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RENAL EXCRETION OF HYALURONIDASE AND CALCIUM IN MAN DURING THE ANTIDIURETIC ACTION OF VASOPRESSINS AND SOME ANALOGUES

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Following the earlier observation of Ginetzinsky (1958) on dogs and rats, it was shown (Dicker & Eggleton, 1960) in man that hyaluronidase was present in urine secreted at a slow rate of flow; it disappeared during a water or alcohol diuresis and reappeared when the diuresis was suppressed by injection of vasopressin. Commercial preparations of vasopressin are extracted either from ox or from hog posterior pituitary glands, or from a mixture of both, and are standardized in terms of pressor units. It is known that the chemical structure of hog vasopressin differs from that of ox: the former being lysine-vasopressin, the latter arginine-vasopressin (Turner, Pierce & du Vigneaud, 1951; Popenoe, Lawler & du Vigneaud, 1952). Arginine-vasopressin exists in man, ox, horse, sheep, rat, dog, monkey, camel and marsupials (Acher & Chauvet, 1953; Light & du Vigneaud, 1958; Acher, Chauvet & Lenci, 1959; Sachs & Barret, 1959; Sawyer, Munsick & van Dyke, 1960), whereas lysine-vasopressin exists in hog and hippopotamus only (Popenoe *et al.* 1952; Heller & Lederis, 1960). Physiologically the two vasopressins differ significantly: van Dyke, Engel & Adamson (1956) showed that one pressor unit of lysine vasopressin has but one sixth of the antidiuretic activity of an equal amount of arginine-vasopressin, when assayed by intravenous injection in dogs. A similar discrepancy between the action of the two vasopressins has been observed in the rat (Nielsen 1958; Thorn, 1957, 1959; Sawyer, 1958). It was therefore thought of interest to compare the effects of lysine- and arginine-vasopressins in man. As analogues of vasopressin and oxytocin were made available to us the investigation was extended, and the relative antidiuretic activity of these compounds was determined in man and correlated with the excretion of hyaluronidase. Since Thorn (1960) had recently shown that an increased calcium excretion accompanied the antidiuresis produced by lysine-vasopressin in dogs, this was also determined in man.

METHODS

Fifteen young healthy subjects were used, some on two or more occasions. After a period of at least 3 hr without food or water, diuresis was induced by ingestion of water, following one or two cups of tea, until the water load was of the order of 14 ml./kg body weight; it was maintained at that level throughout the experiment. When the subject was in full diuresis, an intravenous infusion of one or other compound was given, for a period of 10 min usually. When the effect of the first compound was over, and the diuresis was back to its initial level, another substance was infused in similar fashion, thus allowing comparison of the two in the same individual. All subjects were sitting during the experiment, but stood for micturition: urine was collected at 15 min intervals. The temperature of the room varied between 21 and 23° C.

Calcium in the urine was estimated by the method of Bachra, Dauer & Sobel (1958). Repeated measurements of the same solution showed that amounts of 5–50 μg calcium per sample of 0·1–0·2 ml. could be determined with a reproducibility of 2%. Sodium and potassium were estimated by flame photometer. Total osmolarity of the urine samples was measured by the thermo-electric vapour-pressure method of Baldes (1934) modified as suggested by Krogh (1939). Repeated measurements of the same solution indicated that the mean error of a single determination was about $\pm 2\%$, or $\pm 5\%$ if the concentration was less than 250 m-osmole/l. Hyaluronidase was determined by change in viscosity of hyaluronic acid (Dicker & Eggleton, 1960). The molecular weight of the carbowax used in concentrating the urine samples (15,000–20,000) is close to the pore size of the Visking tubing in which it was contained, and with some batches slight leakage occurred through the tubing. The viscosity of these smaller molecules was, however, unaffected by boiling, unlike the original mixture, so that the boiled sample of urine still served as a satisfactory blank in relation to any change in viscosity induced by hyaluronidase in the unboiled sample. In view of the recent criticism of Berlyne (1961) that hyaluronidase may become adsorbed on dialysis tubing, it should be said that with the Visking tubing used in the present work standard hyaluronidase was recovered quantitatively when added to a large volume of water or dilute urine and concentrated in the usual way.

Drugs used for intravenous administration were: arginine^8-vasopressin, arginine^8-oxytocin, lysine^8-vasopressin, lysine^8-oxytocin, phenylalanine^2-lysine^8-vasopressin and oxytocin. The first three were extracts from ox, chicken and pig pituitary glands, respectively. The last three were synthetic products manufactured by Sandoz, Ltd.

Vasopressin analogues as well as lysine^8- and arginine^8-oxytocin were assayed for their pressor activity according to Dekanski's (1952) method against the Third International Standard for posterior pituitary (Bangham & Musset, 1958). They were injected or infused intravenously in doses stated in pressor units. Oxytocin was administered intravenously in doses stated in oxytocic units.

RESULTS

Arginine^8-vasopressin injections or infusions were given on eleven occasions, lysine^8-vasopressin fifteen times, arginine^8-oxytocin six times, lysine^8-oxytocin five times, phenylalanine^2-lysine^8-vasopressin (= PLV_2) four times and oxytocin twice, with entirely consistent results. A representative comparison of their antidiuretic activities is shown in Fig. 1. Oxytocin (dose of 19 m-u. of oxytocic activity) shows no activity, a result in agreement with those of other observers (Cross, Dicker, Kitchin, Lloyd & Pickford, 1960). Of the other compounds given in a dose of 19 m-u. of

pressor activity, PLV_2 produces negligible antidiuresis, lysine^8-oxytocin appreciably more, arginine^8-oxytocin and lysine^8-vasopressin considerably more and arginine^8-vasopressin most of all. A more quantitative comparison of the last two substances was made in one individual by giving smaller doses in single intravenous injections. The results given in Fig. 2 suggest that arginine^8-vasopressin has approximately twice the antidiuretic activity of lysine^8-vasopressin.

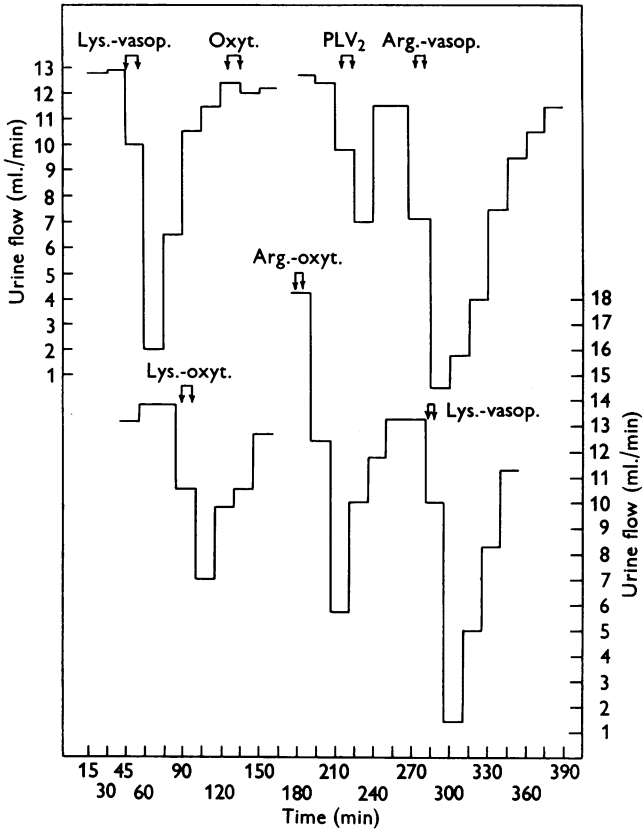

Fig. 1. The relative antidiuretic response to intravenous infusions of 19 oxytocic milliunits of oxytocin and of 19 pressor milliunits of vasopressin and some of its analogues, in four subjects.

Calcium excretion. Calcium excretion varied inversely with the rate of urine flow: it was high at low rates of flow and decreased with the onset of diuresis (Figs. 3 and 4). The higher rate of calcium excretion observed, either at low rates of flow before the onset of diuresis, or during antidiuresis occurred irrespective of the fact that the initial rate of calcium excretion varied widely from one subject to another and on different

occasions in the same subject. The antidiuretic effect following the infusion of vasopressin or one of its analogues was always accompanied by a marked increase of calcium excretion. This agrees with Thorn's (1960) findings in the dog. Moreover, there is a relationship between the magnitude of the antidiuretic effect and the rise of calcium output in the same subject, as may be seen from Fig. 4. Oxytocin which, in the doses used, had no antidiuretic effect did not affect calcium excretion.

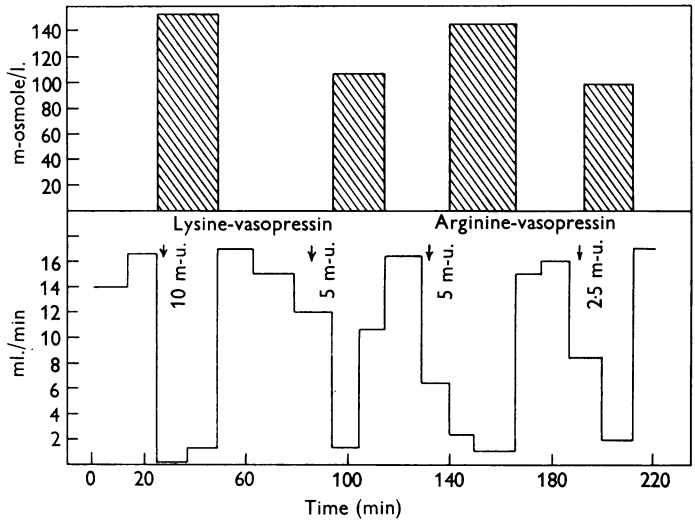

Fig. 2. A comparison of the antidiuretic response to single intravenous injections of varying doses of lysine- and of arginine-vasopressin in one subject. The osmolar concentrations of the urines during antidiuresis are given in the upper part of the figure.

Hyaluronidase concentration. As found previously (Dicker & Eggleton, 1960), hyaluronidase could be detected in the urine at a relatively slow rate of flow, and its concentration was then more or less quantitatively related to rate of flow, as may be seen in Figs. 3 and 5. This relationship was much closer than that between total hyaluronidase excretion and rate of flow, and although individual differences existed they did not obscure an overall relationship, as may be seen from the results shown in Fig. 6.

Na and K excretion. None of the products used increased the excretion of potassium. In regard to sodium excretion, arginine^8-oxytocin alone caused some increase in three out of the six subjects used, in one of whom it was accompanied by an increase in creatinine excretion. Lysine^8-oxytocin produced a decreased excretion of both ions, accompanied by a decreased creatinine output (Fig. 5), suggesting that the antidiuretic

effect observed was partly due to vascular effects. Neither of these two substances had any effect on the blood pressure (measured with a sphygmomanometer) whereas PLV_2 raised both systolic and diastolic pressures.

Total osmolar concentration was measured in a number of experiments and showed the usual inverse relation with rate of urine flow. An example

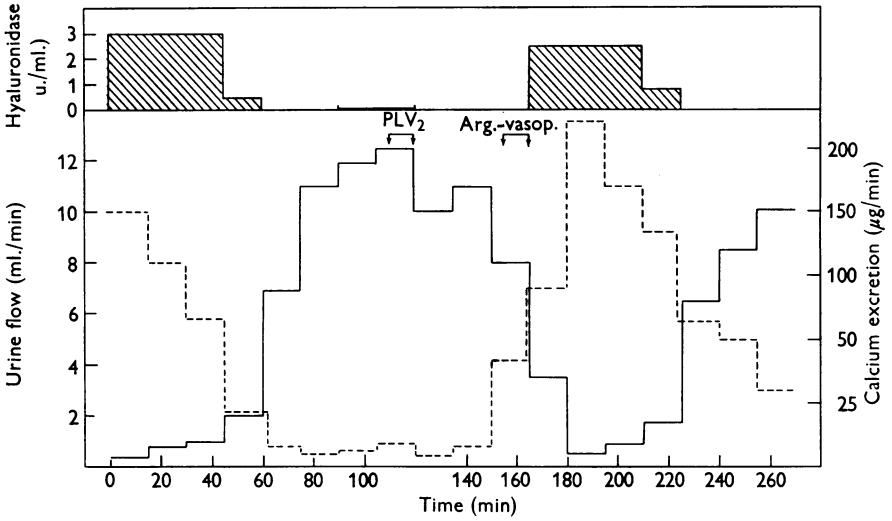

Fig. 3. The relationship between rate of urine flow, Ca excretion and hyaluronidase concentration in the urine before and during water diuresis and after intravenous infusions of 19 pressor m-u. of PLV_2 and of arginine-vasopressin. Ca excretion, – – –.

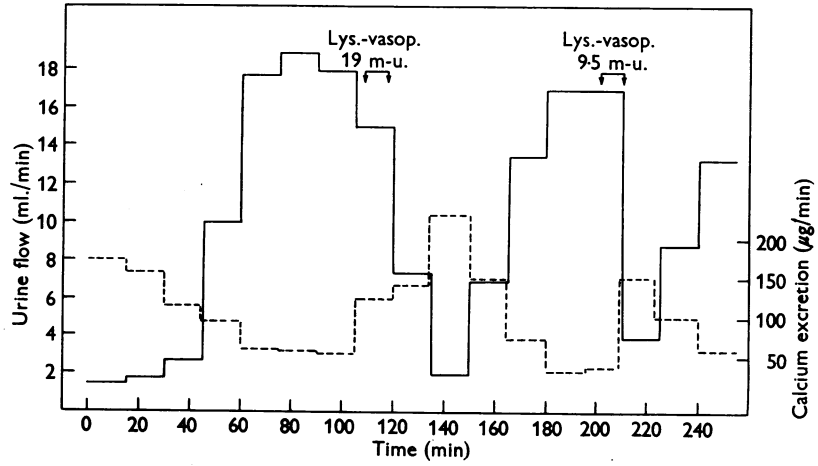

Fig. 4. The relation between calcium excretion and rate of urine flow following intravenous infusions of different doses of lysine-vasopressin. Ca excretion, – – –.

is given in Fig. 2, stressing the similarity of the effects obtained by injections of lysine-vasopressin with those of a half pressor dose of arginine-vasopressin.

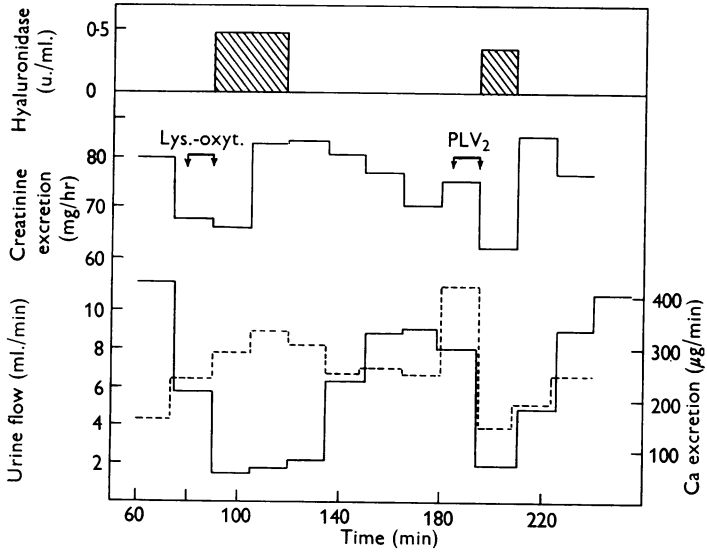

Fig. 5. The effects of large doses (40 pressor m-u.) of lysine^8-oxytocin and of PLV_2 on rate of urine flow, creatinine and calcium excretion, and hyaluronidase concentration in the urine. Ca excretion, – – –.

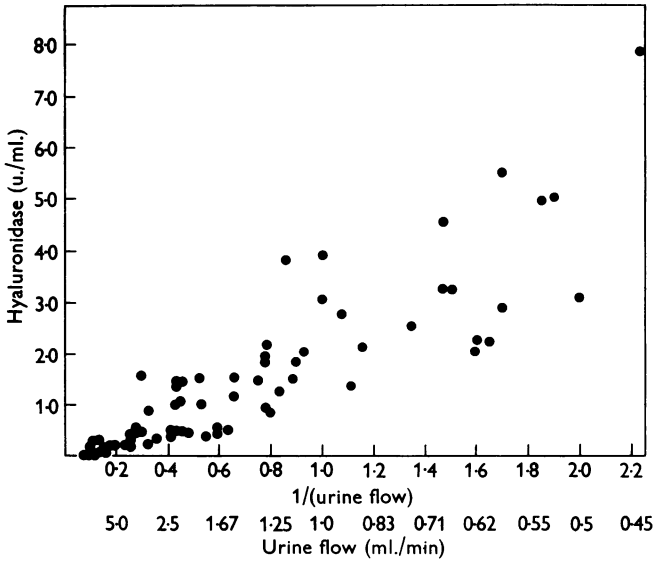

Fig. 6. The relationship between hyaluronidase concentration in the urine and the reciprocal of urine flow in seventeen subjects (seven of the present fifteen and ten others).

TABLE 1. Structural differences in vasopressins and some of their analogues in relation to their antidiuretic activity.

	1	2	3	4	5	6	7	8	9	Antidiuretic activity
Oxytocin	CYS	TYR	ILEU	GLU(NH_2)	ASP(NH_2)	CYS	PRO	LEU	GLY(NH_2)	0
Lysine^8-oxytocin	CYS	TYR	ILEU	GLU(NH_2)	ASP(NH_2)	CYS	PRO	LYS	GLY(NH_2)	+ +
Lysine^8-vasopressin	CYS	TYR	PHE	GLU(NH_2)	ASP(NH_2)	CYS	PRO	LYS	GLY(NH_2)	+ + +
Arginine^8-oxytocin	CYS	TYR	ILEU	GLU(NH_2)	ASP(NH_2)	CYS	PRO	ARG	GLY(NH_2)	+ + +
Arginine^8-vasopressin	CYS	TYR	PHE	GLU(NH_2)	ASP(NH_2)	CYS	PRO	ARG	GLY(NH_2)	+ + + +
Phenylalanine^2-lysine^8-vasopressin (PLV_2)	CYS	PHE	PHE	GLU(NH_2)	ASP(NH_2)	CYS	PRO	LYS	GLY(NH_2)	+

DISCUSSION

The variation of antidiuretic activity in relation to pressor activity of the vasopressins and their analogues examined can be related to the differences in composition of the molecules. In Table 1 they are shown in ascending order of antidiuretic activity. Starting with oxytocin, which in the dose given has no antidiuretic activity, the slight activity of lysine^8-oxytocin is associated with a change of only one amino acid, lysine^8 in place of leucine^8. Considerable further enhancement of activity occurs if either phenylalanine^3 is substituted for isoleucine^3 in this molecule or if leucine^8 of oxytocin is replaced by arginine^8. Introduction of phenylalanine^3 into arginine-oxytocin, giving arginine-vasopressin, approximately doubles its activity. Thus increasing basicity of amino acid, in position 8, would appear to be associated with increasing antidiuretic activity, the effect being enhanced by the presence of phenylalanine^3 in place of isoleucine. This interpretation agrees with the views expressed by Katsoyannis & du Vigneaud (1959). From the observations that the synthetic analogue, leucine^8-vasopressin (= oxypressin) has a low pressor activity (Katsoyannis, 1957), whereas arginine^8-vasopressin has a high pressor activity, Katsoyannis & du Vigneaud (1959) concluded that, 'a strong basic amino-acid in the side chain is one requirement for high pressor activity'. As reported by Acher (1960), the most potent octapeptide in this series of analogues is arginine^8-vasopressin with 600 pressor u./mg, as compared with 300 pressor u./mg for lysine^8-vasopressin, 125 u./mg for arginine^8-oxytocin and 3 u./mg for leucine^8-vasopressin. From the present results it would appear that the antidiuretic activity is even more affected by changes in the molecule, since this has been expressed throughout in relation to pressor activity.

Some indication of the relative importance of amino acids 3 and 8 in the various activities of the analogues may be obtained from comparison of these and other results with our own. Boissonnas, Guttmann, Jaquenoud & Waller (1956) and Berde, Doepfner & Konzett (1957) found that replacement of isoleucine by phenylalanine in oxytocin increased the negligible antidiuretic activity of the latter ninetyfold. A similar replacement in lysine oxytocin also considerably increases its antidiuretic activity. The basicity of amino acid 8, however, seems of even greater importance, for arginine^8-oxytocin has an antidiuretic effect similar to that of lysine^8-vasopressin in terms of pressor units, whereas its pressor activity is only 125 u./mg compared to 300 u./mg for lysine^8-vasopressin.

The analogue phenylalanine^2-lysine^8-vasopressin (= PLV_2 in Table 1) appears to be an anomaly. Its smaller antidiuretic activity may possibly be attributed to the presence of two phenylalanines, in positions 2 and 3,

for tyrosine^2-tyrosine^3-oxytocin has been shown to be an antagonist of oxytocin (Guttmann, Jaquenoud, Boissonnas, Konzett & Berde, 1957).

The definite relationship between hyaluronidase concentration and reciprocal of urine flow shown in Fig. 6 strengthens the suggestion originally made by Ginetzinsky (1958) that it is inherently connected with the antidiuretic action of vasopressin. Berlyne (1960) has criticized Ginetzinky for expressing his results in terms of u./ml. rather than u./min, on the grounds that substances such as creatinine would show a similar relation of decreasing concentration with increasing rate of urine flow when the total output is constant. This is true also in regard to some substances which are largely reabsorbed, as for example sodium and potassium in most of the present experiments. Hyaluronidase, however, is in rather a special category and variability in excretion rate is so great that no convincing relationship with rate of urine flow can be shown. We have already reported such a relationship in any individual experiment, and although there is a day-to-day variation in any one individual, the relationship can be seen in each, as is shown in Fig. 7. At urine flows greater than 1 ml./min there is an unmistakable decrease in hyaluronidase excretion with increasing rate of flow. Fewer determinations were made on the remaining subjects, but a similar relation was found in all of them. No further increase occurs in output per minute at rates of urine flow lower than about 1 ml./min, and this would rather seem to strengthen Ginetzinsky's view that the hyaluronidase appearing in the urine is a spill-over of enzyme not utilized in the walls of the collecting tubules.

Berlyne (1960) finds no such relationship in any of the three subjects studied and his relation between hyaluronidase concentration and rate of flow shows a much wider scatter than that seen in Fig. 6. The most likely cause of this different result is some difference in technique, and although substrate and standard hyaluronidase were of different origin in the two cases dialysis or lack of it would seem to be of greater importance. Ascorbic acid, which reduces the apparent viscosity of hyaluronic acid (McClean & Hale, 1941), is likely to be present in varying amounts in different urines and will vary in amount in the boiled control according to its pH, so that dialysis with subsequent adjustment of electrolyte concentration should provide the more reliable estimate of hyaluronidase content.

Results now presented indicate that the observed increase in urinary calcium is also implicated in the antidiuretic action of vasopressin. Howard, Wilde & Malvin (1960) have shown with the stop-flow technique in dogs that the site of maximum reabsorption of calcium from the tubular fluid is just proximal to that of sodium reabsorption, i.e. proximal to the probable site of action of the antidiuretic hormone. It would seem likely, therefore, that the increased secretion observed under the action of this

hormone is an actual addition of calcium to the tubular fluid. When hyaluronic acid is depolymerized, its contained calcium is replaced by monovalent cations, but whether diffusion of this calcium into the tubular fluid is sufficient to account quantitatively for the observed increase is not known.

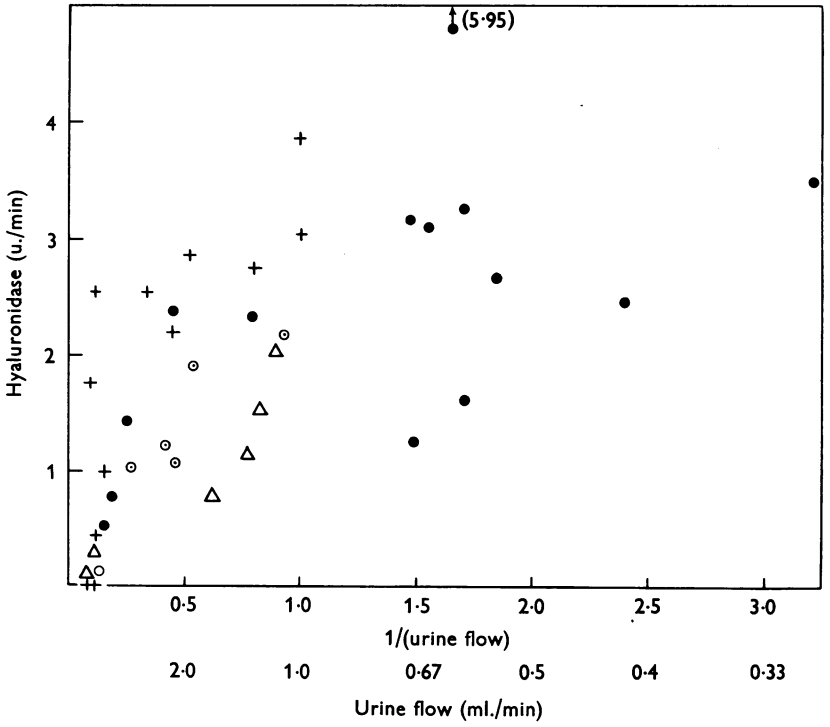

Fig. 7. The relationship between hyaluronidase excretion and the reciprocal of urine flow in four different subjects.

SUMMARY

1. Analogues of oxytocin and vasopressin show different degrees of antidiuretic activity when infused in equipressor amounts intravenously into man during an established water diuresis. Oxytocin has little antidiuretic activity: whereas phenylalanine[2]-lysine[8]-vasopressin, lysine[8]-oxytocin, argine[8]-oxytocin and lysine[8] vasopressin, and argine[8]-vasopressin show increasing activity, in this order.

2. The degree of antidiuretic activity is thus largely determined by the basicity of amino acid[8], with enhancement by phenylalanine[3].

3. The concentration of hyaluronidase found in the urine in these circumstances is quantitatively related to the degree of antidiuresis pro-

duced; antidiuresis is also related to increased excretion of calcium quantitatively in any single experiment.

4. The results support the view that antidiuretic action involves the liberation of hyaluronidase, depolymerizing the intercellular matrix of the tubules in the renal medulla so that the tubular fluid comes into osmotic equilibrium with the hypertonic tissue fluid.

We wish to record our thanks to Messrs Sandoz for the gift of PLV_2, lysine^8-oxytocin and lysine^8-vasopressin and to Professor R. Acher for the gift of arginine^8-oxytocin; to Mrs Margaret Harkness for a supply of hyaluronic acid; to Dr L. E. Bayliss for estimations of total osmolarity and to all the subjects who gave so generously of their time and patience.

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