CIRCULAR PAPER CHROMATOGRAPHY

Part VI:—The quantitative determination of Amino acids

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Summary

A review of the literature on the methods of quantitative analysis of proteins and amino acids by chromatographic methods is presented.

A method for the quantitative determination of amino acids separated by circular paper chromatographic technique has been described.

The method is based on the elution of the colour of ninhydrin-stained bands of amino acids separated on the chromatogram with 75% ethanol containing copper sulphate and comparing the colour intensity with that of known standard solution of amino acid run on the same paper.

The effect of metallic ions, concentration of ninhydrin reagent, different solvents for the reagent, temperature, time of incubation, and area of the bands of amino acids, on the colour intensity of the bands of amino acids has been investigated.

Optimum conditions for an accurate quantitative procedure have been established.

The method has been applied to the quantitative estimation of some of the amino acids present in the hydrolysate of edestin.

The method is simple and accurate and it may be used on a variety of biological materials and requires only common laboratory equipment.

Introduction

The analysis of amino acids has been the subject of intensive research for the past several years and a rather bewildering body of literature has accumulated on the subject. Most of the available data on the amino acid composition of proteins were obtained by chemical methods of isolation. These methods are very laborious, demanding high degree of skill and as such they are not suitable for routine use.

In recent years, however, various micro-methods have been developed which show great promise of being used as routine procedures for amino acid analysis. Of these, isotope dilution method, enzymatic methods, microbiological methods and chromatographic methods are worthy of mention.

The isotope dilution method is the most accurate method known, but it has not been widely used owing to complexity of the apparatus and the difficulty of obtaining the isotopes necessary for the estimation.

In the enzymatic methods for the determination of lysine, histidine, arginine, glutamic acid, tyrosine and ornithine, specific decarboxylase enzyme preparations from bacteria are used (Gale, 1945, 1946, a, b). Only the naturally occurring L-isomers of the amino acid are determined by this method. Protein hydrolysates in which racemisation might have occurred will give low results by this method. As the presence of the D-isomers in protein hydrolysates is not clearly established, the values obtained by enzymatic procedures should be taken as representing the minimum amount only. The methods are rapid and yield accurate results.

Microbiological methods based on the use of micro-organisms to assay amino acids have been widely used by many investigators. The advantage of these methods is that they require small amounts of material. But occasionally erratic and confusing results have been obtained when it has been applied to certain materials of biological origin. It is difficult to obtain accurate and reproducible values by microbiological assay procedures unless great care is exercised in controlling various factors which are at present not well understood and amino acids of highest degree of purity are used. The use of prolonged incubation periods (2—5 days) necessary to obtain consistent results is one of the disadvantages of microbiological methods for use in routine analysis.

Chromatographic Methods.—In recent years chromatographic methods have been widely used for the separation and estimation of

amino acids. Before the advent of partition chromatography, simple and accurate methods were not available for separating amino acids from protein hydrolysates and for routine quantitative determination. The existing methods were quite tedious and unequal to the task of routine analysis. With the development of the technique of partition chromatography on column or paper a new and elegant method became available for the quantitative separation and estimation of amino acids and for the investigation of their role in metabolism. The chromatographic methods can be divided into three groups :—

(*i*) The ninhydrin method of Stein and Moore & Moore and Stein (1948) : In this method the amino acids are separated by starch column chromatography. The method is sensitive, reasonably accurate and reproducible.

(*ii*) Method based on the separation of amino acids using ion exchange resins (Moore and Stein, 1951). 3-6 mg. of the amino acid mixture are required. The method has given quantitative recoveries for all amino acids.

The methods of partition chromatography on column, though accurate and sensitive, require the use of a fraction collector, which is not easily available in ordinary laboratories.

(*iii*) Method of estimation of amino acids after separating them by paper chromatographic technique :—

The classical work of Consden, Gordon and Martin (1944) on paper partition chromatography provided a new technique for the separation and identification of amino acids in various complex biological mixtures and opened up new vistas of research in the nitrogen metabolism of plants and animals. Although the paper chromatographic method as originally described by Consden *et al* was only qualitative, a number of quantitative adaptations of the method have been published in recent years by several investigators. The methods suggested for the quantitative analysis of amino acids

after they have been chromatographed on paper may be placed in the following categories :—

1. Methods based on the visual comparison of the colour intensities of standard and test sample chromatograms stained with ninhydrin reagent and on certain physical measurements which include the fluorescence, light transmission and area of the spot.

2. Isotope dilution analysis of the spots with the aid of radio-active tracer amino acids.

3. Methods based on the elution of the amino acid bands and estimating by the usual methods employing ninhydrin reaction or the copper phosphate method.

4. Methods based on the elution of the colour developed after treatment with ninhydrin.

5. Retention Analysis (Wieland *et al.*, 1948, 1951).

6. Serial dilution method (Auclair and Durreuil, 1952).

Method (4) seems to be promising in view of the work done in this laboratory and elsewhere (Thompson and Steward, 1951).

1. This method in which the colour of the ninhydrin-stained spots is compared with that of standard amounts of amino acids chromatographed simultaneously with the unknowns has been used by Polson (1948), Berry and Cain (1949) and others for the analysis of proteins. However, an inspection of the recovery of added amino acids to urine published by Berry and Cain (1949) shows that the method can only be regarded as semi-quantitative. These methods depend on subjective test and require a large number of standards with each sample.

Fisher *et al* (1948, 1949) reported a quantitative method based on the measurement of the area of the spot after staining with ninhydrin. When a series of dilutions of a mixture of amino acids is run on a paper chromatogram (unidimensional) the size of the spot diminishes regularly with increasing dilution. Different methods have been used to determine the size of the spot.

One method is to measure the extension of the spot in the direction of solvent flow in the case of regular ovoid spots. There is a linear relation between the length of the spots and logarithm of the amino acid concentration. Another method is to outline the spot on the chromatogram by means of a planimeter. These areas are linearly related to the logarithm of the concentration of amino acid present. A third method was the use of a Hilger Photomicrometer to scan a quarter plate negative prepared from the original chromatogram. After plotting the transmission curve the area between the base line and this curve was used for each spot as a measure of the content of the amino acid. This procedure may prove satisfactory if the boundaries of the spot are well defined. It cannot be applied with accuracy, when the spots are not distinctly separated from each other.

This method has also been used by Block (1950) Fromageot (1949, 1950[a & b]) and others for the determination of amino acids.

Bull *et al* (1949) determined the percentage intermission along the chromatogram and this transmission has been plotted on semi-log paper against the distance along the filter paper strips. The areas of the segments above the plotted curve were measured with a planimeter. The areas so determined, are over a limited concentration range a simple function of the concentration of amino acids. The spraying was done on either side of the paper with a 0.4% solution of ninhydrin in butanol-phenol (9 : 1). The strips were heated to 90°C. for 10 minutes. They were then placed in an enclosed steambath for 20 minutes. The colour intensity reached maximum after this treatment, since it was found that the colour increased gradually possibly due to absorption of moisture.

Block (1949, 1950) has extended this method further to obtain the percentage composition of a mixture of amino acids. It was observed that the maximum colour density of each spot was proportional to the concentration of the material in the entire spot. The proportionality of maximum colour to concentration was observed only when aliquots of equal size were applied to the paper. A

procedure has been described by Block (1948) which allows the molecular proportions of each amino acid to be calculated on two-dimensional chromatograms. Although the colour density of any one amino acid may vary from day to day, probably because of uncontrollable laboratory conditions such as temperature, contaminants in the atmosphere, etc., the amount of colour developed by any amino acid remained proportionally constant to the others on the same chromatogram run on the same day. To obtain accurate values a large number of chromatograms were necessary. In a later paper Block (1950) has discussed the choice of paper and solvents, the hydrolysis of sample, development of the colour, special tests for specific amino acids, measurement of the colour density and quantitative estimation of amino acids.

Rockland and Dunn (1949 [a & b] and 1951) have described a method for the quantitative determination of amino acids by direct photometry using unidimensional chromatograms. To determine the concentrations of the amino acids, the filter paper strips were placed in a special sample holder containing an opening of a size just sufficient to enclose the entire area of the coloured spot and the colour intensities were read directly with the aid of a photo-electric colorimeter. The alanine and glycine contents of silk fibroin were estimated by interpolation from the standard curves drawn from plots against concentration of amino acid on co-ordinate paper.

This method can be employed with advantage only when the spots are distinct and do not overlap.

A method for the quantitative determination of amino acids based on the measurements of the colour intensity of the spots photometrically has been described by Redfield and Barron (1952). All common amino acids have been separated on one-dimensional chromatograms with different aliphatic alcohol and water mixtures as developing solvents. The authors have described a modification of the ninhydrin spraying reagent containing stannous chloride, citrate buffer and methyl cellosolve. The effect of small amounts of electrolyte is discussed.

Patton and Chism (1951) have employed the maximum colour density method using the Welch Densichron with a green filter for the analysis of amino acids.

McFarren and Mills (1952) have carried out the quantitative analysis of β-lactoglobulin by separating the amino acids by a series of unidimensional buffered chromatograms. The amino acids have been analysed by direct photometry on the paper.

2. Keston, Udenfriend and Levy (1947, 1950) have reported a new method for the detection and estimation of amino acids, which employs the treatment of the unknown mixture with p-iodo benzene-sulfonyl chloride labelled with radioactive iodine. Paper chromatography was applied to the separation of p-iodo benzene-sulfonyl derivatives from the protein hydrolysate products with a reagent containing I^{131}. The recovery was measured by the use of indicators consisting of known p-iodo benzene-sulfonyl derivatives containing S^{35} which were added in known amounts immediately after forming the I^{131} derivatives of the bands.

3. A polarographic modification of *Kobers method* (1912) for the estimation of amino acids as their copper complexes was described by Martin and Mittelmann (1948). Several micromethods were tried by them. The ninhydrin reaction and the Folin's technique were found to be unsuitable, probably due to the interfering substances apparently present in the paper and the solvents. Finally the technique of Pope and Stevens (1939) was used. The amino acid was added to a suspension of copper phosphate in a phosphate borate mixture, causing the copper to go into solution as the amino acid-copper complex. As the reducing material derived from the paper interfered with the iodometric titration, the copper in the solution after filtration was estimated polarographically.

Klatzkin (1952) estimated the amino acids separated by paper chromatography using Micro-Kjeldahl method. Nitrogen was estimated in a Micro-Kjeldahl flask with side arm which could

hold 3 c.c. of solution. Each sample was heated for 2 minutes with 0.1N NaoH to remove any ammonia absorbed by the paper. After digestion for 12 hours with hot concentrated sulphuric acid, copper and selenium catalyst, the contents were diluted to a known volume and ammonia N was determined by the Conway micro-diffusion method.

Woiwod (1948, 1949) used Pope and Stevens' method except that he determined the copper colorimetrically. A specially prepared copper-phosphate suspension was used to determine the α-amino-N. The presence in filter paper of a substance which inhibits the completeness of complex formation between copper and some amino acids renders the method less reliable (Fowden, 1951).

Jones (1948) and Blackburn (1950) have described methods based on the use of Pope and Stevens' copper phosphate procedure.

Naftalin (1948) located the amino acid spot by spraying with 0.025—0.05% ninhydrin solution in dry butanol. The coloured spots were cut, placed in test tubes and treated with 5% ninhydrin in water-saturated butanol. The test tubes were heated to 80°C. and the colour extracted with 75% acetone and estimated using a Hilger-Spekker spectrophotometer. According to Fowden (1951), the method lacked reproducibility.

The method described by Landua and Awapara (1949) was essentially the same as used by Awapara (1949) except that a modified ninhydrin reagent was used. They extracted the spot marked with a minimum of ninhydrin and developed the colour in a test tube with the Moore and Stein ninhydrin reagent. The use of a 2% ninhydrin solution in methyl cellosolve-water at pH 5 (citrate buffer) which contained stannous chloride as a reducing agent gave more reproducible results.

Fowden (1950, 1951) applied a slightly modified Moore and Stein reagent for the estimation of amino acids separated on filter paper. This method was claimed to be more sensitive than the copper-complex method of Woiwod (1949) which was marred by

the presence in filter paper of a substance which inhibits the complex formation between copper and some amino acids (Fowden, 1949). The method of Awapara (1949) was vitiated by high and variable " paper blanks" due to the presence of absorbed ammonia on the paper. The method of Fowden (1951) can be used for the determination of 1-30 μg. of α-amino-N of an amino acid.

Fowden (1951) also made the significant finding that when chromatograms were dried in hot air at 70-100°C, 20-30% losses of α-amino-N were found for many of the amino acids. When the chromatograms were dried at room temperature after removal of the solvent by washing with ether, the losses were completely eliminated (Fowden and Penney, 1950).

Boissonnas (1950) used ammonia-free solvents for separating the amino acids. An ingenious method for the location of the amino acids was devised by Boissonnas. Ninhydrin (3 grams) in a mixture of 50 c.c. tertiary butanol, 40 c.c. glycerol and 10 c.c. water was applied to paper chromatograms in small points at a distance of 7 mm. with the aid of a stamp equipped with fine metal needles. The viscosity of the reagent prevents its spreading. The spots were located by exposing the paper to infra red radiation. The paper was then sprayed with a 1% solution of KOH in anhydrous methanol. The amino acid spots were then eluted and the colour fully developed using a modified ninhydrin reagent.

Boissonnas (1952) applied this method for the evaluation of amino acids present in urine after treatment with urease followed by desalting of the concentrated urine sample.

Similar procedures based on the colorimetric estimation of amino acids after elution from their chromatograms have been described by Block (1950), Pereira and Serra (1951), Porath (1951) and others.

4. Recently Thompson *et al* (1951 a & b) made a careful and thorough study of the various factors and variables involved in the ninhydrin reaction with amino acids. In their final quantitative procedure, the papers were washed with 0·3N HCl. Sufficient

dilute NaOH was then put on the paper to neutralise the acid. The papers were then thoroughly washed with water, finally treated with phosphate buffer (pH 7.0) and dried. The developed chromatogram was air-dried and sprayed with 2% ninhydrin in 95% ethanol containing collidine and lutidine. Special tanks for developing the ninhydrin colour at 60°C. in a CO_2 atmosphere were used. The spots were cut out and the colour extracted with 50% ethanol. The colour was measured in a colorimeter with a 570 m μ filter.

Wellington (1952) has also described a similar simple, quantitative method for the determination of amino acids in about 300γ of protein.

Bode *et al* (1952) have reported a method for the quantitative estimation of amino acids making use of the reaction of cupric ions with the ninhydrin pigment (Wieland and Kawerau, 1951). This paper appeared after the publication of our preliminary note (Giri *et al.*, 1952). The red colour was eluted with methanol. As these investigators employ relatively high temperatures (80-100°C.) for the drying of the paper, errors due to losses of amino acids cannot be completely eliminated. As recovery experiments are not given, the sensitivity and accuracy of the method as employed by these workers cannot be evaluated.

5. Wieland and coworkers (1948, 1951) have developed a new technique of analysis based on the observation that when strips of filter paper are dipped into a solution, the latter will rise up by capillary action until the front of the solution meets the area containing the substance with which it can react. After further ascent of the solution, a triangular gap is formed. The area of this gap is found to be proportional to the concentration of the substance present on the paper. This principle was applied to the quantitative determination of amino acids separated on paper. When the paper containing the amino acids separated after development with water-saturated phenol, was immersed to a depth of 3 mm. in a solution of copper acetate (0.1%) in tetrahydrofuran containing 10% water and minimum amount of glacial acetic acid to prevent turbidity, the

solution rose up the paper until it reached the area occupied by the amino acids which retained the copper. The triangular copper-free areas above each spot was found to be related to the amount of amino acid present in the spot.

6. Auclair and Durreuil (1952) have described a simple ultramicro method based on the estimation of amino acid needed to give a barely detectable ninhydrin reaction on a 2-dimensional ascending paper chromatogram. Diminishing aliquots of the test solution are used and the lowest concentration to give a detectable spot is selected. It is reported that accurate results can be obtained with amounts of amino acids of the order 0.05 microgram. This method is subject to serious errors due to background coloration and personal equation. A very large number of chromatograms are necessary to minimise the errors making this method tedious and incapable of being easily used for routine analysis.

In this report is described a simple procedure for the quantitative estimation of amino acids separated by paper chromatography. The circular paper chromatographic technique described in Part I of this series of publications (Giri and Rao, 1952) has been adapted to quantitative determination of amino acids. The procedure measures amounts of amino acids in the range of 2-12γ and is accurate to $\pm$ 5%. The method is based on the development of colour of the amino acids bands by spraying the chromatogram with ninhydrin reagent, cutting out the coloured bands, eluting with 75% ethanol containing $CuSO_4$, 5 H_2O and measuring the colour intensity. A brief description of this method has been given in an earlier publication (Giri *et al.*, 1952).

Experimental

Apparatus.—The apparatus used was similar to the one described by Giri and Rao (1952).

For small size chromatograms a six-inch diameter petri-dish was used as the support for the paper (18.5 cms. dia.) covered by a pneumatic trough.

For large size chromatograms, either a perspex basin or a wooden cabinet was used. It consisted of an air-tight cabinet with glass bottom and the lid (glass plate in wooden frame) was hinged on to the side so that it could be opened or closed easily. A square wooden frame with a number of slots for the glass rods, served as a support for the paper. After equilibration, the solvent could be transferred to the small petri-dish at the centre by means of a bent glass tube with a stop-cock and funnel. Provision was also made for the introduction of a thermometer to record the temperature inside the cabinet. It was arranged such that the level of the solvent in the petri-dish was as near the paper as possible to enable a quicker flow of solvent.

Paper.—Whatman No. 1 paper was used in all these experiments. The small chromatograms were run with 18.5 or 24 cms. filter circles. For the larger ones, about 40 cms. diameter paper was used.

Solvent.—n-Butanol (distilled)-acetic acid-water (40 : 10 : 50) was used as the solvent. The lower layer was used for equilibration purposes.

Pipettes.—Capillary tubes cleanly cut on either side were used as micropipettes. They were calibrated by using mercury. A number of determinations were made and the average taken.

Reagents.—1. Ninhydrin reagent: 0.5 per cent ninhydrin dissolved in 95% acetone.

2. Copper sulphate ($CuSO_4, 5H_2O$) dissolved in 75% ethanol to contain 0.05 mg per c.c.

3. Amino acids, stock standard solutions prepared by dissolving accurately weighed amount of each amino acid in iso-Propanol (10%). In the case of difficultly soluble amino acids, a drop of HCl was used.

Procedure.—Known quantities of standard mixtures of amino acids or the test sample to be analysed were transferred to the paper by means of a calibrated pipette (usually 2.5μ l – 20μ l). The outside of the pipette was carefully wiped out by means of a clean filter paper before transferring. The chromatogram was developed using butanol-acetic acid-water as solvent. When the solvent had run the required distance the paper was removed, air-dried and sprayed with ninhydrin under specified conditions described below :

I. *Factors affecting the quantitative procedure.*—(*a*) Elution of the ninhydrin-stained bands :—After preliminary experiments it was found that 75% ethanol was the best solvent for the pigment produced by the ninhydrin reaction.

(b) Effect of metal ions—Kawerau and Wieland (1951) reported a method for the conservation of chromatograms by coupling the blue pigment with a metal ion. All amino acids form red pigments with cupric ions except proline and hydroxyproline which form a yellow metal complex. The following ions were tested by them: Ni^{++}, Cd^{+}, Cu^{++}, Co^{++} and Zn^{++}. Copper nitrate was finally selected for the spray reagent for the conservation of chromatograms.

It was, therefore, considered that the sensitivity of the elution method for quantitative analysis of amino acids could be greatly improved by the addition of one of these ions, which incrcase the colour intensity. The ninhydrin pigment of the amino acids was cut off from the filter paper and eluted with 75% ethanol. The effect of the various metal ions was first qualitatively tested using the alcohol eluate. It was observed that the change in the colour and intensity was not considerable except in the case of Cd^{++} and Cu^{++}. The final colour given by different amino acids was of varying tinge from reddish purple to ochre. The ninhydrin colour given by peptides was also changed to red on addition of copper. The colour was not stable towards acids (HCl etc.) but was normally stable over a period of several hours (48 hrs.). The colour given by cadmium was a brilliant reddish purple.

The spectral absorption curves of the colour of the alcohol eluates of the bands as well as that of the metal complex are illustrated in Fig. I *a*, *b*, *c*. These determinations were made using a Beckmann Quartz Spectrophotometer (Model DU) with 1.00 Cm. lightpath at 24°C. The maximal absorption occurred at 580 mμ, for the blue pigment. For the copper and cadmium complex of the blue pigment it was the same, *i.e.*, at about 520 mμ. It may be noted that the maximum is not sharp, since the concentration of the amino acids was low, the order being the same as used in chromatographic analysis.

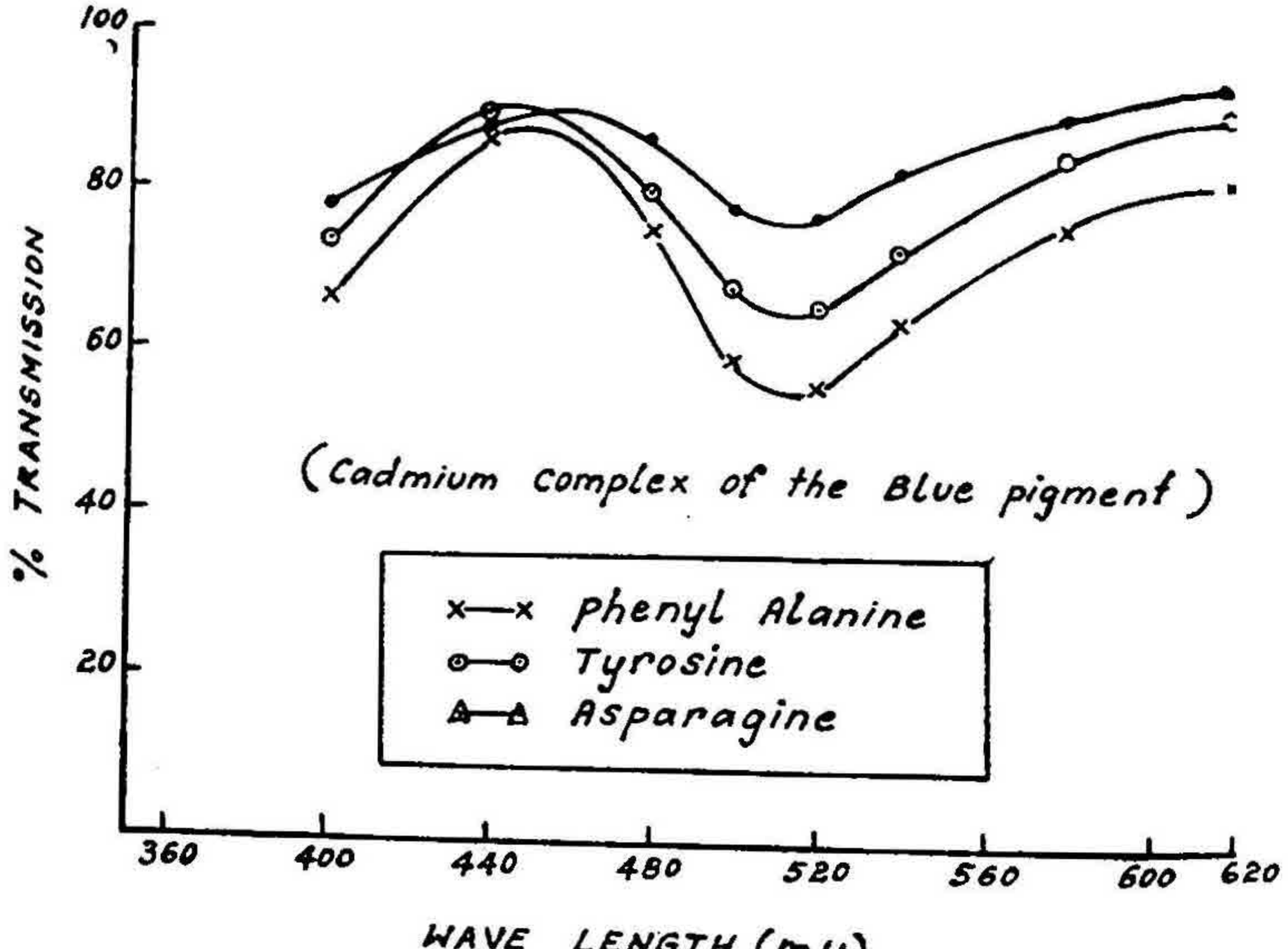

FIG. I (a). Percentage Transmission *Vs.* Wave Length.

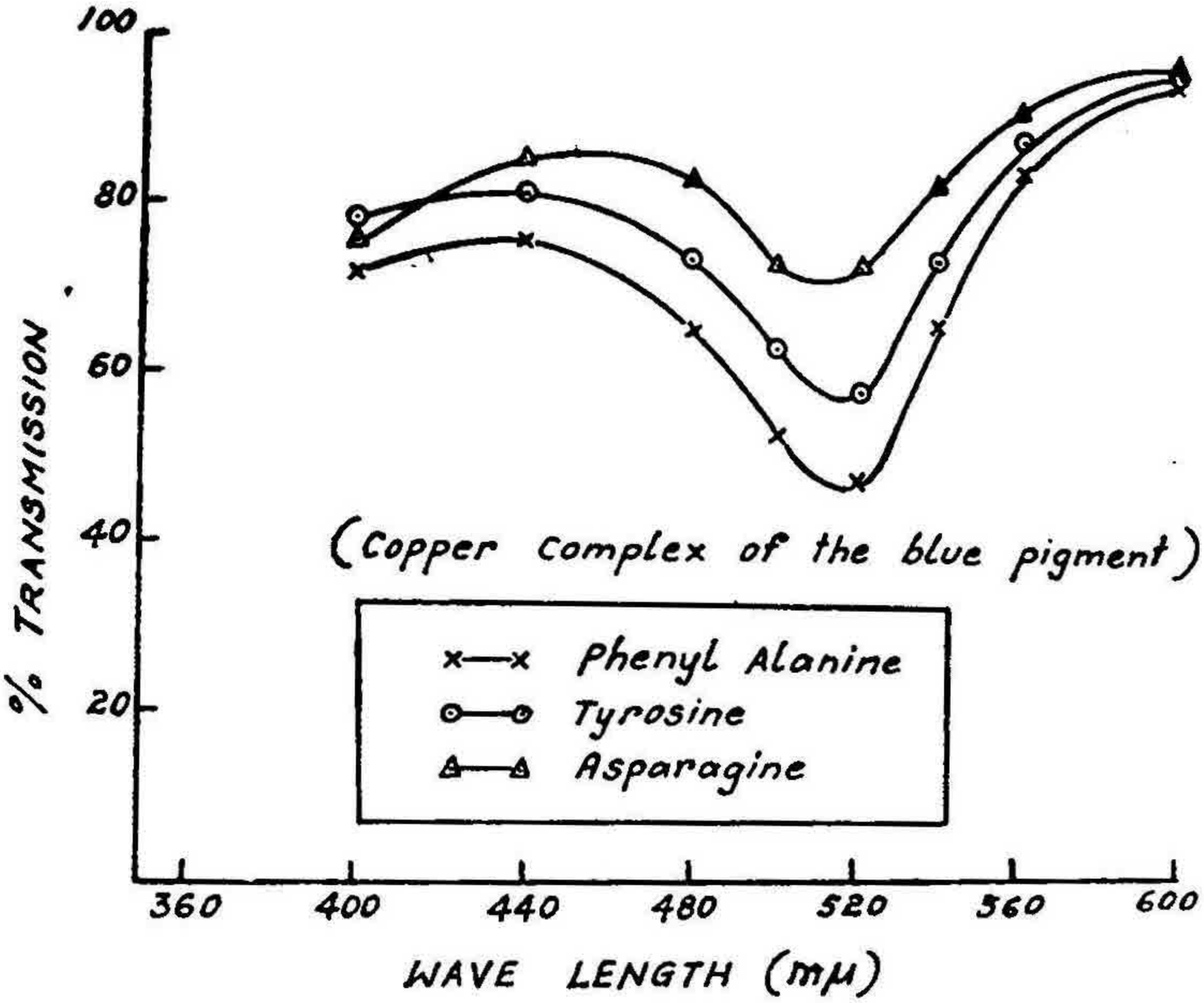

FIG. I (b). Percentage Transmission *Vs.* Wave Length

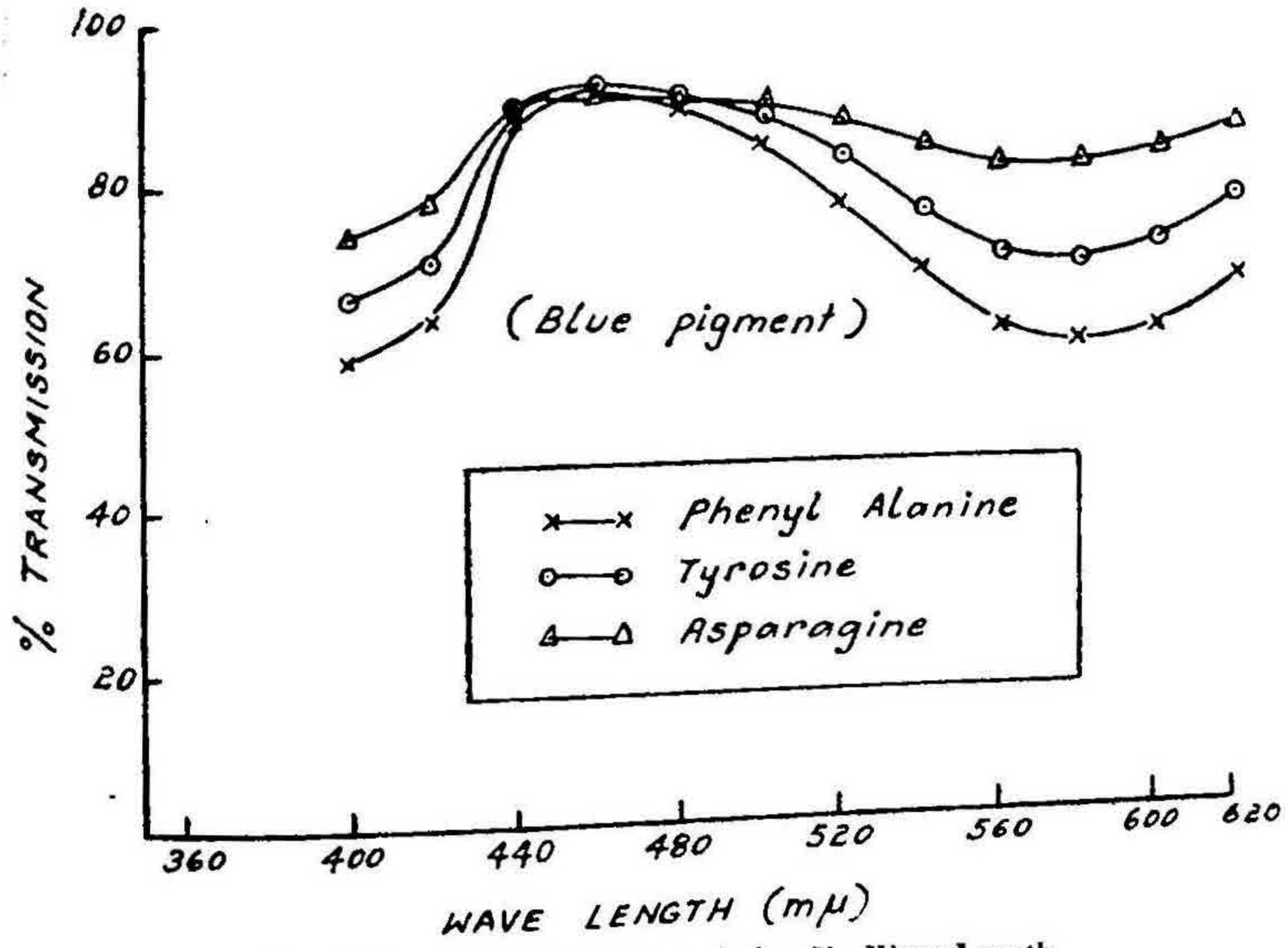

FIG. I (c). Percentage Transmission *Vs.* Wave Length

The green filter No. 54 (520-580 mμ) was therefore chosen for the comparison of the intensities with the Klett-Summerson Photo-electric colorimeter.

(c) Effect of varying concentrations of copper sulphate.—The ninhydrin-stained bands were cut out and appropriate amounts of 75% ethanol added. Varying quantities of $CuSO_4$, $5H_2O$ solutions (1 mg. per c.c.) were added keeping the total volume constant (4 c.c.). The colour intensity was measured. The effect of varying concentrations of copper sulphate on the intensity of the colour of the alcohol eluate is illustrated in Fig. II and Table I. It can be seen that the colour intensity is greatly enhanced by the addition of even small amounts of Cu^{++} as compared with the colour of the blue pigment itself. Also most of the amino acids (5 — 7.5γ) tried showed a maximum colour intensity at a copper concentration of 0.2 mg. $CuSO_4$, $5H_2O$ in 4 c.c. of the solution.

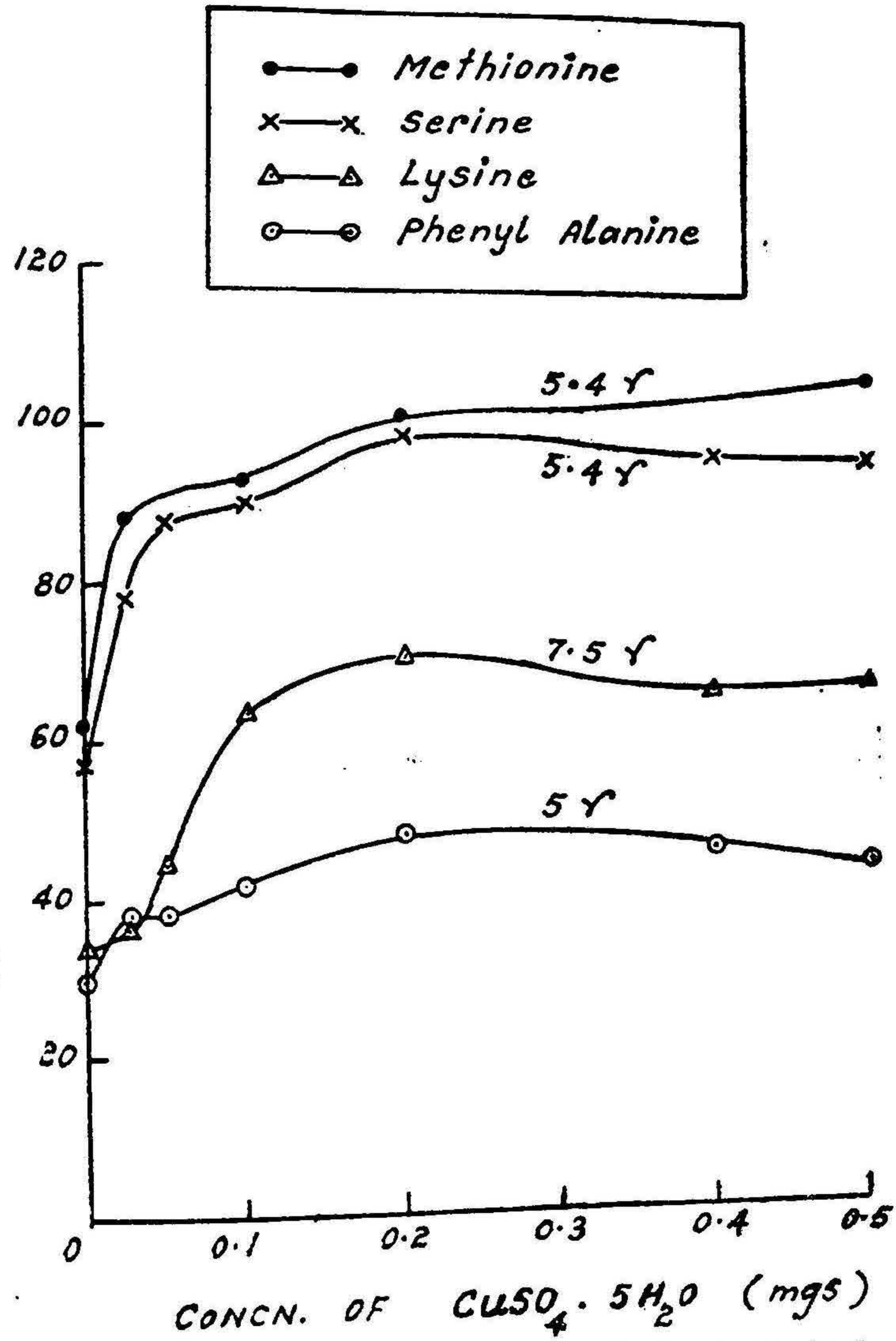

FIG. II. Effect of different concentrations of $CuSO_4$, $5H_2O$ on the intensity of Ninhydrin-stained bands.

Table I

Effect of varying concentrations of $CuSO_4, 5H_2O$ *on the ninhydrin pigment*

Mgs. of $CuSO_4$, $5H_2O$ in 4 c.c. of solution	Colorimeter readings														
	Aspg (16.7γ)	Glu (5γ)	Hi (5γ)	Ty (5γ)	Ph. Al. (5γ)	Leu (5.4γ)	Lys. (7.5γ)	Se. (5.4γ)	AA (5.4γ)	I.L. (5.4γ)	Try (7.5γ)	Al. (5.4γ)	Me (5.4γ)	Val (5.4γ)	Lys. (5.4γ)
0 00	10	39	35	31	30	60	34	57.5	20.5	72	30	23	62	68	31
0.025	24	65	45	38.5	38	76	37	78	27.5	101	40.5	28	88	90	41
0.05	32	72	...	45	38	...	45	88	32	109	45.5	33.5	87	107	39
0.10	34	74	45	50	42.5	99	64	90	30.5	113	49	37	93	110	44
0.20	37	76.5	51	53	48.5	108	71	99	38	119	55.5	33	101	116	59
0.3	37	72	...	...	...	...	70.5	...	34.5	118	54	...	...	...	59
0.4	36	72	47	50	46	...	66	96	35	114	53.5	35	...	...	...
0.6	35	70	...	50	44	110	67	96	32	116	53	34	106	122	58
0.8	...	68	45	53	42	...	65.5	97.5	...	118	...	34	...	...	...
1.00	...	...	53	53	42	108	65	98	...	...	...	34	...	...	...
1.2	...	...	44	51	41	...	65	99	33	...	55	...	...	120	57

Aspg = Asparagine, Glu = Glutamic acid, Hi = Histidine, Ph Al = Phenyl alanine, Leu = Leucine, Lys = Lysine, Se = Serine, AA = Aspartic acid, IL = iso Leucine, Try = Tryptophan, Al = Alanine, Me = Methionine, Val = Valine.

(d) Effect of varying concentrations of Cd^{++} (*as* $CdSO_4\ 3H_2O$).– The method followed was the same as described above. It is clear from Fig. III and Table II, that in the case of cadmium also, the maximum colour is obtained at a cadmium concentration of 0.2 mg. $CdSO_4, 3H_2O$ in 4 c.c. of the solution.

Table II

Effect of different concentrations of $CdSO_4, 3H_2O$ *on the ninhydrin pigment*

Mgs. of $CdSO_4, 3H_2O$ in 4 c.c of solution	Colorimeter Readings					
	Leucine 8.5γ	Arginine 8·5γ	Cystine 8·5γ	Phenyl Alanine 8·5γ	Alanine 8·5γ	Histidine 8·5γ
0·00	94	62	59	52	172	33
0·025	139	90	80	75	248	47
0·05	155	95	94	79	254	50
0·10	160	95	100	84	268	55
0·20	165	100	106	88	274	64
0·40	160	100	105	87	275	64

Comparison of the effects of Cu^{++} *and* Cd^{++}.—The proper choice between Cu^{++} and Cd^{++} was decided by comparing the effects of the two ions under similar conditions. Three aliquots were taken from the alcohol eluate of the blue pigment of various amino acids. One was used as a control. To the others Cu^{++} and Cd^{++} solutions (same concentration by weight) were added. The results presented in Table III show that Cu^{++} is definitely superior to Cd^{++} under identical conditions. Cu^{++} was therefore finally selected for use in the quantitative method.

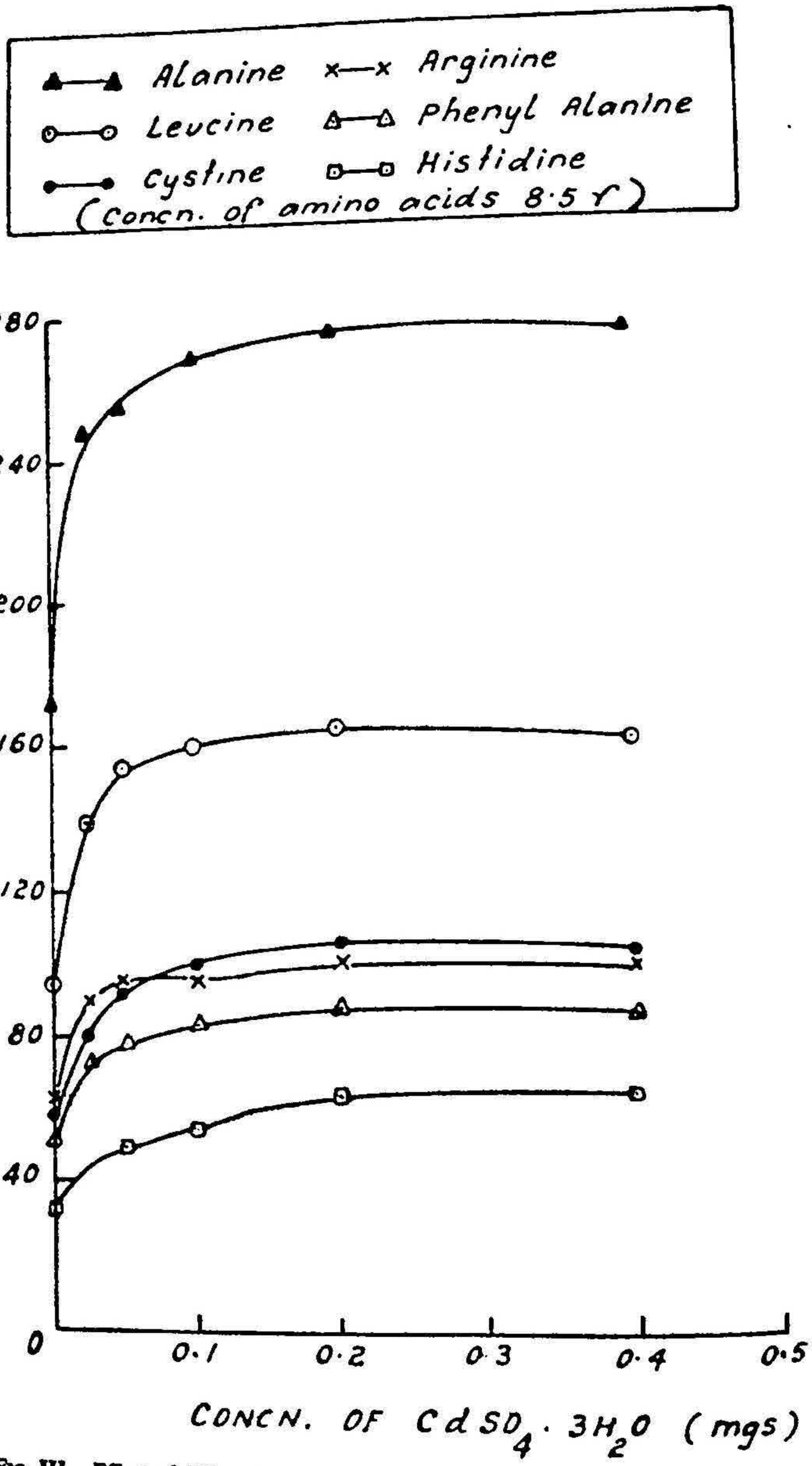

FIG. III. Effect of different concentrations of cadmium sulphate on intensity of Ninhydrin-stained bands.

Table III

Comparison between Cu^{++} *and* Cd^{++}

Addition of 0.2 mg. $CuSO_4, 5H_2O$ or $CdSO_4, 3H_2O$ in 4 c.c. solution.

Sl. No.	Amino acid	Concentration in μg	Colorimeter Readings		
			Control	+Cd++	+Cu++
1.	α-Alanine	3.5	69	87	100
2.	β-Alanine	7.5	51	65	80
3.	α-Amino butyric acid	—	105	125	146
4.	γ-Amino butyric acid	—	59	66	88
5.	Arginine	5	51	58	67
6.	Asparagine	25	48	69	70
7.	Aspartic acid	6	30	35	39
8.	Cystine	–	36	55	60
9.	Glycine	25	123	180	187
10.	Glutamic acid	6	54	65	74
11.	Glutamine	—	43	55	67
12.	Histidine	7.5	37	45	57
13.	Leucine	5	58	63	72
14.	iso-Leucine	7.5	86	108	115
15.	nor-Leucine	7.0	74	94	100
16.	Lysine	17.5	97	135	143
17.	Methionine	6	69	89	102
18.	Ornithine	8	56	65	74
19.	Phenyl Alanine	17.5	99	131	144
20.	Serine	7.5	97	124	135
21.	Taurine	—	25	25	32
22.	Threonine	7.5	66	80	98
23.	Tryptophan	7.5	33	33	50
24.	Tyrosine	10	72	89	106
25.	Valine	5	59	75	76

(e) The effect of concentration of ninhydrin.—The optimum concentration of ninhydrin cannot easily be fixed since the concentration of amino acid also changes. In quantitative work using circular paper chromatography, it was found that good separation can be obtained only when the concentration of the amino acid is less than about 15γ. So the effect of concentration of ninhydrin has been carried out in the case of serine and leucine at concentrations of 5γ and 10γ which are the more usual. The ninhydrin was dissolved in 95% acetone. From Table IV, it can be seen that 0.5% is the optimum concentration and this was used in later experiments. Very high concentrations (4-8%) appear to reduce the colour intensity slightly.

Table IV

Effect of concentration of ninhydrin

% ninhydrin in 95% acetone	Colorimeter Readings			
	SERINE		LEUCINE	
	7·5γ	12·5γ	5γ	10γ
0·1	102	149	87	135
0·25	119	200	88	154
0·5	130	215	89	160
1·0	127	218	90	161
2·0	131	210	89	164

(f) Solvent for ninhydrin.—Thompson *et al* (1951) and Patton and Chism (1951) have mentioned the effect of solvent for ninhydrin. The adverse effect of butanol saturated with water, when used as solvent for ninhydrin has also been referred to. The relative effects of four solvents, *viz.*, 95% ethanol, water-saturated butanol 95% iso-propanol and 95% acetone were studied. All the solvents were distilled in an all-glass apparatus to free them from metal ions. The chromatograms were sprayed with ninhydrin (0.5%) in the appropriate solvent and dried at 65° for 30 mins. To the alcohol eluate 0.2 mg. of $CuSO_4$, $5H_2O$ was added and readings taken in a colorimeter. From Table V, it is clear that acetone and ethanol are better than butanol as solvents for ninhydrin. Since the papers sprayed with acetone dry quicker, it was used as the solvent for ninhydrin in all the experiments. In this connection it is important to note the reported rapid destruction of colour (Thompson *et al.*, 1951) under aerobic conditions when the paper is wet.

Table V

Effect of solvent for ninhydrin

Solvent	Colorimeter Readings			
	SERINE		LEUCINE	
	5γ	8.5γ	5γ	8.5γ
1. Ethanol (95%)	94	149	86	139
2. Butanol (Saturated with water)	72	101	57	107
3. iso-propanol (95%)	103	150	90	139
4. Acetone (95%)	102	149	87	135

(g) Effect of Temperature.—The development of the colour was very slow at room temperature although it was preferred by some of the earlier workers (Patton and Chism, 1951; Dent, 1948). The colour develops rapidly when heated, but at high temperatures, the colour decreases. The optimum temperature was therefore selected by heating the ninhydrin-sprayed chromatograms at various temperatures. The results are presented in Table VI. The colour intensity reaches a maximum at about 65°C, which was used for later experiments.

Table VI

Effect of temperature on the ninhydrin colour intensity (time of heating 30 mins.)

Temperature °C.	Colorimeter Readings			
	SERINE		LEUCINE	
	5γ	10γ	5γ	10γ
40	90	146	91	153
50	96	163	95	165
60	108	174	98	171
70	112	176	96	170
80	100	175	97	172
100	106	178	96	173

(h) Effect of time of keeping the chromatogram at 65°C.—Keeping the temperature constant at 65°C, the effect of time of keeping the paper at this temperature was studied. Several chromatograms were run, sprayed with 0.5% ninhydrin and kept in a thermostatic oven at 65°C. They were removed at known intervals and the colour intensity measured after elution with 75% alcohol and addition of Cu^{++}. The results given in Table VII show that for the concentrations of the amino acids employed, the colour development reaches a maximum at 30 mins. It is to be noted that the background colour increases when the paper is heated for a long time. This will introduce blank errors.

Table VII

Effect of time of development on the ninhydrin colour intensity (temperature 65°C)

Time	Colorimeter Readings			
	Serine		Leucine	
	5γ	12.5γ	5γ	10γ
15 mins.	106	195	93	165
30 mins.	118	216	102	170
45 mins.	120	217	105	168
1 hr.	120	214	100	168
2 hrs.	113	210	100	165
3 hrs,	120	210	100	170
9 hrs.	100	193	91	162
19 hrs.	110	197	94	158
24 hrs.	113	188	93	160

II. *Relation between area of the band, colour intensity,* R_f *values and distance travelled by the solvent front :*

(*a*) *Area of the band in relation to the solvent front :*—It was observed that the area of the coloured band increased with the distance travelled by the solvent front. The intensity of the colour extracted also appeared to increase with the area of the band. The following procedure was adopted to find the relation between the area and the intensity of the colour extracted. Varying concentrations (4.17γ — 20.8γ) of iso-leucine were applied keeping the area constant (0.1138 sq. in.). In a second experiment the amino acid solution was spotted without allowing the drop to dry after each application, thereby increasing the area of the spot. In the third experiment the amino acid solution was spotted side by side each spot corresponding to the area of the first experiment. The spots corresponding to each concentration ofamino acid were cut out after colour development with ninhydrin and eluted with alcohol and the intensity measured. The results are given in Table VIII. From the values given it is clear that the colour intensity increases with increase in the area of the band.

Table VIII

Relation between area of band and intensity of colour extracted.

Concentration of iso-leucine μ g.	I		II		III	
	Area sq. in.	Intensity Col. rdg.	Area sq. in.	Intensity Col. rdg.	Area sq. in.	Intensity Col. rdg.
4·17	0·1138	30	0·1138	34	0·1138	34
8·34	,,	32	0·2018	48	0·2276	63
12·51	,,	29	0·3019	72	0·3414	97
16·68	,,	34	0·3900	88	0 4552	130
20·85	,,	34	0·4712	107	0·5690	150

Following the above observation, an experiment was carried out on a single chromatogram to obtain a relation between the area of the band, the distance travelled by the solvent front and the intensity of the colour extracted. The amino acid spots were arranged in a spiral form at varying distances from the centre of the paper. When the chromatogram was run with the solvent, the amino acids travelled varying distances from their origin. The results are presented in Table IX.

The intensity of the colour extracted increases to a considerable extent with the area of the band. It may also be noted that the R_f values decrease progressively with the increase in the distance of the original drop from the centre of the paper.

Table IX

Relation between area, intensity of the band and the distance of the solvent front

Concentration of amino acids = 10·8 γ in each case. (The size of drop spotted was kept same)

Distance of the original drop from centre in inches.	Distance of the orgl. drop to new spot (ins.)			Distance of orgl. drop to the solvent front (ins.)			Rf values.			Area (Sq. in.)			Intensity, colorimeter readings		
	Threo-nine	Valine	Leu-cine	Threo-nine	Valine	Leu-cine	Threo-nine	Valine	Leucine	Threo-nine	Valine	Leucine	Threo-nine	Valine	Leucine
0.85	1.2	2.4	3.0	3.3	3.8	3.7	0.36	0.63	0.8	0.23	0.71	0.75	120	177	215
1.7	0.7	1.4	1.9	2.9	2.4	2.5	0.24	0.58	0.76	0.19	0.41	0.43	116	172	191
2.1	0.5	1.1	1.7	2.5	2.1	2.5	0.2	0.52	0.68	0.19	0.33	0.42	114	142	180
2.5	0.25	0.90	1.45	1.8	2.0	2.2	0.14	0.49	0.66	0.14	0.29	0.34	93	133	167
2.0	0.15	0.50	0.8	1.4	1.3	1.4	0.12	0.38	0.57	0.14	0.27	0.26	86	120	137
3.3	0.15	0.3	0.4	1.35	0.8	0.8	0.11	0.37	0.50	0.12	0.20	0.17	81	103	119
3.7	0.1	0.2	0.3	0.9	0.7	0.75	0.1	0.28	0.39	0.12	0.14	0.13	78	96	117

III. *Quantitative Procedure.*—The final procedure for the quantitative analysis of amino acids was developed after studying the variables involved in it, as described before and suitably controlling them. For any one set of experiments the distance travelled by the solvent front was the same. This would keep the area of the amino acid band (same concentration) constant within limits. After developing the chromatogram with a suitable solvent the paper was dried in air and sprayed uniformly with 0.5% ninhydrin in 95% acetone. Dipping in the chromogenic reagent can be employed when the papers are not very large. It is also found convenient to add the ninhydrin reagent by means of a pipette taking particular care to see that the paper does not go dry in some regions during the addition. The paper was air-dried and heated in an oven at 65°C. for 30 mins. The paper was removed from the oven and the boundary of the individual amino acid band was carefully marked with a pencil. The bands were cut off carefully, rolled and placed in a test tube. 4 c.c. of 75% ethanol containing 0.2 mg. $CuSO_4$, $5H_2O$ were added. The elution was found to be complete in 10-15 minutes. The filter paper roll was removed by means of a clean glass rod and the intensity of the colour of the solution was determined using a Klett-Summerson photo-electric colorimeter with a green filter (No. 54). The quantity of the amino acid was estimated by means of standard curves drawn for each amino acid under identical conditions. In Table X are given the values for the calibration curves. The values obtained without the addition of Cu^{++} are also given for comparison. A linear relationship between colour and quantity of the amino acid was found over the range 2.5 to 12.5 γ above which concentration the curve slightly deviates from linearity (Fig. IV *a* & *b*).

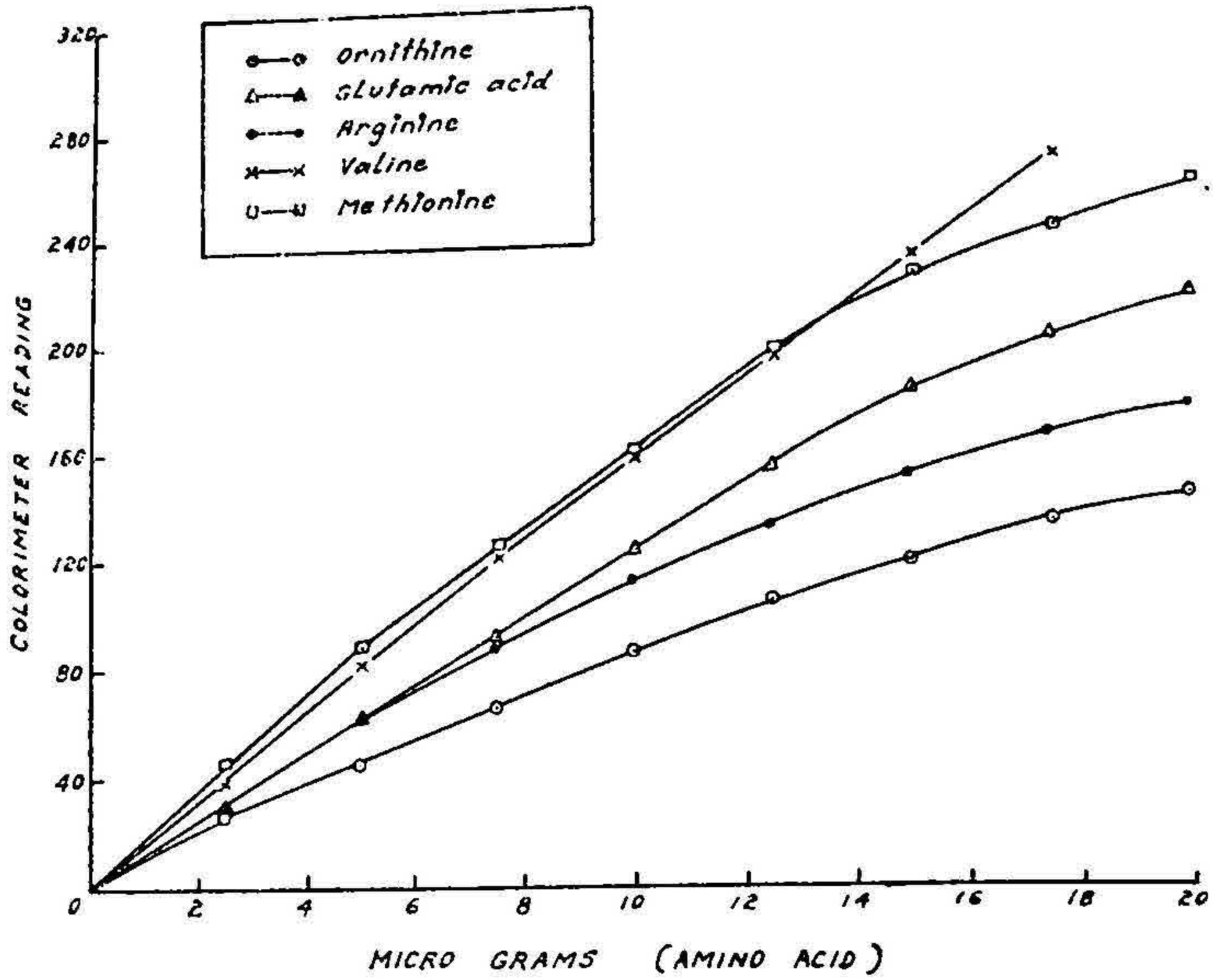

FIG. IV (A). Calibration curves of Amino Acids

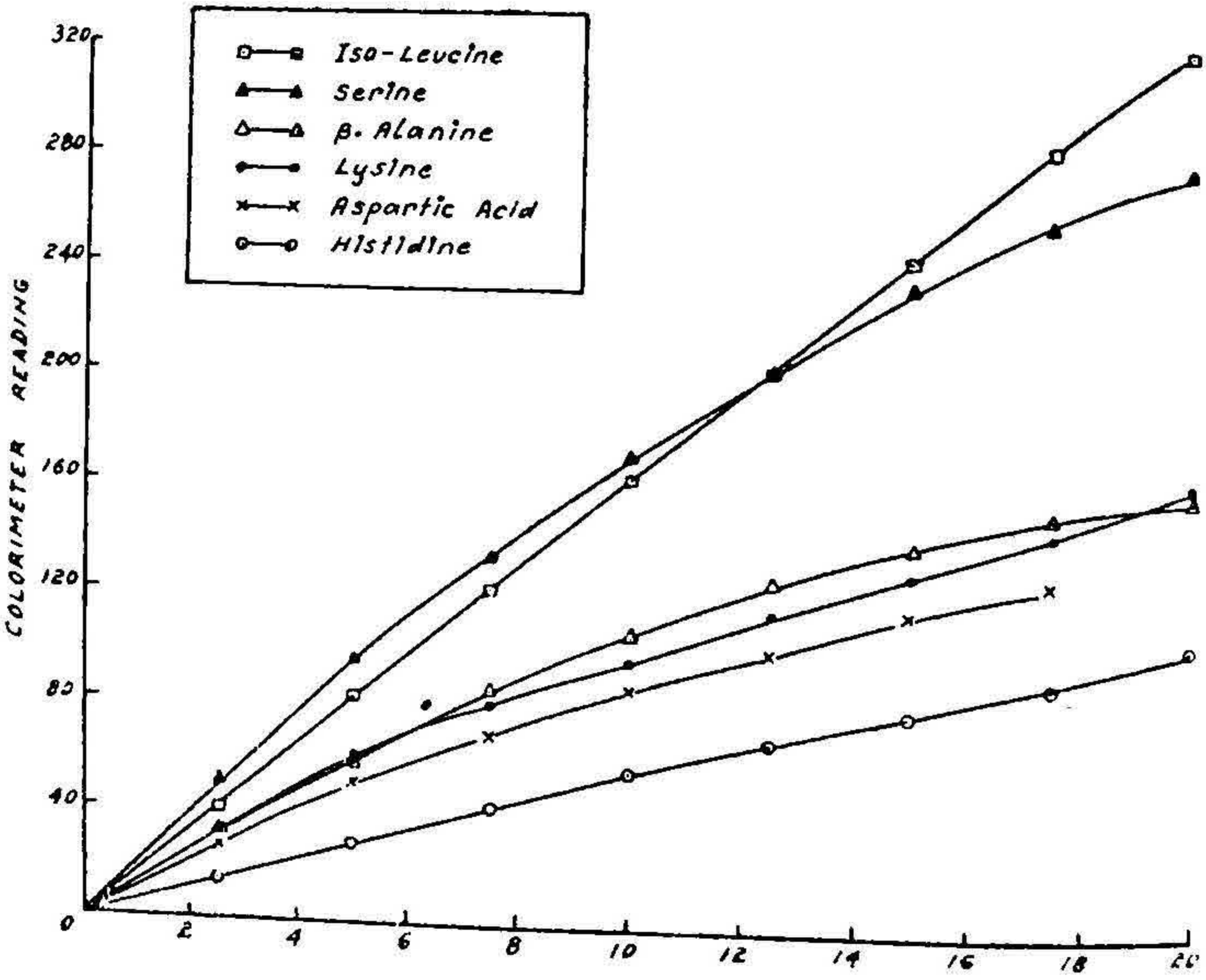

FIG. IV (B). Calibration curves of Amino Acids

Table A

Calibration Curves (with $CuSO_4$, $5H_2O$, 0.2 mg. in 4 c.c.)

No.	Amino Acid			Concentration ($\mu g.$) 2.5	5.0	7.5	10.0	12-5	15.0	17.5	20.0
1	Arginine	$-Cu^{++}$	...	15	27	44	57.5	71	82	89	95
		$+Cu^{++}$	...	28	60	87	111	130	149	162	174
2	Glutamic acid	$-Cu^{++}$	...	20	40	60	80	97	113	130	150
		$+Cu^{++}$	...	28	60	90	122	152	180	199	216
3	Valine	$-Cu^{++}$	...	22	45	66	91	114	136	162	185
		$+Cu^{++}$	...	37	80	120	157	193	230	265	...
4	Ornithine	$+Cu^{++}$	...	25	45	65	85	104	117	131	142
5	Glycine	$+Cu^{++}$	...	37	65	90	105	120	132	144	153
6	Lysine	$-Cu^{++}$	...	16	30	46	59	73	88	103	...
		$+Cu^{++}$	...	31	60	80	95	114	129	144	160
7	Aspartic acid	$-Cu^{++}$	...	17	30	42	54	63	71	...	...
		$+Cu^{++}$	...	27	50	68	85	101	114	125	...
8	Methionine	$-Cu^{++}$	...	24	48	72	96	118	138	152	162
		$+Cu^{++}$	...	45	88	124	159	195	223	239	257
9	Histidine	$-Cu^{++}$	...	9	18	26	35	43	52	60	68
		$+Cu^{++}$	...	14	29	43	57	68	79	90	103
10	β-Alanine	$-Cu^{++}$	...	20	38	55	69	82	93	102	110
		$+Cu^{++}$	...	32	59	84	106	126	139	150	159
11	Tyrosine	$-Cu^{++}$	...	17	30	44	59	75	88	101	...
		$+Cu^{++}$	...	24	48	71	94	116	136	155	...
12	Serine	$-Cu^{++}$	...	27	54	81	103	124	145	164	...
		$+Cu^{++}$	...	50	95	133	170	202	232	256	275
13	Asparagine	$+Cu^{++}$	...	9	16	24	30	36	41	47	52
14	Phenyl Alanine	$-Cu^{++}$	...	14	29	40	54	68	82	96	109
		$+Cu^{++}$	...	23	46	66	85	103	121	139	155
15	Iso-Leucine	$-Cu^{++}$	...	25	50	76	103	126	152	178	204
		$+Cu^{++}$	...	41	82	122	163	202	243	281	317
16	Tryptophan	$-Cu^{++}$	...	10	20	29	37	44	52	58	...
		$+Cu^{++}$	...	14	30	44	57	69	81	92	...
17	Leucine	$+Cu^{++}$	...	40	82	125	162	200	246	290	330
18	Threonine	$+Cu^{++}$	...	32	62	93	123	151	180	200	224

Relative intensities of colour from amino acids.—It was found that equivalent amounts of different amino acids give different intensities of colour with ninhydrin on paper in presence of Cu^{++}. The relative order of intensity of the ninhydrin colour with the amino acids differs from the order given by Moore and Stein (1948) who have carried out the experiment in solution. The relative values for colour intensities at 5×10^{-7} M of amino acid are shown in Table XI.

Table XI

Relative intensities of colour from amino acids at 5×10^{-7} M

Amino acid (5×10^{-7} M)	Colorimeter reading
1. Methionine	123
2. Arginine	115
3. Leucine	106
4. Iso-Leucine	107
5. Serine	98
6. Valine	93
7. Glutamic acid	88
8. Ornithine-HBr	88
9. Tyrosine	85
10. Lysine	78
11. Phenyl Alanine	72
12. Threonine	71
13. Histidine	63
14. Aspartic acid	61
15. Tryptophan	57
16. β–Alanine	52
17. α–Alanine	95

Application to the amino acid analysis of edestin hydrolysate.—The accuracy of the method was tested by carrying out the analysis of a pure protein, edestin, and comparing the values obtained with those reported in literature.

Hydrolysis.—100 mg. of pure edestin (Hoffman La Roche) were hydrolysed with 3 c.c. of 6 N HCl for 24 hours. The acid was removed at 50°C. by means of a Hi-vac Pump. The residue was taken up with glass distilled water and made up to 10 c.c. It was then filtered to get a light brown filtrate. The hydrolysate was preserved in a refrigerator.

Analysis.—Only those amino acids present in the hydrolysate which could be separated clearly on the chromatogram were estimated. A volume of 8.5 μl of the hydrolysate was spotted on the filter paper. Since a larger paper (35 cms. dia.) was found to be suitable for a good separation of the amino acid bands, it was found necessary to run known concentrations of a mixture of known amino acids on the same paper. The values expressed as gm. amino acid per 100 gm. of protein, are given in Table XII. The results obtained by this method are in general agreement with published values, except in the case of leucines, alanine and tyrosine.

Table XII

Analysis of Edestin

Amino acid	% by weight	
	By this method	Literature values. (Tristram, 1949)
Leucines	9.6	12.1 – 13.9
Alanine	3.5	4.3
Histidine	2.94	2.4 – 3.04
Arginine	14.7	14.16 – 16.76
Cystine-Cysteine	2.6	1.1 – 2.1
Tyrosine	6.2	3.7 – 4.49
Phenyl Alanine	4.7	4.2 – 5.71

Recovery experiments.—The method was also checked by carrying out recovery experiments. Known quantities of various amino

acids were added to the edestin hydrolysate (8.5 μ1). After developing the chromatogram, the quantities of each amino acid in the hydrolysate were estimated before and after addition of the known amino acids. It is evident from the results shown in Table XIII that the percentage recovery of added amino acids is, within limits, highly satisfactory.

Table XIII

Recovery experiments.

Amino acid	In Edestin hydrolysate (μ g.)	Amino acid added (μ g.)	Total quantity (μ g.)	Quantity recovered (μ g.)	%Recovery
Leucine ...	6·8	4·25	11·05	11·4	103
Tyrosine ...	5·3	,,	9·55	9·5	99
Cystine ...	2·2	,,	6·45	6·0	93
Phenyl Alanine ...	4·0	,,	8·25	8·0	97
Alanine ...	3·0	,,	7·25	7·4	102
Histidine ...	2·8	,,	7·05	6·7	95

Reproducibility of the method.—Reproducibility of the method was demonstrated by performing a number of determinations on a single sample of amino acid. The colorimetric readings obtained by different experiments for the same sample is given in Table XIV. It is evident that the method gives highly reproducible results.

Table XIV

Comparison of values obtained by Replicate Experiments.
(*Diameter of paper :* 38 cms.)

Amino acid	(Diameter of paper : 38 cms.)				
	Concentration (γ)	Colorimeter readings			
		I	II	III	IV
Leucine ...	6·8	153	153	158	150
Alanine ...	3·0	121	110	111	114
Histidine ...	5·2	65	57	55	54
Arginine ...	12·5	170	180	160	158
Cystine ...	2·2	23	25	27	29
Tyrosine ...	5·3	64	60	57	55
Phenyl Alanine ...	4·0	58	54	49	46

Discussion

The paper chromatographic method described above is simple, rapid and convenient for routine analysis and sufficiently accurate for quantitative determination of the amino acids present in protein hydrolysates and biological fluids. The percentage recoveries of some of the added amino acids from the edestin hydrolysate are found to be satisfactory.

The degree of accuracy obtainable by paper chromatographic methods is generally sufficient for the kind of problems which face the investigator, such as, the amino acid analysis of proteins and nutritional and pathological problems. The results of a single determination obtained by the method outlined here are correct within about 5 to 10 per cent. A higher degree of accuracy is no doubt possible by making a number of replicate determinations and taking the mean value, a considerable increase in the accuracy of the method may be achieved. It is also possible to obtain a rough quantitative estimate of the amino acids present in the test sample by visual comparison of the experimental amino acid bands with a graded series of amino acid standards chromatographed under identical conditions on the same paper. Although the calibration curves drawn for the amino acids show distinct proportionality between the concentration of the amino acids and colour intensity within a limited range, it is desirable to compare the colour intensity of the alcohol eluates of the bands relating to the test sample with that of known standard amino acids spotted on the same paper. This procedure will eliminate any errors due to variations in experimental conditions which often occur when the chromatograms are run on different papers at different times, as it is very difficult to control all experimental conditions. The method can be applied to the estimation of all the amino acids present in protein hydrolysates which separate into individual bands on the chromatogram. In the case of those amino acids which overlap, when the chromatogram is developed with *n*-butanol-acetic acid-water solvent mixture the method cannot be applied for the determination of these amino acids. Investigations are now in progress on the separation of

these overlapping acids when butanol-acetic acid-water is used as solvent, by employing other solvent mixtures. It is hoped that the complete analysis of all the amino acids present in protein hydrolysates, can be achieved by this method when the procedures for the separation of overlapping amino acids are developed.

Most of the quantitative procedures described by other investigators have been carried out by one dimensional paper strip chromatography. It is very difficult to separate the large number of amino acids present in protein hydrolysates by the one dimensional method using one solvent mixture. However, by running a number of chromatograms with different solvent mixtures, it is possible to separate most of the amino acids.

Two dimensional procedure will no doubt separate many of the amino acids, which are difficult to separate by one dimensional procedure. The advantage of improved resolution in two dimensional chromatography is offset by some disadvantages enumerated below :—

1. It has often been observed that even in two dimensional chromatograms some amino acids overlap each other.

2. Although two dimensional chromatograms give better separation, they show poorly defined spots and also irreproducible R_f values.

3. It suffers from the disadvantage that only one sample can be mapped at a time. To run controls it is necessary to prepare separate maps.

4. Losses of amino acids occur during both runs. The amino acids may be decomposed by deamination due to the action of solvents or the atmosphere. Adsorption on paper may also occur resulting in the loss of amino acids. These losses will be more pronounced when the chromatograms are run for a long time and on large sheets of papers as is usually done in two dimensional chromatography.

It is necessary in quantitative work to control all the above factors in order to minimise the losses of amino acids during

chromatography. Considering the disadvantages of the two-dimensional chromatographic technique for use in quantitative analysis, it will be more useful to develop the one-dimensional technique for quantitative studies. Our experience on the application of circular paper chromatographic technique to the separation and quantitative determination of amino acids has shown that this technique possesses some advantages over one dimensional strip chromatographic technique.

The circular paper chromatographic procedure takes less time to complete the chromatogram and therefore the losses of amino acids during chromatography are negligible. The separation of amino acids into distinct narrow bands facilitates accurate cutting of the bands for elution and measurement of colour. All the amino acids present in protein hydrolysates including the overlapping amino acids separated by using butanol-acetic acid-water solvent mixture can be separated by the technique developed recently in this laboratory using different solvents (Giri and Rao, unpublished work). In addition to the advantages mentioned, the circular paper chromatographic method has the virtues of being extremely easy to operate and makes no demand for specially designed equipment.

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