XCI. THE AMIDE NITROGEN OF CASEINOGEN.

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THE work of Kühne [1877] and of many subsequent investigators has established the fact that the digestion of proteins with aqueous extracts of the pancreas, pancreatic juice, or commercial trypsin preparations fails to give complete hydrolysis. The observation of Fischer and Abderhalden [1903], that the proline and phenylalanine of caseinogen, haemoglobin, fibrin, egg albumin, and other proteins occur in a trypsin-stable complex, demonstrated in addition to the above a marked specificity in the action of trypsin. This conception of specific proteolysis was strengthened by later work of the same authors [1905] in an investigation of the action of trypsin on many synthetic peptides.

Very few observations have been made on the liberation of ammonia in the enzymic hydrolysis of proteins. The results of work on acid hydrolysis favour the assumption that the precursors of the ammonia are mainly amides of the dicarboxylic acids. The evidence on this point has been collected by Osborne [1924] and requires no repetition here. It need only be said that the general evidence is based on the rapid liberation of ammonia in the acid hydrolysis of proteins, on the absolute amounts of dicarboxylic acids and ammonia thus set free, and on the presence of asparagine and glutamine in the juices of plants.

Further light on the source of ammonia in the hydrolytic products of proteins is obtained from the following considerations:

(1) Maximum production of amino nitrogen is obtained by hydrolysis with 3 *N* hydrochloric acid for 1½ hours at 150°. Increase of temperature increases the ammonia at the expense of amino groups [Henriques and Gjaldbæk, 1910; Van Slyke, 1912].

(2) The additional ammonia obtained by prolonged acid hydrolysis at 150° does not arise from tryptophan [Andersen and Roed-Müller, 1915] or arginine, but partially from cystine [Henriques and Gjaldbæk, 1910].

(3) More ammonia is liberated by peptic digestion than by tryptic digestion. This is mainly due to the high acidity of the peptic digest [Henriques and Gjaldbæk, 1911, 1912].

(4) Uramino acids are not hydrolysed by trypsin but are readily split by acid hydrolysis. Their presence in the products of enzymic hydrolysis of proteins has not been demonstrated [Andersen and Roed-Müller, 1915; Lippich, 1914].

(5) Levites [1904] could find no free CONH_2 groups in caseinogen.

(6) An imide linkage R-CO-NH-CO-R is improbable because of its resistance to acid hydrolysis.

Inasmuch as the ammonia nitrogen of proteins varies from 5 % to 30 % of the total nitrogen, it is obvious that ammonia is no mean constituent of the protein molecule and further evidence with respect to its association and behaviour is desirable. While engaged in some work requiring a digest of caseinogen, it was observed that prolonged hydrolysis of the protein with an aqueous extract of the pancreas failed to liberate the calculated amount of ammonia. It was therefore felt that a trypsin-stable residue might serve as a convenient material for investigation.

PART I.

In the experiments reported below, ammonia was estimated by rapid aeration for half an hour of a sample saturated with anhydrous potassium carbonate [Van Slyke and Cullen, 1914], Amino nitrogen estimations were made with the macro-apparatus of Van Slyke [1911, 1], and total nitrogen estimations by the Kjeldahl method. Clark and Lubs' buffers and indicators and the Cole-Onslow comparator were used in adjustments of the hydrogen ion concentration. Acid hydrolysis was effected by diluting if necessary to a convenient volume (25 cc. minimum) and refluxing for 16 hours with an equal volume of concentrated hydrochloric acid. The hydrolysate was evaporated to dryness *in vacuo* and subsequent determinations made upon the aqueous solution of the residue. If only the acid hydrolysable ammonia were to be determined it was found convenient to place in combustion tubes, $6'' \times 1''$, 5 cc. samples of suitable concentration and 0.5 cc. of concentrated sulphuric acid. Hydrolysis was then effected by autoclaving at 10 lbs. pressure. After two hours the tubes were removed. To each was added from a wide-mouthed pipette 5 cc. of a warm baryta solution (containing 2.95 g. of crystalline baryta, $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$, per 5 cc.) approximately to neutralise the sulphuric acid contained in the sample. The tubes were thoroughly cooled and the ammonia estimation proceeded with in the usual manner without transfer of the contents.

The enzyme solution used was a weak alcoholic (15 %) extract of fresh minced pig pancreas. Toluene alone was used as an antiseptic.

The liberation of ammonia in the tryptic hydrolysis of caseinogen.

The results of a typical experiment are given in Table I. Digestions were allowed to continue until the free ammonia content became constant and failed to increase after the addition of more enzyme solution.

It will be observed that the ratio Total ammonia/Total nitrogen is constant and in agreement with accepted values for the ammonia content of caseinogen, viz., 10.6 % to 10.7 % of the total nitrogen.

Table I.

300 g. caseinogen + 15 cc. toluene + 150 cc. enzyme solution + 20 g. sodium carbonate + water to 2 litres

Days		Decimilligrams nitrogen per cc. of filtrate				Amino N Total N	Total NH ₃ Total N	Free NH ₃ Total NH ₃	Adjustment to <i>p</i> _H 7.8. cc. added to whole digest
		Total N	Free amino N	Free am- monia	Total am- monia				
0.2	Volume made up to 2 litres	75	16.5	—	—	0.220	—	—	108.6 N soda
1	"	131	—	—	—	—	—	—	123.0 "
2	"	152	55.2	—	—	0.363	—	—	58.0 "
3	"	138	55.9	—	—	0.405	—	—	24.1 "
5	"	136	65.2	—	—	0.480	—	—	0.0 "
7	"	133	67.0	—	—	0.503	—	—	4.2 N HCl
13	"	127	—	7.20	13.4	—	10.55	0.537	8.3 "
	100 cc. enzyme sol. volume made up to 2 litres								
20		133	72.0	8.30	14.3	0.541	10.75	0.581	—
29	Volume made up to 2 litres	113	62.0	7.90	12.4	0.549	10.95	0.636	6.2 N HCl
	100 cc. enzyme solution								
40		105	59.0	7.50	11.1	0.561	10.55	0.675	—
44		106	59.3	7.50	11.3	0.559	10.65	0.663	—

The titration values are of some interest. Assuming that all the phosphorus in caseinogen (0.85 %) is present as orthophosphoric acid, 140 cc. of normal alkali would be required for complete neutralisation of the phosphoric acid in 300 g. of caseinogen. The sodium carbonate initially added is equal to 215 cc. of normal alkali. It would therefore appear that in the early stages of tryptic hydrolysis, the substances liberated were mainly neutral and acid ampholytes. In the later stages of hydrolysis, the tendency of the solution to become more alkaline than *p*_H 7.8 would seem to indicate a relatively great liberation of the hexone bases.

For the sake of brevity, only the final ratios Free ammonia/Total ammonia have been given in Table II.

Table II.

Digest 2		18 days	Free NH ₃ Total NH ₃
"	3	12 "	0.643
"	4	56 "	0.658
"	5	50 "	0.646
"	6	50 "	0.667
"	1	44 "	0.679
			0.663

While the time required for the completion of ammonia production is subject to much variation—the relative constancy of the final ratio is obvious.

Two possible causes of this failure to attain complete hydrolysis of the ammonia precursors might be presented: first, depression of the point of equilibrium by the accumulating end products of tryptic hydrolysis and inactivation of the enzyme, or secondly, the liberation of a trypsin-stable residue. The first possibility is suggested by the work of Abderhalden and Gigon [1907]. In studying the effect of many amino acids upon the cleavage of *d*-alanyl-

d-alanine and glycyl-*l*-tyrosine by pressed yeast juice, they observed marked inhibition by *dl*-leucine, *dl*-valine, and *d*-glutaminic acid—particularly the last. Northrop [1921] in a series of investigations on the inactivation of trypsin, observed the time required to effect a fixed change in the conductivity of a gelatin-trypsin solution by:

- (1) trypsin-hydrolysed gelatin and caseinogen;
- (2) acid-hydrolysed caseinogen;
- (3) alkali-hydrolysed caseinogen.

No inhibition was observed with the totally hydrolysed protein (2) and (3). Positive results were obtained with the trypsin-hydrolysed products.

The second possibility has already been referred to on page 679.

The results recorded in Table III indicate that the failure of trypsin completely to hydrolyse the ammonia precursors in caseinogen, is due not to inactivation of the enzyme or depression of the point of equilibrium, but to the specific nature of the substrate. The tryptic digestion of preparations 9 and 10, each of 1200 g. caseinogen was allowed to continue until the free ammonia content failed to be increased by the addition of fresh quantities of enzyme solution. The digests were then filtered, neutralised to p_H 7.0 and concentrated *in vacuo* at 35°–40° to a volume of 1200 cc. The amino acids of lower solubility which separated out during concentration were filtered off. The final syrup was warmed to 60° and precipitated with four volumes of warm 95 % alcohol. The deeply pigmented oil which immediately settled out was run off through a separating funnel. By this procedure, about one half of the trypsin-stable residue was precipitated. This was dissolved in water and reconcentrated *in vacuo* for the removal of alcohol. The concentrate was finally taken up in water, diluted to five litres, adjusted to p_H 7.8 and re-digested with an activated aqueous extract of the pancreas. As is shown in Table III, no increase in the free ammonia content, and hence no further hydrolysis of the ammonia precursor took place.

Table III. *Redigestion of the alcoholic precipitate.*

Days	Free ammonia	
	Digest 9	Digest 10
0	2.30	3.65
1	2.35	3.60
3	2.37	3.65
5	Fresh enzyme added	Fresh enzyme added
6	—	3.60
7	—	3.70
10	2.35	—

It is therefore concluded that in the tryptic digestion of caseinogen a portion of the ammonia representing approximately one-third of that which may be obtained by total acid hydrolysis, fails to be liberated by the enzyme. The residue isolated after digestion totally resists tryptic hydrolysis (of the ammonia precursor) and in consequence is considered to be structurally different from the trypsin-hydrolysed portion.

PART II.

Isolation Experiments.

The results of many preliminary isolation experiments may be briefly summarised.

1. Phosphotungstic acid quantitatively precipitated the trypsin-stable precursor of the ammonia. The simultaneous precipitation of the hexone bases and the acidity required for its use, were serious limitations. The proline-phenylalanine compound of Fischer and Abderhalden [1903] and possibly other undesirable peptides were also precipitated by this reagent.

2. Alcoholic mercuric chloride gave quantitative precipitation at p_H 7.0 of the ammonia precursor, but the increasing acidity during subsequent treatment with hydrogen sulphide was a source of trouble. The simultaneous precipitation of the hexone bases was also an undesirable feature.

3. Prolonged fractional precipitation with both aqueous and alcoholic mercuric chloride gave no separation.

4. The combined use of lead acetate and alcohol [Mack, 1904] failed to give quantitative precipitation and effected little additional purification if any (see Table V).

5. The known low solubility of copper aspartate and glutamate and the great solubility of the copper salts of polypeptides [Fischer, 1906], led to the use of barium hydroxide [see Kober and Sugiura, 1913], and alcohol as precipitants of the copper salts. The results indicated that no free dicarboxylic acids were contained in the material that had been first precipitated by lead acetate and alcohol. Methods 4 and 5 were each capable of giving a yield of only 30 % to 50 % of the calculated amount. In consequence the use of these in sequence involved a great loss of material.

It was however found that three to seven volumes of 95 % ethyl alcohol would precipitate 50 % to 70 % of the trypsin-stable ammonia precursor. The advantages of its use are that the precipitant may be readily removed, that by precipitating at p_H 6.0–7.0, the free monoamino-monocarboxylic acids (all of which are undissociated in this range) are not precipitated, and that by precipitating with warm alcohol, the precipitate at once separates as a deeply pigmented oil which may be readily extracted at different acidities with ethyl alcohol of various concentrations.

Alcoholic precipitation and extraction.

The redigested solutions from digests 9 and 10 (see p. 682) were neutralised. To each was added a 50 % solution of normal lead acetate until a precipitate ceased to form. This procedure undoubtedly precipitates a portion of the ammonia precursor, but is desirable for clearing the solution. The filtrates were treated with hydrogen sulphide for the removal of lead, filtered free of lead sulphide, and concentrated *in vacuo* for the removal of hydrogen sulphide.

The partially concentrated filtrates were neutralised with soda and again concentrated to a final volume of 1000 cc. Four volumes of warm 95 % alcohol were added. The precipitated syrups of 300–350 cc. were then extracted as follows:

1. With 90 % alcohol—after adding increasing amounts of sodium hydroxide solution.

2. At p_H 7.0 with ethyl alcohol of decreasing strength.

The initial precipitations at neutrality with ethyl alcohol will have given a material free from the monoamino monocarboxylic acids. By the first set of extractions, it was hoped that residual free amino acids, if any, with alcohol-soluble sodium salts (*e.g.* histidine) would be removed. In the second set of extractions, the removal of any free amino acids would be facilitated by the increasing dilution. This procedure is also effective in totally removing the free ammonia. The extractions were effected by rapid shaking in a mechanical shaker in the order shown. Acetic acid was used in acidifying from p_H 11.0 to 7.0, because of the solubility of sodium acetate in alcohol.

Table IV.

	Extraction time	Alcoholic extractive		p_H of solution	Total N	Percent. of total N	
		Volume cc.	Conc. %			Free amino N	Total amino N
1 a	15 minutes	150	90	7.0	100	53.0	78.0
b	"	"	"	"			
c	"	"	"	"			
d	"	"	"	"			
2 a	15 minutes	150	90	9.0			
b	"	"	"	"			
c	"	"	"	"			
d	"	"	"	"			
3 a	15 minutes	150	90	11.0			
b	"	"	"	"			
c	"	"	"	"			
d	"	"	"	"			
4 a	15 minutes	150	95	7.0	100	54.3	79.3
b	"	"	"	"			
c	"	"	"	"			
d	"	"	"	"			
5 a	15 minutes	150	90	7.0			
b	"	"	"	"			
c	"	"	"	"			
d	"	"	"	"			
6 a	15 minutes	150	85	7.0	100	54.3	79.3
b	"	"	"	"			
c	"	"	"	"			
d	"	"	"	"			
7 a	15 minutes	150	80	7.0			
b	"	"	"	"			
c	"	"	"	"			
d	"	"	"	"			

In digest 10 the combined extracts 4 and 7 each of 600 cc. were concentrated to 100 cc. Total nitrogen, free amino nitrogen and total amino nitrogen were estimated as shown.

In Table V are given the results of nitrogen estimations on

1. The extraction product from digest 9.
2. The extraction product from digest 10.
3. The product obtained from digest 4 by precipitating with lead acetate and alcohol.
4. The material obtained by twice precipitating with alcohol the calcium salt of the extraction product from digest 9 and subsequently removing the calcium with oxalic acid.

Table V.

	Total N	Free amino N	Total amino N
1. Extraction product (9)	100	52.5	82.5
2. Extraction product (10)	100	53.3	80.9
3. Lead acetate with alcohol (4)	100	49.0	82.0
4. Calcium salt of (1)	100	50.9	82.5

The nitrogen estimations as given in Table IV and V indicated sufficient constancy in this product of prolonged tryptic digestion of caseinogen to justify a total analysis of the product.

All preparations gave a very marked biuret test.

Analysis. 103 g. of the clear syrupy extraction product 10, representing 40 grains of material dried at 110°, were dissolved in 225 cc. of water; 75 cc. of concentrated sulphuric acid were added. After 24 hours at 100°, the biuret test was negative and hydrolysis was considered to be complete. The solution was diluted with two volumes of water. A warm saturated solution of recrystallised baryta was added until distinctly alkaline to phenolphthalein. Ammonia was removed and estimated by vacuum distillation into standard acid. After two hours' distillation, the excess baryta was neutralised to p_H 7.0 by the addition of sulphuric acid. The barium sulphate was removed by filtration and three times extracted by shaking with one litre of boiling water. The combined neutral filtrates were concentrated to 100 cc.

Continuous extraction with butyl alcohol, as recommended by Dakin [1918], was used for removal of proline and the monoamino monocarboxylic acids. The extraction was effected under diminished pressure to inhibit anhydride formation. The butyl alcohol rapidly became light brown in colour but only a very slight separation of amino acids appeared on the flask at the butyl alcohol surface. The extraction was continued for 30 hours.

The extracted aqueous solution was concentrated *in vacuo* for the removal of butyl alcohol. The residual syrup was dissolved in water and diluted to 200 cc.

The hexone bases were determined by precipitation of a 10 cc. portion with phosphotungstic acid in the usual manner. The washed precipitate was decomposed by shaking with washed amyl alcohol and ether. The individual bases were estimated by the partition method of Van Slyke [1911, 2].

The 190 cc. residue was saturated at -5° with dry hydrogen chloride. The glutaminic hydrochloride was filtered off on asbestos after 24 hours at 0° and washed with 75 cc. of cold concentrated hydrochloric acid. The filtrate and washings were concentrated *in vacuo* to 100 cc. and resaturated as above with hydrogen chloride. After 24 hours the second crop of glutaminic hydrochloride, which was very small in quantity, was filtered off. The combined hydrochloride fractions were dissolved in 90 cc. of water and purified by reprecipitation with hydrogen chloride. The hydrochloride was filtered off after 24 hours and washed with cold absolute alcohol and ether.

The combined filtrates from the glutaminic hydrochloride were evaporated *in vacuo* to a syrup. The residue was dissolved in water, and distilled *in vacuo* with an excess of pure calcium hydroxide. A very small amount of ammonia which escaped the previous alkaline distillation was caught in standard acid and estimated. The excess calcium hydroxide was filtered off and the filtrate and washings were concentrated to 100 cc. The calcium salts were precipitated with alcohol according to the method of Foreman [1914]. The precipitated calcium salts were dissolved in water. The calcium was exactly removed with oxalic acid.

Aspartic acid was separated as the lead salt [Dakin, 1918] and estimated as the copper salt. The solution free from calcium was heated on a water-bath with an excess of freshly precipitated and well washed lead hydroxide. After 15 hours the lead precipitate was filtered off and washed. It was decomposed by boiling with dilute sulphuric acid. Excess of the latter was removed with barium carbonate. The filtrate was concentrated and treated with hot saturated copper acetate. After a few days, deep blue aggregates of copper aspartate separated out.

The filtrate from the lead aspartate was treated with hydrogen sulphide for the removal of lead. The filtrate was freed from hydrogen sulphide, acidified to 5 % with sulphuric acid and precipitated with phosphotungstic acid. After 48 hours the voluminous precipitate was filtered off. Phosphotungstic and sulphuric acids were exactly removed from the colourless filtrate with baryta. Chlorides were removed with silver nitrate and nitric acid. The filtrate, free from chlorides, was made slightly alkaline to litmus with soda. The further addition of silver nitrate and soda until the completion of precipitation brought down a voluminous precipitate. This was suspended in water and treated with hydrogen sulphide for the removal of silver. The filtrate and washings from the silver sulphide were concentrated *in vacuo*. A few colourless crystals separated out after 24 hours. The residual solution of a few cc. was precipitated with alcohol and ether. The flocculent white precipitate was filtered off, dissolved in water and reprecipitated three times. The crystals and the ether precipitate melted at 198° and proved to be *dl*-glutaminic acid. No β -hydroxy-glutaminic acid could be found.

Neither the butyl alcohol-soluble fraction nor the fraction extracted by, but insoluble in, butyl alcohol was in sufficient quantity for analysis.

				Grams	Percent.
Glutaminic acid	...	...	...	21.80	54.50
Lysine	...	...	...	7.30	18.25
Ammonia	...	...	...	0.50	1.25
Arginine	...	...	...	1.55	3.88
Aspartic acid	...	...	...	0.59	1.48
Butyl alcohol solution	...	...	...	0.55	1.38
Butyl alcohol extracted	...	...	...	0.05	0.12
				32.34	80.90

The above analysis shows that if all of the ammonia (0.50 g.) that has been liberated by acid hydrolysis is associated with a single amino acid, then only glutaminic acid and lysine can be considered as parts of the trypsin-stable ammonia precursor. The arginine content is one-fourth of the amount that would be required for the existence of even equimolecular amounts of arginine and ammonia. For the purposes of this investigation, it would therefore appear that the arginine and aspartic acid were impurities.

Evidence will now be presented that this fraction of the ammonia is not associated with the lysine. A portion of the final extraction product from digest 4 was precipitated with phosphotungstic acid, following a preliminary precipitation with alcoholic mercuric chloride. The phosphotungstic precipitate was decomposed by adding gradually a warm solution of baryta with vigorous shaking until the precipitation of barium phosphotungstate and barium sulphate was complete. These were removed by filtration. The residue was twice extracted with boiling water. The combined filtrates were concentrated *in vacuo* to 500 cc. and found to contain 3.34 g. of amino nitrogen. The solution was shaken for 6 hours with an ethereal solution (200 cc.) of 67.5 g. of β -naphthalenesulphonic chloride (25 % excess); 40 % soda was added at intervals of one hour to maintain the contents alkaline to phenolphthalein. A brown syrupy mass remained in the agitation bottle. The clear supernatant liquid was decanted into a separating funnel for the removal of the aqueous layer. The latter was acidified with hydrochloric acid and was placed in the ice chest over night. The white crystalline mass which separated out weighed 5.5 g. and was nitrogen-free. It was probably β -naphthalenesulphonic acid.

The oily residue which separated from the alkaline solution was taken up in 75 cc. of warm 95 % alcohol, and slowly poured with much stirring into 1000 cc. of cold dilute hydrochloric acid. A gummy mass separated and hardened into a granular mass. After three days the aqueous layer was decanted off and the powdered residue washed repeatedly with cold water. Ether extraction of a portion was attempted but was found impracticable owing to softening. The whole was then boiled for half an hour with 1500 cc. water and the hot portion was decanted off. The residue rapidly hardened to a brown transparent mass. Very little material appeared to be dissolved by the water. The dry residue was dissolved in 100 cc. of warm 95 % alcohol and poured slowly into two litres of cold ether. After 24 hours in the ice chest, the brown gummy residue was separated by decantation. On again treating

with alcohol, a small portion of about 1.5 g. failed to go into solution after repeated boiling. The portion readily soluble in 75 cc. alcohol was reprecipitated by slowly pouring into 2500 cc. of water at 0°. The white granular precipitate was dried by vacuum desiccation to constant weight; 8 g. were obtained containing 7.65 % of nitrogen. 5.85 g. of the material were boiled with 200 cc. of 1 : 1 hydrochloric acid for 7½ hours. The yellow supernatant liquid (Solution A) on cooling, was poured off from the brown insoluble portion. The latter was boiled with 100 cc. of water, and dissolved in alcohol. The alcoholic solution was concentrated to dryness by vacuum desiccation and gave a syrupy residue which rapidly hardened to a brittle, granular mass.

0.1022 g. after drying for five hours at 105° weighed 0.1004 g.

The total vacuum-dried product weighed 2.511 g. Duplicate carbon and nitrogen estimations were made.

	% C	% N
Found	59.0	5.28
Calculated for di- β -naphthalenesulphonic deriv. of lysine	59.3	5.33

The formation of the di- β -naphthalenesulphonic derivative of lysine is only possible when the amino groups do not occur in peptide linkage. We may therefore assume one of the following possibilities:

(1) That the lysine contained in the isolation product is free and not in peptide linkage through its carboxyl groups.

(2) That the carboxyl group of the lysine is in ordinary peptide linkage with another amino acid

(3) That the carboxyl group of the lysine is in an imide linkage

(4) That the isolation product contains lysine amide. The first and second assumptions have no bearing on the lysine and ammonia relationship and may be disregarded. A compound of the third type is split by alkaline hydrolysis but is unchanged by boiling with mineral acids [Bergell and Feigl, 1908]. It would not be split by acid hydrolysis. The occurrence of lysine amide is not possible, for the following reasons:

(1) If the amide group were to react [Königs and Mylo, 1908] with β -naphthalenesulphonic chloride, a carboxy-sulphonimide compound would be formed which is resistant to acid hydrolysis, ...CONH—SO₂...

(2) If the amide group were not to react [Bergell, 1914] with β -naphthalenesulphonic chloride, subsequent acid hydrolysis would liberate ammonia in equimolecular amounts. The hydrolysis of 5.85 g. of the β -naphthalenesulphonic derivative produced 8 mg. of ammonia nitrogen in solution A in place of the 69 mg. required by theory.

Solution A containing approximately 3.3 g. (by difference) of the hydrolysed derivative was evaporated to a syrup and dissolved in 200 cc. of boiling water. On cooling the solution the material separated out as oily drops which failed

to crystallise (from the aqueous supernatant layer 100 mg. of glutaminic acid were obtained as the hydrochloride). The material was repeatedly precipitated from alcoholic solution by pouring slowly into a rapidly stirred freezing mixture of ice and concentrated hydrochloric acid, but no crystalline product could be obtained.

The largest amount of ammonia in protein hydrolysis has been found in certain of the vegetable proteins—the prolamines. The fact that the members of this group contain no lysine [Osborne and Clapp, 1907] may therefore be advanced in further support of the view that the ammonia of the trypsin-stable residue in caseinogen is not in direct association with the lysine.

It may now be shown that the ammonia of the trypsin-stable residue is present in amide form. The collected results of the work on amide hydrolysis by tissue enzymes *in vitro* [Gonnerman, 1902, 1903; Bergell and von Wülfing, 1910, 1, 2; Bergell and Brugsch, 1910; Schwarzschild, 1904; Lang, 1904] may be summarised as follows.

Of seventeen different amides and imides the pancreas hydrolysed three and gave negative results with fourteen; the liver hydrolysed thirteen and gave negative results with four, the kidney hydrolysed nine and gave negative results with six.

The work of Lang [1904] on deamidation has been referred to by many investigators. Previous workers, Bergell and v. Wülfing [1910, 1, 2] and Schwarzschild [1904], had reported that asparagine was not deamidised by pancreas extracts. Lang tried asparagine with preparations of the liver, kidney, testes, spleen, and intestinal mucosa. Glutamine was used in one experiment with a liver preparation only. Unfortunately these amides were not used with pancreas extracts. His conclusions were as follows: “Diese Amide (Asparagine and Glutamine) wurden in allen Organen, denen sie zugesetzt wurden, desamidiert.” Subsequent references to Lang’s conclusions have heightened the impression that all animal tissues deamidised these substances, whereas the pancreas would probably have given negative results had it been tried [See Bostock, 1912; Effront, 1917; Cathcart, 1921].

It seemed desirable to investigate the behaviour of the trypsin-stable residue with preparations of the pancreas, liver and kidney. Five g. of each of these organs from the pig were finely minced and ground with washed sand. The ground tissue was washed out with water from time to time until 50 cc. of the suspension were obtained. To the pancreas preparation were added 5 cc. of a similar extract of the mucosa of the small intestine.

To 6 cc. of a 5 per cent. solution of the isolated residue from digest 4 were added 2 cc. of the tissue suspension and 0.5 cc. of toluene. Duplicate experiments were made at initial p_H of 5.6, 6.2, 6.8, 7.4, 8.0, 8.6. The tubes were well-shaken, corked, and kept for four days at 40°.

				Nett cc. (experimental - blank) of 0.1 N ammonia				
p_H				5.6	6.2	6.8	7.4	8.0
Pancreas	...	...	...	0.0	0.0	0.0	0.0	0.0
Liver...	...	...	...	0.05	0.30	0.50	0.65	0.70
Kidney	...	...	...	0.05	0.30	0.30	0.35	0.35
Acid hydrolysis	...	...	—	—	—	1.60	—	—

This experiment again demonstrates the failure of pancreas extracts to effect deamidation of the trypsin-stable residue. It is also possible that the ammonia liberated by the liver and kidney preparations arose from deamination of α -amino groups.

The effect of liver and kidney preparations was then tried on:

- (1) The extraction product from digest 9.
- (2) The extraction product from digest 9 after hydrolysis with 20 % sulphuric.
- (3) Gelatin hydrolysed by acid.

If the ammonia is a product of deamidation, positive results would be obtained only with the unhydrolysed material (1). If the mechanism is one of deamination—the α -amino group of (2) and (3) should be attacked.

The experiments were performed in duplicate at a single initial p_H of 7.4.

				cc. 0.1 N ammonia		Average
2 cc. liver (pig) suspension + 6 cc. water	...	...	...	0.23	0.23	0.23
6 cc. (9) hydrolysate	...	...	...	0.60	0.55	0.58
6 cc. (9) unhydrolysed	...	...	...	0.00	0.00	0.00
6 cc. gelatin hydrolysate	...	...	...	0.60	0.70	0.65
2 cc. liver + 6 cc. (9)	...	...	...	1.85	1.90	1.88
2 cc. liver + 6 cc. (9) hydrolysate	...	...	...	1.00	1.00	1.00
2 cc. liver + 6 cc. gelatin hydrolysate	...	...	...	1.30	1.30	1.30
2 cc. kidney + 6 cc. (9)	...	...	...	1.65	1.55	1.60
2 cc. kidney + 6 cc. (9) hydrolysate	...	...	...	0.80	0.80	0.80
2 cc. kidney + 6 cc. gelatin hydrolysate	...	...	...	0.85	0.90	0.88
2 cc. kidney + 6 cc. water	...	...	...	0.22	0.22	0.22
Acid hydrolysis of 6 cc. (9)	...	...	...	2.15	2.15	2.15
Recalculated				Gross		Blanks
Liver + (9)	...	...	...	1.88	0.23	0.0
„ + (9) hyd.	...	...	...	1.00	0.23	0.58
„ + gelatin...	...	...	...	1.30	0.23	0.65
Kidney + (9)	...	...	...	1.60	0.22	0.0
„ + (9) hyd.	...	...	...	0.80	0.22	0.58
„ + gelatin	...	...	...	0.88	0.22	0.65
						Nett
						1.65
						0.19
						0.42
						1.38
						0.00
						0.01

While slight deamination has been effected by the liver, the results obtained with the kidney preparations indicate marked deamidation and no deamination. The production of ammonia from only the unhydrolysed isolation product is advanced as evidence in support of an amide structure for the ammonia precursor. This is again supported by the analogous behaviour of amides with liver, kidney, and pancreas preparations,—referred to on page 689.

SUMMARY.

1. In the prolonged tryptic digestion of caseinogen about one-third of the total ammonia that may be obtained by acid hydrolysis fails to be liberated by the enzyme.

2. The isolated residue totally resists tryptic hydrolysis of the ammonia precursor and is considered to be structurally different from the portion hydrolysed by trypsin.

3. About one-half to two-thirds of the trypsin-stable residue may be precipitated by ethyl alcohol. Total precipitation is obtained with phosphotungstic acid and alcoholic mercuric chloride.

4. The acid hydrolysate of the alcohol-precipitated and extracted residue consists almost entirely of glutaminic acid and lysine. Eight to ten per cent. of the total nitrogen occurs as ammonia.

5. It is shown that the ammonia contained in the trypsin-stable residue is not associated with the lysine.

6. The behaviour of the material with enzyme preparations from the pancreas, liver and kidney of the pig was investigated. The results confirm the view that the ammonia is present in amide form.

It may therefore be concluded that glutamine, or a glutamine-containing peptide, is present in the tryptic digestion product of caseinogen. While it is probable that the glutamine is an integral part of the caseinogen molecule,—the possibility of the secondary formation of the amide by an enzymic synthesis cannot be neglected.

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