Acta vet. scand. 1967, 8, 50—70.

From the Department of Physiology, Veterinary College of Norway, Oslo.

DETERMINATION AND OCCURRENCE OF HISTAMINE IN RUMEN LIQUOR OF SHEEP *)

By
Ø. V. Sjaastad

High levels of histamine in rumen have been suggested to be of importance in the pathogenesis of some diseases in ruminants (*Dougherty* 1942; *Dain et al.* 1955). However, the literature gives sparse information as to the occurrence and formation of histamine in the rumen of healthy animals. Deamination is normally a major pathway in the degradation of histidine in the rumen (*Lewis* 1955; *Lewis & Emery* 1962). Decarboxylation, resulting in histamine, is not supposed to take place to any considerable extent. *Dain et al.* were not able to detect histamine in the rumen contents of healthy sheep fed hay of good quality. The chromatographic method used was, however, rather insensitive. *Shinozaki* (1957), on the other hand, reported histamine values ranging from 2.9 to 5.6 μg per ml of rumen liquor. The sheep were either fed clover or they were at pasture, and the highest values were found in the grazing periods. It was not mentioned whether the histamine values were expressed as the base or as a salt.

In a preliminary paper, *Stormorken & Sjaastad* (1962) reported large diurnal fluctuations in the concentration of histamine in the rumen, related to the time of feeding. When histamine was extracted from the feeds, the results revealed that the histamine found in the rumen at least partly originated from pre-

*) This investigation has been financially supported by the Agricultural Research Council of Norway. Thanks are due to Miss Liv Burum for skilful technical assistance.

formed histamine in the foodstuffs. No experiments were then performed to decide if formation of histamine also normally takes place in the rumen.

The object of this study has been to examine more closely various methods for assaying histamine in rumen liquor, and the occurrence of histamine in the rumen. Furthermore, experiments were undertaken to examine whether formation of histamine takes place in the rumen of healthy animals.

METHODS

Animals and feeding. Adult, apparently healthy sheep of both sexes of the Dala breed were used in the studies. The animals were kept indoors and fed twice a day, at 7 a.m. and 3 p.m. One meal consisted of hay of good quality and concentrates, the other one of grass silage preserved with either H_2SO_4-HCl or HCOOH. In some experiments fresh grass was substituted for hay. Any residual food remaining 30 min. after the feeding had started was removed. Water ad libitum was allowed.

Sampling. Samples of rumen contents were aspirated according to *Krogh* (1959), and rumen liquor was prepared by immediate centrifugation of rumen contents at 3,000 × g for 2 min. at room temperature.

Determination of histamine in rumen contents. Two different methods were used. 1) In most experiments histamine in rumen liquor was determined by the method used by *Dunér & Pernow* (1956) for the determination of histamine in human urine. Some modifications were introduced, and the procedure was as follows: Twenty-five ml samples of rumen liquor, adjusted to pH 6.5, were passed through Amberlite IRC-50 columns (60 × 10 mm, 4—6 ml per min.). As histamine is both formed (vide infra) and inactivated (*Sjaastad,* to be published) in rumen contents, stress was laid on passing the rumen liquor through the columns as soon as possible after sampling; usually this was accomplished within 10 min. When the level of rumen liquor reached the top of the resin, approximately 5 ml of distilled water were added and passed through the columns at the same flow rate as the liquor. The resin was then washed with 2 × 25 ml of distilled water. Precautions were taken that the resin was always covered by a layer of fluid. 1.2 N-HCl was added to the columns, the volume (in ml) being calculated by multiplying the height of the resin after washing with distilled water (in cm) by a factor of 0.95. This amount of 1.2 N-HCl was in preliminary experiments found to be sufficient to bring the pH of the outflow from the columns down to the desired level at the end of the elution. The flow rate of the 1.2 N-HCl was adjusted to approximately 0.2 ml per min. When the level of 1.2 N-HCl had reached the upper edge of the resin, 10 ml 0.01 N-HCl was added, and the collection of the eluate started. The pH of the effluent was

checked with frequent intervals and the eluates discarded until the check prior to the change to an acid pH. After the pH had changed to acid the flow rate was increased to about 0.5 ml per min. The eluation then continued until all acid had passed through the resin. If not the pH of the eluate at any point had dropped to below 1.5, another 0.5 ml 1.2 N-HCl and 5 ml 0.01 N-HCl were added to the columns. The eluates were stored at 4°C for no longer than 4—5 days. The histamine concentration of the neutralized eluates was measured on isolated guinea-pig ileum, suspended in an organ bath (6 ml) containing Tyrode's solution (37°C) with atropine (0.05 μg/ml) and glucose (1 g/l). Tests were made every 55 sec. and the determination of the unknown was done by bracketing between standards usually not differing more than 15 %. In 22 samples of rumen liquor (15.5—52.8 μg histamine diphosphate/ml) the standard deviation of the method, determined from the difference between duplicates, was 5.9 %. Histamine concentrations as low as 0.01 μg/ml rumen liquor could usually be detected. 2) On some occasions the rumen liquor for practical reasons could not be subjected to ion-exchange chromatography within the desired time after aspiration of rumen contents. After centrifuging, 10 ml aliquots of rumen liquor were adjusted to pH 4.8 and immediately afterwards treated with 4 volumes of ethanol. After vigorous shaking, this mixture was left at 4°C for 30 min, and then filtered. Subsequent to addition of 1 ml N-HCl, providing a pH of approximately 2, the filtrates were stored at 4°C. Within 2—3 days the pH of the filtrates was adjusted to 6.5 and then subjected to ion-exchange chromatography as described above. In 10 samples of rumen liquor (1—2 μg histamine diphosphate/ml) determined in duplicate by this method the standard deviation was 6.2 %.

In vitro formation of histamine in rumen contents. Rumen contents aspirated at different times after feeding a diet poor in histamine, were incubated at 38°C with and without addition of L-histidine. After adding histidine, the pH was readjusted to the level found immediately after aspiration. In some experiments pyridoxal-5-phosphate was added. A gentle stream of N_2 was passed through the incubation vessels, but in order to reduce washing out the gases normally formed in rumen contents, the gas was not allowed to bubble through the incubation vessels. In some pilot experiments a gas mixture containing 95 % N_2 and 5 % CO_2 was substituted for pure N_2 without changing the rate of histamine formation. Samples of the incubation mixture were removed at noted intervals and analyzed for histamine (method 1).

Extraction of histamine from feeds. From 10 g wet weight of silage, hay, grass, or concentrates histamine was extracted by two different methods: 1) In the first part of the study the continuous ether extraction method recommended by *Block & Bolling* (1951) for the extraction of amines was used. The acid ether extracts obtained were vigorously shaken twice with distilled water (1:1 v/v). By this procedure more than 99 % of the histamine was found to be transferred to the water phase. When large amounts of histamine were expected, the bioassay of histamine was performed directly, as small

amounts of ether did not interfere. When small amounts of histamine were expected, the ion-exchange method was used, serving the purpose of removing the ether and concentrating the extracts. 2) Since the ether extraction method gave rather variable yields, it was replaced by extraction with water: Aliquots of 10 g of feeds were 3 times extracted for 15 min. at 70°C with 50 ml of distilled water after adjustment of pH to 4 by N-HCl. After centrifuging, the pooled supernatants were subjected to ion-exchange chromatography or tested for histamine content directly depending on the expected content of histamine.

Histamine values given in this paper are not corrected for losses and are calculated as the diphosphate and represent the mean of duplicates. The variability of histamine values is consistently expressed as standard deviation (s). Mostly the person who performed the bioassay did not know what the specimen tested represented.

Determination of rumen fluid volume was performed according to *Hydén* (1956). Ten g of polyethylene glycol (PEG) was administered by a stomach tube or through a permanent rumen fistula at zero time. Samples of rumen contents were aspirated at intervals of 30 min., the first being taken 30 min. and the last one 4 hrs. after the administration of PEG. The turbidity produced when TCA and $Ba(Cl)_2$ were added to clear rumen filtrates was measured at 650 mμ in a Zeiss spectrophotometer. A standard calibration curve was prepared in each experiment as the extinction of a given concentration varied slightly between experiments. The concentration at the time of administration was extrapolated. The fluid volume of the rumen at zero time was then calculated by dividing the amount of PEG administered by its concentration at zero time.

Hydén found that the turbidity of PEG reached a maximum 4 min. after addition of TCA, whereafter it was stable, especially in the range of PEG concentrations recommended. The wave-length at which the turbidity was measured was not mentioned. In the present experiments maximum turbidity was not reached until approximately 60 min. after the addition of TCA. In 8 experiments the increase in the turbidity from 5 to 60 min. after adding TCA was 75—100 % in the range of PEG examined. The relationship between PEG concentrations (0.1—0.4 mg/ml filtrate) and turbidity was linear 60 min. after the addition of TCA, while measured after 5 min. the extinction of 0.2 mg PEG per ml rumen filtrate was relatively lower than that of 0.1 and 0.4 mg/ml. In the experiments reported in this paper all readings were made exactly 60 min. after addition of TCA, and when necessary samples of rumen liquor were diluted to obtain PEG concentrations of 0.1—0.4 mg/ml filtrate.

MATERIALS

Histamine diphosphate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, USA.

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

Polyethylene glycol (PEG) was bought from British Drug Houses Ltd., England.

Pyridoxal-5-phosphate was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. A final concentration of 1 mg/ml was used.

Ion-exchange resin. Ion-exchange was performed with Amberlite IRC-50 from British Drug Houses Ltd., England. Batches of the resin were prepared according to *Bergstrøm & Hansson* (1951). After transfer of the resin to ion-exchange columns it was treated with the 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.

Aminoguanidine bicarbonate was obtained from L. Light & Co. Ltd., Colnbrook, England.

Antihistamine, "Allergin" (Diphenhydramine chloride), was bought from Nyegaard & Co. A/S, Oslo, Norway.

Osmolarity was measured with "Osmometer" from Knauer, Wissenschaftlicher Gerätbau, West Germany.

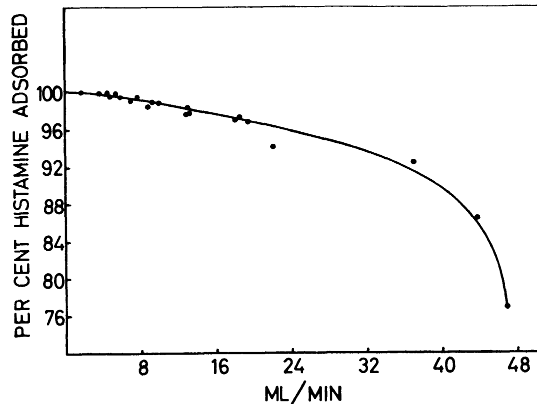
RESULTS

Methodological investigations

Identification of the active substance in extracts of rumen contents and of the feeds was always done by ascertaining that the effect on the guinea-pig ileum was completely counteracted by antihistamine in doses necessary to abolish contractions of the same magnitude produced by histamine standards. In addition, the extracts were also subjected to paper chromatography after the following pretreatment: The acid extracts were evaporated to dryness ex vacuo and the residue was dissolved in small amounts of 10 % isopropanol. After evaporation to 0.5 ml, aliquots were chromatographed on Whatman paper no. I at 20°C using diethylether/ethanol/water/conc. ammonia (5:4:1:0.1) as moving phase. The chromatograms were sprayed with diazotized sulphanilic acid (method of Pauly modified by *Ames & Mitchell* 1952). In extracts from 8 different silages, a Pauly positive spot was found at a Rf 0.48—0.52. The Rf never deviated more than 0.05 from that of histamine standards. Addition of internal histamine standards to the extracts did not result in any additional Pauly positive spot. No spot with approximately the same Rf as acetylhistamine or imidazole acetic acid was found. As further identification, the area expected to contain histamine on an unstained chromatogram of the extracts was eluted with 0.9 g/100 ml NaCl and shown to possess smooth muscle contracting activity, the effect being counteracted by antihistamine. The rest of the chromatograms gave inactive eluates. Similar

results were found for extracts of rumen liquor aspirated after feeding silage. When grass, hay or concentrates were chromatographed, a faintly coloured spot with Rf about 0.50 was observed for some of the extracts, while others did not give any Pauly positive spot at this area.

In addition to the histamine found in the feeds, a chromatographic fraction corresponding to tyramine was invariably found in silage, in quantities visually judged to exceed those of histam-



F i g u r e 1. Adsorption of histamine to Amberlite IRC-50 at different flow rates of rumen liquor through the resin. Size of resin: 60 × 10 mm. Histamine added to rumen liquor: 100 μg histamine diphosphate/ml. The histamine concentration of the effluent was tested without any pretreatment on guinea-pig ileum.

ine. In the other feeds examined, small amounts of tyramine were sometimes demonstrated (unpublished results).

Adsorption of histamine at different flow rates of rumen liquor through the resin. Twenty-five ml aliquots of rumen liquor, to which histamine had been added (100 μg/ml) were passed at different flow rates through the resin. At flow rates of 4—6 ml/min., as used in this study, almost all histamine was adsorbed to the resin (Fig. 1).

Recovery of added histamine by the direct ion-exchange method. Histamine diphosphate in the range of 5—20 μg was added to altogether 35 aliquots (25 ml) of rumen liquor obtained from 5 different sheep fed a diet poor in histamine. The rumen liquor was passed through the Amberlite IRC-50 columns after adjusting the pH to 6.5. The average recovery was 81.1 ± 5.9 %. The mean

recovery was somewhat less when 5 μg was added (15 expts.) than after addition of 20 μg (6 expts.) (77.0 ± 5.3 and 84.5 ± 6.8 %, respectively). This difference is not significant ($P > 0.05$). Higher recoveries were not obtained by increasing the height of the resin to 10 cm.

Recovery of histamine added to rumen liquor treated with ethanol. To 10 ml aliquots of rumen liquor 10 (8 expts.) or 20 μg (16 expts.) of histamine diphosphate was added. After treatment with ethanol and filtration, the pH was adjusted to 6.5 and the filtrates were immediately subjected to ion-exchange chromatography. The average recovery was 71.6 ± 6.4 %. No difference was found between the recoveries after addition of 10 μg and 20 μg (71.1 ± 5.9 and 71.9 ± 6.8 %, respectively).

Table 1. Stability of histamine in ethanol-treated rumen liquor stored at 4°C or 20°C and pH 2. Histamine added: 20 μg.

No. of samples	Days stored	Percentage of added histamine recovered (Mean and s)	
		4°C	20°C
6	3	66.8 ± 3.4	67.7 ± 3.2
6	5	68.1 ± 4.7	57.5 ± 4.3
6	8	66.3 ± 5.9	54.0 ± 4.9

Storage of ethanol-treated rumen liquor. Aliquots of rumen liquor (10 ml) to which 20 μg histamine diphosphate had been added, were treated with ethanol, filtered and stored at 4°C or 20°C, and pH 2. The histamine contents were then determined after storage for different periods of time. The recovery of histamine in filtrates of rumen liquor stored at 4°C for 8 days (66.3 ± 5.9 %, 6 expts., Table 1) was not significantly lower ($P > 0.05$) than the recovery in aliquots subjected to ion-exchange chromatography immediately after treatment with ethanol (71.9 ± 6.8 %, 6 expts.). When filtrates of ethanol-treated rumen liquor were stored at 20°C, a considerable loss of activity was found (Table 1). The recovery of histamine in filtrates stored for 8 days at this temperature (54.0 ± 4.9 %, 6 expts.) was significantly lower ($P < 0.05$) than the recovery of histamine in filtrates passed through the Amberlite-resin immediately after treatment with ethanol (71.6 ± 6.4 %, see above).

When rumen liquor was treated with ethanol, and stored at the pH found after aspiration, a marked decrease in histamine activity was found. After storage at 20°C for 8 days, only 3—10 % of the initial amount was left.

Salt content of eluates from the resin. Since rumen liquor often is very low in histamine concentration, it seemed mandatory that the eluates were as physiological as possible to facilitate the bioassay of histamine. Some experiments were therefore undertaken to examine when the collection of the eluates should be started to obtain the most suitable osmolarity. No rumen liquor was passed through the columns in these experiments. After washing with distilled water HCl was added and the collection of the eluates started at four different stages of elution (see text, Table 2).

Table 2. Osmolarity and salt content of eluates of Amberlite IRC-50 by starting collection at different stages. Height of resin: 6 cm.

Exp. no.	Osmolarity of eluates (milliosmol)				Salt content of eluates calculated as NaCl (milliequivalent)			
	I	II	III	IV	I	II	III	IV
1	580	540	450	430	5.91	3.95	2.85	2.64
2	590	535	448	437	5.93	3.92	2.84	2.79
3	640	560	505	468	6.34	4.08	3.48	3.18
4	648	552	518	465	6.69	4.08	3.99	3.13
5	640	545	515	445	6.40	3.81	3.73	3.00
6	653	540	502	452	6.59	4.04	3.54	3.08

I Whole eluate collected.

II Collection started when 1.2 N-HCl is 1 cm above upper edge of resin.

III Collection started when 1.2 N-HCl is 1 mm above upper edge of resin.

IV Collection started from the check prior to the change to an acid pH.

The eluates were neutralized with N-NaOH and the osmolarity measured. Furthermore, the effect of the eluates on the guinea-pig ileum was examined.

It is evident that the lowest osmolarity was obtained when the collection of the eluates was started just prior to the change to an acid pH (Table 2). Also by this mode of collection the eluates were hypertonic.

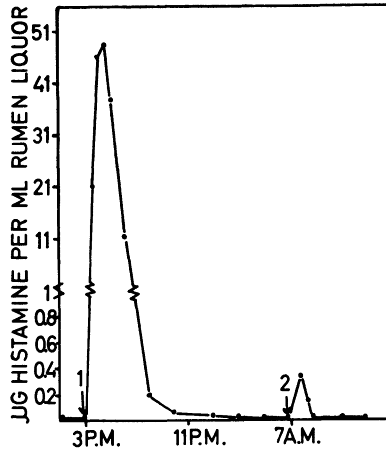
When 1 ml of the eluates in the expt. no. 3, Table 2 was added to the organ bath, eluates I, II, III and IV caused concentrations of the guinea-pig ileum equivalent to 150, 65, 50 and 10 μg of histamine diphosphate. Similar results were found in the other experiments. These results showed that by the method used, addition of large volumes to the organ bath of eluates not containing histamine also caused contraction of the guinea-pig ileum. When 0.5 ml of eluates collected by mode IV (see text, Table 2) was added to the organ bath, contraction of the guinea-pig ileum was not observed. In the following experiments mode IV for collection was used (see Methods) and the volume of eluate added to the bath never exceeded 0.5 ml.

Recovery of added histamine in the ether-extraction method of feeds. To 5 g aliquots of silage, histamine diphosphate varying from 500 to 2.000 μg was added (7 expts.) and the mixture subjected to continuous ether extraction. The extracts were assayed without ion-exchange chromatography. In these experiments the recovery was 76.4 ± 4.8 %. When histamine diphosphate (5—10 μg) was added to 10 g of hay, grass or concentrates, and the extracts obtained were subjected to ion-exchange chromatography, the recovery in 8 experiments was 57.1 ± 14.1 %.

Recovery of added histamine by water extraction of feeds. Mixtures of histamine diphosphate (5—10 μg) and 10 g of hay or concentrates were extracted with water and subjected to ion-exchange chromatography (Methods). The recovery of added histamine in 10 experiments was 78.8 ± 6.3 %.

Diurnal variations of histamine concentration in the rumen

Diurnal variations in the concentration of histamine in the rumen liquor was examined in 10 experiments performed in 5 sheep. Some data from these experiments have already been published in preliminary reports (*Stormorken & Sjaastad* 1962; *Sjaastad & Stormorken* 1963). Feeding with silage invariably resulted in a large increase in the concentration of histamine in the rumen liquor. The maximum increment varied widely. The highest value found was 135 μg per ml rumen liquor. Signs of intoxications after ingestion of silage were never observed. Feeding with hay, concentrates or grass caused a moderate but distinct increase in the histamine concentration. The diurnal variations



F i g u r e 2. Variations in the concentrations of histamine in rumen liquor *in vivo*. Fed at hours indicated by arrows: 1: silage (400 g). 2: hay (about 400 g) and concentrates (about 200 g). Histamine content of the silage: 3500 µg histamine diphosphate/g dry weight.

of histamine concentration in the rumen in one typical experiment are demonstrated in Fig. 2.

Even when silage was fed, the concentration of histamine in the rumen remained high for only a relatively short time, levels below 1 µg/ml being reached in 3—6 hrs. Minute amounts of histamine were, however, usually still found 8—10 hrs. after feeding. After this, the concentrations of histamine in the rumen liquor were in all experiments too low to be detected by the methods used. Subsequent to feeding with hay, grass or concentrates, undetectable levels of histamine in the rumen were reached after 1½—3 hrs., except when some histamine remained in the rumen after a previous feeding of silage.

Extracts of the silages used in the experiments revealed high concentrations of histamine in contrast to extracts from hay, concentrates and grass (*Sjaastad & Stormorken*). In Table 3 are condensed the histamine values for the feeds used both in experiments reported in the preliminary papers and in subsequent studies. Table 3 also includes the histamine contents of 8 silages not used for the feeding experiments (preserved with either HCOOH or H_2SO_4-HCl). All histamine values for silage are the mean of at least 2 samples, as the histamine concentration varied considerably within the same stock. The highest concentration of histamine found in the rumen in the feeding experiments

Table 3. Content of histamine in feeds (μg/g dry weight). The values for hay, concentrates and fresh grass are obtained by extraction with water, and those for silage partly by water extraction and partly by continuous ether extraction.

	Grass silage	Hay	Concentrates	Fresh grass
No. of foodstocks	13	10	9	6
Mean	2080	0.34	0.39	0.45
Range	480—4400	0.10—0.54	0.22—0.50	0.05—0.93

(135 μg/ml) was observed after the ingestion of 950 g of silage containing 3850 μg/g dry weight.

Histamine analyses of the feeds had thus revealed that histamine found in the rumen at least partly originated from preformed histamine in the feed. However, the possibility still existed that a formation of histamine also might take place in the rumen subsequent to feeding. This would be most easily detected with a feed itself containing only small quantities of histamine. Hence, 5 sheep were fed hay or concentrates of which the concentration of histamine had been estimated previously. PEG (10 g) was administered by a stomach tube just prior to feeding. Samples of rumen contents were aspirated at intervals of 30 min. and the concentrations of histamine and PEG were determined. Prior to feeding, the concentrations of histamine in rumen liquor in all experiments were too low to be estimated. Histamine ingested with the food could account for only a small fraction of the histamine calculated to be present in the rumen at the point of time when the concentration of histamine had reached its maximum (Table 4).

Table 4. Formation of histamine in rumen contents *in vivo*.

Exp. no.	Feeding	Histamine in the food (μg) (a)	Rumen fluid volume (l)	Maximum concentration of histamine in rumen liquor (μg/ml)	Maximum amount of histamine in the rumen (μg) (b)	$\frac{a}{b} \times 100$
1	Hay, 700 g	247	8.5	0.27	2295	10.7
2	„ 750 g	262	6.2	0.35	2170	12.1
3	Concentrates, 500 g	215	8.5	0.15	1275	16.8
4	Hay, 500 g	175	5.6	0.18	1008	17.3
5	„ 600 g	240	5.7	0.59	3363	7.1
						Mean 12.8

In vitro formation of histamine in rumen contents

When rumen contents, aspirated at different intervals after feeding hay, were incubated at 38°C for 3—5 hrs., variable results were obtained (Table 5). In rumen contents aspirated 45 min. after feeding had started, the concentration of histamine usually increased during the first hour of incubation. Addition of aminoguanidine to the incubation mixture resulted in slightly higher concentrations of histamine than in controls to which no aminoguanidine had been added (Table 5). In rumen contents aspirated at other intervals after feeding, a small increase in the concentration of histamine could occasionally be detected after incubation. It is noteworthy that the *in vivo* concentration of histamine in rumen contents 45 min. after feeding is rather high. At the other intervals examined, histamine could not be detected *in vivo* (Table 5).

Table 5. *In vitro* formation of histamine in rumen contents aspirated at different intervals after feeding of hay.

Sheep no.	Time of aspiration (hrs. after feeding)	Concentration of histamine in rumen liquor before incubation (μg/ml)	Treatment	Concentration of histamine in rumen liquor after incubation (μg/ml)		
				1 hr.	3 hrs.	5 hrs.
1	¾	0.72	—	0.53	0.04	0.03
1	¾	0.27	—	0.60	0.24	0.03
2	¾	0.19	—	0.52	0.28	0.18
1	¾	0.59	—	1.20	1.25	—
1	¾	0.59	10^{-3} AMG*)	1.60	1.87	—
2	¾	0.10	—	0.02	0	—
2	¾	0.10	10^{-3} AMG	0.10	0	—
2	¾	0.10	$5 . 10^{-3}$ AMG	0.15	0	—
1	3½	0	—	0	0	0
1	3½	0	—	0	0	0
2	3½	0	—	0	0.06	0.12
2	3½	0	—	0	0.01	—
2	3½	0	10^{-3} AMG	0	0.01	0
1	3½	0	—	0	0	0
2	7	0	—	0	0.03	0
2	17	0	—	0.02	0.01	0.01
2	17	0	10^{-3} AMG	0.02	0.02	0.01
1	17	0	—	0	0	0

*) AMG = aminoguanidine.

Addition of L-histidine. Rumen contents, aspirated 3 hrs. after feeding hay, and incubated for 5 hrs. with 4 different concentrations of histidine, resulted in histamine formation at concentrations of histidine higher than 0.1 mg/ml (Fig. 3). Increasing the concentration of histidine from 10 to 20 mg/ml did not greatly increase the histamine formation. Other experiments, however, showed that if the incubation periods were prolonged, the dif-

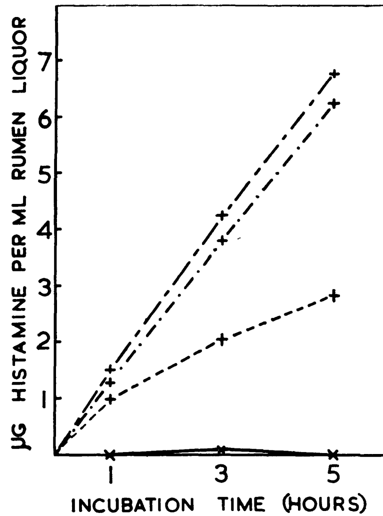


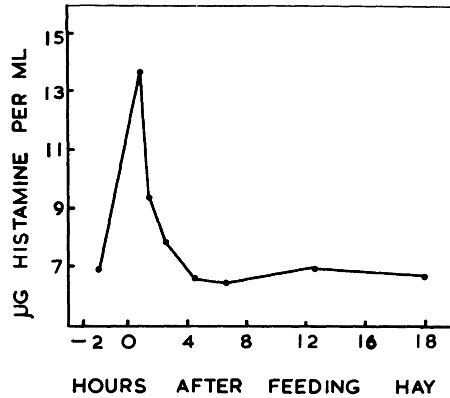
Figure 3. *In vitro* formation of histamine in rumen contents incubated with L-histidine.

—-—-—- 20 mg L-histidine/ml
— · — · — 10 mg L-histidine/ml
- - - - - - - - - 1 mg L-histidine/ml
————— 0.1 mg L-histidine/ml

Incubation period: 1 hr.

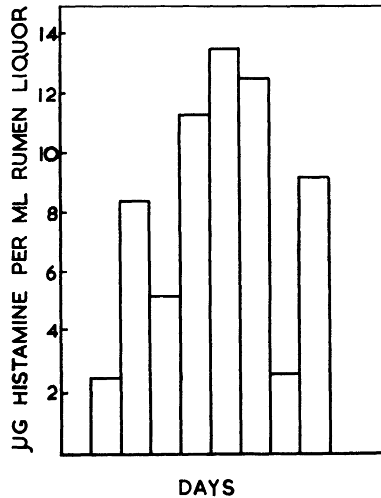
ferences between the rates of histamine formation at histidine concentrations of 10 and 20 mg/ml became large. By using an incubation period of 24 hrs. and an initial concentration of histidine of 20 mg/ml, concentrations of histamine higher than 100 μg/ml were often found. But as the quantities of NaOH necessary for the readjustment of pH after the addition of 20 mg histidine/ml were rather large, a histidine concentration of 10 mg/ml was used in the following experiments in order to avoid high salt concentrations.

In 3 experiments histamine formation was examined in rumen



F i g u r e 4. Variation in the formation of rumen contents *in vitro*. L-histidine added: 10 mg/ml. Incubation period: 1 hr. The curve represents the mean of 3 experiments.

contents aspirated at different intervals after feeding hay, and incubated with histidine for 1 hr. The largest capacity for forming histamine was found in samples of rumen contents a short time after feeding (Fig. 4). Similar results were found if the incubation periods were prolonged to 3 hrs. When rumen liquor aspirated 3 hrs. after feeding was sterile-filtered and incubated



F i g u r e 5. Day to day variations in the *in vitro* capacity of rumen contents to form histamine after addition of L-histidine (10 mg/ml). Rumen contents aspirated 5 hrs. after feeding hay. The experiments fell within a period of one month. Incubation period: 1 hr.

Table 6. The effect of pyridoxal-5-phosphate on the *in vitro* formation of histamine in rumen contents. Concentration of L-histidine: 10 mg/ml.

Sheep no.	Time of aspiration (hrs. after feeding)	Histamine formed after incubation of rumen contents (μg/ml)			
		Without pyridoxal-5-phosphate		Pyridoxal-5-phosphate (1 mg/ml	
		1 hr.	3 hrs.	1 hr.	3 hrs.
1	3	6.1	15.5	9.1	27.2
1	3	4.5	12.2	5.7	21.7
2	6	4.0	7.3	6.3	12.4
2	6	3.7	8.3	5.7	16.3
2	18	6.6	13.7	9.2	20.3
2	18	4.9	12.5	6.3	21.3

in the presence of histidine (2 expts.), histamine formation could not be demonstrated.

One sheep receiving a diet of hay, was studied on 8 different days within a period of 1 month. Rumen samples were aspirated at a fixed time after feeding and incubated with histidine. Considerable daily variations in histamine forming capacity were found (Fig. 5).

Addition of pyridoxal-5-phosphate (1 mg/ml) to rumen contents increased histamine formation (Table 6).

DISCUSSION

A few methods have been reported for the study of histamine in rumen contents (*Dain et al.* 1955; *Shinozaki* 1957; *van der Horst* 1961; *Thorsell* 1961). These methods must be regarded only qualitative or semiquantitative, and no data are available as to their reproducibility and sensitivity. The method used in the present study gave satisfactory reproducibility and recovery of added histamine, and the sensitivity was high.

However, when just detectable concentrations of histamine are determined (about 0.01 μg/ml rumen liquor), the reproducibility of the bioassay is low. Furthermore, the possibility then increases that the contraction of the guinea-pig ileum might be caused by other substances than histamine. The specificity of the bioassay was therefore on these occasions regularly ascertained by adding antihistamine to the organ bath.

Likewise, at low concentrations, inhibiting substances might obscure the presence of or reduce the response to histamine. It is for example well known that high salt concentrations inhibit the response of histamine on the guinea-pig ileum (*Barsoum & Gaddum* 1935). By the method used for ion-exchange in the present study it is therefore of importance to start the collection of the eluates just before the change to an acid pH as this provides the lowest osmolarity. Furthermore, as the rumen liquor is concentrated to the largest extent by this mode of collection, the amount of eluate necessary to add to the bath for producing a measurable contraction is then smaller than when the collection is started at an earlier stage. However, internal standard of histamine should regularly be added to the eluates to test the presence of inhibitory activity. In the present experiments some degree of inhibition was occasionally encountered when low concentrations of histamine were determined. The histamine values given on these occasions therefore represent minimum figures.

Formation of histamine in rumen contents after addition of glucose or other sources rich in carbohydrates has been demonstrated both *in vivo* (*Dain et al.*; *Sanford* 1963) and *in vitro* (*van der Horst*; *Sanford*). Administration of carbohydrates causes lowering of the pH accompanied by a change of the ruminal flora, characterized by enrichment of the lactobacilli (*Krogh* 1959). The lower the pH, the larger the formation of histamine (*Dain et al.*). In this connection it is worth while to mention that *Rodwell* (1953) found several strains within the genus lactobacillus having the ability to decarboxylate histidine. Ingestion of silage with a pH of 3.5—5.0 did not lower the pH in the rumen thus creating conditions favourable for decarboxylation of histidine (*Stormorken & Sjaastad* 1962). It is therefore not very likely that the large increase in the ruminal concentration of histamine after ingestion of silage should partly be due to a large formation of histamine in the rumen.

Sanford did not find any significant increase in the rate of histamine formation *in vitro* when histidine in amounts giving a final concentration of 1.5 mg/ml was added to rumen contents containing 2 % glucose. It was not mentioned what kind of ration the animals were fed. Inconsistent with the findings of *Sanford,* considerable *in vitro* formation of histamine invariably occurred in the present experiments when histidine was added to rumen contents (Figs. 3—5). Although a histidine concentration of

10 mg/ml was used in most of the present experiments, a concentration even lower than that used by *Sanford* caused formation of appreciable amounts of histamine. The discrepancy between our results and those obtained by *Sanford* may be due to differences in the diet and/or the ruminal flora. In this connection it might be pertinent that the lack of histamine formation in sterile-filtered rumen contents showed that histamine probably was formed by bacteria and not by free enzymes. On the other hand, *Werle & Raub* (1948) have shown that histidine-decarboxylating activity may be present in plants.

The interpretation of the *in vitro* experiments with histidine, however, was impeded by the fact that the histamine formed was simultaneously subjected to inactivation, and that the *in vitro* capacity of inactivation also showed variations related to the time of feeding (*Sjaastad,* to be published). This inactivation was, however, never great enough to mask the formation of histamine in experiments where high concentrations of histidine were added to rumen contents.

When sheep are fed twice daily, the total number of rumen bacteria reach maximum values 4—6 hours after feeding (*Moir & Somers* 1957; *Bryant & Robinson* 1961), i.e. at hours when the *in vitro* capacity to form histamine is low (Fig. 4). It is, however, likely that fluctuations in the balance of the specific types of bacteria comprising the total flora also occur.

It is further possible that the increased histamine forming capacity after feeding is due to increased ruminal concentrations of co-factors for the bacterial histidine-decarboxylase. Pyridoxal-5-phosphate has been reported to be a co-factor for histidine-decarboxylase (*Gale* 1946). *Sanford,* on the other hand, was not able to demonstrate increased *in vitro* formation of histamine when this substance was added to rumen contents. In the present experiments, addition of pyridoxl-5-phosphate to rumen contents to which histidine had been added, resulted in considerable increase in the rate of histamine formation (Table 6). The results in Table 6 do not indicate any variation in the effect of pyridoxal-5-phosphate related to the time of feeding. But as only few samples of rumen contents, aspirated on different days, were examined, this possibility still remains open.

The histidine concentration in the above mentioned experiments has been relatively high. No measurements were carried out of the histidine concentrations in the feeds used. According

to *Florkin & Mason* (1963) histidine accounts for about 2 % of the amino-acids in plant proteins. Assuming a crude protein content of 8—10 % in the hay used and a rumen fluid volume of 5 l, 500 g of hay would give a maximum histidine concentration in the rumen of 0.16—0.20 mg/ml, which should be sufficient to allow detectable formation of histamine (Fig. 3). As most of the amino-acids in plants are bound in proteins, and are slowly released by rumen bacteria, the concentration of free histidine probably never reaches so high levels in the rumen as calculated above. Nevertheless, when rumen contents were incubated without addition of histidine, a slight increase in the concentration of histamine sometimes occurred, especially in rumen contents aspirated shortly after feeding (Table 5). When hay was extracted with water (see Methods), detectable amounts of histamine were not left after extraction for 45 min. It is thus not likely that increased histamine concentrations observed during the incubation period are due to liberation of histamine from food particles. This indicates that with the diet used in these experiments, small amounts of histamine were formed in rumen contents in vitro. Furthermore, as the *in vitro* histamine concentration of rumen contents is the net result of histamine formation and inactivation, the real amount of histamine formed was probably higher than estimated. This conclusion is supported by the fact that when the inactivation of histamine was reduced by the addition of aminoguanidine, the increase in histamine concentration upon incubation was somewhat larger than in controls to which no aminoguanidine had been added.

Recent studies have shown that histamine is present in many feedstuffs usually consumed by domestic ruminants (*Fowler* 1962; *Macpherson* 1962; *Stormorken & Sjaastad*). However, when hay or concentrates were fed, only a minor part of the histamine present in rumen after feeding seemed to originate from preformed histamine in the food (Table 4). This supports the *in vitro* experiments in indicating that histamine formation normally takes place in the rumen.

REFERENCES

Ames, B. N. & H. K. Mitchell: Paper chromatography of imidazoles. J. Amer. chem. Soc. 1952, *74*, 252—253.

Barsoum, G. S. & J. Y. Gaddum: The pharmacological estimation of adenosine and histamine in blood. J. Physiol. (Lond.) 1935, *85*, 1—14.

Bergstrøm, S. & G. Hansson: The use of Amberlite IRC-50 for the purification of adrenaline and histamine. Acta physiol. scand. 1951, *22*, 87—92.

Block, R. J. & D. Bolling: The Amino Acid Composition of Proteins and Foods. Charles C. Thomas, Springfield, Illinois, 1951, 576 pp.

Bryant, M. P. & I. M. Robinson: An improved nonselective culture medium for ruminal bacteria and its use in determining diural variation in numbers of bacteria in the rumen. J. Dairy Sci. 1961, *44*, 1446—1456.

Dain, J. A., A. L. Neal & R. W. Dougherty: The occurrence of histamine and tyramine in rumen ingesta of experimentally over-fed sheep. J. Animal Sci. 1955, *14*, 930—935.

Dougherty, R. W.: Analyses of gas and ingesta of bloated cattle. Amer. J. vet. Res. 1942, *3*, 401—402.

Dunér, H. & B. Pernow: Urinary excretion of histamine in healthy human subjects. Scand. J. clin. Lab. Invest. 1956, *8*, 296—303.

Florkin, M. & H. S. Mason: Comparative Biochemistry. Academic Press 1963, *vol. 5*, p. 29.

Fowler, H. D.: Histamine in grasses and clovers. Nature (Lond.) 1962, *193*, 582—583.

Gale, E.: The bacterial amino acid decarboxylases. *In,* F. F. Nord (ed.) Advances in Enzymology. Interscience Publ., New York. Enzymol. 1946, *6*, 1—32.

van der Horst, C. J. G.: Keto-acids and amines in rumen liquid of cattle. Nature (Lond.) 1961, *191*, 73—74.

Hydén, S.: A turbidimetric method for the determination of higher polyethylene glycols in biological materials. Ann. Roy. Agric. Coll. Sweden 1956, *22*, 139—145.

Krogh, N.: Studies on alterations in the rumen fluid of sheep, especially concerning the microbial composition, when readily available carbohydrates are added to the food. 1. Sucrose. Acta vet. scand. 1959, *1*, 74—97.

Lewis, D.: Amino-acid metabolism in the rumen of the sheep. Brit. J. Nutr. 1955, *9*, 215—230.

Lewis, D. & R. S. Emery: Relative deamination rates of amino acids by rumen microorganisms. J. Dairy Sci. 1962, *45*, 765—768.

Macpherson, H. T.: Histamine, tryptamine and tyromine in grass silage. J. Sci. Food Agric. 1962, *13*, 29—32.

Moir, R. J. & M. Somers: Ruminal flora studies. VIII. The influence of rate and method of feeding a ration upon its digestibility, upon ruminal function, and upon the ruminal population. Aust. J. agric. Res. 1957, *8*, 253—265.

Rodwell, A. W.: The occurrence and distribution of aminoacid decarboxylases within the genus lactobacillus. J. gen. Microbiol. 1953, *8*, 224—232.

Sanford, J.: Formation of histamine in ruminal fluid. Nature (Lond.) 1963, *199*, 129—130.

Shinozaki, K.: Studies on experimental bloat in ruminants. 4. Identification of amines from rumen ingesta and passage of histamine across the rumen epithelium. Tôhoku J. agric. Res. 1957, *8*, 149—154.

Sjaastad, Ø. V. & H. Stormorken: Diet and histamine in the ruminant. Nature (Lond.) 1963, *197*, 907—908.

Stormorken, H. & Ø. V. Sjaastad: Diet and histamine in the ruminant. Occurrence of histamine in silage. Arch. int. Pharmacodyn. 1962, *140*, 336—340.

Thorsell, W.: A qualitative chemical method for the demonstration of "Pauly-positive" bases in rumen contents. Acta chem. scand. 1961, *15*, 937—938.

Werle, E. & A. Raub: Über Vorkommen, Bildung und Abbau biogener Amine bei Pflanzen unter besondere Berücksichtigung des Histamins. Biochem. Z. 1948, *318*, 538—553.

SUMMARY

Methods for the determination of histamine in rumen liquor and different feeds are described. The procedures involve adsorption of histamine on Amberlite IRC-50 columns and biological determination of the histamine content of eluates from the ion-exchange resin. By these methods 70—80 % of added histamine were recovered and histamine concentrations as low as 0.01 μg histamine diphosphate per ml of rumen liquor could usually be estimated.

Occurrence and formation of histamine in rumen contents of sheep have been examined with the following results:

1. Feeding invariably increased the concentration of histamine in the rumen, the increments being related to the histamine content of the feed.

2. A small formation of histamine seemed to take place in rumen content both *in vivo* and *in vitro* after feeding hay or concentrates.

3. The *in vitro* formation of histamine in rumen contents was greatly increased when L-histidine (10 mg/ml) was added. The formation of histamine varied widely from day to day and was further related to the time of feeding.

4. Pyridoxal-5-phosphate increased the *in vitro* formation of histamine in rumen content.

ZUSAMMENFASSUNG

Bestimmung und Vorkommen von Histamin im Pansengehalt von Schafen.

Methoden zur Bestimmung von Histamin in der Pansenflüssigkeit und in Futtermitteln werden beschrieben. Die Methoden umfassten eine Adsorbtion des Histamins in Säulen bestehend aus Amberlite IRC-50 und anschliessend eine biologische Bestimmung der Eluate des Ionenaustauschers. Bei diesen Methoden wurden 70 bis 80 % des hin-

zugesetzten Histamins wiedergefunden, und Konzentrationen bis zu 0,01 μg liessen sich gewöhnlich nachweisen.

Das Vorkommen sowie die Bildung von Histamin in dem Pansengehalt von Schafen wurden mit folgendem Ergebnis bestimmt:

1. Die Fütterung verursachte immer eine Steigerung der Histaminkonzentration im Pansen. Diese Steigerung hing von dem Histamingehalt im Futter ab.

2. Eine geringe Bildung von Histamin schien im Pansen sowohl *in vivo* als auch *in vitro* nach einer Fütterung mit Heu oder Kraftfutter stattzufinden.

3. Die *in vitro* Bildung von Histamin in der Pansenflüssigkeit wurde durch eine Beigabe von L-Histidin (10 mg/ml) stark erhöht. Die Histaminbildung variierte sehr von Tag zu Tag und hing ausserdem davon ab wie lange nach der Fütterung der Pansengehalt aspiriert wurde.

4. Pyridoxal-5-Phosphat erhöhte die *in vitro* Bildung von Histamin im Pansengehalt.

SAMMENDRAG

Bestemmelse og forekomst av histamin i vominnhold fra sau.

Det er beskrevet metoder for bestemmelse av histamin i vomsaft og fôrstoffer. Metodene innbefatter adsorbsjon av histaminet til kolonner av Amberlite IRC-50 fulgt av biologisk bestemmelse av eluatene fra jonebytteren. Ved disse metoder ble 70—80 % av tilsatt histamin gjenfunnet og konsentrasjoner ned til 0,01 μg histamin difosfat pr. ml vomsaft kunne vanligvis bestemmes.

Forekomst og dannelse av histamin i vominnhold fra sau er blitt bestemt med følgende resultater:

1. Fôring medførte alltid en økning av histaminkonsentrasjonen i rumen og stigningen var relatert til histamininnholdet i fôret.

2. En liten dannelse av histamin syntes å finne sted i vominnhold både *in vivo* og *in vitro* etter fôring med høy eller kraftfôr.

3. *In vitro* dannelsen av histamin i vomsaft ble sterkt øket ved tilsettning av L-histidin (10 mg/ml). Dannelsen av histamin varierte sterkt fra dag til dag og var videre avhengig av hvilket tidspunkt etter fôringen vominnholdet var aspirert.

4. Pyridoxal-5-fosfat økte *in vitro* dannelsen av histamin i vominnhold.

(Received September 19, 1966).