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Volume 3

METHODS IN PSYCHOBIOLOGY

Advanced Laboratory Techniques in
Neuropsychology and Neurobiology

EDITED BY
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PREFACE

The third volume of this series is devoted in large measure to specialized techniques that are widely used in the fields of psychobiology and neurobiology. The experimental methods described in the following pages form a companion to those presented in Volumes 1 and 2. Many of the procedures presented here hinge directly on a mastery of the more rudimentary techniques dealt with in the earlier volumes. In fact, some aspects of each presentation can be viewed as really specialized extensions of more fundamental methods or bench-top procedures. For example, lesioning or perfusion of brain tissue and electrical recording from given loci all require the development and attainment of certain skills in surgical, stereotaxic, and histological procedures before a research problem is considered or a project is even instigated. Therefore, the reader is urged to become acquainted not only with the special material in the earlier volumes but to become equally familiar with the extent of coverage of their subject matter.

The emphasis in the content of this book, as in the preceding volumes, is on one facet of neurobiology, the mammalian central nervous system. Again, the central or peripheral processes that affect this system in a rat or larger laboratory animal constitute the principal focus of this series. This is due in large part to the enormous amount of research carried out with higher species and the generalizations that, it is hoped, may be drawn to the nervous system of the human.

We are fortunate to have contributions in Volume 3 from scientists who are at the forefront of their respective disciplines. Each author is a specialist in his own right, an active laboratory worker who, because of a close proximity to the day-to-day problems posed by the method, is able to describe in detail the alarmingly large number of factors that constitute the successful undertaking of an experiment in this field.

A special word of gratitude is given to my wife, Marjorie A. Myers, for her constant dedication to the production of this volume and to the countless details associated with it. Thanks are also due to Anne Beaver and Kay Solomon for their assistance in proofreading. The patience and support of the staff of Academic Press, New York, are gratefully acknowledged.

R. D. MYERS

Chapter 1

Anatomical Analysis of Neuronal Connectivity

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I. INTRODUCTION

Cells in most parts of the body directly communicate only with immediately adjacent cells, and it is unusual to find elongated cytoplasmic processes extending for great distances in organs other than the brain. The existence of long processes, often arranged in discrete bundles, is one of the unique features of the brain and spinal cord and permits serial communication between neurons in different parts of the nervous system. It is not surprising, therefore, that the study of pathways formed by these neuronal processes is considered a necessary first step in the analysis of the function of any part of the central nervous system (CNS).

The study of neuronal projections has been termed *hodology* (Greek: *hodos*, path), and several anatomical techniques have been developed to reveal pathways. During the latter half of the nineteenth century histological studies of the brain were concerned largely with establishing the cellular nature of the neuron, culminating in the 1880s with the acceptance of the "neuron doctrine." Although significant contributions to the analysis of long tract systems in the brain were made following the introduction of the Golgi method in 1875, it was the discovery by V. Marchi of a technique whereby one could selectively stain the fragmented myelin sheaths of degenerating axons that permitted an experimental approach in

hodological studies. "Waller's Law" soon became established, stating that following interruption of an axon the distal portion degenerates while the proximal axon and cell body are unaffected (Cajal, 1928). It was later shown by van Gehuchten (1903) that under certain circumstances interruption of the axon may also lead to degeneration of the proximal axon and cell body. The development of new information about the process of neuronal degeneration, together with the introduction of the double silver impregnation method by Bielschowsky (1904), set the stage for many of the methods currently used to study axonal pathways. In the early 1950s Nauta and his colleagues (Nauta and Gygax, 1951, 1954; Nauta and Ryan, 1952; Nauta, 1957) introduced modifications of Bielschowsky's method that resulted in suppression of the staining of normal axons, allowing selective visualization of degenerating fibers. The method of Nauta, along with subsequent modifications of the staining procedure by Fink and Heimer (1967), has been one of the principal techniques available for determining the course and sites of termination of fiber systems in the brain. In recent years, several additional anatomical methods have been added to the armamentarium of the neuroanatomist. The major methods currently employed in the study of neuronal connectivity are based upon one or more of the following principles:

1. Impregnation of degenerating axons and/or terminals with silver salts and the suppression of staining of intact axons (Nauta and Gygax, 1954; Fink and Heimer, 1967)
2. Electron microscopic studies of ultrastructural changes that occur in synaptic terminals of axons that have been interrupted (Colonnier, 1964; Gray and Guillory, 1966; Westrum, 1973)
3. Incorporation of labeled amino acid into protein in the soma; it is then transported along the axon to the terminal (Cowan *et al.*, 1972; Edwards, 1972; Hendrickson, 1975a)
4. Pinocytosis at the synaptic terminal of enzymes or peptides such as horseradish peroxidase or nerve growth factor, and their subsequent retrograde transport to the soma (LaVail and LaVail, 1972, 1974; LaVail, 1975; Stöckel *et al.*, 1974)
5. Histochemical visualization of neurotransmitters or their synthetic or degradative enzymes (Koelle and Friedenwald, 1949; Koelle, 1957; Fuxe *et al.*, 1970; Fuxe and Jonsson, 1973; Hökfelt and Ljungdahl, 1972; Hökfelt *et al.*, 1974)

While there are still other methods available, this list will be sufficient for the purposes of a general discussion. The first two principles enumerated here involve the destruction of neural tissue and depend upon subsequent

axonal degeneration. Since not all parts of the cell degenerate at the same rate, the survival time is a major variable in neuroanatomical experiments employing degeneration methods. The following description applies to several regions of the CNS, but there is considerable variability in the time course of degeneration from region to region.

Following interruption of an axon, the earliest morphological signs of degeneration are seen in synaptic terminals when the tissue is examined with the electron microscope. Two types of degenerative changes have been seen. In most parts of the CNS, compaction of synaptic vesicles together with increasing electron density of the cytoplasm begins within 36 hr of interruption of the axon (Gray and Guillory, 1966). The rate of terminal degeneration depends upon the length of the axonal stump (Vaccarezza *et al.*, 1970). Over the next day or two the synaptic terminal pulls away from its attachment site on the postsynaptic neuron and is engulfed by glial processes. The degenerating terminal will eventually be removed by glial phagocytosis and, to some extent, by ingestion by nearby dendrites (Walberg, 1963). Degenerating terminals may have largely disappeared 5–6 days following severance of the axon. A second, less common, form of terminal degeneration is associated with hypertrophy of the terminal and an increase in the number of neurofilaments (Jones and Rockel, 1973).

The breakup of the axon distal to the site of injury begins several days after axonal interruption and reaches its peak 5–12 days later. Thereafter, degeneration products are gradually removed by phagocytosis. The rate at which axons degenerate depends upon the diameter of the fiber and the location of the cell in the nervous system. Although maximal degenerative changes occur within a week in the dorsal columns following interruption of dorsal root afferent fibers, early stages of axonal degeneration have been seen as late as 100 days (Bignami and Ralston, 1969). In peripheral nerves, degeneration products may persist for only short periods (Cottle and Mitchell, 1966). Apparently phagocytosis by Schwann cells in the periphery proceeds more rapidly than microglial phagocytosis in the CNS.

The extent of retrograde degenerative changes appears to depend upon the distance between the site of axonal injury and the cell body and also upon the presence or absence of collateral branches proximal to the site of injury. The traditional methods of studying retrograde degeneration depend upon the changes in volume of the cell body, the location of the nucleus, and dissolution of Nissl bodies, all of which are signs of the process called *chromatolysis*. Chromatolytic changes and increases in some of the enzymes in the cell body occur progressively, reaching a peak 2–3 weeks following axonal injury (Bodian and Mellors, 1945). The cell

may recover or go on to vacuolization and fragmentation, ultimately being removed by microglial phagocytosis and replaced by the proliferation or hyperplasia of astrocytes. A general scheme of the varying rates of degeneration seen in axon terminals, large and fine axons, and in the cell body is shown in Fig. 1. *The scheme is only intended to show relative differences in the rates of degeneration, and the reader must keep in mind that these may vary in different parts of the nervous system.* One must also remember that interruption of axons may lead to transneuronal changes, i.e., degeneration in the postsynaptic cell (Van Buren, 1963; Valverde, 1967). The extent to which this occurs is variable and may be related to the degree of removal of input to the postsynaptic neuron.

This brief description of the degenerative process is intended to emphasize the importance of time as a variable in the design of anatomical experiments based upon the degeneration principle. Not all of the methods available permit visualization of every part of a neuron. Therefore, in selecting a method to study connectivity in the CNS, it is important that the investigator have a clear idea of the information sought and the degree of detail required. The following questions should be considered in designing neuroanatomical experiments:

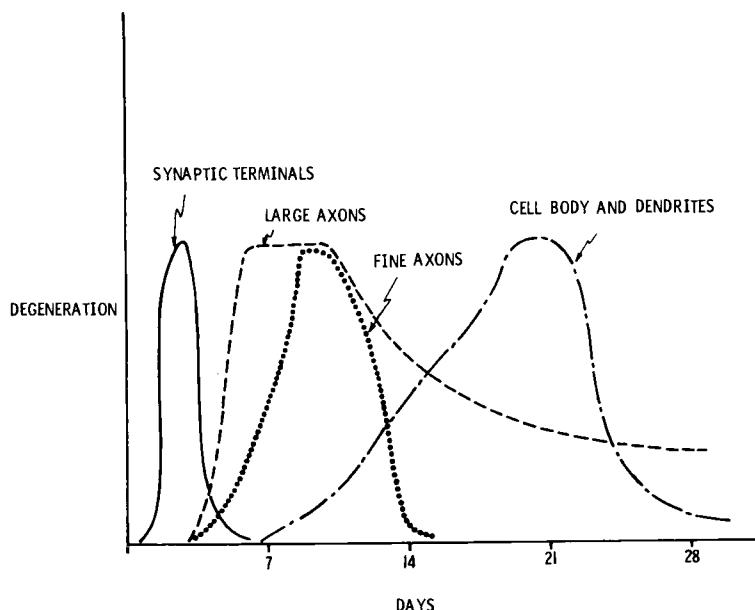


FIG. 1. Schematic representation of the relative time course of morphologic changes in portions of the neuron proximal and distal to injury of the axon. The absolute times will vary in different parts of the nervous system. See text for details.

1. Is it necessary to determine the course of axons as well as their site of termination?
 2. Is it sufficient to define the general nuclear region in which axons terminate, or is it necessary to know whether axons terminate upon dendrites, dendritic spines, or the soma of postsynaptic neurons?
 3. Is it important to characterize the morphological features of axon terminals, such as synaptic vesicle shape or density and patterns of brain postsynaptic thickenings?
 4. Does one wish to characterize the pharmacological nature of the neuronal projection, i.e., cholinergic, noradrenergic, GABAminergic (GABA: γ -aminobutyric acid), etc.?
 5. Is it necessary that the anatomical method selected reveal all of the axons or cell bodies present?
 6. Is it important to distinguish axons passing through a region from axons originating from cells within that region?
 7. Is it necessary to obtain quantitative data about synaptic terminals?
- The answers to these questions will determine the methods that will have to be employed. Let us consider one illustration of an experimental situation in which it is desirable to obtain anatomical information. For the purposes of this discussion, consider three zones of the nervous system containing five types of neurons as illustrated in Fig. 2. The neurons are

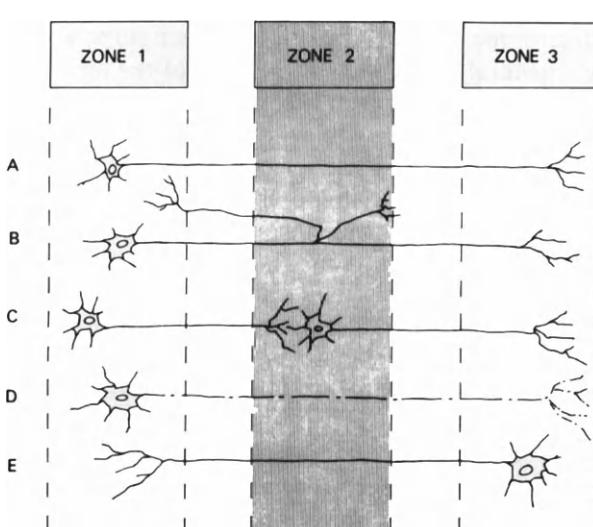


FIG. 2. Simplified schema of several ways in which three zones or regions of the CNS may be interconnected by neurons A to E. See text for discussion of the selection of anatomical methods used in analysis of connectivity.

labeled A to E. Neuron A has a cell body in zone 1 and an axon passing through zone 2 to terminate in zone 3. Neuron B is similar, except that it has collateral branches leaving the axon in zone 2 which run back to terminate in the neuropile of zone 1. Neuron C represents a cell with a shorter axon which synapses upon neurons in zone 2, which in turn project to zone 3. A pharmacologically different cell is represented by D. For example, cell D might represent a monaminergic neuron, whereas cell A synthesizes some other transmitter. Finally, cell E has its cell body in zone 3 with its axon projecting through zone 2 back to zone 1.

Assume a situation in which it has been experimentally observed that electrical stimulation in zone 2 produces stimulus-bound eating which can be prevented by the prior administration of adrenergic-blocking drugs. In this example, zone 1 might represent the septal region, zone 2 the lateral hypothalamus, and zone 3 the midbrain. The further analysis of stimulus-bound eating in this case would lead the investigator to seek information about the neuronal connections of zone 2, as well as the distribution of monaminergic neurons in this region. Since electrical stimulation will activate both cell bodies and axons of passage, it is not important in this example to distinguish between these two possibilities. However, since electrical stimulation of axons will cause action potentials to be propagated antidromically as well as orthodromically, it is important to know whether the axons afferent to zone 2 possess collateral branches in other zones.

The investigator may decide that, for his or her purposes, it is necessary to have only a general knowledge of the sites of the terminal fields of the axons but that it is very important to know the course of the axons. In acquiring this information it would be necessary to use at least three different anatomical methods. The course of axons originating in or passing through zone 2 could be determined by use of one of the silver impregnation methods after a lesion is produced in the region. However, it would be necessary to use groups of animals with different survival periods, since large and small fibers do not degenerate at the same rate.

The presence of monaminergic pathways could be studied using the histochemical fluorescence method. The question of whether or not afferent fibers have collateral branches to other regions of the nervous system is more difficult to answer and may require use of the horseradish peroxidase method in order to determine the location of the cells of origin of the afferent fibers. With this information, use of axoplasmic transport autoradiography in another group of animals would enable the investigator to define the axonal projections of cells located in the regions identified by the peroxidase studies. Even then, the answer may be equivocal and resolved only by electrophysiological testing of the infer-

ences drawn from the anatomical studies. Unless great care is taken in specification of the anatomical questions asked, the resulting information may be of little use in the design of further experiments.

II. ANATOMICAL METHODS

A. Degeneration Methods

1. Anterograde Degeneration

a. *Light Microscopy.* Excellent comprehensive discussions of silver impregnation methods for staining degenerating axons, along with detailed staining protocols, have been provided by Guillory (1970) and by Heimer (1970). The following discussion is intended to serve as an introduction to the literature for those who are not experienced in the use of these techniques.

As mentioned earlier, in some parts of the nervous system the rate of degeneration appears to vary as an inverse logarithmic function of fiber size, so that fine fibers require a longer time to fragment (Van Crevel and Verhaart, 1963). The principal silver methods used for the study of axonal degeneration have evolved from the Bielschowsky (1904) technique. Nauta and Ryan (1952) and Nauta and Gygax (1954) achieved suppression of silver impregnation of normal, intact fibers by pretreating sections in a solution of phosphomolybdic acid followed by immersion in a potassium permanganate solution. The potassium permanganate acts as an oxidizing agent in the pretreatment step. If the tissues have been stored for some period of time in formalin, the time in permanganate can be reduced. This pretreatment permits the impregnation of degenerating fragments of axons and, in some parts of the nervous system, terminals (Guillory and Ralston, 1964; Sprague and Ha, 1964). In other regions it is not always possible to find morphological evidence for the staining of terminals (Nauta, 1957; Szentagothai and Rajkovits, 1959; Eager and Barrnett, 1966).

In the suppressive silver stains it is necessary to depend upon the pattern of distribution of degeneration fragments in order to make judgments about whether one is visualizing axons of passage or the terminal plexus. A pericellular distribution of some degeneration fragments is often considered a sign of terminal degeneration, but it is not always possible to see the soma and dendrites of cells outlined by degenerating fragments. More often, one simply sees an apparently random distribution of degenerating fragments in the neuropile. Since this pattern differs from the more orderly, linear arrangement of fragments along the course of the fiber bundle, it is usually interpreted as the branching of axons

immediately before termination and designated "preterminal" degeneration. Since clear-cut morphological signs of terminal degeneration at the light microscope level are usually not seen with the original technique of Nauta and Gygax, this method is primarily useful as a means for tracing the course of axons and determining the general field of termination of fibers. The chemical basis of silver staining is not well understood, but some rationale for certain steps in the procedure is provided by the studies of Eager and Barnett (1966) and Giolli (1965). The Nauta method stains mainly degenerating axons and/or myelin and is not effective after extraction (Evans and Hamlyn, 1956).

Variability in staining of degenerating synaptic terminals by the Nauta method led to the development of further modifications. Fink and Heimer (1967) introduced two variants of the staining protocol known as procedures 1 and 2. The principal change in procedure 1 is elimination of tissue pretreatment in phosphomolybdic acid; the sections are placed directly in a solution of potassium permanganate and subsequently impregnated in a mixture of silver nitrate and uranyl nitrate. Procedure 2 is basically the same, except that subsequent impregnation uses pyridine-silver rather than a silver-uranyl nitrate mixture.

Both of the Fink-Heimer procedures also revert to the use of ammoniacal silver nitrate solution for the second stage of silver impregnation. This was called for in the original Nauta method but was subsequently replaced by Laidlaw's ammoniacal silver carbonate solution in a modification introduced by Chambers *et al.* (1956). Procedure 2 produces less consistent results, with frequent background staining of glial cells or nonspecific silver deposits, but offers greater latitude in manipulation of the various steps in the staining schedule and is useful in revealing degeneration in some fiber systems which are difficult to impregnate with procedure 1.

It is customary to run a permanganate time series when working with regions of the brain in which one has not had previous experience. Frozen sections of the tissue are cut, generally at 25 μm thickness, and 5–10 adjacent sections are collected in each compartment of a multicompartiment tray. After the block is completely cut, several sections from the region containing the lesion, where it can be reasonably expected that degeneration is present, are removed, and each section is treated in permanganate for a different period of time before one proceeds to the remaining steps in the staining protocol. This permits a reasonable assessment of the optimal time for the pretreatment oxidation step needed to provide good visualization of axonal or terminal degeneration and suppression of normal fiber staining. For the convenience of the reader,

an outline of the steps in the Fink-Heimer (Fink and Heimer, 1967; Heimer, 1970) and Eager (1970) techniques is presented next.

METHOD OF FINK AND HEIMER

Procedure 1

1. Rinse fixed frozen sections in distilled water (three changes, 5 min each).
2. Immerse in 0.05% potassium permanganate for 5–10 min. (Make fresh daily.)
3. Rinse briefly in distilled water.
4. Immerse in bleaching solution for 1 min, or until the sections are completely decolorized. (Make fresh daily.)
5. Rinse in distilled water (three changes, 5 min each).
6. Immerse in Solution A for 30 min.
7. Immerse in Solution B for 60 min.
8. Rinse in distilled water (three changes, 5 min each).
9. Immerse in Solution C for about 1–3 min.
10. Immerse in reducing solution. Agitate quickly until tissue turns brown, then transfer to a second volume of reducing solution for 1–2 min.
11. Rinse in distilled water.
12. Immerse in 0.5% sodium thiosulfate for 1 min.
13. Rinse in distilled water (three changes, 5 min each).
14. Mount from water onto gelatinized slides, blot, dry briefly, dehydrate, clear, coverslip.

Solutions

Bleaching solution

Mix equal parts of freshly prepared solutions of 1% oxalic acid and 1% hydroquinone.

<i>Final volume desired</i>					
	50 ml	100 ml	200 ml	500 ml	1000 ml
<i>Solution A</i>					
Uranyl nitrate	50 mg	100 mg	200 mg	500 mg	1 gm
Silver nitrate	250 mg	500 mg	1 gm	2.5 gm	5 gm
Distilled water	50 ml	100 ml	200 ml	500 ml	1000 ml
<i>Solution B</i>					
Uranyl nitrate	0.087 mg	0.175 mg	0.350 mg	0.875 mg	1.75 gm
Silver nitrate	0.813 mg	1.625 mg	3.25 gm	8.13 gm	16.25 gm
Distilled water	50 ml	100 ml	200 ml	500 ml	1000 ml

Solution C

Add 1.0 ml of the base mixture (below) to 10.0 ml of 2.5% silver nitrate solution and stir until dissolved. (Use this solution within 30 min.)

Base mixture

9 parts (45 ml) 2.5% sodium hydroxide
 6 parts (30 ml) concentrated ammonia water
 (Keep in a small, tightly closed bottle.)

Reducing solution

900 ml water
 75 ml 95% alcohol
 17 ml 10% formalin
 20 ml 1% citric acid

Potassium permanganate solution

50 mg per 100 ml distilled water. (Make fresh daily.)

Procedure 2

1. After a brief rinse in distilled water, soak fixed sections for 5–10 minutes in 0.025% potassium permanganate.
2. Rinse and decolorize sections in bleaching solution.
3. Rinse thoroughly and transfer sections to a 2.5% uranyl nitrate solution for 5–10 min.
4. Rinse thoroughly and transfer sections to a 0.3% silver nitrate solution for 1–2 hr. The addition of 0.2 ml pyridine to 10 ml of the silver nitrate solution may improve the results.
5. Without washing, the sections are transferred for 2–5 min to a freshly prepared ammoniacal silver nitrate solution composed of:

1.5% silver nitrate solution	20 ml
95% ethyl alcohol	12 ml
Strong ammonia water	2 ml
2.5% sodium hydroxide	1.6–1.8 ml

6. Transfer sections without rinsing to the reducing solution described in Procedure 1.
7. Rinse and transfer sections to 0.5% sodium thiosulfate solution for 1 min.
8. If desired, counterstain by immersing sections briefly in a cresylecht violet solution.
9. Rinse, mount, dehydrate, clear, coverslip.

METHOD OF EAGER*Staining Procedure*

1. Rinse fixed frozen sections in distilled water.
2. Immerse in 2.5% uranyl nitrate for 5 min.
3. Immerse in ammoniacal silver for 2–5 min.
4. Transfer sections to reducer for 2–5 min.
5. Rinse in distilled water.
6. Immerse in 0.5% sodium thiosulfate for 2 min.
7. Rinse in distilled water.
8. Dehydrate, clear, and mount.

*Solutions**2.5% uranyl nitrate**Ammoniacal silver*

40 ml 1.5% silver nitrate
 24 ml 95% ethyl alcohol
 4 ml ammonium hydroxide
 3.6 ml 2.5% sodium hydroxide

Reducing solution

810 ml distilled water
 90 ml 100% ethyl alcohol

27 ml 1% citric acid

27 ml 10% formalin

0.5% sodium thiosulfate

The intraaxonal and terminal deposition of reduced silver in degenerating neurons stained with the Fink-Heimer method has been confirmed by electron microscopy (Heimer, 1972). Other variants of the silver impregnation methods will be found in the publications of deOlmos (1969), deOlmos and Ingram (1971, 1972), Kalaha-Brunst *et al.* (1974), and Desclin and Escubi (1975). The Desclin-Escubi method is said to be especially helpful in staining dendritic and soma fragments as well as axonal and terminal degeneration resulting from the cellular breakdown produced in animals treated with antimetabolites. There is some controversy over the identification of retrograde degeneration in Fink-Heimer stained material, and the investigator must be aware of this possibility in interpretation of results (Grant and Aldskogius, 1967; Grant, 1968; Tanaka and Chen, 1974; Kalil, 1975).

Although some investigators have questioned whether silver stains can impregnate degenerating monaminergic fibers, Hedreen and Chalmers (1972), Ibata *et al.* (1973), Shimizu and Ohnishi (1973), Shimizu *et al.* (1974), Simon *et al.* (1974), and McBride and Sutin (1976) have successfully used the Fink-Heimer method to show degeneration in catecholaminergic pathways. The Fink-Heimer techniques work well in the rodent and carnivore but are less reliable in primates. Wiitanen (1969) has modified the Fink-Heimer technique (procedure 2) to effectively stain monkey brain. For those investigators interested in the early maturation of brain pathways, the distinction between short-lived and longer-persisting argyrophilia following CNS lesions may be useful (Leonard, 1975).

b. *Electron Microscopy.* Unequivocal evidence of synaptic terminal degeneration usually requires cytological studies with the electron microscope. Once the general area of termination of a fiber tract has been determined with silver impregnation methods, precise sites of termination of axons upon postsynaptic cells can be determined in ultrastructural studies. The reader will find an excellent review of the morphology of both normal and degenerating synapses in Gray and Guillory (1966), and a brief introduction to some methods of tissue preparation for electron microscopy in Palay and Chan-Palay (1974).

In the cerebral cortex, two classes of synaptic terminals can be distinguished when the morphological arrangement of synaptic vesicles and pre- and postsynaptic membrane thickenings are considered (Gray, 1959; Gray and Guillory, 1966). The type I synapse of Gray forms contacts with

dendritic spines of pyramidal cells in the cerebral cortex and with the dendritic spines of Purkinje neurons in the cerebellar cortex. The junctional membrane thickenings extend over most of the contact area between the terminal and the dendritic spine. Synaptic vesicles are round and clustered close to the presynaptic membrane thickening. The type II synapses of Gray form axosomatic contacts upon pyramidal or Purkinje cells and show only patchy thickenings in the region of contact between pre- and postsynaptic membranes. In contrast to the type I synapse, the synaptic cleft of the type II synapse is not increased, and the postsynaptic thickening is not markedly thicker than the presynaptic thickening. For these reasons, the type I synapse is described as an asymmetrical synapse, and the type II synapse as a symmetrical junction.

In other regions of the nervous system there may be intermediate forms, making it more difficult to characterize synapses as type I or type II according to the scheme of Gray. The synaptic vesicles in some terminals are subject to osmotic deformation, whereas those in other terminals resist deformation. With appropriate preparation of tissues for electron microscopy, terminals may be said to contain either round or flattened synaptic vesicles. The synaptic terminals which resist osmotic deformation usually have features of Gray type I junctions. In certain parts of the nervous system where there are electrophysiologically or behaviorally defined inhibitory pathways, it has been possible to correlate the morphology of synaptic vesicles with an excitatory or inhibitory function of the pathway. It has been found that Gray type II synapses with flattened vesicles occur at sites where inhibitory synapses would be expected [(Uchizono, 1965; Bodian, 1966; Laramendi *et al.*, 1967); see also Palay and Chan-Palay (1974) for a review of this topic], and type I synapses with round vesicles at sites where excitatory synapses occur. It would be unwise to carry this generalization too far until there is additional evidence correlating synaptic morphology with function in many other regions of the nervous system. In molluscan neurons the function of a synaptic transmitter may result in inhibition, excitation, or a combination of both effects, depending upon the postsynaptic membrane receptor mechanism (Kandel and Gardner, 1972). It would, therefore, be unwise to attempt to categorize synapses solely on the basis of the morphology of presynaptic elements.

Following interruption of the axon, degenerative changes in synaptic terminals may take three forms. Most commonly, an increase in the electron density of the cytoplasm of the terminal is observed together with fragmentation of mitochondria and compaction of synaptic vesicles. Only later in the degenerative process does the presynaptic terminal pull away from its postsynaptic attachment site and become engulfed by the

glial processes. The second type of degeneration involves enlargement of the terminal together with proliferation of neurofibrils (Gray and Guillory, 1966; Jones and Rockel, 1973; Westrum, 1973). A third form of terminal degeneration involves swelling of the terminal with disintegration of synaptic vesicles and mitochondria, but it is not accompanied by an increase in the number of neurofilaments. This has been termed "light type" to distinguish it from the "dark type" (i.e., "electron-dense") and filamentous forms of degenerative changes (Walberg, 1972; Westrum, 1973; Fifková, 1975).

As a cautionary note, one must be alerted to the occurrence of spontaneous degeneration, or the appearance of dark profiles in apparently normal tissue (Ibata and Otsuka, 1968; Cohen and Pappas, 1969).

Ultrastructural signs of degeneration have been extremely helpful in providing new information about the organization of the CNS. Although a detailed consideration of the problems in the application of this method is beyond the scope of this chapter, the time of survival following axotomy is extremely important. The light type of degeneration appears to begin earlier than the dark type. Since not all terminals degenerate at the same rate, it is difficult to obtain quantitative information when using a single survival time. In general, degeneration may begin within 24 hours. The terminals become extensively fragmented and removed by phagocytosis within 5 days. After 3 days, terminals in many parts of the nervous system have detached themselves from the postsynaptic cell, making it impossible to determine whether one is dealing with an axodendritic or axosomatic synapse. Thus the degeneration times must be chosen to provide clear evidence of degeneration in the terminal while also being early enough to preserve the contact site with the postsynaptic cell.

2. Retrograde Degeneration

a. *Chromatolysis and Histochemistry.* In addition to plotting the course of degenerating axons distal to the site of axotomy, an investigator may wish to determine the location of the cell bodies from which the proximal segment of the axon arises.

In medium-size and large neurons, the cytological changes associated with the degenerative and regenerative phases following axonal injury are sometimes clearly revealed with routine Nissl stain. These changes usually begin later than do terminal or axonal degeneration and persist for a longer period. However, there is an extreme variability in the time course of the changes in the cell soma. Retrograde reaction in the cell body, termed chromatolysis, depends upon (1) the type and age of the animal; (2) the distance from the cell body at which the nerve is injured; (3) the survival time; and (4) the histologic and functional type of neuron, e.g., somatic-motor neuron or visceral-motor neuron (Geist, 1933). In

very young animals, interruption of the axon is more likely to lead to irreversible degeneration of the proximal portions of the neuron (Brodal, 1940). Retrograde degeneration begins at the cell body and proceeds peripherally toward the site of injury (Cowan *et al.*, 1961). In neurons in which the axon has two or more major branches, severance of some branches may lead to greater chromatolytic reaction in the cell body than does interruption of other branches (Fry and Cowan, 1972).

If the proximal segment of the neuron does degenerate completely, the debris is phagocytized by microglia (Torvik, 1972) and replaced by the proliferation or hyperplasia of astrocytes. When there is a massive loss of cell bodies, such as the dorsal thalamic degeneration seen after extensive lesions in the parietal cortex, the resulting gliosis can be used to help determine the location of the thalamic nuclei projecting to the damaged region of the cortex.

In addition to the morphological changes visible in Nissl stained material, enzymatic changes also occur in the cell bodies of neurons whose axons have been damaged. These enzymes may be visualized using precipitation histochemical techniques. Bodian and Mellors (1945) observed an increase in cytoplasmic acid phosphatase in motor neurons 10–28 days after peripheral nerve damage. In a subsequent study, Hirsch and Oben-chain (1970) used fluorogenic substrates to visualize acid phosphatase activity in motor neurons of the spinal cord anterior horn. These investigators were unable to find differences in enzyme concentration in cell bodies of neurons whose axons were intact and those whose axons had been interrupted; they did find, however, an increase in glucose-6-phosphate dehydrogenase levels in anterior horn motor neurons whose axons had been damaged. Kreutzberg (1963) and Meyer and Cole (1970) examined changes in TPNH-diaphorase¹ activity in neurons undergoing chromatolytic reaction. The latter authors found an increase in enzymatic activity in the perikarya of cells which had axons passing outside of the CNS, whereas those cells which had axons remaining within the CNS showed a decrease in enzyme levels following injury. This brief review shows that while, under certain conditions, a combination of enzymatic and morphological techniques may be useful, great care must be exercised in the interpretation of retrograde degeneration following axotomy.

B. Nondestructive Methods

1. Anterograde Methods

a. Axoplasmic Transport Autoradiography. In the course of studies

¹ TPNH: triphosphopyridine nucleotide (reduced form), also known as nicotinamide adenine dinucleotide phosphate (NADPH, reduced form).

related to the axoplasmic transport of protein, Droz and LeBlond (1963) demonstrated that the systematic injection of tritiated amino acids, such as leucine, resulted in the rapid labeling of the nucleus and cell body of neurons. Peak labeling of the cell body occurred within 30 min. At successively longer periods following the injection of amino acid, autoradiography revealed a progressive movement of radioactivity along the axon. The value of the autoradiographic visualization of transport of labeled proteins along the axon was recognized by Cowan *et al.* (1972) and Edwards (1972). Both publications outline the autoradiographic technique for studying neuronal projections. A more concise description of the procedure will be found in Hendrickson (1975a) and Graybiel (1975).

Basically, small volumes (0.05–0.5 μl) of tritiated leucine or proline are slowly injected through a microsyringe needle or micropipette. Prior to injection, the isotope is usually concentrated by centrifugation under a vacuum. The desired activity, ranging from 10 to 50 μCi of tritiated amino acid per microliter, is achieved by redilution. Since protein synthesis occurs largely in the perikaryon, labeled amino acid is incorporated by cell bodies. This method allows the investigator to study only axonal projections arising from neuronal somata in the vicinity of the injection without the labeling of axons originating elsewhere and passing through the region.

Droz (1973) studied the half-life of proteins which have incorporated labeled amino acid and which are transported along the axon to the synaptic terminal. Axoplasmic transport occurs at a variety of rates, but with a prominent slow phase moving at 1–4 mm/day and a rapid phase traveling 100–400 mm/day (Ochs, 1974; Lasek, 1975). By selection of the appropriate survival time following injection of the labeled amino acid, axonal terminals may be preferentially labeled as a result of the movement of labeled protein carried in the fast phase of axoplasmic transport to the synaptic knobs. With longer survival times, labeled protein moves along with the slow phase of axoplasmic transport, allowing visualization of the axon. Since the half-life of neuronal proteins is usually many days, long survival times will allow demonstration of both synaptic terminations and the course of the axons (Edwards, 1975).

b. *Interpretation.* The problem of interpretation of autoradiographs is not unlike that encountered in silver impregnation of axonal degeneration. Only in limited regions of the nervous system is there a clear-cut morphological pattern of the arrangement of synaptic terminals. When the terminal axon breaks up into many branches distributed throughout a large area of neuropile, it is not always possible to distinguish the label in some axons from that in synaptic terminals. Just as it was necessary to make electron microscopic observations of terminal degeneration in order un-

equivocally to identify sites of termination, electron microscopic autoradiography may be necessary to determine the site and nature of the terminals containing labeled protein (Hendrickson, 1975b).

Some investigators prefer to use tritiated leucine, whereas others feel that tritiated proline results in less nonspecific labeling and is preferentially incorporated by some neurons (Künzle and Cuénod, 1973). Some have dealt with the possibility of this preferential uptake by using a mixture of both amino acids. Leucine has been found equivalent to the mixture of amino acids, at least in brainstem fiber systems (Edwards, 1975). The use of formaldehyde fixation minimizes the occurrence of free, unbound labeled amino acid and is preferable to glutaraldehyde fixation for autoradiography (Peters and Ashley, 1967).

For a general consideration of autoradiographic techniques and associated artifacts, the reader is referred to the monograph of Rogers (1967).

2. Retrograde Methods

a. *Horseradish Peroxidase Uptake.* Horseradish peroxidase [HRP (see Paul, 1963)] was used as a tracer substance in electron microscopic studies by Graham and Karnovsky (1966). This pioneering work led to many ultrastructural investigations of protein uptake in which the peroxidase reaction was used to reveal the sequential events associated with the cellular incorporation of proteins. Although the details vary in different cell systems, exogenous protein is adsorbed upon the plasma membrane, which then forms micropinocytotic vesicles by infolding. The ingested protein is found within membrane-bound vacuoles that may fuse and ultimately become incorporated in acid phosphatase-positive dense bodies (Straus, 1969).

Kristensson and Olsson (1971) reported that intramuscular injection of HRP led to retrograde axonal transport of the enzyme into the soma of the motor neurons supplying the injected region of the muscle. Earlier observations by Zacks and Saito (1969) had shown that exogenous peroxidase is taken up into coated vesicles on the neural side of the neuromuscular junction, but not into synaptic vesicles. The LaVails and others recognized the value of HRP histochemistry for the study of neuronal connectivity and demonstrated the retrograde transport of the enzyme in the CNS (LaVail and LaVail, 1972, 1974; LaVail *et al.*, 1973; LaVail, 1975; Ralston and Sharp, 1973; Nauta *et al.*, 1974; Kuypers *et al.*, 1974). The retrograde transport of HRP probably occurs mainly through endocytosis of the enzyme into coated vesicles, which are then transported back toward the cell body in relation to the smooth endoplasmic reticulum (LaVail and LaVail, 1974; Sotello and Riche, 1974; Nauta *et al.*, 1975).

There is evidence that orthograde transport of HRP also occurs (Lynch *et al.*, 1974; Sotello and Riche, 1974; Hansson, 1973; Sherlock and Raisman, 1975; Snow *et al.*, 1976). The rate of transport of the enzyme may vary with the specific activity of the enzyme, the direction of transport, and the specific neuronal system under study. Optimal visualization of the enzyme has been associated with the slow phase of orthograde transport (Lynch *et al.*, 1974) and with the fast phase of retrograde transport (Hansson, 1973; Sotello and Riche, 1974).

Sherlock and Raisman (1975) found that the more purified form of the enzyme (type VI) was transported more rapidly and also inactivated more rapidly than was the less active (type II) HRP. Because in axons the HRP is packaged in smaller vesicles, the accumulation of the enzyme in axonal terminals following orthograde transport is generally below the limit of resolution of the light microscope and must be studied with the aid of electron microscopy. Retrograde transport, on the other hand, culminates in the fusion of HRP-containing vesicles in the cell body, forming granules visible at the light microscope level.

In order to visualize the exogenous HRP which has been incorporated by synaptic terminals and transported back to the cell body, it is necessary to produce a colored, nonsoluble reaction product. Although fixation with 10% formaldehyde has been used by some workers, most find that an aldehyde mixture consisting of 0.4% paraformaldehyde and 1.25% glutaraldehyde is most effective in the fixing of tissue without producing a marked inactivation of the HRP.

After fixation, the tissue is treated by 3,3'-diaminobenzidine in the presence of hydrogen peroxide. The enzyme then cleaves hydrogen peroxide to form water and permits oxidation of the diaminobenzidine to a colored polymer. This polymerization appears to take place in two steps. The first step involves oxidative polymerization, which is followed by oxidative cyclization to produce a brown phenazine polymer. If the tissue is to be studied with the electron microscope, subsequent treatment with osmium tetroxide is employed (Seligman *et al.*, 1968).

b. *Protocol.* The following protocol is the one currently used in our laboratory and is based upon the methods described by LaVail *et al.* (1973) and Jones and Leavitt (1974).

HORSERADISH PEROXIDASE PROCEDURE

Injection

Add 10 μ l sterile saline to 5 mg HRP (horseradish peroxidase, type VI, Sigma) in a BEEM^{*} capsule; spin down in centrifuge at 8 g to put into solution. The solution should be made up immediately before use and kept at a cool temperature. Intracerebral injection volumes

usually range from 0.05 to 0.2 μ l. To ensure accurate volume administration, only the amount of HRP to be injected is put into an injection micropipette. A small amount of mineral oil is aspirated into the pipette after filling with HRP. After injection the plunger of the microsyringe is pulled back slightly and the pipette is withdrawn from the brain. The latter two steps eliminate HRP spread along the pipette track. Survival times are usually 2–3 days but vary with the system under study.

Perfusion

The volumes listed are those normally used for one cat.

1. Perfuse intracardially with 0.85% saline followed by a solution of 0.4% paraformaldehyde and 1.25% glutaraldehyde. Mix immediately before perfusion:

6 gm paraformaldehyde¹
 37.5 ml 50% glutaraldehyde²
 40.2 gm $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

Add together with enough distilled water to make 1.5 liters. Adjust pH to 7.4 by adding 1.0 *N* HCl.
2. Block and remove brain immediately after perfusion, and store in refrigerator in the glutaraldehyde–paraformaldehyde solution for 24 hr.
3. Transfer brain to 0.1 *M* $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ buffer in 30% sucrose and store 24 hr or overnight in refrigerator. Several changes of the solution are desirable.

13.4 gm $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$
 150 gm sucrose
 Add distilled water to make 500 ml.
 To adjust pH to 7.4, add 1 *N* HCl.

Histology

1. Cut frozen sections into 0.1 *M* phosphate buffer in compartmented tray.

26.8 gm $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$
 Distilled water to make 1 liter
 Add 1 *N* HCl to adjust pH to 7.4.
2. Incubate sections in diaminobenzidine (DAB)–hydrogen peroxide solution for 30 min [50 mg 3,3'-diaminobenzidine tetrahydrochloride per 100 ml Trizma buffer, pH 7.6 (Sigma); add about 2 drops 30% H_2O_2 per 50 ml DAB solution]. The area around the injection site should turn brown almost immediately.
3. Rinse sections in at least two changes of distilled water.
4. Sections can then be mounted and alternate slides stained following normal procedures for frozen sections. It is helpful to scan unstained sections when plotting the distribution of HRP uptake.

Notes

1. Since fixation can partially inhibit the HRP reaction with DAB, it is necessary to limit the storage time of tissue and to rinse sections thoroughly before incubation.

¹ Add paraformaldehyde powder to about 300 ml H_2O ; heat to 65°C, and add NaOH drop by drop until solution clears.

² Use biological grade glutaraldehyde, and filter through activated charcoal before use. The solution must always be kept under refrigeration. If the solution becomes cloudy after addition of the glutaraldehyde, it should be discarded.

2. All solutions (except phosphate buffer) should be prepared immediately before use and refrigerated in the interval between preparation and use.
3. HRP and DAB stock bottles should be stored in the freezer.
4. Bottles of glutaraldehyde and Trizma buffer should be stored in the refrigerator.

WARNING: 3,3'-diaminobenzidine (DAB) is irritating to the skin and *may be carcinogenic*; therefore caution should be used in handling the chemical. The investigator or technician should wear both a respirator with organic vapor filters and rubber gloves; the incubation should be carried out in a hood. Upon completion of tissue incubation, the remaining DAB solution should be deaminated by treatment with bleach (at least 1 ml of sodium hypochlorite per 500 ml DAB) and left in a hood for several hours before disposal in the manner prescribed by the biohazards committee of your institution. The solution should not be disposed of in the sanitary drains. The literature documenting the carcinogenicity of DAB is sparse, and the effectiveness of the bleach treatment in diminishing this carcinogenic action is not known, so it is prudent to treat it as an environmental biohazard.

It is helpful to use dark-field illumination when surveying the section for labeled cells (Kuypers *et al.*, 1974). Endogenous peroxidase will also be visualized by this method, but only unidentified cells bordering regions of the brain in which the blood-brain barrier is deficient (Sherlock and Raisman, 1975) generally show the reaction in the CNS. Red blood cells and leukocytes also contain endogenous peroxidase and will stain if they have not been adequately washed out with saline prior to the perfusion with fixative. Cytochrome *c* and cytochrome oxidase in mitochondria may also react with DAB (Roels, 1974), but this is not likely to be a problem in studies of the nervous system at the light microscope level.

The intracerebral injection of peroxidase is likely to cause changes in capillary permeability above and beyond those normally expected in the inflammatory reaction to insertion of a foreign object into the brain (Clementi, 1970). There is usually a dense brown precipitate at the injection site, and this is surrounded by a less dense, but extensive, halo of tissue. With increased time between the injection of HRP and sacrifice of the animal, the halo is much reduced (Sherlock and Raisman, 1975). Some workers feel that only axon terminals in the central, densely staining region of the injection site take up the enzyme (Bunt *et al.*, 1975). This is consistent with the view that one of the factors in the retrograde cellular incorporation of HRP is the density of the terminal field and the concentration of the enzyme (Jones and Leavitt, 1974; Jones, 1975). There is evidence that some neuronal systems, such as autonomic axons, will not take up HRP (Stöckel *et al.*, 1974); but other workers have been able to demonstrate retrograde transport of the enzyme in autonomic nerves (Ellison and Clark, 1975).

It is now established that damaged axons can take up HRP (Krishnan and Singer, 1973; DeVito *et al.*, 1974; Bunt *et al.*, 1975). At the light

microscopic level, axons containing HRP show a homogeneous light-brown staining, in contrast to the granular nature of the reaction product in the cell body. Every prudent investigator will include control injections to be sure that cell bodies labeled with HRP product are terminating in the region of the injection site rather than sending axons through this region to some other place. The HRP technique has proved to be invaluable in the analysis of connectivity.

Methods introduced in the last ten years have greatly increased our understanding of the connections of CNS neurons. However, the limitations of each of these methods must be carefully considered when designing experiments to detail specific systems. Although we can learn much through the use of currently available techniques, many questions cannot be adequately dealt with, such as the organization of functional subgroups of neurons and the identification of neurons with collateral branches. These and other problems must await the development of new neuroanatomical methods.

REFERENCES

- Bielschowsky, M. (1904). Die Silberimprägnation der Neurofibrillen. *J. Psychol. Neurol.* **3**, 169–188.
- Bignami, A., and Ralston, H. J. (1969). The cellular reaction to Wallerian degeneration in the central nervous system of the cat. *Brain Res.* **13**, 444–461.
- Bodian, D. (1966). Development of fine structure of spinal cord in monkey fetuses. I. The motoneuron neuropile at the time of onset of reflex activity. *Bull. Johns Hopkins Hosp.* **119**, 129–149.
- Bodian, D., and Mellors, R. C. (1945). The regenerative cycle of motoneurons, with special reference to phosphate activity. *J. Exp. Med.* **81**, 469–488.
- Brodal, A. (1940). Modification of Gudden method for study of cerebral localization. *Arch. Neurol. Psychiatry* **43**, 46–58.
- Bunt, A. H., Hendrickson, A. E., Lund, J. S., Lund, R. P., and Fuchs, A. F. (1975). Monkey retinal ganglion cells: Morphometric analysis and tracing of axonal projections, with a consideration of the peroxidase technique. *J. Comp. Neurol.* **164**, 265–286.
- Cajal, S. Raymón y (1928). "Degeneration and Regeneration of the Nervous System." Vol. 1. Oxford Univ. Press, London.
- Chambers, W. W., Liu, C. Y., and Liu, C. N. (1956). A modification of the Nauta technique for staining of degenerating axons in the central nervous system. *Anat. Rec.* **124**, 391–392.
- Clementi, F. (1970). Effect of horseradish peroxidase on mice lung capillaries' permeability. *J. Histochem. Cytochem.* **18**, 887–892.
- Cohen, E. B., and Pappas, G. D. (1969). Dark profiles in the apparently normal central nervous system: A problem in the electron microscopic identification of early anterograde axonal degeneration. *J. Comp. Neurol.* **136**, 375–396.
- Colonnier, M. (1964). Experimental degeneration in the cerebral cortex. *J. Anat.* **98**, 47–53.
- Cottle, M. K. W., and Mitchell, R. (1966). Degeneration time for optimal staining by Nauta technique. A study on transected vagal fibers of the cat. *J. Comp. Neurol.* **128**, 209–222.

- Cowan, W. M., Adamson, L., and Powell, T. P. S. (1961). An experimental study of the avian visual system. *J. Anat.* **95**, 546-563.
- Cowan, W. M., Gotlieb, D. I., Hendrickson, A. E., Price, J. L., and Woolsey, T. A. (1972). The autoradiographic demonstration of axonal connections in the central nervous system. *Brain Res.* **37**, 21-51.
- deOlmos, J. S. (1969). A cupric-silver method for impregnation of terminal axon degeneration and its further use in staining granular argyrophilic neurons. *Brain Behav. Evol.* **2**, 213-237.
- deOlmos, J. S., and Ingram, W. R. (1971). An improved cupric-silver method for impregnation of axonal and terminal degeneration. *Brain Res.* **33**, 523-529.
- deOlmos, J. S., and Ingram, W. R. (1972). The projection field of the stria terminalis in the rat brain. An experimental study. *J. Comp. Neurol.* **146**, 303-333.
- Desclin, J. C., and Escubi, J. (1975). An additional silver impregnation method for demonstration of degenerating nerve cells and processes in the C.N.S. *Brain Res.* **93**, 25-39.
- DeVito, J. W., Clausing, K. W., and Smith, O. A. (1974). Uptake and transport of horseradish peroxidase by cut end of the vagus nerve. *Brain Res.* **82**, 269-271.
- Droz, B. (1973). Renewal of synaptic proteins. *Brain Res.* **62**, 383-394.
- Droz, B., and LeBlond, C. P. (1963). Axonal migration of proteins in the central nervous system and peripheral nerves as shown by radioautography. *J. Comp. Neurol.* **121**, 235-246.
- Eager, R. P. (1970). Selective staining of degenerating axons in the central nervous system by a simplified silver method: Spinal cord projections to external cuneate and inferior olfactory nuclei in the cat. *Brain Res.* **22**, 137-141.
- Eager, R. P., and Barnett, R. J. (1966). Morphological and chemical studies of Nauta-stained degenerating cerebellar and hypothalamic fibers. *J. Comp. Neurol.* **126**, 487-509.
- Edwards, S. B. (1972). The ascending and descending projections of the red nucleus in the cat: An experimental study using an autoradiographic tracing method. *Brain Res.* **48**, 45-63.
- Edwards, S. B. (1975). Autoradiographic studies of the projections of the midbrain reticular formation: Descending projections of nucleus cuneiformis. *J. Comp. Neurol.* **161**, 341-358.
- Ellison, J. P., and Clark, G. M. (1975). Retrograde axonal transport of horseradish peroxidase in peripheral autonomic nerves. *J. Comp. Neurol.* **161**, 103-114.
- Evans, D. H. C., and Hamlyn, L. H. (1956). A study of silver degeneration methods in the central nervous system. *J. Anat.* **90**, 193-203.
- Fifková, E. (1975). Two types of terminal degeneration in the molecular layer of the dentate fascia following lesions of the entorhinal cortex. *Brain Res.* **96**, 169-175.
- Fink, R. P., and Heimer, L. (1967). Two methods for selective silver impregnation of degenerating axons and their synaptic endings in the central nervous system. *Brain Res.* **4**, 369-374.
- Fry, F. J., and Cowan, W. M. (1972). A study of retrograde cell degeneration in the lateral mammillary nucleus of the cat, with special reference to the role of axonal branching in the preservation of the cell. *J. Comp. Neurol.* **144**, 1-24.
- Fuxe, K., and Jonsson, G. (1973). The histochemical fluorescence method for the demonstration of catecholamines: Theory, practice and application. *J. Histochem. Cytochem.* **21**, 293-311.
- Fuxe, K., Hökfelt, T., Jonsson, G., and Ungerstedt, U. (1970). Fluorescence microscopy in neuroanatomy. In "Contemporary Research Methods in Neuroanatomy" (W. J. H. Nauta and S. O. E. Ebbesson, eds.), pp. 275-314. Springer-Verlag, Berlin and New York.

- Geist, F. D. (1933). Chromatolysis of efferent neurons. *Arch. Neurol. Psychiatry* **29**, 88-103.
- Giolli, R. A. (1965). A note on the chemical mechanism of the Nauta-Gygax technique. *J. Histochem. Cytochem.* **13**, 206-210.
- Graham, R. C., and Karnovsky, M. J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**, 291-203.
- Grant, G. (1968). Silver impregnation of degenerating dendrites, cells and axons central to axonal transection. II. A Nauta study on spinal motor neurons in kittens. *Exp. Brain Res.* **6**, 284-293.
- Grant, G., and Aldskogius, H. (1967). Silver impregnation of degenerating dendrites, cells and axons central to axonal transection. I. A Nauta study on the hypoglossal nerve in kittens. *Exp. Brain Res.* **3**, 150-162.
- Gray, E. G. (1959). Axosomatic and axodendritic synapses of the cerebral cortex. An electron microscopic study. *J. Anat.* **93**, 420-433.
- Gray, E. G., and Guillory, R. W. (1966). Synaptic morphology in the normal and degenerating nervous system. *Int. Rev. Cytol.* **19**, 111-182.
- Graybiel, A. M. (1975). Wallerian degeneration and anterograde tracer methods. In "The Use of Axonal Transport for Studies of Neuronal Connectivity" (W. M. Cowan and M. Cuénod, eds.), pp. 175-216. Elsevier, Amsterdam.
- Guillory, R. W. (1970). Light- and electron-microscopical studies of normal and degenerating axons. In "Contemporary Research Methods in Neuroanatomy" (W. J. H. Nauta and S. O. E. Ebbesson, eds.), pp. 77-105. Springer-Verlag, Berlin and New York.
- Guillory, R. W., and Ralston, H. J., III (1964). Nerve fibers and terminals: Electron microscopy after Nauta staining. *Science* **143**, 1331-1332.
- Hansson, H.-A. (1973). Uptake and intracellular bidirectional transport of horseradish peroxidase in retinal ganglion cells. *Exp. Eye Res.* **16**, 377-388.
- Hedreen, J. C., and Chalmers, J. P. (1972). Neuronal degeneration in rat brain induced by 6-hydroxydopamine: a histological and biochemical study. *Brain Res.* **47**, 1-36.
- Heimer, L. (1970). Selective silver-impregnation of degenerating axoplasm. In "Contemporary Research Methods in Neuroanatomy" (W. J. H. Nauta and S. O. E. Ebbesson, eds.), pp. 106-131. Springer-Verlag, Berlin and New York.
- Heimer, L. (1972). The olfactory connections of the diencephalon in the rat. An experimental light- and electron-microscopic study with special emphasis on the problem of terminal degeneration. *Brain Behav. Evol.* **6**, 484-523.
- Hendrickson, A. (1975a). Tracing neuronal connections with radioisotopes applied extracellularly. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 1612-1615.
- Hendrickson, A. (1975b). Technical modifications to facilitate tracing synapses by electron microscopic autoradiography. *Brain Res.* **85**, 241-247.
- Hirsch, H. E., and Obenchain, T. (1970). Acid phosphatase activity in individual neurons during chromatolysis: A quantitative histochemical study. *J. Histochem. Cytochem.* **18**, 828-833.
- Hökfelt, R., Fuxe, K., Goldstein, M., and Johansson, O. (1974). Immunohistochemical evidence for the existence of adrenaline neurons in the rat brain. *Brain Res.* **66**, 235-251.
- Hökfelt, T., and Ljungdahl, A. S. (1972). Histochemical determination of neurotransmitter distribution. In "Neurotransmitters" (I. J. Kopin, ed.), Association for Research in Nervous and Mental Disease, Vol. 50, pp. 1-24. Williams & Wilkins, Baltimore, Maryland.
- Ibata, Y., and Otsuka, N. (1968). Fine structure of synapses in the hippocampus of the

- rabbit with special reference to dark presynaptic endings. *Z. Zellforsch. Mikrosk. Anat.* **91**, 547-553.
- Ibata, Y., Nojyo, Y., Matsuura, T., and Sano, Y. (1973). Nigro-neostriatal projection. A correlative study with Fink-Heimer impregnation, fluorescence histochemistry and electron microscopy. *Z. Zellforsch. Mikrosk. Anat.* **138**, 333-345.
- Jones, E. G. (1975). Possible determinants of the degree of retrograde neuronal labeling with horseradish peroxidase. *Brain Res.* **85**, 249-253.
- Jones, E. G., and Leavitt, R. Y. (1974). Retrograde axonal transport and the demonstration of nonspecific projections to the cerebral cortex and striatum from thalamic intralaminar nuclei in the rat, cat, and monkey. *J. Comp. Neurol.* **154**, 349-378.
- Jones, E. G., and Rockel, A. J. (1973). Observations on complex vesicles, neurofilamentous hyperplasia and increased electron density during terminal degeneration in the inferior colliculus. *J. Comp. Neurol.* **147**, 93-118.
- Kalah-Brunst, C., Giolli, R. A., and Creel, D. J. (1974). An improved silver impregnation method for tracing degenerating nerve fibers and their terminals in frozen sections. *Brain Res.* **82**, 279-283.
- Kalil, K. (1975). A study of so-called "retrograde fine-grain" degeneration in the thalamus. *Brain Res.* **93**, 189-202.
- Kandel, E. R., and Gardner, D. (1972). The synaptic actions mediated by the different branches of a single neuron. In "Neurotransmitters" (I. J. Kopin, ed.), Association for Research in Nervous and Mental Disease, Vol. 50, pp. 91-146. Williams & Wilkins, Baltimore, Maryland.
- Koelle, G. B. (1957). Histochemical localization of acetylcholinesterase in nervous tissue. In "New Research Techniques of Neuroanatomy" (W. F. Windle, ed.), pp. 62-69. Thomas, Springfield, Illinois.
- Koelle, G. B., and Friedenwald, J. S. (1949). A histochemical method for localizing cholinesterase activity. *Proc. Soc. Exp. Biol. Med.* **70**, 617-672.
- Kreutzberg, G. W. (1963). Changes of coenzyme (TPN) diaphorase and TPN-linked dehydrogenase during axonal reaction of the nerve cell. *Nature (London)* **199**, 393-394.
- Krishnan, N., and Singer, M. (1973). Penetration of peroxidase into peripheral nerve fibers. *Am. J. Anat.* **136**, 1-14.
- Kristensson, K., and Olsson, Y. (1971). Retrograde axonal transport of protein. *Brain Res.* **29**, 363-365.
- Künzle, H., and Cuénod, M. (1973). Differential uptake of [³H] proline and [³H] leucine by neurons: Its importance for the autoradiographic tracing of pathways. *Brain Res.* **62**, 213-217.
- Kuypers, H. G. J. M., Kievit, J., and Groen-Klevant, A. C. (1974). Retrograde axonal transport of horseradish peroxidase in rat's forebrain. *Brain Res.* **67**, 211-218.
- Larramendi, L. M. H., Fickenscher, L., and Lemkey-Johnston, N. (1967). Synaptic vesicles of inhibitory and excitatory terminals in the cerebellum. *Science* **156**, 967-969.
- Lasek, R. J. (1975). Axonal transport and the use of intracellular markers in neuroanatomical investigations. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34**, 1603-1611.
- LaVail, J. H. (1975). The retrograde transport method. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34**, 1618-1624.
- LaVail, J. H., and LaVail, M. M. (1972). Retrograde axonal transport in the central nervous system. *Science* **176**, 1416-1417.
- LaVail, J. H., and LaVail, M. M. (1974). The retrograde intraaxonal transport of horseradish peroxidase in the chick visual system: A light and electron microscopic study. *J. Comp. Neurol.* **157**, 303-358.

- LaVail, J. H., Winston, K. R., and Tish, A. (1973). A method based on retrograde intraaxonal transport of protein for identification of cell bodies of origin of axons terminating within the CNS. *Brain Res.* **58**, 470-477.
- Leonard, C. M. (1975). Developmental changes in olfactory bulb projections revealed by degeneration argyrophilia. *J. Comp. Neurol.* **162**, 467-486.
- Lynch, G., Gall, C., Mensah, P., and Cotman, C. (1974). Horseradish peroxidase histochemistry: A new method for tracing efferent projections in the central nervous system. *Brain Res.* **65**, 373-380.
- McBride, R. L., and Sutin, J. (1976). Projections of the locus coeruleus and adjacent pontine tegmentum in the cat. *J. Comp. Neurol.* **165**, 265-284.
- Meyer, D. D., and Cole, J. (1970). Comparison of certain retrograde oxidative reactions after section of axons in central and peripheral nervous systems. *Neurology* **20**, 918-924.
- Nauta, H. J. W., Pritz, M. B., and Lasek, R. J. (1974). Afferents to the rat caudoputamen studied with horseradish peroxidase. An evaluation of a retrograde neuroanatomical research method. *Brain Res.* **67**, 219-238.
- Nauta, H. J. W., Kaiserman-Abramof, I. R., and Lasek, R. J. (1975). Electron microscopic observations of horseradish peroxidase transported from the caudoputamen to the substantia nigra in the rat: Possible involvement of the agranular reticulum. *Brain Res.* **85**, 373-384.
- Nauta, W. J. H. (1957). Silver impregnation of degenerating axons. In "New Research Techniques of Neuroanatomy" (W. F. Windle, ed.), pp. 17-26. Thomas, Springfield, Illinois.
- Nauta, W. J. H., and Gygax, P. A. (1951). Silver impregnation of degenerating axon terminals in the central nervous system: (1) technique; (2) chemical notes. *Stain Technol.* **26**, 5-11.
- Nauta, W. J. H., and Gygax, P. A. (1954). Silver impregnation of degenerating axons in the C. N. S. A modified technique. *Stain Technol.* **29**, 91-93.
- Nauta, W. J. H., and Ryan, L. F. (1952). Selective silver impregnation of degenerating axons in the central nervous system. *Stain Technol.* **27**, 175-179.
- Ochs, S. (1974). Axoplasmic transport: Energy metabolism and mechanism. In "The Peripheral Nervous System" (J. I. Hubbard, ed.), pp. 47-72. Plenum, New York.
- Palay, S. L., and Chan-Palay, V. (1974). "Cerebellar Cortex. Cytology and Organization." Springer-Verlag, Berlin and New York.
- Paul, K. G. (1963). Peroxidases. In "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrback, eds.), pp. 227-274. Academic Press, New York.
- Peters, T., and Ashley, C. (1967). An artefact in radioautography due to binding of free amino acids to tissues by fixatives. *J. Cell Biol.* **33**, 53-60.
- Ralston, H. J., and Sharp, P. V. (1973). The identification of thalamocortical relay cells in the adult cat by means of retrograde axonal transport of horseradish peroxidase. *Brain Res.* **62**, 273-278.
- Roels, F. (1974). Cytochrome c and cytochrome oxidase in diaminobenzidine staining of mitochondria. *J. Histochem. Cytochem.* **22**, 442-446.
- Rogers, A. W. (1967). "Techniques of Radioautography." Elsevier, Amsterdam.
- Seligman, A., Karnovsky, M., Wasserkrug, H., and Hanker, J. (1968). Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmophilic reagent, diaminobenzidine (DAB). *J. Cell Biol.* **38**, 1-14.
- Sherlock, D., and Raisman, G. (1975). A comparison of anterograde and retrograde axonal transport of horseradish peroxidase in the connections of the mammillary nuclei in the rat. *Brain Res.* **85**, 321-324.

- Shimizu, N., and Ohnishi, S. (1973). Demonstration of nigro-neostriatal tract by degeneration silver method. *Exp. Brain Res.* **17**, 133–138.
- Shimizu, N., Ohnishi, S., Toyama, M., and Maeda, T. (1974). Demonstration by degeneration silver method of the ascending projection from locus coeruleus. *Exp. Brain Res.* **21**, 181–192.
- Simon, H., LeMoal, M., Galey, D., and Cardo, B. (1974). Selective degeneration of central dopaminergic systems after injection of 6-hydroxydopamine in the ventral mesencephalic tegmentum of the rat. Demonstration by the Fink-Heimer stain. *Exp. Brain Res.* **20**, 375–384.
- Snow, P., Rose, P., and Brown, A. G. (1976). Tracing axons and axon collaterals of spinal neurons using intracellular injection of horseradish peroxidase. *Science* **191**, 312–313.
- Sotelo, C., and Riche, D. (1974). The smooth endoplasmic reticulum and the retrograde and fast orthograde transport of horseradish peroxidase in the nigro-striato-nigral loop. *Anat., Histol., Embryol.* **146**, 209–218.
- Sprague, J., and Ha, H. (1964). The terminal fields of dorsal root fibers in the lumbosacral spinal cord of the cat, and the dendritic organization of the motor nuclei. *Prog. Brain Res.* **11**, 120–154.
- Stöckel, K., Paravicini, U., and Thoenen, H. (1974). Specificity of the retrograde axonal transport of nerve growth factor. *Brain Res.* **76**, 413–421.
- Straus, W. (1969). The use of horseradish peroxidase as a marker protein for studies of phagolysosomes, permeability and immunology. *Methods Achiev. Exp. Pathol.* **4**, 54–91.
- Szentágothai, J., and Rajkovits, K. (1959). Über den Ursprung der Kletterfasern des Kleinhirns. *Z. Anat. Entwicklungsgesch.* **121**, 130–141.
- Tanaka, D., and Chen, J. Y. C. (1974). Retrograde thalamic degeneration: Observations using a modification of the Fink-Heimer silver impregnation technique. *Brain Res.* **65**, 333–337.
- Torvik, A. (1972). Phagocytosis of nerve cells during retrograde degeneration. An electron microscopic study. *J. Neuropathol. Exp. Neurol.* **31**, 132–146.
- Uchizono, K. (1965). Characteristics of excitatory and inhibitory synapses in the central nervous system of the cat. *Nature (London)* **207**, 642–643.
- Vaccarezza, O., Reader, T., Pasqualini, E., and Pecci-Saavedra, J. (1970). Temporal course of synaptic degeneration in the lateral geniculate nucleus. Its dependence on axonal stump length. *Exp. Neurol.* **28**, 277–285.
- Valverde, F. (1967). Apical dendritic spines of the visual cortex and light deprivation in the mouse. *Exp. Brain Res.* **3**, 337–353.
- Van Buren, J. (1963). Trans-synaptic retrograde degeneration in the visual system of primates. *J. Neurol., Neurosurg. Psychiatry* **26**, 402–409.
- Van Crevel, H., and Verhaart, W. J. C. (1963). The rate of secondary degeneration in the central nervous system. I. The pyramidal tract of the cat. *J. Anat.* **97**, 429–449.
- Van Gehuchten, A. (1903). La dégénérescence dite rétrograde ou dégénérescence Wallérienne indirekte. *Nevraxe* **5**, 1–107.
- Walberg, F. (1963). An electron microscopical study of the inferior olive of the cat. Normal and experimental findings. *Acta Neurol. Scand.* **39**, 308–313.
- Walberg, F. (1972). Further studies on silver impregnation of normal and degenerating boutons. A light and electron microscopical investigation of a filamentous degenerating system. *Brain Res.* **36**, 353–369.
- Westrum, L. E. (1973). Early forms of terminal degeneration in the spinal trigeminal nucleus following rhizotomy. *J. Neurocytol.* **2**, 189–215.
- Wiitanen, J. T. (1969). Selective silver impregnation of degenerating axons and axon termi-

- nals in the central nervous system of the monkey (*Macaca mulatta*). *Brain Res.* **14**, 546-548.
- Zacks, S. I., and Saito, A. (1969). Uptake of exogenous horseradish peroxidase in coated vesicles in mouse neuromuscular junctions. *J. Histochem. Cytochem.* **17**, 161-170.

Chapter 2

Chemical Lesioning: Catecholamine Pathways

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In 1968, Tranzer and Thoenen (see Tranzer and Thoenen, 1968; Thoenen and Tranzer, 1968) provided convincing evidence that 6-hydroxydopamine (6-OHDA) selectively destroyed peripheral noradrenergic terminals. These initial observations not only stimulated additional studies to define the pharmacological properties of 6-OHDA but also led investigators to examine the possibility that 6-OHDA might produce selective destruction of catecholamine-containing neurons in brain when administered directly into the central nervous system (Ungerstedt, 1968; Bloom *et al.*, 1969; Uretsky and Iversen, 1970; Breese and Traylor, 1970). The favorable outcome of these early studies has resulted in the acceptance of the use of 6-OHDA as a tool to evaluate the role of

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catecholamine-containing fibers in the mediation of various physiological, behavioral, and pharmacological responses. The purpose of this chapter will be to present procedures by which 6-OHDA can be administered to destroy catecholamine-containing neurons and to review experimental data demonstrating the application of 6-OHDA to problems encountered in neurobiology and neuropharmacology.

I. PERIPHERAL ADMINISTRATION OF 6-HYDROXYDOPAMINE

Historically, surgical ablation of the peripheral sympathetic nervous system has been used to define its homeostatic role (Cannon and Rosenblueth, 1937) and to elucidate adrenergic mechanisms (see Thoenen, 1972). "Chemical sympathectomy" with 6-OHDA provided a means by which peripheral adrenergic fibers could be destroyed without many of the disadvantages concomitant with the use of surgical denervation. Procedures by which a peripheral sympathectomy can be produced with 6-OHDA are described in Table I.

Studies of the histological effects of 6-OHDA after parenteral administration to adult animals have indicated that adrenergic nerve terminals begin to degenerate within 1 hour of intravenous administration (Tranzer and Thoenen, 1968; Bennett *et al.*, 1970). No morphological alterations in

TABLE I
6-Hydroxydopamine Dosage Schedules Used to Destroy Peripheral Sympathetic Fibers in the Rat

Schedule	Heart norepinephrine (% control) ^a	References
<i>Adult rats</i>		
2 × 34 mg/kg 6-OHDA within 24 hr ^b	7.6	Thoenen and Tranzer (1968)
2 × 68 mg/kg 6-OHDA a week later ^b		
1 × 100 mg/kg 6-OHDA ^b	10.0	de Champlain and Nadeau (1971)
<i>Infant rats</i>		
4 × 50 mg/kg 6-OHDA every other day for 4 days starting on day of birth ^c	8.0	Angeletti (1971)

^aIn adult rats, the analyses of heart norepinephrine were made at least 2 weeks after injection of 6-OHDA. In the case of the infant rats, the heart was analyzed 6 months after treatment.

^bDrug was administered intravenously.

^cDrug was administered intraperitoneally.

smooth muscle cells or in cholinergic nerve endings have been observed, providing evidence that the actions of 6-OHDA are confined to sympathetic neurons (Tranzer *et al.*, 1969). In addition to histological procedures, the degree of destruction of noradrenergic fibers after 6-OHDA treatment can be assessed with other methods. Examples include the measurement of tissue content of norepinephrine (Tranzer and Thoenen, 1968), determination of [³H]norepinephrine uptake into tissue (Jonsson and Sachs, 1970) or assessment of the activity of tyrosine hydroxylase and dopamine- β -hydroxylase (Mueller *et al.*, 1969; Axelrod and Molinoff, 1971).

Although 6-OHDA induces degeneration in peripheral sympathetic nerve terminals, most evidence indicates that 6-OHDA does not affect the cell bodies of the various sympathetic ganglia of mature rats (Laverty *et al.*, 1965) nor the morphology of chromaffin cells in the adrenal medulla (Tranzer and Thoenen, 1968). It is this remarkable resistance displayed by cell bodies of adult animals to the action of 6-OHDA that allows for regeneration of adrenergic terminals into organs innervated by the sympathetic nervous system (Tranzer and Thoenen, 1968; de Champlain, 1971; Jonsson and Sachs, 1972). However, a permanent destruction of the cells in sympathetic ganglia can be achieved by injecting newborn animals intraperitoneally with 6-OHDA (Angeletti and Levi-Montalcini, 1970; see Table I). Because most of the cell bodies in sympathetic ganglia are destroyed when infant rats are treated with 6-OHDA, the possibility of regeneration of peripheral noradrenergic fibers is eliminated.

II. CENTRAL ADMINISTRATION OF 6-HYDROXYDOPAMINE

Early studies of the pharmacology of 6-OHDA given peripherally suggested that this compound did not readily enter the central nervous system unless administered prior to the development of the blood-brain barrier (Angeletti, 1971; Lew and Quay, 1971). Because of this problem, 6-OHDA must be administered into the cerebroventricular system or directly into brain tissue to destroy catecholamine fibers in the central nervous system.

Following either intracisternal or intraventricular administration of 6-OHDA, there is a persistent dose-related reduction of both brain norepinephrine and dopamine (Uretsky and Iversen, 1970; Breese and Traylor, 1970, 1971). Histological as well as biochemical findings have supported the view that this prolonged reduction of brain catecholamines is the result of neuronal degeneration. For example, ultrastructural alterations characteristic of neuronal degeneration have been shown to occur in areas rich in catecholamine-containing neurons after injection of 6-OHDA

(Bloom *et al.*, 1969; Richards, 1971; Ungerstedt, 1971f). Other evidence suggesting destruction of catecholamine fibers by 6-OHDA includes: a loss of specific fluorescence for noradrenergic and dopaminergic neurons as demonstrated by fluorescence microscopy (Ungerstedt, 1971f); a marked reduction of tissue uptake of [³H]norepinephrine (Uretsky and Iversen, 1970); a decrease in tyrosine hydroxylase and dopamine- β -hydroxylase activity (Breese and Traylor, 1970; Reis and Molinoff, 1972); and a reduction in the amount of labeled norepinephrine and dopamine formed from [³H]tyrosine (Breese and Traylor, 1970). Because the aforementioned procedures can be used to assess the degree of destruction of catecholamine fibers following 6-OHDA treatment, it is important that investigators using 6-OHDA as a tool familiarize themselves with these methods.

Several studies have shown that noradrenergic fibers are more sensitive than dopaminergic fibers to the effects of 6-OHDA (Uretsky and Iversen, 1970; Breese and Traylor, 1970). Therefore, special procedures must be employed in order to produce relatively equal destruction of both catecholamine systems. One method (Uretsky and Iversen, 1970) is to administer two 250- μ g doses of 6-OHDA into either the fourth ventricle ("intracisternally") or the lateral ventricle ("intraventricularly"). Another approach is to pretreat rats with 50 mg/kg of pargyline or another monoamine oxidase inhibitor 30 min prior to intracisternal or intraventricular injection of the 6-OHDA (Breese and Traylor, 1970, 1971; Fibiger *et al.*, 1972; Breese, 1975). In the case of the latter approach, an even greater degree of destruction of catecholamine fibers can be obtained if an additional injection is given 1 week after the first dose of 6-OHDA (Breese and Traylor, 1971; Breese *et al.*, 1973b). Effective reduction of brain catecholamine content can also be produced if 6-OHDA is administered intracisternally to developing rats (Breese and Traylor, 1971; Smith *et al.*, 1973). These methods to reduce both catecholamines in brain with 6-OHDA are summarized in Table II.

Ungerstedt (1968) was the first investigator to report the effects of administering 6-OHDA directly into brain tissue. In this work, injection of small amounts of 6-OHDA into the area of the substantia nigra was found to reduce histochemical fluorescence in the striatum (Ungerstedt, 1971f). Although this approach was intended to deplete only dopamine in the forebrain, investigators who measured catecholamine content after injecting 6-OHDA into the substantia nigra found that brain norepinephrine as well as dopamine was reduced after this treatment (Creese and Iversen, 1975; Beese *et al.*, 1975). A selective destruction of the dopamine pathway was not possible by this approach because catecholamine tracts are coincidental in this area of brain (see Section IV).

TABLE II

Schedules of Central Administration of 6-OHDA Used to Destroy Catecholamine-Containing Fibers in the Central Nervous System

Route and schedule	Brain content (% control)		References	
	Norepi- nephrine	Dopamine		
Intracisternal or intraventricular				
<i>Adult</i>				
2 × 250 µg 6-OHDA	20	35	Uretsky and Iversen (1970)	
2 × 200 µg 6-OHDA ^a	12	11	Breese and Traylor (1971); Cooper <i>et al.</i> (1973)	
1 × 250 µg 6-OHDA ^b	—	4	Fibiger <i>et al.</i> (1972)	
<i>Infant</i>				
1 × 100 µg 6-OHDA	15	8	Breese and Traylor (1972); Smith <i>et al.</i> (1973)	
Intracerebral administration				
<i>Adult</i>				
8 µg 6-OHDA into ventral tegmentum ^c	10	5	Ungerstedt (1971a,c)	

^aRats received pargyline (50 mg/kg) 30 min prior to receiving 200 µg 6-OHDA into the fourth ventricle ("intracisternal" injection). A second 200-µg dose of 6-OHDA was administered 7 to 10 days later.

^bRats were given 250 µg of 6-OHDA into the lateral ventricle 30 min after an injection of 50 mg/kg of pargyline (Fibiger *et al.*, 1972). Brain content reported is an estimate based upon tyrosine hydroxylase.

^cThe 6-OHDA was injected into the ventral tegmentum in the area containing the dopamine cell bodies [see Ungerstedt (1971a,c), Cooper *et al.* (1974), or Creese and Iversen (1975) for coordinates]. Catecholamine values are from Creese and Iversen (1975) and represent an estimate of forebrain content after treatment.

III. PREFERENTIAL REDUCTION OF BRAIN NOREPINEPHRINE

In contrast to the catecholamine depletions which occur after injection of 6-OHDA into the area of the substantia nigra, it is possible to obtain a relatively selective reduction of norepinephrine by administering 6-OHDA into the noradrenergic pathways (Ungerstedt, 1971e). At least for the present, direct injection of 6-OHDA into the tegmental structures containing the ascending norepinephrine fibers would seem to be the treatment of choice to permit a selective reduction of norepinephrine in the forebrain (Breese, 1975). Recently, Thierry *et al.* (1975) described a procedure in which 6-OHDA was administered into the brainstem of the developing rat, causing permanent interruption of noradrenergic pathways

TABLE III
Procedures to Permit Preferential Destruction of
Noradrenergic Fibers with 6-OHDA

Procedure	Brain content (% control)		References
	Norepi- nephrine	Dopamine	
<i>Adult rats</i>			
8 µg 6-OHDA into nor- epinephrine pathways ^a	8	100	Ungerstedt (1971a,e)
3 × 25 µg 6-OHDA intracisternally ^b	12	92	Breese and Taylor (1971); Uretsky and Iversen (1970)
<i>Infant rats</i>			
1 × 10 µg 6-OHDA intra- cisternally on day 1 ^a	5	100	Konkol <i>et al.</i> (1975)
2 µg 6-OHDA into nor- epinephrine pathways ^{a,c}	25	100	Thierry <i>et al.</i> (1975)
100 mg/kg 6-OHDA intra- peritoneally on day 1 ^a	18	100	Tassin <i>et al.</i> (1975)

^aValues for catecholamine content refer to amount found in forebrain structures.

^bValues for catecholamine content were obtained from whole-brain samples.

^cRats were 4 days of age when given 2 µg of 6-OHDA in 1 µl solution—delivered to the brain in 5 min (Thierry *et al.*, 1975).

(Table III). While it is perhaps too early to assess the possible utility of this latter technique as a means of investigating the role of noradrenergic fibers in various functions, the procedure has already provided an additional approach to demonstrate the presence of dopamine fibers in cortical areas (Thierry *et al.*, 1975).

In addition to the procedures just described, other methods utilizing 6-OHDA are also available to reduce norepinephrine content in brain with minimal effects on dopamine. Based upon the finding that noradrenergic fibers are more sensitive than are dopaminergic neurons to the cytotoxic effects of 6-OHDA, multiple administrations of a small dose of 6-OHDA (three 25-µg doses spaced at various short intervals) have been used to produce an animal in which brain norepinephrine was depleted with little alteration in dopamine content (Uretsky and Iversen, 1970; Breese and Taylor, 1971). Other investigators have administered 6-OHDA intraperitoneally to neonates and found that norepinephrine content in brain was selectively reduced (Clark *et al.*, 1972; Jonsson and Sachs, 1972; Singh and de Champlain, 1972). However, peripheral treatment of the neonate with 6-OHDA has the disadvantage of producing a peripheral sympathet-

tomy. In addition to peripheral treatment of neonatal rats, intracisternal injection of a small dose of 6-OHDA (10 µg) to a 1-day-old rat has been found to produce a marked reduction of forebrain norepinephrine while causing an increase in norepinephrine content in brain stem and cerebellum. Therefore, the intracisternal injection of a small dose of 6-OHDA to the neonate may provide not only an approach by which to reduce brain norepinephrine but also an important research avenue by which to study fiber growth and sprouting after neural injury (Kostrzewska and Harper, 1975; Konkol *et al.*, 1975).

It is also possible to reduce brain norepinephrine by administering 2,4,5-trihydroxyphenylalanine (6-OH-DOPA) in combination with a decarboxylase inhibitor. When the proper dose of a decarboxylase inhibitor is administered the peripheral sympathetic neurons are protected while still allowing destruction of central noradrenergic neurons (Jacobowitz, 1973; Kostrzewska and Harper, 1975). Nevertheless, the toxicity of 6-OH-DOPA and its relative ineffectiveness compared with the other procedures outlined earlier seem to preclude the use of 6-OH-DOPA as an effective tool for destruction of noradrenergic fibers in the brain (Corrodi *et al.*, 1971; Breese *et al.*, 1975).

IV. PREFERENTIAL DESTRUCTION OF DOPAMINE PATHWAYS

Development of techniques using 6-OHDA to induce a selective reduction of norepinephrine in the brain subsequently led to methods by which dopamine terminals and pathways could be preferentially destroyed with this drug. Based on observations that compounds inhibiting uptake of norepinephrine prevented the destruction of peripheral sympathetic fibers by 6-OHDA, studies were initiated to determine if these compounds might protect noradrenergic fibers from the neurocytotoxic effects of centrally administered 6-OHDA (see Breese, 1975). It was shown that prior administration of uptake blockers, such as desipramine or protriptyline, retarded the destruction of brain noradrenergic fibers without altering the ability of 6-OHDA to destroy dopaminergic neurons (Evets and Iversen, 1970; Breese and Traylor, 1971). The procedure of pretreating animals with desipramine to reduce dopamine selectively has proven to be particularly effective in rats treated during development (Smith *et al.*, 1973). Depletions comparable to those observed after 6-OHDA administration to developing rats can also be obtained in adult rats if two 240 µg doses of 6-OHDA are administered one week apart to desipramine pretreated rats (Cooper *et al.*, 1973). Furthermore, pretreatment of rats with desipramine prior to the infusion of 6-OHDA into the area of the substantia nigra protects noradrenergic fibers from destruction, permit-

TABLE IV
Procedures to Permit Preferential Destruction of
Dopaminergic Fibers with 6-OHDA

Procedures	Brain content (% control)		References
	Norepi- nephrine	Dopamine	
<i>Adult rats</i>			
2 × 200 µg 6-OHDA to desipramine (30 mg/kg) pretreated rats ^a	92	15	Breese <i>et al.</i> (1973a,b) Cooper <i>et al.</i> (1973)
8 µg of 6-OHDA into the ventral tegmentum of rats pretreated with desipramine (30 mg/kg) ^b	97	10	Breese <i>et al.</i> (1975); Kelly <i>et al.</i> (1975)
<i>Infant rats</i>			
1 × 100 µg 6-OHDA to 5-day-old rats pretreated with desipramine (20 mg/kg) ^a	99	10	Smith <i>et al.</i> (1973)

^a Catecholamine values determined in whole brain.

^b Catecholamine values determined in forebrain (Breese and Cooper, unpublished data).

ting a relatively specific destruction of dopaminergic pathways when this route of administering 6-OHDA is chosen (Breese *et al.*, 1975). Examples of these various approaches to reduce dopamine in the brain are described in Table IV.

V. ANIMAL CARE AFTER 6-HYDROXYDOPAMINE ADMINISTRATION

A major problem encountered in 6-OHDA usage is keeping the experimental animals healthy, especially after treatments which produce large depletions of brain dopamine. Several investigators have reported that 6-OHDA treatments producing large reductions of dopamine are accompanied by transient aphagia and adipsia (Ungerstedt, 1971c; Breese *et al.*, 1973a; Fibiger *et al.*, 1972, 1973b). In order to reduce mortality, rats must be fed by oral intubation much like animals with lateral hypothalamic lesions (Teitelbaum and Epstein, 1962). It has been our practice to initiate intragastric feeding of 6-OHDA-treated rats 2 days postinjection, intubating each rat twice daily (see Myers, 1971). In addition, sliced fresh fruit, cookies, and a jar containing ground rat chow mixed with water, Met-

recal, and a solution of sucrose are placed on the floor of the cage. Methods for maintaining a proper nutritional state in aphagic or adipsic animals have been described by other investigators and are adequate for the maintenance of 6-OHDA-treated rats with these symptoms (Teitelbaum and Epstein, 1962; Ungerstedt, 1971c; Ellison, 1972; Myers and Martin, 1973). Bacterial infections of 6-OHDA treated animals can be minimized if they are given antibiotics (e.g., penicillin or tetracycline) at the time of 6-OHDA treatment.

VI. EVIDENCE FOR SPECIFICITY OF 6-HYDROXYDOPAMINE

Most early work suggested that 6-OHDA administered into the ventricular system had relatively selective actions on catecholamine-containing fibers. For example, several laboratories reported that treatment with 6-OHDA did not alter 5-hydroxytryptamine (serotonin) content in brain (Bloom *et al.*, 1969; Uretsky and Iversen, 1970; Breese and Traylor, 1970; Jacks *et al.*, 1972; Smith *et al.*, 1973), alter turnover rate of brain serotonin (Bloom *et al.*, 1969); or affect 5-hydroxytryptophan decarboxylase (Sims and Bloom, 1973) or tryptophan hydroxylase activities (Smith *et al.*, 1973). However, in a few studies the content of serotonin in brain has been found to be reduced after 6-OHDA treatment (Eichelman *et al.*, 1972; Cooper *et al.*, 1973; Breese *et al.*, 1973b). Since high doses of 6-OHDA (more than 250 µg) can acutely reduce serotonin in brain (Breese, unpublished data), it may be advisable to measure serotonin content after 6-OHDA treatment if a depletion of serotonin would be expected to interfere with the interpretation of the experiment. Other studies have shown that choline acetylase content is not affected in treated animals (McGeer *et al.*, 1973; Kim, 1973; Smith *et al.*, 1973). Likewise, treatment with 6-OHDA has been shown to be without effect on the content of γ-aminobutyric acid, glutamic acid, glycine, and several other amino acids in brain (Jacks *et al.*, 1972), providing further support for the view that the actions of 6-OHDA are relatively selective for catecholamine-containing fibers in the rat.

In spite of results suggesting that 6-OHDA has a relatively specific action on catecholamine neurons, recent work suggests that the route of administration of 6-OHDA may have relevance to the amount of nonspecific damage observed. Hedreen (1975) has recently published work comparing the extent of nonspecific damage produced by 6-OHDA after injection into the lateral ventricle versus administration into the fourth ventricle (intracisternal route). From this histological study, it was concluded that 6-OHDA injected into the lateral ventricle resulted in severe nonspecific degeneration near the injection site whereas intracis-

ternal administration of 6-OHDA caused little nonspecific damage. In view of this report, the route by which the 6-OHDA is injected into the brain may prove to be more important than first recognized.

The procedure of administering 6-OHDA into brain tissue has generated considerable controversy concerning whether or not 6-OHDA produced its effects by making a nonspecific lesion. Whereas Ungerstedt (1971e) found that 6-OHDA administered into the brain caused nonspecific damage restricted to the tip of the injection cannula, Poirier *et al.* (1972) and Butcher *et al.* (1974) have concluded that the effects produced by intracerebral application of 6-OHDA were the result of a nonspecific lesion. Other work has not entirely supported the conclusion that 6-OHDA acts by producing a brain lesion (Evans *et al.*, 1975). For example, desipramine has been shown to prevent the destructive effects of 6-OHDA on noradrenergic fibers without altering its neurocytotoxic effect on dopaminergic neurons (Breese *et al.*, 1975). This observation would not be expected if the destructive action of 6-OHDA were nonspecific. Furthermore, Javoy *et al.* (1974) have provided evidence that the degree of nonspecific lesioning produced by intracerebrally administered 6-OHDA is proportional to the dose injected. These findings are in accord with reports from other laboratories (Hökfelt and Ungerstedt, 1973; Cooper *et al.*, 1974; Evans *et al.*, 1975). It can, thus, be concluded that intracerebral injection of 6-OHDA is a *relatively* specific method which can be used to interrupt catecholaminergic fibers so long as the concentration of 6-OHDA is 2 $\mu\text{g}/\mu\text{l}$ or less and total dose does not exceed 8 μg . However, a microinjection of greater than 1 μl into parenchyma is suspect.

VII. EVIDENCE FOR ACTIVATION OF COMPENSATORY MECHANISMS FOLLOWING 6-HYDROXYDOPAMINE TREATMENTS

Early studies using 6-OHDA to examine the role of catecholamine fibers in the maintenance of a variety of behavioral and physiological paradigms often reported that following an initial reduction, the response under examination became essentially normal within 7 to 10 days after treatment. This recovery occurred even though catecholamine content of brain remained chronically depleted. The apparent dissociation of neurochemical and behavioral effects following treatment with 6-OHDA was explained by the suggestion that 6-OHDA-treated rats compensated for the damage to the catecholamine fibers (Breese *et al.*, 1973a,b; Zigmond and Stricker, 1975). Two general findings have provided support for this view: (*a*) studies showing that catecholamine receptors are supersensitive after 6-OHDA treatment (Ungerstedt, 1971b), and (*b*) the obser-

vation that animals recovered from the acute effects of 6-OHDA are more sensitive to the depressant effects of catecholamine-depleting drugs (Breese *et al.*, 1973b; Cooper *et al.*, 1972, 1973; Schoenfeld and Uretsky, 1972).

Support for the idea of enhanced sensitivity of postsynaptic receptors after 6-OHDA can be traced to early work indicating that rats treated with 6-OHDA showed exaggerated responses to L-dihydroxyphenylalanine (L-DOPA). Ungerstedt (1971b) showed that unilateral lesions with 6-OHDA of catecholamine fibers in the nigro-striatal pathway caused an L-DOPA-elicited increase in turning to the side of the lesion and proposed that dopamine formed from L-DOPA was acting directly on the receptor. Several investigators have also shown that L-DOPA-induced locomotor activity is markedly potentiated in animals treated with 6-OHDA to reduce catecholamine content bilaterally (Uretsky and Schoenfeld, 1971; Breese *et al.*, 1973b; Cooper and Breese, 1974; Hollister *et al.*, 1974). While inhibition of dopamine uptake could explain the enhanced response to L-DOPA (Schoenfeld and Uretsky, 1973), increased sensitivity to the actions of the dopamine agonists, apomorphine and ET-495, have also been found after 6-OHDA treatment (Ungerstedt, 1971b). Since apomorphine and ET-495 are not taken up by dopamine neurons, the latter results suggest a change in receptor mechanism(s) occurred to produce the change in sensitivity to these agonists (Ungerstedt, 1971b; Schoenfeld and Uretsky, 1972).

While most work has been associated with alterations of receptor sensitivity in the dopamine system after treatment with 6-OHDA, some other reports have suggested that noradrenergic receptors can also become supersensitive when noradrenergic fibers are destroyed. For example, the increased hypothermia produced by centrally injected α -methylnorepinephrine (Breese *et al.*, 1972) or clonidine (Breese, unpublished data; Zis and Fibiger, 1975) and the enhanced amounts of cyclic adenosine monophosphate (AMP) found in tissue after a norepinephrine stimulus (Palmer, 1972; Kalisker *et al.*, 1973; Huang *et al.*, 1974) suggest supersensitive noradrenergic receptors are also present in 6-OHDA-treated animals. Whether referring to the dopaminergic or the noradrenergic system, changes in the responsiveness of postsynaptic receptors would certainly be a method by which the animal could compensate for the destruction of catecholamine fibers produced by 6-OHDA.

Another line of evidence indicating that rats compensate for damage after 6-OHDA treatment is the observation that treated animals are more sensitive to agents which reduce brain catecholamine content (Breese *et al.*, 1973b; Schoenfeld and Zigmond, 1973; Cooper *et al.*, 1973). The discovery that 6-OHDA-treated rats are more sensitive to cate-

cholamine-depleting agents provided the basis for relating various deficits produced by 6-OHDA to the absence of noradrenergic or dopaminergic neural systems. One approach has been to administer α -methyltyrosine to rats whose brain norepinephrine or dopamine fibers have been preferentially destroyed with 6-OHDA. In addition, a dopamine- β -hydroxylase inhibitor which would reduce norepinephrine synthesis was included in these experiments so that changes related to reduced norepinephrine levels in brain could be more clearly specified. Figure 1 shows results of experiments in which these catecholamine-depleting drugs were administered to 6-OHDA-treated rats performing a shuttle-box-avoidance response or a bar-press response for self-stimulation. In each task, a dose of α -methyltyrosine was given that did not affect responding of control rats. Marked depression of responding was observed in rats treated with 6-OHDA to destroy dopaminergic fibers. In contrast, treatment of rats with the dopamine- β -hydroxylase inhibitor, U-14,624, produced no deficits in animals treated with 6-OHDA to destroy noradrenergic fibers, even though norepinephrine content in brain was greatly reduced by this approach (Breese *et al.*, 1973b).

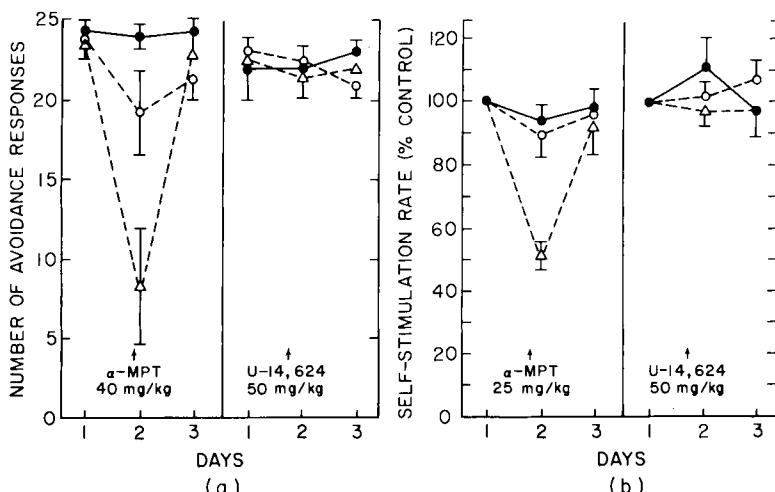


FIG. 1. Shuttle-box-avoidance (a) and self-stimulation (b) responding from control rats (○), dopamine-down treated rats (△), and norepinephrine-down treated rats (●) after α -methyltyrosine (α -MPT) and U-14,624. Doses of α -methyltyrosine (α -MPT) and U-14,624 were administered 4 and 6 hr, respectively, before behavioral testing. Norepinephrine-down treatment consisted of three doses of 25 μ g of 6-OHDA as described in Table III. Desipramine (25 mg/kg) was administered 1 hr before 6-OHDA (200 μ g) to reduce dopamine preferentially (dopamine-down). (From Breese *et al.*, 1973b.)

TABLE V
Selected Studies Using 6-OHDA as a Research Tool

Topic	References
<i>Psychology and physiology</i>	
Anatomical studies	Ungerstedt (1971a); Jacobowitz and Kostrzewa (1971); Jacobowitz (1973); Sachs <i>et al.</i> (1973)
Operant behavior	Breese <i>et al.</i> (1973b), Cooper and Breese (1974); Cooper <i>et al.</i> (1973, 1974); Howard <i>et al.</i> (1974a); Schoenfeld and Uretsky (1972); Peterson and Sparber (1974)
Ingestive behaviors	Ahlsgog and Hoebel (1973); Breese <i>et al.</i> (1973a); Fibiger <i>et al.</i> (1973b); Marshal and Teitelbaum (1973); Stricker and Zigmund (1974); Ungerstedt (1971c); Zigmund and Stricker (1972); Myers and Martin, 1973; Cooper <i>et al.</i> (1974)
Self-stimulation	Breese and Cooper (1975); Cooper <i>et al.</i> (1974); Lippa <i>et al.</i> (1973); Stein and Wise (1971)
Aggressive behavior	Jimerson and Reis (1973); Nakamura and Thoenen (1972); Thoa <i>et al.</i> (1972a,b,c); Eichelman <i>et al.</i> (1972); Sorenson and Ellison (1973)
Reproduction	Grant <i>et al.</i> (1971); MacDonald and Airaksinen (1974)
Temperature regulation	Breese <i>et al.</i> (1972); Simmonds and Uretsky (1970); Nakamura and Thoenen (1971)
Autonomic functions	Haeusler <i>et al.</i> (1972); Williams <i>et al.</i> (1972); Bolme <i>et al.</i> (1974); Howard <i>et al.</i> (1974b)
<i>Psychopharmacology</i>	
Amphetamines	Creese and Iversen (1973, 1975); Fibiger <i>et al.</i> (1973a); Hollister <i>et al.</i> (1974, 1975); Marsden and Guldberg (1973); Peterson and Sparber (1974); Ungerstedt (1971d); Kelly and Iversen (1975)
Dopamine agonists	Schoenfeld and Uretsky (1973); Uretsky and Schoenfeld (1971); von Voigtländer and Moore (1973)
Opiates	Ayhan (1972); Friedler <i>et al.</i> (1972); Nakamura <i>et al.</i> (1973a,b,c); Elchisak and Rosecrans (1973); Blasig <i>et al.</i> (1975)
Neuroleptics	Jalfe and Haefely (1971); Cooper <i>et al.</i> (1972, 1973)

Whether these results indicate a lack of involvement of noradrenergic fibers in the mediation of these behaviors will await future research.

VIII. USE OF 6-HYDROXYDOPAMINE IN NEUROBIOLOGY AND NEUROPHARMACOLOGY

Table V lists several specific studies detailing the use of 6-OHDA as a research tool in the investigation of various behavioral, physiological, and pharmacological responses. It can be seen that 6-OHDA has contributed to the understanding of the anatomical organization of catecholamine-

containing neural systems, their role in behavior, reproduction, temperature regulation, and autonomic function. Major contributions to our understanding of the mechanism of amphetamines, opiates, neuroleptics, and other compounds have also resulted from work with 6-OHDA. For further examination of the specific approach chosen, the reader is referred to particular studies in Table V that may be appropriate to his or her interests.

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REFERENCES

- Ahlskog, J. E., and Hoebel, B. G. (1973). Overeating and obesity from damage to a noradrenergic system in brain. *Science* **182**, 166-169.
- Angeletti, P. U. (1971). Chemical sympathectomy in newborn animals. *Neuropharmacology* **10**, 55-59.
- Angeletti, P. U., and Levi-Montalcini, R. (1970). Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. *Proc. Natl. Acad. Sci. U.S.A.* **65**, 114-121.
- Axelrod, J., and Molinoff, P. B. (1971). Biochemistry of catecholamines. *Annu. Rev. Biochem.* **40**, 465-500.
- Ayhan, I. H. (1972). Effect of 6-hydroxydopamine on morphine analgesia. *Psychopharmacologia* **25**, 183-188.
- Bennett, T., Burnstock, G., Cobb, J. L. S., and Malmfors, T. (1970). An ultrastructural and histochemical study of the short-term effects of 6-hydroxydopamine on adrenergic nerves in the domestic fowl. *Br. J. Pharmacol.* **38**, 802-809.
- Blasig, J., Herz, A., and Gramsch, C. (1975). Effects of depletion of brain catecholamines during the development of morphine dependence on precipitated withdrawal in rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **286**, 325-336.
- Bloom, F. E., Algeri, S., Groppetti, A., Revuelta, A., and Costa, E. (1969). Lesions of central norepinephrine terminals with 6-OH-dopamine: biochemistry and fine structure. *Science* **166**, 1284-1286.
- Bolme, P., Fuxe, K., Nygren, L., Olson, L., and Sachs, C. (1974). Enhanced sensitivity to the noradrenaline receptor stimulating agent, clonidine, following degeneration of noradrenaline pathways; Studies on arterial pressure, heart rate and respiration. In "Dynamics of Degeneration and Regeneration in Neurons" (K. Fuxe, L. Olson, and Y. Zotterman, eds.), pp. 597-602. Pergamon, New York.
- Breese, G. R. (1975). Chemical and immunochemical lesions by specific neurotoxic substances and antisera. In "Handbook of Psychopharmacology" (L. L. Iversen, S. D. Iversen, and S. H. Snyder, eds.), Vol. 1, pp. 137-189. Plenum, New York.
- Breese, G. R., and Cooper, B. R. (1975). Relationship of dopamine neural systems to the maintenance of self-stimulation. In "Neurotransmitters and Behavior" (E. Domino and I. Davis, eds.), pp. 37-56. Edwards Brothers, New York.
- Breese, G. R., and Traylor, T. D. (1970). Effect of 6-hydroxydopamine on brain norepi-

- nephrine and dopamine: Evidence for selective degeneration of catecholamine neurons. *J. Pharmacol. Exp. Ther.* **174**, 413-420.
- Breese, G. R., and Traylor, T. D. (1971). Depletion of brain noradrenaline and dopamine by 6-hydroxydopamine. *Br. J. Pharmacol.* **42**, 88-99.
- Breese, G. R., and Traylor, T. D. (1972). Developmental characteristics of brain catecholamines and tyrosine hydroxylase in the rat: effects of 6-hydroxydopamine. *Br. J. Pharmacol.* **44**, 210-222.
- Breese, G. R., Moore, R. A., and Howard, J. L. (1972). Central actions of 6-hydroxydopamine and other phenylethylamine derivatives on body temperature in the rat. *J. Pharmacol. Exp. Ther.* **180**, 591-602.
- Breese, G. R., Smith, R. D., Cooper, B. R., and Grant, L. D. (1973a). Alterations in consummatory behavior following intracisternal injection of 6-hydroxydopamine. *Pharmacol. Biochem. Behav.* **1**, 319-328.
- Breese, G. R., Cooper, B. R., and Smith, R. D. (1973b). Biochemical and behavioral alterations following 6-hydroxydopamine administration into brain. In "Frontiers in Catecholamine Research" (E. Usdin and S. Snyder, eds.), pp. 701-706. Pergamon, New York.
- Breese, G. R., Smith, R. D., Cooper, B. R., Hollister, A. S., Kraemer, G., and McKinney, W. T. (1975). Use of neurocytotoxic compounds in neuropsychopharmacology. In "Chemical Tools in Catecholamine Research, I" (G. Jonsson, T. Malmfors, and C. Sachs, eds.), pp. 335-342. North-Holland Publ., Amsterdam.
- Butcher, L. L., Eastgate, S. M., and Hodge, G. K. (1974). Evidence that punctate intracerebral administration of 6-hydroxydopamine fails to produce selective neuronal degeneration. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **285**, 31-70.
- Cannon, W. B., and Rosenblueth, A. (1937). "Autonomic Neuro-Effector Systems." Macmillan, New York.
- Clark, D. W. J., Laverty, R., and Phelan, E. L. (1972). Long-lasting peripheral and central effects of 6-hydroxydopamine in rats. *Br. J. Pharmacol.* **44**, 233-243.
- Cooper, B. R., and Breese, G. R. (1974). Relationship of dopamine neural systems to the behavioral alterations produced by 6-hydroxydopamine administration into brain. In "Neuropsychopharmacology of Monoamines and Their Regulatory Enzymes" (E. Usdin, ed.), pp. 353-363. Raven, New York.
- Cooper, B. R., Breese, G. R., Howard, J. L., and Grant, L. D. (1972). Sensitivity to the behavioral depressant effects of reserpine and α -methyltyrosine after 6-hydroxydopamine treatment. *Psychopharmacologia* **27**, 99-110.
- Cooper, B. R., Breese, G. R., Grant, L. D., and Howard, J. L. (1973). Effects of 6-hydroxydopamine treatments on active avoidance responding: Evidence for involvement of brain dopamine. *J. Pharmacol. Exp. Ther.* **185**, 358-370.
- Cooper, B. R., Howard, J. L., Grant, L. D., Smith, R. D., and Breese, G. R. (1974). Alteration of avoidance and ingestive behavior after destruction of central catecholamine pathways with 6-hydroxydopamine. *Pharmacol., Biochem. Behav.* **2**, 639-649.
- Corrodi, H., Clark, W. G., and Masuoka, D. I. (1971). The synthesis and effects of DL-6-hydroxydopa. In "6-Hydroxydopamine and Catecholamine Neurons" (T. Malmfors and H. Thoenen, eds.), pp. 187-192. North-Holland Publ., Amsterdam.
- Creese, I., and Iversen, S. D. (1973). Blockade of amphetamine induced motor stimulation and stereotypy in the adult rat following neonatal treatment with 6-hydroxydopamine. *Brain Res.* **55**, 369-382.
- Creese, I., and Iversen, S. D. (1975). The pharmacological and anatomical substrates of the amphetamine response in the rat. *Brain Res.* **83**, 419-436.

- de Champlain, J. (1971). Degeneration and regrowth of adrenergic nerve fibers in the rat peripheral tissues after 6-hydroxydopamine. *Can. Physiol. Pharmacol.* **49**, 345-355.
- de Champlain, J., and Nadeau, R. (1971). 6-hydroxydopamine, 6-hydroxydopa and degeneration of adrenergic nerves. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **30**, 877-885.
- Eichelman, B. S., Jr., Thoa, N. B., and Ng, K. Y. (1972). Facilitated aggression in the rat following 6-hydroxydopamine administration. *Physiol. Behav.* **8**, 1-3.
- Elchisak, M. A., and Rosecrans, J. A. (1973). Effect of central catecholamine depletions by 6-hydroxydopamine on morphine antinociception in rats. *Res. Commun. Chem. Pathol. Pharmacol.* **6**(1), 349-352.
- Ellison, G. D. (1972). The use of microknives in brain lesion studies and the production of isolated brain-stem islands. In "Methods in Psychobiology" (R. Myers, ed.), Vol. 2, pp. 303-318. Academic Press, New York.
- Evans, B. K., Armstrong, S., Singer, G., Cook, R. D., and Burnstock, G. (1975). Intracranial injection of drugs: comparison of diffusion of 6-OHDA and guanethidine. *Pharmacol., Biochem. Behav.* **3**, 205-217.
- Evetts, K. D., and Iversen, L. L. (1970). Effects of protriptyline on the depletion of catecholamines induced by 6-hydroxydopamine in the brain of the rat. *J. Pharm. Pharmacol.* **22**, 540-542.
- Fibiger, H. C., Lonsbury, B., Cooper, H. P., and Lytle, L. D. (1972). Early behavioral effects of intraventricular administration of 6-hydroxydopamine in rat. *Nature (London), New Biol.* **236**, 209-211.
- Fibiger, H. C., Fibiger, H. P., and Zis, A. P. (1973a). Attenuation of amphetamine induced motor stimulation and stereotypy by 6-hydroxydopamine in the rat. *Brit. J. Pharmacol.* **47**, 683-692.
- Fibiger, H. C., Zis, A. P., and McGeer, E. G. (1973b). Feeding and drinking deficits after 6-hydroxydopamine administration in the rat: Similarities to the lateral hypothalamic syndrome. *Brain Res.* **53**, 135-148.
- Friedler, G., Bhargava, H. N., Quock, R., and Way, E. L. (1972). The effect of 6-hydroxydopamine on morphine tolerance and physical dependence. *J. Pharmacol. Exp. Ther.* **183**(1), 49-55.
- Grant, L. D., Sar, M., Stumpf, W. E., Howard, J. L., and Breese, G. R. (1971). Effects of 6-hydroxydopamine on reproductive functions. *Pharmacologist* **13**, 286.
- Haeusler, G., Gerold, M., and Thoenen, H. (1972). Cardiovascular effects of 6-hydroxydopamine injected into a lateral brain ventricle of the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **274**, 211-228.
- Hedreen, J. (1975). Increased nonspecific damage after lateral ventricle injection of 6-hydroxydopamine compared with fourth ventricle injection in rat brain. In "Chemical Tools in Catecholamine Research, I" (G. Jonsson, T. Malmfors, and C. Sachs, eds.), pp. 91-100. North-Holland Publ., Amsterdam.
- Hökfelt, T., and Ungerstedt, U. (1973). Specificity of 6-hydroxydopamine induced degeneration of central monoamine neurones: An electron and fluorescence microscopic study with special reference to intracerebral injection on the nigrostriatal dopamine system. *Brain Res.* **60**, 269-297.
- Hollister, A. S., Breese, G. R., and Cooper, B. R. (1974). Comparison of tyrosine hydroxylase and dopamine- β -hydroxylase inhibition with the effects of various 6-hydroxydopamine treatments on amphetamine-induced motor activity. *Psychopharmacologia* **36**, 1-16.
- Hollister, A. S., Ervin, G. N., Cooper, B. R., and Breese, G. R. (1975). The roles of monoamine neural systems in the anorexia induced by (+) amphetamine and related compounds. *Neuropharmacology* **14**, 715-723.

- Howard, J. L., Grant, L. D., and Breese, G. R. (1974a). Effects of intracisternal 6-hydroxydopamine treatment on acquisition and performance of rats in a double T-maze. *J. Comp. Physiol. Psychol.* **86**, 995-1007.
- Howard, J. L., Smith, R. D., Mueller, R. A., and Breese, G. R. (1974b). Cardiovascular changes following DOCA/NaCl or conditioning in 6-hydroxydopamine-treated rats. *Pharmacol., Biochem. Behav.* **2**, 537-543.
- Huang, M., Ho, A. K. S., and Daly, J. W. (1973). Accumulation of cyclic adenosine 3', 5'-monophosphate in rat cerebral cortical slices: Stimulatory effect of alpha and beta-adrenergic agents after pretreatments with 6-hydroxydopamine, 2,3,5-trihydroxyphenethylamine and dihydroxytryptamines. *Mol. Pharmacol.* **9**, 711-717.
- Jacks, B. R., de Champlain, J., and Cordeau, J. P. (1972). Effects of 6-hydroxydopamine putative transmitter substances in the central nervous system. *Eur. J. Pharmacol.* **18**, 353-360.
- Jacobowitz, D. M. (1973). Effects of 6-hydroxydopa. In "Frontiers in Catecholamine Research" (E. Usdin and S. Snyder, eds.), pp. 729-739. Pergamon, New York.
- Jacobowitz, D., and Kostrzewska, R. (1971). Selective action of 6-hydroxydopa on noradrenergic terminals; mapping of preterminal axons of the brain. *Life Sci.* **10**, 1329-1342.
- Jalfre, M., and Haefely, W. (1971). Effects of some centrally acting agents in rats after intraventricular injections of 6-hydroxydopamine. In "6-Hydroxydopamine and Catecholamine Neurons" (T. Malmfors and H. Thoenen, eds.), pp. 333-346. North-Holland Publ., Amsterdam.
- Javoy, F., Agid, Y., Glowinski, J., and Sotelo, C. (1974). Biochemical and morphological changes after mechanical or chemical degeneration of the dopaminergic nigro-striatal pathway. In "Dynamics of Degeneration and Growth in Neurons" (K. Fuxe, L. Olson, and Y. Zotterman, eds.), pp. 85-97. Pergamon, New York.
- Jimerson, D., and Reis, D. J. (1973). Effects of intrahypothalamic injection of 6-hydroxydopamine on predatory aggression in rat. *Brain Res.* **61**, 141-152.
- Jonsson, G., and Sachs, C. H. (1970). Effects of 6-hydroxydopamine on the uptake and storage of noradrenaline in sympathetic adrenergic neurons. *Eur. J. Pharmacol.* **9**, 141-155.
- Jonsson, G., and Sachs, C. (1972). Neurochemical properties of adrenergic nerves regenerated after 6-hydroxydopamine. *J. Neurochem.* **19**, 2577-2585.
- Kalisker, A., Rutledge, C. O., and Perkins, J. P. (1973). Effect of nerve degeneration by 6-hydroxydopamine on catecholamine-stimulated adenosine 3',5'-monophosphate formation in rat cerebral cortex. *Mol. Pharmacol.* **9**, 619-629.
- Kelly, P. H., and Iversen, S. D. (1975). The use of 6-hydroxydopamine techniques for studying the pathways involved in drug-induced motor behaviors. In "Chemical Tools in Catecholamine Research, I" (G. Jonsson, T. Malmfors, and C. Sachs, eds.), pp. 327-333. North-Holland Publ., Amsterdam.
- Kelly, P. H., Sevior, P. W., and Iversen, S. D. (1975). Amphetamine lesions of the nucleus accumbens septi and corpus striatum. *Brain Res.* **94**, 507-522.
- Kim, J. S. (1973). Effects of 6-hydroxydopamine on acetylcholine and GABA metabolism in rat striatum. *Brain Res.* **55**, 472-475.
- Konkol, R. J., Breese, G. R., and Cooper, B. R. (1975). Effects of intracisternally administered NGF or anti-NGF on the postnatal development of catecholamine neurons. *Neurosci. Abstr.* **1**, 414.
- Kostrzewska, R. M., and Harper, J. W. (1975). Comparison of the neonatal effects of 6-hydroxydopa and 6-hydroxydopamine on growth and development of noradrenergic neurons in the central nervous system. In "Chemical Tools in Catecholamine Research,

- I" (G. Jonsson, T. Malmfors, and C. Sachs, eds.), pp. 181-188. North-Holland Publ., Amsterdam.
- Laverty, R., Sharman, D., and Vogt, M. (1965). Action of 2,4,5-tri-hydroxyphenylethylamine on the storage and release of noradrenaline. *Br. J. Pharmacol.* **24**, 549-560.
- Lew, G., and Quay, W. (1971). Noradrenaline content of hypothalamus and adrenal gland increased by postnatal administration of 6-hydroxydopamine. *Res. Commun. Chem. Pathol. Pharmacol.* **2**, 307-812.
- Lippa, A. S., Antelman, S. M., Fisher, A. E., and Canfield, D. R. (1973). Neurochemical mediation of reward: A significant role for dopamine? *Pharmacol., Biochem. Behav.* **1**, 23-28.
- MacDonald, E. J., and Airaksinen, M. M. (1974). The effect of 6-hydroxydopamine on the oestrus cycle and fertility of rats. *J. Pharm. Pharmacol.* **26**, 518-521.
- McGeer, E. G., Fibiger, H. C., McGeer, P. L., and Brooke, S. (1973). Temporal changes in amine synthesizing enzymes of rat extrapyramidal structures after hemitransections or 6-hydroxydopamine administration. *Brain Res.* **55**, 229-233.
- Marsden, C. A., and Guldberg, H. D. (1973). The role of monoamines in rotation induced or potentiated by amphetamine after nigral, raphe and mesencephalic reticular lesions in the rat brain. *Neuropharmacology* **12**, 195-211.
- Marshall, J. F., and Teitelbaum, P. (1973). A comparison of the eating in response to hypothermic and glucoprivic challenges after nigral 6-hydroxydopamine and lateral hypothalamic electrolytic lesions in rats. *Brain Res.* **55**, 229-233.
- Mueller, R. A., Thoenen, H., and Axelrod, J. (1969). Increase in tyrosine hydroxylase activity after reserpine administration. *J. Pharmacol. Exp. Ther.* **69**, 74-79.
- Myers, R. D. (1971). General laboratory procedures. In "Methods in Psychobiology" (R. D. Myers, ed.), Vol. 1, 27-65. Academic Press, New York.
- Myers, R. D., and Martin, G. E. (1973). 6-OHDA lesions of the hypothalamus: interaction of aphagia, food palatability, set-point for weight regulation, and recovery of feeding. *Pharmacol. Biochem. Behav.* **1**, 329-345.
- Nakamura, K., and Thoenen, H. (1971). Hypothermia induced by intraventricular administration of 6-hydroxydopamine in rats. *Eur. J. Pharmacol.* **16**, 46-54.
- Nakamura, K., and Thoenen, H. (1972). Increased irritability: A permanent behavior change induced in rat by intraventricular administration of 6-hydroxydopamine. *Psychopharmacologia* **24**, 359-372.
- Nakamura, K., Kuntzman, R., Maggio, A. C., Augulis, V., and Conney, A. H. (1973a). Influence of 6-hydroxydopamine on the effect of morphine on the tail-flick latency. *Psychopharmacologia* **31**, 177-189.
- Nakamura, K., Kuntzman, R., Maggio, A., and Conney, A. H. (1973b). Restoration of morphine analgesia in morphine-tolerant rats after the intraventricular administration of 6-hydroxydopamine. *J. Pharm. Pharmacol.* **25**, 584-587.
- Nakamura, K., Kuntzman, R., Maggio, A., and Conney, A. H. (1973c). Decrease in morphine's analgesic action and increase in its cataleptic action by 6-hydroxydopamine injected bilaterally into caudate and putamen areas; partial restoration by L-DOPA plus decarboxylase inhibition. *Neuropharmacology* **12**, 1153-1160.
- Palmer, G. C. (1972). Increased cyclic AMP response to norepinephrine in rat brain following 6-hydroxydopamine. *Neuropharmacology* **11**, 145-149.
- Peterson, D. W., and Sparber, S. B. (1974). Increased fixed ratio performance and differential d- and l-amphetamine action following norepinephrine depletion by intraventricular 6-hydroxydopamine. *J. Pharmacol. Exp. Ther.* **191**, 349-357.
- Poirier, L. J., Langelier, P., Roberge, A., Boucher, R., and Kitsikis, A. (1972). Non-specific histopathological changes induced by the intracerebral injection of 6-hydroxydopamine. *Neurol. Sci.* **1**, 401-416.

- Reis, D. J., and Molinoff, P. B. (1972). Brain dopamine- β -hydroxylase: Regional distribution and effects of lesions and 6-hydroxy-dopamine on activity. *J. Neurochem.* **19**, 195-204.
- Richards, J. G. (1971). Ultrastructural effects of 6-hydroxydopamine on catecholamine containing neurons in the rat brain. In "6-Hydroxydopamine and Catecholamine Neurons" (T. Malmfors and H. Thoenen, eds.), pp. 151-161. North-Holland Publ., Amsterdam.
- Sachs, C., Jonsson, G., and Fuxe, K. (1973). Mapping of central noradrenaline pathways with 6-hydroxy-dopa. *Brain Res.* **63**, 249-261.
- Schoenfeld, R. I., and Uretsky, N. J. (1972). Operant behavior and catecholamine-containing neurons: prolonged increase in lever-pressing after 6-hydroxydopamine. *Eur. J. Pharmacol.* **20**, 357-362.
- Schoenfeld, R. I., and Uretsky, N. J. (1973). Enhancement by 6-hydroxydopamine of the effects of dopa upon the motor activity of rats. *J. Pharmacol. Exp. Ther.* **186**, 616-624.
- Schoenfeld, R. I., and Zigmond, M. J. (1973). Behavioral pharmacology of 6-hydroxydopamine. In "Frontiers in Catecholamine Research" (E. Usdin and S. Snyder, eds.), pp. 695-700. Pergamon, New York.
- Simmonds, M. A., and Uretsky, N. J. (1970). Central effects of 6-hydroxydopamine on body temperature. *Br. J. Pharmacol.* **40**, 630-638.
- Sims, K. L., and Bloom, F. E. (1973). Rat brain L-3,4-dihydroxyphenylalanine and L-5-hydroxytryptophan decarboxylase activities: differential effect of 6-hydroxydopamine. *Brain Res.* **49**, 165-175.
- Singh, B., and de Champlain, J. (1972). Altered ontogenesis of central noradrenergic neurons following neonatal treatment with 6-hydroxydopamine. *Brain Res.* **48**, 432-437.
- Smith, R. D., Cooper, B. R., and Breese, G. R. (1973). Growth and behavioral changes in developing rats treated intracisternally with 6-hydroxydopamine: evidence for involvement of brain dopamine. *J. Pharmacol. Exp. Ther.* **185**, 609-619.
- Sorenson, C. A., and Ellison, G. D. (1973). Nonlinear changes in activity and emotional reactivity scores following central noradrenergic lesions in rats. *Psychopharmacologia* **32**, 313-325.
- Stein, L., and Wise, C. (1971). Possible etiology of schizophrenia: progressive damage to the noradrenergic reward system of hydroxydopamine. *Science* **171**, 1032-1037.
- Stricker, E. M., and Zigmond, M. J. (1974). Effects on homeostasis of intraventricular injections of 6-hydroxydopamine in rats. *J. Comp. Physiol. Psychol.* **86**, 973-994.
- Tassin, J. P., Velley, L., Stinus, L., Blanc, G., Glowinski, J., and Thierry, A. M. (1975). Development of cortical and nigro-neostriatal dopaminergic systems after destruction of central noradrenergic neurones on foetal or neonatal rats. *Brain Res.* **83**, 93-106.
- Teitelbaum, P., and Epstein, A. N. (1962). The lateral hypothalamic syndrome: Recovery of feeding and drinking after lateral hypothalamic lesions. *Psychol. Rev.* **69**, 74-90.
- Thierry, A. M., Velley, L., Stinus, L., Tassin, J. P., Blanc, G., and Glowinski, J. (1975). Development of the mesocortical and nigrostriatal dopaminergic systems following various 6-hydroxydopamine treatments. In "Chemical Tools in Catecholamine Research, I" (G. Jonsson, T. Malmfors, and C. Sachs, eds.), pp. 205-210. North-Holland Publ., Amsterdam.
- Thoa, N. B., Eichelman, B., and Ng, L. K. Y. (1972a). Shock-induced aggression: effects of 6-hydroxydopamine and other pharmacological agents. *Brain Res.* **43**, 467-475.
- Thoa, N. B., Eichelman, B., and Ng, L. K. Y. (1972b). Aggression in rats treated with dopa and 6-hydroxydopamine. *J. Pharm. Pharmacol.* **24**, 337-338.
- Thoa, N. B., Eichelman, B., Richardson, J., and Jacobowitz, D. (1972c). 6-hydroxydopa depletion of brain norepinephrine and the facilitation of aggressive behavior. *Science* **178**, 75-77.

- Thoenen, H. (1972). Surgical immunological and chemical sympathectomy: their application in the investigation of the physiology and pharmacology of the sympathetic nervous system. In "Handbook of Experimental Pharmacology: Catecholamines" (H. Blaschko and E. Muscholl, eds.), pp. 813-844. Springer-Verlag, Berlin and New York.
- Thoenen, H., and Tranzer, J. P. (1968). Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **261**, 271-288.
- Tranzer, J. P., and Thoenen, H. (1968). An electron microscopic study of selective, acute degeneration of sympathetic nerve terminals after administration of 6-hydroxydopamine. *Experientia* **24**, 155-156.
- Tranzer, J. P., Thoenen, H., Snipes, R. L., and Richards, J. G. (1969). Recent developments on the ultrastructural aspect of adrenergic nerve endings in various experimental conditions. *Prog. Brain Res.* **31**, 33-46.
- Ungerstedt, U. (1968). 6-hydroxydopamine induced degeneration of central monoamine neurons. *Eur. J. Pharmacol.* **5**, 107-110.
- Ungerstedt, U. (1971a). Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiol. Scand.* **367**, 1-48.
- Ungerstedt, U. (1971b). Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. *Acta Physiol. Scand.* **367**, 69-93.
- Ungerstedt, U. (1971c). Adipsia and aphagia after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system in the rat brain. *Acta Physiol. Scand.* **367**, 49-68.
- Ungerstedt, U. (1971d). Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behavior. *Acta Physiol. Scand.* **367**, 49-68.
- Ungerstedt, U. (1971e). Use of intracerebral injections of 6-hydroxydopamine as a tool for morphological and functional studies on central catecholamine neurons. In "6-Hydroxydopamine and Catecholamine Neurons" (T. Malmfors and H. Thoenen, eds.), pp. 315-332. North-Holland Publ., Amsterdam.
- Ungerstedt, U. (1971f). Histochemical studies on the effects of intracerebral and intraventricular injections of 6-hydroxydopamine on monoamine neurons in the rat brain. In "6-Hydroxydopamine and Catecholamine Neurons" (T. Malmfors and H. Thoenen, eds.), pp. 101-127. North-Holland Publ., Amsterdam.
- Uretsky, N. J., and Iversen, L. L. (1970). Effects of 6-hydroxydopamine on catecholamine-containing neurons in the rat brain. *J. Neurochem.* **17**, 269-278.
- Uretsky, N. J., and Schoenfeld, R. I. (1971). Effect of L-DOPA on the locomotor activity of rats pretreated with 6-hydroxydopamine. *Nature (London), New Biol.* **234**, 157-159.
- von Voigtländer, P., and Moore, K. (1973). Turning behavior of mice with unilateral 6-hydroxydopamine lesions in the striatum: Effects of apomorphine, L-DOPA, amantadine, amphetamine and other psychomotor stimulants. *Neuropharmacology* **12**, 451-462.
- Williams, R. B., Eichelman, B., and Ng, L. K. Y. (1972). Depletion of brain amines reverses blood pressure responses to footshock in the rat. *Nature (London), New Biol.* **240**, 276-277.
- Zigmond, M. J., and Stricker, E. M. (1972). Deficits in feeding behavior after intraventricular injection of 6-hydroxydopamine in rats. *Science* **177**, 1211-1214.
- Zigmond, M. J., and Stricker, E. M. (1975). Compensatory changes after intraventricular administration of 6-hydroxydopamine: a neurochemical model for recovery of function. In "Chemical Tools in Catecholamine Research, I" (G. Jonsson, T. Malmfors, and C. Sachs, eds.), pp. 319-326. North-Holland Publ., Amsterdam.
- Zis, A. P., and Fibiger, H. C. (1975). Functional evidence for postsynaptic supersensitivity of central noradrenergic receptors after denervation. *Nature (London)* **256**, 659-661.

Chapter 3

Chemical Lesioning of Indoleamine Pathways¹

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¹ Dedicated to Prof. Rudolf Janzen, M.D., Ph.D., on the occasion of his emeritization as chairman of the Department of Neurology, University of Hamburg.

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I. INTRODUCTION

The complexity of brain architecture has challenged neurobiologists to design methods for localized destruction of cell groups or interruption of axonal pathways. However, even the most restrictive surgical, electrolytic, or electrothermic approach to nucleotomy or tractotomy inevitably damages heterogeneous neuronal, glial, or vascular elements in the brain; the results obtained by such physical lesioning methods must be questioned as to their selectivity and physiological relevance. This is particularly true for loosely arranged neurons with diffuse projections, such as many reticular cell groups. Most of the monoaminergic neurons and their fibers belong to this class of reticular elements, despite the fact that in

some regions of the rat brainstem there are localized condensations of monoamine-containing perikarya, such as the nucleus locus coeruleus.

A fairly diffuse arrangement is also characteristic of serotonergic cell bodies in the brainstem tegmentum and raphe nuclei. Destruction of these cell groups by surgical, electrothermic, or electrolytic means must necessarily result in nonspecific damage to nonserotonergic neurons intermingled with serotonin-containing ones, as well as in interruption of pathways penetrating the lesioned area.

The recently introduced *chemical approach* to axotomy in the central nervous system (CNS) seems, at least partly, to overcome this obstacle. The so-called neurotoxic amine congeners thus offer a possibility for more selective lesioning of monoaminergic pathways, which may be of advantage for functional studies of these types of neurons in the CNS. Recently published results from studies on behavioral consequences of raphe coagulation compared to those using localized injections of 5,7-dihydroxytryptamine (5,7-DHT) strongly support this contention (cf. Lorens *et al.*, 1975; Hole *et al.*, 1976; Srebro and Lorens, 1975).

II. HISTORICAL REVIEW

Thoenen and Tranzer (1968) discovered that the long-lasting depletion of noradrenaline (NA; i.e., norepinephrine) in sympathetic nerves of the mouse produced by systemic injections of 2,4,5-trihydroxyphenylethylamine (i.e., "6-hydroxydopamine," 6-OHDA) (Porter *et al.*, 1963; Laverty *et al.*, 1965) was due to a degeneration of noradrenergic axon terminals. This discovery and subsequent application of this principle to the destruction of dopamine- (DA) and NA-containing neurons in the brain (Ungerstedt, 1968; Uretsky and Iversen, 1969, 1970) established a new method in neurobiology: the method of selective chemical degeneration.

A consideration of the chemical properties of 6-OHDA, a strongly reducing hydroxylated congener of DA having the capacity to form highly reactive quinoid oxidation products, suggested that selective degeneration of neurons containing serotonin (5-hydroxytryptamine, 5-HT) should be possible by injection of an analogous hydroxylated serotonin derivative. The work of Baumgarten *et al.* (1971, 1972a) provided evidence for a long-lasting and rather selective depleting action of 5,6-dihydroxytryptamine (5,6-DHT) on central serotonin neurons, but disclosed at the same time certain limitations of the drug as a tool in neurobiological research, mainly because of nonspecific toxic side effects on nonmonoaminergic neurons (Baumgarten *et al.*, 1972a,b).

In 1972(a) Baumgarten and Lachenmayer reported that 5,7-DHT when

administered intraventricularly (i.v.t.) to adult rats, caused pronounced lesions to the central indoleamine neurons. Subsequently, Baumgarten *et al.* (1973a) showed that 5,7-DHT produces dose-related long-lasting depletion of 5-HT and non-dose-related decreases in brain NA, with few nonspecific toxic side effects.

More recently, it was found that the NA depletion by 5,7-DHT in brain can be antagonized by blockers of NA uptake or by monoamine oxidase (MAO) inhibitors without attenuating its 5-HT-lowering properties (Gershon *et al.*, 1974; Björklund *et al.*, 1975a; Gershon and Baldessarini, 1975; Breese and Cooper, 1975). Thus, this drug could be used for extensive and rather selective lesioning of serotonergic neurons.

III. GENERAL COMMENTS ON THE DRUGS AND THEIR ADMINISTRATION

The drugs 5,6-DHT and 5,7-DHT differ from 5-HT by having an additional hydroxyl group in the benzene nucleus of the indole molecule: 5,6-DHT by an additional OH— in position 6; 5,7-DHT by an OH— in position 7 (Fig. 1). The former is thus an *o*-substituted, the latter an *m*-substituted dihydroxytryptamine (DHT). This difference has consequences for their behavior in aqueous solutions: *o*-DHTs are, at neutral or alkaline pH, very prone to autoxidation and tend to convert into colored quinoid products (red, blue, violet, or brown color), predominantly *o*-quinones. This autoxidation requires oxygen; thus, in oxygen-free solution, 5,6-DHT is quite stable. Both acidification or addition to the vehicle of antioxidants, such as *l*-ascorbic acid, help to prevent oxidation of 5,6-DHT. It follows that 5,6-DHT should be made up freshly before use and dissolved in the presence of biologically tolerable antioxidants.

5,6-DHT² is available as a gray-pinkish creatinine sulfate; its purity can be checked by chromatography or gas chromatography-mass spectroscopy (GC/MS); it should be stored frozen in a desiccator, preferably under nitrogen atmosphere.

5,7-DHT is also available as a creatinine sulfate of pinkish color that should be stored frozen in a desiccator. Temporary storage at room temperature under dry conditions does not cause detectable deterioration. When dissolved in the presence of antioxidants, pure 5,7-DHT gives a slightly yellowish or pinkish color, depending on the concentration used. It may be advisable to keep 5,7-DHT-containing solutions cold before use. Solutions of 5,7-DHT-containing antioxidants can be stored

² Both 5,6-DHT and 5,7-DHT are commercially available from Regis Chemical Co., Morton Grove, Illinois.

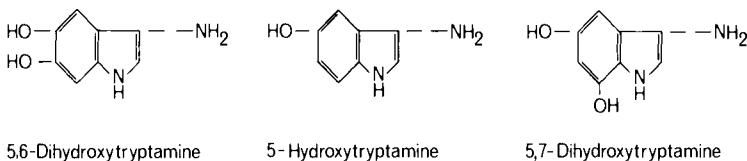


FIG. 1. Structural formula of 5-HT, 5,6-DHT, and 5,7-DHT.

frozen (-30°C) for a few days, but thawing and refreezing should be avoided. In aqueous solutions at neutral or alkaline pH, 5,7-DHT slowly takes on a bluish, reddish, or violet color, indicating formation of quinoid oxidation products. Such deeply colored solutions should not be used for animal work. The purity of 5,7-DHT can be checked by chromatography or GC/MS.

For the synthesis of 5,6-DHT, see Schlossberger and Kuch (1960, 1963), and for the synthesis of 5,7-DHT, see Lee *et al.* (1973).

For both 5,6- and 5,7-DHT, concentrations of ascorbic acid between 0.1 and 0.5 mg per milliliter of vehicle are sufficient for short-lasting protection against oxidation. If higher concentrations of *l*-ascorbic acid are used (1 mg/ml), the pH should be checked and, if lower than 6.5, brought back to 7.3 by addition of alkali.

For newborn animals (e.g., rats), the amount of vehicle per intracister nal (i.c.) or i.v.t. injection should not exceed 10 μl ; for adult rats up to 25 μl are tolerable. For intracerebral injections, the volume should be in the range of 1–4 μl (cf. Agid *et al.*, 1973) and the concentration of neurotoxic indoleamine should be in the range of 1–2 μg per microliter of vehicle. The injection should be made slowly in the case of the intracerebral route of administration, whereas the drugs should be injected quickly if applied i.v.t. or i.c.

If 5,6-DHT is to be administered intravenously (i.v.), animals should be pretreated with an α -adrenergic receptor and/or a serotonin-receptor blocking agent (e.g., phenoxybenzamine, phentolamine, methysergide) to prevent the severe cardiovascular and pulmonary side effects.

In rats, ether anesthesia has been found to give the best results, but short-acting barbiturates are also useful and usually more convenient (cf. Section XIV,C). If large amounts of 5,7-DHT are injected i.c. or i.v.t., animals should be protected against the short-lasting convulsions by intraperitoneal (i.p.) injections of a short-acting barbiturate in doses lower than that required to produce narcosis.

It is advisable to keep animals treated i.c. or i.v.t. with either com-

pound (5,6- or 5,7-DHT) singly or in small groups to avoid exaggerated aggression.

IV. EFFECTS OF INTRAVENTRICULAR ADMINISTRATION OF 5,6- OR 5,7-DIHYDROXYTRYPTAMINE ON CNS MONOAMINE CONTENT IN THE ADULT RAT

A. Effects of 5,6- or 5,7-DHT on Whole Brain and Spinal Cord Monoamines

Eight to twelve days following i.v.t. injection of either 50 μg 5,6- or 5,7-DHT, the whole-brain 5-HT content is reduced to 56% and 48% of the control, respectively. These values reflect maximum serotonin depletion as judged from time-course studies performed on selected brain regions (Baumgarten *et al.*, 1971, 1973a). While there is no significant further depletion of 5-HT content measured in whole brain following doses higher than 50 μg (75 or 100 μg) or following two i.v.t. doses of 50 μg 5,6-DHT given 24 hr apart (Baumgarten and Björklund, unpublished observations), 5-HT content appears to be depleted in a dose-dependent manner at least in some brain regions (e.g., striatum, hypothalamus, forebrain) 10 days after drug administration (cf. Baumgarten *et al.*, 1971). Doses of up to 50 μg 5,6-DHT have either insignificant (Baumgarten *et al.*, 1971) or slightly depletive (Baumgarten *et al.*, 1975a) effects on brain NA and DA after 8–12 days. Time-course studies on the effects of 75 μg 5,6-DHT on whole-brain catecholamine (CA) content indicate, however, that both NA and DA are depleted transiently (DA 40% depletion after 12 hr; NA 25% after 1 hr); DA recovers to near-normal levels within 4 days, whereas NA is back to control levels within 3 hr and increases to values 50% above control after 10 days. This increase in whole-brain NA content following 75 μg 5,6-DHT is no longer detectable at 1 month (Figs. 2 and 3).

Significant reductions in forebrain NA and in striatal DA levels occur 4 days after 100 μg 5,6-DHT is administered to the rat (Baumgarten *et al.* 1972a). The discrepancy between results reported by Baumgarten *et al.* in 1971 and 1975(a) concerning the increase in brain NA levels at 10 days after injection is not easily resolved but may be due to varying purities of the 5,6-DHT samples obtained from different sources. 5,7-DHT, on the other hand, depletes whole-brain 5-HT in a dose-related fashion, and a dose as low as 10 μg causes a significant reduction of brain serotonin content at 10 days (to 78% that of the control). Following 200 μg 5,7-DHT, 5-HT is reduced to 25% that of the control. While DA is not affected, NA is decreased in a dose-dependent manner with doses up to 50 μg ; higher

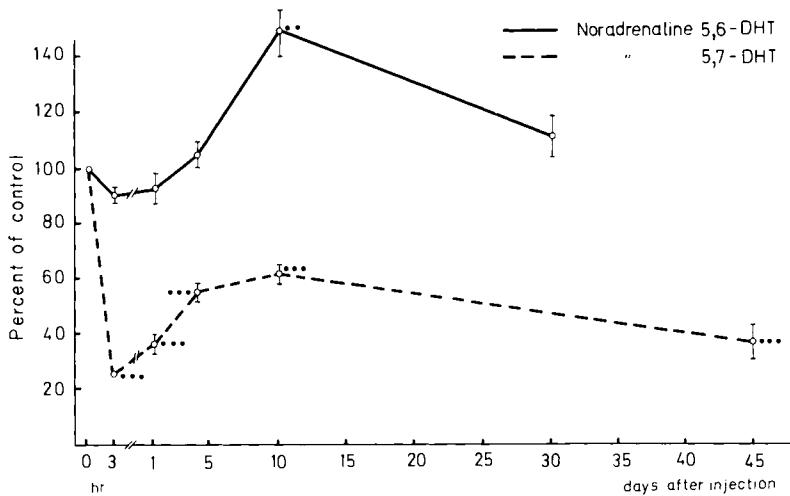


FIG. 2. Time course of depletion and recovery of whole-brain noradrenaline in the rat following i.v.t. 5,6-DHT (75 µg) and 5,7-DHT (200 µg). Bars give means \pm SEM. *0.05 > p > 0.01; **0.01 > p > 0.001; *** p < 0.001. Student's t test. (From Baumgarten *et al.*, 1971, 1973a.)

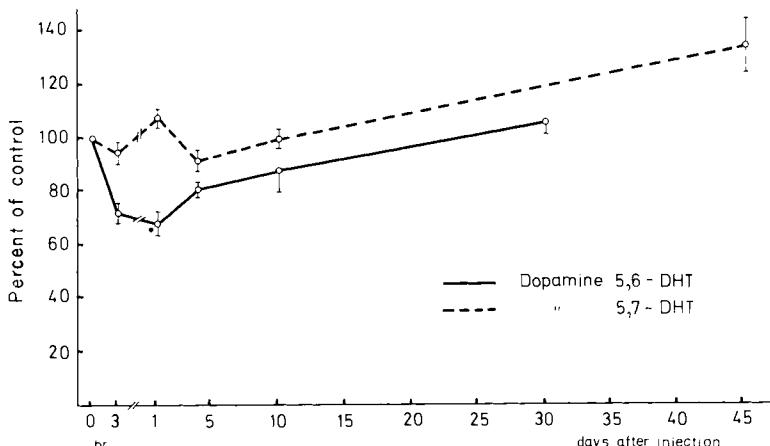


FIG. 3. Time course of depletion and recovery of whole-brain dopamine in the rat following i.v.t. 5,6-DHT (75 µg) and 5,7-DHT (200 µg). Bars give means \pm SEM. *0.05 > p > 0.01; **0.01 > p > 0.001; *** p < 0.001. (From Baumgarten *et al.*, 1971, 1973a.)

doses fail to enhance the NA depletion in brain [(Baumgarten *et al.*, 1973a) see Fig. 4].

Time-course studies performed with 200 µg 5,7-DHT (given i.v.t.) reveal a long-lasting reduction in whole-brain NA for up to 45 days but not in DA. By 45 days, brain DA appears to be increased above control levels [(Baumgarten *et al.*, 1973a) see Figs. 2 and 3].

Figure 5 shows that by 10 days after i.v.t. 5,6-DHT (10,25,50,75 µg), spinal cord 5-HT is maximally depleted by 25 µg of the drug. The percentage of serotonin depletion after 10 µg 5,6-DHT is only moderate (15%). NA is not significantly affected with doses lower than 50 µg. At doses of 75 µg 5,6-DHT, there is a significant elevation of spinal cord NA (Nobin *et al.*, 1973).

Ten days after i.v.t. 5,7-DHT, spinal cord 5-HT content is maximally decreased over a wide range of doses (10–200 µg). The effects of i.v.t. 5,7-DHT on spinal cord NA seem to be correlated with the dose administered (Fig. 6). When a single high dose of 5,7-DHT is given (150 µg) spinal cord NA remains maximally depleted for up to at least 4 months (Björklund *et al.*, 1975b).

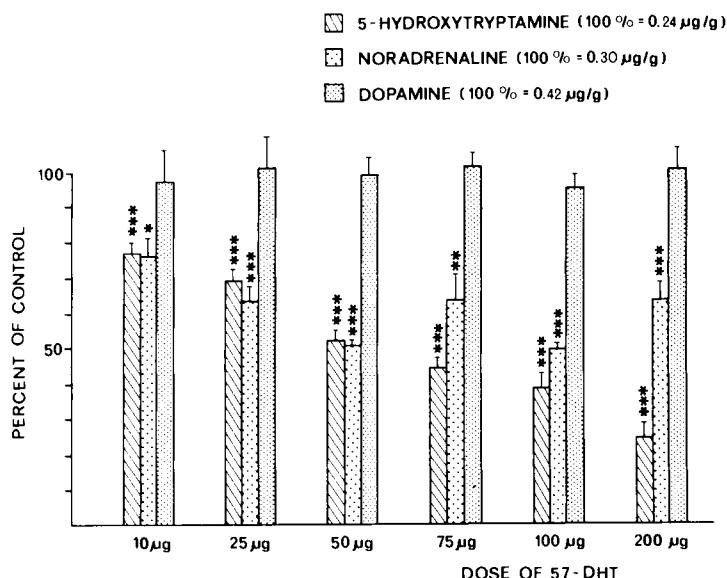


FIG. 4. Dose-response effects of 5,7-DHT on whole brain 5-HT, NA and DA concentrations, 10 days after i.v.t. injection in the rat. Bars give means \pm SEM. For significance levels, see Fig. 2. (From Baumgarten *et al.*, 1973a.)

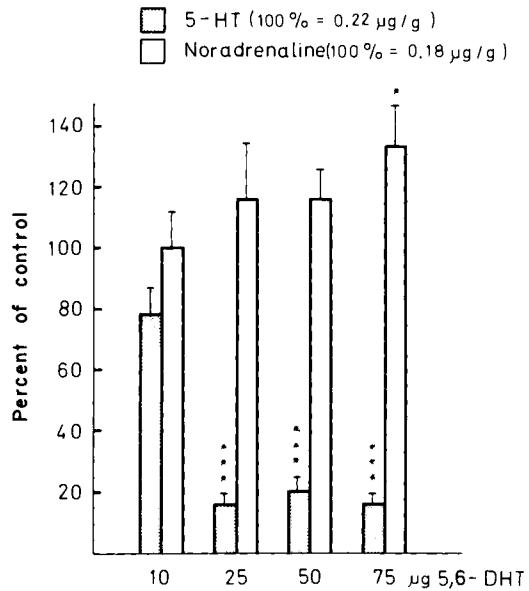


FIG. 5. Dose-response effects of i.v.t. 5,6-DHT on spinal cord 5-HT and NA concentrations, 10 days after injection. Bars give means \pm SEM. For significance levels, see Fig. 2. (Based on data in Nobin *et al.*, 1973.)

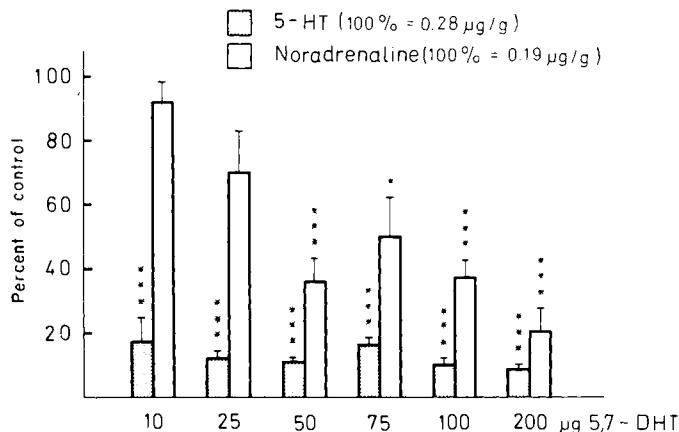


FIG. 6. Dose-response effects of i.v.t. 5,7-DHT on spinal cord 5-HT and NA concentrations in the rat, 10 days after injection of the drug. Bars give means \pm SEM. For significance levels, see Fig. 2. (From Baumgarten *et al.*, 1973a.)

B. Effects of 5,6- or 5,7-DHT on CNS Serotonin Content: Time-Course and Regional Differences

Single doses of 75 µg 5,6-DHT or 200 µg 5,7-DHT (which are the near-maximally tolerable doses of the two compounds) both cause, within 24 hr, pronounced reductions of the 5-HT content in all regions explored (Fig. 7a-g). The extent of depletion following 5,7-DHT (200 µg) exceeds that following 5,6-DHT (75 µg) administration in all regions except the spinal cord. While 5-HT remains severely depleted in spinal cord, hypothalamus, striatum, septum, and the rest of the forebrain for at least 1 month after 5,7-DHT is administered, 5-HT levels recover moderately in all regions analyzed, except in the spinal cord, following 5,6-DHT. From studies using a more restricted dissection of the brain, it is evident that there is no significant recovery of 5-HT in the forebrain and spinal cord beyond 1 month in the 5,7-DHT treated animal, and only a partial recovery of brainstem 5-HT, whereas 5-HT levels continue to rise in many regions after 5,6-DHT (Baumgarten *et al.*, 1971, 1973b, 1974a; Björklund *et al.*, 1975b).

In summary, in the adult rat, 50–75 µg 5,6-DHT given via the lateral ventricle produce significant and long-lasting depletion of brain and spinal cord 5-HT. While 5,6-DHT appears to be selective for serotonin neurons in the spinal cord, brain DA levels are temporarily decreased. Although most studies indicate that brain NA content is hardly affected initially and tends to rise temporarily, the possibility of a very mild direct action of even low doses of 5,6-DHT on brain NA neurons must be considered until further time-course studies on individual brain regions have been carried out. 5,7-DHT produces a dose-related depletion of brain 5-HT in the rat and is a somewhat more potent serotonin depletor than 5,6-DHT. Brain DA levels appear unaffected by 5,7-DHT, but both brain and spinal cord NA are reduced—although not to the same extent as 5-HT.

V. EFFECTS OF INTRACISTERNALE ADMINISTRATION OF 5,6- OR 5,7-DHT ON CNS MONOAMINE CONTENT IN THE ADULT RAT

One injection of 75 µg 5,6-DHT selectively reduces whole-brain 5-HT by 37% at 1 month (Breese *et al.*, 1974a). An additional dose of 75 µg 5,6-DHT, given 7 days later, causes a further reduction in brain 5-HT (to 51% of control at day 30), without affecting the brain CAs.

In whole brain, the administration of 200 µg 5,7-DHT causes a 68% decrease of 5-HT at day 14. There is little further change in the extent of 5-HT depletion for up to 270 days. Brain NA is depleted by 32% at day 30

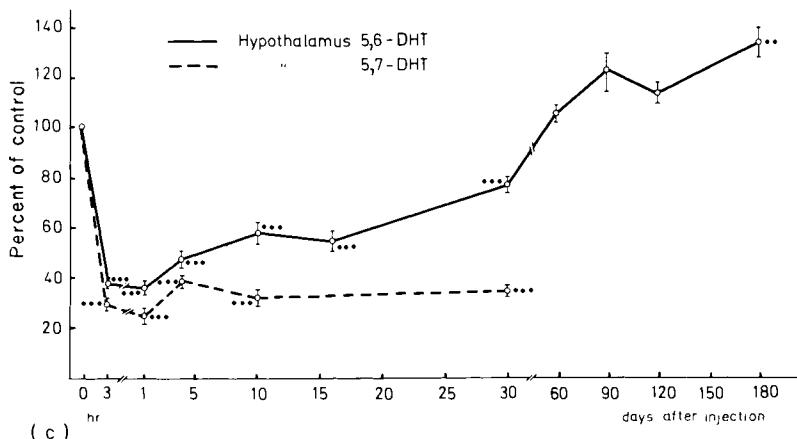
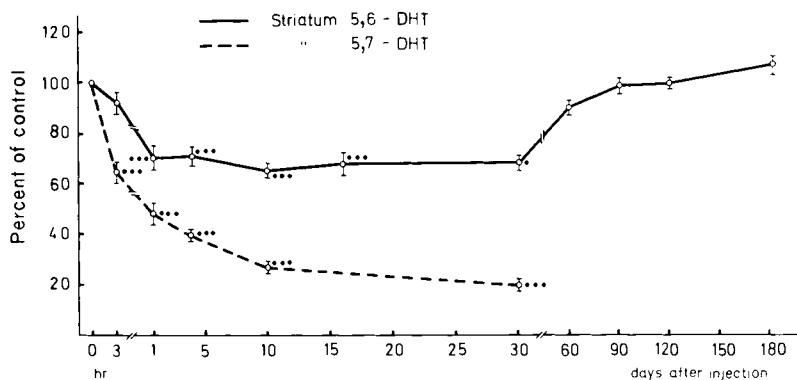
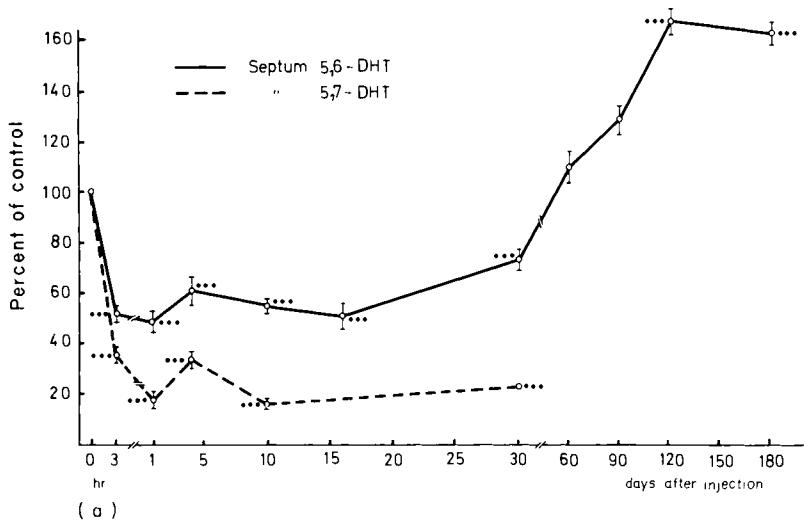


FIG. 7(a-c).

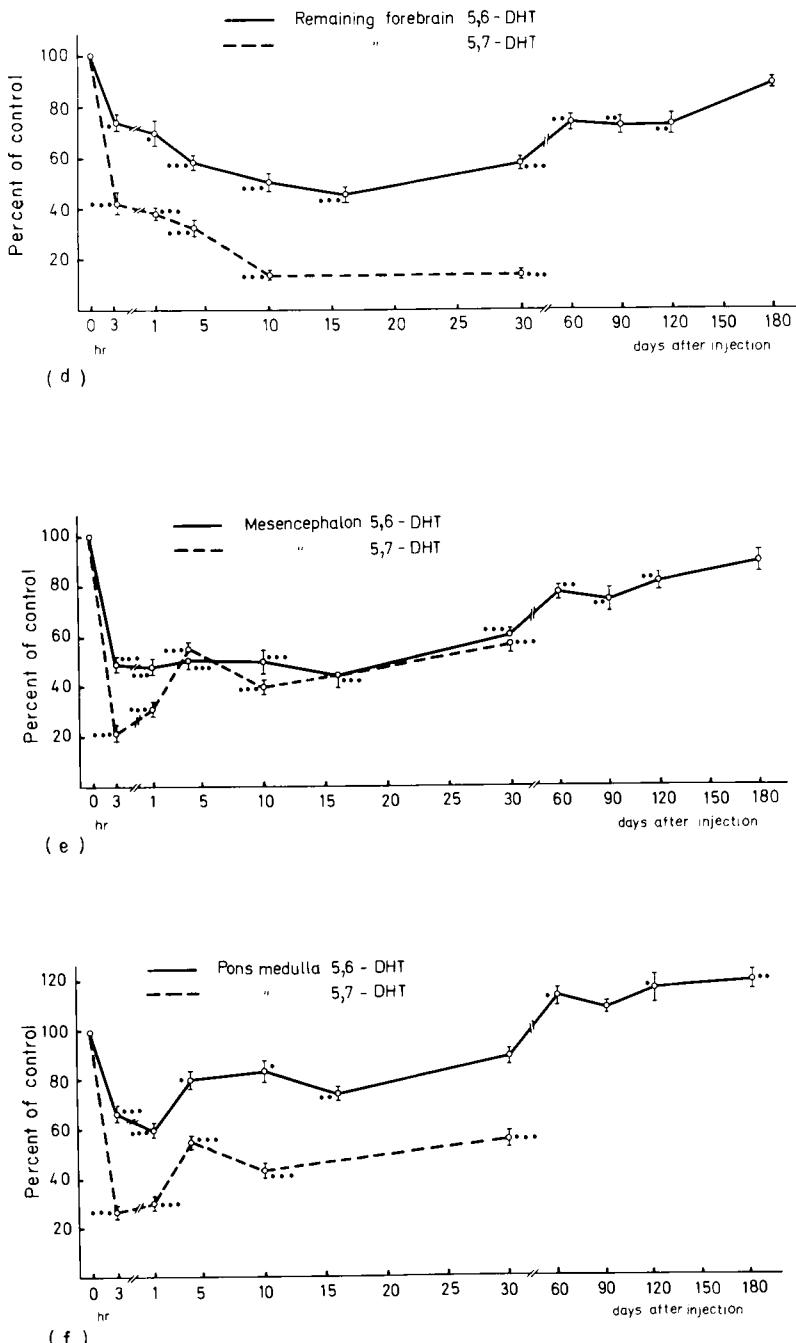


FIG. 7(d-f).

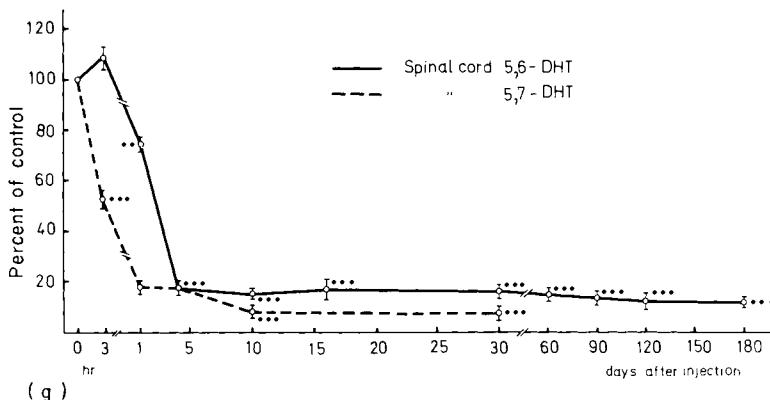


FIG. 7. Time-course effects of i.v.t. 5,6-DHT (75 µg) and 5,7-DHT (200 µg) on regional 5-HT concentrations in the rat: (a) septum; (b) striatum; (c) hypothalamus; (d) remaining forebrain; (e) mesencephalon; (f) pons-medulla oblongata; (g) spinal cord. Bars give means \pm SEM. For significance levels, see Fig. 2. (Based on data in Baumgarten *et al.*, 1971, 1973a.)

and shows a tendency to recover during the subsequent 8 months (9% reduction at day 270). Whole-brain DA content is not significantly altered (Breese and Cooper, 1975). Regional analysis of brain 5-HT content at 1 month after the injection of 200 µg 5,7-DHT indicates that the striatum is profoundly affected (65% reduction) and that the loss of 5-HT in the hypothalamus (55%) and brainstem (33%) is less (Breese and Cooper, 1975).

Data for spinal cord monoamines are available in the paper by Gershon *et al.* (1974). The injection of 65 µg 5,6-DHT lowers spinal cord 5-HT to 18% of the control without changing NA content significantly. Surprisingly, Vogt (1974) failed to obtain significant reduction of spinal cord 5-HT by the i.c. administration of 40 µg 5,6-DHT. The i.v.t. injection of 50 µg 5,6-DHT, on the other hand, was found to reduce the content of 5-HT in the lumbar spinal cord by as much as 87%.

In summary, one i.v.t. dose of 50 µg 5,6-DHT is as potent as two successive doses of 75 µg i.c. 5,6-DHT (given 7 days apart) in reducing whole-brain 5-HT. The i.c. administration of 200 µg 5,7-DHT is nearly as effective in depleting whole-brain 5-HT as 200 µg given via the lateral ventricle. However, regional analysis of 5-HT content following either route of administration indicates that forebrain regions are depleted less with i.c. than with i.v.t. injections of 5,7-DHT. Multiple doses of 5,6- or 5,7-DHT have little or no potentiating effect on brain 5-HT content already depleted by one single high dose of either compound. The i.c.

route of administering 5,6- or 5,7-DHT, although slightly inferior to the i.v.t. route as concerns 5-HT depletion, might provide improved selectivity since side effects on brain CA content are less marked.

VI. EFFECTS OF INTRAVENTRICULAR ADMINISTRATION OF 5,6- OR 5,7-DHT OR 6-OHDA ON MONOAMINE BIOSYNTHETIC ENZYMES

As shown by Victor, Baumgarten, and Lovenberg (1974), the time-dependent depletion and recovery patterns of tryptophan hydroxylase activity are rather complex and in part different from those of 5-HT. This may be illustrated by comparing the time-course characteristics of 5-HT and tryptophan hydroxylase activity in the septum and hypothalamus following the injection of 75/ μ g i.v.t. 5,6-DHT (Fig. 8*a,b*). The main differences concern the extent of early recovery, which is much more pronounced with tryptophan hydroxylase, and the time of onset and peaking of enzyme activity. These differences become less apparent after 10 to 16 days, i.e., at a time when the process of anterograde terminal degeneration is completed and the initial reaction of the neuronal perikarya to the severe axonal damage has leveled off, being replaced by continuing efforts to compensate for the loss of terminals by axonal sprouting. With the exception of the spinal cord, the long-term reduction of tryptophan hydroxylase is only moderate in all brain regions (as with 5-HT) and there are signs of mild recovery from 12 days to 2 months after injection of 75 μ g i.v.t. 5,6-DHT.

The intravenous injection of 150 μ g of 5,7-DHT decreases tryptophan hydroxylase in terminal-rich ventricular or superficial brain regions within 2 days after the injection; the levels are close to the minimum activity occurring in 12 days. In contrast, the depletion of enzyme activity in regions that are cell-body rich and distant from the ventricular regions becomes progressively greater between 2 and 12 days (Baumgarten *et al.*, 1973c).

The specificity of 75 μ g of 5,6-DHT (i.v.t.) and 150 μ g of 5,7-DHT (i.v.t.) has been assessed by measuring their effects on tryptophan-, tyrosine-, and dopamine- β -hydroxylase activity at 10 days after injection and comparing them to those of 200 μ g of 6-OHDA [Baumgarten, Victor, Brunswick, and Lovenberg (unpublished observations), see Fig. 9]. Tyrosine hydroxylase is significantly decreased by 6-OHDA, in all regions analyzed, but not by 5,6- or 5,7-DHT. Except for the midbrain tegmentum, DA- β -hydroxylase is strongly reduced in all CNS regions after 6-OHDA administration. In the spinal cord, tectum, and remaining forebrain, 5,7-DHT decreases DA- β -hydroxylase activity. It is interesting

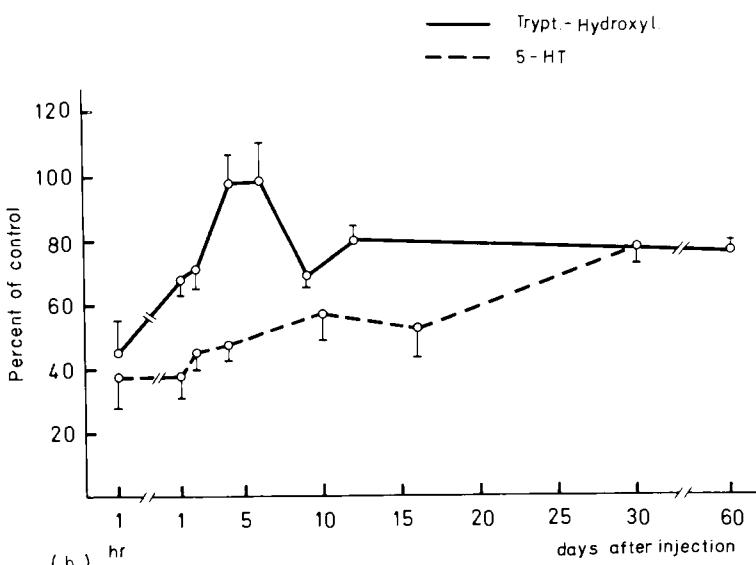
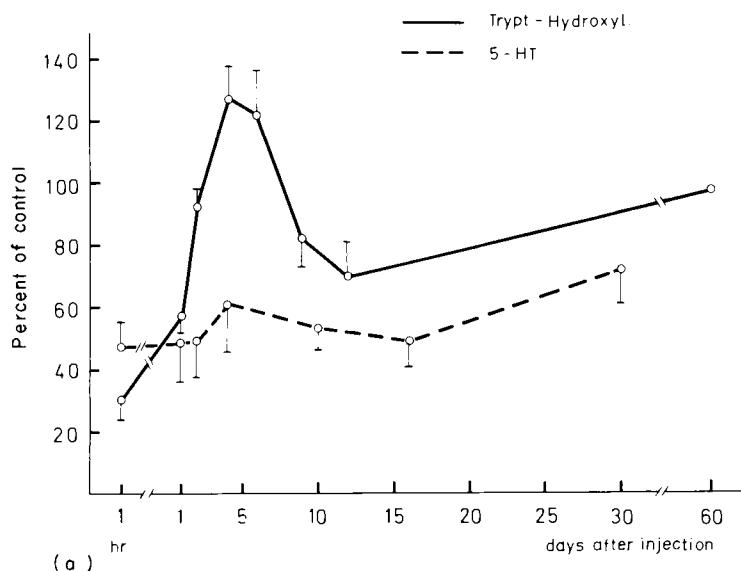


FIG. 8. Time-course effects of i.v.t. 5,6-DHT (75 µg) on regional 5-HT concentration and tryptophan hydroxylase activity in the rat: (a) septum; (b) hypothalamus. Bars give means ± SEM. [Based on data in Baumgarten *et al.* (1971) and Victor *et al.* (1974).]

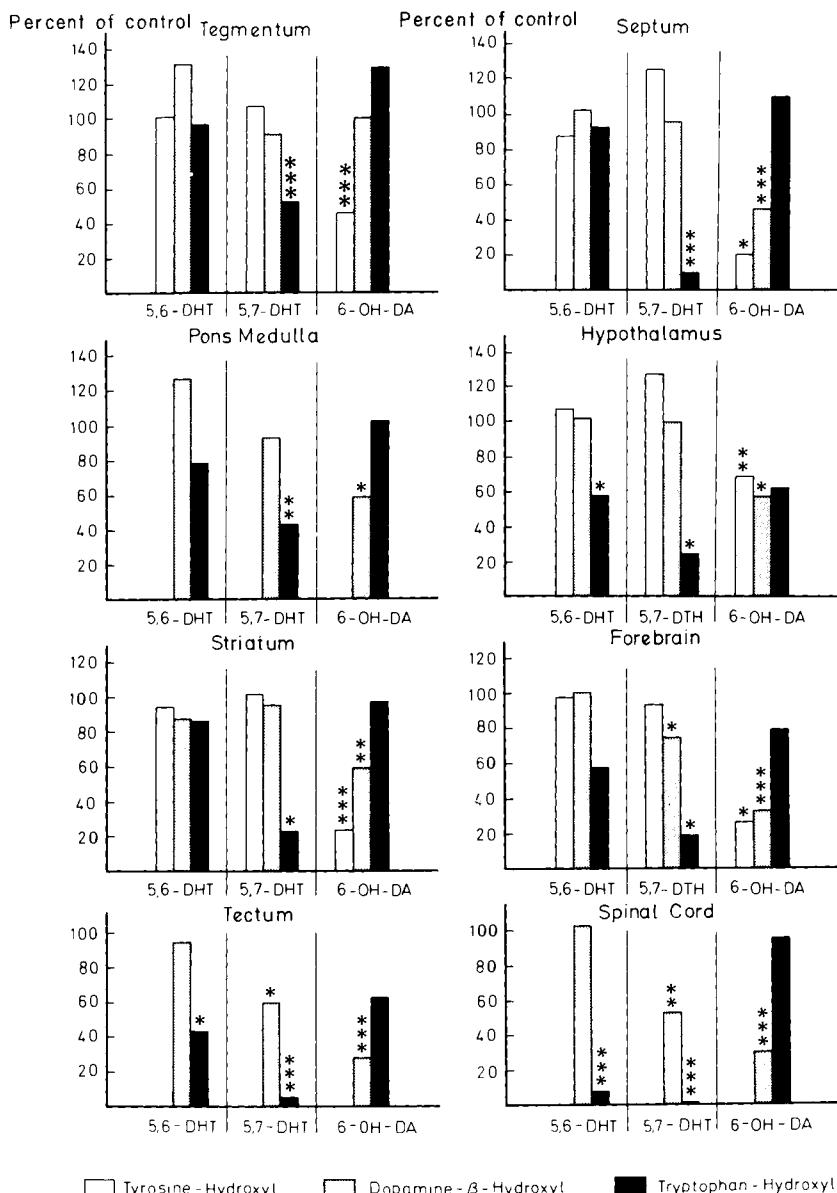


FIG. 9. Effects of i.v.t. 5,6-DHT (75 µg), 5,7-DHT (150 µg) and 6-OHDA (200 µg) on regional tyrosine-, dopamine- β -hydroxylase and tryptophan hydroxylase activity in the rat, 10 days after injection of the drugs. The columns represent the mean of four determinations. For significance levels, see Fig. 2. (Based on unpublished work by H. G. Baumgarten, S. J. Victor, E. Brunswick, and W. Lovenberg.)

to note that 5,6-DHT does not significantly affect DA- β -hydroxylase in the brain and spinal cord. At least in two regions, the hypothalamus and tectum, there is a tendency towards a decrease in tryptophan hydroxylase activity after 6-OHDA administration. At 10 days after injection, 5,6-DHT causes a profound depletion of tryptophan hydroxylase in the spinal cord, but its effects are only moderate or even nonsignificant in all brain regions. In harmony with the data just given, 5,7-DHT severely depletes tryptophan hydroxylase in all CNS regions.

Recently, Renaud *et al.* (1975) studied the effect of 50 μg of 5,6-DHT (i.c.) on the time-course characteristics of tyrosine hydroxylase in selected regions of the rat CNS. Both the cell body-containing noradrenergic region in the brainstem (locus coeruleus) and the terminal-rich frontal cortex revealed a significant transient increase in tyrosine hydroxylase; this is interpreted as signifying a loss of serotonergic inhibition of tyrosine hydroxylase synthesis in noradrenergic neurons of the locus coeruleus system. These results confirm the earlier observations of a rise in tyrosine hydroxylase activity in the pons-medulla (Baumgarten *et al.*, 1972a) and in the NA content of whole brain (Baumgarten *et al.*, 1971) following i.v.t. 5,6-DHT injection.

In summary, available data on monoamine enzymes following i.v.t. administration of 5,6- or 5,7-DHT confirm the selectivity of action of these neurotoxins on the central serotonergic and noradrenergic neurons. However, it must be noted that there are striking differences in the time course and extent of depletion of enzymes when compared to the respective transmitters. This is particularly evident with 5,6-DHT and is in accord with the partial and incomplete axotomy caused by i.v.t. injections of this drug.

VII. EFFECTS OF INTRAVENTRICULAR OR INTRACISTERNAL 5,6- OR 5,7-DHT ON BRAIN AND SPINAL CORD 5-HYDROXYINDOLEACETIC ACID

In adult rats treated with one i.v.t. injection of 50 μg 5,6-DHT, 5-hydroxyindoleacetic acid (5-HIAA) is in most regions not depleted to the same extent as 5-HT (e.g., at 4-5 days); this indicates an accelerated turnover of serotonin in the remaining intact terminals. After 2 months, when the recovery of 5-HT is evident, there is no longer any difference in the extent of residual depletion of 5-HT and 5-HIAA (Baumgarten *et al.*, 1972a). An accelerated turnover of newly formed [^3H]5-HT in 5,6-DHT-treated animals (i.c. injections) is evident in the lower brainstem plus spinal cord after 1 week (Gershon *et al.*, 1974), confirming the aforementioned results.

Following a high i.v.t. dose of 5,7-DHT (150 µg), 5-HT and 5-HIAA are both decreased to a comparable degree within the first month after treatment. By 2 months, recovery of 5-HT supersedes that of 5-HIAA in whole brain (Jacoby *et al.*, 1974), suggesting a decreased turnover in the lesioned 5-HT-containing neurons. Similar relationships are seen in the developing rat brain after treatment with i.c. 5,7-DHT at birth (Lytle *et al.*, 1975).

In summary, measurements of the 5-HIAA/5-HT ratio after administration of DHTs to adult or developing rats might reveal important changes in the turnover of 5-HT in the damaged serotonergic neurons. Available data suggest an increased turnover in the remaining neurons following partial damage by 5,6-DHT but a decreased turnover following more severe axotomy induced by high doses of 5,7-DHT.

VIII. EFFECTS OF INTRAVENTRICULAR INJECTIONS OF 5,6- OR 5,7-DHT ON THE MORPHOLOGY OF CENTRAL MONOAMINE NEURONS

A. Ultrastructural Observations

Ultrastructural evidence for the ability of 5,6- and 5,7-DHT to cause damage and degeneration of central serotonergic preterminal fibers and axon terminals has been presented (Baumgarten *et al.*, 1972b,c; Baumgarten and Lachenmayer, 1972a,b; Møllgard *et al.*, 1977). These studies have also disclosed that 5,6-DHT in high concentrations—such as are found in brain regions close to the injection cannula—may seriously damage the myelin sheath of axons and glial cells, including the ependyma. 5,7-DHT has been reported to be largely devoid of similar nonspecific cytotoxic effects when administered in comparable or even higher doses.

In agreement with histochemical observations (discussed next) on the nonselective damage of central CA-containing fibers by 5,6- and 5,7-DHT, both drugs have been found to cause ultrastructural signs of damage in peripheral noradrenergic axons (Baumgarten *et al.*, 1972d, 1974c).

B. Fluorescence Histochemical Observations

In the CNS, 5,6- and 5,7-DHT cause a characteristic sequence of changes in the fluorescence morphology of monoaminergic neurons (Baumgarten and Lachenmayer, 1972a,b, 1973; Baumgarten *et al.*, 1972c, 1973a, 1974a,b, 1975a; Björklund *et al.*, 1973a,b, 1975b; Nobin *et al.*, 1973). Immediately after injection into the lateral ventricle of adult rats,

many varicose terminal and nonterminal axons located in the periventricular or surface-bordering brain parenchyma are seen to have accumulated high amounts of fluorogenic substances most probably identical with the formaldehyde-induced fluorophores of 5,6- or 5,7-DHT (cf. Björklund *et al.*, 1974; Jonsson *et al.*, 1975). To judge from their topographical pattern of distribution, these 5,6- or 5,7-DHT-accumulating fibers correspond to monoaminergic axons and terminals. This uptake of 5,6- or 5,7-DHT into presumed monoaminergic fibers is followed by a partial long-lasting disappearance of fluorescent indoleamine and, to some extent, CA-containing fibers. This finding is compatible with a rapid degeneration of ventricle or superficial monoamine terminals. Careful fluorescence microscopic investigations indicate that 5,6-DHT (50 or 75 µg i.v.t.) is rather selective in that it reduces the number of indoleamine terminals but not of noradrenaline terminals and affects only those dopamine terminals of the head of the caudate nucleus which are located close to the lateral ventricle. 5,7-DHT (in doses up to 150 µg) causes both 5-HT and NA terminals to disappear, but there is as yet little evidence for a direct effect on DA terminal systems.

By 24 hr after injection of 5,6- or 5,7-DHT, 5-HT terminals can still be demonstrated in centrally located brain regions and in the spinal cord, provided the 5,7-DHT is administered in doses not higher than 75 µg. At this time, many nonterminal axons in the major ascending and descending serotonergic projections reveal brightly fluorescent indoleamine accumulations in variably sized local enlargements of the axons ("droplet swellings") and in the intervaricose thin neurite sections. In the 5,7-DHT-treated brain, similar changes are seen in the ascending NA projections and in the bulbospinal NA pathway. Recent observations (Wuttke *et al.*, 1977; Baumgarten *et al.*, 1977) indicate that 5,7-DHT (100 µg i.v.t., or higher doses) also causes very discrete lesions in some DA axons of the periventricular caudates and septum and of the olfactory tubercle.

With a latency of 2–4 days after injections of 50–75 µg of 5,6- or 5,7-DHT, additional 5-HT terminals disappear from the brain and spinal cord as a result of an anterograde disintegration process initiated by primary lesions to the nonterminal axon bundles. While the process of anterograde terminal degeneration is continuing, signs of sprouting appear at or close to the stumplike, damaged preterminal indoleamine axons, particularly in the lower brainstem. In 5,7-DHT-treated animals, similar axonal sprouting phenomena are noted at the damaged proximal stumps of the noradrenergic fibers (Björklund *et al.*, 1975b).

Regrowth of serotonergic and noradrenergic axons after 5,6- or 5,7-DHT-induced lesions has been analyzed in some detail in the bulbospinal 5-HT pathways and ascending noradrenergic projections (Nobin *et al.*,

1973; Baumgarten *et al.*, 1974a; Björklund *et al.*, 1975b; Björklund and Lindvall, unpublished observations). The regrowth process is very long lasting and results partly in an orderly regeneration of denervated regions and partly in an aberrant growth of newly formed axons into normally sparsely innervated or noninnervated regions of the brain. Some brain regions seem to be permanently deprived of their serotonergic and/or noradrenergic terminals. Since lesions in the descending bulbospinal pathways after 5,6- and 5,7-DHT occur close to the indoleaminergic perikarya in the medulla oblongata, some neurons undergo a slow retrograde degeneration. This is most prominent after high doses of 5,7-DHT, which may account for the less extensive regeneration seen after such treatment (Baumgarten *et al.*, 1974a). Limited regrowth of serotonin axons has also been observed in developing animals injected at birth with i.c. or i.p. 5,7-DHT (Baumgarten, unpublished observations; Sachs and Jonsson, 1975).

5,6- and 5,7-DHT are also capable of destroying peripheral noradrenergic fibers in the rat iris after local application of these drugs into the anterior eye chamber (Lachenmayer and Groth, 1973), or in various sympathetically innervated organs of the mouse after systemic (i.v.) administration of 5,7-DHT (Baumgarten *et al.*, 1974a). These results confirm observations on the limited selectivity of both drugs when applied to the CNS.

In summary, regional effects of i.v.t. or i.c. 5,6- or 5,7-DHT on central 5-HT-, NA-, or DA axons and terminals can be studied by fluorescence microscopic analysis using the Falck-Hillarp technique to visualize all three monoamines (Falck and Owman, 1965), or by the glyoxylic acid method to detect subtle changes in catecholaminergic fiber systems (Lindvall and Björklund, 1974). The latter procedure, which has not yet been applied extensively, should give a precise picture of the selectivity of 5,6- or 5,7-DHT-induced damage in the CNS. Electron microscopic investigations may be useful in revealing the degeneration of monoaminergic fibers and the nonspecific cytotoxic effects of the DHTs.

IX. EFFECTS OF INTRACEREBRAL INJECTIONS OF 5,6- AND 5,7-DHT IN THE ADULT RAT

The local, intracerebral application of neurotoxic drugs, introduced by Ungerstedt (1968, 1971) for lesioning of catecholaminergic systems by means of 6-OHDA, has several advantages when compared to injecting drugs into the cerebrospinal fluid (CSF).

Intracerebral administration of neurotoxins allows a more restricted and, under certain circumstances, more selective lesioning of axon bundles or denervation of individual brain regions. Intraparenchymal

localized injection may help to overcome the general cytotoxicity of 5,6-DHT and other neurotoxins, which is particularly evident and rather widespread with the intraventricular route of administration (Hedreen, 1975).

An important problem in the intracerebral injection technique is to control the extent of unspecific damage caused by the drug. The DHTs are potentially cytotoxic, and selective damage to monoamine systems is achieved only when the tissue concentration of the drug is so low that toxic levels are reached just within those tissue elements that actively concentrate the drug by means of the so-called membrane pump, i.e., the monamine neurons. To meet these conditions, the amount of drug and volume injected have to be titrated for each individual neurotoxic compound in order to obtain an optimum selectivity of the lesion.

As is true for all stereotaxic brain lesions, the nature, localization, and extent of chemical lesions must be checked microscopically. Biochemical controls alone can obviously be entirely misleading. Because the neurotoxic amines have no absolute specificity for the monoamine neurons, it seems likely that extensive damage to these systems is always accompanied by a certain degree of damage also to other brain elements that are exposed to high concentrations of the drug. Such nonspecific effects are particularly hazardous when the neurotoxic drugs are used for functional studies. For further discussion of these problems, see Sotelo *et al.* (1973), Agid *et al.* (1973), and Hökfelt and Ungerstedt (1973). In these papers, the morphological features of lesions produced by 6-OHDA injected locally into the brain substance are described and critically evaluated.

The characteristics of the lesion induced by an intracerebral or intraspinal injection of 5,6-DHT or 5,7-DHT (4 μg in 4 μl vehicle, or 2 μg in 2 μl) have been described by Björklund *et al.* (1973c). Just as is the case after an injection with 6-OHDA, three different zones can be distinguished around the injection site (Fig. 10*a,b*). Centrally, there is a zone of necrotic tissue (zone A), which is of approximately the same size (about 0.5 mm in diameter) after injection of 5,6-DHT, 5,7-DHT, or saline alone. Around the necrosis, there is a zone of apparently damaged tissue (zone B). This zone is about 0.1–0.3 mm wide after 5,7-DHT injection, and broader, about 0.5–1.0 mm, after 5,6-DHT injection. Outside this zone is the area of selective axonal damage. This area is partly pigmented (zone C in Fig. 10*a*), particularly after 5,6-DHT. In their injections into the mesencephalon and the spinal cord, Björklund *et al.* (1973c) estimated the area of selective axotomy in the indoleamine systems to extend approximately 1–1.5 mm outside the area of general tissue damage (zones A plus B). These observations thus indicate that 5,7-DHT is the compound causing

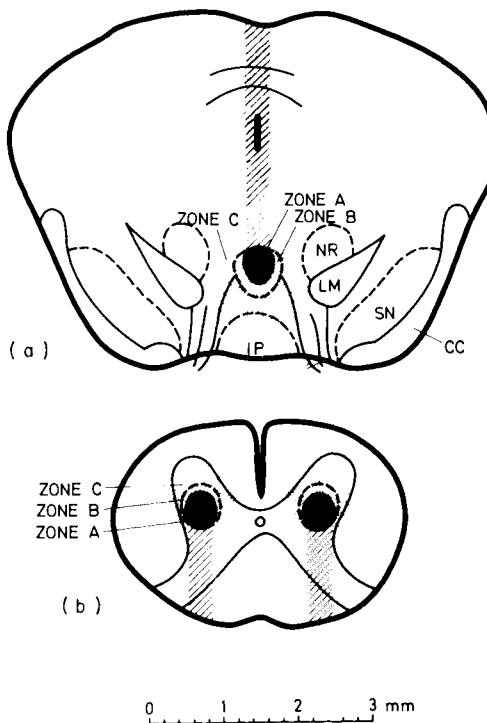


FIG. 10. (a) Position of and tissue damage caused by intracerebral injection of 5,7-DHT (4 µg in 4 µl) in the midline of the ventromedial tegmentum at the level of the emergence of the oculomotor nerve: Zone A, central necrotic zone; zone B, zone of apparently damaged tissue; zone C, pigmented but largely intact tissue. Abbreviations: CC, crus cerebri; IP, interpeduncular nucleus; LM, lemniscus medialis; NR, nucleus ruber; SN, substantia nigra. Cross-hatched area indicates needle track. (b) Position of and tissue damage caused by intraspinal injections of 5,7-DHT into the cervical spinal cord. Each injection was 2 µg in 2 µl. Zones as indicated in (a). Cross-hatched areas represent the needle tracks. (From Björklund *et al.*, 1973c.)

the least direct damage to the tissue. Interestingly, the extent of damage caused by 5,6-DHT is similar to that reported by Sotelo *et al.* (1973) for 6-OHDA.

From studies employing i.v.t. injections of the DHTs (Nobin *et al.*, 1973; Baumgarten *et al.*, 1975a) it seems clear that the axons of the 5-HT neurons are much more sensitive to the toxic actions of the drugs than are the cell bodies. One should therefore choose to place the injections either close to a 5-HT axon pathway or within a terminal region. Results of such injections have been published by Björklund *et al.* (1973c), Hole *et al.* (1976), and Lorens *et al.* (1975), i.e., injections into the major ascending

indoleamine pathways in the ventromedial mesencephalic tegmentum and into the descending bulbospinal indoleamine system, and by Saner *et al.* (1974), i.e., injections into the medial forebrain bundle; both studies demonstrated extensive destruction of the serotonergic input to the rat forebrain. In the experiments of Daly *et al.* (1973) using injections close to the indoleamine cell bodies in the mesencephalon, the denervating effect was clearly less than in the studies in which 5,6-DHT or 5,7-DHT was injected into axon bundles.

An injection of 5,6-DHT or 5,7-DHT (4 µg in 4 µl vehicle) into the ventral mesencephalic tegmentum, at the level of the emergence of the oculomotor nerve (Fig. 10a) produces a very efficient destruction of the ascending 5-HT fiber system (Björklund *et al.*, 1973c). 5,7-DHT, which is more efficient, causes an 85% reduction in the forebrain level of 5-HT and a 90–94% reduction in [³H]5-HT uptake in cortex and hypothalamus. The reduction produced by 5,6-DHT (75% of the 5-HT in the forebrain and 50–70% in [³H]5-HT uptake) is clearly greater than previously obtained with i.v.t. injections (35–55% reduction in 5-HT and 50–53% reduction in [³H]5-HT uptake in different forebrain regions after 75 µg 5,6-DHT); see Baumgarten *et al.* (1971) and Björklund *et al.* (1973a). Saner *et al.* (1974) obtained a 50–55% reduction of 5-HT in the ipsilateral diencephalon and telencephalon after an injection of 2–10 µg 5,6-DHT in 10 µl into the medial forebrain bundle in the lateral hypothalamus. In the spinal cord, a bilateral injection of 5,7-DHT (2 µg in 2 µl on each side) caused an 80% reduction in 5-HT and a 70–94% reduction in [³H]5-HT uptake in the spinal cord portion below the injections.

Although 5-HT axons are the ones most severely damaged by 5,6- or 5,7-DHT injections, neither compound is totally selective for the serotonin neuron systems. Thus, intracerebral or intraspinal 5,7-DHT injections cause significant lesions to the noradrenergic but not the dopaminergic axon systems (Björklund *et al.*, 1973c). 5,6-DHT also lesions DA axons, whereas NA axons seem to escape damage after injections into the medial forebrain bundle or into the ventromedial mesencephalic tegmentum (Daly *et al.*, 1973; Saner *et al.*, 1974; Björklund *et al.*, 1973c). In fact, Saner *et al.* (1974) have shown that 5,6-DHT, injected intracerebrally, can be used for quite efficient lesioning of the DA pathways: one injection of 10 µg of 5,6-DHT into the nigro-neostriatal dopaminergic axon bundles causes an 85% reduction in striatal DA on the injection side. These nonselective actions of 5,7-DHT and 5,6-DHT on the catecholaminergic systems obviously limits their usefulness for selective serotonin lesions in the brain.

It seems quite possible to overcome this obstacle, however, first by selecting a site of injection distant from the major NA or DA pathways. This has been achieved by Lorens *et al.* (1975), who placed the 5,7-DHT

injections in the mesencephalic raphe: with such lesions they report substantial reductions in forebrain 5-HT without any significant drop in NA levels. It seems likely that a similar placement of the 5,6-DHT injection would avoid damage to the major ascending DA systems. Secondly, it might be possible to improve the selectivity for 5-HT neurons with the aid of uptake blockers. Thus, it might be possible—just as is the case after i.v.t. or i.c. injections—to protect the NA neurons from damage by 5,7-DHT through pretreatment of the animal with NA uptake blockers (cf. Hole *et al.*, 1976) or with MAO inhibitors (cf. Section XIV). It should be emphasized, however, that the potential efficiency of this protection has yet to be evaluated for intracerebral injections. Still to be examined also is the dispersion of a large microinjection volume of 2 to 10 μl of neurotoxin (see Myers, 1974, Chapter 2).

X. AFFINITY TO MONOAMINERGIC UPTAKE SITES AS A DETERMINANT OF SELECTIVITY OF ACTION OF NEUROTOXIC TRYPTAMINES

The rationale for the development of drugs acting selectively on monoaminergic neurons is to establish structural configurations as close to the transmitter molecule as possible in order to render them substrates for the selective high-affinity transport mechanism of serotonergic, noradrenergic, or dopaminergic neurons. Both 5,6- and 5,7-DHT fulfill this criterion, since their affinity for the 5-HT uptake sites in brain (IC_{50} values: $6.0 \times 10^{-7} M$ and $4.0 \times 10^{-6} M$, respectively) is rather high and, in the case of 5,6-DHT, close to that of 5-HT itself ($2.0 \times 10^{-7} M$) (cf. Shaskan and Snyder, 1970; Horn, 1973; Horn *et al.*, 1973; Baumgarten *et al.*, 1974b; Björklund *et al.*, 1975c; Baumgarten and Björklund, 1976). Both drugs do, however, compete for uptake into catecholaminergic neurons, although with less efficiency. This affinity to the CA-transport sites is at least partly responsible for the limited selectivity of action of 5,6- and 5,7-DHT on central 5-HT neurons. As will be outlined later (Section XIV), the transport of 5,7-DHT into noradrenergic neurons can be counteracted by pretreatment of animals with blockers of the NA transport mechanism such as DMI without notably affecting their affinity to the transport mechanism of central 5-HT neurons.

XI. UPTAKE IMPAIRMENT AS A DETERMINANT OF THE NEUROTOXIC POTENCY OF TRYPTAMINE ANALOGS

In order to obtain a quantitative measure of the ability of neurotoxic transmitter analogs to impair the membrane-bound monoamine uptake mechanism [known to be an early and reliable sign of damage in

monoaminergic axons (cf. Jonsson and Sachs, 1970)], an *in vitro* test system was designed by Björklund *et al.* (1975c).

Brain slices were exposed to the DHTs for 30–60 min, rinsed in buffer for 20 min, and then incubated for 10 min in buffer containing $0.5 \times 10^{-7} M$ [3H]5-HT or $1.0 \times 10^{-7} M$ [3H]NA. The results obtained with this method indicate that exposure to $10^{-6} M$ (but not $10^{-7} M$) 5,6- or 5,7-DHT for 30 min causes more than 50% reduction of [3H]5-HT uptake. Raising the concentration of the toxins to $10^{-5} M$ or prolonging the exposure time to 1 hr has little potentiating effect on the impairment of [3H]5-HT uptake by either compound. 5,6-DHT, in concentrations ranging from 10^{-6} to $10^{-4} M$, has no effect on [3H]NA uptake, but 10^{-6} or $10^{-4} M$ 5,7-DHT impairs [3H]NA uptake, although not as efficiently as it does [3H]5-HT uptake.

This test system thus reflects the *in vivo* specificity and neurotoxic potency of both drugs. The results also demonstrate that the axonal degeneration induced by the neurotoxic indoleamines is rapid in onset and, at least partly, results from direct action of the drugs on monoaminergic axon terminals. Interestingly, MAO inhibition prior to or during preincubation of brain slices with 5,6- or 5,7-DHT counteracts the impairment of [3H]5-HT uptake (Björklund *et al.*, 1975c).

XII. REDUCTION OF [3H]AMINE UPTAKE AS A MEASURE OF TERMINAL DEGENERATION

Measurements of the rate of uptake of [3H]5-HT into brain slices *in vitro* (Björklund *et al.*, 1973a,c; Baumgarten *et al.*, 1973a, 1975a) have shown that 5,6-DHT has regionally different and rather selective decreasing effects on [3H]5-HT uptake in the rat brain and that 5,7-DHT reduces both regional 5-HT and NA uptake. These measurements were made 10 days after the i.v.t. injection of 75 μg 5,6-DHT or 200 μg 5,7-DHT in the adult rat. A comparison of changes in [3H]5-HT and [3H]NA uptake in the brain with changes in endogenous transmitter content reveals a good correlation between these two parameters and suggests that both are, with certain restrictions, good indicators of the degree of axonal degeneration in the monoaminergic systems. The [3H]NA uptake occurs both in NA and DA axons (see Iversen, 1975), and thus, in regions having NA and DA terminals, the reduction in [3H]NA uptake will reflect degeneration in either or both types of neurons.

In summary, measurements of [3H]5-HT and [3H]NA uptake into brain slices or homogenates of brain regions obtained from animals treated with 5,6- or 5,7-DHT are good measures of the extent of degeneration of serotonergic and noradrenergic terminals caused by these agents in

the brain, provided that the [³H]NA assays are performed on CNS regions having little or no innervation by dopaminergic axons or that the assays of [³H]NA are carried out in the presence of drugs selectively inhibiting the membrane pump of dopaminergic neurons.

XIII. DISTRIBUTION OF [¹⁴C]5,6-DHT IN BRAIN AFTER INTRAVENTRICULAR INJECTION³

Distribution of [¹⁴C]5,6-DHT in the brain was analyzed autoradiographically after i.v.t. injection of 75 µg of the labeled drug. For retention of the label in the brain, vascular perfusion of 4% formaldehyde (using neutralized formaldehyde 40%, diluted 1:9 in 0.9% NaCl) was employed. The radioactivity retained by this fixation method can be considered to represent firmly bound 5,6-DHT or its metabolites (e.g., unchanged 5,6-DHT accumulated in amine storage vesicles of monoaminergic neurons or protein-linked radioactivity derived from covalently bound quinoid oxidation products).

By 1 hr after drug injection, most of the radioactivity is confined to the structures near to the ventricle of the telencephalon, the head and body of the caudate nucleus (Fig. 11a) having by far the highest number of grains. The nucleus accumbens, septi and supracommissural parts of the bed nucleus of the stria terminalis are less intensely labeled, as are the lateral septal nucleus and superficial layer of the hippocampus. In all these structures, the density of silver grains is higher on the side of drug injection than in the contralateral hemisphere. Only a few grains are detected over structures known to carry nonterminal fibers of monoaminergic pathways, such as the internal capsule or the cingulum. There are few or no grains randomly superimposed upon telencephalic periventricular structures known to have few monoaminergic fibers, such as the corpus callosum or anterior commissure. Heavily labeled structures in the remaining CNS include the periventricular hypothalamus, the midbrain periventricular gray matter, and, in particular, some perikarya of the nucleus dorsalis raphe (Fig. 11b), and the periventricular gray matter bordering on the fourth ventricle. High numbers of seemingly randomly distributed grains are noted over structures of the lower brainstem and cerebellum facing the lateral openings of the fourth ventricle.

Significant amounts of label are also incorporated into certain structures of the brain and spinal cord, such as the diagonal band of Broca, the tuberculum olfactoriun, median eminence, the superficial layers of the

³ Data in this section are based on work of L. Lachenmayer, J. Maas, and H. G. Baumgarten (unpublished observations).

superior and inferior colliculi, parts of the occipital cortex, the substantia nigra and interpeduncular nucleus, the perikarya and neuropil of the nucleus raphe pallidus, and, finally, superficial punctate structures in the anterolateral and posterior funiculus of the cervical spinal cord, and the most posterior laminae of the dorsal horn gray matter at the same level of the cord.

In general, this pattern of distribution of grains is retained up to 48 hr after drug injection, but there is an increase in the number of grains confined to periventricular structures in the forebrain, known to have significant numbers of nonterminal 5-HT axons, such as the cingulum (Fig. 11c) and medial forebrain bundle, and a mild decrease in the number of grains confined to areas rich in monoaminergic terminals. After 2 days, an intense labeling of large cells in the pia-arachnoid is noted, and there are signs of nonspecific tissue damage in the head of the left and right caudate and in the left septum.

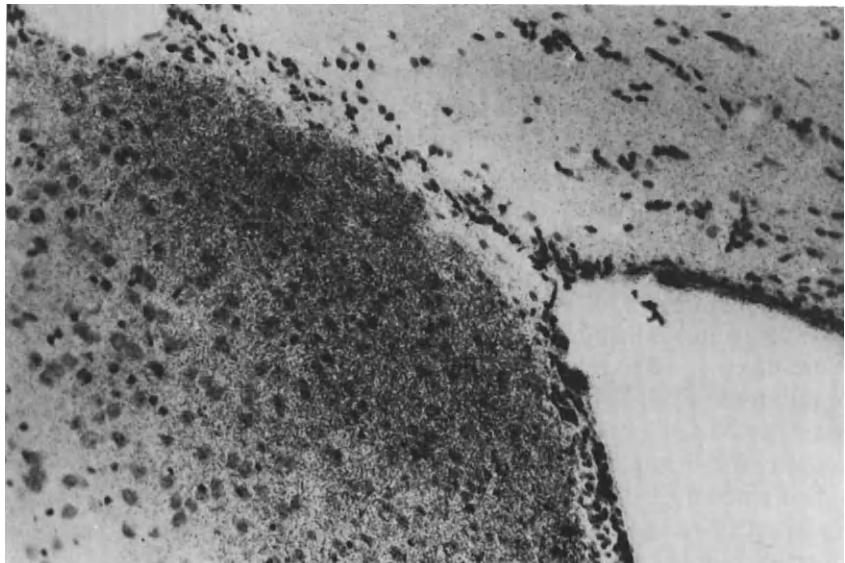
The pattern of distribution of silver grains suggests that 5,6-DHT is preferentially taken up into monoaminergic terminals and axons. Since the exact number and localization of 5-HT terminals is not yet known, it is impossible to estimate how many grains over a given region reflect accumulation of [¹⁴C]5,6-DHT in 5-HT fibers and how many are accounted for by nonselective uptake into dopaminergic or noradrenergic axons. Intense labeling of the caudate is, however, compatible with the efficient nonselective uptake of 5,6-DHT into DA terminals, part of which is always degenerated by i.v.t. 5,6-DHT (Björklund *et al.*, 1973a). The present data also demonstrate that penetration of the drug from the ventricles and subarachnoid space into the brain parenchyma is, in fact, rather limited.

XIV. EFFECTS OF DRUGS ON THE TOXIC ACTIONS OF 5,6-OR 5,7-DHT IN THE ADULT RAT

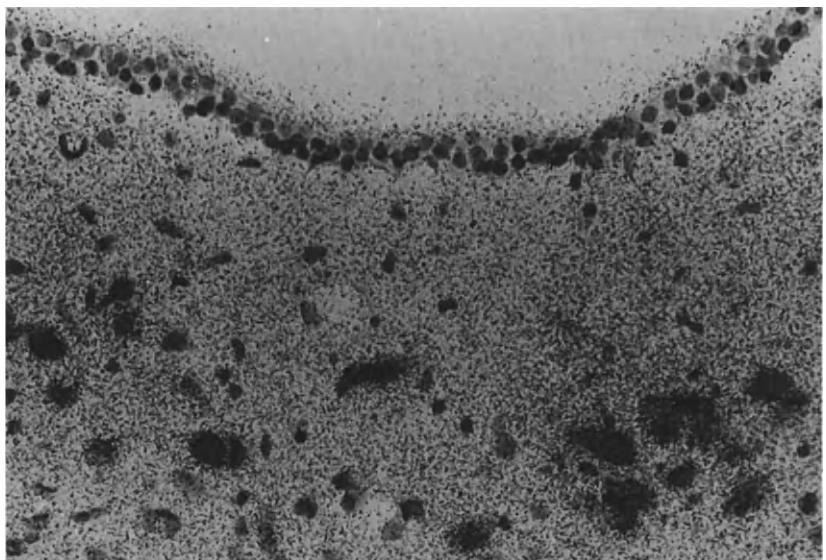
A. Desmethylimipramine (DMI)

The toxic actions of 6-OHDA on central noradrenergic neurons can be attenuated or even blocked by pretreatment of animals with DMI or related drugs, known to inhibit the monoamine transport mechanism of such neurons (see Section II). With this mode of pretreatment, the toxicity of 6-OHDA can, in fact, be rendered fairly specific for dopaminergic neurons.

Several groups of authors have attempted to improve the specificity of action of 5,7-DHT on 5-HT neurons by inhibiting the transport of this compound into noradrenergic neurons by DMI pretreatment (Gershon *et*

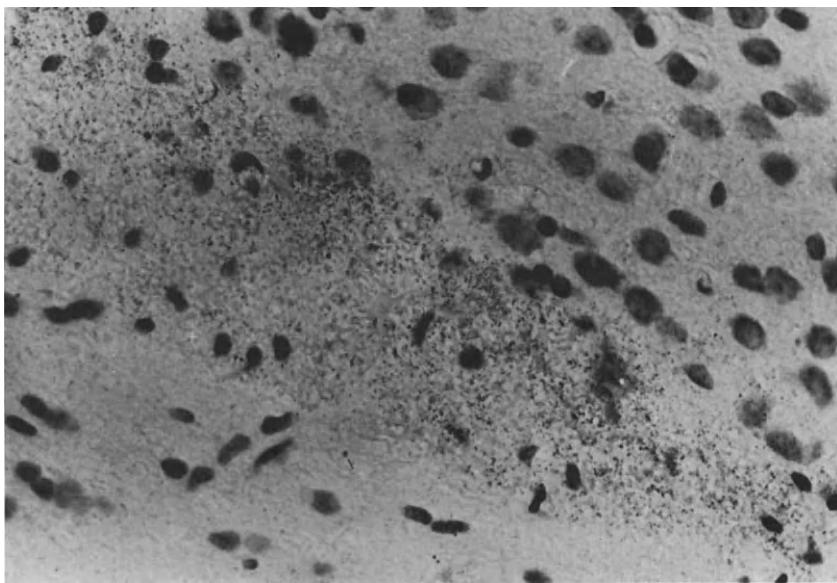


(a)



(b)

FIG. 11(a,b).



(c)

FIG. 11. Radioautograms of transversely cut sections (6 μm) from rat brain after i.v.t. injection of [^{14}C]5,6-DHT (3.14 $\mu\text{Ci}/\mu\text{mol}$): (a) grains over cells and neuropil of the head of the left caudate (injection side), 1 hr after injection of the labeled drug; (b) grains over neuropil and perikarya of the nucleus raphe dorsalis, 1 hr after [^{14}C]5,6-DHT; (c) grains over fibers of the cingulum (level of anterior septum), 24 hr after [^{14}C]5,6-DHT.

al., 1974; Björklund *et al.*, 1975a; Gershon and Baldessarini, 1975; Breese and Cooper, 1975). As shown by Björklund *et al.* (1975a), one intraperitoneal (i.p.) injection of 25 mg/kg DMI, given 45–60 min before i.v.t. injection of 150 μg 5,7-DHT, prevents the depletion of NA produced by 5,7-DHT in all brain regions analyzed after 10 days, whereas the 5,7-DHT-induced reduction in 5-HT content is not affected. However, the uptake of [^3H]NA in slices from rat spinal cord (8–14 days after injection of 5,7-DHT) was found to be significantly reduced by about 20% despite DMI pretreatment, and the uptake of [^3H]NA into slices of medulla oblongata showed a tendency towards supranormal levels. This indicates that even with DMI pretreatment there is minor damage to part of the noradrenergic system (cf. also Gershon *et al.*, 1974). In subsequent studies, Wuttke *et al.*, 1977 and Baumgarten *et al.* (1977) have, therefore, employed a lower i.v.t. dose of 5,7-DHT (100 μg free base) to obtain an even more selective action on central 5-HT neurons. As shown in Fig. 12, DMI pretreatment (25 mg/kg i.p.) appears to protect the central norad-

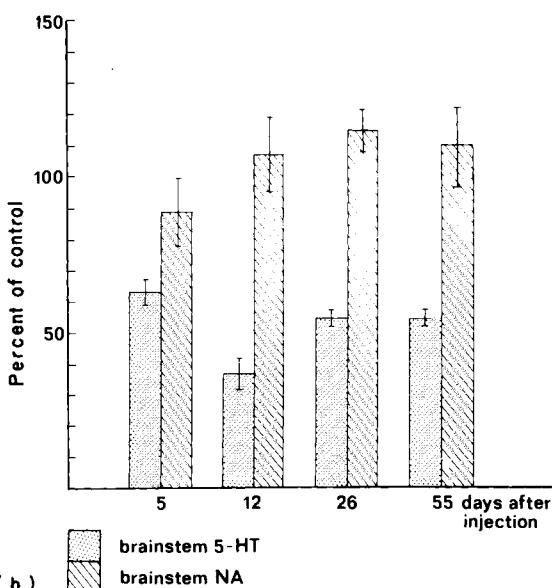
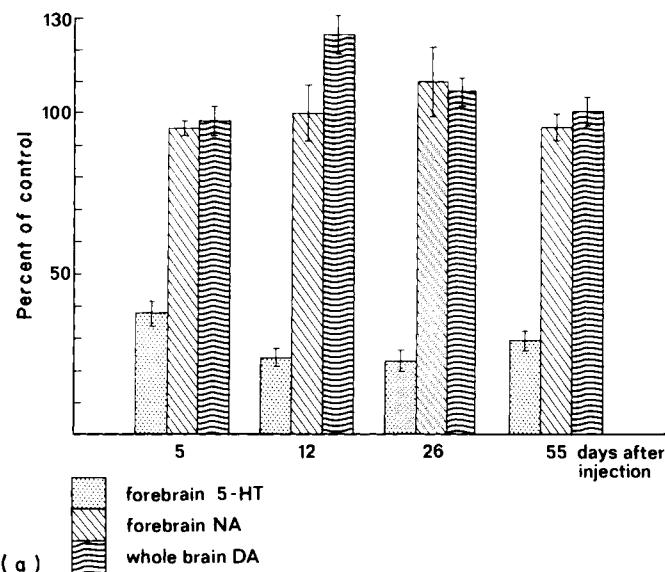


FIG. 12. Time-course effects of i.v.t. 5,7-DHT (100 µg), given to DMI-pretreated adult male rats (25 mg/kg i.p., 1 hr before 5,7-DHT), on forebrain NA and 5-HT and whole-brain DA concentrations (a) and on brainstem NA and 5-HT concentrations (b). Bars give means \pm SEM. (Based on data from Wuttke *et al.*, 1977.)

renergic neurons against damage by 5,7-DHT, and there is no evidence for any decrease in whole-brain DA content 5–55 days after injection. Gershon and Baldessarini (1975) report that in the cerebral cortex of adult rats a high dose of 5,7-DHT (200 µg free base), given i.c., produces a 52% fall in NA and a 72% decrease in 5-HT 10 days after injection; two successive high doses of DMI (25 mg/kg i.p.) given 4 and 1 hr before the 5,7-DHT injection, prevent the depletion of cortical NA, but not of 5-HT. However, time-course studies on individual brain regions would be required to prove that the central noradrenergic neurons are completely protected from damage by this treatment. Additional information is, however, provided by Gershon and Baldessarini (1975): the initial uptake of DA, choline, γ -amino-butyric acid (GABA), glutamic acid, and glycine is unaltered by 5,7-DHT combined with DMI, pointing to a low degree of long-term nonselective toxic effects after this treatment.

B. MAO Inhibition

Recent studies by Breese and Cooper (1975) reveal that MAO inhibition prior to an i.c. injection of 5,7-DHT in adult rats provides an equally effective means of protecting the central noradrenergic neurons against damage by 5,7-DHT. However, it remains to be demonstrated that this protection is complete, and so far no time-course study has been performed. In our *in vitro* test system for evaluating the neurotoxic potency of DHTs (cf. Section XI), MAO inhibition, prior to and during preincubation of rat cortical slices with 5,6- or 5,7-DHT counteracts the impairment of [³H]NA uptake produced by 5,6- and 5,7-DHT (Björklund *et al.*, 1975c). Furthermore, the α -methylated analog of 5,7-DHT (which is MAO resistant) appears to enhance [³H]NA uptake at concentrations at which 5,7-DHT reduces [³H]NA uptake ($10^{-6} M$). It must be noted, however, that α -methylation of 5,7-DHT also attenuates the impairment of [³H]5-HT uptake by 5,7-DHT, although not to the same degree as that of [³H]NA uptake. Considering that α -methyl-5,6-DHT has a markedly lower neurotoxic potency than 5,6-DHT, both *in vivo* and *in vitro*, it is remarkable that there is a small but significant enhancement of the long-term 5-HT depletion *in vivo* in adult rats treated with MAO inhibitors prior to the i.c. administration of 5,6-DHT (cf. Baumgarten *et al.*, 1975a; Björklund *et al.*, 1975c; Breese *et al.*, 1974a). This discrepancy could perhaps be explained by differences in the fate of 5,6-DHT and 5,7-DHT *in vivo* (cf. Section XVI). Application of i.v.t. or i.c. 5,6-DHT to adult rats pretreated with MAO inhibitors provokes acute behavioral disturbances which cause a high incidence of deaths among the treated animals (Baumgarten and Lachenmayer, 1972b; Breese *et al.*, 1974a). This points to a

dramatic enhancement of the nonspecific cytotoxicity of 5,6-DHT and renders this treatment obsolete.

C. Anesthetics

While the type of anesthetic used (ether or barbiturate) has not been found so far to influence the selectivity and potency of 5,7-DHT, there is published evidence to support the idea that sodium pentobarbital might weaken the long-term serotonin-depleting capacity of 5,6-DHT and increase the risk of nonselective toxic side effects (cf. Baumgarten *et al.*, 1972a).

To summarize, then, DMI pretreatment is an effective means of counteracting the selective toxicity of i.v.t. or i.c. 5,7-DHT on central noradrenergic neurons, as is treatment of animals with MAO inhibitors prior to i.c. 5,7-DHT. Both of these treatments do not decrease the long-term 5-HT-depleting capacity of 5,7-DHT. In order to obtain full protection with either mode of pretreatment, doses of the protecting agents and of the neurotoxin have to be well adjusted. The evaluation of the degree of protection afforded by treatment with DMI or MAO inhibitors requires a careful time-course analysis of regional amine content and/or [³H]amine uptake.

Ether anesthesia gives better amine depletion and less postoperative complications than do barbiturates when 5,6- or 5,7-DHT are administered i.v.t. or i.c. For injections of 5,7-DHT, short-acting barbiturates may be used. In MAO inhibited animals, ether is preferable to a short-acting barbiturate, the narcotic action of which is seriously prolonged under the influence of MAO inhibitors.

XV. INTRACISTERNALE, INTRAVENTRICULAR, OR PERIPHERAL ADMINISTRATION OF 5,6- OR 5,7-DHT IN THE DEVELOPING RAT

A. Effects on Monoamine Content

In studies by Breese *et al.* (1974a), 40 µg i.c. 5,6-DHT were found to be extremely toxic in newborn rats (50% mortality within 4 hr); 5-HT was reduced by 31% after 1 month in the brain of the surviving rats. A slightly better depletion of regional 5-HT levels in the brain and less mortality were obtained by Krieger (1975) in rats injected at birth with 25 µg i.v.t. 5,6-DHT. Signs of nonspecific toxic damage were prominent in the brains of most of the 5,6-DHT-injected animals: edema, ventricular dilatation, or hydrocephalus. Sachs and Jonsson (1975) failed to see reductions in [³H]5-HT uptake in brain regions taken from rats injected subcutaneously

(s.c.) with different doses of 5,6-DHT. Again, few animals survived the fourth postinjection day. 5,6-DHT must therefore be regarded as a highly nonspecific and toxic drug in newborn rats.

As revealed in studies by Lytle *et al.* (1975) and Breese and Cooper (1975), 5,7-DHT given i.c. is reasonably well tolerated by newborn rats even when injected in higher doses (50–100 µg). As little as 12.5 µg (free base) 5,7-DHT given to neonates produce a near 40% decrease in whole-brain 5-HT content by 20 days after birth; the extent of 5-HT depletion, expressed as percentage of control levels, remains unchanged until adulthood (at day 240). Dose-related increases in the extent of 5-HT depletion are evident for up to 50 µg 5,7-DHT. While there is a negligible mortality rate in the animals having received 12.5 or 25 µg 5,7-DHT (no difference from vehicle), the death rate increases sharply in those treated with 50 µg. By 240 days, brain NA is decreased by 20–30%, disregarding the dose administered. Brain tryptophan and DA content are not influenced by the treatment (Lytle *et al.*, 1975).

When administered i.c. on day 7 or 14 after birth, 100 µg 5,7-DHT produce a 65% or 54% reduction, respectively, in brain 5-HT content 80 days after birth. This degree of 5-HT depletion in the brain is not clearly better than the aforementioned decrease obtained in the brain of animals given 25 µg 5,7-DHT at birth. The percentage of reduction in brain NA obtained by Breese and Cooper (1975) is similar to that found by Lytle *et al.* (1975).

In the cortex and pons-medulla of rats assayed at 8 weeks of age, in those injected at birth with two s.c. injections of 100 mg/kg 5,7-DHT (given 24 hr apart), 5-HT is decreased to 57% of the control and increased to 137% of the control, respectively (Sachs and Jonsson, 1975).

B. Effects on Biosynthetic Enzymes

Only one study is available in the literature dealing with the effects of i.c. injection of 5,7-DHT on tryptophan and tyrosine hydroxylase in the developing rat (Baumgarten *et al.*, 1975b). In control animals, tryptophan hydroxylase activity rises dramatically after birth in the cell-body-rich midbrain and reaches near-adult levels by 22 days. A multifold increase in the activity levels of this enzyme is also noted in the hypothalamus, but here the peak activity is not reached until 42 days after birth, possibly signifying a continuing axonal arborization and terminal outgrowth during this period. Forty micrograms 5,7-DHT, given i.c. on day 2 after birth, produce a drop in the tryptophan hydroxylase activity, down to almost undetectable levels in the spinal cord, pons-medulla, midbrain, hypothalamus, and the rest of the forebrain. With the exception of the spinal

cord and forebrain, activity recovers to 11–22% of control by 42 days after injection. Tyrosine hydroxylase in the forebrain after 5,7-DHT treatment showed a temporary mild drop, evident only on days 6 and 12 after injection. No significant change in tyrosine hydroxylase was found in the midbrain, which contains most of the dopaminergic perikarya in the rat brain.

The treated animals had long-lasting deficits in weight gain; they showed almost permanent behavioral abnormalities for up to 1 year; and the mortality rate approached 30% in the 5,7-DHT treated group of animals. It seems clear, therefore, that 40 µg i.c. 5,7-DHT is beyond the dose limits at which this drug is free from generally cytotoxic actions in the newborn. The ideal i.c. dosage should be between 25 and 40 µg per animal.

C. Effects on [³H]Amine Uptake

Sachs and Jonsson (1975) have recently reported that 5,7-DHT can be administered peripherally to newborn rats in rather high concentrations, up to 200 mg/kg s.c. Near-maximal effects on 5-HT content and [³H]5-HT uptake are accomplished with repeated application of 5,7-DHT at lower dose levels, 2 × 50–100 mg/kg, given s.c., 24 hr apart. Time-course studies on [³H]5-HT uptake in three selected regions of the rat CNS following 2 × 50 mg/kg s.c. 5,7-DHT, given on the day of birth and 24 hr later, demonstrate an inverse relationship between the terminal-rich cerebral cortex-spinal cord and cell-body-rich pons-medulla plus caudal mesencephalon, with decreases in 5-HT uptake in the former and increases in the latter. By 56 days of age, [³H]5-HT uptake is still elevated in the pons-medulla to 150% of control and almost unchanged in the cerebral cortex between 10 and 20% of control. In the spinal cord, recovery of the temporarily severely depressed 5-HT uptake to 10% that of controls is evident at 14 days of age, and 50% that of controls at 56 days.

DMI (20 mg/kg s.c.) given before 5,7-DHT, 2 × 100 mg/kg s.c., prevents the decrease in [³H]NA uptake provoked by 5,7-DHT in cortex and brainstem but does not antagonize its lowering effects on [³H]5-HT uptake in cortex. The retarded increase of [³H]5-HT uptake in the pons-medulla seen after 5,7-DHT is attenuated but not totally prevented by DMI pretreatment.

A number of questions remain to be answered. What is the long-term fate of [³H]5-HT uptake in various regions after this treatment? Does DMI counteract the effect of 5,7-DHT in the peripheral nervous system of newborn rats? At a certain dose level, are there signs of nonspecific

damage, both centrally and/or peripherally, by 5,7-DHT? Which dose of DMI can be considered devoid of long-term undesired side effects in newborn rats? In all probability, the s.c. route of administration is less traumatizing than the intracranial one and should therefore be explored in more detail in the future.

D. Effects of Drugs on Monoamine Content

As seen in Fig. 13a,b, DMI, given 1 hr before i.c. administration of 5,7-DHT to newborn rats, prevents the depletion of whole-brain NA content noted in animals treated with 5,7-DHT alone. The DMI-mediated protection of the central noradrenergic system against damage by 5,7-DHT is effective on days 12 and 30 after birth [Fig. 13a,b (D. Pettibone and L. D. Lytle, unpublished observations)]. The DMI treatment by itself slightly depresses brain NA and DA content, but not 5-HT content, at 30

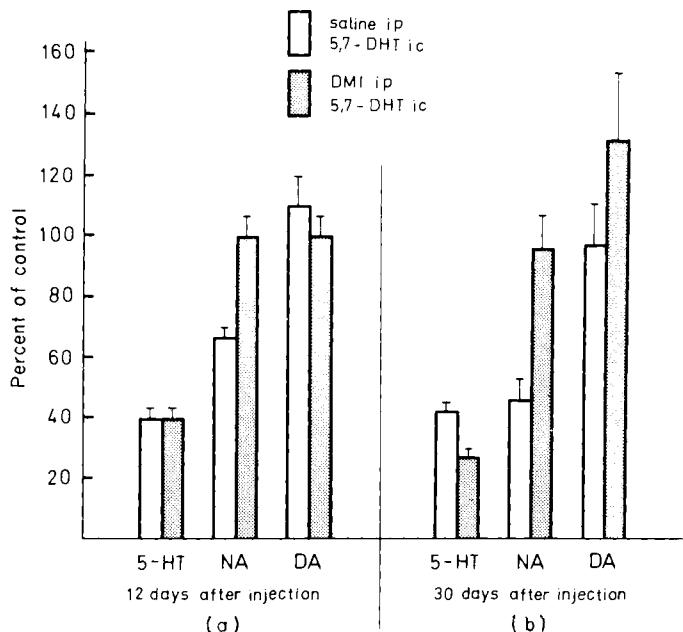


FIG. 13. Effects of i.c. 5,7-DHT (50 µg) or DMI (20 mg/kg i.p.) given 45 min before 50 µg i.c. 5,7-DHT, administered to 2-day old rats, on whole-brain 5-HT, DA, and NA content, assayed (a) 10 and (b) 28 days after drug injection. Bars give means ± SEM. (Based on unpublished data of D. Pettibone and L. D. Lytle.)

days of age. In the absence of uptake and turnover data, whether or not this small reduction in brain CA levels by the tricyclic antidepressants reflects nonspecific damage exerted upon the central catecholaminergic neurons is unknown.

The results presented by Breese and Cooper (1975) are in line with the data just given and reveal that DMI is capable of antagonizing the NA depletion by even higher i.c. doses of 5,7-DHT, i.e., 75 or 100 µg; however, it must be pointed out that these amounts of 5,7-DHT are too high since the mortality rate was rather elevated, especially among rats injected 7 days after birth. DMI also fails to alter significantly the weight gain deficits in 5,7-DHT-injected animals, confirming the notion of nonspecific toxic effects of this treatment. A very important observation by Breese and Cooper (1975) is that, despite the use of high doses of 5,7-DHT, pargyline is capable of blocking NA depletion and of positively influencing both the mortality rate and growth retardation. Provided that this holds true also in time-course experiments, this mode of drug treatment would be the method of choice for selectively decreasing brain 5-HT in the newborn rat. It remains to be shown whether rats younger than 7 or 14 days of age also tolerate this drug combination, and at what dosage.

Finally, as shown by Sachs and Jonsson (1975), DMI also prevents the NA-depleting effect of two successive high doses of 5,7-DHT, 100 mg/kg s.c., given on the day of birth and 24 hr later, without attenuating the long-term 5-HT reduction in the cortex. This route of administration of 5,7-DHT in DMI-treated rats can be employed until 5 days after birth. Thereafter, a blood-brain barrier mechanism develops in the rat that appears to prevent the accumulation of sufficient 5,7-DHT to produce neuronal damage in 5-HT or NA neurons.

In summary, 5,7-DHT in combination with DMI or MAO inhibitors can be considered a rather specific method for producing degeneration of central 5-HT neurons in developing rats. Peripheral, i.v.t., or i.c. administration may all be useful but can be expected to give very different patterns in the extent of depletion and recovery of 5-HT in individual brain regions. It appears that a dose up to 30 µg 5,7-DHT, given i.c., represents the tolerable, single maximal dose which does not cause nonspecific damage to the brain of a newborn rat. The safety limits for i.v.t. and peripheral administration of 5,7-DHT remain to be determined. The tolerable dose can be expected to increase with age. Finally, it remains to be demonstrated that high doses of DMI do not permanently alter the functional state of CNS monoaminergic and, in particular, noradrenergic neurons. 5,6-DHT causes severe nonspecific toxic damage in newborn animals and thus cannot be recommended.

XVI. STRUCTURE-ACTIVITY RELATIONSHIPS AND MOLECULAR MECHANISM OF ACTION OF 5,6- AND 5,7-DHT

As outlined in Section X, 5,6- and 5,7-DHT compete for uptake into 5-HT neurons, though less effectively than does 5-HT itself. The affinity of 5,7-DHT to NA transport is even greater than to 5-HT transport, suggesting preferential action on noradrenergic neurons. The data on NA and 5-HT depletion in brain (cf. Section IV) and on the impairment of [³H]NA and [³H]5-HT uptake *in vitro* (cf. Section XI), demonstrating a preferential action of 5,7-DHT on 5-HT neurons, indicate that the affinity to the NA and 5-HT transport systems does not allow a safe prediction of the neurotoxic potency of this *m*-DHT against NA and 5-HT neurons. A similar lack of correlation pertains to 5,6-DHT, which, according to the data on its affinity to the [³H]NA and [³H]DA uptake mechanisms, should affect noradrenergic and dopaminergic neurons equally well. When administered i.v.t. or directly into the brain, 5,6-DHT lesions dopaminergic neurons much more efficiently than noradrenergic ones. A possible explanation for this unexpected behavior of 5,6-DHT and 5,7-DHT is that the NA transport mechanism operates at a lower capacity or efficiency than do the DA and 5-HT transport mechanisms [cf. Björklund *et al.* (1975c) for a detailed discussion].

A second discrepancy concerns the neurotoxic potency of both drugs on 5-HT neurons: despite the fact that 5,7-DHT has more than six times less affinity toward the 5-HT uptake sites in brain than 5,6-DHT, it is slightly more potent in damaging 5-HT uptake and depleting brain and spinal cord 5-HT. This discrepancy can be resolved by considering the fate of both drugs in the brain, closely connected to their molecular mechanism of action. According to the study of Björklund *et al.* (1975c), [¹⁴C]5,6-DHT accumulates at a high velocity in brain slices, even beyond the time when uptake into 5-HT neurons is reduced due to damage by 5,6-DHT (which can be postulated to develop after about 5 min of exposure of the slices to the drug; cf. Fig. 14). When [¹⁴C]5,6-DHT accumulates in tissue slices devoid of 5-HT fibers, taken from animals previously treated with 5,6-DHT, the amount of radioactivity retained is reduced by only 40–55%, clearly showing that binding of 5,6-DHT occurs to non-serotonergic structures in the brain (Fig. 15). NA fibers do not participate in the extraserotonergic binding of 5,6-DHT since 6-OHDA treatment has little, if any, influence on the amount of radioactivity taken up into the slices (Fig. 15). Most of the radioactivity that accumulates in the brain 3–4 hr after i.v.t. injection of [¹⁴C]5,6-DHT is nonextractable and thus most probably covalently bound to proteins (cf. Baumgarten *et al.*, 1975c,

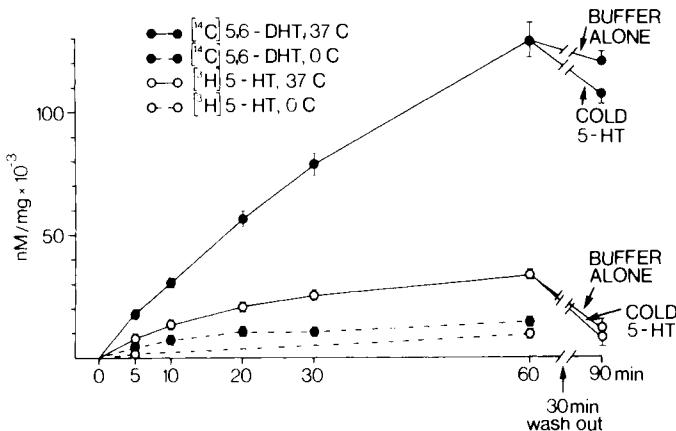


FIG. 14. Time course of accumulation of radioactivity in cortex slices after incubation in $10^{-5} M$ [^{14}C]5,6-DHT or [^3H]5-HT at 37°C or 0°C. Part of the slices, incubated for 1 hr, were washed in fresh buffer or buffer containing $10^{-5} M$ nonlabeled 5-HT for 30 min. Values give means \pm SEM of six to eight observations. (From Björklund *et al.*, 1975c.)

1976c). It appears that this protein binding is mediated through an oxidation product of 5,6-DHT, namely, 5,6-*o*-indolequinone, which has the capacity to undergo addition reactions with nucleophilic groups of proteins; *o*-diphenols and *o*-dihydroxyindoles can be postulated to form *o*-quinones by autoxidation.

As pointed out in Section III, 5,6-DHT rapidly changes color in solutions of biological pH in the absence of antioxidants. This reflects the inherent tendency of this *o*-DHT to become oxidized. When the brain is dissected only 30 min after an i.v.t. injection of 5,6-DHT a brownish precipitate is seen, despite the presence of ascorbic acid in the vehicle, in structures bordering the lateral, third, and fourth ventricle, suggesting that a substantial amount of 5,6-DHT never obtains access to the 5-HT uptake sites. This tendency of 5,6-DHT to become oxidized and to polymerize into pigmented products or to undergo covalent binding to any protein weakens its apparent neurotoxic potency for 5-HT fibers and is most likely responsible for its potentially severe nonspecific cytotoxicity.

5,7-DHT behaves quite differently, since it cannot form highly reactive *o*-quinones but rather *o*- and *p*-quinone imines. Furthermore, in aqueous solutions at pH 7.3, there is an equilibrium between several keto-enol tautomeric forms of 5,7-DHT (Björklund *et al.*, 1975c), some of which probably retain the ability to serve as substrates for the amine uptake sites in brain, as is the case with their oxidation products, the quinone imines. These favorable properties of 5,7-DHT greatly suppress its tendency to establish covalent binding to proteins and thus reduces the risk for non-

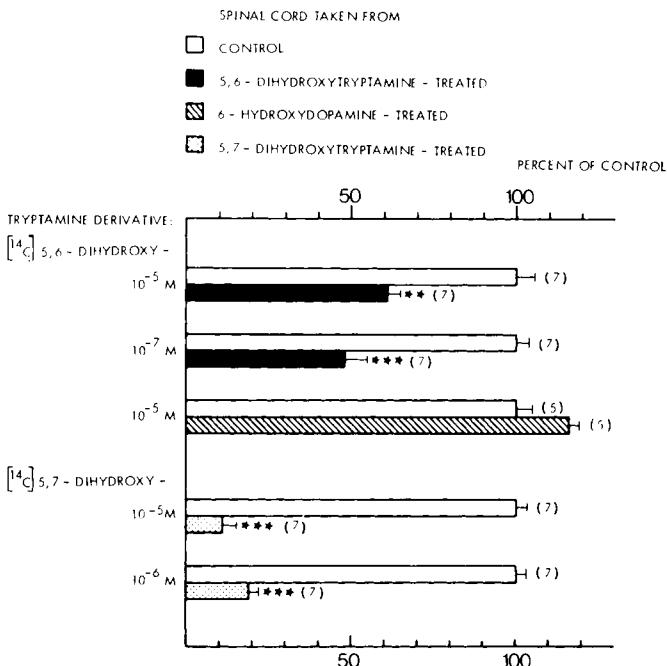


FIG. 15. Retention of radioactivity (from $[^{14}\text{C}]5,6-$ or $[^{14}\text{C}]5,7\text{-DHT}$) in spinal cord slices from control, 5,6-DHT, 5,7-DHT, or 6-OHDA-treated animals during a 10 min incubation at 37°C ; 5,6-DHT, 5,7-DHT, or 6-OHDA were injected i.v.t., 3–4 weeks before the incubation experiments. The values represent active uptake calculated as the uptake measured at 37°C minus uptake at 0°C . Bars give means \pm SEM with numbers of determinations within brackets. Differences from control. For significance levels, see Fig. 2. (From Björklund *et al.*, 1975c.)

selective cytotoxicity. This is clearly reflected in the fact that, in the case of $[^{14}\text{C}]5,7\text{-DHT}$, little nonspecific binding occurs in tissue slices devoid of NA and 5-HT fibers, due to prior treatment of animals with 5,7-DHT (Fig. 15), and in the fact that the rate of accumulation of radioactivity from $[^{14}\text{C}]5,7\text{-DHT}$ is more than 10 times lower than that of the label from $[^{14}\text{C}]5,6\text{-DHT}$ (Fig. 16; cf. also Fig. 14).

These results show that an ideal neurotoxin must have a high uptake site affinity and not too high an autoxidation rate and also that the type of quinoid oxidation product determines whether the incidence of non-specific binding is high or low.

Taking these considerations into account, it should be possible to develop even more selective toxins for central 5-HT neurons. One such example is perhaps α -methyl-5,7-DHT which has a reduced toxicity on

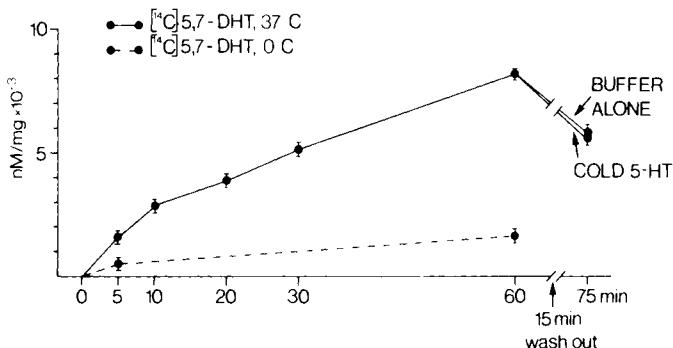


FIG. 16. Time course of accumulation of radioactivity in cortex slices after incubation in $10^{-5} M$ [^{14}C]5,7-DHT at 37°C or 0°C. Some slices, incubated for 1 hr, were washed in fresh buffer or in buffer containing $10^{-5} M$ nonlabeled 5-HT for 15 min. Means \pm SEM of six to eight observations. (From Björklund *et al.*, 1975c.)

noradrenergic neurons but still a reasonably high damaging action of central 5-HT neurons (Baumgarten *et al.*, 1975a; Björklund *et al.*, 1975c).

XVII. NONSPECIFIC ACTIONS OF 5,6- AND 5,7-DHT

5,6- and 5,7-DHT have effects which are not readily explicable on the basis of selective actions on monoaminergic neurons. 5,6-DHT when given i.v.t. in doses up to 75 μg to adult rats causes staining of the brain parenchyma bordering immediately on the lateral, third, or fourth ventricle, a reduction in the weight of the septum on the side of injection, and irregular, focal demyelination in the head of the caudate on the injection side or in the ventral medulla oblongata near the foramina laterales (cf. Baumgarten *et al.*, 1972a,b; Nobin *et al.*, 1973).

If even higher doses are administered i.v.t. to adult rats, signs of generalized brain damage become overwhelming: the animals are paralyzed, have trunk and limb tremor, deficits in thermoregulation, are extremely sensitive to handling or sensory stimulation, and die within 2–4 days. MAO inhibition prior to the i.v.t. injection of 5,6-DHT greatly enhances the toxic side effects even of doses (e.g., 50 μg) which are well tolerated in non-MAO-inhibited animals and is also compatible with the idea that actions of 5,6-DHT on structures other than monoaminergic neurons are involved in these behavioral disturbances.

Injection of 5,6-DHT i.v.t. or i.c. into the newborn rat is complicated by a high incidence of side effects (cf. Section XV). In their recent paper on structure-activity relationships of various tryptamine derivatives, Creveling *et al.* (1975) reported that, in contrast to 5,6- and 6,7-DHT,

5,7-DHT, administered i.v. in concentrations of 400 $\mu\text{mol}/\text{kg}$, did not cause nonspecific damage to mouse heart muscle fibers as judged by electron microscopy.

When more than 50 μg 5,7-DHT are given i.v.t. to adult rats or more than 15 μg 5,7-DHT are given i.c. to newborn rats, tonic-clonic convulsions may develop immediately after the injection that last for 15 to 60 min. Animals treated i.v.t. or i.c. with doses of 5,7-DHT higher than those just cited should be protected against convulsion by means of nonnarcotic doses of a short-acting barbiturate.

A sensitive indicator of nonspecific damage by 5,7-DHT is weight loss or retardation in weight gain. It is, therefore, of utmost interest to control for this parameter and to apply drug combinations which help to avoid this nonspecific effect of high doses of 5,7-DHT, such as pretreatment of animals with MAO inhibitors (cf. Breese and Cooper, 1975). This mode of pretreatment also prevents damage to central noradrenergic neurons by 5,7-DHT.

XVIII. FUNCTIONAL CONSEQUENCES OF CENTRALLY ADMINISTERED DIHYDROXYTRYPTAMINES

A. Effects on Central Mechanisms for Cardiovascular Control

5,6-DHT attenuates development of the neurogenic component of hypertension (caused by baroreceptor deafferentation) in the adult rabbit and lowers mean arterial blood pressure in the normotensive rabbit (Wing and Chalmers, 1974), pointing to a role of serotonin in the central control of blood pressure. The dose of 5,6-DHT administered i.c. (300 μg) was rather selective in that it mainly decreased the 5-HT levels in the rabbit CNS, and affected the NA levels significantly in only two of the regions studied, pons-medulla and cerebellum. The results can be explained by assuming that 5-HT is involved, under normal circumstances, in keeping blood pressure elevated by tonically suppressing an inhibitory descending noradrenergic pathway acting on the preganglionic sympathetic neurons in the spinal cord. Loss of serotonergic inhibitory control of the descending bulbospinal noradrenergic fibers would thus potentiate their inhibitory influence upon the sympathetic neurons.

B. Endocrine Effects

A selective degeneration of serotonergic terminals would represent a highly interesting approach for evaluating the role of serotonin in the control of pituitary secretions. Recent findings by Wuttke *et al.* (1977)

indicate that serum luteinizing hormone (LH) is depressed for more than 4 weeks in adult male rats following i.v.t. 5,7-DHT administration (100 µg) in DMI-pretreated animals, given 25 mg/kg i.p. (Fig. 17). While serotonin is strongly depleted in forebrain and brainstem for up to at least 55 days, there is no significant change in brain CA content throughout this postinjection period, suggesting that 5-HT neurons may have a tonic stimulatory action on LH secretion in the adult male rat. No significant changes were noted in the serum levels of prolactin or follicle-stimulating hormone (FSH). The results from previous studies in which 5,6-DHT was used to assess the role of 5-HT in gonadotropin release (Wuttke *et al.*, 1974) must be regarded as inconclusive in view of the limited specificity of 5,6-DHT and lack of data for 5-HT, NA, and DA turnover in the hypothalamus of treated animals. Similar arguments can be raised against experiments in which i.c. 5,7-DHT treatment (40 µg) was used to clarify the role of 5-HT

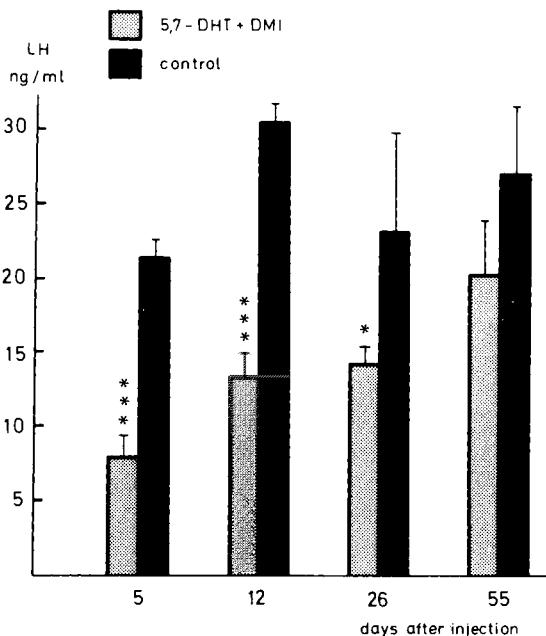


FIG. 17. Time-course effects of i.v.t. 5,7-DHT (100 µg) given to DMI-pretreated adult male rats (25 mg/kg i.p., 1 hr before 5,7-DHT) on serum LH levels. (Based on data of Wuttke *et al.*, 1977.)

in growth hormone (GH) release in the developing rat (Müller *et al.*, 1976), particularly because i.c. 5,7-DHT has, at the dose level used, strong transitory anorexigenic effects in newborn animals, perhaps as a consequence of simultaneous injury to the noradrenergic input of the hypothalamus (Myers, 1974).

Thus, extremely careful correlations of time-dependent changes in amine depletion and recovery with changes in the fluorescence morphology of central monoaminergic neuron systems, changes in activity of the monoaminergic neurons, and time-dependent alterations in receptor sensitivity are warranted to substantiate the fact that changes in serum levels of pituitary hormones are indeed related to functional disturbances in a certain type of monoaminergic neurons. Perhaps the selective interruption of defined monoaminergic input to the hypothalamus by intracerebral administration of neurotoxins represents the safest methodological approach to exploring neuroendocrine interrelationships.

C. Effects on Behavior

Both 5,6- and 5,7-DHT stimulate aggression, abnormal social interaction, and sexual activity in grouped male rats (Baumgarten *et al.*, 1972a; Baumgarten and Schlossberger, 1973; Baumgarten and Lachenmayer, 1972a; da Prada *et al.*, 1972; Breese *et al.*, 1974a; Breese and Cooper, 1975); they induce a long-lasting hyperirritability to various types of sensory stimulation, tactile, auditory, and visual; they appear to lower the pain sensitivity threshold in rats (Baumgarten *et al.*, 1972a; Breese *et al.*, 1974a; Vogt, 1974) and change active avoidance reactions (Breese and Cooper, 1975). While many of these behavioral alterations and abnormalities resemble those produced by *p*-chlorophenylalanine (*p*-CPA.) (cf. Weissman, 1973) and suggest a correlation to a dysfunctioning of the 5-HT system after 5,6-DHT and 5,7-DHT, one would like to see them confirmed using a more specific mode of pretreatment, such as with DMI or MAO inhibitors, plus 5,7-DHT. A similar skepticism may also be justified with respect to studies in which changes in sleep pattern and cortical electroencephalograms (EEG) have been reported following i.v.t. 5,6-DHT administration (Longo *et al.*, 1974). Again, the approach using precisely localized intracerebral injections may prove to be the most satisfactory way for selective lesioning of 5-HT pathways. In such an experiment in which only 1.2–2.5 µg of 5,6-DHT were injected directly into the anterior hypothalamus, food and water intakes were reduced in the rat; recovery of normal ingestion began about 5 days after 5,6-DHT (Myers, 1975).

D. Drug Interaction

Although only a few studies have been performed which use chemical degeneration of central 5-HT fibers to evaluate the role of 5-HT in mediating or modifying effects of drugs that act upon the CNS, this would seem a fruitful application of 5,7-DHT, combined with MAO inhibitors or DMI, in the future. The potency of morphine in producing analgesia in rats has been found to be attenuated by i.v.t. 5,6-DHT treatment, which is consistent with the concept of 5-HT as one of the mediators of raising pain-sensitivity threshold (Vogt, 1974, 1975). The interdependence of morphine and 5-HT transmission is disclosed only if a low dose of morphine is tested in the 5,6-DHT-lesioned animals (Vogt, 1974), and this explains the failure of Bläsig *et al.* (1973) to unravel changes in morphine sensitivity after i.v.t. 5,6-DHT treatment. In mice, Ho *et al.* (1973) have found that 5,6-DHT counteracts the development of both tolerance to and dependence on morphine in mice. Amphetamine-induced motor activity is potentiated in rats treated with i.c. 5,6-DHT (Breese *et al.*, 1974b). In rats, i.v.t. treatment with 5,6-DHT enhances alcohol intake in contrast to that with 6-OHDA, which suppresses preference (Myers and Melchior, 1975; Ho *et al.*, 1974).

E. Thermoregulation

In the monkey (Waller *et al.*, 1976) and rat (Myers, 1975), 5,6-DHT microinjected into the thermogenic zone of the anterior hypothalamus (1.25 µg) produces severe thermoregulatory deficits. However, after two to three weeks, the animals could defend against the heat but not the cold. This confirms that 5-HT pathways serve the heat-production mechanism.

XIX. CHEMICAL LESIONING OF CENTRAL INDOLEAMINE PATHWAYS BY SUBSTANCES OTHER THAN 5,6- OR 5,7-DHT

As recently shown by Baumgarten *et al.* (1975a) and Björklund *et al.* (1975c), several other tryptamine derivatives, in addition to 5,6- and 5,7-DHT, are able to damage central indoleaminergic and catecholaminergic fibers following intraventricular injection in the rat: 4,5-DHT, 6,7-DHT, 5,6-diacetoxytryptamine, and 5,6-dibutyroxytryptamine, *N*-methyl-5,6- and *N*-methyl-5,7-DHT and α -methyl-5,7-DHT. With the exception of 5,6-diacetoxytryptamine and α -methyl-5,7-DHT, none proved more selective in lesioning central 5-HT fibers than 5,6- or 5,7-DHT. 5,6-Diacetoxytryptamine and α -methyl-5,7-DHT were found to be slightly less potent than 5,6- or 5,7-DHT. Work is in progress to find out whether α -methyl-5,7-DHT can be rendered a selective tool for de-

generation of central 5-HT neurons by combining it with drugs inhibiting its transport into central CA fibers.

While the action of systemically injected pCPA on central 5-HT neurons is reversible within about 2 weeks [coinciding with the time required for the production and transport of tryptophan hydroxylase in the rat CNS (Weissman, 1973; Jequier *et al.*, 1967)], recovery of brain tryptophan hydroxylase is much more retarded after treatment of rats with single or repeated doses of *p*-CA (*p*-chloroamphetamine) or *p*-CMA (*p*-chloromethamphetamine) or fenfluramine (Sanders-Bush *et al.*, 1972; Fuller *et al.*, 1975; Gal *et al.*, 1975; Harvey and McMaster, 1975; Harvey *et al.*, 1975; Lovenberg *et al.*, unpublished observations) is consistent with the idea of toxic damage to part of the central 5-HT neurons by these amphetamine derivatives. It has been proposed that the reduction in forebrain 5-HT by *p*-CA is due to a preferential action on the neurons of the B9 cell group according to the classification of indoleaminergic cell groups of Dahlström and Fuxe (1964) (Harvey *et al.*, 1975). Unpublished results by Lovenberg are not in agreement with this suggestion, since tryptophan hydroxylase is initially and preferentially depleted in the terminal-rich areas of brain and only secondarily decreased in the cell-body-rich regions, and because the effects of *p*-CMA are widespread and affect regions of the rat CNS not innervated by the B9 cells.

Finally, colchicine, when given i.v.t. to adult rats, damages serotonergic neurons in the brain, particularly axons and terminals of the bulbospinal 5-HT system (H. G. Baumgarten, A. Rensch, W. Lovenberg, and A. Björklund, unpublished observations). The specificity of action of the neurotoxic amphetamines and of colchicine is still unknown, and these drugs cannot yet be recommended as tools for selective lesions in the central 5-HT fibers.

XX. CONCLUSIONS

5,6-DHT, when given i.v.t. or i.c. to adult rats, has, within tolerable doses (up to 75 µg), limited potency for depletion of 5-HT in most brain regions and also a limited selectivity of action, since the dopaminergic terminals near the ventricles are affected. 5,6-DHT appears, however, satisfactory for extensive and selective lesioning of the serotonin innervation of the spinal cord. Even when applied in low doses, 5,6-DHT has some non-specific toxic side effects on nonmonoaminergic structures in the brain due to the covalent binding of its metabolites to nucleophilic groups of proteins. The intracerebral route of administration is probably the most useful way of administering 5,6-DHT to the brains of experimental animals.

5,7-DHT clearly has less general cytotoxic side effects than does 5,6-

DHT and produces dose-related increases in 5-HT depletion and in the extent of damage to central indoleaminergic fibers. On the other hand, 5,7-DHT is less selective for 5-HT neurons than is 5,6-DHT, since the former affects noradrenergic neurons. When administered i.c. or i.v.t. in high doses to adult rats (150 µg or more), it causes some injury also to ventricular and superficial dopaminergic terminals. The cytotoxicity of i.v.t. or i.c. injected 5,7-DHT on central noradrenergic neurons can be attenuated or even prevented by pretreatment of animals with either DMI or MAO inhibitors, but the degree of protection depends on the dose of 5,7-DHT administered and also on the route of administration.

In the newborn, a rather specific 5-HT reduction in the CNS can be achieved by i.c. or s.c. injection of 5,7-DHT in DMI-pretreated or, preferably, MAO-inhibited animals. Whether the nonspecific effect of high doses of 5,7-DHT, given i.v.t. to adult rats on dopaminergic fibers, can be avoided by injecting the drug intracisternally or by prior administration of blockers of DA transport, e.g., benztropine, remains to be clarified.

The i.c. route of administration of 5,7-DHT, while yielding less efficient and less permanent 5-HT depletion than the i.v.t. route, might have some advantage in that the damage caused by the injection cannula in septum or caudate of i.v.t. injected animals is avoided. This is true also for 6-OHDA, as recently pointed out by Hedreen (1975). 5,7-DHT is also highly suitable for direct intracerebral application. As yet, it is unknown whether DMI or MAO inhibition is capable of protecting central noradrenergic fibers from damage by such localized injections of 5,7-DHT.

When working with chemical lesioning of axonal pathways, we should bear in mind that we are dealing with a methodology that is developing rapidly. With time, new drugs, new modes of application, and new insights into the mechanism of action of these drugs can be expected. The greatest risk today is that we might underestimate the difficulty and the complexity involved in the use of neurotoxic drugs.

REFERENCES

- Agid, Y., Javoy, F., Glowinsky, J., Bouvet, D., and Sotelo, C. (1973). Injection of 6-hydroxydopamine into the substantia nigra of the rat. II. Diffusion and specificity. *Brain Res.* **58**, 291–301.
- Baumgarten, H. G., and Björklund, A. (1976). Neurotoxic indoleamines and monoamine neurons. *Annu. Rev. Pharmacol.* **16**, 101–111.
- Baumgarten, H. G., and Lachenmayer, L. (1972a). 5,7-Dihydroxytryptamine: Improvement in chemical lesioning of indoleamine neurons in the mammalian brain. *Z. Zellforsch. Mikrosk. Anat.* **135**, 399–414.
- Baumgarten, H. G., and Lachenmayer, L. (1972b). Chemically induced degeneration of indoleamine-containing nerve terminals in rat brain. *Brain Res.* **38**, 228–323.

- Baumgarten, H. G., and Lachenmayer, L. (1973). Selektive, chemisch-induzierte Degeneration monoaminerger Neuronensysteme im Säugergehirn: Eine neue Methode für die experimentelle Neuroanatomie und Neuropharmakologie. *Dtsch. Med. Wochenschr.* **98**, 574–577.
- Baumgarten, H. G., and Schlossberger, H. G. (1973). Effects of 5,6-dihydroxytryptamine on brain monoamine neurons in the rat. In "Serotonin and Behavior" (J. D. Barchas and E. Usdin, eds.), pp. 209–224. Academic Press, New York.
- Baumgarten, H. G., Björklund, A., Lachenmayer, L., Nobin, A., and Stenevi, U. (1971). Long-lasting, selective depletion of brain serotonin by 5,6-dihydroxytryptamine. *Acta Physiol. Scand., Suppl.* **373**, 1–15.
- Baumgarten, H. G., Everts, K. D., Holman, R. B., Iversen, L. L., Vogt, M., and Wilson, G. (1972a). Effects of 5,6-dihydroxytryptamine on monoaminergic neurons in the central nervous system of the rat. *J. Neurochem.* **19**, 1587–1597.
- Baumgarten, H. G., Björklund, A., Holstein, A. F., and Nobin, A. (1972b). Chemical degeneration of indoleamine axons in rat brain by 5,6-dihydroxytryptamine. *Z. Zellforsch. Mikrosk. Anat.* **129**, 256–271.
- Baumgarten, H. G., Lachenmayer, L., and Schlossberger, H. G. (1972c). Evidence for a degeneration of indoleamine-containing nerve terminals in rat brain, induced by 5,6-dihydroxytryptamine. *Z. Zellforsch. Mikrosk. Anat.* **125**, 553–569.
- Baumgarten, H. G., Göthert, M., Holstein, A. F., and Schlossberger, H. G. (1972d). Chemical sympathectomy induced by 5,6-dihydroxytryptamine. *Z. Zellforsch. Mikrosk. Anat.* **128**, 115–134.
- Baumgarten, H. G., Björklund, A., Lachenmayer, L., and Nobin, A. (1973a). Evaluation of the effects of 5,7-dihydroxytryptamine on serotonin and catecholamine neurons in the rat CNS. *Acta Physiol. Scand., Suppl.* **391**, 1–19.
- Baumgarten, H. G., Lachenmayer, L., Björklund, A., Nobin, A., and Rosengren, E. (1973b). Long-term recovery of serotonin concentrations in the rat CNS following 5,6-dihydroxytryptamine. *Life Sci.* **12**, 357–364.
- Baumgarten, H. G., Victor, S. J., and Lovenberg, W. (1973c). Effects of 5,7-dihydroxytryptamine on regional tryptophan hydroxylase in rat brain. *J. Neurochem.* **21**, 251–253.
- Baumgarten, H. G., Björklund, A., Lachenmayer, L., Rensch, A., and Rosengren, E. (1974a). De- and regeneration of the bulbospinal serotonin neurons in the rat following 5,6- or 5,7-dihydroxytryptamine treatment. *Cell Tissue Res.* **152**, 271–281.
- Baumgarten, H. G., Björklund, A., Horn, A. S., and Schlossberger, H. G. (1974b). Studies on the neurotoxic properties of hydroxylated tryptamines. In "Dynamics of Degeneration and Regeneration in Neurons" (K. Fuxe, Y. Zotterman, and L. Olson, eds.), pp. 153–167. Pergamon, Oxford.
- Baumgarten, H. G., Groth, H. P., Göthert, M., and Manian, A. A. (1974c). The effect of 5,7-dihydroxytryptamine on peripheral adrenergic nerves in the mouse. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **282**, 245–254.
- Baumgarten, H. G., Björklund, A., Nobin, A., Rosengren, E., and Schlossberger, H. G. (1975a). Neurotoxicity of hydroxylated tryptamines: Structure-activity relationships. I. Long-term effects on monoamine content and fluorescence morphology of central monamine neurons. *Acta Physiol. Scand., Suppl.* **429**, 1–27.
- Baumgarten, H. G., Victor, S. J., and Lovenberg, W. (1975b). A developmental study of the effect of 5,7-dihydroxytryptamine on regional tryptophan hydroxylase in rat brain. *Psychopharmacol. Commun.* **1**, 75–88.
- Baumgarten, H. G., Björklund, A., and Bogdanski, D. F. (1975c). Similarities and differences in the mode of action of 6-hydroxdopamine and neurotoxic indoleamines. In "Chem-

- ical Tools in Catecholamine Research" (G. Jonsson, T. Malmfors, and C. Sachs, eds.), Vol. 1, pp. 59–66. North-Holland Publ., Amsterdam.
- Baumgarten, H. G., Klemm, H. P., Lachenmayer, L., Björklund, A., Lovenberg, W., and Schlossberger, H. G. (1977). Mode and mechanism of action of neurotoxic indoleamines: A review and progress report. *Ann. N. Y. Acad. Sci.*, in press.
- Björklund, A., Nobin, A., and Stenevi, U. (1973a). Effects of 5,6-dihydroxytryptamine on nerve terminal serotonin and serotonin uptake in the rat brain. *Brain Res.* **53**, 117–127.
- Björklund, A., Nobin, A., and Stenevi, U. (1973b). Regeneration of central serotonin neurons after axonal degeneration induced by 5,6-dihydroxytryptamine. *Brain Res.* **50**, 214–220.
- Björklund, A., Nobin, A., and Stenevi, U. (1973c). The use of neurotoxic dihydroxytryptamines as tools for morphological studies and localized lesioning of central indoleamine neurons. *Z. Zellforsch. Mikrosk. Anat.* **145**, 479–501.
- Björklund, A., Baumgarten, H. G., and Nobin, A. (1974). Chemical lesioning of central monoamine axons by means of 5,6- and 5,7-dihydroxytryptamine. *Adv. Biochem. Psychopharmacol.* **10**, 13–33.
- Björklund, A., Baumgarten, H. G., and Rensch, A. (1975a). 5,7-Dihydroxytryptamine: Improvement of its selectivity for serotonin neurons in the CNS by pretreatment with desipramine. *J. Neurochem.* **24**, 833–835.
- Björklund, A., Baumgarten, H. G., Lachenmayer, L., and Rosengren, E. (1975b). Recovery of brain noradrenaline after 5,7-dihydroxytryptamine-induced axonal lesions in the rat. *Cell Tissue Res.* **161**, 145–155.
- Björklund, A., Horn, A. S., Baumgarten, H. G., Nobin, A., and Schlossberger, H. G. (1975c). Neurotoxicity of hydroxylated tryptamines: Structure-activity relationships. 2. *In vitro* studies on monoamine uptake inhibition and uptake impairment. *Acta Physiol. Scand., Suppl.* **429**, 29–60.
- Bläsig, J., Reinholt, K., and Herz, A. (1973). Effect of 6-hydroxydopamine, 5,6-dihydroxytryptamine and raphe lesions on the antinociceptive actions of morphine in rats. *Psychopharmacologia* **31**, 111–119.
- Breese, G. R., and Cooper, B. R. (1975). Behavioral and biochemical interactions of 5,7-dihydroxytryptamine with various drugs when administered intracisternally to adult and developing rats. *Brain Res.* **98**, 517–527.
- Breese, G. R., Cooper, B. R., Grant, L. D., and Smith, R. D. (1974a). Biochemical and behavioral alterations following 5,6-dihydroxytryptamine administration into brain. *Neuropharmacology* **13**, 177–189.
- Breese, G. R., Cooper, B. R., and Mueller, R. A. (1974b). Evidence for involvement of 5-hydroxytryptamine in the actions of amphetamine. *Br. J. Pharmacol.* **52**, 307–314.
- Creveling, C. R., Lundström, J., McNeal, E. T., Tice, L., and Daly, J. W. (1975). Dihydroxytryptamines: Effects on noradrenergic function in mouse heart *in vivo*. *Mol. Pharmacol.* **11**, 211–227.
- Dahlström, A., and Fuxe, K. (1964). Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brainstem neurons. *Acta Physiol. Scand., Suppl.* **232**, 1–55.
- Daly, J. W., Fuxe, K., and Jonsson, G. (1973). Effects of intracerebral injections of 5,6-dihydroxytryptamine on central monoamine neurons: Evidence for selective degeneration of central 5-hydroxytryptamine neurons. *Brain Res.* **49**, 476–482.
- da Prada, M., Carruba, M., O'Brien, R. A., Saner, A., and Pletscher, A. (1972). The effect of 5,6-dihydroxytryptamine on sexual behaviour of male rats. *Eur. J. Pharmacol.* **19**, 288–290.

- Falck, B., and Owman, C. (1965). A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund., Sect. 2* **7**, 1-23.
- Fuller, R. W., Perry, K. W., and Molloy, B. B. (1975). Effect of 3-(*p*-trifluoromethyl-phenoxy)-*N*-methyl-3-phenylpropylamine on the depletion of brain serotonin by 4-chloroamphetamine. *J. Pharmacol. Exp. Ther.* **193**, 796-803.
- Gal, E. M., Christiansen, P. A., and Yunger, L. M. (1975). Effects of *p*-chloroamphetamine on cerebral tryptophan-5-hydroxylase *in vivo*: A reexamination. *Neuropharmacology* **14**, 31-39.
- Gershon, S., and Baldessarini, R. J. (1975). Selective destruction of serotonin terminals in rat forebrain by high doses of 5,7-dihydroxytryptamine. *Brain Res.* **85**, 140-145.
- Gershon, S., Baldessarini, R. J., and Wheeler, S. C. (1974). Biochemical effects of dihydroxylated tryptamines on central indoleamine neurones. *Neuropharmacology* **13**, 987-1004.
- Harvey, J. A., and McMaster, S. E. (1975). Fenfluramine: Evidence for a neurotoxic action on midbrain and a long-term depletion of serotonin. *Psychopharmacol. Commun.* **1**, 217-228.
- Harvey, J. A., McMaster, S. E., and Yunger, L. M. (1975). *p*-Chloroamphetamine: Selective neurotoxic action in brain. *Science* **187**, 841-843.
- Hedreen, J. (1975). Increased nonspecific damage after lateral ventricle injection of 6-OHDA compared with fourth ventricle injection in rat brain. In "Chemical Tools in Catecholamine Research" (G. Jonsson, T. Malmfors, and C. Sachs, eds.), pp. 91-100. North-Holland Publ., Amsterdam.
- Ho, I. K., Loh, H. H., and Way, E. L. (1973). Influence of 5,6-dihydroxytryptamine on morphine tolerance and physical dependence. *Eur. J. Pharmacol.* **21**, 331-336.
- Ho, A. K. S., Tsai, C. S., Chan, R. C. A., Begleiter, H., and Kissin, B. (1974). Experimental studies on alcoholism. I. Increase in alcohol preference by 5,6-dihydroxytryptamine and brain acetylcholine. *Psychopharmacologia* **40**, 101-107.
- Hökfelt, T., and Ungerstedt, U. (1973). Specificity of 6-hydroxydopamine induced degeneration of central monoamine neurons: An electron and fluorescence microscopy study with special reference to intracerebral injection on the nigro-striatal dopamine systems. *Brain Res.* **60**, 269-298.
- Hole, K., Fuxe, K., and Jonsson, G. (1976). Behavioral effects of 5,7-dihydroxytryptamine lesions of ascending 5-hydroxytryptamine pathways. *Brain Res.* **107**, 385-389.
- Horn, A. S. (1973). Structure-activity relations for the inhibition of 5-HT uptake into rat hypothalamic homogenates by serotonin and tryptamine analogues. *J. Neurochem.* **21**, 883-888.
- Horn, A. S., Baumgarten, H. G., and Schlossberger, H. G. (1973). Inhibition of the uptake of 5-hydroxytryptamine, noradrenaline and dopamine into rat brain homogenates by various hydroxylated tryptamines. *J. Neurochem.* **21**, 233-236.
- Iversen, L. L. (1975). Uptake processes for biogenic amines. In "Handbook of Psychopharmacology" (L. L. Iversen, S. D. Iversen, and S. H. Snyder, eds.), pp. 381-442. Plenum, New York.
- Jacoby, J. H., Lytle, L. D., and Nelson, M. F. (1974). Long-term effects of 5,7-dihydroxytryptamine on brain monoamines. *Life Sci.* **14**, 909-919.
- Jequier, E., Lovenberg, W., and Sjöerdsma, A. (1967). Tryptophan hydroxylase inhibition: The mechanism by which *p*-chlorophenylalanine depletes rat brain serotonin. *Mol. Pharmacol.* **3**, 274-278.

- Jonsson, G., and Sachs, C. (1970). Effects of 6-hydroxydopamine on the uptake and storage of noradrenaline in sympathetic adrenergic neurons. *Eur. J. Pharmacol.* **9**, 141–155.
- Jonsson, G., Einarsson, P., Fuxe, K., and Hallman, H. (1975). Microspectrofluorimetric analysis of the formaldehyde-induced fluorescence in midbrain raphe neurons. *Med. Biol.* **53**, 25–39.
- Krieger, D. T. (1975). Effect of intraventricular neonatal 6-OH-dopamine or 5,6-dihydroxytryptamine administration on the circadian periodicity of plasma corticosteroid levels in the rat. *Neuroendocrinology* **17**, 62–74.
- Lachenmayer, L., and Groth, H. P. (1973). Degeneration and regeneration of the adrenergic nerves in the rat iris induced by dihydroxytryptamines. *Virchows Arch. B* **13**, 197–213.
- Laverty, R., Sharman, D., and Vogt, M. (1965). Action of 2,4,5-tri-hydroxyphenylethylamine on the storage and release of noradrenaline. *Br. J. Pharmacol.* **24**, 549–560.
- Lee, F. G. H., Dickson, D. E., Suzuki, J., Zirnis, A., and Manian, A. A. (1973). Synthesis of 5,7- and 6,7-disubstituted tryptamines and analogs. *J. Heterocycl. Chem.* **10**, 649–654.
- Lindvall, O., and Björklund, A. (1974). The glyoxylic acid fluorescence histochemical method: A detailed account of the methodology for the visualization of central catecholamine neurons. *Histochemistry* **39**, 97–127.
- Longo, V. G., Scotti de Carolis, A., Liuzzi, A., and Massotti, M. (1974). A study of the central effects of 5,6-dihydroxytryptamine. *Adv. Biochem. Psychopharmacol.* **10**, 109–120.
- Lorens, S. A., Köhler, C., Srebro, B., and Guldberg, H. C. (1975). Behavioral effects of central 5-HT depletion: comparison of pcpa, 5,7-DHT and electrolytic midbrain raphe lesions. *Exp. Brain Res.* **23** (Suppl.), 130.
- Lytle, L. D., Jacoby, J. H., Nelson, M. F., and Baumgarten, H. G. (1975). Long-term effects of 5,7-dihydroxytryptamine administered at birth on the development of brain monoamines. *Life Sci.* **15**, 1203–1217.
- Møllgard, K., Lundberg, J. J., Lachenmayer, L., Wiklund, L., and Baumgarten, H. G. (1977). Morphological consequences of serotonin neurotoxin administration. Neuron-target cell interaction in the rat subcommissural organ. *Ann. N. Y. Acad. Sci.*, in press.
- Müller, E. E., Baumgarten, H. G., Gil-Ad, I., Udeschini, G., and Cocchi, D. (1976). Effect of indoleamine derivatives administered at birth on growth and plasma growth hormone levels in the rat. *Ergänz. Bd. Anat. Anz.*, in press.
- Myers, R. D. (1974). "Handbook of Drug and Chemical Stimulation of the Brain." Van Nostrand-Reinhold, New York.
- Myers, R. D. (1975). Impairment of thermoregulation, food and water intakes in the rat after hypothalamic injections of 5,6-dihydroxytryptamine. *Brain Res.* **94**, 491–506.
- Myers, R. D., and Melchior, C. (1975). Alcohol drinking in the rat after destruction of serotonergic and catecholaminergic neurons in the brain. *Res. Commun. Chem. Pathol. Pharmacol.* **10**, 363–378.
- Nobin, A., Baumgarten, H. G., Björklund, A., Lachenmayer, L., and Stenevi, U. (1973). Axonal degeneration and regeneration of the bulbo-spinal indoleamine neurons after 5,6-dihydroxytryptamine treatment. *Brain Res.* **56**, 1–24.
- Porter, C., Totaro, J., and Stone, C. (1963). Effect of 6-hydroxydopamine and some other compounds on the concentration of norepinephrine in the hearts of mice. *J. Pharmacol. Exp. Ther.* **140**, 308–316.
- Renaud, B., Buda, M., Lewis, B. D., and Pujol, J. F. (1975). Effects of 5,6-dihydroxytryptamine on tyrosine hydroxylase activity in central catecholaminergic neurons of the rat. *Biochem. Pharmacol.* **24**, 1739–1742.

- Sachs, C., and Jonsson, G. (1975). 5,7-Dihydroxytryptamine-induced changes in the post-natal development of central 5-hydroxytryptamine neurons. *Med. Biol.* **53**, 156-164.
- Sanders-Bush, E., Bushing, J. A., and Sulser, F. (1972). *p*-Chloroamphetamine-inhibition of cerebral tryptophan hydroxylase. *Biochem. Pharmacol.* **21**, 1501-1510.
- Saner, A., Pieri, L., Moran, J., da Prada, M., and Pletscher, A. (1974). Decrease of dopamine and 5-hydroxytryptamine after intracerebral application of 5,6-dihydroxytryptamine. *Brain Res.* **76**, 109-117.
- Schlossberger, H. G., and Kuch, H. (1960). Synthese des 5,6-Dihydroxytryptamins. *Chem. Ber.* **93**, 1318-1323.
- Schlossberger, H. G., and Kuch, H. (1963). Synthese von 5,6-Dihydroxytryptophan und verwandten Verbindungen. *Liebigs Ann. Chem.* **662**, 132-138.
- Shaskan, E. G., and Snyder, S. H. (1970). Kinetics of serotonin accumulation in slices from rat brain: Relationship to catecholamine uptake. *J. Pharmacol. Exp. Ther.* **175**, 404-418.
- Sotelo, C., Javoy, F., Agid, Y., and Glowinski, J. (1973). Injection of 6-hydroxydopamine in the substantia nigra of the rat. I. Morphological study. *Brain Res.* **58**, 269-290.
- Srebro, B., and Lorens, S. A. (1975). Behavioral effects of selective midbrain raphe lesions in the rat. *Brain Res.* **89**, 303-325.
- Thoenen, H., and Tranzer, J. P. (1968). Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine. *Naunyn-Schmiedebergs Arch. Pharmakol.* **261**, 271-288.
- Ungerstedt, U. (1968). 6-Hydroxydopamine induced degeneration of central monoamine neurons. *Eur. J. Pharmacol.* **5**, 107-110.
- Ungerstedt, U. (1971). Histochemical studies on the effects of intracerebral and intraventricular injection of 6-hydroxydopamine on monoamine neurons in the rat brain. In "6-Hydroxydopamine and Catecholamine Neurons" (T. Malmfors and H. Thoenen, eds.), pp. 101-127. North-Holland Publ., Amsterdam.
- Uretsky, N. J., and Iversen, L. L. (1969). Effects of 6-hydroxydopamine on noradrenaline-containing neurons in the rat brain. *Nature (London)* **221**, 557-559.
- Uretsky, N. J., and Iversen, L. L. (1970). Effects of 6-hydroxydopamine on catecholamine-containing neurons in the rat brain. *J. Neurochem.* **17**, 269-278.
- Victor, S. J., Baumgarten, H. G., and Lovenberg, W. (1974). Depletion of tryptophan hydroxylase by 5,6-dihydroxytryptamine in rat brain. Time-course and regional differences. *J. Neurochem.* **22**, 541-546.
- Vogt, M. (1974). The effect of lowering the 5-hydroxytryptamine content of the rat spinal cord on analgesia produced by morphine. *J. Physiol. (London)* **236**, 483-498.
- Vogt, M. (1975). Tryptaminergic neurotransmission. *Proc. Int. Congr. Pharmacol.*, 6th **2**, 3-16.
- Waller, M. B., Myers, R. D., and Martin, G. E. (1976). Thermoregulatory deficits in the monkey produced by 5,6-dihydroxytryptamine (5,6-DHT) injected into the hypothalamus. *Neuropharmacology* **15**, 61-68.
- Weissman, A. (1973). Behavioral pharmacology of *p*-chlorophenylalanine (PCPA). In "Serotonin and Behavior" (J. D. Barchas and E. Usdin, eds.), pp. 235-248. Academic Press, New York.
- Wing, L. M. H., and Chalmers, J. P. (1974). Participation of central serotonergic neurons in the control of the circulation of the unanaesthetized rabbit. A study using 5,6-dihydroxytryptamine in experimental neurogenic and renal hypertension. *Circ. Res.* **35**, 504-513.
- Wuttke, W., Baumgarten, H. G., Fenske, M., and Lachenmayer, L. (1974). Correlation of degenerative processes in the hypothalamus after neurotoxic drug administration with

- serum prolactin and LH and with the corresponding hypothalamic hormones. In "Neurosecretion—The Final Neuroendocrine Pathway" (F. Knowles and L. Vollrath, eds.), pp. 328–329. Springer-Verlag, Berlin and New York.
- Wuttke, W., Björklund, A., Baumgarten, H. G., Lachenmayer, L., Fenske, M., and Klemm, H. P. (1977). De- and regeneration of brain serotonin neurons following 5,7-dihydroxytryptamine treatment: Effects on serum LH, FSH and prolactin levels in male rats. *Brain Res.*, in press.

Chapter 4

Determining Sensory Deficits in Animals

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I. INTRODUCTION

Evaluation of sensation is useful in many different experimental situations. Behavioral methods for sensory testing, which will be discussed in this chapter, have the overwhelming advantage of suitability in the alert, perceiving animal. Such noninvasive testing methods allow the animal to remain unaltered so that it can be tested again at a later time. Behavioral methods, of course, also present the experimenter with a rather formidable disadvantage: that of dealing with a large number of variables generated by awake, self-motivated animals. This disadvantage may be overcome, however, by a choice of behavioral methods and controls appropriate to the information desired.

The various methods available afford access to different types of information. The methods range from the brief, but reproducible, broad survey of all sensory abilities that can be achieved by use of the neurological

examination, to the precise determination of the limits of discrimination within one specialized sensory modality. Although all the methods which will be discussed are applicable for testing animals before and after operative lesions, most of them are equally suitable for determining the effects of drugs, environmental change, and development.

Before discussing testing methods in detail, it is worthwhile to establish the framework of sensory abilities to which the methods may be applied. In general the "five senses" of common parlance, i.e., vision, audition, olfaction, taste, and touch, are recognized. Another sense, the sense of equilibrium, must also be mentioned. Each of the major sensory modalities can be divided according to even more basic modalities, such as flutter-vibration, temperature, pain, and light touch within somesthesia. Each of these subdivisions is served by overlapping but slightly different neural pathways and is perceived by human subjects to be somewhat different in quality from the others.

The modalities may also be divided according to integrative level within the modality. Loss of sensation related to loss of a primary afferent pathway, such as the optic nerve in the visual system, may be considered an elementary or primary sensory loss. A higher-order visual loss, on the other hand, seems to result from certain cortical lesions. For instance, the loss of pattern vision, but not of other visual abilities, is found following inferotemporal cortical lesions in the monkey (Iwai and Mishkin, 1969). Many levels of integration are recognized, and testing methods must be designed with this factor in mind, as well as that of the basic modality within which the testing is to be made.

Methods of sensory testing most commonly used are the neurological examination, conditioning (or training), and sensory-evoked potentials. The neurological examination has the advantage of allowing rather rapid evaluation of many sensory modalities. The examination is essentially a set pattern of observations which has been used, in gradually more complex form, since the nineteenth century (Charcot, 1873); it has often been thought to yield qualitative rather than quantitative results. Conditioning methods have also long been in use. Some have been refined to the point where they provide information which can be dealt with by quite precise scales of measurement and statistical operations (Stevens, 1961).

Success in evaluation of sensation with either of these methods depends on the response of the whole animal—or, in other words, upon the efferent or motor limb, in terms of the simple reflex paradigm, as well as upon the afferent or sensory limb of the neural pathway. If one wishes to isolate the afferent limb for testing, recording sensory-evoked potentials may be quite useful. Sensory-evoked potential recordings, however, do

not indicate the perceptual value that the afferent impulses attain within the subject's sphere of awareness.

The background state of the nervous system is a factor in all sensory testing. The optimal state of the animal for most sensory testing is one of alertness but not of excitement or anxiety. It is therefore best that the animal be accustomed to the test procedure. Then the choice of methods should be made on the basis of the level and quality of the information needed for the purpose of the experiment.

The animal species involved must also be taken into account when choosing methods of testing. In the paragraphs that follow, discussion will center primarily on the methods used for sensory testing in the rat and monkey, including the sensory neurological exam, operant and classical conditioning methods, and evoked potential methods. Since even within these limited topics an immense variety of methodology exists, rather circumscribed examples will be given.

II. THE NEUROLOGICAL EXAMINATION

A. *Uses and Abuses of the Neurological Examination*

The neurological examination is a standard series of tests designed to assay each neural network within the central nervous system (CNS) and to assay each one as much as possible in isolation from the others. In designing a neurological examination for an animal, much may be adapted from the neurological examination used for human patients. In clinical medical neurology, the neurological examination has been elaborated to such a degree that the clinical neurologist is able to localize lesions within the nervous system of his patient with a high degree of accuracy. In order to do this, the neurologist requires approximately 1 hour, a reflex hammer, a tuning fork, a flashlight, a pin, a cotton ball, and two identical bottles containing hot and cold water (see Section of Neurology and Physiology of Mayo Clinic Foundation, 1963). There are numerous subtests for detailed examination of various parts of the nervous system in humans. When the neurological clinician approaches the patient he must exercise judgment in selecting, out of all of the many standard subtests available, those appropriate to the patient's problem (Denny-Brown, 1957). If he were to deploy all available subtests, the clinician would require several days to complete the neurological examination, obviating one of its main advantages—rapid diagnosis.

The usual practice of the neurologist is to assay certain standard functions, such as the functioning of each of the cranial nerves (including

vision, olfaction, audition, and taste), strength and coordination of the limbs, deep tendon reflexes, and somatic sensation. The clinician does this against a background of his assessment of the patient's mental status: motivation, degree of alertness, degree of intelligence, and ability to comprehend the meaning of the examination. After he has investigated all of these functions, the clinician may make a more detailed examination of functions that are abnormal. If for instance there is a loss of somatic sensation, he will proceed to determine if the loss is in the distribution of a specific dermatome, as might be seen after a section of a dorsal root, if the loss is in the distribution of a given peripheral nerve, or whether the loss is of a higher order, possibly related to a lesion in the cerebral cortex.

The medical methods of the neurological examination are useful not only for a rapid assay of a broad range of nervous system functions, but also for a repeatable assay of such functions which does not depend upon learning and memory. Serial examinations may be done, and each will be little affected by the animal's previous experience with the test. Thus, the serial examination can clearly demonstrate change of sensory function over time. One of the difficulties with the neurological examination, of course, is its limited precision and the scale of measurements used (Stevens, 1961). The value of the neurological examination is as a control procedure, covering many variables, when the primary dependent variable is being studied by means which allow more precise scales of measurement.

Many factors need to be taken into account when planning a scheme for sensory neurological examination of an animal. The one factor of prime importance is the animal's state of alertness and motivation. In most species, a state of high motivation may be secured by food or water deprivation sufficient to insure that the animal will consume at least one day's food ration. Food deprivation is effective in monkeys, cats, and dogs, whereas water deprivation is quite effective in rats and mice. When the animal has been deprived of food or water, these substances may be used as rewards during the neurological examination and, therefore, may be used to induce the animal to perform actions which it would not perform spontaneously.

Another important factor during neurological examination of an animal is the fear of the situation. In monkeys, certain postures and facial expressions rather reliably indicate the animal's emotional status (Hinde and Rowell, 1962; Butter *et al.*, 1968), so that the investigator is able to determine whether the animal is "fearful" of the situation. In most species, it is necessary to familiarize the animal with the test equipment and the examining room, as well as the examiner, prior to subjecting the animal to the information-gathering portion of the neurological examina-

tion. The time necessary for the adaptation varies from species to species and from animal to animal, especially among monkeys and cats.

To insure an animal's response, it is important to pick out stimuli which are of interest to the species under consideration. The same is true, of course, for rewards. For example, a hungry monkey will pay close visual attention to small bits of food when they are brought in from the periphery of the visual field. Yet this same monkey will completely ignore similar-sized white paper objects and demonstrate extremely constricted visual fields if white paper objects are used for stimuli. On the other hand, the Long-Evans hooded rat appears to be quite interested in white paper objects and will orient when a white paper object is brought into its peripheral visual field.

Different species demonstrate lack of motivation in different ways. Monkeys, for instance, who may appear at ease in a test situation may demonstrate lack of motivation by simply performing more slowly or by ignoring the sensory stimuli until they are extremely intense. In such instances it may be impossible to determine whether faulty performance is related to inability on the monkey's part or unwillingness. It is best to attempt to use other inducements to get the monkey to perform the response required (such as a more preferred food, or larger pieces of the same food), or to allow the animal to rest and then to repeat the particular subtest at another time.

Aside from going as far as possible to secure the cooperation of the animal by allowing it to become familiar with the test situation and the tester, and by having hunger, thirst, and interesting stimuli as driving forces, other factors must be taken into account. Naturally nocturnal animals, like the tree shrew, may perform best at night and in dim light. Background sensory stimuli, including light and sound, may prove distracting to many animals. Therefore, the neurological exam, like other behavioral tests, should be carried out in a sound-attenuated, lighting-controlled room. A white noise generator may be used to mask whatever sound does come through from outside of the testing room. It is also valuable, before attempting to examine sensory systems, to be certain that there is no mechanical block to sensory reception, such as eyelids adhered shut, or some foreign matter in the external auditory meatus.

The ear bars of many stereotaxic instruments may damage the external auditory meatus and tympanic membrane, thus leading to a nonneural auditory sensory deficit. The motor system which will be utilized by the animal to communicate its response to the sensory stimulus should be briefly examined. A period of ambulation on a table top may be allowed for rats and mice, and for larger animals ambulation in a large cage may be encouraged. Observations of whether the animal is able to turn both head

and eyes in four directions should also be made. If the animal cannot be induced to move freely, or to turn its head or eyes voluntarily, certain reflexes may be utilized to determine the intactness of the motor response (Tilney, 1933). Some useful reflexes for this purpose are the optical righting reflex (Ruch, 1965; Bignall, 1974), the labyrinthine righting reflex (Magnus, 1924), and the hopping reflex (Rademaker, 1931). Other reflexes, such as the tonic neck reflex and the vestibular-ocular reflex, may be useful at times.

Once such factors have been satisfactorily controlled, the sensory examination itself may be carried out. It is generally more useful to test the distance receptors before testing somatosensory receptors. This allows the animal to respond to the more pleasant stimuli at the beginning of the examination and thus does not alter the animal's motivation and cooperation until the end. Vision and audition are the modalities which can be tested most accurately. A satisfactory test of olfaction is more difficult, at least in the monkey, during the neurological examination. Somesthesia, i.e., touch, pain, and temperature, can be tested to a limited degree, and testing of taste and equilibrium have not been part of most animals' neurological exams, but rather are tested by conditioning methods (Macht, 1951; Venura and Cohen, 1972).

Most of the measurements of subtests of the neurological examination are made in a simple nominal scale. That is to say, the phenomena are observed either to be present or not. In general, when more quantitative measures are desired, conditioning methods described in Section II are more appropriate. Some extensions of the neurological test that do not necessarily require conditioning techniques but may provide an ordinal scale of measurement can be used, but with caution.

The obvious way to obtain data which are amenable to statistical handling appropriate to ordinal scales of measurement is to carry out a large number of trials of any given subtest. For instance, if an animal is given 20 trials with each forepaw, using a pseudorandom order to determine when to try left and when to try right, on tactile placing, it is legitimate to compare the median number of successful placings of each paw. Thus, repetitive testing of the same response may be useful in some instances. When such testing is carried out, however, account must be taken of learning by the animal. When monkeys are tested on their ability to empty six food wells of tiny bits of food, and this testing is carried out over a number of days (Deuel, 1977) with measurements of speed, it becomes obvious that the animal is faster on the last day than on the first. Thus, the findings from the first day may not be strictly comparable with those of the last, as practice, even in this simple task, that the animal in fact performs spontaneously, has a measurable effect.

Learning and memory may also have the paradoxical effect of making an animal less efficient in a given task. Some tasks, when carried out over a large number of trials, may require reinforcement with food as the animal adapts to the stimulus and its novelty diminishes. An example is a task of auditory localization as described by Heilman *et al.* (1970). In that task the animal, after a certain number of trials without reward, stops orienting unless a reward is presented after each orientation. As soon as an animal receives reinforcement for performing a task, the laws which govern conditioning are in effect.

In summary, the neurological examination provides a controlled measure for sensory and other deficits which are not the chief dependent variable of the experiment in progress. The neurological examination determines the selective nature of sensory deficit found by more quantitative methods; it is, therefore, a requirement for a truly comprehensive evaluation of sensory deficits following CNS lesions in animals. In any experimental situation, the examination allows for a brief, broad, repeatable evaluation in a short period of time. Neurological examinations have been devised for the cat (Sprague and Meikle, 1965), the kitten (Bignall and Schramm, 1974), the rabbit (Stewart and Riesen, 1972), tree shrew (Snyder *et al.*, 1966), and other animals (Ravizza and Masterton, 1972). The details of these examinations vary from species to species and, of course, with the purpose of the given experiment. The following sections will give a detailed explanation of neurological examinations for monkeys and rats.

B. The Monkey Neurological Examination

The following is a description of the neurological examination as carried out in my laboratory. It has the purpose of providing a very brief, general survey of the monkey's sensory abilities. It is designed to serve as an adjunct to behavioral training and testing that yield quantitative but highly circumscribed information. By using the neurological examination, much additional information of a qualitative nature is gathered concerning the effects of lesions and the recovery process. Without a neurological examination this information would be nonexistent.

1. Visual Fields

For most of the sensory testing, the animal is seated in a primate chair as depicted in Fig. 1. The neckpiece and the waistpiece of the chair restrain the animal but leave the limbs free. Although the chair serves to orient the animal in one direction, considerable freedom of movement is available to the head and forelimbs. Once the animal is comfortable in the

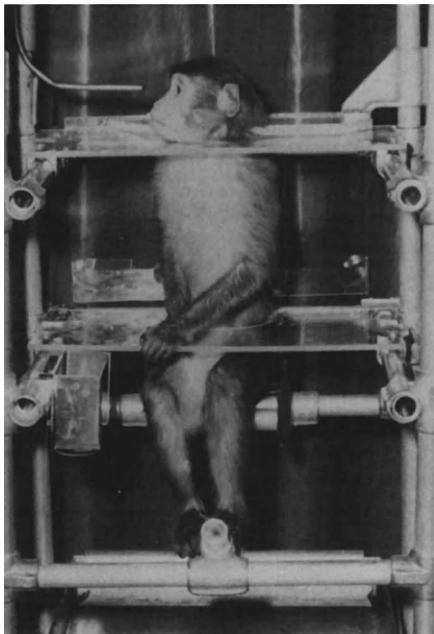


FIG. 1. Rhesus monkey in restraining chair. Examiner has free access to the limbs of the animal. The animal has free access to its mouth with its hands. For somatosensory testing it is only necessary to provide an extended opaque neckpiece, which prevents view of the limbs and body.

chair, tests of visual fields are made. It is necessary for the animal to be hungry for this test, and two experimenters are required. Experimenter one stands facing the animal, holding small bits of food at the animal's eye level just beyond the reach of the animal's head toward its fixation point. When the animal shifts its gaze from the forward fixation point to the piece of food on the moving wire and reaches for it, this indicates that the animal has perceived the food. The position within the visual periphery where the food was when the animal reaches for it is then plotted on a chart of the visual perimeter. Four trials from each of the quadrants of the visual perimeter are adequate in a cooperative monkey to determine whether each of the quadrants of the visual field is intact.

Difficulties with this kind of field testing are created by the animal's turning in the primate chair. If the animal is not cooperative nor motivated for food, it is best to terminate this type of visual field examination and attempt to repeat it at a later time. If cooperation cannot be obtained, and

it is absolutely necessary to estimate the visual fields, response to visual threat may be evaluated (Bannister, 1973).

To perform visual field tests using threat, the animal's eyes must be oriented in one direction before the start of the trial, which consists of bringing a large object or hand rapidly into the periphery of one of the visual field quadrants. The animal usually blinks when the threatening object appears in the field being tested. This is a method that would be unlikely to disclose a visual field defect less obvious than a complete hemianopsia. Other more exacting means of testing visual fields exist and will be discussed in Section III.

The method of testing visual fields gives an estimation of a quadrantic or a hemianopic visual field defect. As described, it is in fact a binocular test, and therefore monocular blindness could be a confounding factor. The test may be performed monocularly by means of blindfolding one eye of the monkey; however, this is difficult in the testing situation. When seated in the primate chair, the animal has access to its mouth and face and will attempt to remove most blindfolds with its hands. It is possible to use an opaque contact lens, an effective occluder, but its placement is an annoying procedure; the awake monkey often will not cooperate on the test when the monocular occluder is in place. Adaptation to an occluder may be carried out but often requires many days, as does adaptation to other devices which allow monocular testing.

When confrontation fields are done monocularly, however, the test can yield information which should lead to localization of lesions in the optic nerve, optic chiasm, and visual cortex. Many standard visual systems maps are available (Walsh, 1957). The visual pathways and the cortical representation of retinal segments in the monkey have been carefully mapped by Hubel and Wiesel (1968, 1970). It is of interest that for a brief period following unilateral frontal cortical lesions in monkeys an apparent hemianopsia may be found (Welsh and Stuteville, 1958; Deuel, 1974), the cause of which is unknown.

2. Visual Acuity

Visual acuity may be tested to some extent in the monkey restrained in the primate chair. A board of dark color upon which are several bits of preferred fruit of varying sizes is held 16 in. from the monkey's eyes. The animal demonstrates accuracy of vision by reaching precisely for a bit of food, removing it from the board and putting it in his mouth. The monkey usually starts with the larger pieces and, if hungry enough, will proceed to the very smallest of pieces. Using this method it must be remembered that monkeys in general lack enthusiasm for picking up very small pieces of food, especially after they have consumed considerable numbers of large

pieces. It is important that the monkey be very hungry if this method of visual acuity testing is to be used; otherwise it will appear to have a gross visual acuity defect. In a hungry, cooperative monkey, binocular testing of visual fields and visual acuity is rapidly done and requires no more than 15 min.

3. Auditory System

During the neurological examination, a test of the animal's auditory attention and ability to localize sounds in space can be made with relative ease. Two examiners are required, one to attract the animal's visual attention forward and the other to stand behind the animal and present the auditory stimuli. The animal orients toward jingling keys, a tuning fork, a snapping sound of low intensity, or clicking sounds. The sound should be presented approximately an inch and a half lateral to and slightly behind the external auditory meatus.

Care must be taken not to (*a*) create air currents that stimulate somatosensory receptors; (*b*) allow the animal to see the stimulus; or (*c*) make other sounds. When the sound starts the animal will immediately turn its head (Heilman *et al.*, 1970). If 10 noises are presented close to each ear in random order, left and right, with variable amounts of time between each presentation, it is reasonable to conclude that the animal has oriented to each noise and that the auditory attention mechanisms are intact.

Within 10 trials it is wise to present three or four different types of noise, partly because different frequencies will be represented, but also because the animal may become accustomed to a single type of noise and not orient toward it. Random order of presentation and variable intervals between presentations must be preserved in order to prevent the animal from anticipating the next stimulus.

Qualitative estimate of sound localization may be gained by presenting stimuli at 30° behind the left ear approximately 10 in. away, as well as close to the auditory meatus. The animal generally looks directly at the source of the sound, and thus demonstrates a differentiation of these two positions. It is important to recognize that monkeys, like humans, have difficulty in localizing sounds on a vertical plane, especially monaurally (Roffler and Butler, 1968; Butler, 1973). Thus noises presented well above or below the monkey's ear often tend to confuse both animal and examiner. If monaural auditory testing is desirable, bone-wax plugs can be placed in one external auditory meatus to provide relative occlusion of sound to one ear while the other is tested. The whole procedure of sound orientation should take less than 10 min with a cooperative animal. When

greater precision than is possible with the foregoing methods is desired, discrimination training is required.

4. Olfactory Responses

Testing the olfactory responses other than simple orienting responses to smell, which are variable in monkeys, is difficult. Monkeys often ignore everything but strong fragrances. Despite their inattention to olfactory stimuli at a distance, it is difficult to avoid the possible confounding of visual orienting responses with olfactory orienting responses when food is being used as a stimulus. Similarly, taste is difficult to evaluate during the course of a rapid neurological examination and, like olfaction, is best tested with conditioning methods (Hoffman, 1961).

5. Somatic Sensation

When the so-called distance receptors have been tested, somatic sensation may be evaluated next. The unconditioned monkey's response to somatic stimuli of various kinds (pin, vibration, touch) is similar. It appears that all such stimuli are understood as noxious by the animal, and no differential information is usually elicited from application of a tuning fork or a pin. Pinprick testing is rather simple. The monkey should not be allowed to view the pin or the area that is about to be pricked. Occlusion of vision is easily accomplished by means of a neckpiece which is opaque and prevents the animal from viewing the lower portions of the body. A safety pin can be used to stimulate, and the animal's withdrawal response noted.

If it is desired to have a measure of the strength of stimulus for touch, Von Frey hairs may be used. The animal's responses are generally withdrawal of the limb stimulated, an angry grimace, plus attempts to grab the stimulus. The responses seem to reflect the animal's degree of annoyance more than the strength of the stimulus.

If dermatome testing is to be carried out, a map of the dermatomal distribution of innervation in the monkey should be followed (see Foerster, 1933). Dermatomal distribution in the monkey is quite similar to that in the human, and human maps are found in most neurology texts. In the monkey it is difficult to test the skin of the face and head without the animal viewing the stimulus. This problem can, however, be avoided by blindfolding.

A test of temperature may also be done by using a warm or cold stimulus and applying it in the same manner as the pin. Since it is impossible to tell whether the animal discriminates between these two temperatures, use of either is permissible. Because the central pathways for pain and temperature are different (Crosby *et al.*, 1962), a deficit in

temperature sensation may be found without a deficit in pin or touch sensation; thus it is worthwhile to test temperature as well, even though the methods used are perforce crude. To differentiate the so-called discriminative or epicritic senses from the so-called protopathic senses (Head, 1920) is difficult in the neurological exam. Thus, a monkey with a dorsal column lesion may not be able to appreciate vibration, but if it feels a tuning fork, whether it is vibrating or not, it is likely to withdraw from it. For this reason it seems best to use conditioning procedures, if the special discriminative submodalities within primary somatic sensation are to be tested.

One ability amenable to testing during a neurological exam is that of localizing somatic sensory stimuli on the body surface. Monkeys will localize slightly noxious stimuli (such as small bulldog clamps) by immediately reaching for the stimulus, removing it, and throwing it (usually at the examiner). This is not as refined as "point localization," but does provide a rough index of the animal's ability to localize stimuli on the body surface. Using the same materials for crude assessment of the animal's ability to distinguish simultaneously applied stimuli bilaterally, we restrain the animal's arms and place one bulldog clip at each side of the animal's body at homologous sites. Occasionally the animal will remove both clips simultaneously, but in general first one clip and then the other is removed in rapid succession. The aforementioned test for the animal's ability to perceive two homologously placed stimuli is not as exacting as conditioning methods, which have also been developed (Eidelberg and Schwartz, 1971).

A kind of stereognosis may be tested in the monkey, by allowing the animal to palpate objects which are out of sight. The monkey will frequently put food directly into its mouth, whereas if the object is a nonfood object, the monkey will try to look at it and, if prevented from looking, will discard it. It is important that the animal cannot see the sensory stimuli but that the animal's hand has access to the mouth. If the animal cannot put the food object in its mouth, no attempt to make a discrimination between food and nonfood objects will be made.

In addition to the primary somesthetic modalities, which can be tested as already described, there are a number of complex sensorimotor responses which may also be tested during the neurological examination. Visual reaching, visual placing, and tactile placing may all be readily assessed. When one of these complex reflexes does not occur, it is possible that the receptor and the afferent pathways (the sensory or stimulus arm of the response) are intact but that the central integrative sector of the response, or the efferent (or motor output) segment of the response is defective. With information already derived, however, from

earlier parts of the neurological examination it may be possible to judge which sector of the response pathway is defective.

Visual reaching is tested rather easily in the primate chair by having the monkey reach in several set positions, such as a left position, a center position, and a right position 12 in. in front of the animal's face at eye level. In a standard primate chair the left and right positions are separated by about 18 or 20 in. and the middle position may be set half way from either end. The animal is presented with bits of food on a wire in each of these positions in a fixed order, which includes 12 trials. The 12 trials are then repeated for a total of 24 reaches in a set. The hand which the animal uses for reaching, the method of grasping the food with the fingers, and the accuracy with which the hand reaches toward the food are all noted.

For tactile (guided) reaching the animal may be presented with a container placed out of sight against the skin of the abdomen. In the container are bits of food. The hungry animal will reach into the container and remove the pieces of food one by one, having found the food by feeling about inside the container. Each hand can be tested separately for this kind of stereognosis and tactile reaching.

Tactile placing is tested in both the feet by blindfolding the animal, suspending the animal in the air, and touching the dorsum of one foot against the edge of a horizontal surface such as a table. The animal will then place the foot on the flat surface. It is important to prevent the animal from anticipating the surface and reaching out for it before the tactile stimulus is delivered: irregular brief intervals of time should be used between placing trials. The only adequate stimulus for the tactile placing response is a touch on the dorsum of the foot. The appropriate response is dorsiflexion of the ankle followed by flexion of the knee, and placement of the foot on the flat surface corresponding to the edge. Tactile placing responses may be absent in numerous pathological conditions, as well as in the intact but uncooperative monkey.

As previously pointed out, somatosensory testing in the monkey requires a cooperative and alert animal. An excited or angry animal may exhibit an inappropriate or absolutely no response to an extremely intense stimulus. At times rhesus monkeys, like other animals, enter into a kind of hypnotic state, in which they respond to no stimulus whatsoever, no matter how noxious (Ingram *et al.*, 1936). In this state of "animal hypnosis" or "catatonia," the monkey's eyes are closed and the monkey may assume any posture in which it is placed. Although there is no single method to determine if the monkey being tested is being mildly uncooperative for a given test, this particular state of catatonia is easily recognizable. We have been in the habit of counting as invalid any lack of response when the animal is exhibiting aggressive behavior, when it is

vocalizing, or when it is in a catatonic state as just described. In addition, even if there are no other manifestations, but the animal does not accept tidbits of food, we consider this adequate justification for discounting the animal's performance. When such contingencies arise, the examination or that part of the examination during which they arose must be repeated.

C. The Rat Neurological Examination

A neurological examination of the rat has been devised by Marshall *et al.* (1971). The indications for performing a neurological examination on a rat are in general similar to those for the monkey. Neurological examination gives a rather rapid, broad survey of the animal's sensory abilities and, to some extent, its motor abilities.

For the performance of a neurological examination on the rat, a recommended protocol is first to allow the animal to roam freely on a flat surface. Observations are then made as to the animal's reactions to edges, corners, and obstacles, as well as the number and direction of free choice turns. The rat characteristically investigates space by using the olfactory sense, with a beating movement of the vibrissae. As the rat explores the free field, it should be established whether the animal can use all four of its extremities freely and with equal frequency, is capable of turning its head freely to both sides, and has intact vibrissal movements. These motor responses may be used later to judge the adequacy of the reception of the sensory stimuli. The rat is then held in the hand of the examiner or placed in a box which restrains the animal but allows for freedom of head movement. If placed on a balanced grid so that any movement will cause the animal to fall, it will remain still. Several of the subtests of this examination are illustrated in Fig. 2.

It is our experience that rats perform better when they are held in the hand of an examiner to whom they are accustomed. To test visual orienting responses, bits of white paper are brought in from behind the animal's head over the eyes. The animal usually turns to the paper as soon as it enters the peripheral visual field. It is feasible to use 10 trials on each side with this stimulus without the animal becoming disinterested. An olfactory stimulus such as shaving lotion or bits of chocolate is next brought forward in the same manner (Marshall *et al.*, 1971). Since the animal orients to the direction of the stimulus, a lack of orientation can reflect disinterest. Attempts with various stimuli should be made, as different animals may be interested in different scents. Even five trials from each direction with a single olfactory stimulus is difficult to complete, since an individual animal may stop orienting to specific olfactory stimuli.

For testing the auditory system, clicks presented close to and behind

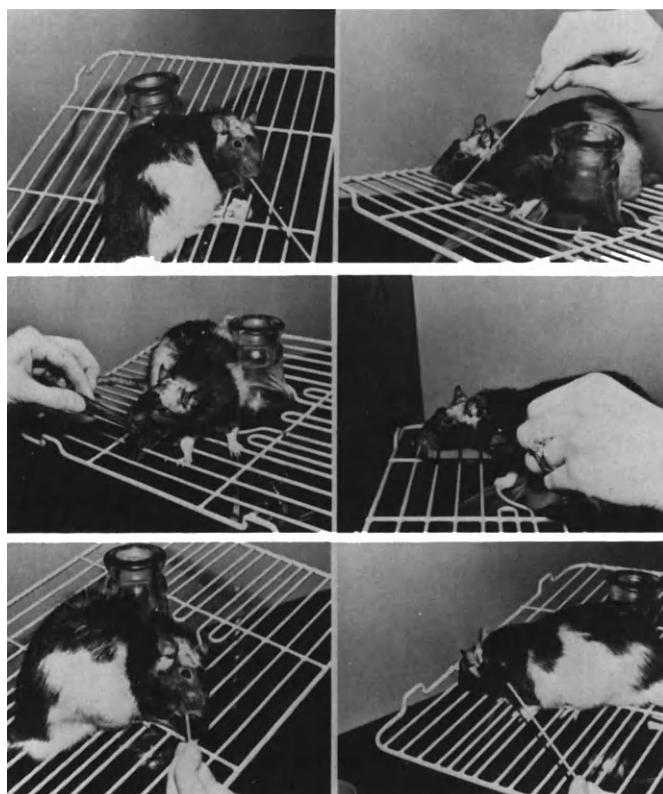


FIG. 2. A rat with unilateral (right) hypothalamic damage shows precise head orientation and biting to various kinds of stimuli (whisker touch, odor, body touch) on the ipsilateral side (*pictures at left*) while neglecting the same stimuli presented contralaterally (*pictures at right*). (From Marshall *et al.*, 1971. Copyright 1971 by the American Association for the Advancement of Science.)

the ear will cause the rat to turn and orient. To test the somatosensory responsiveness of a rat, the animal is held in the hand by the examiner or allowed to stand freely on a grid or table. A pinch with a small pair of forceps is made first on the hind leg, then on the flank, on the forepaw, on the neck, and then on the face. One must always take care that the animal does not view the incoming stimulus. Rats in general not only orient toward the pinching but will most often attempt to bite the forceps. Insensitive skin areas may be determined rather specifically, as long as the stimulus modality is limited to pain. When touch is to be tested, again the animal will orient toward the stimulus. If Von Frey hairs are used, the animal generally orients the most consistently to the heaviest weight of Von Frey hair. The foregoing tests are all field sensitivity tests.

After testing orienting responses to these sensory stimuli, it is valuable to perform placing tests, both visual and tactile. For visual placing, the animal is allowed to view a surface while the head and forelimbs are brought down toward the surface by the examiner. For tactile placing, the animal's viewing of the surface is prevented by holding the chin up with a forefinger. Then both forepaws are brought simultaneously in contact with a horizontal surface. The dorsal surface of the forepaws should be touched. The response should be flexion of the forelimb followed by extension of the foot in the angle directed by the horizontal edge.

If it is important to segregate tactile sensory input to one limb, each limb can be tested alone for this reflex by simply restraining the opposite limb. When either tactile or visual placing is tested, care must be taken to prevent anticipation of and unstimulated reaching for the surface by the animal. An apparatus consisting of prongs protruding from a flat surface is perhaps best used for the evaluation of visual placing. This apparatus insures that the animal uses visual input to guide the placement of the limb on the prong. Placement without guidance is likely to result in the limb's falling into the space between the prongs.

Taste sensation may be grossly tested in the rat by allowing the animal access to solutions of various tastes. If the solution appears to be a nutritive one, the rat will generally drink it (Valenstein *et al.*, 1967; Teitelbaum and Epstein, 1963), whereas if an unpleasant solution is delivered to the mouth, the rat will retract the head and sneeze. Such a test is clearly not quantitative but purely qualitative.

Response to vibrissal stimulation may also be evaluated. This is a special type of somatic sensation carried by the fifth nerve. Much work has been done on the anatomical characteristics of the cortical area where vibrissal sensation is represented. The elaborate arrangement of the "barrel fields," as this parietal cortical region is called, suggests vibrissal sensation is an important sensory modality in the rat and mouse (Van der Loos and Woolsey, 1973).

III. CONDITIONING IN THE EXAMINATION OF SENSORY DEFICITS

Conditioning has been extensively used in animal experimentation since the late 1800s. There are two main types of conditioning, classical and instrumental. Within the instrumental or operant (Thorndike, 1898) conditioning paradigm, the two major subcategories are (1) conditioning with positive reinforcers, which the animal is motivated to obtain, and (2) conditioning with negative reinforcers, which the animal is motivated to avoid. It has long been considered that the neural pathways utilized are somewhat different for each (Kimble, 1961; Russell, 1971). Some inves-

tigators have recently reported that classical conditioning does not require cerebral integrity (Normal *et al.*, 1974), at least in the auditory modality.

The central pathways utilized with operant responses appear to be the same ones used in "conscious" motivated learning (DiCara *et al.*, 1970; Oakley, 1965). For this reason and for others (to be mentioned shortly), operant methods are employed most frequently for the evaluation of sensation. Classical conditioning is less commonly used, but is useful for studying the autonomic components of behavior; it is frequently helpful in avoiding problems with minute-to-minute changes in motivation which are invariably encountered in the early stages of operant conditioning.

A. Operant Conditioning for Sensory Evaluation

Operant conditioning is the method most frequently used for testing sensation in all modalities and in all recognized levels of sensation. In general terms, the advantage of operant conditioning as a means of testing animals for sensory and other abilities is that it takes place in the awake responding animal; therefore, the "subjective" elements of sensation are included in the experimental preparation. With long-term conditioning, stable deficits may be very accurately defined, and ordinal or integral scales of measurement may be achieved. By use of careful experimental design in the stable preparation, precise and reproducible psychophysical measurements may be obtained. Two disadvantages of operant conditioning methods are that they are often too time consuming to be suited for assessing rapidly changing deficits and that they generally assay only one integrated level of one submodality. Also the multiplicity of variables generated by the alert, self-motivated "preparation" must be continuously dealt with when using operant conditioning methods.

1. Vision

Experiments in vision which have been directed at the elementary integrative level include those for determining visual acuity, perception and discrimination of color (Sidley *et al.*, 1965) and visual fields. A simple operant method for testing visual acuity is that used by Weiskrantz and Cowey (1967). These investigators required animals to discriminate striped from solid-colored plaques. When stripes were narrow enough to be chosen as a solid color the threshold of resolution of the stripes was determined. Another primary visual parameter tested by operant means is the difference in brightness of light. Visual fields in the monkey have been studied exactly by the operant conditioning method (Wurtz and Goldberg, 1972). The animal must be trained (for many trials while sitting

in a primate chair) to fix on a central light and to shift its gaze when aware of a second light somewhere in the visual periphery. In the particular experiment cited, the animal's head was restrained while responses of single cells in the superior colliculus were recorded. For accurate visual field testing, however, all that would be necessary would be knowledge of where in the visual periphery the second light was when the monkey first perceived it.

With regard to the testing of more complex visual capacities, again, many methods have been employed. An example of this type of experiment is embodied in the report of Iwai and Mishkin (1969). In that experiment three different kinds of visual discrimination were used: (1) discrimination of patterns, a plus, and a square; (2) discrimination of three-dimensional objects; and (3) a test of visual equivalents. The plus-square discrimination was presented for 30 trials a day in a Wisconsin general testing apparatus (WGTA) until the animal reached the criterion of 90 or more correct responses in 100 trials. This particular experiment entailed preoperative training, then postoperative retraining, and comparison of the two learning scores. After the monkey had learned the discrimination to a 90% criterion it was given small inferotemporal lesions and then retrained to criterion. The pre- and postoperative learning scores were compared for several experimental groups.

The results are of interest in that the animals of some groups showed a deficit in one type of visual discrimination but were normal or relatively less impaired in another type. A second group of lesioned animals was severely impaired on a different type of visual discrimination learning but completely unimpaired on pattern learning. Such a finding is known as a double dissociation of deficits between the groups. The inferences which can be made concerning sensory function of any two given regions of the nervous system which yield doubly dissociated deficits are much stronger than inferences drawn on the basis of simple differences.

The testing of more complex, visually related learning is also amenable to experimental methods. An example of this is the experiment reported by Jones and Mishkin (1972). In that experiment, visual object discrimination as well as the reversal of the object discrimination were tested. Object discrimination appears to have a different behavioral meaning than simple two-dimensional pattern discrimination, light discrimination, or color discrimination. In addition to the object discrimination and its reversal, animals were tested on visual place discrimination. Although both tasks required the visual modality for solution, other abilities of the animals were involved especially in the reversal of position of food reward. Each test is therefore not strictly one for sensation, but rather of a sensory modality, in this case, vision, along with integrative abilities of

a supramodal nature. Thus the discrimination learning method can be useful in testing sensory modalities from the most primary level to the highest integrative level.

2. *Somesthesia*

In testing of the somesthetic system in monkeys, there are several types of experiments which exemplify the levels of information that can be obtained from conditioning studies. Basic modalities within somesthesia mentioned in Section I include vibration, temperature, touch, and pain.

The first example of conditioning for somesthetic testing will concern the tactile submodality. The example given is taken from the experiment by Semmes and Porter (1972), who compared the results of testing tactile discriminations in monkeys with small cortical lesions. In this study, which entailed initial postoperative discrimination learning, animals were first given cortical lesions. A postoperative recovery period of a month was then allowed. Thereafter, tactile abilities were tested, using the apparatus pictured in Fig. 3. The apparatus is described in some detail as it allows for testing of many complex somatosensory abilities.

Before being adapted to the use of the test object, the monkey was trained to reach with the left hand while in the WGTA. This adaptation was easily accomplished by allowing the animal to obtain food only when it reached with its left hand. Usually, one to three half-hour sessions of such reaching were sufficient to accomplish training. Once the left hand was used exclusively, the animal was trained to use the left hand to reach into the tubes of the discrimination apparatus. This was again easily accomplished by moving the bits of food further and further toward the experimenter's end of the apparatus. As soon as the animal had reached into the end of the tube, a side-piece (Fig. 3) was put on the front of the apparatus next to the aperture so that the animal could not, by turning its head or moving it very far to one side of the tube, see anywhere near the end of the tube to the discriminanda. Once the animal had learned to palpate the lever at the end of the tube, remove its hand from a tube in which there was no lever, and place it in the other tube to palpate whether there was discriminanda there or not, testing could be carried on. Initial discrimination was between a plain metal cylinder and one covered with foam rubber. The animal was allowed 30 trials in a single day's session. The positive stimulus, which in the case of the above two discriminanda was the hard metal handle, was presented in the up or down tube. The position of the positive discriminanda was varied in a random order. The criterion on the task was 30 trials out of 30 trials with no errors in a single day's testing session. The animal's learning score was the number of trials required to reach criterion.

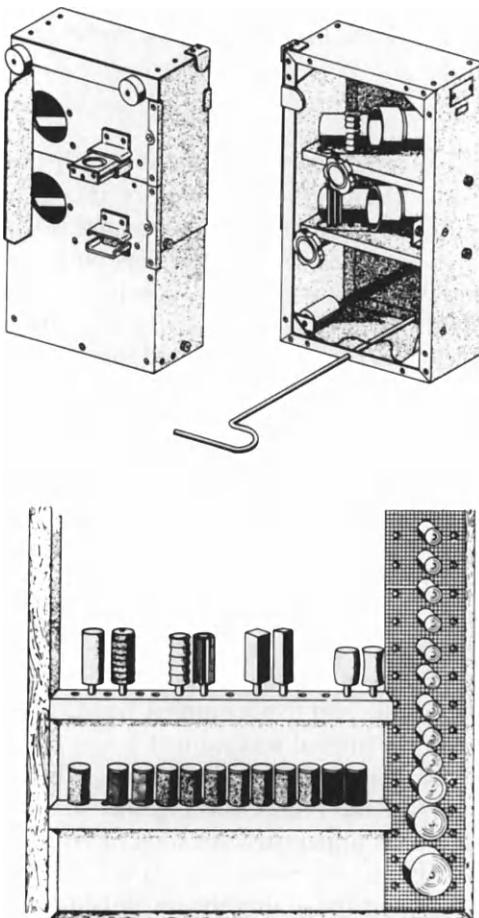


FIG. 3. Apparatus and stimulus objects used for training monkeys on tactal discriminations. *Top left:* monkey's view of discrimination box. Subject reaches between bars of his cage to insert his left arm into one or the other of the tubes; the bend in the tubes (45° plumber's elbows), together with the barrier at extreme left, precludes vision of the stimulus objects. Food drawers are on right, with upper one protruded and containing a peanut. *Top right:* experimenter's view of box. A pair of stimulus objects is shown in place, with the upper one advanced as though the subject had made the correct instrumental pulling response. Small light bulb at inside right is illuminated when either the positive or negative object is pulled forward far enough to barely expose the edge of the food well. Handle inserted through lower frame of box is used to move the box forward and backward on tracks. *Lower sketch* shows some of the stimulus objects used. On upper shelf are, from left to right, Hard-Soft, Horizontal-Vertical, Square-Diamond, and Convex-Concave. At extreme right are a series of cylinders, differing in diameter, used in determining size thresholds. On lower shelf are a series of cylinders, identical in size but covered with different grades of sandpaper, used in determining roughness thresholds. (From Semmes, 1963.)

Other discriminations were presented which were increasingly more difficult, in that the discriminanda became harder and harder to distinguish. (These discriminanda are pictured in Fig. 3.) Next, threshold testing was carried out in these animals with ascending and descending thresholds determined in each test session. When the more finely graded threshold testing method was used in this particular experiment, the learning of two of the animals with precentral lesions became impaired, although they had not been on the discrimination testing. Thus, in an investigation of somatic sensation more complex than tactile discriminations, such as tactile discrimination reversal testing, such a box is also useful (Deuel *et al.*, 1971). Somatosensory reversal, like visual reversal, is beyond the realm of simple sensation but within the realm of sensory modality-linked integrative mechanisms.

A somatosensory submodality which has been most carefully studied by conditioning methods is vibration (Mountcastle, 1975). Several segments of an exacting study have been published in the *Journal of Neurophysiology* (Mountcastle *et al.*, 1972; Talbot *et al.*, 1968). This experiment is particularly good in exemplifying not only the method of studying vibration, but also the use of behavioral testing to obtain true psychophysical measurements. The measurements were made with such a high degree of accuracy that evidence for two different types of receptors involved in the unitary function of vibratory sensation was found. The monkeys in this experiment could detect and respond appropriately to different parameters of tuning forks, with almost the same degree of accuracy as humans if tested over many months. This experimental result was obtained by having the animals trained to great accuracy, having stable response parameters, and testing with only minor alterations of stimulus parameters over multiple trials. The analysis of this experiment was handled with statistical methods appropriate to integral scales of measurement. When data are collected in such a manner, various biases that vary slightly from subject to subject may be factored out (Green and Swets, 1966).

3. Audition

Operant conditioning in the auditory modality, like that in the visual and somesthetic modalities, may be useful in examining residual primary sensory abilities after nervous system lesions, and in testing of more complex auditory-related learning and performance. An example of determination of simple frequency-discrimination thresholds is given in the experiment of Iversen and Mishkin (1973). Monkeys were trained to respond to a low-frequency auditory stimulus and to withhold response to a high-frequency stimulus. The higher stimulus was altered systematically

to provide ascending and descending differences in frequency between the positive and negative stimuli. It was found that animals without their primary auditory cortex had a higher threshold for frequency discrimination than did normal animals but that this deficit was not permanent.

Studies of more complex auditory functions are exemplified by the conditioning experiment of Axelrod and Diamond (1965). Prior to auditory cortex ablation, cats were taught to cross a barrier to avoid shock when a train of clicks was presented to the right ear, and to refrain from crossing the barrier when a train of clicks was delivered to the left ear. Postoperatively, although animals were only mildly impaired in detection of auditory signals, they demonstrated a marked loss of ability to carry out the action appropriate to the ear being stimulated by the train of clicks. Don and Starr (1972) trained monkeys to press a reward bar on the same side as the ear to which a train of clicks was being presented. After mastering this task the same monkeys were required to respond to the reward bar on the side of the ear in which the first or the most intense of binaural clicks was presented. It was found that variations in intensity had little effect upon the animal's choice of side, but that variation in the time interval between the first and second stimuli might alter the animal's performance considerably. The authors were able to separate the effects of varying time intervals from those of intensity on the performance of these monkeys.

B. Classical Conditioning Methods

A classically conditioned reflex is at times extremely useful for evaluating sensory changes. In classical conditioning of the Pavlovian type, the animal's response is not instrumental in changing the reward or punishment (Pavlov, 1928; Bykov, 1957). The sensory modality to be tested is that in which the conditioned stimulus is presented. The unconditioned stimulus should be of a variety or modality which is not contiguous with that of the conditioned stimulus; otherwise great confusion may result.

The orienting response and its autonomic components are generally used as an assay for the adequacy of the sensory input. That is to say, if the dog salivates (the autonomic response which is the behavioral end point), it is likely that the bell is heard. Cautions are given by the Russian school of psychobiologists concerning the relationship of the so-called sensory analyzer (a term more comprehensive than our familiar "sensory system") to the response measured (Sokolov, 1963; Gantt, 1940). This is, in fact, the same caution one must bear in mind when carrying out a neurological examination or operant conditioning. The state of the efferent arm of the response arc (in classical conditioning terms, this means

the portion of the orienting response chosen for the unconditioned response) does not always reflect the state of the afferent arm (in classical conditioning paradigm, the afferent arm would be the sensory system involved in perceiving the conditioned stimulus).

There are other cautions, related to habituation and extinction of the stimulus, which must also be borne in mind when using classical conditioning for testing sensory systems. For a review of this subject and a detailed discussion of methods, Dykman's (1967) chapter in *Methods in Psychophysiology* is an excellent reference.

IV. CORTICAL-EVOKED POTENTIAL TESTING FOR SENSORY DEFICIT

Since both classical and operant conditioning, as well as the neurological exam, depend upon the *response* on an end-point measure, they are not entirely to be trusted in terms of stimulus adequacy. The *response* may not reflect the *signal* as it was actually represented in the nervous system, even at a central level. It is therefore useful to be able to sample the response arc at a point before the efferent portion begins. Evoked potential recording may be useful for such sampling, as amply described in several articles and reviews (Donchin and Lindsley, 1969; Vaughn and Costa, 1964; Thompson and Patterson, 1974).

A physiological stimulus (sound, light, or somatosensory stimulus) is presented to the animal; a recording is made of electrical activity generated by a given brain region. The response to the physiological stimulus is averaged from the ongoing electrical activity of the segment of brain under study. One of the major difficulties in evaluating the results of evoked potential measurements is that sensation has a certain subjective element which is difficult to ascertain from the amplitude or duration of the averaged evoked responses. Indeed, many studies of animals and humans suggest that cortical- and subcortical-evoked potentials vary in amplitude and duration not so much with the strength of the sensory stimulus presented but with the state of arousal or anticipation of the subject matter of the experiment (Donchin and Lindsley, 1966; Eason and Harter, 1969; Chalupa *et al.*, 1976; Carli, 1969).

Although evoked potentials are a useful adjunct to the study of sensation if carried out with classical or operant conditioning methods and the neurological examination, it must be noted that the value of evoked potentials as the sole method for assessing sensation is not clear. After all, a common definition of sensation (Guralnik, 1972) is "the power or process of receiving conscious sense impressions through direct stimulation of the bodily organism." The portion of the definition which is left out

in studying cortical evoked potentials is the "conscious sense impressions." Even though a cortical-evoked potential is recorded from the brain it is not clear just how much conscious perception of the stimulus occurs which elicits the evoked potential. Although sensation may be extensively studied and discussed without reference to factors of conscious perception (Nathan, 1976), the need for a means of assessing conscious perception of the sensory stimulus at issue ultimately arises—and is not satisfied by evoked potential techniques.

V. CONCLUSION

The methods discussed in this chapter are employed for evaluating sensation in animals after lesions, treatment with drugs, electrical stimulation, cooling, or radical changes in environment or development; in general, they are designed to take into account not only the physical properties of the sensory stimulus but also the animal's response to that stimulus. This is of course the essence of sensation as we know it. The study of sensation must include the study of subjective variables. It may be that in the end, the best and most accurate way for testing sensation is a combination of the various methods described in this chapter. However, it is not necessary to the purposes of every experiment to assess every parameter discussed here.

A choice of the method appropriate for the information desired can readily be made. If a broad overview of sensory ability is desired, the neurological exam is in order. If a precise definition of the psychophysical properties of the sine wave of a vibratory fork is to be studied, a long-term conditioning experiment is in order. If the integrity of a peripheral neural pathway is in question, the most appropriate means may be evoked potential studies. Lastly, in the use of all or any combination of these methods, it must always be borne in mind that the animal's state of consciousness and motivation is directly related to the responses which can be elicited when the primary dependent variable of an experiment is a sensory modality.

REFERENCES

- Axelrod, S., and Diamond, I. (1965). Effects of auditory cortex ablation on ability to discriminate stimuli presented to the two ears. *J. Comp. Physiol. Psychol.* **59**, 79-89.
Bannister, R. (1973). "Brain's Clinical Neurology," 4th Ed., pp. 14-19. Oxford Univ. Press, London and New York.
Bignall, K. E. (1974). Ontogeny of levels of neural organization: the righting reflex as a model. *Exp. Neurol.* **42**, 566-573.
Bignall, K. E., and Schramm, L. (1974). Behavior of chronically decerebrated kittens. *Exp. Neurol.* **42**, 519-531.

- Butler, R. A. (1973). The relative influence of pitch and timbre on the apparent location of sound in the median sagittal plane. *Percept. Psychophys.* **14**, 255-258.
- Butter, C., Mishkin, M., and Mirsky, A. (1968). Emotional responses toward humans in monkeys with selective frontal lesions. *Physiol. Behav.* **3**, 213-215.
- Bykov, K. M. (1957). "The Cerebral Cortex and the Internal Organs" (W. H. Gantt, transl. and ed.). Chem. Publ. Co., New York.
- Carli, G. (1969). Dissociation of electrocortical activity and somatic reflexes during rabbit hypnosis. *Arch. Ital. Biol.* **107**, 219-234.
- Chalupa, L., Rohrbaugh, J., Gould, J., and Lindsley, D. (1976). Cortical and subcortical visual evoked potential correlates of reaction time in monkeys. *J. Comp. Physiol. Psychol.* **90**, 119-126.
- Charcot, J. M. (1873). "Le cours sur les maladies du système nerveux faites à la Salpêtrière." Delahaye, Paris.
- Crosby, E., Humphrey, T., and Lauer, E. (1962). "Correlative Anatomy of the Nervous System," pp. 56-111. Macmillan, New York.
- Denny-Brown, D. (1957). "Handbook of Neurological Examination and Case Recording." Harvard Univ. Press, Cambridge, Massachusetts.
- Deuel, R. (1974). Unilateral periarculate lesions cause loss of motor habits. *Prog. Soc. Neurosci.* October 1974, p. 189.
- Deuel, R. (1977). Loss of motor habits after cortical lesions. *Neuropsychologia* **15**, 205-215.
- Deuel, R., Mishkin, M., and Semmes, J. (1971). Interaction between the hemispheres in unimanual somesthetic learning. *Exp. Neurol.* **30**, 123-138.
- DiCara, L. V., Braun, J. J., and Pappas, B. A. (1970). Classical conditioning and instrumental learning of cardiac and gastrointestinal responses following removal of neocortex in the rat. *J. Comp. Physiol. Psychol.* **73**, 208-216.
- Don, M., and Starr, A. (1972). Lateralization performance of squirrel monkey to binary click signals. *J. Neurophysiol.* **35**, 493-500.
- Donchin, E., and Lindsley, D. (1966). Average evoked potentials and reaction times to visual stimuli. *Electroencephalogr. Clin. Neurophysiol.* **20**, 217-223.
- Dykman, R. A. (1967). On the nature of classical conditioning. In "Methods in Psychophysiology" (C. Brown, ed.), Chap. 11. Williams & Wilkins, Baltimore, Maryland.
- Eason, R., and Harter, M. (1969). Effects of attention and arousal on visually evoked cortical potentials and reaction times in man. *Physiol. Behav.* **4**, 283-289.
- Eidelberg, E., and Schwartz, A. S. (1971). Experimental analysis of the extinction phenomenon in monkeys. *Brain* **94**, 91-108.
- Foerster, O. (1933). The dermatomes in man. *Brain* **56**, 1-39.
- Gantt, W. H. (1940). The role of the isolated conditioned stimulus in the integrated response pattern and the relation of pattern changes to psychopathology. *J. Gen. Psychol.* **23**, 3-16.
- Green, D., and Swets, J. (1966). "Signal Detection Theory and Psychophysics." Wiley, New York.
- Guralnik, D., ed. (1972). "Webster's New World Dictionary." World Publ., New York.
- Head, H. (1920). "Studies in Neurology," pp. 333-335. Oxford Med. Publ., London.
- Heilman, K., Pandya, D., and Geschwind, N. (1970). Trimodal inattention following parietal lobe ablations. *Trans. Am. Neurol. Assoc.* **95**, 259-261.
- Hinde, R., and Rowell, T. (1962). Communication by postures and facial expressions in the rhesus monkey. *Proc. Zool. Soc. London* **138**, 1-21.
- Hoffman, P. (1961). The sensory and motivating properties of the sense of taste. In "Nebraska Symposium on Motivation" (M. R. Jones, ed.), pp. 71-110. Univ. of Nebraska Press, Lincoln.

- Hubel, D., and Wiesel, T. (1968). Receptive fields and functional architecture of monkey striate cortex. *J. Physiol. (London)* **195**, 215-243.
- Hubel, D., and Wiesel, T. (1970). Stereoscopic vision in macaque monkey. *Nature (London)* **225**, 41-44.
- Ingram, W., Barris, R., and Ranson, S. (1936). Catalepsy: an experimental study. *Arch. Neurol. Psychiatry* **35**, 1175-1197.
- Iversen, S., and Mishkin, M. (1973). Comparison of superior temporal and inferior prefrontal lesions on auditory and nonauditory tasks in rhesus monkeys. *Brain Res.* **55**, 335-367.
- Iwai, E., and Mishkin, M. (1969). Further evidence on the locus of the visual area in the temporal lobe of the monkey. *Exp. Neurol.* **25**, 585-594.
- Jones, B., and Mishkin, M. (1972). Limbic lesions and the problem of stimulus-reinforcement associations. *Exp. Neurol.* **36**, 362-377.
- Kimble, G. A. (1961). "Hilgard and Marquis' Conditioning and Learning," 2nd Ed. Appleton, New York.
- Lindsley, D. (1969). Achievements, failures and prospects. In "Average Evoked Potentials" (E. Donchin and D. Lindsley, eds.), NASA. Chap. 1, pp. 1-43. U. S. Gov. Printing Office, Washington, D.C.
- Macht, M. B. (1951). Subcortical localization of certain "taste" responses in the cat. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **10**, 88-89.
- Magnus, R. (1924). "Körpersteilung." Springer-Verlag, Berlin.
- Marshall, J., Turner, B., and Teitelbaum, P. (1971). Sensory neglect produced by lateral hypothalamic damage. *Science* **174**, 523-525.
- Mountcastle, V. (1975). The view from within. *Johns Hopkins Med. J.* **136**, 109-131.
- Mountcastle, V., LaMotte, R., and Giancarlo, C. (1972). Detection thresholds for stimuli in humans and monkeys. *J. Neurophysiol.* **35**, 122-136.
- Nathan, P. W. (1976). The gate control theory of pain—a critical review. *Brain* **99**, 123-159.
- Norman, R., Villablanca, J., Brown, K., Schwafel, J., and Buchwald, J. (1974). Classical eyeblink condition in the bilaterally hemispherectomized cat. *Exp. Neurol.* **44**, 363-380.
- Oakley, B. (1965). Impaired operant behavior following lesions of the thalamic taste nucleus. *J. Comp. Physiol. Psychol.* **59**, 202-210.
- Pavlov, I. (1928). "Lectures on Conditioned Reflexes" (W. H. Gantt, transl.). International Publ., New York.
- Rademaker, G. (1931). "Das Stehen, städische Reaktionen, gleichgewichts Reaktionen und Muskeltonus bei Kleinhirnlosentieren." Springer-Verlag, Berlin.
- Ravizza, R., and Masterton, B. (1972). Contribution of neocortex to sound localization in opossum. *J. Neurophysiol.* **35**, 344-356.
- Roffler, S., and Butler, R. (1968). Factors that influence the localization of sound in the vertical plane. *J. Acoust. Soc. Am.* **43**, 1255-1259.
- Ruch, T. (1965). Pontobulbar control of posture and orientation in space. In "Physiology and Biophysics" (T. Ruch and H. Patton, eds.), Chap. 9. Saunders, Philadelphia, Pennsylvania.
- Russell, I. S. (1971). Neurological basis of complex learning. *Brit. Med. Bull.* **27**, 278-288.
- Section of Neurology and Physiology of Mayo Clinic Foundation (1963). "Clinical Examinations in Neurology." Saunders, Philadelphia, Pennsylvania.
- Semmes, J. (1973). Somesthetic effects of damage to the central nervous system. In "Handbook of Sensory Physiology" (A. Iggo, ed.), Vol. II, Chap. 18. Springer-Verlag, Berlin and New York.
- Semmes, J., and Porter, L. (1972). A comparison of effects of precentral and postcentral cortical lesions on somatosensory discrimination in the monkey. *Cortex* **8**, 249-264.
- Sidley, N., Sperling, E., Bedarf, E., and Hiss, R. (1965). Photopic spectral sensitivity in the monkey. Methods for determining and initial results. *Science* **150**, 1837-1839.

- Snyder, M., Hall, W., and Diamond, I. (1966). Vision in tree shrews after removal of striate cortex. *Psychonom. Sci.* **6**, 243-244.
- Sokolov, Y. N. (1963). "Perception and the Conditioned Reflex" (S. W. Waydenfeld, transl.), pp. 22-31. Macmillan, New York.
- Sprague, J. M., and Meikle, T. H. (1965). The role of the superior colliculus in visually guided behavior. *Exp. Neurol.* **11**, 115-146.
- Stevens, S. (1961). The psychophysics of sensory function. In "Sensory Communication" (W. A. Rosenbluth, ed.), Chap. 1, pp. 1-33. MIT Press, Cambridge, Massachusetts.
- Stewart, D., and Riesen, A. (1972). Adult vs. infant brain damage: behavioral and electrophysiological effects of striatectomy in adult and neonatal rabbits. In "Advances in Psychobiology" (G. Newton and A. Riesen, eds.), Vol. 1, pp. 171-211. Wiley, New York.
- Talbot, W. H., Darien-Smith, I., Kornhuber, H., and Mountcastle, V. B. (1968). The sense of flutter-vibration: comparison of the human capacity with response patterns of mechanoreceptive afferents from the monkey hand. *J. Neurophysiol.* **31**, 301-334.
- Teitelbaum, P., and Epstein, A. (1963). The role of taste and smell in regulation of food and water intake. In "Olfaction and Taste" (Y. Zotterman, ed.), pp. 347-360. Pergamon, Oxford.
- Thompson, R. F., and Patterson, M. (1974). "Bioelectric Recording Techniques." Academic Press, New York.
- Thorndike, E. L. (1898). Animal intelligence: an experimental study of the associative processes in animals. *Psychol. Monogr.* **2**, No. 8.
- Tilney, F. (1933). Behavior in its relation to the development of the brain. *Bull. Neurol. Inst. N.Y.* **3**, 252-358.
- Valenstein, E. D., Kalcolewski, J. W., and Cox, V. C. (1967). Sex differences in taste preference for glucose and saccharin solutions. *Science* **156**, 942-943.
- Van der Loos, H., and Woolsey, T. (1973). Somatosensory cortex: structural alterations following early injury to sense organs. *Science* **179**, 395-397.
- Vaughn, H., and Costa, L. (1964). Application of evoked potential techniques to behavioral investigation. *Am. N.Y. Acad. Sci.* **118**, 71-75.
- Venura, T., and Cohen, B. (1972). Vestibulo-ocular reflexes: effects of vestibular nuclear lesions. *Prog. Brain Res.* **37**, 515-528.
- Walsh, F. B. (1957). The visual pathways: diagnosis of lesions situated at various levels. In "Clinical Neuro-ophthalmology," Chap. 1, pp. 1-74. Williams & Wilkins, Baltimore, Maryland.
- Weiskrantz, L., and Cowey, A. (1967). Comparison of the effects of striate cortex and retinal lesions on visual acuity in the monkey. *Science* **155**, 104-106.
- Welsh, K., and Stuteville, P. (1958). Experimental production of unilateral neglect in monkeys. *Brain* **81**, 341-347.
- Wurtz, R., and Goldberg, M. (1972). Activity of superior colliculus in behaving monkey. II. Effect of attention on neuronal responses. *J. Neurophysiol.* **35**, 561-574.

Chapter 5

Assessing the Effects of Early Experience¹

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I. INTRODUCTION

There are several historical roots to modern-day research on the effects of early experience with animals. One branch involves basic descriptions

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of developmental behavior patterns of various animal species. The basic procedure is that of detailed observations with little or no intervention by the investigator, and the prime interest is in an ethological description of the ontogeny of a species. Another approach stems from the interest in testing hypotheses derived from Freudian psychoanalytic theory. These hypotheses could not be put to experimental test with humans but could be investigated experimentally with animals. The third source of stimulation for this field—and the one that has most powerfully influenced present-day research—is the viewpoint that the experiences of the immature and growing organism during its development act to organize behavioral and neurophysiological processes, and that this organization influences the animal throughout its life. This position was put forth in Hebb's classic book, *The Organization of Behavior*, published in 1949.

In order to appreciate the proper procedures and the kinds of research problems which arise in this field, it is first necessary to present a perspective on early experience and developmental research. Since most laboratory research in early experience uses rodents, the rat will be used as the reference animal in the discussion of principles and procedures in this chapter, unless otherwise specified.

II. THE DEVELOPMENTAL CONTINUUM

Figure 1 shows developmental life lines for a female rat and her litter based upon several major biological and behavioral markers. The life lines start with conception and terminate with death. Between these two events are key happenings including birth, weaning, puberty, pregnancy, parturition, and the weaning of young.

The first thing to note is that the newly formed organism receives its full genetic complement from both parents at the moment of conception. This must always be kept in mind, since a change in the genetic substrate may markedly influence the effects of a subsequent environmental variable (i.e., genetic-environmental interactions are likely to occur in early experience research). Also included in Fig. 1 are the time boundaries for three developmental regions which have been experimentally studied: prenatal, infantile, and postweaning. Prenatal effects are those which are brought about by stimulation between conception and birth. The period of infantile stimulation is between birth and weaning. The interval for post-weaning effects starts at weaning but does not have an established upper boundary, though puberty may be used as such a reference point in many studies.

The definitions of these three time periods are somewhat arbitrary but reasonable in that conception, birth, weaning, and puberty represent

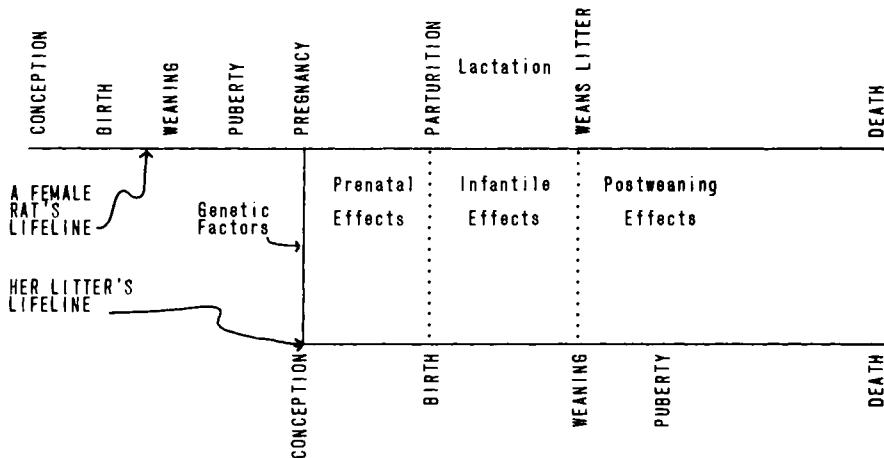


FIG. 1. Developmental life lines for a female rat and her litter, starting with conception and terminating with death.

nodal points of major biobehavioral changes. Also, much of the experimental research has involved studies within these three time zones.

Figure 1 can also be used to illustrate the differences between and the complementary nature of *early experience paradigms* and *developmental paradigms*.

A. Early Experience Paradigms

In its most basic form, an early experience experiment relates stimulation given during one of the three developmental regions shown in Fig. 1 to measures of behavioral and/or biological events in adulthood. The following conditions must be satisfied for an experiment to qualify as an investigation of the effects of early experience:

1. An experimental group receives stimulation during one of the three developmental periods (prenatal, infantile, postweaning) or some combination of them.
2. There are one or more appropriate control groups in the study.
3. An interval of time occurs between the termination of the experimental treatment and the test for the effects of the treatment. All animals must be treated alike during this interval.
4. There must be evidence that the effects, if significant, are due to the "early" experience and not just to prior experience.

The first two conditions listed are the usual requirements of any ex-

perimental study in which one is interested in evaluating the effects of an independent variable, with the added stipulation that the variable must occur some time during the early development of the organism.

The third stipulation—that a gap of time must ensue between the end of the experimental treatment and the test for effects—is necessary to show that the experimental treatment has had relatively long-term (i.e., "permanent") effects. If there were no time interval between the end of the treatment and the measurement of the end points, then any differences obtained between groups could be attributed to the immediately preceding effects of the differing treatments. One of the powerful and exciting phenomena which has come out of early experience research is the documentation that the effects will often influence the animal throughout its lifetime and can also affect the offspring and grandoffspring of female rats.

The final requirement—to show that the effects are due to early experience rather than just prior experience—is necessary in order to establish that the status of the organism's developmental processes (behavioral, endocrinological, physiological, etc.) are an important condition in mediating the effects of the experimental variable. This is particularly important when working with postweaning stimulation since the animal's central nervous system is relatively mature and chronological age may not be relevant. This is what Rosenzweig and his colleagues (see Rosenzweig, 1971) have found in their research on postweaning environmental enrichment and brain changes. Theoretically, the matter of prior versus early experience is not much of an issue when one is working with prenatal or infantile stimulation because so many biological systems are immature. Experimental evidence supporting this position has been reported by Levine and Otis (1958) and Lindholm (1962), who have found that handling and shock prior to weaning have different effects than when administered after weaning.

B. Developmental Paradigms

In an early experience experiment, measures are generally taken in adulthood, at which time most behavioral and biological processes are relatively stabilized. If significant effects are found and the experiment has been properly designed and conducted, then the researcher can relate the adult end points to the independent variables which were manipulated during development. However, this tells the researcher nothing about the *immediate* effects of the independent variables upon behavioral and biological processes. Such information may be quite important, since the

immediate changes brought about by the experimental intervention may be part of the pathway involved in mediating the long-term effects.

In order to study the immediate effects of an experimental procedure, one uses a developmental experimental design in which animals receive the experimental treatment for one or more days during early development (with appropriate control groups) and are then measured immediately or within a day or two thereafter. If this is done at different ages in development, one is able to plot developmental curves for the effects of the experimental treatment relative to the nontreated control. Such data offer suggestions to the researcher concerning possible mechanisms underlying the effects of early stimulation, but do not establish that the changes found immediately after stimulation are the causes of the differences found in adulthood. To isolate causal events in the sciences is always difficult and is doubly so when working with developmental phenomena because of the continuous and discontinuous growth and changes taking place within the maturing organism.

With this background we can now turn to an examination of the problems involved and the procedures needed to conduct valid experimental research using early experience and developmental paradigms.

III. GENERATING ANIMALS FOR EARLY EXPERIENCE RESEARCH

The production and management of subjects for experimental purposes is a serious matter. All too often researchers have made major errors in generating their animals, thus invalidating their research findings. These errors are much less likely to occur today than 10 to 15 years ago, but they still can be found in occasional published journal articles. When I find such an article, I generally skip over it since the data cannot be trusted. I cannot emphasize too strongly the need for very careful consideration of the subject population to be used in early experience studies. In actuality, there are two populations which the careful researcher must worry about: the mothers of the experimental subjects, and the subjects themselves. We will next consider the issues concerned with obtaining a population of mothers. This will be followed by a discussion of procedures required to insure a valid population of experimental subjects.

A. *Obtaining Females to Be Used as Mothers of the Experimental Animals*

It is necessary to start one's research by obtaining a homogeneous population of females whose offspring will be the experimental subjects.

In our laboratory the mothers are always females who have been reared as nonhandled controls (see below for definition of nonhandled controls) for their entire life. In addition, their mothers were nonhandled controls, as were their grandmothers, etc. The need for homogeneous females has been experimentally documented by Denenberg and Whimbey (1963b) and by Denenberg and Rosenberg (1967), who have shown that mothers and grandmothers who have had differential experiences in early life will have offspring and grandoffspring whose behavior and physiology differ solely as a function of the experiences of their mothers and grandmothers.

We breed our own animals, and it is for this reason that we are able to insure such great experiential homogeneity. However, this requires a large laboratory, and many researchers do not have the capability of maintaining their own breeding colony. In that situation, breeders have to be purchased from outside. A common procedure—and one to be avoided at all costs—is to purchase pregnant females, have them shipped to the laboratory where they give birth, and use their offspring as the subjects in the experiment. It is immediately obvious that there must be enormous stresses placed upon an animal when it is removed from its typical environment, packaged into a carton with other animals, sent by truck, rail, bus, or plane to another destination, and, eventually, introduced into a strange laboratory where lighting cycles, food, ambient temperature, cleaning schedules, and cages are probably very different from what these animals were used to in the commercial colony from which they were purchased.

Sufficient research has been done to show that stresses during pregnancy will affect the developing fetus (Joffe, 1969), raising serious questions about the value and validity of any experiment using animals obtained in this manner. To use pregnant animals brought in from another facility is rather like the situation of a chemist who does his assays with dirty test tubes. It may be possible to get some useful information from the assay, but it is highly questionable whether the results have any degree of generality, and certainly one's colleagues would not hold the chemist in much respect. The same thing is true for those who do not know how to generate animals appropriately for early experience studies.

What is to be done, then, if it is necessary to purchase animals? The recommendation here is to purchase adult females who are homogeneous with respect to parity (i.e., all are nulliparous or primiparous) and age (a range of 1 month is reasonable with the rat) and who have not been used in any experiment. After they arrive, they should be allowed 2 to 4 weeks to settle into the new laboratory, after which they can be bred and their offspring used for early experience studies.

B. Arranging Litter Conditions

1. Definition of Age

In most laboratories, maternity cages containing pregnant rats are inspected once or twice a day to check for births. If we find a litter born in the morning, we designate the date of birth as the previous day unless it is apparent from inspection that birth has occurred quite recently. Evidence of a recent birth includes wet or very dark blood on the shavings, and lack of milk in the stomach of the pups. If the pups have been born recently, the date of birth is listed as the date when they were found. The date of birth is always considered to be day 0 in our laboratory.

2. Litter Size

The laboratory rat can give birth to as many as 16 pups, and several research studies have shown that variation in litter size will bring about changes in maternal behavior, offspring body weight, and offspring behavior (Seitz, 1954, 1958). Thus, if litter size is allowed to vary naturally this will result in increased variability and an increased error term, thereby reducing the power of one's experiment and making it more difficult to find significant effects. For these reasons the recommendation is to keep litter size constant.

The median litter size in our laboratory is 10, and we standardize all litters at eight pups. Litters are cut back to eight on day 1 of life and are also sexed at this time. The sex ratio is determined by the nature of the study. In some experiments we maximize males, in others females, and in still other studies we require that each litter contain at least two or three males and two or three females.

If a litter contains less than eight pups or the sex ratio within the litter is unacceptable, we try to avoid using the litter in our experiment. We may foster a female to a litter to bring it up to size if we are only going to use the males in an experiment (and vice versa). The reason for not cross-fostering is that this adds variability to the experimental structure by having pups from different mothers, and thus from differing genetic and prenatal conditions, added to a litter. The principle to follow is to minimize variability whenever possible.

There are times, admittedly, when the number of breeders is low and the researcher cannot afford to discard litters even though the number born within a litter is inadequate. In that instance there is no choice but to foster animals to make up the litter size to eight. If this is done, however, notes should be made concerning what was done to that litter, and the performance of the pups should be examined later to determine whether

they are deviant from the other animals within the same experimental group.

Now that we have considered how to set up our population of breeders and how to standardize litter size, we can turn to a discussion of problems and procedures involved in early experience studies during the three developmental regions specified in Fig. 1.

IV. STIMULATION DURING THE PRENATAL INTERVAL

Even though the developing embryo and fetus is not directly accessible to the researcher, it is possible to carry out experimental investigations of the effects of stimulation during this interval. One can always introduce drugs or other pharmacological agents into the pregnant female at various times of gestation and study the consequences of this upon the offspring. A variation of this procedure is to undernourish or malnourish females during gestation to determine the effects of lack of adequate nutrients upon the offspring's behavior and physiology.

It is not necessary to use such blunt procedures as drugs or food deprivation. Thompson (1957) has shown that "anxiety" during pregnancy will also influence the offspring. Thompson's procedure was to take nonpregnant females, condition them to associate a buzzer with electric shock, and teach them to avoid the shock by running into a safe chamber when the buzzer sounded. When pregnant, the animals were placed in the same apparatus and the buzzer sounded, but the escape door was locked so that the animals could not get out of the chamber which had previously been associated with shock. The animals never received shock in this situation, and Thompson assumed that any disturbance resulting from this had to be due to the psychological variable of anxiety generated by the sound of the buzzer. He cross-fostered his animals at birth and tested them later in life. He found that the animals who, as fetuses, had been carried by mothers that received conditioning during pregnancy were more emotionally reactive than offspring of mothers who had not undergone a conditioning regimen.

In order to establish that an effect is due to a prenatal event, *it is necessary to cross-foster the animals at birth*. If this is not done, one is not able to determine whether the effects are limited to the prenatal period. One should cross-foster all animals to a standardized group of homogeneous mothers in order to insure constancy of postnatal conditions. Thus, all foster mothers should have given birth within 1 to 5 days previously so that their maternal behavior and milk is similar in kind to that of the natural mother. They should be of the same parity and should not vary more than a month in age.

In the vast majority of work cross-fostering is done approximately 24 hours after birth. That is, the natural mother takes care of its own young for the first day of life prior to fostering. This has been considered to be quite adequate until recently, when research has shown that the behavior of the mother toward the pups within the first 12 hours of life can have important effects upon the pups' potential for survival as well as upon its body weight (Denenberg *et al.*, 1963, 1976). Thus, it may be necessary to cross-foster within the first few hours after birth if one is concerned that the behavior of the mother toward its own pups within the first 24 hours could influence their subsequent performance. Probably the best procedure to follow is to cross-foster at 1 day of age to see whether any effects are found. If so, a second experiment could be carried out in which the fostering is done within the first hour or two after birth. If the former finds significant effects and the latter does not, this would suggest that the behavior of the natural mother toward its pups during the first 24 hours was the key event influencing subsequent performance, rather than the prenatal intervention.

V. STIMULATION DURING THE PERIOD OF INFANCY

The two major classes of stimulation administered to animals during the period between birth and weaning involve physical manipulations of the pups themselves and social manipulations of the mother and/or pups. We will consider each in turn.

A. Physical Manipulations

1. Handling

The most common procedure for manipulating animals is a technique called *handling*. This consists of placing each pup separately into a Number 10 tin can partially filled with shavings, leaving the animals in these containers for 3 min., and then returning them to their mother. This procedure is repeated once every day generally between birth and weaning. The use of Number 10 cans is governed by the fact that these are available in plentiful supply at college and university kitchens and can be obtained at no cost. The important thing is to place shavings in the cans so as to standardize the surface upon which the pup is lying. Some laboratories use a series of small boxes made of plywood as their handling containers. It is important to be aware that different containers can affect the temperature of newborn pups. Temperature loss from being placed on a metal surface will be much greater than from being placed on a wood or shavings surface.

If animals are to be handled on day 1 of life, they are placed into the

handling containers immediately after they have been sexed and reduced to a litter size of eight. They are then returned to the home cage and the mother.

In our laboratory we always leave the mother in its home cage and remove the pups by pulling out the sliding tray which is the floor of the cage. In some laboratories the procedure is to remove the mother first, place it into a holding cage, then place the pups into the handling cans. The mother might remain in the holding cage until the pups are returned or it might be placed back into its own nest box immediately after the pups have been placed in the handling containers.

2. Shock

Another common procedure used for the stimulation of animals is to administer electric shock. A serious difficulty with the use of this technique involves changes in the animal's size and electrical characteristics as it develops. For example, the electrical resistance of the pup changes with age. Our procedure has been to use a shocker with a very high internal resistor (2.25 megohms) so that the variable resistance of the pup, when added in series to that of the internal resistor, will have essentially no influence upon the milliamperes of current going through the animal.

3. Controls

Whenever there is a group which is handled or shocked, there needs to be an appropriate control group. At first glance, one would think that the appropriate control for a group which received electric shock is to have another group which is placed into the shock apparatus but does not get any current. But this is similar to a handled group. If the shocked and handled groups do not differ from each other, the only logical interpretation is that the addition of electric shock to the stimulation brought about by handling did not cause a change. This does not answer the question: Does stimulation in infancy have an effect? To answer that question it is necessary to have a nondisturbed control group (often called a nonhandled group). The importance of such a group was first shown by Levine *et al.* (1956) who found that a group of rats which received shock in infancy and a group which was removed and placed into the shock apparatus but did not receive shock (i.e., was handled) both differed significantly from the nondisturbed control group but did not differ from each other.

In order to insure that our nondisturbed controls are indeed undisturbed, we never change the shavings in the cage after a litter has been born. Also, the food hopper is attached to the outside front door of the cage as is the water bottle, so that feeding and watering can be done without opening the cage door. In some laboratories the shavings are

changed once or several times prior to weaning, food is placed inside the cage, either in a tray or on the floor, and water may also be supplied inside the cage. The recommendation is to avoid these procedures since they disturb the mother and her pups by increasing stimulation. Animal husbandry variables, although they appear particularly uninteresting, have been shown by Denenberg and Whimbey (1963a) to significantly influence the effects of early experience variables, and thus precautions should be taken to minimize any disturbance of the nonhandled control litters.

4. Stimulating Part of a Litter

An experimental design which appears logical on paper is one in which some of the animals within a litter receive stimulation while the others remain as undisturbed controls. This is not possible. If any member of a litter is disturbed, one must assume that all the pups will be disturbed. This is because the experimental stimulation given to one animal will change its behavior and physiology and thus will change the nature of its interactions with its littermates and its mother. In turn, the interactions of the remaining littermates and mother will now be different. For these reasons, it is necessary that all animals within a litter be given the same experimental treatment.

If one wishes to try out an experimental procedure which involves differential treatments of animals within a litter, it is necessary to have other litters in the study in which all animals within the litter receive just one treatment. A comparison of those reference litters against the animals that received differential treatment within litters would provide an empirical assessment as to whether the within-litter procedure was appropriate. As always, a nondisturbed control group is necessary as an ultimate reference point.

B. Social Stimulation

The two social units which can be varied within a litter are the mother and the pups. Typically the father is not present in rat litters after it has served the function of "servicing" the female.

1. Pups

The major variable involving the pups is litter size. This has been varied from one through at least 16 pups. This is quite a complex variable since it influences the behavior of the mother, the amount of milk she secretes, and the number of conspecifics with which each pup interacts. The litter size variable has been found to have wide effects upon a variety of behavioral and biological measures (Seitz, 1954, 1958).

2. Mothers

Many different procedures have been used to manipulate the mother. These include varying prior experience (e.g., primiparous versus multiparous), selecting females on the basis of individual difference measures (e.g., emotional versus nonemotional, as measured by an open-field test), and direct experimental intervention (e.g., reducing the food supply during lactation). The list of experimental variables is much too long to catalog, and the ones cited are to give the reader a flavor for the variety involved.

3. Interspecies and Intraspecies Cross-Fostering

Cross-fostering has been used in several interesting ways as a method for varying the nature of the mother. One method has been to foster across species. Most of this work has involved fostering newborn mice to lactating rat mothers (Denenberg, 1970; Denenberg *et al.*, 1964). These two species are sufficiently similar in their maternal behavior and the quality of their milk that the rat can effectively take care of the mouse pup. This procedure of fostering across species breaks the inherent confounding present when one fosters within a species. In this manner one is able to separate the genetic and prenatal effects upon behavior from the postnatal effects. From this work Denenberg and his colleagues were able to show that aggression, activity, passive learning, and the corticosterone response to a novel stimulus were all modified by the experience of being reared by a rat mother.

A variation on this procedure is to foster within a species but across different strains or breeds. For example there are numerous strains of mice, and many of them differ on behavioral parameters. By fostering across strains it is possible to determine whether the behavior of the mother is a critical determiner of these behavioral differences. A good example of this approach is the work of Southwick (1968), who showed that the aggressive behavior of mice from a passive strain was increased if they were reared by a mother from an aggressive strain.

4. Rat "Aunts"

Rosenblatt (1967) has reported that both male and female rats who are exposed to young pups eventually became "sensitized" to them and act maternally toward them by nest building and by retrieving and/or grooming the pups. However, the sensitized female does not have milk for the pups. Denenberg *et al.* (1969) used this technique to generate rat "aunts" to give maternal care to mouse pups while the natural mouse mother was the supplier of the milk.

VI. STIMULATION DURING THE POSTWEANING PERIOD

The most powerful variable we have isolated for influencing animals after weaning revolves around conditions of housing and the concept of an "enriched environment" (also called a free environment). This concept was originated by Hebb (1949): it was his experimental procedure to induce rats to interact with a relatively complex world at the time in development when they were first prepared to cope independently with their world, that is, at the time of weaning. There are various forms of an enriched environment, but all of them have large physical size (up to 4 feet square), and contain a number of "toys" or playthings. Animals reared in such an environment, as compared to control animals reared in typical laboratory cages, are better at a variety of learning, perceptual, and problem-solving measures. These animals also differ on several parameters of brain chemistry and brain morphology, although these physiological changes are not causally related to the differences in learning and perceptual performance (Rosenzweig, 1971; Rosenzweig *et al.*, 1972; Krech *et al.*, 1962).

The opposite extreme of the enriched environment is to house weanling animals singly in small laboratory cages where they receive minimal sensory and perceptual stimulation. This has been shown by Ader (1965) and Ader and Friedman (1964) to increase emotional reactivity.

One major methodological advantage of working with rats after weaning is that litters can be split and different animals from the same litter can be randomly assigned to different treatment conditions. This procedure has consistently been used by Rosenzweig and his associates, who place one male from a litter into isolation, another into laboratory cages with other rats, and still a third littermate into the enriched environment.

For literature reviews of the effects of early experience, see the papers by Denenberg (1969, 1975), Levine (1962), and Russell (1971).

VII. STATISTICAL ANALYSIS OF EARLY EXPERIENCE EXPERIMENTS

An experiment is not complete until the findings have been properly evaluated. An improper analysis of the data can cause one to draw incorrect conclusions about the experiment. In early experience research an improper analysis is frequently made because the researcher uses the wrong statistical model to assess the data. This happens often enough to warrant a discussion of the topic.

The error occurs when whole litters of animals receive the same ex-

perimental treatment and then several or all of the animals from each litter are measured on the same dependent variable. In such a situation the researcher can do one of three things: (1) ignore the litter classification and treat each subject as an independent observation; (2) get a litter mean by taking the average of all animals from the same litter and using this mean in subsequent statistical analyses; or (3) carry out a statistical test to determine whether the individual subject or the litter mean is the proper unit to use for the evaluation of the experiment. If the researcher chooses the first option, this will generally result in an incorrect statistical analysis which may yield invalid results. The second and third options are both statistically correct, with option 2 being more conservative.

The error in the first option is in assuming that each subject from a litter is an independent observation. Animals from the same litter are not expected to be independent of each other because they share (1) a common genetic background; (2) a common intrauterine environment; and (3) common postnatal maternal and social environments. Because of these factors, it is highly likely that there are positive correlations among littermates with respect to many behavioral and biological characteristics. The presence of positive correlations means that littermates are not statistically independent, and to treat them as such will cause differences that are due to chance to appear to be "significant."

How should an early experience design be evaluated? In any statistical design it is necessary to determine the random element and to use that as the source of error variance to evaluate treatment effects. In an early experience design where all animals within a litter are assigned to the same treatment condition, the random element is the litter itself. Thus, the litter becomes the appropriate unit for statistical analysis in evaluating the effects of an experimental treatment. However, even though the animals within a litter were not assigned at random to treatment conditions, they are still a random element since they make up the litter. If there are no significant litter effects, then it is legitimate to use the individual subject as the unit for statistical analysis. The statement that "there are no significant litter effects" is equivalent to stating that there is no correlation among littermates with respect to the end points being evaluated. To put this into somewhat more formal statistical language, we have what is called a *nested* or *hierarchical* design in which there are two random variables: (1) Litters within Treatments, and (2) Subjects within Litters within Treatments. The former term has to be used as the measure of error variance unless a statistical test establishes that this is not a significant source of variability.

We can illustrate these concepts by considering a simple design. Assume that we are interested in the effects of handling in infancy upon

open-field behavior. Five litters are randomly assigned to the handling condition, and five others are nonhandled controls. Let us assume that each litter contains eight subjects with at least three males within each litter. Suppose we were to test three males from each litter in adulthood. Table I shows the usual analysis of variance table for this design, except that we have also included the expected mean squares (EMS) for the design based upon the assumptions that Treatments is a fixed effect and that Litters and Subjects are both random variables. We can see that the EMS for the Treatments contains sources of variance attributable to Subjects and to Litters. In order to evaluate the Treatment effect, it is necessary to have in the denominator of our F test a mean square which contains all terms except that due to the treatment itself. Thus we see from Table I that MS_{trt} must be divided by MS_{lit} to form a valid F test.

However, it may be that the Litter effect is zero. If so, then σ_L^2 disappears from the EMS, leaving us with σ_E^2 for the error term. In order to determine whether the Litter effect is significant, we carry out a preliminary statistical test on the model (Winer, 1971). This test is to divide MS_{lit} by MS_{sub} . If the Litter effect is significant at or beyond the 0.25 level, then MS_{lit} has to be used to test MS_{trt} with 8 degrees of freedom. However, if the Litter effect is not significant at the 0.25 level, we may conclude that its effect is zero and eliminate it from our statistical model. This allows us to pool subjects and yields MS_{sub} as our error term with 28 degrees of freedom.

In our own research we typically find significant litter effects when working with rats, but small or no litter effects when working with mice. Presumably this is because the mice are inbred and extremely homogeneous genetically, whereas there is still significant genetic variability in our rats.

TABLE I
Analysis of Variance Table and Expected Mean Squares for a Nested Design Containing Litters and Subjects within Litters

Source	<i>df</i>	MS	Expected mean squares
Treatment	1	MS_{trt}	$\sigma_E^2 + 3\sigma_L^2 + 15\sigma_T^2$
Litters within Treatment	8	MS_{lit}	$\sigma_E^2 + 3\sigma_L^2$
Subjects within Litters within Treatment	20	MS_{sub}	σ_E^2
<i>Total</i>	29		

In designing an experiment, it is always best to assume that there will be a significant litter effect. The experiment should be set up so that there are sufficient degrees of freedom to make a useful test of the experimental variable based upon litter means alone. For a further discussion of this topic, see the paper by Abbey and Howard (1973).

VIII. USES OF EARLY EXPERIENCE PARADIGMS

Two major uses of early experience experiments have been emphasized, namely, to establish that effects are limited to a certain interval in early life and that the effects are relatively permanent ones. A third use of early experience studies is to obtain information and insights into the key events occurring in ontogeny. For example, experimental studies have shown that manipulating the mother during the period of infancy or administering various forms of stimulation in the infantile period affects behavioral measures of emotionality and the hormone corticosterone from the adrenal gland.

Other studies have shown that the most powerful manipulation one can use with a newly weaned animal is to place it into an enriched environment. This will have a marked effect upon improving problem solving, learning, and perceptual capabilities. These various findings, taken together, suggest that the interval between birth and weaning is primarily involved with the development of affective or emotional behavior, whereas postweaning stimulation has its major impact upon intellectual behavioral processes.

These designs, therefore, establish the phenomenon of early experience and offer suggestions as to the processes involved. However, it is not possible to isolate the mechanisms involved from such a design. In order to do so, it is necessary to consider a different experimental design—a developmental paradigm.

IX. DEVELOPMENTAL PARADIGMS

In a developmental paradigm, we measure the animals at various ages during early development in order to determine trends in behavioral or biological processes and to see how these trends are modified by experimental interventions. There are two kinds of designs that we can use, one involving independent groups of animals which are measured only once, and a second involving repeated measurements for the same groups of animals.

In the first design an experimental treatment is administered to one group of animals daily up until the day of testing, while a control group

receives no experimental intervention until the day of testing. After the animals are measured the complete litter is discarded. The advantage of the design is that each group is "pure" in the sense that it has had no prior test experience before the day of evaluation. However, it is a very expensive design in terms of numbers of litters needed.

A design which avoids that problem is one in which litters of animals are tested repeatedly over several days. The obvious disadvantage is that the prior test experience may have an effect on the behavior which is being measured. Therefore, the repeated measures design cannot be used unless the measurement procedure itself does not influence the behavior being studied. This is often useful when the researcher is making observations but not intervening. For example, consider a situation where animals are manipulated by some experimental procedure daily in infancy (e.g., handling or shock) and then the maternal behavior of the mother toward the pups is evaluated for the 2 hours after the animals are returned to the nest box. If the evaluation of maternal behavior is done by observational techniques which do not affect the mothers or their pups, then the repeated measures design is a valid one giving meaningful results.

X. OTHER FACTORS

There are several other items which should be briefly noted.

A. Critical Period Experiments

Without getting involved in the theory underlying the critical period hypothesis, the experimental question is whether one can isolate a relatively short period of time in development during which a particular treatment has a measurable effect. One approach is to limit the experimental stimulation to one or a few days and have a number of groups which receive the same stimulation at different ages. If one or a few groups show an effect from the experimental treatment while other groups do not, this suggests the possibility of a critical period. [See Denenberg (1968) for a discussion of the experimental conditions required to isolate a critical period and for a review of the literature. Theoretical discussions can be found in Scott (1962), Scott *et al.* (1974), and Denenberg (1964).]

B. Choice of Species

This chapter has focused upon the rat as the major research animal for early experience work. The principles and procedures described herein can also be easily generalized to studies involving mice. One major advantage of using mice is that there are a large number of inbred strains

available, many of which have differing behavioral and biological characteristics. Indeed, there are certain lines and sublines of mice which differ only with respect to one major gene. These animals, then, are excellent biological material if one is interested in studying the interactive effects of the genetic substrate with environmental variables. [For discussion of characteristics of inbred mice, see Green (1966).]

One major disadvantage of working with mice or rats is that the manipulations introduced by the experimenter in a carefully controlled and systematic fashion are not "pure" because the pups are returned to their nest box, where the mother immediately begins interacting with them. Thus, it is impossible, using these species, to determine whether the behavioral and biological differences obtained in the animals are a function of the intervention introduced by the experimenter, the behavior of the mother upon the return of the pups, or some interactive combination of both. The only way to avoid this problem is to use a species where the pups can be separated from the mother, e.g., precocial animals such as guinea pigs or monkeys. However, a major difficulty with these animals is that their central nervous systems are far more advanced in development than are the central nervous systems of altricial species such as rats, mice, and humans. Ideally one would seek an altricial animal in which there is minimum interaction between the mother and her litter. There is one animal which fulfills these conditions—the rabbit. This animal, both in nature and in the laboratory, spends only 3 to 15 min per day with her young for purposes of feeding them (Zarrow *et al.*, 1965; Sorensen *et al.*, 1972). The rest of the time is spent roaming the fields if she is in the wild, or outside the nest box if she is in the laboratory.

C. Nutrition Research

An important question in studies of nutritional deficiency is whether differences in the behavior and biology of animals reared by underfed mothers are brought about by (1) the lack of adequate nutrition through the milk supply; (2) lack of appropriate maternal behavior because of inanition on the part of the mother; or (3) some combination of these events. [For a review of this literature, see Levine and Weiner (1976).] If the underfeeding is restricted to the prenatal interval, then the newborn pups can be cross-fostered to healthy mothers. However, it may be necessary to cross-foster within the first few hours of life to establish firmly that the effects are mediated via prenatal undernutrition rather than immediate postnatal events.

If undernutrition is introduced during the postnatal interval, then there is an inherent confounding of the reduction in milk supply and marked

changes in maternal behavior on the part of the mother. One way to break this confounding is to utilize another species in which feeding and maternal involvement are relatively independent of each other, such as a nonhuman primate, guinea pig, or rabbit. Another approach is to use a rat "aunt," as described previously, to furnish appropriate maternal care while the underfed mother supplies the milk for the pups.

D. Marking of Animals

Typically animals are marked at weaning by ear punching. However, at times it is necessary to identify animals at birth or shortly thereafter (e.g., when one is doing a developmental study). Geller and Geller (1966) have described a procedure for permanent marking of animals by using a 27 ga needle to inject a small quantity of India ink into one or more of the paws of the newborn pup.

XI. CONCLUSION

In conclusion, the suggestions in this chapter will help in designing proper experiments for research on the effects of early experience as well as in avoiding some of the more common pitfalls. However, it is not possible to cover all of the many techniques and procedures of experimentation. Many of these can be gleaned from a reading of the primary literature, but some of the procedures are rather subtle and can best be understood by visiting and/or working in the laboratory of an established investigator.

REFERENCES

- Abbey, H., and Howard, E. (1973). Statistical procedures in developmental studies on species with multiple offspring. *Dev. Psychobiol.* **6**, 329-336.
- Ader, R. (1965). Effects of early experience and differential housing on behavior and susceptibility to gastric erosions in the rat. *J. Comp. Physiol. Psychol.* **60**, 233-238.
- Ader, R., and Friedman, S. B. (1964). Social factors affecting emotionality and resistance to disease in animals: IV. Differential housing, emotionality, and Walker 256 carcinosarcoma in the rat. *Psychol. Rep.* **15**, 535-541.
- Denenberg, V. H. (1964). Critical periods, stimulus input and emotional reactivity: A theory of infantile stimulation. *Psychol. Rev.* **71**, 335-351.
- Denenberg, V. H. (1968). A consideration of the usefulness of the critical period hypothesis as applied to the stimulation of rodents in infancy. In "Early Experience and Behavior" (G. Newton and S. Levine, eds.), pp. 142-167. Thomas, Springfield, Ill.
- Denenberg, V. H. (1969). The effects of early experience. In "Behavior of Domestic Animals" (E. S. E. Hafez, ed.), pp. 95-130. Baillière, London.
- Denenberg, V. H. (1970). The mother as motivator. In "Nebraska Symposium on Motivation, 1970" (W. J. Arnold and M. M. Page, eds.), pp. 69-93. Univ. of Nebraska Press, Lincoln.

- Denenberg, V. H. (1975). Effects of exposure to stressors in early life upon later behavioural and biological processes. In "Society, Stress, and Disease: Childhood and Adolescence" (L. Levi, ed.), Vol. 2, pp. 269-281. Oxford Univ. Press, London and New York.
- Denenberg, V. H., and Rosenberg, K. M. (1967). Nongenetic transmission of information. *Nature (London)* **216**, 549-550.
- Denenberg, V. H., and Whimbey, A. E. (1963a). Infantile stimulation and animal husbandry: A methodological study. *J. Comp. Physiol. Psychol.* **56**, 877-878.
- Denenberg, V. H., and Whimbey, A. E. (1963b). Behavior of adult rats is modified by the experiences their mothers had as infants. *Science* **142**, 1192-1193.
- Denenberg, V. H., Grotta, L. J., and Zarrow, M. X. (1963). Maternal behavior in the rat: Analysis of cross-fostering. *J. Reprod. Fertil.* **5**, 133-151.
- Denenberg, V. H., Hudgens, G. A., and Zarrow, M. X. (1964). Mice reared with rats: Modification of behavior by early experience with another species. *Science* **143**, 380-381.
- Denenberg, V. H., Rosenberg, K. M., Paschke, R. E., and Zarrow, M. X. (1969). Mice reared with rat aunts: Effects on plasma corticosterone and open-field activity. *Nature (London)* **221**, 73-74.
- Denenberg, V. H., Holloway, W. H., and Dollinger, M. J. (1976). Weight gain as a consequence of maternal behavior in the rat. *Behav. Biol.* **17**, 51-60.
- Geller, L. M., and Geller, E. H. (1966). A simple technique for the permanent marking of newborn albino rats. *Psychol. Rep.* **18**, 221-222.
- Green, E. L., ed. (1966). "The Biology of the Laboratory Mouse," 2nd Ed. McGraw-Hill, New York.
- Hebb, D. O. (1949). "The Organization of Behavior." Wiley, New York.
- Joffe, J. F. (1969). "Prenatal Determinants of Behaviour." Pergamon, New York.
- Krech, D., Rosenzweig, M. R., and Bennett, E. L. (1962). Relations between brain chemistry and problem-solving among rats raised in enriched and impoverished environments. *J. Comp. Physiol. Psychol.* **55**, 801-807.
- Levine, S. (1962). The effects of infantile experience on adult behavior. In "Experimental Foundations of Clinical Psychology" (A. J. Bachrach, ed.), pp. 139-169. Basic Books, New York.
- Levine, S., and Otis, L. S. (1958). The effects of handling before and after weaning on the resistance of albino rats to later deprivation. *Can. J. Psychol.* **12**, 103-108.
- Levine, S., and Wiener, S. (1976). A critical analysis of data on malnutrition and behavioral deficits. *Adv. Pediat.* **22**, 113-136.
- Levine, S., Chevalier, J. A., and Korchin, S. J. (1956). The effects of early shock and handling on later avoidance learning. *J. Pers.* **24**, 475-493.
- Lindholm, B. W. (1962). Critical periods and the effects of early shock on later emotional behavior in the white rat. *J. Comp. Physiol. Psychol.* **55**, 597-599.
- Rosenblatt, J. (1967). Non-hormonal basis of maternal behavior in the rat. *Science* **156**, 1512-1514.
- Rosenzweig, M. R. (1971). Effects of environment on development of brain and of behavior. In "The Biopsychology of Development" (E. Tobach, L. R. Aronson, and E. Shaw, eds.), pp. 303-342. Academic Press, New York.
- Rosenzweig, M. R., Bennett, E. L., and Diamond, M. C. (1972). Brain changes in response to experience. *Sci. Am.* February, **226**, 22-29.
- Russell, P. A. (1971). "Infantile stimulation" in rodents: A consideration of possible mechanisms. *Psychol. Bull.* **73**, 192-202.
- Scott, J. P. (1962). Critical periods in behavioral development. *Science* **138**, 949-958.
- Scott, J. P., Stewart, J. M., and De Ghett, V. (1974). Critical periods in the organization of systems. *Dev. Psychobiol.* **7**, 489-514.

- Seitz, P. F. D. (1954). The effects of infantile experiences upon adult behavior in animal subjects: I. Effects of litter size during infancy upon adult behavior in the rat. *Am. J. Psychiatry* **110**, 916-927.
- Seitz, P. F. D. (1958). The maternal instinct in animal subjects: I. *Psychosom. Med.* **20**, 215-226.
- Sorensen, R. M. F., Rogers, J. P., and Baskett, T. S. (1972). Parental behavior in swamp rabbits. *J. Mammal.* **53**, 840-849.
- Southwick, C. H. (1968). Effect of maternal environment on aggressive behavior of inbred mice. *Commun. Behav. Biol.* **1**, 129-132.
- Thompson, W. R. (1957). Influence of prenatal maternal anxiety on emotionality in young rats. *Science* **125**, 698-699.
- Winer, B. J. (1971). "Statistical Principles in Experimental Design," 2nd Ed. McGraw-Hill, New York.
- Zarrow, M. X., Denenberg, V. H., and Anderson, C. O. (1965). Frequency of suckling in the pup. *Science* **150**, 1835-1837.

Chapter 6

Measurements of Body Temperature

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When one sees such results obtained by the sole aid of a little mercury in a glass tube, and reflects that the discovery of the New World was owing to a little bit of iron suspended on a pivot, surely nothing which can supplement or perfect the operation of our senses should be held in slight esteem. (J. B. Biot, ca. 1838)

I. INTRODUCTION: SOME MILESTONES

Measurement of body temperature, as an adjunct to the diagnosis of disease, has been carried out sporadically since the invention of the thermoscope by Galileo Galilei in 1593. Though clinical thermometry has only been an accepted necessity since the mid-nineteenth century, Sanctorius (1614) used a thermometric instrument of his own design, and this, together with his use of a man-balance, gave us our first knowledge of the efficacy of sweating to reduce body temperature. The development of the mercury-in-glass thermometer by G. D. Fahrenheit, 1686–1736, enabled more accurate temperatures to be taken, using a scale of temperature which enabled comparisons to be made between the observations of numerous workers.

Boerheave in the seventeenth century, Van Swieten (1745), and others of this period correlated a raised body temperature with the disease process in a more quantitative way, and noted the association of a raised pulse rate with the raised body temperature. Blagden (1775) demonstrated that body temperature could be kept nearly constant in rooms having dry air temperatures of up to 100°C (212°F). The use of a mercury-in-glass thermometer to study rates of body cooling was carried out by Currie (1797), and he also characterized the body temperature changes in many common disease states. Becquerel and Breschet (1835) used sensitive thermocouples to measure the temperatures of many different sites in the body; they also demonstrated that inflamed parts had higher temperatures than other peripheral sites.

The existence of a diurnal temperature rhythm was documented by Davy (1850) and confirmed by Ogle (1866). The relationship of skin temperature to skin blood flow and the effects of the sympathetic nerves on them was studied by Bernard (1852, 1862), Brown-Séquard (1853), and Schiff (1855).

Once the theoretical basis of the law of conservation of energy had been propounded by Mayer (1842), and the mechanical equivalent of heat experimentally determined by Joule in 1850 [quoted by Wunderlich (1871)], the role of muscular as well as tissue oxidation in body heat production began to receive serious study. Total body calorimetry to determine overall body heat exchanges was carried out in water baths (Liebermeister, 1875), in a huge inverted bell jar (d'Arsonval, 1894), and

in a specially designed room, in which radiant, convective, conductive, and evaporative as well as respiratory heat exchanges could be measured (Atwater and Rosa, 1899, 1903). Further development of these methods by Lefèvre (1911) represents another milestone in thermoregulatory technology, as did the work of DuBois (1939.).

Since the work of Lefèvre (1911), the major advances have been mainly technological sophistication of the methods used by the earlier workers. The gradient layer calorimeter (Benzinger *et al.*, 1963) is an example of one of these. In addition, the use of these techniques has led to the new concepts of the significance of temperature measurements in different body loci and of the role of superficial and deep thermodetector nervous structures in the control of body temperature.

II. THE SIGNIFICANCE OF TEMPERATURE MEASUREMENTS IN VARIOUS BODY LOCI

The problem of which measurement of temperature truly represents "body" temperature has provoked much study. Temperatures measured simultaneously in many sites reveal significant differences between different parts of the body. The hepatic venous temperature (Rowell, 1974) is one of the highest in the body, followed by that measured in the rectum (Grayson, 1951). In newborn infants, blood returning from the brain may be at a high temperature (Cross, 1976, and personal communication). The temperature of the blood in the abdominal aorta may be considerably lower than that in the rectum (Grayson, 1951). Temperatures taken at various sites in the rectum may vary considerably, depending on whether the probe rests on the posterior wall of the rectum over the plexus of veins draining the legs, or on the anterior wall. Temperatures taken within the abdomen in women during laparoscopy (Taylor *et al.*, 1976) demonstrate that the serosal surface of the rectum is usually cooler than the mucosal surface, and that considerable variation of temperature occurs within the pelvis. There is evidence that the gut mucosa is a major source of heat production (Grayson, 1951), and thus intraabdominal temperatures may fluctuate according to the state of mucosal metabolism. The esophageal temperature, measured at heart level, appears to be a close measure of the temperature of the blood leaving the heart (Cooper and Kenyon, 1956).

Temperatures taken under the tongue with the mouth taped shut follow induced rapid changes in deep body temperature more faithfully than do temperatures measured in the rectum (Gerbrandy *et al.*, 1954). Tympanic membrane temperature (Benzinger *et al.*, 1963) and aural canal temperature (Cooper *et al.*, 1964) also follow rapid changes in blood temperature

well. A refinement of the aural canal measurement, in which a servo-heated pad keeps the skin of the outer ear at the same temperature as the aural canal, would appear to give accurate measurements of rapidly changing arterial blood temperatures (Keatinge and Sloan, 1975).

Attempts to estimate hypothalamic temperature indirectly have been made, particularly by the use of tympanic membrane temperature (Benzinger *et al.*, 1963). The validity of such indirect methods varies between species. Hayward (1973) has drawn attention to the rete mirabile at the base of the brain in some species. Here, the blood flowing to the hypothalamus passes through a fine network (rete) of vessels, embedded within a venous sinus, en route to the brain. The venous sinus in part drains blood from the nares and so, in the panting animal, is cooled. Thus, despite a rise in temperature of most of the body core, the hypothalamic temperature remains relatively unchanged.

Despite the variations in temperature that may occur within the body, the concept that there is a central region of the body whose temperature is closely regulated is a valid one. Aschoff and Wever (1958) introduced the idea of a "body core," consisting of the intracranial, intrathoracic, and intraabdominal contents, whose temperature measured over a reasonable period of time is quite constant; and a "shell," made up of the skin and subcutaneous tissues and limbs, whose temperature could be allowed to fluctuate widely in order to preserve the constancy of the core temperature. Of importance, as derived from this concept, is that at any core temperature the total body heat content (or mean body temperature) can fluctuate widely according to the thermal topography of the shell. Muscle temperatures can change rather more widely than it is customarily believed in response to environmental temperature change; they also depend on the balance between metabolic heat production and the induced blood flow changes.

Measurement of skin temperature, usually with one of the electrothermal probes to be described later, is sometimes used as an index of skin blood flow. It is important to bear in mind that the relationship between skin temperature and blood flow is nonlinear, and very imprecise at high skin temperatures (Cooper *et al.*, 1949). The actual measurement of skin temperature itself can be fraught with error. For example, if the skin is exposed to radiation, then any solid device on the surface will shield the area of skin of which the temperature is to be measured. In addition, the probe may have a thermal emissivity quite different from that of skin and thus absorb radiant heat to a greater or lesser extent than the skin. Further, a skin probe may alter the conductive or convective heat loss, as well as prevent evaporation from the region of skin under consideration, so that imprecise measurements are made.

It is therefore important to consider carefully the significance of the temperature to be measured, to estimate the possible errors introduced by the measuring technique, and to choose the method and site most likely to reflect the temperature required and to be related most closely to the dependent physiological variables. Because of circadian rhythms, in scientific investigations in which comparative results are wanted it is desirable to carry out the experimental protocol at the same time each day. For scientific investigations, the only valid approach is direct measurement at the required locus rather than that inferred from indirect measurements.

III. METHODS AND DEVICES: GENERAL CONSIDERATIONS

A. Mercury-in-Glass Thermometers

Most frequently used in clinical practice (and to calibrate other devices) are mercury-in-glass thermometers. There are considerable variations in calibration of clinical and inexpensive laboratory thermometers. Errors may occur due to inexact positioning of the scale in relation to the mercury column, and there may also be variations along the scale due to slight changes in bore of the glass capillary tubing. In addition, fluctuations in the temperature of the exposed stem of the thermometer will alter the bore and hence the reading, and pressure on the bulb will give an apparently high reading. For scientific work, all clinical and laboratory thermometers should be calibrated against a standard thermometer, which itself has been calibrated against a gas thermometer, and which has a certificate giving the true reading and errors at different parts of the scale. Such accurately calibrated standard thermometers are commercially available.

B. Thermocouples

The oldest method of temperature measurement, apart from the liquid-in-glass devices, is the thermoelectric device or thermocouple. The principle (Seebeck, 1821) is that a wire of one substance has a wire of a different material joined to it at each end (see Fig. 3 in Section IV, B, 3,a). If a temperature difference exists between the two junctions of dissimilar metals and the circuit is completed through a galvanometer, a current will flow. The electrical potential difference across the galvanometer, generated by the difference in temperature between the two junctions is proportional to that temperature difference; and although the potential difference is nonlinear over a very wide range, for small temperature differences it approximates linearity. This thermoelectric emf (electromo-

tive force) depends on the nature of the metals used; for example, for copper-constantan junctions at near 20°C it is approximately $45 \mu\text{V}/^\circ\text{C}$, whereas for iron-constantan it is $52 \mu\text{V}/^\circ\text{C}$. The thermocouple has the advantage that it can be made of any gauge wire and thus very fine probes can be used. Simple or sophisticated recording apparatus can be employed (optical galvanometers or electronic recorders); the thermocouples can be made easily in the laboratory, and their calibration remains constant. The difficulties of thermocouple use include the need for a very constant temperature device in which to set the "reference" junction so that the thermoelectric emf will be proportional only to changes in temperature of the "measurement" junction. If melting ice is used for the reference junction, and measurements are made at 37°C, there will be a steady emf of some 1.4 mV existing before further temperature fluctuations induce small (microvolt) changes in the emf. If a galvanometer is used to measure the emf, the 1.4 mV has to be backed off by using a precise and steady voltage source in order to enable the measurements to be made. Alternatively, the reference junction can be immersed in a water bath at about the temperature to be measured, but the provision of such a water bath to allow fluctuations of no more than 0.01°C can be costly. "Stray" or "induced" emf may plague thermocouple circuits under same circumstances. In many commercial recorders, there are electronic reference junctions of varying constancy.

C. Resistance Thermometers

Variation of electrical resistance of a metal with temperature has been used since 1887 (Callendar) as a means of measuring temperature. A coil of fine platinum wire, wound on a form, so that mechanical stresses cannot also modify the electrical resistance of the wire, forms one arm of a modified Wheatstone bridge. The thick platinum wires connecting the resistance element to the bridge may also change their temperature and thus their resistance, and so temperature-compensating arms are included in the circuit. This device forms a most reliable measure of temperature from -40°C to 1200°C .

A modified type of resistance thermometer arose with the use of some semiconductor materials having large coefficients of change of resistance with temperature. A typical value would be $30 \Omega/\text{K}$. These devices are now available in many sizes and of great stability, and enable very sensitive measurement of temperatures. They can be adapted to go into fine needles, into flat disks for skin temperature measurements, or into flexible plastic tubes of varying sizes for probing various body cavities.

The devices are virtually linear in response over *small* temperature ranges only and can form the sensors of portable and robust recorders.

Sensitive sensors for radiant heat are now available to replace the old thermopiles: they are constructed in devices which enable the body surface to be scanned in such a way that a picture is formed with patterns whose color or optical density depends on the surface temperature. Infrared thermography can be used not only to detect the heat emission from vascular lesions but to map the surface radiant heat emission and so locate areas of heat loss (Hayward *et al.*, 1975).

Further development of thermography is underway in order to assess the deep skin temperature without having to use invasive techniques. Another recently devised method of assessing surface temperature, particularly to pick up points of inflammation, consists of spraying the skin surface with cholesteric liquid crystals (Puhl and Golding, 1975). The sprayed surface changes color according to its temperature, and photographs of these areas enable the surface temperature to be measured. The time lag in response of the chemical is 0.2 sec and up, and with further work this technique may have considerable use in estimating the patterns of blood flow in exercise, for example.

Finally, a modification of the thermocouple was introduced by Hatfield (1949) in which a thin disk of silver–tellurium alloy had wafers of copper mesh welded to each surface, thus forming a Cu/(Ag–Te)/Cu thermocouple with a thermoelectric emf of greater than $50 \mu\text{V}/^\circ\text{C}$. Attached to the skin surface, the temperature difference between the two surfaces is proportional to the heat flow through the disk, which has a very low resistance to heat flux. Thus, the thermoelectric emf generated is a measure of the heat elimination from that area of skin and can be useful in assessing skin blood flow.

IV. MEASUREMENT AND RECORDING OF BODY TEMPERATURES

A. Selection and Calibration of Sensory and Recording Equipment

In the measurement of body temperature a variety of techniques are available. In the remainder of this chapter we will first describe some of the body temperature measurement devices, advantages and disadvantages of each, and some principles of operation. We will then describe their use in measuring temperatures at various locations on the body surface or within specific tissues of humans and other animals.

Listed next are points which should be considered when selecting a system for the detection and recording of body temperature:

1. Size of sensor
2. Range of temperature measurement
3. Linearity over the range of measurement
4. Reproducibility of reading by the instrument used
5. Signal processing capability of recording system
6. Safety of the subjects
7. Response latency of the equipment
8. Ease and stability of calibration
9. Suitability for remote reading
10. Type of covering surrounding the sensing element
11. Transfer of heat along wires connecting the device to the indicating apparatus
12. Cost
13. Site of temperature sensor
14. Movement of subject
15. Method of sterilization of materials in contact with tissues
16. Future expansion of system employed

When using any type of temperature-monitoring sensor it is important that it be calibrated properly. In most cases this requires an accurately regulated stirred water bath and a certified thermometer as a reference. The National Bureau of Standards (NBS) will for a fee test a thermometer which is of acceptable quality and issue a certificate of calibration. Information is published (Swindells, 1965) on the type and characteristics of thermometers that NBS will accept for testing and the methods employed. (The appropriate address is included in the appendix to this chapter.) Alternatively thermometers may be purchased from scientific suppliers with NBS certificates of calibration.

To calibrate a temperature sensor in the laboratory it is necessary to have a large, closed-top, electrically heated, well-insulated, stirred water bath located in a temperature-controlled room at 25°C. The calibrated thermometer bulb and the sensor to be tested should be in very close proximity, located in the center of the mass of water at an appropriate immersion depth. After a suitable time has elapsed for equilibration, a minimum of six readings should be taken at uniform intervals throughout the range of measurement. If applicable, one of these should be at the ice melting point. At least two observers should be involved in recording the calibration and the mean observed reading used as the final temperature value.

B. Techniques for the Measurement of Body Temperature

1. Thermoexpansive Devices

Some substances, provided they remain in the same physical state, expand as their temperature increases and contract as it decreases. The coefficient of expansion is the amount of expansion per given rise in temperature. Three devices for measuring body temperature based on this principle are the liquid-bulb thermometer, pressure-type thermometer, and the bimetallic thermometer.

a. *Liquid-Bulb Thermometer.* This is a common means of determining body temperature. Most liquid-bulb thermometers employed in measuring body temperature utilize mercury as the expanding liquid. The clinical mercury thermometer is available in forms for oral and rectal temperature measurements, and special purpose ones are available, e.g., for recording body basal temperature, for determining ovulation time, and "premature" thermometers for subnormal clinical temperature ranges. The normal range for these types of thermometers is from 35° to 42°C with divisions of 0.1°C. They have a constriction in the capillary tubing which prevents the mercury from falling once the thermometer is removed from the body orifice. The thermometer should be inserted a minimum of 3 min before a reading is taken.

The laboratory liquid-bulb thermometer has a greater range than the clinical thermometer and is therefore useful for temperatures which fall outside the normal clinical range. They are available in a variety of ranges (-200° to +600°C), divisions (0.02° to 5.0°C), lengths, immersion depths, expanding liquids used, and protective coverings. The liquid-bulb thermometer may also be used to calibrate other types of thermometers if it is tested and certified by the NBS to meet their requirements for structural integrity and accuracy. This type of thermometer may be used for giving an indication of deep body temperature during endotracheal surgical anesthesia by measuring deep nasal temperature. For a discussion on the characteristics and properties of liquid-in-glass thermometers the reader is referred to Busse (1939), Ween (1968), and Thompson (1968).

A common problem occurring with mercury-bulb thermometers is separation of the column, but it can be reunited. The safest way to do this is to cool the thermometer in an ice-water mixture until all the mercury is drawn into the bulb, tap gently to dislodge gas bubbles, and then rewarm slowly to room temperature. Another method is to warm the bulb until mercury is gradually forced into the expansion chamber located at the top of the capillary, then cool slowly. The column should recede united. One must do this carefully as there is a danger of bursting the bulb.

Advantages of the liquid-bulb thermometers are that they are simple to use, inexpensive, reliable, portable, have a long useful life, and have a linear scale. On the other hand, these thermometers have certain disadvantages: there is no opportunity for remote reading; the value of the reading is subject to observer error; and they break relatively easily. In addition, the clinical thermometer cannot be used to monitor rapidly varying temperatures because the reading lags behind the true temperature. The reservoir bulb must be completely surrounded by the medium to be thermally sensed, and the immersion depth must be appropriate for the thermometer used.

b. *Pressure-Type Thermometer.* This type of temperature monitor can be used for limited distance remote monitoring of body temperature. It is composed of a reservoir bulb connected to a capillary tubing which leads to a "Bourdon" tube. When the sensing bulb's internal pressure is changed as a result of a change of temperature, the pressure change is transmitted through the connecting tube to the Bourdon tube, which is connected to a linkage that turns a pointer on a calibrated dial. The thermometer may be filled with any of the following: a gas such as nitrogen or helium; a liquid such as mercury or xylene; or vapor such as ethyl ether, as long as it stays in the same phase over the range of measurement. This type of thermometer may be useful for measuring temperature during a surgical operation. Hagelsten *et al.* (1972) have reported the clinical application of this type of thermometer.

The main advantages of the pressure-bulb thermometers are that they permit remote reading and are relatively inexpensive. Disadvantages of pressure-bulb thermometers include their large size, the tendency for leakage of filling gas or liquid, the lack of flexibility, and sensitivity to changes in barometric pressure and ambient temperature.

c. *Bimetallic Thermometer.* In this type of thermometer a flat spiral spring is made up of two metal ribbons in contact; each has a different expansion coefficient. The spring will tend to wind or unwind with variations in temperature because of the difference in coefficients of expansion of the metals. This movement may then be transferred to a pointer that moves along a calibrated scale. Several types are available. One type has a stem which can be inserted into a body orifice and the temperature read on a dial. Others are flat and can be used to measure surface temperatures. Huston (1962) gives an account of the characteristics of bimetallic temperature measuring elements.

Factors in favor of bimetallic thermometers are that they are sturdy, unaffected by vibration, easily read, portable, and inexpensive. Drawbacks include the relatively large size of meter, their inflexibility, and change in calibration with rough handling.

2. Thermoresistive Devices

The change in electrical resistance of certain semiconductors and metals with changes in temperature is the basis for thermoresistive temperature transducers. The temperature coefficient of resistance depends upon the type of material used in manufacturing and can be positive or negative. The temperature element is generally placed in a bridge circuit, and the change in resistance is expressed in terms of temperature. In designing bridge circuits, an important consideration is the self-heating effect of the current through the sensing element.

a. *Platinum Wire Thermometer.* This is an accurate, sensitive, and stable temperature sensor. A coil of high-purity platinum wire is wound upon a ceramic core and enclosed in a protective sheath. Platinum wire possesses a linearly positive thermal coefficient. The leads from the coil are connected to one arm of a Wheatstone bridge and suitably calibrated. Increases in temperature will result in increased current through the meter circuit. A typical circuit is shown in Fig. 1.

United Systems Corporation manufactures a series of platinum resistance sensors, the output of which can be digitally displayed on their modules. There are facilities for a calibration check and adjustment if necessary; binary coded digit (BCD) logic output is available also for signal processing. Model 5530 has a range from -100.0° to $+400.0^{\circ}\text{C}$, and a system accuracy of $\pm 0.3^{\circ}\text{C}$ in a digital display with 0.1°C resolution. The time constant for their platinum resistance probes is 2.5 sec or less. Platinum resistance thermometers have the advantages of being relatively resistant to contamination and mechanically and electrically stable; moreover, platinum wire can be highly refined. The drift and error with age and use are negligible, and the instrument is relatively accurate. On the other hand the platinum wire thermometer has a relatively large size and a somewhat slow response time. Leeds and Northrop and also Omega Engineering Inc. have available several different types of platinum resistance thermometer elements.

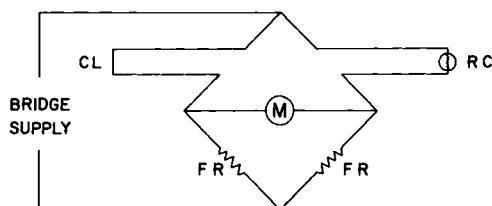


FIG. 1. Bridge circuit for a temperature-dependent resistance coil of platinum wire (RC), consisting of fixed resistors (FR), compensatory leads (CL) and display meter (M).

b. *Thermistors.* Thermistors represent a widely used electrical method of measuring body temperature. The thermistor is a "thermally sensitive resistor." It is composed of a well-aged combination of semiconducting elements consisting of a mixture of metal oxides that usually has a large negative temperature coefficient of resistance. Oxides of manganese, nickel, cobalt, iron, and zinc are used in their manufacture. They are made by compressing proportions of the oxides into disks, rods, or beads under high pressure into a solid mass and then affixing leads to the material. Thermistors come in a variety of sizes and shapes ranging in diameter from 0.006 to 0.1 in. (0.015 to 2.5 mm); they are generally used in bridge circuits. Figure 2 illustrates two arrangements of thermistor-bridge combinations. To improve linearity over a wide range of temperatures, two thermistors of different resistance values may be combined into one composite thermistor. Circuits used with this combination are described by Trolander (1967).

Yellow Springs Instruments Corp. (see the Appendix) manufactures a series of thermistor probes and temperature display instruments. The

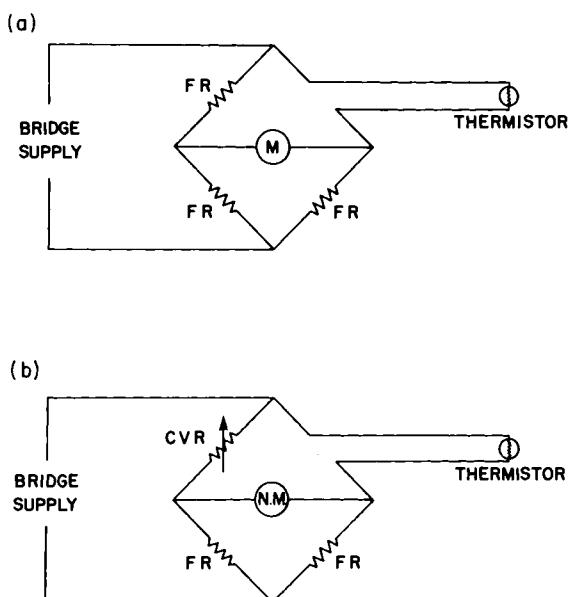


FIG. 2. (a) Direct reading Wheatstone bridge with calibrated meter (M), and fixed resistance (FR). (b) Null-balance Wheatstone bridge with calibrated variable resistance (CVR), null meter (NM), and fixed resistance (FR).

probes in the 400 series are interchangeable and are guaranteed to remain within tolerances of 0.1°C over the range of 0° to 80°C for 1 year. Our experience has shown that standard probes remained within this tolerance for more than 7 years. Time constants for the No. 401 general purpose probe is 7.0 sec. Time constants for other thermistor probes in the series range from 0.3 to 7.0 sec. Approximately five time constants are required for a probe to read 99% of the total change. The probes and leads can be sterilized by conventional methods other than autoclaving or boiling. The standard lead is covered with a flexible vinyl jacket and the length for the 400 series is 10 feet (about 3 m). However, this can be extended to 100 feet and longer, if consideration is taken of potential errors due to lead resistance. This company also manufactures a series of telethermometers and temperature controllers for use with the thermistors. Output facilities for recording the signal are provided.

United Systems Corporation manufactures a series of digital thermometers that can be used with Yellow Springs Instruments Corp.'s thermistor sensors. These units range from two to five channels and have calibration checks and corrections which can be easily made on the front panel. Some models have output connectors with BCD logic for signal processing.

Beckman Instruments Inc. supplies a thermistor coupler for use in their Dynograph recorders. Yellow Springs Instruments Corp.'s thermistor probes may be used as the sensing element. The Dynograph has the advantage of interchangeable couplers so that other physiological variables may be monitored and displayed at the same time.

Temperature-sensing probes may be manufactured by the investigator himself. It is possible to purchase the thermistor elements, and the investigator can then fabricate his own probe and design an electronic circuit suitable for his needs. (Addresses of some companies which sell thermistor elements and display units are included in the Appendix to this chapter.)

Thermistors can rapidly respond to a change in temperature, are sensitive, can be used with long leads, have a long life, and provide the capability of a small sensor. Thermistors directly provide an accurate indication of temperature, may be bought in matched sets, may have thin flexible leads, and are of relatively low cost. Some drawbacks associated with thermistors include some time lag of the reading, a slight change in resistance characteristics as the element ages, and nonlinear variations in resistance; however, this may be compensated for electronically by shunting with a fixed resistor to obtain a linear relationship over a limited range. Variations may occur between unmatched sensors and self-heating may occur, but again this can be avoided by proper electronic design.

TABLE I

Characteristics of Thermocouples Used for Measuring Body Temperature

Junctions	Sensitivity at 20° C ($\mu\text{V}/^\circ\text{C}$)	Range ($^\circ\text{C}$)	Accuracy (%)
Iron-constantan	52	-150 to +1000	± 1.0
Copper-constantan	45	-150 to +350	± 0.5

3. Thermoelectric Devices

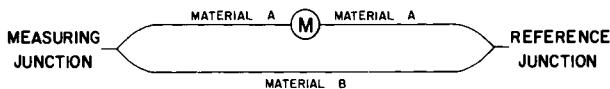
If two dissimilar metals are connected in a closed circuit with their two junctions at different temperatures, a current flows. This phenomenon is known as the Seebeck effect and was first reported in 1821. It involves the absorption of heat by the junction at the higher temperature and the release of heat from the cooler one, forming a potential between the two junctions. The thermal emf responsible for current flow depends on the type of metals involved and is proportional to the temperature differences between the two junctions. One of the junctions is used as a reference junction ("cold") and is strictly maintained at a constant temperature; the other is the sensing junction ("hot"). Combinations of metals used and the characteristics of thermocouples suitable for measuring body temperature are presented as Table I.

Iron-constantan junctions produce a larger potential difference than do copper-constantan junctions, but iron rusts in the presence of moisture. Constantan is an alloy of copper and nickel varying in composition from $\text{Cu}_{50} : \text{Ni}_{50}$ to $\text{Cu}_{65} : \text{Ni}_{35}$. For thermoelectric purposes it is approximately $\text{Cu}_{57} : \text{Ni}_{43}$ for the best thermoelectric power. The signal is measured by using either a precision dc potentiometer, a digital voltmeter, or a galvanometer. Introduction of a third metal (e.g., copper) into the thermocouple circuit does not influence the reading provided both junctions of this conductor are at the same temperature.

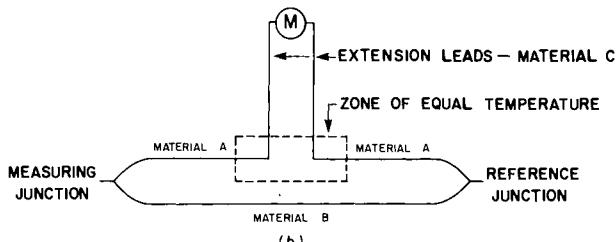
Schematic diagrams of various thermocouple circuits are given in Fig. 3, and components of the circuits are discussed next.

a. *Measuring Junction.* This is the area where the temperature is measured. Techniques for fabricating this junction will be described.

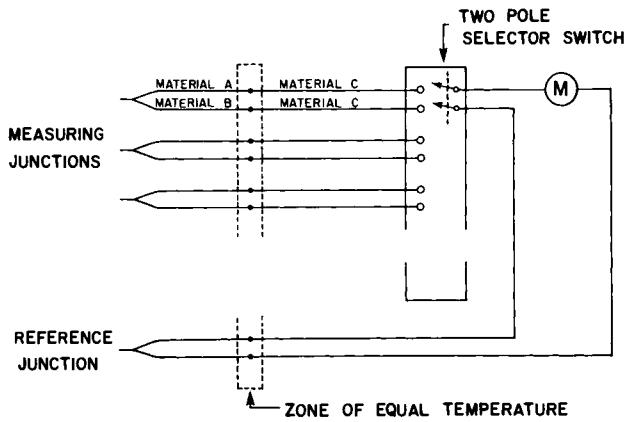
FIG. 3. (a) Basic thermocouple circuit composed of a measuring junction, a reference junction, and a display meter (M). (b) Basic thermocouple circuit with extension leads to a display meter (M). (c) Thermocouple circuit with multiple measuring junctions, one reference junction, selector switch, and display meter (M). (d) Thermopile for sensing average temperature.



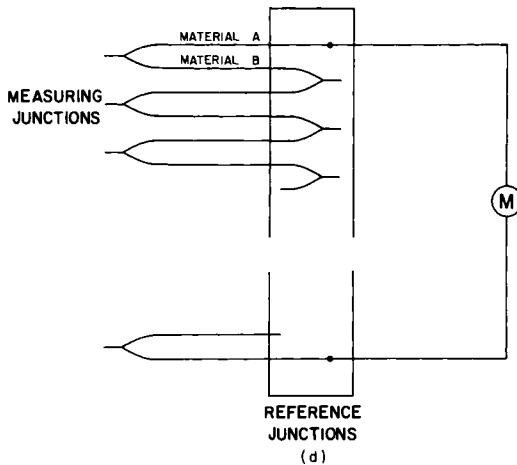
(a)



(b)



(c)



(d)

Thermocouple junctions are easily made in the laboratory. The enamel coating is first removed for a short distance away from the end of the wires. They are then placed in apposition. The wires can be twisted together, or the tip can be spot-welded or hard soldered. The smallest amount of material necessary for the mechanical integrity of the junction should be used in the sensing element. Riley (1949) describes an electrical method of making thermocouple junctions. Before using the junction it should be tested to ensure the integrity and site of the junction. This can be done by observing the deflection of the galvanometer when the junction and leads are slowly lowered and raised in water, paying particular attention to the tip area. The completed junction can be used as is or can be inserted into a protective sheath such as a catheter or hypodermic needle in methods to be described later in this chapter. To increase the sensitivity of thermocouples, a number of thermocouples can be connected in series to form a thermopile.

b. *Selector Switch.* Special thermocouple switches may be purchased. To minimize errors when in use the temperature of the system should be the same as that when the system was calibrated. Errors can be minimized by positioning the switch in the part of the circuit containing the copper extension wires and using all copper switch contacts.

c. *Extension Wires.* All junctions between thermoelectric wires and extension wires must be at the same temperature at both the time of measurement and calibration. Extension wires and thermocouples should be used as matched sets and not interchanged.

d. *Reference Junctions.* In order to measure temperature it is necessary to maintain the temperature of one junction constant while the other is used as the thermal sensor. This can be done in several ways:

1. *Ice bath*—The simplest is to immerse the reference junction in a well-insulated flask containing melting ice.

2. *Mechanical cold junction compensation*—The reference junction is mounted in a small enclosure next to a sensitive reflecting galvanometer of which the suspension is connected to a bimetallic spring. As room temperature changes, the temperature of the reference junction changes, and this is compensated by movement of the bimetallic spring, so that the net effect is that of a constant reference temperature.

3. *Electrical cold junction compensation*—The thermojunction is arranged in series with a resistance bridge circuit in which only one arm is temperature dependent. As the ambient temperature varies, the change in emf can be compensated by the bridge. This is the most practical method of cold junction compensation if portability and reliability are desired.

Electrical cold junction reference systems can be purchased, some of which are battery operated.

e. *Meter.* The display can be a galvanometer, a precision potentiometer, or a digital voltmeter.

Several firms specialize in these kinds of materials. Leeds and Northrup manufactures thermocouple wire, assembled thermocouples, and instruments to be used in thermoelectric thermometry, such as chart recorders, digital displays, scanners and controllers. Omega Engineering Inc. manufactures thermocouple wire, assembled thermocouples, compensators, ice-point references, meters, amplifiers, readouts, controllers, connectors, and rotary switches for use in thermoelectric thermometry. United Systems Corporation manufactures a digital thermocouple thermometer with BCD output, self-check calibration and adjustment, and internal reference junction. For further information on the principles and use of thermocouples, the reader is referred to Roeser (1940), Benedict (1969), Finch (1962), and ASTM technical publication 470 (1970).

Advantages of thermocouples are their small size, long-term stability and ease of fabrication in the laboratory. Further, they respond rapidly; can be made in matched sets; can be fabricated with a narrow sensitive junction point; are relatively tough; can be mounted with ease; and are essentially expendable because of their low cost. The disadvantages of thermocouples are that (1) they provide small voltage output per change in temperature; (2) they must have a reference temperature; and (3) they are quite susceptible to electrical noise.

4. Quartz Crystal Thermometer

A quartz crystal thermometer is based on the principle of the sensitivity of the resonant frequency of a quartz crystal to temperature change. This type of thermometer is valuable because of its high resolution, which can be of the order of 0.0001°C . This is more sensitive than most other types of thermometry systems. Hewlett-Packard manufactures a digital quartz crystal thermometer (Model 2801-A) and a series of temperature-sensing probes calibrated to an absolute accuracy of 0.02°C . This particular model can be equipped with two sensing probes and can indicate the absolute temperature of either probe or the temperature difference between them. BCD logic output is provided.

Advantages of quartz crystal thermometers are their high resolution; the capability of long leads on sensing probes; their linear relationship over a range of measurement; good reproducibility of reading; speed of

response; and long-term stability. The main disadvantage of the quartz crystal thermometer is that it is relatively expensive.

5. pn Junction Diode Thermometry

The *pn* junction diode when forward biased with a constant current displays a voltage drop that varies linearly with temperature. The linearity is a strong feature of this type of transducer. Germanium, silicon, and gallium arsenide diodes have all been used for this purpose. The sensitivity of a silicon diode is about 2.0 mV/°C at a current of 10 μA. Ganfield and Smaha (1971) describe the use of temperature-sensitive diodes in accurately monitoring muscle temperature of the cat. Sensitivity is greater than 0.1°C, and an account is provided for the circuitry and recording of the signal. For further information on the use of *pn* junction diodes in thermometry the reader is referred to Barton (1962), McNamara (1962), Cohen *et al.* (1963), and Sclar and Pollock (1972). The advantages of the *pn* junction diode temperature transducer are its small size, low cost, linearity of measurement, and reproducibility of the temperature coefficient with diodes from the same batch. The main disadvantage of the *pn* junction diode temperature transducer is the need to keep the biasing voltage constant.

6. Chemical Thermometry

The two most commonly applied methods of using chemical means to indicate temperature are liquid crystals and solid-liquid transition.

a. *Liquid Crystals*. Liquid crystal film provides an excellent means of demonstrating skin temperature. Its unique properties were first observed in 1888 by Friedrich Reinitzer in Austria. Liquid crystals is the name given to a group of compounds which are capable of existing in a state between solid and liquid. These compounds display the optical properties of solids and the mechanical properties of liquids. Liquid crystals are classified according to their molecular order as *nematic*, *smectic*, and *cholesteric*.

Nematic liquid crystals have the least ordered molecular arrangement and possess no optical rotary power. Smectic liquid crystals have their molecules arranged side by side in a series of parallel layers. Cholesteric liquid crystal molecules are arranged in layers with the long axis of the molecules lying parallel to the plane of the layers; in adjacent layers, they are aligned about 15 min of arc per layer differential, so that a helical path is traced out through successive layers. It is this unique molecular arrangement that causes light to trace a spiral pattern as it passes through the molecular layers. The molecular arrangement of the two-dimensional layered arrangement of the crystals is strongly temperature

dependent. It is possible to discriminate temperature differences of about 0.1°C . Color changes are reversible, and the speed of response to changing skin conditions is about 0.2 sec.

The range and sensitivity can be varied by mixing together various cholesteric substances and by varying the proportions within the mixture. The solution may be applied to thin films, which are then placed on the surface of the medium to be thermally sensed. The color varies with the angle of view, so it is important to study highly curved surfaces in small sections. The film of crystals does not act as an insulator, and sweating occurs normally from areas where liquid crystals are applied. Liquid crystals may be purchased in solutions which have been proportioned for the temperature range desired with a fast evaporating solvent. For further information on the properties and use of liquid crystals in thermometry the reader is referred to Fergason (1964), Crissey *et al.* (1965), Puhl and Golding (1975).

Advantages of liquid crystals include their capability of indicating temperature at any point on the body, ease of application, and low cost of the crystals. The most obvious disadvantages are that curved surfaces must be studied as small areas and that the observed area must be stationary.

b. *Solid-Liquid Transition Thermometers*. This chemical method for temperature indication is based on the solid-liquid phase transition temperature of certain substances. The substances are manufactured in the form of crayons, lacquers, aerosols, pellets, labels, and matrix strips and are available in a variety of ranges and sensitivities. An example of a chemical possessing this characteristic is a mixture of 1-bromo-2-nitrobenzene and 1-chloro-2-nitrobenzene. This mixture can have a temperature range suitable for body temperature and a differentiation of up to 0.2°C . This is the principle used in the disposable thermometer which will be discussed later in the chapter.

The main points in favor of solid-liquid transition thermometers are that a permanent record can be obtained with some techniques and that they are relatively easy to use. On the less positive side is the consideration that the substances are not reusable, nor are the changes produced in them by a temperature change reversible.

7. Radiation Thermography

All living organisms radiate energy in the form of infrared radiation. The relation between the radiation of wavelength $2.5\text{--}15 \mu\text{m}$ emitted from a surface and the surface temperature forms the basis of radiation thermometry. The thermal patterns that exist on the skin are determined largely by variations in blood flow and the heat locally conducted to the

skin from underlying organs. Instruments are available to measure and record thermal patterns from the body. Detector cells which can be used in these machines include:

1. Radiation thermopile—Series connections of a large number of thermocouples or thin-film thermocouple pairs
2. Thermistor bolometer—Fast response thermistors formed into a sensing element
3. Pyroelectric detector—A change in emf will result if the temperature is changed because of the change in state of polarization of the elements in the detector
4. Golay-cell detector—Consists of a gas-filled chamber connected by tubing to a flexible diaphragm whose outer surface is reflective; the deflections of the diaphragm are suitably transduced and monitored

Scanning (about 4 min for the human body) or nonscanning thermograph units are available. It is possible to determine skin temperature to the sensitivity of 0.1°C . Pigmentations do not alter the skin's emissivity. Hair, because it has no blood vessels, comes to equilibrium with room temperature, so it is best not to scan hairy areas. There are several types of thermograph machines available. One type of thermograph used clinically is a scanning radiometer that uses a thermistor bolometer as a detector and has a resolution of 0.1°C ; it operates in the spectral range of $2\text{--}16 \mu\text{m}$ with a field view of $10^{\circ} \times 20^{\circ}$. The data can be presented in several forms, including analog black and white or color, ten-digit black and white or color, three-digit black and white or color, and isotherm black and white or color. The machine may be calibrated by a thermal scale scanned at the same time and included in the output data. For further information on radiation thermography the reader is directed to articles by Gershon-Cohen (1967), Barnes (1968), Samuel (1969), and Cobbold (1974).

Radiation thermometry has the advantage of being a noncontact method which can scan surfaces or isolated areas with good accuracy. Also, the equipment is easily calibrated. The main drawbacks for most applications are that radiation thermometry is expensive, its operation requires a trained staff, and it requires a lengthy scanning time.

8. Heat Flow

Devices are available which measure the current of heat flowing from a surface. They were first demonstrated by Hatfield (1949). The sensor consists of a thin disk of an alloy of tellurium, coated on its sides with a fine copper gauze. The coatings are connected by copper wires to a galvanometer. When the disk is placed in a current of heat at right angles

to its direction of flow a small difference of temperature is established between the two sides of the disk. This difference in temperature is proportional to both the heat flowing from the surface and thickness of the disk; it is inversely proportional to its thermal conductivity. Differences in temperature between the two copper-tellurium junctions cause a thermoelectric current in the galvanometer circuit. The disks can be individually calibrated to give a caloric output/unit area/time. Heal *et al.* (1970) has developed a telemetry system for use with mobile subjects. Abrams and Stolwijk (1972) designed a heat flow device for measuring the thermal conduction of the vaginal wall of ewes.

9. Telemetry

With the development of miniaturized telemetry circuits, it is now possible to monitor the temperature of mobile organisms continuously over long periods of time. The telemetry systems may be swallowed (Wolff, 1961), surgically implanted, or attached to the surface of the skin with leads to the thermal sensor. The most important consideration in designing temperature telemetry systems is that they be able to measure temperature changes accurately and reliably over extended periods of time, permitting the investigator to be certain that he is obtaining the correct information, unaltered by changes in the characteristics of the electronic components.

Wang (1972) recorded the circadian body temperature of Richardson's ground squirrel in both field and laboratory conditions using a temperature telemetry system implanted in the peritoneal cavity. The telemeter used weighs about 20 gm, has a range of 15 m, and a theoretical battery lifespan of 694 days at 37°C. The signal is received by FM radio receiver at a frequency between 88 and 108 MHz. The frequency of the signal from the transmitter is temperature dependent.

Mitchell and Siegel (1973) developed a telemetry system for measuring deep body temperature by implanting a sealed-end cannulae with an external opening in the abdomen of chickens. When these investigators desired to measure body temperature the bead thermistor was inserted down the cannulae and the leads leading to a transmitter were strapped to the animal's back. By this means, calibration checks could be made before and after monitoring. The system was accurate to $\pm 0.1^\circ\text{C}$.

Advantages of telemetry systems for monitoring temperature include: freedom of movement of the experimental subject; opportunity for continuous monitoring; and availability of the signal for central processing. Some of the disadvantages of telemetry systems are that the location of gastrointestinal radio pills is not easily determined, and there is often a change in calibration over a period of time.

V. TECHNIQUES OF MEASUREMENT OF SPECIFIC TEMPERATURES IN THE BODY

A. Measurement of Rectal Temperature

For the measurement of rectal temperature the following four types of sensors are most commonly used: clinical mercury bulb thermometer, bimetallic dial thermometer with stem, thermocouple with protective covering, and thermistor with protective covering. Rectal temperature is probably the most widely used method of measuring body temperature in the experimental laboratory. This measurement has many characteristics which the investigator should be aware of, if he wishes to use this as an index of body temperature. First, there is a lag between body temperature as measured by other techniques and measured reading. The value may be inaccurate because of interference by feces or by vasculature. Often it is difficult to position the sensor accurately, and the method is esthetically undesirable. One risk is the danger of perforating the rectum or colon.

Mead and Bonmarito (1949) showed that in the human the depth of insertion of the probe into the rectum is critical, as there is a gradient of temperature through the rectum. To determine this, they used a rectal catheter with thermocouples spread at 1-in. (2.6-cm) intervals along its length. In addition, they were able to show by pelvic X-ray examination that there was a random variation in the position of the catheter within the rectum. For further discussion on the measurement of rectal temperature, see Grayson (1951) and Cooper and Kenyon (1956).

1. Clinical Mercury-Bulb Thermometer

The bulb and stem is inserted into the rectum a suitable distance, held there, and then removed and read. This method has the disadvantages of potential breakage, restraint of subject while the bulb is equilibrating, and traumatic insertion. With the clinical mercury-bulb thermometer one cannot measure rapid variations in temperature. In the human an insertion depth of 4 cm is recommended.

2. Bimetallic Dial Thermometer

The bimetallic dial thermometer with a stem is used in a similar manner as the mercury bulb thermometer. It has, however, the advantage of not being fragile, and changes in temperature can be monitored. This is a useful instrument for determining the temperature of an animal in which a glass thermometer is not utilized because of the danger of breakage.

3. Thermocouple with Protective Covering

By enclosing the thermocouple junction within a protective sheath such as a catheter, rectal temperature may be measured accurately. This

method has the advantage of being relatively inexpensive, but it has the disadvantages inherent in the use of thermocouples.

4. Thermistor

The use of thermistor elements as temperature sensors is one of the more widely used methods of continuously monitoring body temperature over periods of time. In our laboratory the rectal temperature of a large variety of animals such as the rat, rabbit, cat, monkey, sheep, and man is routinely monitored using this method.

5. General Procedures in Animal Experimentation

For the first few days the animal is conditioned to the experimental environment by simply bringing it to the laboratory and placing it in the experimental cage or restraint device. Once the animal is acclimated to the surroundings, it is conditioned to the rectal probe. The thermistor probe (Yellow Springs Instruments Nos. 401 or 701) is lubricated with a suitable ointment (e.g., Nupercainal, from Ciba-Geigy Canada, Ltd.), and then gently inserted into the rectum a suitable distance for the animal: rat, 6 cm; rabbit, 7 cm; cat, 10 cm; squirrel monkey, 10 cm; man, 10 cm. The lead is then attached to the tail (in man it can be held in place with the clothing) by two or three wraps of 2-in. wide micropore tape (Minnesota Mining and Mfg. Co.), followed by two or three wraps of 1-in. wide white adhesive tape. The lead is then connected to the recording or display system.

Standard leads with the Yellow Springs Instruments thermistor probes are 10 feet (3m) long; this allows the animal to move in the experimental cage with relative freedom if it is a species which can be conditioned to avoid biting the cable. Some animals, including the rabbit and certain rats, can not always be conditioned to avoid biting the probe. With these animals, restraint in conventional restraining devices may be necessary. Experimental procedures are generally carried out at the same time each day. Figure 4 shows rectal temperature being monitored in two unanesthetized cats. Figure 5 shows rectal temperature being monitored in a group of unanesthetized rabbits.

In our laboratory we use three main recording systems. The Honeywell Electronik-16, a multipoint strip chart recorder, is used in conjunction with an adjustable range unit to record temperatures sensed by means of Yellow Springs Instruments thermistor probe No. 401 and displayed on a Yellow Springs Instruments Model 73 indicating temperature controller. In our system we have the capability to record several temperature sensors, scanned approximately once every minute. Honeywell has other systems available which can accommodate up to 24 temperature channels, with couplers that will accept either thermistors or thermocouples.

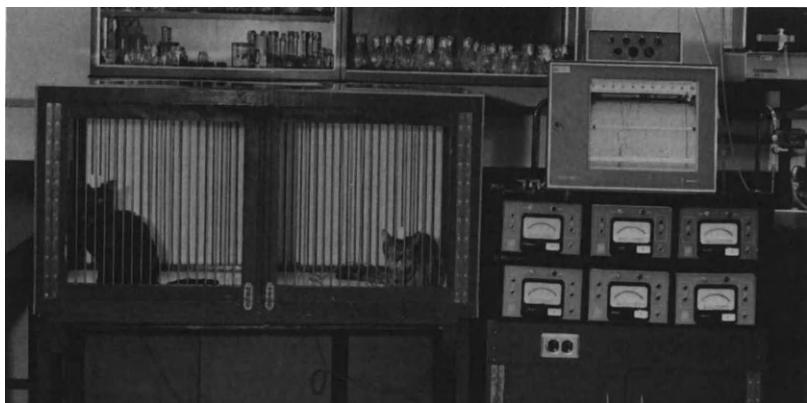


FIG. 4. Rectal temperature of two cats being monitored continuously utilizing Yellow-Springs Instruments No. 401 thermistor probes and displayed on Honeywell Electronik-16 strip chart recorder.

United Systems Corporation produces a temperature data acquisition system composed of several interconnected modules: Model 251A digital thermometer; Model 662A digital clock; Model 636 analog scanner; Model 691 digital printer; Model 672 paper tape punch; Model 625 paper tape punch controller (ASCII code); Wang Model 705-1 microinterface (to



FIG. 5. Rectal temperature of a group of rabbits being monitored continuously with Yellow Springs Instruments No. 701 thermistor probes and processed on United Systems Corporation data acquisition system.

interface with Wang 700 series programmable calculator and peripherals). In our laboratory this system is interfaced to a Wang 700C programmable calculator with plotting output. By suitable programming we can scan up to 50 Thermistors (Yellow Springs Instruments No. 701), perform statistical calculations such as means and variances, and (by using the plotting output) draw graphs of the temperatures and statistical values for individual animals or for groups of animals. In addition, tape output is available for storage of all the data.

The Beckman Dynograph permits the recording of temperature with the use of coupler Model 9858, in conjunction with Yellow Springs Instruments No. 401 thermistor probes. The Beckman Dynograph has the advantage of being very flexible, in that with suitable couplers a wide variety of other physiological functions can be monitored at the same time as temperature.

B. Measurement of Oral Temperature

Measurement of oral temperature is a commonly used index of body temperature, especially in the clinical setting. Thermometers used include the clinical mercury-bulb thermometer, bimetallic dial thermometer with stem, thermocouple, thermistor, and chemical strip. Oral temperature measurement is not used frequently in animal work unless the animal is under anesthesia. Even though the method is simple, the investigator should be aware of some of the weaker features. It is difficult to position the sensor in the same position in the oral cavity each time temperature is taken. Salivation may interfere with the temperature measured, and there may be some interference with respiratory activities. In man, a real disadvantage is that it precludes the need to communicate verbally (with some subjects, this may be an advantage!).

The sensor is generally inserted under the tongue and then slowly moved towards the back of the mouth until the bulb is in a pocket of tissue at the base of the tongue. The mouth is closed and, for accurate recording, taped shut. This is the region of the oral cavity that has the largest arterial blood supply and the highest temperature. There is very little difference in the temperature between the right and left sides of the mouth. When taking oral temperature it is important to obtain information on the person's previous activities such as eating and drinking, which can influence the reading. In a clinical setting, temperature elevations can be created by the patient if the investigator is not observant.

1. Clinical Mercury-Bulb Thermometer

Oral temperature measured with the clinical mercury-bulb thermometer is the most widely used method of measuring body temperature for

clinical purposes because of its simplicity. Because the measurement is a maximum, thermometer variations in temperature cannot be monitored without repeatedly removing the thermometer, shaking the mercury column down, and reinserting it.

2. Bimetallic Dial Thermometer

Dial thermometers with stems may be used to monitor oral temperature. This method has the advantage of monitoring variations in temperature and the option of recording maximum and minimum values.

3. Thermocouple

Thermocouple junctions may be placed inside a protective sheath and then inserted into the oral cavity at the desired point. This method has the advantage of monitoring the temperature continuously, and thus remote monitoring is possible.

4. Thermistor

Thermistor probes may be placed into the oral cavity. In a hospital setting, the leads may go to a central monitoring facility. Portable units with disposable sheaths are available with analog or digital displays. Typically they have a range of 35–42°C, an accuracy of 0.1°C, and—when used with a disposable sheath—a reading time of about 20 sec. These electronic thermometers are becoming more frequently used in the clinical setting because of their accuracy, sensitivity, and the display of varying temperatures.

5. Chemical Strip

Disposable "Fever Strips" are available which are composed of a matrix of indicators, or sensing elements, distributed along a flat surface. Each element has a melting point which varies only slightly (0.2°C) from the previous element in the matrix. The elements are arranged consecutively so that when the temperature is taken, a permanent digital display is recorded; since all elements in the matrix below the measured temperature will change color, the temperature can be read on an adjacent scale. This provides a permanent record of the subject's temperature at the time it is taken.

C. Measurement of Aural Temperature

Measurement of aural temperature can be used as an index of body temperature. This method does not interfere greatly with the normal movement of the subject, and can be connected to a telemetry system.

1. Simple Temperature Sensor in the Aural Canal

This method suffers from the major disadvantage of being seriously affected by local cooling of the aural canal. It is useful, therefore, only for measuring relative changes in temperature and not for measuring absolute body temperature. Either thermistors or thermocouples may be used as the sensing transducer. The ear is first examined with an Otoscope to check for any irregularities; then the thermistor is inserted into the aural canal. A covering such as foam rubber or cotton may be introduced into the external meatus and secured. Another method of doing this is by forming a mold of the external auditory canal and positioning thermistors on the outer surface of this mold in proximity to the skin. For further information, the reader is referred to Cooper *et al.* (1964) and Greenleaf and Castle (1972).

2. Zero Gradient Method

This method of measuring aural temperature provides a means of eliminating the effect of local cooling. This method measures aural canal temperature and provides for servo-controlled heating to keep the outer ear at the same temperature as that of the aural canal. This method has been tested (Keatinge and Sloan, 1975) at various ambient temperatures and wind speeds; in comparison to other methods of measuring body temperature, it has been found to be very accurate and sensitive. It follows changes in central body temperature much more rapidly than rectal temperature and almost as rapidly as esophageal temperature.

In this method, a small thermistor probe is inserted into the aural canal and a servo-controlled heated pad mounted on a headband is placed over the ear to eliminate cooling. The instrument reaches equilibrium in about 10 min (the temperature of the outer ear will be the same as that within the ear canal), and then temperature can be read. The instrument manufactured by Muirhead Limited (see Appendix), can be operated off a lab outlet (mains) or by an internal rechargeable battery. It also has a digital display and facilities for connection to an external recorder. It is simple to use, as there are only two control switches. We use this type of temperature monitor frequently in our laboratory to monitor the temperature of subjects immersed in water to the neck (Cooper *et al.*, 1976). Cross and Stratton (1974) and others have used this type of thermometer on the newborn human infant.

D. Measurement of Tympanic Membrane Temperature

In a method of measuring body temperature that permits utilization of thermistors or thermocouples, a sensing probe is brought into contact

with the tympanic membrane. The tympanic membrane has a large blood supply from the posterior auricular and internal maxillary branches of the external carotid artery. It is thought that this method permits the monitoring of a temperature which represents brain temperature relatively closely. There is a real danger of perforating the tympanic membrane, so care must be taken when positioning the sensor. Generally, the ear lobe is pulled down, and then the sensor is inserted carefully until resistance is felt. The leads should be firmly secured so that no movement can occur after the sensor is in position. Some models of temperature sensors have a brushlike device for holding the probe against the tympanic membrane with a relatively constant force; others have molded earplugs which fit into the ear canal. For further information, the reader is referred to Dickey *et al.* (1970), and Nadel and Horvath (1970).

E. Measurement of Cutaneous Temperature

When one is measuring skin temperature, some precautions must be taken in order to get a true indication of the temperature. The subject should be in a resting basal state, and room temperature must be constant and suitable, with no drafts. The temperature of furnishings or equipment in contact with the subject must be considered as well as their thermal conductivity. Temperature should be recorded at several points, and several readings should be taken at each point. In selecting points, one should check for presence of scars, blood vessels, skin lesions, or other surface abnormalities. The sensor should be in firm and complete contact with the skin. The same force should be exerted on the sensor each time a reading is taken, and on all sensors within a group.

If the temperature is taken at several different points at the same time, a mean skin temperature can be calculated by using weighted coefficients. The general formula would be

$$T_{\text{skin}} = w_1 T_1 + w_2 T_2 + \cdots + w_n T_n$$

Here w is the weighting coefficient at a particular location, and T is the skin temperature at that point. No system has been devised that might be called the perfect method of measuring mean skin temperature. The better systems use temperature measured at a large number of sites, the sites being selected by dividing the body into a number of regions, the area of which can be determined. This area, as a proportion of the whole, is used as the weighting factor. For more complete information on formulas, methods of weighting, and calculation of various mean skin temperatures,

the reader is referred to Hardy and DuBois (1937), Mitchell and Wyndham (1969), Gonzalez *et al.* (1971), and Stitt *et al.* (1971).

1. Bimetallic Dial

In this method the sensing surface of the unit is placed in contact with the skin, and after a suitable period of time the temperature is read. The dial shows variations in temperature and is simple to use; it can be secured and left in place to give a continuous indication of body temperature.

2. Thermistor

Thermistors are available in the form of a disk, one side of which forms part of the sensing element, the other side of which is insulated. When fastened to the skin, thermistors provide a means of continuously monitoring skin temperature. If they are connected electrically (using appropriate resistors) to a monitoring system, mean skin temperature may be displayed by suitably weighting each thermistor. Two thermistors which we use in our laboratory for measuring skin temperature are the Yellow Springs Instruments Nos. 409 and 709.

3. Thermocouple

Thermocouples may be used in a manner similar to that described for the thermistor. They can be very sensitive and are conveniently small, providing a relatively inexpensive means of measuring mean skin temperature when a large number of skin temperature measurement points are made. A fine thermocouple, arranged on a flexible holder like a bowstring, may be used for spot measurements.

4. Liquid Crystals

Normal and abnormal venous patterns are well demonstrated by the liquid-crystal technique. A thermographic map of a subject may be produced under normal conditions, and then changes induced by experimental manipulations may be evaluated. Liquid crystals provide an excellent means of visualizing and quantitating the effects of vasoactive drugs into the skin. They can also show how outside agents such as air temperature and air speed affect the skin.

The skin is thoroughly cleaned to remove all traces of dirt and grease. It is necessary first to apply a black coating such as a mixture of carbon black and polyvinyl alcohol to the skin in order to prevent light transmitted by the film from reflecting back from the skin surface. This must be applied uniformly or else artifacts will appear. The film is either painted or

sprayed onto the skin; again, this should be done carefully, as the accuracy of the temperature display depends partially on the uniformity of the liquid-crystal layer. A color photograph may be taken to reveal the temperature distribution. After the measurements have been made, the coating can be removed with a lukewarm soap solution. The absolute temperature may be determined at any given point by means of a calibration chart.

5. Thermograph

Temperature may be determined by simply pointing the sensor at the area to be measured or by using a scanning system to measure accurately the temperature distribution of the whole body. This method can be employed in the study of vasculature and skin conditions. Scanning time is a balance between resolution desired and avoidance of changes in thermal gradients during the scanning time. Thermography should be done in a closed room held at a constant temperature (21°C). Clothing must be removed from the area to be scanned. Care must be taken to avoid skin-to-skin cross reactions by positioning the body so that no overlapping skin areas are scanned. Appendages should be spread apart.

6. Heat Flow

The heat flow disk is attached to the skin with a suitable adhesive. The skin area should be clean and free of hair.

F. Measurement of Subcutaneous Temperature

By inserting small thermistors or thermocouples into hypodermic needles, subcutaneous temperature may be measured. If a sequential arrangement of thermal sensors is placed along the length of the needle, gradients of temperature may be measured. When one is taking subcutaneous temperature measurements, it is difficult to judge the exact location of the sensing element in the tissue. The investigator must be careful to identify blood vessels, local injury, variations in skin thickness, and movement artifacts. Heat transfer along the needle and environmental temperature must also be considered.

For further information on the construction and use of needle thermal sensors for subcutaneous measurements, the reader is referred to Krog (1954, 1956) and Gray and Axelrod (1953).

Barrett and Myers (1975) have recently reported on a noninvasive method of sensing subcutaneous temperature. They detect with microwave receivers the thermal radiation emitted from depths of up to several centimeters of skin, with a sensitivity of about 0.1°C. They call this technique *microwave thermography*.

G. Measurement of Intramuscular Temperature

Myothermal measurements can easily be made by simply mounting a thermistor or thermocouple near the tip of a hypodermic needle and then inserting it into the muscle tissue. Important features to observe when measuring muscle temperature are movement artifacts at the start and during a contraction, and the existence of thermal gradients within the tissue mass. As with all needle thermosensors, there is local injury to tissue, and it is difficult to localize exactly where the sensor is placed. By X-ray examination, some indication of position can be determined. Movement artifacts can be avoided somewhat by constructing a small barb at the tip of the needle.

Edwards *et al.* (1974) describe the construction of a thermistor probe for myothermal measurements. This probe consists of a thermistor bead embedded in epoxy resin at the end of a nylon tube whose tip is wider than the main shaft of the probe. This serves to anchor it in the muscle. This design has a 95% speed to response in 1.0 sec and a sensitivity of 0.001°C . Edwards and associates' excellent article discusses the construction of the probe, its performance characteristics, and its use in measuring muscle temperature. Issel *et al.* (1972) describe the incorporation of an electrode in the same needle as that holding the thermistor in order to record the electromyogram at the same time as the temperature.

H. Measurement of Intravenous Temperature

By inserting a temperature-sensing element (thermistor or thermocouple) within a flexible catheter and then introducing it into the vascular system, temperature of blood at many points within the body can be measured. The temperature of the blood throughout the vascular system is not constant. Its value at any point depends upon the metabolic level of the tissue through which the blood passes, the temperature of the blood with which it mixes, and the rate of blood flow. This is an invasive technique, and the catheter interferes with normal blood flow down the vessel. There is difficulty in determining exactly where the sensing element is located, and the temperature transducer is subject to vasomotor changes.

With suitable surgery it is possible to use this technique in chronic preparations as well as acute ones. When fabricating the thermistor catheter it is best if the thermistor occupies the geometric center within a protective cage at the tip of the catheter. This is to avoid the possibility of the thermistor lying in contact with the vessel wall. For further information on construction and use of intravascular thermistor catheters, the reader is referred to Afonso *et al.* (1962) and Warren (1974).

I. Measurement of Esophageal Temperature

Esophageal temperature closely follows the temperature of the blood going through the heart. A flexible catheter containing a thermistor or thermocouple is inserted through the nostril and lowered until the sensor is located behind the middle of the lower third of the sternum. It is very difficult to position the sensor in the esophagus of an awake or anesthetized subject atraumatically. There is also significant longitudinal variation in temperature based on the depth of insertion into the esophagus; however, there is little lateral variation in temperature. For further information, the reader is referred to Whitby and Dunkin (1968).

Brittain and Spencer (1964) describe a method of measuring esophageal temperature in small unanesthetized animals—mice, rats, and guinea pigs. These investigators sealed a small thermocouple inside a bent 4.5-cm long 18-ga hypodermic needle. Following insertion of the thermocouple into the esophagus a constant temperature is obtained in 3–4 sec.

J. Measurement of Abdominal Temperature

By surgically implanting temperature sensors into the abdomen, temperatures of various abdominal organs and tissues may be determined. In long-term preparations, the leads can be exteriorized through the skin to a suitable connector for direct connection to a display, or a telemetry system may be used. In short-term preparations, the temperature sensors can be introduced into the abdomen through small incisions and located by direct vision. In experiments in which the subject is under anesthesia, consideration must be given to the total body cooling due to the anesthetic, conducting furnishings, and differences in muscular and vascular tone.

Taylor *et al.* (1976) have developed a technique for measuring thermal gradients in the female human pelvis during pelvic laparoscopy. In this procedure, under general anesthesia, pneumoperitoneum is induced by carbon dioxide gas passing through a needle inserted through the abdominal wall. The laparoscope is then positioned and the thermistor (Yellow Springs Instruments No. 701) is introduced via a manipulator through a second puncture in the abdominal wall and positioned upon the desired organ or tissue under direct laparoscopic control. In addition rectal lumen temperature is measured continuously at a depth of 20 cm using a similarly matched thermistor. Temperatures are monitored on a two-channel United Systems Corporation Digital Thermometer Model 251A. Pelvic organs and tissues studied by using this method include the follow-

ing: serosal surface of the rectum; mesovarium; ovarian artery; external iliac artery; small intestine (serosal surface); and appendix.

The insufflating gas temperature within the abdomen is also measured. This method could easily be adapted for use in animal models. For further information on intraabdominal temperatures, the reader is referred to Grayson and Durotoye (1971) and Fedorov and Shur (1942).

K. Measurement of Brain Temperature

By implanting needles stereotactically with temperature sensors (thermocouples, thermistors; at their tip or arranged consecutively along the needle shaft) accurate measurements of brain temperature may be made. Thermistor needles can be bought commercially or they may be fabricated in the laboratory.

Following standard stereotaxic surgical procedures, the animal is placed in a head holder. An incision is made in the scalp, a hole is drilled through the skull, the dura is punctured if necessary, and the needle thermal sensor is lowered to the desired depth. Dental acrylic is used to secure the needle to the skull and also to two or three stainless steel machine screws (1-72, 3/16 in., from Small Parts Inc., Miami, Florida) previously inserted into the adjacent skull area. The incision is then closed, leaving only the connector visible. After the animal is allowed to recover, recordings may be made after the animal is suitably conditioned. The signal may also be received by telemetry.

Delgado and Hanai (1966) stereotactically implanted copper-constantan thermocouples into various locations in the brain of cats and had a connector fixed to the skull. They monitored brain temperature continuously for periods of up to 3 days on unanesthetized cats housed in an experimental cage.

L. Measurement of Fetal Temperature

Temperature-sensing elements (thermistors, thermocouples) may be surgically implanted into fetal tissue and suitably exteriorized, or the signal can be received via a telemetry system. The sheep is the animal model that is particularly valuable in fetal studies because of its size, ease of handling, and the large amount of information already available about the fetus.

Abrams *et al.* (1969) conducted a long-term study by implanting thermocouples into fetal sheep brain and the maternal aorta at stages between days 122 and 139 of gestation. Following strict aseptic precautions the ewe's abdomen is opened by midline incision. The fetal head is located and shifted to the distal part of the uterine horn. After encircling

the incision site with fine silk sutures, an incision is made to expose the fetal scalp. The periosteum is removed, a hole is drilled through the skull for the thermocouple, and several stainless steel anchoring screws are inserted in the skull. The thermocouple is then positioned in the brain and anchored with dental acrylic spread on the skull and over stainless steel screws. The fetal scalp incision is closed around the acrylic, and as the uterine incision is closed, the fetus is allowed to move; 30 to 45 cm of lead are left free in the amniotic cavity. The lead is exteriorized through a stab wound on the flank and protected there by a cloth pouch.

After recovery from surgery, the fetal brain temperature can be monitored as well as the maternal aorta temperature via thermocouples drawn into sealed polyvinyl catheters and inserted into the abdominal aorta via a femoral artery at the time of surgery. Some of the lambs are delivered by caesarian section; others are delivered naturally, the leads being broken off in the birth process. In all cases, the thermocouples are firmly attached to the skull. In a similar manner, thermal sensors could be positioned into other areas of the fetus, such as the abdomen or rectum, or they could be inserted intravenously. In our laboratory we routinely monitor fetal sheep temperature via a Yellow Springs Instruments No. 402 thermistor probe secured with a suture and positioned in the area to be thermally sensed.

M. Measurement of Cellular Temperature

Hill (1932), using an elegantly constructed apparatus, showed that the temperature increase in a nerve during the passage of an impulse was 7×10^{-8} °C. He used the sciatic nerve of the Hungarian frog, mounted on a thermopile composed of 150 couples of iron-constantan junctions. The thermopile was mounted on a frame inside a glass tube, which was then well insulated from environmental temperature changes. The signal from the thermopile was monitored on a galvanometer and recorded on a "plate camera."

Hodgkin and Katz (1949) reported the effect of temperature on the electrical activity of the giant axon of the squid. The axon was placed in a seawater bath with the recording and stimulating electrodes appropriately located. The temperature was varied by replacing the seawater in the bath and then measured with a thermometer.

VI. CONCLUDING REMARKS

For the routine measurement of body temperature the traditional clinical thermometer has historically been the most widely used method. In the future, with the development of inexpensive integrated circuits, small

electronic devices using interchangeable sensors and digital displays will play a greater role in the monitoring of body temperature. Telemetry systems to monitor temperature continuously deep within the body core will also find more and more use. Because of the variations in temperature throughout the body of an experimental subject, it is extremely important to think out carefully the method of measurement of temperature and the site at which it is measured.

REFERENCES

- Abrams, R., Caton, D., Curet, L. B., Crenshaw, C., Mann, L., and Barron, D. H. (1969). Fetal brain-maternal aorta temperature differences in sheep. *Am. J. Physiol.* **217**, 1619-1622.
- Abrams, R. M., and Stolwijk, J. A. J. (1972). Heat flow device for vaginal blood flow studies. *J. Appl. Physiol.* **33**, 144-146.
- Afonso, S., Herrick, J. F., Youmans, W. B., Rowe, G. G., and Crumpton, C. W. (1962). Temperature variations in the venous system of dogs. *Am. J. Physiol.* **203**, 278-282.
- Aschoff, J., and Wever, R. (1958). Kern und Schale warmhaushalt des Menschen. *Naturwissenschaften* **45**, 477.
- Atwater, W. O., and Rosa, E. B. (1899). A new respiration calorimeter and experiments on the conservation of energy in the human body. [Quoted in Lefèvre, J., "Chaleur Animal et Bioénergétique," Paris, Masson (1911), p. 165.]
- Atwater, W. O., and Rosa, E. B. (1903). "Experiments on the Metabolism of Matter and Energy in the Human Body." Office of Exp. Stns., U.S. Dep. of Agric., Washington, D.C. [Quoted in Lefèvre, J., "Chaleur Animal et Bioénergétique," Paris, Masson (1911), p. 165.]
- Barnes, R. B. (1968). Diagnostic thermography. *Appl. Opt.* **7**, 1673-1685.
- Barrett, A. H., and Myers, P. C. (1975). Subcutaneous temperatures: A method of noninvasive sensing. *Science* **190**, 669-671.
- Barton, L. E. (1962). Measuring temperature with diodes and transistors. *Electronics* **35**, 38-40.
- Becquerel and Breschet (1835). *Ann. Sci. Nat., Zool.* **iii**, **iv**, **ix**.
- Benedict, R.P. (1969). Thermoelectric thermometry. In "Fundamentals of Temperature, Pressure, and Flow Measurements," Chap. 7, pp. 53-98. Wiley, New York.
- Benzinger, T. H., Kitzinger, C., and Pratt, A. W. (1963). The human thermostat. In "Temperature: Its Measurement and Control in Science and Industry" (J. D. Hardy, ed.), Vol. 3, Part 3, Chap. 56, pp. 637-665. Reinhold, New York.
- Bernard, C. (1852). De l'influence de système nerveux grand sympathique sur la chaleur animale. *C. R. Acad. Sci.* **34**, 472.
- Bernard, C. (1862). Experimental researches on the vascular and calorific nerves of the great sympathetic. *C. R. Acad. Sci.* **40**, 228.
- Biot, J. B. (1838) [Quoted in Wunderlich, C. A. (1871), "On the Temperature in Diseases. A Manual of Medical Thermometry" (W. B. Woodoran, transl.), 2nd ed., pp. 28-29. New Sydenham Soc., London.]
- Blagden, C. (1775). *Phil. Trans. R. Soc. London* p. 111. [Quoted in Wunderlich, C. A. (1871), "On the Temperature in Diseases. A Manual of Medical Thermometry" (W. B. Woodoran, transl.). New Sydenham Soc., London.]
- Boerheave, H. (1708) [Quoted in Wunderlich, C. A. (1871). "On the Temperature in

- Diseases. A Manual of Medical Thermometry" (W. B. Woodoran, transl.), p. 20. New Sydenham Soc., London.]
- Brittain, R. T., and Spencer, P. S. J. (1964). Measurement of body temperature in conscious small laboratory animals by means of an oesophageal thermocouple. *J. Pharm. Pharmacol.* **16**, 497-499.
- Brown-Séquard, C. E. (1853). "Experimental Researches Applied to Physiology and Pathology." [Quoted in Wunderlich, C. A. (1871). "On the Temperature in Diseases. A Manual of Medical Thermometry" (W. B. Woodoran, transl.), p. 44. New Sydenham Soc., London.]
- Busse, J. (1939). Liquid-in-glass thermometers. In "Temperature, Its Measurement and Control in Science and Industry," Vol. 1, pp. 228-255.
- Cobbold, R. S. C. (1974). Temperature transducers. In "Transducers for Biomedical Measurements," Chap. 5, pp. 57-113. Wiley, New York.
- Cohen, B. G., Snow, W. B., and Tretola, A. R. (1963). GaAs *p-n* junction diodes for wide range thermometry. *Rev. Sci. Instrum.* **34**, 1091-1093.
- Cooper, K. E., and Kenyon, J. R. (1956). A comparison of temperature measured in the rectum, oesophagus on the surface of the aorta during hypothermia in man. *Brit. J. Surg.* **44**, 616-619.
- Cooper, K. E., Cross, K. W., Greenfield, A. D. M., Hamilton, D. M. K., and Scarborough, H. (1949). A comparison of methods for gauging the blood flow through the hand. *Clin. Sci.* **8**, 217-234.
- Cooper, K. E., Cranston, W. I., and Snell, E. S. (1964). Temperature in the external auditory meatus as an index of central temperature changes. *J. Appl. Physiol.* **19**, 1032-1035.
- Cooper, K. E., Martin, S., and Riben, P. (1976). Respiratory and other responses in subjects immersed in cold water. *J. Appl. Physiol.* **40**, 903-910.
- Crissey, J. T., Fergason, J. L., and Bettenhausen, J. M. (1965). Cutaneous thermography with liquid crystals. *J. Invest. Dermatol.* **45**, 329-333.
- Cross, K. W., and Stratton, D. (1974). Aural temperature of the newborn infant. *Lancet* **ii**, 1179-1180.
- Currie, J. (1798). "Medical Reports on the Effect of Water. Cold and Warm, as a Remedy in Fever and other Diseases," 2nd ed. J. M'Creery, Liverpool.
- d'Arsonval (1894). Anémo-calorimètre. *Arch. Physiol.* **360**.
- Davy, J. (1850). On the temperature of man within the tropics. *Phil. Trans. R. Soc. London* p. 360. [Quoted in Lefèvre, J. (1911). "Chaleur Animale et Bioénergétique," p. 124. Paris, Masson.]
- Delgado, J. M. R., and Hanai, T. (1966). Intracerebral temperatures in free-moving cats. *Am. J. Physiol.* **211**, 755-769.
- Dickey, W. T., Ahlgren, E. W., and Stephen, C. R. (1970). Body temperature monitoring via the tympanic membrane. *Surgery* **67**, 981-984.
- DuBois, E. F. (1939). Heat loss from the human body. *Bull. N.Y. Acad. Med.* **15**, 142-173.
- Edwards, R. H. T., McDonnell, M. J., and Hill, D. K. (1974). A thermistor probe for myothermal measurements in man. *J. Appl. Physiol.* **36**, 511-513.
- Fedorov, N. A., and Shur, E. I. (1942). The role of the viscera in regulating the temperature of the body of an animal under physiological and pathological conditions. *Am. J. Physiol.* **137**, 30-38.
- Fergason, J. L. (1964). Liquid crystals. *Sci. Am.* **211**, 77-83.
- Finch, D. I. (1962). General principles of thermoelectric thermometry. In "Temperature, Its Measurement and Control in Science and Industry" (C. M. Herzfeld, ed.), Vol. 3, Pt. 2, pp. 3-32. Reinhold, New York.

- Ganfield, R. A., and Smaha, L. A. (1971). Temperature measurement in cats with a chronically implanted sensory device. *Physiol. Behav.* **7**, 924–930.
- Gerbrandy, J., Snell, E. S., and Cranston, W. I. (1954). Oral, rectal and oesophageal temperatures in relation to central temperature control in man. *Clin. Sci.* **13**, 615–624.
- Gershon-Cohen, J. (1967). Medical thermography. *Sci. Am.* **216**, 94–102.
- Gonzalez, R. R., Kluger, M. J., and Hardy, J. D. (1971). Partitional calorimetry of the New Zealand white rabbit at temperatures 5–35°C. *J. Appl. Physiol.* **31**, 728–734.
- Gray, R. S., and Axelrod, A. E. (1953). Application of thermistor to measurement of subcutaneous temperatures during hydrothermal injury in the rat. *Proc. Soc. Exp. Biol. Med.* **83**, 269–272.
- Grayson, J. (1951). Observations on the temperature of the human rectum. *Brit. Med. J.* **ii**, 1379–1382.
- Grayson, J., and Durotoye, A. O. (1971). Effect of environment on temperatures in the viscera of the dog. *Int. J. Biometeor.* **15**, 176–180.
- Greenleaf, J. E., and Castle, B. L. (1972). External auditory canal temperature as an estimate of core temperature. *J. Appl. Physiol.* **32**, 194–198.
- Hagelsten, J. O., Jessen, K., and Rosendal, T. (1972). Clinical application of an outdoor-indoor thermometer. *Anaesth. Analg.* **51**, 863–864.
- Hardy, J. D., and DuBois, E. F. (1937). The technique of measuring radiation and convection. *J. Nutr.* **15**, 461–475.
- Hatfield, H. S. (1949). A heat flow meter. *J. Physiol. (London)* **111**, 10P–11P.
- Hayward, J. (1973). The anatomy of heat exchange. In "The Pharmacology of Thermoregulation" (E. Schönbaum and P. Lomax, eds.), pp. 22–41. Karger, Basel.
- Hayward, J. S., Eckerson, J. D., and Collis, M. L. (1975). Thermal balance and survival time prediction of man in cold water. *Can. J. Physiol. Pharmacol.* **53**, 21–32.
- Heal, J. W., Ingram, D. L., and Legge, K. F. (1970). Measurement of heat loss from the skin by means of radiotelemetry. *J. Physiol. (London)* **210**, 123 P.
- Hill, A. V. (1932). A closer analysis of the heat production of nerve. *Proc. R. Soc., Ser. B* **111**, 106–164.
- Hodgkin, A. L., and Katz, B. (1949). The effect of temperature on the electrical activity of the giant axon of the squid. *J. Physiol. (London)* **109**, 240–249.
- Huston, W. D. (1962). The accuracy and reliability of bimetallic temperature measuring elements. In "Temperature, Its Measurement and Control in Science and Industry" (C. M. Herzfeld, ed.), Vol. 3, Pt. 2, pp. 949–957. Reinhold, New York.
- Issel, W., Lahoda, F., and Ross, A. (1972). Simultaneous recording of temperature and action potentials in human voluntary muscle. *Electroencephalogr. Clin. Neurophysiol.* **37**, 561–562.
- Keatinge, W. R., and Sloan, R. E. G. (1975). Deep body temperature from aural canal with servo-controlled heating to outer ear. *J. Appl. Physiol.* **38**, 919–921.
- Krog, J. (1954). Improved needle thermocouple for subcutaneous and intramuscular temperature measurements in animals and man. *Rev. Sci. Instrum.* **25**, 799–800.
- Krog, J. (1956). Thermistor hypodermic needle for subcutaneous temperature measurement. *Rev. Sci. Instrum.* **27**, 408–409.
- Lefèvre, J. (1911). "Chaleur Animale et Bioénergétique." Masson, Paris.
- Liebermeister, C. von (1875). "Handbuch der Pathologie und Therapie des Fiebers." Vogel, Leipzig.
- McNamara, A. G. (1962). Semiconductor diodes and transistors as electrical thermometers. *Rev. Sci. Instrum.* **33**, 330–333.
- Mayer, J. R. (1842). "Bemerkungen über die Käfte der unbelebten Natur." Wohner und Liebig's Annalen, Weinheim, Germany.

- Mead, J., and Bonmarito, C. L. (1949). Reliability of rectal temperature as an index of internal body temperature. *J. Appl. Physiol.* **2**, 97-109.
- Mitchell, B. W., and Siegel, H. S. (1973). Measuring body temperature by telemetry using a removable probe in an implanted cannulae. *J. Appl. Physiol.* **35**, 925-927.
- Mitchell, D., and Wyndham, C. H. (1969). Comparison of weighting formulas for calculating mean skin temperature. *J. Appl. Physiol.* **26**, 616-622.
- Nadel, E. R., and Horvarth, S. M. (1970). Comparison of tympanic membrane and deep body temperatures in man. *Life Sci.* **9**, 869-875.
- Ogle (1866). On the diurnal variations in the temperature of the human body. *St. George's Hosp. Rep., London* **1**, 221.
- Puhl, J., and Golding, L. A. (1975). Mapping body heat with liquid crystals. *Physician Sports Med.* pp. 47-53.
- Riley, J. A. (1949). A simple method for welding thermocouples. *Science* **109**, 281.
- Roeser, W. F. (1940). Thermoelectric thermometry. *J. Appl. Phys.* **11**, 388-407.
- Rowell, L. B. (1974). Human cardiovascular adjustments to exercise and thermal stress. *Physiol. Rev.* **54**, 75-159.
- Samuel, E. (1969). Thermography—some clinical applications. *Bio-Med. Eng.* **4**, 15-19.
- Sanctorius, S. (1614). [Quoted in Wunderlich, C. A. (1871). "On the Temperature in Diseases. A Manual of Medical Thermometry" (W. B. Woodoran, transl.), p. 20. New Sydenham Soc., London.]
- Schiff (1855). "Untersuchungen zur Physiologie des Nerven Systems Frit Berücksichtigung der Pathologie." pp. 124-228.
- Sclar, N., and Pollock, D. B. (1972). On diode thermometers. *Solid-State Electron.* **15**, 473-480.
- Seebeck (1821). [Quoted in Smith, C. J. (1932). "Intermediate Physics." Arnold, London.]
- Stitt, J. T., Hardy, J. D., and Nadel, E. R. (1971). Surface area of the squirrel monkey in relation to body weight. *J. Appl. Physiol.* **31**, 140-141.
- Swindells, J. F. (1965). Calibration of liquid-in-glass thermometers. *Natl. Bur. Stand. (U.S.), Monogr.* **90**.
- Taylor, P. J., Cooper, K. E., and Malkinson, T. (1976). Temperature gradients at various points within the human pelvis, as measured during laparoscopy. *J. Reprod. Med.* **16**, 163-166.
- Thompson, R. D. (1968). Liquid-in-glass thermometers. Principles and Practices *Instrum. Soc. Am. Trans.* **7**, 87-92.
- Trolander, H. W. (1967). The measurement of biological temperatures. In "Methods in Psychophysiology" (C. C. Brown, ed.), pp. 95-113. Williams & Wilkins, Baltimore.
- Van Swieten (1745). "Commentaries on Boerhaave's Aphorisms." Leydn.
- Wang, L. C. H. (1972). Circadian body temperature of Richardson's ground squirrel under field and laboratory conditions: A comparative radio-telemetric study. *Comp. Biochem. Physiol.* **43A**, 503-510.
- Warren, D. J. (1974). A thermistor probe for measurement of blood temperature and cardiac output in small animals. *Cardiovasc. Res.* **8**, 566-569.
- Ween, S. (1968). Care and use of liquid-in-glass laboratory thermometers. *Instrum. Soc. Am. Trans.* **7**, 93-100.
- Whitby, J. D., and Dunkin, L. J. (1968). Temperature differences in the oesophagus. *Brit. J. Anaesth.* **40**, 991-995.
- Wolff, H. S. (1961). The radio pill. *New Sci.* **12**, 419-421.
- Wunderlich, C. A. (1871). "On the Temperature in Diseases. A Manual of Medical Thermometry" (W. B. Woodoran, transl.), 2nd ed. New Sydenham Soc., London.

Appendix

Electrical temperature sensors and displays

United Systems Corporation
918 Woodley Road
Dayton, Ohio 45403

Yellow Springs Instrument Corporation
Yellow Springs, Ohio 45387

Beckman Instruments Inc.
1117 California Avenue
Palo Alto, California 94304

Fenwal Electronics
P.O. Box 585
63 Fountain Street
Framingham, Massachusetts 01701

Zero-gradient aural thermometer

Muirhead Limited
154-160 Croydon Road
Beckenham, Kent
United Kingdom

Tympanic membrane thermometer

Radiation Systems Inc.
1755 Old Meadows Road
McLean, Virginia 22101

Quartz thermometer

Hewlett-Packard
1501 Page Mill Road
Palo Alto, California 94304

Thermoelectric supplies

Omega Engineering Inc.
Box 4047
Springdale Station
Stamford, Connecticut 06907

Leeds and Northrup International
North Wales, Pennsylvania 19454

Calibration of thermometers

United States Department of Commerce
National Bureau of Standards
Washington, D.C. 20234

Liquid-in-glass thermometers

Available from most scientific and hospital supply houses.

Chemical temperature indicators

Paper Thermometer Co., Inc.
10 Stagg Drive
Natick, Massachusetts 01760

Liquid crystals

Eastman Organic Chemicals
Eastman Kodak Company
Rochester, New York 14650

Liquid Crystals Products, Inc.
724 West Algonquin Road
Arlington Heights, Illinois 60005

Minnesota Mining and
Manufacturing Co.
P.O. Box 33800
St. Paul, Minnesota 55133

Poly Sciences, Inc.
Paul Valley Industrial Park
Warrington, Pennsylvania 18976

BDH Chemicals
350 Evans Avenue
Toronto, Ontario M8Z 1K5

Portable digital thermometers

Measurement Science Corporation
P.O. Box 338
Brigham City, Utah 84302

Portable infrared thermometer

Mikron Instrument Co., Inc.
P.O. Box 211
Ridgewood, New Jersey 07451

Miscellaneous Thermometers

Brooklyn Thermometer Co., Inc.
90 Verdi Street
Farmingdale, New York 11735

Chapter 7

Experimental Studies of Sleep in Animals¹

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I. INTRODUCTION

In this chapter we do not plan to discuss in detail the technical aspects of electroencephalography, polygraph machines, electronic circuits, types and uses of electrodes, and the like. The technical aspects of

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electroencephalography and the related electronic circuits are covered fully in many bioelectronic texts (e.g., see Thompson and Patterson, 1974; Brown *et al.*, 1973; Bureš *et al.*, 1967), while the historical aspects of studies of the electrical activity of the brain and related methodology are excellently rendered by Brazier (1960). We will outline only the most salient features of polygraph recording, discuss the setup and operation of polygraph machines, and provide some general information regarding the interpretation of the written encephalographic record. This background will facilitate discussions of normal polygraphic recording and alterations of electroencephalographic activity occurring with changes in the vigilance states, including changes induced by manipulating the brain electrically or chemically.

In previous chapters in this series, in particular Chapters 6 and 7 in Volume 1, there are adequate discussions relating to the presumed origin of brain waves, preparation and uses of electrodes, polygraphic recording techniques, and some types of analysis of electroencephalographic activity. Our main role here will be to review how sleep-waking activity can be induced or altered by electrical or chemical stimulation of the brain and how the vigilance states of sleep and waking can be "scored" and developed into quantitative "profiles" which serve as sensitive indices of altered brain functioning. We will further outline how and why sleep deprivation experiments are used and discuss pharmacological manipulation of sleep states as an indicator of chemical organization of the brain.

II. BACKGROUND

In 1875 Caton published the initial account of the recording of functionally significant electrical activity from the cerebral cortex of an experimental animal. The amplitude of these electrical oscillations was so low, i.e., in the order of microvolts, that Caton's (1875) discovery is all the more amazing, since it was made almost 50 years before suitable electronic amplification became available for the laboratory. In 1924 Berger carried out the first recordings of electrical activity from the human brain, and these were first published in 1929. Berger (1929) was the first to use the word *electroencephalogram* in describing these brain potentials in man. He noted that brain waves were slow in sleep and states of depressed function and observed the marked increase in voltage of brain electrical activity that accompanies a convulsive seizure.

Adrian and Matthews (1934) put electroencephalographic studies on a firmly established basis by localizing certain rhythms, especially the prominent alpha rhythm, to the parieto-occipital region and also observed the alpha rhythm-disrupting effects of photic stimulation. Later

it was shown that any type of attention or concentration on objects in the visual field tends to block the alpha rhythm. This effect has since been demonstrated in many types of animals as well as in man, and was one of the earliest observations to be made in electroencephalography. The physiological basis of this phenomenon was not, however, explained until the discovery of the "arousing" influence on the cortex of the reticular-activating system (Moruzzi and Magoun, 1949), wherein a clear relationship between electroencephalographic changes and levels of consciousness was first demonstrated. In the past 25 years unambiguous associations of electroencephalographic activity with states of vigilance have been demonstrated, as have precise relations of brain waves to specific states and stages of sleep.

III. ORIGIN AND SIGNIFICANCE OF BRAIN WAVES

Electrical activity of the brain, as recorded by the electroencephalogram (EEG), is thought most likely to represent a fluctuation in the membrane potential of the dendritic networks of the cerebral cortex. Recent evidence indicates that the EEG may be the sum of the extracellular potentials produced by numerous small, slow wave generators located on the nerve cell membranes in the cerebral cortex (Elul, 1968). This conclusion is based upon the premise that activity from deep subcortical areas is too attenuated at the surface to be recordable. The cortex in most areas generally contains six distinct layers and, although these vary considerably in architecture in different regions, the six-layered arrangement is generally clearcut over most neocortical areas. There is considerable evidence that the uppermost granular layer of the cortex probably makes the greatest contribution to the surface-recorded EEG.

The significance of cerebral electrical activity is interpreted largely in terms of the establishment of empirical, clinical correlations. For example, if a certain type of electrical activity is observed to arise from the brain in particular conditions, or certain states of vigilance, and not in others, the investigator records this fact; and, when he sees a similar type of activity again, he will match this with those conditions which have previously given rise to this same type of activity in the past. This essentially amounts to correlating types of electrical activity of the brain under behavior states of the experimental subject.

It is by no means clearly understood why certain states of vigilance produce one type of electrical activity of the brain and various other conditions produce another. However, studies of the origins of EEG activity (e.g., see Elul, 1972) are an active area of research today. As indicated, even though the underlying mechanisms of the EEG are not at

present adequately identified, the correlation of EEG activity with specific levels of awareness or consciousness is so clear as to make use of EEG in identifying vigilance states, i.e., sleep-waking activity, perfectly adequate even without understanding its physiological origins.

IV. TECHNIQUES, METHODS, AND INSTRUMENTATION

The voltage of electrical activity generated in the brain is so minute that it must be greatly amplified by a special apparatus designed for this purpose. In the case of recording EEG activity the Model 78 series Grass polygraph (see Fig. 1) provides the investigator with a high performance ac (alternating current) amplifier which combines a high gain preamplifier and driver amplifier in one rackmount. This system, based on the latest solid state technology, ensures stability in recording clinical or research EEG activity. The output connections can be used to drive the pens of a polygraph or permit direct connection to tape recorders, computers, frequency analyzers, oscilloscopes, etc. In the case of polygraphic recording, sufficient energy is produced to drive the pens which write out a record of the brain waves on a continuous strip of paper as it moves under them at standard speed. A whole series of stages of amplification (that required in electroencephalography may be on the order of a million to one) is required for each pen recording the brain waves that arise from the neighborhood of the electrodes to which the chain of amplifiers is connected.

In these types of experiments an animal, for example, a cat or rat, is implanted with a full electrode array (Fig. 2) for recording cortical EEG activity, deep brain activity (usually hippocampal EEG), eye movements, neck electromyographic (EMG) activity and ponto-geniculo-occipital (PGO) waves (the latter are easily recorded in cats but not in rats). After surgical recovery the animal is placed in a recording chamber (Figs. 3 and 4) for acclimatization, usually 2-3 days in a living chamber with food and water available *ad libitum*. It is then hooked up for recording baseline information from all channels. The pen movement from each channel is recorded in the form of oscillations in the ink line being written out on the moving sheet of paper driven at a set speed under the pen.

Electroencephalographic machines are constructed so that two connections may be made to each channel of amplification at the input end, i.e., at the end of which the wires leading from the electrodes placed on the animal are connected. The usual practice is to hook one electrode to each connection and, in this manner, the channel of amplification measures the difference in voltage between the two electrodes connected to it. Thus, a given channel measures the difference in potential or voltage

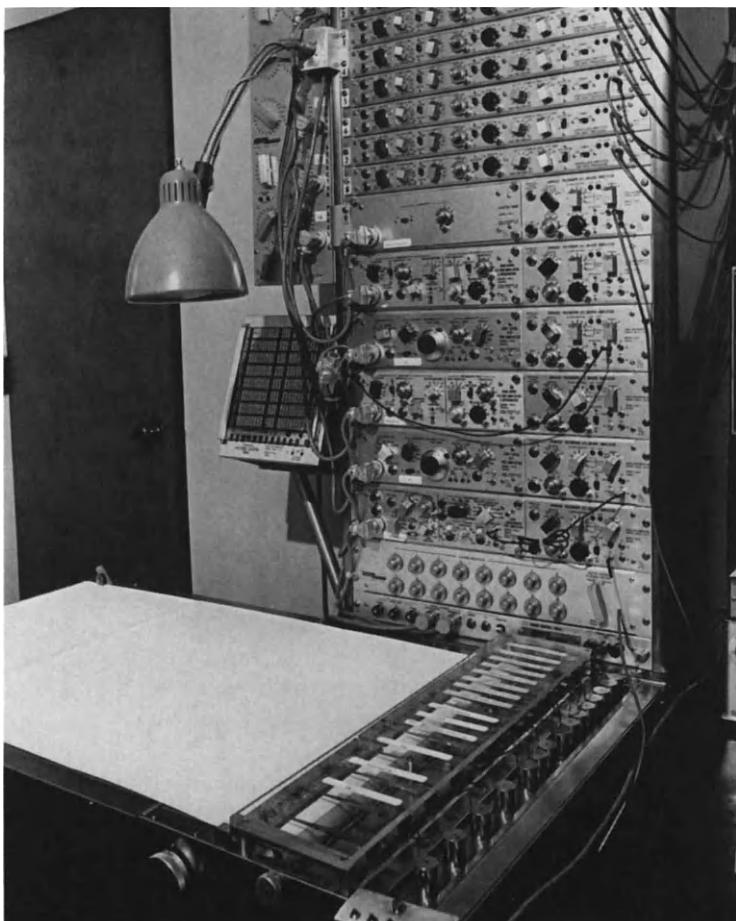


FIG. 1. Modern solid state polygraph recorders such as the Grass Model 78 shown here provide a great deal of flexibility. A maximum of 16 channels can be accommodated in the console shown. If fewer channels are used, a tape recorder or cathode ray oscilloscope can be accommodated in the rack panel console of this unit. The writer unit has excellent pen writer characteristics and up to 12 speeds ranging from 1.5 to 100 mm/min. In addition, a 23-position pushbutton electrode selector is available and is shown in the left center portion of the figure. Above this unit, a master electrode selector switch is shown which provides the investigator with a great deal of flexibility in viewing the electrical events occurring between numerous electrodes.

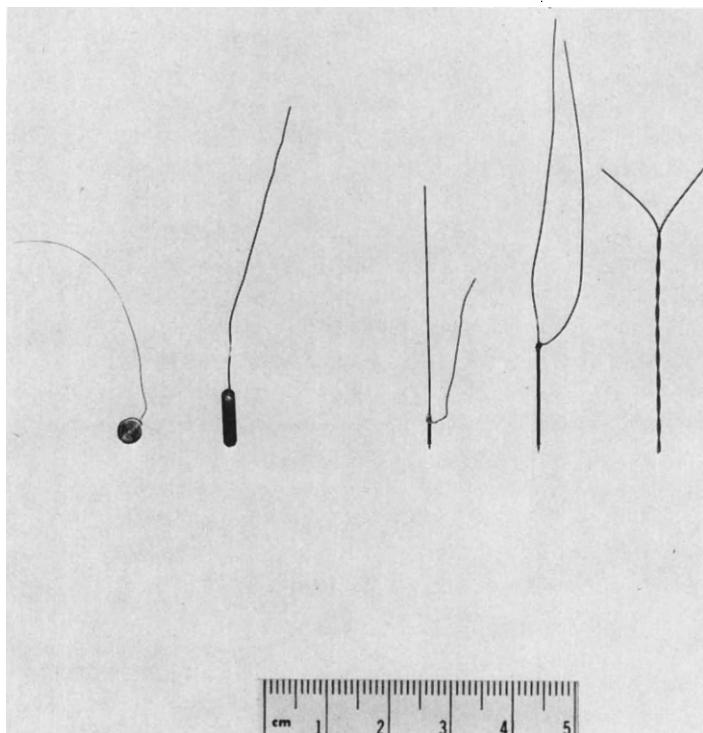


FIG. 2. Electrode types used in recording of electrographic activity to define the vigilance states. The two electrodes to the left are coiled and flat type electromyographic (EMG) recording electrodes. To the right are (left to right) short and long bipolar concentric electrodes and twisted bipolar electrode. The latter two are of sufficient length for recording deep-brain EEG activity.

between the electrodes or groups of electrodes that are connected to each of its two grids.

Brain waves are often distorted by the apparatus used to amplify and record them; thus, it is of great importance that the investigator be continuously aware of the type and extent of distortion which may occur with the particular type of apparatus used in his laboratory. Since the electrical apparatus used in electroencephalography is usually designed specifically for the purpose intended, the amount of distortion that occurs when one is recording the type of electrical activity generated by the brain is usually negligible.

Any electrical wave has certain characteristic terms in which it may be described. A brief discussion of these terms is necessary, since they enter

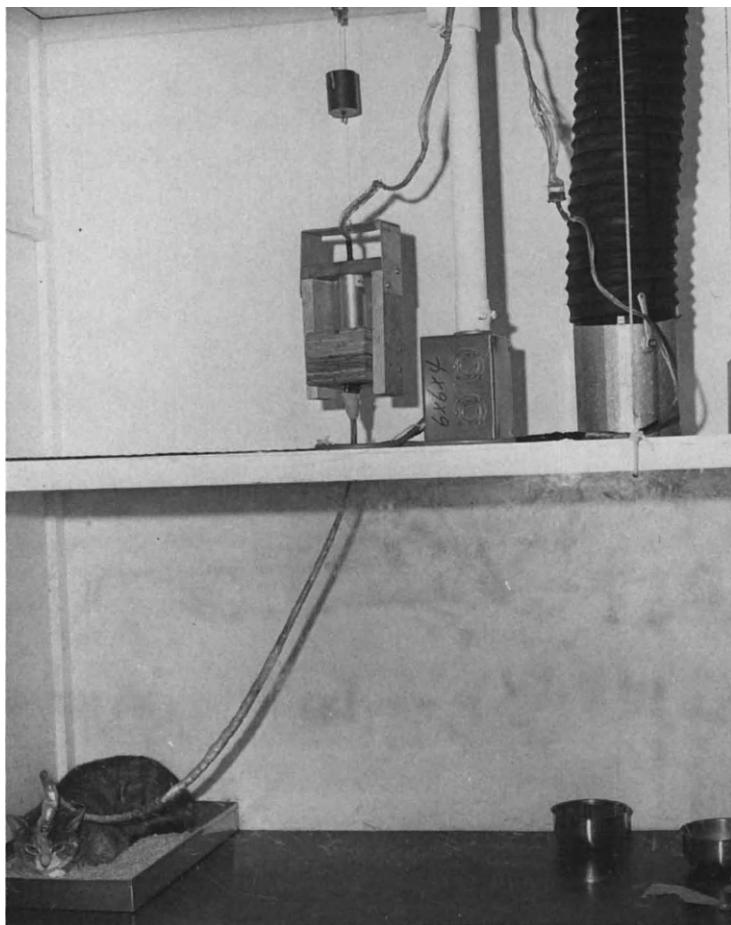


FIG. 3. Recording chamber for cat. This large living chamber is fronted with a roll-up plexiglass so that the animal's behavior can be seen at all times through a peephole in the main door to the chamber. Food and water and a litter tray are always present. The entire room is both sound insulated and electrically shielded. The slip-ring system is framed in a wooden protective device and is suspended by a delicately balanced counterweight system above the chamber. Leads from the slip ring are shown looped above the chamber and enter a pipe conduit which leaves the room above the chamber and crosses overhead to the polygraph machine outside the chamber. The cable from the slip ring to the cat enters the chamber through a hole in the top and plugs into the female socket secured to the head of the cat. With such a device the cat can freely turn about without danger of becoming coiled in the leads. The counterweight system moves with the animal to approximately full extension (as shown) when animal goes into the corners of the chamber. The black accordian duct system is a ventilation device for the chamber area. Cats may live in this type of chamber for many weeks, since it is large enough for exercise. Food and water are changed once a day, as is the litter pan.

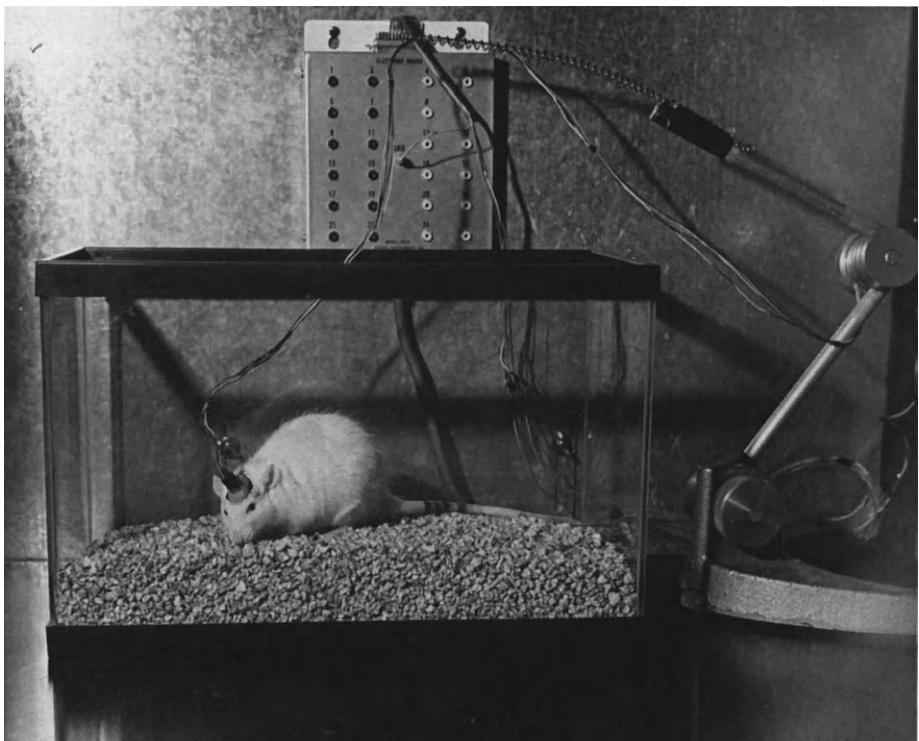


FIG. 4. One type of recording chamber used for rats. This plexiglass chamber is excellent for small animals. A recoiling boom system is devised in this case to prevent coiling of the cables when the animal turns about. This system is quite adequate for some types of vigilance-state recording in the rat.

into the consideration of scoring of sleep-waking states and in interpreting the patterns of activity written out by the polygraph machine. One of these is the *frequency*, which is the rate of repetition (per second) of the wave form in question. Another is *amplitude*, which is synonymous with voltage and, in general, is a measure of the height of the wave in question. *Wave form* refers to the shape characteristics of the wave. *Phase* refers to the relationship in time and with respect to sign (positivity or negativity) between two waves recorded in different, usually adjacent, channels.

If the relationship is such that when one of the waves is moved directly up or down it can be superimposed on the other, the two waves are said to be in phase. If, on the other hand, the waves fail to coincide with each other, then they are said to be out of phase to whatever extent they fail to overlap. This is important, since activity in several channels may need to

be simultaneously compared in gross visual scoring of the record. Superimposing of waves is made easier by the fact that EEG paper is ruled with closely spaced vertical lines.

Three main types of distortion may occur in electroencephalography and must always be under consideration by the investigator carrying out studies of the sleep-waking states: (1) frequency distortion; (2) phase distortion; and (3) amplitude distortion. In addition, there are other more complicated varieties, such as distortion due to electrical activity generated *de novo* in the cable from the subject and/or amplifier itself, all of which is termed *noise*. Apart from being aware of distortions which the apparatus may occasionally produce, the investigator must also consider possible artifacts. In reality, distortion is one form of artifact. When we speak of artifacts it is generally implied that the electrical activity is introduced into the input of a given channel of amplification but does not come from the brain itself. Such things as eye and body movement, contraction of the scalp muscles, and even the electrical activity of the heart can produce differences in potential between the electrodes and the electrode groups connected to the two grids of a channel of the polygraph.

It is important that, at the beginning of every recording session, the polygraph be calibrated. This is described fully in the manuals supplied with each polygraph machine (for example, by the Grass Instrument Company, whose polygraphs are widely used in animal research). Calibration is done by introducing an electrical signal of known voltage across the input (i.e., between grids 1 and 2) of each channel. Then, each channel is adjusted so that the deflection of its pen in response to this calibration signal is the same as that of all of the other pens. It is usual to repeat the calibration of the EEG machine, after the record has been run, in order to make sure this has not been changed.

As we will see, the vigilance states of sleep and wakefulness are characterized variously in different species by a whole series of electrographic indicators. These are well defined for each particular species and have been described in the literature for the experimental animals most commonly used, i.e., rats and cats. This characterization and standardization of electrographic indicators has reached its zenith in human encephalographic recordings, but this is a highly specialized field, well beyond the scope of this chapter. Suffice it to note that sleep is characterized in humans and animals by various sequences of events; these are becoming more and more sharply delineated as more knowledge about the physiological origins and development of the vigilance states is becoming available. It is also important to note that the transition from wakefulness to sleep varies significantly from subject to subject; though the description of the various stages of sleep applies in general, some variation from

these is considered normal. As indicated in the following discussion, preexperimental recordings (baseline records) establish a "profile" against which manipulative measures can be compared.

V. THE ELECTROENCEPHALOGRAM: SYNCHRONIZATION AND DESYNCHRONIZATION

The EEG is actually a graphic plot of voltage as a function of time, and the nature of these wave forms is influenced by a variety of physiological and pathological conditions. A basic EEG in an undisturbed animal is said to be a record of "spontaneous" brain electrical activity. Actually the term *spontaneous* is a misnomer and is merely intended to denote activity present without any deliberate attempt to stimulate the brain. Naturally, even when an investigator does not deliver specific sensory stimuli, there are many influences constantly infringing upon the brain and influencing its electrical activity.

The EEG also displays a variety of amplitude and wave durations. Although amplitude is quite variable, it is clear that the EEG voltages are in the microvolt range, usually between 5–75 μ V, in scalp or brain surface records of animals. The EEG voltages are biphasic, i.e., they have both positive and negative polarities, and EEG wave durations often range from less than 1 per second to more than 100 per second, depending upon the recording apparatus and physiological conditions. There is no conspicuous periodicity, that is, waves of a given duration do not occur with any regularity. Moreover, the record reveals much superimposition of smaller waves upon larger, usually slower ones. This superimposition is actually the result of "compounding" of different frequencies which results because numerous cortical generators are producing different potentials at the same time.

As already described, there is a large body of evidence that supports the hypothesis that the EEG is a summation of the voltages produced by the membrane potential fluctuations of individual neurons. It is clear that some speculative correlations of the time course of membrane potential changes can be given with regard to EEG waves. If many neurons are being activated by transmitters in a random time pattern, the sum of these membrane potentials would result in an EEG that has many short-duration, low-voltage waves. This pattern is referred to as *desynchronized*, and it is usually associated with alert behavior—the so-called aroused or activated pattern. Conversely, if many neurons are activated in unison, the sum of their membrane potentials would create an EEG with slow, high-voltage waves. This pattern is called *synchronized*, and it is usually associated with sedation or sleep behavior.

Many areas of the brain exhibit regional differences in membrane potential differences and in their associated EEG characteristics. For example, the EEG from the human occipital cortex often contains rhythmic activity of 8–12 waves per second and the hippocampus often exhibits activity of 4–7 per second, whereas the amygdala frequently shows discharges of 40 per second. Similar variations occur in all experimental animals so far studied. Other questions, beyond the scope of this chapter, involve the ways in which various EEG generators are coupled with each other and how they mutually interact. The EEG represents a summed composite of all the excitatory postsynaptic potentials and inhibitory postsynaptic potentials within the pickup range of the electrodes but does not directly reveal the extent of the active inhibition (inhibitory postsynaptic potentials). For example, a desynchronized EEG could result simply from excitatory postsynaptic potentials or from decreased inhibitory postsynaptic potential activity (disinhibition), or both. A synchronized EEG obviously would indicate a decrease in excitatory postsynaptic potential activity and probably would suggest an increase in inhibitory postsynaptic potential activity (active inhibition), which might serve to pace certain circuits synchronously.

These types of considerations are important in assessing sleep-waking activity mechanistically or physiologically but do not necessarily enter into "matching" EEG waves with a given behavioral arousal state in the continuum from full or aroused waking to the deepest phases of sleep. Since it is preferable not to deal with purely "correlative" data, however, it is essential in describing the induction of sleep that the underlying neuronal processes, biochemical substrates, and anatomical bases of the sleep-waking states be presented at least in outline form.

VI. INDUCTION OF SLEEP

A. *Electrical Stimulation of the Brain*

Some of the earliest descriptions of induction of sleep and sleeplike states date from the classical work of Hess (1954), who electrically mapped large sectors of the brain, especially the diencephalon, and invoked "adynamias" and other behaviors resembling sleep states. Since this work, done before the elucidation of the different states and phases of sleep, was not accompanied by polygraphic recordings, little can be said about the specific vigilance states that were affected by these procedures. The lesion work of Nauta (1946) had identified so-called sleep and waking centers in the hypothalamus along the trajectory of the medial forebrain bundle in the preoptic–basal forebrain areas and posterolateral hypo-

thalamus, respectively; with the development of electrical stimulation and, later on, chemical stimulation of the brain, it was thus natural that these manipulations would center on the hypothalamus and its rostral projections into the basal forebrain zones.

In the early experiments dealing with electrical stimulation of the brain in relation to states of sleep and wakefulness or states of "consciousness," cortical synchronization was of special interest because of its similarity to the spontaneously occurring cortical spindle bursts associated with sleep and other states of central nervous system suppression. One of the most important of these studies was carried out by Sterman and Clemente (1962a), who stimulated subcortical areas bilaterally and recorded the EEG activity from several cortical areas in immobilized adult cats. An immediate and sustained cortical synchronization was evoked upon stimulation of the preoptic–basal forebrain zone just rostral to the optic chiasm; this zone was termed the "basal forebrain synchronizing area." The ventral zone of the diagonal band of Broca was found to be the most effective site from which this cortical synchronization was induced. A second focus inducing cortical synchronization was found in the medial and basal amygdaloid nuclear areas.

These studies indicated that complex interactions were occurring between the reticular formation and the basal forebrain synchronizing area. It is important in these types of studies to emphasize the relevance of stimulation parameters as well as the time required for sleep induction. Thus, from this zone stimulation parameters of 1–3 V, 0.5 to 0.75 msec duration were effective in the wide frequency range of 5 to 250 cycles/sec. Latencies to sleep varied from 5 sec to as long as 3 min (average of 30 sec) and were clearly related to the stimulation, i.e., were stimulus bound. It is well to emphasize here that in studies of this type several factors such as time of day, time of feeding, light cycle, and number of previous stimulations must always be considered as affecting the capacity of brain stimulation to induce sleep. Generally, the first stimulations are the most effective, while in subsequent stimulations various "resistances" to sleep induction often appear to develop. It is also essential to note that the synchronizing response may be most easily achieved when the cortex exhibits an "alerted" or "activated" pattern. Hence, the prevailing vigilance state of the animal should always be carefully defined at the time of initiation of the stimulation.

Sterman and Clemente (1962b) extended these studies to nonimmobilized cat preparations in order to determine the behavioral response to stimulation in the preoptic–basal forebrain area. Bilateral stimulation of this area in the behaving cat resulted in both the behavioral and EEG manifestations of sleep in every animal tested. The transition of alert

waking behavior to sleep occurred rapidly, usually in less than 1 min, and took place either during one prolonged stimulation period or during several short periods of stimulation. Both low- and high-frequency stimulation (5–250 cycles/sec) were effective in producing the postural adjustments, EEG patterns, and response reversibility observed in the onset of natural sleep. Bilateral stimulation was just as effective in producing full-fledged behavioral sleep from this zone as it had been in producing cortical synchronization in immobilized animals. What is interesting in these findings is the induction of sleep by stimulation of an area long thought to have a suppressor influence upon several important systems, i.e., somatic, autonomic, and endocrine, all of which are suppressed naturally in physiological sleep. Of interest in the discussion to follow is that Hernández-Péón *et al.* (1962, 1963) induced sleep in this same basal-preoptic zone by stimulating it chemically with cholinergic compounds.

The relationship between the basal forebrain synchronizing zone and the other brain system most often related to cortical synchronization, the midline thalamic system, requires a great deal of further analysis as well as study of other forebrain areas, especially the caudate system. Hernández-Péón (1962) confirmed the behavioral induction of drowsiness and sleep with electrical stimulation of the lateral preoptic area and this "mapping" led to the beginnings of "chemical" tracing of a comprehensive "sleep pathway" consisting of ascending and descending components which appeared to converge upon lower brainstem centers concerned with sleep. The electrocortical synchronizing influence of the anterolateral hypothalamus in unanesthetized, immobilized cats has been confirmed in many recent studies.

It appears obvious that the neural substates of sleep must accomplish a broad, integrative process. It is difficult to believe that all aspects of this complex global process can be fully comprehended in terms of release of a few neurohumoral secretions, as might be presumed from recent biochemical approaches to studies of the sleep states which have concentrated almost exclusively on lower brainstem aminergic systems such as the raphe complex and locus coeruleus. There is strong evidence that the basal forebrain structures play an essential role in this integration, since activation of this system directly induces sleep. As is known from the classic study of Nauta (1946), lesions placed in the basal forebrain region produce a profound and often lethal suppression of sleep. Furthermore, neuroanatomical and electrophysiological studies have established the convergence of neural pathways from the frontal and limbic cortex, thalamus, hypothalamus, and brainstem tegmentum upon this basal forebrain region. The basal forebrain region, in turn, sends recip-

roating fibers to many of these same structures and also gives rise to prominent fiber systems projecting into the medial thalamus and brainstem tegmentum.

The level of cortical activation (desynchronization) or of cortical synchronization may be largely regulated by dually active systems: the reticular activating system, on the one hand, and the basal forebrain-cortical synchronizing system, on the other. Possibly the two systems act reciprocally, perhaps through relays in limbic circuits and in the diffusely projecting thalamo-cortical system, but further electrophysiological and morphological studies will be needed to unravel these complex relationships.

In addition to the very effective forebrain synchronizing and sleep-inducing mechanism, lower regions of the brainstem also contain mechanisms which are capable of exerting a generalized EEG synchronizing effect and of inhibiting reflexes in the brainstem. For example, Bonvallet *et al.* (1954) and Magnus *et al.* (1961) produced cortical synchronization by either increasing visceral afferent discharge through carotid sinus distention or by low-frequency stimulation of the tractus solitarius and adjacent reticular structures of the lower brainstem. Thus, there is considerable evidence suggesting that EEG synchronization, behavioral suppression, and sleep are each capable of being initiated by stimuli from the extreme ends of the nervous system, forebrain to hindbrain.

With respect to the mechanisms operating in the onset of normal, physiological sleep, considerable evidence indicates that "passive reticular deactivation," normally brought about by the reduction of sensory stimuli in the activating system through sleep-preparatory behavior, sets the stage for the overt expression of a potently active forebrain (and probably lower brainstem) inhibition which leads directly to the initial stages of sleep. These observations support the interpretation that the processes of suppression and behavioral inhibition, just like excitation, are active basic functions of the central nervous system.

In later studies Alnaes *et al.* (1973) produced electrocortical synchronization, associated behaviorally with light sleep, by low-frequency (6 cycles/sec) stimulation of the posterior part of the subcallosal region and the orbital gyrus of the frontal lobe in unanesthetized, but relaxed, cats. This effect was, however, weaker (higher stimulation thresholds) than that obtained with basal forebrain stimulation. High-frequency stimulation (30–100 cycles/sec) of the same areas produced electrocortical desynchronization and behavioral arousal. These data are compatible with the view that the subcallosal and orbital portions of the frontal cortex give rise to descending systems capable of modulating the excitatory state of sleep-producing mechanisms in the diencephalon or lower brainstem.

Thus, electrocortical desynchronization and "light sleep" may be produced not only by subcortical stimulation but also directly from the cerebral cortex. The latter effect is most likely mediated through the sleep-producing mechanisms of the lower brainstem and diencephalon by way of fiber connections known to exist between the subcallosal and orbital cortex and the subcortical areas. Thus, in terms of organization of sleep systems, the subcortical areas inducing synchronization and behavioral sleep appear to be influenced not only by ascending impulses but also by descending impulses from the cortical level.

It is also a well-established fact that reticulo-thalamo-cortical systems play significant roles in arousal, alerting, and attention. High-frequency stimulation of the mesencephalic reticular formation, or of the midline, so-called nonspecific, thalamic nuclei, will arouse a sleeping animal or alert a waking animal and cause desynchronization or activation of ongoing electrocortical activity (Moruzzi and Magoun, 1949; Monnier *et al.*, 1960). On the other hand, low-frequency stimulation of the midline thalamic nuclei produces inattention, drowsiness, and sleep, and is associated with slow waves and spindle bursts in the EEG (Akert *et al.*, 1952; Monnier *et al.*, 1960). With respect to pathways, Skinner and Lindsley (1967) studied the effects of interrupting or blocking the nonspecific thalamo-cortical system, by lesions or cryogenic means, in an effort to further delimit it anatomically and functionally. Specifically, this system was shown to involve the rostral thalamus, the anterior thalamic radiations (inferior thalamic peduncle), and the orbital cortex. Thus, the nonspecific thalamo-cortical system encompasses the dorsal medial nucleus of the thalamus and other portions of the midline nuclear group which project, via the inferior thalamic peduncle, to the lateroventral and orbital surface of the frontal lobe. The nonspecific thalamo-cortical system, although influenced by projections from the ascending reticular formation, appears, therefore, to be mainly anchored in the nonspecific nuclei of the thalamic midline nuclear group. Projections of this system converge and become somewhat spatially restricted in the forebrain, since lesions or reversible blocking by cryogenic means show that a very sharply delimited focus is found in the region of the inferior thalamic peduncle. Thus, several forms of cortical synchronization can be abolished by blockade of the inferior thalamic peduncle (Skinner, 1971). This thalamo-cortical system has been shown to project to the orbitofrontal region, which, in the cat, is mainly granular cortex.

One functional role of the midline nuclear group of the thalamus, mediated by the projections of the nonspecific thalamo-cortical system, is that of synchronization of electrocortical activity. The intactness of this system appears to be essential for the continuance of spindle bursts,

which, along with slow waves, are characteristic of slow-wave sleep. It is of interest that the orbito-frontal cortex, which receives these projections, is the principal link in this system for synchronizing and regulating electrocortical activity.

B. Chemical Stimulation of the Brain

In the past 20 years there have been new developments in methods of studying the brain that have involved direct microchemical stimulation of particular nuclei and nerve tracts, direct injection of chemicals into the ventricular system, and push-pull perfusion techniques. These types of studies have been widely used to analyze the "chemistry of behavior" (see Myers, 1974), and we will briefly review various ways in which these techniques have been used to provide data relating to the induction of sleep and elucidating the biochemical bases of the states of vigilance.

Some of the earlier studies in this regard were carried out by Hernández-Péón *et al.* (1962) and, later, by Cordeau *et al.* (1963). In the study of Hernández-Péón and associates, devices termed exploring "chemitrodes" (Fig. 5) were implanted along the pathway of the limbic-midbrain circuits in cats. Microamounts, usually 1–5 µg of a crystalline chemical agent (acetylcholine, carbachol, norepinephrine, γ-aminobutyric acid (GABA), or another putative neurotransmitter) were tamped into the chemitrode while the animal was under behavioral observation and was hooked up for polygraphic recording (Fig. 3). The details of tamping these agents with the chemitrodes (and of the use and construction of chemitrodes) are given in Hernández-Péón *et al.* (1963), Bronzino *et al.* (1972c), and Morgane *et al.* (1972). In the studies of Hernández-Péón *et al.* (1962, 1963) several hundred brain loci were "mapped" and the sleep sites were related to a so-called cholinergic pathway in the medial forebrain bundle and other well-defined neuronal systems in the brain.

An extensive limbic cholinergic pathway involved in sleep was charted in detail (Fig. 6) and was found to parallel closely the trajectory of the limbic-midbrain circuit of Nauta (1958). Cholinergic stimulation in an area extending from the limbic-midbrain area all the way forward to the limbic-forebrain zones, following the medial forebrain bundle, were areas where cholinergic stimulation induced behavioral and electrographic signs of sleep. These effects were shown to be clearly demarcated in a highly defined zone, since the Hernández-Péón group used exploring cannula devices that were capable of moving up or down in the brain in 1-mm stages (Fig. 5) for stimulation at multiple points.

In many instances chemical stimulation just a millimeter above a

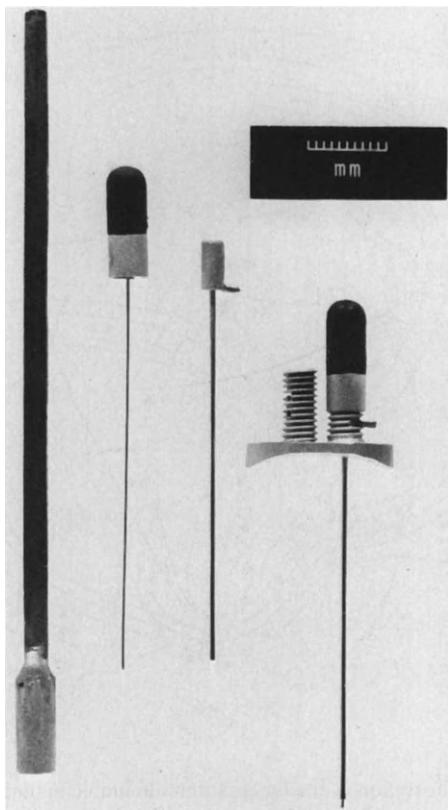


FIG. 5. Exploring chemitrode system for stimulating several brain areas 1 mm apart. The components are (left to right): the stereotaxic driving device, tamping stylet, exploring guide with locking notch and skull plate showing one threaded barrel with two notches (left) and a second barrel onto which the screw cap with tamping stylet has been placed. Detailed construction and use of this device is given in Morgane *et al.* (1972).

“sleep” zone induced arousal, rage, or other behavior, as did placements below the sleep zone. Hence, the effects were clearly specific and localized to sharply defined zones in the trajectory of these nerve pathways. It appeared that this neuronal system was essentially an inhibitory descending hypnogenic circuit, since atropine blockade in a cannula posterior to a cholinergic stimulation point inhibited the sleep-inducing effects of acetylcholine or carbachol, whereas cholinergic stimulation through a similar cannula placed anterior to a cholinergic “sleep site” had no such effect. In most experiments it was found that within 3 min following deposition of cholinergic crystals through the chemitrode, cats began to show behavioral and EEG evidence of drowsiness which pro-

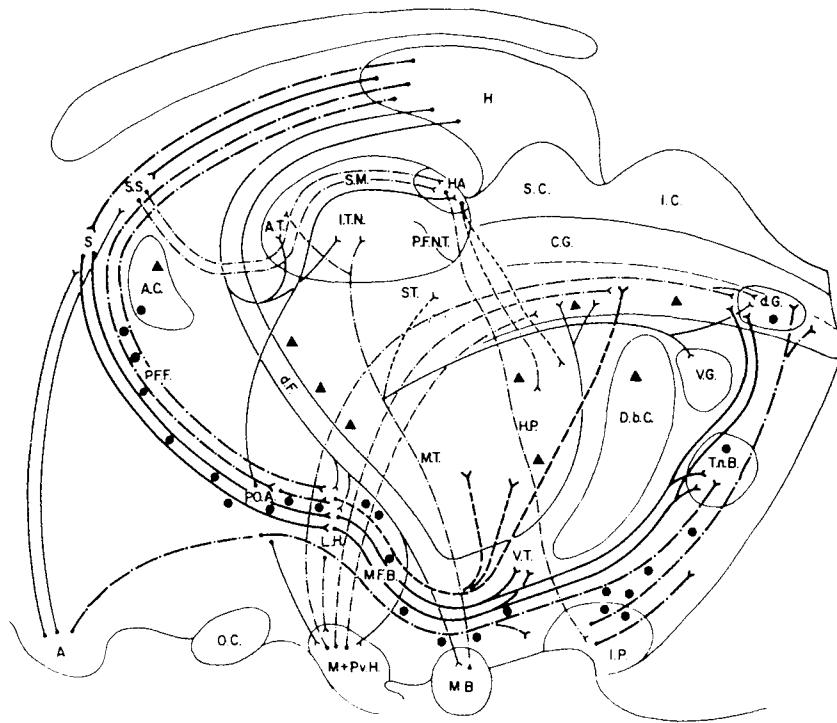


FIG. 6. Schematic illustration of the limbic forebrain-limbic midbrain circuitry designating the loci from which sleep (●) and arousal (▲) were elicited by cholinergic stimulation. Abbreviations: H, hippocampus; HA, habenular nucleus; S.C., superior colliculus; I.C., inferior colliculus; C.G., central gray; d.G., dorsal tegmental nucleus of Gudden; S.T., subthalamic region; V.G., deep tegmental nucleus of Gudden; T.n.B., central tegmental nucleus of Bechterew; D.b.C., decussation of brachium conjunctivum; I.P., interpeduncular nucleus; V.T., ventral area of Tsai; H.P., habenulointerpeduncular tract; M.B., mamillary body; M + Pv.H., medial and periventricular hypothalamic regions; O.C., optic chiasm; A, amygdaloid nucleus; M.F.B., medial forebrain bundle; M.T., mamillothalamic tract; d.F., descending fornix column; L.H., lateral hypothalamus; P.O.A., preoptic area; P.F.F., precommissural fornix fibers; A.C., anterior commissure; S, septum; S.S., supracommissural septum; A.T., anterior thalamic nucleus; I.T.N., intralaminar thalamic nuclear areas; P.F.N.T., parafascicular nucleus of thalamus; S.M., stria medullaris. From Hernández-Péon *et al.* (1963).

gressed to full-fledged slow-wave sleep alternating with periods of REM (rapid eye movement) sleep. Other agents, including norepinephrine or GABA, had no such sleep-inducing effects. Most of this work, therefore, indicated a predominately cholinergic organization of these components of central hypnogenic mechanisms.

In the study of Cordeau *et al.* (1963) acetylcholine and epinephrine (adrenaline) were injected into three main levels of the brainstem, i.e., the rostral pontine and caudal mesencephalic tegmentum, the caudal pontine reticular formation, and the bulbar reticular formation. Liquid injections were carried out in the amount of 20 μ l over a period of 100 sec. A total of 20 μ g of acetylcholine was injected in both sleeping and waking animals. Epinephrine injections in the mesencephalic and rostral pontine reticular formation were invariably followed by behavioral arousal and electrocortical desynchronization. Injections of 20 μ g of acetylcholine in the bulbar reticular formation of awake but quietly resting cats were usually followed by behavioral sleep and electrocortical synchronization.

In these studies the slowing of the EEG trace began some 60–120 sec following the end of the injection. During the synchronization of sleep following acetylcholine injections, the cats were aroused easily by knocking on their cages, but such induced arousal was usually short lasting, and the animals returned to sleep immediately upon cessation of the stimulus. Qualitatively similar results were obtained when acetylcholine was injected in the caudal pontine reticular formation and in the rostral pontine or caudal mesencephalic tegmentum. When acetylcholine injections were made in already sleeping cats, no changes were observed other than a further slowing of electrocortical activity. In some animals the EEG changes obtained from bulbar and mesencephalic loci following the injection of acetylcholine proceeded to the so-called REM or "activated-sleep" patterns. Cordeau *et al.* defined their most appropriate stimulation sites for induction of sleep with acetylcholine as being those more caudal and close to the midline, whereas the effects of epinephrine were more pronounced in rostral brain areas.

Although direct chemical stimulation of the brain has come under some criticism because of the use of high concentrations of stimulating agents and the widespread distribution of active sites in the brain, low concentrations of cholinergic agents have also been found to be effective in inducing sleep. Although some spread cannot be directly controlled, there is clearly a high degree of specificity in that the use of the exploring cannula technique has shown that points as little as a millimeter dorsal, ventral, lateral, or medial to a critical zone are not sleep sites. In later studies, after the more precise definitions of the two states of sleep were established, Hernández-Péón *et al.* (1967) emphasized that acetylcholine injected in various brain sites elicits all the behavioral and electrographic manifestations of both patterns of sleep (slow-wave sleep and REM sleep) from well-defined cortical areas, as well as from the anterior limbic zone, among others. Electrolytic lesions destroying the medial forebrain bundle at the preoptic region prevented sleep previously elicited by acetylcholine

stimulation of the same frontal and anterior limbic cortical areas. These studies lend support to the view that the hypnogenic fronto-limbic cortical areas have functional connections with the limbic-midbrain hypnogenic circuit forming part of a corticofugal descending limb of a "cholinergic" sleep system.

Yamaguchi *et al.* (1964) tamped crystalline acetylcholine into areas of the reticular formation of cats in more rostral zones than those investigated by Cordeau and also produced synchronized cortical and subcortical activity 5–15 min after application. Behavioral sleep and its EEG correlates were induced by cholinergic stimulation (approximately 30 µg) of the nucleus centralis medialis, preoptic region, and brainstem reticular formation, whereas norepinephrine elicited arousal and EEG activation. These findings provide additional support for the existence of some type of "balance" between adrenergic arousing and cholinergic sleep-inducing elements in various subcortical areas of the brain.

George *et al.* (1964) microinjected carbachol in total volumes of 1–2 µl into the ponto-mesencephalic reticular formation in conscious cats and induced atonia, cortical desynchronization, and synchronization of hippocampal activity, i.e., a full-fledged behavioral and electrographic REM sleep state. These effects were found to be antagonized by atropine injected either focally into the brain or given systemically. These findings also provide further evidence for the existence of a cholinergic sleep mechanism in this area of the reticular formation. In contrast to the findings of Cordeau *et al.* (1963) that light sleep (spindling) was never induced by such stimulation, it might be remembered that the differences could be correlated with the small volumes and quantities of drugs used by George *et al.* Hence, the volume factor is always an important methodological consideration to keep in mind in carrying out studies of this type.

More recently, McKenna *et al.* (1974) and Amatruda *et al.* (1974) have chemically stimulated the gigantocellular tegmental fields of the reticular formation (their electrophysiological studies had already indicated that these cells are active in REM sleep, possibly interacting with the locus coeruleus neurons in the generation of the REM state). These authors found that 3–9 µg of carbachol injected via a cannula system in a 3 µl volume induced REM sleep only from the specific area of the gigantocellular tegmental field 1–2 mm lateral to the midline. The enhancement of REM sleep in long-term polygraphic recording was found to be 3.5 times baseline levels: REM sleep, defined by EMG suppression and EEG desynchronization, lasted an average of 75 min [the REM periods induced by George *et al.* (1964) lasted only 45–60 min], but often, with larger doses of 9 µg, REM sleep would extend into the third or fourth hour. Interest-

ingly, in these experiments the first injection into the REM enhancement area of the gigantocellular tegmental fields produced less impressive REM enhancement than subsequent injections into the same fields. In these types of experiments, concerned with inducing sleep, it is important to reemphasize the importance of considering the state of arousal of the animal at the time of injection, the light cycle, relation to feeding schedules, hydrodynamics of the delivery system, and exact histological controls so that "sleep sites" are clearly related to the cellular and fiber architecture of the region under study.

The aminergic hypothesis of sleep has received the most widespread attention in recent years (see Cordeau, 1970; Jouvet, 1972; Morgane and Stern, 1974). Thus, a large number of studies indicate that serotonin may play a significant role in the generation of slow-wave sleep whereas norepinephrine appears to be involved in the generation of REM sleep. These hypotheses have been supported by a wide variety of lesioning and pharmacological studies. However, placing biogenic amines directly into many areas of the brain has not given clear-cut results, and these aminergic hypotheses have not been integrated with the cholinergic findings just described. Also, a variety of theories involving the possible role of precursors or metabolic products of the biogenic amines as being "sleep factors" have arisen. For example, some earlier work had tended to indicate involvement of the tryptophols in sleep, but Morgane and Stern (1973) showed that these agents, when injected intraventricularly in cats, produced behavioral and electrographic signs of arousal. Several other studies have indicated the involvement of tryptophan, the amino acid precursor of serotonin, in the induction of sleep—but, again, the results are still controversial [see the review by Jouvet (1972)].

Cross-perfusion experiments (Myers, 1967) also represent a type of chemical stimulation of the brain in the sense that agents presumably produced by a given physiological-behavioral state are shunted back to another animal's brain (ventricles, specific nuclei or tracts), the assumption being that they are specific behavior-inducing substances (sleep factor, feeding factor, satiety factor, etc.). Chemical stimulation of the brain, by perfusing materials that came from the brain of an animal in a given behavioral state, is fraught with difficulties and subject to many errors of interpretation. Most of these have been reviewed by Myers (1974) in the *Handbook of Drug and Chemical Stimulation of the Brain* and in Volume 2 of this series, Chapter 7.

Essentially, as with direct chemical stimulation of the brain with biogenic amines or other substances occurring naturally in the brain, these substances may be inducing pharmacological effects rather than physiological ones. Diffusion is impossible to control, and the brain

processes influenced to "induce" behavior are difficult to demonstrate. No matter how precise these stimulations are in terms of locus, volume, or microamount, it should be emphasized that, just because a chemical stimulation or cross-perfused agent is "associated" with the development of a behavioral or electrographic state, that agent may not be *the* normal physiological agent that induces that state. Hence, cholinergic induction of sleep in a given system does not mean that the normal transmitter for sleep is acetylcholine, even if other known neurotransmitters do not induce sleep in that particular system. It points up the fact that iontophoretic, pharmacological, and histochemical studies should then be carried out in such a system to attempt to define the normal transmitters. As is well known, this is almost impossible in the central nervous system considering the present state of technology.

Given the foregoing considerations, the Myers' cross-perfusion type of experiment has also been used to study sleep. Drucker-Colin *et al.* (1970) carried out a series of studies using push-pull cannula techniques to identify the so-called hypnogenic inhibitory transmitter by perfusing the midbrain reticular formation during sleep and waking in cats. Cats were sleep-deprived by the island technique (see Section VII,B) in order to insure maximal sleep development. Then brain perfusate from a sleeping donor cat was perfused into the midbrain reticular formation of an awake recipient cat, after which the latter began to show the typical behavior preparatory attitude of sleep. It became quiet, lay down, closed its eyes, showed high-voltage spindle bursts in the frontal cortex, and later curled up with the head down. Gradually the spindles appeared more frequently, and within 3 to 17 min after injection of the perfusate the recipient cats showed a continuous high-voltage slow-wave EEG and were behaviorally asleep. The first periods of fast-wave sleep, with a desynchronized EEG, flat EMG, high-voltage rhythmic theta activity in the entorhinal cortex, and bursts of eye movement, had a latency of appearance which varied from 10 to 30 min. The injection of the "waking perfusate" into the midbrain reticular formation of a recipient cat never induced sleep.

The results obtained with the cross-perfusion method suggest that it is possible to extract from the midbrain reticular formation of a sleeping cat a perfusate which, when injected into the same area of an awake recipient animal, leads to all the behavioral and electrophysiological manifestations of sleep. In later studies Drucker-Colin (1973) showed that perfusate extracted from the mesencephalic reticular formation of a sleeping donor cat increased the duration and decreased the latencies of slow-wave sleep in recipient cats whereas "awake" perfusate completely inhibited the appearance of REM sleep. The entire concept of a possible sleep transmitter substance has recently been reviewed by Drucker-Colin (1976).

Attempts to chemically identify a so-called sleep substance have been

carried on largely by Pappenheimer and his group, and by Monnier. In Pappenheimer's latest reviews (Pappenheimer *et al.*, 1974, 1975, 1976) he indicates that progress has been made toward isolating, concentrating, and identifying these natural "sleep-inducing" factors. He notes that the factor he has obtained from the cerebrospinal fluid of sleep-deprived goats (which, when injected into rats, is followed by "sleep") appears to have the properties of a small peptide in a molecular weight range of 350 to 700, although its amino acid composition has not been established. If the chemical structure of "sleep factor" obtained from cerebrospinal fluid of sleep-deprived goats turns out to be identical with that obtained from dialyzates of cerebral venous blood of rabbits during stimulation of thalamic "sleep centers" (Monnier and Schoenenberger, 1974), this will greatly add generality and physiological significance to a phenomenon which has already been shown not to be species specific. The gradual increase of "sleep factor" concentration in cerebrospinal fluid during the course of sleep deprivation (Fencl *et al.*, 1971) does suggest that the substance plays some role in the induction of normal sleep, but this is an area still fraught with many unresolved difficulties.

Monnier and his group have been carrying out similar studies by stimulating a sleep-inducing region of the thalamus of rabbits and withdrawing venous blood from the brain of the sleeping animals. The blood from the donor animals was dialyzed to remove large molecules, and the purified fraction of the resulting "sleep dialyzate" was injected into the brain of recipient rabbits. Within 5–10 min of the injection, the EEG showed an approximate doubling of delta-wave activity (slow waves, less than 5 per second) in rabbits given the active substance, whereas there was no appreciable change in the delta activity in rabbits given dialysate from the control donors. These results indicate that the so-called sleep factor is a peptide containing seven or more amino acids, with a molecular weight between 355 and 1500 (most likely, about 700), and this factor may also be involved in normal humoral regulation of sleep. Further characterization of this factor has recently been carried out (Monnier *et al.*, 1975). It remains to be seen whether the sleep factors of Pappenheimer and of Monnier are the same or different substances. At present it appears that they are two separate agents, which indicates that there may be multiple sleep factors produced in the brain.

C. Light Cycle Effects

It is important to point out that sleep states can be manipulated by altering the light-dark cycles. Hence, control of this most important variable is essential in all sleep studies. The light cycle should always be reported in detail in relation to the sleep cycle, and the sleep profiles

should be developed so that each state is shown in both the light and dark phases of the cycle. Sleep profile values should thus be reported separately for the lights-on and lights-off part of the day, or else lights-on/lights-off ratios should be given.

This brings up another important method of inducing sleep states by manipulation of the light-dark cycle, namely, lights-off induction of REM sleep. Although the rat is primarily a nocturnally active species, it has been found that, by repetitively alternating short periods (less than 30 min) of light and dark, rats will redistribute most of their REM sleep periods into the dark portion of the cycles (Lisk and Sawyer, 1966; Rechtschaffen *et al.*, 1969). This phenomenon has also been seen in the cat (Chamblin and Drew, 1971). Thus, if a study requires repetitive observation of REM sleep episodes, one way to maximize their occurrence in a predictable fashion is to place the subjects in a 10 min light-10 min dark cycle for several hours. Other variations in the light-dark schedules shown to be effective are 5 min light-5 min dark or 25 min light-5 min dark.

VII. SLEEP DEPRIVATION EXPERIMENTS

Some remarks on REM deprivation are in order here, since this is one major method used to study the functional properties of REM sleep. Naturally, REM deprivation produces the well-known rebound of REM, so that if one is interested in studying REM from any standpoint, it is easiest to do this when REM is most intense and is prolonged by previous deprivation. Also, since we are discussing chemical induction of sleep, including REM sleep, one way to build up the REM potential, or REM-inducing factor, would be to deprive an animal of REM and cross-perfuse fluid from key areas of its brain into the brain of another animal. In chemical studies one might, during the REM-rebound period, withdraw and measure chemical agents, such as biogenic amines, or their metabolites, or other putative neurotransmitters in order to see if they are regionally altered in the brain during the full-blown REM state as opposed to slow-wave sleep or waking.

Methods for producing sleep deprivation in animals vary with the species, intended duration of the deprivation, and the type of deprivation desired, i.e., total sleep deprivation or only REM sleep deprivation. Procedures are currently available for deprivation of total sleep, slow-wave and REM sleep, or for REM sleep selectively. There is no method for producing selective deprivation of slow-wave sleep. This is due to the fact that REM sleep is always preceded by a period of slow-wave sleep and any deprivation of slow-wave sleep would result in substantial reductions in REM sleep as well. Attempts have been made to circumvent this

problem by comparing the effects of periods of sleep characterized by high amounts of slow-wave sleep and little REM sleep to periods consisting mainly of REM sleep (Fowler *et al.*, 1973). For example, in man the first few hours of a night's sleep consist mainly of slow-wave sleep, whereas the last few hours have much less slow-wave sleep and a large amount of REM sleep. However, this first half versus the last half of the night design confounds the effects of slow-wave sleep versus the REM sleep with time of day, temperature cycles, and hormone secretion patterns (Weitzman *et al.*, 1975). This method of selecting periods of high slow-wave sleep or REM sleep is not readily done in animals because their sleep cycles are not patterned in the same way as those of man.

The more commonly employed methods for producing deprivation of total sleep time or of just REM sleep in the rat and cat will be described next.

A. Total Sleep Deprivation

Comparatively few studies of total sleep deprivation exist in animals, and those which have been conducted generally failed to measure polygraphically the amount of sleep occurring during the deprivation procedure. It is thus difficult to quantify the effectiveness of previously employed sleep deprivation procedures. Basically, three approaches have been attempted:

1. *Wakefulness produced by enforced exercise.* Continuous walking can be produced by placing the subject, either rodent or cat, on a motor-driven treadmill or activity wheel. Bast and Loevenhart (1927) observed that rabbits placed on a motor-driven wheel were sleep deprived for only the first several days and then appeared to sleep while actually tumbling in the apparatus. In order to overcome these difficulties procedures were modified so that failure to walk on the treadmill or activity cage resulted in being immersed in water. For example, Licklider and Bunch (1946) employed a motor-driven cylinder (4 in. diameter, 3½ in. wide, rotating at 7½ in. per hour) which was half immersed in water. Failure to maintain walking would result in the animal being driven into the water. Other activity wheel dimensions and rotation rates for rats and/or mice have been described by Rust (1962), Webb (1962), and Kavanau (1962). The procedures employed by the latter investigator attempted to minimize the physical effort expended by the subjects in the sleep deprivation chamber.

2. *Repeated administration of stimulant drugs.* In rats, an injection of 10 mg/kg of *d*-amphetamine sulfate every 4–6 hours will prevent most sleep from occurring for up to 5 days (Levitt, 1966). Obviously, interpretation of results of experiments conducted on such sleep-deprived rats must

attempt to identify possible pharmacological changes due to chronic amphetamine administration (e.g., changes in cardiovascular status or in central nervous system catecholamine chemistry), apart from the sleep loss experienced by the subjects. Because of the high degree of potential confounding of results due to post-amphetamine drug-withdrawal effects with those of sleep-deprivation effects, the use of chronic amphetamine injections for inducing sleep loss should be pursued with great caution.

3. *Systematic administration of other drugs to reduce sleep time.* The effect of a wide variety of systematically administered pharmacological agents on sleep patterns in animals has been extensively reviewed by Jouvet (1972), King (1971), and Morgane and Stern (1974). As summarized in these reviews, certain classes of psychoactive drug tend to decrease total sleep time, e.g., 200–400 mg/kg *para*-chlorophenylalanine (pCPA), while others have a more selective suppressing effect on REM sleep, e.g., 1–10 mg/kg of imipramine. The interested reader should consult these reviews for a summary of drug-induced changes in sleep.

B. REM Sleep Deprivation

Two methods are commonly employed: arousals produced by the experimenter, and the so-called flowerpot or island techniques.

1. Arousal

In the experimenter arousal method, a cat or rat bearing chronically implanted electrodes for sleep-waking recording [i.e., EEG, EMG, and EOG (electrooculogram), as previously described] is placed in a recording chamber and connected to the polygraph. After obtaining baseline sleep-waking data the experimenter begins continuous monitoring of the polygraph output for evidence of the onset of a REM episode. As soon as a REM period is identified, usually in 5–10 sec after REM sleep onset, the subject is awakened by an arousing stimulus, such as tapping on the recording chamber, shaking the subject, presentation of a loud noise, or an air blast.

When conducted for more than a few hours, this procedure is laborious and requires significant manpower, especially for around-the-clock monitoring of the polygraphs. More importantly, the REM deprived subjects tend markedly to increase the frequency of occurrence of REM episodes after only 4–8 hr of deprivation. This results in the accumulation of hundreds of 5–10 sec REM epochs, which, when totalled together, represent a considerable fraction of the normal REM time.

One way to reduce this effect and lower the amount of time spent monitoring the EEG is to place the subject on a treadmill moving slowly at a speed of 2 m/min (for cats) with a water-filled container at the end to

enforce walking (Dement *et al.*, 1969). A speed of approximately 13 cm/min for rats for 16 hr/day has been used by Licklider and Bunch (1946). During this 16-hr period the subject can obtain only brief periods of slow-wave sleep, with no REM sleep. It is advisable to obtain sample polygraph recordings to verify that no REM is occurring during these 16-hour periods. Then, for the remaining 8 hr of each day the subject is placed in a sleep-recording chamber and the experimenter arousal method for REM episodes is employed. Control subjects receive the same 16-hr treadmill-activity cage treatment, and for the next 8 hr are given the same number of awakenings from slow-wave sleep as the experimental subjects receive from REM sleep. The control animals have experienced the same 16-hr sleep deprivation-stress paradigm as the experimental animals but are allowed to have undisturbed REM sleep for 8 hr/day, which is sufficient to give the normal daily allotment of REM sleep. The control animals also experience arousal from sleep to the same extent as do the REM deprived animals.

Several investigators have succeeded in automating the EEG-monitoring method by constructing computer-based or hard-wired automatic discriminators of sleep stage occurrence. REM sleep is then identified using continuous EEG, EMG, and EOG input from polygraphs. Some description of this type of scoring by automated procedures is given in a later section, but many of the technical details are beyond the scope of this chapter. However, descriptions are available from several recent methodological papers dealing with mice (Mitler and Levine, 1970), rats (Kohn *et al.*, 1974; Branchey *et al.*, 1974), and primates (Berger and Meier, 1966; Larsen *et al.*, 1972). Once the REM state is identified as having occurred, delivery of an arousing stimulus, such as a loud noise, stimulation of the reticular-activating system through implanted electrodes, or vibration of the recording chamber, are effective methods for producing arousal from REM sleep.

2. Flowerpot or Island Technique

This popular method for deprivation of REM sleep was first described by Jouvet *et al.* (1964) and later by Morden *et al.* (1967). Basically, the method consists of placing a cat, rat, or mouse (Fishbein, 1970) on a small platform, typically the base of an inverted cement-filled flowerpot, which is surrounded by water in an escape-proof chamber (Fig. 7). This island should be small enough to prevent the subject from fully curling up on its side during sleep. Should the subject enter REM sleep while positioned on the island the muscular atonia which accompanies REM sleep would result in its falling off the platform into the water. In reality, after the first few hours in the deprivation chamber, few subjects fall into the surround-

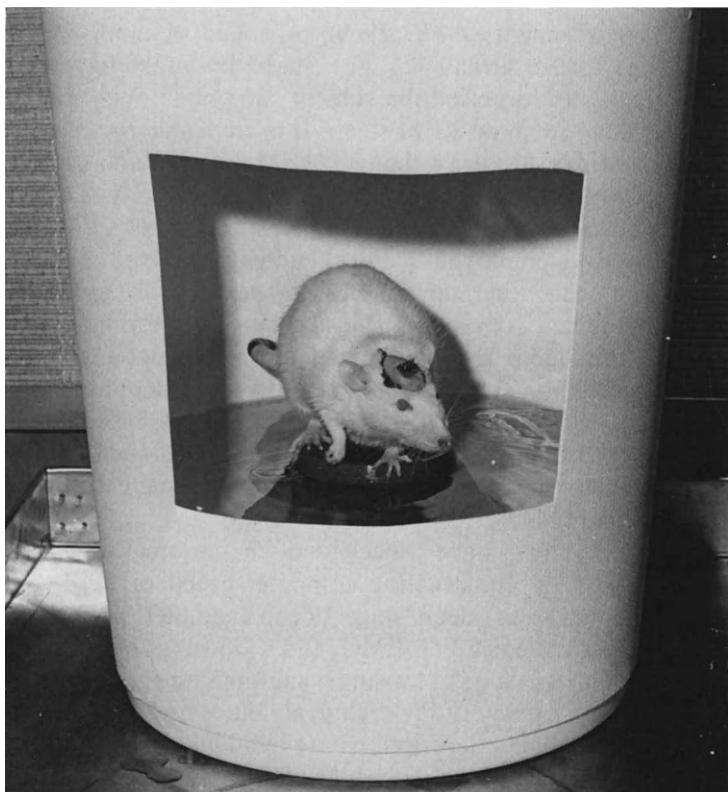


FIG. 7. Photograph of rat on inverted flower pot in plastic chamber. This "island" is small enough to prevent the animal from curling up and going into REM sleep.

ing water. The advantages of this method are that it does not require round-the-clock EEG monitoring, is procedurally simple, and does not require electrode implantation in each subject. This technique also provides relatively selective deprivation of REM sleep and can be readily adapted to depriving large numbers of subjects at the same time. However, to be assured that REM sleep does not occur, monitoring could be easily carried out in this situation as well. The disadvantages of this procedure are that there is some concomitant loss of slow-wave sleep, and a marked stress reaction occurs in subjects that are REM deprived in this way.

The flowerpot technique has been reported to produce from 50 to 100% deprivation of REM sleep (Mark *et al.*, 1969; Mendelson *et al.*, 1974), depending, in part, upon the size of the island. The size of the deprivation platform is related to the size of the subject, e.g., a 5–6 cm diameter

platform is used for a 200-gm rat with a water depth of 5–10 cm extending to 1 cm from the island level. A 2–3-kg adult cat requires a 12 cm diameter platform with 2–3 cm deep water located about 1 cm below the platform level. In the case of the cat, the pan is usually placed in a large cage to prevent the animal from escaping.

For rats or mice, food can be given from an overhead wire mesh screen during the time spent on the flowerpot. In the case of cats, they should be removed from the deprivation chamber at least twice each day for at least 30–60 min for exercise and feeding. If closed-circuit television equipment is available, monitoring of the condition of the animal can be carried out on a reasonably regular basis in the laboratory without disturbing the animal. Rats can be readily maintained in the REM deprivation apparatus for 5 days or longer with a mortality rate in the 5-day condition of no more than 10%. The deaths which occur appear to be due to exhaustion and hypothermia resulting from repeated escape attempts and swimming. Dement *et al.* (1969) report that cats can be maintained in the deprivation chamber for as long as 1 month without markedly adverse effects.

For rats, the islands and water can be contained within a hard plastic wastebasket (see Fig. 7) or else a galvanized steel wash basin. For both rodents and cats clean water should be introduced every 1–2 days to maintain generally sanitary conditions and reduce odors. To eliminate the need for constant cleansing of the deprivation chambers some investigators have constructed a continuous flow system in which fresh running water constantly flushes the deprivation chambers. The influx of fresh water equals the rate of efflux, and a safety overflow drain prevents the water levels from rising above the top of the animal's platform.

The main disadvantage of the flowerpot technique for REM sleep deprivation is the chronic stress that is produced by this procedure. Actually, it is unclear to what extent the stress responses reported after such REM deprivation are due to the loss of REM sleep or to the deprivation procedures themselves. The stress response of rats that are REM deprived with the flowerpot method is reflected in a daily weight loss of 5–10 gm, even though food is available *ad libitum*, and in adrenal hypertrophy and thymus atrophy (Stern and Hartmann, 1972).

Two stress control procedures have generally been advocated. The first is the use of "large" island controls (12 cm diameter for rats) in the deprivation chamber. This larger platform permits the subjects to curl up and enter REM sleep without falling in the water, yet exposes the subjects to restraint of movement and dampness. However, some reports indicate that the large platform control animals also exhibit substantial amounts of REM sleep deprivation, often in the 50% range (Mark *et al.*, 1969; Mendelson *et al.*, 1974).

An alternative stress control procedure consists of twice daily immersions of rats in 17°C water, depth of 10 cm, for two 1-hr sessions, and the restriction of food intake to produce a weight loss comparable to that of the REM deprived subjects (Stern and Hartmann, 1972). These controls are given *ad libitum* sleep for the remaining 22 hr of the day. The cold-water-stress control rats show an endocrine stress response at least equal to that of the REM deprived condition but show no diminution in REM sleep. A comparison of the effectiveness of the large island control with the cold-water immersion control treatments has been described by Mendelson *et al.* (1974). The stress response of the cat to the flowerpot deprivation has not been evaluated. It is strongly recommended that any series of REM deprivation studies include a subset of polygraphically recorded subjects to verify the extent of REM and slow-wave sleep deprivation being produced.

VIII. PHARMACOLOGICAL STUDIES OF SLEEP

Sleep is one of the major electrographic and behavioral states that has been subjected to a wide variety of pharmacological manipulations. This is due to the fact that the sleep states have been so closely identified with neurochemical systems in the brain that are altered by pharmacological agents. Since chemical anatomy has come to the forefront in studying the organization of aminergic and other pathways in the brain, pharmacological methods related to the manipulation of specific aminergic systems have been widely used in the study of sleep [see the reviews by Jouvet (1972) and Morgane and Stern (1974)]. We will point out in this review some common considerations that should be taken into account in pharmacological studies of the sleep states.

Most pharmacological sleep studies usually fall into one of two categories: (1) use of a drug with known neurochemical effect(s) on the brain to investigate the neurochemistry of the sleep-waking mechanisms (e.g., does reduction in the synthesis of brain serotonin by *para*-chlorophenylalanine reduce sleep?); (2) purely descriptive studies conducted without regard to underlying mechanisms, or a preclinical evaluation of the potential sleep-altering effects of a given agent (e.g., what is the effect of aspirin or flurazepam on sleep?).

When the investigator attempts to demonstrate that the neurochemical action of a drug is responsible for the observed change in sleep, the strength of such arguments can be greatly increased by showing blockage or reversal of the drug-induced sleep changes by other pharmacological agents known to block or reverse the particular neurochemical event in question. For example, 5-hydroxytryptophan, a serotonin precursor, re-

verses both the sleep-disrupting effects and the serotonin-depleting effects of *para*-chlorophenylalanine in the cat (Jouvet, 1972).

Cats and rats are the most widely selected species for pharmacological studies of sleep in animals. Both species have adequate skull sizes for electrode implantation, do not require special handling or restraint, and sleep about one-half to two-thirds of the time during the day. This amount of spontaneous sleep permits an assessment of both increases or decreases from baseline sleep time following pharmacological treatment. Rats are not selected if the purpose of the study is to examine the physiology or pharmacology of PGO spikes, since these events cannot be readily observed in this species (Stern *et al.*, 1974).

Monkeys, while having sleep characteristics more similar to those of man than do rats or cats, have the marked disadvantage of requiring a restraint device when using a cable-based EEG recording system. The restraint can produce abnormal sleep patterns and thus confound any interpretations of postdrug sleep changes. A telemetry system of data collection would obviate these difficulties, but such a system is not readily available to the average laboratory. The rabbit is, generally speaking, a poor subject to use in sleep studies because of the prolonged adaption time required for REM sleep to occur. However, the female rabbit displays a remarkable hormone-coital-dependent REM sleep pattern (Sawyer and Kawakami, 1959), which is not as readily seen in other species. Submammalian species, for the most part, do not show REM sleep [see the review of Tauber (1974)], and only a few pharmacological studies have been conducted in this group.

Pharmacologists on occasion assess the effects of a test compound on the duration of barbiturate "sleeping time" in mice, i.e., the duration following the intraperitoneal (i.p.) injection of 100 mg/kg of sodium hexobarbital versus an injection of hexobarbital plus the test agent; following this, mice fail to stand erect or show loss of righting reflexes. Since barbiturates are metabolized primarily by the liver, the level of activity of certain drug-metabolizing enzymes can be assessed by measuring the duration of action of barbiturates. Actually, barbiturate sleeping time is a misnomer since barbiturates do not induce normal sleep. Instead, they produce a nonarousable narcotic or anesthetic state in which the cortical EEG consists of continuous high-voltage, slow-wave and spindle activity with a total suppression of REM sleep. At present, there is no evidence that barbiturates activate the normal physiological mechanisms of sleep.

The design and conduct of an experiment aimed at evaluating the effect of pharmacological agents on sleep require the investigator to provide the following:

1. *Experimental subjects*, preferably six or more, bearing chronically

implanted electrodes for obtaining at least cortical EEG, eye activity, and neck muscle activity. Although visual observation of the behavior of a subject is sufficient to distinguish periods of activity from inactivity, such observation poorly discriminates REM sleep from slow-wave sleep or relaxed waking with eyes shut from slow-wave sleep. There is no substitute for electrographic recording when studying sleep.

2. *A recording chamber and polygraph system* (see Figs. 1, 3, and 4).

3. *Sufficient recording paper.* When operating on a modest budget the cost of recording paper, which ranges from \$5 to \$20 per pack, depending on its width, and the number of channels being recorded, can be a limiting factor. By reusing paper on the reverse side and selecting a slower paper speed, such costs can be minimized considerably. In general, a paper speed of less than 2.5 mm/sec will not allow adequate resolution of the EEG wave form and will reduce the accuracy of scoring of the records. In the past a number of studies have employed recording sessions of only 2-3 hr duration. This is inadequate, because during the first 30-60 min following connection to a recording cable the animal is usually awake and readapting to the recording situation or to the effects of prior handling. Also, 2-3 hour recordings do not permit assessment of a full time course of drug action. Thus, such sessions can be too short a sample to reflect accurately the true changes in sleep-waking patterns.

4. *Selection of the proper experimental design.* Fortunately, most sleep studies in animals lend themselves to "within subject" experimental design, i.e., each subject provides its own baseline and postdrug results. This experimental design has the obvious advantage of reducing intersubject variability in assessing the sleep effects of a given drug.

The typical protocol for a pharmacological study of sleep involves: (a) surgical implantation of electrodes with a postoperative recovery period of at least 2 weeks or, preferably, longer; (b) two to four adaptation sessions in the recording chamber followed by two to four baseline sessions; (c) initiation of the treatment sequences, which involves the establishment of the dose-response curve. This requires a minimum of three doses plus placebo, vehicle, or other pharmacological control injections. It is advisable to conduct another set of baseline runs after completion of the drug series to insure that no major long-term adaptational changes have occurred. For drugs believed to have a duration of action of a few hours, successive administrations should be several days apart and, preferably, separated by a week or more. Drugs with long-term effects (days to weeks) require intervals of several weeks (or months) between doses.

As in other areas of psychopharmacology the route of administration of a drug can importantly determine the magnitude and nature of the sleep

effects observed. In general, intravenous (i.v., commonly given in the tail vein in rats, or femoral vein in cats) or intraperitoneal (i.p.) injections give the most rapid onset of action and are the most widely employed. Subcutaneous (s.c., usually under the skin of the back or the neck) and intramuscular (i.m., usually in the hind limb) injections give a slower onset, but show more prolonged duration of effect than do the i.v. or i.p. routes. Oral administration in food, water, or by direct intubation tends to give the slowest onset of effect and smallest magnitude, because of the slow time course of gastric and intestinal absorption. Also, there are the additional factors of partial digestion and/or fecal excretion of the drug. Sleep-waking recordings should commence immediately after drug administration, since the latencies of the drug effects may be extremely short. The investigator should make periodic observations of the behavior of the subject all during the recording period, preferably at 15–30 min intervals, so as to confirm the validity of the EEG-behavior relationship and to verify that disassociations do not exist between EEG and behavior (as do those after anticholinergic agents such as atropine).

Once the polygraph records from the baseline and drug sessions are obtained they should be categorized into periods of waking, slow-wave sleep, and REM sleep by experienced scorers (see Section IX,A) who do not have knowledge of the treatment for a particular session. If more than one scorer is used, then periodic cross-scorer reliability checks, whereby both individuals score the same record, should be conducted in order to minimize interscorer variability. In general, categorization of the records into 5–10 sec epochs will provide adequate resolution of short periods of waking, slow-wave sleep, and REM sleep. A less laborious method is to score only every 30 sec, or in the extreme case, every minute.

In addition to computing the percentage of recording time spent in waking, slow-wave sleep, and REM sleep, valuable descriptive information of sleep characteristics can be obtained from: (1) the latency from drug administration to the first episode of slow-wave sleep and/or REM sleep; (2) duration of the individual slow-wave sleep and REM episodes; (3) sleep cycle length for periods of continuous sleep, i.e., time from onset of one REM period to the start of the next; (4) percentage of total sleep time spent in slow-wave sleep or REM sleep; (5) the change in occurrence of a particular correlate of a sleep state, e.g., the number of eye movements per minute during REM sleep, the number of PGO spikes in REM or slow-wave sleep, the occurrence of cortical EEG spindles; and (6) the number and length of arousal periods. The development of a so-called sleep profile quantitating many of these various measures is indicated in Table I.

An important aspect of the analysis of sleep data in pharmacological

TABLE I

Effects of Anterior Raphe Lesions and *para*-Chlorophenylalanine (PCPA) on Sleep Profiles^a

	Means and Standard Deviation		
	A (average values from Jouvet, Sterman, and Morgane)	B (effects of raphe lesions)	C (effects of PCPA 150 mg/kg i.p.)
Waking/total recording time	28.8 ± 5.5	82.6 ± 9.6 ^b	87.3 ± 8.3 ^c
Total sleep/total recording time	71.2 ± 4.8	17.4 ± 4.5	12.7 ± 3.6
Slow wave sleep/total recording time	54.8 ± 5.9	2.8 ± 1.3	2.1 ± 1.2
Fast wave sleep/total recording time	16.3 ± 2.3	14.6 ± 2.7	10.6 ± 2.2
Slow wave sleep/total sleep	74.6 ± 5.4	16.1 ± 2.5	16.5 ± 3.2
Fast wave sleep/total sleep	25.3 ± 4.3	83.9 ± 5.1	83.5 ± 6.5
Mean duration of fast wave sleep episodes (minutes)	5.7 ± 1.4	19.3 ± 3.2	17.4 ± 4.8

^a Sleep-waking profile of the cat showing baseline values in column A and the effects on these states of raphe lesions (column B) and the drug *para*-chlorophenylalanine [PCPA (column C)]. Raphe lesions and PCPA both reduce levels of serotonin in the brain, but by quite different mechanisms. The sleep-waking profile is a sensitive indicator of these changes in brain chemistry, as is indicated by comparisons of baseline recordings (column A) with those following manipulation (columns B and C).

^b 6-day means of 5 cats beginning 36 hours after operation.

^c 6-day means of 5 cats beginning 36 hours after PCPA administration.

studies is that of the time-response curve. Many drugs have a short duration of action, i.e., less than 4 or 5 hr. If the investigator is employing recording sessions of 8-hr duration and the entire drug effect is present for only the first few hours, he may not observe a change from baseline when the data is computed over the full 8-hr session. Sleep is under strong homeostatic regulation and drug-induced decreases or increases in sleep time are likely to be compensated for once the drug effects have abated. This consideration is especially pertinent to REM sleep since a 30–50% suppression in REM time for the first 3 hr after drug administration can be readily compensated for by a 30–50% increase in the last 5 hr of an 8-hr session.

Obviously, should drug effects last longer than the 8-hr daytime recording session, then overnight recordings are required. These may or may not require considerable laboratory manpower. Overnight data can be obtained without the continued presence of an investigator. The data can be electronically stored on an FM tape recorder, using the slowest tape

speed available, by feeding the polygraph amplifier output into the tape and turning the polygraph paper drive and pen motors off. The tapes can be replayed through the polygraph at a later time to provide a write-out or paper copy of the recordings. The playback can be made at two to four times real time. A playback factor higher than 4 tends excessively to distort the write-out of the EEG signals, since most pen motors have difficulty following signals much above 60 Hz. Tapes played back by a factor of 4 convert the original 15-Hz signals into 60-Hz output.

A consideration related to the duration of drug action on sleep is the desirability of measuring the effects of chronic drug administration and subsequent withdrawal. Limited numbers of such studies with hypnotic agents and other psychopharmacological agents in humans and animals show that prolonged drug administration can result in either loss of effect of the drug, in terms of failure to alter sleep patterns (Kales *et al.*, 1974), or a prolonged and abnormal sleep pattern during a withdrawal even if during the chronic administration period sleep was not disturbed (Oswald, 1969). Unfortunately, most investigators do not evaluate this important aspect of drug action on sleep.

Sleep, especially the REM state, is very susceptible to disruption by drugs which exert peripheral side-effects. Thus, when postdrug decreases in REM time are observed it behooves the investigator to attempt to determine whether peripheral side-effects are responsible. Obviously, it is exceedingly difficult to eliminate the contribution of all possible side-effects, but major aspects of autonomic nervous system functioning can be examined. For example, changes in heart rate, blood pressure, respiration, temperature, salivation, diarrhea, or irritation at the site of injection can be investigated, assuming that such effects have not already been described in the pharmacological literature with regard to the particular drug being tested.

IX. SLEEP DATA PROCESSING TECHNIQUES

A. Manual Scoring of Polygraphic Records

In classical electrophysiological experiments, the processing of data may be relatively simple but often very tedious. It is based mostly on the marking of intervals on the recorded curve and on measuring their voltage and time coordinates, the sizes and changes of which are evaluated in relation to the conditions of the experiment. Some recordings, such as the EEG, are often evaluated simply on the basis of the overall qualitative impression they give or, at most, on a quasi-quantitative basis. We cannot go into detail on the scoring of polygraphic records to establish sleep-

waking "profiles" but will briefly describe some of the salient features of this hand-scoring technique and will follow this with an outline of one type of computer analysis of EEG activity. In this way, the main features as to how "sleep" data are handled in studies of sleep-waking physiology can be understood.

Hand scoring of the various indicators of the sleep-waking states, i.e., EEG activity from cortex, hippocampus, other brain loci (depending on how many recording channels one has), eye movements, neck EMG activity, and PGO activity, involves scanning segments of a continuous record run over 8 to 24 hr in the usual condition. Since polygraph paper has ruled vertical lines and the paper speed can be set to run at a known rate, we know the time represented between each set of vertical lines and on an entire single sheet of polygraph paper. It is important to note that it takes several indicators to assure adequate definition of the different states we standardly score, i.e., waking, slow-wave sleep (SWS), and REM sleep (see Fig. 8).

Although these are the three main states, waking can be further divided into waking 1 and waking 2, i.e., "aroused" waking or "relaxed" waking, and SWS can be divided into SWS 1 and SWS 2 depending on predominance of spindles in SWS 1 and large, slow waves in SWS 2. In waking the

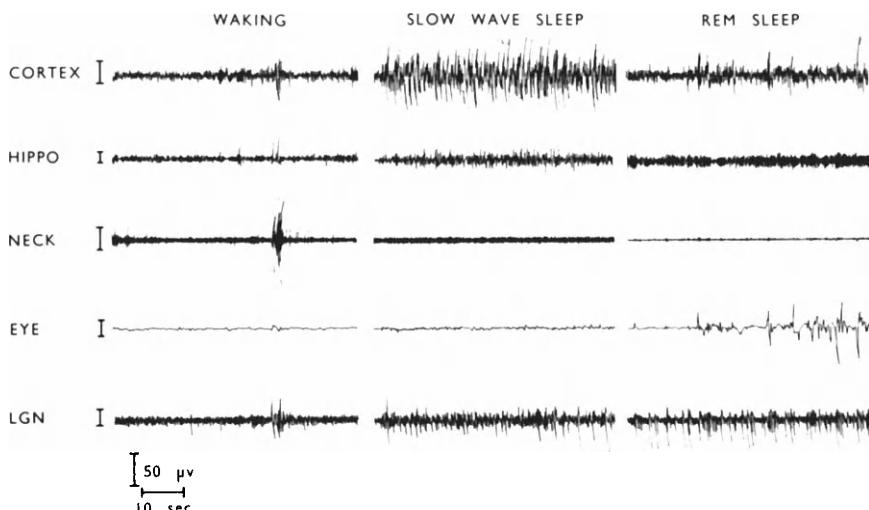


FIG. 8. Polygraphic recording showing the five principal indicators of the vigilance states [neocortical EEG, hippocampal EEG, neck EMG, eye movements, and lateral geniculate (LGN) PGO spikes]. This record represents a short sample of waking, slow-wave sleep, and REM sleep in the cat. Note that calibration voltage and time are indicated at base of record. See the text for a general description of each of the three states.

cortex shows low voltage, fast activity, the eyes move rapidly in small excursions, there are few or no PGO spikes, and the neck EMG is active. The hippocampus shows a low-voltage, fast activity or a type of waking theta. In SWS the cortex shows spindles or slow waves, the eyes again are quiet, there are no PGOS, and the neck EMG is reduced in amplitude from the waking EMG while the hippocampus also shows large slow waves. In REM sleep the cortex again shows low-voltage, fast activity, often indistinguishable by eyeball scoring techniques from waking, the eyes show bouts of rapid large excursions of movement, the lateral geniculate (LGN) shows PGO spikes in bursts and clusters, the hippocampus shows a distinct theta activity, and the neck EMG becomes isoelectric or "flat" (Fig. 8).

The investigator or scorer spreads out the record and on a score sheet marks the time spent in each state using the page number on each polygraph sheet. Fractions of each sheet are marked, since different papers have various divisions per one heavy line, and there are various heavy lines, usually 10 major divisions per page. Thus, if the waking stage 1 begins on page 644 at the first heavy line it is marked as beginning at 644.0, and if it ends at the fifth heavy line it is marked 644.5. Then, if the animal goes into SWS1 at 647.1 and then back to W2 at 651.0, it is marked as shown in Fig. 9. The scorer then goes back and forth across the score sheet as the changes occur, until the entire record is scored. Then sums are made for each page, and the percentage in each stage is then calculated. These percentages, when tabulated, result in what we term a "sleep profile" (see Table I).

Obviously, there are so-called transition periods that occupy a large part of the record and give the most problems in scoring. For example, "drowsiness" is sometimes scored as a separate category, but it is agreed that a decision must be made by the investigator as to defining this as either sleep or waking. Transition periods give the most trouble in scoring and, naturally, are subject to much individual interpretation. These are decided upon by certain criteria established in the laboratory. As yet, overall criteria for defining the vigilance states and stages in various species other than man are not standardized. Attempts are being made to develop more adequate standardization of the sleep states in rats and cats. This means, of course, that the electrode sites have also to be as standardized in animals as they are in human electroencephalography. In any experiment involving continuous polygraphic recording the animal is placed in the recording chamber (Fig. 3) for an acclimatization period before any recording is done. Then, several "baseline" recording sessions are carried out which give the comparative, unmanipulated quantitative record against which we measure changes produced by experimental manipulation of the animal.

Animal No. _____ Date _____

Scorer _____ Experimental Condition _____

Record Box No. _____ Paper Speed _____

EEG Page No. _____					No. of Pages per Stage												
W1	W2	SWS1	SWS2	REM	W1	W2	SWS1	SWS2	REM								
644.0	644.5	647.1					0.5	2.6	3.9								
651.0	651.5					0.5	2.9										
[etc., approx.					20 or 25 lines for usual record sheet]												
Sum of this page:																	
Sum of total pages:																	
Percentage of total time: [normal cat profile] = 17 12 20 35 16																	

FIG. 9. Example of a sleep-score sheet.

B. Computer Analysis of Electroencephalographic Signals

In order to analyze the electrical signals obtained from the experimental preparation, a magnetic tape recorder (Fig. 10) is usually employed. The primary advantages of such an arrangement are as follows: (1) Data can be stored and analyzed at a later time. (2) The time base of the recorded data may be expanded if the investigator is interested in the subtle details of the electrical signal immediately after the stimulation, or it can be contracted if he is interested in analyzing a long record in a relatively short period of time; for example, an EEG record that lasts 15 min can be played back faster to speed up the analysis time. (3) Only those parts of the experiment which were important and critical need to be analyzed and emphasized. Both large computers (Fig. 11) and portable (Fig. 10) tape recorders are commercially available. A typical tape recorder which can



FIG. 10. A typical light-weight portable instrumentation tape recorder like the Hewlett-Packard Model 3960 shown here can be used in a large assortment of applications, such as data acquisition and reproduction, formerly provided by larger and more expensive recorders. Operation is enhanced by properly selecting direct recording or FM recording electronics, depending upon the type of data to be recorded. Using such a recorder with four record-reproduce channels and three speeds (15/16, 3-3/4, and 15 in./sec), the investigator can record the bioelectric events, including the EEG, of interest during the conduct of an actual experiment. At a later time the investigator can play these data into a computer for analysis.

be employed is a Hewlett-Packard model capable of recording seven channels of data on 2400 foot reels of 0.5-in. magnetic tape (Fig. 10). This tape transport can be operated at tape speeds of 3.75, 7.5, 15.0, and 30.0 in./sec. Record-reproduce amplifiers mounted on individual printed circuit boards are usually available, each of which can be inserted into any of the seven channels of the tape recorder. These amplifiers may be a direct



FIG. 11. The Digital Equipment Corporation PDP-12 computer is commonly found in many neuroscience laboratories. In the system shown here the analog-to-digital converters are located at the left of the keyboard console, and the magnetic tape typically used for storing the digitized data and processed information is located directly above the cathode ray tube display screen. In addition, there is an IBM compatible magnetic tape system (located at the right) so that data may also be stored on these tapes for analysis using larger computers.

record-reproduce amplifier that is usually capacitor coupled (i.e., is an ac amplifier) and has a lower cutoff frequency of 50 Hz and an upper cutoff frequency determined by the speed of the magnetic tape. In addition, FM record-reproduce amplifiers are available that employ a frequency-modulated carrier system permitting the recording of signal frequencies

down to zero Hz. The carrier frequency and the upper cutoff frequency of these amplifiers are also determined by the speed of the magnetic tape. In recording the EEG, FM record-reproduce amplifiers are appropriate, since the frequency response of these amplifiers is from 0–50 Hz. However, in analyzing evoked responses the direct amplifier is necessary, since it has a frequency response of up to 20 kHz.

The general purpose computer, such as the PDP-12 (Peripheral Data Processor (Fig. 11), has had an enormous impact in the neuroscience laboratory and has enabled the investigator's results to become more quantitative and statistical. Several types of general purpose laboratory computers available today now provide neuroscientists with great flexibility. With these, alterations in such bioelectrical events as single and multiunit activity, evoked potentials, and the gross electrical activity (EEG) obtained from subcortical as well as cortical areas of the brain can be analyzed utilizing a variety of mathematical techniques. As a matter of fact, the only limitations upon the type of analysis that can be done lie in the memory capacity of the computer and the imagination of the computer programmer.

The researcher can interact with the computerized data provided to him in several ways. First, he can operate the computer in a "real time" mode which permits modifications in the experimental parameters to be made during the actual experiment. Based upon the processed information fed back to him, the investigator can explore more carefully those effects which dramatically affect the preparation. In this way, he can optimize the positive results obtained from each experiment. Secondly, if he is interested in recording the data during the experiment and processing it at a later time, the information can be stored on magnetic tape and analyzed at a later time on the computer. Using this approach, it is possible to monitor carefully the data being analyzed and perhaps even to perform more than one type of mathematical analysis on the same data. This type of operation is commonly employed and allows the researcher the opportunity to "save" the electrical events that take place during his experimental procedures and reanalyze them at any time or when it appears that another type of analysis might provide an insight otherwise not possible. This approach therefore maximizes the variety of analysis techniques that may be used on the data available from a single experiment. Since it is impossible to describe the entire spectrum of data processing techniques used in sleep-waking EEG analyses in this chapter, emphasis is placed here upon one type of EEG analysis using a general purpose computer.

The general purpose laboratory computer described here is based on a Digital Equipment Corporation PDP-12 digital computer system which is

widely used. This system consists of the PDP-12 computer, an eight-channel multiplexer, a 10-bit analog-to-digital converter providing a resolution of one part out of 1024, printer, digital magnetic tape recorders, and a Calcomp plotter. A schema of interconnections of this system is shown in Figure 12.

In analyzing the EEG, the first step is to transform the bioelectric event, i.e., the analog (continuously varying physical entities such as voltage and current) EEG signal into the appropriate digital format (or number) which the computer can utilize in order to perform the various calculations desired. This is accomplished by the analog-to-digital converter (A-D converter). The electrical signal, which must be amplified or attenuated to be within a specified range (-1 to +1 V), is supplied directly from the experimental setup or from the tape recorder. The input data

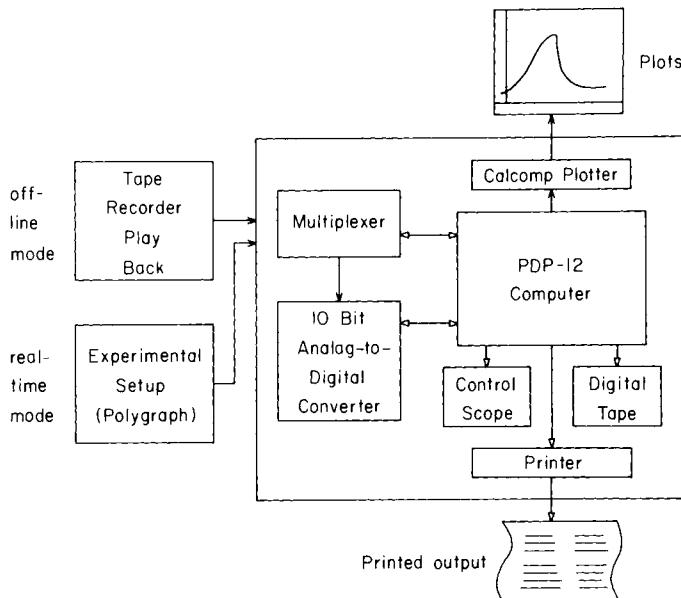


FIG. 12. A typical data analysis system providing various optional input-output modalities. Input to the computer can come directly from the experimental animal so that the data, e.g., EEG, may be analyzed as events actually occur; or input may come from a tape recorder which has this same type of information stored on magnetic tape for analysis upon completion of the experiment. In either case, these analog signals representing bioelectric events are converted into a digital format by the analog-to-digital converter while the multiplexer permits more than one channel of data to be processed at the same time. The processed data can then be presented to the investigator on the screen of a cathode ray oscilloscope, in the form of a graph on the Calcomp plotter, as a table of numbers on the digital printer, or simply stored for further use on the magnetic tape system available.

may consist of information from one to eight channels at a time. A multiplexer or electronic switching device allows more than one channel to be "viewed" by the computer simultaneously.

These input data are then sampled by the computer's A-D converter approximately every 8 msec in each channel. This results in a sample rate per channel of 128 per sec. Since the Nyquist frequency is the reciprocal of twice the sampling period of 8 msec, this provides a Nyquist frequency of 62.5 Hz. This sampling frequency is twice the maximum frequency of interest in the EEG and, as it is above 60 Hz, also reduces any line frequency variation. Usually, each EEG record is divided into a number of epochs which may be 4, 8, or 16 sec in duration. If a 16-sec epoch is selected, for example, and the sampling interval is 8 msec, as indicated above, then 2048 individual samples are provided for each channel during that epoch. The digitized information obtained using the A-D converter is then stored on digital magnetic tape or in the memory of the computer to be used again, depending upon the type of analysis methods the investigator has decided to employ.

Of the possible methods available for analysis of the EEG, one technique in particular, i.e., *spectral analysis*, has received a great deal of attention in recent years. Since the EEG is an expression of the electrical processes in neurons beneath the recording electrodes, it contains time variations in both amplitude and frequency. They change naturally with the various states of vigilance and are altered by physiological or pharmacological manipulation. Spectral analysis provides an effective means to quantitate any frequency shifts that might occur. An excellent discussion of automated analysis of the EEG, including an extensive bibliography on the subject, has been published by Gevins *et al.* (1975).

Spectral analysis is based upon the theorem that any repetitive signal can be considered to be the sum of sinusoidal components whose frequencies are integral multiples of the basic repetition frequency. Fourier-transform analysis involves the calculation of the amplitudes and phase angles of these components. With the development of the Fast Fourier Transform—an algorithm for the machine calculation of complex Fourier series—by Cooley and Tukey (1965), spectral analysis algorithms have been developed and incorporated into computer programs that are readily available today (DECUS PROGRAM LIBRARY) and permit these calculations to be done in a reasonably short time.

Autospectral analysis, comparing electrical activity in one channel of the EEG with itself, involves the calculation of the squared magnitude of the Fourier spectrum and is the quantity produced by most spectral analyzers or computer programs. Because it is proportional to the power of a signal, it is commonly called Power Spectral Density (PSD). It gives exactly the same information as the autocorrelation function; in fact, it is

the Fourier transform of the autocorrelation function, and it can be calculated in that way (Blackman and Tukey, 1958).

Using these programs, the digitized data are analyzed two channels at a time. Since each epoch is 16 sec long, it represents a fundamental frequency of $\frac{1}{16}$ or 0.0625 Hz. Since the Nyquist frequency is 62.5 Hz, within each 0.5 Hz interval below 62.5 Hz there will be found 8 harmonic frequencies (i.e., 8×0.0625 Hz = 0.50 Hz). The program is therefore designed to sum the variances in successive groups of 8 sinusoids. This process is done for each channel of data. The program also computes the real and imaginary parts of the average cross-product between the corresponding coefficients computed from each channel.

The autospectral analysis for each channel in 0.5 Hz increments is often normalized so that the area under the curve (power versus frequency) from 0.5 to 62.5 Hz is set to 1. The same normalization is done to the magnitude of the cross-power spectrum. The phase angle is computed by taking the arc tangent of the imaginary part of the cross spectral analysis divided by the real part. This is done for each 0.5-Hz increment.

The coherence function, which is a measure of the similarity between two wave forms, is defined as

$$\text{coherence } (f) = \sqrt{W(f)_{12}^2 / W(f)_1 \times W(f)_2}$$

where $W(f)_1$ is the autopower of channel 1; $W(f)_2$, the autopower of channel 2; $W(f)_{12}$, the cross-power of channel 1 with channel 2. This function can also be computed for each 0.5-Hz increment. Since the coherence function is a measure of the degree of similarity of the electrical signals present in the two channels, a value of 1 would indicate that the two signals are exactly alike while 0 would indicate that they are completely dissimilar.

These spectral measures of the EEG, i.e., autocorrelation (comparing the electrical activity in one channel of EEG with itself) and cross-correlation (comparing the electrical activity present in two different EEG channels) have been widely used in order to quantitate EEG activity for statistical purposes. For example, power spectrum studies and autocorrelation analyses have been employed in our laboratory to do the following: (1) *Quantitate the frequency shifts in the EEG due to pharmacological manipulation;* in a series of experiments, direct application of serotonin to the region of the area postrema clearly "slowed" the EEG, as was evident by the shift to low frequencies as seen by the power spectrum presentation [Fig. 13 (Bronzino *et al.*, 1972a)]. (2) *Characterize the electrical nature of the EEG during each state of vigilance;* it was clearly

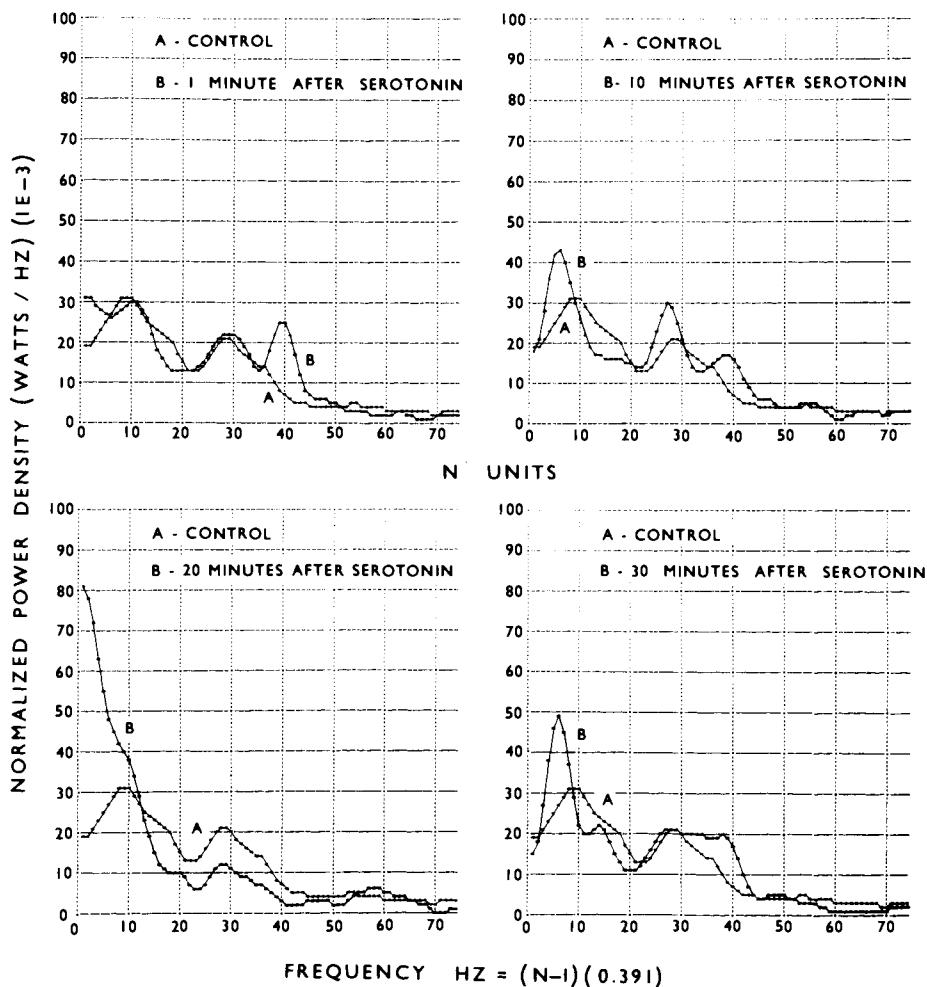


FIG. 13. Power spectral density analyses of EEG activity. In this instance these analyses are used to study quantitatively the effects of pharmacological manipulations of the brain. Thus, serotonin ($3\mu\text{g}/\text{ml}$) was applied directly to the area postrema of a cat. The variable N used by the Fast Fourier Transform on an IBM 1130 computer indicated the n th term in the Fourier transform. This figure represents the format actually presented by the computer printer available. Control curve (A) on each plot used for comparison was calculated from the EEG record 1 min before application of serotonin. There was a significant increase in the low-frequency components of the EEG with the maximum effect occurring 20 min after application of serotonin. After 30 min, the effect was receding, since curve B is once again approaching control values.

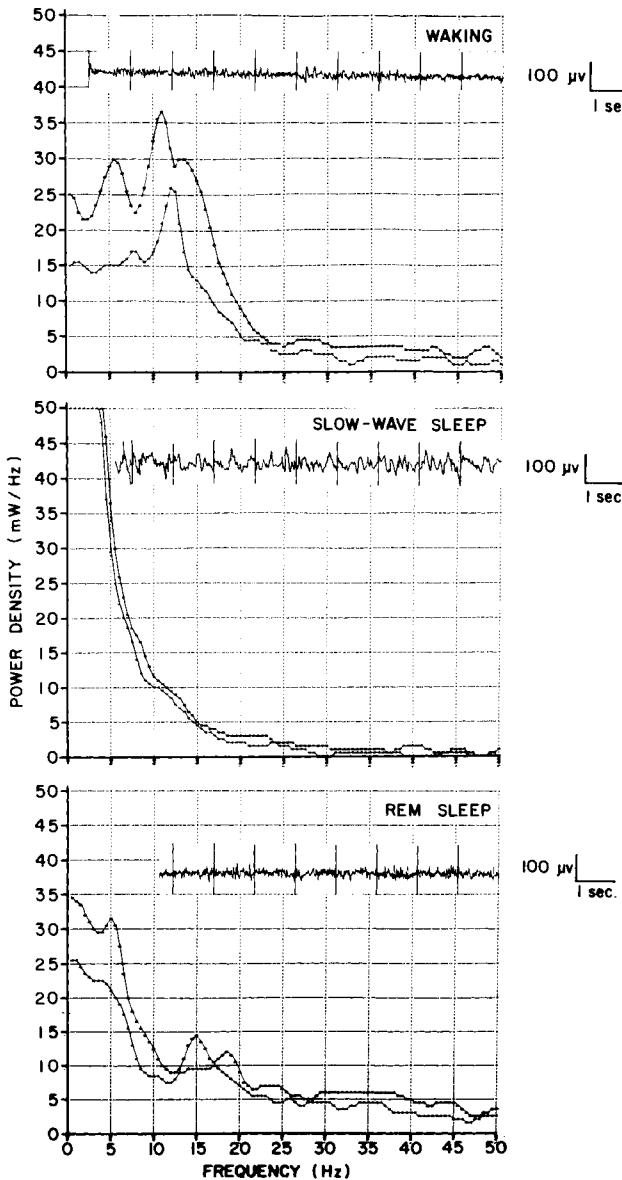


FIG. 14. Examples of power spectral density analyses used to quantitate the electrical characteristics of the EEG during the various vigilance states of the cat. This figure represents the computer printout of the normalized power density spectra (area under the curve is equal to 1) of the cortical EEG [$(\text{mW}/\text{Hz}) \times 100$] from a single cat during waking, slow-wave sleep, and REM sleep. The two power spectral curves for each state represent the ± 1.0 SD away from the mean (mean values not shown). A sample of the corresponding EEG record for each vigilance state is given alongside the representative power density spectral curve.

demonstrated that each vigilance state had its own electrical signature and therefore its distinctive power spectral plot pattern that clearly distinguished it from the pattern in other states of vigilance [Fig. 14 (Bronzino *et al.*, 1972b)]. (3) Denote the transitional states of sleep as the experimental subject shifts from one sleep state to another. These transition periods have caused confusion in manual scoring and classification, but power spectral representation has provided quantitative assessment of the nature of the transitional periods so they could be better defined as a definite vigilance state, i.e., waking, slow-wave sleep, or REM sleep. Autospectral analyses are also used to distinguish states that could not be defined by hand-scoring or eyeballing methods, namely, EEG differences such as in the cortical desynchronization seen in REM sleep versus waking. The power spectral approaches do not, however, lend themselves to analysis of long records of many hours duration, since they are time consuming; rather, they are best applied to critical segments of records where finer discriminations are needed.

REFERENCES

- Adrian, E. D., and Matthews, B. H. C. (1934). The interpretation of potential waves in the cortex. *J. Physiol. (London)* **81**, 440-471.
- Akert, K., Koella, W., and Hess, J. R., Jr. (1952). Sleep induced by electrical stimulation of the thalamus. *Am. J. Physiol.* **168**, 260-267.
- Albert, I., Cicala, G. A., and Siegel, J. (1970). The behavioral effects of REM sleep deprivation in rats. *Psychophysiology* **6**, 550-560.
- Alnaes, E., Kaada, B., and Wester, K. (1973). EEG synchronization and sleep induced by stimulation of the medial and orbital frontal cortex in cat. *Acta Physiol. Scand.* **87**, 96-102.
- Amatruda, T., Black, D., McKenna, T., McCarley, R., and Hobson, J. A. (1974). The effects of carbachol injections at two brain stem sites. In "Sleep Research" (M. Chase, W. Stern, and P. Walter, eds.), Vol. 3, p. 38. Brain Information Serv., Los Angeles, California.
- Bast, T. H., and Loevenhart, A. S. (1927). Studies in exhaustion due to lack of sleep. I. Introduction and methods. *Am. J. Physiol.* **82**, 121-126.
- Berger, H. (1929). Über das Elektrenkephalogramm des Menschen. *Arch. Psychiat.* **87**, 527-570.
- Berger, R. J., and Meier, G. W. (1966). The effects of selective deprivation of states of sleep in the developing monkey. *Psychophysiology* **2**, 354-371.
- Blackman, R. B., and Tukey, J. W. (1958). "The Measurement of Power Spectra." Dover, New York.
- Bonvallet, M., Dell, P., and Hiebel, G. (1954). Tonus sympathique et activité électrique corticale. *Electroencephalogr. Clin. Neurophysiol.* **6**, 119-144.
- Branchey, M., Brebbia, D. R., Kohn, M., and Litchfield, D. (1974). Results of studies using an on-line automatic analyzer of sleep stages in the rat. *Electroencephalogr. Clin. Neurophysiol.* **37**, 501-506.
- Brazier, M. A. B. (1960). "The Electrical Activity of the Nervous System." Macmillan, New York.

- Bronzino, J. D., Morgane, P. J., and Stern, W. C. (1972a). EEG synchronization following application of serotonin to area postrema. *Am. J. Physiol.* **223**, 376-383.
- Bronzino, J. D., Brusseau, J., Morgane, P. J., and Stern, W. C. (1972b). Power spectrum analysis of EEG synchronization following application of serotonin to area postrema. *Ann. Biomed. Eng.* **1**, 246-253.
- Bronzino, J. D., Morgane, P. J., Stern, W. C., and Bottaro, S. (1972c). A new design for an exploring chemode. *Electroencephalogr. Clin. Neurophysiol.* **32**, 195-198.
- Brown, P., Maxfield, B. W., and Moraff, H. (1973). "Electronics for Neurobiologists." MIT Press, Cambridge, Massachusetts.
- Bureš, J., Petrán, M., and Zachar, J. (1967). "Electrophysiological Methods in Biological Research." Academic Press, New York.
- Caton, R. (1875). The electrical currents of the brain. *Brit. Med. J.* **ii**, 278.
- Chamblin, M. H., and Drew, W. G. (1971). The effects of lights-off stimulation on the circadian distribution of REM sleep in the cat. *Commun. Behav. Biol.* **6**, 111-114.
- Cooley, J. W., and Tukey, J. S. (1965). An algorithm for the machine calculation of complex Fourier series. *Math. Comput.* **19**, 267-301.
- Cordeau, J. P. (1970). Monoamines and the physiology of sleep and waking. In "Physiology of L-Dopa and Dopamine" (A. Barbeau and F. McDowell, eds.), pp. 369-383. Davis, Philadelphia, Pennsylvania.
- Cordeau, J. P., Moreau, A., Beaulnes, A., and Laurin, C. (1963). EEG and behavioral changes following microinjections of acetylcholine and adrenaline in the brain stem of cats. *Arch. Ital. Biol.* **101**, 30-47.
- Dement, W., Ferguson, J., Cohen, H., and Barchas, J. (1969). Nonchemical methods and data using a biochemical model: The REM quanta. In "Psychochemical Research in Man: Methods, Strategy, and Theory" (A. J. Mandell and M. P. Mandell, eds.), pp. 275-325. Academic Press, New York.
- Drucker-Colín, R. (1973). Crossed perfusion of a sleep inducing brain tissue substance in conscious cats. *Brain Res.* **56**, 123-134.
- Drucker-Colín, R. (1976). Is there a sleep transmitter? *Prog. Neurobiol.* **6**, 1-22.
- Drucker-Colín, R., Rojas-Ramírez, J., Vera-Trueba, J., Monroy-Ayala, G., and Hernández-Péón, R. (1970). Effect of crossed-perfusion of the midbrain reticular formation upon sleep. *Brain Res.* **23**, 269-273.
- Elul, R. (1968). Brain waves: Intracellular recording and statistical analysis help clarify their physiological significance. *Data Acquis. Process. Biol. Med.* **5**, 93. Proc. Rochester Conf.
- Elul, R. (1972). The genesis of the EEG. *Int. Rev. Neurobiol.* **15**, 227-272.
- Fencı, V., Koski, G., and Pappenheimer, J. (1971). Factors in cerebrospinal fluid from goats that affect sleep and activity in rats. *J. Physiol. (London)* **216**, 565-589.
- Fishbein, W. (1970). Interference with conversion of memory from short-term to long-term storage by partial sleep deprivation. *Commun. Behav. Biol.* **5**, 171-176.
- Fowler, M. J., Sullivan, M. J., and Ekstrand, B. R. (1973). Sleep and memory. *Science* **179**, 302-304.
- George, R., Haslett, W., and Jenden, D. (1964). A cholinergic mechanism in the brainstem reticular formation: Induction of paradoxical sleep. *Int. J. Neuropharmacol.* **3**, 541-552.
- Gevins, A., Yeager, C., Diamond, S., Spire, J., Zeitlin, G., and Gevins, A. (1975). Automated analysis of the electrical activity of the human brain. *Proc. IEEE* **63**, 1382-1399.
- Hernández-Péón, R. (1962). Sleep induced by localized electrical or chemical stimulation of the forebrain. *Electroencephalogr. Clin. Neurophysiol.* **14**, 419-430.
- Hernández-Péón, R., Chávez-Ibarra, G., Morgane, P. J., and Timo-Laria, C. (1962). Cholinergic pathways for sleep, alertness, or rage in the limbic midbrain circuit. *Acta Neurol. Latinoam.* **8**, 93-96.

- Hernández-Péón, R., Chávez-Ibarra, G., Morgane, P. J., and Timo-Iaria, C. (1963). Limbic cholinergic pathways involved in sleep and emotional behavior. *Exp. Neurol.* **8**, 93–111.
- Hernández-Péón, R., O'Flaherty, J., and Mazzuchelli-O'Flaherty, A. (1967). Sleep and other behavioral effects induced by acetylcholine stimulation of basal temporal cortex and striatal structures. *Brain Res.* **4**, 243–267.
- Hess, W. R. (1954). "Diencephalon—Autonomic and Extrapyramidal Functions." Grune & Stratton, New York.
- Jouvet, D., Vimont, P., Delorme, F., and Jouvet, M. (1964). Étude de la privation sélective de la phase paradoxale de sommeil chez le chat. *C. R. Séances Soc. Biol. Paris* **158**, 756–759.
- Jouvet, M. (1972). The role of monoamines and acetylcholine containing neurons in the regulation of the sleep-waking cycle. *Ergeb. Physiol., Biol. Chem. Exp. Pharmakol.* **64**, 166–307.
- Kales, A., Bixler, E. O., Tan, T., Scharf, M. B., and Kales, J. D. (1974). Chronic hypnotic-drug use. Ineffectiveness, drug-withdrawal insomnia, and dependence. *J. Am. Med. Assoc.* **227**, 513–517.
- Kavanau, J. L. (1962). An improved method for deprivation of sleep. *J. Appl. Physiol.* **17**, 375–377.
- King, C. D. (1971). The pharmacology of rapid eye movement sleep. *Adv. Pharmacol. Chemother.* pp. 1–91.
- Kohn, M., Litchfield, D., Branchey, M., and Brebbia, D. R. (1974). An automatic hybrid analyzer of sleep stages in the rat. *Electroencephalogr. Clin. Neurophysiol.* **37**, 518–520.
- Larsen, L. E., Rusconi, E. H., McNew, J. J., Walter, D. O., and Adey, W. R. (1972). A test of sleep staging systems in the unrestrained chimpanzee. *Brain Res.* **40**, 319–343.
- Levitt, R. A. (1966). Sleep deprivation in the rat. *Science* **153**, 85–87.
- Licklider, J. C. R., and Bunch, M. E. (1946). Effects of enforced wakefulness upon the growth and maze-learning performance of white rats. *J. Comp. Psychol.* **39**, 339–350.
- Lisk, R. D., and Sawyer, C. H. (1966). Induction of paradoxical sleep by lights-off stimulation. *Proc. Soc. Exp. Biol. Med.* **123**, 664–667.
- McKenna, T., McCarley, R., Amatruda, T., Black, D., and Hobson, J. A. (1974). Effects of carbachol at pontine sites yielding long duration desynchronized sleep episodes. In "Sleep Research" (M. Chase, W. Stern, and P. Walter, eds.), Vol. 3, p. 39. Brain Information Serv., Los Angeles, California.
- Magnus, J., Moruzzi, G., and Pompeiano, O. (1961). Electroencephalogram-synchronizing structures in the lower brain stem. *Ciba Found. Symp. Nature Sleep* pp. 57–85.
- Mark, J., Heiner, L., Mandel, P., and Godin, Y. (1969). Norepinephrine turnover in brain and stress reactions in rats during paradoxical sleep deprivation. *Life Sci.* **8**, 1085–1093.
- Mendelson, W. B., Guthrie, R. D., Frederick, G., and Wyatt, R. J. (1974). The flower pot technique of rapid eye movement (REM) sleep deprivation. *Pharmacol. Biochem. Behav.* **2**, 553–556.
- Mitler, M. M., and Levine, R. (1970). Sleep analysis and a simple technique for selective deprivation of low-voltage fast-wave sleep in a species of deermouse *P. M. Bairdi*. *Psychophysiology* **7**, 112–120.
- Monnier, M., and Schoenenberger, G. (1974). Neuro-humoral coding of sleep by the physiological sleep factor delta. In "Neurohumoral Coding of Brain Function" (R. D. Myers and R. Drucker-Colín, eds.), pp. 207–232. Plenum, New York.
- Monnier, M., Kalberer, M., and Krupp, P. (1960). Functional antagonism between diffuse reticular and intralaminar recruiting projections in the medial thalamus. *Exp. Neurol.* **2**, 271–289.
- Monnier, M., Schoenenberger, G., Dudler, L., and Herbert, B. Production, isolation and

- further characterization of the "sleep peptide delta." In "Sleep 1974" (P. Levin and W. P. Koella, eds.), pp. 41-46. Karger, Basel.
- Morden, B., Mitchell, G., and Dement, W. (1967). Selective REM sleep deprivation and compensation phenomena in the rat. *Brain Res.* **5**, 339-359.
- Morgane, P. J., and Stern, W. C. (1973). Effects of serotonin metabolites on sleep-waking activity in cats. *Brain Res.* **50**, 205-213.
- Morgane, P. J., and Stern, W. C. (1974). Chemical anatomy of brain circuits in relation to sleep and wakefulness. In "Advances in Sleep Research" (E. Weitzman, ed.), Vol. 1, pp. 1-131. Spectrum Publ., Flushing, New York.
- Morgane, P. J., Bronzino, J. D., and Stern, W. C. (1972). An exploring chemitrode device for direct chemical stimulation of the brain. *J. Appl. Physiol.* **32**, 138-142.
- Moruzzi, G., and Magoun, H. W. (1949). Brain stem reticular formation and activation of the EEG. *Electroencephalogr. Clin. Neurophysiol.* **1**, 455-473.
- Myers, R. D. (1967). Transfusion of cerebrospinal fluid and tissue bound chemical factors between the brains of conscious monkeys: A new neurobiological assay. *Physiol. Behav.* **2**, 373-377.
- Myers, R. D. (1974). "Handbook of Drug and Chemical Stimulation of the Brain." Van Nostrand-Reinhold, New York.
- Nauta, W. J. H. (1946). Hypothalamic regulation of sleep in rats. *J. Neurophysiol.* **9**, 285-316.
- Nauta, W. J. H. (1958). Hippocampal projections and related neural pathways to the mid-brain in the cat. *Brain* **81**, 319-340.
- Oswald, I. (1969). Human brain protein, drugs and dreams. *Nature (London)* **223**, 893-897.
- Pappenheimer, J. (1976). The sleep factor. *Scientific American* **235**, 24-29.
- Pappenheimer, J., Fencl, V., Karnovsky, M., and Koski, G. (1974). Peptides in cerebrospinal fluid and their relation to sleep and activity. In "Brain Dysfunction in Metabolic Disorders" (F. Plum, ed.), Vol. 53, pp. 201-210. Raven, New York.
- Pappenheimer, J., Koski, G., Fencl, V., Karnovsky, M., and Krueger, J. (1975). Extraction of sleep-promoting factors from cerebrospinal fluid and from brains of sleep-deprived animals. *J. Neurophysiol.* **38**, 1299-1311.
- Rechtschaffen, A., Dates, R., Tobias, M., and Whitehead, W. E. (1969). The effect of lights-off stimulation on the distribution of paradoxical sleep in the rat. *Commun. Behav. Biol.* **3**, 93-99.
- Rust, L. D. (1962). Changes in bar pressing performance and heart rate in sleep-deprived rats. *J. Comp. Physiol. Psychol.* **55**, 621-625.
- Sawyer, C. H., and Kawakami, M. (1959). Characteristics of behavioral and electroencephalographic after-reactions to copulation and vaginal stimulation of the female rabbit. *Endocrinology* **65**, 622-630.
- Skinner, J. E. (1971). Abolition of several forms of cortical synchronization during blockade in the inferior thalamic peduncle. *Electroencephalogr. Clin. Neurophysiol.* **31**, 211-221.
- Skinner, J. E., and Lindsley, D. (1967). Electrophysiological and behavioral effects of blockade of the non-specific thalamocortical system. *Brain Res.* **6**, 95-118.
- Sterman, M. B., and Clemente, C. D. (1962a). Forebrain inhibitory mechanisms. Cortical synchronization induced by basal forebrain stimulation. *Exp. Neurol.* **6**, 91-102.
- Sterman, M. B., and Clemente, C. D. (1962b). Forebrain inhibitory mechanisms: Sleep patterns induced by basal forebrain stimulation in the behaving cat. *Exp. Neurol.* **6**, 103-117.
- Stern, W. C., and Hartmann, E. L. (1972). Reduced amphetamine lethality following chronic stress. *Psychopharmacologia* **23**, 167-170.

- Stern, W. C., Forbes, W. B., and Morgane, P. J. (1974). Absence of ponto-geniculo-occipital (PGO) spikes in rats. *Physiol. Behav.* **12**, 293-295.
- Tauber, E. S. (1974). Phylogeny of sleep. In "Advances in Sleep Research" (E. D. Weitzman, ed.), Vol. 1, pp. 133-172. Spectrum Publ., Flushing, New York.
- Thompson, R. F., and Patterson, M. M. (1974). "Bioelectric Recording Techniques (Electroencephalography and Human Brain Potentials)." Academic Press, New York.
- Webb, W. B. (1962). Some effects of prolonged sleep deprivation on hooded rats. *J. Comp. Physiol. Psychol.* **55**, 791-793.
- Weitzman, E. D., Boyar, R. M., Kamen, S., and Hellman, L. (1975). The relationship of sleep and sleep stages to neuroendocrine secretion and biological rhythms in man. *Recent Prog. Horm. Res.* **31**, 399-441.
- Yamaguchi, N., Ling, G. M., and Marcynski, T. (1964). The effects of chemical stimulation of the pre-optic region, nucleus centralis medialis, or brain stem reticular formation with regard to sleep and wakefulness. *Recent Adv. Biol. Psychiat.* **6**, 9-20.

Chapter 8

Induction and Measurement of Tremor and Other Dyskinesias

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I. INTRODUCTION

The rationale for chemical stimulation of the central nervous system (CNS) has been presented earlier by Myers (1971). However, the use of

chemical stimulation of the brain for the purpose of producing tremors and other abnormal motor activities needs further elaboration.

In brief review, it is well known that diseases of the basal ganglia, the caudate, putamen, and globus pallidus (the striatum) in particular are associated with abnormal movement disorders such as Huntington's chorea. Other nuclei are also known to be involved in movement disorders. Among the best known examples are the loss of neurons from the zona compacta of the substantia nigra in Parkinson's disease, and the association of lesions of the subthalamic nucleus with ballismus (Brodal, 1969; Denny-Brown, 1962).

Early attempts to produce experimental models of these diseases in animals were largely unsuccessful. Lesions rarely produced reliable results; however, some investigators have been successful. Poirier (1960) developed a tremor model in monkeys by placing lesions in the midbrain, and involuntary movements were induced in cats by lesions in the caudate nucleus (Liles and Davis, 1969). Carpenter *et al.* (1950) produced involuntary movements in monkeys by means of lesions in the subthalamic area. However, these models have not been widely adopted by other investigators. A more promising approach to the problem has recently been introduced by Poirier *et al.* (1973), who have combined lesions and drug stimulation so as to consistently produce circus movements in cats.

Electrical stimulation of the extrapyramidal areas of the brain also proved to be rather unproductive (Brodal, 1969). Turning or circling was produced in cats by stimulation of the caudate nucleus, but tremor has rarely been produced by this approach.

A. Basis for Chemical Induction of Tremor

There are certain problems associated with the rather nonspecific stimulus of electrical current. Electrical stimuli may excite fibers of passage as well as neuronal perikarya near the electrode tip. In addition, electrical stimuli excite both excitatory and inhibitory systems. The technique of chemical stimulation of discrete loci in the CNS can overcome many of these problems and provide additional information about neural mechanisms. This is due to the fact that one can elect to stimulate specific neuronal receptor populations, i.e., cholinergic, dopaminergic, serotonergic, etc. Thus, Connor *et al.* (1966) and Dill *et al.* (1968b) were able to use cholinergic drugs to produce tremors in cats and rats, respectively, by injecting these drugs directly into the caudate nucleus.

B. Anatomical and Biochemical Considerations

A few years ago it was noted that there was a marked deficiency in the putative inhibitory neurotransmitter, dopamine, in the caudate and puta-

men nuclei of deceased patients with Parkinson's disease. It was also known that anticholinergic drugs were beneficial in the treatment of the disease. This led Barbeau (1962) to postulate a balance of cholinergic and dopaminergic activity in the striatum (caudate, putamen, and globus pallidus), a shift in favor of cholinergic activity would result in the symptoms of Parkinsonism, i.e., tremor and rigidity. This concept is an oversimplification, but it provides a basis for understanding the production of tremor by cholinergic stimulation of the striatum. It is interesting to note that Connor *et al.* (1967) could halt the cholinergic drug-induced tremors by subsequent injection of dopamine, apparently restoring the balance.

Recent evidence strongly suggests the presence of more than one type of dopamine receptor in this area (Klawans, 1973; Costall and Naylor, 1975) and a heterogeneous distribution of these receptors within the striatum (Cools, 1974). These complexities of the striatum provide a fertile field of study by means of chemical stimulation techniques. Judicious use of specific receptor agonists and antagonists coupled with careful evaluation of the resulting motor activities will greatly aid in understanding these neural mechanisms.

II. METHODS OF CANNULATION

The basic principles of stereotaxic technique (Pellegrino and Cushman, 1971) and methods of cannulation of the brain for chemical stimulation have been described earlier (Myers, 1971). Additional details and the specific approaches to the problem utilized in our laboratory will be presented in this chapter. The major difference in our technique is the use of the single cannula. No guide cannula is employed since all drugs are injected through this cannula, which is subsequently flushed with saline. This permits the use of cannulae of very small diameter, resulting in minimal damage to small neural structures.

A. Rats

For most of our work on the rat we have selected the central portion of the caudate/putamen nucleus, as this site is quite sensitive to cholinergic stimulation with carbachol. Coordinates for targets in the rat brain are taken from the atlas of the rat brain by Pellegrino and Cushman (1967) and are presented in Table I.

In preparation of the surgical procedure, the rat is anesthetized with sodium pentobarbital, 35 mg/kg, plus atropine sulfate, 10 mg/kg, given intraperitoneally (i.p.). Atropine is used to reduce secretions of the respiratory tract. We then routinely make a small cut in the ventral part of the ear with a pair of scissors as shown in Fig. 1. This aids in visualizing the

TABLE I
Atlas Coordinates (in millimeters) with Respect to Ear Bar Zero
as the Reference Point

Animal	Target	Anterior	Lateral	Vertical
Rat	Caudate-putamen	7.8	3.0	1.5
	Globus pallidus	6.6	3.5	0.0
	Nucleus accumbens	9.2	1.5	0.5
Cat	Caudate	14.0	5.0	16.5
	Globus pallidus	11.5	9.8	10.2
	Entopeduncular nucleus	11.0	5.5	8.5
Squirrel monkey	Caudate	13.0	2.8	18.3
	Medial globus pallidus	10.5	4.5	12.8
	Lateral globus pallidus	10.5	6.5	15.0
	Putamen	12.0	7.5	17.0

auditory meatus, thus speeding up correct insertion of the ear plugs. The rat is then placed in the stereotaxic machine (Kopf Model 900), a midline incision about 3 cm long is made, the skull scraped clean, and the bregma visualized. Most of our stereotaxic procedure utilizes the bregma as a point of reference, rather than reading ear bar zero each time. However, if problems occur this step should not be omitted (see Chapter 10). The

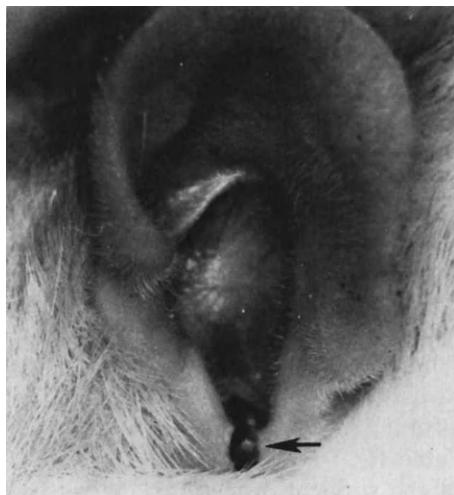


FIG. 1. A cut made in the ventral area of the rat's ear to expose the external auditory meatus is indicated at the arrow.

point of entry into the skull for each cannula is determined, and the holes for them are drilled with a Dremel moto-tool (drill bit size is No. 56). Four additional holes are drilled for the four screws (No. 0-80 $\frac{1}{8}$ -in. flat-head stainless steel) used to secure the dental acrylic and cannulae. These screws are then forced into the hole with a jeweler's screw driver. The diameter of the hole is slightly smaller than that of the screw, thus insuring that the screw is threaded into the bone of the skull.

Cannulae are cut from a length of 26-ga stainless steel tubing (Tubesales, Inc.), to the desired length with an abrasive disk and Dremel moto-tool. Stainless steel stylets of the type used to clean blood pipettes are cut at this time to match each cannula. The sides of the cannula are roughened at the point where it is anticipated that it will be secured by the acrylic cement.

The cannula is lowered into its proper position, and dental acrylic is applied to the cannula while it is secured by the stereotaxic holder. We use paper matches as brushes to apply the acrylic. The paper end of the match is dipped first in the liquid component of the acrylic, then into the powder. The resulting small blob of semiliquid acrylic is transferred to the cannula at the point where it enters the skull. Additional acrylic is painted onto the cannula and adjacent screws until the screws are covered with acrylic (Fig. 2). The mass of acrylic is smoothed by painting with the liquid or a very thin liquid-powder mixture. As work with the acrylic proceeds, acrylic builds up on the brush (hence, the advantage of using discardable matches). If more than one cannula is being implanted, care is taken not to cover over the drill holes for the remaining cannulae. As soon

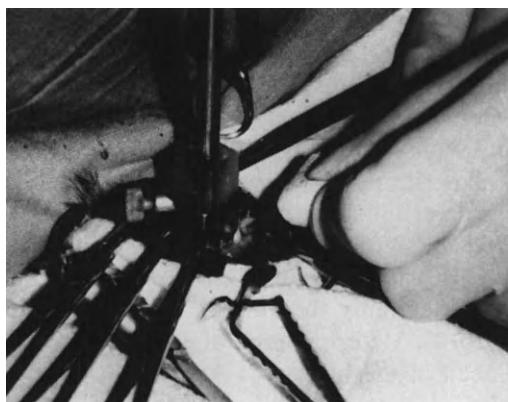


FIG. 2. Cannulation of the squirrel monkey brain. Dental acrylic is being applied to the cannulae with a paper match held with sterile gauze.

as the last application of acrylic to the cannula has hardened (about 3 min), the cannula holder is removed and the next cannula implanted. In order to protect the cannulae from bending and to prevent the loss of stylets, a cylindrical barrier made from a plastic embedding capsule (electron microscopic) or a short length of the barrel of a disposable syringe is placed over the cannula(e) and secured to the acrylic mass with additional acrylic. The wound is closed by pursing up the skin around the plastic cylinder with a few interrupted sutures (No. 00 silk suture). Stylets previously cut to match the length of each cannula are inserted while the animal is still in the stereotaxic instrument. The animal is now removed from the instrument and given postoperative care until recovery from the anesthetic is assured.

B. Cats

There are important areas of investigation that involve nuclei too small to be successfully cannulated in the rat. The entopeduncular nucleus is an excellent example. In the rat and cat, this nucleus is homologous to the primate medial segment of the globus pallidus, a critical component in the neural pathways under consideration. Thus cats are used for studies of such structures.

1. Preparation of the Animal

The cats are anesthetized with sodium pentobarbital, 25 mg/kg i.p., and placed in the stereotaxic machine (Kopf Model 1730 equipped with a Model 1760 micromanipulator) as described by Pellegrino and Cushman (1971). Dopram (doxapram hydrochloride) is kept on hand as a respiratory stimulant for use in case of pentobarbital overdose. The dosage is 4 mg/kg given intramuscularly (i.m.).

Sterile operating procedure is used throughout. The incision of the scalp is now made, oriented to best suit the needs of the preparation. We usually cannulate sites bilaterally, finding it best to make a transverse incision. The skull is scraped clean with a periosteal elevator, and a skull reference point is determined with the first cannula in the holder. Ear bar zero has been previously determined with this cannula. The skull reference for our work is usually equal to ear bar zero in the anteroposterior (AP) and lateral planes; only the vertical reading differs. This reference point is essential for the determination of ear bar zero values for cannulae subsequently placed in the holder.

2. Cannula Placement

Coordinates for targets in the cat brain are taken from the atlas of the cat brain by Snider and Niemer (1961) and are presented in Table I. The

positions where the cannulae enter the skull are determined, and appropriate holes for the cannulae and screws are drilled as already outlined for the rat (Section II,A). One should be certain that the second table of bone has been penetrated when drilling holes for the cannulae. Cannulation proceeds as outlined for the rat (No. 4-0 silk suture is used for cats). Recovery is carefully monitored.

C. Squirrel Monkeys

Squirrel monkeys, *Saimiri sciureus*, weighing between 400 and 700 gm and in good health are anesthetized lightly with sodium pentobarbital, 20 mg/kg i.p., so that lateral radiographs of the skull can be made. Ether should not be used for anesthetic purposes with these animals because respiratory tract production of mucus is stimulated, frequently resulting in strangulation. The Frankfurt plane is established, from which the height of the skull is determined. The skull length is also measured (making sure to correct for radiographic enlargement) and, if the skull size differs markedly from the atlas, proportioned corrections in the stereotaxic coordinates can be made. Coordinates for targets in the squirrel monkey brain are presented in Table I. We use *The Stereotaxic Atlas of the Squirrel Monkey Brain* by Emmers and Akert (1963).

Once the coordinates of the cannulation targets have been determined and several days recovery from any previous anesthesia allowed, one can proceed with the surgical preparation. Needless to say, sterile procedures should be used. A sterile instrument pack is prepared in advance. The pack should include the required surgical instruments, gauze sponges, towels, the precut cannulae and stylets, a few stainless steel screws for the skull, the drill bit, and a jeweler's screw driver. The drill bit may be sterilized in 1:750 zephiran chloride solution. The acrylic and paper match applicators are not sterilized, as experience has shown this to be unnecessary. The matches are held in sterile gauze (see Fig. 2).

Cannulae are placed as in the previous procedures. Sodium pentobarbital, 25 mg/kg i.p., is used for general anesthesia. Dopram is used as a respiratory stimulant, as just described for cats. No. 4-0 silk suture is used to close the wound, and sutures are removed after 2 weeks.

III. METHODS OF INTRACRANIAL INJECTION

A. Drug Solutions

Drugs are routinely dissolved in sterile saline in such concentrations that the desired amount of drug can be administered in a volume of 2–5 µl.

This can be a problem with drugs having low water solubility. Adjustments in pH may help, but extremes, such as pH less than 5.0 or more than 8.0, should be avoided. Propyleneglycol appears to be a safe organic solvent in some cases, but careful controls should accompany its use.

B. Injection Equipment

Injections are made by means of a micrometer-driven syringe. The micrometer drive is a Kopf Model 1202, and the syringe is the type used in gas-liquid chromatography (Hamilton). The syringe is filled with the drug solution; PE 20 polyethylene tubing is attached and filled with the drug solution. All air bubbles must be removed. The stylet is removed from the cannula, and the tubing attached. Injections are then made at a rate of 1 $\mu\text{l}/5$ sec up to a maximum of 5 $\mu\text{l}/5$ sec. One minute is allowed for equilibrium of intracranial pressure and diffusion of the solution into the neural tissue before the tubing is removed. A similar syringe filled with sterile saline is now attached, and 1 μl injected immediately, followed 1 min later with a second 1 μl injection. This procedure flushes the cannula dead space (1 $\mu\text{l}/20$ mm). The stylet is then replaced.

C. Calibration of Injection Equipment

The micrometer-driven syringe is calibrated by filling the syringe with distilled water, advancing the micrometer drive, and weighing the water delivered onto the weighing pan of an analytical balance. One microliter of distilled water weighs 1 mg. Micrometer readings yielding 10 μl or more are used in order to increase weighing accuracy and decrease the error due to loss by evaporation.

D. Animal Restraints

Rats are simply hand held during stylet removal and connection of tubing. The animal is placed in a small circular plastic open-top cage during the injection procedure and allowed freedom of movement. Cats are hand-held during the entire process.

Squirrel monkeys require a special restraint device during the intracranial injection process. The device used here is a shallow wooden V-shaped board lined with plastic sponge material and provided with two dividers, one to slip around the neck and one to restrain the animal at the waist. The wrists are tied to the sides of the device with cord. A monkey in the holder during intracranial injection is shown in Fig. 3.

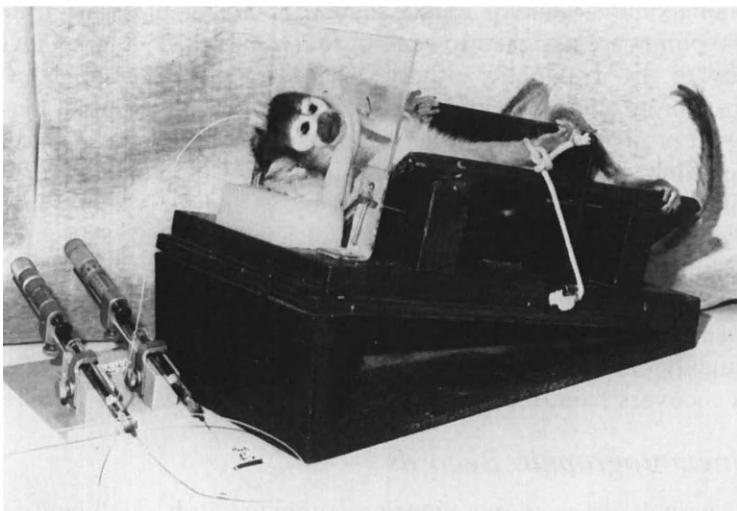


FIG. 3. Squirrel monkey being restrained during the intracranial drug injection process. One microinjector contains the drug; the other, physiological saline.

IV. EVALUATION OF MOTOR ACTIVITY IN RATS

A. Ranking of Dyskinesias in Rats

Immediately following intracranial injection each rat is placed in a clear plastic cage for observation. The time of the first appearance of abnormal motor activity, e.g., contralateral forelimb tremor, is recorded, and the latency from time of injection determined. Each type of activity and estimates of intensity are noted. Cholinergic stimulation of the striatum in rats typically produces contralateral limb movements (Dill *et al.*, 1968b). In addition to limb tremor, other dyskinetic events may occur. These are listed in the following paragraphs.

We have ranked the dyskinetic activity of rats according to the expression $R = S + I + D$, where rank R equals the arithmetic sum of S , the total number of dyskinetic signs, plus I , the rank of intensity, and D , the rank of duration. Each separate dyskinetic sign is given one point. Such signs include contralateral forelimb tremor, ipsilateral forelimb tremor, chewing, grimacing, neck tremor, contralateral hindlimb tremor, rearing, bilateral forelimb tremor, sialorrhea, neck torsion, and generalized convulsions. Thus S values can vary from 1 to 11.

Two points are assigned for each 5 min of duration up to 20 min, beyond

which a maximum value of 10 is assigned. Thus D values vary from 2 to 10. Two points are assigned to each of four subjectively ranked levels of intensity of the dyskinetic activity. These are (*a*) *minimal*, defined as weak tremor or other activity usually limited to a small group of muscles, e.g., wrist or digits; (*b*) *mild*, obvious brisk tremors involving muscles of an entire limb, neck etc.; (*c*) *moderate*, tremors or other activity of strong intensity; and (*d*) *severe*, characterized by strong to violent dyskinetic activity with periodic generalized convulsions. The values for I range from 2 to 8, giving a maximal possible rank of 29.

Animals displaying maximum rank values, i.e., convulsions for 30 min, are given sodium pentobarbital, 18 mg/kg i.p., for humanitarian reasons. This quickly blocks the dyskinesia, which is no longer present when the animal recovers from the anesthetic.

B. Cinematographic Records

We routinely make 16-mm cinematographic records of all new experiments and the consequent motor or behavioral activity. We have found film records to be indispensable in reviewing and analyzing motor phenomena. A small chalk board records pertinent data such as rat number, cannula location, drug and amount, time after injection, and date of the study. Two flood lights are used to light the subject and chalk board. Tri-X reversal black and white film is generally used to record rat activity, whereas color film is used for cats and monkeys. The camera is tripod mounted and set at 24 frames per second (sound speed). Sound speed permits us to slow down the action by projecting at silent speed, 16 frames per second, a helpful aid in studying complex movements.

C. Tremor Recording

Tremor rates and activities have been determined by means of a magnetic tremor recorder (Dill *et al.*, 1968a). This technique is based on the principle that an electromotive force (EMF) is generated in a conductor that cuts the lines of a magnetic field. In this case, the magnetic field is generated by a small magnet on the limb of the rat, and the conductor is a coil of copper wire placed under the rat's observation cage. The magnet is cut from a piece of rubber impregnated with magnetized metal particles such as found in cabinet door catches. The weight of the magnet is about 150 mg. Movement of the rat's limb and the magnet produces electrical signals in the coil associated with each change in direction of movement. These signals are amplified and fed into an ink-writing oscillograph (Gilson) and recorded on paper. An example of rat tremor is shown in Fig. 4. The amplifier used in this study (Gilson Module CH-CBPP) has a linear

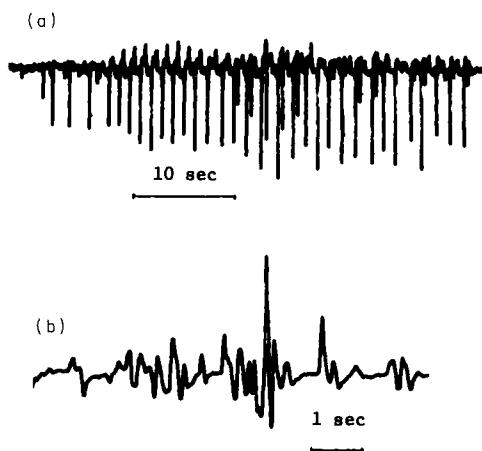


FIG. 4. Activity records made with the magnetic recorder described in the text. (a) A record made from the contralateral forelimb of a rat injected in the caudate/putamen with 1 μ g carbachol. The movement was a rhythmical flexion of the limb. (b) A record of irregular involuntary movement in the contralateral forelimb of a rat after intracaudate injection of 1 μ g carbachol.

response from dc to 17 Hz, beyond which attenuation begins. A 60-Hz signal is attenuated 20 dB, reducing the need for extensive shielding. Most movements occur at frequencies below 17 Hz.

The signals may also be fed into an automated counter designed to accumulate the signals and display or print out the sums at predetermined intervals. We currently use a modular Hewlett-Packard counter with visual display and a digital-to-analog converter (Module Nos. 5301A, 5300A, and 5311B, respectively). The signals from the converter are fed into an ink-writing oscillograph to produce histograms. Such units may be used for activity measurements. In short-term studies, i.e., tremor measurements where the animal is distracted by the ongoing dyskinesias, the magnet may be taped to the forelimb with cellophane tape. However, in long-term studies of normal animals it is necessary to implant the magnet beneath the skin. To reduce local tissue irritation we coat the magnets with nylon (Cutex clear fingernail polish). After the small incision is healed, experimental studies may begin. The advantage of this method is the freedom of movement allowed by absence of recording leads or radio telemetry devices on the animal.

D. Circling

Circling is another motor activity seen after chemical stimulation of the striatum, the globus pallidus in particular (Springer and Dill, 1975). Cir-

cling is evaluated by placing an affected animal in an open field and counting the complete circles made by the animal in one direction within a specified time interval, usually 2 to 5 min. Some rats are hypokinetic and may require a stimulus such as a tail pinch in order to get them moving. If they consistently turn to one side or the other without completing a circle, then the investigator may wish to refer to this as turning rather than circling. The important factor here is accurate description of the phenomenon and the conditions leading to its demonstration.

E. Catalepsy

Catalepsy is also seen in rats following chemical stimulation of the striatum (Springer and Dill, 1975) and nucleus accumbens (Dill *et al.*, 1975). Neuroleptic drugs and some cholinergic agents may produce this response. We test for catalepsy by first placing the forelimb on a wooden block 3 cm high. Retention of the limb in this position for a minimum of 10 sec is considered a minimal response. The hindlimb is also tested in this manner. If the animal is cataleptic by this test, we place the forelimb and subsequently the high limb on a block 9 cm high. A rat showing this response is seen in Fig. 5. Ranking methods can be devised based on the number of limbs showing cataleptic response, the height of the test block, and the length of time the limb remains on the block. Be sure to test both sides of the animal, as one side may be more involved than the other following a unilateral drug injection.

Evaluation of changes in muscle tone and reflex activity in the limbs of rats is difficult. One may flex and extend the limbs of the animal and

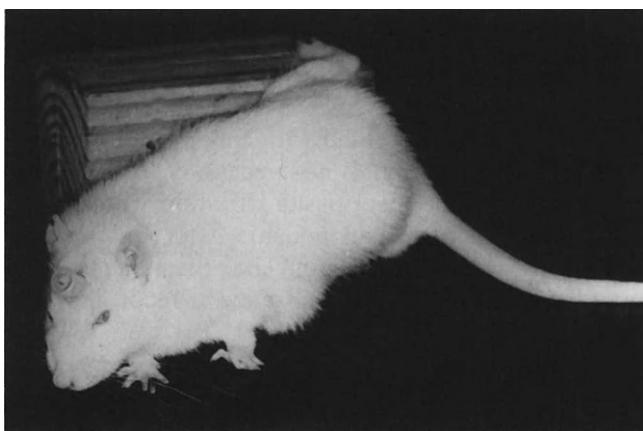


FIG. 5. A rat showing the signs of catalepsy. The block is 9 cm high.

assess the tone subjectively. However, struggling of an alert animal obscures much of the activity needed for evaluation. Quantitative electromyography would be most helpful, but these techniques are difficult to apply to rats (see Section V,A).

V. EVALUATION OF MOTOR ACTIVITY IN CATS AND SQUIRREL MONKEYS

Our experience with abnormal motor responses in cats induced by chemical stimulation of the CNS is rather limited, but a few remarks follow. Cholinergic stimulation of the caudate and entopeduncular nuclei has resulted in contralateral circling (Springer and Dill, unpublished results). Associated with the circling movements is a pronounced increase in muscle tone of the contralateral paraxial muscles, especially in the neck. These tonic changes are readily demonstrated by manual muscle testing, i.e., gently pushing the cat's head from side to side. Cinemato-graphic records have been made of this activity.

A. Quantitative Electromyography in Cats

The recording of electrical activity of a muscle associated with its contraction (electromyography) can greatly aid in the interpretation of changes in muscle activity produced by chemical stimulation of the striatum. However, interpretation of electromyographic (EMG) records is difficult for the novice and is rarely quantitative. We have overcome this problem in our laboratory in the following way.

Fine wire electrodes are prepared from No. 0025 polyurethane enamel wire by cleaning 1 mm from one end and bending a small hook (1 mm) at this end. The hook ends of two wires are inserted into a 27-ga needle. The needle is inserted into the muscle under study and then withdrawn, leaving the electrodes behind. At the end of the recording period, a sharp tug on the electrode straightens the hook and the wire is easily pulled out (Basmajian and Stecko, 1962).

The electrodes are implanted in the muscles to be studied and the cat placed in a canvas sling located in a shielded room. Shielded cables are attached to the electrode and fed into a Hewlett-Packard EMG high-gain amplifier (Model Sanborn 350-2700C). The EMG activity is displayed on oscilloscopes (Sanborn 780-6A Visoscope) for monitoring. The amplified EMG signals are then fed into a thermal stylus recorder (Sanborn 7700 series) and also into the Hewlett-Packard counter previously described with the tremor recorder. The digital-to-analog converter output is fed back into the recorder, where a second channel records a histogram of the EMG activity parallel to the recording of the spike activity (Fig. 6).

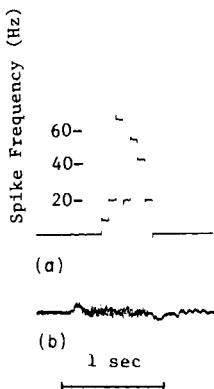


FIG. 6. (a) Parallel histogram of spike frequency produced by the equipment described in the text. (b) A burst of muscle activity recorded by the usual electromyographic methods.

Calibration of the histogram produces rapid quantitation of the EMG activity.

B. Squirrel Monkeys

Chemical stimulation of the striatum in squirrel monkeys has provided a promising model for the study of motor disorders. In our study of squirrel monkeys (Murphrey and Dill, 1972; Campbell and Dill, 1974), we have followed the same basic plan used for rat observations. Clear plastic observation cages 30 cm wide by 28 cm deep by 60 cm high were made and provided with a perch 22 cm from the floor. The perch consisted of a metal bar $\frac{1}{4}$ in. in diameter.

Immediately following intracranial injection, the animal is transferred to the observation cage and careful records made of abnormal movements. Cinematographic records are used extensively. In some cases the animal is periodically removed and manual muscle tests are performed (Campbell and Dill, 1974). These tests are carried out by placing the monkey on a padded test board where it is loosely restrained by a strap around the waist. This board is flat and has no dividers to hinder limb or trunk movement. The investigator normally wears welder's gloves to provide protection from the bite of these small monkeys.

Electromyographic records have been made from extensor and flexor muscle groups of the squirrel monkey during the involuntary movements produced by cholinergic stimulation of the caudate and putamen (Campbell, 1973). Fine-wire electrodes were implanted in the muscles of the awake animal. The animal was kept in the holder used for intracranial

injection. This procedure reduced the likelihood of the electrode's being pulled out by the animal.

C. Pharmacological Verification of Receptors

The specificity of the chemical stimulus can be verified by selecting specific receptor antagonists to the drug used. The blocking agent may be injected intracranially along with the chemical compound. Absence of the motor response previously shown to occur with the chemical stimulus alone is evidence for a specific response. If the blocking agent is poorly soluble, then it may be administered systemically prior to the intracranial injection or even during the resulting motor response. This approach has the advantage of flexibility in timing but lacks the specificity of site of action afforded by direct intracranial injection. It is possible to make two or more intracranial injections sequentially, i.e., agonist followed by antagonist, but one may encounter problems associated with injections of cumulatively large volumes. For a complete discussion of the subject of receptor identification, the reader should consult a text of pharmacology.

VI. VERIFICATION OF CANNULA PLACEMENT

Upon completion of the study, the precise location of the cannula tip should be determined histologically. The animal is anesthetized (ether for rats; pentobarbital for cats and squirrel monkeys), the thorax opened, and the arterial system perfused with formalin-ammonium-bromide fixative (Armed Forces Institute of Pathology, 1960). We use this fixative as it produces fixed brain tissue which sections very nicely. After a suitable volume of fixative has flowed through the animal, perfusion is stopped, the head is removed, all cannulae are removed with auto-mechanics pliers, and the brain is removed from the skull. The brain is stored in a labeled jar of fixative until required for further processing. We use routine paraffin methods and stain with a modified Wiegert stain or hematoxylin and eosin (Armed Forces Institute of Pathology, 1960).

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REFERENCES

- Armed Forces Institute of Pathology (1960). "Manual of Histologic and Special Staining Technics." McGraw-Hill, New York.

- Barbeau, A. (1962). The pathogenesis of Parkinson's disease: A new hypothesis. *Can. Med. Assoc. J.* **87**, 802-807.
- Basmajian, J. V., and Stecko, G. A. (1962). A new bipolar indwelling electrode for electromyography. *J. Appl. Physiol.* **17**, 849.
- Brodal, A. (1969). "Neurological Anatomy in Relation to Clinical Medicine." Oxford Univ. Press, London and New York.
- Campbell, K. M. (1973). Studies on the function of the globus pallidus in the squirrel monkey (*Saimiri Sciureus*). Ph.D. Thesis, Baylor University, Dallas, Texas.
- Campbell, K. M., and Dill, R. E. (1974). Trunk rigidity and limb hypotonia produced in squirrel monkeys by direct cholinergic stimulation of the globus pallidus. *Exp. Neurol.* **42**, 555-565.
- Carpenter, M. B., Whittier, J. R., and Mettler, F. A. (1950). Tremor in the rhesus monkey produced by diencephalic lesions and studied by a graphic method. *J. Comp. Neurol.* **92**, 293-332.
- Connor, J. D., Rossi, G. V., and Baker, W. W. (1966). Analysis of the tremor induced by injection of cholinergic agents into the caudate nucleus. *Int. J. Neuropharmacol.* **5**, 207-216.
- Connor, J. D., Rossi, G. V., and Baker, W. W. (1967). Antagonism of intracaudate carbachol tremor by local injections of catecholamines. *J. Pharmacol. Exp. Ther.* **155**, 545-551.
- Cools, A. R. (1974). The transsynaptic relationship between dopamine and serotonin in the caudate nucleus of cats. *Psychopharmacologia* **36**, 17-28.
- Costall, B., and Naylor, R. J. (1975). Neuroleptic antagonism of dyskinetic phenomena. *Eur. J. Pharmacol.* **33**, 301-312.
- Denny-Brown, D. (1962). "The Basal Ganglia and Their Relation to Disorders of Movement." Oxford University Press, London and New York.
- Dill, R. E., Dorman, H. L., and Nickey, W. M. (1968a). A simple method for recording tremors in small animals. *J. Appl. Physiol.* **24**, 598-599.
- Dill, R. E., Nickey, W. M., and Little, M. (1968b). Dyskinesias in rats following chemical stimulation of the neostriatum. *Tex. Rep. Biol. Med.* **26**, 101-106.
- Dill, R. E., Dorris, R. L., and Thonnard-Phillips, I. (1975). 3-Methoxytyramine, mescaline, *N,N'*-dimethyltryptamine: Effects of injection into the nucleus accumbens and neostriatum of rats. *Neurosci. Abstr.* **1**, 260.
- Emmers, R., and Akert, R. (1963). "A Stereotaxic Atlas of the Brain of the Squirrel Monkey *Saimiri Sciureus*." Univ. of Wisconsin Press, Madison.
- Klawans, H. L. (1973). The pharmacology of tardive dyskinesias. *Am. J. Psychiat.* **130**, 82-86.
- Liles, S. L., and Davis, G. D. (1969). Athetoid and choreiform hyperkinesias produced by caudate lesions in the cat. *Science* **164**, 195-197.
- Murphy, D. L., and Dill, R. E. (1972). Chemical stimulation of discrete brain loci as a method of producing dyskinesia models in primates. *Exp. Neurol.* **34**, 244-254.
- Myers, R. D. (1971). Methods for chemical stimulation of the brain. In "Methods in Psychobiology" (R. D. Myers, ed.), Vol. 1, pp. 247-280. Academic Press, New York.
- Pellegrino, L. J., and Cushman, A. J. (1967). "A Stereotaxic Atlas of the Rat Brain." Appleton, New York.
- Pellegrino, L. J., and Cushman, A. J. (1971). Use of the stereotaxic technique. In "Methods in Psychobiology" (R. D. Myers, ed.), Vol. 1, pp. 67-90. Academic Press, New York.
- Poirier, L. J. (1960). Experimental and histological study of midbrain dyskinesias. *J. Neurophysiol.* **23**, 534-545.
- Poirier, L. J., Langelier, P., and Boucher, R. (1973). Spontaneous and L-DOPA induced circus movements in cats with brain stem lesions. *J. Physiol. (Paris)* **66**, 735-754.

- Snider, R. S., and Niemer, W. T. (1961). "A Stereotaxic Atlas of the Cat Brain." Univ. of Chicago Press, Chicago, Illinois.
- Springer, P., and Dill, R. E. (1975). Motor effects of cholinergic stimulation of the globus pallidus in cats. *J. Neurosci. Res.* 1, 143-149.

Appendix

Cannula material

Tubesales
Rm. 308, Arlington Bank and Trust Bldg.,
Arlington, Texas 76010

Screws

Albany Products Co., Inc.
3046 W. 77th Street
Chicago, Illinois 60652

Microinjector

David Kopf Instruments
7324 Elmo St.
Tujunga, California 91042

Stylet material (blood pipettes) and polyethylene tubing

Clay-Adams, Inc.
141 East 25th St.,
New York, New York 10001

Syringe for microinjection

Hamilton Co.
P.O. Box 7500
Reno, Nevada 89502

EMG equipment and counter

Hewlett-Packard
195 Page Mill Rd.
Palo Alto, California 94306

Dental acrylic (Shur-Weld)

Modern Materials Manufacturing Co.
St. Louis, Missouri

Wire for EMG electrodes

Driver Harris
Harrison, New Jersey 07029

Chapter 9

Hormone Administration: Peripheral and Intracranial Implants¹

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I. INTRODUCTION

With the recent growth of behavioral endocrinology, hormone administration is increasingly used by psychologists and endocrinologists as an independent variable. Among the many possible methods of hormone treatment, the implantation of hormone depots is particularly useful—either systemic implants when stable blood levels are needed, or intracerebral implants when local effects in specific brain regions are desired.

While the methods discussed here are applicable to the administration of many hormones, drugs, and other agents, this chapter will concentrate on steroid hormones, the most widely investigated hormones in psychobiological studies. This account is based on experience in this laboratory with Long-Evans rats, except when otherwise stated. The hormone most often referred to is testosterone (T).

¹ Supported by grants MH 21178 and HD 0078.

II. SYSTEMIC ADMINISTRATION

A major objective of systemic hormone treatment is to produce blood hormone concentrations proportional to the dose administered. In addition to choosing a convenient and efficient method of implementing this aim, it is highly desirable, for optimal control of this experimental variable, to produce blood hormone titers which are stable over time.² A barrier to attainment of this objective is the fact that most hormones are rapidly metabolized and/or excreted once they enter the circulatory system.

A. Injections

Because of the lipid affinity of steroid hormones, they can be dissolved or suspended in oil and administered subcutaneously or intramuscularly. This method prolongs hormone effectiveness, but because of the short half-life of hormonal steroids in the circulation, either multiple injections or very large doses must be used to maintain effective blood levels throughout most of the day. The effectiveness of a single daily injection may also be prolonged by using synthetic analogs of the steroid hormones, many of which are more potent, specific in action, or convenient for administration than are the natural steroids. Chemically, these analogs range from minor modifications of the molecule to nonsteroidal compounds of great biological activity. However, the possibility always exists that the physiological or behavioral effects of the analogs may differ from those of the endogenous hormones.

The usual compromise between natural hormones and the more convenient or effective analogs is to use steroid esters. These esters are formed by condensation of a fatty acid (e.g., acetic or propionic) with one of the hydroxyl groups on the steroid molecule. The increased duration of effectiveness shown by these esters on subcutaneous injection is assumed to be due to their slow release from the oil vehicle and/or from fat stores in which they are retained. Apparently, the fatty acids are cleaved from the steroid molecule either before or shortly after entering the circulatory system. Consequently, the active form is presumably the unesterified steroid.

Although the duration of effectiveness of the esters is two to four times that of the natural hormones, the blood level following a single daily

² Intravenous injection, the method of choice when rapid effects are desired, is seldom used in psychobiology, because behavioral effects often require blood levels and/or testing which are continued for longer periods. This method requires the use of solvents such as alcohol or propylene glycol, or emulsifiers such as polysorbate 80 (Green *et al.*, 1970).

injection is not constant but is assumed to peak a short time after injection and then to decline slowly.

In order to validate this assumption for one hormone, we have recently measured plasma testosterone levels in castrated male rats following daily subcutaneous injections of testosterone propionate (100 $\mu\text{g}/100 \text{ gm}$ body weight in 0.1 ml sesame oil). As can be seen in Fig. 1, when blood samples were obtained at intervals after the first injection (4 hr after castration: day 0) a high peak in plasma T was found 3 hr after the injection. Testosterone levels just prior to each daily injection (i.e., at the lowest point) increased over the first 2 days and then remained fairly constant for the rest of the experiment (Fig. 2). Blood samples obtained at various times after the last injection (day 7) again showed a peak at 3 hr (Fig. 1). These data showing daily peaks in plasma T following subcutaneous injection of the hormone indicate that quantitative estimation of effective hormone titers resulting from such treatment is not feasible

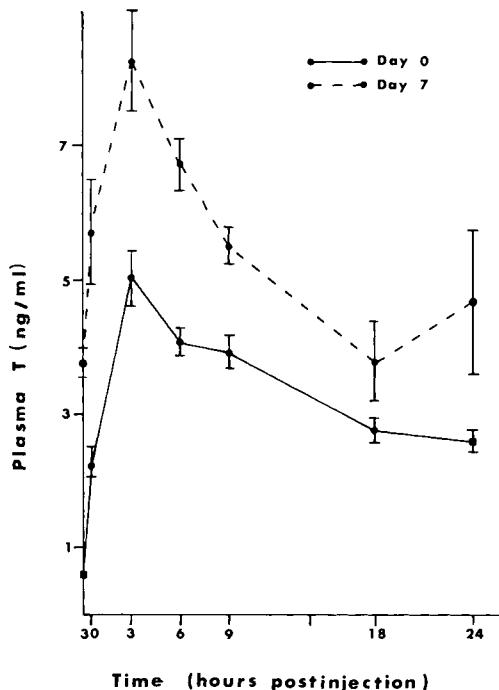


FIG. 1. Plasma testosterone (T) immediately following daily subcutaneous injections of T-propionate in adult castrated male rats: first injection (day 0), 4 hr after castration; eighth injection (day 7). Means \pm SE shown in this and subsequent figures; $N = 5$; 0 and 24 hr values also shown in Fig. 2.

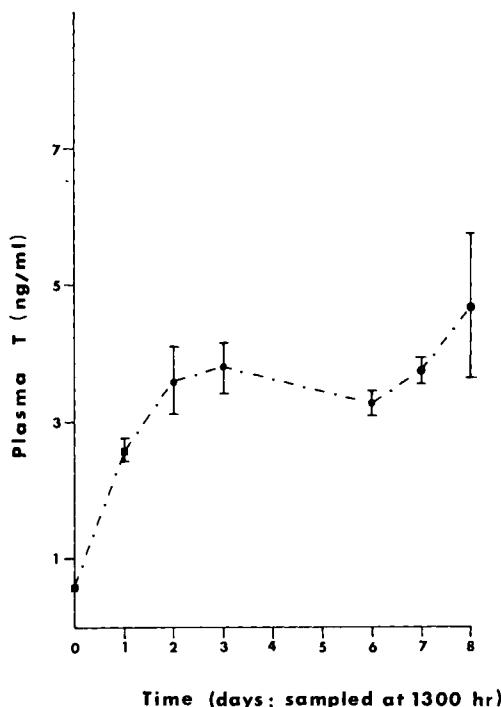


FIG. 2. Plasma testosterone 24 hr after daily subcutaneous injections of T-propionate in adult castrated male rats (first injection after day 0 sample); data from same animals as shown in Fig. 1.

because it is not known whether target tissues respond to the peak titer, the integrated daily level, some combination of both, or other possibilities.

To circumvent the problem of fluctuating blood levels induced by daily hormone injections, administration of large depots of crystalline steroid hormones can be utilized. While in the past the rather crude method of implanting pure hormone pellets has been used, the recent introduction of controlled release devices provides a convenient, sophisticated, and versatile technique for production of hormone levels which remain stable over long periods of time.

B. Controlled Release Capsules

1. Introduction

The production of certain polymers which are both compatible with tissue and permeable to drugs has led to the development of controlled-

release devices for steroid hormone administration. These fall into two basic categories: (a) those in which the hormone is embedded in the polymer (matrix type); and (b) those in which the hormone depot is surrounded by a polymeric membrane (reservoir type). Of the two, only the latter type is theoretically capable of maintaining a constant rate of hormone release for prolonged periods of time.

Although the capsule membrane can be made from a vast number of polymers and polymer combinations, silicone rubber has been found to be most useful with respect to steroid permeability and tissue biocompatibility. The availability of silicone rubber tubing [Silastic—i.e., polydimethyl siloxane with up to 25% siliceous earth fillers (Roseman, 1972)] has made the fabrication of controlled release capsules very easy and to date most studies have employed Silastic devices. This section will therefore concentrate on the methodology of Silastic reservoir-type controlled release capsules as used for the administration of steroids.

2. Factors Influencing the Rate of Steroid Release from Silastic Capsules

The basic theory of the operation of Silastic capsules is that the contained steroid dissolves in the capsule wall, possibly first in a very thin fluid layer on the inside of the capsule. It then diffuses through the wall in a nonsolvated form (passing down a thermodynamic activity gradient according to Fick's first law), and then leaves the wall to dissolve in the solution around the outside of the capsule. The rate at which the steroid leaves the capsule is directly proportional to the effective capsule surface area, the concentration gradient (inside versus outside the capsule), and the solubility of the steroid in Silastic; the rate is inversely related to capsule wall thickness. Of these factors, the solubility of the steroid in Silastic is often the rate-limiting factor. Although not an absolute relationship, it appears that the more polar a steroid is (i.e., the more hydroxyl groups it has), the lower will be its rate of release. For example, the polar adrenal glucocorticoids, corticosterone and cortisol, are not effectively delivered to the circulation from Silastic capsules due to their low solubility in Silastic as well as to the high blood levels required for physiological effects. Fabrication of capsules from polymers with permeability characteristics different from Silastic could provide a convenient method for these highly polar steroids.

3. Capsule Construction

We have found Silastic tubing (Dow Corning) measuring 3.18 mm o.d. and 1.57 mm i.d. suitable for the administration of androgens, progesterone, and estrogens to rodents and have used this size exclusively in the work presented in this chapter. The capsule is sealed at both ends with wood plugs which serve three functions: (a) they provide an area of

nonreleasing surface for adhesive application; (b) they impede the diffusion of steroid through the ends of the implant, thereby making the capsule surface area directly proportional to the length of the releasing surface; and (c) they provide an indicator of improper sealing of the implant in that they turn a darker color when wet (see Section II,B,5).

A cylindrical piece of wood (2.2-mm diameter Tomac or Puritan wood applicators) is inserted 5 mm into one end of a section of tubing which has been cut 10 mm longer than the desired length of releasing surface. The applicator is then cut flush with the end of the tubing by using a sharpened pair of wire cutters. The implant is filled either by tamping the crystalline steroid into the open end of the tubing or by using an 18-ga needle (bevel removed) as a funnel, depending on the physical properties of the steroid crystals used. The crystals should be packed firmly enough to give even contact with the internal capsule wall but without causing any deformation of the tubing. For steroid packing, we use an 18-ga spinal needle (trochar cemented in place and bevel removed). The implant is filled to within 5 mm of its open end and then occluded with another piece of dowling, which is again cut flush with the end of the tubing. We use only one applicator for each implant, so that the flat, manufactured ends contact the hormone.

After packing and insertion of the second wood plug, the capsule is wiped off with ethanol-dampened tissue paper and its ends are sealed with Medical Adhesive Silicone Type A (Dow Corning) and allowed to cure at room temperature for 24 hr. Implants are then given a second coat of adhesive, taking care on both applications not to allow the adhesive to contact the releasing surface. Following another 24-hr curing, implants are rinsed with approximately 30-ml absolute ethanol and wiped dry with a clean tissue to remove steroid adhering to the outside of the implant. The capsules are now ready for *in vitro* incubation (which will be described later).

As controls, we use empty capsules constructed in the same manner. Since steroid hormones can be biologically effective at very low concentrations (10^{-12} gm/ml) and implants are filled with milligram quantities (10^{-3} gm), extreme care is needed to avoid contaminating the control implants and/or steroid assays performed in the same laboratory, as well as cross contamination of capsules containing different steroids. For these reasons, individual sets of materials and equipment used for construction of capsules are kept separately for each hormone and for control implants. Implants are made in a specified location as far away from glassware storage as possible and on disposable absorbent pads which are replaced after each use. Laboratory coats are changed and hands thoroughly washed following construction of implants.

4. Filling Media

Since the rate of steroid release from a Silastic implant depends on the concentration gradient across the wall of the capsule, any filling medium which changes concentration over time will change the release rate of the implant. Use of hormone crystals with no filling medium (as already described) has been found to provide a release rate as good as or better (depending on the steroid used) than a suspension of crystals in sesame oil or liquid Silastic (Kratochvil *et al.*, 1970). Carboxymethyl cellulose was found to be an unacceptable filling medium, because it diffused out of the capsules implanted *in vivo*. However, the use of suspensions (crystals suspended in oil or water) would in most cases provide adequate release rates and is the method of choice when using expensive steroids. Wood plugs are not used when capsules are filled with solvent.

Another method of crystal loading is to dissolve the steroid in ethanol and then fill the tubing with this solution. After sealing the implant, the ethanol is allowed to evaporate through the capsule wall (it does so without modifying the membrane structure), thus depositing a layer of crystals on the inside of the implant. A possible disadvantage to the use of crystals for filling implants (Kratochvil *et al.*, 1970) is that, as the steroid is depleted from the capsule, parts of the inner wall may lose contact with steroid crystals, thus producing a fall in release rate. This criticism, we feel, would apply more to implants filled by the ethanol method than to those packed with crystals.

A filling medium such as cholesterol can also be used to reduce release rates by lowering the concentration gradient of hormone across the membrane. The use of the long-acting propionic ester of testosterone in Silastic capsules has been found to be more effective in stimulating biological responses than is testosterone (Berndtson *et al.*, 1974). This may also apply to other hormones and may be due to retention in body fat, producing a depot effect (see Section II,A), a greater release rate from Silastic capsules, or reduced clearance from the blood.

5. Incubation Procedure

If it is desirable to produce circulating hormone levels which are stable from the time of capsule implantation, an *in vitro* incubation must be used prior to *in vivo* implantation. As shown in Fig. 3, unincubated capsules implanted subcutaneously produce initial plasma testosterone levels three times as high as when incubated capsules are used. This is probably due to the fact that (even with the ethanol cleaning procedure), some hormone still remains on the outside of the implant and is rapidly delivered to the circulatory system. Even if perfect cleaning of the outside of the implant were feasible, however, preincubation would still be necessary because

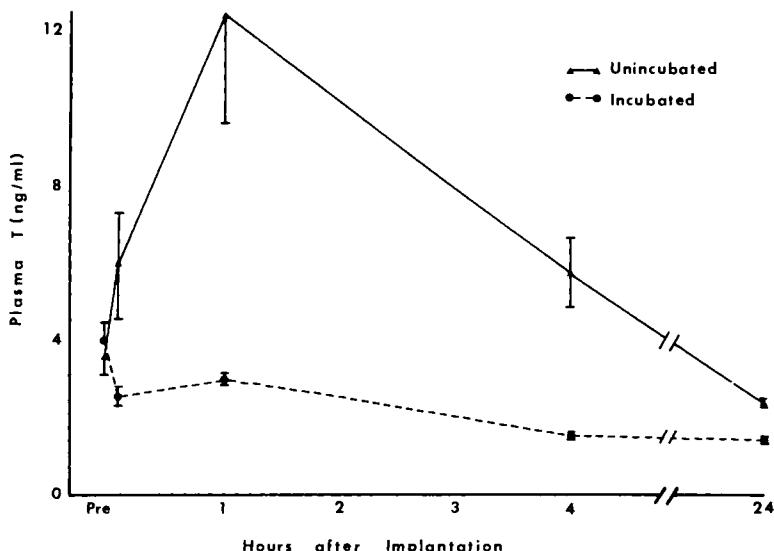


FIG. 3. Plasma testosterone in castrated adult male rats following subcutaneous implantation of incubated ($N = 10$) and unincubated ($N = 5$) Silastic T-filled 20 mm capsules. Pre: T level just prior to simultaneous castration and capsule implantation.

time is required to establish a steroid concentration gradient within the membrane. Thus, theoretically the implant would release hormones at a rate below the eventual steady-state level while the first steroid molecules are diffusing across the capsule wall ("lag time").

Our incubation procedure, established empirically, consists of placing implants in screw-top glass vials containing 0.01 M phosphate buffered saline at a volume of about 2 ml for every millimeter of capsule length. The vials are then placed in a water bath (preferably in a shaker bath with moderate agitation) at 37°C for 2 days, and the buffered saline is aspirated and replaced with fresh medium three times each day. To avoid cross-contamination, care must be taken to change the aspirating pipette when one is simultaneously incubating hormone-containing and empty (control) implants at the same time.

A final incubation medium change is effected just prior to implantation. This is to avoid transferring steroid-laden medium with the implant. Each capsule is then checked for leakage (as evidenced by darkening of the wood plugs), and those which have leaked are discarded.

6. *In vivo* Release Rate

The *in vitro* rate of steroid release from properly preincubated Silastic capsules remains constant until the steroid reservoir is depleted. How-

ever, *in vivo* release rates have been found to be only 50 to 80% of the *in vitro* rate, and furthermore the *in vivo* rate tends to fall slowly over time (Benagiano *et al.*, 1970).

We have measured plasma T levels in adult male rats (400–600 gm) at various times after castration and simultaneous implantation of Silastic capsules filled with testosterone. Table I shows the mean plasma T levels 3, 21, and 76 days following subcutaneous implantation of capsules of various lengths in groups of 9 to 11 castrated animals. The T levels produced by these implants were linearly related to capsule length, but by 76 days had dropped to approximately 75% of those found during the first 3 weeks. That this decline continues is evidenced by plasma levels measured in 8 rats 220 days after implantation, which were found to be around 40% of the values obtained during the first 2 weeks. It has been postulated that this apparent decrease in the *in vivo* release is due to encapsulation of the implant by fibrous connective tissue (Ermini *et al.*, 1973). Such a diffusion barrier would create a pocket of fluid around the implant containing high levels of the steroid (boundary layer), decreasing the concentration gradient across the membrane, and producing a drop in release rate. Arguments for the existence of such a boundary layer are (*a*) the observation in our laboratory that a transient rise in plasma testosterone follows the removal of subcutaneous capsules, and (*b*) the finding that long-term implants showing reduced release rates will release testosterone at the initial rates when removed and reimplanted in another animal (Benagiano *et al.*, 1970). Whether the site of capsule implantation can affect hormone release rate has not been extensively examined, but in rabbits it has been shown that subcutaneous and intraperitoneal placement of capsules filled with testosterone produced circulating levels which did not differ significantly (Stratton *et al.*, 1973).

TABLE I
Plasma Testosterone (ng/ml) 3, 21, and 76 Days after Castration and
Subcutaneous Implantation of Silastic T-Filled Capsules of
Various Lengths in Adult Male Rats^a

Time	Silastic capsule length (mm) ^b				
	2	5	10	30	60
Day 3	0.34 ± .03	0.58 ± .10	0.74 ± .04	1.87 ± .11	3.20 ± .13
Day 21	0.19 ± .02	0.55 ± .01	0.74 ± .03	1.98 ± .08	3.13 ± .19
Day 76	0.17 ± .02	0.31 ± .01	0.47 ± .03	1.36 ± .08	2.78 ± .21

^a N equals 9 to 11 per group.

^b Capsule is 3.18 mm o.d., 1.57 mm i.d.; crystal filled.

TABLE II

Plasma Testosterone 3 Weeks after Castration and
Subcutaneous Implantation of T-Filled Silastic Capsules
of Various Lengths in Adult Male Mice^a

Hormone	Silastic capsule length (mm) ^b					
	0	2	4	8	16	32
Plasma T (ng/ml)	<0.5	1.18 ± .08	1.79 ± .11	3.52 ± .28	6.72 ± .66	11.28 ± .03

^a N equals 2 or 3 per group.

^b Capsule is 3.18 mm o.d., 1.57 mm i.d.; crystal filled.

Table II shows another dose-response relationship for plasma T versus capsule length in the mouse (C57BL/6S). These data from the rat and mouse are presented to show that the rate of hormone release is closely related to the physical and chemical properties of the capsules. However, it must be recognized that appropriate capsule dimensions for the production of various physiological effects must be determined for each species, steroid, and biological end point examined. Among the other species studied are rhesus monkeys (Karsch *et al.*, 1973), rabbits (Stratton *et al.*, 1973), sheep (Cunningham *et al.*, 1975), and guinea pigs (Bullock, 1970). In large experimental animals such as dogs (Simmons and Hammer, 1973), achievement of requisite hormone levels may require implantation of such considerable total capsule length that is necessary to implant several capsules in each subject. An indication that the number of capsules implanted does not seem to influence the rate of delivery in rats is seen in Table III. Subcutaneous implantation of either one, two, or four individual capsules (with a total capsule length of 20 mm) produced equivalent plasma T levels.

TABLE III

The Effects of Subcutaneous Multiple or Single Silastic T-Filled
Capsules with the Same Total Capsule Length on
Plasma Testosterone in Castrated Adult Male Rats

Size of T capsules (mm)	Number of capsules	Total capsule length	Plasma T (ng/ml)	Number of animals
5	4	20	1.49 ± .04	14
8, 12	2	20	1.46 ± .10	5
20	1	20	1.32 ± .06	9

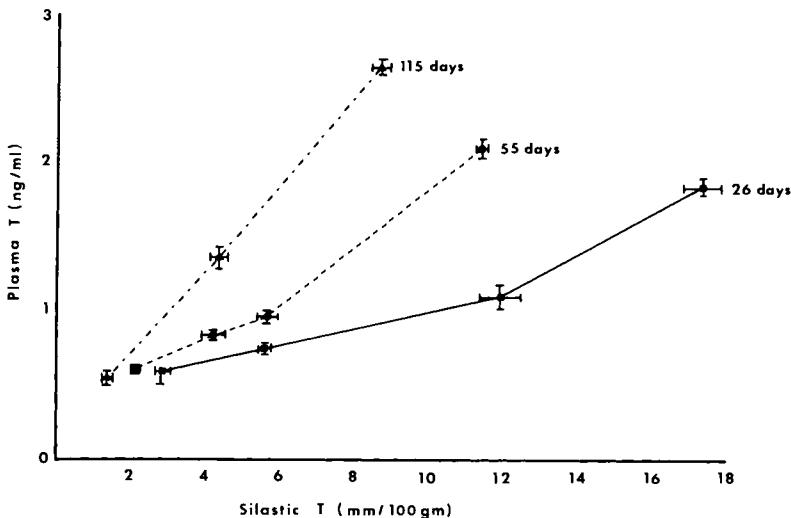


FIG. 4. Plasma testosterone in adult (115 day), pubertal (55 day), and immature (26 day) male rats 5 days after castration and subcutaneous implantation of Silastic T-filled capsules of various lengths. Data expressed in terms of capsule length (mm Silastic T per 100 gm body weight); vertical bars, SE plasma T; horizontal bars, SE capsule length; $N = 5$ or 6 per group.

Extrapolations made on the basis of size or age within a given species can also be hazardous, as seen from the results of the following study. Prepubertal, pubertal, and adult male rats were castrated and implanted with Silastic T-filled implants of various sizes. Five days later the animals were autopsied and blood samples collected and analyzed for testosterone. Figure 4 shows the dose-response relationship between Silastic length (adjusted for body weight) and plasma T. It is obvious that correction for body weight does not suffice for prediction of plasma T levels in younger animals by extrapolation of data collected on adults.

III. INTRACEREBRAL IMPLANTATION OF CRYSTALLINE STEROIDS

A. Introductory Remarks

The widespread view that the important influences of hormones on behavior are mediated in large part by direct action of the hormone on the brain has led to many attempts to localize specific intracerebral hormone-sensitive areas. Autoradiographic and radioactive uptake studies have shown that certain behavior-relevant hormones (particularly steroids) have specific patterns of uptake in the brain. However, the

concentration of radioactivity in a given brain area, or even the finding of specific receptors, says nothing about the relevance of that area for any particular behavioral or physiological hormone-dependent system, and the accumulation of radioactivity may even be devoid of functional significance.

The most direct approach to the problem of localization involves the effort to influence behavior by application of hormones to specific brain regions. In Volume 1 of this series, Myers (1971) has already discussed the rationale of chemical stimulation of the brain and various methods used. This section will be limited to a more detailed description of methods for intracerebral implantation of compounds in solid form and, more specifically, to crystalline steroids. Such implants have been used in a variety of species including the cat (Sawyer, 1963; Harris and Michael, 1964), rat (Lisk, 1960; Davidson, 1966), rabbit (Davidson and Sawyer, 1961a), dog (Davidson and Sawyer, 1961b), monkey (Everitt and Herbert, 1975), hamster (Ciaccio and Lisk, 1973-1974), chicken (Gardner and Fisher, 1968; Barfield, 1969), dove (Hutchison, 1967), duck (Gogan, 1968), and guinea pig (Morin and Feder, 1974) for behavioral studies as well as to localize brain sites of endocrine feedback mechanisms.

B. Types of Implants and Carrier Tubes

With the exception of the double cannula, we shall describe only simple implants easily constructed from materials readily available in the laboratory. These fall into three categories: (1) the pellet type, either (*a*) fixed tube or (*b*) tube removed; (2) the fused type; and (3) the double cannula type, fused or tamped. Implant types 1 and 2 are easily made and differ only in whether the steroid is ejected into the brain as a "pellet" (1), providing a larger surface area for absorption, or fused to the lumen of the tube (2), providing a smaller area. In type 1, the carrier tube may be cemented to the skull and left *in situ* throughout the experiment to help keep the steroid in place and aid localization (*a*); or it may be removed at the time of implantation (*b*). Double cannulae (type 3) are the most versatile, allowing for intracerebral application of hormones for any specified length of time, with minimal disturbance to the animal during placement and removal.

The carrier tubes are generally made from stainless steel hypodermic needle tubing, although glass capillaries have also been used (Döcke *et al.*, 1968). The steel tubing can be purchased in 2-3 m lengths and cut to size (about 4 cm lengths for hypothalamic implants in adult rats), with a sanding disk attached to a hobby motor (Dremel) tool. One end of the tube is sanded flat, and the edges are gently bevelled or rounded. The bore of

the tube is then reamed out using the tip of a disposable syringe needle of the same gauge as the implant tube. This is necessary because the walls of the tube tend to collapse at the point of cutting and sanding, and metal filings can occlude the tube.

1. Pellet Type

Pellet implants require a plunger or stylet for ejection of the pellet. This is made from stainless steel suture wire, whose dimensions are approximately 4 ga smaller than the gauge of the tubing, i.e., a 20-ga implant requires a 24-ga stylet. The stylet is inserted into the cut bevelled end of the carrier tube until it is just flush with the other end. A handle for ejection of the hormone pellet is formed by bending the wire at an angle greater than 90° to the tube. If the handle is insufficiently angled, the stylet may protrude beyond the tip of the tube and pellet placement will be inaccurate. The tube with stylet inserted is then sanded flat and the edges gently rounded so that the stylet is the exact length of the tube (excluding the handle). The stylet is removed and the bore of the tube is again reamed out to insure that the sanding has not reduced the inner dimensions. Because of the necessity for precise fitting, each tube should have its own stylet. Tubes and stylets are cleaned by immersion first in alcohol (90–100%) and then in ether, and then allowed to dry completely.

The empty tube with the stylet inserted is then weighed, filled with steroid, and reweighed to determine pellet weight. A highly sensitive Mettler microbalance or similar instrument is used since pellet weights are often 100 µg or less, and implants are always handled with forceps during filling and weighing. The tubes are filled by gently tapping them (with stylet raised) into a mound of crystalline steroid. Since different steroids have different packing characteristics, a variable number of taps will be required, e.g., testosterone propionate requires about one-third as many taps as cholesterol to make the same 200-µg pellet in a 20-ga tube. The stylet is then gently lowered until a few crystals are ejected to establish that the pellet is not packed so tightly as to hinder ejection. Implants are then carefully wiped off and reweighed. If the amount of hormone is too great, the stylet may be lowered further to eject some of the contained hormone, but if there is not enough, it is preferable to eject the pellet completely, raise the stylet, and start over again.

Finally, the tip is coated with a thin film of saturated sucrose solution. This temporarily seals off the carrier tube so that crystals are not knocked loose during the implantation procedure. If different compounds are being used, care must be taken to keep the sucrose solution for each compound separate in order to eliminate cross-contamination. The completed implants are allowed to dry overnight and then used.

For implantation into the brain, stereotaxic methodology is used (Pellegrino and Cushman, 1971). If the pellet implants are of the fixed type, they are secured to the cranium by means of dental cement and a stainless steel screw (Allen hex-socket set screw No. 0-8 NF \times 1/8 Allen point cup for rats), which serves as an additional point of solid attachment for the dental cement. The screw is inserted with a small Allen wrench into a hole drilled in the skull a short distance from the implant and threaded by means of a tap. All traces of periosteum should be removed from the skull, and bleeding points stopped before dental cement is placed around the implant, extending to cover the screw. The cement is allowed to dry thoroughly, and then the pellet is ejected by lowering the stylet with forceps, pushing or pulling gently down on the handle.

The excess tubing extending above the dental cement is cut off with a sanding disk, preferably while held by the electrode carrier. The rough surface of the tube is covered with another layer of dental cement, usually in the form of a knob to facilitate removal of the implant at the time of autopsy. The skin is sutured over the implant. Screws can be removed, cleaned with acetone, and saved for reuse. Pellet-type implants are not recommended for narrow tubing sizes (>22 ga) because of insufficient rigidity of the stylet wire for ejection of the pellet, but 24-ga tubing can be used with care.

Tube-removed pellet-type implants can be used when a large number of animals have to be prepared in a limited period of time or if presence of the tube is considered to interfere with the experiment. The carrier implant tube and stylet are removed from the head once the pellet has been ejected, but while the head is still held in the stereotaxic instrument, the hole in the cranium is filled with Gelfoam and the wound sutured. Care must be taken to prevent the pellet from being dragged back along the implant track as the tube is being removed. This can be done by turning the stylet handle back and forth several times once the pellet has been ejected, thereby knocking the pellet free from the tube. The empty tube should always be checked after removal for adhering crystals. In the rat, this type of implantation can be performed in about 10 min, whereas the fixed type requires 20–30 min due to the time required for drying of the dental cement. A modification of the tube-removed implant has been used in neonatal rodents (Nadler, 1972). The steroid is mixed with carbon in this case to facilitate localization of implant site in adulthood.

2. Fused Implants

Fused implants can be used only for compounds whose melting points are appreciably below their decomposition points, e.g., many steroids, but not proteins. The tubes are cut, sanded, cleaned, etc., as described

previously. A disposable capillary pipette is broken off so that the tip end forms a shallow scoop. A small amount of the crystalline steroid is taken up into the scoop and then passed quickly several times through or near a bunsen burner flame so that the steroid just melts.

The tubes are filled by placing one end into the molten steroid and allowing them to be filled by capillary action. If the color changes, this indicates decomposition and the hormone is discarded. The carrier tubes are allowed to cool and then cleaned carefully under a dissecting microscope. The outside of the tube is scraped with a razor blade and then wiped clean with ether, to ensure that the hormone is limited only to the inner diameter of the tubing. Sometimes implants are prepared by allowing the melted hormone to form a "blob" or sphere covering the tip of a tube (Lisk, 1969; Hutchinson, 1971; Everitt and Herbert, 1975). Another method which has been used (Dörner *et al.*, 1968) is to aspirate an alcoholic solution of the steroid into glass capillary tubes and implant following evaporation of the alcohol and recrystallization of the steroid.

Fused implants, even when cemented to the skull as described for the fixed-tube, pellet type (1a), may be removed after varying lengths of time, providing that no screw is used. The animal is placed back into the stereotaxic instrument, and the implant is removed by means of a direct pull on the cement "knob" with pliers or nail clippers. The hole in the cranium is filled with Gelfoam and the skin resutured.

3. Utilization of Implant Types and the Double Cannula

Fused implants can be used when more precise localization is required, since the area of brain tissue exposed can be precisely limited to that of the bore of the tubing. Pellet implants are used when relatively large doses are required for study of a diffuse neuronal system or an insensitive one. The dose of hormone administered can also be reduced by diluting the steroid hormone with cholesterol. This can be achieved by thorough mixing of the solid steroids with a mortar and pestle or by mixing after melting them together. These diluted mixtures can be used in both fused and pellet-type implants.

The double cannula is used when it is desired to expose brain tissue to hormone for limited periods of time in unanesthetized, "undisturbed" animals. It can be purchased or constructed as described by Myers (1971). This type of implant consists of an outer guide tube with dust cap and an inner hormone-bearing tube. The guide tube or cannula is placed stereotactically into the brain using the appropriate coordinates but is cut to a length such that it does not impinge upon the area to be studied. It must, however, be sufficiently long to serve as an accurate guide for the inner tube, which is inserted at a later time.

The outer cannula is secured to the skull by dental cement, and three hex-socket screws are triangulated around the cannula. Extreme care must be taken to ensure that the implant adheres firmly to the skull (i.e., a very dry skull and two or three coats of dental cement). The outer guide cannula is then closed by means of a brass screw cap. The animal is handled daily and the cap checked.

After recovery from surgery and habituation to handling and cap removal, the inner hormone-bearing tube can be inserted into the opened guide tube without anesthesia or apparent stress to the rat. Occasionally, guide cannulas become blocked, but they can be reopened using a hypodermic needle clean-out wire. Animals which appear to be traumatized by this procedure should not be used for experimental purposes that day. The inner cannula can be filled with crystalline hormones by tamping or by fusion. Once an inner cannula has been used for a particular hormone, it can be reused, but preferably only with the same hormone. The inner implant tube may be replaced at regular intervals to counter depletion of the hormone (Christensen and Clemens, 1974).

C. Problems of Interpretation

The major problems encountered when using intracerebral implants of crystalline compounds are (1) the extent of diffusion of the drug away from the site of implantation, and (2) the degree of "leakage" (i.e., absorption and release into the systemic circulation). In the case of steroid implants, crystalline ³H-labeled estrogen has been implanted into the hypothalamus of rats (Palka *et al.*, 1966) and ¹⁴C-labeled stilbestrol in cats (Michael, 1961) using 27-ga implants. These workers found that radioactive material diffused some 1-2 mm from the implant site, with radioactivity dropping sharply with increasing distance. Evidence of systemic leakage from intracranial implants has also been obtained in several studies (Kierniesky and Gerall, 1973; Lisk, 1967).

Implantation of the hormone into multiple brain sites around the suspected anatomical loci provides a control for diffusion. If diffusion is not a major factor only implants in the active site should be effective. This assumes, however, that no substantial systemic release occurs or, if it does, that leakage is equal from all brain sites. The problem of systemic release may be difficult to deal with, however, especially in light of recent observations showing that very low circulating levels of androgen from a constant release capsule can maintain sexual behavior in the male rat (Damassa *et al.*, 1977). In the past, evidence of stimulation of male (Davidson, 1966) or female (Harris and Michael, 1964; Lisk, 1962) reproductive structures, for instance, has been used as an index of peripheral

release of the gonadal steroids. With the exception of the vaginal smear (affected by estrogen), however, this approach is only applicable to a single estimation of hormone leakage at the time of autopsy (barring biopsies). Moreover, since weight changes in accessory sexual glands, uteri, etc., may not be obvious, histological studies may be required, producing results that are not readily quantifiable. This method also requires prior study of the relative sensitivities of the morphological response and the relevant behavioral response to the circulating hormone, for if the latter is more sensitive, behaviorally relevant titers of hormone may be missed by relying on morphological criteria.

In our laboratory, the question of systemic release has been extensively examined in regard to the localization of testosterone-sensitive brain areas and the restoration of sexual behavior in the castrated male rat, by the direct approach of sequential measurement of the circulating testosterone (Smith *et al.*, 1977). Using crystalline testosterone propionate implants (200 µg) of the fixed-tube, pellet type, it was found that there is differential hormone release into the circulatory system depending on implant location, with medial basal hypothalamic and pituitary implants resulting in substantially higher levels than those in other hypothalamic regions and cortex. Secondly, release was high in the first few days (in the low normal range for intact male rats) but then diminished rapidly to very low levels by 1 week (Fig. 5). When brain loci showing release rates which were not significantly different (cortex, anterior hypothalamus-medial preoptic area continuum, and posterior hypothalamus) were examined for the restoration of sexual behavior, systemic release did not correlate with the effectiveness of the brain implant (Fig. 6). Sexual accessory gland weights were not different from those of castrate controls at autopsy 4 weeks after implantation.

This study demonstrated that there is substantial systemic release of hormone, at least during the initial period, if testosterone implants of a size necessary to obtain behavioral and physiologic results (Davidson, 1966; Lisk, 1967) are used. The finding that release differs among different brain areas indicates that the differential effectiveness of implants in different regions is not a sufficient control for systemic release. While the conclusion that specific androgen-sensitive behavior-regulating areas exist in the rat brain was upheld in this study, the data suggest that systemic release, particularly in the early postimplantation period, is sufficient to constitute a possible source of experimental error. The findings of Kiernesky and Gerall (1973), for instance, suggest that *bilateral* testosterone propionate implants may "leak" sufficient hormone to preclude anatomic localization for sexual behavior.

In light of these considerations, it is strongly recommended that plasma

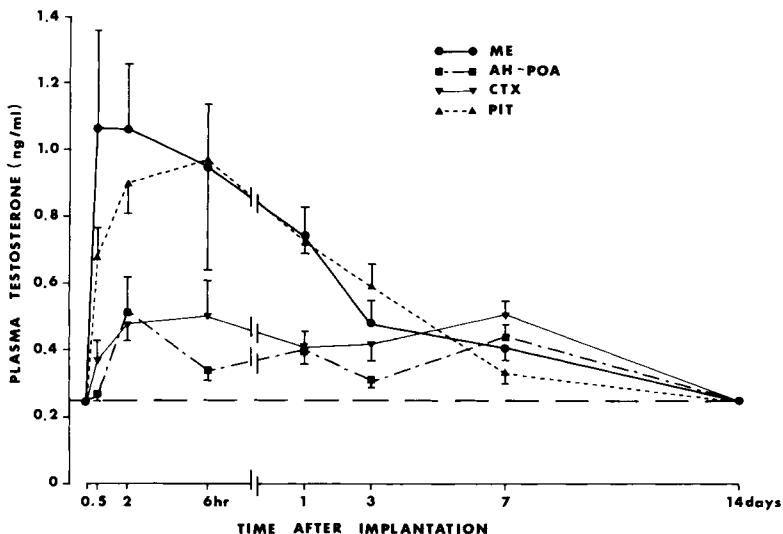


FIG. 5. Plasma testosterone following intracerebral implantation of T-propionate in various brain areas: ME, median eminence ($N = 11$); AH-POA, anterior hypothalamus-preoptic continuum ($N = 11$); CTX, cerebral cortex ($N = 14$); and PIT, anterior pituitary ($N = 7$). Broken line indicates lowest T concentration measurable in this experiment, based on the volume of plasma assayed.

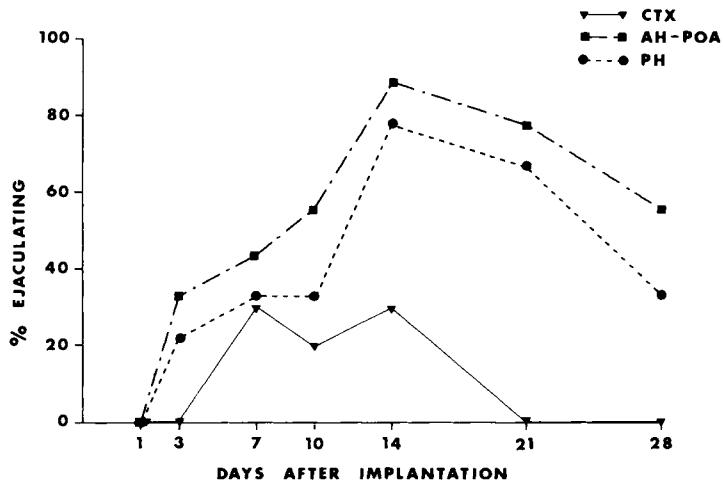


FIG. 6. Percentage of adult castrated rats showing complete ejaculatory patterns following intracerebral implantation of T-propionate. Mean plasma T levels were not significantly different between the groups at any time; plasma samples were obtained immediately after behavior tests were completed: CTX, cerebral cortex ($N = 10$); AH-POA, anterior hypothalamus-preoptic continuum ($N = 9$); and PH, posterior hypothalamus ($N = 9$).

levels of the agent implanted be monitored whenever possible, in order to obtain accurate data on systemic release in terms both of static release from different regions and of temporal patterns of release over the duration of the experiment. Attention should be given to the site of blood sampling for determination of released hormone, since we have found that jugular vein blood contains about twice as much testosterone as does cardiac blood in the initial period following testosterone implantation in the basal hypothalamus. This is presumably because intracranial drainage is mainly via the jugular vein (Smith *et al.*, 1977).

Finally, experiments should be designed so that the results are not confounded by the destruction or irritation of tissue by insertion of the implant. The possibility that an effect of hormone implantation may be due to production of a lesion is easily controlled by placement of implants containing an inert material, cholesterol being the agent of choice when steroid hormones are being studied. Somewhat more difficult to control is the possibility that optimal responses from hormone implants are precluded by damage to relevant behavior-controlling areas caused by the implant. Though intrahypothalamic testosterone implants have never produced full restoration of male sexual behavior, it has been shown that this was not due to lesion production by the implant. Moderate doses of systemically administered testosterone restore full patterns of sexual behavior in all subjects implanted with cholesterol in the relevant preoptic-anterior hypothalamic and posterior hypothalamic regions (Davidson and Bloch, 1969). Nevertheless, this possibility must be considered in other experiments where intracerebral hormone replacement partially or completely fails to restore behavior.

REFERENCES

- Barfield, R. J. (1969). Activation of copulatory behavior by androgen implanted into preoptic area of male fowl. *Horm. Behav.* **1**, 37-52.
- Benagiano, G., and Ermini, M. (1972). Continuous steroid treatment by subdermal polysiloxane implants. *Acta Eur. Fertil.* **3**, 119-130.
- Benagiano, G., Ermini, M., Chang, C. C., Sundaram, K., and Kincl, F. A. (1970). Sustained release hormonal preparations. 5. Absorption of 6-methyl-17 α -acetoxyprogesterone-4,6-diene-3,20-dione from polydimethylsiloxane implants *in vivo*. *Acta Endocrinol. (Copenhagen)* **63**, 29-38.
- Berndtson, W. E., Desjardins, C., and Ewing, L. L. (1974). Inhibition and maintenance of spermatogenesis in rats implanted with polydimethylsiloxane capsules containing various androgens. *J. Endocrinol.* **62**, 125-135.
- Bullock, D. W. (1970). Induction of heat in ovariectomized guinea pigs by brief exposure to estrogen and progesterone. *Horm. Behav.* **1**, 137-143.
- Christensen, L. W., and Clemens, L. G. (1974). Intrahypothalamic implants of testosterone or estradiol and resumption of masculine sexual behavior in long-term castrated male rats. *Endocrinology* **95**, 984-990.

- Ciaccio, L. A., and Lisk, R. D. (1973-1974). Central control of estrous behavior in the female golden hamster. *Neuroendocrinology* **13**, 21-28.
- Cunningham, N. F., Saba, N., and Millar, P. G. (1975). Release of progesterone from silicone rubber implants *in vitro* and the effects of the implants on plasma progesterone levels in sheep. *J. Reprod. Fertil.* **43**, 555-558.
- Damassa, D. A., Smith, E. R., and Davidson, J. M. (1977). The relationship between circulating testosterone levels and sexual behavior. *Horm. Behav.* In Press.
- Davidson, J. M. (1966). Activation of the male rat's sexual behavior by intracerebral implantation of androgen. *Endocrinology* **79**, 783-794.
- Davidson, J. M., and Bloch, G. J. (1969). Neuroendocrine aspects of male reproduction. *Biol. Reprod., Suppl.* **1**, 67-92.
- Davidson, J. M., and Sawyer, C. H. (1961a). Effects of localized intracerebral implantation of oestrogen on reproductive function in the female rabbit. *Acta Endocrinol. (Copenhagen)* **37**, 385-393.
- Davidson, J. M., and Sawyer, C. H. (1961b). Evidence for an hypothalamic focus of inhibition of gonadotropin by androgen in the male. *Proc. Soc. Exp. Biol. Med.* **107**, 4-7.
- Döcke, F., Dörner, G., and Voight, K.-H. (1968). A possible mechanism of the ovulation-inhibiting effect of chlormadinone acetate in the rat. *J. Endocrinol.* **41**, 353-362.
- Dörner, G., Döcke, F., and Moustafa, S. (1968). Differential localization of a male and a female hypothalamic mating centre. *J. Reprod. Fertil.* **17**, 583-586.
- Ermini, M., Carpino, F., Russo, M., and Benagiano, G. (1973). Studies on sustained contraceptive effects with subcutaneous polydimethylsiloxane implants. 3. Factors affecting steroid diffusion *in vivo* and *in vitro*. *Acta Endocrinol. (Copenhagen)* **73**, 360-373.
- Everitt, B. J., and Herbert, J. (1975). The effects of implanting testosterone propionate into the central nervous system on the sexual behaviour of adrenalectomized female rhesus monkeys. *Brain Res.* **86**, 109-120.
- Gardner, J. E., and Fisher, A. E. (1968). Induction of mating in male chicks following preoptic implantation of androgen. *Physiol. Behav.* **3**, 709-712.
- Gogan, F. (1968). Sensibilité hypothalamique à la testostérone chez le Canard. *Gen. Comp. Endocrinol.* **11**, 316-327.
- Green, R., Luttge, W. G., and Whalen, R. E. (1970). Induction of receptivity in ovariectomized female rats by a single intravenous injection of estradiol-17 β . *Physiol. Behav.* **5**, 137-141.
- Harris, G. W., and Michael, R. P. (1964). The activation of sexual behaviour by hypothalamic implants of oestrogen. *J. Physiol. (London)* **171**, 275-301.
- Hutchison, J. B. (1967). Initiation of courtship by hypothalamic implants of testosterone propionate in castrated doves (*Streptopelia risoria*). *Nature (London)* **216**, 591-592.
- Hutchison, J. B. (1971). Effects of hypothalamic implants of gonadal steroids on courtship behaviour in Barbary doves (*Streptopelia risoria*). *J. Endocrinol.* **50**, 97-113.
- Karsch, F. J., Dierschke, D. J., Weick, R. F., Yamaji, T., Hotchkiss, J., and Knobil, E. (1973). Positive and negative feedback control by estrogen of luteinizing hormone secretion. *Endocrinology* **92**, 799-804.
- Kierniesky, N. C., and Gerall, A. A. (1973). Effects of testosterone propionate implants in the brain on the sexual behavior and peripheral tissue of the male rat. *Physiol. Behav.* **11**, 633-640.
- Kratochvil, P., Benagiano, G., and Kincl, F. A. (1970). Sustained release hormonal preparations. 6. Permeability constants of various steroids. *Steroids* **15**, 501-511.
- Lisk, R. D. (1960). Estrogen-sensitive centers in the hypothalamus of the rat. *J. Exp. Zool.* **145**, 197-208.

- Lisk, R. D. (1962). Diencephalic placement of estradiol and sexual receptivity in the female rat. *Am. J. Physiol.* **203**, 493-496.
- Lisk, R. D. (1967). Neural localization for androgen activation of copulatory behavior in the male rat. *Endocrinology* **80**, 754-761.
- Lisk, R. D. (1969). Estrogen: Direct effects on hypothalamus or pituitary in relation to pituitary weight changes. *Neuroendocrinology* **4**, 368-373.
- Michael, R. P. (1961). An investigation of the sensitivity of circumscribed neurological areas to hormonal stimulation by means of the application of oestrogens directly to the brain of the cat. *Reg. Neurochem. Reg. Chem., Physiol. Pharmacol. Nerv. Syst., Proc. Int. Neurochem. Symp. 4th, 1960* pp. 465-480.
- Morin, L. P., and Feder, H. H. (1974). Inhibition of lordosis behavior in ovariectomized guinea pigs by mesencephalic implants of progesterone. *Brain Res.* **70**, 71-80.
- Myers, R. D. (1971). Methods for chemical stimulation of the brain. In "Methods in Psychobiology" (R. D. Myers, ed.), Vol. 1, pp. 247-280. Academic Press, New York.
- Nadler, R. D. (1972). Intrahypothalamic exploration of androgen-sensitive brain loci in neonatal female rats. *Trans. N.Y. Acad. Sci.* **34**, 572-581.
- Palka, Y. S., Ramirez, V. D., and Sawyer, C. H. (1966). Distribution and biological effects of tritiated estradiol implanted in the hypothalamo-hypophysial region of female rats. *Endocrinology* **78**, 487-499.
- Pellegrino, L. J., and Cushman, A. J. (1971). Use of the stereotaxic technique. In "Methods in Psychobiology" (R. D. Myers, ed.), Vol. 1, pp. 67-90. Academic Press, New York.
- Roseman, T. J. (1972). Release of steroids from a silicone polymer. *J. Pharmacol. Sci.* **61**, 46-50.
- Sawyer, C. H. (1963). Induction of estrus in the ovariectomized cat by local hypothalamic treatment with estrogen. *Anat. Rec.* **145**, 280 (Abstr.)
- Simmons, J. G., and Hamner, C. E. (1973). Inhibition of estrus in the dog with testosterone implants. *Am. J. Vet. Res.* **34**, 1409-1419.
- Smith, E. R., Damassa, D. A., and Davidson, J. M. (1977). Plasma testosterone and sexual behavior following intracerebral implantation of testosterone propionate in the castrated male rat. *Horm. Behav.* In press.
- Stratton, L. G., Ewing, L. L., and Desjardins, C. (1973). Efficacy of testosterone-filled polydimethylsiloxane implants in maintaining plasma testosterone in rabbits. *J. Reprod. Fertil.* **35**, 235-244.

Chapter 10

Chronic Methods: Intraventricular Infusion, Cerebrospinal Fluid Sampling, and Push-Pull Perfusion

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I. INTRODUCTION

In some instances, the fundamental mechanism underlying a normal or pathological process can be understood only if the development of a biochemical change in the nervous system is traced over time. Currently, the issue of chronicity has become vital to many questions raised in the neurosciences. If a scientist utilizes a procedure that allows the investigation of the chronic rather than acute preparation, progressive, temporal influences of neurochemical factors in the function of a given system in

the brain may be ascertained. This chapter describes three of these approaches.

With one method, the metabolic and chemical dynamics of the brain are altered directly over some specified period of time, without undue influence on organ systems outside of the brain. This is achieved through the method of continually infusing a solution containing a drug or other substance into the cerebral ventricle of the conscious animal. Two other approaches make use of chronic sampling techniques. Samples of cerebrospinal fluid (CSF) can be collected repeatedly at given intervals. Alternatively, samples of perfusate can be obtained from circumscribed regions of the brain. In these ways, assays of endogenous activity of specific humoral factors in the brain can be undertaken repeatedly under different experimental conditions.

A. Rationale

The principle of chronicity in brain research has several points of justification. Perhaps foremost is the fact that the animal can be used as its own control. This is particularly advantageous when difficult techniques are used, surgery and histology are costly, and the species (e.g., primate) is of limited availability. To compare values of a given substance under both control and experimental conditions in the same brain may provide unequivocal results. If the test animal is killed for the purpose of brain assay, the opportunity for such a comparison is lost.

Similarly, metabolic changes in the brain associated with prolonged drug treatment can be assessed at regular intervals. Thus, the accumulated effects of a given drug can be identified that are not so easily recognized following a single injection of a drug. Further, by the chronic intracranial delivery of a compound, the pathological development of a neurological disorder may be simulated experimentally. Finally, the repeated sampling of the activity of a chemical substrate in a particular area of cerebral tissue, following a lesion or other manipulation, can help to differentiate the neurochemical basis of one structure from another.

B. Specific Precautions for Chronic Experiments

Many pertinent considerations are of concern when an animal is maintained for extensive investigation over a prolonged period. Health is a principal one. In our laboratory, neither surgery nor an experiment is undertaken if signs of disease or ill-health are manifest. In the rat, a consistent pattern of food and water intake is a major index of general physical condition. Growth rate, as reflected by steady increases in body weight and the maintenance of a set weight in an adult, are also indicators

of the animal's state of health. Respiratory ailments, whether chronic or acute, rule out at once the usage of a rat in an experiment.

In the cat, the presence of ticks, fleas, bloody or mucus-laden stools, ear mites, parasitic worms (sometimes diagnosed by abnormal protraction of the cat's nictitating membrane), mange, or bacterial disease also negate the usage of the animal until the specific ailment is remedied.

Complementing physical considerations is the factor of demeanor or general behavior. For example, in the infrahuman primate, the level of aggressiveness, the frequency of threat behavior, and the ease with which vocalization and threat are elicited are observed carefully. In a restrained monkey, close watch for abrasions or lesions is paid to areas of skin that are in contact with the chair. Also, the appearance of the animal's fur, frequency of defecation and urination, and condition of the stools are likewise monitored for sudden changes.

1. *Surgical Aspects*

During the early stages of surgical anesthesia, a hypothermia of 33° to 35°C may be induced intentionally for hemostasis by not insulating the animal from the cool surface of a surgical table or stereotaxic instrument. As surgery nears completion, the animal is rewarmed by a heating pad placed underneath its trunk. If the dose of the anesthetic given is sufficiently low, shivering, vasoconstriction, and other thermoregulatory responses serve to return the animal's body temperature rapidly to a normal level. One advantage of intense postoperative heat production seems to be the rapidity of the animal's recovery from surgical anesthesia, due presumably to the rate at which the anesthetic is metabolized.

Aseptic precautions should always be exercised in the chronic preparation. Even in certain strains of laboratory rat, heretofore thought to be resistant to bacterial infection, cerebral tissue may culture bacteria following an intracranial implant. Whether or not this decline in resistance to pathogens is due to persistent genetic inbreeding, surgical instruments should be sterilized and the cannula or electrode kept in a Zephiran or other sterilizing solution.

Rigid aseptic procedures are mandatory during neurosurgery with the cat or monkey. These include the use of a head cap, mask, sterilized gown and drapes, and sterilized instruments in the operative field (see Meyer and Meyer, 1971).

2. *Postoperative and Temporal Factors*

For the first 24 to 48 hours after a cannula or chemitrode is implanted, the blood-brain barrier may be partially penetrable at the site of tissue damage [see review in Myers (1975)]. During this period, an antibiotic that does not ordinarily cross the barrier may be efficacious at the site of the

implant. By lowering a microinjection needle through the cannula or by perfusing a tissue site once a day, the integrity of the blood-brain barrier is not reinstated. Postoperatively, then, antibiotic therapy can be continued with benefit.

An important point in all experiments utilizing chronic preparations is the frequency of usage. Nearly every investigator (including the author) has typically believed that injections or perfusions of brain tissue done at frequent intervals are contraindicated because of general trauma to the tissue. In the last 2 years, we have found that the experimental usage of an animal on a daily or bidaily basis is not injurious. In fact, on histological examination, the deposition of glia or unwanted fibrous material around the site of an implant is somewhat retarded. More important, the tissue surrounding the tip of the implanted cannula retains its reactivity to a chemical substance. Overall, a specific site in the brain can be tested frequently.

II. METHOD OF CHRONIC INTRAVENTRICULAR INFUSION

Historically, the method of injecting a drug or other compound directly into an animal's cerebral ventricle has been a powerful tool. The intradisciplinary interface between the pharmacology of the central nervous system (CNS) and the physiology or behavior of an animal has been broadened through usage of this method [see reviews in Schain (1960); Feldberg (1963); Winterstein (1961); Myers (1974)]. New theories have evolved concerning the CNS mechanisms that control respiration, drinking, feeding, emotional behavior, body temperature, hormone release, autonomic function, and motor responses.

In the early 1960s, the method of acute intraventricular injection was taken one step further. A procedure was devised whereby a chemical could be injected repeatedly into the cerebrospinal fluid, around the clock, for days on end (Myers, 1963). In this way, the chemical milieu of the structures that form the walls of the cerebral ventricles could be chronically altered in a controlled fashion. The rationale essentially is that protracted rather than acute effects of a centrally active compound may be examined. Not surprisingly, some substances require hours or even days to exert their effect upon a metabolic process.

Until recently, the chronic method of intracerebral drug delivery was used primarily to study drug-seeking behavior in the rat and monkey (cf. Myers *et al.*, 1972a,b). For example, compounds infused into the cerebral ventricle over many days were found to alter the selection and ingestion of a solution of alcohol, water, or other fluid. Even though an intraventricular injection is made automatically every 15 min, day and night, an

animal can be maintained in perfect health for as long as 60 days (Myers and Veale, 1972).

The following sections describe: the procedure for setting up a chronic infusion system, special surgical considerations, the maintenance of ventricular patency, how the infusion site is verified, and practical problems and pitfalls.

A. Selection of Cannula and Swivel

A concentrically designed cannula is the most satisfactory for the chronic delivery of a chemical substance into the ventricle.

1. Rat

An early design of a system for chronic intraventricular infusion in the rat is illustrated in Fig. 1. A 26-ga stainless steel syringe needle is im-

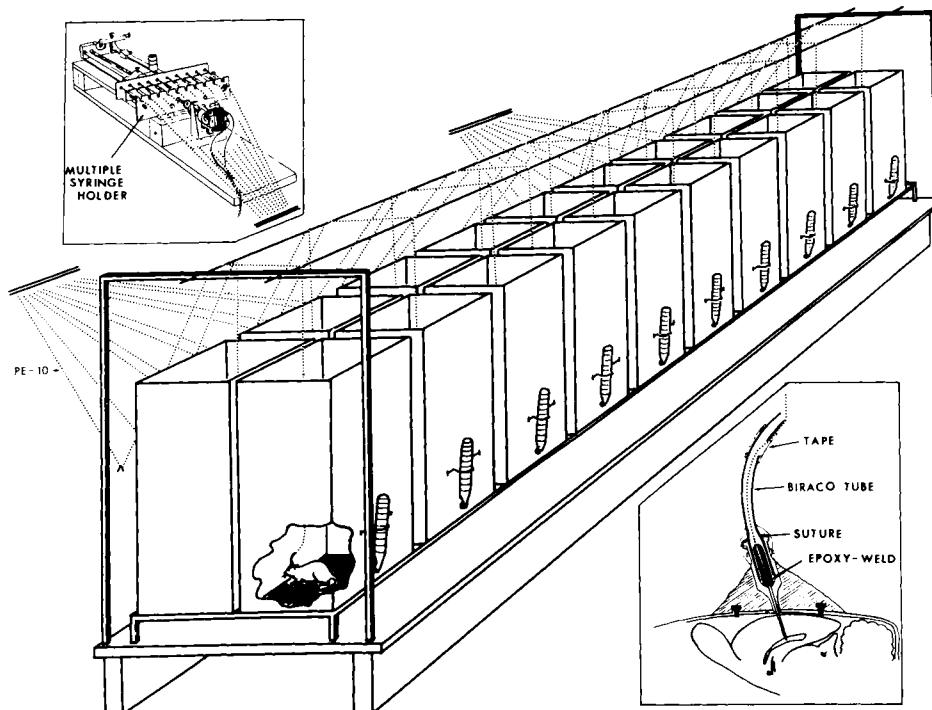


FIG. 1. A series of open-top animal chambers for chronic intracranial infusion of fluids through PE-10 tubing. *Left inset:* one of the infusion pumps fitted with ten 0.25-ml syringes, and programmed to deliver 2 μ l of fluid intracranially every 15 min. *Right inset:* details of skull-tubing junction providing flexible yet durable connection for long-term studies. (From Myers, 1963.)

planted with the tip resting in one lateral ventricle and held in place by cement and stainless steel anchor screws inserted into the rat's calvarium (Myers *et al.*, 1967). A 32-ga injector needle attached to polyethylene (PE-10 or PE-20) tubing is inserted into the guide needle to the depth of the ventricle (Fig. 1, *right*). To protect the junction of the needle and the PE tubing, larger, flexible tubing (Biraco) is inserted into the hub of the syringe needle and tied to the tip of the needle with ordinary 2-0 suture. Then the PE tubing is threaded through a small hole in the caudal portion of this sleeve and held in place with surgical adhesive tape. Damage to the cannula implant is thus avoided when the rat tucks its head under its body as it sleeps. Also, when the animal washes and grooms, the outer, protective tubing prevents the rat from grasping the thin PE tubing that will carry the drug solution. As long as they tend to retain their shape, several kinds of resilient plastic tubing can be used satisfactorily to hold the PE tubing away from the animal's head (Fig. 1, *right*).

In a preparation in which the drug solution has to be replaced once or twice a day, the cannula shown in Fig. 2 is more suitable because the injector needle can be easily removed. A plastic base is fashioned from the needle end of a 1-ml disposable tuberculin syringe (Becton-Dickinson). The outer surface of this base is threaded with a $\frac{3}{16}$ -in. dye. A length of 22-ga stainless steel needle tubing is mounted inside of the plastic base to serve as a guide tube. The guide tube is then cemented in place with a non-tissue-reactive epoxy resin (Araldite). The protective cap of the syringe is used also to protect the cannula; the inside of this cap is simply threaded with a $\frac{3}{16}$ -in. tap.

The injector cannula is a 28-ga length of needle tubing pushed through an undersized hole drilled in another plastic syringe cap. The cap is similarly threaded with a $\frac{3}{16}$ -in. tap and then screwed onto the base. In its final position, the injector needle should extend 1 mm beyond the tip of the guide tube, and thus penetrate the ventricle. Silastic, Biraco, or other flexible tubing is fitted onto the cap so that the needle and PE tubing are protected. As already described, the PE-10 or PE-20 tubing is threaded through a small hole cut in the caudal portion of the protective tubing and held in place by adhesive tape (Fig. 2).

An important feature of the cannula configuration is the extent to which the injector cannula is screwed down tightly to the cannula base. The tip of the injector cannula should extend sufficiently far so as to reach the lumen of the ventricle. It is best to construct two caps of identical measurements so that a substitute is immediately available should one become damaged.

Comparison of the weights of the two cannulae presented in Figs. 1 and 2

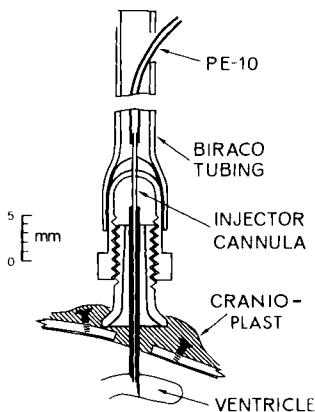


FIG. 2. An injector cannula for making injections into the ventricle is kept in place by screwing the cap assembly onto the guide cannula base. The guide cannula is fashioned in the same way as that described in Fig. 1 and is held in place by Cranioplast cement packed in and around the anchor screws. Biraco or other plastic tubing is cemented over the cap, and PE-10 tubing containing the solution for the microinjection is run through a hole in this tubing, which protects the PE tubing and injector cannula. (From Myers *et al.*, 1967.)

are as follows: the stainless steel cannula is approximately 3 gm, whereas the plastic cannula weighs 0.75 gm.

a. *In-Line Swivel*. To permit the rat complete freedom of rotation in its cage, a leak-proof swivel is inserted in the tubing line. A miniaturized swivel, with near-zero dead space, is desirable for the chronic infusion procedure. With a large swivel, there may be problems of (a) hysteresis in the delivery of the solution in a microliter volume; (b) accumulation of stale drug solution in the swivel; and (c) disconnections of the PE tubing because of the weight factor.

The Bainbridge-Wright swivel (see Bainbridge and Wright, 1965) or one based on a similar design principle is the most trouble-free and reliable. The swivel can be constructed either from a Plexiglas (Perspex) or Teflon rod measuring 3.2 mm in diameter. A diagram of the parts and the assembled Bainbridge-Wright swivel is presented in Fig. 3. If the diameter of the rod is slightly larger (an advantage in the machining process) the internal dimensions should nevertheless be kept in the same proportions as in Fig. 3. One modification of the Bainbridge-Wright design is the omission of the mercury filler used as a sealant. In some swivels, tiny beads of mercury are found to escape into the PE tubing and eventually reach the animal's brain. If the machining is done punctiliously, the fit of

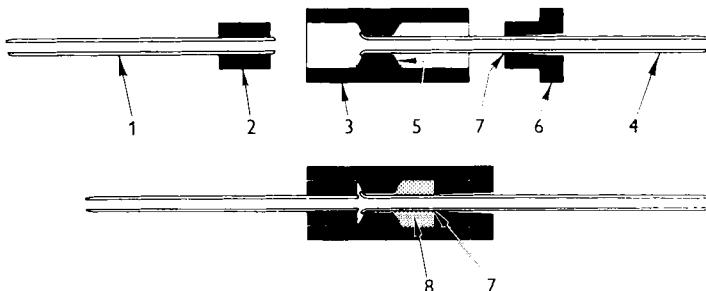


FIG. 3. The swivel is made from a 1/8-in. (3.2-mm) diameter Perspex rod. The stainless-steel inlet tube (1) is a tight fit in a Perspex plug (2), which is a tight fit in the body (3). The outlet nozzle (4) has a flanged inner end and is a loose running fit in the central partition (5). The flanged bush (6) is a tight fit in the other end of the body and has a taper hole reamed in it which, at its smallest diameter (7), is a light interference fit (torque about 0–2 g/cm) on the outlet tube. The swivel is filled with mercury (8) by injecting it into the inlet tube, with the outlet tube closed. A pressure of more than 20 lb/in.² is required to drive out the mercury through the gap (at 7) around the outlet tube. (From Bainbridge and Wright, 1965 with permission of Cambridge University Press.)

the parts of the swivel is so snug that leakage of the drug solution is uncommon.

The position of the swivel in the PE-10 or PE-20 tubing line is not particularly crucial. For ease of reach to verify free rotation, it is placed halfway between the animal's cannula base and the pulley (cf. Fig. 7 in Section II,C,1). Sometimes a small length of 3-0 suture tied around the junctures of the PE tubing and ends of the swivel enhance the tightness of the joints. Also, a small strip of adhesive tape wrapped around these junctions prevents the PE tubing from pulling away from the swivel.

Commercially available swivels (e.g. Lehigh Valley Electronics) or other designs based on the Epstein–Teitelbaum model (see Epstein and Teitelbaum, 1962) are not recommended. For this application, they are too heavy or possess a large dead space. Commercial swivels are used principally for intravenous or intragastric intubation, in which case the swivel size is not vital.

b. *Cranial Swivel*. An infusion cannula, which incorporates a swivel within the pedestal affixed to the rat's skull, has been developed by Khavari (1970). The design principle is straightforward; the PE tubing–injector needle assembly is stationary, and the animal is simply rotating around it. The cannula–swivel unit, illustrated in Fig. 4, is machined from either a nylon or Teflon rod 8.0 mm in diameter. A guide cannula, cut from either 21- or 22-ga stainless steel, thin-walled needle tubing is fitted into a base that is later cemented onto the rat's skull. The injector needle consists of a 27-ga tube that is center-mounted within the core of a Teflon

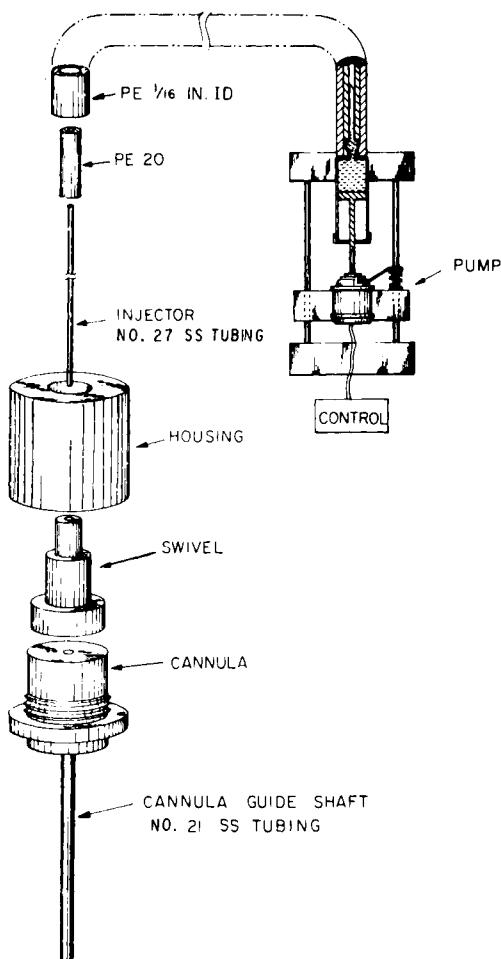


FIG. 4. Exploded view of the cannula-swivel unit is shown together with the liquid pump. The 27-ga injector tubing is friction fitted through the 21-ga cannula guide shaft into the ventricle. (From Khavari, 1970.)

cylinder. This cylinder, depicted in Fig. 4, rotates and thus serves as the swivel. Therefore, after the injector needle is lowered inside the guide tube, with the tip resting in the cerebral ventricle, the injector likewise swivels inside of the guide tube.

As shown in Fig. 4, a threaded hollow cap (housing) with a hole drilled in its center is screwed onto the cannula base over the rotating Teflon swivel. This cap holds the swivel and its injector needle securely in place.

An outer protective tubing (Silastic) through which the solution-carrying PE tubing is threaded is friction fitted onto the top of the Teflon cap. Since Teflon or nylon is used in the fabrication of the swivel, the need for lubrication is of course eliminated. Thus, problems with the rotation of this swivel such as binding or freezing are not encountered.

For long-term infusion, the Khavari swivel should be modified to prevent the outer protective tubing from disconnection, as shown in Fig. 5. Once the protective tubing is fitted snugly onto the top flange, a 27-ga pin, cut from needle tubing, is pushed horizontally through a previously drilled hole. The ends of the pin are clipped back with wire cutters. Whenever the swivel is unscrewed for flushing of the system, a new pin is simply inserted (Fig. 5) in the same hole.

2. Chronic Cannula for the Primate

The method for the chronic administration of a drug into the cerebral ventricles of a monkey is a far more direct procedure technically. Since the primate is seated in a restraining chair, it does not require a swivel, pulley, or other arrangement. However, stringent aseptic procedures make the technique difficult for the experimenter in that a single mistake with a monkey can lead to a fulminating meningitis or some other disorder.

By following the method described for primate neurosurgery (Myers *et al.*, 1972b), a modified Collison cannula is implanted aseptically with the tip resting just above the lateral or third ventricle. Or two cannulae are simultaneously implanted, usually bilaterally. Stereotaxic coordinates

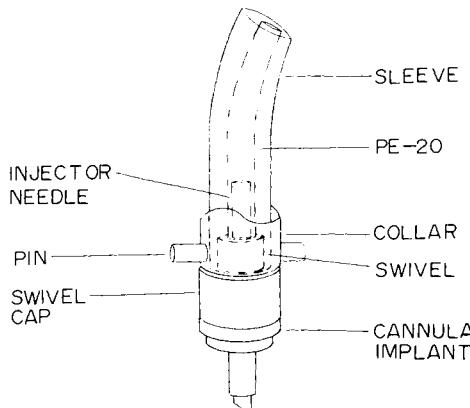


FIG. 5. A Khavari swivel shown in its completed form as it would rest on the rat's cranium. Note the pin inserted through the collar swivel to hold the protective sleeve firmly in place during long-term infusion.

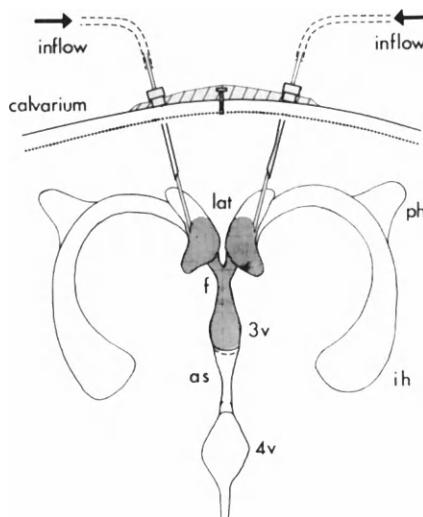


FIG. 6. Diagrammatic representation in the coronal plane of the procedure for the bilateral infusion of the cerebral ventricles of the unanesthetized monkey. The shaded area indicates the region reached immediately by the infusion. Cranioplast cement retains the cannula hubs in position together with the anchor screw placed on midline. Abbreviations: *as*, aqueduct of Sylvius; *f*, foramen of Monroe; *ih*, inferior horn of lateral ventricle; *lat*, lateral ventricle; *ph*, posterior horn of lateral ventricle; *3v*, third ventricle; *4v*, fourth ventricle. (From Myers *et al.*, 1972b.)

(Section II,B,2) are selected to maximize inflow of the drug solution and outflow of CSF (Myers, 1967). Figure 6 illustrates the positioning of cannulae above the anterior portion of the lateral ventricle. Note that the inner injector cannula penetrates the ventricle at a site as close as possible to the foramen of Monroe. As seen in Fig. 6, a stainless steel anchor screw(s) is inserted in the calvarium so that Cranioplast cement can hold the infusion cannulae firmly in place.

Several modifications of this procedure are easily adopted. For example, unilateral instead of bilateral injections can be made or a unilateral infusion can be interrupted with the simultaneous sampling of CSF accomplished by the tapping of another ventricle through a contralaterally positioned cannula (Section IV,A).

B. Implantation Procedures

For the very experienced neurosurgeon, a Collison or other intraventricular cannula may be implanted in the cat or other species without the

use of a stereotaxic instrument. The cannula can be positioned by hand, but this is accomplished only after much practice. For all chronic preparations, it is recommended that stereotaxic procedures always be employed so as to maximize the accuracy of cannula placement.

1. *Stereotaxic Rationale*

Within the ventricular lumen, the region in which the cannula tip ultimately rests is a critical factor with respect to the volume of solution to be infused and the maintenance of patency. In the cat or monkey, the stereotaxic coordinates for the tip of the infusion tube are commonly selected on the basis of their proximity to the foramen of Monroe (Carmichael *et al.*, 1964; Myers *et al.*, 1971). In the rat, however, coordinates are chosen that are as much as 1 mm distant from the foramen. The reason is that the size of the ventricular space is somewhat greater in other coronal planes. Thus, the dimension of the ventricle of a small animal constitutes the chief criterion for the selection of anterior, posterior, and lateral stereotaxic coordinates.

a. *Selecting the Coordinates.* Table I presents the range in millimeters of optimal stereotaxic coordinates for gaining entry into the lateral, third, or fourth cerebral ventricle for the purpose of infusing a drug or collecting CSF. The three commonly used species for which these coordinates have been determined are the rat, cat, and monkey. To enhance the utility of this table, some special adjustments in each of the precise coordinates selected may be in order. The variation in the body weight of individual animals and differential cranial dimensions, particularly in the younger animal, are the main reasons.

In various species of macaque monkey, including *Macaca mulatta*, *M. nemestrina*, *M. iris*, and *M. fascicularis*, the spatial position of the ventricular cavities are *relatively* constant. Nevertheless, the neurosurgeon often must adjust a coordinate, sometimes during surgery, because of the width of the particular animal's skull or the location of its coronal suture.

b. *Special Caution.* External bony landmarks such as the bregma, lambda, or midline suture should not be used as primary reference points for stereotaxic surgery. Although laboratory workers tend to use these landmarks as a quick and easy shortcut procedure, accuracy is often sacrificed. For example, the bregma of a rat is a wandering intercept of two skull sutures, which varies anterior to posterior by as much as 0.8 mm. In the cat, the corresponding coronal suture varies between 18 to 21 mm rostral to stereotaxic or interaural zero. The usefulness of these landmarks lies only in their means as an approximate external check of a given set of stereotaxic coordinates that has been established by the

TABLE I

Range of Optimal Stereotaxic Coordinates in Millimeters for Placing a Chronically Indwelling Cannula with the Tip Resting in the Lateral, Third, or Fourth Cerebral Ventricle of the Rat, Cat, and Monkey^a

		Rat ^b	Cat ^c	Monkey ^d
Lateral ventricle	AP	5.4 to 6.2	11.5 to 13.5	13.0 to 16.0
	Lat.	1.2 to 2.0	3.0 to 4.5 ^e	3.0 to 5.0 ^e
	Hor.	+1.5 to +2.5	+6.5 to +8.5	+11.0 to +16.0
	"Dura"	-2.8 to -3.8	-11.5 to -13.5	-14.0 to -19.0
Third ventricle	AP	5.5 to 6.5	12.0 to 13.5	12.5 to 15.5
	Lat.	0.0	0.0	0.0
	Hor.	-1.0 to -3.0	+3.0 to -4.0	+9.0 to -2.0
	"Dura"	-7.0 to -9.0	-17.0 to -24.0	-21.0 to -32.0
Fourth ventricle	AP	P 3.0 to 4.0 ^f	P 0.5 to 2.0 ^f	P 6.5 to 9.5 ^f
	Lat.	0.0	0.0	0.0
	Hor.	-4.0 to -5.0	+1.0 to +3.0	-5.5 to -8.5
	"Dura"	-4.0 to -5.0 ^g	-16.0 to -18.0 ^h	-35.5 to -38.5

^a For lateral ventricular cannulae, a lateral angle of 5° to 6° off vertical is often used particularly in the cat and monkey. The "Dura" measure refers to the distance (in millimeters) that the tip of the cannula should rest below the surface of the exposed dura mater.

^b De Groot (1959) atlas. Measures always based on stereotaxic zero *not* bregma.

^c Jasper and Ajmone-Marsan (1961) atlas.

^d Atlases of Olszewski (1952) and Winters *et al.* (1969). For macaques.

^e Position of tip when cannula is implanted at 5° to 6° angle.

^f P signifies posterior, i.e., in millimeters caudal to stereotaxic zero.

^g Below cerebellar meninges.

^h Bony tentorium must be bypassed, necessitating an angled placement.

interaural reference zero. Thus, should doubt arise as to whether the ear bars of the stereotaxic instrument actually rest within the animal's ear canals, bony landmarks do indeed serve the purpose of general validation.

The coordinate system of König and Klippel (1963) is never used for the adult male rat because of anatomical discrepancies. This atlas is based upon the brain of the female rat of approximately 150-gm body weight. Clearly, coordinates derived therefrom are not useful when an infusion cannula, chemitrode, or other device is to be implanted in a fully grown male rat that may weigh up to 500 gm. One can validate this caution quite simply by attempting to implant the tip of a needle in the tractus diagonalis in each of two rats—one female weighing 150 gm, one male weighing 450 gm—using the König and Klippel coordinates.

2. Anchoring the Cannula

Before a cannula is lowered into the brain of the rat, four stainless steel anchor screws are inserted equidistantly from each other around the craniotomy hole. The screw holes are drilled slightly undersized at a vertical angle of 5° to 8° to the surface of the skull. Once the screws are covered with Cranioplast cement, the cannula–cement–screw pedestal can be dislodged only if the bone around the screws is damaged or displaced. If the array of anchor screws is inserted in the vertical plane, they may pull out since the bone could then offer little horizontal resistance.

In the cat or monkey two or three large anchor screws (3/8-in. 1-72) are placed close to midline, and Cranioplast cement is flowed between the screws and the cannula array. When a pedestal is placed over the cannula array on the calvarium, two or three screws are inserted through holes previously drilled in the edge of the pedestal cap and are screwed to the skull (see Fig. 6). To prevent the protective cap from being unscrewed by a monkey, a tiny setscrew can be run through the cap and threads of the pedestal.

3. The Issue of Postoperative Recovery

Within several days after the operation, fibrous and glial tissue begin to encapsulate the tip of the guide tube. This interval corresponds to the period during which the integrity of the blood–brain barrier is restored following surgical intervention (Myers, 1975). Naturally, this tissue provides a seal which enlarges and becomes firm and resilient with the passage of time. In fact, if 7 to 10 days elapse before an injection is made, the infused solution usually takes the line of least resistance and flows dorsalward, up the external shaft of the guide tube and into the subarachnoid space. Again, this is readily verified by tracing the path of movement of a solution of dye injected at the tip of the guide tube implanted for 10 days. Johnson and Epstein (1975) have shown that glial growth may prevent the dispersion of a microliter droplet into the parenchyma in the region immediately surrounding the tip of an injector cannula.

4. Overcoming Cannula Occlusion

Several avenues are available by which the problem of cannula occlusion can be overcome. First, the guide tube is always implanted approximately 1 mm, or slightly more, dorsal to the ependymal surface of the ventricle. According to a method described previously (Myers, 1971), the tip of the injector needle is cut 1.0 to 1.5 mm longer than the guide tube. Then, the injector always penetrates the glial mass directly. By first sharpening the injector needle at a bevel angle of 45° to 60°, the scar tissue surrounding the

guide can be punctured rather than torn. A localized hemorrhage is thus prevented.

Second, as elaborated upon in Section II,A,1, the ventricle of an animal used for chronic infusion procedures is tapped as soon as possible post-operatively. However, a period of 2 to 3 days may elapse before an animal is connected to the infusion system for chronic injections. If the animal fails to resume its preoperative intake of food and water or shows an unstable body temperature, the postoperative recovery period is extended. But with each passing day the serious trade-off against the maintenance of ventricular patency increases.

Also of grave concern is the possibility that elevated intracranial pressure occurs when a drug solution is infused for a prolonged period. However, there is presently no evidence whatsoever that the multiple infusions of *microliter quantities* of a solution produce either a regional or disseminated hydrocephalus. Histological analysis of brains of the rat and monkey in which infusions were delivered for up to 30 days generally reveals no consistent enlargement of the ventricular lumen. When a control solution such as artificial CSF or a five-ion solution is infused similarly (Myers, 1972), no notable behavioral or physiological change (including signs of aphagia, hypodipsia, hypo- or hyperthermia) is produced. Nonetheless, whenever the method of chronic infusion is employed, daily surveillance of each animal's vital signs, locomotor behavior, food and water intakes, body temperature, excreta, and appearance of its fur coat is mandatory.

C. Intraventricular Delivery of the Solution

1. Pump

The heart of the chronic infusion method is the pump system that accurately propels a set of syringe plungers into their respective barrels. Several infusion pumps of high quality are available commercially (Harvard Apparatus Company; Palmer Ltd.), and are well worth the purchase price.

To save on cost, it is possible to modify the standard base of a Harvard Apparatus pump. The base consists of the mount for the machined lead screw and the plunger–pusher syringe-holder assembly. But it is necessary to affix to the lead screw a motor (General Electric Telechron synchronous) that has the desirable properties of low rotational inertia, minimal hysteresis, and near instantaneous start and stop characteristics. Ordinarily, our infusion pumps are modified to accommodate up to ten 0.25- or 1.0-ml tuberculin syringes for simultaneous infusion of 10 animals (Fig. 1, left).

To do this, the bolts on the end plate of the Harvard pump are removed and a piece of 1-mm-thick sheet aluminum measuring 25 cm by 5 cm is positioned horizontally and bolted to the end plate. This piece of aluminum serves to hold one end of the syringe barrel and is notched out earlier with 1.0 cm by 0.5 cm semicircles (half-holes) placed equidistantly 1 cm apart. Each notch will accommodate the needle end of a 0.25-ml syringe (Becton-Dickinson). If a 1.0-ml tuberculin syringe is used for the infusion of larger volumes, a correspondingly larger semicircular hole must be drilled similarly in the aluminum plate (Fig. 1, *left*).

To cradle each syringe barrel at the plunger end, a 3-mm-thick sheet of Plexiglas is drilled out in precisely the same way and with dimensions corresponding to those of the aluminum plate. The aluminum holder can serve as the drilling jig for the Plexiglas. These two plates are fastened together with 6-cm-long bolts. Alternatively, a set of 6 to 10 syringes can be placed in a cradle, as illustrated in Fig. 7, fabricated by furrowing rounded slots in rectangular Plexiglas blocks.

The "pusher plate" that impels the syringe plungers simultaneously can be cut from either a Plexiglas or aluminum sheet measuring 3 cm by 2.5 cm. The pusher plate is bolted to the steel saddle that rides upon the lead screw. As the lead screw is turned by the motor, the plate advances so that the plunger of each syringe is driven identically (Fig. 7).

2. How to Select and Calibrate the Syringe

Only glass syringes are used for a chronic infusion system. Every syringe is matched in terms of volume to insure that each animal receives the same dose of infused compound. To calibrate a given syringe, it is filled with distilled water, mounted on the pump, and its needle connected via PE tubing to a 10- or 50- μ l syringe (Hamilton). The pump is activated until the distilled water moves a measured distance within the Hamilton syringe. The pump is repeatedly activated and the motor's speed, and the running time adjusted to deliver the same volume every time the motor is actuated. Usually, 1.0 μ l is used as the standard calibration volume.

Note that it is improper to calibrate a syringe on the basis of the number of millimeters that a monitor bubble moves down a length of PE tubing. Although this procedure is sometimes used (unwisely), unsuspected variation in the internal diameter of PE tubing from batch to batch invalidates this calibration procedure.

Prior to usage, the glass plunger is laid beside its respective barrel, on aluminum foil; both are wrapped and heated for 90 min in an oven at 150°C to render them pyrogen free. A plastic disposable syringe is never used under any circumstances for the chronic infusion of a solution into an animal's brain. The reasons are as follows: (1) the plastic may give off

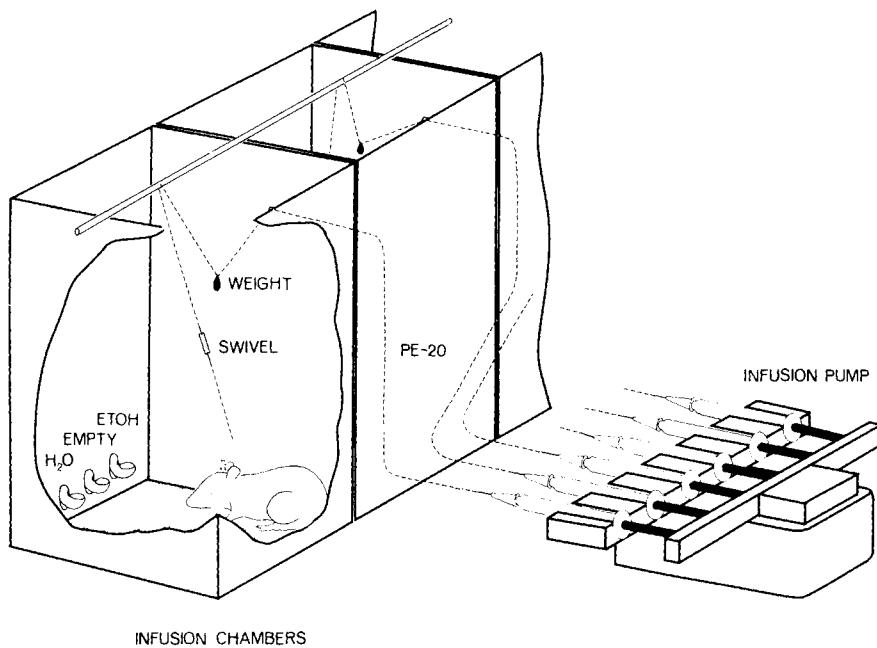


FIG. 7. Schematic diagram of the experimental method for simultaneous chronic infusion of solutions into the cerebral ventricles of rats. A multisyringe infusion pump is programmed to deliver 2 μ l, over an 86 sec interval, every 15 min around the clock. Each 1.0-ml calibrated syringe is connected via PE-20 tubing to a cannula which is permanently implanted in the lateral ventricle. An in-line, miniature, watertight swivel enables the animal to rotate freely, and a tension weight prevents the infusion tubing from becoming loose enough for the rat to be able to bite it. An ethanol solution and water are offered according to the three-bottle, two-choice, self-selection procedure. (From Myers *et al.*, 1972a, with permission of Pergamon Press.)

biologically active hydrocarbons (Bowery and Lewis, 1968) into the solution, which in turn can exert marked inhibitory or excitatory pharmacological effects on brain tissue; (2) uneven resistance caused by the rubber gasket present on the tip of the plunger may cause the pusher plate to move in a jerking fashion and give unequal volumes; (3) the rubber seal of a disposable syringe plunger is not always fitted tightly, or it may become pliable and then leak; (4) in spite of the volumetric scale printed on each barrel, plastic syringes vary in their volume, and different animals may receive different volumes of infusate.

3. Selection of Tubing

Greater accuracy and reliability of infusion is achieved in the delivery of a solution into the cerebral ventricle if PE tubing of the smallest

possible diameter is utilized. Ordinarily, either PE-10 or PE-20 intramedic tubing (Clay-Adams) is preferred (Figs. 1 and 7). When PE or Teflon tubing of small internal diameter is employed, unwanted factors such as system hysteresis, compressibility of the fluid, elasticity, and porosity of the tubing line are attenuated. Polypropylene, Tygon, Vivosyl, and other types of tubing are not recommended.

a. *Eliminating Bubbling.* A bubble or series of bubbles of air in the PE tubing lines, swivel, or syringe constitutes a serious hazard. Sources of bubbling include: (1) dissolved air that comes out of solution, particularly if the solution has been kept at a temperature that is not the same as that of the room; (2) nonairtight syringe; (3) positioning of the multiple-syringe pump too high above the level of the animal, resulting in an air-siphoning effect; (4) air drawn in through micropores in the wall of the PE tubing; (5) a leaky connection at the swivel or junction between the syringe needle and tubing line.

To overcome the bubble problem, several alternatives may be explored. First, the carrier solution used for preparing the infusate, such as pyrogen-free artificial CSF, should stand at room temperature for several hours. Second, stopcock grease (Halocarbon 25-55 or Dow-Corning) deposited on the shaft of the plunger after it has been inserted several cm into the barrel provides a leak-proof seal at the plunger-barrel junction. Third, by placing the infusion pump at the same level as the floor of the animal's cage, the suction effect is prevented whereby air is drawn into the tubing system (Fig. 7). Fourth, the ends of the PE 10 or 20 tubing that connect to the injector cannula and swivel should not be jagged or frayed but are cut sharply and cleanly.

b. *Tension Weight on Tubing.* As shown in Fig. 7, a tension weight must be placed over the PE tubing to keep the tubing taut as the animal bobs its head. Onto a piece of copper wire, sinkers from a fishing line or other metal weights are threaded, and then the wire is twisted over the PE tubing. A suitable counterweight ranges between 30 and 40 gm.

4. *Verification of Inflow*

It is essential that the actual infusion of the test solution into the ventricle be verified to ensure that there are no leaks. Usually once a day, the cap holding the injector cannula is unscrewed from the pedestal and removed so that the entire system can be examined. To do this, the remote programming equipment is activated so that the infusion pump is switched on. The volume of solution expelled, per unit time, at the tip of the injector needle is estimated visually by holding the microliter droplet above a millimeter rule. A more precise volumetric determination is made

by connecting the injector needle to a 10- or 50- μl Hamilton syringe by a short piece of PE tubing. Once the infusion pump is activated, the volume which is infused into the animal's brain is verified accurately as it moves into the syringe barrel. Ordinarily, an air bubble is not introduced purposely into the tubing line for monitoring purposes. However, the movement of a 1- or 2-mm bubble in the PE tubing line gives another validation of inflow. Caution should be exercised, however, because very minute bubbles may adhere to the side of the tube; when this occurs, a false indication is given that the infusion solution is not flowing. Overall, witnessing the outflow of a measured droplet at the tip of the injector needle is the most satisfactory procedure.

a. *Replacing the Infusate.* At the same time that the infusion system is temporarily shut down, a freshly prepared solution should be flushed into the infusion system. Ordinarily, this is done only once a day. If the particular drug in the injection solution is subject to autoxidation, is thermally labile, or degrades on exposure to light, a new solution may have to replace the old more often. Depending on the drug used, ascorbic acid (0.1–0.3 mg/ml) or another retardant of chemical breakdown can be added to the infusion medium so that the pH of the solution is lowered.

b. *Repositioning of Injector Needle.* Of utmost importance is the precision with which the injector needle is repositioned within the guide cannula. In fact, the reliability of a sequence of infusions is entirely dependent upon maintaining the injector needle at a constant depth. At the time of surgery a test injection is made to establish the patency of the ventricle. This is done by lowering an injector needle, attached to CSF-filled PE tubing, to the intended horizontal coordinate. By observing carefully the rate at which artificial CSF flows through the tubing into the ventricle, the final vertical position of the needle is adjusted until inflow is maximal. A record of this depth is noted, and the position is not varied during the chronic infusion experiment.

The intracerebral orientation of the bevel of the injector needle should also be maintained. To do this, external marks (scratch or notch) are placed on the needle, the needle cap, and pedestal. Then, each time the needle is reinserted, the cap is screwed onto the pedestal in an identical position until the marks are perfectly aligned. If, when the system is checked, the depth of the injector needle varies from day to day, the possibility increases that the infused solution will disperse up the shaft of the guide tube into the subarachnoid space, rather than into the cerebral ventricle itself. Such a seemingly small detail as this may be the difference between a successful experiment and a failure.

D. In Situ Validation of Cannula Patency

Two exceedingly important aspects of the method of chronic infusion are (*a*) empirical determination of the presence of infusion solution in the ventricle itself, and (*b*) dispersion characteristics of the test solution within the ventricular lumen or a portion of it.

1. Invalidity of Histology

Unexpectedly, the serial sectioning for subsequent histological examination of the brain in the region where the cannula rests is of little value. For instance, if the brain is sectioned on a microtome in the coronal plane, the lesion reflecting the guide tube is usually visualized quite clearly; in fact, the zone of necrotic tissue may encroach upon the ventricle. However, if the tip of the guide tube were encapsulated with a glial or fibrous mass, the injected solution would not penetrate and pass through this mass. Thus, a histological section would not distinguish the penetrability of the glial mass, nor reveal the actual path taken by the infused solution.

2. Dye Substitution Method

Alternatives to histological analysis for validating the chronic infusion procedure include several dye substitution methods as pioneered by Feldberg and his co-workers (cf. Feldberg, 1963). Essentially, a marker dye such as bromophenol blue, Evans blue, or India ink is used as a substitute for the infusion medium. For instance, bromophenol blue stains the parenchyma rather distinctively because of its particular chemical properties; or Evans blue is utilized if the structural elements of the subarachnoid spaces are to be visualized (Da Silva and Sproull, 1964).

After a chronic infusion sequence has been terminated, the animal should be taken immediately to the lab bench, and its own injection system used for the validation procedure. A 25–50% solution of India ink is loaded into the injector system. Then, the injector needle is placed exactly in the same position as that during the period of chronic infusion, by aligning the marks on the cap and pedestal. A 2- to 5- μ l infusion of dye is given in the ventricle of the rat, whereas a volume of 100 to 200 μ l is used for the monkey. The injection interval should be the same as that of the chronic infusion.

The animal is anesthetized immediately, and the brain is perfused with formalin solution following standard procedures. A catheter is placed retrograde in the descending aorta; the heart is clamped by curved mosquito forceps with care taken not to occlude the aortic arch; exsanguination is accomplished by incising both external jugular veins. As soon as the brain is fixed, the calvarium is removed carefully by rongeurs. With a broad spatula used as an elevator, the brain is taken out of the skull

quickly after the cranial nerves are sectioned. The reason for this is that bromphenol and other dyes may be bleached out if the tissue is kept in formalin or other fixative.

3. Exposure of the Ventricular Cavities

After the brain is placed on its ventral surface on a moistened towel or filter paper, the ventricular cavities are exposed in sequential steps. With a new scalpel blade, an incision is made carefully along the dorsal surface of the cortex in the rostral to caudal direction, parallel to midline. The point of entry of the blade is very anterior, about 2 mm off the midline. Once the internal location of the anterior horn of the lateral ventricle is approximated, the blade is then drawn back medially. Then, as the inferior horn is approached, the caudal direction of the incision turns more laterally. With practice, this dissection procedure enables one to expose the entire ventricular cavity of the cerebral hemisphere with a single stroke of the scalpel blade (Myers, 1972). The septum, caudate nucleus, and hippocampus are particularly easy to visualize in the rat and monkey. A pencil sketch of the regions reached by the dye should be made at once before proceeding further. The same procedure is followed to expose the contralateral ventricle of the remaining hemisphere. Another pencil sketch is then made.

After the tissue of the two hemispheres is replaced in the natural position with a blunt probe, the third ventricle is exposed. To do this, the point of the scalpel is inserted between the two retracted hemispheres directly upon the rostral portion of the corpus callosum. Again, a rostro-caudal incision is made with a single cut along the midline. Thus, the entire third ventricle, the cerebral aqueduct, and fourth ventricle are exposed in a bilaterally symmetric fashion. Now that all of the internal subhemispheric surfaces of the ventricular cavity are revealed, a pencil sketch again will portray the extent to which the tracer dye has diffused.

One helpful, time-saving practice is to use a standard stenciled diagram of the cerebral ventricles of the particular species in use. In this way, a permanent record can easily be made of the structures reached by the dye with consistency and without the necessity of redrawing the ventricles.

E. Controls, Daily Checks, and Data Analysis

Important controls must of necessity be undertaken in all experiments in which the chronic infusion method is employed.

1. Pharmacological Validation

One pharmacological procedure commonly used in conjunction with this method is the systematic variation in the concentration of the drug

infused in the brain. To derive a dose-response curve of the effect of the drug, it is usually essential to show that a given response is augmented as the concentration infused is increased. If a reported effect cannot be produced reliably by an infused substance, the dose is typically doubled or halved, or the volume infused into the ventricle is doubled or even quadrupled. The chronic infusion of a single dose of only one drug in a set volume under one condition is not an appropriate experimental strategy. Simply put, the dose may not be in the range of efficacy for the particular animal of the specific strain employed.

Another important pharmacological control is the parallel infusion of a compound that is related chemically to the structure of the infused test substance. A compound that forms a part of the metabolic pathway of the test compound is a justifiable alternative. For example, in experiments on the effect of intraventricular norepinephrine on feeding or motor behavior, dopamine is often compared with its parent catecholamine (Myers, 1974). An "inactive" optical isomer of a compound (e.g., *d*-norepinephrine) can also act as a control to contrast the effect of the active isomer (e.g., *l*-norepinephrine). In studies involving the chronic infusion of ethanol, either acetaldehyde or paraldehyde are often used for comparison (Myers and Veale, 1969).

The latency as well as magnitude of a response to a known test compound typically provides an internal validation of the infusion system. That is, if a solution is actually delivered to the cerebral ventricle, a well-defined, already documented response occurs. For example, norepinephrine infused acutely in the rat will produce several easily observed effects reliably if the tip of the cannula does indeed rest in the lateral cerebral ventricle (see Myers, 1974). This sort of simple control test is particularly valuable if there is any question of reproducing an effect which reportedly occurs.

2. Physiological Controls

A sham-operated or nonimplanted group of animals must be included in experiments as controls for the procedure itself. A second control group is required in which the same volume of an artificial CSF is infused intraventricularly on an identical schedule. A third control is also used in which the test drug is infused into the brain substance or subarachnoid space rather than the ventricle; an *anatomical control* is thus provided. Parenthetically, 0.9% saline cannot be used as a control vehicle as a substitute for artificial CSF. Sodium chloride possesses potent central actions of its own, particularly on the CNS processes underlying the intake of fluids and the regulation of body temperature (Andersson, 1971; Myers, 1974).

In summary, the control infusion of an ionically balanced CSF serves to test the multiple effects of the presence of an attached cranial pedestal, the constant pull of the PE tubing, the surge in intraluminal pressure occasioned by each ventricular infusion, and the transient disequilibrium in the electrolyte concentration in the animal's own CSF.

3. Genetic Characteristics

The problems constantly posed by differences in strain and species of animal are not unique to the action of a drug on the ventricular structures. In experiments in which a central effect of a compound is examined over a long period, the genetic characteristics of a particular strain become an important factor. It is known already that inbred strains of laboratory rat differ widely in their selection and intake of various fluids, including ethyl alcohol, following the peripheral or central administration of substances known to alter substrate metabolism (Myers and Melchior, 1977a). Therefore, two or more strains should be investigated so that a more comprehensive, accurate interpretation of a research result can be deduced.

4. Cautions in Interpreting Results

The use of more than one or two animals in a group is mandatory, primarily because of anatomical differences in the way that an individual animal's ventricle is reached by an injection. Factors that influence one's results are (*a*) the specific position or placement of the indwelling injector cannula in the ventricle; (*b*) subtle anatomical irregularities in the dimensions of the ventricular cavity from one animal to the next; (*c*) kinetics of inflow in a specific preparation with respect to direction, penetration, and dispersion of the infused solution; and (*d*) partial occlusion or total loss of patency of the ventricular cavity.

When the infused compound reaches the amygdala, septum, and thalamus but not the hippocampus, the animal's behavioral or physiological responses are different than when the converse is true. Further, should the tip of the cannula become gradually occluded by neuroglia, a part of the infused solution diffuses up the external shaft of the guide cannula and enters the subarachnoid space where it may (or may not) have an observable effect. Thus, the anatomical patterning of the verifying dye solution is the most critical aspect for interpreting the result of a chronic infusion study.

For these reasons, it is often difficult to use an averaging statistic such as a mean or median measure that groups data without taking into consideration the anatomical differential in dispersion of the infused solution. In fact, it is possible to have a self-cancellation effect if the infusion medium flows rostrally in half of the animals and caudally in the rest. Again,

sketches of the cannula position and of the structures reached by the injection often clarify a puzzling result.

III. METHODS FOR CHRONIC SAMPLING OF CEREBROSPINAL FLUID AND CEREBRAL TISSUE

Postmortem analysis of the level of an endogenous substance and/or its metabolites in the entire brain, or large portions thereof, disregards a cardinal principle of functional neuroanatomy. Analyses of this sort underscore the misconception that the brain is an amorphous conglomerate of chemically undifferentiated cells. That is, whole brain assays are tantamount to one's equating some exceedingly diverse structures: cerebellum with the hypothalamus; the visual cortex with the caudate nucleus; or Broca's area with the olfactory bulb. Morphology notwithstanding, such an equivalence is physiologically inconceivable and scientifically invalid.

Repeated sampling of the chemical milieu of an animal's brain is one alternative to this whole-brain approach. For example, samples of CSF collected from discrete regions provide a gross estimate of the chemical characteristics of the structures lining the walls of the ventricle—the brain's internal milieu. Or by "washing" or perfusing a circumscribed region of brain by means of push-pull cannulae, local changes can be detected in the chemical activity within a given nucleus or fiber system. In this section, the procedures as well as the disposition of the collected samples of these fluids will be considered.

A. Collecting Fluid from the Cerebral Ventricle

For the chronic sampling of CSF, a large animal such as the cat or monkey is always preferred, simply because of the size of the brain. The surgical procedures are identical to those used for implanting a guide cannula of a push-pull perfusion system (Myers, 1972); a 35 to 55 mm long guide tube is cut from 17- or 18-ga stainless-steel tubing and implanted as described next.

1. Cannulation of the Ventricle

The guide cannula is positioned stereotactically usually at a 6° to 10° angle. It is held in place in one of two ways: (1) by a cranial pedestal, or (2) by affixing it to the hub of a Collison cannula which is either cemented or screwed into the cranium. The stereotaxic coordinates for maximal success in obtaining patency of the cannula and maintaining outflow of CSF are presented in Table I.

If the tip of the guide tube is implanted 0.5 to 1.5 mm dorsal to the lateral ventricle, the CSF tapping needle is used for penetrating the

ependymal wall of the lateral or third ventricle. The needle is cut from 20-ga stainless-steel tubing and lowered through the guide tube. In approximately 50% of the brains used for repeated sampling of CSF, a channel of tissue is eventually opened up which communicates with the tip of the guide tube. Thus, CSF flows directly into the guide tube from the ventricle; this eliminates the necessity of puncturing the ependymal wall each time a sample is required. The procedure for maintaining this open needle–ventricle channel is now described.

2. Tapping Procedures

Three types of CSF tapping procedures are used. To avoid local trauma it is ordinarily best to settle upon one procedure and disregard the others until the first one fails.

Flow of CSF from the guide tube is initiated immediately after the animal has recovered from surgery. The ventricle is opened with a special 20-ga needle, the tip of which is sealed by touching a tiny amount of stainless steel solder to the lumen at the end (Fig. 8b). This tip is then blunted by rounding it off with a fine file or carborundum paper. As shown

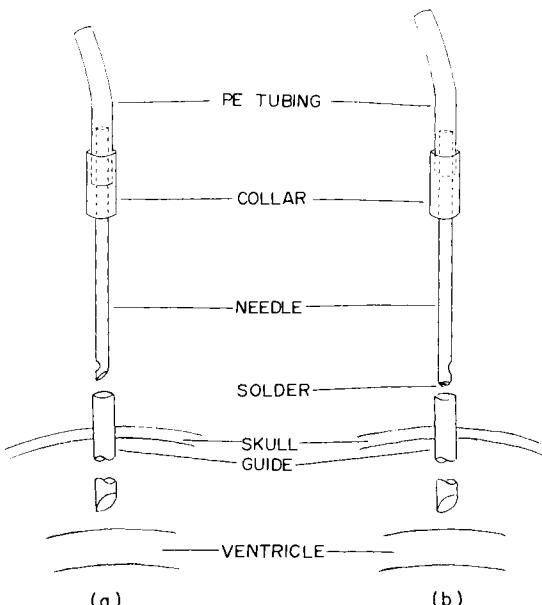


FIG. 8. Ventricular tapping needles with side opening at their base with the tip (a) open to permit CSF outflow or (b) sealed in case of occlusion. The needle shown in (b) is used when considerable tissue must be penetrated to reach the ventricular lumen.

in Fig. 8(a,b,), a semicircular side-opening is placed just above the droplet of solder; this is cut by a jeweler's file.

The tapping needle is shimmed by a length of either PE, Silastic, or stainless-steel tubing. This spacer prevents the needle from penetrating beyond the ventral surface of the ventricle, once the exact depth has been established. The tapping needle is connected to a strand of PE-60 tubing about 75 cm in length. A 1 cm length of Silastic tubing (Dow-Corning No. 602-155) is fitted over the junction of the PE tubing and needle. The Silastic collar serves as an airtight seal when the tapping needle is inserted into the guide tube to its fullest extent. The position of the collar is shown in Fig. 8(a,b) in which the tapping needle is ready for insertion within the ventricle.

The type of tapping needle depicted in Fig. 8(a) has a tip that is beveled sharply at 45° to 60°; but *not sealed* by solder. Again, a side opening cut approximately 1.5 mm above the middle of the bevel provides two lumens for entry of the CSF.

The third method for tapping, although most direct, can be used only if the tip of the guide tube is in open contact with the ventricle. This occurs often after a tissue channel is cleared by repeated penetrations with the tapping needle (Fig. 9). In this case, at the instant that the stylet is removed, CSF begins to bubble out (or almost gush) from the end of the open guide tube. Care must be taken to position the PE tubing-Silastic sleeve swiftly over the guide opening so that nearly all of the fluid can be

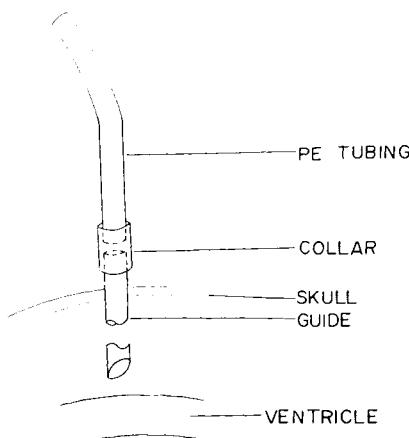


FIG. 9. Cerebrospinal-fluid collection tube fitted over the end of an indwelling guide tube in which a channel to the ventricular lumen is open. The collar forms a seal to prevent leakage of CSF as it is collected in the PE tubing, once or twice each day.

collected. Because of the typically high intraluminal pressure head, CSF usually flows directly into the collecting tube without one's having to resort to siphoning.

It should be emphasized that when the animal is not in use, a Silastic sleeve or collar must always be placed as a seal over the junction of the indwelling stylet and guide tube. This prevents pernicious leakage of CSF between 12 to 24 hour sampling intervals.

3. Preparation of the Tapping Needle

The tapping needle and length of PE collecting tubing are always stored in 70% ethyl alcohol. They are rinsed by using a sterilized disposable syringe and flushing with 10–15 ml of 0.9% pyrogen-free saline.

Before the stylet is removed from the guide tube, the PE collecting tubing is first filled with 0.9% saline. Then, the open end of the PE tubing is clamped shut with a Halstead or other mosquito forceps. This keeps the solution in the tubing.

For monitoring the rate of CSF flow, an air bubble 1 cm in length is introduced in the tubing line at the needle end by momentarily releasing and then reclamping the forceps. The monitor bubble also indicates the precise volume collected by the distance traversed. To determine volume, a length of PE-60 collecting tube is marked off with small pieces of adhesive tape before an experiment is begun. It is convenient to place the tape along the tube at intervals calibrated at 50- μ l volumes. As the bubble moves past each 50- μ l marker, the rate of collection over a given interval is calculated per unit time.

As soon as the PE tubing is filled with the volume of CSF desired (e.g., 200 μ l), the end of the tube is again clamped with mosquito forceps to prevent further emptying of the ventricle. Then, at the instant that the collecting tube is removed from the guide tube, the indwelling stylet is replaced.

4. Collecting a CSF Sample

After the tapping needle is inserted into the guide tube of the cat or monkey, the Silastic tubing sleeve is inspected to ensure that there is a snug fit over the guide. The forceps are then released. If the CSF flows out rapidly from the ventricle under its own head of pressure, the forceps must be held ready to clamp off the end of the collecting tube. If the rate of CSF flow is low, the speed of collection can sometimes be enhanced by lowering the end of the collecting tube below the level of the animal's ventricle. To avoid possible inflow of saline in the collecting tube, which must be avoided at all costs, the level of the tube must be watched closely. As much as 200–500 μ l of CSF can be collected from the monkey or cat within 8 to 30 sec. Taps are made at least once every 24 hours.

In those cases in which CSF flows out of the guide tube without tapping, the Silastic-sleeve seal should be checked for leakage twice a day. When an animal persistently loses CSF, it usually becomes sickly, aphagic, lethargic, and may ultimately succumb. Although the etiology of this CSF-depletion syndrome is unknown, absence of intracerebral pressure provided by CSF is presumably to blame.

B. Repeated Perfusion with Push-Pull Cannulae

The method of push-pull perfusion (Myers, 1972) in a chronic preparation permits the sampling of chemical substances in a circumscribed area of brain tissue. It is also highly useful for the controlled application of drugs, over a specified period of time, to a particular structure or region. The procedure has contributed some basic knowledge to the field called "chemical neuroanatomy" (cf. Morgane, 1975).

1. Utility of Localized Sampling

Push-pull perfusions are used to study the effect of two or more kinds of experimental manipulation; the site is perfused also under control conditions. Recently, the use of both single and double isotope labeling methods has enabled the investigator, during the course of an entire experiment, to trace the activity of a compound which is taken up into endogenous pools (Martin and Myers, 1975). Accordingly, once a push-pull perfusion site is labeled with a ^{14}C or ^3H compound, a washout curve of radioactivity is obtained by collecting samples for 5–10 min and repeating the perfusion every 10–30 min. At the midpoint of a washout curve, an experimental condition is altered during the collection of one sample and any change in its radioactivity is ascertained.

2. Precautions in the Perfusion Technique

Repeated perfusions of this sort require several sterile precautions. For example, after the cap covering the polystyrene pedestal is unscrewed, a large cotton sponge soaked in 70% ethanol is stuffed inside the cap. Between perfusions, when the cap is screwed onto the pedestal, the sponge is removed but the cap is not dried. Residual ethanol vapor sterilizes the surface of the cap and the inside surfaces of the pedestal, including the tips of the protruding guide tubes.

After removal from the guide tube, each concentric push-pull cannula assembly is placed on a 4 × 4 in. gauze sponge saturated with 70% ethanol and covered with a second 4 × 4 in. gauze sponge also soaked in the ethanol. Special care is taken, moreover, that the tips of the push-pull cannulae are neither touched by the fingers nor bumped against a surface that is not sterilized.

3. Repeated Perfusions

For repeated testing of the neurochemical activity of a given tissue site, the push-pull cannula assembly is always lowered to the same depth. In order to reduce any movement of the push-pull assembly, should an animal shake its head, a tightly fitted Silastic collar is positioned over the shim so that as the push-pull assembly is lowered into the brain, the collar fits snugly on the guide. The principle is the same as that shown in Fig. 8.

When signs of occlusion are noted (bubbling), the push-pull perfusion must be terminated immediately. Otherwise, the anatomical integrity of the site will be destroyed. If the fluid on the pull (outflow) side of the system shows signs of discoloration due to bleeding, or if bubbles of air begin to appear in the line, the push-pull pump is switched off quickly. Usually, back-flushing of the pull side of the tubing line with artificial CSF will expel the bit of tissue, dust particle, or other matter that causes a temporary occlusion. If a back-flush does not unclog the system, then each tubing connection, the rubber diaphragm in the cap of the push-pull assembly, and the pull syringe are examined to pinpoint the source of leakage. If the plunger of the pull syringe does not fit perfectly within the barrel, stopcock grease is also used to coat the plunger for a watertight seal.

4. Maintaining Viability of the Site of Perfusion

A series of push-pull perfusions is ordinarily carried out at successive depths, usually in 0.5- to 1.0-mm steps below the tip of the guide tube. Once an active site of chemical release is found, that site should be used repeatedly for as many experiments as possible until all evidence of its viability has vanished. Only then is the push-pull cannula assembly lowered to the next successive depth. The reason for this is that once a locus has been penetrated and perfused, or labeled by microinjection of a radionuclide, glial deposits tend to wall off the neurons that are responsible for the activity of that site. Thereafter, the use of this perfusion site is impractical.

5. Advantage of Frequent Usage

Experience gained in our laboratory has shown unequivocally that a push-pull perfusion site remains viable *only* if used with great frequency. Contrary to a clinical sort of expectation in relation to tissue trauma, experiments carried out on a daily basis enhance the reactivity of a perfusion site; similarly, the quantitative release of an endogenous substance will persist if so used. Under no circumstance, should the site be left untouched for more than 48 hr. If this happens, an encapsulating wall of fibrous material or glia is deposited within the small channel that is

produced by the push-pull cannulae. Such a mass of "scar" tissue quickly inactivates the physiological responsivity of the locus of perfusion. In some instances, two independent sets of push-pull perfusions can be undertaken even on the same day (McCaleb and Myers, 1976). Surprisingly, the rate of stability of an effluxing endogenous compound is much greater when an interval of less than 48 hr elapses between perfusions.

C. Handling and Disposition of the Collected Samples

The analysis of the constituents under study in a sample of CSF or push-pull perfusate depends largely upon the type of chemical or biological assay to be performed. As a general principle, samples should be assayed as soon after collection as possible. Many factors associated with degradation, autoxidation, thermolability, and crystallization contribute to the changes that are possible in the structural nature of the sample.

After CSF is collected, it is drained quickly into a 1.0-ml minireaction vial (Regis) chilled earlier in a 0°C freezer. The sample is placed under a stream of nitrogen, where it is dried. If the sample cannot be assayed on line, it is frozen instantaneously by placing the vial in a dry ice-acetone bath. As estimated by gas chromatographic analyses, samples of CSF that have been stored at -20° to -60°C in a low-temperature freezer (Revco) seem to maintain consistent activity for at least 4 weeks. However, using a bioassay method, we have noted vastly different values of biogenic amines, with thawing and refreezing of ventricular fluid. A third alternative is to transfer the CSF to a centrifuge tube and lyophilize it under vacuum by freeze-drying. The disadvantage of this procedure is that some of the lyophilized particles are lost due to suction into the vacuum orifice.

Push-pull perfusates are also assayed immediately upon collection. If the volume of perfusate collected is sufficiently large so that part of the sample enters the pull syringe, then a small polyethylene bag containing crushed ice should be placed over the pull syringe. In this way, the sample is collected under conditions of relatively low temperature and the degradation of some constituents is retarded. Once the solution is expelled by back-flush into a collection vial kept on ice, the perfusate is assayed at once. Generally, samples of push-pull perfusate are not frozen for later assay because of the low amount of biological activity typically detected in perfusate. Further, when radiolabeled compounds are used, freezing and thawing tend to enhance the formation of metabolites of certain biological substances released within the brain parenchyma.

The problem of reducing the degradation of endogenous products is a difficult one. Ascorbic acid or sodium EDTA (ethylenediamine-tetra-acetate) can be included in the push-pull perfusion medium at the time that it

is prepared. Usually ascorbic acid is added in a sufficient quantity (0.3–1.0 mg/ml) to the artificial CSF so that the solution is brought to pH 3.0–4.0. On occasion, the solution in the pull tubing can be acidified with 0.1 N hydrochloric acid to bring the pH even lower if the constituent under study is particularly labile at higher pH.

IV. CONCLUDING COMMENT

Considering the general principles of chronicity underlying both intraventricular infusion as well as the repeated sampling of CSF and tissue, several important points stand out. One conclusion to be drawn concerning all of these methods is that frequent usage of the preparation is advisable, preferably every 24 hr. In this way, the unwanted formation of glia, fibrous, and other material at the tip of an implanted cannula will be retarded. Scrupulous attention paid to sterile procedures will help to prevent infection and other serious complications as well as the deposition of foreign material that promotes a tissue reaction at the cannula tip. Minimizing the exposure of the tapping needle or infusion needle to air will help in this respect. The use of ethanol in the protective cap of a pedestal and for the sponging procedures is equally essential.

Interpretation of a Result

It should be emphasized once again that the use of a mean statistic to analyze data accumulated with these methods must be done with great caution. Vast morphological differences in the infusion or perfusion loci may lead to an inappropriate conclusion or gross misinterpretation. Grouping of data, therefore, can be justified only if there is an incontrovertible commonality of anatomical regions or test sites which in themselves can be grouped according to morphological principles. Otherwise, a self-cancellation or other artifact can result.

If a discrepancy in an observation arises, one possibility is that a methodological difference constitutes the basis of the inconsistency. Clearly, considering the material presented in this chapter, it is easy to conceive of a plethora of procedural reasons why inconsistent results may be obtained. To illustrate, if a solution of alcohol is infused chronically into the ventricle of a rat, the animal's preference for alcohol should increase. However, if the solution ejected from the tip of the cannula does not enter the cerebral ventricle after all, the rat will not drink alcohol above a control intake. Naturally, direct evidence through visualization of the dispersion of the perfusion solution (see Section II,D) is always necessary in order to demonstrate ventricular patency. A study completed in our laboratory has revealed that tetrahydropapaveroline (THP),

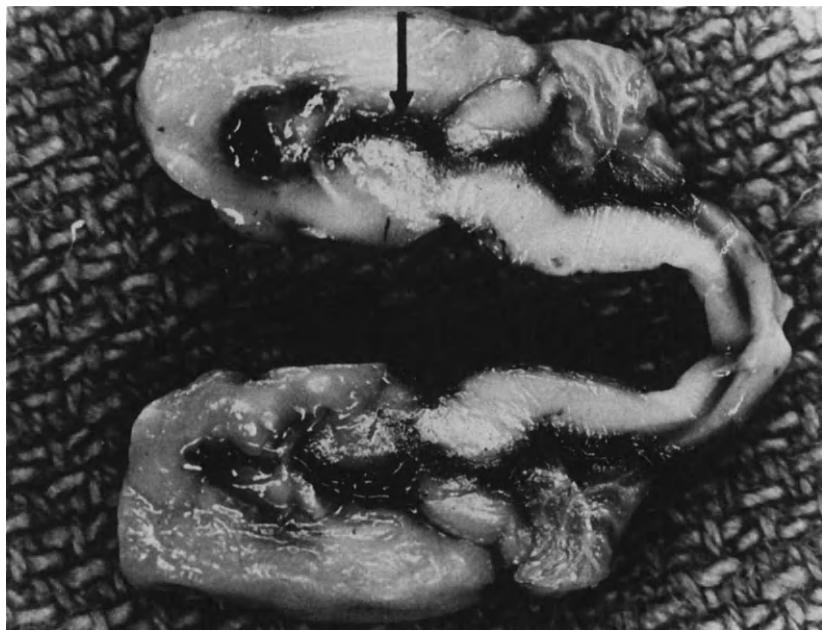


FIG. 10. Dispersion of 10 μ l of India ink after its injection into the lateral ventricle of the rat. The rat was killed by anesthetic, the calvarium removed, and the brain dissected along the midline as described in the text.

a condensation product of ethanol and catecholamine metabolism, when infused chronically into the cerebral ventricle of the rat, causes a remarkable preference for ethyl alcohol (Myers and Melchior, 1977b). But excessive drinking of alcohol is seen only in some animals. The reason for this was shown in postmortem examination using the dye injection-ventricular exposure method. When ethanol was rejected, the THP solution failed to enter the rat's ventricle but passed dorsalward into the subarachnoid space or into the parenchyma. Figure 10 illustrates a rat's brain in which dye is visible in the lateral and third ventricle as well as in the aqueduct of Sylvius and cisterna magna.

Mastery of the difficult neurosurgical and neurochemical techniques described here requires time, patience, pilot study, and successive attempts. When the techniques are mastered and internal validation of one's own result is achieved, then a given method can be used to investigate successfully some of the immensely complex, functional processes of the central nervous system.

REFERENCES

- Andersson, B. (1971). Thirst and brain control of water balance. *Am. Sci.* **59**, 408-415.
- Bainbridge, D. R., and Wright, B. M. (1965). A multi-channel long-term infusion system for small animals. *J. Physiol. (London)* **177**, 6P-8P.
- Bowery, N. G., and Lewis, G. P. (1968). Pharmacological activity in polyvinyl chloride (PVC) tubing. *Br. J. Pharmacol.* **34**, 207P.
- Carmichael, E. A., Feldberg, W., and Fleischhauer, K. (1964). Methods for perfusing different parts of the cat's cerebral ventricles with drugs. *J. Physiol. (London)* **173**, 354-367.
- Da Silva, F. H. L., and Sproull, D. H. (1964). Systemic absorption of adrenaline from the cerebral fluid spaces of the cat. *J. Physiol. (London)* **171**, 494-503.
- De Groot, J. (1959). The rat forebrain in stereotaxic coordinates. *Proc. K. Ned. Akad. Wet., C* **52**, 1-40.
- Epstein, A. N., and Teitelbaum, P. (1962). A watertight swivel joint permitting chronic injection into moving animals. *J. Appl. Physiol.* **17**, 171-172.
- Feldberg, W. (1963). "A Pharmacological Approach to the Brain from its Inner and Outer Surface." Arnold, London.
- Jasper, H. H., and Ajmone-Marsan, C. (1961). Diencephalon of the cat. In "Electrical Stimulation of the Brain" (D. E. Sheer, ed.), pp. 203-231. Univ. of Texas Press, Austin.
- Johnson, A. K., and Epstein, A. N. (1975). The cerebral ventricles as the avenue for the dipsogenic action of intracranial angiotensin. *Brain Res.* **86**, 399-418.
- Khavari, K. A. (1970). Chemical microinjections into brain of free-moving small laboratory animals. *Physiol. Behav.* **5**, 1187-1189.
- König, J. F. R., and Klippel, R. A. (1963). "The Rat Brain: A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem." William & Wilkins, Baltimore, Maryland.
- McCaleb, M. L., and Myers, R. D. (1976). Noradrenergic activity in the hypothalamus of the rat is altered by local glucose, insulin or 2-deoxy-D-glucose delivered by push-pull perfusion. *Proc. Neurosci. Abstr.* **II**, Part 1, p. 304.
- Martin, G. E., and Myers, R. D. (1975). Evoked release of [¹⁴C]norepinephrine from the rat hypothalamus during feeding. *Am. J. Physiol.* **229**, 1547-1555.
- Meyer, P. M., and Meyer, D. R. (1971). Neurosurgical procedures with special reference to aspiration lesions. In "Methods in Psychobiology" (R. D. Myers, ed.), Vol. 1, pp. 92-130. Academic Press, New York.
- Morgane, P. J. (1975). Anatomical and neurobiochemical bases of the central nervous control of physiological regulations and behaviour. In "Neural Integration of Physiological Mechanisms and Behaviour" (G. Mogenson and F. Calaresu, eds.), pp. 25-67. Univ. of Toronto Press, Toronto.
- Myers, R. D. (1963). An intracranial chemical stimulation system for chronic or self-infusion. *J. Appl. Physiol.* **18**, 221-223.
- Myers, R. D. (1967). Transfusion of cerebrospinal fluid and tissue bound chemical factors between the brains of conscious monkeys: A new neurobiological assay. *Physiol. Behav.* **2**, 373-377.
- Myers, R. D. (1971). Methods for chemical stimulation of the brain. In "Methods in Psychobiology" (R. D. Myers, ed.), Vol. 1, pp. 247-280. Academic Press, New York.
- Myers, R. D. (1972). Methods for perfusing different structures of the brain. In "Methods in Psychobiology" (R. D. Myers, ed.), Vol. 2, pp. 169-211. Academic Press, New York.
- Myers, R. D. (1974). "Handbook of Drug and Chemical Stimulation of the Brain." Van Nostrand-Reinhold, New York.

- Myers, R. D. (1975). Blood-brain barrier: Techniques for the intracerebral administration of drugs. In "Handbook of Psychopharmacology" (L. L. Iversen, S. D. Iversen, and S. H. Snyder, eds.), Vol. 2, pp. 1-28. Plenum, New York.
- Myers, R. D., and Melchior, C. L. (1977a). Alcohol and alcoholism: Role of serotonin. In "Serotonin in Health and Disease" (W. E. Essman, ed.). Spectrum, New York. In press.
- Myers, R. D., and Melchior, C. L. (1977b). Alcohol drinking: abnormal intake caused by tetrahydropapaveroline in brain. *Science* **196**, 554-556.
- Myers, R. D., and Veale, W. L. (1969). Alterations in volitional alcohol intake produced in rats by chronic intraventricular infusions of acetaldehyde. *Arch. Int. Pharmacodyn. Ther.* **180**, 100-113.
- Myers, R. D., and Veale, W. L. (1972). The determinants of alcohol preference in animals. In "The Biology of Alcoholism" (B. Kissin and H. Begleiter, eds.), Vol. 2, pp. 131-168. Plenum, New York.
- Myers, R. D., Casaday, G., and Holman, R. B. (1967). A simplified intracranial cannula for chemical stimulation or long-term infusion of the brain. *Physiol. Behav.* **2**, 87-88.
- Myers, R. D., Yaksh, T. L., Hall, G. H., and Veale, W. L. (1971). A method of perfusion of cerebral ventricles of the conscious monkey. *J. Appl. Physiol.* **30**, 589-592.
- Myers, R. D., Evans, J. E., and Yaksh, T. L. (1972a). Ethanol preference in the rat: Interactions between brain serotonin and ethanol, acetaldehyde, paraldehyde, 5-HTP and 5-HTOL. *Neuropharmacology* **11**, 539-549.
- Myers, R. D., Veale, W. L., and Yaksh, T. L. (1972b). Preference for ethanol in the rhesus monkey following chronic infusion of ethanol into the cerebral ventricles. *Physiol. Behav.* **8**, 431-435.
- Olszewski, J. (1952). "The Thalamus of the *Macaca mulatta*: An Atlas for Use with the Stereotaxic Instrument." Karger, Basel.
- Schain, R. J. (1960). Neurohumors and other pharmacologically active substances in cerebrospinal fluid: A review of the literature. *Yale J. Biol. Med.* **33**, 15-36.
- Winters, W. D., Kado, R. T., and Adey, W. R. (1969). "Stereotaxic Brain Atlas for *Macaca nemestrina*." Univ. of California Press, Berkeley.
- Winterstein, H. (1961). The actions of substances introduced into the cerebrospinal fluid and the problem of intracranial chemoreceptors. *Pharmacol. Rev.* **13**, 71-107.

APPENDIX

Syringes

Becton-Dickinson and Company
Rutherford, New Jersey 07070

Hamilton Company
Reno, Nevada 89510

Polyethylene and other tubing

Clay-Adams
Division of Becton-Dickinson and Company
Parsippany, New Jersey 07054

Portex
Portland Plastics, Ltd.
Hythe, Kent, England

LKB-Products A B
Bromma 1, Sweden

Dow-Corning Corporation
Medical Products Division
Midland, Michigan 48640

Stainless steel tubing

Popper and Sons, Inc.
New Hyde Park, New York 11040

Pedestals or 32-oz polyethylene bottles
Scientific Products
Chicago, Illinois 60646

Pumps

Harvard Apparatus Company
Millis, Massachusetts 02054

Motors

Hurst
Princeton, New Jersey
North American Phillips Controls Corp.
Cheshire, Connecticut 06410

Stopcock grease

Dow-Corning Corporation
Midland, Michigan 48640

Halocarbon Products Corporation
Hackensack, New Jersey 07601

Implantable pump (see note below)

Alza Corporation
Palo Alto, California

Note on Implantable Pumps

Commercially available devices that are implanted subcutaneously are now used for the continuous systemic administration of a chemical substance. Their usage for the delivery of a small volume of a drug or other solution into the cerebral ventricle of a small animal also has been suggested. Nicknamed an osmotic "mini-pump" (Alzet), the capsule-like device operates on the principle that extracellular fluid passes slowly through a porous membrane, thus causing an internal sac which holds the drug to collapse at a steady rate. This action thereby forces out the solution from the sac as osmotic equilibrium is approached. If the "pump" would be connected by means of polyethylene tubing to a cannula resting in the cerebral ventricle, the drug solution would conceivably be forced out into the CSF spaces. On the surface, this sort of device would appear promising, but one should not adopt it for experiments without a keen awareness of its limitations and experimental hazards.

Cautions. Irrespective of the high cost factor per single pump, there are seven serious drawbacks to their usage. (1) Only a single flowrate, which cannot be varied, is possible with the design. (2) Because the delivery of the solution is a continuous infusion rather than a series of pulsed injections, a gradual build-up of glia and fibrous material around the cannula could occlude the tip, thus preventing the steady flow of solution from the sac. (3) The limited duration of the pump's life, at most 7 days, makes it useful only for relatively short-term experiments. (4) Because the design represents a virtually closed system, no flexibility is provided for changing a solution in the middle of an experiment, should the dose of the compound be too high or its delivery too rapid. If the discontinuation of infusion is called for because of symptoms of ill-health in the test animal, a second surgery would be required. (5) The verification of inflow and dispersion of solution through the tissue, and the determination of structures reached by the infusate are all difficult, if not impossible. The reason is that a marker dye cannot be loaded into the pump, after 7 days of infusion, because the pump is not reusable. (6) Only compounds that are stable at the temperature of an animal's body (e.g., 37–38°C) for 7 days can be used. That is, relatively thermolabile substances which include those endogenous compounds of neurochemical interest, such as biogenic amines, transmitters, their precursors, metabolites, etc., are excluded from investigation. (7) *In situ* validation of the patency of the infusion system, including the pump, tubing line, cannula, and connections, at any given time in an experiment, is not feasible.

Chapter 11

Dietary Constituents and Self-Selection Procedures: Solid Foods

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I. CONCEPTUAL BACKGROUND

Most animals can maintain stable health and nutritional balance in the face of great dietary diversity. This is accomplished by the simple fact that many natural foods contain a balance of essential nutrients and partly by the capacity of the body to meet its needs by the degradative and synthetic processes of intracellular metabolism. In addition, however, animals can exhibit substantial adaptive behavioral change to sustain the health of the body. For instance, many animals make appropriate dietary choices, both qualitative and quantitative, when required to consume essential nutrients from separate sources; and they can learn whether new foods sustain health or produce illness. This chapter summarizes some of the major techniques and theoretical considerations to be heeded in dietary self-selection experiments as well as in research assessing the effects of specific nutrients on physiology and behavior.

A mere consideration of the number of dietary constituents necessary for sustaining health (see Table I in Section II) indicates the great number of dietary manipulations that have been done and could still be done in this area of research. But, of course, progress in this field is not made simply by testing the behavioral effects of the possible permutations of the constituents of food, but rather by the search for answers to broad conceptual questions. Despite the great number of behavioral experiments that have been done using the basic technique of modifying dietary constituents, most work can be categorized under three broad headings:

1. *To what extent can animals maintain health and growth when required to ingest the individual constituents of a balanced diet separately?* These are the classic cafeteria-type experiments which are still best exemplified by the pioneering studies of Richter (1942–1943). The complexity of these studies has ranged from those where only two choices are offered [often a carbohydrate and protein diet as in the studies of Collier *et al.* (1969a) and Leshner *et al.* (1971, 1972)] to those requiring a choice between the major nutrient categories [fats, proteins, carbohydrates, a composite multivitamin mixture, and a composite salt mixture as in the studies of McDonald *et al.* (1963)]. In other studies, not only are the macronutrients presented separately, but also the individual vitamins and minerals [e.g., studies by Richter *et al.* (1945) employed a choice among 15 substances].

Presently the upper limit to the number of choices with which an animal can still maintain balanced intake is an open question, but it is clear that, under favorable conditions (i.e., group housing, or choice of sufficiently palatable nutrient sources), rats can sustain apparently normal growth with a minimum of 11 components presented separately [protein, carbohydrate, fat, cod liver oil as the source of fat-soluble vitamins, bakers' yeast as a source for B vitamins, NaCl, KCl, calcium lactate, Na₂HPO₄, MgCl₂, and water as in the Young (1944) study]. A great deal of existing work in this area along with important theoretical and methodological considerations has been reviewed by Lat (1967) and should be read by everyone pursuing work in the field.

Not only have self-selection experiments been done to determine how well animals can choose healthy diets but also how well they reject diets which may not be compatible with normal growth. The most prominent representative of this class of experiments are those where the amino acid composition of available diets has been manipulated so as to be either deficient or excessive in certain essential amino acids. Generally, it has been observed that animals rapidly learn to reject such diets even in preference to protein-free diets which support growth and life less well

than the imbalanced food. This work has been thoroughly reviewed by Harper *et al.* (1970).

2. *When experimentally deprived of specific nutrients, can animals make appropriate dietary choices to restore health?* The paradigmatic experiments run by Harris *et al.* (1933) demonstrated that thiamine-deprived rats can make appropriate dietary choices to alleviate the vitamin deficiency. A thorough analysis of how rats are able to do this has been presented by Rozin and Kalat (1971). It appears that thiamine-deficient rats, and possibly all animals with severe nutritional deficits, exhibit a propensity to sample new foods at well-spaced intervals, and if an animal happens to consume a thiamine-rich diet, it associates the rapid restoration of health with the sensory properties of the recently eaten food; henceforth it exhibits a strong preference for the new health-giving food.

The novelty with which the general paradigm of depriving animals of specific nutrients can be used is indicated by a study of Nance and Kilbey (1972), demonstrating that rats whose body serotonin has been depleted by administration of the tryptophan hydroxylase inhibitor *para*-chlorophenylalanine would make the appropriate dietary choice of the serotonin precursor 5-hydroxytryptophan to restore normal levels of this amine in the body.

3. *When deprived of or replenished with specific nutrients, how are the behavior, physiology, and biochemistry of an animal modified?* We are what we eat. This has always been a fascinating though somewhat esoteric proposition; but in the past few years research in this area has blossomed, and major advances in our knowledge of how brain and behavioral processes are regulated by nutrients may be forthcoming in the future. Work in the area is presently ranging from the effects of protein malnutrition on brain physiology and intelligence to the control of brain transmitter levels by essential amino acids in the diet (Fernstrom and Wurtman, 1971). The degree to which diet can control brain functions was recognized from early observations on the variety of mental dysfunctions that could result from vitamin deficiencies; but the systematic analysis of the subtlety of dietary participation in the control of brain function is only recently becoming a fruitful and provocative area of controlled experimental inquiry.

The rest of this chapter will highlight selected methodological considerations that should be heeded in pursuing research requiring the use of dietary manipulations—whether in self-selection experiments or those assessing the effects of specific diets on the behavior and physiology of animals. Since there is no such thing as a generally accepted methodology

in this area of research, the main aim will be to share some general methodological and theoretical considerations that are important in such dietary research. Specific procedures, of course, would be determined by the nature of each inquiry and should be straightforwardly deduced from the hypotheses being tested.

II. PREPARATION OF DIETS

Before initiating a study in which dietary constituents are varied, it behoves each investigator to anticipate the type of journal to which the work might be submitted. This is because many journals devoted exclusively to nutrition research insist that the dietary constituents be capable of being defined precisely; this normally necessitates the extensive use of purified nutrients. To the contrary, most behavioral and neuroscience journals unhesitatingly publish work which employs crude nutrient sources, for which the nutrient composition might only be generally specifiable. Interestingly, the choice of pure versus crude nutritional components might be an important methodological consideration, since there is some evidence that rats might thrive better on certain crude nutrient sources than on purified ones (Collier *et al.*, 1969b).

Preparation of various experimental diets is greatly facilitated by the existence of companies devoted to distributing dietary components for animal experimentation. In addition to manufacturing a large variety of special diets routinely (for example, diets lacking individual minerals and vitamins), such firms also willingly prepare special recipes on a contract basis. Two prominent companies providing these services are Nutritional Biochemicals Corp. of Cleveland, Ohio, and General Biochemicals of Chagrin Falls, Ohio (see the Appendix).

Naturally, the type of diet which is to be used will depend on the species being studied and the question being asked. The experimenter should above all be familiar with the nutritional requirements and idiosyncrasies of the experimental animal being studied. General nutritional requirements of a great number of species can be found in the handbook *Metabolism* (Altman and Dittmer, 1968), published by the Federation of American Societies for Experimental Biology. An especially fine summary of the needs of common experimental animals is to be had in a pamphlet entitled *Nutrient Requirements of Laboratory Animals* (1972).¹ A useful chapter specifically on the nutritional requirements of the laboratory rat has been written by McCoy (1949). For detailed information, the above publications should be consulted. Herein, I will only

¹ This pamphlet (No. 10) is put out by the National Academy of Sciences and is available for about \$5 from the Academy Publishing Office at 2101 Constitution Avenue, Washington, D.C. 20418.

summarize general principles for the preparation of experimental diets, with most of the presented facts applying specifically to the laboratory rat.

First, attention has to be paid to the distribution of macronutrients (fats, proteins, and carbohydrates) and the levels of micronutrients (vitamins, minerals, and trace elements) in the diet. Unless nutrient deficits are being studied, the available diet(s) should be able to sustain the health and growth of the animal at certain normative levels. In general, the biologically useful potential energy value of proteins and carbohydrates can be estimated to be 4 kcal/gm, and that of fat as 9 kcal/gm. Although these values are adequate for estimating the energy content of prepared diets, it should be noted that the exact values will vary slightly depending on the specific nutrients employed. Furthermore, it is difficult to specify the biologically useful caloric values of foods precisely because physical procedures (bomb calorimetry) which do yield exact gross energy values do not account for the incomplete absorption (digestible energy) and nutrient utilization (metabolizable energy) which occurs in living organisms. For instance, though the physical energy content of most carbohydrates is about 5 kcal/gm, the actual biological value of glucose, for instance, is 3.8 kcal/gm. Although it is difficult to measure the actual energy that an organism extracts from complex foods, as a general rule of thumb the metabolizable energy of most balanced rat diets is about 80 to 90% of the empirically measured gross energy content. For diets employing natural foods, the nutrient compositions and energy equivalence estimates can be obtained from the aforementioned handbook (*Metabolism*).

Well-balanced diets should be capable of providing both for the energy needs of an animal as well as the specific micronutrients (cf. Table I) required for the smooth flow of metabolic processes. For the laboratory rat, a diet which will maintain good health and growth should contain 12–40% protein, 3–40% fat, and 20–70% carbohydrate, though it should be noted that there is no specific bodily requirement for carbohydrate as there is for protein and fat. The most common source of protein in experimental diets is casein, which is milk protein and of "high biological value" since it contains all essential amino acids [those that cannot be synthesized by the body (Table I)] in the correct proportions. The only amino acid which may need supplementing is L-methionine, and it may be supplemented as 0.25% of the diet.

Care should always be taken to supplement protein sources of "low biological value" (proportionately low in some essential amino acid) with missing amino acids; otherwise growth failure will ultimately occur. In general, both fat and carbohydrates serve primarily as energy sources in

the diet and, thus, they are relatively interchangeable but for the fact that a minimal level of fat is required to serve as a source and carrier of fat-soluble vitamins and essential fatty acids. Conversely, too high a level of fat will tend to predispose the animal to ketosis. A minimal level of ongoing carbohydrate metabolism (oxaloacetic acid production) is necessary for fats to be completely combusted in the Krebs cycle. The most common sources of fat in experimental diets are vegetable oils and lard, whereas the traditional carbohydrate sources are starch, sucrose, and glucose.

Caloric density of diets can, of course, be varied easily by the ratios of fats to carbohydrates as well as by addition of nonnutritive fillers. The two most commonly employed fillers to reduce the caloric density of diets are kaolin, a powdered clay that comes in various densities, and Alphacel (Nutritional Biochemicals), which is a finely granulated cellulose powder. It should be remembered that both fillers reduce the palatability of diets. Mineral oil can also be used to dilute diets, but making them more greasy will also tend to make them more palatable. The only possible shortcoming is that mineral oil will tend to impede absorption of fat-soluble vitamins and, thus, appropriate supplementation may be advisable.

A great deal of self-selection research has focused on animals' capacity to adjust their intakes of micronutrients. The minimal daily requirements of micronutrients which rats have to consume in their diets are listed in Table I. The need for other trace elements such as aluminum, arsenic, boron, bromine, and fluorine has not been conclusively demonstrated. In addition to the indicated vitamins, the rat also needs inositol, folic acid, biotin, and vitamin C, but under normal circumstances sufficient quantities of all of these can be synthesized endogenously and need not be provided in the diet. In experiments not requiring manipulation of individual micronutrients, balanced diets may be prepared simply by adding commercial salt and vitamin preparations to the chosen mixture of macronutrients. For instance, all the mineral and trace element requirements can be met by adding 4% of USP XIV salt mixture, while vitamins can be provided by adding 2% of Vitamin Diet Fortification Mixture (both sold by Nutritional Biochemicals Corp.).

In addition to considerations of nutritional balance, investigators should pay close attention to the sensory properties of the foods they prepare. Taste, texture, and odor of foods are all capable of modifying food intakes, food choices, and feeding patterns of experimental animals. For instance, the mere addition of fat to the rats' diet during daytime can induce feeding primarily during the day rather than at night (Panksepp and Krost, 1975), and prolonged feeding of extremely fat-rich diets (64%) has been used successfully to make rats obese (Mickelsen *et al.*, 1955).

TABLE I
Essential Nonenergy Nutrient Needs of a Mature Laboratory Rat

Nutrient	Minimum daily requirement (units indicated)
Essential amino acids	
Histidine	35 mg/kg
Isoleucine	180 mg/kg
Leucine	110 mg/kg
Lysine	60 mg/kg
Methionine	90 mg/kg
Phenylalanine	50 mg/kg
Threonine	85 mg/kg
Tryptophan	30 mg/kg
Valine	120 mg/kg
Essential fatty acids	
Arachidonic acid	25 mg
Linoleic acid	25 mg
Linolenic acid	25 mg
Vitamins	
Water-soluble	
Thiamine (B ₁)	10 µg
Riboflavin (B ₂)	25 mg
Nicotinic acid	15 mg
Pyridoxine (B ₆)	12 µg
Pantothenic acid	80 µg
Vitamin B ₁₂ (cyanocobalamin)	50 ng
Choline	3 mg
Fat-soluble	
Vitamin A (retinol)	200 IU/kg
Vitamin D	3 IU
Vitamin E (α -tocopherol)	1 mg
Vitamin K	1 mg
Minerals, ions, and trace metals	
Sodium	10 mg
Chlorine	5 mg
Calcium	60 mg
Magnesium	4 mg
Potassium	15 mg
Phosphorus	50 mg
Iodine	5 µg
Iron	250 µg
Copper	50 µg
Manganese	500 µg
Cobalt (as constituent of vitamin B ₁₂)	400 ng
Zinc	40 µg
Chromium	5 µg

Although there are no set rules that one should employ to control the sensory properties of a diet, it is the responsibility of each investigator to attempt to demonstrate that results which are ascribed to differential nutrient content of food are not actually due to some differential sensory attractiveness of diets. Palatability considerations can become especially important when a large number of purified nutrient sources are made available to animals in self-selection experiments, since isolated nutrients often are unpalatable.

III. SOME USEFUL DIETS

If, in assessing the preference of animals for individual macronutrients, one provides individual dishes containing fats, proteins, and carbohydrates, the procedure would bias intake for the most palatable choice. Though it might be impossible to equate such diets precisely in terms of sensory qualities, it is certainly advisable to try to approach that goal. Possible recipes for such equicaloric diets are presented in Table II. In addition to the individual macronutrients, various nonnutritive additives are included to equalize sensory qualities—saccharin for taste, almond or any other extract for odor, mineral oil and cellulose powder for texture.

TABLE II
Ingredients (in grams) for Each Kilogram of a Balanced Macronutrient Diet, Individual Macronutrient Diets, and a Comparable No-Calorie Diet^a

Ingredients	Balanced diet	Carbo-hydrate diet	Fat diet	Protein diet	No-calorie diet
Corn starch	113	550	—	—	—
Sucrose	112	125	—	—	—
Corn oil	110	—	300	—	—
Casein	225	—	—	675	—
USP XIV salts	40	40	40	40	40
Vitamin Diet					
Fortification Mixture	20	20	20	20	20
Mineral oil	165	265	110	200	350
Saccharin	0.75	0.75	3	3	3
Cellulose (Alphacel)	224	—	524	60	585
Almond extract	3	3	3	3	3
Energy (kcal/gm)	2.7	2.7	2.7	2.7	2.7
Amount eaten during 3 days (gm/day)	23.4	24.3	20.4	17.5	25.8

^aFrom Panksepp (1971).

Furthermore, since equivalent micronutrients are added to each diet, the individual mixtures are nutritionally identical but for the energy sources.

In single diet tests, rats eat large quantities of each diet, but consumption of the carbohydrate mixture is highest and of the protein diet the least (Panksepp, 1971). Whether these differences are due to differential effects of the nutrients or residual taste qualities is unclear, but the readiness with which all diets are eaten does indicate their potential usefulness over purified components in choice experiments. Certainly it is advisable in all self-selection experiments to try to make components equally palatable, so that differential consumption can truly be ascribed to the bodily consequences of the nutrients rather than to differential taste acceptabilities.

Although diet preparation, i.e., accurate weighing and even mixing, is ultimately a straightforward procedure and requires no belaboring, one kind of diet deserves more attention than it has yet received. This is one which is essentially free of calories but still sufficiently palatable to be eaten. Not only might such diets ultimately prove useful as therapeutic aids in treating obesity (the saccharin-sweetened diets on the market are, of course, approaching this goal), but the more extensive use of such diets in laboratory experiments should provide insights into the fundamental mechanisms which control feeding. Naturally, it would be impossible to pursue such lines of inquiry unless acceptable energy-free diets could be prepared, and two studies indicate that this is possible.

In the work with equicaloric single macronutrient diets already discussed, a comparable no-calorie diet was also concocted (Table II), and it was consumed in greatest quantities during the 3 days of access. Of course, this probably did not reflect a preference for the diet but merely the accruing force of deprivation. Taylor and Bruning (1967) have also manufactured a nonnutritive diet which rats consume readily when it is provided as the only source of food. This diet, summarized in Table III, was found by humans to be indistinguishable from an energy-rich diet (4.0 kcal/gm) made from the same flavorings but with nutritive bulk. Nonnutritive food pellets which will fit conventional pellet dispensers may also be obtained in various flavors from the P. J. Noyes Company.

To determine whether such nonnutritive diets could compete effectively against nutritive diets, I have tested the preference for one relatively tasty, energy-free diet made of a blend of light kaolin (60%), mineral oil (34%), saccharin (1%), and Marmite (5.5%). The last substance is a rich, meaty-tasting, low-energy yeast spread manufactured in England. As summarized in Fig. 1, the energy-poor diet was consumed in substantial amounts even when animals had free access to their normal commercial laboratory chow. Indeed, during the first day, animals ate more

TABLE III
Recipe for Nonnutritive Diet and Equipalatable Caloric Diet^a

Nonnutritive diet (0 kcal/gm)	Nutritive diet (4.0 kcal/gm)
Methyl cellulose	50.0 gm
Vaseline petroleum jelly	63.0 gm
Calcium carbonate	35.0 gm
Saccharin	4.2 gm
Mineral oil	28.0 gm
Diet-Pepsi syrup	47.0 ml
Water	23.0 ml
	Methyl cellulose 50.0 gm
	Crisco 43.0 gm
	Casein 20.0 gm
	Sucrose 33.0 gm
	USP XIV salt 4.0 gm
	Vitamins 2.2 gm
	Diet-Pepsi syrup 47.0 ml
	Water 55.0 ml

^aFrom Taylor and Bruning (1967).

nonnutritive food than chow. Although this maladaptive behavior was corrected within a few days in both groups, substantial intake of the experimental diet continued for the duration of the experiment. In fact, during the 8 days when animals were given a choice, the rats ate on the average 40% of their daily intake from the nonnutritive diet; the diet was even consumed during the first meal following 24 hours of starvation (Fig. 1).

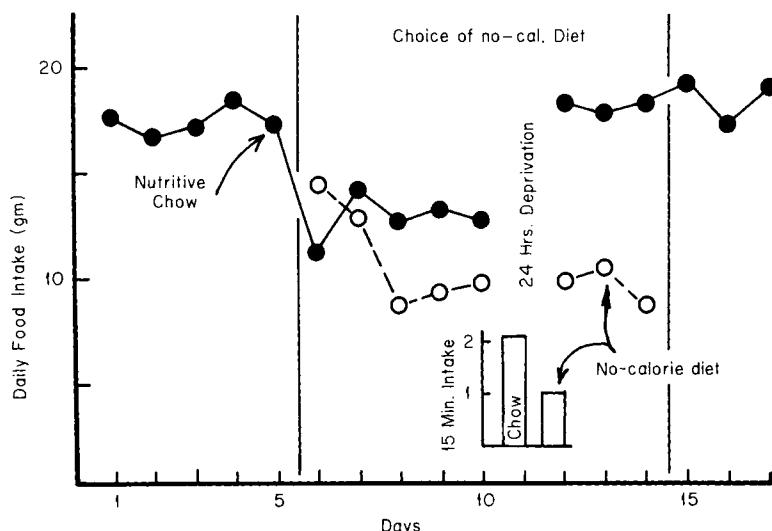


FIG. 1. Average daily food intakes of eight rats during 8 days of choice between their normal nutritive maintenance chow (Diet 41-B) and a palatable nonnutritive diet. Intake during first 15 min following a day of starvation is summarized in insert.

IV. PROCEDURAL CONSIDERATIONS IN SELF-SELECTION EXPERIMENTS

Many of the general procedures to be used in feeding experiments have been summarized by Falk (1971) in a previous chapter in Volume 1 of this series and will not be discussed here. Rather, specific experimental considerations relatively unique to self-selection experiments will be emphasized. Six major issues should be considered in designing such research:

1. The number of choices to be given should, of course, be based on the experimental question asked. When one is trying to test the limits of this behavioral capacity, the number of choices is increased; on the other hand, when assessing effects of specific experimental treatments, one usually employs fewer choices. For instance, many experiments which have assessed how environmental or hormonal states modify food choices have only used two diets—one rich in carbohydrates, the other in proteins.
2. The use of social housing could be considered. What such a procedure would lose in experimental control it might gain in the increased capacity of animals to make fine dietary discriminations. Social facilitation appears to broaden the behavioral repertoire of animals, probably by eliciting imitative behaviors. A striking example of this has been observed in young rats reared by parents who had conditioned aversions to a poisoned food. The pups rejected that same food without ever consuming it (Galef and Clark, 1971).
3. The manner of food presentation is important. It is advisable to randomize the position of the various food dishes on successive days so that position habits are discouraged. Further, to get accurate measurements of ingestion with solid foods, feeding dishes should be so arranged as to discourage spilling, either by having lids with eating access holes or by being placed within larger spillage collection vessels. The difficulty of accurately measuring micronutrient intakes has been noted often. Rats have a predilection for spilling distasteful items and, with the already relatively small daily intakes of those items, accurate measures would be hard to obtain. In such circumstances, the use of solutions might promote accurate recording.
4. If, in the course of an experiment, it is found that animals are not making adequate choices, the use of training procedures should be considered. Usually this entails giving animals the components which they have failed to ingest individually until consumption occurs. This will provide the animal an opportunity to learn the metabolic consequences of ingesting each food.

5. How long animals are tested is important to any interpretation of the results of self-selection experiments, especially since the evidence that animals make adequate dietary selections is based on the lack of deviation of the selections from the growth baseline of a group of animals receiving a single balanced diet. Differential effects are easy to interpret, but if one wishes to argue from negative evidence, the argument is always strengthened by having tested animals for long periods. Typically, self-selection experiments run anywhere from 30 to 120 days. Naturally, if one is studying differential behaviors of independent groups, it is sufficient to run experiments only for as long as it takes to demonstrate statistically conclusive differences.

6. Finally, an issue which has already been alluded to is the taste of diets. The generality of results is always limited by the specific conditions employed, and if it is the case that animals fail to make appropriate selections of dietary constituents, the failure need not be due to a lack of capacity but merely to the fact that the choices are so unpalatable as to preclude adaptive behavior. For instance, dry casein as the protein source appears to be quite distasteful to a rat, whereas when casein is wetted its intake increases considerably (Lat, 1967).

V. GENERAL PROCEDURES FOR CONDITIONED AVERSION AND PREFERENCE EXPERIMENTS

Since the pioneering work of Garcia and Koelling (1966) and Revusky and Bedarf (1967), it has been recognized that animals made ill soon after eating new foods will tend to refuse those foods on subsequent occasions. The procedures for such experiments are extremely simple and require no elaborate explication. The main point is that food to be used as a conditioned stimulus should have distinct sensory characteristics. With solid foods, this is most easily accomplished by spiking them with distinct odors, i.e., with any of the multitude of confectioners' essences sold in supermarkets. As far as is known, any new odor will suffice. Once given access to the novel diet for a time adequate for a meal (30–60 min); animals are injected by an appropriate route (usually intragastric, intra-peritoneal, or subcutaneous) with the illness-inducing agent. The most commonly employed poisons are lithium chloride and apomorphine; the former is preferable since it is cheaper, more published data are available, and no Bureau of Narcotics and Dangerous Drugs license is required for its use. Of course, a great number of other substances could be used, including amphetamine, imbalanced amino acid mixtures, chlorpromazine, atropine, ethylene diamine, or fenfluramine.

Animals can also be induced to exhibit conditioned preferences for

distinct diets which are paired with the alleviation of nutrient deficits and imbalances. In one of the more extensive series of studies along these lines, protein-starved rats tube fed with balanced amino acid mixtures before receiving distinctively odored diets exhibited a preference for the paired diet on subsequent occasions (Booth and Simson, 1971; Simson and Booth, 1973). In these experiments, the balanced amino acid injections were given just prior to food access. Weaker effects were obtained with different temporal pairings. To obtain the best conditioning, the general principle is that the alleviation of illness should come as close to the actual ingestive experience as possible.

VI. INTERPRETATIVE CONSIDERATIONS

Essentially, most self-selection experiments are asking whether animals are equipped to make certain kinds of dietary discriminations, namely, those tending to preserve health or ward off illness. Since each food is a multidimensional substance—with taste, odor, texture, and metabolic effects—it is difficult to accept conclusions that animals cannot make the necessary discriminations. One is always left with questions such as the following: Might a discrimination have been made if the sensory qualities of the diets were changed? Might a discrimination have been made if the number of dietary choices were reduced? Might a discrimination have been made if the animal were forced to experience the effects of a certain diet? Of course, the dilemma of handling negative effects is present in all areas of research, but it is especially problematic where precise, well-accepted experimental procedures are the exception rather than the rule. In research areas where investigators can use traditional, well-validated techniques, negative results obtained by skilled investigators are generally considered to be as meaningful and compelling as positive ones.

The specific methodological concern one faces in experiments in which animals successfully self-select nutrients is the evaluation of whether a demonstrated appetite for a nutrient is innate or learned. In practice, this is such a thorny question that it has been resolved with any degree of assurance for only thiamine and sodium appetites. Therapeutic thiamine intake appears to be largely learned, since animals can cope only with the selection procedure if a limited number of choices are made available (Harris *et al.*, 1933). Moreover, when given the choice between a thiamine-enriched and thiamine-deficient diet, rats will often choose the deficient diet if it had been health sustaining (i.e., thiamine replete) previously in the animal's history (Rozin, 1968). To the contrary, sodium appetite appears to truly qualify as an innate appetite. The conclusive set of experiments, executed by Kriechhaus and Wolf (1968) and Kriechhaus

(1970), demonstrated that rats suddenly depleted of sodium would seek out the place where sodium had once been located, even though they had only consumed the sodium while in a replete state. These studies are well worth reading for the fine attention applied to important matters of methodological detail.

Although procedures used in conditioned aversion experiments are quite standardized, it should be noted that the procedure can be used with different degrees of sensitivity. This is especially important in testing substances which might have only mildly aversive effects. Essentially, the sensitivity of the test is determined by the level of deprivation at which animals are tested. The hungrier they are, the less likely they are to develop conditioned aversion. The animal that is just ready to eat provides the most sensitive baseline for aversive effects. Whether this is also the case with conditioned acceptance paradigms is yet to be determined.

Also it is worth keeping in mind that agents which may produce conditioned rejection of foodstuffs can theoretically produce the effect by means of conceptually distinct mechanisms. For instance, besides learning that certain foods make them ill, animals can learn how satiating a food is (LeMagnen, 1969) and also to gauge subsequent food intake by such learned cues. Although the issue of separating conceptually different types of conditioned rejections is yet to be adequately addressed in the literature, one reasonable way to attack the problem would be to assume that diets paired with agents which produce true conditioned aversions should be strongly rejected by both starved and nondeprived animals in choice situations, whereas diets paired with agents which produce conditioned satiety might actually be preferred by hungry animals even though less might be eaten.

VII. FUTURE RESEARCH

Of course, there are many experiments left to be done in the area of dietary self-selection, most of them having to do with the generality of certain demonstrated phenomena. For instance, work on the ability to acquire specific appetites for diets replete in thiamine (Rozin and Kalat, 1971) is yet to be extended to the remaining vitamins, not to mention ions and trace elements. The manner in which hormones, physiological states, and environmental conditions modify self-selection still leaves a large number of conditions unstudied. Besides such straightforward problems, however, there are many novel questions that remain almost untouched.

A basic question that remains unanswered is the extent to which ani-

mals will work to maintain nutritional balance. We have little idea to what extent animals having calorically adequate but nutritionally imbalanced diets will strive to obtain the missing nutrients. Recently, Collier (1975, personal communication) has initiated the study of such issues and is observing that rats given free access to carbohydrate diets will emit thousands of responses daily to obtain a protein ration.

The question of how behavior patterns of animals change when deprived of specific nutrients remains open for study. Lat (1967) has done some pioneering work in this area by demonstrating that the carbohydrate/protein ratio of the food that an animal ingests is determined by the temperament of an animal—highly excitable animals consuming more carbohydrate, and unexcitable animals more protein. Conversely, the temperament of the animals was also capable of being modified by the nutrient ratios in the diets. Clues to the possible molecular cause of these changes might be derived from the findings of Fernstrom and Wurtman (1971) that dietary tryptophan can control levels of brain serotonin, a putative synaptic transmitter that appears to control the reactivity of animals. The possibility that other essential amino acids can also modify behavior is suggested by the severe psychological changes in humans on diets lacking phenylalanine-tyrosine (Williams, 1971). By depleting the essential amino acid precursors, such a diet should decrease brain norepinephrine and dopamine levels.

The area of dietary causes and amelioration of disease has become a popular topic for discussion, what with the severe mental symptoms accompanying hypoglycemia and the unverified possibility that vitamins might be useful in treating psychiatric disorders (Pauling, 1968). All the essential vitamins, ions, and trace elements are cofactors in metabolic reactions in the brain, and there is hardly any systematic work on the behavioral disorders which may ensue from a lack of the individual micronutrients. There is also now some preliminary evidence indicating that certain food contaminants may precipitate abnormal behaviors. For instance, dietary lead can precipitate hyperactivity in rodents which can be reduced by centrally acting drugs and, thus, may be an important clue to the genesis of hyperkinesis in human children (Silbergeld and Goldberg, 1974).

Finally, little is known of the brain mechanisms which participate in any of the behavioral phenomena that have been discussed. There is a study indicating that the perception of amino acid deficiencies is elaborated via the pyriform cortex (Leung and Rogers, 1971), but little else. Clearly, work on the interrelations among the brain, behavior, and diet is still in its infancy.

REFERENCES

- Altman, P. L., and Dittmer, D. S., eds. (1968). "Metabolism." Fed. Am. Soc. Exp. Biol., Bethesda, Maryland.
- Booth, D. A., and Simson, P. C. (1971). Food preferences acquired by association with variations in amino acid nutrition. *Quart. J. Exp. Psychol.* **23**, 135-145.
- Collier, G., Leshner, A. I., and Squibb, R. L. (1969a). Dietary self-selection in active and non-active rats. *Physiol. Behav.* **4**, 79-82.
- Collier, G., Leshner, A. I., and Squibb, R. L. (1969b). Self-selection of natural and purified dietary protein. *Physiol. Behav.* **4**, 83-86.
- Falk, J. L. (1971). Determining changes in vital function: Ingestion. In "Methods in Psychobiology" (R. D. Myers, ed.), Vol. 1, pp. 301-331. Academic Press, New York.
- Fernstrom, J. D., and Wurtman, R. J. (1971). Effect of chronic corn consumption on serotonin content of rat brain. *Nature (London), New Biol.* **234**, 62-64.
- Galef, B. G., and Clark, M. M. (1971). Social factors in the poison avoidance and feeding behavior of wild and domestic rat pups. *J. Comp. Physiol. Psychol.* **75**, 341-357.
- Garcia, J., and Koelling, R. A. (1966). Relation of cue to consequence in avoidance learning. *Psychon. Sci.* **4**, 123-124.
- Harper, A. E., Benevenga, N. J., and Wohlheuter, R. M. (1970). Effects of ingestion of disproportionate amounts of amino acids. *Physiol. Rev.* **50**, 428-558.
- Harris, L. J., Clay, J., Hargreaves, F. J., and Ward, A. (1933). Appetite and choice of diet. The ability of the vitamin B-deficient rat to discriminate between diets containing and lacking the vitamin. *Proc. R. Soc. London, Ser. B* **113**, 161-190.
- Kriekhaus, E. E. (1970). "Innate recognition" aids rats in sodium regulation. *J. Comp. Physiol. Psychol.* **73**, 117-122.
- Kriekhaus, E. E., and Wolf, F. (1968). Acquisition of sodium by rats: Interaction of innate mechanisms and latent learning. *J. Comp. Physiol. Psychol.* **65**, 197-201.
- Lat, J. (1967). Self-selection of dietary components. In "Handbook of Physiology, Sect. 6: Alimentary Canal, Vol. 1: Food and Water Intake" (C. F. Code and W. Heidel, eds.), pp. 367-386. Am. Physiol. Soc., Washington, D.C.
- LeMagnen, J. (1969). Peripheral and systemic actions of food in the caloric regulation of intake. *Ann. N.Y. Acad. Sci.* **157**, 1126-1156.
- Leshner, A. I., Collier, G. H., and Squibb, R. L. (1971). Dietary self-selection at cold temperatures. *Physiol. Behav.* **6**, 1-3.
- Leshner, A. I., Siegel, H. I., and Collier, G. (1972). Dietary self-selection by pregnant and lactating rats. *Physiol. Behav.* **8**, 151-154.
- Leung, P. M. B., and Rogers, Q. R. (1971). Importance of prepyriform cortex in food-intake response of rats to amino acids. *Am. J. Physiol.* **221**, 929-935.
- McCoy, R. H. (1949). Dietary requirements of the rat. In "The Rat in Laboratory Investigations" (E. J. Farris and J. Q. Griffith, eds.), pp. 68-103. Lippincott, Philadelphia.
- McDonald, D. G., Stern, J. A., and Hahn, W. W. (1963). Effects of differential housing and stress on diet selection, water intake and body weight in the rat. *J. Appl. Physiol.* **18**, 937-942.
- Mickelson, O., Takahashi, S., and Craig, C. (1955). Experimental obesity. I. Production of obesity in rats by feeding high-fat diets. *J. Nutr.* **57**, 541.
- Nance, D. M., and Kilbey, M. M. (1972). Self-selection of 5-hydroxytryptophan in D1-*para*-chlorophenylalanine treated rats. *Physiol. Behav.* **8**, 545, 547.
- "Nutrient Requirements of Laboratory Animals" (1972). Pamphlet No. 10. Natl. Acad. Sci., Washington, D.C.

- Panksepp, J. (1971). Effects of fats, proteins, and carbohydrates on food intake in rats. *Psychon. Monogr. Suppl.* **4**, 85-95.
- Panksepp, J., and Krost, K. (1975). Modification of diurnal feeding patterns by palatability. *Physiol. Behav.* **15**, 673-677.
- Pauling, L. (1968). Orthomolecular psychiatry. *Science* **160**, 265-271.
- Revusky, S. H., and Bedarf, E. W. (1967). Association of illness with prior ingestion of novel foods. *Science* **155**, 219-220.
- Richter, C. P. (1942-1943). Total self-regulatory functions in animals and human beings. *Harvey Lect. Ser.* **38**, 63-103.
- Richter, C. P., Schmidt, E. C. H., and Malone, P. D. (1945). Further observations of the self-regulatory dietary selection of rats made diabetic by pancreatectomy. *Bull. Johns Hopkins Hosp.* **76**, 192-219.
- Rozin, P. (1968). Specific aversions and neophobia as a consequence of vitamin deficiency and/or poisoning in half-wild and domestic rats. *J. Comp. Physiol. Psychol.* **66**, 82-88.
- Rozin, P., and Kalat, J. W. (1971). Specific hungers and poison avoidance as adaptive specializations of learning. *Psychol. Rev.* **6**, 459-486.
- Silbergeld, E. K., and Goldberg, A. M. (1974). Lead-induced behavioral dysfunction: An animal model of hyperactivity. *Exp. Neurol.* **42**, 146-157.
- Simson, P. C., and Booth, D. A. (1973). Effects of CS-US interval on the conditioning of odour preferences by amino acid loads. *Physiol. Behav.* **11**, 801-808.
- Taylor, C. J., and Bruning, J. L. (1967). Effects of nonnutritive bulk on eating behavior. *J. Comp. Physiol. Psychol.* **64**, 353-355.
- Williams, H. L. (1971). The new biology of sleep. *J. Psychiat. Res.* **8**, 445-478.
- Young, P. T. (1944). Studies of food preference, appetite and dietary habit. II. Group self-selection maintenance as a method in the study of food preferences. *J. Comp. Psychol.* **37**, 371-391.

Appendix

Foods and food processing

General Biochemicals
Laboratory Park
Chagrin Falls, Ohio 44022

The P. J. Noyes Co.
101 Main St.
Lancaster, New Hampshire 03584

Nutritional Biochemicals Corp.
ICN Life Sciences Group
26201 Miles Rd.
Cleveland, Ohio 44128

Drugs and biochemicals

Aldrich Chemical Co., Inc.
940 W. St. Paul Ave.
Milwaukee, Wisconsin 53233

Calbiochem
10933 N. Torrey Pines Rd.
La Jolla, California 92037

Sigma Chemical Co.
P.O. Box 14508
St. Louis, Missouri 63178

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