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Biochem. J. (1961) **79**, 663

Inactivation of Arginine Vasopressin by Rat-Kidney Slices

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(Received 7 October 1960)

Previous studies have indicated that the mammalian kidney can inactivate as well as excrete vasopressin. Only 5–10% of a dose of vasopressin injected intravenously into rats can be recovered from the urine (Heller, 1952; Ginsburg, 1954), although the kidneys have been found responsible for removing approximately half of the injected hormone from the circulating blood (Ginsburg & Heller, 1953; Dicker, 1954; Crawford & Pinkham, 1954; Lauson & Bocanegra, 1960). There is suggestive evidence *in vivo* that this unexcreted vasopressin is destroyed by the kidney (Heller & Zaidi, 1957). At the present time, however, little is known about the nature of the cellular or enzymic processes involved in this inactivation of vasopressin. In the present paper we have attempted *in vitro* to develop a system useful for such investigations. Also reported are studies in which vasopressin, labelled *in vivo* with [³⁵S]cystine, has been used in an effort to study the manner in which this polypeptide hormone is inactivated by rat-kidney slices.

EXPERIMENTAL

Materials. Arginine vasopressin was purified as described by Sachs (1960a) with an estimated potency of 300–400 i.u./mg. This material showed only a single component when subjected to paper ionophoresis (Sachs, 1960a) or paper chromatography (Heller & Lederis, 1958). Oxytocin was highly purified synthetic oxytocin with a

stated potency of 450 i.u./ml. from Sandoz Co., Hanover, N.J., U.S.A. Heparin was from Upjohn Co., Kalamazoo, Mich., U.S.A. Phenoxybenzamine hydrochloride (Dibenzylamine) was from Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A.

Preparation of kidney slices. Male Sprague-Dawley rats weighing from 200 to 350 g. were stunned by a blow on the head and killed by decapitation. A midline incision was made and both kidneys, freed from perirenal fat and decapsulated, were placed in ice-cold Cross & Taggart (1950) buffer containing 0.01M-sodium acetate. Longitudinal sections of 0.5 mm. thickness were cut with a Stadie-Riggs microtome; the first slice on each side of the kidney was rejected and the second slice placed in fresh ice-cold buffer. Each slice weighed about 75 mg. and contained both cortical and medullary tissue. These operations were performed at 6°.

Incubation procedure. Unless otherwise stated, each incubation flask contained four kidney slices in 4 ml. of Cross & Taggart buffer (containing about 300 mg. of tissue). Slices and buffer were initially shaken for 30 min. at 25° under O₂, after which time the total wet weight of the slices was determined and the slices were transferred to 4 ml. of fresh buffer. Vasopressin (3 i.u.) was added and the incubation continued, under the same conditions, for a further time interval ranging from 30 to 120 min. During this second incubation period, measured portions of the incubation fluid were periodically taken for assay; the samples were pipetted into 5% trichloroacetic acid to stop any enzymic reaction. The trichloroacetic acid was subsequently removed by ether extraction (4 × 3 vol.). Ether dissolved in the aqueous phase was evaporated in a stream of N₂ at 40°, and the samples were assayed for pressor activity.

Assay of pressor activity. The method of Dekanski (1952) was used except that Dibenzylamine (1 mg./100 g. body wt.) was injected intramuscularly 12–24 hr. before the beginning of assay. Anaesthesia was induced by intraperitoneal

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injection of a 25% (w/v) solution of urethane (0.4 ml./100 g. body wt.). A (2 by 2) dose assay was used in duplicate on all occasions.

Preparation of [^{35}S]vasopressin. [^{35}S]Cystine was prepared as described by Sachs (1960a) and purified by chromatography on Dowex 50 (Hirs, Moore & Stein, 1954). The purified [^{35}S]cystine was treated with thioglycolic acid, and subsequently 2×10^8 counts/min. of the reduced material were infused continuously into the third ventricle of an anaesthetized, 15 kg. male dog, over a period of 8 hr. After the infusion was complete, the animal was killed and the hypothalamus and neurohypophysis were excised, combined and used for the isolation of [^{35}S]vasopressin as described by Sachs (1960b).

The purification steps included: (1) homogenization of the tissue in 0.1 N-acetic acid; (2) precipitation of proteins with 10% (w/v) trichloroacetic acid; (3) addition of 300 i.u. of highly purified carrier arginine vasopressin to the trichloroacetic acid-soluble extract (which contained 18 i.u. of pressor activity); (4) removal of trichloroacetic acid with ether and passage of the aqueous extract through a small Dowex XE-64 resin (H form); (5) elution of the hormone with pyridine-acetate, and, after removal of solvent *in vacuo*, chromatography of vasopressin on a carboxymethylcellulose ion-exchange column (0.9 cm. $\times$ 30 cm.); (6) rechromatography of the biologically active peak on Dowex XE-64 (ammonium form) with a continuous-gradient elution procedure; and finally (7) adsorption and elution of the active peak on a small (2.0 cm. $\times$ 1.0 cm.) column of Dowex XE-64 (H form). The amount of [^{35}S]vasopressin obtained was approx. 123 i.u. and contained a total of 370 counts/min. Previous experiments (Sachs, 1960a, b) have shown that [^{35}S]vasopressin obtained at this stage of purification was essentially pure.

Radioactive materials were plated at infinite thinness on stainless-steel planchets, and counting was performed with a thin window Geiger-Müller tube and scaler (Nuclear Instrument and Chemical Corp., Chicago, Ill., U.S.A.).

Buffers. Those used were: citrate-phosphate buffer (McIlvaine, 1921), consisting of 0.1 M-citric acid mixed with 0.2 M-disodium hydrogen phosphate; 2-amino-2-hydroxy-methylpropane-1:3-diol (tris) buffer (Michaelis, 1930), consisting of 0.2 M-tris mixed with 0.2 M-hydrochloric acid; borax-sodium hydroxide buffer (Clark & Lubs, 1948), consisting of 0.05 M-borax mixed with 0.2 M-sodium hydroxide.

RESULTS

Recovery and estimation of vasopressin from incubation media. It was first necessary to determine whether vasopressin was stable in Cross-Taggart buffer and to determine the percentage of vasopressin which was lost due to transference of samples to trichloroacetic acid and subsequent ether extraction. Accordingly, 3 i.u. of vasopressin in 4 ml. of Cross-Taggart buffer was incubated and samples were transferred to trichloroacetic acid at the beginning, and after 60 min., of incubation at 25°. Half of the samples contained bovine serum albumin (2.5 mg./ml.) in addition to vasopressin, to ascertain whether the presence of protein would lower the efficiency of the extraction procedure.

No difference in recovery could be detected between any of the samples. The recovery of vasopressin from 16 experiments was $88.9 \pm 2.9\%$ (mean $\pm$ S.D.), and all future vasopressin determinations were corrected on the basis of this recovery.

Time course of vasopressin disappearance during incubation with rat-kidney slices. The rate of disappearance of vasopressin from the incubation medium in the presence of kidney slices (approx. 75 mg./ml.) was studied under the standard conditions (see Experimental section). The rate of disappearance of the hormone from the medium essentially follows a first-order reaction (Fig. 1). An apparent first-order velocity constant was calculated for each of four such experiments. These apparent first-order rate constants have been corrected to represent those obtained from 75 mg. of tissue/ml. of incubation medium and have been given the symbol k_{75} ; the mean k_{75} value equalled $0.0109 \pm 0.0005 \text{ min.}^{-1}$. The validity of the correction applied for kidney-slice wt./ml. of incubation medium is demonstrated below.

Necessity for the presence of the kidney slice during vasopressin disappearance. In order to exclude the possibility that soluble enzymes liberated into the incubation medium by the kidney slice were responsible for inactivation of vasopressin, the following control experiments were performed. Kidney slices (approx. 75 mg./ml.) were incubated under standard conditions for 60 min. in the absence of vasopressin. The slices were removed from the medium, vasopressin was added (0.75 i.u./ml.), and the incubation was continued for a further

Fig. 1. First-order plot of vasopressin disappearance during incubation with rat-kidney slices. C_0 , Initial vasopressin concentration; C_t , vasopressin concentration at any time during incubation. Each point is the mean of four experiments. Each flask contained 3 i.u. of vasopressin, 70–80 mg. of slices/ml., in a final volume of 4 ml. of buffer, incubated under O_2 at 25° for the time shown.

60 min., during which time samples were taken for assay at 20 min. intervals. Less than 10 % of the added vasopressin was lost during this 60 min. period, whereas, when slices were present, over 50 % of the added vasopressin was lost during the first 60 min. incubation. Soluble enzymes prepared by incubating kidney slices at 37° (instead of 25°), which were capable of inactivating vasopressin, were shown to be stable under the standard incubation conditions.

Effect of various amounts of kidney tissue on the rate of vasopressin disappearance. The disappearance of vasopressin incubated under standard conditions in the presence of various amounts of kidney slices was determined over a period of 2 hr. The apparent first-order rate constant was calculated for the different amounts of kidney tissue used. Fig. 2 shows a linear relationship between the apparent rate constant and the amount of kidney tissue used throughout the range studied, 0–150 mg. of kidney slice/ml. Unless otherwise stated, subsequent experiments employed 280–320 mg. of tissue in the incubation medium. The rates of hormone disappearance were corrected to that expected from using 300 mg. of kidney tissue.

Effect of vasopressin concentration on the rate of vasopressin disappearance. The rate of vasopressin disappearance was measured in a series of experiments where various concentrations of hormone (0.45–15.3 i.u./ml., or approx. 1.04–35.3 μ M respectively) were incubated with slices under standard conditions. In Table 1 are shown the k_{75} values calculated for each vasopressin concentration.

At low vasopressin concentrations (0.5–2.0 i.u./ml.), the apparent rate constant remained unchanged, but as the concentration of hormone was increased above 3 i.u./ml., the k_{75} values fell progressively. A Lineweaver–Burk plot gave half-maximal velocity at a vasopressin concentration of 23.8 i.u./ml. (or 55 μ M, assuming that 1 mg. of vasopressin is equivalent to approx. 400 i.u.).

Unless stated otherwise, the standard incubation flask contained an initial vasopressin concentration of 0.75 i.u./ml. (1.7 μ M). This choice of vasopressin concentration had the advantage of being within the substrate concentration range (0.5–2.0 i.u./ml.) where the apparent rate constant was unchanging

and maximal. Also, greatest percentage loss of vasopressin occurred under these conditions, so enabling high accuracy in the determination of k_{75} values.

Effect of pH on the loss of vasopressin during incubation with rat-kidney slices. The medium used (Cross & Taggart) for the incubation of slices and vasopressin contained 8 mM-phosphate buffer. The initial pH of the complete standard system was 7.15 and remained unaltered throughout an experiment. At more acid or alkaline pH values, it was necessary to increase the buffer strength to 0.04 M to maintain a constant pH during the course of the incubation. Increasing the buffer strength from 8 mM to 0.04 M at pH 7.15 did not affect the rate of disappearance of vasopressin. In addition, replacing the phosphate buffer with either citrate-phosphate or tris buffer (both at pH 7.15) did not affect the rate of loss of vasopressin from the incubation medium. However, at pH 4.0 and pH 5.5 (citrate-phosphate) little or no loss of vasopressin occurred after 40 min. incubation in the presence of slices. On the other hand, at pH 7

Fig. 2. Effect of changing the weight of kidney tissue on the apparent first-order rate constant. The apparent rate constant (k) is calculated from the slope of a plot as shown in Fig. 1; the slope was the best straight line calculated from four experimental points. Each flask contained 3 i.u. of vasopressin and 2–8 kidney slices in 4 ml. of buffer, incubated under O_2 at 25° for 120 min. Samples were taken for assay at 40 min. intervals.

Table 1. *Effect of vasopressin concentration on the k_{75} values*

Each flask contained four kidney slices and various amounts of vasopressin in 2 ml. of Cross & Taggart buffer and was incubated under O_2 at 25° for 60 min. k_{75} values were calculated from three experimental points. i.u./ml. $\times 2.307 \times 10^{-3} = \mu$ moles/ml.; this is based on the assumption that each mg. of vasopressin is equivalent to 400 i.u.

Initial vasopressin concn. (i.u./ml.)	0.45	0.89	1.88	3.23	5.79	11.3	12.7	15.3
$10^3 k_{75}$ (min. ⁻¹)	1.03	0.98	1.06	0.93	0.75	0.57	0.41	0.31

and 8 (phosphate or tris buffer) or pH 9 and 10 (borax-sodium hydroxide buffer), from one-third to one-half of the vasopressin was lost from the incubation fluid after 40 min. of incubation. There appeared to be no significant difference in the amount of vasopressin lost over the pH range 7-10. Control experiments showed that the loss of vasopressin at the higher pH values (pH 9 and 10) was not due to soluble enzymes liberated into the incubation medium; negligible quantities of hormone were lost after 40 min. of incubation with medium (pH 9 or 10) previously incubated with slices alone.

Effect of temperature on the rate of vasopressin disappearance from the incubation medium. Under the standard incubation conditions at 25°, k_{75} for vasopressin disappearance was consistently reproducible: $0.0109 \pm 0.0005 \text{ min.}^{-1}$ (four experiments). Fig. 3 shows that at 7° the rate of vasopressin loss, though still first-order, was much slower. The mean k_{75} , calculated from four experiments, was $0.0026 \pm 0.004 \text{ min.}^{-1}$, which was significantly different from the k_{75} determined at 25°. The Q_{10} (that is the factor by which the apparent velocity constant is altered by a 10° change in temperature) calculated from the two mean k_{75} values was approx. 2.0. Control experiments in which vasopressin (0.75 i.u./ml.) was incubated for 60 min. with buffer previously incubated at 7° with kidney slices showed no detectable loss of vasopressin.

When kidney slices were incubated with vasopressin under standard conditions at 37° (instead of 25°), the pressor activity disappeared rapidly

from the incubation medium during the first 60 min. However, it could be shown that slices incubated at 37° for 60 min. (instead of at 25°) liberated heat-labile (100°; 2 min.) material into the incubation medium capable of inactivating vasopressin. Such media inactivated as much as 66% of added vasopressin over a period of 60 min. at 25°.

Effect of inhibitors on vasopressin loss from the incubation medium. A number of substances, which have been previously shown to inhibit either the energy-yielding and transport mechanisms of kidney cells or the activity of a variety of proteolytic enzymes, were tested for their ability to inhibit the inactivation of vasopressin by kidney slices.

Dinitrophenol at a concentration of either 0.1 or 1 mM (in the presence or absence of acetate) did not affect the rate of loss of vasopressin from the incubation medium; 1 mM-*p*-chloromercuribenzoate gave a 30% inhibition; 1 mM-diisopropyl phosphorofluoridate (preincubated with slices), soya-bean trypsin inhibitor (1 mg./ml.), 0.04 M-ethylene-diaminetetra-acetate and 0.2 M-phosphate (pH 7.15), did not appear to affect the rate of loss of vasopressin. On the other hand, oxytocin, a peptide with a structure similar to vasopressin, showed a marked ability to inhibit the rate of loss of vasopressin from the incubation medium. Under standard conditions (0.75 i.u. of vasopressin/ml.), the addition of synthetic oxytocin (15 i.u./ml.) inhibited the rate of loss of vasopressin by about 40-50%. The mean k_{75} for vasopressin loss under these conditions was $0.0059 \pm 0.00025 \text{ min.}^{-1}$ (four experiments). If oxytocin were destroyed when incubated with vasopressin in the presence of kidney slices the inhibition of vasopressin destruction would be even greater since, for each removed portion of the incubation medium assayed for vasopressin, a constant correction had been made for the pressor activity of oxytocin, assuming each i.u. of oxytocin had 0.95% of the pressor activity of an i.u. of vasopressin.

Fig. 3. Effect of temperature on the rate of vasopressin disappearance during incubation with rat-kidney slices. ○, Incubation temperature, 25°; ●, incubation temperature, 7°; C_0 and C_t are as for Fig. 1. Each point is the mean of four experiments. Incubation conditions are given in the legend to Fig. 1.

Fate of vasopressin incubated with kidney slices

Experiments were carried out to determine whether vasopressin which disappeared from the incubation medium was taken up and stored by the kidney slice. Parallel incubations were performed under standard conditions in flasks containing either the complete standard system or slices and buffer with no added vasopressin. After 60 min. at 25°, samples were taken from the incubation media (in order to determine the amount of hormone lost) and the slices were simultaneously removed from the incubation fluid and homogenized with 10% (w/v) trichloroacetic acid. The homo-

genates were centrifuged and the precipitated proteins washed several times with 5% (w/v) trichloroacetic acid; the washings were combined with the first trichloroacetic acid supernatant. Solutions were treated with ether in order to remove trichloroacetic acid, then the aqueous extracts were assayed for pressor activity. Both the control (slices incubated alone) and test (slices incubated with vasopressin) extracts contained barely detectable amounts of pressor activity, 25.3 and 27.8 milliunits/300 mg. of kidney tissue respectively (mean of three experiments). These figures have been corrected for vasopressin recovery of 72.7%, which was the mean recovery (three experiments) in which kidney slices were homogenized with trichloroacetic acid in the presence of known quantities of vasopressin, and the extracts treated as described above.

The small amount of pressor activity (which probably was not vasopressin) recovered from kidney slices incubated with vasopressin would account for only 2.2% of the hormone lost during the 60 min. incubation period. Apparently then, vasopressin, incubated with kidney slices, was transformed into some inactive product (or products).

Quantitative ninhydrin determinations on paper and ion-exchange chromatography of the incubation fluid (before and after incubation) failed to detect the presence of newly formed amino groups or peptide material resulting from the inactivation of vasopressin. However, this inability to demonstrate any peptide- (or amide-) bond splitting of vasopressin was in all likelihood due to the presence of relatively large quantities of ninhydrin-reactive material arising from the kidney slice. Experiments were therefore initiated with [^{35}S]-vasopressin as substrate, in order (1) to confirm the biological recovery experiments, and (2) to obtain some information about the chemical properties of the inactivation product or products. Accordingly, [^{35}S]-vasopressin (123 i.u.; 370 counts/min.) was incubated in 5.3 ml. of the standard buffer which contained 1.5 g. of kidney slices. Before incubation, a zero-time sample (30 i.u.; 91 counts/min.) was removed and added to 2 vol. of 15% (w/v) trichloroacetic acid. After 30 min. at 25° a measured portion was removed from the incubation medium for assay in the usual manner; the remainder of the incubation fluid was poured into 10 ml. of 15% (w/v) trichloroacetic acid. The slices were separately treated with trichloroacetic acid as described above. All the trichloroacetic acid supernatant solutions and the washings were combined, extracted with ether and adjusted to pH 5.0 with dilute sodium hydrogen carbonate. The zero-time sample was treated in a similar manner. These two aqueous extracts (obtained at

zero time and after 30 min. of incubation) were each passed through a Dowex XE-64 (H form) ion-exchange column (0.9 cm. $\times$ 2.0 cm.). Acetic acid [50 ml. of 0.25% (v/v)] was passed through the column, followed by 40 ml. of pyridine-acetic acid solution [30% (v/v) pyridine, 4% (v/v) acetic acid] and finally 25 ml. of 0.2N-hydrochloric acid. Each of the effluent fractions was assayed for vasopressin and radioactivity. The results are: (a) amount of [^{35}S]-vasopressin removed at zero time, 30 i.u., 91 counts/min.; (b) amount of vasopressin in the zero-time sample eluted from Dowex XE-64 with pyridine-acetic acid, 21 i.u., 70 counts/min.; (c) amount of [^{35}S]-vasopressin incubated with kidney slices, 93 i.u., 279 counts/min.; (d) amount of vasopressin activity remaining after 30 min., 60 i.u.; (e) amount inactivated, 33 i.u.; (f) amount of vasopressin eluted from Dowex XE-64 with pyridine-acetic acid, 44 i.u., 184 counts/min.

The other effluent fractions did not contain either vasopressin activity or radioactivity.

In addition there were no detectable counts above background (13.7 counts/min.) associated with the kidney-slice proteins, which were precipitated and washed with trichloroacetic acid and then ether, plated, and counted.

Apparently all the recovered counts which included [^{35}S]-vasopressin and ^{35}S -labelled inactivation products were adsorbed on Dowex XE-64 (a cation-exchange resin) at pH 5.0 and could be eluted with pyridine-acetic acid solution. The zero-time sample gave identical results except that the specific activity of the recovered [^{35}S]-vasopressin remained essentially unchanged from the specific activity of the original starting material.

DISCUSSION

Both crude and partially purified fractions of mammalian kidney extracts (Heller, 1952; Dicker, 1960) have been shown to be capable of inactivating vasopressin *in vitro*. Presumably, these extracts contain several enzymic activities (Dicker, 1960) directed towards either the disulphide or amide bonds contained in the vasopressin molecule. Further, it has been suggested that these enzymes are responsible for the inactivation of vasopressin *in vivo*. In view of the demonstration that many tissues contain a spectrum of hydrolytic enzymes whose activities become apparent only after disruption of the cell or particles, or both, contained within the cell (de Duve, 1959), there is a reasonable possibility that such enzymes present in tissue extracts may either have no relationship to or mask similar enzymic activities operative in the intact cell. Therefore, in order to study the manner in which the mammalian kidney inactivates vasopressin, it was decided to use rat-kidney slices in

these investigations. Such preparations *in vitro* have proved to be of value in studies concerned with renal tubular transport (Taggart, 1958). In this paper are the first phases of these studies where we have attempted to define some of the properties and requirements for the inactivation of arginine vasopressin *in vitro*, by rat-kidney slices.

It was expected (and subsequently demonstrated) that the incubation of kidney slices could under some circumstances give rise to the presence of enzymes in the incubation medium capable of inactivating vasopressin. Since such a situation would be analogous to the studies conducted by Dicker (1960) with cell-free preparations, it was essential to find conditions where the intact slice was solely and intimately associated with the disappearance of vasopressin activity from the incubation medium. Under our standard incubation conditions, which included the use of slices previously incubated in buffer alone, and an incubation temperature of 25°, it could be shown that this requirement was essentially fulfilled.

Aerobic phosphorylation, an important requirement for active transport by the kidney cell, was apparently not a rate-limiting step in the inactivation of vasopressin. Some of the observations on the rate of vasopressin inactivation by kidney slices may be summarized: the overall velocity followed first-order kinetics; the apparent first-order rate constant was proportional to kidney-slice concentration and decreased with increasing substrate concentration; the vasopressin concentration which gave half-maximal velocity was approximately 55 μM ; and a 10° rise in temperature (over the range studied) gave an approximate doubling of the reaction rate. These results are reminiscent of the properties of a number of well-characterized proteolytic enzyme systems (cf. Elkins-Kaufman & Neurath, 1948; Neurath & Schwert, 1950; Adams & Smith, 1951). However, supplementing the incubation medium or pre-incubating slices with a number of reagents (previously shown to inhibit the action of a variety of such proteases or enzymes, or both, capable of inactivating vasopressin) failed to affect the rate of disappearance of vasopressin from the incubation medium. Nevertheless, the inability of these inhibitors to affect the rate of inactivation of vasopressin in a crude system does not preclude the possibility that such enzymes may play an important role in the inactivation of vasopressin by the kidney cell. In any event, valid conclusions concerning the kind of enzymic activities involved would require an identification of the reaction products.

The possibility remained that the vasopressin which disappeared from the incubation medium was taken up and stored by the kidney slice in a

manner analogous to that observed *in vivo* with adrenocorticotrophic hormone (Richards & Sayers, 1951) and other foreign proteins (Oliver, MacDowell, Moses & Lee, 1954), although vasopressin storage seemed unlikely (Heller & Zaidi, 1957). Trichloroacetic acid extracts of slices previously incubated with vasopressin did not contain significant quantities of pressor activity. The extraction procedure used was previously shown to be effective in the recovery of vasopressin from brain tissue (Sachs, 1960a), from blood (Weinstein, Berne & Sachs, 1960) and from kidney slices homogenized in the presence of vasopressin and trichloroacetic acid.

Also, the biological recovery experiments were confirmed by the use of [^{35}S]vasopressin prepared biosynthetically. Speculations on the precise nature of the inactivation products would at this point be premature.

SUMMARY

1. Highly purified arginine vasopressin, in the presence of rat-kidney slices, was shown to disappear at a rapid rate from the incubation medium.

2. The disappearance of vasopressin from the incubation medium was not due to the presence of degradative enzymes in the medium which were liberated from either broken or intact cells.

3. The apparent first-order rate constant was consistently reproducible with slices taken from different animals on successive days and was shown to be proportional to the weight of kidney tissue.

4. Above 3 i.u. of vasopressin/ml., the apparent k_{75} fell progressively as the concentration of hormone was increased; half-maximal velocity was achieved at a concentration of 55 μM .

5. Below pH 6.0, little or no loss of vasopressin occurred, whereas the optimum rate of disappearance of hormone was observed over the pH range 7–10.

6. The mean k_{75} values were found to be $0.0109 \pm 0.0005 \text{ min.}^{-1}$ at 25° and $0.0026 \pm 0.0004 \text{ min.}^{-1}$ at 7°.

7. Dinitrophenol (0.1 or 1 mM), diisopropyl phosphorofluoridate (1 mM), trypsin inhibitor (1 mg./ml.), ethylenediaminetetraacetate (0.04M), and phosphate (0.2M) did not affect the rate of loss of vasopressin from the incubation medium; *p*-chloromercuribenzoate (1 mM) and oxytocin (15 i.u./ml.) induced 30 and 50% inhibition respectively.

8. The vasopressin which disappeared from the incubation medium could not be recovered from the kidney slice and had apparently undergone inactivation. This was confirmed by the use of [^{35}S]vasopressin prepared biosynthetically.

This work was supported in part by grants from the Air Force Office of Scientific Research [Contract No. AF

49(638)-764], from the National Institutes of Health, United States Public Health Service (No. A2650) and from the Foundation for the study of Diabetes and Related Metabolic Disorders. The authors would like to express their gratitude to Dr Ned Redgate of the Dept. of Physiology for the performance of the operative procedure involving the infusion of [³⁵S]cystine into the third ventricle of the dog.

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Acidic Peptides of the Lens

7. THE PREPARATIVE ENZYMIC SYNTHESIS OF OPHTHALMIC ACID*

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(Received 14 December 1960)

Ophthalmic acid (γ -glutamyl- α -amino-*n*-butyryl-glycine) is a peptide isolated from calf lens (Waley, 1958). This analogue of glutathione has been used in studies on *Hydra* (Cliffe & Waley, 1958a), on the metabolism of γ -glutamyl peptides (Cliffe & Waley, 1961a), on the glyoxalase reaction (Cliffe & Waley, 1959, 1961b) and on embryological development (Lallier, 1959). The need for a method of producing ophthalmic acid is not altogether met by the synthesis reported by Waley (1958), which is fairly lengthy and requires the expensive L- α -amino-*n*-butyric acid. The enzymic synthesis of ophthalmic acid from the constituent amino acids (Cliffe & Waley, 1958b; 1961a), in which DL- α -amino-*n*-butyric acid is used, has now been adapted to the production of this tripeptide.

The source of enzyme for the synthesis is rabbit liver, supplemented by lens (Cliffe & Waley, 1961a). The main problem is the isolation of the ophthalmic

acid. This has been accomplished by ion-exchange chromatography. The method was based on the carrier-displacement technique (Buchanan, 1957). The separation of aspartic acid and glutamic acid was effected by Buchanan by adsorption on an anion-exchange resin in the acetate form; this column was then fitted over another column that contained the resin in the lactate form. When both columns were eluted with aqueous citric acid, the glutamic acid and aspartic acid emerged in that order, separated by a band of lactic acid ('the carrier'). When the same method was tested on a mixture of glutamic acid and ophthalmic acid, separation was effected, although lactic acid did not intervene between the amino acid and the peptide. Evidently ophthalmic acid is not acidic enough to be strongly retained by the lactate column. This trial experiment was carried out with about 5 mg. of ophthalmic acid and 50 mg. of glutamic acid on a column of 0.25 cm.² cross-sectional area. The fractions that contained the ophthalmic acid were extracted with amyl alcohol, and with ethyl

* Part 6: Cliffe & Waley (1961a).

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