B and 2E, show that the distribution of urea is approximately equal between serum and corpuscles of the blood, which confirms the results found by Bang[1] and older investigators.

Summary.

Values are given for the normal urea content of the blood and tissues of a number of species of vertebrates.

In vertebrates whose end-product of nitrogenous metabolism is urea, the kidneys are much higher in urea content than are the other tissues in which the urea content is about the same as that of the blood.

Hens injected with alanine do not show any increased amount of urea in the blood and tissues, indicating that urea is not one of the stages in the intermediary metabolism of amino acids in the hen. The kidneys of the hen have the same urea content as the other tissues, showing that urea is not present to any considerable extent in the kidney excretion of the hen.

URBANA, ILL.

[CONTRIBUTION FROM THE LABORATORY OF THE NORTHWESTERN UNIVERSITY MEDICAL SCHOOL.]

ON THE ASSUMED DESTRUCTION OF TRYPSIN BY PEPSIN AND ACID.[2]

BY J. H. LONG AND MARY HULL.

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This question is approached from two general standpoints. In one case it is a purely physiological one without reference to any therapeutic application whatever; in the second case the importance of the question comes from its bearing on the administration of ferments as remedies in disorders of digestion. Both points of view are interesting and important. In this laboratory the question comes up now as part of a problem dealing with the second point of view, and mainly through queries suggested by certain investigations prosecuted by the Council on Pharmacy and Chemistry of the American Medical Association, and particularly this question: What is the fate of the so-called pancreatins, administered by the mouth, in passing through the stomach? As digestive remedies have these preparations any real value? Do they, in any way, inhibit or diminish the activity of the pepsin in the stomach, or, on the other hand, are the components of the pancreas preparations themselves weakened or destroyed by the pepsin?

In part the queries suggested here are comparatively simple and easily

[1] *Biochem. Z.*, **72**, 104 (1915).

[2] This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

answered. In several papers published from this laboratory[1] satisfactory answers have been given to some of the questions, and much of the older literature is quoted in them. But others of the points involved are evidently far from simple, although at first they would seem susceptible of easy solution. Their study involves the consideration of numerous subsidiary questions, some of which have not yet been fully answered. It is easy to arrange experiments *in vitro*, in which one group of ferments may appear to be readily destroyed by another, but it does not necessarily follow from these that under the conditions obtaining in the body a like result should be expected. In many of the published investigations the methods employed have been so narrowly limited as to fail to answer the last practical question, although doubtless giving correct conclusions for the arbitrarily chosen conditions of the experiment. For example, Mellanby and Woolley[2] in a recent publication have come to the conclusion that trypsin and steapsin are mutually antagonistic and that the latter is rapidly destroyed by the former. This may be true under some conditions, but scarcely under those which obtain practically. In our laboratory we have frequently made pancreatic extracts and mixtures in which both ferments exist and persist through a relatively long period. The stability and mutual action of ferments depend on a multiplicity of conditions which are properly balanced in the animal body, but which appear to present enormous difficulties in the duplication *in vitro*. Concordant and consistent results are, therefore, hard to secure.

In former work, and especially in the papers from this laboratory, referred to above, we believe sufficient evidence has been presented to show that the starch-converting ferment of the pancreas, the amylopsin, is so sensitive to the action of traces of acid and acid plus pepsin that its passage through the stomach must appear extremely doubtful under practical conditions. At the very beginning of the gastric digestion and before enough acid has been secreted to combine with the ingested proteins, amylopsin administered will doubtless persist and some of it may pass through into the duodenum. But with increasing hydrogen-ion concentration the conditions for its existence become less and less favorable.

It is not our intention to discuss in this place the behavior of amylopsin or steapsin, as the facts for the first seem to be pretty well established, while for the second many of the data necessary are as yet lacking. We shall confine our attention to the question of tryptic activity under a number of conditions with reference to peptic digestion.

In the papers quoted above[3] the fact is brought out that trypsin may

[1] Long and Johnson, THIS JOURNAL, **35**, 897, 1188 (1913); Long and Muhleman, *Arch. Intern. Med.*, **13**, 314 (1914).

[2] *J. Physiol.*, **48**, 287 (1914).

[3] Long and Johnson, and Long and Muhleman, *Loc. cit.* The literature on this point is discussed.

be incubated with considerable quantities of hydrochloric acid without suffering appreciable loss of strength. In some of these cases of incubation through one-half hour the weight of acid was greatly in excess of the weight of the active tryptic ferment employed. These results have been secured so often and in solutions containing from 100 to 160 mg. of actual acid and 50 mg. of pancreas powder, containing trypsin and not trypsinogen, in 50 cc. of solution, that we are at a loss to understand the statements of Mellanby and Woolley[1] that, while trypsin withstands the action of weak acid pretty well, it is so quickly destroyed in acid of 0.2% strength that nothing can be said of the action of pepsin plus the acid on trypsin. In some of our experiments we have employed trypsins of more than the usual activity, but then the weights were so small that their protein content could not bind much of the acid.

These experiments showed, further, that the addition of pepsin to the acid worked a rapid destruction of the trypsin in some cases, while in others no such destruction was observed. The fate of the trypsin seemed to depend on the relation of the amount of protein present, and this practical point was left for further investigation. Meanwhile somewhat similar findings have been reported by Edie,[2] while Serono and Palozzi[3] reach the conclusion that pepsin and acid of 0.2% strength do not destroy the pancreatic enzymes. In Edie's experiments, in which Benger's ferment solutions were mostly employed, the behavior of small amounts of pepsin with large amounts of trypsin and fibrin in acid solution is studied. Under the conditions there can be no tryptic digestion, it is assumed, but a marked diminution in the extent of the peptic digestion was recognized. This cannot be attributed to any destructive action of the trypsin on pepsin, but the hypothesis is advanced that the trypsin exerts its effect by combining with specific groups of the protein complex in such manner as to prevent the pepsin from attacking them in the normal way. The practical bearings of the observations are somewhat remote because of the relative strengths of the solutions from which the results were obtained.

In investigating the action of pepsin on trypsin small volumes of solutions of the latter were allowed to act on much larger amounts of pepsin in presence of fibrin and sodium carbonate. Under these conditions there could be no peptic digestion of the fibrin, but the normal tryptic action appeared to be distinctly retarded. The same explanation is offered as in the other case. The pepsin is here assumed to combine with the protein in such manner as to protect the groups which the trypsin should attack. It will be seen that this is not quite the problem which

[1] *J. Physiol.*, **47**, 339 (1913–14).

[2] *Biochem. J.*, **8**, 84, 193 (1914).

[3] *Chem. Zentr.*, **84**, I, 1212 (1913); *Maly's Jahrb.*, **43**, 393 (1913).

we have on hand, since we wish to determine the action of pepsin on trypsin in acid solution. These investigations represent the physiological rather than the therapeutic side of the problem. Edie's work agrees fully with ours in showing the comparative stability of trypsin in presence of acid alone.

Serono and Palozzi appear to have worked with glycerol extracts of the pancreas, of their own preparation, obtained by aid of high pressure. These glycerol extracts when mixed with acid to make 0.2% strength could be incubated through 24 hrs. at the body temperature without losing their proteolytic activity. Nothing is said about the amount of protein present in the extracts which might serve as a protection to the trypsin by combining with the acid, but the impression is given that pepsin is practically without action on trypsin.

We have gone over these experiments at some length because they are the latest in the literature, and because, further, they leave the question not much nearer a solution than it was when opposite views were expressed by Ewald, Engesser, Mays and others[1] years ago. Ewald drew the conclusion from his experiments that trypsin is destroyed by digestion with pepsin and hydrochloric acid. But from the conditions under which his tests were made this result would of necessity follow without proving the point at all. For example, he incubated 700 mg. of pepsin and the same weight of trypsin, the Engesser preparation, with 100 cc. of 0.3% hydrochloric acid through three hours at 40°. As will appear below, destruction of trypsin would quite naturally result with these conditions. Engesser maintained that the preparation contained the "zymogen" rather than the finished trypsin, and for this reason should be very resistant. It appears, however, that he used relatively lower amounts of acid by his method of testing then did Ewald. Mays, noting the discrepancy between these results and the lack of agreement with earlier work of Kuehne on the behavior of trypsin, pointed out that the ferment is weakened or destroyed by acids, and that the rapidity of this destruction is hastened by presence of pepsin. He came to the conclusion, generally not admitted, that trypsin digests protein in acid solution when sufficient excess of protein is present, but overlooked the real condition under which trypsin appears to be stable when acid and pepsin are present. These three papers gave a basis for views which are often advanced, even down to the present time.

We have attacked the problem of the conditions of trypsin destruction in two ways. First by a series of experiments *in vitro* where a number of varying conditions with reference to strength of acid, amount and kind of protein present and resultant hydrogen-ion concentration were

[1] Ewald, *Z. klin. Med.*, **1**, 615 (1879); Engesser, *Ibid.*, **2**, 192 (1880); Mays, *Maly's Jahrb.*, 10, 298 (1880).

considered, and secondly through animal experiments on dogs with duodenal fistulas or Pawlow pouches, by aid of which the products of reactions might be withdrawn and tested. In a considerable number of cases the stomach contents have been withdrawn by a tube and examined as to the extent of the action. In this paper only the first group of experiments will be reported.

The Pepsin-Trypsin Reaction in Vitro.

In these experiments the pepsin employed was an active commercial product which fully satisfied the requirement of the U. S. Pharmacopoeia, in the digestion of egg albumin. In the preliminary work we used the standard product of Fairchild Brothers and Foster and in the later tests the standard pepsin of Armour and Company. As tryptic preparations we have employed a variety of products. In much of our earlier work we used glycerol extracts of the pancreas of our own preparation. Some of these were freshly made and some had been on hand through long periods, to provide for a possible activation, which, however, did not seem to occur. We finally found it most convenient for comparisons to employ two brands of commercial trypsins from the laboratories of the firms whose pepsins we used. In most of the preliminary trials a product of Fairchild Brothers and Foster was used which had been in the laboratory some time and had lost part of its original activity. Later two other samples from the same firm were drawn in for comparison. In the larger number of routine tests the Armour and Company product was used in the digestions, and where convenient these digestions have been frequently carried out in presence of a phosphate mixture giving a hydrogen ion concentration of $P_H = 7.7$. But there are some drawbacks in the use of this buffer solution and the usual sodium carbonate medium found more frequent application. In each of the trial experiments of the short table below 3 g. of prepared moist, flake fibrin, yielding about 900 mg. of dry fibrin, was used in the substrate. This was mixed with 100 mg. of trypsin in 50 cc. of water, plus 50 cc. of approximately 0.4% sodium carbonate. The 100 cc. of mixture was protected by toluene in each case and incubated through 8 hrs. at 38°. Then a slight excess of hydrochloric acid was added and the liquids boiled to expel carbon dioxide. They were then made faintly alkaline to litmus and received an addition of 10 cc. of neutral formaldehyde. The final titrations with 0.2 *N* sodium hydroxide followed, with the results below. In each case a blank test was made with trypsin added from a boiled solution. The soluble nitrogen found in the blank test comes in part from the amino nitrogen of the ferments and in part from the effect of the long incubation. The hydrogen concentration at the end of the incubation was found to be essentially the same for all the mixtures, *viz.*, $P_H = 8.1$, which is a favorable concentration for activity.

TABLE I.—STRENGTH OF TRYPSINS.

Trypsin No.	1.	2.	3.	4.
NaOH required in actual test....	12.45 cc.	13.00 cc.	12.80 cc.	11.75 cc.
NaOH required in blank test....	3.90 cc.	3.80 cc.	3.70 cc.	3.80 cc.
	8.55 cc.	9.20 cc.	9.10 cc.	7.95 cc.

In the next few tests, preliminary trials, trypsins 1, 2 and 3 were used; later, in the longer trials, trypsin 4.

It was to be expected that pepsin and trypsin preparations would furnish a certain amount of amino nitrogen in the digestions. The extent of this is suggested by the next tests, where the amount of the ferments used was the same as in the subsequent practical trials.

Series A. Preliminary Tests.

Pepsin and Trypsin without Additional Protein.

In each of the following experiments 25 mg. of pepsin, 500 mg. of trypsin in 50 cc. of water and varying amounts of 0.1 N hydrochloric acid were made to a constant volume of 100 cc. and incubated through three hours at 38° in small flasks. The liquids were neutralized by the addition of the proper amount of sodium carbonate, well shaken to remove carbon dioxide, and then made up to 200 cc. in each case. These volumes were divided into two equal portions and one 100-cc. portion was boiled in each case to kill the ferments. To all the flasks enough sodium carbonate was added to make the hydrogen concentration the same, *viz.*, $P_H = 8$, as determined by preliminary trials. This required nearly 4 cc. of normal carbonate. The flasks were returned to the incubator and kept at 38° through 20 hrs., after which they were all made slightly acid to remove carbon dioxide on boiling, and brought back to litmus neutrality with 0.2 N alkali. In each case 10 cc. of neutralized formaldehyde were added and the usual titration made with 0.2 N NaOH. In the table below the results of the titrations are given, those with the unboiled trypsin being designated by A and those with the boiled trypsin by B.

TABLE II.—PEPSIN-TRYPSIN DIGESTION WITHOUT ADDED PROTEIN.

Vol. of 0.1 N HCl.....	50 cc.	40 cc.	30 cc.	25 cc.	22.5 cc.	20 cc.	15 cc.
A. cc. of 0.2 N NaOH.....	4.4	5.0	5.1	5.2	6.4	6.6	6.8
B. cc. of 0.2 N NaOH.....	4.4	4.2	4.2	4.2	3.8	3.8	4.3
	0.0	0.8	0.9	1.0	2.6	2.8	2.5

It is evident that in the first few titrations the effect of the trypsin is practically negligible. The amino acid nitrogen from the peptic digestion and other causes is small and nearly constant, as shown by the blank titrations opposite B. However, when the amount of acid used at the start is below 25 cc. we seem to have a slightly larger weight of liberated amino acid nitrogen. This is possibly due to the self digestion of the

trypsin not destroyed in the acid digestion. The titration value in a flask containing 25 mg. of pepsin, 500 mg. of trypsin and 25 cc. of 0.2 *N* HCl and titrated without incubation was only 2.5 cc. of 0.2 *N* NaOH. It is important to note that the blanks from the action of the acid and pepsin alone are around 4 cc. always.

To test the effect of larger and increasing weights of pepsin in the digestion of fibrin with a constant weight of trypsin present the next series of digestions was carried out at 38°. In each case 3 g. of moist fibrin, equivalent to 0.9 g. of the dry substance, was taken with 500 mg. of trypsin in a volume of 100 cc. of 0.1 *N* HCl. Different weights of pepsin were added, as shown below. The mixtures were digested through three hours. Then enough carbonate was added to neutralize and leave an alkalinity of $P_H = 8$, after which a further incubation of three hours was carried out. The properly neutralized liquids were titrated with alkali after the formaldehyde addition in the usual way. The results are shown below.

TABLE III.—PEPSIN-TRYPSIN DIGESTION, WITH PROTEIN.

No.	Pepsin added.	Conditions varied.	Cc. of 0.2*N* NaOH reqd
A	None	.....	5.5
B	50 mg.	.....	14.0
C	100 mg.	.....	15.0
D	100 mg.	3 hrs. acid digestion only	16.2
E	150 mg.	.....	14.5
F	150 mg.	both ferments killed	4.0

In A we have the effect of the preëxisting amino acid groups and possibly a slight digestion through the residual trypsin which may have survived the acid digestion in presence of the fibrin. In B and C we have a characteristic pepsin digestion. The flake fibrin disappeared completely so that the opalescent liquid could be easily titrated. In D there could have been no tryptic digestion as the incubation was stopped at the end of the three-hour acid period. The larger alkali volume used in titration has probably no significance. In E the conditions are the same as in B and C, but in view of the result in D it does not appear probable that we have any tryptic digestion here, and, therefore, not in B and C. F serves as a general control for the amount of amino nitrogen made soluble in the incubation alone, or as coming from the ferments themselves. This value subtracted from A leaves a remainder so small as to suggest that the assumed tryptic activity in the latter case must be practically negligible. It was pointed out above that trypsin survives a short acid digestion at this temperature very well, but it is evident that with or without pepsin present an incubation of three hours' duration in a medium containing 365 mg. of hydrochloric acid and 900 mg. of dry fibrin is sufficient to destroy the trypsin. This amount of dry fibrin can bind about 60 mg.

of the acid at the most, to the extent of rendering it inert as regards the ferment. Under tests B, C, D and E it is shown that the amount of amino nitrogen liberated by 150 mg. of pepsin from the given weight of fibrin is not essentially greater than the amount liberated by 50 mg. With the active pepsin used it is likely that the same result would be secured by even a much smaller weight of pepsin.

In the continuation of the preliminary experiments we employed smaller weights of pepsin and acid with the constant weight of 3 g. of moist fibrin and 500 mg. of trypsin in a total volume of 100 cc. Each mixture was incubated through 3 hrs. at 38°, the acid neutralized and the reaction brought to $P_H = 8.1$ by sodium carbonate. At this point a further 3 g. of fibrin were added and the incubation continued through 20 hrs. at the same temperature. The contents of each flask was made slightly acid and boiled to drive out carbon dioxide, then brought to litmus neutrality, treated with 10 cc. of neutralized formaldehyde and finished in the usual manner. The results are shown in the table below. In half of the experiments the trypsin was killed by boiling in part of the water used for solution and these experiments are designated by the letters A′, B′, C′ and so on.

TABLE IV.—PEPSIN-TRYPSIN DIGESTION WITH FIBRIN.

No.	Vol. 0.1*N* HCl. Cc.	Wt. of pepsin. Mg.	Vol. of 0.2*N* NaOH. Cc.	A—A′, B—B′, C—C′, etc. Cc.
A	50	25	25.3	
A′	50	25	17.4	7.9
B	30	25	26.8	
B′	30	25	16.8	10.0
C	25	25	29.3	
C′	25	25	16.2	13.1
D	20	25	28.6	
D′	20	25	14.3	14.3
E	15	25	36.5	
E′	15	25	13.5	23.0
F	oo	oo	39.1	
F′	oo	oo	7.5	31.6

Tests F and F′ at the bottom of the table are straight trypsin digestions carried out through the 20-hr. period only, in the weak alkaline medium, with the ferment in F′ killed at the outset. In A′, B′, C′, D′ and E′ we have peptic digestions in presence of diminishing amounts of acid. In all the peptic digestions where not less than 25 cc. of acid had been used the fibrin solution was practically complete in the three hours, and in the second, or alkaline digestion, in cases where not over 20 cc. of the acid had been used in the first digestion, the solution went so far that no neutralization precipitate formed when the excess of carbonate was finally destroyed by acid. In all cases the formaldehyde titration was made on the filtered liquid, with exclusion of the neutralization precipitate.

It is seen that in test F′ of the table we have a blank of 7.5 cc. of 0.2 *N* alkali, and following an incubation in which the pepsin was omitted and the trypsin killed. This is larger than was found in Table III, No. F, where a blank of 4 cc. of the alkali is given. The trypsin weights were the same in the two cases. The conditions are not the same, however, since in Table IV we have a 20-hr. digestion period against 6 hrs. for Table III. Some greater solution may follow the longer digestion, and it is, of course, possible that in Table IV the trypsin had not been completely destroyed by the preliminary boiling. It has been pointed out that the resisting power toward heat is greater than was formerly assumed.

Aside from this slight discrepancy it is evident that a tryptic action is present in some of the mixtures of Table IV. With the larger weights of acid employed the peptic activity is marked, and that is shown in A and A′, where the difference between the results of the two titrations is not large. The total protein is not sufficient to bind all the acid, and we have in consequence destruction of most of the trypsin. In the following tests, with diminishing weights of acid, the peptic digestions become gradually less, as shown by B′, C′, D′ and E′, while the combined digestions are correspondingly increased. The trypsin appears to be less and less injured until we have in E a very marked action, approximating the straight tryptic digestion shown in F. The acid of E is not sufficient to bring about a marked peptic digestion on the one hand, or to weaken the trypsin on the other.

Series B. Fibrin Digestion.

The experiments just discussed show beyond question that trypsin is able to withstand the action of pepsin and hydrochloric acid under some conditions, while under other conditions there is, apparently, a destruction of the trypsin in acid solution. More detailed experiments are necessary to bring out the facts, and these follow. Fibrin was used for some of these later experiments, but casein and meat, both raw and cooked, have also been used. In what follows a weaker trypsin was employed.

The general method of experimentation was essentially the same as described above, with this modification. After incubating the mixture of 25 mg. of pepsin, 500 mg. of trypsin, protein and acid, made up to 100 cc., through a period of three hours, and neutralizing then exactly with sodium carbonate, the liquid was made up to 200 cc. This volume was divided into two equal portions, one of which was boiled to kill any trypsin left. Both portions were brought to a P_H concentration of approximately 8.1 with carbonate and each received an addition of 3 g. of moist fibrin. All the flasks holding the mixtures of the series were incubated through 20 hrs. at 38°, made then slightly acid with hydrochloric acid, boiled to remove carbon dioxide and throw out any neutralization precipitate and the contents filtered. The filtrates were brought to litmus neutrality

treated with formaldehyde and titrated with 0.2 N NaOH in the usual manner.

In these experiments, as distinguished from those of Table IV, there was only one flask incubated for each acid concentration, and in the division for the tryptic digestion in a 100 cc. volume the trypsin concentration would be just half what it was in the earlier experiments, for the new amount of fibrin added. A wider range of acid concentrations in the preliminary digestion was further chosen. In the tables below the results for the unboiled half of the incubated liquid are shown by A, B, etc., while the boiled portions are designated by A′, B′, etc.

The preliminary acid digestion mixtures contain, in the stronger concentrations, enough acid to saturate the protein, while in the lower concentrations this is not the case. The actual P_H values for several mixtures, from which others may be interpolated, are given below. The measurements were made at the end of the three-hour digestion period.

Vol. of 0.1 N HCl in 100 cc.	P_H.	C_H.	Vol. of 0.1 N HCl in 100 cc.	P_H.	C_H.
50 cc.	1.81	0.01550	25 cc.	2.58	0.00263
35 cc.	2.24	0.00575	15 cc.	3.18	0.00066

In mixtures containing 25 cc., or less, of the acid the *free* acid present is equivalent to that which dissociates from such protein compounds on solution in water.[1] Such concentrations of acid do not affect the trypsin in any marked degree, it appears. In fact, there may be even a little tryptic digestion in concentrations not greatly different from this, as will be shown in some of our work to follow, and as indicated by experiments of Michaelis and Davidsohn.[2] As stated, the acid values were found at the end of the three-hour digestion period, which is preferable to measurement at the outset. In making such mixtures some time elapses before an equilibrium is reached, as the combination between acid and protein is slow, unless they are ground together.[3] The initial concentrations might appear somewhat greater for this reason, and for the further reason that in a digestion like this the products formed in three hours would combine with a little more of the original acid.

In the later digestion in the slightly alkaline medium a hydrogen concentration of P_H = 8.0 to 8.2, at the start, was found satisfactory. This was secured, in the present series of experiments, by the addition of something less than 4 cc. of normal carbonate for each 100 cc. of digesting liquid. No marked change in the alkalinity follows in a 20-hour digesting period, as illustrated by the short table below. Four mixtures were made up as in the regular acid digestions and carried through to the final di-

[1] Long, THIS JOURNAL, **37**, 1333 (1915).

[2] *Biochem. Z.*, **36**, 280 (1911).

[3] Long and Hull, THIS JOURNAL, **37**, 1593 (1915).

gestion in alkaline medium. The hydrogen concentrations were found after the 20-hour period, as well as just before the beginning of incubation.

Vol. of 0.1*N* HCl in prelim.	50 cc.	22.5 cc.	20 cc.	15 cc.
P_H before incubation.....	8.0	8.0	8.15	8.15
P_H after incubation.....	8.6	8.4	8.3	8.3

While the alkali concentrations formerly recommended for tryptic digestions, equivalent to about 0.5% of sodium carbonate, were too high for most mixtures, we have found that something less than half of this gives a good result. The limits need not be as narrow as claimed for the optimum activity by Michaelis and Davidsohn,[1] *viz.*, $P_H = 7.68$. Indeed, their own experiments seem to show that the rate of tryptic digestion is not much less at $P_H = 9$. In the practical formaldehyde titration employed to measure the results there is some advantage in using the carbonate rather than phosphate mixtures. With these a longer preliminary treatment is necessary to bring the mixtures into condition for titration.

TABLE V.—PEPSIN-TRYPSIN DIGESTION WITH FIBRIN.

No.	Vol. of 0.1*N*HCl. Cc.	Vol. of 0.2*N* NaOH. Cc.	A—A′, etc.	General appearance.
A	50	8.4		All fibrin gone after peptic digestion. No digestion later
A′	50	8.4	0.0	As for A
B	45	7.5		As under A
B′	45	7.0	0.5	As under A
C	40	8.2		Peptic digestion only
C′	40	7.6	0.6	
D	37.5	7.7		Peptic digestion only
D′	37.5	7.4	0.3	
E	35	8.4		Peptic digestion only
E′	35	7.5	0.9	
F	32.5	8.0		Peptic digestion complete. A little change in second digestion
F′	32.5	7.3	0.7	Loss of fibrin in tryptic digestion
G	27.5	9.8		
G′	27.5	7.5	2.3	
H	25	11.0		Tryptic digestion
H′	25	7.0	4.0	
I	22.5	15.3		Tryptic digestion
I′	22.5	7.6	7.7	
J	20	15.6		Marked tryptic digest
J′	20	7.5	8.1	
K	15	17.4		Marked loss of fibrin in second digestion
K′	15	8.0	9.4	
L	00	27.5		Complete tryptic digestion
L′	00	8.6	18.9	
M	00	26.4		Complete tryptic digestion
M′	00	7.2	19.2	

[1] *Loc. cit.*

With these explanations of general relations which hold for all the mixtures to follow we present this table as showing the results of the digestions under the modified conditions.

L, L′, M, and M′ are straight tryptic digestions through 20 hrs. only, and with 3 grams of fibrin only. For the mixtures with boiled trypsin we have here rather large blanks, which come from the gradual solution or hydrolysis of fibrin through long contact with the weak alkaline liquid, and in which the protein dissolved is not thrown out by the final neutralization. But they come in part from the greater weight of soluble trypsin used here, as in these digestions the 500 mg. of trypsin was not divided into two portions, finally, as in the other cases. With this explanation it appears that the effect in K is almost as strong as in the L and M tests.

In comparing with Table IV it must be kept in mind that in that series the 100 cc. volumes with 500 mg. of trypsin were not divided at the end of the acid digestion, as here. In comparing the results in the above table among themselves it is evident that we have for the incubations with the higher acid concentrations peptic digestion only, plus the slight hydrolysis effect. As the acid concentration falls the higher titrations following the tryptic digestions become very apparent and the increase can be explained only through the activity of the residual trypsin. The H, I, J, K and L volumes differ from the H′, I′, J′, K′ and L′ volumes only in this that the ferment was killed in the latter cases and not in the former. In the first set of flasks the trypsin, in part at least, must survive the acid incubation where this acid is diminished in activity by combining to a certain degree with the fibrin.

Attention must be called to the fact that the volumes of alkali used in the titrations of the contents of the flasks in which the trypsin had been killed before the final incubation are very constant throughout. This is interpreted as indicating the effect of the primary peptic digestion. But this view does not fully describe the situation. Even where there is no peptic digestion there is always some hydrolysis by reason of the long incubations, first in acid and then in alkaline medium, and the products remain in solution after the usual neutralization.

Series C. Casein Digestion.

In a manner similar to that shown for the fibrin digestions we have carried out a series of digestions with casein as the substrate, using in each case 1 g. of the pure dry substance in the preliminary incubations. The casein employed was made in the laboratory by the usual Hammarsten process and was light and easily soluble in the proper amount of weak alkali. A second gram was added for the tryptic digestions after the division of the original volume into two halves. Enough sodium carbonate was added to each flask to make a soluble salt of this casein and then the usual further amount to bring the reaction to the desired P_H value of

about 8.1. In the table below the boiled trypsin flasks are indicated by the prime letters, as before.

TABLE VI.—PEPSIN-TRYPSIN DIGESTION WITH CASEIN.

No.	Vol. of 0.1*N*HCl. Cc.	Vol. of 0.2*N*NaOH. Cc.	A—A′, etc. Cc.	General appearance.
A	50	16.0		In all of the flasks the casein became pretty thoroughly digested in the acid incubation.
A′	50	11.6	4.4	
B	45	14.9		
B′	45	11.8	3.1	
C	40	15.5		After the tryptic digestion all the flasks appeared clear. Heavy neutralization ppts. came down in the blank flasks and in A, B and C; not much in D and E. Nothing in F, G, H.
C′	40	11.6	3.9	
D	35	22.4		
D′	35	11.7	10.7	
E	30	23.9		
E′	30	11.4	12.5	
F	25	26.8		
F′	25	12.0	14.8	
G	20	26.0		
G′	20	12.0	14.0	
H	15	25.8		
H′	15	11.9	13.9	
I	∞	20.0		
I′	∞	8.4	11.6	

The survival of the trypsin is clearly shown in the tests with the liquids containing the lower acid concentrations. The I experiments show the effects of tryptic digestion plus hydrolysis through the alkali in the long incubation. It is evident that this incubation can in time accomplish a part of the effect which is reached in the much shorter peptic digestion, as far as the amino acid titration is concerned. There appears to be some tryptic digestion in the flasks with even the highest acid concentrations, and this may be due to a greater initial combination of the protein with acid, leaving the latter less active in its trypsin destroying power. This will be illustrated in discussing the behavior of meat below. There is a second possibility here which must be referred to, and that is this that the trypsin may combine with the excess of protein in such a manner as to be protected more or less perfectly from the pepsin-acid action. This view will be taken up later.

Series D. Meat Digestion.

Three sets of tests have been carried out here, two with raw meat and one with cooked meat, and under the same general conditions as have been described for the fibrin and casein. The meat used in the raw condition was thoroughly washed to remove blood and easily soluble salts and then pressed out to eliminate the excess of water. In each case 4 g. were taken to make up the substrate and mixed with pepsin, trypsin and acid to make up a volume of 100 cc. as before. The digestions, neutraliza-

tions, division of the volume and addition of more meat for the tryptic digestions were carried out as before. These two sets of trials with raw meat were made six months apart and are reported separately to show about what degree of constancy may be expected under the conditions of the tests.

TABLE VII*a*.—PEPSIN-TRYPSIN DIGESTION WITH RAW MEAT.

No.	Vol. of 0.1*N*HCl. Cc.	Vol. of 0.2*N*NaOH. Cc.	A—A′, etc. Cc.	General appearance.
A	50	20.0		After the peptic digestion but little meat or other residue remained in any case, but there was always a precipitate on neutralization. After the tryptic digestion there was some meat left in all cases, in the unboiled as well as in the boiled flasks.
A′	50	11.2	8.8	
B	40	19.8		
B′	40	11.3	8.5	
C	35	20.6		
C′	35	11.1	9.5	
D	25	21.7		
D′	25	11.5	10.2	
E	23	23.8		
E′	23	11.3	12.5	
F	20	24.6		
F′	20	11.1	13.5	
G	20	25.0		
G′	20	11.0	14.0	

TABLE VII*b*.—PEPSIN-TRYPSIN DIGESTION WITH RAW MEAT.

No.	Vol. of 0.1*N*HCl. Cc.	Vol. of 0.2*N*NaOH. Cc.	A—A′, etc. Cc.	General appearance.
A	50	20.2		In the peptic digestion there was a practical disappearance of all the meat. The neutralization precipitate was small. In the following tryptic digestion there was meat left in all cases, from the second gram added.
A′	50	12.0	8.2	
B	45	21.0		
B′	45	13.4	7.6	
C	40	19.8		
C′	40	11.8	8.0	
D	35	20.5		
D′	35	13.0	7.5	
E	30	19.5		
E′	30	11.8	7.7	
F	25	21.7		
F′	25	12.0	9.7	
G	20	24.8		
G′	20	12.0	12.8	
H	15	28.8		
H′	15	11.5	17.3	
I	25	32.4		
I′	25	19.5	12.9	100 mg. of pepsin used.

At first sight it is apparent from these tables that a marked liberation of amino acid nitrogen occurs in the mixtures with the higher hydrochloric acid concentrations. To some extent this was noticed in the casein digestions, as distinguished from those with fibrin, but the dry casein

weights were a little larger than the dry fibrin weights which might account for a somewhat greater binding of acid. But such an explanation does not hold here where the action is pronounced. The four grams of moist, raw meat used in the initial incubations contained ordinarily less than a gram of actual protein. In grinding different lots of meat and washing as described, the Kjeldahl determinations have given amounts of nitrogen corresponding to a range of 0.9 g. to 1.0 g. of protein. A mean of 0.95 g. may be taken for the pulp employed here, which is a little greater than the fibrin weight. The acid-binding power of this meat is not greater than was found for the fibrin, as the following results of experiments conducted as were those with fibrin show. In each incubation 4 g. of meat with constant amounts of pepsin and trypsin and varying acid were employed.

Vol. of 0.1 *N* HCl in 100 cc.	P_H.	C_H.	Vol. of 0.1 *N* HCl in 100 cc.	P_H.	C_H.
50 cc.	1.74	0.0182	25 cc.	2.51	0.0031
35 cc.	2.06	0.0087	15 cc.	2.94	0.0011

In fact, the residual ion concentration, while small, appears to be greater than with the liquids secured from the fibrin experiments, from which it follows that the apparent tryptic activity in this case cannot be attributed to a lower hydrogen ion value, as was suggested above as a possibility.

It is well known that trypsin has a marked tendency to attach itself to certain protein groups and the meat proteins may have this binding and protecting power in a degree more marked than obtains with fibrin or casein. This brings us back to the hypothesis of Edie,[1] advanced to account for lowered pepsin activity in presence of trypsin, but the binding power of the protein may be just as important in holding and protecting the ferment as it is in holding the acid. In the one case we have a lowered hydrogen-ion concentration, while in the other the specific group of the ferment on which the acid may act destructively may be the one through which the ferment is linked to the protein. With high acidity this linkage would be broken with following destruction of the ferment. On the other hand, in the case of the low hydrogen-ion concentration, the stimulating value alone of these ions might come into play, with consequent solution of the protein linked up with the ferment.

The digestion tests with cooked meat were made in the same manner and with the same proportions. Since meat loses water in cooking the 4 g. taken for each mixture must represent a somewhat larger weight of real protein than is the case with the moist meat. The acid binding power would be correspondingly greater, which seems to show in some of the experiments.

[1] *Loc. cit.*

TABLE VIII.—PEPSIN-TRYPSIN DIGESTION WITH COOKED MEAT.

No.	Vol. of 0.1*N*HCl. Cc.	Vol. of 0.2*N*NaOH. Cc.	A—A′, etc. Cc.	General appearance.
A	50	17.9		Most of the flakes of meat disappeared during the peptic digestion, but a fine sediment settled. A precipitate formed on neutralization which was larger than with the raw meat.
A′	50	10.0	7.9	
B	45	18.5		
B′	45	9.3	9.2	
C	40	17.6		
C′	40	9.3	8.3	In the appearance of the flasks after the second digestion there was not much difference between the boiled and unboiled ferment portions.
D	35	21.2		
D′	35	9.0	12.2	
E	30	19.8		
E′	30	8.7	11.1	The trypsin ferment was killed in both I and I′.
F	25	23.5		
F′	25	9.7	13.8	
G	20	25.1		
G′	20	10.0	15.1	
H	15	24.7		
H′	15	9.3	15.4	
I	15	9.5		
I′	15	9.5		

We have here the same general situation noticed in the digestions of the raw meat samples. In the trials with the weaker acid in the substrate the persistence of the trypsin is very apparent, while in the other cases the residual digestion is smaller but constant. In two experiments to determine the hydrogen concentration at the end of the peptic digestion of cooked meat under the conditions as obtaining above we found these results for liquids filtered from the flasks made up with 4 g. of cooked meat and 50 cc. and 25 cc. of the 0.1 *N* acid in 100 cc.

50 cc. flask..... $P_H = 2.03$ $C_H = 0.00927$

25 cc. flask..... $P_H = 2.61$ $C_H = 0.00245$

These degrees of acidity are so extremely small that we can well understand how the tryptic activity may persist to some extent. As pointed out in a previously quoted paper[1] peptic digestion begins to slow down at a concentration of hydrogen ions in the neighborhood of $P_H = 2.60$. The acid present is pretty well bound to the protein in these cases and not enough is free to seriously interfere with the trypsin.

In the discussions above the effects observed at the end of the initial incubation are spoken of as due to "peptic" digestion. This expression is used largely for convenience and includes the effects of simple incubation in producing hydrolysis under the conditions. The ordinary protein bodies when brought into solution in any manner show some acid groups by the formaldehyde titration, but in some cases the apparent effect may be due to the protein split products in the ferments themselves. It was formerly held that in the ordinary short pepsin digestion not many

[1] Long, THIS JOURNAL, **37**, 1333 (1915).

peptide bonds are loosened in such a manner as to show anything in this titration. This view seems to have been held, for example, by Soerensen[1] himself, who introduced the general method. But enough work has been done since to demonstrate the value of the scheme in just such studies as the above. In a paper by J. Christiansen[2] some interesting information is given as to the extent of the preformed acid groups in some of the commercial ferments and the extent of the liberation of new groups by self digestion. The time element comes in here as an important factor.

But we are not concerned with the extent of the real peptic splitting in the initial incubation, or how much of the carboxyl measured here, by the titration after addition of formaldehyde, is due to a splitting just completed, as distinguished from the carboxyl of the ferments, etc. It is the *increase* of the carboxyl in the second incubation which interests, and under the conditions as brought out there seems to be no question as to the part taken by the trypsin here. All the results secured point in the same direction and to the conclusion that the pancreatic ferment persists through the acid incubation, provided this acid is sufficiently bound by protein to bring the hydrogen-ion concentration down to certain values.

This fact must have an important bearing on the answer to the practical question proposed at the beginning of the paper, which amounts to this: Will trypsin administered by the mouth persist in the stomach and retain sufficient activity to aid in proteolytic digestion in the duodenum? It is evidently true that trypsin when given in relatively large amount and in presence of protein possesses the degree of resisting power requisite for this. Not only does trypsin appear to resist the action of pepsin and acid under the conditions described above but it seems further likely that it is able to bring about some degree of digestion in acid solution which is more marked with some proteins than with others. This kind of activity toward fibrin seems to be less marked than with casein or meat, which may depend on some peculiar binding power or configuration in the fibrin complex as distinguished from the others.

It has been pointed out above that the work of Michaelis and Davidsohn[3] seems to indicate some slight degree of tryptic activity in low acid concentrations, while the same conclusion evidently follows from the studies of Mays[3] already quoted. That tryptic digestion, while greatly impeded in hydrochloric acid solutions, may go on very well in presence of lactic acid or weak acetic acid was shown in the investigations of Lindberger[4] who attempted to account for the protein digestion in the acid duodenal

[1] *Biochem. Z.*, **7**, 45 (1907); **21**, 289 (1909).

[2] *Ibid.*, **46**, 50 (1912).

[3] *Loc. cit.*

[4] *Maly's Jahresb.*, **13**, 280 (1883). From the Swedish. Reviewed at length by Hammarsten.

tract of the dog. At that period the relation of acidity to hydrogen-ion concentration was not known. Weak lactic acid furnished the proper medium for the purpose. The commonly accepted view that trypsin is readily destroyed by acids and that it can exert its peculiar behavior only in alkaline solutions follows, in part, from the assumed alkaline character of the pancreatic juice which is able to neutralize completely the acid chyme and leave a marked degree of alkalinity. Many recent observations have shown that the alkalinity of the pancreatic juice is often less than formerly considered "normal." The mixed duodenal fluid in dogs is frequently found to be not alkaline at all. It is also possible, as has indeed been frequently suggested, that for the initial solution and superficial splitting of the protein through trypsin a greater degree of alkalinity is called for than is favorable in the subsequent deep-seated loosening of peptide bonds. If the initial hydrolysis is accomplished through the action of pepsin and acid a nearly neutral medium might furnish the optimum condition for the later cleavage.

In recent papers by Long and Fenger[1] it has been shown that the press juice of the pancreas of hogs, sheep and cattle is distinctly acid and constantly so. More recent observations of Dr. Fenger of the Research Laboratories of Armour and Company and Mr. Nelson of this laboratory, as yet unpublished, have shown that in the juice of the duct of hogs, noted immediately after killing, the reaction is as often acid as alkaline. This undoubtedly bears some relation to the time of last feeding, but the result cannot be ascribed to the presence of traces of acid chyme which might have penetrated the duct from the duodenum. When expressed in terms of hydrogen-ion concentration the mixed duodenal fluid may, perhaps, be more often alkaline than acid, but the degree of alkalinity is so slight and so much lower than usually assumed that there appears to be no physiological necessity for the assumption that trypsin can act *only* in an alkaline medium, or that it is readily destroyed by weak acids of a concentration of physiological importance.

Coming now to a practical side of the discussion this observation is in order. While it is undoubtedly true that trypsin is able to withstand incubation with pepsin and acid in presence of protein sufficient to give a certain low resultant concentration of hydrogen ions, and while it is true that a certain degree of tryptic digestion may be possible in a medium in which the hydrogen-ion concentration is apparently greater than 10^{-7} it does not follow that the practical value of tablets or powders containing a few milligrams of trypsin along with pepsin is worth considering. In the above experiments we have dealt with relatively large weights, and even greater amounts might have to be administered to have much effect in the conditions of the stomach. The most important factor in this

[1] THIS JOURNAL, 37, 2213 (1915); 38, 1115 (1916).

connection is not the absolute weight of hydrochloric acid or pepsin present but the relation of one to the other, and the resultant hydrogen-ion concentration.

Summary.

It has been shown in previous papers from this laboratory, as well as by other investigators, that trypsin may be incubated with HCl of P_H = 1.5 through half an hour or longer without appreciable loss of strength. In presence of pepsin the tryptic power is rapidly lost.

However, if sufficient protein is likewise present the acid, in combining with it, is unable to destroy in the same degree. When the acid concentration is reduced in this manner to P_H = 2.6, or below, tryptic activity persists, even through several hours at the temperature of the body. This is a practical condition which very commonly obtains in the human stomach. An active tryptic ferment would unquestionably pass with the chyme, in part at least, into the duodenum where the P_H value is quickly reduced to 6.5, or lower, and there be able to produce a normal proteolytic digestion of some degree.

From the above experiments it appears further likely that some actual protein splitting is accomplished by trypsin at a P_H concentration of 1.8 with certain types of proteins. The rapidity of this proteolysis must be slight, however, and the practical importance low.

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CHICAGO, ILL.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, No. 268.]

EXPERIMENTS UPON THE AMYLASE OF ASPERGILLUS ORYZAE.

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The amylase produced by the fungus *Aspergillus oryzae* has been known to science since about 1875 after having been used empirically in Japan for centuries. It forms the chief active constituent of taka-diastase, introduced by Takamine in 1898,[1] which is prepared by growing the fungus on wheat bran, extracting with water and precipitating by the addition of alcohol in such quantity as to give a concentration of 70% alcohol by volume.

The present study was undertaken with the object of purifying the amylase as far as practicable and comparing its nature and properties

[1] *J. Soc. Chem. Ind.*, **17**, 118.