Brit. J. Pharmacol. (1954), **9**, 31.

IDENTIFICATION OF THE STABLE ANTIDIURETIC SUBSTANCE ("STABLE ADS") OF SERUM WITH 5-HYDROXYTRYPTAMINE

BY

V. ERSPAMER AND G. SALA

From the Institute of Pharmacology, University of Bari, Italy

(RECEIVED JULY 28, 1953)

Rat serum contains, according to Ginsburg and Heller (Ginsburg and Heller, 1951; Heller, 1952; Ginsburg and Heller, 1953), two antidiuretic factors. The first, called "unstable antidiuretic substance" ("unstable ADS"), disappears on standing and is most abundant in heart or jugular serum—that is, in serum obtained from blood returning from the head: its posterior pituitary origin seems probable. The second factor, provisionally called "stable antidiuretic substance" ("stable ADS"), is not inactivated on standing, is found in arterial serum, seems to originate during coagulation—the same amount of plasma tested by the same method did not modify urine flow significantly—and to manifest its antidiuretic action only when injected subcutaneously (Ginsburg and Heller, 1953): its origin and nature are uncertain.

On the basis of unpublished observations, Ginsburg and Heller (1950) reported that they were unable to identify the "stable ADS" with 5-hydroxytryptamine (serotonin, enteramine).

This seemed surprising, since all the characteristics of the "stable ADS" noted above are shared by 5-hydroxytryptamine. Moreover, rat serum, in which "stable ADS" was first found, contains quantities of 5-hydroxytryptamine which are sufficient to influence the diuresis of hydrated rats. In fact the average 5-hydroxytryptamine content of rat serum is 0.97 μg./ml. (Erspamer and Faustini, 1953) and the minimum antidiuretic dose of this substance, by the subcutaneous route, is 0.4 μg. per 100 g. of body weight (Erspamer and Ottolenghi, 1953).

When given intraperitoneally 5-hydroxytryptamine is 5 to 10 times less effective than when injected subcutaneously (Erspamer and Ottolenghi, 1953). Ames and Van Dyke (1952) found that 4 or 6 μg. serotonin given intravenously had no antidiuretic effect in rats.

The experiments now to be described were designed to determine whether 5-hydroxytryptamine is identical with Ginsburg and Heller's "stable ADS."

METHODS

Blood was obtained by decapitation, except in dogs and in man, when it was taken from the femoral artery or from the antecubital vein. After collection it was kept for 2 to 3 hours at room temperature and then for a further 15 to 20 hours in a refrigerator. The serum was separated by centrifugation.

Serum samples were obtained from 20 human beings, 15 rats, 10 rabbits, 3 dogs, 3 cats, 2 goats, and 10 hens.

In some experiments native serum was compared with acetone extracts of serum, but in most experiments acetone extracts only were studied. To prepare these extracts, serum was treated with 4 volumes of acetone, was left standing overnight in the refrigerator and then filtered. The solvent was evaporated under reduced pressure just before use, and was then brought to the desired volume with distilled water. The acetone extracts keep indefinitely and thus offer homogeneous material for several successive experiments.

Estimations of antidiuretic activity were made on unselected adult albino rats of both sexes, weighing from 150 to 270 g. In all, 180 groups of four animals each were used. Two doses of water, the first of 2.5 ml./100 g., and the second of 5 ml./100 g., were given by stomach tube with an interval of 3 hours.

Serum, serum extracts, 5-hydroxytryptamine solutions (Farmitalia, S.p.A.) and control saline were injected subcutaneously (1 ml./100 g. rat) immediately after the second dose of water. The urine output was measured for seven hours thereafter, at intervals of 30 to 60 min.

RESULTS

Effect of Serum and of Acetone Extracts of Serum on Water Diuresis.—In rats and rabbits we have made separate assays of serum, of acetone extracts of serum, and of serum proteins precipitated by acetone and dissolved in water.

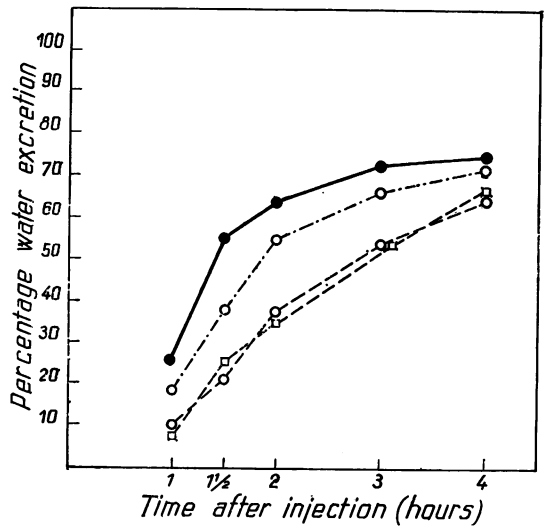

FIG. 1.—The antidiuretic effect of rabbit serum compared with that of the acetone extract of the same serum. Unanaesthetized rats. Subcutaneous injections. ●———● 1.0 ml. saline per 100 g.; ○----○ 1.0 ml. serum per 100 g.; ○-·-·-·-○ 0.1 ml. serum per 100 g.; □-·-·-□ acetone extract corresponding to 1.0 ml. serum per 100 g. Each point of the curves is the mean of results from 3 groups of 4 animals each.

Fig. 1 clearly shows that sera and their acetone extracts possess the same antidiuretic activity. The antidiuretic principle of serum has therefore passed completely into the acetone.

The moderate antidiuretic action provoked by the acetone precipitate was not further investigated, but it may be due to breakdown products of serum proteins (cf. oxytocic action of pepsitensin, as described by Croxatto, Rojas, and Barnafi, 1950).

Because of these first results only acetone extracts of serum were used in the subsequent experiments.

Antidiuretic Action of Extracts Prepared from Sera of Different Species.—Extracts of dog, cat, goat, rabbit, rat, hen, and human serum were compared with one another and with solutions of 5-hydroxytryptamine. The results are shown in Tables I and II.

Quantitative estimations of 5-hydroxytryptamine on the rat uterus (Erspamer, 1940a) gave, in terms of μg. of 5-hydroxytryptamine base per ml. of serum, the following values for the different extracts: man, 0.16; dog, 0.18; cat, 5.60; goat, 2.20; rabbit, 4.30; rat, 1.05; hen, 2.70.

Having previously demonstrated that the antidiuretic action of 5-hydroxytryptamine is only approximately proportional to the dose (Erspamer and Ottolenghi, 1953), we now find that for the majority of the sera examined there is a satisfactory agreement between antidiuretic effect and 5-hydroxytryptamine content. Extracts of cat and hen serum are exceptions; these have much less antidiuretic action than one would expect from their 5-hydroxytryptamine content.

It is difficult to account for these discrepancies. It may be that serum contains substances which retard the absorption of 5-hydroxytryptamine from the site of injection, or substances which, without modifying its absorption, interfere directly with its renal effects. Further work is necessary to clarify this point.

Tables I and II show that, of the various species tested, the extract of rabbit serum had the most powerful antidiuretic effect. Doses of this extract corresponding to 0.1 ml. of serum per 100 g. of body weight caused a significant reduction in urine flow. Extracts of rabbit serum were therefore used for experiments on inactivation and inhibition of the antidiuretic substance.

Antidiuretic Action of the Rabbit Serum Extracts after Treating with Diazonium Salts and with Guinea-pig Intestine Homogenates. — Diazonium salts (Erspamer, 1940b, 1948) and extracts of guinea-pig intestine (Erspamer, 1942) have been shown to inactivate crude enteramine extracts. The following liquids were therefore given subcutaneously to rats in doses of 1 ml. per 100 g.:

TABLE I

THE EFFECT OF SUBCUTANEOUS INJECTION OF SERUM EXTRACTS FROM DIFFERENT ANIMAL SPECIES (AMOUNTS OF EXTRACT EQUIVALENT TO 1·0 ML. SERUM PER 100 G. OF BODY WEIGHT) ON THE WATER DIURESIS OF HYDRATED RATS

Species from which Serum Extract was Prepared	No. of Rats	Percentage Water Excretion					
		1 hr.	1½ hr.	2 hr.	3 hr.	4 hr.	7 hr.
Nil (saline control)	40	29·2	54·0	62·5	73·2	78·0	93·0
Man	8	35·0	61·2	70·0	72·0	76·0	87·0
Dog	24	29·3	55·7	67·0	77·8	78·3	91·0
Cat	16	21·0	43·0	54·0	69·0	73·0	85·0
Goat	16	19·0	37·0	47·0	57·0	64·0	79·5
Rabbit ..	24	3·2	14·7	26·0	48·2	57·3	80·3
Rat	8	20·5	39·0	53·0	63·0	72·5	92·5
Hen	8	21·0	33·0	53·0	62·5	65·0	86·0

TABLE II

THE ANTIDIURETIC EFFECT OF DIFFERENT DOSES OF THE ACETONE EXTRACT OF RABBIT SERUM

	No. of Rats	Percentage Water Excretion				
		1 hr.	1½ hr.	2 hr.	3 hr.	4 hr.
Saline	40	29·2	54·0	62·5	73·2	78·0
0·05 ml. serum/100 g. ..	16	28·0	51·5	60·5	69·0	76·0
0·1 „ „ „ ..	16	19·3	41·8	58·3	66·5	71·0
0·2 „ „ „ ..	24	13·6	35·5	52·3	65·3	70·0
0·5 „ „ „ ..	32	6·6	22·2	37·8	53·2	68·8
1·0 „ „ „ ..	24	3·2	14·7	26·0	48·2	57·3
0·004 mg. 5-hydroxytryptamine/100 g. ..	48	8·7	25·7	39·7	55·3	66·8

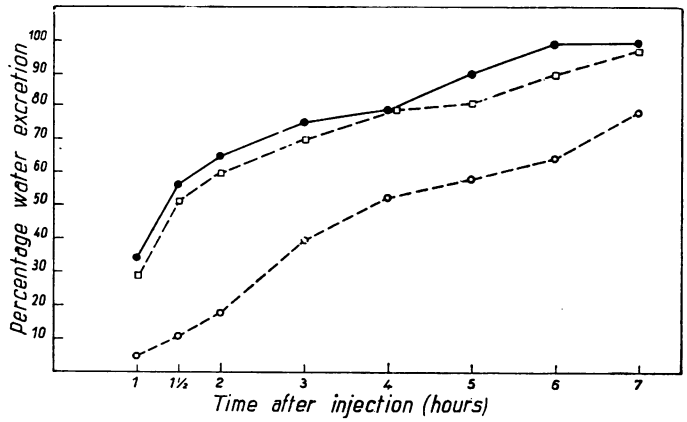

FIG. 2.—The antidiuretic effect of rabbit serum extract, 1.0 ml. per 100 g., before (○- - -○) and after (□- - -□) treating with diazotized *p*-nitroaniline. ●———● 1 ml. saline per 100 g. Each point of the curves is the mean of results from 2 groups of 4 animals each.

(a_1) *Serum Extract Treated with Diazotized* p-*Nitroaniline.*—The extract from 30 ml. rabbit serum was concentrated under reduced pressure, then brought to 21 ml. with distilled water; 4.5 ml. of diazotized *p*-nitroaniline and 4.5 ml. of 2% sodium bicarbonate were added. A reddish-yellow colour was produced.

(a_2) *Control Saline.*—21 ml. saline was substituted for the serum extract.

(b_1) *Serum Extract Treated with Homogenate of Guinea-pig Intestine.*—The extract corresponding to 20 ml. of serum was evaporated under reduced pressure and the dry residue taken up with 4 ml. of 0.07 M phosphate buffer at *p*H 7.4. After adding 4 ml. of a homogenate of guinea-pig small intestine diluted to 1/4 with phosphate buffer, the liquid was kept for 1 hour in a water bath at 39° C. and oxygen was bubbled through. 30 ml. acetone was now added. After standing in a refrigerator overnight the liquid was filtered, freed from the acetone by distillation under reduced pressure, and then brought to 40 ml. with distilled water (1 ml. liquid =0.5 ml. serum).

(b_2) *Serum Extract Treated with Guinea-pig Intestine Homogenate and Methylene Blue.*— The sample was treated exactly as above with the sole difference that the addition of the intestine homogenate was preceded by that of 1 ml. of 0.002 M methylene blue.

(b_3) *Control.*—Treated as b_1, but with saline substituted for serum extract.

Figs. 2 and 3 show the results of these experiments. It will be seen that treatment with both diazonium salts and guinea-pig intestine homogenate

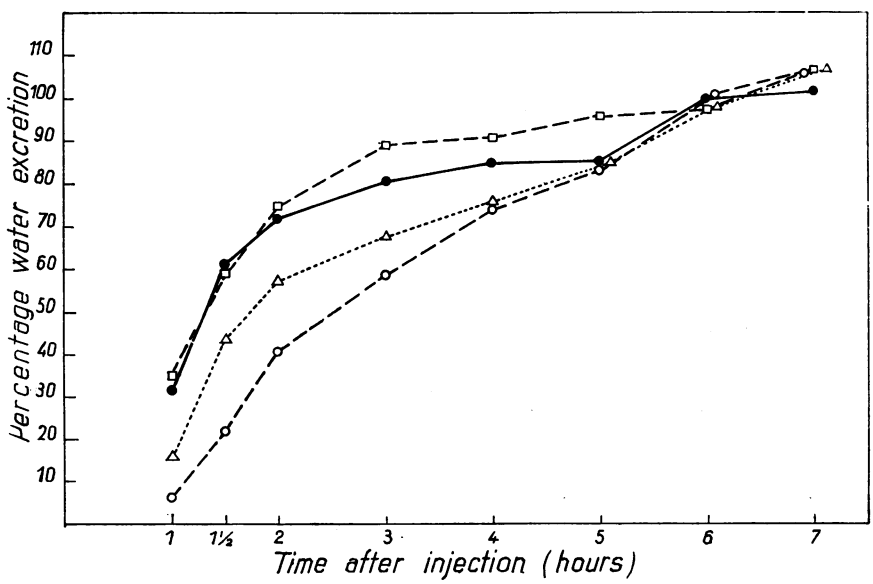

FIG. 3.—Inactivation of the extract of rabbit serum by homogenates of guinea-pig small intestine. ●———● 1.0 ml. saline per 100 g.; ○- - -○ untreated extract corresponding to 0.5 ml. serum per 100 g.; □- - -□ the same quantity of extract after incubation with homogenate of guinea-pig intestine; △.....△ the same quantity of extract after incubation with intestine homogenate + methylene blue. Each point of the curves is the mean of results from 4 groups of 4 animals each.

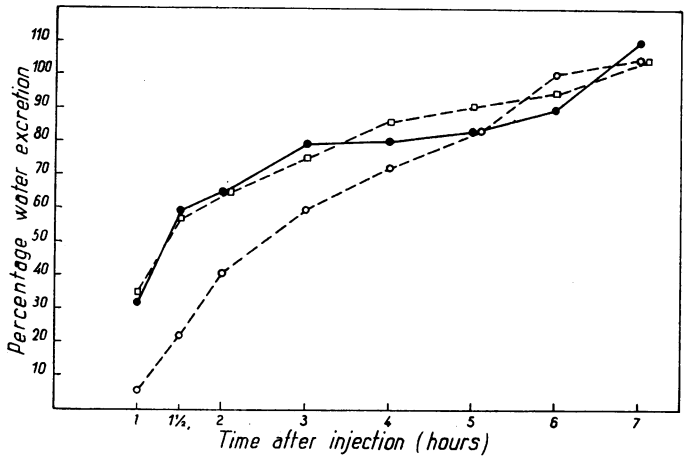

FIG. 4.—The antidiuretic effect of rabbit serum extract, 0·5 ml. per 100 g., on normal rats (○- - - -○) compared with that on rats pretreated with 10 mg./kg. of dibenamine intraperitoneally (□- - - -□). ●——● 1.0 ml. saline per 100 g. Each point of the curves is the mean of results from 3 groups of 4 animals each.

completely destroys the antidiuretic activity of the serum extracts, exactly as it destroys the spasmogenic and the antidiuretic activity of crude enteramine extracts and of pure 5-hydroxytryptamine (Erspamer, 1940b, 1942, 1948, 1953a).

It is very probable that the inactivating agent in the intestine homogenate is amine oxidase. This view is supported by the observation that the disappearance of the antidiuretic effect of serum is at least partly prevented by adding methylene blue —a known inhibitor of amine oxidase (Blaschko, 1952)—to the homogenate.

Controls carried out on the atropinized oestrous uterus of the rat showed that the liquids a_1 and b_1 had no stimulant action, while liquid b_2 had about 30% of the activity of the untreated serum extract. The incomplete inhibition of amine oxidase by methylene blue can be ascribed to the long duration of the inactivating treatment, combined with the very high content of enzyme in the guinea-pig small intestine.

Antidiuretic Effect of the Rabbit Serum Extract in Rats Treated with Dibenamine.—Dibenamine is one of the most powerful antagonists of 5-hydroxytryptamine (Erspamer, 1952, 1953b; Fingl and Gaddum, 1953). Three groups of 4 animals each were injected intraperitoneally with 0.5 ml. of 0.2% dibenamine hydrochloride solution per 100 g. of body weight, 3 hours before the administration of the rabbit serum extract (0.5 ml./100 g.). Fig. 4 shows that treatment with dibenamine produced a complete inhibition of the antidiuretic effect of the serum extract.

Influence of the Route of Administration on the Antidiuretic Effect of the Rabbit Serum Extract.—Fig. 5 shows that the subcutaneous route is much more effective than the intraperitoneal one. The decrease in urine flow was more pronounced after 0.2 ml. of serum extract given subcutaneously than after 1 ml. intraperitoneally. Doses of extract corresponding to 0.5 ml. of serum were completely ineffective by the intraperitoneal route.

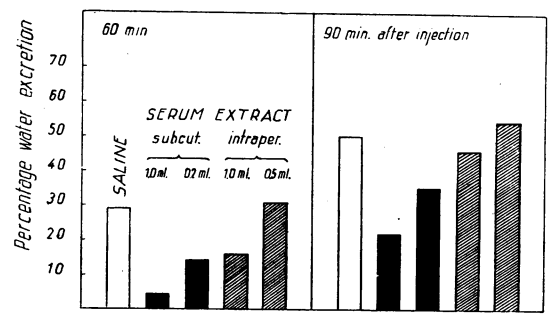

FIG. 5.—Influence of the route of administration on the antidiuretic effect of rabbit serum extract. The doses shown were given per 100 g. of body weight. Each column represents the mean of results from 2 groups of 4 animals each.

An identical behaviour has been described for 5-hydroxytryptamine by Erspamer and Ottolenghi (1953).

Preliminary Experiments on the Mechanism of the Antidiuretic Action of Serum.—Three groups of 4 rats were injected subcutaneously with a dose of extract corresponding to 1 ml. of rabbit serum per 100 g. of body weight and, at the same time but in a different site, with 0.5 ml./100 g. of a 4% solution of creatinine (200 mg./kg.). Creatinine was also administered to three control groups.

Fig. 6 shows that the extract of rabbit serum, besides reducing urinary flow, also reduces (though apparently for a shorter period) the renal excretion of creatinine. This signifies that, in the mechanism of the antidiuretic action of serum, decreased

glomerular filtration must play an important if not an exclusive part.

Since rabbit serum, given in subcutaneous doses of 2 ml./100 g. or less, does not modify the systemic blood pressure of the intact, unanaesthetized rat, we conclude that the decrease in filtration rate is due to constriction of the afferent glomerular arterioles causing a decrease in intraglomerular pressure.

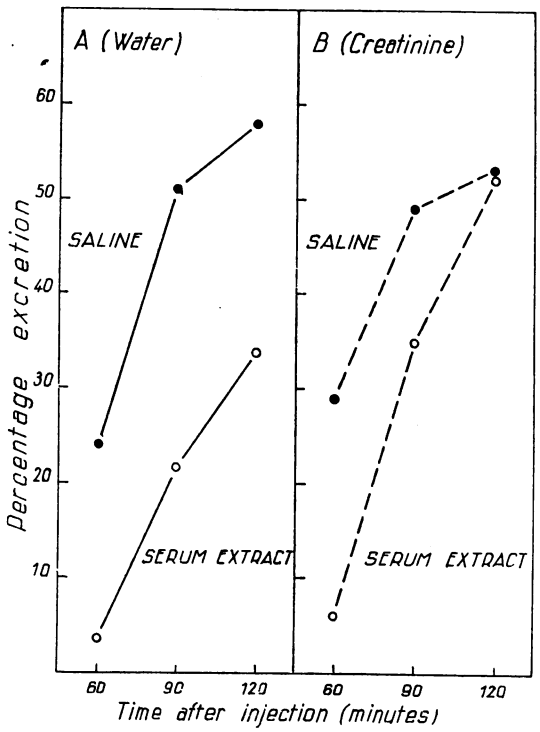

FIG. 6.—Comparison between the renal excretion of water (5 ml./100 g.) and that of creatinine (200 mg./kg.) after subcutaneous injection of extract corresponding to 0.5 ml. rabbit serum per 100 g. Each point of the curves is the mean of results from 2 groups of 4 animals each.

The vascular point of attack of 5-hydroxytryptamine in the rat kidney has been emphasized by Erspamer and Ottolenghi (1953).

DISCUSSION

The findings of Dicker and Ginsburg (1950) and of Ginsburg and Heller (1951, 1953) on the presence of a stable antidiuretic factor ("stable ADS") in rat serum were completely confirmed. Furthermore, it was demonstrated that the same factor is contained in rabbit, cat, goat, and hen serum, often in very considerable quantity.

The "stable ADS" has the following characteristics: (*a*) it is apparently present only in serum and not in plasma, since it becomes detectable by our methods only after coagulation; (*b*) it is more active after subcutaneous injection than after administration by other routes; (*c*) it may be extracted from serum by acetone; (*d*) it is completely destroyed by treating the serum with diazonium salts and with homogenates of guinea-pig small intestine (amine oxidase), but it is not attacked by serum enzymes; (*e*) it is ineffective when administered after dibenamine; (*f*) finally, it provokes antidiuresis by a decrease of glomerular filtration rate caused by an afferent vasoconstriction. All these characteristics of "stable ADS" are shared by 5-hydroxytryptamine.

It should be added that 1 ml. rat serum when given per 100 g. rat contains more than the minimum antidiuretic dose of 5-hydroxytryptamine. The antidiuretic effect of serum from different species is, with a few exceptions, satisfactorily proportional to its 5-hydroxytryptamine content, as established by bioassay on the atropinized oestrous uterus of the rat. Human serum has very little 5-hydroxytryptamine; its content per ml. is lower than the minimum antidiuretic dose per 100 g. of rat. Dog serum is similar. Rabbit serum is very rich in 5-hydroxytryptamine, and has a powerful antidiuretic action.

All these results strongly support the identity of the "stable ADS" with 5-hydroxytryptamine, and no observations exist, as far as we know, which are unfavourable to this conclusion.

From the recent work of Erspamer and Faustini (1953), the hydroxytryptamine content of the serum of more than 30 animal species, including practically all the domestic and laboratory ones, is now known. It is easy to predict, with good approximation, the antidiuretic action of these sera in hydrated rats. One can presume that only sera with a 5-hydroxytryptamine content above 0.3–0.4 μg./ml. are likely to elicit a detectable antidiuretic response.

Heller (1952) found that the "stable ADS" was usually present in rat serum, but not invariably so. The occasional failure to detect this substance may now be explained by our finding that the 5-hydroxytryptamine content of rat serum varies considerably—from 0.57 to 1.75 μg./ml. in 35 serum samples. When 1 ml./100 g. is injected subcutaneously into rats, the lower values nearly coincide with the minimum antidiuretic dose of pure 5-hydroxytryptamine.

Work is in progress to investigate the changes in the 5-hydroxytryptamine content of serum under various experimental and pathological conditions.

Summary

1. Serum and acetone extracts of serum from normal rats, rabbits, cats, goats, and hens have a marked antidiuretic effect when injected subcutaneously into hydrated rats. The presence in serum of an antidiuretic factor of non-pituitary origin ("stable ADS" of Ginsburg and Heller, 1953) is confirmed.

2. The antidiuretic factor is inactivated *in vitro* by diazonium salts and by homogenates of guinea-pig small intestine (amine oxidase), and its action is blocked *in vivo* by dibenamine. Its activity is much more conspicuous after subcutaneous injection than after intraperitoneal administration.

3. Preliminary experiments on the renal excretion of creatinine by rats show that the antidiuretic effect of serum extracts may be ascribed mainly to a reduction in glomerular filtration rate produced by constriction of afferent glomerular arterioles.

4. Since (1) the "stable ADS," like 5-hydroxytryptamine, apparently originates in serum during coagulation, (2) the two substances share many chemical and pharmacological characteristics, (3) their mechanism of renal action appears to be the same, and (4) serum from different animal species shows an antidiuretic action which is satisfactorily proportional to its 5-hydroxytryptamine content, it seems justifiable to postulate the identity of the stable antidiuretic substance, of serum and of serum extracts, with 5-hydroxytryptamine.

References

Ames, R. G., and Van Dyke, H. B. (1952). *Endocrinology*, **50**, 350.

Blaschko, H. (1952). *Pharmacol. Rev.*, **4**, 415.

Croxatto, H., Rojas, G., and Barnafi, L. (1950). *Bol. Soc. Biol. Santiago*, **8**, 84.

Dicker, S. E., and Ginsburg, M. (1950). *Brit. J. Pharmacol.*, **5**, 497.

Erspamer, V. (1940a). *Arch. exp. Path. Pharmak.*, **196**, 343.

—— (1940b). Ibid., **196**, 366.

—— (1942). Ibid., **200**, 43.

—— (1948). *Acta pharmacol., Kbh.*, **4**, 213.

—— (1952). *Ricerca scient.*, **22**, 1568.

—— (1953a). Unpublished observations.

—— (1953b). *Arch. int. Pharmacodyn.*, **93**, 177.

—— and Faustini, R. (1953). *Naturwiss.*, **40**, 317.

—— and Ottolenghi, A. (1953). *Arch. int. Pharmacodyn.*, **93**, 177.

Fingl, E., and Gaddum, J. H. (1953). *Fed. Proc.*, **12**, 320.

Ginsburg, M., and Heller, H. (1950). Quoted by Dicker, S. E., and Ginsburg, M.(1950).

—— —— (1951). *J. Physiol.*, **115**, 43*P*.

—— —— (1953). *J. Endocr.*, **9**, 274.

Heller, H. (1952). *Ciba Found., Coll. on Endocr.*, **4**, 463.