A CRITICAL STUDY OF THE HYDROLYSIS OF PROTEINS WITH ENZYMES AND INORGANIC REAGENTS

by

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OBJECT OF THE RESEARCH

The results presented in this thesis are those secured by a study of the methods of protein hydrolysis. Work was started while the author was an undergraduate at the Massachusetts Institute of Technology, and the subject which it was planned to study was then considerably wider in scope. It was intended that a study be made of the amino acid content of animal feedstuffs. After the proportion of amino acids present in a foodstuff had been determined, attempts were to be made to build up a "synthetic" animal food from waste vegetable materials which would be combined in such a way as to furnish the necessary proportions of amino acids.

The results obtained during this first period were presented in an undergraduate thesis in 1924. P. K. Bates, who collaborated on the larger part of the undergraduate work, also submitted a thesis in that year, and Helen Jones, Margaret Kennard, and Warren Center reported at the same time in the section of Advanced Biological Chemistry. These reports are on file at the Massachusetts Institute of Technology.

The preliminary work made it very clear that the problem, as originally outlined, presented too broad an aspect for a single research. Therefore, it was narrowed down, and the four years of graduate work which have since been gi-

ven to it were devoted to one point alone, namely, the hydrolysis of the proteins.

The reason why endeavor should be concentrated on this one point is easily understood. No complete hydrolysis of a protein has yet been made. The greatest degree of hydrolysis that has ever been attained is in the case of gelatin, where it has proved possible to carry the hydrolysis 90% to completion (41). From this figure values drop until we find cases in which hydrolysis can be carried only 60% to completion (41). Losses during hydrolysis run, then, from 10% to 40%, dependent upon the substance hydrolyzed and the methods and reagents used, before any system of analysis is applied to the hydrolysate. Until such losses are prevented, protein analysis remains uncertain.

Incomplete protein analysis occurs in two ways, first, through the Law of Mass Action, and second, through the formation of humin. As yet there is no method known of avoiding the effects of the Law of Mass Action, for under the conditions of hydrolysis the end products remain soluble and reactive, and therefore equilibrium is reached while the conversion of total nitrogen to amino nitrogen is still incomplete.

Humin formation is caused by a union of amino groups with carbohydrate under the conditions of hydrolysis. In enzyme hydrolyses no humin is formed, acid hydrolyses may produce it in large amounts, and alkaline hydrolyses are practi-

cally certain to increase the amount of humin over that formed from the same protein when hydrolyzed with acid.

The important factor in the production of humin during acid hydrolysis is the amount of carbohydrate present. If the carbohydrate exists as carbohydrate groups in a conjugated protein, there is no chemical method by which it may be removed without destruction of the amino acids which make up the rest of the protein molecule. If the carbohydrate is not in chemical combination but rather in a physical mixture, as in the case of the cereals and the grains, it can be removed. It is only reasonable to expect that such treatment will reduce materially the amount of humin formed.

What part oxidative reactions may play in humin formation is not known, but it is believed that oxygen is necessary to its formation. If there were any means of preventing oxidation, humin formation should be almost entirely eliminated.

The following pages, after a brief survey of the general literature relating to protein hydrolysis, are devoted to the data secured in the study of the points just mentioned. The results have been almost wholly negative, but it is hoped that they may prove valuable in showing the next man what to avoid.

SUMMARY OF THE GENERAL LITERATURE

The literature bearing specifically on protein hydrolysis is sparse. That which has appeared is devoted to a study of the kinetics of the reaction. The literature which has appeared in an effort to better the accepted methods of converting protein nitrogen to amino nitrogen is negligible. In that respect, then, if in no other, this thesis enjoys a position that is unique.

The first time that proteins were hydrolyzed in the laboratory was in 1820 when Braconnot (9) was successful in hydrolyzing protein by boiling with acid. In 1839, Mulder (48) obtained essentially the same results by treatment with alkalies.

The work of these two men is of historical importance and nothing more, for neither one had a definite concept of the chemical nature of the changes which took place during their experiments. It was not until 1902 that the theory of peptide linkage was first advanced by Hofmeister (38). The study of the products of protein hydrolysis was begun three years later by Siegfried (68), who treated the amino acid mixtures with calcium hydroxide and carbon dioxide in the cold, thus forming carbamino acids which may be decomposed with a precipitate of calcium carbonate on heating.

No means had yet been devised to successfully follow the progress of hydrolysis, that is, the degree to which total nitrogen is converted to amino nitrogen. In 1909, Mathieu (44) attempted to use Siegfried's method (68) to follow the progress of hydrolysis, but the results obtained were not very satisfactory. In 1908, however, Sorensen (69) devised the formol titration method which measures the amount of carboxyl set free. Henriques and Gjaldbak (36, 37) in 1911 applied the titration to follow the enzymatic hydrolysis of proteins, and this work furnished the first definite chemical evidence that amino acids are united for the most part in the protein molecule through the peptide linkage.

The method next developed for determining the degree of hydrolysis was that of Van Slyke (77, 80) in 1911, and this was further perfected until by 1918 a highly accurate apparatus was made available for the determination of aliphatic amino nitrogen. The most recent development in methods for determining the degree of hydrolysis is that introduced by Foreman (17) in 1920. It consists essentially of an improved Sorensen (71) method.

The two methods which have been established for determining the degree of hydrolysis are dependent upon the liberation of one or the other of two groups, carboxyl and amino, which enter into the peptide linkage. In addition to the amino nitrogen and to the nitrogen which is bound as humin, nitrogen is also converted during hydrolysis to the form of ammonia.

These facts bring us of necessity to a consideration of the ways in which nitrogen may be combined in the protein molecule.

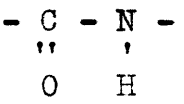
NITROGEN LINKAGE IN THE PROTEIN MOLECULE

The degree to which ammonia is formed during protein hydrolysis is dependent to a large extent upon the conditions of the reaction. Nasse (49) in 1872 was the first to point out that the nitrogen which gives rise to ammonia must be differently bound in the protein molecule than that which becomes available as amino nitrogen. The nitrogen which is converted to ammonia during hydrolysis is now known as amide nitrogen, and the work of Osborne and Nolan (59), in 1920, demonstrated with reasonable certainty that the ammonia comes from the amides of dicarboxylic acids, provided that the conditions least favorable to the formation of ammonia from other sources are satisfied (90).

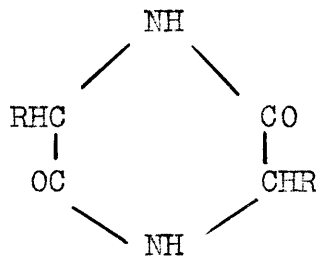
There are still other linkages of nitrogen in the protein molecule which cannot be split like the peptide linkage to give amino and carboxyl groups. Those which are definitely proved are the guanidine group of arginine, the imidazole group of histidine, and the indole ring of tryptophane (86). According to Fischer and Abderhalden (16), proline can enter into peptide linkage not only with its carboxyl group but also with its imino nitrogen group. Other types of linkages which have been suggested are the uramino linkage (4) and the thiopeptide linkage (39).

Lloyd (41) states that the possible linkages within the protein molecule are four in number. These are tabulated as follows:

1. Peptide linkage:



2. 2:5 diketopiperazine linkage:



3. The phosphorous linkage which may, according to Lloyd, be bound in the peptide linkage. There is also the possibility that phosphorous is bound in some unknown manner (64).

4. The sulfur linkage which is also of unknown constitution. Walker (88) has applied a modified nitroprusside reaction for the sulphydryl group and studied the results which proved to be uniformly positive for all disulfides tested. The application of the test to ovalbumin led to a confirmation of the suggestion advanced by Harris (33) that in the case of ovalbumin the sulphydryl group - SH formed on denaturization of the ovalbumin does not have as a precursor a disulfide linkage - S - S - , for the application of the test to native ovalbumin gives a negative result.

It is particularly important to keep in mind the

fact that while Fischer and Abderhalden (16) are generally credited with having established the existence of the peptide linkage through their isolation of numerous polypetides from partially hydrolyzed proteins, they did not determine whether or not all the amino acids are united in the peptide linkage. We know that they are not, but we do not know the proportions in which the amino acids are distributed in the various types of linkage. Neither do we know exactly the proportion of total nitrogen which is bound in peptide linkage. But if we ignore for the moment the nitrogen which may be lost as ammonia or bound in humin, we can say that by far the greater portion of the remaining nitrogen is bound in peptide linkage.

This is particularly important because the only two methods available for following the progress of hydrolysis are dependent upon the breaking of the peptide bond. There is no method known at present by which imino nitrogen peptide linkage, as in the case of proline (16), may be detected in the protein molecule (86).

It must be realized that in measuring the carboxyl groups or the amino groups set free during hydrolysis, we can never obtain a measurement which would equal 100% of the total nitrogen because of the other linkages which are known to exist. It is, therefore, not strictly correct to take such measurements and express the ratio between them and the total nitrogen as the per cent conversion, or degree of hydrolysis. The ratio should be expressed not between the value for amino

nitrogen and the total nitrogen but between the value for amino nitrogen and the total nitrogen which would be available as amino nitrogen provided hydrolysis were complete. Unfortunately, that value has never been determined for any protein. Unless the total nitrogen value is chosen as a basis for per cent computation, some other figure must be chosen which is equally arbitrary. The total nitrogen value as been taken accordingly as the basis on which the degree of hydrolysis, or per cent conversion of total nitrogen to amino nitrogen, should be calculated in this work.

While the per cent conversions thus obtained are not quantitative, they are nevertheless comparable. An increase in amino nitrogen has been taken, then, as meaning an increase in the per cent conversion.

Amino nitrogen may be measured in two ways, either directly by means of the Van Slyke apparatus or indirectly through establishing the titration value for the corresponding carboxyl groups which were liberated. The next step is to consider in detail the different methods by which amino nitrogen may be determined.

METHODS FOR AMINO NITROGEN DETERMINATION

Methods of Van Slyke and Sorensen -

These two methods were the first ones available for determining the degree to which a protein is hydrolyzed. The method of Sorensen preceded that of Van Slyke by about three years (Cf. page 5, this report). While both methods have their faults, it is probable that the liability to error in the hands of an experienced operator is very nearly equal.

One of the difficulties encountered with the Van Slyke method is that most of the proteins and many of their hydrolytic products are precipitated in the nitrous acid solution. Wilson (92) believes it possible that some of the material is occluded by the precipitation and thereby the length of time necessary for the reaction is increased. Van Slyke and Birchard (84) studied this point. They tried increasing the time of the reaction from 2-5 minutes to 20-30 minutes, but they were uncertain whether this gave good results because of the possibility of hydrolysing some of the protein in the reaction vessel. They decided finally that no hydrolysis occurred, because analysis of peptides of varying composition and containing up to fourteen amino acids yielded theoretical results.

Abderhalden and Kramm (1), in analyzing digestion

mixtures of proteins by Van Slyke's method found that great differences in results were obtained according to whether the reaction was run for 5 minutes or for 10 minutes. This they believed due to hydrolysis of some of the easily split peptones, although in accord with the findings of Van Slyke and Birchard (84) no hydrolysis had been noted in previous work with pure polypeptides. However, they did not consider this latter point proved because of insufficient data.

Hart and Sure (34) also are in doubt as to whether or not protein cleavage products higher than amino acids are hydrolyzed during the course of the Van Slyke determination. The degree to which this factor may influence the accuracy of the determinations made in this work is considered further on under the discussion of the methods for amino nitrogen determination (Cf. page 16, this report).

White and Thomas (90) made a comparison of the methods of Van Slyke and Sorensen and found that the results obtained with the Van Slyke method were parallel with those obtained with the Sorensen method but slightly lower. These workers apparently use the 5 minute reaction period during their determinations, but they are not definite on this point. Rogozinski (67) and Andersen (3) both noted variations between the two methods but came to the conclusion that the Van Slyke method was the more satisfactory. Northrop (55) believes that in absolute determinations the Van Slyke determination is the more accurate but favors the Sorensen method

for comparative experiments where hydrolytic changes are to be measured as it is more accurate and much more rapid.

Method of Foreman -

The method devised by Foreman (17) for amino nitrogen determination is a titration method and consists essentially of an improved Sorensen (71) method. The method differs from that of Sorensen in that the titration is run in alcoholic instead of aqueous solution. The advantage is that ammonia, liberated during the reaction with formaldehyde, does not form an ionizable compound with the phenolphthalein used as indicator provided the concentration of alcohol is kept above 80%. Besides being more exact, the method has an additional advantage in that it is applicable to alcoholic extracts of protein hydrolysates. This is not true of the Van Slyke apparatus because of the volatility of the alcohol, which may introduce an error.

Morrow (47) states that Foreman's method is preferable to Sorensen's. Davies (12) found Foreman's method entirely satisfactory.

The Ninhydrin Reaction -

Harding and MacLean (29, 30) have developed a colorimetric method for determining protein hydrolysis by measurement of the amino acid alpha nitrogen. The reaction is run in the presence of pyridine between the amino acids and triketohydrindene hydrate, and is essentially the ninhydrin reaction. Harding and MacLean found a close correlation between the Van

Slyke determination and the colorimetric method. They also mention that both corresponded well with Sorensen's method.

Discussion of the Methods for Amino Nitrogen Determination -

Of the four methods outlined, the Van Slyke method was selected as the one by which amino nitrogen determinations would be made during this work. The reasons for this are not hard to understand.

The method of Harding and MacLean (29, 30) was discarded arbitrarily. It has never been mentioned by any other worker and only twice by its originators. Lacking corroboration, the method did not appear to be a suitable one, particularly since others were available on which the data was voluminous by comparison. Another drawback to this method is that it is based on a colorimetric determination. Protein hydrolysates obtained through the action of acids or bases tend to be highly colored, and in many cases the color is a true color and in solution from which it cannot be removed by an adsorbing agent. Such colors would tend to interfere so seriously with the colorimetric determination as to render it valueless.

For the purposes of the present report, Foreman's method may also be disregarded, the reason being that the method did not receive any attention from Jones and Kennard (40) who collaborated in the earlier part of this investigation. This is difficult to understand, as one of the problems which they wished to solve was whether the Van Slyke me-

thod or the Sorensen method was the more suitable for amino nitrogen determinations on protein hydrolysates. Their decision was in favor of the Van Slyke determination. Foreman's method was brought to its present stage in 1920, while Jones and Kennard did not start work until 1924. Why they should have ignored Foreman's method is not known.

Jones and Kennard's (40) unfavorable report on the Sorensen method, coupled with satisfactory experience with the Van Slyke apparatus during the preliminary work, led to the choosing of the Van Slyke method for all subsequent determinations. When the method of Foreman came to the writer's attention it was inadvisable to change the method of determining amino nitrogen as a great deal of work had already been completed on the basis of the Van Slyke determination.

If there were any reason for assuming that either of the titration methods was superior to the Van Slyke determination, there would have been good reason for abandoning it, but such is not the case. Authorities appear to be about equally divided in their preference for one method or the other (1, 3, 12, 34, 47, 55, 67, 84, 90, 92).

The Van Slyke determination is subject to errors, and theoretically, these may take place in two ways (92). Results may be too high, due to hydrolysis of the protein with nitrous acid, or they may be too low due to the insolubility of certain proteins in nitrous acid. There is also the possibility that unknown and slow reacting groups may

contribute to give high results. It is very unlikely, however, that these factors have any practical significance, particularly in this work where the method was confined entirely to determinations on protein hydrolysates.

In the first place, hydrolysis is definitely a function of temperature, and while deaminization takes place during the determination and so upsets the equilibrium that has been established, the reaction is run at room temperature. This means that whatever hydrolysis does take place must be extremely small. The experimental data furnished by Van Slyke and Birchard (84) offers confirmation of this line of reason.

The insolubility of proteins in nitrous acid presents a serious difficulty in cases where the ratio of protein to protein derivatives is very high. Such is not the case, however, with satisfactory protein hydrolysates. No case can be called to mind where, if the per cent conversion of total nitrogen to amino nitrogen was in excess of 30%, any difficulty was experienced with insolubility of the sample. With hydrolysates of lower value, the difficulty often could be overcome by dilution of the sample before adding to the reaction mixture. It should also be kept in mind that hydrolyses reaching values of 30% or less for per cent conversion are undeserving of serious consideration, and therefore errors which are introduced through insolubility of the hydrolysate in the nitrous acid mixture are not important.

The possibility of the effect of unknown groups is

one that cannot be ignored. Nevertheless, we know that the greater part of the nitrogen of the protein molecule is convertible to alpha amino nitrogen. Of that which remains, we have a fairly definite idea as to the type of linkage which exists (Cf. pages 7-10, this report). Knowing this, it is possible to estimate to what degree those linkages will affect the Van Slyke determination. The slow reacting groups also deserve consideration. These are factors, however, that affect the method from the strictly quantitative viewpoint. They do not affect it when employed only for comparative results as has been done in this work. There has been nothing of a quantitative nature about the determinations, for the results obtained were compared on an arbitrary basis of per cent conversion and not against a standard whose composition was definitely known.

It has, therefore, been concluded that the Van Slyke amino nitrogen determination furnished a satisfactory method for determining the degree of hydrolysis of protein. Because all the experimental data is dependent upon the determinations made with the Van Slyke apparatus, it seems advisable to consider the apparatus and its method of use at this point.

THE VAN SLYKE METHOD FOR THE GASOMETRIC DETERMINATION OF ALIPHATIC AMINO NITROGEN

References -

For detailed information regarding the set-up of the apparatus and its method of use, it is best to consult Van Slyke's original papers (77, 79, 81, 83).

Apparatus -

The apparatus necessary for the proper carrying out of the Van Slyke amino nitrogen determination is obtainable in two sizes, one relatively large, which is known as the macro size, the other much smaller, known as the micro size. Both apparatus are to all intents and purposes identical. The only difference between them is with respect to size. The micro apparatus employs the same reagents and is operated in the same manner as the macro apparatus, but the quantities of reagents used with the micro apparatus are, of course, smaller.

The micro apparatus has the following advantages over the macro apparatus. First, less material need be taken as a sample for running the determination. Second, the determination can be performed in a shorter time due to the smaller quantities of reagents involved. A micro apparatus was used for all the determinations made in this work.

These differences from the apparatus diagrammed by

Van Slyke (79) should be noted, however. First, connected to the deaminizing bulb, D, is a second two c.c. burette, C. This is used only for the addition of capryl alcohol, the sample being introduced by means of the burette B. The capryl alcohol burette can be distinguished from the sample burette by the fact that the sample burette is connected to the deaminizing bulb by means of a two way stopcock, while the capryl alcohol burette is connected by means of a one way stopcock.

Second, the gas burette, F, is of three c.c. total capacity and is graduated to one-hundredths of a c.c.

Third, the cylindrical vessel A is of 15 c.c. capacity and has two marks, one at 2.22 c.c., the other at 8.88 c.c.

Fourth, the deaminizing bulb D is of 10 c.c. capacity and has two marks, one at 11.1 c.c., the other at 4 c.c.

Operation -

The method of operating outlined by Van Slyke was followed strictly. The deamination time was taken as three minutes because the determinations were run at 20-25°C. practically without exception. The time interval was measured accurately by means of a timer. The Hempel pipette was given two shakings, one for two minutes and one for one minute after passing the gas from the pipette to the gas burette and back again. This procedure was found sufficient to remove all traces of nitric oxide.

Identically the same procedure was followed for the blank determinations.

Limits of the Reaction -

The reaction is complete enough for measurements that are intended for comparison and not for strictly quantitative results, as every known amino acid reacts quantitatively with one and only one nitrogen atom except lysine, which reacts with two, and proline and oxyproline, which do not react at all. All the amino acids react with all their nitrogen except tryptophane, which reacts with one-half; histidine with one-third; arginine with one-quarter; proline and oxyproline with one. The foregoing estimations by Van Slyke (77) have been accepted apparently, for there is nothing in the literature to contrevert them. For confirmation, there is only the work of Hart and Sure (34) who agreed with Van Slyke on the reaction of lysine with nitrous acid, differing only in that they thought 15 minutes or even 10 minutes was sufficient to get all the reactive nitrogen if the temperature was above 30°C. instead of the 30 minutes recommended by Van Slyke (77).

Testing of the Apparatus -

For the testing of the apparatus, Van Slyke recommends a two c.c. sample of Kahlbaum's leucine which is made up so as to be equivalent to 20 mgms. of leucine (81). An accuracy of 0.005 mgm. is claimed when less than two c.c. of gas is measured, while with more the accuracy is limited to 0.01 mgm. (81).

In this work the apparatus was tested simply by drawing a measured amount of air into the gas burette. This was then passed over into the deaminizing bulb. The apparatus was put through all the manipulations of a true determination and at the end, if the volume of air corresponded within ±0.01 c.c. of the original volume, the apparatus was considered to be in perfect shape.

Every precaution must be taken against air leaks as the very smallest of these will seriously affect the accuracy of the determinations. Wilson (91) frequently reground the stopcocks with powdered emery and greased the stopcock at the upper end of the gas burette after every three or four determinations. The precautions observed during this work were not as extreme, but particular care was taken that the stopcocks were well lubricated and the rubber connections tight. The apparatus was frequently tested as described above.

It was not found necessary to use stethoscope tubing as suggested by Van Slyke (83). The regulation small bore pressure tubing has proved entirely satisfactory, provided it was a good fit.

Blank Determinations -

With the micro apparatus the residual gas obtained on a blank determination should amount usually to 0.06 to 0.12 c.c. In any case, it must be under 0.2 c.c., otherwise the nitrite should be rejected (81). While these are the limits for the blanks specified by Van Slyke, it has proved impossible

to keep within them during the course of this work. The difficulty of keeping these limits led naturally to a search of the literature to determine whether other workers had experienced difficulty with the blank determinations. As a result of this survey but one reported case was found where difficulty was encountered with checking the blank determinations (63). Strangely enough, those workers who report difficulty with checking true determinations claim to have obtained satisfactory blanks. The difficulty which has been encountered with checking true determinations during this work will be dealt with at a later point (Cf. page 25, this report).

Not only has it been impossible to check the blank determinations for the author, but within the last few months a thesis student, supplied with an entire new Van Slyke micro apparatus, came independently to the conclusion that the blanks are difficult if not impossible to check.

Reilly and Pyne (63), whose report furnishes the only published case of failure to check the blanks, tested a number of samples of sodium nitrite but found that the blanks remained very large. Using the micro apparatus and Kahlbaum's nitrite, they obtained the set of values for the blank shown in Table 1. In contrast to these values is given a tabulation of ten blank determinations made during this work. During the series of ten determinations performed here, temperature and pressure were to all practical purposes constant, and the apparatus was tested by the method described (Cf. page 20,

this report) before each blank determination. Therefore, it is to be noted that there were eleven tests mad for the ten blanks performed, and as the tests were all satisfactory, the apparatus was guarranteed against mechanical defect. Whether Reilly and Pyne observed these precautions is not known.

TABLE 1

Determination No.	c.c. gas R. B. P.	c.c. gas R. and P.
1	0.39	0.46
2	0.25	0.46
3	0.27	0.59
4	0.39	0.44
5	0.29	0.48
6	0.27	0.45
7	0.28	
8	0.27	
9	0.23	
10	0.28	

The above values for the blanks in this work were obtained with J. T. Baker's nitrite, while Reilly and Pyne were using Kahlbaum's nitrite. The author has tested four other brands of nitrite, including Powers-Weightman-Rosengarten nitrite recommended by Van Slyke (81). None of these have furnished any more satisfactory set of blank determina-

tions than the one given above.

The driving motor used through all the determinations was an induction motor and therefore maintained a very nearly constant speed. Reilly and Pyne make no mention of the motor used. They do claim, however, to have analyzed the blank gas in a Bone and Wheeler apparatus, the analysis showing that the gas consisted entirely of nitrogen. They found that recrystallization of the nitrite led to only slightly lower values for the blanks and did not in any way improve their ability to secure checks. The procedure finally adopted by Reilly and Pyne was to run an amino nitrogen determination and follow it immediately with a blank, taking care that in the two cases the volume of nitric oxide evolved was the same. It was found in this work, however, that blanks could not be checked by this method.

In view of the fact that the apparatus was mechanically satisfactory, being gas tight and subject to shaking at a nearly constant speed, one cannot avoid being forced to the conclusion that the variation encountered in the blanks is one inherent in the determination. Necessarily this means that it is unavoidable. Further strength is lent this view by the fact that an independent research man using a separate apparatus, and class students using the same apparatus as was employed in this work, have obtained essentially the same results with the blank determinations over a period covering two years. The degree to which the inherent variation of the blanks may af-

fect the accuracy of the true determinations will be considered at a later point (Cf. page 31, this report).

Check Determinations -

The discussion just presented on the blank determinations makes it clear that true determinations can never be checked more closely than the limits imposed by the variations of the blank determinations. Strangely enough, however, it has been found that true determinations vary over much wider limits than can be accounted for by the variations of the blanks, and this fact led to a further search of the literature to determine whether other investigators had encountered the same difficulty.

Satisfactory checks are either implied or clearly stated by several of the workers previously mentioned under the discussion on the methods for determining amino nitrogen (3, 44, 67, 90). In addition, Dernby (A) and Avery and Cullen (B) tacitly accept the possibility of checking the true determinations.

But DeBord (C) finally abandoned the Van Slyke method because of the failure to obtain consistent control analyses, finding in one particular series of tests that the variation was as high as 18.4%.

A. Dernby, K. G. J. Biol. Chem. 35, 179(1918).

B. Avery, O. T. and Cullen, G. E. J. Exp. Med. 32, 547(1920).

C. DeBord, G. G. J. Bacteriol. 8, 7(1923).

The results presented by Lamson (D) are particularly interesting for they cover several hundred determinations and were produced by two men. In general, the results obtained were quite irregular. Modifications which were applied to the method gave no perceptible improvement.

In Table 2 is given the tabulation of a series of ten determinations made on Hydrolysate No. 581. The necessary data on this hydrolysate is as follows:

HYDROLYSATE NO. 581

The raw material consisted of 20 gms. of dried Proco (Cf. page 36, this report). To this was added 20 gms. of stannous chloride and 200 c.c. 90% by volume concentrated hydrochloric acid. The mixture was boiled under reflux for four hours, heat being supplied with a direct flame. The mixture was cooled under the tap, filtered, and stoppered tightly. It then stood from May 14 to June 15, when 10 c.c. was withdrawn and diluted volumetrically to 100 c.c. The determinations were run on this dilution.

D. Lamson, R. W. J. Bacteriol. 9, 307(1924).

Van Slyke Check Determinations on 1 c.c. Samples from Hy. No. 581

Det. No.	Mgms. Amino N per c.c.	Mgms. Total Amino N	% Conversion
1	7.52	1504.	63.2
2	9.47	1894.	79.6
3	7.37	1474.	61.8
4	8.78	1756.	73.8
5	7.92	1584.	66.5
6	8.09	1618.	67.9
7	7.29	1458.	61.2
8	6.82	1364.	57.3
9	8.16	1632.	68.6
10	7.82	1564.	65.7

In Table 2, the results given in the first column represent mgms. of amino nitrogen in 1 c.c. of Hydrolysate No. 581. An examination of the set of values in this column will give an idea of how the determinations fluctuate among themselves. It must be remembered that the values for the first column are but for 1 c.c. of the hydrolysate, and to determine the amount of amino nitrogen in the entire hydrolysate it is necessary to multiply the values of the first column by the total volume, 200 c.c. Thus are derived the values for the total amino nitrogen contained in the hydrolysate which are given in the second column. The per cent con-

version is the ratio of the total amino nitrogen to the total nitrogen, and a study of the figures in the third column of Table 2 gives an idea of how this value may fluctuate due simply to variations in the determination.

The reason for the variation in check determinations was thought at first to lie in the strength of mineral acid that was run in with the sample. It will be remembered that the hydrolysate tested was originally 90% concentrated hydrochloric acid by volume. The hydrolysate, after dilution, had its strength of mineral acid reduced to approximately 9% concentrated hydrochloric acid by volume. Calculating from this on the basis that the concentrated acid was 37.5% hydrochloric acid gas by weight, that the specific gravity was 1.19, and that the molecular weight of hydrochloric acid is 36.46, the normality of the hydrolysate taken for samples was 1.1 N. Van Slyke states that the normality of the mineral acid run in with the sample should never exceed 0.5 N (77). Accordingly, another portion of the same hydrolysate was taken and diluted so that the normality of the acid was slightly below 0.5 N. The results obtained in a series of ten determinations exhibited the same irregular and wide variation as those given in Table 2. Finally, a hydrolysis was run similar to No. 581 in every respect save that the acid strength was reduced to 0.5 N. The results obtained with a series of ten check determinations were no better.

It now seemed reasonably certain that the strength of mineral acid run in with the samples during the check determinations had no effect on the constancy of the determinations. To settle this point definitely a solution containing about one gram of Difco Bacto-Peptone was made up in 100 c.c. of distilled water, and this was taken for a series of ten check determinations. The results varied widely as in the previous case and are tabulated in Table 3 in conjunction with those obtained with Hydrolysate No. 581 so as to illustrate the chancy and wide variation which is common to both sets of check determinations. Putting these two sets of determinations in one table, however, does not mean that they are to be compared on a quantitative basis for no such relationship exists between them.

The fact that not only hydrolysates produced by weak and strong mineral acid (hydrochloric) but also an enzyme hydrolysate give check determinations that are irregular and widely divergent makes it reasonably certain that in the check determinations previously mentioned the strength of mineral acid introduced with the sample was not a factor. Yet Van Slyke (80) neutralized his hydrolysates before running the amino nitrogen determinations. No reason for this is given. Greenberg and Burk (28) also neutralized the hydrolysates, giving as a reason that they wished to prevent further hydrolysis. As the hydrolysates were cooled to room temperature before neutralizing, this reason hardly seems a valid one.

TABLE 3

VAN SLYKE CHECK DETERMINATIONS

	Mgms. Total Amino N	
Determination No.	Difco Bacto-Peptone	Hydrolysate No. 581
1	18.72	1504.
2	20.36	1894.
3	25.71	1474.
4	25.55	1756.
5	20.69	1584.
6	23.95	1618.
7	21.54	1458.
8	18.95	1364.
9	22.86	1632.
10	24.91	1564.

Note: As mentioned before, there is no quantitative relationship between these two sets of figures. In the case of the Bacto-Peptone the original determination was multiplied by 100 to give the total amino nitrogen, while the original determinations on Hydrolysate No. 581 were multiplied by 2,000 to obtain this value. The table illustrates simply that the Bacto-Peptone determinations vary widely, and the values for Hydrolysate No. 581 are given purely to refresh the memory.

Now, Van Slyke (80) had a reason for removing the

hydrochloric acid from the hydrolysate in view of the subsequent treatment which it was to receive. Greenberg and Burk (28), apparently, did not. The question therefore arises as to whether or not it is necessary to neutralize the filtrates before running the Van Slyke determinations. There does not appear to be any good reason for so doing, but the point should be settled by experiment. Unfortunately this matter did not come to the writer's attention until after the laboratory work had been completed and there has not yet been any further opportunity for trying it out.

The fact remains that the Van Slyke determinations cannot be checked closely whether run in blank or run on hydrolysates produced through the action of a mineral acid or of an enzyme. The reason for this is at present unknown.

Effects of Blanks on the True Determinations -

It was mentioned previously that the true determinations vary over a wider range than the blank determinations (Cf. page 25, this report). Referring to Table 4, there is a tabulation given of the series of ten blank determinations first shown in Table 1 with the equivalent values in milligrams of amino nitrogen for the gas volumes evolved.

An examination of Table 4 will show that seven out of the ten blanks listed had values falling withing the limits of 0.1350 - 0.1600 mgms. If the usual variation in the blanks is taken as coming within these two values, there is a range of 0.0250 mgms. over which the blanks may be expected to vary

TABLE 4

Van Slyke Blank Determinations

Determination No.	c.c. Gas	mgms. Equiv. Amino N
1	0.39	0.2159
2	0.25	0.1384
3	0.27	0.1495
4	0.39	0.2162
5	0.29	0.1608
6	0.27	0.1495
7	0.28	0.1546
8	0.27	0.1490
9	0.23	0.1266
10	0.28	0.1541

normally. In order to understand the effect of this variation upon final results, it is necessary to multiply the value by 2,000, because this is the factor by which it is necessary to multiply in every case to obtain the value for total amino nitrogen in a hydrolysate. If, then, the usual variation in the blanks is multiplied by this factor, a value of 50 mgms. is the result, and accordingly it can be said that the value for total amino nitrogen of a hydrolysate will vary over a range 50 mgms. wide due to the blanks alone. In order to prevent misunderstanding, this fact may be stated in another way: The value for total amino nitrogen of a hydrolysate may vary

±25 mgms. due to the effect of the blank variation and nothing else. That is what may be expected ordinarily. A further reference to Table 4 shows that the maximum and minimum values for the series of ten blanks are 0.2100 and 0.1200 mgms. respectively. The difference between these values is 0.0900 mgms., which gives a value of 180 mgms. when multiplied by the factor 2,000. The greatest variation in the value for total amino nitrogen of a hydrolysate will, then, be on the order of ±90 mgms., and this is caused by the blank alone.

A similar procedure is followed in the case of the peptone values and those obtained for Hydrolysate No. 581. The two sets of results from the determinations will be found by referring back to Table 3. In the case of the peptone solution, the milligrams of total amino nitrogen correspond to those contained in one gram of peptone, and this is in turn closely equivalent to the amount of solid in 10 c.c. of Hydrolysate No. 581. The factor used in the case of the peptone was 20 instead of 2,000 to reduce the two to the same terms. In the case of Hydrolysis No. 581 there was no need of supplying any additional factor, as 2,000 had already been used to calculate the value for total amino nitrogen.

In Table 5 will be found the results of following the above procedure. The significant thing is that in both usual error and maximum error, when the three sets of determinations are put upon the same basis, the blanks vary least, the peptone solution is intermediate, while the acid hydroly-

TABLE 5

Errors Compared on Basis of Total Amino Nitrogen

	Blank	Difco Bacto-Peptone	Hy. No. 581	
Usual	±25	±60	±100	Usual
Max.	±90	±70	±275	Max.

sate exhibits the greatest degree of variation of all. It must, therefore, be understood that in the case of the peptone and Hydrolysate No. 581 a variation occurs which is far greater than could be accounted for by the effects of the blanks alone.

The fact that there is a greater variation in the true determinations than in the blanks is established but the reasons for it are not yet known. It is particularly unfortunate that there is a variation to deal with greater than that which can be accounted for by the blanks, because on the basis of 1500 mgms. of total amino nitrogen, which is a fair value for a hydrolysate of the type of No. 581, a variation of ±25 mgms. introduced by the blank - Cf. Table 5 - would mean a variation in the per cent conversion value of only about ±1½%. With the maximum error supplied by the blank the per cent conversion would be affect to the extent of ±3%. Compare these variations in percent conversion which are to be expected if only the blank variation is operative with those actually obtained as illustrated by the

last column of Table 2. Such a comparison furnishes conclusive evidence that the large errors found in the true determinations are to be blamed not upon the apparatus, not upon the operator, but upon the method itself.

Method of Securing Results -

The question now naturally arises - if the Van Slyke determinations could not be checked how was it possible to do work of even a comparative nature using this method as a basis of measurement? This problem was dealt with in the following manner:

Examination of a very large number of check determinations revealed that if three determinations were obtained which checked closely, the average of the three would come very close to the average of a large number. There were exceptions to this, but these were infrequent. The number of determinations taken for checking had to be limited because of the time consumed, and from that standpoint three checks was nearly the limit. If there were any reason for questioning a value so obtained it was, of course, run over.

There were, therefore, never less than three determinations run for any value which was taken as a final result. Frequently there were many more. The limits to which the original determinations were required to check were such as to keep the variations in the per cent conversion values within $\pm 5\%$. It was not practical to check any closer than this with the method of measurement available, neither was

it necessary; for if the experimental work yielded the results desired the per cent conversion would be increased to a degree where it would rise above and no longer be obscured by errors of measurement.

RAW MATERIALS

The materials which have been taken as subjects for hydrolysis have varied. During the undergraduate work, yellow corn meal was used for at that time the predominant idea was to develop a system of analysis that could be applied to feeds and grains with good results (60). This material was retained during the graduate work until the enzyme hydrolyses were completed.

When acid hydrolyses were started, corn meal was abandoned because, with the large number of runs to be made, the work of preparing corn meal - i. e. removal of fat and starch - presented a problem in that so much time was consumed by this one step. During the following year a commercial egg flake preparation, marketed under the trade name of Keith's Egg Flakes, was used in the hydrolyses. This product was chosen because it furnished a fairly uniform, soluble, native protein, suitable for repeated experiments over a long period of time. At this stage of the investigation it was felt that one form of protein, from the hydrolytic standpoint, was as suitable as another.

A commercial serum albumin preparation, trade name "Proco," was used in the last year of research. The reasons for choosing this were that it ran a little more constant

than the egg flake preparation and could be dried readily to constant weight.

PRELIMINARY TREATMENT

Need of Treatment -

In the case of proteins such as egg flakes or blood serum no preliminary treatment is necessary. They are carbohydrate-free save for the carbohydrate radicles of conjugated proteins which cannot be removed chemically without destruction of the protein. Egg flakes contain practically no fat, while the fat content of "Proco" was shown by analysis to be 0.02%.

With a cereal or grain, however, the need for purification of the protein becomes imperative. Gortner and Blish (20) and Dowell and Menaul (13) have shown that carbohydrate in acid hydrolysis will cause a drop in the amino nitrogen and an increase in humin. What effect fat may have is not known, but this is easily removed and should always be done as it is advantageous to have the protein in a pure state, especially if the hydrolysate is to be analyzed.

Removal of Fat -

For fat extraction an apparatus was used whose detailed description may be found in the writer's undergraduate thesis (60). Briefly, it consisted of a cylinder within a cylinder, the inner cylinder being supported upon the outer one. Ether vapors passed up the space between the two cylin-

ders and was refluxed back to the top of the inner cylinder. The condensed ether percolating through the material in the inner cylinder constituted the extractive process.

The extractions were run for 24 hour periods. To determine if nitrogen was lost during this treatment, fifteen extracts were combined and concentrated. Duplicate Kjeldahl determinations on the concentrate failed to reveal any nitrogen. Almost all fat was removed by this treatment as a second run with a fresh solvent gave but negligible amounts of fat. About 100 c.c. of solvent were required to 125 gms. of corn meal in the inner cylinder.

Removal of Starch -

Center (11), following the technique suggested by Hamilton (31), employed trichloracetic acid for starch extraction. Center relied upon centrifuging to separate the trichloracetic acid from the extracted meal. This method is unsatisfactory, for while a sharp line of demarcation exists between the meal and the supernatant, the meal is so light as to make it impossible to drain off more than half the supernatant. The centrifuge treatment also limits the amounts, which is undesirable as the process requires much time. Using the standard 250 c.c. centrifuge bottle on a two place head, one is limited to about 200 gms. in weight of the meal before extraction. Modifying the method as described below, five to ten kilos weight of the original meal can be run through in practically the same amount of time.

The modified method is as follows: Six extractions were made with 2% trichloracetic acid. The extraction periods were of one-half hour each and the ratio of liquid volume to weight of original meal was as ten to one in all extractions. That is, for 400 gms. of the original meal 4,000 c.c. of 2% trichloracetic acid were used for each one of the six extractions.

Each extraction was carried out on a boiling water bath. The extraction was put into the boiling bath cold, and by the end of the half hour period had come up to 75-80°C. Care must be taken not to boil the extractions as trichloracetic acid decomposes on boiling. The extractions were stirred with a motor driven stirrer. Some difficulty was always encountered with the first extraction as the starch made the solution so thick that the stirrer soon ceased to operate. The first extraction, therefore, was stirred only during the first few minutes.

At the end of the extraction the liquid and meal were poured into a cloth bag. This was put into a meat press and pressure applied gradually. The material cannot be squeezed powerfully enough with the hands. The press is essential, and the more force that can be applied, the better. The bags do not last long due to the effect of the acid, but if washed out after using they are good for from thirty to fifty pressings.

It is extraordinary to watch the change in the ex-

pressed liquid. That obtained from the first extraction is soupy while that obtained from the sixth appears like water. Extractions were not run until the expressed liquid was negative to iodine in potassium iodide. It is very doubtful if this method would ever give a liquid that was starch free. Six extractions were used because it was found that this number was necessary to remove the greatest part of the starch, while more did not give any perceptible improvement in the finished product.

After the sixth extraction the protein is washed with a large volume of water on filter cloth and dried at 110°C. No nitrogen is lost during the extractive process. Combined filtrates from three runs, making eighteen extractions, were taken down to dryness, the dry product appearing physically to be exactly like corn starch, but nitrogen free. The low temperature and comparatively short period of time, coupled with the fact that 2% trichloracetic acid is not a particularly strong acid, are all unfavorable to hydrolysis, hence this was to be expected.

The protein content of the treated meal was calculated from duplicate Kjeldahl determinations which were made on the separate products obtained from each of three extractions. The Kjeldahl, or total nitrogen, value was multiplied by $6\frac{1}{4}$ to give an approximation of the protein content which, by this method, was determined to be 97.6%. The cellulose content of corn meal is on the order of 2%, accord-

ing to bulletins of the Amherst Agricultural Experiment Station and of the U. S. Government. Consequently, about 0.5% of the product of extraction is unaccounted for, and this is believed due to residual starch and fat.

The corn meal protein thus prepared was used during all the enzyme work. It was given up for the acid hydrolyses because of the time needed to prepare it. However, some of the preparation was hydrolyzed with hydrochloric acid and gave a hydrolysis as smooth and free from humin as that obtained with an animal protein such as egg flakes or serum albumin.

This was to be expected, because the work of Gortner (18) demonstrated that the amount of carbohydrate present determined the amount of humin formed. In Table 6 are reproduced figures, furnished by Gortner, which show the relation between the addition of increasing amounts of carbohydrate and the amount of humin formed.

Table 6 furnishes an idea of the losses that will be encountered due to humin formation if the proportion of carbohydrate to protein is very high. Gortner found in addition that an equal weight of carbohydrate in proportion to the protein raised the humin nitrogen to double the value without carbohydrate in practically every case. Where the ratio of carbohydrate to protein is about three to one, which is true in the case of corn meal, it is interesting to speculate on what the binding of nitrogen as humin would be

TABLE 6

Increase in Humin with Increased Amount of Carbohydrate

(Figures of R. A. Gortner)

Increasing Amounts of Carbohydrate Added to 3 Gms. Fibrin

Gms. Swedish Filter Paper Added	% Increase in Humin N over Original with No Carbohydrate
1.5	51.94
3.0	84.53
6.0	127.7
9.0	160.4

provided the material were not extracted. The foregoing facts make it clear that vegetable proteins, at least, require a preliminary treatment before hydrolyzing in order to obtain a satisfactory hydrolysis.

Drying to Constant Weight -

The materials taken for hydrolysis were in all cases first dried to constant weight. This was accomplished at 70°C. and 29.5 ins. vacuum. By this means any charring of the material due to excessive heat was avoided.

After removal from the vacuum chamber the material was transferred immediately to a desiccator and kept there until ready for use.

HYDROLYSIS WITH ENZYMES

Reasons for Choosing the Enzyme Method -

The chief reason for selecting enzymes as a means of hydrolyzing proteins was that such hydrolyses were free of humin formation. Consequently, enzymes were the hydrolytic agents employed by the writer during undergraduate work, and when that was completed it was thought advisable to continue with a study of this method of hydrolysis for a longer period in the hope that a satisfactory technique could be developed.

While it was recognized that in using enzymes there would be no difficulties encountered from humin formation, it was also known that practically no previous workers had reported enzymes as being as satisfactory hydrolyzing agents as mineral acids. It was the writer's belief, however, that if the proper attention was given to the conditions of the reaction, satisfactory results could be obtained. The manner in which these conditions were worked out and the degree of success obtained with them will be discussed in the following pages.

Enzymes Used -

During the undergraduate work two proteolytic enzymes were employed, Difco Pepsin 1:3,000, and Fairchild's

Pancreatin. These were later supplanted by Difco Pepsin 1:10,000 and Fairchild's Trypsin. The trypsin, according to information furnished by Fairchild Bros. and Foster, had a tryptic power of ten thousand Roberts units, determined by the meta-casein reaction originally proposed by Sir William Roberts. The more powerful preparations were used because it was soon found necessary to keep the nitrogen added with the enzyme to a minimum; and the more potent the enzyme preparation, the more nearly could this ideal be realized.

It was not possible to obtain preparations more potent than these from commercial houses. No attempt was made to prepare more powerful enzymes because it was felt that if a satisfactory method of hydrolysis were developed it should make use of the materials readily available. Also, the preparation of more powerful enzymes would constitute a large piece of research in itself.

Aside from the two enzymes, pepsin and trypsin, no others were employed in this work. The individuality of erepsin is doubtful, but in any case there must have been a goodly proportion of the so-called erepsin in the trypsin preparations which were used. Both pepsin and trypsin were necessary, however, for the writer (60) had previously shown that not only did trypsin produce a large increase in amino nitrogen but also that it acted upon some material which was untouched by pepsin. This latter fact corroborated the work of Northrop (56).

Conditions of the Reaction - Temperature -

Hydrolyses with both enzymes were run at 49-50°C. While this is the optimum temperature for peptic activity, trypsin has its optimum temperature at 45°C. The slight increase over the optimum for trypsin was not sufficient to be harmful, however.

The hydrolyses were run in a large water jacketed incubator. The volume of water was large, and consequently, once the incubator was up to temperature, the regulation was good. The variation of reaction mixtures when in the incubator was never more than ±0.5°C. after once coming up to temperature.

Conditions of the Reaction - H-ion Concentration -

While it had long been recognized that there was a vaguely defined zone of H-ion concentration in which pepsin and trypsin were most active, Northrop (51) brought the proper emphasis to bear upon this point. His work had to do with the kinetics of the action of enzymes, and he concluded that it was only over a very narrow range of H-ion concentration that the maximum rate of conversion would be obtained.

The writer was studying, not rates, but per cent conversion. The H-ion concentration was found to be optimum only within narrow limits for maximum conversion (60). The H-ion concentration was one of the conditions of the reaction which had to be most strictly fulfilled, for any deviation from the optimum value was immediately reflected in a decrease

in the per cent conversion.

The pH value for pepsin was taken as 1.8 and the value for trypsin as 8.0. The hydrolyses, whether in the case of pepsin or trypsin, were started at optimum value. At 1½ hour intervals a small sample was withdrawn and the pH determined electrometrically. The sample was returned to the hydrolysis and acid or alkali added as necessary to bring back the pH value to the optimum for the enzyme in question.

A reference to the writer's undergraduate thesis (60) will prove helpful for the method followed is given there in detail. Less alkali or acid, as the case might be, was required at each back titration, and during the final 1½ hour interval the pH value underwent no perceptible change. Similarly, amino nitrogen determinations made simultaneously with the pH determinations showed a slackening off in enzymic activity as the end of hydrolysis was approached.

Conditions of the Reaction - Time -

The time for the completion of the reaction was previously determined under conditions of optimum reaction and temperature (60). It was found possible to secure the utmost peptic activity in 4½ hours, while tryptic activity came to an end after a second period of six hours duration. Provided other conditions were carefully controlled, it was extraordinary how closely one could check these time limits again and again.

Addition of more enzyme after the optimum time had

expired, although pH and temperature were still at optimum, did not increase the corrected amino nitrogen value. The total amino nitrogen was increased, but after this had been corrected for the amount of amino nitrogen produced by autolysis of the enzyme, the amino nitrogen showed a drop. This was also true if the hydrolyses were allowed to stand beyond the optimum time without addition of further enzyme.

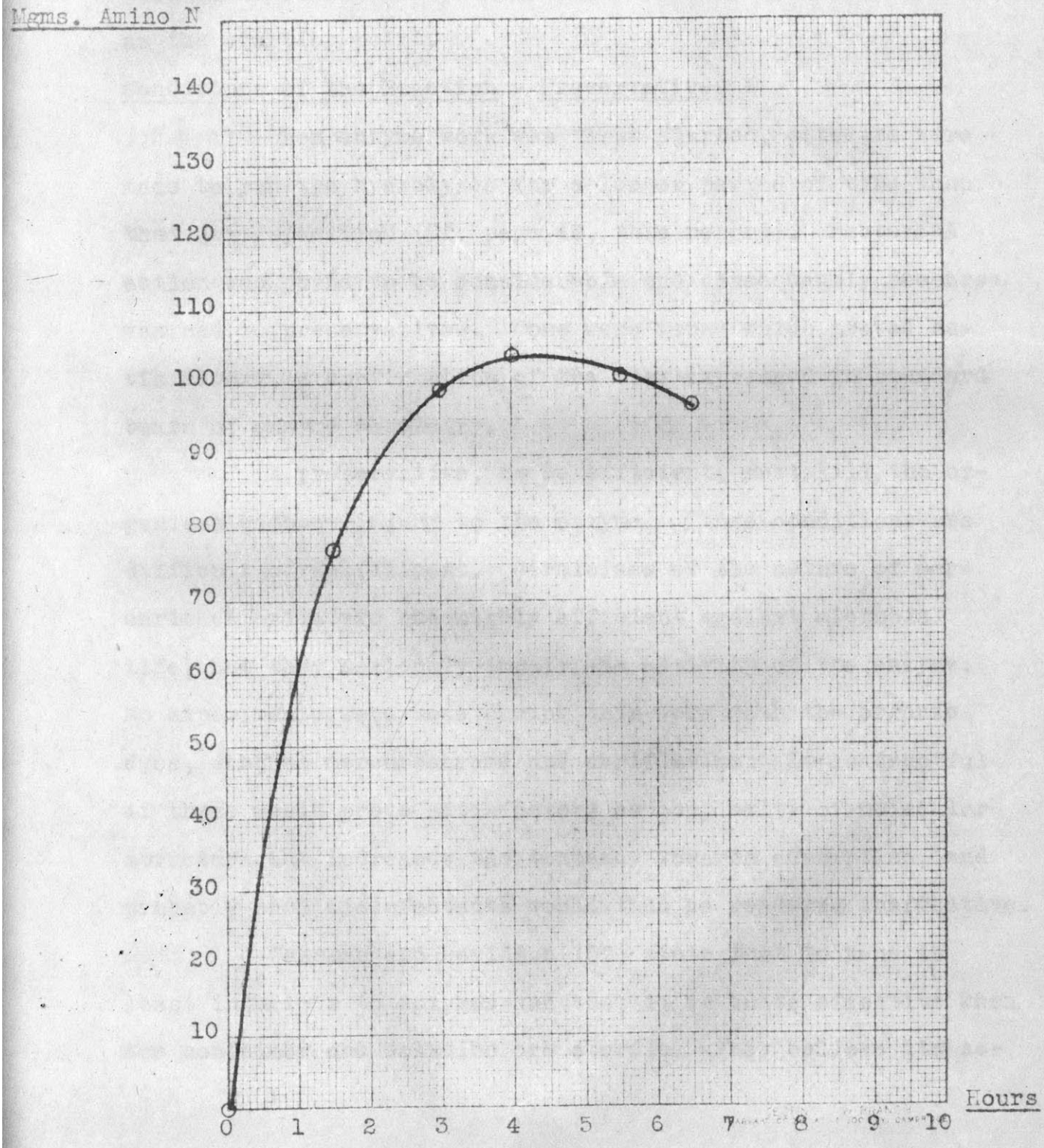
The facts just stated were found to be true in the case of both pepsin and trypsin. A reproduction of a curve obtained with one of the hydrolyses run during the writer's undergraduate work is shown in Figure 1, page 49a. The curve shows that the amino nitrogen (corrected) reaches a maximum in the case of pepsin after 4½ hours have elapsed. Then there is a loss of amino nitrogen as shown by the downward trend of the curve. The reason for this loss will be discussed at a later point (Cf. page 50, this report).

Conditions of the Reaction - Ratio of Menstruum to Substrate -

Northrop (51) had already called attention to the fact that there was an optimum concentration of menstruum and substrate at which hydrolysis would take place at the highest rate. The writer (60), studying not rates but per cent conversion, found that in the case of corn meal the best conversions were obtained when the ratio of liquid to corn meal protein was as ten is to one. This condition was fulfilled in all subsequent hydrolyses and was, of course, one reason why corn meal protein was retained as a raw material for all the

FIGURE 1

TOTAL AMINO NITROGEN PRODUCED DURING PEPTIC HYDROLYSIS PLOTTED AGAINST PER CENT CONVERSION



enzyme work even after the idea of setting up a system of analysis for feeds and grains had been abandoned, because this ratio, while true in the case of corn meal protein, must have been established anew for any other protein which was selected as the starting point.

Conditions of the Reaction - Preservatives -

When enzyme work was first started, attempts were made to run the hydrolyses for a longer period of time than that just specified (Cf. page 49, this report). Bacterial action was found to be considerable and consequently recourse was had to preservatives. None were found which proved satisfactory, a confirmation of the view expressed in standard texts of enzyme chemistry.

A preservative, to be efficient, must kill the organisms without injury to the enzyme. Those conditions are difficult of fulfillment. Germicides of the nature of mercuric chloride are undeniably efficient against microbial life, but they seriously impair the activity of the enzyme. No experiments were made during this work with the organic dyes, such as mercurochrome and acriflavine. It is doubtful if these would prove satisfactory as complexity of molecular structure but increases the tendency towards adsorption, and probably such disinfectants would thus be rendered ineffective.

Waksman and Davidson (87) state that toluene is least injurious to enzymes and that it is quite effective when the container and solution are sterile. They believe its ac-

tion due to the film formed over the surface of the liquid by toluene and not to any inherent germicidal power. The writer used Erlenmeyer flasks for the enzyme hydrolyses which were stoppered with cotton plugs. These were sufficient to keep contamination out of the apparatus. Toluene, therefore, was not used, but in the earlier experiments it had been found useless as a preservative. The difficulty lay with the enzyme preparations which were heavily contaminated. Even though the other materials and the container were sterile, bacteria were introduced with the enzyme, and there did not appear at the time to be any ready means of avoiding this difficulty. The nature of the bacteria and the effects due to their presence are discussed at a later point (Cf. page , this report). So far as preservatives are concerned, it is sufficient to state that no material was found which met the two necessary requirements - toxic to microorganisms and benign to enzymes.

Conditions of the Reaction - Amount of Enzyme -

In the earlier work (5, 60) it was found that some of the conversions obtained with enzyme hydrolyses were running over 100%. This was patently absurd, and it appears that the difficulty was that no allowance had been made for the amino nitrogen evolved by the autolysis of the enzyme preparation.

Accordingly, samples of pepsin and trypsin were taken and autolyzed under the same set of conditions employed

during hydrolysis. It should be clearly understood that in this control determination a combination of the two enzymes, pepsin and trypsin, was used. The reason was that this is the manner in which these enzymes are employed in the true determination. A description of the procedure used in the control determination where the enzymes are autolyzed applies equally well to the procedure followed during the true determinations.

The number given to the control determination is hydrolysis No. 110. This was not the only control hydrolysis run, but is taken arbitrarily as a representative of the determinations run for control. In the entire report this practice has been followed, and the hydrolyses which are selected for illustration are but isolated representatives of a large group. The procedure for hydrolysis No. 110 is as follows:

HYDROLYSIS NO. 110

10 gms. of Difco 1:10,000 pepsin were added to 150 c.c. of hydrochloric acid, pH 1.8, and incubated at 49-50°C. for $4\frac{1}{2}$ hours. The incubator used in this determination and all subsequent work was similar to that previously described. At the end of the $4\frac{1}{2}$ hour period, the mixture was boiled and allowed to stand over night. The

next morning the mixture was back titrated to pH 8.0, determinations being made electrometically to determine when the proper pH value was reached. Incubation was now carried out at 49-50°C. for six hours. The mixture was again boiled and allowed to stand over night. Van Slyke determinations were run on the hydrolysate the next morning, and the total amino nitrogen was found to be 876 mgms. As there were 10 gms. of pepsin and 10 gms. of trypsin used during the course of the digest, making 20 gms. of combined enzymes, it was calculated that 876÷ 20 = 43.8 mgms. of amino nitrogen for each gram of combined pepsin and trypsin added to the mixture. The value thus determined was used in a subsequent series of hydrolyses, Nos. 112 - 117 inclusive, to calculate the true value of amino nitrogen.

The true value was determined by subtracting the correction for the amino nitrogen produced by autolysis of the enzymes from the value for total amino nitrogen found in the hydrolysate.

The value now having been established for the autolysis of combined pepsin and trypsin, a series of hydrolyses, Nos. 112 - 117 inclusive, were run to determine the amount of enzymes which in proportion to a fixed amount of menstruum and substrate would give maximum hydrolysis with

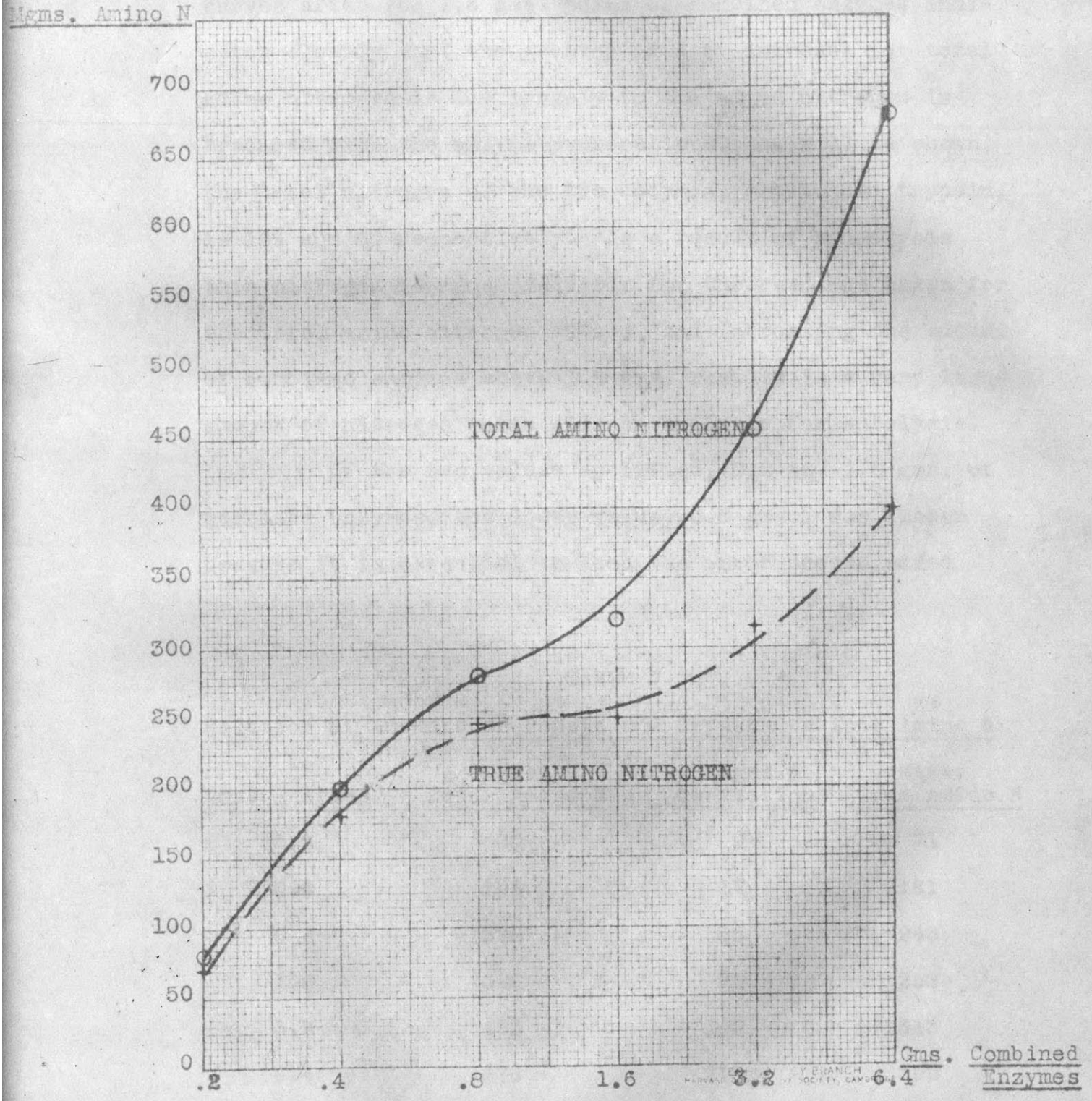
a minimum amount of enzyme preparation. This procedure was necessary because it was desired to keep the amount of nitrogen added with the enzyme preparations as low as would be consistent with a maximum conversion. The raw material taken as a source of protein was the same throughout the series, 10 gms. of corn meal protein which was prepared as previously described. The only factor varied during the series of hydrolyses, Nos. 112 - 117 inclusive, was the amount of combined enzyme added. All other conditions remained unchanged.

The results obtained are given in Table 7. A curve plotted from the figures given in this table is illustrated in Figure 2. The upper curve is for total amino nitrogen produced. The lower curve represents the effect of the amount of combined enzymes after a correction has been applied for the autolysis of the enzyme preparations, this correction having been established by the procedure followed in hydrolysis No. 110.

Study of either the table or the curves indicates that under the conditions specified the optimum amount of combined enzymes lies between 0.8 and 1.6 gms. per 10 gms. of corn meal protein to 150 c.c. total volume of menstruum. Below this range the production of true amino nitrogen, which is the value that must be carried to a maximum, is very small. Above it, the production is increased but to a much less extent than that increase obtained with the

FIGURE 2

MGMS. AMINO NITROGEN PLOTTED AGAINST AN INCREASING WEIGHT OF ENZYME PREPARATIONS



value for total amino nitrogen. The divergence of the curves after the 1.6 gms. point of combined enzymes indicates clearly that the greater rate of increase for total amino nitrogen is due largely to the added nitrogen introduced with the enzyme preparations. As will be shown, the total nitrogen of the two enzymes, pepsin and trypsin, is 16% and 6% respectively. As a result of hydrolysis this nitrogen is made available for the readings taken for the total amino nitrogen values, and increasing the amount of combined enzymes above 1.6 gms. results in a very large amount of nitrogen which will be available for autolysis.

Of the two values specified, 0.8 and 1.6 gms. of combined enzymes, the lower value, 0.8 gms., was chosen because it is essential to keep the error due to added

TABLE 7

Relation of Amounts of Pepsin and Trypsin to True Amino N

Gms. Enzymes	Mgms. Total Amino N	Mgms.# Correction	Mgms. True Amino N
0.2	80	9	71
0.4	198	17	181
0.8	278	35	243
1.6	322	70	252
3.2	453	140	313
6.4	678	280	398

(#) Note: For calculating correction Cf. page 52, this report.

nitrogen as low as possible, and the 0.8 gms. of combined enzymes gave a value for true amino nitrogen practically equal to that produced by 1.6 gms. of combined enzymes. This is readily apparent because 0.8 gms. of combined enzymes gives a value of 243 mgms. of true amino nitrogen while 1.6 gms. of combined enzymes gives a value of only 252 mgms. of true amino nitrogen. Because the desired result was practically the same in both cases, the value of 0.8 gms. of combined enzymes was chosen as this value would cut in half the error due to added nitrogen as compared with the error introduced by 1.6 gms. of combined enzymes.

Strength of the Enzyme Preparation -

There is no question but that the strongest obtainable enzyme preparations must be used if a satisfactory hydrolysis is to be obtained. The reasons for this may be summarized as follows:

First, the stronger the preparations, the less will be required to give maximum hydrolysis. Therefore, the less the error introduced by nitrogen added with the enzymes.

Second, authorities on Enzyme Chemistry (Beatty, Bayliss, Cohnheim, Effront, Euler) agree remarkably well on one point - that the end products of enzyme action inhibit the activity of the enzyme, probably through uniting with it, the union being either adsorptive or chemical.

Regarding the first point, there is given in Table 8 the values for total nitrogen obtained with Difco Pepsin 1:3,000 and 1:10,000, and Fairchild's Pancreatin and Trypsin.

These values indicate that whether the enzyme preparation is strong or weak the amount of nitrogen added with a unit weight will be considerable. The advantage lies with the stronger preparation, however, because a smaller amount need be taken to accomplish the desired result. The importance of this fact has already been demonstrated, Table 7, and the resulting curve plotted from this table in Figure 2, indicate clearly that a certain amount of enzyme is necessary to effect a satisfactory conversion of protein nitrogen to amino nitrogen. By this statement is meant, not the amount of enzyme preparation, but the amount of active substance, for it is the active substance which hydrolyzes and not the inert material. Keeping in mind, then, that a minimum amount of active

TABLE 8

Per Cent Total Nitrogen of Enzyme Samples

Pepsin		Trypsin	
1:3,000	1:10,000	"Pancreatin"	"Trypsin"
16.0%	15.5%	6.3%	6.0%

material is necessary, it follows that if the required amount of active material can be secured and the amount of inert material reduced, this would be theoretically approaching to the ideal condition for enzyme hydrolysis. Unfortunately, such a thing as a pure enzyme has never been obtained. The best strengths of pepsin and trypsin commercially obtainable are 1:10,000 and 10,000 Roberts units respectively, and these preparations, being the strongest, have consequently been employed in all enzyme hydrolyses run during the work covered by this report. The only exception to this statement is the case where the weaker preparations have been run under the same set of conditions as the stronger preparations in order to secure a comparison of efficacy of the two different strengths of hydrolyzing agents. It is not practical to carry the two proteolytic enzyme preparations, pepsin and trypsin, beyond the maximum strength of 1:10,000 and 10,000 Roberts units respectively, because while stronger preparations have been made they are not available commercially and to attempt to prepare substances of greater proteolytic power would constitute a piece of research that would cover a wider field than that covered by this entire report.

With regard to the second point, inactivation of the enzyme by the end product, Table 9 demonstrates that this consideration is more than theoretical because the data obtained and given in this table indicates it to be

an experimental fact. Two hydrolyses, Nos. 116 and 118, were run together. No. 116 contained 3.2 gms. of combined pepsin and trypsin, 1.6 gms. of each enzyme which makes a total for the two of 3.2 gms. of the 1:10,000 and 10,000 Roberts units respectively, while No. 118 contained the same amounts of the weaker enzymes, 1:3,000 pepsin and Fairchild's Pancreatin. Other conditions of hydrolysis, time, temperature, reaction, amount and kind of substrate, and total volume were the same; and these were identical with the conditions specified under Hydrolysis No. 110. In both Hydrolyses, Nos. 116 and 118, the amounts of enzyme preparation used was greatly in excess of that required for the given set of conditions, as only 0.8 gms. of the combined enzymes was necessary to effect maximum conversions. This condition was created purposely because it was to be expected, as long as the amount of enzymes was greatly in excess of the maximum, that hydrolysis with the weaker preparation would give a higher value for total amino nitrogen than the hydrolysis run with the stronger preparation. The reason is that in the case of the weaker

TABLE 9

Mgms. Total Amino Nitrogen Produced	
1:3,000 Pepsin and Pancreatin	1:10,000 Pepsin and Trypsin
312	453

preparation more nitrogen was available, not in enzymic form, but as extraneous matter which was hydrolyzable to some extent. The values given in Table 9 show that the total amino nitrogen produced in the hydrolysis run with the weaker enzyme preparations was considerably less than that obtained in the other hydrolysis where the preparations were much more active. From this fact it is possible to deduce one or both of two things - either the extraneous material added with the weak preparation adsorbed its share of the active enzyme and so checked the hydrolysis prematurely; or else, there being less enzyme added in the case of the weaker preparation (for the weight of the preparations was the same in both cases) the lesser amount was adsorbed out to such an extent that the hydrolysis could not proceed. Whatever the reason, the fact remains. The more active preparations are to be preferred, not only because a given weight of the preparation will push a hydrolysis under an optimum set of conditions than the same weight of a weaker preparation, but also because the error due to added nitrogen can be cut down through using less of the preparation.

It should also be mentioned that the type of curve shown in Figure 2 where the amount of enzyme is plotted in one case against the total amino nitrogen produced, and in another against the true value for amino nitrogen, could be obtained only if the assertion of the standard text

writers that the end products of enzyme action inactivate the enzyme were true. This conclusion can be drawn because a study of the first part of the curve for true amino nitrogen indicates no satisfactory reason as to why it should flatten out and reach a maximum of efficiency, as compared to the amount of enzyme added, at the 0.8 - 1.6 gms. point unless a factor entered in which restricted the rate of enzyme activity. The curve reveals that such a factor does exist. In accord with theoretical consideration this factor must consist of adsorption of the active enzyme on hydrolytic products of protein. It must be remembered that when a comparatively large amount of enzyme preparation is added, such as 3.2 gms. under the conditions of hydrolysis stated, there is also added a large amount of inert material. This material, by the very nature of the preparation of proteolytic enzymes, consists entirely of protein degeneration products, probably in the nature of peptones or lower in the scale of protein degradation. These substances are quite as good enzyme adsorbents as those produced by the action of the enzyme on the protein substrate. A large amount of enzyme, and particularly of enzymes of lower strength, results in a large amount of protein derivatives being added to the hydrolysis mixture when this amount is considered proportionally in relation to the amount of substrate and volume of menstruum, which in all the experimental work conducted with enzymes was 10 gms. of corn meal protein and 150 c.c.

total volume.

The deductions given above are in accord with the work of Northrop (53) who found that the rate of digestion of protein by pepsin is not proportional to the total concentration of pepsin. It is as well to make clear at this point that the writer was concerned chiefly not with rates of protein hydrolysis, but with effecting maximum conversions. Because of the effects of bacterial action, the rate is an important factor which cannot be neglected; for if enzyme hydrolyses are not carried to their maximum within a minimum of time, the effects of bacteria are highly detrimental. Nevertheless, the chief object of this research, regardless of the hydrolyzing agent used, was to effect the maximum possible conversion. In this respect the work done and the data presented are unique, because previous investigators, such as Northrop (50-54) and Greenberg and Burk (28) have dealt with the kinetics of protein hydrolysis. Such work is valuable in that a study of the rates of conversion obtainable is bound to furnish an idea of the efficiency of hydrolysis. It does not follow necessarily that a high rate of conversion will carry the hydrolysis to a greater degree of completion than a slower one, but the time factor is important in hydrolysis with both enzymes and inorganic reagents.

Time is important in enzyme conversion because of the effects produced by bacteria. As will presently be

shown, bacterial action results in the loss of amino nitrogen. The detrimental effects of bacterial action increase as time goes on. It is, therefore, important in enzymic conversions that the time taken be kept to a minimum so as to reduce bacterial action as much as possible.

The time factor is also important in acid hydrolysis. Henriques and Gjaldbak (35) study the conditions for the complete hydrolysis of proteins and found that when proteins are hydrolyzed with acid, both amino nitrogen and ammonia increase up to a certain point at which the amino nitrogen attains its maximum. If the hydrolysis is carried beyond this point a transformation of amino nitrogen into ammonia follows, indicating a deaminization of some amino acid or acids. Consequently, they define the end point of hydrolysis as that point at which the amino nitrogen reaches a maximum with the least possible formation of ammonia. It should be noted that this end point of hydrolysis which has just been defined is not complete hydrolysis. In fact, since complete hydrolysis has never yet been attained, the two points do not coincide. The time factor, however, does enter in here, because if the time of boiling be prolonged beyond that necessary to reach the end point of hydrolysis a loss will occur as deaminization results in the freeing of amino groups as ammonia.

Van Slyke's (80) data also indicates that prolonged heating results in a loss of amino nitrogen. After $1\frac{1}{2}$ hours

heating at 150°C., the amino nitrogen content was 78.3 per cent, while after three hours heating at the same temperature the value for amino nitrogen dropped to 73.4 per cent. The work of Gortner (18-26), in which the effects of carbohydrate, aldehydes, and ketones on humin formation were studied, also indicates that time is an important factor in hydrolysis with mineral acids, because prolonged boiling resulted in an increase in the amount of humin formed.

The rate at which hydrolysis takes place is, then, a factor which will determine to a greater or less extent the success of the conversion. The highest rate obtainable is desirable. To that extent the studies on the kinetics of protein hydrolysis are valuable in determining what combination of reagents will be most satisfactory in effecting maximum conversion.

The work which has been completed during this research has been conspicuously successful in that the conversions have reached their maximum within a minimum of time. The data previously presented shows that peptic hydrolyses reached their maximum after $4\frac{1}{2}$ hours, while tryptic hydrolyses required but 6 hours to come to completion. These periods of time are in sharp contrast to those employed by earlier investigators where the time intervals employed extended over a period of weeks. As will later be shown, the conversions secured with acid hydrolyses have also reached their maximum in an extremely short period of

time.

Because of the time factor, the stronger enzyme preparations must also be used because the data presented indicates that a higher rate of conversion is secured with a larger amount of enzyme than could be secured with less than the optimal range of 0.8-1.6 gms. of enzyme preparation per 10 gms. corn meal protein in 150 c.c. total volume.

The Law of Mass Action - Per Cent Conversions -

It is known that hydrolyses with proteolytic enzymes come to an end long before the total conversion of the protein into amino acids has been effected. It is a question as to whether this effect is due to the kinetics of the Law of Mass Action or to inactivation by the end products. The writer favors the inactivation theory, for the experimental evidence obtained in this work supports it. The data obtained and illustrated in Figure 2, where a comparison is shown between the different rates of production of true amino nitrogen compared to total amino nitrogen, indicates that it is the adsorptive factor which is at work. This is also true of the comparative hydrolyses shown in Table 9.

Because the same protein can be hydrolyzed to a greater extent with mineral acid than with enzymes, the view that an equilibrium has been reached in enzyme hydrolysis is untenable, for there is no reason why equilibrium should be reached sooner in one case than in the other; no

reason, that is, attributable to the workings of the Mass Law, which is the only theory that can explain the end of hydrolytic action on the basis that an equilibrium has been reached. Equilibrium is, therefore, explainable only on the basis of inactivity of the enzyme by the end products.

The best per cent conversions which the writer was able to obtain with enzyme hydrolyses were on the order of 45 per cent. This was found to be the maximum value obtainable in spite of the fact that the conditions have been made most favorable for enzyme action. That is, time, temperature, reaction, ratio of menstruum to substrate, and concentration of the enzyme were all optimal for a fundamental basis of 10 gms. of corn meal protein per 150 c.c. total volume. It is not a good conversion compared to that which may be obtained with the same protein when treated with mineral acid, as the conversion in that case has been found to range from 60 to 70 per cent if the acid concentration and time were optimum.

There appeared to be no further modification of the conditions of reaction which would increase the conversion obtainable with enzymes above 45 per cent, because these conditions had already been determined by experiment to be the best obtainable. It was decided, however, that while the conversions were coming to completion more because of inactivation of the enzyme by protein degeneration products than by workings of the Mass Law, it would be worth while

to attempt the removal of these hydrolytic products from the reaction mixture. If this were done, detrimental effects of the end products would be minimized if due to the Mass Law. If due to adsorption, an addition of further enzyme should produce a greater degree of hydrolysis, because as the protein degeneration products were removed there would be less to adsorb the active enzymes.

Dialysis -

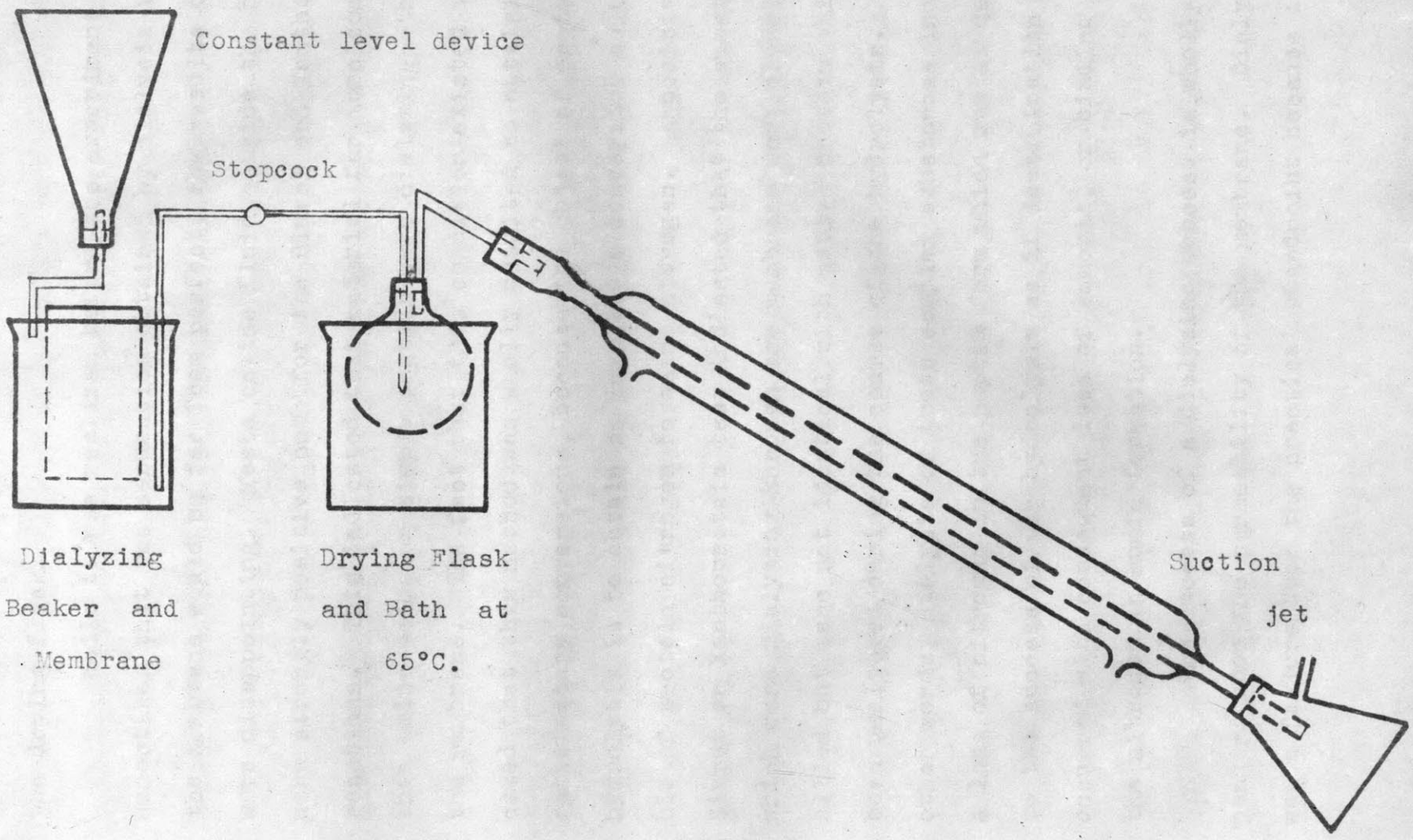
Unfortunately, one is limited in dealing with enzyme hydrolyses by the fact that conditions of the reaction can undergo hardly any variation without an almost immediate departure from the optimum which is necessary for the most efficient enzyme action. Furthermore, no combination of reagents or adsorbents were found which could be added to the reaction mixture without destroying the activity of the enzymes which, because of their susceptibility to inactivation by the usual chemicals, cannot come into contact with them without impairment or total destruction of their activity. Adsorbing agents will combine with the enzyme equally as well as protein degeneration products because of the similarity which exists between them. It appeared, therefore, that dialysis was the only means by which the end products could be removed from the sphere of the reaction.

Accordingly an apparatus was devised of which a diagram is given in Figure 3. A large collodion membrane

was used within which the hydrolysis was run. Into this membrane was put the necessary combination of reagents for the most efficient enzymic conversion, namely, 10 gms. corn meal protein, 150 c.c. of menstruum, 0.4 gms. of first pepsin and then 0.4 gms. trypsin when the reaction had been shifted to pH 8.0. The membrane containing the hydrolysis mixture was suspended in a beaker filled with hydrochloric acid at pH 2.0 for the peptic hydrolyses, and the acid in the beaker was replenished by a constant level device. The liquid in the beaker, which was outside of that contained in the collodion membrane, was sucked slowly through a tube which terminated in a fine capillary. Suction was applied at the end of the apparatus by means of a suction pump. The jet drawn through the capillary struck the sides of a flask which was immersed in a water bath kept at 65°C. Because of the fineness of the capillary, the jet obtained was very small, and as the spray struck the warm sides of the flask, the temperature plus a vacuum of 29.5 ins. was sufficient to dry the dissolved material at once. This material had come into solution by dialysis through the membrane. The apparatus was so designed that from 3800 to 4000 c.c. of pH 2 hydrochloric acid would be drawn down from the supply in the optimal peptic conversion time of four and one half hours, or in the case of trypsin the same volumes of pH 8 sodium hydroxide would be drawn through in six hours. Regulation of the rate of flow was controlled by means of a

FIGURE 3

APPARATUS FOR CONTINUOUS DIALYSIS OF ENZYME DIGESTIONS



stopcock in the line leading from the dialyzing beaker to the drying flask.

While it was realized before the experiment was undertaken that the separation obtained by dialysis through the membrane would be far from perfect, the results obtained were disappointing. Tests on the fluid outside the membrane were strongly positive both for the Biuret and Xanthoproteic reactions. This indicated that material far more complex than amino acids or simple peptids were dialyzing through the membrane. The fact that this condition existed indicated that this method was wholly unsuited to obtaining satisfactory conversions, because the object of proteolytic hydrolysis is to obtain as complete a conversion as possible of protein nitrogen to amino nitrogen. A positive Biuret or Xanthoproteic test indicated that the substances which were dialyzing through the membrane were closely allied but were not identical with native protein which had been unaltered during the course of the hydrolysis. In other words, dialysis of these complex substances indicated a loss of nitrogen in the peptid form which was as damaging to the success of the hydrolysis as if deaminization had occurred with consequent loss of ammonia, or binding of the nitrogen in humin formation.

The success of a dialyzing process is wholly dependent upon the permeability of the membrane. Dialysis was a failure from the practical standpoint because of the

difficulty of securing a membrane which would be satisfactory. There can be no question but that a simple dipeptid such as glycyl glycine has a smaller molecular size than a complex amino acid such as tryptophane. The type of dialysis which was used in these experiments is a physical phenomenon, and it is dependent upon differences in molecular size to effect a separation of one substance from another. On the physical basis, therefore, no membrane exists which can refuse the passage of the physically small dipetid yet allow the large amino acid to go through.

Neither does it appear that electrodialysis would be any more successful because this process is also dependent upon a physical basis for separation of substances. There also is the difficulty introduced in this type of procedure by electric current, which might exert a detrimental upon the enzyme, even inhibiting its activity entirely. There is, however, the possibility that the mutual solubilities of the substances which it is desired to separate could be utilized for the removal of the hydrolytic products, provided a membrane were used in which the amino acids were soluble and all other protein degeneration products were not. The success of the dialyzing procedure if this principle is to be followed would also be wholly dependent upon the selection of a proper membrane. Granted that such a membrane could be found, nevertheless, all the disadvantages common to the enzyme method would still be retained,

that is, the difficulties presented by bacterial action, which if brought about by bacteria introduced with the enzyme preparation, and by the nitrogen which necessarily must be added with the enzyme preparation. In view of these difficulties, the dialyzing procedure was abandoned.

Buffer Salts -

When it was found necessary to back titrate enzyme hydrolyses in order to keep the reaction at or near the optimal value, it was thought that the use of buffers to maintain the reaction would be a decided advantage. Accordingly, hydrolyses were run in which buffer solutions were employed to give proper H ion concentration. For a value of pH 2.0 a potassium chloride and hydrochloric acid buffer was used, while for pH 8.0 the buffer was made up of monopotassium phosphate and sodium hydroxide. The pH 2.0 buffer was made up using 3.73 gms. of potassium chloride and 1.55 c.c. of concentrated hydrochloric acid made up to one liter with water. The pH 8.0 buffer was made up with 6.8 gms. of monopotassium phosphate and 2 gms. of sodium hydroxide, also made up to one liter with water. These are the concentrations given by Clark in the text on H ion concentration. The buffers were not made up using carefully purified fifth molecular solutions, but the amount of dry substances specified was used and the pH of the resulting solution determined electrometrically. According to whether a buffer was on the acid or alkaline side of the

desired point, alkali or acid, or potassium chloride or acid was added to bring the buffer solution to the proper value. The buffers thus prepared were used in the hydrolyzing mixtures full strength, that is, the requirement of 150 c.c. of menstruum was fulfilled by adding to the mixture 150 c.c. of buffer solution.

The first series of hydrolyses run with the potassium chloride hydrochloric acid buffer showed that there was no enzyme action, for there was absolutely no conversion of protein nitrogen to amino nitrogen, and this is attributable to the toxic effect of the high concentration of chloride ion on the enzyme. The tryptic hydrolyses, on the other hand, ran through to the maximum point successfully, the time being required to effect the maximum conversion still being six hours. Apparently, then, sodium and phosphate ions are not toxic to trypsin.

It was not feasible to employ buffer solutions in either the peptic hydrolysis or tryptic hydrolysis because in the case of pepsin the enzyme was rendered inactive, while in the case of trypsin the reaction of the buffer changed. This change was not as rapid as that noted where sodium hydroxide alone was used, but during the six hour interval consumed by tryptic hydrolysis, the change was considerable as the buffer dropped from pH 8.0 to pH 6.7 to 6.8 in all cases. This change in the reaction of the buffer is not surprising for it must be remembered that the

hydrolytic products, of which a considerable amount are liberated during tryptic hydrolysis, also have the properties of buffers. Furthermore, protein hydrolysates are distinguished by the fact that they are highly buffered, and there is no question but that the buffering effect of the hydrolytic products was so great as to completely overcome the effect of the phosphate sodium hydroxide solution, because the final pH reached at the end of tryptic hydrolysis was the same as that which would have been attained if no buffer had been used.

In view of these facts, therefore, it was decided that the buffer solutions specified were totally unsuited to hydrolyses with pepsin and with trypsin, for in the case of pepsin, the enzyme action was inhibited entirely, and in the case of trypsin the reaction did change. There was also a further undesirable feature in that in the case of either buffer a large amount of extraneous material had been introduced in the form of buffer salts, which would render the hydrolysate difficult if not impossible to treat subsequently for indentification and estimation of the individual amino acids.

Bacterial Action -

The fact that bacteria are present in enzyme hydrolyses and that they were added with the enzyme preparations has already been mentioned. That the enzyme preparations were responsible for contamination by bacteria was demonstrated to

be so. Control hydrolysis were run which were the same in every respect as hydrolyses run for true conversion with the exception that no enzymes or combination of enzymes are added. These controls were carefully sterilized in an autoclave at 20 lbs. pressure for one hour so that there could be no question as to the sterility of the controls. These control hydrolyses remained unchanged in all respects for periods which extended up to and including three months. That no change had taken place was evinced by the fact that Van Slyke determinations on the liquid in the controls were negative. Filtration of the menstruum from the substrate and subsequent analysis for ammonia are also negative, and total nitrogen determinations on the dried substrate corresponded with those made before the substrate was introduced into the control flask. It was evident, therefore, that no change had taken place, and the controls consequently were proved to be sterile.

To certain of the sterile controls pepsin was added in the optimum of 0.4 gms., and to others trypsin was added in the same amount. These hydrolyses were then incubated for the optimum time of four and one half hours and six hours respectively. Back tritration in both cases was accomplished by using sterile solutions and sterile pipettes. It is apparent, therefore, that any contamination which occurred must have resulted from the introduction of the enzyme preparations.

After incubation all flasks were allowed to stand at room temperature. It was very soon evident that putrefactive

changes had set in. Within two hours there was a decided drop in the total amino nitrogen from the maximum value which had been obtained, and after 18 hours the appearance and odor of the hydrolysates were conclusive evidence that decomposition had begun. The bacteria found in the hydrolysates were in cases of both pepsin and trypsin very large and extremely motile rods. They could be readily found under the high dry and were the largest the writer has ever seen. It is evident that they were thermophiles as hydrolyses contaminated with these organisms gave evidence of putrefaction much more quickly if the temperature was maintained at 49-50°C. than at 37°C. or room temperature. They could not have formed spores that were highly resistant, as merely bringing a hydrolysate to a boil was sufficient to insure sterility. No attempt was ever made to identify the organisms. The fact of their presence was sufficient.

The enzyme preparations could have been sterilized by making up a glycerine extract or passing substances in solution through a Berkfeldt filter. In the case of glycerine, the water which dilutes the glycerine, is the solvent for the enzyme, not the glycerine itself; hence glycerine preparations are invariably weak. The enzyme would also be weakened by filtration due to adsorption on the pores of the filter. The necessity of a strong enzyme preparation has already been emphasized.

Loss in Amino Nitrogen -

Referring back to Figure 1, it will be noted that the curve for production of amino nitrogen plotted against time rises to a maximum at the 41/2 hour point and then assumes a downward trend. When this was first noted it was thought there had been an error in the measurements. Checks with other hydrolyses proved, however, that this drop was always to be expected. The only variation was in the rapidity with which it took place, and this did not extend over a wide range.

The decrease, then, had to be accepted as a fact. One explanation which can be given is that after equilibrium had been reached the reaction tended to reverse itself and the protein decomposition products were recombined with a consequent loss in free amino nitrogen. There are many workers who claim that the proteolytic enzymes are able to effect a synthesis of complex products from simpler derivatives. Borsook and Wasteneys (6, 7, 8) have presented the most recent work on this subject. Their papers deal with the synthesis of protein by pepsin from peptic digestion mixtures. Taylor (75, 76) claims to have synthesized protein with trypsin. This success was met with after an earlier failure (74). Satisfactory results were finally obtained because the trypsin which was used was claimed to be non-hydrolyzable. Abderhalden and Rona (2) state that they were unsuccessful in synthesizing protein with trypsin. Robertson (66) claimed to have secured

a synthesis with pepsin, and in addition stated that the greater the concentration of the enzyme, the greater the synthesis obtained.

The drop in amino nitrogen can also be explained by bacterial action. Protein cleavage products are an ideal food for bacteria. The hydrolysates were heavily contaminated with bacteria which were introduced with the enzyme preparations. On multiplying, they utilized the protein as a source of nitrogen, and there is no doubt but that the simpler derivatives would be taken first because most readily assimilable.

Of the two explanations, the second seems the more tenable, particularly when one considers the evidence offered in support of the idea of protein synthesis by means of the hydrolytic enzymes, pepsin and trypsin. Borsook and Wasteneys (6, 7, 8) make no mention of aseptic precautions other than the addition of small amounts of chloroform. Considering the length of time that their "syntheses" were run and the temperature at which these were maintained, it would appear very likely that the so-called synthesis was in reality an effect arising from bacterial activity. The enzymes used by these workers certainly were not sterile, and in the cases where they inactivated the enzyme, in order to demonstrate that it was the synthesizing agent, they also practically sterilized it. No action was observed with an enzyme thus treated, which is not surprising if we adopt the view that the bacteria contaminating the enzyme preparation were dead. As for temperature

effects, these workers found that at 80°C. no synthesizing action was obtained, but it was noted at temperatures up to this point. It is remarkable that pepsin, which has an optimum of 49-50°C. in its hydrolytic action, should exhibit such a marked change in temperature requirements for a synthetic action. It is even more remarkable that the limit-value of 80°C. should coincide with the upper temperature limit for thermophilic bacteria. Borsook and Wasteneys (6, 7, 8) finally point out that the reaction took place best at pH 4.0. That, however, is not damaging to the belief that their results were due to bacterial action rather than to any synthesis because the organisms encountered by the writer were extremely active at pH 1.8 to 2.0.

Taylor's (75) claim that the trypsin prepared by him from the liver of a large Pacific Coast clam was non-hydrolyzable seems very doubtful. Resistance to autolysis, under favorable conditions of temperature and reaction, is not a characteristic of proteolytic enzymes. The only aseptic precaution observed by Taylor was the addition of toluene to the synthesizing mixtures. Five months were required for the production of two grams of "protamin" from 400 gms. of amino acids. After the five month interval the contents of the container were stated to have been tested bacteriologically with negative results, but the nature of the tests is not given; moreover there was ample time for bacterioautolysis. Taylor was, apparently, the only worker who gave the bacteriological

aspect serious consideration, but not knowing the type of bacteriological tests employed by him, it is difficult to tell whether or not his synthesizing mixtures were truly sterile. It has been the experience of the writer that toluene does not prevent growth of bacteria.

Robertson (66) makes no mention whatsoever of aseptic precautions. He worked with a pepsin of which no identification was given other than to name a commercial brand. At the time that his work was carried on (1908-09) it is doubtful if anything better than a 1:3,000 pepsin was obtainable commercially. Therefore, the claim that the degree of synthesis obtained was proportional to the concentration of the enzyme reaches what is almost an absurdity, for a pepsin preparation of 1:3,000 or less is so impure as to make it impossible to raise the concentration of the enzyme in solution without adding a large amount of extraneous material. The effect of this is to change the relation of concentrations between menstruum, substrate, and enzyme, which means that three variables are introduced instead of one. To be dealing with three variables, and ascribe the effects obtained as due to one, is not reasonable.

The discussion just presented on methods of determining the synthetic action of protolytic enzymes makes it clear that this fact is not yet established so definitely that it can be relied on. Furthermore, even if it were proved to be so, this would not mean necessarily that it had any

bearing upon the hydrolyses run during this work, because these were contaminated with bacteria. It seems, therefore, most likely that the loss in amino nitrogen noted after the optimum time had elapsed was due to bacterial action.

Importance of Optimum Time -

The foregoing discussion emphasizes particularly the importance of the time factor in enzyme hydrolyses. To put it plainly, from the moment the hydrolysis is started it is a race between the enzyme and the bacteria. While the latter are outdistanced at the start, they are sure to win unless the hydrolysis is sterilized at the proper time. That marks, of course, the end of all enzymic activity.

How much the tapering off of the curve shown in Figure 1, page 49a, is due to the effects of bacterial action is impossible to say. The effect should be relatively small compared with that produced by adsorption of the enzyme on the end products, because during the first part of the hydrolysis the production of amino nitrogen is very rapid. During the last stages, although temperature and reaction are still at the optimum, the amino nitrogen increases much more slowly. If the enzyme were still active the reaction should continue longer at the higher rate because the hydrolysis can be carried to further limits by other means. In four to six hours time the effects of bacteria should not have become so great as to account for much of the decrease in the production of amino nitrogen, but let that come to a standstill and every hour that

passes makes the destructive effects of bacteria cumulative.

Increase in Acidity - Decrease in Alkalinity -

Another phenomenon, in addition to the loss of amino nitrogen, which is noted as a result of bacterial action, is the increase in acidity or decrease in alkalinity of the hydrolysate as determined electrometrically. That is, after the optimum time has elapsed, the acidity increases as the value for amino nitrogen decreases.

The acid may come from carboxylic acids which are set free after deaminization of amino acids by bacteria. Also, it may be hydrochloric acid liberated after the amino group was lost, originally bound as an amino hydrochloride. Probably both these factors are operative.

No experiments were made to determine which of these two factors was responsible. The increase in acidity was taken as supplying further confirmation that the loss in amino nitrogen was due to deaminization of protein hydrolytic products through bacterial action.

Reasons for Abandoning the Enzyme Method -

The experimental data supplied in the foregoing pages makes it evident that hydrolysis with enzymes, even under the best conditions obtainable, is unsatisfactory. The reasons why successful conversions cannot be obtained may be summarized as follows:

(1) Addition of nitrogen with the enzymes.

(2) Low conversion compared to that obtainable

with the same protein when treated with mineral acid.

(3) Bacterial action.

The difficulty encountered in nitrogen added with the enzyme can be minimized by using enzymes of extreme purity, provided the technique is available to secure them. Some nitrogen, however, must always be added because to the best of our knowledge and belief enzymes are nitrogeneous in nature. A correction for added nitrogen can be made by running a control determination on the enzyme preparations alone, but there is no guarantee that the value thus obtained will apply when the enzyme is introduced into a mixture in which hydrolysis of other proteins takes place. If the error in correction is thrown on an amino acid which is present in the protein studied, the error in results and conclusions will not, either qualitatively or quantitatively, be as serious as if the protein were entirely lacking in any or all of the amino acids to which the correction must be applied.

Neither does it appear that the conversions obtainable with combinations of pepsin and trypsin as hydrolyzing agents can be pushed to any further degree than 45 per cent conversion of total nitrogen to amino nitrogen.

Conditions for hydrolysis were maintained at the optimum, which resulted in a high rate of conversion, but it does not appear practical to alter these conditions in

any manner which would assist in carrying the conversion to a higher degree without impairing the efficiency of the enzyme.

The bacteria present in enzyme preparations constitute another drawback to this method of protein hydrolysis. Their removal cannot be accomplished without weakening the enzyme preparation, and above all this must be avoided. In view of these facts, therefore, it was deemed advisable to give up enzymes as hydrolytic agents for proteins.

HYDROLYSIS UNDER PRESSURE

Reasons for Pressure Treatment -

After experimenting with dialysis as a means by which better conversion could be obtained with enzymes, the possibilities of increasing the per cent conversion of enzyme hydrolyses seemed about exhausted. Before abandoning the enzyme method entirely, however, it was though advisable to give some hydrolyses subsequent treatment with inorganic reagents. There were two possible objects to be gained by such treatment. First, humin formation might be greatly minimized compared to that obtained with hydrolysis by the inorganic reagent alone. Second, the per cent conversion might be raised above that obtainable with either hydrolyzing agent alone.

Pressure treatment was selected because of the satisfactory results reported by Van Slyke (80) at 150°C. for $1\frac{1}{2}$ hours, which appeared to be very promising, as a good conversion had been secured in a short time.

Procedure -

Enzyme hydrolyses were run following the procedure outlined previously. At the end of tryptic hydrolysis the hydrolysate was boiled and allowed to stand. It was then subjected to autoclaving. The acid autoclavings run with 0.1 N hydrochloric acid, while 0.1 N sodium hydroxide was

used in the alkaline autoclavings. The period of autoclaving was two hours, measured from the time the autoclave had come up to temperature. The steam pressure used was 20 lbs. which is approximately equivalent to 126°C. Van Slyke determinations were run on the hydrolysate after it had cooled, and again after neutralization to pH 7.0.

A few hydrolyses were also run using acids other than hydrochloric acid. These will be distinguished from the others by consideration in a separate table. It was also found necessary to determine whether or not the losses in amino nitrogen which were found were losses as ammonia. This was determined by attaching a trap to the container

TABLE 10

Hydrolysis No. 211

Effect of Autoclaving on Amino Nitrogen Content

Procedure	Mgms. Total Amino Nitrogen
End of enzyme hydrolysis	284
1. Acid autoclaving	243
Neutralization	252
2. Alkaline autoclaving	261
Neutralization	241
3. Acid autoclaving	174
Neutralization	186
4. Alkaline autoclaving	198
Neutralization	181

which was filled with 0.1 N sulfuric acid. Nesslerization of the contents of the trap gave an idea of the amount of ammonia produced, if any. No quantitative determinations were run on the trap contents.

Results -

Hyrdolyses Nos. 211 and 212 give one a very good picture of the results secured by the treatment outlined above. Note that the treatments with acid and alkali were alternated, and that neutralization and a subsequent determination of amino nitrogen followed each autoclaving and determination.

TABLE 11

Hydrolysis No. 212

Effect of Autoclaving on Amino Nitrogen Content

Procedure	Mgms. Total Amino Nitrogen
End of enzyme hydrolysis	308
1. Alkaline autoclaving	150
Neutralization	144
2. Acid autoclaving	149
Neutralization	150
3. Alkaline autoclaving	103
Neutralization	96
4. Acid autoclaving	100
Neutralization	98

A study of the results indicates that the per cent conversion is not increased by subsequent autoclaving of enzyme hydrolysates. In cases where there is not a definite loss, there is no gain. It was to be expected that there would be losses resulting from the treatment with alkali, and it will be seen that these losses are very great. Peculiarly enough, however, they occur only if the alkaline autoclaving is applied first. If it follows an acid autoclaving the value for total amino nitrogen is not decreased but instead increases slightly. The writer can suggest no reason as to why this should be so.

One must keep in mind, however, that during the autoclaving the effect of neutral salts was bound to affect the course of the reaction. The salt is formed as a result of the neutralization of the hydrolyses. It is, of course, sodium chloride, and during either acid or alkaline autoclavings supplies a common ion. Stieglitz (72), Northrop (54), and Falk (15) all emphasize the importance of neutral salts in hydrolysis and the peculiar effects which may be expected from them. In the case of Hydrolysis No. 211 a greater amount of sodium chloride will always be present when the alkaline autoclaving is run than in Hydrolysis No. 212. Probably this condition is responsible for the difference.

That the nitrogen which was lost was evolved as ammonia was easily shown by using traps filled with 0.1 N

sulfuric acid which was Nesslerized after an autoclaving was completed. The trap contents gave heavy precipitates in the case of hydrolyses similar to No. 212 after the alkaline autoclavings. In all other cases the Nessler color was slight. No quantitative determinations were made, for it was readily apparent that the loss in amino nitrogen which was encountered in hydrolyses of the 212 type was due to deaminization and subsequent evolution of the amino groups as ammonia. This was in accord with what would be expected.

Experiments were also conducted using acids other than hydrochloric. The results obtained us are given in Table 12.

TABLE 12

Effect of Acid on Autoclaving

Total Amino Nitrogen Before Autoclaving 250 Mgms.

0.1 N Sulfuric Acid	0.1 N Acetic Acid	0.02 N Oxalic Acid'
263	246	228

There is nothing in these results to indicate that the method would give a sizable increase in per cent conversion. The autoclave treatment retained all the disadvantages inherent to the enzyme method. It failed in the

desired purpose of increasing per cent conversion. It did not reduce humin formation materially. The alkaline hydroyses were rich in humin, but the acid hydrolyses had but very little black insoluble humin. What humin appeared in these hydrolyses was for the most part soluble and gave a clear red to dark red, almost black, color to the solution. These conditions are little, if any, better than those encountered with acid hydrolysis by the ordinary methods. Steam pressure treatment of enzyme hydrolysates accordingly was abandoned.

HYDROLYSIS WITH ACID

Reasons for Acid Hydrolysis -

Acid hydrolysis was the final method studied. It was undertaken only after the previous research had indicated that enzymic hydrolysis, either in combination with inorganic reagents or alone, was unsatisfactory.

It was known that difficulties would be encountered with humin formation. However, a preliminary treatment had already been devised which removed the carbohydrate in physical union with protein. This was further amplified as described under the procedure on "Preliminary Treatment". It was known that this would prove helpful in the case of many of the vegetable proteins. The object of the investigation was, therefore, to devise a method which would prvent humin formation through the combination of aldehydic constituents and tryptophane.

Choice of an Acid -

The acid selected for all subsequent work was hydrochloric acid. One reason for choosing it was that Jones and Kennard (40) had run a series of experiments using both hydrochloric and sulfuric acids. They found that on neutralization the precipitate of barium sulfate obtained with sulfuric acid could not be filtered out. Hydrochloric acid, on the other hand, can be removed by distillation under high

vacuum. This process is doubly advantageous in that no salts are introduced into the hydrolysate as a result of neutralization.

Jones and Kennard (40) also found that under the same set of conditions hydrochloric acid gave a greater degree of hydrolysis than sulfuric acid. This agrees with data furnished by Greenberg and Burk (28) whose curves show that there is a wide difference in the results obtained with the two acids under like conditions of concentration, temperature, and time. Vickery (86) and Osborne and Jones (57) also found that sulfuric acid is a less efficient hydrolyzing agent than hydrochloric acid.

The choice lay between these two acids, for not only are they the best known of the mineral acids but they are both strong acids. That is, they furnish a high concentration of H ions, which is important in hydrolysis as it is these ions which catalyze the reaction.

There was another factor which favored hydrochloric acid, and this was the most important of all. It was planned to run some hydrolyses in conjunction with reducing agents. A common and easily applicable reducing agent is stannous chloride. It is also a strong reducing agent. To use this material it was necessary to employ hydrochloric acid so that the anions would be the same, and this was the deciding factor in choosing hydrochloric acid as the hydrolyzing agent.

Causes of Humin Formation -

Gortner (18) and his collaborators demonstrated that carbohydrate was an important factor in humin formation. The only published work that has ever question Gortner's findings is a paper by McHargue (45). A study of this paper makes it plain to one who has had any experience with protein hydrolysis that the statements made are ill-advised.

Gortner's first work established the fact that carbohydrate contributed to humin formation, and up to certain limits the amount of humin bore a relation to the amount of carbohydrate present. This is not true, however, when the proportion of carbohydrate to protein becomes very great, as the proportionality is then lost although an increase in humin will be noted. It appeared that the carbohydrate effected humin formation in that aldehydic or ketonic fractions, derived from the carbohydrate under the conditions of hydrolysis, combined with tryptophane to produce black insoluble humin. The soluble humin, it is believed, arises through combination of these carbohydrate derivatives with other amino acids, probably cystine and tyrosine.

Gortner demonstrated that aldehydes and ketones entered into humin formation because these substances when boiled with tryptophane yielded a substance which was physically similar to the humin obtained on acid hydrolysis.

Furthermore, hydrolysis of a protein lacking in tryptophane decreased the formation of black insoluble humin to a marked degree.

Choice of a Reducing Agent - Stannous Chloride -

It occurred to the writer that if hydrolyses were run in conjunction with reducing agents, the formation of humin should be reduced if not eliminated. Mann (43) states on page 86 that "The formation of melanoidin depends on oxidation...." It was this phrase which furnished to the writer the clue that reducing agents might be helpful. A thorough survey of Chemical Abstracts from 1912 to 1927 did not indicate that any other worker had tried out the idea in recent years. However, Gortner and Blish (20) state that they have no way of explaining what happens when carbohydrate is absent and humin is formed unless tryptophane is oxidized to indol aldehyde. They add that traces of some other amino acid may be oxidized to the corresponding aldehyde. In a later paper, Gortner and Holm (25) reiterate the belief that humin formation may be due to oxidizing reactions.

Because of the emphasis laid on oxidation the writer ran two hydrolyses, Nos. 371 and 372, to one of which stannous chloride had been added and to the other potassium dichromate. The following conditions were observed in both cases: 20 grams egg flakes, 200 c.c. of 10% concentrated hydrochloric acid by volume, refluxed

with direct flame for $15\frac{1}{2}$ hours. To No. 371 was added 20 grams potassium dichromate, and to No. 372, 20 grams stannous chloride. The difference between the two hydrolyses was surprising. No. 371 began to char almost immediately, and this was accompanied by frothing. At the end of the hydrolysis there was not a large residue, it being very finely divided and almost impossible to filter, clogging even cloth. The color of the solid and of the liquid was black. No. 372, on the other hand, underwent no charring or frothing, and at the end was crystal clear and deep maroon color. While hot, there were some black oily drops on the surface of the liquid which tended to coalesce. On cooling, these presented a small, black, gummy mass characteristic of humin. The mass when dried to constant weight weighed 0.12 gram.

The Van Slyke determinations on these hydrolyses were most conclusive, the per cent conversions being as follows:

No. 371	No. 372
2.3%	38.2%

In view of the observations just noted, it was concluded that a reducing agent exerted a beneficial effect compared to that produced by an oxidizing agent. A second pair of hydrolyses were now run, Nos. 373 and 374. Conditions were the same as above as to time, acidity, total volume, and protein. To No. 373 nothing further was added

while to No. 374 the dosage of stannous chloride was again 20 grams. The per cent conversions obtained were as follows:

No. 373	No. 374
32.6%	38.9%

Hydrolysis No. 373 certainly contained more humin than No. 374. From this fact and from the per cent conversions, it was apparent that the stannous chloride exerted a beneficient effect. These experiments were run on May 14 and 15, 1926, and indicated to the writer that it would be well worth while to retain stannous chloride as a reducing agent during the subsequent work. It was, accordingly, used in all hydrolyses from this point on.

During the fall of 1927, it came to the writer's attention that Dr. M. X. Sullivan had presented a paper dealing with the hydrolysis of proteins in a reducing atmosphere. Correspondence with Dr. Sullivan (73) indicated that this worker had used both stannous chloride and titanous chloride, finding the latter more satisfactory because more easily removed from solution. At the time of writing, Dr. Sullivan stated that the effects of the reducing agent on the general amino acid content are not yet known.

In consequence, the writer does not know whether his original use of stannous chloride has priority or not. The matter is not of great importance, but it should be plainly understood that no claim to priority is made for the

use of stannous chloride in preventing humin formation.

Concentration of Hydrochloric Acid -

The first series of experiments were run to determine the concentration of hydrochloric acid which would give the best hydrolysis. Time, total volume, weight of protein and of stannous chloride were kept constant. The temperature varied somewhat with the barometer and increasing concentration of the acid. The fluctuations were over a range of from 102 to 114°C. It was always noted, when temperature readings were taken that there was a gradual increase as the hydrolysis was proceeding. The figures given in Table 13 will indicate the nature of this change.

TABLE 13

Increase in Temperature During Acid Hydrolysis

No. of Hours	No. 500	No. 501	No. 502	No. 503
1	102.0°C.	103.5°C.	106.0°C.	108.0°C.
2	102.5°C.	104.0°C.	107.0°C.	109.0°C.
3	102.5°C.	104.5°C.	107.5°C.	109.0°C.
4	102.8°C.	104.8°C.	107.5°C.	109.5°C.

The hydrolyses tabulated in Table 13 differed only in concentration which for Nos. 500, 501, 502, and 503 was 10%, 20%, 30%, and 40% of concentrated hydrochloric acid by

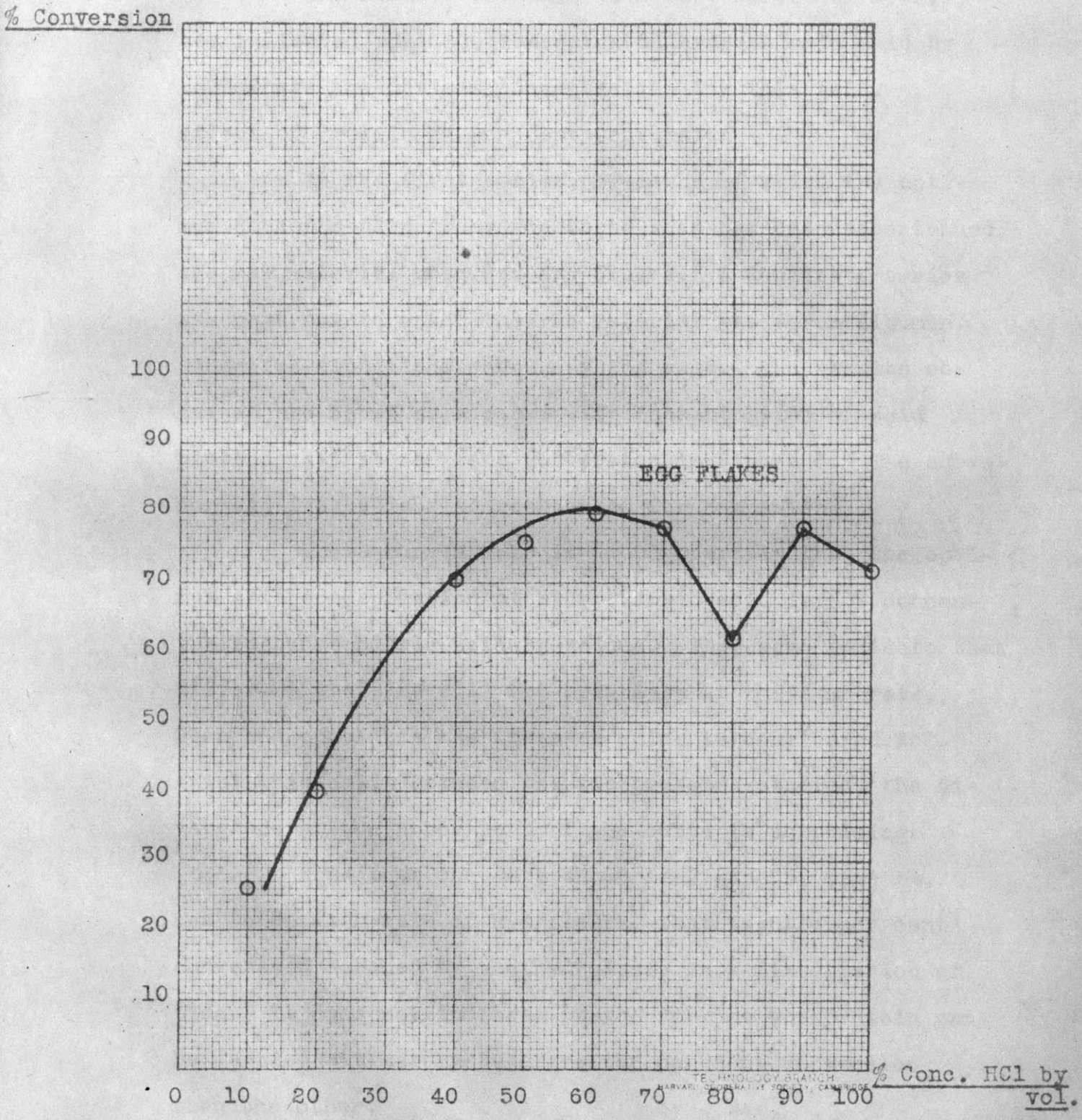
volume respectively.

The time selected was four hours, and hydrolyses were boiled under a reflux with a direct flame. At the end of the four hour period the hydrolyses were allowed to cool for five minutes and were then cooled quickly under the tap.

As a result of the first series of experiments a set of results was obtained which is shown as a curve with concentration of acid plotted against per cent conversion in Figure 4. It will be noted that maximum conversion is secured at the concentration of 60% of concentrated hydrochloric acid by volume. Beyond this point values fluctuate widely. A concentration of 60% of concentrated hydrochloric acid by volume corresponds very closely to 20.2% of hydrochloric acid gas which forms a constant boiling mixture. Below this concentration of gas water is distilled off faster than the gas. This, of course, is returned by the reflux. Above this concentration the gas is distilled off faster, but this is not returned by the reflux, passing through the condenser instead. It was noted that as the concentration was run up from 60% to 100% concentrated hydrochloric acid by volume, fuming became progressively worse. The fuming would account for the erratic results secured above 60% concentrated hydrochloric by volume because the concentration of acid would be varied over a wide range.

FIGURE 4

CONCENTRATION OF HCl PLOTTED AGAINST PER CENT CONVERSION



The results obtained with this series of hydrolyses indicated that 60% concentrated hydrochloric acid by volume was the optimum concentration for this acid.

Effect of Protein on Per Cent Conversion -

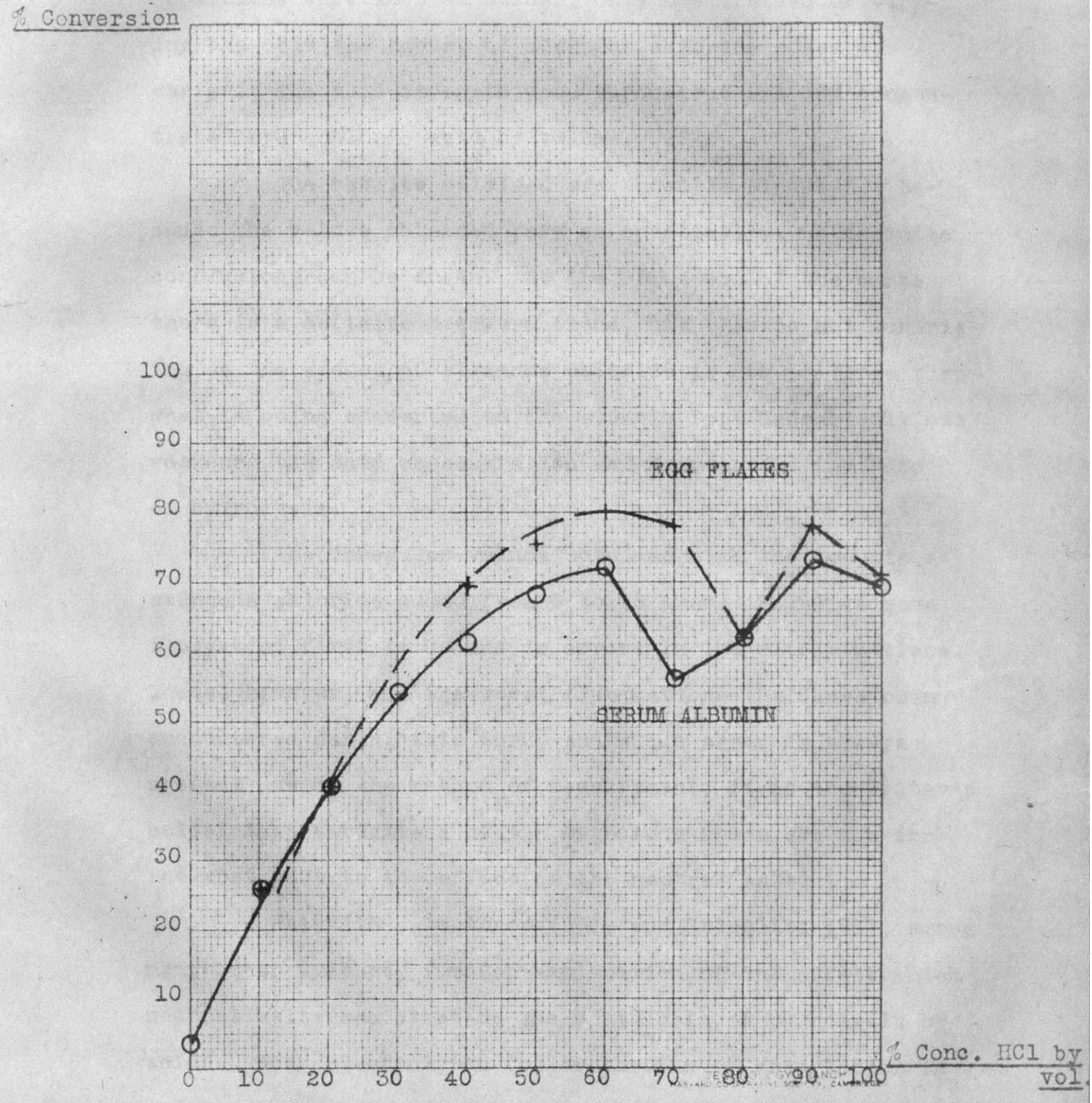
In the first series, by means of which the optimum concentration of hydrochloric acid had been ascertained, the raw material used was egg flakes. A duplicate series was next run in which the raw material was serum albumin. Otherwise conditions were kept the same. The results obtained are shown as a curve with concentration of acid plotted against per cent conversion in Figure 5. The curve for egg flakes is also reproduced for comparison.

These curves make it doubly certain that the optimum acid concentration of hydrochloric acid is 60% concentrated hydrochloric acid by volume. They also indicate that different proteins will not hydrolyze at the same rate. When one considers the likeness which is considered to exist physically between the two proteins studied, the divergence at the upper part of the curve is surprising.

Note that the data shown in Figure 5, page 98a, has to do with rate of hydrolysis, that is, the per cent conversion reached by the hydrolysis in a fixed period of time. It does not indicate whether or not one protein can be carried ultimately to a greater per cent conversion than the other.

FIGURE 5

CONCENTRATION OF HCl PLOTTED AGAINST PER CENT CONVERSION



Effect of Stannous Chloride on Per Cent Conversion -

A series of hydrolyses was next run in which all conditions were kept as before, only now instead of varying the acid the amount of stannous chloride added was varied. The acid strength used throughout was 60% concentrated hydrochloric acid by volume.

The results obtained are shown in Figure 6. Because the values obtained vary so erratically, no definite conclusions can be drawn. At the right end of the curve there is a definite downward trend, but this is not surprising as the amount of stannous chloride is now so large that what is being converted to the stannic form undoubtedly has weakened the acid concentration and so retarded the rate of hydrolysis.

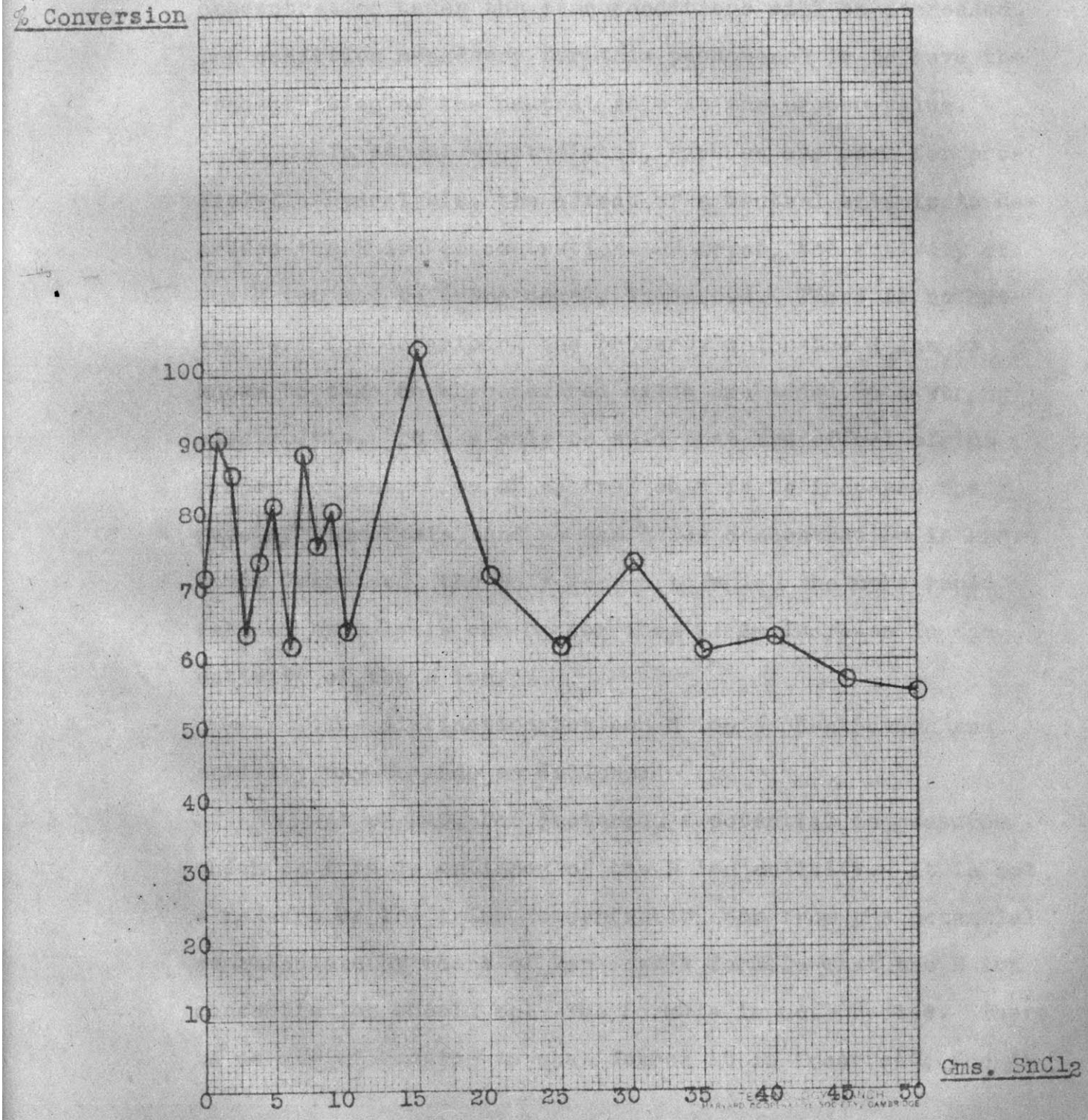
The irregular values obtained with the amounts of stannous chloride added from 0 to 20 grams inclusive were thought at first to be due to errors in the determinations. These, however, are the worst fluctuations that have been encountered during this work, and while error is always present due to the method of measurement, it is the writer's belief that variations of the magnitude shown are due for the most part to the effect of the neutral salt.

Falk (15), Northrop (55), and Stieglitz (72), among many other writers, furnish explanation of the effect which neutral salts can exert on the contalysis of hydrolysis by acids. Weak electrolytes follow the Law of Mass Action in

FIGURE 6

GMS. OF $SnCl_2$ PLOTTED AGAINST

PER CENT CONVERSION



that if a salt be added to an acid which has an anion common to that acid, the H ion concentration is decreased. This is also true of strong electrolytes where the H ion concentration under the same conditions will be decreased. The condition necessary for this phenomenon is to have the concentration of the neutral salt at the proper value.

In strong electrolytes, such as are used for proleolytic hydrolysis, the effect of a neutral salt is to decrease the H ion concentration. However, the activity of the H ion may be tremendously increased. There is no better term for describing the property which the H ion is known to take on when neutral salts are added to a strong electrolyte. It can only be said that the effect of the proper concentration of neutral salt is to increase the rate of hydrolysis, and as the H ion concentration is known to be decreased, the only reason to which the more rapid rate of hydrolysis can be ascribed is an increase in the activity of the H ion.

The distinction between H ion concentration and activity may be made as follows:

At pH 2.0, for instance, a potential is measured which in turn is an index of the H ion activity. It is not a measure of the H ion concentration for from the potential we calculate by means of Van Enst's formula what the H ion concentration should be. The formula is not precise. There is no way of knowing to what degree it is incorrect, for it

is impossible to compare what is obtained from the potential reading, which is something definitely known, to the concentration of H ion, which is something indefinitely known. In the case of dilute acids, the concentration and the activity of H ions probably are similar, but in strong acids there are no rules that can be applied. Hence, if a neutral salt is added, and the assumption is made that the H ion concentration is decreased, there is also a perfect right to assume that the H ion activity was increased.

The experimental data presented demonstrates beyond question that stannous chloride benefits hydrolyses conducted with hydrochloric acid. Not only is humin formation materially reduced but the rate of hydrolysis is increased. It appears reasonable, because of the stress that has been laid by Mann (43) and Gortner (20,25) on the part which oxidations play in humin formation, that the reduction in humin noted during the course of these experiments is due to the reducing property of stannous chloride. The increase in rate of hydrolysis can, however, be attributed only to the increased activation of the H ion, which is brought about through the addition of a neutral salt. It appears, therefore, that stannous chloride enjoys a dual role in that humin formation is reduced and the rate of hydrolysis increased.

DISCUSSION

It is evident from the data which has been presented that the results obtained during the course of this research were largely of a negative character. They are, however, none the less valuable because they are of assistance in showing any worker, who desires to continue in this field of research, the technique which is unsuccessful. It is to be regretted that more ground was not covered during this research. One must keep in mind, however, that the difficulty experienced with the method for determining the degree of protein hydrolysis made the work much slower than it otherwise would have been, for the necessity of repeated checks consumed a large amount of time. It must also be remembered that the cases which have been cited, where specific hydrolyses have been referred to by number, are but representative of a large number of determinations. All the data obtained in this work was secured as a result of no less than duplicate determinations, and there were many instances where determinations were made in triplicate, while the work on the Van Slyke method for amino nitrogen determination was run in series of ten so that the experimental error would be minimized in so far as was possible.

Of the negative results secured, it is evident that protein hydrolysis by means of enzymes cannot be made

successful with the means which at present are at hand. The difficulty presented by the nitrogen added with the enzyme preparations is one which can be overcome only by using an enzyme of extreme purity, and even under these conditions the error can never be wholly eliminated because proteolytic enzymes are nitrogenous in character. The contamination of enzyme hydrolyses by bacteria also constitutes so serious a drawback in this method of hydrolysis as to indicate that the method should be abandoned. The only means available for sterilizing enzyme preparations, either by treatment with glycerine or filtration through a porcelain filter, are bound to result in a lessening of enzyme activity. Above all this is to be avoided, because up to a definite ratio which can be experimentally established between the concentration of enzyme, menstruum and substrate, the amount of amino nitrogen produced is a direct function of the enzyme added. Therefore, it is not possible to use a concentration of enzyme below the optimum and secure an efficient conversion, but if the enzyme is weakened by any process of sterilization, the amount necessary to reach the optimum concentration of active material will be so great as to make it prohibitive because of the large amount of nitrogen added.

The final difficulty which stands in the way of successful enzymic conversion is adsorption of the active material on the hydrolytic products. Such adsorption is

known to exist, for in no other way can the reaching equilibrium at a lower per cent of conversion than that obtainable with the same protein when treated with mineral acids be explained. Here, then, is one positive fact which can be adduced from the experimental data presented, namely, that protein degeneration products inhibit the activity of proteolytic enzymes and that this phenomenon is due probably to adsorption.

Of the other positive data presented, one of the most important advances which has been made in this research is the successful preparation of a vegetable protein which was almost wholly free from fat and starch. The corn meal which was studied, after extraction with ether and trichloracetic acid, was found to have a protein content slightly in excess of 97 per cent, which meant, if the average value of two per cent for cellulose content be accepted, that the residual starch and fat could not amount to more than one per cent at the utmost. The ability to prepare vegetable proteins which will be free from the fat and carbohydrate which exists in physical union with them is important. The amount of carbohydrate present determines the degree of humin formation and until the technique with stannous chloride was essayed, the only possible means of averting humin formation was to reduce the amount of carbohydrate to a minimum.

The use of stannous chloride in acid hydrolysis

was another positive fact adduced by this research. There can be no question but that the use of this salt in combination with hydrochloric acid is decidedly beneficial. The first experiments run indicate that the favorable action is due to the reducing powers of this substance, as it will be recalled that similar hydrolyses run in which an oxidizing agent was used instead of stannous chloride, have a value of only 2.3 per cent for per cent conversion as opposed to 39 per cent obtained with the hydrolyses using stannous chloride, all other conditions of reaction being the same except for the matter of oxidant and reductant.

It is further evident that stannous chloride must produce some effect in acid hydrolysis other than that which can be accounted for by its reducing properties. The erratic variations extending over a wide range which are to be noted, when the amount of salt added is changed only one gram at a time, indicate that the salt is producing an effect in the hydrolysis which is explainable only on the basis of increased activity of the H ion. Stannous chloride has, therefore, the ability not only to reduce in the case of acid hydrolyses, but also to exert the effect of a neutral salt. From this it would appear that the concentration of stannous chloride is highly important in determining the rate and possibly the degree of per cent conversion. Experiments are planned which will demonstrate whether or not the variations obtained with small increases and

decreases in the amount of stannous chloride present are due to the introduction of a common anion.

This type of treatment with a reducing agent appears to be the only method known at the present time by which humin formation in hydrolyses with inorganic reagents can be minimized. It has long been an established fact that the customary boiling with acids under reflux resulted in a large proportion of the protein nitrogen being lost as humin nitrogen. This research further showed that the degree to which the protein had already been hydrolyzed did not affect the formation of humin to any marked degree, because the pressure treatments which were given to enzyme hydrolysates with inorganic reagents contained large amounts of humin. So far as the writer has been able to determine, this research furnishes the first case in which an agent of the nature of stannous chloride was employed to reduce humin formation.

The research as brought to its present stage, then, indicates that hydrolysis with enzymes or with enzymes plus inorganic reagents is unsuccessful. Considerable promise is shown, however, by treatment with mistures of hydrochloric acid and stannous chloride, for not only is humin formation reduced but the conversions obtained have proceeded at a higher rate. Therefore, it is recommended that further work which is undertaken on protein hydrolysis be conducted only along the lines of hydrolysis with mineral acids, and in conjunction with the proper amount of a reducing agent

of the nature of stannous chloride.

A survey of the literature on protein analysis made it appear that a better method of hydrolysis must be evolved. Experimental evidence supports this assumption. A study of the literature on the subject of protein hydrolysis alone indicates that while a large amount of work has been done which all bears more or less directly upon the kinetics of the reaction, practically no data is available on the all important point of securing high conversions of total nitrogen to amino nitrogen. Of the methods for amino nitrogen determination, it would appear that there is but little to choose between improved titration methods or the Van Slyke method for determining amino nitrogen. The Van Slyke method was taken as the means for determining the degree of hydrolysis in this research, and in accord with the data presented by other workers it was found that determinations which checked closely could not be obtained. This fact is believed due to some inherent deficiency in the reaction, since the check determinations run on acid hydrolysates varied over a wider range than those performed on peptone solutions, and this obviated the possibility of error on the part of the apparatus or operator. Neither does it appear that any of the unknown groupings in the protein molecule would effect the reaction seriously. Of the four linkages recognized, other than the peptid linkage, these are present but to a very small extent and they

are only slightly or not at all reactive with nitrous acid.

While hydrolysis with enzymes is entirely free from humin formation, the difficulties inherent to that method make it unsuitable. The effects due to added nitrogen and bacterial action, and the additional fact that enzyme conversions cannot in the case of the same protein be carried to as high a degree as that which can be reached by treatment with mineral acids, make it apparent that hydrolysis with enzymes is unsatisfactory. Because of the nature of the difficulties presented, there does not appear to be any means of circumventing them, and as the conditions for enzymic activity have been carefully established and maintained at the optimum during this research, the degree of conversion which has been secured with the combination of pepsin and trypsin probably can never be pushed above the maximum value of 45 per cent, unless some means is devised for removing the hydrolytic products as they are formed.

Dialysis was tried out in an attempt to remove the end products. The type of dialysis used, however, was dependent upon molecular size to effect a separation of substances. Consequently it was unsatisfactory, for in so far as spacial dimensions are concerned, the simple dipeptid is likely to be smaller than the complex amino acid.

Chloride ions were demonstrated as toxic to pepsin, sodium and phosphate ions as untoxic to trypsin, as the

buffers which were made up containing one of the two combinations mentioned either completely inhibited or did not affect enzyme activity. Buffer solutions were found unsatisfactory, being either toxic or else subject to change in reaction due to the buffer effect of the hydrolytic products.

In accord with the data presented by Northrop (55), it was determined that protein hydrolysis is dependent upon the enzyme concentration, provided the amount of inert material added with the active substance is not so great as to act as an additional adsorbent to inhibit enzyme activity. The experimental data also tends to show that the syntheses of complex products from simpler protein derivatives by pepsin or trypsin, as claimed by previous workers, is very unlikely. In view of the lack of precautions taken on their part to insure sterility of the reaction mixture, it is probable that the decreases in amino nitrogen and the other effects observed by them were due to nothing more than bacterial action. Preservatives were found of no use in protecting enzyme hydrolyses against bacterial decomposition. The effects of bacterial action were found to be a decrease in the amount of total amino nitrogen and an increase in acidity.

The treatment of previously digested proteins by inorganic reagents was shown to be valueless, as in the case of alkalies, deaminization appeared to as great an extent as if there had been no previous hydrolysis, while

in the case of acids, the gain in per cent conversion was negligible.

Stannous chloride was found to benefit hydrolyses run with hydrochloric acid. The optimum concentration of hydrochloric acid, under the conditions of a total volume of 200 c.c., 20 gms. of stannous chloride and 20 gms. of egg flakes or serum albumin, were found to be 60 per cent concentrated hydrochloric acid by volume. Two physically similar proteins, egg flakes and serum albumin, were found to have under similar conditions of hydrolysis rates of decomposition that were not the same, but differed by as much as 15 per cent at the end of four hours.

Experiments run with varying concentrations of stannous chloride indicated that the concentration of the salt increased the activity of the H ion. Other work indicated that humin formation was kept down because of the reducing properties of this salt. Therefore, it would seem that stannous chloride plays a dual role in protein hydrolysis, and it is hoped that in the near future data can be obtained which will prove whether or not the salt possesses both properties, or acts merely as a reducing agent alone.

CONCLUSIONS

1. Hydrolysis with proteolytic enzymes can, ununder the proper conditions, be run through in a fairly short time - a matter of hours rather than weeks and months.

2. Even though the conditions be optimum, the conversions secured with enzymes are not as great as those that can be obtained with mineral acids. Data tends to show that this is due to adsorption of the enzyme on the hydrolytic products.

3. Because of the low conversions, coupled with the problems presented by introduction of nitrogen and bacteria with the enzyme preparations, enzyme hydrolyses are unsatisfactory compared to hydrolyses with mineral acids.

4. Treatment of enzyme hydrolysates with alkalies under pressure results in deaminization with evolution of ammonia. With acids, no gain is noted over the original per cent conversion attained with enzyme. Moreover, humin formation appears not to be minimized.

5. The use of stannous chloride in acid hydrolyses was established as beneficial, for it both increased the rate of per cent conversion and decreased humin formation.

6. The optimum amount of stannous chloride under

hydrolysis with 60% concentrated hydrochloric acid by volume was 20 gms. stannous chloride to 20 gms. egg flakes or serum albumin in a total volume of 200 c.c.

BIBLIOGRAPHY

The bibliography is arranged alphabetically according to the names of authors. In cases where two or more writers have collaborated on one paper, reference is made but once. The policy has been to set down the name of the author first which was published first over the paper in question. The only exception to this rule is when a large number of papers have been presented under the direction of one man and the name of one of the collaborators has been published first. Under these circumstances the paper is listed under the name of the man who directed the work.

Each reference is given an arbitrary number and it is by this number that the reader must identify the reference in the text.

The abbreviations employed for the journals from which the references were taken are those accepted by the American Chemical Society in the "List of Periodicals Abstracted by Chemical Abstracts," 1926.

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