STUDIES ON ACID HYDROLYSIS OF PROTEINS

A Thesis

by

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Submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

McGill University

May 1948

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation of the assistance received from the following:

1. Dr. W. D. McFarlane for helpful criticism and encouragement during the course of this study.
2. Dr. R. H. Common for interest shown and for criticism during the latter part of this work.
3. Dr. D. W. Woolley, Rockefeller Institute for Medical Research, New York, for microbiological assays of amino acids.
4. Dr. H. B. Woodruff, Merck and Company Inc., Rahway, N. J., for microbiological assays of amino acids.
5. Dr. J. H. Quastel for his assistance in supplying a polarimeter.
6. Dr. A. E. Ledingham, Dominion Rubber Company, Guelph, Ontario, for proximate analyses.

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GENERAL INTRODUCTION

At the present time all known methods for the hydrolysis of proteins are subject to serious limitations. Enzymatic hydrolysis is slow and incomplete, alkalis racemize the optically active amino acids and destroy cystine and arginine, and acid hydrolysis is accompanied by humin formation and tryptophane destruction.

The destruction of tryptophane during acid hydrolysis is not, in all cases, due to the direct action of the acid since pure tryptophane is relatively stable in hot dilute sulphuric and hydrochloric acids. The destruction must, therefore, be due to some other constituent or constituents of the protein. Consequently, there is some reasonable prospect that tryptophane destruction can be avoided if the harmful effects of these other agents can be eliminated.

The formation of humin, and the errors and difficulties resulting therefrom, has been a problem of fundamental importance since the earliest attempts to study the constitution of proteins. The significance of tryptophane destruction, however, has been emphasized in recent years through the increasing demand for pure amino acids in nutritional and bacteriological investigations. Despite continued discovery of, and improvements in, synthetic processes, commercial proteins are still an important source of pure amino acids for these purposes. In addition, complete protein hydrolysates for both

oral and parenteral administration, are receiving widespread clinical use. These should be high in amino nitrogen and contain all of the essential amino acids in sufficient quantity. Improved methods of protein hydrolysis are, therefore, desirable from both a fundamental and practical viewpoint.

Some reagents have been used to prevent humin formation, particularly reducing agents. Unfortunately, their presence in the final hydrolysate is as troublesome, for most purposes, as the humin which would ordinarily be present, and no degree of tryptophane preservation has ever been reported following their use.

The principal objective in this study has been to preserve tryptophane during the acid hydrolysis of proteins. The problems of tryptophane destruction and humin formation are so intimately linked that their solution must be sought simultaneously. Since reducing agents have been used to prevent humin formation, this seemed to be the best initial approach, but it was considered highly desirable that some new reducing agent be employed, which would not be objectionable from the standpoint of its presence in the final hydrolysate. Preliminary experiments indicated that hydrogen, if it could be applied in the nascent form, offered some promise of success. The problem then evolved into a search for a method whereby this reagent could be applied efficiently during the acid hydrolysis of proteins, and a study of the degree of tryptophane retention and prevention of humin formation which could be attained by such a procedure.

HISTORICAL INTRODUCTION

The use of nascent hydrogen to preserve tryptophane and prevent humin formation during the hydrolysis of proteins with acid has not previously been reported. In consequence, very few previous works have any direct bearing on the subject of this study in the narrowest sense. The research on acid hydrolysis in general, however, is a series of attempts to employ this type of catalyst to achieve the maximum degree of hydrolysis with a minimum of inconvenience and undesirable features in the final hydrolysate.

Since nearly all studies on the chemical constitution of the proteins involve hydrolysis, the literature on this general subject is extremely extensive. In this review an attempt has been made to cover only the major contributions on the general features of the acid-hydrolysis reaction, such as kinds of acid employed, rates of hydrolysis and hydrolyzable linkages, and to emphasize more particularily the work on the losses of amino acids during hydrolysis and the phenomenon of humin formation and tryptophane destruction.

1. Acids Used

One of the first reports on the use of acid for the hydrolysis of protein was made by Braconnot (18) in 1820. He boiled glue with dilute sulphuric acid and obtained a substance which he called glycocoll, now commonly called glycine. The first use of hydrochloric acid was reported by Bopp (17)

in 1849. Hlasiwetz and Habermann (69) in 1871 carried out hydrolysis with hydrochloric acid in the presence of stannous chloride to prevent humin formation.

Fischer et al (42) in 1901 and ensuing years, used both sulphuric and hydrochloric acid in the development of the peptide linkage theory, but considered the use of stannous chloride with hydrochloric acid unnecessary.

Several attempts have been made to employ acids, other than hydrochloric or sulphuric, generally with unsatisfactory results. Hugounenq and Morel (78) used an 8 per cent solution of hydrofluoric acid. Biuret tests were negative after heating the mixture in a lead vessel on a steam bath from forty-eight to one-hundred hours. Less humin substances were formed and the acid could be readily removed by calcium carbonate. Later (79) they found that complete hydrolysis had not been attained and recommended the use of 20 per cent hydrofluoric acid, but even this concentration did not produce complete hydrolysis. Finally (80), after using 15, 20, 30 and 35 per cent acid, they concluded that an inverse relationship existed between the degree of hydrolysis attainable and the concentration of hydrofluoric acid, and that a very long period of heating in 15 per cent acid was necessary for a high degree of hydrolysis.

Kossel and Kutscher (85) used hydriodic acid for hydrolysis. This demethylates methionine, as was shown by Barger and Coyne (7), and this principle is used in the

hydrolysis of proteins by Baernstein (6) in the determination of methionine. Hess and Sullivan (68) report that no humin is formed when hydriodic acid is used as the hydrolytic agent for a number of proteins.

Pfannl (113) and Weizmann (147) used alcohol saturated with gaseous hydrogen chloride but only slight hydrolysis was obtained.

Zelinsky and Sadikov (151) found that formic acid of various strengths was unsuitable for the hydrolysis of proteins even at 180°C. in an autoclave. The action of 10 per cent formic acid on gelatin yielded some glycine and a variety of other complex products. Further investigations (152), using 50 per cent formic acid, showed the hydrolysate to contain a mixture of complex polypeptides and amino acid anhydrides. Acetic acid gave similar results. Some condensation occurred when glycine was heated with formic acid. Much less humin was formed with these acids than with stronger acids. Sadikov (119) attempted the hydrolysis of protein by autoclaving with carbonic acid and with oxalic acid. An abiuret (sic) product resulted containing no free amino acids.

Gilson et al (51) prepared hydrolysates of casein suitable for bacteriological media by heating 100 g. of casein with 150 g. of oxalic acid and 300 cc. of water in the autoclave at fifteen pounds pressure for forty hours.

2. Studies on the Rate of Hydrolysis

The early measurements of the extent of hydrolysis were

based on the biuret reaction, the precipitate with phosphotungstic acid, or the isolation of amino acids (78, 85, 152). The discovery of the formol titration principle by Sørensen (130), and its application to the study of acid hydrolysis by Henriques and Gjaldbaek (66), provided a more accurate method. The latter authors found that, when proteins are hydrolyzed with acids, the amino nitrogen and the ammonia increase to a certain point where amino nitrogen attains its maximum. Beyond this point some of the amino nitrogen is transformed into ammonia. They concluded that the most certain way to attain the end point was to heat the protein with 3 N hydrochloric acid in the autoclave at 150°C. for one and one-half hours.

Van Slyke (141), after developing his rapid nitrous acid method for the determination of amino nitrogen (139), found that the same maximum amino nitrogen was obtained by heating at 100°C. for forty-eight hours with 6 N hydrochloric or by autoclaving at 150°C. with 3 N hydrochloric for one and one-half hours.

Vickery (146) followed the liberation of amino nitrogen during hydrolysis of wheat gliadin with sulphuric and hydrochloric acids and was the first to point out that acid hydrolysis is a continuous process, proceeding from first to last without marked interruption due to the existence of stable complexes and is, therefore, distinguishable from enzymatic hydrolysis. He also found that hydrochloric acid gave a slightly higher

rate of hydrolysis than sulphuric.

Greenberg and Burk (59) studied the hydrolysis of gelatin and silk fibroin by hydrochloric and sulphuric acids and confirmed that hydrochloric acid is a slightly more rapid catalyst. From data on the rates with various acid concentrations, and over a temperature range of 50°C., they deduced that the catalytic effect of acids on protein hydrolysis is proportional to the thermodynamic activity of the hydrogen ion. The hydrolysis of these proteins, as measured by the increase in amino nitrogen, was found to conform to the equation for a second-order reaction. A mathematical relationship was found to exist between the change in reaction velocity with change of temperature.

Dunn (36) found the hydrolysis of casein fitted a first-order reaction equation best, but in a more extensive study Nasset and Greenberg (103) found that the hydrolysis of this protein conformed to the equation for a second-order reaction. They established a mathematical relationship for the change of velocity constants with temperature which permitted the prediction of the course of casein hydrolysis at a given temperature and with acid of known activity. This relationship is:

$$\log Ka = 0.0307T - 5.61$$

where Ka is the velocity constant at unit hydrogen ion activity and T is the temperature in degrees centigrade.

Recently, Bird and Minor (10) have found that a higher amino nitrogen content can be obtained after hydrolysis of

crude proteins with hydrochloric acid at 75 or 80°C. for one hundred and forty-eight hours than at 105°C. for twenty-four hours.

Royal (117) has found that the hydrolysis of corn protein proceeds at room temperature in 35 per cent hydrochloric acid if sufficient time is allowed. After one year amino nitrogen represented 7.45 per cent of the weight of protein, and after three years 8.20 per cent.

Steinhardt and Fugitt (132) found that certain organic acids of high molecular weight such as cetylsulfonic, dodecylsulfonic and p-diphenylbenzene-sulfonic, are capable of breaking amide and peptide bonds of wool and egg albumin at a rate one humdred times faster than hydrochloric acid of the same concentration. The differences in hydrolytic effectiveness among these acids parallel differences in the affinities of their anions for protein. A maximum rate of amide hydrolysis was attained at a low concentration of the acid, where the anion concentration was stoichiometrically equivalent to the sum of the amino plus the amide groups. They, therefore, attributed the catalytic effect to the anion. The sodium salts of these acids greatly increased the rate of hydrolysis of amide and peptide bonds by hydrochloric acid when the concentration of hydrochloric was 0.05 N. The catalytic effect of the organic anions in breaking peptide bonds did not reach a maxium as concentration was increased, due to the large number of peptide bonds present. The influence of the catalytic-

ally-active anions, on both amide and peptide bonds, is attributed to their ability to promote the combination of hydrogen ions with these weakly basic groups in dilute acid solution.

The effect of these anions was noticeable only at very low hydrogen ion concentrations. When the hydrogen ion concentration was high, the catalytic effect of the anion was not apparent due to the decreased dissociation of the organic acid and to the masking effect of the high rate of hydrolysis produced by the hydrogen ions alone.

The application of the ninhydrin reaction to the measurement of the carboxyl groups of free amino acids by Van Slyke et al (143), has made it possible to follow the course of hydrolysis by the liberation of free amino acids, since the carboxyl groups of peptides will not react. Frost and Heinsen (50) used this method, in conjunction with the nitrous acid method for amino groups, to study the hydrolysis of casein and blood fibrin by 2.8 N sulphuric acid. From the total amino nitrogen by the nitrous acid method, and the free amino acid nitrogen (obtained from the ninhydrin carboxyl values), it is possible to calculate the average length of the peptides present at any stage of hydrolysis. From their data it is apparent that at 60 per cent hydrolysis of casein, as measured by amino nitrogen, only 28 per cent of the amino acids are free, and the average peptide size of the remainder is that of a tripeptide. At 83 per cent hydrolysis, 57 per

cent of the amino acids are free, and the average size of the remaining particles is slightly greater than a dipeptide.

These authors noticed a decrease in the rate of hydrolysis in the final stages which was greater than could be expected on the basis of peptide bond concentration effect. They attributed this to the lowering of the effective acid concentration by the binding of a large fraction of the acid anion and cation by the increased number of amino groups. This was noticeable only with low acid concentration. White and Sayers (148) similarily found that a maximum of 30 per cent hydrolysis of casein was obtained with 0.25 N hydrochloric acid in seventeen hours. Continuing the hydrolysis for twenty-four or forty-eight hours actually resulted in a decrease in amino nitrogen.

Sillivan and Hess (135) found that the rate of liberation of cystine from proteins during hydrolysis with hydrochloric acid is greatly accelerated by the addition of titanous chloride.

3. Acid-Hydrolyzable Linkages in Simple Proteins

Liebig (86), after his discovery of tyrosine in 1846, was probably the first to consider proteins as complex substances built up through the linkage of amino acids, but the actual form of the linkage was not fully confirmed until the synthesis of peptides by Fischer (42, 43), who termed it the peptide bond.

Nasse (101) pointed out that the nitrogen which gives

rise to ammonia on hydrolysis of proteins must be bound differently than the nitrogen found in the amino groups of amino acids after hydrolysis. Osborne et al (111) showed the correlation between the ammonia yield on hydrolysis and one of the carboxyl groups of the total glutamic and aspartic acid content. Osborne and Nolan (112) later found that, when protein was hydrolyzed with dilute acid, a further acidity appeared in the solution which was closely equivalent to the ammonia liberated, and which was best explained by the hypothesis that ammonia was derived from amides of the dicarboxylic acids.

The discovery of proline by Fischer (41), and its synthesis into peptides by Fischer and Abderhalden (44), showed that linkage could occur through its imino group and that such peptides are hydrolyzed by acids.

These are the only confirmed acid-hydrolyzable carbon-nitrogen bonds in simple proteins.

4. Losses of Amino Acids Other Than Tryptophane

Roxas (118) found that tyrosine, cystine, arginine, lysine, and histidine entered into the formation of humin when boiled with 20 per cent hydrochloric acid in the presence of carbohydrate. Tyrosine and cystine did not lose the reactivity of their amino nitrogen toward nitrous acid. Dowell and Menaul (35) found similar results when furfural was added to pure amino acids in hydrochloric acid. In these studies the

amount of carbohydrate added in proportion to the amino acid was much greater than would be found even in many crude protein preparations and thus the results may have over-emphasized the losses which can occur.

Lugg (89) showed that tyrosine was completely stable when heated alone in 7 N sulphuric acid, but losses up to 54 per cent could occur in the presence of carbohydrate with the formation of humin.

In the investigation of the origin of acid-soluble humin, Gortner (54) and Gortner and Holm (56, 57) found the amount of soluble humin to be dependent on the amounts of tyrosine and aldehyde present during the hydrolysis. Their conclusions are responsible for the general belief that the darkly-colored soluble substances formed during acid hydrolysis are largely due to tyrosine destruction.

Very little precise information is available on the losses of tyrosine encountered during acid hydrolysis since many of the commonly-used analytical methods are designed for the determination of tyrosine and tryptophane in the same hydrolysate and, consequently, employ alkali as the hydrolytic agent (14).

Block et al (15) found that several proteins gave more phenylalanine after hydrolysis with 5 N sodium hydroxide than with 8 N sulphuric acid, 20 per cent hydrochloric acid, or 57 per cent hydriodic acid. The higher values obtained after alkaline hydrolysis were also confirmed by Knight and Stanley (84) and by Fontaine, Olcott and Lowy (48), who

found up to 10 per cent more after alkali hydrolysis. Knight and Stanley (84) observed that the presence of large amounts of tryptophane in the protein results in high phenylalanine values after acid hydrolysis and a correction must, therefore, be applied. Bolling and Block (16), in a further investigation, added phenylalanine to lactoglobulin before hydrolysis and found that only 87 to 95 per cent of the added phenylalanine could be recovered after hydrolysis. No mention is made of the cause of such losses.

Losses of the basic amino acids have also been attributed to the action of acids during hydrolysis, but to a lesser degree. Hunter and Dauphinee (81), in the development of the arginase method for arginine determination, found five hours hydrolysis with hydrochloric acid to be optimum. Longer periods gave lower arginine values but the effect was attributed to racemization, not destruction.

Schein and Berg (124) found that autoclaving casein with 8 N sulphuric acid resulted in considerable racemization of lysine and destruction of arginine, but not until the minimum time for complete hydrolysis had been greatly exceeded.

While Roxas (118) showed that the free basic amino acids were capable of entering into humin formation when heated alone with carbohydrate and acid, the conditions he employed were highly artificial, the amount of carbohydrate used was five times the weight of amino acid in some cases.

Tristram (138), working with purified leaf-proteins

hydrolyzed by sulphuric acid, found losses in arginine and histidine which he attributed to humin formation. Gortner (55), in contrast, stated that adding histidine to zein did not increase the humin nitrogen, but as Osborne and Jones (110) pointed out, zein forms little humin on hydrolysis.

Knight (83) reported that, when small amounts of histidine were added to tobacco mosaic virus before acid hydrolysis, only 34 per cent to 64 per cent of the added histidine could be recovered after hydrolysis.

The losses of the sulphur-containing amino acids which can occur during acid hydrolysis have received considerable study. Bailey (5) found that cystine added to edestin could be almost completely recovered, but the absolute values for cystine varied among samples, from which he concluded that cystine in peptide linkages may be more readily destroyed than free cystine. He reports that 30 per cent of the cystine and 20 per cent of the methionine could be lost on hydrolysis with 5 N hydrochloric acid in fifteen hours. Some sulphur was found in the insoluble humin.

Bolling and Block (16), hydrolyzing β-lactoglobulin with a mixture of hydrochloric and formic acids, found no loss of cystine, but add that this protein is devoid of carbohydrate.

Lugg (88) concluded, from a detailed investigation, that some cystine can be lost by adsorption on insoluble humin, and that the loss of cysteine, by condensation with impurities in the presence of acids, can be quite large. In the presence of

large amounts of carbohydrate, a considerable amount of cystine is lost and the destruction of cysteine is virtually complete. He discourages the use of stannous chloride in the presence of carbohydrate and hydrochloric acid, as it not onl fails to prevent the loss of cysteine, but it also converts the cystine to cysteine, a form in which it is more easily destroyed. In further investigations, using leaf-proteins, Lug (90) found hydrolytic losses of cysteine to be minimized by hydrolysis with 57 per cent hydriodic acid from six to eight hours. Hess, Sullivan and Palmer (67) further confirmed the value of using hydriodic acid, and also found 20 per cent hydrochloric acid plus one per cent titanous chloride, or sulphuric acid under nitrogen, to be beneficial.

McFarlane, Fulmer and Jukes (94) found a higher cystine content in caseinogen and egg proteins following tryptic digestion than after sulphuric acid hydrolysis.

Ratner and Clarke (115) offer an explanation of the larg losses of cysteine in the presence of carbohydrate. They sho that formaldehyde reacts with cystine to yield thiazolidine-4-carboxylic acid and conclude that aldehydic substances, produced from carbohydrate by the action of acids, could act similarily during acid hydrolysis.

Halwer and Nutting (65), in an investigation of the cystine content of some crystalline proteins and keratins, employ two modified procedures for hydrolysis with hydrochloric acid One consisted of adding cysteine to the protein and acid and

hydrolyzing in a sealed tube, after evacuating and filling with carbon dioxide. The second method was essentially that of Brand and Kassel (19), who added urea in the proportion of 2 g. to 0.1 g. of protein, and hydrolyzed by refluxing with hydrochloric acid while passing a stream of carbon dioxide through the flask. Cysteine added to proteins could not be completely recovered in either case, but the procedures did give partial improvement over ordinary methods. When cysteine was added to β-lactoglobulin, a crystalline protein containing no carbohydrate, all of the cysteine could not be recovered after hydrolysis, but a mixture of pure amino acids simulating the composition of lactoglobulin could be subjected to the hydrolytic procedure without loss of cysteine. This, and other experiments, indicated the presence of a destructive agent which was not a carbohydrate. Urea prevented the formation of insoluble humin, and most of the soluble humin except in the case of the keratins.

Hydrolytic losses of the dicarboxylic acids, imino acids, hydroxy amino acids, and several aliphatic mono-amino monocarboxylic amino acids, have not been studied extensively, due largely to the inaccuracy of analytical methods. The recent advent of microbiological methods is furnishing more information with respect to some of these amino acids, although the specificity of most organisms for the natural isomers of the amino acids creates a difficulty in distinguishing between racemization and complete destruction. There is also th

possibility that the organism can utilize partial decomposition products. The foregoing studies have all employed chemical measurements, but those now to be discussed are based on microbiological assays.

Hac, Snell and Williams (63) autoclaved several pure proteins with 10 per cent hydrochloric acid for periods up to thirty-six hours and found no great changes in glutamic acid assay values after autoclaving for ten hours. Casein gave a value of 21.8 per cent at four hours and 21.7 per cent at thirty-six hours.

Horn, Jones and Blum (77) obtained the same value for the methionine content of several foods and proteins after hydrolysis with 20 per cent hydrochloric acid for eighteen hours as after hydrolysis with papain, indicating that methionine is not readily destroyed by acid hydrolysis. The colorimetric method of McCarthy and Sullivan (93) was used and this method does not require complete hydrolysis of the protein.

Stokes et al (133) determined the amounts of threonine, histidine, methionine and phenylalanine in some relatively crude protein materials after hydrolysis with 10 per cent hydrochloric acid in an autoclave at 15 lbs. pressure. Maximum amino acid values were obtained after hydrolyzing for a period of five hours. Extending the hydrolysis time to thirty hours caused only minor losses, seldom exceeding 1 per cent, due to destruction or racemization. They concluded that these amino

acids are quite stable during acid hydrolysis. Similar conclusions with respect to the stability of valine and arginine in casein were made by McMahon and Snell (95).

Gunness, Dwyer and Stokes (62) conducted microbiological assays for tyrosine on several crude proteins with a high carbohydrate content. The apparent tyrosine content was much higher following alkaline hydrolysis than after acid hydrolysis but, with relatively pure protein, the values on the acid hydrolysates were higher in four samples out of six.

5. Humin Formation and Tryptophane Destruction

While the presence of an acid-unstable indole compound in protein was suspected for many years, its identity was not established until the isolation of tryptophane from a tryptic digest by Hopkins and Cole (76) and the elucidation of its structure by Ellinger and Flamand (40) in 1907.

The name "humin" was first applied to the darkly-colored substances formed during acid hydrolysis by Osborne and Jones (110). They suspected that these substances were a mixture of secondary decomposition products of different constituents of the protein from the observation that tryptophane and histidine gave darkly-colored products with carbohydrate in acids, whereas zein, a protein which contained no tryptophane and little histidine, produced very little humin. Prior to this time (123, 127) these dark insoluble substances were called "melanins" or "melanoidins". To avoid confusion, Gortner (53)

endorsed the name "humin" to distinguish the substances in question from "the dark pigments which occur normally or pathologically in the animal body, skin, hair or feathers".

Van Slyke (140) boiled a solution of tryptophane in 6 N hydrochloric acid for twelve hours and obtained only a slightly colored solution and concluded that tryptophane was not responsible for humin formation. However, Gortner and Blish (55) showed that zein, when hydrolyzed with acid, produced negligible amounts of humin, but when tryptophane was added large amounts of humin formed. When tryptophane and carbohydrate were heated in hydrochloric acid, 86 per cent of the tryptophane-nitrogen was lost as humin nitrogen, but when histidine was substituted for tryptophane only 0.5 per cent of its nitrogen was lost in the humin. They postulated that the reaction was probably the condensation of an aldehyde with the imino group of the indole nucleus.

Gortner (54, 55) found that the nitrogen content of the insoluble humin, formed during the hydrolysis of relatively pure proteins, corresponded to the tryptophane nitrogen, if the optimum amount of aldehydic substances were provided. He proposed this principle as the basis for a quantitative determination of tryptophane, but further work (56, 70) indicated that the results were influenced by the varying carbohydrate content of protein samples. Although tryptophane was found to be the only amino acid chemically combined in the formation of insoluble humin, other amino acids were occluded

or adsorbed to varying degrees, particularly in the presence of carbohydrate. Holm and Greenbank (72) succeeded in applying this principle in the quantitative determination of tryptophane. When p-dimethylamino benzaldehyde was added to the protein in the correct amount, the nitrogen content of the insoluble humin corresponded to the tryptophane nitrogen of the protein. This method has not met with general approval.

Gortner (54) distinguished between acid-insoluble and acid-soluble humin nitrogen. The nitrogen content of the filtered and washed insoluble material was termed acid-insoluble humin nitrogen. After removal of the hydrochloric acid from the filtered hydrolysate, the ammonia was removed by making alkaline with excess calcium oxide and aerating. The colored material in the hydrolysate was found to be strongly adsorbed on the excess calcium oxide. The nitrogen adsorbed on the calcium oxide was determined and expressed as acid-soluble humin nitrogen. Using pure amino acids, Gortner established that tryptophane reacts with several aldehydes to form insoluble humin while tyrosine formed insoluble humin with benzaldehyde but only soluble humin with formaldehyde and trioxymethylene. Gortner and Holm (56, 70), using fibrin and gelatin with or without added pure amino acids, obtained evidence that a similar phenomenon occurred during the hydrolysis of proteins, tyrosine increasing the soluble humin and tryptophane increasing the insoluble humin. They also established that the amino group of tryptophane is not involved in the humin-forming

reaction. Humin formed from pure tryptophane and trioxymethylene in hydrochloric acid was dried, pulverized and suspended in water. Amino nitrogen determinations on this suspension accounted for almost 48 per cent of the total nitrogen, indicating only 4 per cent loss of amino nitrogen.

In a further study, Holm and Gortner (71), using the colorimetric method of Folin and Denis (46), followed the destruction of pure tryptophane during boiling with 6 N hydrochloric acid. They found over 80 per cent of the tryptophane remained after twenty-four hours and approximately 50 per cent after six days. Ammonia was also determined and it was apparent that, under these conditions, deamination of tryptophane occurs to the extent of 15 per cent in twenty-four hours and approximately 27 per cent in six days.

Gortner and Norris (58) studied the hydrolysis of proteins in the presence of the ketones, acetone and acetophenone. Neither produced any appreciable increase in soluble or insoluble humin and hence they concluded that the unknown agent reacting with tryptophane to form humin is not a ketone.

After the preparation of over forty samples of humin by condensation of indole derivatives with aldehydes, and also by acid hydrolysis of proteins, Burr and Gortner (21) were unable to reach a definite conclusion regarding the mechanism of the indole reaction with aldehydes to form humin. Humins formed during acid hydrolysis of proteins had no fixed composition but their molecular weights were in the range of 500 to 1000,

resembling artificial humins in this respect. The order of reactivity of the hydrogens in the indole nucleus was found to be β, α, imino, hence with tryptophane where the alanine side chain occupies the β position, the α hydrogen of tryptophane is likely to be involved. The analysis of humin formed from benzaldehyde and tryptophane indicated an approximate ratio of one benzaldehyde to two tryptophane molecules without the loss of water. When trioxymethylene was used, the ratio was three molecules of aldehyde to one of tryptophane.

Homer (74) investigated the reaction between various aldehydes and tryptophane and concluded that two types of reaction were possible:

(i) Formaldehyde or glyoxylic acid reacts directly with the amino group of tryptophane in aqueous solution to give crystalline derivatives.

(ii) Tryptophane, due to its imino group, will react with formaldehyde and trioxymethylene in the presence of a condensing agent to form colored substances which are insoluble in most solvents.

In further work (75) she found that while pure tryptophane was relatively stable in hot sulphuric acid, the presence of ferric or cupric sulphate caused rapid destruction of the tryptophane.

Zeleny and Gortner (150) found that the hydrolysis of casein with acids in the presence of formaldehyde resulted

in an abnormally high ammonia content due to the deamination of amino acids. Experiments with pure tyrosine, glutamic acid, alanine and cystine showed that deamination occurred with all these amino acids in the presence of formaldehyde under the conditions employed.

Van Veen and Hyman (145) discovered djenkolic acid, a condensation product of cystine and formaldehyde, in the protein of the djenkol bean following hydrolysis with barium hydroxide. This new amino acid was hydrolyzed by sulphuric acid to give cysteine and formaldehyde. Lillevik and Sandstrom (87) showed that this compound was hydrolyzed by 20 per cent hydrochloric acid, at a rate similar to that obtained with proteins under the same conditions. They found that the formaldehyde released during the hydrolysis of this compound in the presence of tryptophane or gelatin was twice as effective in forming humin as the equivalent amount of formaldehyde added directly, and suggested that the rate of liberation in the first case may be an explanation. Analysis of the humin indicated a ratio of three molecules of aldehyde to one of tryptophane, which is the same ratio as Burr and Gortner (21) obtained with indoles and trioxymethylene. They suggest that this naturally-occurring compound may be a general factor in humin formation. Thiazolidine-4-carboxylic acid will behave similarily according to Ratner and Clarke (115).

Riesser, Hansen and Nagel (116) obtained acetaldehyde in yields up to 1.7 per cent from the alkaline hydrolysis of

casein. The yield was somewhat greater when the distillation and hydrolysis was performed in an atmosphere of nitrogen rather than air, from which they concluded that the acetaldehyde was not an oxidation product and did not arise from carbohydrate. When the protein had previously been hydrolyzed with acids and the hydrolytic products subjected to alkaline hydrolysis, no acetaldehyde was obtained. They concluded that the acetaldehyde had been lost in humin formation during the acid hydrolysis.

In addition to the reducing agents previously mentioned as being effective against humin formation, (15, 67, 68, 69, 85, 88, 135), Sahyun (120) found that zinc dust or zinc chloride added to hydrochloric acid is also effective. No preservation of tryptophane has been reported following the use of these reducing agents nor has any precise conclusion been reached as to the role of these reducing agents in preventing the formation of humin. Narita (100) states that, after hydrolysis with hydrochloric acid in the presence of stannous chloride, the removal of the tin by hydrogen sulphide resulted in the adsorption of arginine, histidine, cystine and tryptophane on the stannous sulphide. He does not mention whether tryptophane was present in traces or in quantity.

Nitschmann and Lauener (105) found that, when casein was treated with formaldehyde in acid solution, some of the formaldehyde was irreversibly bound to the exposed indole ring. Attempts to distil off the formaldehyde by heating with 2 N sulphuric acid resulted in liberation of the tryptophane by

hydrolysis which then condensed with aldehyde to form humin.

Fraenkel-Conrat et al (49), working with gramicidin which contains 40 per cent tryptophane, found that in weak alkali the indole ring bound formaldehyde strongly on either the imino nitrogen or the ∝carbon, giving a methylol group. In strong alkali this reaction was reversible but acid liberated only part of the formaldehyde and caused a breakdown of the indole ring. Crystalline gramicidin could be hydrolyzed without the formation of humin, but the presence of formaldehyde led to a loss of about 25 per cent of the nitrogen in the insoluble humin.

Olcott and Fraenkel-Conrat (107), in a study of the formation and loss of cysteine during the acid hydrolysis of crystalline proteins, carried out the reaction in Thunberg tubes using 6-7 N sulphuric and hydrochloric acids. After introducing the acid and protein, the tubes were evacuated, refilled with carbon dioxide and re-evacuated. Hydrolysis was carried out at 100°C. in an air oven, or at 125°C. in an autoclave. They observed that crystalline proteins, which contained no detectable sulphydryl groups in the intact protein, frequently yielded some cysteine after acid hydrolysis. This had also been observed by Brand and Kassel (19). Olcott and Fraenkel-Conrat heated cystine with acid under the conditions of hydrolysis and found negligible conversion of cystine to cysteine, except with sulphuric acid at 125°C. Tryptophane,

under the same conditions, showed losses of only 5 per cent in 18 hours, except with sulphuric acid at 125°C. When cystine was added to the crystalline protein, considerable cysteine was formed. Since these proteins had a high tryptophane content, they heated pure cystine and tryptophane under the same conditions and observed considerable formation of cysteine accompanied by large losses of tryptophane. They concluded that the conversion of cystine to cysteine supplied the oxidation necessary for the destruction of tryptophane. In proof of this, tryptophane added to cystine-containing proteins resulted in the formation of larger amounts of cysteine. No humin formation accompanied the loss of tryptophane when heated in the presence of cystine and acid.

The presence of serine and pyruvic acid also resulted in tryptophane destruction, but with many other amino acids tryptophane was relatively stable, recoveries of from 88 to 96 per cent being obtained after 18 hours at 100°C. in 6 N acid. Gramicidin, which contains no cystine or serine, could be hydrolyzed without appreciable loss of tryptophane, whereas such a procedure was unsuited to most other proteins. Pyruvic acid has been identified among the acid hydrolysis products of proteins (99) and is considered to arise from decomposition of serine (33), or possibly from cystine (29). Tryptophane was quite stable when heated with acids in evacuated Thunberg tubes, but 82 per cent was destroyed if heated for the same length of time while exposed to the atmosphere.

Witkop and Graser (149) found that, when tryptophane in acetic acid solution was subjected to ozone, the double bond of the pyrole ring was rapidly broken. After the addition of 1 N hydrochloric acid to this solution and heating, formic acid was split off. Precipitation with mercuric sulphate yielded kynurenine sulphate.

White and Sayers (148) have made the only recorded attempt to retain tryptophane during acid hydrolysis of a non-crystalline protein. Using commercial casein, they were able to prepare hydrolysates containing up to 70 per cent of the original tryptophane which were suitable for parenteral administration to small animals. This retention of tryptophane was obtained largely at the sacrifice of the degree of hydrolysis. The recommended procedure is to hydrolyze the casein with 0.25 N hydrochloric acid for seventeen hours, at which time a maximum of 30 per cent hydrolysis is obtained. The retention of tryptophane obtained under various conditions of acid concentration and time is reported. The most remarkable feature of their results is the retention of 55 per cent of the original tryptophane after the attainment of 98 per cent hydrolysis with 5.5 N sulphuric acid.

In summary, it has been observed that tryptophane is relatively stable if heated alone in sulphuric or hydrochloric acids, but if oxidizing agents such as molecular oxygen, ozone, cystine or cupric and ferric salts are present, rapid destruction of tryptophane occurs. In addition, the effect of

reducing agents in preventing humin formation is further evidence that oxidation is involved. The presence of aldehydes or aldehyde precursors is also responsible for rapid tryptophane destruction and humin formation, presumably by the formation of condensation products in the presence of strong acids. The exact mechanism of the reaction of tryptophane with aldehydes in the presence of strong acids is still obscure. It is the opinion of some that the imino group of tryptophane is the one involved in the initial step of the reaction while others favour the ∝ carbon and still others suspect that either or both may be involved. Further evidence that condensation with an aldehyde is involved is provided by the fact that the presence of urea is beneficial, the polymerization of this agent with formaldehyde being well known.

While knowledge concerning tryptophane destruction is increasing, little real progress is being made with respect to humin formation, and the state of knowledge is still best summarized in the words of Mitchell and Hamilton (98), 1929, who state:

> "Most of the accumulated data ... point to the conclusion that the artificial humin formed during the acid hydrolysis of proteins is not a simple condensation product but is due to a condensation followed by a re-arrangement or oxidation, or both, with the ultimate formation of an extremely resistant molecule or molecules. The nature of the chemical reactions involved and the structural configuration of the humin molecule or molecules formed still require elucidation".

MATERIALS

Commercial casein rather than pure casein was used throughout most of this study, not only for the sake of economy, but more particularly to test the procedure employed under conditions favorable for a reasonably high degree of humin formation and tryptophane destruction. All analyses listed below are reported on a moisture-free basis.

1. Casein

a. Commercial

This casein was supplied through the courtesy of the Champlain Milk Products Company, Limited, Stanbridge, Quebec, and was prepared by lactic acid precipitation. Nitrogen and tryptophane analyses gave the following values:

Nitrogen	-	13.3 per cent
Tryptophane	-	1.06 per cent

b. Partially purified casein

One kg. of commercial casein was subjected to the purification procedure of Van Slyke and Baker (144). This purification consisted of two re-precipitations, thorough washing with water, one washing with ethanol and a final washing with ethyl ether.

With such a relatively large amount of casein it

is difficult to achieve a high degree of purity as is shown in the analyses:

Nitrogen - 14.5 per cent

Tryptophane - 1.20 per cent

2. Blood Fibrin

This product was purchased from Fisher Scientific Company, Limited, Montreal, Quebec. The following values were obtained on analysis:

Nitrogen 14.9 per cent

Tryptophane - 2.38 per cent

ANALYTICAL METHODS

1. Tryptophane

a. Shaw and McFarlane Method

The colorimetric glyoxylic acid method of Shaw and McFarlane (129) was used to measure the tryptophane recovery in nearly all experiments. The rapidity and accuracy of this method greatly reduced the labor involved in carrying out the large number of tryptophane analyses.

Aliquots of the acid hydrolysate withdrawn from the hydrolysis flask were pipetted while hot into sufficient 50 per cent sodium hydroxide to give a final alkali concentration of 20 per cent. The samples thus obtained could then be stored for a few hours before the analyses were carried out without danger of further destruction of tryptophane due to exposure of the hot acid solution to atmospheric oxygen.

The alkaline samples were heated on the steam bath for twenty minutes as in the normal application of the Shaw and McFarlane method. This was later found to be unnecessary except for samples withdrawn early in the course of hydrolysis. Such samples contained some undissolved denatured protein which dissolved more slowly in alkali than is normally the

case with the native casein. It was deemed advisable, however, to treat all samples similarily, regardless of degree of acid hydrolysis, and the twenty minute heating period in 20 per cent alkali was applied to all samples.

At each time of sampling, additional aliquots of the acid hydrolysate were pipetted into Kjeldahl flasks for total nitrogen determination. From the tryptophane and nitrogen content of the original casein and the tryptophane and nitrogen content of aliquots of acid hydrolysate at various stages of hydrolysis, the tryptophane percentage was calculated and expressed as tryptophane, per cent of original.

b. Block and Bolling Modification of Millon-Folin Method (11)

This method was used in one experiment only, to permit comparison with results obtained using the Shaw and McFarlane method.

Aliquots of the acid hydrolysate (5 cc. containing 300 mg. casein approximately) were pipetted into 30 cc. Pyrex test tubes containing sufficient 60 per cent sodium hydroxide to give a final alkali concentration of 5.0 N. The tubes were equipped with reflux condensers and placed in an oil bath at 120°C. for five hours. From this point the analysis was carried out in the usual manner, employing photoelectric colorimetry.

2. Tyrosine

The Block and Bolling modification of the Millon-Folin

method (11) was used for the determination of tyrosine. Samples of the acid hydrolysate were treated as described under the determination of tryptophane (1. b.), since this method is designed for the determination of tryptophane and tyrosine in the same aliquot of hydrolysate.

3. Total Nitrogen

a. Macro-Kjeldahl

The Kjeldahl-Gunning-Arnold method (3) was used with mercuric oxide as catalyst.

b. Micro-Kjeldahl

The method of Pregl (114) was modified to employ the digestion mixture of Campbell and Hannah (22) and the boric acid absorber as used by Stover and Sandin (134).

4. Amide Nitrogen

The procedure used was essentially that recommended by Van Slyke (141).

Aliquots of the hydrolysate were made alkaline with magnesium oxide and aerated under partial vacuum for thirty-six hours, and the ammonia absorbed in 0.1 N sulphuric acid.

5. Amino Nitrogen

After removal of the amide nitrogen, the residues were neutralized with sulphuric acid and amino nitrogen determined by the manometric method of Van Slyke (139). Since some

experiments were not continued to the completion of hydrolysis, an aliquot of each hydrolysate was subjected to an additional period of hydrolysis by simple refluxing until a twenty-four hour period of hydrolysis had been completed. The amino nitrogen value obtained at this time, after removal of the amide nitrogen, was used as a basis for calculating the percentage hydrolysis at each time of sampling.

EXPERIMENTAL RESULTS

Part I

Preliminary Experiments

INTRODUCTION

In the general introduction to this study, preliminary experiments were mentioned which indicated that nascent hydrogen could be employed to prevent humin formation and tryptophane destruction. In the following experiments an account will be given of the discovery of this principle and of various methods employed to apply it with the greatest efficiency. The problem, at this stage, was largely mechanical in nature and the results reported do not reflect the amount of time spent on this phase of the work. Tryptophane destruction is so intimately related to humin formation that in most cases the appearance of the hydrolysate was sufficient to indicate whether or not any measure of success had been achieved. In consequence, little in the way of exact quantitative data is reported.

EXPERIMENTAL

1. Activation of Hydrogen by Platinum Foil

A 250 cc. two-necked flask, equipped with reflux condenser, was used in this experiment. A hydrogen-carrying tube was made by sealing a 40 mm. length of 12 mm. bore glass tubing to a length of 3 mm. bore glass tubing. The small bore portion of the tubing was inserted up through the centre of the reflux condenser, so that the wide-mouthed end projected into the reaction flask, Fig. 1.

A piece of platinum foil, approximately 12 sq. cm. in size, was coated heavily with platinum black, rolled into a coil, and placed into the wide-mouth of the hydrogen-carrying tube. The foil was adjusted to project approximately 1 cm. beyond the lower end of the tube.

The protein and acid were added to the flask, 2 g. of commercial casein and 60 cc. of 6 N sulphuric acid. The depth of the hydrogen tube was adjusted so that the wide mouth was slightly below the surface of the solution. When hydrogen was passed through the tube, part of the surface of the foil was washed alternately with acid and hydrogen as the bubbles escaped. The action was thus similar to the common hydrogen electrode. The flask was heated in a glycerine bath maintained at 120°C. At the same time a control experiment was carried out in an identical manner, but without the platinum foil.

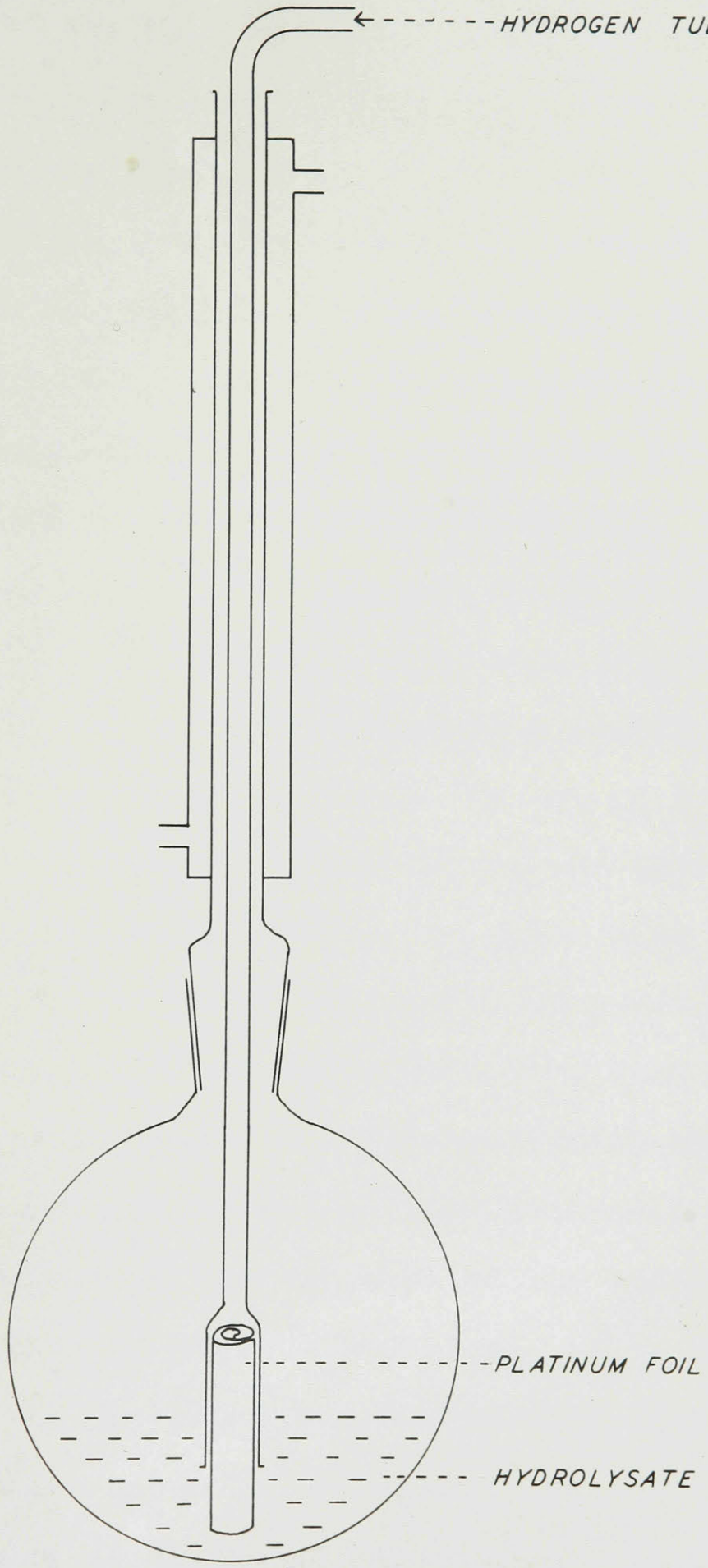

FIGURE I

APPARATUS USING HYDROGEN ACTIVATED BY PLATINUM

Samples of the hydrolysate were withdrawn at intervals, examined visually for humin formation, and tested qualitatively for tryptophane with the Hopkins-Cole test (76). The hydrolysate obtained in the experiment in which the hydrogen had been partially activated by the platinum was definitely lighter in color than the control, and contained no insoluble humin. The glyoxylic acid test remained positive, but with diminishing intensity, up to nine hours. The control gave a negative test for tryptophane after three hours.

Modifications of this procedure were tried, consisting of replacement of the platinum foil by a freshly platinized piece at hourly intervals and also the use of a larger piece of foil. These changes produced only minor improvements.

This preliminary experiment indicated that the use of nascent hydrogen in the prevention of humin and preservation of tryptophane offered some promise of success, provided this reagent could be employed in a more efficient manner. Greater efficiency in the application of this principle obviously depended upon increasing the exposure of the hydrolyzing mixture to the action of nascent hydrogen.

According to Grubb and Van Cleave (60) the half-life period of hydrogen atoms is 0.2 seconds at room temperature and at a pressure of 40 mm. of mercury. It is, therefore, highly unlikely that any practical means could be found whereby externally activated hydrogen could be introduced into the reaction mixture. Methods are thereby limited to those in which

nascent hydrogen is produced in the immediate presence of the hydrolysis mixture. The problem, therefore, developed into a search for suitable methods whereby this could be achieved.

2. Hydrogenation

At the outset, hydrogenation offered only remote possibilities of success for several reasons:

(i) Choice of catalyst is severely limited since the catalyst and carrier must both be resistant to hot mineral acids.

(ii) Proteins are in themselves notorious catalyst poisons through their film-forming properties.

(iii) A complex substance such as commercial casein and its hydrolytic products, is a source of a variety of catalyst poisons, chief of which are probably sulphur compounds.

Nevertheless, there remained the possibility that only a slight amount of catalytic activity might be sufficient to produce the desired results. The following experiment was designed to test this possibility.

A Burgess-Parr laboratory catalytic apparatus, Illinois Model, commonly used in hydrogenation of fats, was adapted for this purpose. An all-glass hydrogenation vessel was devised from a 250 cc. centrifuge bottle, provided with a ground glass stopper, inlet tube for hydrogen, and sampling tube.

Platinum on an asbestos carrier was selected as a

suitable catalyst. It was prepared from platinic chloride and acid-washed powdered asbestos, heated in the presence of formaldehyde, as directed by Berkman, Morrell and Egloff (9). Additional reduction by a hydrogen stream at 1000°C. was found necessary to reduce the platinum salt completely.

Since the reaction was to be carried out under pressure, a higher temperature could be used than in normal refluxing. This permitted the use of more dilute acid and a shorter hydrolysis period without lowering the degree of hydrolysis attained.

The hydrolysis of 2 g. of commercial casein in 50 cc. of 2.5 N sulphuric acid was carried out, using 2 g. of the catalyst, a hydrogen pressure of 50 lbs. per sq. in., and a temperature of 130°C. for five hours. The reaction flask was agitated vigorously by the mechanical shaker.

Some difficulties were encountered in the execution of this experiment:

(i) The vapor pressure of water at 130°C. is 39 lbs. per sq. in., and that of 2.5 N sulphuric acid is only slightly less. Thus, the effective hydrogen pressure over the surface of the liquid was approximately 12 lbs. per sq. in.

(ii) There was negligible, if any, consumption of hydrogen and, without a flow of hydrogen through the inlet tube, small variations in temperature caused a back flow of hydrogen. The hydrolysate splashed

up into the inlet tube and the back flow of hydrogen forced a considerable amount of the hydrolysate out of the flask.

After filtering off the catalyst, the hydrolysate was somewhat brown in color but still considerably lighter than an ordinary hydrolysate. It is highly probable, however, that the finely-divided catalyst adsorbed much of the colored material. The hydrolysate gave a negative Hopkins-Cole test.

In view of the technical difficulties encountered and the unsatisfactory results obtained, hydrogenation in this way, using a metallic catalyst, was abandoned as a possible solution to the problem.

3. Electrolytic Reduction

The process of electrolytic reduction has seldom been employed in research problems of a biochemical nature, but in this work it was particularily well suited to the purpose for several reasons.

(i) Conductivity would be adequately provided by the highly acid medium.

(ii) All of the hydrogen liberated by electrolysis would be initially in the nascent state and this would not be influenced by the presence of protein.

(iii) In such a strongly acid medium the protein, peptide and amino acid molecules possess a positive charge and any migration under the influence of the current would be towards the cathode. This would be

particularly advantageous since it would minimize diffusion through the diaphram separating the anode and cathode compartments.

The major disadvantage was likely to be the difficulty of applying electrolytic reduction in quantitative analytical procedures unless a diaphram was available which was completely impermeable to amino acids, or unless the analytical results were calculated on a nitrogen basis.

Since electrolytic reduction offered a further means of producing nascent hydrogen in-situ, it was applied, in a relatively crude manner at first, to create the reducing conditions apparently essential for the prevention of humin formation and the preservation of tryptophane. The initial experiment was planned and conducted as follows:

A 250 cc. two-necked boiling flask was used. A platinum crucible cover, approximately 16 sq. cm. in size, was placed on the bottom of the flask to serve as a cathode. This cathode was connected by a platinum wire to a mercury-platinum junction, inserted through a rubber stopper in the side neck of the flask, to permit external contact, Fig. 2. A piece of platinum wire, sealed into a glass tube containing the external connection and suspended through the reflux condenser tube, served as an anode. This platinum wire anode was also used to suspend an alundum extraction thimble (10 mm. x 70 mm.). This thimble acted as a porous diaphram surrounding the anode. The alundum cup provided the separate anode compartment

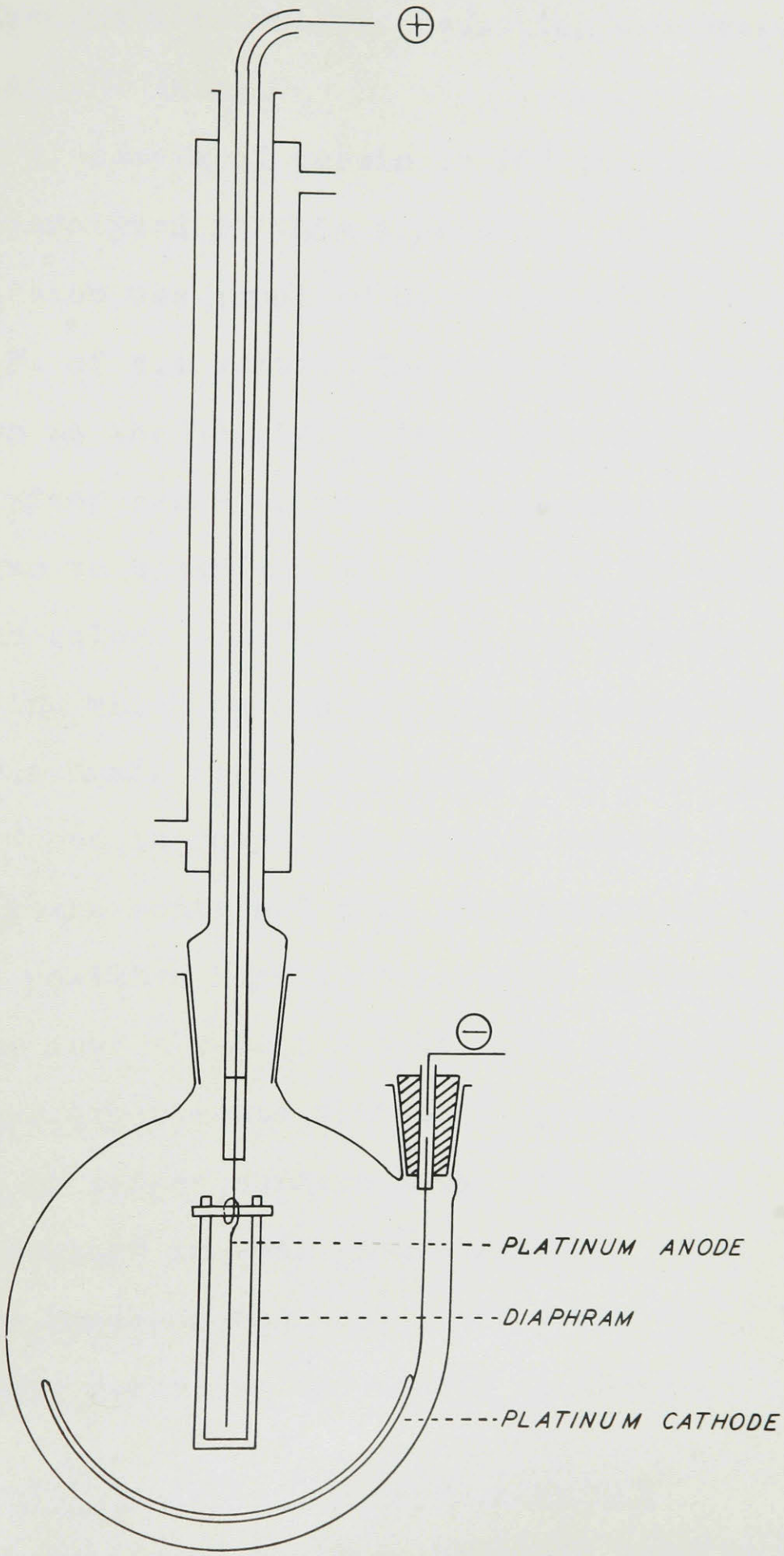

FIGURE 2

ELECTROLYTIC REDUCTION APPARATUS

commonly used in electrolytic reduction processes to avoid oxidation at the anode.

A 3.0 g. sample of casein in 100 cc. of 6 N sulphuric acid was hydrolyzed in this apparatus. Simultaneous electrolytic reduction was supplied by a direct current of 0.2 amperes at an E.M.F. of 4.4 volts. The hydrolysate became pink and later brown as the boiling point was approached. Within one-half hour after reaching the boiling point the hydrolysate slowly began to clear and at the end of two hours had only a faint amber color. The hydrolysis was continued for twelve hours, during which period the hydrolysate slowly darkened. No insoluble humin formed and the amount of soluble humin was greatly reduced in comparison with a control hydrolysate. The hydrolysate contained some tryptophane, as indicated by a definitely positive Hopkins-Cole test, whereas the control hydrolysate gave a negative test after five hours.

Further experiments with this apparatus, using a platinum cathode of larger surface area, and later a lead-strip cathode, resulted in small improvements. While the hydrolysate always became reasonably clear after about two hours boiling, some darkening invariably followed.

4. Electrolytic Reduction Under Pressure

As was mentioned earlier, pressure permits the use of less concentrated acids and greatly decreases the time required for the attainment of complete hydrolysis. It was,

therefore, considered likely that the combination of these features with electrolytic reduction should facilitate attainment of complete hydrolysis with a minimum of humin formation and tryptophane destruction.

The apparatus used was similar to that shown in Fig. 3, except for the cathode, and was devised as follows:

A 250 cc. centrifuge bottle was used as a pressure flask. Due to their shape and strong construction, these bottles will withstand considerable internal pressure. All bottles used were first subjected to a pressure of seventy pounds per sq. in., in order that they could be used at thirty pounds pressure with some degree of safety.

A condenser tube of thick-walled Pyrex tubing was fitted to the bottle using a rubber stopper. This condenser tube was flared slightly at the lower end so that it would not be forced out by the pressure. The rubber stopper also carried two mercury-platinum junctions for anode and cathode connections. A lead-strip cathode (1 x 30 cm.), coiled in the form of a helix (not illustrated), was placed in the flask. An alundum thimble was again used as an anode compartment, being placed in the centre of the helix formed by the cathode, and resting on the bottom of the flask. The lead cathode was connected to one mercury-platinum junction and the platinum anode to the other. A metal clamp was used to prevent the stopper from being forced out of the flask.

The upper end of the condenser tube was connected to a

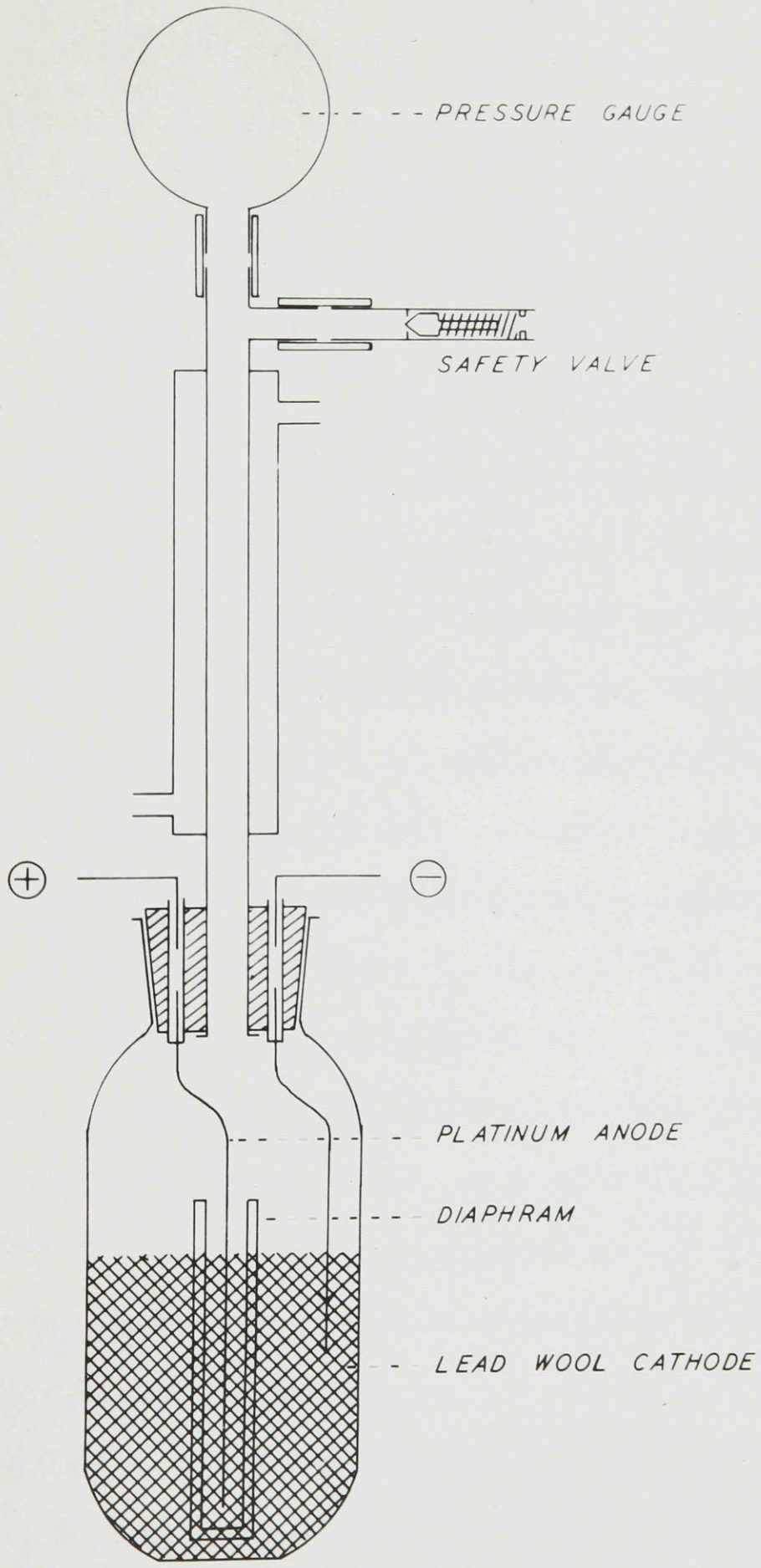

FIGURE 3

ELECTROLYTIC REDUCTION UNDER PRESSURE

pressure gauge and a safety valve. It was essential that the safety valve should have a narrow operating range, for any sudden drop in pressure caused vigorous boiling of the hydrolysate with consequent foaming. A suitable safety valve was adapted from an automobile tire valve and stem of the Schraeder type, used in reverse position, so that the pressure built up in the bottle served to open the valve in the same manner as air forced into a tire. The pressure required to open these valves ranges from 10 to 50 lbs. per sq. in., and one can be selected which responds to the required pressure. Such a safety valve is quite sensitive and maintained an almost constant pressure in the bottle.

The hydrolysis of 3 g. of commercial casein was carried out in this apparatus using 50 cc. of 25 per cent sulphuric acid at a temperature of 130°C. and a pressure of 30 lbs. per sq. in. This temperature was maintained by a constant temperature glycerine bath. Electrolytic reduction was provided by a current of 0.4 amperes at an E.M.F. of 6.2 volts. The hydrolysis was allowed to proceed for three hours after reaching maximum temperature.

This experiment produced the most favorable results so far obtained. The hydrolysate contained a very small amount of soluble humin and the glyoxylic acid test was strongly positive.

A second experiment was conducted with the apparatus in which 50 g. of lead wool was substituted for the lead-strip

cathode. This produced excellent results. The large surface area afforded by this amount of lead in such form was obviously responsible for the improvement. The hydrolysate so obtained was almost water-clear in color. A small quantity of the hydrolysate gave a strong Hopkins-Cole test, the color being characteristic of the test with pure tryptophane.

The odor of these acid hydrolysates was noticeably different from a normal acid hydrolysate. They possessed a pungent odor which could not be characterized but was presumably due to alcohol resulting from the reduction of aldehydic substances in the casein.

In all of these electrolytic reduction experiments the solution in the anode compartment, after completion of hydrolysis, was black in color, and devoid of tryptophane. This confirms the prevailing hypothesis that oxidation favours humin formation and tryptophane destruction.

Sufficient progress had now been made to warrant quantitative determination of tryptophane in the acid hydrolysate. A 1.0 g. sample of commercial casein was hydrolyzed in this apparatus under conditions described above. The Shaw and McFarlane method (loc. cit.), for the estimation of tryptophane, was applied directly to the acid hydrolysate in this instance, but with unsatisfactory results. The blank determination, in which no glyoxylic acid was present, developed a violet color of identical shade to the test determination but of lesser intensity. Reading the color of the test determination agains

such a blank showed a low tryptophane content, 0.30 per cent. Repetition of the determination gave similar results. Apparently, some unreduced aldehyde remained in the acid hydrolysate which was capable of fulfilling the function of the glyoxylic acid.

A second aliquot of the hydrolysate was made alkaline with sodium hydroxide and heated for twenty minutes. This overcame the difficulty encountered in the attempt to carry out the analysis directly on the acid hydrolysate. A tryptophane content of 0.63 per cent was obtained, representing a recovery of 57 per cent of the original tryptophane.

One factor which decreased the apparent recovery of tryptophane was the loss of a certain fraction of the hydrolysate by diffusion into the anode compartment. Assuming equal concentration on both sides of the diaphram, up to one-sixth of the hydrolysate could have been lost in this way.

This apparatus proved that nascent hydrogen could be employed to prevent humin formation entirely and to preserve a fair percentage of the original tryptophane. The assumption was made at this stage that the compound responsible for the color with the glyoxylic acid reagent actually was tryptophane and not a decomposition product arising from tryptophane during the hydrolysis. It was realized that the presence of tryptophane would eventually have to be verified by isolation. Since isolation of tryptophane would require the hydrolysis of at least 100 g. of casein, a larger scale apparatus

would be necessary for this purpose.

In an attempt to achieve this, a battery of four cells, each constructed as depicted in Fig. 3, was set up and connected in parallel. The condenser tubes of all four were connected to a manifold so that the same safety valve and pressure gauge served for all four flasks. Each flask was charged with 10 g. of commercial casein and 100 cc. of 25 per cent sulphuric acid, and the hydrolysis carried out with simultaneous electrolytic reduction as in previous experiments.

This did not prove too successful. One or two of the cells would produce clear hydrolysates while the others would have varying degrees of humin formation. This was largely attributable to the different resistances among the four cells, but other details of their construction may also have had effect.

5. Design and Construction of Large Scale Apparatus

Throughout the course of preliminary experiments it became apparent that certain features would have to be incorporated into the design of a more efficient apparatus. These features are enumerated below:

(i) Some agitation of the solution would have to be provided. Electrolytic reduction can take place only at the cathode surface since it is there only that the hydrogen is in the monatomic form.

Once bubbles form at the cathode surface or escape from this surface, such hydrogen no longer has effective reducing activity. Stirring or other agitation is therefore essential for rapid interchange of the material at the cathode surface and a consequent rapid rate of reduction.

(ii) Oxygen should be excluded from the atmosphere above the solution. Molecular oxygen is a fairly strong oxidizing agent and its exclusion is know to reduce humin formation (107). In addition, some ozone is produced at the anode and this also would be conducive to humin formation and tryptophane destruction.

(iii) The diaphram separating the anode compartment should be of as low porosity as possible without creating too high a resistance for the passage of electrolyte ions.

(iv) The cathode should be large in surface area to facilitate a rapid rate of reduction.

A considerable amount of time was spent in attempting to construct an experimental model of such an apparatus to operate at a pressure of approximately 30 lbs. per sq. in. The original intention was to employ a lead vessel of approximately 1500 cc. capacity enclosed in a brass air-tight jacket. The lead vessel was to serve as the container for the hydrolysate and also as the cathode.

The oxygen and hydrogen were to be confined in separate compartments, each with a safety valve adjusted to maintain a balance in pressure between both compartments to avoid a pressure differential across the porous diaphram separating the anode and cathode compartments.

The technical difficulties encountered proved insurmountable, particularly since it was not deemed advisable to make any extensive expenditure for specialized equipment at this stage. It was, therefore, decided to abandon the idea of employing high pressure, at least until the possibilities of operation at atmospheric pressure had been fully investigated.

A simplified all-glass apparatus was constructed and, after several trial experiments and minor modifications, was considered satisfactory for a systematic study of the conditions required for minimum humin formation and tryptophane destruction. The apparatus in its final form is illustrated by a schematic diagram in Fig. 4, and is constructed as follows:

A two-litre three-necked flask, carrying two standard ₸ 29/42 ground glass openings and a central ₸ 45/50 ground glass opening, is used as a hydrolysis flask. A ground glass condenser, bearing a ₸ 29/42 ground glass opening at the top, is fitted to the centre opening. The shaft of the stirrer passes up through the centre of this condenser and is connected to the steel shaft of the stuffing-box assembly which fits the

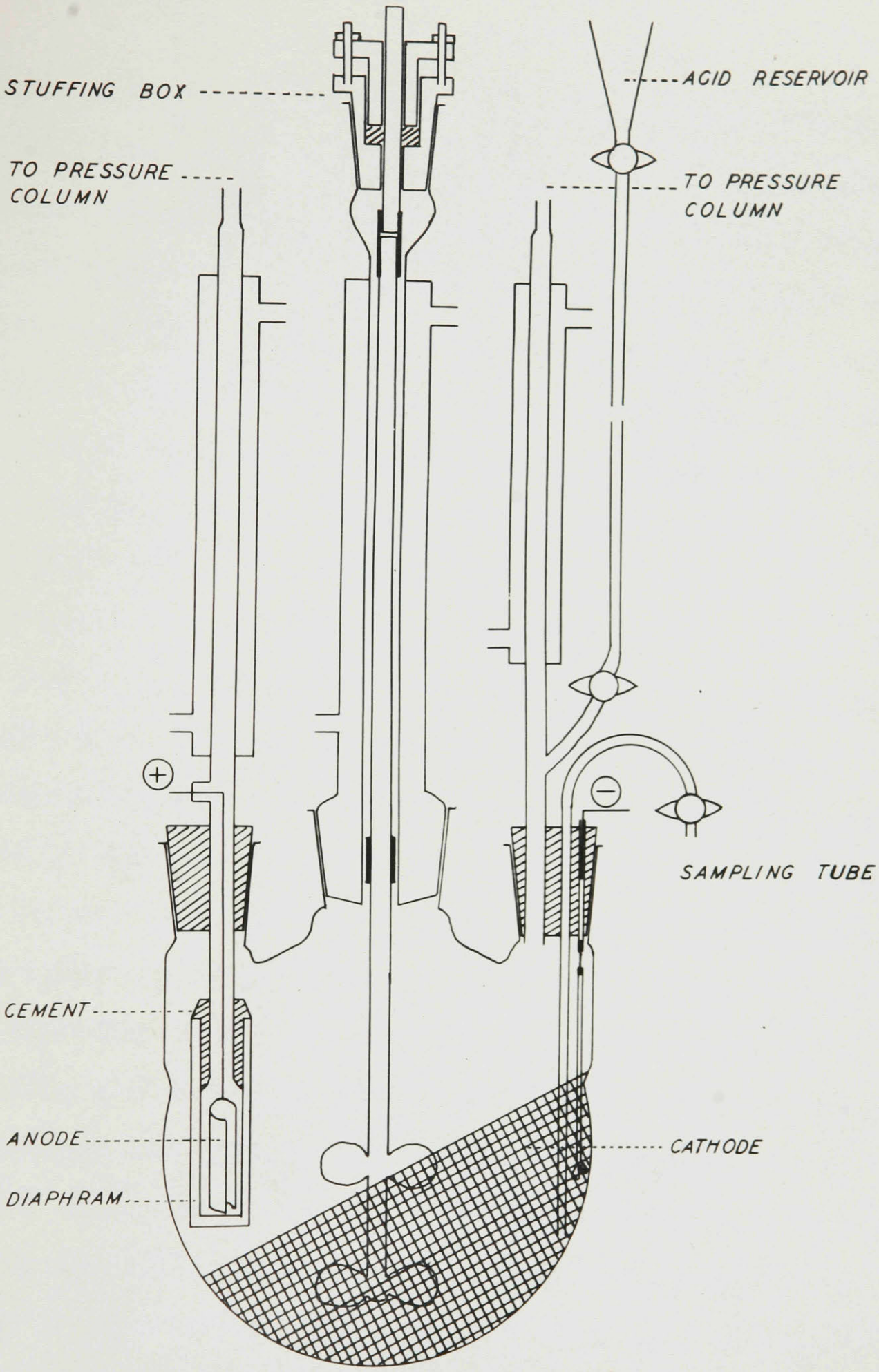

FIGURE 4

APPARATUS FOR HYDROLYSIS WITH SIMULTANEOUS ELECTROLYTIC REDUCTION

top ground glass opening. The connection is made with a short length of Tygon tubing. This central condenser need not be more than about 20 cm. in length, its purpose being to prevent any of the acid hydrolysate from coming into contact with the steel stuffing-box due to splashing or foaming. This prevents harmful corrosion of the metal and also avoids contamination of the hydrolysate by small amounts of ferric sulphate. As mentioned earlier, such iron salts act catalytically in humin formation (75).

The stirrer shaft is of Pyrex tubing, 8 mm. outside diameter. The inside diameter of the condenser tube is 12 mm. Vibration of the stirrer shaft is prevented by a short collar (about 2 cm.) of Tygon tubing around the shaft near the lower end of the condenser tube. When constructed in this way the stirrer will operate smoothly at high speed, despite the long distance between the blades and the power source.

To form the blades of the stirrer, short lengths of Pyrex tubing are welded to the shaft at the lower end and then flattened and shaped with broad tweezers while hot. The blades should be as large as the flask opening will permit. They should be twisted at an angle, depending on the direction of rotation of the motor, so that the hydrolysate will be thrown downwards onto the cathode at the bottom of the flask.

Blades are attached at three levels on the stirrer shaft, approximately 2.5 cm. apart. The stirrer is driven by a 1/30

H. P. electric motor at a speed of approximately 1600 R.P.M. An induction type motor is preferred since it avoids the danger of sparks which might ignite any hydrogen in the vicinity. Since efficient stirring is essential, the construction of the stirrer is extremely important.

The anode assembly is placed in one of the side ₸ 29/42 openings. Ground glass interchangeable joints are preferable but rubber stoppers will serve almost as well. This anode assembly consists of a condenser, a platinum anode and a porcelain cup. A platinum wire is sealed into the condenser tube near the lower end and extends downward to the anode which is just below the end of the condenser tube. A piece of thin sheet platinum, about 3 cm. x 4 cm. is used as the anode. The porcelain cup was adapted from a porcelain Berkfeld bacterial filter of extremely fine porosity. The upper glazed part of the filter was removed with a file or saw, leaving the lower part as a cup, outside dimensions being 23 mm. x 100 mm. with walls 4 mm. thick. The lower end of the condenser tube is flared to fit the inside of the porcelain cup and is then inserted into the cup to a distance of approximately 50 mm. The space between the condenser tube and the inside of the cup is then filled with acid-resistant cement, composed of barium sulphate, 4 parts, powdered asbestos, 1 part, sodium fluosilicate, 1 part, and sufficient sodium silicate solution to make a free-flowing paste (24). The cement should be drawn into the side walls of the procelain cup with suction to make

a gas-tight joint. The level of the acid during operation lies somewhere in the condenser tube above the seal, and there is extremely small possibility that any oxygen can escape into the main part of the flask.

The cathode consists of approximately 200 g. of lead wool, placed in the form of a mat on the bottom of the flask. Care should be taken to distribute the lead wool evenly and over as large an area as possible. This lead wool is connected by a lead-strip to a mercury-platinum junction inserted through a rubber stopper in the remaining neck of the flask. This rubber stopper also carries a reflux condenser, sampling tube, and tube for addition of strong acid to the flask, the purpose of the latter will be discussed in detail elsewhere.

When the electrolysis is proceeding, the hydrogen and the oxygen are evolved in separate compartments. Each passes up a separate condenser tube and can be disposed of in a suitable manner.

It has been found convenient to conduct the hydrolysis under a slight pressure, one to two pounds per sq. in. This permits the reaction to be carried out at the normal boiling point under atmospheric pressure by proper adjustment of the heating-bath thermostat. Due to the slight pressure, the solution is not actually boiling and yet is held at 106°C. or slightly more, which is the normal boiling point in simple refluxing. This pressure also facilitates sampling without

permitting the entry of atmospheric oxygen since the stopcock of the sampling tube only need be turned and the hydrolysate is forced out.

The pressure is obtained by two columns of water, contained in lengths of large-bore glass tubing approximately 4 feet high. The oxygen is bubbled through one and the hydrogen through the other. As rubber tubing would not withstand the action of ozone, small quantities being evolved at the anode, plastic tubing was used in connections between the anode condenser and the water column. "Tygon" brand plastic tubing was found very satisfactory.

Small amounts of antifoam, such as Dow-Corning Silicone Antifoam, will completely eliminate the need for this pressure system. Successful experiments have been conducted without pressure or antifoam agents, although vigorous boiling combined with high current density, must be avoided in such cases.

Direct current is provided by two six-volt storage batteries. An ammeter and a variable resistance, (0 to 8 ohms) are placed in the circuit to permit observation and control of current. The hydrolysis flask is heated by an oil bath equipped with a thermostatically controlled electric heating coil.

SUMMARY

1. The discovery has been made that nascent hydrogen is an efficient agent for the prevention of humin during hydrolysis of casein with sulphuric acid.

2. Nascent hydrogen also retards the destruction of tryptophane.

3. Electrolytic reduction has been found to be the most practical and efficient means of generating nascent hydrogen in a protein hydrolysate.

4. Apparatus has been designed for future experiments in which the hydrolysis of protein with sulphuric acid can be carried out with simultaneous electrolytic reduction. The apparatus consists, essentially, of a two-compartment cell, a platinum anode and a lead-wool cathode. The anode and anode compartment are small in relation to the cathode and cathode compartment. Vigorous stirring must be provided.

EXPERIMENTAL RESULTS

Part II

The Effect of Certain Variables on the Efficiency of the Reduction Process

INTRODUCTION

The apparatus designed on the basis of information obtained in preliminary experiments was tested in several series of experiments in order to evaluate the optimum conditions for highest efficiency. Within each set of experiments, all variables, with the exception of the one under examination, were strictly controlled, in order that differences in the results could be attributed to the variable in question. Tryptophane retention and the prevention of humin formation were the criteria used to evaluate the optimum conditions.

Some conditions were selected without experimental evaluation. In such cases the reasons for the choice are given.

EXPERIMENTAL

1. Cathode Size

A series of experiments was conducted to establish the optimum cathode size for tryptophane preservation and the prevention of humin formation. All variables except cathode size were maintained strictly constant throughout the series. A 100 g. sample of commercial casein and 1500 cc. of 6 N sulphuric acid were used. A current flow of 4.0 amperes at an E.M.F. of 12.0 volts was arbitrarily chosen. Cathodes consisting of 25, 50, 100 and 200 g. of lead wool were used. The results are summarized graphically in Fig. 5.

Samples of the hydrolysate were withdrawn at intervals and analyzed for tryptophane and total nitrogen. The amounts of soluble humin present were compared by visual observation only.

Doubling the size of the cathode from 25 to 50 and from 50 to 100 g. resulted in increased tryptophane retention but the successive increments became smaller. The slope of the curve, Fig. 5, levelled out more rapidly with the larger cathodes. No further improvement in tryptophane preservation was obtained by increasing the cathode size above 100 g. The amount of soluble humin was small even with the 25 g. cathode and, unlike the tryptophane values, decreased with each increase in cathode size. With the 200 g. cathode barely perceptible traces of color were present in the hydrolysate.

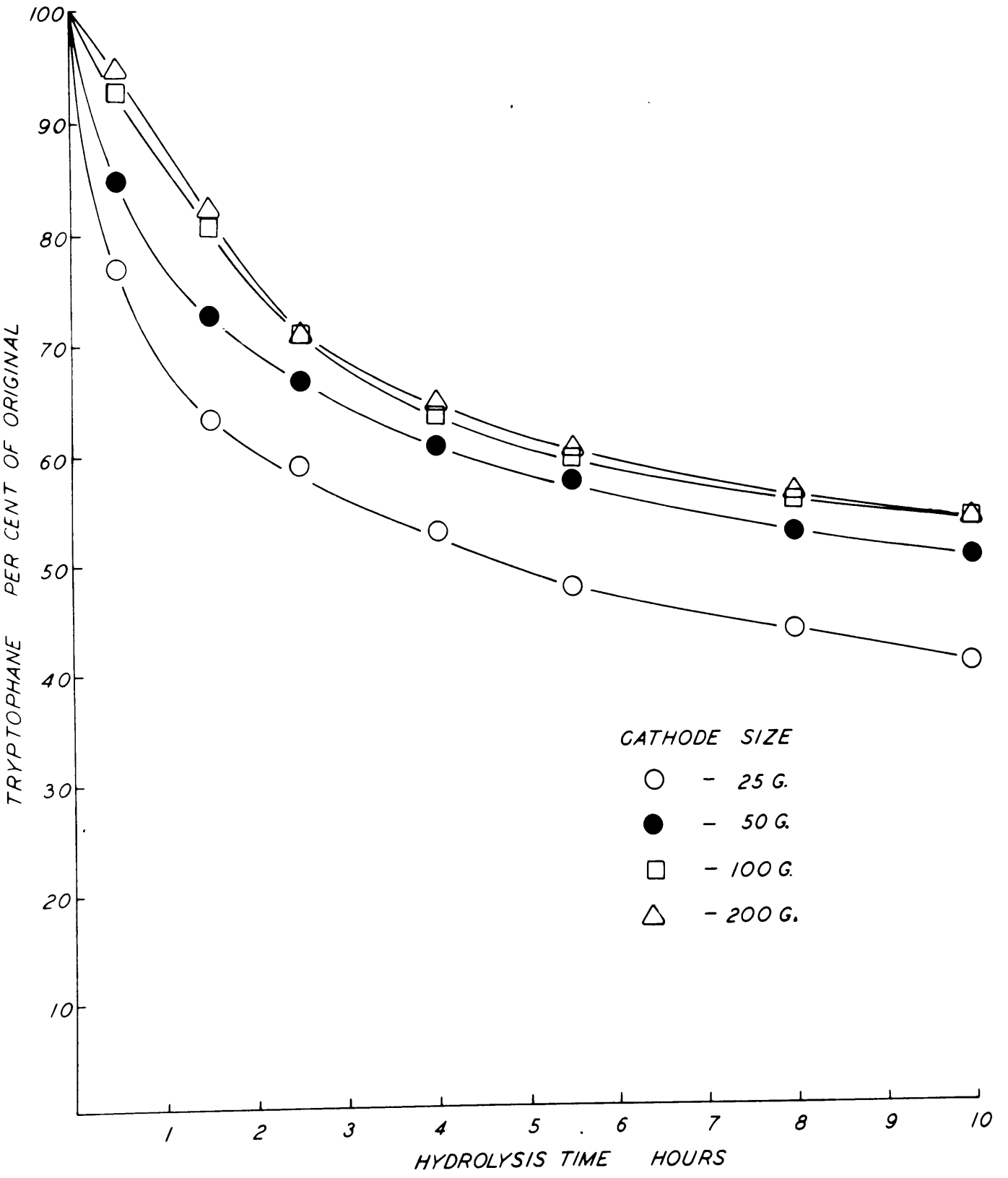

FIGURE 5

EFFECT OF CATHODE SIZE

It is surprising that the recovery of tryptophane did not increase further with cathodes over 100 g. since the rate of reduction should be influenced by this factor. Two possible explanations can be advanced. When arranging the lead-wool cathode in the flask, it is desirable to spread the material in the form of a mat on the bottom and also up around the sides of the flask as far as possible. The area of the inside surface of the flask which can be covered by 200 g. of lead wool is only slightly greater than by 100 g., because any attempt to bring the wool up high on the sides of the flask results in collapse of that section due to its own weight. Thus, the mat is thicker but not appreciably larger. A second factor is the observable tendency for hydrogen to be evolved in larger amounts at points closest to the anode. This is particularly noticeable with the larger cathodes, since they extend over a greater portion of the bottom of the flask, and there is a much greater range of distance from various points on the cathode surface to the anode.

The tendency for hydrogen to be evolved at points nearest to the anode shows a defect in the design of the apparatus, but this is not easily circumvented with laboratory apparatus. In conventional electrolytic reduction processes (109) the anode and cathode are frequently arranged in concentric ring formation or as parallel plates. This is hardly feasible in this application but could be achieved, to some

extent, by placing the anode in the centre neck of the flask. This would necessitate moving the stirrer to one of the side necks and would result in lower efficiency of agitation. Also, unless the flask had a side neck of larger diameter, the size of the stirrer blades would be severely limited.

From these experiments it is concluded that 200 g. is the optimum size of cathode for both tryptophane retention and prevention of humin formation in this apparatus and under the other conditions employed. The optimum size of cathode for any other apparatus will naturally depend on the size and design of the apparatus.

While lead wool is satisfactory as a cathode material, it would greatly facilitate the general application of the process if a metal or metal-lined flask could be employed, using the container itself as the cathode. The feasibility of this would depend on the surface area of the inside of the flask relative to that of the optimum weight of lead wool.

The surface area corresponding to 1 g. of lead wool was calculated from measurements of diameter, length and weight of a large number of single strands of lead wool. The average surface area per g. of lead wool was thus found to be approximately 2.5 sq. cm. The inside surface of the two-litre flask employed was approximately 700 sq. cm. Thus, 100 g. of lead wool corresponds in surface area to slightly over one-third of the inner surface of the flask and that of 200 g. to a little over two-thirds. It should, therefore, be

feasible to employ a lead-lined container. This relationship, by coincidence, was favorable in this size of flask. In smaller flasks the relationship would be more favorable but in larger flasks the cathode size would become less adequate as the size of the vessel was increased.

2. Current Density

A series of experiments, similar to that described in the preceding section, was conducted to establish the optimum current density. A 200 g. cathode was used and all other conditions remained the same, except that the current was varied from one to eight amperes. On the basis of the calculated surface area of 200 g. of lead wool, the current density was thus varied from 0.2 to 1.6 amp. per sq. dm., at an E.M.F. of 12.0 volts. The results are summarized graphically in Fig. 6.

Increases in current density produce increases in tryptophane recovery but the increments become successively smaller. It is questionable whether any increase was obtained between 0.8 and 1.6 amp. per sq. dm. The colour of the hydrolysate improved with increasing current density in similar manner.

When the current density was increased, larger volumes of hydrogen escaped from the cathode surface. In consequence, the reducing action of this hydrogen was lost and this would account for the smaller improvement with each successive in-

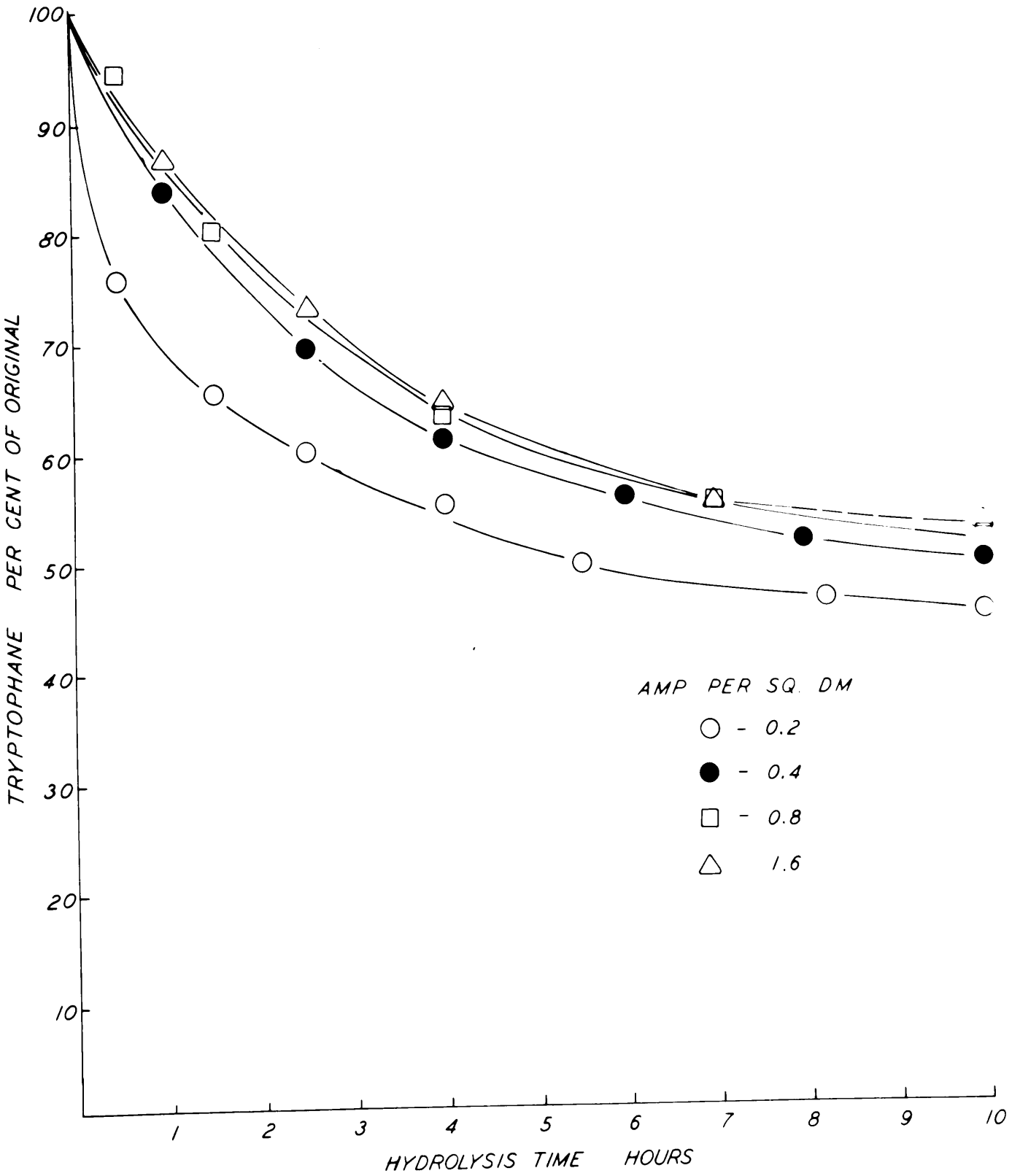

FIGURE 6

EFFECT OF CURRENT DENSITY

crease in current density.

While the best results were probably obtained with the highest current density, the large volume of hydrogen evolved introduced serious danger of foaming in the early stages of hydrolysis. For this reason, 0.8 amp. per sq. dm. was selected as the optimum current density for future experiments.

3. Cathode Composition

According to the theory of electrolytic reduction, the rate of reduction of many organic compounds depends upon the nature of the cathode material (52). Different metals have different hydrogen over-voltages and, consequently, vary in their ability to bring about reduction of the compound.

The choice of a cathode material for use in a highly acidic medium is very limited, being confined to metals low in the electromotive series and to those slowly attacked by acid. By fortunate coincidence, lead has a high hydrogen over-voltage and, in addition, lead sulphate is almost insoluble in sulphuric acid. Platinum has one of the lowest hydrogen over-voltages of all metals and mercury one of the highest. Amalgams, in general, also possess high hydrogen over-voltage.

In order to compare the efficiency of different cathode materials, all other variables must be constant. This

created difficulty in comparing lead wool with any other metal. As a result, little information was obtained on this point.

An experiment was designed to compare the efficiency of lead with lead-mercury amalgam. After completing one hydrolysis experiment, the lead-wool cathode was washed thoroughly with water and then converted into a mercury-lead amalgam without removing it from the flask. This was accomplished by filling the flask with dilute sulphuric acid and adding approximately 50 cc. of a 15 per cent solution of mercuric sulphate in dilute sulphuric acid. The mercuric ions were then electrolytically reduced by a low current for approximately fifteen minutes. The lead cathode took on the silvery sheen of mercury-lead amalgam within a few seconds. After removal of the electrolyte and thorough washing, the cathode was ready for use.

A hydrolysis experiment was conducted with this cathode and the results compared with those obtained using the cathode before amalgamation. No measurable difference in tryptophane recovery was found. The hydrolysate was very clear and free from color while in the hydrolysis flask but began to develop color within a few seconds after removal from the flask and after exposure to the air. This color formation was much more rapid, and of a different type, from that which forms in hydrolysates after several days exposure to the atmosphere, following the use of a

lead cathode. After the use of the mercury-amalgam cathode the color resembled that of brightly polished copper, rather than the reddish-brown shade forming after long standing, following the use of lead alone. A similar color was observed when mercuric sulphate was added to other clear hydrolysates.

In some cases (137), the efficiency of a cathode is increased by reversing the polarity of the cell and removing some of the metal from the electrode surface by electrolytic oxidation. In this way, small particles of other metals responsible for lowering the over-voltage, can sometimes be removed from the cathode material.

A lead-wool cathode was purified by this procedure, but an electrolyte of dilute ammonium hydroxide was used, rather than sulphuric acid, since lead oxide is more readily dissolved in alkaline solutions. A hydrolysis experiment with this purified cathode showed no improvement.

On the basis of this limited information, it appears that cathode composition does not influence the preservation of tryptophane to any appreciable extent. The choice of material is limited largely by other conditions, particularily solubility and surface area. Graphite or platinum might serve equally well if a cathode of sufficient area could be obtained, but these materials are not readily available in the desired form.

4. Protein:Acid Ratio

A third series of four experiments was conducted in which only the amount of protein was varied. From 25 g. to 200 g. of casein was used in 1500 cc. of 6 N sulphuric acid. The volume of acid per g. of casein was thus varied from 60 cc. to 7.5 cc. or, as it is commonly expressed, the protein:acid ratio ranged from 1:60 to 1:7.5, when the quantity of protein is expressed in grams and acid in cubic centimeters.

The results are reported in Table 1, and summarized graphically in Fig. 7.

TABLE I

Effect of Protein:Acid Ratio

Hydrolysis Time Hours	Tryptophane, Per Cent of Original			
	Ratio 1:60	Ratio 1:30	Ratio 1:15	Ratio 1:7.5
0.5	90.1	86.5	84.9	82.0
1.5	81.8	-	74.8	-
2.0	-	-	-	68.0
2.5	-	70.1	-	-
4.0	68.0	62.5	61.0	57.7
5.5	63.4	59.0	56.1	50.1
8.0	61.0	56.4	52.8	48.0
10.0	59.1	55.6	51.7	46.1

Tryptophane recovery is inversely related to protein:

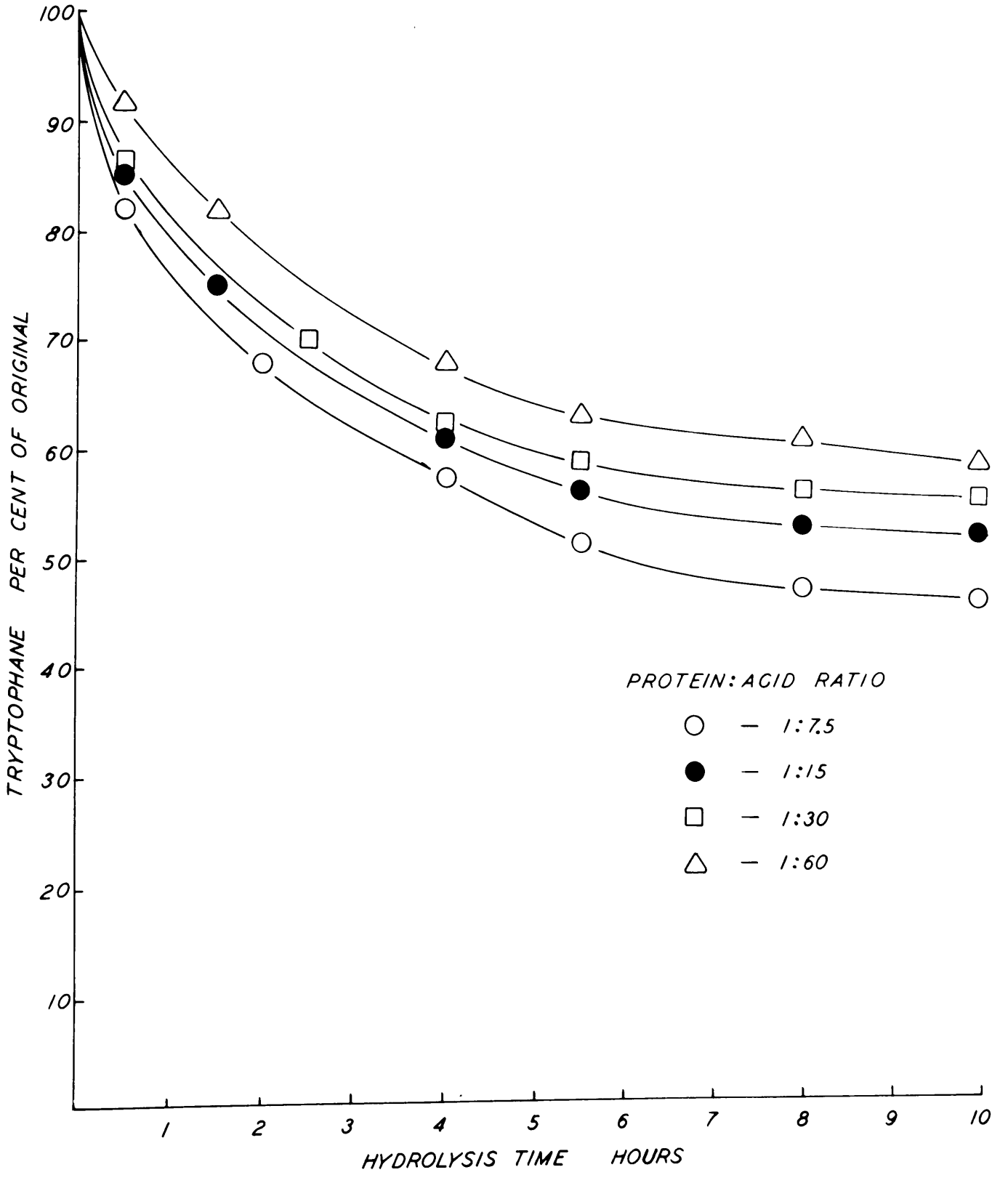

FIGURE 7

EFFECT OF PROTEIN:ACID RATIO

acid ratio. This would be expected from the fundamental concept of reaction velocities, based on the law of mass action.

Where A = agent responsible for tryptophane destruction

T = tryptophane

H = nascent hydrogen

the rate of destruction will be given by:

$$r_1 = k\,[A][T]$$

and the rate of reduction will be given by:

$$r_2 = k_1\,[A][H] = k_2\,[A] \text{ since } [H] \text{ is constant.}$$

When the concentration of protein is doubled, the concentrations of A and T are doubled and thus r_1 is increased to a greater extent than r_2.

5. Acid Concentration, Temperature, and other Variables

Acid concentration and temperature undoubtedly influence the rate of tryptophane destruction, but they also affect the rate of hydrolysis to such an extent that a small decrease in either greatly increases the time required for complete hydrolysis (103). In addition, high pressure apparatus would be required if temperatures above the normal boiling point were employed and it is only under these conditions that very dilute acids could be used effectively. As was mentioned earlier, it is difficult to employ electrolytic reduction at high pressures with laboratory apparatus. However, such a study should yield useful and interesting

information.

Throughout this study 6 N acid and boiling temperatures were employed since these conditions are frequently used in hydrolysis for analytical and other purposes.

Some other variables, such as efficiency of stirring and porosity of the porcelain anode compartment, will affect tryptophane recovery and humin formation but need little experimental evaluation. Stirring should be as vigorous as possible. The porcelain diaphram should be difficultly permeable to the amino acids but should not offer such a high resistance that it prevents adequate current flow. Size of anode is unimportant, again provided that it is not so small that it limits the current flow below the optimum level.

6. Choice of Acid

It is generally recognized that choice of acid is confined to hydrochloric acid or sulphuric acid if a high rate and degree of hydrolysis is to be attained.

Sulphuric acid was chosen in preference to hydrochloric acid because the latter possesses disadvantages as an electrolyte for electrolytic reduction under the required conditions. These disadvantages are listed below:

(i) The chlorides of most metals are fairly soluble and this creates difficulty in obtaining suitable electrodes.

(ii) The reaction between hydrogen and chlorine,

catalyzed by light, introduces a serious danger of explosion. This was particularly true in the preliminary experiments where the atmosphere above the hydrolysate would be a mixture of equal amounts of hydrogen and chlorine. This danger is eliminated if the two gases are evolved and maintained in separate compartments, but there can be no absolute assurance that small amounts of either do not pass through the diaphram.

(iii) Isolation of tryptophane by the commonly employed methods (30, 108) requires that the tryptophane be in a solution of sulphuric acid.

(iv) The removal of chloride ions from the final hydrolysate for analytical or other purposes is more difficult than the removal of sulphate ions. The removal of hydrochloric acid is usually accomplished by distillation, generally under reduced pressure. This would result in further destruction of tryptophane since electrolytic reduction could not be applied conveniently under reduced pressure.

SUMMARY

1. It has been established experimentally that the degree of tryptophane preservation and prevention of humin which can be obtained during the hydrolysis of casein by sulphuric acid with simultaneous electrolytic reduction is affected by the area of the cathode surface, the current density, and the ratio of protein to acid.

2. The optimum cathode size is 200 g. of lead in the form of lead wool. This quantity of lead wool has a calculated surface area of approximately 500 sq. cm. Such a cathode is considered to be optimum only for apparatus constructed according to the specifications previously described.

3. The optimum current density for the preservation of tryptophane and the prevention of humin formation by electrolytic reduction at a lead cathode is 0.8 amp. per sq. dm. of cathode surface in this apparatus.

4. Tryptophane recovery and humin prevention are inversely related to the protein:acid ratio. The destruction of tryptophane and the reduction of the agent or agents responsible are two competing reactions. Increasing the amount of protein increases the rate of tryptophane destruction to a greater extent than it increases the rate of reduction since the concentration of the reducing agent, nascent hydrogen, remains unchanged.

5. In view of the limited experimental evidence obtained, it is impossible to conclude that the composition of the cathode affects the degree of humin prevention and tryptophane preservation attainable. No difference in either was obtained through the use of lead, lead-mercury amalgan, and purified lead cathodes. The possibility remains that the use of other cathode materials, particularily those with lower hydrogen over-voltage, would yield different results.

6. Other variables likely to affect the efficiency of the process have been discussed and reasons given for the selection made in this study.

EXPERIMENTAL RESULTS

Part III

A Study of Conditions Required for the Maximum Tryptophane Preservation and Prevention of Humin Formation

INTRODUCTION

In the following experiments a critical study was made of the degree of tryptophane retention which could be attained by the use of electrolytic reduction.

The apparatus designed and developed in preceding experiments was used without further modification. Attempts were made to increase the degree of tryptophane retention to the maximum level possible. Emphasis was also placed on examination of the rate of tryptophane destruction and the rate of hydrolysis in an effort to obtain further information on the nature of substances responsible for destruction of tryptophane.

No modification was employed which would result in the presence of difficultly removable substances in the final hydrolysate, since such substances might prove undesirable whether the hydrolysate was used for analytical or other purposes. Modifications were also avoided which would decrease the rate of hydrolysis to such an extent that complete, or almost complete, hydrolysis could not be attained within one day.

EXPERIMENTAL

1. Control Experiment

This experiment was conducted in the same apparatus as electrolytic reduction experiments except that the anode and cathode were removed. The atmosphere above the hydrolysate was thus a mixture of air and water vapor, as is normally the case in simple refluxing.

Samples were withdrawn at intervals and analyzed for tryptophane and amino nitrogen. The percentage of tryptophane retained and the degree of hydrolysis obtained were calculated. The results are reported in Table II.

TABLE II

Control Experiment

Hydrolysis Time Hours	Tryptophane Per Cent of Original	Hydrolysis Per Cent of Total
1.0	64	25.7
1.5	57	58.3
2.5	48	74.6
4.0	40	85.1
5.5	28	92.2
7.5	21	96.2
10.0	15	98.1
13.0	9	99.2

It is apparent that a large fraction of the tryptophane,

almost 50 per cent, was destroyed in the first two hours of hydrolysis. The results, plotted graphically, Fig. 8, show a contrast between the curves for tryptophane preservation and hydrolysis, one being almost the reverse of the other. The degree of hydrolysis, however, does exceed the degree of tryptophane destruction. At first glance, the conclusion might be drawn that hydrolysis is the rate-controlling reaction and that destruction of tryptophane follows upon its liberation. Amino nitrogen values, however, give slight indication of the percentage of free amino acids and, unless tryptophane was liberated preferentially, most of it would still exist in peptide form at the end of two hours hydrolysis. From the data of Frost and Heinsen (50), it can be calculated that at 60 per cent hydrolysis, measured by amino nitrogen, approximately 28 per cent of the amino acids are completely liberated. In this experiment 45 per cent of the tryptophane has been destroyed at this stage. Since the indole nucleus is not involved in any known linkage of amino acids, and since the α carbon or imino group of the indole ring is considered the vulnerable point in the destruction of tryptophane (21, 74), there is strong likelihood that tryptophane can be destroyed while it is still an integral part of the protein molecule. The similarity in the shape of these two curves may be due to one or more of the factors enumerated below:

(i) The destruction of tryptophane may be due to some

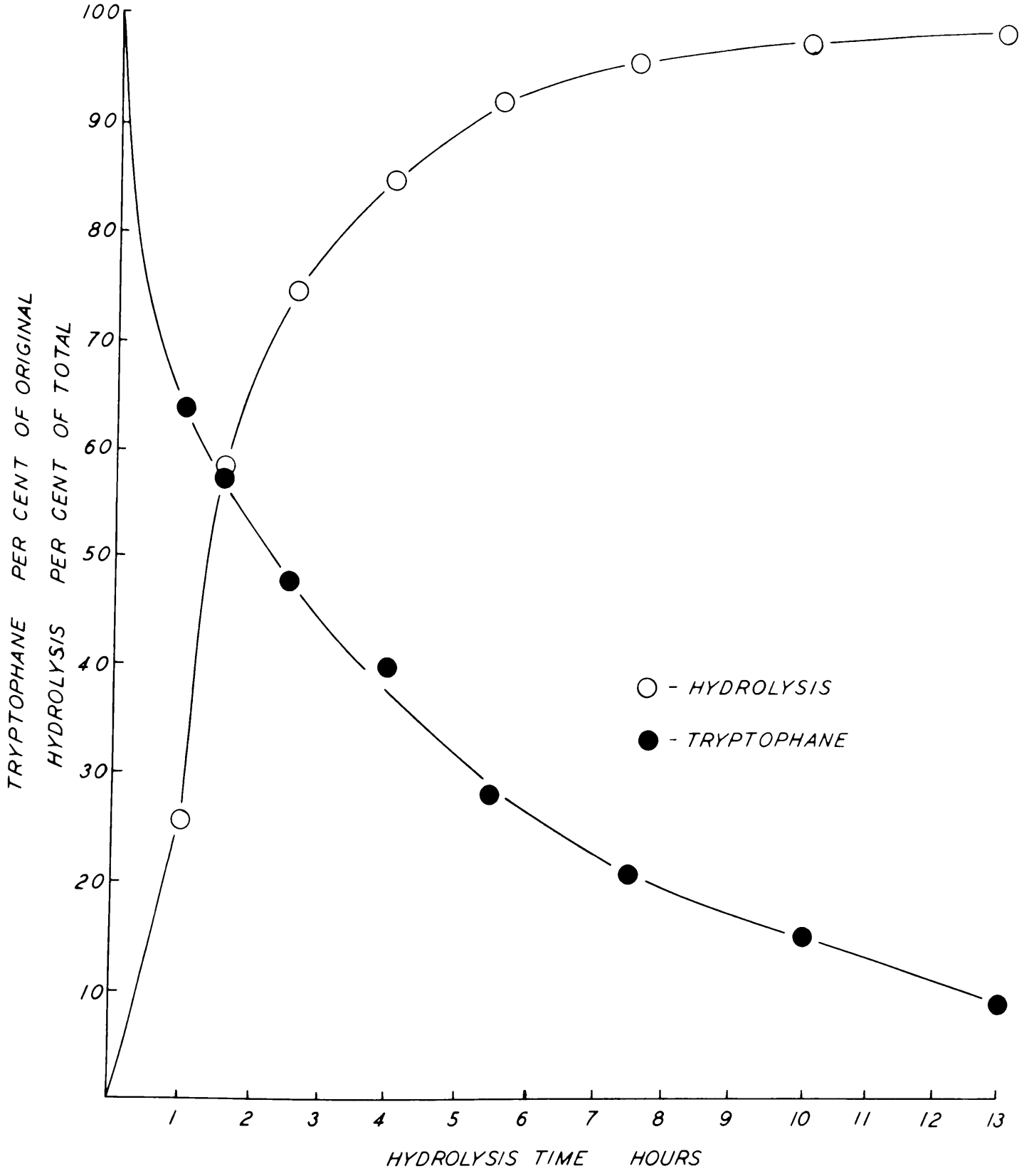

FIGURE 8

CONTROL EXPERIMENT

free impurity in the casein and its combination with the indole nucleus is favoured by the same conditions that favour the hydrolysis of the protein.

(ii) The agent responsible for tryptophane destruction may not be free, but may be readily liberated from the protein molecule by hydrolysis.

(iii) The destruction of tryptophane may be due to oxidation by atmospheric oxygen.

In all cases this type of curve would be expected, the rate naturally depending on the concentration of the reacting substances.

Spontaneous decomposition of tryptophane cannot be considered since pure tryptophane is relatively stable in sulphuric acid (75, 107). The possibility of oxygen being the sole agent responsible is extremely unlikely since Homer (75), and Lugg (89) took no precaution to exclude oxygen and yet found tryptophane fairly stable when refluxed with acid.

A considerable amount of insoluble humin was formed under the conditions existing in this experiment and the filtered hydrolysate was black in color as is normally expected after acid hydrolysis.

2. Electrolytic Reduction

The anode and cathode were replaced in the flask and the same reagents were employed as in the control experiment. Electrolytic reduction was provided by a current of 4 amp. at an E.M.F. of 12 volts.

The mixture of acid and casein turned light pink in color as boiling point was approached. This pink color darkened to a light brown during the first hour and then disappeared. Except for colloidal particles of fat, the hydrolysate was thereafter quite clear and transparent. If the colloidal fat was removed by filtration or ether extraction, the hydrolysate, when viewed in test tube quantity, contained barely perceptible traces of color and was difficult to distinguish from water on this basis. When viewed in larger quantities, some color was apparent, ranging from light yellow to a very light amber.

The degree of humin prevention obtained by this procedure was much superior to that obtained through the use of stannous chloride or urea. A photographic comparison of the humin content of several hydrolysates is presented in Fig. 9.

The degree of hydrolysis and the percentage of the original tryptophane were determined at intervals and the results are reported in Table III.

The electrolytic reduction resulted in the preservation of tryptophane to a considerable degree, 52 per cent of the tryptophane being present after fourteen hours of hydrolysis as compared with less than 9 per cent in the control experiment. While some destruction of tryptophane does take place, it occurs chiefly in the early stages of hydrolysis. Of the total 47.6 per cent destroyed, 35 per cent is lost in the first four hours and only 1.6 per cent in the last

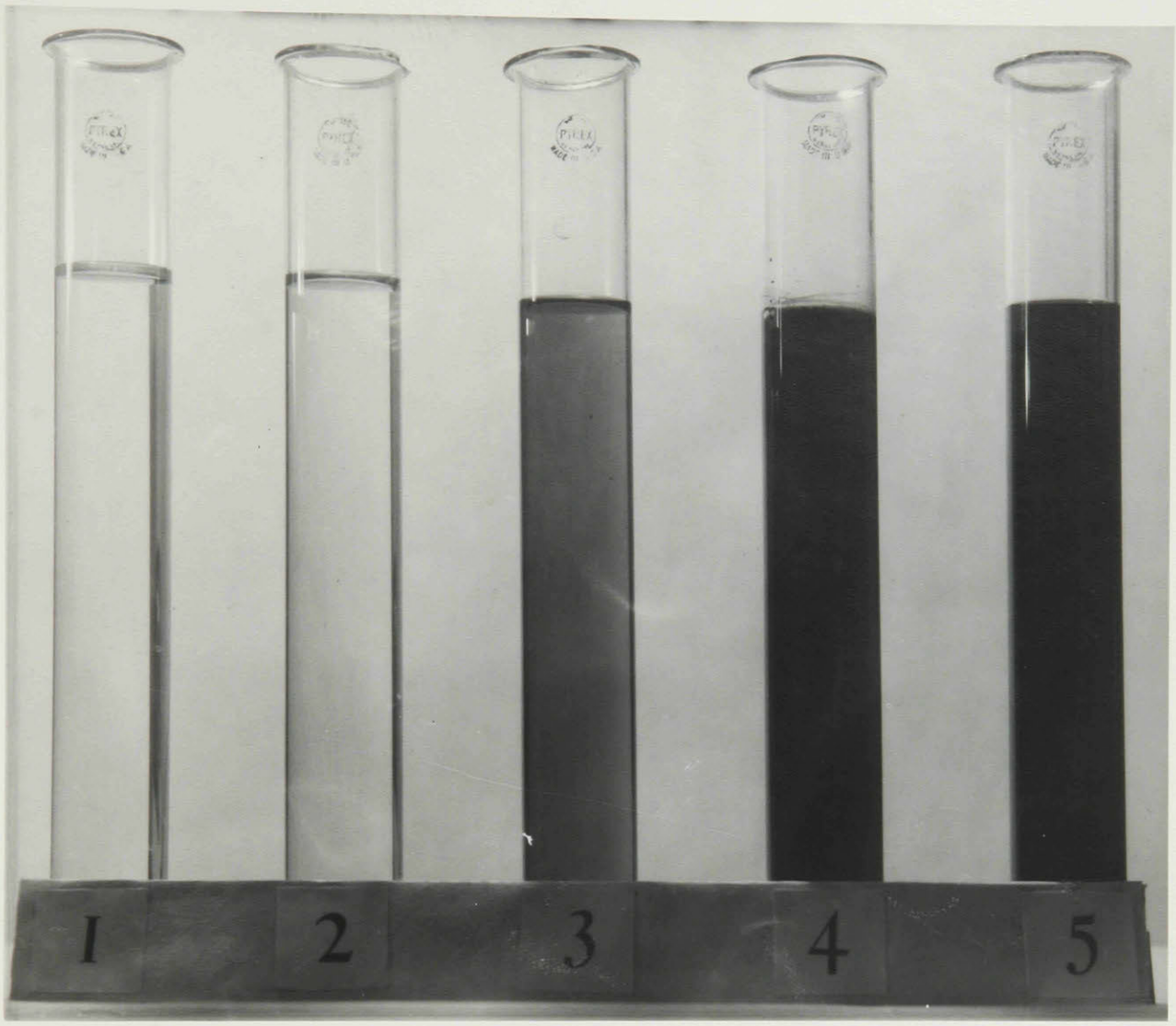

Fig. 9

1. Water.

2. Electrolytically reduced sulphuric acid hydrolysate.

3. Hydrochloric acid hydrolysate with equal amounts of casein and stannous chloride.

4. Sulphuric acid hydrolysate with equal amounts of casein and urea.

5. Control sulphuric acid hydrolysate.

NOTE: A protein:acid ratio of 1:15 in 6 N acid was used in all hydrolysates.

four hours. This levelling-off effect is well illustrated by the graph, Fig. 10.

TABLE III

Hydrolysis with Simultaneous Electrolytic Reduction

Hydrolysis Time Hours	Tryptophane Per Cent of Original	Hydrolysis Per Cent of Total
0.5	95.0	14.1
1.5	83.0	57.2
2.5	71.0	75.1
4.0	65.0	85.6
5.5	59.4	91.4
8.0	56.5	96.8
10.0	54.0	97.7
12.0	53.0	98.2
14.0	52.4	98.9

The rate of hydrolysis is not significantly affected by electrolytic reduction. This indicates that the action of nascent hydrogen differs from that of titanous chloride which is reported to increase the rate of hydrolysis, as measured by the rate of liberation of cystine (135).

Since the reaction is carried out in an atmosphere of hydrogen, none of the destruction can be attributed to atmospheric oxygen. Approximately one hour is required to heat the solution to boiling point. During this period sufficient hydrogen should be generated by the electrolysis to sweep all

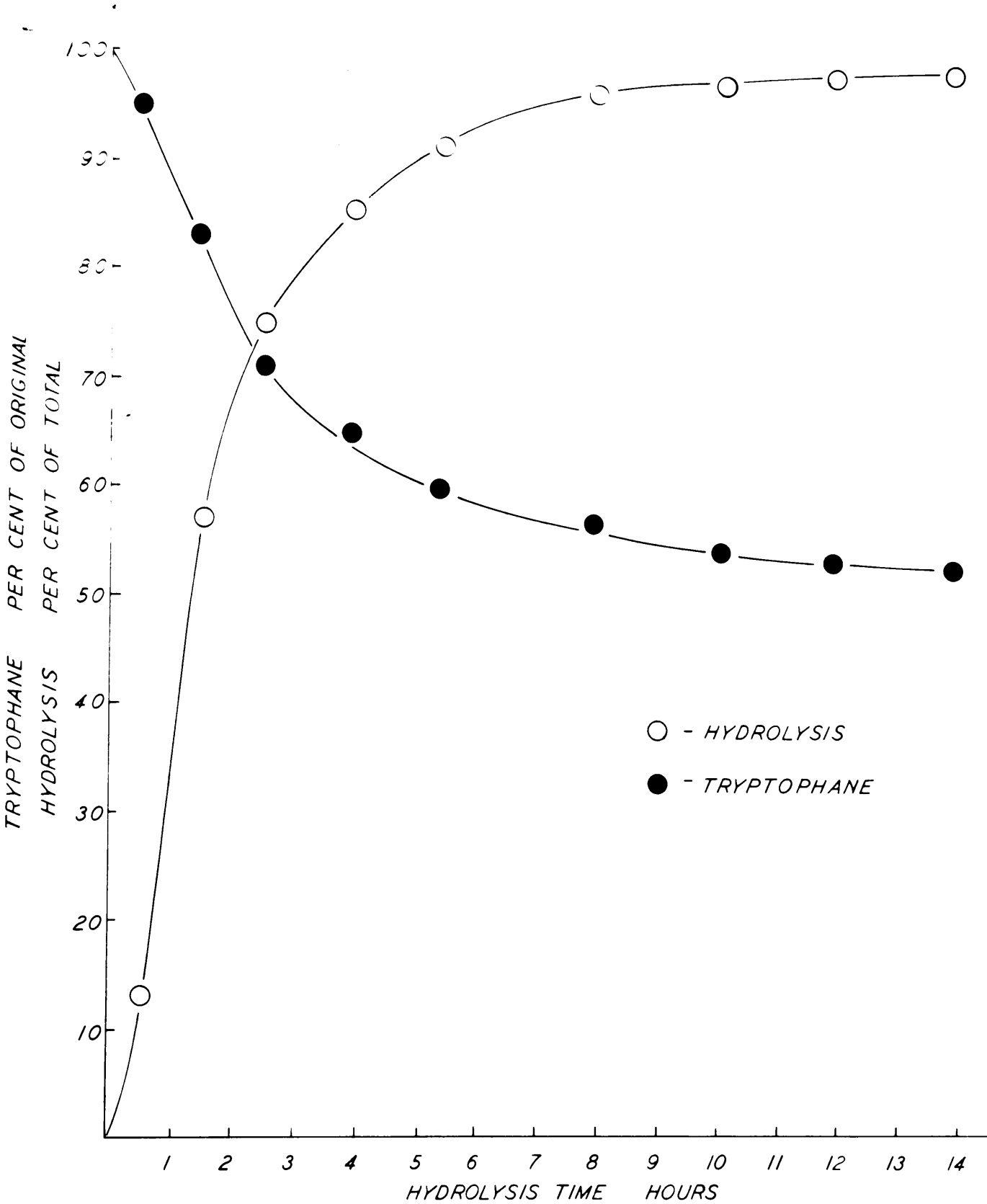

FIGURE 10

HYDROLYSIS OF CASEIN WITH SIMULTANEOUS ELECTROLYTIC REDUCTION

of the oxygen out of the flask.

Electrolytic reduction is not a rapid means of bringing about reduction, since it depends upon all of the material coming in contact with the cathode surface. For this reason, it is highly probable that part of the tryptophane destruction which does occur results from failure of the electrolytic reduction to dispose of the harmful agents rapidly enough to avoid some combination with tryptophane.

3. Preliminary Reduction in Weak Acid

This experiment was designed in an attempt to eliminate at least a part of the tryptophane destruction in the initial stages of hydrolysis. Instead of 1500 cc. of 6 N sulphuric acid, 1020 cc. of 0.5 N sulphuric acid was used at the beginning of the hydrolysis reaction. One hour after boiling point had been reached, sufficient 18 N sulphuric acid (about 485 cc.), was added to bring the acid strength up to 6 N. This strong acid was added dropwise, from a separatory funnel, Fig. 4, over a period of two hours. Electrolytic reduction was proceeding during the entire period of hydrolysis. The results of this experiment are given in Table IV.

As expected, this treatment did result in improved tryptophane recovery. The weak acid is less conducive to tryptophane destruction and apparently permits reduction of a larger fraction of the deleterious agents before they can react

TABLE IV

Preliminary Reduction for One Hour in 0.5 N Acid

Hydrolysis Time Hours	Tryptophane Per Cent of Original
3.25	90
5.25	81
7.50	73
9.25	71
11.50	70

with tryptophane. The addition of strong acid, however, is accompanied by destruction of tryptophane. Insufficient time was allowed for complete hydrolysis but it is apparent, from the way in which the tryptophane values level off, that a high degree of hydrolysis can be obtained with less destruction of tryptophane.

4. Prolonged Reduction in Weak Acid

The improvement obtained in the preceding experiment warranted additional study of the initial weak acid treatment. In this experiment conditions were similar to those of Experiment 3, except that the hydrolysis was allowed to proceed for five hours with 0.5 N acid, and the period over which the strong acid was added was also increased to five hours.

The results are reported in Table V, and the results

of the one hour and five hour weak acid treatments compared graphically in Fig. 11.

TABLE V

Preliminary Reduction for Five Hours in 0.5 N Acid

Hydrolysis Time Hours	Acid Normality	Tryptophane, Per Cent of Original	Hydrolysis, Per Cent of Total
2.0	0.5	99.0	10.7
4.0	0.5	97.0	16.8
5.0	0.5	96.4	18.8
7.0	1.2	95.1	24.9
9.5	2.2	84.5	44.3
11.5	5.1	76.0	69.5
13.0	6.0	74.1	85.6
14.5	6.0	73.2	92.7
16.0	6.0	72.7	97.6

While destruction of tryptophane was delayed, it was not eliminated, as is clearly shown in Fig. 11. The tryptophane recovery levels off at slightly over 70 per cent and the prolonged weak acid treatment appears to have only small advantage over the shorter period. Destructive agents appear to be released during hydrolysis and, consequently, this fraction of the tryptophane destruction cannot be completely avoided by prolonging the reduction in weak acid. The curves in Fig. 11 show how the period of rapid tryptophane

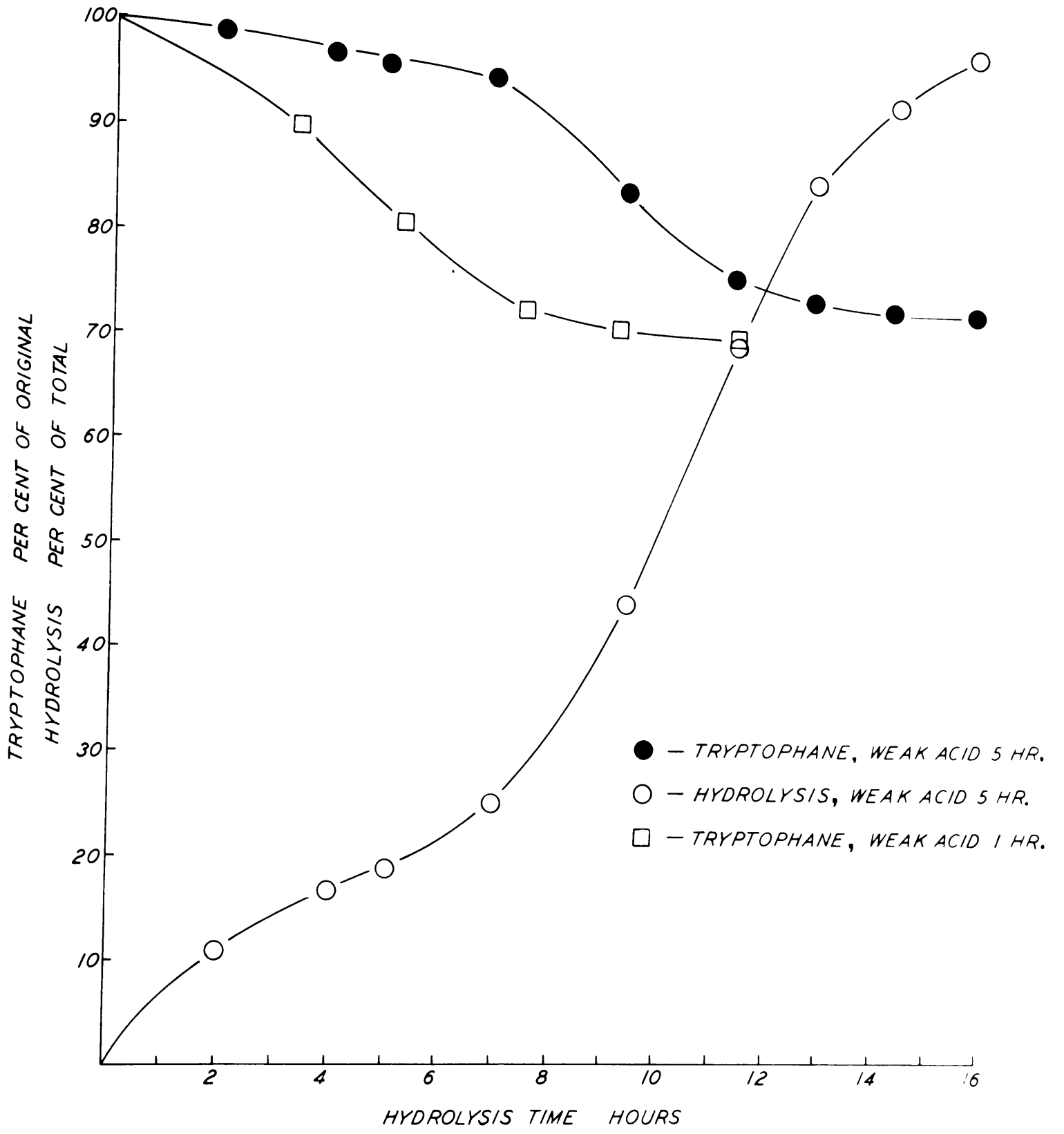

FIGURE 11

THE EFFECT OF A PRELIMINARY REDUCTION PERIOD IN 0.5N ACID

destruction coincides with a high rate of hydrolysis.

There is a possibility that the aldehydic agents assumed to be responsible for tryptophane destruction, require the action of relatively strong acid to convert them into a readily reducible form. For example, it might be that a sugar, hexose or pentose, is more susceptible to reduction after conversion to a furfural type compound by the action of stronger acid. However, Gunn (61) used electrolytic reduction to convert sugars directly to polyhydroxy alcohols in sulphuric acid solution, although Creighton (26) states that in sulphuric acid the products are pentose and formic acid.

5. Destruction of Tryptophane in Unhydrolyzed Protein

The association between rapid hydrolysis and rapid tryptophane destruction rendered it advisable to establish more definitely whether or not hydrolysis is prerequisite for the destruction of tryptophane. The following experiment was designed to obtain more information on this point.

Experiment 4, using a five-hour initial weak acid treatment, was repeated. The first sample was withdrawn at the end of the five-hour weak-acid treatment. The strong acid was then added slowly and additional samples of hydrolysate taken 1.25 and 2.50 hours later. These samples were large (100 cc. of hydrolysate) in order to permit the large number of analyses which had to be carried out. The tryptophane and total nitrogen content of each sample was established.

The amide nitrogen was removed, the residues made up to standard volume and tryptophane, total nitrogen, and amino-nitrogen analyses again conducted. Aliquots were then transferred to viscose casings and dialyzed in running tap water for sixty hours. At the end of this time no measurable amount of nitrogen escaped through the membrane when allowed to stand in a beaker of cold water for one hour. The dialysis was thus considered to have been exhaustive.

The non-dialyzable portion was made up to standard volume and total nitrogen, amino nitrogen, and tryptophane again determined.

From the results, the percentage of the original total nitrogen (exclusive of amide nitrogen), amino nitrogen and tryptophane in the non-dialyzable fraction was calculated, and also the ratio of tryptophane to total nitrogen in both dialyzable and non-dialyzable fractions. The results are shown in Table VI.

In the interpretation of these data, it is necessary that at least two assumptions be made:

(i) Assume that hydrolysis of the peptide bonds occurs purely at random, no particular amino acid being liberated more rapidly than others. If this is true, the composition of both dialyzable and non-dialyzable fractions could be the same as the hydrolysate before dialysis. Unfortunately, it is highly probable that this assumption is incorrect,

TABLE VI

Dialysis Experiment

Sample	Tryptophane (I) Per Cent of Original	Hydrolysis Per Cent of Total	Non-Dialyzable Fraction				Mg. Tryptophane per g. N (4)	
			Total (2) Nitrogen Per Cent	Amino (2) Nitrogen Per Cent	Trypto- (2) phane Per Cent	Amino (3) Nitrogen Per Cent Total N	Dialyzable Fraction	Non-Dialyzable Fraction
1	95	22.4	68.8	48.6	70.1	9.0	79.6	84.2
2	90	33.0	36.9	17.0	38.0	10.0	78.5	82.1
3	83	50.4	10.8	5.6	14.7	12.6	73.2	75.0

(1) Based on analyses before removal of amide N

(2) Percentage of the total found before dialysis but after removal of amide N

(3) Amino N as per cent of the total N in the non-dialyzable fraction

(4) Compare with the value of unhydrolyzed casein, 90 mg. of tryptophane per g. of nitrogen when allowance is made for 10.2 per cent amide N.

but little is known of the relative rates of liberation of various amino acids during acid hydrolysis. Despite this disadvantage, it is still possible that the tryptophane:total-nitrogen ratio in both fractions would be approximately the same if the nitrogen content of the more rapidly liberated components was approximately the same as that of casein.

(ii) Assume that the viscose dialysis casing separates the fractions on the basis of size only and does not exercise a selective action in favour of certain amino acids or peptides. This factor should be minimized by the extremely long dialysis period.

Admittedly, these assumptions make the interpretation less valid than is desirable, but disregarding the possible errors, the information provided by these data gives additional evidence on some points.

In all samples the percentage of the total nitrogen lost on dialysis is greater than the percentage of the total tryptophane lost, but the differences are not great. This indicates a slightly higher percentage of tryptophane in the non-dialyzable fraction.

The mg. of tryptophane per g. of nitrogen is consistently greater in the non-dialyzable fraction, indicating that destruction occurs more slowly in the larger fragments.

This ratio in both fractions is definitely lower than the ratio for unhydrolyzed casein. Consequently, tryptophane is destroyed before complete liberation. The lower rate in the larger fragments is probably due to such factors as steric hindrance and relatively slower mobility of these larger particles. The difference between the tryptophane: nitrogen ratio in the two fractions is lowest in Sample 3. As expected, the average peptide size in the non-dializable fraction is slightly smaller in this sample as shown by the higher value for amino nitrogen as a percentage of the total nitrogen.

The values for amino nitrogen, as per cent of the total nitrogen, in the non-dialyzable fraction show a small increase as hydrolysis proceeds. Apparently, the range in peptide size, within this fraction, becomes smaller, as would be expected. Since the variations in amino nitrogen as per cent of total nitrogen are not large in the three samples in this fraction, it appears that the viscose membrane is reasonably efficient in separating the peptides on the basis of size.

6. Hydrolysis of Purified Casein

A quantity of commercial casein was partially purified according to the procedure described in the section on Materials. A 100 g. sample was hydrolyzed in 1500 cc. of 6 N sulphuric acid under conditions identical to those of Experiment 2, where 100 g. of commercial casein was used.

Tryptophane recoveries are reported in Table VII and the results compared graphically with those of Experiment 2 in Fig. 12.

TABLE VII

Hydrolysis of Purified Casein

Hydrolysis Time Hours	Tryptophane, Per Cent of Original
1.0	97
2.0	95
3.0	87
4.5	75
6.0	70
8.0	68
10.0	67
12.0	67

The outstanding feature of these data is the slower rate of tryptophane destruction in the first two hours of hydrolysis. There is also a marked improvement in the final tryptophane recovery over that obtained with commercial casein under identical conditions.

The curve for purified casein in Fig. 12 shows a lag phase in the destruction of tryptophane. This is definite evidence that a large part of the early destruction of tryptophane, as found with commercial casein, is due to

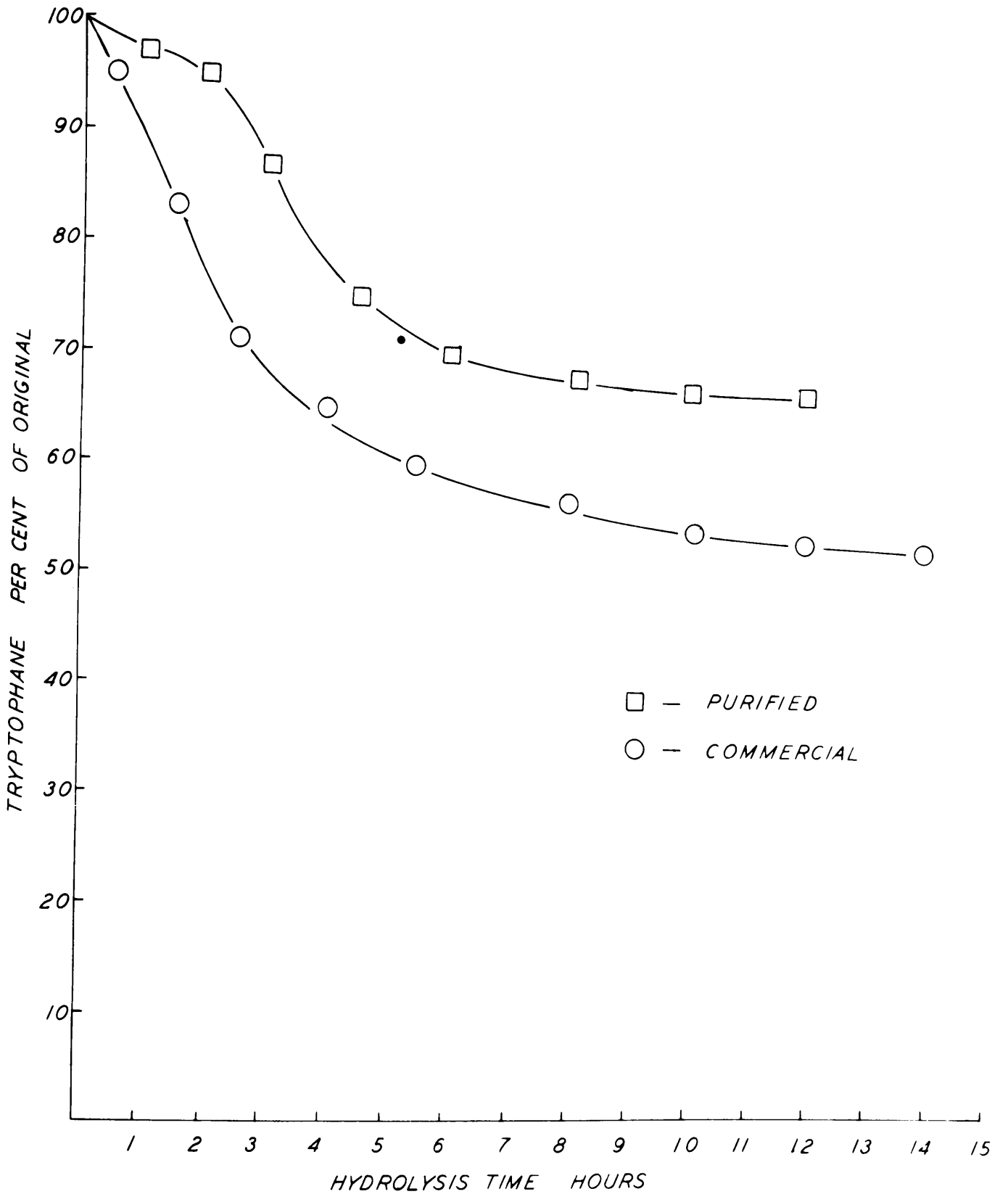

FIGURE 12

THE EFFECT OF PURIFICATION OF THE CASEIN

removable impurities. It is further evidence that the agents responsible for that destruction which cannot be eliminated by preliminary reduction in weak acid, are released from the protein molecule during hydrolysis.

It must be remembered that a high degree of purity was not obtained in the purification of this casein. Moisture, fat and ash-free casein has a nitrogen content of 15.5 per cent whereas the product obtained here contained 14.5 per cent nitrogen. Further purification might have extended the lag phase in tryptophane destruction.

7. Effect of Casein Purification and Preliminary Reduction in Weak Acid

The conditions employed in this experiment were similar to those employed in Experiment 3. The 100 g. of purified casein was hydrolyzed, with simultaneous electrolytic reduction, for two hours in 0.5 N sulphuric acid. Strong acid was then added to bring the concentration to 6 N. Results are reported in Table VIII.

These conditions produced the highest degree of tryptophane preservation obtained by any modification of conditions. Initial destruction of tryptophane was minimized by purification and the weak acid treatment. Destruction in the later stages was also reduced by maintaining a slow rate of hydrolysis during this critical period.

The results of this entire series of experiments are

TABLE VIII

Hydrolysis of Purified Casein with Preliminary Reduction in 0.5 N Acid

Hydrolysis Time Hours	Tryptophane Per Cent of Original
2	98.7
4	95.0
6	84.2
8	81.1
10	79.8
12	78.8
14	78.2

summarized and compared in a composite graph, Fig. 13.

From this and preceding experiments considerable evidence has been gathered in support of a theory that tryptophane destruction is due to two general types of agent. One is a free impurity or readily-hydrolyzable component of the protein which is responsible for rapid destruction in the early stage of hydrolysis. The second type appears to be an integral part of the protein molecule and its linkage is hydrolyzed with difficulty.

The first type might consist of agents such as furfuraldehyde or a derivative formed by the action of the acid on carbohydrate. Some carbohydrate is undoubtedly present in the free state in commercial casein but there is

the possibility that some sugar residue, such as a pentose from the paranucleic acid reported in casein (34, 73), may be present. This type of impurity is probably responsible for the preliminary browning during the hydrolysis of casein with 6 N sulphuric acid as encountered in Experiment 2, and associated with a rapid loss of tryptophane.

In view of the recent findings of Olcott and Fraenkel-Conrat (107), the second type may be an amino acid. As mentioned elsewhere, these workers found that cystine and serine cause destruction of tryptophane when either is heated with tryptophane in 6 N sulphuric acid. The destruction due to these amino acids would likely follow their liberation. It is also noteworthy that these authors found that the destruction of tryptophane by cystine was not accompanied by humin formation. The destruction accompanying the addition of strong acid, after preliminary reduction in weak acid, in the foregoing experiments also takes place without humin formation or darkening of the solution to any significant degree.

Other workers (29, 33, 70, 87) have also suspected that the agents responsible for tryptophane destruction are (at least in part) liberated during hydrolysis. Further evidence of this is apparent in the fact that loss of tryptophane occurs even during the hydrolysis of crystalline proteins (49, 107).

The possibility cannot be overlooked, however, that the destruction of tryptophane accompanying the addition of strong

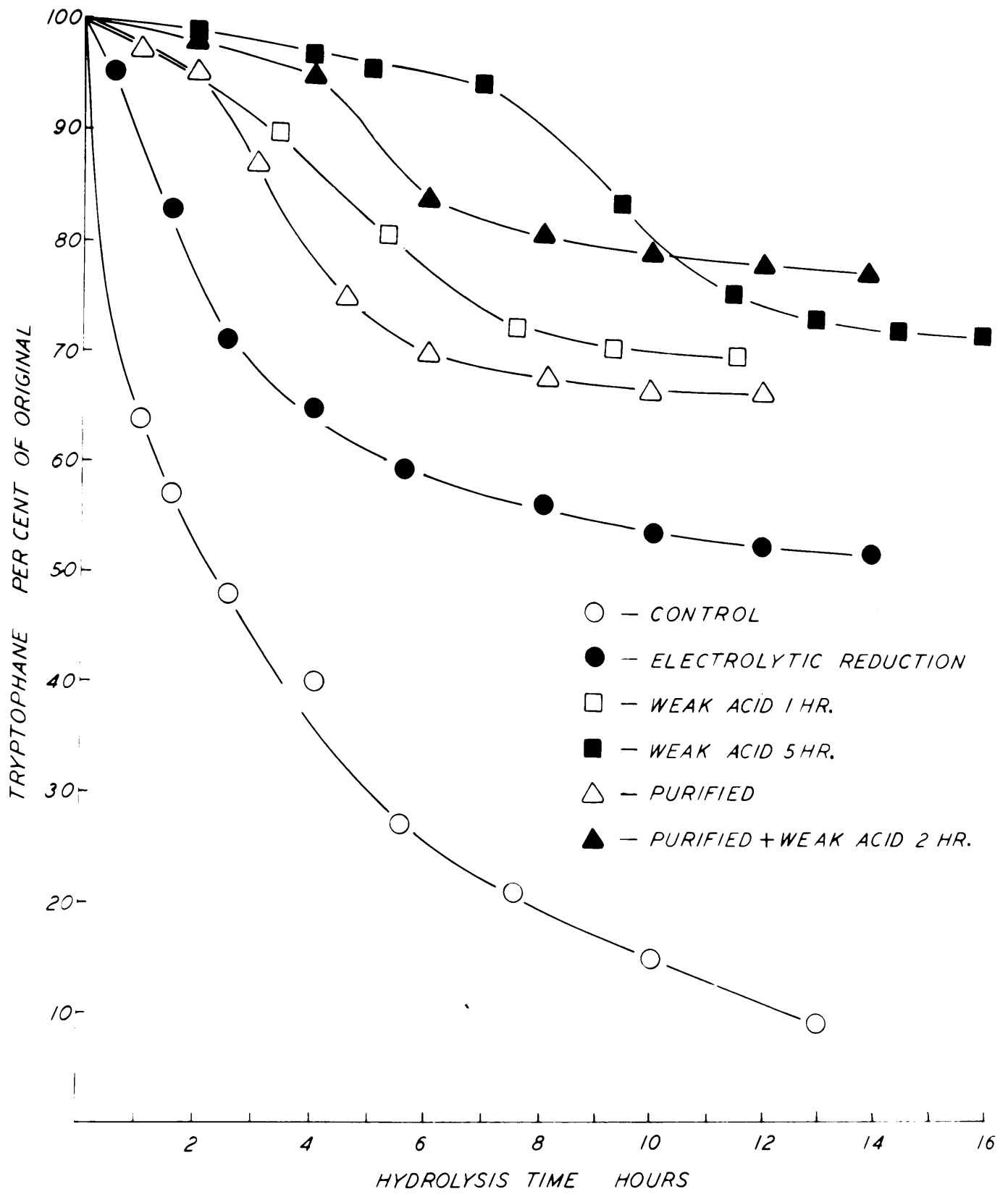

FIGURE 13

COMPOSITE GRAPH

acid, may be due to the further alteration or breakdown of impurities which were rendered harmless in the preliminary reduction period. Since such reactions generally require oxidation, this should be minimized here. Also, if this was the case, one would expect more improvement to result from purification of the casein.

8. Effect of Added Phosphoric Acid

Phosphoric acid is sometimes used as a film coating on the inside of atomic hydrogen generators of the discharge tube type (131). It has the property of retarding the recombination of hydrogen atoms to produce molecular hydrogen. In this experiment it was hoped that it would exercise a similar effect at the cathode surface, thus increasing the efficiency of the reduction.

The conditions employed in this experiment were similar to those of Experiment 3. A 100 g. sample of commercial casein and 1020 cc. of 0.5 N sulphuric acid, also containing 100 cc. of 85 per cent phosphoric acid, were used as a starting mixture. After two hours of hydrolysis, the strong sulphuric acid was added slowly in the next two hour period.

The results are shown graphically in Fig. 14. Destruction of tryptophane occurs to a slightly greater extent than it does in the absence of phosphoric acid. The destruction follows a curve similar to that obtained in other experiments.

9. Effect of Added Toluene

The object of this experiment was to add an immiscible solvent to the hydrolysate in expectation that some of the aldehydic compounds, formed by the action of the acid on carbohydrate, would be more soluble in the organic solvent. This might retard the rate at which they combine with tryptophane and result in improved recovery.

The solubility of furfural in 6 N. sulphuric acid was compared with its solubility in several organic solvents. Toluene was selected on the basis of its high boiling point, immiscibility with water, and its ability to dissolve furfural.

An experiment was conducted employing conditions identical to those of Experiment 8, except that phosphoric acid was omitted and 50 cc. of toluene added instead. The toluene formed an emulsion with the other components, due to the vigorous stirring, but separated readily on standing.

This treatment did not result in improved tryptophane recovery. The results are compared graphically with those of Experiment 8 in Fig. 14. Further investigation of the use of immiscible solvents appeared unwarranted.

One interesting point arises from the comparison of these data in Fig. 14. For reasons unknown, the curves for hydrolysis do not coincide. Hydrolysis proceeds more rapidly in the presence of toluene during the first five hours and less rapidly thereafter, the two curves crossing at this point.

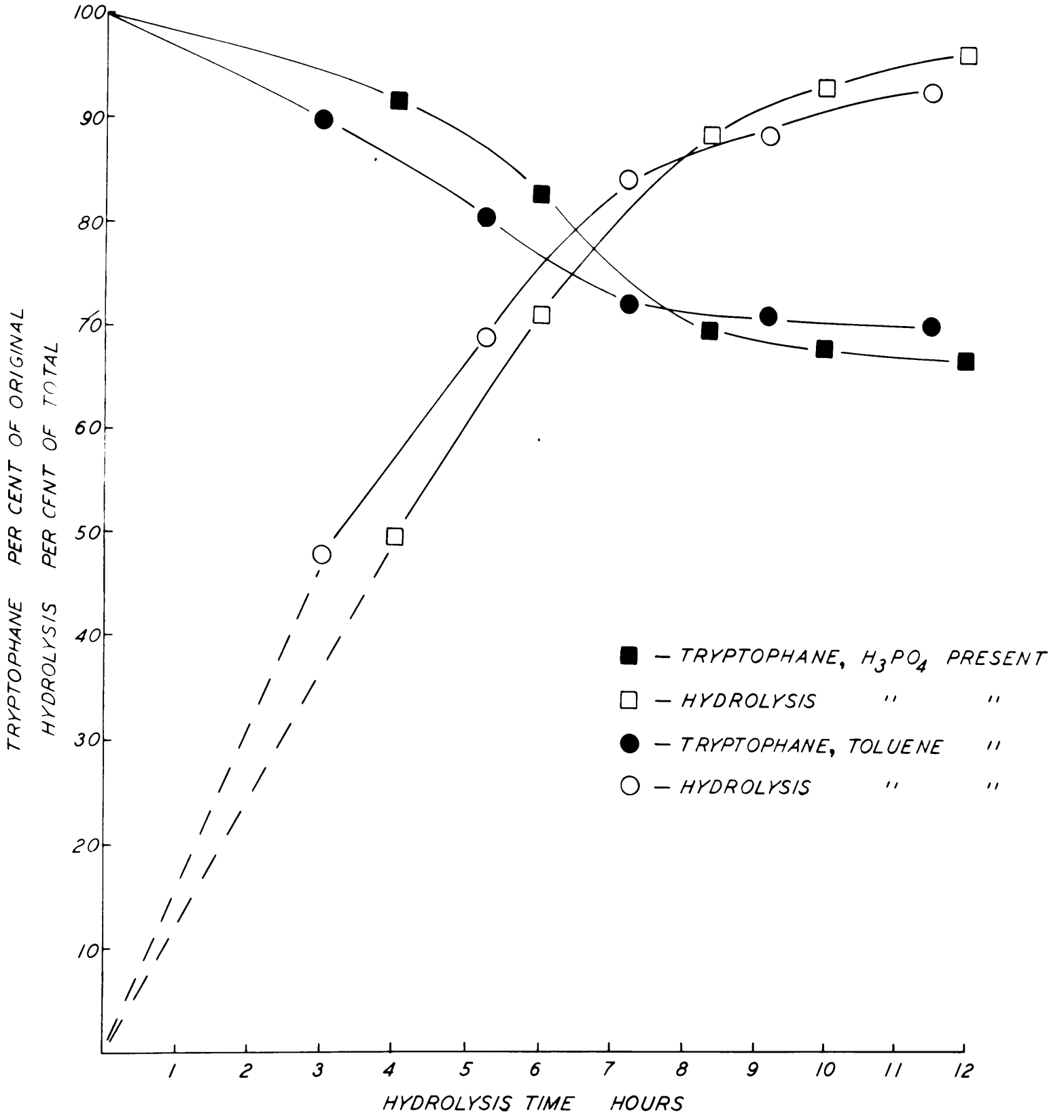

FIGURE 14

THE EFFECT OF THE ADDITION OF PHOSPHORIC ACID OR TOLUENE

The curves for tryptophane preservation cross similarily, and at approximately corresponding points. In view of information obtained in earlier experiments, the relationship found here between hydrolysis and the destruction of tryptophane appears more than coincidental.

10. Effect of Ether Extraction of Casein

The presence of colloidal fat particles in the hydrolysate was mentioned previously. The possibility that the presence of fat, or some decomposition product resulting therefrom, might be instrumental in tryptophane destruction could not be overlooked. To investigate this, a quantity of commercial casein was extracted with low-boiling petroleum ether for ninety hours.

This product produced a hydrolysate of excellent color and free from colloidal fat particles. Apart from this, no other improvement was obtained. Tryptophane recoveries were identical to those obtained with commercial casein.

11. Hydrolysis of Blood Fibrin

This protein was used to test the efficiency of the procedure on a protein other than casein. A 100 g. sample of fairly pure beef-fibrin was used. The original intention was to use 1020 cc. of 0.5 N sulphuric acid in the initial period but this was found unsuitable. The fibrin formed a gel in this quantity of 0.5 N acid which was resistant to stirring and, consequently, resulted in inefficient reduction. To

overcome this, sufficient strong acid was added, at the time of gel formation, to bring the acid strength to 2.0 N. This hastened hydrolysis and consequent solution of the protein. It also lowered the protein:acid ratio which helped to prevent gel formation. After two hours hydrolysis and reduction in 2.0 N acid, the remainder of the 18 N acid was added slowly to bring the normality to 6.0. Unfortunately, this change in conditions does not facilitate comparison of the results with those obtained with casein. The original plan could have been adhered to if a smaller amount of fibrin had been used, but this again would have prevented rigid comparison with casein hydrolysis.

No insoluble humin formed in this experiment but the hydrolysate was not as free from color as a casein hydrolysate. The color of the first sample withdrawn was a light reddish-brown and the intensity of this color did not change throughout the entire course of hydrolysis. It may be possible that the fibrin contained coloring substances not arising from the formation of soluble humin.

The analytical results are presented in Table IX and Fig. 15. It will be noticed that 72.3 per cent of the original tryptophane still remained after 12.5 hours hydrolysis, and the attainment of 99.2 per cent complete hydrolysis. The utility of the procedure is thus not confined to casein.

TABLE IX

Hydrolysis of Blood Fibrin

Hydrolysis Time Hours	Tryptophane, Per Cent of Original	Hydrolysis Per Cent of Total
1.5	95.4	23.8
3.0	90.3	51.5
4.5	-	69.5
6.5	80.7	82.9
8.5	76.7	92.0
10.5	73.6	96.7
12.5	72.3	99.2

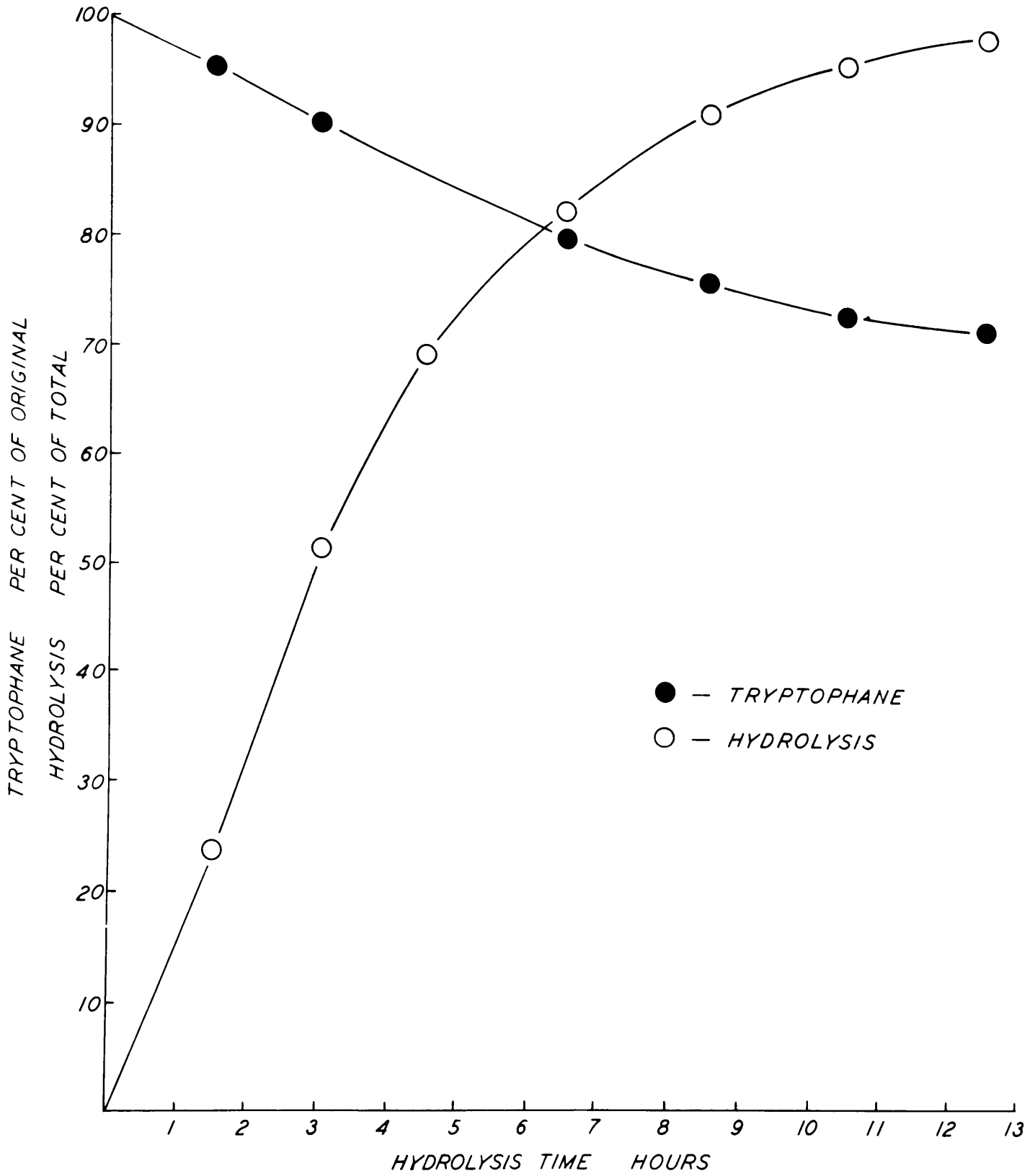

FIGURE 15

HYDROLYSIS OF FIBRIN WITH SIMULTANEOUS ELECTROLYTIC REDUCTION

SUMMARY

1. Under ordinary conditions of refluxing with 6 N sulphuric acid, only 10 per cent of the original tryptophane in commercial casein remains after thirteen hours hydrolysis and the attainment of 99 per cent complete hydrolysis. Some insoluble humin and large amounts of soluble humin are formed.

2. When simultaneous electrolytic reduction is supplied 52 per cent of the original tryptophane remains in the final hydrolysate after fourteen hours hydrolysis, under comparable conditions, and the attainment of 99 per cent complete hydrolysis.

3. Approximately 70 per cent of the tryptophane originally present in commercial casein can be preserved during hydrolysis by sulphuric acid with simultaneous electrolytic reduction if the hydrolysis is started with 0.5 N acid and allowed a preliminary reduction period of one hour or more before the gradual addition of strong acid during the second and third hours. Prolonging the reduction period in weak acid to five hours resulted in a further small improvement.

4. Some of the agents responsible for tryptophane destruction can be removed by purification of the casein. Partially-purified casein, after hydrolysis by 6 N sulphuric acid with simultaneous electrolytic reduction, contained

67 per cent of the original tryptophane. Commercial casein hydrolyzed under identical conditions contained 52 per cent.

5. When purified casein is used in conjunction with preliminary reduction in weak acid, the final hydrolysate contains approximately 78 per cent of the tryptophane originally present. The comparable value obtained with commercial casein is 70 per cent.

6. The formation of insoluble humin during the acid hydrolysis of casein can be entirely prevented by simultaneous electrolytic reduction. The formation of soluble humin is reduced to negligible proportions, the hydrolysate containing barely perceptible traces of color.

7. The degree of humin prevention obtained by this procedure with casein, is much superior to that obtained through the use of stannous chloride or urea when these compounds are used in amounts equal to the weight of casein.

8. The rate of hydrolysis is not appreciably affected by simultaneous electrolytic reduction.

9. Evidence has been presented showing that tryptophane can be destroyed while linked in relatively large peptides but the rate of destruction is not as rapid as when the tryptophane is free or in smaller peptides.

10. The results indicate that the destruction of tryptophane is due to two general types of agent, one a free impurity or readily-hydrolyzable component and the other a difficultly-hydrolyzable component of the protein which is liberated under approximately the same conditions as the amino acids and hence may be an amino acid.

11. Tryptophane preservation and humin prevention, by the procedure developed, can be attained in proteins other than casein. Up to 72 per cent of the tryptophane originally present in blood fibrin remains in the hydrolysate after attainment of over 99 per cent complete hydrolysis.

12. Fat as it occurs in casein, is not responsible for the destruction of tryptophane under the conditions employed.

13. The addition of phosphoric acid or toluene does not increase the degree of humin prevention or tryptophane preservation.

EXPERIMENTAL RESULTS

Part IV

General Experiments on Hydrolysates

INTRODUCTION

The development of a modified procedure for the hydrolysis of proteins introduces many problems, particularily since protein is such a complex biological substance. One of the greatest dangers lies in the possible effect of the procedure on each of the amino acids present. In this study it was not found possible to investigate the fate of each amino acid without neglecting the original objective, to preserve tryptophane, but whenever possible, some attempt was made to obtain further information in this respect.

The following section is devoted to the investigation of various features of these humin-free tryptophane-containing acid hydrolysates of casein.

EXPERIMENTAL

1. Isolation and Identification of Tryptophane

Claims for the preservation of tryptophane in acid hydrolysates require substantiation in addition to quantitative determination by colorimetric methods, since most color reactions are non-specific. The following isolation experiment was carried out to verify the presence of tryptophane in the acid hydrolysate.

A total of 350 g. of commercial casein was hydrolyzed in four lots of approximately 90 g. each. A two-hour initial reduction period in 0.5 N sulphuric acid was used before increasing the acid strength to 6 N to achieve complete hydrolysis. A fourteen-hour hydrolysis period was used.

The hot acid hydrolysate was siphoned directly into a sufficient volume of an ice and water mixture to give a final solution containing 7 per cent sulphuric acid. Cooling the hydrolysate to a low temperature with ice had two advantages:

(i) It minimized loss of tryptophane which would probably occur upon exposure of the hot hydrolysate to atmospheric oxygen.

(ii) It resulted in the formation of a lighter-colored precipitate when the mercuric sulphate was added to this solution.

The procedure followed from this point on was a combination of some features of the Onslow (108) and Dakin (30) methods. A brief outline of the procedure is given

below:

> After reducing the concentration of sulphuric acid in the hydrolysate to 7 per cent, a solution of mercuric sulphate in 7 per cent sulphuric acid is added to the extent of 30 g. of mercuric sulphate for each 100 g. of casein used. After standing for twenty-four hours or more in the refrigerator, the supernatant liquid is siphoned off and the residual precipitate collected by filtration with suction. The precipitate is washed five times with 1.5 per cent mercuric sulphate in 7 per cent sulphuric acid and then twice with water. The precipitate is then suspended in distilled water and barium hydroxide added until the solution is permanently alkaline to phenolphthalein. The suspension is mechanically stirred and a stream of hydrogen sulphide passed in until the mercury is completely precipitated. After filtering off the mercury sulphide, the barium hydroxide is removed with sulphuric acid by careful adjustment to the precipitation end point. The filtrate, after removal of the barium sulphate, is concentrated under reduced pressure to approximately 100 cc. This concentrate is then extracted with n-butyl alcohol in a continuous liquid-liquid extractor under reduced pressure for thirty-six hours. The water is removed from the butyl alcohol extract by repeated vacuum distillations, followed by additions of pure butyl alcohol, until no further tryptophane is obtained. The crude product is crystallized from 60 per cent ethyl alcohol.

Some difficulties were encountered when the procedure was applied to the acid hydrolysate. One of the most serious was the rapid darkening of the solution when the mercuric sulphate was added. This was probably due to the mercuric sulphate acting as an oxidizing agent when placed in the highly reduced medium. If the temperature of the hydrolysate was very low 1° to 10°C., the amount of colored compound formed was considerably less than if the solution

was warm. In the latter case the hydrolysate became an intense reddish-brown in a few seconds.

A considerable amount of this colored material was present in the precipitated mercury-tryptophane complex. Following decomposition of the mercury salt by hydrogen sulphide and filtering, most of the colored material was present in the filtrate. It was also soluble in butyl alcohol and was extracted along with the tryptophane. This colored substance was not readily removed by decolorizing agents and a heavy application of Norit A was necessary to produce a reasonably clear solution. This was particularily unfortunate since tryptophane is strongly adsorbed on activated carbon. Sahyun (122) used this property as a means of separating tryptophane from other amino acids in enzyme digests.

A second problem was encountered due to the co-precipitation of a large amount of cysteine or cystine by the mercuric sulphate. No investigator has reported such difficulty in the normal isolation of tryptophane from enzymatic digests. Onslow (108) examined the precipitate produced by mercuric sulphate in enzymatic hydrolysates of caseinogen and found that several amino acids were precipitated in addition to tryptophane. He crystallized histidine, aspartic acid, glutamic acid, leucine and tyrosine and suspected the presence of cystine since the precipitate gave a faint test for sulphur.

Under conditions of electrolytic reduction, cystine

will be reduced to cysteine and as such must be more readily precipitated by mercuric sulphate. After decomposing the mercury precipitate with hydrogen sulphide, and concentrating the filtrate to a small volume, approximately 0.8 g. of amorphous grey precipitate separated out. This precipitate gave strongly positive nitroprusside and sulphur tests. When this precipitate was dissolved in dilute hydrochloric acid and adjusted to pH 4.8 with sodium acetate, 0.32 g. of cystine was obtained. This was identified by melting point, 252°C., and by the characteristic hexagonal plate-like crystals when viewed under the microscope. Since this yield accounted for less than half of the total precipitate, it was concluded that some cysteine was also present. It is highly probable that the compound precipitated by the mercuric sulphate was cysteine and in subsequent manipulations part of the cysteine was oxidized to cystine. Andrews and Wyman (2) found that mercuric sulphate will form a precipitate when added to cystine in sulphuric acid solution, but examination of the precipitate revealed that it was a mercury-di-cysteine compound, a mercury atom being attached to the sulphur atoms of two cysteine residues. It thus seems reasonable to conclude that cysteine would be readily precipitated from the hydrolysate by mercuric sulphate.

After the removal of as much of this sulphur-containing amino acid as possible by aeration to convert the cysteine to

cystine, concentrating the solution, and chilling in the refrigerator, the tryptophane concentrate was extracted with butyl alcohol. Crude tryptophane recovered from the butyl alcohol extract amounted to 3.41 g. This represents 0.98 per cent of the original casein and a higher yield than could be expected on the basis of quantitative determination. This discrepancy arises from the impurities present in this crude product.

The crude product was dissolved in 100 cc. of hot 60 per cent ethyl alcohol and decolorized by boiling with 1.0 g. of Norit A. This undoubtedly resulted in some loss of tryptophane but was essential to remove the coloring material mentioned earlier. The first crop of crystals was very small and contained some cystine and a minute quantity of another amino acid which was later identified as aspartic acid.

Additional crops of crystals were obtained by successive concentration, adjustment of alcohol percentage, and chilling. A total of 1.73 g. of crystalline tryptophane was obtained, representing 0.49 per cent of the original casein. The mother liquor became viscous and gum-like when concentrated further and undoubtedly contained additional tryptophane which could not be recovered. The yield was also reduced by unavoidable losses during the many manipulations. Such losses can seriously decrease the yield when the total quantity involved is small.

The product of the first crystallization was re-crystal-

lized four times to give a product of high purity. The final yield was then approximately 0.8 g.

The melting point of this product was found to be 262°C. in an open capillary tube. The melting point of Eastman Kodak l-tryptophane under the same conditions was 263-264°C. Mixed melting point of the two samples was 262-263°C. Considerable darkening of the crystals occurred with both samples as the melting point was approached, and decomposition accompanied melting. These melting points are considerably lower than those reported in the literature. Melting points reported for l-tryptophane range from 278° (96) to 295° (23). Dunn and Brophy (37) show how widely differing melting points of amino acids can be obtained under different conditions and found that special apparatus and carefully standardized conditions are necessary to obtain reliable melting points. Schmidt (126) indicates that the melting point is an unreliable criterion for establishing the purity or identity of amino acids.

Proximate analyses were carried out on the purified tryptophane and the results are reported in Table X.

These results prove beyond doubt that tryptophane has been obtained from an acid hydrolysate of casein. The values for hydrogen are slightly high, probably attributable to traces of water, although the tryptophane was dried in vacuo at 70°C. for 48 hours and stored over phosphorus pentoxide for two weeks prior to analysis. The high hydrogen value

cannot be attributed to reduction since even one additional atom of hydrogen would have increased the value by 0.5 and lowered the others by the same amount.

TABLE X

Proximate Analyses of Tryptophane

Element	Found Per Cent	Theoretical Per Cent
Carbon (1)	64.77	64.69
	64.69	
Hydrogen (1)	6.05	5.93
	6.10	
Nitrogen	13.70	13.72
	13.80	

(1) Carbon and hydrogen analyses by courtesy of Dr. A. E. Ledingham, Dominion Rubber Company Limited, Guelph, Ontario.

Optical rotation measurement showed that no racemization had occurred. The specific rotation, $[\alpha]_D^{26} = -32.1^\circ$ in water.

Other values for l-tryptophane, reported in the literature, for a water solution and using sodium light are: -31.5° at 20 C. (39), -32.1° at 20°C. (8), and -33.20° at 25°C. (32).

2. Comparison of Methods for Tryptophane Determination

Of the many methods available for the determination of tryptophane, Block and Bolling (13) recommend four as most likely to give reliable results. Two of these are the Shaw and McFarlane method (loc. cit.) and the Block and Bolling modification of the Folin-Ciocalteu method (101). The Folin and Ciocalteu method (45) was used by White and Sayers (loc. cit.) in their study of the destruction of tryptophane during acid hydrolysis, and they obtained considerably higher tryptophane retention by simple refluxing than was obtained in the control experiment in the present study. It was, therefore, considered advisable to compare the tryptophane recoveries obtainable by these two methods. The Block and Bolling modification of the latter method was used here because it is adapted for photoelectric colorimetry whereas the original procedure employs visual colorimetry.

The two methods were first applied on casein which had not been hydrolyzed with acid. Two samples of casein were used, commercial and purified, as described in the section on Methods.

For the Block and Bolling method, the samples were hydrolyzed for five hours with 20 per cent sodium hydroxide and for the Shaw and McFarlane method they were heated for twenty minutes in 20 per cent sodium hydroxide as the respective methods require. The Shaw and McFarlane method,

however, was also applied to the samples hydrolyzed for five hours in alkali to test for losses during the hydrolysis. The results are reported in Table XI.

TABLE XI

Comparison of Methods for the Determination of Tryptophane in Casein

Method	Sample	
	Commercial	Purified
Block and Bolling	1.07	1.14
Shaw and McFarlane	1.06	1.20
Shaw and McFarlane (five-hour alkali hydrolysate)	0.95	0.99

The results obtained with the two methods agree reasonably well when applied as the authors recommend, but serious losses are indicated by the Shaw and McFarlane method during five-hours hydrolysis with 20 per cent sodium hydroxide. Shaw and McFarlane, during the development of their method, encountered similar losses following hydrolysis with 20 per cent sodium hydroxide.

The following experiment was conducted in order to compare the tryptophane recoveries obtained by these two methods in an acid hydrolysate produced under conditions of electrolytic reduction. A 100 g. sample of commercial casein was hydrolyzed with 1500 cc. of 5.0 N sulphuric acid with simultaneous electrolytic reduction. The main object was to obtain

a series of samples showing varying degrees of tryptophane destruction. In order to make this destruction more gradual 5.0 N acid was used.

The analyses were carried out as described in the section on Methods and the results are reported in Table XII and compared graphically in Fig. 16.

TABLE XII

Comparison of Methods on Acid Hydrolysates

Hydrolysis Time Hours	Tryptophane, Per Cent of Original		Difference Units Per Cent
	Shaw & McFarlane Method	Block & Bolling Method	
1.0	84.5	97.2	12.7
2.0	77.7	93.5	15.8
3.5	72.7	91.6	18.9
5.5	69.5	87.5	18.0
8.0	63.7	84.5	20.8
12.0	62.3	81.0	18.7
15.0	61.0	79.2	18.2

The results with the Block and Bolling method are consistently higher. Fig. 16 illustrates that the rapid destruction in the initial stages shown by the Shaw and McFarlane method is not shown by the Block and Bolling method.

A consideration of the color-producing reactions which form the basis of the two methods provides some evidence as to their relative reliability. While the exact mechanism of

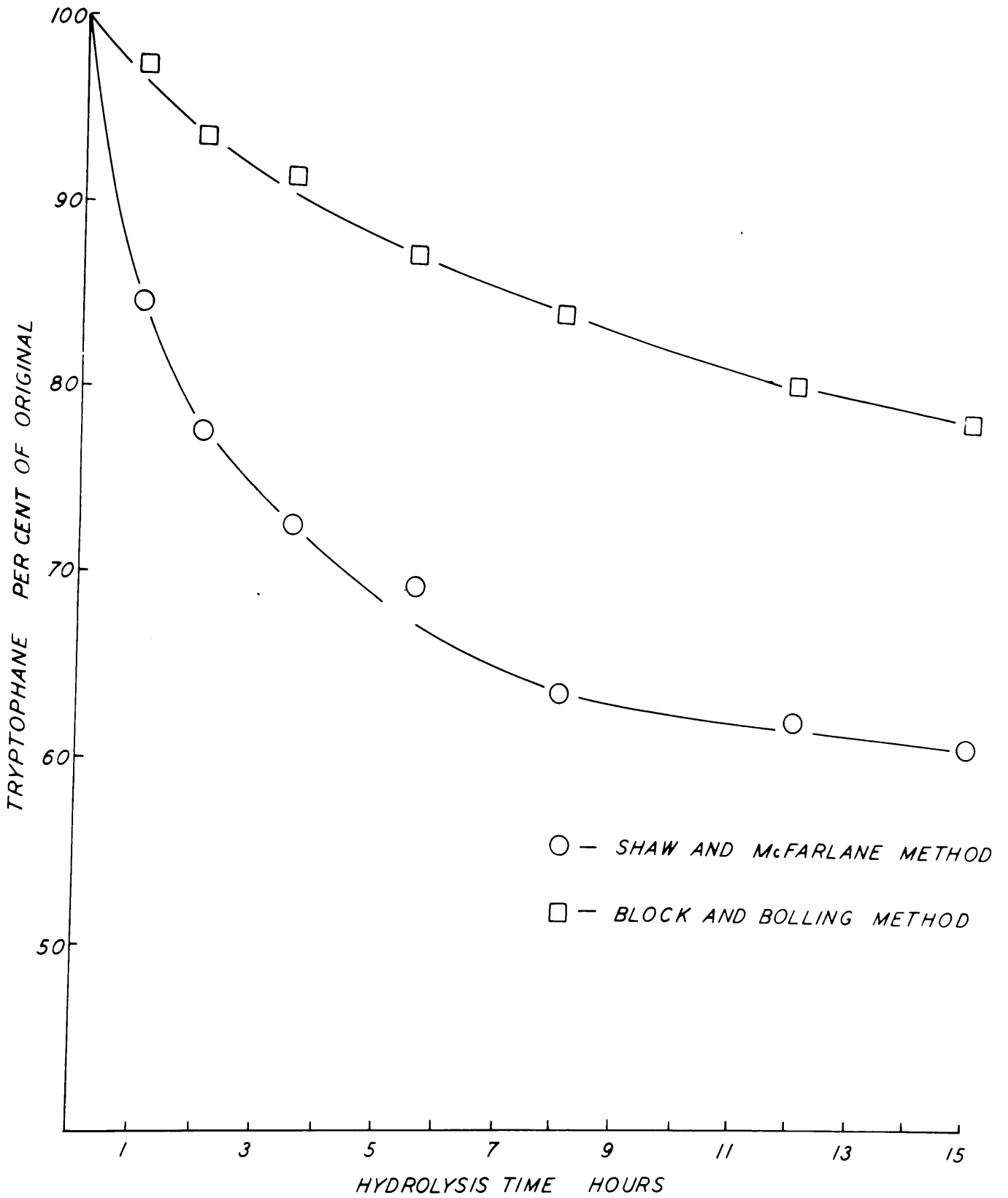

FIGURE 16

COMPARISON OF METHODS ON ACID HYDROLYSATES

the reaction is rather obscure, the blue color produced by the action of tryptophane on Folin's phenol reagent, phosphomolybdictungstic acid, is attributed to the reducing action of tryptophane (45). Schild and Enders (125) found that Folin's phenol reagent gives the color, even after mercuric sulphate precipitation, with melanoidin formed from the condensation of glucose and glycine, and also with purine and pyrimidine compounds, acetone, indole and its derivatives, pyrol, pyruvic acid and other substances.

Folin and Looney (47), early in the development of the method, employed phosphomolybdic acid which can be used for the estimation of tryptophane, tyrosine and cystine in similar manner. On the applicability of the method to acid hydrolysates they state:

> ".... it seems to be generally agreed that the tryptophane survives better under alkali than under acid hydrolysis. The humin formation in acid hydrolysis ... constitutes an added reason against this form of hydrolysis for tryptophane determinations although we are not quite sure to what extent tryptophane really loses its reactivity with the phenol reagent by virtue of such humin formation".

Apparently, they suspected that decomposition products of tryptophane, leading to humin formation, are capable of producing the color with the phenol reagent. This effect is probably emphasized in the humin-free acid hydrolysate produced by electrolytic reduction since the tryptophane destroyed does not become a component of a colored complex humin compound.

In the glyoxylic acid reaction, as used in the Shaw and McFarlane method, color production depends on a condensation reaction between the aldehyde group and some reactive point in the indole ring. If the indole ring has been broken or has entered into a prior condensation with some aldehyde during hydrolysis, a positive reaction with glyoxylic acid would not be expected. In any event there has not been the same criticism of the specificity of the glyoxylic acid reaction. In view of these considerations the results obtained with the Shaw and McFarlane method are considered more reliable.

This comparison of the two methods provides at least a partial explanation for the high recoveries of tryptophane obtained by White and Sayers (loc. cit.) after hydrolysis with sulphuric acid. Their recoveries are much higher than those obtained in the Control Experiment, Part III, where no electrolytic reduction was used.

Some of their results are given in Table XIII and compared with those obtained in the control experiment in this study. The results are not strictly comparable since White and Sayers used 5.5 N sulphuric acid and 1 g. of casein to 20 cc. of acid while in the present work 6 N sulphuric and 1 g. of casein to 15 cc. of acid were used. However, even after liberal allowance is made for differences arising from these factors, the values obtained by White and Sayers are considerably higher.

TABLE XIII

Comparison of White and Sayers' Results with Author's

Hydrolysis Period Hours	Tryptophane, Per Cent of Original			Extent of Hydrolysis Per Cent		
	White & Sayers	Author's	(I)	White & Sayers	Author's	(I)
3	71.7	43.0		74.5	79.0	
6	53.1	26.0		91.0	93.0	
8	54.8	20.0		98.0	96.5	

(I) Values obtained by graphical interpolation since exact hours of sampling did not coincide with those of White and Sayers.

It is highly probable that the differences in tryptophane recovery are largely due to the analytical method used, since White and Sayers used the method of Folin and Ciocalteu. White and Sayers actually report a higher tryptophane recovery after eight hours hydrolysis than after six.

3. Effect of Electrolytic Reduction on Amino Acids other than Tryptophane

The hydrolysate gives strongly positive tests when any of the usual qualitative color reactions, specific for individual amino acids, are applied. Since only a few of the amino acids can be detected in this way and since the tests are quite sensitive, this does not yield much information.

A brief consideration of the structural groups present in various amino acids and their susceptibility to

reduction indicates that many of the amino acids should not be affected by electrolytic reduction.

Amino groups and hydroxyl groups are already in a state of maximum reduction. The carboxyl group is highly resistant to reduction by any chemical means, as Neuberg (104) found that reduction of the carboxyl group to an aldehydic group was possible only by converting the amino acids to their hydrochloride esters and reducing by sodium amalgam. Brockman (20) states that the reduction of any carboxyl group is difficult to achieve by electrolytic reduction. The unsaturated rings of the aromatic amino acids also possess high stability towards reduction.

Cystine is undoubtedly reduced to cysteine. Nascent hydrogen, obtained by adding zinc dust to the acid hydrolysate, is used to reduce cystine to cysteine in some quantitative analytical procedures (106, 136). Some possibility exists that methionine may be affected, yielding either homocysteine and methane or ∝-amino butyric acid and methyl mercaptan. The latter does not occur since the mercaptan would be detected readily by its odour, the former remains a possibility.

With the basic amino acids, the more complex structures of histidine and arginine make prediction of their behavior difficult. Hall (64) mentions, without further comment, that there is some evidence that arginine is affected by stannous chloride when it is used to reduce humin formation. On the

other hand, hydriodic acid, a strongly reducing acid which also prevents humin formation, is sometimes used to obtain protein hydrolysates from which all of the basic amino acids are isolated on a quantitative basis (85). The splitting of arginine into ornithine and urea requires oxidation and, in any event, the presence of either urea or guanidine would result in a higher ammonia content of the hydrolysate. This does not occur, as will be shown later.

As previously mentioned, time did not permit investigation of the fate of each amino acid without neglecting the initial objective but wherever possible, attempts were made to obtain further information in this respect.

(a) Determination of Tyrosine. The Block and Bolling method, (loc. cit.) used for the determination of tryptophane in the foregoing experiment, is designed for the simultaneous determination of tyrosine on the same aliquot of hydrolysate, using a modification of the Millon-Nasse reaction (97, 102). A considerable portion of the tyrosine can be destroyed during the acid hydrolysis of impure proteins (89) and it was, therefore, considered advisable to determine the tyrosine content of the series of samples obtained in the foregoing experiment. The results are reported in Table XIV.

The tyrosine content of the original casein, determined following alkaline hydrolysis alone, was 5.68 per cent. It is quite apparent that electrolytic reduction does not cause any destruction of tyrosine and, in view of the destruction

normally encountered during acid hydrolysis, it is highly probable that some destruction has been avoided. Another sample, hydrolyzed for fourteen hours with 6 N sulphuric acid, using electrolytic reduction, also showed no destruction of tyrosine.

TABLE XIV

Effect of Electrolytic Reduction on Tyrosine

Hydrolysis Period Hours	Tyrosine (1) Per Cent
1.0	5.67
2.0	5.70
3.5	5.60
5.5	5.67
8.0	5.63
12.0	5.67
15.0	5.70

(1) The acid hydrolysate was subjected to further hydrolysis for five hours in 20 per cent sodium hydroxide.

It cannot be concluded that electrolytic reduction would eliminate all destruction of tyrosine during hydrolysis with 8 N sulphuric acid for twenty hours, as is often used for analytical procedures, but there is definite indication that such reduction would be an improvement over

normal procedures.

(b) Microbiological Assays. Samples of hydrolysate were submitted to outside laboratories where microbiological assays for certain amino acids were being carried out as a routine procedure. The first hydrolysate was prepared from 100 g. of commercial casein, hydrolyzed as described in Experiment 3, Part III, page 88. The acid hydrolysate was neutralized with barium hydroxide, the precipitate washed with boiling water, and the filtrate and washings concentrated in vacuo to the point where some amino acids began to crystallize out. These were brought back into solution with a little water and the sulphuric acid-barium sulphate precipitation carefully adjusted to the end point. The solution was clarified with Norit A, filtered, and the remainder of the water removed in a freeze-drying apparatus. The residue was pulverized and dried further in a vacuum oven at 70°C. for forty-eight hours.

Preparation of the hydrolysate in this powdered form facilitated shipment and avoided bacterial decomposition, thus permitting the analysis to be carried out at any convenient time.

The only assay conducted on this hydrolysate was for glutamic acid [1]. A value of 17.8 per cent was obtained.

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(1) Courtesy of Dr. D. W. Woolley, Rockefeller Institute for Medical Research, New York, N. Y.

Block and Bolling (12), tabulating the values reported for glutamic acid in casein, show that they range from 11.0 per cent to 24.2 per cent, and submit 22.8 per cent as the most probable value, calculated on the basis of 16 per cent nitrogen in casein. The hydrolysate submitted for assay contained 13.1 per cent nitrogen. When calculated to a 16.0 per cent nitrogen basis the glutamic acid content was 21.8 per cent.

A second powdered hydrolysate was prepared in similar manner to the first with the exception that 100 g. of partially-purified casein was used rather than commercial casein. Clarification with charcoal was not necessary and consequently was not employed. Microbiological assays for thirteen amino acids were obtained on this hydrolysate and the results are reported in Table XV, column 1. The most probable values for each of these amino acids in casein, according to Block and Bolling (12) are given in the second column, on the basis of 16.0 per cent nitrogen in casein. In column 3 the microbiological assays are given after a positive correction for the lower nitrogen content of the hydrolysate and a negative correction for the low glutamic and aspartic acid values.

The outstanding feature of these results is the low glutamic acid content and the almost complete absence of aspartic acid. While the possibility of partial destruction of these amino acids cannot be eliminated entirely, it is highly probable that these low values are due to the adsorp-

TABLE XV

Microbiological Assays on Hydrolysate (1)

Amino Acid	Micro-biological Assay (2)	Most Probable Value (Block and Bolling) (3)	Corrected Micro-Biological Assay (4)
Arginine	3.0	4.1	3.1
Lysine	6.6	6.9	6.9
Histidine	2.9	2.5	3.0
Isoleucine	5.8	6.5	6.0
Leucine	10.2	12.1	10.6
Valine	7.0	7.0	7.3
Threonine	3.9	3.9	4.1
Methionine	3.1	3.5	3.2
Phenylalanine	6.7	5.2	7.0
Glutamic Acid	12.0	22.8	12.5
Aspartic Acid	0.17	6.3	0.18
Tryptophane	1.1	1.8	1.2
Tyrosine	7.5	6.4	7.8

(1) These microbiological assays were obtained through the courtesy of Dr. H. B. Woodruff, Microbiological Department, Merck and Company, Rahway, N. J.

(2) Based on the weight of dry powdered hydrolysate, 13.1 per cent nitrogen.

(3) Values for casein on the basis of 16.0 per cent nitrogen in casein.

(4) To compare with the Block and Bolling values, the microbiological assays are multiplied by 16/13.1 to correct for nitrogen and by 84/100 to compensate for differences due to the loss of glutamic and aspartic acids.

tion of these amino acids on the barium sulphate during the neutralization process. Approximately 1050 g. of barium sulphate was formed in the neutralization of the hydrolysate and thorough washing of this quantity of precipitate was difficult to accomplish. This was particularly true since it was desirable to limit the amount of water used to a minimum and avoid the necessity of filtering and evaporating excessive amounts of water.

The much higher glutamic acid assay obtained on the first hydrolysate is further evidence that the losses of the dicarboxylic acids in the second hydrolysate are due to adsorption. This can be explained only by more thorough washing of the precipitate in the first case, since the procedures were otherwise identical.

The correction applied to the assays to compensate for the loss of glutamic and aspartic acids brings the general level of the other amino acid values into close proximity with those reported by Block and Bolling. This correction would not be justified if the apparent loss of the dicarboxylic acids was not due to complete removal. Destruction during hydrolysis would not have resulted in the removal of the decomposition products or their nitrogen, except in the unlikely event that such products were volatile. If this negative correction was not applied, correction for nitrogen content alone would increase the values for the other amino acids to levels considerably above those reported by Block

and Bolling.

The formation of insoluble salts of glutamic acid and aspartic acid with barium is a well-known reaction (31). Lyman et al (91), also found microbiological assays for glutamic and aspartic acids to be much lower following hydrolysis by sulphuric acid than after hydrolysis by hydrochloric acid and they attribute the difference to adsorption on the barium sulphate during neutralization of the sulphuric acid. Chibnall et al (25) also experienced serious losses of aspartic acid and glutamic acid due to adsorption on barium sulphate.

Apart from the dicarboxylic acids, the microbiological assays for the other amino acids are reasonably close to the expected values. The histidine, valine, threonine, phenylalanine and tyrosine assays are higher than the most probable values, while the assays for arginine, isoleucine, leucine and methionine are lower. The assay for tryptophane confirms a high degree of retention during the acid hydrolysis procedure. The uncorrected assay for tryptophane of 1.1 per cent on the hydrolysate is slightly higher than the value of 0.96 per cent obtained on this hydrolysate using the Shaw and McFarlane colorimetric method.

A rigid comparison of the microbiological assays with the values submitted by Block and Bolling is not justified since the former were obtained on a dried hydrolysate after many manipulations in which adsorption of amino acids or loss of ammonia could occur, while the latter values are tabulated

from the work of many authors and are presented by Block and Bolling with considerable reserve since most methods are subject to error. The microbiological assays do, however, provide considerable evidence favouring the conclusion that, with the possible exception of aspartic acid, these amino acids are not seriously affected by the electrolytic reduction procedure. Considerable more work would be necessary before absolute conclusions could be made.

4. Amide Nitrogen

Hydrolysates produced under conditions of electrolytic reduction have an amide nitrogen content identical to those produced by normal refluxing. Many determinations were conducted and the average value for both types of hydrolysate was 10.2 per cent of the total nitrogen. This is in excellent agreement with values reported by Van Slyke (142), Crowther and Raistrick (28), Dunn and Lewis (38), and others.

This provides some evidence that arginine is not affected since any destruction of the guanidine group would be expected to produce an increase in the ammonia content.

5. Amino Nitrogen

The maximum amino nitrogen, exclusive of amide nitrogen or ammonia, represented 70.3 per cent of the total nitrogen in the hydrolysates produced with electrolytic reduction. The value obtained on normal hydrolysates, using an identical procedure, was 70.1 per cent. These values were used as

a basis for the calculation of the extent of hydrolysis at any given time. A five-minute reaction period was used in all cases.

Van Slyke (141) found the amino nitrogen in a casein hydrolysate to be 72.6 per cent of the total nitrogen, while Osborne and Guest (109) reported 71.7 per cent, using a five-minute reaction period with nitrous acid. Dunn (36), in a thorough investigation, found the amino nitrogen in casein to be 70.0 per cent of the total nitrogen, using the Van Slyke method (139), and a ten-minute reaction period.

Apart from the work cited above, there is a serious lack of standardization of methods employed to determine the maximum amino nitrogen in casein hydrolysates and its relation to the total nitrogen. In consequence, wide variations exist among the values for maximum amino nitrogen used in calculation of the extent of hydrolysis.

In the Van Slyke manometric method most differences arise through the interference of ammonia resulting from amide linkages. White and Sayers (loc. cit.) do not mention taking precautions in this respect and used 74.8 per cent as the maximum amino nitrogen as per cent of total nitrogen. Frost and Heinsen (50) used 76 per cent amino nitrogen, expressed as per cent of the total nitrogen minus the ammonia nitrogen. They also considered that about 30 per cent of the ammonia was measured as amino nitrogen. Calculation, on the basis of this information, shows that amino nitrogen represented approxi-

mately 65.8 per cent of the total nitrogen.

Failure to correct for the measured ammonia introduces serious errors into values reported for amino nitrogen and the degree of hydrolysis. Amide linkages are readily hydrolyzed by acids, the maximum value being obtained after only one hour in 6 N hydrochloric acid (36). The errors are particularly large when the degree of hydrolysis is low. The following example illustrates the magnitude of error: A sample containing 20 mg. total nitrogen will contain approximately 14 mg. (70 per cent) amino nitrogen after complete hydrolysis. When 10 per cent hydrolysis has been attained, 1.4 mg. of amino nitrogen will be present, but, unless precautions are taken, 30 per cent of the ammonia will also be measured, amounting to 0.6 mg. of nitrogen, in addition to the amino nitrogen (82), giving an apparent amino nitrogen content of 2.0 mg. The error is thus approximately 43 per cent with respect to the absolute amount of amino nitrogen. The percentage error decreases as the amino nitrogen increases, but it is always appreciable. The error in the calculated degree of hydrolysis is only slightly smaller in the early stages of hydrolysis but it disappears entirely at complete hydrolysis.

Nasset and Greenberg (loc. cit.) did not consider the interference of ammonia when studying the order of the hydrolysis reaction, using casein and mineral acids, nor did Greenberg and Burk (loc. cit.). This is surprising since

the velocity constants obtained for the initial stages of hydrolysis were too high. Recognition of the effect of ammonia, from the rapid hydrolysis of amide linkages, would have improved this irregularity in their results.

Applications of the Sørensen formol titration procedure (loc. cit.), to establish the ratio of amino nitrogen to total nitrogen, and the degree of hydrolysis, have also produced discordant results. The discrepancies here arise from the pH limits chosen by various workers. Sahyun (121) added formaldehyde at pH 6.0 and titrated with alkali to pH 9. He thus obtained a value of 82 for ∝-amino nitrogen as per cent of total nitrogen in a neutralized acid hydrolysate. Although not mentioned by the author, it is assumed that the ammonia was eliminated during the neutralization process. It should also be remembered that the formol titration procedure measures the imino groups of proline and hydroxyproline whereas the nitrous acid method does not.

The Association of Official Agricultural Chemists (4) recommend adding the formaldehyde at pH 8 (end point for phenolphthalein) and titrating with alkali to pH 8 after the shift in the dissociation equilibrium of charged and uncharged amino groups. This procedure naturally gives lower values. During the present work this method was used on one occasion and gave a value of 60.0 for amino nitrogen as per cent of total nitrogen at complete hydrolysis of casein.

Ammonia also interferes in the formol titration (4) and

should be considered in estimating the amount of amino nitrogen and the extent of hydrolysis.

In the present work the errors discussed above were reduced to a minimum by complete removal of the ammonia before determination of amino nitrogen. While this is considerably more laborious, the results are more reliable than those obtained in the presence of ammonia.

Another alternative which should produce equally reliable results is the method proposed by Irving, Fontaine and Samuels (82) in which a correction is applied for the amount of ammonia measured by the Van Slyke nitrous acid method.

6. Comparison of Powdered Acid Hydrolysate with Enzyme Hydrolysates

Powdered hydrolysates for oral administration are usually prepared by enzyme hydrolysis since this obviates the necessity of replacing amino acids which are destroyed in other methods of hydrolysis. As is generally known, hydrolysates produced by normal acid hydrolysis must be supplemented with tryptophane from other sources.

The dried acid hydrolysate, of partially purified casein, prepared for microbiological assay in an earlier experiment, was compared with two enzyme digests of casein prepared for pharmaceutical purposes. While it was not intended to carry out an extensive study on the preparation of powdered hydrolysates, a brief comparison of these hydrolysates was

deemed worthwhile as a point of interest. Analyses for total nitrogen, amino nitrogen and tryptophane were conducted and the results are reported in Table XVI.

TABLE XVI

Comparison of Acid Hydrolysate
and Commercial Enzyme Preparations from Casein

	Total Nitrogen Per Cent	Amino Nitrogen Per Cent	Trypto-phane Per Cent
Acid Hydrolysate	13.2	8.6	0.96
N-Z Amine (Sheffield Farms, Inc., N. Y.	13.5	5.6	0.87
Enzyme Hydrolysate (Mowat & Moore, Montreal)	13.1	5.1	0.84

The tryptophane content of the acid hydrolysate is remarkably high in comparison with the enzyme hydrolysates. This can be explained, in part, by the loss of the dicarboxylic amino acids as was shown by the microbiological assays on the acid hydrolysate. After applying the correction factor to compensate for this, (footnote 4, Table XV), the tryptophane value would be 0.81 per cent. For an acid hydrolysate, this still compares favourably with the enzyme hydrolysate since, theoretically, no tryptophane should be lost during enzyme hydrolysis. The history of these enzyme digests is not known but there is a possibility that some

tryptophane has been destroyed in manipulations following digestion, particularly in the drying process. This loss was minimized in the acid hydrolysate by employing a freeze-drying procedure.

As would be expected, the acid hydrolysate contains a much higher percentage of amino nitrogen. This is considered advantageous from a nutritional standpoint (92).

The odor of the acid hydrolysates is undetectable while that of enzyme hydrolysates, generally, is unpleasant. Acid hydrolysates are also characterized by superior flavor.

7. Lead content of Hydrolysate

The utility of lead as a cathode material is favored by the low solubility of lead sulphate in sulphuric acid. The solubility of lead sulphate in water and aqueous solutions of sulphuric acid was investigated by Crockford and Brawley (27) and they report the solubility of lead sulphate in water at 25°C. to be 44.5 p.p.m. In 20 and 30 per cent sulphuric acid the solubility drops to 1.2 p.p.m., but rises again in sulphuric acid concentrations over 75 per cent. Reagent grade concentrated sulphuric(I) acid contains up to 5 p.p.m. of lead. Calculation shows that, after dilution to 6 N, the concentrated acid can supply approximately sufficient lead to saturate the solution with respect to lead sulphate

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(I) Mallinckrodt reagent

at 25°C.

In addition, other factors favour a low lead content in the final hydrolysate. Since the lead wool is used as a cathode in the reduction process, lead ions would have to enter the solution against a high electrical potential. It is thus possible that the electrolytic reduction could result in a lower lead content than would be obtained in the complete absence of lead wool, but the precise behaviour of the lead ions under these conditions, and in the presence of protein, cannot be predicted.

Low lead content of the hydrolysate is also favoured by the high adsorption capacity of barium and calcium sulphates for lead sulphate, where those compounds are the end products of neutralization (128).

The powdered hydrolysates, previously described, were tested qualitatively for lead with several reagents. The only test which yielded a questionable result was the diphenylthiocarbazone test, all others were negative. The same doubtful test with diphenylthiocarbazone was obtained on the unhydrolyzed casein.

There is little doubt that the lead content is low enough to avoid interference during the application of analytical methods for the estimation of the amino acids. Whether or not the lead content of the hydrolysate exceeds levels for nutritional purposes would require further investigation, but this too is considered unlikely.

SUMMARY

1. Tryptophane has been isolated from commercial casein following hydrolysis by sulphuric acid. The yield of crude tryptophane obtained by butyl alcohol extraction was 0.98 per cent. The yield of crystalline tryptophane was 0.49 per cent. Since the residual mother liquor contained considerable tryptophane which could not be recovered by crystallization, the maximum possible yield is considered to be intermediate between these two values.

2. This tryptophane has been identified by melting point, proximate analyses, crystal structure and optical rotation. The specific rotation $[\alpha]_D^{26} = -32.1^\circ$ in water, showing no racemization.

3. Two methods for the quantitative determination of tryptophane in acid hydrolysates have been compared. The method of Folin and Ciocalteu, as modified by Block and Bolling, gives higher values than the method of Shaw and McFarlane. The latter method is considered more accurate when applied to these hydrolysates.

4. Tyrosine is not affected by electrolytic reduction. The results indicate that the electrolytic reduction may act beneficially through prevention of humin formation, eliminating losses which ordinarily occur during acid hydrolysis.

5. Microbiological assays indicate that arginine, lysine, histidine, isoleucine, leucine, valine, threonine, methionine, phenylalanine, glutamic acid and tyrosine are not seriously, if at all, affected by electrolytic reduction. The assay for aspartic acid is extremely low but this is believed to result from extensive adsorption on the barium sulphate during neutralization of the hydrolysate, rather than from destruction.

6. Amide nitrogen and amino nitrogen values are unaffected by electrolytic reduction.

7. A powdered hydrolysate has been produced by acid hydrolysis of partially-purified casein which compares favorably, in tryptophane content, with some commercial preparations prepared by enzyme digestion.

8. With the exception of the diphenylthiocarbazone reagent, the powdered hydrolysate gives negative qualitative tests for lead. The test with diphenylthiocarbazone is inconclusive.

CLAIMS TO ORIGINAL RESEARCH

1. The discovery that nascent hydrogen is an effective agent for the prevention of humin formation during the hydrolysis of proteins with sulphuric acid.

2. The discovery that when humin formation is prevented by the use of nascent hydrogen the extent of tryptophane destruction is reduced during hydrolysis with sulphuric acid.

3. The design and construction of apparatus whereby nascent hydrogen, developed electrolytically at a cathode surface, can be efficiently employed under conditions suitable for the acid hydrolysis of proteins to prevent the formation of humin and reduce the destruction of tryptophane.

. The development of a procedure for the acid hydrolysis of proteins, employing simultaneous electrolytic reduction, and including a preliminary reduction period in weak acid, which permits the attainment of a high degree of hydrolysis with the preservation of up to 70 per cent of the original tryptophane content of commercial casein, or up to 78 per cent with purified casein, as measured by colorimetric method.

. The isolation of tryptophane for the first recorded time

following the hydrolysis of a protein with acid and in a yield of 0.49 per cent from commercial casein.

6. The ascertainment that tryptophane is not racemized to any appreciable extent during the hydrolysis of casein with 6 N sulphuric acid for a minimum of twelve hours.

7. The presentation of new evidence that tryptophane loses its chromogenic activity with glyoxylic acid to a greater extent than it does with Folin's phenol reagent during acid hydrolysis, thereby producing discordant analytical results which must be interpreted with caution.

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