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An Experimental Approach to the Quantification of Ionographic Value

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AN EXPERIMENTAL APPROACH TO THE QUANTIFICATION OF IONOGRAPHIC VALUES

by

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APPROVAL SHEET

The dissertation submitted by Matthew Charles Urbin has been read and approved by a committee of five members of the Graduate Faculty.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosphy.

May 18, 1954

Date

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LIFE

Matthew Charles Urbin was born in Chicago, Illinois, January 17, 1926.

He was graduated from St. Michael's High School, Chicago, June 1943; from September 1943 to June 1944 he attended Loyola University; from June 1944 he served in the Army of the United States; honorably discharged, returned to Loyola University in June 1946. He graduated in June 1949 with the degree Bachelor of Science in Chemistry. He began work for his Master of Science degree in Biochemistry at the Graduate School of Loyola University in January 1950 and received the degree in February 1952.

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He is co-author of the following publications: "Measurement of Ion Migration on Paper in an Electric Field. Transference Numbers of Nickel and Copper Sulfates". Science, 112, 227 (1950); "Relation of Movement to Time in Ionography", J. Amer.

Chem. Soc., 73, 1893 (1951); "Isoelectric Points of Amino Acids and Proteins by Ionography", Fed. Proc. 10, 218 (1951); "Ionography: Some Aspects of Ion Migration on Paper in an Electric Field", J. Colloid Science, 6, 236 (1951); "Ionography; Electromigration on Wet Surfaces", M.S. Thesis, Biochemistry, Loyola University, February 1952; "Ionography in the Study of Amino Acids and Proteins", Abstracts, XIIth International Cong. Pure and App. Chem., New York, N.Y. Sept., 1951 p. 67; "Behavior of Amino Acids and Some Derivatives in Simultaneous Crossed-Current Ionography", Fed. Proc. 12, 243 (1953); "Electromigration in Stablizied Electrolytes. I. The Development of the Technique", Clin. Chemist, 5, 17-23 (1953); "Electromigration in Stablizied Electrolytes. II. Factors Influencing Mobility", Clin. Chemist, 5, 35 (1953); "Electromigration in Stabilized Electrolytes. III. Specific Applications", Clin. Chemist, 5, 51, (1953).

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CHAPTER I

INTRODUCTION

The last four year has witnessed a rapid growth in the field of ionography. There have been approximately three hundred and forty papers published in this period of time, yet, of all these publications only a few have been quantitative in nature. The need still exists, as it did four years ago, to investigate certain phases of the quantitative aspects of the technique. In only two laboratories have investigators been interested in mobility determinations and none have achieved values for mobilities which approach those determined in non-stabilized electrolytes. Little work has been done on the direct determination of the amounts of material present in the various zones of the developed ionogram, and only one worker has used an automatic scanning device. No attempts have been made to determine whether molecular weight determinations can be made using the technique of ionography or any modification thereof.

The purpose of this dissertation was to study, experimentally, these quantitative aspects of a most important problem. A method by which free solution electrophoretic mobilities can be approached, using ionography, is given. A method for

the direct scanning of ionograms to estimate the amounts of various substances present is presented. A system will be described which appears theoretically to offer an approach to the determination of molecular weights. The system was tested experimentally and found to hold for at least one series of compounds.

CHAPTER II

MOBILITY DETERMINATIONS

A great mass of work has been done in the field of paper electrophoresis. Most of the work has been of a resolving or preparative nature, and all but a few workers have ignored the measurement of mobilities. Mobility may be defined as the directed Brownian movement of a particle in solution due to the imposition of an electrical field, or it may be considered as the movement per unit electrical force per unit time. It is classically thought of as the movement a particle undergoes in solution, in centimeters in a potential field of one volt per centimeter, in the course of one second. However, none of these definitions gives an exact idea of the physical reality of mobility. First they give no insight into the variance of mobility with a change in the indifferent ions in the solution, nor do they give any idea as to the accuracy of such measurements by the instruments presently available. Thus we must first consider some of these difficulties before we can discuss the determination of mobilities in paper electrophoresis, or ionography as McDonald, Urbin, and Williamson (62) have named this adaption of electrophoresis in stabilized media. Also

since the mobility work to be discussed in this dissertation is to be compared to mobilities calculated from non-stabilized moving boundary electrophoresis it is necessary to consider some of the inaccuracies found in this method of measurement.

It is not the purpose here to consider moving boundary electrophoresis in its entirety, but rather only insofar as errors are introduced in the calculation of mobilities. The method of studying mobilities in the classical moving boundary method is well demonstrated by Tiselius's method (82). The system is essentially a U tube method using the refractive index or the scanning method for the detection of the boundary. In either the refractive index or the scanning method curves are photographed and developed to indicate peaks of electrophoretically pure components. This is not meant to be a comprehensive description of the instrument, which can be found in almost any text on biophysics. The main considerations here are the sources of error in calculating mobilities. One, concerning the cell itself is due to the fact that the ascending boundary is moving into a salt concentration and the descending boundary is moving into a region just vacated by the ascending boundary resulting in different mobilities for the ascending and descending boundaries. Other difficulties of the cell will be discussed under difficulties of all the methods in general. An error also may arise in the process

of photographing the boundaries. Development time in the method is critical due to film shrinkage which need not be great to influence mobilities of migrants which have moved less than one centimeter. The human factor still remains in measuring the boundary peak which may be easy in cases of concentrated boundaries but can be extremely difficult in the case of diffuse boundaries.

On a final analysis of moving boundary discrepancies those errors should be discussed which are general to all techniques, including those in stabilized electrolytes. One of these is the dependence of mobility on the buffer ions per se; that is, it is noted that if pH, ionic strength, and all other factors are held constant and only the type of buffer used is varied that different mobilities will be observed. This would seem to indicate a reaction of the buffer with the so called indifferent ions. This has been such a factor that mobilities as a physical chemical index lose much importance unless a standard buffer system be set down. Also the fact that it is physically impossible to achieve the same conductivity for the migrant as for the buffer solution indicates another source of error in mobility measurements.

Errors also exist in stabilized media, many of which are the same, such as those due to the buffer. Others are unique to the system itself such as the introduction of a non-mobile

component to the system, the stabilizer itself. This latter factor has been comprehensively treated by Marbach (52). It becomes obvious that ionography cannot be expected to compare more favorably to the moving boundary method than the moving boundary method does to itself; that is the inherent errors in the system of measuring mobilities make an error of ten per cent well within experimental accuracy.

At this point it becomes necessary to defend the concept of mobility lest it seem completely meaningless. Under defined conditions, that is, set buffer specifications, mobilities can be determined with a reasonable accuracy. Mobility measurements aid in determining the electrophoretic homogeneity of a system. They provide an excellent method for the determination of isoelectric points, or isoionic points. Also they enable one to study the effect of the buffer system upon a particular substance.

Mobility determinations on paper may be thought of from different aspects. McDonald and co-workers (51,53,54,55,56,57, 58,59,60,63,64,65,79,86) have emphasized the importance of ionographic mobilities. The criteria for ionographic mobilities is a linear movement with respect to both time and potential gradient. However, as demonstrated by Marbach (52) these are necessary but not sufficient to define ionographic mobilities. According to Marbach the mobility is dependent upon the ratio of electrolyte to paper as well as the factors of potential

gradient and time. Therefore, mobilities must, to be reported as mobilities, be determined under a known equilibrium of buffer to paper. The work to be presented here is in confirmation of this general hypothesis. Marbach (52) has given further emphasis to ionographic mobilities in his work to "convert", rather than correct as did Kunkel and Tiselius (39,83,84), these mobilities to unstabilized moving boundary mobilities. The distinction here is quite important since to correct indicates that the ionographic mobilities are wrong, which is far from the case, rather they are not in most cases identical with the free solution mobilities. It is part of the purpose of this dissertation to show that free solution mobilities can be achieved using the Ionograph without the necessity of using a conversion factor.

A few researchers have been interested in correlating ionographic mobilities with free solution mobilities. Kunkel and Tiselius (39) have attempted to correct paper electrophoretic mobilities to those obtained in non-stabilized electrophoresis. According to Kunkel and Tiselius the expression for field strength or potential gradient, is not equal to the current flow through the paper, divided by the cross sectional area times the specific conductivity. In other words they say that the potential gradient measured from one end of the paper strip to the other is not a "true" measure of the potential gradient. This basically is a contradiction of Ohm's law as pointed out

by Marbach (52). Marbach found that there is no need to explain this basic inequality, of Kunkel and Tiselius, by using "a tortuous channel". In their experiments Kunkel and Tiselius used the specific conductivity of the buffer solution instead of the specific conductivity of the buffer-paper system. Marbach on the other hand, used a specially constructed conductivity cell in which he measured the conductivity of the buffer-paper system. He regarded the decrease in conductivity of the buffer as a decrease in the thermodynamic activity of the buffer ions due to the presence of the paper fiber. That this hypothesis is sound, from a thermodynamic point of view, becomes evident when the effect of an increase in mass in a solution upon the electrochemical potential of the indifferent ions is considered. It should be mentioned here that the hypothesis of Kunkel and Tiselius and that of Marbach both work experimentally; however, the hypothesis proposed by Kunkel and Tiselius must be considered as an empirical conversion factor while that proposed by Marbach is on sound theoretical grounds. Another treatment of "mobility" which should be mentioned here is that of Macheboeuf, and associates (47,49,50) in which use is made of an apparatus of the type described by Durrum (14). Essentially this consists of two tumblers, covered with lucite plates which seal their tops and support an inverted L-shaped glass rod. The horizontal portion of the glass rod served to support the apex of the filter paper strips, which were draped symmetrically over it,

with the ends of the strip passing through slots in the Lucite covers and extending into the buffer solution in the tumblers below. A third tumbler was inverted to cover the paper draped over the glass rod and a spot of test solution applied to the paper at the apex. A potential was then applied across the carbon electrodes which were inserted directly into the tumblers containing the buffer solution. Instead of trying to eliminate or minimize evaporation of water from the buffer solution on the paper strip during the course of a paper electrophoresis experiment Macheboeuf attempted to regulate and use this phenomenon to advantage. As previously mentioned his apparatus was similiar to that described by Durrum (14) except that at the sides of the paper strip he replaced the top tumbler with a protecting shield which was perforated to allow regulation of evaporation. The top of this shield however, was left unperforated and was made of asbestos to absorb any water that condensed there; the purpose of this design was to offset the possibility of water falling back onto the paper surfaces as droplets, after it had once evaporated. Under these conditions, then, there are three factors which must be considered during the electrophoresis experiments: (1) $\underline{e}$, the electrophoretic displacement velocity of an ion with respect to the water that surrounds it, (2) e, the velocity of the displacement of water due to electroosmosis, and (3) E, the velocity, at a point x, of the displacement of water occasioned by evaporation.

Since water moves up into the paper from both ends to replace that which is evaporated, there is obviously a lateral line through the paper along which the liquid flow is zero. If there were no electroosmosis, this line would be midway between the two ends; the effect of electroosmosis, however, shifts the line of zero liquid flow toward the negative electrode. If V represents the total velocity of an ion with respect to the paper at a point x, then $V = \underline{e} + e + E$. If the pH of the buffer solution is higher than the isoelectric point of an amphoteric migrant, then the migrant will move toward the positive electrode, that is $\underline{e}$ is positive. As stated above the effect of e is negative and E is negative in that portion of the paper between the line of zero liquid flow and the positive electrode and positive between the zero flow line and the negative electrode. The value for e is constant, as is the value $\underline{e}$ for any particular species of migrant. The absolute value E increases proportionately as the distance from the line of zero flow. Thus, a sufficient condition, at any point x, for zero ion velocity with respect to the paper is $E + e = \underline{e}$. Under these conditions then, each protein species comes to rest at a characteristic point along the paper electrophoresis diagram, depending on the value $\underline{e}$ for each species. The value of this technique cannot be in obtaining mobilities since Macheboeuf's evaluation is certainly not what is usually meant by mobility;

however, it may have some value, though the advantages over the horizontal strip methods are few and the disadvantages are many.

The method used by the author to determine the ionographic mobilities reported in this dissertation is that of McDonald, and associates (53,58,62,63), or descriptively, it is a system of horizontal paper strips in a closed system. A photograph of the instrument is shown in figure 1. A schematic diagram of the instrument is given in figure 2. Briefly it may be described as follows; Paper strip, F. is held horizontally in frame, J; the end clamps, H, are movable, thus allowing for variable length paper strips or wide sheets; if necessary the end clamps may each be reversed in position and glass plates of any desired length placed on the frame. In any case, the ends of the paper strips or sheets dip into the buffer solution contained in the buffer vessels, E. These, in turn, are connected by means of the inverted U-tubes, C, to the electrode vessels, D. The U-tubes are filled with agar-stabilized electrolyte solution. Platinum electrodes, A, which are connected to a regulated source of electrical power, dip into the electrode vessels. The solution level in each of the buffer vessels, E, is maintained at a constant height throughout a run, by a glass siphon tube, L, of small bore whose ends dip into the buffer solution. (The siphon is optional and as will be shown later may well

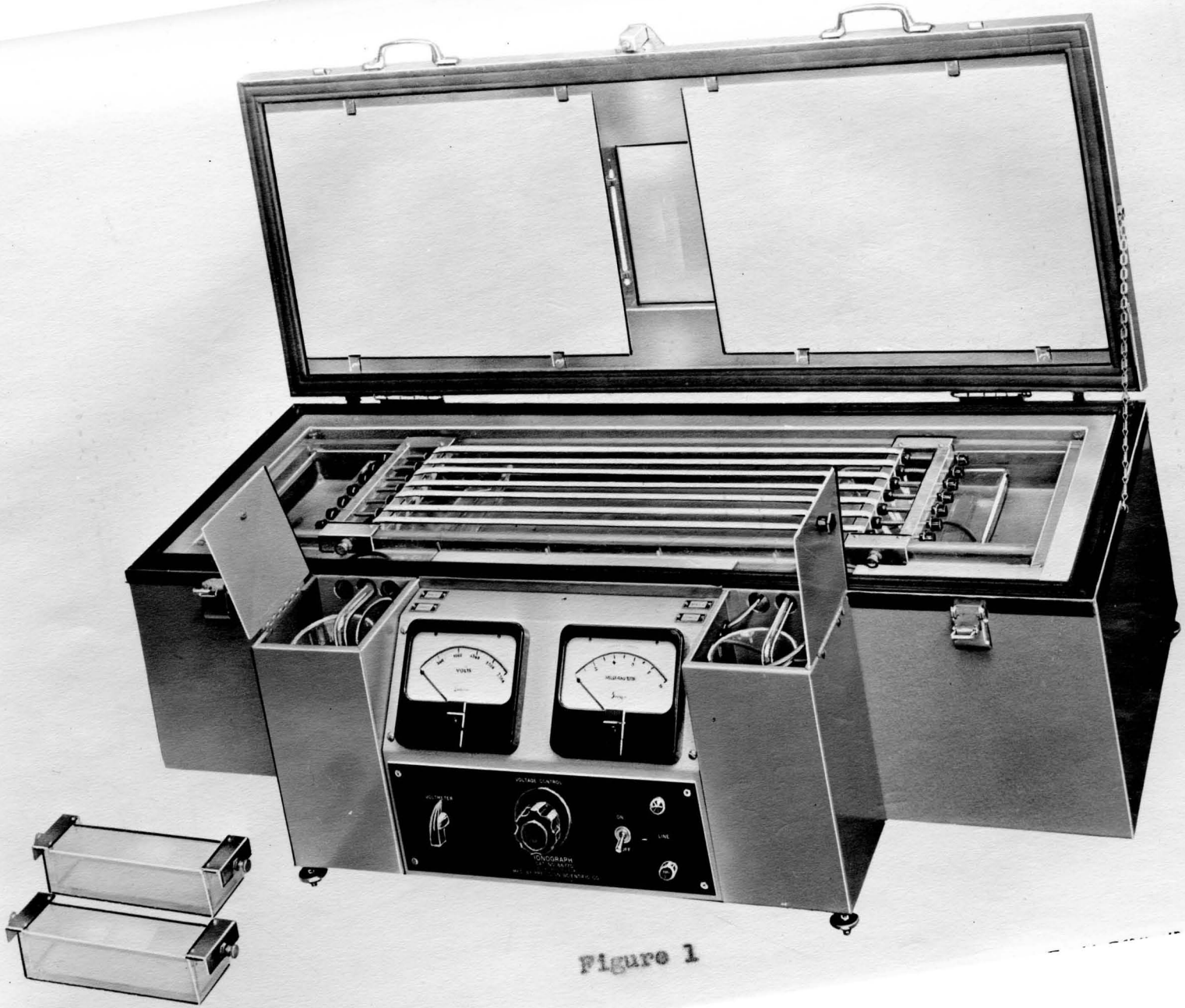


Figure 1

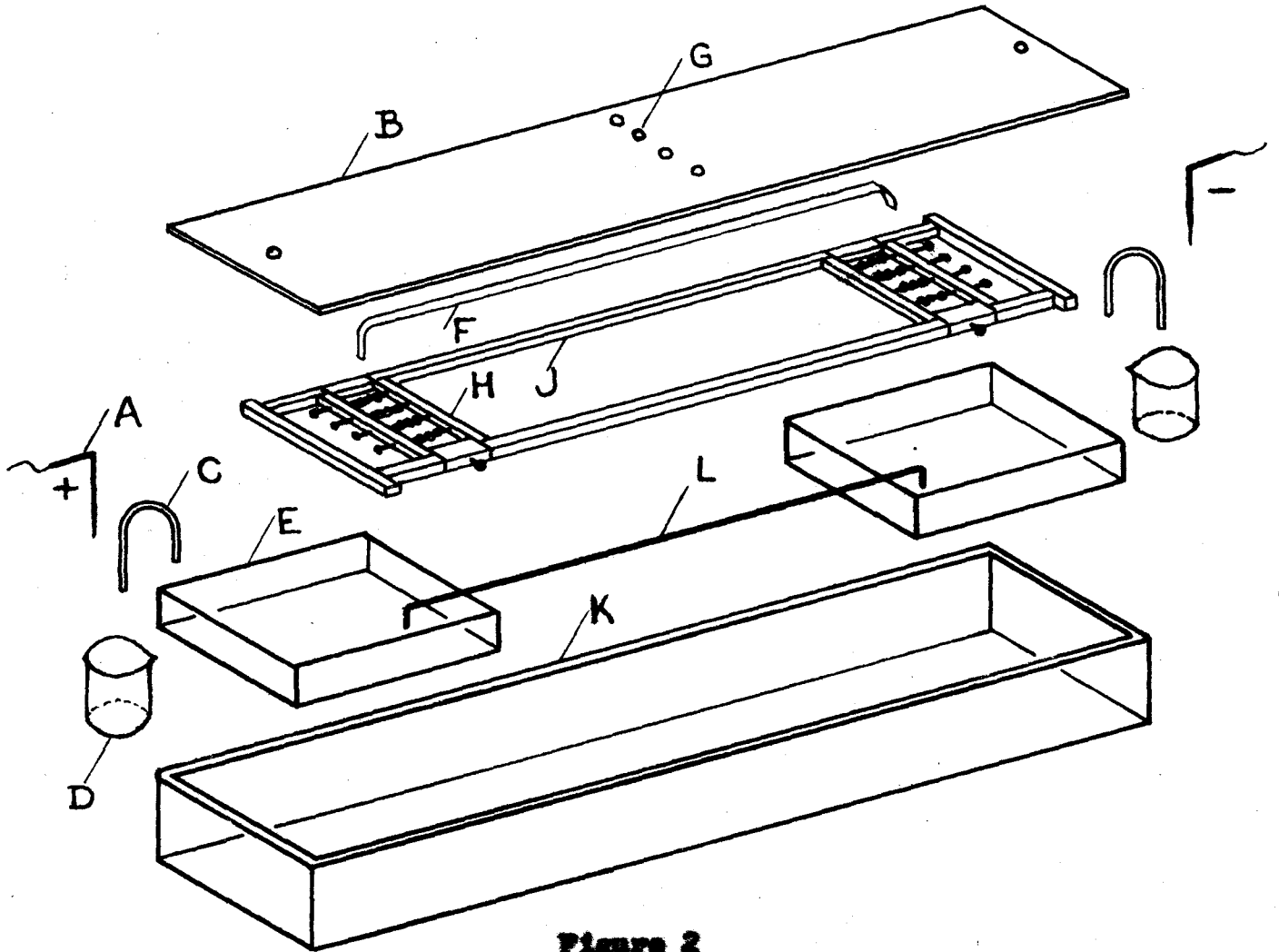


Figure 2

A Schematic Diagram of the Ionograph

prove to be an undesirable factor in mobility work. Marbach (52) has derived a phenomenological equation for the effect of the siphon, that is, his equation demonstrates the effect of hydrostatic pressure on electroosmosis. It would be expected that electroosmosis remains at a maximum with the siphon in place; without the siphon however, if sufficient time is allowed before beginning a run, for equilibrium - conditions to be reached as regards the moisture content of the paper - electroosmosis becomes a minimum). Except for the electrode vessels, D, and salt-bridges, C, all components of the apparatus are inclosed within the container, K, which is covered by the lid, B. The migrant is added to the paper strips as a thin streak at right angles to their length, by means of a micropipet. The open space within the container is maintained at a minimum by proper design of the apparatus, and by pouring water into the container, K, up to the level of the buffer vessels. Liquid can be circulated through the walls of the double walled chamber to maintain a constant temperature. The platinum electrodes and electrode vessels are separated by means of agar salt-bridges, from the buffer vessels into which the ends of the paper strip dip, thus protecting the migrating material from unfavorable pH changes due to accumulation of electrode products.

Normally for ionographic mobilities the criteria that must be satisfied are that the section of filter paper through which

the migrant moves must be reasonably uniform with respect to water sheath, ionic composition, potential gradient and temperature. An indication of sufficient uniformity, as regards these factors is a consistent milliammeter reading, as well as a linear movement of the migrant with time. For low ionic strength, that is 0.01 or less, this method of obtaining mobilities is excellent. However, to approach free solution mobilities these conditions are not sufficient, since at low ionic strength values of the buffer, the factors which tend to make ionographic mobilities differ from free solution mobilities become larger. An example of this is the fact that as the ionic strength is lowered, electroosmosis tends to become larger, or the proportionate effect of the paper on the indifferent ions becomes greater in these solutions.

Therefore, with the above in mind samples of various proteins, along with their free solution electrophoresis patterns , were procured from Armour and Company, Chicago, Illinois.

Hereafter in this dissertation these mobilities will be refered to as the Armour values, rather than using the sesquipedalian phrase of free solution electrophoretic values. The proteins obtained were; bovine serum albumin, Armour value 6.66×10^{-5} cm/sec/volt/cm, in veronal buffer, pH 8.6, ionic strength 0.1, and run at $4^{\circ}C$; beta lactoglobulin, Armour value 5.62×10^{-5} cm/sec/volt/cm, in phosphate buffer, pH 7.7, ionic

strength 0.2, and run at 4°C; egg albumin, Armour value 5.69 X 10^{-5} cm/sec/volt/cm, in phosphate buffer, pH 7.7, ionic strength 0.2, and run at 4°C; bovine gamma globulin, Armour value 1.54 X 10^{-5} cm/sec/volt/cm, in phosphate buffer, pH 7.7 ionic strength 0.2, and run at 4°C.

An important factor in determining mobilities is the wattage, that is, amperes times volts, which must be dissipated along the strip usually either by convection, or evaporation. With a desire to keep the wattage factor low an operating potential gradient of two volts per centimeter was chosen. The buffer conditions were essentially the same as those used in the free solution determinations and the temperature was 4°C. The length of the strips from buffer vessel to buffer vessel was fifty centimeters. No great variation in strip wetness was noted as the ratio of buffer to paper (E and D number 613) remained constant within experimental error giving a ratio of 2.20 to 1.00. However, it was noted that the migrants did not move linearly with time, for as the length of an experiment increased the movement per unit time interval decreased with time. Now if evaporation were too great it would be expected that a decrease of mobility with time would result. The decrease in mobility would not be expected to be a linear function with respect to time. Nevertheless, on ploting the mobility, figure 3, for bovine serum albumin it was found that a fairly linear

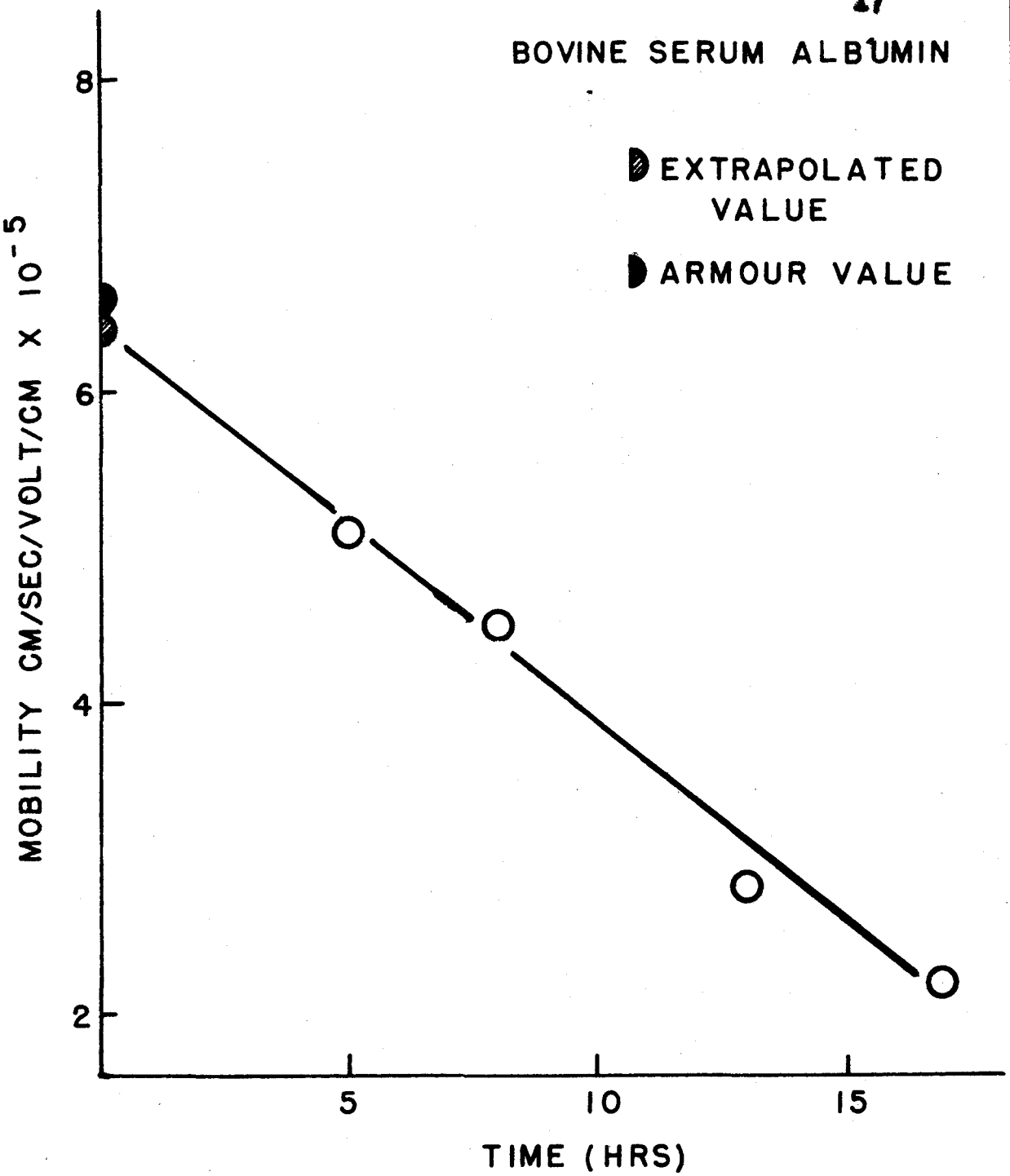


Figure 3

Extrapolated Mobility for Bovine Serum Albumin

relationship resulted up to twenty eight hours. From a theoretical consideration it was now thought that an extrapolation of mobility versus time to zero time might approximate the Armour value.

Briefly, the reasoning was this: the decrease in mobility with time would most probably be due to evaporation and a washback effect of buffer coming up the paper strip from the buffer vessels. Evaporation necessitates a drying effect, if the buffer evaporated is not replaced by an equivalent amount of buffer from the end vessels. Now since no great change in the "buffer to paper" ratio took place, it could be said that while evaporation occured the effect was such that the evaporated water was replaced by an equivalent amount of water from the end vessels. Replacement of the evaporated water definitely seems to have taken place, since a fairly linear relationship resulted in plotting mobility against time. Thus the extrapolation to zero time gave an ionographic mobility of 6.40×10^{-5} cm/sec/volt/cm, which most certainly is within experimental error of the 6.66×10^{-5} cm/sec/volt/cm given by Armour. Next, a phosphate buffer system of pH 7.7, ionic strength 0.2 was used, to study egg albumin, beta lactoglobulin, and gamma globulin. The experiments were run at 4°C using a paper strip length of fifty centimeters. Again as was noted in the case of bovine serum albumin a non linear relationship of movement

with time resulted. However, a plot of the mobility of these proteins against time gave fairly linear plots, with the exception of gamma globulin whose mobility is too low to give any accurate results in an experiment of this type. The extrapolation of the mobility versus time curve, figure 4, of egg albumin gave an ionographic mobility of 5.88×10^{-5} cm/sec/volt/cm which was within the experimental error of Armour's value of 5.69×10^{-5} cm/sec/volt/cm. In the case of beta lactoglobulin the extrapolation of the mobility versus time curve, figure 5, gave an ionographic mobility of 5.80×10^{-5} cm/sec/volt/cm which once again was certainly within the experimental error of Armour's value of 5.62×10^{-5} cm/sec/volt/cm. A comparison of the extrapolated ionographic values with those obtained by Armour are given below.

Protein	Armour Mobility in cm/sec/volt/cm	Extrapolated Ionographic Mobility in cm/sec/volt/cm
Bovine Serum Albumin	6.66×10^{-5}	6.40×10^{-5}
Beta Lactoglobulin	5.62×10^{-5}	5.88×10^{-5}
Egg Albumin	5.69×10^{-5}	5.80×10^{-5}

The major result of these experiments was a demonstration of the feasibility of getting free solution mobilities using the Ionograph. It is reasonable to assume that if one can extrapolate back to zero time and achieve free solution mobilities

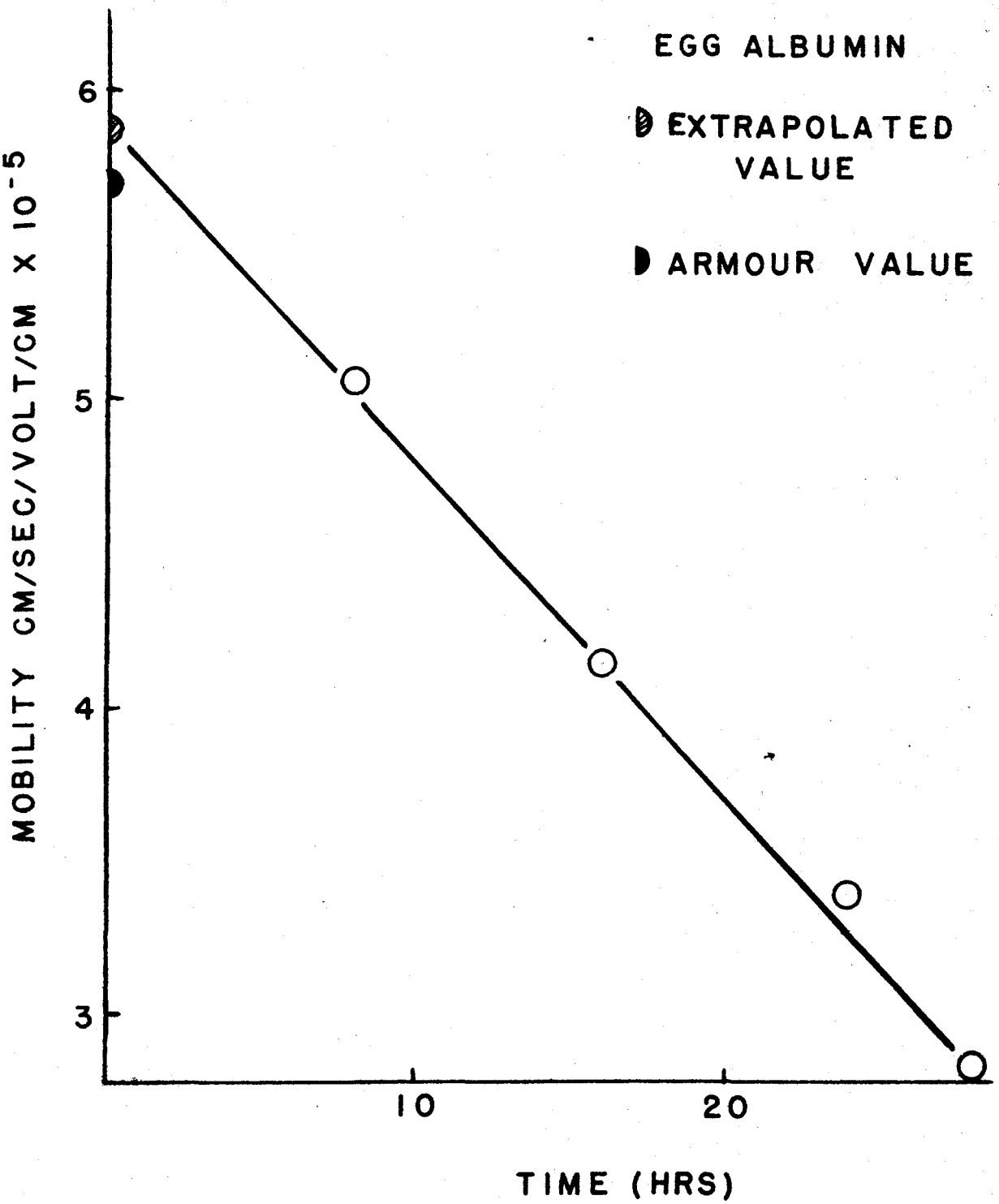


Figure 4

Extrapolated Mobility for Egg Albumin

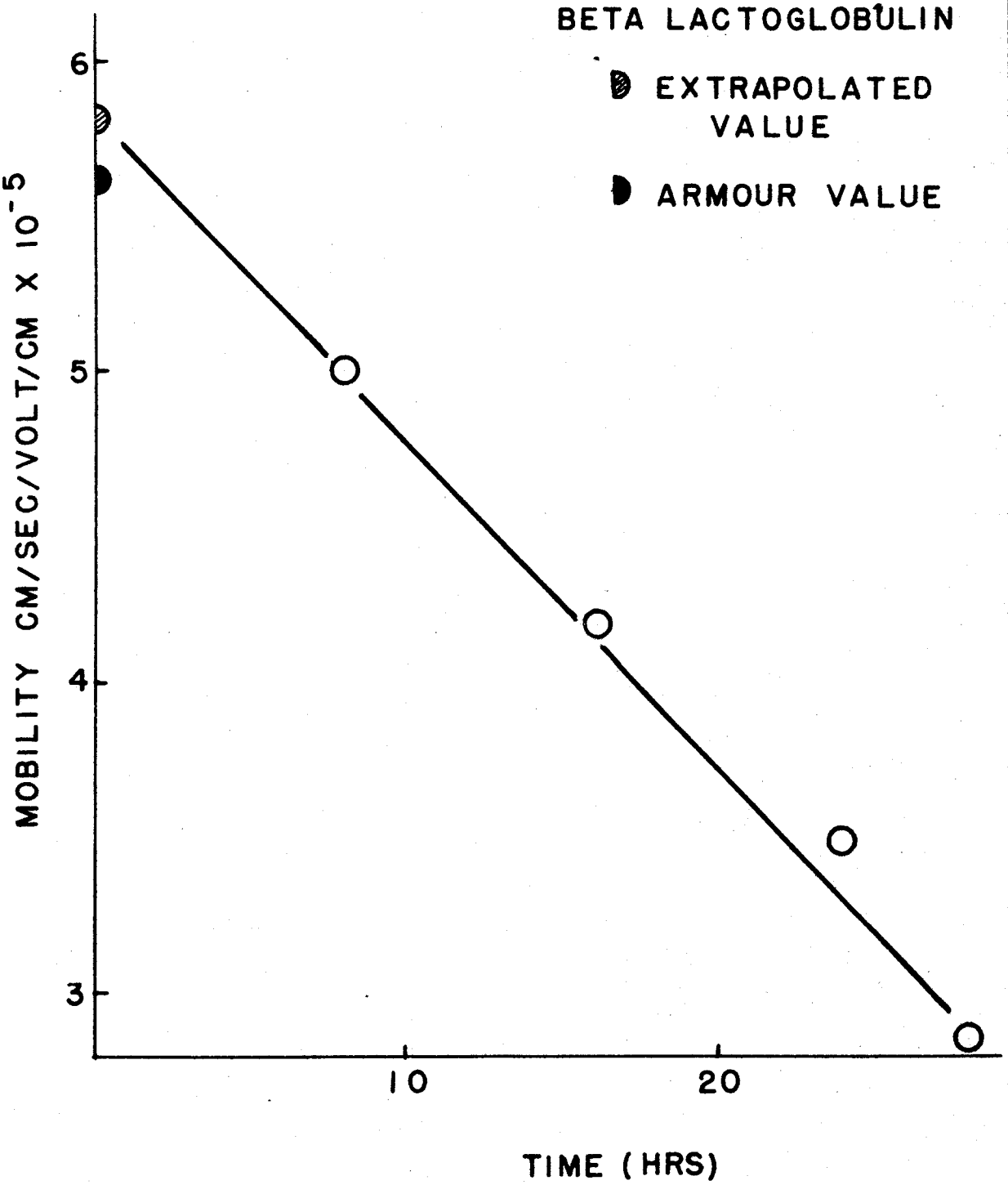


Figure 8

Extrapolated Mobility for Beta Lactoglobulin

then perhaps conditions exist which allow the calculation of these mobilities directly. It was thought that two factors would aid in achieving free solution mobilities. Firstly, a decrease in the wattage expended which would be accomplished by decreasing the voltage since the wattage is equal to the product of the voltage and amperage. Secondly, a decrease in the length of the paper strips was made. As to the first factor of wattage, a decrease of one quarter in voltage would result in one quarter amperage decrease or a one sixteenth decrease in wattage. This of course means a decrease in the evaporation. Therefore, the replacement of evaporated buffer is a minimum as is its effect on the mobility of a migrant. As to the second factor, shortening the paper strips from fifty centimeters to twenty centimeters means an added decrease in the washback effect. This is so since less water will travel up the strip simply because there is less strip to keep wet. These conditions are empirically defined, since it required several unsuccessful experiments before they were achieved.

Under the condition of a potential gradient of one half volt per centimeter, using a veronal buffer pH 8.6, ionic strength 0.1, at 4°C and allowing an equilibrium time of twelve hours the migration of bovine serum albumin was studied. The passage of sufficient time for equilibrium to be established was found to be critical; twelve hours was chosen partly for

convenience, since it permits an experiment to be set up and to equilibrate over night. The time of the experiment was varied from six, twelve, eighteen, and twenty four hours. From the results it was evident that the movement was linear with time, giving a mobility for bovine serum albumin of 6.94×10^{-5} cm/sec/volt/cm. This result was well within the experimental error for Armours value of 6.66×10^{-5} cm/sec/volt/cm. It should be stated here that the variation in ionographic mobilities for all the proteins studied was about 0.30×10^{-5} cm/sec/volt/cm, which is much better than the ten per cent error found in calculating mobilities in free solution electrophoresis, since the deviation would in this case be 0.67×10^{-5} cm/sec/volt/cm. The experimental conditions for the beta lactoglobulin studies were a phosphate buffer pH 7.7, ionic strength 0.2, at 4°C and allowing an equilibrium time of twelve hours. The time of the experiment was varied from six, twelve, eighteen, and twenty four hours with a resulting linear movement of the migrant with time. The calculated mobilities gave 5.60×10^{-5} cm/sec/volt/cm, with a deviation of 0.39×10^{-5} cm/sec/volt/cm. The Armour value is 5.62×10^{-5} cm/sec/volt/cm. The fact that these particular values are so very close must necessarily be considered fortuitous since the experimental error involved in any method of measurement would belie such reproducibility. The next protein studied was egg albumin in a phosphate buffer,

pH 7.7, ionic strength 0.2, at 4°C and allowing an equilibrium time of twelve hours. Experiments were run varying the time from six, twelve, eighteen, and twenty four hours with a resulting linearity of movement with time. Mobilities calculated for egg albumin were 5.86×10^{-5} cm/sec/volt/cm, with a deviation of 0.30×10^{-5} cm/sec/volt/cm. This also compared well with the Armour value of 5.69×10^{-5} cm/sec/volt/cm. The next protein studied, namely gamma globulin, presented the real challenge. Of all the materials investigated this was expected to present the greatest difficulty. Gamma globulin was studied in a phosphate buffer, pH 7.7, ionic strength 0.2, at 4°C, allowing an equilibrium time of twelve hours. Due to its small movement, twenty four hour runs were the only ones made on it. A mobility of 1.51×10^{-5} cm/sec/volt/cm, with a deviation of 0.30×10^{-5} cm/sec/volt/cm, was obtained; this compared favorably with Armour's value of 1.54×10^{-5} cm/sec/volt/cm. A comparison of the direct ionographic values with those obtained by Armour are given below.

Protein	Armour Mobility in cm/sec/volt/cm	Direct Ionographic Mobility in cm/sec/volt/cm
Bovine Serum Albumin	6.66×10^{-5}	6.94×10^{-5}
Beta Lactoglobulin	5.62×10^{-5}	5.60×10^{-5}
Egg albumin	5.69×10^{-5}	5.86×10^{-5}
Bovine Gamma Globulin	1.54×10^{-5}	1.51×10^{-5}

In summary, the mobility of four proteins, bovine serum albumin, beta lactoglobulin, egg albumin, and gamma globulin were determined by means of the ionographic technique. It was found that under certain specific conditions the mobility values agreed, within experimental error, with those determined by the moving boundary method in non-stabilized electrolytes.

Actually the results make the method appear much better than it really is. It is a long procedure, requiring anywhere from twenty four to thirty six hours to determine mobilities. It must be carried out at around four degrees centigrade and the attainment of equilibrium conditions as regards the moisture content of the paper strip, before applying the migrant, seems to be absolutely necessary, The length of the strip, twenty centimeters, has certain advantageous features, in as much as it is a very convenient size to work with on the Ionograph. However, the real problem is the "wetness" of the strip. At present the strips are wetted by allowing the buffer solution to flow on the strip from a pipette. This procedure is certainly not ideal, since the wetness from strip to strip will vary regardless of the care taken in applying the buffer. Of course the equilibration time of twelve hours allows the equilibrium wetness - a ratio of buffer to paper, of 2.20 to 1.00, by weight - to be approached. A series of criteria are given here which must be met if meaningful mobilities are to

be calculated using this variation of the ionographic technique. (1) The buffer conditions such as pH, ionic strength, and proper temperature must be right - as described earlier in this chapter. (2) The voltage must be low, so as to reduce evaporation to a minimum. The voltage given here, of one half volt per centimeter, may be an upper limit, as a voltage of one volt per centimeter has been shown to be too high. Enough time must be allowed for the experiment, to permit sufficient migration, in order to avoid potentially serious errors in measuring the distance of migration. (3) The strips must be brought to a proper wetness by a good procedure of wetting, and a sufficient equilibration time. The criteria for determining proper wetness are: (a) a check of the constancy of the "buffer to paper" ratio, which should remain constant; (b) the mobilities to be achieved on three distinct portions of each strip. This is done in the following manner. The strip is marked at a point midway between the buffer vessels, and at points three and a half centimeters on either side of the midway point. Migrant is applied at all three points and if the migration distance of all points is not within experimental error the determination is discarded. The reason for discarding these experiments is that there is obviously another factor affecting the movement besides the potential gradient. In most of the experiments which were discarded the migrants from the two end points would move in

toward the midway point; this can be interpreted only as a water shift, which most certainly invalidates any calculation of mobility. The most glaring weakness in the method is the necessity for maintaining the level of wetness above a certain critical level.

A study was also made on the effect of the nature of the paper on the mobility of a migrant. Three types of paper were studied, namely Eaton and Dikeman numbers 613 and 248, and Cremer and Tiselius Munktells. The buffer to paper ratios were calculated in the following manner. The ionograms, were allowed to equilibrate from two to twenty four hours. The paper strip was then removed from the Ionograph and weighed on an analytical balance. The weighing bottle and strip were then dried in an oven at 110°C for three hours. The weighing bottle was cooled and weighed again; subtraction of this weight from the first value gave the amount of water contained in the paper strip. The dried paper strip was then weighed in another weighing bottle of predetermined weight. By dividing the weight of the water by the weight of the paper the wetness ratios were calculated. The following were the ratios:

Paper	Ratio
Eaton and Dikeman number 613	2.20
Eaton and Dikeman number 248	1.80
Munktells, Cremer and Tiselius	2.20

No large variations were found in the wetness after a two hour equilibration time. The deviation was found to be about plus or minus 0.05 in the ratio of buffer to paper. These results cannot be interpreted as indicating that a two hour equilibrium time alone is a sufficient criterion on which to initiate mobility studies. An additional criterion, namely "uniform movement of the migrant in three different spots on the strip" must also be met. It was interesting to note that the uniformity of the wetness, as the portion of the strip cut out to calculate the wetness ratio was varied, held rather well. In some cases smaller portions of paper were removed and no serious variation resulted when comparing these ratios with those from longer portions of the paper strip. The buffer conditions for all strips were the same, namely, a veronal buffer, pH 8.6, ionic strength 0.1, and the temperature of the experiment 4°C. The total length of the strips, from buffer vessel liquid level to buffer vessel liquid level, was twenty centimeters. The potential gradient was one half volt per centimeter.

After determining the wetness ratios for these different papers, a study of the mobility of bovine serum albumin on these same papers was undertaken. The bovine serum albumin had an Armour value of 6.66×10^{-5} cm/sec/volt/cm. The duration of the experiments were varied from six, twelve, and eighteen hours, with all papers yielding a linear movement with respect

to time. The results of these determinations were:

Paper	Ratio	Mobility in cm/sec/volt/cm
Eaton and Dikeman number 613	2.20	6.94×10^{-5}
Eaton and Dikeman number 248	1.80	3.90×10^{-5}
Munktells, Cremer and Tiselius	2.20	6.94×10^{-5}

It is not the purpose of this dissertation to make a definitive determination of the effect of the effect of wetness, since Marbach (52) has made a comprehensive study of this subject. However, it is interesting to note that the wetness ratio of the Eaton and Dikeman number 613 and the Cremer and Tiselius Munktells were the same and the mobility values of bovine serum albumin obtained when using these papers were also alike. On the other hand, the Eaton and Dikeman number 248 had a comparatively lower wetness and showed a correspondingly lower mobility value.

The wetness factor in relation to mobility is an important one, and one which has for the most part been overlooked. It is obvious that mobility measurements depend heavily upon the wetness ratio. What the lower limit of this ratio is would be very interesting to know, but its determination seems next to impossible since almost any method used is fraught with error. It appears to be a case of a cat chasing its tail. Regardless of this the most important conclusion to be reached here is that

a proper wetness can be obtained using certain paper, thus affording conditions under which mobilities can be calculated which compare favorably with those determined in non-stabilized electrolytes.

CHAPTER III

THE AUTOMATIC SCANNING RECORDER

This chapter will concern itself with a discussion of the recording scanner itself, and the following chapter will deal with the theory, literature, and use of recording scanners.

A photograph of the Automatic Scanning Recorder manufactured by W.M. Welch Co., Chicago, Illinois, is shown in figure 6. Essentially the unit consists of six components: (1) a Bausch and Lomb grating monochromator; (2) the Welch blue sensitive photoelectric probe; (3) the Welch designed motor driven feed system made to accommodate paper strips; (4) the Welch Densichron amplifying unit; (5) the Welch logarithmic amplifier; and (6) a Brown Electronik strip chart recorder.

The unit was specially designed to make direct measurements of materials on filter paper strips. The instrument will be described here, with the emphasis being on the features which make it particularly adaptable for the measurements it was designed to make. The first unit of the instrument is the Bausch and Lomb grating monochromator. The unit is made so that different light sources can be used, such as a mercury arc

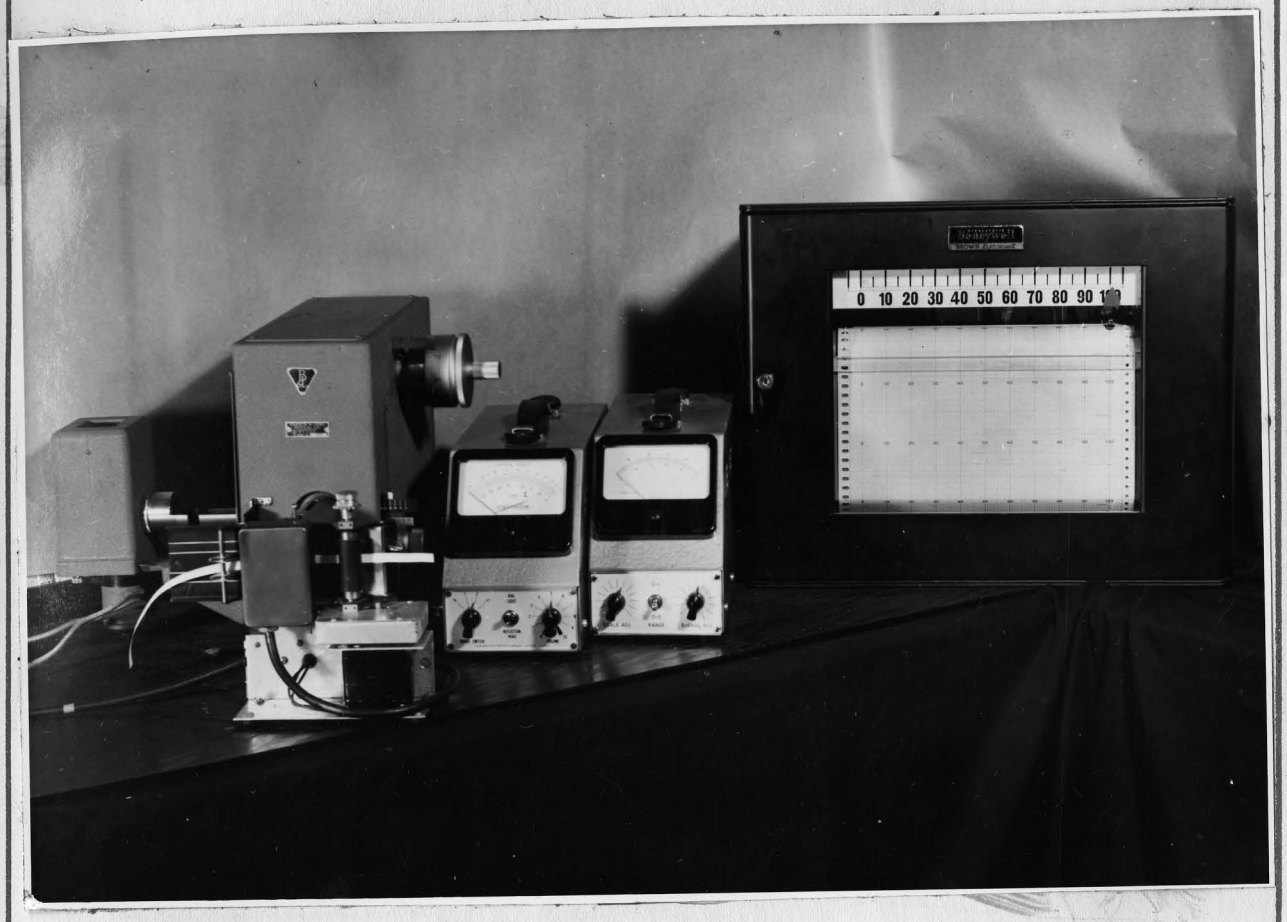


Figure 6

The Automatic Scanning Recorder

or a tungsten filament. An entrance slit permits adjustment of the width of light entering the grating system; it can be varied from zero to one centimeter. The grating in the instrument is of the reproduction type blazed for the ultraviolet of the first order. There is overlapping of orders which is characteristic of any grating system. All lenses in the system are quartz and therefore transmit energy throughout the scale range. As a wave length control allows a chopping of the spectrum, it should be zeroed and calibrated. The exit slit permits adjustment of the band width of the spectral lines desired. The purity of the light emerging from the exit slit is controlled by the basic dispersion of the system and the widths of the slits being used. One hundred per cent purity can be achieved only if a discontinuous light source is used, such as a mercury arc source. If a continuous source is used one hundred per cent purity can never be achieved, as in the case of the tungsten filament source. Upon leaving the grating system the light is then focused through a quartz lens on a slit of six millimeters in length and one millimeter in width. The paper is guided past this slit, whose slit length permits the use of paper of eight millimeters width. A motor-driven feed system pulls the paper past the slit, the motor speed is mechanically synchronized with the rollers of the strip chart recorder. Holding the paper against the slit is the Welch blue sensitive photoelectric probe. The

photoelectric probe is the heart of the instrument and requires special consideration.

One of the difficulties associated with the use of photoelectric cells as radiation detectors results from the amplification of the direct current, DC, space current which flows between the electrodes of the cell. The current may be as low as 10^{-12} amperes and it is directly proportional to the light flux incident upon the cathode of the phototube. If DC amplification is used there results an instability caused by a zero drift which makes frequent recalibration necessary. The problem then, is to modulate the space current so that alternating current, AC, amplification may be used, since AC amplification is very stable. The Densichron magnetic-modulation system conveniently provides a solution for the problem of amplification by modulating the space current through the use of an alternating magnetic field. Essentially the system is this. A quantity of light will strike the cathode of the phototube and release a proportionate number of electrons. These electrons will flow steadily to the anode if the source is constant, but in order to use an AC amplifier they must be converted to an AC signal. The phototube is, therefore, placed in a magnetic field produced by an alternating current. The result is that when the magnetic field is a maximum, the electrons will not flow in the phototube from the cathode to the anode. Thus an alternating current

output results from the phototube which is directly proportional to the light intensity. This in turn permits the use of an AC amplifier of extremely high gain, thus avoiding the instability of a DC amplifier.

The AC current from the phototube is then amplified by the Welch Densichron amplifying unit. Now since the light being transmitted by the phototube, due to Beer's law, is a logarithmic function, the next stage of amplification is a logarithmic amplifier. This is another important feature of this instrument. The logarithmic amplifier changes the logarithmic function to a linear function. Lastly, the amplified current is fed from the logarithmic amplifier to the Brown Electronik strip chart recorder.

The purpose for which the instrument has been developed is to relate the concentration of a material on filter paper with the optical density. Briefly from Beer's law:

$$\log \frac{I}{I_o} = -kc$$

where,

I is the amount of light passing through the substance into the photoelectric tube.

I_o is the amount of light incident upon the system

$-k$ is a constant which is a function of wavelength, and of the length of the path of light as it passes

through the substance

c is the concentration of the substance through which the light passes.

It would be expected that a linear relationship would result between optical density, $-\log I/I_o$, and concentration c. However, it should be remembered that Beer's law is a limiting law, having its best results in dilute solution, and depending for its applicability in all solutions upon the homogeneity of the system through which the light passes. It was with these facts in mind that the instrument was to be tested as to its ability to respond to a homogeneous system.

The system chosen to check the instrument was as follows. Cellophane which was homogeneously coated with gelatin was procured from Ansco, Binghamton, New York, emulsion number C 18-628-J3. The gelatin was then cut into a section eight inches by five inches. This section was then dyed in a 1% solution of brom phenol blue dissolved in 95% ethyl alcohol which is saturated with mercuric chloride. The dyed gelatin was then washed using a saturated solution of mercuric chloride as the wash and keeping the brom phenol on the acid side by washing with dilute hydrochloric acid. After the gelatin seemed to retain a certain minimum amount of color, noted by the fact that no brom phenol appeared in the wash solution it was air dried. After drying the section was cut into strips seventeen

centimeters long and one centimeter wide; this gave twelve strips of these dimensions. The strips were then checked on the automatic scanner for homogeneity of dye uptake by the gelatin. It was noted that the optical density of a single strip was 0.1 optical density units. The consistency of the readings on each strip and from strip to strip was very good. It was of course fortuitous that the optical density for each strip came out exactly 0.1 optical density units. Now since each strip was homogeneous it was possible to test the ability of the instrument to measure a homogeneous system by placing the strips behind each other and noting the response of the unit. The results of this experiment are shown in figure 7. The instrument responded excellently for as each additional strip was added the optical density increased 0.1 optical density unit. It could now be concluded that the instrument, if used for a homogeneous system, could be expected to respond in such a manner as to obey Beer's law.

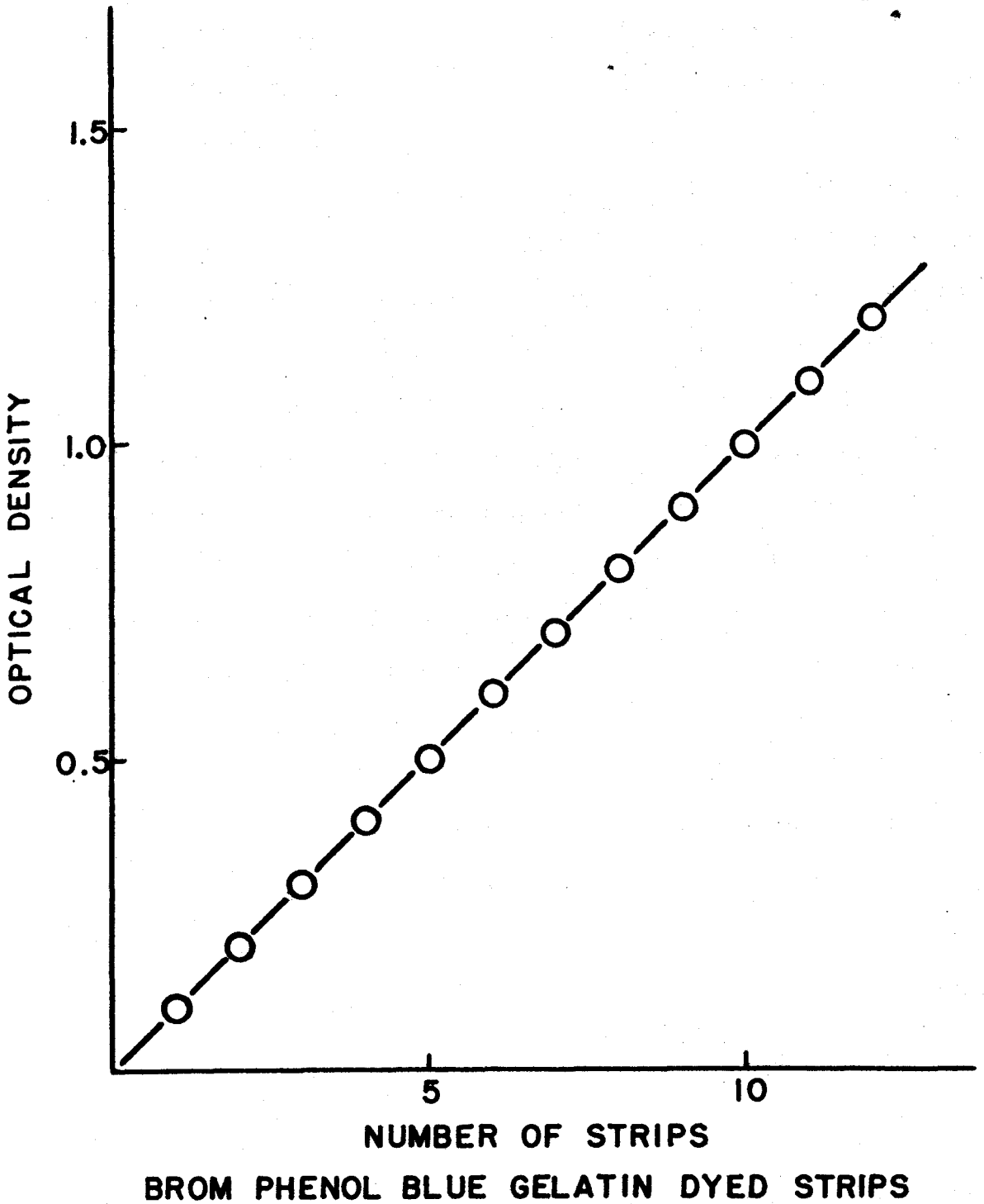

1.5
1.0
0.5
OPTICAL DENSITY
5
10
NUMBER OF STRIPS
BROM PHENOL BLUE GELATIN DYED STRIPS

Figure 7

CHAPTER IV

APPLICATION OF THE AUTOMATIC SCANNER

The first investigation undertaken with the automatic scanning recorder was to determine a suitable paper for the studies to be made subsequently. Four papers and glass fiber ribbon (Fiberglas woven tape, continuous filament, tight-weave, B; 3/8 inch width; 0.007 inches thickness) were studied first microscopically, and then as to the optical density variation on the scanner. The materials were Cremer and Tiselius Munktell, Eaton and Dikeman numbers 248,613, and 950, and the glass fiber of a tight weave. The microscopic examination showed the glass fiber to have serious holes where the horizontal weave crossed the vertical weave. Cremer and Tiselius Munktell being a thick paper looked very good under the microscope, as did Eaton and Dikeman numbers 248, and 950. Eaton and Dikeman number 613 showed some holes in the paper under the microscope though they did not seem to be abundant. Strips of these materials were now run through the scanner to observe what variations occured in optical density due to the stabilizing medium itself. The Cremer and Tiselius Munktell and Eaton and Dikeman number 613

showed about the same deviations, namely 0.02 optical density units. The glass fiber ribbon was excellent showing practically no variation in optical density. The Eaton and Dikeman number 950 was the best of the papers tried, but unfortunately it binds brom phenol blue readily and therefore had to be discarded as a possible stabilizing medium. The Eaton and Dikeman number 248 was by far the worst material studied since it showed variation in optical density of 0.04 optical density units. It would seem that the two best materials, from a practical point of view were the Cremer and Tiselius Munktells and the Eaton and Dikeman number 613. The Eaton and Dikeman number 613 was chosen as the stabilizing medium due to its availability in rolls of eight millimeters width, while the Munktells came in sheet form necessitating the use of a paper cutter to achieve the desired widths. This could later be a source of error when measuring the concentrations of various materials on the strips, since the width would not always be exact enough, and would, therefore, cause variations in the amount of migrant apparently present.

Since the automatic scanning recorder was found capable of responding to a homogeneous system, it was unfortunate that the practical systems which were to be studied and which were stabilized with paper were not homogeneous in nature. This first became apparent when a solution of one gram per cent of brom phenol blue and various serial dilutions of it were

studied. The results of this experiment are demonstrated in figure 8. It should be obvious that the system does not conform to Beer's law and thus Beer's law should not be applied in this instance. An observation of this curve would seem to indicate that second order effects are important especially above an optical density of 0.4. As the concentration of brom phenol blue increases the reaction of photons does not apparently occur molecule for molecule of brom phenol blue due to second order effects. It may be said that the current in the phototube is not proportional to the concentration. It was felt that perhaps some relationship could be derived which would take into account the second order effects. The following hypothesis is prosposed.

$$\text{the rate of absorption} = kc\beta \qquad (1)$$

where,

k is a constant of proportionality

c is the concentration of the material

β is the fraction of light transmitted

However, we know that if β is the fraction of light transmitted then $1 - \beta$ = the fraction of light absorbed, α. Now equation (1) may be rewritten

$$\text{the rate of absorption} = kc(1 - \alpha) \qquad (2)$$

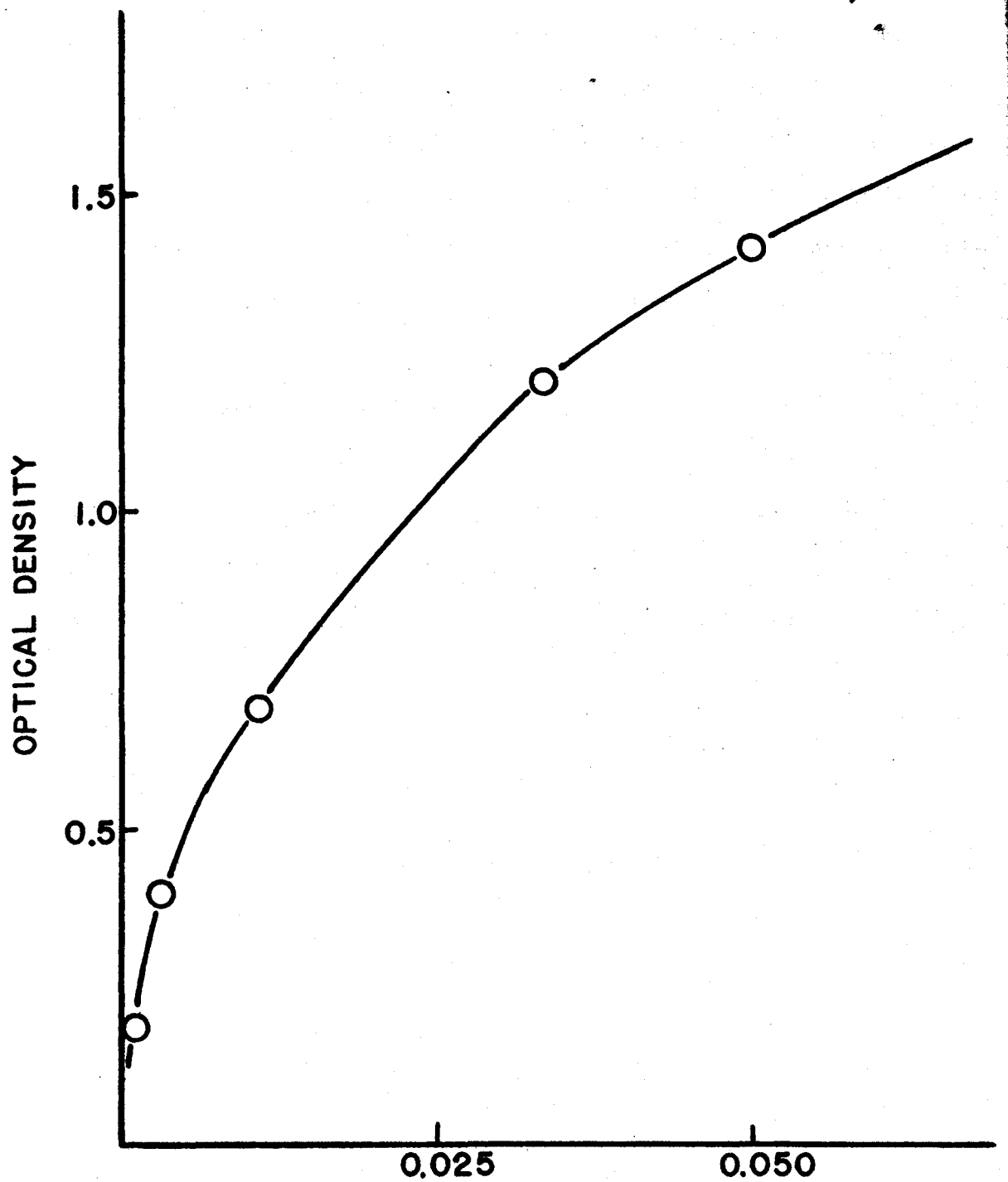

1.5
1.0
0.5
OPTICAL DENSITY
0.025
0.050
CONCENTRATION GRAM %
BROM PHENOL BLUE

Figure 8

This equation merely states that the rate of absorption of photons is a function of the concentration and the transmitted light. Now the activated molecules will dissipate the energy imparted by the photon as kinetic energy. And we may say that the:

$$\text{rate of dissipation} = k'\alpha \qquad (3)$$

where k' is a constant of proportionality. Or more simply that the rate of dissipation is dependent upon the fraction of light which has been absorbed.

In the steady state condition, the rate of absorption is equal to the rate of dissipation or:

$$k'\alpha = kc(1 - \alpha) \qquad (4)$$

$$k'\alpha = kc - kc\alpha \qquad (5)$$

rearranging (5) and factoring α

$$\alpha(k' + kc) = kc \qquad (6)$$

or,

$$\alpha = \frac{kc}{k' + kc} \qquad (7)$$

However, since α is the fraction which is absorbed then:

$$\alpha\ 100 = \% \text{ absorption} = a \qquad (8)$$

substituting a/100 for α then:

$$a = \frac{100\ kc}{k' + kc} \qquad (9)$$

multiplying through by $\frac{1/k'}{1/k'}$:

$$a = \frac{100\ k/k'\ c}{1 + k/k'\ c} \qquad (10)$$

Since both k and k' are constants we may say that $k/k' = K$ and equation (10) becomes:

$$a = \frac{100\ Kc}{1 + Kc} \qquad (11)$$

Equation (11) is an equation for a hyperbola and may be made linear by the following method. Taking the reciprocal of (11):

$$\frac{1}{a} = \frac{1 + Kc}{100\ Kc} = \frac{1}{100K}\frac{1}{c} + \frac{1}{100} \qquad (12)$$

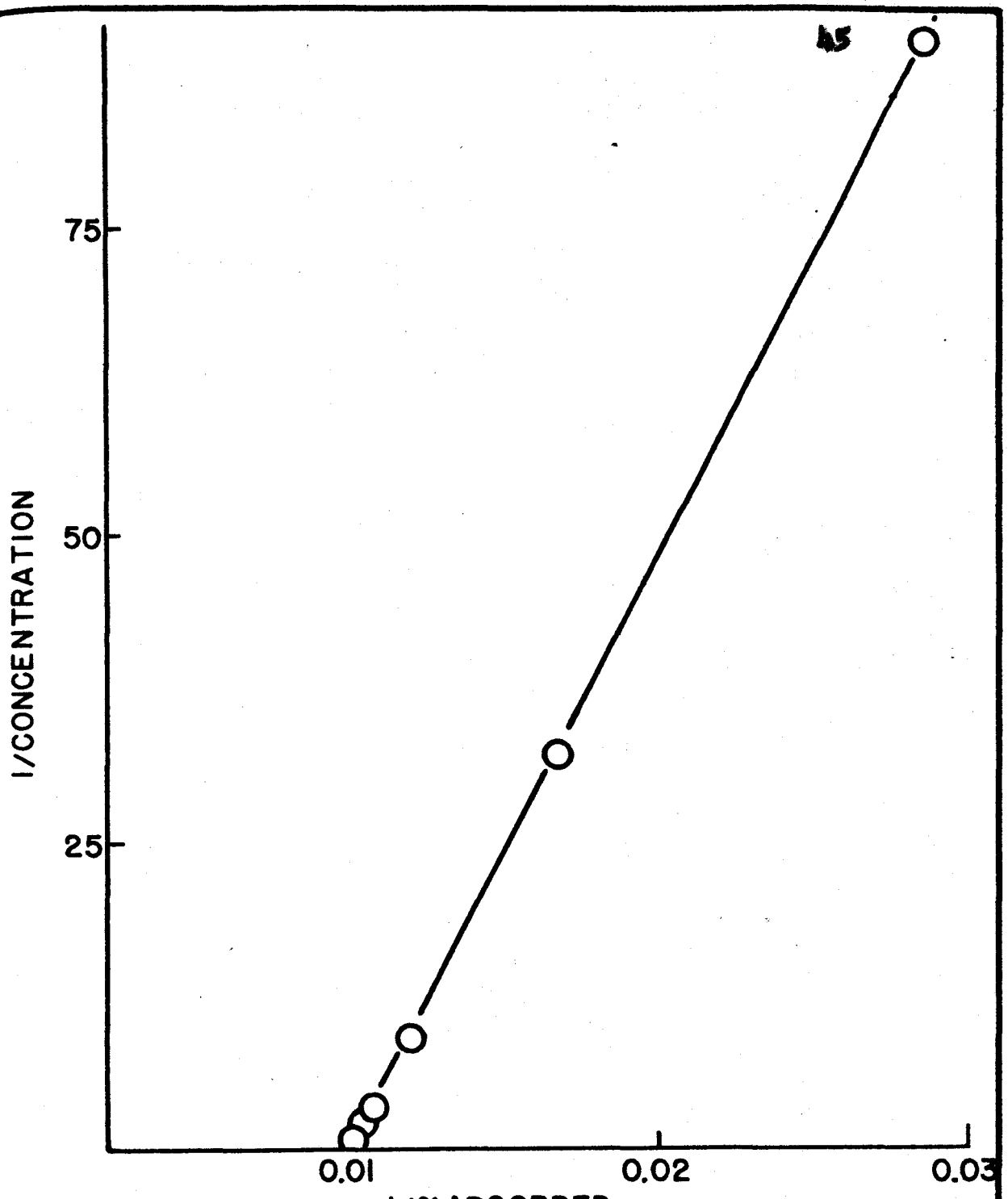
$$y = \quad m \quad x + \quad b$$

Therefore, a plot of 1/a versus 1/c should give a straight line having a slope of 1/100K and an intercept of 0.01. This equation fits the actual data of the brom phenol blue absorption very well, as shown in figure 9. As seen in the graph the intercept of the curve is 0.01. Another manner in which the curve may be made linear is to multiply equation (12) by c:

$$c/a = 0.01c + 1/100K \qquad (13)$$

$$y = m\ x + \quad b$$

A plot of this equation with c/a plotted against c gives a



BROM PHENOL BLUE STANDARD CURVE

Figure 9

linear curve as demonstrated in figure 10. In this case the slope of the line experimentally is 0.01. An important application of this curve will be shown later when a discussion of the literature is made.

An interesting limiting case of equation (11) occurs when c is small and 1 is very much greater than Kc then:

$$a = 100Kc \qquad (14)$$

Therefore, at low concentrations the concentration would be a linear function of the per cent absorption.

Equation (14) is interesting, since it can also be derived from Beer's law. The derivation is briefly this: a is equal to 1 - t, where a is the fraction of light absorbed and t is the fraction of light transmitted. Beer's law is:

$$kc = \text{Optical density} = -\log 100t = -\log 100(1 - a) \qquad (15)$$

and expanding (15) by a MacLaurin series:

$$\text{Optical density} = -\log 100(1 - a) = a + 1/2\ a^2 + 1/3\ a^3 + \ldots \qquad (16)$$

or for small values of a the higher powers may be neglected as an approximation in comparison with the first power and:

$$-\log 100t = -\log 100(1-a) = a$$

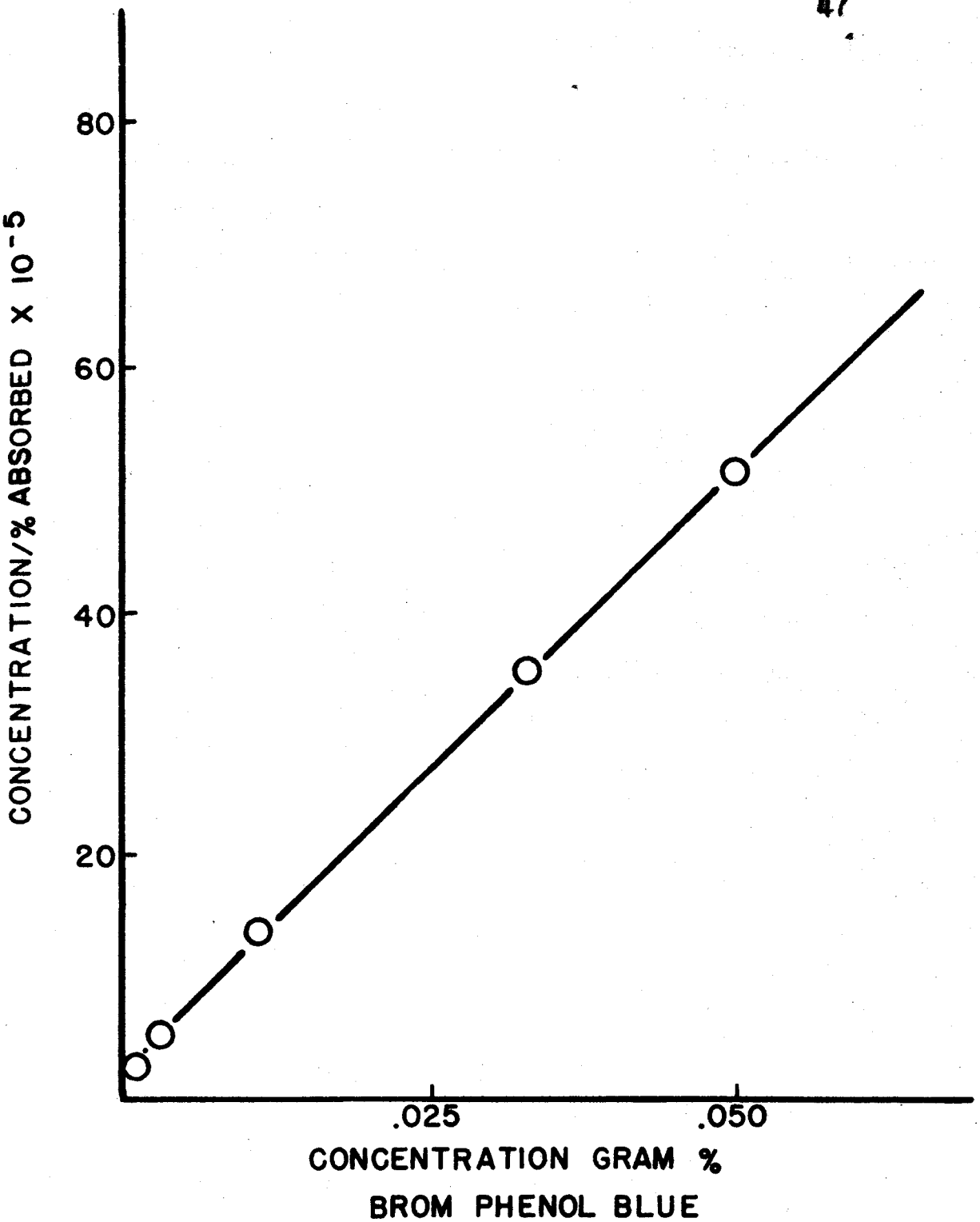

80
60
40
20
CONCENTRATION/% ABSORBED X 10⁻⁵
.025
.050
CONCENTRATION GRAM %
BROM PHENOL BLUE

Figure 10

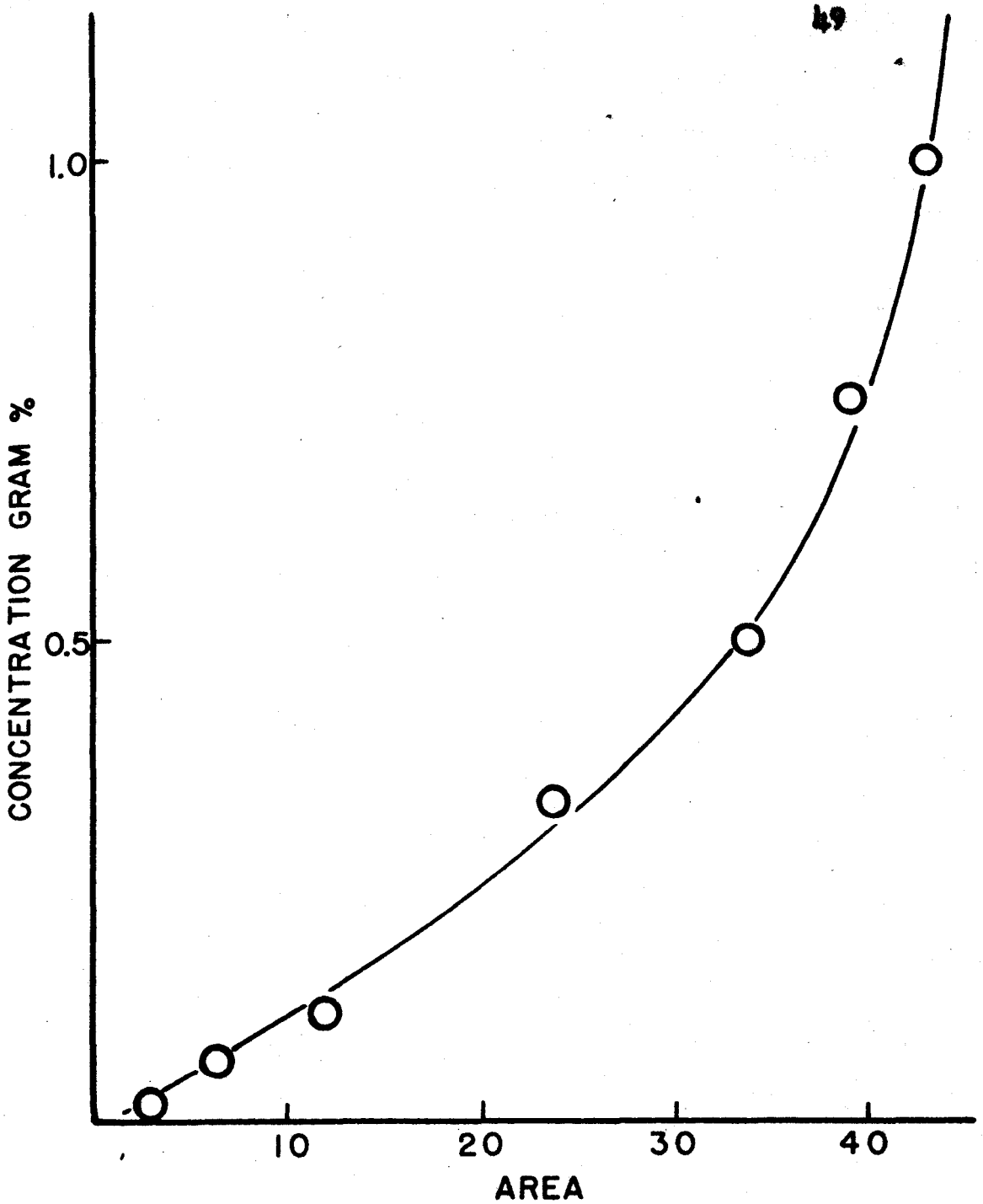
therefore, for small values of a:

$$a = kc \qquad (17)$$

Equation (17) is identical to equation (14) previously derived for small values of a.

A further extension of this hypothesis can be made if the area under the curve is considered as a function of per cent absorption. This assumption is reasonable if the system being studied consists of the same spread of material on the paper; that is, if two different concentrations of a substance are observed on a strip, the width of the bands must be the same for both substances. Further credulousness is given to the area being a function of the per cent absorbed, by an observation of the graph of area versus concentration, which like the concentration versus per cent absorbed curve, is a hyperbola, as shown in figure 11. This curve can also be made linear by plotting the reciprocal of the concentration against the reciprocal of the area under the curve, as illustrated in figure 12.

Early work in ionography on the determination of the amount of a substance present on an ionogram usually involved the elution of the migrant from the paper strip, followed by the use of standard procedures of analysis. Turba and Enenkel (85) eluted the blood proteins from the paper strip after they had been stained with brom phenol blue and measured them spectrophotometricày. Cremer and Tiselius (10), on eluting the proteins



BROM PHENOL BLUE CURVE

Figure 11

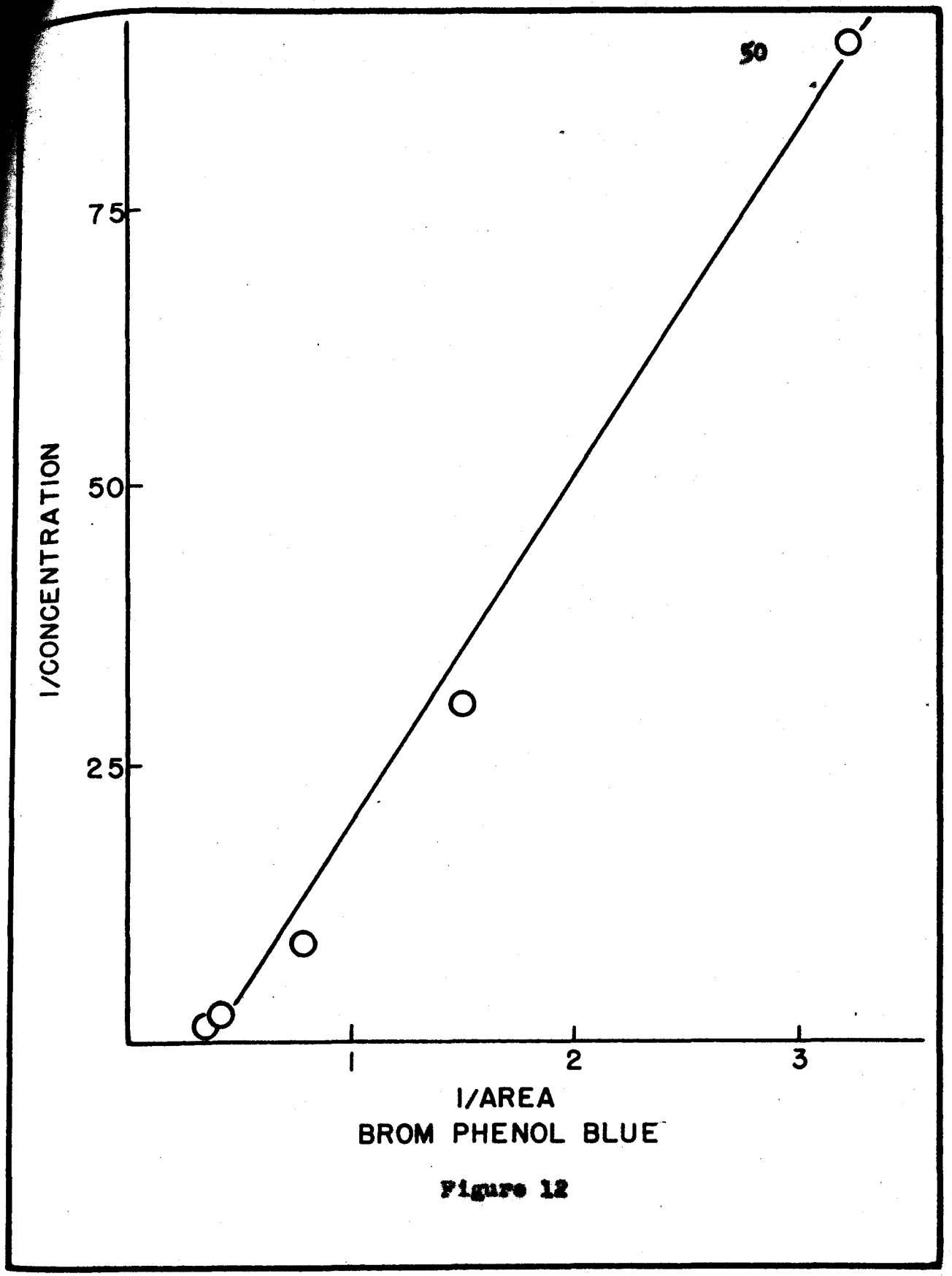


Figure 12

found that paper electrophoresis gave results which compared well with free solution measurements. Other authors have successfully used this technique (14,15,39). Levin and Oberholzer (44, 45) eluted the blood proteins and determined the nitrogen of the various fractions by using a Kjeldahl analysis. They also used the dye elution method and found that both methods gave results which were in satisfactory agreement with free solution electrophoresis. An interesting variation of the elution method is that of Homolka (29). Homolka extracted the albumins with four millimeters of 0.9 % sodium chloride and the globulins with two millimeters of 0.9 % sodium chloride. To each of these solutions he added equal volumes of Brdicka's colbaltic solution and did a polarographic analysis of these solutions at 1.5-1.7 volts. He found his method gave good results when compared with free solution electrophoresis. Sternberg (30) first treated his blood proteins with riboflavin solution, thus causing them to fluoresce. In this way, he circumvented the need for staining the strips, and he simply measured the fluorescence of the riboflavin coupled protein. Latner (43) has worked out a method of visual evaluation by the area of spread of the fraction. His report on the method, however, is fragmentary and he himself seems to lack confidence in the work.

Instruments on which to analyze paper strips have been described by several authors in the literature. Block (5) and

Bull, Hahn, and Baptist (9) have used Photovolt densitometers to measure paper chromatograms. Cook, Harris, and Warren (12) have described an instrument which can measure the amounts of materials in any of the conventional stabilizing media, such as paper, gelatin, starch, etc. Griffiths (25) describes an apparatus for reading electrophoretically separated zones on paper strips, for which he claims good accuracy. Latner (41,42) has used both reflectance and absorbance measurements, concluding that absorbancy measurements are much more accurate than the reflectance measurements. Parke and Davis (68) used an automatic spectrophotometer on paper-strip chromatograms. Rottger (75) has used several different instruments.

Bull, Hahn, and Baptist (9) used a Photovolt densitometer and calculated the amounts of various amino acids present on chromatograms. Bull found that plotting area/concentration against area a linear plot resulted, for arginine, serine, valine, glutamic acid, leucine, threonine, alanine, and lysine. Bull's equation:

$$A/C = -kA + k' \qquad (18)$$

where,

C is the concentration

A is the area

k and k' are constants

can be related to equation (13). Since the area is a function

of absorption equation (13) can be written:

$$C/A = K_2C + K_1 \qquad (19)$$

Now, on dividing (19) by C and K_1, and multiplying by A, it is found that:

$$A/C = -AK_2/K_1 + 1/K_1 \qquad (20)$$

In other words the equation is identical to the empirical equation found by Bull. Block (5) has attempted to evaluate some amino acids and amines on chromatograms. He found that proline and cystine did not give linear results when optical density was plotted against concentration. His curves seem to be hyperbolas, but his main concern is that linearity does not exist. Latner (40) claims a linear relationship of optical density with concentration according to Beer's law, when measuring albumin and gamma globulin up to 0.95 grams per cent. However, Latner gives no experimental data at all, and no real analysis can be made of his findings.

McFarren, Brand, and Rutkowsky (66) have calculated the concentrations of glucose and galactose on paper chromatograms. They found a linear relationship was obtained by plotting the logarithm of the concentration against optical density, which is already in a logarithmic form. Mathematically the center portions of any hyperbola can be described by a log-log plot.

Paladini and Leloir (72) have used ultraviolet absorption to measure adenosine at 260 millimicrons; like McFarren and co-workers, they plotted optical density against the logarithm of the concentration, with a resulting linear plot.

Block (5) found in his densitometric measurements that he achieved linear relationships for glycine when he plotted concentration against per cent transmission. The same observation was made by Brown and Marsh (7) in their work on pyridoxine hydrochloride. From figure 13 it is obvious for large values of per cent transmittance, the optical density and per cent transmittance are linear functions, so that what is linear for one will necessarily be linear for the other. This cannot, as stated by Brown and Marsh, be used as an exception to Beer's law, since for very low concentrations (of below fifty per cent transmission) concentration would be linear with either optical density, or per cent transmission. In most cases the data that various workers have derived has been good. A variety of analytical methods were employed to describe their data, and all have, in the final analysis, agreed. There have however, been several papers in which the author's make statements which they fail to substantiate, such as the work previously mentioned by Latner (40). A paper by Griffiths (24) makes the claim that Beer's law is applicable for measurements of serum proteins on filter paper by making the measurements on a colorimeter. As evidence of the

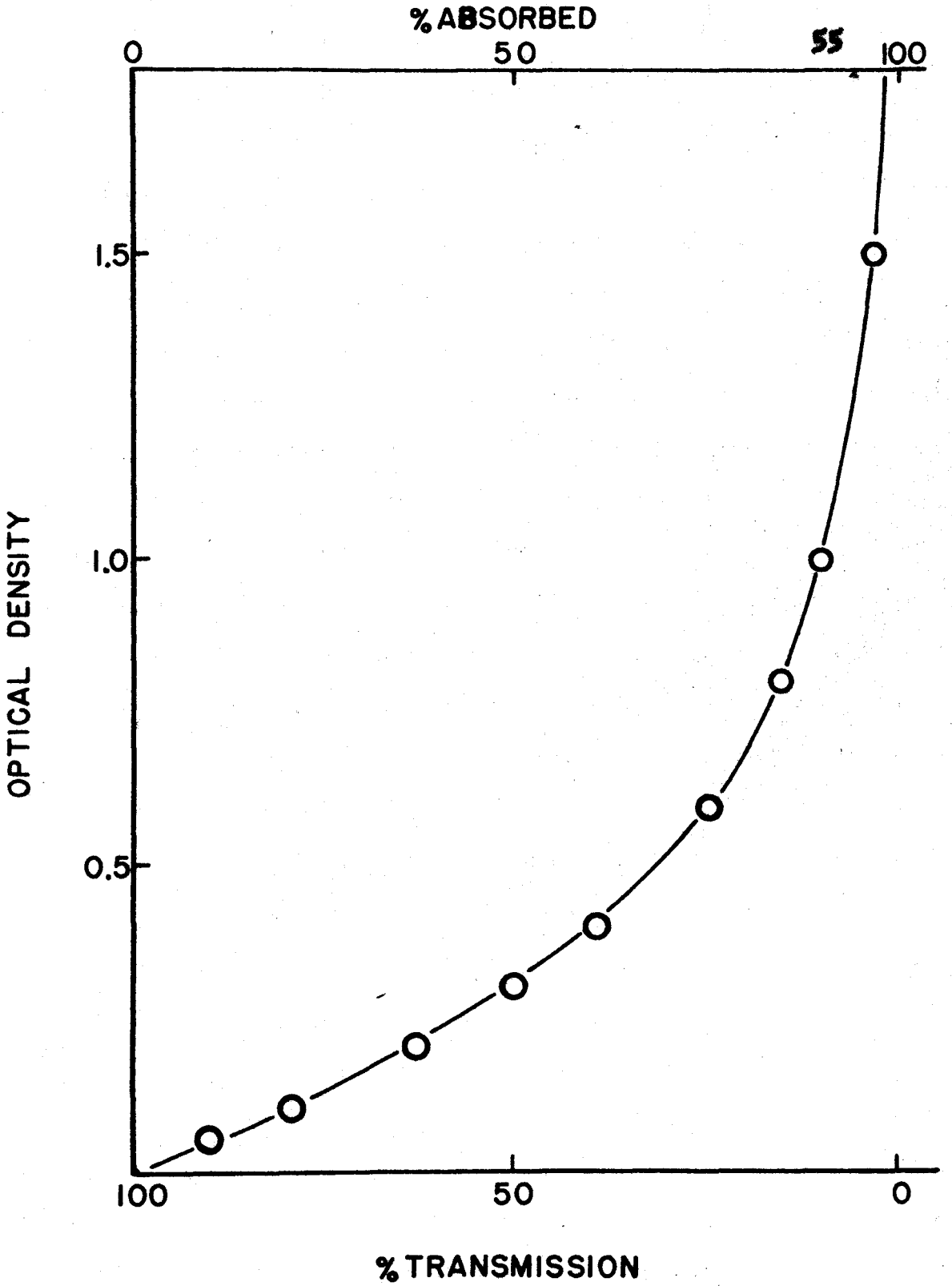

% ABSORBED
0
50
100
1.5
1.0
0.5
OPTICAL DENSITY
100
50
0
% TRANSMISSION

Figure 13

questionable quality of this report, it may be mentioned that he maintains that Beer's law is applicable on the basis of the fact that when he plots transmission against the amount of serum, he achieves a linear relationship. However, on examining his curve it can only be concluded that he has not plotted what he has said he plotted. If he plotted transmission against concentration the slope of his curve is of the wrong sign. It is work of this type which almost defies analysis.

An excellent paper has appeared recently by Crook, Harris, Hassan, and Warren (11). These authors have used a photometer to measure the concentrations of various dyes and the blood proteins on filter paper. In their work on naphthalene black and azocarmine, they found that by plotting concentration against optical density a hyperbola resulted. This they made linear by considering it a rectangular hyperbola. They used a standard form, namely plotting the reciprocal of the photocell current against the reciprocal of the concentration, giving a straight line. They, however, feel that essentially their work indicates a deviation from Beer's law, rather than the non-applicability of Beer's law. Why they do not consider Beer's law as non-applicable is not apparent, since their results would seem to rule it out completely. Probably a very good contribution of this paper is their use of a calibration curve for correcting high peaks for deviation of the curve from Beer's law.

Some photometric evaluations have been reported using ultraviolet wavelengths to analyze amounts of various materials on paper strips. Kimbel (33) used ultraviolet of 254 millimicrons by putting the paper strip in front of photographic paper and then evaluating the blackening on the paper using a colorimeter. He also found that using a wavelength of 280 millimicrons caused a large variation in his results. However, Bracco (6), using the same type of technique as Kimbel, used a wavelength of 280 millimicrons and obtained satisfactory results. Hashimoto and Mori (28), used ultraviolet of wavelength 254 millimicrons for the qualitative determination of flavanoids.

An important application of this study of the amounts of substances present on a filter paper strip, is its adaptability, to the determination of albumin globulin ratios, when used in conjunction with paper electrophoresis. Numerous workers have studied A/G ratios using paper electrophoresis (1,2,3,13,16,17, 20,21,22,23,26,27,32,35,46,48,67,76,78,80,81,87,88,89,90,91). Most of these workers have used elution methods, or if they used photoelectric methods, have not calculated concentrations but rather compare the albumin to the globulin on a percentage basis. Several authors (4,37,91) have found that using standard free solution electrophoresis and ionography comparable A/G ratios could be calculated. Sommerfelt (77) has reported that he found differences of 2-10 % in blood protein fractions using

paper electrophoresis on the same sample of blood. There are, needless to say, numerous applications of A/G ratios.

A primary application, of course, is as a clinical tool, which is what most of the above mentioned authors were interested in. For instance, Poli and associates (71) have studied the effect on the electrophoretic pattern of liver diseases, using ionography. Boussemart and Marchand (8) have studied the effect of nursing on the serum proteins of an infant. Roberts and Brunish (74) studied the effect of injecting C^{14} labeled amino acids into normal and hepatectomized rats. The serum proteins from the respective animals were separated and the radioactivity of the components determined. They concluded that the liver is directly involved in the formation of albumin and alpha globulin but that gamma globulin is not immediately dependent on the liver. Knedel (34) used paper electrophoresis to study the reaction of drugs on the various proteins in the blood. Koiw and Gronwall (36) made a study of the protein bound carbohydrates using fuchsin sulfite solution which caused the protein bound carbohydrates to appear as violet-bands. Hugentobler, Wunderly, and Schneider (30) first seperated the proteins by paper electrophoresis, then degraded them to amino acids and analyzed the amino acids using chromatography. Kanzow (31) studied the effect of leukemia on the serum proteins, and found the effect was that of a general acute inflamation. Frunder and Bornig (18) studied

the serum proteins before and after lipid extraction and found their results agreed well with those found in free solution electrophoresis. Pluckthun and Matthes (70) made a comparative study of the proteins of the blood and of the cerebrospinal fluid and found that the concentrations in these fluids were very similiar.

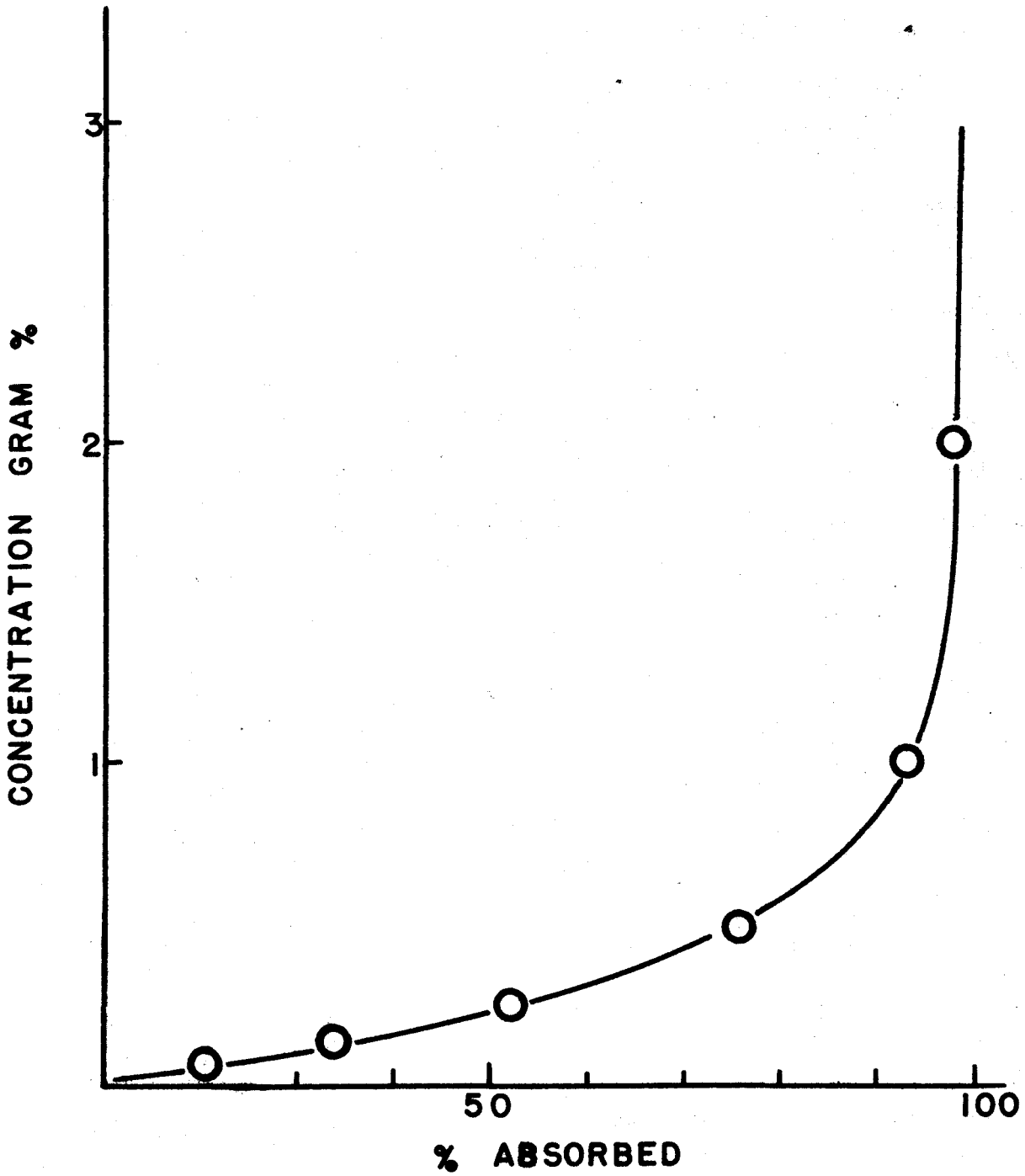
The Welch automatic scanning recorder was used to test several applications. Standard curves were calculated for albumin and globulin, and blood samples were analyzed.

The standard curves are calculated in the following way. An albumin sample of four gram per cent, and a globulin sample of three gram per cent are made. These samples are then diluted by serial dilution, each sample is one-half of the next larger sample. Seven samples of each protein were made giving a range for the albumin of 4.000 to 0.062 grams per cent, and 3.000 to 0.047 grams per cent for the globulin. Strips of Eaton and Dikeman number 613 paper were wetted, and two and five lambda volumes of each of the fourteen standard solutions were applied. The strips were oven dried and then stained in 1% brom phenol blue in 95% ethyl alcohol saturated with mercuric chloride. The following was the wash procedure in the case of all proteins. After staining with brom phenol the strips were placed upon a vertical glass sheet and dilute hydrochloric acid was poured on them. They were allowed to stand approximately a minute, after

which time they were washed with a solution of saturated mercuric chloride, in a wash bottle, until the brom phenol blue began to change from yellow to blue. They were again brought to the acid side of the indicator by dilute hydrochloric acid, and the procedure of standing and washing was repeated. This procedure resulted in a very clear background which is a necessity for use on the automatic scanner. The strips, after washing was complete, were dried. The wavelength of the monochromator was set at 585 millimicrons and the strips were recorded on the strip chart recorder. The optical density for the albumin and globulin was converted to per cent absorption. This was plotted against concentration and the curves which resulted were found to be hyperbolic. For albumin see figure 14, and for the globulin curve see figure 15. These curves were then made linear according to the formula (12).

$$1/a = 1/100K \quad 1/c + 0.01$$

For the albumin a plot of 1/a versus 1/c gave a linear curve with the intercept (see figure 16) approximately 0.01. The globulin also gave a linear curve when 1/a was plotted against 1/c with the intercept approximately 0.01 as shown in figure 17. These curves were used later as one possible technique of evaluating the serum blood proteins. Next the areas under the curves were evaluated. Two methods were used:(1) the areas were measured



ALBUMIN ABSORPTION

Figure 14

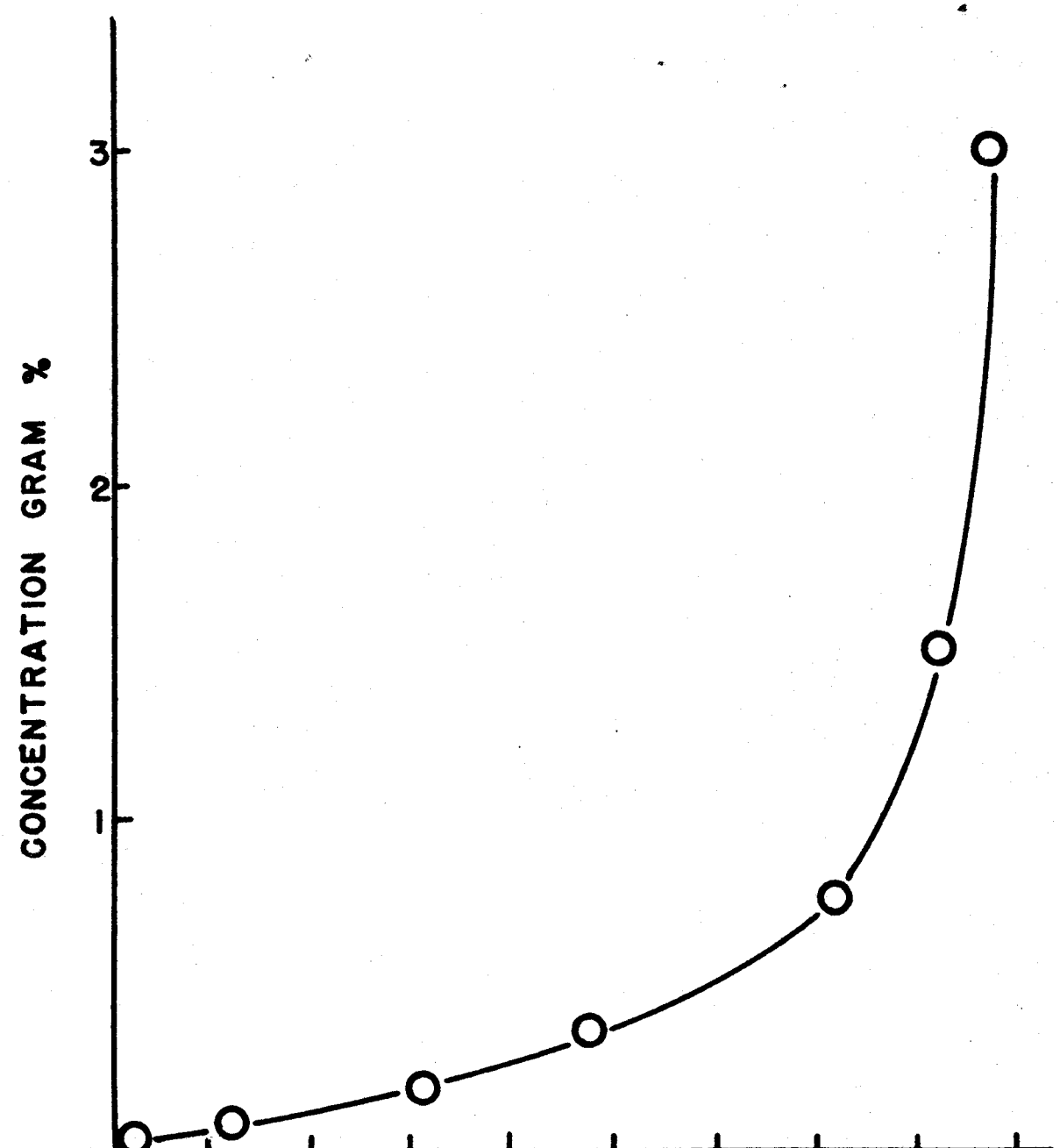

CONCENTRATION GRAM %
3
2
1
50
100
% ABSORBED
GLOBULIN ABSORPTION

Figure 15

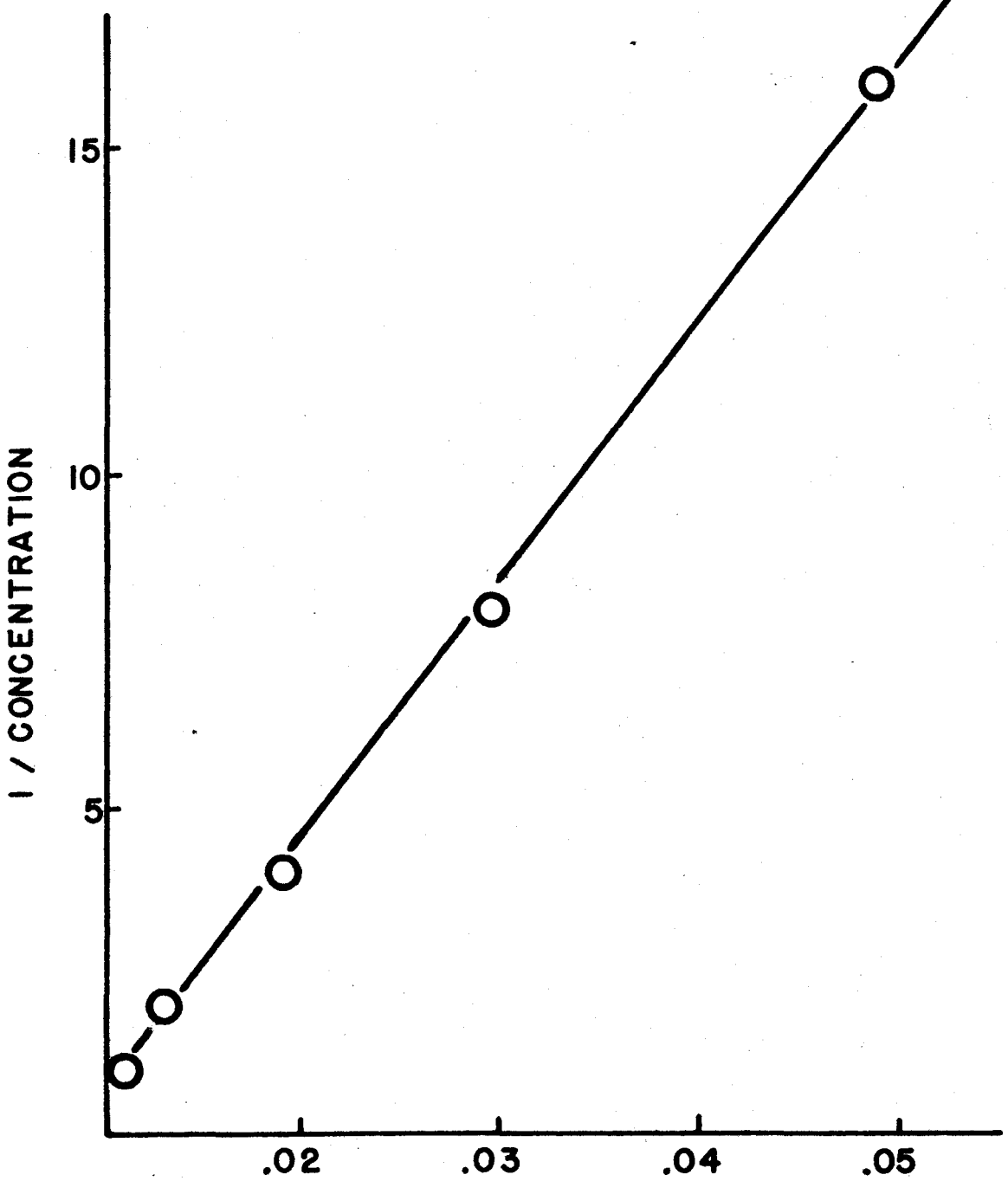
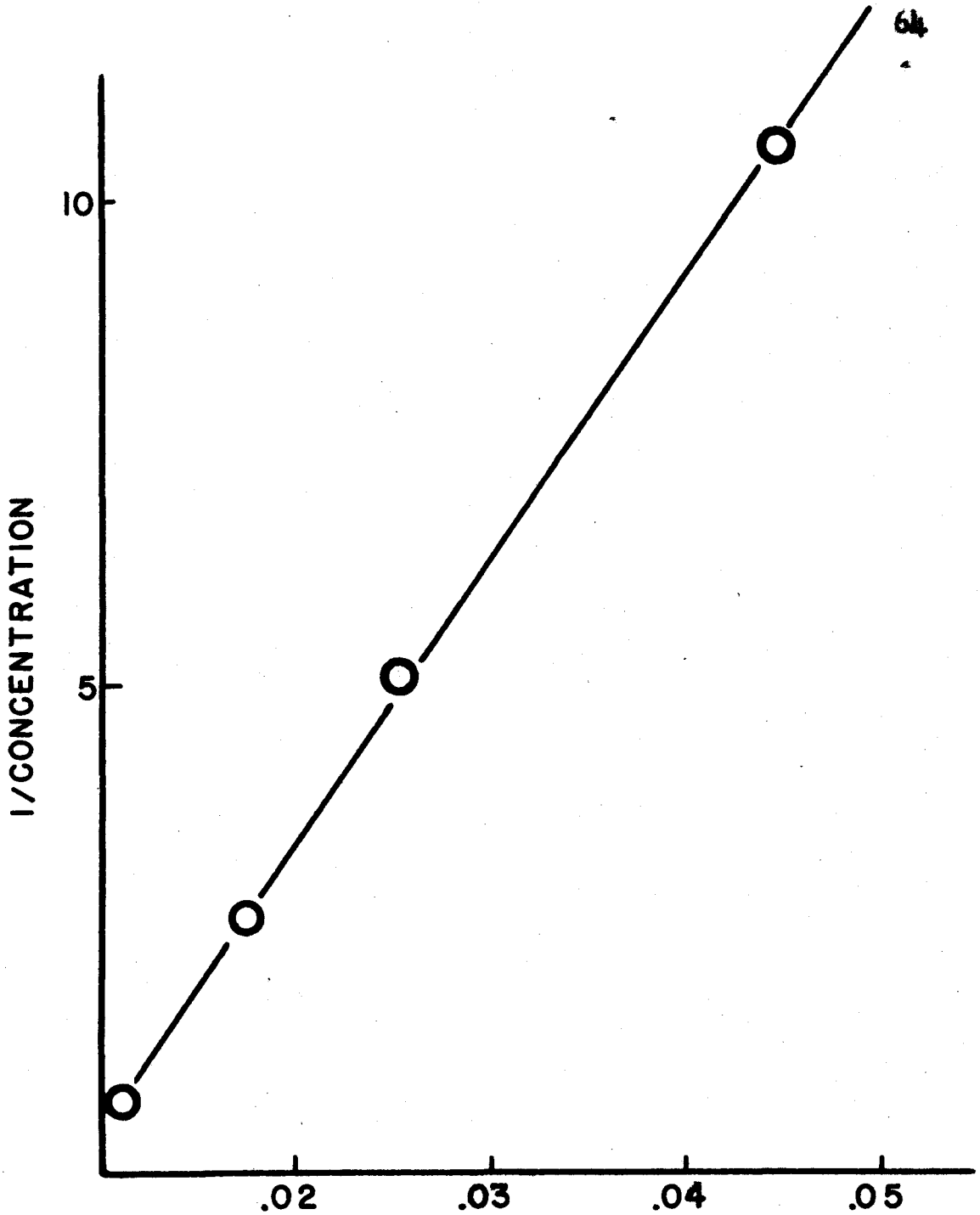

15
10
5
1 / CONCENTRATION
.02
.03
.04
.05
1 / % ABSORBED
ALBUMIN STANDARD CURVE

Figure 16



GLOBULIN STANDARD CURVE

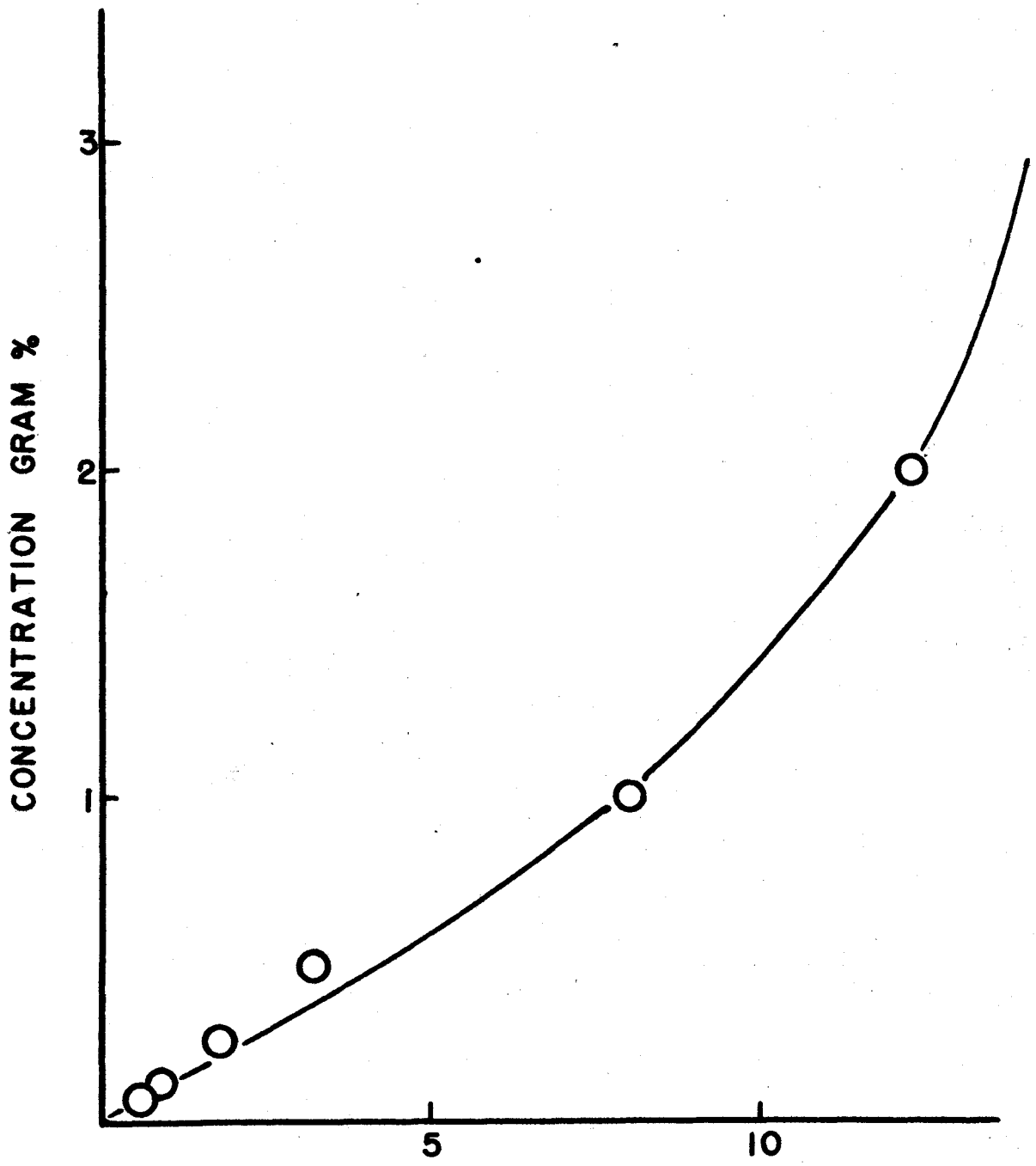
Figure 17

using a planimeter and (2) the area under the curve was cut out and weighed on an analytical balance; from a knowledge of the weight of a one square centimeter area of the same paper, it was possible to calculate the area under the curve. The values calculated by these two methods agreed within five per cent, and the values used in calculating the data for the curves is an average of the two methods. The areas under the curve for the five lambda and the two lambda runs compared well, with a difference no greater than five per cent resulting. The albumin curve of area versus concentration, figure 18, and globulin curve, figure 19, are both hyperbolic in nature and can be made linear by the following formula:

$$1/A = 1/K_1 \quad 1/c + 1/K_2$$

The albumin data was plotted and gave a linear curve, see figure 20, as did the globulin data, figure 21. These curves are used later to calculate the A/G ratios.

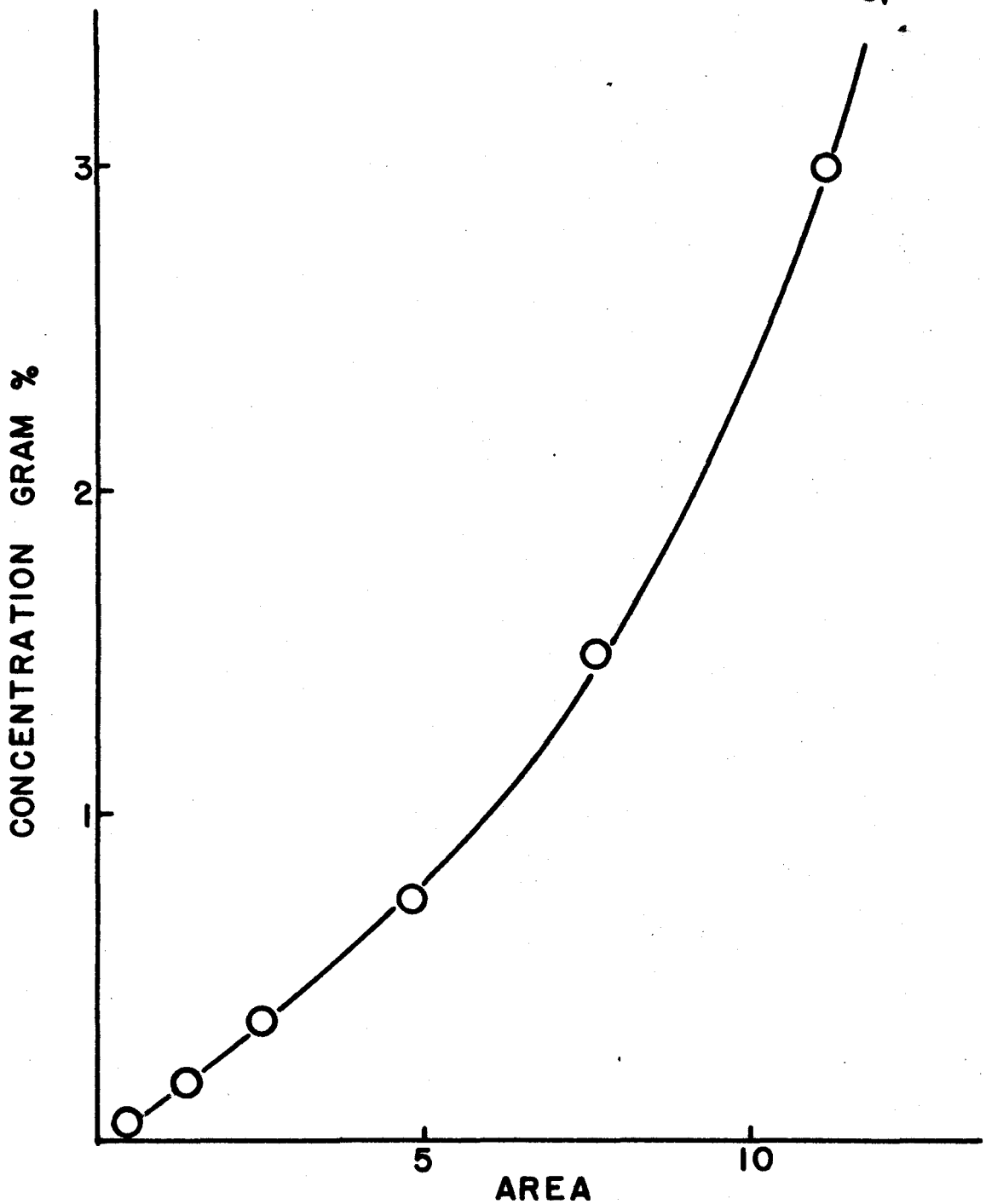
Three different human blood samples were run (two normal and one pathological - rheumatic fever with mitral valvuritis and myocarditis) on the Ionograph, and the serum proteins separated. The conditions were, a veronal buffer system pH, 8.6, ionic strength 0.05, eight volts per centimeter potential gradient, three hours duration of an experiment, with an equilibration time of one hour. The buffer to paper ratio was 1.90 to 1.00 at a temperature of 20-25°C. These conditions were



AREA UNDER CURVE VS CONCENTRATION

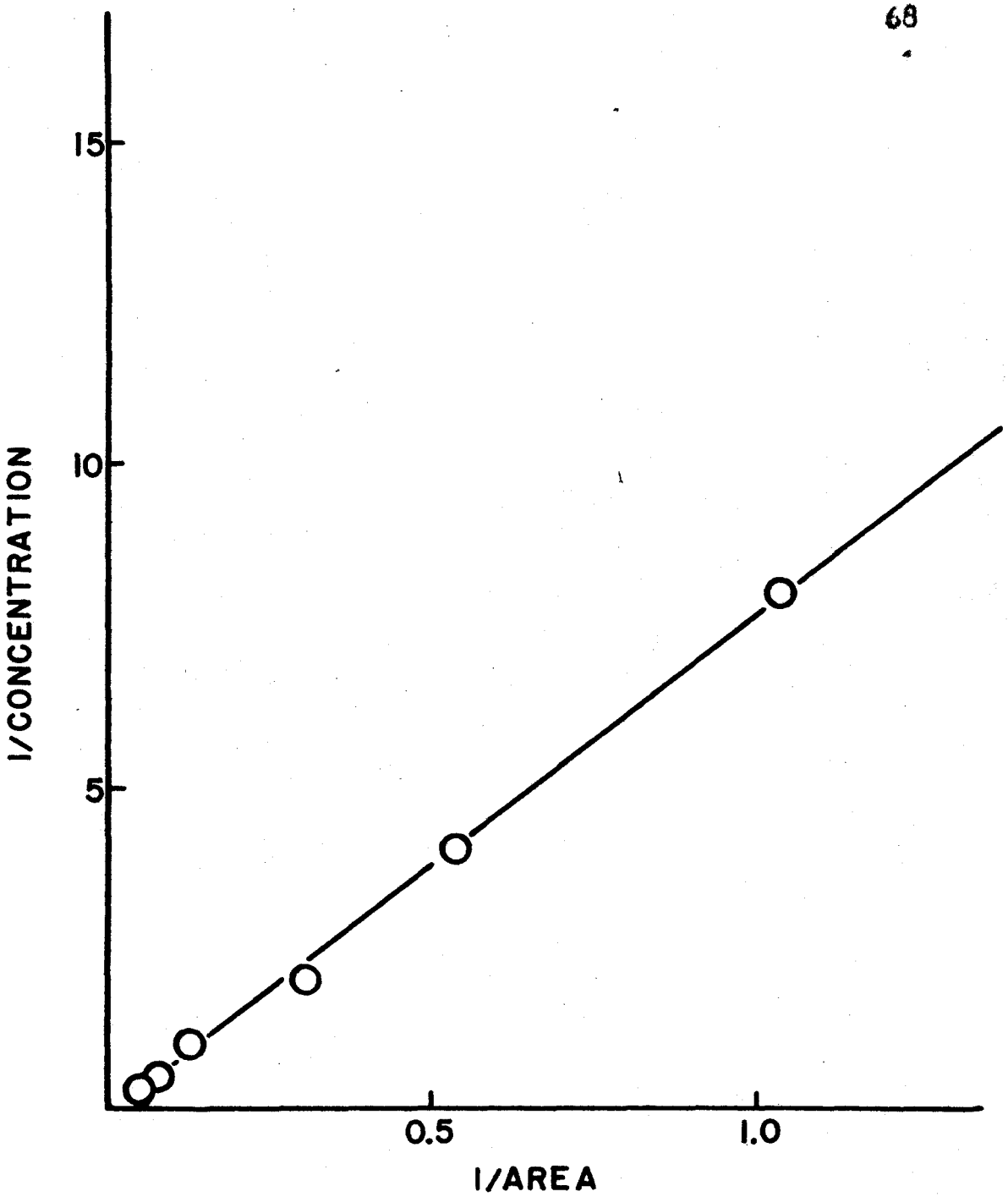
ALBUMIN

Figure 18



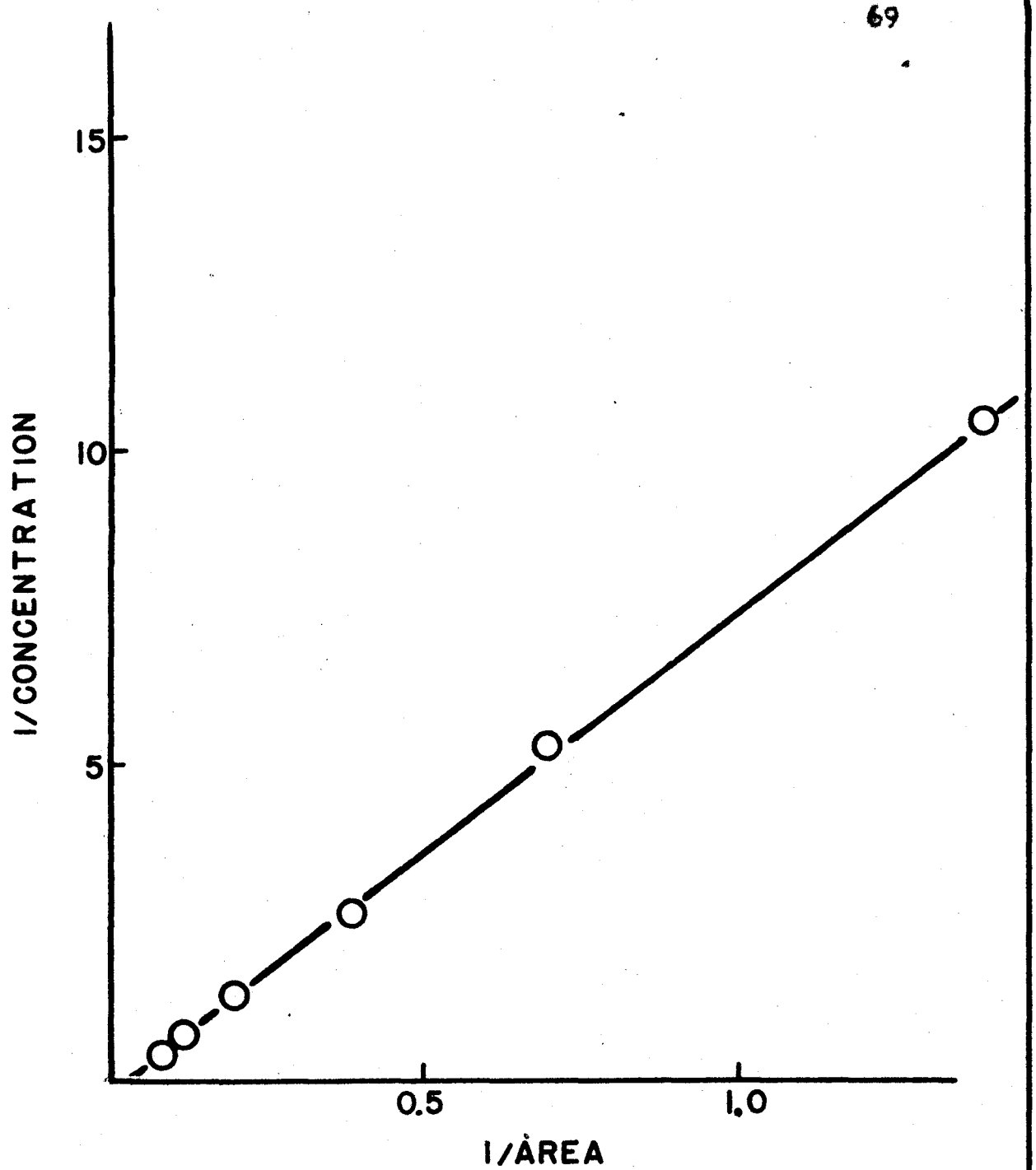
AREA UNDER CURVE VS CONCENTRATION GLOBULIN

Figure 19



ALBUMIN CURVE

Figure 20



GLOBULIN CURVE

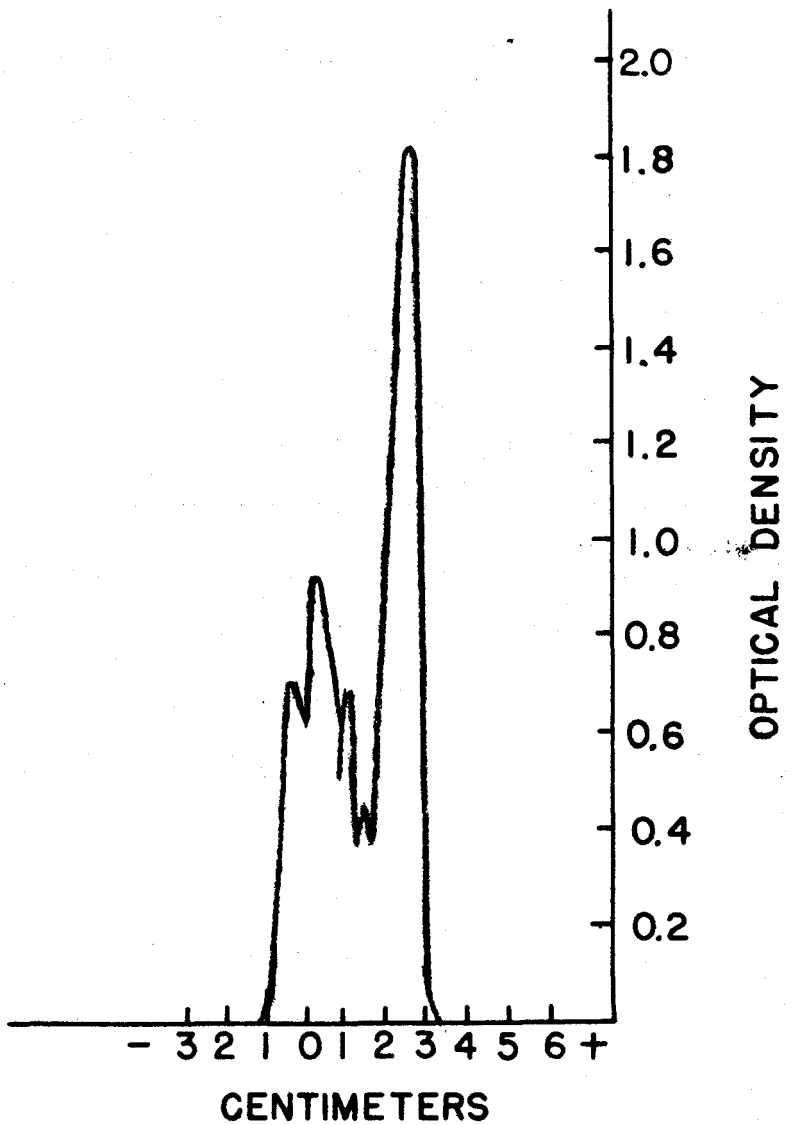
Figure 21

found to give excellent separations into the five components usually found in free solution electrophoresis in a veronal buffer. Seven strips were run on each sample.

After separation by ionography, the samples were dyed with brom phenol blue and analyzed using the automatic scanning device. The per cent absorption of each peak was noted, and the area under each of the curves determined. Figure 22 shows a typical blood protein pattern as given on the strip chart recorder. The data for the percent absorption and the area under the curve are given in table I.

The data was analyzed in the following manner. The data representing the per cent absorption could give the correct protein concentrations if the bands move homogeneously, and maximum absorption is not the result of the band either becoming more concentrated or less concentrated. During the course of a run a fraction might spread or it might possibly be compressed so that in either of these cases the absorption would be no measure of the concentration. However, if the band remains homogeneous during a run, then the percent absorption would become an index of the concentration.

It is important here to note that Cremer and Tiselius (10) observed the uptake of dye by the albumin to be 1.6 times as great as by the globulin. A comparison of the standard absorption curves, figure 16 and 17, show the albumin uptake



A TYPICAL DENSITOMETER PATTERN OF BLOOD PROTEINS SEPARATED BY IONOGRAPHY

Figure 22

Table I

BLOOD PROTEIN DATA

Sample Number	Protein Component	Optical Density	Area	% Absorbed
1	Albumin	1.82	13.56	98.5
	α_1	0.44	2.00	63.7
	α_2	0.72	4.47	81.0
	β	0.92	7.15	88.0
	Gamma	0.71	7.33	80.5
2	Albumin	1.74	15.14	98.2
	α_1	0.60	4.65	74.9
	α_2	0.68	7.32	79.2
	β	0.68	7.55	79.2
	Gamma	0.57	6.06	73.1
3	Albumin	1.92	16.00	98.8
	α_1	0.72	3.87	81.0
	α_2	0.96	6.93	89.1
	β	1.22	8.08	94.0
	Gamma	1.19	10.43	93.6

to be 1.5 times as great as globulin. However, a comparison of figure 20 and 21 show that the area under the curve of albumin is the same as the area under the curve for globulin, when comparing equal concentrations of either protein. It must be concluded that the original assumption that the area was a function of the amount of dye absorbed was not true. It becomes necessary then to correct the albumin area curve by a factor of 1.5. In table II are listed the values of the protein concentrations as calculated from the absorption data. However, these concentrations are probably not correct due to the fact that the criteria set previously for the validity of absorption being a function of concentration are not met in an electrophoretic separation of blood proteins. In table III are listed the values of the protein concentrations as calculated from the data representing the area under the curve. These values are probably the better values, except for the albumin value which is 0.67 fraction of its true value, due to the 1.5 in uptake of the dye for albumin when compared with globulin.

The A/G ratios for samples one and three, which are normals, are then 1.095 and 1.035, respectively, while the A/G ratio for the patholigical sample is 1.275. These values are in good agreement with those calculated by the method of free solution electrophoresis. The ionographic method described here is to be preferred to salt fractionation since the values determined

Table II

BLOOD PROTEIN DETERMINATION BY % ABSORBED

Sample Number	Protein Component	1/% Absorbed	1/Concentration	Concentration
1	Albumin	0.0101	0.25	4.00
	α_1	0.0156	2.21	0.47
	α_2	0.0123	1.33	0.75
	β	0.0113	0.87	1.15
	Gamma	0.0124	1.38	0.72
2	Albumin	0.0101	0.25	4.00
	α_1	0.0133	1.59	0.63
	α_2	0.0126	1.43	0.70
	β	0.0126	1.43	0.70
	Gamma	0.0136	1.69	0.59
3	Albumin	0.0101	0.25	4.00
	α_1	0.0123	1.33	0.75
	α_2	0.0112	0.87	1.15
	β	0.0106	0.59	1.69
	Gamma	0.0106	0.59	1.69

Table III

BLOOD PROTEIN DETERMINATION BY AREA UNDER THE CURVE

Sample Number	Protein Component	1/Area	1/Concentration	Concentration
1	Albumin	0.077	0.39	2.60
	α_1	0.416	3.01	0.33
	α_2	0.223	1.46	0.68
	β	0.140	0.80	1.25
	Gamma	0.136	0.77	1.30
2	Albumin	0.067	0.27	3.70
	α_1	0.214	1.39	0.72
	α_2	0.136	0.77	1.30
	β	0.132	0.75	1.33
	Gamma	0.165	1.01	1.00
3	Albumin	0.062	0.25	4.00
	α_1	0.259	2.44	0.41
	α_2	0.144	0.83	1.20
	β	0.123	0.68	1.47
	Gamma	0.095	0.40	2.50

using the salt fractionation method are much too high. Ionography seems to offer an excellent tool for the clinic, yielding, as it does, good A/G ratios and at the same time giving the concentrations of the albumin; α_1, α_2, β, and gamma globulins.

CHAPTER V

ELECTROACCELERATION STUDIES

Molecular weight determinations based on electrophoretic measurements have been tried but thus far no one has been sucessful. It is thought that a migrant moving in a solution achieves a terminal velocity so rapidly that it is impossible to calculate the acceleration of the particle before it reaches its terminal velocity.

If a particle were being accelerated in an electric field under a constant force then it would be possible to calculate its molecular weight.

The following equations are defined for a case of constant acceleration.

$$v = ds/dt \qquad (1)$$

$$a = dv/dt \qquad (2)$$

$$a = d^2s/dt^2 \qquad (3)$$

where, v is the velocity

t is the time

s the distance

a the acceleration

Integrating (2) with respect to t:

$$\int d^2s = a \int dt^2$$

$$ds = at\ dt + c_1\ dt \qquad (4)$$

Taking the differential dt

$$v = ds/dt = at + c_1 \qquad (5)$$

Now to evaluate c_1, let $v = v_o$ at $s = 0$; $t = 0$

$$v_o = 0 + c_1 \qquad (6)$$

Or measuring a from $t = 0$; $s = 0$

$$v = at + v_o \qquad (7)$$

Now Newton's second law of motion stated mathematically is

$$F = ma \qquad (8)$$

where, F is the force

m is the mass

a is the acceleration

From equation (7)

$$a = v\text{-}v_o/t \text{ or if } v_o = \text{zero}$$

then,

$$a = v/t \qquad (9)$$

Substituting (9) into (8)

$$F = mv/t \qquad (10)$$

And substituting (1) into (10)

$$F = \frac{m\ ds}{t\ dt} \qquad (11)$$

On integrating (11) with respect to s

$$F\int tdt = m\int ds$$

$$\frac{Ft^2}{2} = ms \qquad (12)$$

Now if the time is set the same for each migrant in any one series of studies then,

$$\frac{Ft^2}{2} = \text{a constant } K \qquad (13)$$

Substituting (13) into (12)

$$K = ms \quad \text{or} \quad m = K/s \qquad (14)$$

It is obvious, from equation (14), that on plotting m versus 1/s a straight line should result.

An instrument was developed to explore the practical validity of the above hypothesis. A photograph of the instrument

is shown in figure 23. It consists of two units, a rectifier and an enclosed chamber, and is presented in more detail by the schematic diagram, in figure 24. The instrument has four electrodes, D, two of which are connected to the positive terminal of the rectifier and two to the negative terminal of the rectifier. Four electrode vessels, E, are connected by agar-salt bridges to the four buffer vessels, G. A sheet of filter paper (Eaton and Dikeman number 613), ten inches by ten inches, B, is enclosed in an air tight chamber A. The sheet of filter paper is connected to the buffer vessels by means of four small tabs of Munktell paper, three inches by one inch. The Munktell paper is used for the tabs since it acts as a better conductor than Eaton and Dikeman 613 and will therefore, cause a smaller voltage drop across the tabs. Now if a potential is applied to the electrodes from the rectifier, this potential is equal in both directions; that is, two forces of equal potential act at an angle of 90° to each other. In this case, the migrant M, will move off at a forty-five degree angle. It is, essentially, thought that the hypothesis $m = K\ 1/s$ will hold for this instrument.

Due to the influence of molecular volume on mobility, in all electroacceleration studies it was necessary to use, for comparison purposes, only families of compounds. Five 2,4 dinitrophenyl derivatives of amino acids were the first group

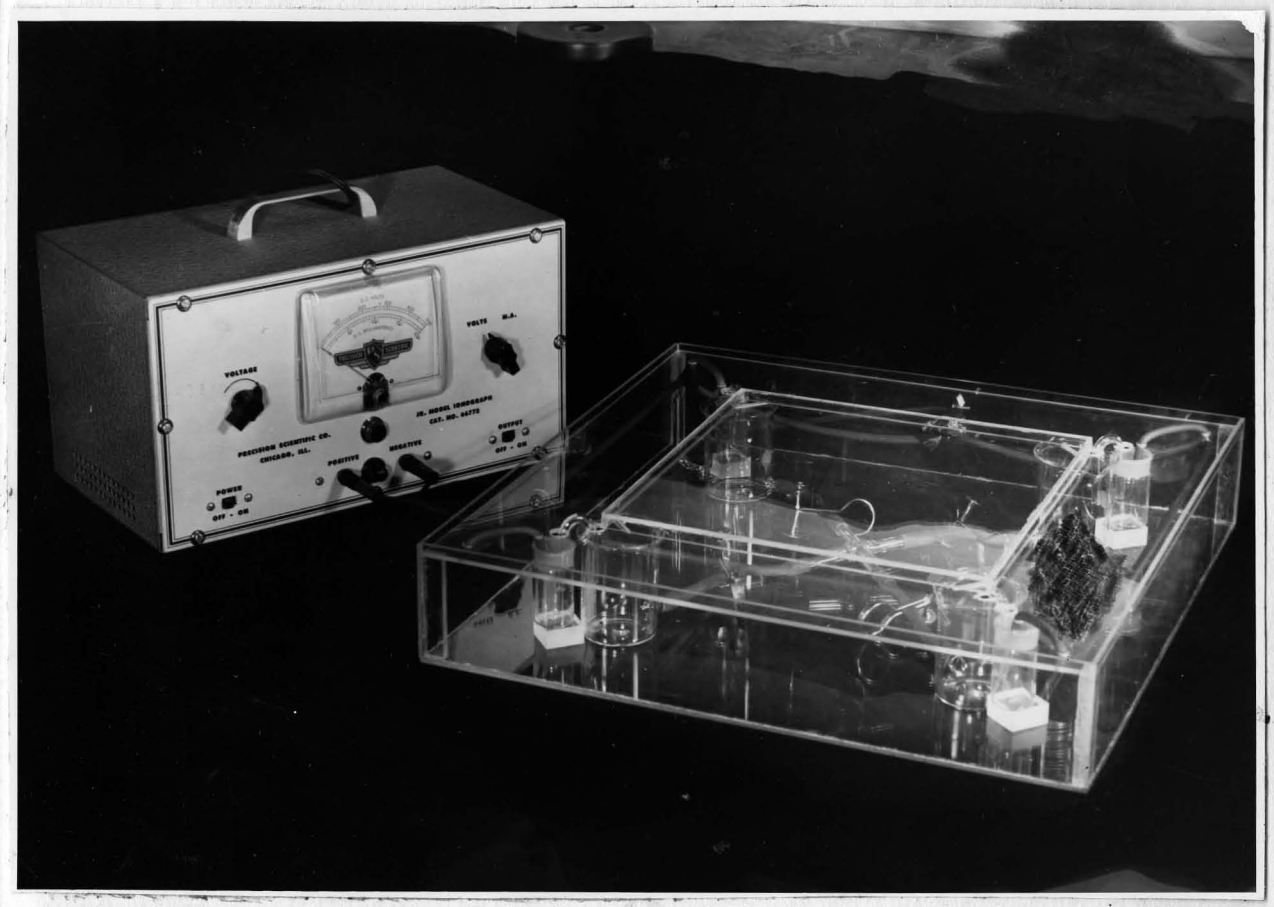


Figure 23

The Electroacceleration Instrument

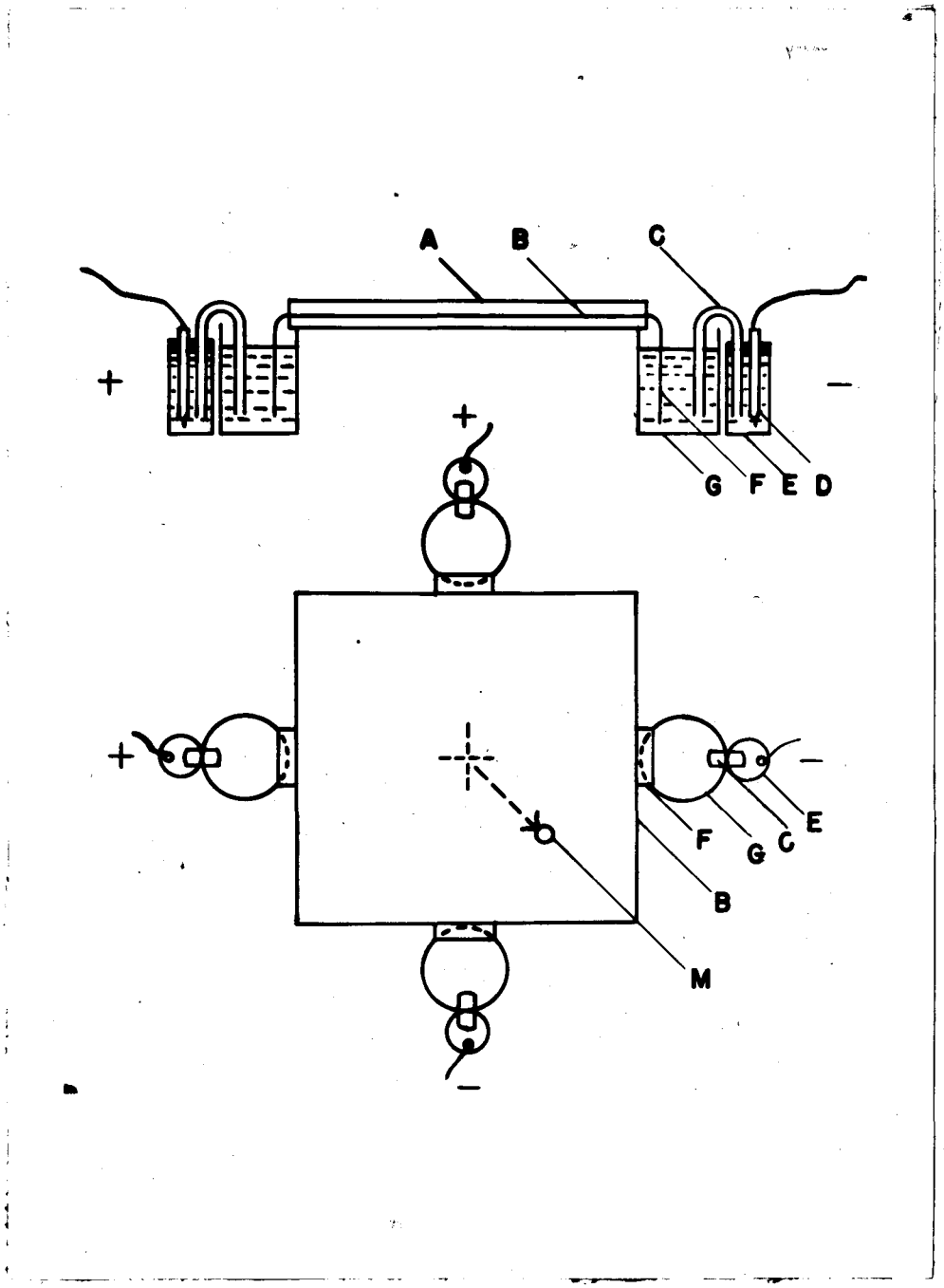
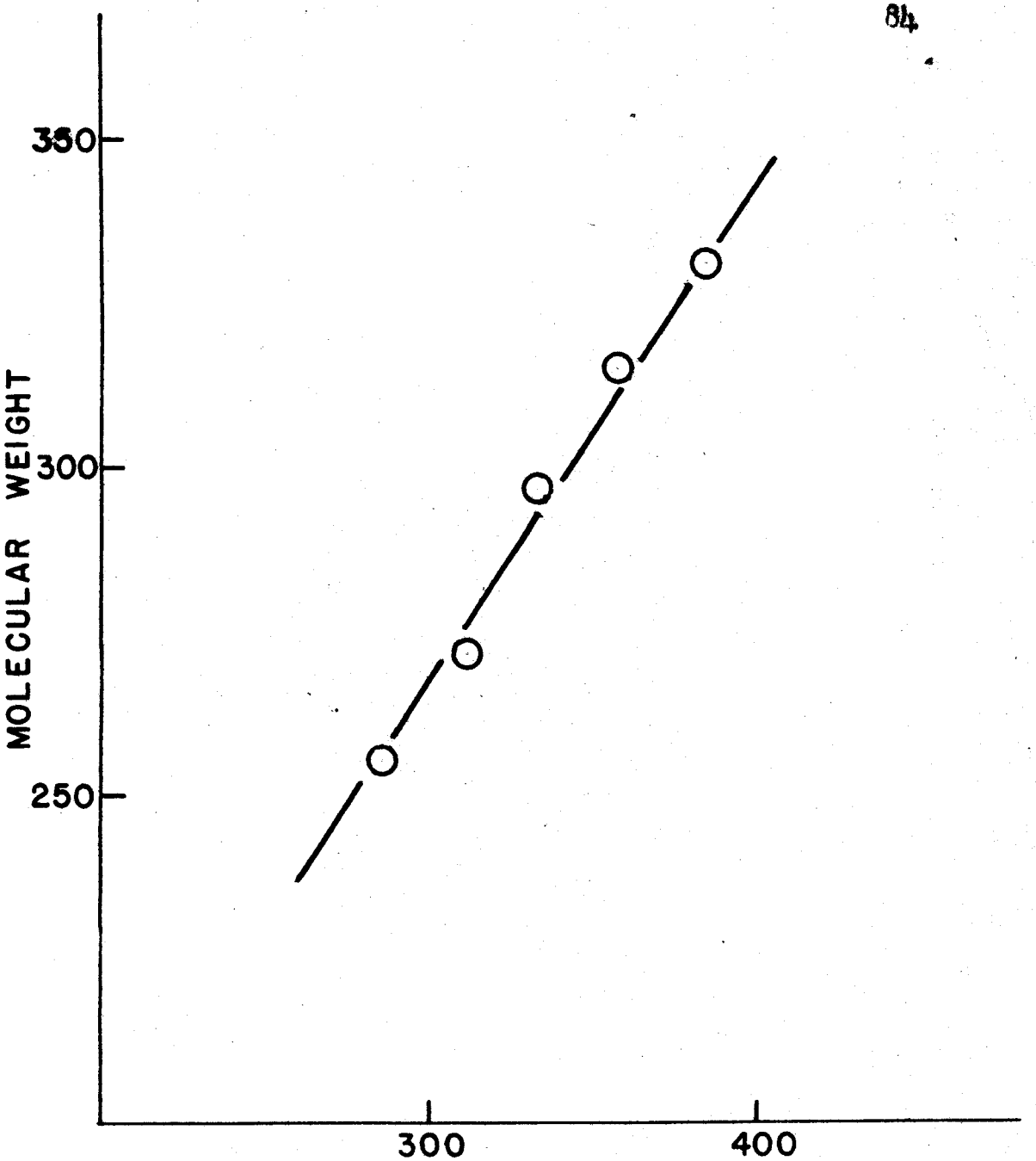


Figure 24

A Schematic Diagram of the Electroacceleration Instrument

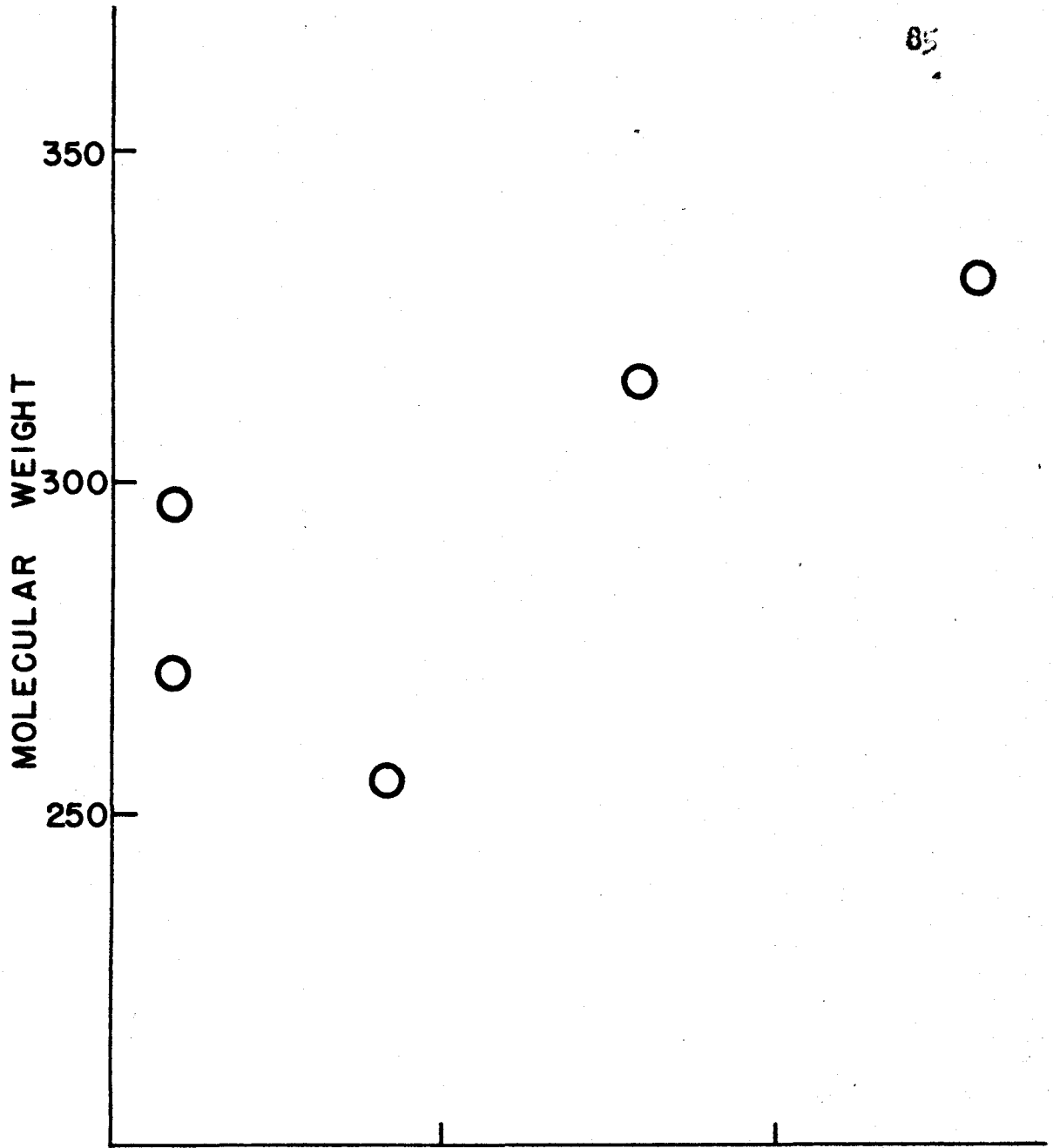
chosen for study. These were the derivatives of alanine, serine, leucine, methionine, and phenylalanine. The conditions of the experiments were a veronal buffer, pH 8.6, ionic strength 0.025; the potentials, applied at ninety degrees to each other, were 200 volts in both directions. The duration of an experiment was two hours, and the temperature was 20-25° C. Since these compounds are all colored, their migration can be readily observed. The instrument was allowed an hour equilibration time. When the molecular weights were plotted against the reciprocal of the distance moved a linear relationship resulted, as shown in figure 25. However, when these compounds were run under identical conditions, except that a potential is applied in only one direction, no relationship results, as shown in figure 26. It was the success of these experiments that raised hope for the more general application of the method.

In the next experiments, a group of twelve amino acids having similar isoelectric properties were used as migrants. The buffer conditions were 0.01 M HCl at a pH 2.1; this pH was necessary to remove all the amino acids from their isoelectric regions. The duration of an experiment was three hours, and a potential of 200 volts was applied in both directions. The migrants then move at a forty-five degree angle with respect to the side of the paper square. However, no relationship could be found between their movement of the amino acids and their



CROSSED-CURRENT MOVEMENT
OF DINITROPHENYL DERIVATIVES
OF AMINO ACIDS

Figure 25



IONOGRAPHIC MOVEMENT
OF DINITROPHENYL DERIVATIVES
OF AMINO ACIDS

Figure 26

respective molecular weights.

At this point, a comment is in order regarding a note by McDonald and Urbin (61). They reported the existence of a relationship between the movement and the respective molecular weights for the dinitorphenyl derivatives and the same series of amino acids reported here. The amino acids are being studied under adverse conditions and the reproducibility of the results is difficult. These authors are now of the opinion that the earlier results on the amino acids must be placed in the category of fortuitous data.

CHAPTER VI

SUMMARY

A. Mobility Determinations: Bovine serum albumin, beta lactoglobulin, egg albumin, and gamma globulin of known free solution mobilities were studied using the Ionograph. It was noted that at higher potential graidents the migrants moved with decreasing velocity; however, if the mobilities were plotted against time a linear relationship resulted which allowed extrapolation to zero time and gave values which corresponded closely to those calculated by moving boundary electrophoresis. It was deduced from this work that under proper conditions mobilities could be calculated directly by ionography. It was found that by using very low potential gradients and maintaining proper equilibrium conditions, the mobilities for bovine serum albumin, beta lactoglobulin, egg albumin, and gamma globulin could be obtained directly using ionography.

B. Automatic Scanning Device: A new automatic scanning device was devised and used to measure the amounts of material on ionograms. The ability of the instrument as a densitometer was first tested by using gelatin strips stained with brom

phenol blue, and increasing the number of gelatin strips tested at one time to see if the instrument would respond according to Beer's law. The results indicated that the instrument responded properly to Beer's law if presented with a homogeneous system. However, when standardization of the instrument began it was apparent in measuring different concentrations of brom phenol blue on paper that Beer's law was not applicable in this case. The curve resulting from plotting optical density versus concentration was a hyperbola. When the per cent of light absorbed was plotted against concentration, the curve, a hyperbola, appeared as a sorption curve, similar to those found for sorption of a gas on a solid. The curve was made linear and a mathematical and theoretical description for the curve were devised. Standardization curves for albumin and globulin were experimentally calculated. It was found that concentration could not be calculated from the per cent of light absorbed but that the area under the curve must be calculated in order to obtain concentration. The uptake of dye by albumin was found to be 1.5 times as great as that for globulin. As a consequence of this fact, when calculation of A/G ratios are being made, a correction must be made for the albumin. The A/G ratios are being made, a correction must be made for the albumin. The A/G ratios calculated using the technique of ionography were found to be in good agreement with those normally found in free solution electrophoresis. The concentrations of albumin, the alpha globulins,

, beta globulin and gamma globulin may also be obtained simultaneously.

C. Electroacceleration Studies: An instrument was developed to study electroacceleration of migrants. The theory of this migration, as to relationship to mass, is discussed. Several 2,4 dinitrophenyl derivatives of amino acids are found to substantiate the theory. However, the work with several amino acids yielded no concrete results due to physical and chemical difficulties encountered in their investigation.

TABLE IV

EXTRAPOLATED MOBILITY OF BOVINE SERUM ALBUMIN

Buffer: veronal (barbital-sodium barbital)

Ionic strength: 0.1

pH: 8.6

Potential gradient: 2v/cm

Temperature: $4^{\circ}C$

MOBILITY ARMOUR: 6.66×10^{-5} cm/sec/volt/cm

EXTRAPOLATED MOBILITY: 6.40×10^{-5} cm/sec/volt/cm

EXPERIMENTAL VALUES

MOBILITY	TIME
5.10×10^{-5} cm/sec/volt/cm	5 hrs
4.50×10^{-5} cm/sec/volt/cm	8 hrs
2.80×10^{-5} cm/sec/volt/cm	13 hrs
2.20×10^{-5} cm/sec/volt/cm	17 hrs

TABLE V

EXTRAPOLATED MOBILITY OF EGG ALBUMIN

Buffer: Phosphate

Ionic strength: 0.2

pH: 7.7

Potential gradient: 2v/cm

Temperature: 4°C

MOBILITY ARMOUR: 5.69×10^{-5} cm/sec/volt/cm

EXTRAPOLATED MOBILITY: 5.88×10^{-5} cm/sec/volt/cm

EXPERIMENTAL VALUES

MOBILITY	TIME
5.60×10^{-5} cm/sec/volt/cm	8 hrs
4.15×10^{-5} cm/sec/volt/cm	16 hrs
3.40×10^{-5} cm/sec/volt/cm	24 hrs
2.84×10^{-5} cm/sec/volt/cm	28 hrs

TABLE VI

EXTRAPOLATED MOBILITY OF BETA LACTOGLOBULIN

Buffer: Phosphate

Ionic strength: 0.2

pH: 7.7

Potential gradient: 2v/cm

Temperature: 4°C

MOBILITY ARMOUR: 5.62×10^{-5} cm/sec/volt/cm

Extrapolated Mobility: 5.80×10^{-5} cm/sec/volt/cm

EXPERIMENTAL VALUES

MOBILITY	TIME
5.00×10^{-5} cm/sec/volt/cm	8 hrs
4.20×10^{-5} cm/sec/volt/cm	16 hrs
3.50×10^{-5} cm/sec/volt/cm	24 hrs
2.84×10^{-5} cm/sec/volt/cm	28 hrs

TABLE VII

GELATIN STRIPS DYED WITH BROM PHENOL BLUE.
MEASURED DENSITOMETRICLY AT 585 MILLIMICRONS

OPTICAL DENSITY	NUMBER OF STRIPS
0.1	1
0.2	2
0.3	3
0.4	4
0.5	5
0.6	6
0.7	7
0.8	8
0.9	9
1.0	10
1.1	11
1.2	12

TABLE VIII

BROM PHENOL BLUE ABSORPTION
AT 585 MILLIMICRONS

CONCENTRATION	OPTICAL DENSITY	% ABSORBED
0.1000	1.840	98.6
0.0750	1.660	97.8
0.0500	1.400	96.0
0.0333	1.200	93.7
0.0111	0.690	80.0
0.0033	0.400	60.0
0.0011	0.185	35.0
0.0003	0.075	16.0
0.0001	0.025	6.0

TABLE IX

BROM PHENOL BLUE ABSORPTION DATA
FOR THE STANDARD CURVE

CONCENTRATION	1/CONCENTRATION	% ABSORBED	1/% ABSORBED
0.1000	10.0	98.6	0.0101
0.0750	13.3	97.8	0.0102
0.0500	20.0	96.0	0.0104
0.0333	30.0	93.7	0.0107
0.0111	90.0	80.0	0.0120
0.0033	320.0	60.0	0.0167
0.0011	900.0	35.0	0.0286
0.0003	3000.0	16.0	0.0625
0.0001	9000.0	6.0	0.1667

TABLE X

BROM PHENOL BLUE ABSORPTION DATA
MEASURED AT 585 MILLMICRONS

CONCENTRATION	% ABSORBED	CONC./% ABSORBED $X10^{-4}$
0.1000	98.6	1.010
0.0750	97.8	0.766
0.0500	96.0	0.522
0.0333	93.7	0.356
0.0111	80.0	0.139
0.0033	60.0	0.056
0.0011	35.0	0.032
0.0003	16.0	0.021
0.0001	6.0	0.016

TABLE XI

BROM PHENOL BLUE CONCENTRATION
AND AREA UNDER THE CURVE DATA

CONCENTRATION	AREA	OPTICAL DENSITY
0.1000	4.32	1.840
0.0750	3.90	1.660
0.0500	3.39	1.400
0.0333	2.37	1.200
0.0111	1.21	0.690
0.0033	0.65	0.400
0.0011	0.30	0.185
0.0003	0.14	0.075
0.0001	0.06	0.025

TABLE XII

BROM PHENOL BLUE CONCENTRATION
AND AREA UNDER THE CURVE DATA

CONCENTRATION	1/CONCENTRATION	AREA	1/AREA
0.1000	10.0	4.50	0.222
0.0750	13.3	3.96	0.252
0.0500	20.0	3.37	0.296
0.0333	30.0	2.38	0.420
0.0111	90.0	1.23	0.810
0.0033	300.0	0.66	1.511
0.0011	900.0	0.31	3.244
0.0003	3000.0	0.12	8.512
0.0001	9000.0	0.05	19.867

TABLE XXXI

RELATIONSHIP OF OPTICAL DENSITY TO
% TRANSMISSION AND % ABSORBED

OPTICAL DENSITY	% TRANSMISSION	% ABSORBED
1.80	1.6	98.4
1.50	3.2	96.8
1.00	10.0	90.0
0.80	15.8	84.2
0.60	25.1	74.9
0.40	39.8	60.2
0.30	50.1	49.9
0.20	63.1	36.9
0.10	79.4	20.6
0.05	89.1	10.9
0.00	100.0	0.0

OPTICAL DENSITY == - log % Transmission

% Transmission == 100 - % Absorbed

TABLE XIV

ALBUMIN ABSORPTION DATA

CONCENTRATION GLOBULIN	OPTICAL DENSITY	% TRANSMISSION	% ABSORBED
4.000	2.05	0.9	99.1
2.000	1.75	1.8	98.2
1.000	1.20	6.3	93.7
0.500	0.63	23.4	76.6
0.250	0.32	47.8	52.2
0.125	0.18	66.0	34.0
0.062	0.10	79.4	20.6

TABLE XV

GLOBULIN ABSORPTION DATA

CONCENTRATION GLOBULIN	OPTICAL DENSITY	% TRANSMISSION	% ABSORBED
3.000	1.55	2.8	97.2
1.500	1.10	7.7	92.3
0.750	0.74	18.2	81.8
0.375	0.37	42.6	57.4
0.187	0.23	58.9	41.1
0.094	0.11	77.6	22.4
0.047	0.06	87.1	12.9

TABLE XVI

ALBUMIN STANDARD CURVE DATA

CONCENTRATION	1/CONCENTRATION	% ABSORBED	1/% ABSORBED
4.000	0.25	99.1	0.0101
2.000	0.50	98.2	0.0102
1.000	1.00	93.7	0.0107
0.500	2.00	76.6	0.0130
0.250	4.00	52.2	0.0191
0.125	8.00	34.0	0.0294
0.062	16.00	20.6	0.0485

TABLE XVII

GLOBULIN STANDARD CURVE DATA

CONCENTRATION	1/CONCENTRATION	% ABSORBED	1/% ABSORBED
3.000	0.33	97.2	0.0103
1.500	0.67	92.3	0.0108
0.750	1.33	81.8	0.0122
0.375	2.67	57.4	0.0174
0.187	5.33	40.0	0.0250
0.094	10.67	22.4	0.0446
0.047	21.33	12.9	0.0775

TABLE XVIII

ALBUMIN CONCENTRATION AND AREA
UNDER THE CURVE DATA

CONCENTRATION	1/CONCENTRATION	AREA	1/AREA
4.000	0.25	15.81	0.063
2.000	0.50	12.31	0.081
1.000	1.00	8.00	0.125
0.500	2.00	3.22	0.310
0.250	4.00	1.85	0.540
0.125	8.00	0.96	1.041
0.062	16.00	0.54	1.851

TABLE XIX

GLOBULIN CONCENTRATION AND AREA
UNDER THE CURVE DATA

CONCENTRATION	1/CONCENTRATION	AREA	1/AREA
3.000	0.33	11.01	0.090
1.500	0.67	8.15	0.122
0.750	1.33	4.86	0.205
0.375	2.67	2.55	0.392
0.187	5.33	1.43	0.699
0.094	10.67	0.72	1.388
0.047	21.33	0.41	2.439

TABLE XX

TWO DIMENSIONAL MOVEMENT OF THE

DINITROPHENYL AMINO ACID DERIVATIVES

DINITROPHENYL DERIVATIVE	MOVEMENT (S)	1/S	MOLECULAR WEIGHT
Alanine	3.5cm	.286	255
Serine	3.2cm	.312	271
Leucine	3.0cm	.333	297
Methionine	2.8cm	.357	315
Phenylalanine	2.6cm	.384	331

TABLE XXI

IONOGRAPHIC MOVEMENT OF THE

DINITROPHENYL AMINO ACID DERIVATIVES

DINITROPHENYL DERIVATIVES	MOVEMENT (S)	1/S	MOLECULAR WEIGHT
Alanine	1.2cm	.833	255
Serine	1.3cm	.769	271
Leucine	1.3cm	.769	297
Methionine	1.1cm	.909	315
Phenylalanine	0.9cm	1.111	331

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