Isolated Animals	Aggregated Animals		
	Dose mg/kg	Mortality Ratio† %		Dose mg/kg	Mortality Ratio† %	Dose mg/kg
DL-Dopa						
	500	0/30	0	80	0/30	0
	700	8/64	12	150	20/91	22
	1400	7/42	16.6	700	31/50	62
	2800	21/30	70			
DL-Amphetamine	14	1/65	1.5	2.5	0/20	0
	30	2/25	8			
	50	13/40	32	5	13/32	40
	80	24/40	60			
	100	27/30	90	14	19/20	95
L-Ephedrine	50	0/20	0			
	100	9/48	18.7			
	200	22/55	40	18	6/20	30
	280	31/40	77	25	20/20	100
				5	1/20	5
				10	2/20	10
				15	17/20	85
				25	20/20	100

\* MAOI: Iproniazid was injected in a single dose of 200 mg/kg, i.p., 4 hours before testing, except in the case of ephedrine where iproniazid was administered daily at a dose of 100 mg/kg during the 5 successive days preceding testing.

$$\dagger \text{Ratio} = \frac{\text{Number of deaths}}{\text{Number of animals treated}}$$

icity was observed in isolated animals as well as in aggregated mice. Table IV summarizes the results relevant to DL-Dopa, amphetamine, and ephedrine (see also Fig. 3).

It is obvious that pretreatment with iproniazid aggravated the toxicity of various sympathomimetic amines and of DL-Dopa, to a considerable extent. Similar results were observed with other MAO inhibitors, such as pheniprazine (P.I.H.) and tranylcypromine.

It is interesting to note that, although aggregation of animals increased the toxicity of ephedrine, these animals did not manifest the symptoms of psychomotor hyperexcitability, of rage, and of antisocial aggressiveness observed under similar conditions with amphetamine.

However, when the animals were treated with iproniazid

(100mg/kg daily, during five days) and then injected with ephedrine, their behaviour was very much like that observed with amphetamine. Similar results were obtained with tyramine, although, with this substance, iproniazid had to be given for several days in order to induce behavioural changes. This is in contrast to the potentiation of the absolute toxicity, where a single injection of iproniazid suffices. Dopamine behaved similarly to tyramine.

The point of interest of these observations is that treatment with MAO inhibitors may cause striking behavioural alterations in animals given certain sympathomimetic substances that, by themselves, do not produce obvious psychical excitement.

#### *V) Thyroid Hormones and Toxicity of Sympathomimetic Substances*

Thyroid hormones are able to modify both the G.T. as well as the absolute toxicity of various sympathomimetic amines (10-12).

The action of thyroxine is shown in three ways, viz: 1) potentiation of the "absolute" toxicity of certain sympathomimetic amines; 2) enhancement of the G.T. phenomenon; 3) reversal of the protective effect conferred by reserpine against induced G.T.

**Potentiation of the Absolute Toxicity of Various Sympathomimetic Amines by Thyroxine.** Previous administration of thyroxine enhanced the absolute toxicity of certain sympathomimetic amines, to a considerable extent (Table V); as can also be seen from Table V, this increase in toxicity was selective to a certain degree. Thus, the toxicity of ephedrine and amphetamine increased 14- and 15-fold; the toxicity of DL-Dopa and dopamine, 3- and 4-fold; the toxicity of tyramine increased only twice, and that of noradrenaline 4-fold. The toxicity of mepyramine (an antihistamine with convulsant effects, used for purposes of comparison) remained practically unaffected.

**Aggravation by Thyroxine of G.T. Induced by Various Sympathomimetic Amines.** In addition, pretreatment with thyroxine aggravated the G.T. induced by various sympathomimetic amines (Table V). It should be stressed that this effect was obtained with a relatively low dose of thyroxine. The aggravation of the G.T. was already evident after 3 days of treatment. Prolongation of

TABLE V

 POTENTIATION BY THYROXINE OF THE ABSOLUTE TOXICITY  
 OF SYMPATHOMIMETIC AMINES, CATECHOLAMINE PRECURSORS AND MAO INHIBITORS

	Control Animals			Thyroxine-pretreated Animals					
	Doses mg/kg	Mortality (x)	%	Thyroxine 0.2 mg/kg		Thyroxine 0.2 mg/kg			
				Days of Treat.	Mortality (x)	%	Days of Treat.	Mortality (x)	%
DL-Amphetamine (Sulfate)	14	1/65	1.5	—	—	—	—	—	—
	30	2/25	8	—	—	—	—	—	—
	50	13/40	32	—	—	—	—	—	—
	80	24/40	60	—	—	—	—	—	—
	100	14/20	70	—	—	—	—	—	—
	5	—	—	6	10/10	100	—	—	—
	8	—	—	5	7/10	70	—	—	—
	—	—	—	—	—	—	—	—	—
L-Ephedrine (Hydrochloride)	100	9/48	18.7	—	—	—	—	—	—
	200	22/55	40	—	—	—	—	—	—
	280	Controls	31/40	77	—	—	—	—	—
	20	—	—	—	—	—	5	8/10	80
	40	—	—	6	4/10	40	3	9/10	90
	50	1/20	5	3	4/10	40	—	—	—
	60	—	—	5	6/10	60	2	6/11	55
3,4 Dihydroxy- phenylalanine (DL-Dopa)	500	0/30	0	—	—	—	—	—	—
	2800	Controls	21/30	70	—	—	—	—	—
	700	8/64	12	5	3/10	30	3	6/10	60
	1400	7/42	16.6	3	9/10	90	—	—	—
L-Noradrenaline (Hydrochloride)	20	53/90	58	—	—	—	—	—	—
	40	14/20	70	—	—	—	—	—	—
	10	13/60	21	3	3/10	30	3	5/10	50
	15	5/20	25	8	17/20	85	3	15/20	75
Tyramine (Hydrochloride)	650	1/30	3.3	—	—	—	3	6/10	60
	800	7/15	46	—	—	—	—	—	—
	900	12/20	60	—	—	—	—	—	—
	1000	20/20	100	—	—	—	—	—	—
	500	—	—	—	—	—	3	6/11	54
Pheniprazine	100	6/40	15	3	12/20	60	—	—	—
	150	24/40	60	—	—	—	—	—	—
	170	18/20	90	—	—	—	—	—	—
	50	—	—	—	—	—	5	5/10	50
	75	—	—	3	3/10	30	3	5/15	33
	75	—	—	—	—	—	5	10/10	100
	—	—	—	—	—	—	—	—	—
Mepyramine	75	6/22	27	—	—	—	—	—	—
	70	Control	—	—	—	—	—	—	—
	100	19/30	63	3	3/10	30	—	—	—
	—	—	—	—	—	—	—	—	—

(x) Mortality =  $\frac{\text{Number of deaths}}{\text{Number of animals treated}}$ .

treatment with thyroxine increased the susceptibility of the animals to the sympathomimetic amines even more. In this respect, the action of thyroxine is analogous to that of certain MAO inhibitors. But there is another aspect in which thyroxine behaved like an MAO inhibitor, namely, the capacity of thyroxine to reverse the protective action of reserpine against G.T. induced by sympathomimetic amines.

**Reversal by Thyroxine of the Protection Conferred by Reserpine Against Amphetamine-induced G.T.** Previous treatment with thyroxine abolished the protection regularly conferred by reserpine against the G.T. induced by amphetamine, as is shown by the data reported in Figure 4. It should be emphasised that, under identical conditions, the action of chlorpromazine remained unaffected (Figs. 4 and 5).

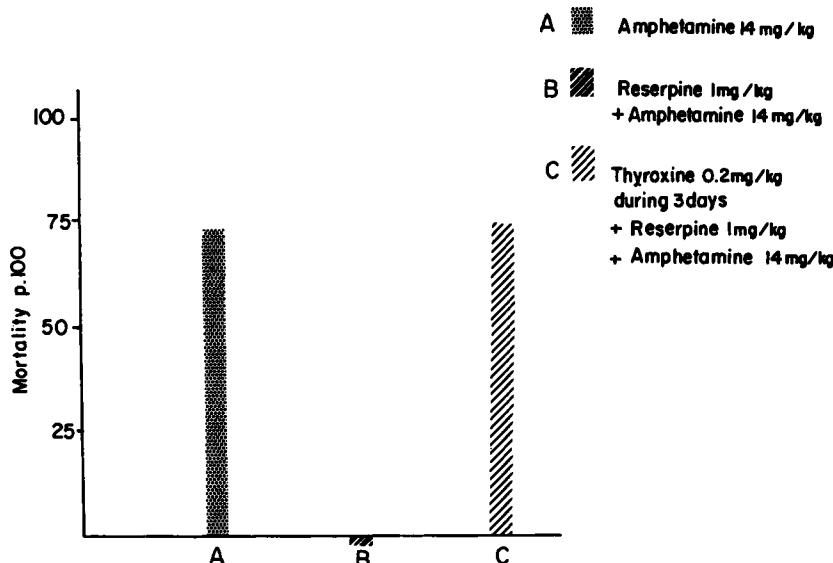


FIGURE 4. Abolition by thyroxine of the protective action of reserpine against the G.T. induced by amphetamine. *A* = Mortality caused by the injection of 14 mg/kg amphetamine in aggregated mice. *B* = Idem after previous administration of 1 mg/kg reserpine. *C* = Idem as in *B* in animals pretreated during 3 days with thyroxine (0.2 mg/kg/day). Note the abolition of the protective effect of reserpine against the G.T. induced by amphetamine in animals receiving thyroxine.

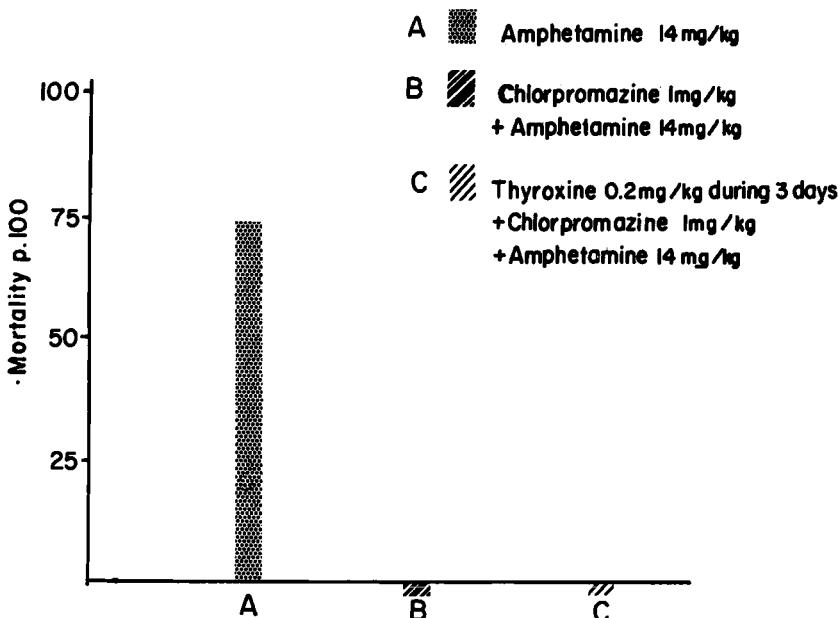


FIGURE 5. The effect of chlorpromazine on the G.T. induced by amphetamine in control and thyroxine-pretreated animals. *A* = Mortality caused by the injection of 14 mg/kg amphetamine in aggregated mice. *B* = Idem after the administration of 1 mg/kg chlorpromazine. *C* = Idem as in *B* in thyroxine-pretreated animals. Note that the protective action of chlorpromazine remains unaltered in thyroxine-pretreated animals.

#### **VI) The Mechanism of Death in G.T.**

In G.T., death occurred within one to two hours of administration of a fraction of the dose of the substance that was lethal for isolated animals. What was the cause of the death in these animals? Our investigations indicate that the death of the animals was caused by severe and extensive myocardial necrosis. Figures 6 and 7 illustrate the myocardial lesions observed in animals that succumbed in the G.T. experiments.

**G.T. and Modifications of Cerebral and Cardiac Monoamines.** There is a great deal of evidence that emotional stress is correlated with changes in brain monoamines. It was, therefore, thought to be worthwhile to investigate the modifications of the monoamine levels in the various experimental conditions reported above.



FIGURE 6. Myocardium of a mouse that died in a G.T. trial, 80 minutes after injection of 14 mg/kg amphetamine. Note the extensive necrosis of almost the whole left ventricle.

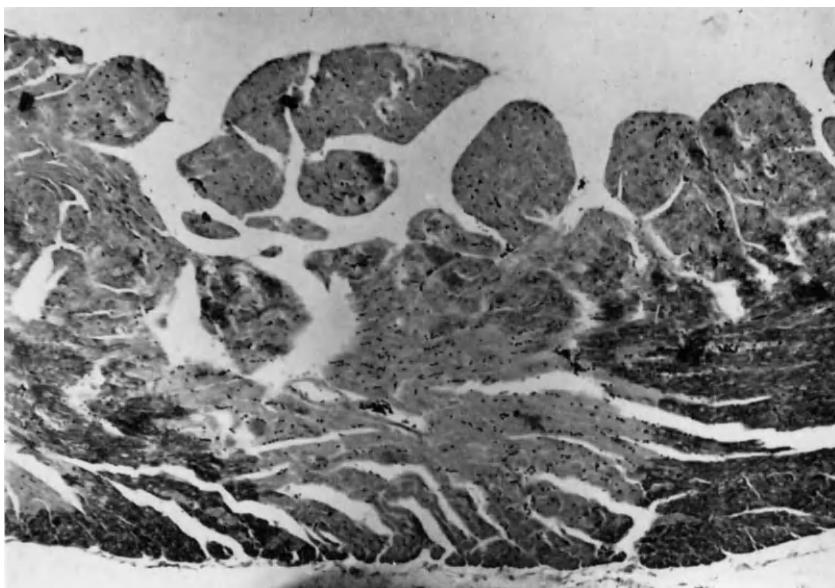


FIGURE 7. Myocardium of a mouse that died in a G.T. assay, 145 minutes after the injection of 14 mg/kg amphetamine. Note the almost full destruction of the myocardial fibers in a region of the right ventricle.

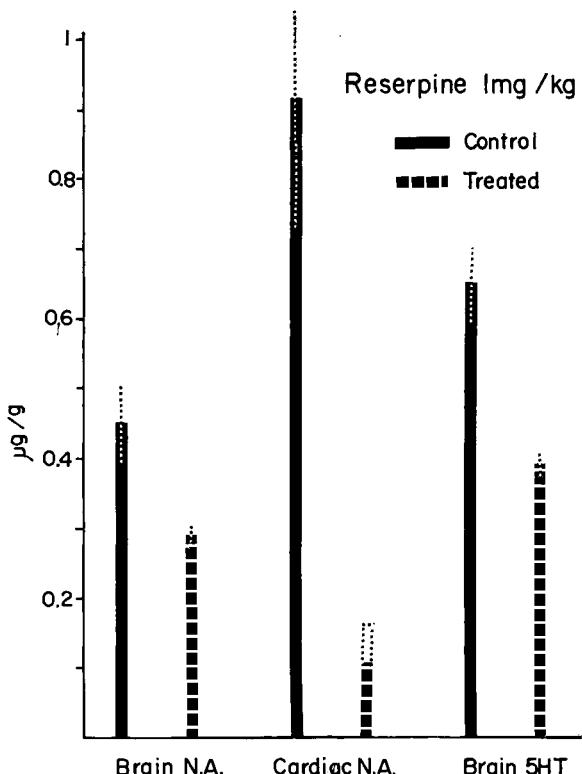


FIGURE 8. Effect of reserpine (single injection of 1 mg/kg) on brain and cardiac N.A. and brain 5-HT.

**Action of Reserpine.** Treatment with reserpine regularly produced a fall in the brain N.A. and 5-HT. The reduction was grossly of the same magnitude for both amines (Fig. 8). The depletion of cardiac N.A. was found to be much more marked than that observed in the brain.

**Action of MAO Inhibitors.** Treatment with MAO inhibitors (iproniazid, isocarboxazide, Pargyline) raised both the N.A. and 5-HT levels in the brain, the increase in the latter being significantly higher than that of the former. Following 5 days of administration of 100 mg/kg/day of iproniazid (Fig. 9), the N.A. level was increased by about 45%, while that of 5-HT was doubled. Surprisingly, the cardiac N.A. store remained unchanged.

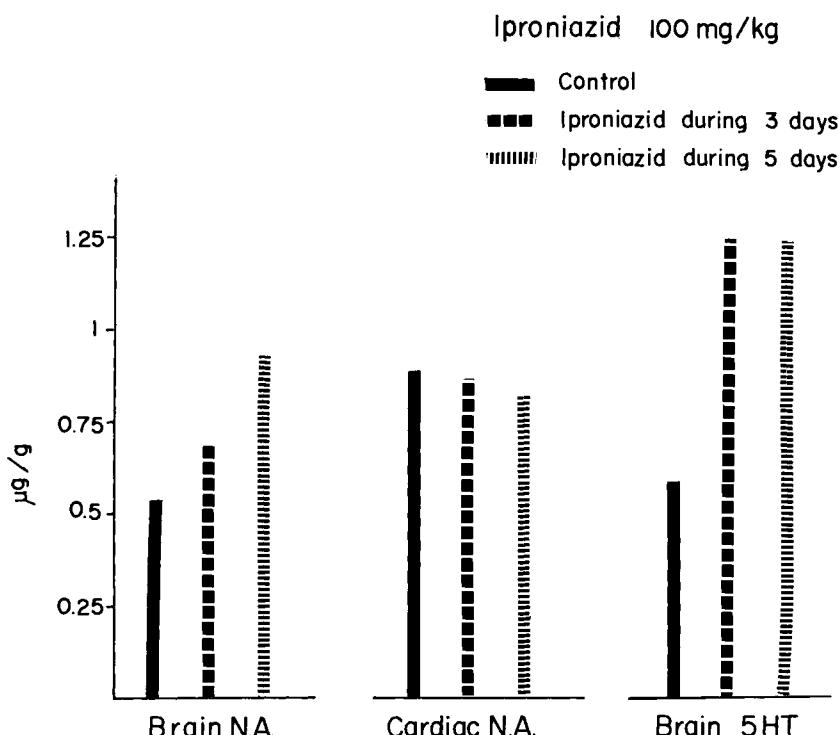


FIGURE 9. Effect of cumulative administration of iproniazid (100 mg/kg/day), administered during 3 or 5 days, on brain and cardiac N.A. and on brain 5-HT.

**Action of Amphetamine.** It has been reported above that the emotional behaviour of the animals receiving moderate doses of amphetamine varied strongly as a function of various parameters, such as: aggregation, ambient temperature, and, as will be seen later, genetic factors.

How was this correlated with the changes in the brain and heart monoamine levels?

1) *Aggregation.* There were definite differences (Fig. 10) in the brain monoamine changes, depending on whether the animals were kept in groups or isolated. In aggregated animals, i.e., animals submitted to severe emotional stress, the brain N.A. was decreased by about 40%, while the 5-HT level was raised by about 40%.

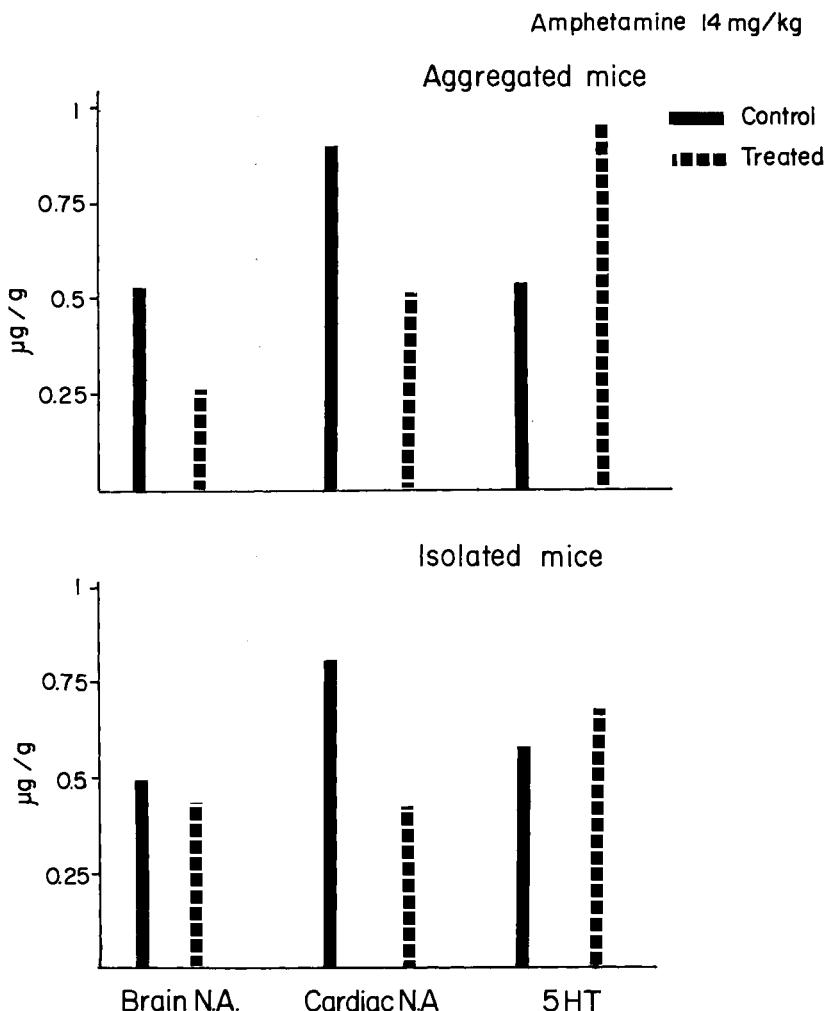


FIGURE 10. Modifications in brain and cardiac N.A. and in brain 5-HT in mice receiving 14 mg/kg amphetamine. *Upper part:* aggregated animals; *Lower part:* isolated animals.

In isolated animals receiving the same dose of amphetamine, neither the N.A. nor the 5-HT showed significant fluctuations in the brain. The fall in the cardiac N.A. was of about the same magnitude as that seen in aggregated and isolated animals.

2) *Ambient Temperature.* Another evidence of a correlation between changes in brain monoamines and emotional behavior in

animals receiving amphetamine was provided by a study of the thermal parameter. As shown above, the emotional paroxysm occurred in aggregated animals only when the ambient temperature was maintained around 27°C. If the temperature was lowered, the phenomenon became less and less evident. Below 10°C, the emotional symptoms were practically absent and the mortality rate was nil. Figure 11 compares the changes in brain N.A. levels as related to the mortality rate under these experimental conditions. A fall in brain N.A. occurred only at 27°C, i.e., in conditions where aggregation provoked violent emotional stress and a high percentage of mortalities. No changes were observed at low ambient temperature, where aggregation did not produce emotional stress.

3) *Genetic Factors.* Our recent observations have shown that the peculiar amphetamine effect in aggregated mice may vary with the strain of animals. This finding suggested that the emotional, amphetamine-induced paroxysm may be controlled by a genetic factor. Among the various strains investigated—"Swiss" (outbred), C<sub>57</sub>Bl, C<sub>3</sub>H, CBA (inbred)—only the last-mentioned showed a behavioural difference. When treated with a dose of amphetamine that in the other strains of aggregated animals evoked

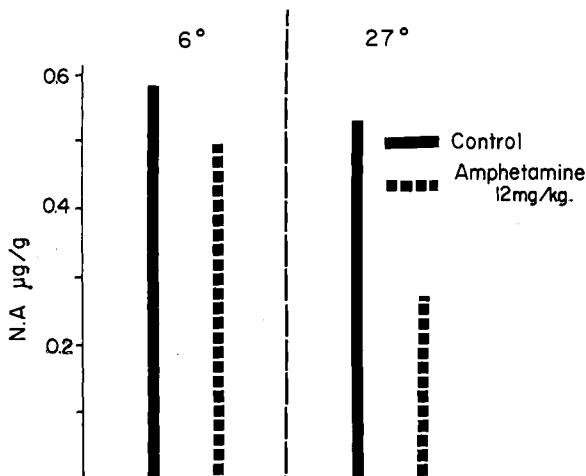


FIGURE 11. Modifications in brain catecholamines in aggregated mice receiving an identical dose of amphetamine (12 mg/kg), when the ambient temperature was kept at 6°C (left) or at 27°C (right).

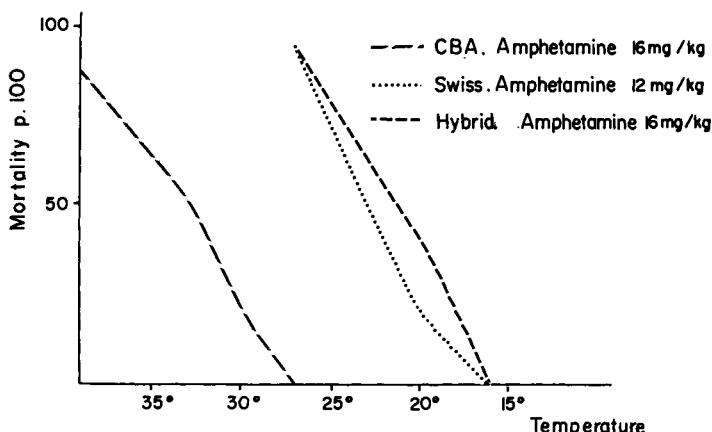


FIGURE 12. Mortality curves in aggregated mice receiving an equi-active dose (expressed in mortality rates) of amphetamine as a function of ambient temperature. *Right:* Swiss strain. *Left:* CBA strain. It is observed that the critical ambient temperature in the CBA strain is significantly higher than in the "Swiss" strain.  $F_1$  hybrids—(Swiss  $\times$  CBA) $F_1$ —behaved like the parental "Swiss" strain.

severe emotional stress in 100% of the cases and a mortality rate of 70%, the CBA mice did not display any of the characteristic symptoms and the mortality was nil. The first impression was that this strain was genetically refractory to the aggregation effect. However, when the phenomenon was further investigated, it evolved that G.T. did occur but at a different ambient temperature. As shown in Figure 12, the optimal ambient temperature at which the CBA mice displayed the usual emotional symptoms when treated with a moderate dose of amphetamine was 35°C instead of 27°C. What is likely to be controlled by the genetic factor is not the phenomenon on the whole, but the thermal optimal parameter. It is worthy of note that the (Swiss  $\times$  CBA) $F_1$  hybrids behaved like the "Swiss" parental strain. When the changes in the brain and heart N.A. were compared in the two strains of mice at the respective optimal temperature (Fig. 13), it was seen that at 27°C only the "Swiss" mice manifested a fall in brain and heart N.A. levels, while no changes were noted in the CBA mice at that temperature. But when the ambient tempera-

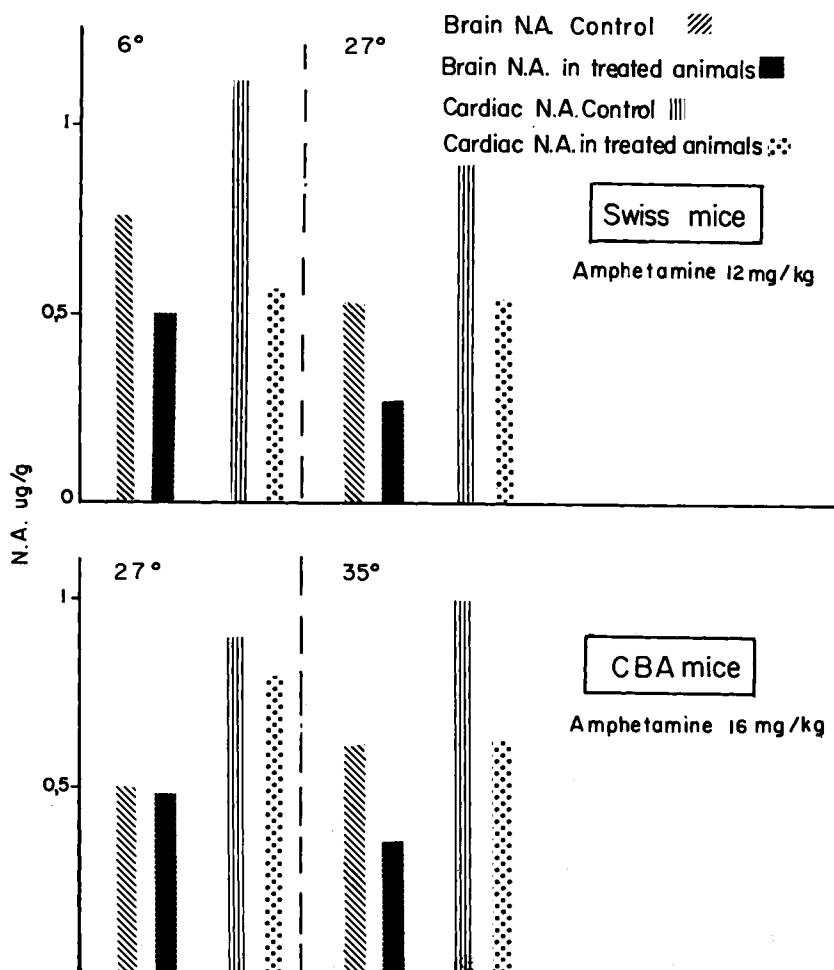


FIGURE 18. Changes in brain and cardiac N.A. in control and in amphetamine-treated aggregated mice of two genetically different strains at various ambient temperatures. *Upper:* Swiss mice. *Lower:* CBA mice. In this figure are presented data obtained at two extreme ambient temperatures; at one of these, no G.T. was observed, while at the other, the lethality due to aggregation was maximal. Note that the genetic factor controls the optimal critical temperature.

ture was raised to 35° C, the same reductions in the brain and heart N.A. levels were observed in the CBA-strain mice.

### Discussion

G.T. was induced by various psycho-stimulating substances, such as sympathomimetic agents, catecholamine precursors, or MAO inhibitors. Not all sympathomimetic agents displayed this property: amphetamine, DL-Dopa, DL-metatyrosine induced G.T. regularly, while ephedrine, tyramine, dopamine, adrenaline and noradrenaline were almost lacking in this property.

In the MAO-inhibitor group, the same unexpected discrepancies were noted. Among the hydrazine derivatives, pheniprazine and nialamide induced G.T., while iproniazid and hydrazine-2-octane were inactive. Tranylcypromine, a non-hydrazine MAO inhibitor, is one of the most potent in this respect. In contrast, Pargyline (N-benzyl-N-methyl-2-propynylamine hydrochloride, kindly supplied by Dr. Richards of Abbott Laboratories), another non-hydrazine MAO inhibitor (7) proved to be inactive in our hands in the acute assay.

Accordingly, it appeared to be impossible to link G.T. either with a definite chemical structure or with a specific pharmacological class. It seemed, nevertheless, that most of the substances able to induce G.T. possessed the property of inhibiting MAO. We shall come back to this problem later.

The disorders induced in aggregated mice by the active substances appeared to be an expression of exaggerated reactions to stimuli resulting from the presence of other animals. This abnormal behaviour was characterized by irritability, motor hyperexcitability, aggressiveness, rage, and fighting.

It is generally accepted that emotional stress is related to brain monoamine metabolism (2, 4, 5). In our investigations, the content in brain catecholamines regularly fell in animals treated with amphetamine and submitted to aggregation. It may be recalled that Moore (14) made similar observations. Moreover, Quinn and Brodie (15) found a fall in brain catecholamines in the emotional paroxysm induced by morphine in cats, while no change was observed in rabbits, where morphine has a sedative effect.

Extensive myocardial necrosis was the cause of death in G.T. (13). The myocardial lesions might be related to the cardiac strain resulting from the motor hyperactivity. However, it is doubtful that this is the only cause, since animals receiving mepyramine, which also pro-

duces hyperkinesia and convulsions, did not show cardiac lesions. Whether this phenomenon was related to the impairment of catecholamine metabolism and, especially, of those located in the heart, as evidenced in our experiments, is a matter of speculation. It was reported by Selye and Bajusz (16) and confirmed in this laboratory that injection of high doses of N.A. also produced myocardial necrosis, in mice. Whatever the cause may be, treatment with reserpine or chlorpromazine completely prevented the cardiac injury.

It seemed that the myocardium was a critical organ in this respect in mice. Halpern and Desnoyers (unpublished) observed that in aggregated rats the toxicity of amphetamine was not increased. Hyperactivity and nervous stimulation were induced by amphetamine, but not aggressiveness, rage and fighting. Myocardial necrosis did not occur in aggregated rats.

Severe hyperthermia reaching 40°C to 42°C occurred regularly in animals subjected to G.T. Here again, motor hyperactivity is certainly an important factor; but it should be recalled that hyperthermia has also been reported in humans who have taken large overdoses of MAO inhibitors (7), indicating a possible central effect.

The mechanism of the potentiation of the toxicity of sympathomimetic amines by various MAO inhibitors is under investigation. The pharmacological properties of these substances suggest a process in which synthesis of monoamines is involved. An argument in favour of such a concept is the observation made by combining a MAO inhibitor with ephedrine. Although ephedrine increased the mortality rate to some extent in aggregated mice, it was unable to elicit the characteristic behavioural paroxysm observed with amphetamine. However, if a MAO inhibitor was injected prior to ephedrine, then the behaviour of ephedrine-treated animals was indistinguishable from those treated with amphetamine.

It is tempting to attribute the differences observed with these two chemically closely related amines to the differences in their inhibitory action on MAO. Amphetamine was found to possess a strong MAO-inhibitory action "in vitro" (1). In our unpublished experiments with liver MAO, amphetamine was, on a molar basis, about 10 times as potent as iproniazid. In contrast, ephedrine was found to be inactive in the highest concentrations used. This suggests that, when combined with a MAO inhibitor, ephedrine acquired amphetamine-like properties.

Thyroxine increased, consistently but unequally, the toxicity of sympathomimetic amines. Thus, the toxicity of amphetamine was in-

creased 16 times, that of ephedrine 14 times, that of DL-Dopa 4 times, and that of N.A. 4 times, while the toxicity of tyramine was not significantly changed. The toxicity of MAO inhibitors was also enhanced by thyroid hormones, but our present data do not allow generalisation. It should be emphasized that potentiation of the toxicity of these substances was obtained in most of the experiments with a relatively moderate dose of thyroxine: 0.2 mg/kg per day, given during three days. This treatment did not apparently affect the general condition of the animals. It goes without saying that the phenomenon could be amplified and considerably aggravated by higher doses of thyroxine and/or more prolonged treatment.

Similar results have been observed in rats (Halpern and Desnoyers, unpublished). Thus, pretreatment with 1 mg/kg thyroxine for 10 days lowered the LD<sub>50</sub> of amphetamine in isolated rats from 70 mg to 3 mg. On the other hand, thyroidectomy or treatment with propyl-thiouracil raised the LD<sub>50</sub> of amphetamine to 130 mg/kg.

The potentiation of the toxicity of adrenergic amines by thyroid hormones, whether in isolated or aggregated animals, seems to be a general phenomenon.

Two additional points relevant to the action of thyroid hormones deserve discussion. Firstly, in G.T. induced by adrenergic amines, the ambient temperature is one of the critical factors (6, 8). Constant results with high mortality rates were only observed when the animals were maintained at 27°C. Lowering the ambient temperature below 20°C abolished the phenomenon. This feature is often ignored or neglected by many investigators and is responsible for conflicting results published in the literature.

It was found that the importance of the environmental temperature was markedly mitigated, not to say abolished, by thyroxine. G.T., with a similar mortality rate for the same dose of amphetamine as in the control group kept at 27°C, has been obtained in thyroxine-treated animals at 16°C. This observation once again raises the problem of the role of central hyperthermia in G.T., a problem that is now under investigation.

The second point concerns the abolition by thyroxine of the protective effect afforded by reserpine against G.T., while the protective action of chlorpromazine remained unaffected. This was one of the striking features in the action of thyroxine, which, in this respect, behaved similarly to MAO inhibitors.

Does this mean that the thyroid hormone acts by a similar mechanism? We have no consistent evidence in favour of such an interpretation. Administration of thyroxine did not affect the brain catechol-

amine content, while a considerable increase was observed when a MAO inhibitor was given under the same conditions. The mechanism by which thyroxine enhances the toxicity of certain adrenergic amines remains a matter of speculation. One can hesitate between two hypotheses: potentiation of the toxicity of a pharmacological agent by a hormone or an eventual increase in the cellular toxicity of the thyroid hormones by the pharmacological agent.

The last point concerns the correlation between the emotional paroxysm observed in aggregated animals receiving a low dose of amphetamine and the monoamine levels in the brain. Our data based on a great number of experiments indicated that the brain-N.A. stores regularly decreased in emotionally stressed animals. Under the same conditions, the 5-HT levels regularly increased. It is tempting to consider that there is a correlation between the brain monoamine changes and the emotional paroxysm. That these changes were not relevant to the action of amphetamine itself was quite clear from the data obtained in solitary animals receiving the same doses of amphetamine and also in animals maintained at a lower ambient temperature. It is worthwhile to stress the influence of genetic factors on the optimal temperature at which the emotional paroxysm was elicited. Here again, the fall of catecholamines was closely related to the emotional stress and independent of the ambient temperature. The question that arises is whether the changes in the brain monoamines were the cause or the consequence of the emotional stress. It is impossible, for obvious reasons, to answer this question directly, today. However, the absence of the emotional stress in animals whose monoamines were depleted by reserpine, and its aggravation in animals whose brain N.A. levels had been raised by pretreatment with MAO inhibitors, favour the first concept. But with regard to the respective role of N.A. and of 5-HT in this emotional expression, here again, the evidence was only conjectural.

The questions raised in this study are very complex. They are situated at a cross-road where major problems meet, such as the impact of the social environment on emotional stress, the correlation between brain monoamine metabolism and emotional behaviour, the synergistic action between thyroid hormones and adrenergic substances, stress and myocardial necrosis.

### **Abstract**

Group toxicity (G.T.), characterized by emotional stress and a high mortality rate in aggregated mice, has been found to be evoked by certain sympathomimetic amines, by catecholamine precursors, and certain MAO

inhibitors. Extensive myocardial necrosis was found to be the main cause of the sudden death.

The inhibition of this phenomenon by reserpine and chlorpromazine has been investigated. MAO inhibitors reversed the protective action of reserpine, but did not affect that of chlorpromazine. No relationship has been found between the ability of a substance to induce G.T. and to reverse the protective action of reserpine.

MAO inhibitors potentiated the absolute toxicity and the G.T. of sympathomimetic amines and of catecholamine precursors.

Thyroid hormones in moderate doses considerably aggravated the absolute toxicity and G.T. of adrenergic amines, of MAO inhibitors, and of catecholamine precursors. Moreover, thyroxine was found to abolish the protective action afforded by reserpine against G.T.

Brain and cardiac N.A. stores were consistently decreased in amphetamine-treated animals. Under the same conditions, the 5-HT levels were regularly raised. The changes in brain monoamines were observed only in aggregated animals and not in the solitary animals, while the changes in cardiac monoamines were independent of the social environment.

There is a body of evidence to suggest that the changes in the brain monoamines were related to emotional stress and were dependent of other ambient factors, although the respective role of N.A. and of 5-HT in this emotional expression was only conjectural.

### Abrégé

La toxicité de groupe (T.G.) est caractérisée par un paroxysme émotionnel et par une mortalité élevée chez les animaux mis en groupe dans certaines conditions expérimentales.

La propriété d'induire la T.G. a été trouvée avec certaines amines sympathomimétiques, avec des précurseurs de catécholamines et certains inhibiteurs de la MAO (IMAO).

La mort paraît être due à une nécrose extensive du myocarde. Les modalités de l'inhibition de ce phénomène par la réserpine et la chlorpromazine ont été étudiées. Les IMAO inversent l'action protectrice de la réserpine mais non celle de la chlorpromazine contre la T.G. Aucune relation n'a pu être établie entre l'aptitude d'une substance à induire la T.G. et celle à inverser l'action protectrice de la réserpine.

Les IMAO potentialisent la toxicité absolue et la T.G. des substances adrénergiques et des précurseurs des catécholamines. Les hormones thyroïdiennes administrées à des doses modérées aggravent considérablement la T.G. et la toxicité absolue des amines sympathomimétiques, des IMAO et des précurseurs de catécholamines.

La thyroxine lève la protection conférée par la réserpine contre la T.G. mais non pas celle de la chlorpromazine. Le paroxysme émotionnel provoqué par la T.G. s'accompagne d'une diminution des taux de N.A. cérébrale et

cardiaque et d'une élévation parallèle de la 5-HT cérébrale. Les modifications des monoamines cérébrales ne s'observent que chez les animaux agrégés et non pas chez les animaux isolés ayant reçu le même traitement. Les modifications de la N.A. cardiaque sont indépendantes de l'impact social.

Il y a des raisons d'admettre que les modifications des monoamines cérébrales sont en rapport avec le paroxysme émotionnel et sont indépendantes des autres facteurs environnants, bien que le rôle respectif de la N.A. et de la 5-HT dans l'expression émotionnelle demeure encore conjectural.

### Acknowledgements

The authors wish to express their sincere thanks to Dr. Angus E. Stuart (Dept. of Pathology, Edinburgh) and to Dr. B. B. Brodie (N.I.H., Bethesda) for their valuable criticism and help in the preparation of this manuscript. This study was supported by grant No. 61-FR-064 attributed by the Délégation Générale à la Recherche Scientifique et Technique.

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**F. C. MacIntosh:** I should like to thank all the speakers for their heroic efforts to distill their massive experimental work into 15 minutes apiece. I have been invited to discuss the paper by Dr. Halpern; but in view of the passage of time, I think I must confine my remarks to one observation and one question. The observation is that Dr. Halpern's fascinating studies underline a lesson that we have learned from Dr. Selye, namely that you can get quite unexpected and significant results when you push the dosage of familiar drugs up close to the toxic level. This is something that Dr. Selye has done repeatedly. The question I should like to ask Dr. Halpern, if he has time to answer at the end, is: what, if any, is the role of heat stroke in myocardial damage? If, as he has pointed out in his earlier papers, the temperature of his animals gets up to 40 or 42°C, which is pretty close to Dr. Rocha e Silva's threshold for irreversible tissue damage, is it possible that the heart is getting "cooked" by

general hyperpyrexia plus its own heat production, and is it possible that some degree of fever is also a prerequisite for the drug action—presumably a central adrenergic one—that underlies this extraordinary emotional behaviour?

**B. Halpern:** Dr. MacIntosh, in your question there are two different points. First, one has to consider this extraordinary, peculiar emotional behaviour that is developed by the animals receiving sympathomimetic amines or monoamine oxidase inhibitors and aggregated under adequate experimental conditions. This is a fundamental pharmacological effect, the mechanism of which is certainly of great interest and of even greater complexity.

The second point concerns the mechanism of death. Death of an animal requires a very great deal of physiological disorders. You were asking about the role of pyrexia. There is no doubt that these animals have their central temperature raised to a high level reaching 41-42°C and sometimes more. There are several causes of this hyperthermia: MAO inhibitors have been shown to produce hyperthermia by their central action; moreover, hyperthermia is also caused in these animals by the extraordinary muscular hyperactivity and agitation observed under these conditions. Therefore, one can suspect that the myocardial necrosis may result from this hyperthermic effect. Although this can be considered as a secondary problem, we have nevertheless tried to find out the possible role of hyperthermia as a cause of the cardiac death. You realize that it is difficult to reconstruct, with some isolated elements, such a complex situation, especially when the results with these isolated elements are negative. I have the feeling that hyperthermia is not the main factor. When hyperthermia is produced in *isolated* animals by hyperthermic drugs or by repeated electric stimulation of the cage, the animals do not show any evidence of myocardial necrosis. On the other hand, myocardial necrosis can be produced in the absence of hyperthermia by injecting large doses of catecholamines, namely noradrenaline, as has been shown by Selye and Bajusz and confirmed in our laboratory. Therefore, I would conclude that the myocardial necrosis that leads to the death of the animals under the conditions described, results from the interplay of several factors: physical strain, hyperthermia and impairment in the balance of the heart catecholamines regulating the coronary supply.

## On the Participation of Polypeptides and Biogenic Amines in the Acute Inflammatory Reactions

M. ROCHA E SILVA

FOURTEEN years ago we met in Montreal at Dr. Selye's home, to discuss the *Mechanism of Inflammatory Reactions* (38). I remember that the most heated discussions dealt with the question of the possible mediators of the inflammatory reactions. By that time, histamine was very much in disfavour and Menkin, who was present at the meeting, declared himself its Public Enemy No. 1; serotonin was increasing in importance, since it had been shown that this new and fashionable principle could be 200 times more potent than histamine in producing an increase in capillary permeability in the rat (73). We know now that the rat and the mouse are about the only species in which serotonin produces its effects on vascular permeability. On the same occasion (28), there was held in Montreal, I think on the same day, the First Symposium on Polypeptides Acting upon Plain Muscle, and bradykinin figured in it, already a highly promising representative of the group. At that time, we had only a vague feeling that bradykinin could be a factor in enhancing vascular (capillary) permeability, since the first incompletely purified preparations did enhance vascular permeability in guinea pigs and rats. We now know that bradykinin is about 200 times as active as histamine in increasing vascular permeability in rabbits and guinea pigs and much more active than histamine and serotonin in the other species studied, including man.

Before proceeding with this presentation, I would like to stress that what has been gained in the last 15 years of research in the field of vasoactive polypeptides is the conviction that we have now a possible operational scheme to deal with the problem of the chemical mediation of acute inflammatory reactions.

We knew, for instance, that proteolytic enzymes (proteases)

present in tissues and in blood plasma would play an important role in the release of mediators of the inflammatory reaction. However, histamine would fit badly, and serotonin still worse, into all attempts to schematize the inflammatory process on the basis of activation or participation of tissue proteases.

We now know that the polypeptides offer a better possibility, from an operational point of view, of introducing plasma and tissue proteases into the root of the inflammatory phenomena.

### **Histamine and Inflammatory Reactions**

In the experimental animal, there are many ways to produce the vascular manifestations that mimic the inflammatory phenomena, from a single increase of vascular permeability, which can be revealed locally by the seepage of a dye bound to plasma proteins, such as trypan blue, Evans blue, Geigy blue, etc., to the increase of volume of the rat's paw, after injection of dextran, ovoalbumin (ovo-mucoid), carrageenin or irritants like formol, or substances that may be mediators of the inflammatory reaction, such as serotonin and bradykinin. It is known that histamine, in spite of the fact that it produces a positive blueing test, is incapable of eliciting rat paw edema by subcutaneous injection. The antihistamines are able to modify the intensity of the dextran or ovoalbumin edema, but are ineffective in carrageenin or formol edema. In 1960, we described another type of edema produced by immersion of the hind paw of the rat in a water-bath at 45° C for 30 min (65). We knew that under these conditions histamine is not liberated or only in negligible amounts. For the release of amounts of histamine sufficient to explain at least part of the reaction, the temperature should be increased to 53-57° C. It should be noted that the mast cells are stabilized at 45° C for 30 min and even so potent a histamine liberator as compound 48/80 will liberate only small amounts of histamine. Moreover, potent antihistaminics like Antergan and Benadryl had no effect on the "thermic edema" produced by heating the rat's paw at 45° C for 30 min. By a special perfusion method, which we have called "coaxial perfusion," it was possible to demonstrate the liberation of a substance that could not be distinguished from bradykinin (65).

The fact that histamine cannot explain all the vascular reactions that take place at the site of inflammation does not exclude its participation in the genesis of these phenomena. There is a considerable volume of evidence to show that histamine is liberated under many different conditions: by heating, by the action of bacterial toxins or animal venoms, in anaphylactic and peptone shock, and others.

On the other hand, antihistamines are not equally effective upon all histamine actions. It is true that, in certain circumstances, the ineffectiveness of antihistamines may prove that histamine is not the mediator and that a completely different mechanism has to be considered to explain some physio-pathological phenomena. This is so, for instance, with the mediation of the vasodilation by antidromic stimulus of sensitive nerves, or when histamine has been considered in order to explain functional hyperemia after intensive muscular work or prolonged anoxia in a section of the cardiovascular system. In such cases, it has been convincingly demonstrated that the phenomenon is produced even when the organism does not respond to the vascular action of injected histamine.

### **Intrinsic Histamine**

We do not agree with Schayer (75-77) when he wants to revive the idea of "intrinsic histamine," using the name of "induced histamine" or "neoformed histamine" in order to explain the microcirculatory phenomena of inflammation, by local production of undetectable amounts of histamine completely resistant to large doses of antihistamines. This would confer fantastic pharmacological properties on histamine to explain such important phenomena as the delayed inflammatory reactions. I am of the opinion that the mechanism of action of this neoformed histamine does not fit into the usually accepted scheme for the active substances of the tissues.

### **Insufficiency of the Histamine Mediation**

When we began our studies on the mediators of the inflammatory response (4, 5, 67, 68), histamine was the only substance among these found in the organism whose capillary-permeability-increasing effect had been demonstrated beyond doubt. The effect of

Menkin's "leukotaxine" (50, 51), presented as a new possibility at that time, could be explained, as with other endogenous or exogenous products, by a histamine liberation in the rabbit's skin, in the well-known trypan blue test. Leukotaxine continues to be an interesting possibility, but its chemical identity has never been unequivocally established.

We know also that a number of proteolytic enzymes, like trypsin, papain, those present in snake venoms, may produce a positive trypan blue test. There were also indications that histamine-liberating substances could give a positive test and it was thought that this could be due to a local liberation of the histamine present in the skin of the rabbit (68) or the guinea pig (53).

There were some unexpected findings, like that of the action of *Bothrops jararaca* venom producing a very strong increase of capillary permeability and liberation of only small quantities of histamine, as well as the effect described by myself (60) that kallikrein produced a substantial increase of capillary permeability in the trypan blue test but did not liberate histamine by perfusion of the isolated guinea-pig lung.

So, in the beginning of the 1940's, one could say that while histamine was the only substance obtained in pure form able to produce a marked increase in capillary permeability, the discovery of other mediators that could also play a role in the characteristic vascular reaction of the first phase of inflammation was highly desirable. Menkin's leukotaxine could not satisfy all biochemical and pharmacological requirements for the identification of a new principle, owing to its hypothetic character and also because of the difficulty of its identification by unspecific biological tests.

On the other hand, after the appearance of potent antihistaminics through the fundamental work of French pharmacologists and chemists (Bovet, Staub, Halpern and others) it became evident that histamine could explain only a small part of the inflammatory reactions. Even as regards the anaphylactic and allergic phenomena, the use of antihistaminics has shown the limitations of the histamine theory. I mention here the volume on "Histamine" published recently by Springer-Verlag, as volume XVIII of *Heffter's Handbuch der experimentellen Pharmakologie* (64).

At the end of the decade 1940-1950, two events took place that

were of great consequence in the explanation of the mechanism of the inflammatory vascular reactions. One was the discovery of serotonin by Page's group in Cleveland and its identification with 5-hydroxytryptamine (58). The other event, which I will discuss more thoroughly, was the discovery of bradykinin in our laboratory at the Instituto Biológico in 1948-49 (66).

### **Participation of 5-Hydroxytryptamine (Serotonin)**

As regards serotonin, I do not believe that it can have a universal significance to explain the vascular phenomena of inflammation. We know now that serotonin is only active in rodents (rats and mice) and that it has practically no effect on the capillary permeability of rabbits and guinea pigs. Serotonin can be of importance, though, as a mediator of other manifestations of the inflammatory reaction, as for instance pain, and eventually as a histamine liberator (3, 22). It should be stressed that the participation of serotonin in the pathogenesis of certain types of edema, like the one produced in the rat by ovoalbumin, was suggested in view of the inhibition displayed by substances such as Dibenamine and Dibenzyline (73), which are not specific antagonists of serotonin. These substances also decrease the edema produced by heating the rat's paw at 45° C for 30 min, a phenomenon that has been attributed to the local formation of bradykinin (65). Recent experiments have shown that Dibenamine and Dibenzyline also inhibit the bradykinin effects on the isolated guinea pig ileum (70). The most specific antiserotonins, such as LSD 25 and BOL 148, have no effect on the intensity of the edema produced by heating the rat's paw at 45° C (thermic edema).

### **The Kinin System**

As regards the polypeptides of the bradykinin group, the possibilities are much more evident. Together with bradykinin, a whole system was discovered, which can be activated in conditions leading to inflammation. Expressed in cybernetic terms, very likely to please my friend Selye, one can say that the bradykinin system is the richest from an operational standpoint to explain many details of the inflammatory reactions. It should also be noted that

this system has the advantage of *being complicated* and one knows that inflammatory reactions are very complicated.

There are in addition other relationships of the kinins to the inflammatory reactions. At the root of the phenomena leading to inflammation, there are proteolytic reactions that should be activated and we all agree that the inflammatory reactions take place as a consequence of activation of enzymatic systems where proteases play an important role.

In Figure 1, it can be seen that there are many common points between the mechanism of bradykinin liberation and those factors that may release an inflammatory reaction. The whole system may be put into motion by an activation of an enzyme system present in plasma and this activation may take place by simple contact of plasma with glass. It has been demonstrated that treatment of plasma or serum with glass beads (ballotini) can liberate up to one third to one half of the total bradykininogen present in normal plasma (15, 39, 48). At some points, activation of the kinin system comes into gear with the coagulation phenomena and it has been accepted that activation of bradykinin in plasma follows activation of the Hageman factor, which also plays a role in certain phases of blood coagulation (39, 46, 47).

The enzymatic system in plasma can also be activated by heating in acid medium (pH 2.0). This is indeed a rather drastic treatment involving the addition of HCl to plasma and maintain-

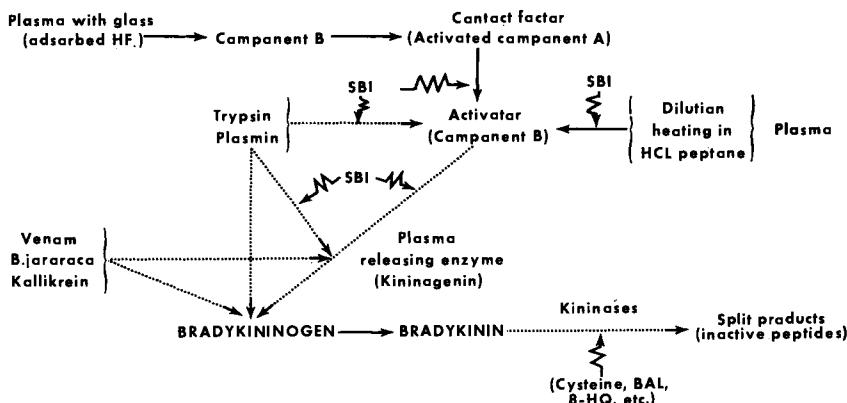


FIGURE 1

ing it in a boiling water bath for a few minutes. We know that, by this treatment, activation of the endogenous enzymatic system takes place, since the pH must return to 7.4 after the heat treatment, to allow the spontaneous release of bradykinin. Furthermore, addition of the soya bean trypsin inhibitor (SBTI, or SBI) stops this process of release, indicating that a protease in plasma is the active agent for the kinin release (69).

Following a similar procedure, Elliott *et al.* (18) were able to isolate methionyl-lysyl-l-bradykinin, which indicates that after heating in acid medium there occurs activation of an enzyme displaying the ability to split a peptide bond "substrate-methionine." Similarly, we could assume that the kininogenins able to release lysyl-bradykinin (kallidin) probably specifically break the methionyl-lysyl bond. Experiments performed in this laboratory have shown that kallikreins (pancreas-kininogenins) are able to split the synthetic substrate benzoyl-methionine-methylester (BMME), besides BAME, which is the specific substrate for trypsin; and that is also split by all enzymes that have been found to release bradykinin (32-34, 56).

It is easy to see in the diagram of Figure 1 that all pathways leading to the release of bradykinin from bradykininogen can be blocked by SBI, which indicates that we are dealing with protease activities. Among the enzymes present in plasma and which can be related to the release of bradykinin, we must bear in mind plasmin, which has been shown to release bradykinin directly (35) or through the intermediary of the plasma kininogenin (82). We know very little on the nature of the latter besides the fact that it can be activated by contact with glass, by acetone or by the drastic treatment of heating at pH 2.0 as mentioned before. It might correspond to what the Germans have called plasma kallikrein, plasma *kininogenase* by others (52, 56). We prefer the more descriptive name of *kininogenin*.

### The Probable Mediators of the Acute Inflammatory Reactions

Coming back to the question of the mediator of the inflammatory reaction, we might say that at this moment there exist at least three possible mediators of the vascular reactions occurring in the inflammatory phenomenon: histamine, bradykinin and serotonin.

TABLE I

RELATIVE POTENCIES OF POSSIBLE ENDOGENOUS MEDIATORS,  
AS WELL AS OF THEIR RELEASERS, TO INCREASE VASCULAR  
PERMEABILITY (7, 17, 27, 81, 83)

<i>Factors of Permeability</i>	<i>Guinea Pig</i>	<i>Rat</i>	<i>Rabbit</i>
Bradykinin . . . . .	2,000,000 to 10,000,000	10,000 to 100,000	700,000
Histamine . . . . .	32,200	1,400	37,000
5-HT . . . . .	60	16,200	—
Histamine liberators:			
Compound 48/80 . . . . .	3,500	6,800	30
Polymyxin B . . . . .	1,120	14,900	0
Kininogenins:			
Kallikreins . . . . .	240	70	1,130
Trypsin . . . . .	2,560	900	600
Guinea pig DPF . . . . .	38,700	1,100	130
Leukotaxine . . . . .	320	120	80

Table I shows that bradykinin and its releasers (kininogenins) are far the most potent agents in increasing vascular (capillary) permeability, followed by histamine and by serotonin. As indicated before, serotonin could explain the inflammatory vascular reactions only in rats and mice, since it is completely inactive in guinea pigs and rabbits. It is also possible that serotonin acts through a release of histamine from rat and human skin. Serotonin might play a role as a mediator of the pain sensation, since it has been shown by Sicuteri *et al.* (79) that the association of both serotonin and bradykinin can reinforce each other in the production of pain, when injected intravenously in man.

Consequently, as regards the vascular phenomena of inflammation, bradykinin and histamine can be considered the main candidates for the chemical mediation in all species of animals and in man. We have already discussed the advantages offered by polypeptides from an operational point of view. However, the final proof will probably depend upon the obtention of specific anti-bradykinin agents.

#### Experimental Proof of the Participation of Kinins in the Inflammatory Reaction

It would be impossible to discuss here all the evidence that has been presented by Lewis, Collier, Schachter, Wilhelm and others

in favor of the participation of the kinin-forming systems in the inflammatory phenomena. The bibliographic listing at the end of this paper supplies the pertinent references. What I would like to present here is rather a summary of the present possibilities of a rational attack on the problem.

The most convincing method would, of course, be the demonstration of an increase in the content of free bradykinin in the blood in individuals subjected to an inflammatory process. But, as for histamine, it is very difficult to demonstrate such an increase, owing to the high diffusibility of the polypeptides, and also to the fact that bradykinin is very rapidly destroyed in the circulating blood. These two obstacles could, of course, be partially overcome, and bradykinin has been recovered from circulating blood after an intravenous infusion (21, 37). It has even been possible to detect increased levels of bradykinin in conditions of anaphylactic shock (41), after injection of adrenaline (14), or under conditions of shock produced by proteolytic enzymes, such as by trypsin, Nagarse and also by kallikrein (13, 59).

### **Release of Bradykinin in the Interstitial Spaces**

To explain the negative results in experiments of this type we have to consider that bradykinin could be released outside of the vessels and, therefore, it would be necessary to find means to demonstrate its presence in the interstitial space. This has been done successfully in the case of thermic edema, where free bradykinin has been obtained by the coaxial perfusion method described elsewhere (61, 62, 65). Another direct method has been used by Edery and Lewis (16), who demonstrated the appearance of kinin-forming enzymes in the effluent lymph from the dog's leg subjected to several conditions, of which heating at 80° C during 15 sec was the most severe. They also observed an increase in the bradykinin-forming enzyme level after arterial injection of histamine; see also Lewis (42). Mention must also be made of the results published by myself and Rosenthal (72) on the presence of bradykinin and histamine in Selye's air pouch (78) on the rat's back, after heating to 96° C for 15 sec.

From these experiments, one can deduce a mechanism operating in a vicious circle where bradykinin formed in the interstitial

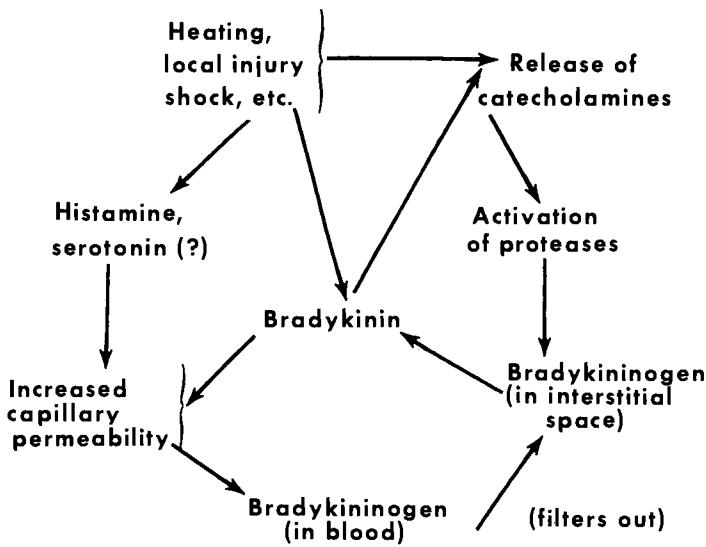


FIGURE 2

space could increase vascular permeability or determine an augmented filtration of plasma bradykininogen, which in turn would increase the local process of kinin activation, according to the scheme presented in Figure 2. It is evident that substances like histamine or serotonin (in the rat and mouse) may participate in this cycle, increasing the bradykininogen content at the site of the lesions.

### Indirect Methods

Besides these direct and more convincing methods, we must consider other indirect possibilities of demonstrating the participation of kinins in the inflammatory reactions. These possibilities will be described as follows:

a) By utilization of *enzyme inhibitors* to inhibit bradykinin release. This procedure has been shown to be very useful *in vitro* because SBTI strongly inhibits the action of endogenous kininogens. Unfortunately, SBTI does not act on pancreatic and urinary kallikrein and is only very slightly active on plasmin itself. The *in vivo* use of an anti-kallikrein substance, Trasylol, has been shown to be of limited application in hemorrhagic pancreatitis

(26, 40, 74); it has also been used in cases of burns (11). However, Trasylol cannot be considered a universal inhibitor of proteases that might participate in the process of bradykinin formation and actually, in the case of release by trypsin, it has very little effect. More recently, Van Arman and his co-workers (2) demonstrated the effect of SBTI in reducing the local edema produced by carrageenin in the rat's paw.

b) It has been verified that proteolytic enzymes, which might have a bradykininolytic effect, increase the resistance of the organism to the inflammatory reactions. This is the case, for instance, of chymotrypsin, papain, and of a protease from *Aspergillus orizae*. It is interesting that the effect of these enzymes can be seen even when they are given orally (49) and it has been shown that bradykinin itself and fibrinolysin can increase the threshold of bradykinin action in the trypan blue test or in the formation of edema (12, 36). This phenomenon is probably due either to a tachyphylactic effect of bradykinin, such as has been observed in its action on the guinea pig bronchioles and pain production (39), or to a general resistance of the organism by secretion of anti-protease agents, which slows down the activation of the kinin forming system. In the case of chymotrypsin, we must also bear in mind the possibility of destruction of the formed bradykinin by this protease. The same reasoning could be applied to other enzymes from microbes and from plants, which may rapidly destroy free bradykinin.

c) By the use of inhibitors of kininases, it is possible to protect the released bradykinin. It is known that bradykinin is destroyed by enzymes displaying the specificity of chymotrypsin or by carboxypeptidases, of which carboxypeptidase-B might be the plasma enzyme responsible for bradykinin inactivation (19). There are now a number of substances that protect bradykinin and potentiate its action on biological structures by inhibiting the activity of the destroying enzymes. The most potent of these substances of known structure is BAL (20, 24). Other compounds that protect SH-groups, such as cysteine, thioglycollic acid, or those that bind heavy metals, like 8-hydroxyquinoline (8-HQ) and Versene (EDTA) are also able to protect bradykinin from the enzymatic actions of plasma kininase. Most of these compounds, such as

BAL, thioglycollic acid and, to a certain degree, cysteine, potentiate the effects of bradykinin *in vitro* and *in vivo* (19, 20, 24, 25, 55). 8-HQ, however, protects bradykinin from destruction by plasma enzymes, but has no potentiating action as regards biological structures sensitive to bradykinin. For this reason, 8-HQ can be used to protect bradykinin in the different preparations or biological extracts. Using this substance, Carvalho and Diniz (6) were able to demonstrate the formation of bradykinin when rupturing the lysosomes of the rat kidney, by the presence of kininogenins in these sub-cellular structures. We have to mention also the "Bradykinin-potentiation factor" (BPF) extracted from the venom of *Bothrops jararaca* (23-25). It is interesting from the biological point of view to note that the venom of *B. jararaca*, which contains such a potent bradykinin releasing protease, also contains a very potent factor that increases its effect upon almost all biological preparations, not only *in vitro* but also *in vivo*. After the injection of some milligrams of the factor into the dog and the cat, sub-threshold doses of bradykinin become enormously active and the effects of higher doses are potentiated in such a manner as to last many minutes or even half an hour. In the dog, the potentiation is around 20-40 fold the initial activity. After BPF the effect of bradykinin on the arterial pressure of the dog, compared with eledoisin, which is normally much more active than bradykinin, becomes equipotent (25). The effect of bradykinin upon capillary permeability is also increased after treatment with BPF (23). Thus, this factor is a very interesting tool to explore the participation of bradykinin in normal and pathological phenomena (and these are currently being investigated in our laboratory), in active hyperemia, in hemorrhagic pancreatitis, in anaphylactic shock, etc. (1, 23).

d) Finally, one can think of inhibiting bradykinin effects by anti-bradykinin agents. I would like to begin with the important studies of Collier and his co-workers (7-10) in England, who have demonstrated the antagonistic effects of antipyretics of the salicylate group, of Amidopyrine and phenylbutazone against guinea pig bronchospasm produced by intravenous injection of bradykinin (7). In spite of the fact that these antipyretics do not antagonize the capillary-permeability increasing activity of brady-

kinin or the thermic edema (65), there seems to be a correlation between the inhibiting effect upon the bronchioli and the analgesic activity of those compounds. It has been further demonstrated that the non-narcotic analgesics, such as Aspirin, salicylate, phenylbutazone and others, effectively inhibit the visceral pain elicited by injections of bradykinin into the splenic artery of the dog (31, 43-45). This correlation between the analgesic action and anti-bradykinin property of the non-narcotic analgesics could be of significance in the understanding of the anti-inflammatory mechanism through which they act. It has been found, for instance, in experiments on local inflammation produced in the rat's paw, that acetylsalicylic acid, while alleviating the hyperesthesia of the inflamed area, had little effect on the intensity of the edema, as measured by the increase in volume of the paw (29).

### **Research on Anti-bradykinin Agents**

The problem now is to find specific antagonists of bradykinin in the same way as the antihistaminics are antagonists of histamine, atropine is of acetylcholine, and the lysergic acid derivatives are antagonists of serotonin. There are strong indications to lead us to believe that in the near future the anti-bradykinins will probably be found. We already have compounds that display some antagonistic actions towards bradykinin and which have shown to be partially effective in reducing the intensity of the thermic edema ( $45^{\circ}\text{ C}$ ) produced in the rat's paw by heating at  $45^{\circ}\text{ C}$  for 30 min (65).

This edema is interesting, because at that temperature histamine is not released. The mast cells are even stabilized and a potent releaser, like 48/80, is no longer able to release any more than minute amounts of histamine. High doses of antihistaminics or anti-serotonins have no action upon this type of edema (65). Through the method of coaxial perfusion, it has been possible to demonstrate the formation of bradykinin *in loco*. One can, therefore, admit that this type of edema is produced by the endogenous liberation of bradykinin to the exclusion of histamine and serotonin.

Among the substances whose action have been assayed on this edema, only a few were able to reduce its intensity. These sub-

TABLE II

CORRELATION BETWEEN ANTAGONISTIC ACTION OF DRUGS UPON  
MEDIATORS AND THEIR INHIBITORY EFFECT ON "THERMIC EDEMA"  
(44-45° C) (30, 54, 57, 65, 70, 80)

<i>Drugs</i>	<i>Antagonistic Action upon</i> <i>Hista-</i> <i>mine</i>	<i>Sero-</i> <i>tonin</i>	<i>Acetyl-</i> <i>choline</i>	<i>Adrenal.</i>	<i>Bradyk.</i>	<i>Inhibitory</i> <i>Effect on</i> <i>"Thermic</i> <i>Edema"</i>
Atropine .....	+	+	++++	-	-	-
Mepyramine ...	+++	+	±	-	-	-
Benadryl ....	++	+	+	±	-	-
Phenergan ....	+++	+	++++	++	+	+
Chlorpromazine +	+	+	+++	+++	++	++
Cyproheptadine +++	+++	++	+	+++	+++	+++
Imipramine ...	++	+	++*	++	++	++
Dibenamine ...	++	++	++	++	++	++
Dibenzyline ...	+++	++	+	++++	+++	+++
LSD 25 .....	+	+++	+	-	-	-
Reserpine .....				deplet.		+++
Cocaine .....				potent.	-	-
Salicylates .....						-
Phenylbutazone						-
Amidopyrine ..						-

\* Only in high doses.

stances are listed in Table II, where a correlation between the anti-bradykinin or bradykininolytic effects upon the guinea pig ileum and the antagonistic action against thermic edema can be seen (70, 71).

It is evident that we are far from possessing a specific antib Bradykinin, because all the compounds tested display more or less pronounced antihistamine or anti-serotonin properties. But, according to Table II, their eventual antihistaminic or anti-serotonin or anticholinergic actions do not seem to interfere with the effect upon thermic edema, because neither the potent antihistaminics, such as Neo-antergan or Benadryl, nor the anti-serotonins, such as LSD 25 or BOL 148, nor atropine or substances with marked atropinic action have a definite effect upon the development of thermic edema. What should be stressed is the remarkable correlation between the anti-bradykinin action and the ability to reduce the edema of the paw seen with some of these compounds.

I would like to repeat here what I said during a Conference of

the New York Academy of Sciences: "It seems useless to try to find more potent antihistaminics or anti-serotonin agents on the assumption that by increasing their potency we might at last counteract the effect of released histamine or serotonin. The ones we have now are potent enough to inhibit histamine or serotonin within a large margin of safety" (63). On the other hand, the anti-inflammatory actions, as can be seen in Table II, do not parallel the antihistamine or anti-serotonin effects. In my opinion, future research should be directed towards anti-kinins and we do hope to find very potent ones in the near future.

### Abstract

Observations that have been accumulating during the past 15 years in the field of research on vasoactive polypeptides have led us to the conviction that an operational scheme can provide a basis for further investigating the problem of the chemical mediation of acute inflammatory reactions. The major components of this scheme are histamine and its analogues, serotonin, and the polypeptides of the bradykinin group. It is also recognized that proteolytic enzymes present in tissues and in blood plasma play an important role in releasing mediators of inflammatory reactions. The present paper discusses the experimental data that support these views.

### Abrégé

Les observations accumulées depuis 15 ans dans le domaine de la recherche sur les polypeptides vasoactifs nous ont mené à la conviction que l'on dispose maintenant d'un schéma opérationnel très utile pour étudier le rôle des médiateurs chimiques de l'inflammation aiguë. L'histamine, sous ses diverses formes, la sérotonine et les polypeptides du groupe de la bradykinine en sont les principales composantes. Nous savons, en outre, que du point de vue opérationnel, les polypeptides offrent une possibilité très naturelle pour introduire les protéases plasmatiques et tissulaires dans la racine même des phénomènes inflammatoires. Ces divers aspects sont discutés dans notre exposé à la lumière de plusieurs observations expérimentales.

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**R. Hoene:** Having the honour of discussing Dr. Rocha e Silva's paper "On the Participation of Polypeptides and Biogenic Amines in the Acute Inflammatory Reactions," I would like to commend the comprehensiveness of the presentation and the painstaking experimental work. Today, we certainly are permitted to have a "clinical bradykinin concept." We may speculate that bradykinin-like substances are active mediators of *acute* inflammatory reactions in man and that, perhaps, the bradykinin system represents the terminal pathway for a variety of "delayed" or *chronic* forms of inflammation.

The high hopes of the Pharmacologist in the development of anti-bradykinin substances may become a reality and we are justified in expecting clinical benefits from such substances in situations where tissues are reacting by inflammation to allergic, thermal, chemical, irradiation, and a variety of other forms of injuries. We Clinicians hope that Pharmacology will offer us the "Anti-Bradykinins" in as specific a form and as free of side effects as possible. Yet, we may be heading for a disappointment similar to our unfulfilled expectations of years ago, when antihistamines were thought to be doing much more than they were, later on, actually shown to be capable of. If the clinical potentialities of anti-bradykinins should prove to be less than expected, no blame should rest on Pharmacologists like Dr. Rocha e Silva, in whose presentation today any pretentious claims are absent. We will remain grateful to him for having unravelled important segments in the pathway of inflammatory reactions. We know that such reactions are complicated, and that, in specific situations, certain pathways will be followed to the exclusion of others. We may find that under certain conditions, as with histamine, bradykinin may occur only incidentally and that then, of course, anti-bradykinins may be ineffective in changing the course of inflammatory reactions.

There are certain intriguing aspects in the possibility of depleting

tissues of their content of bradykinin activators or precursors. Perhaps such a condition was partially responsible for what 15 years ago, at Dr. Selye's Institute, we described as "Topical Tissue Unresponsiveness." A few of you may recall that when tissues are irritated, they lose inflammatory reactivity to a subsequent irritating stimulus. In that way, a thermally pre-irritated rat paw can be shown to resist even a necrotizing thermal injury later on. The same can be demonstrated on the rabbit ear. Pre-irritation of a rat paw by dextran or egg-white protects and cross-protects against the subsequent inflammatory reaction due to egg-white or dextran, but not due to heat. Pre-irritation by heat failed to protect against a subsequent dextran edema or against the inflammatory reaction due to injected kaolin. Apparently, every irritant has its own specific pathways by which it induces the reaction pattern that eventually develops. With different irritants, the pathways may be similar or part of them may be common to a group of irritants. If, for instance, by way of a "Topical Tissue Unresponsiveness" one stimulus induces a cross-protection against other subsequent irritants, we may assume that the first stimulus acted through pathways shared by the subsequent irritant. Failure of an irritant to protect against inflammatory reaction to a subsequent different irritant would indicate that the reaction pathways of the two different irritants were non-identical. We thought, at that time, that the results of cross-protection experiments would foreshadow some difficulties for a concept that tries to relegate inflammatory mechanisms to the effects of only a few common chemical agents.

Pharmacologists, fortunately, will be kept restless and intrigued by "Inflammatology" for years to come. As is evident, our investigations originated with, and centered around, mediators of acute inflammation as they presented themselves: histamine, serotonin, and the bradykinin substances. Perhaps a more technologically oriented research would have started out to investigate the effects of anything capable of changing the physico-chemical condition of the cell structure; and here we should remember that the cell surface not only contains protein that may provide precursors of histamine or bradykinin, but also fat and carbohydrate matter that may be changed directly by corresponding enzymes or other influences. For example, in the instance of a bee sting or cobra bite, lecithinase will split the lecithin of the cell surface, thereby changing the cell structure, and setting free lysolecithin, a very necrotizing substance. Perhaps bacterial enzymes could act likewise. I may mention in this connection the chemical similarity between pneumococcal polysaccharides and

the ground substance, a situation that could offer pneumococcal enzymes all sorts of possibilities in our joints and tissue spaces. Following this train of thought, there is the possibility that intravenously injected dextran or egg-white, because of chemical similarity to ground substance structures in the paws or the ears of the rat, may be split by enzymes found especially in these localities, inflammation resulting directly. All this seems to indicate that we may not have to invoke the bradykinin-histamine systems as essential pathways of inflammation, although certainly histamine and bradykinins may originate in all sorts of inflammatory reactions. In this connection, and as an opening question to Dr. Rocha e Silva, I wonder how we should explain the effect of reserpine in inhibiting the thermal edema in the rat, in view of the absence of anti-bradykinin activity of this substance. Also, I wonder whether the decrease in bradykininogen found under certain experimental conditions can be shown to be a "limiting factor" in inflammation.

**M. Rocha e Silva:** I have to say that Dr. Hoene picked the two best questions that could be raised in this presentation. The first is the effect of reserpine. That is very difficult to explain, though reserpine was very active in reducing this thermic edema and there is always the possibility of explaining this by a reduction of the amount of catecholamines present in the tissue; but we have no direct evidence for that. Anyway, it seems that the catecholamines might come into the picture through a vicious circle as I showed in Figure 2 of my presentation.

For the second question, if bradykininogen has been estimated in an inflammatory reaction; I would like to mention the shocks produced by many enzymes, such as trypsin, Nagarse, Padutin, as shown by Corrado *et al.* (*Biochem. Pharmacol.*, 15:959, 1966). In such cases, the bradykininogen in plasma can go to zero, starting from normal levels. We were able to see the effect of an enzyme producing very strong shock: the bradykininogen does not fall very much, but if the amount given is enough to produce death, the bradykininogen content also goes to zero. Also, kallikrein, from urine or pancreas (Padutin), produces shock and a very low level of bradykininogen. With pronase, another enzyme that also produces shock, the plasma bradykininogen goes to zero when the animal is going to die.

Well, according to many authors, these are models of inflammation, like the shock produced by enzymes and many other irritant substances. Another situation in which inflammation is produced, as I have shown, is heating to 42.44°C, where the bradykininogen content is also decreased in the circulation.

## **Pharmacology of Aggression\***

H. LABORIT

THE words used in the title first require definition. We understand, by the term "aggression," the action of mechanical, thermal, chemical or radiation energy capable of causing typical disturbances in a living organism by producing abnormal physiological and biological values. We shall consider today only acute aggressions, i.e., of great intensity but short duration, leaving aside chronic aggressions of lesser intensity but of longer duration. As far as the term "pharmacology" is concerned, we may define it as the experimental study of the activity induced by the introduction into a healthy organism of substances that appear in fluids and tissues of another organism subjected to aggression: for instance, the pharmacology of catecholamines, of kinins. We have preferred to accept the term "pharmacology" in its therapeutic sense and we shall, therefore, consider the therapeutic pharmacology of post-aggressive disturbances, primarily. Now, the acceptance of this term permits us to conceive a pharmacological action as an inhibition of the characteristic symptoms of post-aggressive disorders: vasopressor therapy against hypotension, buffering substances to control, for instance, acidosis, i.e., a symptomatic pharmacology. We believe that it is not too ambitious, today, to envisage a pathogenic therapeutic pharmacology aimed at controlling and correcting mechanisms triggered off by aggression. But it must then be understood that the problem is primarily a study of the principal mechanisms activated by the aggression. This aggression generally causes two different although closely related types of phenomena. On the one hand, its local action will cause complex and limited tissue disorders seen at the very site of its application, but which may secondarily have a general repercussion. We shall call this *lesion phenomena*. On the

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\* This paper was presented in French.

other hand, the aggressed organism as a whole will trigger off a no less complex system of reactions that will influence physiological balance in its totality. We shall call this *reaction phenomena*. Moreover, the part played by each of them in the over-all behavior of an organism subjected to aggression varies according to the type of aggression and the previous condition of the organism. The elements that form the total picture are sufficiently coherent to permit, right from the start, a schematization that constitutes a basis on which a pharmacological study can be initiated.

## I. Reaction Phenomena

### *Catecholamine Liberation*

We shall not define the well-established concept of the liberation of catecholamines following an aggression, again. Its importance was recognized only at the time when Weil-Malherbe and Bone defined the precise methods of quantitative determination in biological fluids (93). Since then, the number of studies demonstrating the increase in the epinephrine level of peripheral blood following the most diverse aggressions has become too large to be listed. Epinephrine and norepinephrine, therefore, play a very important role in aggression (20).

It must be kept in mind that the mechanism of epinephrine liberation calls upon the ascending spinal pathways and originates either in the aggressed areas or at the level of the baro- or chemoreceptors. We must also remember the part played by the reticular formation of the brain-stem and by the hypothalamus. Renal participation, by means of the angiotensin system, in the vasomotor response to aggressions seems, in itself—if we accept the most recent studies (33, 77)—to result from the liberation of catecholamines from their granules.

The general response of the organism to aggressions appears in fact to be dominated by the activation of the adrenal-sympathetic system and the liberation of catecholamines.

### *The Consequence of Catecholamine Liberation*

According to the level of the phenomena observed, vasomotor repercussions and others more metabolic in appearance can be

distinguished in particular, since a vasomotor phenomenon is also a metabolic phenomenon.

#### *A) Vasomotor Disturbances*

Vasomotor disturbances are predominant at the level of abdominal viscera.

The concept has now been fully recognized that, after any type of aggression, the mesenteric circulation, the intestinal circulation, and that of the hepatic portal system, and of the spleen, pancreas and kidneys, all are subjected to considerable disturbance. In collaboration with Leterrier, using a technique that was elaborated in our laboratory (57), we measured the hepatic and renal output during a hemorrhagic aggression in a rabbit, directly. We also observed the mesenteric circulation, under the same circumstances, by means of direct microscopy (58, 60), as had previously been done by Zweifach (96) on the mesoappendix of the rat. Figure 1 shows that supra-hepatic and renal output collapses right at the start of a hemorrhage produced with the Wiggers' technique, which maintains arterial pressure at 25 mm Hg. After

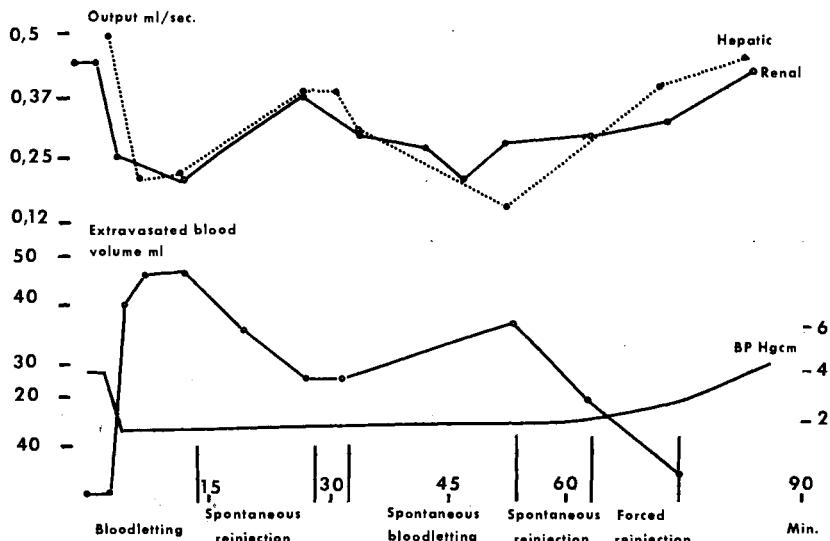


FIGURE 1. Evolution of hepatic and renal circulatory flow during a reversible hemorrhagic shock in the rabbit.

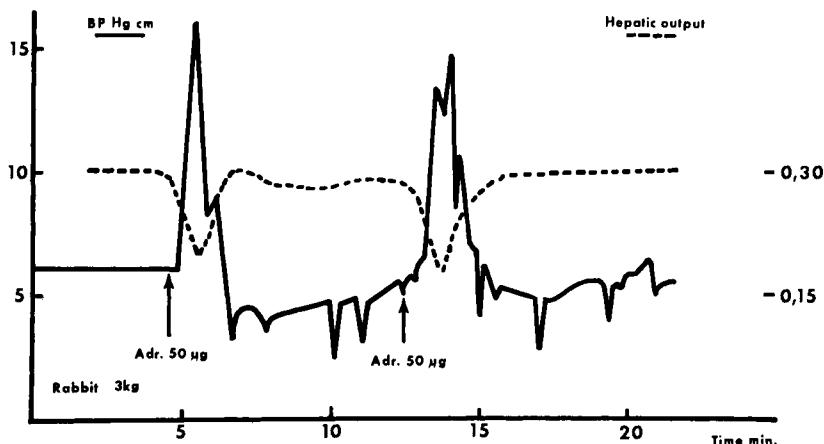


FIGURE 2. Variations of hepatic circulatory flow and of femoral arterial pressure following injection of 50  $\mu\text{g}$  of adrenaline in the rabbit.

a single hemorrhage, the progressive restoration of hepatic circulation can be obtained within about 1 hour. But maintenance of a low systemic pressure will not permit such restoration. The decrease in output is due both to the decrease in the circulating blood mass and to an active vasoconstriction of nervous or humoral origin (97).

Vasoconstriction can be revealed by direct observation. The flow of venous blood returning to the intestine is slowed down to such an extent that it causes intravascular agglutination (sludge). The decrease in oxygen saturation of portal and hepatic venous blood has been demonstrated and also the decrease in oxygen consumption of the liver and intestine (82).

In animals, continuous epinephrine or norepinephrine perfusion in parapathophysiological doses (2  $\mu\text{g}/\text{kg}/\text{min}$ ) induces a state of irreversible shock within 2 to 4 hours (19, 38, 68). Attempts to establish what happens to hepatic and renal circulation under epinephrine perfusion (57) revealed that a considerable decrease of output resulted from only a single injection of 50  $\mu\text{g}$  in the rabbit (from 0.6 ml/sec to 0.4 ml/sec) or of 200  $\mu\text{g}$  (from 0.6 ml/sec to 0.25 ml/sec) (Fig. 2). Observation of the mesenteric circulation enabled us to observe, in the latter case, a complete arrest of capillary circulation for about 30 sec.

A perfusion of 10  $\mu\text{g}/\text{kg}/\text{min}$  of norepinephrine induces mesenteric sludge within 30 to 40 min, in the same manner as does a hemorrhage, causing a hypotension of 35 mm Hg. Sludge is produced at an average lactacidemia of 88.6 mg/100 ml (51).

Finally, under the influence of a norepinephrine perfusion capable of restoring arterial pressure to its normal level, there is an increased drop in hepatic and renal output, already much depressed by the hemorrhage (although the reinjection of the shed blood has in principle restored the original blood mass).

These observations enable us to incriminate the adrenal sympathetic reaction as being at the origin of the vasomotor disturbances that appear in abdominal viscera following an aggression. This responsibility is confirmed by the favorable therapeutic action of agents that are able to decrease the intensity of such disturbances: sympatholytics, ganglioplegics, neuroplegics. We shall study them later as major pharmacological agents in the prevention of post-aggressive disorders.

### ***The Consequences of Splanchnic Vasomotor Disturbances***

The result of vasomotor disorders in the splanchnic area is the appearance of necrotic and hemorrhagic intestinal lesions of the mucosa, of the wall, and of the mesentery. These lesions, constant during irreversible hemorrhagic shock, are the same during endotoxin shock (17, 70). Animals that recover after prolonged hemorrhagic shock under the effect of sympatholytic therapy do not present such lesions (85).

**1) The Role of Microbial Toxins.** Fine *et al.* (22) presented experimental data to support the role of microbial toxins that penetrate through the damaged intestinal wall; probably bacterial polysaccharides. However, sterilisation of the intestine with systemically unabsorbed antibiotics only prolongs survival but does not prevent death (26). This has since been confirmed by many research workers as well as by us. Moreover, the behavior of germ-free animals towards aggression is identical to that of normal animals (98). But Fine *et al.* (22) believe that these animals have a deficient reticular-endothelial system. Besides, in addition to the microbial toxins, certain substances, such as the ammonium liberated by the ammoniogenetic intestinal flora and which can

no longer be transformed into urea by a hypoxic liver, are also involved (50).

**2) The Role of the RES.** As early as 1952—having accepted that serum cholinesterase has a reticular-endothelial origin and having observed a constant decrease in this enzymatic activity after aggressions—we presented the hypothesis of an RES inhibition (37). It was possible to confirm that bacteria injected intravenously during aggression syndromes are not destroyed (79), that the bacteriostatic and phagocytic index of the granulocytes decreases (80), and that animals whose RES is inhibited with Thorotrast die of a hemorrhagic shock that is not otherwise lethal (22).

Many authors believe that this RES inhibition during aggression syndromes is the result of a toxin discharge of intestinal origin. We shall see in studying the metabolic consequence of adrenergic reaction to aggression the experimental evidence that can be presented in support of the role of hyperlactacidemia as the major cause of RES inhibition, and also that of the lysosomes in phagocytosis and their relationship to the adrenergic reaction.

**3) The Role of Hyperlactacidemia and Sludge.** The decrease in hepatic and renal output and in the  $O_2$  consumption of these organs, as a consequence of the adrenal sympathetic reaction to aggression, reduces the capacity to transform ammonium into urea, and lactic acid into glycogen. This conclusion was also reached by Cain (13). We will see the degree of importance of hyperlactacidemia following aggression. Later, we shall also describe the experimental evidence for our belief that it is at the origin of sludge in the mesenteric microcirculation (51).

### B) Metabolic Disturbances

We will only consider the most significant disturbances from the pathogenic point of view, and accept as characteristic those that appear after a hemorrhage induced by Wiggers' method.

**1) Major Disturbances—Lactacidemia and Pyruvicemia.** Lactacidemia and pyruvicemia increase substantially during shock (Figs. 3 and 4). But the import of the  $\frac{\text{lactate}}{\text{pyruvate}}$  ratio is well known and also that of the excess lactate calculated with Hucka-

bee's formula (29):  $XL = (Lt - Lo) - (Pt - Po) \times \frac{Lo}{Po}$ , in which  $XL$  = excess lactate;  $Lt$  and  $Pt$  = lactate and pyruvate level at time  $t$ ;  $Lo$ ,  $Po$  represent the level at time 0.

The evolution of  $XL$  during hemorrhagic shock in the dog is demonstrable by Wiggers' double hemorrhage method (53). At the time of irreversibility it reaches a level of 3.7 mM/l, which is close to the level found by Broder and Weil (12) in man: 4.0 mM/l. But we must emphasize one primary fact, i.e., progress

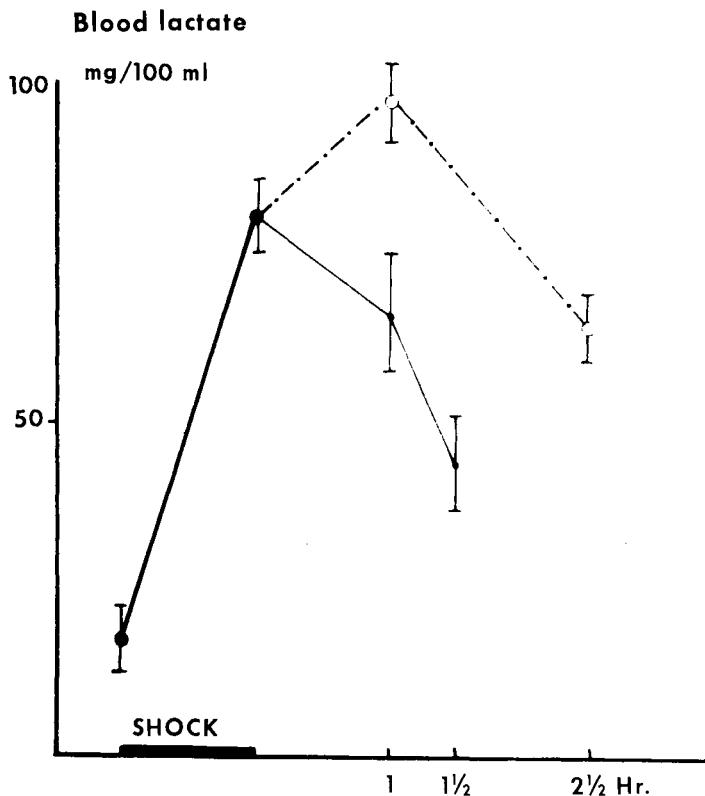


FIGURE 3

FIGURES 3, 4, 5 and 6. Evolution of blood lactate, pyruvate, sugar and potassium during the course of irreversible hemorrhagic shock in the dog. The mean values derive from 22 animals. *Full lines*: controls. *Bars and dots*: animals treated with DHA starting at the stage of irreversibility.

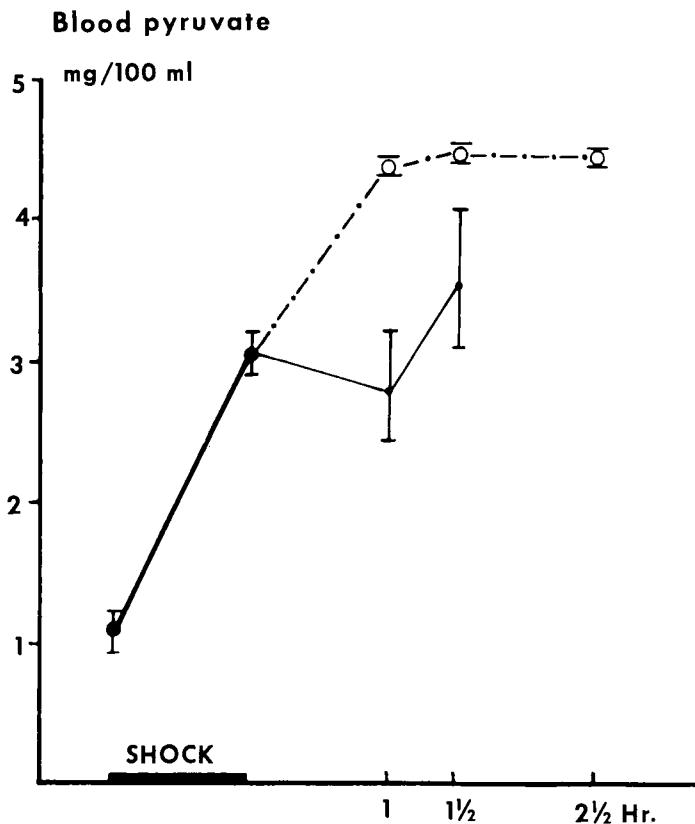


FIGURE 4

toward death is accompanied by a drop in lactacidemia, which is relatively more important than that observed in pyruvicemia and which, as death approaches, produces a decrease and a return to normal of excess lactate, a phenomenon that still lacks interpretation and therefore decreases the import of excess lactate as a prognostic element.

*Glycemia.* The evolution of the results of severe aggressions proceeds in two stages. A primitive hyperglycemia followed by hypoglycemia that some authors (89) consider as a good sign of irreversibility (Fig. 5).

*Kaliemia.* Kaliemia is an expression of glycolysis, such as hyperglycemia, and also of anoxia and of cellular depolarization in

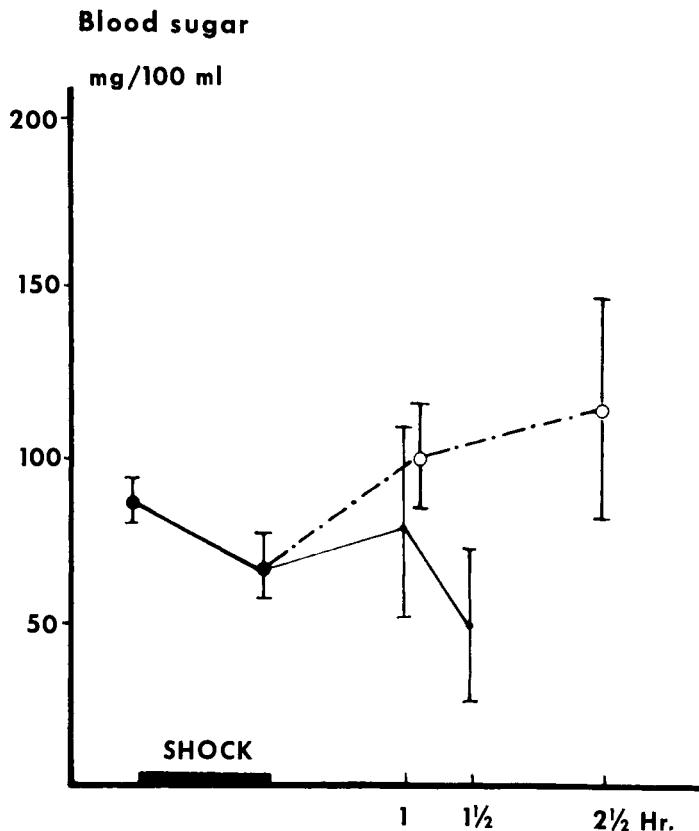


FIGURE 5

general; it is constant. The absence of diuresis explains why hyperkaliemia continues to increase right up until death (Fig. 6).

$pCO_2$  (Fig. 7). Metabolic acidosis and hyperventilation are at the origin of the drop in the  $pCO_2$ . But while arterial  $pCO_2$  decreases progressively right from the beginning of aggression, the venous  $pCO_2$ , on the other hand, increases during the first stage. This increase in venous  $pCO_2$  may be produced by the liberation from the tissues of fixed acids (principally free lactic and fatty acids). Since venous blood is taken before it passes through the lungs, the venous  $pCO_2$  increases as long as the bicarbonate reserve can be maintained. From the moment the venous  $pCO_2$  in its turn, drops, we believe that the condition can be considered

as normally irreversible. We shall see that the  $\text{pCO}_2$  drop was the element that, in our study, proved to be most resistant to therapy. It should be noted that the drop in circulation velocity cannot be considered responsible, because, although it continues to increase as death approaches, the venous  $\text{pCO}_2$  in turn collapses.

*pH* (Fig. 8). While arterial pH follows a trend similar to that of the arterial  $\text{pCO}_2$ , venous pH, in contrast to venous  $\text{pCO}_2$ , collapses at an early stage. However, forced retransfusion of shed blood momentarily raises the arterial and venous pH, but does not prevent progress toward death. The same is observed with injection of buffering substances (THAM, sodium bicarbonate), which, in our study, did not prevent the fatal onset; the results of

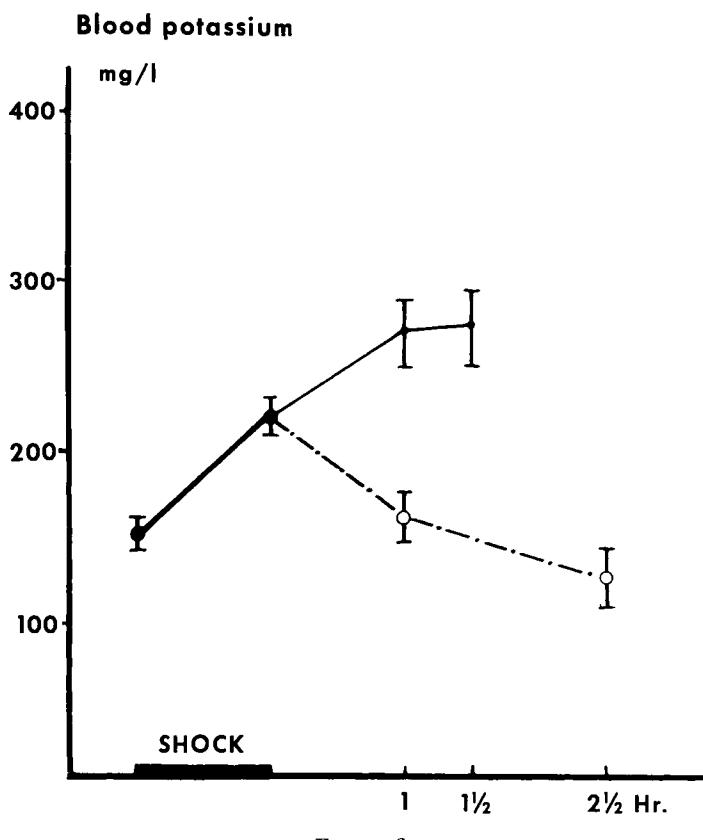


FIGURE 6

Nelson *et al.* (67) and Selmonoski *et al.* (81) did not differ from these. Sodium bicarbonate failed to improve cardiovascular activity by restoring the pH of the dog in hemorrhagic shock (8).

**2. Other Biological Investigations. The Enzymatic Activity of Blood Serum**—We have already mentioned the decrease in cholinesterase activity (49). We have also tried to establish the variations in blood serum transaminase (SGOT and SGPT) that are often increased after aggressions, since they appear to express a state of cellular damage (54a). Similarly, we shall come back in a moment to the problem of the increase in acid phosphatases, which raises the question of the role of the lysosomes. However, we must mention right here that we have been able to record in the rat an increase of these enzymes following an i.p. injection of

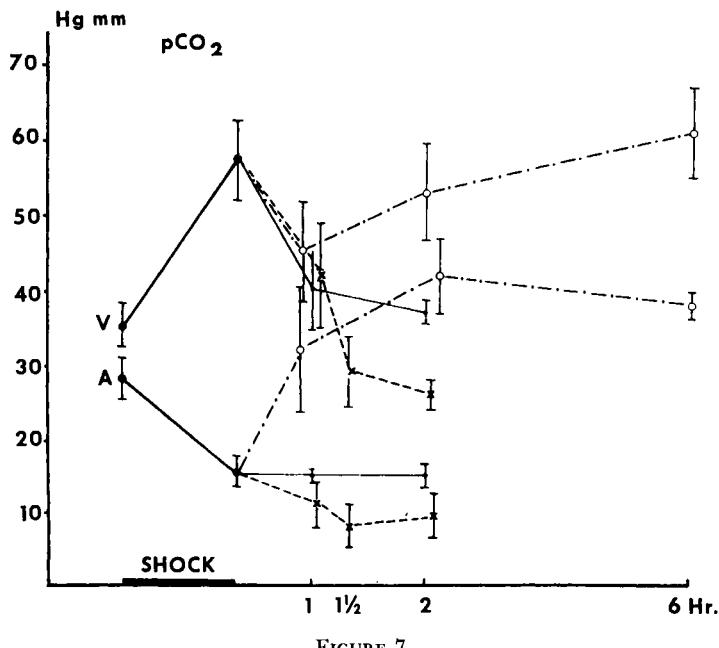


FIGURE 7

FIGURES 7 and 8. Evolution of arterial (A) and venous (V) pCO<sub>2</sub> and pH in dogs subjected to irreversible hemorrhagic shock. *Full lines:* controls. *Interrupted lines:* animals treated with DHA at the stage of irreversibility. *Bars and dots:* animals treated with DHA + acetazolamide + sodium bicarbonate, starting at the stage of irreversibility.

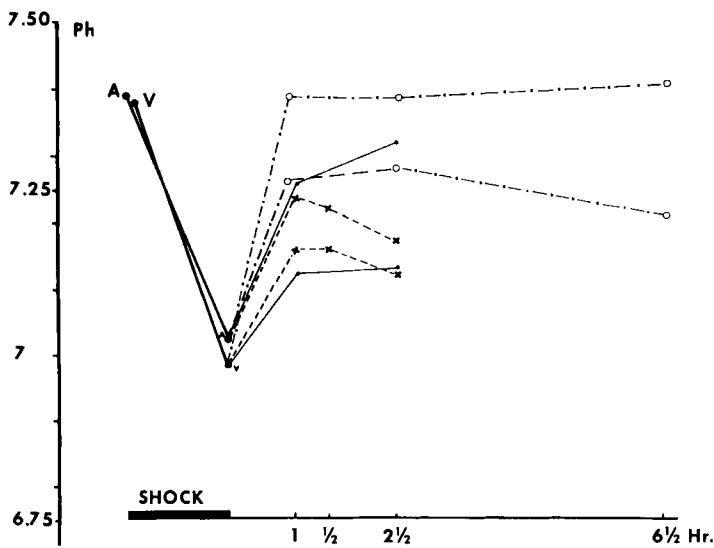


FIGURE 8

a dose of 1 mg/kg of epinephrine (42a). Moreover, investigation of the lipid metabolism shows a marked increase in free fatty acids, which is undoubtedly related to epinephrine liberation.

### C) Synthesis of the Pathogenesis of Reaction Phenomena

The metabolic disturbances that we have just described, and which last from 2 to 3 hours, can be produced by a single perfusion of epinephrine at a dose of 2 to 6  $\mu\text{g}/\text{kg}/\text{min}$ . Epinephrine, like the catecholamines in general, constitutes, with anoxia, the essential factor of hyperlactacidemia. Doses as small as 0.9  $\mu\text{g}/\text{kg}/\text{min}$  of epinephrine increase lactacidemia from 18 mg/100 ml to 38 mg/100 ml (64). Hyperlactacidemia, hyperglycemia, hyperkalemia, an increase in the free fatty acid level, can all be considered as direct or indirect consequences of adrenal sympathetic activation. As far as acidosis is concerned—expressed by the drop in the pH and  $\text{pCO}_2$  and by the bicarbonate reserve—it is obviously the consequence of hyperlactacidemia and of the increases in free fatty acids, although hyperventilation produced by acidosis does play an initial part in hypocapnia.

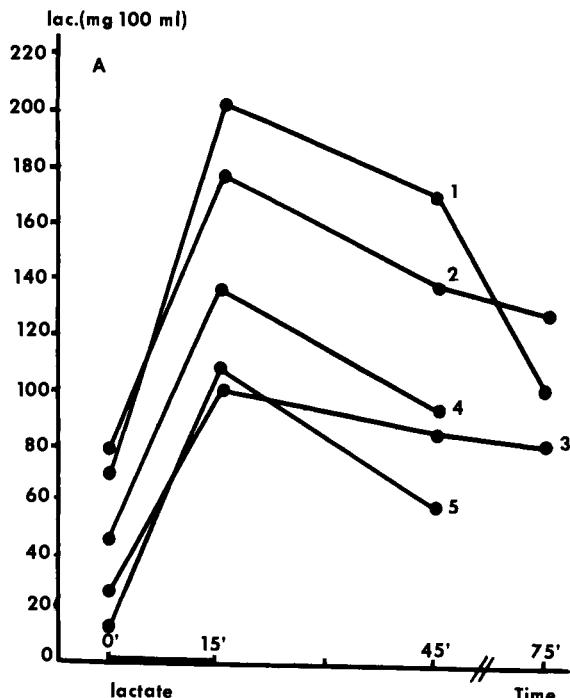


FIGURE 9. Evolution of blood lactate in the rabbit after perfusion with sodium DL-lactate (DL ratio = 45-65) during 15 min at a dose of 40 mg/kg/min of a pure 60% solution.

But, if the catecholamines are at the origin of metabolic acidosis, they are also the cause of its maintenance, owing to hypoxia, and of the depression of the metabolic activity of the liver that inhibits the onset of neoglucogenesis from lactic acid. In fact, a perfusion of sodium lactate in the normal animal, in the absence of any catecholamine liberation or injection or of any hyperventilation, is accompanied by rapid metabolization (43) (Fig. 9).

Experimentally, however, we have observed that there seems to be yet another factor responsible for the maintenance of hyperlactacidemia at a high level over a long period of time, i.e., the drop in the  $pCO_2$ .

Indeed, it is known that a single hyperventilation can produce hyperlactacidemia (29) and can even change a gaseous alkalosis into a metabolic acidosis. We were able to demonstrate that hy-

perventilation with a mixture enriched with  $\text{CO}_2$  cannot restore lactacidemia to its normal level on a stable basis. There has to be an associated adrenolytic (chlorpromazine), which leads us to believe that acidosis, which is at first metabolic then gaseous after a supply of  $\text{CO}_2$  (16, 67), must necessarily intervene as a factor of catecholamine liberation (45) (Fig. 10).

But, if from the vasomotor viewpoint catecholamine discharge is particularly dangerous because of splanchnic hypoxia and the ensuing lesions, from the metabolic viewpoint it would appear

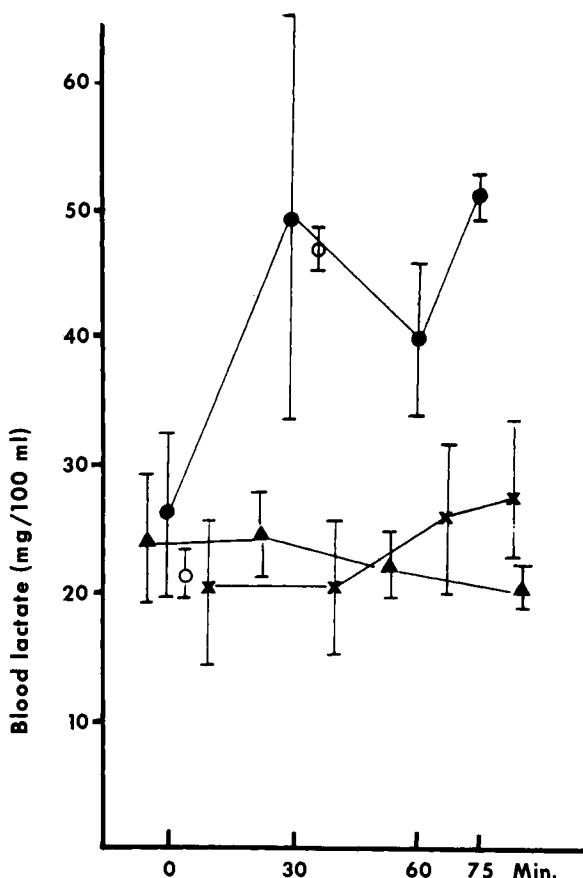


FIGURE 10. Variation of the blood lactate in the rabbit after hyperventilation during 75 min. *Black dots:* 5 controls. *White dots:* 14 controls. *Triangles:* 5 animals treated with acetazolamide and chlorpromazine. *Crosses:* 5 animals treated with acetazolamide alone.

that the hyperlactacidemia it produces must be held responsible for the major disturbances observed. Excess lactate does not appear to us to be only the consequence of hypoxia, but also the cause of other essential disturbances in the progress toward irreversibility.

### ***The Role of Stable Hyperlactacidemia***

We have observed experimentally that starting at a certain concentration of lactic acid in the extracellular fluids (in our opinion about 80 mg/100 ml) there occur functional disturbances independent of the pH and obtainable with continuous perfusion of Na lactate; these disturbances are also seen following various aggressions.

**a) Intravascular Aggregation of Microcirculation (Sludge).** We have been able to produce experimental sludge with the factors generally known to cause it (hemorrhage, epinephrine perfusion) and we have noted its appearance when lactacidemia reaches about 80 mg/100 ml. In this case, an adrenolytic (chlorpromazine, hydergine) can inhibit it, but not the sludge caused by perfusion of sodium lactate or of ethyl alcohol (NAD reducer). A molecule as closely related to lactic acids as pyruvic acid (NAD oxidant), not only does not cause sludge, but, to a certain extent, inhibits its production by lactate (51).

**b) RES Inhibition.** We have mentioned that this inhibition is part of the reaction to severe aggression, and we have considered its possible toxin origin. In the rabbit, we used the technique of Halpern *et al.* (25) based on the kinetics of disappearance from the blood of carbon particles injected intravenously. This test supplies a constant "K" [the phagocytic index of Biozzi *et al.* (9)], which is the slope of the carbon elimination curve in relation to time and represents the phagocytic activity of RES cells. Figure 11 a and b shows that the rate of phagocytosis decreases for a period that can reach up to 20 min in animals that have received an injection of sodium DL lactate at a dose of 0.9 g/kg, which produces hyperlactacidemia at about 80 mg/100 ml (in L lactate) (51).

**c) Increase in Sedimentation Rate.** After perfusion of DL lactate

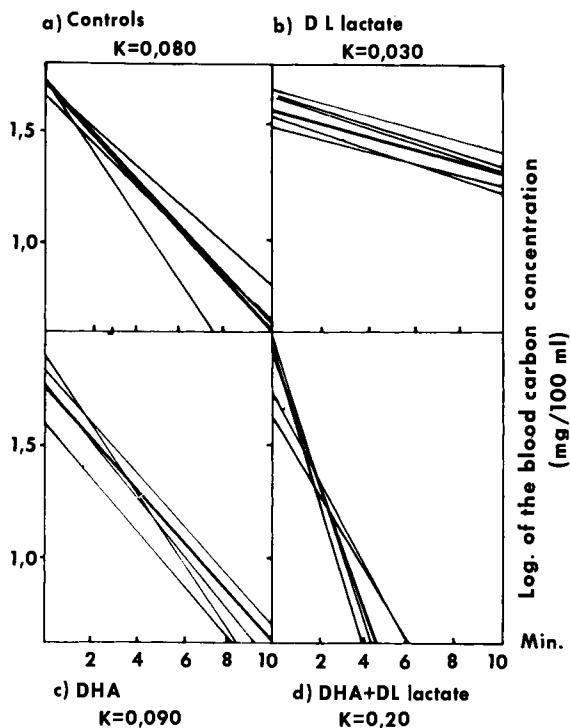


FIGURE 11. Variations of the phagocytic index (constant K) in the rabbit. a) control animals: i.v. injection of 10 ml physiologic saline 10 min before injecting 4 mg of carbon per 100 g body-weight. b) animals subjected to the same conditions, but receiving instead of physiologic saline, an injection of 10 ml sodium DL-lactate at a dose of 0.9 g/kg. c) same arrangement with DHA: 1 g/kg (10 ml). d) animals given 5 ml of DHA (1 g/kg) and, 10 min later, 5 ml of sodium DL lactate (0.9 g/kg) and, 10 min later, carbon injection.

in the rabbit at a dose of 0.9 g/kg, the sedimentation rate increases by 40 to 70% at the end of the 15th min. The lactate added to the blood *in vitro* does not change the sedimentation rate, a finding that demonstrates the existence of an intermediary factor liberated in the plasma, probably of hepatic origin (51) (Fig. 12).

**d) Cerebral Edema.** The elaboration of a simple but precise technique to measure true cerebral edema by determining the specific gravity of a cerebral cortex fragment (which is normally

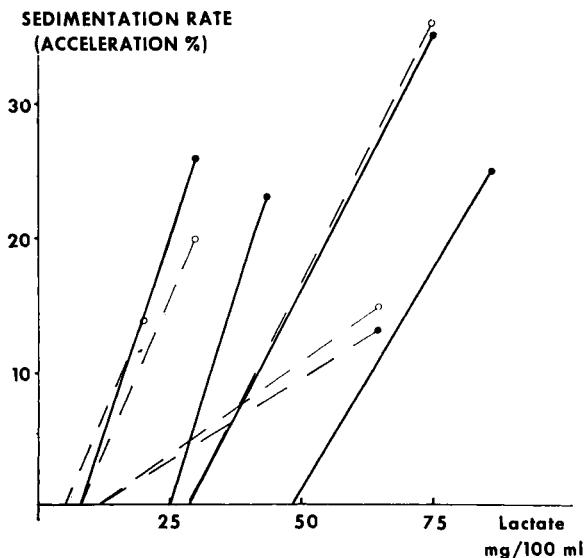


FIGURE 12. Percentage of acceleration of sedimentation rate in relation to the elevation of blood lactate by the end of a perfusion of 0.8 g/kg of sodium DL-lactate during 15 min in the rabbit. *Full line:* readings following 2 hours of sedimentation. *Interrupted line:* following 4 hours.

1.041) has enabled us to state that all the factors causing catecholamine liberation are also factors of cerebral edema (52). But here again, the catecholamines do not appear to interfere as such, but by means of the hyperlactacidemia that they induce. Na lactate is in itself a factor of cerebral edema as soon as lactacidemia reaches about 80 mg/100 ml. We had already observed (59) that during non-hypercapnic hypoxia the brain retains lactic acid, while the cerebral venous lactacidemia becomes less marked than arterial lactacidemia as soon as the latter reaches a level of about 80 mg/100 ml. Simultaneously, the brain acquires glycogen. Figure 13 shows the variation in cerebral specific gravity caused by hypercapnia (50% CO<sub>2</sub> + 50% O<sub>2</sub>) in the conscious rabbit, with or without acetazolamide and chlorpromazine protection (44).

*e) Endarterial Lesions.* We have been able to demonstrate, with Ancla *et al.* (2), using the electron microscope, lesions of the aorta wall of the rabbit after hyperventilation (for 1 hour) or sodium lactate perfusion (0.9 g/kg) similar to those shown

earlier by Ancla *et al.* (3) after epinephrine injection. These lesions are characterized by edematous vesicles in the endothelial cells and the smooth muscles of the aorta wall.

**f) Hyperlactacidemia and Hypocapnia.** As early as 1908, Henderson had already expressed the opinion that hypocapnia was at the origin of irreversibility of shock (27). It is certain that hyper-ventilation, which accompanies the onset of the reaction to aggressions, is in itself a factor of hypocapnia, the latter being primarily a consequence of metabolic acidosis. But we have also mentioned that, conversely, if it is prolonged, hypocapnia becomes a factor of metabolic acidosis as well as of ventilatory alkalosis. It would appear, therefore, that there exists a reciprocal effect between these two elements, metabolic acidosis causing hypocapnia and the latter inducing a secondary metabolic acidosis.

During severe aggressions such as hemorrhagic shock we were able to observe that once irreversibility was reached, the use of buffering substances, while restoring the pH to normal did not restore the  $pCO_2$  to its original value. In such cases, the administration of  $CO_2$  ( $CO_2$  5%;  $O_2$  95%) does not improve the cardio-

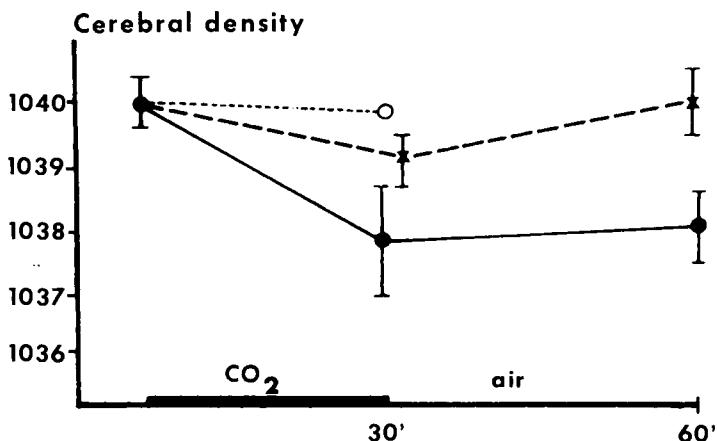


FIGURE 13. Cerebral density variations (edema measurement) observed in a rabbit subjected to hypercapnia lasting 30 min (50%  $O_2$  + 50%  $CO_2$ ) and then returned to the normal atmosphere. a) Full line: controls. b) Bars and crosses: animals receiving 1 mg/kg chlorpromazine. c) Dots and white circles: animals receiving 1 mg/kg chlorpromazine + 10 mg/kg of acetazolamide.

vascular condition (8, 53). In our opinion, this may perhaps be because of its transformation into bicarbonate in the intracellular environment under the action of carboanhydrase. We have, therefore, used acetazolamide (Diamox) to restore the  $\text{pCO}_2$ .

#### D) Biological Interpretation of the Phenomena Observed.

Without discussing the metabolic mechanism of action of catecholamines in detail, we must mention that their glycolysis and lypolysis activating effect appears to depend on 3'5'AMP synthesis, which they increase (97). The activation of phosphofructokinases appears also to participate in the increase of glycolysis (94). This would be the reason for increased lactic synthesis. We have just indicated that we attach special importance to hyperlactacidemia, not as a simple consequence of activated glycolysis, but as the apparent cause of irreversible disturbances.

(a) We are tempted to explain this by stressing that the reaction between pyruvic and lactic acid is an equilibrium reaction controlled by lactic dehydrogenase. It may be expected that, starting from a certain lactic acid serum level, the reaction can either be reversed—as it is actually physiologically in neoglucogenesis—or be self-inhibiting, since some isozymes of lactic dehydrogenase (isozyme 1) respond to the accumulation of a substrate.

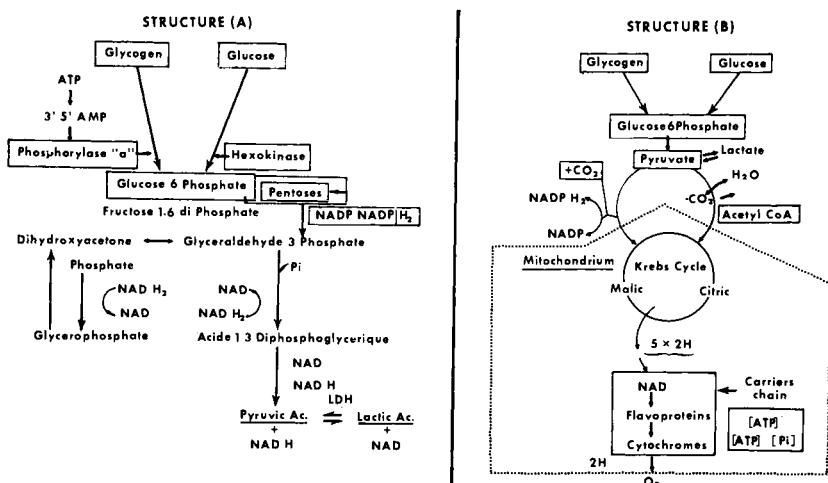


FIGURE 14

Numerous facts show that certain cells have an essentially glycolytic metabolism: they are poor in mitochondria and in succinic dehydrogenase, rich in lactic dehydrogenase and damaged only very slowly by anoxia. We have named them "A" structures to distinguish them from "B" structures, which, conversely, are essentially oxidative, react to anoxia and to inhibitors of the tricarboxylic cycle, and are rich in mitochondria and succinic dehydrogenase (Fig. 14) (40, 41). By inhibiting NADH<sub>2</sub> oxidation, hyperlactacidemia could inhibit glycolysis in "A" structures and, consequently, the only source of ATP synthesis. Hence, the impossibility to reject sodium (15), the depolarization and cell edema.

The astrocytes, certain smooth muscle fibers, and phagocytic cells may be classified as "A" type cells. This would offer an explanation for the fact that hyperlactacidemia can be a causal factor of cerebral edema (intraglial), of intravascular aggregation (sludge, of RES inhibition and of wall lesions of the aorta).

(b) But it is also important to mention that the maintenance of the tricarboxylic cycle functions demands the carboxylation of pyruvic into malic and oxaloacetic acids. It can well be assumed that this basic carboxylation requires a certain partial pressure of intratissular CO<sub>2</sub>. Since as soon as the pCO<sub>2</sub> drops below a figure which we believe to be around 15 mm Hg carboxylation is no longer possible, the arrest of the oxidative processes would be at the origin of glycolysis activation. This is the case in hyperventilation. It is also the case in metabolic acidosis, which then constitutes the beginning of a vicious circle, since the tricarboxylic cycle appears to be, on the other hand, an essential source of metabolic CO<sub>2</sub>.

We now understand why it is not sufficient to buffer acidosis in an attempt to restore the pCO<sub>2</sub>; why irreversible shock is accompanied by hypoglycemia, since carboxylation of pyruvic acid and the passage through the tricarboxylic cycle are an indispensable step of neoglucogenesis; and finally why lactate excess decreases as shock develops. Indeed, it cannot be imagined that this could result from its increased utilisation as a substrate in the tricarboxylic cycle. It can, therefore, only be produced by a secondary decrease of glycolysis. In this hypothesis, hyperlactaci-

demia would, indeed, be the cause of an inhibition both of glycolysis and of oxidative processes, and therefore, of over-all metabolic inhibition that characterizes the states of irreversible shock.

#### **E) The Lysosomes and the Theory of Toxicity**

Since the discovery by de Duve *et al.* (18) in 1955 of the cytoplasmic fraction that they called lysosomes, a considerable number of publications have appeared concerning these lysosomes. They have been thoroughly studied during phagocytosis, where they intervene to produce the digestion of phagocytosed particles by means of the proteolytic enzymes contained in them. Phagocytosis appears to develop in two stages: one, inclusion, depending on glycolysis; the other, digestion, depending on the pentose pathway (83). The paucity of mitochondria and of phosphorylating oxidative processes of the polynuclear leukocyte proves the absence of any tricarboxylic cycle participation in this activity. This is, therefore, an "A" structure and, as mentioned, we were able to demonstrate a phagocyte inhibition caused by hyperlactacidemia. It would, thus, be an inhibition of the first glycolytic stage. But numerous authors believe that the increase in the number of oxidizing phenomena at the end of the pentose pathway is at the origin of permeabilization through lipoperoxidation of lysosomal membranes. If the inclusion of particles can take place in anaerobiosis (30), digestion would only be possible in aerobiosis. All the factors of aggression capable of increasing the liberation of free radical forms (pro-oxidants) would, therefore, also be capable of causing the breakdown of lysosome membranes: ionizing radiations (95), carbon tetrachloride intoxication (84, 74), oxygen under pressure (24). Numerous antioxidants, on the other hand, have a protective action on lysosomal membrane: vitamin E and ubiquinone, chlorpromazine and promazine (71), Phenergan and Nupercaine (85). But it should be noted that anoxia also causes the breakdown of the lysosomal wall.

Besides, as indicated by Limperle (62), a differentiation can be made between the reticular-endothelial action of the phagocytic function and the antitoxic function. We would be tempted to place the latter as dependent on the pentose pathway, which is at

the origin of the synthesis of drug metabolizing enzymes (72).

We have tried to determine the state of the lysosomes during the aggression syndromes (hemorrhagic shock) by the indirect method of determination of SGOT and SGPT activity in blood serum (45) and of a more specifically intralysosomal enzyme, acid phosphatase.

It was found that acid phosphatase rises during irreversible hemorrhagic shock in the rabbit. It increases from  $3.39 \pm 0.27$  at the beginning of shock (a high figure related to the preparation of the animal) to  $4.14 \pm 0.33$  at the time of irreversibility. Pre-treatment with oral neomycin 12 hours earlier does not produce any significant changes:  $2.82 \pm 0.15$  before as compared with  $4.47 \pm 0.41$  after shock. We further observed in the rat that, after the injection of 1 mg/kg of epinephrine i.p., acid phosphatases increase from  $1.09 \pm 0.088$  to  $3.17 \pm 0.42$ . It would therefore appear that the catecholamines are capable of increasing the fragility of the lysosome membrane. On the other hand, no change is noted after lactate perfusion, which produces a lactacidemia of about 80 mg/100 ml.

It can thus be assumed that in the course of aggressions numerous causes of increased fragility of lysosomal membrane are to be considered. Some are connected with the type of aggression in itself; others constitute different toxic factors probably of intestinal origin or originating, for instance, from the traumatized site; but, in addition, a very general factor, the adrenal-sympathetic reaction to aggression, seems to be involved. Lysosomal membrane lysis probably plays an important part in both general and local proteolysis, in which connection it will be mentioned again. But it represents additional support for the use of antioxidants (58) and neuroleptics such as chlorpromazine (48) in the prevention of various aggression syndromes.

## II. Lesion Phenomena

The difficulty encountered when defining these phenomena results from the fact that they may be more or less localized in a certain organ or tissue, as in the case of traumatic, hemorrhagic or burn aggressions, or they can be more generalized, as in the case of toxic or infectious aggressions.

These local phenomena lead to cellular or intercellular edema caused by a complex chain of biochemical and vascular events. At times this results in a loss of fluid, which is sufficiently large to reduce the circulating blood to a dangerous level. These local phenomena are of greatest importance in certain forms of aggression, such as those caused by loosening of a tourniquet, crushing of a limb, or extensive burns.

*a) Water and Electrolyte Disturbances.* Only after Moon (65) had shown the importance of local fluid losses in the traumatized area, and precise measurements of the quantities normally contained could be taken (23), was it possible to demonstrate the reduction in the circulating volume during shock conditions without hemorrhage. Moreover, fluid replacement therapy increases local edema (10). Simultaneously, Tabor and Rosenthal (91) demonstrated that  $\text{Na}^+$  accumulates in the traumatized area while, conversely,  $\text{K}^+$  escapes. Thus, hyponatremia and hyperkaliemia are present in all aggression syndromes, whether hemorrhagic (31), traumatic (73), following arterial occlusion (4), or due to asphyxia (21), to give only a few examples. More generally, it can be inferred that any metabolic disturbance, whether toxic or anoxic, would cause a loss of cellular  $\text{K}^+$  and an increase in cellular  $\text{Na}^+$ . Besides, it was the desire to maintain or restore the  $\frac{[\text{K}_t]}{[\text{K}_e]}$  and  $\frac{[\text{Na}_e]}{[\text{Na}_t]}$  ratios by activating metabolism that motivated us to recommend the repolarizing combination, insulin + glucose, in the treatment of aggression syndromes associated in certain cases with a potassium salt (39).

*b) The Role of the Connective Tissue and of Tissue Hormones.* Actually, exchanges between the circulating fluids, blood and lymph, and the parenchymatous cells most often take place through the intermediary of the connective tissue, with its ground substance in which the collagen and elastic fibers can be identified and where the fibroblast and mastocyte cells are immersed. The ground substance is essentially composed of mucopolysaccharides, neutral heteropolysaccharides, and glycoproteins, to which are attached certain blood constituents (water, enzymes, mineral salts, albumins, globulins, vitamins, hormones, etc. . . ).

Mucopolysaccharides result from the metabolic activity of the fibroblast, which produces the synthesis of the hexosamine and

of glycuronic acid. A few years ago, we presented a hypothesis (42) concerning the metabolic mechanism of action of the principal pharmacological and hormonal agents on the fibroblast, which, in turn, directs the biological equilibrium of the connective tissue. This hypothesis was centered on the reducing or oxidizing action as regards NADP and NAD coenzymes, or their mutual transhydrogenation. The effect of the hormones on the enzymatic synthesis of the fibroblast must also be taken into account. It appears that one of the major effects of the mucopolysaccharides is the maintenance of an electronegative environment around the parenchymatous cells.

As regards the mastocyte, it contains two amines with strong positive charges, histamine and serotonin, which are neutralized by heparin with a strong negative charge.

An aggression, and more generally any substance with positive charge, causes the "explosion" of the mastocyte, which then liberates its granules into the ground substance. Heparin then combines with the positive charges that have penetrated into it, and forms complexes that are taken up and digested by the fibroblasts. Serotonin and histamine liberated from the heparin complex will then act on the arteriolar-cellular system, producing hyperpermeability and disturb its motricity (28). But a certain number of polypeptides with low molecular weight also participate in the local vasomotor phenomena, among which we were particularly interested in kallidin, decapeptide and bradykinin, nonapeptide, following the work conducted by Rocha e Silva *et al.* (76). These kinins are hypotensive and algogenous. It should be noted that Rocha e Silva (75) suggested that the catecholamines *in vivo* could activate proteases and may possibly be at the origin of a process liberating bradykinins or other active peptides, which themselves are factors of capillary vasodilation and of the breakdown of mastocytes. A recent work performed by Tsuneo and Movat (92) shows that burn lesions on the abdomen of the rabbit are accompanied by the penetration into the connective tissue of polynuclear leukocytes that are seen to be altered and degranulated under the electron microscope. Their lysosomes are broken down, and the authors report that the liberated hydrolases increase vascular permeability, cause the breakdown of the masto-

cytes with consequent liberation of histamine. The liberated hydrolases can also effect the kininogens directly and form active kinins, which, in turn, could liberate non-specific peptides capable of increasing capillary permeability. Here, it is worthy of note that we have observed a substantial increase of acid phosphatases in blood serum, under the influence of a single epinephrine injection. The anti-inflammatory role of glucocorticoids and of reducing corticoids may be due to their reducing activity on NADP as also to their protection of lysosome membranes. Their presumed favorable activity in shock, which is proposed by some authors (61), but still open to question, seems, in fact, to be principally apparent in endotoxin shock.

### **III. Pharmacology of Aggression**

The sequence of events that we have presented permits us to group the numerous theories proposed for the interpretation of the physiopathology of acute aggression: A Nervous Theory, since the basic element is the neuro-autonomic reaction to aggression; a Neuro-humoral Theory, since catecholamines play a predominant part in it; a Vasomotor Theory, since splanchnic vasoconstriction is often an essential stage; also a Toxic Theory, since toxins of intestinal origin influence the evolution toward death. Toxic, also, if the resorption of acid metabolites, of kinins, of vasomotor tissue hormones originating from the aggressed tissue are considered; toxic and neuro-humoral, if attention is focussed on the breakdown of lysosomal membranes, RES inhibition and sludge; a theory of fluid losses and of water and electrolyte stockage in the traumatized areas.

The rather theoretical distinction made between lesion and reaction phenomena, which are in fact closely imbricated, enables us in practice, nevertheless, to make a distinction between therapy directed to the lesion (traumatic, hemorrhagic) and to the aggressive agent (toxic, infectious), and general nonspecific therapy.

But we must admit that, as a whole, pharmacological therapeutics in general also have a favorable local effect, synergistically, as we shall see later. And finally, we must make a distinction between prophylactic therapy and curative therapy.

#### A) **Prophylactic Pharmacology**

It is understood that such pharmacology must essentially inhibit the liberation or the action of catecholamines, taking into account the importance of the latter in the triggering off of the mechanism that leads to irreversibility. Since we are acquainted with the nervous pathways activated by the adrenosympathetic response to aggression, it can easily be imagined that the agents selected are those that tend to inhibit these pathways at various levels of their evolution, although very often these agents also act at different levels.

a) **Adrenolytics.** The above physiopathogenic comments help us to understand why some of the effects of epinephrine should be preserved while others are to be inhibited. We do not agree fully with Alquhist's terminology (1) for we believe that the response of an organ to the catecholamines depends more on its metabolic structure (A, B or C structure) than on hypothetical receptors, which, moreover, one is obliged to assume are numerous (37). But, taking into account the present widespread use of this terminology, we shall speak of  $\alpha$  and  $\beta$  receptors, and of  $\alpha$  and  $\beta$  effects. Now, it seems certain that the  $\alpha$  effects (vasoconstriction) are particularly harmful in epinephrine activity. Indeed, if hyperlactacidemia is considered as a  $\beta$  effect, we have seen that, on the contrary, it is the probable secondary inhibition of glycolysis it causes that should be corrected therapeutically.

This obliges us to turn to the so-called  $\alpha$  inhibitors. Among these, Dibenamine (phenoxybenzamine) has been studied by Nickerson (69), and has been used by Baez *et al.* (6); tolazoline (Priscoline) and phentolamine (Regitine) have a stimulating inotropic and chronotropic action on the myocardium that limits their clinical use (63). Other  $\alpha$  adrenolytics, such as azapetine, the benzodioxanes, yohimbine, that cause some side-effects are not used in current therapy.

Finally, some phenothiazines should be mentioned here. Among these are chlorpromazine and certain ergot derivatives (one of which is hydergine) possessing the characteristics of  $\alpha$  inhibitors; these, however, are referred to in greater detail in the section dealing with neuroleptics.

It can be understood, nevertheless, why under these conditions compounds such as the  $\beta$  inhibitors (DCI, pronethalol, propranolol) can have only very specific indications in post-aggressive conditions. The same holds true for agents such as reserpine that produce depletion in the store of catecholamines, in particular, at the level of the myocardium (14), the more so since restoration of such stores is slow. This is also the case with  $\alpha$  methyldopa, an efficient inhibitor of dopadecarboxylase (87). With regard to guanethidine and bretylium, their mechanism of action is still very unclear; so their use cannot yet be considered in aggression therapy.

**b) Ganglioplegics.** Inhibition of the influx transmission at the level of the ganglions, which is a relay of the sympathetic chain, leads to effects that resemble those of the inhibition of mediators at nerve endings. Although an attempt has been made to associate them with tetraethylammonium in prophylactic treatment of acute aggressions (34, 35), the discovery of hexamethonium and of related compounds has, of late, permitted better control of adrenergic activity. Some authors have studied the protective action of hexamethonium in hemorrhagic shock (5). Its protective action is, in general, accepted, but it is used in the therapy of acute aggression only to obtain controlled hypotension.

**c) Neuroplegics.** This category comprises agents, which, although possessing a peripheral adrenolytic activity, are essentially interesting because of their central activity.

*Chlorpromazine*, among the phenothiazine derivatives, is the most widely used since its introduction in therapeutics (48). Some of the earlier phenothiazines (promethazine, diethazine) possess interesting, similar properties and are still being used, but the indications for their use are more specialized. Chlorpromazine inhibits or even reverses the  $\alpha$  effect of injected epinephrine. It depresses the carotid-sinus pressure reflex. It also exerts an antagonistic action on histamine and serotonin. We have seen that it protects the lysosome membrane. It possesses only a slight anticholinergic effect, and appears to be a relatively weak ganglionic inhibitor. Its action is essentially central. At the level of the cerebral-spinal axis, among its complex activities, particular stress is laid on its depression of hypothalamic functions: a hypothermic-facilitating action, inhibition of hypertension caused by stimula-

tion of the pressor areas of the hypothalamus. It also depresses the activity of the limbic system and that of the corpus striatum, which may explain its action on the extrapyramidal system. But its main activity appears to be on the reticular formation of the cerebral stem. Although its mechanism of action in this area is still open to question, it appears to depress the function of the collateral sensory pathways (11). Killam and Killam (32) believe that it stimulates the reticular formation, but in turn, would activate the filtering effect of this formation on the influx of the stimuli that it receives from the collaterals of the sensory pathways.

*Ergot Alkaloids*, and in particular a mixture of three alkaloids (ergocornine, ergocristine, ergokryptine) known as hydergine, are also  $\alpha$  adrenolytics that reverse the pressor effect of epinephrine and the stimulation of the sympathetic. But although dehydrogenated, the direct action of these derivatives on the vessel muscles could still be that of all ergot derivatives, i.e., a constricting effect, and it would be their central activity, in particular, on the vaso-motor center that would be responsible for the sympatholytic (78) and depressive effect on vasomotor reflexes (7). It should be noted that while chlorpromazine has a depressive action on the postrema area, hydergine has a stimulating effect. The former is antiemetic, and the latter can facilitate vomiting.

In short, it would, therefore, appear that in the pharmacological therapy of acute aggression the neuroplegics should be chosen primarily to inhibit the epinephrine reaction. In aiming at the central relay of the peripheral influx, it would appear that the depression of this reaction as a whole and of its peripheral consequences would be safer and more harmonious. Thus, since we proposed the use of chlorpromazine to achieve this aim, numerous studies have justified such reasoning.

*The criticisms* formulated are mostly based on the hypotension caused by neuroplegics. But we believe that this hypotension is only dangerous when concomitant transfusion is not given to fill the vascular areas liberated from vasoconstriction (36) and when the neuroplegic is administered too rapidly, before the vascular system has been able to adapt itself to the transfused blood.

*d) Antihistaminics.* The above-mentioned drugs often possess

antihistaminic properties. But we believe that in view of their synergistic central activity, other derivatives of the phenothiazines, such as promethazine (Phenergan), should also be used, since we have observed that histamine frequently plays a part locally.

e) *Antikininins.* For the time being, these substances have only a limited action in acute post-aggressive syndromes. The best known at present are the salicylates. Conversely, a few years ago, we synthetized a derivative of pyridazone (*Ag 246*, or morpholino-2-ethyl-4-methyl-6-phenyl-3-pyridazone hydrochloride), which has been shown to have a strong antibradykinin effect on numerous organs (56). This compound, with a marked central neuroplegic action, also affords a strong analgesic effect without ventilatory depression. In addition, it is also an antagonist of serotonin and histamine. Its study, which has not yet been completed, already permits us to foresee its potential for use in man in the prevention of organic reactions to aggressions. The interest presented by the combination of a neuroplegic with an antihistaminic and an analgesic, as was achieved in what we call the "lytic cocktail" (chlorpromazine + promethazine + pethidine), has recently been extended to neuroleptanalgesia. Such a compound as *Ag 246* would appear, to a certain extent—once certain drawbacks have been eliminated (ventilatory depression of morphinomimetic analgesics)—to offer, *per se*, all the advantages afforded by the combination of substances.

f) *Lysosome Protectors.* We have already mentioned some of these: vitamin E, hydrocortisone. Here, once again, we find the derivatives of phenothiazine and in particular chlorpromazine and promethazine. But it is probable that in the case of a number of antioxidants this property still remains to be discovered. It is interesting to note that the same agents that, from a general viewpoint, are able to minimize the adrenal-sympathetic reaction to aggression are also liable at the cellular level to offer a certain degree of protection by limiting vascular hyperpermeability and the local inflammatory phenomena.

### **B) Curative Pharmacology**

Once an aggression has induced irreversible disturbances in the organism, application of the preceding pharmacological therapy,

even when combined with hypothermia, has only a limited effect. However, since irreversibility is not easily established in clinical practice, such treatment should still be attempted. Experimentally, the curative results of treatment can only be determined in otherwise 100% lethal shock.

**Acidosis.** Acidosis can be corrected in a relatively simple manner with buffering substances (THAM, sodium bicarbonate); but as already mentioned, this corrective therapy in no way improves the survival rate.

**Restoration of Glycolysis.** We have already mentioned that in our opinion it is not so much a decrease in hyperlactacidemia that should be achieved, since the latter decreases progressively in the course of evolution toward death, but on the contrary, reactivation of glycolysis, which we believe is inhibited by hyperlactacidemia. It is probable that the good results that we have obtained experimentally, and which have been confirmed clinically on a

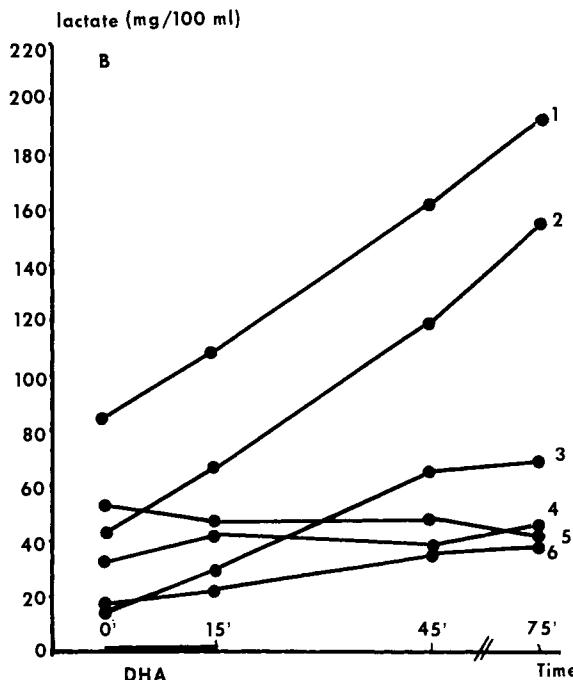


FIGURE 15. Blood lactate variations in the rabbit after perfusion with DHA at a dose of 66 mg/kg/min during 15 min.

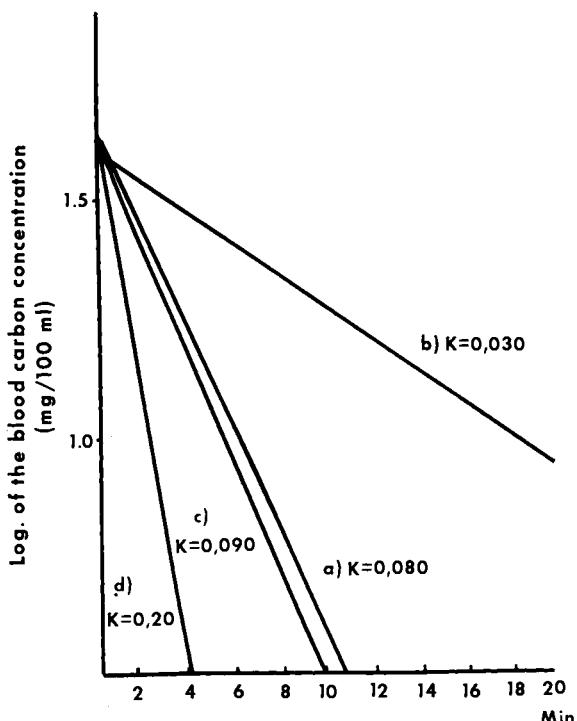


FIGURE 16. Mean variations of phagocytic activity (constant K) in the rabbit.  
 a) normal. b) after perfusion with sodium DL-lactate. c) after perfusion with DHA. d) after perfusion with DHA and, subsequently, with sodium DL-lactate.

large scale with a combination of a 30% glucose solution and insulin, are the results of such an action, although such a combination also possesses a strong splanchnic vasodilating activity and restores the hepatic and renal output in the animal in hemorrhagic shock (58). But since then, we have been using dihydroxyacetone to this end, which, when phosphorylated, can oxidize NADH<sub>2</sub>, producing  $\alpha$ -glycerophosphate, and reactivate glycolysis. Figure 15 shows the progressive and prolonged hyperlactacidemic-inducing action of this substance when injected intravenously (43). When perfused at a dose of 3 g/kg at the time of irreversibility of shock in the dog, it reduces the decrease in lactacidemia, maintains a strong hyperpyruvicism and thereby reduces lactate

excess (Figs. 3, 4, 5 and 6). It is remarkably efficient in restoring arterial pressure, both in shocked man and animals; it acts efficiently on sludge in the mesenteric vessels, on experimental cerebral edema; and it restores phagocytic activity inhibited by lactate perfusion (55) (Figs. 7, 8 and 16).

**Restoration of  $pCO_2$ .** We have already mentioned that a supply of  $CO_2$  added to the inhalation mixture is sufficient to restore correct cardiovascular functions; we suggested that this was the result of an increase in the  $\frac{[CO_3H_1]}{[CO_3H_2]}$  ratio due to the presence of tissue carboanhydrase, which, in the case of depletion of bicarbonate reserves, is forced to function in the direction  $CO_2 + H_2O \rightarrow CO_3H_2$ . Conversely, we imagine that since  $CO_2$  seems indeed to be liberated as such by the metabolic pathways, the inhibition of the carbonic anhydrase may possibly permit an increase in its cellular concentration, to improve pyruvate carboxylation, and consequently, tricarboxylic cycle operation. In fact, the administration of 10 to 30 mg/kg of Diamox to the animal in irreversible shock, in which the  $pCO_2$  has collapsed to below 15 mmHg, rapidly restores, in a stable manner, the venous and arterial  $pCO_2$  (Fig. 7).

**Proteolytic Antienzymes.** If such compounds as EACA, Trasylol and Iniprol, which inhibit the action of certain proteolytic enzymes, have proven to be effective in certain syndromes such as acute hemorrhagic pancreatitis, they have, on the other hand, and in spite of the probable breakdown of lysosomes observed during hemorrhagic shock, been disappointing when used in hemorrhagic shock (46).

### Conclusion

We purposely chose the study of the physiopathogenesis of acute organic reaction to aggression, since it is clear that therapeutic pharmacology can only be truly effective from a pathogenetic but not a systematic viewpoint. In this, we believe that we may be justified in proposing a pharmacologic means of control in which the principal difficulties encountered, when it is applied therapeutically, possibly reside only in the semantic context of the term "means of defense." Indeed, it often tends to

depress rather than to stimulate these so-called means of defense, which then take on the significance of defense of our autonomous motor activity, defense by means of escape or fight, rather than direct defense of the life of our tissue as such.

We have also intentionally neglected the endocrine aspect of this reaction, as this has been described in detail by Selye and his School.

### **Abstract**

The author discusses the consequences of a short but acute aggression, and the pharmacological control of its pathogenic effects. He makes a distinction between lesion and reaction phenomena.

#### **1) Reaction Phenomena**

The author briefly reviews the mechanism of catecholamine liberation and its consequences: *a) Vasomotor disturbances*, predominantly at the level of abdominal viscera, causing intestinal lesions. The latter are at the origin of microbial toxin resorption that may well participate in the inhibition of the reticular endothelial system. Splanchnic vasoconstriction is accompanied by sludge in the mesenteric microcirculation, a depression of hepato-renal metabolism and output, maintaining a high lactacidemia. *b) Metabolic disturbances*. The author then discusses the significance of hyperlactacidemia and of lactate excess that decreases with approaching death; of initial hyperglycemia and secondary hypoglycemia; of increasing hyperkaliemia, while the venous  $pCO_2$  first rises and then collapses. The increase in serum transaminases and in acid phosphatases, and the drop in serum cholinesterase activity are also considered. *c) On the basis of these observations, a mechanism of the pathogenesis is proposed.* It attributes the main role to hyperlactacidemia in the disastrous effect of the adrenal-sympathetic reaction. It is shown experimentally that hyperlactacidemia may well be responsible for sludge, reticular-endothelial inhibition, increase in sedimentation rate, cerebral edema, and aortic endarterial lesions. Finally, it appears to be largely responsible for hypocapnia. Thus, hyperlactacidemia would have two major consequences at the metabolic level: on the one hand, the inhibition of glycolysis, starting from a concentration of about 80 mg/100 ml, a phenomenon that is essentially detrimental to cell structures in which the glycolytic cycle is predominant (astrocytes, endothelial and phagocytic cells, certain smooth muscle fibers); on the other, the drop of the  $pCO_2$  to below 15 mm Hg, which may inhibit the carboxylation of pyruvic acid into malic and oxaloacetic acid, and consequently, the maintenance of the tricarboxylic cycle. *d) The lysosomes* provide a new element to the mechanism of toxic lesions. But the intestinal antibiotic therapy applied before hemorrhagic shock does not hinder an increase in serum acid phosphatase level, and a single

injection of epinephrine causes a rise in the level of these serum enzymes similar to that observed during shock. The increase in oxidative processes under the action of catecholamines is considered to be at the origin of the increase in permeability of lysosomal membranes, and an attempt is made to distinguish between phagocytic and antitoxic processes at the metabolic level. The perfusion of lactate does not have any effect on acid phosphatase.

## 2) *Lesional Phenomena*

The author briefly reviews the local water and electrolyte disturbances and the role of the connective tissue; that of the negative electric charge of mucopolysaccharides; also the role of fibroblasts and of mastocytes, of the liberation of histamine, serotonin, and heparin from their granules, and that of kinins and of the lysosomes of PMN leukocytes after diapedesis.

## 3) *The Pharmacology of Stress*

On the basis of this physiopathogenic picture, the author proposes: *a) Prophylactic therapy*, which aims, primarily, at controlling the adrenal-sympathetic reaction. He discusses the use of adrenolytics, ganglioplegics and neuroplegics, in addition to that of the antihistaminics and antibradykinins. Finally, the author considers the use of lysosomal protectors and of non-resorbable intestinal antibiotics. *b) Curative Therapy*. It should be noted that the control of pH by buffers does not increase the survival rate following irreversible shock. The resorption of glycolysis appears essential: hypertonic glucose and insulin, or better still dihydroxyacetone, are able to achieve this.

The restoration of the  $pCO_2$  is more difficult to achieve through maintenance of cardiovascular functions. Acetazolamide can produce this. The use of proteolytic antienzymes to oppose the action of liberated lysosomal hydrolytic enzymes did not improve the results during hemorrhagic shock.

## Abrégé

L'auteur discute des conséquences d'une agression aiguë de courte durée et des implications pharmacologiques dans sa pathogénie. Il établit une distinction entre la lésion et les phénomènes réactionnels.

## 1) *Phénomènes réactionnels*

L'auteur passe en revue les mécanismes de libération des catécholamines et ses conséquences: *a) troubles vaso-moteurs* prédominants au niveau des viscères abdominaux et provoquant des lésions intestinales. Il s'ensuit une résorption de toxines microbiennes avec inhibition du système réticulo-endothélial. La vasoconstriction splanchnique et les altérations microcirculatoires qui en découlent ont pour effet de déprimer le métabolisme hépato-rénal responsable du maintien d'une lactacidémie élevée. *b) troubles*

**métaboliques**; l'auteur discute des effets délétères de l'hyper-lactacidémie, des variations extrêmes de la glycémie et de l'accroissement du potassium sanguin. L'accroissement des transaminases et des phosphatases acides dans le sérum, ainsi que la chute de la cholinestérase sérique, sont également pris en considération. *c)* l'auteur s'appuie sur ces observations pour proposer un **mécanisme pathogène** par lequel l'effet désastreux de l'hyper-lactacidémie sur la réaction sympathico-surrénalienne joue un rôle de premier plan. Il considère en outre que les lysosomes participent à la genèse des lésions toxiques.

### **2) Phénomènes lésionnels**

L'auteur passe brièvement en revue les altérations du métabolisme hydro-minéral et la participation des éléments du tissu conjonctif, de leur produit de sécrétion, de même que ceux des leucocytes qui entrent en diapédèse.

### **3) La Pharmacologie du stress**

En se fondant sur ce tableau physiopathogénique, l'auteur propose une thérapeutique prophylactique destinée à contrôler plus particulièrement la réaction sympatho-surrénalienne. Il propose, en outre, une thérapie curative, qui aurait pour effet de corriger la résorption de la glycolyse, soit par l'administration de glucose et d'insuline ou encore mieux de dihydroxy-acétone.

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**G. Heuser:** I have long admired Dr. Laborit's work, which cuts across so many disciplines and has had such an impact not only in basic research but also in clinical medicine. Rather than discuss the very complex problem of metabolic and pharmacological aspects of shock, a subject so admirably covered by Dr. Laborit, I should like to bring up a philosophical point in relation to his career and discuss clinical investigation as "opposed" to basic research. This "opposition" has been of increasing concern to me as I have tried to make my way in both "camps." Both Selye and Laborit studied medicine and became physicians. Both left the practice of medicine or surgery and have devoted their lives fully to animal research. Why? They must have felt that they could make a greater contribution to medicine where questions can be asked more freely, answers tested with greater accuracy, and significance appreciated with more ease by using statistics. And, of course, the history of medical science supports the notion that, in order for clinical medicine to advance, basic research has to be encouraged. I do not dispute this notion.

However, I should like to make the point that our present academic structure has a tendency to separate the basic investigator from the clinician. In spite of many exceptions, this separation is generally very real indeed and very much to be deplored. The man in basic research loses much, since he is deprived of an opportunity to witness the drama of disease and thus be exposed to all basic questions it raises. But the man in clinical medicine and his patients lose more, since they are not exposed to the often more original thinking and approach and the knowledge of the basic scientists. The discipline and demands inherent in the medical curriculum are such that the product, the M.D., is a highly intelligent but nevertheless usually more uniform individual than the Ph.D., who can as a rule retain much more of his individuality throughout his curriculum. This

makes for a situation in which an every-day communication between basic scientists and physicians is not easily established and maintained. Advances in thinking and approach, therefore, reach the bedside only with considerable delay.

I believe that clinicians and basic investigators should be given much more opportunity to communicate with each other at the bedside and in the laboratory, by sharing a greater part of their curriculum, by having joint appointments and responsibilities in a clinical and basic science department, by being geographically closer and having laboratories on the same floor or in the same building. This would not interfere with the necessary independence of the basic scientist, but would certainly improve communication between him and the clinician.

Let me conclude with the hope that some of the young Selyes and Laborits of the future will find clinical research more attractive than it has been in the past and will find it worthwhile to be close to it as consultants or to be in it as clinical investigators.

## Serum Aminopexy\*

JEAN-LOUIS PARROT

### Introduction

THE toxicity of gastrically introduced histamine is markedly increased by prior administration of a diamine, putrescine (17). Following ingestion of 400 mg/kg of histamine dihydrochloride, the otherwise untreated animal survives for 30 minutes to 3 hours and it is noted that a large part of the histamine is bound by the tissues. The level in the various tissues of this bound histamine, as measured by Barsoum and Gaddum's method (1) modified by Code (4), and expressed as  $\mu\text{g}$  histamine dihydrochloride per g wet weight, is: gut 120, lung and liver 60, kidney 25, adrenals 10 (12). Gut studies have shown that ingestion of 4 mg/kg of a diamine, spermine phosphate, subsequent to histamine ingestion, abolishes this binding.

When ingested in the presence of a diamine, histamine cannot be bound by the gastric juice mucin or the various tissues. Instead, it remains in the general circulation, the blood histamine level is increased and the toxic dose of histamine is decreased (13). Administration of 40 mg of histamine dihydrochloride, per os, in the guinea-pig does not increase the blood level of histamine above the normal value of 600  $\mu\text{g}/\text{l}$ . But when 200 mg/kg putrescine hydrochloride are added, the histamine blood level is increased to 3,000 at 30 minutes and still remains high (about 1,800  $\mu\text{g}/\text{l}$ ) after 2 hours.

The LD<sub>50</sub> of histamine dihydrochloride is 300 mg/kg per os. But the addition of 150 mg/kg putrescine dihydrochloride (a dose that is non-toxic in itself, the LD<sub>50</sub> being 1200 mg/kg) decreases the LD<sub>50</sub> of histamine dihydrochloride to 30 mg/kg.

In 1949, the first step in the fate of histamine was elucidated:

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\* This paper was presented in French.

the binding of histamine by tissues and mucin in an inactive biological form. This first non-enzymatic process takes place prior to the slow destruction of histamine by histaminase. Diamines such as putrescine or spermine act as competitors of this binding.

Following these investigations, we attempted to determine whether human serum was also able to bind histamine. Our efforts resulted in the demonstration of serum histaminopexy. We shall therefore describe the different techniques used, the results obtained in normal subjects and in non-allergic and allergic patients, and the different studies concerning the mechanism involved in this phenomenon, as well as the role of the endocrine glands.

### Techniques for the Evaluation of Aminopexy

#### A) Pharmacologic

Serum aminopexy occurs with amines, such as histamine, 5-hydroxytryptamine, and acetylcholine. These amines are all bound by the normal serum, but not by that of the allergic patient. Since the biological test for histamine is the easiest to perform and gives the most constant results, we have always used it in our investigations. All our results, therefore, and those of other authors, have been obtained with the use of histamine. The technique is as follows: using the isolated guinea-pig ileum, we compare the contractions induced by a histamine solution alone and in mixture with serum diluted 1:20. When the serum of normal subjects is used, it is observed that a part of the histamine does not act on the biological test (Fig. 1). The serum histaminopexy is measured by the percentage of the bound histamine. On the other hand, the serum of allergic patients does not modify the histamine-induced contraction, i.e., amine is not bound by the serum (Fig. 2).

#### B) Physical

**1. Equilibrium Dialysis.** Introduction of an equal concentration of histamine to the inside and the outside of a dialysis bag containing normal serum results in an increased concentration inside the compartment. But when serum from an allergic patient

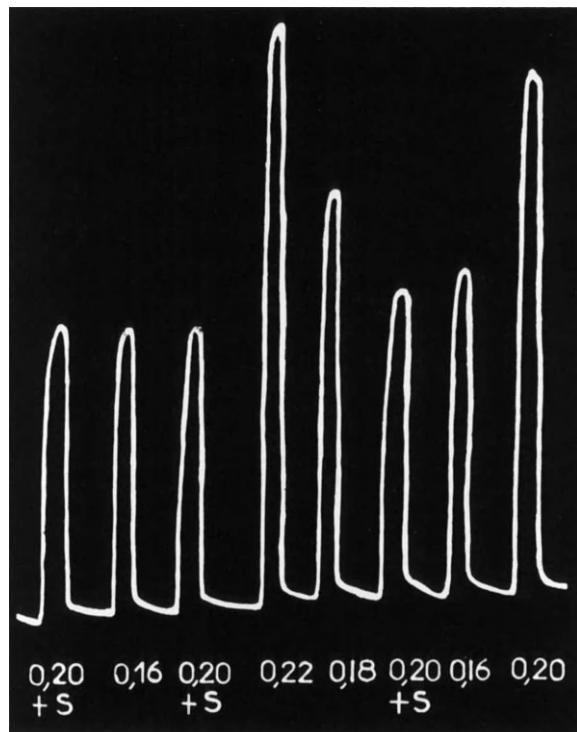


FIGURE 1. Normal human serum or serum from non-allergic patient: Histaminopexy 20%.

FIGURES 1 and 2. *Pharmacological technique for evaluation of histaminopexy (isolated guinea-pig ileum).* The numbers refer to the amount of histamine dihydrochloride (in  $\mu\text{g}$ ) added to the bath (8 ml), either alone, or mixed with serum (S) diluted 1:20.

is used, the inner and outer concentrations of histamine remain equal. The final concentration of histamine can be measured by various methods: bioassay, colorimetry, and radiation count.

**2. Ultrafiltration.** When histamine is added to normal serum, ultrafiltration ( $2 \text{ kg/cm}^2$  during 10 to 15 hours) reveals that the concentration of histamine is lower in the ultrafiltrate (19).

**3. Ultracentrifugation.** Wallenfels *et al.* (23) showed that some of the histamine added to normal human serum is carried down with the proteins during ultracentrifugation (40,000 rpm for 8 hours at  $0^\circ \text{ C}$ ). In contrast to this, all of the histamine is found

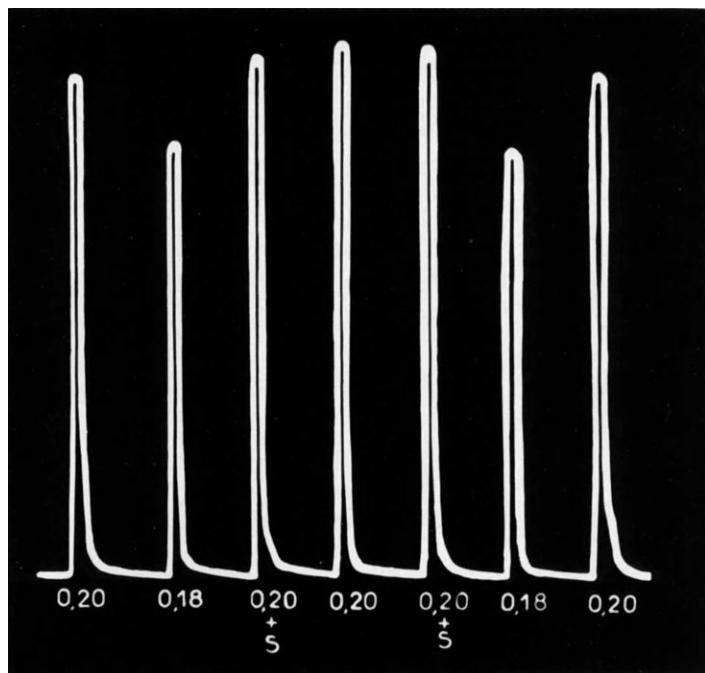


FIGURE 2. Serum from allergic patient. Histaminopexy absent.

TABLE I  
TECHNIQUES FOR EVALUATION OF SERUM HISTAMINOPEXY

<b>A—PHARMACOLOGICAL:</b>	Manual	Laborde <i>et al.</i>	(10)
	Automatic	Craps and Inderbitzin	(5)
		Flavian <i>et al.</i>	(8)
<b>B—PHYSICAL:</b>			
a) Equilibrium dialysis and histamine evaluation by:	Bio-assay Colorimetry Spectrophotometry Radiation count	Parrot <i>et al.</i> Parrot <i>et al.</i> Parrot and Laborde Klamerth Parrot <i>et al.</i>	(20) (14) (18) (9) (15)
b) Ultrafiltration:		Neugebauer and Schmid Parrot and Mordelet-Dambrine	(11) (19)
c) Ultracentrifugation:		Wallenfels <i>et al.</i>	(23)

in the supernatant when serum from allergic patients is used.

The techniques for evaluating serum histaminopexy are shown in Table I.

### Serum Histaminopexy Studies in Man

The results of these studies are summarized in Tables II and III.

**1. Normal Subjects and Non-allergic Patients.** Our findings, which have been confirmed by many authors, showed an average serum histaminopexy of 28% (range: 22-33).

**2. Allergic Patients.** Here, we studied two different types of patients and found that histamine was either permanently absent, or low or absent during the crisis only.

The first group corresponded to atopy, such as asthma, rhinitis, urticaria and Quincke oedema, migraine and diathetic eczema. In these cases, histaminopexy was found to be permanently absent (Table III, A). The same results were found by other authors (2,668 sera, in 90% of which histaminopexy was low or absent).

The second group included cases of tissue-type allergy, such as contact eczema, and various diseases, such as gastroduodenal ulcer, ulcerative colitis, and rheumatic fever. In these cases, histaminopexy was found to be low or absent during a crisis, and

TABLE II  
HISTAMINOPEXY IN NORMAL SUBJECTS AND NON-ALLERGIC PATIENTS

	No. of Cases	Histaminopexy $\leq 10\%$ No. of Cases	Histaminopexy Average Value %
A. 1. Carefully selected .....	79	0	33
Random sampling .....	225	16	27
Blood donors .....	564	58	23
2. Carefully selected .....	201	8	—
Allergic progenitors .....	30	12	—
B. 1. Carefully selected .....	100	0	27.5
Random sampling .....	133	15	—
2. Selected .....	160	13	22

A. Normal subjects: 1. Adults; 2. children.

B. Non-allergic patients: 1. Adults; 2. children. The same results were found by other authors (normal adults: 1,340 sera, in 11% of which histaminopexy was low or absent; non-allergic patients: 1,713 sera, in 39% of which histaminopexy was low or absent).

TABLE III  
SERUM HISTAMINOPEXY IN ALLERGIC PATIENTS

	<i>No. of Cases</i>	<i>Histaminopexy <math>\leq 10\%</math> No. of Cases</i>	<i>%</i>
<b>A. Atopy</b>			
—Bronchial asthma .....	498	434	85
—Urticaria .....	126	115	91
—Rhinitis .....	51	47	92
—Quincke oedema .....	55	44	80
—Migraine .....	86	79	92
—Diathetic eczema .....	170	132	78
<b>B. Tissue-type allergy</b>			
—Contact eczema .....	170	132	78
—Gastro-duodenal ulcer .....	96	82	86
—Ulcerative colitis .....	52	49	94
—Rheumatic fever .....	63	61	90

normal or subnormal between crises (Table III, B). The same results were found by other authors (546 sera, in 80% of which histaminopexy was low or absent).

### Discussion

The clinical findings resulted from studies on almost 12,000 serum samples, carried out by many investigators. We are therefore able to state that: if the serum histaminopexy is normal, there are 90 probabilities out of 100 that the patient's disease is non-allergic in etiology.

Nevertheless, the results of such tests are controversial. Using the automatic apparatus of Boura *et al.* (3), Craps and Inderbitzin (5), failed to confirm our findings, while Flavian *et al.* (8) and Wodniansky (24, 25) used it with success. The serums studied by some authors exerted a toxic effect on the guinea-pig ileum. Pautrizel *et al.* (21) and Cruchaud *et al.* (6) questioned the reliability of the test; on the other hand, Pellerat *et al.* (22) found it reliable and Wodniansky (24, 25) published a statistical analysis of the pharmacological technique (500 serums each studied three times by three different workers in a double-blind test).

### ***Role of Mineral Ions***

**A) Experiments In Vitro.** We showed, by equilibrium dialysis, that normal serum histaminopexy cannot be demonstrated in the absence

of cations (Ca or Mg). On the other hand, K or Cu inhibits this ability of the serum to bind histamine.

**B) Experiments In Vivo.** In the normal rat or guinea-pig, a single injection of KCl abolishes serum histaminopexy for a number of days. On the other hand, in adrenalectomized rats, where serum histaminopexy is absent, a single injection of CaCl<sub>2</sub> or MgCl<sub>2</sub> restores serum histaminopexy to normal for a number of days.

Infusion of Ca-gluconate or MgCl<sub>2</sub> in the asthmatic patient restores the serum histaminopexy values to normal and relieves the clinical symptoms.

### **Action of Neuraminidase**

Neuraminidase unbinds N-acetylneuraminic acid from the proteins and disappearance of the serum histaminopexy ensues. However, this acid, per se, does not bind histamine or inhibit the normal serum histaminopexy. It is probable that N-acetylneuraminic acid plays an important part in serum histaminopexy by conferring specific histamine binding sites to the gamma globulins.

### **Humoral Control of Serum Histaminopexy**

The serum histaminopexy of normal rats shows an average value of 25% (100 animals).

a) *Pituitary:* Histaminopexy is not detectable in the serum of hypophysectomized rats; it can be restored by administration of ACTH or cortisone, but not by desoxycorticosterone acetate or growth hormone.

b) *Adrenal Cortex:* Serum histaminopexy disappears in adrenalectomized rats for at least three months (80 animals), but can be completely restored by administration of cortisone acetate, desoxycorticosterone acetate, or aldosterone (200 animals).

### **Pharmacological Studies**

Some drugs were studied for their ability to restore serum histaminopexy in adrenalectomized rats given saline to drink. Intraperitoneal injection of 200 mg/kg Na-salicylate completely restores histaminopexy. 4909 R.P., a phenothiazine drug with no antihistaminic but with anti-inflammatory and anti-shock activity, affords partial restoration. On the other hand, mepyramine, a specific anti-histaminic drug, failed to restore the serum histaminopexy. Colchicine 0.4 mg/kg exerts an effect that lasts for more than a week. Phenyl-

butazone 50 mg/kg produces its maximum effect between 6 and 8 hours after injection. All these drugs act directly, their effect being independent of their antihistaminic activity.

### Abstract

In studies on clinical manifestations of humoral allergy, two etiologic factors are to be found. One, the background, is hereditary; the other, sensitization, is acquired. The serum of normal subjects is able to bind amines, such as histamine, 5-hydroxytryptamine and acetylcholine. The decrease or the disappearance of this binding capacity seems to be one of the manifestations connected with the allergic terrain.

Serum histaminopexy can be demonstrated as follows: when mixed with normal serum, the effect of histamine on the isolated guinea-pig ileum is decreased. This phenomenon is not due to some antihistaminic activity of the serum, nor can it be explained by a partial destruction of the histamine. The serum of the allergic patient does not bind amines, i.e., its aminopexy, or histaminopexy, is absent or low. These results were confirmed by 50 groups of investigators (12,000 individual evaluations), but were not confirmed by 5 other groups.

The ability of the serum to bind histamine can be shown by various other processes, such as equilibrium dialysis, ultrafiltration and ultracentrifugation. If a mixture of histamine and normal serum is ultracentrifuged, a part of the histamine is carried down with the proteins; but if serum of an allergic patient is used, all of the histamine remains in the supernatant.

A bivalent cation, such as Ca, Mg, or Zn, must be present for the demonstration of histaminopexy; on the other hand, K or Cu inhibits this capacity of the serum to bind histamine. Neuraminic acid appears to play some role, since neuraminidase inhibits histaminopexy. Serum histaminopexy is absent in hypophysectomized rats, but is restored by ACTH. It is restored in adrenalectomized animals by corticosteroids and anti-inflammatory agents, such as salicylate, antipyrine, colchicine and phenylbutazone.

Serum aminopexy still requires further research before the mechanism and the meaning of this phenomenon can be clarified.

### Abrégé

Dans les études portant sur les manifestations cliniques de l'allergie dite humorale, deux facteurs étiologiques généraux sont à considérer. L'un, le terrain, est héréditaire; l'autre, la sensibilisation, est acquise.

Le sérum de sujets normaux est capable de capter certaines amines telles que l'histamine, la 5-hydroxytryptamine et l'acétylcholine. La diminution ou l'absence de cette propriété paraît liée au terrain allergique.

L'histaminopexie sérique peut être mise en évidence de la manière suivante: en présence de sérum normal l'action de l'histamine sur l'iléon

de cobaye est diminuée; or ce phénomène ne peut être imputé à une action antihistaminique du sérum, au sens pharmacologique classique de ce terme; il n'est pas dû non plus à une destruction partielle de l'histamine. Si le sérum provient d'un sujet allergique, il ne modifie pas l'action pharmacologique de l'histamine, ou la modifie fort peu.

Ces résultats ont été confirmés par au moins 50 équipes de chercheurs (ce qui représente au moins l'étude de 12000 sérums), mais n'ont pas été retrouvés par 5 autres équipes.

La propriété du sérum de capter l'histamine a pu être mise en évidence par divers autres procédés tels que équilibre de dialyse, ultrafiltration et ultracentrifugation: si un mélange de sérum normal et d'histamine est soumis à l'ultracentrifugation, une partie de l'histamine sédimente avec les protéines; mais si le sérum provient d'un sujet allergique, l'histamine se retrouve totalement dans le surnageant.

Un cation bivalent, tel que Ca, Mg ou Zn doit être présent pour que l'histaminopexie se manifeste. D'autre part K ou Cu diminue l'histaminopexie sérique.

L'acide neuraminique semble jouer un rôle dans le phénomène ici décrit, car l'action de la neuramidinase fait disparaître celui-ci.

L'histaminopexie sérique est nulle chez le rat hypophysectomisé, mais elle est restituée par l'action de la corticostimuline. Elle est également restituée chez le rat surrénalectomisé par certains corticostéroïdes et par des agents anti-inflammatoires tels que le salicylate, la colchicine et la phénylbutazone.

Ces diverses constatations expérimentales méritent de nouvelles recherches.

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**P. Sacra:** I promise to cut this discussion as short as possible in view of the time. It is my privilege to discuss the paper presented

by Doctor Parrot, whose interesting experiments are conducive to speculation regarding the role played by histamine in various pathological conditions and its possible physiological function.

I found Doctor Parrot's pharmacological and clinical data very useful in connection with some of our own findings. In 1964, we reported that, in animals, several effects of histamine, such as increased capillary permeability (Adamkiewicz, V. W., and P. J. Sacra: *Can. J. Physiol. Pharmacol.*, 43:877, 1965) and gastric secretion (Adamkiewicz, V. W., and P. J. Sacra: *Arch. Internat. Physiol. Biochim.*, 74:21, 1966), the blood pressure response and smooth-muscle contractions (Adamkiewicz, V. W., and P. J. Sacra: *Federation Proc.*, 26:224, 1967), can be inhibited or abolished by increased blood sugar levels. Also, in 1965, we reported that the toxic action of the histamine liberator 48/80 depends on the glycemic level at the time of its injection; a five-fold decrease in toxicity and an almost three-fold increase in the survival time of the animal was observed as the blood sugar was raised from hypo- to hyperglycemic levels (Sacra, P. J., and V. W. Adamkiewicz: *Arch. Internat. Pharmacodyn.*, 156:255, 1965). At that time, all these results were interpreted as an autohaptenic effect of free glucose in the body at the site of histamine action; but now, in view of Doctor Parrot's findings, I realize that there is another possible explanation: that the change in blood sugar levels is followed by a change in the level of a specific blood protein and that this protein may, in turn, inactivate free histamine.

Doctor Parrot has postulated here and in previous communications that histaminopexy is due to the binding of histamine with a gamma globulin, and that  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions are essential for such binding. He has also stated that acquired histaminopexy depends on the presence of a thermolabile agent that loses this binding property on heating to 56°C for 30 minutes. In this connection, it comes to mind that these two prerequisites are very similar to those encountered in some of the steps leading to complement fixation. It has been pointed out by Keller in his monograph on allergy (*Tissue Mast Cells in Immune Reactions*, Karger, Basel, 1966) that, in some biological systems, a component of complement plays an important role in the liberation of histamine from cells. I would very much like to learn Doctor Parrot's opinion as to whether complement, or any of its components, plays a role in histaminopexy. If it does so, is the binding of histamine a mechanism by which the body's defences neutralize that amine, thus rendering it innocuous; or is this binding part of the physiological role of histamine itself, thus inactivating

certain of the body's components, which, if allowed to react unhampered with cells, would lead to cell destruction and tissue injury? In closing, I would like to congratulate Doctor Parrot on his interesting presentation and for shedding some further light on the still poorly understood physiological role of histamine. I would also like to mention that, although 60 years have passed since histamine was synthetized, its physiological role is still an enigma, despite extensive research by so many investigators. In contrast, compare the successful elucidation of the physiological action of the endocrines and of the General Adaptation Syndrome, due in so large a part to the contributions of Doctor Selye; and it did not take him all of his 60 years to accomplish only that.

**J. L. Parrot:** Je remercie le Dr. Sacra de sa très intéressante intervention. Je pense comme lui que le rôle des sucres en général intervient dans ces phénomènes. Je n'ai pas eu l'occasion d'étudier le glucose, mais il est certain que si l'on compare l'héparine, la mucine et l'acide neuraminique qui est déterminant dans la captation de l'histamine par la plasmapexine, on est orienté vers le rôle général d'une molécule sucrée.

Je n'ai parlé, dans mon exposé, que de ce que l'on pourrait appeler le pouvoir histaminopexique inné, et dans ce cas on peut affirmer que le complément ne joue pas de rôle, car si l'on prend un sérum humain normal, on peut lui faire subir un chauffage à 56° pendant 20 minutes, son pouvoir histaminopexique n'a pas changé. Mais vous avez fait allusion au pouvoir histaminopexique que j'ai appelé acquis: on l'obtient chez les malades atteints d'atopie par un traitement qui consiste à injecter un mélange de gamma-globuline humaine et d'histamine; ce traitement a été mis au point au préalable par des expériences sur l'animal. Là, la situation est tout à fait différente, et vous avez raison de penser au complément; le sérum d'un de ces malades avant le traitement ne captait pas d'histamine; après le traitement il acquiert le pouvoir de capter l'histamine; si on lui fait alors subir un chauffage à 56° pendant 20 minutes, on constate qu'il a perdu ce pouvoir histaminopexique acquis; et, bien plus, on peut rétablir ce pouvoir histaminopexique acquis en ajoutant à ce sérum qui a été chauffé, un sérum de cobaye frais qui lui apporte du complément. Je n'insiste pas sur le détail technique de ces expériences: il fallait utiliser un sérum de cobaye qui, par lui-même, était incapable de capter l'histamine. Nous avons eu recours à un sérum de cobaye qui se trouvait au début du scorbut; le pouvoir histaminopexique était tombé à zéro. Dans ce cas, on peut rétablir

le pouvoir histaminopexique acquis d'un sérum humain. Voilà des faits que nous avons observés, Mme Laborde-Burtin et moi et qui sont tout à fait favorables à l'hypothèse que vous avez soulevée: il est possible que dans ce pouvoir histaminopexique acquis le complément joue en effet un rôle.

## **Brain Plasticity: Hormones and Stress**

PAOLA S. TIMIRAS and ANTONIA VERNADAKIS

### **Introduction**

ONE of the most characteristic features of the brain as a tissue and as an organ is its *plasticity*, which is reflected particularly in its ability to be influenced by the external and internal environment. Although this plasticity persists throughout life, the developing brain is the more impressionable and, consequently, more vulnerable to changes in its surrounding milieu.

The embryonic fetal and early postnatal periods are the most critical growth periods for the central nervous system (CNS). Although the prenatal environment is considered to be relatively constant compared with the instability of the post-natal environment, relatively small changes in the internal environment of the fetus may not only result in perinatal death, but may also play a fundamental role in the etiology of neurological disease. The regulatory influence of environmental factors is also operative, but to a lesser degree, during postnatal development, especially during the first two years and at puberty. A better understanding of the physiological development of the brain will help to explain neurological abnormalities, as well as to provide a basis for direct treatment and effective prevention. The present paper discusses effects of hormones and environmental factors, such as hypoxia, X-radiation and sensory stimulation or deprivation, on the biochemical and physiological development of the CNS.

### **Hormones**

The regulatory influence of hormones on CNS development is dependent upon the stage of maturation of the neural tissue at the time of hormonal administration or deficiency, and on the affinity of specific CNS structures for specific hormones (25).

A prime example of critical age and hormonal specificity is the

role of *neonatal androgens* on the differentiation of the hypothalamus. It is well known that the hypothalamus at birth is potentially feminine in function, i.e., at sexual maturation it will show cyclic activity. If, however, androgens are secreted or administered neonatally, the feminine hypothalamus will acquire a continuous, tonic pattern of activity characteristic of the male adult hypothalamus. The transformation of the hypothalamus from a potentially feminine to a masculine type of secretory activity is age-dependent and androgen-specific (11).

Administration of *cortisol* and *estradiol* at critical ages markedly increases the sensitivity of the developing CNS to electrical stimulation. In the rat, the spinal cord is functionally mature at birth compared with the brain, which is relatively immature. The brain develops rapidly in the first 22 days of age (at weaning) and reaches complete maturity at 60 days (after sexual maturation). The critical age period for cortisol to increase developing neural activity is between the eighth and sixteenth day of age (28), whereas for estradiol, the critical age period is between the fourth and the eighth day of age (8, 9). Although the mechanisms by which these hormones accelerate brain maturation are not well understood, evidence shows that their action may be mediated by changes in myelinogenesis and electrolyte distribution (1, 2, 18). It is possible that these hormones selectively influence CNS structures in relation to their developmental sequence.

Hormonal specificity is also demonstrated by the fact that *desoxycorticosterone*, unlike cortisol and estradiol, does not exert any effect on the developing CNS (27). Relatively large doses of testosterone administered to newborn rats do not accelerate the development of neural activity (11); however, a deficiency of testosterone (neonatal castration) results in delayed functional development and altered sexual behavior. Testosterone seems to have a "permissive" rather than a regulatory influence on CNS development (25).

Little is known about the role of hormones on CNS development before birth. Most studies have been concerned with the prenatal development of endocrine functions, with the interrelationship between the hypophysis and other endocrines in fetuses, and with the effects of fetal endocrines on fetal growth. Hormones

have been administered to animals during pregnancy, but the study of their effects on the offspring has provided little information on CNS development. Some understanding of the possible role of hormones on prenatal CNS growth has been obtained from *CNS organ tissue culture* studies. For example, the presence of estradiol and cortisol in the culture medium has a favorable influence on the maintenance and growth of cerebellar explants removed from chick embryos (23). These *in vitro* observations are supported by *in vivo* studies with chick embryos, in which estradiol and cortisol were shown to accelerate brain myelogenesis (6).

There is ample evidence, based on anatomical, electrophysiological and behavioral studies, that *thyroid hormones* participate in the maturation processes of the CNS, both in animals and man. Anatomical studies of the rat have shown that following thyroidectomy at an early age, cerebral cortical cells are reduced in size and in the number and extent of branching of their processes. This anatomical impairment correlates with retarded development of electrical and behavioral activity. The cerebral hypoplasia and hypofunction described in cretinoid animals can be ascribed, in part, to impaired protein synthesis. Studies *in vivo* and *in vitro* have shown a reduction in cerebral RNA concentration per cell (3) and a depressed cerebral protein turnover (5), which reflect a reduced rate of protein synthesis and, consequently, a lower functional activity of the cells. Protein synthesis can be restored, however, if thyroxine is administered within the first twelve days after birth. In the foregoing study, changes in the ionic distribution also were observed in the cerebral cortex of thyroid-deficient rats (5). A deficiency in the Na-K pump would result in a depressed uptake of labeled amino acid, because of the characteristic sensitivity of the brain protein-synthesizing system to ionic environment.

In neonatally hypothyroid rats, reduction in protein synthesis is accompanied by a decrease in acetylcholinesterase (AChE) and cholinesterase (ChE) activity (4). AChE has been used as evidence for the presence of acetylcholine; therefore, a reduction of AChE implies alteration of synaptic transmission. Since ChE activity is predominantly present in glial cells, a reduction of this enzyme

suggests that the function of glial cells may be altered in hypothyroid animals.

### **Stress: Environmental Changes**

#### **A. Anoxia and Hypoxia**

Clinical studies have implicated anoxia before and during birth among the principal causes of cerebral palsy and mental retardation, but as yet most of them have provided no substantial proof of the suspected relationship, and follow-up studies of children with histories of adverse birth conditions continue to indicate that neurological abnormalities are not commonly encountered.

Hypoxia, such as results from exposure to high altitude, induces alterations in CNS development of long-term postnatal duration, as was revealed experimentally with first and second generation rats born and raised at 3,800 meters at the White Mountain Research Station (7, 10). Delayed brain maturation and altered CNS function is accompanied by other signs of retarded growth as well as by general and specific metabolic derangements. Among the neurochemical changes is an increase in carbonic anhydrase activity in the neocortex and hypothalamus. Animals born and raised at high altitude also show a slower increase in AChE activity during development, which points to disturbances of synaptic activity as a contributing factor in delayed CNS maturation (15).

Impaired maturation of the hypothalamo-hypophyseal system at high altitude may be correlated with the low protein levels in the hypothalamus. The decreased hypothalamic function might be responsible for pituitary hypotrophy, and hence retardation of body growth, and hypofunction of the adrenals and gonads (15). Such neuroendocrine changes directly and indirectly influence the animals' adaptation to and survival in the hypoxic environment.

#### **B. Ionizing Radiation**

During the past ten years, there has been increasing interest in the effects of ionizing radiation on the nervous system, both with respect to malformation induced during early development, and functional and structural changes occurring in later stages. It has

been thought for some time that, whereas the embryonic brain is very radiosensitive, the adult brain is relatively radioresistant. However, extensive evidence is accumulating which shows that both the developing and adult CNS undergo structural, biochemical, functional, and behavioral changes after radiation. The magnitude of the damage induced by radiation depends upon the age of the animal and the level of CNS maturity, and also upon dose, dose-rate, CNS structure, cell specificity (e.g., neurons vs. glial cells) and site of radiation (e.g., whole-body vs. head). It has been established that primitive neuroblasts and spongioblasts are selectively damaged by ionizing radiation. It has also been demonstrated that radiation produces specific and predictable anomalies in the nervous system.

Various studies on the functional development of the CNS have shown that prenatal and neonatal irradiation of rats increases the incidence of audiogenic seizures, accelerates the development of electroconvulsive responses, and alters spontaneous cortical rhythms (21, 22). One interpretation of these changes may be that radiation has lowered the threshold of some neuronal elements by blocking the development of inhibition to those elements. Inhibitory systems in rats undergo rapid maturation during the first two weeks after birth; this period, then, may be critical for these systems in terms of their susceptibility to radiation (24). Additional evidence that radiation can affect the development of inhibitory elements in various CNS areas is provided by studies of drugs that are known to act selectively on excitatory and inhibitory systems. For example, early postnatal radiation of rats lowers the dose of strychnine required to produce convulsions (24). Since strychnine is known to block postsynaptic inhibition at various levels of the CNS, a lower convulsive dose for strychnine in irradiated animals represents decreased inhibition, which lowers the threshold at which neuronal elements respond to stimulation (21, 22).

The morphological factors underlying the functional impairment induced by prenatal and early postnatal radiation have been extensively discussed by several investigators. Some of the biochemical alterations that may contribute to changes in neural activity after radiation are changes in lipids, ionic environment,

and enzymatic activity. Radiation during gestation increases glycolipid concentration in the brain of the offspring during postnatal development (20). This increase in lipids may be attributed to postradiation proliferation of glial cells implicated in lipid metabolism of the CNS. The increase of glial cells following prenatal irradiation has been described morphologically, and is substantiated by the finding that DNA content is higher postnatally in the brain of irradiated than control rats (20). Decreases in the Na and Cl content of the cerebral cortex in irradiated animals offer further evidence of the attendant increase in glial cells (17).

In view of the radiosensitivity of the synapse (16), it might be expected that radiation will affect neurotransmitter substances. In fact, impairment of cholinergic transmission after prenatal and postnatal radiation has already been observed (13, 14).

In most of the studies discussed, the alterations in CNS development were produced with relatively *low* radiation doses, insufficient to cause abnormalities in other systems or to decrease survival. These observations, therefore, emphasize the radiosensitivity of the CNS and its increased vulnerability at critical fetal and postnatal periods of development.

### **C. Sensory Stimulation and Restriction**

The concept that increased or reduced levels of stimulation accelerate or delay the development of CNS structures is not new. It is also well known that the growth and maintenance of neural structures are dependent upon the functional demands placed upon them by stimulation. A number of experimental studies have substantiated some of the speculative ideas on this subject. Evidence from laboratory studies with animals seems to confirm that those receiving some form of sensory stimulation or deprivation between birth and maturity differ from controls in their adult behavior and physiology.

Stimulation in the form of daily minimal electroshock, administered from two days of age until adulthood, increases DNA content and ChE activity of the cerebral cortex (26). Accepting the view that neurons remain constant in number, DNA increase

can only represent increase in glial cells. Further evidence for the increased number of glial cells is afforded by the observation of higher ChE activity in the cerebral cortex. An increase in ChE activity in the cerebral cortex is also observed in rats handled at early infancy as well as in rats exposed to complex environments and training activities (26). There is also an increase in AChE activity in the hypothalamus of rats electroshocked daily from two days of age until adulthood (26).

Because of the sensitivity of the hypothalamus to intrinsic and extrinsic stimuli during the neonatal period, an increase in AChE activity may be of special physiological significance, inasmuch as it suggests increased activity in cholinergic mechanisms. In such animals, sexual maturation occurs at an earlier age than in controls. Since hypothalamo-hypophyseal systems are immature in infancy, increased afferent input during this period may accelerate maturation of the nervous pathways involved with sexual function. Accelerated hypothalamic maturation may also explain the adrenal hypertrophy and the increased plasma levels of adrenocortical steroids observed in rats that have been electroshocked or handled from infancy (26).

Sensory *restriction* as well as stimulation may alter the development of cholinergic systems, although with opposite results. Deprivation of light from birth until weaning inhibits development of AChE and choline acetylase activities in some CNS structures concerned with vision, e.g., superior colliculi and lateral geniculate bodies (12). Impairment of the development of cholinergic systems of the optic lobes has also been demonstrated in chicks hatched in the dark (19).

Mothers stressed during pregnancy by visual, auditory, or electrical stimuli produce progeny with observable abnormalities of behavior, metabolism and growth patterns. For example, postnatal development proceeded at a slower rate in the offspring of rats electroshocked at critical periods during pregnancy (unpublished observations). It is possible that the increased amounts of circulating adrenocortical steroids produced in the mother by electroshock may cross the placenta, thereby influencing fetal development and resulting in long-lasting postnatal effects.

### Abstract

It is concluded that hormones and stressing factors directly influence growth and maturation of the CNS. Hormones accelerate the process of myelination, act on mechanisms of cholinergic transmission, affect brain electrolyte distribution, and regulate brain protein synthesis. Hypoxia and radiation alter CNS function by selectively impairing synaptic activity and the development of inhibitory systems. Changes in glial-neuronal interrelations are implicated in the mechanisms of action of radiation and sensory-stimulation.

### Abrégé

Nous pouvons conclure, à la lumière des travaux que nous avons passés en revue, que les hormones et le stress exercent une influence directe sur la croissance et la maturation du système nerveux central. Les hormones accélèrent le processus de myélinisation, influent sur les mécanismes de la transmission cholinergique, affectent la distribution des électrolytes et régularisent la synthèse des protéines du cerveau. L'hypoxie et l'irradiation altèrent le système nerveux central par une diminution sélective de l'activité synaptique et le développement de systèmes inhibiteurs. Les changements dans les interrelations gliales-neuronales sont impliqués dans les mécanismes d'action de l'irradiation et de la stimulation sensorielle.

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## Sleep-wakefulness Patterns and Hormones

GUNNAR HEUSER

THE physiology of sleep has recently become a fascinating field of interest, particularly since various stages of sleep can now be distinguished and a number of centers for sleep have been defined (for recent reviews see 10, 15, 25, 26, 37, 38).

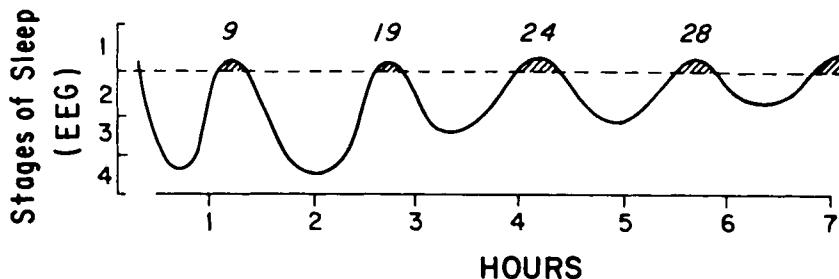
In this paper, I will introduce the subject of sleep by briefly discussing its more conventional aspects (behavior, electroencephalogram, electromyogram, electro-oculogram, electrocardiogram). I will then go on to discuss recent methods used and the results obtained in my laboratory.

### *The Stages of Normal Sleep*

As animals and man fall asleep, they may go through an early stage (as yet poorly defined neurophysiologically) of twitching and disintegration of thought while they enter light sleep.

Deep sleep is known to occur in two separate forms: slow wave sleep (SWS) and paradoxical sleep. SWS is characterized by an electroencephalogram (EEG) with high amplitude slow waves in a quiet animal that appears deeply asleep. By contrast, paradoxical sleep is characterized by intermittent twitching movements (vibrissae, ears, paws, tail), irregular respiration and heart rate, complete relaxation of certain muscle groups, and intermittent rapid eye movements (REM). The EEG during this stage of sleep is very similar to that of alertness and attention. It is desynchronized and of low amplitude.

Synonyms for paradoxical sleep are: deep sleep, D(ream)-state, REM sleep, low voltage fast sleep, rhombencephalic sleep, and desynchronized sleep. All these designations refer to only *one* aspect of this stage of sleep. Since any one of these aspects may or may not be present during certain forms of this stage (17), I prefer to call it paradoxical. This term refers to the fact that some



— Stage 1 REMP Dream Period

FIGURE 1. Typical curve depicting normal sleep pattern in man. Note that the deep stages of sleep are reached more frequently during the early hours of sleep (3, 4), whereas paradoxical sleep (stage 1—REM) time becomes progressively longer during the late hours (see ref. 37).

parameters found in this phase of sleep are similar to those found during the waking state (and yet, the animal is asleep).

During a night's sleep, SWS usually alternates with paradoxical sleep. The percentage distribution of stages is quite characteristic for any given species including man (Fig. 1) and is rather rigidly adhered to. If a certain percentage of SWS or paradoxical sleep is not reached during a given night (e.g., during sleep deprivation), the loss is made up the following night.

When man is awakened from SWS, he usually does not recall dreaming. By contrast, dreams are usually reported when awakening occurred out of paradoxical sleep. The conclusion that dreaming takes place only during paradoxical sleep is probably not justified. For instance, sleep walking and talking were reported to start during SWS (24). The following interpretation is probably closer to the truth: events (e.g., dreaming, sleep walking) during SWS are recalled with difficulty, whereas events (e.g., dreams) during paradoxical sleep are more easily recalled.

#### *Neuronal (Unit) Discharges During Sleep*

Electrophysiological methods for the exploration of the brain include: impedance measurements, DC recording of slow changes, conventional electroencephalographic recording of events in the

intermediate frequency range, and recording of fast events, i.e., discharges of individual cells.

One of the cardinal problems in neurophysiology is whether or not a given area in the brain is more or less active during a given experimental situation. Impedance, DC and EEG recordings do not answer this question. Conventional EEG recordings are at best a measure of hypersynchrony of large cell populations. Furthermore, signals can be transmitted into dead tissue from which EEG recordings can readily be obtained.

By contrast, the firing rate of individual cells (units) is directly related to local neuronal activity. Over the years, studies of discharges of individual cells with microelectrodes have become highly sophisticated and have contributed much to basic neurophysiology. However, most are limited by the fact that they were done in "acute" preparations and that it is difficult to generalize findings obtained from the behavior of a single cell. "Acute" preparations are unphysiological preparations indeed. This is true whether they are anesthetized or curarized (and artificially ventilated) or sectioned (e.g., encephale isole).

It would thus appear that recording of electrical discharges of "multiple units" in a chronically implanted freely moving animal should yield more meaningful information than any other method. This type of recording can be accomplished by setting electronic filters in such a way that they reject the low frequency range of the conventional EEG and accept only the fast frequency range of individual neuronal discharges. The frequency and amplitude changes can then be electronically integrated and be expressed as "tonic," "background," or "averaged activity," or as "unit envelopes" (2, 5, 6, 36, 39, 47, 48). Usually, the write-out (a curve) is in parallel with other parameters studied (e.g., conventional EEG, EKG, etc.): an upward deflection of the curve reflecting an increase in frequency and amplitude of the electrical discharges of the total population of neurons (units) studied in a given area of the brain.

Investigators who have used this method have reported that neuronal activity decreases during SWS, is high during the quiet alert stage, and is highest during paradoxical sleep. These changes were seen in some but not all areas of the brain (36, 48). By con-

trast, in the corpus callosum, tonic activity was lowest during paradoxical sleep (3). Other authors (33) described multiple unit bursts during SWS but no definite decrease or increase of unit activity during paradoxical sleep, in the area of the brain they studied.

The advantage of the averaging technique is that conventional macroelectrodes can be used: their large uninsulated tip lies in close proximity with a great number of cells, the discharges of which are then integrated. A further advantage is that this technique can easily be employed in the chronically implanted animal. The disadvantage of this method is that individual detail is lost: no single cell can be examined in detail and cells with different amplitudes of discharge cannot be compared. This comparison should be possible, since a given cell supposedly always discharges with the same amplitude.

The amount of detail lost in averaged recordings becomes immediately apparent when one examines reports of *single* cell recordings during SWS in the anesthetized or immobilized (curare, encephale isole) preparation (1, 8, 30, 32, 40, 43, 46). Here, not only slowing of frequency but also grouping of discharges (which were at times related to the slow waves) were reported in certain areas of the brain, while the frequency was high and discharges were regular during the more alert stages. In this type of preparation, it is also possible to relate unit activity with DC shifts (13, 39) or to the EEG (7).

Some authors have obtained recordings of single cell discharges in unrestrained animals by "fishing" for cells with a roving micro-electrode inserted via remote microdrive control. Slowing and grouping of unit firing was described in some areas of the brain during SWS (12, 21-23, 34) or after anesthetics (29), but was not described in other areas (4, 11).

In studying these reports, it becomes apparent that the firing patterns of different cells can only be compared by recording first from one and later on from another cell. This is a definite disadvantage. Figure 2 illustrates how much more meaningful a comparison becomes when simultaneous recording of two cells is accomplished. In this case, the two cells responded differently to different stimuli (31).

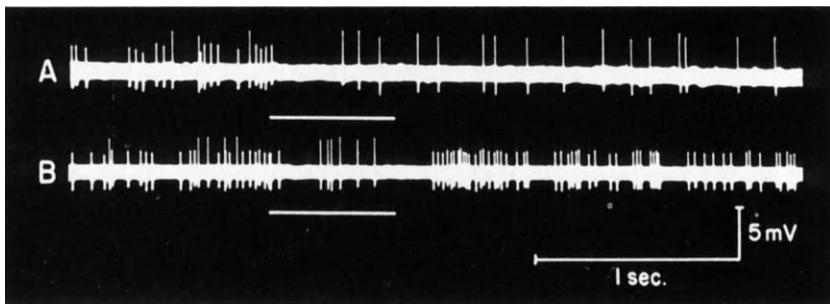


FIGURE 2. Opposite effects by two different odors on the same cells recorded in the external plexiform layer of the olfactory bulb. Rabbit under Urethane. White signals indicate the olfactory stimulation applied through the ipsilateral nostril. Two cells of different amplitude, recorded simultaneously. *A*: Olfactory stimulation with amyl acetate completely inhibits the small unit and facilitates the large one. *B*: Olfactory stimulation with clove oil briefly facilitates and subsequently inhibits for a long period the large unit. The small unit is inhibited during the stimulation and facilitated afterwards.

(From Mancia *et al.*: *Arch. Ital. Biol.*, 100:449, 1962.)

In view of these data, it becomes apparent that recording from two or more cells in a chronically implanted animal would be ideal. We believe that we have accomplished this. Our method and first results are discussed below.

We (in cooperation with Dr. Viliam Jonec of the Institute of Endocrinology, Slovak Academy of Sciences, Bratislava, Czechoslovakia) began by implanting cats under pentobarbital anesthesia with insect-pin electrodes that were insulated except for the tip (diameter of  $50\ \mu$ ). After a recovery period of 1 to 2 weeks, these animals were studied in a sound-attenuated box. Electrical activity of the brain was led through a cable to an EEG machine and an oscilloscope. The signals were amplified and filtered appropriately, so that units could be recorded in parallel with a conventional EEG, both units and EEG thus originating from the same point. The neuronal discharges were also fed into a four-window integrator circuit that was specifically developed for the purpose. With the aid of this circuit, a certain amplitude of unit discharges can be selected and the frequency of the discharge in that amplitude range (window) can be electronically counted and distinguished from the count in the other windows.

At first we obtained recordings from a "forest" of units, but soon found that the number of recorded cells could be decreased to a more informative range if we monitored neuronal discharge during implantation of electrodes. Thus, with the help of a micro-drive, we slowly advanced the electrode tip and selected a position that yielded records from only a few cells. Figures 3-6 illustrate our results and at the same time the flexibility of this approach.

With this new approach, the following questions can now be asked and answered:

- 1) Is there indeed a differential change in the firing rate of different cells (e.g., high vs. low amplitude) during certain conditions? Do some cells then slow down whereas others speed up? Or do they slow down and/or speed up at different rates?

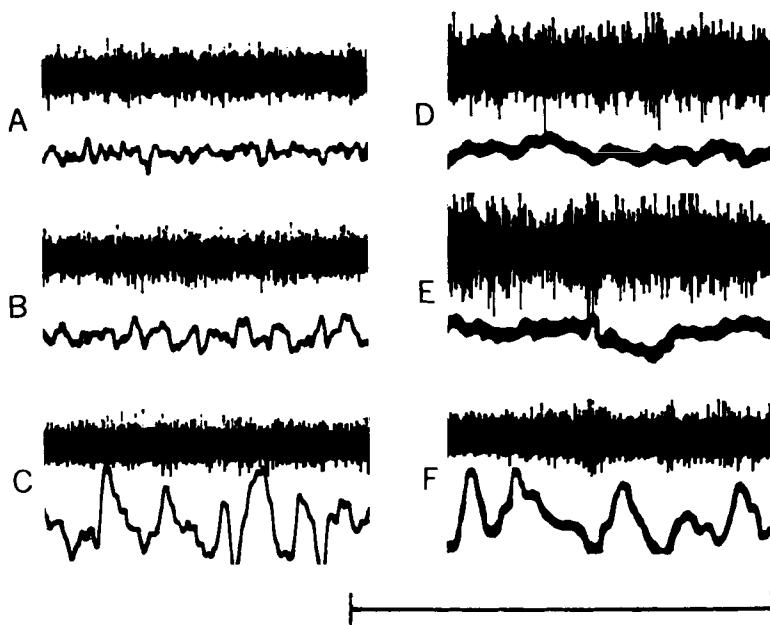


FIGURE 3. Parallel recording of conventional EEG and "forest" of multiple units from the same site in the unrestrained cat. *A* and *D*: Paradoxical sleep. *B* and *E*: Alert. *C* and *F*: During SWS, the multiple unit record shows no change in amplitude on the left, whereas there is a marked decrease on the right. No grouping of unit discharges is seen in these examples. The cat is still alive. Tracings on the left were derived from an electrode aimed at the ventromedial hypothalamus, tracings on the right from an electrode aimed at the pericentral gray.

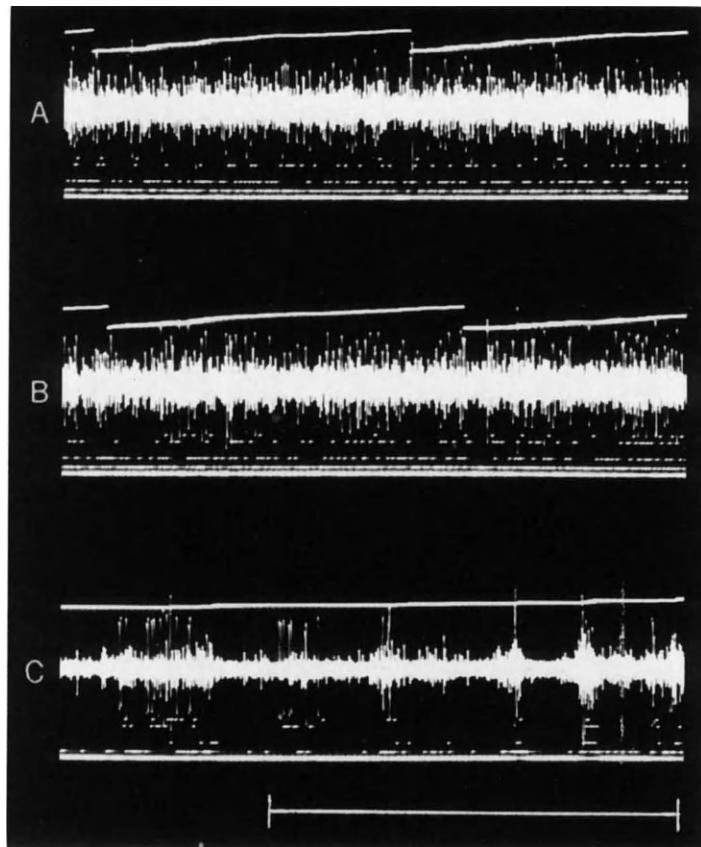


FIGURE 4. Multiple unit recording in unrestrained cat has here reached a point where we can distinguish more detail. *A*: Alert. *B*: Paradoxical sleep. *C*: SWS during which characteristic grouping (in "clusters") of high and low amplitude units can be seen at this site (electrode aimed at reticular formation).

Below the unit record, note the horizontal line above which dots in four different planes are seen. The dots closest to this line represent discharges of units within a predetermined low amplitude range ("window"). The upper rows are dots corresponding to unit discharges within a progressively higher amplitude range (always measured from zero line up). Our "Four-Window-Circuit" will be described elsewhere in detail. This amplitude pattern of discharges can be put on tape, fed into a computer, and analyzed. A cumulative recording of discharges in any given amplitude range (window) can also be obtained as illustrated in the ascending lines above the unit record. They count, here out of the second lowest window, to a peak of 50 discharges and then automatically reset to zero. If enough unit detail is thus provided, statistical and computer analysis of neuronal firing patterns becomes possible. Time base: 1 sec.

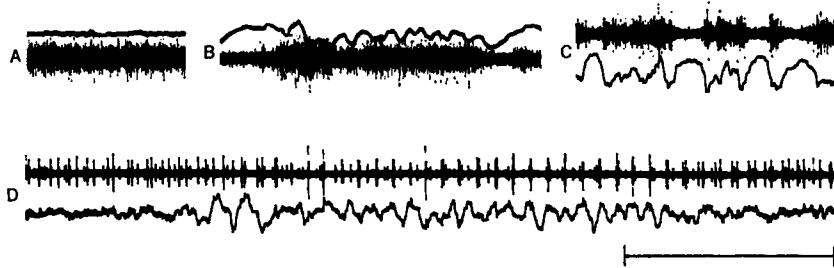


FIGURE 5. Parallel recording of EEG and multiple units from the same site in unrestrained cats with examples, from various areas of the brain, of the possible relationship between EEG and unit discharge during SWS. *A*: Alert. *B*: SWS (same cat as in *A*). Note that EEG shows a downward shift during slow wave EEG activity and cluster discharge of units. *C*: SWS in another chronically-implanted cat. The EEG is displaced downward during unit discharge and upward during quiescence of unit activity. *D*: SWS in another freely moving cat. Unit recording shows fewer units and more detail. The parallel EEG appears unrelated at first, but later falls in step with the grouped unit discharge. Electrodes were aimed at the dorsal fornix in *A* and *B*, at the anterior suprasylvian gyrus in *C*, and at the anterior hypothalamus in *D*. Time base: 1 sec.

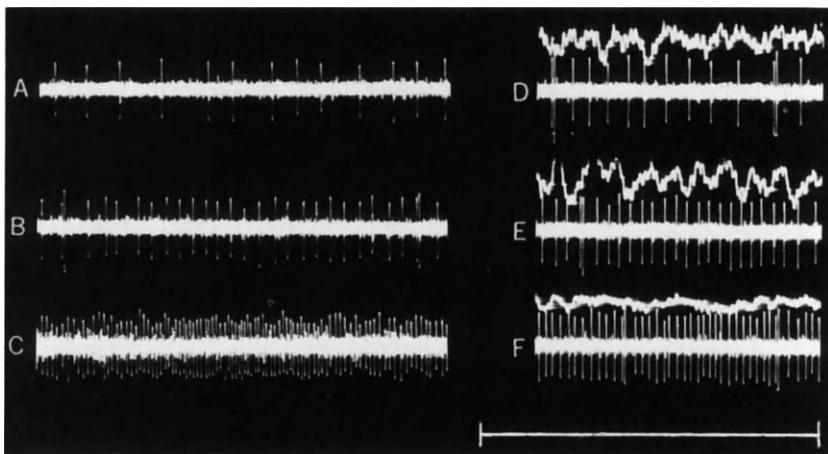
2) Is there grouping (with clusters) of cell discharges during certain conditions? If so, do all cells participate?

3) Is the timing of a discharge of a given cell influenced by the preceding firing pattern of other cells?

4) Under what conditions and in what areas of the brain are EEG and unit discharges related, if recorded in parallel from the same site? This brings up the interesting question whether an unrelated EEG pattern has originated at a distance and has invaded the recording site without changing unit activity, whereas a related EEG might originate at the site of recording.

5) Are there centers in the brain that can be pinpointed as subserving a well-defined function? For instance, is there an area in the hypothalamus in which unit populations increase their firing rate during ACTH release, while other areas maintain their original rate of discharge?

This and other questions come to mind and make multiple unit recording in the freely moving animal a fascinating new tool in neurophysiology and neuroendocrinology. We plan to employ



**FIGURE 6.** Unit activity (electrode aimed at posterior hypothalamus) in a freely moving cat which is very drowsy in *A*, moderately alert in *B*, and fully alert and about to move in *C*. Note increasing frequency of discharge. In *D* and *E*, the cat was in SWS. The record in *D* shows a quiet period between two spindle bursts. Note that unit firing is more frequent during the actual spindle burst (*E*). The discharges are even faster during paradoxical sleep (*F*), and comparable to the alert record in *C*. Note coupling (lower amplitude unit followed within a short interval by a higher amplitude unit), in *B*, *D*, *E* and *F*. Time base: 1 sec.

this technique extensively and have just started to use it in the study of neuroendocrinological feedback mechanisms. Reports on hypothalamic unit activity in "acute" preparations are already available (9, 42, 45), and will be compared with our results in chronically implanted animals.

#### ***Sleep and CNS Depression***

Normal sleep has been so well defined in recent years that the definition of abnormal sleep patterns has now become feasible. In addition, comatose conditions of varying etiology will have to be distinguished from sleep.

**Sleep is normal if:**

- a) The subject can be aroused to full alertness.
- b) All stages of sleep are present and their distribution falls into the normal range for a given age and sex.

**Sleep is abnormal if:**

- a) The subject can be aroused but not necessarily to full alertness.
  - b) All stages are present, but their distribution does not fall within the normal range.
- CNS depression (e.g., anesthesia, coma) is present if:
- a) The subject cannot be aroused (or be aroused only with great difficulty).
  - b) The successive stages of sleep cannot be recognized (e.g., absence of paradoxical sleep).

For the clinically oriented investigator, this distinction of physiological sleep from abnormal sleep and CNS depression is very important, since disease and drugs may interfere with normal sleep.

Barbiturates, for instance, markedly reduce the paradoxical phase of sleep, thus inducing abnormal rather than normal sleep. In high doses, they induce CNS depression. When barbiturates are withdrawn, paradoxical sleep occurs in excess (subjectively often experienced as nightmares) for up to 5 weeks. This, of course, makes it very tempting for the subject to return to the sleeping pill habit (35).

It should be noted that, depending on the dose (or drug or hormone) or the severity of a disease, the spectrum of changes can extend from normal sleep to abnormal sleep to CNS depression. Progesterone, for instance, induces near-normal sleep in low doses, while anesthesia is induced with high doses (17). Hypothyroidism leads to progressively abnormal sleep (27) until, in severe cases, hypothyroid coma ensues.

These endocrine influences on sleep will now be discussed in more detail.

### ***Steroid Hormones and Sleep***

Most biologically active steroids are also CNS-active. This was initially shown by Selye (41) in 1941 when he discovered steroid-induced anesthesia. Reports on the CNS-effects of steroids followed in rapid succession. When Woodbury (49) reviewed the field in 1958, he included 618 references.

Recently, we have added and investigated a number of new effects:

1) A spectrum of recurrent seizure activity (tonic, tonic-clonic, myoclonic, falling spells), preceded by mild sedation with slow waves, after single doses of dehydroepiandrosterone and 11-desoxycortisol (16-19).

2) Sleep after local (directly into the preoptic region of the forebrain) or systemic (i.p.) administration of progesterone to unrestrained freely moving chronically implanted cats (17, 20). This sleep is characterized by recurrent episodes of slow wave and paradoxical sleep. Paradoxical sleep time is equal to or somewhat increased over that seen in normal control runs.

Similar results were obtained in preliminary studies on human volunteers.

Steroid hormones undoubtedly contribute to the physiology and pathology of sleep. Until sleep is investigated in animals or in man in whom both adrenals and gonads have been removed, we will not know if these hormones are essential for physiological sleep.

### ***Thyroid Function and Sleep***

It is well known that patients with hypothyroidism show slowing of mentation and motor activity, and complain of feeling cold and sleepy. In addition, they are often depressed and have crying spells. We (27) studied the sleep patterns of such patients before and after treatment with desiccated thyroid. It was found that stages 3 and 4 (the deepest stages of SWS) were markedly reduced in hypothyroid patients. As treatment progressed, the sleep pattern began to approach that of normal adults in that age group.

These results are of particular interest, since psychiatrically depressed patients were also reported to have a reduction in stages 3 and 4 sleep. Whether this reduction of stages 3 and 4 is a non-specific effect of disease, or whether it indicates a similarity between the depression of thyroid and manic depressive disease, remains to be investigated.

### ***The Hypothalamic Hypophyseal System and Sleep***

Extensive lesions in the hypothalamus of experimental animals and extensive hypothalamic disease in man have both been reported to induce marked sedation. This sedation has not been

neurophysiologically defined. In other words, it is at present not known whether this sedation represents normal or abnormal sleep or CNS depression (as defined above). Recently, a patient with a large hypothalamic tumor (inoperable) and marked somnolence was referred to me. This bedridden young female had a markedly abnormal EEG with predominant slow wave activity that made evaluation of sleep stages very difficult. Rapid eye movements did, however, occur and the patient could be aroused when sensory stimulation was strong enough. In the meantime, two more patients with hypothalamic somnolence were referred to me. They are ambulatory and a more detailed study of their sleep patterns will be undertaken.

Hypothalamic somnolence may well be a purely neuronal event. It should be noted, however, that a number of pituitary hormones induce sedation if administered in high doses (28). Of these, oxytocin originates in the hypothalamus, whereas anterior lobe hormones can easily reach this part of the brain. Since a hypothalamic lesion most likely changes the blood brain barrier in that area and probably disturbs neuroendocrine function, it could well lead to somnolence on that basis.

### ***Other Endocrine Glands and Sleep***

The effects of parathyroid or pancreatic disease on sleep have not thus far been investigated. It can be anticipated, however, that sleep patterns are modified, as is consciousness, when calcium or glucose levels change markedly.

### **Abstract**

The neurophysiology of sleep is briefly reviewed. It is pointed out that the new technique of multiple unit recording in unrestrained chronically implanted animals reveals some fascinating changes in neuronal firing patterns in different stages of sleep. Our technique for and results with multiple unit recording during sleep are described. The effect of hormones on sleep is then reviewed. It is concluded that the total endocrine environment contributes to the physiology and pathology of cerebral function and sleep-wakefulness.

### **Abrégé**

L'aspect neurophysiologique du sommeil est brièvement discuté. Il est démontré que la technique nouvelle d'enregistrement pluricellulaire par

l'implantation de microélectrodes en demeure chez des animaux libres de leurs mouvements, révèle des changements considérables dans la distribution temporelle des décharges neuroniques au cours des différents stades du sommeil. Notre technique d'enregistrement pluricellulaire et les résultats obtenus au cours du sommeil sont décrits. Ensuite, l'action des hormones sur le sommeil est discuté. En conclusion, il appert que l'activité endocrinienne au total influe sur la physiologie et la pathologie du fonctionnement cérébral et de l'alternance éveil-sommeil.

### Acknowledgements

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## **Endocrine Factors in the Etiology of Peptic Ulcer**

ANDRÉ ROBERT

**T**HREE seems to be no doubt that without acid and pepsin in the stomach, peptic ulcer does not form. However, the vast majority of people do secrete acid and pepsin and only a very small percentage (less than 10%) ever develop an ulcer; it is clear, then, that by itself the acid-pepsin complex rarely succeeds in inducing the disease and that other factors are necessary. Two main areas have been particularly explored in the last three decades and found to play an important role in the etiology of peptic ulcer, namely, the psychological status and hormones. Although it is neither in the scope of this presentation nor within our competence to discuss the emotional components of ulcer disease, it may be useful to briefly state the main observations that have been gathered. On a statistical basis, ulcer patients have been found to be of a characteristic personality type. They are usually tense and exhibit an infantile oral craving to be loved, to be fed. Because of this subconscious desire to be loved, they refrain from expressing openly their aggressiveness for fear of endangering the close relationship they have maintained with persons with whom they live or work. Such overt animosity would result, they think, in loss of affection from these people. Consequently, these patients are torn between their want for love and the repudiation by the adult ego of such wishes. According to Alexander (1), the gastric hypersecretion seen in most patients with duodenal ulcer would be related to the oral desire to be fed. The subconscious anticipation of food would chronically stimulate the stomach to secrete acid and pepsin in unusually large amounts.

The endocrine factors in ulcer formation are multiple and complex. It seems that most glands do influence (stimulate or inhibit) gastric secretion and/or motility. Among the excitatory hormones, the most prominent are gastrin, insulin and glucocorticoids. The list of inhibitory hormones is longer and includes most of the

gastrointestinal hormones (secretin, pancreozymin, cholecystokinin, enterogastrone), glucagon, epinephrine and vasopressin. We will limit our discussion, however, to the role of the following endocrine glands in ulcer formation: adrenal, pituitary and ovary, and to the syndrome of polyendocrine tumors.

### A. Adrenal Hormones

#### *1. Clinical Studies*

Patients with Addison's disease secrete little or no acid and pepsin (50, 51) and they rarely, if ever, suffer from peptic ulcer (4, 30, 48). However, with the advent of corticoids, several cases of peptic ulcer were reported in patients with Addison's disease during the course of glucocorticoid therapy (18, 21, 23), which also promoted gastric secretion (50, 51). These observations suggested that glucocorticoids can induce ulcer formation. This view was later substantiated by numerous reports of ulcers in patients treated with glucocorticoids for chronic diseases such as arthritis, lupus erythematosus and asthma. The incidence of steroid-induced ulcers in humans ranges from 10-25% of treated cases (42).

Glucocorticoids also increase acid and pepsin secretion in subjects with intact adrenals. This was first demonstrated by Gray and his co-workers in 1951 (20). In the following years, this observation was challenged by some investigators who could not reproduce it either in man or in animals. However, Gray had insisted that the rise in acid and pepsin took place only after several days and sometimes weeks of continued treatment, a fact that has been overlooked in several studies (15, 25, 34). Although there are a few exceptions (5, 16, 29), Gray's observations have now been amply confirmed, both in man (8, 26, 28) and in dogs (9, 12, 12a, 33, 36, 49, 57). Concomitant with the rise in acid and pepsin, the number of parietal cells increases during treatment with glucocorticoids (12, 19, 25, 26, 36). The increase in parietal cell mass is of such magnitude (50%) that it may account for the rise in acid secretion.

#### *2. Experimental Studies*

We have studied the gastric effects of glucocorticoids in rats and were able to produce peptic ulcers within a short period of

treatment (38). The procedure consists in fasting the animals for three days while injecting a glucocorticoid (e.g., prednisolone) once a day. At the end of treatment, the animals are sacrificed, their stomachs are dissected out, opened along the greater curvature and examined with a 2 X magnifier for the presence of ulcers (so-called "steroid-induced ulcers"). These are located in the glandular portion of the stomach (the corpus) and are multiple, deep and bleeding. The duodenum is not ulcerated. The rat is peculiar in this respect; no technique is known to produce duodenal ulcers in this species. The antrum (a triangular segment of the glandular portion located on the lesser curvature with the apex at the pylorus) is never ulcerated. In such animals treated with ulcerogenic doses of corticoids, gastric secretion is not increased but actually markedly inhibited and the degree of inhibition increases with the duration of treatment (40). Perhaps the most significant finding concerning the etiology of steroid-induced ulcers is the marked reduction in mucus formation after corticoid therapy. The decrease in acid may in part be due to the neutralizing effect of blood oozing from the ulcers, but the reduction of mucus is very specific. There is less mucus not only in gastric juice (concentration and output) (39, 40) but also in the mucosa itself [as shown histochemically by the PAS technique (42) and chemically by determining the hexosamine content of the tissue (40)]. Since, under the conditions of our experiments, the ulcers cannot be blamed on hyperacidity (there is actually less acid), and since mucus formation is practically arrested by the hormone, the ulcers may be due in large part to the loss of mucus. Steroid ulcers have since been produced in other species (9) and, in the dog, glucocorticoids were also found to reduce formation of mucus (31).

Mucus has often been considered as a protective substance for the gastroduodenal mucosa. It appears in gastric juice both as droplets extruded from the cells and as whole mucus cells shed into the lumen. Both the physical (adhesiveness, viscosity, adherence to mucosal lining) and chemical (acid combining power, antipeptic activity) properties of mucus make it an ideal material to counteract the irritating effect of gastric juice and of any irritant introduced into the stomach. In fact, mucus may consti-

tute the natural defense against any ulcerogenic agent. It is to be recalled also that mucus cells are the only dividing cells of the gastric mucosa. Mitoses are never seen in parietal and zymogenic cells and the latter two types are believed by some investigators to originate from mucus cells. The turnover rate of mucus cells is very high; it was calculated that in the rat corpus, the whole surface epithelium is renewed every three days and the mucus neck cells in six and a half days (52). It is also significant that steroid-induced ulcers appear in the corpus (which contains the three types of gastric cells: parietal, zymogenic and mucus) but never in the antrum (which contains only mucus cells). The antrum may be protected because the concentration of mucus in its mucosa is very high [twice that of the corpus (39, 41)] and also because of the rapid turnover rate of the mucus cells (the antral mucosa may be renewed in less than 24 hours) (42).

**Conclusions.** Glucocorticoids tend to elevate gastric secretion of acid and pepsin in some species, but they markedly reduce mucus formation. These steroids can also be ulcerogenic. Although the pathogenesis of these ulcers is not well understood, the following hypotheses are thought to be the most plausible.

1. Steroid-induced ulcers are due to a diminution in mucus formation by gastroduodenal mucosa. This reduction renders the mucosa more vulnerable to damage.
2. The anti-inflammatory property of glucocorticoids is detrimental to the gastroduodenal mucosa in that it hampers healing of superficial erosions that normally take place. The anti-connective tissue property of these steroids, especially when directed against the lamina propria, would prevent repair of otherwise innocuous defects.
3. The stimulation of acid and pepsin secretion together with the increase in parietal cell mass following glucocorticoids are probably not, in themselves, of enough magnitude to cause an ulcer; but they still may start the necrotic process because of the associated mucolytic and anti-inflammatory properties (mentioned in 1 and 2) of the steroids.

### **B. Pituitary Hormones**

The pituitary exerts a profound influence on the stomach. After hypophysectomy, all cells involute markedly and gastric

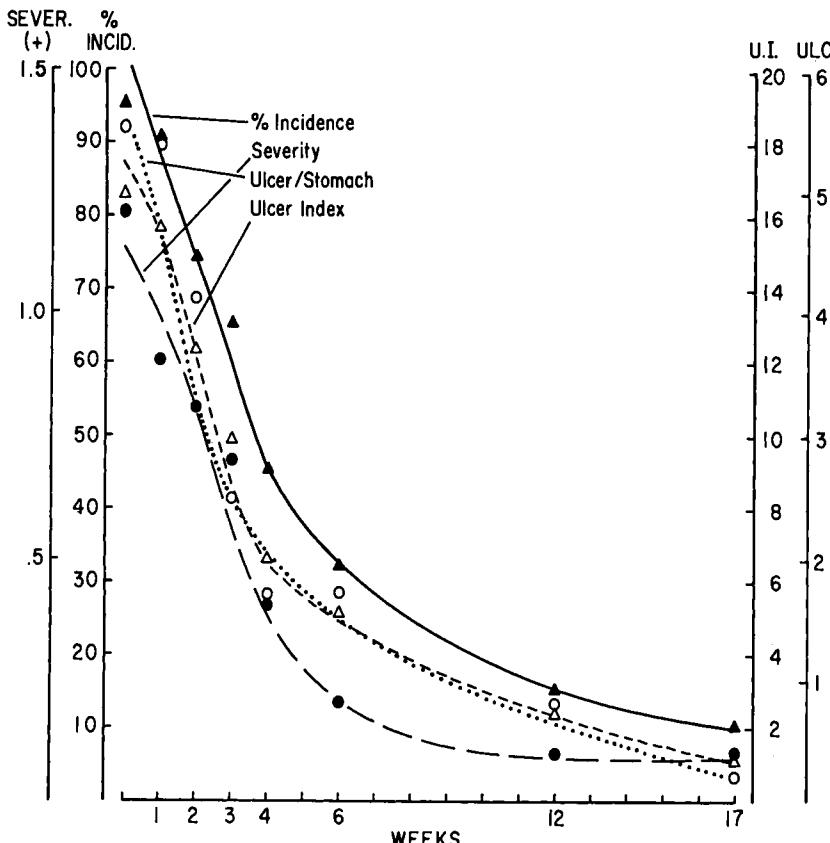


FIGURE 1. Effect of hypophysectomy on steroid-induced ulcers. *Abscissa:* intervals since hypophysectomy. *Sever.:* severity of ulcers from scale 0 to 3+. *Incid.:* % incidence of animals with ulcers. *U.I.:* ulcer index. *ULC.:* No. of ulcers per stomach. (A. ROBERT, J. P. PHILLIPS, and J. E. NEZAMIS: *Proc. Soc. Exper. Biol. Med.*, 121:992, 1966.)

secretion is reduced to very low levels. However, no single pituitary hormone or individual hormone of pituitary-dependent glands (adrenals, thyroid, gonads) can restore the morphology or the secretion to normal (2). It appears, therefore, that the gastric changes seen after hypophysectomy result from the lack of more than one pituitary hormone.

We recently observed that hypophysectomized animals were resistant to steroid-induced (e.g., prednisolone) ulcers and that the degree of resistance increased with the interval between hypo-

physectomy and steroid treatment (43) (Fig. 1). Complete resistance occurred at 17 weeks post-hypophysectomy. Hypophysectomy did not affect other properties of prednisolone; for instance, the degree of body weight loss and of spleen atrophy caused by the steroid was the same as in intact animals. The progressive refractoriness to ulcer formation was not accompanied by a cor-

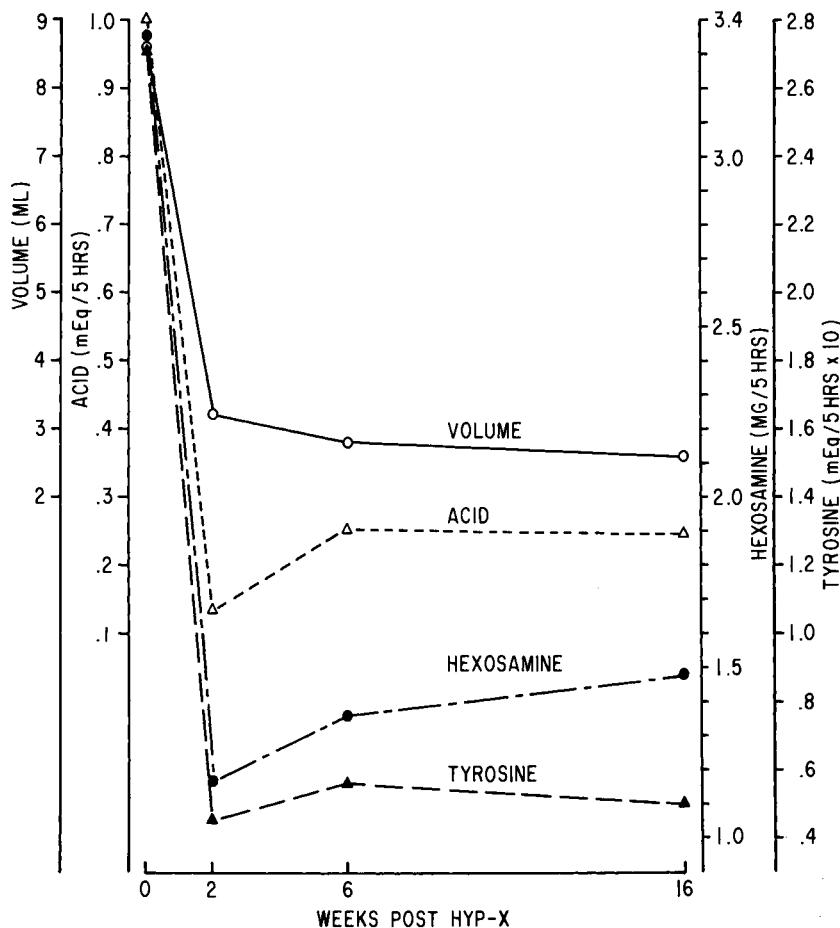


FIGURE 2. Effect of hypophysectomy on gastric secretion. Gastric juice is collected, in fasted rats, five hours after pylorus ligation. "Hexosamine": used as an estimate of mucus content. "Tyrosine": amount of tyrosine liberated from hemoglobin substrate after 10 minutes incubation with gastric juice; this indicates pepsin content. Number of animals per group: intact, 11; hypophysectomized for 2 weeks, 8; for 6 weeks, 9; for 16 weeks, 6.

responding decrease in gastric secretion, since the latter was already maximally inhibited two weeks after hypophysectomy (Fig. 2); the progressive resistance, therefore, appears to be related to some undefined property of the gastric mucosa itself.

In order to find out whether the failure to produce steroid-induced ulcers after hypophysectomy was due to the lack of one or more of the known pituitary hormones, several of these were administered to hypophysectomized rats for 7 days, after which challenging (ulcerogenic) doses of prednisolone (5 mg a day) were given. The results are summarized in the next six paragraphs.

### 1. Hypophysectomy + ACTH

ACTH (8 U.S.P. units of a long-acting preparation, subcutaneously, twice a day) restored the capacity of the stomach of hy-

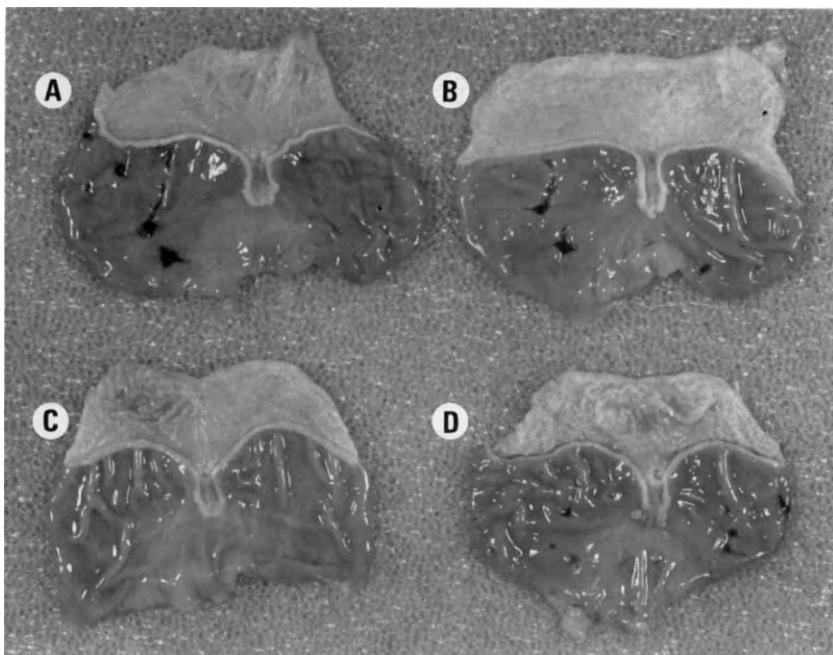


FIGURE 3. Effect of ACTH on steroid-induced ulcers in hypophysectomized rats. A. *Intact + prednisolone*, and B. *Intact + prednisolone + ACTH*. Ulcers located in the corpus. The antrum (central portion) is not affected. C. *Hyp-X + prednisolone*. No ulcer. D. *Hyp-X + prednisolone + ACTH*. Ulcers are present, as in intact animals.

pophysectomized animals to develop steroid-induced ulcers (Fig. 3), although the low values of gastric secretion characteristic of hypophysectomy were not normalized. Therefore, the pro-ulcer effect of ACTH was not due to increased digestive power of gastric juice.

## **2. Hypophysectomy + Low Doses of Prednisolone**

To find out whether ACTH was sensitizing hypophysectomized animals to steroid ulcers by stimulating the adrenals to secrete glucocorticoids, low doses (300 µg/day) of prednisolone were administered for a week prior to treatment with high, ulcerogenic doses of the same prednisolone (5 mg/day). In this experiment, no ACTH was given. This pretreatment with "physiological" doses of a corticoid did not permit steroid ulcers to develop nor did it influence gastric secretion. These results suggested that the pro-ulcer property of ACTH was not due to release by the adrenals of steroids of the prednisolone type. At this point, we were tempted to conclude that the effect of ACTH was not mediated through the adrenals.

## **3. Hypophysectomy + ACTH + Adrenalectomy**

In order to settle this important point, rats were hypophysectomized and 6 weeks later adrenalectomized. A week later, they were given ACTH (8 U.S.P. units twice a day) for one week, after which challenging doses of prednisolone were administered. Under these conditions, that is, in the absence of the adrenals, ACTH no longer sensitized to steroid ulcer formation in hypophysectomized animals. It was clear then that the pro-ulcer effect of ACTH was not direct but adrenal-mediated. However, the adrenal hormone(s), stimulated by ACTH and responsible for the ulcer abetting effect of the latter, does not appear to be a glucocorticoid, since prednisolone could not duplicate the action of ACTH. The role of mineralocorticoids was ruled out in other experiments, when hypophysectomized rats, treated with desoxycorticosterone plus ulcerogenic doses of prednisolone, failed to develop ulcers. It was tentatively concluded that ACTH may favor ulcer formation by stimulating secretion of more than one adrenal hormone or of corticosterone, the natural corticoid for the rat.

#### 4. Hypophysectomy + Triiodothyronine ( $T_3$ )

We wanted to know if pituitary hormones other than ACTH (especially TSH, STH and prolactin) could restore the ability of hypophysectomized rats to steroid ulcer formation. Instead of using TSH itself, we gave  $T_3$  (3  $\mu$ g daily for 7 days) to hypophysectomized rats fed *ad libitum*. When challenged with ulcerogenic doses of prednisolone, these animals developed ulcers whereas hypophysectomized rats not treated with  $T_3$  did not (44) (Fig. 4). As in the case of ACTH,  $T_3$  restored the sensitivity to steroid ulcer formation without altering gastric secretion. It appears, therefore, that both hormones favor steroid ulcer formation by acting on gastric tissue rather than gastric secretion. Although a

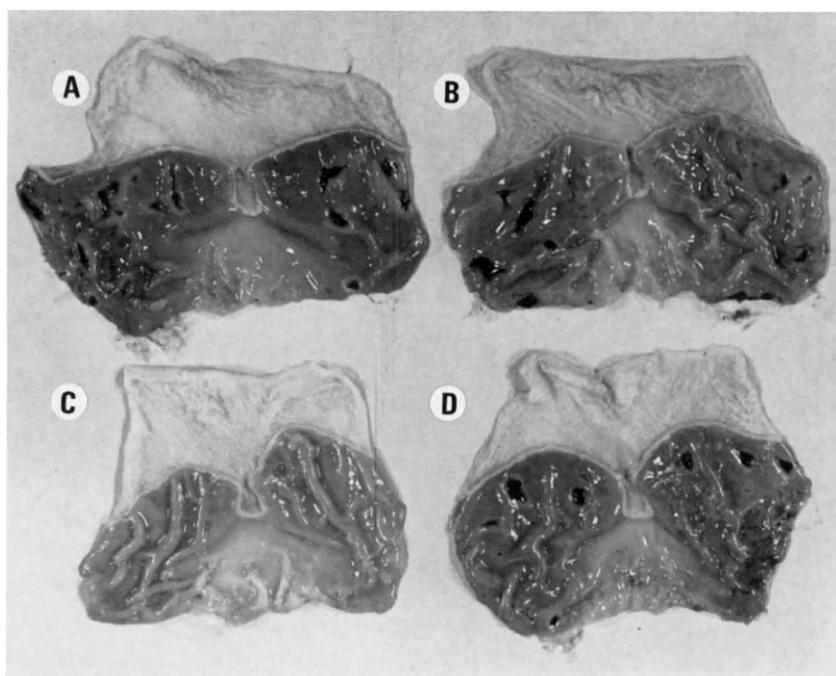


FIGURE 4. Effect of triiodothyronine ( $T_3$ ) on steroid-induced ulcers in hypophysectomized rats. A. *Intact + prednisolone*, and B. *Intact + prednisolone +  $T_3$* . Numerous steroid-induced ulcers in the corpus, none in the antrum. C. *Hyp-X + prednisolone*. No ulcers. D. *Hyp-X + prednisolone +  $T_3$* . Presence of ulcers as in intact animals.

remote possibility, it would be interesting to find out whether  $T_3$  requires also the presence of the adrenals to exert its pro-ulcer effect, as is the case for ACTH.

### **5. Hypophysectomy + STH**

Bovine STH (1.5 mg/day) was administered for one week to hypophysectomized animals (165 g) and then ulcerogenic doses of prednisolone were injected for 4 days. Animals were fed *ad libitum*. Not only did STH fail to restore the ability of hypophysectomized rats to develop steroid ulcers, but these were reduced even further by the hormone (Table I) (45). This anti-ulcer effect is the more remarkable because STH was found to stimulate acid secretion in hypophysectomized rats (Table I) (45). There-

TABLE I  
STEROID-INDUCED ULCERS AND GASTRIC SECRETION IN RATS  
AFTER HYPOPHYSECTOMY AND STH

	Hypophysectomized			Intact		
	I Vehicle (5 mg/day)	II Prednis- olone mg/day)	III STH (1.5 mg/day)	IV Prednis- olone + STH	V Vehicle	VI Prednis- olone
No. of animals .....	8	20	8	24	10	10
Ulcers						
Incidence % .....	0	60	0	21	0	100
Severity 0-3+ .....		.8		.2		1.3
No. per stomach .....		3.9		1.3		8.0
ULCER INDEX .....		10.7		3.6		19.3
No. of animals .....	18		20		10	
Gastric juice						
Volume (ml) .....	2.4 (18)		5.7†		8.2†,‡	
Total acid (mEq/5 hrs) .....	.291 (8)		.625*		.871†	
Hexosamine (mg/5 hrs) .....	1.07 (8)		1.49*		2.15†,‡	
Pepsin (tyrosine: mEq/5 hrs × 10) ....	.406 (13)		.608		1.516†,‡	

Figures shown in parentheses indicate number of samples used for a given determination. In Group I the low volumes of gastric juice in many animals did not permit determination of all components.

† P <.01 when compared with values of Group I.

‡ P <.01 when values of Groups III and V are compared.

\* P <.05 when compared with values of Group I.

(Modified after A. ROBERT, J. P. PHILLIPS and J. E. NEZAMIS: *Am. J. Digest. Dis.*, 11:546, 1966.)

fore, we have here an unusual case where an agent stimulates acid formation in the stomach while preventing ulcers. Perhaps the clue to this paradox resides in the increase in mucus formation also produced by STH in hypophysectomized animals. This abundant, newly formed mucus may have counteracted the ulcerogenic property of prednisolone. When, however, STH was administered to intact animals in other experiments, steroid-induced ulcers were not inhibited. It appears, therefore, that the anti-ulcer property of STH is manifest when ulcers are not too severe, as in the case of recently hypophysectomized animals, but that such activity cannot be demonstrated against severe ulcers, as in the case of intact rats. Also, the normal secretion of ACTH and TSH, shown earlier to favor ulcer formation, may have counteracted the anti-ulcer property of STH in intact animals.

The anti-ulcer property of STH had previously been observed in experiments in which ulcers were produced in the rat by injecting a small volume of formaldehyde into the gastric wall (37). These ulcers normally heal within 48 hours. If, however, the animal has been treated with cortisone, a large crater forms with a necrotic base and no tendency to heal. STH succeeded in counteracting the pro-ulcer effect of cortisone so that 48 hours after injection of formaldehyde the lesion was maximal in the cortisone-treated rats whereas it had practically healed in the animals receiving cortisone plus STH. This anti-ulcer effect of STH was ascribed to the stimulation of connective tissue promoted by this hormone. The resulting scar tissue may have prevented further penetration of the necrotic process.

#### **6. Hypophysectomy + Prolactin**

Bovine prolactin (7.5 mg/day of a purified preparation) did not restore sensitivity of hypophysectomized animals to steroid ulcer formation nor did it reduce these ulcers.

**Conclusions.** From the foregoing, it is clear that a close relationship exists between the pituitary gland and the stomach. In general, one can conclude that a hypofunction of the pituitary reduces both gastric secretion and the sensitivity to ulcer formation. This end result appears to be directly related to the atrophy of the gastric mucosa. On the other hand, hyperfunction of the

pituitary, at least as far as ACTH and TSH are concerned, can, in animals, sensitize to ulcerations. The pathways by which ACTH exerts its pro-ulcer activity has not yet been defined. The protective effect of STH against experimental ulcers is not understood either, but the mucigogue action of this hormone and its known anabolic property seem, at the present, to be the most likely hypotheses.

### C. Sex Hormones

Several observations suggest that sex hormones play an important role in the etiology of peptic ulcer.

#### *1. Sex Incidence of Peptic Ulcer*

Peptic ulcer is predominant in males. The ratio of males to females is 4:1 for duodenal ulcers and 2:1 for gastric ulcers (6). These ratios, however, hold true only between the age of puberty and the onset of menopause. In children of 12 years or less, both sexes are equally prone to ulcers (47) whereas the incidence of ulcers among women increases sharply at the time of menopause (10). These two variations in ulcer incidence point to ovarian hormones as exerting a beneficial effect. This conclusion has been confirmed by a recent clinical study in which an estrogen was given therapeutically (see next paragraph).

#### *2. Effect of an Estrogen on Peptic Ulcer*

Truelove (53) selected a group of 80 male patients with active duodenal ulcers, radiologically positive. Half of these received 0.5 mg of stilbestrol twice a day for 6 months, whereas the other half did not. During the period of treatment, the ulcer recurred in 12 of the controls but in only one of the stilbestrol-treated patients. Similarly, in only four controls did the crater disappear as opposed to twenty of the stilbestrol-treated patients. All the subjects were followed for 5 years, after which the results were reassessed. Table II indicates that the benefits observed in the stilbestrol-treated patients at the end of the 6 months of treatment were still present 5 years later. It was found further that the best results with stilbestrol were obtained in patients with a short history of ulcer (less than 10 years duration). This study appears

TABLE II

LONG-TERM RESULTS IN RELATION TO TREATMENT WITH STILBESTROL

	<i>Stilbestrol Group</i>	<i>Control Group</i>
Prolonged clinical remission with radiological healing .....	24 (61.5%)	11 (27.5%)
Symptoms persisting and/or radiological evidence of ulcer crater present .....	6 (15.4%)	11 (27.5%)
Treated by partial gastrectomy (including 1 patient who died while awaiting operation) ....	9 (23.1%)	18 (45.0%)
Total	39* (100%)	40 (100%)
Significance or difference between stilbestrol group and control group	Highly significant $P < 0.01$	

\* The patient who was lost sight of during the follow-up period has been excluded.

(From: S. C. TRUELOVE: *Brit. J. Med.*, 2:559, 1960.)

very significant to us in that it demonstrates that doses of estrogen not much in excess of the normal secretion observed in women tend to cure an active ulcer and to prevent its recurrence even long after cessation of treatment. These results strongly suggest that one important reason why the incidence of peptic ulcer is lower in women is that their content in circulating estrogen is higher than in men. In the same connection, Sandweiss *et al.* (47) was able to reduce by half the incidence of Mann-Williamson ulcers in the dog by administering human chorionic gonadotrophin, in spite of the fact that this treatment did not reduce gastric acid secretion.

### 3. Pregnancy and Peptic Ulcer

It is common knowledge among gastroenterologists that ulcers are relieved by pregnancy, to the point that it is a rarity to find a pregnant woman with an ulcer (47). The beneficial effect occurs early in pregnancy (probably during the first 3 to 4 weeks) and persists until term. Soon after delivery, however, recurrences appear. In a study of ulcer patients who became pregnant (total of 344 pregnancies), symptoms had recurred in about half the cases by the end of the third month post-partum and in three-fourths of the subjects at the end of 6 months; after 2 years, almost

every patient had suffered a recurrence (10). In other patients (also pregnant but who had never had an ulcer), gastric secretion (basal as well as histamine stimulated) was found to be the same as in non-pregnant subjects (11). Therefore, the beneficial effect of pregnancy cannot be ascribed to a reduced secretion of acid.

It is not clear what factor(s) formed during pregnancy is endowed with anti-ulcer property. Since administration of estrogens to male duodenal patients was beneficial (44) and in view of the lower incidence among females during the fertile period of their life, that is, when they secrete estrogens, one could ascribe the therapeutic effect of pregnancy to estrogens as well. Although estrogens are indeed elevated during pregnancy, so are many other hormones (gonadotrophins, progesterone, corticoids). Also, the psychological lift often seen in pregnant women, characterized by a feeling of accomplishment, may contribute to the improvement of such a psychosomatic disease as peptic ulcer.

In the rat, the acute gastric lesions (edema and hemorrhagic necrosis) produced by a single injection of polymyxin B, a mast-cell discharger, were prevented when the compound was administered in late pregnancy (27). If, however, the fetuses were surgically removed while the placenta was left *in situ*, the full lesion developed. The authors found that the stomach was still completely protected if only one fetus had been left. This suggested that the protective agent was elaborated only if the fetus was present; however, protection against polymyxin lesions persisted during lactation. Two female rats were then united by parabiosis and one made pregnant; injection of polymyxin to both partners produced no lesion in either rat, a result indicating that the protective factor elaborated by the pregnant partner was present in the blood and was carried to the other animal. The authors concluded from these ingenious experiments that "the fetus may produce an agent which is not in itself protective, but induces the elaboration of the protective agent by the mother. Similarly, the act of suckling may in some way produce the protective agent in the mother" (27).

Although the gastric lesions produced in the rat by polymyxin are of a very special type, which may bear no resemblance to

human peptic ulcer, it remains that this type of ulcer was also benefited by pregnancy.

Finally, reserpine was found to be ulcerogenic in pregnant rats and guinea pigs but not in nonpregnant animals (56). This observation indicates that pregnancy is not beneficial for all kinds of gastric lesions.

#### **4. Effect of Sex on Gastric Secretion**

The output of gastric acid and pepsin, whether basal or histamine stimulated, is much higher in men than in women (14, 54, 55). Similarly, the total parietal cell mass is 40% greater in men (13).

**Conclusions.** Although definite conclusions on the role of sex hormones cannot be reached with certainty, the following observations point to a connection between peptic ulcer and ovarian hormones.

1. Ulcer incidence in women is highest when ovarian secretion is at its lowest (before puberty and at menopause).
2. Ulcers are relieved during pregnancy, a condition characterized by marked increase in secretion of ovarian hormones.
3. Estrogen administration is beneficial to male duodenal ulcer patients.

More research on this aspect of ulcer disease should be done as it is likely to yield important information on its pathogenesis and its therapy. In particular, the following questions could be asked:

1. Is the incidence of ulcer in women ovariectomized between the age of 20 and 40 higher than in non-operated women?
2. Are oral contraceptives (usually containing a mixture of progesterone-like steroid and estrogen) beneficial to patients with ulcer?
3. Can the therapeutic effect of estrogens be confirmed in males? If so, what is their mode of action?
4. What is the anti-ulcer agent present in the blood during pregnancy?

#### D. Syndrome of Polyendocrine Tumors

Several authors have reported an association of peptic ulcer and hyperparathyroidism and suggested a cause-effect relationship (17, 24, 46). However, it now appears that the parathyroid is only one of several endocrine glands that can become adenomatous in the same patient, often with development of peptic ulcer. Except in the case of the Zollinger-Ellison syndrome (58) caused by a non-beta cell pancreatic tumor, which secretes enormous amounts of gastrin (22), the mechanism of ulcer formation in the syndrome of polyendocrine tumors is not understood. The glands most frequently involved are the pancreas, the parathyroids, the pituitary and the adrenals (3, 7, 32) and the syndrome is often familial.

#### General Conclusion

The dictum "no acid, no ulcer" which has been repeated for decades may still be true but is an oversimplification of the etiology of peptic ulcer. One also needs ink to write, but a pen filled with ink may never be used to write. A decision must be made to mobilize the hand muscles in such a way that letters will be formed, and a meaningful message has to be thought of. Similarly, accessory, albeit essential, elements conducive to peptic ulcer do exist and seem to consist in a large part of hormones assisted by the proper psychological factors. Enough already is known on the interaction of hormones in this disease to be able to predict that they will eventually have their place in its treatment.

#### Abstract

The stomach is a target organ for many hormones, and some appear to play an important role in the genesis of peptic ulcer. *Glucocorticoids* tend to increase secretion of acid and pepsin and can produce ulcers, in man and animals, when given at either high doses or for long periods of time. These steroid-induced ulcers appear to be due to the marked reduction in mucus formation induced by the corticoids rather than to the rise in gastric acidity. The mucus cell appears to be extremely important for the integrity of the gastric mucosa. 1) It secretes mucus, a viscous, adherent material that protects against physical and chemical damage of irritants. 2) It is the only cell of the mucosa that divides. 3) The turnover of the mucus cell mass is very rapid—3 to 6 days for the corpus, 24 hours or less for the antrum. Consequently, any agent, such as glucocorticoids, influencing the mucus cells

or their secretory products is likely to affect the natural defense of the mucosa.

Removal of the pituitary reduces all functions of the stomach and produces atrophy of its secretory cells; it also prevents the formation of steroid-induced ulcers. These changes are not due to the lack of one particular pituitary hormone, because none, when given alone, can restore gastric reactivity to normal. It appears that ACTH, TSH and STH need to be present for maintenance of gastric integrity. ACTH or TSH suffices to restore sensitivity of hypophysectomized animals to formation of steroid ulcers, even when these hormones are given at doses too low to stimulate gastric secretion. STH, on the other hand, inhibits these ulcers, while, paradoxically, it stimulates acid secretion. The protection afforded by STH may be due to the anabolic (growth stimulation of gastric mucosa) and mucigogue properties of this hormone.

Several observations suggest that female *sex hormones* are beneficial for peptic ulcers. 1) Males are affected four times more frequently than females and also secrete more gastric juice. 2) Before puberty (ovarian function dormant), no such sex difference exists. 3) At menopause (decreased ovarian function), the ulcer incidence suddenly rises. 4) During pregnancy (large quantities of ovarian hormones secreted), ulcers regularly disappear, to recur only after parturition. 5) Stilbestrol was reported to produce lasting remission in male duodenal patients. These results suggest strongly that estrogens possess anti-ulcer activity; however, other factors, as yet unidentified, may contribute to the lower ulcer incidence in the conditions listed above.

A familial syndrome of *polyendocrine tumors*, involving the pancreas, the parathyroids, the pituitary and the adrenals, is often associated with peptic ulcer. The pathogenesis of the latter in such cases is not known.

### Abrégé

Plusieurs hormones influencent la fonction et la morphologie de l'estomac, et certaines semblent jouer un rôle important dans la genèse de l'ulcère peptique. Les *glucocorticoïdes* augmentent chez plusieurs espèces animales, dont l'homme, la sécrétion d'acide et de pepsine et peuvent produire des ulcères après administration prolongée ou à forte dose. Ces ulcères aux stéroïdes semblent résulter d'une diminution marquée de la formation de mucus, produite par les corticoïdes, plutôt que de la stimulation d'acide gastrique. Les cellules à mucus semblent être en grande partie responsables de l'intégrité de la muqueuse gastrique. En effet, 1) elles sécrètent du mucus, substance visqueuse et adhérente qui protège contre les effets nocifs d'irritants physiques et chimiques; 2) parmi les cellules de la muqueuse gastrique, la cellule à mucus est la seule qui se multiplie; 3) le renouvellement de la masse de cellules à mucus est très rapide—de trois à six jour pour le corpus, moins de 24 heures pour l'antrum. En conséquence, tout agent qui, comme les corticoïdes, modifie les cellules à mucus ou leurs produits de sécrétion, est susceptible d'influencer la défense naturelle de la muqueuse.

L'ablation de l'*hypophyse* diminue toutes les fonctions de l'estomac et produit une atrophie des cellules sécrétrices. L'hypophysectomie, de plus, inhibe la formation d'ulcères aux stéroïdes. Ces changements ne sont pas dus à l'absence d'une hormone pituitaire particulière car aucune, administrée isolément, ne réussit à rétablir la réactivité normale de l'estomac. Il semble toutefois que l'ACTH, la TSH et la STH soient nécessaires pour le maintien de l'intégrité gastrique. L'ACTH et la TSH suffisent à restaurer la sensibilité d'animaux hypophysectomisés à la formation d'ulcères aux stéroïdes, même quand ces hormones sont administrées à des doses trop faibles pour stimuler la sécrétion gastrique. La STH, au contraire, inhibe ces ulcères tandis que, paradoxalement, cette hormone stimule la sécrétion d'acide gastrique. La protection par la STH est vraisemblablement due à une action anabolique (stimulation de la croissance de la muqueuse gastrique) et à un effet mucogogue.

Plusieurs observations indiquent que les *hormones sexuelles* femelles exercent une action anti-ulcéreuse. 1) L'ulcère peptique est quatre fois plus fréquent chez l'homme que chez la femme. 2) Avant la puberté (alors que la fonction ovarienne est latente), il n'y a pas de différence sexuelle dans la fréquence. 3) A la ménopause (fonction ovarienne diminuée), la fréquence d'ulcère augmente. 4) Pendant la grossesse (alors que les hormones ovaraines sont sécrétées en grandes quantités), les ulcères guérissent régulièrement pour réapparaître après l'accouchement. 5) On a rapporté que le stilbestrol produit des rémissions prolongées chez des patients mâles souffrant d'ulcère duodénal. Il ressort de ces faits que les estrogènes semblent doués d'une activité anti-ulcéreuse; cependant, il peut exister d'autres facteurs, jusqu'à maintenant non identifiés, qui contribuent à réduire la fréquence d'ulcère peptique dans les conditions ci-dessus mentionnées.

Un syndrome de *tumeurs polyendocrinianes*, souvent familial et incluant le pancréas, les parathyroïdes, l'hypophyse et les surrénales, est souvent associé à la présence d'ulcère, dont la pathogénie reste incomprise.

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**D. J. Ingle:** I am not an authority on the genesis of peptic ulcer, but take special interest in the studies of Dr. Robert for three reasons. First, I have a general interest in the role of hormones, especially those of the adrenal cortex, in the etiology of diseases. The development of this interest was greatly influenced by the research and concepts of Professor Hans Selye and his students. Second, in our earlier studies on hypercorticalism produced in rats by overdosing them with either corticoids or with ACTH we observed the occurrence of ulcers in the G-I tract. Third, I spent twelve happy years at the University of Upjohn—this is the name supposed by

scientists of the Soviet Union whom I once visited—and Dr. Robert, who came later to be a professor in the same laboratories, had the good fortune to acquire as a research associate Mr. James E. Nezamis, who was my first laboratory associate at Upjohn and the best that I have known.

Our first observation that hydrocortisone was ulcerogenic came in 1945. In a study of the diabetogenic activity of cortisone and hydrocortisone, Ingle *et al.* (*Endocrinology*, 37:341, 1945) observed that 2 of 5 intact male rats that were tube-fed and given diabetogenic doses of steroid died during the experiments and were found to have deep ulcers in the glandular portion of the stomach. The cecum of one rat had several ulcers, two of which penetrated the wall. We never again observed a steroid-induced ulcer in the cecum. A year later, Ingle *et al.* (*Endocrinology*, 39:32, 1946) observed an ulcer in the glandular portion of the stomach of an intact, male, tube-fed rat in which hypercorticalism was induced by the frequent injection of ACTH. In a subsequent study, Ingle *et al.* (*Am. J. Physiol.*, 166:165, 1951) administered ACTH by continuous subcutaneous injection to 17 rats and observed ulcers in the glandular portion of the stomach in 12 of them. The lesions varied from multiple tiny ulcers to a few large deep ulcers that almost penetrated the mucosa. In some instances, considerable amounts of blood were lost into the gut from these lesions. During the same year, Ingle *et al.* (*Am. J. Physiol.*, 166:171, 1951) found stomach ulcers in 21 of 22 rats overdosed with cortisone acetate. In these and later studies it was found that ulcers can be induced in almost all normal rats given 10 mg or more of cortisone or of hydrocortisone for 10 days or longer. I emphasize that these observations were made during studies of some of the general metabolic and tissue changes during experimental hypercorticalism and we did not do systematic studies of the ulcerogenic effects of glucocorticoids. It is interesting that ACTH but not prednisolone will sustain the capacity of hypophysectomized rats to develop steroid-induced ulcers. The biologic effects of the individual glucocorticoids are not precisely identical and Dr. Robert offers the plausible hypothesis that either the secretion of more than one corticoid or the secretion of a corticoid differing somewhat from prednisolone in its biologic properties may be necessary to sustain responsiveness. I am reminded that we could normalize the work performance of adrenalectomized rats with beef adrenal extract, but not with any individual corticoid; and I make the old-fashioned suggestion to Dr. Robert that large doses of adrenal cortex extract

be tested in the hypophysectomized rat. I also note that 8 U.S.P. units of ACTH daily is a large dose and may well induce a state of severe hypercorticalism; if ACTH is given by constant injection, even 1 U.S.P. unit per rat per day will cause hypercorticalism.

Many of the great diseases are affected by the presence and absence of several hormones and by non-hormonal factors. We have spent some time studying experimental diabetes and listing means of causing or exacerbating glycosuria and means of ameliorating glycosuria. Under one condition or another, most hormones are capable of causing exacerbation of glycosuria. Dr. Robert has reviewed studies that seem to support the suggestion that peptic ulcer is among those diseases that can be prevented or ameliorated by adrenal cortical insufficiency. Are there any case reports of patients with history of peptic ulcer who have subsequently developed Addison's disease? Studies of coexisting diabetes mellitus and Addison's disease contributed greatly to the understanding of the clinical physiology of both diseases. Does there seem to be an increase in the incidence of peptic ulcer in patients with Cushing's disease? Dr. Robert has noted the reports of ulcers in patients treated with large doses of glucocorticoids for the control of some of the inflammatory diseases. It has been claimed by some gastroenterologists that corticoids only exacerbate gastric ulcers and do not cause them to appear in patients who have no history of ulcers.

I would be interested to learn the thoughts of Dr. Robert on the question as to whether the increased release of glucocorticoids during severe stress plays a role in the production of gastric ulcers in either laboratory animals or in man. Can stress-produced ulcers be made to occur in the absence of the adrenal cortices?

Years ago I was among the laboratory investigators who described corticomimetic effects of estrogens. Although estrogens do not sustain life in adrenalectomized animals, large doses will mimic some effects of corticoids on electrolyte balance, nitrogen balance, and are even glycogenic and diabetogenic. These effects could be demonstrated in the absence of the adrenal glands, but not generally in the absence of the corticoids. The relationship between the actions of estrogens and corticoids is complex and confusing, because estrogens can modify the rates of secretion of corticoids and can modify their metabolism and possibly their peripheral actions. I am interested to note the findings of Dr. Robert that estrogens do not mimic the effects of glucocorticoids on gastric ulcers; they seem to confer some protection.

Although I have no reason whatever to doubt that psychological states play an important role in the etiology of peptic ulcer, I am chary of the generalizations of psychoanalysts, including those of Franz Alexander, about the personality type who is likely to develop ulcers and the psychological bases of their emotional tensions. These hypotheses have not been rigorously tested by controlled experiments and do not meet the requirements for proof that have been found necessary to build a body of knowledge in the biological and physical sciences. I close my remarks by congratulating Dr. Robert and his associates on their highly original and careful laboratory studies on the role of hormones in the genesis of peptic ulcer.

**A. Robert:** I would like to thank Dr. Ingle for his very pertinent remarks. We owe much to Dr. Ingle for his pioneering work in the field of adrenal hormone physiology. In particular, he and his group reported for the first time, more than twenty years ago, on the ulcerogenicity of corticoids and ACTH in experimental animals. Their careful observations have since been confirmed in many laboratories.

Dr. Ingle mentioned that since hypophysectomized animals can be sensitized to steroid-induced ulcers with ACTH but not with physiological doses of prednisolone, one could possibly duplicate this effect of ACTH by administering an adrenal cortical extract rather than prednisolone alone. This point is well taken, since the steroid mixture contained in such an extract may indeed correspond more closely to the various adrenal hormones stimulated by ACTH administration. We recently performed such an experiment in which we treated hypophysectomized rats with adrenal cortical extract (Lipo-Adrenal Cortex at various doses including those that induced body-weight loss. When, however, the animals were challenged with high doses of prednisolone, they failed to develop steroid ulcers. It appears, therefore, that in our experimental model the pro-ulcer effect of ACTH is due either to the release of adrenal steroids not present in the right proportion in the cortical extract, or to a factor secreted by the adrenal upon ACTH stimulation but not present in the extract used. As to the question of whether an ulcer patient who becomes an Addisonian would be relieved of his ulcer, I do not know of any published report on this subject. Such information would be very instructive.

The question of the incidence of steroid-induced ulcers in humans is an important one. Reported statistics go all the way from none to 31% of treated cases. I believe that this wide range of reported incidence of steroid-induced ulcers is related mostly to the care with which these ulcers were sought by the clinician. It must be recalled

that these ulcers are often silent, their symptoms often masked, and that even perforations have occurred without distress to the patient. It is significant that most reports of a high incidence of steroid-induced ulcers in humans were derived from studies in which the patients were systematically examined radiologically (Robert, A., and J. E. Nezamis: *Arch. Pathol.*, 77:407, 1964). From these observations, it is concluded that steroid-induced ulcers develop in susceptible subjects, provided the dose of steroid is high enough and administered chronically. Available evidence indicates that although corticoids can reactivate ulcers, they can also induce formation of ulcers *de novo*. This point has been stressed by several investigators, among whom, Dubois *et al.* (*Am. J. Gastroenterol.*, 33:435, 1960) found that in a series of 63 patients treated chronically with various corticoids (mostly for lupus erythematosus), ulcers developed in 17 cases (27%), of which only one had a previous history of the disease. Moreover, the fact that no radiological abnormality was seen before the treatment started (except for the case with an ulcer history) supports the authors' conclusion that the ulcers were causally related to the steroid treatment.

Dr. Ingle inquired about stress ulcers in humans. Since adrenal steroids are ulcerogenic, and since during stress adrenal steroids are secreted in large amounts, can exposure to stress in man lead to peptic ulcer as a consequence of adrenal hyperfunction? The answer to the first part of the question (can stress induce ulcers in man?) appears to be established, since it has been repeatedly reported that intense stress (burns, prolonged surgery, fractures, cardiac infarct, brain trauma) is often followed by acute ulcers, appearing within 10 days of the stressful stimulus and usually lasting only a few days—although ulcers due to burns (Curling's ulcer) can be fatal. It is not known, however, whether these stress ulcers are adrenal dependent. The fact that, in animals, stress ulcers (e.g., due to cold, spinal-cord transection) appear after adrenalectomy as well (Selye, H.: *Stress*, Acta Inc., Medical Publishers, 1950, p. 689) does not support such a hypothesis.

**R. Guillemin:** First of all, I would like to congratulate Dr. Robert for his very elegant studies. I would like to make a remark that will also be a question to him. All commercial preparations of ACTH do contain, within the limits set by the United States Pharmacopeia, non-negligible quantities of vasopressin. Could it be that chronic treatment with commercial ACTH vs. chronic treatment with the cortical extract might "sensitize" to production of gastric ulcers because of its vasopressin contamination, which of course might

produce constriction of small vessels? What is the effect of synthetic 24-peptide-ACTH or any other synthetic ACTH in a chronic treatment with similar doses?

**A. Robert:** First, to relieve Dr. Guillemin's conscience, I would like to say that our ACTH was made by Upjohn; therefore, it is one of the purest on the market. However, I grant that it still probably contains traces of vasopressin and that the point is well taken; we have not used either vasopressin as a sensitizer or synthetic ACTH. It could be.

**B. Halpern:** The problem of the aetiology of gastric ulcer will certainly not be solved tonight. Having myself had a very long experience in treating patients with corticosteroids in chronic disease, like certain allergic chronic disease, I have seen in the last 15 years hundreds of patients who have been taking steroids for several years. I agree with Dr. Robert in certain aspects that the frequency of gastric ulcers in man chronically treated for years with steroids is greater than is quoted in the literature, for the simple reason that many of them remain undiscovered. We know of many cases of silent ulcers that were revealed by systematic x-ray examination of the patients. But it is also true that at least half of chronically treated patients do not develop gastric ulcers detectable by radiology. Therefore, gastric ulcer in patients given chronically at least therapeutical doses of steroids is not an obligatory consequence like in animals studied by Dr. Robert. Probably the dosage is not the same, if one considers the weight of a stomach of a rat and the weight of a stomach of a man. On the other hand, there is a fundamental difference between the spontaneous human ulcer and the ulcers obtained in steroid-treated patients. We know of many patients who have developed gastric ulcers through prolonged steroid therapy and who recovered rapidly when the therapy was stopped. Usually, the ulcer was healed and healed definitively. We are dealing here with a type of ulcer that is different from the spontaneous gastric ulcer such as is observed in humans.

The third point I would like to mention is the histamine ulcer with which I am a little familiar. Hans Selye will remember, when he came to Paris in 1946, I showed him stomachs of guinea pigs with perforations that I had induced unintentionally in animals receiving high doses of histamine under the protection of the powerful phenothiazine-derived antihistamines. These lesions could be obtained after only about 48 hours' administration of histamine. Is there any relation between steroid- and histamine-induced ulcers as to their mechanism?

## Blood Sugar Levels and Allergies

VINCENT W. ADAMKIEWICZ

I SHALL not attempt on this occasion to review the investigations into the relationships between allergies and the metabolism of sugars. Such relationship has often been observed and reported in the past, mostly as incidental additions to other themes; but no systematic study of it appears to have been made.

I shall simply start with the year 1957 when Andres Goth (22) in Dallas, and I (7) in Montreal, observed almost simultaneously, but quite independently, that the dextran-anaphylactoid reaction in rats depends on the sugar level in their blood. The anaphylactoid reaction, also called inflammation, and considered as not immunological in nature, was discovered by Professor Hans Selye in 1937 (38). The date of the discovery may now safely be revealed, since the present discourse is a contribution of one of his students to the celebrations of Professor Selye's sixtieth birthday. He introduced the appellation "anaphylactoid" in honor of his own mentor, Professor A. Biedl from Prague, who together with R. Kraus used that term to describe the peptone shock they observed in dogs (16). Speaking of R. Kraus, it is useful to recall in passing that, in 1897, he also discovered the precipitin reaction between antiserum and antigen (25) considered as a classical example of an immunologic interaction. The anaphylactoid and immunologic phenomena, having had an almost common historical origin, subsequently led divergent scientific existences. Evidence adduced here points to additional links between them.

### Glycemia and the Dextran Anaphylactoid Reaction in Rats

Anaphylactoid reactions are produced by a single injection of various substances (24), of which dextran has certain advantages.

TABLE I

EXPERIMENTAL PROCEDURES THAT POTENTIATE OR INHIBIT THE  
DEXTRAN-ANAPHYLACTOID REACTION IN RATS

<i>Potentiation</i>	<i>Hypo-glycemia</i>	<i>Reference</i>	<i>Inhibition</i>	<i>Hyper-glycemia</i>	<i>Reference</i>
1. Pretreatment with dextran .....	?	(9)	1. Antihistaminic drugs .....	?	(24)
2. Thyroid hormone administration .....	?	(39)	2. Inhibitors of 5-hydroxytryptamine .....	?	(32)
3. Adrenalectomy .....	Present	(24)	3. Glucocorticoïd-like steroids .....	Present	(24)
4. Fasting .....	Present	(1)	4. D-glucose administration .....	Present	(3)
5. Insulin administration	Present	(7)	5. Alloxan diabetes .....	Present	(2)
6. Chlorpropamide administration .....	Present	(39)	6. Certain simple sugars administration (Present)	(1)	
7. Hypoglycemic drugs administration	Present	(6)			

It is a polymer of glucose—the common physiologic sugar—and it is available in a fairly pure chemical form.

West and his collaborators (39) obtained evidence that the reaction of rats to dextran is a genetic trait regulated by a dominant allele. It is a fact that Sprague-Dawley rats and most other strains are susceptible to dextran, but Wistars are less so and the highly inbred Wistar-Furth not at all (21).

Genetics apart, the dextran reaction has been known to be potentiated or inhibited by a number of apparently unrelated procedures listed in Table I. A glance at this Table reveals that procedures resulting in hypoglycemia potentiate the reaction, whereas those resulting in hyperglycemia inhibit it. Not only the hyperglycemia due to an increase of circulating glucose, but indeed hyperconcentration of other mono- and disaccharides displays inhibiting effects (Fig. 1). On the other hand, equimolar amounts of non-sugars are ineffective in general (Fig. 1) (1).

The inhibition by hyperglycemia is ephemeral. It lasts as long as the hyperglycemia and disappears when the excess sugar is eliminated. This can be observed in rats injected simultaneously with dextran (120 mg/Kg, i.v., 6% w/v in saline, Abbott, M. W.  $\approx$  70,000) and with a hyperglycemic dose of glucose (80 mmol/Kg

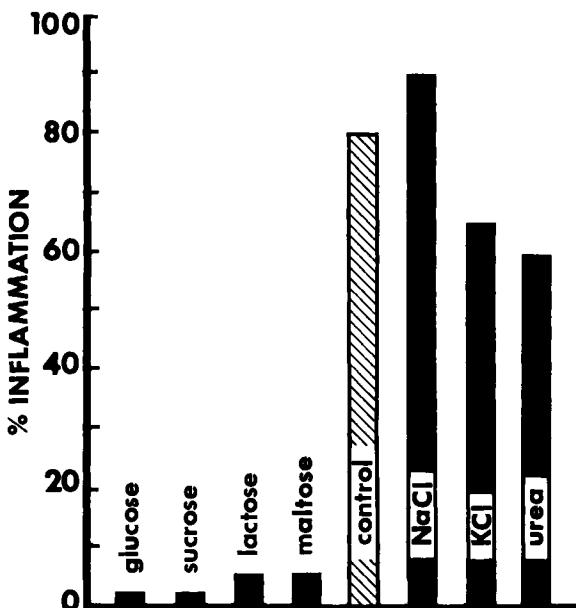
**INHIBITION BY SUGARS OF DEXTRAN ANAPHYLACTOID REACTION (RATS).**

FIGURE 1. Inhibition by sugars of the dextran anaphylactoid reaction in Sprague-Dawley rats. The severity of anaphylactoid reaction is expressed as "% inflammation" at 2 hours after injection of 120 mg/Kg dextran, i.v., in normoglycemic rats. All other substances were injected s.c. in equimolar doses. (From: ADAMKIEWICZ, V. W., and P. J. SACRA: *Fed. Proc.*, 26:224, 1967.)

per os or s.c.) (3). The animals that should normally react within 60 minutes after the dextran show no sign of reaction for two to three hours and up to ten hours if reinjected with the glucose. Only when the excess sugar has been removed from the circulation, the anaphylactoid reaction appears quite suddenly. The animals scratch; their nose, ears, paws and ano-genital regions (called target organs) become warm, red, and conspicuously swollen. The same may be shown with alloxan diabetic rats that are refractory to dextran. But within two hours after lowering their blood sugar with insulin they become susceptible again (Fig. 2) (2).

There exists, in fact, an inverse relationship between the inten-

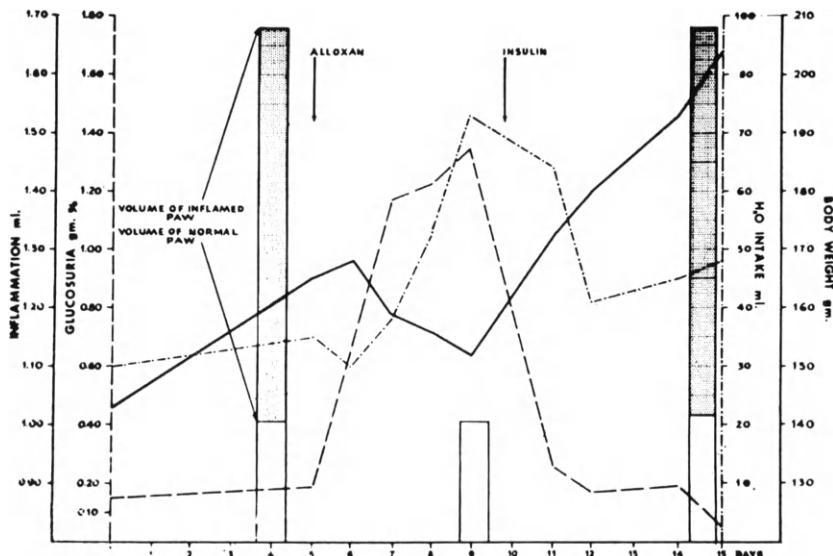


FIGURE 2. Inhibition of the dextran anaphylactoid reaction by alloxan diabetes, and restoration by insulin therapy. The first four days are a control period. A group of Sprague-Dawley rats that grew normally (—), drank a constant amount of water (----), and showed no glucosuria (---), underwent a reaction following a systemic injection of dextran, as judged by the increased volume of a swollen hind paw (shaded rectangle). On the fifth day, alloxan was administered (arrow) and diabetes developed as judged by body weight, water consumption and glucosuria changes. The paws did not swell following dextran (open rectangle). On the tenth day insulin therapy was started (arrow) and the signs of diabetes disappeared. The hind paw became swollen following dextran injection. (From: ADAMKIEWICZ, V. W., and L. M. ADAMKIEWICZ: *Am. J. Physiol.*, 197:377, 1959.)

sity of the dextran reaction and the level of blood sugars (Fig. 3). This relationship does not result from some osmotic change in hyperglycemic animals, since changing the osmosis with non-sugars is without effect (3). Neither is it induced by some metabolite of glucose, since subcutaneously injected equimolar doses of sucrose, which is practically non-metabolisable by this route, yield effects similar to glucose (Fig. 1) (1). What appears to occur, therefore, is a competition between the glucose molecules of the dextran and the circulating free sugars for some receptors in the animal (4). These receptors when combined with the dextran

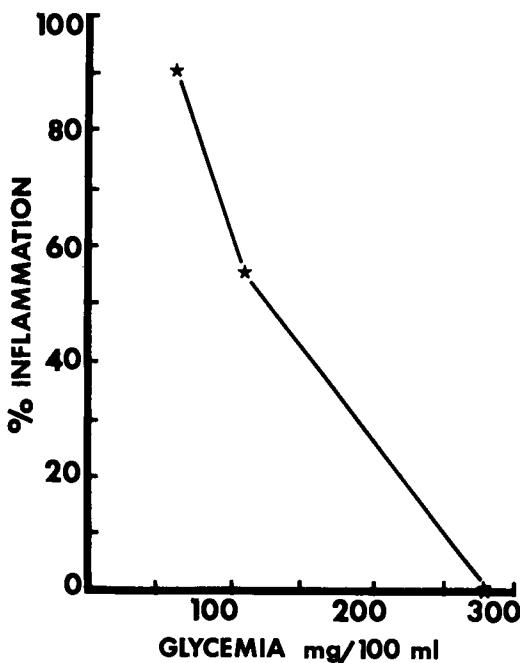
**INVERSE RELATIONSHIP OF ANAPHYLACTOID REACTION TO GLYCEMIA (RATS).**

FIGURE 3. The intensity of anaphylactoid reaction produced in Sprague-Dawley rats by a constant dose of dextran (120 mg/Kg, i.v. M.W.  $\approx$  70,000) varies inversely with blood sugar levels. (From: ADAMKIEWICZ, V. W., and L. M. ADAMKIEWICZ: *L'Union Méd. du Canada*, 94:1264, 1965.)

trigger the anaphylactoid reaction. But when saturated with excess free glucose or with excess analogous sugars, they do not react with dextran, thus appearing to display greater avidity for simple sugars than for polysaccharides. Should this be the case, glucose would exert an auto-haptenic-like inhibition with respect to dextran, akin to haptenic inhibitions of classical immunology (4, 5, 28).

Such effects would be surface phenomena subject to the criteria of the physical-chemistry of surfaces. In the case of the dextran-glucose competition for the surface of some receptors, a Langmuir adsorption isotherm may in fact be derived. It can be shown by means of this that the intensity of the dextran reaction should be

inversely proportional to the concentration of glucose in the blood.

- $V_g$  = velocity of glucose adsorption.
- $V_d$  = velocity of dextran adsorption.
- $V'g$  = velocity of glucose desorption.
- $V'd$  = velocity of dextran desorption.
- $S_g$  = fraction of surface covered with glucose.
- $S_d$  = fraction of surface covered with dextran.
- $C_g$  = concentration of glucose  
(blood sugar level).
- $C_d$  = concentration of dextran  
(dose injected).

The fraction of the surface (receptors) covered with glucose and with dextran is:  $S_g + S_d = S$ .

Therefore, the fraction of the surface that remains free is:  $1 - S$ .

The speed of adsorption of glucose on the free surface is proportional to:  $V_g \approx (1 - S) C_g$ .

The speed of adsorption of dextran on the free surface is proportional to:  $V_d \approx (1 - S) C_d$ .

Adsorption is a reversible process. The glucose and dextran that are adsorbed may also desorb.

Therefore, the speed of desorption of glucose is proportional to:

$$V'g \approx S_g.$$

The speed of desorption of dextran is proportional to:

$$V'd \approx S_d.$$

When the speeds of adsorption and desorption of glucose and dextran reach equilibrium we have:  $V_g = V'g$  and  $V_d = V'd$ .

$$\text{Therefore: } \frac{V_g}{V_d} = \frac{V'g}{V'd}$$

$$\text{By substituting: } \frac{(1 - S) C_g}{(1 - S) C_d} = \frac{S_g}{S_d} \text{ and } \frac{C_g}{C_d} = \frac{S_g}{S_d}$$

It was postulated that the anaphylactoid reaction is triggered after combination of dextran with the receptors.

Therefore, the intensity of reaction is proportional to:  $\approx S_d$ .

Consequently, it is also proportional to:  $\approx C_d$ .

And with respect to glucose the intensity of reaction becomes proportional to:

$$\frac{1}{Cg}$$

It is, however, indeed premature to rely too strictly on equations in this system. The Langmuir isotherm, for example, is based on the Law of Mass Action. No proof can be obtained at present that the Law itself applies here (4).

### TARIF, The Transferable Anaphylactoid Reaction Inducing Factor

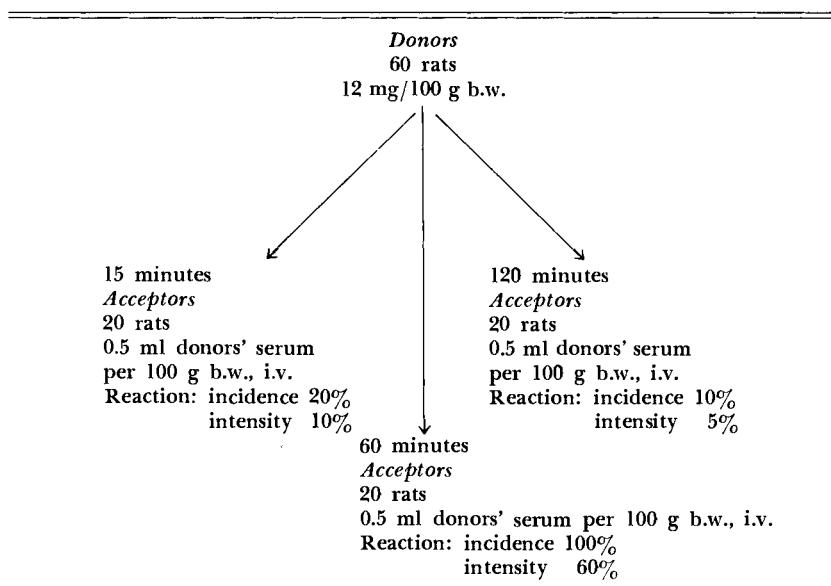
The dextran reaction may be transferred passively from one lot of rats—the Donors—to another lot—the Acceptors. The transfer is possible only during a restricted period of time in the course of the reaction (9). Serum taken about 60 minutes after the injection of dextran into donors (12 mg/100 g, i.v.) and reinjected into the acceptors (0.5 ml/100 g, i.v.) produces in them the same reaction as that seen in the donors, consisting of the conspicuous swelling of the target organs. However, serum taken from the donors at some other time, 15 or 120 minutes after dextran, results in little or no reaction in the acceptors (Table II).

If the donors and the acceptors are made hypoglycemic with insulin (20 u/Kg, s.c., Crystalline-Insulin-Zn, Connaught), the passive transfer may be performed three times, at hourly intervals, from Donors to Acceptors I, then from Acceptors I to Acceptors II, etc. (12). Each successive lot of animals undergoes the anaphylactoid reaction in turn. However, the incidence and intensity subside progressively to disappear in Acceptors IV (Table III, experiment 1). Only two such transfers are possible with serum from normo-glycemic donors, and only one from hyperglycemic donors, the acceptors being hypoglycemic, throughout (Table III, experiments 2, 3 and 4) (12).

At each of the consecutive transfers, the original serum of donors becomes diluted about 20 times by the extracellular fluids of each acceptor. Let us suppose that the dextran injected into the first donors remains intact in their fluids. The amount present one

TABLE II

## PASSIVE TRANSFER OF DEXTRAN-ANAPHYLACTOID REACTION IN SPRAGUE-DAWLEY RATS



(From: ADAMKIEWICZ, V. W., and L. M. ADAMKIEWICZ: *L'Union Méd. du Canada*, 95:1155, 1966.)

hour later should then be equal to:  $\frac{12 \text{ mg}}{20 \text{ ml}} = 0.6 \text{ mg/ml}$ , in a rat of 100 g body weight, or 0.3 mg in 0.5 ml of transferred serum. After the second transfer the amount injected into Acceptors III would be:  $0.3 \text{ mg} \times \frac{0.5 \text{ ml} \times 0.5 \text{ ml}}{20 \text{ ml} \times 20 \text{ ml}} = 0.00018 \text{ mg}/100 \text{ g, i.v.}$  But, so small a dose of dextran is insufficient to trigger the anaphylactoid reaction, even in hypoglycemic rats (9). This and other evidence (9, 12) prompted us to postulate that the anaphylactoid reaction is mediated by a factor, the TARIF. This factor, which could consist of a complex between dextran and a physiologic molecule, is formed in the donor rats within some 60 minutes and induces the anaphylactoid reaction in them. Upon transfer, it induces the reaction in the acceptors. The factor is destroyed *in vivo* at a rate such that two hours after its formation too little remains for passive transfer into normo-glycemic acceptors. Hypo-

glycemic acceptors, however, are more sensitive to it and may detect it for longer periods of time. On the other hand, when frozen *in vitro* it keeps for weeks.

The production of this factor is inhibited by glucose (12), because rats with low blood sugar levels make more of it than hyperglycemic rats, three consecutive transfers being possible with the

TABLE III  
GLYCEMIC STATES AND THE CONSECUTIVE PASSIVE TRANSFERS OF  
ANAPHYLACTOID-REACTION-INDUCING FACTOR

Donors*	Acceptors* I	Acceptors* II	Acceptors* III	Acceptors* IV
<i>Experiment 1: Hypoglycemic donors to hypoglycemic acceptors.</i>				
Glycemia mg/100 ml .....	55 ± 4	65 ± 3	62 ± 3	62 ± 3
Anaphylactoid Reaction:				
Incidence % .....	100	100	66	16
Intensity % .....	70	56	32	5
<i>Experiment 2: Normoglycemic donors to hypoglycemic acceptors.</i>				
Glycemia mg/100 ml .....	116 ± 4	60 ± 2	60 ± 2	60 ± 2
Anaphylactoid Reaction:				
Incidence % .....	100	100	83	0
Intensity % .....	45	65	34	0
<i>Experiment 3: Hyperglycemic (glucose) donors to hypoglycemic acceptors.</i>				
Glycemia mg/100 ml .....	216 ± 15	64 ± 3	64 ± 3	—
Anaphylactoid Reaction:				
Incidence % .....	66	83	0	—
Intensity % .....	29	42	0	—
<i>Experiment 4: Hyperglycemic (diabetic) donors to hypoglycemic acceptors.</i>				
Glycemia mg/100 ml .....	437 ± 57	55 ± 4	55 ± 4	—
Anaphylactoid Reaction:				
Incidence % .....	0	83	0	—
Intensity % .....	0	53	0	—
<i>Experiment 5: Hypoglycemic donors to hyperglycemic (glucose) acceptors I, to hypoglycemic acceptors II.</i>				
Glycemia mg/100 ml .....	55 ± 4	195 ± 7	55 ± 7	61 ± 5
Anaphylactoid Reaction:				
Incidence % .....	100	33	83	0
Intensity % .....	67	12	37	0
<i>Experiment 6: Hypoglycemic donors to hyperglycemic (diabetic) acceptors I, to hypoglycemic acceptors II.</i>				
Glycemia mg/100 ml .....	61 ± 2	486 ± 28	55 ± 3	55 ± 3
Anaphylactoid Reaction:				
Incidence % .....	100	0	66	0
Intensity % .....	70	0	27	0

\* Six rats per group.

(From: ADAMKIEWICZ, V. W., and P. J. SACRA: *Can. J. Physiol. Pharmacol.*, 44:615, 1966. Reproduced by permission of the National Research Council of Canada.)

serum of the former and only one with that of the latter (Table II, experiments 1, 2 and 3). The allergic-like effect of this factor also depends on blood sugar levels, since the injection of TARIF-containing serum into hyperglycemic Acceptors I does not result in anaphylactoid reaction, but reinjection of the serum from such non-responding Acceptors I into a new lot of Acceptors II that are hypoglycemic leads to a reappearance of the reaction (Table III, experiments 4 and 6) (12). Glucose therefore appears to be involved at both ends of the life-cycle of TARIF. It inhibits its production in the donors, probably by an autohaptenic-like effect. It inhibits the anaphylactoid reaction TARIF produces at the other end in the acceptors, by a mechanism whose elucidation must await further knowledge of TARIF's structure. At any rate, this last inhibition is a reversible process as shown by experiments 4 and 6 of Table III.

It has been suggested by some investigators that TARIF may be identical with free dextran (26, 27). However, in their experiments, the strain of rats and other conditions differed from those reported above, and no attempt was made at more than one consecutive passive transfer. On the other hand, Polushkin (33), using the Prausnitz-Küstner test, demonstrated the presence of a reagin-type antibody against egg-white in the serum of albino rats. Such rats, when injected with a single dose of egg-white, undergo an anaphylactoid reaction undistinguishable from that induced by dextran.

Anaphylactoid reactions have not been considered truly immunological and have been classed together with certain other hypersensitivities under the heading of "non-immunological equivalents of hypersensitivity reactions" (17). The reason is mainly that they do not require pre-sensitization and are not accompanied by the presence of identifiable antibodies. However, several of the anaphylactoid agents do probably react with pre-existing "natural antibody" (33) and elicit a conventional immune response. Furthermore, complement-containing guinea-pig serum potentiates the dextran anaphylactoid reaction (8) while the inhibiting effect of hyperglycemia may also be reproduced in the following classical immunological situations.

### Anaphylaxis and Immune Hemolytic Anemia

Normal rats and mice are resistant to horse-serum or egg-white anaphylactic shock. It is, however, sufficient to lower the blood sugar levels of these animals (insulin, or 24-hour fast) to obtain high anaphylactic mortality and an exacerbation of the specific anaphylactic lesions in the gut and elsewhere (13). Should the hypoglycemic state be neutralized by administrations of glucose, the animals regain their original resistance and the anaphylactic lesions subside (Table IV). An inverse relationship occurs in mice between the intensity of the shock and the blood sugar levels (Fig. 4), similar to that for the dextran reaction in rats (Fig. 3). In the case of anaphylaxis, the change of the glycemic status is brought about at a time when antibody production is fully completed. Therefore, the inhibitory effect of the blood sugars

TABLE IV

AGGRAVATION OF THE ANAPHYLACTIC SHOCK IN THE RAT BY HYPOGLYCEMIC STATES

<i>Experiment, Group and Treatment</i>	<i>Number of Rats</i>	<i>% Mortality of Rats</i>	<i>Number of Rats</i>	<i>Intensity of Lesion in the Ileum</i>
<i>Experiment 1—Horse-serum anaphylactic shock</i>				
G I, normo-glycemic controls .....	20	0	10	1.84 ± 0.16
G II, fasting hypoglycemic state .....	20	50	10	2.37 ± 0.24
G III, fasting hypoglycemic state neutralized with glucose .....	20	0	10	1.65 ± 0.26
G IV, hyperglycemic with glucose .....	20	0	10	1.05 ± 0.18
<i>Experiment 2—Horse-serum anaphylactic shock</i>				
G II, insulin hypoglycemic state .....	20	40	10	2.40 ± 0.29
G III, insulin hypoglycemic state neutralized with glucose .....	20	0	10	1.87 ± 0.17
<i>Experiment 3—Egg-white anaphylactic shock</i>				
G I, this is a control group that was not sensitized but which was in an insulin hypoglycemic state, and challenged .....	20	0	—	—
G II, normo-glycemic controls .....	20	0	—	—
G III, insulin hypoglycemic state .....	20	60	—	—
G IV, insulin hypoglycemic state neutralized with glucose .....	20	0	—	—

(From: ADAMKIEWICZ, V. W., P. J. SACRA, and J. VENTURA: *J. Immunol.*, 92:3, 1964.)

**INVERSE RELATIONSHIP OF ANAPHYLACTIC  
SHOCK TO GLYCEMIA (MICE).**

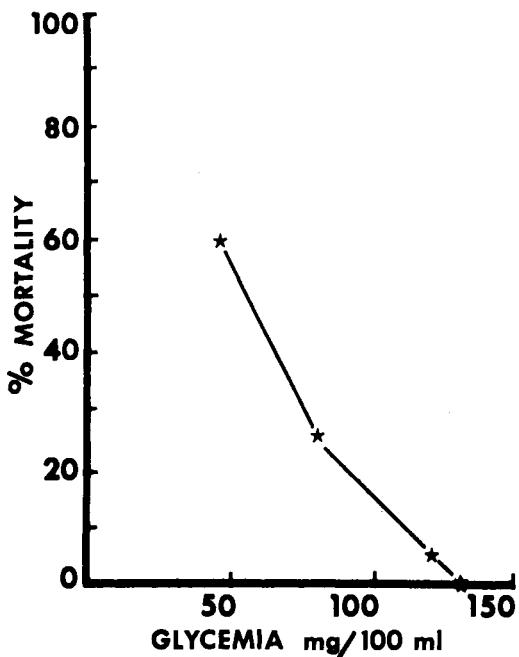


FIGURE 4. The intensity (% mortality) of anaphylactic shock produced by a constant dose of horse-serum in mice varies inversely with blood sugar levels. (From: ADAMKIEWICZ, V. W., and L. M. ADAMKIEWICZ: *L'Union Méd. du Canada*, 94:1264, 1965.)

must alter the antigen-antibody complex formation or its sequel. Sanyal *et al.* (36) reported that lowering the blood sugar with insulin in rats had no effect on subsequent "in vitro anaphylaxis" (Shultz-Dale reaction). This would suggest that the inhibiting effect of blood sugar occurs in the circulation.

Similar observations may be made in mice and rats with hemolytic anemia, following inoculation with specific rabbit anti-erythrocyte sera (14). The dose of hemolytic antiserum required to produce an LD<sub>50</sub> in mice (within 24 hours) increases directly with the blood sugar levels obtaining at the time of inoculation. Conversely, the mortality following a fixed dose of antiserum is

**INTERRELATIONSHIPS OF FIXED DOSE, FIXED EFFECT AND GLYCEMIA DURING IMMUNE ERYTHROCYTOLYSIS (MICE).**

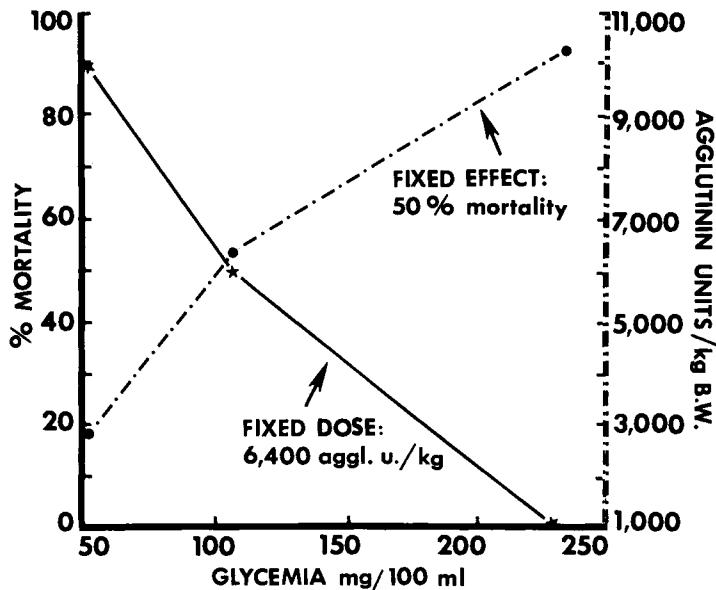


FIGURE 5. The intensity (% mortality) of immune hemolytic anemia (—) produced in mice by a constant dose of rabbit antimouse erythrocyte serum varies inversely with blood sugar levels. But the dose of antiserum required to produce a constant effect (- - -) varies directly with the blood sugar level.

in inverse relationship to the blood sugar levels (Fig. 5). Furthermore, a small dose of antiserum (1,000 agglutinin units/Kg, i.p.) produces little or no mortality in hypoglycemic mice, but the resulting anemia is severe and protracted (10 days). The erythrocyte count falls from the normal of  $9 \times 10^9/\text{ml}^3$  to about  $4 \times 10^9/\text{ml}^3$ . On the other hand, the same small dose in hyperglycemic mice results in mild anemia ( $8 \times 10^9$  erythrocytes/ $\text{ml}^3$ ) of short duration (2 days), (11, 14), (Fig. 6).

In the case of immune hemolytic anemia, unlike in the anaphylactoid and anaphylactic reactions, an upper limit for blood sugar is reached beyond which no further protection occurs (11, 14). This happens in the mouse at some 225 mg glucose/100 ml. Such hyperglycemia still inhibits a dose of antiserum equal to 10,200 agglutinin units/Kg, but higher doses are fully effective inde-

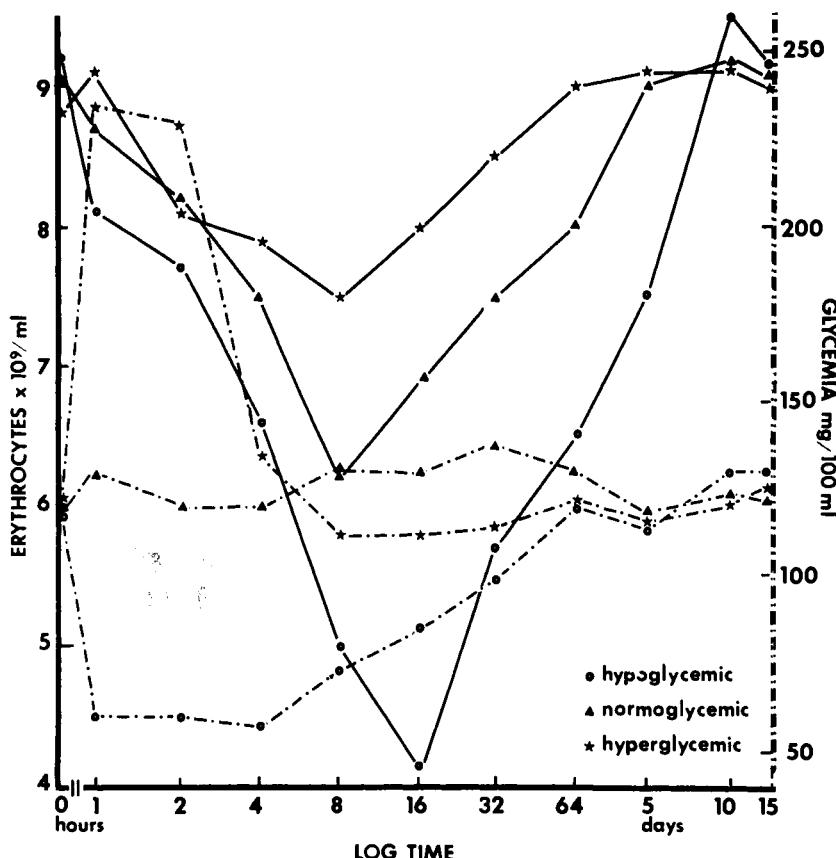


FIGURE 6. Patterns of hemolytic anemia produced by a fixed dose of rabbit antimouse erythrocyte serum, in hypo- (○), normo- (△) and hyperglycemic (\*) mice. Blood sugar levels (-----) were measured at intervals before and after the glycemic state was changed by insulin or glucose administration at 1 hour. At that time, antiserum was also injected. Note that the number of erythrocytes (—) decreases most markedly and recovery is slowest in the hypoglycemic mice. (From: ADAMKIEWICZ, V. W., and P. J. SACRA: *Fed. Proc.*, 26:224, 1967.)

pendently of any blood sugar levels. It thus appears that the number and kinds of receptors that react with the hemolytic antiserum, or with some product of the antigen-antibody reaction, are large. High doses of anti-serum find enough receptors to react with under any circumstances. But, in anaphylactoid and anaphy-

lactic reactions, the number and kinds of the receptors are limited. They become totally unavailable in hyperglycemia.

Various mechanisms could be postulated to explain the inhibition of immune hemolytic anemia in the presence of sugar in the blood *in vivo*. Some mechanisms would require a direct participation of glucose, others the participation of different molecules whose concentration in the blood would be synchronized to the sugar levels. The hemolytic antiserum, of course, hemolyses and agglutinates erythrocytes also *in vitro*, and provides a convenient means for the study of these reactions. However, neither hemolysis nor agglutination of washed rat-erythrocytes is modified by an *in vitro* incubation with the specific antiserum mixed with complement and with various concentrations of glucose (0 to 200 mg/100 ml), (Fig. 9), (11). The dynamics of hemolysis of whole blood taken from hypoglycemic rats and subsequently incubated *in vitro* with antiserum and complement to which various glucose concentrations have been added also remains unaltered (Fig. 7). Glucose, therefore, does not appear to directly inhibit the hemolytic anemia.

The amount of circulating endogenous insulin is synchronized to the blood sugar levels. However, this hormone is unlikely to be involved in the inhibition, because rats and mice overdosed with it so as to become hypoglycemic are as sensitive to the hemolytic antiserum as are the fasted hypoglycemic animals that have low insulin levels.

As in the case of dextran reaction, the inhibitory effect of the hyperglycemic state on immune hemolytic anemia is ephemeral. This may be observed in rats given glucose per os from whom samples of blood are taken at hourly intervals thereafter. The speed of hemolysis is then measured in the samples *in vitro* by addition of antiserum and complement. Sugar levels are simultaneously determined. About one hour after the *in vivo* hyperglycemic peak is reached the speed of the *in vitro* hemolysis slows down considerably. Six hours later as hyperglycemia subsides normal speed tends to resume (Fig. 8). Although the inhibitory effect of hyperglycemic blood is transitory, the resultant neutralization of antiserum is permanent. This is seen in agglutinin hyperglycemic mice injected with the small dose of antiserum

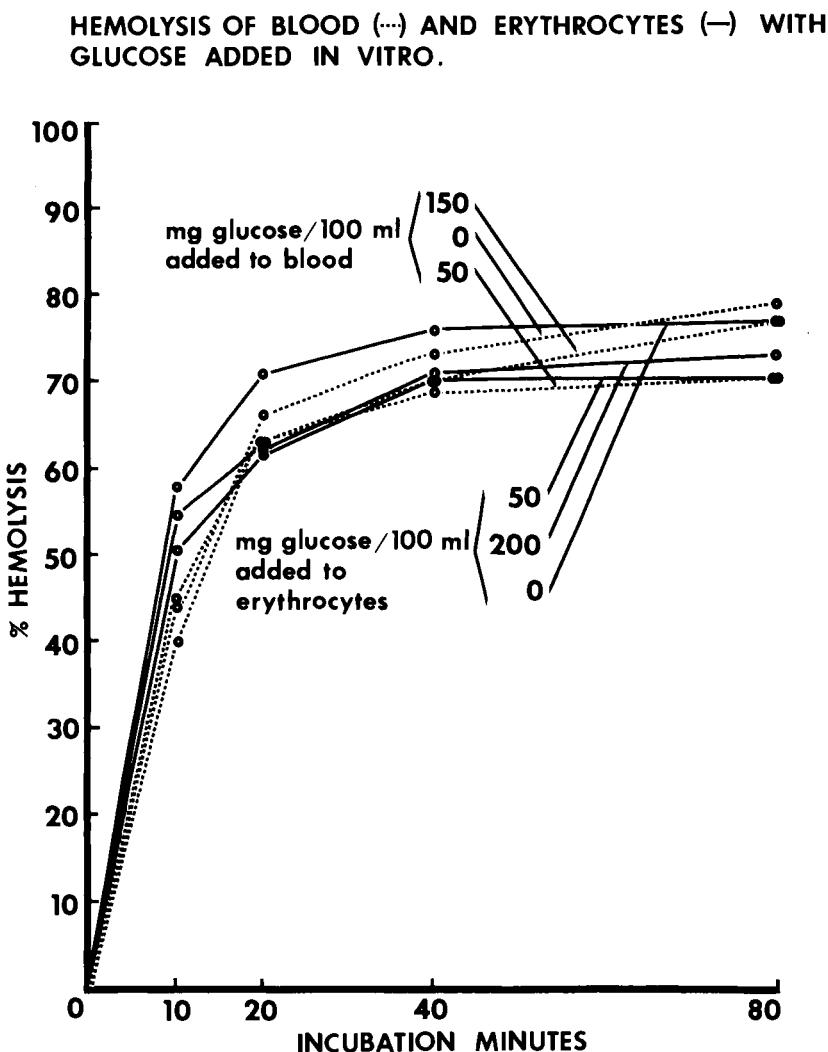


FIGURE 7. In vitro dynamics of immune hemolysis of blood (.....) and erythrocytes (—), following addition of rabbit anti-rat erythrocyte serum, complement, and various concentrations of glucose. Blood and erythrocytes were obtained from insulin-hypoglycemic rats. Note similarity of hemolysis curves despite variations in the amounts of glucose added.

**INHIBITION BY HYPERGLYCEMIA OF IMMUNE HEMOLYSIS OF WHOLE BLOOD OF NORMAL RATS.**

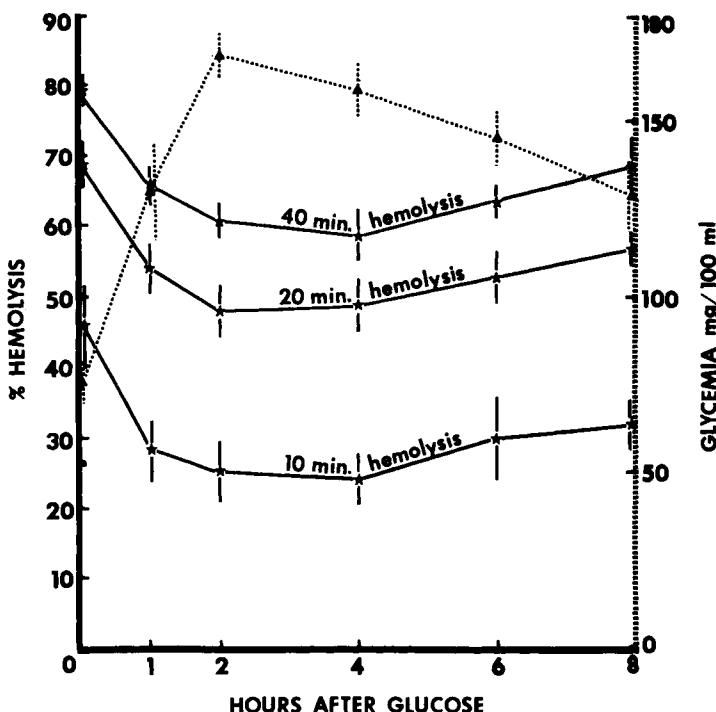


FIGURE 8. Transitory inhibition by hyperglycemia (.....) of the speed of hemolysis of whole blood (—) of rats. At 0 hours, 80 mmol glucose/Kg was administered per os. Samples of whole blood were taken at the 0 hours and at hourly intervals thereafter for 8 hours. The speed of hemolysis in samples was determined by addition of rabbit anti-rat erythrocyte serum and complement *in vitro*. The value of hemolysis was determined after incubations of 10, 20, and 40 minutes. Note that hemolysis is inhibited about 2 hours after glucose administration, irrespective of the length of subsequent incubation. The inhibition corresponds to peak hyperglycemia. Inhibition and hyperglycemia decrease at 8 hours.

(1,000 units/Kg) referred to before (Fig. 6), (14). Blood sugar returns to normal levels within six hours in these mice but the activity of antiserum remains permanently inhibited as judged by the low grade and short duration of the resulting anemia. The dynamics of *in vitro* hemolysis of whole blood depends on whether

**HYPERGLYCEMIC INHIBITION OF IMMUNE HEMOLYSIS OF  
WASHED ERYTHROCYTES (—) AND WHOLE BLOOD (···)**

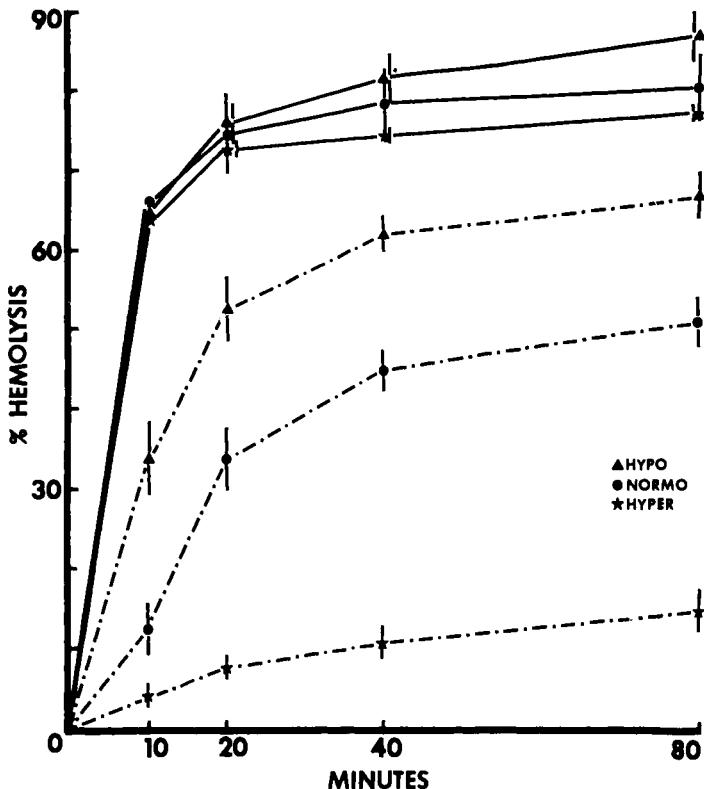


FIGURE 9. Dynamics of *in vitro* immune hemolysis following addition of rabbit anti-rat erythrocyte serum and complement to washed rat-erythrocytes or to whole rat blood. The washed erythrocytes (—) were obtained from hypo- ( $\Delta$ ), normo- ( $\circ$ ) or hyperglycemic (\*) rats. No significant difference in the speed of hemolysis is noted. However, the speed of hemolysis of whole blood (···) from hypoglycemic rats is much faster than in hyperglycemic animals. The erythrocytes and whole blood were obtained at the second hour after administration of insulin or glucose.

the blood originates from hypo-, normo- or hyperglycemic animals (not to be confused with the unchanging pattern of hemolysis of whole blood to which various concentrations of glucose have been added *in vitro*). Whole blood from hyperglycemic animals hemolyses several times more slowly than whole blood from

hypoglycemic rats (Fig. 9). However, washed erythrocytes hemolyse at the same speed independently of their hypo- or hyperglycemic origin (Fig. 9).

It seems clear then that hyperglycemic whole blood contains a transient excess or lack of something that is directly involved in hemolysis. As pointed out already, glucose and insulin are not "it." Could it be the complement: *in vitro* incubation of complement-containing hemolysing systems with various concentrations of glucose does not affect the dynamics of hemolysis. However, these experiments were conducted in an excess of complement and do not exclude the possibility that the concentration of circulating complement *in vivo* is synchronized to blood sugar levels.

Although the effects of various glycemic states on immune hemolysis are quite striking, we failed to demonstrate any such effect on the agglutination reaction using various experimental set-ups.

### Histamine and Compound 48-80

Drugs that inhibit immune reactions display multivalent effects. The antagonists of purine, pyrimidine, folic acid, and especially the alkylating agents, modify fairly specifically any one of the following immunological events: the antibody induction period, primary and anamnestic productions, tolerance and delayed hypersensitivity (37). They also display anti-inflammatory activity against non-specific as well as against the specific immune inflammations that result from delayed or immediate hypersensitivity (37). Somewhat analogous is the multivalent anti-allergic effect triggered by the presence of glucose in blood. This presence inhibits TARIF production as well as its allergic-like action. It interferes with the antigen-antibody combination or its sequel in anaphylaxis and in experimental immune anemia. The presence of glucose in blood also triggers an anti-inflammatory activity.

Endogenous histamine released in immediate hypersensitivity is partly responsible for the subsequent inflammatory changes. It may be demonstrated that the activity of exogenous histamine depends on blood sugar levels. Rats are injected intravenously with Evans blue and by stomach tube with a hyperglycemic dose of glucose (80 mmol/Kg). Every hour thereafter, a "spot test"

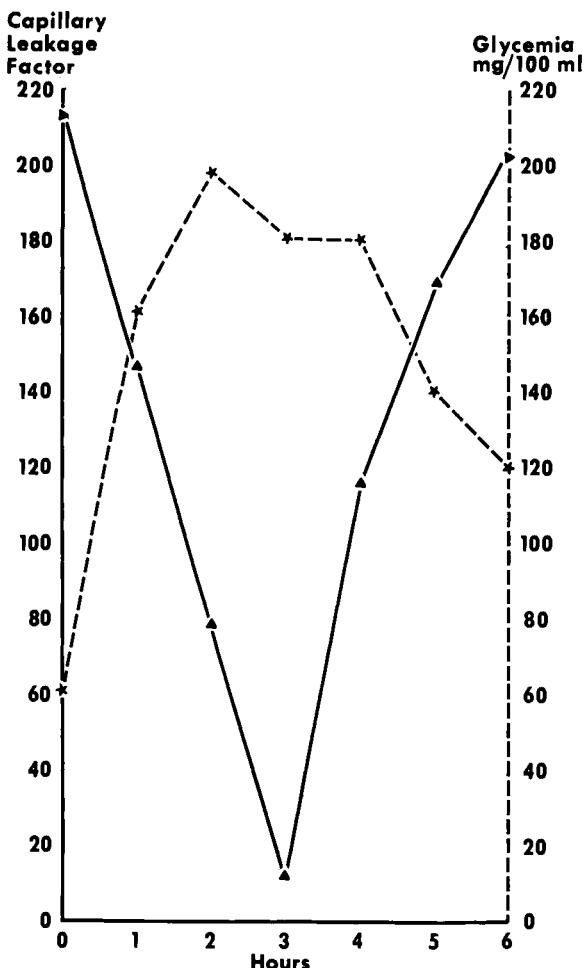


FIGURE 10. Time relationship between blood sugar levels (---) and the capillary leakage (—) in the skin as induced by histamine in rats. 80 mmols glucose/Kg were administered per os. At hourly intervals thereafter, blood sugar levels were determined and a histamine "spot test" was performed in the skin. At the third hour, when hyperglycemia was at a peak, topical injection of histamine (0.02 micromols) induced practically no capillary leakage. (From: ADAMKIEWICZ, V. W., and P. J. SACRA: *Can. J. Physiol. Pharmacol.*, 43:877, 1965. Reproduced by permission of the National Research Council of Canada.)

is performed by intradermal injection of 0.02 micromols of histamine in 0.05 ml solution (10). This dose equals about twice the physiological concentration of skin-histamine, and induces capillary leakage. The blue-colored plasma seeps out and a colored spot forms, the dimensions of which may be measured. The size of this spot becomes smaller as the blood sugar levels increase in the rat. One hour after the peak hyperglycemia is reached (three hours after sugar administration) practically no leakage occurs around the histamine (Fig. 10). As hyperglycemia subsides, the spot reappears and is again full size six hours after sugar administration. The transient inhibition by hyperglycemia in this experiment is identical with that of the dextran-reaction and of the hemolytic anemia.

The specificity of this inhibition may further be studied in normo-glycemic, Evans-blue injected rats treated intradermally with 0.01 to 0.02 micromols of histamine mixed with serial dilutions of glucose, or other simple sugars and control substances (10). The optimal inhibitory molar ratio of glucose to histamine is 16:1. Higher or lower molar ratios, as well as equimolar control substances, do not inhibit the histamine. Various other simple sugars display specific although distinct optimal inhibitory molar ratios (Table V).

Goth *et al.* showed both *in vivo* (22) and *in vitro* (20) that glucose inhibits the endogenous histamine release resulting from the interaction of dextran with tissues. Beraldo *et al.* (15) and Poyser and West (34), using the "spot test," produced capillary leakage by intradermal injection of dextran (15, 34), yeast mannan (34), ovomucoid (34), and zymosan (34). The leakage was inhibited by diabetes (15) and by mixing the polysaccharides with certain simple sugars (34) that were either identical with those of the polysaccharides or were related to them by common stereochemical features, and would thus be expected to exert a haptenic inhibition (28). However, endogenous histamine was also involved in the capillary leakage induced by the topical polysaccharides, since antihistaminics were inhibitory (34). Glucose and the other simple sugars, besides acting as inhibitory haptens towards the respective polysaccharides, could also therefore inhibit more directly the histamine action, as described in the paragraph above,

TABLE V  
OPTIMAL MOLAR RATIOS OF TOPICAL SUGARS THAT INHIBIT CAPILLARY  
LEAKAGE FOLLOWING INTRADERMAL DEPOSITION OF 0.01  $\mu$ MOL HISTAMINE  
(RESULTS EXPRESSED AS "LEAKAGE FACTOR")

$\mu$ mols of sugar, or control sub- stance, added to .	0	0	0.005	0.01	0.02	0.04	0.08	0.16	0.32	0.64	1.28
CAPILLARY LEAKAGE FACTOR											
Saline control . . . . .	17 ± 5	—	—	—	—	—	—	—	—	—	—
D-Glucose . . . . .	—	300 ± 12	—	—	—	262	—	144 ± 10*	60 ± 10*	170 ± 10*	—
D-Arabinose . . . . .	—	206 ± 7	—	—	156	157	199	102 ± 10*	52 ± 10*	121 ± 9*	188
L-Arabinose . . . . .	—	206 ± 7	—	—	187	164	190	103 ± 16*	57 ± 13*	116 ± 9*	152
L-Xylose . . . . .	—	206 ± 7	—	—	196	170	123 ± 17*	180	198	165	175
Sucrose . . . . .	—	206 ± 7	196	120 ± 11*	73 ± 6*	99 ± 6*	150 ± 8	171	205	218	210
Urea . . . . .	—	250	250	243	197	244	228	—	244	—	—
NaCl . . . . .	—	200	206	182	199	180	240	195	206	—	—
Molar ratio of sugar (or control substance) to . . . . .	0 : 0	0 : 1	0.5 : 1	1 : 1	2 : 1	4 : 1	8 : 1	16 : 1	32 : 1	64 : 1	128 : 1

\* These values are statistically different from the histamine control,  $P < 0.001$ .

(From ADAMKIEWICZ, V. W., and P. J. SCARA: *Can. J. Physiol. Pharmacol.* 43:877, 1965. Reproduced by permission of the National Research Council of Canada.)

the two inhibitions resulting in the over-all decrease of capillary leakage.

Instead of by polysaccharides, endogenous histamine may be discharged by compound 48-80, a synthetic polymer containing trimeric formaldehyde. This compound injected into rats produces a reaction analogous to the dextran inflammation, discharges endogenous histamine, a lipase (23) and other toxic substances. In sufficient doses, it causes death. The LD<sub>50</sub> of this drug is directly related to the blood sugar levels obtaining at the time of injection (35). In hyperglycemic rats, the LD<sub>50</sub> (7.3 mg/Kg) is six times higher than in hypoglycemic animals (1.22 mg/Kg). Conversely, a fixed dose of the drug produces mortality effects that vary inversely with the blood sugar levels (Fig. 11). These relationships are analogous to what was stated for the dextran anaphylactoid reaction, for anaphylaxis, and especially for

**INTERRELATIONSHIPS OF FIXED DOSE, FIXED EFFECT  
AND GLYCEMIA FOLLOWING COMPOUND "48-80" (RATS).**

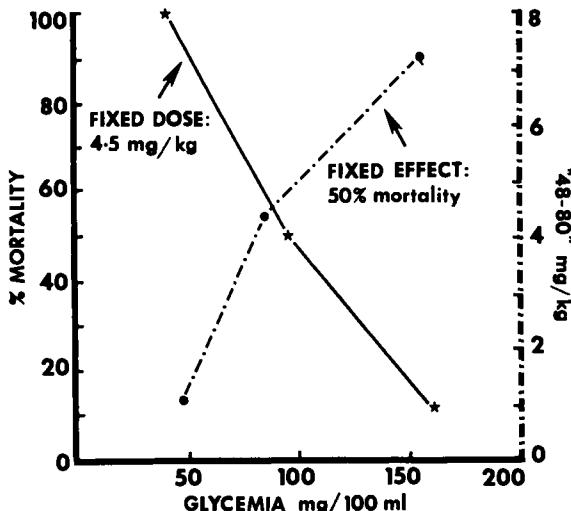


FIGURE 11. The mortality of rats (—) following a fixed dose of compound 48-80 varies inversely with blood sugar levels. But the dose of compound 48-80 necessary to produce a constant mortality (----) increases with the blood sugar levels. (From: ADAMKIEWICZ, V. W., and P. J. SACRA: *Fed. Proc.*, 26:224, 1967.)

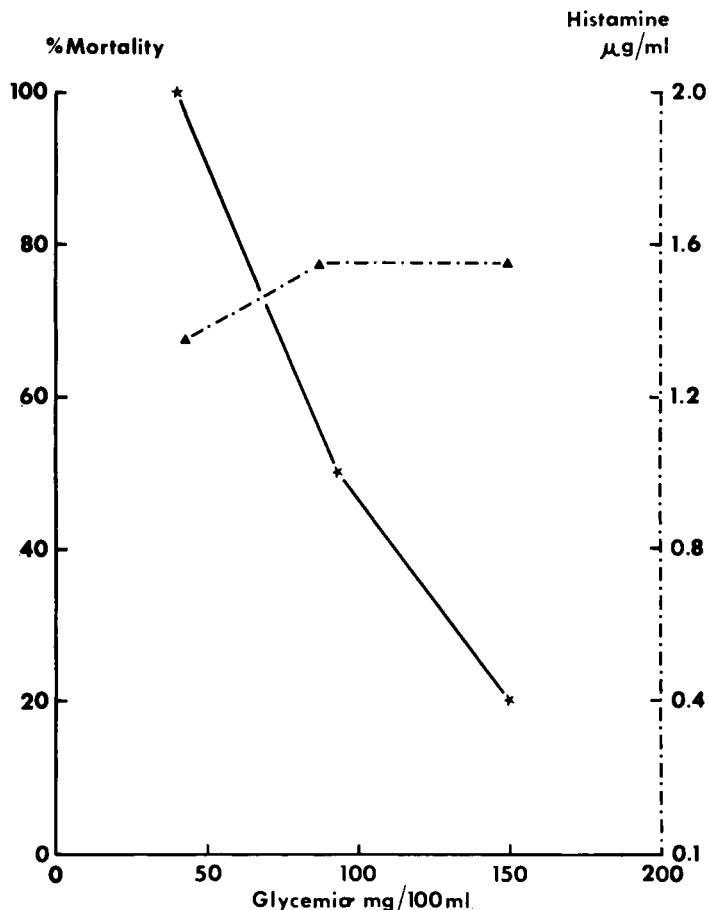


FIGURE 12. The mortality of rats (—) following a fixed dose of compound 48-80 varies in an inverse relationship with the blood sugar levels. But the amount of endogenous histamine (----) liberated by the fixed dose is independent of glycemia variations. (From: ADAMKIEWICZ, V. W., and L. M. ADAMKIEWICZ: *L'Union Méd. du Canada*, 94:1264, 1965.)

hemolytic anemia (Fig. 5). Although higher blood sugars inhibit the lethal effect of 48-80, they do not change the amount of endogenous histamine the drug discharges (19, 22, 35), (Fig. 12). One would be tempted to assume, therefore, that glucose protects against the lethal action of compound 48-80 by inhibiting the toxicity of the endogenous histamine the compound liberates. This is unlikely,

however, because the LD<sub>50</sub> of exogenous histamine is unaffected by blood sugar variations. It must be assumed, therefore, that the protection by glucose against the toxicity of 48-80 results either from interference with some reaction between the drug and a receptor or from interference with the lethal action of a substance, other than histamine, but liberated by the drug. The same reasoning also applies to the inhibition by glucose of the lethal effect of anaphylaxis.

### The Oscillations of Blood Sugars and Susceptibility to Allergy

Blood sugar levels of normal animals, like any other physiological function, oscillate around a base-line. The wave length of this oscillation in man is about 2 to 4 hours and the amplitude about 40 mg glucose/100 ml (31). Similar oscillations have long been noted in animals after stoppage of a glucose infusion. Normal mice

**VARIATION OF BLOOD SUGAR LEVELS IN NORMAL MICE DURING FOUR DAYS. GLYCEMIA AT NOON EACH DAY WAS TAKEN AS 100%, ITS MEAN VALUE WAS  $133 \pm 12$  mg %.**

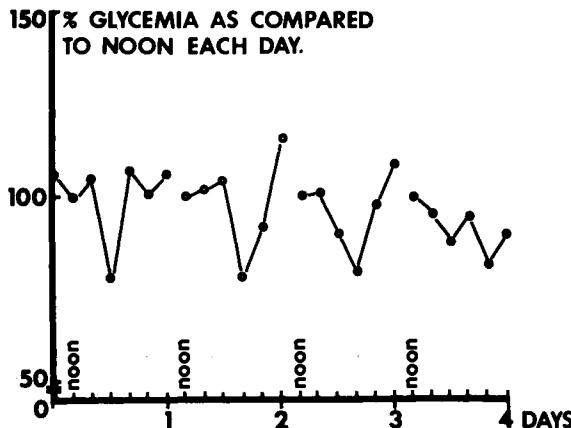


FIGURE 18. Diurnal variations of blood sugar levels of normal mice fed ad libitum during four 24-hour periods. The blood sugar level at noon each day was taken as 100%. Each point represents average value from ten mice. Note that normal mice go through a "spontaneous" hypoglycemic period between 8 and 12 p.m. each day.

display a blood sugar oscillation that hints at the existence of a circadian rhythm (Fig. 13). The hypoglycemic phase lasts from 8 to 12 p.m., and coincides with the night-feeding period of these nocturnal animals. The difference between the hypo- and hyper-phases may reach as high as 50 mg/100 ml.

**GLYCEMIA CHANGES FOLLOWING DIFFERENT DOSES OF ANTISERUM (agg. u./kg) IN MICE.**

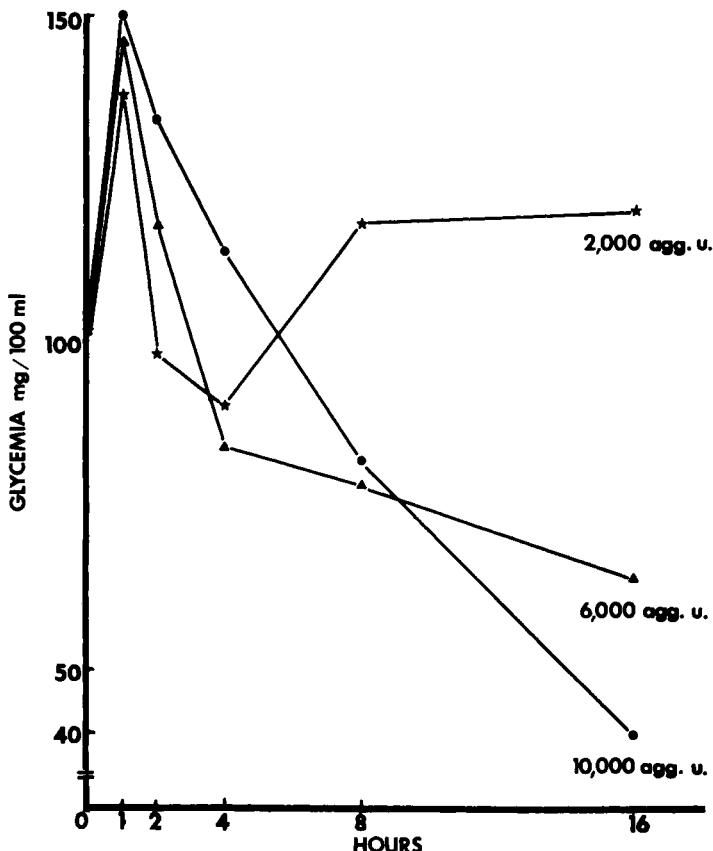


FIGURE 14. Biphasic blood sugar level variation in mice injected with rabbit antimouse erythrocyte serum. Following each dose of antiserum, the first phase is a hyperglycemia that returns to normoglycemia at about the fifth hour. The second phase is a hypoglycemia when the dose of antiserum is 6,000 agglutinin units/Kg, or more. This is followed by death. With smaller doses of antiserum, the second phase is a normo- or secondary hyperglycemia accompanied by survival of the mice.

The susceptibility to small doses of certain allergens or antigens may conceivably depend, therefore, on whether the animals are in their hypo- or hyperglycemic phase. In turn, the differential susceptibility would become critical in the evaluation of clinical and other tests that make use of small amounts of such antigenic material. Long (29), for example, suggested that the tuberculin reaction is related to the glycemic state.

On the other hand the ability of animals to raise blood sugar levels by up to 50% may serve as a first-line defense against certain invading allergens or antigens. Mikami, in 1925 (30), noticed that rabbits overdosed with diphtheria toxin undergo a biphasic change in blood sugar levels. An initial hyperglycemia is followed by secondary hypoglycemia and death within 24 hours.

A similar biphasic change is seen in mice injected with hemolytic antiserum (Fig. 14). The primary phase is a hyperglycemia that is independent of the dose of antiserum injected. It reaches a peak some two hours after inoculation and returns to normal some three hours later. This may be interpreted as an attempt on the part of the animal at neutralization of the antiserum or of some product of antigen-antibody reaction, since hyperglycemic mice and rats tolerate doses of certain antigens or allergens several times higher than normo-, or hypoglycemic mice. On the other hand, the secondary phase depends on the initial dose of antiserum. Thus, larger doses that lead to mortalities produce secondary hypoglycemia. The cause of this is not known, although it could result in part from the anorexia of the animals overdosed with the antiserum or, in the case of the Mikami experiment, with diphtheria toxin. However, it is intriguing that after a small initial dose of antiserum the secondary phase is not a hypoglycemia but a normo- or hyperglycemia, and this is followed by the survival of the animals (Fig. 14).

### Conclusions

For some time, studies in immunology have centered on antigens, antibodies, and their interaction, to the detriment of other physiological aspects, although early investigators, R. Kraus for example, set their sights on the response of the entire animal in an immunological situation.

Physiological glucose, an ubiquitous and active molecule, obviously modifies, directly or indirectly, the course of anaphylaxis, immune hemolytic anemia, immune hemolysis, as well as the sensitivities to polysaccharides, histamine, and drugs such as compound 48-80. Admittedly, the influence of glucose is not very marked when the dose of the antigenic or allergenic material that invades the "milieu intérieur" is large. However, massive invasion is rare. The common occurrence, rather, is a constant invasion by minute amounts of such material. Physiological processes engaged in the neutralization of this material constitute what is known in immunology as "natural resistance" (18).

"Natural resistance" is defined as "non-specific resistance determined by physiological conditions subject to variation from one animal to another and within a single individual at different times" (18). Thus defined, it comprises physical and chemical barriers presented by the epithelium, age, state of nutrition, as well as the several antibacterial substances of normal body fluids and tissues (properdin, phagocytin, lysozyme, etc.) and intermediates of immune reactions such as the complements.

This is indeed a heterologous group of phenomena in need of clarification. I think the anti-allergic effect of physiological glucose belongs in this group. It is significant that glucose as a factor of "natural resistance" makes all the difference between the survival and death of an animal subjected to the experimental conditions described herein.

### **Abstract**

Increasing the blood sugar from the hypoglycemic level of 50 mg/100 ml to the hyperglycemic value of 250 mg/100 ml inhibits or suppresses the following allergic and analogous reactions in rats and mice: 1) anaphylactoid inflammation; 2) anaphylaxis; 3) experimental immune hemolytic anemia; 4) immune hemolysis; 5) the toxicity of histamine and of compound 48-80.

The anaphylactoid reaction of rats to dextran may be transferred passively. It is mediated by a blood factor, the production and anaphylactoid action of which are both inhibited by glucose in the blood.

The following mechanisms are postulated for the multivalent anti-allergic effect of the hyperglycemic state: 1) autohaptenic inhibition by glucose of isologous or homologous polysaccharidic allergens; 2) general interference by glucose in antigen-antibody reactions or their sequels *in vivo*; 3) interference in these reactions by unidentified factors whose level in the blood is synchronized to blood sugar levels.

The anti-allergic effect of the hyperglycemic state is part of the natural immunological resistance.

### Abrégé

L'élévation de la glycémie à partir de sa valeur hypoglycémique de 50 mg/100 ml vers une valeur hyperglycémique de 250 mg/100 ml s'accompagne d'une inhibition ou d'une suppression des réactions allergiques, ainsi que de quelques réactions analogues suivantes chez le rat et la souris: 1) l'inflammation anaphylactoïde, 2) l'anaphylaxie, 3) l'anémie hémolitique immunitaire, 4) l'hémolyse immunitaire, 5) la toxicité de l'histamine et du composé 48-80.

L'inflammation anaphylactoïde au dextran chez le rat se prête à un transfert passif. Elle est causée par un facteur sanguin dont la formation ainsi que l'action anaphylactoïde sont toutes deux inhibées par le glucose sanguin.

Les mécanismes suivants sont proposés pour expliquer l'effet anti-allergique multivalent de l'état hyperglycémique: 1) inhibition autohapténique par le glucose d'allergène polysaccharidique iso- et homologue, 2) interférence plus générale du glucose dans l'interaction antigène-anticorps et dans les réactions qui suivent cette interaction *in vivo*, 3) interférence dans ces réactions par des facteurs encore non identifiés dont le niveau sanguin serait synchronisé à la valeur de la glycémie.

L'effet antiallergique de l'état hyperglycémique fait partie de la "Résistance immunologique naturelle."

### Acknowledgements

This study was supported by Grant MT-640 from the Medical Research Council of Canada, and Grant AM-05684 from the U.S. Department of Health, Education and Welfare.

### Addendum

The term allergy (literally: altered energy) as used originally by von Pirquet in 1906 signified an altered capacity for reaction, particularly but not exclusively in relation to immune responses. It is used here in its broad original meaning and encompasses hypersensitivity reactions as well as the "non-immunological equivalents of hypersensitivity reactions" (20).

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**R. Veilleux:** I should like to congratulate Doctor Adamkiewicz for his very good presentation. His studies have undoubtedly provided anaphylactoid inflammation with a new and hitherto unsuspected dimension. So I am personally very happy to have had this opportunity of hearing the present summary of his work.

My own interest in anaphylactoid inflammation goes back to the years 1961 to 1963. This was the time when Doctor Selye had just developed an experimental system through which it had become possible to calcify, at will, practically any organ or part of the body of the rat (Selye, H.: *Calciphylaxis*, University of Chicago Press, Chicago, 1962). This led to the development of many different syn-

dromes, one of which is characterized by selective calcification of the lips and forelegs of the rat after the administration of certain metallic compounds (Selye, H., et al.: *Brit. Med. J.*, ii:1194, 1961). I demonstrated, at that time, that this characteristic localization of calcium was conditioned by the previous occurrence of an anaphylactoid inflammation (Veilleux, R.: *Rev. Can. Biol.*, 22:15, 1963) that was, in fact, elicited by the dextrin fraction (Veilleux, R.: *Brit. J. Pharmacol.*, 21:235, 1963) of the compound injected. This shed further light on the role played by the metallic fraction of the injected substance, namely iron, which I then considered as the seeder or primer of the calcium deposition (Veilleux, R.: *Thèse*, Université de Montréal, 1963; *Acta Histochem.*, 17:343, 1964). At the same time, I was following with great interest Doctor Adamkiewicz's work concerning the transferability of anaphylactoid oedema. The main objection then was that this might be the result of a mere transfer of dextran from one animal to another. But, as we have just seen in these consecutive transfer experiments, the problem is not quite that simple. In fact, the evident effect of different glycemic states on anaphylaxis, on immune hemolysis, and on the anaphylactoid inflammation, together with the presence of an antibody in the serum of rats allergic to egg-white, constitute a good justification for a complete re-evaluation of what Doctor Adamkiewicz has called "the physiology of sugars" in regard to hypersensitivity in general.

I think it can be safely predicted that these studies have just provided a way that will undoubtedly stimulate much further investigation to definitely prove "the inadvisability of severing the links between the anaphylactoid and immunologic phenomena." I shall conclude, therefore, by saying that it is quite stimulating to see how such an apparently simple reaction as anaphylactoid oedema could have acquired such extent and scope in the hands of one of us.

**B. Halpern:** Je vais me permettre de dire un mot, en tant qu'immunologue. Je suis surpris d'entendre que le Dr. Adamkiewicz emploie le mot "allergène" pour le dextran et le 48-80. Il est impérieux dans toute science, et l'immunologie est une science exacte, d'utiliser les termes conformément à leur définition. Or, le terme "allergène" ou "antigène" a une signification précise. Un antigène est une substance qui provoque une réaction immunologique spécifique, soit sous forme de synthèse d'anticorps, soit sous forme d'hypersensibilité retardée. Or, on n'a jamais démontré la présence d'anticorps ni pour le dextran ni pour la polyvinylpyrrolidone, qui est un autre histaminolibérateur, et encore moins pour le 48-80 qui est une substance syn-

thétique de faible poids moléculaire. Bien qu'on ignore encore le mécanisme par lequel ces substances déterminent une libération de médiateurs chimiques, elles n'agissent certainement pas par un processus immunologique. De ce fait, il ne convient pas de les dénommer "allergènes."

**V. W. Adamkiewicz:** Monsieur le Professeur Halpern, je crois que les questions de nomenclature posent en effet une difficulté en allergologie. Malheureusement, les mots expriment rarement ce que l'on croit qu'ils expriment avec autant de clarté qu'ils n'en donnent l'impression. L'allergie, c'est surtout une entité clinique caractérisée, du reste assez vaguement, par des critères cliniques. Ce qui la déclenche se nomme un allergène. Donc allergène est un terme générique s'appliquant à tout déclencheur d'allergie. Une des sous-classes d'allergènes, peut-être la plus importante, consiste en des antigènes. Une autre se compose encore toujours de facteurs physiques et chimiques divers tels: le froid, la chaleur, la formaldéhyde, le dextran, etc. L'allergie déclenchée par les allergènes-antigènes découle d'une aberration du mécanisme immun. Celle qui est déclenchée par les allergènes non-antigéniques procède d'un mécanisme en voie d'être élucidé.

Je concevais, quoique je n'en verrais pas l'utilité immédiate, de faire de l'allergène un synonyme d'antigène. Le sens du mot allergie se restreindrait alors seulement à l'aberration du mécanisme immun déclenché par un allergène qui est un antigène. Par contre, tout ce qui se passerait quand l'allergène ne serait pas un antigène cesserait d'être allergique. Ainsi le dextran ne causerait pas d'allergie chez le rat et deviendrait alors un: allergénoïde, antigénoïde, allergoïdogène, anaphylactoïdogène, etc. Mais de faire du mot allergène un synonyme d'antigène requerrait une redéfinition préalable du mot allergie lui-même.

## **Antineoplastic Effects of BCG and Other Agents\***

PAUL LEMONDE

DURING the past years, we have been studying the influence of various environmental and physiological factors on the evolution of neoplasms, particularly of viral tumors (12-17). What is the origin or the rationale of this work? I was struck by the fact that in cancerous inbred strains of animals, which serve as subjects for research on cancer, a high proportion of individuals succumb to cancer, but a certain number resist the disease and die, later, of diverse causes. For example, in mice of the Ak strain, about 85% of the animals die of lymphoid leukemia. These 85% are most valuable, for the purpose of studying leukemia; they have enabled investigators to make fundamental discoveries on leukemia, on the development of this disease, the biochemistry, chemotherapy, hormonal influences, etc. Thanks to them, Gross found the viral etiology of mouse leukemia. But the other 15%, which are neglected as being of no interest, have intrigued me. Why do they not develop leukemia? They are of the same homozygous strain, live in the same cages, the same rooms, breathe the same air, eat the same food as their congeners: why do they escape their destiny? These 15% appear to be as interesting as the others, if not more so. They are an evidence that Nature has effective means of combatting cancer. Another indication of this is afforded by the so-called spontaneous regressions. If we knew the mechanisms by which these animals resist the cancerous process, we could try to imitate Nature and apply her methods. Hence, I have attempted to unravel some of the factors that could operate in that resistance.

### **Materials and Methods**

Mice of the Ak strain were used; they were from our own colony, which is derived from a pair given to us by Dr. L. Gross

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\* This paper was presented in French.

and is maintained by inbreeding. The status of leukemia in these animals is as shown in Table I. The incidence is higher and the disease occurs earlier in females.

Mice of our C3Hf inbred strain were also employed. In these, the incidence of mammary tumors is nil, for they were cleared of the milk factor by foster nursing. Leukemia occurs in 1-2% of the animals, and liver or digestive tract tumors in about 10-15%. The latter appear in older mice, kept for more than 15 months, i.e., beyond the age limits within which the animals are used in experiments.

Golden hamsters used were from the stock raised in this laboratory, but outbred.

The strain of polyoma virus employed was isolated a few years ago and characterized by its tumor-inducing activity *in vivo*, cytopathic effects *in vitro*, hemagglutination of guinea-pig red blood cells and neutralization by specific antiserum (17).

### Experiments and Results

In previous communications, it was reported that fighting in males (12), pregnancy in females (13) and BCG (14, 16) counteracted spontaneous mouse leukemia. In males, leukemia develops later and with a lower incidence than in females, as mentioned before (Table I). This sexual difference is usually ascribed to sex hormones, on the grounds that estrogens promote or androgens oppose leukemia, as is indeed the case. However, other factors may have something to do with it. Males of the Ak line, as in several other strains of mice, fight each other, ferociously, often to the death. This behavior may have a bearing on leukemia.

TABLE I  
LEUKEMIA IN SUBLINE OF AK MICE

Sex	Number of Mice	Incidence of Leukemia	Survival Time (Avg. Days $\pm$ S.E.)	
			All Mice	Mice with Leukemia
♂♂ .....	1013	87.8%	337.0 $\pm$ 3.5*	323.7 $\pm$ 3.1*
♀♀ .....	1053	94.8%	281.6 $\pm$ 2.8	278.6 $\pm$ 2.6

\* P < 0.001.

TABLE II  
FIGHTING AND LEUKEMIA IN MALE Ak MICE

Groups	Number of Mice	Incidence of Leukemia	Survival Time (Avg. Days $\pm$ S.E.)	
			All Mice	Mice with Leukemia
Controls: isolated . . . . .	40	39 = 98%	295.3 $\pm$ 13.8*	286.7 $\pm$ 11.0*
Fighting: subordinate ..	88	80 = 91%	350.4 $\pm$ 13.4	343.1 $\pm$ 16.1
dominant . . . . .	30	25 = 83%	375.2 $\pm$ 21.3	355.9 $\pm$ 16.9

\* Significantly different from other groups:  $P < 0.01$ .

In a first experiment (12), two groups of mice were formed: some were placed in individual cages at the age of two months and could not fight; the others were mice that lived 2-6 to a cage and fought often. The fighting males had less leukemia and lived longer than the isolated animals; when leukemia did develop, it occurred later (Table II). In addition, the winners, which the psychologists call the dominants, developed leukemia later and to a lesser degree than did the losers or subordinates. These subordinates were intermediate between the dominants and the non-fighters, and the latter intermediate between the fighters and the females.

In females, the development of leukemia was significantly delayed following pregnancy, and the more pregnancies there were, the more was leukemia deferred. There was no decrease in the incidence (13).

A third factor studied was BCG, i.e., tubercle bacilli of the Calmette-Guérin strain. On transplanted leukemia, BCG was found to exert a counteracting influence (16): in animals previously inoculated once with live BCG, the weight and general condition were better, the local tumor remained smaller than in the controls and even regressed completely in some individuals, and survival was prolonged. Killed BCG had no effect.

As regards spontaneous leukemia, its incidence was lowered and the survival time was significantly prolonged in Ak mice given a single injection of live BCG (15, 16) (Table III). This experiment was repeated later, with even better results, as seen in Table IV (14). Old and his co-workers have reported that spontaneous leukemia was delayed in mice given BCG (20); the pres-

TABLE III  
SPONTANEOUS LEUKEMIA Ak MICE GIVEN BCG

Groups	Incidence of Leukemia	Survival Time (Avg. Days $\pm$ S.E.)	
		All Mice	Mice with Leukemia
Controls (12 ♂♂, 8 ♀♀) .....	19 = 95%	292.5 $\pm$ 18.6*	299.8 $\pm$ 18.1
BCG (12 ♂♂, 8 ♀♀) .....	14 = 74%	371.8 $\pm$ 24.8	353.4 $\pm$ 26.9

\* P < 0.02.

TABLE IV  
EFFECT OF COMBINED FACTORS ON SPONTANEOUS  
LEUKEMIA Ak MICE

Groups	Number of Mice	Incidence of Leukemia	Survival Time (Avg. Days $\pm$ S.E.)	
			All Mice	Mice with Leukemia
♀♀-	Controls .....	28	27 = 96%	280.9 $\pm$ 18.6
	BCG .....	18	14 = 78%	333.1 $\pm$ 27.2
	Fasting, pregnancy, and BCG .....	10	5 = 50%	358.2 $\pm$ 45.0
♂♂-	Controls .....	32	29 = 91%	312.3 $\pm$ 17.9
	BCG .....	20	10 = 50%	411.6 $\pm$ 26.1
	Fasting, fighting, and BCG .....	10	0 = 0%	448.8 $\pm$ 33.0

ent experiments confirm this finding and show, moreover, that the incidence is also decreased. Incidentally, it might be noted that not only transplanted tumors are impeded under the influence of BCG, as is often believed and written, but at least two types of spontaneous neoplasms (16, 20) as well as one type induced by virus inoculation (16, 17) are also inhibited.

I then attempted to find out whether combining various factors could further improve resistance. In a first experiment, fasting was associated with fighting or pregnancy, Saxton and his associates having shown that fasting hindered leukemia in mice (22). It was indeed found that the incidence of leukemia was decreased in mice managed in this way and that the life span was significantly prolonged, owing to the retardation of leukemia. These effects were more pronounced than with either factor alone (14).

In a subsequent experiment, three factors were combined: BCG

was added to fasting and fighting or pregnancy (14). In this case, the incidence of leukemia fell from 96 to 50% in the females, the appearance of the disease and death were postponed by 12 weeks (Table IV). In the males, leukemia was reduced to 0%, and the animals died, late, of degenerative diseases. The objection could be raised that this striking result was obtained with only a small number of animals, but the experiment was repeated on a larger scale and the results of this new trial, which is now terminating, are similar. It is an established fact: such a combination of factors remarkably inhibits spontaneous leukemia in mice.

In order to extend and if possible to generalize these results, we tested the effect of BCG on another type of virus-induced neoplasm, namely polyoma. A first experiment showed that the action of polyoma virus was counteracted by previous administration of BCG in hamsters (16). In a more extensive experiment (17), intended not only to confirm the first but also to explore the mechanism of the antipolyomatous effect, it was again found that BCG exerted an inhibiting action: incidence decreased by half, survival time more than doubled (Table V). The same experiment was carried out in mice and yielded similar results (17).

With the purpose of finding out whether BCG operates by starting an infectious process or merely by the presence of bacterial bodies or products, we have studied the influence of heat-killed BCG and of a lipopolysaccharidic extract of tubercle bacilli ("wax D") on polyoma tumors in hamsters. Again the live bacilli exerted a protective action, as in the two preceding experiments; killed BCG and the extract had a similar but lesser effect; since the experiment is not yet completed, I cannot say how much this effect will be significant.

TABLE V  
EFFECT OF BCG ON POLYOMA IN HAMSTERS (IN PART)

Groups	Incidence of Polyoma	Survival Time (Days)	
		Avg. $\pm$ S.E.	50%
Polyoma virus . . . . .	98%	124.9 $\pm$ 31.6*	74
BCG + Polyoma virus . . . . .	48%	267.8 $\pm$ 27.7	270

\* P < 0.01.

TABLE VI

## B. PERTUSSIS AND SURVIVAL TIME IN HAMSTERS WITH POLYOMA

Groups	Number of Hamsters	Survival Time (Avg. Days $\pm$ S.E.)
Polyoma virus . . . . .	15	37.6 $\pm$ 1.9*
B. pertussis + Polyoma virus . . . . .	46	58.9 $\pm$ 5.5

\* P < 0.001.

Finally, assuming that the action of BCG was perhaps not particular to this bacillus, but possibly the consequence of infections in general, or at least of a certain number of infections, we wanted to examine the influence of other microorganisms. In this connection, it may be recalled that there have been often reported clinical cases of cancer in which spontaneous regressions were associated with infectious diseases, erysipelas, typhoid fever, etc. Several bacteria have also been found to counteract experimental tumors, e.g., *Lactobacilli* (15), *Corynebacterium parvum* (8, 25). For our part, we have studied the whooping cough bacillus, *Bordetella pertussis*. Why this one? Because if BCG, as it appears, acts by stimulating immune functions, *pertussis* has also been found to stimulate these functions (19). In addition, *pertussis* has been shown to oppose a transplanted mouse sarcoma (S-180) (18).

In a first trial, we tested *pertussis* on polyoma in hamsters, under various experimental conditions. A protective effect was obtained when the bacilli were administered by the intraperitoneal route before the polyoma virus: the survival time was significantly increased (Table VI), and this was true whether live or killed *pertussis* was inoculated: 17 of the 46 hamsters received killed, the other 29 live bacilli, and the survival was the same in both cases, 59 days. The incidence of tumors was not changed: over 80% in both groups. A repeat experiment on a larger scale is still in progress; the results to date are encouraging: the death rate is 72% in the controls and only 12% in the animals given *pertussis*. A similar experiment was set up to investigate the effect of *pertussis* on spontaneous leukemia, but it is not yet sufficiently advanced to yield any results.

## Discussion

This discussion will be an attempt to elucidate the mechanisms of the results reported.

With respect to fighting, the repressive action of this factor on leukemia may be attributed to two mechanisms. The first is increased androgen secretion. The fighting instinct is under the influence of androgens. The animals that constitutionally produce more testosterone are more bellicose. Compelling their companions to fight in self defense, they stimulate androgen secretion in them. There ensues a reciprocal stimulation of testosterone on fighting and of fighting on testosterone. Now, androgens are antileukemic, as has been demonstrated in many ways (5, 11, 21). The second mechanism is an augmentation of corticosteroids of the "gluco-corticoid" type. The agitation, constant fights and resulting wounds provoke an overproduction of corticosteroids. These hormones are known to be antileukemic: they are used in clinical practice to retard the evolution of leukemia. It may be questioned whether fighting as such is at play here or whether the effect is merely due to social pressure or crowding, as is thought by some (4). For factual reasons discussed elsewhere (15), I am inclined to assert that fighting is the main factor.

As to pregnancy, there is no question of estrogens or progesterone in its effect against leukemia: the former hormones are proleukemic (5, 11, 21) and the latter has no effect on mouse leukemia (21). Here again, corticosteroids are probably the acting mechanism. They are produced in increased amounts during pregnancy, as is well known. It was from the observation that arthritis subsided in women during pregnancy that Hench *et al.* proposed the use of cortisone in the so-called collagen diseases (9). Thus, one pregnancy in the mouse amounts to one treatment with corticosterone, two pregnancies to two periods of treatment, and so on.

As regards fasting, the same mechanism is liable to operate. In addition, underfeeding hampers the growth of tumors by depriving them, like other tissues, of nutritive materials necessary to their development.

With respect to BCG, the situation is different, for this organism gives rise to no serious conditions in the mouse. In the hamster, it brings on a more severe disorder, which must be treated if it is not to degenerate into a progressive infection. Some investigators, particularly Biozzi, Halpern and their associates (2, 7, 20) have reported that BCG counteracts the development of certain transplanted tumors. Inspired by their observations and prompted by my previous work on the

relationships between hormones, infections, the reticulo-endothelial system and tumors, I undertook to study the effect of BCG on neoplasia. It appears that this organism acts by stimulating the reactions involved in immunity: antibody production and phagocytic activity. In this connection, it may be recalled that mycobacteria are ingredients of immunity adjuvants, such as Freund's adjuvant. That BCG possesses such a stimulating power has been shown by many investigators (1, 3, 6, 10, 20, 23), using carbon clearance from the blood, histochemical evaluation of phosphatases in phagocytic cells, or measurements of antibodies against various antigens (bacteria, red blood cells, viruses, albumin, etc.). We in turn have found such a stimulation in our experiments with polyoma. With a view to analysing the mechanism of protection by BCG, we titrated three types of antiviral antibodies in the animals and found that all three types were increased in hamsters as well as in mice given BCG (17). This finding confirmed, in another biological system, the fact that BCG stimulates antibody formation. However, it does not explain the protective effect afforded against polyoma tumors. Antiviral antibodies are not the cause of the protection against viral tumors. Other investigators have seen this fact in experiments done under other conditions. We drew the same conclusion from this experiment. On what basis? From the observation that, paradoxically, the animals developing more antibodies were less protected. The hamsters with no antibodies lived the longest, whereas those showing the highest titers of antibodies died the earliest. In parallel the animals that showed no polyoma changes were among those with little or no antibodies, while those having the largest amounts of antibodies exhibited as a rule the most serious changes (17). So, if BCG does not protect through antiviral antibodies, how does it work? It is logical to think that it protects by stimulating antitumoral immunity. It is known that cells transformed into cancer cells, either by viruses or by chemical carcinogens, produce new antigens, specific of cancerous tissue as such, which in turn induce the formation of tumoral antibodies. This immunity can be demonstrated in various ways; but since the first method by which it was evidenced was the resistance to isologous transplantation, we used this method first, to study the influence of BCG. If polyoma virus is inoculated into adult mice, or in small doses in hamsters, it induces no tumors, but the animals become resistant to later implantation of isologous polyoma tumor cells. Accordingly, we tested the resistance to isologous transplantation of polyoma tumors, in hamsters previously inoculated with the virus and having received BCG or not. We did obtain with

TABLE VII

EFFECT OF BCG AND POLYOMA VIRUS ON TRANSPLANTED  
POLYOMA TUMORS IN HAMSTERS

Groups	Number of Hamsters	Size of Tumors at Death (Mean Diameter, mm) (Avg. $\pm$ S.E.)		Metastases Incidence	Importance <sup>†</sup> (Avg. $\pm$ S.E.)
		(Avg. $\pm$ S.E.)	(Avg. $\pm$ S.E.)		
Virus + Implantation . . . . .	12	65.4 $\pm$ 4.1*	65.4 $\pm$ 4.1*	67%	26.9 $\pm$ 6.3**
BCG + Virus + Implantation	12	40.0 $\pm$ 3.2	40.0 $\pm$ 3.2	40%	6.8 $\pm$ 3.6

<sup>†</sup> An index, from 0 to 54, taking into account the number, dissemination, and size of metastases.

\* P < 0.001.

\*\* P < 0.02.

BCG an increase in resistance that was manifested by a slowing down of tumor development and a great decrease in the incidence and importance of metastases (Table VII). However, the incidence of tumors was not changed, nor was the survival prolonged. New tests are in progress, with a view to bringing out more clearly any immunological action of BCG.

It is likely that a stimulation of phagocytosis, either by multiplication or by activation of phagocytes, or by an increase of opsonizing antibodies, or by all of these means, has something to do with the protective effects of BCG.

Regarding *pertussis*, it probably acts by a mechanism similar to that of BCG (19), but this remains to be established.

As I said at the beginning, the object of this study was to look for factors that could inhibit cancer and to explain how tumors develop in some individuals and not in others. We have indeed found several agents that, alone or combined, succeed in checking at least some forms of neoplastic processes. These factors seem to fall into two classes: some acting rather through corticosteroids, the others by stimulating immunity. The former are useful in leukemia or similar neoplasms, because they attack, by atrophy, the very tissues that leukemia affects by hyperplasia. They are probably less beneficial in other types of neoplasia. The two classes do not work necessarily in agreement, since, for example, "gluco-corticoids" can depress the organs and functions of immunity.

It is remarkable that by combining what can be called natural factors, such as pregnancy, fasting, fighting and mild infections, an inhibition of cancer can be achieved. There is more to it than a mere

laboratory curiosity. Fighting and malnutrition are not proposed as preventive measures against cancer, and more research must be done before encouraging pregnancy for the same purpose; but physical exercise, which also hinders tumor development, and frugality, if not fasting, might be worth a trial. In any case, research in this field can lead to a better understanding of natural defences and then to the use of such defences in preventing, arresting or curing cancer.

### Abstract

The incidence of spontaneous leukemia was decreased and its appearance delayed in Ak mice, following the administration of tubercle bacilli of the BCG strain. The same effects were obtained on neoplastic changes induced by polyoma virus in hamsters and mice. These effects are ascribed to a stimulation of immune functions, on the basis of the evidence reported by other workers and of the experiments described here.

Fasting, fighting in the males, and pregnancy in the females were found to counteract spontaneous leukemia in Ak mice. This effect is attributed to corticosteroid hormones and also to catabolism for fasting and to androgens for fighting. When such factors were combined, e.g., fasting, fighting and BCG, there resulted an inhibition of spontaneous leukemia that could go so far as complete prevention. Whooping cough bacilli, *B. pertussis*, were also found to counteract the development of polyoma changes in hamsters.

### Abrégé

Le BCG a diminué l'occurrence et retardé l'apparition de la leucémie spontanée chez la souris Ak et des néoplasmes induits par le virus du polyome chez le hamster et la souris. Cet effet est attribué à une stimulation des fonctions de l'immunité, tant à cause des observations faites par d'autres auteurs que des constatations expérimentales rapportées ici.

Le jeûne, la bataille chez les mâles et la gestation chez les femelles contrecarrent la leucémie chez les souris Ak. cet effet est attribué aux hormones cortico-surrénaliennes, et de plus au catabolisme pour le jeûne et aux androgènes pour la bataille. Si l'on associe les facteurs susdits, par exemple jeûne, bataille et BCG, on obtient une inhibition de la leucémie qui peut aller jusqu'à la suppression complète. Le bacille de la coqueluche, *B. pertussis*, a lui aussi contrecarré le développement du polyome chez le hamster.

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**J. L. Riopelle:** Monsieur le Président, M. le Prof. Selye, Mesdames, Messieurs, lorsque tout à l'heure, à la fin de la communication du Dr. Lemonde, le Dr. Halpern a confirmé ses observations du poids de sa très grande autorité, je me suis dit que c'est lui qui devrait être à ma place pour faire des commentaires et ajouter ses expériences personnelles à ce que vient de nous exposer notre confrère. Je ne sais pas si c'est une chose un peu téméraire de la part d'un discutant d'inviter le président à faire des commentaires et je voudrais poser simplement une seule question au Dr. Lemonde. Il est de plus en plus d'usage potir la thérapeutique du cancer d'être polyvalente; on associe toutes sortes de traitements et il arrive assez souvent que l'on traite de telle façon que l'on attente peut-être aux propriétés immunologiques de l'hôte; et j'allais demander, justement, pour rendre la question plus générale, si le Dr. Lemonde a déjà eu l'occasion d'étudier des facteurs qui favorisent l'évolution du cancer.

**P. Lemonde:** Professor Riopelle asked whether, in our work, we

had studied factors that enhance cancer. The answer is yes. I have investigated the effect of growth hormone (GH), or somatotrophin (STH), in leukemia. The idea was that GH, being lymphotropic, could enhance spontaneous lymphoid leukemia. Years ago, Selye reported that STH stimulated extramedullary and extranodular hemopoiesis (Selye, H.: *Annual Report on Stress*, p. 304, Acta Inc., Montreal, 1951). On the other hand, Moon and his co-workers reported that in rats, after a prolonged treatment with GH, lymphosarcomas developed in the lungs of a few animals (Moon, H. D., et al.: *Cancer Res.*, 10:297, 1950); to my knowledge, no confirmation of this result has appeared. In mice, no effect of GH on the incidence of tumors was observed (Moon, H. D., et al.: *Cancer Res.*, 12:448, 1952). Later on, I myself treated mice over a long period with GH; I obtained giant mice, weighing up to 70 g, but no tumors of any sort. More recently, I looked for a possible effect of GH on spontaneous leukemia in the Ak mouse, a natural carrier of leukemia. I found, indeed, that the hormone increased leukemia in three ways: higher incidence, earlier appearance, more severe changes (Table I). The severity of lymphoid tumors (thymic, mesenteric, axillary and inguinal) was evaluated by weight and these tumors were significantly more developed in GH-treated animals than in controls injected with an inert protein. The findings in the males are shown in Table I; those for females were similar, though less significant. It should be noted that these results were obtained with a relatively short treatment, 30-50 days, administered to young mice (10 weeks), several months before leukemia developed. Yet this sufficed to induce an enhancement of leukemia, later. One may then ask if a temporary episode of GH overproduction in a young person could not result later in the development of leukemia or the evocation of a latent leukemia. This is an interesting subject for an epidemiologist: to seek the association that may exist between leukemia incidence and body stature. I am

TABLE I  
GROWTH HORMONE AND SPONTANEOUS LEUKEMIA IN MALE Ak MICE

Groups	Number of Mice	Incidence of Leukemia	Survival Time (Avg. Days $\pm$ S.E.)	Weight of Lymphatic Organs (mg, Avg. $\pm$ S.E.) In Mice That Died of Leukemia
Controls .....	12	75%	385.0 $\pm$ 44.5	668.4 $\pm$ 57.9
Growth hormone .....	12	100%	261.2 $\pm$ 16.9	1127.1 $\pm$ 151.8
Significance of differences:			P < 0.02	P < 0.02

no epidemiologist myself, but I have looked into the possible relationships between acromegaly and leukemia. I found, indeed, in the medical literature, several cases of acromegaly in which were reported not only a hypertrophy of hemopoietic organs, but leukosis, lymphocytosis, and even lymphosarcomas and leukemia. There are cases of acromegaly in which the patients died of leukemia. Unfortunately, acromegalics are treated by endocrinologists. The latter generally have a biochemical turn of mind; they report elaborate data on protein and sugar metabolism, phosphatemia, phosphatasemia, creatinuria and so forth, but no hematology as a rule. It is possible then that an association between acromegaly and a leukemoid syndrome or diathesis is really more frequent than would appear from the literature.

Another agent that I suspect to be proleukemic is norethynodrel; but this I report with all reserves, because I have carried out a preliminary experiment that gave only faint indications. I said that pregnancy delayed leukemia and, on the basis of Rudali's paper, that this effect was not due to progesterone. However, I still had doubts about this and wished to conduct another experiment along this line. Instead of progesterone, I used a synthetic progestative, norethynodrel, because: 1°) it is more active; 2°) its use is becoming more widespread these days. Forty female Ak mice were treated with this compound, in maize oil, by stomach tube, 5 days a week, from the age of 2 months until they were 1 year old. Controls received oil only. As a result, there was a separation of the survival curves: animals receiving norethynodrel died of leukemia earlier. However, the separation was slight; in other words, the difference between the two groups was not significant. But this was perhaps due to an unexpected event that interfered with the experiment: the compound apparently has a very bad flavor, at least for mice, and whereas controls given oil made no protest when the tube was introduced, test animals briddled up, writhed, bit, and tried to resist the insertion of the tube. This constituted an aggression, a stress, and I have shown by many examples that stress retards leukemia. This retardation may have brought the two curves closer to each other (pushing the curve of test animals towards that of controls). In another experiment, which is in preparation, a bad-tasting substance will be added to the oil of the controls; thus, the curves will probably move away from each other (the curve of controls moving farther right, away from that of test animals) and a significant difference will perhaps appear between the two groups. If such a result does occur, it will be obvious that norethynodrel accelerates leukemia.

**B. Halpern:** I would like to say a few words about personal research in this field, which of course are relevant to the data presented by

Dr. Lemonde. I think that there is a very important problem, a general one, which is implicated in the so-called "natural resistance." It is a common observation in tumorigenesis, whether induced or spontaneous, that even in isogenic strains, and Dr. Lemonde observed it also in spontaneously leukemic AkR mice, that a certain percentage of animals resist or escape the disease. This is also true in human beings. We don't know exactly what the natural resistance is; but it is a fact and a fact that is, I think, very important. For about 20 years, my associates and I have been attempting to understand what mechanisms are implicated in the so-called "natural resistance." By a series of investigations we came to the conclusion that the reticulo-histiocytic system is mainly responsible for the so-called natural resistance. Let me give an example: If a sarcoma type J is grafted in a C<sub>57</sub>Bl mouse and the histological changes in the immediate vicinity are studied, one can see, within the following days, a histiocytic reaction and a general stimulation of the RES as measured by the phagocytic activity of various colloids. While the tumor is developing, the local histiocytic reaction is weakening and the phagocytic activity of the RES is decreasing until the death of the animal. The picture is quite different in animals pretreated with *Corynebacterium parvum* or with BCG, which have been shown to be potent stimulants of the RES. In animals so treated, about 50% overcome and destroy the tumor. The destruction is due to a marked afflux of histiocytes around the tumor. The cells invade the tumor and destroy the malignant cells by some increase either in their recognition capacity or in their aggressiveness against the tumoral cells. This phenomenon is likely to be related to a stimulation of the intrinsic activity of the cells rather than to the increase of circulating antibody, although antibody synthesis is also promoted in animals so treated. The results are even more striking in the Ehrlich's ascites tumor. When *Corynebacterium parvum* was administered prior to the intraperitoneal graft of the cancerous cells, 80 to 90% of definite survivors were obtained while 100% of the control animals succumbed (Halpern, B., et al.: *Nature*, 215:400, 1967.) Dr. Lemonde's results, obtained with BCG in the leukemic AkR mice, are an extension of our observation. These findings have of course raised an understandable interest among clinicians. Our own observations and those made by others suggest that bacterial substrates, which stimulate the RES, depress to a certain degree the invasiveness of malignant tumors. It is likely that these favorable effects would be interpreted as resulting from the raised level of the "natural resistance" of the individual.

## **Thymus and Inflammation**

L. HEILMEYER, H. KASEMIR and L. KERP

THE question whether the thymus plays a role in the inflammation process is still unsolved. The few investigators dealing with this problem have obtained different results. Csaba *et al.* (1) seemed to prove that the formalin arthritis in adult rats increased slightly when thymectomy had been performed three days prior to the beginning of the test. On the other hand, Girerd and Di Pasquale (4) did not find an increase of formalin paw-oedema in younger thymectomized rats in which an inhibition of the development of granuloma around cotton pellets and of the volume of exudate in granuloma pouches was, however, evident. In the case of wound healing as a model of inflammation, Fisher and Fisher (3) could not observe any difference between neonatally thymectomized and control rats with regard to the increase of firmness of healing cuts as well as to the retraction of punch defects of the skin.

In view of these conflicting results, our own experiments were carried out using the formalin paw-oedema, the carrageen paw-oedema, and the cotton pellet method with carrageen soaked pellets.

### **Materials and Methods**

Inbred male and female mice of the Swiss-S-albino and C57BL strains were used. Thymectomy and sham-operations were performed within the first 20 hours after birth. At the age of 40 days, the inflammation tests were performed. After the tests had been finished, the completeness of thymectomy was checked histologically. Small remainders of thymus were found in approximately 50% of the animals.

### **Results**

The following results were obtained:

1. No difference was found between the weight of formalin paw-

oedema of neonatally thymectomized and non-operated C57BL mice (16 complete and 30 incomplete thymectomies, 43 controls).

2. The findings were the same for carrageen paw-oedema in Swiss-S-albino mice, which showed no differences between the groups (26 complete and 24 incomplete thymectomies, 35 controls).

3. On the other hand, neonatal thymectomy in Swiss-S-albino mice strongly inhibited the formation of granuloma around carra-

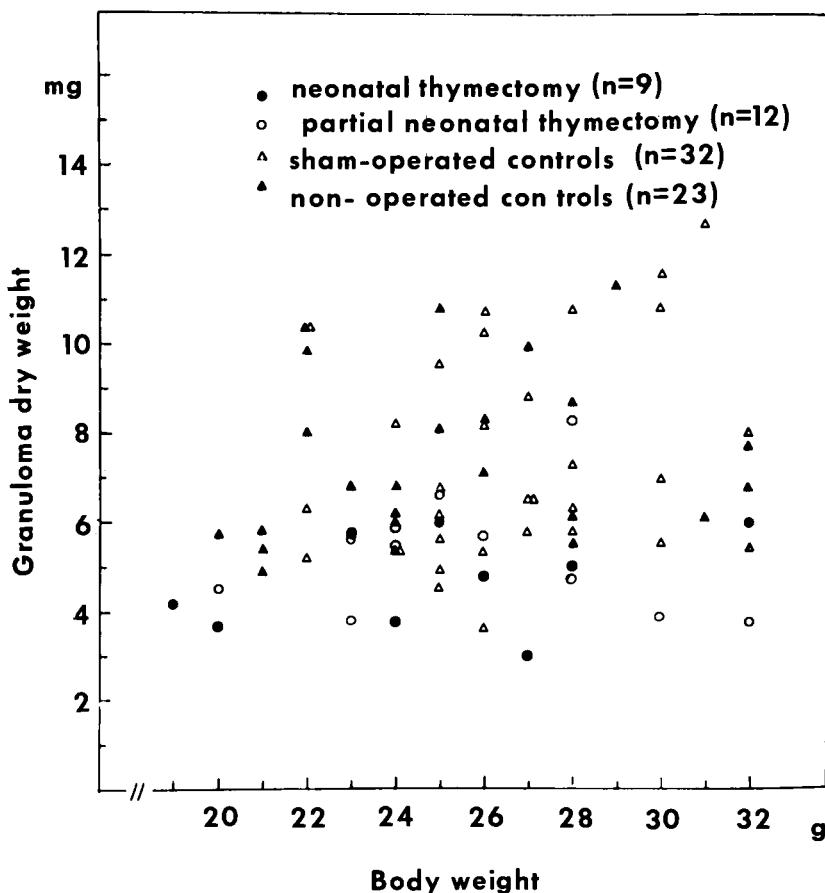


FIGURE 1. Effect of complete and incomplete thymectomy on the development of granuloma around carrageen-soaked cotton-pellets. Swiss-S-albino mice. Dry weight of cotton pellets 2 mg. Thymectomy within 20 hours after birth, implantation of the pellets at age of 40 days, and removal 15 days later.

geen-soaked cotton pellets. In comparison with sham-operated (34 animals) and non-operated controls (23 animals), the wet weight and dry weight (Fig. 1) of the granuloma in the groups of completely (12 animals) and incompletely (13 animals) thymectomized mice were considerably decreased ( $p < 0.0005$ ). No differences were found between completely and incompletely thymectomized mice or between sham-operated and non-operated controls.

Hitherto, there has been no explanation for this influence of the thymus on the formation of granuloma. The recent findings of Dukor and Miller (2) can probably be considered an analogy to this phenomenon, as these authors described a decrease of mitotic indices of liver regenerates of mice thymectomized neonatally or at the age of six weeks.

### **Abstract**

Using inbred mice (C57BL, Swiss-S-albino), paw-oedema tests with formalin or carrageen and the cotton-pellet method with carrageen-soaked pellets were performed in order to study the influence of the thymus on the inflammatory response. Neonatal thymectomy was carried out within 20 hours after birth; simultaneously sham-operated and non-operated mice served as controls. All tests were initiated at the age of 40 days. Neither formalin paw-oedema nor carrageen paw-oedema were affected by neonatal thymectomy. The wet weight as well as the dry weight of the granuloma in the cotton-pellet test were markedly decreased in completely and incompletely thymectomized mice.

### **Abrégé**

L'influence du thymus sur la réaction inflammatoire chez des souris consanguines de souche C-57BL, Swiss-S-albino, fut étudiée par l'épreuve de l'oedème de la patte provoqué par de la formaline ou de la carragénine ainsi que par la méthode du "pellet" de coton imbiber de carragénine. La thymectomie néonatale fut effectuée au cours des 20 heures qui ont suivi la naissance; des animaux témoins non-opérés ou ayant subi une opération simulée furent utilisés. Les animaux étaient âgés de 40 jours au moment des épreuves. La réaction oedémateuse de la patte à la formaline ou encore à la carragénine fut inchangée par la thymectomie néonatale. Par ailleurs, le poids humide ou sec du granulome imbiber de carragénine était considérablement diminué aussi bien chez la souris complètement qu incomplètement thymectomisée.

### **Acknowledgements**

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

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*R. F. Strelbel and S. Levine:* As shown by Heilmeyer *et al.*, different models of inflammation in the mouse are not equally influenced by prior neonatal surgical thymectomy. Thus, Heilmeyer reported that neonatal thymectomy in mice does not alter the inflammatory process associated with formalin paw-edema and carrageen paw-edema. On the other hand, it is of interest that Heilmeyer was able to demonstrate that the thymectomized mouse is protected against inflammation associated with the carrageen-soaked cotton pellet technique.

Neonatal surgical thymectomy is well known to cause a suppression of the lymphocyte population in the lymph nodes, spleen, and peripheral blood (Arnason, B. G., *et al.*, *J. Exptl. Med.*, 116:177, 1962). In relating this information to Heilmeyer's results, it is conceivable that the suppression of inflammation around carrageen-soaked cotton pellets is dependent upon a dearth of circulating lymphocytes in his neonatally thymectomized mice. On the other hand, since Heilmeyer's formalin paw-edema and carrageen paw-edema techniques produced inflammation in thymectomized mice, it is possible that the latter models of inflammation are not dependent on the circulating lymphocyte for their induction.

In regard to the role of the lymphocyte in Heilmeyer's models of inflammation, it would be of interest to ascertain how a heterologous anti-lymphocyte antiserum affects each of his models. If anti-lymphocyte antiserum, as in the case of neonatal surgical thymectomy, suppresses inflammation associated with the carrageen-soaked cotton pellet technique, it would lend further support to the possibility that the lymphocyte is an important factor in the production of inflammation by the latter method. The thymus and its relation to inflammation has been approached in our laboratory in quite another way. We have studied the effects of neonatal thymic calcification

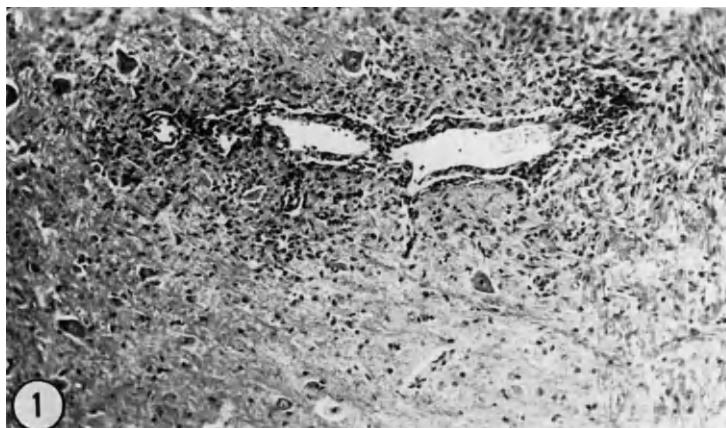
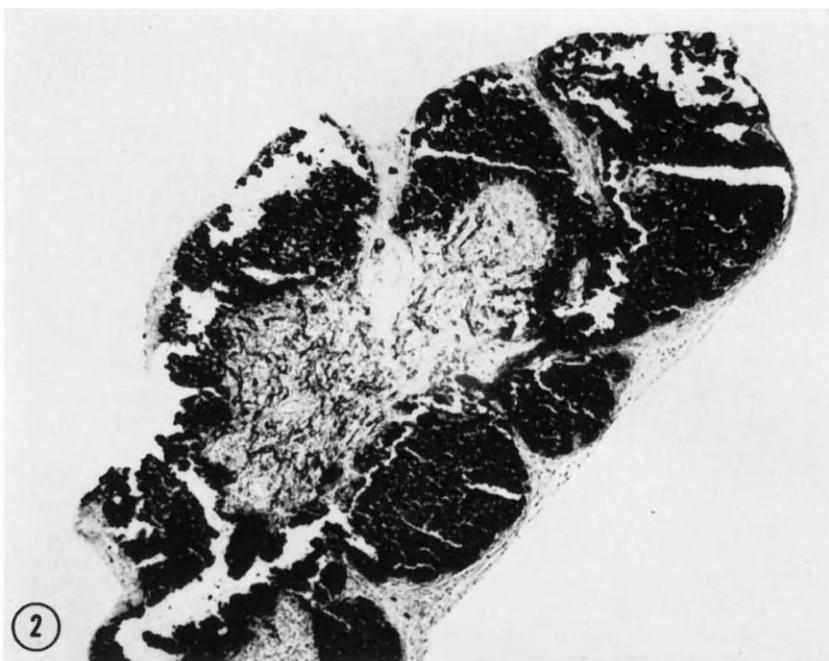


FIGURE 1. Perivascular infiltration of mononuclear inflammatory cells in spinal cord of rat with EAE. Hematoxylin-eosin stain  $\times 100$ .

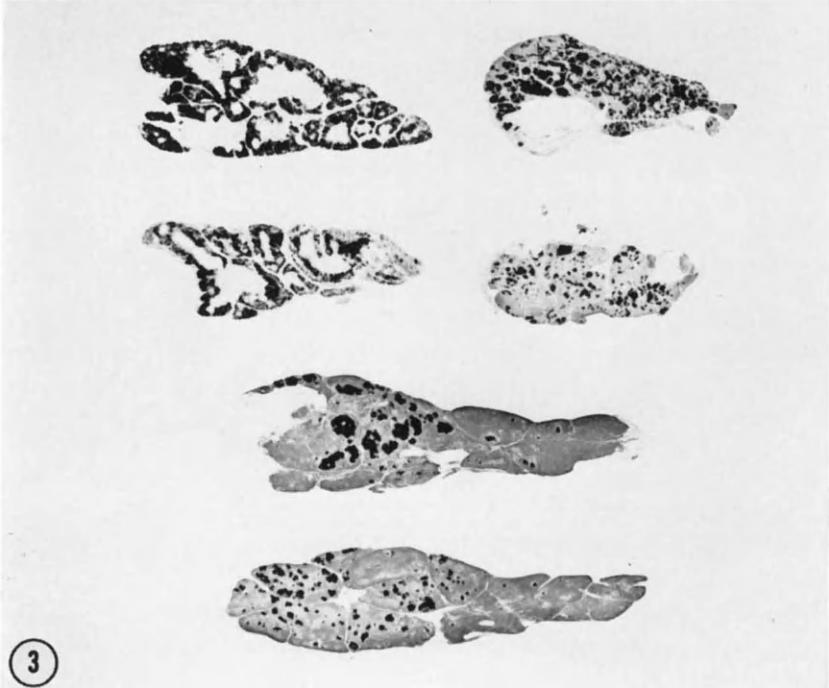
(chemo-ablation of the thymus) on the development of experimental allergic encephalomyelitis (EAE), a delayed hypersensitivity reaction that constitutes still another form of inflammation. EAE in the rat is characterized clinically by weakness and subsequent paralysis, especially of the tail and hind limbs. Histologically, the salient feature is perivascular inflammation that consists of mononuclear infiltrates in the hind brain and spinal cord. Figure 1 is an illustration of typical EAE lesions in the spinal cord. It should be noted that it has been previously reported that neonatal surgical thymectomy is effective in suppressing EAE in the rat (Arnason, B. G., et al.: *J. Exptl. Med.*, 116:177, 1962; Arnason, B.G., et al.: *Nature*, 194:99, 1962), as is antilymphocyte antiserum (Waksman, B. H., et al.: *J. Exptl. Med.*, 114:997, 1961).

In order to induce chemo-ablation of the thymus, we used a variation of the technique originally described by Selye and Padmanabhan (*Endocrinology*, 24:179, 1962). Instead of adult female rats we used neonatal rats (Strebel, R. F., et al.: *Proc. Soc. Exptl. Biol. Med.*, 118:617, 1965). These animals were injected on the sixth day of life with dihydrotachysterol (DHT) and subsequently challenged with triamcinolone (Triam), a thymolytic glucocorticoid, on the seventh day (Table I, group 3). Maximal calcification, which appeared to be sufficiently severe to constitute a chemo-ablation of the thymus, occurred within one week after this dual treatment (Fig. 2).

To test the functional effects of neonatal rat thymic calcification



(2)



(3)

on ultimate immunologic competence, susceptibility to EAE was determined in 8-week-old animals with a history of thymic calcification (Strebel, R. F., et al.: *Exptl. Med. Surg.*, 24:20, 1966). To induce EAE, the rats were injected in the hind foot pad with guinea-pig spinal cord tissue emulsified in Freund's complete adjuvant.

The inoculated animals were graded (arbitrary scale of 1-4) for clinical evidence of EAE over a subsequent 21-day period and for histologic lesions at necropsy. Several control groups were included in the experiment (Table I). These comprised: untreated animals (group 1), animals that were surgically thymectomized on the 6th day post-partum (group 2), animals that were given only Triam on the 7th day post-partum (group 4) and animals that were given Triam on the 6th day post-partum followed by DHT 3 days later (group 5). The experimental arrangement for this last control group was devised to use the same drugs as in the main experimental group (group 3) but administered in a reversed sequence that would fail to produce thymic calcification.

Although the neonatal thymic calcification appears severe enough to constitute a chemo-ablation of the thymus (Fig. 2), the data in Table I indicates that neonatal thymic calcification (group 3) had no effect on the WBC count, lymphocyte population in lymphoid tissue, and susceptibility to EAE (clinical and histological). The neonatal thymic destruction (calcification) was apparently not sufficiently severe to alter the functional capacity of the thymus when subsequently evaluated at maturity (8 weeks of age). At sacrifice, the animals in group 3 manifested a small amount of residual thymic damage as evidenced by some focal calcium deposits and the collapse of some lobules. Aside from this relatively mild pathology, the rats of group 3 regained ample viable thymic tissue during the 10-week-



FIGURE 2. Thymic calcification in a neonatal rat treated with DHT and Triam. Calcification is predominantly in cortex, but there is some calcium in walls of blood vessels of medulla. von Kossa  $\times$  150. (After Strebel, R., et al.: Courtesy *Proc. Soc. Exptl. Biol. Med.*, 118:617, 1965.)

FIGURE 3. Left upper: Two thymuses, 5 days after Triam. There are bands of calcium that follow the cortical contours of each lobule. Right upper: Two thymuses, 2 weeks after Triam. The calcific masses are denser and have become aggregated into nodules. Below, two thymuses, 4 weeks after Triam. The calcified material is very dense and well aggregated, but the outstanding change is the tremendous regeneration of cortical tissue. Phloxine-von Kossa stain  $\times$  3. (After Levine, S., et al.: Courtesy *Exptl. Mol. Pathol.*, 6:237, 1967.)

TABLE I  
EFFECT OF NEONATAL THYMIC CALCIFICATION ON EAE AND  
LYMPHOCYTE POPULATION IN BLOOD AND LYMPHOID TISSUE

Group	Neonatal Treatment <sup>1</sup>	No. of Rats	Thymic Calcification (Scale 0-3)	EAE <sup>2</sup> Readings (Scale 1-4)	Clinical Histologic Rats	Blood Counts 8 Weeks of Age			Lymphocyte Population 8 Weeks of Age (Noes/ and Spleen)
						No. of Rats	WBC/cmm	% Lymphs	
1	None	30	0	2.2	1.4	6	16,200	77	Normal
2	Surg. thymectomy (6th day)	34	0	1.0	0.7	6	11,700	66	Low
3	DHT (6th day) + Triam (7th day)	81	0.5	2.2	1.3	10	15,200	80	Normal
4	Triam (7th day)	12	0	2.5	1.1	4	14,400	80	Normal
5	Triam (6th day) + DHT (9th day)	52	0	2.5	1.4	8	18,700	82	Normal

<sup>1</sup>The post-partum day for each treatment is indicated.

<sup>2</sup>Animals inoculated for EAE at 8 weeks of age. Clinical readings represent average severity of EAE during the ensuing 21-day observation period. Histologic readings represent average severity of spinal cord lesions on the 22nd post-EAE inoculation day.

period intervening between the induction of neonatal thymic calcification and the end of the EAE observation period (11th week of life). Further experiments (Levine, S., *et al.*: *Exptl. Mol. Pathol.*, 6:237, 1967) in our laboratory, using the combined treatment outlined above for the induction of thymic calcification, showed a dramatic regeneration of thymic cortex and progressive loss of thymic calcification within four weeks after Triam challenge (Fig. 3).

In contrast to the above results, and in confirmation of the work of others (Arnason, B. G., *et al.*: *Nature*, 194:99, 1962), surgically thymectomized animals (group 2) manifested a suppression of EAE, both clinically and histologically. The blood count for these animals was within the normal range for the rat, but total count and lymphocyte percentage were lower than in any of the other groups. Moreover, histologic examination of lymphoid tissue (lymph nodes and the spleen) revealed a relative decrease in the lymphocyte population in the popliteal and mesenteric lymph nodes (especially primary lymphoid masses) and in the splenic white pulp (especially around the arterioles). Of the original 36 surgically thymectomized neonates, only two were found to have residual fragments of thymic tissue at autopsy and these two were eliminated from the experiment. Control animals (groups 1, 4, and 5) did not manifest a suppression of EAE and presented normal blood counts and a normal lymphocyte population in lymphoid tissues. No sex differences were noted in regard to any of the treatments. Thus, unlike neonatal surgical thymectomy, neonatal thymic calcification is ineffective in preventing the destructive inflammatory lesions associated with the EAE delayed hypersensitivity response.

In summary, we may say that the chemical method of thymic ablation in the rat is not sufficiently destructive to prevent EAE, a delayed hypersensitivity reaction that is apparently dependent on the circulating lymphocyte. The early (neonatal) destruction seen in the thymus appears severe enough to constitute a chemo-ablation of the thymus, but enough viable cells are spared to allow a reconstitution of the thymic cortex. Our failure to alter the course of a delayed hypersensitivity reaction via chemo-ablation of the neonatal thymus should not deter others from further refining the technique of neonatal thymic calcification. If the latter technique can be improved, it would be of interest to replicate our attempt to suppress the inflammatory reaction associated with EAE and also to attempt to suppress non-immunologic inflammatory responses such as those used by Heilmeyer *et al.*

## The Renin-angiotensin-aldosterone System in Renal Hypertension

G. M. C. MASSON

EARLY evidence for a possible causal relationship between renin and renal hypertension was based on three main observations: one made in 1898 by Tigerstedt and Bergman (42), who noted that the intravenous injection of saline extracts of kidneys elicits a rise in arterial pressure and who attributed this pressor effect to a substance which they called renin; the second one by Goldblatt *et al.* (14), in 1934, who caused hypertension by partial constriction of the renal artery; and the third one in 1939 by Braun-Menendez *et al.* (5) and by Page and Helmer (34), who simultaneously demonstrated that renin is an enzyme which is not directly pressor but acts on a globulin present in blood to liberate a pressor substance, angiotensin. There was apparently sufficient evidence to suggest that partial constriction of the renal artery caused hypertension through the release of renin and subsequent formation of angiotensin. To remove any doubt about this mechanism, angiotensin was then called hypertensin by some (5). The kidney would therefore act as a typical endocrine gland and hypertension would be an expression of hyperactivity. Despite greater knowledge on the chemistry, physiology and pharmacology of renin and angiotensin, this view, for lack of direct evidence, still remains a hypothesis.

Present evidence is derived from determinations of the renin concentration in kidneys and plasma, from the effects of antirenin on hypertension, from the effects of chronic injections of angiotensin and renin, and from determinations of aldosterone.

### **1) Renin Content of Kidneys in Renal Hypertension**

Renal renin is elevated in a kidney with the renal artery constricted and low in the contralateral kidney (16, 35). Since an in-

crease in renin content presumably reflects an increase in secretion (33), it can be assumed that the increase in content is at the origin of the hypertension. However, this conclusion is nullified by the observation that an increase in renin content does not necessarily result in hypertension and that, conversely, hypertension may occur even when the renin content remains normal, as is seen when contralateral nephrectomy is performed simultaneously with clipping of the renal artery (36). In some way, the contralateral kidney is necessary for the increased renin concentration.

## 2) *Plasma Renin and Angiotensin in Renal Hypertension*

There seems to be general agreement that "pressor activity," angiotensin and renin in plasma are elevated during the early and malignant phases of hypertension (18, 39, 41), but not during the chronic phase (4, 39). The period during which renin is released may last about one week after constriction of the renal artery. As noted in the case of the renin content of kidneys, there is no parallelism between the plasma renin concentration and the degree of hypertension. Thus, Brown *et al.* (7) observed that in sodium depleted dogs constriction of the renal arteries results in hypertension without a significant increase in plasma renin, and that salt loading in hypertensive dogs reduces plasma renin but not blood pressure levels. Assuming that renin and angiotensin levels parallel each other, one explanation is that salt has inverse effects on renin secretion and on cardiovascular reactivity to renin. The decrease in renin secretion caused by salt loading, which would be expected to cause a fall in arterial pressure, is compensated by an increase in reactivity to renin and vice versa. Lever and Robertson (21) reported that renin in the peripheral blood was elevated in rabbits with renal hypertension as well as in some rabbits which did not become hypertensive in spite of a clamp on the renal artery. It may be that we are attaching too much significance to renin, which is after all only the initial component of a series of enzymatic reactions leading to the formation of angiotensin II. Determinations of angiotensin II, although more desirable, are not yet possible on small blood samples.

### **3) Effects of Antirenin on Hypertension**

Remission of hypertension has been reported in dogs with chronic renal hypertension, following treatment with heterologous or acetylated homologous antirenin (11). The hypertension recurred following cessation of treatment, with subsequent disappearance of the renin antibodies. Although these experiments are considered crucial in demonstrating the role of the renal pressor system, they were performed in only a few dogs. No report has been presented that similar treatment would prevent hypertension or be effective during the acute phase and, furthermore, since renin has not been isolated as a pure substance, it is likely that the antirenin preparations contain antibodies against renin as well as against the impurities present.

### **4) Production of Hypertension with Angiotensin and Renin**

Since single intravenous injections of renin and of angiotensin caused a pressor response, it was to be expected that the intravenous infusion of these agents would elicit a sustained rise in pressure. Elevated arterial pressures have been maintained for periods up to 3 to 4 months in rabbits; blood pressure fell at once upon cessation of treatment. No vascular lesions were noted. Increasing the rate of infusion gave a greater initial rise which was shortly followed by a return to normal levels in spite of the continuation of the infusion (2, 6). More significant results have been obtained by infusing minute amounts of angiotensin or by the subcutaneous injection of kidney extracts rich in renin. Dickinson and Lawrence (12) showed that infusion of angiotensin, in doses which initially were non-pressor, caused a gradual and sustained increase in arterial pressure. Masson and his collaborators (26-28) later showed that extracts of endocrine kidneys, which contain large amounts of pressor material, crude extracts of hog kidneys or semipurified hog renin to which retarding substances had been added, also caused hypertension and vascular disease when administered subcutaneously to uninephrectomized rats. These animals show the same changes as those with a clip on one renal artery: decrease in renin in the remaining kidney similar to that found in the kidney contralateral to the clipped kidney, nephro-

sclerosis, vascular disease, and hypertrophy of the zona glomerulosa. The exact mechanisms responsible for the hypertension induced by renin and angiotensin are not known. It is not due to an accumulation of angiotensin, since the half-life of this peptide is only about half an hour (17). There is evidence for self potentiation of the effects of angiotensin, since nonpressor doses cause hypertension. Masson *et al.* (30) have also shown that on repeated injections of renin, pressor responses to renin and angiotensin become progressively greater. This increased sensitivity may result from electrolyte changes or participation of nervous factors.

In summary, one can assume that the development of renal hypertension results from a definite but still unknown pattern in renin secretion, this may be more important than the amounts of renin secreted.

### 5) Determinations of Aldosterone

Methods for determination of renin are not specific. Those for the determination of angiotensin require large amounts of blood, which limit their usefulness. Unlike these two substances, aldosterone has been definitely identified in plasma or urine, where it can be measured by accurate and specific chemical methods. Angiotensin is a potent and specific stimulus of aldosterone secretion (10, 13). In view of the functional relationship existing between the renal pressor substances and aldosterone it has been suggested that this renal-adrenal interaction constitutes a well integrated and specific hormonal system (1). Thus, since renin through angiotensin stimulates aldosterone secretion, aldosterone secretion rates should be elevated in renal hypertension if the hypertension is due to excess production of renin. The data are conflicting on this point, even when clinical observations are included. In dogs with benign hypertension, aldosterone secretion rates are normal; but they are increased during malignant hypertension (8, 31). In rats, secretion rates are elevated when the kidney contralateral to the clipped kidney is present, and normal in the absence of the contralateral kidney (40). Aldosterone secretion parallels renin content in the clipped kidney. Thus, the contralateral untouched kidney plays a critical but mysterious role.

Normal values have been reported in patients with mild hy-

pertension due to unilateral renal stenosis (19, 38). In the more acute or severe forms (9, 19), both renin and aldosterone levels can be markedly elevated. Such patients, like those with primary aldosteronism, show hypoalkalosis. It is significant to note that, whether or not these hormonal abnormalities are demonstrable, this form of hypertension can be corrected by appropriate surgery.

### **Discussion**

From these observations, it is apparent that there is not necessarily a direct relationship between the occurrence of renal hypertension and the amounts of circulating renin, angiotensin or aldosterone. Hypertension may be present in spite of normal levels of renin or aldosterone. On the other hand, hypertension is not always present when renin and aldosterone levels are elevated, as during sodium deficiency or in patients with secondary aldosteronism associated with edema and ascites. Thus, more significance should be attached to vascular reactivity or the response of the vascular system to a given blood level of angiotensin than to angiotensin levels.

Mechanisms that increase cardiovascular reactivity to angiotensin may be metabolic or neurogenic. The metabolic mechanisms are related to the sodium state; sodium retention resulting from excess salt in the diet or administration of desoxycorticosterone enhances pressor responses to exogenous renin or angiotensin (15, 24), and sodium depletion has the opposite effects (3, 37). In human subjects, as well as in animals, constant prolonged infusion of small doses of angiotensin causes a gradual increase in arterial pressure and less and less angiotensin is required to obtain the same pressor response. This led Ames *et al.* (1) to suggest that an increase in vascular reactivity is related to concomitant sodium retention and intravascular accumulation of sodium ions. This may be true in human subjects but not in experimental animals, in which angiotensin causes natriuresis instead of sodium retention. To reconcile this inconsistency, it may be more reasonable to assume that angiotensin, whatever its renal effect, elicits a more subtle change such as a redistribution of sodium ions within the arterial wall. The increase in vascular reactivity would explain the development of hypertension by amounts of angiotensin too small to be detected and too small to stimulate aldosterone secretion.

That neurogenic mechanisms are capable of modifying vascular reactivity to angiotensin has been demonstrated by the increased response to renin and angiotensin following resection of the sinoaortic

buffer nerves (29). It has also been noted that section of the buffer nerves enhances renal but not adrenal hypertension (20, 29). Participation of the nervous system during infusion of minute amounts of angiotensin has been suggested by the observation that the vaso-depressor response to Trimetaphan becomes progressively more pronounced as arterial pressure increases (43). We have previously proposed (28) that angiotensin has two main effects on blood vessels: an acute vasoconstriction due to a direct action on vascular smooth muscles, and a hypertensive effect which is indirect and delayed and which, as its name indicates, is mainly responsible for hypertension. Whether this hypertensive effect results from changes in sodium distribution or from nervous mechanisms or from both remains to be seen.

The case of malignant hypertension is more clearly defined. There is general agreement that it is associated with high levels of renin and angiotensin and of aldosterone (19). This form of hypertension represents a derangement in the renin-angiotensin-aldosterone endocrine

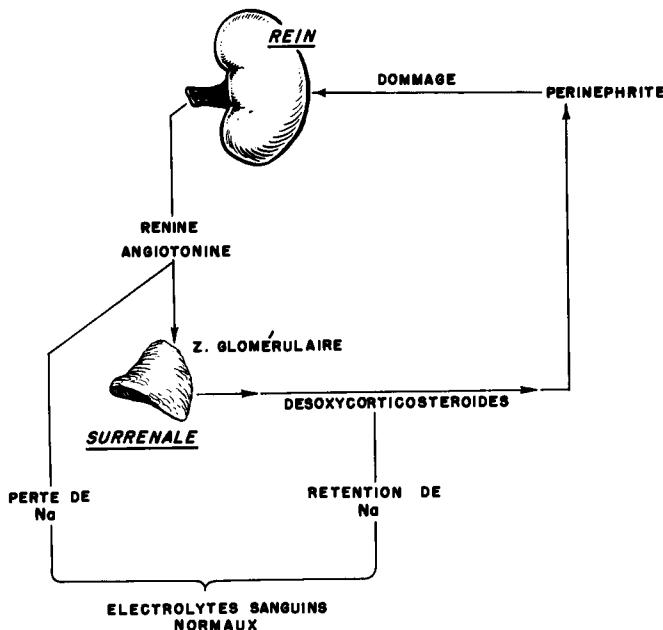


FIGURE 1. Proposed renal-adrenal relationships in renal hypertension. (From MASSON, et al.: *Compt. Rend. Congrès de Cardiologie*, Paris, J. B. Baillière, 1950.)

system, in which the negative feed-back mechanisms are no longer operating. Interactions between angiotensin and aldosterone would then accelerate vascular deterioration and impair further renal function. Such a vicious cycle (Fig. 1), already described in 1950 by Masson *et al.* (22), was based on the following observations. Knowing that desoxycorticosterone causes hypertension and vascular disease, and that injection of renin and renal hypertension are associated with a stimulation of the adrenal zona glomerulosa, source of desoxycorticosteroids, we postulated that severe renal hypertension resulted from an interaction between renal and adrenal hormones. This was demonstrated by the observation that injections of renin to desoxycorticosterone-treated rats elicit a syndrome reminiscent of malignant hypertension. Later on, under the same experimental conditions, the syndrome was produced by substituting angiotensin for renin (23) and aldosterone for desoxycorticosterone (25). In the doses used, each of these agents caused either slight or no elevation of arterial pressure. Experimental and clinical observations have since supported this hypothesis.

### **Abstract**

Based on results obtained from determinations of the renin concentration in kidneys and plasma, from the effects of antirenin, from the effects of chronic injections of angiotensin and renin, and from determinations of aldosterone, an evaluation of the role of the renal pressor system in renal hypertension has been presented. Malignant renal hypertension seems to be definitely associated with increased amounts of angiotensin and of aldosterone; interaction between these two hormones may be at the origin of the acute vascular disease. Although the role of this system in benign hypertension is still not clear, data suggest that it plays a critical role during the early phase, and only a permissive role during the later phase. The sodium ion may intervene by increasing vascular reactivity to angiotensin; sodium redistribution in the vascular wall may have more significance than excess body sodium. The role of the nervous system in increasing the reactivity to angiotensin and in enhancing renal hypertension remains to be more clearly defined.

### **Abrégé**

A partir de données sur la concentration de la rénine dans les reins et le plasma, sur les effets de l'anti-rénine, sur les effets hypertenseurs d'injections prolongées de rénine et d'angiotensine, et sur la sécrétion d'aldostérone, nous avons cherché à évaluer le rôle du système presseur rénal dans l'hypertension d'origine rénale. Il apparaît définitivement établi que l'hypertension maligne est associée à une forte élévation de la rénine, de l'angiotensine et de

l'aldostérone plasmatique; des interactions entre angiotensine et aldostérone semblent être responsables des lésions vasculaires aiguës et de l'évolution rapide de l'hypertension. Bien que le rôle de l'angiotensine dans l'hypertension bénigne ne soit pas encore bien défini, les résultats présents suggèrent qu'elle joue un rôle critique pendant la phase aiguë et seulement un rôle secondaire pendant la phase chronique. L'ion sodium est important, parce qu'il augmente la réponse vasculaire à l'angiotensine; toutefois il se peut que ce ne soit pas tant la quantité qui compte que sa distribution dans les parois des vaisseaux sanguins. Le rôle du système nerveux dans le développement de l'hypertension rénale reste encore à préciser.

### Acknowledgements

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**J. M. Rojo-Ortega:** I want to congratulate Dr. Masson for his masterly presentation on the renin-angiotensin-aldosterone system in renal hypertension. I would like to present briefly, in this discussion, a simple method for producing experimental hypertension in rats. About four years ago, we performed some experiments (unrelated to hypertension), in collaboration with Dr. M. Miranda, in Dr. Selye's laboratory. We observed that, after "complete ligation of the aorta"

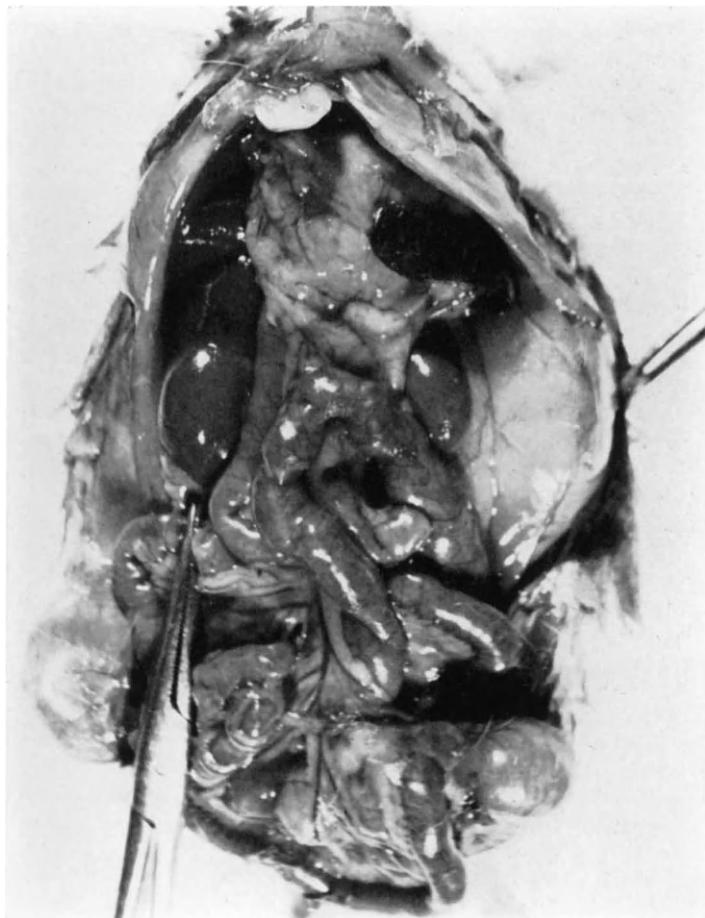
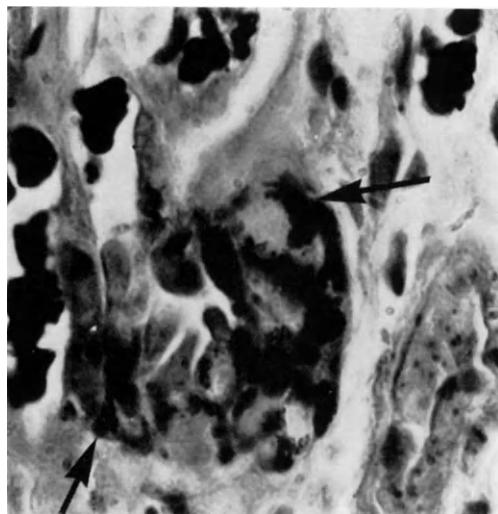


FIGURE 1. Macroscopic view of the abdominal cavity, showing the smaller left kidney as compared with the right one. Note the normal appearance of the intestines 14 days after complete aortic ligature.

between the two renal arteries, adult rats developed atrophy of the left kidney accompanied by hypertrophy of the right one (Fig. 1) and frequent infarctoid heart lesions. Later, we developed this simple method in relation to body weight. We observed that some animals developed a picture similar to that described by Selye and Stone as an "endocrine kidney." This experimental method gave excellent results in animals over 300 g, about 80% becoming hypertensive within a month.

FIGURE 2. Afferent arteriole showing numerous granulated cells (Arrows). "Endocrine kidney" after three weeks. Modified Bowie stain  $\times$  1250.



These experimental models enabled us to study the juxtaglomerular (JG) complex. Here, we found an increase in JG cell granularity (Fig. 2), glucose-6-phosphate dehydrogenase activity of the macula densa (Fig. 3), and plasma renin activity, in spite of the fact that no urine is formed by the "endocrine kidney." No correlation was found between the blood pressure and the various parameters studied.

At the level of the macula densa, since the tonicity or the sodium concentration of the tubular fluid has been emphasized as one of

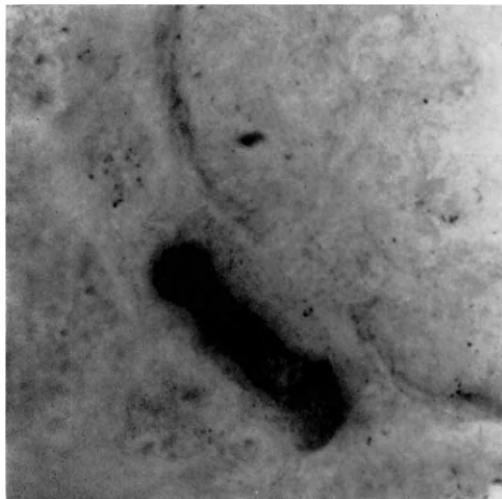


FIGURE 3. High activity of the glucose-6-phosphate dehydrogenase in the macula densa. "Endocrine kidney" after three weeks.  $\times$  670.

the stimuli for the cytological changes occurring in the JG complex and for renin release, our results strongly suggest that such a stimulus does not play any major role in the "endocrine kidney." These studies were supported by grant No. MT-1740 from the Medical Research Council of Canada.

## **Studies on the Pathogenesis of Adrenal-regeneration and Methylandrostenediol Hypertension**

FLOYD R. SKELTON and ALEXANDER C. BROWNIE

**T**WO types of experimental endocrine hypertensive disease have been described, whose pathogenesis has intrigued students of this subject since they were first reported.

The first type is that which occurs in uninephrectomized, salt-treated rats during regeneration of the adrenal cortex after enucleation of one adrenal and removal of the other (44). Despite the fact that blood pressure elevation occurs during active regeneration when the mass of cortical tissue is considerably below normal (13, 48), we have been convinced that the function of this regenerating gland plays a primary role in the development of the hypertensive state (49). This belief has been supported by the observations that when adrenal regeneration is interrupted by such procedures as hypophysectomy (46), the presence of the opposite adrenal (46), or by the administration of corticosterone (47) or testosterone propionate (25), the hypertension fails to develop. When synthesis of steroid hormones by the regenerating gland has been inhibited by either amphenone B (8) or diethyl stilbestrol (51) the occurrence of hypertensive disease is also obviated. Transplantation of the enucleated adrenal into the portal circulation, thus routing adrenal venous blood through the liver before it enters the general circulation, also prevents the occurrence of hypertension and vascular disease (10). The observation that delayed regeneration of the adrenal cortex by transplanting the capsule subcutaneously (9) or by leaving the contralateral adrenal gland in place for a period of time before removing it (9) is accompanied by a concomitant delay in the production of the hypertensive disease has added further support to the conviction that some functional activity of the regenerating adrenal is concerned in the development of the hypertension.

The second type is that produced by the administration of

methylandrostenediol [ $17\alpha$ -methyl- $\Delta^5$ -androst-5-ene- $3\beta$ ,  $17\beta$ -diol (MAD)] to uninephrectomized rats drinking 1% sodium chloride solution (43). Since this compound is an androgen devoid of mineralocorticoid activity (16), it was originally thought that the observation cast some doubt on Selye's hypothesis that hypertensive disease could result specifically from mineralocorticoid hypersecretion by the adrenal. Subsequent studies of Salgado and Selye (38, 39) showed that in the absence of the adrenal glands, MAD did not produce hypertension and cardiovascular renal lesions. This led them to postulate that MAD interfered with adrenal cortical activity in such a way that excessive amounts of mineralocorticoid hormones were secreted and these in turn induced the hypertensive syndrome.

Many studies have been done to characterize the secretion pattern of regenerating adrenals in the hope of explaining adrenal-regeneration hypertension and these have been considered at length in a recent review (52). On the other hand few reports concerning steroid biogenesis by adrenals from MAD-treated rats have appeared (7, 37). Our studies in these areas have been limited very largely to an examination of the *in vitro* conversion of progesterone to corticosteroids by normal and regenerating adrenal glands and by adrenals from rats treated with MAD. Since Saffran has shown (36) that MAD is converted by the adrenal into methyltestosterone [ $17\alpha$ -methyl- $17\beta$ -hydroxy androst-4-ene-3-one (MT)], our attention has been directed towards a similar examination of the steroid biogenesis by adrenals from rats treated with this compound. In addition to these biochemical studies, the adrenal tissues have been examined electron microscopically in attempts to associate, at least, the functional changes with the ultrastructural appearance of the cortical cells.

### **Materials and Methods**

In the studies on adrenal regeneration, immature female Holtzman rats have been used. All rats have had one kidney and one adrenal removed, have been fed Purina laboratory chow and allowed to drink 1% sodium chloride solution *ad libitum*. Rats in experimental groups had the remaining adrenal enucleated according to the procedure of Ingle and Higgins (19).

In the MAD and MT studies, immature male and female Holtzman rats were used. All rats were uninephrectomized, fed Purina laboratory chow and given 1% sodium chloride solution to drink *ad libitum*. Rats in experimental groups were injected subcutaneously daily with 10 mg of MAD, either as a micro-crystalline suspension in distilled water or in solution in corn oil. MT was given at the same daily dosage as a subcutaneous injection in corn oil. Control rats received injections of equal amounts of the vehicle.

Adrenal homogenate incubations and steroid analyses were carried out according to previously described procedures (6). In summary, these consisted of short-term incubation of adrenal homogenates with progesterone-4-<sup>14</sup>C in the presence of added fumarate with or without supplemental NADP. Biosynthetic products such as corticosterone, 18-hydroxy-11-desoxycorticosterone (18-OH DOC) and 11-desoxycorticosterone (DOC) were then isolated by paper chromatography and quantitated.

For electron microscopy, adrenals were prepared by two procedures. In one, the rats were killed by decapitation and the adrenal glands removed immediately and minced in 4.5% glutaraldehyde buffered with collidine or sodium phosphate to pH 7.3. After 1½ hours, the tissue fragments were removed and washed for 10-20 hours in buffer at pH 7.3, followed by fixation for an additional 1½ hours in 3% osmium tetroxide buffered with collidine or sodium phosphate to pH 7.3. The tissue was then washed for 15 minutes in 3 changes of collidine buffer and transferred into 70% ethanol. After dehydration in graded concentrations of alcohol and propylene oxide, the tissue fragments were embedded in Epon or Maraglas.

In the other procedure, the adrenal gland was perfused for 10 minutes with 1% glutaraldehyde buffered with collidine or sodium phosphate to pH 7.3, at the previously determined systolic blood pressure of the rat. The gland was then removed, placed in 4.5% glutaraldehyde, and cut under a dissecting microscope into blocks representing specific zones. The tissue was embedded in Epon or Maraglas as for the minced tissue.

Sections were cut on a Porter-Blum ultramicrotome and stained for 2 minutes in saturated uranyl acetate solution followed by 10

minutes in lead citrate. The sections were examined in a Siemen's Elmiskop I electron microscope.

## Results

### *Adrenal Regeneration Hypertension*

**a) Steroid Biogenesis.** In young male or female rats, a significant elevation of systolic blood pressure can be seen as early as two weeks after enucleation; by five weeks after enucleation, hypertension is marked and accompanied by severe vascular disease in the kidney, heart, pancreas, mesentery and brain (3). In the present investigation, a comparative study was made of the ability of normal and regenerating adrenals to metabolize progesterone-4-<sup>14</sup>C at intervals of 2, 3 and 5 weeks after enucleation; 50 mg of control and regenerating adrenal tissue was used per incubation and incubations lasted for 10 minutes in the presence of added fumarate alone and fumarate plus NADP.

The results are shown in Figure 1. It can be seen that adrenal gland homogenates from all control rats formed more corticosterone than DOC when incubated only with fumarate; when incubated with fumarate plus NADP, the preponderance of corticosterone over DOC was overwhelming. In contrast, the regenerating adrenal gland homogenates produced predominantly DOC with little corticosterone when incubated with fumarate alone. When fumarate and NADP were used, the 3rd- and 5th-week regenerated adrenals produced corticosterone and DOC comparable to that produced by control adrenals. However, there was little effect of NADP on the activity of regenerating adrenals removed at 2 weeks. These studies indicate that 21-hydroxylation takes place readily in adrenal homogenates when 3- and 5-week regenerating adrenals were used, whereas 11 $\beta$ -hydroxylation proceeds in a manner comparable to that of control glands only when the homogenates are supplemented with NADP.

**b) Electron Microscopy of Regenerating Adrenals.** The experimental conditions of uninephrectomy and increased sodium chloride intake, which are conducive to the development of hypertension during adrenal regeneration, also bring about marked atrophy of the zona glomerulosa of adrenals from control rats and virtual absence of this zone in regenerated glands (45). For this reason, our attention has been directed primarily to the ultra-

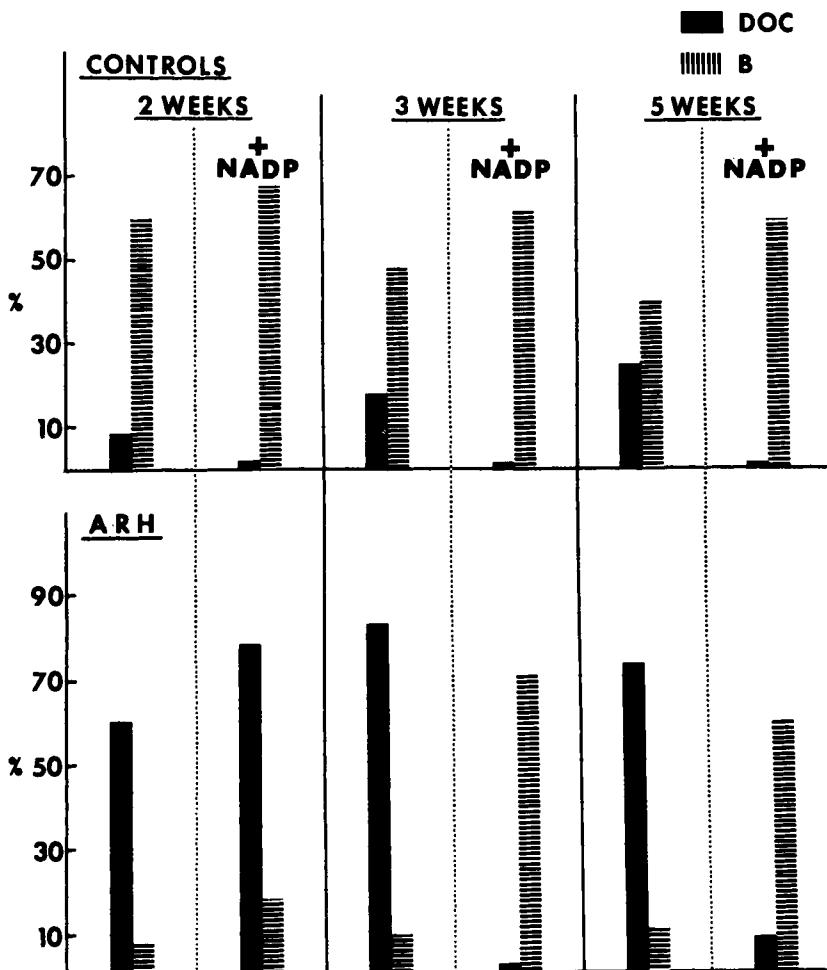
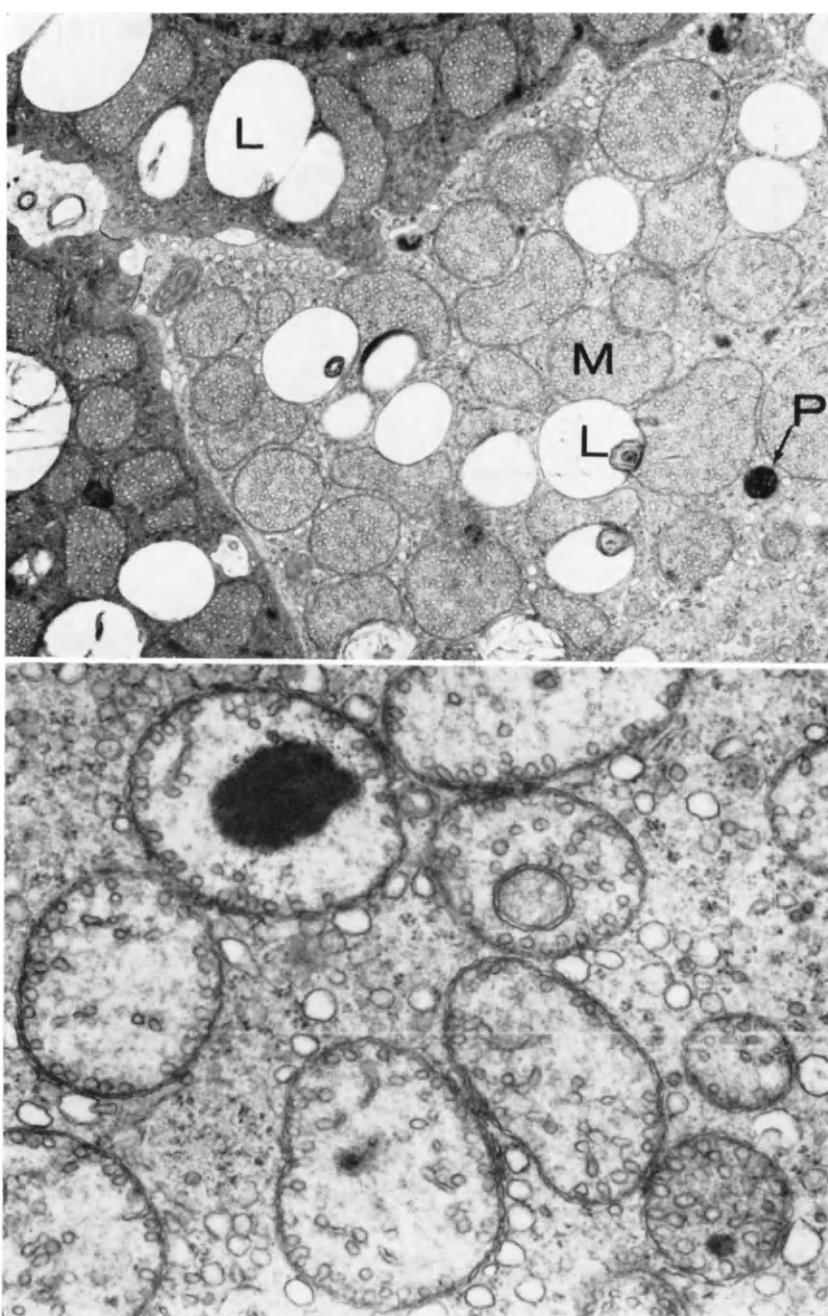


FIGURE 1. Biosynthesis of corticosterone (B) and 11-desoxycorticosterone (DOC) from progesterone-4- $^{14}\text{C}$  by normal and regenerating adrenal gland homogenates (ARH), at 2, 3 and 5 weeks post operation. Progesterone-4- $^{14}\text{C}$  ( $0.2 \mu\text{c}$ ) was incubated with 50 mg of control and regenerating adrenal tissue in 5 ml of Kreb's-Ringer- $\text{HCO}_3$  buffer containing sodium fumarate (100 mg%), with or without 1 mg NADP.

structural characteristics of zona fasciculata cells from both regenerating and control adrenals. Since 1960, a number of reports on the ultrastructure of the adrenal cortex of the rat have appeared (26, 27, 29, 34, 35, 40, 60) so that no attempt will be made here to duplicate the descriptions contained in those references. Further-



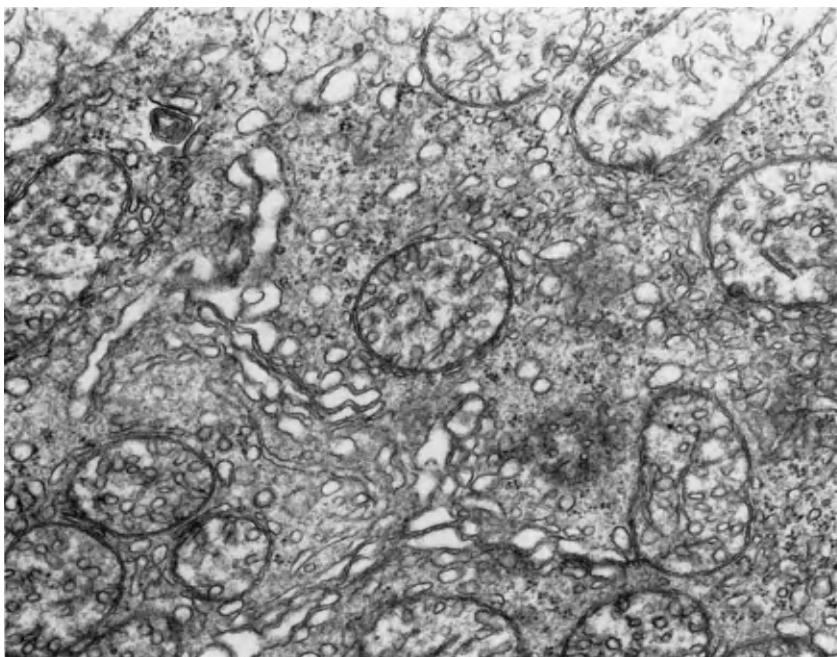


FIGURE 4. Regenerating adrenal cortical cell 5 weeks after enucleation. Mitochondria are increased in number and their content of vesicles also is greater than at 2 weeks of regeneration. Endoplasmic reticulum dilated and contains flocculent material.  $\times 22,000$ .

more, our interest in the ultrastructure of the regenerated adrenal cortical cells has centered around the mitochondria, since these organelles are known to play a vital role in adrenal steroid hormone biosynthesis (5, 24, 54).

It is worth pointing out that the normal zona fasciculata is

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FIGURE 2. Electron micrograph of adrenal cortex from a control rat showing portions of 3 zona fasciculata cells—2 dark cells on the left and 1 light cell on the right. The vacuoles (L) represent lipid droplets and the smaller electron dense structures (P) are pigment bodies. Note the large number of mitochondria (M) with tightly packed vesicles.  $\times 5,500$ .

FIGURE 3. Regenerating adrenal cortical cell 2 weeks after enucleation. Mitochondrial population is decreased. Vesicular cristae of mitochondria also reduced in number and arranged around periphery. Sparse but dilated endoplasmic reticulum present.  $\times 28,000$ .

composed of cords of cells arranged about sinusoids and that "light" and "dark" cells can be recognized. Mitochondria are generally round to oval and contain closely packed vesicles or short tubulo-vesicular cristae (Fig. 2). In the regenerating adrenals two weeks after enucleation, the most apparent difference from control glands was the reduced number of "dark" cells and in the regenerated cells themselves a decreased number of mitochondria. These latter had a swollen appearance with abundant electron lucid matrix (Fig. 3). The number of mitochondrial vesicles was greatly reduced and they were predominantly in a peripheral position. In addition, the characteristically smooth endoplasmic reticulum of adrenal cortical cells was dilated and cytoplasmic lipid vacuoles were virtually absent. Electron dense material (lipid?) was present in the central matrix of some mitochondria.

With increasing time after enucleation, the regenerated cortical cells came more and more to possess a normal ultrastructural appearance (Fig. 4). Mitochondria increased in number but continued to show peripherally arranged vesicles in many, although this characteristic became more difficult to observe as the number of vesicles increased with time.

### ***MAD Hypertension***

**a) Steroid Biogenesis.** In these studies, the MAD-treated rats and their controls were killed at 5, 12, 19, 25 and 32 days, treatment being given on only 5 days of each week. Homogenates of the adrenal glands were incubated in the presence of progesterone-4-<sup>14</sup>C for 10 minutes with added fumarate and NADP. The results are shown in Figure 5.

It can be observed that at all time intervals control adrenal homogenates converted practically all of the progesterone-4-<sup>14</sup>C to corticosterone and 18-OH DOC. In contrast, the adrenals from MAD-treated rats, while metabolizing the progesterone-4-<sup>14</sup>C to the same extent as control adrenals, formed predominantly DOC with relatively little corticosterone and 18-OH DOC.

**b) Electron Microscopy of Adrenals from MAD-Treated Rats.** At the present time, no ultrastructural changes have been observed in adrenals from rats treated with MAD for 5 days. After

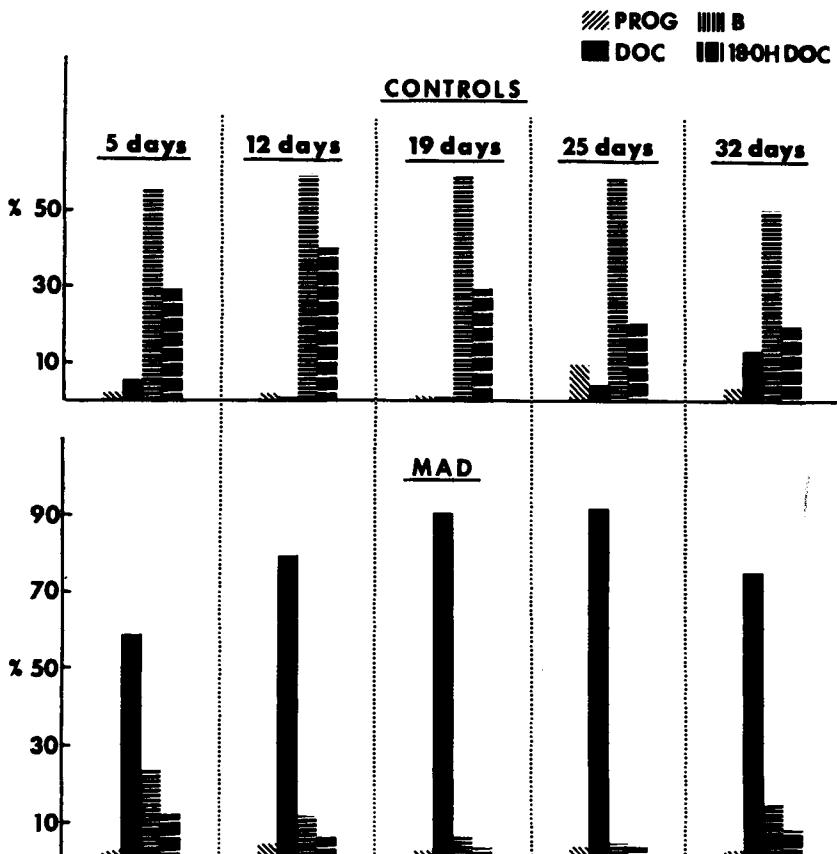


FIGURE 5. Biosynthesis of corticosterone (B), 11-desoxycorticosterone (DOC) and 18-hydroxy-corticosterone (18-OH DOC) from progesterone-4- $^{14}\text{C}$  (PROG) by adrenal gland homogenates from control and MAD-treated rats at 5, 12, 19, 25 and 32 days. Incubation of 35 mg adrenal tissue with 0.2  $\mu\text{c}$  progesterone-4- $^{14}\text{C}$  in Kreb's-Ringer-Phosphate buffer (pH 7.4) containing sodium fumarate (100 mg %); 1 mg NADP added to incubation flasks. Incubation for 10 minutes at 37°C.

12 days, however, electron microscopic abnormalities have been found, and with increasing duration of MAD administration the changes have shown a general increase in severity and a more widespread distribution. Nevertheless, it is difficult to construct a rigid chronology of the alterations, since the sensitivity of individual rats to MAD differs considerably and various combinations

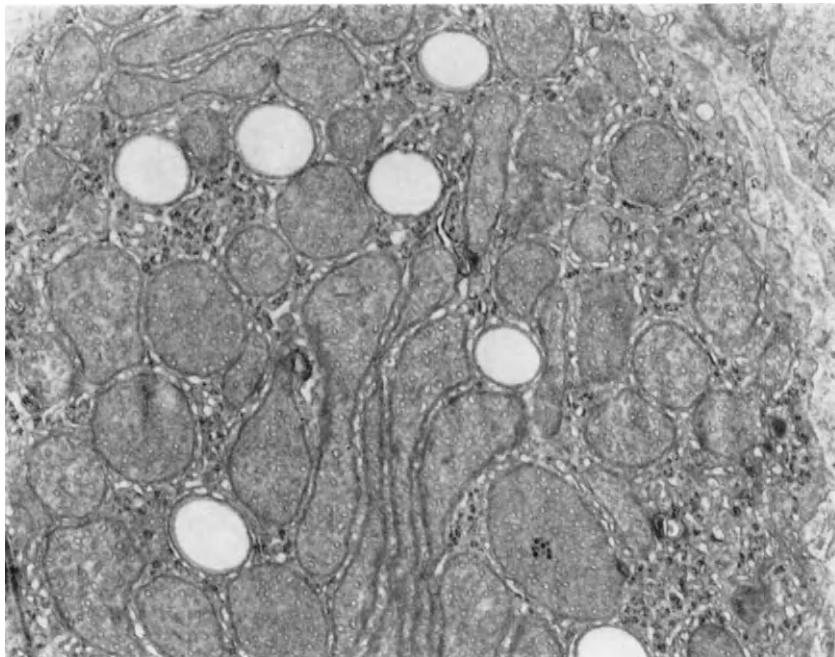
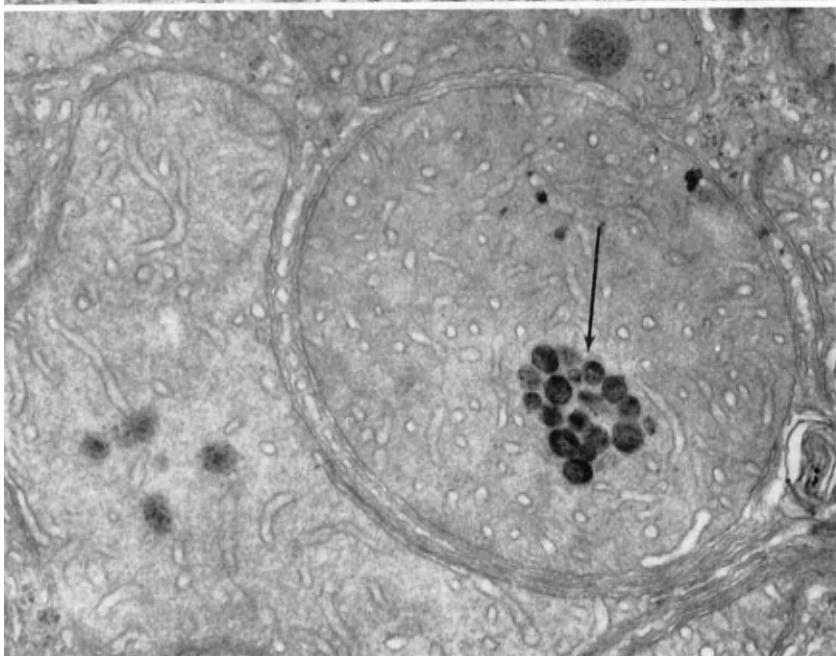
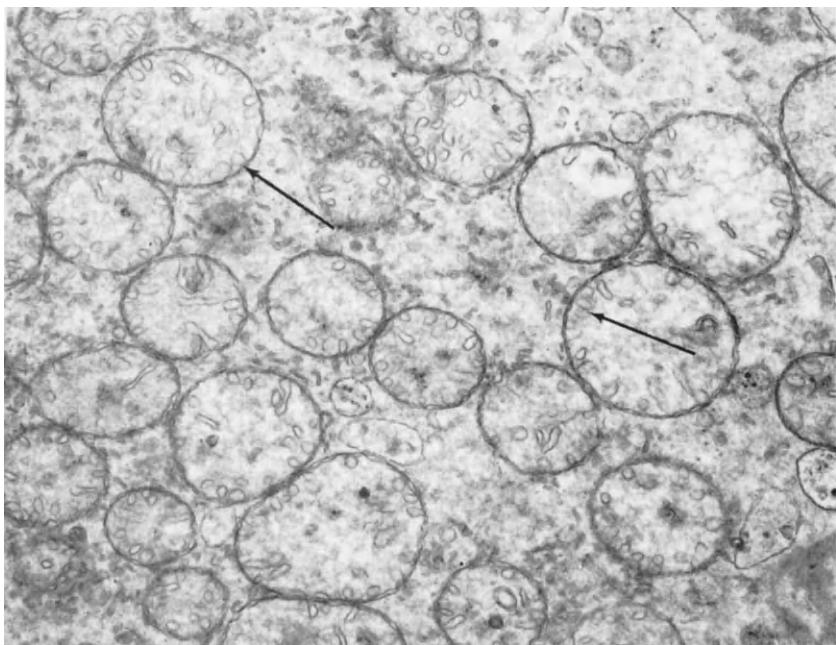


FIGURE 6. Mitochondria of zona fasciculata cell in adrenal cortex of MAD-treated rat (12 days) showing elongated and irregular appearance.  $\times 9,000$ .

of changes have been found in adrenals of rats treated for different periods of time. It appeared that the earliest detectable change occurred in the mitochondria, which became swollen or enlarged and were often of irregular shape (Fig. 6). External limiting membranes appeared less well-defined than normal and the number of vesicles decreased. Tubular cristae having a clear continuity with the internal limiting membrane became more characteristic and as the mitochondrial appearance was further simplified these showed a peripheral arrangement (Fig. 7). The matrix of the mitochondria became more prominent and often contained electron-dense material or laminated structures (Fig. 8).

FIGURE 7. Mitochondria of zona fasciculata cell in adrenal cortex of MAD-treated rat (32 days) illustrate loss of internal structure and frequent peripheral localization of residual tubulo-vesicular cristae (*arrows*).  $\rightarrow$   $\times 22,000$ .

FIGURE 8. Electron dense structures (*arrow*) in mitochondrion of zona fasciculata cell of adrenal from MAD-treated rat (32 days).  $\times 35,000$ .



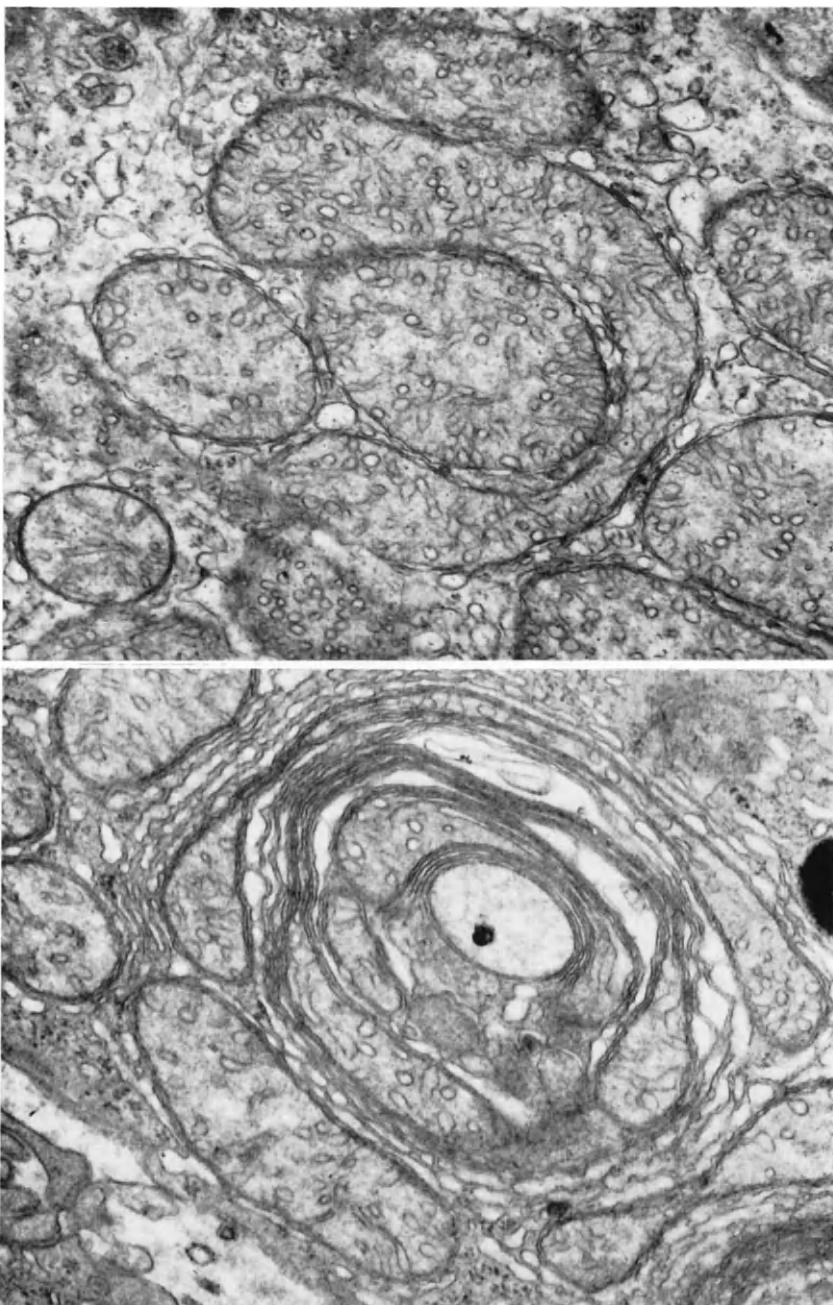


FIGURE 9. Irregular C-shaped mitochondrion in zona fasciculata cell of MAD-treated rat (32 days) enclosing adjacent mitochondrion.  $\times 22,000$ .

FIGURE 10. Complex mitochondrial and membranous structure in zona fasciculata cell of MAD-treated rat (32 days).  $\times 29,000$ .

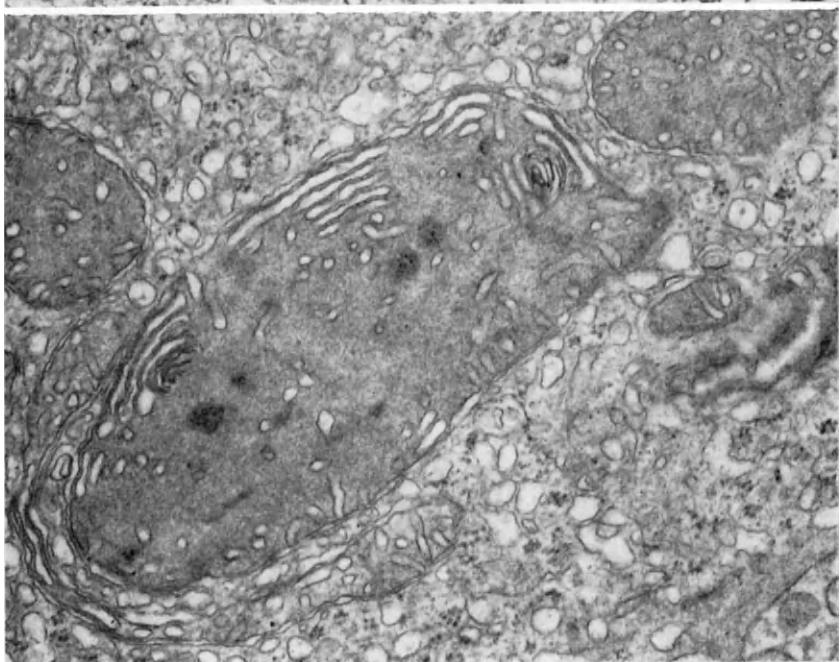
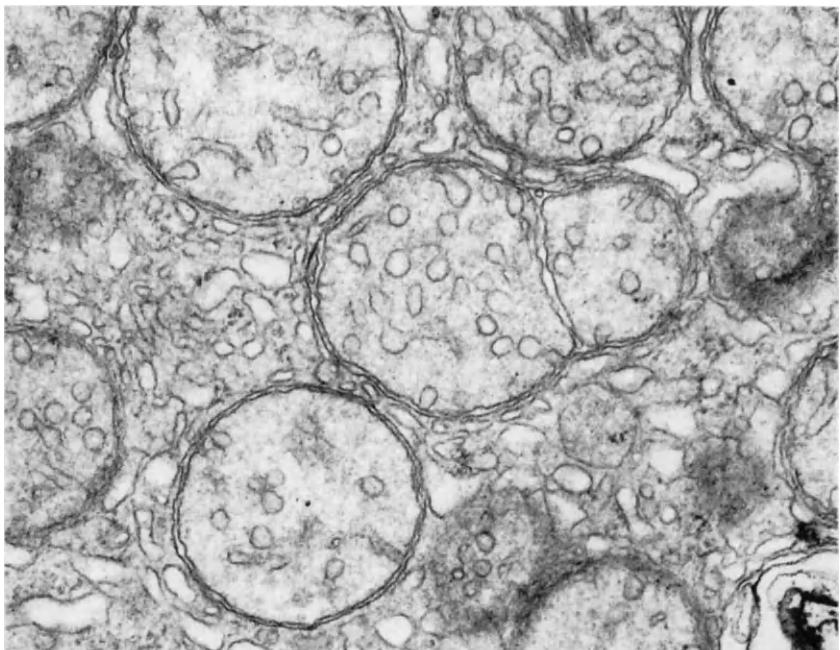


FIGURE 11. Reduced number of tubular and vesicular structures in mitochondria of adrenal zona fasciculata cell from MAD-treated rat (32 days).  $\times 48,000$ .

FIGURE 12. Mitochondrion showing tubular structures arranged parallel to external limiting membranes. Matrix is dense and contains short tubules and a few vesicles (32 days).  $\times 30,000$ .

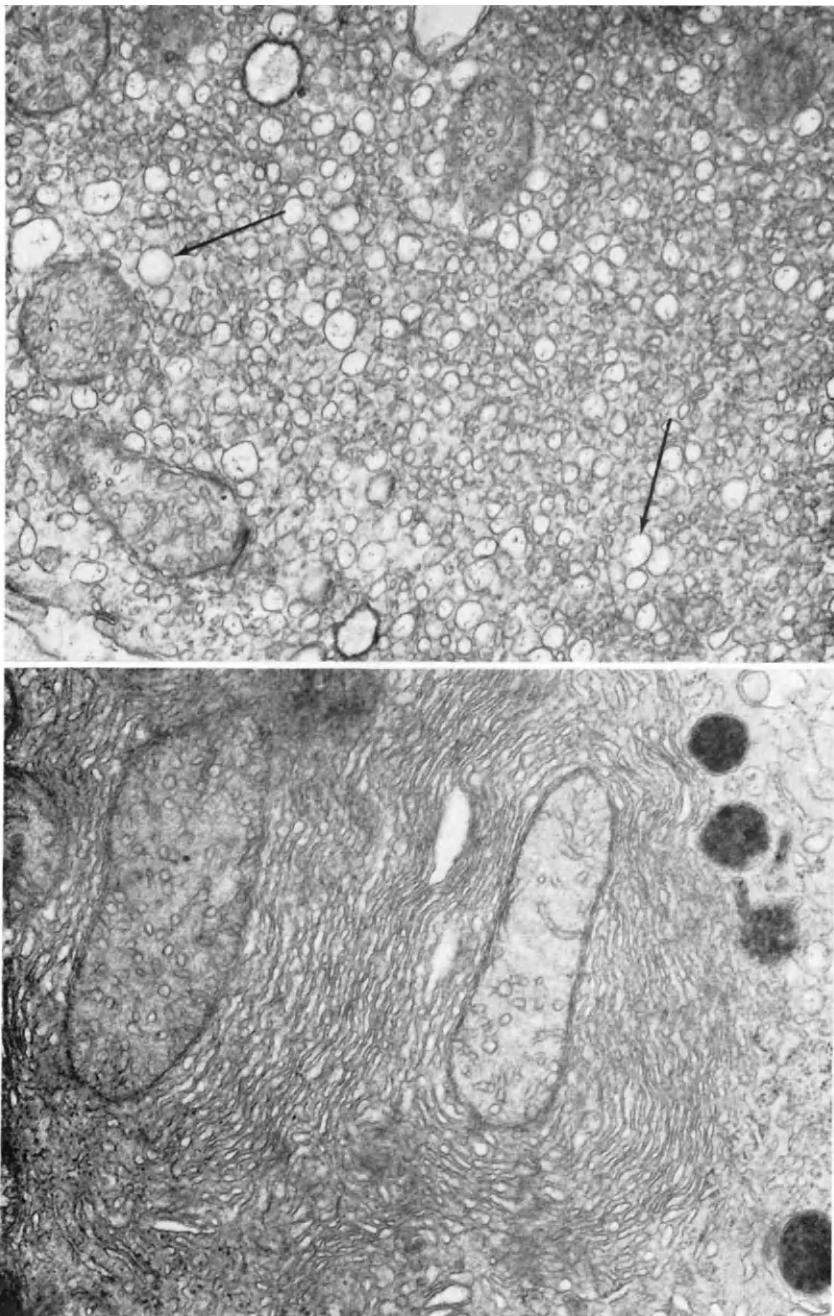


FIGURE 13. Focal increase in amount of smooth endoplasmic reticulum showing some dilated cisternae (arrows) and few mitochondria (32 days).  $\times 22,000$ .

FIGURE 14. Increased endoplasmic reticulum of zona fasciculata cell arranged as whorls about central mitochondria. MAD-treated (32 days).  $\times 24,000$ .

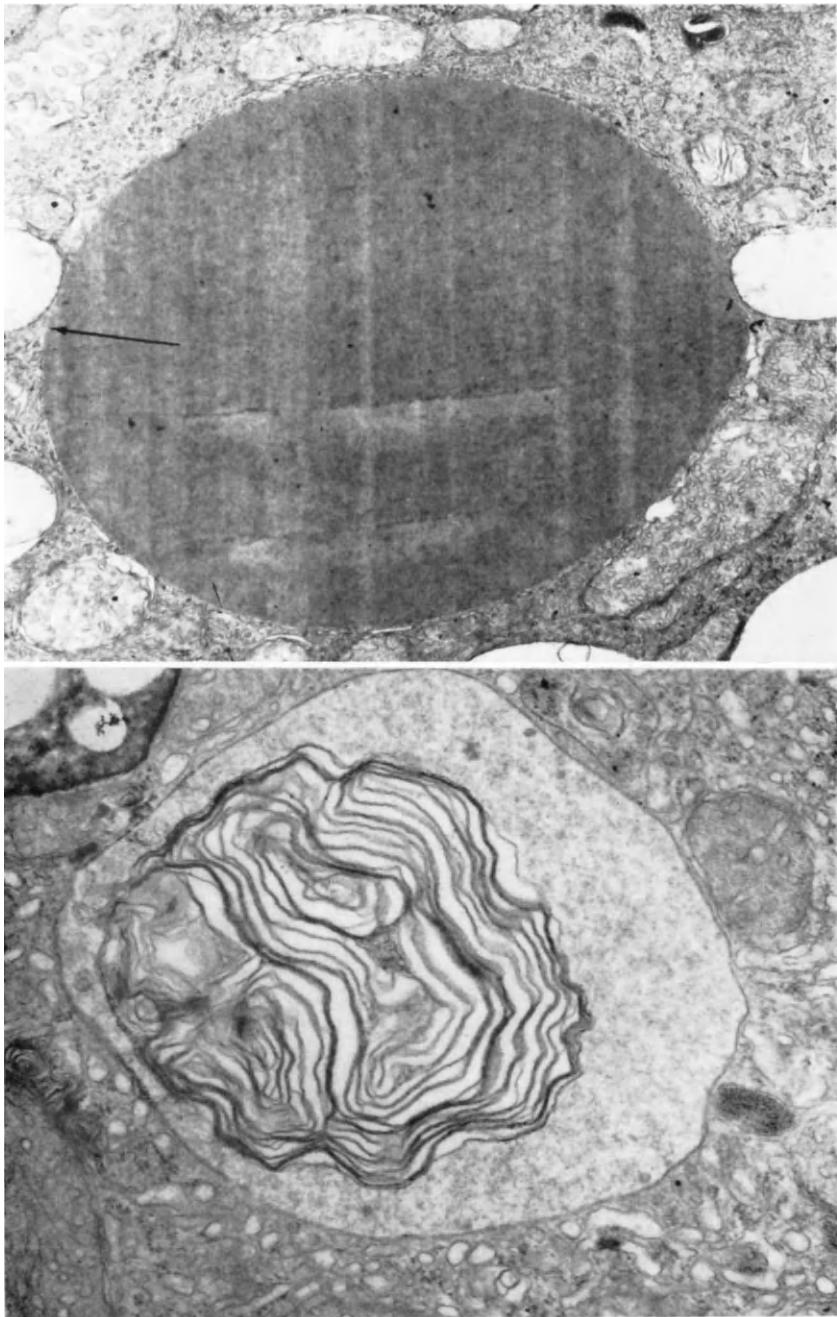


FIGURE 15. Electron dense body in cytoplasm of adrenal zona fasciculata cell from MAD-treated rat (25 days). There is no definitive limiting membrane about much of this structure, whereas other segments show a single thin membrane (arrow).  $\times 14,000$ .

FIGURE 16. Vacuole surrounded by a single membrane containing both flocculent and membranous material in a zona fasciculata cell of adrenal MAD-treated rat (25 days).  $\times 27,500$ .

One of the most striking changes was the enlargement, elongation and bizarre appearance of many of the mitochondria. These mitochondria assumed many shapes, ranging from simple irregularities (Fig. 9) to complicated intertwining of several mitochondria (Fig. 10). The internal structure of these mitochondria also showed considerable variation. In some, there was a prominent matrix with few tubular cristae (Fig. 11), whereas others contained parallel arrays of tubules often arranged parallel to the limiting membranes at one or more locations in the mitochondrion (Fig. 12). This often gave a frayed appearance to the mitochondrion and the membranes merged almost imperceptibly with the surrounding smooth-surfaced endoplasmic reticulum.

Another striking change in these adrenals was an increase in the smooth-surfaced endoplasmic reticulum (Fig. 13). In some cells, this was seen as a focal mass of irregularly arranged tubules, some of which were dilated while others were collapsed. In other cells, the endoplasmic reticulum assumed a more structured pattern seen as whorls of parallel membranes arranged around a central vacuole, electron-dense body, or mitochondrion (Fig. 14).

A prominent change in the cells of MAD-treated rats as seen under the light microscope is the presence of bodies of variable density often referred to as "colloid" or "hyaline" globules (38, 43). These have been seen with the electron microscope, also, and differ in their appearance from dense homogeneous masses (Fig. 15) to vacuoles containing granular, electron-lucid material (Fig. 16). Characteristically, these are rather late changes and have a complex morphogenesis that will be described more fully in another communication (21).

### ***MT Experiments***

**a) Steroid Biogenesis.** Recent studies of Saffran (36) have shown that MAD is converted by the adrenal cortex into MT and the suggestion has been made that this compound is responsible for the altered steroid biogenesis by the adrenal. If this is so, then similarly altered steroid biogenesis should result from both *in vivo* administration of MT to rats and *in vitro* addition of this androgen to incubations of normal homogenized adrenals. We have

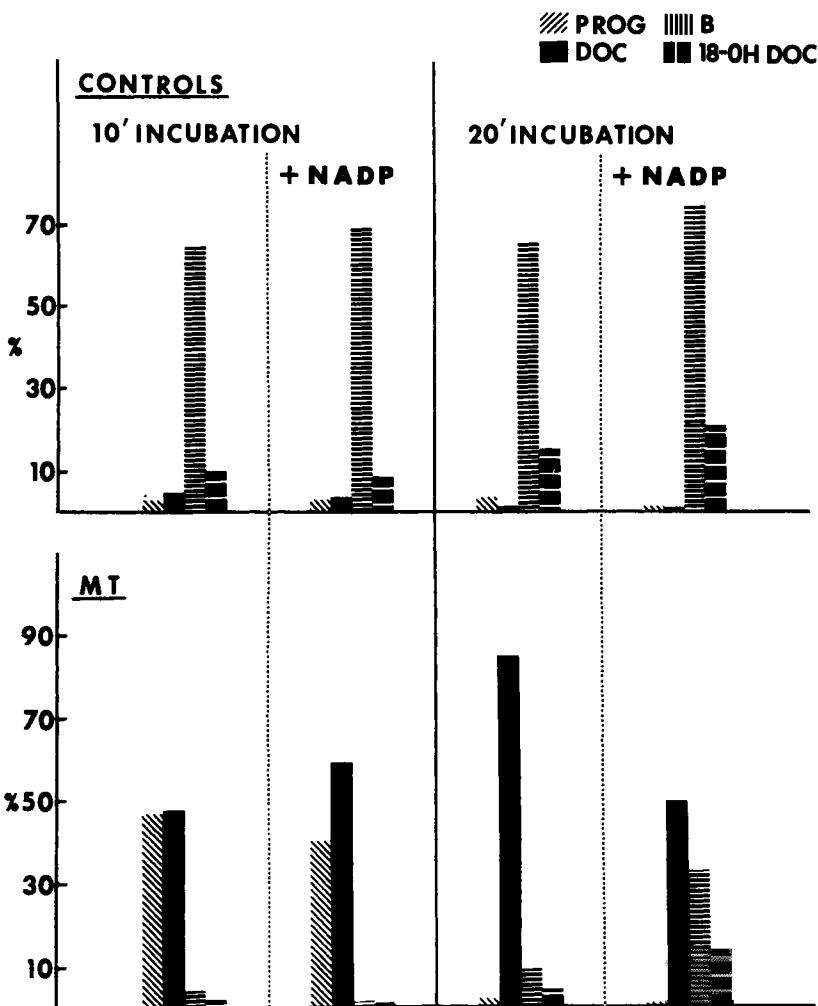


FIGURE 17. Biosynthesis of corticosterone (B), 11-desoxycorticosterone (DOC) and 18-hydroxy-corticosterone (18-OH DOC) from progesterone-4- $^{14}\text{C}$  (PROG) by adrenal gland homogenates from control and MT-treated rats at 6 weeks. Incubation of 35 mg of adrenal tissue with 0.2  $\mu\text{c}$  of progesterone-4- $^{14}\text{C}$  in 5 ml Kreb's-Ringer-Phosphate buffer (pH 7.4) containing 100 mg % sodium fumarate. Incubations for 10 and 20 minutes at 37°C, with and without added NADP (1 mg).

examined both these circumstances and the results are shown in Figures 17 and 18.

After six weeks of MT administration, it can be seen that the ability of adrenal gland homogenates to metabolize progesterone-4-<sup>14</sup>C was markedly impaired as compared with control adrenal

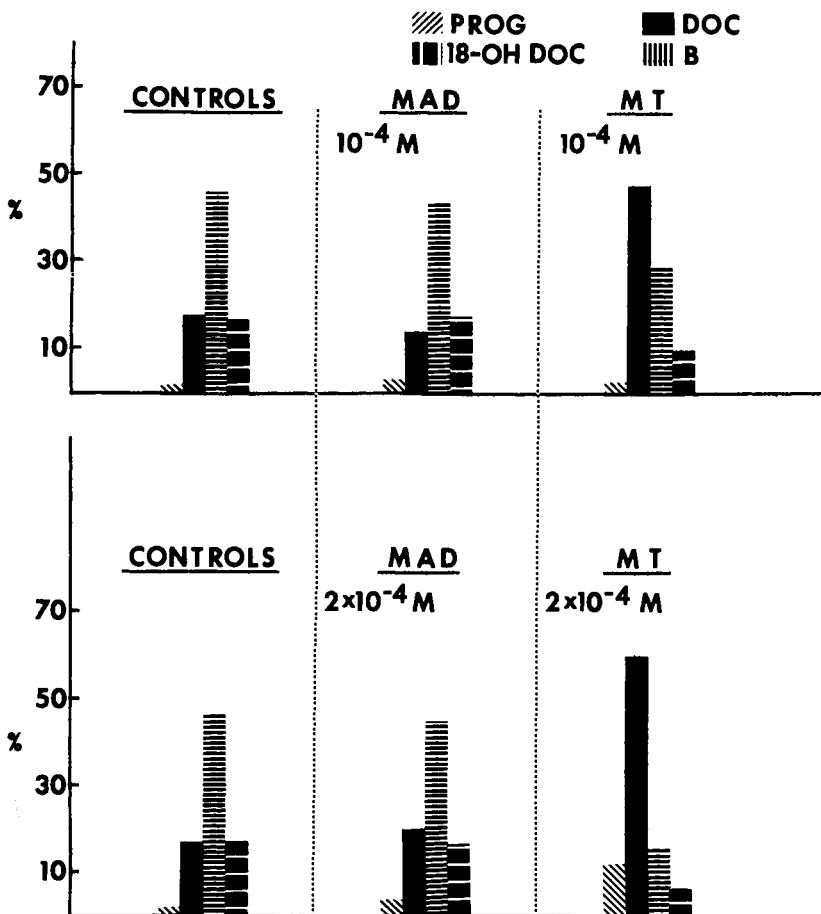


FIGURE 18. Biosynthesis of corticosterone (B), 11-desoxycorticosterone (DOC) and 18-hydroxy-corticosterone (18-OH DOC) from progesterone-4-<sup>14</sup>C (PROG) by normal adrenal gland homogenates to which MAD and MT were added directly to the *in vitro* incubation flasks. Incubation of 35 mg of adrenal tissue with 0.2  $\mu$ c of progesterone-4-<sup>14</sup>C in 5 ml Kreb's-Ringer-Phosphate buffer (pH 7.4) containing 100 mg % sodium fumarate. Incubation for 10 minutes, with and without MAD or MT added in 0.1 ml ethanol.

homogenates, when incubation was for 10 minutes either with or without added NADP. On the other hand, 20-minute incubations of adrenals from MT-treated rats showed metabolism of progesterone equal to that of control gland incubations. Under both incubation conditions, however, corticosterone formation was markedly impaired in the incubations of adrenals from MT-treated rats and the major metabolic product was DOC.

When MAD and MT were added directly to the incubation flasks of homogenates of normal adrenal glands, corticoid biosynthesis was unaffected by MAD whereas MT caused a decreased formation of corticosterone and 18-OH DOC and an increased accumulation of DOC.

**b) Electron Microscopy of Adrenal Glands from MT-treated Rats.** Only preliminary observations can be reported at the present time on this aspect of our studies. In general, the changes found in the adrenals of rats that had been injected with MT for 6 weeks bore many similarities to those observed in the adrenals of MAD-treated animals. Thus, the mitochondria of the zona fasciculata cells showed tubular or tubulo-vesicular cristae rather than the vesicles of normal fasciculata cells. Mitochondrial size varied considerably, many being enormously enlarged and of irregular shape. Intertwining of several mitochondria was frequently seen. The smooth endoplasmic reticulum was increased in amount, dilated, and formed into focal whorls of parallel membranes with a central mitochondrion or vacuole. Electron dense bodies and large vacuoles containing flocculent material also were present. As in the adrenals of MAD-treated rats, these appeared to correspond to the "hyaline" or "colloid" bodies and cytoplasmic vacuoles seen under light microscopic examination of the tissue.

### Discussion

It is interesting that two such different experimental conditions as adrenal cortical regeneration and MAD administration should produce hypertensive vascular disease and that increased mineralocorticoid secretion by the adrenals should be invoked as the common pathogenetic mechanism (39, 49). In both cases, the suggestions to this effect were based, primarily, on the indispensability of adrenal tissue for development of hypertensive disease (39, 48) and, secondarily, on the similarity between these syndromes and that produced

by exogenous administration of desoxycorticosterone acetate (38, 49). At the time these hypotheses were put forth, little direct evidence was available to support them and in the period immediately following their promulgation experimental data tended to negate their validity. For example, corticosterone secretion was measured in adrenal venous blood from regenerating adrenals and found to be lower than in corresponding control rats (2, 17, 23, 58). Peripheral blood level of corticosterone also has been determined at various times during adrenal regeneration and only at 16 days was it slightly above normal (12). Indeed, the level reached was far below what has been found necessary for corticosterone to produce hypertension even under the most favorable circumstances (18). Corticosterone production has been studied *in vitro* and again the output of regenerated adrenals has been found to be below or about the same as that of control glands (1, 6, 22, 41, 51).

Aldosterone secretion by regenerating adrenals has been studied also by *in vivo* and *in vitro* methods similar to those employed for corticosterone and uniformly found to be below that of normal control adrenal glands (1, 2, 14, 22, 23, 41). Rapp (31) measured the mineralocorticoid activity of steroids extracted from adrenal venous plasma from both regenerating and intact adrenals and found that bioassayed sodium-retaining activity was always less in rats with regenerating adrenals than in controls, irrespective of whether the animals were drinking tap water or 1% saline. Somehow, this does not seem to agree with the observations that aldosterone antagonists such as SC-5233 (53) and SC-9420 (30) prevent the development of adrenal-regeneration hypertension, observations that would suggest a pathogenetic role for sodium-retaining steroids.

The most logical evaluation of this data seems to be that neither corticosterone (50) nor aldosterone overproduction can account for the development of adrenal-regeneration hypertension. Other adrenal steroids have been sought, therefore, in attempts to solve this perplexing problem. It is interesting to note that Masson *et al.* (23) found DOC in incubations of 30-day regenerated adrenals in larger amounts than in control adrenal incubations. Macchi and Wyman (22) reported that DOC production of regenerated adrenal autografts tended to be higher than in control glands and identified 18-OH DOC and 18-OH corticosterone in incubates of normal and regenerated adrenals; but they were unable to demonstrate any differences in the production of these compounds. On the other hand, La Plante *et al.* (20) found increased production of 18-OH DOC by regenerated adre-

nals when these glands were incubated with progesterone-4-<sup>14</sup>C. Furthermore Birmingham *et al.* (1) have reported that regenerated adrenal glands when incubated *in vitro* had a greater capacity to respond to long-term ACTH stimulation with an increased production of corticosterone and 18-OH DOC than did normal adrenals. These data prompted Dr. Birmingham and her co-workers to suggest that 18-OH DOC may be playing some important role in the pathogenesis of adrenal-regeneration hypertension.

The fact that adrenal-regeneration hypertension develops during regeneration of the cortical tissue not after this reparative process has been completed, always has suggested to us that if a derangement in steroid biosynthesis is involved in the genesis of the hypertensive disease it should be looked for prior to and at the time the blood pressure elevation is occurring. Furthermore, the amounts of steroid that must be sought, identified, and quantitated are very small and this difficulty alone has severely limited many of the investigations that have been done in this field. For these reasons, we chose to study the *in vitro* biosynthesis of corticosteroids from added radioactively-labelled precursor at several time intervals during the regeneration of the adrenal. The accumulation of DOC in large amounts in the incubation of regenerating but not of control adrenals when fumarate alone was used as cofactor indicates to us that 21-hydroxylation of progesterone readily occurs in regenerating adrenals but that 11 $\beta$ -hydroxylation of the DOC formed thereby is abnormally low as compared with control glands.

The 11 $\beta$ -hydroxylation of corticosteroids is a mitochondrial function (5, 24, 54) and like 21-hydroxylation (33) requires NADPH as cofactor (15, 55). The resumption of normal steroid biogenesis by incubates of regenerating adrenals removed at 3 and 5 weeks when fumarate and NADP were used as cofactors suggests to us that in regenerating adrenals there may be a deficiency in NADPH generation. In normal adrenals, sufficient NADPH is generated in the presence of fumarate alone to allow normal steroidogenesis to go on, but in the regenerating adrenal this is not so. Thus, the concentration of NADPH plays a vital role in the pattern of steroidogenesis, there being sufficient cofactor available in regenerating glands to allow efficient 21-hydroxylation of progesterone to DOC, but virtually no 11 $\beta$ -hydroxylation of the DOC to corticosterone, so that DOC accumulates in large amounts. Several studies (28, 42) have indicated the relationship between NADPH concentration and the nature of the adrenal cortical hormones formed in normal rat adrenals. These work-

ers found that when NADPH was low progesterone was converted to DOC, which then accumulated and corticosterone production was reduced.

The ultrastructural appearance of the mitochondria of regenerating adrenal cortical cells correlates in a reasonable fashion with the biochemical observations. Thus, the most severe biochemical lesion was observed after 2 weeks of glandular regeneration and at this time the mitochondria were fewest in number in the regenerating cells and had the smallest number of vesicular cristae with the largest amount of matrix. As regeneration progressed through 3 and 5 weeks, the severity of the biochemical lesion decreased and during this time the number of mitochondria per cell increased and the internal structure of these organelles returned towards a more normal appearance. Rapid regeneration of new cells and cell structures such as occurs in regenerating adrenal glands requires large amounts of NADPH (59). Thus, there may be a limited availability of this essential cofactor to support corticosteroid biogenesis, especially during the most active period of cell multiplication. It is also possible that the simplification of mitochondrial structure reduces the number of sites for enzymatic reactions, thus limiting  $11\beta$ -hydroxylation. Another factor here may be the concentration of the mitochondrial cytochrome P 450. In any event, a correlation seems to exist between the restitution of more normal mitochondrial structure and a return toward more normal corticosteroid biogenesis as regeneration proceeds.

In contrast to the situation with regenerating adrenals, the adrenals from MAD-treated rats showed progressively greater derangement of corticoid biosynthesis the longer MAD was given. This was seen in both the greater accumulation of DOC and the decreased ability of the adrenal homogenates to metabolize the added progesterone- $4^{-14}\text{C}$ . Unlike the regenerating glands, however, the addition of NADP to the homogenates of adrenals from MAD-treated rats was unable to enhance corticosterone production and reduce DOC accumulation to the levels found for control adrenals. Thus, while the defect in steroidogenesis is manifest as an inadequacy of  $11\beta$ -hydroxylation, it is not due to a deficiency in NADPH production as it is in the regenerating adrenal. Of importance here may be the observation (4) that adrenal mitochondria isolated from MAD-treated animals have a much lower cytochrome P 450 concentration than control adrenal mitochondria.

The recent disclosure by Saffran (36) that MAD is metabolized by the adrenal cortex to MT has raised the question of whether MAD

directly affects corticosteroid biogenesis or whether its effects are mediated via the MT formed in the adrenal. The results reported in this communication show that MAD added directly to adrenal homogenates was without effect on corticoid biosynthesis, whereas equal amounts of added MT blocked the production of corticosterone and brought about the accumulation of DOC in the incubation medium. The systemic administration of MT, not only duplicated the effects of MAD on *in vitro* adrenal corticoid biosynthesis, but also produced hypertension and cardiovascular renal lesions indistinguishable from those produced by MAD.

The ultrastructural changes in the adrenals of MAD-treated rats showed some parallelism with the functional changes. The earliest detectable morphologic alterations were seen at 12 days and the most marked abnormalities were present at 32 days. Our preliminary observations with the electron microscope on the adrenals from MT-treated rats showed changes that were similar to those produced by MAD. It should be borne in mind that both MAD and MT caused similar effects on the *in vitro* corticoid biosynthesis of adrenals from rats that had been injected with these steroids, whereas only MT inhibited 11 $\beta$ -hydroxylation when these compounds were added directly to incubated adrenal homogenates. It appears, therefore, that MAD has to be converted to MT in order to obtain inhibition of corticosterone biosynthesis and accumulation of DOC. The question is whether the inhibition of 11 $\beta$ -hydroxylation brings about the structural changes in the adrenal cortex of the treated animals or whether the structural changes mediate the deficiency of 11 $\beta$ -hydroxylation via some effect of MAD and MT on the pituitary. It is also possible that the changes represent entirely separate phenomena, one functional and the other structural, which are brought about by unrelated mechanisms.

It is noteworthy that giant mitochondria similar to those we have observed in MAD- and MT-treated rats have been reported by Volk and Scarpelli (57) in hypophysectomized rats. Similarly, DeRobertis and his associates (11, 34, 35) have shown that mitochondria after hypophysectomy contain tubular infoldings and greatly reduced vesicular cristae, thus suggesting that the characteristic organization of mitochondria of zona fasciculata cells is dependent on ACTH. It is perhaps possible that these morphologic changes would be accompanied by abnormalities in steroid biogenesis such as we have found after MAD and MT treatment, although they have not been specifically looked for. Against this possibility is the fact that hypertensive

disease does not develop after hypophysectomy and, indeed, this operation prevents the development of adrenal-regeneration hypertension (46). The hyperplasia of smooth endoplasmic reticulum is reminiscent of a cytoplasmic change in hepatic cells that accompanies the administration of toxic substances such as barbiturates (32). In the case of the liver, this is related to an increase in enzymes involved in drug metabolism. It is difficult to postulate a direct effect of MAD or MT on these membranous components of adrenal cortical cells or to relate the change to a metabolic action of the cells. Similar changes in endoplasmic reticulum have been reported by Volk and Scarpelli (56) in adrenal cortical cells of rats treated with triparanol and they concluded that these structures may reflect disturbed steroid biogenesis. The "colloid globules" of light microscopy that were seen as electron dense bodies in the electron microscope are presently considered to represent an end-stage in degenerative phenomena occurring in the cell cytoplasm.

### Abstract

Using adrenal homogenate preparations, corticosteroid biosynthesis has been studied in regenerating adrenals and adrenals from rats treated with MAD and MT. It has been found that corticosteroid synthesis is deficient in the regenerating gland, especially during the early stages. The abnormality of biosynthesis that was apparently related to decreased availability of NADPH for  $11\beta$ -hydroxylation resulted in DOC accumulation. Electron microscopically, the regenerating adrenal cortical cells showed decreased mitochondrial vesicles, which increased in number as regeneration progressed.

Administration of MAD for as short a time as 5 days led to decreased corticosteroid synthesis from added progesterone in adrenal homogenates. Almost complete inhibition of corticosterone and 18-OH DOC formation accompanied by the accumulation of DOC occurred with more prolonged MAD treatment, even in the presence of added NADP. Similarly, abnormal corticosteroid biosynthesis was found in the adrenals of rats injected with MT for six weeks. MT produced inhibition of  $11\beta$ -hydroxylation with accumulation of DOC and decreased amounts of corticosterone when added directly to adrenal incubations, but MAD did not. It appears that MAD must be converted to MT to produce these effects. Both MAD and MT produced similar ultrastructural changes in adrenal cortical cells when chronically administered. The mechanism by which these alterations are produced is not known nor has the precise relationship between the functional and morphologic changes in the adrenal been established by these studies.

However, the results do suggest that both adrenal-regeneration and MAD hypertension may be caused by an abnormality in corticosteroid biosynthesis characterized by excessive DOC production.

### Abrégé

A l'aide d'un homogénat de parenchyme surrénalien, nous avons étudié la biosynthèse des corticostéroïdes chez des rats dont les surrénales sont en régénération ou après traitement avec des dérivés méthylés de l'androstènadiol (MAD) et de la testostérone (MT). Il fut observé que la synthèse des corticostéroïdes est déficiente dans les surrénales en régénération, particulièrement durant la phase précoce. L'anomalie de biosynthèse, qui est apparemment reliée à une déflection de NADPH pour la  $11\beta$ -hydroxylation, résulte d'une accumulation de désoxycorticostérone (DOC). L'examen des cellules du cortex surrénalien au microscope électronique a révélé une diminution des vésicules mitochondrielles, lesquelles augmentent en nombre au cours de la régénération.

L'administration de MAD durant une brève période de 5 jours a eu pour effet de diminuer la synthèse des corticostéroïdes à partir d'un homogénat de surrénales auquel nous avions ajouté de la progestérone. Nous avons observé une inhibition presque complète de la formation de corticostérone et de 18-OH désoxycorticostérone s'accompagnant d'accumulation de désoxycorticostérone par suite de l'administration prolongée de méthylandrostènadiol et ce, même en présence de NADP surajouté. De façon comparable, nous avons observé une biosynthèse anormale de corticostéroïdes dans des surrénales de rats injectés avec du MT durant 6 semaines. Le MT a pour effet de produire une inhibition de la  $11\beta$ -hydroxylation avec accumulation de désoxycorticostérone et une baisse en teneur de corticostérone lorsque ajouté directement au milieu d'incubation; le MAD, cependant, n'a pas exercé d'effet comparable. Il s'avère que le MAD doit être converti en MT pour produire de tels effets. Le MAD, tout autant que le MT, produisent des changements infrastructuraux dans les cellules du cortex surrénalien, lorsque administrés de façon chronique. Le mécanisme par lequel ces altérations sont induites est inconnu, et il n'est pas davantage possible, à la lumière de ces études, d'établir une relation entre les modifications fonctionnelles et structurales de la glande surrénalienne.

Toutefois, nos résultats laissent supposer que l'hypertension expérimentale consécutive à la régénération surrénalienne ou à l'administration de MAD est due à une anomalie de la biosynthèse des corticostéroïdes caractérisée principalement par une production excessive de désoxycorticostérone.

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**I. H. Page:** Adrenal regeneration and methylandrostenediol hypertension have been looked upon by most of us in the field as curiosities. But the more I have watched Floyd Skelton's analysis of the problem, the more impressed I am that he might discover a critically important truth about one of the naturally occurring varieties. The crux of the matter seems to be to explain why the functioning of a regenerating adrenal gland is critical in the development of hypertension. When adrenal regeneration is interrupted by hypophysectomy or the presence of the contralateral normal adrenal gland, hypertension does not develop. Also, when synthesis of steroid hormone by the regenerating gland is inhibited by amphenone B, no hypertension occurs. Methylandrostenediol has no "mineralo-corticoid" activity, the latter being a felicitous term coined by the person we are honoring today. This steroid produces hypertension only in the presence of the adrenals, leading to the suggestion that it interferes with normal adrenal cortical secretion in such a way that excessive mineralocorticoids are secreted and hypertension is thus elicited.

It is to the problem of corticoid biosynthesis that Skelton and Brownie address themselves with gun and camera; no pun intended! A study of synthesis during the early stages of regenerating gland

homogenate showed an abnormality, seemingly related to decreased availability of reduced triphosphopyridine nucleotide (NADPH) for 11-beta-hydroxylation, thus resulting in desoxycorticosterone accumulation. This was accompanied by decreased mitochondrial vesicles, but which increased in number as regeneration progressed. Administration of methylandrostenediol or methyltestosterone also led to decreased corticosteroid synthesis and accumulation of desoxycorticosterone. Thus, it is suggested that both adrenal regeneration and methylandrostenediol hypertension result from excessive desoxycorticosterone. The latter had originally been shown by Selye to produce hypertension.

I am bound to say I think Skelton must have planned these experiments to come to fruition just in time for the Selye celebration. But I rather doubt that he schemed so carefully that this would be the outcome. We should be grateful that these two bizarre experimental methods involving adrenal regeneration and methylandrostenediol now seem to fall into line with the other odd one resulting from desoxycorticosterone. I say "odd one" because, so far as I know, no one has shown that there is enough desoxycorticosterone to produce hypertension clinically. So far as I know, it has not been shown to occur in the dog. Dogs are resistant to the hypertensive action of desoxycorticosterone. Nor has subtotal adrenalectomy increased the severity of hypertension in patients. Nonetheless, perhaps we should now *re-examine* the question, if only because I well remember the days when renovascular hypertension did not occur in patients! And this was due chiefly to the fact that pathologists, bless their financially independent souls, failed to look carefully at the renal vessels.

Right or wrong, it is a great relief to have a specific chemical lesion to explain these experimental hypertensions. The chances of being right are pretty good with a man like Skelton. The most convincing data are those showing accumulation of desoxycorticosterone in large amounts during incubation of adrenal regeneration homogenates but not in control adrenals when fumarate alone was used as co-factor, indicating that 21-hydroxylation of progesterone occurs readily in the former but that 11-beta-hydroxylation of the desoxycorticosterone formed is abnormally low compared with controls.

Thus, on balance, it looks as though Skelton has found the key to this curious problem. And when you have keys it is a good idea to find out what they unlock. It also shows that if you live long enough, you are almost sure to see Hans Selye stick his head out and quite justifiably say, "I told you so!"

**M. Cantin:** I should like to ask one question of Dr. Skelton. If I remember correctly, MAD hypertension is accompanied by the appearance of PAS-positive granules in the adrenal cortex, particularly in the glomerulosa. I would like to know what these correspond to under the electron microscope.

**F. R. Skelton:** There are two changes that are seen by light and electron microscopy in the adrenal cortical cells of MAD-treated rats: vacuole formation and PAS-positive granules or colloid bodies in the cytoplasm. The vacuolar change, we believe, is due to degeneration of mitochondria. With continuing administration of MAD, the vesicular cristae decrease in number in the mitochondria, which ultimately appear in the electron microscope as empty, double membrane-bound sacks. Our observations suggest that these abnormal mitochondria fuse together becoming progressively larger. The vacuole so formed may occupy much of the cytoplasm or there may be multiple such vacuoles in the cell. The morphogenesis of the colloid of hyaline bodies we believe to be quite different. At first there are degenerative changes in the cytoplasm, which are seen as a fuzziness or loss of definition of the agranular endoplasmic reticulum. Such areas become progressively denser, although of irregular shape and without a limiting membrane. As the area becomes denser it appears to acquire a limiting membrane by compression of the surrounding agranular endoplasmic reticulum. The result is a dense, PAS-positive hyaline mass suspended in the cytoplasm. Multiple such bodies may be seen in a single cell or only one large granule may be present. A paper on this aspect of adrenal cortical cell changes in MAD-treated rats was published recently (Levine, A. J., and F. R. Skelton: *Am. J. Pathol.*, 51:831, 1967).

**W. S. Hartroft:** Dr. Skelton, if I saw PAS-positive droplets by light microscopy, say where there is normally some fat and where you have produced hemorrhages, I would look to see if any of this was ceroid. The electron microscopic description you have given is what we think ceroid looks like and this is a distinct possibility.

**F. R. Skelton:** We have not examined extensively the composition of these globules, but several years ago Masson *et al.* (*Endocrinology*, 56:541, 1955) reported that the colloid material was glycoprotein or mucoprotein and did not react with fat stains. Our results are in agreement with these findings, all of which would suggest that the globules are not ceroid.

## **High Blood Pressure in Rats Protected Against Acute Post-enucleation Adrenal Insufficiency and Its Bearing on the Etiology of Adrenal-regeneration Hypertension**

C. E. HALL, O. B. HOLLAND,\* and O. HALL

**R**ATS given a high salt intake ultimately develop hypertensive vascular disease (5, 7, 16, 19), particularly if a kidney is removed (9, 12). Under the latter circumstances, the process is greatly accelerated and enhanced by enucleation of the adrenal glands (4, 11, 14, 20). The etiology of adrenal-regeneration hypertension (ARH) has thus far eluded definition, although numerous hypotheses to account for it have been proposed. Available evidence indicates a subnormal rate of hormone secretion by regenerated glands (2, 6, 15, 17, 24), which is difficult to harmonize with hypertension. Faced with this contretemps it has been suggested that perhaps an abnormal ratio of steroids is secreted, or that some unusual hormone is elaborated by such adrenals. In any event, the syndrome is of some theoretical importance because of the possibility that a similar glandular dysfunction underlies essential hypertension (10).

Skelton (21) has shown that postoperative administration of 1 mg/day of corticosterone following enucleation will prevent adrenal-regeneration hypertension, and Grollman (8) has reported that if a mixture of 1 mg of cortisone acetate and 1 mg of desoxycorticosterone acetate (DCA) is given on the day of operation and the dosage diminished by 10% of these amounts each succeeding day for 9 more days, the same effect is obtained. Although Skelton's results could be attributed to steroid suppression of anterior pituitary function and Grollman ascribed his own to correction of adrenal insufficiency in the immediate postoperative period, both sets of data could be explained by either postulate. Corticosterone undoubtedly corrected or ameliorated adrenal insuf-

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\* Fellow of the Life Insurance Medical Research Fund.

ficiency while suppressing the pituitary in the first instance, and the steroid mixture probably suppressed pituitary function, perhaps at a critical phase of regeneration, while correcting adrenal insufficiency in the second. However, if its etiology is to be established, it is undesirable to have two equally plausible theories, both of which are unlikely to be correct, held accountable for the hormonal prevention of ARH. The present experiments were designed to determine whether or not a common *modus operandi* could be assigned to the two hormonal methods of blocking development of the disorder.

### Experiment 1

#### *Materials and Methods*

Fifty-four female rats of the Houston-Cheek strain, a Sprague-Dawley derivative, weighing 60-80 g, were divided into four groups. Group 1 consisted of fourteen animals subjected to right nephroadrenalectomy and left adrenal enucleation. Group 2 consisted of sixteen similarly operated rats given 1 mg cortisone acetate and 1 mg DCA on the day of operation, that dosage being reduced by 10% of the original dosage each day thereafter for 9 more days. Group 3 consisted of fourteen similarly operated animals given no hormone until the 8th postoperative day, on which day they were given 1 mg cortisone acetate and 1 mg desoxycorticosterone acetate, the dosage then being diminished by 10% of that amount on each succeeding day for 9 more days. Group 4 consisted of ten rats subjected only to right nephroadrenalectomy. The hormones, given subcutaneously, were suspended in a saline vehicle, containing 0.5% carboxymethylcellulose and 0.4% polysorbate, of the type used in commercial steroid hormone suspensions. All groups were given 1% NaCl solution to drink and Purina laboratory chow *ad libitum*. Animals were individually caged in temperature-controlled quarters. Blood pressure was measured periodically in unanesthetized animals by tail plethysmography, and systolic pressures above 150 mm Hg were regarded as hypertensive.

Surviving animals were killed with ether on the 29th day and various tissues and organs were excised and placed in neutral

10% formalin. Those to be weighed were removed, trimmed, blotted dry and weighed on an analytical balance.

### Results

It soon became evident that hormone treatment at this dosage was poorly tolerated by adrenal-enucleated rats. In both of the steroid injected groups, deaths began to occur in the week following institution of therapy. Over the ensuing week or ten days, the mortality curve in the two groups ran parallel until about half of each group had died. There were no deaths among untreated enucleated rats, and only one among controls during the experiment. The mortality curves are shown in Figure 1.

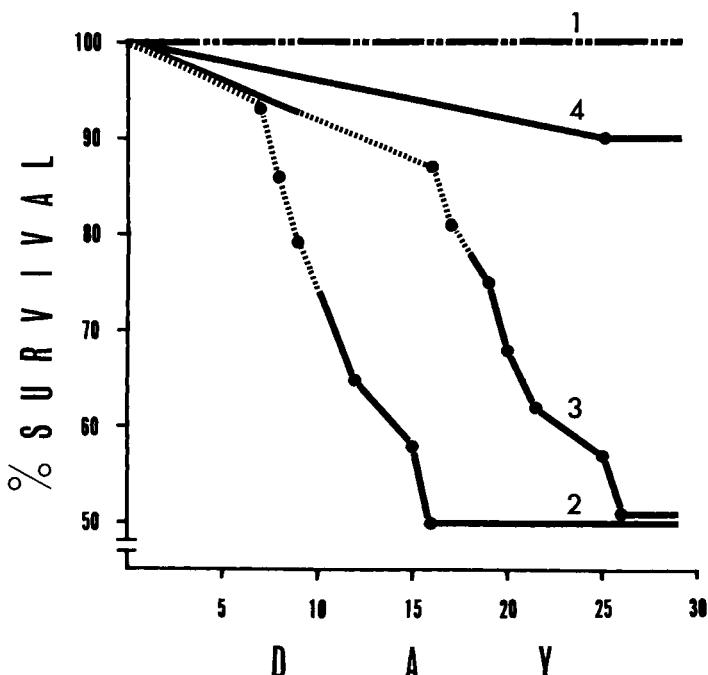
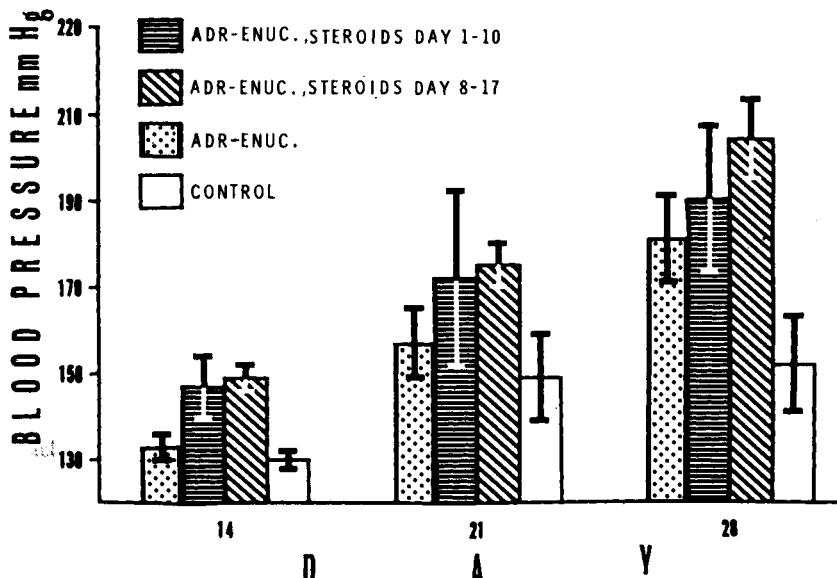


FIGURE 1. Mortality rate in adrenal-enucleated and control rats: (1) untreated enucleated rats; (2) adrenal enucleates given DCA and cortisone for the first ten postoperative days; (3) enucleates given DCA and cortisone for ten days after allowing a week of adrenal insufficiency; and (4) controls. The period of hormone treatment is indicated by cross hatching on the respective slopes. Note the close parallelism in the response of hormone-treated groups once deaths begin.

On the 14th day, when the first blood pressure estimation was made, one of the fourteen untreated enucleated rats was hypertensive. Four of the nine survivors given hormone treatment from the day of operation, but in which treatment had been discontinued four days earlier, were hypertensive. The difference between the group average pressures was significant ( $P < .05$ ). Six of the fourteen rats in which replacement therapy had been delayed for a week, but were at this time still receiving hormone, were also hypertensive. None of the controls had elevated pressures. By a further week, six of the fourteen untreated enucleated rats had become hypertensive, as were six of the seven survivors given replacement therapy from the day of operation, nine of the eleven surviving in the group allowed a week of insufficiency before therapy was instituted, and three of the ten controls, one of which had a pressure of 210 mm Hg and manifested a periodic twitching of the head and neck indicating cerebrovascular damage. By the 28th day, eleven of the untreated enucleated rats were hypertensive, the average pressure being  $181 \pm 10$  mm Hg. All save six of the enucleated group protected against postoperative adrenal insufficiency had died, and five of the survivors were hypertensive, the average pressure being  $190 \pm 17$  mm Hg. Eight of the sixteen animals afforded delayed replacement therapy had died and all of the survivors were hypertensive, the average pressure being  $204 \pm 9$  mm Hg. The hypertensive control rat that had presented a tic in the previous week had since died, but hypertension had developed in another. Three of the nine control rats were hypertensive, one having reached 210 mm Hg, and the group pressure stood at  $152 \pm 11$ . The results are compared in Figure 2.

At necropsy of the fourteen untreated adrenal-enucleated animals, cardiac lesions were grossly evident in four and nephrosclerosis in two: none had visible polyarteritis. In the group given replacement treatment from the day of operation, four of the six had heart lesions, two had renal lesions, and three had polyarteritis of mesenteric arteries. All of the eight rats given delayed hormonal treatment had relatively severe cardiac lesions, half of them had grossly discernible nephrosclerosis, and three had polyarteritis.

The terminal body weight of each of the adrenal-enucleated



**FIGURE 2.** Blood pressure response in untreated and hormonally supported adrenal-enucleated rats and their controls. Hypertension begins sooner in the former and the group average pressure is generally higher.

groups was below that of controls ( $P < .05$  or better), but the two hormone-treated groups were more severely retarded than were animals subjected to enucleation only ( $P < .05$ ).

*Organ Weights:* The regenerated adrenal glands in all three groups were significantly smaller than the single hypertrophied adrenals of unilaterally adrenalectomized controls ( $P < .001$ ), those of untreated rats being slightly though not significantly larger than those of hormone-injected animals.

Average heart weight of untreated enucleated animals, three of which had remained normotensive, did not exceed that of unilaterally adrenalectomized controls, three of which had become hypertensive. The greatest cardiac enlargement was found in the two groups of hormone-treated enucleated rats, whose hearts were larger than in either untreated enucleated rats ( $P < .05$ ) or controls ( $P < .01$  or better).

The kidneys of untreated enucleated rats were larger than those of controls ( $P < .05$ ), but were further enlarged by hormone treatment. Those of rats given hormones from day 8 to 17 were

larger than in any other group ( $P < .05$  or better). Those given treatment from the outset were smaller than these ( $P < .05$ ), larger than controls ( $P < .01$ ), but not significantly larger than in untreated enucleated rats ( $P > .10$ ).

Brain weight followed a pattern similar to that of the hearts. Hormone-treated enucleated rats had enlarged brains, heavier than those of either enucleated-untreated rats ( $P < .05$ ) or controls ( $P < .01$ ), although there was no significant difference between the last two groups themselves. The data are given in Table I.

The toxicity of the hormone combination, taken together with the fact that neither is elaborated in significant quantities by the rat adrenal and the failure to ameliorate adrenal-regeneration hypertension under either circumstance, frustrated the purpose of the experiment and led us to approach the problem in another way.

## Experiment 2

Because of the high mortality caused by cortisone and DCA, it was decided to repeat the experimental design, but to substitute

TABLE I  
PRINCIPAL FINDINGS IN UNINEPHROADRENALECTOMIZED-  
ADRENAL-ENUCLEATED RATS AND THEIR CONTROLS

	<i>Data</i>	<i>Group 1</i>	<i>Adrenal Enucleated</i>	<i>Group 3</i>	<i>Controls</i>
					<i>Group 4</i>
Hormones Injected .....		None	1-10th Day	8-17th Day	None
No. Rats					
Initial .....	14	14	16	10	
Final .....	14	6	8	9	
Body Wt g					
Initial .....	69 ± 1*	69 ± 2	70 ± 1	71 ± 1	
Final .....	158 ± 6	132 ± 14	127 ± 10	176 ± 3*	
No. Hypertensive .....	11	6	8	3	
Organ Weight					
Liver } g/100 g Body Wt	5.98 ± 0.18	6.58 ± 0.57	6.84 ± 0.34*	5.33 ± 0.07*	
Brain }	1.00 ± 0.05	1.42 ± 0.19*	1.45 ± 0.15*	0.87 ± 0.02	
Kidney }	1180 ± 38	1291 ± 68	1490 ± 53*	985 ± 64*	
Heart }	476 ± 20	571 ± 33*	595 ± 32*	441 ± 19	
Thymus }	217 ± 19	227 ± 17	170 ± 21	199 ± 18	
Adrenal	18.8 ± 1.0	17.1 ± 1.7	17.6 ± 1.3	27.1 ± 1.0	

\* Mean ± SEM.

Italic figures differ from controls  $P < .05$ .

\* Differ from untreated enucleated rats.

corticosterone as the replacement hormone. This hormone has the advantages of being the principal steroid secretion of the rat adrenal, of possessing appreciable intrinsic mineralocorticoid and glucocorticoid activity, and of being known to suppress the development of adrenal regeneration hypertension when given continuously postoperatively in a dose of 1 mg per day (21).

### ***Materials and Methods***

Fifty-four female Houston-Cheek rats, 75-95 g, were divided into six equal groups. Those of group 1 and 2 were subjected to right nephroadrenalectomy, group 1 remaining untreated and group 2 being given 1 mg/day of corticosterone subcutaneously, postoperatively. Group 3 and 4 were right nephroadrenalectomized and left-adrenal enucleated. Group 3 received no further treatment, but rats of group 4 were given 1 mg/day of corticosterone as in group 2. Groups 5 and 6 were operated similarly to groups 3 and 4; group 5 received 1 mg of corticosterone on the day of operation, this dosage being reduced by 10% of that amount each day thereafter so that after 10 days no further hormone was given. Group 6 received no hormone treatment until the 8th postoperative day, when they were treated for 10 days precisely as had been animals of the previous group. Hormone was suspended in distilled water and given subcutaneously. All animals were given 1% NaCl solution to drink and Purina laboratory chow *ad libitum*.

Animals were housed as in the preceding experiment. Blood pressures were periodically taken in the same manner and subjected to the same criteria as previously. Twenty-four-hour fluid consumption taken on two consecutive days in each mid-week for the first month of the experiment and the average computed from these figures was considered as representative of the group for that week.

The animals were killed with ether on the 43rd day and organs taken for weight and histology were processed as described in the foregoing experiment. Pathologic lesions observed microscopically were graded arbitrarily on a 0 to 3+ scale and the severity for the group expressed as a percentage of the theoretical maximum possible, e.g., 24 for an eight-member group.

## Results

Untreated enucleated rats drank more saline than controls in every period save the first, whereas hormone-treated enucleated rats drank slightly less in every period except the last; the latter drank about the same volume as did unilaterally adrenalectomized rats given corticosterone. After the second week of the experiment, polydipsia was significantly greatest in the group that had received

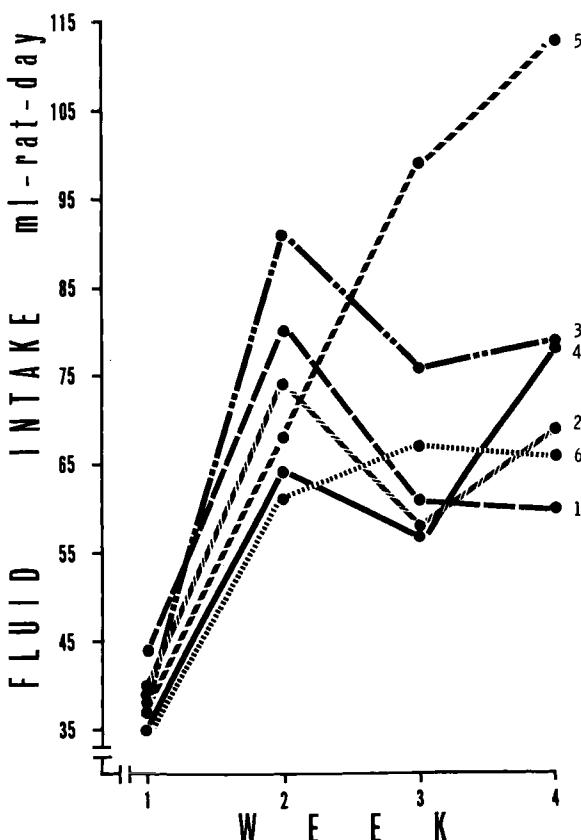


FIGURE 3. Fluid consumption during the experiment: (1) controls; (2) controls given corticosterone throughout; (3) adrenal enucleates; (4) adrenal enucleates given corticosterone throughout; (5) adrenal enucleates given corticosterone for the first ten postoperative days; and (6) adrenal enucleates given corticosterone for ten days following a week of uncontrolled adrenal insufficiency.

corticosterone for the first ten postoperative days, and only in this group was a progressively greater intake apparent. The results are shown in Figure 3.

Hypertension developed as anticipated in untreated, adrenal-enucleated rats. While in the second week they were all normotensive, by a further week eight of the nine were hypertensive, and on the 32nd day all were. The severest response was encountered in the group given corticosterone for the first ten post-operative days. On the 14th day these, too, were entirely normotensive, but by a further week all were hypertensive. The difference in severity is best exemplified by the findings on the 32nd day. At this time, the blood pressure in the untreated enucleated group was  $200 \pm 12$  mm Hg, but only three of nine rats had pressures exceeding 200 mm Hg; in the group given corticosterone for ten days postoperatively the pressure was  $230 \pm 7$  mm Hg and ten of the eleven rats had pressures above 200 mm Hg. The difference was significant ( $P < .05$ ) in this period, though not in any of the others.

Corticosterone given to enucleated rats from the 8th to 17th day did not have the same effect. The group average pressure was less than that of untreated enucleated rats in each of the periods, although significantly so only at the third week,  $177 \pm 9$  mm Hg versus  $157 \pm 4$  ( $P < .05$ ). The pressures of rats treated 8-17 days postoperatively, as compared with those treated in the first ten days, were significantly lower in each period after the 2nd week ( $P < .001$  on the 21st and 32nd days and  $P < .05$  on the 41st day). Continuous postoperative corticosterone administration to enucleated rats prevented the early hypertension exhibited by similarly operated untreated rats. In none of the periods did the pressures of untreated or corticosterone-treated unilaterally adrenalectomized rats differ from each other or from enucleated rats given hormone continuously. However, the late developing hypertension that eventually began to occur in controls, as distinguished from quickly developing adrenal-regeneration hypertension, affected all three of these groups to the same degree. At the last reading, hypertension affected eight of nine untreated controls ( $164 \pm 5$  mm Hg), seven of nine corticosterone-treated controls ( $172 \pm 13$  mm Hg), and six of nine enucleated rats given

continuous steroid treatment ( $165 \pm 5$  mm Hg). The response is shown in Figure 4.

There were no deaths during the first month of the experiment. One rat died in the 8-17 day corticosterone-treated enucleated group on the 33rd day as did one of the untreated enucleated rats on the 41st day, and two others on the 43rd day, which prompted the decision to end the experiment on that day.

At necropsy, it was evident that untreated enucleated rats, and

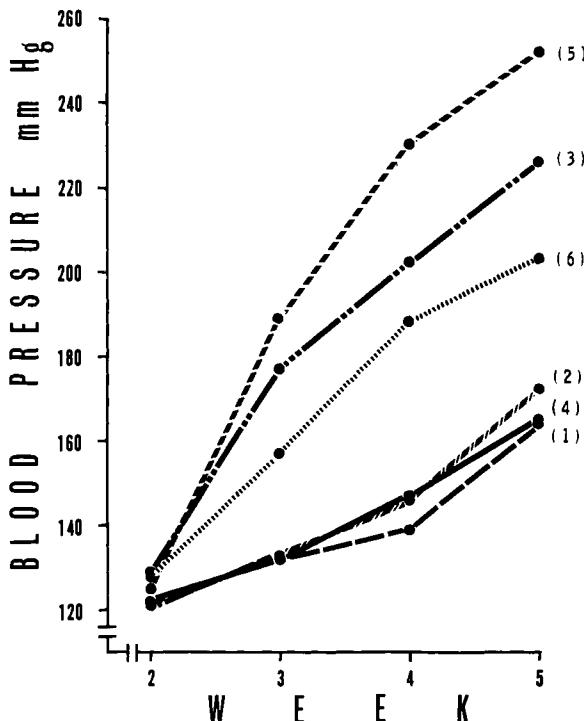


FIGURE 4. Blood pressure response in: (1) untreated controls; (2) controls given corticosterone throughout; (3) untreated enucleated rats; (4) enucleates given corticosterone throughout; (5) enucleates given corticosterone for the first ten postoperative days; and (6) enucleates given corticosterone for ten days following a week of adrenal insufficiency. Note that the hypertensive response is greater when acute insufficiency is hormonally prevented, but not if hormone treatment is delayed for a week. Continuous hormone treatment prevents the occurrence of early adrenal-regeneration hypertension in enucleates, but in controls it neither induces hypertension nor prevents the late rise in blood pressure associated with continued salt excess.

both enucleated groups given hormone for 10 days, weighed less than the other groups, which did not differ among themselves. Examination of visceral organs revealed that the adrenals in rats given corticosterone, both enucleated and control, were somewhat atrophic. Gross kidney and cardiac lesions were visible only in the untreated enucleated rats and in the two ten-day hormone-treated groups. In the first of these, the two that had died that day showed such lesions (as had the one which died earlier): the remainder appeared entirely normal. Ten of the eleven given hormone from day 1-10 and two of eight treated from day 8-17 were visibly affected.

Untreated adrenal-enucleated rats had enlarged hearts and kidneys and smaller adrenals than controls; there was no effect upon liver or spleen; and the thymus glands were smaller, although not significantly so ( $P < 0.10$ ). Continuous corticosterone treatment inhibited cardiac enlargement, and minimized thymus atrophy. It also reduced adrenal size to 48% of that in untreated enucleated rats. Given to control unilaterally adrenalectomized animals, its only effect was to reduce the magnitude of compensatory hypertrophy, the glands averaging 26% less in weight. Corticosterone given for the first ten postoperative days, on the other hand, enhanced organ response. The liver and spleen were significantly enlarged as compared with the size in either controls or untreated enucleated rats, and hearts and kidneys were also far larger than in any other group. This group also exhibited the greatest impairment of growth and was the only group to display significant thymus atrophy ( $P < .05$ ). The same quantity of hormone given after a week of insufficiency had less effect. The liver, but not the spleen showed significant enlargement; the hearts and kidneys were almost exactly the same weight as in the untreated enucleated rats; the thymus and adrenals were not demonstrably affected.

*Histologic Findings:* Vascular lesions were observed only in enucleated rats, untreated or given hormone for ten days. They were absent from animals given hormone throughout the experimental period. Two untreated enucleated rats showed necrotizing vascular lesions in the heart and adrenal cortex, the latter associated with cortical necrosis; four had moderate to severe nephrosclerosis, and one had mesenteric polyarteritis. Similar adrenal lesions or poly-

TABLE II  
EFFECT OF CORTICOSTERONE IN UNILATERALLY NEPHRECTOMIZED RATS SUBJECTED TO REMOVAL OF ONE ADRENAL WITH OR WITHOUT CONTRALATERAL ADRENAL ENUCLEATION

	Data	Adrenal Enucleation						Unilateral Adrenalectomy	
		None	Day 1-10	Day 8-17	Daily	None	Daily	Daily	Daily
Corticosterone Given . . . . .	None								
No. Rats									
Initial		9	11	9	9	9	9	9	9
Final		8†	11	8	9	9	9	9	9
Body Wt g									
Initial		<i>83 ± 2*</i>	<i>81 ± 2</i>	<i>82 ± 2</i>	<i>83 ± 2</i>	<i>82 ± 2</i>	<i>83 ± 3</i>		
Final		<i>176 ± 9</i>	<i>164 ± 6</i>	<i>183 ± 5</i>	<i>195 ± 4</i>	<i>203 ± 3</i>	<i>195 ± 3</i>		
Organ Wt/100 g Body Wt									
Liver (g)									
Initial		<i>4.95 ± 0.42</i>	<i>6.60 ± 0.31<sup>a</sup></i>	<i>5.43 ± 0.17</i>	<i>5.03 ± 0.11</i>	<i>4.91 ± 0.12</i>	<i>4.82 ± 0.15</i>		
Final		<i>1055 ± 71</i>	<i>1351 ± 60<sup>a</sup></i>	<i>1054 ± 40</i>	<i>985 ± 25</i>	<i>888 ± 21</i>	<i>828 ± 47</i>		
Kidney }									
Heart }									
Spleen }									
Thymus }									
Adrenal }									
Vascular Lesions									
Heart									
% Incidence . . . . .		<i>18.2</i>	<i>81.9</i>	<i>9.1</i>	<i>0</i>	<i>0</i>	<i>0</i>		
% Severity . . . . .		<i>14.8</i>	<i>58.6</i>	<i>7.4</i>	<i>0</i>	<i>0</i>	<i>0</i>		
Kidney									
% Incidence . . . . .		<i>45.5</i>	<i>91.0</i>	<i>36.4</i>	<i>0</i>	<i>0</i>	<i>0</i>		
% Severity . . . . .		<i>40.8</i>	<i>69.7</i>	<i>18.5</i>	<i>0</i>	<i>0</i>	<i>0</i>		
Adrenal									
% Incidence . . . . .		<i>18.2</i>	<i>54.6</i>	<i>0</i>	<i>0</i>	<i>0</i>	<i>0</i>		
% Severity . . . . .		<i>18.5</i>	<i>24.2</i>	<i>0</i>	<i>0</i>	<i>0</i>	<i>0</i>		
Polyarteritis									
% Incidence . . . . .		<i>9.1</i>	<i>36.4</i>	<i>0</i>	<i>0</i>	<i>0</i>	<i>0</i>		
% Severity . . . . .		<i>3.7</i>	<i>15.2</i>	<i>0</i>	<i>0</i>	<i>0</i>	<i>0</i>		

† Includes two rats that died on last day. Lesion data based on all nine.

\* Mean  $\pm$  SEM.

Italic figures differ from untreated controls  $P < .05$  or better.

<sup>a</sup> Differ from untreated enucleated rats  $P < .05$  or better.

arteritis were not seen in any of the group given hormone from the 8th to 17th days, but renal and cardiac lesions were present in one and three animals, respectively. Animals hormonally protected against insufficiency for the first ten days were most severely affected. Four of the eleven had polyarteritis, six had focal adrenal necrosis, nine had cardiac lesions, and ten had nephrosclerosis. The histologic characteristics of the lesions observed have been adequately described in the literature. The incidence, and the severity, established by arbitrary criteria as described, are synopsized in Table II.

### **Discussion**

The concept that ARH depends upon a sensitization of the vascular system by adrenal insufficiency during the immediate post-enucleation period would have been supported by the finding that the condition could only be prevented by giving steroids in this period. On the other hand, if treatment instituted after a period of insufficiency had been allowed proved equally efficacious, that theory would have had to be abandoned in favor of another, such as suppression of the anterior hypophysis. The discovery that the correction and adrenal insufficiency by either DCA and cortisone or corticosterone enhanced the hypertensive response was unexpected and requires analysis.

DCA and cortisone, unlike corticosterone, increased the hypertensive response even when treatment was delayed for a week. The data, although initially surprising to us, confirm Ledingham's (13) observation that when DCA and cortisone are given together there is reinforcement of the hypertensive effect of each, and together they constitute a mixture inimical to the survival and growth of rats with adrenal insufficiency. The effect of these two steroids may then be interpreted as indicating that, under the circumstances in which they were given, ARH was aggravated, or, as seems more likely to us, that steroid hypertension was induced in rats that merely happened to have been adrenal enucleated.

Why the response should have been diametrically opposed to what has been reported to occur (8) is unclear. The hormone solvent system used in that study was not given, but if the one that was used permitted a delayed absorption, the acute overdosage symptoms encountered in this study might have been avoided; furthermore, a delayed absorption might have extended the period of action beyond the time that injections were stopped, and thus perhaps have favored pituitary suppression.

In the second experiment, corticosterone in the dosage used did not induce hypertension in controls, but prevented ARH in enucleated rats when given continuously. Therefore, the observed enhancement of ARH when it was given for the first ten days postoperatively, which clearly indicated that hypertension was aggravated rather than suppressed by abolition of the period of adrenal insufficiency, cannot be attributed to a direct hypertensogenic effect of the steroid. This was the only group to demonstrate a progressively severe polydipsia and to show splenic enlargement and thymus atrophy, and had the greatest growth impairment, the severest vascular lesions, and the largest livers, hearts and kidneys. The only animal of this group without vascular lesions had a blood pressure of 174 mm Hg on the 32nd day, at which time the others were all above 210 mm Hg.

The same quantity of hormone given after a week of adrenal insufficiency had no demonstrable effect. On the 22nd day, when hypertension became evident, these animals had significantly lower pressures than either untreated enucleated animals ( $P < .05$ ) or those given hormone for the first ten postoperative days ( $P < .001$ ). Thereafter, the pressures continued to average lower, but the difference was not significant.

When salt intake is high, ARH begins to appear late in the second or in the third postoperative week. For the first five or six postoperative days, adrenal insufficiency is evident and it is probably during the second week that the adrenal becomes able to secrete the hormones that cause ARH. This ability is suppressed by continuous corticosterone treatment at a dose level sufficient to interfere with adrenal regeneration but insufficient to cause hypertension directly. Given for the first ten postoperative days, it prevents adrenal insufficiency, but aggravates ARH and does not interfere with regeneration in a manner that is detectable by differences in glandular weight as long after cessation of treatment as in the present instance. Possibly by preventing the hypotension of adrenal insufficiency, it provides a higher baseline pressure upon which hormones from the regenerating adrenal impinge. Whatever pituitary suppression occurs at this time is probably inconsequential, because there is little adrenal cortical tissue to be affected thereby. Given from the 8th to 17th day, when the bulk of adrenal tissue is larger, the pituitary suppression might well play a more significant role. This was suggested by the lower pressures exhibited by this group throughout the experiment as compared with untreated enucleated rats or those given hormone for the first ten days postoperatively.

Given continuously postoperatively, corticosterone effectively prevented the saline polydipsia, growth impairment, early hypertension, cardiac enlargement, and vascular lesions seen in untreated enucleated rats, and interfered with the regeneration of adrenal glands as indicated by their atrophic condition. However, rats given corticosterone continuously, whether adrenal enucleated or only unilaterally adrenalectomized, did develop a late hypertension at precisely the same rate and with the same intensity as did untreated controls. This condition, which is probably best considered as uncomplicated salt hypertension, was essentially unaffected by hormone treatment in contrast to the considerable effect of such treatment upon ARH. Continuous hormone treatment failed to completely prevent kidney enlargement in adrenal-enucleated rats and inhibited adrenal regeneration to a greater extent than adrenal hypertrophy, suggesting that the mechanisms underlying the two might be somewhat different.

The findings in both experiments negate the notion that post-enucleation adrenal insufficiency sensitizes the vascular system to the cortical hormones or that corrective therapy will prevent ARH. The present experiments indicate that, in fact, such treatment enhances rather than prevents ARH, suggesting that postoperative insufficiency has, if anything, a protective effect. Had 5 mg/day of corticosterone been used in the second experiment rather than the 1 mg dosage, it seems likely that treatment of enucleated rats in the 8-17 day period would have led to results similar to those obtained with DCA and cortisone, for the higher dosage of corticosterone directly causes hypertension (21).

References to the supposed sensitization of the vascular system during the period of post-enucleation insufficiency as the basis of adrenal-regeneration hypertension continue to appear (18), although the present evidence and other data (3, 23) fail to support the notion. Since the total quantity of steroid secreted by the gland is subnormal, an absolute excess of a particularly hypertensive steroid is unlikely. It would seem that the etiology of this condition depends upon an abnormal ratio or imbalance between hormones normally secreted.

The data confirm Skelton's observation that 1 mg/day of corticosterone will prevent adrenal-regeneration hypertension, and the efficacy of such treatment would seem to depend upon pituitary suppression, since it clearly cannot be imputed to prevention of adrenal insufficiency. Whether the unsuppressed regenerating cortex secretes a ratio of steroids having predominantly a mineralocorticoid effect as suggested by Skelton (22) or principally a glucocorticoid effect as proposed by Birmingham *et al.* (1) remains to be demonstrated.

### Abstract

The role of postoperative adrenal insufficiency in the genesis of adrenal-regeneration hypertension was evaluated by using either a mixture of DCA and cortisone or corticosterone alone. In each case, the hormones were either administered in a declining amount for the first ten days postoperatively, or the same hormone regimen was followed after first allowing a period of adrenal insufficiency to supervene. In the case of corticosterone, continuous therapy during the experiment was also employed.

Prevention of postoperative adrenal insufficiency by either means resulted in the development of higher blood pressures, greater enlargement of organs responding to hypertension, and more severe cardiovascular lesions in adrenal-enucleated rats. When treatment was instituted after a period of adrenal insufficiency, the same effect was obtained with DCA and cortisone, but not with corticosterone. Continuous corticosterone treatment inhibited adrenal regeneration and prevented adrenal-regeneration hypertension, but not the more delayed development of high blood pressure as among controls associated with continuous consumption of saline.

The conclusions derived from these studies were that the inhibitory effect of continuous corticosterone treatment upon adrenal-regeneration hypertension probably depends upon suppression of the hypophysis and hence impairment of adrenal regeneration. Either DCA and cortisone or corticosterone given in a manner designed to prevent postoperative adrenal insufficiency aggravates the response, perhaps because such treatment prevents the associated hypotension. Given a week later, the principal effect of corticosterone appears to be to cause slight pituitary suppression, without exerting the direct hypertensive effect seen with DCA and cortisone. The fact that continuous corticosterone treatment prevented adrenal-regeneration hypertension but did not affect the development of salt hypertension, as seen in controls, is further evidence that adrenal enucleation does not merely increase the hypertensive effect of salt excess in rats.

### Abrégé

Nous avons évalué le rôle de l'insuffisance surrénalienne post-opératoire dans la genèse de l'hypertension qui survient au cours de la régénération de la surrénales, en utilisant soit un mélange de DCA et de cortisone, soit de la corticostérone seule. Dans les deux cas, les hormones furent administrées soit en quantités décroissantes pendant les dix jours qui ont suivi l'intervention, soit en maintenant le même régime hormonal à la suite d'une période préalable d'insuffisance surrénalienne. Dans le cas de la corticositérone, on a aussi éprouvé la thérapie continue pour la durée de l'expérience.

La prévention de l'insuffisance surrénalienne post-opératoire par l'un ou l'autre des moyens susmentionnés a eu pour effet d'accroître les pressions artérielles, d'augmenter le volume des organes affectés par l'hypertension et de produire des lésions cardiaques plus graves chez les rats dont les surrénales

étaient énucléées. Lorsque le traitement fut entrepris, après une période d'insuffisance surrénalienne, nous avons noté que la DCA et la cortisone exerçaient un effet comparable, mais non la corticostérone. La corticostérone administrée de façon continue a entravé la régénération surrénalienne, sans toutefois empêcher le développement plus tardif de l'hypertension artérielle due à la consommation continue d'eau salée chez les témoins.

Sur la base de nos observations, il nous est permis de conclure que l'action inhibitrice de la corticostérone en traitement continu, sur l'hypertension par régénération surrénalienne, est probablement attribuable à une action suppressive au niveau de l'hypophyse et, par conséquent, à une altération de la régénération surrénalienne. La DCA et la cortisone, ou la corticostérone, administrées d'une façon adéquate en vue d'empêcher l'insuffisance surrénalienne post-opératoire, exagèrent la réponse des animaux d'expérience, peut-être en prévenant l'hypotension associée à cette condition. Une semaine après l'opération, l'administration de corticostérone a pour effet principal d'induire une faible suppression hypophysaire, sans action hypertensive directe, comme nous l'avons observé avec la DCA et la cortisone. Le fait que la traitement continual avec la corticostérone empêche l'hypertension au cours de la régénération surrénalienne mais n'influence aucunement le développement de l'hypertension à l'eau salée, tel que constaté chez les témoins, constitue une preuve additionnelle que l'énucléation surrénalienne n'augmente pas simplement l'action hypertensive d'un excès de solution saline chez les rats.

### Acknowledgements

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**M. Nadasdi:** The experiments of Hall *et al.* shed some new light on the pathogenesis of adrenal-regeneration hypertension (ARH). Ever since the first description by Skelton in 1955 (*Proc. Soc. Exptl.*

*Biol. Med.*, 90:342, 1955), this experimental hypertensive disease has remained an enigma. The hypertensive syndrome produced by the presence of a regenerating adrenal is in many ways reminiscent of the hypertensive syndrome generated by mineralocorticoids, particularly desoxycorticosterone (DOC) and a high-salt intake (Selye, H.: *Brit. Med. J.*, i:203, 1950): they both depend upon unilateral nephrectomy and salt. Hypophysectomy and thyroparathyroidectomy prevent both syndromes (Salgado, E.: *Endocrinology*, 55:377, 1954; Chappel, C. I., et al.: *Proc. Soc. Exptl. Biol. Med.*, 98:23, 1958). While these similarities are quite obvious, it is puzzling that the same type of hypertensive disease can be produced in two completely opposite ways: by overdosage with corticoids on one hand and by the lack of them on the other. Deficient hormone secretion in the regenerating gland has been demonstrated by several authors (Brogi, M. P., and C. Pellegrino: *J. Physiol.*, 146:165, 1959; Ingle, D. J., and R. E. Harris: *Am. J. Physiol.*, 114:657, 1935), and the development of hypertension has been attributed to this fact. In the present experiment, DOC and cortisone produced severe hypertensive disease in adrenal-enucleated animals. This finding is not entirely unexpected, considering that similar treatment would give the same results in animals with either intact or completely removed adrenals. Inclusion of such groups in the present experiment could have clarified this point in support of the authors' statement (with which I agree) that they induced steroid hypertension in incidentally adrenal-enucleated rats. Based upon their experimental results, the authors reject the hypothesis that the regenerating adrenal sensitizes the vasculature to injury by corticoids and that corrective therapy prevents ARH. This interpretation depends upon what we consider "corrective" therapy. Clearly, the continuous administration of 1 mg of corticosterone after enucleation prevented ARH. In my opinion, this therapy can be labelled as "corrective," judged by the diminution of adrenal regeneration and the correction of the growth curve. The effect of 1 mg daily of corticosterone is comparable with that exerted by an intact contralateral adrenal. These experiments reveal an interesting time relationship between adrenal regeneration and the development of hypertension. It seems obvious that the protective action of corticosterone requires the continuous administration of the hormone in the post-operative period, although the animals are invariably normotensive during the first two weeks, whether treated or not. Apparently this is the critical period during which the adrenal acquires its capacity to produce a vasculotoxic agent. Corticosterone, when started after the

first week, cannot prevent the adrenal from exerting its pathological effect, but merely tempers its severity. Conversely, the cessation of corticosterone treatment after the first week gives a sudden spurt to the noxious action of the adrenal. This seems almost to be a rebound phenomenon after an initial period of suppression. It is unlikely that the hypertensive action of the gland is related to its "regular" hormone secretion, since it has been shown to be subnormal for about four weeks after enucleation (Masson, G. M. C., *et al.*: *Endocrinology*, 62:229, 1958). It is also unlikely to be caused by the lack of adrenocortical principles, since in that case hypertension would be expected to develop much earlier after the intervention.

Little attention has been paid so far to the possible role of the adrenal medulla. Evidently, uniadrenalectomized, adrenal-enucleated rats are completely deprived of their adrenal medulla and, hence, of their major source of epinephrine. An experiment using adrenal-enucleated plus contralateral adrenal-decorticated (instead of adrenal-ectomized) animals would show whether an imbalance of endogenous catecholamines, such as a shift of the epinephrine/norepinephrine ratio, could be the stimulus for the malfunction of the adrenocortical remnant, resulting in hypertension and vascular disease. This is conceivable, since catecholamines are known to have a potentiating effect upon steroid hypertension. The authors postulate, justifiably, that an imbalance of cortical hormone secretion is the main factor in ARH. It has been shown that there is a marked decrease of the aldosterone/corticosterone ratio in regenerating adrenals (Brogi, M. P., and C. Pellegrino: *J. Physiol.*, 146:165, 1959), aldosterone being decreased to a much greater extent than corticosterone. While aldosterone can induce mild hypertension in rats, it does not produce any morphological cardiovascular defects. This characteristic of aldosterone makes it perhaps a more suitable agent than DOC for the correction of steroid "imbalance" in an attempt to prevent ARH. The fact that corticosterone can protect against the hypertensive action of a regenerated adrenal seems to contradict the previous finding that amphenone can do the same (Chappel, C. I., *et al.*: *Endocrinology*, 60:677, 1957). This latter substance blocks adrenal cortical secretion without interfering with cortical cell regeneration. Since it suppresses both gluco- and mineralocorticoid secretion (Renold, A. E., *et al.*: *New Engl. J. Med.*, 256:16, 1957), it provides no conclusive evidence either for or against the pathogenetic role of steroid imbalance. However, it nullifies the importance of ACTH, for amphenone further increases anterior pituitary secretion through the

suppression of the remaining adrenocortical activity. It would be interesting to see if aldactone, a compound that blocks mineralocorticoid action without interfering with its secretion, would have an effect on ARH similar to that of amphenone.

Among the many hypotheses concerning the mechanism of ARH, there is one that has been entertained in a number of publications, not unlike in the presently discussed paper. The intriguing question of some unknown principle(s) in the regenerated adrenal was raised almost a decade ago (Masson, G. M. C., et al.: *Endocrinology*, 62:229, 1958; Skelton, F. R.: *Endocrinology*, 62:365, 1958). It is tempting to postulate that this unknown factor is similar in nature to that produced by the ischemic kidney. Twenty-two years ago, Dr. Selye developed a technique for the production of experimental renal hypertension, whereby the left kidney of a rat is transformed into an exclusively endocrine organ, subsequent to the cessation of urine excretion, tissue perfusion remaining sufficient for cell survival (*Nature*, 158:131, 1946). The result is called the "endocrine" kidney and it produces a cardiovascular syndrome similar to that of ARH. Its action is not mediated through the adrenal, since adrenalectomy does not interfere with it (Nadasdi, M.: *J. Endocrinol.*, 22:1, 1961). The endocrine kidney, like the regenerated adrenal, is under the influence of the pituitary.

What, then, could be the common factor in the hypertensive syndromes of the regenerated adrenal and the endocrine kidney? Since both organs have a common embryonic origin, it is possible that their cells have similar functional potentials that can be evoked by different stimuli. In the case of the kidney, deprivation of its normal excretory function may awaken a dormant cellular ability present since the time of differentiation of the Wolffian duct. The adrenal cortex, whenever its cells are forced to regenerate, may react in a similar way. In both cases, the result would be identical: the production, under pituitary regulation, of an aberrant substance with vasculotoxic properties (Nadasdi, M.: *Rev. Can. Biol.*, 24:213, 1965). Such a hypothesis could be proven only through the isolation of a chemical substance from both the atrophic kidney and the regenerated adrenal. The results of the work under discussion offer some possibilities of correlating renal and adrenal hypertension. Since ARH takes about two weeks to develop, while the production of the endocrine-kidney syndrome needs only a few days, it would be interesting to examine the simultaneous effect of an endocrine kidney and a regenerated adrenal. Would they potentiate or antagonize

each other's hypertensive effect? Would the endocrine kidney modify adrenal regeneration and, vice versa, would adrenal enucleation interfere with the transformation of the ischemic kidney? By answering these questions we could come somewhat closer to a unifying concept of hormonal and renal hypertension.

**C. E. Hall:** There are a lot of questions that really would depend upon setting up experiments that have not yet been set up; so I don't know if one could really predict exactly what would happen. I may say that one of the things that has always intrigued me (this is not the answer to the question, but I think it bears much more closely on what Dr. Page had to say) is that, in trying to determine how much of a given steroid will produce hypertension, the argument is constantly made that the dose given was much larger than the adrenal cortex can secrete, for example, or the amount needed is larger than the adrenal gland is known to contain, or something of that sort. I was much intrigued by a paper of Dr. Gross's some years ago in which he pointed out that one gets more intense mineralocorticoid effects by pellet implantation of steroid than by administering an oil solution of steroid, even though the absorption is much greater from the oil solution than from the pellet. One cannot, therefore, relate quantities to response, precisely. It is perhaps a matter of the rate at which the hormone gets delivered to the target organ over a period of time rather than the total amount absorbed per 24 hours, or something of that nature. Dr. Skelton's pertinent recent results, for example, indicate that the regenerating adrenal gland puts out more DOCA. I should think that the most effective way of getting a mineralocorticoid to the target organs would be by the blood stream and from the adrenal rather than from a pellet or from an oil solution depot. Thus, it seems to be quite possible that, although it is not putting out vast quantities of DOCA in comparison with what could be injected, the adrenal is, nevertheless, putting out quite sufficient to do whatever is causing the high blood pressure with which these rats have to contend. Since we have had some experience with aldosterone hypertension recently, I may say that we have had no difficulty in producing very similar hypertensive lesions with aldosterone, at first with 250  $\mu\text{g}/\text{day}$ , although later on we found that 100  $\mu\text{g}/\text{day}$  is just as effective and, I suspect, lots less would do the same thing. So, in our hands we find that aldosterone does exactly what DOCA does, which is what one would expect it to do if it is sodium that is doing the dirty work.

Now, another thing I would like to comment upon and which also

bears on Dr. Page's remarks is that, frequently, those of us who work with the rat have dogs thrown at us. It is my contention that the dog is totally unsuited to this kind of study. The dog has an adjustable glomerular filtration rate; the rat does not and man does not. Rat and man can be made to retain salt; the dog cannot, but dumps it. The rat and man have isotonic or hypotonic glomerular filtrate in the distal nephron; the dog always has hypotonic fluid in the distal nephron under any circumstances. I think as far as salt effects are concerned, and as far as mineralocorticoid effects are concerned, the dog just cannot be used for this purpose and it is not surprising, therefore, that the dog would not develop adrenal-regeneration hypertension, for example. It will also not develop DOCA-hypertension or aldosterone hypertension very easily. I think one has to adapt the experimental animal to the particular study needed, and I think the rat is very useful for this kind of study; but the dog is not. Now, getting around to Dr. Nadasdi's question as to what would happen if one had an endocrine kidney and an enucleated adrenal simultaneously in a given animal, I would be prepared to lay a small bet that one would get a very hypertensive animal under these circumstances; I have not prepared any such animal and I could not guarantee it, but the bet is still good. (Remark from audience: not a very good bet!)

**W. S. Hartroft:** I wonder whether an animal could stand all that. This is one of those experiments you can plan on paper but you end up with dead rats the next day.

# A Radioautographic Survey of the Formation and Fate of Connective Tissue Giant Cells in Selye's Granuloma Pouch

LEONARD F. BÉLANGER and PIERRE DROUIN

## Introduction

THE origin and fate of the cells that take part in the mechanism of inflammation are still a mystery. This is particularly true of the multinucleated giant cells (1, 31). It is also true of other giant cells, those which are observed in the vicinity of bone tissue (osteoclasts) and those which are characteristic of specific neoplasms of bone or connective tissue (giant cell tumours, osteoclastomas (1, 10, 16, 19)). The tracer methodology has been applied in recent times to this problem, producing a variety of interpretations (4, 13, 24-26, 29, 30).

The present survey is an application of the integration process of radioautography (2, 3) to a time-study of  $H^3$ -thymidine-labeled cells located in the wall of granuloma pouches produced by the method of Selye (20, 21). Multinucleated cells (osteoclasts) located along trabecular bone in the vicinity of the teeth have been observed, concurrently.

## Materials and Methods

A dorsal "granuloma pouch" (20, 21) was produced in 24 male Sprague-Dawley rats according to the following schedule: on day 1, injection of 25 ml of air and 0.5 ml croton oil; on day 4, second dose of 0.5 ml croton oil along with 0.5 ml of mazola oil containing 20% of lycopodium spores.

On day 12, the animals were injected intraperitoneally with a single 1 ml dose of  $H^3$ -thymidine containing 100  $\mu$ c of tritium. At that time, the average weight of the animals was 200 g. The rats were then killed in groups of 4, at intervals of 2 hr, 1, 2, 3, 4 and 8 days. The pouch was dissected and fixed in a mixture of 75 parts ethanol, 20 parts formaldehyde, and 5 parts glacial acetic

acid (AFA) for 24 hr. Portions of the wall were then cut off and embedded in paraffin. Sections of approximately  $7 \mu$  were then coated (2, 3) with fluid nuclear emulsion (NTB<sup>3</sup> Eastman Kodak Co.) for radioautography. These preparations were exposed for 2 months and then photographically processed and post-stained with haematoxylin and eosin at 4°C (2).

### Observations

#### *Injection Site*

In our early attempts at stimulating the production of giant cells, the lycopodium spores were introduced inside the air-fluid chamber of the granuloma pouch. Two weeks after the injection most of the spores were found in groups of various sizes, surrounded by granulocytes (Fig. 2). Frequently, a thin layer of basophilic material (Fig. 2), positive to the Von Kossa reaction for calcium salt (17), was located between the granular elements and the spores. The latter were at that time in various degrees of disintegration. A small proportion of the spores were also found deeper in the wall, in an area populated mostly by large, clear mononuclear cells. Some of the spores in that site were located within the eosinophilic cytoplasm of multinucleated giant cells.

In most of the experiments reported here, the lycopodium spores were injected in two or more sites within the thickness of

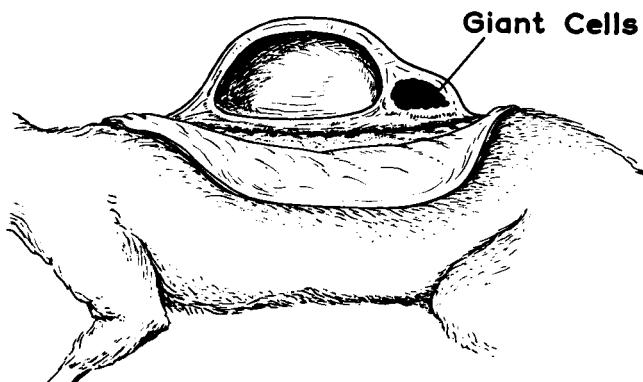


FIGURE 1. Diagram showing best site for production of giant cells, following intra-mural introduction of lycopodium.

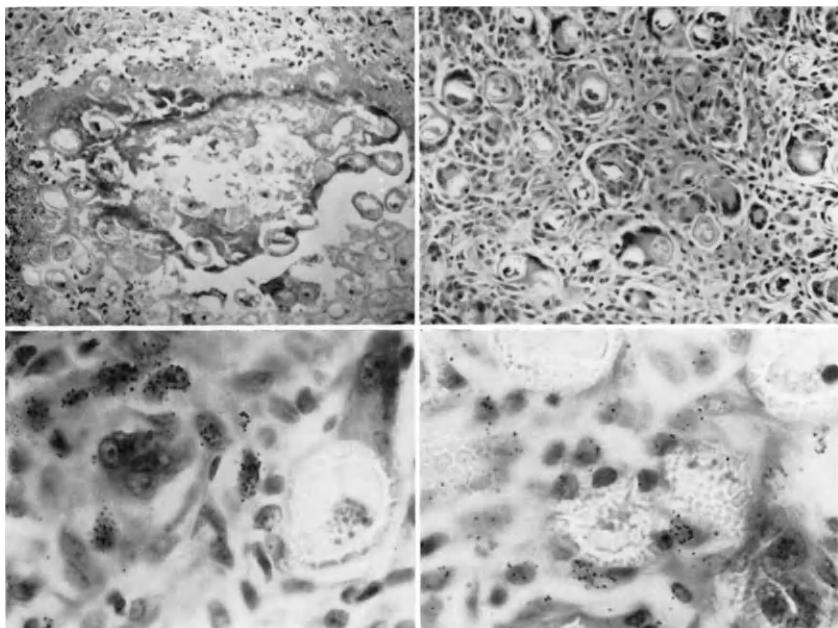


FIGURE 2. Reaction to deposition of lycopodium in the pouch cavity: accumulation of granulocytes, gradual dissolution of spores, deposition of salt.  $\times 100$ .

FIGURE 3. Reaction to intra-mural injection of lycopodium. The spores have been segregated individually inside multinucleated giant cells.  $\times 100$ .

FIGURE 4. Two-hr stage: several mononuclear cells labeled; giant cells not labeled.  $\times 1,000$ .

FIGURE 5. Three days: one or more nuclei of giant cells labeled.  $\times 1,000$ .

the wall. This method produced a much improved yield of giant cells (Fig. 3). The largest concentration was always obtained in the caudal portion of the wall of the granuloma pouch (Fig. 1).

#### **Radioautography**

**1) Granuloma Pouch Constituents.** The time factor involved in the completion of this manuscript did not allow for actual counts of labeled cells. Consequently, the present report will consist of proportional estimates. Two hours after intraperitoneal introduction of radioactive thymidine, some of the larger mononuclear cells of the wall were labeled (Fig. 4). Practically none of the giant cells (Fig. 4) and none of the granular cells or small

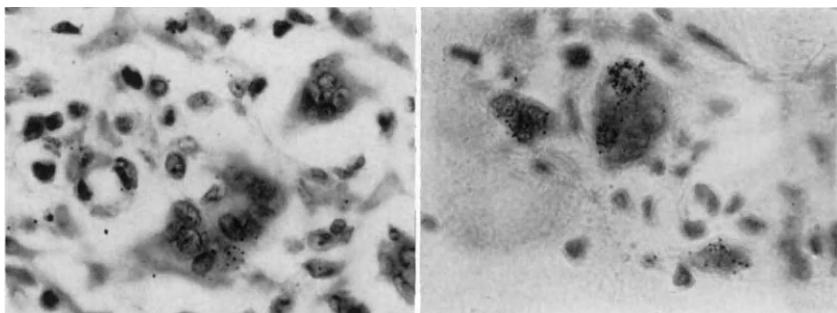


FIGURE 6. Eight days: practically no more label present.  $\times 1,000$ .

FIGURE 7. Osteoclasts in peri-alveolar bone at 3 days post-labeling: one or more nuclei contain  $H^3$ -thymidine.  $\times 1,000$ .

basophilic elements (lymphocytes and plasma cells) were labeled. After 24 hr, a few endothelial cells produced a record of radioactivity. A scattering of label over single nuclei was also observed amongst the giant cells.

After 2 and 3 days, the proportion of labeled giant cells was remarkably increased. Some of these had apparently only one labeled nucleus out of the 5 to 8 that could be seen in the same section. In other instances, 2 or 3 nuclei exhibited a radioautographic record. Labeling of all the nuclei of individual giant cells was never observed.

After 8 days, practically no nuclei were labeled in giant cells; occasionally, single nuclei with a few gains above them were observed (Fig. 6). Most of the mononuclear cells were also devoid of radioactivity. A few small, round cells (lymphocytes?) produced a radioautographic record.

**2) Alveolar Bone and Marrow.** In the vicinity of the roots of the molar teeth, a large number of osteoclasts are generally observed. At 2 hr and 1 day, practically none of these were labeled, although a fair number of ovoid or fusiform cells in the immediate vicinity exhibited the presence of  $H^3$ -thymidine. In the marrow, a large proportion of the hemopoietic elements were also radioactive (Fig. 8). Some megakaryocytes (or megakaryoblasts) were faintly labeled throughout their large, tormented nucleus (Fig. 8, M).

At 2, 3 and 4 days, many of the osteoclasts showed the presence

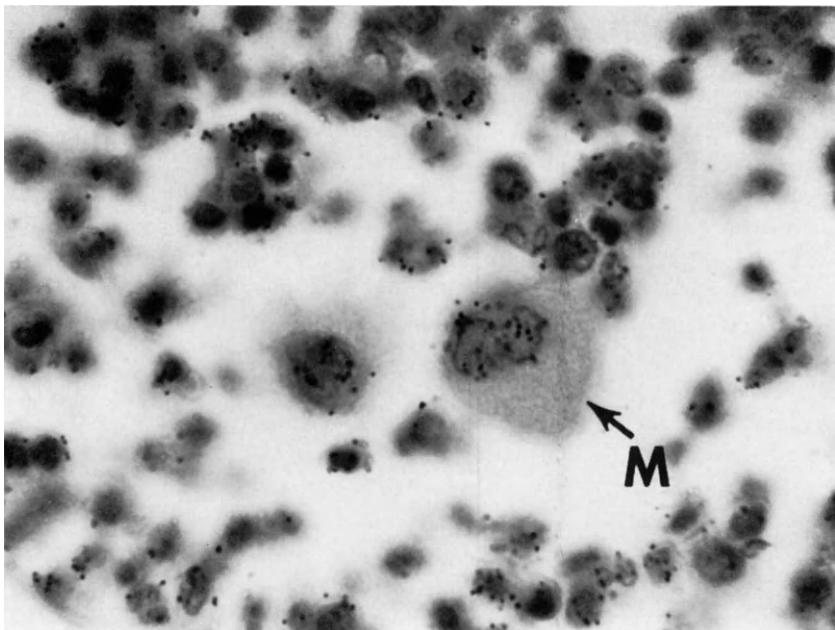


FIGURE 8. Megakaryocyte (M) at 1 day post-labeling: nucleus lightly labeled throughout; a large proportion of the other marrow elements are labeled.  
 $\times 1,000$ .

of radioactive thymidine in one or more of their nuclei (Fig. 7) but never in all of the nuclei. At this stage, a small number of the osteoblasts and odontoblasts were labeled. The proportion of radioactive marrow elements had greatly decreased.

At 8 days, there was practically no radioactivity recorded over the hard tissues, except for the occasional fusiform cells and small round cells. In the jaw as well as in the granuloma pouch, many mast cells were present, but not a single instance of labeling of these cells was recorded.

#### Discussion and Conclusions

**1) Experimental Production of Giant Cells.** It appears from the above report that the granuloma pouch technique, which has already proved so useful in the study of the mechanism of inflammation (20-22), is an excellent procedure for the production of giant cells, providing that the foreign body particles, the lycopodium spores in the present case,

are introduced intra-murally and preferentially caudad to the air sac (Fig. 1), where it seems that the local conditions are more favourable. In our hands, however, the duration of the experiment was limited to a maximum of 30 days. At that time and even earlier, spontaneous calcification of the pouch occurred along the inner portion of the granular layer (Fig. 2). In some instances also, purulent ulcerations developed, with ensuing general ill-health of the animals. Spontaneous calcification of inflammatory lesions has already been recognized (14, 18, 23). Experimental calcification of the granuloma pouch by the calciophylactic process has also been reported by Selye (23).

**2) Giant Cell Formation.** The main purpose of the present experiments was to investigate the origin and fate of the giant cells. The classical authors are generally in agreement on the facts that the nuclei of these cells vary greatly in shape, size and number (1) but that they practically never show mitotic figures. A recent study by Silverman and Shorter (24) revealed that this is true, even after administration of colchicine. Consequently, new nuclei must be acquired by amitosis or by "annexation" of pre-existing adjacent elements. Silverman and Shorter (24) have presented an excellent case for this possibility, through the appearance of labeled nuclei in the giant cells subsequent to that of mononuclear cells in the immediate vicinity ("histiocytes").

In the present series, the time involved in the appearance of labeled nuclei in giant cells was comparable to that reported by Silverman and Shorter (24) and, consequently, also favours the annexation theory. Equally, the rapid disappearance of the labeled nuclei was observed in both instances.

#### ***Nature and Origin of the Labeled Mononuclear Precursors***

The origin of the mononuclear cells of the inflammatory lesion from circulating blood cells has been recently postulated on the basis of radioautographic studies of labeled DNA. However, there are differences of opinion as to the cells involved. While Kosunen *et al.* (13) consider the medium-size and large-size lymphocytes as the blood precursors of inflammatory histiocytes, Spector and Coote (25), Spector and Lykke (26), and Spector *et al.* (27) favour the monocyte.

There is no doubt that histiocytes are numerous in the inflammatory lesion, but are they involved in the formation of multinucleated giant cells? An interesting fact reported by Spector and Lykke (26) seems to militate against this concept: cells laden with carbon particles do not divide.

In a discussion of the inflammatory mechanism, Selye (22) states

that "mature connective tissue cells are less capable of transformation into entirely different elements than are the dedifferentiated more embryonic cells."

Cronkite *et al.* (6) in studying the dynamics of haemopoietic proliferation in man and mice, with H<sup>3</sup>-thymidine label, have postulated that "a primitive proliferation pool for formation of new mesenchymal cells of all types" must be maintained throughout life. It is quite possible that in the present series of experiments as well as in that of Silverman and Shorter (24), the relatively small proportion of labeled mononuclear cells consist of "primitive cells" (6). At 8 days, while most of the label has disappeared, the fusiform cells in which a strong label persists may also be "primitive cells" still in their G<sup>2</sup> phase, quiet, slow living, guardians of the past.

#### ***Origin of the Osteoclasts***

The present series of experiments allow a time element comparison between the appearance and disappearance of H<sup>3</sup>-thymidine in the nuclei of giant cells and in those of osteoclasts. This time factor is *remarkably comparable*. Furthermore, the progressive occurrence of one and then of two or more labeled nuclei in both locations is a notable coincidence.

In a previous survey of H<sup>3</sup>-thymidine incorporation in chick bone, which included groups taken at 2 hr to 16 days (4), the proportion of labeled osteoclasts had also been found to be largest at 4 days. Again, these observations seem to be in accord with the annexation theory of multinucleated cell formation.

Most of the recent authors are in agreement on that point, but they diverge as to the nature of the cells involved. Fischman and Hay (7) consider that "mononuclear leucocytes, probably monocytes," are the precursors. Tonna at one time considered that these were osteoblasts (29), but later on stated that "osteoclasts arise from fusion of osteoblasts, preosteoblasts and a combination of those cells in different stages of development" (30). Young (32) considers the precursors as primitive but already predestined "osteoprogenitors." Kember (11) as well as Bélanger and Migicovsky (4) are in favour of the concept of "persistence of a primitive proliferating pool for formation of non-mesenchymal cells of all types" (6).

#### ***The Nature of Giant Cells and Osteoclasts***

On the basis of enzymic and behavioural similarities, several authors (8, 9, 12, 19) have recently postulated that connective tissue giant cells

and osteoclasts might represent similar entities in different locations. The present report adds the argument of comparable formative mechanism to this intriguing concept.

### ***Maturation of Megakaryocytes***

According to Bessis (5), these giant mononuclear cells of the marrow "become hyperplastic as a result of an increase in size and shape of their nuclei without this being accompanied by a division of their cytoplasm." The present observations do not add to this statement or subtract from it.

A major problem remains unsolved. Since DNA is metabolically stable (15, 28), the disappearance of label from most areas with time (Fig. 6) can only be explained by cell migration or cell death. However, the fate of the multinucleated giant cells either in the granuloma pouch or in the bone remains a mystery. The existence of pycnotic nuclei in these cells might indicate that while the nuclei have a limited life span, the cell itself goes on.

### **Abstract**

A large number of giant cells have been produced by injecting lycopodium spores into the wall of pre-established "granuloma pouches." A radioautographic survey following a single intraperitoneal tracer dose of  $H^3$ -thymidine has revealed that the earliest labeled cells in the granuloma are fusiform or ovoid mononuclear elements located between the giant cells. One or more labeled nuclei of the giant cells appeared at later intervals of 1 to 4 days. Mostly all radioactive nuclei seemed to have disappeared after 8 days.

A parallel survey of periodontal bone has shown a similar pattern in the appearance and disappearance of labeled nuclei in osteoclasts.

These observations are in accord with the annexation concept of giant cell formation; they also add to the already reported similarities between multinucleated giant cells of the connective tissue and of the bone.

### **Abrégé**

De nombreuses cellules géantes ont envahi la paroi du granulome de Selye, à la suite d'injections de spores de lycopode.

Des observations d'ordre radioautographique ont permis de reconnaître le cycle d'apparition et de disparition des noyaux marqués au tritium dans le granulome et également dans l'os mandibulaire. Ce cycle s'est avéré comparable dans les cellules géantes conjonctives et dans les ostéoclastes, ajoutant ainsi aux analogies déjà connues entre ces deux entités.

### Acknowledgements

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**B. Messier:** Radioautographically speaking, Dr. Bélanger is my father. When I joined the Histology Department at McGill some years ago, the coating technique of Bélanger and Leblond was a rising sun. At that time, I proposed the dipping step as a modification to his original technique; but basically, his contribution remains. It is with filial devotion, therefore, that I dare make a few comments here.

For those of you who may not be familiar with the technical implications of the thymidine- $H^3$  tracer technique, I would like to make the following comments. The tritium label emits very weak beta par-

ticles, so weak that they barely travel more than  $1\mu$  away from their source. Actually, this is the reason why tritium radioautographs offer such good resolution. This advantage, however, has some drawbacks. Indeed, any tritium label located in the histological section more than  $1\mu$  away from the photographic emulsion will not register and can lead to a false negative reaction.

In Dr. Bélanger's experiment, histological sections of the wall of the granuloma pouch were prepared at  $7\mu$ . When such sections were covered with photographic emulsion, only the upper  $1\mu$  of the section could contribute to the radioautographic image. In multinucleated giant cells, therefore, all radioactive nuclei located deeper in the section were interpreted as unlabelled. Since these multinucleated cells are so large, it might be worthwhile to make serial sections at  $2\mu$  to favor the radioautographic detection of all labelled nuclei. Thus, the apparent sporadic labelling observed at 2-4 days after thymidine-H<sup>3</sup> injection might turn out to be a more uniform and extensive process. The "annexation" theory mentioned by Dr. Bélanger may acquire further acceptance if a smoother progression of radioactive nuclear "annexation" could be demonstrated.

The second comment I wish to make concerns the proposed parallelism between osteoclasts and connective-tissue giant cells. Dr. Bélanger demonstrated the remarkable similarity in the labelling behavior of these two cell types. But, is this the only morphological or functional similarity between them. How would other labelled precursors behave? Similarly, relevant histochemical reactions could disclose further aspects of the apparent formative resemblance of osteoclasts and connective-tissue giant cells. Since so much has yet to be learned about multinucleated giant cells, it seems worthwhile to follow up the evidence of the nuclear annexation theory reported by Dr. Bélanger.

**L. F. Bélanger:** I use or abuse my privilege as Chairman now to thank Dr. Messier for his comments and his very kind thoughts. Indeed, if in all laboratories there were people who would understand the procedures that they have learnt and improve on them with such understanding as Dr. Messier has done, you would figure that this sort of indoctrination is worth a lot of papers that one may write for the outside world. Dr. Messier is perfectly right—in relation to the limited accuracy of this type of experimentation; but as Claude Bernard and Dr. Selye himself have taught us many times, one cannot always go into the greatest of details—sometimes the ensemble of a phenomenon can be observed by a cruder type of approach. In the present case, recognizing the deficiencies of my experimental approach,

I still think that the conclusions are valid, because under comparable conditions we observed curves that were remarkably similar. There are probably many aspects of this problem worth investigating further. Dr. Messier, thank you again.

## **Peripheral Vasodilatation and Thymic Tumors in Magnesium-Deficient Rats**

P. BOIS

THE original communications of McCollum and his associates (32, 43) on magnesium deficiency in rats described in detail the unexpected peripheral vasodilatation induced by the deficiency. The spectacular convulsive seizures occurring in the deficient animals were also carefully studied by the same workers (33), who viewed a possible relationship between the two phenomena, suggesting that the vasodilatation was the result of a vasomotor spasm (32). The signs and symptoms of magnesium deficiency were also related to deficiencies of the vitamin-B complex (22, 59). However, the work of Sullivan and Evans (55) did not confirm this claim. These, as well as other earlier investigations on magnesium deficiency, have been reviewed by Follis (18).

The time of appearance, duration and intensity of the peripheral vasodilatation is closely related to the age of the experimental animal. In very young rats, as reported by Kruse *et al.* (32), erythema develops rapidly within 3 to 5 days, is generalized, and lasts for 5 to 7 days. In older animals, it is delayed for as much as 12 to 14 days (42, 53); here, the duration and intensity is also more variable, the longest delay occurring in hypophysectomized rats (29). Few investigations have been made on the nature of the peripheral vasodilatation and Welt (60) and MacIntyre (38), in their recent reviews, state that the cause is still unknown.

Lowenhaupt *et al.* (36), studying the changes in perivascular connective tissue of magnesium-deficient rats, noticed the presence of eosinophilic infiltrations. However, it was the investigations of Kashiwa and Hungerford (30) that revealed the marked eosinophilia and neutrophilia associated with the peripheral vasodilata-

tion of magnesium deficiency. This observation was confirmed later on by other workers (47, 58, 63). A close association was observed by Kashiwa (29) between the drop in serum magnesium level, hyperemia and eosinophilia. The possible relationship between mast cell degranulation and histamine liberation during the early phase of magnesium deficiency was originally proposed by Bélanger *et al.* (3); Hungerford and Karson (27) further noted a significant reduction in the number of peritoneal mast cells during that same phase of the deficiency. Finally, a marked increase in plasma and urinary histamine was observed during the phase of vasodilatation in deficient rats (4, 8, 61). An indirect demonstration of the participation of histamine in the peripheral vasodilatation came from studies in rats treated with a histamine liberator, compound 48/80 (7). It was shown that treatment with 48/80, inducing mast cell degranulation and histamine liberation prior to magnesium deficiency, prevented the onset of hyperemia and skin disorders in the deficient animals (7, 58). A good parallelism was also found between eosinophilia, hyperemia and histamine increase (58). These findings led to the conclusion that dietary magnesium-deficiency in rats induced an endogenous histamine liberation with mast cell degranulation, resembling the effects of 48/80 (7, 8). Similar conclusions were also reached by Hungerford (26). However, the situation is not entirely comparable, since during magnesium deficiency, mast cell degranulation in tissues is not parallel to the increase in plasma histamine (4) except in the peritoneal fluid (27). Very little mast cell degranulation can be found in subcutaneous tissue during the first weeks of magnesium deficiency (3, 4, 31) and it is only after 4 to 5 weeks that a significant decrease in mast cells can be established (3, 4).

Nonetheless, we felt that the peripheral vasodilatation due to magnesium deficiency in rats was more closely related to histamine liberation than to histamine overproduction. Furthermore, liberation of other amines such as 5-hydroxytryptamine is also probably involved, as was suggested by J. F. Riley (personal communication).

Besides peripheral vasodilatation, another unexpected finding occurring in magnesium-deficient rats created a new interest.

Tumors of the thymus developed after chronic periods of magnesium deficiency (5). It was shown in numerous experiments that such tumors, lymphoma or lymphosarcoma, appeared only in rats fed a magnesium-deficient diet for at least 2 months (11). These tumors have been described in previous reports (6, 10, 11); briefly, they always develop initially in the thymus, produce metastases and are transplantable in non-magnesium-deficient animals. A report on a study made during the past three years gives an incidence of approximately 18 to 19% in rats surviving for an average of 64 days on a magnesium-deficient diet (11). Various hypotheses were suggested as to the relationship of these tumors to magnesium deficiency. The possible involvement of some unknown carcinogen in the diet seemed remote, since no tumor ever developed in control animals fed the same diet supplemented with magnesium. A comparable observation had been made by Jasmin (28) and a recent report describes the occurrence of myeloid leukemia in magnesium-deficient rats (44). The only constant factor in these observations is chronic lack of magnesium in the diet.

Since the first reports of McCollum and Orent (43), Kruse *et al.* (32) and Cramer (15) in the early thirties, numerous magnesium-deficient diets have been described in the literature, variations usually being made in the composition of the salt mixture. Most of our experiments on histamine levels (4, 8) and thymic tumors (6) in magnesium deficiency were performed with a deficient diet modified after Van Reen and Pearson (57) and Bélanger *et al.* (3) (Table I). The Ca:P ratio of this diet is higher than usual, a factor reported to accentuate the symptoms of magnesium deficiency (14, 17, 56), although a high phosphorus level is probably more deleterious especially with regard to the production of renal calcifications (12, 25, 35, 45, 48). There is no doubt that the signs and symptoms of magnesium deficiency can be influenced by the relative concentration of other cations in the diet (14, 20, 49). As stated by Chutkow (13), hypomagnesemia *per se* does not appear to be etiologically important; perhaps the peripheral vasodilatation results from the imbalance of minerals in the absence of magnesium and could be studied from this viewpoint. Another question is the

possible role of the early increase in plasma histamine in the later induction of tumors of the thymus. These two aspects have been investigated further in the present report.

### **Materials and Methods**

Three experimental series were performed with male Sprague-Dawley rats, using the magnesium-deficient and control diets described in Table I.

***Experiment 1. Effect of Various Single Mineral Deficiencies on Plasma Histamine.*** The salt mixture of the diet was modified and made deficient in calcium, potassium, sodium, or iron, as shown in Table II. In each case, the substituted element was replaced by an equal amount of alphacel. Groups of 12 rats of 100 g average body-weight were given the respective diets and 6 rats per group were killed after 6 days and 12 days. Plasma histamine was measured according to the technique described by Lowry *et al.* (37).

***Experiment 2. Effect of Deficiencies in Various Minerals, on Serum Calcium and Magnesium, and Plasma Histamine.*** Diets were prepared with no mineral content and various salts were added separately or in combination as shown in Table III, other elements being replaced by alphacel. After 14 days, serum calcium was measured by a modified colorimetric method (54), serum magnesium by atomic absorption spectrophotometry (16), and plasma histamine as previously described (37).

***Experiment 3. Effect of Parathyroidectomy on Serum Calcium and Magnesium, and Plasma Histamine, in Magnesium-deficient Rats.*** Parathyroidectomy was performed surgically in male Sprague-Dawley rats weighing 150 to 180 g. Only rats with a serum calcium of less than 9.0 mg/100 ml were used for the experiment; sham-operated controls were also included as shown in Table IV. Three days after surgery, the rats were divided into groups of 15; 6 of each group were sacrificed after 14 days for histamine, magnesium and calcium determinations as in experiment 2, and the remainder were kept on the diets for 3½ months before being sacrificed. At autopsy, tissues were fixed in

TABLE I  
BASIC MAGNESIUM-DEFICIENT DIET\*

Constituents	%	Salt Mixture	%	Mineral Content Calculated mg/100
Casein .....	24.2	CaCO <sub>3</sub>	45	Ca 1197
Gelatin .....	2.5	NaCl	20	P 372
Methionine .....	.3	K <sub>2</sub> HPO <sub>4</sub>	18	Na 472
Dextrose .....	60.	CaHPO <sub>4</sub>	12.2	K 487
Corn oil .....	5.	Fe-citrate	4.	
Salt mixture .....	6.	MnSO <sub>4</sub>	0.6	
Vitamin mixture** .....	2.	KI	0.13	
		Zn-carbonate	0.035	
		CuSO <sub>4</sub>	0.035	

\* Control diet is supplemented with 800 mg MgSO<sub>4</sub>/100 g of diet.

\*\* Obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

TABLE II  
EFFECT OF VARIOUS SINGLE MINERAL DEFICIENCIES ON PLASMA HISTAMINE IN THE RAT

Diets and Groups	Plasma Histamine μg/100 ml Plasma	
	6 Days*	12 Days*
Control diet .....	1.5 ± .14	1.6 ± .15
Mg deficient .....	14.1 ± 1.8**	20.6 ± 3.1**
Ca deficient .....	2.0 ± .11	1.9 ± .17
K deficient .....	2.1 ± .20	2.2 ± .18
Na deficient .....	1.8 ± .14	2.1 ± .14
Fe deficient .....	1.9 ± .16	1.6 ± .12

\* 6 rats per group.

\*\* P < 0.01.

Bouin-Holland and stained with May-Grunwald and hematoxylin-phloxine-orange G for histological study.

### Results

**Effect of Single Mineral Deficiencies.** Except for magnesium, as shown in Table II, none of the mineral deficiencies studied produced any change in plasma histamine or peripheral vasodilatation. On the other hand, the increase in histamine observed in the magnesium-deficient group in comparison with other groups was highly significant.

TABLE III  
EFFECT OF DEFICIENCIES IN VARIOUS MINERALS ON SERUM CALCIUM AND MAGNESIUM AND PLASMA HISTAMINE IN RATS

<i>Groups* and Diets*</i>	<i>Bodyweight Gm/g</i>	<i>Ca mg/100 ml</i>	<i>Mg mg/100 ml</i>	<i>Histamine μg/100 ml Plasma</i>
I Control diet .....	+ .88	11.0 ± .34	2.3 ± .1	1.4 ± .12
II Mg-deficient diet** .....	+ .51	12.8 ± .16	0.8 ± .07	12.8 ± 2.3
III CaCO <sub>3</sub> as only mineral .....	- .9	8.8 ± .29	1.7 ± .04	1.1 ± .86
IV CaHPO <sub>4</sub> as only mineral .....	- .8	9.4 ± .86	2.0 ± .02	1.7 ± .17
V K <sub>2</sub> HPO <sub>4</sub> as only mineral .....	+ 18	6.9 ± .30	1.3 ± .06	3.1 ± .1
VI NaCl as only mineral .....	+ 1	5.6 ± .43	2.0 ± .02	1.5 ± .21
VII CaCO <sub>3</sub> + CaHPO <sub>4</sub> as only mineral .....	- .5	10.1 ± .18	1.9 ± .11	1.9 ± .11
VIII CaCO <sub>3</sub> + CaHPO <sub>4</sub> + K <sub>2</sub> HPO <sub>4</sub> ** as only mineral .....	+ .27	11.1 ± .22	0.7 ± .03	7.0 ± 1.2
IX CaCO <sub>3</sub> + CaHPO <sub>4</sub> + K <sub>2</sub> HPO <sub>4</sub> * as only mineral .....	+ .42	11.4 ± .43	0.7 ± .09	10.2 ± 2.1

\* 8 rats per group. Diets of groups III-IX contained normal amounts of casein, corn oil, dextrose and vitamin mixture.

\*\* Peripheral vasodilatation in all animals.

TABLE IV

## EFFECT OF PARATHYROIDECTOMY ON SERUM Ca AND Mg AND PLASMA HISTAMINE IN Mg-DEFICIENT RATS

<i>Groups* and Diets</i>	<i>Vasodi-latation</i>	<i>Ca mg/100 ml</i>	<i>Mg mg/100 ml</i>	<i>Histamine µg/100 ml Plasma</i>
Sham-operated, control diet . . . . .	0	10.2 ± .19	2.4 ± .03	1.8 ± .17
Sham-operated, deficient diet . . . . .	+++	11.9 ± .27**	0.6 ± .08	9.9 ± 1.0**
Parathyroidectomy, control diet . . . . .	0	8.2 ± .49	2.0 ± .12	2.6 ± .14
Parathyroidectomy, deficient diet . . . . .	0	7.6 ± .28	0.5 ± .12	3.8 ± .29

\* 6 rats per group.

\*\* P &lt; 0.01.

**Effect of Deficiencies in Various Minerals.** The results are reported in Table III. Magnesium-free diets containing only one of the following: calcium, calcium phosphate, potassium phosphate or sodium chloride, did not induce any increase in plasma histamine. Furthermore, serum magnesium in these groups was close to the level seen in control animals. Histamine increase and peripheral vasodilatation developed only with diets containing mixtures of calcium phosphate and potassium in the absence of magnesium; serum magnesium also dropped to deficient levels under these dietary conditions.

**Effect of Parathyroidectomy.** The results shown on Table IV reveal that parathyroidectomy significantly reduced the increase of plasma histamine produced by the magnesium deficiency. Peripheral vasodilatation was not observed in this group. The increase in blood calcium produced by the deficient diet was abolished by parathyroidectomy. Serum magnesium did not appear to be influenced by removal of the parathyroid gland.

In the second part of the experiment, three rats in the non-parathyroidectomized group died after 95 days on the magnesium-deficient diet. A large 2.8 g tumor of the thymus was found in one of them (Figs. 1 and 2). Four rats of this group had died earlier of convulsive seizures and the two surviving animals were killed at the end of the experiment. They exhibited pleural effusion and ascites associated in some cases with pitting white oedema of the paws and a large oedematous tongue. No other tumor was found in this group. In the parathyroidectomized

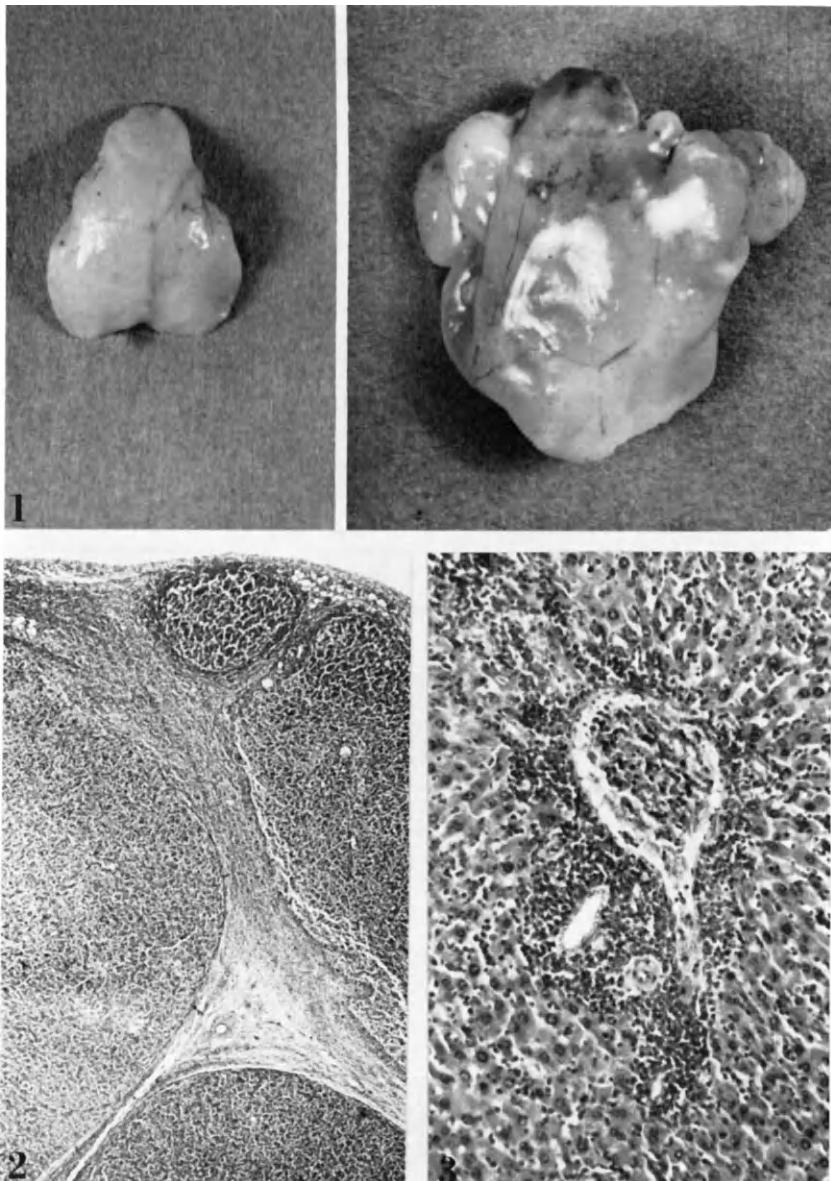


FIGURE 1. On the left, thymus of control animal fed the magnesium-supplemented diet for three months. On the right, tumor of thymus found after the same period on the magnesium-deficient diet.

FIGURE 2. Low power view of the tumor. Note complete replacement of normal architecture of the thymus by the neoplastic cells. May-Grunwald  $\times 50$ .

magnesium-deficient group, one rat died after  $3\frac{1}{2}$  months; this animal presented an enormous tumor of 5.2 g, which extended into the thoracic cavity and had eroded the large blood vessels, producing massive hemorrhage. Numerous metastases (Fig. 3) and leukemia were found. The lymphoid tumor was of the same type as the one observed in the non-parathyroidectomized animal. Histological sections of the thyroid region did not reveal the presence of any parathyroid remnant. No tumors were seen in any of the other animals.

### Discussion

The present investigations show that, among the various mineral deficiencies tested, only lack of magnesium can produce an increase in plasma histamine with peripheral vasodilatation in rats. However, dietary deficiency of magnesium *per se* is not sufficient to cause these symptoms. The histamine increase with peripheral vasodilatation can be reproduced only when potassium, calcium and phosphate are present in the magnesium-deficient diet. Plasma magnesium also reaches its lowest levels under these conditions. Previous investigations had shown that lack of carbohydrates and lipids in the diet did not interfere with the peripheral vasodilatation of magnesium deficiency (9). Although only a limited number of mineral mixtures deficient in magnesium have been investigated in this study, it appears that dietary potassium and calcium are important factors in the development of vasodilatation, potassium probably being the more important element. Schrader *et al.* (49) and Forbes (20) observed that rats fed a diet deficient in both magnesium and potassium showed no erythema or peripheral vasodilatation. Delayed and variable hyperemia was also reported with a similar diet (41, 51). On the other hand, the association of high potassium with low magnesium accelerated the onset of magnesium-deficiency symptoms according to Colby and Frye (14) and Seta *et al.* (51). The level of dietary phosphorus does not appear to affect the rate of appearance of the symptoms of magnesium deficiency (12), but Forbes (19) did not observe any erythema with a diet low in calcium and phosphate as well as magnesium. It might be concluded from these observations that the presence of normal or

←

FIGURE 3. Higher power showing metastatic infiltration of portal space of the liver in parathyroidectomized rat with thymic lymphosarcoma; numerous neoplastic cells are also present in the blood of the portal vein. HPO  $\times 120$ .

larger than normal amounts of potassium, calcium and phosphate in the diet in the absence of magnesium is one of the causal factors in the development of peripheral vasodilatation. The amount of food intake is probably also involved (W. S. Hartroft, personal communication).

Serum calcium is increased by magnesium deficiency, as has already been reported by other workers (1, 2, 23, 24, 35, 40, 52, 62), and this hypercalcemia is abolished by parathyroidectomy (23, 24, 34). Our present results concur with these observations. After parathyroidectomy, the low serum calcium of magnesium-deficient rats was associated with a decreased plasma histamine without vasodilatation. Conversely, the non-parathyroidectomized animals with limited mineral supplements, where peripheral vasodilatation was observed, showed an increase in serum calcium as well as in plasma histamine. These findings suggest that peripheral vasodilatation and increased plasma histamine in magnesium-deficient rats are related to some extent to the increase in serum calcium. It should be stated, however, that the inhibitory effect of parathyroidectomy on plasma histamine as well as peripheral vasodilatation is related to the age of the animals. In young rats, such an effect is less significant (9).

The development of tumors of the thymus in magnesium-deficient rats is still difficult to explain. Dietary deficiencies in general induce an involution of lymphoid tissue rather than a proliferation through the non-specific activation of the hypophysio-adrenal axis. On the other hand, in the case of magnesium depletion, adrenal secretion of corticosterone, rather than being stimulated, is decreased (21, 47) and secretion of aldosterone, a corticoid with antithymolytic activity (50), is increased (21). Therefore, tumors of the thymus develop in animals under the influence of high aldosterone secretion (21) and increased parathyroid activity (23, 24, 34, 39). The possible influence of such hormonal stimulation on tumor induction is not known.

Recent electron microscopic investigations have shown that viral-like particles are present in some of these tumors; however, transplantation by cell-free extracts has not been successful to-date (10, 11). Another hypothesis could be that some relationship might exist between the disturbances in serum calcium during magnesium deficiency and the mitotic activity of lymphoid cells. The recent observations of Perris and Whitfield (46) revealed that injected calcium could induce a marked stimulation of mitotic activity in thymocytes of the rat. If this change occurs in the thymocytes of magnesium-deficient rats, it could be related to the eventual tumor development. On the other hand, the

presence of a tumor in one of our parathyroidectomized animals would not support this contention. It should be noted that, except for the hypocalcemia recorded on the 14th day, we do not have any data on the level of serum calcium in this animal at the end of the experiment. The possibility of the activation of a small ectopic gland cannot be excluded, although no hyperemia was observed.

Finally, since parathyroidectomy abolished the substantial increase in histamine with vasodilatation during the early phase of magnesium deficiency, this phenomenon would not appear to be an essential factor for the later development of tumors of the thymus.

### Abstract

Dietary magnesium deficiency was studied in intact and parathyroidectomized rats with regard to peripheral vasodilatation and the development of thymic tumors. Serum calcium and magnesium as well as plasma histamine were measured and the effects of diets containing various salt mixtures were investigated.

The presence of calcium phosphate and potassium in the absence of magnesium in the diet is one of the causal factors in the development of peripheral vasodilatation and increase in plasma histamine; other salt mixtures or mineral deficiencies do not have such an effect.

Parathyroidectomy significantly reduced the increase in serum calcium and plasma histamine produced by magnesium deficiency; peripheral vasodilatation was not observed under these conditions. Conversely, vasodilation is related to some extent to the increase in serum calcium after magnesium depletion.

A thymic lymphosarcoma was found at the end of the experiment in one of the intact and one of the parathyroidectomized rats on the magnesium-deficient diet. It would appear that the substantial increase in histamine with vasodilatation during the early phase of magnesium deficiency is not an essential factor for the later development of tumors of the thymus.

### Abrégé

La vasodilatation périphérique et les tumeurs du thymus produites par la diète carencée en magnésium, ont été étudiées chez le rat normal et parathyroïdectomisé. Les effets de diètes contenant divers mélanges de minéraux ont été analysés d'après les variations du calcium et du magnésium sérique ainsi que de l'histamine plasmatique.

Une des causes de l'apparition de la vasodilatation périphérique et de l'augmentation de l'histamine plasmatique, est la présence de calcium, de phosphate et de potassium dans la diète dépourvue de magnésium. On ne retrouve pas d'effets semblables avec d'autres mélanges de minéraux dans

la diète. Une diminution significative de l'augmentation du calcium sérique et de l'histamine plasmatique, induite par la carence en magnésium, a été observée après la parathyroïdectomie. La vasodilatation périphérique n'apparaît pas dans ce cas. Inversement, la vasodilatation dépend dans une certaine mesure de l'augmentation du calcium sérique durant la carence en magnésium.

Un lymphosarcome thymique a été observé à la fin de l'expérience chez l'un des animaux normaux et l'un des parathyroïdectomisés ayant reçu la diète carencée en magnésium. Il semblerait que l'augmentation marquée de l'histamine avec vasodilatation durant les premières semaines de la carence en magnésium, ne soit pas un facteur essentiel au développement ultérieur de tumeurs du thymus.

### Acknowledgements

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**P. Jean:** Doctor Bois has presented us with interesting results underlining the importance of dietary potassium, calcium and phosphate, as well as of serum calcium, in the development of peripheral vasodilation in magnesium deficient rats. In addition, his results suggest that an early increase in plasma histamine is not essential for the development of thymic tumors. Those findings furnish us with another chapter in the experimental pathology of magnesium deficiency. Among the preceding chapters, many were written by Doctor Bois, who has combined the classical morphological approach with a more refined methodology: the study of mast-cell behavior, determination of blood, urine and tissue histamine, measurement of serum calcium and magnesium, histochemistry and electron microscopy have all contributed to a better understanding of magnesium deficiency. The production of thymic tumors has held our attention. In his first description of such tumors (Bois, P.: *Nature*, 204:1316, 1964), Doctor Bois proposed the following hypothesis: the chronic lack of magnesium at the cellular level might increase the removal of magnesium out of the nucleus into the cytoplasm, thus causing chromosomal aberrations and cell mutation. In order to examine this possibility, chromosome analyses of 5 primary thymic tumors were made and the results are listed in Table I. Almost

TABLE I  
CHROMOSOMES OF PRIMARY THYMIC TUMORS  
IN MAGNESIUM-DEFICIENT RATS

Tumor	Number of Cells	Percentage of Cells Containing the Indicated Chromosome Counts				
		40	41	42	43	44
T4a	8	0	0	100	0	0
T5a	36	3	9	88	0	0
T4	93	0	5	95	0	0
T5	124	3	8	88	0	1
T6	35	0	6	94	0	0

all the cells have 42 chromosomes, which is the normal diploid number for the rat. The only morphological change was seen in tumor T5, which is characterized by the presence of a marker chromosome (Fig. 1). It was concluded that major chromosomal changes are not necessary for the induction of thymic tumors in magnesium-deficient rats. However, any minor changes or genic mutations would have escaped our

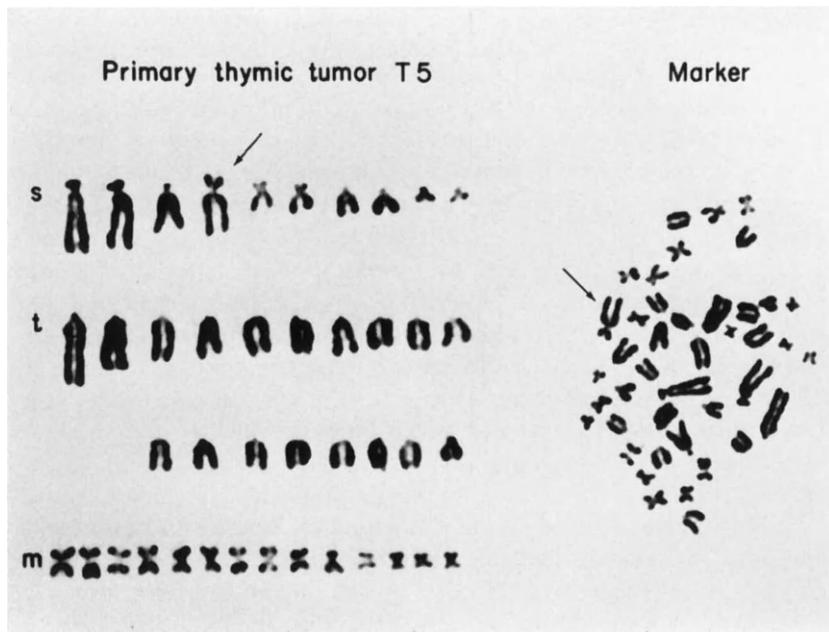


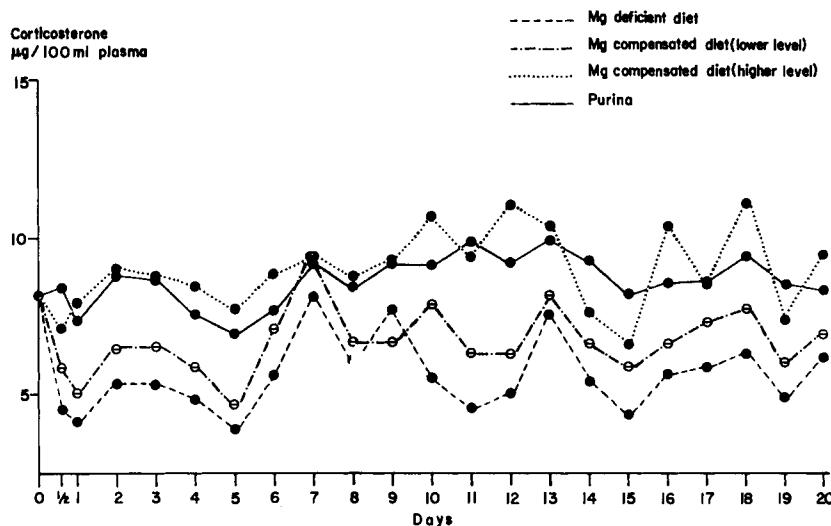
FIGURE 1. Karyotype of thymic tumor T5. Note the presence of a marker chromosome (arrow) replacing one chromosome  $s_2$ .

attention. A study of the literature (Jean, P., and P. Bois: *Rev. Can. Biol.*, 26:207, 1967) shows that tumors of viral origin in rats, mice, rabbits and hamsters are mostly diploid, while tumors produced by chemical, physical or hormonal agents are mostly aneuploid. Thus, the results of our cytogenetic study are compatible with the presence of virus-like particles observed in some of the thymic tumors of magnesium-deficient rats.

Corticosterone secretion and eosinophilia during magnesium deficiency were studied by Doctor Richer and Doctor Veilleux, respectively. Those two workers happen to be with us today and I hope they will agree to make comments on that subject in the course of the general discussion.

**C.-L. Richer:** I welcome this occasion to present a slide that illustrates our latest results on corticosterone levels in magnesium deficiency, as Doctor Jean has just mentioned. The solid line represents the daily corticosterone levels of the Purina fed animals; the interrupted line the levels found in the animals receiving the magnesium-deficient diet described by Bois. The corticosterone levels are decreased in the deficient group. This decrease is statistically significant for every experimental day except for the ninth. On this day, and to a lesser degree on the seventh and eighth days, individual measurements show

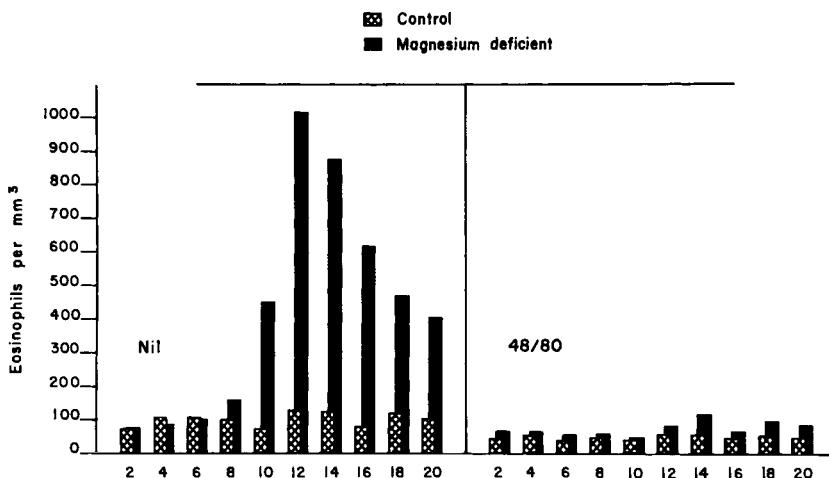
EFFECT ON RAT PLASMA CORTICOSTERONE  
OF VARIOUS LEVELS OF DIETARY MAGNESIUM



that, in most animals, corticosterone is discharged into the plasma for a very short period of time. The summation of a large number of results, not always coinciding, tends to flatten the curve and to mask this transient rise. Besides the results obtained from the magnesium-deficient group and the Purina fed animals, we see the mean plasma corticosterone levels of two control groups receiving synthetic diets containing respectively 600 mg and 1 g of magnesium sulphate per 100 g of diet. The results obtained with the diet containing the lower level of supplement illustrate a second point that we consider important, namely, that a diet containing a supplement sufficient to prevent magnesium-deficiency symptoms may still be inadequate to restore the adrenal function to normal levels.

**R. Veilleux:** In addition to Dr. Richer's studies on corticosterone, we have studied the eosinophilic response of magnesium deficiency and found that, like hyperemia, it is completely abolished by the administration of 48/80 for a period of 20 days prior to the deficiency state. This is illustrated in the following slide (technical details of experimental procedure can be found in *Rev. Can. Biol.*, 25:241, 1966). It therefore seems quite clear that the three main features of magnesium deficiency in the rat, namely: histamine discharge, hyperemia and eosinophilia, are perfectly synchronous and intimately linked with one another.

**ACTION OF 48/80 ON THE EOSINOPHILIA  
OF MAGNESIUM DEFICIENCY IN THE RAT**



The nature of this link is not yet completely understood, but the chain of events would seem to take place in the following manner: For some as yet unknown reasons, magnesium deficiency provokes mast-cell degranulation and histamine is discharged into the tissues. This leads to peripheral vasodilation and marked eosinophilia. When a critical level of histamine is reached it would trigger an adrenal response of very short duration, which abruptly abolishes both the eosinophilic and the hyperemic response. Thereafter, the histamine level returns to approximately normal values.

## Action of Hormones on the Progression of Magnesium Deficiency Syndrome in Rats

G. JASMIN\*

THE essentiality of magnesium in biological processes has been recognized for a number of years (34). Acute magnesium deprivation by dietary means in laboratory animals was shown to interfere with normal body growth and to generate neuromuscular disorders and eventual generalized convulsions, better known as "magnesium tetany" (26). Early signs of magnesium deficiency comprise one additional feature that seems specific to the rat. Depending upon their age, rats kept on a sufficiently low magnesium diet develop, within 4 to 8 days, a hyperemic cutaneous reaction most evident in the acral regions, which may subside in a recurrent fashion. Concomitantly, dermal mast cell degranulation occurs, indicating that the hyperemic phenomenon results from histamine liberation (4). Experimental magnesium deficiency in rats also elicits a rise in blood leukocytes with a predominance of eosinophils during the hyperemic phase (17).

The symptomatology of acute magnesium deficiency in rats has been well explored since the original description of Kruse *et al.* (26). Besides the early erythema, leukocytosis, and nervous disorders that end in convulsions and death, a series of regressive lesions are particularly evident at the level of the kidney, myocardium, and dermo-epidermic structures. The pathology of chronic magnesium deficiency in rats, on the other hand, has been little investigated for the reason that young animals receiving a regimen lower in magnesium than 1 mg per 100 g cannot survive longer than 22 to 30 days; they all succumb to tetanic seizures. By raising the magnesium content of the diet to a level of 2 mg per 100 g (14), or else by using adult rats over 200 g initial body weight, it is possible to carry

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\* Research Associate of the Medical Research Council of Canada.

out long-term experiments with little or no convulsive episodes, but the acute signs of magnesium deficiency do not occur and, except for a more or less severe nephrotic change, no untoward effects are apparent.

In the course of our experiments on wound healing during magnesium deficiency in rats (19, 21) we were able to observe that dextrose or sodium chloride given in the drinking water as a 1% solution or the daily oral administration of calcium gluconate during the third week of magnesium deficiency greatly alleviates the neuro-muscular symptoms and, thereby, increases the survival rate of the animals. It is the object of the present report to describe the progression of the magnesium deficiency syndrome with special reference to the chronic pathologic changes, including the development of thymic tumors, and the influence of endocrine glands on the development of these manifestations.

### Materials and Methods

The experiments were carried out with female Sprague-Dawley rats (Sprague-Dawley Farm, Madison, Wisconsin) with an initial body weight varying between 70 to 100 g. They were fed a magnesium-deficient diet supplied by Nutritional Biochemical Corp., Cleveland, Ohio. The two major components of this synthetic diet are dextrose (58%) and casein (25%), supplemented with oil, essential minerals, and vitamins; the magnesium content does not exceed 1 mg per 100 g of diet. The animals were caged in a quiet room, and were given distilled water to drink, in which was added dextrose or sodium chloride (reagent grades) in a 1% concentration.

Glandular extirpations were performed under ether anesthesia. The pituitary gland was removed by suction after trephining, using the parapharyngeal route. Somatotrophic hormone (STH) (Endocrinology Study Section, Bethesda) was injected subcutaneously in the form of an aqueous suspension, once daily, at the dose of 2 mg for the duration of the experiment. The adrenals were excised through the standard lumbar approach, a substitution dose of 0.5 mg of desoxycorticosterone acetate (DOCA) (Ciba, Montreal) or cortisol acetate (Pfizer Canada)

in the form of a microcrystal aqueous suspension being injected subcutaneously, once daily, for the duration of the experiment. The parathyroids were selectively destroyed with a thermocautery after blunt dissection of the upper poles of the thyroid gland. Thyroidectomy (including the parathyroids) was carried out surgically, taking care not to damage the recurrent nerve. Thyroxine (Tx) (L-Thyroxine Sodium, British Drug Houses) was injected subcutaneously in the form of an aqueous suspension, once daily, at a dose of 0.5 µg for the duration of the experiment. Adrenal enucleation was performed through a small incision made in the capsule of the gland, using negative pressure to extrude the glandular tissue. Except for the hypophysectomized rats, which were used 8 days postoperatively, all other animals deprived of their endocrine glands were given the magnesium-deficient diet 48 hours after surgery. Data pertaining to the number of animals in each group and the duration of experiments are given in the appropriate section of the Tables of results. The rats were weighed once a week; the erythema was recorded daily, according to an arbitrary scale of 0 to 4, the highest value corresponding to the maximal possible reaction, both in intensity and extent.

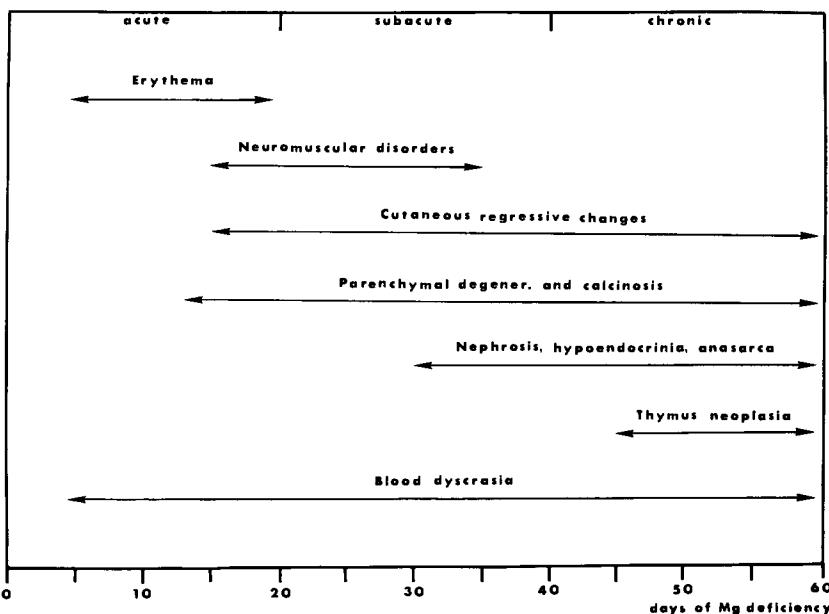
Blood cytology was performed according to the standard methods used in clinical practice. Each sample was obtained by making a small incision at the level of the tail. Eosinophil counts were carried out on blood diluted 1:20 in a WBC pipette with phloxine diluting fluid (phloxine 0.5 g, propylene glycol 50 ml, distilled water 50 ml) and cells were counted under a microscope, using a double-sided 0.2 mm Fuchs-Rosenthal counting chamber. Differential white blood cell counts were made on smears stained with May-Grunwald Giemsa. At autopsy, organs were fixed in formalin or Susa solution, sectioned in paraffin, and stained with hematoxylin-phloxine-saffron (HPS), azure B and eosin, Sudan black B and the Von Kossa silver reaction for calcium.

## Results

### *Clinical State*

The chronology of the symptoms of magnesium deficiency is outlined in Figure 1. Depending upon their occurrence and

Fig 1. OCCURRENCE OF MAGNESIUM DEFICIENCY SYMPTOMS IN RATS



main duration, the pathologic events were grouped under three successive periods of 20 days, which were designated acute, subacute, and chronic stages, respectively.

The erythema reaction develops after 4 days of magnesium deprivation (Table I) and culminates in the majority of animals between the 8th and 10th day; it progressively disappears during the following 10 days, with some periodic recrudescence. The redness is more prominent in the ears, the paws, and the tail, but involves the entire cutaneous surface. This allergic-like dermatitis is accompanied by intense itching and a striking rise in blood eosinophils (Table II).

With regression of the erythema, the animals become more and more erethistic and soon develop convulsive seizures, mainly during the daytime, when overmanipulated or subjected to an audiogenic stimulus. The most critical period occurs between the 20th and 30th day, during which time a high proportion of animals die of suffocation as a result of repeated convulsive seizures. The most efficient palliative measure consists in the

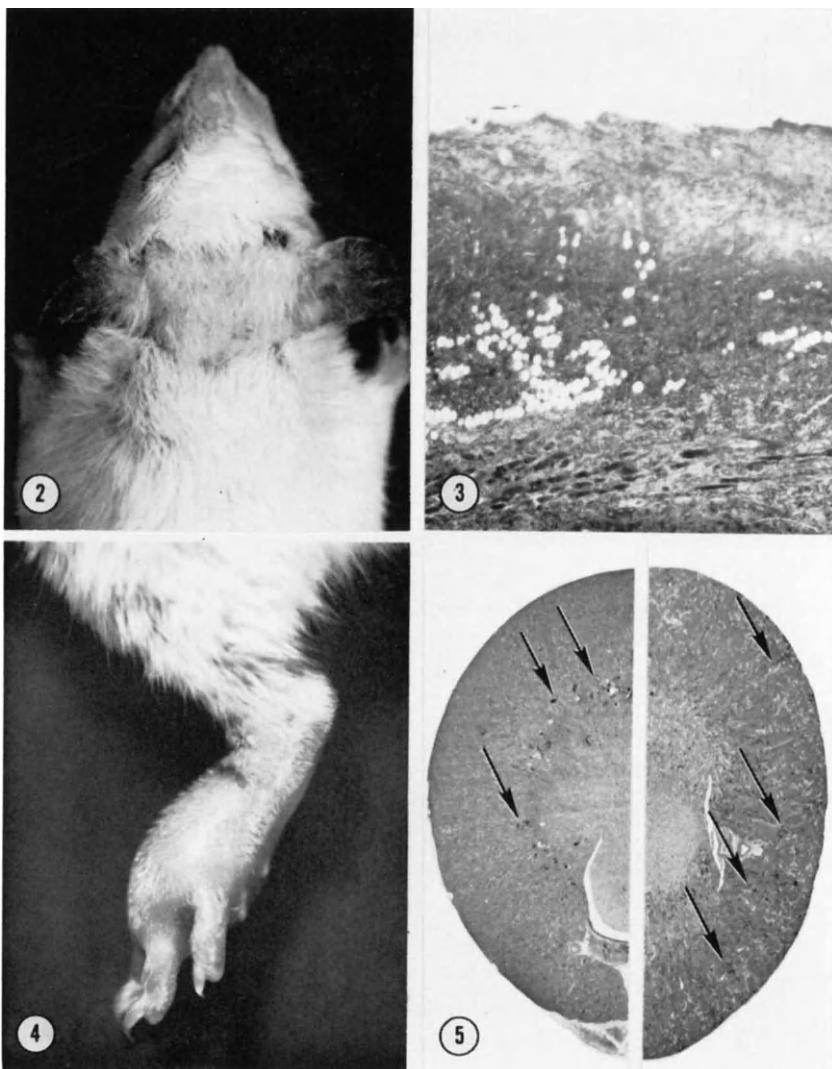


FIGURE 2. Cutaneous regressive changes in the region of the scalp in a rat after 30 days of magnesium deficiency. In this particular region, the skin becomes atrophic more rapidly, as evidenced by the hair loss, the shrivelled aspect and crust formation.

FIGURE 3. Dermo-epidermic degenerative changes characterized by the disappearance of the epithelial layer, hair follicles, and sebaceous glands, with infiltration of plasma and polynucleated cells. HPS  $\times 80$ .

FIGURE 4. Swollen hind paw in a rat after 40 days of magnesium deficiency. The edema reaction will sustain until the death of the animal.

systematic administration of a concentrate of dextrose and gelatin by stomach tube at mid-day. Calcium gluconate (Sandosten, Sandoz, Montreal) given by the same route, as a 10% solution, also proved to be beneficial. It was further observed that the mere fact of increasing the water intake was salutary, as was simultaneous augmentation in food consumption. Sodium chloride or dextrose in a 1% concentration was therefore added to the drinking water in most of our experiments.

During the subacute phase of experimental magnesium deficiency, the fur of the animals becomes progressively thinner with evidence of a defect in the regenerating capacity. The hair loss is particularly prominent in the region of the scalp (Fig. 2) and the neck; the epilated areas show multiple, well-circumscribed and adherent crusts that, in many instances, become generalized throughout the skin. Such cutaneous regressive changes (Fig. 3) parallel other parenchymal degenerative lesions in the liver, kidney, and myocardium, which will be discussed later.

With the disappearance of convulsions, the animals become progressively lethargic and edematous (Fig. 4); this third phase, properly designated the "nephrotic phase," gives rise to the chronic signs of magnesium deficiency. It is characterized by an arrest in body growth, water retention, anemia, and regressive tissue changes with lowered resistance to infection. More severely affected animals will die earlier with liver steatosis or multiple abscesses in the lungs and the liver.

With sufficient care, it is possible to prolong the survival of the animals up to 90 days, but a high proportion will die on or about the 65th day (see second group in Table IV) with respiratory insufficiency and severe anasarca. The subcutaneous tissue of the declivous regions is highly edematous and both the peritoneal and thoracic cavities are filled with fluid that is often milky in appearance. This fluid, when stained as a film with



FIGURE 5. Nephrocalcinosis at two different stages of magnesium deficiency.  
a) *On the left:* between the 20th and 30th day, the calcific deposits more often locate at the cortico-medullary junction (*arrows*). b) *On the right:* after 60 days or more, the degenerative changes and calcinosis involve the cortical area (*arrows*). HPS  $\times 10$ .

Giemsa, shows a large amount of medium-sized lymphocytes and a few erythrocytes. But the most striking finding upon opening the thoracic cavity is the presence of a tumorous mass that fills the entire mediastinum; by its texture and color it can easily be identified as the thymus gland.

### ***Pathologic Findings***

Among the animals that die from convulsions or are killed during the sub-acute stage of magnesium deficiency, a good number show myocardial or renal degenerative calcifying lesions.

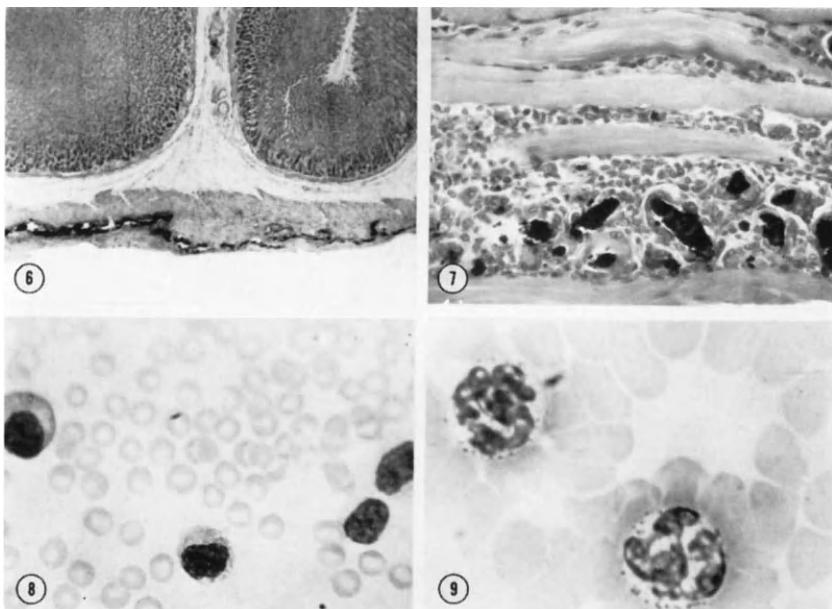


FIGURE 6. Low magnification of the calcific degenerative changes often visible at the level of the longitudinal outer muscle layer of the stomach during magnesium deficiency. HPS and Von Kossa  $\times 100$ .

FIGURE 7. Skeletal muscle calcinosis with formation of giant-cell granuloma in the gastrocnemius during experimental magnesium deficiency. HPS and Von Kossa  $\times 200$ .

FIGURES 8 and 9. Peripheral blood smears from thymic-tumor-bearing rats at a late stage of magnesium deficiency. *Left side:* atypical lymphocytes similar to those seen in viral diseases. Giemsa  $\times 1000$ . *Right side:* immature basophilic leukocytes similar to those found in myelocytic leukemia. Giemsa  $\times 1800$ .

The calcific deposits are often detectable with the naked eye, either at the apex or the base of the heart; the renal lesions, on the other hand, are apparent only after sectioning the organ in the hilar region and are usually located in the cortico-medullary junction (Fig. 5a). Microscopic examination of the kidney slices reveals that the ascending limb of Henle is primarily involved; however, at later stages the nephrocalcinosis extends to other segments of the nephron, namely the proximal tubules in the cortical region (Fig. 5b). Several other sites of dystrophic calcinosis are detected in the course of chronic magnesium deficiency. These are, by decreasing frequency, the gastrointestinal tract, the skeletal muscle, the skin, the lungs, the adrenals, and the ovaries (Figs. 6, 7, 19 and 20).

There is a great deal of difference in the size and weight of the thymic tumors; they may vary between 1.2 to 9.9 cm<sup>3</sup> and weigh up to 3.3 g. It seems obvious that the neoplastic transformation of the thymic glands occurs stepwise in such a way that the tumor changes may be limited to one lobule (Figs. 10 and 12) or else involve the entire right or left lobe with little or no visible alterations in the contralateral part of the gland. In the majority of the cases, however, especially when animals survive more than 60 days, the thymus is wholly neoplastic (Fig. 11). Depending upon the degree of anemia, the tumor, which occupies almost half of the thoracic cavity, is more or less yellow in color, well encapsulated, and little adherent by its anterior surface to the sternum. On the other hand, the posterior structures, namely the trachea, the esophagus, and aorta (Fig. 13), are embodied in the neoplasm, which often infiltrates the mediastinum and in some instances the parietal pleura and intercostal muscles.

Histologically, the tumors exhibit different cellular patterns all consistent with the various maturation stages of lymphopoiesis (37); these could be related to the progression of the neoplastic process. The cytologic characteristics of the lymphoid cells are best evidenced with the azure B-eosin staining method. The circumscribed or lobular type of tumors are composed essentially of uniform sheets or large lympho-reticular cells with a pale nucleus, prominent nucleolus, and an intensely basophilic cyto-

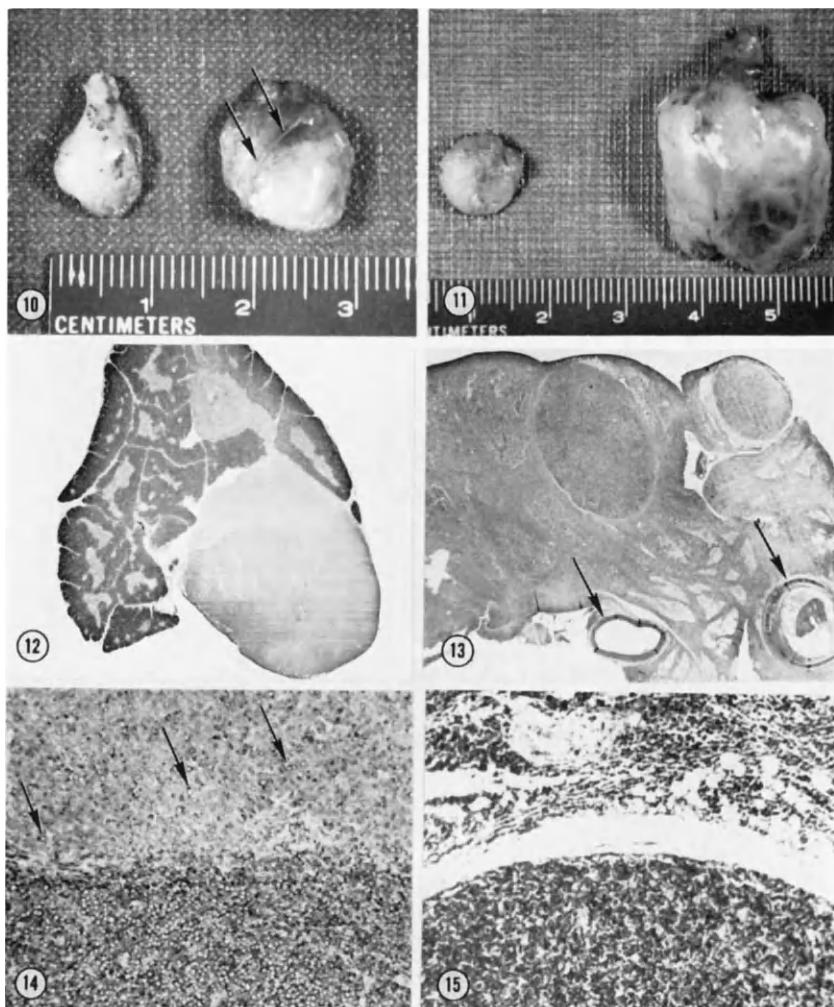


FIGURE 10. Macroscopic appearance of a thymic tumor found in a rat killed after 45 days of magnesium deficiency. In comparison with a normal gland (*left*), note that the tumor growth is restricted to the left lobe (*arrows*). FIGURE 11. Macroscopic appearance of a wholly neoplastic thymus in a rat that died after 65 days of magnesium deficiency. The neoplastic thymus on the right side is approximately 10 times larger than the normal, partly involuted gland on the left.

FIGURE 12. Low magnification of the thymic tumor shown in Figure 10. At this stage, the neoplastic lesion is lobular in nature. HPS  $\times 8$ .

FIGURE 13. Low magnification of the thymic tumor shown in Figure 11. The

plasm having a polygonal outline. The mitotic figures in these early forms of tumors are numerous and very few vessels and connective tissue elements can be evidenced. Similar immature cells tend to infiltrate the surrounding non-neoplastic lobules (Fig. 14) and can also be recognized in the cortical zone of all the other lobules of the residual gland; they are usually absent in the medulla.

A second variety of tumor, which has reached a higher degree of maturation, exhibits in a nodular arrangement (Fig. 13) and lymphoid cell proliferation seems less rapid as judged by the low mitotic index; the nodules are well-partitioned by connective tissue septa infiltrated with scattered plasma cells, histiocytes, and mast cells. These circumscribed areas are variable in size, well-vascularized, and often infiltrated by adipose cells most probably deriving from the perithymic brown fat. The lymphocytic cells are assembled in a more or less compact fashion (Fig. 15) and exhibit all intermediate stages of maturation, ranging from the typical lymphoblast with light chromatin and more or less abundant cytoplasm up to the medium type of lymphocytes with a smaller and darker nucleus and scanty cytoplasm. It appears that the capacity for differentiation of the cells cannot exceed this stage. There are very few reticular cells in these nodular formations.

Other lymphatic organs are found to be atrophic. In a large proportion of the tumor-bearing animals, the lymph nodes as a rule are small and fibrous, their cortical follicles being disorganized and the sinuses filled with plasma cells. The splenic parenchyma also exhibits various degrees of fibrotic changes,

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neoplasm invades the surrounding structures, such as the trachea and esophagus (*arrows*) and has a nodular aspect. HPS  $\times 25$ .

FIGURE 14. Higher magnification of the early lobular type of thymic tumor seen in Figure 12, in a junctional region between normal and neoplastic tissue. Note that the dark lympho-reticular cells, with a pale nucleus from the neoplastic lobule, infiltrate the cortical region of a normal lobule (*arrows*). Azure B-eosin  $\times 125$ .

FIGURE 15. Homogeneous aspect of dark nucleated lymphoblastic cells in a tumor nodule and a stromal tissue of the well-developed thymic neoplasm seen in Figure 13. Azure B-eosin  $\times 125$ .

TABLE I  
ACTION OF GLANDULAR EXSTIRPATION AND HORMONAL SUBSTITUTION TREATMENT UPON THE DEVELOPMENT OF ERYTHEMA  
IN MAGNESIUM-DEFICIENT RATS

Groups	Number of Rats	Number of Reactors	Erythema			
			Average Day of Eruption	Ultimate Day of Incidence	Mean Duration (Days)	Mean Severity 4 Days after Onset (Grade 0-4)
Control .....	14	0				
Deficient intact .....	20	20	4	30	15	3.0
Deficient Pthy X .....	16	15	8	32	17	2.0
Deficient Thy-Pthy X .....	20	10	6	47	5	0.4
Deficient Thy-Pthy X + Tx* .....	18	18	4	26	9	3.0
Deficient Adr X + DOCA* .....	12	12	4	45	16	2.3
Deficient Adr X + cortisol* .....	8	3	46	53	4	1.0
Deficient Adrenoc. ....	9	9	6	17	8	2
Deficient Hypo X .....	12	8	19	36	3	3.3
Deficient Hypo X + STH* .....	8	8	4	30	22	0.7
						4

\* Tx: 5  $\mu$ g sc/day; DOCA and cortisol: 0.5 mg sc/day; STH: 2 mg sc/day.

depending upon the severity of anemia in the deficient animal. The white pulp is hypoplastic and the red pulp is loaded with hemosiderin cells and erythroblasts. The liver, apart from being congested, is occasionally infiltrated with large mononucleated cells of the lymphoblastic or erythroblastic series, in the periportal areas and rarely in the sinusoids.

#### ***Endocrine Action Upon Development of Erythema***

In order to determine the influence of internal secretions upon the development and magnitude of the erythema reaction resulting from magnesium deficiency, an assessment was made on the basis of the different criteria outlined in Table I. In comparison with the intact rats, the number of reactors was reduced by 50% following thyro-parathyroidectomy, by 60% following adrenalectomy and cortisol substitution, and by 35% following hypophysectomy. Moreover, in the two latter groups, the onset of eruption was considerably delayed and when erythema did appear it was of low grade severity, the lowest values being recorded in the thyro-parathyroidectomized animals. On the other hand, substitution therapy with either thyroxine, STH or DOCA entirely restored the sensitivity of animals to levels comparable to intact magnesium-deficient rats. Interestingly, adrenal-enucleated rats proved to be more reactive than the intact deficient rats, with a resultant shortening in the duration of erythema.

#### ***Endocrine Action Upon White Blood Cell Count***

The blood cellular changes listed in Table II concern the eosinophil counts, which were made on the 5th and the 10th days as they could be related to the occurrence and disappearance of the erythema. Also included are the differential leukocyte counts, which were made on the 10th day of magnesium deficiency.

As was to be expected on the basis of previously published reports (23), there was a marked rise in blood eosinophils that coincided with the erythema reaction occurring in intact magnesium-deficient rats. The high values recorded on the 5th day

TABLE II  
ACTION OF GLANDULAR EXTRIPATION AND HORMONAL SUBSTITUTION TREATMENT ON  
WHITE BLOOD CELL COUNT IN MAGNESIUM-DEFICIENT RATS

Groups	Number of Rats	Eosinophil Count / mm <sup>3</sup>		Differential Leukocyte Count (%), Day 10	
		Day 5	Day 10	Polynuclears	Mononuclears
Control	14	123 ± 16*	152 ± 24	38	62
Deficient intact	20	1602 ± 210	501 ± 44	58	42
Deficient Pthy X	8	319 ± 80	399 ± 66	33	67
Deficient Thyr-Pthy X	24	117 ± 14	695 ± 172	45	55
Deficient Thyr-Pthy X + Tx**	10	810 ± 118	334 ± 67	64	36
Deficient Adr X + DOCA*	6	461 ± 180	611 ± 28	38	62
Deficient Adr X + cortisol**	8	21 ± 4	58 ± 11	51	49
Deficient Adrenud.	8	2355 ± 692	1116 ± 386		
Deficient Hypo X	14	210 ± 74	264 ± 42	16	84

\* : Mean ± SEM.

\*\* : Tx: 5 µg sc/day; DOCA and cortisol: 0.5 mg sc/day.

diminished by two-thirds on the 10th day and it is assumed that the count would have reached normal levels on the 15th day, at which time the hyperemic cutaneous reaction tends to disappear in most animals. The eosinophil count was raised three-fold in parathyroidectomized rats and was found to be slightly higher on the 10th day in the same group. This seems to correlate quite well with the duration of the erythema reaction, which lasted considerably longer than the average time registered in other groups (cf. Table I).

In the thyro-parathyroidectomized rats, eosinophilia occurred only on the 10th day of magnesium deficiency. This delayed manifestation is consistent with the fact that such animals are rather resistant to tissue histamine release, as is reflected by the low incidence and severity of the erythema reaction (cf. Table I). Thyroxine treatment, on the other hand, exerted a promoting action on magnesium-deficiency-induced eosinophilia. Conversely, cortisone treatment in adrenalectomized rats caused eosinopenia, as expected. Adrenal enucleated rats, which already showed the most severe erythema, also had the highest rise in blood eosinophils. Hypophysectomized rats exhibited a two-fold eosinophilia on the 5th and 10th days, despite a lack of demonstrable erythema by that period.

The percentage of polynucleated cells in Giemsa-stained smears of the peripheral blood was found to be relatively increased in all groups of deficient rats, except in adrenalectomized DOCA-treated and in hypophysectomized rats. Beside their relative diminution in number, the mononucleated cells were qualitatively different, with a higher proportion of large lymphocytes than in normal controls. The considerable increase of mononuclears in hypophysectomized rats under the present experimental conditions was also noteworthy.

The peripheral blood of rats bearing a thymic tumor exhibited changes similar to those encountered in myeloproliferative disorders. Atypical large lymphocytes similar to myeloma cells were seen more often and a few animals showed immature basophilic leukocytes similar to those found in myelocytic leukemia (Figs. 8 and 9).

TABLE III

ACTION OF GLANDULAR EXTRIPATION AND HORMONAL SUBSTITUTION TREATMENT UPON THE DEVELOPMENT OF TISSUE CALCINOSIS FOLLOWING 30 DAYS' MAGNESIUM DEFICIENCY IN RATS

Groups	Number of Rats with Calcification	Heart		Kidney	
		Incidence	Severity (Grade 0-4)	Incidence	Severity (Grade 0-4)
Deficient intact . . . . .	7/16	6	1.5	5	1.3
Deficient Pthyro X . . . . .	6/6	3	0.8	4	2.0
Deficient Thyr-Pthyro X . . . . .	4/6	1	1.0	4	1.8
Deficient Thyr-Pthyro X + Tx* . . . . .	5/6	5	3.0	2	0.5
Deficient Adr X + DOCA* . . . . .	7/7	4	1.6	7	2.2
Deficient Adr X + cortisol* . . . . .	4/6	2	2.0	3	1.2
Deficient Adr-enucl. . . . .	12/14	6	1.0	12	1.8
Deficient Hypo X . . . . .	0/8	0	0	0	0
Deficient Hypo X + STH* . . . . .	4/6	2	1.0	4	1.1

\* Tx: 5 µg sc/day; DOCA and cortisol: 0.5 mg sc/day; STH: 2 mg sc/day.

### *Endocrine Action Upon Development of Heart and Kidney Calcinosis*

The myocardial and renal calcific changes following 30 days of magnesium deficiency were abolished by adrenal enucleation and hypophysectomy; this preventive effect was counteracted by STH substitution therapy (Table III). In comparison with the controls, the occurrence of heart and kidney calcification was little influenced by glandular extirpation. The heart lesions were found to be particularly severe in thyro-parathyroidectomized thyroxine-treated rats and, to a certain degree, following adrenalectomy and cortisol substitution. The kidney lesions, on the other hand, seemed to be aggravated by parathyroidectomy and following adrenalectomy with DOCA substitution. After 30 days of magnesium deficiency, the soft tissue calcification also involved many other organs not indicated in the Table of results and, more especially, the muscle layers of the gastrointestinal tract and of the large arteries.

### ***Endocrine Action Upon the Survival Rate and the Development of Thymic Tumors***

Thyro-parathyroidectomized rats, which were highly resistant to the acute and subacute effects of magnesium deficiency, also survived longer and at a higher rate by comparison with all other animals, including the intact magnesium-deficient group (Table IV). Conversely, thyroxine administration considerably reduced the survival time of animals deprived of their thyroid and parathyroid glands, which averaged 30 days. Adrenalectomized DOCA-treated rats also tolerated the deficient diet badly, with a mean survival time of 37 days; the differences in the other groups were not significant.

As regards their number and development, the tumors occurred with an incidence of 40% in intact magnesium-deficient rats, the majority appearing before 65 days of dietary deficiency. Parathyroidectomy had little or no preventive action on the emergence of tumors; these developed at a slower rate but with an incidence of 25%. In the thyro-parathyroidectomized rats, which best tolerated the magnesium-deficient diet, the percentage of tumors was very low (5%). It is possible that this percentage would have increased had the period of observation been extended beyond 90 days. On the other hand, the early, high mortality seen in the thyroxine-thyro-parathyroidectomized rats did not allow for tumor development in these animals as the tumors never develop before the 45th day; this also seems to be the case for adrenalectomized DOCA-treated rats. Following adrenalectomy and cortisol treatment, no thymic tumors were demonstrable, even though the survival time was longer in this group. Finally, the highest incidence of tumors (50%), with the shortest time of appearance, was observed in adrenal-enucleated rats.

### ***Histopathology of the Endocrine Glands***

With accumulating evidence that endocrine secretions influence the course of the magnesium deficiency syndrome, investigations were carried out on the histologic aspect of the glandular

TABLE IV  
ACTION OF GLANDULAR EXTRIPATION AND HORMONAL SUBSTITUTION TREATMENT ON BODY WEIGHT GAIN, SURVIVAL AND OCCURRENCE OF THYMIC TUMORS FOLLOWING 90 DAYS OF MAGNESIUM DEFICIENCY IN RATS

Groups	Number of Rats	Initial Body Weight (g)	Weight Gain (g)	Mean Survival Time (Days)	Number of Survivors on 90th Day	Thymic Tumors Incidence	Average Day of Development
Control	14	94	+ 150	14	7	8 (40%)	59
Deficient intact	20	90	+ 58	65	7	2 (25%)	80
Deficient Pthy. X	8	96	+ 65	66	2	1 (5%)	87
Deficient Thyr-Pthy. X	20	90	+ 32	69	13	1 (5%)	87
Deficient Thyr-Pthy. X + Tx*	18	86	+ 31	30	0	0	
Deficient Adr X + DOCA*	12	100	+ 49	37	1	0	
Deficient Adr X + cortisol*	8	100	+ 17	58	1	0	
Deficient Adr-enud.	8	126	+ 19	59	3	4 (50%)	54

\* Tx: 5 µg sc/day; DOCA and cortisol: 0.5 mg sc/day.

tissue after 60 days of experimental magnesium deficiency in order to correlate the chronic pathologic changes with the glandular alterations.

**Anterior Pituitary.** Using either aldehyde-thionin (11) or colloidal iron in combination with PAS, we were able to observe cytologic changes, especially in animals bearing thymic tumors. There was a definite increase in the number of basophils, which exhibited signs of hypersecretion. The thyrotrophic thionin-positive cells were degranulated and showed one or more vesicles in their cytoplasm (Fig. 16), changes that are compatible with hypothyroidism. The gonadotrophin cells reacting to both iron and PAS were regressive and showed typical Golgi halo in their cytoplasm, such changes being compatible with hypogonadism.

**Thyroids.** In most animals, these glands were slightly increased in size and upon histologic examination they showed distended follicles filled with colloid and lined by a low cuboidal epithelium. We also observed a high incidence of ultimobranchial bodies (Fig. 17) and it remains to be seen whether the fact that these structures were present in an unusually high number has any functional meaning with regard to the blood calcium level.

**Parathyroids.** On histologic examination, the glandular parenchyma was seen to be dissected by connective tissue septa, imparting an unusual trabecular aspect to the gland. The interstitial fibrosis was particularly marked in deficient animals that were replaced on a normal diet after 45 days (Fig. 18). Such a reparative process is suggestive of a functional disturbance of the gland.

**Adrenals.** After 50 days of experimental magnesium deficiency, the adrenal glands were generally enlarged, but their yellow or brownish color merely indicated that they were not hyperfunctional. Microscopically, all glands exhibited more or less severe regressive changes consisting in hydropic degeneration of cells with marked involution of the fasciculata. In some instances, the parenchyma was highly hemorrhagic, with replacement of the medulla and the greater part of the cortex by erythrocytes; some adrenals also presented focal necrosis with calcific deposits (Fig. 19).

**Ovaries.** The ovaries frequently showed involutive changes characterized by the presence of basophilic muco-substance within

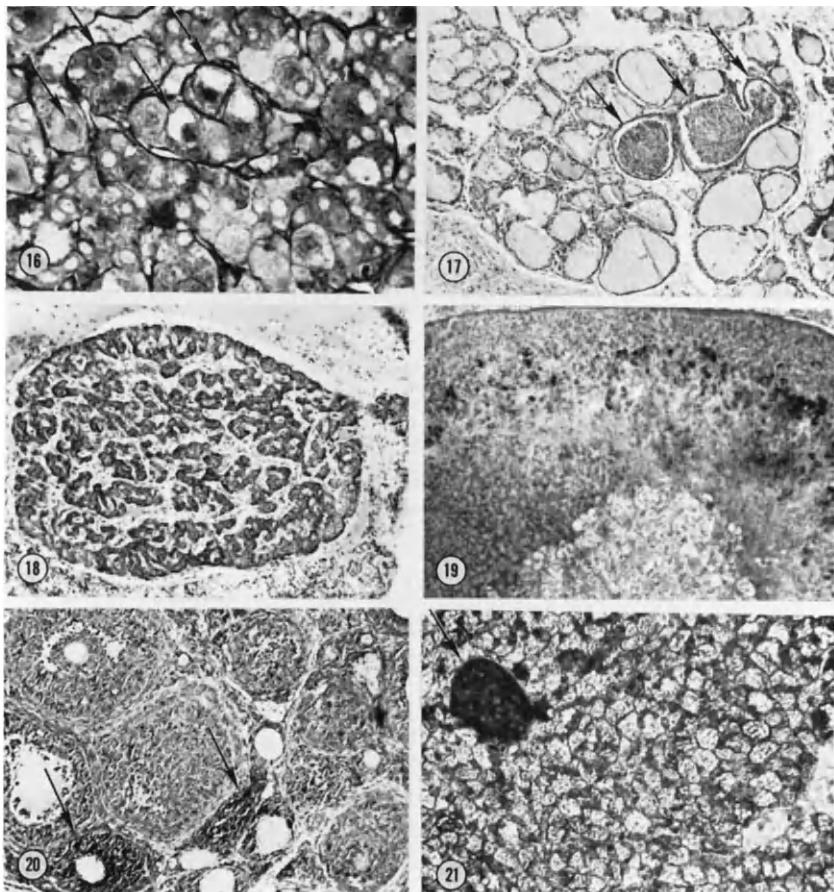


FIGURE 16. Anterior pituitary cytologic changes after 60 days of magnesium deficiency. The thyrotrophic thionin-positive cells are vesiculated (*arrows*) and the gonadotrophic basophils are regressive and show a typical Golgi halo in their cytoplasm (*arrows near left margin*). Aldehyde-thionin  $\times 500$ .

FIGURE 17. Histologic aspect of the thyroid gland in rats after 60 days of magnesium deficiency. The relatively distended follicle contains a large amount of colloid and is lined by a low epithelium. Note the hyperplastic ultimobranchial bodies (*arrows*). HPS  $\times 100$ .

FIGURE 18. Histologic aspect of a parathyroid from a rat fed magnesium-deficient diet during 45 days and a regular regimen thereafter. The glandular epithelium is dissected by connective tissue septa, imparting an unusual trabecular aspect to the gland. HPS  $\times 100$ .

FIGURE 19. Histologic aspect of the adrenal after 45 days of magnesium deficiency. Note the regressive changes characterized by hydropic degeneration and calcific deposits in the fasciculata. HPS  $\times 60$ .

dilated hydropic follicles, some being partly or completely calcified (Fig. 20).

**Hibernating Glands.** Since the brown adipose tissue normally surrounding certain organs, such as the kidney, the adrenals, the aorta, and the thymus, appeared to be overdeveloped, we undertook investigations of the interscapular brown fat (hibernating gland) in animals that were deprived of magnesium for a period of 60 days. When dissected and weighed, the gland proved to be increased in weight. Histologic studies revealed that the fat cells were hyperplastic and their cytoplasm was partly degranulated (Fig. 21).

### Discussion

In their original description, Kruse *et al.* emphasized the importance of recourse to a refining procedure in the preparation of a low-magnesium diet (26). They were able to demonstrate in prepuberal animals a "spectacular series of events" when the magnesium content of the deficient diet was lower than the critical level of 1.8 parts per million. Their description included some of the acute and subacute manifestations described here, such as cutaneous hyperemia, hyperexcitability, convulsive seizures, and cutaneous lesions. Renal changes with calcific deposits were subsequently described by Cramer (10) and, thereafter, a series of conflicting reports were published on the mechanisms underlying the pathologic changes observed in the course of magnesium deficiency (27, 32, 38, 40, 42, 43). With the exception of Greenberg *et al.* (13), who showed that prolonged magnesium deprivation eventually leads to nephrotic changes with a progressive diminution of blood serum proteins, there has been, to our knowledge, no description of the chronic manifestations of magnesium deficiency.

While investigating the influence of hormones on the course and the development of experimental deficiency symptoms, it soon became evident that the occurrence of neuro-muscular disorders was a limiting

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FIGURE 20. Histologic aspect of the ovaries after 45 days of magnesium deficiency. Note the calcific deposits in the degenerating follicles (*arrows*).

HPS and Von Kossa  $\times 100$ .

FIGURE 21. Histologic aspect of the hibernating gland after 60 days of magnesium deficiency. There is a marked diminution of lipid droplets with proliferation of stromal tissue and formation of an epithelial islet (*arrows*).

Sudan black B  $\times 250$ .

factor to carrying out long-term experiments in rats. The so-called magnesium tetany has been ascribed to cytologic alterations in the mid-brain, the pons (14) or the cerebellar tissue (2). Although we were also able to detect lesions in the Purkinje cells, these could not be related to the susceptibility of animals to develop tetany. With increasing data arising from biochemical studies on the parenchymal cell alteration in animals depleted of magnesium, it is more likely that the functional disturbances as much as the morphologic changes derive from the fact that magnesium exerts a great influence on the ionic cell balance.

The biochemical findings of MacIntyre and Davidsson are particularly relevant in this respect. These workers showed a slight but definite hypercalcemia concomitant with the fall in magnesium and, at the same time, alterations in the sodium-potassium balance in the tissue (29). These findings are substantiated by recent observations indicating that acute hypermagnesemia induces changes in the cellular concentration of calcium, particularly in the skeletal muscle and myocardium, suggesting that the competition between calcium and magnesium binding to cell membranes may have a regulatory influence on the influx of blood electrolytes (41). In agreement with Jenkinson (22), we are inclined to believe that a magnesium deficit at the level of the neuro-muscular synapses may generate disorders in the transmission of nervous impulses, with increased production or release of acetylcholine.

There are a number of observations to suggest a relative state of hyperparathyroidism during experimental magnesium deficiency, e.g., increased intestinal calcium absorption (1, 8, 9), hypophosphatemia with elevated phosphaturia. On the other hand, the diminution in alkaline phosphatase activity (35), the absence of bone resorption (33), as well as the occurrence of hypomagnesemia in clinical hypoparathyroidism (16) do not support the assumption that the parathyroid gland plays a dominant role in the magnesium deficiency syndrome. Should this gland become hyperactive, this most likely occurs during the early phases of magnesium deficiency, when there is a rise in blood calcium that progressively returns to normal values after 20 days (35).

Another valuable contribution to the understanding of acute magnesium-deficiency symptoms in the rat is the tissue mast cell degranulation with a secondary rise in blood histamine and eosinophils (4, 6, 7, 18, 21, 23). The role of histamine in the development of the erythema seems indubitable; its long-term effect on the progression of

the magnesium-deficiency symptoms in rats remains to be elucidated. It is our belief that a lack of histamine regenerative capacity is largely responsible for tissue regressive changes, namely the cutaneous lesions with hair loss and the involutive changes seen in most organs including the endocrine glands (20).

The thyroid hypoplasia together with the changes in the pituitary cytology and the generalized edema provide evidence that chronic magnesium deficiency is associated with a negative protein balance largely imputable to the existing nephrosis (13) and possibly to a defect in protein synthesis (28, 31). Electrophoretic studies carried out on a series of animals after prolonged magnesium deprivation revealed that the blood proteins drop to values as low as 5.1% (unpublished).

Finally, the more unpredictable aspect of these studies on chronic magnesium deficiency in rats is the development of thymic tumors. Since our original description (20) and similar observations reported by Bois (5), there has been no report in the literature regarding these tumors. Several postulates can be put forward to explain the neoplastic transformation of the thymus gland (12, 24, 25, 30, 44); but in our view, myeloproliferative disorders play an important role in the genesis of the tumors. Repeated cytologic studies on the peripheral blood of our animals revealed that there is a sustained leukocytosis in the course of magnesium deficiency, most probably as a result of granulocytic hyperplasia of the bone marrow. In this connection, it is of interest to note that a transmissible chronic granulocytic leukemia following leukocytosis due to magnesium deprivation has been reported in the rat (3). Acute hypomagnesemia has also been reported in children suffering from acute leukemia (36). Whether the neoplastic changes are promoted by the endocrine dysfunction that is morphologically evident in chronic magnesium deficiency remains to be seen. It should also be mentioned that extramedullary lymphoid and myeloid proliferation often occurs in rats subjected to a severe and prolonged catabolic impulse (39).

### **Abstract**

The progression of magnesium deficiency symptoms has been investigated in rats, with the object of elucidating factors contributing to their genesis. The pathologic events can be grouped under three successive periods and analyzed with regard to the hormonal status of the animals. The acute phase, which occurs between the 5th and the 20th day of magnesium deficiency, is characterized by erythema with a sharp increase in blood eosinophils, a

moderate neutrophilia, and erythema. These acute signs have been related to tissue-histamine liberation; they are prevented by hypophysectomy, thyro-parathyroidectomy, and cortisol substitution therapy after adrenalectomy. The second phase occurs between the 20th and the 40th day and is characterized by convulsive seizures, cutaneous regressive changes, and soft tissue calcification. These changes were attributed to modifications in tissue electrolytes resulting from hypomagnesemia, namely potassium depletion; they are totally prevented by hypophysectomy. The third phase is characterized by extensive renal damage with signs of nephrosis, severe blood dyscrasia, and the development of thymic tumors. These manifestations result from the hypo- and possibly dysproteinemia associated with hypoendocrinia and myeloproliferative disorders. The structural alterations that take place in the endocrine glands during this late stage are described.

### Abrégé

Notre recherche avait pour objet d'étudier la progression des symptômes de carence en magnésium chez le rat et les facteurs qui influent sur leur développement. Nous avons cherché à déterminer plus particulièrement le rôle des sécrétions endocrines dans la genèse des lésions. Compte tenu de leur apparition et de leur durée, les diverses manifestations furent groupées sous trois étapes successives. Les manifestations aiguës surviennent entre le 5e et le 20e jour de la déficience en magnésium et se caractérisent par un érythème, une augmentation des éosinophiles du sang circulant avec une neutrophilie modérée et de l'éréthisme. L'ensemble de ces changements fait suite à une libération tissulaire d'histamine. Ils sont prévenus par l'hypophysectomie, la thyro-parathyroïdectomie et l'administration de cortisol après surrénalectomie. La deuxième phase survient entre le 20e et le 40e jour; elle se caractérise par des convulsions, des lésions régressives de la peau et de la calcinose des tissus mous. Ces modifications seraient liées à des altérations tissulaires des électrolytes, en particulier du potassium et ce, par suite de la chute du magnésium sanguin. Elles sont entièrement prévenues par l'hypophysectomie. La troisième phase se caractérise par une atteinte rénale avec des signes évidents de néphrose, une dyscrasie sanguine et l'apparition de tumeurs thymiques. Ces manifestations, selon nous, font suite à une hypo- et possiblement dysprotéinémie associée à de l'insuffisance endocrinienne et à des troubles myéloprolifératifs. Les modifications structurales observées au niveau des glandes endocrines semblent révélatrices en ce qui concerne la pathogénie des lésions tardives de déficience en magnésium chez le rat.

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**H. Selye:** Quand j'étais tout petit, ma mère, pour m'amuser, avait donné des oeufs de canard à couver à une poule. Quelques jours seulement après que les petits canards sont sortis de leur coquille, ils se mirent à nager au bord du Danube. Ils le firent sans effort et très bien, tandis que la poule, n'osant s'avancer dans l'eau, restait là, les regardant avec admiration mais non sans crainte. Eh! bien, aujourd'hui, je comprends ses sentiments. Elle devait se dire: "Comment, c'est moi qui ai couvé ça, des petits qui savent nager dans ces ondes dangereuses? Où l'ont-ils appris? Certainement pas de moi; je ne suis jamais arrivée à l'apprendre moi-même. Ils ont l'air bien sûrs d'eux, mais est-ce que, vraiment, ils ne sont pas en danger? Mon Dieu, s'ils se noyaient, je ne saurais leur venir en aide!"

Mr. Chairman, it is with this childhood experience as a background that I would like to comment on the papers we just heard. Doctor Messier has spoken of Professor Bélanger as his spiritual father, while the latter has very generously acknowledged what little guidance I could offer him during the first formative years of his scientific career. Let me trace the family tree further back. It was at the beginning of the Century that my own spiritual father, Professor Biedl (in collaboration with F. Krauss), described the severe shock that develops in dogs following intravenous injection of peptone. He called it "*anaphylactoid shock*," because it resembled anaphylactic shock and yet differed from the latter in that its production required no previous

sensitization. It was later found that anaphylactoid shock is accompanied by *mast-cell degranulation* and a massive *discharge of histamine*. The finding remained a laboratory curiosity that had little effect upon medicine in general—but it was intriguing. It so happened that later, as a graduate student, I had the privilege of working with Professor E. V. McCollum (at The Johns Hopkins University), who did the original experiments on *magnesium deficiency* with Kruse that have just been cited. I did not participate in this work except during weekends (as you pointed out at the dinner last night, it was always my strength to be in the lab on Saturdays and Sundays); so I looked after the rats during the weekends and noted their cutaneous hyperemia and scratching as a passive spectator. It turned out later—thanks to the work of Professor Bélanger and his group—that this magnesium deficiency is also somehow related to mast-cell discharge and histamine liberation. Yet, otherwise, Biedl's anaphylactoid shock and the magnesium-deficiency syndrome appeared to have little in common.

In 1937—while trying to produce stress with the most outlandish agents—I happened to inject egg-white intraperitoneally into some rats that promptly developed the very acute and striking acral edema and hyperemia that was to occupy so many of us during the subsequent thirty years. In honor of Biedl we called it "*anaphylactoid edema*." This term emphasizes that the response is also similar to but not identical with anaphylaxis, being characterized primarily by a Quincke edema-like swelling of the face and paws rather than by severe shock. [Let me point out here in parentheses that I fully agree with Professor Halpern that it would be confusing if we were to refer to the eliciting mast-cell dischargers (such as egg-white, polymyxin or compound 48-80) as "antigens."] When first observed, this anaphylactoid edema appeared to be "a mere laboratory curiosity" and we were so preoccupied with stress research at the time that we did not follow it much further. It was not until about ten years later that Drs. J. Léger and G. Masson began to study this reaction in depth; and indeed, Dr. Léger made it the subject of his Ph.D. thesis. Here again, chance was good to us, because Professor Mauricio Rocha e Silva happened to be at our Institute as our first "Claude Bernard Visiting Professor" and suggested that we should use the then recently discovered *antihistamines* (which we owe to Professor Halpern) to see whether they would prevent the anaphylactoid edema. As you all know, they did. This finding was especially important, because it came just after the discovery by O'Leary and Farber at the Mayo Clinic that antihistamines often

give spectacular relief from urticaria and angioneurotic edema in man. These findings called attention to the close relationship between these clinical syndromes and anaphylactoid edema. Thereupon, the experimental model became one of the most commonly used pharmacologic tests for the assay of histamine antagonists of potential clinical value.

In 1954 Benditt *et al.*, and in the following year Jasmin and Richer, brought forth evidence that the *mast cells* (which are especially numerous in the shock organs of anaphylactoid edema) discharge their granules under the influence of various anaphylactoidogenic agents, and that the characteristic edema presumably results from a topical release of histamine and related compounds from the mast cells. Soon afterwards, independently and almost simultaneously, Goth, Adamkiewicz, and their respective co-workers noted that *insulin* hypoglycemia increases, whereas *alloxan diabetes or glucose* administration decreases, the anaphylactoidogenic activity of dextran. By that time, numerous investigators in many laboratories throughout the world had become interested in the mechanism and implications of this reaction. Between 1962 and 1966, the co-operative effort of many members of our Institute revealed that combined treatment with anaphylactoidogenic agents and drugs producing calciphylaxis or calcergy can cause selective *calcification in the anaphylactoid shock organs*. On the other hand, conjoint administration of anaphylactoidogenic agents and drugs producing the thrombohemorrhagic phenomenon (THP) elicit an *anaphylactoid purpura* in these same characteristic locations. Furthermore, anaphylactoidogenic compounds increase the tendency of the rat to respond with massive necrosis to topical irritation, a phenomenon referred to as *acute conditioned necrosis (ACN)*. Since these same anaphylactoidogenic agents also delay the *tissue clearance* of such dyes as phenolsulfonephthalein, we suspect that the predisposition to chemically induced necrosis is due to a prolonged contact between tissues and injected irritants whose absorption is delayed during anaphylactoid edema. In other words, these more recent observations revealed that, depending upon conditions, the anaphylactoid edema can produce *qualitatively different lesions*, such as: simple serous inflammation with edema, calcification, thrombohemorrhagic lesions, or necrosis. Under certain circumstances, it can even be associated with massive calcification of the autonomic nerves. Thus, it often determines not only the development but also the selective *localization* of changes in one or the other site. Now we learn from Professor Rocha e Silva that *bradykinin* may also play an important part in the anaphylactoid reaction, while Professors Bélanger, Bois, and Jasmin show that this

response—or at least modifications of it—may play a role even in lymph-cell production, the development of *thymus tumors*, and many other responses that we would never have connected in any way with Biedl's anaphylactoid shock or with anaphylactoid edema. It has also become evident that beside the originally used egg-white, there are innumerable agents that can produce anaphylactoid edema, including even dietary magnesium-deficiency (as shown by the preceding papers) and certain platinum salts (as shown by Professor Parrot and his co-worker Doctor Saindelle). Instead of giving an extensive bibliography here, I should like to refer those interested in further detail to my book *Anaphylactoid Edema* (Warren H. Green, Inc., St. Louis, 1968), which discusses nearly a thousand references dealing with this subject.

In closing, let me thank the organizing committee for having brought together here so many of the investigators whom I had occasion to mention in this brief historical outline of our knowledge about anaphylactoid edema, namely: V. W. Adamkiewicz, L. F. Bélanger, P. Bois, B. Halpern, G. Jasmin, J. Léger, G. Masson, J. L. Parrot, C. L. Richer, and M. Rocha e Silva. I for one, will certainly be influenced in my research for years to come by the many suggestions that I have received from these colleagues.

**B. Halpern:** Mes chers Collègues, quelqu'un a dit que la différence entre un génie et un homme ordinaire c'est que le génie voit les choses là où l'homme simple ne les voit pas. J'ai toujours été étonné par la fécondité de l'esprit et par la façon de voir choses qui sont propres à Hans Selye. Il vient d'esquisser d'une façon aussi magistrale, comme il a l'habitude de le faire, le survol de tant de problèmes, dont chacun pourrait constituer une source de longues recherches pour des équipes entières. Hans Selye vient de rappeler ses travaux sur la réaction anaphylactoïde chez le rat qui, à certains égards, rappelle l'oedème angioneurotique de Quincke chez l'homme. Les problèmes biopathologiques sont toujours plus complexes qu'on ne le pense. On connaît, dans le cadre de l'oedème angioneurotique, une forme familiale qui revêt généralement une allure grave car, du fait de sa localisation pharyngée, elle peut être mortelle. Il y a environ 15 ans j'ai rapporté, avec M. Pasteur Vallery-Radot, l'observation d'une famille où, dans trois générations, sept personnes, tous des mâles, étaient morts par asphyxie; le huitième descendant de cette famille est arrivé, un jour, à l'Hôpital Broussais, avec un mouchoir ensanglanté devant la gorge. Ce malade, qui était sujet à ces crises d'oedème et qui avait vu mourir son père et d'autres membres de sa famille au cours d'un tel accès par asphyxie, s'était

tranché la gorge avec un rasoir pour échapper à la mort. Nous n'avons pas compris, à cette époque, l'étiologie de cette affection et ses relations avec les facteurs génétiques. Car indiscutablement il y avait une cause génétique. Dans cette famille on a dénombré la maladie de Quincke à travers les descendants de trois générations, tous de sexe masculin. Eh bien, tout récemment, on vient de découvrir que, dans cette forme familiale d'oedème qui est, comme on l'a vu, parfois dramatique, voire aussi mortelle, il existe effectivement un défaut génétique. Ce défaut génétique se traduit par le manque d'une protéine qui bloque le G<sup>1</sup> du complément. Lorsqu'on injecte le G<sup>1</sup>, qui a été isolé de malades atteints de cette affection, on observe immédiatement, à l'endroit de l'injection, un oedème important alors que cette protéine ne produit rien chez les autres individus normaux. On a trouvé récemment un autre défaut génétique qui concerne le G<sup>3</sup> et qui contrôle le métabolisme de la kallikréine. Chez ces malades, l'injection de kallikréine par voie intradermique produit également un oedème local qui ressemble tout à fait à celui qui apparaît spontanément. Ainsi, le problème de la réaction anaphylactoïde devient très complexe, en tout cas en pathologie humaine. Aux faits connus il faut ajouter aussi des facteurs encore inconnus. Je pense, en particulier, au magnésium qui peut jouer un rôle important, car nous savons que le magnésium est nécessaire pour l'activation du complément. Or, les fractions du complément sont de véritables enzymes. Chez les individus mentionnés, l'injection des fractions non inhibées peut provoquer des manifestations locales ou des manifestations générales.

Ceci étant dit, je voudrais poser une ou deux questions à ceux qui viennent de parler, notamment au Dr. Bois et au Dr. Jasmin, au sujet de ce syndrome provoqué par la déficience en magnésium. Quand vous dites que cette vasodilatation périphérique est liée à l'hyperhistaminémie, je voudrais vous demander si la 5-hydroxytryptamine ne joue pas un rôle dans ce syndrome de vasodilatation périphérique, chez les animaux carencés en magnésium. Nous savons que, chez le rat, la 5-hydroxytryptamine est beaucoup plus active dans ses propriétés vasopérmeabilitrices et, d'autre part, nous savons que, quand on injecte le dextran ou un autre histamino-libérateur, on observe, ainsi que nous l'avons montré, à la fois une libération d'histamine et de 5-hydroxytryptamine. Etant donné que la 5-hydroxytryptamine est environ 100 fois plus active sur les capillaires que l'histamine chez le rat, je me demande s'il n'y a pas là une mise en liberté de deux libérateurs qui pourraient être synergiques. Je voudrais aussi vous

demander si les antihistaminiques sont capables de modifier ce phénomène. Enfin, je voudrais vous demander s'il s'agit ici d'un phénomène du type "idiosyncrasie," autrement dit d'un effet qui est propre à une espèce, ainsi qu'il en est du dextran ou de la polyvinyl-pyrrolidone qui n'agissent respectivement que chez le rat ou chez le chien. Encore une question: Est-ce que la thymectomie prénatale modifie, de quelque manière, l'apparition de ce syndrome et de son évolution? Autrement dit, est-ce que le thymus est un "target organ" ou est-ce que c'est un organe qui commande ce syndrome?

**G. Jasmin:** Je laisserai le soin au Dr. Bois de discuter plus en détails les dosages d'histamine et de sérotonine dans le sérum et les urines durant la phase initiale de déficience en magnésium au cours de laquelle l'hyperémie cutanée fait son apparition. Je dois dire, cependant, que nos déterminations de l'acide indole-acétique urinaire effectuées au cours de cette période, se sont avérées peu concluantes quant au rôle de la sérotonine.

En ce qui concerne l'action des antihistaminiques, nous avons constaté qu'ils réduisaient l'intensité et la durée des rougeurs sans les prévenir totalement et ce, à des doses suffisamment élevées. Bien que la réaction hyperémique fût atténuée, les phases subséquentes qui caractérisent le syndrome de déficience en magnésium ne furent pas pour autant modifiées. Cette diminution d'intensité ne se compare pas à celle qui résulte de l'hypophysectomie, de la thyroïdectomie ou encore de l'administration de la cortisone (cf. Tableau I de ma présentation).

Le facteur espèce soulève sans doute un aspect intéressant dans ces études. La souris, par exemple, que nous avons étudiée plus particulièrement, ne montre jamais d'hyperémie cutanée et, que je sache, c'est également le cas pour la plupart des autres animaux de laboratoire soumis à l'action d'une diète synthétique déficiente en magnésium. Déjà Orent et coll. avaient noté que la symptomatologie initiale était moins évidente chez le chien (*Am. J. Physiol.*, 101:454, 1932). La réaction hyperémique cutanée du rat s'expliquerait par la proportion élevée des mastocytes, comme nous l'avions déjà démontré au cours de la réaction anaphylactoïde (*Rev. Can. Biol.*, 15:107, 1956).

Nous n'avons pas étudié l'influence de la thymectomie prénatale sur l'évolution du syndrome de déficience en magnésium chez le rat. Nous avons constaté, cependant, que l'ablation du thymus, chez nos animaux carencés dont le poids initial moyen était de 70 g, ne changeait en rien la séquence des événements que nous venons de décrire, à part bien entendu, l'apparition des tumeurs du thymus.

In brief, it can be said that antihistamines lower but do not prevent the hyperemic cutaneous reaction in the magnesium-deficient rat. As far as the species factor is concerned, we were unable to reproduce the early erythema changes in other laboratory animals; the phenomenon, to our way of thinking, is probably related to the mast-cell content in the skin of the rat. The magnesium deficiency syndrome has not yet been investigated in thymectomized new-born animals; in young adult rats, however, ablation of the thymus did not influence the sequence of the pathologic events.

**P. Bois:** Nous avons déterminé la sérotonine dans le sang de quelques-uns de ces animaux et nos résultats indiquent une toute légère augmentation à peine mesurable. Je crois que la sérotonine est impliquée dans ce phénomène d'hyperémie, mais comme il est plus simple du point de vue technique de déterminer l'histamine, nous avons plutôt utilisé cette dernière comme index d'évolution. Tel que l'a mentionné le Dr. Jasmin, on n'observe pas ce changement chez d'autres espèces comme la souris ou le chien. La carence en magnésium chez le chien, que nous avons étudiée un peu plus longuement, ne provoque aucun phénomène hyperémique. La thymectomie à la naissance n'a pas modifié l'évolution du phénomène; toutefois, il est difficile de maintenir des animaux thymectomisés pendant assez longtemps avec des régimes carencés; il ne nous a donc pas été possible d'en faire une étude aussi prolongée que nous l'aurions voulu.

## **The Role of the Adrenal Cortex in the Pathogenesis of a Spontaneous, Hereditary Congestive Heart Failure**

EÖRS BAJUSZ

THE role of the adrenal cortex in the pathogenesis of congestive heart failure has been debated for more than 15 years. It was shown in 1950 that the urine of patients with congestive failure contained a substance promoting sodium retention (21). This substance was subsequently identified as aldosterone and the hypothesis has been advanced that its hypersecretion precipitates or intensifies the formation of edema in congestive heart failure (29). Support for this concept has been claimed in numerous reports showing elevated aldosterone in the urine of patients with heart failure (11, 24, 26, 40); it has also been observed that a marked increase in the secretion of this steroid occurs in dogs with congestive heart failure produced experimentally by either progressive pulmonary stenosis or tricuspid insufficiency (12).

However, the literature on the role of aldosterone in congestive heart failure is increasingly confusing, as shown by recent review papers (22, 28, 31, 39) and original contributions (14, 34, 41). Whether an elevated aldosterone secretion is a primary cause of fluid retention, a contributing factor, or merely an associated change which accompanies edema cannot be ascertained from the data available at present.

The existence in an inbred strain of Syrian hamsters of a genetically transmitted (or conditioned), degenerative cardiomyopathy that terminates in congestive heart failure offers unique opportunities to study the sequence of events leading to cardiac insufficiency and to analyze the importance of the adrenal cortex and other extra-cardiac factors in the mechanism of edema formation. This hereditary disease, which has a 100% incidence in the pedigreed BIO 14.6 strain of hamsters, is apparently a primary myopathy (25). The degenerative lesions which occur in the

striated muscles are definitely noninflammatory in nature. The gross and histopathologic abnormalities seen in the middle-aged or older myopathic hamsters, except those of the cardiac and skeletal muscles, are consistent with long-standing passive venous congestion, and this progressive cardiovascular insufficiency may be regarded as the ultimate cause of death (7).

The congestive heart failure develops relatively slowly and progressively in these myopathic animals through three distinct stages. During the first stage (between the 25th and 65th days of age), myocardial lesions characterized by spotty myolysis occur; during the second stage (ranging between the 55th and 85th days of age, approximately), cardiac hypertrophy and dilatation develop; during the third stage (usually after the 80th day of age) the hypertrophy is gradually replaced by extended dilatation of the cardiac chambers and, concurrently, the peripheral manifestations of generalized venous congestion become increasingly marked. Myocardial degeneration is progressive until about the 60th to 70th days of age, when compensatory phenomena and healing processes begin to occur in the affected hearts; only a very few fresh lesions appear in the cardiac muscle of the older animals. Nevertheless, the myocardial degeneration itself seems to be responsible for the development of systemic circulatory insufficiency in the myopathic hamsters (6).

The experiments to be reported here were undertaken to establish the influence exerted by adrenalectomy and/or the injection of corticoids upon the spontaneous development of myocardial degeneration and congestive heart failure in the hamster.

### **Materials and Methods**

The myopathic hamsters used were those of the BIO 14.6 inbred line. Each experimental group consisted of 10 animals. Randomly selected hamsters of approximately the same age were subdivided into groups, the mean initial body weight being identical for each group in a given experimental series. Since myocardial lesions occur earlier in the females than in the males of this strain, and because progression of the subsequent development of congestive heart failure is more rapid in the former than in the latter

(6), hamsters of the same sex were usually included in the groups of each experiment. The animals were kept under identical, controlled-housing conditions in air-conditioned rooms; Guilford hamster chow supplemented by oats and wheat germ as well as tap water were offered *ad libitum*.

Bilateral *adrenalectomies* were performed under light ether anesthesia on the first day of the experiment. *NaCl* (1.0%) served as drinking fluid for animals not receiving corticoid supplements after adrenalectomy. *Triamcinolone* (9 $\alpha$ -fluoro-16 $\alpha$ -hydroxyprednisolone diacetate) (Lederle Laboratories), *hydrocortisone acetate*, or *COL* (Nutritional Biochemicals), *desoxycorticosterone acetate*, or *DOC* (Nutritional Biochemicals) and *aldosterone acetate* (Sigma Chemical Co.) were injected as microcrystal suspensions in 0.2 ml of physiologic saline, twice daily, subcutaneously, continuously throughout the experiments. The total daily doses given of each of these steroids are indicated in the Tables. The control animals always received the solvent by the same route of administration.

After death—either spontaneous or by sacrifice with chloroform—each animal was autopsied; the gross pathological findings were recorded, and specimens were taken for routine histology. These always included the heart, liver, and a hind-leg muscle (anterior tibialis). The histological sections were stained with hematoxylin-eosin. Morphological observations on the skeletal muscle sample merely served to ascertain the presence of the hereditary myopathy in doubtful cases when the myocardium was not afflicted by the disease.

The *myocardial lesions* which develop spontaneously in the BIO 14.6 hamsters are only occasionally visible by gross inspection or under the dissection microscope, but they can be readily graded on histological preparations because of their focal nature. The severity of myocardial lesions was assessed on an arbitrary scale of 0 to 4; 0 designates no lesion; grade 1, mild lesions; grades 2 and 3, moderate lesions; grade 4, severe lesions. The following morphological criteria served as a basis for this grading: grade 1, 1 to 3 small focal areas of degeneration, each focus involving not more than 10 adjacent muscle fibers or fiber-segments; grade 2, 4 to 6 small foci and/or not more than 2 larger areas of myoly-

sis; grade 3, 7 to 12 small and/or 3 to 5 large areas; grade 4, more than 12 small and/or more than 5 larger focal lesions. Confluent and diffuse myocardial involvements were approximated in terms of small focal lesions and graded accordingly.

To estimate the severity of *congestive heart failure*, the degree of generalized edema was assessed and complemented by histopathological studies of the liver as well as by observations on the presence of pleural effusion and pulmonary congestion. From studies on the progression of various peripheral manifestations of congestive heart failure in the hamster, the following grading scale was established (6) and used in the present investigations: *Mild heart failure (grade 1)*: a moderate degree of subcutaneous edema restricted to the dependent parts; absence of a measurable amount of exudate in the body cavities; liver enlargement accompanied by dilatation of centrilobular veins and of sinusoids, but with no structural alterations. *Moderate heart failure (grades 2 and 3)*: diffuse subcutaneous edema; liver pathology consisting of hemorrhages and necroses around the central veins; mild pleural effusion (grade 2). The additional occurrence of measurable amounts of exudates in the abdominal and chest cavities (not exceeding a total of 2.5 ml) with marked pulmonary congestion was considered to represent a more advanced (grade 3) stage of moderate failure. In such cases, the liver pathology was usually diffuse and showed progressive destruction of the parenchymal tissues. *Severe heart failure (grade 4)*: severe generalized edema involving the subcutaneous areas of the entire body (including the facial parts), together with ascites, hydrothorax and hydropericardium. In such severe cases, the majority of skeletal muscle groups, especially those of the hind legs and abdominal wall, were markedly swollen. The mesenteries of intestines and the pancreas were highly edematous and the measurable exudates amounted to well over 2.5 ml. There were diffuse necrotic and fibrotic lesions present in the congested liver, and hemosiderin-laden macrophages ("heart-failure cells") were seen consistently in the lungs.

## Results

***Effects of Adrenalectomy and Corticoid Overdosage upon the Development of Myocardial Pathology (Table I).*** Our first objec-

tive was to establish the influence of adrenalectomy and that of corticoid administration upon the spontaneous development of myocardial lesions, since the extent and intensity of heart muscle pathology was shown to influence the subsequent development of congestive heart failure in the myopathic animals (5). Male hamsters, 34 ( $\pm 6$ ) days of age, with a mean initial body weight of 71 g (range: 59 to 78 g) were used. The experiment was terminated on the 35th day after initiation of treatments, thus, at 69 days of age. It should be noted that earlier studies have shown that the spontaneous myocardial degeneration reaches its maximal severity in hamsters between their 50th and 80th day of age (6).

It can be seen from the data summarized in Table I that the administration of large doses of glucocorticoids, such as triamcinolone (group 3) and COL (group 4), mineralocorticoids, such as DOC (group 5) and aldosterone (group 6), or the combination of both these types of corticoids (group 7), did not significantly influence the development of cardiac pathology (compare with group 1). Nevertheless, in the groups treated with glucocorticoids the mortality rate was constantly reduced (groups 3, 4 and 7) when compared with the untreated controls (group 1) or with the groups receiving mineralocorticoid treatment (groups 5 and 6).

The result obtained in the adrenalectomized animals demands special consideration because of the high mortality (group 2).

TABLE I  
EFFECTS OF ADRENALECTOMY (ADR-X) AND CORTICOID OVERDOSAGE UPON THE DEVELOPMENT OF MYOCARDIAL PATHOLOGY IN THE HAMSTER

<i>Group</i>	<i>Treatment</i>	<i>Myocardial Lesions Severity<sup>1</sup> (Grades: 0-4)</i>	<i>Incidence (%)</i>	<i>Mor- tality (%)</i>
1 . . . . .	None	2.9 $\pm$ 0.23	100	20
2 . . . . .	Adr-X + NaCl	1.2 $\pm$ 0.39	60	100
3 . . . . .	Triamcinolone, 250 $\mu$ g	3.2 $\pm$ 0.31	100	0
4 . . . . .	COL, 1.0 mg	3.4 $\pm$ 0.18	100	0
5 . . . . .	DOC, 1.0 mg	2.7 $\pm$ 0.24	100	30
6 . . . . .	Aldosterone, 200 $\mu$ g	3.0 $\pm$ 0.27	100	20
7 . . . . .	COL, 1.0 mg + DOC, 1.0 mg	3.1 $\pm$ 0.24	100	0

<sup>1</sup> Mean  $\pm$  standard error.

Since the average survival time was 18 days (range: 9 to 23 days) for this group, it was necessary to use an additional control group with identical age-range, and maintained similarly on 1.0% NaCl. In this latter group, only 50% of the hamsters revealed the presence of myocardial lesions with an average severity of 1.5. It may be concluded from these observations that neither adrenalectomy nor the administration of large doses of corticoids significantly influences the spontaneous occurrence and progression of myocardial pathology in the myopathic hamsters. The myocardial lesions consisted of focal myolyses in various stages of evolution and were morphologically identical in animals of all groups.

***Effect of Varying Doses of Corticoids upon Development of Congestive Heart Failure in Adrenalectomized Hamsters (Table II).*** The purpose of the second experimental series was to observe the effects of varying doses of triamcinolone (a glucocorticoid), aldosterone (a mineralocorticoid), and the combination of these two types of corticoids upon the development of congestive heart failure in adrenalectomized hamsters. We were especially interested in determining whether the presence of aldosterone is essential to the onset and development of generalized cardiac edema and whether any interaction between the two types of corticosteroids can be demonstrated under the present experimental conditions. Female myopathic hamsters, 43 ( $\pm 5$ ) days of age, with a mean initial body weight of 87 g (range: 73 to 94 g) were employed. Such animals usually show a mild to moderate degree of cardiac pathology without cardiac enlargement or any other signs of heart failure (6). The experiment was of 52 days' duration; thus, the survivors were killed when they were, on an average, 95 days old. Preliminary studies were performed to establish the minimal daily dose of triamcinolone and that of aldosterone sufficient to keep the adrenalectomized myopathic hamsters alive. This minimal maintenance dose proved to be 50  $\mu\text{g}/\text{day}$  in the case of triamcinolone, and 20  $\mu\text{g}/\text{day}$  in the case of aldosterone. In order to observe the effect of hormonal excesses, daily doses of each corticoid ten times higher than the minimal maintenance doses were administered.

The results summarized in Table II indicate the presence of moderate to severe degrees of congestive heart failure in 70% of

TABLE II

EFFECTS OF VARYING DOSES OF CORTICOIDS UPON THE DEVELOPMENT OF CONGESTIVE HEART FAILURE IN ADRENALECTOMIZED (ADR-X) HAMSTERS

<i>Group</i>	<i>Treatment</i>	<i>Congestive Heart Failure Mor- Severity<sup>1</sup> (Grades: 0-4)</i>	<i>Incidence (%)</i>	<i>Mortality (%)</i>
1 . . . . .	None	2.9 ± 0.36	70	30
2 . . . . .	Adr-X + Triamcinolone, 50 µg	1.2 ± 0.41	60	0
3 . . . . .	Adr-X + Aldosterone, 20 µg	1.5 ± 0.30	70	40
4 . . . . .	Adr-X + Triamcinolone, 50 µg + Aldosterone, 20 µg	0.4 ± 0.17	30	0
5 . . . . .	Adr-X + Triamcinolone, 500 µg	3.6 ± 0.18	100	50
6 . . . . .	Adr-X + Aldosterone, 200 µg	1.3 ± 0.28	80	30
7 . . . . .	Adr-X + Triamcinolone, 500 µg + Aldosterone, 200 µg	2.6 ± 0.21	90	20

<sup>1</sup> Mean ± standard error

the intact, untreated controls (group 1). Interestingly enough, about an equal degree of generalized venous congestion developed in the adrenalectomized hamsters receiving maintenance doses of either triamcinolone (group 2) or aldosterone (group 3), while concurrent administration of small doses of both corticoids exerted a significant preventive effect (the differences between groups 2 and 4 and between groups 3 and 4 =  $p < 0.001$ ).

Injection of large doses of triamcinolone to adrenalectomized animals resulted in the development of much more severe congestive failure (group 5) than did the administration of small, maintenance doses of the same steroid (difference between groups 2 and 5 =  $p < 0.001$ ; large doses of aldosterone failed to exert such aggravating action (compare group 6 with group 3). Finally, the degree of venous congestion was less severe in animals treated with excessive amounts of both triamcinolone and aldosterone (group 7) than in those which received large doses of the glucocorticoid alone (difference between groups 3 and 7 =  $p < 0.05$ ).

The mortality rate was the highest in the highly edematous animals treated with large doses of triamcinolone (group 5), while small doses of this steroid exerted a definitely beneficial effect in this respect (see groups 2 and 4).

Histological observations on hearts indicated the presence of a comparable degree of cardiac pathology in all groups.

TABLE III

EFFECT OF VARYING DOSES OF CORTICOIDS UPON THE SEVERITY OF EDEMA  
IN ADRENALECTOMIZED (ADR-X) HAMSTERS WITH HEART FAILURE

Group	Treatment	Congestive Heart Failure Mortality <sup>a</sup> (Grades: 0-4)	Incidence (%)	Mortality (%)
1 . . . . .	None <sup>b</sup>	1.6 ± 0.37	60	—
2 . . . . .	None <sup>c</sup>	2.8 ± 0.21	90	40
3 . . . . .	Adr-X + Triamcinolone, 50 µg	2.7 ± 0.19	80	20
4 . . . . .	Adr-X + Aldosterone, 20 µg	3.8 ± 0.17	100	60
5 . . . . .	Adr-X + Triamcinolone, 50 µg + Aldosterone, 20 µg	1.5 ± 0.32	70	10
6 . . . . .	Adr-X + Triamcinolone, 500 µg	3.5 ± 0.16	100	0
7 . . . . .	Adr-X + Aldosterone, 200 µg	3.6 ± 0.18	100	60
8 . . . . .	Adr-X + Triamcinolone 500 µg + Aldosterone, 200 µg	2.3 ± 0.27	100	30

<sup>a</sup> Mean ± standard error.<sup>b</sup> Control group killed on first day of experiment.<sup>c</sup> Control group killed at termination of experiment.

**Effects of Varying Doses of Corticoids upon the Severity of Edema in Adrenalectomized Hamsters with Heart Failure (Table III).** In view of the unexpected observations made in the previous experimental series in which the influence of pretreatment with corticoids upon the spontaneous development of congestive heart failure was studied, it was deemed necessary to establish the effects of the same steroids on hamsters already in heart failure.

Female myopathic hamsters, 75 ( $\pm 6$ ) days of age, with a mean initial body weight of 108 g (range: 89 to 117 g) were used. Groups 1 and 2 served as intact, untreated controls; group 1 was killed on the first day of the experiment in order to determine the severity of congestive heart failure normally present, while group 2 was sacrificed on the 35th day in order to establish the degree of venous congestion at the termination of the entire experimental series. All animals in groups 3 to 8 were adrenalectomized and treated with corticoids, as indicated in Table III.

It is obvious from the data summarized in this Table that, in animals with heart failure, maintenance doses of triamcinolone resulted in the development of less severe edema and other manifestations of congestive heart failure than did maintenance doses

of aldosterone (difference between groups 3 and 4 is significant at the level  $p < 0.001$ ). Among all the groups in this series, the smallest degree of venous congestion was observed in those adrenalectomized animals treated with small doses of both triamcinolone and aldosterone (group 5). The differences between groups 3 and 5 as well as between groups 4 and 5 proved to be statistically highly significant ( $p < 0.001$  in both cases).

The results obtained in the remaining groups of this series were, to a large extent, identical with those seen in comparable groups of the previous series (compare groups 6 to 8 in Table III with groups 5 to 7 in Table II). Thus, the injection of large doses of triamcinolone elicited a much more severe congestive failure than did the administration of small (maintenance) doses of the same steroid and, here again, large doses of aldosterone failed to exert such aggravating effect. The degree of edema was less severe after concurrent treatment with triamcinolone and aldosterone (group 8) than after the administration of excessive amounts of either triamcinolone alone (group 6) or aldosterone alone (group 7). Nevertheless, this protective effect of the combined mineralocorticoid and glucocorticoid treatment proved to be more obvious at the level of maintenance doses (group 5) than at the level of excessive doses (group 8).

The highest mortality rates occurred in the animals treated with aldosterone (groups 4 and 7), while those receiving large doses of triamcinolone all survived the 35-day period of observation (group 6). This last-mentioned finding is at variance with the result obtained in the previous experimental series (see Table II), where the mortality amounted to 50% in the comparable group.

The onset, intensity and healing of myocardial lesions normally seen in myopathic hamsters of the age range used here (group 2) was not influenced by any of the treatments (groups 3 to 8), as evidenced by histological analyses.

### **Discussion**

Since earlier studies have shown that the administration of various corticoid hormones exerts a marked influence upon the development of a wide variety of experimentally induced cardiac necroses (4, 35),

it is surprising that the spontaneous, hereditary myocardial lesion normally seen in the BIO 14.6 myopathic hamster is singularly resistant to the overdosage of adrenocortical hormones (see Table I). In this connection, it should be recalled that in these genetically afflicted hamsters the myocardial pathology is characterized by spotty myolysis; these spontaneous lesions are indistinguishable on histological preparations from those elicited by the administration of adrenaline or noradrenaline (7). In fact, treatment with catecholamines markedly enhances both the speed of development and intensity of cardiac lesions in the myopathic hamster (5); yet the administration of corticoids (either mineralocorticoids or glucocorticoids) is effective only in aggravating the severity of the catecholamine-induced heart muscle lesions (36) and not the histologically identical spontaneous myocardial pathology of the hamster. There is little doubt that the prolonged administration of large doses of corticoids—as given in the first experimental series of the present studies—induces potassium depletion. Therefore, the resistance of the spontaneous heart disease of the hamster to corticosteroids is even more interesting, since our earlier studies have shown that a marked and rapidly fatal aggravation of this myocardial pathology occurs within a few days after a reduction in dietary potassium intake (5). The present experiments were not performed with the intention of analyzing the reasons for the above discrepancies. In any event, the fact that neither adrenalectomy nor the administration of corticoids influenced the onset and development of the spontaneous myocardial pathology in the hamster provided an opportunity to study the role of the adrenal cortex in the pathogenesis of congestive heart failure which invariably occurs in these diseased animals.

It is important to stress that in one of our experimental series the adrenals were surgically removed and the corticoid treatments were initiated at a time when heart failure was not yet present in the myopathic animals (see Table II), while the other, otherwise comparable, study was started in animals already showing mild to moderate degrees of congestive failure (see Table III). Thus, the results of the first-mentioned investigations should be interpreted as indicating the influence of varying amounts of a mineralocorticoid, a glucocorticoid, or both types of corticoids, upon the onset of generalized venous congestion. On the other hand, the findings in the latter experimental series merely indicate the effects of the induced corticoid imbalances upon the maintenance and progression of edema and other manifestations of congestive heart failure.

It is clear from the observations described in the present paper that the presence of aldosterone or any other mineralocorticoid hormone is not essential to the appearance of congestive heart failure in the myopathic hamster. Equal degrees of edema and other manifestations of passive venous congestion have been seen in adrenalectomized animals maintained on small doses of either a glucocorticoid (triamcinolone), or a mineralocorticoid (aldosterone). However, when animals already in heart failure were adrenalectomized and injected with maintenance doses of corticoids, the presence of aldosterone resulted in a more severe circulatory congestion than that caused by the presence of triamcinolone; this suggests an increased sensitivity of the hamster with cardiac failure to the edema-promoting action of aldosterone. It is also significant that in both experimental series the effect of aldosterone proved to be not dependent upon the injected amounts of this steroid; the groups receiving merely maintenance doses revealed the same degree of congestion as those treated with excessive doses.

There are several observations in the literature which may be cited to support the assumption that aldosterone does not play a primary role in the development of cardiac edema and that this conclusion may be applicable, not only to the spontaneous heart disease of the hamster, but also to similar conditions occurring in man.

Despite the well-established fact that hypersecretion of aldosterone occurs in some patients with congestive heart failure, the plasma and urinary levels of this corticoid appeared to be within the normal range in more than half of the cases studied (34, 39, 41). It was reasoned that if aldosterone were playing a primary role in the edema of congestive heart failure, then the administration of anti-aldosterone compounds to such patients should induce diuresis. However, only inconsistent and largely disappointing results were obtained with therapeutic trials using either spiro lactones, which are capable of blocking the action of aldosterone on the renal tubules, or amphetamine, which interferes with the biosynthesis of adrenocorticoids (2, 33, 34, 37). Moreover, observations on untreated patients with Addison's disease complicated by congestive heart failure (15) may be taken to suggest that the edema of congestive heart failure can occur in the absence of appreciable amounts of mineralocorticoids. It is also known that hypersecretion of aldosterone in primary aldosteronism (13) and the exogenous administration of mineralocorticoids to normal subjects (1, 16, 27, 32) do not produce edema, even when the sodium intake is maximally increased.

That the presence of some aldosterone is necessary for edema formation has been assumed on the basis of observations made in patients with ascites following adrenalectomy (23, 30) and from findings on dogs with experimentally induced heart failure (17-20). Negative water and sodium balance resulted when the adrenals were removed from edematous humans and dogs, and a positive balance associated with fluid accumulation in the abdominal cavity resulted when mineralocorticoids were given. These findings were interpreted to indicate that the amount of a mineralocorticoid sufficient to maintain an adrenalectomized man or dog in sodium balance is required for the formation of edema in certain diseases. However, a closer analysis of the above-mentioned studies suggests that probably only a reduction in, and not a complete disappearance of, edema ensued in the subjects following adrenalectomy and that this latent edema was aggravated by mineralocorticoid administration. This interpretation, if applicable, would be more compatible with the results of the present studies. Unfortunately, NaCl supplements (thus, no hormone therapy) failed to maintain the lives of adrenalectomized hamsters with heart disease for an adequate period; hence, the question whether corticoids are really essential for edema formation cannot be answered. Nevertheless, it is clear that in the adrenalectomized hamster, maintenance doses of a glucocorticoid are as effective in permitting the occurrence and progression of generalized venous congestion as are maintenance doses of a mineralocorticoid.

It has been shown by various groups of investigators that in dogs with heart failure the renal tubules are abnormally sensitive to exogenous mineralocorticoids (9, 10, 38), and some of our observations may be explained on this basis. In the adrenalectomized hamster already in heart failure, maintenance doses of aldosterone resulted in a more rapid progression of venous congestion than did maintenance doses of triamcinolone. However, positive correlation between the degree of edema and the amount of corticoid given was observed only in the case of triamcinolone; this suggests that the circulating amount of aldosterone *per se* is of little importance in this respect.

One of the most satisfactory results of the present investigations is the demonstration that the degree of generalized venous congestion was constantly less marked when triamcinolone and aldosterone were both administered to the adrenalectomized animals than when either of these corticosteroids was injected alone. The fact that an interaction between glucocorticoids and mineralocorticoids is important in many physiological and pathological processes has been repeatedly

demonstrated (3). Furthermore, it has been suggested that imbalance between the concentrations of glucocorticoids and mineralocorticoids (normal glucocorticoid secretion and high mineralocorticoid secretion), rather than absolute levels of the individual steroids, may be a significant factor in fluid retention (8). It is also known that in clinical edematous states the administration of glucocorticoids often results in diuresis (34). Moreover, it should be kept in mind that not only the mineralocorticoids but also the glucocorticoids exert effects on the electrolyte metabolism; there are observations to indicate that glucocorticoids are apparently a vital part of the mechanisms which maintain the internal water and electrolyte balance of the organism, although the precise role they play in this respect has not yet been established. In any event, in adrenalectomized animals the administration of glucocorticoids in addition to mineralocorticoids is necessary for the return of the renal function to normal (3).

Our observations permit the conclusion that maintenance of cardiopathic hamsters on a proper mineralocorticoid-glucocorticoid balance is of preventive value as regards the subsequent development of congestive heart failure, a finding worthy of further exploration.

### **Abstract**

The existence in an inbred strain of Syrian hamsters of a genetically transmitted (or conditioned), degenerative cardiomyopathy that terminates in congestive heart failure offers a unique opportunity to study the importance of the adrenal cortex in the mechanism of edema formation and of other manifestations of generalized venous congestion of cardiac origin. Since it is believed that the myocardial degeneration itself is responsible for the subsequent occurrence of congestive heart failure in these diseased animals, it is of special importance that neither adrenalectomy nor the administration of corticosteroids, such as triamcinolone, hydrocortisone acetate, aldosterone, and desoxycorticosterone acetate exerted any significant influence upon the onset, progression and healing of the spontaneous heart-muscle lesions.

Since adrenalectomized hamsters with the spontaneous heart disease could not be kept alive for a sufficient period by NaCl supplements alone, the question whether corticoids *per se* are essential for edema formation remained unanswered. Nevertheless, it is clear from the observations described that the presence of aldosterone or any other mineralocorticoid is not essential to the appearance of congestive heart failure in this hamster. Equal degrees of edema and other manifestations of passive venous congestion were seen in adrenalectomized animals maintained on small doses of either a glucocorticoid (triamcinolone) or a mineralocorticoid (aldosterone). However, in adrenalectomized animals already in heart failure, the administra-

tion of aldosterone resulted in a more severe circulatory congestion than did treatment with maintenance doses of triamcinolone; this suggests an increased sensitivity to the edema-promoting action of the mineralocorticoid. Thus, while hamsters with heart failure are able to accumulate edematous fluids in the absence of mineralocorticoids, they develop more complete fluid retention in the presence of small amounts of aldosterone. A positive correlation between the degree of generalized venous congestion and the amount of corticosteroids administered was seen in animals injected with triamcinolone, but not in those treated with aldosterone alone.

One of the most interesting results of the present investigations was the demonstration that the degree of generalized venous congestion was constantly less marked in the adrenalectomized animals when triamcinolone and aldosterone were both administered than when either of these corticoids was injected alone. Moreover, it appears reasonable to suggest that the maintenance of the cardiopathic hamsters on a proper mineralocorticoid-glucocorticoid balance is of preventive value as regards the subsequent development of congestive heart failure. The possible significance of these observations in the light of pertinent literature data has been discussed.

### Abrégé

L'existence d'une souche consanguine de hamsters de Syrie, ayant une cardiomyopathie par transmission génétique (ou conditionnée) qui évolue invariablement vers la décompensation cardiaque, nous fournit une occasion unique d'étudier le rôle du cortex surrénalien dans la formation d'oedèmes et des autres manifestations associées à une congestion veineuse généralisée d'origine cardiaque. Comme il est établi que la dégénérescence du myocarde est elle-même responsable de l'apparition subséquente de la défaillance cardiaque chez ces animaux malades, il s'avère important que ni la surrénalectomie ni l'administration de corticostéroïdes, comme la triamcinolone, l'acétate d'hydrocortisone, l'aldostérone et l'acétate de désoxycorticostérone n'exercent aucune influence sur l'apparition, la progression et la guérison des lésions spontanées du muscle cardiaque.

L'impossibilité de maintenir en vie pour une période suffisamment longue avec un supplément de chlorure de sodium des hamsters surrénalectomisés, ayant une maladie cardiaque spontanée, soulève la question du rôle essentiel des corticoïdes dans la genèse de l'oedème. Toutefois, il semble évident, en se basant sur nos observations, que la présence de l'aldostérone ou tout autre minéralo-corticoïde n'est pas essentielle à la genèse de la défaillance cardiaque chez ces hamsters. L'oedème et les autres manifestations reliées à la congestion passive veineuse étaient en effet également démontrables chez des animaux surrénalectomisés maintenus avec de faibles doses d'un glucocorticoïde (triamcinolone) ou encore d'un minéralo-corticoïde (aldostérone). Cependant, chez les animaux surrénalectomisés déjà décompensés, l'administration d'aldostérone a eu pour effet de produire une congestion circulatoire plus grave que celle qui

résulte de l'administration de triamcinolone à doses de maintien. Cette observation laisse supposer une sensibilité accrue à l'action oedématogène du minéralo-corticoïde. Par conséquent, bien que des hamsters défaillants soient capables d'accumuler du liquide d'oedème en l'absence de minéralo-corticoïde, leur potentiel de rétention s'en trouve accru par la présence de petites quantités d'aldostérone. Il fut possible d'établir une relation définitive entre le degré de congestion veineuse généralisée et la quantité de corticos-téroïde injecté aux animaux traités par la triamcinolone, mais non chez ceux ayant reçu de l'aldostérone seule.

L'observation qui nous a paru la plus intéressante au cours de ces recherches, se rapporte au fait que le degré de congestion veineuse généralisée était toujours moindre chez les surrénalectomisés lorsque la triamcinolone était associée à l'aldostérone que lorsque chaque corticoïde était injecté seul. Au surplus, il semble raisonnable de supposer que le maintien des hamsters cardiopathes par une balance appropriée de minéralo-glucocorticoïdes a une valeur préventive, à tout le moins en ce qui concerne le développement éventuel de la défaillance cardiaque. La signification possible de ces observations est discutée en regard des données de la littérature sur cette question.

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**J. M. Dieudonné:** En utilisant un matériel animal remarquablement propice à cette étude, monsieur Bajusz a exploré le rôle de l'aldostérone dans la genèse des oedèmes d'origine cardiaque. Ses conclusions furent négatives. La seule suggestion d'effet en fut une d'accentuation d'oedème déjà existant dans le cas spécifique d'animaux

surrénalectomisés en décompensation. Monsieur Bajusz a également insisté sur le fait que, chez les animaux cardiomyopathes, l'administration conjointe des hormones substitutrices inhiba de manière plus prononcée le développement des oedèmes que l'administration séparée de chaque hormone.

A titre de physiologiste intéressé à la mécanique ventriculaire, il m'est difficile d'exposer une quelconque opinion circonstanciée sur les effets ioniques de l'interaction hormonale surrénalienne, que ce soit par exemple sur le volume plasmatique, la pression artérielle ou sur la résistance artériolaire d'animaux surrénalectomisés. Dès lors, toute interprétation des phénomènes observés ne peut être que globale, ainsi que nous l'a, de fait, proposée monsieur Bajusz.

La genèse des oedèmes cardiaques s'explique par des facteurs purement mécaniques. En 1948, à partir d'études sur territoire vasculaire isolé de chien ou de chat, Pappenheimer et Soto Rivera (*Am. J. Physiol.*, 152:471, 1948), ont réussi à décrire la pression capillaire en fonction des paramètres hémodynamiques artériolaires et veinulaires selon la formulation suivante:

$$P_c = \frac{R_v P_a + R_a P_v}{R_a + R_v}$$

Dans cette équation, P et R sont pression et résistance tandis que les désinences a et v se réfèrent à artériolaire et veinulaire. Vu que les résistances et pressions veinulaires sont nettement inférieures à leurs équivalents artériolaires, il s'ensuit qu'une augmentation de pression veinulaire aura pour effet d'augmenter la pression capillaire de filtration, aux dépens de celle de réabsorption, de manière beaucoup plus prononcée qu'une augmentation équivalente de pression artériolaire. Mais, dans les cas de pression veinulaire augmentée, par exemple, dans les parties déclives lors d'une défaillance cardiaque, l'oedème en voie de formation par suite de la prédominance de la filtration capillaire tendra à se stabiliser à un certain degré, un peu à la manière d'un thermostat, selon le degré d'accroissement de la pression interstitielle causé par l'oedème lui-même et qui tendra à diminuer la pression effective de filtration mais aussi selon, avec un effet opposé, l'efficacité du drainage lymphatique. Ce schéma général ne laisse aucune place à une intervention hormonale hypothétique dans la genèse des oedèmes cardiaques. Ceci ne veut pas dire qu'une intervention hormonale ne puisse jamais exister de fait mais tout simplement qu'à l'heure actuelle on ne voit pas la nécessité d'admettre cette

intervention. Il faudrait pour cela montrer que dans les conditions de défaillance cardiaque contrôlée par quelque paramètre hémodynamique, l'oedème n'apparaîtra pas en l'absence de la seule hormone soupçonnée. Il convient de noter à cet égard que l'extirpation des surrénales non seulement supprime la source des hormones corticales et médullaires, mais aussi modifie certainement les données hémodynamiques inhérentes à la défaillance cardiaque. En conclusion, l'interprétation globale proposée par monsieur Bajusz semble justifiable et s'insère très bien dans le cadre classique de la microcirculation.

## **The Cause of Coronary and Cerebral Artery Thrombosis in Man**

P. CONSTANTINIDES

**I**T is rather astonishing that although coronary thrombosis represents the most frequent cause of death for middle-aged adults in our society, our concepts of the pathogenesis of this deadly accident have been, until very recently, purely speculative.

Apart from certain epidemiological correlations with sex, smoking, hypertension and blood lipids, the only two definite facts known about coronary thrombosis were: (a) that it occurs almost exclusively in arteries with advanced fibrous atherosclerosis—practically never in normal vessels, and (b) that it is very frequently accompanied by hemorrhages within the plaques of these atherosclerotic arteries. However, the actual mechanism through which thrombosis is initiated in atherosclerotic vessels remained shrouded in mystery.

It is, thus, not surprising that various theories sprang up, each attempting to explain this phenomenon in a different way. Coronary thrombosis was attributed in turn to stasis, hypercoagulability of the blood, the rupture of tiny capillaries that grow into plaques from the lumen, and breaks or ulcerations of atheroma surfaces—although none of these theories had yet been proven to be true.

The concepts of stasis, hypercoagulability and capillary hemorrhages were purely hypothetical; breaks and ulcerations were very convincing as inducers of thrombosis whenever they were found, but unfortunately they had so far been found in only a minority of all the cases studied—in about 25% or less of all the published series. We must consider, however, that most of the histological studies of the past were done with just a few random sections or, at best, with large-interval step-serial sections (every 1 or 5 mm), so that under these circumstances only the

biggest breaks would be detected—all the small ones could be missed.

Unfortunately, until recently, experimental work contributed little to the testing of these theories, because we did not know how to reproduce, in animals, the specific anatomical environment in which human thrombosis occurs, i.e., advanced fibrous atherosclerosis.

Seven years ago, we developed in our laboratory a technique for the production of lesions with all the features of highly advanced human atherosclerosis in rabbits. These experimental atheromata had a thick fibrous (collagenic) capsule, crystalline gruel, calcification, capillarization, lymphocytic infiltration and giant cells, exactly like their human counterparts (7).

We then found that we could induce thrombi in the aorta and the coronaries of such rabbits if we exposed them to a sudden episode of hypertension combined with the injection of chemicals that caused hypercoagulability and attacked the endothelium, e.g., with the combination of i.v. epinephrine and i.p. Russel's viper venom (8, 9).

Now, the most remarkable thing about the thrombi obtained in this manner was that they reproduced precisely the characteristics of human coronary thrombosis; they could only be induced in animals whose arteries exhibited fibrous atherosclerosis or other wall injury—never in rabbits with normal arteries—and they were intimately associated with plaque hemorrhages (4).

Histological study showed that most of the hemorrhages had developed through breaks of plaque surfaces by an inrush of blood from the lumen (4). It was evident that our experimental maneuver had stretched and cracked the rigid collagenic wall of the atherosclerotic vessels and that the resulting breaks were sealed by thrombi after first allowing some blood to penetrate into the wall. It was also probable that we could not elicit such breaks in normal arteries because their wall was elastic enough to stretch without cracking. (For documentation see Figures in Refs. 4, 8 and 9.)

Inevitably, the next question that arose was whether human coronary thrombosis was initiated by cracks of atheroma surfaces

as well. To find out, we made for the first time a complete serial section study of 20 consecutive cases of coronary thrombosis that came for autopsy at Barnes Hospital in St. Louis three years ago. We discovered that every thrombus was anchored in cracks of the surrounding atherosclerotic wall and that most of the underlying plaque hemorrhages could be traced to an entry of blood through the same breaks (2, 3, 5).

Our results have since been confirmed by three other investigators (1, 10, 11) and, moreover, we obtained the same findings when we made a serial section study of ten consecutive cases of cerebral artery thrombosis last year in Vancouver (6). (For documentation see Figures in Refs. 5 and 6.)

We conclude that thrombosis in the major arteries of the heart and the brain is initiated primarily by breaks of the atherosclerotic or fibrosed wall of these vessels, even though the speed of development, the ultimate size, and the persistence or disappearance of the resulting thrombi may well depend on other systemic factors.

Having established that, our next most important problem is to find what causes these breaks of human plaque surfaces. Theoretically, fractures of the atherosclerotic arterial wall could be the result of any one or a combination of the following four factors: 1) sudden changes of intra-arterial pressure that would crack the wall of an altered, fragile artery; 2) changes in blood chemistry leading to the accumulation of certain chemicals that attack the endothelial lining or "unzipper" intercellular junctions, promoting wall disintegration (e.g., enzymes, amines, antigen-antibody complexes); 3) changes in the plaque itself, leading to increased fragility or spontaneous disintegration of the plaque capsule, e.g., a gradual cell depopulation resulting in the breakdown of the collagen and polysaccharide produced and maintained by the cells, molecular ageing of the plaque collagen such as increasing cross-linkages and contraction, etc.; and 4) external forces acting on the brittle artery, such as the rhythmic bending and torsion of the proximal coronaries with every heart beat.

More research with experimental models and their human prototypes will probably solve this last problem in future years.

### Abstract

Certain systemic precipitating factors were found to induce, in the arteries of animals exhibiting advanced atherosclerosis, large thrombi attached to hemorrhagic fibrous plaques, just as they occur in spontaneous human coronary thrombosis. Histological study indicated that hemorrhages had taken place through breaks of the plaque surfaces from the lumen, and that the thrombi developed as physiological seals over these fissures.

To find out whether a similar fissure mechanism is responsible for the thromboses and associated plaque hemorrhages that occur in human arteries, complete serial section studies of the occluded arterial segments from 20 consecutive cases of coronary thrombosis and from 10 consecutive cases of cerebral artery thrombosis were made.

It was discovered that all thrombi were caused by breaks of the atherosclerotic or fibrous arterial wall, and that most hemorrhages had taken place through the same breaks from the lumen.

It is concluded that thrombosis in the large arteries of the human heart and brain is initiated by the fracture of the altered wall of these vessels, even though the size and the fate of the resulting thrombi may well depend on other factors.

### Abrégé

Nous avons constaté que certains facteurs précipitants systémiques provoquent chez les animaux dont les artères montrent des lésions d'athérosclérose avancée, de gros thrombi greffés sur des plaques fibreuses et hémorragiques, tout comme dans les thromboses coronaires spontanées de l'homme. Une étude histologique nous a révélé que les hémorragies ont pris naissance au site de rupture des plaques et que ces thrombi tiennent lieu de ciment physiologique en regard de la lumière des vaisseaux.

Afin de vérifier si un processus similaire de fissures contribue à la production de thromboses associées aux plaques hémorragiques présentes dans les artères chez l'homme, des coupes séries d'artères obliterées furent étudiées chez 20 cas de thrombose coronaire et 10 cas de thrombose des artères cérébrales. Il fut observé que tous les thrombi résultaient d'une rupture de parois artérielles athérosclerotiques fibreuses et que, dans la plupart des cas, les hémorragies originaient au site de ces mêmes ruptures à l'intérieur de la lumière des vaisseaux. Nous concluons que les thromboses dans les gros vaisseaux du myocarde et dans le cerveau font suite à des fractures de leurs parois altérées, même si la dimension et le devenir de ces thrombi peuvent aussi dépendre d'autres facteurs.

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**Y. Lemire:** Since the pathogenesis of coronary (and cerebral) artery occlusion is largely unknown, most of our therapeutic measures seem to be purely empirical. This is the main reason why clinicians are looking forward with keen interest to new developments that could throw some light on the mechanism of this disease. At the time of diagnosis, a coronary patient usually presents in his arteries some diffuse, severe, and probably irreversible changes that started very early in life. At this late stage, the most we can expect in trying to correct the factors possibly correlated with the disease is to delay its fatal issue. Of course, this is far from being satisfactory.

Clinical research for coronary heart disease has now a tendency to be prospective or cross-sectional, consisting of long term studies of various parameters such as age, sex, diet, physical fitness, blood pressure, and other biologic and psychologic data. Applied to a large group of initially normal subjects, these studies go on for years and will possibly reveal some of the predisposing factors in those subjects who will develop coronary disease during the observation period. Such a project concerning 1,000 civil employees, all of them French Canadian, was undertaken 5 years ago at the Montreal Heart Institute, but to draw definite conclusions from these studies would be premature.

Whatever the interest of clinical surveys, the principal approach to the problem lies in experimental and basic research concerned with the underlying causes of atherosclerosis and thrombosis, as well as with the role of mechanical, biologic, and hematologic factors involved in this histopathologic process. The production, in animals, of vascular atherosclerosis and thrombosis comparable to the lesions observed in man is the first prerequisite before any progress in the understanding of the disease can be made. Dr. Constantinides' demonstration that arterial thrombosis is initiated primarily by breaks of atherosclerotic fibrosed plaques was achieved by him in experimental animals and later confirmed in almost all of the cases of cerebral and coronary thrombosis he studied in man. It was known that breaks in the atheroma surface could cause thrombosis in arteries. But the merit of Dr. Constantinides' work is that it demonstrates clearly that a very high percentage of coronary and cerebral thrombosis in man can be initiated by fissures of atherosclerotic plaques. A constant finding according to Dr. Constantinides is the association between thrombosis and the intimal hemorrhage said to be produced by intrusion of circulating blood through the ulcerated atherosclerotic plaque. It is known that atherosclerotic changes of the vascular intima lead to the formation of new capillaries derived in part from vasa vasorum and in part from the lumen of the artery itself, and many authors hold that intimal hemorrhages result directly from the rupture of capillary ramifications in atherosclerotic plaques. Therefore, there is some disagreement as to whether the intimal hemorrhages initiate the intimal fissure and subsequent thrombosis, or whether the intimal hemorrhages represent the entrance of blood from the arterial lumen by way of the fissure.

Whatever its mechanism, intramural coronary hemorrhage is of interest for the cardiologist treating coronary patients with anticoagulants. Is this treatment liable to increase the frequency of intraparietal hemorrhage? Could this bleeding in atheromatous plaques lead to more frequent episodes of coronary occlusion in anticoagulant-treated patients? Histologic studies available in this domain have failed to reveal any aggravation of the condition in patients subjected to anticoagulant therapy. This has also been confirmed indirectly by a great number of clinical studies conducted on a large series of patients treated with anticoagulant drugs during the past 20 years. The majority of such studies resulted in data attesting to a notable improvement in the prognosis of coronary patients. We share this opinion, on the basis of a retrospective study of 1,237

coronary patients subjected to long or short term anticoagulant therapy in our Institute during the last 10 years. In the first group of 240 subjects with impending myocardial infarction (or sub-acute coronary disease), as well as in the other 997 cases of acute myocardial infarction, we were able to observe a reduction in the mortality rate as well as a decrease in thromboembolic complications. In addition, none of the relatively few investigators who have concluded that anticoagulants are of no significant value in coronary disease have reported that it can aggravate the course of the disease.

The complete understanding of the interrelations between atherosclerosis and thrombosis is essential if we are to succeed in our attempts to treat coronary disease. Dr. Constantinides' paper on the initiating mechanism of coronary and cerebral thrombosis in man appears to be a valuable scientific contribution in this field. Nevertheless, for clinicians, the most interesting part of this study would be the next step. In order to be able to administer appropriate therapy, we need to know what causes this breakage of the plaque surface. In the meantime, reduction of blood lipids by dietary manipulations, to possibly decrease further evolution of the atherosclerotic plaques and perhaps also the susceptibility to thrombosis, would seem to be, like anticoagulants, the only treatment presently available.

**P. Constantinides:** Capillary hemorrhage was mentioned as a possibility to produce hemorrhages in the plaques. This idea was initiated by Dr. Patterson, who was the first pathologist to point out that thromboses are associated with hemorrhages. But then he went on to explain this association in the wrong way. He thought that it was the capillaries of the plaque that ruptured, causing the thrombi. I must say that I don't believe that, because it was brought up by Dr. Lemire. I must say that nobody has proven that capillary hemorrhage has caused the thrombi or even breaks of the plaque surface—not even the initiator of this theory. I was the first one to do complete serial sections and I found unequivocal capillary hemorrhages in four cases, but in none of these four cases was there a thrombus over the capillary hemorrhage. When there was a thrombus, there was a break—but no capillary hemorrhage. If there was an association I should have picked it up, because I sectioned the whole vessel from one end to the other. Neither did any of the other investigators (Prof. Sinapis, Dr. Chapman or Dr. Friedman) find any association between thrombi and capillary hemorrhages. They did step-serial sections in another 80 cases.

# **Sensitization by Renal Hypertension for the Production Of Thrombohemorrhagic Lesions with Endotoxin**

MARC CANTIN

THE generalized Sanarelli-Shwartzman reaction, which can be easily elicited in rabbits by two properly spaced injections of endotoxin, is notoriously difficult to induce in the rat by a similar method (1, 19). The administration of endotoxin in the latter species is followed by the occurrence of hepatic and pulmonary thrombi as well as by renal congestion, but not by renal cortical necrosis and thrombosis, which are considered the hallmark of the generalized Shwartzman phenomenon (3, 12). In the rat, it is only under certain conditions such as pregnancy (19) or with the concomitant administration of a high molecular weight acidic polymer, sodium polyanetholsulfonate (6), that a single injection of endotoxin results in thrombohemorrhagic lesions analogous to those seen in the rabbit.

The object of this communication is to describe renal cortical necrosis and thrombohemorrhagic lesions of various organs, produced by a single intravenous injection of endotoxin in rats rendered hypertensive by partial ligation of the aorta between the renal arteries. The implications of these observations are discussed.

## **Materials and Methods**

Ninety-four female rats of the Sprague-Dawley strain (Robidoux Animal Laboratory Farm, St-Constant, P.Q.) with a mean initial body weight of 199g (range 190-210g) were divided into eight groups and treated as indicated in Tables I and II. Throughout the experiments, the animals were maintained on Purina Laboratory Chow (Purina Company of Canada) and tap water.

The operations were performed under ether anesthesia on the first day of the experiments. In the majority of animals (Table I, groups 3 and 4; Table II, groups 1 to 4) an acute type of hyper-

tensive vascular disease was produced by aortic stenosis. The technique consists in partial ligation of the aorta (using a silk thread and the stylet of a No. 21 subcutaneous injection needle) between the origins of the renal arteries, and ligation of the left ureter. Because of the aortic ligation, the renal arterial pressure becomes equal to or lower than filtration pressure; the left kidney thus loses its excretory function and is transformed into a purely endocrine organ, a situation analogous to that obtained with a Goldblatt kidney (11, 17). In other animals (Table I, group 2), the left kidney was removed. Here also, the aorta was ligated as outlined above, in order to obtain non-hypertensive rats in otherwise comparable circulatory conditions.

Starting on the ninth day, the animals were individually housed in metabolism cages. The twenty-four-hour urine output (from the ninth to the tenth day) of the various groups of animals can be found in the Tables. Blood pressure was recorded on the tenth day, under ether anesthesia, by inserting a No. 22 injection needle, connected to a mercury manometer, into the left common carotid artery. Immediately afterwards, the animals were injected with *Shigella flexneri* endotoxin (Difco) in 1 ml of water in the jugular vein, at the dosages indicated in the Tables. All experiments were terminated twenty-four hours after the injections by killing the survivors with chloroform. At autopsy, the lesions were estimated in terms of an arbitrary scale in which 0 = no lesion, 1 = just detectable, 2 = moderate, and 3 = most severe lesions. The accuracy of these readings was verified by examination of histological preparations. The means of all these observations with their standard errors are listed in the Tables. Specimens of the affected organs were fixed in Susa solution saturated with picric acid for subsequent embedding in paraffin. The hearts were fixed in 10% formalin for twenty-four hours, weighed on an analytical balance, and then transferred to a solution of Susa saturated with picric acid. All sections were stained according to the following techniques: hematoxylin and phloxine, periodic acid Schiff, and phosphotungstic acid hematoxylin (PTAH).

### Results

The animals bearing an endocrine kidney lost weight, developed hypertension, and had a greater urine flow than either

TABLE I  
INFLUENCE OF RENAL HYPERTENSION ON THE PRODUCTION OF THROMBOHEMORRHAGIC LESIONS BY ENDOTOXIN

Group	Treatment*	Survival Ratio†	Final Body Weight (g)‡	Urine Flow (ml per Day)	Blood Pressure (mm HG)	Weight (mg 100g)	Heart Hemorrhage			Adrenal Necrosis			Cortical Necrosis and Thrombosis and Thrombosis (Right Kidney)		
							In- tensity (0-3)	In- tensity (0-3)	In- tensity (0-3)						
1	.....	10:10	215 ± 6.1	13 ± 1.6	105 ± 3.2	448 ± 17.1	0	0	0	0	0	0	0	0	0
2	Endocrine kidney removed	11:11	209 ± 7.3	15 ± 1.3	114 ± 4.5	456 ± 16.9	0	0	0	0	0	0	0	0	0
3	Endocrine kidney .....	5:13	162 ± 8.9	46 ± 6.1	162 ± 4.1	540 ± 23.4	0.8 ± 0.42	31	1.5 ± 0.29	77	1.7 ± 0.33	77			
4	Endocrine kidney .....	5:12	164 ± 5.1	39 ± 4.9	—	550 ± 25.2	0.7 ± 0.39	25	1.3 ± 0.33	58	1.8 ± 0.30	84			

\* In addition to the treatments listed in this column, all the animals received 300 $\mu$ g of endotoxin per 100g body weight.

† The values for final body weight, urine flow, blood pressure, and heart weight in groups 3 and 4 are statistically significant ( $P < 0.01$ ) in comparison with those of groups 1 and 2.

absolute controls (Table I, group 1) or rats in which the left kidney was removed at the time of surgery (Table I, group 2). The heart weight, which is considered a reliable index of diastolic hypertension, was higher in endocrine-kidney-bearing rats than in other groups of animals.

In the hypertensive animals, administration of endotoxin resulted in diarrhea, prostration, evidence of shock and oliguria. In several rats, the urine became dark red. The control animals only became prostrated for a short period.

Since some of the effects of endotoxin might have been altered by ligation of the left carotid artery following blood pressure recording, a group was added in which blood pressure was not taken (group 4). As shown in Table I, both groups of rats bearing an endocrine kidney and injected with 300 µg of *S. flexneri* endotoxin per 100g body weight presented essentially similar lesions whether the carotid artery was ligated or not: they developed a high incidence of unilateral (right) renal cortical necrosis and thrombosis (from 77 to 84%) and of bilateral adrenal cortical necrosis and hemorrhage (from 58 to 77%), with a lesser incidence of cardiac hemorrhages (from 23 to 31%). On the other hand, the injection of proportionately the same quantity of endotoxin to normal animals or to those in which the left kidney was removed did not have any of these effects.

The intensity of lesions in the right kidneys was generally proportional to the length of the survival period following the injection of endotoxin. In the animals that survived less than ten hours, no lesions could be macroscopically detected. In several instances, however, the glomerular capillaries were filled with fibrin deposits, and small foci of tubular necrosis were evident. In the animals that survived more than ten hours, grayish irregular patches of cortical necrosis became visible. When the survival period exceeded twenty hours, the surfaces of the right kidneys in which cortical necrosis was observed exhibited the generalized mottled appearance (Fig. 1) characteristic of that lesion in rabbits. Microscopically, the cortices showed either focal or diffuse involvement. The cytoplasm of cortical tubules exhibited all degrees of changes from hyaline degeneration to complete disaggregation. The nuclei were pycnotic, karyorrhectic, or absent. The medul-

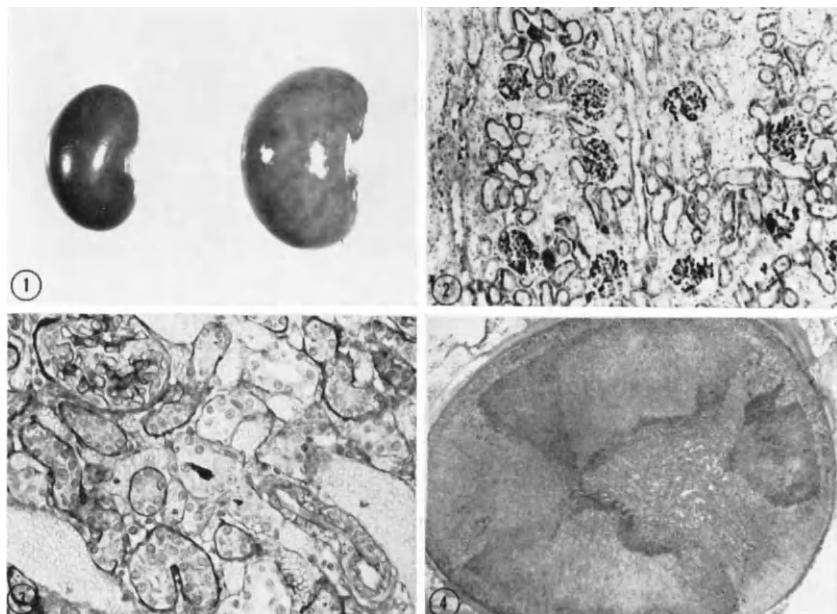


FIGURE 1. Kidneys of a hypertensive rat killed twenty-four hours after injection of endotoxin. Mottled appearance of the right kidney due to cortical necrosis. The left (endocrine) kidney, although in this case not markedly atrophic, shows no sign of necrosis.

FIGURE 2. Section through infarcted right kidney. Fibrin thrombi are present in every glomerulus. Poorly delimited areas of tubular necrosis.

(PTAH  $\times 75$ .)

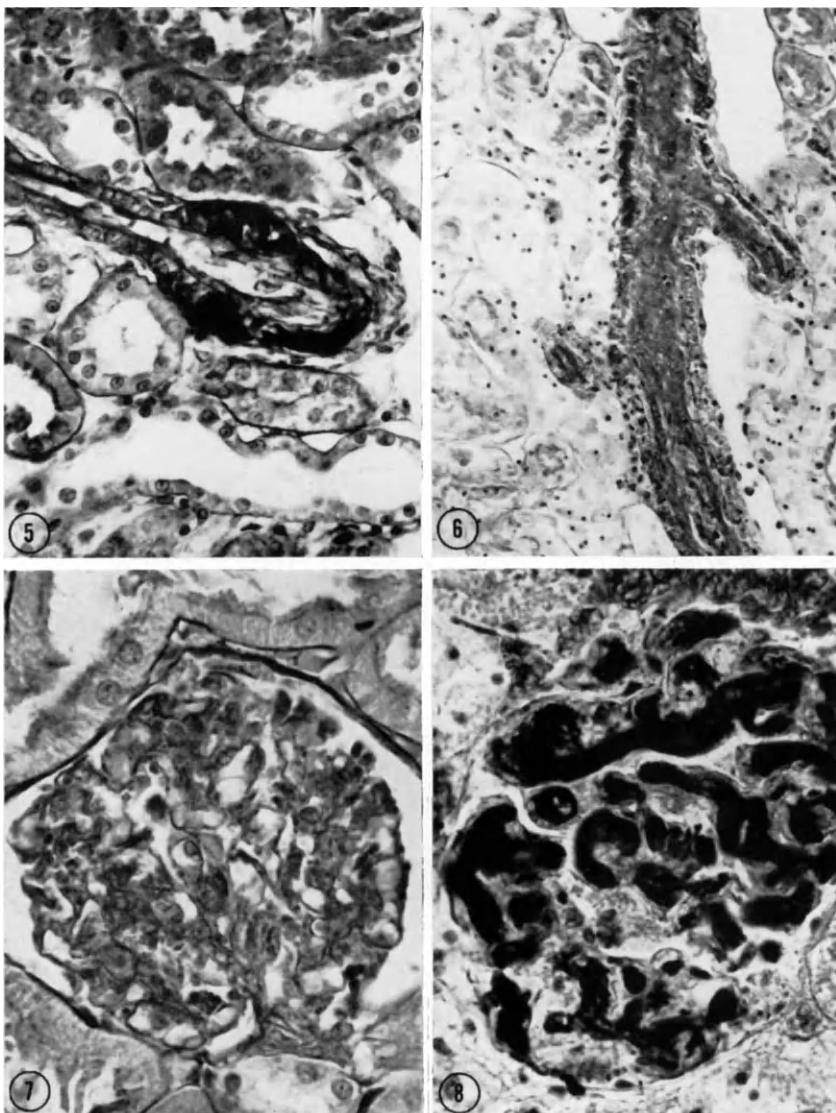
FIGURE 3. Congested endocrine kidney of animal given endotoxin. Atrophy of glomerulus (*upper left*) and of tubules. Absence of arterial lesions (*lower right*), fibrin thrombi, or necrosis. (PAS  $\times 200$ .)

FIGURE 4. Adrenal of a hypertensive, endotoxin-treated rat; large foci of hemorrhage and necrosis partially surrounded by dark rim of leukocytes. The medulla and part of the glomerulosa are spared. (HP  $\times 25$ .)

lary tubules were filled with casts. The necrotic process extended to the walls of arteries and arterioles, which were also infiltrated by red blood cells, and to glomeruli, which were often hemorrhagic. Fibrin thrombi were found in arteries, in arterioles, including afferent glomerular arterioles, and in glomerular cap-

FIGURE 5. Focal medial necrosis of arterial wall of kidney contralateral to an endocrine kidney. No other treatment. (PAS  $\times 300$ ). →

FIGURE 6. Cortical necrosis of the right kidney. Large fibrin thrombus filling



the lumen of an artery. The wall shows fibrinoid necrosis in the left upper portion and necrosis with hemorrhage in its lower portion. Surrounding tubules are necrotic. (PAS  $\times 300$ .)

FIGURE 7. Enlarged glomerulus from the kidney contralateral to an endocrine kidney. No other treatment. Focal thickening and disruption of membranes, with dense deposits of PAS-positive material. (PAS  $\times 600$ .)

FIGURE 8. Glomerulus from a necrotic kidney. Fibrin thrombi occlude most of the capillaries. (PTAH  $\times 630$ .)

illaries (Figs. 2, 6, and 8). In contrast, the left (endocrine) kidney of these animals, apart from the well-known atrophy, showed only congestion without evidence of either thrombosis or necrosis.

In the animals that died less than ten hours after injection, the wall of the small intestine was often focally or entirely dark red. The atrophic thymus of the animals that died early was also hemorrhagic. Microscopically, the vessels of the intestinal mucosa were dilated and filled with red blood cells. A series of alterations ranging from epithelial desquamation, to focal areas of necrosis and hemorrhage, to complete necrosis of the entire wall were present; however, no thrombi could be detected in the vessels. In the animals that survived longer than ten hours, no intestinal or thymic lesions were seen.

The adrenals were grossly hemorrhagic. Under the microscope they showed large foci of necrosis and hemorrhage surrounded by polymorphonuclear leukocytes. The medulla and often part of the glomerulosa were spared (Fig. 4). Fibrin thrombi were less abundant than in the kidneys and were located in the sinusoids of the glomerulosa or of the external fasciculata. The hearts in some instances showed large foci of hemorrhage that completely dissected the wall of a ventricle, particularly the right ventricle, and protruded on the external surface (Fig. 9). Histologically, in the areas of hemorrhages, groups of necrotic muscle fibers were present. The capillaries were dilated and filled with either fibrin thrombi or with red blood cells (Figs. 10 and 12).

In the controls (groups 1 and 2), none of these changes were observed. The kidneys of these animals occasionally exhibited a degree of congestion similar to that seen in endocrine kidneys. In the four groups of this experiment, foci of hepatic necrosis and small areas of pulmonary congestion and hemorrhage were noticed at autopsy. The hepatic lesions consisted of small patches of centrolobular necrosis in which sinusoidal fibrin thrombi could be identified. In the lungs, apart from focal congestion, hemorrhage and edema, fibrin thrombi were infrequently found in small veins.

There was, generally, a relationship between the height of the blood pressure and the intensity of renal necrosis and thrombosis following injection of endotoxin. The most hypertensive animals,

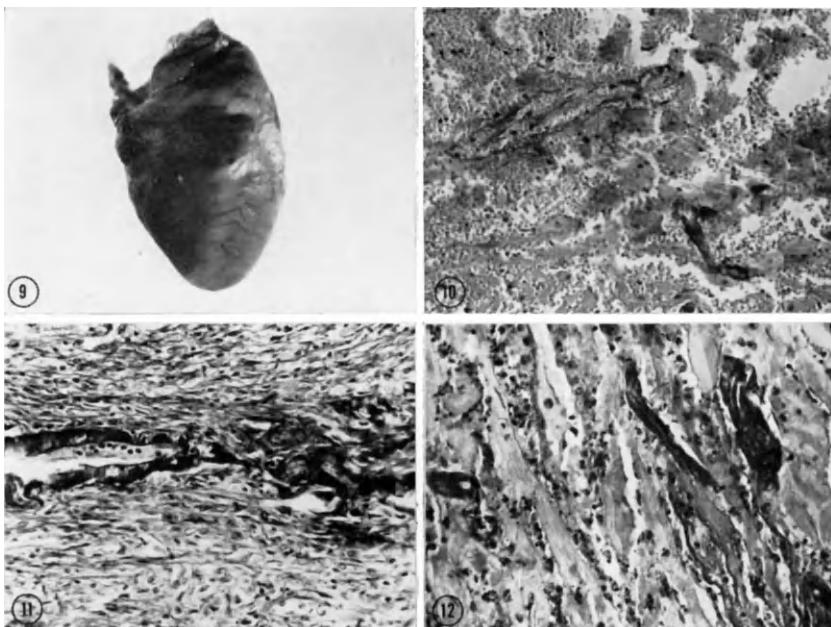


FIGURE 9. Large, triangular focus of hemorrhage dissecting the right ventricle and protruding on the surface. Smaller area of hemorrhage above and to the right.

FIGURE 10. Transverse section through area of myocardial hemorrhage in endotoxin-treated rat. Fibrin thrombi in capillaries (*lower right*). Fibrin threads in a larger vessel (*upper left*) whose wall is focally ruptured. Several muscle fibers are necrotic. (PAS  $\times 100$ .)

FIGURE 11. Heart of a hypertensive, otherwise not treated, animal. Transverse section showing fibrinoid necrosis of arterial wall and deposition of similar material in myocardium. Surrounding muscle fibers are replaced by inflammatory and fibrous tissue. (PAS  $\times 100$ .)

FIGURE 12. Fibrin thrombi and muscle necrosis near an area of hemorrhage in myocardium of endotoxin-treated rat. (PAS  $\times 200$ .)

presumably because they died earlier, showed less extensive renal lesions, while the rats in which blood pressure was lower and survival longer presented more severe lesions.

During microscopic examination of necrotic kidneys it soon became evident that the extent of glomerular and vascular damage precluded an exact appraisal of the underlying nephrosclerosis. In order to evaluate the degree of vascular damage induced by the presence of an endocrine kidney alone, a second experiment

TABLE II  
EFFECT OF VARIOUS DOSES OF ENDOTOXIN ON THE PRODUCTION OF THROMBOHEMORRHAGIC LESIONS IN HYPERTENSIVE RATS

Group	Treatment*	Survival Ratio	Final Body Weight (g)	Urine Flow (ml per Day)	Blood Pressure (mm Hg)	Heart Weight (mg/100g Body Weight)	Incidence (%)	Intensity (0-3)	Adrenal Incidence (%)			Cortical Necrosis and Thrombosis (Right Kidney) (%)		
									Heart Hemorrhage	In-Hemorrhage	In-Adrenal	Cortical Necrosis	Necrosis and Thrombosis	
1	.....	16:16	171 ± 6.3	47 ± 6.8	166 ± 4.3	544 ± 16.7	0.1 ± 0.09	12	0	0	0	0	0	
2	Endotoxin 600 µg .....	2:10	166 ± 5.2	43 ± 4.7	160 ± 4.1	560 ± 26.3	0.8 ± 0.40	30	1.3 ± 0.34	60	1.9 ± 0.34	80		
3	Endotoxin 300 µg .....	6:12	163 ± 7.7	40 ± 4.2	158 ± 3.9	572 ± 24.1	0.5 ± 0.26	25	1.4 ± 0.40	58	1.6 ± 0.35	66		
4	Endotoxin 100 µg .....	8:10	165 ± 7.5	48 ± 5.3	163 ± 5.3	546 ± 27.3	0.2 ± 0.13	20	0.4 ± 0.16	40	0.2 ± 0.13	20		

\* Endotoxin was injected at the doses indicated, irrespective of body weight. All the animals bore an endocrine kidney.

was performed. Other groups were added to this experiment to determine the effects of various doses of endotoxin in hypertensive rats. As can be seen in Table II, the injection of 600 $\mu$ g of endotoxin (group 2) to rats bearing an endocrine kidney induced lesions roughly similar in incidence and severity to those seen in the animals of the first experiment. There was a slight decrease in renal and cardiac lesions following the administration of 300 $\mu$ g of endotoxin (group 3), while the smallest dose induced alterations in only a few animals (group 4).

The endocrine-kidney-bearing animals that were not injected with endotoxin (group 1) did not develop any of the above-mentioned lesions, except for microscopic foci of hemorrhage in the myocardium. All the lesions of hypertensive vascular disease (8) were, however, present in these animals. The heart showed typical hypertensive changes in all cases (Fig. 11). Cardiac lesions of the same type were also present in all other animals of both experiments in which the aorta was ligated. Nephrosclerosis was present in ten of the sixteen hypertensive control rats. As far as could be ascertained by examination of arteries and arterioles, a similar proportion existed in rats bearing an endocrine kidney and injected with endotoxin. Out of thirty-five animals that received the larger doses of endotoxin (Table I, groups 3 and 4; Table II, group 2) seven did not show any thrombotic renal lesions. Among these non-reactive animals, two had normal right kidneys, while in five rats nephrosclerosis was present. Briefly, the affected right kidney (Table II, group 1) showed irregularity of the cortical surface, moderate dilatation of cortical tubules, the presence of casts in three cases, focal hyalinization and even fibrinoid necrosis of arteries and arterioles, including the afferent glomerular arterioles (Fig. 5). Several glomeruli showed changes typical of early hypertensive disease (11, 16). They were enlarged, their basement membranes were focally thickened and disrupted, and their pattern obscured by deposits of PAS-positive material (Fig. 7). There was no evidence of either thrombosis or fibrosis. The periadrenal and adrenal capsular arteries and arterioles as well as the mesenteric and intestinal vessels showed periarteritic and necrotic lesions in several instances.

### Discussion

These observations demonstrate that in rats suffering from hypertensive vascular disease induced by unilateral renal ischemia, the intravenous injection of *S. flexneri* endotoxin produces contralateral renal cortical necrosis and thrombohemorrhages and necrosis in various organs, while the ischemic kidney is spared. The general incidence of renal lesions (80%) observed after injections of the larger doses of endotoxin in hypertensive rats is comparable to that seen in the course of the generalized Sanarelli-Schwartzman phenomenon in the kidneys of pregnant rabbits after a single injection of endotoxin (14) and greater than that reported in pregnant rats given the same type of treatment (19). The morphology of renal, adrenal, and other lesions found in these hypertensive, endotoxin-treated rats is likewise similar in several aspects to that observed in either rabbits or rats in the course of the generalized Schwartzman phenomenon. It thus appears that renal hypertension, like pregnancy, can sensitize or "prepare" the rat to the potentially thrombotic and vasculotoxic action of endotoxin.

It is likely that this "preparation" is due to endothelial damage and to alterations in blood clotting mechanisms. The presence of thrombi in blood vessels of various organs has been reported in late experimental hypertension (18). Fibrin thrombi are also an important component of the pathologic anatomy of acute hypertensive vascular disease induced by administration of renin to uninephrectomized rats pretreated with desoxycorticosterone and sodium chloride (10). Such disseminated clotting in experimental hypertension is thought to be secondary to endothelial damage and elevation of fibrinogen and platelets induced by adrenal steroids (12). This pathogenesis is probably relevant to our observations. On the other hand, the intraperitoneal administration of two doses of endotoxin, in rats rendered hypertensive for a period of one month by uninephrectomy and given combined treatment with desoxycorticosterone and sodium chloride, resulted in the deposition of fibrin in glomeruli, without renal cortical necrosis or other extra-renal lesions (7). While this difference in reactivity may depend on variations in techniques, it may also be due to more profound changes in blood clotting or in other factors in primary renal hypertension.

A number of factors may have played a role in the protection of the left (endocrine) kidney against the effects of endotoxin. The absence of vascular lesions, the atrophy of the parenchyma, the

locally diminished blood pressure may each have participated. It is known, for instance, that, presumably owing to a change in the local vascular system, unilateral decapsulation protects the rabbit kidney against endotoxin-induced renal cortical necrosis (4). The same type of protection is also afforded by cutting the nerve supply to one kidney (15).

The present experiments seem to demonstrate that, once hypertensive vascular disease is established, bacterial endotoxin may be of great importance in the production of thromboses. Like pregnancy (13), hypertension could, in the course of certain infections, sensitize human beings to the production of thrombotic lesions. In fact, a possible human counterpart to the present experimental situation has been described (2). A hypertensive patient who died after an infectious episode showed, at autopsy, unilateral renal cortical necrosis and thrombosis with malignant nephrosclerosis. The much smaller contralateral kidney, presumably the cause of the hypertension owing to severe narrowing of the renal artery, was free of parenchymal necrosis and of lesions of its vessels. The adrenal apoplexy reported in some hypertensive patients could also have a similar pathogenesis (5, 9).

### **Abstract**

In rats suffering from hypertensive vascular disease induced by unilateral renal ischemia (endocrine-kidney technique), the intravenous administration of a single dose of bacterial endotoxin elicits cortical necrosis of the contralateral kidney, adrenal apoplexy, and cardiac, thymic and intestinal hemorrhages. The ischemic kidney is not affected by the thrombotic process. The incidence and morphology of these lesions are comparable in several aspects to those elicited by endotoxin in pregnant rabbits or rats. Mention is made of possible human counterparts of this experimental situation.

### **Abrégé**

Chez des rats atteints des lésions vasculaires de l'hypertension artérielle induite par ischémie rénale unilatérale (technique du rein endocrinien), l'injection intraveineuse d'une seule dose d'endotoxine bactérienne est suivie de l'apparition de nécrose corticale du rein contralatéral, d'apoplexie surrénalienne et de lésions hémorragiques du myocarde, du thymus et de l'intestin grêle. Le rein ischémique n'est pas affecté par le processus thrombotique. L'incidence et la morphologie de ces lésions sont comparables en plusieurs points à celles induites par administration d'endotoxine chez la lapine ou la rate en gestation. Certains cas de nécrose corticale rénale et d'apoplexie surrénalienne chez des patients hypertendus sont examinés à la lumière de ces résultats.

### Acknowledgements

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**B. Tuchweber:** It is well known that the rat is notoriously resistant to bacterial endotoxins and that the generalized Sanarelli-Shwartzman reaction (SSR), which can be easily elicited in rabbits, is difficult to produce in the rat by the classical treatment of two properly spaced injections of endotoxin. It has been shown that pregnant rats respond to a single i.v. injection of endotoxin with lesions similar to those characteristic of the generalized Shwartzman reaction. In addition, other treatments, e.g., sodium polyanethol sulfate, can also prepare animal species for a similar response. Rats rendered hypertensive by combined treatment with desoxycorticosterone plus unilateral nephrectomy and dietary sodium-chloride supplements and chronic administration of *E. Coli* endotoxin exhibit the same changes as those seen in the generalized SSR, particularly occlusive glomerular lesions associated with fibrinoid degeneration of glomerular basement membranes in about 70% of the animals; but the absence of renal cortical necrosis should be noted. Dr. Cantin has shown in his remarkable work that in rats rendered hypertensive by unilateral renal ischemia (the endocrine-kidney-technique) and given a single i.v. injection of bacterial endotoxin, it is possible to produce cortical necrosis of the contralateral kidney and cardiac, pulmonary and intestinal hemorrhages.

I should now like to present the results of experiments conducted in our laboratories showing that lead acetate greatly increases the susceptibility of the rat to bacterial endotoxins. A single intravenous injection of 5 mg of lead acetate per 100 g body weight, normally well-tolerated in the rat, increases the sensitivity of this animal to endotoxins of many gram-negative bacteria about 100,000 times above normal. The first experiments indicated that 1  $\mu$ g of bacterial endotoxin, e.g., shigella flexneri, given i.v. followed immediately by lead acetate can cause 100% mortality. The accompanying organ lesions were localized in the kidney, spleen, hepatic hilum, heart,

lung and thymus. There was renal cortical necrosis, intense venous engorgement of the medulla, and fibrin thromboses in the glomerular capillaries. Often, hemorrhagic necrosis of the medium-sized and small arterioles as well as occasional thrombus formation in the afferent glomerular vessels were seen. The spleen showed hemorrhages with thromboses of the veins; hemorrhage was seen in the hepatic hilum, thymus and heart, but less frequently. The highest mortality rate and most intense lesions were obtained with 1  $\mu\text{g}$  of bacterial endotoxin. The mortality was still high at the 0.1  $\mu\text{g}$  level and even the 10 and 1 nanogram doses of endotoxin were no better tolerated by lead-sensitized rats than were 100  $\mu\text{g}$  amounts in normal controls. In order to determine whether the sensitizing effect of lead is specific, a number of additional predominantly metallic compounds were tested, many of which are known to share certain pharmacological actions of lead in producing calcification or else they affect blood clotting or the reticulo-endothelial system. Among a large number of metals tested, only scandium chloride and thorium nitrate approximated, although none equalled, the sensitizing potency of lead acetate.

We also determined whether non-microbial reticulo-endothelial-blocking agents would also become highly toxic in animals pretreated with lead acetate. None of the RES-blocking agents tested, e.g., Thorotrast, Collargol, etc., acquired the usual toxicity after treatment with lead acetate. It is difficult to explain the mechanism of action of lead acetate in these experiments. It has long been known that RES-blocking agents can increase sensitivity to bacterial endotoxins in the rat; hence, it is tempting to assume that lead acts through the same mechanism. However, none of the agents tested equalled the extraordinarily sensitizing action of lead acetate. It is possible to assume that the blocking effect of lead on the RES system is far greater than that of all the other agents tested. It is also highly probable that lead interferes with the defensive function of some other organs, such as the adrenals, or with certain metabolic phenomena, such as detoxification of endotoxin by the liver.

## Influence of Corticoids on Coagulation and Thrombosis in Rat: Possibility of a Serum-protein Related Effect

S. RENAUD\*

ALTHOUGH it appears to be well documented that the administration of glucocorticoids inhibits cholesterol-induced atherosclerosis in the rabbit (3, 5, 9), physicians seem to be reluctant to use corticoids in patients with coronary disease, probably for the following reasons: 1) In animals, corticoid administration usually results in an increase in serum cholesterol, although the atherosclerotic lesions are inhibited (5). 2) In man, elevated levels of corticoids have been held to be responsible for the severe atherosclerosis observed in Cushing's Syndrome. 3) Thromboembolisms have been reported to occur during or after corticoid therapy (2).

Recently, we have shown that the administration for four to seven days of ACTH or glucocorticoids, but not mineralocorticoids, to hyperlipemic rats prevents the production of endotoxin-initiated thrombosis (12). This preventive effect could be related to a marked prolongation of the clotting time and to a considerable increase in the percentage of  $\alpha$ -lipoproteins, but not to a stimulation of the fibrinolytic activity. Furthermore, we have found that it was not necessary to utilize pharmacologic doses of corticoids to obtain a beneficial effect. Triamcinolone included in the hyperlipemic diet of rats for a four-week period, at a dosage not affecting the body weight, had the same effect on thrombosis, coagulation, and lipoproteins as did high doses of hydrocortisone (15). In man, preliminary experiments have indicated that triamcinolone administered once a day (6 to 10 mg per Kg) for a period of two to three months induced changes in the clotting time and the lipemia that were essentially similar to those observed in the rat (15).

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\* Scholar of the Medical Research Council.

It is well known that heparin is able to prolong the clotting time, increase the level of  $\alpha$ -lipoproteins, and prevent thrombosis. Since it has been reported in man (8) that administration of glucocorticoids can increase the protamine titer of the blood, the beneficial effect of corticoids on the production of thrombosis in the rat could be due to the *in vivo* release of heparin-like substances. Our aim in the present study was to substantiate or disprove this hypothesis—by investigating the effect of protamine on heparin- and corticoid-treated rats—by further analyzing the similarities in the blood changes induced by heparin and corticoid therapy. In the course of these experiments, unexpected relationships were observed between the level of certain serum proteins and lipoproteins and the clotting time, in animals with and without corticoid administration. Thrombosis was induced, as in previous experiments (11, 12, 13), by an intravenous endotoxin injection in hyperlipemic rats. The results obtained will be reported in the present communication.

### **Materials and Methods**

Holtzman male rats with an initial body weight of 150-170 g were used for this study, the exact number of animals actually utilized being listed in the corresponding Table. The rats were housed in air-conditioned quarters and given, *ad libitum*, tap water and the following hyperlipemic diet: butter 38, casein 11, cellulose 15, cholesterol 5, salt mixture 4, sodium cholate 2, sucrose 23, vitamin mixture 2 (weight %), as reported in detail elsewhere (11, 12, 13). The cellulose (alphacel), the salt-mixture (Wesson), and the vitamin mixture (vitamin diet fortification mixture) were all purchased from Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A. After 9 weeks of this dietary feeding, certain groups were injected subcutaneously, once a day, for 7 days, with distilled water alone (0.5 ml) (group 1, Table I and Table II), or with an aqueous suspension of hydrocortisone acetate (groups 4 and 5, Table I, and group 2, Table II) or of desoxycorticosterone acetate (group 3, Table II), both hormones being given at the dose of 15 mg per Kg. Blood samples were taken 2 hours after the injection on the last day of the treatment. After 10 weeks of dietary feeding (groups 2 and 3, Table I, and group 4,

Table II), a single dose of heparin (Depo-heparin, 25 mg/Kg) was injected subcutaneously, after dilution as follows: Depo-heparin (Upjohn Co.) 200 mg, gelatin 2 g, dextrose 1 g, and distilled water, quantity sufficient to 24 ml. Saline alone (groups 2 and 4, Table I) or saline containing protamine sulfate (15 mg/Kg) (groups 3 and 5, Table I) was injected intravenously 10 minutes before blood sampling.

Finally, after 17 hours' fasting, blood was removed in siliconized syringes from the jugular vein of all the rats, under ether anesthesia. On 1 ml of the blood, collected in a syringe containing sodium citrate, the plasma clotting time after recalcification was determined in duplicate as reported previously (13). On another 2 ml of blood, serum protein and lipoprotein determinations were performed by paper electrophoresis, as described in detail elsewhere (13), and by the biuret method for total protein and albumin (after sodium sulfate precipitation). For the induction of thrombosis, immediately after withdrawal of blood for laboratory tests the rats were given, by the same needle, 1 ml per 100 g body weight of saline containing 0.04 mg of a *Salmonella typhosa* (0901, Boivin type, Difco Laboratories) lipopolysaccharide. Every animal was autopsied, the survivors being killed with chloroform 24 hours after the endotoxin injection. The red hepatic infarcts were evaluated macroscopically and graded in terms of an arbitrary scale of 0 to 3. The macroscopic readings were verified by histologic examination of the left hepatic lobe.

### Results and Discussion

Intravenous injection of a *S. typhosa* lipopolysaccharide in hyperlipemic rats injected with distilled water only (group 1, Tables I and II) resulted in a high mortality rate and the production of thrombosis comparable in severity in the two experiments (1.3, Table I as compared with 1.4, Table II). The thromboses recorded here were those occurring primarily in the large hepatic veins as described previously (11, 12, 13). They are occlusive thromboses, giving rise to large multiple red hepatic infarcts, easily seen macroscopically. In confirmation of our earlier findings (10, 12), heparin (group 2, Table I and group 4, Table II) administered subcutaneously in repository form was markedly effective in preventing thrombosis. Intravenous administration of protamine sulfate (15 mg/Kg) to heparin-treated rats (group

TABLE I

INFLUENCE OF PROTAMINE ON THE HEPARIN AND HYDROCORTISONE  
PREVENTIVE EFFECT ON THROMBOSIS

Treatment	Distilled Water	Heparin	Heparin	Hydro-	Hydro-
		+ Saline	+ Protamine	+ Saline	+ Protamine
<i>Group</i>		1	2	3	4
Number of animals .....	12	6	6	10	16
Plasma clotting time (sec) ...	155±7	298±17	205±15	197±24	219±16
Lipoproteins ( $\alpha + \alpha_1$ %) ....	24±1.8	36±1.7	28±2.5	34±2.0	36±2.4
Triglycerides (mg %) ....	148±5	83±5	82±5	124±4	113±4
Thrombosis: severity (0-3) ...	1.3	0.5	0	0	0
Mortality .....	58	50	17	10	0

Results = Mean ± S.E.

*Treatment:* Distilled water or hydrocortisone (acetate) (15 mg/Kg) once a day subcutaneously, for 7 days, the last injection 2 hours before blood removal. Heparin (repository) (25 mg/Kg) once only, subcutaneously, 1 hour before blood removal. Saline and protamine sulfate (15 mg/Kg) intravenously, 10 minutes before blood removal.

*Group comparison*—Plasma clotting time: 3 vs. 2 p < 0.01; 5 vs. 4 p > 0.05.

$\alpha + \alpha_1$  lipoproteins: 3 vs. 2 p < 0.01; 5 vs. 4 p > 0.05.

3, Table I), 10 minutes before blood removal and the endotoxin injection, considerably decreased the clotting time and the percentage of  $\alpha + \alpha_1$  lipoproteins. This result is, therefore, concordant with the well-known effect of protamine as an inhibitor of heparin. In contrast to this, protamine administered at the same dosage to hydrocortisone-treated rats (group 5, Table I) failed to reduce the clotting time and the percentage of  $\alpha + \alpha_1$  lipoproteins. Preliminary experiments had shown that doses of protamine ranging from 5 to 20 mg/Kg did not further influence the effects of hydrocortisone on the clotting time or the incidence of thrombosis. Therefore, these experiments do not support the hypothesis that glucocorticoids could act on the coagulation and the lipoproteins through the liberation of heparin-like substances in the blood.

In the experiment reported in Table II, thrombosis was markedly prevented in group 2 treated with hydrocortisone, as in previous experiments (12). It should also be noted that in addition to the prolongation of the clotting time, the  $\alpha + \alpha_1$  lipoproteins were significantly increased by the corticoid treatment as compared with control group 1, and this at the expense of the  $\alpha_2 + \beta$  fractions. In the present experiment and several others (13, 14), the  $\alpha + \alpha_1$  and  $\alpha_2 + \beta$  fractions

were considered together for the sake of simplicity, since the  $\alpha$  lipoproteins do not appear to be more closely related to thrombosis or atherosclerosis than the  $\alpha_1$  lipoproteins. In addition, the  $\alpha_2$  lipoproteins seemed to be as much related to thrombosis as was the  $\beta$  fraction.

In this experiment, in addition to the lipoproteins, the protein fractions were determined by paper electrophoresis and also by sodium sulfate precipitation. It can be seen in Table II that, after hydrocortisone treatment (group 2), the albumin +  $\alpha_1$  globulin percentages, as determined by electrophoresis, were significantly increased at the expense of the  $\alpha_2 + \beta$  globulin. The total proteins were also markedly increased as compared with group 1, the increase being mostly due to what is referred to as albumin in the sulfate precipitation technic. An increase in the albumin content of the serum has already been reported in the rat (1) after glucocorticoid therapy.

The administration of desoxycorticosterone to hyperlipemic rats does not seem to decrease the susceptibility to shock, since the mortality rate in group 3 treated with this hormone was slightly higher than in group 1, in confirmation of previous experiments (12). How-

TABLE II  
INFLUENCE OF CORTICOIDS AND HEPARIN ON SERUM PROTEINS AND LIPOPROTEINS IN RELATION TO THROMBOSIS

Treatment	Distilled Water	Hydrocortisone	Desoxycorticosterone	Heparin
<i>Group</i>	1	2	3	4
Number of animals .....	15	15	6	15
Plasma clotting time (sec) .....	141±9	164±9	143±20	401±41
Proteins				
Total proteins (g %) .....	7.7±0.1	8.9±0.25	7.3±0.3	7.9±0.26
Albumin (g %) .....	3.8±0.05	4.9±0.2	3.5±0.2	3.9±0.1
Albumin + $\alpha_1$ globulin (%) ..	38±1.8	46±1.6	36±2.1	41±1.4
$\alpha_2 + \beta$ globulin (%) .....	52±2.5	43±1.6	55±2.4	45±2.0
Lipoproteins				
$\alpha + \alpha_1$ (%) .....	26±2.2	38±1.8	22±1.7	36±2.2
$\alpha_2 + \beta$ (%) .....	61±2.4	52±2.7	69±1.4	51±1.7
Thrombosis: severity (0-3) .....	1.4	0.3	0.7	0.3
Mortality % .....	60	13	67	27

Results = Mean ± S.E.

Treatment: Hydrocortisone (acetate) and desoxycorticosterone (acetate) (15mg/Kg) once a day subcutaneously, for 7 days. Heparin (repository) (25 mg/Kg) once only, subcutaneously, 1 hour before blood removal.

Group comparison—Plasma clotting time: 2 vs. 1 p < 0.001.

Albumin +  $\alpha_1$  globulin: 2 vs. 1 p < 0.001.

$\alpha + \alpha_1$  lipoproteins: 2 and 4 vs. 1 p < 0.001.

ever, here, in this small experiment, the severity of thrombosis was decreased as compared with the control group 1, although the clotting time was the same. In the present experiment, there was no increase in the  $\alpha + \alpha_1$  lipoproteins or in the total proteins and in the albumin fraction but, rather, a slight decrease in the desoxycorticosterone-treated rats. Therefore, it seems that, not only the effect on thrombosis, lipoproteins, and coagulation, as has previously been shown (12), but also the effect on protein is linked with glucocorticoid activity.

In the group treated with heparin, and despite the fact there was an increase in the  $\alpha + \alpha_1$  lipoproteins similar to that seen in the hydrocortisone-treated rats and a marked increase in the clotting time, no significant difference was noted in the protein level between this group 4 and the control group 1. In addition, using the paper electrophoresis post-stained technic, we were able to observe that, in the heparin-treated rats, the increase was seen mostly in the  $\alpha_1$  lipoprotein

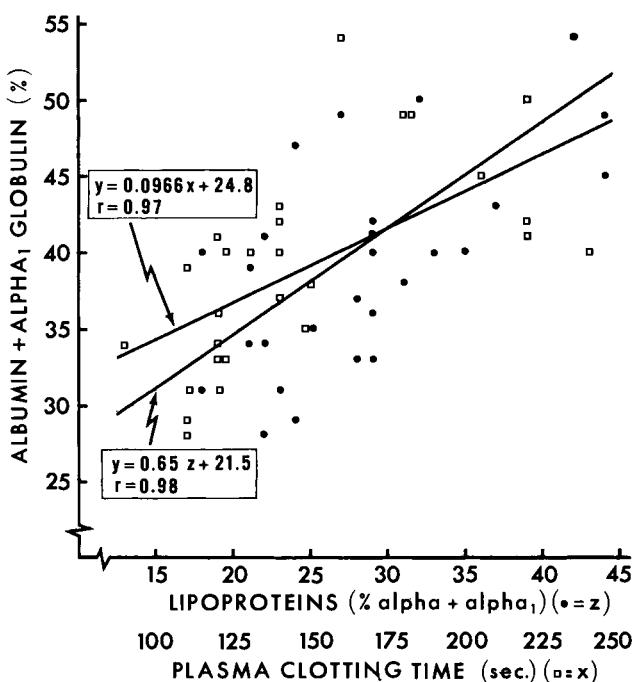


FIGURE 1. Relationship between the serum level of albumin +  $\alpha_1$  globulin and that of  $\alpha + \alpha_1$  lipoprotein, and the plasma clotting time in normal and corticoid-treated rats.

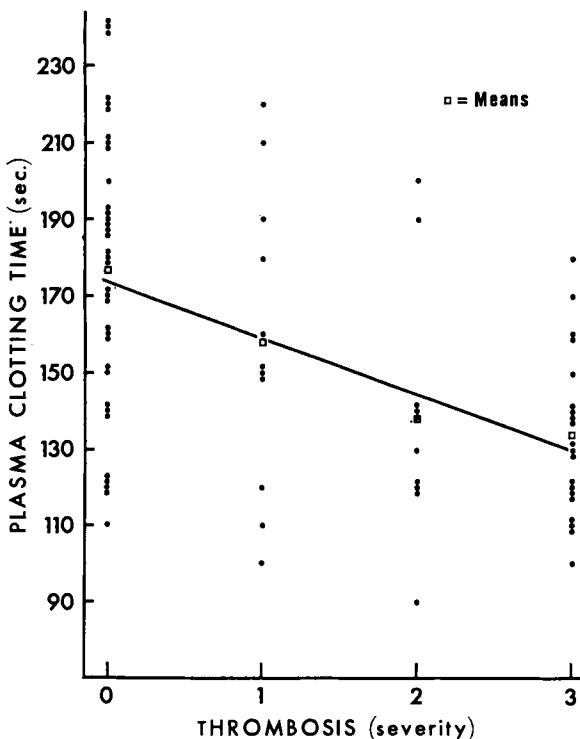


FIGURE 2. Relationship between the plasma clotting time and the severity of thrombosis in normal and corticoid-treated rats.

fraction, while with hydrocortisone the increase occurred mainly in the  $\alpha$  fraction. With the present electrophoresis technic, in contrast to observations made with pre-stained sera (12), the  $\alpha$  lipoproteins in both heparin- and hydrocortisone-treated rats were not migrating ahead of the albumin zone when compared with the proteins of the same animal. In addition, the serum triglyceride level was much more decreased by the heparin than by the hydrocortisone treatment.

Utilizing the pooled data of the present experiments but excluding the heparin-treated rats, there was a close correlation between the percentage of the albumin +  $\alpha_1$  globulin fraction and that of  $\alpha + \alpha_1$  lipoproteins ( $r = 0.98$ ), and also between the percentage of albumin +  $\alpha_1$  globulin and the plasma clotting time ( $r = 0.97$ ) (Fig. 1). In addition, when 0.15 ml of an albumin solution (25 g/100 ml human albumin, Connaught Medical Research Laboratory, Toronto, Canada) was added to 0.85 ml of human serum and incubated at

37° C for 10 minutes, we consistently observed an increase of 25 to 30% in the clotting time that could not be reproduced by the addition of saline alone. Although this preliminary study was carried out on the plasma of 5 patients only, it confirms the results obtained by other investigators on platelet aggregation (7). In addition, albumin is known to interact with free fatty acids (4) and to inhibit their promoting effect on platelet aggregation *in vitro* (7) and on thrombosis when injected intravenously (6). With the pooled data of the present experiments, still excluding the heparin-treated rats, it appears that there is a close relationship between the clotting time and the severity of thrombosis (Fig. 2), although the correlation coefficient could not be calculated under these conditions. Therefore, it seems reasonable to assume that, under the conditions of the present study, most of the hydrocortisone effect on coagulation and thrombosis was related to an increase in serum albumin, probably resulting from stimulation of albumin synthesis in the liver (1).

### Abstract

While the intravenous injection of a gram-negative bacteria endotoxin in a laboratory-chow fed rat induces the formation of only microthrombi in the hepatic sinusoids, the same injection in a rat fed certain types of hyperlipemic diets for several weeks initiates the formation of occluding thrombosis of the large hepatic veins, giving rise to multiple red hepatic infarcts. This experimental model has been utilized to study the effect of corticoids on lipemia, coagulation and thrombosis in the rat. We showed that when a glucocorticoid was injected to rats for four to seven days it resulted in a prolongation of the plasma clotting time, in an increase in the percentage of  $\alpha$  lipoproteins, and in a marked prevention of thrombosis. These effects were somewhat similar to those of heparin. A mineralocorticoid, such as desoxycorticosterone, was not able to reproduce these effects. In order to investigate further the similarity between the effects of heparin and those of hydrocortisone, protamine was injected intravenously 10 minutes before blood removal and the endotoxin injection, in heparin- and corticoid-treated rats. Only in the group treated with heparin were the clotting time and the percentage of  $\alpha$  lipoproteins affected by the injection of protamine. In addition, the level of total proteins was increased in the hydrocortisone but not in the desoxycorticosterone- or heparin-treated rats. This increase in total proteins was solely due to an increase in the albumin or in the albumin +  $\alpha_1$  globulin fraction depending on the method of analysis utilized. In this experiment, a correlation was found between the percentage of albumin +  $\alpha_1$  globulin and that of  $\alpha + \alpha_1$  lipoproteins in serum and of the plasma clotting time. In addition, when albumin was added *in vitro* to plasma, it markedly increased the clotting time. Since the clotting time appears to be connected with the severity of thrombosis, the effect of a glucocorticoid, such

as hydrocortisone, on the lipoproteins, the clotting time, and the production of thrombosis does not seem to result from the liberation in blood of heparin-like substances, but rather from the increase in blood of certain protein fractions, mostly albumin.

### Abrégé

Tandis que l'injection intraveineuse d'endotoxine de bactérie gram négative chez un rat nourri de "Laboratory Chow" ne produit que la formation de microthrombi dans les sinusoides hépatiques, la même injection chez un rat nourri pendant plusieurs semaines de certains types de régimes hyperlipémiants, déclenche la formation de volumineuses thromboses occlusives des veines hépatiques, responsables de multiples infarcissements. Utilisant ce modèle expérimental, nous avions déjà montré que lorsqu'un glucocorticoïde était injecté à des rats pendant une période de 4 à 7 jours, on observait une prolongation du temps de coagulation du plasma, une augmentation du pourcentage des lipoprotéines  $\alpha$  et une prévention marquée des thromboses. Ces effets paraissaient semblables à ceux obtenus par l'administration d'héparine. Par contre, un minéralocorticoïde comme la désoxy-corticostérone, ne pouvait reproduire ces effets.

Dans les expériences rapportées ici, nous avons analysé plus avant les similitudes entre l'action de l'héparine et celle de l'hydrocortisone, en observant les effets de l'injection intraveineuse de protamine avant la prise de sang et l'injection de l'endotoxine, chez des animaux traités par l'héparine ou par ce corticoïde. C'est seulement dans le groupe traité avec l'héparine que le temps de coagulation et le pourcentage des lipoprotéines  $\alpha$  ont été diminués par la protamine. Par contre, seulement les animaux traités par l'hydrocortisone ont présenté une augmentation du niveau des protéines totales du sérum. Cette augmentation était due à la seule augmentation de l'albumine ou de l'albumine et de la globuline  $\alpha_1$  selon la méthode d'analyse utilisée. Dans ces expériences, on a trouvé une corrélation entre le pourcentage des fractions albumine + globuline  $\alpha_1$  et celui des lipoprotéines  $\alpha + \alpha_1$  du sérum, de même qu'entre les fractions albumine + globuline  $\alpha_1$  et le temps de coagulation du plasma. De plus, *in vitro*, l'addition d'albumine ou plasma augmentait considérablement le temps de coagulation. D'autre part, puisque nous avons aussi observé que le temps de coagulation semblait être relié à la sévérité des lésions de thromboses, l'effet de l'hydrocortisone sur les lipoprotéines, le temps de coagulation et la production de thromboses ne paraît pas résulter de la libération de substances semblables à l'héparine mais plutôt de l'augmentation dans le sang de certaines protéines, principalement de l'albumine.

### Acknowledgements

This work was supported by Grant MT-1444 from the Medical Research Council and by a Grant from the Quebec Heart Foundation.

The author is particularly indebted to Dr. J. Champagne, Ph.D., Chief of the Biochemical Laboratory, Institut de Cardiologie de Montréal, for the total proteins and albumin analysis performed in this study by the biuret method. The co-operation of the companies Merck Sharp & Dohme of Canada and Ciba of Canada, in supplying, respectively, the hydrocortisone and the desoxycorticosterone utilized in this study, is gratefully acknowledged.

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**G. Gabbiani:** There is no doubt that the influence of ACTH or glucocorticoids on thrombotic or thromboembolic lesions is a very controversial problem that is worthy of the greatest attention because of its pathophysiological and clinical implications. As Dr. Renaud points out, one of the reasons why physicians are reluctant to use corticoids in patients with coronary disease is the appearance of thromboembolism during or after corticoid therapy. It might be added that, in numerous animal experiments, the administration of ACTH or corticoids has been shown to facilitate the production of thrombotic lesions. The literature is replete with examples of inhibition or aggravation of thrombohemorrhagic phenomena by glucocorticoids. The local Sanarelli-Shwartzman phenomenon (SSP) produced by two injections of meningococcal endotoxin in rabbits can be inhibited by cortisone or ACTH given a few hours before the provocative injection. Cortisone does not influence the thrombohemorrhagic lesions produced by a single intravenous (i.v.) injection of endotoxin plus Liquoid or other acidic polymers, yet this response is prevented by heparin. Conversely, the thrombohemorrhagic lesions induced by intracutaneous epinephrine and i.v. endotoxin are prevented by cortisone but not by heparin. The thrombohemorrhagic lesions elicited in chick embryos by a single endotoxin injection can also be prevented by various glucocorticoids. On the other hand, glucocorticoids can sensitize for the production of thrombohemorrhagic lesions. Cortisone prevents the development of resistance to the local SSP that normally occurs following repeated courses of two injections of endotoxin. Both in the rabbit and in the hamster, a single i.v. injection of endotoxin produces bilateral renal cortical necrosis with hyaline thromboses in the glomerular capillaries, following pre-treatment with cortisone. Furthermore, under these conditions, intracutaneous injection of endotoxin can elicit both the general and local forms of the SSP. Prolonged treatment with ACTH or cortisone also aggravates the generalized SSP produced by two properly spaced i.v. injections of endotoxin (for bibliography see: Selye, H.: *Thrombohemorrhagic Phenomena*, Thomas, Springfield, 1966).

To these findings we can add some experiments carried out in our

laboratory, which indicate that in rats sensitized by the combined administration of 9 $\alpha$ -fluorocortisol and a sodium salt, a single injection of colloidal carbon produces bilateral cortical necrosis of the kidney with precipitation of fibrin in the glomerular capillaries. When given alone as pretreatment, fluorocortisol is almost inactive as a conditioning agent; however, when administered together with a sodium salt (a combination known to be highly effective in predisposing for the production of a particular type of cardiac necrosis), the hormone is highly effective in sensitizing for the production of glomerular lesions following the administration of colloidal carbon (Gabbiani, G.: *Med. Exptl.*, 11:209, 1964). In rats pretreated with ACTH, 9 $\alpha$ -fluorocortisol or restraint, a single i.v. injection of thorium-dextrin produces thrombohemorrhagic lesions with necroses in the adrenals and liver, as well as hyaline glomerular capillary thrombosis in the kidneys (Gabbiani, G., et al.: *Endocrinology*, 77:177, 1965). These changes represent a reliable experimental model of suprarenal changes with characteristics similar to those of the Waterhouse-Friderichsen syndrome and the SSP. The simultaneous

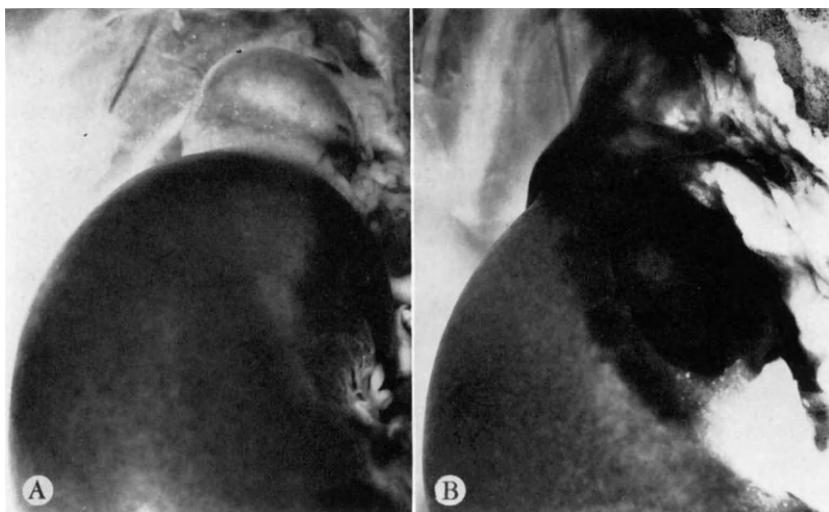


FIGURE 1. Thrombohemorrhagic lesions in the adrenals produced by thorium-dextrin and restraint. *A*. After i.v. injection of thorium-dextrin alone, the gland has a normal appearance. *B*. After combined treatment with thorium-dextrin i.v. and restraint, hemorrhagic spots are visible on the surface of the adrenal, and the bleeding spreads into the surrounding connective tissue. (After Gabbiani et al.: *Endocrinology*, 77:177, 1965.)

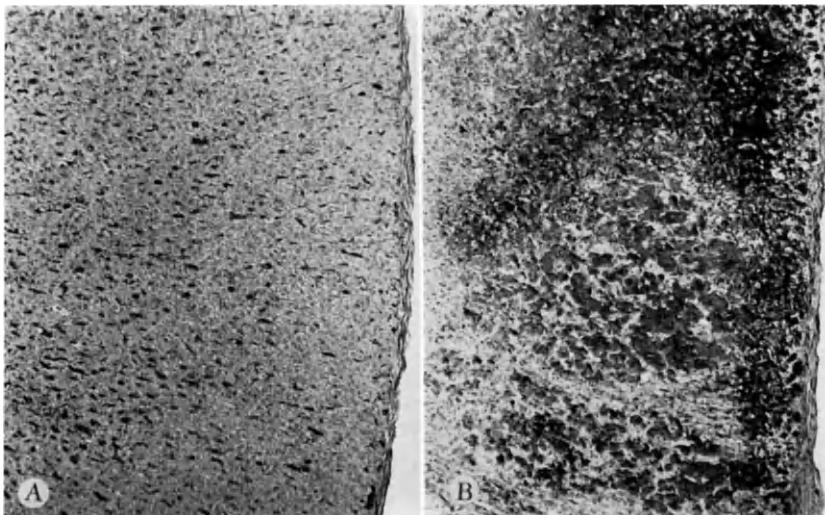


FIGURE 2. Histologic aspect of adrenal and liver under various experimental conditions. *A*. After an i.v. injection of thorium-dextrin alone, thorium granules are present in the RES cells. *B*. Lesions induced by thorium-dextrin i.v. in an animal sensitized with fluorocortisol. The necrotic area is replete with thorium-dextrin granules and surrounded by a hemorrhagic halo. Susa, phosphotungstic acid, aldehyde-fuchsin and alcian blue.  $\times 120$ . (After Gabbiani *et al.*: *Endocrinology*, 77:177, 1965.)

presence of kidney lesions considered typical of the SSP further supports this view.

All these facts suggest that treatment with glucocorticoids or ACTH can either enhance or inhibit the production of thrombotic lesions, depending upon the experimental conditions. In our investigations, we have only studied the morphologic changes of the affected organs. The biochemical studies of Dr. Renaud suggest that the level of albumins may be important in determining the protection afforded by glucocorticoids against the experimental production of thrombosis. We think that further research along these lines, using the speaker's interesting model in comparison with others, would undoubtedly help to elucidate this problem.

**S. Renaud:** I should like to thank Dr. Gabbiani for his discussion of my paper; but also would like to add a few words concerning the data available in the literature on the predisposing effect of corticoids on thrombotic phenomena. Thromboembolisms have been reported to occur following long-term treatment with corticoids, but

most of the authors refer to a study made by Cosgriff in 1951. In this study, it was shown that among 700 patients treated with ACTH or cortisone for a long period, 28 patients presented thromboembolic complications. However, the incidence of thrombotic phenomena in a similar group of patients without hormonal therapy was not available. In contrast to this, other investigators have emphasized that corticoids could be administered safely, even to cardiac patients. At any rate, we have observed a beneficial effect afforded by the prolonged administration of glucocorticoids, on coagulation in man and animals and on thrombosis in rats. Nevertheless, when corticoids were administered for several months at a constant dosage, we also observed that this beneficial effect could eventually be lost. It is only by a careful study of corticoids under various conditions of administration and at various dosages that the effect of these powerful substances will be completely elucidated.

### **Final Comments**

**H. Selye:** Well, we have come to the close of this event and the time has come to thank you all. However, before doing so, I would like to address all my former students as their professor and give them one last lecture. I will probably never have the occasion again to have all of you—almost my whole academic family—as a captive audience in one auditorium, and I hope you will forgive me if I cannot resist this temptation. The time is late, the lecture will be short. But I will try to summarize in it the principal lesson I would like you to derive from all our daily Laboratory Rounds, Staff Conferences, and all the postgraduate lectures I gave you. It's a lecture of four words: "*Never neglect laboratory curiosities.*" If you look back upon the highlights of this meeting, they all grew out of "laboratory curiosities." Whether the original finding was made in our Institute or later, quite independently, in your own laboratories, if the subject was really new, it first presented itself marked as a freak observation. We look on a thing as a "laboratory curiosity" because it does not fit into the classical structure of our knowledge. It is an oddity, difficult to explain and without any obvious application; that is to say, a singular unique fact whose origin and future cannot be appraised. Even in science, every age has its fashions and idiosyncrasies. Some forty years ago, when I started out on my scientific career, the whole of basic research was considered a kind of "laboratory curiosity," an unusual pastime for admittedly intelligent and sometimes even learned men with an odd taste for things that really do not matter. It was

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**H. Selye:** Well, we have come to the close of this event and the time has come to thank you all. However, before doing so, I would like to address all my former students as their professor and give them one last lecture. I will probably never have the occasion again to have all of you—almost my whole academic family—as a captive audience in one auditorium, and I hope you will forgive me if I cannot resist this temptation. The time is late, the lecture will be short. But I will try to summarize in it the principal lesson I would like you to derive from all our daily Laboratory Rounds, Staff Conferences, and all the postgraduate lectures I gave you. It's a lecture of four words: "*Never neglect laboratory curiosities.*" If you look back upon the highlights of this meeting, they all grew out of "laboratory curiosities." Whether the original finding was made in our Institute or later, quite independently, in your own laboratories, if the subject was really new, it first presented itself marked as a freak observation. We look on a thing as a "laboratory curiosity" because it does not fit into the classical structure of our knowledge. It is an oddity, difficult to explain and without any obvious application; that is to say, a singular unique fact whose origin and future cannot be appraised. Even in science, every age has its fashions and idiosyncrasies. Some forty years ago, when I started out on my scientific career, the whole of basic research was considered a kind of "laboratory curiosity," an unusual pastime for admittedly intelligent and sometimes even learned men with an odd taste for things that really do not matter. It was

extremely difficult to obtain money for basic research, because medicine was considered to be an applied science and its object was to cure the patient. "What's the use," we were told, "of looking for an enzyme in the liver or a pigment in a butterfly's wing, when others are trying to cure disease?"

Truly new developments in medicine almost always grow out of basic laboratory research. To mention but a few examples of such discoveries made during my lifetime right in this country, think of the discovery of insulin by Banting and Best, the sex chromatin by Murray Barr, calcitonin by D. H. Copp. Today, it is no longer necessary to defend basic research; but there is still a great tendency to look down upon "mere laboratory curiosities" as findings that amuse more than instruct. And that is what they are, as long as you do not follow them up to unravel their mechanism or at least to connect them with other things already known. Then suddenly they cease to be "laboratory curiosities." But don't forget that the oddity was the seed. You can do a lot for a plant by caring for it, but to get a new plant, you need a seed. Some seeds do, some don't, grow into useful plants. That is the risk you must take; but if you have an odd new seed, don't throw it away for being worthless as it is; try to plant it.

Now I should like to thank all of you who made this meeting possible. I shall mention no names, because the time is late and, if I cited everyone who helped, I would have to keep you here for quite a while yet. So let me thank only groups of people: All past Claude Bernard Professors and other distinguished scientists who came from as far away as France, England, Germany, Brazil and Japan—to mention just a few countries represented here—to honor us with their presence and raise the scientific level of our meeting. The members of the Organizing Committee, who gave so generously of their time for months in preparation of this event. Last, not least, I also want to thank all my former and present pupils for having made this a truly memorable event—and for being what they are.

I was particularly touched by the fact that, through the very special style you gave this reunion, I can see that you have really come to know me. Many of the scientific contributions were indeed first rate, quite comparable to the best I hear at the large international congresses, and you managed to steer clear of all priority squabbles (even those that are usually slipped in between the lines). Nobody tried to show *who* is right but *what* is right, an important point that we emphasized so often during your postgraduate days when we spoke of the guidelines on public speaking and the writing of articles on

scientific subjects. The social events that you organized were characterized by warmth and fellowship, without any of the clowning and horseplay that usually goes on at class reunions. I thank you from the bottom of my heart for all this.

In the course of my long scientific career, I have had the usual share of academic honors, medals, and prizes that accumulate in this profession if you live long enough; but I can assure you that, today—as I look back upon these years—I consider this meeting as my most memorable reward. It shows me that although I have been awfully rough with many of you at times, and have always driven you hard, somehow you all got to be somebodies and apparently you did not mind my spartan education, because otherwise you would not have given me this wonderful party. Thank you.

**B. Halpern:** Un jour, en traversant la salle d'un grand laboratoire américain, j'ai vu une inscription sur la porte: "Chercheur, il est tard dans la journée et tu n'as encore rien vu." Mon cher Selye, il est encore très tôt dans votre vie et vous avez vu déjà tant de choses. Au nom de mes collègues français et en mon nom personnel je voudrais vous remercier de nous avoir permis de vivre quelques heures enrichissantes et inoubliables. Merci pour le festival Selye.

**P. Constantinides:** Doctor Selye, there isn't much to say after your beautiful Good-Bye. I want to tell you that I am grateful to you, like all of us, for what you have given us, by letting us watch your mind in action, and for showing us that the mind can triumph over the machine, because you discovered a terribly important physiological mechanism in the 20th Century with the simplest means, something that Vesalius could have discovered had he had the mind for it. The second thing I want to thank you for is for bringing me here to Canada. You brought me and many other people, hundreds of scientists, and we are very grateful for this and very proud to be members of this country that has a great role to play in the whole world as a mediator nation and as a scientific nation.

**N. Carey:** It is a real pleasure to be invited to attend this Conference in honour of Hans Selye and a very great privilege to be given the opportunity to voice my appreciation in a few closing words. It is especially memorable to have just witnessed the standing ovation he has received from such a distinguished gathering. In 1936, it may well have seemed impossible to Hans Selye that some thirty years later he would be standing on Mont Tremblant surveying the rich harvest that has sprung from the seed of his laboratory curiosity. The exceptionally high standard of the first paper, read at this meeting

by my good friend Roger, has been brilliantly sustained throughout. This more than all else must on such an occasion be the highest compliment Hans Selye could both wish for and deserve. There are also the absent friends, those laboratory and clinical investigators working with meticulous care, their creative talent too often limited only by a contemptuous budget, who, were they also present, would salute Selye and wish him many happy returns. When the loneliness of the laboratory is accentuated by frustration and despair, it is worth remembering that those who will benefit embrace five continents, although we all know it is really the intellectual challenge that is the spur. Observation and correlation were the first two principles Selye impressed on me when I came to the Institute in 1948. I joined a happy family. My brothers and sisters comprised many nationalities and it is a great joy to be able to meet most of them again. Those unable to be present are sadly missed by us all. About that time, Selye and Stone had just induced myeloid metaplasia with crude anterior pituitary extract. Every medical student soon learns the problems facing medicine and for me these included especially the pulmonary cripple and the leukaemic child. Since the highest concentration of histamine in the blood under any circumstances occurred in myelogenous leukemia, we decided to look at this aspect. The bioassay method being so delicate, I thought it simpler and indeed fitting to enlist the technical assistance of Bram Rose's laboratory at McGill. The results were interesting, though certainly not startling. Circumstances then intervened and I returned to England. To observe and correlate become the more vital the closer one approaches the bedside. As the Sister tutors are wont to say, anyone can save a patient from a bad doctor but no one can save him from a bad nurse. The leap from the laboratory to the bedside is also of fundamental importance, as Doctor Heuser illustrated so clearly in his address and the late Philip Hench demonstrated so brilliantly.

Attending this superbly organised Conference and meeting again my friends and colleagues has been a wonderful experience that I will remember for many years. For the past ten years I have been working in the family practice. Though the history of medicine is studded with the names of illustrious men, like Mackenzie and Jenner, who engaged in general practice, nowadays there is little time to listen, to talk, to read and to reflect. In fact, I feel I have briefly emerged from the salt mines and spent two days at the Ritz. In the last decade, sitting in a cramped surgery in South East London

while Selye has been standing in his laboratory overlooking the beautiful city of Montreal, I have often thought we both could be saved a lot of trouble if man would only write and dispense his own prescription for stress—peace, courtesy and his labour held in honour. On this happy occasion, however, the only thought in all our minds is simply *Hans Selye, ad multos annos, vivat.*

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