J. Physiol. (1939) 96, 293–301 612.492.8:612.463.1

THE APPARENT AUGMENTATION OF PITUITARY ANTIDIURETIC ACTION BY VARIOUS RETARDING SUBSTANCES

By R. L. NOBLE,[1] H. RINDERKNECHT and P. C. WILLIAMS[2]

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1

(*Received* 14 *April* 1939)

Anselmino & Hoffman [1931] reported the presence of postuitary[3] antidiuretic hormone in the blood and Teel & Reid [1939] in the urine of eclamptics, though the findings of the former workers have been disputed [Byrom & Wilson, 1934; Melville, 1937]. The excretion of the hormone in the urine of dehydrated animals seems well established [Gilman & Goodman, 1937; Ingram, Ladd & Benbow, 1938; Boylston & Ivy, 1938]. A patient with symptoms suggestive of postuitary hyperfunction [Jones, 1938] was shown to have pressor and antidiuretic activity in his urine [Noble, Rinderknecht & Williams, 1938].

In this paper we wish to draw attention to the objections to quantitative assays based on the length of antidiuresis following the *subcutaneous* injection of impure extracts. So far, all the antidiuretic assays quoted, with the exception of those of Melville [1937] have been performed with subcutaneous injection. It has been shown that the presence of salts of certain metals in postuitary extracts may prolong the absorption and antidiuretic activity when given subcutaneously [Dodds, Noble, Rinderknecht & Williams, 1937]. This work has been extended and it has been found that certain anions, organic compounds and substances extracted from blood and urine may have a similar action.

[1] Work done during tenure of Leverhulme Fellowship, Royal College of Physicians.

[2] Working with a grant from the Medical Research Council.

[3] Throughout this paper the term postuitary will be used to refer to the posterior lobe of the pituitary gland.

Methods

The postuitary extract (PP) used was made from acetone dried ox pituitary posterior lobe by the method of Kamm, Aldrich, Grote, Rowe & Bugbee [1928]. Assayed on spinal cats it had an activity of approximately 10 pressor units per mg. It was kept in the cold in a solution made slightly acid with acetic acid and when required was diluted to form a solution containing 10 μg./c.c. either with water or with the solution whose augmenting activity was to be tested.

The antidiuretic test was carried out by the method of Burn [1931]. A batch of four adult male rats (average weight about 200 g.) was starved overnight and in the morning each rat was given 10 c.c. water by mouth and a simultaneous subcutaneous injection of 0·2 c.c. (2 μg. PP) of the solution to be tested. The rats were then placed in a metabolism cage and the volume of urine excreted was measured every 15 min. When the diuresis was completed the measurements were graphed and the time elapsing between the administration of the water and the time at which half the total volume of urine was excreted was read off. This time is referred to throughout as the 50 % excretion time.

Results

The 50 % excretion time when water alone was given with no simultaneous injection was $87 \pm 3{\cdot}2$ min. (standard error of the mean of 25 determinations). Heller [1937] and Boylston & Ivy [1938] gave corresponding values of 86 ± 11 and $87 \pm 2{\cdot}8$ respectively. When the postuitary extract PP was injected alone the 50 % excretion time was $134 \pm 2{\cdot}2$ (85 tests), but when combined with the augmentor substances this figure was higher. The standard deviation of the individual observations in the two cases was 16 min. and 20 min. respectively.

Metallic ions

The 50 % excretion times when the postuitary extract was injected in solutions of various metallic salts are given in Table I. Consideration of the results obtained showed that the metals could be roughly divided into four groups. The first group were diuretic by themselves and so overrode the antidiuretic action of the injected PP. These metals were palladium, mercury, silver, gold and uranium. The second and largest group comprised those metals which either had no prolonging action or whose action gave a 50 % excretion time differing by less than 40 min. (twice the standard deviation) from that given by PP alone. Such a

degree of prolongation could not be regarded as significant. The third group comprising trivalent iron, cobalt and manganese gave definite augmentation but only in the comparatively high concentration of 4 %. (This concentration was adopted for the initial tests as control experiments with injections of sodium chloride solutions showed that solutions with higher concentrations exerted an antidiuretic action by themselves, presumably caused by the hypertonicity of the injection.) With the third group, however, a very marked degree of augmentation was found. The metals concerned were zinc, nickel, and cadmium. In the table is given, for purposes of comparison, the dose of PP that would be required by itself to produce such an antidiuretic effect. It can be seen that the augmentation with 1 % zinc or nickel acetate was equivalent to a twenty-fold increase in the dosage of PP. In the case of cadmium such an increase was obtained with as low a concentration as 0·1 %. These prolonged 50 % excretion times could only be judged approximately, since the

TABLE I. Augmentation of pituitary antidiuresis by metallic salts

Group	Injection		50 % excretion time (min.)	Approx. dose PP required to produce similar antidiuresis (μg.)
1	(No injection—water alone)		87	—
	2 μg. PP + Palladium chloride	4 %	53	—
	,, Mercuric acetate	4 %	60	—
	,, Silver lactate	4 %	73	—
	,, Gold chloride	4 %	84	—
	,, Uranyl acetate	4 %	103	—
2	,, (PP alone)		134	—
	,, Ferrous sulphate	4 %	116	—
	,, Titanous chloride	4 %	122	—
	,, Sodium alum	4 %	132	2
	,, Chromium trichloride	4 %	139	—
	,, Stannous chloride	4 %	146	3
	,, Iron alum	4 %	150	—
	,, Lead acetate	4 %	155	4
	,, Calcium chloride	4 %	161	—
	,, Thorium chloride	4 %	162	5
	,, Copper sulphate	4 %	171	—
	,, Magnesium chloride	4 %	173	—
3	,, Ferric chloride	4 %	187	12
	,, Cobalt acetate	4 %	198	15
	,, Manganese chloride	4 %	201	—
	,, Dialysed iron		230	25
4	,, Zinc acetate	0·1 %	260	30
	,, ,,	1·0 %	370	40
	,, Nickel acetate	0·02 %	133	2
	,, ,,	0·1 %	233	24
	,, ,,	1·0 %	360	40
	,, Cadmium acetate	0·01 %	190	13
	,, ,,	0·1 %	300	35

diuresis was not completed in the 8–10 hr. that the experiment was followed.

Control experiments in which the PP and augmentor substances were given in different sites produced no prolongation neither did the metallic solutions by themselves have any antidiuretic effect. (It should be mentioned that nickel and cadmium acetates in a concentration of 5 % were toxic and did have antidiuretic action. The animals so injected usually died. With concentrations of 1 % and below there were no antidiuretic effects and the animals survived.)

Organic substances and anions

Apart from the metallic ions above, some organic substances (gelatine, various protamines and amino-acids) were found to have augmenting activity. Some were insoluble and were consequently given in suspension. The different mode of augmentation brought into play by such procedure will be discussed below. The results are recorded in Table II.

TABLE II. Augmentation of pituitary antidiuresis by organic solutions and anions

Substance	Concentration %	Condition	50 % excretion time (min.)
Salmine	1·0	Suspension	198
	2·5	Suspension	267
	5·0	Suspension	230
	10·0	Suspension	199
Clupeine	2·5	Suspension	245
	5·0	Suspension	273
Glutamic acid	1·0	Solution	178
	5·0	Solution	210
	10·0	Solution	211
	15·0	Solution	253
Arginine	1·0	Solution	230
	2·5	Solution	221
	5·0	Solution	222
Tyrosine	1·0	Suspension	159
	2·5	Suspension	209
	5·0	Suspension	163
Gelatine	1·0	Suspension	189
Potassium ferrocyanide	0·1	Solution	195
	1·0	Solution	257
	2·5	Solution	257
Sodium citrate	1·0	Solution	184
Sodium nitrite	2·0	Solution	216
Sodium cyanate	1·0	Solution	190
2 μg. PP alone	—	—	134±20

Also in this table are given the results obtained with certain metallic salts whose action must be due to the anions present since the sodium

and potassium ions when combined with acetate or chloride had no augmenting action. These salts were soluble; the most active was ferrocyanide.

It can be seen from the table that the action of these substances was not so pronounced as that of the three most active metals. Also there was a tendency for the augmentation to reach a maximum and not to vary very greatly with increasing concentration. In fact, in the cases of salmine and tyrosine, increasing the concentration beyond a certain value produced a decrease in augmentation.

Extraction of prolonging substances from blood and urine

Salicylsulphonic acid precipitation has been used as a method for the extraction of gonadotrophic material from pregnant mare serum [Rinderknecht, Noble & Williams, 1939]. This procedure precipitated most of the serum proteins while leaving the hormone complex in the supernatant fluid. An attempt was made to adapt this method to the extraction of added PP from blood. Preliminary experiments showed the PP to be extracted together with the augmentor substances, since there was a more than 100 % recovery when assayed by the antidiuretic test.

The treatment was as follows: 40 c.c. of horse blood were diluted to 80 c.c. with distilled water and precipitated with 5 c.c. of 33·3 % salicylsulphonic acid. The precipitate was removed after centrifugation, washed with 40 c.c. of water and centrifuged again. The combined supernatants were concentrated to 20 c.c. and dialysed for 48 hr., centrifuged free from insoluble material and concentrated to a small volume. This final product when injected with 2 μg. PP gave a prolonged antidiuresis (B 5*a*) though by itself it had no antidiuretic action. In another experiment 40 c.c. of horse serum were similarly treated and the final volume evaporated to dryness. A brown powder weighing 50 mg. was obtained. This product in a concentration of 6 mg. (roughly equivalent to 5 c.c. serum) in 1 c.c. also gave augmentation (B 11*a*) while its ash did not (B 11*g*), suggesting that the augmentation was due to the organic content. These results are given in Table III.

Other active augmentors derived from blood and tissues were the salicylsulphonic acid precipitate from horse serum which was very active (BP 1) particularly as a suspension (BP 2) and a hydrochloric acid hydrolysate of lean beef (MH 1). Horse serum itself had prolonging action.

The extraction of postuitary hormones from urine may also be effected by simultaneous extraction of augmentor substances. It has been shown [Noble *et al.* 1938] that extracts from the urine of a patient with symptoms

suggestive of postuitary hyperfunction contained antidiuretic and pressor factors, the antidiuretic potency, however, being greater than the pressor. (This discrepancy was most easily explained by the augmentation in the antidiuretic assay.) This extract was made by a not very successful method depending on the process of Kamm *et al.* [1928] for the extraction of posterior pituitary glands. A method for the extraction of hormones from urine (British Patent No. 454,778 (15 May 1936)) has been used in slightly modified form for the extraction of postuitary hormones from urine.

Method of extraction. To 100 c.c. of urine adjusted to *p*H 5, 1 c.c. of 2*N* zinc sulphate was added. 1·125 c.c. of 2*N* $K_4Fe(CN)_6$ were then added drop by drop with vigorous stirring. After cooling in the ice-chest the supernatant was decanted and the remaining mixture centrifuged. The precipitate was extracted subsequently with three 25 c.c. portions of 80 % alcohol containing 1 % ammonia. A clear extract was obtained which was evaporated to a small volume *in vacuo* at 15°. The resultant solution was acidified and had a deep brown colour. Further purification could be effected by removal of some of the contaminants by precipitation with 10 volumes of absolute alcohol. This procedure effected a 70–90 % recovery as measured by pressor tests on spinal cat. Amounts of postuitary extract as small as one unit per litre could be recovered and as a routine the final product was evaporated to 1 % of the volume of the original urine.

When a known amount of pituitary principle was added to urine and this method of extraction used it was found that the antidiuretic assay showed a yield of more than 100 %. Evidently augmentor substances had been extracted as well. (Urine N in Table III is the extract from

TABLE III. Augmentation of pituitary antidiuresis by substances extracted from blood, urine and tissues

Starting material	Extract	Concentration %	Dose	Equivalent dose starting material	50 % excretion time
—	—	—	Water alone	—	87
—	—	—	2 μg. PP alone	—	134
Horse blood	B 5*a*	*ca.* 1	0·5 c.c. + 2 μg. PP	4 c.c.	236
Horse blood	B 5*a*	*ca.* 1	0·5 c.c. alone	4 c.c.	97
Horse serum	B 11*a*	0·6	0·2 c.c. + 2 μg. PP	1 c.c.	195
Horse serum	B 11*g*	—	0·4 c.c. + 2 μg. PP	1 c.c.	155
Horse serum	BP 1	1·25	0·4 c.c. + 2 μg. PP	—	205
Horse serum	BP 2	1·25	0·4 c.c. + 2 μg. PP	—	260
Horse serum	—	—	0·4 c.c. + 2 μg. PP	0·4 c.c.	203
Lean beef	MH 1	2·5	0·4 c.c. + 2 μg. PP	0·01 g.	216
Human urine	N	—	0·2 c.c. + 2 μg. PP	2 c.c.	223
Human urine	N	—	0·2 c.c. alone	—	75

a normal sample. By itself it had no antidiuretic activity but when injected with 2 μg. PP there was definite prolongation.) This finding suggests that the figures given for the excretion of antidiuretic hormone following dehydration [Gilman & Goodman, 1937; Boylston & Ivy, 1938] or hypertonic saline ingestion [Bundschuh & Kuschinsky, 1939] may be exaggerated.

Discussion

The activity of zinc and nickel in prolonging the hypoglycaemic effects of insulin were reported by Scott & Fisher [1935]. Since that date the prolonging action of zinc has been shown to apply to gonadotrophic hormones [Fevold, Hisaw & Greep, 1936], diphtheria antitoxin [Rosenthal & Kamlet, 1937], histamine [Dodds *et al.* 1937], and adrenaline [Schwab, 1937]. The activity of organic substances in this respect is also marked. Protamine [Hagedorn, Jensen, Krarup & Wodstrup, 1936], gelatine [Broun & Schwab, 1937] and tannic acid [Broom & Bavin, 1937] all prolong the action of insulin, while benzoic acid enhances the effect of oestradiol [Emmens, 1939] and fatty acids the effects of testosterone [Parkes, 1936]. It is obvious from the diversity of substances augmented that the action is not specific to any one injected substance and the most likely explanation of the augmentation is a delay in the absorption from the site of injection. None of the above effects are obtained when the augmentor and augmented substances are injected in different sites or given together intravenously. In the case of pituitary antidiuretic hormone it has been found that various degrees of activity were obtained when the same dose of postuitary extract was given in different volumes; whereas 2 μg. PP injected in 0·2 c.c. gave a 50 % excretion time of 122 min., while if contained in 1·0 c.c. the time was 177 min. To explain this augmentation it must be assumed that the slower permeation, due to the lower concentration and to the wider distribution of the vasoconstriction owing to the larger volume, outweighs the greater local vasoconstriction caused by the higher concentration in the smaller volume.

The delay in absorption may be caused by two factors. The augmenting substances may act directly on the tissues at the injection site rendering them relatively impermeable or the postuitary extract may be adsorbed on to the augmenting substance if the latter is injected in suspension. These two factors are illustrated by a series of experiments with zinc in various forms. Zinc acetate solution 0·1 % with PP gives a 50 % excretion time of 268 min., while the same concentration of insoluble zinc carbonate hardly prolongs at all (167 min.). The same concentration

of insoluble zinc ferrocyanide, which we know from the urine extraction method adsorbs the antidiuretic principle, gives a prolongation with a 50 % excretion time of 212 min. This shows that in the case of zinc the delayed adsorption is primarily due to a direct action on the tissues. Increasing the concentration of the soluble augmentors generally gives a limit to the prolongation obtained but since the higher concentrations have an antidiuretic action by themselves, presumably due to their hypertonicity, the results become difficult to assess. With the augmentors given in suspension (salmine and tyrosine) increasing the concentration reduced the prolonging activity. This was presumably due to the absorption being so delayed that the hormone hardly acted at all. Similar effects were reported for the augmentation of insulin by Bavin & Broom [1937]. The practical conclusions to be derived from these results are that any assay of the antidiuretic potency of an impure extract by subcutaneous injection cannot be regarded as quantitatively exact.

The addition of augmenting substances to an extract containing only a small amount of antidiuretic activity makes it possible to increase the qualitative sensitivity of the test. Such an example is demonstrated by a series of experiments recorded in Table IV.

TABLE IV. Increasing qualitative sensitivity of antidiuretic test

Dose of PP μg.	50 % excretion time with: PP alone min.	50 % excretion time with: PP + 5 % Zn acetate min.
2·0	134	—
0·5	97	173
0·2	84	124
0·04	—	142
0·02	—	88
0·00	87	—

It is seen that the minimal effective dose of PP given alone was greater than 0·5 μg. but the addition of 5 % zinc acetate increased the sensitivity of the test more than ten times. Also it is obvious that an assay under these conditions cannot be quantitatively accurate since with added zinc 0·04 μg. of PP caused a longer antidiuretic effect than 0·5 μg. of PP alone.

Finally it may be said that the results recorded above seem to indicate that biological assay of any impure substance by subcutaneous injection may be open to the same objections. A particularly misleading conclusion may be drawn from a decreased assayed potency which may be given by what is really a purer product, but free of augmenting substance.

SUMMARY

Various cations, anions, and organic substances when added to posterior pituitary extract, prolonged the antidiuresis obtained by subcutaneous injection.

The most active substances were zinc, nickel, cadmium and ferrocyanide.

When posterior pituitary principles were extracted from blood and urine and tested by the antidiuretic assay, an apparently greater than 100 % recovery was obtained owing to the presence of such augmenting substances. Similarly the addition of such augmenting substances made it possible to increase the sensitivity of the qualitative test for antidiuretic hormone.

The augmentation was presumably due to delayed absorption from the injection site.

A method of extracting posterior pituitary principles from large volumes of urine is described.

We are grateful to Messrs Boots Pure Drug Co., Ltd., for supplies of salmine and clupeine and to Prof. E. C. Dodds for his interest and criticism.

REFERENCES

Anselmino, K. J. & Hoffman, F. [1931]. *Arch. Gynaek.* **147**, 604.
Bavin, E. M. & Broom, W. A. [1937]. *Quart. J. Pharm.* **10**, 327.
Boylston, G. A. & Ivy, A. C. [1938]. *Proc. Soc. exp. Biol., N.Y.*, **38**, 644.
Broom, W. A. & Bavin, E. M. [1937]. *Quart. J. Pharm.* **10**, 334.
Broun, D. & Schwab, H. [1937]. *Paris Med.* **27**, 212.
Bundschuh, H. E. & Kuschinsky, G. [1939]. *Klin. Wschr.* **18**, 251.
Burn, J. H. [1931]. *Quart. J. Pharm.* **4**, 517.
Byrom, F. B. & Wilson, C. [1934]. *Quart. J. Med.* **3**, 361.
Dodds, E. C., Noble, R. L., Rinderknecht, H. & Williams, P. C. [1937]. *Lancet*, **2**, 309.
Emmens, C. W. [1939]. *Spec. Rep. Ser. med. Res. Coun., Lond.* No. 234.
Fevold, H. L., Hisaw, F. L. & Greep, R. [1936]. *Amer. J. Physiol.* **117**, 68.
Gilman, A. & Goodman, L. [1937]. *J. Physiol.* **90**, 113.
Hagedorn, H. C., Jensen, B. N., Krarup, N. B. & Wodstrup, I. [1936]. *J. Amer. med. Ass.* **106**, 177.
Heller, H. [1937]. *J. Physiol.* **89**, 81.
Ingram, W. R., Ladd, L. & Benbow, J. T. [1938]. *Amer. J. Physiol.* **123**, 107.
Jones, E. I. [1938]. *Lancet*, **1**, 11.
Kamm, O., Aldrich, T. B., Grote, I. W., Rowe, L. W. & Bugbee, E. P. [1928]. *J. Amer. chem. Soc.* **50**, 573.
Melville, K. I. [1937]. *J. exp. Med.* **65**, 415.
Noble, R. L., Rinderknecht, H. & Williams, P. C. [1938]. *Lancet*, **1**, 13.
Parkes, A. S. [1936]. *Lancet*, **2**, 674.
Rinderknecht, H., Noble, R. L. & Williams, P. C. [1939]. *Biochem. J.* **33**, 381.
Rosenthal, L. & Kamlet, J. [1937]. *Proc. Soc. exp. Biol., N.Y.*, **36**, 474.
Schwab, H. [1937]. *C.R. Acad. Sci., Paris*, **205**, 628.
Scott, D. A. & Fisher, A. M. [1935]. *J. Pharmacol.* **55**, 206.
Teel, H. M. & Reid, D. E. [1939]. *Endocrinology*, **24**, 297.