Discussion and Summary.

The following conclusions seem to be justified from the authors' experience with the persulfate methods, as well as from the results obtained:

1. That the process of digestion is shortened considerably in the macro method, thereby decreasing the danger of the loss of nitrogen by prolonged heating.
2. That the oxidation is rapid and complete, so far as the ordinary constituents of the urine are concerned. This is indicated by the agreements with the Arnold-Gunning method.
3. That the entire procedure may be carried out in a small fraction of the time required by the ordinary Kjeldahl process, or one of its modifications.
4. That the accuracy is not inferior to the Arnold-Gunning method, though a closer scrutiny and a greater number of determinations must be made to put this beyond question.
5. That the micro method yields results quickly. These agree well with the standard method employed, considering the inherent possibilities of error.
6. It is necessary that the persulfate salt used be free from nitrogen and that all traces of calcium and magnesium be eliminated from the distilled water used in making the dilutions.

NEW ORLEANS, LOUISIANA.

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

ON THE OPTIMUM REACTION IN TRYPTIC DIGESTION. I.

BY J. H. LONG AND MARY HULL.

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Tryptic activity was long supposed to be possible in a medium of slightly alkaline reaction only. This assumption was in part based on the view commonly held that in man the pancreatic juice is rather markedly alkaline, and that this reaction, along with that from the bile, is imparted to the upper intestinal tract where pancreatic proteolysis takes place. Evidence accumulated, however, to show that the balance between the reaction of the chyme as it passes the pylorus and the several secretions poured into the duodenum is so nicely adjusted that on a mixed diet a nearly neutral condition in the small intestine follows, and indeed throughout its whole extent.[1] Something depends on the character of the food, however, as certain foods, through their digestion products, are potentially acid, while others may be alkaline. Proteins, as amphoteric elec-

[1] *Zentr. Physiol.*, 16, 33, 146 (1902); Cohnheim, "Die Physiologie der Verdauung und Ernaehrung," p. 94 (1908).

trolytes, function in both directions, but in some cases one reaction is much more marked than the other.

Following observations on fluids collected from duodenal fistulas several investigators attempted to discover by tests *in vitro* the condition most favorable to tryptic digestion. According to Schierbeck[1] the reaction most favorable for digestive ferments in general, excluding pepsin, is that of a weak alkali solution supersaturated with carbon dioxide, or nearly neutral, for which condition we have $P_H = 7$, expressed in modern terms. Kanitz[2] was perhaps the first to attempt to express the optimum reaction in tryptic digestion in terms of the important factor of hydrogen or hydroxyl concentration. He placed the favorable degree of alkalinity as that of a solution 0.014 to 0.005 N with reference to [OH] ions, which value is probably too high in any case, and certainly too high for some proteins.

Michaelis and Davidsohn[3] have given the most recent and accurate determination of the degree of dependence of tryptic activity on the hydrogen-ion concentration of the substrate medium, employing the gas chain method in the measurement of the reaction. They point out, as has been recognized by other workers in the same field, that such experiments can be made only by the aid of media so constituted as to behave as energetic buffers, since a protein substrate itself combines with acid or alkali (or both) and thus alters the reaction. But in their experiments a number of important points have been left out of consideration, and they have apparently assumed that all proteins exhibit the same behavior, which may be far from the truth. As our own experiments have frequently brought us face to face with the question of the relation of tryptic activity to reaction we have undertaken a fuller study of some of the factors involved.

It appears from the tables given by Michaelis and Davidsohn that they calculated from the composition of the buffer solution itself its probable reaction, and then found it by actual determination, with the protein added, before incubation. The explanations given are not very clear on this point. Between the calculated and observed values there is often a wide divergence, due to the combining power of the protein, which in their experiments was a solution of peptone. A second determination may profitably be made here and that is on the mixture left at the end of the digestion, because under certain degrees of ion concentration a very considerable change may take place during the digestion, unless a rather excessive concentration of the buffer medium is employed. With such an excess of salts in the substrate solution it is doubtful if a really normal reaction may be assumed to occur.

[1] *Scand. Arch. Physiol.*, **3**, 344 (1891).

[2] *Z. Physiol.*, **37**, 75 (1902).

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

Our method of experimentation was in general this: We followed the digestion of dried and finely powdered fibrin and dry casein powder in liquids of known composition with definitely varied hydrogen-ion concentration. In each individual test we employed 50 cc. of the liquid, 1 g. of the protein and 0.1 g. of the active trypsin ferment, in this case the product of Fairchild Brothers and Foster. The digestions were carried out through a period of three hours at 40°, the mixtures being shaken at definite intervals. For a number of reasons we preferred to employ the solid proteins in the substrates rather than a soluble peptone as used by Michaelis and Davidsohn, notwithstanding the fact that somewhere more regular results might be obtained with a body of the latter type. But in such compounds the first stages of digestion are already passed and it was our wish to follow the proteolysis from the beginning. The use of the solid and insoluble protein, would, in addition, correspond more nearly to the conditions obtaining in the animal body.

In this work we desired, further, to note the effects in media more acid than solutions of primary potassium phosphate, on the one hand, and more alkaline than solutions of secondary sodium phosphate, on the other. We secured the desired range by making the following three groups of mixtures: In the first group we mixed 125 cc. of 0.1 *M*[1] KH_2PO_4 solution with decreasing volumes of 0.1 *N* HCl, and made each volume resulting up to 250 cc. with fresh distilled water. The HCl volumes were 50 cc., 40 cc., 30 cc. and so on, giving in the completed mixtures a hydrogen concentration running from $P_H = 2.37$ to $P_H = 4.81$, as measured by the gas chain method.

In the second group we mixed varying proportions of 0.067 *M* primary and secondary phosphate solutions to make 100 cc. and then diluted to 200 cc. with pure water. The P_H range here was from 6.21 to 9.48.

For the third group we used mixtures of 0.067 *M* secondary phosphate and 0.1 *N* NaOH to make 100 cc. and then diluted to 200 cc., securing in this way a P_H range of 10.81 to 11.86.

On mixing protein with a solution of secondary phosphate and allowing to stand for the production of a condition of equilibrium, which is hastened by shaking, the supernatant liquid becomes less acid, while, conversely, protein added to the primary phosphate diminishes its acidity. For a secondary phosphate solution used in a preliminary trial the change in the P_H value was from 9.88 to 7.27, while for a primary phosphate solution the change, in becoming less acid, was from $P_H = 5.15$ to 6.24. From protein bodies, as amphoteric electrolytes, we should expect this peculiar behavior. It is exhibited much more strikingly in some of the solutions of greater acidity or alkalinity used below.

The change does not stop here, however. On allowing some of these

[1] *M* is used for molal concentration in this article.

mixtures to digest in the thermostat through three hours there is a further variation in the same direction. That is, during the digestion the acid solutions become still less acid, probably through the liberation of more amino groups, or more amino than carboxyl groups. The more strongly alkaline solutions become less alkaline in the digestion, while in mean ranges the change is not great. All of these points are best brought out by the full tables in which the results are given in condensed form.

The hydrogen-ion concentrations were all measured by the gas-chain method and at 20°. We wish here to call attention to a practical matter of importance where a large number of tests have to be made. For some time back we have been using electrolytic hydrogen for the saturation of the platinum black plates because of its cheapness, and especially because of its convenience in use. An extremely pure grade of hydrogen made by electrolysis of an alkali solution, sodium hydroxide, between iron electrodes is obtainable in large cities, and this requires nothing more than washing through two or three bottles containing a strong solution of sodium pyrogallate, the alkali in considerable excess, to make it quite safe for use. Much time is saved in this way as tanks of hydrogen containing 100 or 500 cubic feet under a pressure of 1800 pounds to the square inch are obtainable and are not too large for easy handling. Furnished with a proper reducing valve a very uniform flow of the gas is secured, and enough to last for weeks or months, even. In the tanks we have used, the only initial impurity seemed to be a trace of oxygen, easily removed.

In the measurement of the digestive effect we employ the formaldehyde titration with the necessary preliminary removal of the phosphates. This is done by the addition of barium chloride and hydroxide to the 50 cc. of incubated mixture at the end of the three-hour period, and enough to produce a distinct alkaline reaction to phenolphthalein. After making the volume to 100 cc. a portion is filtered off, from which 50 cc. are brought to litmus neutrality by aid of hydrochloric acid, and titrated with phenolphthalein and 0.2 N sodium hydroxide after the addition of the neutral formaldehyde. A double digestion is made in all cases, the second one containing the same weight of buffer, protein and trypsin as the first, but with the ferment killed by preliminary boiling. With this blank the various operations are carried through just as with the active solution and the titration result is subtracted from the first result to get the actual fermentation effect. Finally, this value is doubled to secure the net result for the whole volume taken. These doubled titration values are given in the tables to follow. A small error is involved in the process as no allowance is made for the volume of the precipitate formed when the barium solutions are added. From additional tests we know that this error is quite small, and besides it is found in all the results to essentially

the same degree, which leaves them strictly comparable, and this is all that is required for our purpose.

In the tables below, the first column shows the composition of the fluid in which the gram of fibrin or casein plus the tenth gram of trypsin is digested. In the second column is recorded the [H] value of the buffer solution before the addition of the protein and ferment. This could be calculated with a fair degree of accuracy in most cases but has always been directly determined. The third column shows the change which has taken place by the addition of the protein substances to the buffer. This new [H] value was measured only after the mixtures had stood at room temperature an hour to allow for complete combination, which for solid substances is not immediate. The portion tested was filtered off in these and the following measurements. In the digestion experiments separate 50 cc. portions were incubated for the electrometric tests and for the titrations, the results of which are shown in the fourth and fifth columns:

TABLE I.—FIBRIN DIGESTION.

Composition of mixtures.	P_H of original liquid.	P_H after adding protein.	P_H after digestion.	Cc. 0.2 *N* NaOH for titration.
10 cc. 0.1 *N* HCl, 25 cc. 0.1 *M* primary phosphate, 15 cc. water	2.37	3.34	3.82	0.4
8 cc. HCl, 25 cc. phos., 17 cc. water	2.45	3.77	4.20	1.3
6 cc. HCl, 25 cc. phos., 19 cc. water	2.64	4.28	4.65	9.1
4 cc. HCl, 25 cc. phos., 21 cc. water	2.96	4.84	5.13	11.7
2 cc. HCl, 25 cc. phos., 23 cc. water	3.24	5.38	5.65	13.1
0 cc. HCl, 25 cc. phos., 25 cc. water	4.81	5.85	6.00	12.7
2.5 cc. 0.067 *M* sec. phos., 22.5 cc. 0.067 *M* prim. phos., 25 cc. water	6.21	6.43	6.17	15.8
5 cc. sec. phos., 20 cc. prim. phos., 25 cc. water	6.54	6.47	6.27	15.9
7.5 cc. sec. phos., 17.5 cc. prim. phos., 25 cc. water	6.77	6.59	6.46	15.5
10 cc. sec. phos., 15 cc. prim. phos., 25 cc. water	6.95	6.80	6.70	15.7
15 cc. sec. phos., 10 cc. prim. phos., 25 cc. water	7.28	7.01	6.91	18.7
20 cc. sec. phos., 5 cc. prim. phos., 25 cc. water	7.68	7.25	7.11	18.8
22.5 cc. sec. phos., 2.5 cc. prim. phos., 25 cc. water	8.03	7.41	7.27	19.0
25 cc. sec. phos., 25 cc. water	9.48	7.61	7.34	20.0
25 cc. 0.067 *M* sec. phos., 2 cc. 0.1 *N* NaOH, 23 cc. water	10.81	8.01	7.38	20.7
25 cc. sec. phos., 4 cc. NaOH, 21 cc. water	11.21	8.32	7.57	20.0
25 cc. sec. phos., 6 cc. NaOH, 19 cc. water	11.43	8.49	7.71	15.5
25 cc. sec. phos., 8 cc. NaOH, 17 cc. water	11.63	8.70	7.89	13.3
25 cc. sec. phos., 10 cc. NaOH, 15 cc. water	11.79	8.98	8.05	13.8
25 cc. sec. phos., 12 cc. NaOH, 13 cc. water	11.86	9.17	8.28	11.8
25 cc. sec. phos., 15 cc. NaOH, 10 cc. water	....	....	....	9.1
25 cc. sec. phos., 20 cc. NaOH, 5 cc. water	....	....	....	6.7

Several points of importance appear at a glance from these tables. We find in both cases a very marked change in the [H] concentration of the solutions on adding the protein. In the case of the fibrin this follows through the stronger acid mixtures down to a hydrogen-ion value of about

$P_H = 6.5$. Here the addition of protein does not change the reaction and evidently because the dissociating hydrogen and hydroxyl ions of the protein must be in equilibrium with the corresponding ions of the buffer solution. At this point the relative acidity of the phosphate mixture is about 14, because these two concentrations follow from the observations: $[H] = 3.2 \times 10^{-7}$, and $[OH] = 0.23 \times 10^{-7}$. This must show approximately the relative acidic and basic dissociation constants of the fibrin, and it follows that as weaker and weaker buffer solutions, in [H], are employed the reaction of the supernatant liquid must grow progressively less alkaline, relatively. With sufficient fibrin used the P_H value of the mixture may be brought to the point where the reacting ions were first in equilibrium.

TABLE II.—CASEIN DIGESTION.

Composition of mixtures.	P_H of original *liquid.*	P_H after adding *protein.*	P_H after *digestion.*	Cc. 0.2 *N* NaOH for *titration.*
10 cc. 0.1 *N* HCl, 25 cc. 0.1 *M* primary phosphate, 15 cc. water	2.37	3.14	3.43	2.2
8 cc. HCl, 25 cc. phos., 17 cc. water	2.45	3.43	3.71	7.2
6 cc. HCl, 25 cc. phos., 19 cc. water	2.64	3.93	4.05	9.0
4 cc. HCl, 25 cc. phos., 21 cc. water	2.96	4.28	4.37	10.3
2 cc. HCl, 25 cc. phos., 23 cc. water	3.24	4.78	4.72	12.2
0 cc. HCl, 25 cc. phos., 25 cc. water	4.81	5.21	4.98	14.9
1.25 cc. 0.067 *M* sec. phos., 23.75 cc. 0.067 *M* prim. phos., 25 cc. water	5.90	....	5.04	20.0
2.5 cc. sec. phos., 22.5 cc. prim. phos., 25 cc. water	6.21	5.55	5.43	20.5
5 cc. sec. phos., 20 cc. prim. phos., 25 cc. water	6.54	5.76	5.73	20.0
10 cc. sec. phos., 15 cc. prim. phos., 25 cc. water	6.95	6.04	6.12	20.0
15 cc. sec. phos., 10 cc. prim. phos., 25 cc. water	7.28	6.30	6.54	19.1
20 cc. sec. phos., 5 cc. prim. phos., 25 cc. water	7.68	6.65	6.69	18.0
25 cc. sec. phos., 25 cc. water	9.48	6.99	6.95	17.0
25 cc. 0.067 *M* sec. phos., 2 cc. 0.1 *N* NaOH, 23 cc. water	10.81	7.04	7.08	15.8
25 cc. sec. phos., 4 cc. NaOH, 21 cc. water	11.21	7.13	7.15	13.6
25 cc. sec. phos., 6 cc. NaOH, 19 cc. water	11.43	7.32	7.27	14.0
25 cc. sec. phos., 8 cc. NaOH, 17 cc. water	11.63	7.42	7.37	14.2
25 cc. sec. phos., 10 cc. NaOH, 15 cc. water	11.79	7.54	7.49	12.9
25 cc. sec. phos., 12 cc. NaOH, 13 cc. water	11.86	7.73	7.61	12.8

During the digestion of the fibrin, as pointed out above, the changes which take place by simple mixing become accentuated and the tables show clearly the extent of the change. The behavior of the buffer mixtures having a P_H value of 6.2 to 6.9 shows that the amino and carboxyl groups are developed in practically equal proportions within this range, and keep in equilibrium with the ions of the phosphate solutions. For the most strongly acid and alkaline mixtures, however, the alteration is very marked and in the case of the acid the total loss of [H] is about 0.2 mg. for the gram of fibrin digested.

In a general way the behavior of casein with the same solutions is similar. At the outset the 50 cc. of acid buffer solution become less acid, although not to the same extent shown by the fibrin. On the other hand the most strongly alkaline of the reinforced phosphate solutions lose their alkalinity to a marked degree and approach the neutral stage. This condition must follow because casein is an uncommonly acid protein and able to form pretty stable salts with alkalies. The acidity of the casein is so pronounced that a neutral condition, after digestion, is not reached until one of the alkaline media is employed.

The digesting medium and the protein are not in initial equilibrium at the same point found for the fibrin, but at a point of distinctly greater acidity. The tables do not show this exactly but it is approximately at $P_H = 4.9$, which indicates a marked excess of hydrogen ions. The acidic dissociation is enormously greater than was indicated for the fibrin, and this undoubtedly has some bearing on the quality of digestion. In the last alkaline phosphate solution the hydrogen-ion concentration increases from 1.4×10^{-12} to 2.4×10^{-8}, and at the expense of the casein.

The greatest interest, however, attaches to the extent of the digestion in the different media. For the fibrin there is practically no action in the acid mixture of P_H = about 3.7, but at 4.28 there is already a marked proteolysis. This is a [H] concentration of 5.2×10^{-5}. According to Michaelis and Davidsohn,[1] trypsin is quite inert at a hydrogen concentration of 10^{-4}, but this statement is based on the behavior of one protein only. The digestive activity increases to a P_H concentration of about 7.5, and remains nearly at this level through $P_H = 8.3$, after which there is a decrease in activity. The first potential value corresponds to $C_H = 3 \times 10^{-8}$ and there is really but little variation in the extent of digestion in three hours between concentrations of 10^{-8} and 5×10^{-9}. The authors quoted carried their observations through much shorter periods, usually from 10 to 60 minutes, and occasionally to 90 minutes. They assume that the optimum reaction for trypsin is found at $C_H = 10^{-8}$, but from their tables this value does not appear more likely than 10^{-7} or 10^{-10}. In fact, with their observations, as with ours, there is a considerable range in which a marked difference in activity is not apparent. In this respect our results stand in fairly good agreement.

A consideration of the casein table brings out other singular facts. As with the fibrin the amount of digestion in the buffer solutions of greatest hydrogen concentration is but slight. The rate of digestion increases to the solution where the P_H value after mixing is 5.2. Then there is a sudden jump in digestive activity when the potential value becomes about 5.5, and this brings a practical maximum as with the fibrin. It is of course probable that the spring is not as sudden as appears in the table; a fuller

[1] *Loc. cit.*

range of potential and digestion results at this point would undoubtedly show a gradual rather than a sudden change within the narrow limits. But from the table results the optimum concentration for the casein is between 3×10^{-6} and 5×10^{-7}, a degree of acidity greatly in excess of that found best for the fibrin. Michaelis and Davidsohn seem to draw the conclusion from their investigations that the optimum reaction value for trypsin is the same for all proteins. This apparently is not the case. Our results for fibrin are in close agreement with theirs for peptone (peptonum siccum, Riedel), but this peptone is said to be a fibrin product and agreement might therefore be expected.

With the more alkaline media the casein went into partial solution before digestion. At the end of the three-hour period some of the protein in solution was dissolved by the alkali only and was filtered out as a neutralization precipitate. This behavior was likewise shown by the higher blanks obtained by the casein.

From the behavior of the casein we are inclined to believe that there may be for each kind of protein a distinct reaction optimum for these ferments which work near the neutral point. This would not hold for pepsin, the behavior of which is very different, inasmuch as it acts in a relatively strong acid medium. But it might hold for papain, the activity of which is not very marked, but which is shown, apparently, on both sides of the neutral point. These optimum conditions will have to be worked out by experiment for different types of protein bodies. There is the further possibility that different trypsins may exhibit a difference in behavior in this respect, since what we call trypsin is in all probability far from being a simple and well-defined substance. In the preparations isolated by different processes there may be several distinct ferments of closely related behavior, a possibility suggested by a variety of observations.[1]

While the digestion of casein is slight in the medium of $P_H = 3.14$ or $C_H = 7.2 \times 10^{-4}$ it is appreciable and too great to warrant the statement of Michaelis and Davidsohn that trypsin is inert at a hydrogen-ion concentration of 10^{-4}. Our results with casein were so unexpected that we were led to repeat the whole series of tests. The new findings were clear cut and in complete agreement with those first secured. All these observations suggest that there may be some appreciable tryptic digestion of certain proteins in the stomach, although the time factor here is undoubtedly an important one. At the low degree of acidity at first obtaining trypsin in the stomach might possibly digest some protein and the action would be arrested only by the accumulation of hydrochloric acid to a [H] concentration above 10^{-4}. At these lower degrees of acidity we note the so-called rennin reaction, as it occurs in milk.

[1] See Long and Barton, This Journal, 36, 2164 (1914)

In all these proteolytic reactions, as in enzymic changes in general, there must be at least three factors, the substrate, the ferment, and the acid or alkali accelerator. Temperature may enter as a fourth modifying factor. Now it is evident that with a difference of molecular structure the proteins may fall apart with different degrees of facility, and this fact must enter to modify the extent or conditions of ferment action. It is not the ferment alone which must be considered as dependent on the reaction of the medium, but the ferment in relation to the substrate. These two constitute a new complex and the optimum reaction may therefore be expected to vary with the substrate. From this point of view other proteins are now under investigation.

Conclusions.

Trypsin acts on fibrin and fibrin peptone most energetically at a hydrogen-ion concentration between 10^{-8} and 5×10^{-9}. Our work on the former leads to results closely agreeing with those of Michaelis and Davidsohn for the latter.

For casein the optimum hydrogen concentration is distinctively greater, and within the limits 3×10^{-6} to 5×10^{-7}. In addition to this the digestion proceeds at a degree of acidity much greater than that for the beginning of the fibrin digestion.

It is probable that for each type of protein substance there is a distinct range for the optimum activity. It is suggested that it may be the enzyme plus the substrate rather than the enzyme alone which is affected by the reaction.

CHICAGO, ILL.

[CONTRIBUTION FROM THE OTHO S. A. SPRAGUE MEMORIAL INSTITUTE AND THE PATHOLOGICAL LABORATORY OF THE UNIVERSITY OF CHICAGO.]

THE MECHANISM OF THE NINHYDRIN REACTION.

A CONTRIBUTION TO THE THEORY OF COLOR OF SALTS OF ALLOXANTINE-LIKE COMPOUNDS.[1]

BY J. M. RETINGER.

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Ninhydrin is the name given by Abderhalden to triketohydrindenehydrate, which because of its peculiar color reaction with amino acids and amines, is used by him as indicator in his dialysis method for detection

[1] The experimental work upon which this article is based was completed at the University of Leipzig in 1913 under the direction of Prof. A. Hantzsch and appears among the publications of that institution. Out of courtesy to Prof. Hantzsch with whom it is impossible to communicate at present, the author does not feel justified in quoting in detail from the protocols.

After submission of this paper for publication Prof. J. Stieglitz called my attention to a publication by G. N. Lewis in the Proceedings of the National Academy of Sciences, 1916, p. 586–92, in which a very similar idea for color production in special cases