The presence of a bile alcohol of the cyprinol type is in agreement with the view (e.g. Romer, 1945) that the coelacanth, although now marine, had a long history of freshwater life, for this kind of bile alcohol has so far been found only in fishes whose evolution is believed to have taken place in fresh water.

SUMMARY

1. Dioxan-trichloroacetic acid cleavage of the bile salts of the coelacanth, *Latimeria chalumnae* Smith, gave as principal product latimerol (3 β ,7 α ,12 α ,26,27-pentahydroxycholestane), a little cyprinol (3 α ,7 α ,12 α ,26,27-pentahydroxycholestane) and a very small proportion of a third (unidentified) bile alcohol. Alkaline hydrolysis of the bile salts gave a little latimerol, but chiefly anhydrolatimerol and a 'bile acid' fraction not characterized.

2. Anhydrolatimerol on mild chromic oxidation was converted into dehydroanhydrocyprinol, similarly obtained from anhydrocyprinol.

3. Allomerization (at C-5) of ethyl 7 α ,12 α -dihydroxy-3-oxocholanoate with Raney nickel in cumene, followed by reduction with sodium borohydride in pyridine, led to the isolation of the digitonin-precipitable ethyl 3 β ,7 α ,12 α -trihydroxyallocholanoate, which was converted by hydrolysis and chromic oxidation into 3,7,12-trioxoallocholic acid. The infrared-absorption spectrum of ethyl 3 β ,7 α ,12 α -trihydroxyallocholanoate showed all the principal bands between 9.0 and 15.0 μ given by latimerol.

4. The bile salts of *Latimeria* are thus shown to be (a) the most primitive (i.e. the nearest chemically to

cholesterol) of any so far examined, and (b) to be indicative (by virtue of their being of the cyprinol chemical type) of a freshwater history for the coelacanth.

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The Formation and Distribution of Methylamine in the Ruminant Digestive Tract

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Although it has long been known that ammonia is produced during ruminal fermentation (Mangold & Schmitt-Kramer, 1927; Lenkeit & Becker, 1938; Wegner, Booth, Bohstedt & Hart, 1941; Harris, Work & Henke, 1943), it was not until McDonald (1948) demonstrated its absorption from the rumen and subsequent return as urea in the saliva that the

significant role of ammonia in rumen metabolism was fully appreciated. Since that time the formation, absorption and utilization of ammonia have received a great deal of attention, and it is now well established that ammonia is of major importance in the nitrogen metabolism of the ruminant animal.

In most of the previous work ammonia was estimated by the distillation of rumen liquor under alkaline conditions followed by acid-base titration,

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and was in fact an estimation of total volatile base, the usual methods being modifications of that of Conway (1947).

During an investigation on amino acids present in the rumen, by using chromatography on sulphonated polystyrene resin by the method of Spackman, Stein & Moore (1958), an unknown base was found to be normally present in the rumen contents. The base was eluted immediately after ammonia, was volatile and was subsequently identified as methylamine, and, although its concentration in rumen contents was relatively low, further studies appeared to be warranted in view of the established importance of its lower homologue, ammonia. A point of practical significance that emerged during this work was that methylamine is estimated as ammonia in the conventional analytical techniques and may lead to appreciable over-estimates of rumen ammonia concentrations.

METHODS AND MATERIALS

Experimental animals

Three adult Clun Forest and one Soay sheep with permanent cannulated rumen fistulae were used. The cannula was a modification of that described by Jarrett (1948). Sheep 1 also had a permanently cannulated fistula of the duodenum placed immediately caudal to the pyloric sphincter; samples collected from this fistula were considered to be abomasal digesta. Rumen contents were also obtained from four Shorthorn $\times$ Hereford steers with permanent rumen fistulae (Harrison, 1961).

The sheep were kept indoors and given a ration of crushed oats (200 g.) and chopped hay (800 g.) once daily.

For the greater part of the year the cattle were housed indoors and given a variety of rations at 8.30 a.m. and 4.30 p.m. daily. After a change in diet, at least a week was allowed to elapse before experiments were carried out. During the summer months they were out at pasture but were brought indoors and placed on the indoor feeding regime before experiments were done. Water was available to all animals *ad libitum*.

Chemical methods

Total volatile base. Centrifuged rumen fluid (2–5 ml.) was vacuum-distilled with saturated potassium metaborate solution (10 ml.) in an apparatus similar to that described by Pucher, Vickery & Leavenworth (1935). The distillation flask was immersed in a water bath at 45°, and the receiving flask, which contained 5 ml. of 0.0143 N-HCl with Tashiro's indicator (Conway, 1947), was cooled in ice. A slow stream of air was bubbled through the apparatus and quantitative distillation of ammonia and methylamine was obtained in 15 min. The acid in the receiver was back-titrated with 0.0143 N-NaOH (carbonate-free), and the total volatile base calculated.

After titration the distillate was acidified and used for the separate estimation of ammonia and methylamine.

Ammonia and methylamine. An automatic amino acid analyser with a 30 cm. $\times$ 0.9 cm. column of 8% cross-linked

sulphonated polystyrene resin (30 μ diam. beads) was used. The apparatus was similar to that described by Spackman *et al.* (1958), except that the column effluent and the ninhydrin reagent were metered in 1 ml. volumes by a syringe unit before entering the heating coil. Extinctions were measured at 570 m μ with 3 or 1.5 mm. of fluid depth in the colorimeter tube.

The ninhydrin reagent was a modification of that described by Moore & Stein (1954), potassium cyanide being used instead of hydriindantin (Troll & Cannan, 1953; Yemm & Cocking, 1955; Chibnall, Mangan & Rees, 1958). Ninhydrin (10 g. in 750 ml. of 2-methoxyethanol) was mixed with 250 ml. of 4M-sodium acetate buffer, pH 5.5, and layered with 2–3 cm. of liquid paraffin B.P. Then 1 ml. of 0.01M-KCN was added through the paraffin layer and the reagent mixed, the inclusion of air bubbles being avoided, and stored away from light.

The ion-exchange resin was prepared in the form of micro-beads by a slight modification of the method of Pepper, Paisley & Young (1953). Before polymerization the styrene-divinylbenzene-benzoyl peroxide mixture was dispersed in the aqueous CM-cellulose phase by using a top-drive macerater (Townson and Mercer Ltd.), care being taken to prevent air being sucked into the mixture. The dispersion was followed microscopically and continued until most of the globules were 10–20 μ in diameter. The suspension was stable and did not aggregate during polymerization at 80°. Subsequent sulphonation (Topp & Pepper, 1949) caused an increase of about 50% in bead diameter. The silver catalyst was effectively removed from the resin by several washes with 1N-HNO₃. The purified beads were fractionated for size by the hydraulic method of Hamilton (1958).

The column was eluted with 0.35M-sodium phosphate buffer, pH 7.7, at 50°. The displacement volume for ammonia was 63 ml. and that for methylamine was 81 ml. At a flow rate of 40 ml./hr. ammonia emerged after 94 min. running time and methylamine was completely eluted after 135 min. The only bases detected by ninhydrin in the distillates were ammonia and methylamine, and as the column required no regeneration it was possible to carry out four analyses over a period of 9 hr. Integration of the peaks was carried out as described by Spackman *et al.* (1958). With this method it was possible to determine as little as 1 μ g. of ammonia N or methylamine N in the presence of a large excess of the other. The normal loading of the column was 50–100 μ g. of N.

When large quantities of methylamine were present, the ammonia in the total-volatile-base distillates was absorbed on yellow mercuric oxide as described by Pugh & Quastel (1937), and the methylamine in the supernatant estimated as described for total volatile bases.

Polyethylene glycol. This was estimated by the turbidimetric method of Hyden (1955).

RESULTS

Identification of methylamine. (a) Chromatography. The base was chromatographed on sulphonated polystyrene resin with 0.35M-sodium phosphate buffer, pH 7.7, at 50° on a 20 cm. column, and with 0.35M-sodium citrate buffer, pH 4.25, at 30° on a high-resolution 50 cm. column.

The base was eluted from both columns in the same position as methylamine, i.e. between ammonia and ethylamine.

(b) Preparation. Freshly withdrawn bovine rumen fluid was cooled with ice and centrifuged at 10000g for 30 min., and 2 l. of the cell-free liquor adjusted to pH 10 with sodium hydroxide and steam-distilled into dilute hydrochloric acid. The distillate was evaporated to dryness *in vacuo*. The mixed hydrochlorides were extracted with anhydrous methanol, in which ammonium chloride is sparingly soluble. After enrichment by several extractions the final product contained more than 30 % of the unknown.

Enriched hydrochloride (25 mg.) was dissolved in 15 ml. of water, 5 ml. of saturated sodium bicarbonate solution and 40 ml. of ethanol. 1-Fluoro-2,4-dinitrobenzene [2 ml. of a 10 % (w/v) solution in ethanol] was added and allowed to react for 1 hr. at room temperature. Aq. 10 % (w/v) glycine (5 ml.) was then added and allowed to react with the excess of fluorodinitrobenzene for 1 hr. This reaction mixture, after the addition of 5 ml. of 10 % (w/v) sodium carbonate solution, was extracted three times with benzene, and the benzene layer was then washed with water, dried with anhydrous sodium carbonate and concentrated *in vacuo* to small volume. The concentrate was chromatographed on a column (4 cm. × 15 cm.) of alumina ('aluminium oxide M.F.C.'; 100–200 mesh; Brockman activity I–II; Hopkin and Williams Ltd.), with benzene–0.5 % (v/v) ethanol as eluent. Relatively small amounts of dinitrophenol and dinitroaniline were present, and the fraction containing the main product was evaporated to dryness *in vacuo* and crystallized from ethanol. Yellow needle-shaped crystals were obtained, m.p. 174.5–175.5° (uncorr.) unchanged by admixture with authentic *N*-methyl-2,4-dinitroaniline (Found: C, 42.0; H, 3.9; N, 21.6. Calc. for C₇H₇N₃O₄: C, 42.6; H, 3.6; N, 21.3 %).

Paper chromatography with butanol–acetic acid–water (4:1:5, by vol.) gave one spot, *R_F* 0.88, identical with that of *N*-methyl-2,4-dinitroaniline.

Concentration in the rumen. Ammonia and methylamine concentrations in the rumen contents of sheep and cattle receiving different diets were estimated by the chromatographic method. The samples were collected at random regardless of the feeding times of the animal. The results are shown in Table 1.

Production of ammonia and methylamine during feeding. The rate of formation of ammonia and methylamine was studied in the rumen of sheep and cattle housed indoors and receiving normal maintenance diets.

(a) Sheep 1, which had permanent rumen and duodenal cannulae, received 800 g. of hay chaff and 200 g. of crushed oats once daily and usually ate the whole of this ration within 2 hr. Samples of rumen and duodenal contents were collected at intervals throughout a 24 hr. period and were immediately cooled in ice and centrifuged at 10000g to obtain a clear liquor for analysis.

In the rumen, the ammonia concentration increased markedly during the feeding period, declined to a minimum 6–8 hr. later, and during the ensuing 16–18 hr. gradually increased to the pre-feeding level (Fig. 1). The ammonia concentration in the abomasal contents followed a similar pattern. In contrast, the methylamine concentration in both the rumen and abomasal contents rose steadily for 6–8 hr. after the commencement of feeding and then rapidly decreased; 5 hr. later methylamine was absent from the contents of both organs and remained so for the rest of the 24 hr. period. In both the ruminal and abomasal contents the maximal concentration of methylamine was found when the ammonia concentration was minimal.

(b) Steer 1, with a permanent rumen cannula, was maintained on a daily ration of dried lucerne meal (3 lb.), beet pulp (2.5 lb.) and mangolds

Table 1. *Ammonia and methylamine concentrations in the rumen contents of cattle and sheep on various diets*

Experimental details are given in the text.			
Animal	Diet (daily ration)	Concn. of ammonia (mg. of N/l.)	Concn. of methylamine (mg. of N/l.)
Shorthorn × Hereford steer 2	Diet A [dried lucerne meal (3 lb.) + dried beet pulp (2.5 lb.) + mangolds (10 lb.) + hay (5 lb.)]	86.2	0.0
Shorthorn × Hereford steer 2	Diet A	15.0	13.6
Shorthorn × Hereford steer 4	Diet A	8.8	13.5
Shorthorn × Hereford steer 3	Free grazing at pasture	26.2	10.1
Shorthorn × Hereford steer 3	Long hay	17.7	3.1
Shorthorn × Hereford steer 1	Long hay	34.4	1.7
Clun Forest sheep 1	Hay chaff (1200 g.) + crushed oats (200 g.)	96.1	13.1
Clun Forest sheep 3	Hay chaff (1000 g.) + crushed oats (100 g.)	60.7	4.4
Soay sheep 4	Hay chaff (400 g.)	128.5	16.5

(10 lb.) divided into two feeds per day. The ammonia and methylamine concentrations in the rumen contents were determined as in the sheep experiment. The results (Fig. 2) agreed closely with those obtained in the sheep, the methylamine peak occurring some time after feeding, by which time the ammonia concentration had fallen to a low level.

Effect of glucose on the rate of disappearance of ammonia and methylamine from the rumen. (a) Sheep 2 was kept without food overnight and three control samples of rumen contents were withdrawn at intervals the following morning. There was no methylamine in these samples. Then 2.41 g. of methylamine hydrochloride and 12.5 g. of polyethylene glycol (mol.wt. 4000) in 250 ml. of water were administered through the rumen cannula and three further samples of rumen digesta were taken at hourly intervals. Then 50 g. of glucose in 250 ml. of water was added to the rumen and two

further samples were taken. The polyethylene glycol was used as a reference substance, and by extrapolation it was possible to calculate the volume of fluid in the rumen at the start of the experiment thus:

$$(V + v)x = 12.5$$

where V was the rumen volume, v the volume of the polyethylene glycol solution added and x the concentration of polyethylene glycol at zero time as obtained by extrapolation. The calculated rumen volume was 4.56 l., and hence the estimated concentration of methylamine in the rumen contents at zero time was 10.9 mg. of N/100 ml. Fig. 3 (a) shows that methylamine disappeared from the rumen at a faster rate than did the inert marker substance. The addition of glucose caused an immediate fall in the ruminal ammonia concentration, but did not accelerate the rate of disappearance of methylamine.

(b) Steer 2 was kept without food overnight and a control sample of rumen liquor withdrawn the following morning. Then 11.3 g. of methylamine hydrochloride in 1 l. of water was administered through the rumen cannula and four samples were withdrawn at timed intervals (Fig. 3b). Then 500 g. of glucose in 1 l. of water was added to the rumen and three further samples of rumen liquor were taken at hourly intervals.

Fig. 1. Concentrations of ammonia (□) and methylamine (○) in the rumen (—) and duodenum (---) of a sheep over a normal 24 hr. period. A, Feed. Experimental details are given in the text.

Fig. 2. Concentrations of apparent ammonia N (total volatile base) (●) and methylamine N (○) in the rumen of a steer as a result of feeding. A, Feed. Experimental details are given in the text.

Fig. 3. (a) Effect of glucose on the concentrations of ammonia N (●) and methylamine N (○) in the rumen of a sheep. Polyethylene glycol (□) was used to measure the rumen volume. A, Methylamine + polyethylene glycol added; B, glucose added. (b) Effect of glucose on the concentrations of ammonia N (●) and methylamine N (○) in the rumen of a steer. A, Methylamine added; B, glucose added. Experimental details are given in the text.

As in the sheep experiment the addition of glucose to the rumen resulted in a marked increase in the rate of disappearance of ammonia from the rumen but had no effect on the rate of disappearance of methylamine.

Effect of soluble protein on the production of ammonia and methylamine in the rumen. Steer 3 was kept without food overnight and then given a ration of dried lucerne meal (1.5 lb.), beet pulp (1.25 lb.) and mangolds (5 lb.). Samples of rumen contents were withdrawn at intervals for 4.75 hr. Then 100 g. of soluble casein in 1 l. of water was administered through the rumen cannula and samples were taken for a further 3.5 hr. In agreement with experiments described above, ammonia was produced rapidly during feeding and thereafter fell to a low value (Fig. 4). After the addition of casein there was rapid production of ammonia, the increase in concentration being approximately the same as that which occurred after feeding. The methylamine concentration in the rumen fluid increased steadily for 5 hr. after the commencement of feeding and then decreased almost to zero in the remaining 3 hr. Casein was added when methylamine was judged, from a previous experiment, to be at maximum concentration, but it did not prevent the rapid disappearance of methylamine from the rumen contents.

Passage down the digestive tract. Methylamine hydrochloride (2.41 g.) and polyethylene glycol (12.5 g.) in 250 ml. of water were administered to sheep 2 through its rumen fistula and the animal was then fed. Samples of rumen content were taken at hourly intervals, and after 3 hr. a further 2.41 g. of methylamine hydrochloride in 250 ml. of water was added to the rumen; 30 min. later the animal was anaesthetized with an intravenous injection of pentobarbitone (Nembutal; Abbott Laboratories) and the abdominal viscera were exposed through a mid-line incision. Samples of portal and peripheral venous blood were removed and the animal was exsanguinated. The entire digestive tract was removed and digesta were collected from the rumen, omasum, abomasum, duodenum and jejunum, ileum, caecum, large colon and small colon. These were stored at -20° until analysed.

Polyethylene glycol was present in the contents removed from all parts of the digestive tract, indicating a rapid movement of digesta from the rumen to the large intestine (Table 2). Although work by Smith (1958) has indicated that polyethylene glycol estimations on digesta removed from the lower part of the digestive tract may be inaccurate, the rapid flow of digesta from the rumen in this experiment is confirmed by the loss of polyethylene glycol from the rumen, the concentration falling to one-half the original in 3 hr. (Fig. 5).

Methylamine was found in considerable amounts in the rumen, omasum and abomasum, with somewhat smaller amounts in the duodenum-jejunum and ileum (Table 2). Only a trace was present in the caecum and none in the large or small colon. Methylamine was also absent from the peripheral and portal venous blood and from the faeces. Polyethylene glycol:ammonia and polyethylene glycol:methylamine ratios showed that ammonia was produced in appreciable amounts in the upper part of the small intestine and in the caecum. There was no evidence that methylamine was produced anywhere except in the rumen.

Fig. 4. Production of ammonia N (●) and methylamine N (○) in the rumen of a steer as a result of feeding and the addition of soluble protein to the rumen. A, Feed; B, casein added. Experimental details are given in the text.

Fig. 5. Passage of methylamine down the digestive tract of a sheep. The concentrations of apparent ammonia N (total volatile base) (○), methylamine N (●) and polyethylene glycol (■) in the rumen before sampling the contents of the digestive tract are shown. A, Methylamine + polyethylene glycol added. Experimental details are given in the text.

Table 2. *Analysis of the contents of the digestive tract of a sheep after the administration of polyethylene glycol (12.5 g.) and methylamine hydrochloride (4.82 g.) to the rumen*

Experimental conditions are given in the text.

Organ	Concn. of polyethylene glycol (mg./100 ml.)	Concn. of ammonia		Concn. of methylamine	
		(mg. of N/100 ml.)	(mg. of N/100 mg. of polyethylene glycol)	(mg. of N/100 ml.)	(mg. of N/100 mg. of polyethylene glycol)
Rumen-reticulum	156	6.9	4.4	9.2	5.9
Omasum	192	17.5	9.1	9.6	5.0
Abomasum	164	11.2	6.8	5.1	3.1
Duodenum-jejunum	135	39.8	29.5	2.8	2.1
Ileum	232	27.3	11.8	2.4	1.0
Caecum	114	19.4	17.0	0.1	0.1
Large colon	138	22.0	15.9	0	0
Small colon	116	13.1	11.3	0	0

DISCUSSION

The presence in rumen contents of a volatile base, methylamine, which is not distinguished from ammonia by the conventional analytical methods, raises the question of the validity of much of the work reported in the literature insofar as rumen ammonia concentrations are concerned. Fortunately, however, under normal feeding conditions the methylamine concentration in the rumen does not usually exceed 15 mg. of N/l., and the value for rumen ammonia concentrations is not likely to be grossly in error. In the experiment illustrated in Fig. 1, which is typical for stall-fed sheep, the error in apparent ammonia concentration was only 2.5 % at peak concentration immediately after feeding, although 6 hr. later the error had increased to 16.8 %. This degree of error, although undesirable for quantitative work, would not invalidate any of the general principles now established for the metabolism of ammonia in the rumen. In certain circumstances, e.g. when excessive amounts of readily available carbohydrate are added to the diet and there is a marked depression in rumen ammonia concentration (McDonald, 1952; Annison, Chalmers, Marshall & Synge, 1954), the true ammonia concentration might be overestimated by as much as 50 %.

The behaviour of methylamine in the rumen, where it builds up to a maximum concentration and then disappears within a few hours, is typical of an active intermediary metabolite and may be compared with the transient appearance of free amino acids at low concentration in the rumen when protein is fed to the animal. There is, however, very little information available on the biochemical reactions of methylamine, and it is not possible to evaluate its role in rumen metabolism at the present time. Although glycine can be decarboxylated by *Pseudomonas fluorescens* to yield methylamine (Emerling & Reiser, 1902), Gale (1940) has shown

that decarboxylation of amino acids by bacteria to produce amines occurs only in acid media, at pH values lower than those normally found in the rumen.

It is possible that methylamine may be converted into formic acid in the digestive tract, since Schievelbein & Werle (1957) have shown that this occurs in the liver of cattle and rabbits. Appreciable amounts of formic acid are found in the blood of ruminants and in the contents of the small intestine (Annison, 1954). Moreover, though only trace amounts of formic acid are present in rumen digesta (Annison, 1954), methylamine is present in greater concentration in the fore-stomach. It would appear therefore that, if methylamine is one of the precursors of formic acid in the ruminant, the major site of conversion must be the small intestine.

Although methylamine is produced in the rumen of animals given a variety of diets, it has been shown that protein is probably not the direct source of methylamine, since soluble casein, when added to the rumen, produced large quantities of ammonia but not methylamine. Moreover, since the addition of glucose accelerated the utilization of ammonia but not of methylamine by the rumen micro-organisms, it would appear that the origin and metabolic pathways of the two bases are different.

SUMMARY

1. Methylamine has been found in the rumen fluid of sheep and cattle in amounts as high as 16 mg. of N/l., and identified by chromatography and conversion into *N*-methyl-2,4-dinitroaniline.

2. Methylamine appeared in the rumen after a variety of diets had been given, and disappeared completely some hours later.

3. As with ammonia, part of the methylamine was metabolized in the rumen, but unlike ammonia the rate of utilization was not increased by the addition of glucose.

4. Large quantities of ammonia but not methylamine were produced when soluble casein was added to the rumen. The maximum methylamine: ammonia ratio found was 1.53.

5. Methylamine was present in the contents of the digestive tract as far caudal as the ileum, but unlike ammonia did not appear to be produced in any region except the rumen.

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Fractionation of the Fibroin of *Bombyx mori* with Trypsin

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It is now well established that hydrolysis of dissolved *Bombyx mori* fibroin with chymotrypsin gives rise to an insoluble precipitate and a mixture of soluble peptides. The precipitate represents about 60 % of the protein by weight and is composed of sequences of amino acid residues in which glycine alternates with serine and alanine (Lucas, Shaw & Smith, 1957). The mixture of soluble peptides has also been shown to contain a substantial proportion of peptides in which glycine residues occupy alternate positions and are associated with residues of alanine, valine and tyrosine (Lucas, Shaw & Smith, 1962). Thus enzymic hydrolysis with chymotrypsin has proved to be a fruitful approach in studies of the structure of the fibroin molecule.

Less attention has been paid to the action of other proteolytic enzymes on fibroin. The first account of the hydrolysis of a solution of fibroin by

a purified trypsin preparation was given by Drucker, Hainsworth & Smith (1953). They obtained a 'tryptic precipitate', which they said contained 70 % of the total protein nitrogen, and a mixture of soluble peptides with a mean chain length of 5.0. Later, Waldschmidt-Leitz & Zeiss (1955) in a similar experiment found that the precipitated material represented 80 % (by weight) of the fibroin and that the soluble peptides had a mean chain length of about 7.

More recently, an account of a series of experiments by Zuber (1961) described the hydrolysis of a solution of fibroin by crystalline trypsin in the presence of hydrocinnamic acid, and recorded that 83 % of the protein nitrogen was precipitated in the form of a gel, while the peptides remaining in solution had a mean chain length of 31. Zuber (1961) investigated the gelatinous precipitate by hydrolysing it further with chymotrypsin, and