COLORIMETRIC METHOD FOR THE ESTIMATION OF AMINO ACID ALPHA-NITROGEN

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A COLORIMETRIC METHOD FOR THE ESTIMATION OF AMINO ACID ALPHA-NITROGEN.

At present there are three methods in use for the determination of amino acid alpha-nitrogen. The first, that of Sörensen,[1] depends upon the fact that when a freshly prepared, neutral solution of formalin (10 cc. of 35 per cent formalin in 20 cc. of water, and enough tenth-normal NaOH to make it just neutral), is added to a solution of amino acid the following reaction results:-

$$R.CHNH_2.COOH + H.C\overset{O}{=}H = \begin{matrix} R.CH\text{-}N=CH_2 \\ | \\ COOH \end{matrix} + H_2O,$$

and the carboxyl group may be titrated by means of standard alkali to a rose pink color - the number of cc. of alkali used being an index of the amino nitrogen present in solution. Although this is a rapid, easy method, it is neither very accurate nor delicate.

The second, that of Van Slyke,[2] is the method now generally accepted for the determination of nitrogen in amino groups. In this method, advantage is taken of the quantitative decomposition of amino groups by nitrous acid as represented in the following equation:-

$$R.NH_2 + HNO_2 = R.OH + H_2O$$

The nitrogen so evolved is collected and measured. In its latest development, the microchemical form,[3] the method will estimate 0.5 mgm. of nitrogen with an accuracy of one per cent, and its application to the determination of amino acid alpha-nitrogen in blood and tissues has yielded in its author's hands very valuable and interesting results.

The third method, due to Kober and Sugiura,[4] involves the reaction between amino acids and cupric hydrate. A known quantity of

carefully prepared cupric hydrate is added to the amino acid solution wherein a quantity of cupric hydrate equivalent to the amino nitrogen is dissolved away, with the formation of a copper complex. After filtering, the unattacked cupric hydrate is dissolved in potassium bicarbonate and titrated with standard solutions of iodine and sodium thiosulphate of very low concentration. This method possesses the advantage over the others previously mentioned in that by its means alpha and beta amino acids may be estimated. Although it has not yet been as widely applied as the others, it has yielded satisfactory results with blood and urine. An accuracy of one part in 500,000 is claimed by the authors. Very recently, it has been utilised with success in the determination of amino nitrogen in soils.[5]

It was felt, however, that if an independent method for the estimation of the alpha-nitrogen of the amino acids, especially if the sensitiveness of the reaction could be increased beyond that of the Van Slyke method without loss of accuracy, could be evolved, it would not only be valuable but necessary as the time approached for a study of the chemistry of the single cell.

It soon became apparent that such a hope could be fulfilled only by the quantitative application of a color reaction of amino acids; and of all such, the most probable seemed the reaction with triketohydrindene hydrate.

In an endeavor to prepare 1:2-diketohydrindene from alpha-hydrindone Ruhemann[6] found that in the presence of potassium hydroxide or sodium carbonate para-nitroso-dimethylaniline attacks both methylene groups of the alpha-hydrindone. This condensation product readily decomposes under the influence of mineral acids

and yields the hydrate of triketohydrindene: $C_6H_4\begin{matrix}CO\\CO\end{matrix}>C(OH)_2$. The solid readily dissolves in hot water, and, on cooling the concentrated solution, crystallises in colorless prisms, which turn red at 125°C and melt and decompose at 239-240°C. The compound, after drying in a vacuum desiccator, yielded the following results on analysis:

$C_9H_6O_4$

	Theory.	Found.
Carbon	60.67%	60.71%
Hydrogen	3.37	3.41

The skin is colored a deep purple by its aqueous solution - a property which renders it similar to alloxan; which, in constitution slightly resembles triketohydrindene. Its solution also reduces Fehling's and ammoniacal silver solutions. If kept for a short time in dilute ammonia the solution of triketohydrindene becomes reddish-violet and no longer reduces silver solution.

If potassium hydroxide solution (15 per cent) is added to the triketone, the crystals turn yellow, then dissolve to a solution, which, at first is yellow, but later becomes colorless at ordinary temperature. A different result is attained, however, if the triketone be warmed at once after the addition of the alkali; under these conditions, a deep blue solution is produced, whose color is permanent on boiling, but becomes colorless on dilution with water, and then no longer reduces Fehling's solution

FORMULA.

The formula for triketohydrindene hydrate is unquestionably as represented, namely, with the elements of water attached to the 2-ketonic grouping of triketohydrindene.[7] The union of

water with any other ketonic group of the triketone would produce a colored compound owing to the proximity of two ketonic groups, whereas triketohydrindene hydrate is colorless as is 1:3-diketohydrindene: $C_6H_4\begin{<}CO\\CO\end{>}CH_2$

This formula finds further support in the fact that the hydrate, under the influence of phosphorus pentachloride, is transformed into the colorless 2:2-dichloro-1:3-diketohydrindene,

$$C_6H_4\begin{<}CO\\CO\end{>}C\ Cl_2.$$

The unstable cyanohydrin $C_6H_4\begin{<}CO\\CO\end{>}C(OH).CN$ is also produced by the interaction of the hydrate and hydrocyanic acid.

REACTION WITH POTASSIUM HYDROXIDE.

The reaction with potassium hydroxide proceeds in three distinct stages, which are indicated by color changes. When the alkali is added to the crystals, they turn yellow at first, then dissolve to form a yellow solution, which subsequently becomes blue even at the ordinary temperature if the alkali is concentrated. The blue color, however, rapidly disappears on dilution with water to yield a colorless solution.

The colorless alkaline solution representing the last phase of the reaction contains the potassium salt of ortho-carboxy-mandelic acid, $CO_2H.C_6H_4.CH(OH).CO_2H$, because, on treatment with dilute sulphuric acid phthalide-carboxylic acid is produced:

$$\begin{matrix}CO\ .\ O\\ \\ C_6H_4\end{matrix}\diagdown CH.COOH.$$

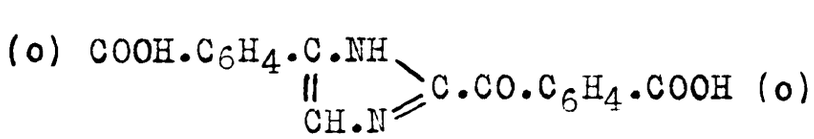
Therefore, under the influence of alkali, the five-carbon ring of triketohydrindene has been opened with the formation of phenyl-glyoxal-o-carboxylic acid, $COOH.C_6H_4.CO.CHO$ which finally under-

goes the change to phthalide-carboxylic acid. A similar reaction is the change which phenyl-glyoxal undergoes by the action of alkalies to mandelic acid: $C_6H_5.CO.CHO \longrightarrow C_6H_5.CH(OH).COOH$. Thus the formation of phenyl-glyoxal-o-carboxylic acid is to be regarded as the first change which the alkali causes in the triketone; and this view is further supported by the fact that the yellow alkaline liquor reduces Fehling's and ammoniacal silver solutions. The explanation of the intermediate phase of the reaction, characterised by the blue color of the alkaline solution, is difficult owing to the unstable nature of the color and its passage into the final stage of the reaction.

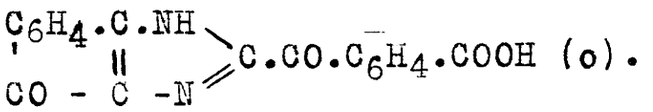
ACTION OF AMMONIA ON THE TRIKETONE (loc. cit.).

If an aqueous solution of the mixture of both substances be kept for a short time, it turns a deep reddish-violet, and no longer reduces silver nitrate. On the addition of dilute hydrochloric acid a red solid is precipitated, which has the empirical formula $C_9H_5O_2N$. From a study of the action of ammonia on phenyl-glyoxal the constitution of the substance is shown to be double that of the empirical formula.

As the reducing action of triketohydrindene hydrate can only be explained by the opening of the five-carbon ring with the formation of phenyl-glyoxal-o-carboxylic acid, the action of ammonia may be explained, as in the case of phenyl-glyoxal, by the assumption that the aldehydo-acid, first condenses to the dicarboxylic acid,



which subsequently loses water and yields:



ACTION OF TRIKETOHYDRINDENE HYDRATE ON AMINO ACIDS.

Ruhemann's first announcement of the behavior of triketohydrindene hydrate towards amino acids[8] records the development of an intense blue color when a slightly warmed aqueous solution of triketohydrindene hydrate and glycine are mixed; this blue colored solution gradually deposited a dark solid. Similar behavior was shown by all the alpha-amino acids obtainable; cystine, however, responded to the test only on boiling, owing to its sparing solubility in water. The blue color was perceptible on warming glycine with a solution which contained one part of triketohydrindene hydrate in 15,000 parts of water. Of the beta-amino acids, only two were examined, namely, beta-aminopropionic and beta-amino-beta-phenylpropionic acids; with these the color reaction took place less readily, and, in the case of the latter was far less intense than in the corresponding alpha-amino acids. No coloration, however, was produced by the triketone in solutions of aromatic amino acids which contain the amino group in the nucleus (for example, ortho-amino benzoic acid), nor did it occur with substituted amino acids, such as phenylglycine and hippuric acid, even on boiling aqueous solutions of the mixtures.

A positive reaction was obtained with peptone, and this, together with the above facts showed the occurence in peptones of compounds containing the free amino group of amino acids.

Normal human urine also produced the same coloration on warming with an aqueous solution of the reagent thus indicating

the presence of a protein-like substance in urine.

RELATION TO ALLOXAN.

In the reaction of triketohydrindene hydrate with amino acids to produce colored products, although great experimental difficulties are presented in the isolation of these, nevertheless, the fact, as established, that their formation is accompanied by the production of aldehydes, indicates a similarity in the behavior between triketohydrindene hydrate and alloxan. The results of Strecker's[9] experiments showed that when aqueous solutions of a mixture of alloxan and an amino acid are warmed together, carbon dioxide is evolved, and, simultaneously, aldehydes and murexide are formed. Thus,alloxan, acting as an oxidising agent, transforms alanine into acetaldehyde,and leucine into valeraldehyde. With glycine, however, Strecker was not able to prove the production of formaldehyde, although in other respects the reaction was analogous to that of the other amino acids; he explained this apparent exception by assuming that the formaldehyde underwent oxidation to formic acid and carbon dioxide.

Piloty and Finckh[10] repeated Strecker's work on the interaction of alloxan and glycine, using a mixture of concentrated solutions at 80°C. Under these conditions, a murexide color is produced, carbon dioxide evolved, and, on rapid cooling, a crystalline product is obtained, which has the color of murexide and which they described as glycine purpurate. On the other hand, if the mixture, instead of cooling, was heated until the murexide color disappeared, an insoluble amorphous, substance was deposited, and from the mother liquor was obtained a yellow, crystalline solid, which they regarded as uramiloacetic acid,

$CO\begin{matrix}NH.CO\\NH.CO\end{matrix}>CH.NH.CH_2.COOH,H_2O$, while no reference was made to the production of formaldehyde.

The apparently abnormal behavior of glycine towards alloxan induced Hurtley and Wootton[11] to repeat the earlier experiments. Their investigations confirmed the production of isovaleraldehyde from leucine and of acetaldehyde from alanine; also, when alloxan was allowed to act upon alpha-amino-butyric acid, propaldehyde was shown to be produced.

In studying the action of glycine on alloxan the reactions were observed in concentrated and dilute solutions. When a concentrated solution of the two substances was heated, no formaldehyde was produced, but the amorphous substance and the uramiloacetic acid of Piloty and Finckh were obtained. When molecular proportions of alloxan and glycine in dilute solution were distilled formaldehyde was identified in the distillate. In this experiment the amorphous substance was not obtained, although the presence of murexide was established and evolution of carbon dioxide observed.

The reaction seemed to be as follows; the glycine loses carbon dioxide and ammonia, $\begin{matrix}CH_2 - NH_3\\ |\quad\quad |\\ CO - O\end{matrix} = >CH_2 + CO_2 + NH_3$ whilst the methylene group thus liberated partially reduces the alloxan to dialuric acid, and is itself mostly converted into formaldehyde. The unchanged alloxan and dialuric acid form alloxantin, which, with ammonia forms murexide. The presence of the reducing substance is ascribed to the transformation of murexide into the substance closely allied to uramil, and its subsequent condensation with the dialuric acid and formaldehyde. Further,

the action of alloxan was tried on tyrosine, tryptophane, cystine and glucosamine, all of which gave a very positive murexide color. Its action on peptone, gelatin and casein revealed a weak murexide color on warming with the first two, but no color with casein.

Since triketohydrindene hydrate exhibits a similarity to alloxan with respect to its behavior with amino acids, the various results seem to indicate that the blue coloration is preceded by the formation of a substance closely resembling alloxantin.[12] A compound of this type is actually produced when an aqueous solution of triketohydrindene hydrate is treated with hydrogen sulphide thus:

$$2\ C_9H_6O_4 + H_2S = C_{18}H_{10}O_6 + H_2O + S$$

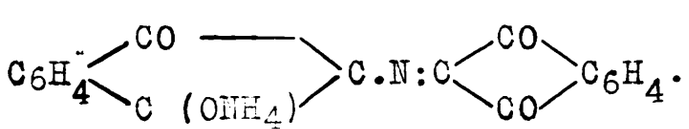
To this substance Ruhemann gave the name hydrindantin. It yields a blue coloration with amino acids; moreover, alloxantin and hydrindantin are very much alike in the behavior of their blue salts under the influence of oxygen. When this gas is passed through the blue solution of hydrindantin in potassium or sodium hydroxide the color disappears in a short time, and the alkaline liquor then contains the salt of o-carboxymandelic acid, which, in turn, by the action of acid yields phthalidecarboxylic acid, which is also produced by the action of alkali on triketohydrindene hydrate. From this fact it is presumed that the formation of the acid from hydrindantin is preceded by the production of triketohydrindene hydrate.

The similarity in constitutional formulae would also support the view of a probable close ~~relationship~~ resemblance in the properties of triketohydrindene hydrate and alloxan ~~recp-~~

reaction which underlies the production of the blue color, since it indicates that triketohydrindene hydrate must first be reduced by the amino acid (and the latter oxidised to aldehyde) before the color can be formed. This intermediate product is, in all probability, hydrindantin, since this readily yields the blue color with amino acids.

CONSTITUTION OF THE BLUE COLORING MATTER.

Owing to the ease with which hydrindantin is transformed into the analogue of murexide, together with the results of Strecker's experiments regarding the formation of murexide by the interaction of amino acids and alloxan, the supposition was advanced that the blue color reaction given by triketohydrindene hydrate with proteins and their degradation products, was due to the production of the ammonium salt of diketohydrindylidenediketohydrindamine,



Evidence of a confirmatory nature for this assumption was based upon the results of an examination of the action of alanine on the triketone. When warm concentrated aqueous solutions of equal weights of these reagents were mixed, a deep blue solution resulted, together with the formation of acetaldehyde and evolution of carbon dioxide, and the separation of a dark, bluish-brown solid. After collecting and washing, this solid material was found to be partially soluble in boiling water, and the blue filtrate, when treated with ammonium chloride, yielded a reddish-brown precipitate. On analysis, and transformation into the corresponding acid the compound thus isolated proved to be the analogue of murexide.

CHARACTERISATION OF THE AMMONIUM SALT OF DIKETOHYDRINDYLIDENEDIKETOHYDRINDAMINE.

In cases where only small amounts of amino acids are present in solution it became not only advisable, but necessary that some system of definitely qualifying this blue coloring matter be evolved. This condition has been fulfilled by Harding,[13] who has been able to do this in three ways:

(1) The coloring matter gives a broad absorption band in the visible spectrum when viewed in dilute solution; this band eliminates almost entirely the whole of the yellow.

(2) A very important feature is the fact that the color which is blue in daylight, appears purple when observed in artificial light. (As this purple color closely resembled a dilute solution of potassium permanganate, it was hoped to utilise this as a permanent colorimetric standard - a fact which will be dealt with later).

(3) The color was found to be resistant to mild oxidation. The passage of a rapid current of air for five minutes through the colored solution apparently had no effect. This experimental fact is interesting in contrast to the respective behavior of the blue salts of hydrindantin and alloxantin towards oxygen (page 9).

Further, the blue salt of hydrindantin does not manifest any absorption band in the visible spectrum; neither does it undergo any visible change in color when compared in artificial and day light. Thus one is enabled to differentiate the two blue solutions, which might otherwise be very easily confused.

The analogy with the murexide reaction is enhanced by the fact that murexide exhibits an absorption band similar to that

of the ammonium salt of diketohydrindylidenediketohydrindamine.

MECHANISM OF THE REACTION.

As has already been pointed out, the action of alanine on the triketone results in the oxidation of the amino acid to acetaldehyde and carbon dioxide, accompanied by the corresponding reduction, and condensation with ammonia of the triketohydrindene hydrate.

The mechanism of the reaction whereby the amino acid functions as a reducing agent, is considerably enlightened by an assumption of the dissociation theory of Dakin and Dudley[14] who have proved that all amino acids in dilute solution dissociate into ammonia and the corresponding glyoxal. Thus alanine gives ammonia and methyl glyoxal:

$$CH_3.CHNH_2.COOH = CH_3.CO.CHO + NH_3.$$

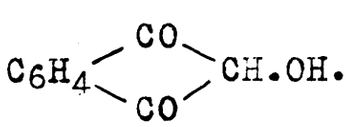
Since the glyoxals are very powerful reducing agents, reducing ammoniacal silver oxide and Fehling's solution even in the cold, it is supposed that methyl glyoxal would reduce the triketone, while simultaneously undergoing oxidation to pyruvic acid,

$$CH_3.CO.CHO + O = CH_3.CO.COOH,$$

which by decarboxylation, would yield acetaldehyde,

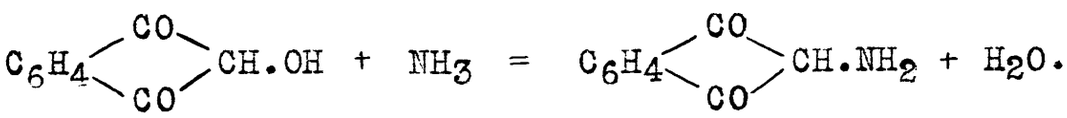
$$CH_3.CO.COOH = CH_3.CHO + CO_2.$$

Hence, the two products of dissociation of the amino acid would be both utilised - the glyoxal reducing the triketohydrindene hydrate, and the ammonia condensing with the product of reduction. The triketone would undergo reduction by glyoxal to hydroxydiketohydrindene,

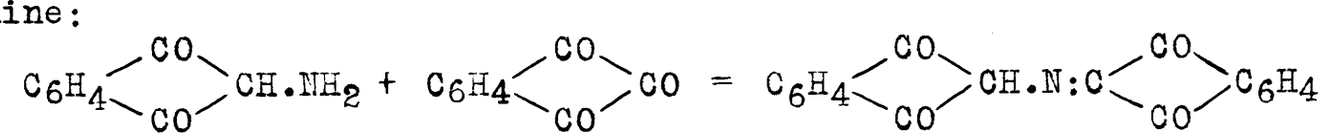


It is then believed that ammonia reacts upon this to produce

diketohydrindamine,



This compound, readily oxidises to the analogue of murexide, readily condenses with aldehydes and probably condenses with triketohydrindene hydrate to form diketohydrindylidenediketohydrindamine:



from which the ammonium salt is produced, thus forming the blue coloration.

This reaction cannot be applied, however, with ammonium salts alone, as the requisite reducing agent is not provided since the neutral ammonium salts have no reducing power. The reaction with ammonium salts will, however, be taken up in detail at a later stage of this treatise (page 42).

DELICACY OF THE REACTION.

The discovery of Ruhemann regarding the development of an intense blue color as a result of the interaction of triketohydrindene hydrate and amino acids having the amino group in the alpha position was corroborated and amplified by Abderhalden and his collaborators, who applied the reaction to the detection of pregnancy and cancer. Triketohydrindene hydrate is now a commercial product under the name of "ninhydrin". In the course of the application of ninhydrin as a reagent for proteins and protein cleavage products, Abderhalden and Schmidt[15] used a solution made by dissolving 0.1 grm. reagent in 300 cc. of water. They found that glycine and alanine gave a positive reaction with this at a concentration of one part of amino acid in 10,000

parts of water, while tyrosine reacted positively at about 1:5000.

The sensitiveness of the reaction is thus indisputable - a fact which is even more strikingly illustrated in a later communication by the same authors[16]. The solution of ninhydrin in this case was of one per cent strength; and the following table is given showing the dilutions at which the amino acids are detectable:

Glycine	1:65,000
d-Alanine	1:26,000
d-Valine	1:15,000
l-Leucine	1:35,000
d-Glutaminic acid	1:22,000
Asparagine	1:19,000
dl-Phenyl-Alanine	1:26,000
l-Histidine	1:79,000
α-Aminobutyric acid	1:16,000

In studying the fate of amino acids during absorption Abderhalden and Lampe[17] had utilised the reaction in a roughly quantitative way, but their results were not placed upon a strictly quantitative basis.

The ninhydrin reaction served as a means of experimental support for Abderhalden's theories on specific defensive ferments. The specific character of the ninhydrin reaction from the chemical point of view met with strong criticism from two sources. Halle, Loewenstein and Pribram[18] claimed that when the triketone and glycerol were heated to boiling for half a minute a blue color was produced, and many alcohols and ketones responded in a similar

manner. Sugars also gave a positive reaction, and, in all cases, the addition of alkali intensified the blue color. Secondly, Neuberg[19] found that several ammonium salts and organic bases reacted positively towards ninhydrin. Some of these results were directly contradictory to Abderhalden and Schmidt's earlier work.

In the production of the blue color characteristic of this reaction it is not easy to explain how such substances as glycerol, levulose, alanine, ammonium acetate and ethylamine can give the identical reactions with triketohydrindene hydrate. Halle, Loewenstein and Pribram noted two blue colors, namely, one caused by amino acids and another produced by alcohols, ketones and aldehydes. The first color could be produced with amino acids in a vacuum in absence of oxygen and was not intensified by the addition of alkali, whereas the latter color production required the presence of oxygen and became more intense when alkali was added.

The blue coloration given by the action of the hydrates of the metals of the alkalies on hydrindantin has already been discussed (page 9). This blue color, however, was rapidly decolorised by mild oxidation. Further consideration of these important phenomena will be taken up later.

OTHER METHODS OF AMINO ACID DETECTION AND ESTIMATION.

Glycine and Chloral Hydrate:

Owing to the fact that chloral hydrate contains two -OH groups attached to a single carbon atom just as in triketohydrindene hydrate, it was thought that an analogous color reaction for amino acids might be developed by the use of chloral hydrate. Watkins[20] investigated this possibility and found that

a glycine solution (1:5,000) when boiled for five minutes with chloral hydrate, yielded a dark red color; while a faint amethyst color was still evident at a glycine concentration of one part in 10,000. The chemistry of the colored compound has not been determined.

Colorimetric Determination of Histidine:[21]

The histidine is first isolated by Kossel's method. 1.5 cc. of a mixture of 1 per cent sulphanilic acid in HCl and a 0.5 per cent solution of sodium nitrite are added to 10 cc. of the histidine solution and into this are introduced 3 cc. of 10 per cent sodium carbonate. The total volume of the mixture being kept constant, the color obtained is then compared ~~with~~ in a Duboscq colorimeter with a standard histidine solution which has been prepared under similar conditions.

Estimation of Tyrosine by Bromination:

Plimmer and Eaves[22] evolved a method for the determination of small quantities of tyrosine (0.01 - 0.04 grm.) by using a modification of Millar's method. A more dilute solution of sodium bromide is employed; an excess of the reagent is added and the unabsorbed halogen can then be titrated with standard thiosulphate, using potassium iodide with starch as indicator. Histidine and tryptophane interfere by reason of their absorption of bromine.

Folin and Denis' Estimation of Tyrosine:[23]

The "Phenol Reagent" is prepared by boiling 750 grms. water, 100 grms. sodium tungstate, 20 grms. phosphomolybdic acid and 50 cc. of 85 per cent phosphoric acid for two hours under a

reflux condenser, cooling and diluting to one liter. 2 cc. of this solution gives a maximum color with 1 mgm. tyrosine. Nitrates interfere with the reaction; the blue color appears in the cold. The reagent reacts with all hydroxybenzene compounds and detects one part of tyrosine in 1,000,000 parts of water.

Complement to the Ninhydrin Reaction:[24]

To 10 cc. of the amino acid solution 0.2 cc. of aqueous solution of triketone was added, the mixture was brought to boiling and maintained at this temperature exactly one minute. In addition to the characteristic bluish-violet coloration, a large absorption band in the yellow-green region of the spectrum may be noticed. The complementary reaction was effected by using 10 cc. of the above solution, and, after 2 drops of glacial acetic acid were added the mixture was vigorously shaken with 2 cc. chloroform. The aqueous layer became decolorised, while the chloroform acquired an orange color together with the corresponding absorption spectrum.

E X P E R I M E N T A L.

PART I.

On account of the very positive color developed by alpha-amino acids when treated with ninhydrin in aqueous solution, together with the delicacy of the reaction as unquestionably shown by Abderhalden and Schmidt, the technique of these qualitative tests suggested a means of applying the reagent quantitatively in an endeavor to estimate minute quantities of amino acid alpha-nitrogen in solution.

The preliminary investigations began with a study of the reaction in aqueous solution between pure amino acids and

ninhydrin in equimolecular amounts. The amino acid solutions in every case were made by dissolving 0.5 grm. of the sample in distilled water and diluting to 500 cc.

One cc. of this 0.1 per cent solution of amino acid was then treated with the volume of a 0.1 per cent ninhydrin solution containing an equimolecular weight. The reacting volumes involved are given in the table showing the relative volumes of equimolecular quantities and excess. The problem now became to determine the time at which the maximum development of blue coloring matter took place. This could be accomplished by immersing a series of test-tubes, prepared as above, in a boiling constant-level water bath, removing them one by one at stated intervals (usually five minutes), diluting to a known volume (100 cc.) with distilled water, using the color produced in the test-tube first removed as standard, and comparing the others against it in a Duboscq colorimeter. At first the comparisons were carried out in daylight; the values thus obtained were so hopelessly at variance that duplicate determinations could not even be approximated. Hence, the manifold advantages of a dark room were utilised both for the preparation of the colors and subsequent comparison; more consistent readings were obtained by the use of a 25 watt tungsten lamp as the source of illumination. The lamp was placed in a conical, semi-opaque shade, the outer end of which was covered with a sheet of tissue paper to diffuse the light; and the whole placed about a foot away from the colorimeter. In this way a strongly and evenly illuminated field was obtained on the white reflector of the colorimeter only, and thus the eye was free from any other disturbing influences of light during the determinations.

Even under these conditions, which greatly improved one's ability to make concordant readings, the results continued to be very irregular, with the use of equimolecular amounts of the two active materials. In some cases - asparagine and aspartic acid - no reliable results were obtainable. The factors, which may have contributed to the failure of the experiments from a quantitative point of view, were the rapid fading of the coloring matter, the presence of traces of impurity, and the sensitiveness of the reaction to heat.

It was soon found, however, that a slight excess of triketohydrindene hydrate enabled one to approach a solution of the problem of the time of the maximum development of the coloring matter in dilute solution. The same method of procedure was adopted as that previously described, except that the test-tubes were now stoppered with absorbent cotton instead of being left open to the air as heretofore. The amount of amino acid used in each determination was 1 cc. of 0.1 per cent solution; the volumes of ninhydrin representing the equimolecular amounts and the volumes required to produce a slight excess are tabulated below:

Amino acid.	Volume of 0.1 per cent ninhydrin containing equimolecular amount.	Volume of the same solution used to produce a slight excess.
Alanine	2 cc.	2.25 cc.
Aspartic acid	1.3 "	2.0 "
Glutaminic acid	1.2 "	2.0 "
Asparagine	1.2 "	1.5 "
Glycine	2.4 "	3.0 "
Leucine	1.4 "	2.0 "
Tyrosine	1.0 "	1.5 "

It will be apparent from the above that the increase of

ninhydrin employed to represent the excess is not a constant value. This was purposely adopted to serve as a guide for the use of an excess of the reagent in future experiments.

The coloring matter in the test-tube first removed was used as standard and the others were read against it. The series of curves in Figure I shows the relative amounts of coloring matter plotted against the time, each amino acid being recorded separately.

It will be noticed that the time of the development of the maximum amount of color is different for each amino acid, thus precluding the use of this technique as a method of estimating amino acid alpha-nitrogen in a mixture of amino acids. No attempt was made to compare one amino acid against another, as such a series of experiments would have served no useful purpose at that time. Moreover, the actual amounts of color produced in the cases of aspartic and glutaminic acids and asparagine were so small that the reaction liquid was diluted to only 50 cc. instead of 100 cc. Also the colors produced by aspartic acid and asparagine were of a pronounced yellowish tint and could not be compared with the reddish-violet given by alanine or glycine.

Figure I - page 22.

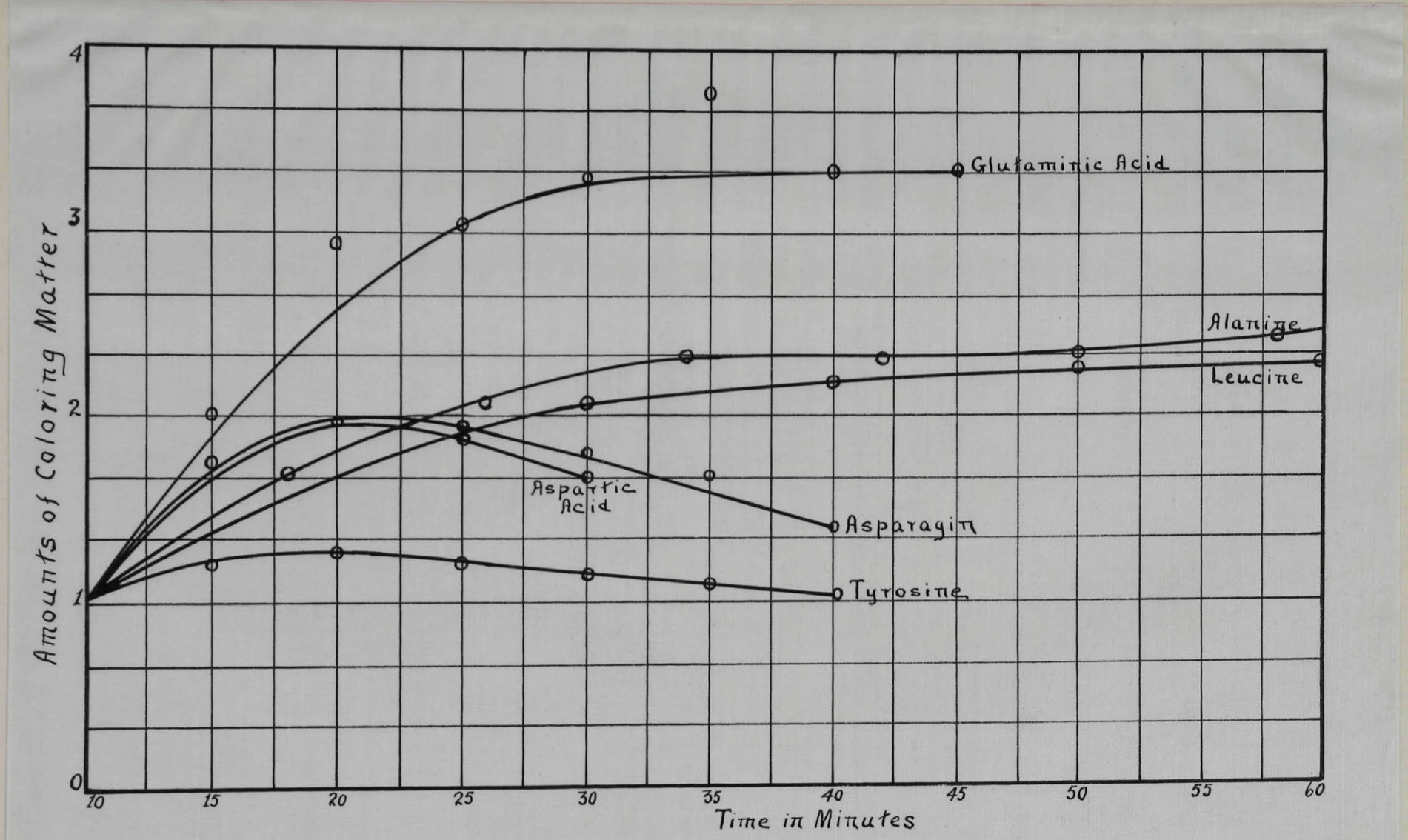

Amounts of Coloring Matter
0
1
2
3
4
10
15
20
25
30
35
40
45
50
55
60
Time in Minutes
Glutaminic Acid
Alanine
Leucine
Aspartic Acid
Asparagin
Tyrosine

Figure I

Some of the values from which the foregoing curves were plotted are shown below. In the instance of asparagine a noteworthy feature is the results which were obtained directly after the experiment was completed (a), and those procured after standing 24 hours in the dark room (b). When an attempt was made to get similar comparative results with glutaminic acid, the colors were found to have completely faded overnight. With the other amino acids examined, the colors were found to be less fugitive, although a slow rate of fading was noticeable about two hours after development. Experiments proved the measurement of the colors an invariable necessity within at least two hours from the time of actual color production.

Color standard was 10 minute sample.

Amounts of coloring matter of Amino acids.

Time in Minutes	Tyrosine	Asparagine (a)	Asparagine (b)	Glutaminic acid	Leucine	Glycine
15	1.20	1.70	1.45	2.00		1.25
20	1.27	1.98	1.87	2.94	1.80	1.26
25	1.20	1.94	1.77	3.03		1.26
30	1.15	1.78	1.57	3.28	2.05	1.28
35	1.11	1.70	1.57	3.77		1.21
40	1.04	1.38	1.36	3.33	2.16	1.13

HERZFELD'S METHOD.

During the progress of this investigation Herzfeld[25] devised and published a method for the estimation of the group $-CH\begin{matrix}NH_2\\COOH\end{matrix}$, using the ninhydrin reaction as a basis. The method was to evaporate the amino acid and excess of triketohydrindene hydrate to dryness on a water bath, dissolve the purple colored residue in a little alcohol with a drop or two of ammonium

hydroxide, make up to a known volume, and determine the amount of coloring matter by measuring its extinction coefficient in a spectrophotometer. This method of preparing the coloring matter in a quantitative way has been repeated, estimating it, however, by a Duboscq colorimeter instead of measuring the extinction coefficient in a spectrophotometer. The former method is much more rapid, and at present is the only one which could be successfully applied in hospital laboratories to the study of amino acid excretion in pathological conditions. The substitution of the Duboscq colorimeter for the spectrophotometer gave results which were unsatisfactory. It was found, however, that by heating varying amounts of the amino acid with an excess of ninhydrin the different amounts of the amino acid could be estimated with moderate accuracy, using a fixed amount of the same amino acid as standard. The following figures illustrate this point.

Amino acid	cc. of 0.1 per cent solution.	Colorimeter Readings Found:	Calculated:
Glycine	1.0 Standard	Set at 2.00 cm.	
	1.5	1.51	1.33
	2.0	1.00	1.00
	3.0	0.68	0.66
Alanine	1.0 Standard	Set at 2.00 cm.	
	1.5	1.17	1.33
	2.0	0.99	1.00
	3.0	0.63	0.66
	4.0	0.48	0.50
Aspartic acid	1.0 Standard	Set at $\frac{3}{4}$.00 cm.	
	1.5	1.89	2.00
	2.0	1.51	1.50
	3.0	0.95	1.00

Amino acid	cc. of 0.1 per cent solution	Colorimeter Readings Found:	Calculated:
Glutaminic acid	1.0 Standard	Set at 2.00 cm.	
	1.5	1.33	1.33
	2.0	1.01	1.00
	3.0	0.69	0.66

Thus it will be seen that varying amounts of glycine can be estimated by using the color produced by a known amount of glycine as standard. The same is true for alanine, and aspartic and glutaminic acids.

When, however, it was attempted to estimate alanine, or aspartic and glutaminic acids, using glycine as standard, the method gave totally erroneous results.

Glycine as Standard (1 cc. contained 0.187 mgm. N_2).

Colorimeter set at 2.00 cm.

Amino acid	Colorimeter Reading	Nitrogen per cc. Found:	Calculated:
Alanine	1.02	0.366 mgm.	0.157 mgm.
Glutaminic acid	2.46	0.152 "	0.095 "

In the case of aspartic acid the color produced by the reaction was more difficultly soluble in alcohol relative to the glycine pigment, and of such a pronounced reddish shade that it was found impossible to matbh it against the bluish-violet color of the standard.

Assuming all the amino nitrogen of the glycine to be decomposed, the above relation between glycine and alanine and glutaminic acid, shows that some of the color produced must be due to the ammonia. Thus it will be seen that this method of estimating amino acid nitrogen fails completely.

In applying the method to obtain the preceding results it was found that the dry residue after evaporation must be allowed to dissolve thoroughly in the alcohol before adding the ammonia and diluting to the required volume with water.

The action of ammonia towards this color is especially noteworthy, in two series of experiments which were carried out with glycine and ninhydrin. In Series I three drops of concentrated ammonia were used in the production of the pigment, while Series II consisted of a repetition of the first without the use of ammonia. Nineteen hours after the development of the color in Series I very little change was noticeable in the colors as compared with the values obtained immediately after the experiment. After standing several days in the dark room, these colors did not appear to fade appreciably when observed without the aid of a colorimeter. In Series II where no ammonia was used at all, the first three samples were observed to have faded to a light yellow color in a space of three days, whereas the fourth of this set had acquired a yellowish-red color somewhat resembling that of potassium dichromate.

From these considerations it would seem that the presence of ammonium hydroxide stabilises the colors to a certain extent, although the colors undoubtedly fade slowly at approximately the same rate, as shown by the daily preparation of fresh standards during such a period, when all the colors will be found to grow gradually weaker.

REACTION OF AMINO ACIDS WITH TRIHETCHYDRINDENE HYDRATE IN LARGE EXCESS.

In the next series of experiments there was determined the

time of the maximum development of color between the different amino acids and the triketone, using a large excess of the latter in high concentration. The necessity of a high concentration to produce quantitative amounts of color in this reaction had been pointed out by Herzfeld,[27] and a series of experiments confirmed this conclusion.

For the purpose of ascertaining the minimum amount of ninhydrin solution of high concentration necessary for the entire decomposition of the amino acid alpha-nitrogen, the following determinations were carried out:

I. 1 cc. glycine (0.1 per cent) and 1.cc. 1 per cent ninhydrin
II. " " " " 0.75 " " "
III. " " " " 0.50 " " "
IV. " " " " 0.30 " " "

The four test-tubes with the above contents were immersed in a boiling constant-level water bath for a period of ten minutes; comparisons were made as follows:

With I as standard and the colorimeter set at 2.0 cm.

II was found to read 1.99 cm.
III " " " " 1.97 "
IV " " " " 2.31 "

Since sample Number III furnishes a reading indicative of a complete reaction with the amino nitrogen, it will be quite evident that 0.5 cc of 1 per cent ninhydrin solution is the most practical quantity to decompose the nitrogen contained in 1 cc. of a 0.1 per cent amino acid solution.

Then there were heated together in a boiling constant-level water bath test-tubes containing 1 cc. of 0.1 per cent solution of amino acid and 0.5 cc. of 1 per cent solution of

triketohydrindene hydrate for periods of 5, 10, 15, and 20 minutes. The amount of color produced in the test-tube heated for five minutes was taken as standard, and the results were plotted as before, each amino acid being considered separately (Figure II).

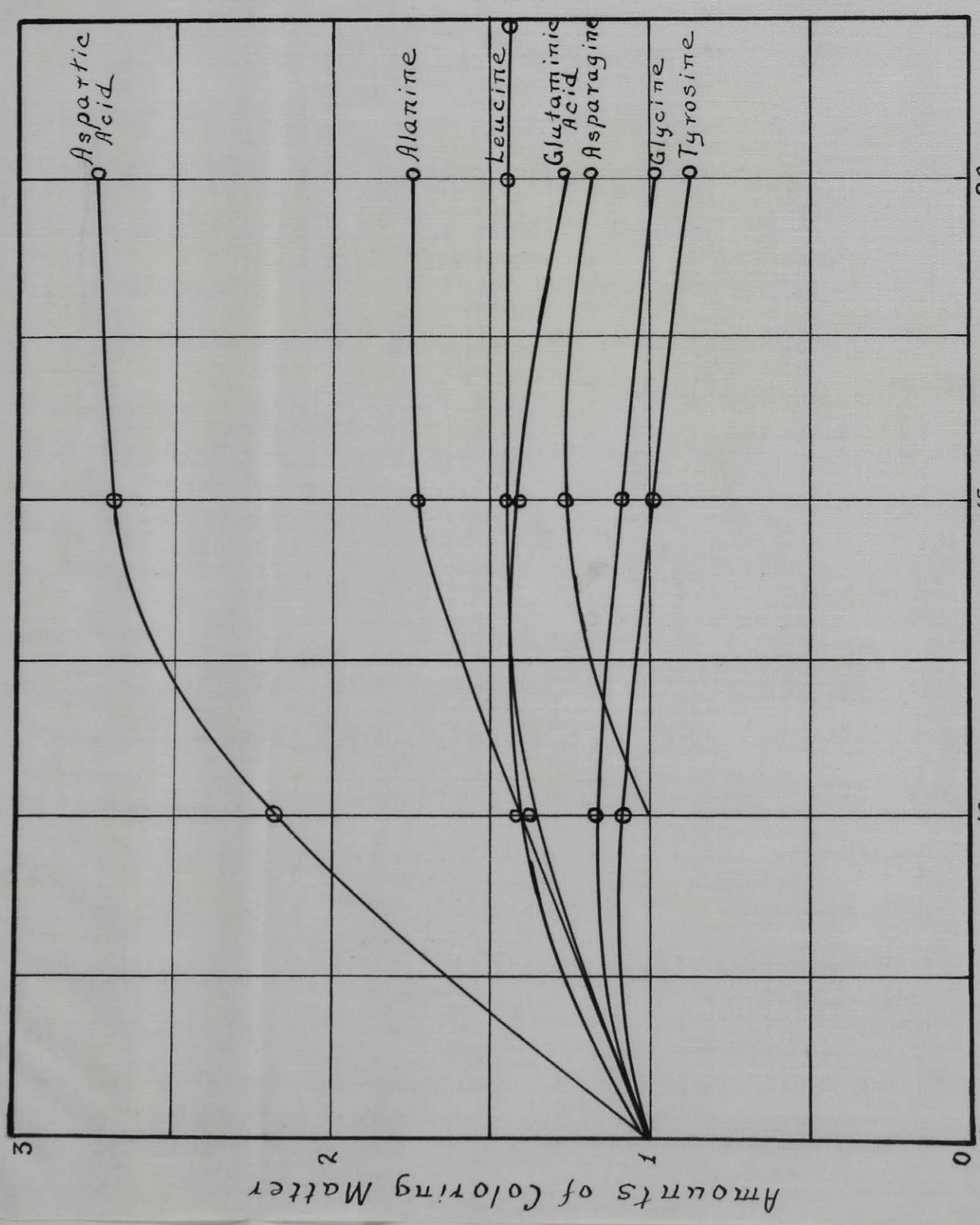


Figure II

TABLE TO CORRELATE FIGURE II.

Five minute standard.

Amounts of Coloring Matter.

Time in Minutes	Aspartic acid	Alanine	Leucine	Glutaminic acid	Glycine	Tyrosine
10	2.19	1.31	1.37	1.39	1.17	1.08
15	2.70	1.74	1.43	1.41	1.08	0.98
20	2.74	1.75	1.43	1.29	0.99	0.90

The effect of the higher concentration of the triketone was at once readily apparent. The amounts of color produced in the same time were much larger, and concordant experiments were easily obtained except in the case of asparagine. It will also be at once noticed that the times of maximum development of color with the different amino acids are much shorter than in the first series of experiments and lie closer together. Thus, in the first series, the times vary from twenty minutes (tyrosine) to seventy minutes (alanine), whereas in the second series the variation is only from ten minutes (glycine) to twenty minutes (aspartic acid and tyrosine). The other amino acids possess the time of maximum development of color at fifteen minutes. The colors with aspartic acid and asparagine, however, were still very weak and still possessed a strong yellowish tinge which rendered their comparison with the other acids extremely difficult. Other irregularities, too, were noticeable. The colors produced by aspartic and glutaminic acids faded very much more rapidly than those produced by the other amino acids. As these two acids differed from the others only in the presence of a second carboxyl group, thus rendering them more acidic in character, the disappearance of the coloring matter on standing was put down to this cause.

Ruhemann had pointed out that long contact with acids causes a decomposition of the coloring matter, giving a colorless solution, and experiments executed in connection with this research have proved the strong inhibitory effect of small amounts of organic acids on the production of the ammonium salt of diketohydrindylidenediketohydrindamine from the interaction of alanine and the triketone.

In order to test this assumption, a third series of experiments was carried out in presence of a base which would neutralise the acidity of the second carboxyl group of aspartic and glutaminic acids. Such a base should not be strong enough to hydrolyse the triketohydrindene hydrate, as happens with the hydroxides of the alkali metals (see page 4), and should not interfere with the reductions and condensations which take place in the reaction. Experiments proved that although ammonium hydroxide decreased the rate of fading of the color it could not be used as a means of assisting in the production of the maximum color. The base chosen was pyridine, since it was not ionised to such an extent as ammonium hydroxide.

INTERACTION OF AMINO ACIDS AND TRIKETOHYDRINDENE HYDRATE IN LARGE EXCESS IN PRESENCE OF PYRIDINE.

One cc.of a 0.1 per cent solution of the amino acid was added to 0.5 cc. of a one per cent solution of triketohydrindene hydrate, and 0.2 cc. of pure, freshly distilled pyridine was added, and the mixture heated in a boiling water bath for varying intervals of time as in the two previous series of experiments. The estimations of the relative amounts of coloring matter were carried out in a manner similar to the former experiments, each acid being compared with itself only. The curves

in Figure III show the results obtained. They prove quite clearly that the addition of the pyridine has achieved the desired result. They establish that the reaction between amino acids and triketohydrindene hydrate in presence of pyridine takes place rapidly, and reaches a maximum amount of coloration which remains constant for some minutes, except in the case of aspartic acid. This time of maximum development of color is constant at about twenty minutes. Moreover, the addition of pyridine has enormously increased the actual amounts of coloring matter produced, and the colors now did not fade appreciably in a short period of time. In the curves obtained with glycine and alanine 0.5 cc. of pyridine was used.

TABLE TO CORRELATE FIGURE III.

Color standard - 5 minutes.

Amounts of Coloring Matter:

Time in Minutes	Alanine	Aspartic acid	Leucine	Glutaminic acid	Glycine	Tyrosine
10	1.43	1.35	1.25	1.23	1.15	1.11
15	1.70	1.40	1.31	1.25	1.19	1.13
20	1.66	1.44	1.31	1.25	1.20	1.17

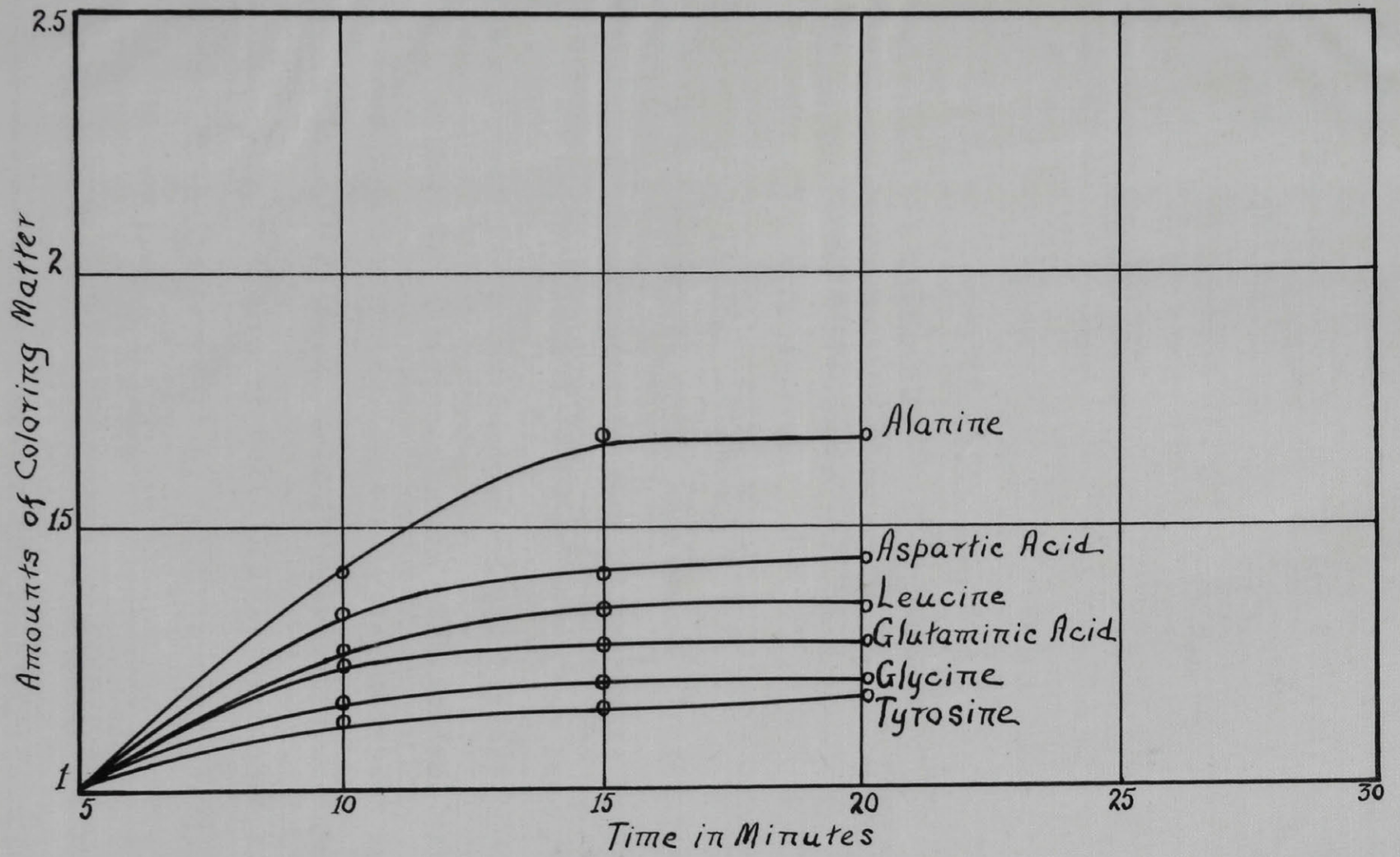

2.5
2
1.5
1
Amounts of Coloring Matter
Alanine
Aspartic Acid
Leucine
Glutaminic Acid
Glycine
Tyrosine
5
10
15
20
25
30
Time in Minutes

Figure III

At this juncture fresh amino acid solutions were prepared of such a strength as to contain 0.0001 gram amino acid alpha-nitrogen per cc. The various weights of the pure amino acids dissolved in 500 cc. of distilled water required to provide a solution such that one cc. contained 0.1 mgm. alpha-nitrogen, are given herewith:

Glycine	0.2678 gram.
Alanine	0.3178 "
Leucine	0.4678 "
Tyrosine	0.6464 "
Aspartic acid	0.4750 "
Glutaminic acid	0.5250 "
Cystine	0.4285 "
Tryptophane	0.7286 "
Phenylalanine	0.5893 "

A determination was then made of the relative amounts of coloring matter produced by four of the amino acids, whose purity was established, and solutions of which had been made in accordance with the foregoing equivalent quantities.

An alanine solution of this strength was taken as standard for these determinations. This solution had been made from Kahlbaum's pure alanine, freshly recrystallised and found by Kjeldahl determination to contain 15.79 per cent nitrogen as compared with the theoretical percentage of 15.73.

The following table will illustrate the experiment and the results. Being in equivalent amounts, they should produce the same amount of coloring matter and thus give the same colorimeter reading.

Amino acid	N_2 per cc.	Pyridine (pure)	Triketone 1.0 per cent	Colorimeter reading
	mgm.	cc.	cc.	
Alanine	0.1	0.2	0.5	1.00
Glycine	0.1	0.2	0.5	0.99
Aspartic acid	0.1	0.2	0.5	1.17
Glutaminic "	0.1	0.2	0.5	1.01

The ratio determination of several other amino acids against alanine furnished satisfactory results with the exception of histidine and cystine. The sample of the former was histidine hydrochloride, and the abnormal results were ascribed to the presence of hydrochloric acid. This proved to be the case, for, after neutralisation with sodium hydroxide the determinations were much more reliable.

Great difficulty was experienced in matching the cystine color against alanine, owing to the reddish appearance of the former. The sulphur of the cystine is believed to be responsible for this anomaly.

Aspartic acid is shown to be still a little low in amount of coloring matter produced when compared with the other acids. For this reason some amino acid solutions were made still more dilute, namely, so that one cc. contained 0.05 mgm. amino alpha-nitrogen. The ratios then obtained with alanine as standard and the colorimeter set at 1.0 cm. were:

Glycine	1.01
Aspartic acid	1.06
Glutaminic "	1.00

Amino acid Ratios in presence of Possible Disturbing Influences:

Alanine standard (1.0 cc. contained 0.05 mgm. N_2).

Colorimeter set at 2.0 cm.

Amino acid	5% Glucose	Sodium Urate (saturated)	2% Urea	0.1% Creatinine
Alanine	2.05	1.87	3.03	2.18
Glycine	1.77	1.76	2.27	2.06
Aspartic acid	2.14	1.91	3.28	2.10
Glutaminic "	1.79	1.89	2.80	2.19

Urea thus appears to exert the greatest inhibitory effect, whereas sodium urate and creatinine do not interfere to an irremediable extent. The effect of glucose, considering the strength of its solution and consequent reducing power, is not as great as might well be expected.

DETERMINATION OF AMINO ACID ALPHA-NITROGEN IN VARIOUS AMINO ACIDS AND COMPARISON WITH THE METHOD OF VAN SLYKE.

A series of determinations was then made of the amino acid alpha-nitrogen in various amino acids, and the results were compared with theory in the case of the pure acid. A parallel series of determinations was made by the method of Van Slyke and the results were compared with the colorimetric determinations. In the case of acids whose purity was doubtful, the method of Van Slyke served as a standard. The results are expressed in mgm. of nitrogen per cc.

Amino acid	Nitrogen per cc.		
	Theory	Colorimeter	Van Slyke
	mgm.	mgm.	mgm.
Glycine	0.186	0.189	0.189
Alanine	0.100	0.101	0.102
Valine	0.201	0.201	0.196
Leucine		0.104	0.102
α-Amido-n-caproic	0.160	0.164	0.161
Phenylalanine	0.128	0.126	0.126
i-Tyrosine		0.084	0.087
Tryptophane		0.090	0.092
Histidine		0.085	0.089
Aspartic acid	0.105	0.103	0.104
Asparagine	0.175	0.178	
Glutaminic acid	0.158	0.157	0.157

The results show quite clearly the excellent agreement of the colorimetric method with theory and with the method of Van Slyke. It must be pointed out that the solutions of the amino acids were made of just sufficient concentration to be estimated in the Van Slyke micro apparatus. To determine the amount colorimetrically, it was necessary to dilute those solutions from two to four times, so that the actual error of determination is less than appears in the table.

The estimation of cystine, however, by this method gave results which were much too low. Moreover, there developed an intense red color, which rendered a comparison with the standard alanine impracticable. As a consequence of this, it would be impossible to estimate the amino acid alpha-nitrogen in a

mixture of amino acids containing a large proportion of cystine without a large error. Such cases, however, are not very common, and it is not likely that the amount of cystine, occurring in the hydrolysis mixture obtained from the majority of proteins, will introduce any serious error. To determine this point and to see if the method would estimate the alpha-nitrogen in the proportions in which they occur in a native protein, an estimation of the amino acid alpha-nitrogen in ereptone was made and compared with the method of Van Slyke.

EREPTONE

Method.	Nitrogen per cc.	Nitrogen.
	mgm.	per cent
Colorimeter	0.132	8.5
Van Slyke	0.139	9.0

It will be seen that the colorimetric method gives figures slightly lower than the Van Slyke method, but not low enough to seriously invalidate the result. The difference, however, is capable of easy explanation. The colorimetric method gives too low a result in presence of cystine. The Van Slyke method gives a result a little too high in the cases of glycine and cystine. These three facts are sufficient to explain the difference of 0.5 per cent.

DETERMINATION OF NINHYDRIN CONCENTRATION FOR THE ESTIMATION OF MIXED AMINO ACID ALPHA-NITROGEN.

EREPTONE: Alanine standard (1.0 cc. contained 0.05 mgm. N_2)

Strength of ninhydrin	Nitrogen value per cc. Found:	Corrected for dilution:	Van Slyke:
1.0 per cent	0.0394 mgm.	0.118 mgm.	0.139
2.0 " "	0.0438 "	0.131 "	
3.0 " "	0.0442 "	0.132 "	

These determinations were carried out on a solution of ereptone which was the original diluted 1:2 after the Van Slyke determination had been made.

As a result of these and other experiments it was decided to reduce the amount of amino acid alpha-nitrogen per cc. to 0.05 mgm.; to reduce the pyridine to 1.0 cc. of a 10 per cent aqueous solution, and to increase the triketohydrindene hydrate to 1.0 cc. of a 2.0 per cent solution.

METHOD OF ESTIMATION OF AMINO ACID ALPHA-NITROGEN BY USE OF TRIKETOHYDRINDENE HYDRATE.

One cc. of the solution to be estimated, containing not more than 0.05 mgm. of amino acid alpha-nitrogen and neutral to phenolphthalein, is mixed with 1 cc. of a 10 per cent aqueous solution of pure pyridine and 1 cc. of a freshly prepared 2.0 per cent solution of triketohydrinene hydrate and heated in a rapidly boiling constant-level water bath for twenty minutes. At the end of that time the test-tube is removed, cooled under running water, and diluted to a suitable volume, usually 100 cc., but if the amino acid alpha-nitrogen is very small in amount, a correspondingly smaller dilution should be used. The solution of coloring matter thus obtained is compared with the standard color in the usual way, in a Duboscq colorimeter.

PREPARATION OF A STANDARD COLOR.

The standard solution is prepared by dissolving 0.3178 of a gram of pure, freshly crystallised alanine in a liter of distilled water. Such a solution contains 0.05 mgm. of amino acid alpha-nitrogen per cc. To prepare the standard color, 1 cc. of the standard alanine solution is heated for twenty minutes in a boiling water bath with 1 cc of a 10 per cent solution of pyridine

and 1.0 cc. of a 1 per cent solution of triketohydrindene hydrate, (the employment of a more concentrated solution of the triketone gives no further increase in the amount of coloring matter in the case of alanine). At the end of that time the contents of the tube are cooled and diluted to 100 cc. This solution of coloring matter is used as a standard and will keep for twelve hours.

The standard solution of alanine is stable for three months. Attempts were made to prepare a permanent standard color without success. A preparation of the pure coloring matter was made according to the directions of Ruhemann,[28] and a dilute solution of it made in water. It was found, however, that when the dried coloring matter was used, it was not as freely soluble in water as the freshly prepared substance, and that an aqueous solution of the pure coloring matter faded rapidly; it was much more stable in the presence of pyridine, but even then at the end of three months it had faded completely. There was thus no advantage to be gained in using a solution of the coloring matter prepared per se. The standard color prepared as directed is little or no trouble to effect along with the determinations themselves.

A series of determinations of the amino acid alpha-nitrogen in peptones from various sources was next carried out in order to test the efficacy of the method when dealing with partial hydrolysis products of proteins. The accompanying table shows the results to be in good agreement with those obtained by the Van Slyke method.

Origin of Peptone	Nitrogen per cc.	
	Colorimeter.	Van Slyke.
Meat (Merck)	0.062 mgm.	0.061 mgm.
"E carne" (Schuchardt)	0.035 "	0.031 "
Precipitated by alcohol (Schuchardt)	0.025 "	0.021 "
Witte	0.067 "	0.067 "

The early experimental results described in this treatise (page 34) have shown that although some of the amino acids could be accurately estimated when the solution contained 0.1 mgm. of nitrogen per cc., yet other amino acids could be estimated only when the nitrogen was 0.05 mgm. per cc. This value is taken as the maximum value. To determine the minimal value, two series of experiments were performed, one on an alanine solution, the other on an ereptone solution. A standard solution of alanine and a solution of ereptone were diluted by known amounts of water and colorimetric estimations made of the amino acid alpha-nitrogen present in the diluted solutions. The coloring matter so produced was diluted to a suitable volume so that the colorimetric readings did not differ from the standard by large amounts. The theoretical values given against the results obtained for ereptone solutions were procured by calculating from the mean result of the colorimetric and Van Slyke determinations given on page 37.

DETERMINATION OF LIMITS.

Standard Alanine (1 cc. contained 0.05 mgm. N_2).

Colorimeter set at 1.0 cm.

Dilutions	Original	1 in 2.	1 in 4.	1 in 8.	1 in 16.
ALANINE:					
Theoretical Values	0.100 mgm.	0.05	0.025	0.0125	0.0062
Van Slyke	0.102 "	-	-	-	-
Colorimeter	0.100 "	0.05	0.025	0.0117	0.0050
EREPTONE:					
Theoretical Values	0.135 "	0.067	0.033	0.016	0.008
Van Slyke	0.139 "	-	-	-	-
Colorimeter	0.132 "	0.065	0.032	0.016	0.008

Thus it may be seen that the method is accurate over a range of 0.05 mgm. to 0.005 mgm. per cc. Whether amounts less than 0.005 mgm. can be estimated, has not been determined. At that concentration the amount of coloring matter produced is so small that it can only be measured with difficulty. For this purpose, test-tubes graduated to 20 cc. were employed and found to be very satisfactory. These test-tubes were used for the production of the color, and, after the requisite twenty minutes in the boiling water bath, were diluted to the 20 cc. volume for the determination of the coloring matter.

Qualitatively, it is possible to detect as little as 0.001 part of a mgm. of amino acid alpha-nitrogen in 1 cc. of solution. This, in the case of alanine, means the detection of one part of the amino acid in a little over 1,500,000 parts of water.

From the results obtained with ereptone (page 37), and the various peptones (page 40), the method can be used for the

determination of the amino acid alpha-nitrogen set free in the hydrolysis of proteins. Its application to the analysis of urine and blood, however, is still attended with difficulties, ammonium salts and urea causing disturbances in the reaction.

Thus it will be seen that:

(1) a method has been devised for the estimation of amino acid alpha-nitrogen colorimetrically;

(2) that this method as compared with the Van Slyke will estimate minute quantities of amino acid alpha-nitrogen with an accuracy equal to that of the ~~former~~ latter;

(3) it cannot be employed for the estimation of cystine owing to a secondary reaction which develops a red color;

(4) it is applicable to the determination of amino acid alpha-nitrogen (in neutral solution) set free in all protein hydrolyses.

QUANTITATIVE CONSIDERATIONS OF THE REACTION BETWEEN TRIKETOHYDRINDENE HYDRATE AND AMMONIUM SALTS.

As has already been pointed out in this theme (page 5), Ruhemann described his results concerning the interaction of ammonium hydroxide and the triketone. The results of Neuberg showed that many ammonium salts gave a positive reaction with ninhydrin and were in direct contradiction to the earlier observations of Abderhalden and Schmidt. This point is extremely important, on account of the fact that if ammonium salts do react with triketohydrindene hydrate, the reaction is no longer a specific test for the detection of amino acids. Herzfeld also reported that ammonium carbonate and ammonium oxalate reacted positively. These facts led to some independent investigations in this laboratory along similar lines. At first the results were very confusing.

The uncertainty which had developed in view of these discrepancies has been elucidated by Harding,[29] and Harding and Warneford.[30] These authors have examined the action of nearly twenty ammonium salts of various types, and have obtained uniform results, by employing quantitative methods. The standard ammonium salt concentration adopted for this research was one per cent. Those ammonium salts which failed to give the characteristic blue coloration at this concentration were therefore classed as negative.

At this concentration the ammonium salts of weak acids react positively; whereas the ammonium salts of strong mineral acids react only at a very high concentration (almost approaching saturation). When the concentration was made very low (1 cc. containing 0.05 mgm. ammonium nitrogen), negative results were obtained, with the exception of ammonium sodium hydrogen phosphate.

These results also show why Ruhemann obtained a positive color reaction in applying the ninhydrin reaction qualitatively to urine; his erroneous conclusion that urine must contain a protein-like substance (loc. cit. pages 6 -7), can now be readily explained by the presence of the ammonium salts.

The next point investigated was the behavior of the ammonium salts in presence of pyridine towards the triketone. For these experiments a uniform concentration of 1 cc. = 0.05 mgm. ammonium nitrogen was used, the reaction carried out in exactly the same way as for $\overset{\alpha\pi}{\wedge}$ amino acid alpha-nitrogen determination, and the colors compared with the customary standard alanine solution.

Working in this way it was found that the decomposition

of ammonium nitrogen was constant at about 0.018 mgm. per cc., or approximately 36 per cent of the total.

Thus it will be seen that under the proper conditions, all ammonium salts are capable of giving the ninhydrin reaction. Such conditions appear to be a faintly alkaline medium - either furnished by the ammonium salts themselves or by pyridine. The color in all cases has been identified as the ammonium salt of diketohydrindylidenediketohydrindamine.

The authcrs suggest, as a working hypothesis, that this type of ninhydrin reaction may be ~~applied~~ explained by the intermediate formation of glyoxal (loc. cit.). With pyridine the reaction is the same for all ammonium salts, irrespective of the acid radicles. Without pyridine, however, only the ammonium salts of weak organic acids and ammonium sodium hydrogen phosphate are positive; in other words, those salts which are easily hydrolysed. Ruhemann has proved that the action of alkalies upon the triketone is the opening of the ring with the formation of phenyl-glyoxal-o-carboxylic acid (page 4). This undergoes condensation with ammonia and a second molecule of triketone to form the ammonium salt of diketohydrindylidenediketohydrindamine.

The influence of reducing agents on the decomposition of ammonium nitrogen is strikingly shown in the following table of a partial list of results obtained by these investigators.

Ammonium salt.	Glucose (0.5%).	Glucose (5.0%).
Chloride	0.025 mgm.	0.032 mgm.
Nitrate	0.027 "	0.031 "
Acetate	0.02b "	0.032 "
Benzoate	0.026 "	0.032 "

Dialuric acid was also found to exert a very important influence upon the decomposition of ammonium nitrogen. A cold saturated solution of dialuric acid raises the decomposition to nearly 90 per cent of the theoretical. Another solution of this acid, known to contain considerable hydrindantin, did not exhibit such a marked effect. The colors produced in this case were of a red shade and difficult to match. From this and further experiments with pure hydrindantin the conclusion was drawn that these troublesome reddish-yellow colors were created by an excess of hydrindantin, which does not decompose readily to give the ammonium salt of diketohydrindylidenediketohydrindamine under the influence of ammonia.

It will thus be seen that the ninhydrin reaction as applied as a colorimetric method for the determination of amino acid alpha-nitrogen cannot be employed for the evaluation of physiological fluids or under any circumstances where the ammonium salts are likely to be present in admixture.

REACTION OF ORGANIC BASES WITH TRIKETOHYDRINDENE HYDRATE.

As with ammonium salts, a uniform concentration of one per cent was also adopted for the bases, and depending upon whether or not the bases gave a blue coloration with ninhydrin at this concentration have they been classified as reacting positively or negatively. Of the amides, urea, ureides and urethane were found to be generally negative. In the case of primary bases all of the reactions were positive and, moreover, the results agreed individually with Neuberg's observations.

Bases of the type $R\!>\!CH.NH_2$ and $\begin{matrix} R \\ R \\ R \end{matrix}\!>\!C.NH_2$ were found for the most part to be negative with the exception of glucosamine.

Secondary amines, except piperazine and adrenaline, which

latter oxidises very easily, were mainly negative, while all tertiary amines examined, also failed to respond to the test. Compounds containing an imino group such as guanidine, creatine and creatinine were found to be negative.

PART II.

APPLICATION TO THE HYDROLYSIS OF PROTEINS BY PANCREATIC ENZYMES.

It was claimed that this method could be used as a means of following protein hydrolysis, and this division of the treatise contains the evidence in support of such a belief.

THE STANDARD ALANINE SOLUTION.

The standard alanine solution is made as previously directed by dissolving 0.3178 grm. of freshly crystallised alanine in water and making the volume up to one liter. Its stability varies a little. Thus one solution was found to be stable for a period of three months, while a second only gave about 95 per cent of the theoretical amount of standard color at the end of that time. In consequence, it has been found advisable to check the stability of the standard alanine by comparing it from time to time with a freshly prepared solution.

THE STANDARD COLOR.

Several further attempts have been made to prepare a permanent standard color, but without success. The direct preparation of the ammonium salt of diketohydrindylidenediketohydrindamine by the interaction of a mixture of ammonium acetate and carbonate upon hydrindantin[31] is anything but quantitative, and conditions have not been found under which the blue coloring matter is absolutely stable.

In the endeavor to prepare artificial standards from mixtures of various dyestuffs and coloring matters, the only partially successful experiment was a mixture of phenolphthalein and thymolphthalein in alkaline solution. This matched the standard alanine color perfectly, but possessed no advantages over it, as it was even less stable. The standard color is therefore prepared as previously directed; 1 cc. of 10 per cent aqueous solution of pyridine may replace the 0.5 cc. recommended, if it is so desired.

The stability of the standard color seems to vary. The minimum time of stability of the standard color which has been observed is three hours; the maximum is twelve. Hence a standard color which has been prepared over a period longer than three hours cannot be recommended.

The production of the color in the standard, and hence in the determinations, is in all probability absolutely quantitative, and that it cannot be increased by the use of excess of either pyridine or triketohydrindene hydrate has been established by the following series of observations.

1 cc. of standard alanine used in each experiment:

1 cc. pyridine concentration	1 cc ninhydrin concentration	Colorimeter Reading.
per cent	per cent	
5	1	1.07
10	1	1.00
10	2	1.01
15	1	0.98
25	1	1.00

The use of 1 cc. of 5 per cent aqueous solution of

pyridine, however, gives too low a result. This is interesting as 0.5 cc. of 10 per cent aqueous solution of pyridine gives the maximal value.

MAXIMAL AND MINIMAL AMOUNTS OF AMINO ACID ALPHA-NITROGEN DETERMINABLE BY THE METHOD.

These two points were fixed at 0.05 mgm. and 0.005 mgm. nitrogen per cc. Judging by experience with the various proteins cited in this section, the method gives accurate results with 0.08 mgm. of amino acid alpha-nitrogen per 1 cc. When dealing with mixtures of the amino acids it appears quite a safe procedure to estimate the alpha-nitrogen in a higher concentration than is possible with some of them by themselves, and this is, of course, to be expected. Even this value is probably too low, but it is preferable to err on the safe side. The lower limit, however, is perhaps too low for practical convenience in this field of work. It is possible to obtain accurate results at that concentration of nitrogen, but they seem to be extremely susceptible to error, and if possible, it is not advisable to use a limit lower than 0.01 mgm. amino acid alpha-nitrogen per cc. Even then great care must be taken in the determination.

THE CASE OF CYSTINE.

As previously pointed out, cystine is the one amino acid among those examined which fails to give quantitative results. Furthermore, it has not been possible to devise any suitable modification whereby this amino acid can be estimated by the colorimetric process. The colors produced are always very red and very low in amount, due to the formation of either excessive amounts of hydrindantin, or sulphur derivatives. Judged by the Van Slyke

method as standard, however, it does not seem to interfere in the estimation of the amino acid alpha-nitrogen in the majority of hydrolysed proteins, thus bearing out the original contention.

METHOD OF ESTIMATION OF AMINO ACID ALPHA-NITROGEN BY THE COLORIMETRIC PROCESS.

One cc. of the solution to be estimated, containing between 0.08 and 0.01 mgm. of amino acid alpha-nitrogen and neutral to phenolphthalein, is mixed with 1 cc. of 10 per cent aqueous solution of pure pyridine, and 1 cc. of a freshly prepared two per cent solution of triketohydrindene hydrate (ninhydrin), in a test-tube, and stoppered lightly with an absorbent cotton tampon. The test-tube is placed in a rapidly boiling constant-level water bath for a period of twenty minutes, then removed, cooled by immersion in cold water, and diluted to a suitable volume, which must be arranged so that the colorimetric readings do not differ greatly from the reading of the standard color. The comparison is made in a Duboscq colorimeter, placed in a dark room, and illuminated as already described (page 19).

The standard color is prepared concurrently by similarly heating 1 cc. standard alanine solution, 1 cc. of 10 per cent aqueous pyridine, and 1 cc. of one per cent solution of triketo-hydrindene hydrate, for a period of twenty minutes, cooling and diluting to 100 cc. It is important that the water bath used in heating the solutions shall be large and of constant level, otherwise the heating may become uneven and irregular results obtained. Too much emphasis cannot be laid upon this point, because the non-agreement of two series of results was traced directly to this cause.

APPLICATION OF THE METHOD TO THE STUDY OF THE HYDROLYSIS OF PROTEINS BY PANCREATIC ENZYMES.

In order to determine experimentally the application of the colorimetric method to a study of proteoclasis, the hydrolysis of various types of proteins by pancreatic enzyme has been followed by its means, and the results obtained compared with those acquired by the Sörensen and Van Slyke methods. The use of the pancreatic enzymes, as proteoclastic agents, gives results which are not complicated by other factors, such as high acidity or the production of ammonia,[32] and thus makes all determinations simpler and less subject to error. The hydrolysis was allowed to proceed for three or four days in presence of toluene until the amino acid alpha-nitrogen had become almost constant. It is to be noted, nevertheless, that in no case did the values ever become absolutely constant, though the rate of hydrolysis became very small, yet it never entirely ceased; even at the end of 216 hours the hydrolysis of casein was still proceeding.

The proteins in these experiments were serum albumin and globulin (Schuchardt), gluten (Schuchardt), fibrin (ox blood), gelatin, casein (purified), nucleoprotein (Schuchardt), Witte peptone and peptone "e carne" (Schuchardt). They were used in solutions of approximately 0.5 per cent, and to 100 cc. of the protein solution were added 1 cc. of the pancreas preparation, 1 cc. of 0.5 per cent sodium carbonate solution, and 2 cc. of toluene. Several such solutions were made and all incubated together at 38 - 39° C.

The enzyme solutions used were two in number. The first

was a preparation of the pancreas by Fairchild Brothers and Forster, and sold under the name of "holadin" and was used in either a 0.2 or a one per cent solution. This preparation is supposed to contain trypsin. It was found, however, that although it was active towards casein, gelatin, and peptones, it was entirely inactive towards serum albumin and globulin, thus corresponding in its properties to erepsin rather than to trypsin. Subsequently it was ascertained that its proteoclastic powers could be extended to fibrin and to gluten. There is undoubtedly, even yet, much uncertainty and confusion existing about the activities of these two classes of proteoclastic enzymes, and the age of the sample may have been the cause of such a result. Nevertheless, as the enzyme was inactive towards albumin and globulin, it is preferable to class it as the pancreatic erepsin discovered by Vernon[33] rather than as trypsin. It did not belong to the autolytic class of enzymes producing large quantities of ammonia. This point has been examined experimentally and there were found the following amounts of ammonia produced by the action of this enzyme on a 0.5 per cent solution of protein in 100 hours; these were then compared with the amounts of amino acid alpha-nitrogen.

100 cc of the protein solution (0.5 per cent).	Ammonia nitrogen.	Amino acid alpha-nitrogen.
Casein	0.67 mgm.	22.7 mgm.
Witte peptone	0.28 "	24.3 "
Peptone "e carne"	0.44 "	17.0 "

The small amounts of ammonia are thus insignificant compared with the quantities of amino acid alpha-nitrogen.

The second enzyme preparation was an alcoholic extract

of pig's pancreas prepared according to the directions given by Cole.[34] This agreed in its proteoclastic properties with trypsin.

At the end of stated intervals the flasks containing the hydrolysis experiments were removed from the thermostat, boiled for about fifteen seconds, and the amino acid alpha-nitrogen content was determined by the three aforementioned methods.

The colorimetric method was applied, as already prescribed, directly to the solution. The alkalinity caused by the presence of the sodium carbonate was found to be too small to affect the results.

In the Van Slyke method the micro form of apparatus[35] was used. Care was taken in the standardisation of the apparatus and the use of reagents.

To apply the Sörensen method, 20 cc. of the solution, neutral to phenolphthalein, were withdrawn, excess of neutral formaldehyde was added, and the mixture then titrated with tenth-normal sodium hydroxide, until a deep pink solution resulted, the color of which was permanent a full five minutes.

The general results can best be seen by an inspection of the curves shown in Figures IV to XI.

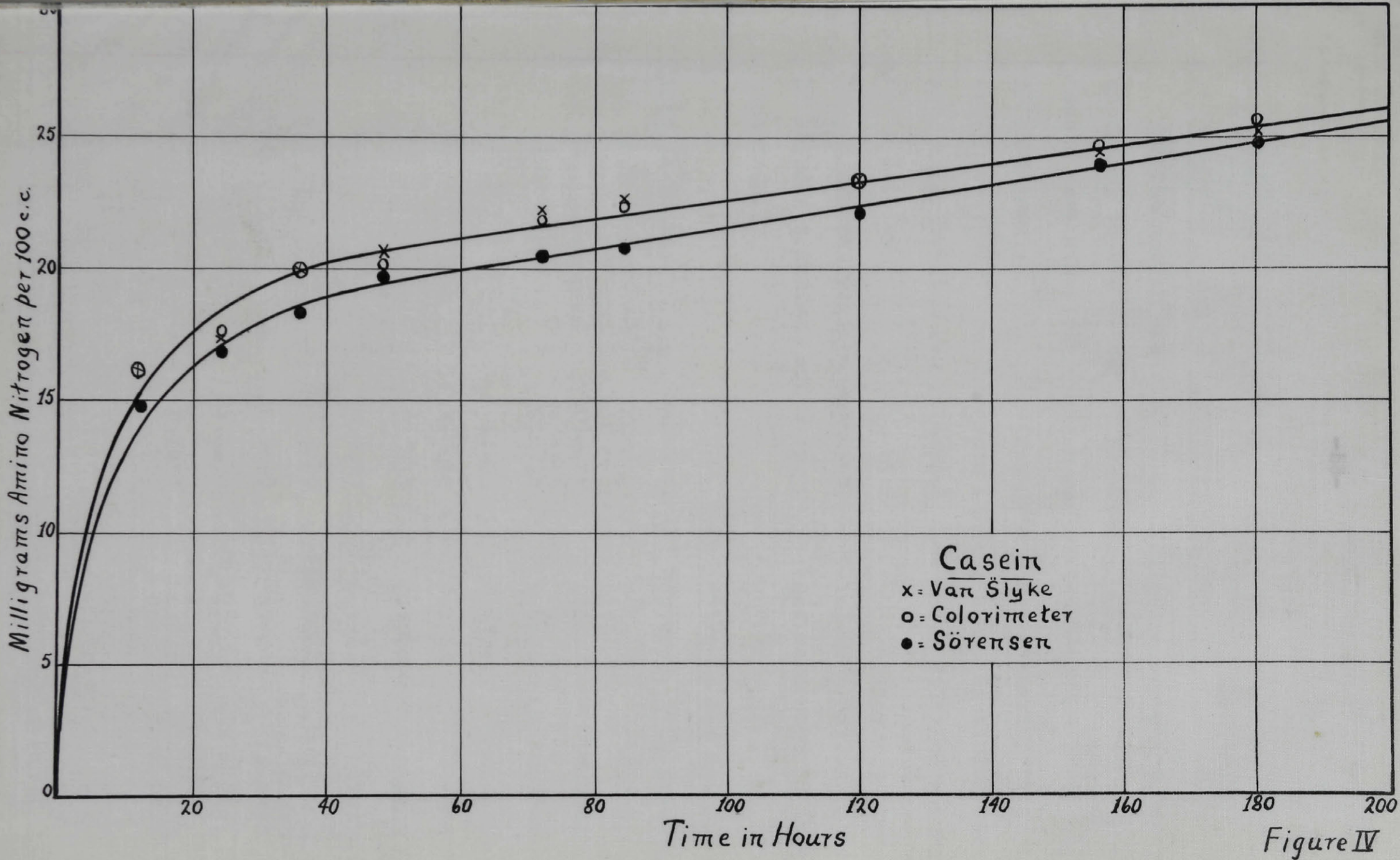

Milligrams Amino Nitrogen per 100 c.c.
0
5
10
15
20
25
20
40
60
80
100
120
140
160
180
200
Time in Hours
Casein
x = Van Slyke
o = Colorimeter
● = Sörensen

Figure IV

CASEIN (FIGURE IV).

The casein employed in the following digestion was purified according to the method of Robertson.[39] 5.6 grams of this pure casein were dissolved in 44.8 cc. of tenth-normal sodium hydroxide in pursuance of Walters' directions[40] for preparing basic sodium caseinate (0.4%). To this were added 300 cc. distilled water, and, after boiling and filtering, the clear filtrate was made up to 1400 cc.

100 cc. protein solution, 1 cc. of 0.5% Na_2CO_3, 1 cc. of 1% holadin solution, and 2 cc. of toluene were used in each experiment.

	Mgm. Amino Nitrogen per cc.		
Time in Hours.	Van Slyke.	Sörensen.	Colorimeter.
0.5	0.0386	0.0297	0.0384
12	0.1628	0.1484	0.1638
24	0.1748	0.1696	0.1754
36	0.1991	0.1838	0.2000
48	0.2068	0.1987	0.2082
72	0.2211	0.2050	0.2180
84	0.2257	0.2121	0.2222
120	0.2329	0.2263	0.2326
156	0.2440	0.2403	0.2462
180	0.2510	0.2474	0.2564
216	0.2657	0.2615	0.2632

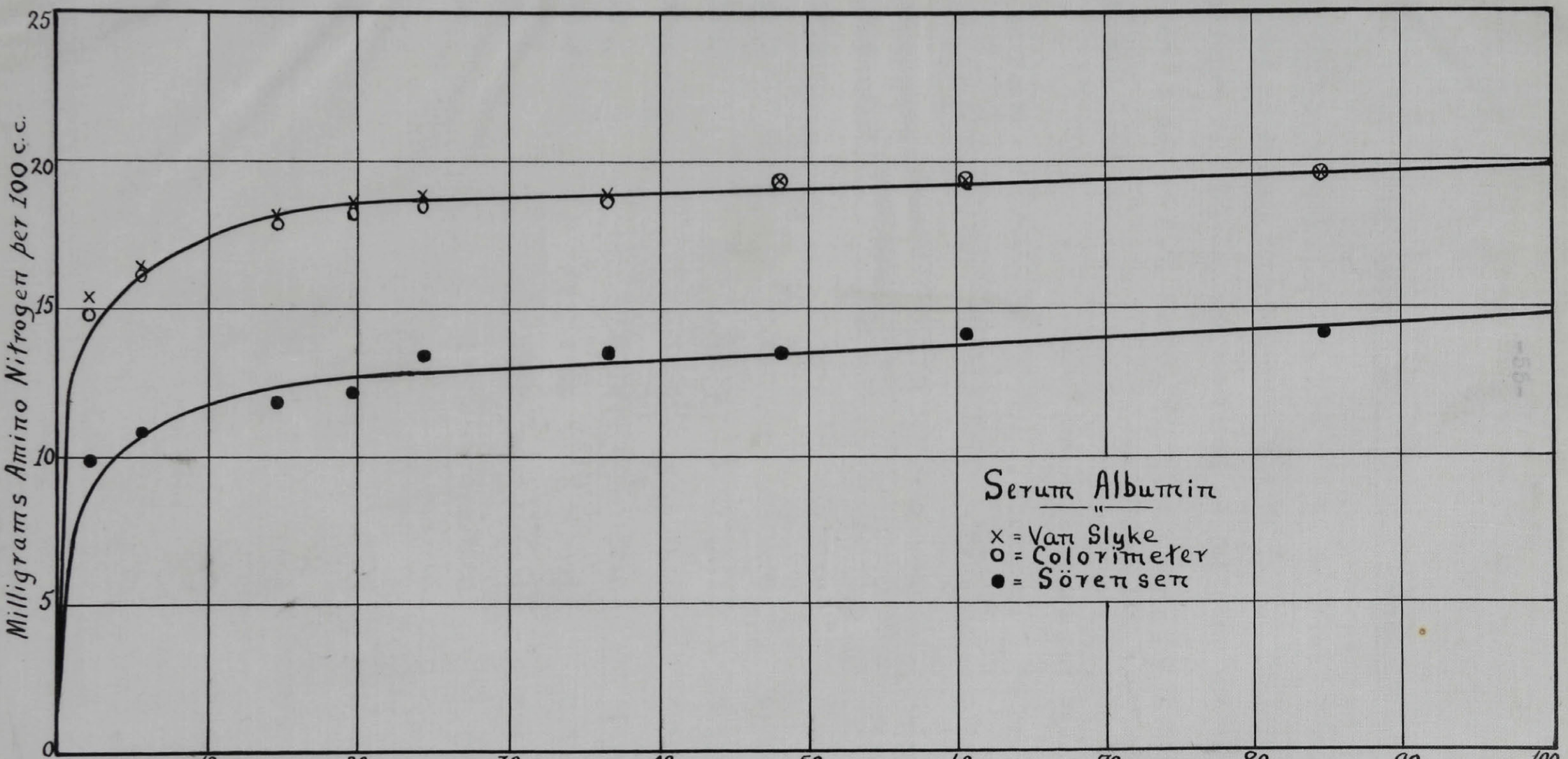

Milligrams Amino Nitrogen per 100 c.c.
25
20
15
10
5
0
10
20
30
40
50
60
70
80
90
100
Time in Hours
Serum Albumin
"
× = Van Slyke
o = Colorimeter
● = Sörensen

Figure V

SERUM ALBUMIN AND GLOBULIN (FIGURE V).

In the endeavor to prepare a 0.5% solution, 6 grams were dissolved in dilute NaCl solution and filtered. The clear filtrate was diluted to 1200 cc. and employed as follows: 100 cc.protein solution, 1 cc. of 0.5% Na_2CO_3 solution, 1 cc. pancreatic extract, and 2 cc. of toluene.

	Mgm. Amino Nitrogen per cc.		
Time in Hours.	Van Slyke.	Sörensen.	Colorimeter.
0	0.0612	0.0282	0.0200
2	0.1538	0.0991	0.1471
5.5	0.1638	0.1061	0.1622
14.5	0.1812	0.1169	0.1786
19.5	0.1841	0.1204	0.1818
24	0.1880	0.1346	0.1832
36.5	0.1880	0.1346	0.1872
48	0.1915	0.1346	0.1922
60.5	0.1922	0.1417	0.1930
84.5	0.1950	0.1417	0.1952
114.5	0.1997	0.1558	0.1976

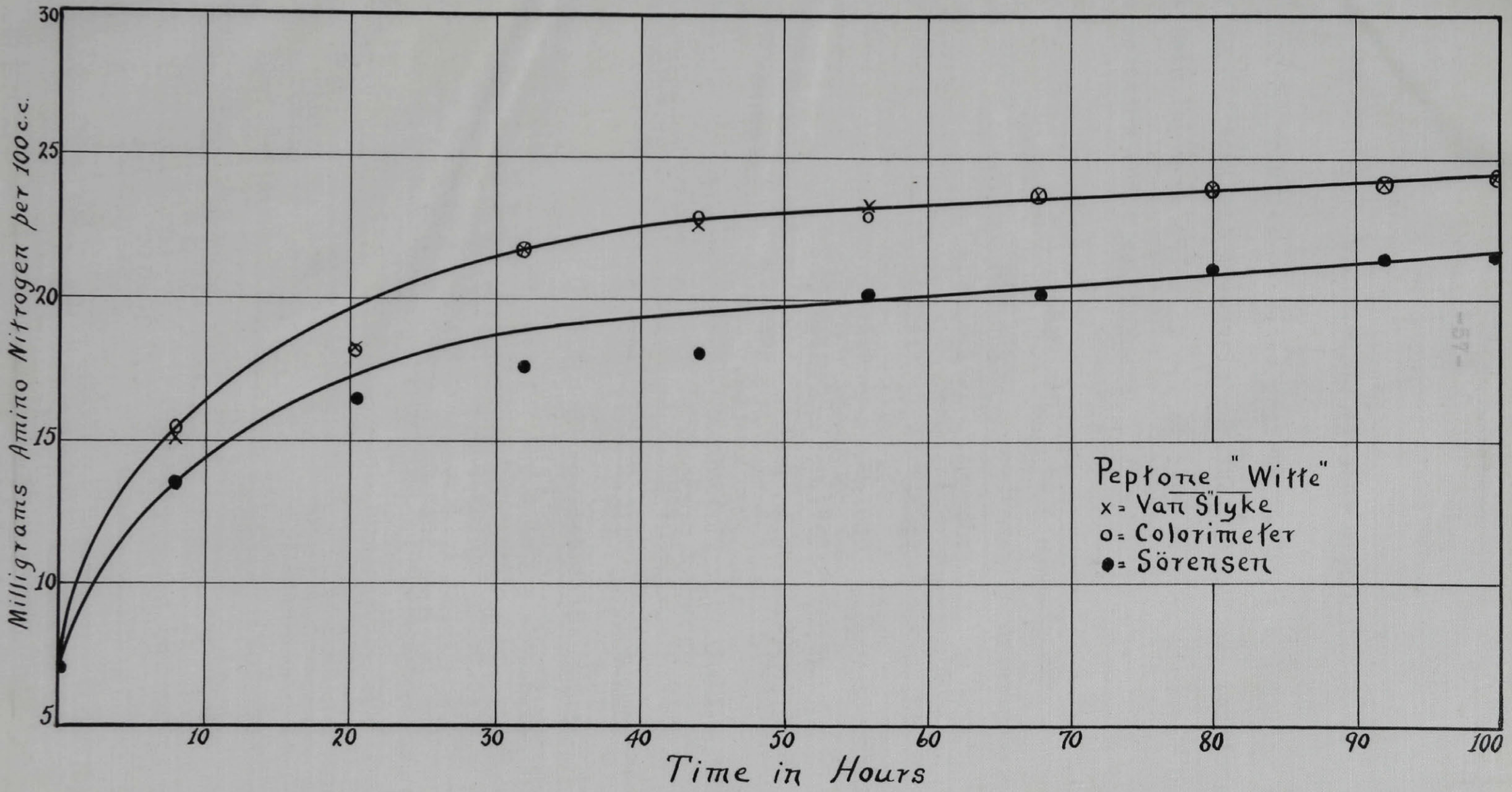

Milligrams Amino Nitrogen per 100 c.c.
30
25
20
15
10
5
10
20
30
40
50
60
70
80
90
100
Time in Hours
Peptone "Witte"
x = Van Slyke
o = Colorimeter
● = Sörensen

Figure VI

PEPTONE (WITTE). (FIGURE VI).

A 0.5% solution of Witte peptone was made by boiling and filtering off a slight insoluble residue. To 100 cc. portions of this solution were added: 1 cc. of 0.5% Na_2CO_3, 1 cc. of 0.2% holadin solution and 2 cc. toluene.

	Mgm. Amino Nitrogen per cc.		
Time in Hours	Van Slyke	Sörensen	Colorimeter
0	0.0734	0.0707	0.0719
8	0.1511	0.1357	0.1538
20.5	0.1518	0.1640	0.1801
32	0.2165	0.1753	0.2150
44	0.2267	0.1809	0.2273
56	0.2332	0.2036	0.2296
68	0.2379	0.2036	0.2381
80	0.2396	0.2112	0.2409
92	0.2417	0.2149	0.2424
104	0.2441	0.2149	0.2439
128	0.2451	0.2205	0.2438
140	0.2467	0.2262	0.2462

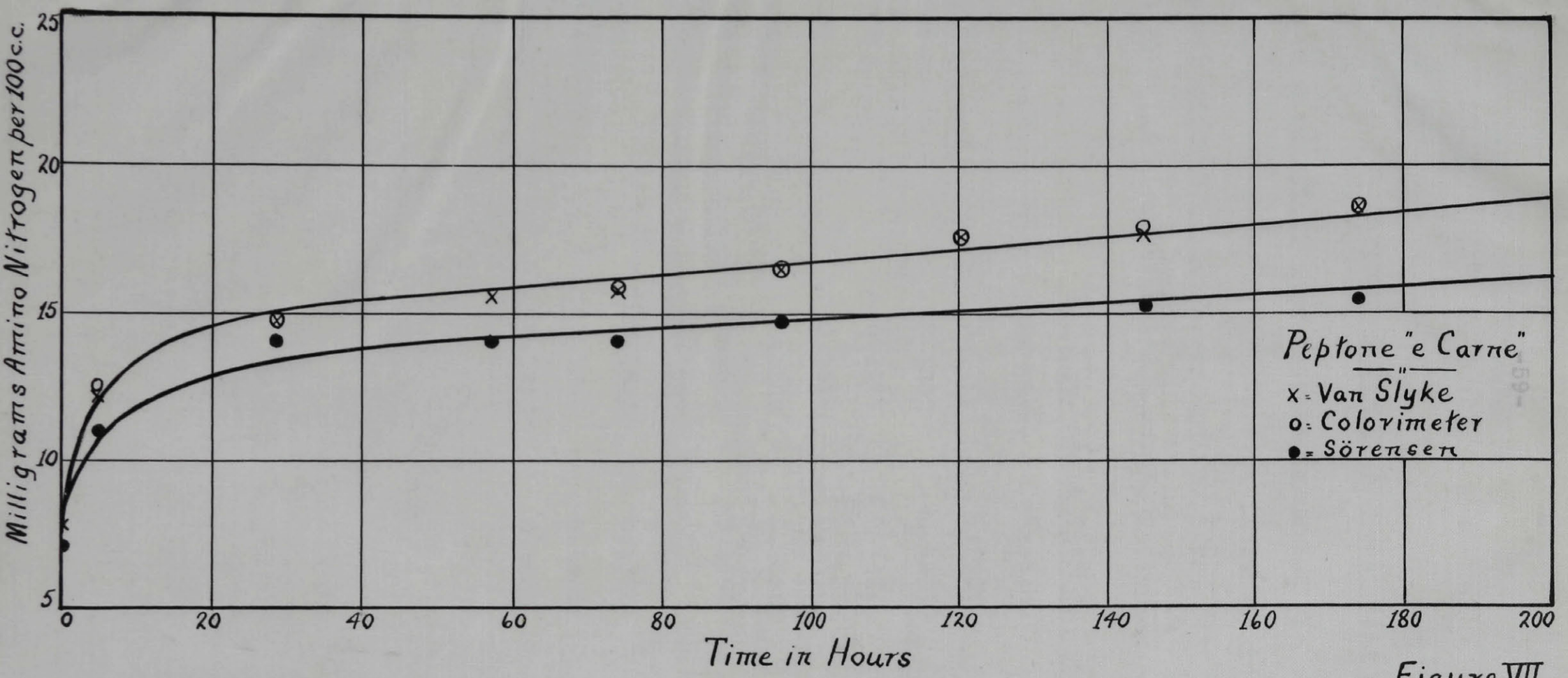

Milligrams Amino Nitrogen per 100 c.c.
25
20
15
10
5
0
20
40
60
80
100
120
140
160
180
200
Time in Hours
Peptone "e Carne"
—"—
x = Van Slyke
o = Colorimeter
● = Sörensen

Figure VII

PEPTONE "E CARNE" (FIGURE VII).

A 0.5% solution of Peptone "e carne" was prepared by boiling and filtering off the slight insoluble residue. To 100 cc. portions of this solution were added: 1 cc. 0.5% Na_2CO_3, 1 cc. of 0.2% holadin solution and 2 cc.of toluene.

	Mgm. Amino Nitrogen per cc.		
Time in Hours	Van Slyke	Sörensen	Colorimeter
0	0.0773	0.0707	0.0807
5	0.1232	0.1201	0.1250
29	0.1477	0.1414	0.1471
57	0.1552	0.1414	0.1666
74	0.1569	0.1414	0.1586
96	0.1694	0.1484	0.1666
120.5	0.1749	———	0.1754
145	0.1762	0.1520	0.1786
174	0.1860	0.1555	0.1852
218.5	0.1922	0.1555	0.1952

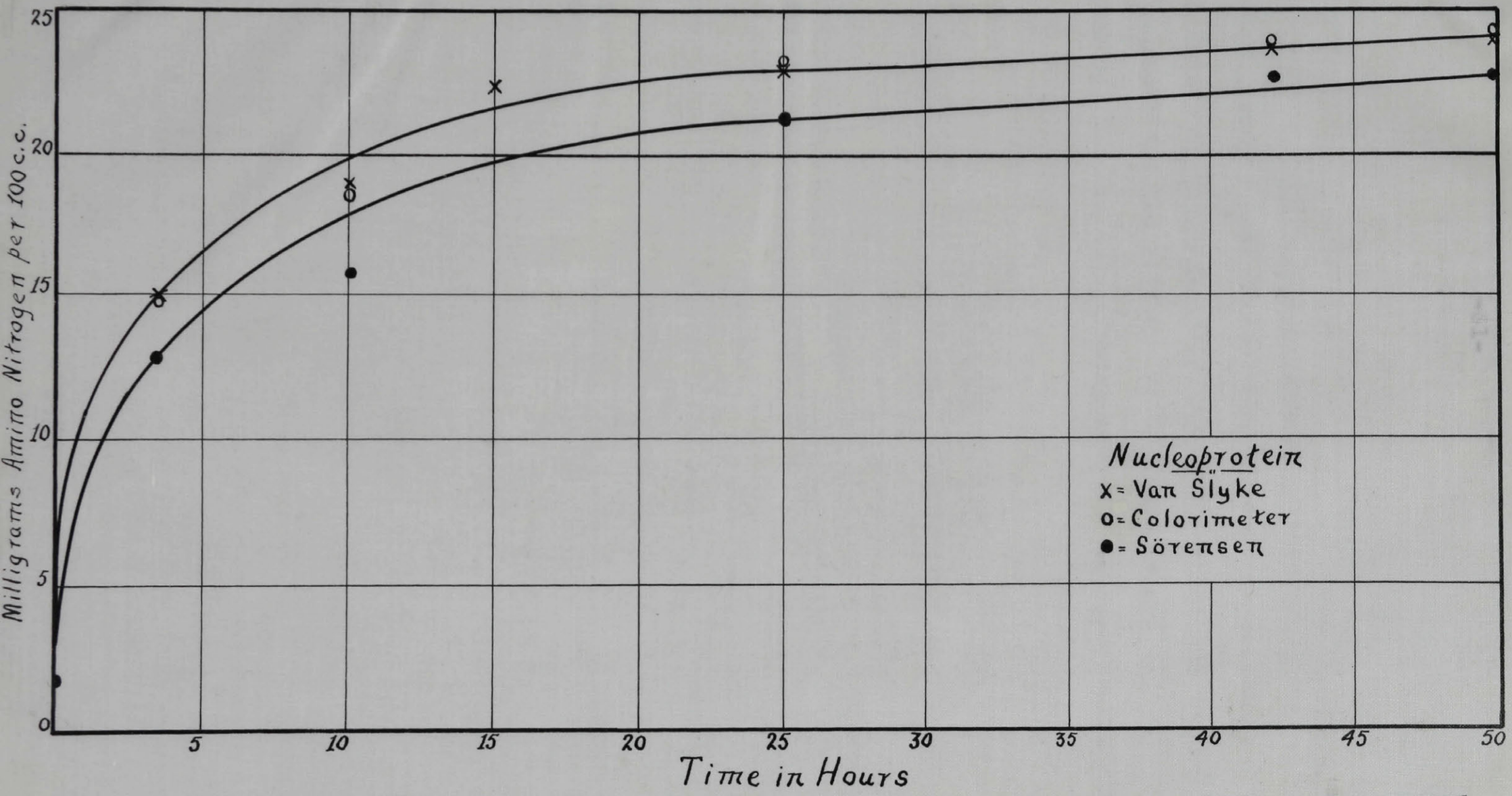

Milligrams Amino Nitrogen per 100 c.c.
25
20
15
10
5
0
5
10
15
20
25
30
35
40
45
50
Time in Hours
Nucleoprotein
x = Van Slyke
o = Colorimeter
● = Sörensen

Figure VIII

NUCLEOPROTEIN (FIGURE VIII).

2.5 grams of Nuclein (Schuchardt) were dissolved in 60 cc. of 0.5% Na_2CO_3 by boiling. After filtering, a residue of 0.2370 gram remained, and the filtrate was diluted to 500 cc.

50 cc. samples were treated with 0.5 cc. pancreatic extract and 2 cc. of toluene.

	Mgm. Amino Nitrogen per cc.		
Time in Hours.	Van Slyke.	Sörensen.	Colorimeter.
0	0.0174	0.0177	0.0176
3.5	0.1500	0.1275	0.1275
10.25	0.1895	0.1558	0.1851
15	0.2244	—	—
25.25	0.2297	0.2125	0.2322
42.25	0.2371	0.2267	0.2388
55.25	0.2397	0.2267	0.2418

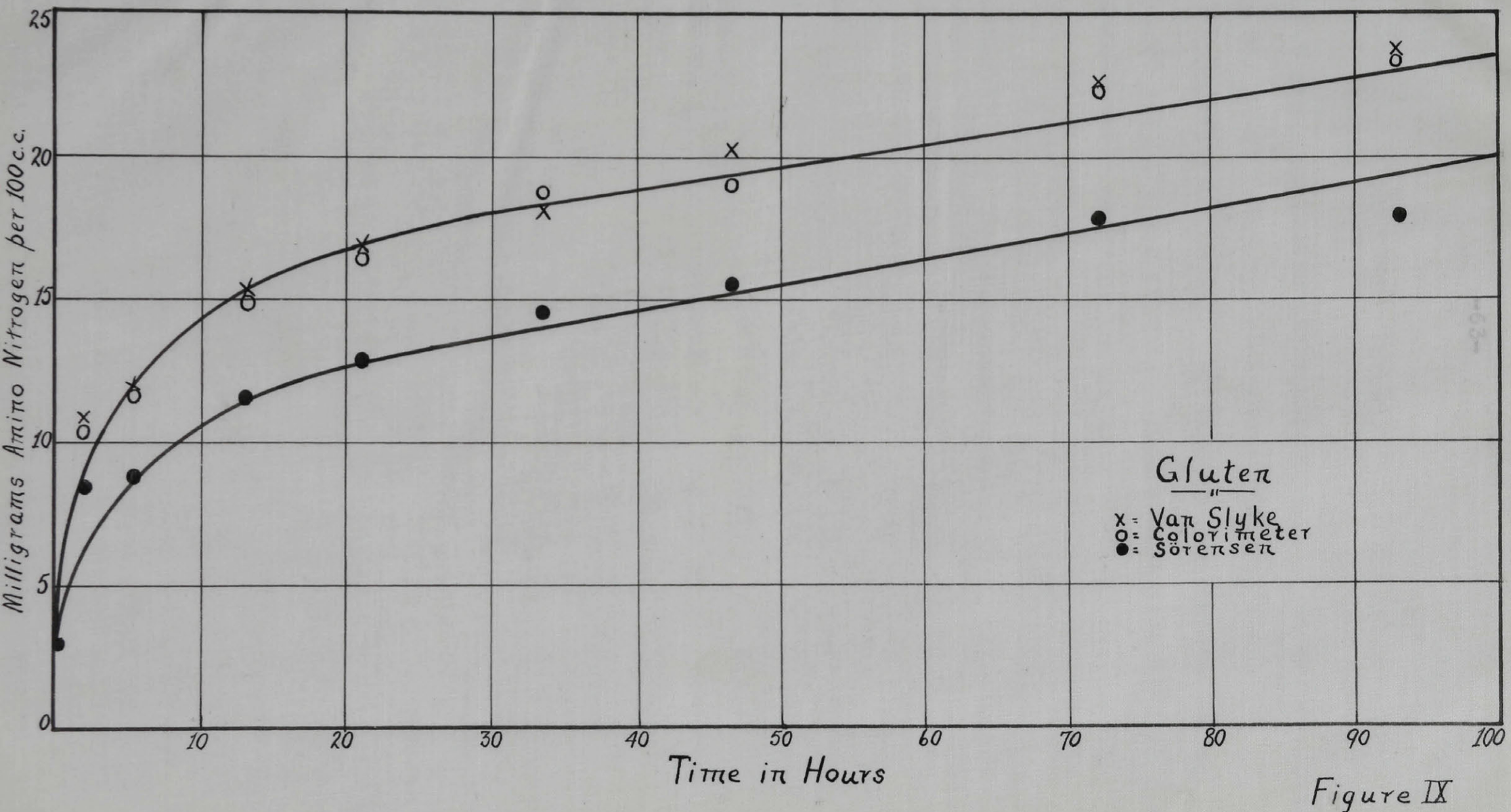

Milligrams Amino Nitrogen per 100 c.c.
25
20
15
10
5
0
10
20
30
40
50
60
70
80
90
100
Time in Hours
Gluten
"
x = Van Slyke
o = Colorimeter
● = Sörensen

Figure IX

GLUTEN (FIGURE IX).

2.5 grams of Gluten (Schuchardt) were dissolved in 60 cc. of 0.5% Na_2CO_3 at ordinary temperature and, after filtering off a slight insoluble residue, the solution was made up to 500 cc. volume. 50 cc. portions of this solution were treated with 0.5 cc. pancreatic extract and 2 cc. of toluene.

	Mgm. Amino Nitrogen per cc.		
Time in Hours	Van Slyke	Sörensen	Colorimeter
0	0.0313	0.0283	0.0293
2	0.1091	0.0850	0.1020
5.5	0.1182	0.0855	0.1150
13	0.1536	0.1154	0.1492
21	0.1673	0.1275	0.1640
33.5	0.1803	0.1452	0.1886
46.5	0.2036	0.1558	0.1899
72	0.2260	0.1771	0.2238
93	0.2390	0.1771	0.2343
100	0.2421	0.1842	0.2418

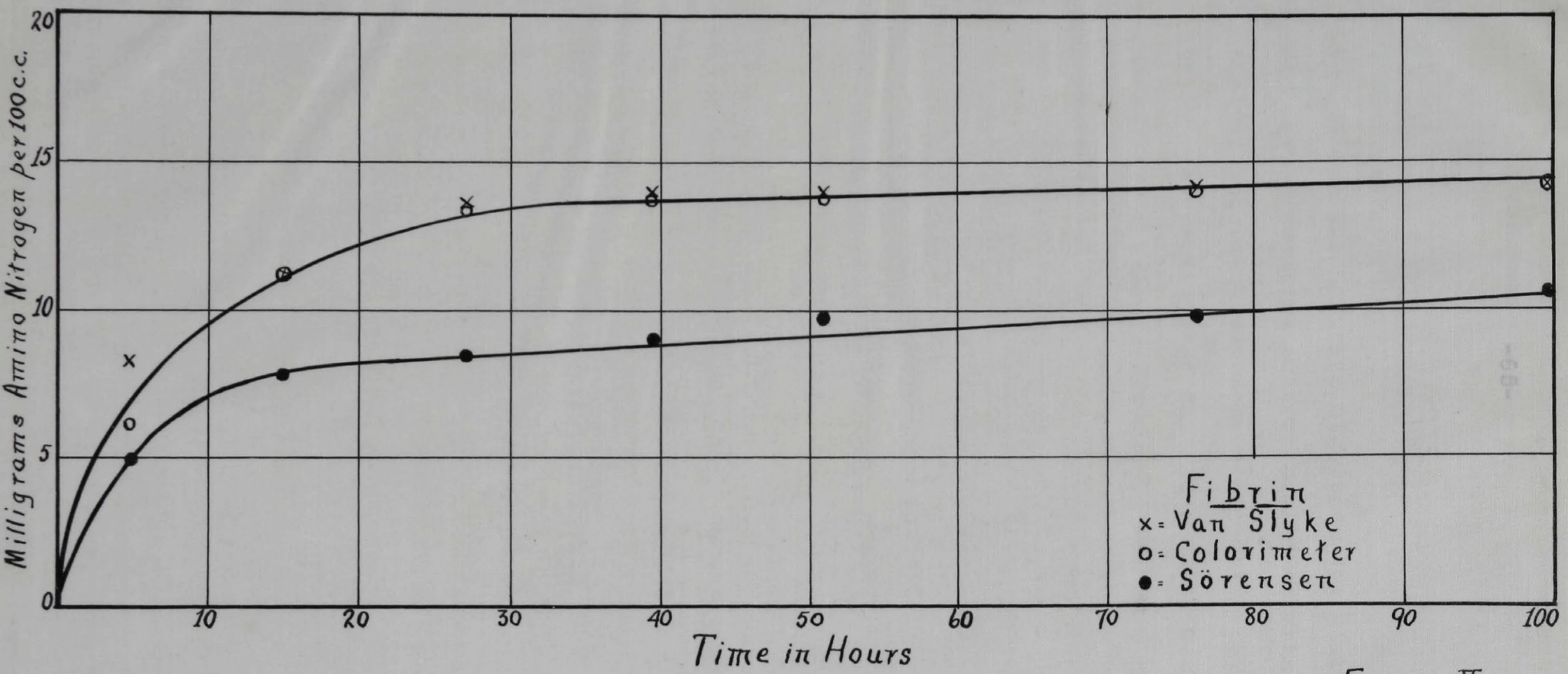

Milligrams Amino Nitrogen per 100 c.c.
0
5
10
15
20
10
20
30
40
50
60
70
80
90
100
Time in Hours
Fibrin
x = Van Slyke
o = Colorimeter
● = Sörensen

Figure X

FIBRIN (FIGURE X).

The fibrin used in this digestion was obtained from ox-blood, and, after removing a large excess of moisture by filter paper, analysis proved the presence of 46.98% H_2O.

To 100 cc. portions of distilled water were added 0.5 gram fibrin, 1 cc. of pancreatic extract, 1 cc. of 0.5% Na_2CO_3, and 2 cc. of toluene.

	Mgm. Amino Nitrogen per cc.		
Time in Hours	Van Slyke	Sörensen	Colorimeter
5	0.0833	0.0495	0.0602
15	0.1110	0.0777	0.1111
27	0.1356	0.0848	0.1338
39.5	0.1389	0.0919	0.1374
51	0.1392	0.0989	0.1388
76	0.1416	0.0989	0.1408
99.5	0.1423	0.1060	0.1424
124	0.1469	0.1131	0.1444
148.5	0.1477	0.1133	0.1471

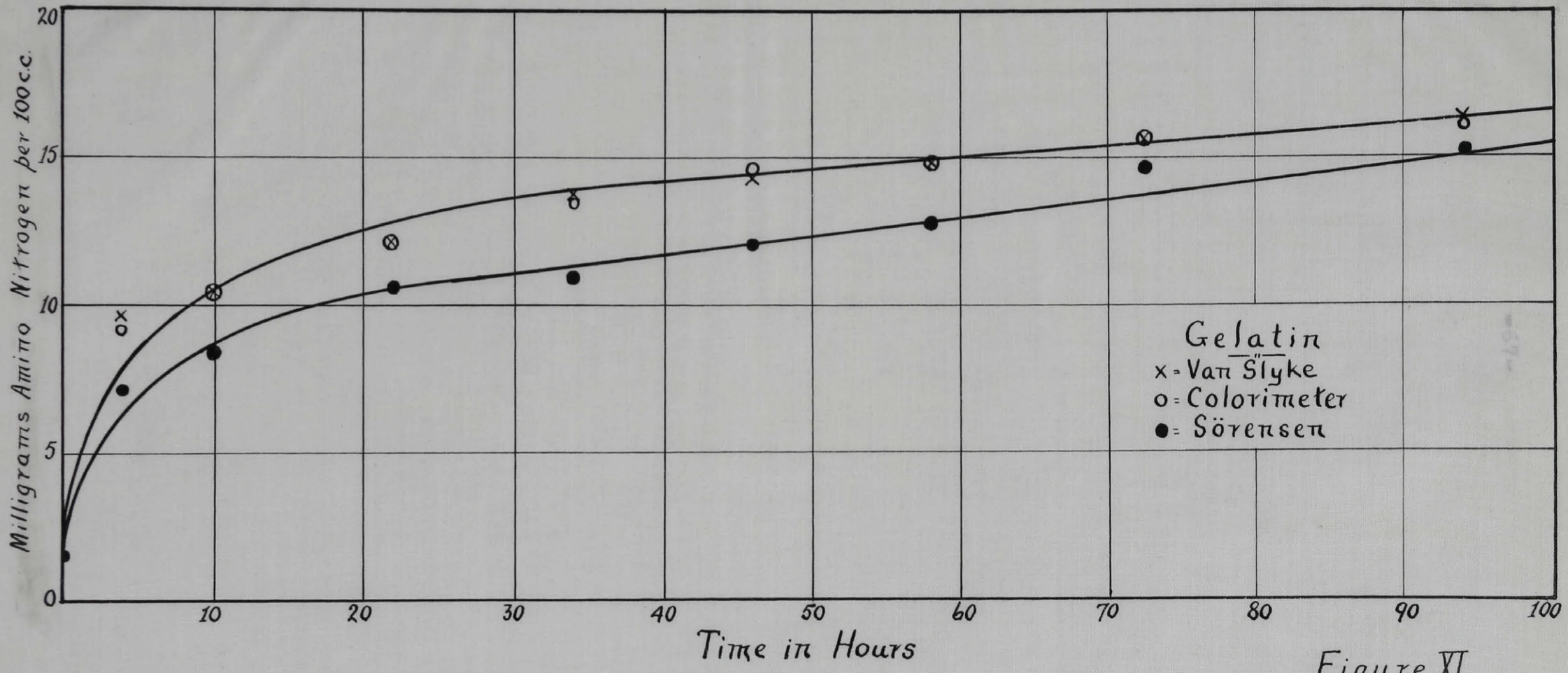

Milligrams Amino Nitrogen per 100 c.c.
0
5
10
15
20
10
20
30
40
50
60
70
80
90
100
Time in Hours
Gelatin
x = Van Slyke
o = Colorimeter
● = Sörensen

Figure XI

GELATIN (FIGURE XI).

6 grams of pure gelatin (isinglass) were dissolved in boiling water and, after filtering, the solution was diluted to 1200 cc.

To 100 cc. portions of this solution were added 1 cc. of 0.5% Na_2CO_3, 1 cc. of 1% holadin solution and 2 cc. of toluene.

	Mgm. Amino Nitrogen per cc.		
Time in Hours.	Van Slyke.	Sörensen.	Colorimeter.
1	0.0352	0.0245	0.0320
4	0.0986	0.0707	0.0921
10	0.1031	0.0848	0.1021
22	0.1204	0.1060	0.1212
34	0.1373	0.1095	0.1351
46	0.1440	0.1201	0.1462
58	0.1489	0.1272	0.1497
72.5	0.1561	0.1484	0.1562
94	0.1636	0.1520	0.1614
119.5	0.1698	0.1520	0.1666
142	0.1725	0.1555	0.1708

DISCUSSION OF RESULTS.

The results of the experiments show quite clearly that the colorimetric method is applicable to a study of proteoclasis. The striking agreement of the method with the Van Slyke, and the general agreement of the two with the Sörensen method show that it estimates quantitatively the hydrolysis of the peptide chain. Qualitatively, all three methods give the same information, and can be used to determine the presence of peptide groupings in an unknown substance or mixture, or to detect the presence of a proteoclastic enzyme when acting on a peptide substrate. The agreement of the colorimetric and gasometric methods is to be expected, for both give theoretical results with aqueous solutions of pure amino acids, except in the case of glycine with the gasometric method and cystine with both methods. Both methods yield identical results when applied to peptone solutions obtained from various sources. A reference to the experimental figures, however, will reveal the fact that with some native proteins and during the early stages of hydrolysis of these proteins the two methods do not give identical results. This is shown in the following table, very clearly in the case of the mixture of serum albumin and globulin, and of fibrin.

Protein	Time	Nitrogen per cc.	
		Van Slyke	Colorimeter.
	hrs.	mgm.	mgm.
Serum albumin and globulin	0.0	0.0612	0.0200
Gluten	0.0	0.0313	0.0293
Fibrin	5.0	0.0833	0.0602
Gelatin	1.0	0.0352	0.0320
Casein	0.5	0.0386	0.0384
Nucleoprotein	0.0	0.0174	0.0176
Witte peptone	0.0	0.0734	0.0719
Peptone "e carne"	0.0	0.0777	0.0807

As the hydrolysis proceeds, however, the figures begin to come into agreement and at the end of 15 hours they read:

	Van Slyke	Colorimeter
	mgm.	mgm.
Serum albumin and globulin	0.1812	0.1782
Fibrin	0.1110	0.1111

The significance of the discrepancy is not apparent. It cannot be due to the partial interaction of the omega-amino group of lysine with nitrous acid and not with triketohydrindene hydrate, otherwise the same nonconformity would show itself with casein and Witte peptone. For the present it can only be ascribed to some physical characteristic of the proteins mentioned. The agreement of the results of the two methods with the remaining proteins in the very early stages of hydrolysis and with the peptones makes it extremely probable that the colorimetric process estimates the amino group alpha to a carboxyl group, even when the latter is bound in a peptide linking, as amide nitrogen gives no ninhydrin reaction. The point, however, requires fuller experimental investigation with purified peptones and synthetic polypeptides.

On comparing the three methods of following protein hydrolysis in very dilute solution, and where the results are not complicated by the presence of large amounts of ammonia, it is evident that the colorimetric or the Van Slyke method is to be preferred. The Sörensen method, nevertheless, is much the simplest to carry out, though its range of application does not allow it to be used in extremely dilute solutions of proteins.[36] There should be mentioned in this connection, however, a recent

paper by Clementi,[37] who, by an improved and accurately calibrated burette, has made the Sörensen method sensitive with solutions containing less than 1 milligram of amino acid alpha-nitrogen per cc. The most sensitive method is undoubtedly the colorimetric process, and it is also relatively simple in manipulation. Nevertheless, it is inapplicable in strongly acid or alkaline solutions, and will probably find its greatest usefulness in the study of protein hydrolysis in neutral or faintly alkaline media. The gasometric method, owing to its susceptibility to correction, for the nitrogen of amino groups other than the alpha amino group of amino acids, is the most accurate of the three. The ninhydrin reaction is also given by a number of amines (loc. cit.), but these are capable of classification and fall into one or two well defined groups.[38]

SUMMARY.

The colorimetric method for the estimation of amino acid alpha-nitrogen can be used to follow the hydrolysis of proteins by pancreatic enzymes. The results agree with those obtained by the Van Slyke method.

In conclusion, the author wishes to express his sincere thanks to Professors R. F. Ruttan and V. J. Harding: to the former, for his constant kindly interest throughout the period of this investigation, and especially for numerous helpful suggestions which led up to the application of the method; to the latter, in collaboration with whom part of the research was carried out, for his careful direction in rendering a task, fraught with many difficulties, more certain of consummation.

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