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Rapid quantitative analysis of basic amino acids by ion exchange resin paper chromatography

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RAPID QUANTITIVE ANALYSIS OF BASIC AMINO ACIDS BY ION EXCHANGE RESIN PAPER CHROMATOGRAPHY

by

John Ennis Coutant UC 1966

Senior Thesis Submitted
in Partial Fulfillment
of the Requirements of Graduation

DEPARTMENT OF CHEMISTRY

UNION COLLEGE

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This Thesis

Submitted by

John Ennis Coutant

to the

Department of Chemistry of Union College

in partial fulfillment of the requirements of the degree of

Bachelor of Science with a Major in Chemistry

is approved by

Robert W Schaefer

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I

HISTORICAL BACKGROUND

HISTORICAL BACKGROUND

In partition chromatography a solid is used to hold a film of liquid and the distribution of a substance is between the liquid phase and another immiscible liquid phase. Because most substances have a slightly different distribution coefficient between two immiscible solvents, a series of successive extractions will cause a separation. This is the predominant factor in paper chromatography. The paper itself is an inert support holding a stationary aqueous phase. As the immiscible solvent flows past an area containing the solute, by capillary flow, partition occurs. When the immiscible solvent reaches an area not containing any solute, partition again occurs resulting in a transfer of the solute from its point of application to a point some distance down the paper. This method of separation, which has been developed to cover the separations of many organic and biological compounds, has some important limitations concerned with the fast separation and quantitative analysis of the separated products. The flow rate of the organic developing solvent is slow (2-3 cm./hr.) and the organic solvent is commonly phenol, which presents problems in its use because of its corrosive nature. Also the amount of

solute which can be separated per unit area of the paper is extremely small. This amount is usually in the order of 1 to 10 microliters of a 0.01 molar solution of the solute. Amounts of this size require special apparatus for the measuring and application of the solution. Because the amount of solute is so small, the experimental error in its analysis is increased.

In ion-exchange chromatography the distribution of a substance is between a solution and a salt of a high-molecular weight ion exchanger. The method of separation is the same as partition chromatography except that the ion exchanger replaces the stationary liquid phase and the solute must have ionic characteristics. Ordinarily the ion exchange resin is packed into a column, a solution containing the solvent is poured through, and then a solution (its nature depending on the resin, solute, etc.) allowed to flow through the column at a fixed rate, and equal amounts of the solution which has passed through the column are collected successively. Each individual sample is analyzed and the different solutes which came off the column at different times are grouped for the total amount of each. The limitations concerned with this method for rast separation and quantitative analysis are again concerned with speed and equipment needed. The time required to

prepare a uniform column is not great, but the collection of many samples and the analysis of each is. The collection is equal volumes of samples also calls for rather complicated equipment. The experimental error is also increased if each sample is analysed individually.

Both paper and ion exchange chromatography have provided methods of analysis with results that were previously impossible or too laborious. A technique which would combine the advantages of both methods yet eliminate their disadvantages was the object of a great deal of research to incorporate the resin as an integral part of the paper. This has been accomplished by "loading" micropulverized resins into a slurry of high quality alpha-cellulose pulp and malting the m xtrue into chromatography paper. These papers are homogeneously loaded sheets containing about 45-50% resin by weight. At present these paper sheets are available with four different types of ion exchange resin - weak and strong cation exchangers and weak and strong anion exchangers.

The use of ion exchange resin loaded paper has several advantages including: use of a shorter length of paper, a higher capacity and concentration effect due to the high concentration ef the ion exchange resin per unit area of paper, the use of relatively simple aqueous-buffer solutions as the developing solvent, and the possibility of attaching differ-

ent type papers end to end for a one step separation of groups of compounds containing different degrees of acidic and basic ions in the same solution.

A great deal of work involved with separations on these papers has been done by Tuckerman, Osteryoung, and Nachod (1)(2), especially with the amino acids. There also has been some work done in this area by Myhre and Smith (3) and also by others whose work was concerned with inorganic separations.

The paper used in this project was a high molecular weight polystyrene nuclear sulfonic acid. This resin is supplied in the sodium form and when it is converted to the acid form, it is similar in acidity to sulfuric acid. Aside from it's behavior as a strong acid it is quite chemically inert. A typical reaction for this resin is:

$$RzSO_3H + Na^+ + Cl^- \rightleftharpoons RzSO_3Na + H^+ + Cl^-$$

where Rz is the copolymer resin of styrene and divinylbenzene (See Figure 1).

Roberts and Kolar have devised a method (4) for the quantitative determination of the basic amino acids. This method involves the separation of the amino acids on the paper and spraying them with a ninhydrin reagent. The density of the color of the spot is related to the concentration of the

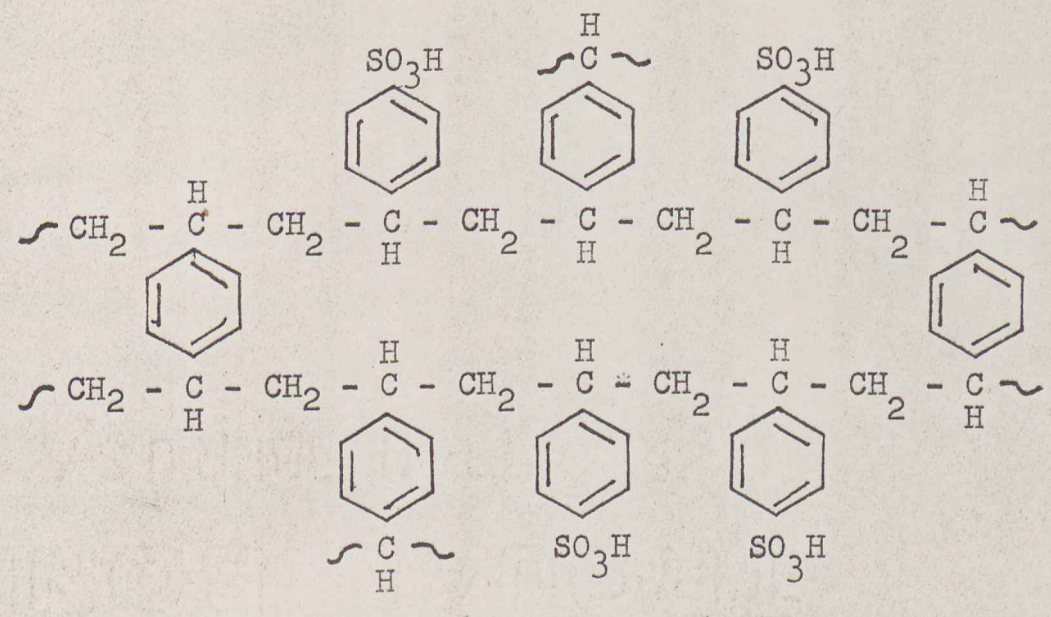


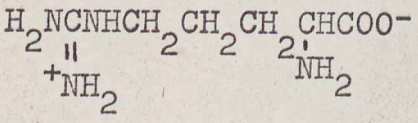
Figure 1

A Polystyrene Sulfonic Acid

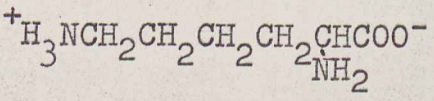
amino acid in the original solution. However this method suffers the same limitations with the exception of time and developing solvent as paper chromatography. Only very small amounts of solution may be applied to the paper and the accuracy of this method is not great.

All the amino acids are alpha-amino carboxylic acids. In addition to the carboxyl group and the amino group, some contain a second basic group which may be an amino group (lysine), a guanidino group (arginine), or the imidazole ring (histidine) (See Figure 2). These are called the basic amino acids and because of this property lend themselves to a separation involving an ion exchange resin of the acidic type.

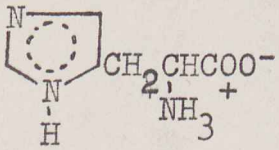
The purpose of this project was to develop a method of analyzing amino acids quantitatively that could be performed by an undergraduate in introductory courses in quantitative analysis. The formost thought in developing the procedure was to keep it as simple and straight forward as possible. In connection with this was to use only apparatus which was easily obtainable and simple to use. The use of ion exchange papers seemed to offer the best solution. They were proven effective in the separation of amino acids of the basic type (1) (4) and allowed a separation to be performed in a length of a lab period. The work done out-



(+) - Arginine



(+) - Lysine



(-) - Histidine

Figure 2

The Basic Amino Acids

lines a method of analysis and separation which could be performed in two consecutive lab periods with reproducable results.

II

DESCRIPTION OF APPARATUS

DESCRIPTION OF APPARATUS

In keeping with the purpose of the project, the apparatus needed is readily available or made. The use of decending chromatography requires a container of some type to keep the atmosphere saturated with the developing solvent. For this project the container consisted of a large glass cylinder with a ground glass top. A glass support in the center held a weighing bottle which was filled with the developing solvent and some glass rods over which the paper strips were extended. This apparatus is diagramed in Figure 3.

The ion exchange resin papers were purchased from the Scientifica Division of H.Reeve Angle & Co., Inc., of New Jersey. The paper used in this project was Reeve Angle Grade SA-2 Amberlite ion exchange resin loaded paper which contains Amberlite IR-120 resin (strong acid). The package used was Control A-7802. These papers come in the form of paper sheets which were cut into strips 2.5 cm. wide. Since the determination of the R_f values was not necessary, the end of the strip was serrated allowing the solvent to drip off the end and thus utilize a shorter strip of paper. The papers are supplied with the resin in the Na^+

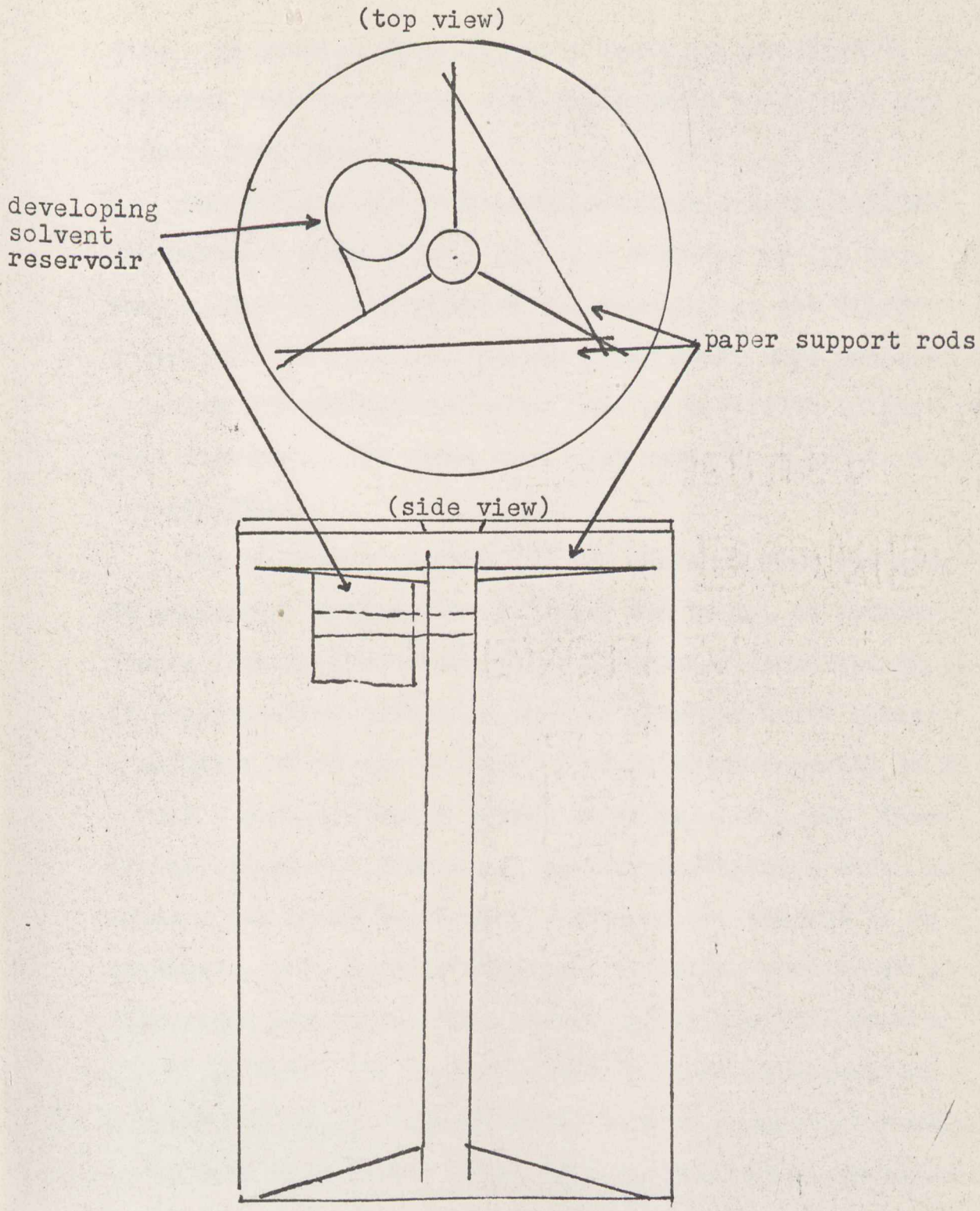


Figure 3

Decending Chromatography Chamber

form. To prevent streaking, the resin is converted to the hydrogen form by washing with acetic acid buffer (1) and rinsing with water.

The amino acids were purchased from the Nutritional Biochemicals Corporation, and were supplied as the free base except for lysine which was supplied as the dihydrochloride. The solutions of the amino acids were prepared by weighing the crystalline amino acid on a torsion balance and then dissolving the amino acid with water in a 10 ml. volumetric flask.

The ninhydrin reagent (5) was prepared from one gram of ninhydrin (Eastman White Lable) and 20 mg. of hydrindantin (K&K Laboratories, Inc.) which were dissolved in 25 ml. of peroxide-free methyl cellosolve (Eastman White Lable). A solution of 40 ml. of $SnCl_2 \cdot 2H_2O$ (Baker Reagent) in 25 ml. of 0.2 M sodium citrate buffer of pH is also made. These two solutions are then mixed and any precipitate which forms after a few hours is removed. Because the reagent is so sensitive, care must be exercised in making sure no amino containing substance contaminates either the glassware or the solutions. The reagent should be kept refrigerated. However the only method of being sure of consistant results is to make the reagent fresh from the individual components each time it is to be used.

The absorption was measured on a Bausch and Lomb Spectronic 20. The plot of wavelength vs absorption (Figure 5) of the ninhydrin reaction product was produced on a Perkin-Elmer Model 202 recording spectrophotometer.

III

PROCEDURE

PROCEDURE

Portions of the solution to be analyzed were withdrawn with a 50 microliter pipette and applied to the ion paper strip. If the area of application of the spot on the strip is kept small, the effect of the spot trailing or streaking will be kept at a minimum. This was accomplished by applying very small amounts of solution and drying the resulting spot with a heat lamp before another portion was applied. The amino acid solution spot was applied about 6 cm. from the end of the ion exchange paper strip (See Figure 6) to allow the end to be immersed in the developing solvent. The solvent front traveled from the reservoir, over a glass supporting rod, and down the paper strip passing over the initial position of the amino acid(s), and then down to the end where it dripped off the serrated end. At the end of a certain period of time the strips were removed and allowed to dry.

To initially find the position of the amino acid spots, the paper strips were sprayed with a solution of 1 gram of ninhydrin in a 100 ml. solution of absolute ethanol and then allowed to develop in an oven at 60-70°C for 15 minutes. The location of the amino acid spot is then revealed as a

blue or purple spot. The distance from the origin of the leading and trailing edge of the spot is then recorded for future reference. Those samples which were run for analysis are marked for the location of the desired amino acid and this section was cut from the paper strip. This piece of paper containing the separated amino acid was placed in a test tube along with 2 ml. of the ninhydrin reagent. The test tube was then placed in a boiling water bath and shaken for a period of 10 minutes. After this the colored reagent reaction product was transfered to a 25 ml. volumetric flask and the test tube containing the paper was washed five times with 2 ml. portions of water and twice with 2 ml. portions of acetone. The flask was then diluted to volume and the solution mixed. This solution was then measured for absorption.

IV

EXPERIMENTAL RESULTS AND CONCLUSIONS

EXPERIMENTAL RESULTS AND CONCLUSIONS

The first concern was the actual separation of the amino acids on the papers themselves. The initial series were made with 5 microliter application of 0.01 molar solution of the three amino acids. These strips were developed for a period of an hour resulting in a separation of the spots, but not to the degree that the individual spot could be physically cut out. The separations were repeated with runs of two and three hours. The developing time of three hours produced separations of sufficient magnitude to be cut apart (See Figure 4).

The next problem was the spectophotometric analysis of the reacted reagent. The wavelength at which the blue pigment of the ninhydrin reagent reaction (See figure 5) was absorbed was determined with a recording spectophotometer, the Perkin-Elmer 202 (See Figure 6). To see if Beer's law plot could be achieved, reactions were run with ninhydrin and solutions of amino acids.

A series of known solutions of amino acids were prepared by dissolving enough amino acid in water in a 10 ml. volumetric flask to get approximately a 0.01 molar solution (See Table I). For the first series, 50 microliters of the

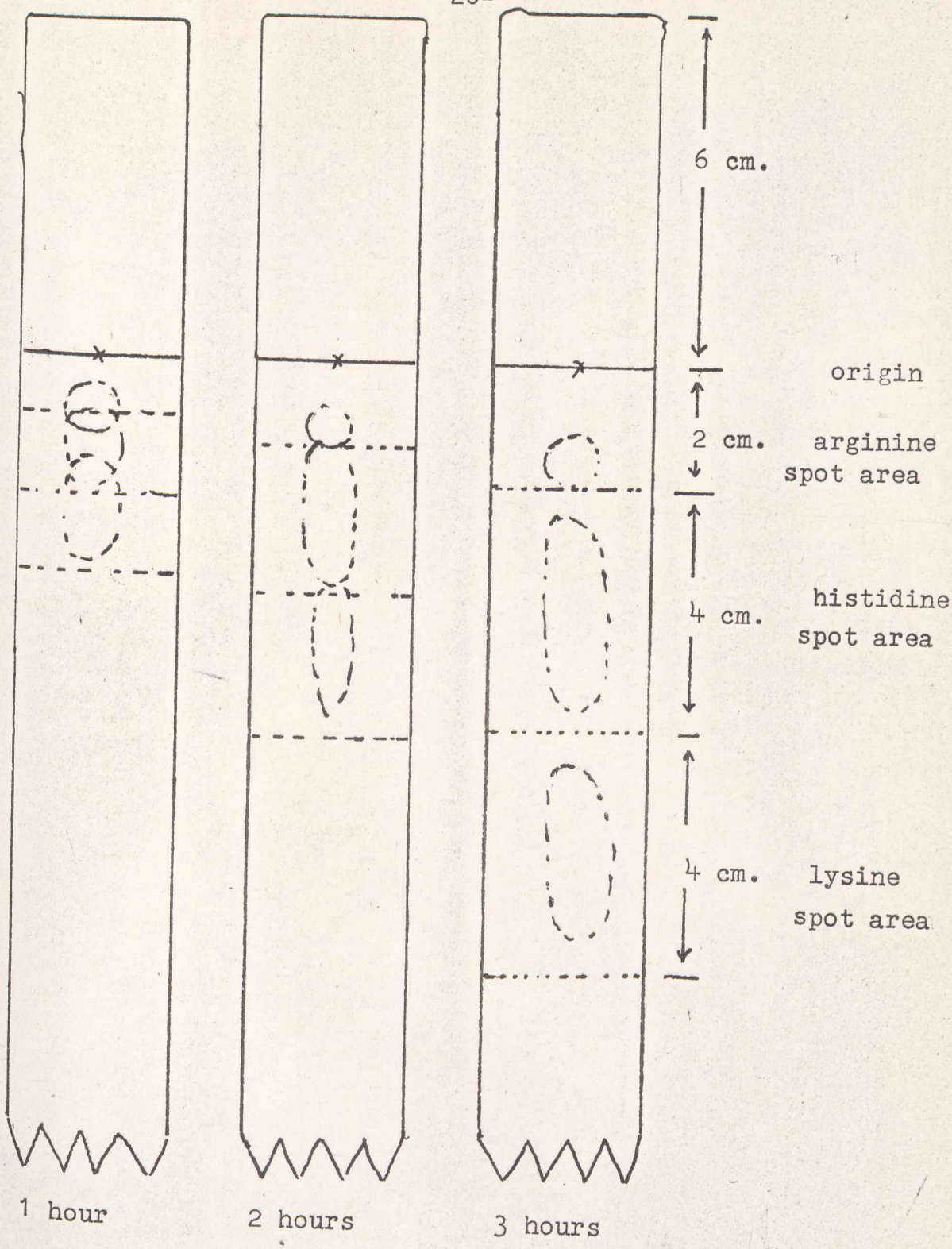


Figure 4

Developing time results

Actual size

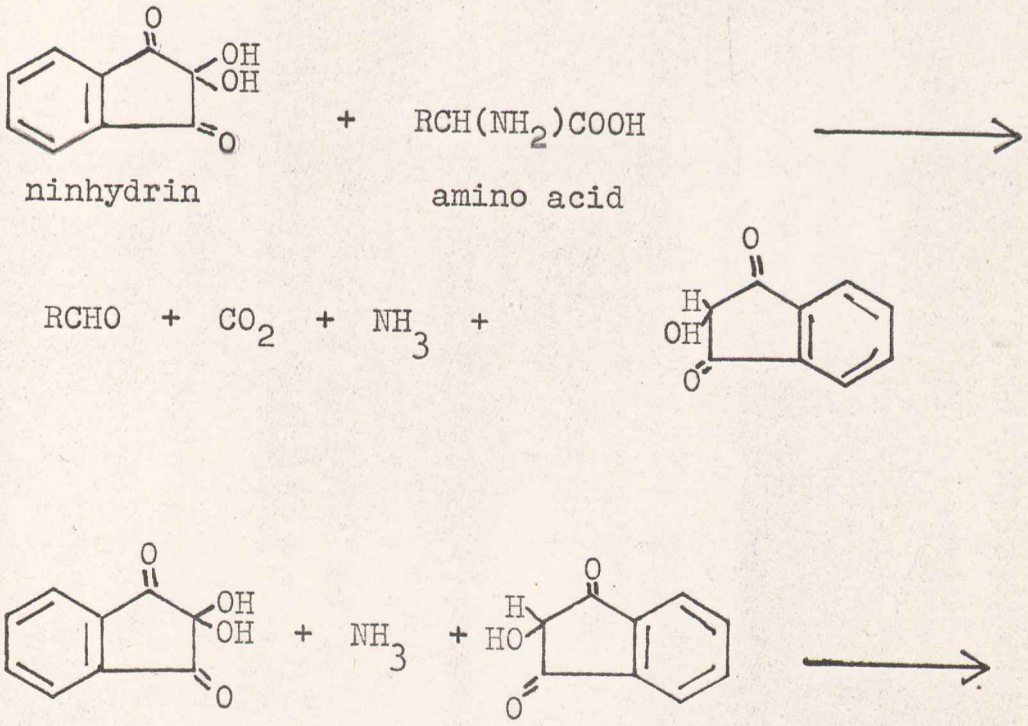
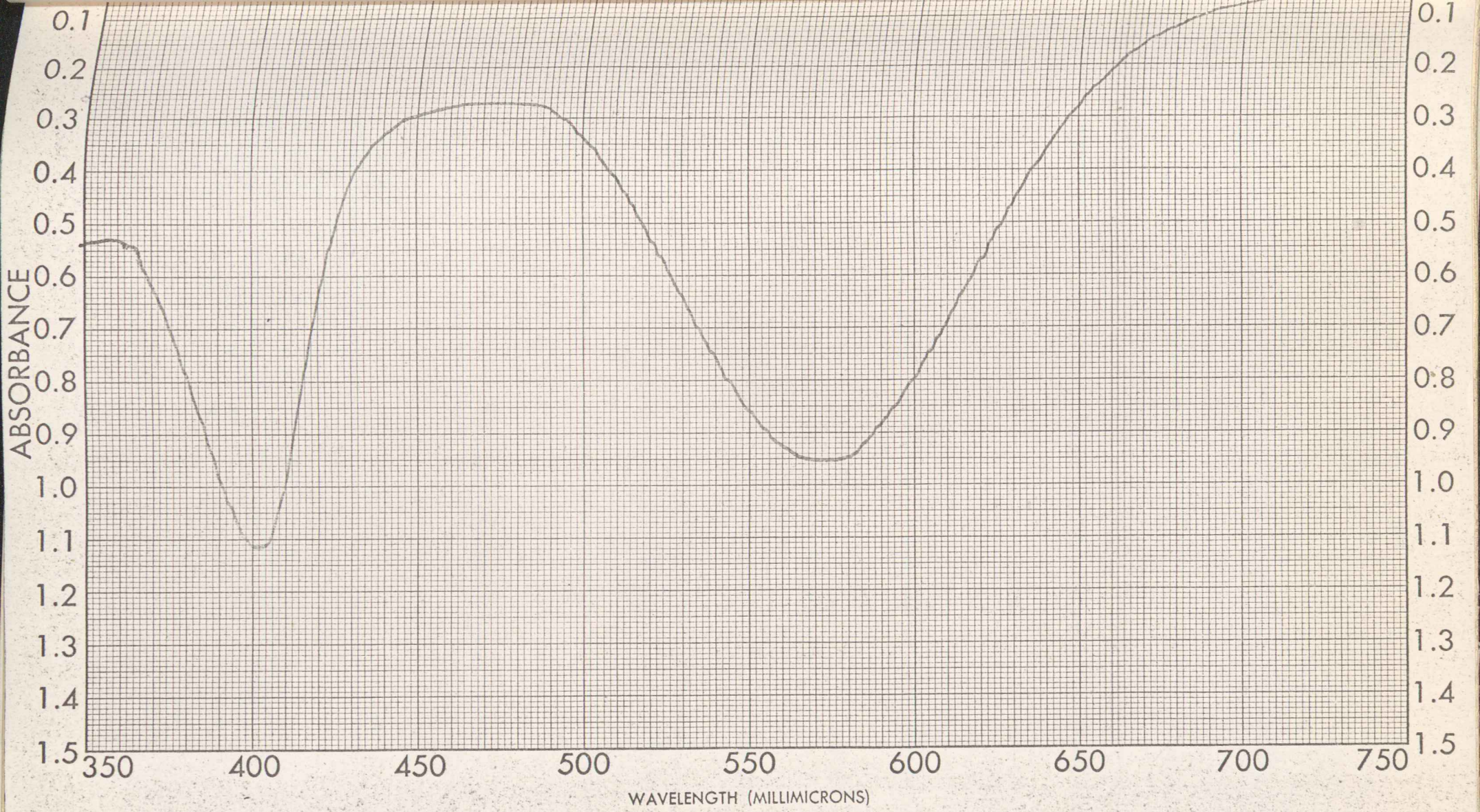


Figure 5

Reaction of Ninhydrin with Amino Acids



SAMPLE ninhydrin - histidine reaction product	CURVE NO. Figure 6	SCAN SPEED Fast	OPERATOR JEC
	CONC.	SLIT 40	DATE 12/9/65
ORIGIN	CELL PATH	REMARKS	
SOLVENT water	REFERENCE water		

PART NO. 202-1512

PERKIN-ELMER

Sample	Amino acid(s)	wt. in mg.	vol. soln.
I	histidine	15.42	10 ml.
II	histidine	20.30	10 ml.
III	arginine	17.70	10 ml.
	histidine	15.98	
IV	arginine	15.36	
	histidine	5.30	10 ml.
	lysine	10.40	
V	arginine	14.80	
	hystidine	10.30	10 ml.
	lysine	11.60	
VI	arginine	16.08	
	histidine	19.64	10 ml.
	lysine	10.80	

samples was reacted with solid ninhydrin in water. The results are recorded in Table II and the results plotted in Figure 7. The results from this series did not produce a straight line without allowing for considerable error. A second series was run using 50 microliter samples and running the reaction in the ninhydrin reagent itself (See Figure 8). The next step was to spot 50 microliter samples on the paper and react the paper with the ninhydrin reagent. (See Table IV and Figure 9) These results came close to those of the second run (Series 3).

A series of separations was then performed on certain strips developed to find the position of the spots, and these spots were cut out and developed (See Table V). This series (4) produced seemingly unrelated points. However the closest agreement to earlier series (2 and 3) seems to arise when a fresh amount of the ninhydrin reagent was prepared. With this in mind a final series (5) was made with three different concentrations of amino acids. These results are found in Table VI and Figure 10. The resulting curve is in close agreement with Beer's law and would support that assumption that resulting separation of the amino acid was quantitative.

Number	Sample	Amount diluted to	Adsorption	conc. histidine (mg/ml)
1	I	10	0.620	.00771
2	II	10	1.50	.01015
3	II	25	0.55	.00406
4	I	25	0.365	.00308

Series I

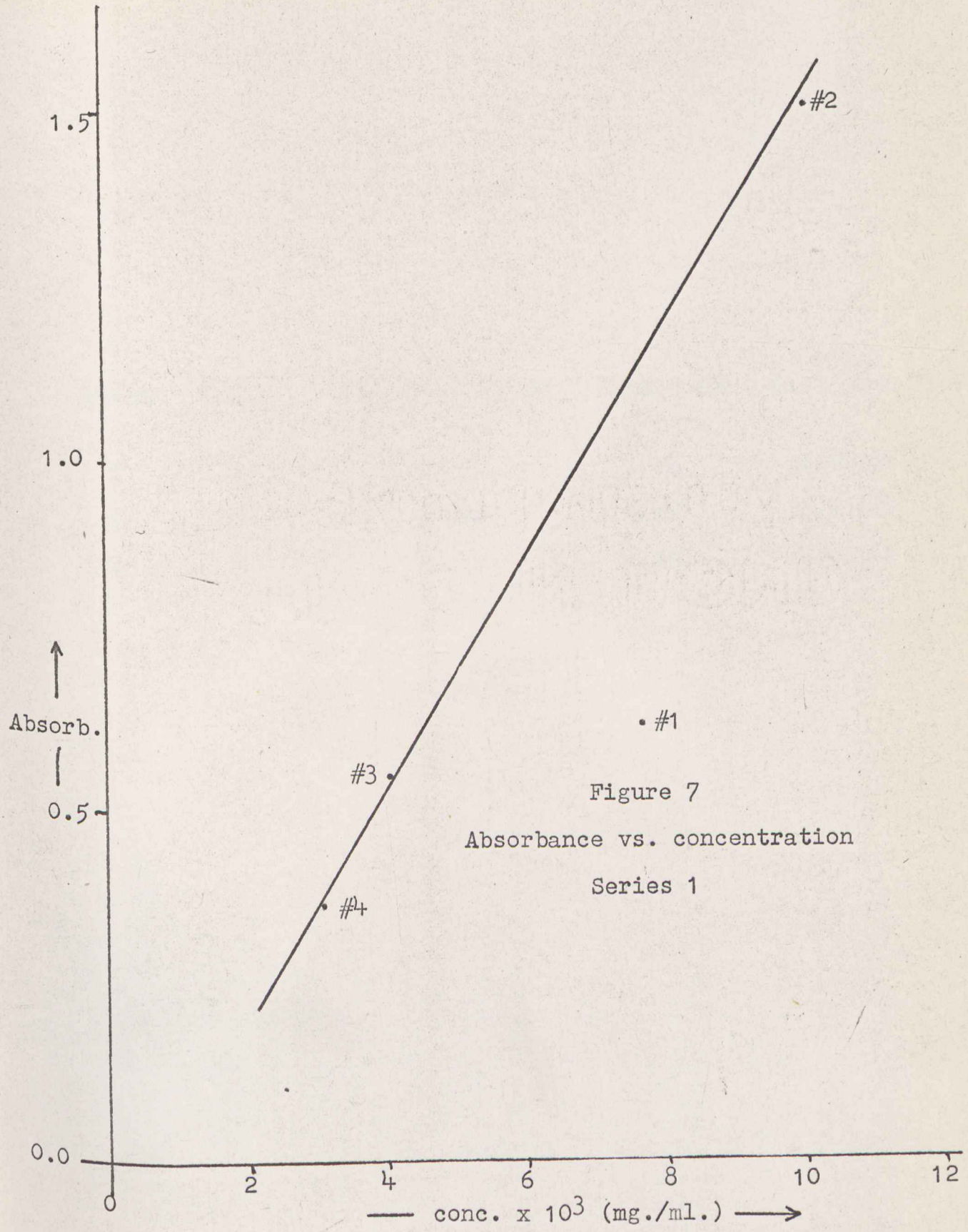


Figure 7

Absorbance vs. concentration

Series 1

Number	Sample	Amount diluted to	Adsorption	conc. histidine (mg/ml)
5	I	25 ml.	0.54	0.00308
6	II	25 ml.	0.725	0.00406
7	I	50 ml.	0.270	0.00154

Series II

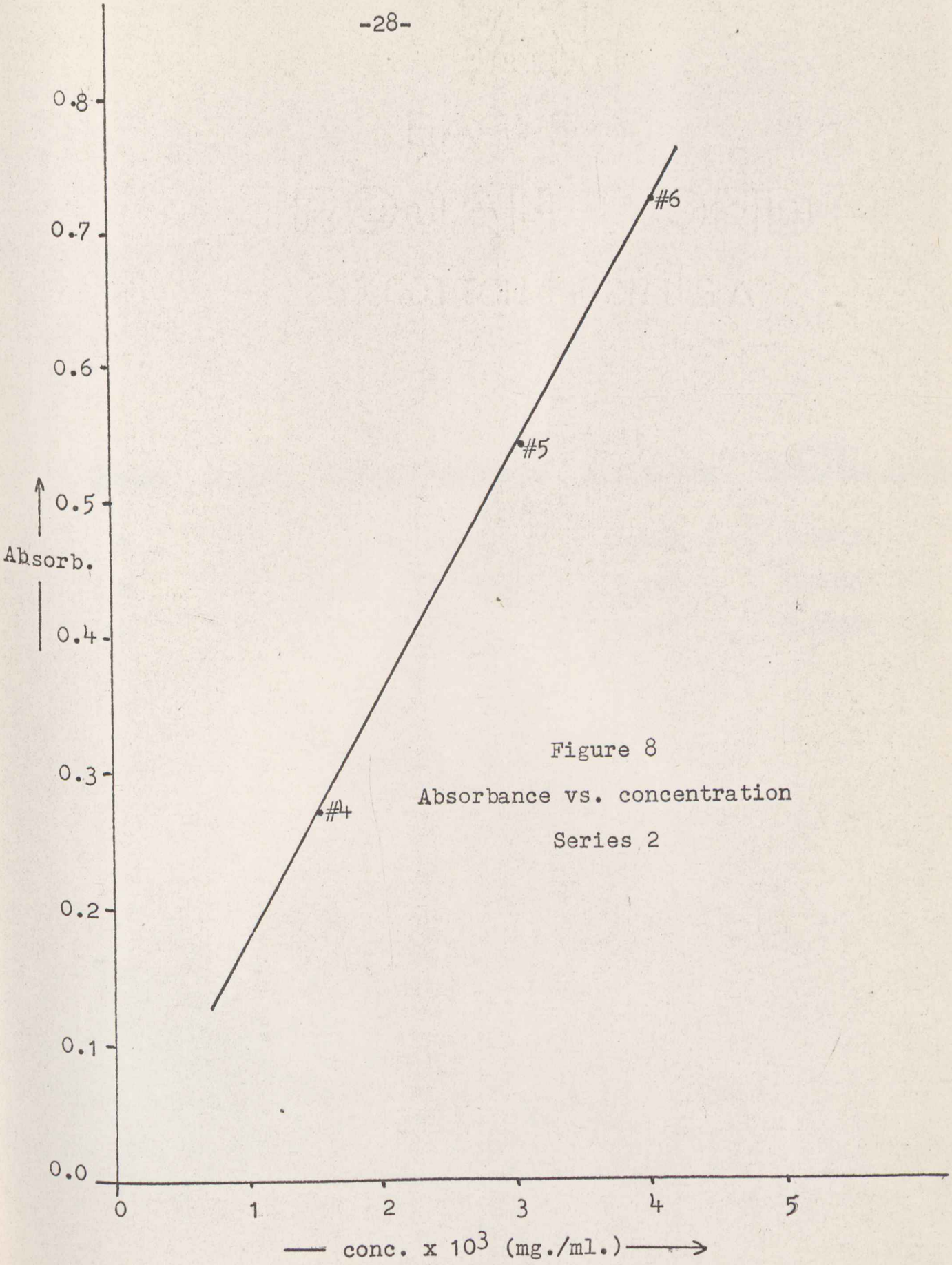


Figure 8

Absorbance vs. concentration

Series 2

Number	Sample	Amount diluted to	Adsorption	conc. histidine (mg/ml)
8	I	50 ml.	0.255	0.00154
9	I	50 ml.	0.220	0.00154
10	I	25 ml.	0.540	0.00308

Series III

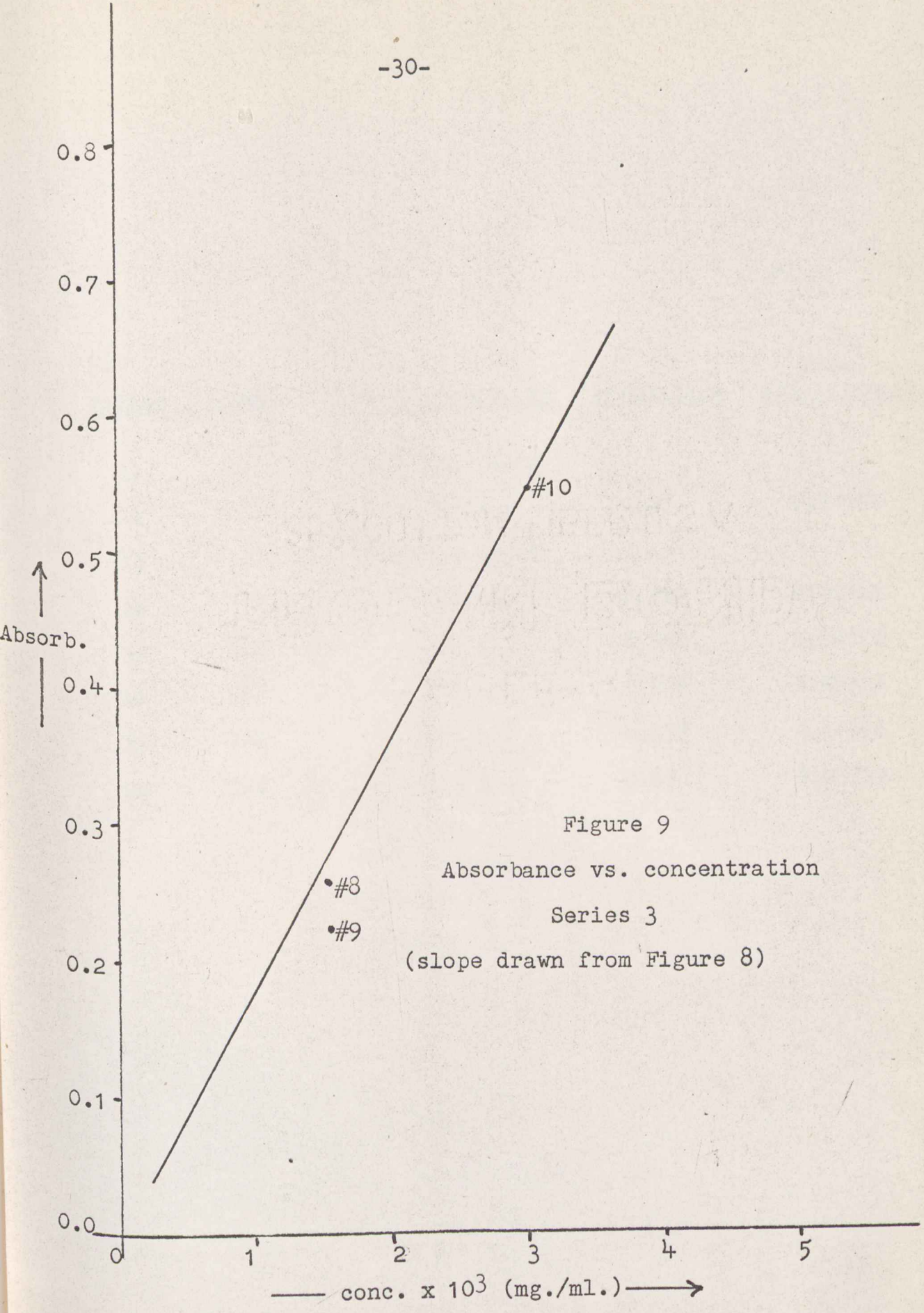


Figure 9

Absorbance vs. concentration

Series 3

(slope drawn from Figure 8)

Number	Sample	Amount diluted to	Adsorption	conc. histidine (mg/ml)
11	II	Position check		
12	II	25 ml.	0.07	0.00406
13	I	Position check		
14	I	10 ml.	0.285	0.00711
15	III	10 ml.	0.205	0.00799
16	III	25 ml.	0.255	0.00319
17	III	25 ml.	0.258	0.00319
18	III	25 ml.	0.45	0.00319

Table V

Series IV

Number	Sample	Amount diluted to	Adsorption	conc. histidine (mg/ml)
19	IV	25 ml.	0.122	0.00106
20	V	25 ml.	0.225	0.00203
21	V	25 ml.	0.260	0.00203
22	VI	25 ml.	0.430	0.00392

Table VI

Beer's Law Plot

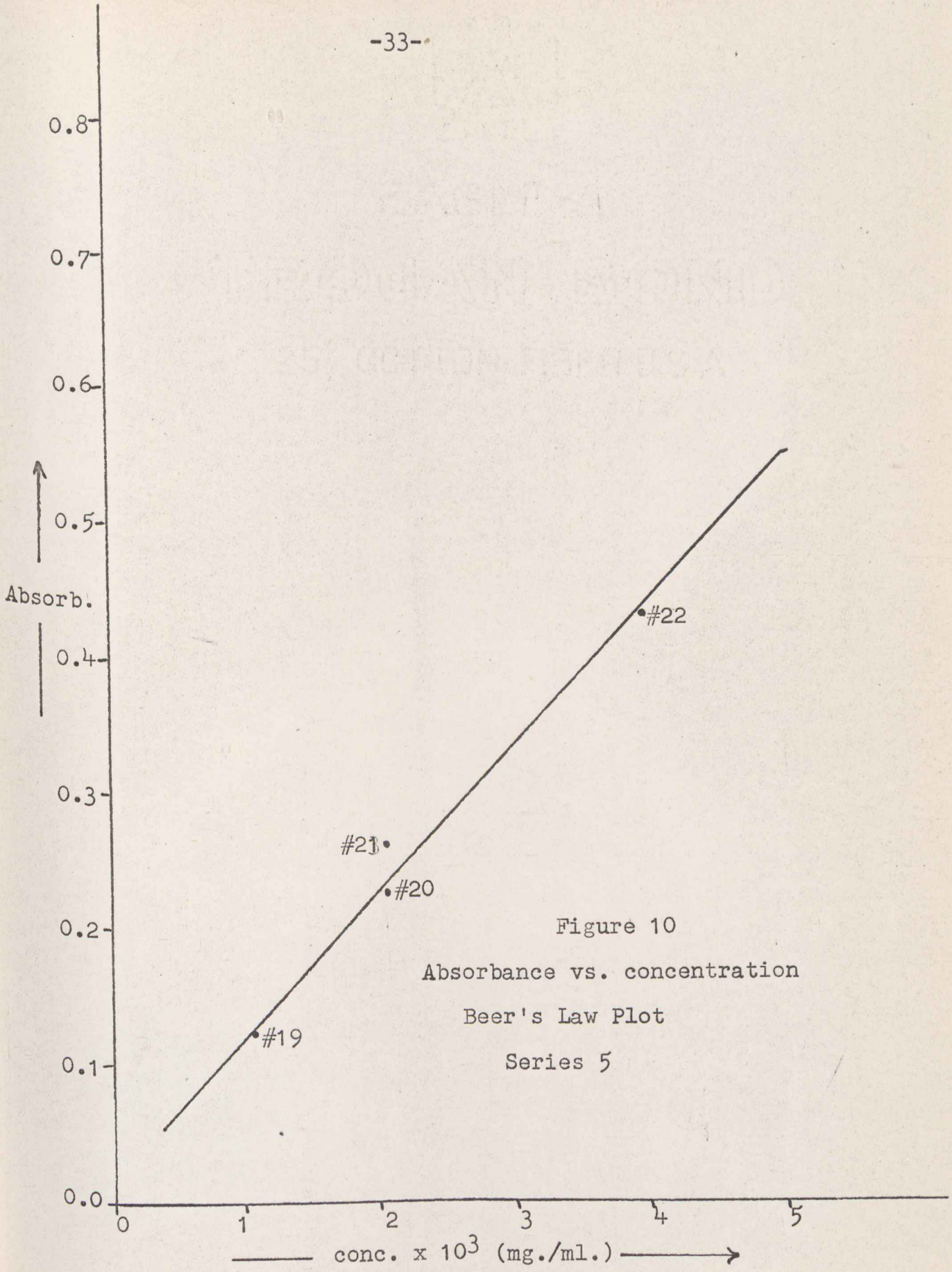


Figure 10

Absorbance vs. concentration

Beer's Law Plot

Series 5

V

SUMMARY

SUMMARY

The actual process by which the amino acids are separated is still a matter of speculation concerning the extent to which the resin and the paper contribute individually to the separation. The low pK_a value of the Amberlite IR-120 resin and the relatively high pH of the developing solvent raise the question of whether an acid-base reaction is the main factor in a separation with the strong acid paper. The use of a weak acidic type of ion exchange paper (Reeve Angle Grade WA-2, containing Amberlite IRC-50 ion exchange resin of the type $RzCOO-H^+$) which has a pK_a in the range of the developing solvent would probably enhance this factor in the separation. There is no question that the ion exchange resin does have an effection the separation. This is supported by a comparison of the R_f values achieved by paper chromatography and ion exchange paper chromatography. The values for one type of paper are opposite from those values for the other type (1) (5).

All the work done in this project was concerned only with the use of histidine in order to have a comparison and check on the results. It should be possible to substitute either of the other two basic amino acids in its place with-

out much change in the procedure. However the wavelength of maximum absorption of the ninhydrin reaction product should be checked along with its position on the ion exchange paper strip for each amino acid.

This separation procedure might be extendable to the acidic and neutral types of amino acids by the use of an ion exchange paper containing a basic ion exchange resin. The problem here is that the resin in these papers contain a free amine group which would react with the ninhydrin reagent. This might be avoided by eluting the separated amino acid before reacting it with the ninhydrin reagent.

Although the separations and analysis of amino acids that were achieved in this project were reproducable and fast enough to make the procedure feasible, much work could still be done to streamline and improve the procedure.

The use of ion exchange paper chromatography is a new field, having only started in 1957, and promises to become as important as paper chromatography for a great number of uses including both those covered by paper and ion exchange chromatography and those areas yet unexplored.

VI

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