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A METHOD FOR THE ASSAY OF VERY SMALL AMOUNTS OF ANTIDIURETIC ACTIVITY WITH A NOTE ON THE ANTIDIURETIC TITRE OF RATS' BLOOD

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The most sensitive of the published tests (see Table 1) for estimating antidiuretic activity are those of Jeffers, Livezey & Austin (1942) on the anaesthetized rat, and of Heller & Blackmore (1952) on the non-anaesthetized mouse. The sensitivity of the rat preparation was 20 μU/rat, that of the mouse about 10–50 μU/10 g. Both these groups of observers estimated the antidiuretic activity while the water load was declining and the blood concentration was correspondingly increasing. The method described below is a development of that of Jeffers *et al.* (1942), differing from it essentially in keeping the water load of the rat constant. Each dose of the test material is thus distributed in the same volume of extracellular fluid.

Table 1. Assay methods for posterior pituitary antidiuretic activity

Test animal	Water load	Anaesthesia	Sensitivity	Authors
Dog	Twice, 250 ml./dog	None	0·25–0·5 mU/dog	Theobald, 1934; Samaan, 1935
Dog (hypophysectomy)	100 ml./dog	None	0·2–0·3 mU/dog	Hare, Melville, Chambers & Hare, 1945
Rabbit	100 ml. followed by 50 ml./kg	Paraldehyde	0·5 mU/rabbit	Walker, 1939
	25 ml. of 20 % glucose and 50 ml. 0·9 % NaCl intravenously	Morphine + urethane	0·2–0·3 mU/rabbit	Fugo & Aragon, 1947; Lindquist & Rowe, 1949
Rat	5 ml./100 g	None	2·3 mU/100 g	Burn, 1931
	3 times 5 ml./100 g	None	0·4 mU/100 g	Ginsburg, 1951
	2·5 ml./100 g followed by 5·0 ml./100 g	None	0·5–1 mU/100 g	Ham & Landis, 1942
	5 ml. of 12 % alcohol/100 g followed by 3 ml./100 g	Alcohol	0·02 mU/rat	Jeffers *et al.* 1942
Mouse	1 ml./mouse intraperitoneally	None	40 mU/mouse	Glaubach & Molitor, 1932
	5 ml./100 g intravenous	None	20 mU/10 g	Nelson & Woods, 1934
	3 times 5 ml./100 g	None	0·010 mU/10 g	Heller & Blackmore, 1952

Where not specified, water was administered in oral doses. mU = milliunit.

METHODS

Experimental procedure. Female rats (body weight about 200 g), deprived of food for 18 hr but allowed free access to water, were given an oral dose of 5 ml./100 g of tepid water followed 45 min later by 5 ml./100 g of a 12% ethanol solution. While the rat was in alcohol anaesthesia, the bladder was catheterized and the bulk of it tied off so as to decrease its dead space, and an external jugular vein was cannulated with polyethylene tubing. When the urine excretion reached 3–4 ml./100 g body weight, a second oral dose of a 2% ethanol solution 3–4 ml./100 g body weight was given by stomach tube which was left *in situ*. Occasionally, owing to depression of normal reflexes by ethanol, the second dose was partly aspirated in the lungs; there was little or no diuresis in such animals, and some of them had to be discarded (Ames & van Dyke, 1952). When the rat had excreted a further 2 ml. urine/100 g and its water load (water load = amount of water administered minus that of urine excreted) was about 8 ml./100 g body weight, it was placed in a holder attached to a balance (Boura & Dicker, 1953). The jugular vein was connected to a set of three syringes (one tuberculin syringe containing 0·9% (w/v) NaCl solution, and two 'Agla' micrometer syringes filled with the standard and unknown solutions) and the bladder catheter and the stomach tube connected respectively to the outflow and inflow mechanism of the apparatus described previously (Boura & Dicker, 1953). This ensured the recording of urine flow and the maintenance of a constant water load. The rat was fed with a 2% ethanol solution, *not* with water. To cancel out extra-renal water losses, the stomach inflow mechanism was adjusted to deliver 0·103 ml. for each 0·1 ml. of urine excreted. The experiment was conducted in a thermo-regulated room kept at 23° C.

Drugs. Pitressin (Parke, Davis and Co.) solutions were used throughout for the estimation of the antidiuretic effects.

Definition. mU = milliunit; micro-units (μU) = activity of 10^{-7} ml. of Injectio Vasopressini B.P.

RESULTS

With a water load of about 8 ml./100 g the rate of urine flow remained remarkably regular for hours. For instance, in a rat of 205 g the rate of urine flow remained unchanged (within a range of $\pm 10\%$) for 5 hr at 0·25 ml./min. Neither intravenous injections of 0·1 ml./100 g of 0·9% (w/v) NaCl solution (Fig. 1) nor external disturbances such as noises of various intensity, squeezing or prodding or even cutting off the tip of the tail, had any significant antidiuretic effect.

Intravenous injections of known amounts of vasopressin

Between the time of injection and the onset of an antidiuretic effect there was a mean time lag of 2 min. Magnitude and duration of antidiuretic responses were correlated with amounts of vasopressin injected. Repeated injections of similar amounts of vasopressin at intervals of 50–60 min produced comparable antidiuretic responses (Fig. 2). When given at shorter intervals, however, they resulted in tachyphylaxis (Fig. 3). The lowest dose of vasopressin which had a significant antidiuretic effect was 3·5 μU/100 g. The highest dose which had a discriminating effect was 50 μU/100 g (Fig. 4).

Quantitative estimation of an antidiuretic effect. In the calculation of the antidiuretic potency of a solution, the actual duration of the effect was not

taken into account; it was replaced by an empirical time factor of 10 min. The antidiuretic potency of a solution was calculated as follows: number of drops (or of 0·1 ml.) of 'urine' (Boura & Dicker, 1953) excreted (a) in the 10 min lasting from the 8th min before to the 3rd min after the injection, and (b) in the 10 min lasting from the 3rd to the 13th min after the injection. The antidiuretic potency (α) being expressed as:

$$\alpha = \frac{b}{a} \times 100,$$

where a and b = the number of drops (or of 0·1 ml.).

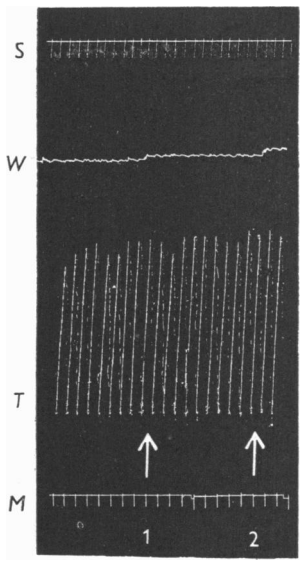

Fig. 1. Effect of intravenous injections of 0·9 % (w/v) NaCl solution. At (1), 0·3 ml.; at (2) 0·6 ml. There was no antidiuretic effect. Rat weight, 325 g. *S*, signal marking each 0·1 ml. urine excreted. *W*, weight line recorded by balance (note that each injection had an effect on the weight of the preparation); *T*, Thorp impulse counter; *M*, time in minutes.

As an example, values for α in a rat injected with 10 μU vasopressin/100 g were 62 and 59; in another rat injected with 20 and 30 μU/100 g, values for α were 38 and 29 respectively; while in a third rat injected with 5 μU/100 g, α was 78 (Fig. 2).

Values for α in twenty rats which had received sixty-five injections of amounts of vasopressin varying between 3·5 and 50 μU/100 g (Fig. 4) show first that the antidiuretic response increased linearly with the logarithm of the dose; secondly, that the comparable amounts of vasopressin produced comparable responses (α) irrespective of whether the injections had been given in the same or in different animals.

As all rats showed a comparable antidiuretic response when injected with comparable doses of vasopressin, the approximate antidiuretic potency of an unknown solution could be read from Fig. 4. For instance, a solution which reduced the urine flow by about 40% ($\alpha = 60$) had an antidiuretic potency of

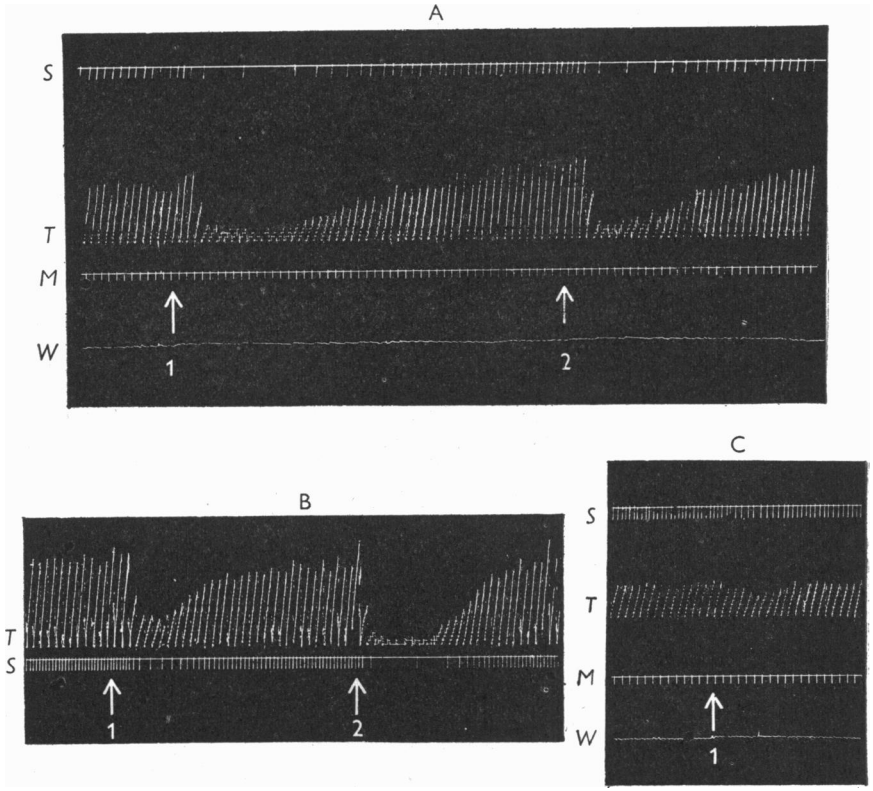

Fig. 2. Antidiuretic effects produced by intravenous injections of vasopressin. *S*, signal marking each 0·1 ml. of urine excreted. *T*, Thorp impulse counter; *M*, time in minutes; *W*, weight line. A, rat, 255 g. Two injections of 10 μU vasopressin/100 g in the same animal given at an interval of 50 min. The rate of urine flow increased after the effect of first injection was over. Drops, 16/0·1 ml. B, rat, 275 g. At (1), 20 μU vasopressin/100 g. At (2), 30 μU vasopressin/100 g. Drops, 12/0·1 ml. The rate of urine excretion was higher than in rat A. C, rat, 230 g. At (1), 5 μU vasopressin/100 g. Drops, 8/0·1 ml.

about 10 μU vasopressin/100 g. The actual assay, however, consisted of the injections of four doses, two of the standard and two of the unknown, the ratio, high to low dose, being the same for standard and unknown. Fig. 5 represents graphically the results of such an experiment: the regression lines relating the values of α to the logarithm of the doses of standard and unknown were parallel.

Estimation of the antidiuretic potency of serum and plasma of rats. Rats hydrated with 5·0 ml. water/100 g body weight were anaesthetized with ether, chloralose or ethanol. Blood was taken 60–70 min after water administration by severance of the head and was thus mainly arterial. Two doses of heparinized plasma or serum (1·0 and 0·5 ml./100 g) were injected into the test animal and the responses compared with those of two dilutions of standard solution of vasopressin. In contrast with what had been observed after

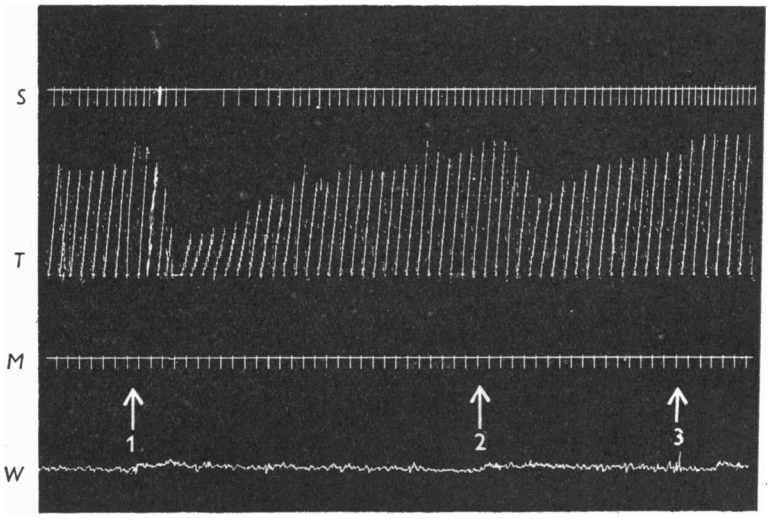

Fig. 3. Repeated injections of 10 μU vasopressin/100 g, showing tachyphylaxis. Time interval between injections: 30 min between (1) and (2), 17 min between (2) and (3). The third injection had no antidiuretic effect. *S*, signal marking each 0·1 ml. of urine excreted; *T*, Thorp impulse counter; *M*, time in minutes; *W*, weight line.

subcutaneous injections (Dicker & Ginsburg, 1950), there was no difference between the effects produced by plasma or serum. During ether anaesthesia the antidiuretic potency of systemic blood serum was equivalent to between 100 μU and 200 μU vasopressin/ml. In chloralose anaesthesia the antidiuretic potency of serum or plasma was between 20 and 30 μU vasopressin/ml. During alcohol anaesthesia, however, no appreciable (i.e. less than 3·5 μU/ml.) antidiuretic activity could be detected in either plasma or serum (Fig. 6). These results agree with those of Ames & van Dyke (1952).

DISCUSSION

Some of the well known and best tried methods for estimating small amounts of antidiuretic activity are given in Table 1. It shows that in non-anaesthetized laboratory animals the sensitivity of the preparation depends mainly on their

degree of hydration: for instance, the sensitivity of rats to vasopressin increases tenfold when their water load is increased from 5 ml./100 g (Burn, 1931) to 15 ml./100 g (Ginsburg, 1951). The same obtains in mice (Nelson &

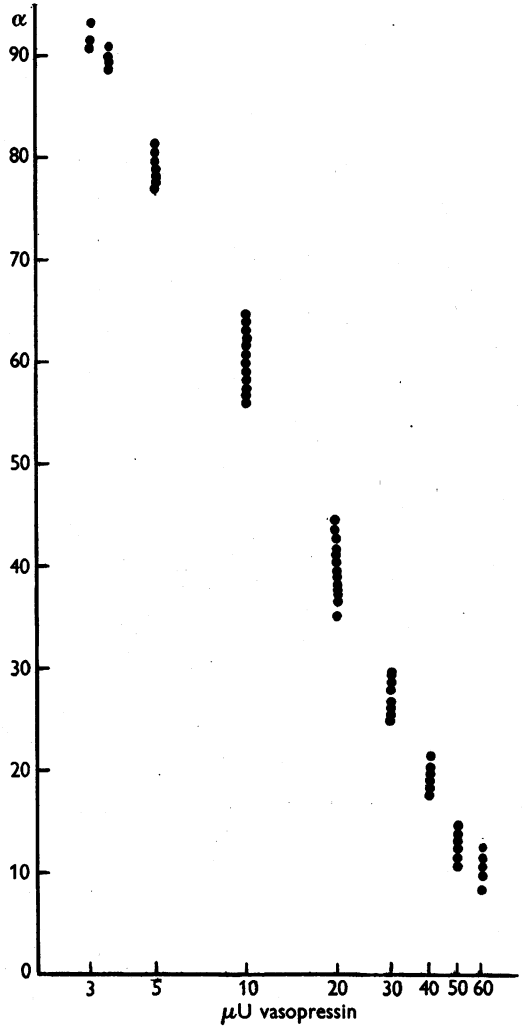

Fig. 4. Log dose-response regression line giving values for α (=antidiuretic effect; see text) after sixty-five injections in twenty rats. Ordinate: values for α. Abscissa: log doses of vasopressin (μU/100 g). Note that there was no differential effect between 3 and 3·5 μU/100 g and between 50 and 60 μU/100 g.

Woods, 1934; Heller & Blackmore, 1952). This could be explained if the greater dilution of body fluids produced a more complete suppression of the level of endogenous posterior pituitary antidiuretic hormones. It is curious to note that in spite of this observation none of the authors attempted to

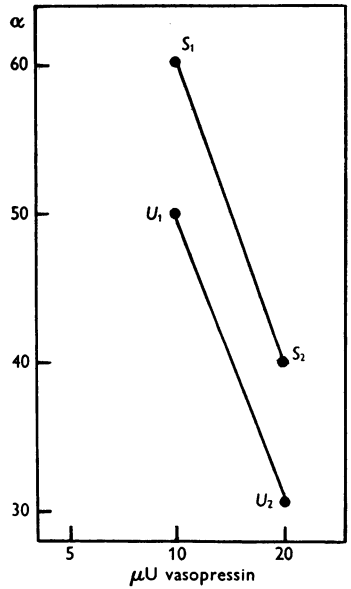

Fig. 5. Abscissa: log dose in micro-units of vasopressin. Ordinate: antidiuretic effect, α. S_1 and S_2 are the responses for the low and high doses of standard. U_1 and U_2 are the responses for low and high doses of unknown. Estimated potency ratio was 1·38; the actual potency ratio was 1·40.

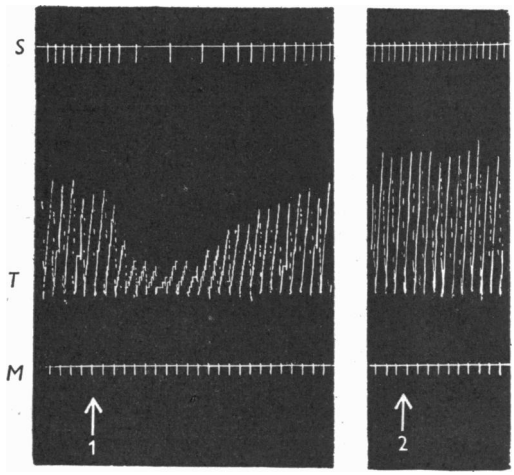

Fig. 6. Injections of serum from rats killed under ether or ethanol anaesthesia. Rat, 200 g. (1) 0·2 ml. serum from a rat killed under ether anaesthesia; (2) 0·8 ml. serum from a rat killed under ethanol anaesthesia. *S*, signal marking each 0·1 ml. of urine excreted; *T*, Thorp impulse counter; *M*, time in minutes.

maintain a constant water load in their preparation and were, as a matter of fact, estimating antidiuretic potency while the blood dilution was decreasing; that is while the endogenous posterior pituitary antidiuretic hormone might have been exerting its normal effect.

In this series of experiments, the effect of ethanol was added to that of a constant water load. According to van Dyke & Ames (1951), ethanol suppresses the excretion of endogenous antidiuretic hormone. The fact that no antidiuretic activity was found in the systemic blood of rats anaesthetized with ethanol would confirm that hypothesis. It was shown, however, by both Eggleton (1942) and van Dyke & Ames (1951), that in man as well as in dog the diuretic response to the administration of one dose of ethanol disappeared when repeated doses were given. In the present series of experiments where the rats were continuously fed with ethanol the rate of urine flow remained regular for several hours.

O'Connor (1950) reported that in one of his hypophysectomized dogs the same dose of vasopressin always had the same effect. This, however, was not so in the other dogs. In a series of twenty rats investigated the antidiuretic responses to the same dose of vasopressin were not only similar in the same animal, but in all animals.

In contradiction with Dicker & Ginsburg's (1950) findings Ames & van Dyke (1952) have shown that plasma of rats killed in ether anaesthesia had a marked antidiuretic potency. Ames & van Dyke's (1952) findings have now been confirmed. The contradiction between previous (Dicker & Ginsburg, 1950) and present findings can be explained by the difference in the methods of assaying and in the sensitivity of the preparations. Dicker & Ginsburg's (1950) rats were injected subcutaneously, and the assays estimated during a falling water load; the maximum sensitivity of their preparation was 400 μU vasopressin/100 g (Ginsburg, 1951). In the method now used the injections were given intravenously, and the water load was kept constant; the maximum sensitivity was 3·5 μU vasopressin/100 g.

SUMMARY

1. In rats under ethanol anaesthesia and kept with a constant water load, the smallest dose of vasopressin injected intravenously which had an antidiuretic effect was 3·5 μU/100 g body weight. The largest dose of vasopressin which had a differential effect was 50 μU/100 g.

2. Similar antidiuretic effects were obtained with comparable amounts of vasopressin, whether injected in the same or in different animals. There was a linear relationship between antidiuretic effects and log doses of vasopressin injected.

3. A means for calculating the antidiuretic titre of unknown solutions has been described. It allows the estimation of the antidiuretic potency by a four-point assay method.

4. The antidiuretic potency of blood plasma (or serum) of rats killed under ether, chloralose or alcohol anaesthesia have been estimated and expressed in terms of vasopressin. No antidiuretic activity could be detected in rats killed in alcohol anaesthesia. Some antidiuretic activity (about 30 μU vasopressin/ml.) was found in blood of rats anaesthetized with chloralose; while blood of rats in ether anaesthesia contained up to 200 μU vasopressin/ml. plasma.

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