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Factors affecting the antidiuretic actions of the 18-monoacetate of (+)-aldosterone and of a substance secreted by heart muscle, in rats

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The method for the assay of a steroid-like substance secreted by the heart (HS) in terms of the biological activity of the 18-monoacetate of (+)-aldosterone (18MA) has been improved. Rats were equipped with ascending carotid, descending external jugular and bladder polyethylene cannulae and the urethra was ligated, under pentobarbitone anaesthesia, 24–36 h before use. Assays were made in restrained animals during sustained water diuresis induced by an intravenous infusion of glucose, 5%, and NaCl, 0·9%; 2: 1 at 9 ml h^{-1}. Antidiuretic responses (measured as the area under the curve) to ascending carotid injections of 18MA and of HS, made at 0·01 ml s^{-1}, maximum 15 s, were obtained from single dilutions of HS and 18MA: urine flow per min was recorded from drop (0·025 ml) counters. Denervation of the carotid sinus above the carotid cannula did not influence the antidiureses. Data obtained were analysed by normal statistical procedures. Ascending carotid injections of 18MA and HS did not influence mean systemic arterial pressures, the clearances of creatinine and of PAH or the urinary outputs of Na^+ and K^+. The antidiuresis produced was compatible with the release of ADH by HS and by 18MA. Marked seasonal variations in threshold sensitivity to 18MA and in the slopes of the log dose-effect curves for the antidiuretic actions of 18MA are described. Variations both in the threshold sensitivity and in the log dose-effect curves for the antidiuresis caused by 18MA occur between rats of different strains.

The 18 monoacetate of (+)-aldosterone (18MA) has been used as a standard of reference for the assay of a physiologically active steroid-like substance (HS) liberated by the heart into the blood stream, *in vitro* and *in vivo* (Lockett, 1967; Lockett & Retallack, 1970a, b: 1971). HS resembles 18MA in its chromatographic properties (Ilett & Lockett, 1968; Lockett & Retallack, 1972) but is weight for weight more active and is more stable in aqueous solution, than 18MA (Lockett & Retallack, 1971). Nonetheless, HS cannot be differentiated from 18MA in biological tests because the slopes of the log dose-effect curves for the two compounds do not differ (Lockett & Retallack, 1970a). Consequently, minute amounts of HS can be estimated biologically in terms of known quantities or concentrations of 18MA. The assay preparation used was a rat in water diuresis; HS and 18MA were injected through an indwelling ascending carotid arterial cannula; the resultant decrease in urine flow was measured.

The immediate purpose was to make a more detailed investigation of the antidiuretic actions of HS and of 18MA in the rat by measurement of the effects of intracarotid injection of these compounds on various parameters of renal function. A

study was also made of the log dose-effect curves for the antidiuretic effect of 18MA, of seasonal variations in these curves and of differences in the curves provided by Wistar, D. A. and Lewis strains of rats.

METHODS

Over 200 male inbred Wistar rats, 180–210 g, 3 Lewis and 3 DA male rats, 240–250 g and 4 Sprague-Dawley crosses, 180–200 g, were used.

Techniques. Anaesthesia was induced by pentobarbitone-sodium (12 mg ml^{-1}, i.p., 0·4–0·6 ml per 100 g). The urethra was ligated and the fundus of the bladder cannulated with polyethylene tubing through a midline 1·5 cm incision, which was closed around the protruding cannula. Saline filled left ascending carotid and left descending external jugular polyethylene cannulae were inserted through an anterior midline cervical incision. These cannulae were exteriorized through the skin at the back of the neck before the anterior incision was closed. A fine, polyethylene 10, descending left carotid cannula was inserted additionally, and was similarly exteriorized in 4 animals. Denervation of the left carotid sinus was performed under a dissecting microscope in 4 rats before cannulation of the artery: successful denervation of the sinus was demonstrated by abolition of the tachycardia (recorded from needle electrodes through coupler No. 9857B amplifier Type 474A, by a Beckman RS pen recorder) which resulted from occlusion of the left carotid artery central to the sinus, for 45 s, before sinus denervation. Animals were placed in restraint cages while still under anaesthesia; an infusion of 1 part 0·9% NaCl, 2 parts 5% glucose, 0·375 ml h^{-1} was delivered continuously through the jugular cannulae until the assay started. A black opaque cloth shielded the animals from light.

The production of sustained water diuresis and the recording of urine flow. The speed of the jugular infusion was increased to 9 ml h^{-1}. Urine flow was recorded as drops (adjusted to 0·025 ml at 6 drops min^{-1}) per min by means of drop counters, thermionic valve units and Thorpe impulse counters (C. F. Palmer Ltd) which wrote on smoked drums, and were returned to base at 1 min intervals by a time clock (C. F. Palmer Ltd.). Stable rates of urine flow are attainable only under *quiet* conditions.

Measurement of antidiuretic activity. A steady water diuresis recorded by a Thorp impulse counter appears as a series of regularly rising steps (one step per drop) on a slowly moving smoked drum: the recorder returns abruptly to base every 60 s. When a graph is plotted showing drops per min (2 drops cm^{-1}) as ordinates against time (2 min cm^{-1}) as abscissae, inhibition of water diuresis appears as an area under a curve (Fig. 1). The total area under the curve measured in mm^2 and then divided by 25 expresses the magnitude of the antidiuresis as the total number of drops withheld. This value is termed the antidiuretic score. The total score then measures a water retention of score $\times$ 0·025 ml. Dose-effect curves are obtained by plotting the antidiuretic scores as ordinates against log ng 18MA injected as abscissae (Fig. 1). The adoption of this simple and uniform method of scoring assures rapid valid comparison of variations in sensitivity between animals and in the slopes of different log-dose effect curves. The slopes (values of b) of the linear log-dose effect curves were calculated as regression lines and the mean squares of the error of the estimates were determined by variance analysis (Emmens, 1948). The degrees of freedom associated

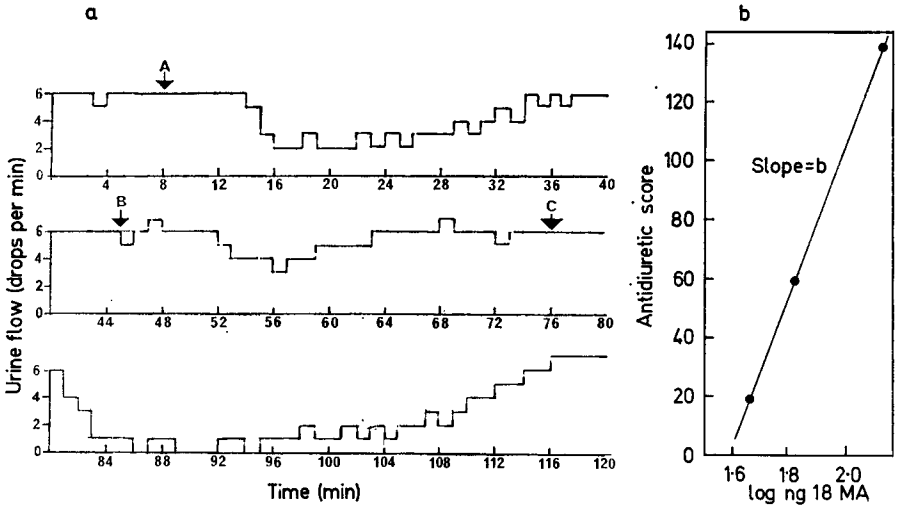

FIG. 1. Assessment of antidiuretic assays. a, the urine flow in drops per min is plotted as ordinate against time as abscissae: each response is scored as the area under the curve. The doses of 18MA were: A, 69 ng; B, 46 ng; C, 138 ng, all in 0·1 ml and given at 0·1 ml s^{-1}. b, the log dose-effect curve is derived by plotting antidiuretic scores as ordinates against log dose as abscissa.

with linearity (in variance analysis) were used to determine the standard deviations of single observations, standard errors of the means and the appropriate value of t. The significance of differences between means found within single experiments and those found within or between groups of animals has throughout been determined by variance analysis and is quoted as significant, highly significant or very highly significant when $P < 0{\cdot}05$, $P < 0{\cdot}001$, $P < 0{\cdot}001$, respectively.

The influence of the rate of injection on the antidiuresis caused by a fixed dose of 18*MA*

Three concentrations of 18MA in 0·9% NaCl were administered by ascending carotid injection at a standard rate of 0·01 ml s^{-1} for 10 s (3 experiments) or 15 s (3 experiments) in the order of a 3 × 3 Latin square to establish a log dose-effect curve for the antidiuretic action of 18MA on restrained Wistar rats in sustained water diuresis. Between each line of the square the fixed dose volume of the most concentrated solution of 18MA was administered at half speed (0·005 ml s^{-1}), then at

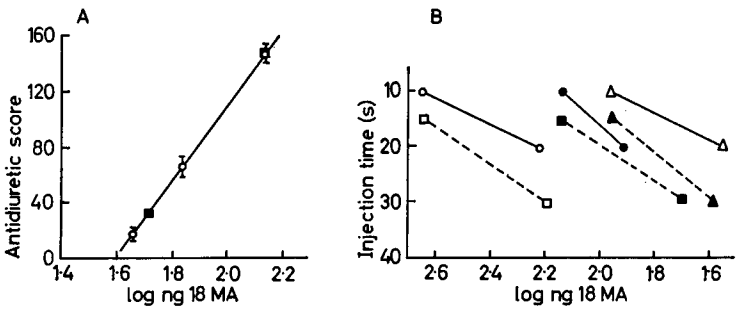

FIG. 2. Effects of halving the rate of the ascending carotid injections of fixed doses of 18MA on the magnitude of the resultant antidiuresis. A. Mean log dose effect curve (○) for 18MA obtained by using a fixed dose vol (0·1 ml) injected in 15 s. The mean effects of a single dose level injected throughout 15 s (□) are contrasted with the effect of the same dose injected throughout 30 s (■). B. The reduction in the effect of a single dose of 18MA caused by halving the rate of its injection is shown for each of 6 similar experiments.

standard rate and then again at half speed. The antidiuretic effect of each dose was measured. Log dose-effect curves were constructed from the data obtained at the standard injection rate of 0·01 ml s^{-1} (Fig. 2A). The effect of the highest concentration of 18MA when administered at half speed was measured (as ng 18MA required to produce equivalent antidiuresis when injected at standard rate) by reference to the log dose-effect curve (Fig. 2A). The results of the 6 experiments are shown in Fig. 2B. Halving the injection speed reduced the apparent potency of 18MA as an antidiuretic agent, highly significantly, in every experiment.

The effect of dosage by duration of injection on the dose-effect curve for the antidiuretic action of 18MA

In each of 3 experiments the dose-effect curve for the antidiuretic action of 18MA obtained by the ascending carotid injection of a single solution of 18MA at 0·01 ml s for 5, 10 and 15 s was contrasted with the curve resulting from administration of the same doses of 18 MA each dissolved in 0·1 ml and injected in 10 s. Thus, in one experiment, for example, doses A, B and C consisted, respectively, of 46, 92 and 138 ng 18MA, each in 0·1 ml and delivered in 10 s. Doses D, E and F were administered by injection of 0·92 μg 18MA ml^{-1} at 0·01 ml s^{-1} for 5, 10 and 15 s, respectively. The order of dosage was as follows:—A, B, C, D, E, F; B, C, A, E, F, D; C, A, B, F, D, E. The antidiuretic effect of each dose was measured. The dose-effect curve for the multiple dilution method was constructed from responses to A, B and C: the curve for the single dilution technique from responses to D, E and F. Both curves showed linear regression of the antidiuretic response on log dose of 18MA. Differences found between these curves in 3 experiments are shown in Table 1.

Table 1. *Minimum (threshold) doses of 18MA producing reproducible action and the slopes of the dose-effect curves obtained for the antidiuretic action of 18MA, given by ascending carotid injection to rats in sustained water diuresis at 0·01 ml s^{-1}, using single, in contrast to multiple, dilutions of 18MA (see text).*

	Threshold dose 18MA (ng)		Value of b ± Smt, $P < 0·05$	
Wistar Rat	Multiple dilution technique	Single dilution technique	Multiple dilution technique	Single dilution technique
X	30	23	290 ± 24·0	202 ± 11·1
Y	42	34	213 ± 13·0	104 ± 15·8
Z	40	23	350 ± 45·3	203 ± 37·6

In each case the steeper curve was found by the multiple dilution method and the lower dose providing reproducible threshold effect by the single dilution procedure. The latter method is now invariably used for assay purposes both because of this lowered detection limit and because of the considerable conservation of valuable material which has resulted from its use.

The use of 18MA as reference standard for assay of HS

Dry 18MA is stored in ampoules under nitrogen at 0° and is dissolved in 0·9% NaCl immediately before use since 18MA decomposes in aqueous solution; these solutions are cooled in ice-water and are used within 1 h of preparation. Provided

that these precautions were observed, 18MA served as a satisfactory standard of reference for assays of HS. 18MA and HS both caused antidiuresis in the restrained rat when administered by ascending carotid injection during a sustained water diuresis. Since the slopes of the dose-effect curves for HS and 18MA were parallel, HS was assayed in terms of activity equivalent to x ng 18MA (Lockett & Retallack, 1970a).

The procedure used for these assays was the single dilution technique described above. The maximum volume injected was 0·15 ml and the rate was always 0·01 ml s^{-1}; thus the maximum duration of the injection was 15 s.

The preparation of HS for bioassay has been described (Lockett, 1967; Ilett & Lockett, 1967; Lockett & Retallack, 1970a, b; 1972). HS is more stable in aqueous solution but dissolves less readily than 18MA. Solutions of HS in 0·9% NaCl can be stored at 5 to 7° for 36 h without detectable loss of activity.

Renal function. Creatinine 2% and *p*-aminohippuric acid (PAH) 0·5% in 0·9% NaCl were added to the infusion fluid, 0·5 ml to 100 ml, 4 h before renal function tests, during which the drop counters were disconnected and urine fell from the cannula directly into small tubes. Concentrations of Na^+ and K^+ in the urine were estimated by flame photometry and of creatinine and PAH by Technicon autoanalysis. Creatinine and PAH in plasma from heart blood, taken at the end of the experiment, were estimated as previously described (Davey & Lockett, 1960). Since it is imperative that haemorrhage and emotional disturbances do not occur during assays of ADH, the plasma levels of creatinine and PAH found in these terminal blood samples were used to calculate their renal clearances. The error so introduced was probably small for these substances had been infused at a low constant rate for a minimum period of 3 h 56 min before urine collections began. Full equilibration, with the production of near constant plasma concentrations of these substances was, therefore, almost certainly already complete.

Mean arterial pressure was recorded by means of an E & M pressure transducer coupled to a Nesco pen recorder.

RESULTS

Effects of HS and of 18MA on renal function and mean arterial pressure, in the rat

Effects of 18MA, administered by ascending carotid injection, on mean arterial pressure and on renal function, were examined in four rats, under restraint, and during sustained water diuresis. Corresponding studies were made in two of these preparations using a selected dose of HS. HS derived from blood collected from the coronary sinus of a cat under chloralose anaesthesia was used in one experiment, sheep arterial HS in the other. In each case a suitable dose of 18MA or of HS was selected for use and was similarly administered three times at 50–60 min intervals. The antidiuretic response to the first and third doses were recorded. The second dose was used for measurement of other parameters of renal function and of the blood pressure; serial 5 min collections of urine were made before the second administration of the dose and throughout the response to it. Fig. 3 shows data from one experiment which is typical of all. The mean systemic arterial pressure was not influenced by ascending carotid injections either of HS or of 18MA in 0·1 ml delivered at 0·01 ml s^{-1}. The antidiureses caused by repeated doses of these substances were reproducible. Neither 18MA nor HS influenced the clearances of creatinine and of PAH or the urinary outputs of sodium (Na) and potassium (K) during sustained water diuresis.

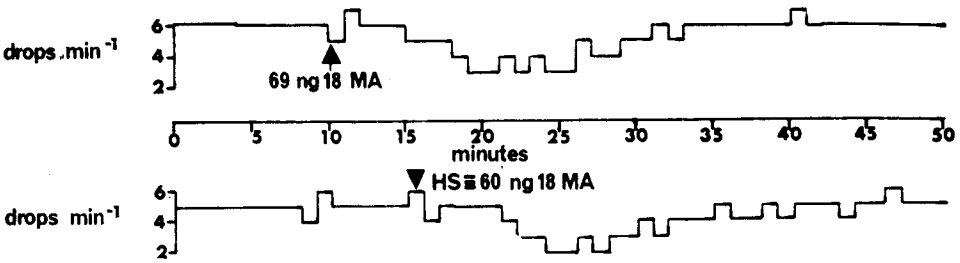

FIG. 3. The influence of 18MA (above) and of HS (below) on renal function in rats. All measurements during sustained water diuresis. The plots of urine flow were obtained by drop recorder during responses to 69 ng 18MA (a) and to HS-activity equivalent to 60 ng 18MA (b).

Diminishing responses to fixed doses of 18MA and of HS were characteristic of the onset of an osmotic diuresis. Occasionally, the standard infusion of 2 parts 5% glucose and 1 part 0·9% NaCl at 9 ml h^{-1} used to produce and to sustain water diuresis, has caused more than trivial glycosuria.

Effects of surgical intervention under pentobarbitone anaesthesia on the induction of water diuresis and on the antidiuretic action of 18*MA*

Experiments undertaken to determine the most suitable post-operative period for assays of HS in rats pre-equipped with indwelling carotid, external jugular and bladder cannulae yielded interesting information which is summarized in Table 2.

The rate of development of water diuresis, impaired at 6–12 h, was maximally slowed 12–18 h post-operatively, whereas the level of water diuresis developing was most markedly reduced at 6–12 h. Glucosuria developed during infusion of 1 part 5% glucose, 2 parts 0·9% NaCl most readily in the 6–12 h post-operative period. Threshold sensitivity to the diuretic action of 18MA was greatly increased and the slope of the log dose response curve markedly depressed 12–18 h after operation.

Table 2. *Effects of surgery under intraperitoneal pentobarbitone anaesthesia on the induction of water diuresis and on the antidiuretic action of* 18*MA in Wistar rats.*

Parameters tested	Hours after induction of anaesthesia 6–12	12–18	24–36
Hours for development of steady diuresis	3·32 ± 0·14 (11)*	4·26 ± 0·29 (15)**	2·75 ± 0·18 (26)
Maximum urine flow ml h^{-1} in water diuresis	5·46 ± 0·31 (7)**	6·10 ± 0·23 (15)**	9·08 ± 0·07 (26)
Maximum tolerated infusion rate without frequent onset of osmotic diuresis (1 part 0·9% NaCl, 2 parts 5% glucose)	4·5 ml h^{-1}	6·0 ml h^{-1}	9·0 ml h^{-1}
Threshold dose 18MA for production of antidiuresis in nanograms	42 ± 3·6 (7)	71 ± 3·1 (15)**	44·2 ± 3·7 (26)
Slope of the log dose-effect curve for 18MA (single dilution method)	148 ± 8·5 (7)**	111 ± 4·7 (15)**	....204 ± 9·3 (26)

All values shown are means ± s.e., followed by the number of animals (in parentheses). Significant differences of means from those of the 24–36 postoperative period has been examined by Student's *t*-test * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$.

Table 3. *Values for potency of solutions of HS in terms of biologically equivalent concentrations of* 18*MA* (*ng ml*$^{-1}$) *determined under various circumstances.*

	Condition of the assay rat				
	Hours post-operative			slight osmotic diuresis	sinus denervated
HS Sample	6–12	12–18	24–36		
A	421 ± 14·2		410 ± 16·5		
B		398 ± 8·4	404 ± 9·1		
C	416 ± 23·6		422 ± 14·8		
D		502 ± 21·7			509 ± 18·7
E	488 ± 9·6		474 ± 12·6		482 ± 15·4
F			440 ± 17·3		449 ± 8·7
G			620 ± 21·8	608 ± 31·6	617 ± 15·4
H		434 ± 22·0		446 ± 15·7	
J			448 ± 19·5	437 ± 21·3	

The values shown are means ± s.d. of single observations, multiplied by t at the $P = 0{\cdot}05$ level of probability. Each mean was determined on not less than 3 observations made by the single dilution assay technique at 2 or more dose levels.

Since estimates of the potency of solutions of HS in terms of biological activity equivalent to x ng 18MA ml^{-1} were unaffected by the post-operative period in which they were made, and by the occurrence of a slight osmotic diuresis, it is evident (Table 3) that the antidiuretic actions of 18MA and of HS were similarly influenced by surgery under pentobarbitone anaesthesia and by osmotic diuresis.

Denervation of the left carotid sinus did not affect antidiuretic responses to ascending injections of HS and of 18MA made through the ascending cannula in the left carotid artery. The slopes of the dose-effect curves and the threshold sensitivities in the denervated animals were found distributed within the normal range.

Seasonal changes in the antidiuretic action of 18*MA*

Seasonal changes in the antidiuretic effect of 18MA have been studied in the inbred strain of Wistar rats readily available in Western Australia. Dose-effect curves for 18MA were repeatedly determined by the single dilution technique in 1970 and 1971. The results of these experiments are shown in Fig. 4. December and January are the hottest months of the year and are very dry: the slopes of the log dose-effect curves for the antidiuretic effect of 18MA were very steep and the minimum repeatedly effective (threshold) dose was high. These findings persisted into February until climatic conditions changed: the humidity rose, rather abruptly and remained relatively high after a series of thunderstorms supervened. The slopes of the log dose-effect curves for the antidiuretic action of 18MA decreased equally abruptly,

Table 4. *A comparison of the antidiuretic action of* 18*MA in Wistar, Lewis and DA rats.*

Strain of rat	Threshold dose ng 18MA	Slope of the log dose
Lewis (3)	73·3 ± 8·3	145·9 ± 41·2
DA (3)	60·0 ± 15·0	142·0 ± 10·9
Wistar (4)	32·5 ± 4·0	214·4 ± 9·9

The values are means ± s.e. All measurements were made in July, 1972 by the single dilution technique. Numbers of animals are shown in parentheses.

and so did the threshold dose. These parameters showed no further significant change in the humid autumn months of March and April and the winter months. Rain was intermittent in May and June and was heavy in July. In the very early spring (August) the slopes of the log dose-effect curves showed only a slight tendency to rise and rainy days were still frequent: it was still relatively cold (about 55° F). In September, as regular rain ceased, the air rapidly became dry and the light strong, the slopes of the log dose-effect curves rose abruptly and remained very steep throughout late spring and summer. By contrast, the threshold dose of 18MA did not alter significantly before the end of October, when the temperature is usually in the seventies with an occasional warm (85° F) day. In the very dry heat of December, the mean threshold dose of 18MA was double that found in the autumn, winter and spring.

Variations in the antidiuretic effect of 18MA amongst strains of rats

Comparison was made between the log dose-effect curves for the antidiuretic action of 18MA during the first 3 weeks of July, 1972, by the single dilution technique. Lewis rats and DA rats were obtained from the Victorian seaboard of Australia. These Lewis and DA rats were studied in the second and third week after transporta-

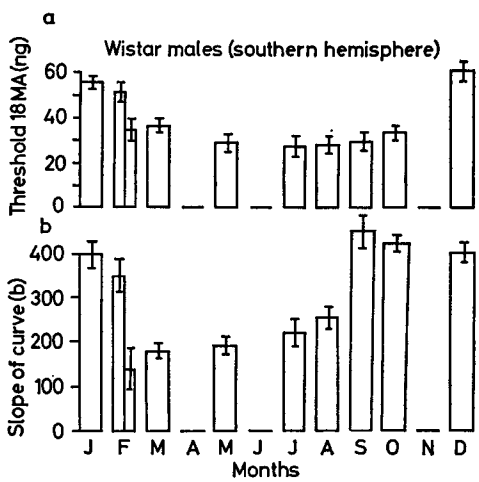

FIG. 4. Seasonal variations in antidiuretic responses to 18MA administered by ascending carotid injection (0·01 ml s^{-1} for a maximum of 15 s) to rats in sustained water diuresis. Southern hemisphere. The heights of the rectangles show mean values contributed by groups of not less than 8 rats, the inset bars are standard errors.

tion to Western Australia by air. The small number of observations made (Table 4) were enough to show that the doses of 18MA required to produce threshold antidiuretic action in both the DA and the Lewis rats were approximately double those needed by West Australian Wistar rats of the same weight (240–255 g). Moreover, the slopes of the log dose-effect curves for 18MA were significantly steeper in the Wistar than in the Lewis and the DA strains.

DISCUSSION

The practical contribution made by these experiments has been an improvement in the assay of HS in restrained rats during steady water diuresis by means of the antidiuresis resulting from ascending carotid injections. Also 18MA has again been shown a valid reference standard provided suitable precautions are taken to overcome the ease with which 18MA decomposes in aqueous solution.

Points of theoretical interest have also arisen from this work. The antidiureses caused by HS and by 18MA were due only to retention of water: glomerular filtration rate, effective renal plasma flow, mean systemic arterial pressure and the urinary outputs of Na^+ and K^+ were not significantly affected. These observations are compatible with the assumption that HS and 18MA release ADH from the neurohypophysis. Previous work in the cat showing that the threshold effect of 18MA on urine flow is abolished by hypophysectomy (Lockett, 1969) supports this assumption. Inhibition of the release of ADH by a rise of pressure in the carotid sinus is postulated (Lemaire, Mazer & Allegrini, 1961). However, the release of ADH by 18MA and by HS is not mediated through this mechanism since the antidiuretic effects of ascending carotid injections of these two substances are unaffected by section of the corresponding sinus nerve. No information is yet available to indicate whether the release of ADH by HS and by 18MA is initiated in the hypothalamus and/or in the neurohypophysis.

The mechanism of ADH release by 18MA and HS has been shown predominantly dependent on the concentrations of these two substances that reach the effector site since the intensity of the antidiuresis produced by a fixed dose is so markedly influenced by the rate of its injection.

The seasonal changes found in the antidiuretic effects of 18MA are remarkable. Temperature, humidity and intensity of light vary greatly with season in Western Australia. The temperature in our animal house is controlled, but the rats are exposed to changes in humidity and to the intensity and duration of daylight. The marked decrease in the threshold dose of 18MA synchronized with the sudden onset of freak high humidity and thunderstorms in mid-February, 1971. This threshold dose persisted through the more humid months of autumn, winter and early spring, but rose suddenly as excessively dry conditions were re-established. It is possible, therefore, that the changes in the threshold dose may be related to changes in humidity. The halving and doubling of the mean threshold dose for the strain might indicate that the number of molecules of ADH released per molecule of 18MA also halves and doubles. It is of interest, in this connection, that thunder has been shown to liberate oxytocin from the neurohypophysis in this same strain of rats (Ogle & Lockett, 1966).

The marked changes occurring in the slope of the dose-effect curve for the antidiuretic action of 18MA synchronize more nearly with change in the intensity and/or duration of daylight than with change in humidity. The remarkable dissociation found in early summer between the sudden occurrence of increase in the threshold dose of 18MA and increase in the slope of the dose-effect curve suggests a mechanism more complex than a simple doubling or halving of the molecules of ADH released per molecule of 18MA in excess of threshold concentration. Seasonal variations in renal sensitivity to ADH may be involved, for this phenomenon has been observed in rats in the northern hemisphere (Heller, Herdan & Zaidi, 1957). Seasonal variations in renal sensitivity to ADH have not yet been measured in West Australian Wistars.

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