In order to show the accuracy of duplicate determinations by the modified method, several products were analyzed for citric acid. The results are shown in Table IV. For the sake of comparison, the table of results given in the 1912 report of the A. O. A. C. is also reproduced.

These results seem to warrant the conclusion that the modified method, if followed rigidly, will give much more satisfactory results than the original one. It is hoped that other workers will give the proposed method a trial, as a trustworthy method for determining citric acid in plant products is needed.

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AN EXAMINATION OF CERTAIN METHODS FOR THE STUDY OF PROTEOLYTIC ACTION.

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There is no lack of methods for the study of proteolytic action but, in general, each method has been devised to meet the needs of a particular line of investigation, or to determine whether or not a commercial pepsin or trypsin complies with the standard arbitrarily established for it. As a preliminary to research upon the proteases, it seemed desirable to study some of these methods with reference to their accuracy and delicacy and their adaptability to proteases of more than one type. The methods studied were: The determination of total digested nitrogen, the increase of amino nitrogen as measured by the Van Slyke method, the acidimetric titration of digestion products, the change in electrical conductivity, and in rotation of polarized light, the Mett method, and the biuret and ninhydrin reactions. The work of Long and Barton[1] published soon after the present investigation was begun, made it unnecessary for us to include certain other well-known methods in our comparison. We have used commercial pepsins and trypsins of good quality and have arranged our experiments with a view to ascertaining to what extent the various methods will permit comparisons of these two types with each other. In the following pages typical results obtained by each of the methods tested are presented very briefly, the work on the ninhydrin reaction being further abbreviated in view of the investigations of Van Slyke and his associates[2] and of Harding and MacLean,[3] the results of which have become available while our studies were in progress.

[1] THIS JOURNAL, **36**, 2151 (1914).

[2] *Proc. Soc. Expt. Biol. Med.*, 11, 154 (1914); *J. Am. Med. Assoc.*, **65**, 945 (1915); Harvey Society Lectures, 1915–16.

[3] *J. Biol. Chem.*, 20, 217 (1915); **24**, 503 (1916); **25**, 319, 337 (1916).

Mett Method.

This method which has long been popular because of the convenience of observing results, and which is the only one in which we have used egg white as substrate, may be described first.

Utilizing the improvements proposed by Cobb[1] and introducing slight modifications in the interest of greater accuracy, we have carried out this method as follows:

Glass tubing of 2.5 to 3 mm. bore is cut into 20 cm. lengths and washed successively with cleaning mixture, water, alcohol, and ether. The ends are then drawn to a capillary and broken off. The white of a fresh egg is thoroughly cut with scissors, filtered through cheesecloth, placed under vacuum for an hour to eliminate air and prevent the subsequent formation of air bubbles, and then sucked up into the prepared glass tubes and the ends of the latter sealed by fusing. The egg-white is coagulated by immersing the tubes in water heated at 95°. In doing this the tubes are laid horizontally upon a wire gauze about 1 cm. below the surface of the water, the temperature of 95° is maintained for 15 minutes after the introduction of the tubes, then the source of heat is removed and the whole allowed to stand until the water is cold, lest by cooling too rapidly a large part of the tube be rendered useless by retraction of the egg-white from the glass walls. In view of the experience of Nirenstein and Schiff,[2] we have always used tubes less than three days old. In making the digestion test, 20 cc. portions of the solution of pepsin in 0.05 *M* HCl[3] (or of trypsin in 0.05 *M* Na_2CO_3[3]) were placed in flat bottomed cylindrical jars of 5 to 6 cm. diameter, the depth of the solution being thus about 1 cm.; the tubes of coagulated egg-white were cut into 2 cm. lengths with smooth, square ends, and two of these lengths immediately placed in each of the jars containing the enzyme solutions and the whole enclosed in a thermostat kept at 40°. At the end of the 20 hours allowed for the digestion test the tubes were removed and the length of column digested was measured four times at each end of both tubes, making 8 measurements on each tube or 16 measurements on the pair of tubes by which each enzyme solution was tested. These measurements were made under a microscope fitted with a mechanical stage and vernier (Leitz No. 141) and a micrometer eyepiece. Under these conditions it is believed that the error in case of pepsin digestions should never be greater than 0.1 mm. in any one reading[4] and that the average of the 16 readings taken for each enzyme solution is reasonably accurate to the second decimal place (expressed in mm.). It is this final average of 16 measurements which appears under the heading of "column digested" in Table I.

In the case of pepsin the Mett method with digestion for 20 hours at 40°, permitted measurements of activity of as little as 2 parts of the commercial enzyme in 1,000,000 of the test solution or 0.04 mg. in the 20 cc. used for a test, thus showing a fairly high degree of delicacy. For quantitative comparisons, however, this method is open to the objection that the observed result of the enzyme action increases only very slowly with the amount of enzyme present, unless this amount be very small. This is

[1] *Am. J. Physiol.*, 13, 448 (1905).

[2] *Arch. f. Verdauungskrankh.*, 8, 599 (1903).

[3] These being the concentrations of acid and alkali which in our experience are most favorable to the activity of pepsin and trypsin, respectively.

[4] In trypsin digestions the alkalinity of the solution affects the surface of coagulated eggwhite and greatly increases the error in the reading as explained beyond.

TABLE I.—RESULTS OF EXPERIMENTS BY THE METT METHOD (20 HOURS).

Concentration.	Enzyme in test solution. Wt. (mg.).	Column digested (in millimeters). Pepsin I.	Pepsin II.	Pepsin III.	Trypsin II.
25 : 1000	500	....	....	....	3.5
16 : 1000	320	....	....	....	2.8
9 : 1000	180	7.26	6.64	7.14	2.1
4 : 1000	80	5.32	5.31	6.04	1.1
3 : 1000	60	....	....	....	0.4
2 : 1000	40	....	....	....	0.0
1 : 1000	20	3.28	3.37	3.89	...
1 : 2000	10	2.53	2.57	(4.04)	...
1 : 4000	5	2.02	1.96	2.50	...
1 : 8000	2.5	1.44	1.51	1.84	...
1 : 16000	1.25	0.98	....	....	...
1 : 32000	0.625	0.63	....	....	...
1 : 64000	0.312	0.40	....	....	...
1 : 128000	0.156	0.15	....	....	...
1 : 256000	0.078	0.11	....	....	...
1 : 512000	0.039	0.05	....	....	...
Blank	None	0.00	0.00	0.00	...

shown by the data of Table I and perhaps more clearly by the curves in Fig. 1. Here the upper solid line (A), which on the scale here used appears nearly vertical, is what would represent a direct linear relationship between the amount of pepsin present and length of column digested. Curve B represents the square root relationship predicated by the Schütz-

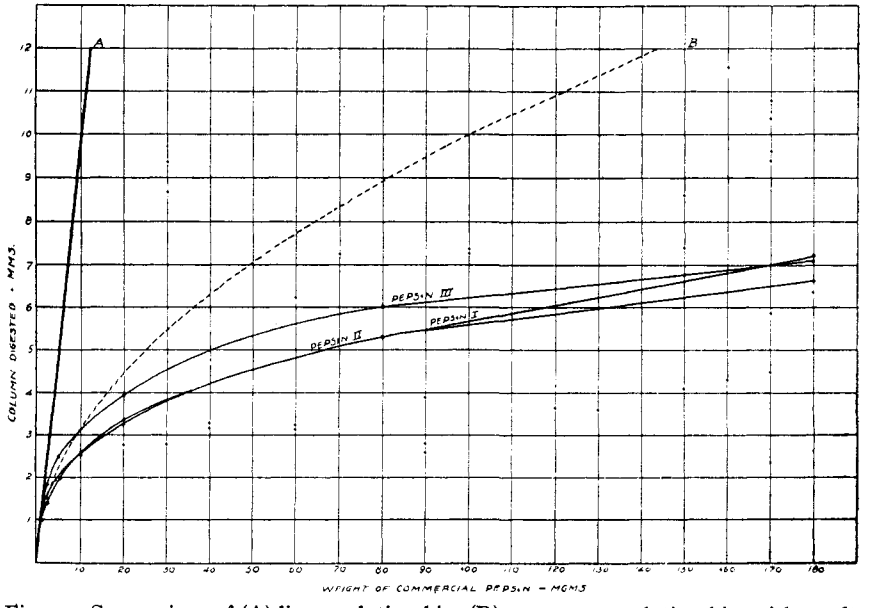

Fig. 1.—Comparison of (A) linear relationship, (B) square-root relationship, with results shown by Mett method in testing three commercial preparations of pepsin.

Borissow rule,[1] and Curves I, II, and III show the results actually obtained with the three pepsins represented in Table I.

Our results like those of Nirenstein and Schiff[2] and of Cobb[2] indicate that the Schütz-Borissow rule fails before the pepsin reaches a sufficient concentration to digest 4 mm. of the column in 20 to 24 hours. With increasing concentrations of enzyme the discrepancy increases rapidly. Taking the best result obtained among the three pepsins tested, 80 mg. actually digested only as much as should have been digested by 36 mg. according to the Schütz-Borissow square root rule, or as would be digested by 6 mg. if the observed result were in direct arithmetical proportion to the amount of enzyme present, which is obviously the desired relationship in quantitative testing of enzymes and is very nearly realized in the testing of amylases and in some of the methods available for testing proteases as will appear below. In Fig. 2 the results obtained with the smaller

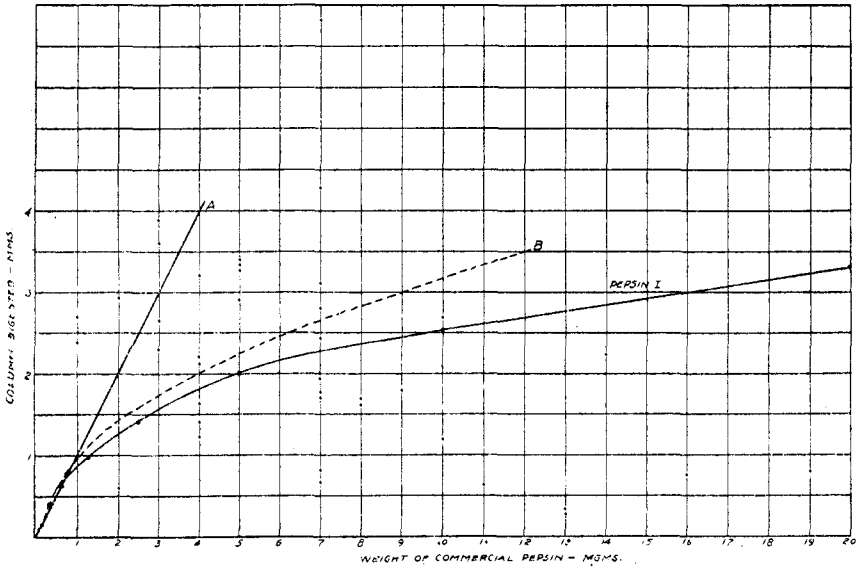

Fig. 2.—Comparison of linear and square-root relationships with results shown by Mett method in testing very small amounts of pepsin.

amounts of Pepsin I are plotted in the manner of Fig. 1, but on a larger scale in order to permit observations of the relationships in low concentrations of enzyme. Here it will be seen that the curve of actual results and the two "theoretical" curves are hardly distinguishable during the first millimeter of digestion but that to digest two millimeters, which in the case of a linear relationship would require 2 mg. and according to the square

[1] E. Schütz, *Z. Physiol. Chem.*, **9**, 577 (1885); J. Schütz, *Ibid.*, 30, 1 (1900).

[2] *Loc. cit.*

root rule should have required 4, actually required 5 mg.; while 10 mg. actually accomplished only what should have been done by 6.5 mg., according to the square root rule.

Hence it appears that the observed result in this method is approximately proportional to the amount of pepsin only at the very lowest concentrations with which of course the length of column digested is too short for any high degree of accuracy in measurement.

With trypsin all the measurements are likely to be inaccurate, because the alkali of the solution acts upon the coagulated albumin, causing a jelly-like meniscus which is very difficult to read. Moreover, the results show plainly that this method is not well adapted to the study of trypsin because egg albumin, even when coagulated, is a relatively resistant substrate for this enzyme.

Casein Substrate for all Following Methods.

In all of the experiments hereinafter described, casein[1] was used as substrate. For pepsin digestions the casein was suspended in 0.05 *M* HCl. For trypsin digestions solutions of sodium caseinate, of the slight alkalinity best suited to tryptic activity, were prepared as follows:

Ten grams of casein were dissolved in a mixture of about 200 cc. of distilled water and 8 cc. of molar sodium hydroxide. The mixture was slowly heated to 40° to aid solution and then rapidly heated to 85–90° to destroy any traces of proteolytic enzyme which might be contained in the casein. The solution was cooled and made up to 500 cc. For each determination, 50 cc. of this solution were placed in a 100 cc. flask, diluted to about 90 cc., warmed to 40° in a thermostat, constant to 0.01°, the enzyme added, and the volume made up to 100 cc. During the digestion the flasks were protected from the rays of light from the lamps in the thermostat. The enzyme action was stopped at stated intervals by pouring the digestion mixture (100 cc.) into 25 cc. of 20% sodium sulfate solution and then adding 5 cc. of half-molar hydrochloric acid. After standing for an hour, the undigested casein was filtered off and aliquot portions of the filtrate used for the measurement of proteolysis by the various methods already mentioned, such as titration, amino nitrogen, total nitrogen, etc.

In the pepsin experiments, 1 g. of casein was weighed out for each flask and dissolved in 0.05 *M* hydrochloric acid, the solution warmed, enzyme added and the volume made up to 100 cc. At the end of the digesion period, the undigested casein was precipitated by pouring the digestion mixture into 25 cc. of 20% sodium sulfate solution. After standing one hour, the casein was filtered out and portions of the filtrate analyzed as in the case of trypsin.

Total Nitrogen of Digestion Products.

In the filtrate obtained after precipitation of undigested casein by sodium sulfate and hydrochloric acid as described above, nitrogen can readily be determined by the Kjeldahl method and thus a measure obtained of the total of digestion products which have passed the early proteose stage. In using this as a method of studying proteolytic action of enzymes, "blank tests," in which all conditions and manipulations are the same except for the addition of the enzyme, must always be made and the nitro-

[1] Kahlbaum's "Casein nach Hammarsten."

gen thus found deducted. When relatively large amounts of enzyme preparation are used the soluble nitrogen thus introduced must also be allowed for. The figures given in the tables which follow are in all cases for the nitrogen found in the filtrates *in excess* of that found in the "blank." Table II shows the amounts of nitrogen digested by different weights of commercial pepsins and trypsins acting for a fixed length of time. Table III shows the results of a fixed weight of enzyme acting for different lengths of time.

TABLE II.—TOTAL NITROGEN OF DIGESTION PRODUCTS FORMED BY DIFFERENT WEIGHTS OF ENZYMES ACTING UPON 1 G. OF CASEIN IN A DIGESTION MIXTURE OF 100 CC. AT 40°.

	Half-hour digestions.		20-hour digestions.	
Mg. of commercial enzyme.	Pepsin I. Mg. of nitrogen digested.	Trypsin I. Mg. of nitrogen digested.	Pepsin I. Mg. of nitrogen digested.	Trypsin I. Mg. of nitrogen digested.
0.001	0.2	0.2	0.5	1.4
0.002	0.7	0.6	1.8	2.2
0.01	0.9	0.9	7.0	9.1
0.05	1.7	1.7	23.8	41.6
0.1	3.0	3.3	37.9	71.9
0.5	9.6	13.6	65.3	132.5
1	19.3	28.3	89.8	133.2
2	30.2	55.2	96.5	134.1
5	51.1	97.6	106.9	133.5
25	93.3	132.9	117.4	134.0
50	(89.2)	133.6	114.0	135.2
100	109.7	133.4	121.9	135.3

TABLE III.—TOTAL NITROGEN OF DIGESTION PRODUCTS.

Amounts Formed in Different Times by 1 Milligram of Enzyme Acting on 1 Gram of Casein at 40°.

Time of digestion. Hours.	Pepsin I. Mg.	Trypsin I. Mg.	Trypsin II. Mg.	Apparent value of Trypsin I in percentage of that of Trypsin II as affected by time of digestion.
1/2	19.8	36.1	16.6	217
1	31.2	61.4	32.0	192
2	44.4	98.4	59.5	165
3	55.6	119.1	81.9	145
4	62.3	128.4	100.3	128
6	73.9	133.4	120.2	111
8	79.8	135.8	128.4	106
21	97.4	138.4	136.5	101
48	107.4	138.0	136.4	101

Comparing the delicacy of this method as shown by the data of Table II with that of the Mett method as shown in Table I, we find that in 20-hour digestions with Pepsin I the smallest amount of this commercial enzyme which was demonstrable by the Mett method was about 0.04 mg., while by the present method 0.001 mg. could be demonstrated with an equal degree of certainty and 0.01 mg. gave a yield of digestion products

sufficient for fairly accurate quantitative measurement. With trypsin, to which the Mett method has been seen to be poorly adapted, the present method gives excellent results. In the case of the commercial enzymes here used the present method permitted positive demonstration of trypsin at quite as great dilution as of pepsin and with increasing amounts of enzyme in the digestion mixtures the trypsin showed regularly higher proteolytic values than did equal weights of the pepsin obtained from the same manufacturer at the same time.[1]

The value of this method for quantitative comparisons is shown by the fact that, allowing for the probable errors of experiment, the amount of digested nitrogen increases in direct proportion to the amount of enzyme used up to about 20–25 mg. of nitrogen in the case of pepsin, or 40–60 mg. of nitrogen in the case of trypsin, quantities large enough to be determined by the Kjeldahl method with a high degree of accuracy.

A comparison of the data for Trypsins I and II in Table III illustrates the importance of avoiding too long a time (or too large a quantity of enzyme) in making comparisons of enzyme activities. Trypsin I has about twice the activity of Trypsin II, as may be seen by comparing the amounts of total digested nitrogen for 30 minutes or for 1 hour of digestion. But when the digestion is too prolonged the product of the weaker enzymes steadily approaches that of the stronger until at the end of 8 hours the enzyme which really has twice the activity appears but little more active. If, on the other hand, we compare the lengths of time required to digest a given amount of nitrogen, we find that, up to quite high amounts, the times required by Trypsin I and Trypsin II are approximately as 1 : 2. This may be seen by reference to the data of Table III, or (perhaps better), the curves in Fig. 4, from which it will be seen that the production of any given amount of total digested nitrogen (even up to 128 mg.) requires almost exactly twice as long with Trypsin II as with Trypsin I.

Amino Nitrogen of Digestion Products (Van Slyke Method).

In our experiments with this method, 2 cc. portions of the filtrates obtained as already described, which contained the digestion products formed by the action of pepsin or trypsin on casein, were analyzed for amino nitrogen by the Van Slyke method[2] in the "micro" apparatus, using the new 3 cc. gas buret recently described. Our experience leads us to emphasize the importance of frequent "reagent blank" determinations in order to

[1] It should, however, be noted in this connection that the commercial pepsin contained only 2.25% of nitrogen while the commercial trypsin contained 10.95% of nitrogen. Undoubtedly considerable amounts of inert material were present in both samples. It cannot be inferred that the digestive activities of pure pepsin and pure trypsin would bear the same quantitative relation to each other as do these commercial products. On the other hand, the commercial products used in our experiments have as high proteolytic activity as any which we have yet found in the market.

[2] *J. Biol. Chem.*, 9, 185 (1911); 12, 275 (1912); 16, 121 (1913); 23, 407 (1915).

detect deterioration of any of the substances used, especially the caprylic or heptyl alcohol which is introduced to prevent frothing. The determinations were carried out in all respects as described by Van Slyke, and the amounts of amino nitrogen found in the 2 cc. portions of filtrate were corrected for "blank" then multiplied by 62.5 or 65 to obtain the total amino nitrogen formed[1] by the action of the enzyme in each case. The data so obtained are given in Table IV.

TABLE IV.—AMINO NITROGEN (MEASURED BY VAN SLYKE METHOD) FORMED BY DIFFERENT WEIGHTS OF ENZYME ACTING UPON 1 GRAM OF CASEIN AT 40°.

	Half-hour digestions.		20-hour digestions.	
Mg. of commercial enzyme.	Pepsin I. Mg. of amino nitrogen.	Trypsin I.	Pepsin I. Mg. of amino nitrogen.	Trypsin I.
0.001	0.0	0.0	0.0	0.5
0.002	0.0	0.0	0.2	0.7
0.01	0.0	0.0	0.3	1.3
0.05	0.0	0.0	1.7	4.6
0.1	0.2	0.7	3.3	8.1
0.5	0.7	2.0	5.5	21.0
1	1.5	3.3	7.0	26.7
2	3.0	5.4	9.4	31.3
5	4.6	11.0	11.9	36.1
25	8.9	27.1	14.8	44.9
50	11.2	35.1	15.8	50.2
100	14.1	41.0	17.8	58.2

If the data of Table IV, especially those for the very small amounts of enzyme, be compared in detail they appear somewhat irregular, probably because of the multiplication of unavoidable errors of observation by the large factor (62.5 or 65). The general trend of the results is, however, quite regular, as may be seen from Fig. 3, where the total and amino nitrogen of the products of half-hour digestions are shown together. The amino nitrogen formed in a given experiment is of course much smaller in amount than the total nitrogen of digestion products (unless the experiment be pushed far beyond the limits of those here considered) and after all of the casein has been converted into digestion products and the total nitrogen of digestion products can no longer increase, the amino nitrogen continues to rise as the proteoses, peptones and peptids are split to yield amino acids. Hence this method, in addition to measuring a change which is more definite in its chemical nature, has also the advantage of enabling one to follow the later stages of proteolysis. On the other hand, the very first stages of proteolysis are more readily detected by determining the total nitrogen of digestion products.

The amino nitrogen of digestion products was also determined in the

[1] The enzyme acts in a volume of 100 cc., but the sulfate solution and standard acid added at the end of the digestion period bring the total volume to 130 cc. in the case of trypsin experiments and 125 cc. in the case of pepsin.

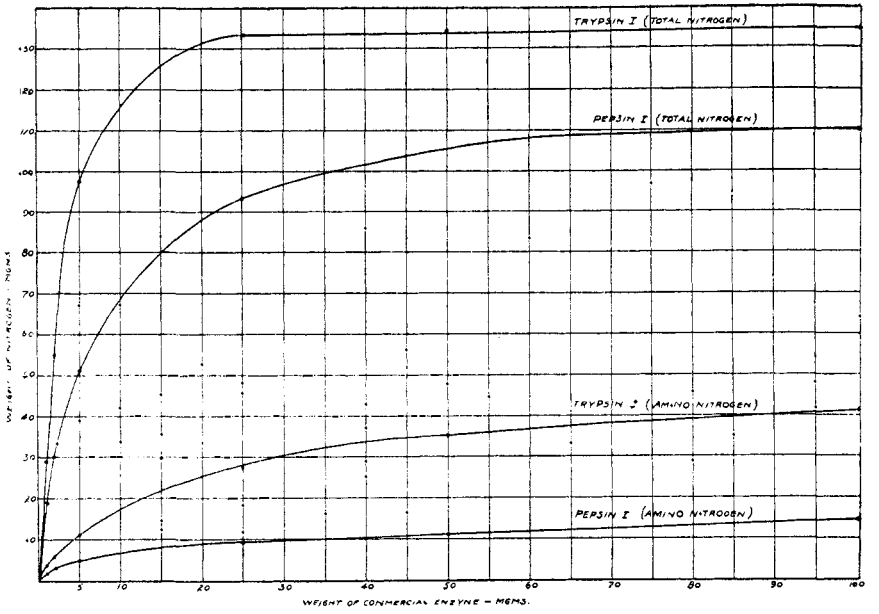

Fig. 3.—Comparison of activity of Pepsin I and Trypsin I as shown by total nitrogen of digestion products (upper two curves), and by increase of amino nitrogen (lower two curves).

experiments (of which the total nitrogen of digestion products was shown in Table III) in which fixed amounts of enzyme were allowed to act for different lengths of time. The results thus obtained are shown in Table V, and those for the trypsins graphically in Fig. 4. As would be expected, the amino nitrogen of digestion products continues to increase long after the total nitrogen of digestion products has reached its maximum and is, therefore, applicable to the study of much later stages of the digestion. Comparing the two trypsins, I and II, we find them bearing the same relation to each other here as in Table III. The results of the determination of both total and amino nitrogen for digestion products of Trypsins I and II at different time intervals are shown together in Fig. 4.

TABLE V.—AMINO NITROGEN OF DIGESTION PRODUCTS (VAN SLYKE METHOD)
Amounts Formed in Different Times by 1 Mg. of Enzyme Acting on 1 G. of Casein at 40°.

Time of digestion. Hours.	Pepsin I. Mg.	Trypsin I. Mg.	Trypsin II. Mg.	Time of digestion. Hours.	Pepsin I. Mg.	Trypsin I. Mg.	Trypsin II. Mg.
1/2	0.71	3.02	1.48	8	6.12	22.00	17.41
1	1.56	6.50	2.98	21	8.93	25.71	24.53
2	3.17	10.85	6.01	48	10.55	29.55	29.47
3	3.82	14.10	9.39	72	10.60[1]	31.84	.....
4	4.53	17.25	11.23	120	11.08[1]	33.70	.....
6	5.23	19.63	14.34	168	12.31[1]	35.61	.....

[1] These solutions contained thymol as antiseptic.

Here again we find striking evidence of the importance of reasonably short digestion periods in comparing the activities of two enzyme preparations. As already explained and as shown by the figures for 30 minutes' or for 1 hour's digestion as given in Table V, the activity of Trypsin I is about twice that of Trypsin II, but when the length of the digestion period is prolonged the difference between the two samples appears less

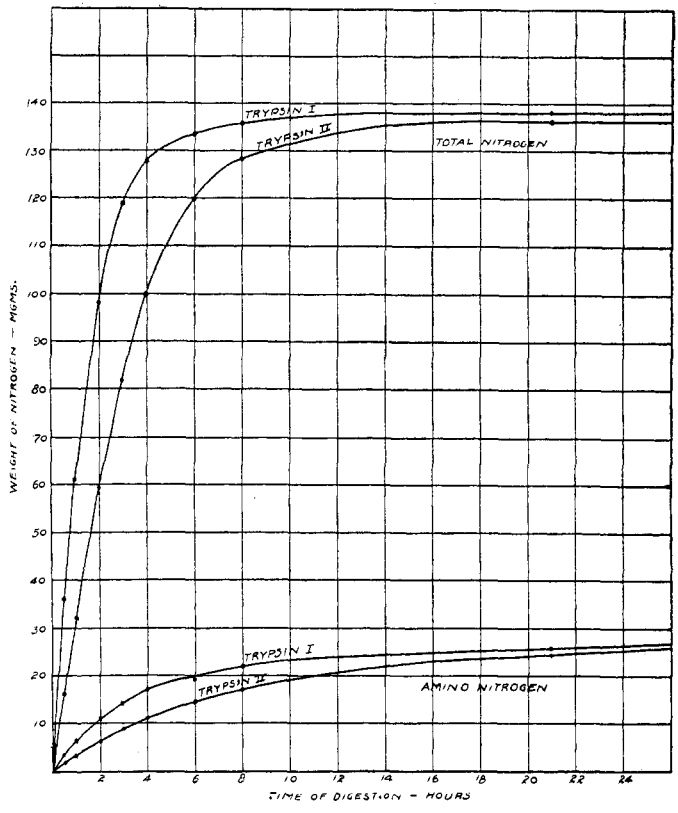

Fig. 4.—Comparison of activities of Trypsins I and II as measured by total nitrogen of digestion products (upper two curves) and by increase in amino nitrogen due to action of enzyme on substrate (lower two curves).

and less and by the end of about 20 hours they appear to be equally active. Thus in a comparison in which the time of digestion is too long a difference of 100% in the activity of two enzyme preparations may be entirely obscured. Unless the amount of protease present is exceedingly minute or the proteolytic activity very feeble the time of digestion should be preferably not be longer than 30 minutes or one hour.

If, instead of comparing the products obtained in a given length of time, one compares the length of time required for the formation of a given

amount of the product, the true relative values of the two trypsins can be traced through longer periods of time and to larger yields of product. Thus 14 mg. of amino acid nitrogen were formed by Trypsin I in three hours, by Trypsin II in six hours; 17 mg. of amino acid nitrogen required in the case of Trypsin I four hours, of Trypsin II eight hours. Even in this case, however, very long periods would be apt to give seriously misleading results, because when the reaction has become very slow the unavoidable errors in determining the total product would necessarily be large as compared with the small hourly increment.

Titration of Acid Cleavage Products (Volhard-Löhlein Method).

Thomas and Weber[1] estimated the activity of trypsin by allowing it to act upon casein as in the general procedure above described and weighing the undigested casein precipitated at the end of the digestion period. Volhard[2] tested pepsin, and Löhlein[3] trypsin, by titrating the filtrate. In 1912, Miss Ruth, working with one of us, obtained promising results from this method and we have therefore included it in the present comparison.

In most of the above experiments for which the total nitrogen or amino nitrogen of the filtrate has been determined, a portion of the filtrate (usually 50 cc.) has been titrated with tenth-molar sodium hydroxide with thymolphthalein as indicator. The amount of alkali thus required, above that required in the "blank," depends upon the carboxyl groups of the digestion products plus a little of the hydrochloric acid used in the digestion or added with the sulfate solution to precipitate the undigested casein; for the acidity of the "blank" filtrate does not account for all of the hydrochloric acid which was added, indicating that some of it is held by the undigested casein. In the pepsin tests this hydrochloric acid is liberated as the digestion proceeds. In the trypsin tests the more complete the digestion of the casein, the less hydrochloric acid will it hold when precipitated at the end of the digestion period. The fact that this method measures something more than the liberation of amino acids suggested that it might yield information somewhat different from that obtained from the Van Slyke and Sörensen methods.[4] In so far as it depends upon the titration of carboxyl groups, it is particularly appropriate that this method should accompany the use of casein as substrate on account of the high proportion of dibasic monoamino acid shown by Osborne and others to result from the hydrolysis of casein.[5]

[1] *Centrbl. f. Stoffw.-u. Verd.-Krankh.*, **2**, 365 (1901).

[2] *Biochem. Z.*, **2**, 231 (1903).

[3] *Beitr. Chem. Physiol.-Path.*, **7**, 120 (1905).

[4] We have accepted the results of Harding and MacLean (*Loc. cit.*) as sufficiently indicative that the results of the Sörensen method run parallel with those of the Van Slyke and so have not included the Sörensen method in our comparative experiments.

[5] Osborne and Guest, *J. Biol. Chem.*, **9**, 352 (1911).

The results obtained by this method are shown in Tables VI and VII, where (as in the preceding tables) the figures given are in all cases calculated for the total solution so as to indicate the work done by the amount of enzyme stated. Some of the filtrates were opalescent, making the end point of the titration somewhat uncertain. This method appears somewhat less delicate than the determination of total nitrogen; about as delicate but not so regular in its results as the determination of amino nitrogen in the filtrate. It is about equally delicate for pepsin and trypsin (referring of course to the commercial preparations here used), so that in 20-hour digestions both can be detected with certainty when present in quantities of 0.01 mg. and usually when as little as 0.002 mg. is present.

TABLE VI.—ACIDITY OF CLEAVAGE PRODUCTS (VOLHARD-LÖHLEIN METHOD) FORMED BY ACTION OF DIFFERENT WEIGHTS OF COMMERCIAL ENZYMES UPON 1 G. OF CASEIN AT 40°.

	Half-hour digestions.		20-hour digestions.	
Mg. of commercial enzyme.	Pepsin I.	Trypsin I.	Pepsin I.	Trypsin I.
	Acidity over that of "blank," in terms of cc. 0.1 *M* NaOH.			
0.001	0.0	0.0	0.0	0.2
0.002	0.1	0.0	0.1	0.2
0.01	0.1	0.0	0.6	1.2
0.05	0.2	0.0	2.4	5.8
0.1	0.3	0.6	4.2	11.0
0.5	0.9	1.3	8.5	22.5
1	1.9	3.0	12.1	26.0
2	3.3	6.1	13.1	27.7
5	6.1	12.1	15.3	30.1
25	12.3	25.6	18.5	33.9
50	12.4	28.6	18.7	35.6
100	16.0	30.6	21.1	37.8

TABLE VII.—ACIDITY OF DIGESTION PRODUCTS RESULTING FROM ACTION OF 1 MG. OF COMMERCIAL ENZYME UPON 1 G. OF CASEIN FOR DIFFERENT LENGTHS OF TIME AT 40°.

Time of digestion. Hours.	Acidity over that of "blank"—cc. of 0.1 *M* NaOH.		
	Pepsin I.	Trypsin I.	Trypsin II.
1/2	1.75	4.43	2.02
1	2.83	8.18	4.10
2	4.50	13.58	7.81
3	5.83	18.00	10.83
4	6.75	19.56	14.37
6	8.58	22.81	18.40
8	9.33	23.98	21.52
21	12.25	26.20	25.80

The general trend of these results (Tables VI and VII) is very nearly parallel with that for the total nitrogen of digestion products (Tables II and III). While the determination either of total, or of amino, nitrogen of digestion products is preferable both in delicacy and on theoretical

grounds, the convenience of this titration method makes it worthy of further study as a rapid approximate method for the measurement of proteolytic action.

The Increase of Electrical Conductivity.

In view of the prominence given by Bayliss[1] to the conductivity method in his study of tryptic action, we have determined the increase of electrical conductivity resulting from the action of different amounts of trypsin upon casein substrates prepared in the manner already described. In this work the spiral Kohlrausch bridge of Leeds and Northrup was used with telephone buzzer. The solutions were contained in Freas conductivity cells having electrodes about 1 cm. square and about 1 cm. apart. The results of typical experiments are shown in Table VIII. Experiments were also made in which the enzyme was allowed to act upon the substrate in a conductivity cell, so arranged that determinations of conductivity could be made from time to time without interruption of the digestion. Table IX shows the results of a typical experiment of this type.

TABLE VIII.—INCREASE IN ELECTRICAL CONDUCTIVITY RESULTING FROM ACTION OF DIFFERENT WEIGHTS OF COMMERCIAL TRYPSIN UPON CASEIN SUBSTRATE AT 40°.

	Increase in reciprocal ohms.			
	Half-hour digestions.		20-hour digestions.	
Mg. of commercial enzyme.	Trypsin I.	Trypsin II.	Trypsin I.	Trypsin II.
	(1)[2]	(2)[2]	(3)[2]	(4)[2]
0.002	0.00	0.00	0.00	0.00
0.01	0.00	0.00	0.16×10^{-4}	0.00
0.05	0.04×10^{-4}	0.00	0.47×10^{-4}	0.10×10^{-4}
0.1	0.07×10^{-4}	0.12×10^{-4}	0.81×10^{-4}	0.29×10^{-4}
0.5	0.21×10^{-4}	0.18×10^{-4}	2.26×10^{-4}	1.24×10^{-4}
1	0.37×10^{-4}	0.25×10^{-4}	3.03×10^{-4}.	1.62×10^{-4}
2	0.66×10^{-4}	0.40×10^{-4}	3.44×10^{-4}	2.37×10^{-4}
5	1.26×10^{-4}	0.84×10^{-4}	3.99×10^{-4}	3.91×10^{-4}
100	5.58×10^{-4}	....	11.62×10^{-4}	....
250	8.82×10^{-4}	....	15.65×10^{-4}	....

This method is very convenient (as Bayliss has previously shown) for following the time curve of tryptic digestions, but offers no special advantage in comparing the effects of different amounts of enzyme. For the purposes of our investigations it has seemed preferable on theoretical grounds to depend chiefly upon those methods in which the effect measured can be expressed in definite chemical terms, *viz.*, as total nitrogen or as amino nitrogen of digestion products formed by the action of the enzyme.

[1] *Arch. d. Sciences Biologiques*, 11, 261 (1904); *J. Physiol.*, **36**, 221 (1907).

[2] Conductivity of "blanks" solutions: (1) 6.54×10^{-4}; (2) 6.48×10^{-4}; (3) 6.72×10^{-4}; (4) 6.48×10^{-4}.

TABLE IX.—INCREASE OF ELECTRICAL CONDUCTIVITY RESULTING FROM ACTION OF 1 MILLIGRAM OF COMMERCIAL TRYPSIN UPON 1 GRAM OF CASEIN SUBSTRATE FOR DIFFERENT LENGTHS OF TIME AT 40°.

Time of digestion. Hours.	Increase of conductivity of digesting solution. Trypsin I (1).[1] Reciprocal ohms.	Trypsin II (2).[1] Reciprocal ohms.
1/2	0.30×10^{-4}	0.24×10^{-4}
1	0.63×10^{-4}	0.54×10^{-4}
2	1.06×10^{-4}	0.80×10^{-4}
3	1.23×10^{-4}	1.02×10^{-4}
4	1.36×10^{-4}	1.21×10^{-4}
5	1.51×10^{-4}	1.33×10^{-4}
6	1.61×10^{-4}	1.46×10^{-4}
7	1.75×10^{-4}	1.56×10^{-4}
9	2.06×10^{-4}	1.76×10^{-4}
24	3.22×10^{-4}	2.95×10^{-4}

The Polariscopic Method.

Most investigators who have employed the optical method for the study of proteolysis have made use of an optically active di-, tri-, or poly-peptid as substrate. Abderhalden[2] tests for proteolytic action by polariscopic observations upon mixtures of blood serum and peptone prepared from tissue protein. In order to test the usefulness of this method for studies of the action of enzymes on typical protein, we have examined portions of the digestion filtrates obtained as described in connection with the four preceding methods, in comparison with those of the "blank" tests to determine what, if any, change had occurred in the optical rotatory effect of the substrate solution as the result of the action of the enzyme. A very delicate polariscope,[3] reading to hundredths of an angular degree, was employed. Readings were usually made in a 220 mm. tube and when made in shorter tubes are calculated to this basis for comparison. The "blank" filtrate solutions were always levorotatory and the result on slight or moderate proteolysis was to increase this levorotation; but with further digestive hydrolysis the levorotation was diminished. The results as shown in Table X are always averages of at least 3 or 4 readings.

The optical method shares with the titration and conductivity methods the advantage of rapidity, the relative convenience of these three methods depending upon the nature of the investigation and the equipment and arrangement of the laboratory. It also affords a delicate means of detecting the action of very small amounts of proteolytic enzyme; but the fact that the change in rotation does not proceed progressively with the amount of enzyme or the time during which a given amount of enzyme

[1] Initial conductivity, (1) 6.56×10^{-4}; (2) 6.56×10^{-4}.

[2] "Abwehrfermente," 4th Ed., pp. 327–362.

[3] For the opportunity of using this instrument, which belongs to the Harriman Research Laboratory, we are indebted to Professor J. M. Nelson.

acts must necessarily interfere seriously with the use of this method for quantitative comparisons.

TABLE X.—CHANGE IN OPTICAL ROTATION OF FILTRATE RESULTING FROM ACTION OF DIFFERENT AMOUNTS OF COMMERCIAL ENZYMES UPON CASEIN SOLUTIONS AT 40°.

	Half-hour digestions.		20-hour digestions.		
Mg. of commercial enzyme.	Pepsin I.	Trypsin I.	Pepsin I.	Trypsin I.	Trypsin II.
	Change in terms of angular degrees in 220 mm. tube.				
0.001	0.00	0.00	0.00	0.00	....
0.002	0.00	0.00	—0.01	—0.03	—0.01
0.01	0.00	0.00	—0.07	—0.12	—0.07
0.05	0.00	0.00	—0.31	—0.56	—0.24
0.1	—0.02	—0.02	—0.52	—1.00	—0.55
0.5	—0.10	—0.10	—0.88	....	....
1	—0.24	—0.32	—1.18	—1.33	—1.29
2	—0.39	—0.60	—1.31	—1.30	—1.21
5	—0.66	—1.09	—1.42	—1.09	—1.02
25	—1.15	—1.26	—1.45	....	....
50	....	....	—1.34	....	....
100	....	—1.15	—1.33	....	....

The Biuret Reaction.

The biuret test was applied to the filtrates obtained from the peptic and tryptic digestions of casein and the colors obtained were found to pass gradually from a faint violet to a rose-red according to the extent of the digestion, showing that the test may serve for rough comparisons of proteolytic power within the range covered by these color changes.

Five cc. portions of the filtrates were placed in small porcelain dishes and to each portion one cc. of a nearly saturated solution of sodium hydroxide, and 0.15 cc. (three drops) of a 1% solution of copper sulfate were added. The solutions were stirred and allowed to stand at least fifteen minutes in order to obtain a full development of color.

The readings are expressed as nearly as possible according to the Milton Bradley Standard Color Charts, as given by Mulliken in his *Identification of Pure Organic Compounds*. In the cases of the strongest digestions, the color is better described as rose-red, than as any shade on the color charts.

The results of these tests are shown in Table XI.

By comparison of these data with those given in previous tables, it will be seen that the application of the biuret reaction, while superior to the Mett method, is a less delicate means of detecting proteolysis than most of the quantitative measurements already described and is capable of showing differences only in a much narrower range of enzyme concentrations. For the purposes of our investigations the method did not seem sufficiently promising to warrant further study.

TABLE XI.—COLORS OBTAINED ON APPLYING BIURET REACTIONS TO FILTRATES FROM DIGESTIONS OF CASEIN WITH DIFFERENT AMOUNTS OF COMMERCIAL ENZYMES.

Mg. of commercial enzyme.	Half-hour digestions. Trypsin I. Color reading.	20-hour digestions. Trypsin I. Color reading.	20-hour digestions. Pepsin I. Color reading.
0.000	Violet[1]	Violet[1]	Violet[1]
0.002	Violet[1]	Red-violet[1]	Violet[1]
0.01	Violet[1]	Red-violet[1]	Red-violet[1]
0.05	Violet[1]	Violet-red[1]	Red-violet[1]
0.1	Violet[1]	Red[1]	Violet-red[1]
1	Red-violet[1]	Rose-red	Violet-red[1]
2	Violet-red[1]	Rose-red	Red[1]
5	Red[1]	Rose-red	Rose-red
25	Rose-red	Rose-red	Rose-red
50	Rose-red	Rose-red	Rose-red
100	Rose-red	Rose-red	Rose-red

The Triketohydrindene Hydrate (Ninhydrin) Reaction.

The filtrates from several of the experiments with casein substrate above described have been subjected to the ninhydrin reaction of Abderhalden and to Herzfeld's modification of this test. As this part of our work was done before the results of the investigations of Harding and MacLean[2] became available we did not have the opportunity to include their colorimetric method based on this reaction in our comparison; but from the striking agreement of their results with those which they obtained by the Van Slyke method it would seem that these methods may be regarded as giving practically identical information in the study of proteolysis induced by pancreatic enzymes.

In our experiments the ninhydrin tests were performed as follows: 5 cc. portions of the filtrates to be tested were dialyzed against 15 cc. of water for 16 hours at 37°, through the specially prepared "Abderhalden" parchment thimbles (No. 579A) of Schleicher and Schüll; the dialysates were then neutralized with sodium hydroxide and 10 cc. portions tested with 0.2 cc. of a 1% solution of ninhydrin: (1) According to Abderhalden, by adding the reagent to the aqueous dialysate and boiling for one minute; (2) according to Herzfeld,[3] by evaporating the dialysate and reagent to dryness and taking up the residue with 10 cc. of absolute alcohol.

Typical results are shown in Table XII.

The Herzfeld modification is plainly much more delicate than the test as described by Abderhalden—perhaps too delicate for the clinical purpose which the latter chiefly emphasizes. Thus Abderhalden[4] says:

"Schwierigkeiten machen einzig und allein rötliche und braungelbe Farbtöne. Sie haben nichts mit der eigentlichen Ninhydrinreaktion zu tun."

[1] In these cases the color was of only slight intensity approximating the "Tint 2" of the Standard Color Chart.

[2] *J. Biol. Chem.*, **20**, 217 (1915); **24**, 503 (1916).

[3] *Biochem. Z.*, **59**, 249 (1914).

[4] "Abwehrfermente," 4th Ed., page 275.

TABLE XII.—COLORS OBTAINED ON APPLYING NINHYDRIN REACTION TO FILTRATES OBTAINED FROM DIGESTIONS OF CASEIN WITH DIFFERENT AMOUNTS OF COMMERCIAL ENZYMES.

Weight of commercial enzyme. Mg.	Half-hour digestions. Trypsin I. Color reading of:		20-hour digestions. Trypsin I. Color reading of:		Pepsin I. Color reading of:	
	Water soln. (Abderhalden).	Alcohol soln. (Herzfeld).	Water soln. (Abderhalden).	Alcohol soln. (Herzfeld).	Water soln. (Abderhalden).	Alcohol soln. (Herzfeld).
0.000	Yellow[1]	Yellow[1]	Yellow[1]	Yellow[1]	Tests negative (colorless to pale yellow)	Yellow-orange[1]
0.002	Yellow[1]	Yellow[1]	Yellow[1]	Yellow[1]		Orange[1]
0.01	Yellow[1]	Yellow[1]	Yellow[1]	Orange[1]		Red-orange[1]
0.05	Yellow[1]	Orange-yellow[1]	Yellow[1]	Orange-red[1]		Red-orange[1]
0.1	Yellow[1]	Red[1]	Orange[1]	Violet-red to Red-violet[3]		Violet-red[2] to Violet-red[3]
1.0	Yellow[1]	Violet-red to Red-violet[1]	Violet to Red-violet[3]	Red-violet[3]		Violet-red to Red-violet[3]
2.0	Yellow[1]	Violet-red to Red-violet[2]	Violet to Red-violet[3]	Red-violet[3]		Violet[3]
4.0–5.0	Orange-yellow[1]	Violet-red[3]	Violet to Red-violet[3]	Red-violet[3]		Violet[4]
25.0	Orange-red[1]	Red-violet[3]	Violet-red to Red-violet[3]	Red-violet[3]		Violet[4] to Red-violet[3]
50.0	Red-violet[2]	Red-violet[3]	Violet-red to Red-violet[3]	Red-violet[3]		Violet[4] to Red-violet[3]
100.0	Red-violet[2]	Red-violet[3]	Violet-red to Red-violet[3]	Red-violet[3]		Violet[4] to Red-violet[3]

[1] These colors were pale, approximating "Tint 2" of the Standard Color Chart; [2] "Tint 1;" [3] "Normal Tone;" [4] "Shade 1;" [5] "Shade 2;" [6] "Broken Tone (Medium)."

However, this may be from a clinical point of view, our experiments show that the solutions which gave an orange color in aqueous solution, contained a substance capable of reacting with ninhydrin because they gave a violet color when Herzfeld's conditions were used to make the test more sensitive. For example, the digestion containing 0.1 mg. of Trypsin I gave the color Orange, Tint 2, when the test was carried out in aqueous solution, and a color between Violet-red and Red-violet, Normal Tones, in alcoholic solution. Many other examples could be cited to the same effect.

But even with the increased delicacy given by the Herzfeld modification the ninhydrin test is, in our hands, a less delicate, and certainly a much less definite, means of demonstrating slight proteolytic action than is the determination of total nitrogen of digestion products or of amino nitrogen by the method of Van Slyke.

Summary.

Eight methods for the study of proteolytic action have been examined: The Mett method, the determination of total nitrogen of digestion products, the measurement of increase of amino nitrogen by the Van Slyke method, the titration of acidity of digestion products, the increase of electrical conductivity, the polariscopic method, and the biuret and ninhydrin reactions.

The results obtained in comparable experiments upon typical commercial pepsin and trypsin are so tabulated as to permit detailed comparisons of the delicacy, accuracy and applicability of these methods. (The comparisons thus made possible are too numerous to be concisely summarized.)

In general it may be said that the quantitative determination of the total nitrogen or the amino nitrogen of the digestion products (or both) appears to be more delicate as a means of detecting proteolysis than either the biuret or the ninhydrin reaction and more delicate, accurate, and generally applicable as a means for its measurement than any of the other quantitative methods here studied.

The results emphasize the importance, in quantitative comparisons, of so limiting the amount of enzyme preparation and the time of its action as to keep within the region in which the velocity of hydrolysis is directly proportional to the enzyme concentration (Fig. 4.).

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CORRECTION.

In the article by C. S. Hudson and J. K. Dale in the July number of This Journal (p. 1434, line 3 from bottom), entitled "The Isomeric