"A STUDY OF THE REACTION BETWEEN COPPER AND DITHIOCARBAMIC ACID, AS APPLIED TO THE DETERMINATION OF COPPER AND AMINO ACIDS"

A Thesis

By

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GENERAL INTRODUCTION

The reaction between copper and dithiocarbamic acid derivatives produces a brown coloured complex which absorbs light at 435 m.u. When this reaction is applied to the determination of copper, it provides a very sensitive method that can detect, and measure accurately, concentrations as low as 0.1 μ.g. of copper. For this particular determination, the dithiocarbamic acid derivatives function as the reagent.

It should be possible, however, to apply the same reaction to the determination of dithiocarbamic acid derivatives by using the copper component as the reagent. If the determination was limited to the dithiocarbamic acids as such the method would have little biological value since these acids do not seem to occur in nature. But since amines can be converted into the corresponding dithiocarbamic acids, and these in turn into the brown coloured copper complexes, the reaction could become a measure of the amines. The amino acids, having the characteristic amino group, should possess this property of amines.

One of the important applications of the determination of copper is in relation to the keeping quality of foods. Milk powder was given special attention because of its increasing importance as a food. It is generally agreed that concentrations of copper of the order of 10 p. p.m. will favour the development of off-flavours in milk powder in a relatively short time. It is recognized also that varying amounts of copper may be introduced into the milk depending on the conditions of production, transportation and processing. The danger of contamination with copper is further increased during the present war since old equipment is being used to procure maximum production.

It becomes important then that the copper content of the milk be carefully controlled during processing. In the determination of copper in foods the existing methods for destroying organic matter, involving ashing or acid digestion, are detailed and time consuming and are unsuitable for routine analysis. Furthermore, dry ashing may cause a loss of copper and the 'reagent blank' in the acid digestion method has a high copper content.

The method described in this thesis is the result of an attempt to provide the dry-milk industry with a rapid, simple and accurate method for the extraction and estimation of copper in milk powder. The direct extraction of the copper from the milk powder would be the most desirable procedure, since it does not require any special apparatus.

Another application of the dithiocarbamic acid reaction was made in an attempt to fractionate and estimate quantitatively the natural occurring monoaminomonocarboxylic acids. Separation was attempted by an adsorption technique although the neutral amino acids do not show pronounced differences in adsorption properties. It was thought that if one of the reactive groups in the amino acid molecule was blocked by chemical combination, the other would become active towards an adsorbant. Two different derivatives were used in an attempt to verify this assumption - the copper salt and the copper salt of the dithiocarbamic acids of the amino acids. Copper combines with the carbonyl group to form the undissociated salt leaving the amino group free, whereas the sulfur of the sulfur derivative combines with the amino group.

These compounds were specially chosen because after separation, they would exhibit special properties that could be used for their quantitative determination. In the case of the simple copper salts, a copper

estimation would serve to estimate the amount of the amino acid present, while the brown colour of the copper salt of the sulphur derivative would permit colorimetric measurement.

PART I

MICRO-ESTIMATION OF COPPER IN MILK POWDER

REVIEW OF THE LITERATURE

The reaction of copper with sodium diethyl-dithiocarbamate to form a golden-brown coloured complex, was first utilized in the determination of the copper content of water, by Callan and Henderson (3) and later applied to biological materials by McFarlane (21). The sensitivity of the reaction permits measurement, with a photoelectric colorimeter, of concentrations as low as 0.10 µg. of copper per ml. According to Greenleaf (12) the reaction deviates slightly from Beer's Law due to an optical effect.

Many modifications of McFarlane's original method have been proposed, most of which are concerned with the interference by Fe, Ni, Co and Bi which also react with the carbamate reagent. To overcome the interference by iron, which is most commonly encountered in food analysis, Haddock and Evers (13) recommend citric acid instead of sodium pyrophosphate and Greenleaf (12) extracts with dithizone to remove Ni, Co and Bi. All these methods employ ashing or wet digestion to destroy organic matter but dry ashing may cause a loss of copper and wet digestion introduces a high reagent blank which decreases the accuracy of the method (12).

EXPERIMENTAL

1. - Analytical methods

As a check on the results obtained in the course of this work, copper determinations were also made by the method of Kerr (16) as follows:

> 2.5 - 5.0 g. of milk powder were ashed in a furnace at 450°C. The ash was dissolved in 15 ml. of 6 N HCl and transferred to a separatory funnel; 20 ml. of a 50% solution of citric acid were added followed by a slight excess of NH_4OH (I/I) and the colour was developed with 2 ml. of a 1% solution of sodium diethyldithiocarbamate. The coloured complex was extracted with 25 ml. of isoamyl alcohol and the colour intensity measured in a Coleman spectrophotometer at a wave-length of 435 mu. The amount of copper was estimated by reference to a calibration curve. A blank determination was made on all the reagents.

By this procedure, duplicate analysis of standard solutions of copper, gave satisfactory results. For purposes of comparison with the method evolved in this thesis, determinations of copper were also made on samples of milk powder by the wet digestion method of Eden and Green (6), as follows:

> 1 g. of milk powder was introduced into a 8" x 1" pyrex test tube etched at approximately 25 ml; 1 ml. H_2SO_4 (A.R.), 3 ml. $HClO_4$ (Cu-free) and two glass beads were added, and the mixture gently heated over a micro-burner. Water was driven off by gentle boiling and heating was continued until rings of darkened mixture began to ascend the tube. The flame was then turned out, the tube allowed to cool for about a minute and 3 ml. HNO_3 (A.R.) carefully added. On heating, the dark brown liquid lightened in colour, passing through orange to colourless. Stronger heating was continued for a few minutes after the colourless stage to drive off most of the residual $HClO_4$, leaving a final volume of about 1 ml., i.e. the quantity of H_2SO_4 originally added.
>
> The combustion residue in the tube was diluted with 5-10 ml. water and 2 ml. 50% ammonium citrate added, followed by 5 ml. ammonia (S.G. 0.880). Water was then added to the 25 ml. mark.
>
> 2 ml. of 1% solution of sodium diethyldithiocarbamate were added followed by 5 ml. of isoamyl alcohol. The tube was closed

with a clean rubber bung and vigorously shaken for ½ min. The intensity of the colour was measured in a spectrophotometer.

II. - Extraction of copper as copper - diethyldithiocarbamate

The first attempt to extract copper from milk powder was made by refluxing the sample with a chloroform solution of sodium diethyldithiocarbamate as follows:

> 2.5 g. of milk powder were weighed into a round-bottomed flask and to it was added 5 ml. of a saturated solution of sodium diethyldithiocarbamate reagent in chloroform. The mixture was refluxed for 20 minutes, cooled, and the content of the flask was transferred to a filter. The flask was rinsed twice with 5 ml. portions of chloroform and the washings were added to the filter. The filtrate was made up to 25 ml. and the intensity of the colour was measured in the spectrophotometer.

Since chloroform is a fat solvent and the milk fat has a yellow colour similar to the colour of the copper carbamate complex, it was necessary to carry out a blank determination omitting the carbamate reagent. The results were disappointing, being only about 10% of the values obtained by ashing. In a subsequent experiment, when a chloroform solution of copper diethyldithiocarbamate was refluxed alone, it was proved that the copper complex had not been affected by heat.

The above experiment was repeated with a known amount of copper added to the milk powder. Failure to recover the added copper would indicate its adsorption or chemical combination with reactive groups in the milk proteins. The results showed that under the conditions of the experiment a portion of the added copper was not extracted i.e. 35 µg. of copper were added and 27 µg. recovered.

In an investigation of the cooked flavour of heated milk, Gould Sommer (11) suggested that the copper might be combined with the free

SH groups of the milk proteins. It is possible therefore that, in the above experiment, part of the added copper formed an undissociated complex with the milk protein under the influence of refluxing. In the determination of acorbic acid, Mapson (19) used formaldehyde to prevent the interference of SH groups. The effect of this reagent and of other reagents on the extraction of copper from milk powder and on the reaction between copper and sodium diethyldithiocarbamate was next investigated.

III. - Factors influencing the extraction of copper from milk powder

A. - FORMALDEHYDE

The influence of formaldehyde on the copper carbamate reaction was first studied by shaking 50 ml. of a chloroform solution of copper carbamate with 2 ml. of a 37% solution of formaldehyde and refluxing the mixture for 20 minutes. Another aliquot of the same copper carbamate solution in chloroform was refluxed in the absence of formaldehyde. The colour intensity was measured before and after refluxing. The results indicated that formaldehyde had no effect on the copper carbamate reaction so its use in the determination of the copper content of milk powder was investigated as follows.

2.5 grams of milk powder were refluxed for 20 minutes with 15 ml. of an extracting solution prepared by shaking 50 ml. of a chloroform solution of the carbamate reagent with 2 ml. of a 37% solution of formaldehyde. The mixture was cooled and transferred to a filter. The flask was rinsed twice with 5 ml. portions of chloroform and the rinsings were used to wash the milk powder on the filter paper. The combined filtrates

were diluted to 25 ml. and the colour intensity was measured in the spectrophotometer. To compensate for the colour of the fat, a blank was prepared in the same manner as the test but omitting the carbamate reagent.

The results obtained by this procedure were still unsatisfactory, the amount of copper extracted being only about 50% of the value obtained by the ashing method. Nevertheless, comparison of these results with the results obtained in the previous experiment shows that formaldehyde did markedly increase the extraction of copper from milk powder and it was of interest to determine if this was really due to the action of formaldehyde on free-SH groups. The experiment was therefore repeated but with a known amount of copper added to the extraction solution. The added copper plus the copper in the 2.5 gram of milk powder amounted to 31.5 μ.g. of which 25 μ.g. were recovered in the analysis. Apparently, the copper is retained by the powder notwithstanding the presence of formaldehyde.

B. - SODIUM CYANIDE

Sodium cyanide is a protein denaturating agent and increases the amount of free-SH groups. Consequently, its presence should decrease the recovery of copper. An experiment was first carried out to determine if sodium cyanide influenced the copper carbamate reaction. The colorimeter readings on standard solutions of copper carbamate in acetone, with and without added sodium cyanide, were found to be the same and the readings were not affected by refluxing for 20 minutes. The effect of sodium cyanide on the extraction of copper from milk powder was next determined as follows:

To 2.5 grams of milk powder in a round-bottomed flask was added 50 ml. of a saturated ether solution of sodium cyanide and the carbamate reagent. The mixture was refluxed for 20 minutes, filtered, and the ether evaporated. The ether-soluble residue was dissolved in 10 ml. of isoamyl alcohol and the intensity of the colour compared with a blank prepared in identical manner but omitting the carbamate reagent.

In this instance, the recovery of copper was about 70%, indicating that denaturation of the protein with sodium cyanide and hence increasing the number of free SH groups did not produce the expected effect; namely, a higher retention of copper. In the light of these results with sodium cyanide or formaldehyde, it was concluded that free SH groups were not responsible for the low extractibility of the copper. The retention of added copper, in the form of copper carbamate, by milk powder was probably due to physical adsorption rather than chemical combination. The low extractibility of copper in milk powder, per se, was probably due partly to adsorption and partly to inadequate contact between the reagent and the protein molecules. In the last experiment it was observed that the presence of sodium cyanide changed the physical nature of the milk powder, the sodium cyanide acting as a swelling or hydrating agent. This hydration of the milk proteins probably accounts for the higher extraction of copper; apparently, a better contact between the solid and liquid phase is provided and hence the amount of copper extracted is increased.

IV. - Extraction of copper as copper thiocyanate

According to Hofmeister, as quoted by Getman and Daniels (10), CNS^- has the highest hydrating activity of all the anions tested; the effect of the anions on the hydration of proteins being arranged in the following order of decreasing activity:

$$CNS^- > I^- > Br^- > ClO_3^- > Cl^- > C_2H_3O_2^- > SO_4^-$$

Potassium thiocyanate is very soluble in acetone or water so, in these experiments, it could be tested at high concentrations. Furthermore, CNS^- combines with copper to form an undissociated compound which has a yellow colour in aqueous solution and a red colour in acetone solution. It appeared therefore that potassium thiocyanate might be an ideal swelling agent and specially suitable for the extraction of copper from a material such as milk powder.

In the following experiments it was decided to use skim milk powder in place of whole milk powder which had been used exclusively in the preceding experiments. It was felt that in this way the preliminary experiments could be simplified by not having fat present. The extracting reagent employed was a solution of potassium thiocyanate in acetone to which approximately 2% of water was added. Three concentrations of thiocyanate were tested, namely, 3, 5, and 8%. The procedure adopted was as follows:

2.5 grams of skim milk powder were refluxed for 15 minutes with 30 ml. of the extracting reagent. The solution was then decanted on to a filter paper and the residue washed with three 10 ml. portions of the extracting reagents and the washings added to the filtrate. The acetone

was then removed by distillation, the residue dissolved in 20 ml. of redistilled water and the solution transferred to a separatory funnel. To this was added 20 ml. of a 50% solution of citric acid (freed from copper by extraction with the carbamate reagent) and the solution made slightly alkaline, about pH 8.5, by the addition of (1 - 1) NH_4OH., 2 ml. of a 1% solution of the carbamate reagent were added and the copper carbamate was extracted with 15 ml. of isoamyl alcohol. The extract was diluted to 25 ml. and the colour intensity measured in the spectrophotometer. A blank was prepared by carrying out the determination in the absence of the skim milk powder.

The concentration of KCNS had no apparent effect on the degree of swelling, in all cases the powder increased in volume and had the appearance of a spongy, gelatinous mass. However, the results showed that the concentration of KCNS had a marked effect on the recovery of copper, the extraction of copper being quantitative at concentrations of 5% or above. To ascertain if KCNS affected the copper carbamate reaction, the copper content of a standard solution of copper sulphate was determined in the presence of 2.5 g. KCNS. The results showed that KCNS did not affect the reaction.

A. - Influence of fat

It is evident that the presence of fat would interfere in this procedure since it would be extracted by the acetone. In the next experiment the fat was removed at the final stage of the determination just before adding the carbamate reagent by extracting the solution with 10 ml. portions of isoamyl alcohol until the extract was colourless.

The citric acid was not purified by the usual procedure employing the carbamate reagent since in the presence of the excess carbamate reagent, copper carbamate would be formed prior to the extraction with isoamyl alcohol and would be soluble in this solvent. Three different whole milk powders were analyzed by this procedure using unpurified citric acid. Recoveries of 100% and 98% were obtained with two of the powders but the third gave a low recovery of only 70%. As was expected, the blank had a high degree of colour due to the fact that the citric acid was not purified.

To permit the use of citric acid purified with the carbamate reagent the above procedure had to be slightly modified. After distilling off the acetone, and before adding the citric acid, the aqueous solution was freed from fat by extraction with isoamyl alcohol. The purified citric acid was then added, the solution made alkaline and the colour developed with the carbamate reagent was extracted with isoamyl alcohol. By this procedure, the copper content of the blank was reduced from about 13 to 3μg. per ml., but the recovery of copper was low and variable, i.e., from 50 to 83%. When the aqueous solution was made alkaline before extracting the fat the apparent recovery of copper was too high, due to the fact that the fat soluble pigments were not completely extracted from alkaline solution. It was also indicated that copper was lost by extracting with isoamyl alcohol from neutral solution, probably being soluble as copper thiocyanate.

It appeared from these findings that in order to remove the milk fat from the test solution, without loosing any copper, a new reagent had to be found. The copper complex formed with this reagent had to be

insoluble in at least one organic solvent. Geiger and Muller (9) found that dihydroxyethyldithiocarbamic acid, unlike sodium diethyldithiocarbamate, forms a complex with copper which is insoluble in some organic solvents. If the citric acid was purified with the new carbamate reagent and the milk pigments extracted with carbon tetrachloride for example, the presence of an excess of the new carbamate reagent would not interfere, thus the difficulties in the preparation of a satisfactory blank, referred to above, would be overcome.

B. - Preparation and properties of the 'new carbamate reagent' dihydroxyethyldithiocarbamic acid

Dihydroxyethyldithiocarbamic acid, was prepared by Geiger and Muller (9) from diethanolamine and carbon disulphide in methyl alcohol solution. We found that the reagent was very unstable in alcohol and developed a deep red colour on standing for a few days. The reagent is stable in aqueous solution so we have prepared it using water in place of methyl alcohol, i.e., 0.2 g. carbon disulphide were shaken with 100 ml. of redistilled water until completely dissolved, then 2 g. of diethanolamine were added. In determinations with the new carbamate reagent, Geiger and Muller employed three drops of a 10% solution. However, this high concentration of the reagent may affect the deionizing action of citric acid on iron. Eden and Green (6) used 2 ml. of a 0.5% solution of the reagent but we have found that an intermediate concentration was most satisfactory, namely, 2 ml. of a 2% solution.

The reaction of the new carbamate reagent with copper is comparable to the reaction with sodium diethyldithiocarbamate and the copper complex has the same golden brown colour. With the two reagents,

the intensity of the colour with a given amount of copper is the same and the reaction shows the same deviation from Beer's Law (Table I and Fig. I). However, the solubility of the copper salt of the new carbamate reagent is quite different, it is insoluble in chloroform, carbon tetrachloride and petroleum ether slightly soluble in ethyl ether and very soluble in ethyl alcohol, acetone, water and especially isoamyl alcohol.

TABLE I. - Galvanometer deflections obtained with various known concentrations of copper

Concentration of copper (u.g./ml.)	Galvanometer readings
0.6	70.0
1.1	53.0
1.6	42.5
2.1	34.0
2.6	27.0

C. - Determination of copper with the new carbamate reagent.

Three different whole milk powders were analyzed by the procedure described above but employing the new reagent. The fat was extracted with petrol ether at the final step in the procedure, i.e., after the reagent was added. A very low reading in the blank equivalent to 0.5 μg. of copper per ml. was obtained. The recoveries of copper in the three samples, expressed as percentage of the value obtained by the ashing method, were 71%, 88% and 98%. Further experiments showed that the variability in the results was due to the amount of water present in the acetone. The water content of the

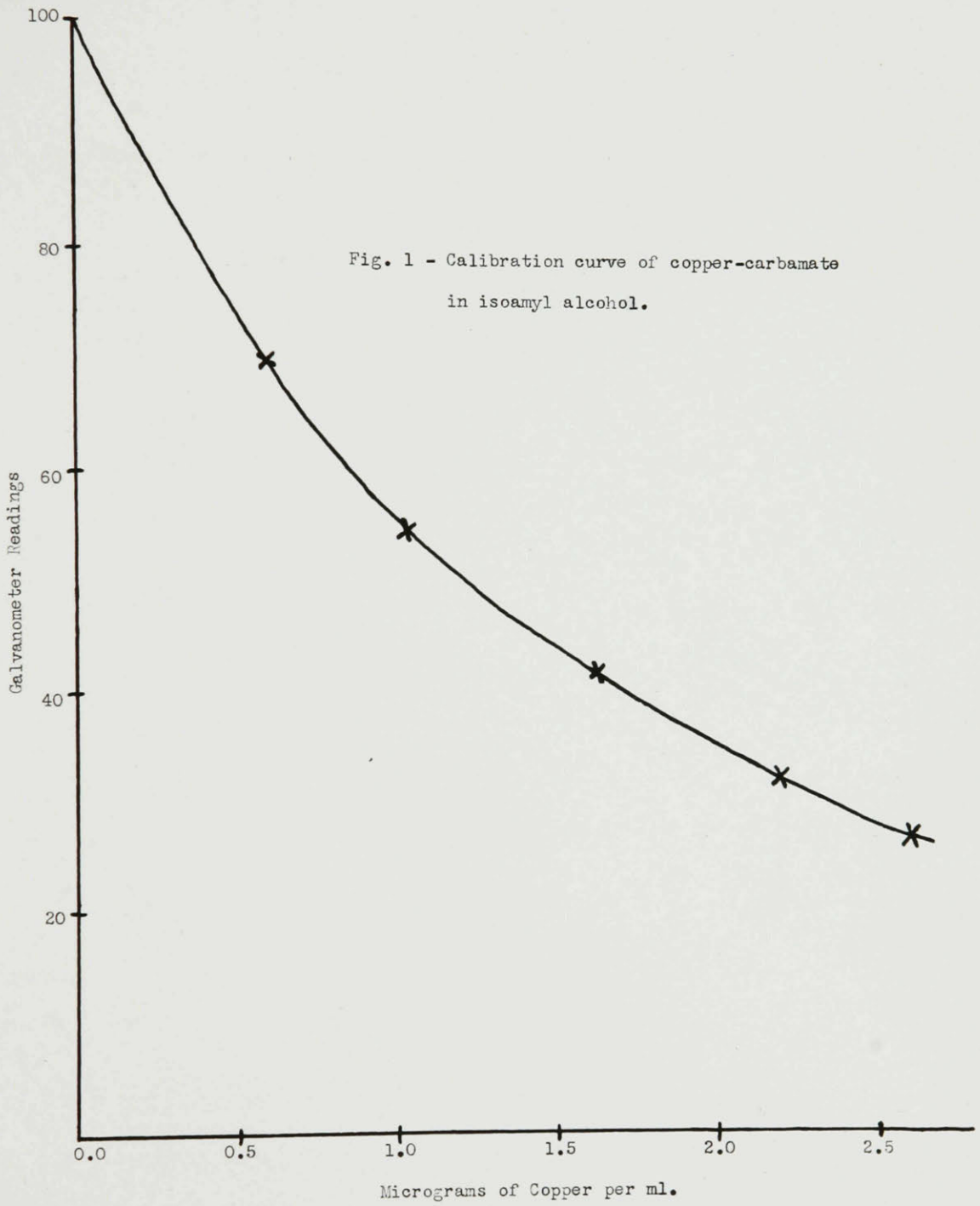


Fig. 1 - Calibration curve of copper-carbamate in isoamyl alcohol.

acetone had not heretofore been controlled and this would be expected to be an important factor in the swelling or hydration of the milk proteins. The amount of water in the acetone was increased from about 2% to 5% and the extracting reagent was now a 5% solution of KSCN in 95% acetone. Reproducible results were finally obtained by the procedure described in detail below.

REAGENTS

1. - EXTRACTING SOLUTION: 5 g. potassium thiocyanate were dissolved in 100 ml. 95% acetone.

2. - AMMONIUM CITRATE SOLUTION: Citric acid (50 gm.) was dissolved in 330 ml. water and 70 ml. of concentrated ammonium hydroxide was added. To this solution in a separatory funnel was added 2 ml. of the modified carbamate reagent, and the coloured copper complex was extracted with 15 ml. portions of isoamyl alcohol. Usually four extractions were sufficient.

3. - NEW CARBAMATE REAGENT: 0.2 g. of carbon disulphide were dissolved in 100 ml. water, with the aid of vigorous shaking, and to the solution was added 2 gm. of diethanolamine.

4. - REDISTILLED WATER: Distilled water was redistilled in an all glass still.

PROCEDURE:

A. - EXTRACTION: 2.5 g. milk powder were weighed into a 125 ml. Erlenmeyer flask with ground-glass joint, and 30 ml. extracting solution added. The material was refluxed for 10 minutes on a water bath. The clear extract was decanted into a 125 ml. Erlenmeyer flask and the residue was washed successively with one-10 ml. and two 5-ml. portions of

extracting solution. The extracts were combined and the acetone distilled off. The residue was dissolved in 30 ml. ammonium citrate solution and the contents of the flask were transferred to a 125 ml. separatory funnel. The flask was rinsed with 30 ml. of the same solution, and this was added to the contents of the separatory funnel.

B. - COLOUR REACTION: 2 ml. carbamate reagent were added and after mixing, the milk pigments were removed by extracting twice with 10 ml. portions of petrol-ether. Finally the copper carbamate was extracted by shaking for one minute with 10 ml. isoamyl alcohol and the aqueous layer was discarded. To clarify the isoamyl alcohol extract a further .5 ml. of isoamyl alcohol were added.

C. - COLORIMETRY: The colour intensity was measured in a Coleman Spectrophotometer with the instrument set at 435 mu, and previously adjusted to 100% transmittency with pure isoamyl alcohol in the colorimeter tube. The copper content of the reagents was determined by a 'blank' estimation in which the entire procedure was carried through, but without milk powder, and starting with 50 ml. extracting solution. The copper content of the 'test' and 'blank' solutions was determined by reference to a calibration curve and the copper content of the sample calculated by difference.

RESULTS:

The copper content of six different whole milk powders was determined by this extraction method and by dry ashing (16) with the results shown in Table II. It will be seen that when the methods are applied to milk powders with a low copper content the results by ashing are considerably lower than those obtained by extraction.

TABLE II. - Copper content of whole milk powders as determined by extraction, dry ashing and wet digestion methods

Sample No.	Copper - μ.g. per g. milk powder		
	Extraction	Dry Ashing	Wet Digestion
1 (a)	1.2	0.8	1.4
(b)	1.2	0.5	1.2
(c)	1.2	0.7	1.2
2	0.9	0.6	-
3	0.9	0.7	-
4	2.0	1.8	-
5	2.8	2.2	-

Identical results were obtained in triplicate determinations on the same sample, by the extraction method and the value agrees closely with those obtained by the 'wet digestion' method but the results by 'dry ashing' are lower and more variable (Table II).

Known amounts of copper were added to portions of the same milk powder and the samples analyzed by the extraction method with the results shown in Table III. Satisfactory recoveries of the added copper were obtained.

TABLE III. - Recovery of varying amounts of copper added to a milk powder

Sample No.	Copper - μ.g. per g. milk powder		
	Added	Recovered	% Recovered
1	2.0	1.9	98
2	6.0	6.0	100
3	14.0	13.2	94
4	20.0	18.8	94

D. - Modified method employing visual colorimetry

Since many 'control' laboratories in powdered milk plants may not be equipped with a photoelectric colorimeter it was thought advisable to modify the method to permit visual colour comparison with a series of standards. The intensity of the colour of copper carbamate in isoamyl alcohol decreases slowly on standing and hence these solutions are unsuitable for permanent standards. It was found, however, that the colour shade of a dilute aqueous solution of Alizarin Yellow G was indistinguishable from the colour of copper carbamate in isoamyl alcohol and the colour was quite stable. The colour standards were prepared as follows:

A sample of Alizarin Yellow G (British Drug Houses) was purified by dissolving in absolute ethyl alcohol, filtering, and evaporating the solution to dryness under reduced pressure. 0.1103 g. of the powdered alcohol-soluble Alizarin Yellow G was dissolved in redistilled water, with the aid of heat, and the solution diluted to 100 ml. in a volumetric flask. A 1 ml. aliquot of this stock solution was transferred to a 100 ml. volumetric flask and diluted to volume with redistilled water. The colorimeter reading on this standard solution was equivalent to a copper carbamate solution containing 1 p.p.m. Cu.

A series of colour standards was prepared by selecting ten-matched test tubes (150 x 18 m.m.). Aliquots of the standard Alizarin solution, 1 to 10 ml., were pipetted into the test tubes and each diluted to 10 ml. with redistilled water, employing a Mohr-type pipette. The contents of each tube were mixed with a swirling motion, the tubes stoppered and placed in order in a test tube rack backed by a sheet of white paper or a piece of white frosted glass.

A 5 g. sample of milk powder was taken for analysis and the extraction of copper was carried out in the manner described above. The final isoamyl alcohol solution of the coloured copper salt was transferred to a test tube of the same dimensions as those used to prepare the colour standards.

The colour matching was carried out in daylight, the operator (standing with his back to the window and holding the test tube rack level with the eye) fitted the sample solution into the standard series until the position of closest match was obtained. Assuming that the sample tube matched tube No. 8 in the series (8 ml. standard Alizarin solution plus 2 ml. water) the copper content of the milk powder was: 8/10 x 15/5 = 2.4 parts per million.

The colour standards covered a range of 0.3 to 3 p.p.m. of copper in intervals of 0.3 p.p.m., when a 5 g. sample of milk powder was taken for analyses. The sample tube could be matched between a pair of standard tubes so that the error should not exceed 10%.

SUMMARY OF PART I

A detailed account is given of experiments to develop a method for determining the copper content of milk powder, which would be simpler and more convenient for routine 'plant' control operations than present methods involving ashing and wet digestion. Two different extracting agents were studied, namely, sodium diethyldithiocarbamate and potassium thiocyanate.

Procedures based on the first extracting agent gave unsatisfactory results due to adsorption of copper by the milk proteins. A successful method, based on the second extracting agent, has been developed in which the copper is extracted with potassium thiocyanate dissolved in 95% acetone and the copper content of the extract determined with a 'new' dithiocarbamate reagent dihydroxyethyldithiocarbamic acid - instead of sodium diethyldithiocarbamate.

The results are higher than those obtained by ashing and agree closely with the results obtained by wet digestion. It is doubtful if the proposed method has any advantages over the 'wet digestion' method for general analysis but it may be preferred in some circumstances.

The method can be further simplified by employing visual colorimetry with Alizarin Yellow G as the colour standard. This would be advantageous for routine analysis in milk powder plants not equipped with a photoelectric colorimeter.

PART II

STUDIES ON THE SEPARATION AND ESTIMATION OF THE NEUTRAL AMINO ACIDS

REVIEW OF THE LITERATURE

Emil Fisher (7) showed that the ethyl esters of certain amino acids could be separated by fractional distillation. This method of separation was later found to be unsatisfactory by Osborne and Jones (23) . Dakin (4)[-5] observed that the monoamino monocarboxylic acids could be extracted from a concentrated aqueous solution by warm n - butyl alcohol. Johns and Jones (14) found alamine in the aqueous residue and glutamic acid in the n - butanol extract.

Brazier (2) fractionated the dry copper salts of the amino acids into three groups by successive extraction with different solvents, as follows:

A. - The copper salts of valine, proline and isoleucine are soluble in dry methanol and the copper salts of leucine and tyrosine are partially soluble.

B. - The copper salts of alanine, tyrosine, glutamic acid, histidine, arginine, lysine and glysine are insoluble in dry methanol, but are soluble in water.

C. - The copper salts of leucine, phenylalanine and aspartic acid are insoluble in cold water and dry methanol.

The two diamino acids, arginine and lysine, may be separated from the other amino acids by chromatographic adsorption on neutral aluminum oxide, as reported by Wieland (28)[-29]. Turba (25) fractionated the basic amino acids into the individual components by the use of a combination of bleaching earths - "Floridin XX Extra" and "Filtrol Neutrol".

Wieland (28[-29]) also observed that the dicarboxylic amino acids could be quantitatively separated from the neutral and basic amino acids by adsorption on acid-treated aluminum oxide. This was confirmed by Turba and Richter (26) who also showed that the fractionation of mixtures of aspartic and glutamic acids with the same adsorbent could be carried out.

Wachtel and Cassidy (27), Schramm and Primosigh (24) working with charcoal found that the neutral aromatic amino acids can be separated from the neutral aliphatic amino acids. The first two authors reported some decomposition of phenylalanine and tyrosine. In the presence of formaldehyde, glycine and serine can be adsorbed on acid-treated aluminum oxide as shown by Schram and Primosigh (24). While studying the reaction of azobenzene 4 - carbonylchloride and amino carboxylic acids, Karrer, Keller and Szönyi (15) noticed that the products, dissolved in 95% ligroin - 5% benzene, could be adsorbed on a column of basic $ZnCO_3$ - the zones on the column, in descending order, being glycine, alanine, leucine and valine.

EXPERIMENTAL

I. - SEPARATION OF THE COPPER SALTS OF THE AMINO ACIDS BY ADSORPTION METHODS

A. - Preparation of the copper salts of amino acids

The copper salts of l-alanine, l-tyrosine, l-proline, l-leucine and dl-valine were prepared as follows: 500 mg. each of l-alanine, dl-valine, l-proline; 25 mg. of l-leucine and 125 mg. of l-tyrosine were dissolved separately in 50 ml. of boiling water. Copper carbonate powder was added to each solution until effervescence ceased. The solutions were filtered while hot and the residues washed with boiling water. The filtrates were allowed to cool to room temperatures, and then placed in the ice box for a few days.

A good crop of crystals were obtained from tyrosine, valine, leucine and alanine. The copper salt of proline, being highly soluble in water even at low temperatures, did not crystallize out. The proline solution was concentrated and the salt was granulated with acetone. The copper salts were finally washed with acetone and dried.

B. - Response of the copper salts of the amino acids to the ninhydrin reagent

The ninhydrin test was used as a spot test to follow the course of adsorption of the copper salts of the amino acids. It was found that the ninhydrin reagent did not give a positive test with the copper salts of the amino acids unless the solution was boiled in the presence of

HCl before the reagent was added and a relatively large amount of ninhydrin must be present. Under these conditions, the sensitivity of the test was not decreased.

C. - Preparation of silica-gel

The silica-gel was prepared by the following procedure as described by Martin and Synge (20) - 500 mls. of commercial water-glass were mixed with one litre of water and three drops of methyl red indicator added. Concentrated HCl was slowly added, with constant agitation, until the indicator remained red. The mixture was allowed to stand for 24 hours, filtered and the silica-gel washed with distilled water until colourless. It was aged on the filter for three days, dried at 110°C and finally activated by heating for three hours at 600 - 700°C. Subsequent experience showed that different batches of silica-gel varied in their adsorption properties, depending upon the amount of HCl used for precipitation. This factor is difficult to control as the indicator end-point is indefinite.

D. - Adsorption of the copper salts of the amino acids on various adsorbants

I. - Adsorption on silica-gel - It was desirable to employ a copper-free adsorbant so that copper estimations could be used to determine the amino acids after their fractionation as the copper salts. Since silica-gel is a synthetic adsorbant, it can be purified during its preparation. The object of the first experiment was to determine if the copper salts of amino acids

were adsorbed on a column of silica-gel and if they vary in their rates of elution.

Five ml. aliquots of aqueous solutions of tyrosine, valine, proline and alanine salts, each containing one mg. per ml., were passed through separate columns of silica-gel. The columns were eluted successively with 5, 10, and 15 mls., of water. The washings were collected separately and analyzed for their amino acid and copper content using ninhydrin and sodium diethyldithiocarbamate, respectively. The determinations were not quantitative but only relative values were obtained by visual comparison of the colours.

The data given in Table IV indicates that the rates of elution of the salts varied appreciably.

TABLE IV - Relative values of ninhydrin test on water eluates

Amino acid salts	Successive washings		
	(5 ml.)	(10 ml.)	(15 ml.)
Tyrosine	3*	4	0
Valine	2	3	0
Proline	1.5	3	0
Alanine	1	2.5	2

* The original solution was given an arbitrary colour value of 10.

It is important to note that the tests for copper were negative.

In a second experiment with alanine and valine using a different batch of silica-gel, the results in Table V were obtained.

Again the values are only relative.

TABLE V - Relative values of ninhydrin test on water eluates

Amino acid salts	Successive washings			
	1.(5ml.)	11.(10 ml.)	111.(10 ml.)	1V.(10 ml.)
Alanine	Trace	4.5	4.5	Trace
Valine	Trace	3.0	5.0	Trace

Once again the copper tests were negative. When the blue coloured solution of amino acid salts passed through the adsorption column, a blue coloured layer formed at the top. It appeared that the salts were adsorbed since, on washing, the blue colour passed very slowly down the column. However, since the eluate did not contain copper, the copper salts of the amino acids must have dissociated on the adsorbant. If dissociation is involved, a non - dissociating solvent, such as absolute methanol, should be used for elution.

In an attempt to elute the copper salts with absolute methanol, 5 ml. of both alanine salt and valine salt solutions were passed through separate columns. The columns were dried by applying vacuum and then washed successively with eight (10 ml.) portions of absolute methyl alcohol.

The eluates were collected separately and analyzed for copper and amino acids. The results are given in Table VI .

TABLE VI - Relative values of ninhydrin and copper tests on methanol eluates

Amino acid salts	Tests	Successive washings (T = traces)						
		1	11	111	1V	V	V1	V11
Alanine	Ninhydrin	2	2	2.5	1	½	T	T
	Copper	0	2	2.5	1	T	T	T
Valine	Ninhydrin	3	3	T	T	T		
	Copper	0	3	T	T	T		

The results show that with the exception of the first washing, the copper salts were eluated by methyl alcohol without decomposition. However, the procedure would not be expected to effect a separation of the amino acids, since the rates of elution differed only slightly and water had to be used as the initial solvent.

2. - Adsorption on Permutit - In this experiment silica-gel was replaced by Permutit to see if better adsorption could be obtained. Ten ml. of an aqueous solution of alanine salt were passed through a column of Permutit and the column was eluated with water as before. The ninhydrin and the copper tests showed that practically no adsorption or decomposition took place. The experiment was repeated with valine salt with the same results.

3. - Adsorption on copper carbonate - $CuCO_3 - Cu(OH)_2$. It was expected that, when solutions of pure amino acids are passed through a column of copper carbonate, the free amino acids would be adsorbed by forming loose bonds with copper. To confirm this assumption, an adsorption column was prepared with copper carbonate ground to 100 mesh and through it was passed 5 ml. of an aqueous solution of alanine (1 mg./ml.) . The column was eluted with 50 mls., and 5 mls., of water. The eluate had a blue colour, showing the alanine had been converted to the copper salt. The second washing with 5 ml. of water was free from amino acid or copper.

This experiment was repeated with leucine. Since the copper salt of leucine is insoluble in water, it was expected that it would not be eluted with water. However, it was found that the copper salt of leucine was not formed under these conditions, the leucine being washed through the column. When a hot solution of leucine was passed through the column, part of the leucine was converted to the copper salt, but it was eluted with water.

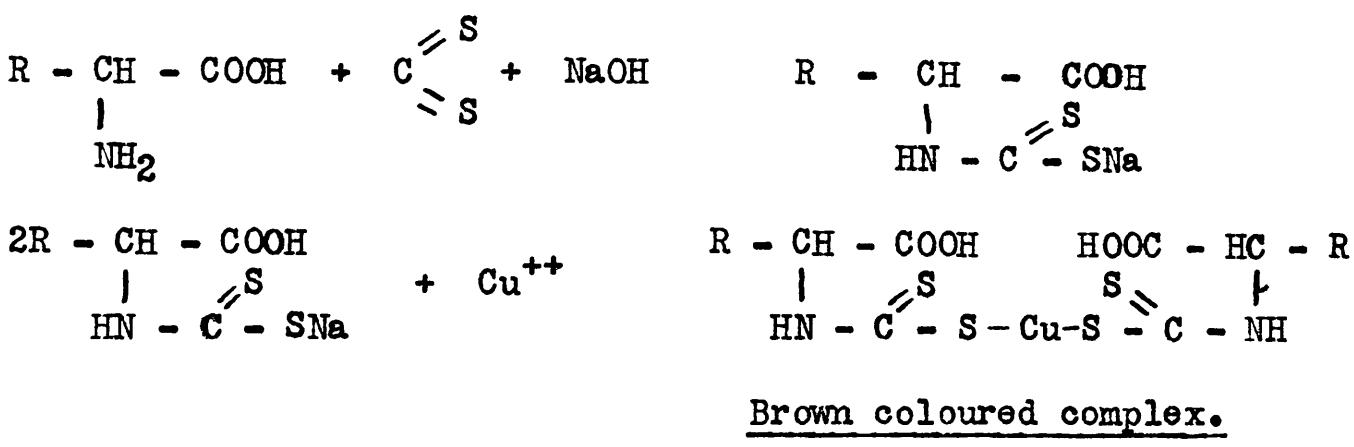
It is interesting to note that although no adsorption took place, a column of copper carbonate could be used to prepare the copper salts of amino acids.

4. - Adsorption on copper oxides - In a similar experiment cupric and cuprous oxides were used, instead of copper carbonate, to study

the adsorption of alanine and valine. No marked differences were observed between the behaviour of copper carbonate and cupric oxide. Cuprous oxide, unlike the others, transformed only a small part of the alanine and valine into their copper salts. It was found also that cuprous oxide did not adsorb the free amino acids.

II. - DETERMINATION OF AMINO ACIDS AS THE COPPER SALTS OF A SULPHUR DERIVATIVE-DITHIOCARBAMIC ACID

It is known that primary or secondary aliphatic amines form a dithiocarbamic acid salt, or a derivative, on the addition of carbon disulphide in alkaline solution (18). It is also known that these dithiocarbamic acids combine with copper to give a brown-coloured compound which can be estimated colorimetrically (3). Since they contain a primary and secondary amine group, this reaction should be given by amino acids, as follows: -

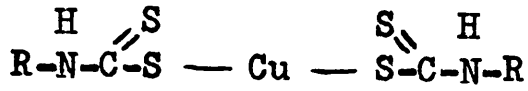


Brown coloured complex.

These reactions have been carried out with alanine and glycine by Fisher (8), Körner (17), Andreash (1), but these workers were mainly interested in qualitative tests.

The first experiment on the adsorption properties of this derivative of amino acids gave promising results indicating that this property of carboxydithiocarbamic acids might offer a means of separating and determining amino acids, especially the momoamino-monocarboxylic acids. The differential solubility of these compounds in various solvents might also be utilized. It was, therefore, decided to study the conditions under which the sulphur derivative is formed.

For purposes of comparison, the amino acids concentrations were expressed as Moles x 10^{-8} . This choice is justified by the fact that the colour is due to the group:



and is not influenced by the nature of the radicle.

A. - Reaction between carbon bisulfide and the amino acids

The amino acids studied were l - tyrosine, l - tryptophane, dl - phenylalanine, l-histidine (HCl), d-arginine (HCl), l-methionine, l-proline, l-hydroxy proline, glutamic acid (HCl) aspartic acid, glycine, alanine, dl-serine, threonine, valine, norleucine, leucine, and isoleucine. Standard solutions of these amino acids, each containing 63.0 moles 10^{-8} per ml., were prepared. To 1 ml. of each solution was added 1 ml. of buffer solution, 5 ml. of 95% ethyl alcohol, 2 ml. of 15% carbon disulphide in 95% ethyl alcohol and finally 1 ml. of copper sulfate solution (50 p.p.m. of Cu). The solutions were shaken and the intensity of the colours measured in a spectrophotometer. A blank was prepared containing all the reagents.

The percentage yield was calculated from the galvanometer reading by reference to a calibration curve prepared with standard solutions of copper diethyldithiocarbamate. In preparing the calibration curve an excess of the reagent (sodium diethyldithiocarbamate) was used to insure complete combination with copper.

The results given in Table VII indicate that pH had a marked influence on the yield of the copper carboxydithiocarbamates and that the

TABLE VII - Yield of copper dithiocarbamates of amino acids. (Results expressed as percentage of the theoretical yield - Maximum values are underlined.)

pH	4.0	4.4	4.8	5.2	5.6	6.8	7.0	7.6	8.0	8.4	8.8	9.0	9.4	10.0
l-Tyrosine	1	1	4.5	10	19	4.5	22	22	22	17	12	12	5	5
l-Tryptophane	1	1	5	10	20	3.	21	25	25	20	18	10	6	5
dl-Phenylalanine	4.5	4.5	6.0	12	20	2.0	23	36	26	28	19	11	9	15
l-Histidine(HCL)	0	1	1	3	6	0	7	7	7	7	7	7	7	4.5
d-Arginine(HCL)	2	1.5	4.5	8	10	1.5	8	9	7	7	8	6	-	-
l-Methionine	5	7	7	7	10	7	12	9	14	12	13	11	13	14
l-Proline	2.5	2.5	8	15	38	4.5	40	38	38	38	38	34	34	20
l-OH Proline	3	4	7	15	23	4.5	34	40	42	45	39	36	40	25
Glutamic acid(HCL)	2.5	2.5	2	2	3.5	3.0	8	24	28	26	18	16	13	18
Aspartic acid	8	7	7	6.5	9	3.5	9	18	19	20	16	16	10	13
Glycine	6	6	9	16	17.5	4.5	23	31	28	24	14	17	16	16
Alanine	-	-	-	9	12	6	20	31	29	24	14	13	15	-
dl-Serine	4	4	7	11	20	9	17	17	13	10	13	11	-	-
Threonine	-	-	-	16	18	12	16	20	11	6	8	8	9	-
Valine	-	-	-	7	12	6	6	20	16	11	6	11	9	-
Norleucine	-	-	-	6	9	4.5	9	18	10	10	17	11	12	-
Leucine	-	-	-	10	16	12	13	19	12	7	6	8	7	-
Isoleucine	-	-	-	12	18	10	15	12	11	4.5	8	7	8	-

effect was the same for all the amino acids studied. In all instances the yield increased up to pH 5.6 and then suddenly dropped at pH 5.8 . The maximum values were always obtained at about pH 7.6 with the exception of isoleucine, which was at pH 5.6 . It was evident that under the conditions of the test, the yield of the copper carboxy-dithiocarbamates was generally low. Hydroxyproline which is the highest, yielded only 45% of the theoretical amount.

B. - Study of the dithiocarbamic acid reaction

I. - Order of the reaction

It was next decided to determine the order of the reaction to establish the conditions which would give a higher yield. The rate of the reaction was measured with proline at pH 7.0 . At this pH the reaction is sufficiently slow to permit relatively accurate measurements.

The procedure employed was the same as described above with the exception that the intensity of the colour was measured at intervals of 2.5 seconds until 15 readings had been made and then at 5 - second intervals until two minutes had elapsed. The amount of the product formed at each time interval was determined from its copper content and was expressed as 'copper combined'. The concentration was then plotted against time as shown in Fig. 2 - (Table VIII).

TABLE VIII - Data showing the rate of the reaction between carbon-disulfide, proline and copper

Time (Sec.)	Conc. of copper Combined (μg./ml.)	Time (Sec.)	Conc. of copper Combined (μ.g./ml.)
10	.23	55	.86
12	.27	60	.90
15	.31	65	.93
17	.36	70	.95
20	.41	75	.97
22	.44	80	.99
25	.51	85	1.01
27	.56	90	1.03
30	.61	95	1.05
32	.66	100	1.06
35	.69	105	1.07
37	.72	110	1.08
40	.75	115	1.09
42	.78	120	1.10
45	.81		

The curve (Fig. 2) is not characteristic of either a zero, first, second or third order reaction. A later experiment revealed that the concentration of copper is an important factor. If the amount present is greater than the theoretical requirement, the reaction is retarded. In this experiment, 100 μ.g. of Cu. were used, whereas the theoretical amount of copper was 20 μ.g.

The next step in the study of the reaction was to investigate the properties of solutions of the product. It was assumed that when an excess of copper was present, it would combine with almost all the undecomposed dithiocarbamic acid.

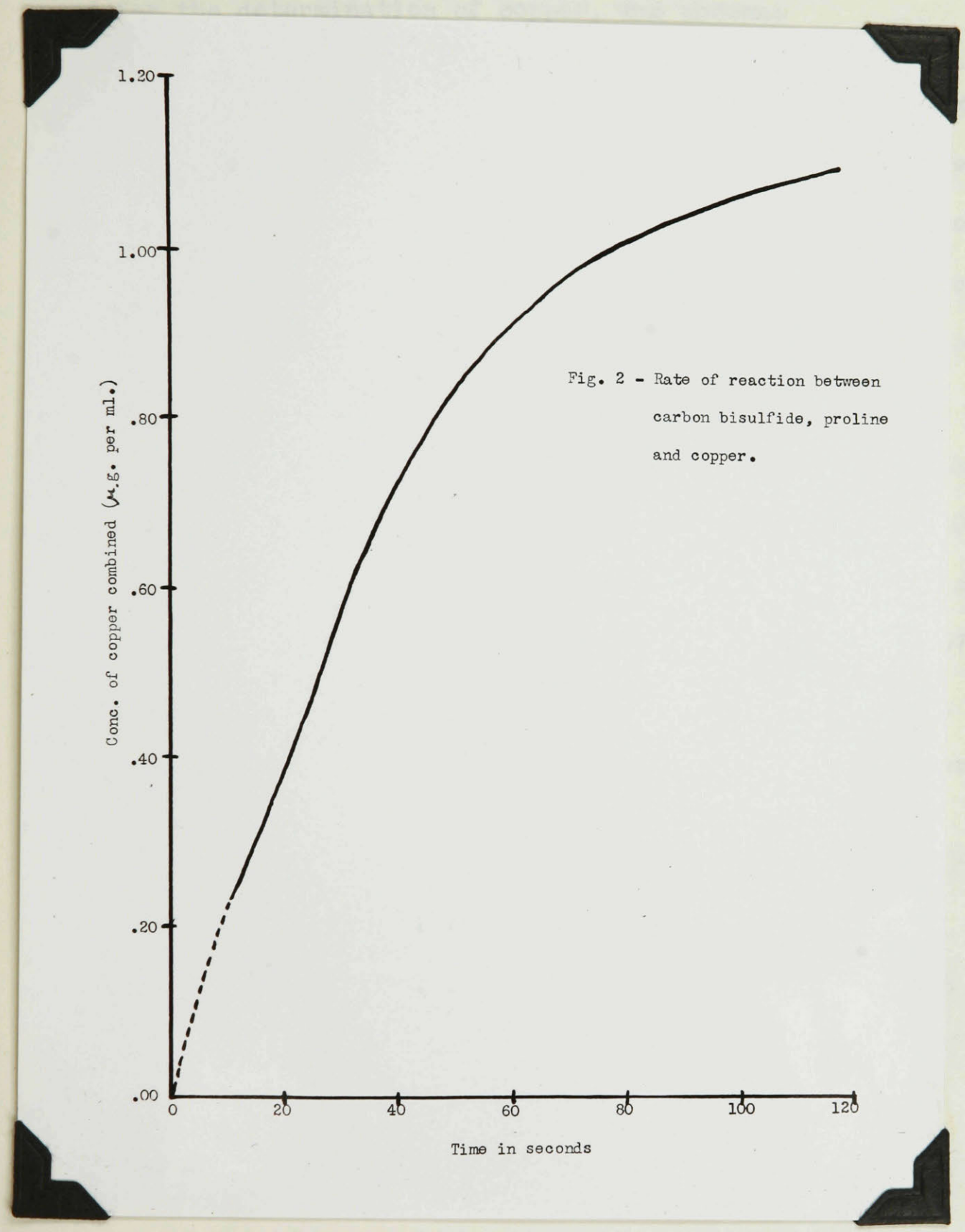


Fig. 2 - Rate of reaction between carbon bisulfide, proline and copper.

2. - Properties of solutions of sodium diethyldithiocarbamate

To study the reaction a preformed dithiocarbamate could be used to advantage and sodium diethyldithiocarbamate, a reagent commonly used for the determination of copper, was chosen.

a) - Influence of concentration of sodium diethyldithiocarbamate:

In a series of experiments various concentrations of sodium diethyldithiocarbamate were allowed to react with a fixed amount of copper and the concentrations of the product were measured colorimetrically. The results were plotted against the original concentration of sodium diethyldithiocarbamate. A standard aqueous solution of sodium diethyldithiocarbamate was prepared and by using the proper dilutions, solutions containing 2,36,11.8, 21.24, 47.2, 118.0, and 236 moles x 10^{-8} per ml. were obtained. To 10 ml. of each of these solutions was added 1 ml. of a copper solution containing 157.3 moles x 10^{-8} per ml., and the coloured product was extracted with 10 ml. of chloroform and diluted to permit colorimetric measurements.

TABLE IX - Influence of concentration of sodium diethyldithiocarbamate on its reaction with copper.

Concentration of sodium diethyldithiocarbamate (Moles x 10^{-8} per ml.)	Concentration of copper combined (Moles x 10^{-8} per ml.)
11.5	6.0
21.5	13.3
46.6	31.5
118.5	83.3

The results (Fig. 3 - Table IX) showed that the concentration of copper dithiocarbamate increased with the concentration of dithiocarbamic acid in such a way as to give a straight line when one component was plotted against the other. However, it was evident that the reaction did not go to completion. When the percent-reaction was plotted against the original concentration, as in Fig. 4, Curve A, it could be seen that the percent-reaction increased rapidly at low concentrations and slowly at higher concentrations. If it is assumed that the sodium diethyldithiocarbamate which did not react with copper was decomposed in some manner or other, and if the percentage decomposition is plotted against the original concentration, it is found that the curve obtained is characteristic of a hydrolytic reaction. (Fig. 4, curve B).

The same experiment was repeated with 95% ethyl alcohol as the solvent and the percentage decomposition was found to be higher (about 85%) and not influenced by concentration.

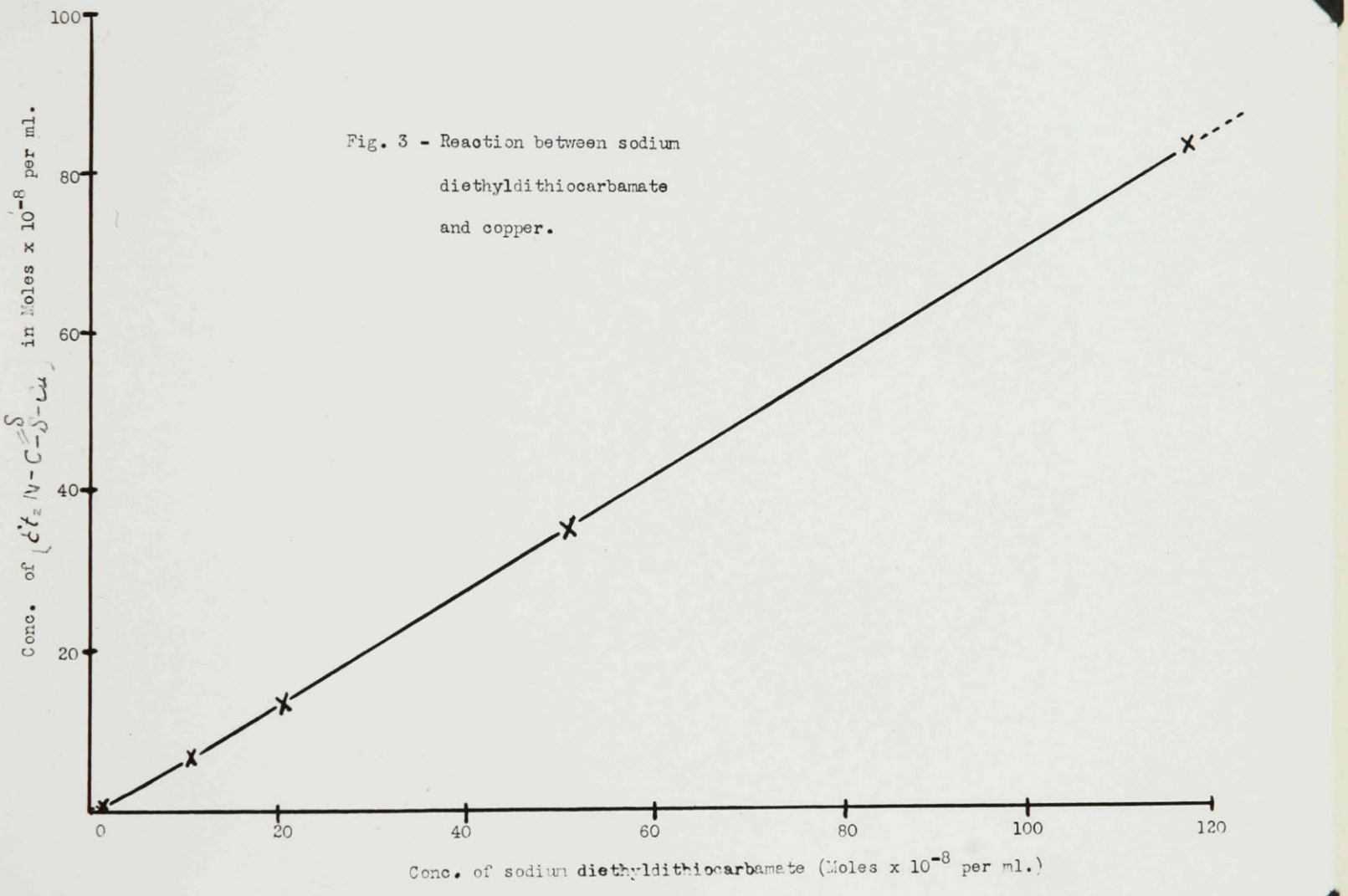
b) - Possible mechanisms of the decomposition:

According to Levi (18), dithiocarbamates yield carbon disulphide and the corresponding amine, when heated in aqueous solution. It is possible, therefore, that decomposition occurred by the same mechanisms at room temperatures. The reaction involved may be:

I
$$Et_2N - C(=S) - SNa \rightleftharpoons \left[Et_2N - C(=S) - S\right]^- + Na^+$$
$$\left[Et_2N - C(=S) - S\right]^- + HOH + Na^+ \rightleftharpoons Et_2NH + CS_2 + NaOH$$

II
$$Et_2N - C(=S) - SNa + HOH \rightleftharpoons Et_2NH + OH - C(=S) - SNa$$
$$OH - C(=S) - SNa \rightleftharpoons CS_2 + NaOH$$

Fig. 3 - Reaction between sodium diethyldithiocarbamate and copper.



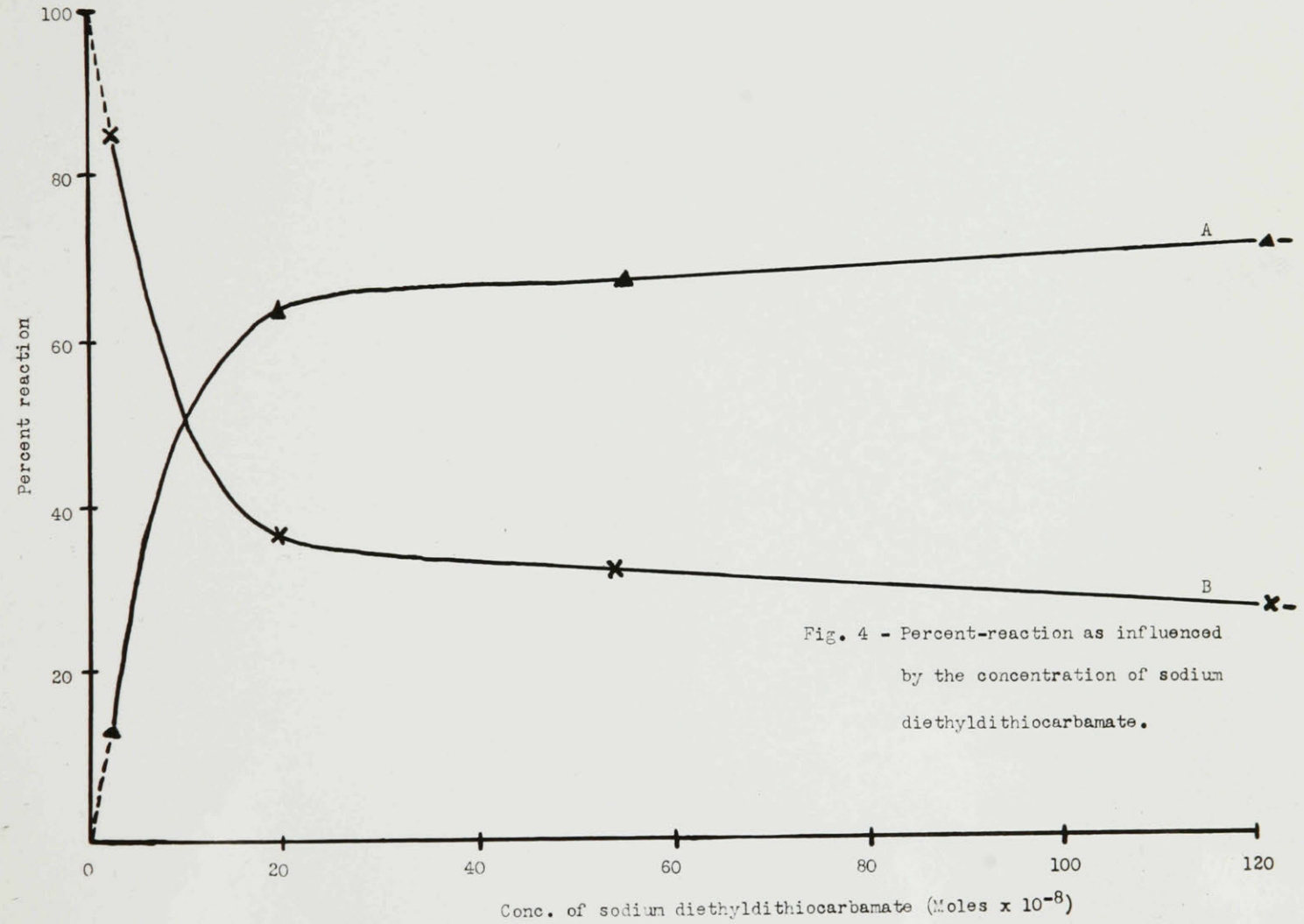


Fig. 4 - Percent-reaction as influenced by the concentration of sodium diethyldithiocarbamate.

If one or other of these reversible reaction occurs, it should be possible in practice to shift the equilibrium by the addition of an excess of one of the products of the reaction. If as in the first mechanism, the dissociation of the sodium-ion is needed for subsequent hydrolysis, the addition of an excess of sodium-ion should push the reaction to the left and thus prevent decomposition.

c) - Influence of excess sodium ion:

Aqueous solutions of NaOH containing 0.10, 10,000 and 100,000 moles x 10^{-8} per ml., were prepared and to 100 ml. of each was added 20 mgs. of dithiocarbamate (12,000 moles x 10^{-8}). These solutions were then diluted to a concentration of 12 moles x 10^{-8} of dithiocarbamate per ml., with fresh solutions of NaOH of the corresponding strengths. The percentage decomposition of dithiocarbamate was determined by the procedure described above. The results in Table X indicate that the decomposition was slightly reduced by increasing the concentration of sodium-ions.

TABLE X - Influence of sodium-ion concentration on the decomposition of sodium diethyldithiocarbamate

Conc. of sodium-ion in moles x 10^{-8}/ml.	% Decomposition
0	45.6
10	42.0
10,000	40.0
100,000	40.0

However, this slight influence of sodium-ion cannot be taken as evidence in favour of the first reaction mechanism which requires the dissociation of sodium-ions prior to hydrolysis. On the contrary, the evidence could favour the second mechanism where dissociation of sodium-ion occurs in the last step.

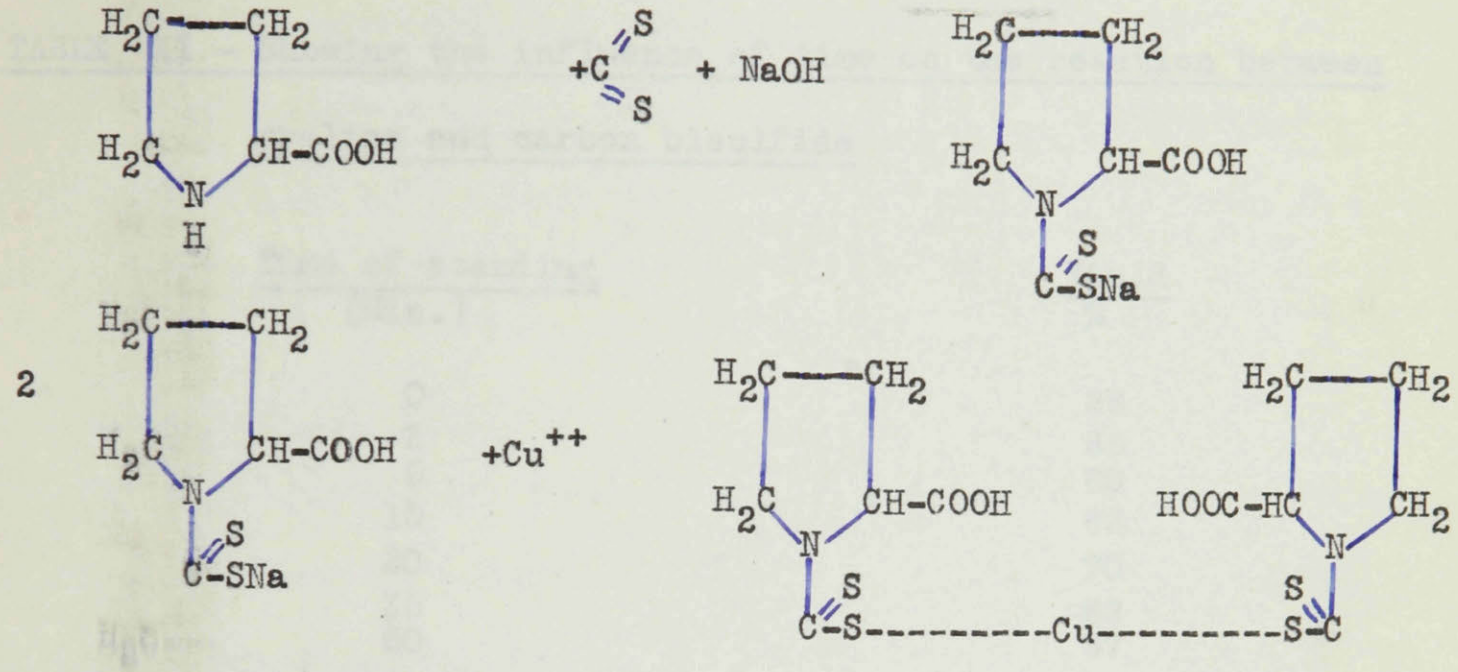
In an attempt to prove that the second mechanism occurs, the proceeding experiment was repeated in the presence of sodium thionthiolate (OHCSSNa) . This salt was prepared by refluxing a normal solution of NaOH with an excess of CS_2 for 30 minutes. The solution was diluted and used to replace the NaOH in the first experiment.

The results showed that sodium thionthiolate interfered with the reaction between sodium diethyldithiocarbamate and copper. Copper dithiocarbamate could not be extracted from solutions containing 10,000 and 100,000 moles x 10^{-8} per ml., amd at a concentration of 10 moles x 10^{-8} per ml., the results indicated 56% decomposition of the original dithiocarbamate. Therefore, the formation of sodium thionthiolate is not involved in the decomposition. Notwithstanding the lack of evidence, mechanism 1 was accepted temporarily as explaining the formation and decomposition of dithiocarbamic acids.

C. - Determination of proline as a dithiocarbamate derivative

Proline and hydroxyproline gave the highest yields of copper dithiocarbamate, 40 and 45% respectively. Since there is no sensitive method for determining proline and since the present reaction seemed promising as the basis of a micro-method, it was decided to direct the present work into this field. Furthermore, the knowledge acquired could be generalized and applied to other amino acids.

The copper dithiocarbamate derivative of proline is formed in two steps: (a) the addition of CS_2 to proline to give 1-carboxypyrrole-dithiocarbamic acid and (b) the combination of two 1-carboxypyrrole-dithiocarbamic acid molecules with one atom of copper, as follows:



Some of the factors which might influence these reactions were studied in the following experiments.

1. - Reaction time

Since the second step in the reaction is instantaneous, the influence of time was studied only on the first step and this was done as follows:-

To 1 ml. of a standard solution of proline in a test tube, was added 1 ml. buffer solution (pH 7.6), 5 ml. of 95% ethyl alcohol and 2 ml. of a 15% solution of CS_2 in 95% ethyl alcohol. After shaking, the solution was allowed to stand for a certain length of time and then the colour was developed by adding 1 ml. of a copper solution (25 p.p.m. of Cu). The intensity of the colour was measured immediately after the last addition. A blank was prepared containing all the reagents and was used to adjust the colorimeter to 100% transmittancy.

Seven tubes were prepared, in order to determine the amount of 1-carboxypyrrole dithiocarbamic acid formed, at seven different intervals of time. The copper solution was added after 0,2,5,10,20, 30 and 60 minutes. The results are given in Table XI .

TABLE XI - Showing the influence of time on the reaction between proline and carbon bisulfide

Time of standing (Min.)	Yield %
0	88
2	85
5	80
10	82
20	70
30	68
60	67

The results (Table XI) showed that the amount of dithiocarbamic acid formed, as determined by this method, decreased with increasing time. The highest value was obtained when copper was added immediately following the addition of the CS_2 solution, and the colour was stable for at least 5 minutes.

2. - Influence of the concentration of CS_2

In the preceeding experiment the final concentration of CS_2 was 3%. In this experiment the concentration of CS_2 was varied from 0.0038%, which was the theoretical concentration needed for complete reaction, to 3.8% in order to determine the influence of the CS_2.

The results showed that the yield of copper dithiocarbamate decreased when the concentration of CS_2 was below 2%. The yield was

constant with 2 and 4% solutions of CS_2, therefore a 4% solution was used in subsequent work.

3. - Influence of temperature

Tests were carried out at 5, 10, 20 and 38°C. A definite increase in yield was obtained in a temperature range 5° to 20°C., but at higher temperatures only the speed of the reaction was increased. Prolonged heating at 38°C caused more rapid fading of the colour after the maximum had been reached.

4. - Influence of copper

None of the factors so far studied gave more constant results nor did they increase the yield. A careful study was next made of the influence of copper on the reaction.

In the first experiments, 100 µ.g. of copper were added to the test solution, but later, 25 µ.g. of copper were used and the amount kept constant while other factors were varied. The change from 100 µ.g. to 25 µ.g. Cu. (which is 5 µ.g. more than the theoretical required) increased the yield from 40% to 85 - 88%.

Increasing amounts of copper, from 12.5 to 125 µ.g. were added to the test solution and the yield of copper dithiocarbamate determined in each case. The results are presented in Table XII .

TABLE XII - Yield of copper 1-carboxypyrroledithiocarbamate as influenced by the amount of copper present

Amounts of copper (μ.g.)	Yield %
12.5	49
20.0*	83
25.0	82
30.0	72
40.0	61
50.0	52
100.0	42
125.0	39

* Theoretical amount for complete reaction

From the results in Table XII, it was evident that the amount of copper was highly critical. Maximum values were obtained with 20 μ.g. Cu. and the yield was reduced with increasing amount of copper. It was also noted that the maximum yield was obtained when the amount of copper corresponded to the theoretical required for complete reaction with dithiocarbamic acid. Thus, in an actual determination when the amount of proline is unknown, the quantity of copper reagent required would also be unknown. A study was next made of other copper salts in the hope that this difficulty might be overcome.

a) - Nature of the copper salt

Copper sulfate, which was used in the preceeding experiments, was highly dissociated. It was thought that less highly dissociated copper salts such as copper stearate, lactate and tartrate would have less effect on the reaction.

Copper stearate

This salt was added to the test solution in the form of an ethyl ether solution. The yields of copper dithiocarbamate were less than those obtained with copper sulfate. This may have been due to the interference by stearic acid or to a slowing of the reaction.

Copper lactate

This salt was prepared by boiling a lactic acid solution with copper carbonate and purifying the copper lactate by recrystallization from water. Aqueous solutions containing 20 to 100 μ.g. copper per ml. were tested. Copper lactate influenced the speed of the reaction; maximum yield (83%) was obtained after three hours standing, but the range of maximum values was not extended. The rate of the reaction was too low and too variable for it to be of value in an analytical method. (Table XIII) .

TABLE XIII - Influence of copper lactate on the yield of copper 1-carboxypyrroledithiocarbamate

Amounts of copper (μ.g.)	Yield %		
	0 Hr.	3 Hrs.	12 Hrs.
20 *	72	83	79
30	57	66	74
40	19	-	74
50	8	-	60
100	8	-	60

* Theoretical amount

Copper tartrate

This salt was prepared from tartaric acid and copper carbonate and purified by recrystallizing from water. The following concentrations were employed - 20, 28.5, 47.5 and 142 µ.g. copper per ml. The yields obtained are given in Table XIV .

TABLE XIV - Effect of varying amounts of copper, in the form of copper tartrate, on the yield of copper 1-carboxypyrroledithiocarbamate

Amount of copper (µ.g.)	Yield %	
	1 min.	2 min.
20	91.0	91.0
28.5	68.0	88.0
47.5	68.0	85.0
142.0	Turbid	Turbid

The use of copper tartrate gave a higher yield (91%) of copper dithiocarbamate in a shorter time. The rate of the reaction and the yield was reduced by increasing amounts of copper. Although copper tartrate gave better results than copper lactate and copper sulfate, it did not offer a satisfactory solution of the problem. The optimum range of copper concentrations was extended appreciably, but at the expense of accuracy.

b) - Method of adding copper

In the preceeding experiments, it was found that the colour intensity increased with increasing amounts of copper until the theoretical amount required for the complete reaction was reached

and then decreases. Although the reaction could not be controlled or stabilized, it could still be utilized for the quantitative determination of proline. With an unknown quantity of proline, it should be possible to determine the maximum point of the reaction by slowly increasing the amount of copper and progressively measuring the intensity of the colour.

This procedure was tried and found satisfactory. An experiment was carried out with two concentrations of proline (7.22 and 3.61 μ.g. per ml.) as follows:-

One ml. of each proline solution was transferred to a test tube and to each was added 1 ml. of buffer solution (pH 7.6), 5 ml. of 95% ethyl alcohol and 2 ml. of 20% CS_2. After mixing, a copper solution (142 p.p.m. Cu.) was immediately added drop by drop with shaking and the colour measured after the addition of each drop. The number of drops added and the corresponding readings were recorded. A reagent blank was prepared and used to adjust the colorimeter to 100% transmittancy.

The yields were calculated after correcting for the change in volume. The results are given in Table XV .

TABLE XV - Yield of copper 1-carboxypyrroledithiocarbamate as affected by the number of drops of copper sulfate added

Conc. of proline μ.g./ml.	No. of drops	Amount of copper μ.g.	Galvanometer readings	Yield %
7.22	1	6.45	69	22.6
	2	12.90	44	59.1
	3	19.35	32	86.7
	4	25.80	32	89.5
	5	32.25	33	86.6
	6	38.70	34	84.3
3.61	1	6.45	72	40.7
	2	12.90	61	65.4
	3	19.35	55	82.2
	4	25.80	51	94.5
	5	32.25	54	86.7
	6	38.70	56	80.6

The addition to proline solutions of increasing amounts of copper in increments of 6.45 μ.g. gave a galvanometer reading, corresponding to a maximum yield, after the fourth addition. Since the maximum yields were obtained with a larger amount of copper than the theoretical amount needed, it is possible that the maxima found were not the true values. It is to be expected that different results would be obtained with varying increment of copper.

A graphical method was used to determine the probable error due to the variable relation of copper to proline. A graph was prepared by plotting the amount of added copper in μ.g. against the amount of copper 1-carboxypyrroledithiocarbamate formed, expressed as μ.g. of copper.

The results obtained gave two straight lines (Figs. 5, Table XVI), each having a constant slope. When extrapolated the lines met at a point corresponding to a greater amount of copper dithiocarbamate than was determined colorimetrically. This point was considered to indicate the maximum yield of the reaction. At a level of 7.22 μ.g. of proline per ml. (Fig. 5A) the maximum yield was 91.2% instead of 89.5% as determined. This corrected maximum coincided with the theoretical amount of copper and the same value was obtained in an earlier experiment using 20 μ.g. of copper as copper tartrate.

These deductions were not equally applicable to the results with 3.61 μ.g. of proline per ml. The corrected maximum corresponded to 24.6 μ.g. of copper which was more than twice the amount needed. (Fig. 5B).

TABLE XVI - Amount of copper 1-carboxypyrroledithiocarbamate obtained with successive additions of copper

Level of proline μ.g.	Added copper μ.g.	Copper 1-carboxypyrroledithiocarbamate as μ.g. of copper
7.22	6.6	4.5
	13.2	11.8
	19.7	17.4
	26.3	17.9
	33.0	17.4
	39.6	16.9
3.61	6.6	4.0
	13.2	6.5
	19.7	8.2
	26.3	9.4
	33.0	8.7
	39.6	8.0

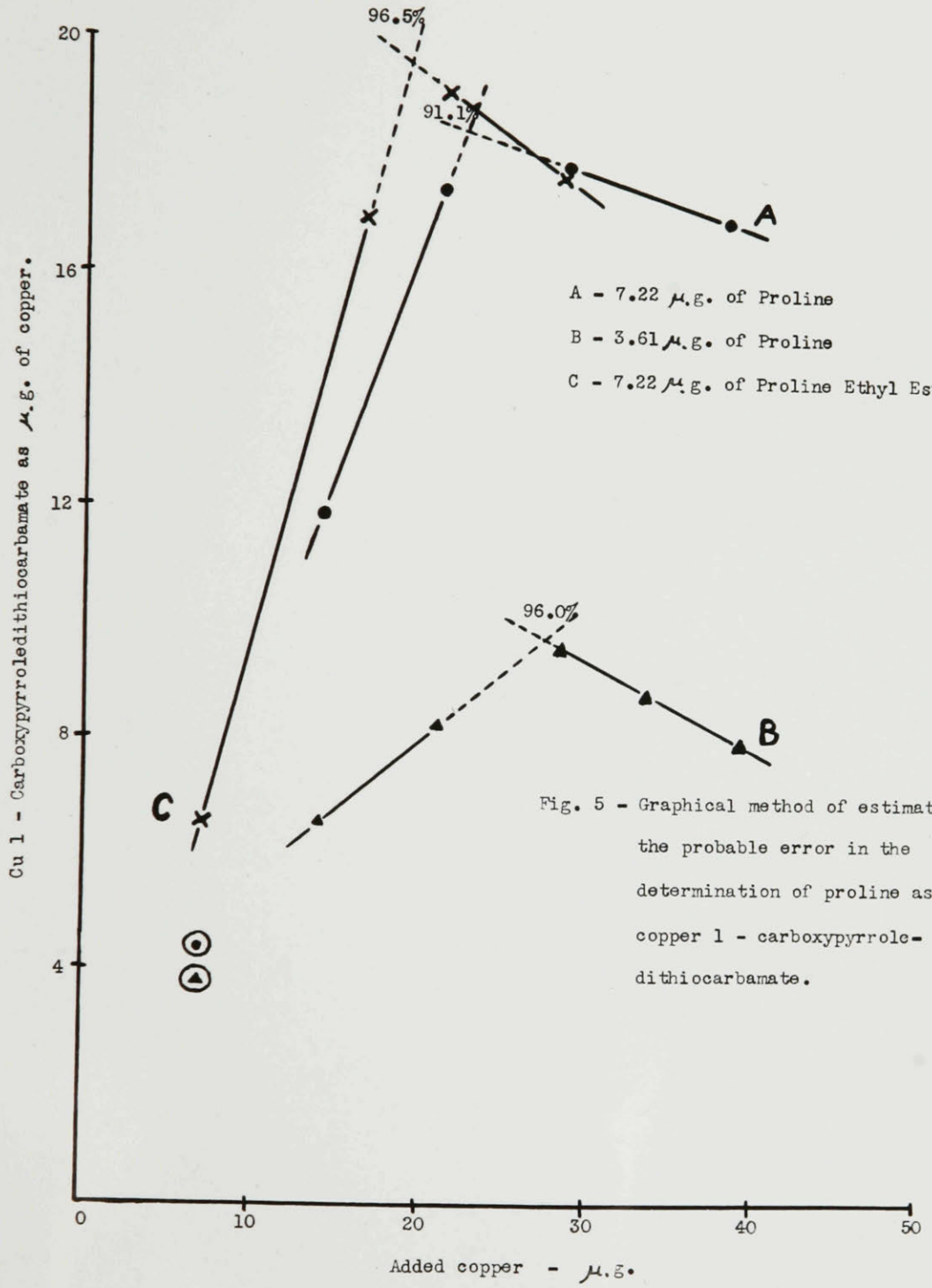


Fig. 5 - Graphical method of estimating the probable error in the determination of proline as copper 1 - carboxypyrrole-dithiocarbamate.

The graphs indicated that the error was related to the increment amount of copper added and also to the concentration of proline. When copper was added in amounts of 6.56 μ.g., the error was approximately 2 and 4% for concentrations of 7.22 and 3.61 μ.g. of proline per ml., respectively.

In the actual determination of proline the error could be reduced to an insignificant value by adding the copper in sufficiently small amounts, or, if desired, a final correction could be made by the graphical method. The corrected yields obtained by the above procedure were 91.2% and 94.7% with concentrations of proline of 7.22 and 3.61 μ.g. per ml., respectively.

An attempt was made to increase the yield by using the ethyl ester of proline instead of proline as the carboxyl group of proline was the only active group which could have interfered in the dithiocarbamate reaction. This modification in procedure was applied as follows: -

7.22 mgm. of proline were dissolved into 25 ml. of absolute ethyl alcohol saturated with dry HCl gas. The solution was evaporated to dryness on a steam bath and the residue was dissolved in 100 ml. of 95% ethyl alcohol. One ml. of this solution was used for a determination and copper was added in increments of 3.23 μ.g. instead of 6.45 μ.g. as used previously.

The results presented in Fig. 5C indicated a significant increase in the yield of copper dithiocarbamate, i.e., from 91.2% to 95%.

c) - Replacement of the buffer solution by a sodium citrate solution

In a last attempt to stabilize the reaction and thus to dispense with the graphical method, a solution of sodium citrate was used to replace the buffer solution. In sufficiently high concentration, the citrate anion has a slight deionizing effect on copper salts and should restrict the action of the metal ion on the copper dithiocarbamate. Furthermore, it is known to combine with iron and so prevent the interference of the latter in the reaction of copper with dithiocarbamic acid. Thus three advantages would accrue from the use sodium citrate - it would provide the cation necessary for the reaction, prevent the interference of iron and finally deionize the excess copper introduced in the test.

In the first series of experiments, three solutions of sodium citrate containing 0.33; 1.0; and 1.5 gm. respectively, of the salt in 100 ml. of solution were prepared and 2 ml. of each of these solutions were transferred to separate colorimetric tubes. 5 ml. of 95% ethyl alcohol were added followed by 1 ml. of proline solution (50 p.p.m.) and 2 ml. of the carbon bisulfide solution. The copper reagent was added dropwise as before and colorimetric measurements were made after the addition of each drop. A blank determination was made which included all the reagents.

The results obtained with the three levels of sodium citrate were very low, yields of 58 to 69% of copper dithiocarbamate being obtained. In a similar experiment, solutions of sodium citrate adjusted to pH 7 with dilute HCl were used instead of the solutions of the pure salts

which had a pH of about 8.0 . With this last modification, yields of 99.5 to 100% were obtained and the amount of copper was less critical, the maximum reading being intended over a range of 3 to 5 drops of copper reagent.

Since this procedure gave satisfactory results, it was used in the preparation of a calibration curve for the estimation of proline.

5. - Calibration curve for the estimation of proline

Colorimeter readings were made with various concentrations of proline, ranging from 1 to 10 µ.g. per ml. The maximum galvanometer readings were plotted against the concentration of proline and the curve obtained, deviated slightly from Beer's law (Fig. 6, No.2 - Table XVII). The Beer's law constant calculated from $K = \frac{2 - \log G}{C}$ was found to be 0.073 ± 0.007 .

TABLE XVII - Maximum galvanometer readings obtained with various concentrations of proline and hydroxyproline in the dithiocarbamic acid reaction

	Concentration of proline or hydroxyproline µ.g./ml.	Galvanometer readings
Proline	1.0	82.0
	2.5	63.0
	5.0	44.5
	7.0	34.0
	9.5	22.5
Hydroxyproline	1.0	87.5
	2.5	74.0
	5.0	59.0
	7.0	48.0
	9.5	36.5

6. - Determination of proline in the presence of hydroxyproline

The above procedure for the determination of proline cannot be applied directly to protein hydrolysates since most of the amino acids also give a colour reaction of varying intensity. It is evident therefore, that proline would have to be isolated before this method could be applied.

Proline and hydroxy proline can be separated from the other amino acids, by Dakin's butyl alcohol extraction method (4), but the further separation of proline from hydroxyproline is more difficult.

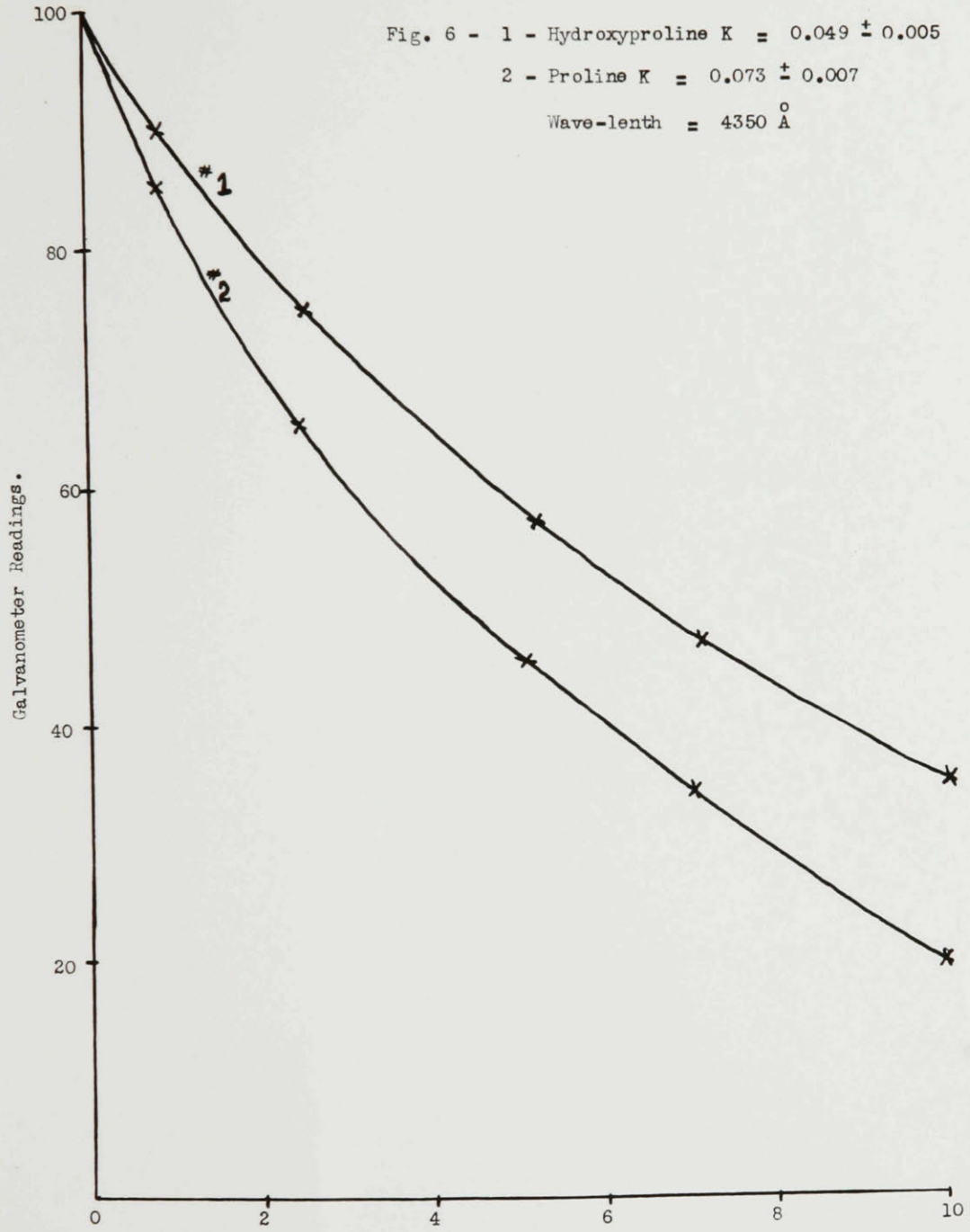
A sensitive colorimetric method has been devised by McFarlane and Guest (22) for the determination of hydroxyproline in the presence of proline. Hydroxyproline is oxidized by sodium peroxide in the presence of copper, to give a stable red chromogen which, in hot acid solution, can be condensed with isatin to form a stable red complex. The reaction is not given by proline. This method could be used in conjunction with the present method for the simultaneous estimation of proline, and hydroxyproline in protein hydrolysates.

However, equivalent amounts of proline and hydroxyproline do not give the same colour intensity in the dithiocarbamic acid reaction, as shown in the calibration curves presented in Fig. 6 . Having determined the amount of hydroxyproline present (McFarlane and Guests method), the proline equivalent in terms of the dithiocarbamic acid reaction could be obtained from a graph prepared by plotting the two calibration curves, on the same scale (Fig. 6). Finally, if this value is subtracted from the total colour value given by proline plus hydroxyproline, the amount of proline present would be obtained.

Fig. 6 - 1 - Hydroxyproline K = 0.049 ± 0.005

2 - Proline K = 0.073 ± 0.007

Wave-lenth = 4350 Å



This method of calculation is, however, based on the assumption that in a mixture, proline and hydroxyproline give the same colour intensity in the dithiocarbamic acid reaction as they would give independantly. This was tested experimentally by applying the reaction to several known mixtures of proline and hydroxyproline and calculating the amount of proline present in the manner just described.

The results given in Table XIV show that the calculated proline value depends on the amount of hydroxyproline present or, in other words, the presence of proline influences the reaction between hydroxyproline and carbon bisulfide or vice-versa.

TABLE XVIII - Calculated recovery of proline as determined by the dithiocarbamic acid reaction, in presence of hydroxyproline

Composition of solution (μ.g.)		Amount of proline recovered. (μ.g.)	% Recovery
Proline	Hydroxyproline		
0.0	5.00	0.00	-
0.50	4.50	0.56	112%
1.00	4.00	1.10	110%
2.50	2.50	2.60	104%
4.00	1.00	4.08	102%
4.50	0.50	4.54	100%
5.00	0.00	4.95	99%

From the two calibrations curves (Fig.6) it is possible to derive another curve representing the 'proline equivalent' of hydroxyproline at various concentrations (Fig. 7, Table XIX). It will be seen, that the 'proline equivalent' of hydroxyproline, decreases when the concentration

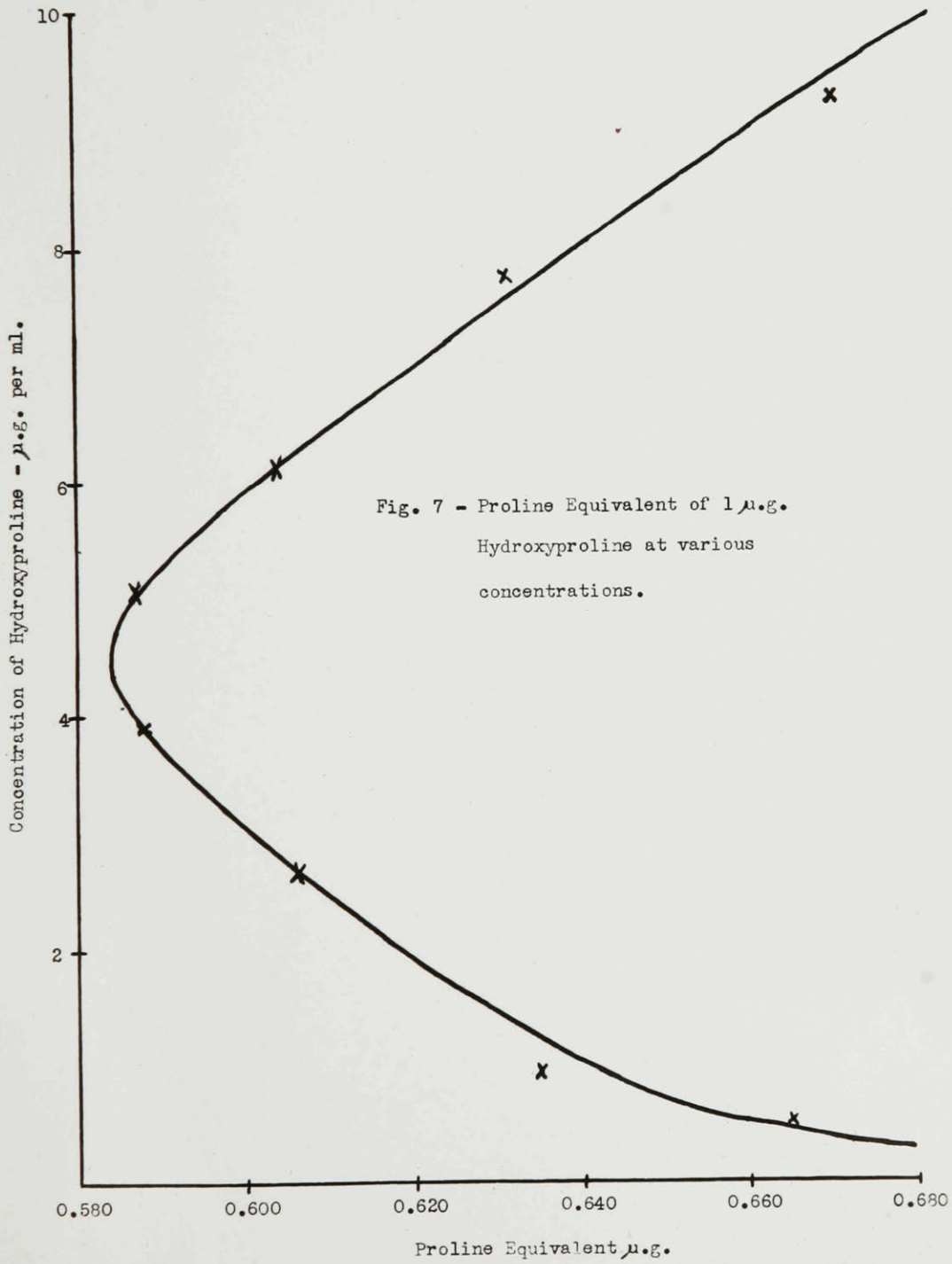


Fig. 7 - Proline Equivalent of 1 µ.g. Hydroxyproline at various concentrations.

of hydroxyproline in the dithiocarbamic acid reaction is increased until a minimum value is obtained at a concentration of 4.5 µ.g. of hydroxyproline per ml. Therefore, the conversion factor will vary with the concentration of hydroxyproline.

When proline is present, the curve might be displaced or in other words, the 'proline equivalent' of hydroxyproline would be changed. We may therefore, assume an 'effective concentration' of hydroxyproline which would be the sum of the concentrations of proline and hydroxyproline. This 'effective concentration' would be used to determine the conversion factor. Due to the fact that the 'effective concentration' of hydroxyproline is equivalent to, or higher than the actual concentration of hydroxyproline, it appears that the conversion factor will be lower if it is based on the 'effective concentration' instead of the actual concentration of hydroxyproline - this is true up to an 'effective concentration' of 4.5 µ.g. of hydroxyproline per ml.

TABLE XIX - Proline equivalent of hydroxyproline at various concentrations

Concentration of hydroxyproline µ.g./ml.	Proline equivalent
0.5	0.666
1.0	0.633
2.5	0.610
4.0	0.587
4.5	0.585
5.0	0.587
7.0	0.613
8.5	0.655
10.0	0.680

The results reported in Table XIV are calculated, using a conversion factor based on the actual concentration of hydroxyproline. If they are recalculated with a conversion factor based on the 'effective concentration', the percentage recovery of proline will be higher, when the actual concentration of hydroxyproline is low, and will remain the same at higher concentrations. Therefore, employing the 'effective concentration' instead of the actual concentration in the determination of the conversion factor will not compensate for the high recovery of proline obtained with mixtures of proline and hydroxyproline.

It may therefore be concluded, that, before the procedure can be successfully applied to protein hydrolysates a simple method will have to be devised for separating proline from the other amino acids, especially hydroxyproline.

SUMMARY OF PART II

The problem of separating and estimating the neutral monoamino-monocarboxylic acids in protein hydrolysates has been investigated. A study has been made of the adsorption properties of the copper salts on columns of silica-gel, permutit and copper carbonate. The result indicates that, insofar as these particular adsorbents are concerned, this procedure will not provide a satisfactory method of separating the individual neutral amino acids.

The reaction involved in the preparation of the copper salts of the dithiocarbamic acid derivatives of the monoamino-monocarboxylic acids have been studied with a view to separating the amino acids as sulphur derivatives by chromatographic methods. The derivatives proved to be unstable and for these reasons the study of their adsorption properties was not attempted.

Special attention has been given to the reaction between proline and carbon bisulphide to form the dithiocarbamic acid derivative. The results obtained indicate that this reaction can be utilized as the basis of a sensitive micro-method for the colorimetric determination of proline. Amounts of proline of the order of one μ.g. per ml. can be determined by the procedure described. Before the method can be applied successfully to protein hydrolysates, a procedure will have to be found for the quantitative separation of proline from the other amino acids, and especially hydroxyproline.

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