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INACTIVATION OF THE ANTIDIURETIC ACTIVITY OF VASOPRESSIN BY TISSUE HOMOGENATES

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It has been shown in a previous paper that the incubation of vasopressin with kidney or liver slices results in a partial inactivation of its antidiuretic activity (Dicker & Greenbaum, 1954). The present investigation was undertaken with a view to securing more information on the mechanism of inactivation. A preliminary account of this work has already been published (Dicker & Greenbaum, 1955).

METHODS

Male albino rats of an average weight of 250 g (range: 180–290 g) were used both for the preparation of tissue homogenates and for the antidiuretic assays. For the preparation of tissue homogenates rats were killed by dislocation of the cervical vertebrae. The tissue samples taken were the kidneys, a piece of the central lobe of the liver, the upper part of the duodenum, the spleen, or a piece of the quadriceps muscle. These were chilled and homogenized in 9 vol. of 0·25 M sucrose solution in a Potter & Elvehjem homogenizer (1936) thus giving a tenfold diluted tissue suspension. Full homogenates were separated into fractions by centrifuging at 0° C, first at 600 *g* for 10 min, then at 34,000 *g* for 30 min, in a Sorval high-speed centrifuge. The three fractions were (1) nuclei and cellular debris; (2) the particulate fraction, which consisted mainly of mitochondria and larger microsomes; and (3) the supernatant fluid.

Incubations of tissue homogenates (or their fractions) with vasopressin were made in Mg- and Ca-free Krebs–Ringer phosphate buffer solution, pH 7·4 (NaCl 0·154 M, 100 parts; KCl 0·154 M, 4 parts; KH_2PO_4 0·154 M, 1 part; phosphate buffer (pH 7·4) 0·1 M, 12 parts; gassed with O_2) at 37° C. At the end of the incubation period the reaction was stopped by plunging the tubes into boiling water for 3 min. Some of the samples were acidified by addition of 3% (v/v) acetic acid, before they were heated, the final concentration of the acetic acid being 0·25%. As some of the experiments resulted in the preparation of up to thirty-six samples, these were frozen and stored at −15° C until they could be assayed. Control experiments had shown that preparations frozen at −15° C retained their antidiuretic activities unchanged for periods up to 5 weeks.

For the assays of antidiuretic activity, rats under ethanol anaesthesia were prepared as described by Dicker & Greenbaum (1954). Their water loads were kept constant during the whole experiment (Boura & Dicker, 1953). Each assay consisted of the intravenous injection of four doses, two of standard and two of test material, the ratio of high to low doses being the same for standard and unknown. Each dose was given once in each group of four doses, its position within the group being chosen at random. The assessment of the antidiuretic effect for each dose was done according to the method described previously (Dicker, 1953).

Drugs. Pitressin (Parke, Davis and Co.) was used for the incubation with tissue homogenates and as standard for the estimation of antidiuretic effects.

Definitions. mU = milliunit; μU = microunit = activity of 10^{-7} ml. Vasopressin B.P. The antidiuretic activity of the drug Pitressin has been referred to throughout as 'vasopressin'. S_1 = supernatant fluid obtained after centrifuging the tissue suspension at 600 *g*; it contained mitochondria and microsomes, but no nuclei or cellular debris. MM = the particulate fraction = mitochondria and the larger microsomes; S_2 = supernatant fluid remaining after the mitochondria and the larger microsomes have been removed. Although this fraction (S_2) still contained the smaller microsomes, it is referred to as the 'particle-free' supernatant fluid.

RESULTS

The validity of the results depended primarily on the accuracy of the method for assaying the antidiuretic activities of the preparations. This was, therefore, tested first. In a series of six assays in which both standard and test solutions had the same concentration of vasopressin, the mean amount of antidiuretic activity of the test solution represented 99% (range: 88–110%) of its stated potency (Table 1). In another series of six assays, where the test solution

TABLE 1. Assays of test solutions containing known amounts of vasopressin

	Stated potency of test solutions (μU/0·1 ml.)	Standard solution of vasopressin (μU/0·1 ml.)	Results of individual assays (μU/0·1 ml.)	Mean and S.E. (μU/0·1 ml.)	Mean of estimated potency expressed as percentage of stated potency
I	20·0	20·0	21·5 17·6 22·0 20·6 17·5 19·6	19·8±0·79	99
II	15·0	20·0	18·8 16·4 15·6 16·0 16·6 14·4	16·3±0·61	109
III	5·0	20·0	2·0 2·6 4·8 5·4 4·4	3·8±0·93	76

Each assay was estimated as a '2 and 2 doses' assay, comprising two groups (see text). In III, a test solution of 5 μU/0·1 ml. was compared with a standard of 20 μU/0·1 ml., to imitate conditions of samples of very low antidiuretic activity.

differed in concentration from that of the standard, the mean amount of the estimated activity was 109% (range 96–125%) of its stated potency (Table 1). In these twelve assays the volumes of both unknown and standard solutions injected intravenously did not exceed 0·1 ml./100 g body weight. However, in cases where the antidiuretic activity of the test material was very low, larger volumes of 0·2 and 0·4 ml./100 g had to be injected. This procedure adversely affected the accuracy of the assay. In a control series of five such assays, the

mean of the estimated potency was only 76% (range: 40–108%) of its stated activity (Table 1). No differences were found whether the test material had been acidified by addition of acetic acid (final concentration: 0·25%) or not. The validity of these control assays was limited, however, by the fact that all test solutions of vasopressin were made in isotonic NaCl solution *without* tissue homogenates. For reasons which will be given later, control assays of stated antidiuretic activity with tissue homogenates could not be performed.

Preliminary experiments

Kidney, liver, spleen and duodenum homogenates were prepared so that equal volumes contained equal amounts of wet weight of tissue. The supernatant fluid, S_1, prepared from each tissue, was incubated with amounts of vasopressin (2·0–10·0 mU) for various times. At the end of the incubation period, the tubes were plunged into boiling water for 3 min, and the supernatant fluid was assayed for its antidiuretic activity. In all experiments (Fig. 1) there was a decrease of antidiuretic activity during the first 2 hr, after which it was not possible to determine whether its level remained constant or continued to decrease slowly, since the concentration of antidiuretic activity was too low for accurate estimation. The residual antidiuretic activity after 2 hr incubation was about 6% of the initial amount for all tissue homogenates investigated. This was less than that found after kidney *slices* had been incubated with similar amounts of vasopressin for a period of 3 hr (Dicker & Greenbaum, 1954). Inactivation of vasopressin by liver homogenate, however, was faster than for the other homogenates; the level of 6% of the original activity was reached in less than 1 hr (Fig. 1). The decrease of antidiuretic activity of vasopressin by tissue homogenates was independent of the presence of O_2. In a series of four experiments, no assayable difference in the rate of its decrease could be found whether the mixture had been incubated in the presence of O_2, N_2 or KCN.

As assays of samples of very low activity lacked accuracy (Table 1), it was of importance to determine whether the antidiuretic activity found after vasopressin had been incubated with either tissue *slices* or homogenates was really different. Vasopressin (10 mU in 10 ml. of the modified Krebs–Ringer solution) was incubated with 250 mg of kidney *slices* for 4 hr. At the end of the incubation period the slices were removed and two samples of 2·0 ml. of the supernatant fluid, obtained after centrifuging at 600 *g*, each with an antidiuretic activity of 400 μU, were mixed either with 50 mg of fresh kidney *slices*, or with 0·5 ml. kidney homogenate (representing 50 mg of tissue). After a further incubation period of 1 hr, the supernatant fluid of the mixture with kidney *slices* had an antidiuretic activity of 382 μU/2·0 ml., whereas the other had decreased to 80 μg/2·0 ml.

In contrast with what had been observed with kidney, liver, spleen and

duodenum homogenates, when vasopressin was incubated with muscle homogenate there was an almost immediate decrease of the antidiuretic activity, to about two-thirds of its initial titre, after which it remained unaltered during the whole period of incubation up to 2 hr.

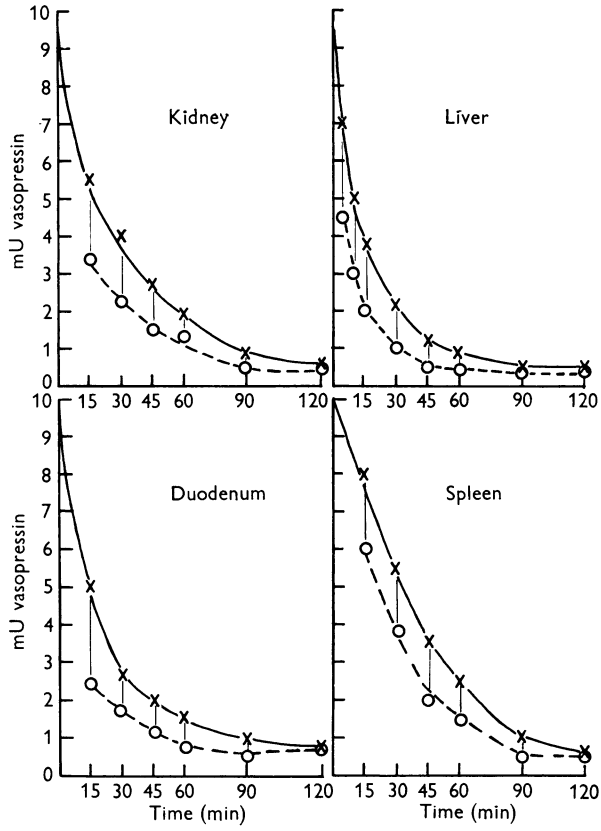

Fig. 1. Inactivation of vasopressin by tissue homogenates. ×——×, results obtained with preparations to which 3% acetic acid (final concentration: 0·25%) has been added before boiling. ○ - - - ○, results obtained with preparations boiled only. On Y axis, antidiuretic activity (mU vasopressin).

Protein binding of vasopressin

Heller & Urban (1935) have shown that tissue suspensions 'adsorbed' vasopressin and they claimed that it could be eluted by simple heating at 100° C. Fromageot & Maier-Hüser (1951) and Maier-Hüser, Clauser, Fromageot & Plongeron (1953) showed repeatedly that elution of bound vasopressin would occur only when heated in the presence of acetic acid. This was confirmed.

Full homogenates of kidney, liver, spleen, duodenum and muscle were shaken with known amounts of vasopressin for 10 sec, plunged into boiling water for 3 min, and centrifuged for 3 min. In all these experiments the antidiuretic activity of the supernatant fluid was about one-third only of its original titre. However, addition of acetic acid (final concentration: 0·25%) to the mixture before boiling resulted in the full recovery of the antidiuretic activity (Table 2).

TABLE 2. Binding of vasopressin by muscle homogenate

Preparation	Estimated antidiuretic activity: After boiling only (mU)	Estimated antidiuretic activity: After addition of acetic acid before boiling (mU)
10·0 mU vasopressin (vol. = 5·0 ml.) + 0·5 ml. muscle homogenate	6·80	—
	6·60	—
	7·00	10·20
	7·20	9·95

Muscle homogenate in 0·25 M sucrose solution, dilution 1:10. Vasopressin was added to 0·5 ml. of the homogenate, shaken for 10 sec and plunged into boiling water, with or without acetic acid, for 3 min. Acetic acid = 0·5 ml. of 3% acetic acid in isotonic NaCl solution (final concentration 0·25%).

To determine whether the binding of vasopressin occurred on the proteins of the supernatant fluid or on those of the residue, kidney homogenates mixed with vasopressin were divided by centrifuging at 600 *g* into a supernatant phase (S_1) containing the small particles (mitochondria and microsomes) and a residue (R) comprising the nuclei and cell debris as well as a small number of intact cells. The residue was extracted by the combined action of acetic acid solution (final concentration 0·25%) and boiling, the period of boiling extending from 3 to 30 min (Fromageot & Maier-Hüser, 1951). In some experiments the residue was extracted once only; in others up to three times. The supernatant fluid (S_1) was either boiled only or acidified before boiling. From the twenty experiments recorded (Table 3), each experiment being repeated two to four times, it will be seen that there is little evidence to support Heller & Urban's (1935) view that the residue played an important role in the binding of vasopressin.

In spite of the fact that the supernatant fluid (S_1) had been boiled only, antidiuretic activity was always greater than that recovered from the residue. When boiled *without* acetic acid the mean amount of antidiuretic activity of S_1 was 33% (range 24–45%) of that of the original amount of vasopressin (Table 3, Expts. 1–5). When, however, acetic acid had been added before boiling the mean amount of antidiuretic activity recovered from the supernatant fluid, S_1, was 88 and 85% of that of the original amount of vasopressin (Table 3, Expts. 6 and 7).

TABLE 3. Binding of vasopressin by kidney homogenate

	Description of preparations and their treatment			Estimated antidiuretic activity (mU)		Recovery of antidiuretic activity as percentage of initial amount of vasopressin
	A	B	C			
I.	Vasopressin (12·5 mU) brought to the boil and mixed with kidney homogenate (2)	Supernatant fluid; boiled	—	3·00	3·60	29
		Residue + acetic acid; boiled	—	0·60		
II.	Same as I (4)	Supernatant fluid; boiled	—	3·75	4·59	37
		Residue + acetic acid left to stand for 30 min; boiled	Supernatant fluid	0·55		
		—	Residue + acetic acid left to stand for 30 min; boiled	0·29		
III.	Vasopressin (12·5 mU) mixed with kidney homogenate, brought to the boil in 1 min 20 sec, maintained boiling for 1 min 40 sec	Supernatant fluid; boiled	—	3·40	4·02	32
		Residue + acetic acid left to stand for 30 min; boiled	—	0·62		
IV.	Same as III (2)	Supernatant fluid; boiled	—	4·60	5·31	42
		Residue + acetic acid left to stand for 30 min; boiled	Supernatant fluid	0·55		
		—	Residue + acetic acid left to stand for 30 min; boiled	0·16		
V.	Vasopressin (7·5 mU) shaken for 10 sec with kidney homogenate; then centrifuged for 3 min (600 *g*) (4)	Supernatant fluid; boiled	—	3·38	3·52	47
		Residue + acetic acid; boiled	—	0·14		
VI.	Vasopressin (7·5 mU) shaken for 10 sec with kidney homogenate + acetic acid; centrifuged for 3 min (600 *g*) (2)	Supernatant fluid; boiled	—	6·58	6·83	91
		Residue + acetic acid; boiled	—	0·25		
VII.	Vasopressin (7·5 mU) shaken for 10 sec with kidney homogenate + acetic acid; boiled; then centrifuged (600 *g*) (2)	Supernatant fluid	—	6·40	6·64	89
		Residue + acetic acid; boiled	—	0·24		
VIII.	Vasopressin (7·5 mU) shaken for 10 sec with kidney homogenate; left to stand at room temperature for 3 min; then suspended in acetic acid and centrifuged (2)	Supernatant fluid; boiled	—	5·05	5·25	70
		Residue + acetic acid; boiled	—	0·20		

All kidney homogenates in 0·25 M sucrose solution, dilution 1:10; volumes used = 0·62 ml. The separation of the mixtures into supernatant fluid and residue was effected by centrifuging at 600 *g*. All boilings lasted 3 min. Acetic acid solutions were made in isotonic NaCl solution (final concentration: 0·25%). The estimated amounts of antidiuretic activity are given as means; number of experiments in parentheses.

In most of these experiments (Table 3) there was an unavoidable time lag which might have allowed some enzymic destruction. For instance, it took 1 min 20 sec to bring the preparation to 100° C (Expts. 3 and 4); or it took 3 min for centrifuging the preparation before it was plunged into boiling water (Expts. 5 and 6). As the velocity of an enzymic reaction is greatest at the beginning, these delays may have explained why the total recovery of the antidiuretic activity of the combined residual and supernatant fraction was not 100%, even when heated in the presence of acetic acid. This explanation

TABLE 4. Binding of vasopressin by kidney homogenate and its fractions

Preparations	Duration of shaking (sec)	Treatment	Estimated antidiuretic activity (mU)	
7·5 mU vasopressin (5 ml.)				
+0·62 ml. kidney homogenate	30	Boil	5·10	5·10
		Acetic acid, boil	6·95	7·74
+0·62 ml. nuclei suspension	30	Boil	6·60	—
		Acetic acid, boil	7·70	—
+0·62 ml. mitochondria suspension	30	Boil	6·70	6·60
		Acetic acid, boil	8·10	7·95
+0·62 ml. microsomes suspension	30	Boil	6·60	6·50
		Acetic acid, boil	7·66	7·80
+0·62 ml. supernatant (=S_2)	30	Boil	5·56	4·25
		Acetic acid, boil	7·65	6·85

Kidney homogenate was first centrifuged at 600 *g*: nuclei and cell debris fraction; remainder centrifuged at 8500 *g*: mitochondrial fraction; remainder centrifuged at 33,000 *g*: microsomal fraction and 'particle-free' supernatant fluid (=S_2). Each fraction of nuclei, mitochondria and microsomes was resuspended in its original volume of sucrose. The experiments were repeated, using modified Krebs–Ringer solution instead of 0·25 M sucrose solution, with similar results.

was suggested by the following experiment (Table 3, Expt. 8) where a mixture of kidney homogenate and vasopressin was allowed to stand at room temperature for 3 min before being acidified and centrifuged, so that at least 7 min elapsed before the enzymic action could be stopped. The recovery of the antidiuretic activity from the residue was of the same order as in Expts. 5–7 (Table 3); that from the supernatant fluid, however, was markedly lower.

To investigate this problem further a full kidney homogenate was divided by differential centrifuging into its components: nuclei, mitochondria, large microsomes and the so-called 'particle-free' supernatant fluid, S_2. Each fraction was resuspended in its original amount of sucrose solution and equal volumes of each were shaken with an equal amount of vasopressin for 30 sec and then divided into halves, of which one was boiled only, but the other was acidified with acetic acid and then boiled. The results of the assays, done in duplicate (Table 4), showed that all the acidified samples had an antidiuretic titre higher than that of the corresponding half which had been boiled only. On the assumption that the difference between the antidiuretic activity of the acidified and non-acidified halves represented the amount of vasopressin bound,

it will be seen (Table 4) that the full homogenate and the particle-free supernatant fluid (S_2) had bound comparable amounts of vasopressin, 30 and 31% of the original amount respectively; whereas the amounts of vasopressin bound by the nuclei, the mitochondria and larger microsomes were 16, 19 and 16% only. Similar results were obtained in experiments performed on liver homogenate. It is thus clear that the proteins of the 'particle-free' supernatant fluid (S_2) were able to bind vasopressin more effectively than the other fractions. Investigation of the binding property of the supernatant fluid (S_2) revealed the following points. First, there was a linear relationship between amounts of vasopressin and volumes of supernatant fluid. In a series of experiments, increasing volumes (from 0·25 to 2·0 ml.) of S_2, prepared from a kidney homogenate, were mixed with 10 mU vasopressin: the amounts of vasopressin bound increased from 0·75 to 6·2 mU. Secondly, though there were variations in the amounts of vasopressin bound by supernatant fluids from a specific tissue, each tissue seemed to have a characteristic capacity for binding vasopressin. In three series of experiments in which 0·5 ml. of S_2 from liver, kidney, spleen and muscle homogenates had been mixed with the same amount of vasopressin for the same length of time (10 sec), the mean amount of vasopressin bound was

for liver: 4·95 mU (range: 4·45–5·9),
for kidney: 2·2 mU (range: 1·9–2·25),
for spleen: 2·5 mU (range: 2·0–3·0),
for muscle: 1·75 mU (range 1·6–1·9).

Thirdly, the difference between the capacity for binding vasopressin by S_2 from different tissues could not be explained by the difference in their protein concentration, as their nitrogen concentrations proved to be of the same order of magnitude. The nitrogen concentration of a series of supernatant fluid samples was: liver 1·20, kidney 1·17, spleen 1·03, and muscle 0·88 mg N/ml. Relating the amounts of vasopressin bound with those of nitrogen present, it could be calculated that 1 mg of the supernatant fluid (S_2) of liver, spleen, muscle and kidney bound 10·0, 6·0, 4·4 and 4·0 mU vasopressin respectively (Fig. 2).

Enzymic inactivation of vasopressin

Reconsidering now the rates at which the antidiuretic activity of vasopressin decreased when incubated with tissue homogenates (Fig. 1) it would seem that they represented a combination of protein binding and of enzymic inactivation. To determine the latter, samples of vasopressin incubated with tissue homogenates were divided into halves, one of which, but not the other, was acidified before boiling. After separating the residue from the supernatant fluid by centrifuging, the latter was injected intravenously and assayed for its antidiuretic activity. The difference between the estimations of the acidified

and non-acidified samples was taken to be equal to the amount of antidiuretic activity which had been bound but not inactivated. With the exception of muscle homogenates, which did not appear to inactivate vasopressin appreciably, homogenates of liver, kidney, spleen and duodenum showed the phenomenon of binding coupled with an enzymic process of inactivation (Fig. 1).

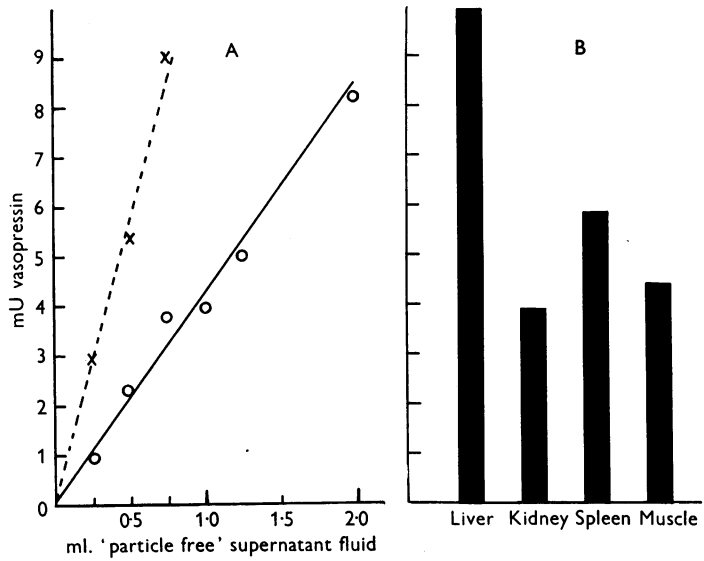

Fig. 2. Binding of vasopressin by 'particle-free' supernatant fluid (S_3) of tissue homogenates. (A) Amounts of vasopressin bound by 'particle-free' supernatant fluid of kidney homogenate (O——O) and of liver homogenate (× - - - ×). (B) Amount of vasopressin bound per mg N of the 'particle-free' supernatant fluid of liver, kidney, spleen and muscle homogenates. All samples of S_3 were shaken with 10 mU vasopressin for 10 sec and then boiled.

The amount of vasopressin bound was greatest immediately after it had been mixed with the tissue homogenate, i.e. at the onset of the incubation; it decreased progressively with time until the amounts present were too small to be assayed. This occurred when both the curve of inactivation and that of binding + inactivation met or ran so closely to each other that they were indistinguishable. This level was reached in 60–90 min by liver homogenate, and in about 120 min by kidney, spleen or duodenum homogenate. Prolonging the incubation periods up to 5 hr did not seem to produce any further significant decrease of the residual antidiuretic activity. This could be the result of a state of equilibrium between enzyme and substrate, a loss of activity of the enzyme with time, or it could represent the transformation of vasopressin into another substance of lower antidiuretic activity. To test the latter hypothesis the standard amount of tissue homogenate, and hence that of enzymic material, was increased from 50 to 100 mg of tissue, but the

amount of vasopressin remained unchanged (=10 mU). After 3 hr of incubation, the antidiuretic activity was so low that it could no longer be assayed (Table 5), thus indicating that the assumed final value of 5–6% of the original antidiuretic activity was *apparent* only and was not constant, as described previously (Dicker & Greenbaum, 1955).

To study the distribution of the enzyme responsible for the inactivation of vasopressin a kidney homogenate was fractionated by centrifuging into (1) nuclear and cellular debris, (2) mitochondria and microsomes, and (3) the 'particle-free' supernatant fluid. Each fraction was resuspended in the original volume of 0·25 M sucrose, and an equal volume of each fraction was incubated with 10 mU of vasopressin for various lengths of time. At the end of each incubation period duplicate samples were taken, one of which was boiled only,

TABLE 5. Inactivation of vasopressin by various amounts of kidney homogenate

Amounts of vasopressin (mU)	Kidney homogenate		Equivalent weight of tissues (mg)	Incubation time (hr)	Estimated antidiuretic activity in mU	
	Volume (ml.)	Dilution				
10·0	0·5	1:10	50	3	0·45	0·51
10·0	0·5	1:5	100	3	Traces	Traces
10·0	1·0	1:10	100	3	Traces	Traces
20·0	0·5	1:10	50	3	0·84	0·85
20·0	0·5	1:5	100	3	0·44	0·55
20·0	1·0	1:10	100	3	0·41	0·52

Kidney homogenate in 0·25 M sucrose. After incubation, all samples were acidified with 3% acetic acid in isotonic NaCl solution and boiled for 3 min. Similar experiments were made using kidney homogenate in modified Krebs–Ringer solution, with similar results.

while the other was acidified with acetic acid before boiling. After 3 hr of incubation the antidiuretic activity present in the mixture of vasopressin and of nuclei and cellular debris was very much the same as at the beginning of the experiment. The antidiuretic activity of the boiled moiety remained at about 8·0 mU, that of the acidified at about 9·5 mU. This suggested that apart from its capacity for binding, the nuclear fraction did not contain any marked amount of enzymic activity. However, when the supernatant fluid (S_2) had been incubated for 2 hr, the antidiuretic activity present in the acidified sample was less than 5% of its original value; after 3 hr of incubation it was too low to be estimated quantitatively. As for the antidiuretic activity present in the mitochondrial and microsomal fraction, the activity in an acidified sample after 3 hr of incubation was still of the order of 30% of its original value (Fig. 3). This activity, however, could be reduced by adding at the end of the second hour of incubation equal volumes of either a fresh sample of mitochondria and microsomes or of the supernatant fluid (S_2): after a further incubation of 2 hr, the antidiuretic activities of the acidified halves were 1·0 and 0·15 mU respectively (Table 6).

To determine whether the difference in the rates of inactivation of vasopressin by the particulate fraction (MM) and the supernatant fluid (S_2) was due either to different enzymes or to different concentrations of the same enzyme, the following experiments were performed. Two samples each of 10 mU vasopressin were incubated, one with 0·5 ml. of the particulate fraction, the other with 0·5 ml. of S_2 which had been diluted 2·5 times. After an incubation of

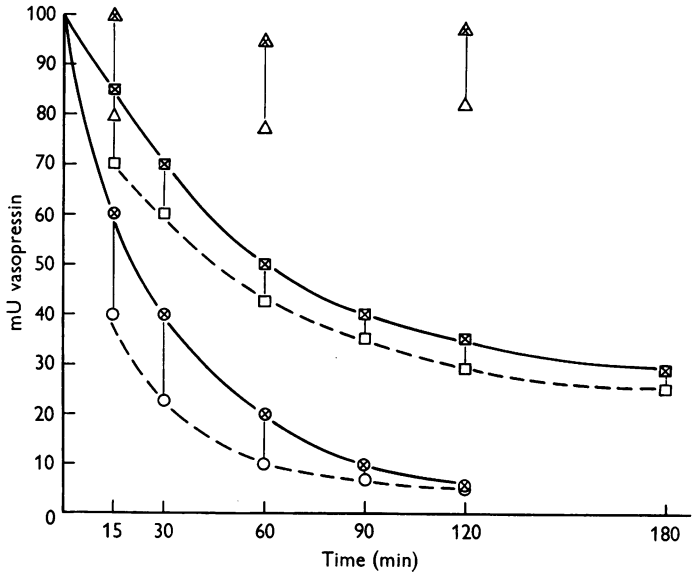

Fig. 3. Inactivation and binding of vasopressin by nuclei and cellular debris (⩓, △), by mitochondria and microsomes (⊠, □), and by 'particle-free' supernatant fluid (⊗, ○) of kidney homogenate. Continuous line, rate of inactivation; broken line, rate of inactivation + binding.

TABLE 6. Inactivation of vasopressin by mitochondria and microsomes and by 'particle-free' supernatant fluid

Preparations	Length of first incubation period (hr)	Preparations	Length of second incubation period (hr)	Treatment	Estimated antidiuretic activity (mU)
10·0 mU vasopressin + 0·5 ml. MM	2	—	—	Boil	3·75
				Acetic acid, boil	4·40
10·0 mU vasopressin + 0·5 ml. MM	2	+ 0·5 ml. MM	2	Boil	0·84
				Acetic acid, boil	1·00
10·0 mU vasopressin + 0·5 ml. MM	2	+ 0·5 ml. S_2	2	Boil	Traces
				Acetic acid, boil	0·15

Kidney homogenate in 0·25 M sucrose solution (dilution 1:10). Nuclei and cellular debris centrifuged off at 600 *g*. Supernatant divided after centrifuging at 34,000 *g* into 'particle-free' supernatant fluid (= S_2) and mitochondrial and microsomal fraction (= MM), which was suspended in original volume of sucrose solution. Acetic acid = 3% acetic acid in isotonic NaCl solution.

15 min the preparations were acidified, boiled and assayed. The antidiuretic activities of both preparations were of the same order of magnitude: 8·2 and 8·0 mU. In the second experiment, a mixture of 0·5 ml. MM and 0·5 ml. S_2, diluted 2·5 times, was incubated for 15 min with 10 mU vasopressin, and the antidiuretic activity of the acidified preparation compared with that of a preparation of 1·0 ml. of MM incubated for the same time with the same amount of vasopressin. Their antidiuretic activities were 4·6 and 4·9 mU, respectively.

TABLE 7. Inactivation of vasopressin by mitochondrial and microsomal fraction and by 'particle-free' supernatant fluid at various dilutions

Amounts of vasopressin (mU)	Kidney homogenate fractions volume (ml.)	First incubation period (min)	Second incubation period (min)	Estimated antidiuretic activity after incubation (mU)
10·0	+0·5 MM	15	—	8·2
	+0·5 S_2 (1/2·5)	15	—	8·0
	+1·0 MM	15	—	4·8
	+0·5 MM +0·5 S_2 (1/2·5)	15	—	4·6
	+1·0 S_2 (1/2·5)	15	—	4·5
	+0·5 MM	15 +0·5 S_2	15	2·5

Kidney homogenate in 0·25 M sucrose, dilution 1:10. Cell debris and nuclei were spun down at 600 *g*. Remainder centrifuged at 34,000 *g* and separated into mitochondrial and microsomal fraction (=MM) and 'particle-free' supernatant fluid (S_2) which was used as such or after being diluted ×2·5 with 0·25 M sucrose. After incubation, *all* samples were acidified with 3·0% acetic acid in isotonic NaCl solution and boiled for 3 min.

Finally, the antidiuretic activity of a mixture of 10 mU vasopressin and 1·0 ml. S_2 diluted 2·5 times and that of a preparation of the same amount of vasopressin and of 1·0 ml. MM both incubated for 15 min, were found to be of the same order, i.e. 4·5 and 4·8 mU (Table 7). These experiments point to the possibility of the same enzymic system existing in both the particulate fraction and the supernatant fluid, though its concentration would be approximately 2·5 times as great in the latter as in the former.

DISCUSSION

Some of the results presented in this paper seem difficult to reconcile with those described previously (Dicker & Greenbaum, 1954). For instance, whereas incubation of vasopressin with kidney *slices* resulted in a decrease of the antidiuretic activity to a constant level of about 20% of the initial activity, there is now evidence that when treated with kidney *homogenate* the inactivation of vasopressin proceeds until no assayable activity remains. In other words, the assumption of a steady value of partial inactivation does not hold when vasopressin is incubated in the presence of kidney homogenate instead of kidney slices. No attempt has been made to elucidate this difference: it may be that homogenization of tissues liberates enzymes which would not be brought into action when the cells are intact as in slices.

The incubation of vasopressin with different tissue homogenates has made it clear that processes of inactivation exist in tissues as different as spleen, duodenum, liver and kidney. Muscles, however, do not seem to exhibit the same property. Evidence that vasopressin could be inactivated by liver and kidney slices (Dicker & Greenbaum, 1954) and by kidney homogenate (Birnie, 1953; Dicker & Greenbaum, 1955) has been described previously. It could be inferred from some of Birnie's (1953) experiments that spleen possessed the same activity. Duodenum homogenate can now be added to this list. It is difficult to see any reason why duodenum or spleen should intervene in the metabolism of a hormone whose ultimate function seems to lie with the kidney. On the other hand, the fact that vasopressin can be inactivated by a ubiquitous enzyme system may help ultimately to determine its mode of action.

All tissue homogenates examined, including muscle, have the property of binding vasopressin when mixed with it. Once bound, vasopressin cannot be released by simple heating. The present investigation fully supports that of Fromageot & Maier-Hüser (1951) who found that elution took place in acid solution only. The property of binding is shown by all the cellular components though with different intensities: the so-called 'particle-free' supernatant fluid being more efficient than the mitochondria and microsomes. Though binding took place with all tissue homogenates, the amount of vasopressin bound was not the same in all. When compared with the normal concentration of the solution it could be shown that the 'particle-free' supernatant fluid of liver homogenate had a much greater binding capacity than that of muscle or spleen, and that the 'particle-free' supernatant fluid of kidney homogenate was the least active of all. The conditions of the experiments did not give any indication whether similar bindings occurred *in vivo*.

Once vasopressin was bound by the cellular proteins a slow process of inactivation took place. This occurred with homogenates (or their fractions) of kidney, liver, spleen and duodenum, but not with that of muscle. It could be stopped by plunging the preparation into boiling water for 3 min. The inactivation did not seem to be affected by the presence or absence of O_2, and was not stopped by potassium cyanide. In those tissues where an enzymic activity was found, its highest concentration was in the 'particle-free' supernatant fluid, though it was present also in the mitochondrial and microsomal fraction; the concentration in the former appeared to be about 2·5 times that found in the particles. However, it is not possible at this stage to ascertain whether it is the same enzyme which acts in both 'particle-free' supernatant fluid and in the mitochondrial and microsomal fraction. Nor is it possible to say whether the same enzyme exists in kidney, liver, spleen and duodenum. The experiments of van Dyke (1955) have shown two possible sites of inactivation of the vasopressin molecule: first, by cleavage of the S—S bond and secondly, by the

splitting of the peptide bond between the carboxyl group of arginine and the amino group of glycinamide. Other sites are also potentially available. This problem is now being investigated.

SUMMARY

1. Kidney, liver, duodenum, spleen and muscle homogenates were incubated with various amounts of vasopressin. After centrifuging, the supernatant fluid, either acidified with 3% acetic acid solution before boiling, or boiled only, was assayed for its antidiuretic activity.

2. When kidney or liver homogenates were mixed with vasopressin and boiled *immediately*, there was a significant decrease of the antidiuretic activity. This could be prevented by acidifying the preparation before boiling. By separating full homogenates into their constituents: (*a*) nuclei and nuclear debris, (*b*) mitochondria and microsomes, (*c*) 'particle-free' supernatant fluid, it could be shown that the binding of vasopressin was least with the nuclei, intermediate with the mitochondria and microsomes and greatest with the 'particle-free' supernatant fluid.

3. The 'particle-free' supernatant fluid of liver bound more vasopressin than that of kidney, muscle or spleen, whether expressed as vasopressin bound per unit of wet weight or per mg of nitrogen.

4. There was a greater concentration of the enzymic activity responsible for the inactivation of vasopressin in the 'particle-free' supernatant fluid of kidney than in the mitochondria and microsomes. No significant enzymic activity could be detected in the nuclear fraction.

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