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HYALURONIDASE AND ANTIDIURETIC ACTIVITY IN URINE OF MAN

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Until recently the action of the antidiuretic hormone has been described as restoring 'water balance by promoting the reabsorption of the osmotically free water left by the distal reabsorption of Na. Under the action of the hormone this water is reabsorbed; in its absence this water is excreted' (Smith, 1956). Wirz (1956) has suggested an explanation of the mechanism of water reabsorption. He, and later Gottschalk & Mylle (1959), showed by micropuncture study in the rat that fluid in the first half of the distal tubule was hypotonic to plasma under all conditions of hydration, but that in dehydrated animals it became isotonic in the second half of the distal tubule and hypertonic in the collecting ducts. In the kidney producing a concentrated urine the tubular fluid comes into osmotic equilibrium first with the cortical tissue and later, in the collecting tubules, with the medullary tissue which is known to be increasingly hypertonic towards the papilla. According to Wirz's theory, the essential action of vasopressin is to increase the permeability to water of the distal parts of the nephron and collecting ducts. In 1958 Ginetzinsky found that the urine of several mammals contained hyaluronidase: this disappeared during water diuresis, but was present during osmotic diuresis, in the dog. In a histological study in rats Ginetzinsky observed that the cement substance between the cells of the collecting tubules reacted as hyaluronic acid when the animals were water-loaded, but as its polymerization products when they were dehydrated. He concluded that when stimulated by the antidiuretic hormone, the cells of the collecting tubules secrete hyaluronidase, which in turn depolymerizes the mucopolysaccharide complex of the basement membrane of the tubules, hence making 'the structures separating the tubule lumen from the interstitial tissue permeable to water. The hypotonic fluid in the tubules then follows the osmotic gradient and undergoes facultative reabsorption' (Ginetzinsky, 1958). This was a new approach to a process which had hitherto been difficult to envisage. It seemed, therefore, of interest to test Ginetzinsky's hypothesis by comparing the excretion of hyaluronidase and antidiuretic activity in urine under various conditions in man.

METHODS

The human subjects were eight normal adults (four men and four women), eight new-born male infants, one patient suffering from diabetes insipidus and two children with an inborn renal disease known as 'nephrogenic diabetes insipidus'. The adult patient with diabetes insipidus was a man of 27 years, whose condition was controlled by intramuscular injections of 10 u. Pitressin tannate administered every other day. When seen, the treatment having been interrupted for 48 hr, the patient was excreting urine at an average rate of 14 ml./min. The children with 'nephrogenic diabetes insipidus' were two cousins, aged 4 and 17 respectively. Both had been treated with chlorothiazide. 'Nephrogenic diabetes insipidus' is a hereditary disease transmitted through the mother in the same way as haemophilia, and characterized by an inability of the kidneys to respond to vasopressin, and hence to concentrate urine, though the post-pituitary gland appears to be unaffected.

Diuresis, when required, was induced by the ingestion of water in varying amounts or of ethanol (35–45 ml. in 200 ml. water). Antidiuresis in hydrated subjects was obtained by intramuscular injections of 500 m-u. vasopressin. In all cases urine was collected without catheterization. In new-born infants the sample of urine collected was that which was first voided after birth, before the first feed and thus at a time when the baby was dehydrated.

Estimation of hyaluronidase in urine. Hyaluronidase was determined by the change in viscosity induced when incubated with hyaluronic acid by the method of McClean & Hale (1941) with certain modifications. In order to minimize the action of interfering substances such as ascorbic acid and to concentrate the hyaluronidase, the urine samples were first taken nearly to dryness by exposure to dialysis tubing containing polyethylene glycol, as described by Kohn (1959), and reconstituted with water to represent a urine flow of 0·1–0·15 ml./min. Since the original rate of urine flow was unknown in the new-born infants, the samples were concentrated three- to eightfold, according to the volume available.

0·2 ml. hyaluronic acid + 0·05 ml. reconstituted urine + 0·05 ml. citrate buffer pH 4·6 (containing 8 % NaCl) was incubated at 34° C for 90 min and the viscosity then determined at room temperature. The hyaluronidase concentration was obtained from a curve derived from different amounts of the standard hyaluronidase preparation (Light and Co; 500 u./mg) under the same conditions. Boiled urine produced little or no change in the viscosity of the hyaluronic acid. The latter was a preparation of mixed mucopolysaccharides from human umbilical cord and was not of uniform viscosity. The solution used was of such a concentration that, diluted as indicated above, it took approximately twice as long (110 sec) in the viscosimeter as water (55 sec). The time was reduced to 101, 91 and 79 sec by 0·5, 1·0 and 2·0 u. hyaluronidase respectively. A difference of < 5 sec between the unknown and hyaluronic acid was not considered quantitatively significant. The absolute values varied with day-to-day variations in room temperature, but this was steady to $\pm 0{\cdot}5°$ C during any one set of readings.

Identification of the viscosity-reducing material in urine as an enzyme, apart from its destruction by boiling, was provided by a typical curve of activity versus time. Its action at 30, 60 and 90 min incubation was 33, 55 and 68 % of that at 3·5 hr, in comparison with 59, 72 and 79 % for the standard preparation (bull testis). Its identity as a hyaluronidase was further substantiated by inhibition of its viscosity-reducing action by Suramin to the same degree as that of the standard preparation.

Estimation of antidiuretic activity in urine. The method was that described by Dicker (1953), using rats under ethanol anaesthesia and kept with a constant water load of 8·0 ml./100g body weight. The estimations were done as a (2 + 2) doses assay, with a commercial preparation of vasopressin as standard, and the antidiuretic activity expressed in terms of the standard used. By a suitable modification in the timing of hydration (Thorn, 1957), the sensitivity of test animals was increased, with the result that the smallest amount of antidiuretic activity assayable was that corresponding to 0·025 m-u. vasopressin/ml. Urine samples containing antidiuretic activity were treated with sodium thioglycollate. In all

but one case (subject Wh. Table 1) this resulted in the complete inactivation of the antidiuretic activity, suggesting that the latter was of neurohypophysial origin.

The commercial preparation of vasopressin used for injections as well as for assays was Pitressin (Parke, Davis and Co; batch LZ 231 A): it contained lysine vasopressin only, as shown by paper chromatography. It was labelled as containing 20 u. pressor activity/ml.: in this paper in stating both doses and results of assays 1 u. means 0·05 ml. of the preparation.

RESULTS

Water and alcohol diuresis. The results of eight experiments on adult subjects are given in Table 1. In all experiments the concentration of both antidiuretic activity and hyaluronidase fell dramatically at the height of the diuresis and increased again as the urine flow returned to its resting value, 40–90 min later. The absolute excretion rate of both hyaluronidase and antidiuretic activity followed the same course in all experiments. There was, however, a greater variation in the excretion of the antidiuretic activity at similar rates of urine flow (0·12–10·0 m-u./min), not only in different individuals but in the same individual on different occasions. Variation occurred also in hyaluronidase excretion but to a much less degree (0·52–4·42 u./min).

TABLE 1. Excretion of antidiuretic activity and hyaluronidase before, during and after water and alcohol diuresis

Subject	Urine flow (ml./min)	Antidiuretic activity (m-u./ml.)	Antidiuretic activity (m-u./min)	Hyaluronidase (u./ml.)	Hyaluronidase (u./min)
		Water diuresis			
Wh.	0·38*	10·64	4·04	0·90	0·34
	14·80*	0·53	7·80	0·00	0·00
	1·23*	8·10	10·00	1·20	1·48
Dic.	3·00	1·82	5·46	0·85	2·55
	9·00	0·00	0·00	0·28	2·52
	1·00	2·40	2·40	3·85	3·85
Im.	0·86	4·75	4·10	2·20	1·90
	6·13	0·00	0·00	0·00	0·00
Eg.	0·68	3·35	2·28	4·60	3·13
	5·60	0·00	0·00	0·14	0·78
	0·63	5·17	3·25	2·00	1·26
		Alcohol diuresis			
Gi.	0·90	0·12	0·11	1·32	1·19
	11·50	0·00	0·00	0·00	0·00
	1·50	0·11	0·16	1·15	1·73
Di.	9·00	0·00	0·00	0·00	0·00
	1·00	0·12	0·12	3·05	3·05
Dia.	1·16	1·60	1·86	3·80	4·42
	7·70	0·37	2·85	0·00	0·00
	1·14	1·25	1·43	1·50	1·71
Eg.	0·53	0·10	0·53	5·00	2·65
	0·61	0·00	0·00	2·20	1·32
	0·44	0·11	0·05	7·80	3·44

* Sodium thioglycollate destroyed about 45% only of the antidiuretic activity of these urine samples.

Injection of vasopressin. Injection of vasopressin into hydrated subjects is known to reduce the rate of urine flow, and it was of obvious interest to see whether this was accompanied by an increase in excretion of hyaluronidase. An intramuscular injection of 500 m-u. vasopressin was made, therefore, into three normal subjects and one patient with diabetes insipidus. The results given in Table 2 show that hyaluronidase concentration in the urine and its total output were considerably increased in all four subjects during 65–90 min following the injection. The antidiuretic activity excreted in the urine during this period amounted to 4½–11 % of that of the injected material.

TABLE 2. Excretion of hyaluronidase and antidiuretic activity following an intramuscular injection of vasopressin into normal subjects

Subject	Time after injection (min)	Urine flow (ml./min)	Hyaluronidase excretion (u./ml.)	Hyaluronidase excretion (u./min)	Antidiuretic activity (m-u./ml.)	Antidiuretic activity Total (m-u.)
Mrs B.	—	2·08	0·41	0·85	0·00	—
	Vasopressin 500 m-u. intramuscularly					
	0–55	0·62	2·25	1·40	0·81	27·6
	55–70	1·33	1·40	1·86	0·76	15·2
E.	—	3·95	0·36	1·42	0·00	—
	Vasopressin 500 m-u. intramuscularly					
	7–30	1·28	1·80	2·30	0·11	3·4
	30–50	0·59	5·50	3·25	0·64	8·0
	51–90	0·68	3·23	2·20	0·44	11·9
Mrs C.	—	1·24	0·84	1·04	0·00	—
	Vasopressin 500 m-u. intramuscularly					
	0–45	1·51	1·54	2·33	0·30	20·1
	45–85	0·93	2·74	2·55	0·98	36·3
H.H. (diabetes insipidus)	—	14·00	0·00	—	0·00	—
	Vasopressin 500 m-u. intramuscularly					
	0–30	2·30	1·40	3·22	0·31	21·5
	30–75	3·45	0·45	1·55	0·14	21·7

The results of an intramuscular injection of vasopressin in two cases of nephrogenic diabetes insipidus (Table 3) stand in sharp contrast with those given in Table 2. The rate of urine flow was actually increased after the injection of vasopressin, possibly owing to an increase in filtration rate, as suggested by an increase in creatinine excretion from 14 to 16·5 mg/hr. There was no hyaluronidase in the urine, either before or after the injection of 500 m-u. vasopressin but a far higher proportion of the injected antidiuretic activity was excreted: 47 and 80 % in 65–75 min as compared with 5–15 % in normal individuals.

New-born infants. In eight new-born infants appreciable amounts of antidiuretic activity were found in the urine, accompanied by hyaluronidase.

The results given in Table 4 show only concentrations, as the estimations were made on the first sample of urine voided after birth, and rates of flow were therefore unknown. Though the concentration of antidiuretic activity is relatively low in comparison with that of resting urine flow in most of the adults, that of hyaluronidase falls within the adult range. As in the adults, there is a wide variation in the ratio of antidiuretic activity to hyaluronidase concentration.

TABLE 3. Excretion of hyaluronidase and antidiuretic activity following the injection of vasopressin in cases of nephrogenic diabetes insipidus

Subject	Time after injection (min)	Urine flow (ml./min)	Hyaluronidase (u./ml.)	Antidiuretic activity (m-u./ml.)	Antidiuretic activity Total (m-u.)
B.B. 4 yr	—	1·26	0·00	0·00	—
	—	2·93	0·00	0·00	—
	Vasopressin 500 m-u. intramuscularly				
	0–45	4·04	0·00	1·18	215
	45–75	3·75	0·00	1·62	182
D.C. 17½ yr	—	3·33	—	—	—
	Vasopressin 500 m-u. intramuscularly				
	0–82	3·93	0·00	0·73	235
	82–130	3·37	0·00	0·48	78

TABLE 4. Excretion of antidiuretic activity and hyaluronidase in new-born babies

Subject	Antidiuretic activity m-u./ml.	Hyaluronidase u./ml.
Bot.	0·025	4·0
H.	0·025	0·7
Bog.	0·056	3·3
Ba.	0·074	1·7
F.	0·150	5·2
Mu.	0·237	5·3
S.	0·875	5·0
Ma.	0·900	4·0

DISCUSSION

The results presented suggest that there is some relation between antidiuretic activity in the urine and hyaluronidase liberation in the kidney, as suggested by Ginetzinsky (1958), and are in apparent conflict with the negative findings on man of Berlyne (1960), who found no relationship between hyaluronidase excretion and rate of urine flow. Berlyne, however, gives little detail, and presumably only one analysis was made on each individual in water, mannitol or potassium diuresis. Of his water-diuresis subjects, only one had a urine flow greater than 2 ml./min. This, in conjunction with the wide individual variation in hyaluronidase excretion both in resting urine and in response to ingestion of water, as seen in Table 1, would appear to offer sufficient explanation of his apparently negative results.

The increase in hyaluronidase excretion following intramuscular injection of vasopressin, both in normal subjects and in a case of diabetes insipidus, strengthens the suggestion that hyaluronidase is concerned in the production of a concentrated small volume of urine. The lack of any increase following a similar injection in two cases of nephrogenic diabetes insipidus further suggests that vasopressin can have no antidiuretic action other than by liberating hyaluronidase and affords strong support to Ginetzinsky's conclusion that the action of vasopressin is to render more permeable to water the collecting tubules in their hypertonic surroundings. The genetically-controlled missing link is clearly somewhere in the vasopressin–hyaluronidase probable chain of reactions, but cannot at present be more accurately located.

The failure of new-born infants to produce as concentrated a urine as adults cannot be ascribed to such an inability. They excrete both antidiuretic activity and hyaluronidase in appreciable concentration. Heller (1944) found that, whereas in infants a few days old an injection of posterior pituitary extract caused some reduction in urine flow with increase in its concentration, two new-born infants so tested gave no response. Their urine was more concentrated than that of the older infants and their kidneys were presumably responding maximally to endogenously produced vasopressin. Heller's results taken in conjunction with our own suggest that vasopressin and its tubular action necessary for the production of concentrated urine in the adult are already present in the new-born baby. The most likely cause of failure of the new-born infant to produce a concentrated urine is failure to develop hypertonicity of the renal medulla which surrounds the collecting ducts in the adult. The kidney of the new-born child is known to be immature in many respects and it would not be surprising if the loop of Henle 'counter-current' system is not fully developed.

The lack of any regular quantitative relation between the excretion rates of antidiuretic activity and hyaluronidase requires some explanation. The antidiuretic activity appearing in the urine is presumably related to vasopressin as in all but one case (see Table 1) it was inactivated by sodium thioglycollate; it appears to be filtered at the glomerulus, whereas hyaluronidase probably spills over from collecting-tubule cells, as suggested by Ginetzinsky on histological grounds. In the normal subject only some 10–15 % of injected vasopressin is recovered from the urine, whereas in the case of nephrogenic diabetes insipidus up to 80 % was excreted in the urine during 65 min following the injection. It is tempting to postulate that vasopressin actually gets destroyed during its stimulation of hyaluronidase liberation from the tubule cells. In such a complex situation, it would be unreasonable to expect any quantitative relation between the excretion rates of the two substances.

SUMMARY

1. Following the ingestion of water or alcohol, antidiuretic activity and hyaluronidase concentrations in the urine fell at the height of diuresis and increased again with restoration of normal rate of urine flow. The total excretion of hyaluronidase followed the same pattern.

2. Injection of vasopressin in two cases of nephrogenic diabetes insipidus caused no excretion of hyaluronidase, in contrast with three normal subjects and a case of diabetes insipidus, in whom a large excretion of the enzyme occurred.

3. The nephrogenic diabetes insipidus patients excreted 50–80% of the injected antidiuretic activity in contrast with the normal 5–15%.

4. The urines of eight new-born babies contained appreciable concentrations of both antidiuretic activity and hyaluronidase.

5. It is concluded that the antidiuretic hormone acts on the kidney by liberating hyaluronidase and that the resultant decrease in rate of urine flow is probably due to increased permeability of the collecting tubules, allowing the concentration of their contents to come into osmotic equilibrium with their hypertonic surroundings.

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