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DETERMINATION AND OCCURRENCE OF FREE AND CONJUGATED HISTAMINE IN URINE OF SHEEP*)

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Anrep et al. (1944) were the first to demonstrate adequately the occurrence of histamine in normal urine. Both free histamine and a conjugated form which could be converted to free histamine by acid hydrolysis of the urine were determined. In carnivora, the conjugated form preponderated. Low concentrations of histamine were found in the urine of herbivora (0.02—0.2 μg histamine diphosphate per ml) and most of it, if not all, was present as free histamine. The only ruminants included in the investigation of *Anrep et al.* were the water-buffalo, the camel and the llama.

While the literature offers sparse information about urinary excretion of histamine in ruminants, the levels of histamine in whole blood have been rather well examined. Some of the data obtained have been regarded as being indicative of the role of histamine in the pathogenesis of some diseases (*Seekles* 1961, *Nilsson* 1963). The validity of blood analyses as a parameter of changes in the liberation and/or formation of histamine in the body may, however, be strongly questioned. No relationship has been demonstrated between the dose of parenterally injected histamine to man and the blood level of the substance (*Rose* 1940, *Adam* 1950). Depending on which species is in question, most of the histamine in whole blood is present either in basophils, in eosinophils or in platelets. It has been demonstrated that the histamine both in basophils and in platelets is partly,

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or entirely, synthesized within the cells (*Schayer & Kobayashi* 1956, *Lindell et al.* 1961). It is therefore probable that the level of histamine in whole blood mainly reflects the number of the specific histamine containing cells. In this connection it is of interest that a high correlation has been demonstrated between the number of basophils and the whole blood level of histamine in man (*Valentine et al.* 1955, *Code & Mitchell* 1957).

Even when histamine is infused at a rate producing pronounced biological effects, the plasma levels do not increase detectably (*Adam et al.* 1954). A high correlation, however, has been demonstrated between the amounts of histamine injected and the increments in urinary free histamine (*Adam et al.*). These authors also showed that urinary free histamine probably would be a sensitive indicator on the formation and/or liberation of histamine in the body since injection of histamine in doses just causing detectable biological effect (increased gastric secretion) also resulted in increased levels of urinary free histamine.

Accordingly, in conditions in which involvement of histamine is to be considered determinations of urinary histamine would probably be a better way of approach than histamine analyses of whole blood or plasma. Since the literature gives no information as to the normal excretion of histamine in sheep, such examinations were undertaken.

When histamine is administered by mouth, a low free/conjugated histamine ratio is found in the urine of most species (*Anrep et al., Millican et al.* 1949). The high free/conjugated histamine ratio found in the urine of ruminants observed by *Anrep et al.* therefore suggested that urinary histamine does not originate from the digestive tract to any large extent in this group of animals. In sheep histamine seems, however, to be both formed and conjugated by the contents of the forestomachs (*Sjaastad,* 1967 a, b). Experiments were therefore undertaken to examine, if the ratio free/conjugated histamine in the urine of sheep is similar to that observed by *Anrep et al.* in other herbivorous animals.

MATERIALS AND METHODS

Animals and feeding

The 24-hr. urinary excretion of free and conjugated histamine was examined in 6 adult sheep of the Dala breed (4 males and

2 females) and 5 Scottish Blackface (1 male and 4 females). The animals were fed at 7 a.m. and 3 p.m., and the daily ration consisted of hay (0.6—0.7 kg), concentrates (about 0.3 kg) and kohlrabi (about 0.5 kg). Water was freely available.

Collection of urine

In the male sheep a rubber sling covering the ventral part of the abdominal wall was used. Before securing the sling, the wool was clipped and the abdominal wall properly washed. From the most ventral part of the sling a plastic tube led to a 2-l plastic flask containing 250 ml of N-HCl, usually providing a pH lower than 2.0. Urine specimens with pH higher than 3.0 were discarded. Preliminary experiments showed that urine could be stored at 20°C and a low pH for 24 hrs. without any significant change in histamine activity. In the females, urine was collected by housing the sheep in metabolism cages of the type described by *Duthie* (1959).

Determination of histamine

For the determination of free and total (free + conjugated) histamine the method of *Dunér & Pernow* (1956) was used with some minor modifications:

Free histamine was determined essentially in the same way as free histamine in rumen liquor (*Sjaastad* 1967 a). The method is briefly as follows: After filtration, the pH of the urine was adjusted to 6.5 (Merck's indicator paper) and 100-ml samples were passed through Amberlite IRC-50 columns (150 × 10 mm, 4—6 ml/min.). The columns were then washed with 2 × 25 ml of distilled water. Precautions were taken that the resin was always covered by a layer of fluid. Histamine was eluted with 1.2 N-HCl (approximately 0.2 ml/min.), the volume of acid (in ml) being calculated by multiplying the height of the resin (in cm) after washing with distilled water, by a factor of 0.95. When the level of 1.2 N-HCl had sunk to the top of the resin, 10 ml of 0.01 N-HCl was added and collection of the eluate started. The pH of the effluent was checked at frequent intervals and the eluates discarded until the check prior to the change to an acid pH. When this change in pH had taken place, the flow rate was increased to about 0.5 ml/min. Elution was then continued until all acid had passed through the resin.

Total histamine (free + conjugated). After adjusting the pH to 6.5, 100 ml of the urine specimens were precipitated with 200 ml of acetone (technical grade). The mixtures were shaken and then filtered into evaporation flasks which contained 7 ml of 2 N-HCl providing a pH of the filtrates lower than 3.0. The filtrates were evaporated ex vacuo on a boiling water bath until 2—3 ml were left, whereafter the residues were dissolved in 10+5+5 ml conc. HCl and boiled under reflux for 2 hrs. The hydrolysates were evaporated to complete dryness ex vacuo. The residues were dissolved in approximately 50 ml of distilled water, and after adjusting the pH to 6.5 (Radiometer, Copenhagen) the samples were centrifuged at 4000 × g for 10 min. Distilled water was added to the supernatant fluid to a final volume of 100 ml, and histamine was extracted by passage through Amberlite IRC-50 columns (200 × 10 mm). After washing with distilled water, 1.2 N-HCl was added to the columns, followed by 15 ml of 0.01 N-HCl. The amounts of 1.2 N-HCl were calculated as for free histamine. A coloured band was usually seen in front of the acidified part of the resin. When this band reached the bottom of the resin, the collection of the eluate was started. When no coloured band was present, the pH was checked with frequent intervals after the addition of 0.01 N-HCl. Collection of the eluate was then started from the time of the check prior to the change to an acid pH.

The acid eluates of free and total histamine were stored at 4°C until determination of histamine concentration; this was carried out not later than 5 days after the extraction procedure. The histamine concentrations of the neutralized eluates were estimated on guinea-pig ileum suspended in an organ bath (6 ml) containing Tyrode's solution and atropine (0.05 µg/ml). The determination of the unknown was done by bracketing between histamine standards, the standards being chosen so they usually differed less than 15 % in histamine content. The coefficient of variation of the method was calculated from the formula $\sqrt{\frac{\Sigma d^2}{2(n-1)}} \cdot \frac{100}{\bar{x}}$, where d is the difference between duplicate determinations, n the number of duplicate determinations and $\bar{x}$ the mean 24-hrs. excretion of free or conjugated histamine. In 20 specimens of urine (free histamine 5.3—12.7 µg base/24 hrs., conjugated histamine 6.7—20.6 µg/24 hrs.) the coefficients of variation of the methods for estimation of free and conjugated

histamine were 6.3 and 12.0 %, respectively. The histamine values are recorded in terms of the base, they represent the mean of duplicates and they are uncorrected for losses due to the extraction procedure.

Amberlite IRC-50 (Standard grade) was obtained from British Drug Houses Ltd., England. Batches of the resin were prepared according to the principle outlined by *Bergström & Hansson* (1951). After transfer of the resin to the ion-exchange columns, it was treated with 0.5 M Na-phosphate buffer (ph 6.5) until the pH of the effluent from the columns was the same as that of the buffer.

Histamine diphosphate and *N-acetylhistamine* were bought from Nutritional Biochemicals Corp., Cleveland, Ohio, USA.

Antihistamine Allergin® (diphenhydramine chloride) was purchased from Nyegaard & Co., A/S, Oslo, Norway.

L-histidine-monochloride-monohydrate was obtained from Sigma Chemical Co., St. Louis, USA.

RESULTS

Free histamine (control of method)

Adsorption of histamine at different flow rates of urine through the resin. 100 ml samples of urine adjusted to pH 6.5, to which histamine had been added (100 µg histamine diphosphate/ml), were passed through the resin at different flow rates. The histamine concentration of the percolate was assayed directly on guinea-pig ileum. At flow rates of 4—6 ml/min., as used in this study, almost all histamine was adsorbed to the resin (Fig. 1). When aqueous solutions of histamine were passed through the resin, about 98 % of the histamine was adsorbed even at flow rates of 25 ml/min.

Recovery of added histamine using different amounts of ion-exchange resin. Histamine diphosphate (5—100 µg) was added to 100-ml samples of urine and adsorbed on Amberlite IRC-50 columns of different heights (6, 10 and 15 cm). With columns of 6 cm the recovery was low and variable (Table 1). By increasing the height of the resin to 15 cm, higher and less variable recovery was obtained (Table 1). The recovery, when 5 or 10 µg histamine diphosphate were adsorbed on 10 cm columns (7 expts.), was not significantly lower than when 100 µg (6 expts.) were added (72.0 ± 15.9 and 82.5 ± 4.0 %, respectively, $P > 0.05$).

Conjugated histamine (control of method)

Temperature during hydrolysis. By the method of *Dunér & Pernow* (1956) conjugated histamine is converted to free hista-

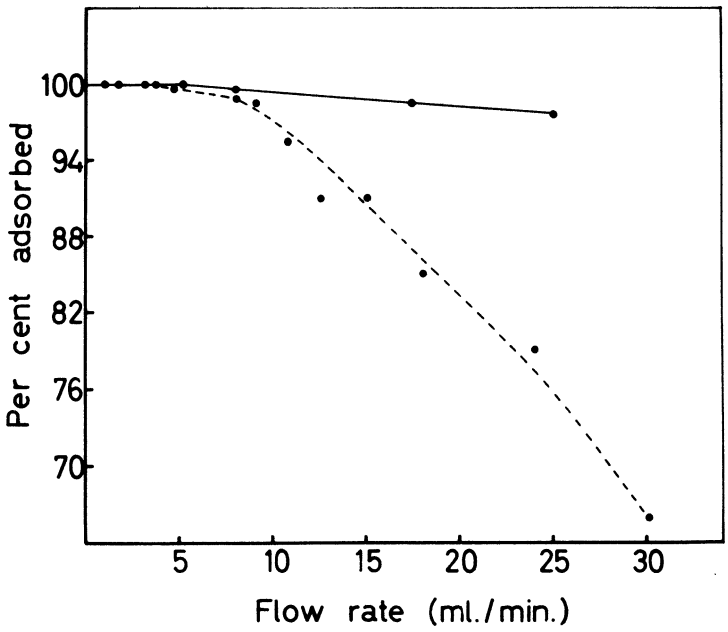

Figure 1. Adsorption of histamine on Amberlite IRC-50 columns (150 × 10 mm) at different flow rates of urine (·————·) and distilled water (·------·) 100 ml of each. 100 μg histamine diphosphate per ml were added. The histamine concentration of the percolate was assayed directly on isolated guinea-pig ileum.

Table 1. Recovery percentage of added histamine using different amounts of ion-exchange resin. Histamine added: 5—100 μg histamine diphosphate.

	Size of resin (mm)		
	60 × 10	100 × 10	150 × 10
n	19	15	12
mean ± s	52.7 ± 15.4	79.5 ± 12.5	88.8 ± 5.8

mine by hydrolysing concentrated and acetone-treated urine samples under reflux in 10 N-HCl on a boiling water bath for 1½ hrs. In our hands this procedure resulted in a low recovery of N-acetylhistamine (40—80 μg) added to urine (29.7 ± 5.1 %, 10 expts.). Boiling under reflux on a hot plate (temperature 107—112°C) greatly increased the recovery (79.4 ± 12.5 %, 7 expts.).

Decarboxylation of histidine takes place at temperatures above 145°C. Although such temperatures were never registered during hydrolysis in the present experiment, it is conceivable that they might occur locally if charring takes place. In 2 experiments 10 mg of L-histidine were added to urine extracts before hydrolysis. The quantities of total histamine in these samples did not differ from that in 2 controls. No detectable decarboxylation of histidine had thus taken place in these experiments.

Loss of free histamine during evaporation. The method of *Dunér & Pernow* for conjugated histamine involves evaporation of acetone-treated urine to dryness ex vacuo at pH 6.5 in a boiling water bath. Since histamine is unstable at alkaline pH, some free histamine might be lost during this stage of the procedure. The effect of pH on the stability of added histamine diphosphate (200 μg) during evaporation was therefore examined in 3 experiments. Losses of free histamine seemed to take place when the evaporation was carried out at pH 6.5 (Table 2). To avoid losses, the pH should be kept as low as 3.0 (Table 2).

Table 2. The effect of pH on the stability of free histamine during evaporation of acetone-treated urine. Histamine added per 100 ml urine: 200 μg histamine diphosphate. The pH of the treated urine was adjusted with N-NaOH and evaporated ex vacuo at 80—90°C. The residues were dissolved in Tyrode's solution and neutralized, whereupon the histamine content was assayed directly on guinea-pig ileum. The figures are obtained by subtracting the blind values for free histamine (determined by ion-exchange chromatography).

Expt. no.	Histamine found after evaporation (% of added)				
	pH 6.5	pH 5	pH 4	pH 3	pH 2
1	69 , 71	75	83	95	94
2	63 , 62	67	74	98	100
3	67	72	78	97	96

Recovery of N-acetylhistamine. Conjugated histamine in urine is believed to be identical to N-acetylhistamine (*Tabor & Mosettig* 1949). The recovery of N-acetylhistamine added to urine was increased by increasing the height of the columns from 6 to 20 cm (Table 3). The recovery was not further increased by extending the period of hydrolysis or by further increasing the amount of resin.

Table 3. Recovery percentage of added N-acetylhistamine using different amounts of ion-exchange resin. N-acetylhistamine added: 40—400 μg/100 ml of urine.

	Size of resin (nm)		
	60 × 10	120 × 10	200 × 10
n	15	12	50
mean ± s	51.3 ± 7.2	66.7 ± 9.6	77.3 ± 9.6

Excretion of free and conjugated histamine in the urine of normal sheep

In 11 healthy adult sheep the 24-hrs. urinary excretion of free and conjugated histamine were 9.8 ± 9.0 μg histamine base/24 hrs. and 15.1 ± 9.9 μg/24 hrs., respectively (Table 4). There was no obvious difference in the excretion in the males and females.

Table 4. The urinary excretion of free and conjugated histamine in 11 healthy sheep.

Sex	Sheep no.	Urinary excretion of histamine (μg base/24 hrs.)	
		free	conjugated
m	1	9.5	13.6
m	2	34.2	37.1
f	3	10.4	26.1
m	4	5.3	13.5
m	5	4.2	8.9
f	6	2.1	2.9
m	7	8.8	19.3
f	8	10.9	15.2
f	9	14.5	16.4
f	10	4.5	9.1
f	11	3.0	3.9
mean ± s		9.8 ± 9.0	15.1 ± 9.9

In 1 sheep (no. 2, Table 4) the excretion of both free and conjugated histamine deviated much from the figures obtained for the rest of the group. However, when re-examined about 1 year later, the excretion of both free and conjugated histamine

in this sheep (9.3 µg/24 hrs. and 20.4 µg/24 hrs., respectively) fell within the range obtained for the remaining animals.

In 3 animals examined for a longer period of time, the intra-individual variation in histamine excretion seemed to be of a similar order of magnitude as that encountered between animals (Table 5).

Table 5. Intraindividual variation of urinary excretion of free and conjugated histamine in 3 sheep. The study was done over a period of 1½ years.

Sheep	Number of expts.	Urinary excretion of histamine (µg base/24 hrs.)	
		free histamine (mean, s and range)	conjugated histamine (mean, s and range)
4	19	5.3 ± 2.0 (1.3—9.9)	13.5 ± 6.7 (2.1—22.2)
5	13	4.2 ± 2.2 (1.0—9.3)	8.9 ± 6.1 (3.6—24.2)
7	7	8.8 ± 4.3 (14.5—14.8)	19.3 ± 16.3 (4.7—48.3)

The mean conjugated/free histamine ratio in the present material was 1.5 (range 1.1—2.6, Table 4). If the values for free and conjugated histamine had been corrected for analytical losses, the mean conjugated/free ratio in the urine would have been 1.7.

DISCUSSION

Anrep et al. (1944) found that the urine of herbivorous animals contained substances which by the method used by them, interfered with the biological assay of histamine. By modifying the method of *Dunér & Pernow* (1956), free histamine in the urine of sheep could be determined with satisfactory reproducibility and on an average about 90 % of histamine added to urine was recovered. However, in order to arrive at an accurate estimate of the recovery of histamine added to urine, 5 µg or more of histamine diphosphate were added to each 100 ml aliquot of the urine specimens. The concentration of free histamine naturally present in the urine of sheep is, on the other hand, sometimes lower than 1 µg histamine diphosphate/100 ml of urine. Larger volumes of urine extracts had therefore to be added to the organ bath to obtain desired responses when the normal excretion was determined, than when the recovery of added histamine was examined. It might thus be wrong to conclude

that histamine naturally present in the urine is equally accurately determined as histamine added to urine. The true values for histamine in the urine of sheep might therefore be somewhat higher than the present results indicate. However, occasional addition of internal standards of histamine to the urine extracts showed that they did not contain interfering substances in amounts greatly disturbing the bioassay.

In contrast to the findings of *Anrep et al.* in other herbivorous animals, the sheep examined in the present study excreted larger amounts of conjugated histamine than of free histamine. Generally, it is believed that conjugated histamine found in the urine is of dual origin, it is partly being formed by bacteria in the intestines (*Urbach* 1949, *Wilson* 1954) and partly it is being formed in tissues (*Millican* 1953, *Wilson, Schayer* 1956). Conjugated histamine consists partly, or entirely, of N-acetylhistamine (*Tabor & Mosettig* 1949). In sheep conjugated metabolites of C^{14}-histamine are not detectable in the urine subsequent to intravenous injection of C^{14}-histamine (*Eliassen*). Disregarding the possibility that endogenous histamine is treated differently from intravenously injected histamine, this fact indicates that conjugated histamine in urine of sheep is mostly, or entirely of exogenous origin. Histamine formed and conjugated by the contents in the forestomachs (*Sjaastad* 1967 a, b) does probably to a considerable extent contribute to the conjugated histamine in the urine. Since the in vitro formation of histamine by rumen contents was increased by addition of L-histidine (*Sjaastad* 1967 a), the amounts of conjugated histamine in the urine are probably to some extent depending on the amount of protein in the diet fed.

In vitro experiments have indicated that small amounts of conjugated histamine (not necessarily N-acetylhistamine) are formed in the liver of sheep (*Sjaastad* 1967 c). The failure to demonstrate the presence of conjugated metabolites in the urine after injection of C^{14}-histamine might be due to the possibility that the amounts of conjugated histamine formed in vivo are too small to be detected in the urine by the technique used by *Eliassen*.

Orally administered histamine does not substantially increase the amounts of free histamine in the urine (*Sjaastad* 1967 d). With some reservations for the possibility that histamine formed

in the digestive tract is treated differently from histamine given by mouth, this fact suggests that most urinary free histamine in sheep originates from histamine formed in the tissues.

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SUMMARY

1. By modifying the method of *Dunér & Pernow* (1956) the urinary excretion of free and conjugated histamine was examined in 11 healthy adult sheep.

2. The mean excretion of free histamine was 9.8 ± 9.0 (s) μg base/24 hrs. (uncorrected for losses during extraction). When individual means were used, the range was: 2.1—34.2 μg base/24 hrs. The intraindividual variations were of the same order as the interindividual variations.

3. The mean excretion of conjugated histamine was equivalent to 15.1 ± 9.9 (s) μg histamine base/24 hrs. In all animals the ratio conjugated/free histamine was above 1. The interindividual as well as the intraindividual variation for conjugated histamine was of the same order as for free histamine.

SAMMENDRAG

Bestemmelse og forekomst av fri og konjugert histamin i urin fra sau (får).

1. Utskillelsen av fri og konjugert histamin med urinen hos 11 voksne, normale sauer ble undersøkt med en metode etter *Dunér & Pernow* (1956) noe modifisert.

2. Ekskresjonen av fritt histamin var i middel 9.8 ± 9.0 (s) μg base/24 timer (ukorrigert for tap under ekstraksjonen). De indivi-

duelle middelverdier varierte fra 2.1 til 34.2 µg base/24 timer. De intraindividuelle og de interindividuelle variasjoner var av samme størrelsesorden.

3. Utskillelsen av konjugert histamin var i middel ekvivalent med 15.1 ± 9.9 (s) µg histamin-base/24 timer. Hos alle dyr var kvotienten konjugert/fri histamin større enn 1. Både de intra- og interindividuelle variasjoner for konjugert histamin var av samme størrelsesorden som for fri histamin.

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