Kjeldahl flask. Talcum is added, and the volume is made up to about 150 cc. Eighty to 100 cc. are distilled into another 300 cc. Kjeldahl flask containing about 50 cc. of cold water. An apparatus similar to, but somewhat larger than that recently described by Bok,[5] is satisfactory. To remove acetone and aldehydes the distillate is treated with 10 cc. of a saturated $HgSO_4$ solution in N H_2SO_4 and 5 cc. of saturated NaOH. It is then slowly distilled until about 80 cc. have been collected. The volume is diluted to the 100 cc. mark and an aliquot is analyzed.

Fifty cc. of standard $KMnO_4$ (0.1 or 0.01 N) and approximately 10 cc. of 5N NaOH are placed into an Erlenmeyer flask. This is heated 10 minutes in the boiling water bath. The flask is momentarily removed and the solution containing alcohol is run in by pipette below the surface. Heating is continued for 30 minutes. The solution is cooled. It is acidified by 10 cc. of 10 N H_2SO_4 and allowed to stand a few minutes. An excess of KI is then added and the iodine is determined by standard thiosulfate. Blank determinations are run simultaneously. The difference between the blank and the unknown represents the volume of standard $KMnO_4$ used up by the oxidation. Within the following limits, and under the conditions described above, 1 mg. is oxidized by 2.38 cc. 0.1 N $KMnO_4$ (limits 0.4 to 8 mg.). 0.1 mg. is oxidized by 2.38 cc. 0.01 N $KMnO_4$ (limits 0.04 to 0.4 mg.).

The end points are very sharp. The maximum error is about $\pm 3\%$.

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Rate of Ammonia Liberation in Tryptic and Peptic Digestion of Casein.

IDA KRAUS-RAGINS.

From the Department of Physiological Chemistry, University of Chicago.

The first properly controlled quantitative study of the rate of liberation of ammonia in tryptic digestion was publised by Hunter and Smith,[1] who discussed the early literature on this phase of the subject and that need not be repeated here. They found that in tryptic digestion ammonia was liberated very slowly and that amidolysis took place very much less rapidly than peptide hydrolysis.

[5] Bok, J. C., *J. Biol. Chem.*, 1931, **93**, 645.

[1] Hunter, A., and Smith, R. Q., *J. Biol. Chem.*, 1924, **62**, 649.

In 10 days of digestion at the time when 65% of the total bound nitrogen was liberated, only about 25% of the total potential ammonia was liberated. The authors compared their results with those of Henrique and Gjoldback[2] and pointed out that whereas the trypsin used by the latter was much less active in peptide hydrolysis it was a good deal more potent in the liberation of amide groups. They concluded that amidolysis is probably due to a separate enzyme and that a pure trypsin might produce no ammonia from proteins.

Zunz,[3] in a quantitative study of the course of peptic digestion on casein and albumin, reported that a little more than one-third of the available ammonia in casein was liberated in 15 days. Henrique and Gjoldback[2] reported 7.2% in 30 days and 9% in 108 days No control of HCl + casein was shown. Effront[4] reported 5.0% in 15 hours and calls attention to the fact that 2-3 gm. HCl per liter eventually causes the formation of amide nitrogen. "The quantity of nitrogen thus formed is much smaller than is formed in the presence of pepsin hydrochloride, and very often this fact is not taken into account."

Having on hand highly active pepsin* and trypsin† preparations it was thought worth while to make a parallel study of the rate of liberation of ammonia by these enzymes.

Experiment I. An approximately 5% solution of Hammersten's casein was prepared by suspending 50 gm. in 500 cc. distilled water and adding N/10 NaOH or 1% Na_2CO_3 solution until completely dissolved. The pH of this solution was then adjusted to 8 and made up to volume. The solution was clear and it was not necessary to filter. The methods of sampling, concentration of enzyme, controls, preservatives, and incubation were those of Hunter and Smith.[1]‡

[2] Henrique, V., and Gjoldback, J. K., *Z. Physiol. Chem.*, 1911, **75**, 363.

[3] Zunz, E., *Z. Physiol. Chem.*, 1898, **28**, 151.

[4] Effront, J., Biochemical Catalysts in Life and Industry, New York, John Wiley and Sons, 1917, 242.

* Pepsin prepared according to the method of Thomas L. Meekin (unpublished University of Chicago thesis, 1925) had an activity of 10X that of the Armour and Company U.S.P. pepsin 1-3000.

† Difco trypsin 1:110, commercial product, had the same activity as the purified product of Robert W. Bates (unpublished University of Chicago thesis, 1931). Activity comparison was made by the refractive index method and found to be 10X as active as the Parke, Davis and Company's pancreatin U.S.P.

‡ Conditions for aeration were established as follows: a peptone solution and an ammonium sulfate solution respectively were aerated for 30 minutes, one hour, and one and one-half hours. The results were identical and therefore the 30-minute aeration was used throughout this work.

TABLE I.

Cross results:	Cas. digest* % total N as NH2 D	NH3 D	Cas. control % total N as NH2 C	NH3 C	Net results % total N as NH2 D-C	NH3 D-C	Author % total hydrolysis Peptide groups	Author Amide groups	Hunter and Smith Peptide groups	Hunter and Smith Amide groups
Orig. solution			4.17	0.075†						
After 1 hr.	33.16	0.32	4.25	0.075	28.99	0.245	42.53	2.41	44.8	2.6
" 24 hr	42.12	0.50	4.59	0.20	37.53	0.30	55.2	2.95	56.0	7.7
" 5 days	48.05	2.24	6.26	0.44	41.79	1.80	61.46	17.74	63.2	21.2
" 10 days	50.71	3.15	8.71	0.96	42.00	2.19	61.78	21.59	65.4	25.2
" 88 days	56.40	5.88	14.51	2.12	41.89	3.76	61.62	37.07	64.2	40.5
" 144 days		6.50		3.10		3.40		33.52		
Complete hydrolysis by acid			72.15	10.45	67.98	10.38				

* Enzyme control is not shown but correction was made.

† The figures are averages of triplicate determinations. In one case an additional 0.25 gm. of trypsin was added, but no effect was noted.

In comparing the net results obtained with those taken from Hunter and Smith, it is apparent that although the percentages are somewhat lower both in peptide and amide hydrolysis, yet in general the data are confirmatory. The trypsin preparation used had the same order of proteolytic and amidolytic activity as that used by Hunter and Smith. As pointed out by the former authors the amide hydrolysis falls far behind the peptide hydrolysis not only in absolute velocity but in the rate with which the hydrolytic equilibrium is approached. The proteolytic activity is greatest in the first hour and reaches an equilibrium in 5 days (61.46%, and in 10 days, 61.78%) amino nitrogen is liberated, after which time the increase may be accounted for by the spontaneous hydrolysis of the substrate in the control. On the other hand, the enzymatic production of ammonia continues slowly but constantly. The 88th day marks a gain of 52% over the 5th day. Thus on the 88th day, 37% (corrected) of the total potential amide nitrogen was liberated. An apparent equilibrium was attained after that. The spontaneous amidolysis of the substrate in the control on the 144th day was greater than in the digest. If calculated without correcting for the protein control of the total available ammonia nitrogen, 62.2%, (or two-thirds) is obtained and one-third is probably in a trypsin-stable complex reported by Luck.[5]

Experiment II. The rate of liberation of ammonia in tryptic and peptic digestion was followed simultaneously: the same concentrations of enzyme and substrate were used. No measures were taken

[5] Luck, J. M., *Biochem. J.*, 1924, **18**, 679.

to stop hydrolysis in the case of pepsin. Aliquot portions were taken, allowed to come to room temperature and then quantitative measurements were made for analysis.

A. 1. 5 gm. casein suspended in 250 cc. N/10 HCl + 0.125 gm. purified pepsin.§

2. 5 gm. casein suspended in 250 cc. N/10 HCl + 0.0 gm. purified pepsin.

B. 1. 5 gm. casein dissolved in N/10 NaOH neutralized with HCl and adjusted to same pH as A.1 + 0.125 gm. purified pepsin.

2. Same as B.1, but no pepsin.

C. 1. 5 gm. casein dissolved in 250 cc. 0.4% Na_2CO_3 + 0.125 gm. trypsin.[4]

2. 5 gm. casein dissolved in 250 cc. 0.4% Na_2CO_3 + 0.0 gm. trypsin.

TABLE II.
% of total nitrogen as ammonia nitrogen.

Time of Digestion	A 1	A 2	A 3(1-2)	B 1	B 2	B 3(1-2)	C 1	C 2	C 3(1-2)
5 hr.	0.05	0.05	0	0.04	0.05	—0.01	0.10	0.05	0.05
24 hr.	0.50	0.50	0	0.719	0.719	0.0	0.47	0.05	0.42
7 days	3.36	0.983	2.37	4.31	1.95	2.36	2.43	0.62	1.71
16 days	5.32	3.10	2.22	6.60	3.96	2.64	2.69	0.89	1.80
25 days	6.16	4.29	1.87	7.25	5.50	1.75	4.14	1.70	2.44
52 days	7.71	6.21	1.50	8.12	7.19	0.93	5.03	2.28	2.75
83 days	8.28	7.34	.94	8.84	8.53	0.31	6.01	3.10	2.91
109 days	8.95	8.04	.91	9.45	6.63	0.82(?)	7.05	3.78	3.27
120 days	9.65	9.05	.60				7.10	3.88	3.22

The only difference in A and B was that the casein in B was first dissolved in NaOH and then neutralized and adjusted to the same pH as A. This treatment rendered the casein more flocculent and, as the results indicate, the liberation of ammonia is slightly more rapid both in the control and in the digest as compared with A.

Table II shows that although pepsin hastened the liberation of ammonia from casein, N/10 HCl at 37°C. will liberate practically the same amount if sufficient time is allowed. Thus in 120 days the difference between pepsin-HCl and HCl alone is 0.60%. The effect of pepsin begins to decrease after the 7th day. In the case of trypsin (column C) the course is quite different. Trypsin at pH 8 definitely liberated ammonia from casein (C-1) more rapidly than was liberated by pH 8 and no trypsin (C-2). An apparent equilibrium was reached in 109-120 days when no more ammonia

§ Enzyme controls were run and corrections for ammonia in pepsin and trypsin were made.

was liberated in either flask and at this point the ammonia concentration in C-1 was almost twice that in C-2, thus showing definitely an enzymatic effect. The data with pepsin may be interpreted that pepsin had no amidolytic activity. The catalytic effect may be due to the proteolysis, *i. e.*, during peptic hydrolysis nitrogenous compounds were formed which gave off ammonia more easily under the conditions of the experiment or "internal anhydrides"[6] might have opened exposing amide groups which were hydrolyzed more easily by the HCl. In the control tube with no peptic hydrolysis the action of HCl took longer. Thus while the effect of pepsin might be explained on the basis of the physical consistency of the substrate and the proteolytic properties, these factors did not enter into the case of trypsin. As was pointed out in Experiment I, proteolysis ceased although amidolysis continued till equilibrium was established when about two-thirds of the amide groups were split off.

Hunter and Smith[1] mentioned that erepsin was no more efficacious than trypsin in the production of ammonia from peptone. They show no data but state "unfortunately the erepsin preparations were not very active." Luck[5] showed that the trypsin-stable complex, which he isolated from a trypsin-casein digest, when incubated with pigs kidney-tissue extract liberated ammonia. This suggested the possibility of hastening the liberation of ammonia from a casein-trypsin digest by adding kidney-tissue extract.**

Experiment III. A casein-trypsin digest and casein control 30 days old were available. These were used as follows: 4 flasks, D and D′ each containing 100 cc. digest, and C and C′ each containing 100 cc. casein control. 50 cc. of kidney extract was added to D′ and C′; to D and C 50 cc. of distilled water was added.

It is evident from Table III that the kidney-extract was active;

TABLE III.

Time of Digestion	Casein Digest		Casein Control		Casein Digest +kidney-ext.		Casein Control +kidney-ext.*	
	mg. of N in 100 gm. of solution as				mg. of N in 100 gm. of solution as			
	D		C		D′		C′	
	NH_2	NH_3	NH_2	NH_3	NH_2	NH_3	NH_2	NH_3
0 hr.	332.94	45.92	81.16	11.9	332.90	45.94	81.16	11.92
1 day	332.90	47.81	83.2	12.1	593.7	52.92	194.1	20.2
5 days	333.0	49.00	88.0	14.0	621.0	55.44	319.9	26.04

* Correction for kidney-extract control was made.

[6] Cohen, E. J., *Physiol. Rev.*, 1925, 5, 349.

** To 10 gm. fresh dog-kidney finely minced and ground with washed sand was added 50 cc. distilled water. The suspension was filtered through 2 layers of cheese-cloth. One part of extract was added to 2 parts of digest.

also, that the amidolysis as well as the proteolysis was greater in C′ than in D′ (greater concentration of "peptide substrate"). In no way, however, did these results show what caused amidolysis. They merely confirmed that ammonia was liberated along with amino nitrogen but at a much lower rate. The liberation of amino nitrogen was due to trypsins or erepsins.††

Experiment IV. Retarding effect of antiseptics. Lang[7] found that toluol is especially effective in retarding the formation of ammonia. To test the effect of tricresol or toluol (antiseptics used in these experiments) Experiment I was repeated with no preservative, *i. e.,* the protein solutions were prepared the same as in Experiment I but no toluol was added. The control as well as the solution to which the trypsin was added and thoroughly mixed were at once filtered aseptically through a sterile Berkefeld filter into sterile flasks and incubated at 37°C. Aliquot samples were taken for analysis, using bacteriological technique, sterile pipettes, etc., at time intervals as indicated. The results were identical with those of Experiment I. Thus, whatever the amidolytic activity in trypsin, it was not affected by the preservative used under the conditions of these experiments.

Summary. 1. In tryptic digestion the liberation of ammonia was due to an enzymic activity associated with the trypsin preparation used, whereas in peptic digestion the ammonia liberation was due to the acidity only. 2. The more rapid liberation at first in a pepsin digest than in the control can be explained on the basis of the physical and chemical change in the substrate due to peptic proteolysis. 3. Kidney-extract did not hasten ammonia liberation. The aminolytic and amidolytic activity in the extract was high and seemed to follow the same course as with trypsin. 4. The concentration of antiseptic used under the conditions described did not retard either activity.

The author wishes to express her appreciation to Professor F. C. Koch for his helpful criticisms and suggestions.

†† It was shown in Experiment I that a further addition of Difco trypsin produced no further effect. Hence the action in the kidney-extract might have been due to a different trypsin or to some erepsin. Erepsin is said to be a mixture of enzymes which act on polypeptides and dipeptides. The action in this experiment is on casein. The addition of the kidney extract to a pepsin-HCl digest and control showed no activity, *i. e.,* all kidney-extract enzyme activity was destroyed at that pH.

[7] Lang, S., *Beitr. Z. Chem. Physiol.*, 1904, **51**, 321.