PAPER CHROMATOGRAPHY OF PROTEINS

FRANKLIN

PAPER CHROMATOGRAPHY OF PROTEINS

FRANKLIN

PAPER CHROMATOGRAPHY OF PROTEINS

by

Arthur Edmund Franklin

A thesis submitted to the Faculty
of Graduate Studies and Research
of McGill University in partial
fulfilment of the requirements for
the degree of Doctor of Philosophy.

McGill University
Montreal

May, 1951

Acknowledgments

I am very grateful to Professor J.H. Quastel, Director of the Montreal General Hospital Research Institute, for his direction and guidance during the period of this work.

My sincere thanks to Canada Packers, for their three-year fellowship, without whose help this work could not have been carried out. My thanks also to the National Cancer Institute for a grant towards this work.

My thanks to the Montreal General Hospital and the Montreal General Hospital Research Institute for their co-operation.

I am indebted to Professor C.P. Leblond and Dr. J. Gross, of the Department of Anatomy, McGill University, to Dr. Roy L. Swank, of the Montreal Neurological Institute, and to Dr. J.S. Foster, Director of the Radiation Laboratory, and Mr. F. Eadie, for their collaboration during a great part of this work.

I wish to acknowledge the technical assistance of Mrs. Mary Hersey, Miss Rose Mamelak and Miss Aagot Grimsgaard during the studies on Multiple Sclerosis. I also wish to acknowledge the work of Mr. Leo Diamond, of the Montreal General Hospital, for doing the photographic work during part of these studies.

My thanks to the Cutter Laboratories, Berkeley, California, for the samples of the Cohn Protein Fractions, and to the Atlas Powder Co. for their supplies of synthetic detergents.

I wish to thank Miss Thelma Watts for the typing of this manuscript.

TABLE OF CONTENTS

Chapter 1

HISTORY OF CHROMATOGRAPHY

"The invention of a new, specialized laboratory procedure brings about rapid conquests in new fields of science and technology; finally it exhausts itself and is replaced by a still more practical method. The method of chromatographic adsorption, invented by the talented Russian botanist, Prof. M. Tswett, makes possible spatial separation of the components of a mixture. It is just now at the beginning of a brilliant development; it offers a simple experimental procedure to the investigator, especially in the field of both pure and applied organic chemistry, of biochemistry and physiology."

L. Zechmeister. 1938.

Chromatography is probably the most recent analytical technique to be adopted by laboratories in almost every part of the world. The extremely simple apparatus that is required, a covered container, a cylinder of filter paper and a simple spray, has made the technique of paper chromatography available to every laboratory. The rapid, efficient and accurate results which have been obtained, have resulted in its application to a wide variety of problems. A complex mixture of rare earths can be separated into spectroscopically pure components in a single operation. This was formerly only possible after about a thousand recrystallizations. A protein hydrolysate can be chromatographed in one procedure, to show the presence of all the amino acids that can be demonstrated by any other known technique. It is due to facts such as these, that this technique has been so widely applied in almost every field of applied and pure research.

The original ideas in paper chromatography are to be found in a paper by Schonbein in 1861 (1), in which he described the separation

of substances on filter paper. According to this method, one end of strips of filter paper were placed in solutions of the materials to be resolved. As the liquid was drawn into the filter paper by capillary forces, the substances gradually separated from one another, and formed a series of bands.

The real start of chromatographic analysis must however be credited to Schonbein's pupil, Goppelsroeder, who carried out numerous researches on capillary analysis prior to 1906 (2). His successful work was carried out on strips of filter paper or textile materials, since his experience with solid adsorbents was unsuccessful. Reed, in 1893, first published a technique for chromatography on columns, having separated alkaloids from organic sources on a column of powdered kaolin (3). Tswett, who was probably influenced by Goppelsroeder, introduced the idea of column development with a pure solvent, and thus made the technique a potential analytical tool (4).

In 1906, Tswett described the separation of a mixture of pigments on an adsorbent of powdered calcium carbonate. He stated that the adsorbent, which is saturated with one substance, is still able to take and bind a certain amount of another. However, at the same time, substitutions can also take place. The petroleum ether solution of the plant pigments was resolved on the adsorbent according to the adsorption sequence, since the more strongly adsorbed pigments displaced the weaker adsorbed ones and forced them down the column. This separation became practically complete if a stream of pure solvent was passed through the adsorbent column. The adsorp-

tion phenomenon was not restricted to the chlorophyll pigments, and he also investigated lecithin, alkannin, prodigiosin, sudan, cyanin, solanorubin and acid derivatives of the chlorophylls.

Carotenes passed directly through the column with no adsorption at all, and bands of other pigments moved slowly down the column. When a good separation had occurred, the adsorbent was extruded from the column, and the bands separated from each other with a knife. Pigment fractions were washed from the adsorbent by means of alcohol. Thus, by a single operation, Tswett showed the components of a mixture separated from each other.

The bands of substances observed by Goppelsroeder on filter paper were analogous to those observed by Tswett on the calcium carbonate column. Only in the last decade has it been realized that the principles involved in this so-called 'capillary analysis' are identical with those in 'chromatographic analysis'.

At the same time that these experiments were being carried out in Europe, Day in America (5) in 1897 wrote: ". . . by experimental work, it may easily be demonstrated that if we saturate a limestone, such as the Trenton limestone, with the oils characteristic of that rock, and exert slight pressure upon it, it is easy to change it in its color to oils similar in appearance to the Pennsylvania oils, the oil which first filters through being lightest in color and the following oils growing darker". By 1908, Day had induced Gilpin and his collaborators (6) to carry out detailed experiments, by means of which they showed that if crude oil is forced upward through a column of Fuller's earth, the following sequence can be noted from top to

bottom: saturated aliphatic hydrocarbons, then aromatics and unsaturated substances, and finally nitrogen and sulfur compounds, and that amounts of each substance increased towards the bottom 'because of selective adsorption'.

Palmer in 1922 (7) carried out extensive chromatographic studies, and it is evident from his remarkable monograph on carotenoids that he realized the importance of the method. In 1931, Kuhn and Lederer (8,9) resolved plant carotenes into several components, fulfilling Tswett's twenty-one year old prophecy that 'very likely carotene is not a chemical entity but a mixture of two or more homologues which it may be possible to separate from each other by means of adsorption methods . . .'.

Liquid-liquid partition chromatography was introduced with success by Martin et al in 1941 (10,11,12,13). In this technique, the adsorbent is essentially a support for a static liquid phase, while a mobile liquid phase flows through the adsorbent. The authors used specially prepared silica gel as the adsorbent, and an aqueous phase as the non-mobile component. The acetoamino acids were separated on this adsorbent when the mobile phase of butanol-chloroform mixtures was saturated with the aqueous phase. On the other hand, Consden et al (14) in 1944, made use of filter paper strips for the resolution of mixtures, in a manner similar to that described by Goppelsroeder. Here, the non-mobile phase was water (in the cellulose of the filter paper) and the mobile phase was phenol or collidine, which was saturated with water.

Late in 1950, James and Martin (15) introduced a variation of

partition chromatography, in which they presented a technique for liquid-gas partitioning of a mixture. The non-mobile phase in this case was liquid paraffin-stearic acid on an adsorbent of kieselguhr. A stream of nitrogen was forced through the kieselguhr as the mobile phase. This was used to obtain an improved separation of the volatile fatty acids.

Consden, Gordon, Martin and Synge appear to have stimulated the current interest in chromatographic analysis. Column and paper chromatography have been applied to the resolution of complex mixtures in almost every field of industrial and pure research. Within the last decade, well over a thousand papers have been published on chromatography, and with the continued interest being shown in this technique, it is certainly probable that the technique will be vastly improved, and applied as a routine laboratory tool for many years to come.

Chapter 2

INTRODUCTION TO CHROMATOGRAPHY

"A good technique sometimes renders more service to science than the elaboration of highly theoretical speculations".

Claude Bernard

OBJECTIVES.

The evident advantages of chromatographic analysis have been recognized in many fields due to its relatively simple technique, and the speed with which results can be obtained. Among the aims of the technique are: resolution of mixtures into their constituents; determination of the homogeneity of chemical substances; comparison of substances suspected of being identical; purification of substances; concentration of materials from dilute solutions; recognition and control of technical products; determination of molecular structure; combination with electrophoretic separations; regeneration of substances from complex addition compounds.

PRINCIPLES.

The method of separating substances by processes based upon solubility relationships is based on one of two main principles:

I. The phenomenon of Adsorption. This is essentially a device which permits the spatial separation of differently adsorbed materials (e.g. plant pigments) on a single adsorbent (e.g. calcium carbonate). On the column, the constituents with a high affinity for the adsorbent displace those having a weaker affinity. Thus the substances arrange themselves on the column in the order of their adsorption affinities,

and the bands move down the column on washing because the adsorption is reversible.

II. Partitioning between two solvents. Separations depend on differences of solubility of the substances to be separated between two liquid phases. If, for example, the two liquid phases are butanol saturated with water, then it is evident that oxalic acid will be present in a greater amount in the aqueous phase, and another organic acid, such as fumaric acid, will be present in greater amount in the butanol phase. This difference in distribution between the two phases permits their separation either on a column or on a paper chromatogram.

COMPONENTS OF THE CHROMATOGRAPHIC SYSTEM.

I. Adsorbent. In column chromatography, a vertical glass tube is packed with an adsorbent. The choice of adsorbent is largely empirical, but it should possess certain properties, namely, it must be insoluble in the solvents used, it must not react with the substances filtered through the column, it must have a certain amount of adsorption activity to hold the constituents of the mixtures to be analyzed, it should be colorless to facilitate the observation of zones, it must be reasonably priced and recoverable, and it must be such that identical results can be obtained.

No universal adsorbent has yet been found, nor has a perfect adsorbent for any given purpose been developed. Some examples of adsorbents, listed in the increasing order of their activation, are: sucrose, starch, inulin, magnesium citrate, talc, sodium carbonate, potassium carbonate, calcium carbonate, calcium phosphate, magnesium

silicates, activated alumina, charcoal, magnesia and Fuller's earth. The adsorption capacities of all solids depend upon the solvents used for the adsorption. Adsorption is greatest from saturated hydrocarbons, less from unsaturated and cyclic hydrocarbons, chlorinated hydrocarbons, ketones and esters, and still less from alcoholic solutions and nitrogenous bases, and least from acid and basic ones. According to Zechmeister and Cholnoky (16), the particles of most adsorbents vary in size from one to ten microns.

The adsorptive affinity of an adsorbent depends chiefly upon the structure of the substance to be analyzed; the more polar the substance is, the more strongly will it be adsorbed. The common functional groups, in approximate order of decreasing polarity are carboxyl, hydroxyl, nitro, azo, carbonyl, amino and phenyl. The presence of conjugated systems considerably augments the adsorptive affinity, and the adsorbability of compounds with the same functional group increases with the molecular weight.

II. Developing Solvent. When a solution to be analyzed has been placed on the adsorbent, a solvent, or mixture of solvents, is poured over the column to separate the constituents of the mixture. The process is known as development, and the solvents used during this process are called the developing solvents.

The choice of a developing solvent is determined to a large extent by the solubility of the materials to be adsorbed and by the activity and insolubility of the adsorbent in the solvent chosen. The following solvents are arranged in approximate order of their effect on adsorption (adsorption is greatest from those materials listed

first in the series): petroleum ether b.p. 30-50°, 50-70°, 70-100°, carbon tetrachloride, cyclohexane, carbon disulfide, ether, acetone, benzene, toluene, esters of organic acids, alcohols, water, pyridine, organic salts, mixtures of acids or bases with water, alcohol and pyridine. The former are more likely to be used as developing solvents, the latter as eluants, depending upon the activity of the adsorbent and upon the nature of the adsorbed compounds. It must be pointed out that the pH of a developing solvent is of the utmost importance, as a relatively small change in pH may mean the difference between strong adsorption and complete elution.

III. R_F and R_M Values. R_F values are used in paper chromatography to designate the position to which a substance will move under specified conditions. They are expressed as the ratio of the distance to which a substance moves to the distance to which the solvent moves. A substance which moves with the solvent front thus has an R_F value of unity. The R_F values for any given substance will vary depending on the type of developing solvent used, the nature of the filter paper, and to a lesser extent on the temperature at which the experiment is carried out. R_M values were proposed in 1950 by Bate-Smith and Westall (17) and are expressed as $\log\left(\frac{1}{R_F} - 1\right)$. These values vary directly with the partition coefficient* and also, in many cases, change by equal increments with each successive addition of a particular substituent group, and thus are likely to be more useful for

*The partition coefficient is expressed as the ratio of the amount of solute present in the organic phase to the amount of solute present in the aqueous phase.

many purposes than the R_F values.

METHODS.

I. Ion Exchange. The practical difference in the function of an adsorbent proper and an exchange adsorbent is that, in the latter instance, the process necessarily involves a release of some constituent from the solid surface to the solvent. Synthetic resin, 8-OH quinoline, permutit, zeolite and amberlite are some of the adsorbents used. The relative adsorption affinities of ions appear to depend primarily on valence, degree of hydration and basicity (18). It was found possible to separate, in a comparitively small number of operations, various mixtures of rare earths into spectroscopically pure substances. To obtain a similar result by older methods would require as many as a thousand fractional recrystallizations. Diphosphopyridine nucleotide has recently been separated from yeast in a purified state by adsorption on Norit, followed by chromatography on Dowex-2 ion exchanger (199).

II. Boundary Method. Tiselius (19) introduced the chromatographic boundary method, in which a solution is forced upwards through an adsorbent, under conditions which make possible a continuous optical characterization of the emerging liquid by means of a device which records the refractive indices. The individual compounds have characteristic retention volumes and show a sudden break-through. The technique required elaborate apparatus and an advanced technique. A great disadvantage is the effect of small changes in temperature, which give rise to convection currents, thus making an accurate optical record difficult to be obtained.

III. Partition Chromatography.

A. Liquid-Liquid Chromatography. This method has probably received most attention since the introduction of the chromatographic technique. A solid column is essentially a support for a static liquid phase, while a mobile liquid phase flows through the adsorbent. Here, a great number of consecutive partitions of the substance occur between the two liquid phases, and chromatographic separations, either on paper or in columns, depend on repeated partitions of the solute between the liquid phase on the adsorbent and the mobile developing solvent. This repeated partition can only take place when there is a dynamic equilibrium among the solute, the adsorbent and the solvent. A single solute molecule migrates in an adsorption column only while it is in solution. When it is held by the adsorbent, it remains stationary as the solvent flows past. The stronger it is held by the adsorbent, the slower it migrates. If two solute molecules differ in their affinity for the adsorbent, they will migrate through an adsorption column at different rates.

(i) Column Chromatography. Martin and Synge in 1941 (10) first used silica gel as the adsorbent, and butanol-water as the mobile-non-mobile phase for the separation of the acetoamino acids. Later, Moore and Stein, in 1944, demonstrated that free amino acids could be separated on potato starch columns (20). Column chromatography has been applied to the determination of organic acids in fruit by Isherwood in 1946 (21), who used specially prepared silica gel. Since this time, column chromatography has been widely applied to many fields with notable success. The advantages of this type of chromatography are that

the constituents of a mixture can be recovered in a pure state and then can be estimated by the usual chemical techniques.

(ii) Paper Chromatography. Consden, Gordon and Martin (14) followed up their brilliant work on column chromatography by adapting their partition technique to filter paper, which replaced silica gel as the adsorbent. The developing solvents passed over it in the same manner as in column development. Single dimensional chromatography implies that one solvent only is used to separate the mixture applied to the paper, whereas two dimensional chromatography indicates that a second solvent is run at right angles to the direction of flow of the first solvent. In this manner, very minute quantities of very complex mixtures can be separated in a simple and effective manner, something that would be almost impossible by any other method. Dent published an amino acid map, which showed the separation of the more commonly encountered amino acids, using phenol as the developing solvent in the first dimension, followed by collidine in the second dimension (22). Position of the resulting amino acids was shown by spraying the paper with a ninhydrin solution and gentle heating. The Consden, Gordon and Martin paper technique is known as the descending method, due to the fact that the paper strip was suspended from a small trough which contained the developing solvent. A capillary ascending technique was introduced by Williams and Kirby (23) in 1948, in which a cylinder was formed by the filter paper, and placed in a covered dish, which contained the developing solvent.

(iii) Alumina-impregnated filter paper chromatography. Datta et al (24) used filter paper dipped in colloidal alumina to separate

Vitamin A alcohol, Vitamin A esters, retinene and other Carr-Price chromogens.

(iv) Electrophoresis on paper. Voltage applied to filter paper chromatograms was described by Haugaard and Kroner in 1948 (25), in which a piece of buffer-dipped filter paper with metal electrodes woven into the paper was used. They were able to achieve a one dimensional separation of amino acids which usually required a two dimensional treatment. The charged amino acids moved towards the oppositely charged pole, while the neutral amino acids remained in the centre of the chromatogram. Biserte (26) reported a slightly more elaborate apparatus for the separation of amino acids and peptides on paper under the influence of an electric field.

(v) The Chromatopile. Mitchell and Haskins in 1949 (27) demonstrated the separation of a mixture of adenine, tryptophan, phenylalanine, p-aminocinnamic acid and anthranilic acid on a chromatopile. The mixture to be analyzed was taken up by fifty sheets of filter paper having a diameter of 9 cm. Forty sheets of untreated paper were placed on top of them. These ninety sheets composed the upper part of the chromatopile, in which the total number of sheets of filter paper was 865. They were placed in a battery jar under slight compression, and developed with an acid-butanol mixture for 28 hours. Recovery of the separated components was 223 out of an original 250 mg. of mixture. This method was later used to separate the constituents of taka-diastase (28) by the same authors. The advantage of the chromatopile is that relatively large quantities of substances can be recovered from a mixture in an almost quantitative manner. Zechmeister in 1951

(203) described the preparation of a column packed with precision-cut filter paper discs, which are cut to fit the glass tube being used.

(vi) Continuous development chromatography. Continuous development in paper chromatography was introduced by Miettinen and Virtanen (29), who used a thick pad of cotton wool or cellulose tissue to soak up the developing solution from the end of the filter paper. The longer period of development permitted the separation of the slower moving fractions.

(vii) Large Scale Paper Chromatography. Yanofsky et al (30) suggested the use of a revolving drum, like a kymograph, to apply to the paper a relatively large aliquot (five ml as opposed to the conventional 0.01-0.02 ml) with a micropipette in less than fifteen minutes, and at the same time maintain a narrow band. This allowed the recovery of chromatographically pure substances in a rapid, effective manner. An even, narrow band eliminates any variation in movement of the band, since it is known that the rate of movement depends to some extent on the initial concentration of the substance to be analyzed.

(viii) Reversed Phase Partition Chromatography. The more commonly used type of partition chromatography, as described before, uses an adsorbent which holds the aqueous phase, while the organic phase flows over it. This is excellent for the separation of water soluble materials, but is impractical for those that are water insoluble. Therefore, Partridge and Swain in 1950, (31) introduced a reversed phase partition chromatogram in which the less polar solvent is held on an inert support, e.g., cellulose acetate, rubber powder or kieselguhr

impregnated with silane. The authors used a commercial chlorinated rubber (Alloprene) for the separation of 2:4-dinitrophenyl derivatives of amino acids, and partitioned them between butanol and an aqueous buffer.

Winteringham et al in 1950 (187) studied the metabolism of the radioactive bromine analogue of DDT by reversed phase partition chromatography. Vaseline-coated filter paper was used to hold the non-mobile phase, and the development was carried out with an ethanol-water-ammonia mixture. In 1951, Partridge et al (196) described the separation of alkaloids on a powdered glass column, using a similar technique.

(ix) Counter-Current Distribution. In 1944 Craig (32,33) also introduced a new technique for the study of the relative distribution of constituents between two liquid phases, which he termed 'counter-current distribution'. A total of twenty tubes was used, which might be considered as a series of separatory funnels. Two liquids were mutually saturated with each other and placed in each tube, the upper part of which was made to fit over the lower part of the adjacent tube, thus a series of nineteen equilibria or 'plates' were accomplished. He described the technique as a method and apparatus for the precise study of the distribution characteristics of small amounts of organic compounds between two liquid phases, being well suited for fractionation, proof of homogeneity and identification of amino acids and peptides. Barry et al in 1951 (198) have described the separation of normal fatty acids having a chain length of five to eight carbon atoms, by use of a counter-current technique.

B. Liquid-Gas Partition Chromatography. The preceding sections show examples of liquid-liquid partition chromatograms, but James and Martin in 1950 (15) introduced a liquid-gas partition chromatogram. The ratio of vapor pressure of two members of a homologous series is usually greater than the ratio of partition coefficients between two liquid phases, therefore, a gas-liquid partition chromatogram should lead to a better separation of the two compounds. This is the case for the volatile fatty acids, when using a chromatogram containing a static liquid phase and a mobile gas phase of nitrogen. In general, the substances investigated emerged from the column in order of the boiling points, except in cases where the energy of association with the stationary solvent differed markedly from the energy of association of the pure substances. Kieselguhr was used as the support for the stationary liquid phase, which was liquid paraffin-stearic acid, and a stream of nitrogen was applied as the gaseous phase.

IV. Location of Substances. In the case of substances chromatographed on the column, the problem of location or detection of the material being investigated is easier than with paper chromatography, for in the latter, much smaller quantities can be dealt with. Thus, for all practical purposes, the smallest quantities of substances that may be detected by chromatography is directly related to the sensitivity of the reaction used to locate the zones and spots.

A. Ultra-violet light. Some substances such as the flavanoid pigments, riboflavin, rutin, etc. are naturally fluorescent, while others such as sugars, may be sprayed with materials to cause fluorescence (34,35). Ultra-violet light is used to detect such substances.

B. Brush or Spray Method. Routine chemical tests that are of a sensitive nature may be used for the detection of spots or zones; ninhydrin for amino acids (36), ammoniacal silver nitrate for sugars (37), pH indicators for organic acids (21), hydrogen sulfide for metals (38), m-dinitrobenzene for progesterone (39), Carr-Price reactions for Vitamin A and similar polyenes (24), sulfanilic acid for purines (40,41) and dichlorophenol indophenol for ascorbic acid (42).

Zechmeister and Rohdewald showed the position of enzymes on powder columns by painting a longitudinal streak with a brush carrying the solution of the corresponding substrate. After a brief incubation period, a second brush applied a color reagent for detection of the enzymic cleavage product (200).

C. Bio-autographic technique. The bio-autographic technique has proven to be very useful for the detection of substances such as Vitamin B_{12} (43), and penicillin (44). The eluant or filter paper strip is placed against an innoculated agar plate, and the area of growth or inhibition of growth of the organism can be observed.

D. Radioactivity Detection. Substances possessing radioactivity can be detected by the use of a Geiger-Müller tube (45) or by placing the chromatogram against X-ray film, thus obtaining a radio-autograph (46).

E. Electronic Image Converter. Harvalik (47) introduced the electronic image converter, which enabled observations of textures and color reactions when the adsorption column was illuminated by an infra-red source. The converter employed an image tube which converted infra-red to visible light, and also permitted observations

with ultra-violet light.

V. Estimations of Substances. In the case of column chromatography, estimations are relatively easy, as the substances eluted may be estimated in routine chemical procedures. The amounts applied in paper chromatography are so minute that their estimation constitutes a real problem. Some ingenious methods have been employed for quantitative determination with varying success.

A. Colorimeter. Amino acids may be allowed to react with ninhydrin under controlled conditions, then measured colorimetrically, with errors of 3-5% in 0.1 mg. quantities (36). α-keto acids may be estimated photometrically as their 2,4-dinitrophenylhydrazones (48).

B. Spectrophotometer. Lacourt et al (49) estimated nickel and cobalt, and later aluminum, iron and titanium quantitatively (50). Awapara measured amino acids (51) spectrophotometrically. Vischer et al (52) measured the separated nucleotides quantitatively, and adenine and guanine were estimated by Edman et al (53). Gage and Wender estimated rutin and quercitin (54).

C. Bio-autography. Penicillin was determined by Hooper et al (44), who incubated trays which had been innoculated with Staphylcoccus aureus. The percent penicillin present was determined from the maximum widths of inhibition zones of two strips with different volumes of penicillin solutions placed on one tray.

D. Bio-assay. Woodruff and Foster (55) detected 0.002γ of Vitamin B_{12} by microbiological assay.

E. Planimeter. Fisher et al (56) measured the area of amino acid spots with a planimeter, and noted that the area was linearly

related to the logarithm of the amount of amino acid present. A 2% error was detected when 0.3-3.0γ of amino nitrogen was applied to the paper. This method can also be used for sugars.

F. Planimeter-Colorimeter. Histidine and tyrosine were estimated by Bolling et al (57) by treatment in an alkaline solution with freshly diazotized sulfanilic acid to produce a red color. The concentration of the amino acid was found to be proportional to the area of the spot multiplied by the color intensity.

G. Scanner. Fosdick and Blackwell (58) showed that a scanning instrument could be used for quantitative one dimensional paper chromatography. Amino acids could be estimated by the transmission of light through ninhydrin-sensitive spots. A radioactivity scanning device for paper chromatograms was devised by Boursnell (45) which had an accuracy of about 99%.

H. Micro-estimations. Hawthorne (59) estimated sugars by elution from filter paper chromatograms by the Willstatter and Schudel method with an accuracy of 95% for 40γ of sugar, and about 10γ of sugar could be estimated by the Lindstrom-Lang method.

APPLICATIONS OF CHROMATOGRAPHY.

In spite of many hindrances, more than a thousand papers have appeared during the last decade, in which successful chromatographic experiments were reported. These many data make it possible to evaluate the scope of the work. It would be impractical to attempt to list here the many aspects of the applications, but some of the trends of chromatography will be mentioned.

I. Amino Acids. Amino acids have probably received the greatest amount of attention, since the technique allows simultaneous examination of all the known amino acids. Work (60,61) has shown the presence of hitherto unknown amino acids in bacterial extracts. Protein hydrolysates have been separated both by column analysis and paper chromatography, and the results are in excellent agreement with each other. Studies have been made on hydrolysates of gramicidin by Gordon et al (62), circulin by Peterson and Reineke (63), silk by Polson (64), a wide variety of proteins by Tristram (65), Bence-Jones protein by Roberts et al (66), oxytocic hormone by Pierce and du Vigneaud (67), tobacco mosaic virus by Stepka and Takahashi (68), ferritin by Gabrio and Tishkoff (69) and enzyme digests of insulin by Phillips (70). Consden and Gordon have studied partial hydrolysates of wool (71), and identified thirteen dipeptides, concluding that most of the mono-amino-monocarboxylic acids were combined both through their amino and carboxyl groups to residues of cystine. Culture filtrates and extracts of micro-organisms have been chromatographed in metabolism studies by Woiwood, Work (72,73). Woiwood (74) has shown that tyrosine is present in the toxin, but not in the toxoid hydrolysate of diphtheria, and that there were no other major differences. The application to pathological conditions was carried out by Dent, who showed an amino aciduria in the Fanconi Syndrome, which was due to lowered renal thresholds (22). The structure and sequence of amino acids in insulin was studied by Sanger (75), who used 1:2:4-fluorodinitrobenzene to identify the free amino groups, and separation of the complexes on silica gel. Phillips and Stephen (76) have extended

Sanger's work, using paper chromatography to separate the peptides of insulin (after chymotrypsin digestion), which varied in size up to hepapeptides.

II. Thyroxin and its Analogues. The uptake of I^{131} by the thyroid has been shown by several groups of workers. Tishkoff et al (109) have shown the presence of several iodinated amino acids in thyroid hydrolysates of rats injected with I^{131}. Gross et al (46) have shown the presence of iodide, thyroxine, di- and mono-iodotyrosine in unhydrolyzed extracts of thyroid, using paper chromatography. Three unidentified, iodinated compounds were also detected in the thyroid. The same authors have shown the presence of thyroxin and iodide in plasma extracts, which led to a postulation of the nature of the circulating thyroid hormone.

III. Organic Acids. Fatty acids by Peterson and Johnson (77), bile acids by Siebermann (78), organic acids by Isherwood (21) and Lugg and Overell (79) and α-keto acids by Cavallini et al (80) have been separated, and have been shown to play an active role in elucidating the structural composition and importance of organic acids in plants and animals. Henderson and Hirsch (81) showed the presence of quinolinic acid in the study of tryptophan metabolism, and Norris and Campbell (82) have shown that gluconic acids occurred as intermediates in the metabolism of Pseudomonas aeruginosa.

IV. Antibiotics. A great deal of work has been carried out on the study of penicillin by Hooper et al (44) and Boon et al (83), separating complex commercial mixtures into its component parts, and thus discovering which penicillin zone had the most effective anti-

biotic activity. Other studies have been made on the detection and degradation products of chloromycetin by Smith and Worrel (84), the metabolism of radioactive pentobarbital by Roth et al (85), the detection of streptomycin by Carter et al (86), neomycin by Swart et al (87) and grifolin by Hirata and Nakanishi (88).

V. Metals. Lederer has shown the separation of many inorganic ions on paper, among them being the noble metals (89) and the chloride group anions (90). Burstall et all have carried out extensive work in showing the separation of ten groups of metals (91).

VI. Nucleic acid Structures. Nucleic acid structures have been studied with considerable success, both qualitatively and quantitatively. Ribonucleotides were separated by Chargaff et al (52,92). Purine and pyrimidine mixtures were resolved on paper by Holiday and Johnson (93) and on starch columns by Edman et al (53). Crammer (94) was able to detect on paper 0.01γ flavine nucleotides by ultra-violet light.

VII. Steroids. Steroids have presented many difficulties in chromatography due to their low solubility in aqueous solvents and their complex structure, as well as the difficulty encountered in establishing the position of very small quantities on the chromatogram. Bush in 1950 (95) showed that a separation of steroids could be achieved on alumina impregnated filter paper. Other studies have been carried out on progesterone by Haskins et al (39), urinary steroids by Lieberman et al (96), ketosteroids by Zaffaroni et al (97), cis- and trans-stilbenes by Zechmeister and McNeely (98), androgens by Johnston (99), adrenocortical hormones by Zaffaroni et al (100) and corticosteroids

by Burton et al (201). These separations have been very valuable in studies on metabolism. McMahon et al in 1950 (189) have detected and separated extremely small quantities of sterols on filter paper, the detection being made with antimony pentachloride in chloroform.

VIII. Carbohydrates. Extensive studies on the separation of closely related sugars have simplified the examination of many solutions in the fields of synthesis and analysis. Successful chromatography has been carried out on hexoses by Partridge and Westall (101), tetra-, tri-, and dimethyl fructoses by Bell and Palmer (102), sugar alcohols, glycosides, reducing sugars and non-reducing sugars by Pascu et al (103), polyhydric alcohols by Hough (104), sugar phosphates by Cohen and Scott (105), and unmethylated sugars by Boggs et al (106). Applications to metabolism have been particularly successful, by showing the formation of intermediate products in photosynthesis. Radioactive sucrose produced by algae exposed to $C^{14}O_2$ was shown by Calvin and Benson (107), and the formation of radioactive glucose and fructose by green leaves exposed to $C^{14}O_2$ was shown by Udenfriend and Gibbs (108). Gluconic and 2-keto gluconic acids were shown to be intermediates in glucose oxidation by Norris and Campbell (82).

IX. Vitamins. Chromatography of vitamins has received surprisingly little attention, probably due to the difficulty of locating the substances. However, with the introduction of the bio-autographic technique, this application will probably receive extensive attention. Studies on vitamins have been carried out on pyridoxine derivatives by Winsten and Eigen (110), ascorbic acid by Mapson and Partridge (42), riboflavin and its photolytic decomposition products by Hais and

Pecakova (111), Vitamin B_{12} and related growth factors by Winsten and Eigen (43), and Vitamin A by Eden (112).

X. Other Applications. Many other fields have received the benefits of the chromatographic technique. A few random applications only will be mentioned: the sex attractant of the female Gypsy Moth by Haller et al (113), vanillin by Bland (114), pH indicators by Lederer (115), adrenaline by Shea (116), radioactive urea by Schulman and Keating (117), tobacco alkaloids by Porter et al (118), adulterants in red wine by Mohler and Hammerle (119), creatine and creatinine by Maw (120), rutin, quercitin and isoquercitin by Howard, Gage and Wender (54,121), isotopes of potassium and lithium by Taylor and Urey (122), carcinogen (3:4-benzpyrene) from shale oil by Berenblum and Schoental (123), helvolic acid by Chain et al (124), carotenoids of milk by Thompson et al (125) and Vitamin K_1 by Binkley et al (126).

Chapter 3

SOME THEORETICAL ASPECTS OF CHROMATOGRAPHY

It has been realized for a long time that the chromatogram is closely analogous in its mode of operation to distillation and fractionating columns. Any fundamental theory should try to give a picture of the concentration of the solute at any time and place on the column, and of the way in which the resolution depends on the length of the column.

Relatively little attention has been directed towards the elucidation of the theoretical aspects of chromatography, and of this work, column chromatography has received almost all of the attention. It has been assumed that the theoretical considerations of paper partition chromatography are similar to, or identical with, those of column analysis.

Two mutually saturated solvents are used in partition chromatography to effect a resolution of a mixture. Such a resolution is brought about by the differences in distribution of the components of a mixture between the two phases. By means of the partition coefficient, usually designated by the symbol γ, the separation and behaviour of substances on a column can usually be predicted. The relation of the partition coefficient to the rate of movement of the zone may be calculated by the method of Consden et al (14). The rate of movement of the band is expressed as the 'R' value (in column chromatography), or as the 'R_F', or rate of flow value (in paper chromatography). The R value is expressed as the ratio of the move-

ment of the zone to the movement of the advancing front of solvent.

Investigations have been undertaken to determine quantitatively the manner in which 'R' depends on the variables: column position, initial volume, initial concentration and rate of flow of solvent. Martin and Synge (10) have indicated that 'R' is independent of the column position and depends, to some extent, on the initial volume and the initial concentration applied. Austin and Shipton (127) have shown, within the limits of their study, that 'R' is independent of the rate of flow of the developing solvent through the column when a constant value of rate of flow is obtained. Weil-Malherbe (128) has carried out the analogous study, in which the volume of filtrate collected before the solute first appears in the filtrate is used as a measure of the rate of movement of a zone. LeRosen (129) concluded, that in agreement with qualitative statements by other workers, the rate of movement of the front edge of the chromatographic zone on the columns, relative to that of the solvent, was found to be independent of column position, and to vary with the initial concentration, and to a less extent, with the initial volume. Theoretical calculations are in rough agreement with the observed rate of movement of these zones. The rate of movement of the trailing edge of the zones, relative to the solvent, is practically independent of column position, initial concentration, or initial volume; however, this boundary is difficult to measure because of its diffuse nature.

The diffusion of solute from one position on the column to another, must be assumed to be negligible. Peters (130) has shown that diffusion is constant through a given column except when the

ratio of the concentrations of the solution entering and leaving the column differs greatly from unity. Diffusion may be considered to be constant for the chromatograms without introducing any serious error.

A second assumption must be made, that at equilibrium the distribution ratio of one solute between the two phases must be independent both of the absolute value of its concentration, and of the presence of other solutes.

Robinson (131) described how, on developing a chromatogram in which the distribution isotherms between the moving and stationary phases for the substances undergoing analysis are linear, relatively sharp bands will result. Deviations of the isotherms from linearity leads to much spread bands with sharp fronts and elongated 'tails', while deviations in the opposite sense, when they occur, give conversely spread bands, with rear sharp edges and diffuse 'fronts'. The band rate of each substance is determined by its effective distribution isotherm between stationary particles and moving liquid.

Wilson (132) has presented a theory that may be applied to adsorption chromatograms. The adsorption isotherm has been used to express the relation between the adsorbent and the adsorbate at equilibrium. He has assumed that equilibrium is attained as an adsorbate passes through the adsorption column, that the volume of the interstices between the particles of adsorbent are negligible, and that the effects of diffusion can be neglected. On these assumptions, he has derived a differential equation that relates the migration of a band of a single adsorbed substance to the volume of solvent that

is passed through the column. He has also derived an equation to express the conditions that prevail at the boundaries of the band. When these equations are applied to the interpretation of the formation of several bands on the column, considerable difficulty is encountered because the adsorption isotherm does not take into account the effect of one adsorbate upon the adsorbability of others at varying concentrations of each one. Wilson's theory accounts qualitatively for the separations effected in chromatographic analysis, for the uniformity of the bands and for the sharpness of the bands. The theory predicts that small quantities of each substance passing through the column will remain on the adsorbent in the upper portions of the tube. It also requires that the substance with the lowest adsorbability will form the lowest band. Comparison of the adsorption isotherms of lauric and stearic acids with their separability on columns has confirmed this last requirement for several adsorbents.

Hanes and Isherwood (188) have concluded that paper chromatography need not necessarily be an adsorption phenomenon or a partitioning system, as indicated by the classical approach to these studies. They have focussed attention on the fact that in paper chromatography, the stationary phase must consist of cellulose with imbibed water, and that this imbibed water is different in important properties from liquid water. Thus it would seem an over-simplification to regard the stationary phase in such systems as a liquid phase. The problem of the mechanism of the chromatogram centers on how this water-cellulose complex holds solute molecules in competi-

tion with the flowing solvent. Such factors as size and shape of molecule, number, position and character of hydrophilic groups, will be the determinants of the specific distribution of solutes between the two phases in a given system. It may be noted that they make no reference to adsorption or partition in considering this mechanism, but state that it would seem to depend upon the formal definition of these terms whether the mechanism proposed should be considered to be adsorption on, or solution in, the water-cellulose complex.

Chapter 4

PAPER CHROMATOGRAPHY OF SOME CRYSTALLINE PROTEINS

INTRODUCTION.

The striking success of paper chromatography in the separation of relatively small molecular weight substances led to the use of this technique in a very wide range of applications. However, the highest molecular weight to be chromatographed was the insulin 'core', having a molecular weight of about 5,000, which resulted from chymotrypsin digestion (76). Therefore, it seemed possible that chromatography could be extended to the study of high molecular weight substances, such as the proteins and enzymes.

In order to accomplish this task, it was necessary to evolve a completely new technique to handle such complex structures as the proteins. It was realized that the classical types of chromatography, i.e., the use of organic solvents as developing solvents, could not be used. Also, a means of detection of small quantities of protein had to be investigated, since the usual protein tests were either insensitive to very small quantities, or the reagents were so concentrated as to make their use on paper chromatograms impractical. Furthermore, it was necessary to find a set of developing solutions that would not cause denaturation or loss of specific properties of the protein or enzyme.

It is known that the benzidine reagent has long been used for the detection of blood in medico-legal tests, due to its sensitivity to hemin, which is one part in 1,500,000. Thus, if a protein could

combine with hemin, the problem of detection would be made easier. Secondly, it is also a well known fact that proteins are insoluble in many organic solvents, thus the partitioning of proteins between an aqueous phase and an organic phase is not possible. Since proteins are soluble in aqueous solutions, it seemed reasonable to use buffers and salt solutions as developing solutions for proteins, in combination with the adsorptive capacity of the filter paper.

Thus, in this chapter, a new technique is presented for the paper chromatography of proteins, using hemin as an 'indicator' for the proteins whenever possible, and using aqueous buffer and salt solutions as developing solutions. It is also shown that the chromatographic results are in excellent agreement with the electrophoretic results, and that proteins and enzymes do not lose specific properties during this technique.

Concurrent with the chromatography of proteins reported from this Institute (46,133,134,135,136,137), came the reports of other researchers who were continuing along similar lines. Cochran (138) caused the movement of the tobacco mosaic virus on paper, detected it with the Sakaguchi-arginine reaction, and noted that the virus retained its infectivity during this process. Riley (139) applied chromatography to segregation studies of the agent of Chicken tumor 1 (Rous Sarcoma Virus), and was able to remove approximately 99% of the virus of a highly potent extract by passage through 0.5 grams of diatomaceous earth. Later Riley et al (140) extended the application to sub-cellular components in the microscopically visible range, as found in pigmented mammalian tumors. Gordon et al (141) showed

the electrophoretic migration of molecules such as hemocyanin, ferritin and hemoglobin in agar jelly. The proteins were recovered unchanged. Mitchell et al (28) described the use of the chromatopile for fractionation of an enzyme mixture such as taka-diastase. Variations in salt concentration and pH were shown to have a great influence on the movement of the enzymes (adenosine deaminase, amylase and phosphatase). Jones and Michael (142) showed that acid dyes have an affinity for protein fibres, but not for cellulose, thus making possible the detection of proteins. The union of antigen and antibody was demonstrated on filter paper by Castaneda (143). The responses produced so far have agreed with the usual agglutination reactions. Wynn and Rogers have recently shown (144) that several proteins had an R_F value of unity in phenol and zero in collidine, and that the area of the spot was approximately proportional to the concentration of the protein. Swingle and Tiselius (197) have described the chromatography of phycoerthyrin on an adsorbent of tricalcium phosphate.

MATERIALS AND METHOD.

Protein Solutions. Ten mg crystalline protein is dissolved in 1 ml distilled water or physiological saline, and to this is added 0.04 ml 0.3% hemin solution. Aliquots of 0.02 ml are applied to the paper for chromatography.

Hemin Solution. A stock solution of hemin is prepared by dissolving 30 mg hemin in 10 ml 3% sodium bicarbonate solution.

Benzidine Solution. The reagent is prepared as follows:

Equal volumes of saturated alcoholic benzidine solution and 3% hydrogen peroxide are mixed, and made acid with glacial acetic acid. Fresh solution must be prepared daily.

Developing Solutions. A search for suitable developing solutions has resulted in the exclusive use of aqueous solutions of salts and buffers. For buffers, 50 ml of a M/5 solution of the salt is adjusted to the desired pH with a N/5 solution of acid or alkali, and the volume made up to 200 ml. In the case of acetate-HCl buffers, normal solutions are used. Salt solution concentrations are usually in the region of 1-2%.

Technique. The technique of capillary ascent (22) and the use of Whatman No. 1 filter paper is used exclusively throughout this work. 0.01-0.02 ml of the protein-hemin solution is placed on the lower left hand corner of the filter paper about one inch from the sides, and allowed to dry. The paper is then formed into a cylinder, being stapled so that the edges do not touch. It is then placed in a dish, which contains the developing solution, and covered with a bell jar to enable the formation of a saturated atmosphere. When the solution has travelled a suitable distance, which requires about one hour for 20 cm of filter paper, the paper cylinder is removed and air dried at room temperature. The paper is then unstapled, and restapled at right angles to the original direction. The cylinder is then placed in the second developing solution in the same manner as before, and the solution is allowed to move about 15 cm up the paper. Once again, the paper is air-dried, then streaked with the benzidine reagent, using a small paint brush, and photographed

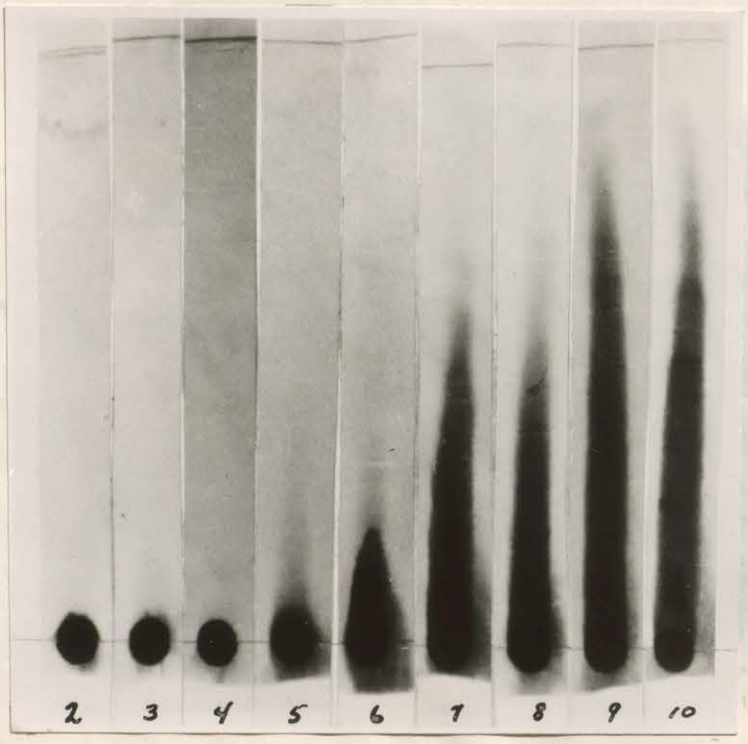


Figure 1

A composite photograph of the hemin control is illustrated, in which 0.02 ml. 0.3% hemin was diluted to 0.5 ml. with distilled water, and 0.02 ml. aliquots were chromatographed. The developing solutions were aqueous buffers from pH 2 to pH 10 at one pH unit intervals.

It was observed that hemin did not move appreciably until pH 7, and that there was no characteristic formation during its migration.

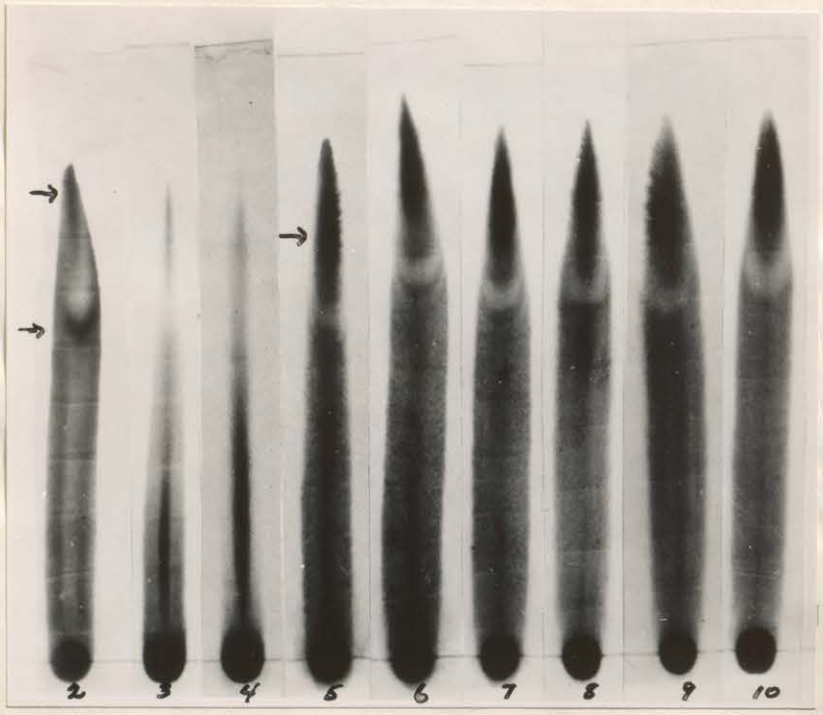


Figure 2

The migration of a bovine albumin-hemin complex on paper chromatograms is illustrated. Development was carried out with aqueous buffers which varied from pH 2 to pH 10 at one pH unit intervals.

It was observed that there were two fractions present in the leading edge of the chromatogram at pH 2, and that movement was least at pH 3 and 4. There was a well-defined leading fraction from pH 5 to pH 10.

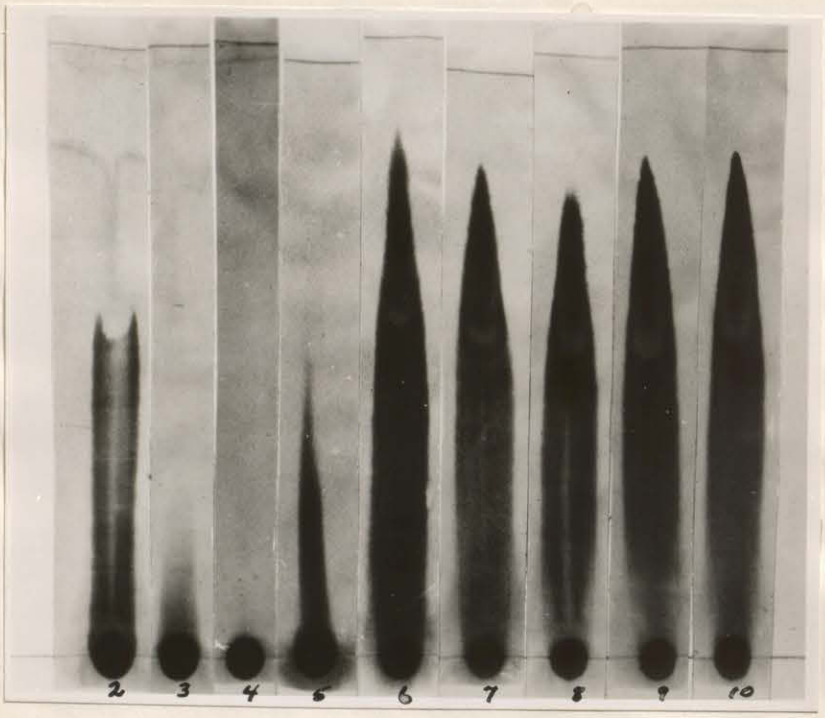


Figure 3

The migration of a β-lactoglobulin-hemin complex on paper chromatograms is illustrated. Development was carried out with aqueous buffers which varied from pH 2 to pH 10 at one pH unit intervals.

It was observed that combination with hemin was incomplete at pH 2, and that movement was least at pH 3, 4 and 5. There were well-defined leading fractions present from pH 6 to pH 10.

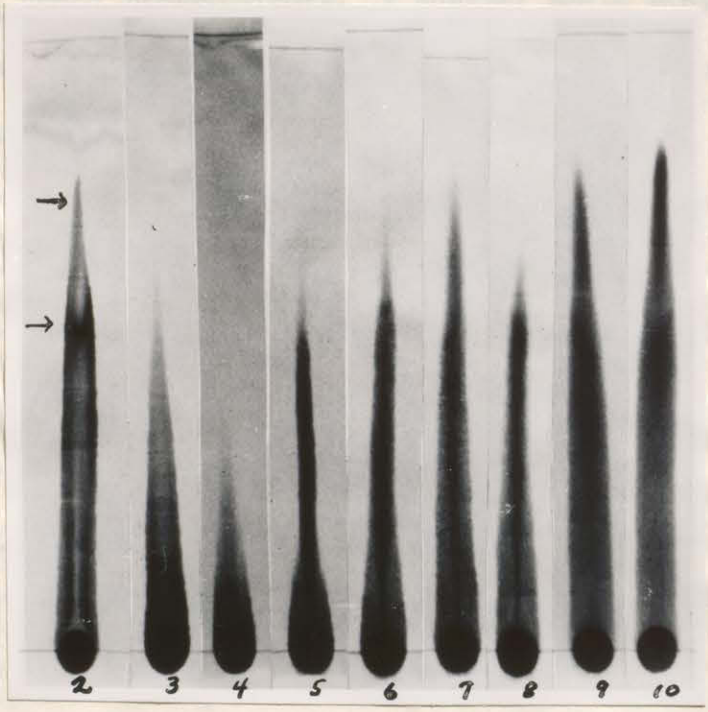


Figure 4

The migration of a γ-globulin-hemin complex on paper chromatograms is illustrated. Development was carried out with aqueous buffers which varied from pH 2 to pH 10 at one pH unit intervals.

It was observed that there were two fractions present at pH 2, and that movement was least at pH 3 and 4. Fairly distinct leading fractions were present at higher pH values.

within a few seconds. This slight delay is essential to ensure optimum color development of the protein-hemin complex.

OBSERVATIONS.

Control hemin chromatograms. A control hemin chromatogram, in which 0.02 ml 0.3% hemin was diluted to 0.5 ml with water, showed that movement was nil up to pH 5, very slight at pH 6, and that it increased very rapidly above this value (Fig. 1). Some developing solutions, among them being those that contained bicarbonate, glucose, sucrose, glycine, methionine, alanine and urea allowed maximum or near maximum movement of the hemin, indicating that combination with hemin had taken place.

The protein chromatogram. A long, rather narrow finger was formed if the protein combined with hemin. The color intensity varied along the finger, depending on the pH with which it was developed. Bovine albumin-hemin at pH 2 showed two intensely colored fractions inside the finger (Fig. 2). It may be shown that the greatest portion of the protein is concentrated in the leading fraction, and only traces remain in the finger.

One dimensional chromatography resulted in the formation of a yellow-colored solution front, which fluoresced in ultra-violet light, and at pH 2, two separated bands appeared at the leading edge of the chromatogram. Two dimensional chromatograms resulted in a series of villus-like structures appearing in the second dimension, which appeared to be due to the salts used in the developing solutions. The presence of proteins on the paper tended to depress the

full movement of these structures that were in the immediate vicinity of the protein.

It is of interest to note that very low concentrations of proteins, such as less than 80γ of bovine albumin, moved only in the first dimension. Therefore, 100-200γ of protein has been arbitrarily chosen as the minimum optimum quantity to be applied to the paper for chromatography.

Protein-hemin complexes. It has been found that some proteins, such as egg albumin, pepsin, papain, diastase and urease did not combine with hemin. However, if hemin was added to the solution of these proteins, chromatographed, and streaked with the benzidine reagent, it was noticed that the hemin remained at or near the point of origin (below pH 7). If the paper was allowed to stand for some time at room temperature, it was found that the background colored (to a varying degree, depending on the nature of the salt in the developing solution), and a colorless spot appeared. It will be shown later that this colorless region actually contained the protein. Presumably, the protein retards background color development and so produces a colorless 'spot'. Casein and β-lactoglobulin gave rise to colorless spots or fractions up to about pH 5, and to deep blue fractions at higher pH values. γ-globulin and bovine albumin gave rise to colored fractions over the entire pH range (Figs. 2,4).

Intensity of color development. It was observed that quantities of protein from about 80γ to 200γ showed different intensities of color development, indicating that the combination between protein and hemin varied with the concentration of the protein over this

range, since the amount of hemin added was constant. Above 200 γ protein, the increase in color intensity was not visually apparent. It seems probable that this could be used as a semi-quantitative means of estimation of the quantity of protein on the chromatogram.

Effect of pH on protein movement. The variation of protein movement with change in pH appeared to be much the same for all the proteins studied. At very acid values, e.g. pH 2, there usually appeared a well-defined leading section. In the region of pH 4-5, there was often little or no movement, and where movement did occur, no well-defined leading fraction occurred. Above this pH, a large, well-defined spot appeared, which was quite constant in size. The R_F values depended upon pH in a similar way, being least around pH 4, which is near the isoelectric point of some of the proteins. R_F values and sizes of the spot were remarkably constant from pH 6 and upwards.

RESULTS.

Bovine albumin-hemin complex. The R_F value of bovine albumin-hemin complex was least in the region of pH 4. At pH 2, two separated fractions appeared inside the finger. This was also true at pH 3, but the spots were smaller and less intensely colored. From pH 5-12, there was a well-defined leading fraction which was quite constant in size and position (Fig. 2).

In two dimensional chromatograms, when citrate buffer was used in the first dimension, and phthalate buffer was used in the second dimension, various results were obtained. At pH 4, there was move-

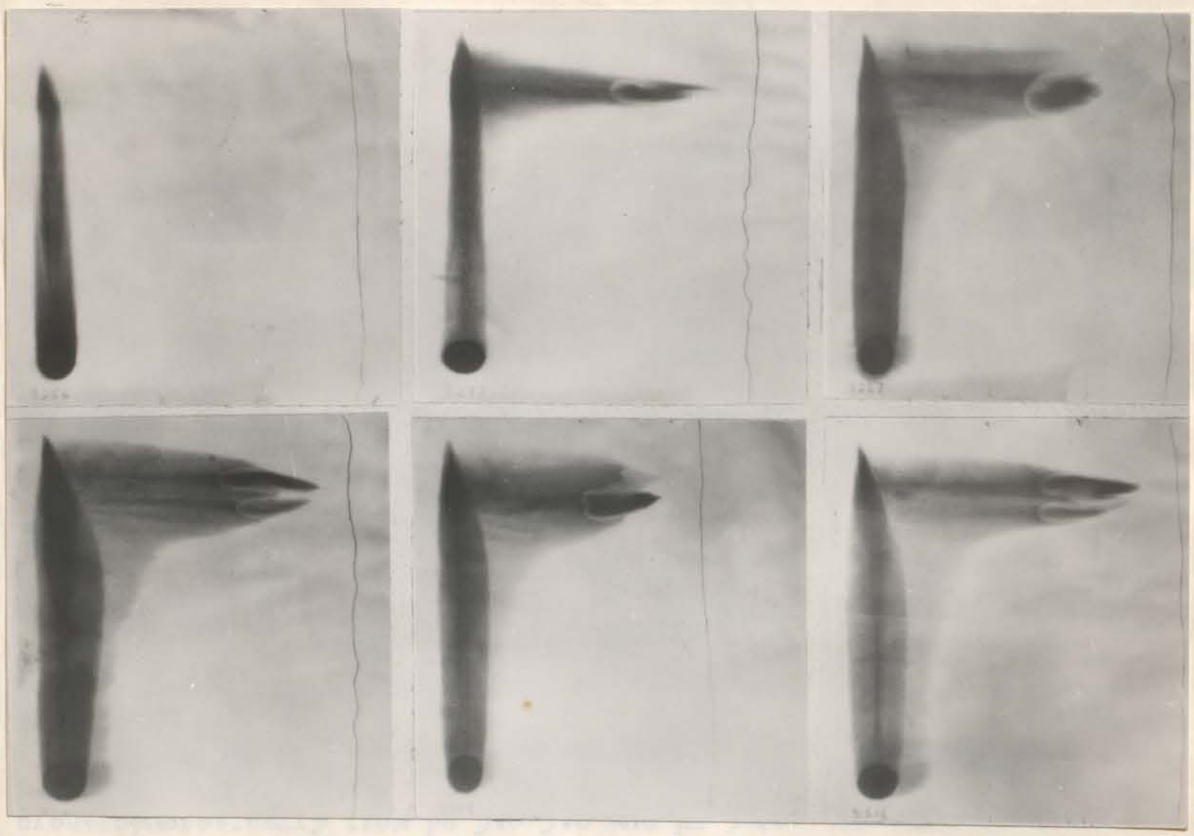


Figure 5

Two-dimensional chromatograms of a solution of bovine serum albumin are illustrated, which were chromatographed in aqueous buffers at pH 4, 4.5 and 5 (top row), and pH 5.5 and 6 (first two figures in the second row). The buffer used in the first dimension was citrate buffer, and the buffer used in the second dimension was either phthalate or acetate buffer. The last picture in the second row represents the protein chromatographed at pH 6, but after the protein solution had remained in the refrigerator at 0° C. for more than 36-48 hours.

It was observed that the protein was homogeneous at pH 4.5, 5.0 and 6.0. Movement occurred one-dimensionally only at pH 4.0. At pH 5.5, two fractions appeared. At pH 6.0, after the protein had remained in solution for some time, two fractions also appeared.

ment in the first dimension only. At pH 5.5, two separated fractions appeared, but at pH 5,6 and 7 only single fractions occurred (Fig. 5). This was also true if citrate buffer was used in the first dimension, and followed by acetate buffer in the second dimension. It was also observed that if bovine albumin was allowed to remain in solution for a period of more than 36-48 hours, multiple fractions, sometimes as many as four, appeared. Various combinations of buffers have shown the separation of these fractions at pH 6 (Fig. 5).

It was apparent from these results, that a freshly prepared crystalline bovine albumin behaved as a homogeneous protein at pH 6-7, whereas two components were present in the region of pH 5.5.

A private communication from Dr. G. Perlmann, who kindly supplied these samples, stated that this protein was homogeneous electrophoretically from pH 3.0-3.8 and pH 5-10. In the region of the isoelectric point, the protein separated into two distinct electrophoretic components. This phenomenon seems to be a property of most serum albumins and indicates that crystalline albumin preparations contain several closely related proteins. Paper chromatography appears to confirm and may possibly extend the results of electrophoretic analysis.

β-lactoglobulin-hemin complex. It was found that the combination between β-lactoglobulin and hemin was not complete if the pH was 5 or less, i.e., the leading fraction of the chromatogram was colorless. From pH 6 and up, the complex showed as an intense blue color when streaked with the benzidine reagent. Thus the formation of a complex between this protein and hemin was dependent upon pH.

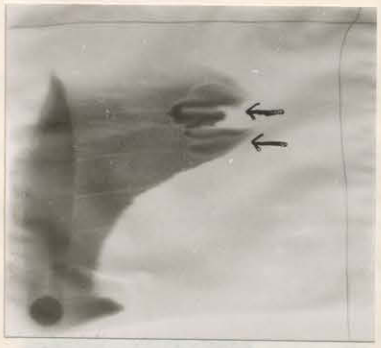


Figure 6

A two-dimensional chromatogram of β-lactoglobulin is illustrated. Development was carried out with M/20 citrate buffer at pH 6.5 in the first dimension, and M/20 phthalate buffer at pH 6.5 in the second dimension.

It was observed that this protein showed the presence of several fractions which were not completely separated from each other under these conditions.

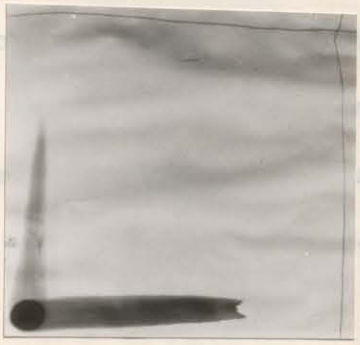


Figure 7

A two-dimensional chromatogram of γ-globulin is illustrated. Development was carried out with a M/20 phthalate buffer at pH 6.0 in the first dimension, and 2% tartaric acid in the second dimension.

It was observed that the main protein component moved only in the second dimension, while the minor fraction moved only in the first dimension.

R_F values showed much less variation than those of bovine albumin. The R_F value was least around pH 4, and greatest at pH 6, where the value was 0.86. All other values were close to 0.80 (Fig. 3).

Two dimensional chromatograms between pH 6 and 7 demonstrated that this crystalline protein was not homogeneous. The use of citrate buffer in the first dimension, and tartrate, phthalate or acetate buffer in the second dimension, showed that there were a number of fractions in this sample. However, a complete separation under these conditions has not yet been achieved (Fig. 6).

Dr. Perlmann, who also supplied this sample, stated that this protein was found to be a mixture, there being two distinct electrophoretic components in the pH range of 3.7-5.0, and also between pH 6 and 7.

Human γ-globulin-hemin complex. It appeared that γ-globulin, at the concentrations used, did not combine with hemin completely over the entire pH range. At each pH value, some combination did take place, but it was evident that from pH 3-8, there was a portion of the protein that did not combine, the amount of which decreased with an increase in pH.

At pH 2, γ-globulin gave rise to two separate fractions inside the chromatogram finger, in much the same manner as that shown by bovine albumin, although the fractions were smaller in size. Movement was least at pH 4. R_F values were 0.78 at pH 2 and 7, and increased to 0.81 at pH 10 (Fig. 4).

A two dimensional analysis showed an excellent separation of two components of γ-globulin, the separation occurring at right angles.

Figure 8

The separation of a mixture of papain and casein, when 2% succinic acid was used as the developing solution, is illustrated.

On the left, papain, which did not combine with hemin (the hemin was left at the point of origin), and on the right, casein which did combine with hemin, is illustrated. In the centre, it will be observed that the movement of the papain was depressed in the presence of casein, although the two proteins were still separated.

The first developing solvent may be phthalate buffer at pH 6, 5% ammonium sulfate or 2% oxalic acid, followed by 2% tartaric acid in the second dimension (Fig. 7).

This protein, according to Dr. Perlmann, consisted of one main electrophoretic component (96% of the total protein content), and showed a marked boundary spreading, indicating lack of homogeneity.

Protein Mixtures. One dimensional chromatograms rarely separated a pair of proteins. We have succeeded, however, in separating components of a mixture of papain and casein, using 2% succinic acid as the developing solution (Fig. 8). The movement of the papain was apparently retarded by the presence of casein. Other proteins have been run in combination with the preparations of papain and diastase, but no separations were effected.

Enzyme Preparations. Some preliminary work has been done on the movement of enzyme solutions. Preparations of pepsin, papain, malt diastase (commercial preparations from British Drug Houses), and urease (Urease-Dunning tablets) were used, and it was found that hemin did not combine with any of them. After streaking the paper with the benzidine reagent and allowing the paper to stand for a time, it was observed that the malt diastase and papain preparations gave rise to very well-defined, colorless spots. The urease preparation produced a vertical streak, but the pepsin preparation failed to show any detectable spot. The papain preparation showed R_F values of 0.81 up to pH 3, and increasing to 0.90 at pH 8-12. The malt diastase preparation showed a very constant R_F value over the entire pH range, varying only between 0.90 and 0.95, whereas the R_F value of

urease was 0.78 at pH 2, increasing to 0.85 at pH 3, and 0.92 at pH 12. It seems likely that paper chromatography will be of service in the evaluation and examination of commercial enzyme preparations.

Urease Chromatography and Manometric Estimation. The question arose as to whether enzymes would retain their catalytic activities if they were subjected to the chromatographic technique. A series of chromatograms were therefore set up for this purpose, the enzyme selected being urease.

The enzyme solution was prepared by grinding twenty 25 mg. Urease-Dunning tablets in a small mortar in 2 ml of a 2% cysteine-glycine solution that had been adjusted to pH 6. It was then centrifuged for 15 minutes, and the supernatant solution was used for chromatography. Aliquots of 0.04 ml were applied to the paper by placing 0.02 ml on the paper, allowing it to dry, and adding another 0.02 ml. The developing solution was prepared by dissolving 2 grams cysteine hydrochloride in water, adjusting the pH to 6 with caustic soda solution, making the volume to 100 ml, and then adding 1 gram glycine. This reagent should be prepared just prior to use. The solution was allowed to advance 20-22 cm. The paper was then removed and allowed to stand at room temperature for a few minutes. While the paper was still damp, it was cut into 3 cm. sections, starting 0.2 cm. from the bottom of the paper. The boundaries were marked 7 mm. on each side of the point of application of the aliquot.

Each section of the filter paper was then cut into four parts, all of which were immediately placed in a Warburg manometric vessel with 3 ml. acetate buffer at pH 5. 0.2 ml. 10% urea was placed in

the side arm. As a control, a ten times dilution of the original supernatant was used (diluted with physiological saline) and 0.4 ml was placed in the Warburg vessel with 2.6 ml. buffer and 0.2 ml. urea in the side arm. A period of 30 minutes was allowed for the contents of the vessel to attain thermal equilibrium (37°). The urea in the side arm was then tipped into the vessel, and carbon dioxide output was measured manometrically in the usual manner for one hour.

The results showed the existence of a distribution curve of urease on the paper strip. Traces of enzyme were left at the point of origin, but a fair proportion ascended the paper, in which the maximum urease activity was between 6 and 12 cm. from the origin. On either side of the maximum, there was a drop in enzymic activity (Fig. 9). If a parallel chromatogram was streaked with the benzidine reagent, it was found that the greatest extent of the colorless region on the colored background was 6-12 cm. from the origin.

On assessing the activity of the urease over the entire strip, by adding together the activities of the various parts of the strip, it was found that the total urease activity over the entire strip showed a recovery of 85% of that expected from the amount of urease placed on the paper. Another assessment of the total urease activity over the entire strip showed a recovery of 110%, the assessment being made from a calibration curve previously prepared, relating activity (rates of carbon dioxide production) to the quantity of urease. These results showed that urease activity was not diminished within experimental error by this technique, and that the movement of urease on filter paper could be followed.

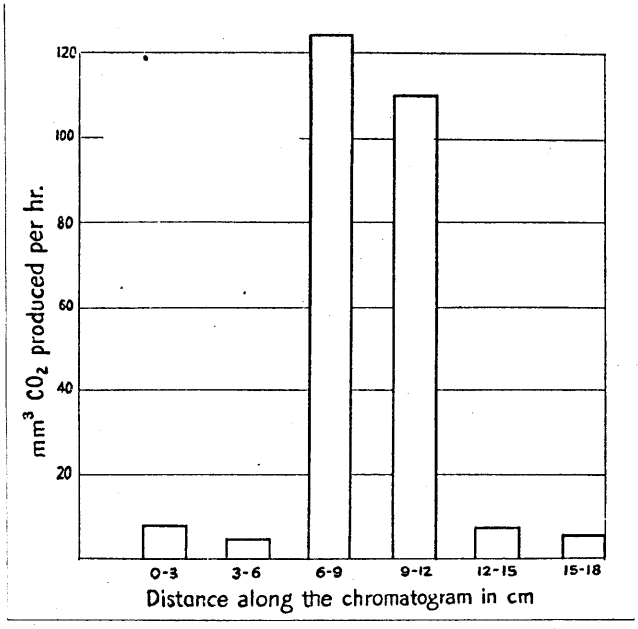


Figure 9

The activity of urease along a strip of filter paper after chromatography is shown in this graph.

Urease was chromatographed one-dimensionally on filter paper, then the strip was cut into 3 cm sections, and each section was placed in a Warburg vessel for manometric estimation.

It was observed that the site of greatest urease activity coincided with the position of the characteristic 'colorless spot' of urease, which was shown when the chromatogram was streaked with the benzidine reagent.

Egg Albumin. Crystalline egg albumin was used, and like enzyme preparations, it did not combine with hemin, but appeared as a colorless spot on a colored background (Fig. 10). It was barely detectable below pH 4, at which point it had an R_F value of 0.60, increasing to 0.75 at pH 9.

The identity of the protein present in the colorless spot was shown by the ability of the egg albumin to form a precipitin with a specific antiserum produced in a rabbit by albumin injections*. Precipitin formation was produced only by solution of the substance contained in that part of the paper strip believed to contain the protein, as determined by the streaked control.

These results proved that a solution of crystalline egg albumin may be chromatographed under the given experimental conditions, the position of the protein being indicated by a well-defined colorless region on a colored background. The protein present in this region reacted typically with an ovalbumin antiserum.

Human Plasma. A few experiments were carried out on the chromatography of human plasma. The results showed the presence of a complex mixture of hemin-reacting proteins, when chromatographed at pH 6 and 7, using citrate buffer followed by phthalate buffer. It was estimated that between 6 and 10 protein fractions appeared under these conditions.

DISCUSSION.

Color development with protein-hemin complexes was generally

*This antiserum was kindly prepared by Professor E.G.D. Murray, of the Department of Bacteriology, McGill University.

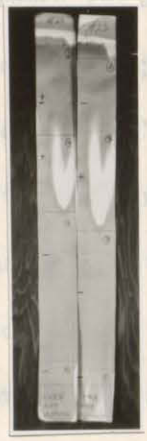


Figure 10

Egg albumin, which was developed in distilled water, pH 6.4, is illustrated in this chromatogram.

Egg albumin did not combine with hemin, but its position on the chromatogram was illustrated by streaking the paper with the benzidine reagent, which allowed the protein to show up as a colorless spot.

Only the colorless spot gave a positive precipitin test when sections of the filter paper were eluted and mixed with the antiserum, indicating that the colorless spot represented the position of the unchanged egg albumin.

blue, but may be purple, brown or green. Some salts, e.g. magnesium sulfate and sodium phosphate, interfered with color development. Citrate, glucose, trisodium phosphate and potassium hydrogen phthalate at some pH values gave rise to a deep purple color.

Background color development varied considerably, depending on the ease with which the benzidine reagent was oxidized. Acid pH values tended to retard development of the background color, while alkaline pH values gave rise to a deeply colored background. Photographs were made of the chromatograms at the optimal development of color, generally within 20 seconds of streaking.

The use of organic solvents gave unsatisfactory results. n-Butanol saturated with water allowed extremely little movement of the proteins and favored the formation of an immediate, deeply colored background on application of the benzidine reagent. Aqueous solutions of ethylene glycol, propylene glycol, ethanol or acetone induced maximum or near maximum movement. All proteins tested moved about the same distance under these conditions.

It has not been found necessary to allow the solution front to advance beyond 20-25 cm., which required from 60-90 minutes. The composition and shape of the chromatogram was determined within the first 20 minutes, and further development only resulted in a more easily evaluated fraction. A complete two dimensional chromatogram may be obtained within five hours.

The chromatography of proteins with this technique appears to be useful as a new method for showing homogeneity and heterogeneity of protein samples. Since results are in good agreement with those

of electrophoresis, it would seem that this technique could be used in conjunction with electrophoretic analysis, and possibly, to extend it. The technique is also valuable, since specific properties of proteins, such as serological properties of ovalbumin, and the enzymic properties of urease, are apparently not altered. It may be ultimately possible to isolate enzymes from complex mixtures such as homogenates, and study the properties of these enzymes without the effects of other substances normally present.

The result of the protein chromatogram appears to be a relationship between the solubility characteristics of the protein, or protein complex, and the adsorptive effect of filter paper. This was apparent when a protein such as bovine albumin was chromatographed with a series of buffers at various pH values. Movement of the protein was least in the region of pH 4, which is near the isoelectric point of the protein, and increased with higher pH values. This was also true with some of the other proteins tested. It is also of interest to note that pH values of 7 and higher show no appreciable differences on the chromatogram for that particular protein. The use of various types of filter papers also gave different results, probably due to the differences in adsorptive capacity. Some of the Schleicher and Schuell filter papers allowed no protein movement in the second dimension, even at higher pH values.

The failure to repress the ionization of the protein molecules during chromatography probably explains the inability of the protein to travel as a discrete spot. It is a well known fact that molecules, such as organic acids, move as streaks in a developing solvent in

which the ionization of the acids has not been repressed. However, in an acid medium, organic acids travel as well-defined spots, and can therefore be separated from each other from a mixture of acids on a one dimensional chromatogram.

It is also of interest to note that a protein need not necessarily combine with hemin to detect it chromatographically. As in the case of the enzymes tested, and egg albumin, it was noticed that the presence of the protein appeared to depress the oxidation of the benzidine reagent on the paper, leaving a colorless spot at the location of the protein.

The use of the conventional R_F values used in paper chromatography appears to be of doubtful value in describing the movement of a protein. Although it may be used when discussing single dimensional chromatograms, the R_F value is not being used to describe two dimensional movements. It appeared, in almost every case, that the trailing edge of the protein in the second dimension was quite distinct, but the leading edge was often quite diffuse, due to the presence of the salts which had been moved up in the second dimension, and collected at the leading edge.

Since some metals will combine with proteins, it was considered possible that this would be a sensitive means of detection. Copper (detected with diethyldithiocarbamate) and manganese (detected with formaldoxime hydrochloride) were combined with proteins, but were not found to be as satisfactory as detection with hemin.

Swingle and Tiselius have recently reported on the chromatography of phycoerythrin (a fluorescent red protein from a sea weed)

on an adsorbent of tricalcium phosphate. In this paper, mention was made of the published portion of the work described in this chapter. They referred to 'paper chromatography of proteins under conditions not involving salting out, although experiments by one of us (S.M.S.) indicate that such chromatograms may be artifacts resulting from the combined effects of viscous resistance to flow offered by the protein spot, and the irreversible adsorption of small quantities of protein in an immobile tail' (197).

Not knowing the nature of their experiments in arriving at this observation, it is difficult to assess the evidence upon which this statement is based. It does not seem probable to us that the results of our chromatograms are due to artifacts, since agreement with results of electrophoresis is good, and we do not get the same results when chromatographing unrelated proteins. There is little doubt that there is an effect of viscous resistance to flow offered by the protein spot, but this is considered to be characteristic of the protein, and therefore to be of advantage in determining the mobilities of various proteins. Furthermore, we have not observed an irreversible adsorption of proteins, since both egg albumin and urease can be eluted from the filter paper to give their characteristic serological and enzymic reactions. It is, however, possible that smaller quantities than were used in these experiments, could be irreversibly adsorbed.

SUMMARY.

(1) A new technique has been described for the paper chromato-

graphy of proteins such as bovine albumin, β-lactoglobulin, γ-globulin, papain, pepsin, diastase, egg albumin and urease. The proteins were combined with hemin, wherever possible, which acted as a 'marker', and detected with the benzidine reagent. Aqueous solutions of buffers or salts were used as developing solutions.

(2) Chromatographic results of bovine albumin, β-lactoglobulin, and γ-globulin were in agreement with results of electrophoresis.

(3) It was shown that egg albumin did not lose its serological specificity during chromatography, as shown by the precipitin reaction.

(4) It was shown that urease did not lose its specific enzymic properties, which could be estimated manometrically, when chromatographed on filter paper.

Chapter 5

PAPER CHROMATOGRAPHY OF PROTEIN MIXTURES AND BLOOD PLASMAS

INTRODUCTION

The movement of proteins on filter paper has been described in the previous chapter, which showed that chromatographic results were in good agreement with electrophoretic analyses, and that proteins such as egg albumin and urease did not lose their specific properties during this technique. Thus, it seemed possible that chromatography of proteins could be extended to the separation of complex mixtures, such as plasmas. Since some very preliminary experiments on chromatography of human plasma indicated heterogeneity, further work was necessary to improve the separations.

It had been noticed that, in the chromatography of plasma, incomplete separations of the fractions had occurred. Therefore, new developing solutions were investigated, to see if a better resolution of the mixture could be achieved. Furthermore, the use of synthetic detergents was considered, to see if the addition of a surface active agent would aid in this resolution.

The use of new developing solutions and surface active agents was then applied to the chromatography of some crystalline protein fractions, as well as human, rat, guinea pig and horse plasmas.

(Mrs. Mary Hersey assisted with the testing of new developing solutions and the effects of various surface active agents on the chromatography of protein mixtures.)

MATERIALS AND METHOD

Developing Solutions. M/10 sucrose solution is used as the developing solution in the first dimension, when this is followed by M/10 sodium potassium tartrate in the second dimension.

Plasma Solutions. Plasmas are taken with a heparinized syringe (1/10 ml 1% heparin-Na salt for 10 ml blood) from a fasting animal. The blood is then centrifuged for 15 minutes at 2500 r.p.m. The plasma is pipetted into culture tubes in 0.5 ml quantities, and 0.02 ml 0.3% hemin is added. If a surface active agent is to be used, it is generally added to the test tube first to facilitate its solution. The contents of the tube are now well mixed, and 0.02 ml aliquots are applied to the paper, each sample being tested in duplicate.

Technique. Whatman No. 1 filter paper is cut into eight inch squares, and 0.02 ml of the plasma solution is placed in the lower left hand corner. After being air-dried, the paper is formed into a cylinder, and stapled so that the edges do not touch. The cylinder is then placed in a pyrex dish which contains the developing solution. When the solution has reached the top of the paper, it is removed and air-dried. The cylinder is then restapled at right angles to the original direction of flow, and placed in the second developing solution. It is only necessary to allow the solution to move about two-thirds of the distance up the filter paper. After the chromatogram has dried, it is streaked with freshly prepared benzidine reagent, as has been previously described. The entire technique can be carried out easily in 5 to 6 hours.

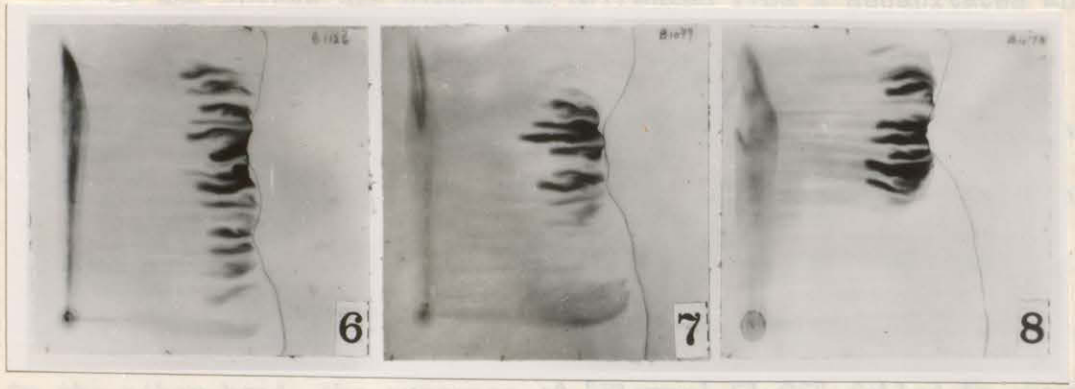


Figure 11

Comparison of the protein chromatograms of human plasma, rat plasma and guinea pig plasma is illustrated. M/10 sucrose was used as the developing solution in the first dimension, and M/10 Na K tartrate in the second dimension.

No. 6. Human plasma with 5 mg "Elvanol 31-31" per 0.5 ml plasma
No. 7. Rat plasma with 5 mg "Elvanol 31-31" per 0.5 ml plasma
No. 8. Guinea pig plasma with 5 mg "Elvanol 31-31" per 0.5 ml plasma.

The plasma of these three different species were seen to give characteristic patterns when chromatographed under identical conditions.

OBSERVATIONS

Comparison of Human, Rat and Guinea Pig Plasmas. Heparinized plasmas of different animals showed different patterns on the chromatogram when they were chromatographed under similar conditions.

Rat and guinea pig blood was collected from a decapitated animal in a test tube containing heparin, and centrifuged for about 15 minutes. 0.5 ml plasma was mixed with 0.02 ml 0.3% hemin, and various amounts of surface active agent was added to a number of such samples. Using 5 mg "Elvanol 31-31" as surface active agent, evident differences between the plasma chromatograms appeared (Fig. 11). On the other hand, the presence of "Elvanol 51-05" failed to show any obvious difference. "Elvanol 54-22" yielded a much better separation of the constituents of human plasma than was obtained with either rat or guinea pig plasma. "Span 20" gave excellent separations of the protein components, but whilst failing to show a marked difference between human and rat plasma, it gave a distinctive pattern with guinea pig plasma. "NNO" gave a good separation of the constituents of each of the plasmas, but the differences between the plasma patterns were not very striking. "Tween 20", "Tween 81" and "Tween 85" are the best surface active agents to use when examining human plasmas.

Chromatography of Pure Proteins and Protein Mixtures. A number of chromatograms has been made of preparations of purified proteins and protein fractions isolated from blood. These preparations were kindly presented by the courtesy of the Cutter Laboratories, and consist of fractions of blood plasma isolated according to the

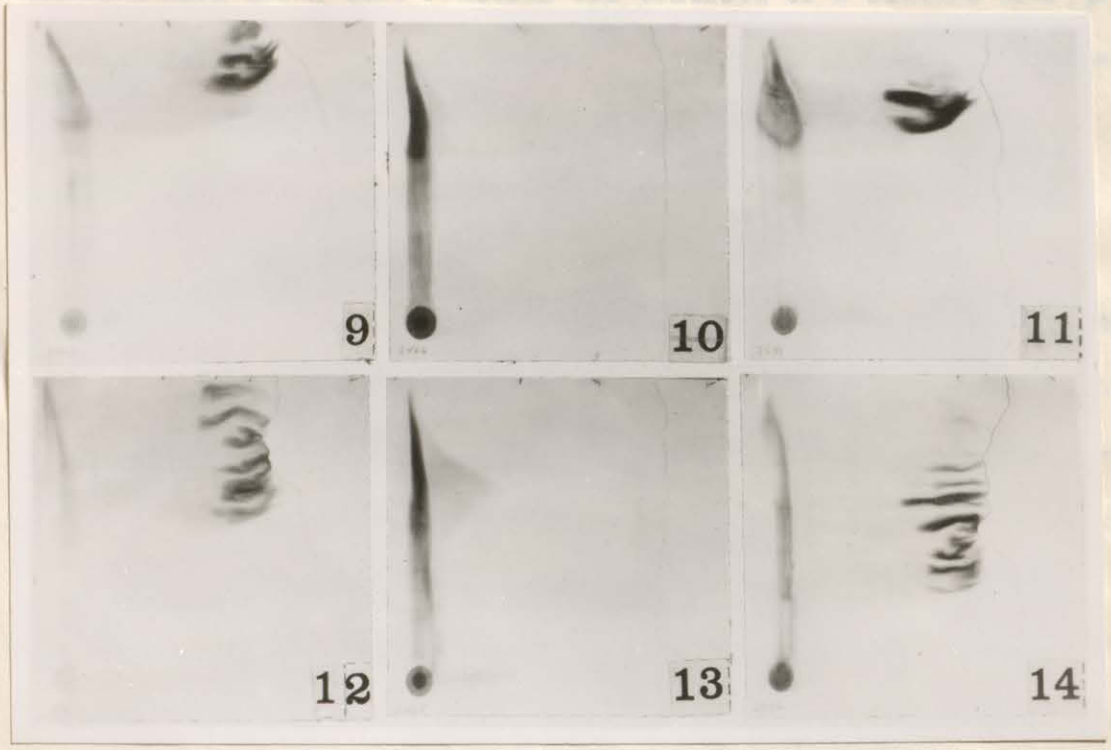


Figure 12

Some protein fractions and a protein mixture are illustrated. M/10 sucrose solution was used as developing solution in the first dimension, and M/10 Na K tartrate in the second dimension.

No. 9. Cohn Fraction V (16 mg) dissolved in 0.5 ml 0.85% NaCl
No. 10. Cohn Fraction III (8 mg) dissolved in 1.0 ml saline.
No. 11. Mixture of Cohn Fractions: 22 mg V, 4 mg II and 5 mg IV-3,4 dissolved in 0.5 ml 0.85% NaCl.
No. 12. As in No. 9 with 0.01 ml "Tween 85" per 0.5 ml solution.
No. 13. As in No. 10 with 0.01 ml "Tween 85" per 0.5 ml solution.
No. 14. As in No. 11 with 0.01 ml "Tween 85" per 0.5 ml solution.

The addition of globulins to albumins did not result in the appearance of new fractions, but altered the existing pattern of the albumins.

procedures of Dr. Cohn and his colleagues (145). Electrophoretic analyses have shown that Fraction III consists of a mixture of α, β, and γ-globulins, and that of Fraction V is largely albumin. Chromatograms of Fractions V and III are shown in Fig. 12. It will be noticed that the chromatograms of Fraction V indicate the presence of more than one protein, whilst those of Fraction III show little movement in the second dimension. The chromatograms did not resemble those of blood plasma. When mixtures of the fractions were taken in such proportions and in such quantities as to constitute the proportions and amounts of albumin and globulin in blood plasma, chromatograms were obtained which resembled those of normal blood plasma. This is shown in Fig. 12. The evidence from a number of chromatograms of protein mixtures indicates that an interaction between proteins occurs, whereby the chromatogram of a mixture of proteins is not necessarily that to be expected from a simple addition of the chromatograms of the proteins taken separately.

This fact is also illustrated by the results of some preliminary experiments carried out with mixtures of proteins with hemoglobin and cytochrome C. Chromatograms indicated that both these substances formed associations with other proteins, but further experiments are required to decide whether the conditions of the chromatographic technique may result in sufficient dissociation of the hemin from hemoglobin or cytochrome C to produce hemin complexes with the other proteins present (Fig. 13).

Comparison of normal and hyper-immunized horse serum. Through the courtesy of the Department of Microbiology and Hygiene of the

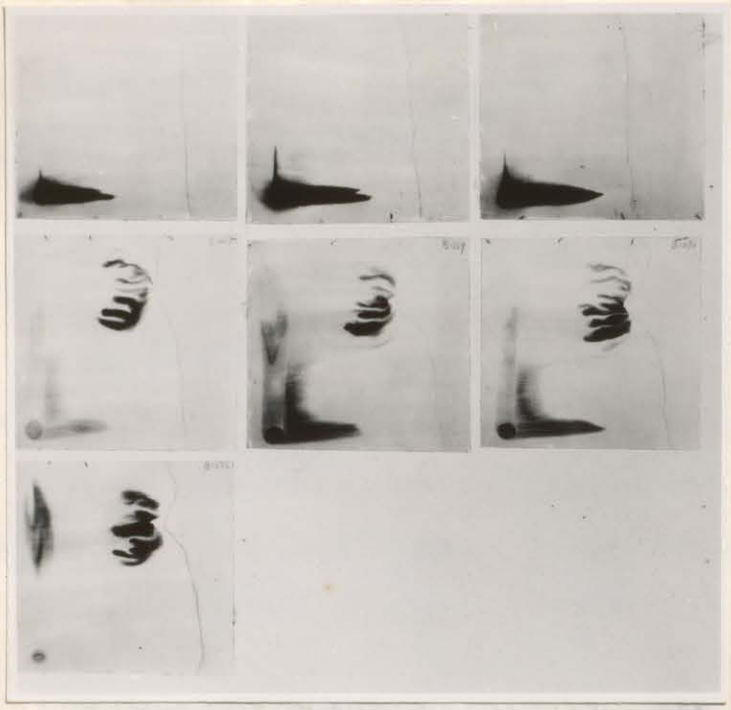


Figure 13

Combination of cytochrome C and hemoglobin with the plasma proteins is illustrated. M/10 sucrose solution was used as developing solution in the first dimension, and M/10 Na K tartrate in the second dimension.

The upper series indicates the movement of three concentrations of 1% cytochrome C solution. The second series indicates the combination of these three concentrations of cytochrome C with human plasma. The single chromatogram at the bottom indicates the chromatogram of human plasma and hemoglobin, obtained by lysis of the red blood cells.

There was a non-specific combination of cytochrome C and hemoglobin with human plasma, giving a pattern similar to the addition of hemin to plasma.

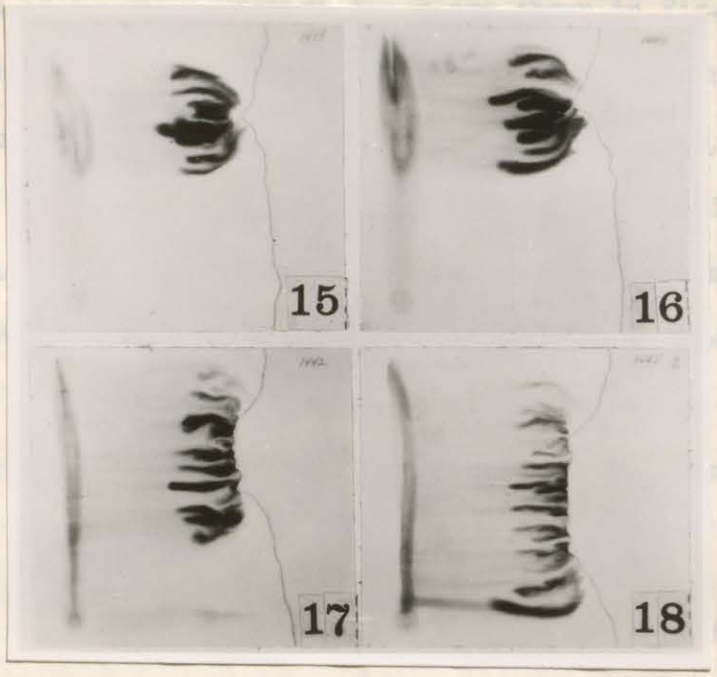


Figure 14

Comparison of normal and hyperimmunized horse serum is illustrated. M/10 sucrose was used as the developing solution in the first dimension, and M/10 Na K tartrate in the second dimension.

No. 15. Normal horse serum.
No. 16. Horse serum immunized against diphtheria toxin.
No. 17. As in No. 15 with 0.01 ml "Tween 85" per 0.5 ml serum.
No. 18. As in No. 16 with 0.01 ml "Tween 85" per 0.5 ml serum.

There were differences in the chromatograms between normal and hyperimmunized horse serum, the latter having fractions not present in the normal serum.

University of Montreal, specimens of normal horse sera and the sera of hyper-immunized horses (immunized against diphtheria toxin) have been obtained. Chromatograms of the two sera both with and without the addition of a surface active agent are shown in Fig. 14. For the experiment, 0.01 ml "Tween 85" was added to a mixture of 0.02 ml 0.3% hemin and 0.5 ml serum, and chromatographed with M/10 sucrose solution in the first dimension, and M/10 sodium potassium tartrate in the second dimension. It will be seen that the chromatograms of the sera (both with and without the addition of the Tween) showed differences from each other. The hyper-immunized horse serum indicated the existence of fractions not present in the normal serum.

DISCUSSION

It was found that citrate buffer and phthalate buffers at pH 6 and 7 could be used to partially separate the fractions present in plasmas, but due to the poor buffering effect at these pH values, it was considered desirable to investigate the problem of developing solutions further. M/10 sucrose solution was found to be very efficient for use in the first dimension, when this was followed by M/10 sodium potassium tartrate in the second dimension. This combination is used in all the chromatograms described in this chapter. Other combinations gave good results, e.g., dextrose, lactose or maltose in the first dimension, and nicotinate, malate, succinate, fumarate, mandelate, acetate or citrate in the second dimension. Certain amino acids have also given good results when used as developing solutions, e.g., M/10 methionine solution followed by M/10

acetyl glycine, or M/10 cysteine solution followed by M/10 sodium glutamate solution.

It is possible that the substances used in the developing solutions in the first dimension, such as sucrose, form loose complexes with the plasma proteins. These complexes appear to have different mobilities on paper, thus resulting in a more efficient separation of the protein components.

When a plasma is chromatographed in the manner that has been described, the various protein components did not entirely separate, and tended to remain bunched together. This is illustrated in Fig. 15. It was found that if certain surface active substances were added to the plasma before chromatography, good separations of the protein fractions occurred. The following substances yielded excellent results: sodium alginate, the "Elvanols", "Spans", "Tweens", "BRIJ" and "NNO". Results with sodium tauroglycholate were relatively poor. The various surface active substances did not give identical results, both their structures and their concentrations being important in determining the pattern of the protein chromatogram.* It has been made a matter of practice to make chromatograms of blood plasma, or protein mixture, in the presence of different concentrations of a selected surface active agent. Usually, 0.01 to 0.06 ml

*Elvanols: A group of polyvinyl alcohols produced by hydrolysis of polyvinyl acetates of varying degrees of polymerization (Dupont)
Span 20: Sorbitan monolaurate
NNO: Glycerol mannitan laurate
BRIJ 30: Polyoxyethylene lauryl alcohol
Tween 20: Polyoxyethylene sorbitan monolaurate
Tween 81: Polyoxyethylene sorbitan monooleate
Tween 85: Polyoxyethylene sorbitan trioleate

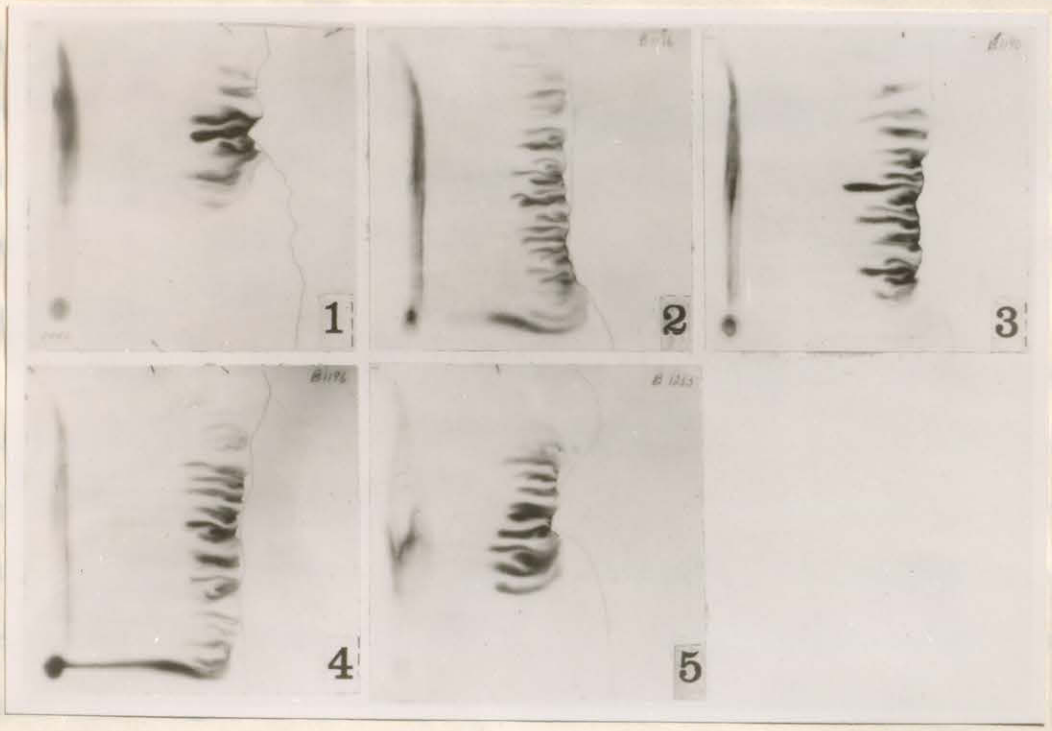


Figure 15

The effect of some surface active agents on the chromatography of human plasma is illustrated. M/10 Sucrose was used in the first dimension, and M/10 Na K tartrate as the developing solution in the second dimension.

No. 1. Human plasma control.
No. 2. Human plasma with 0.03 ml "Span 20" per 0.5 ml plasma.
No. 3. Human plasma with 0.03 ml "NNO" per 0.5 ml plasma.
No. 4. Human plasma with 0.05 ml "BRIJ 30" per 0.5 ml plasma.
No. 5. Human plasma with 0.04 ml "Tween 20" per 0.5 ml plasma.

It will be noticed that these surface active agents had a spreading effect on the human plasma pattern. The detergents also appeared to have had effects which differed from each other.

of a selected surface active agent was added to 0.5 ml plasma before the addition of the hemin, and subsequent chromatography. The separation of the plasma constituents, as facilitated by the presence of the surface active agents, is shown in Fig. 15. High concentrations of surface active agents must not be added; in their presence, the movement of the proteins on the filter paper appeared to be greatly depressed.

Once again, it would appear that plasma proteins combine with surface active agents in such a manner as to give various mobilities on paper. Large excesses appear to form insoluble complexes, possibly analogous to the lipoproteins. Thus it would seem that these chromatographic results are due to the formation of plasma protein-sucrose-detergent complexes, which have characteristic mobilities on filter paper.

It must be emphasized at this point, that the use of synthetic detergents will not lead to a method for the isolation of proteins from complex mixtures on filter paper. Detergents have been shown to cause denaturation of proteins, and our use of detergents with protein mixtures is merely to accentuate differences which may exist between protein complexes.

The spreading of the protein pattern, as seen by the use of synthetic detergents, can also be demonstrated by the use of other substances, e.g., ricinoleate can be shown to have a similar, though not so pronounced effect (see Chapter 9). It is probable that natural surface active substances, such as the bile salts, behave in an analogous manner, but this has not been investigated.

Loomeijer (146) has recently reported that serum proteins may be fractionated using 'Desogen', a quaternary ammonium detergent (methylphenyldodecyltrimethylammonium methosulfate). Serum can be separated into α, β and γ-globulins, as well as albumin, and sometimes the β-fraction can be divided into two or three minor fractions. The identity of the various fractions was proven by electrophoresis.

Dialysis of human plasma against running water did not seem to affect the pattern of the plasma chromatogram. It was concluded, therefore, that the dialyzable material of the plasma had little or no influence on the movement of the proteins on the filter paper.

The choice of filter papers seems to be very important, as it appeared that not all animal plasmas gave the best results with the same filter paper. Human and rat plasmas gave the best chromatograms with Whatman No. 1 filter paper, but guinea pig plasma gave the best results when using S&S 589. When comparing the plasmas of various species, however, Whatman No. 1 paper has been consistently used.

With any given plasma, or protein mixture, excellent duplication of results has been obtained. It is necessary to be strictly accurate in the measurement of the surface active agent added to the plasma, but if due care is taken, there is no difficulty in obtaining good duplication with any sample of a protein mixture.

The differences observed between human, rat and guinea pig plasma are in agreement with Moore's observation that 'the sera of different species of the animal kingdom differ widely in their

content of albumins and of the different types of globulins' (192). It has also been observed that differences are found in the plasmas of individuals of different ages.

The interaction of proteins has been apparent in much of the work on paper chromatography. The interaction between cytochrome C and hemoglobin with the plasma proteins is evident in Fig. 13. The movement of human albumin alone is seen in Fig. 12 (No. 12), and the addition of physiological concentrations of globulins to it did not increase the number of protein fractions, but only altered the movement of the albumin. It will be observed that there were seven fractions in each case (compare with No. 14). Thus, it would seem, that there has been a reaction between the albumins and the globulins to produce a complex that moves in a manner which is different to its component fractions.

This loose combination of proteins that has been observed is in agreement with the statements made by Hawk et al (194). "Studies of the behaviour of the serum globulins led Sørenson, some years ago, to conclude that euglobulin and pseudoglobulin were reversibly combined in a loose combination. Subsequent investigations of other proteins led to the result that highly purified preparations of serum albumin, casein and gliadin were each found to consist of mixtures of an unknown number of proteins of similar character combined in a reversible manner. Such proteins, according to Sørenson, represent 'reversibly dissociable component systems'. In serum, for example, we probably have not only such component systems such as albumins and globulins, but also more complex systems in which these proteins

are combined in varying proportions not only with each other, but with other serum constituents, such as the lipids. In protoplasm, instead of relatively inert, independent substances, there are probably complex systems composed of protein, lipid and carbohydrate in equilibrium with each other, and constantly shifting in response to changes in environment".

SUMMARY

(1) A technique for the two dimensional chromatography of blood plasma and protein mixtures on filter paper is described.

(2) Separations of protein constituents were greatly facilitated by the addition of surface active agents such as the "Tweens" or "Spans". The technique at present adopted is to add Tween 81 or Tween 85 to the plasma before chromatography, and to develop the chromatograms with M/10 sucrose solution in the first dimension, and M/10 sodium potassium tartrate solution in the second dimension.

(3) Differences between the protein patterns of human, rat and guinea pig plasmas were noted.

(4) With protein mixtures, associations of proteins may occur that may be detected by chromatography.

(5) The sera of hyperimmunized horses (as against diphtheria toxin) yielded chromatograms which differed from, and exhibited fractions not present in, chromatograms of normal horse sera.

(6) The presence of several closely related protein fractions in a crystalline human albumin preparation has been observed.

Chapter 6

THE EFFECT OF BOMBARDMENT ON PROTEINS

INTRODUCTION

It has been observed for several years that protein structure may be profoundly affected by exposure to bombardment of various types of irradiation. The first observations on the effect of ionizing particles on albumin were published by Fournau in 1923 (147), who noticed that egg albumin solutions were coagulated by α-rays. Svedburg and Brohult (148) studied the action of α-rays on solutions of human serum albumin by means of the ultracentrifuge. They reported the finding of low molecular weight substances and general inhomogenization of the protein. These effects were attributed to an indirect reaction, since they did not appear when the irradiation was carried out at the temperature of liquid air. These authors also showed the splitting of hemocyanin (149), which has a molecular weight of 6.7×10^6, was attributed to the direct action of the rays; concluding that the small albumin molecules are extremely stable so long as secondary chemical effects were excluded, whereas the large hemocyanin molecule was readily split in two by irradiation. Sanigar et al (150) irradiated a solution of serum with 29,000 roentgens of x-rays and found no effect. Clark (151) carried out experiments on albumin solutions using ultraviolet light for irradiation, and observed an increase in light absorption and flocculation. He reached the conclusion that the denaturation effect, which preceded flocculation, was independent of temperature, and

therefore, the reaction was direct. Carruthers (152) concluded that it was apparent that the results of ultraviolet irradiated albumin solution have shown the same general effect as that observed for α-irradiated solutions, i.e., increased absorption, evidence of disruption of the molecules, and aggregation products. Lea (153) described experiments on radiation of enzymes, viruses and genes, which has yielded valuable information on the destruction of the functional ability of these substances. He has developed a theoretical basis for quantitative analysis of results which has shown agreement with experimental observations on enzymes and viruses. The theory is based on the assumption that a single ionization will destroy the activity of a virus, etc., but the extent of molecular deformation is usually not considered. Carruthers (152) described the decomposition of crystalline bovine serum albumin into cleavage products, which varied in size from effectively non-centrifugable material to apparently normal albumin. This was attributed to the direct action of the ionizing α-rays, since there was an inappreciable effect in the heat-treated control sample. There was a correlation between the average size of the particles and the radiation dose. Alpha particle radiation of 8×10^7 to 5×10^9 roentgens was obtained from slow neutron capture in boron mixed with albumin samples.

McLean and Giese (190) have recently postulated that one of the primary actions of ultraviolet light on proteins was to hydrolyze the peptide bonds adjacent to the ultraviolet chromophores tyrosine, tryptophan and phenylalanine. Extensive irradiation of egg albumin resulted in a small but significant increase in the

number of acidic groups, although no significant change was found in the number of amino or basic groups.

It seemed reasonable to expect that we could show alterations in protein structure and appearance of cleavage products with the chromatographic technique, so a series of experiments were carried out on the proton bombardment of crystalline and bovine albumin solution in the cyclotron.

MATERIALS AND METHOD

Bovine albumin was supplied for proton bombardment in the cyclotron either as a 1% saline solution or in the crystallized state. Bombardment was carried out under three main conditions: (a) under a copper dome, and cooled by ice or liquid nitrogen, (b) in a small diameter aluminum tube with no cooling, (c) in sealed glass tubes placed above Co^{60} and cooled with ice. Controls were treated in the same manner, but without exposure to irradiation. In some cases, cysteine was added to the protein solution to test the protective ability of cysteine against protein denaturation.

Bombardment of proteins was carried out under conditions which varied from 20-80 MEV, a maximum current of 0.5 microamperes, and time of bombardment which varied from less than 15 seconds to 21 minutes. Exposure to Co^{60} was for seven days, resulting in a dosage of 5,000 roentgens.

Each sample was chromatographed under protein conditions, i.e., using M/20 citrate buffer pH 6 as the developing solution in the first dimension, and M/20 phthalate buffer pH 6 in the second di-

mension; and also under amino acid conditions, i.e., a uni-dimensional analysis, using n-amyl alcohol (20)-95% ethanol (60)-10N ammonium hydroxide (20) as the developing solution. In the former instance, the protein was combined with hemin, and its final position determined by streaking with the benzidine reagent (133), and in the latter instance, the chromatogram was sprayed with 0.1% ninhydrin in water-saturated n-butanol.

RESULTS

The chromatograms of the bombarded proteins were compared with controls, as described in Chapter 4. In every case, there was a significant decrease in protein solubility, thus resulting in a chromatogram in which there was little or no movement from the point of origin. In the case of proteins which had been bombarded in aluminum tubes, the color of the protein crystals varied from white, grey, and increasingly darker shades of brown along the length of a single 25 mg sample. The solubility of the protein was nil or extremely slight, and when the protein had been colored a dark brown, a characteristically unpleasant odor was apparent when saline was added to try to dissolve it. The inclusion of cysteine in the protein solution during bombardment did not protect the protein from losing its solubility characteristics.

No amino acids or other ninhydrin sensitive substances were found to be liberated from the protein during bombardment. In cases

(Mr. Frank Eadie built the cells for proton bombardment, and supervised the bombardment of the protein samples.)

where the protein had been highly discolored, there were no ninhydrin sensitive spots left at the origin. This would indicate that even the protein was absent.

In the small number of experiments that were carried out, it was found that quantitative recoveries of the crystalline protein after bombardment was not possible, even though the protein was sealed off during this procedure. Recoveries averaged 64% in 20-25 mg samples, although recoveries ranged from 50-89%.

SUMMARY

Bovine albumin was irradiated with protons in both the dry and dissolved state in the cyclotron and with Co^{60}. No effects other than a decreased solubility of the protein was observed by the chromatographic technique.

Chapter 7

PAPER CHROMATOGRAPHY OF IODINATED AMINO ACIDS IN UNHYDROLYZED THYROID AND PLASMA

INTRODUCTION

De Robertis (154) demonstrated the presence of a proteolytic enzyme in the colloid of the thyroid follicle. He postulated that this enzyme causes the breakdown of thyroglobulin with the release of biologically active fragments, which are of low enough molecular weight to diffuse out of the follicle, and which presumably constitute the thyroid hormone. The only compound of small molecular weight found so far in the unhydrolyzed thyroid is thyroxin (155). In the present work, the technique of paper chromatography has been used to confirm this result, and to study the production of other iodinated amino acids which might be formed during the breakdown of thyroglobulin. The appearance of these substances in the circulation has been studied by observing their presence in n-butanol extracts of plasma. In addition, chromatographic analysis of whole plasma has supplied evidence of the nature of the circulating thyroid hormone.

Although methods had been presented for the chromatography of iodinated compounds on paper by Fink et al (156) and Trikojus (157), it was not considered that the separations were good enough to carry out elution experiments and isotope dilution analyses. Thus, new sets of developing solvents were investigated, and a new set of conditions are described in this chapter, that allows excellent

separation of the iodinated amino acids, and also shows the presence of hitherto undetected iodinated substances.

MATERIALS AND METHODS

Female albino rats weighing 150-200 grams and sustained on Remington's low iodine diet No. 342 (158), to which 10% Brewer's yeast had been added, were injected subcutaneously with about 100 microcuries of carrier-free radioactive iodide (NaI^{131}). After 48 hours the animals were anaesthetized with ether and exsanguinated with a heparinized syringe via the inferior vena cava.

Chromatography of n-butanol extracts of thyroid and plasma. The thyroids were removed immediately, ground in 1 ml of ice-cold saline in a chilled mortar, and extracted three times with an equal volume of n-butanol. Similarly, the plasma was extracted three times, first with a double volume, and then twice with an equal volume of butanol. In both cases, the combined butanol extracts were reduced to dryness in vacuo at room temperature. The dry residue was taken up in distilled water, 0.1 ml and 0.2 ml being used for the thyroid and plasma residues, respectively.

Aliquots of 0.02 ml of this solution were then chromatographed by the capillary ascent method (23), with or without the addition of the following carriers: DL-thyroxine, DL-diiodotyrosine, DL-diiodothyronine, and DL-monoiodotyrosine.

(Dr. J. Gross and Miss J. Cambron supervised the care of the animals and their subsequent injection with I^{131}. They carried out the preparation of the thyroid and plasma extracts. Radioautographic procedures were carried out in the darkroom of the Department of Anatomy, McGill University.)

The aliquots were placed in the lower left hand corner of ten inch by twelve inch Whatman No. 1 filter paper sheets and dried. The developing solvent used for the first dimension was the upper layer obtained after shaking n-butanol with 2N formic acid in a separatory funnel. For the second dimension, the upper layer of a mixture of 4 parts n-butanol and 1 part dioxane shaken with 5 parts of 2N ammonium hydroxide was used. After overnight development for each dimension, the papers were removed and dried in a current of air. They were then applied to Eastman No-Screen X-ray film, with a sheet of cellophane intervening, and thus exposed for varying periods of time. The duration of exposure depended on the activity present, as determined with a Geiger probe monitor. After autography, (Figs. 16,17, 18), the papers were dried for 30 minutes at 100° C., sprayed with ninhydrin (0.1% ninhydrin in chloroform with the addition of 0.1% collidine), and dried at 100° C. again. The colors obtained with ninhydrin were rather pale, but were sufficiently clear to locate the added carrier amino acids. By this technique, it was possible to obtain a complete separation of the four iodinated amino acids and of iodide as well.

Chromatography of the Plasma Proteins. The plasma proteins were combined with hemin, in the manner described in Chapters 4 and 5, and chromatographed at pH 7, using M/20 citrate buffer in the first dimension, and M/20 phthalate buffer in the second dimension. After the paper had dried, it was streaked with the benzidine reagent, and the chromatogram photographed immediately.

This method was used with radioactive plasma obtained from the

radio-iodide-treated animals described. 0.02 ml plasma was applied directly to the paper. After two-dimensional chromatography, the paper was placed in contact with X-ray film for autography, and then treated with benzidine reagent for localization of the proteins (Fig. 19).

The remaining plasma was extracted with butanol, and the butanol divided into two portions which were dried in the usual manner. The first portion was dissolved in 0.1 ml 4% sodium chloride, of which 0.01 ml was chromatographed with the butanol buffers and autographed (to act as a control on each batch of radioactive plasma that was produced). Another 0.01 ml aliquot was chromatographed with buffers and autographed (Fig. 20). Finally, the second portion of the dried butanol extract was dissolved in non-radioactive plasma obtained from another rat, mixed with hemin, chromatographed with buffers and autographed (Fig. 21).

Confirmatory experiments with plasma proteins not containing hemin were carried out by the addition of non-radioactive DL-thyroxine. 0.1 mg thyroxine was mixed with 0.02 ml radioactive plasma, chromatographed with buffers and the location of the thyroxine determined by spraying with ninhydrin.

OBSERVATIONS AND RESULTS

Thyroid and plasma fractions of ten animals were chromatographed and autographed. The autographs (Fig. 16) of the butanol extract of thyroid, with added carriers, showed the presence of spots corresponding to monoiodotyrosine, diiodotyrosine, thyroxine

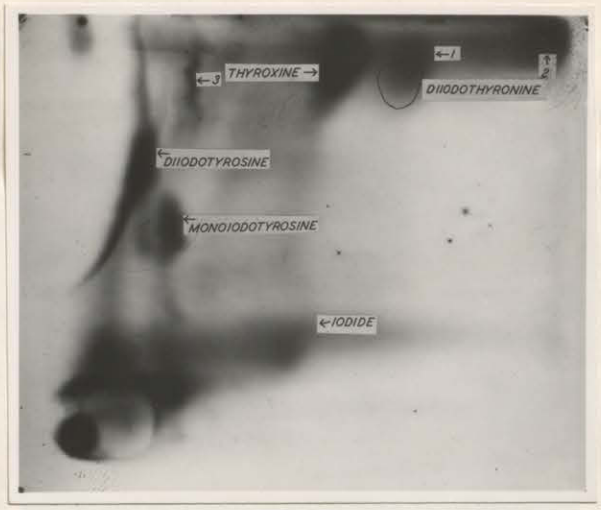


Figure 16

An autograph of a two-dimensional chromatogram of the entire butanol extract of the fresh thyroid taken from an adult rat given 100 microcuries of carrier-free NaI^{131} subcutaneously, 48 hours previously is illustrated. Twenty micrograms each of non-radioactive DL-monoiodotyrosine, DL-diiodotyrosine, DL-thyroxine and DL-diiodothyronine were added to the butanol extract. The paper was developed vertically with a butanol-formic mixture and horizontally with a butanol-dioxane-ammonia mixture. The material was deposited on the lower left hand corner. Some spots are due to thyroxine, diiodotyrosine, monoiodotyrosine and iodide; others are due to unidentified substances, indicated by the numbers 1, 2 and 3.

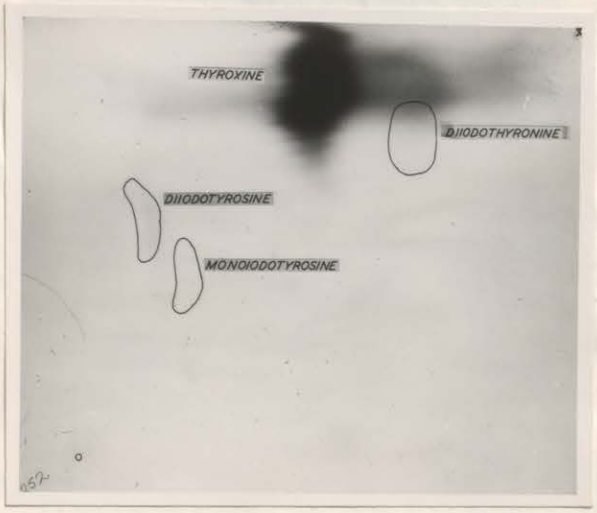


Figure 17

An autograph of the entire butanol extract of 2.5 ml of the plasma of the same animal as in Figure 16 is illustrated. The same carriers were added. An intense thyroxine spot is visible. The much less intense spot next to it is probably due to the substance causing spot 1 in Figure 16.

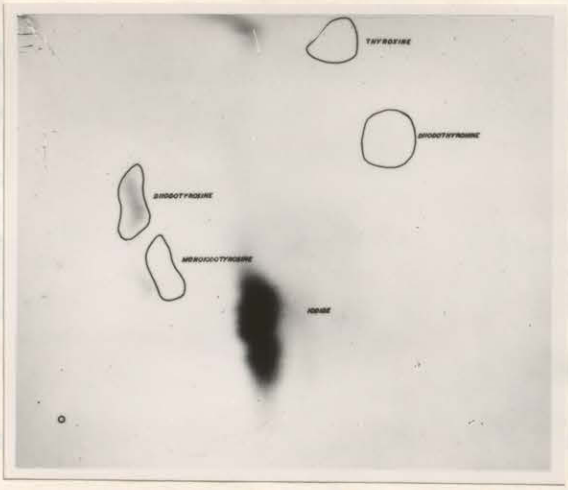


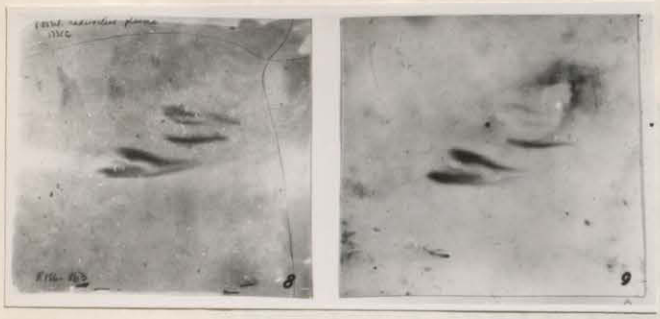
Figure 18

An autograph of a control chromatogram of a mixture containing carrier-free NaI^{131}, and 20 micrograms each of the non-radioactive DL-monoiodotyrosine, DL-diiodotyrosine, DL-thyroxine and DL-diiodothyronine is illustrated. Besides an intense spot due to iodide, a faint spot is visible in the location of diiodotyrosine. Two spots due to unidentified substances are also present. When the marked intensity of the iodide spot is compared with the low intensity of all other spots, it is apparent that only a very small percentage of the iodide present exchanged with diiodotyrosine and none at all with thyroxine.

and iodide; all of which were found in the ten animals investigated. These spots were similar in location and intensity when no carrier was used, and therefore, the presence of these substances cannot be due to an interchange between the labelled iodide and the carriers. This was borne out by control experiments in which tracer iodide was chromatographed with carriers (Fig. 18). In addition, several compounds, whose identification are unknown, were found more or less regularly throughout the series of thyroid chromatograms. Thus, of the unknowns marked 1, 2 and 3 (Fig. 16), compound 1 was found in ten thyroids, and the other two components were found in seven of the preparations.

Thyroxine made up the greater part of the radioactivity of the butanol extract of plasma (Fig. 17). By using the entire plasma extract and exposing for a long period of time, it was possible to distinguish faint spots corresponding to iodide in five plasma samples and to diiodotyrosine in six samples. Both substances were found together in extracts of five samples of plasma. The results were identical whether or not carriers were added to the plasma extract.

A possible source of error lay in the production of artifacts by the radiochemical effect of large doses of radioactivity used. However, it could be demonstrated that the same amino acids were present in the thyroid and plasma of two rats, in which the radioactivity of the injected radio-iodide was only one microcurie, a dose which has been shown to have no effect on the physiological processes of the thyroid (159). A very long exposure had to be



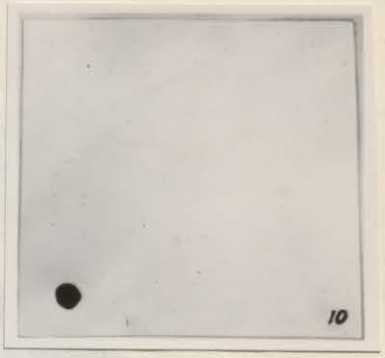


Figure 19 Figure 20

Figure 19. A chromatogram of 0.02 ml of the plasma of a rat given 100 microcuries of carrier-free NaI^{131} subcutaneously 48 hours previously is illustrated. A small amount of hemin was added to the plasma, and the location of the proteins was indicated by reaction with benzidine. The pencil lines indicate the leading edges of the developing solvents. The photograph on the right is the radioautograph corresponding to the chromatogram on the left. The radioactive material of the plasma is arranged in a pattern similar to that of the proteins.

Figure 20. The radioautograph of the butanol extract of another aliquot of the same plasma as used in Figure 19, and chromatographed with buffers as in Figure 19 is illustrated. By the method used in Figure 17, this extract was found to consist wholly of radioactive thyroxine. The radioactive thyroxine did not move from the point of origin.

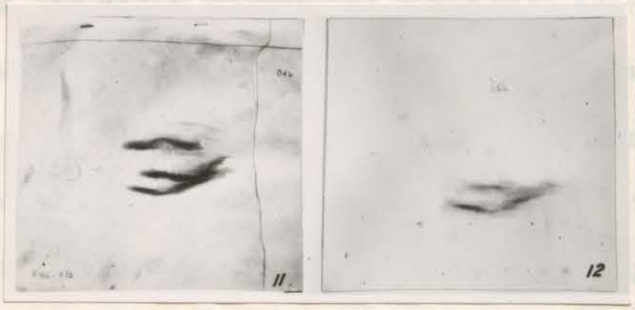


Figure 21

On the left, a chromatogram of 0.02 ml of the non-radioactive plasma is illustrated. Hemin was added to the plasma and combined with an aliquot of the butanol extract used in Figure 20, chromatographed with buffers as in Figure 19, and streaked with the benzidine reagent. The protein pattern is apparent. On the right, the radioautograph corresponds to the chromatogram on the left. The radioactive thyroxine added to non-radioactive plasma moved with the proteins, giving a pattern similar to that obtained with radioactive plasma, which is shown in Figure 19.

used to detect the minute amounts of radioactivity present in the chromatographic spot obtained under these conditions.

Nature of the circulating thyroid hormone. When an aliquot of whole plasma from an animal given radioactive iodide was mixed with hemin, and chromatographed with buffer solutions for development, the distribution of the proteins, as shown by the benzidine reagent, corresponded to the location of the plasma radioactivity (Fig. 19), indicating that the radioactivity had moved with the plasma proteins. When the radioactivity extracted by butanol was chromatographed with butanol developers, it proved to consist almost entirely of radio-thyroxine. However, when chromatographed with buffers, this radio-active thyroxine remained at the point of origin (Fig. 20). Nevertheless, if the radio-thyroxine was added to non-radioactive plasma, it was displaced along with the plasma proteins (Fig. 21), in a manner similar to that of the original radioactive plasma (Fig. 19).

The same results were obtained when non-radioactive thyroxine was chromatographed with plasma that did not contain hemin, indicating that the presence of hemin was not necessary for plasma-thyroxine combination.

DISCUSSION

These results demonstrate that, besides thyroglobulin and iodide, the thyroid gland contains small amounts of monoiodotyrosine, diiodotyrosine and thyroxine (Fig. 16). It has been shown that the presence of these amino acids is not due to exchange phenomena (Fig.

18) or to radiochemical action. The presence in the thyroid of these amino acids, which are known to be components of thyroglobulin, lends support to the hypothesis that thyroglobulin undergoes proteolysis by the enzyme system described by De Robertis.

The thyroxine found in the plasma is most likely of thyroid origin. This was indicated by experiments showing that the butanol-soluble radioactivity of plasma is extremely low in thyroidectomized rats (160). The passage of thyroxine into the circulation has previously been explained by a gradient of thyroxine concentration in thyroid tissue and plasma (155). It may be pointed out further that the diffusion of thyroxine from the thyroid would tend to correct the increase in osmotic tension due to proteolysis in the colloid. The failure to find significant amounts of other iodinated amino acids in plasma may be explained by their destruction within the follicle and a re-utilization of their iodine by the gland.

In the plasma, thyroxine has been found combined with proteins. This combination may be severed easily by butanol, but was reconstituted when thyroxine was placed in contact with the plasma proteins (Fig. 21). Furthermore, since plasma proteins may combine with amounts of thyroxine well above the physiological doses (100 γ in 0.01 ml), it may be assumed that under physiological conditions the thyroxine secreted from the thyroid combines with the plasma proteins as it is released.

The nature of the thyroxine complex in plasma is obscure. However, iodine has been found in the albumin fraction of plasma (161, 162,163), and to a somewhat lesser extent in the globulin fraction

(161,162).

SUMMARY

(1) When adult female rats on a low iodine intake were sacrificed 48 hours after an injection of carrier-free radioiodide, butanol extracts of unhydrolyzed thyroid and plasma analyzed by radioautography of two dimensional chromatograms revealed the presence of six radioactive compounds besides iodide and thyroglobulin. Of these, three were identified as thyroxine, diiodotyrosine and monoiodotyrosine.

(2) Three unidentified, iodinated substances have been found more or less consistently in dried butanol extracts of unhydrolyzed thyroid.

(3) In the blood plasma, practically all the butanol-extractable radioactivity was present as thyroxine, with a very low amount of diiodotyrosine.

(4) When the whole plasma was analyzed by radioautography of two dimensional protein chromatography, it was shown that the location of the radioactive material corresponded to that of the plasma proteins. Although thyroxine solutions showed no movement from the origin under these conditions, radioactive thyroxine dissolved in non-radioactive plasma was displaced along with the plasma proteins and gave a pattern very similar to that of the radioactive plasma itself.

It was concluded that thyroxine, after its release by the thyroid gland, circulates in combination with plasma proteins, thus

constituting the circulating thyroid hormone. The complex thus formed can be split with butanol and reconstituted in vitro.

Chapter 8

THE BINDING OF THYROXINE TO PLASMA PROTEINS

INTRODUCTION

It was shown in Chapter 7 that thyroxine combined with plasma proteins, after being liberated by the thyroid gland into the circulation. The thyroxine-protein complex thus formed was readily split by the action of butanol and reconstituted by adding thyroxine to plasma. This chapter describes some of the factors controlling the combination of thyroxine with plasma proteins. For this purpose, radioactive thyroxine was added either to whole plasma or to various purified plasma protein fractions. The mixtures were then chromatographed on paper, and the location of proteins on the paper was compared with that of radio-thyroxine on radio-autographs of the paper. In this manner, the association of thyroxine with given proteins was ascertained, and the mobility of thyroxine-protein complexes was examined.

METHODS AND MATERIALS.

Since thyroxine may be separated from the complex formed with plasma proteins by the action of butanol (46), the radio-thyroxine used in these experiments was obtained by butanol extractions of the blood of rats on a low iodine diet given 100 microcuries of radio-iodine 48 hours previously. The method, which has already been described (164), was completed by a check on the thyroxine content of the extract by chromatography with butanol solvents (46).

The dose of radio-thyroxine used in each experiment in vitro was calculated as previously indicated (164) and found to be 0.001 micrograms. Further purification of the radio-thyroxine was effected in some of the experiments as follows: the whole radio-thyroxine containing extract was chromatographed two dimensionally with acid and alkaline butanol respectively (46), a procedure which restricts thyroxine to a well defined area of the paper. This area was cut out of the paper, and eluted in water-saturated butanol, the volume of which was then reduced in vacuo, thus supplying a purified thyroxine freed from the other butanol soluble components of plasma.

Radio-thyroxine which had thus been purified was used in the first series of experiments (Fig. 22, Nos. 1-6). The radio-thyroxine was added to either whole human plasma or a mixture of the following plasma protein fractions: 120γ of Fraction II (γ-globulin), 588γ of Fraction V (albumin), 72γ of Fraction IV-1 (mainly α-globulin), 120γ of Fraction IV-3,4 (α and β-globulin, and albumin), and 12γ of Fraction VI (fringe fraction) added in amounts proportional to those present in plasma. This mixture was dissolved in 0.85% sodium chloride and chromatographed two dimensionally on paper by the method described in Chapters 4 and 5.

An experiment was also carried out under similar conditions to find out whether the amount of butanol soluble material present with radio-thyroxine in the unpurified butanol extract of plasma influenced the chromatographic pattern. 0.02 ml of plasma containing radio-thyroxine was chromatographed two-dimensionally as above after addition of the dried butanol extract from 0.1 ml of plasma, i.e.,

five times the normal quantity. The chromatogram was exposed to X-ray film for autography (Fig. 22, No. 6).

In a second series of experiments (Fig. 23, Nos. 7-18), the influence of changes in the medium on the chromatograms of the thyroxine-plasma protein complex was further examined by using various concentrations of sodium chloride and also Ringer solution. In this experiment, the samples were chromatographed on paper in a single dimension with M/20 citrate buffer at pH 7. Three different sets of conditions were used, namely, plasma removed from a rat given 100 microcuries of radio-iodine 48 hours previously (hereafter referred to as radioactive plasma), plasma from an uninjected rat to which radio-thyroxine had been added in vitro (hereafter referred to as reconstituted plasma), and a mixture of purified plasma protein fractions similar to that used in the first experiment. Three aliquots of each were used, a control (Fig. 23, Nos. 7,11,15) and two others to which enough sodium chloride was added to raise the concentration respectively to 1% and 4% (Fig. 23, Nos. 9,13,17). In addition, two aliquots of the plasma protein mixture to which radio-thyroxine had been added were dissolved in Ringer and Ringer-glucose respectively. After chromatography, all samples were exposed to X-ray films (Fig. 23, Nos. 8,10,12,14,16,18).

In a third series of experiments (Fig. 23, Nos. 19-30), controls were carried out with radio-iodide. Uni-dimensional chromatography with M/20 citrate buffer at pH 7 was carried out with samples containing tracer radio-iodine and either whole plasma (No. 21), the same plasma protein mixture as above (No. 23), or individual plasma

protein fractions, namely 120γ of Fraction IV-3,4 (No. 25), 294γ of Fraction V (No. 27) and 180γ of Fraction VI (No. 29). The paper chromatograms were then radioautographed (Nos. 20,22,24,26,28,30).

In a fourth series of experiments (Fig. 23, Nos. 31-40), the binding capacity of separate plasma protein fractions and thyroxine was systematically examined. Uni-dimensional chromatography with M/20 citrate buffer at pH 7 was carried out with samples containing radio-thyroxine and 200γ of each of the available purified protein fractions, namely Fraction II (No. 33), Fraction III, Fraction IV-1 (No. 35), Fraction IV-3,4 (No. 37), Fraction V (No. 39) and Fraction VI (No. 41). Reconstituted plasma was also run simultaneously (No. 31). The paper chromatograms were then radioautographed (Nos. 32, 34,36,38,40,42). This experiment was repeated using the following developing solutions: 2% glycine, acetate buffer at pH 6, M/5 sodium acetate, M/10 sucrose, phthalate buffer at pH 6 and 2% tartaric acid. Only the results obtained with 2% tartaric acid are illustrated (Fig. 23, Nos. 43-54). The series carried out with tartaric acid also included samples containing mixtures of globulins such as (72γ of Fraction IV-1 and 120γ of Fraction IV-3,4 - Nos. 51,52) or (200γ of Fraction II, 72γ of Fraction IV-1 and 120γ of Fraction IV-3,4) and mixtures of globulins and albumin (60γ of Fraction IV-1 and 294γ of Fraction V - Nos. 53,54) or 120γ of Fraction IV-3,4 and 294γ of Fraction V) or (200γ of Fraction II, 72γ of Fraction IV-1, 120γ of Fraction IV-3,4 and 294γ of Fraction V).

In a fifth series of experiments, the behaviour of the complex

formed by the binding of the individual proteins to radio-thyroxine was examined in two-dimensional chromatograms. The available protein fractions (II, IV-1, IV-3,4, V and VI) were used singly (Fig. 24, Nos. 55,57,59) or in combination. The amounts of proteins were at the same concentration as those found in plasma. The paper chromatograms were then radio-autographed (Nos. 56,58,60).

RESULTS

Identity of the location of plasma proteins and thyroxine on chromatograms. In the first experiment, radio-thyroxine purified by elution from paper after chromatography, was added to plasma protein fractions mixed in amounts proportional to those of plasma. The chromatography of this material with buffers made it possible to show that the proteins moved to a location (Fig. 22, No. 1) identical with that of the radioactivity (No. 2). The superimposition of the two spots indicated that thyroxine must have combined with the plasma proteins and remained bound to them while they were displaced on the paper. It may be noted that some substances (presumably including unbound hemin) remained situated in the first dimension (No. 1), but these did not bind radio-thyroxine.

Purified radio-thyroxine similarly combined with proteins of whole human plasma, since there was an exact coincidence between the protein spot (Fig. 22, No. 3) and radio-thyroxine (No. 4). When a large amount of crude butanol extract of plasma was added to radio-thyroxine, there was a more widespread distribution of plasma proteins (No. 5). The combination of radio-thyroxine with proteins

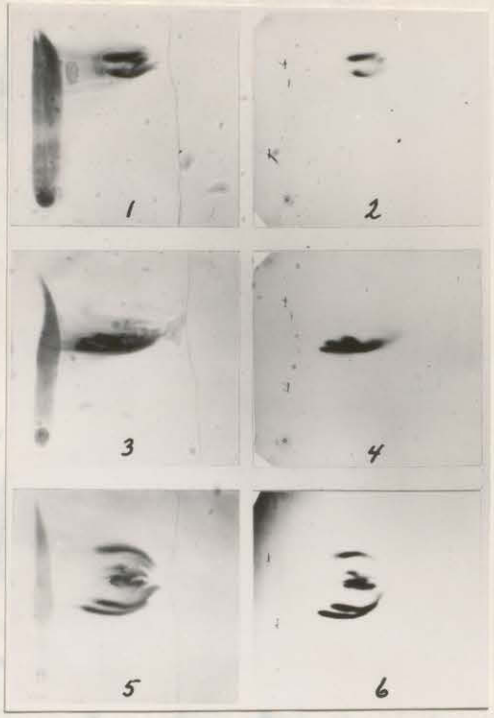


Figure 22

The two dimensional chromatograms of proteins on the left are to be compared with their radioautographs on the right.

Nos. 1 and 2. Combination between mixed protein fractions from human plasma and purified radio-thyroxine. The location of the proteins in the upper centre of No. 1 corresponds to the location of the radioactivity in the upper centre of No. 2.

Nos. 3 and 4. Combination between human plasma (0.02 ml) and purified radio-thyroxine. The location of the protein in No. 3 corresponds to that of the radioactivity in No. 4. Also, the pattern obtained here was somewhat similar to that given by protein mixtures (Nos. 1 and 2).

Nos. 5 and 6. Radio-thyroxine containing rat plasma (0.02 ml) to which has been added five times the normal quantity of dried plasma butanol extract, The increase in the amount of butanol extract produces a spreading of the protein pattern (No. 5) with a corresponding spreading of the radioautographic image due to thyroxine.

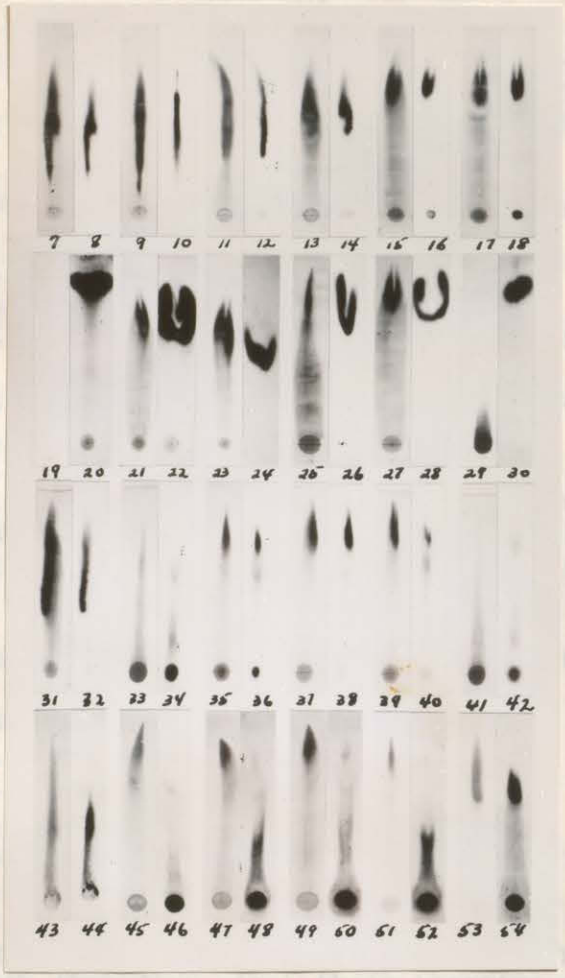


Figure 23

Each one of the four rows of pictures illustrates an experiment. The odd-numbered pictures represent chromatograms, and the even-numbered ones, radioautographs.

Nos. 7-18. Effect of 4% NaCl on the combination of radio-thyroxine with plasma or plasma protein mixture, as shown on citrate buffer chromatograms.

Nos. 7, 8. Radioactive rat plasma.

Nos. 9,10. Radioactive rat plasma in which enough NaCl had been added to raise the concentration to 4%.

Nos. 11,12. Reconstituted rat plasma.

Nos. 13,14. Reconstituted rat plasma in which enough NaCl had been added to raise the concentration to 4%.

Nos. 15,16. Mixture of plasma protein fractions and radio-thyroxine in 0.85% NaCl.

Nos. 17,18. Mixture of plasma protein fractions and radio-thyroxine in 4% NaCl. The protein sites and radio-thyroxine location coincide in all cases. Therefore, the combination of thyroxine with plasma proteins is not affected by raising the NaCl concentration.

Nos. 19-30. Behaviour of radio-iodine in the presence of plasma or plasma proteins on citrate buffer chromatograms.

Nos. 19,20. Radio-iodide alone.
Nos. 21,22. Radio-iodide added to plasma.
Nos. 23,24. Radio-iodide added to a mixture of plasma protein fractions.
Nos. 25,26. Radio-iodide added to Fraction IV-3,4.
Nos. 27,28. Radio-iodide added to Fraction V.
Nos. 29,30. Radio-iodide added to Fraction VI.

While radio-iodide alone moved to the top of the paper, its progression was limited by all protein fractions but VI. Furthermore, the location of the radio-iodide tends to surround from below the protein sites, but the protein and radio-iodide spots never coincide.

Nos. 31-42. Combination between single protein fractions (0.2 mg.) and radio-thyroxine as compared to that of plasma when chromatographed with citrate buffer at pH 7 as the developing solution.

Nos. 31,32. Whole plasma.
Nos. 33,34. Fraction II.
Nos. 35,36. Fraction IV-1.
Nos. 37,38. Fraction IV-3,4.
Nos. 39,40. Fraction V.
Nos. 41,42. Fraction VI.

It will be noted that the whole plasma proteins, Fractions IV-1, IV-3,4 and V combine with thyroxine since their location on the chromatogram coincides with that of radio-thyroxine. Fractions II and VI show no displacement, nor does the added thyroxine.

Nos. 43-54. Combination between single protein Fractions (0.2 mg) and radio-thyroxine as compared to that of plasma when chromatographed with 2% tartaric acid as the developing solution.

Nos. 43,44. Whole plasma.
Nos. 45,46. Fraction II.
Nos. 47,48. Fraction IV-3,4.
Nos. 49,50. Fraction V.
Nos. 51,52. Mixture of globulins (IV-1 and IV-3,4).
Nos. 53,54. Mixture of globulins and albumin (IV-3,4 and V).

It will be noted that, in the presence of tartaric acid, there is combination of thyroxine with plasma proteins, no combination with single protein fractions (except for a slight one in the case of albumin) or with the mixture of globulins, but a good combination with the mixture of globulins and albumin, the one most closely resembling plasma.

remained unaffected, since the pattern of radioactivity distribution (No. 6) still coincided with that of the plasma proteins (No. 5).

Control experiments. The second series of experiments demonstrated that radioactive plasma from a rat given radio-iodine 48 hours previously gave a chromatographic pattern (Fig. 23, Nos. 7, 8) which was not modified by addition of sodium chloride up to a concentration of 4% (Nos. 9, 10). Reconstituted plasma obtained by adding radio-thyroxine to plasma showed a similar pattern (Nos. 11, 12) which was not affected by the addition of sodium chloride (Nos. 13, 14). Similarly, the complex obtained by adding radio-thyroxine to a mixture of plasma proteins (Nos. 15, 16) remained the same in the presence of sodium chloride (Nos. 17, 18). Finally, no change in pattern was obtained when Ringer or Ringer-glucose was used as solvent for plasma protein mixtures.

Since at all times iodide is present with thyroxine in the circulating plasma, it was necessary to understand its behaviour. A third experimental series was designed to determine how radio-iodide was displaced on chromatograms in the presence of plasma proteins. A first control with iodide alone showed that this substance moved to the top of the paper (Fig. 23, Nos. 19, 20). In presence of the proteins, movement also occurred but was depressed in such a way that the radio-iodide spot appeared to follow the trailing edge of the protein spots (Fig. 23, Nos. 21-28), in contrast to the radio-thyroxine spots which superimposed directly on the protein sites. This behaviour of radio-iodide was observed on mixing it with whole plasma (Nos. 21, 22), or a purified protein mixture (Nos. 23, 24), or

separate protein fractions, namely, Fractions II, IV-3,4 (Nos. 25, 26) and V (Nos. 27, 28). The location of radio-iodine in the case of Fractions IV-1 and VI (Nos. 29, 30) was the same as the iodide control (Nos. 19, 20).

Combination of thyroxine and individual protein fractions. Mixtures of radio-thyroxine with individual purified protein fractions were chromatographed in the fourth series of experiments. The results obtained using citrate buffer as the developing solution (Fig. 23, Nos. 31-42) were similar to those obtained when the developing solutions were 2% glycine, acetate buffer at pH 6, M/10 sucrose, phthalate buffer at pH 6 or M/5 sodium acetate. They demonstrated that a combination with thyroxine took place with Fractions IV-1 (Nos. 35, 36), IV-3,4 (Nos. 37, 38) and V (Nos. 39, 40). Therefore, both globulin and albumin fractions showed ability to combine with thyroxine.

With 2% tartaric acid as the developing solution, a fair proportion of the protein moved from the origin with all fractions (Nos. 45, 47, 49), except Fraction VI. However, under these conditions, radio-thyroxine did not move appreciably as may be seen in the case of Fractions II (No. 46), IV-3,4 (No. 48), and V (No. 50). Since migration occurred with whole plasma (Nos. 43, 44), a series of protein mixtures were then examined. No combination of thyroxine appeared to occur with mixtures of globulins, namely, Fractions II, IV-1, and IV-3,4 or Fractions IV-1 and IV-3,4 (Nos. 51, 52). Thyroxine, however, combined with mixtures containing albumin, namely, Fractions II, IV-1, IV-3,4 and V (Nos. 53, 54). The migration of

thyroxine in all mixtures containing Fraction V may indicate that thyroxine has the highest affinity for the albumin component of blood plasma.

The predominant role of Fraction V was illustrated in the fifth experiment, which consisted of a series of two-dimensional chromatograms of thyroxine mixed with separate protein fractions. These were carried out to see which thyroxine-protein complex would be distributed according to a pattern similar to that obtained with whole plasma. Under these conditions, when single protein fractions were examined, only the pattern obtained with Fraction V (Fig. 24, Nos. 55, 56) was similar to the pattern obtained with protein mixtures (Fig. 22, Nos. 1, 2) or with whole plasma (Fig. 22, Nos. 3, 4). No such pattern was obtained with Fractions II, VI, IV-1 (Fig. 24, Nos. 57, 58) or IV-3,4 (Fig. 24, Nos. 59, 60), although some combination was present in the last case.

DISCUSSION.

It was previously shown that addition of radio-thyroxine to plasma produced a radio-thyroxine-protein complex (46). The same complex was identified in the plasma of rats sacrificed 48 hours after an injection of radio-iodine, that is, after an interval of time sufficient for the thyroid of the animal to place labelled thyroxine into the circulation. It was concluded that the circulating thyroid hormone consisted of thyroxine attached to plasma proteins (46). In this work, the formation of a thyroxine-protein complex remaining bound in the course of chromatographic migration was confirmed with the help of a highly purified radio-thyroxine (Fig. 22,

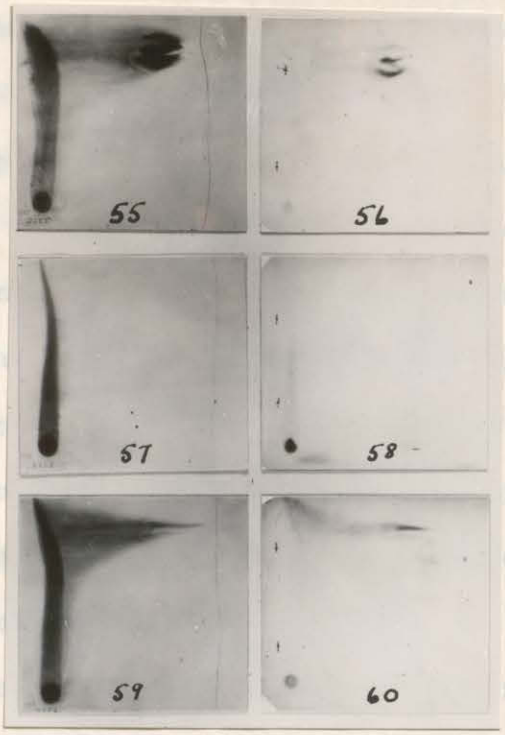


Figure 24

The two-dimensional chromatograms of single purified protein fractions on the left are to be compared with their radioautographs on the right.

Nos. 55 and 56. Mixture of Fraction V (Albumin) and radio-thyroxine. The location of the albumins in the upper center of No. 55 corresponds to the location of the radioactivity in No. 56.

Nos. 57 and 58. Mixture of Fraction IV-1 (Globulin) and radio-thyroxine. There is no evidence of displacement or combination. The radioactivity has remained at the origin.

Nos. 59 and 60. Mixture of Fraction IV-3,4 and radio-thyroxine. There is evidence of a slight displacement of radio-thyroxine in combination with this fraction.

Nos. 3, 4), as well as with crude preparations similar to those previously used (Fig. 23, Nos. 11,14,31,43,44). All these pictures, obtained by addition of radio-thyroxine to plasma, were similar to those found with the plasma of rats given radio-iodine 48 hours earlier (Fig. 23, Nos. 7, 10), a fact confirming that the same thyroxine-protein complex was involved in both cases. An indirect confirmation was also provided by the fact that iodide, the only other form in which iodine is present in the circulation, did not combine with proteins in the same manner as thyroxine (Fig. 23, Nos. 19-30).

The combination between thyroxine and plasma proteins did not require the presence of other blood components, since it was readily obtained by placing purified thyroxine in presence of a mixture of purified plasma protein fractions. Here too, the combination was maintained as thyroxine migrated on the chromatograms along with the plasma proteins (Fig. 22, Nos. 1, 2).

The pattern of protein distribution was unaffected by changes in the concentration of sodium chloride up to 4% (Fig. 23, Nos. 7-18), or by the use of Ringer or Ringer-glucose as solvent for the proteins. However, the pattern was more widespread when the proteins were associated with a large amount of plasma butanol extract (Fig. 22, No. 5). Since the same effect was obtained by the addition of detergents (134), it was likely that the butanol extract contained surface active agents which controlled the spreading of the protein pattern. At any rate, whatever the protein distribution obtained with plasma, the radio-thyroxine distribution was always superimposed upon it. Therefore, the protein-thyroxine complex was not modified by the sodium

chloride concentration, Ringer solution, or by the surface active agents of plasma.

When a combination of separate plasma protein fractions with thyroxine was examined, a mobile thyroxine-protein complex was formed with the globulins (Fraction IV-1; Fig. 23, Nos. 35, 36, and Fraction IV-3,4; Nos. 37, 38) and albumin (Fraction V; Nos. 39, 40). Of these, only the albumin showed a mobility similar to that of the thyroxine-carrier of plasma (Nos. 55-60). It is likely, however, that the globulins shared in the thyroxine carrying ability of albumin, since the use of tartaric acid as a developing solution revealed that a mixture of globulins and albumins produced a better combination with radio-thyroxine than any single protein fraction. Some caution must, however, be used in interpreting the results obtained with this low pH solution.

The persistence of association between albumin or globulin on one hand, and thyroxine on the other hand, through chromatography with a variety of developing solutions at pH values, indicates the existence of a strong bond between thyroxine and these proteins. It has been emphasized previously that the bond may be broken with butanol (46), and is easily reconstituted by association. Therefore, the binding is not by way of covalent linkages, such as peptide bonds, but is rather ionic or by van der Waal's forces. The positive results obtained at various pH values favor the latter mechanism.

SUMMARY.

(1) Thyroxine combined with whole plasma proteins. The combin-

ation was sufficiently strong to persist during chromatographic analysis. Butanol secured a dissociation of the thyroxine-protein complexes.

(2) Thyroxine combined with a mixture of Cohn's plasma protein fractions. The chromatographic pattern was similar to that of the proteins of whole plasma. This combination with proteins occurred with crude or purified radio-thyroxine; and the addition of salts, glucose, etc., did not appear to affect it.

(3) Thyroxine also combined with some of the separate purified plasma protein fractions, namely, Fractions V (albumin), IV-1 and IV-3,4 (α and β-globulins).

(4) Of the purified plasma protein fractions, which combined with thyroxine, only Fraction V (albumin) carried thyroxine according to a pattern similar to that seen with plasma. The addition of other proteins appeared to promote further combination with the thyroxine.

Chapter 9

THE COMBINATION OF SMALL MOLECULAR WEIGHT SUBSTANCES WITH PROTEINS

INTRODUCTION.

It is not many years since plasma proteins were conceived to be only inert colloids, being concerned with equilibrium in the blood stream. Now plasma proteins are known to be able to bind small molecules of physiological importance.

Within recent years, the combination of many substances with isolated protein fractions, or whole plasma, has been shown by a variety of techniques, such as spectrophotometry, electrophoresis, ultrafiltration, manometric techniques and dialysis-equilibrium experiments. Electrophoretic studies by Ballou et al have shown that a combination existed between serum albumin and the lower fatty acids (165), and later studies by the same workers were carried out on the quantitative studies on serum albumin combination with the lower fatty acids by ultrafiltration (166). They determined that the amount of combination with fatty acids increased markedly with an increase in chain length of the acid. Under conditions in which the combination with butyrate was slight, the combination with caprate was almost complete. A combination between proteins and synthetic detergents was shown by Putnam and Neurath (167), who stated that the protein-detergent complex is soluble in an excess of the detergent, apparently due to the formation of a complex around the protein molecule, whose surface is covered by the anionic groups of the detergent. Soluble compounds of protein and detergent were also

formed in alkaline solution. Their presence can be proven electrophoretically. Combinations with egg albumin and certain buffer anions, such as citrate, phthalate and acetate was shown by Smith (168), calcium ions and plasma proteins by ultrafiltration by Greenberg and Gunther (169), and some organic anions (by dialysis-equilibrium), such as phenyl butyrate, phenyl acetate, cinnamate, hippurate, phenoxyacetate, nitrophenolate and picrate with bovine serum albumin was shown by Teresi and Luck (170). Serum albumin has been shown to combine with phenol red (by ultrafiltration) (171), and also with methyl orange, although combination did not occur with γ-globulin (by dialysis-equilibrium) (172). An interaction between human serum albumin and ferriprotophorphyrin IX was shown by Rosenfeld and Surgenor (173). Quastel showed that certain amino compounds could protect the enzyme urease from the toxic action of some basic dyes, which indicated an enzyme-amino acid combination (174). Lea and Hannan showed that as much as 7% of a reducing sugar could be firmly bound to a protein, almost entirely at the free amino groups, under conditions of neutral pH and 37° (175). The combination of human serum albumin and penicillin was shown by dialysis experiments (176), and later between crystallized bovine serum albumin and penicillin, but not between penicillin and ribonuclease (177). The binding of sulfonamides with serum albumin was shown by equilibrating the drug between a plasma protein solution and a buffer solution (178). Eakin et al (193) observed that avidin of egg proteins would combine with biotin to produce a biotin deficiency.

Stadie et al (202) demonstrated that certain substances which

are metabolically active combine with considerable rapidity with elements of tissue structure with sufficient firmness to resist the dissociating action of washing and, in combined form, exert characteristic effects on tissue metabolism. They also demonstrated the combination of epinephrine and 2,4-dinitrophenol with tissue structures, which, in bound form, brought about a diminution of glycogen synthesis from glucose.

The use of paper chromatography to show a combination between radioactive thyroxine and plasma proteins was shown in Chapters 7 and 8. This chapter is to demonstrate that paper chromatography can be utilized to show the combination between many substances having a relatively small molecular weight, such as amino acids, fatty acids, sugars, vitamins, some carcinogens and other molecules of physiological importance, and the plasma protein fractions.

MATERIALS AND METHODS.

Three concentric cylinders of filter paper are used to increase the number of samples to be chromatographed at the same time. Whatman No. 1 filter paper is cut into sheets; 18 x 10, 15 x 10, and 11 x 10 inches. Samples to be analyzed are placed at 1.5 inch intervals at a distance of one inch from the bottom of the paper.

0.02 ml aliquots of the protein solution is placed on the paper, allowed to dry, then 0.01 ml of the test solution is applied on top of the dried protein spot. There have been no differences observed whether the test solution was mixed with the protein solution in the test tube or on the filter paper.

For two-dimensional chromatography, 0.02 ml aliquots of the protein-test substance solution is placed in the lower left hand corner of the filter paper, and chromatographed in the manner described previously (134).

Developing Solutions. The developing solutions are of an aqueous nature, and are freshly prepared. They include: 2% tartaric acid, M/10 sucrose, M/10 sodium potassium tartrate, M/5 sodium acetate, M/20 phthalate buffers at pH 5 and 6, M/20 citrate buffer at pH 6 and 1% glycine, for single dimensional analyses. For two dimensional chromatography, two combinations of developing solutions are used: (a) M/10 sucrose as the developing solution in the first dimension, when this is followed by M/10 sodium potassium tartrate in the second dimension, and (b) M/20 citrate buffer pH 6 as the developing solution in the first dimension, when this is followed by M/20 phthalate buffer pH 6 in the second dimension.

Protein Solutions. 10 mg of the Cohn protein fraction is dissolved in 1 ml 0.85% saline, to which is added 10 mg of the test substance per 0.5 ml of protein solution, unless stated otherwise. If mixed on the paper, 0.01 ml of the test solution is applied on top of 0.02 ml of the protein solution.

Blood is removed from normal humans with a heparinized syringe, and centrifuged at 2500 r.p.m. for 20 minutes. The plasma is used immediately.

If the test substance, such as ricinoleate, stearate, biotin, pantothenate, etc. is not easily detectable on filter paper, hemin is added to the protein solution (0.02 ml 0.3% hemin per 0.5 ml protein

solution) and changes in the protein pattern, due to the test substance, are observed.

Test Substances. Whenever possible, 10 mg of the test substance is dissolved in water, neutralized and the volume made to 0.5 ml. If the substance must be dissolved in alcohol, (e.g. dimethyl yellow) then the corresponding amount of alcohol is added to the protein solution control. Substances such as adenylic acid and riboflavin are dissolved in dilute bicarbonate solution.

Detection of Test Substances.

(a) Hemin. If hemin has been added to the protein solution, the chromatogram is streaked with the benzidine reagent, as described previously (133).

(b) Natural color. Bilirubin and dimethyl yellow retain their natural color during chromatography in the presence of protein solutions. The dimethyl yellow chromatograms can also be sprayed with dilute acid to get a more detectable pink color.

(c) Fluorescence. Some substances possess a natural fluorescence in ultraviolet light, while others can be caused to fluoresce by spraying with the appropriate reagent. Those substances which may be detected by fluorescence include: riboflavin, ascorbic acid, dehydroascorbic acid, pyridoxine and benzpyrene. Thiamine and thiamine pyrophosphate (co-carboxylase) can be caused to fluoresce by spraying the chromatogram with the Thiamine Reagent (dissolve 30 mg potassium ferricyanide in 100 ml 15% NaOH).

(d) Ninhydrin. 0.1% ninhydrin in water-saturated n-butanol can be used for the detection of the amino acids, as well as glutamine,

muscle adenylic acid and glucosamine.

(e) 0.2M m-phenylenediamine dihydrochloride (in 76% ethanol). After spraying, the chromatogram is heated for 5 minutes at 105° and some sugars will show up as dark brown on a light brown background. It can be used for glucose, fructose, maltose, sucrose, xylose and ribose.

(f) Alkaline permanganate. 1% potassium permanganate containing 2% sodium carbonate can be used to detect inositol, mannitol, glucose, fructose, maltose, sucrose and xylose. These substances give a yellow color on a pink background, but since the color development is very transient, it is only used where other reagents fail.

(g) Ammoniacal silver nitrate. The chromatograms can be sprayed with a solution containing equal volumes of 0.1 N silver nitrate solution and 5N ammonia solution, and placed in an oven for 5-10 minutes at 105°. This reagent can be used to detect glucose, fructose, maltose, inositol, xylose, mannitol and ascorbic acid.

(h) Heat. Alloxan turns red when placed in an oven at 100° for 5 minutes. In the presence of fresh plasma, it turns red within a few minutes at room temperature.

(i) Substances which were undetectable included: calcium pantothenate, adenosine triphosphate, sodium ricinoleate, sodium stearate and biotin. Phlorizin did not fluoresce, but its position on the chromatogram could be seen when exposed to ultraviolet light.

RESULTS.

Combination with Cohn Protein Fractions. The following substances

combined with Fraction I only, when 2% tartaric acid was used as the developing solution: tryptophan, phenylalanine, cysteine, arginine, histidine, glutamine (these substances did not show a combination with any fraction when M/10 sucrose and M/10 Na K tartrate were used as developing solutions), sucrose, maltose, inositol, ribose, xylose, glucosamine, dehydroascorbic acid, pyridoxine, and alloxan (these latter substances showed no combination with any fraction when M/5 sodium acetate, citrate buffer pH 6 and phthalate buffer pH 6 were used as developing solutions).

Glycine, glutamate, methionine, lysine and proline showed combination with some or all of Fractions I, II and III, depending on the type of developing solution used, which were the same as above.

Riboflavin showed combination with all the protein fractions when M/10 sucrose and M/10 Na K tartrate were used as developing solutions, but no combinations with any fraction was observed when 2% tartaric acid was used.

Glucose, fructose, mannitol and ascorbate showed combination with some or all of Fractions I, II and III when the following developing solutions were used: 2% tartaric acid, M/5 sodium acetate, citrate buffer pH 6 and phthalate buffer pH 6. No combination with Fractions IV-1, IV-3,4 or V occurred under these conditions.

Thiamine and adenylic acid showed combinations with one or more of each of the protein fractions when the following developing solutions were used: 2% tartaric acid, M/10 sucrose, M/10 sodium potassium tartrate, M/5 sodium acetate, phthalate buffer pH 5 and 1% glycine.

Thiamine pyrophosphate showed combination with every protein fraction under every condition used in this chapter.

Phlorizin showed a slight combination with Fraction I when 2% tartaric acid, M/10 sucrose and M/10 sodium potassium tartrate were used as the developing solution, but no combination occurred with other fractions under these conditions.

Dimethyl yellow did not combine with any of the protein fractions under any of the conditions mentioned.

Benzpyrene showed combinations with Fractions IV-1, IV-3,4 and V when the following developing solutions were used: 2% tartaric acid, M/10 sucrose, M/10 sodium potassium tartrate, M/5 sodium acetate, phthalate buffer pH 5 and 1% glycine. No combinations with Fractions I, II or III were observed under these conditions.

Bilirubin showed combinations with Fractions IV-1, IV-3,4 and V when 2% tartaric acid, M/10 sucrose and M/10 sodium potassium tartrate were used as developing solutions. Combinations with Fractions II and III occurred when the latter two developing solutions were used, but no combination occurred with Fraction I under any of these conditions.

Combination with whole plasma. Cysteine, tryptophan, arginine, phenylalanine, histidine, glutamine, methionine, lysine, proline, glycine, glutamate, glucosamine, bilirubin, dimethyl yellow, alloxan, adenylic acid and benzpyrene appeared to combine with the plasma proteins in a non-specific manner when chromatographed with M/10 sucrose in the first dimension, and M/10 sodium potassium tartrate in the second dimension. The inclusion of 0.01 ml Tween 85 per 0.5

ml plasma did not affect this combination. The resulting chromatograms very closely resembled those of the plasma protein-hemin chromatograms.

Glucose, fructose, maltose, sucrose, inositol, xylose, mannitol and ribose could not be detected with the sugar reagents when chromatographed in the presence of the plasma proteins, using citrate buffer pH 6 in the first dimension, and phthalate buffer pH 6 in the second dimension. However, all the sugar-plasma protein complexes showed chromatograms which were altered from the normal, indicating that a probable combination had taken place.

Riboflavin, thiamine, thiamine pyrophosphate, ascorbate, dehydrophosphate and pyridoxine combined with the plasma proteins in a non-specific manner when chromatographed two-dimensionally with sucrose/tartrate or citrate/phthalate buffers at pH 6.

Biotin and calcium pantothenate were not detectable, and their presence in plasma protein chromatograms did not alter the pattern when compared to a control pattern.

Since fatty acids are not easily detectable on paper chromatograms, hemin was added to the plasma proteins, and comparisons were made with the plasma protein control. Stearate in relatively high concentrations (20 mg/0.5 ml plasma) decreased the mobility of the plasma proteins. Linseed oil (0.02, 0.04 and 0.06 ml / 0.5 ml plasma) caused the plasma proteins to move as one fraction only, which is considerably less than the control pattern. Neither stearate nor linseed oil had a surface active effect, i.e., no ability to cause a spreading of the protein pattern. Ricinoleate showed a

Figure 25

The figure on the left illustrates a typical combination between a non-protein substance and a protein fraction. On the extreme left of this illustration is the control test substance, then the non-protein-protein complex in duplicate, and finally the control protein in duplicate.

The figure on the right illustrates a typical non-combination between a non-protein substance and a protein fraction, in the same order as the illustration on the left.

Figure 26

The effect of sodium ricinoleate on the movement of plasma proteins is illustrated.

Human plasma (left), the presence of 3 mg sodium ricinoleate per 0.5 ml plasma (centre) and 10 mg sodium ricinoleate per 0.5 ml plasma (right) was chromatographed with hemin, and developed with M/10 sucrose in the first dimension and M/10 sodium potassium tartrate in the second dimension.

It was observed that sodium ricinoleate had a slight surface active effect (centre), but larger quantities depressed the movement of the proteins (right).

significant surface active effect when 3-5 mg per 0.5 ml plasma was used (almost identical as with the addition of 0.01 ml Tween 85), although higher concentrations (10-20 mg / 0.5 ml plasma) decreased the mobility of the plasma proteins. (Fig. 26).

DISCUSSION.

A combination was assumed to have taken place between the test substance and the protein, only when the complex was superimposable on the chromatogram of the control protein (Figure 25). Non-combinations were assumed when the test substance remained at the point of origin and the protein moved, or when both the test substance and the protein moved, the result not being superimposable on the control protein chromatogram (Figure 25). In this case the protein appeared to hinder the movement of the test substance, particularly when the latter had an R_F value of unity, and the test substance formed a partial halo around the trailing edge of the protein. This was particularly striking when observing the movement of the proline-protein complex, in which proline gave a yellow color with ninhydrin and the protein gave a purple color. If any combination occurred when this 'halo effect' was observed, it was not possible to detect it under these conditions. A non-combination resulted in a yellow halo around a purple streak, and a combination gave rise to a third color, usually blue in this case.

It will be observed that most of these substances combined with Fractions I, II and III, particularly Fraction I (glycine, glutamate, methionine, lysine, tryptophan, phenylalanine, cysteine, arginine,

histidine, glutamine, proline, glucosamine, inositol, xylose, sucrose, maltose, glucose, fructose, mannitol, ribose, riboflavin, ascorbate, dehydroascorbate, pyridoxine, alloxan and phlorizin). Some substances showed combinations mainly with Fractions IV-1, IV-3,4 and V (bilirubin and benzpyrene). Adenylic acid showed a more general combination with the protein fractions. Thiamine and thiamine pyrophosphate, particularly the latter, showed combinations with many fractions under a wide variety of chromatographic conditions. Dimethyl yellow failed to combine with any of the protein fractions under these conditions.

A very striking observation is that, in spite of the fact that combinations between these substances and the proteins occurred only slightly, and under certain conditions, combination with whole plasma occurred in every case. Dimethyl yellow, for example, did not move with any individual fraction, but migrated with the whole plasma. In most cases, the two-dimensional chromatogram closely resembled that of the protein-hemin chromatogram which has been described previously.

The greater combining ability of Fraction I that has been observed, may be due to the fact that this fraction contains some of all the plasma protein fractions, although fibrinogen makes up the greater part (145). Since whole plasma showed a remarkable ability to combine with all the test substances, it seems possible that a protein-protein complex is far more efficient for binding power than a single protein. The interaction of proteins has been evident in much of the work carried out in protein chromatography. It will also

be recalled that the binding ability of whole plasma for radioactive thyroxine is greater than the sum of the binding capacities of the individual protein fractions (137). Thus, it seems reasonable to assume that a mixture of plasma proteins has a greater combining power for some molecules of physiological importance than that of its component fractions.

Haurowitz has observed (191) that ". . . one can expect that γ-globulin, the isoelectric point of which is near to pH 7, will combine to form saltlike compounds with albumins, whose isoelectric point is close to pH 4.7 . . . We cannot exclude the possibility, therefore, that the native blood plasma contains only a small number of proteins and that many of the fractions found are formed by combination of these basic proteins with lipids, carbohydrates, other proteins, or certain ions".

Combination of proteins with non-protein substances, or prosthetic groups, in vivo, lead to the formation of protein complexes, such as the lipoproteins, glucoproteins, phosphoproteins, metalloproteins and chromoproteins. On the other hand, a very unstable relationship exists in the enzyme-substrate complex. In between these extremes, there exist complexes which are reversible, such as the thyroxine-plasma protein combination (46). This latter type probably represents the nature of most of the combinations observed in this chapter.

Sodium ricinoleate at a concentration of 3-5 mg per 0.5 ml plasma appears to have a significant surface active effect, i.e., it has a spreading effect, resulting in the separation of 8-9 fractions

from plasma. This is about the same spreading effect as 0.01 ml Tween 85 per 0.5 ml plasma (Tween 85 is polyoxyethylene sorbitan trioleate) (134).

Since the plasma proteins combined with all of the test substances under these relatively mild chromatographic conditions, it would seem to indicate that probably physiological substances of a small molecular weight do not exist in the plasma as such, but circulate in the blood stream as a loose combination with the protein components. This could be in a manner similar to that of the circulating thyroid hormone, in which thyroxine appears to be loosely bound to the proteins of the plasma (46).

Haurotwitz (191) has described the nature of the forces operating between proteins and other substances. They include: (a) ionic groups, which have bonds between the protein and organic or inorganic ions that are due to electrostatic forces, (b) polar non-ionic groups, which have dipole-dipole bonds which are effective between hydroxyl, sulfhydryl and amino groups, and include the hydrogen bond, (c) non-polar groups, which have bonds which are attributed to short period oscillations of electrons in which the attractive forces are very weak.

There are also intermediary types of intermolecular bonds. Electrostatic forces are effective not only between two ions or two dipoles, but also between an ion and adjacent dipoles. Intermediary types of linkages may also exist between polar and non-polar groups.

The hydroxyl, sulfhydryl, amino and other non-ionic polar or polarizable groups of the protein molecules combine with polar groups

or with ionic groups of other molecules by means of electrostatic forces. Many drugs such as the sulfonamides are bound to protein molecules by means of such bonds. The steric arrangement of the binding polar groups has a great influence on the strength of the bonds. The mutual attraction between antigens and their specific antibodies is due to this type of linkage as well as the mutual attraction of complementarily shaped surfaces.

The non-polar hydrocarbon group probably combines with the non-polar groups of the proteins, i.e., with the aliphatic side chains of alanine, valine, leucine, and isoleucine, with the benzyl group of phenylalanine and with the CH_2 groups of the pyrrolidine ring of proline. By means of these non-polar groups, proteins combine with fats and fatty acids, and also with simple hydrocarbons.

Przylecki et al (195) observed that the tendency of tyrosine and arginine to combine with polysaccharides was outstanding. Therefore, it was suggested that the polysaccharides were bound to these amino acids of the protein component.

This wide range of forces existing between proteins and other substances may go a long way in explaining the combinations observed in this work, which vary with the nature of the chromatographic conditions. The nature of the salts used in the developing solutions and the pH of the medium could profoundly influence the binding ability of the proteins for these various substances. The non-specificity of the binding of these substances with whole plasma proteins could then be explained by the vast number of groups present in a protein mixture which are available for this wide range of

binding power.

It must be emphasized that these observations are of a preliminary nature only, since experimental conditions included a very narrow range of protein and non-protein concentration. The effects of variations in these concentrations, as well as other experimental conditions, are being studied at the time of submission of this thesis (March, 1951).

SUMMARY.

(1) The combination of molecules of physiological importance and of small molecular weights, with plasma proteins and plasma protein fractions has been investigated by paper chromatography, using a wide variety of chromatographic conditions. This includes cysteine, tryptophan, arginine, phenylalanine, histidine, glutamine, methionine, lysine, proline, glycine, glutamate, stearate, ricinoleate, linseed oil, glucose, fructose, mannitol, sucrose, maltose, inositol, xylose, glucosamine, ribose, riboflavin, ascorbate, dehydroascorbate, pyridoxine, biotin, pantothenate, thiamine, thiamine pyrophosphate, bilirubin, dimethylaminoazobenzene, benzpyrene, adenylic acid, alloxan, and phlorizin. They have been combined with the Cohn Protein Fractions I (mainly fibrinogen), II (mainly γ-globulin), III (mainly β-globulin), IV-1 (mainly α-globulin), IV-3,4 (α and β-globulins) and V (mainly albumin), as well as human plasma. Developing solutions used were: 2% tartaric acid, M/10 sucrose, M/10 sodium potassium tartrate, M/5 sodium acetate, M/20 phthalate buffers at pH 5 and 6, M/20 citrate buffer pH 6 and 1% glycine.

(2) It has been observed that in most cases, combination appeared to occur only with certain proteins, which probably depended on the nature of the experimental conditions. However, combination with whole human plasma appeared to be more complete than with the individual protein fractions, and in a non-specific manner.

(3) Sodium ricinoleate appeared to have a surface active effect, i.e., allowed a spreading of the plasma protein pattern.

(4) This work indicated that substances of physiological importance may exist in the blood stream as loose complexes with the plasma proteins, and circulate with them.

Chapter 10

EFFECTS OF FAT MEALS AND HEPARIN ADMINISTRATION ON BLOOD PLASMA COMPOSITION AS SHOWN BY PAPER CHROMATOGRAPHY

INTRODUCTION.

The ability to show differences between rat, human and guinea pig plasma, as described in Chapter 5, indicated that changes in an animal under various biological conditions could probably be followed with this technique. Thus, a series of experiments were set up, in collaboration with Dr. R.L. Swank, of the Montreal Neurological Institute, using the dog as the test animal to show the effects of fat meals and intravenous heparin administration on blood plasma composition.

Swank showed in a previous study that six to nine hours after a large fat meal, and immediately after intravenous injections of a small amount of heparin, the chylomicra and red blood cells have a tendency to aggregate (179). The clustering of chylomicra after intravenous heparin has been observed by Weld (180). These changes were frequently attended by a decrease in the hematocrit, and by an increase in the sedimentation rate in lipemic plasma. Heparin has also been observed (181) to cause a significant reduction in the number of chylomicra in the plasma. The maximum aggregation of the chylomicra and red blood cells after a fat meal did not develop

(Dr. Swank supervised the feeding of fat meals to the dogs and the removal of plasma from them. Miss Aagot Grimsgaard carried out determinations of the hematocrit, sedimentation rate and chylomicron counts).

three hours after the fat meal when the chylomicra were most numerous, but six to nine hours afterwards when the number of chylomicra were rapidly decreasing (179). It was therefore suggested that this alteration in the suspension stability of the blood elements was due to a change in the factors in the plasma responsible for the maintenance of stability. It was presumed that the chylomicra competed with the red blood cells for these factors and a temporary deficiency resulted. Because of the possibility that the factors in question are protein in nature, preliminary studies of the total proteins, albumin, globulin and fibrinogen were carried out. The studies showed no very consistent or significant changes in these factors apart from a possible slight reduction in the total proteins (Table I).

MATERIALS AND METHODS.

Two male and four female dogs weighing from 10 to 21 kg. were studied. A total of 23 experiments were performed in these six animals. Cream fat (relatively saturated) and a mixture of cod liver oil and linseed oil (relatively unsaturated) were tested; the test meals consisting of four grams of fat per kilogram of body weight. The maintenance diet of the dogs was beef heart and fox chow. Prior to the fat tests, all dogs were fasted for 18 hours unless otherwise indicated. All bloods were withdrawn from one of the leg veins and prevented from clotting by heparin-sodium salt (Connaught Laboratories). Occasionally isotonic ammonium and potassium oxalate were used as anti-coagulants to provide controls on the heparin. 0.3 ml heparin (10 mg/ml) was injected intravenously. Observations were

made of the number of chylomicra, hematocrit, sedimentation rate, protein fractions (total protein, albumin, globulin and fibrinogen), and non-protein nitrogen. In the present experiments, the hematocrit and sedimentation rates were used in Table I to indicate changes in the aggregation tendency of the red blood cells because these measurements are probably more dependable and less subjective than the changes in dark field illumination (179).

The paper chromatographic technique as described in Chapter 5 was used. Four sets of conditions were used to examine the plasma. One condition did not include the surface active agent, and the others used 0.02 and 0.03 ml of Tween 81, and 0.03 ml Tween 85 per 0.5 ml of the test plasma, to which had been added 0.02 ml 0.3% hemin solution. 0.02 ml aliquots were applied to the paper, and developed with M/10 sucrose solution in the first dimension, and M/10 sodium potassium tartrate in the second dimension. The use of 0.01 ml Tween 85 and 0.01 and 0.03 ml of BRIJ 30 did not show up changes in the protein patterns, and were discarded.

RESULTS.

The effect of intravenous administration of heparin in fasting dogs. Heparin was given intravenously to four dogs and five tests were made. In every instance, a significant change in the protein pattern of the paper chromatograms was observed. The changes under one set of conditions in two dogs are shown in Fig. 27 (Nos. 1, 2 and 3, 4). The changes in the hematocrit, sedimentation rate and chylomicra counts before and ten minutes after the intravenous heparin are

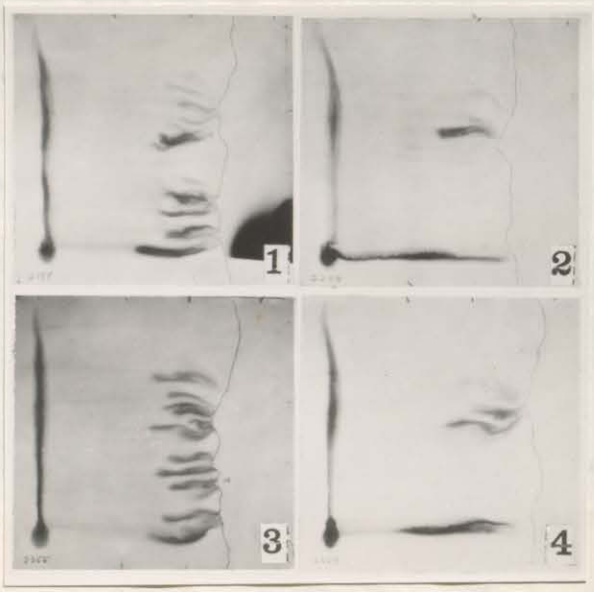


Figure 27

The comparison between the protein chromatograms of fasting dog plasma (Nos. 1 and 3) and 10 minutes after intravenous heparin (Nos. 2 and 4) is illustrated. 0.03 ml Tween 81 was used with 0.5 ml plasma. M/10 sucrose solution was used as the developing solution in the first dimension, and M/10 Na K tartrate as the developing solution in the second dimension.

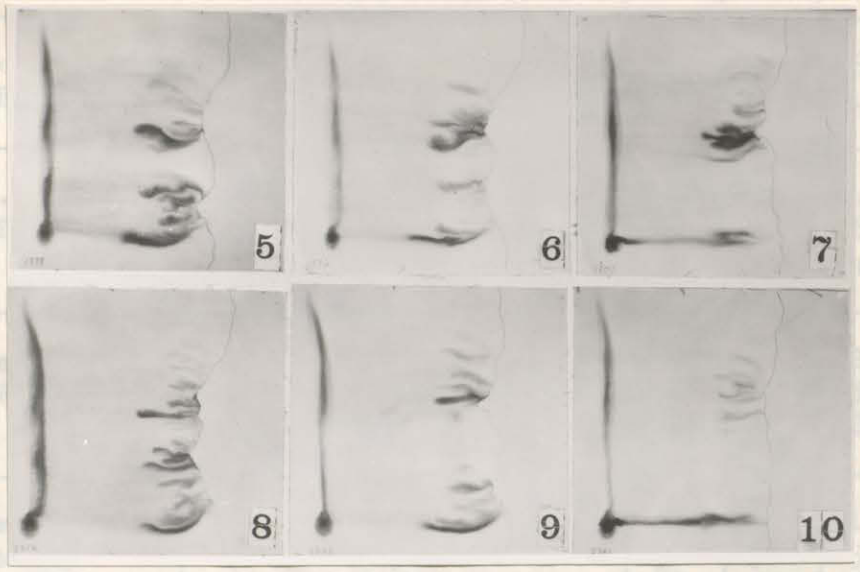


Figure 28

The effect of fasting dog plasma (Nos. 5 and 8), 6 hours after a high fat meal (Nos. 6 and 9), and ten minutes after the intravenous administration of heparin (Nos. 7 and 10), on the protein chromatograms is illustrated. 0.03 ml Tween 81 was used with 0.5 ml plasma. M/10 sucrose solution was used as the developing solution in the first dimension, and M/10 Na K tartrate in the second dimension.

Table I

		Hem.	Sed.	C.M.	Chrom.
A. F.F.F. (Female)	Fasting	49	3	1	0
	After heparin	47	1	0	++
B. Beagle (Female)	Fasting	50	1	6	0
	After heparin	47	3	1	++
C. F.F.F. (Pregnant)	Fasting	40	28	4	0
	Cream lipemia 6 hours	39	26	60	+
	After heparin	37	3	40	++
D. Beagle (Female)	Fasting	49	1	2	0
	Cream lipemia 6 hours	48	2	32	+
	After heparin	47	2	20	++
E. Farmer (Male)	Fasting	50	3	1	0
	Cream fat 3 hours	50	3	55	0
	Cream fat 6 hours	48	5	32	+
	Cream fat 9 hours	44	9	6	++
F. Farmer (Male)	Fasting	52	4	9	0
	Cod liver oil and linseed oil	54	2	62	+
		52	3	50	+
		52	4	25	0

The hematocrit (Hem.) and sedimentation rates (Sed.) on whole blood, chylomicron (C.M.) counts on plasma, and estimations of the changes in the plasma proteins (Chrom.), in the series of experiments described in the text. Changes in the chromatograms are indicated arbitrarily by 0 (normal), + (slight but definite change), and ++ (marked change).

shown in Table I (A and B) for these same dogs. They suggest only a slight reduction in the hematocrit in each of these two experiments, although in other experiments, more marked decreases in the hematocrits were seen (179).

One additional experiment showed that the changed protein patterns observed in the plasma after intravenous heparin may return to normal in less than one hour. In this experiment, the initial changes in the red blood cells and chylomicra were slight. This was frequently the case (179) when the dogs had been fasted for 24 to 36 hours.

Effect of a large cream fat meal with subsequent heparin administration. A large cream fat meal, with subsequent heparin administration, was given to six dogs on eight occasions. Six hours after the fat meal, seven plasmas showed definite paper chromatographic changes, presumably due to the fat absorption alone. In all eight tests, heparin was injected six hours after the fat meal was given, and post-heparin samples of plasma were obtained ten minutes later. All post-heparin bloods exhibited further alterations in the plasma protein patterns. The paper chromatograms for two such tests are shown in Figure 28 (Nos. 5,6,7 and 8,9,10). It is interesting to note that the changes produced by fat absorption alone appear to be intermediate between the fasting and post-heparin records in these experiments. The hematocrit showed a progressive, but slight decrease during each of these experiments (Table I; C and D), and in experiment C a marked reduction in the sedimentation rate was observed after the injection of heparin. The dog used in this experi-

ment was pregnant and parturition occurred two weeks later. Similar changes in the sedimentation rate have been observed before in males, and in pregnant and non-pregnant females (179). The reduction in the chylomicron counts following heparin is also to be noted.

Effects of single, large fat meals. The effects of fat feeding alone was observed five times with two dogs. Plasmas were taken at 0, 3, 6 and 9 hours after the fat meal. In two experiments, cream fat, and in three others, a mixture of cod liver oil and linseed oil was used. The three tests with the mixture of very unsaturated fats all showed changes in the protein patterns at three hours, with maximum changes at six hours (Fig. 29: Nos. 15, 16, 17, 18). The chromatograms returned to very nearly normal at nine hours. With the relatively saturated fat, one dog exhibited no change; the other exhibited marked changes at six and particularly at nine hours after the fat meal (Fig. 29: Nos. 11, 12, 13, 14). The dog which exhibited marked changes in his plasma proteins also exhibited a marked drop in the hematocrit and an increase in the sedimentation rate at nine hours, at a time when the chylomicron count had returned to about normal (Table I: E). In the test with the unsaturated fat (Table I: F), no significant change occurred in the hematocrit or sedimentation rate.

Effects of separation of chylomicrons from plasma. The effect of separating the plasma into a very nearly chylomicron free portion (which is clear), and a cloudy portion containing most of the chylomicra was observed in two experiments. The samples were taken three and six hours after the fat meal and the plasma was centrifuged

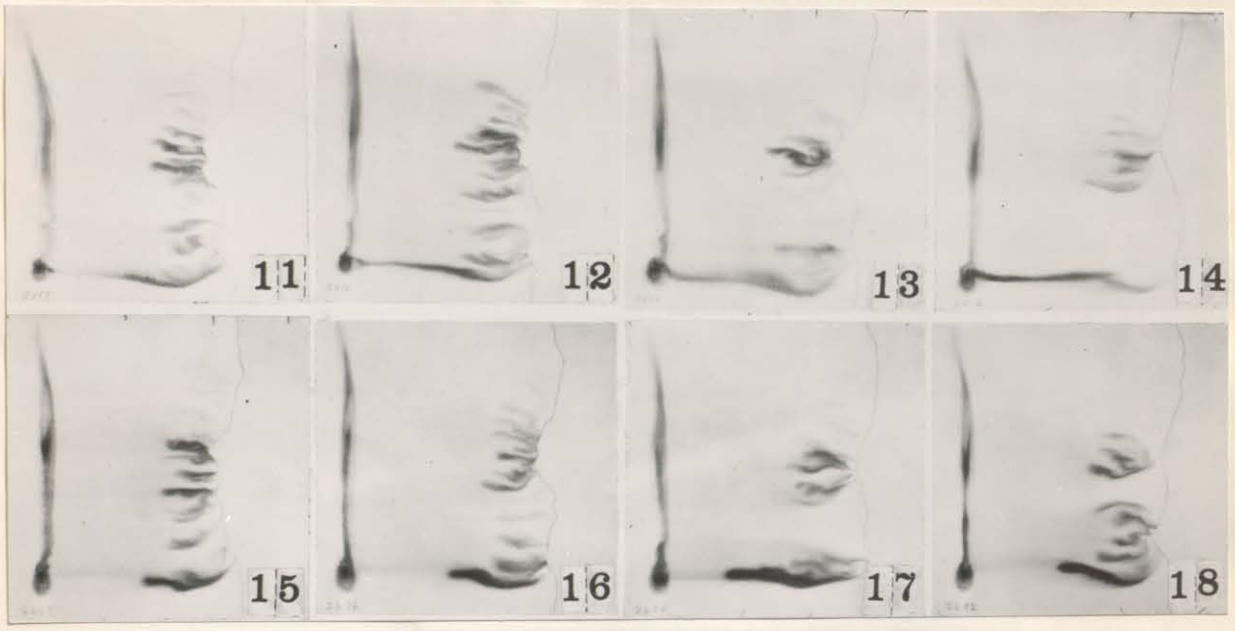


Figure 29

The differences between fasting plasma (Nos. 11 and 15), three hours (Nos. 12 and 16), six hours (Nos. 13 and 17), and nine hours (Nos. 14 and 18) after the feeding of a high fat meal is illustrated. 0.03 ml Tween 81 was used with 0.5 ml plasma. M/10 sucrose was used as the developing solution in the first dimension, and M/10 Na K tartrate in the second dimension.

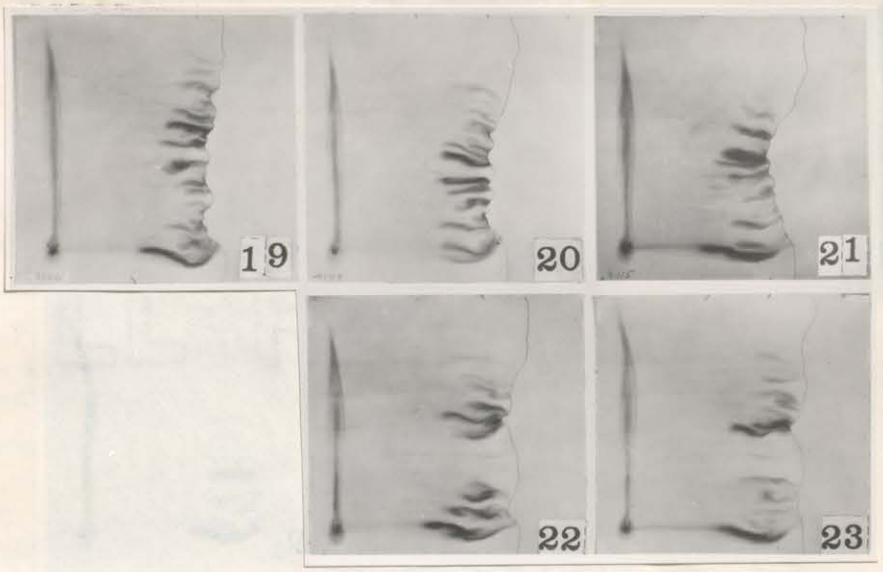


Figure 30

The effect of high speed centrifugation on the plasma of dogs which have been fed a high fat meal is illustrated. No. 19 is the chromatogram of a fasting plasma, No. 20 of the clear fraction of the plasma of a three hour lipemia, No. 21 is the same at six hours. No. 22 is the lipemic portion at three hours and No. 23 is the same at six hours. 0.03 ml Tween 81 was used with 0.5 ml plasma. M/10 sucrose solution was used as the developing solution in the first dimension, and M/10 Na K tartrate in the second dimension.

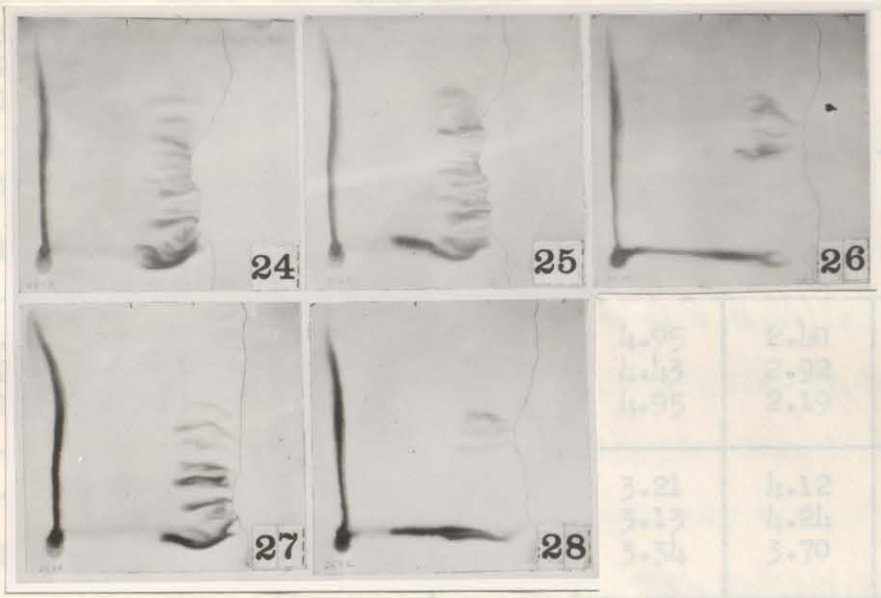


Figure 31

The effect of the intravenous injection of Tween 81 on a fasting and a lipemic dog is illustrated. Nos. 24 and 27 show the protein chromatograms of fasting dog plasma. No. 25 is a six hour lipemia, and No. 26 is ten minutes after the intravenous injection of Tween 81 given after six hours of lipemia. No. 28 shows the effect of intravenous Tween 81 on a fasting dog. 0.03 ml Tween 81 was mixed with 0.5 ml plasma in vitro to illustrate these differences. M/10 sucrose solution was used as the developing solution in the first dimension, and M/10 Na K tartrate in the second dimension.

Table II

	Sample	NPN	TP	ALB	GLOB	FIB
Brownie Feb. 27, 1950 (female)	1 2 3	20.4 18.1 16.7	8.00 8.00 7.76	4.28 4.14 3.70	3.27 3.86 4.06	--- --- ---
Brownie Sept. 18, 1950 (female)	1 2 3	110.0 101.0 48.6	6.79 6.74 6.74	2.70 2.56 2.84	4.09 4.08 3.90	.430 .480 .490
F.F.F. Feb. 27, 1950 (female)	1 2 3	33.2 20.8 21.6	7.35 7.35 7.14	4.95 4.43 4.95	2.40 2.92 2.19	--- --- ---
F.F.F. Sept. 18, 1950 (female)	1 2 3	64.2 66.9 50.9	7.33 7.38 7.04	3.21 3.13 3.34	4.12 4.24 3.70	.613 .302 .631
Farmer Sept. 21, 1950 (male)	1 2 3	101.0 71.3 58.4	6.29 6.50 5.98	3.34 3.45 3.84	2.95 3.05 2.14	.396 .310 .332
Farmer Sept. 25, 1950 (male)	1 2 3 4	17.6 18.4 18.0 19.6	Fasting 10 min. after heparin 60 min. after heparin 150 min. after heparin			Chrom 0 + 0 0

The non-protein nitrogen (NPN), albumin (ALB), globulin (GLOB) and fibrinogen (FIB) for a series of experiments described in the text. Changes in the chromatograms (Chrom) are indicated by 0 or + as for Table I.

at about 13,000 r.p.m. (R.C.F. 20,000 x gravity) to separate the chylomicra. This gave two samples of equal volume, one clear and the other very cloudy, for protein analysis. It can be seen in Figure 30 that the cloudy portion of plasma (Nos. 22,23) exhibits definite and similar changes at both three and six hours, and that lesser changes are to be observed in the clear plasma at six hours (No. 21) and none at three hours (No. 20).

Effects of intravenous administration of Tween 81. The effect of Tween 81 injected intravenously was tested twice in the same dog. Both times 5 ml of Tween 81, diluted to 20 ml with distilled water, was injected; once with the dog fasting (Fig. 31: Nos. 27,28) and subsequently four hours after a fat meal (Fig. 31: Nos. 24,25,26). During the first test, the dog became listless, very weak, and collapsed a few minutes after injection of the Tween, but did not lose consciousness. During the next test, two weeks later, the dog was physically unaffected by the Tween injection, which was given at the presumed height of lipemia. In both cases, the injection of Tween 81 altered the chromatograms considerably.

Due to the fact that the clustering of chylomicra was much greater in plasma than whole blood (179), red blood cells from two normal humans were washed with a volume of normal saline equal to the removed plasma, and chromatograms of this saline were made. A trace of a protein fraction was observed in each experiment, which was believed to be an albumin.

Analyses of protein fractions and of non-protein nitrogen. The non-protein nitrogen (six experiments), the total proteins, albumin

and globulin (five experiments), and fibrinogen (three experiments) were determined before a fat meal, six hours after a fat meal, and ten minutes after injection of heparin given at six hours after the fat meal (Table II). The total proteins were reduced an average of approximately 0.25 grams per cent after four of the five injections. The albumin, globulin and fibrinogen varied, but not consistently. The non-protein nitrogen fell 20 to 60% in five of the six experiments. Part of this drop occurred at the end of six hours after the fat meal, but the greater part during the ten minutes after the injection of heparin. The greatest non-protein nitrogen change occurred when the fasting nitrogen was highest; and no change occurred in one animal with a very low initial non-protein nitrogen.

DISCUSSION.

The change in the protein pattern in the chromatograms described in this chapter is probably not due to an overall reduction in the plasma proteins, since these changed so little. More likely, specific fractions were affected selectively, in a way that altered the protein complex, and consequently its distribution in the chromatogram.

The fact that changes in the chromatograms may be accompanied by increased clumping of the red blood cells and chylomicra, by a decreased hematocrit, and by changes in the sedimentation rate raises the question as to whether the protein complexes in question are associated with maintenance of the stability condition of blood. The protein changes were sometimes attended by little or no evidence of

physical change in the blood, such as no significant change in the hematocrit and sedimentation rate, indicating that there is a critical level for these protein complexes beyond which the emulsification of the blood elements becomes unstable. The absence of increases in the globulin and fibrinogen is of interest since these factors are increased under certain circumstances in which the sedimentation rate is also increased (182).

The similarity of the changes in the protein patterns, and in the physical characteristics of the blood, both after a high fat meal and after intravenous heparin, and the apparent cumulative nature of these changes suggest that their mechanisms are similar. It has already been suggested (179) that the chylomicra which appear in the blood stream after a fat meal may, by competing with the red blood cells, produce a deficiency in the so-called emulsification factors. This deficiency may last no longer than three to six hours as indicated by changes in the physical state of the blood (179), and in the protein pattern. The action of heparin is not clear, but it may be that this substance, either directly or indirectly, causes changes in the plasma by surface active forces resulting in alterations in the protein fractions. The duration of this change may be less than one hour. Anderson and Fawcett (183) have shown that the surface tension of plasma decreases after injections of heparin effective in clearing the lipemia. These authors ascribe this to the formation of a surface active heparin-phospholipid complex.

The paper chromatogram observations on lipemic plasma, subjected to very fast centrifugation, indicate that the changes in the protein

patterns are not due to the presence of the chylomicra alone. It is also evident that a very marked lipemia produces additional changes in the paper chromatogram resulting in protein pattern similar to those seen after intravenous heparin. As further evidence that the presence of chylomicra was not the sole cause of the protein changes, it is to be noted that the maximum changes in the proteins and physical characteristics of the blood were observed in the cream fat test in one dog (Table I: E) nine hours after the fat meal, when the number of chylomicra had returned nearly to normal; at three hours when the lipemia was maximum no such changes in the protein patterns were observed.

The evidence would indicate that the administration of heparin, as well as a high fat meal, modifies the surface active substances in the plasma with the result that a marked change occurs in the protein pattern seen in the paper chromatograms. This is noted also in the effects due to administration of a surface active agent such as Tween 81. It has been shown recently (184) that heparin inhibits the action of hyaluronidase, possibly by competition with hyaluronic acid for the enzyme. Conceivably the change in the protein chromatogram of plasma after heparin administration is due to some similar association with a protein fraction.

SUMMARY.

(1) Changes in the plasma protein patterns of dogs after high fat meals, and after the intravenous administration of heparin or Tween 81, have been demonstrated, using the paper chromatographic

technique.

(2) Concurrent reductions in the hematocrit, and changes in the sedimentation rate were observed after these procedures. The chylomicron counts were decreased after intravenous heparin.

(3) The evidence indicates that the surface active substances present in the blood are modified after the above administrations, thus affecting the protein patterns of the paper chromatograms.

(4) Very small reductions in the total proteins occurred after high fat meals or intravenous injections of heparin. Much greater reductions were found to occur in the non-protein nitrogen.

Chapter 11

PAPER CHROMATOGRAPHY OF BLOOD PLASMAS IN MULTIPLE SCLEROSIS

INTRODUCTION.

The preceding chapter has shown that the changes in the protein pattern of dog plasma following high fat meals and intravenous heparin administration can be shown with the paper chromatographic technique. It seemed reasonable to assume, therefore, that changes in the protein pattern of human plasma could also be demonstrated, and possibly to be of use in the diagnosis of pathological conditions, or to follow the progress of a patient during clinical treatment. Dr. R.L. Swank, of the Montreal Neurological Institute, has collaborated on this problem, and with his aid, a number of patients who have multiple sclerosis were followed during the period of exacerbation and remission. Significant changes in the plasma protein pattern have been demonstrated.

Previous observations by Swank have indicated the possibility that the degree of severity of multiple sclerosis is related directly to the amount of daily fat intake (185). This observation has stimulated studies of the effects of high fat meals on the composition of the blood of normal men and of dogs. First, it was shown that six to nine hours following high fat meals, the red blood cells and chylomicra in normal humans and dogs have a tendency to aggre-

(Dr. Swank was in charge of the clinical reports of the patients with multiple sclerosis, and supervised the blood transfusions. Miss Aagot Grimsgaard recorded changes in sedimentation rate, hematocrit and chylomicron counts. Mrs. Mary Hersey and Miss Rose Mamelak aided in chromatography of the plasmas of patients with multiple sclerosis).

gate (186). Secondly, these changes in the physical stability of the blood were found to be accompanied in the dog by alterations in the plasma protein pattern of the paper chromatogram (Chapter 10).

MATERIALS AND METHODS.

Plasmas were obtained from a group of intensively studied patients with multiple sclerosis, who were followed at the Neurological Clinic of the Royal Victoria Hospital, Montreal. All bloods were obtained from fasting patients and were prevented from clotting by heparin-sodium salt. The bloods were obtained at different intervals of time, and when possible, during exacerbations and remissions of the disease. From one to eight tests were made on each patient. All of the 26 patients had well established disease; most of them had been admitted to the Montreal Neurological Institute for diagnosis and treatment; and all but one were ambulatory. Some of the patients were on low fat diets currently being used in this clinic to treat patients with multiple sclerosis.

A total of eleven normal subjects was also studied. Three of these were studied at weekly intervals over a period of six weeks, and as a control for those multiple sclerosis patients receiving the low fat diet, these three subjects were put on the same low fat diet for a period of three weeks.

The technique was followed as described in Chapter 5, using Whatman No. 1 filter paper. Seven sets of conditions were used to examine the plasma of each normal and each patient. One condition did not include the surface active agent, and the others included 0.02, 0.04 and 0.06 ml Tween 81, and 0.01, 0.03 and 0.05 ml Tween

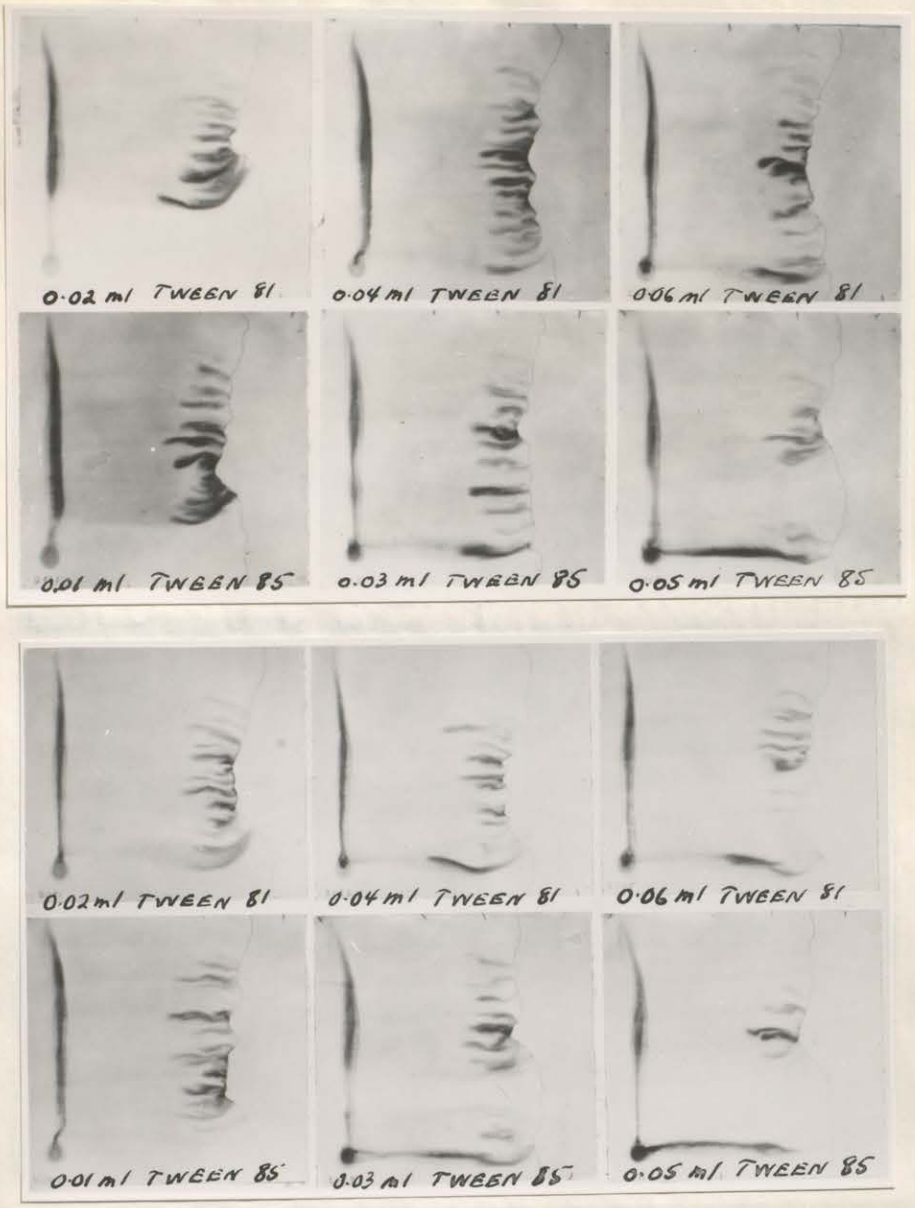


Figure 32

Two series of chromatograms of a series of six of a normal healthy 33 year old woman is used to illustrate a set of normal plasma protein chromatograms. The upper set represents the plasma on July 10 (normal diet) and the lower set represents the plasma on July 24 (low-fat diet). The illustrated chromatograms show increasing concentrations of Tween 81 (0.02, 0.04 and 0.06 ml per 0.5 ml plasma) in the top row, and increasing concentrations of Tween 85 (0.01, 0.03 and 0.05 ml per 0.5 ml plasma) in the bottom row of each series. M/10 sucrose was used as the developing solution in the first dimension, and M/10 Na K tartrate in the second dimension.

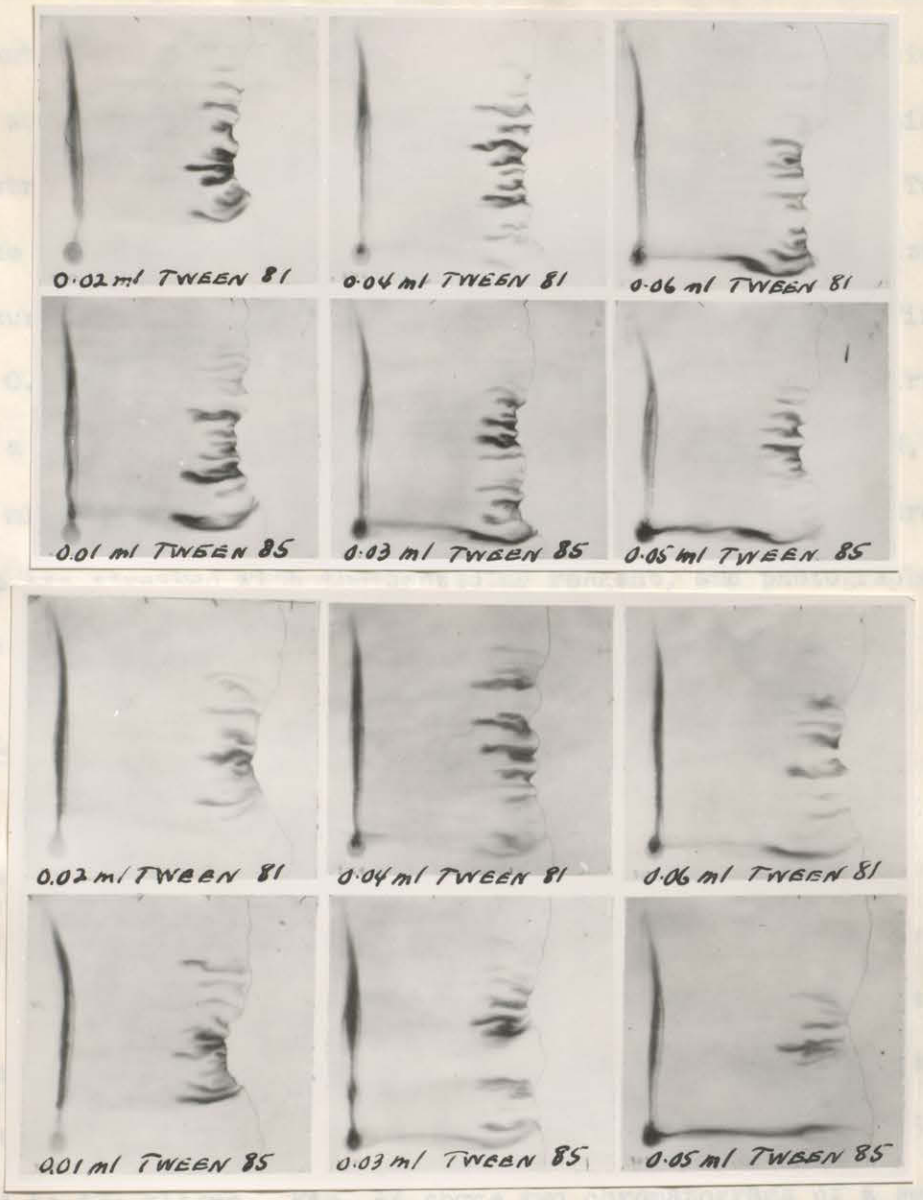


Figure 33

Two series of chromatograms of a series of six of a normal, healthy 29 year old man is used to illustrate a set of normal plasma protein chromatograms. The upper set represents the plasma on July 3 (normal diet) and the lower set represents the plasma on July 24 (low-fat diet). The illustrated chromatograms show increasing concentrations of Tween 81 (0.02, 0.04 and 0.06 ml per 0.5 ml plasma) in the top row, and increasing concentrations of Tween 85 (0.01, 0.03 and 0.05 ml per 0.5 ml plasma) in the bottom row of each series. M/10 sucrose was used as the developing solution in the first dimension, and M/10 Na K tartrate solution in the second dimension.

85 per 0.5 ml plasma and 0.02 ml 0.3% hemin solution. The developing solvents used were M/10 sucrose solution in the first dimension, and M/10 sodium potassium tartrate solution in the second dimension. The illustrated chromatograms show increasing concentrations of Tween 81 in the top row and Tween 85 in the bottom row. Mixing is easier if the surface active agent is placed in a small culture tube first, then 0.5 ml of plasma is added, and these are thoroughly stirred with a glass rod. Then 0.02 ml 0.3% hemin solution is added, and 0.02 ml aliquots are applied to the paper. The resulting chromatograms are streaked with the benzidine reagent, and photographed, as previously described.

RESULTS.

Protein patterns observed in healthy, young adults. Fig. 32 shows two chromatograms of a series of six taken at weekly intervals from a healthy woman 33 years of age. The protein pattern in this subject varied very little from week to week during this period. The maximum variation consisted of an absence of several central fractions in the chromatograms obtained when 0.03 ml Tween 85 was added to the plasma. Fig. 33 shows two chromatograms of a series of six taken at weekly intervals from a healthy man 29 years of age. Even greater consistency of the protein pattern was shown in this series of tests. Nine other controls (six women, three men) were examined. They showed similar protein patterns with no great variations upon repeated testing. When 0.05 ml Tween 85 was used with the plasma, few protein fractions were observed in either the con-

trol or the experimental subjects.

The chromatograms of normal, healthy persons appeared to vary to some extent from time to time. The variations, however, the causes for which are as yet unknown, have not affected the general protein pattern as observed in normal individuals. Generally speaking, the normal pattern indicated the presence of a large number of fractions in all conditions of examination with the exception of the higher concentrations of the surface active agents. (Figs. 32,33). It is possible that in normal women these variations are greater than in men, but insufficient material prevents any conclusions on this point. The relationship of the menstrual cycle to the plasma proteins may be of importance in the apparent greater variation observed in women, but this has not yet been studied.

Protein patterns observed in Multiple Sclerosis Patients. Of a total of 81 chromatograms taken from 26 patients with multiple sclerosis, 31 tests in 17 patients showed distinct abnormalities, and one or more tests in each of four patients indicated probable abnormalities. In five patients all the chromatograms were normal, despite the occurrence in two of them of mild exacerbations of disease during the period of study. In one of the latter two patients, the chromatograms were carried out four days after, and in another case, one day after the onset of mild retrobulbar neuritis (as evidenced by a sudden partial loss of vision with return to former vision in about two weeks).

In three patients, striking changes were observed in the chromatogram during exacerbations of disease. These changes were pre-

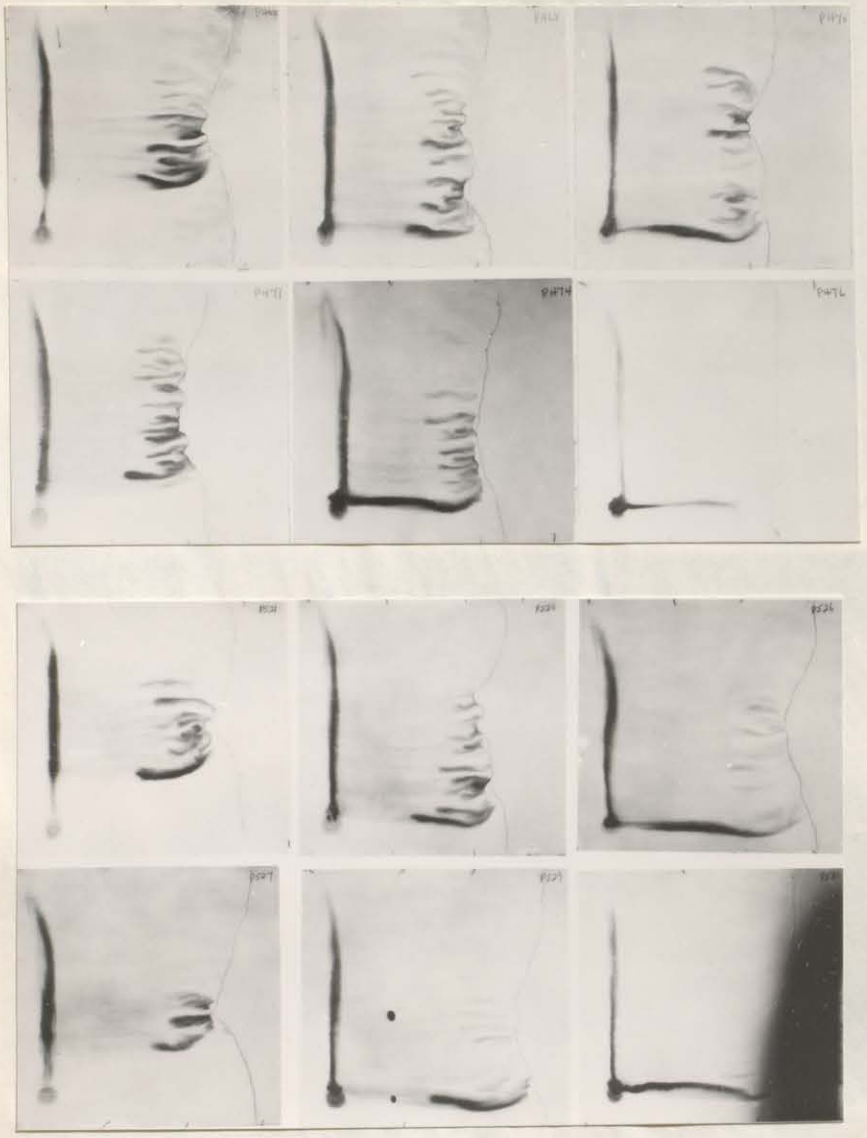


Figure 34

The above two series of chromatograms illustrate the plasma protein pattern of a male patient with multiple sclerosis. The upper series was made from plasma obtained on April 6, during a routine check prior to an exacerbation. The lower series illustrates the plasma protein pattern during an exacerbation (April 13).

Compare with the normal protein patterns shown in Figures 32 and 33.

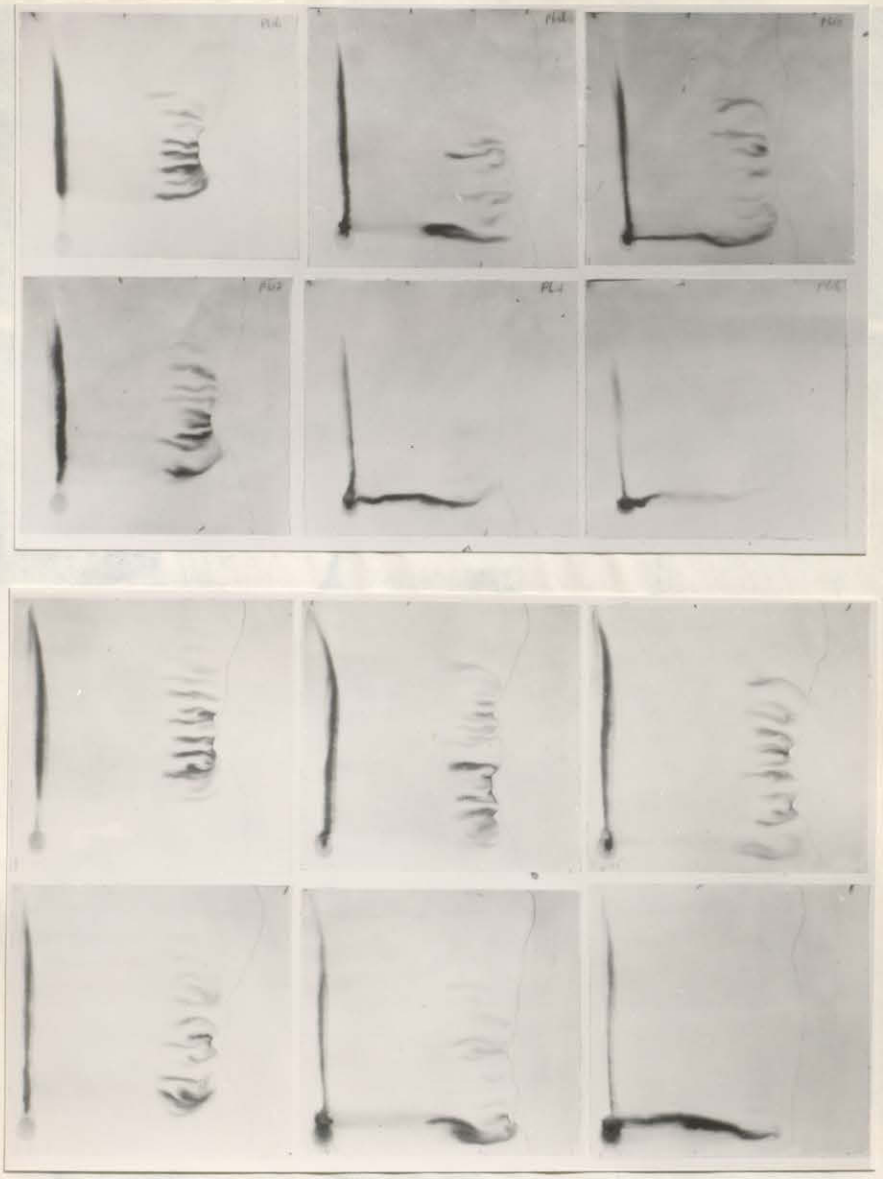


Figure 35

The above two series of chromatograms continue to illustrate the changes in the same patient with multiple sclerosis, as shown in Figure 34. The upper series represents the plasma protein pattern during the remission period (April 21), which follows the exacerbation. The lower series represents the plasma protein pattern during a routine check on this patient (June 26).

Compare with Figure 34, and also with the normal protein patterns shown in Figures 32 and 33.

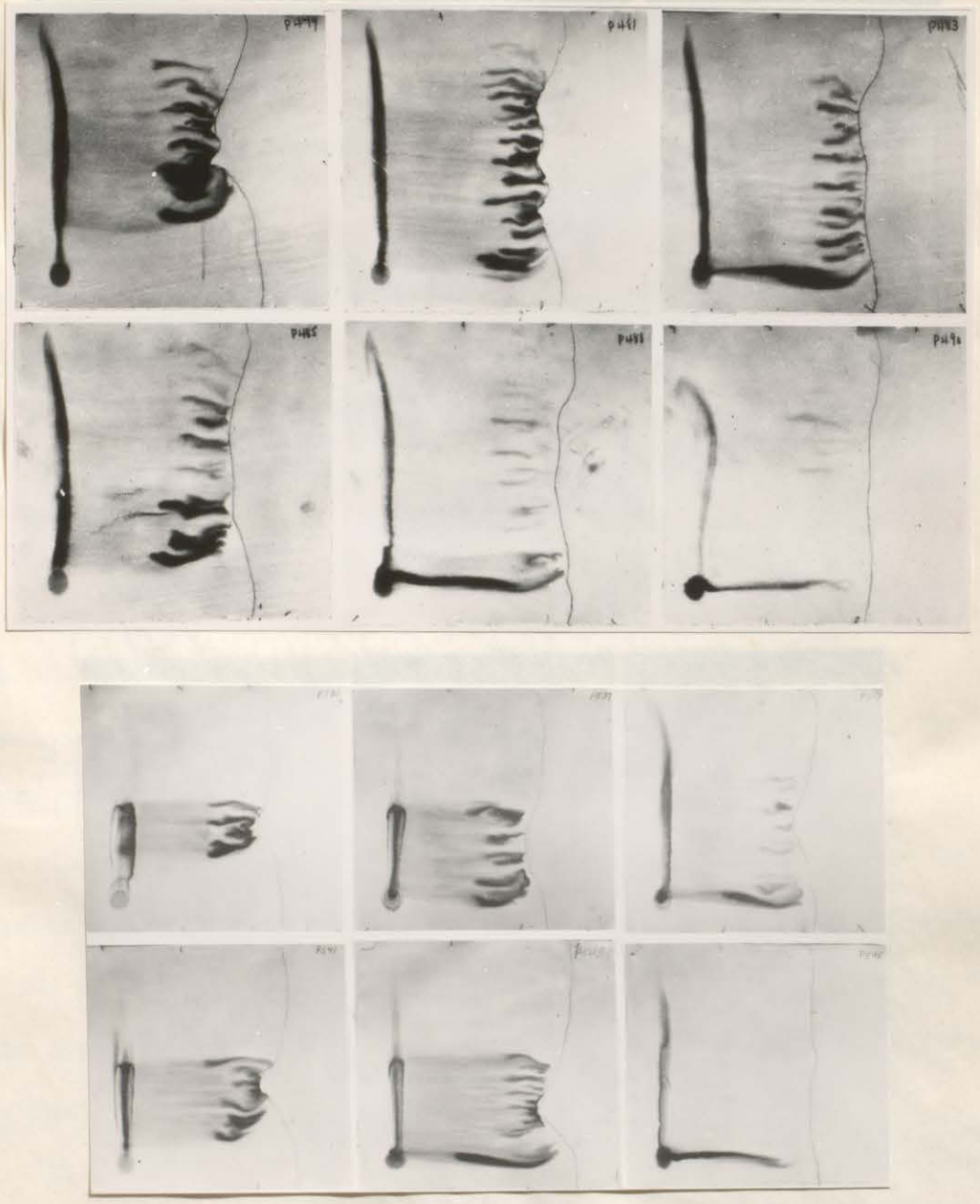
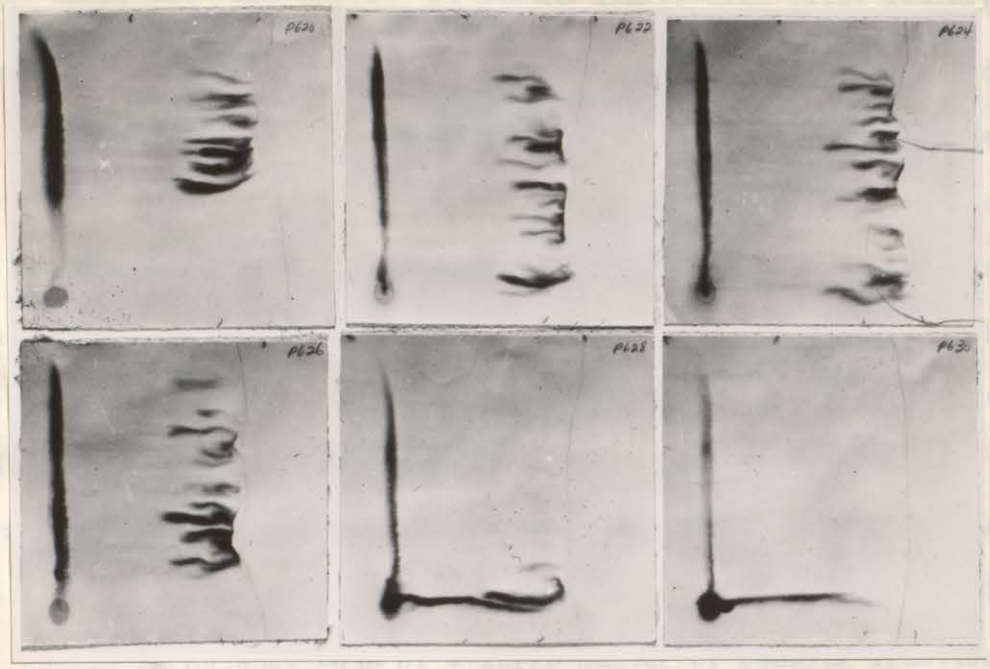


Figure 36

This series of chromatograms also illustrate the changes in plasma protein patterns of a male patient with multiple sclerosis. The upper series was made from plasma taken on April 6, prior to an exacerbation. The lower series of chromatograms was made from plasma taken on April 14, three days after the onset of an exacerbation.

Compare with the normal protein patterns of Figures 32 and 33.



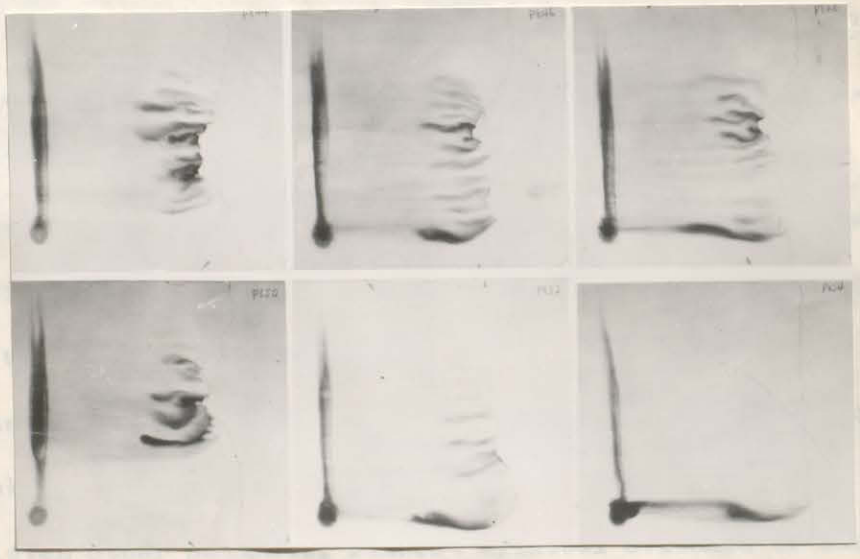


Figure 37

The above two series of chromatograms continue to illustrate the changes in the same patient with multiple sclerosis, as shown in Figure 36. The upper series of chromatograms represent the plasma protein pattern during the remission period (April 14) of the disease. The lower series of chromatograms represents the plasma protein pattern during a routine check on this patient on May 18.

Compare with Figure 36, and also with the normal protein patterns shown in Figures 32 and 33.

ceded by normal (two cases), or nearly normal (one case) chromatograms, and were followed by a return of the chromatograms towards (or to) normal during the subsequent clinical remissions. These patients were not on a low fat diet when the chromatograms before and during the exacerbations were carried out. During the period of remission, however, they were given the low fat diet. An example of the changes in the chromatograms in one patient is shown in Figs. 34 and 35. The chromatogram prior to exacerbation was essentially normal. Five to six days after the onset of exacerbation, and while the neurological signs were still increasing, the chromatogram (Fig. 34) showed an absence both of a large number of central fractions, and of some of the upper fractions (compare with Figs. 32,33). During clinical remission, eight days and eleven weeks (Fig. 35) later, the chromatograms returned towards normal.

Another example of the protein pattern changes during an exacerbation is shown in Figs. 36 and 37. It will be noted that many of the upper fractions are absent during exacerbation (Fig. 36) and that these gradually reappear in the subsequent two chromatograms, one week and five weeks (Fig. 37) later.

Eleven other patients, without clinical evidence of exacerbation of disease, exhibited changes which were considered distinctly abnormal in one or more chromatograms. All of these patients suffered from a relatively active disease as judged by the occurrence of frequent and severe exacerbations during the preceding years. Three additional patients with steadily progressive disability con-

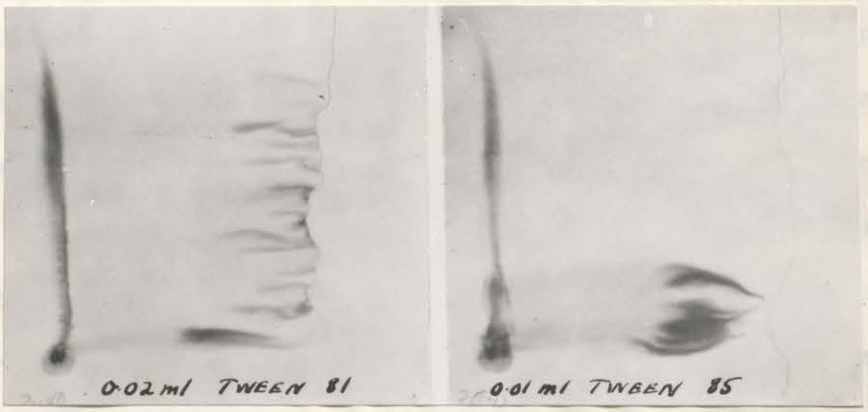


Figure 38

This represents two chromatograms of the plasma protein pattern of a male patient with steadily progressive symptoms of multiple sclerosis (June 30). Only the 0.02 ml Tween 81 and 0.01 ml Tween 85 per 0.5 ml plasma conditions are illustrated.

Compare with the normal protein pattern distribution as shown in Figures 32 and 33.

sisting of mixed ataxia and spasticity displayed definite alterations of the protein pattern in the chromatograms (see, e.g. Fig. 38).

Effects of Blood Transfusions. Preliminary experiments have indicated that, following transfusion, the protein pattern tends to revert to normal. This matter is being investigated.

DISCUSSION.

The significance of the changes which have been observed in the plasma proteins of patients with multiple sclerosis is not known. The limited observations suggest that these changes usually do not precede a clinical exacerbation of the disease by an appreciable interval of time. It is difficult to assess the time interval, however, because the exact time of the onset of an exacerbation cannot be established with certainty. Neither do the changes in the protein pattern continue to be present for many weeks after the onset of the exacerbation.

If the pattern of the plasma proteins shown by paper chromatography is secondary to, and determined by the amount of nervous tissue undergoing change, then it is reasonable to assume that small lesions (e.g., mild retrobulbar neuritis) might not alter the protein pattern. On the other hand, extensive lesions might occur in the so-called silent areas of the brain and produce marked changes in the chromatograms, and yet be unaccompanied by symptoms.

The chromatograms may reflect changes in the blood which occur concomittantly with, or even prior to, the development of lesions in the nervous system. It may also be considered that the proteins

Figure 39

A series of chromatograms of the plasma proteins of a patient (Mrs. G.) with multiple myeloma is illustrated. The upper series represents a routine check, and the lower series was done two weeks later, during which cortisone was given as treatment.

It will be observed that the chromatograms of this patient differ greatly from the normals (compare with Figures 32 and 33). A distinctive fraction occurs at the top of the chromatograms in the first series, which disappears after cortisone treatment.

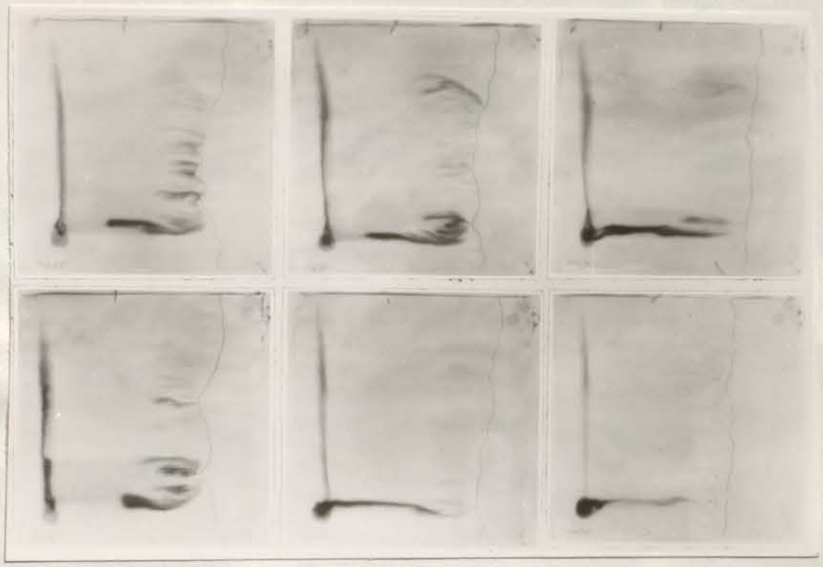


Figure 40

This series of chromatograms of the plasma proteins represents a routine check on a multiple myeloma patient (Mr. D.)

It will be observed that the plasma of this patient lacks many of the fractions present in normal plasma (compare with Figures 32 and 33.

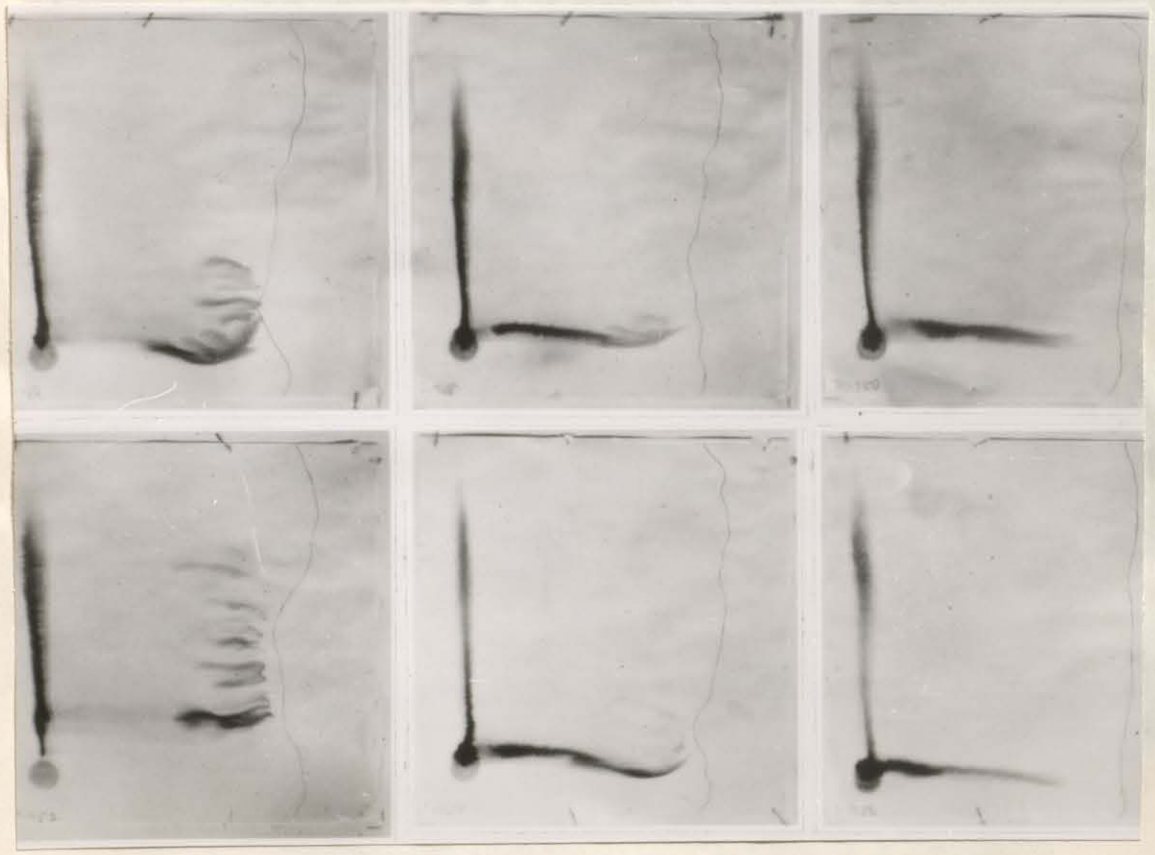


Figure 41

This series of chromatograms represents the plasma proteins during a routine check on a patient (Mr. T.) with liver cirrhosis.

It will be observed that the plasma of this patient lacks many of the fractions present in normal plasmas (compare with Figures 32 and 33).

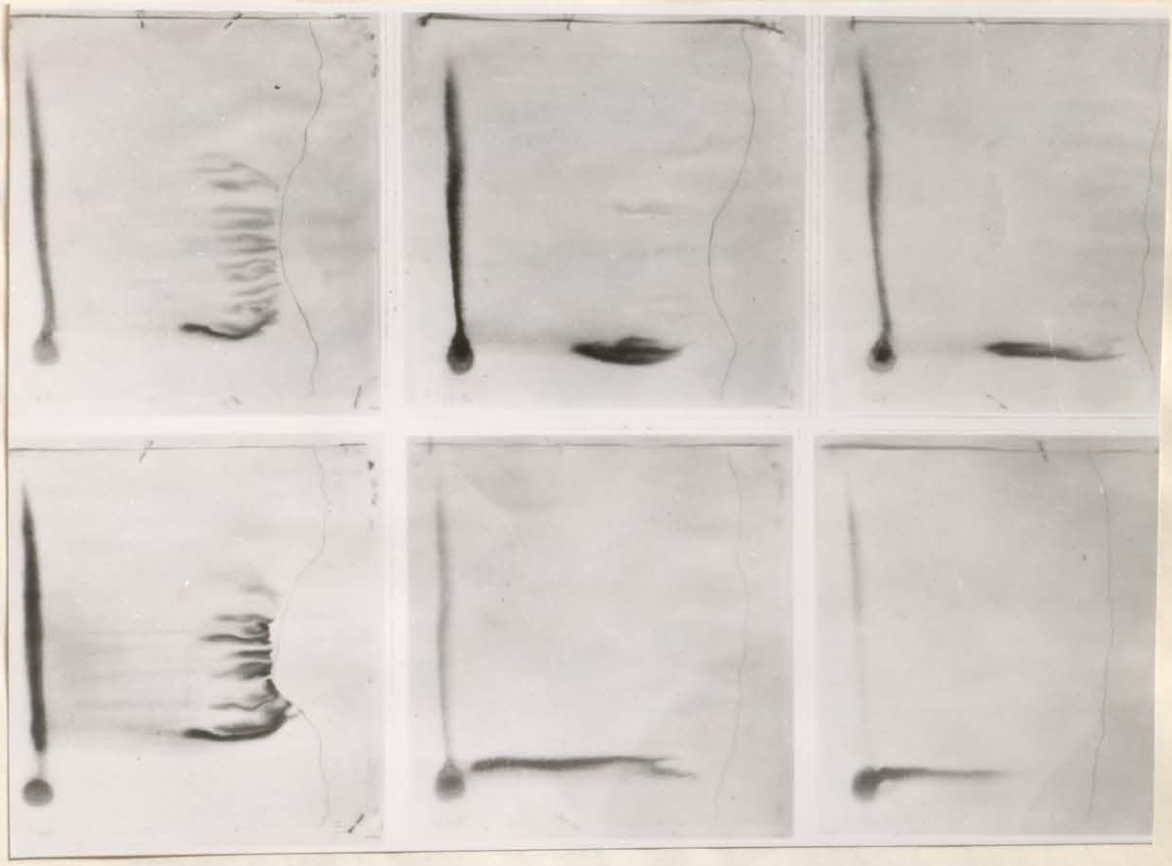


Figure 42

This series of chromatograms represents the plasma proteins during a routine check on a patient (Mr. H.) with portal cirrhosis.

It will be observed that the plasma of this patient lacks many of the fractions present in normal plasma (compare with Figures 32 and 33).

might be modified by chemical changes attending the occurrence of these lesions.

The abnormalities in the plasma protein chromatograms do not appear to be specific for multiple sclerosis. Some preliminary experiments were carried out on patients of the Montreal General Hospital, who had multiple myeloma or liver cirrhosis, and the resulting chromatograms showed very distinct abnormalities. It was observed that a protein fraction sometimes occurred in the chromatograms of plasma of patients who had multiple myeloma, which was not present in normal plasma, or plasma of patients with multiple sclerosis or liver cirrhosis (Fig. 39). In one case, this fraction disappeared after cortisone treatment (Fig. 39). Another series of plasmas of a patient with multiple myeloma is illustrated in Figure 40. Other pathological conditions that showed abnormalities in the plasma protein pattern include Baker's cyst, syphilis, carcinoma of the rectum, carcinoma of the stomach, carcinoma of the esophagus, ventral hernia and pancreatic abscess.

Unpublished observations by Swank have shown that sedimentation rates of patients with multiple sclerosis (determined at weekly or monthly intervals) are frequently abnormally high, but the evidence so far has not established a clear correlation between such changed sedimentation rates and the abnormal chromatograms shown by these patients. No attempt was made to correlate the plasma protein patterns with the Lange reaction in the spinal fluid.

SUMMARY

(1) Using the paper chromatographic technique, significant changes in the protein patterns have been demonstrated in patients with multiple sclerosis. We are unable to state at present the relationship of these changes to the activity of the disease, but our evidence would seem to indicate that they may occur concomitantly with, or immediately following the onset of exacerbation. Furthermore, the changes in the protein patterns revert towards normal during remission.

(2) The abnormalities in the plasma protein chromatograms do not appear to be specific for multiple sclerosis, since very abnormal patterns have also been observed in multiple myeloma, liver cirrhosis and some other pathological conditions.

CLAIMS TO ORIGINAL RESEARCH

(1) A new chromatographic technique has been developed to study the movement and separation of proteins and protein mixtures on filter paper, using hemin as a 'marker', and development of the chromatogram with aqueous salts and buffers.

(2) Agreement of chromatographic results with those of electrophoresis in the case of crystalline proteins such as bovine albumin, β-lactoglobulin and γ-globulin has been observed.

(3) Egg albumin can be detected by serological methods after chromatography.

(4) Urease can be demonstrated by manometric techniques after chromatography.

(5) A method has been described which shows the ability of a protein to form a hemochromogen in the presence of hemin.

(6) Separation of protein components of plasmas on filter paper, using synthetic detergents as a means of accentuating the differences between various protein fractions, has been described.

(7) Differences between the plasma protein patterns of humans, rats and guinea pigs by the chromatographic technique have been demonstrated.

(8) The sera of hyperimmunized horses (as against diphtheria toxin) yielded chromatograms which differed from, and exhibited fractions not present in, chromatograms of normal horse sera.

(9) The presence of several closely related protein fractions in a crystalline human albumin preparation has been observed.

(10) With protein mixtures, associations of proteins may occur

that can be detected by chromatography.

(11) It has been shown that proton bombardment of bovine albumin does not cause cleavage of the protein molecule, but does cause a decrease in solubility of the protein.

(12) Confirmation of the presence of thyroxine and iodide in the unhydrolyzed thyroid of the rat has been demonstrated using a radioactive technique.

(13) In the blood plasma of a rat, practically all the butanol-extractable radioactivity was found to be present as thyroxine, with a very low amount of diiodotyrosine and iodide.

(14) The presence of diiodotyrosine and monoiodotyrosine has been demonstrated in the unhydrolyzed thyroid of the rat using radioactive iodide.

(15) Presence of three, unknown, iodinated substances has been shown in the unhydrolyzed thyroid of the rat, using radioactive iodide.

(16) The nature of the circulating thyroid hormone as a thyroxine-plasma protein complex has been postulated.

(17) It has been shown that thyroxine will combine with a mixture of Cohn's plasma protein fractions in a manner similar to that with the proteins of whole plasma. The presence of physiological salts or glucose did not affect this combination, but the combination was severed by butanol at 0° C.

(18) Thyroxine combined with Cohn Fractions V (albumin), IV-1 and IV-3,4 (α- and β-globulins), but only albumin carried thyroxine according to a pattern similar to that seen with plasma. The

addition of other proteins appeared to promote further combination with the thyroxine.

(19) The combination of molecules of physiological importance and having a small molecular weight, with plasma proteins and plasma protein fractions, has been demonstrated. It has been shown that combination is superior in the presence of whole plasma when compared to the combination with the individual plasma fractions. The postulation is that small molecules do not exist as such in the blood stream, but circulate as loose complexes with the plasma proteins.

(20) Changes in the plasma protein patterns of dogs after high fat meals, and after the intravenous administration of heparin or Tween 81, have been demonstrated, using the paper chromatographic technique. The evidence indicated that the surface active substances present in the blood were modified after these administrations, thus affecting the protein patterns of the chromatograms.

(21) Significant changes in the plasma protein patterns have been observed in patients with multiple sclerosis. These changes appeared to occur concomittantly with, or immediately following the onset of exacerbation, and tended to revert towards, or to, normal during the remission period.

(22) Distinct abnormalities in the plasma protein patterns have been demonstrated in patients with multiple myeloma, liver cirrhosis and portal cirrhosis.

BIBLIOGRAPHY

1. Schonbein, C.F. : Verh. Naturforsch. Ges. Basel 3 249 (1861)

2. Goppelsroeder, F. : "Anregung zum Studium der auf Capillaritats und Adsorptionserscheinungen beruhenden capillaranalyse". (Basel). 1906.

3. Reed, L. : Proc. Chem. Soc. 9 123 (1893)

4. Tswett, M. : Ber. Deutsch. Botan. Ges. 24 316 (1906)

5. Day, D.T. : Proc. Am. Philos. Soc. 36 112 (1897)

6. Gilpin, J.E., Schneeberger, P. : Am. Chem. J. 50 59 (1913)

7. Palmer, L.S. : Carotenoids and Related Pigments. The Chemical Catalog Co., New York, 1922.

8. Kuhn, R., Lederer, E. : Ber. Deutsch. Chem. Ges. 64 1349 (1931)

9. Kuhn, R., Winterstein, A., Lederer, E. : Z. Physiol. Chem. 197 141 (1931)

10. Martin, A.J.P., Synge, R.L.M. : Biochem. J. 35 1358 (1941)

11. Gordon, A.H., Martin, A.J.P., Synge, R.L.M. : Biochem. J. 37 79 (1943)

12. Gordon, A.H., Martin, A.J.P., Synge, R.L.M. : Biochem. J. 38 65 (1944)

13. Synge, R.L.M. : Biochem. J. 38 285 (1944)

14. Consden, R., Gordon, A.H., Martin, A.J.P. : Biochem. J. 38 224 (1944)

15. James, A.T., Martin, A.J.P. : Biochem. Soc. 290th Meeting 1950

16. Zechmeister, L., Cholnoky, L. : Die Chromatographische Adsorptionsmethode. Berlin. 1938.

17. Bate-Smith, E.C., Westall, R.G. : Biochim. et Biophys. Acta 4 436 (1950)

18. Ketelle, B.H., Boyd, G.E. : J. Am. Chem. Soc. 69 2800 (1947)

19. Tiselius, A. : Adv. Colloid Sc. 1 81 (1942)

20. Stein, W.H., Moore, S. : J. Biol. Chem. 176 337 (1948)

21. Isherwood, F.A. : Biochem. J. 40 688 (1946)

22. Dent, C.E. : Biochem. J. 41 240 (1947)

23. Williams, R.J., Kirby, H. : Science 107 481 (1948)

24. Datta, S.P., Overell, B.G., Stack-Dunne, M. : Nature 164 673 (1949)

25. Haugaard, G., Kroner, T.D. : J. Am. Chem. Soc. 70 2135 (1948)

26. Biserte, G. : Biochim. et Biophy. Acta 4 416 (1950)

27. Mitchell, H.K., Haskins, F.A. : Science 110 278 (1949)

28. Mitchell, H.K., Gordon, M., Haskins, F.A. : J. Biol. Chem. 180 1071 (1949)

29. Miettinen, J.K., Virtanen, A.I. : Acta Chem. Scand. 3 459 (1949)

30. Yanofsky, C., Wasserman, E., Bonner, D.M. : Science 111 61 (1950)

31. Partridge, S.M., Swain, T. : Nature 166 272 (1950)

32. Craig, L.C. : J. Biol. Chem. 155 519 (1944)

33. Craig, L.C., Golumbic, C., Mighton, H., Titus, E. : J. Biol. Chem. 161 321 (1945)

34. Phillips, D.M.P. : Nature 161 53 (1948)

35. Chargaff, E., Levine, C., Green, C. : J. Biol. Chem. 175 67 (1948)

36. Naftalin, L. : Nature 161 763 (1948)

37. Partridge, S.M. : Nature 158 270 (1946)

38. Lederer, M. : Anal. Chim. Acta 2 261 (1948)

39. Haskins, A.L., Sherman, A.I., Allen, W.M. : J. Biol. Chem. 182 429 (1950)

40. Woodhouse, D.L. : Biochem. J. 44 185 (1949)

41. Woodhouse, D.L. : Arch. Biochem. 25 347 (1950)

42. Mapson, L.W., Partridge, S.M. : Nature 164 479 (1949)

43. Winsten, W.A., Eigen, E. : J. Biol. Chem. 181 109 (1949)

44. Hooper, I.R., Johnson, D.L., Taylor, H.D. : Fed. Proc. 8 207 (1949)

45. Boursnell, J.C. : Nature 165 399 (1950)

46. Gross, J. Leblond, C.P., Franklin, A.E., Quastel, J.H. : Science 111 605 (1950)

47. Harvalik, Z.V. : Anal. Chem. 22 1149 (1950)

48. Cavallini, D., Frontali, N., Toschi, G. : Nature 164 792 (1949)

49. Lacourt, A., Gillard, J., Van Der Walle, M. : Nature 166 225 (1950)

50. Lacourt, A., Sommereyns, G., Degeyndt, E., Baruh, J. Gillard, J. : Nature 163 999 (1949)

51. Awapara, J. : J. Biol. Chem. 178 113 (1949)

52. Vischer, E., Magasanik, B., Chargaff, E. : Fed. Proc. 8 263 (1949)

53. Edman, P., Hammarsten, E., Low, B., Reichard, P. : J. Biol. Chem. 178 395 (1949)

54. Gage, T.B., Wender, S.H. : Fed. Proc. 8 293 (1949)

55. Woodruff, H.B., Foster, J.C. : J. Biol. Chem. 183 569 (1950)

56. Fisher, R.B., Parsons, D.S., Morrison, G.A. : Nature 161 764 (1948)

57. Bolling, D., Sober, H.A., Block, R.J. : Fed. Proc. 8 185 (1949)

58. Fosdick, L.S., Blackwell, R.Q. : Science 109 314 (1949)

59. Hawthorne, J.R. : Nature 160 714 (1947)

60. Work, E. : Biochim. et Biophy. Acta 5 204 (1950)

61. Work, E. : Bull. Soc. chim. biol. 31 138 (1949)

62. Gordon, A.H., Martin, A.J.P., Synge, R.L.M. : Biochem. J. 37 86 (1943)

63. Peterson, D.H., Reineke, L.M. : J. Biol. Chem. 181 95 (1949)

64. Polson, A. : Science 105 603 (1947)

65. Tristram, G.R. : Biochem. J. 40 721 (1946)

66. Roberts, E., Ramasarma, G.B., Lewis, H.B. : Proc. Soc. Exp. Biol. Med. 74 237 (1950)

67. Pierce, J.G., du Vigneaud, V. : J. Biol. Chem. 182 359 (1950)

68. Stepka, W., Takahashi, W.N. : Science 111 176 (1950)

69. Gabrio, B.W., Tishkoff, G.H. : Science 112 358 (1950)

70. Phillips, D.M.P. : Biochim. et Biophy. Acta 3 341 (1949)

71. Consden, R., Gordon, A.H. : Biochem. J. 46 8 (1950)

72. Woiwood, A.J. : J. Gen. Microbiology 3 312 (1949)

73. Work, E. : Biochim. et Biophy. Acta 3 400 (1949)

74. Woiwood, A.J. : Nature 163 218 (1949)

75. Sanger, F. : Biochem. J. 39 507 (1945)

76. Phillips, D.M.P., Stephen, J.M.L. : Nature 162 152 (1948)

77. Peterson, M.H., Johnson, M.J. : J. Biol. Chem. 174 775 (1948)

78. Siebermann, H., Siebermann-Martyncewa, S. : J. Biol. Chem. 165 359 (1946)

79. Lugg, J.W.H., Overell, B.T. : Australian J. Sci. Res. Ser A 1 98 (1948)

80. Cavallini, D., Frontali, N., Toschi, G. : Nature 163 568 (1949)

81. Henderson, L.M., Hirsch, H.M. : J. Biol. Chem. 181 667 (1949)

82. Norris, F.C., Campbell, J.J.R. : Can. J. Res. 27C 253 (1949)

83. Boon, W.R., Calam, C.T., Gudgeon, H., Levi, A.A. : Biochem. J. 43 262 (1948)

84. Smith, G.N., Worrel, C.S. : Arch. Biochem. 28 1 (1950)

85. Roth, L.J., Leifer, E., Hogness, J.R., Langham, W.H. : J. Biol. Chem. 178 963 (1949)

86. Carter, H.E., Clarke, R.K., Dickman, S.R., Loo, Y.H., Skell, P.S., Strong, W.A. : J. Biol. Chem. 160 337 (1945)

87. Swart, E.A., Hutchison, D., Waksman, S.A. : Arch. Bioch. 24 92 (1949)

88. Hirata, Y., Nakanishi, K. : J. Biol. Chem. 184 135 (1950)

89. Lederer, M. : Nature 162 776 (1948)

90. Lederer, M. : Science 110 115 (1949)

91. Burstall, F.H., Davies, G.R., Linstead, R.P., Wells, R.A. : J. Chem. Soc. Feb. 1950 p. 516

92. Chargaff, E., Magasanik, B., Doniger, R., Vischer, E. : J. Am. Chem. Soc. 71 1513 (1949)

93. Holiday, E.R., Johnson, E.A. : Nature 163 216 (1949)

94. Crammer, J.L. : Nature 161 349 (1948)

95. Bush, I.E. : Nature 166 445 (1950)

96. Lieberman, S., Fukushima, D.K., Dobriner, K. : J. Biol. Chem. 182 299 (1950)

97. Zaffaroni, A., Burton, R., Keutmann, E.H. : J. Biol. Chem. 177 109 (1949)

98. Zechmeister, L., McNeely, W.H. : J. Am. Chem. Soc. 64 1919 (1942)

99. Johnston, C.D. : Science 106 91 (1947)

100. Zaffaroni, A., Burton, R., Keutmann, E.H. : Science 111 6 (1950)

101. Partridge, S.M., Westall, R.G. : Biochem. J. 42 238 (1948)

102. Bell, D.J., Palmer, A. : Nature 163 846 (1949)

103. Pascu, E., Mora, T.P., Kent, P.W. : Science 110 446 (1949)

104. Hough, L. : Nature 165 400 (1950)

105. Cohen, S.S., Scott, D.B.M. : Science 111 543 (1950)

106. Boggs, L., Cuendet, L.S., Ehrenthal, I., Koch, R., Smith, F. : Nature 166 520 (1950)

107. Calvin, M., Bassham, J.A., Benson, A.A. : Fed. Proc. 9 524 (1950)

108. Udenfriend, S., Gibbs, M. : Science 110 708 (1949)

109. Tishkoff, G.H., Bennett, R., Bennett, V., Miller, L.L. : Science 110 452 (1949)

110. Winsten, W.A., Eigen, E. : Proc. Soc. Exp. Biol. Med. 67 513 (1948)

111. Hais, I.M., Pecakova, L. : Nature 163 768 (1949)

112. Eden, E. : Biochem. J. 46 17 (1950)

113. Haller, H.L., Acree, F., Potts, S.F. : J. Am. Chem. Soc. 66 1659 (1944)

114. Bland, D.E. : Nature 164 1093 (1949)

115. Lederer, M. : Science 112 504 (1950)

116. Shea, S.M. : Nature 165 729 (1950)

117. Schulman, J., Keating, R.P. : J. Biol. Chem. 183 215 (1950)

118. Porter, W.L., Naghski, J., Eisner, A. : Arch. Bioch. 24 461 (1949)

119. Mohler, H., Hammerle, W. : Z. Unters. Lebensm. 70 193 (1933), 73 186 (1936)

120. Maw, G.A. : Biochem. J. 43 139 (1948)

121. Howard, W.L., Gage, T.B., Wender, S.H. : Arch. Biochem. 25 74 (1950)

122. Taylor, T.I. Urey, H.C. : J. Chem. Phys. 5 597 (1937), 6 429 (1938)

123. Berenblum, I., Schoental, R. : Brit. J. Exp. Path. 24 232 (1943)

124. Chain, E., Florey, H.W., Jennings, M.A., Williams, T.I. : Brit. J. Exp. Path. 24 108 (1943)

125. Thompson, S.Y., Kon, S.K., Mawson, E.H. : Biochem. J. 36 xvii (1942)

126. Binkley, S.B., MacCorquodale, D.W., Thayer, S.A., Doisy, E.A. : J. Biol. Chem. 130 219 (1939)

127. Austin, C.R., Shipton, J. : J. Counc. Sci. Ind. Res. 17 115 (1944)

128. Weil-Malherbe, H. : J. Chem. Soc. 303 (1943)

129. LeRosen, A.L. : J. Am. Chem. Soc. 69 87 (1947)

130. Peters : Indust. Engng. Chem. 14 476 (1922)

131. Robinson, F.A. : The Analyst 71 251 (1946)

132. Wilson, J.N. : J. Am. Chem. Soc. 62 1583 (1940)

133. Franklin, A.E., Quastel, J.H. : Science 110 447 (1949)

134. Franklin, A.E., Quastel, J.H. : Proc. Soc. Exp. Biol. Med. 74 803 (1950)

135. Swank, R.L., Franklin, A.E., Quastel, J.H. : Proc. Soc. Exp. Biol. Med. 75 850 (1950)

136. Swank, R.L., Franklin, A.E., Quastel, J.H. : Proc. Soc. Exp. Biol. Med. 76 183 (1951)

137. Gross, J., Franklin, A.E., Leblond, C.P., Quastel, J.H. : (in process of publication)

138. Cochran, G.W. : J. Phytopathology 37 850 (1947)

139. Riley, V.T. : Science 107 573 (1948)

140. Riley, V.T., Hesselbach, M.L., Fiala, S., Woods, M.W., Burk, D. : Science 109 361 (1949)

141. Gordon, A.H., Keil, B., Sebesta, K. : Nature 164 498 (1949)

142. Jones, J.I.M., Michael, S.E. : Nature 165 685 (1950)

143. Castaneda, M.R. : Proc. Soc. Exp. Biol. Med. 73 46 (1950)

144. Wynn, V., Rogers, G. : Aust. J. Sci. Res. B3 124 (1950)

145. Cohn, E.J., Strong, L.E., Hughes, W.L., Mulford, D.J., Ashworth, J.N., Melin, M., Taylor, H.L. : J. Am. Chem. Soc. 68 459 (1946)

146. Loomeijer, F.J. : Nature 166 951 (1950)

147. Fournau, A. : Kolloid Z. 33 89 (1923)

148. Svedburg, T., Brohult, S. : Nature 143 938 (1939)

149. Svedburg, T., Brohult, S. : Nature 142 830 (1938)

150. Sanigar, F.B., Krejci, L.E., Kraemer, E.O. : Biochem. J. 33 1 (1939)

151. Clark, J.H. : J. Gen. Physiol. 19 199 (1935)

152. Carruthers, J.A. : Doctoral Dissertation, McGill University. April, 1949.

153. Lea, D.E. : "Actions of Radiation on Living Cells". Cambridge University Press. 1946.

154. De Robertis, E. : N.Y. Acad. Sci. 50 279 (1949)

155. Leblond, C.P., Gross, J. : J. Clin. Endocrinol. 9 149 (1949)

156. Fink, R.M., Dent, C.E., Fink, K. : Nature 160 801 (1947)

157. Hird, F.J.R., Trikojus, V.M. : Aust. J. Sci. X 185 (1948)

158. Remington, R.E. : J. Nutrition 13 223 (1937)

159. Skanse, B. : J. Clin. Endocrinol. 8 707 (1948)

160. Gross, J. : Doctoral Dissertation. McGill University. Page 174 1949.

161. Riggs, D.S., Lavietes, P.H., Man, E.B. : J. Biol. Chem. 143 363 (1942)

162. Taurog, A., Chaikoff, I.L. : J. Biol. Chem. 176 639 (1948)

163. Treverrow, V. : J. Biol. Chem. 127 737 (1939)

164. Gross, J., Leblond, C.P. : J. Biol. Chem. 184 489 (1950)

165. Ballou, G.A., Boyer, P.D., Luck, J.M. : J. Biol. Chem. 159 111 (1945)

166. Boyer, P.D., Ballou, G.A., Luck, J.M. : J. Biol. Chem. 167 407 (1947)

167. Putnam, F.W., Neurath, H. : J. Am. Chem. Soc. 66 692, 1992 (1944)

168. Smith, E.R.B. : J. Biol. Chem. 108 187 (1935)

169. Greenberg, D.M., Gunther, L. : J. Biol. Chem. 85 491 (1929-30)

170. Teresi, J.D., Luck, J.M. : J. Biol. Chem. 174 653 (1948)

171. Smith, W.W., Smith, H.W. : J. Biol. Chem. 124 107 (1938)

172. Klotz, I.M., Walker, F.M., Pivan, R.B. : J. Am. Chem. Soc. 68 1486 (1946)

173. Rosenfeld, M., Surgenor, D.M. : J. Biol. Chem. 183 663 (1950)

174. Quastel, J.H. : Biochem. J. 26 1685 (1932)

175. Lea, C.H., Hannan, R.S. : Nature 165 438 (1950)

176. Chow, B.F., McKee, C.M. : Science 101 67 (1945)

177. Klotz, I.M., Urquhart, J.M., Weber, W.W. : Arch. Biochem. 26 420 (1950)

178. Davis, B.D. : Science 95 78 (1942)

179. Swank, R.L. : (in process of publication)

180. Weld, C.B. : Proc. Canad. Physiol. Soc. p. 39 (1948)

181. Hahn, P.F. : Science 98 19 (1944)

182. Fahreus, H. : Physiol. Rev. 9 241 (1929)

183. Anderson, N.G., Fawcett, B. : Proc. Soc. Exp. Biol. Med. 74 768 (1950)

184. Astrup, T., Alkjaersy, N. : Nature 166 568 (1950)

185. Swank, R.L. : Am. J. Med. Sci. 220 421 (1950)

186. Swank, R.L. : Am. J. Physiol. (in press)

187. Winteringham, F.P.W., Harrison, A., Bridges, R.G. : Nature 166 999 (1950)

188. Hanes, C.S., Isherwood, F.A. : Nature 164 1107 (1949)

189. McMahon, J.M., Davis, R.B., Kalnitsky, G. : Proc. Soc. Exp. Biol. Med. 75 799 (1950)

190. McLean, D.J., Giese, A.C. : J. Biol. Chem. 187 543 (1950)

191. Chemistry and Biology of the Proteins. F. Haurowitz. Academic Press, Inc. 1950.

192. Moore, J.H. : J. Biol. Chem. 161 21 (1945)

193. Eakin, R.E., Snell, E.E., Williams, R.J. : J. Biol. Chem. 140 535 (1941)

194. Hawk, P.B., Oser, B.L., Summerson, W.H. : Practical Physiological Chemistry. 1948.

195. Przylecki, S., Kasprzyk, K., Rafalowska, H. : Biochem. Z. 286 360 (1936)

196. Partridge, M.W., Chilton, J. : Nature 167 79 (1951)

197. Swingle, S.M., Tiselius, A. : Biochem. J. 48 171 (1951)

198. Barry, G.T., Sato, Y., Craig, L.C. : J. Biol. Chem. 188 299 (1951)

199. Nielands, J.B., Akeson, A. : J. Biol. Chem. 188 307 (1951)

200. Zechmeister, L., Rohdewald, M. : Enzymologia 13 388 (1949)

201. Burton, R.B., Zaffaroni, A., Keutmann, E.H. : J. Biol. Chem. 188 763 (1951)

202. Stadie, W.C., Haugaard, N., Marsh, J.B., Hills, A.G. : Amer. J. Med. Sci. 218 265, 275 (1949)

203. Zechmeister, L. : Science 113 35 (1951)

"Effects of Fat Meals and Heparin Administration on Blood Plasma Composition as shown by Paper Chromatography" by Roy L. Swank, A.E. Franklin and J.H. Quastel was published in Proc. Soc. Exp. Biol. Med. 75 850 (1950). Reprints have not yet been received from the publisher.

"Paper Chromatography of Blood Plasmas in Multiple Sclerosis" by Roy L. Swank, A.E. Franklin and J.H. Quastel was published in Proc. Soc. Exp. Biol. Med. 76 183 (1951). Reprints have not yet been received from the publisher.

Reprinted from SCIENCE, October 28, 1949, Vol. 110, No. 2861, pages 447–451.

Paper Chromatography of Proteins and Enzymes[1]

A. E. Franklin[2] and J. H. Quastel

Montreal General Hospital Research Institute, Montreal, Canada

The original work of Consden, Gordon, and Martin (*1*) on the paper chromatography of substances of biological importance has been extensively developed, and a large literature has accumulated which cannot be adequately summarized here. So far as we are aware, however, there is no report of the separation of protein molecules by paper chromatography.

We wish to report preliminary results of an investigation of the paper chromatography of proteins and enzymes. Our work shows that it is possible to study the movements of proteins on paper, and to determine the conditions under which proteins may be separated by this means. It is also possible by the same means to bring about separation of the components of enzyme mixtures, and to study enzyme activity, manometrically, on paper.

Our work has been greatly facilitated by the use of hemin as a "marker," the presence of a protein-hemin complex on paper being easily shown by the benzidine-hydrogen peroxide reagent. We employ buffer and aqueous salt solutions, instead of nonaqueous solvents, as developing agents.

The technique employed is that of Williams and Kirby (*2*), which involves chromatography by capillary ascent. Whatman No. 1 sheet filter paper has been used throughout this work. When drying is complete, the paper is streaked with freshly prepared benzidine reagent, using a small paint brush. Color development is immediate and intense, and should be photographed almost immediately, as a background color develops gradually and this tends to obscure the color from the protein-hemin complex.

Protein solutions. Ten mg crystalline protein is dissolved in 1 ml of distilled water or saline and to this is added 0.02 ml of 2.0% hemin, which has been dissolved in 3% sodium bicarbonate solution. Aliquots of 0.01–0.02 ml are used for chromatography.

Developing solutions. A search for suitable developing solutions has resulted in the exclusive use of aqueous solutions of salts and buffers. For buffers, 50 ml of a M/5 solution of the salt is adjusted to the desired pH with a *N*/5 solution of HCl or NaOH and the volume is made up to 200 ml. In the case of acetate-HCl buffers, normal solutions are used. Salt solution concentrations are usually in the region of 1–2%.

The use of organic solvents gave unsatisfactory results. *n*-Butanol saturated with water allowed extremely little movement of proteins and favored the formation of an immediate, deeply colored background on application of the benzidine reagent. Aqueous solutions of ethylene glycol, propylene glycol, ethanol, or acetone induced maximum or near maximum movement. All proteins tested moved about the same distance under these conditions.

Benzidine reagent. The preparation is made as follows: Equal volumes of saturated alcoholic benzidine solution and 3% hydrogen peroxide are mixed and made acid with glacial acetic acid. Fresh reagent must be prepared daily. Poor color development results if the hydrogen peroxide stock solution has decreased in strength.

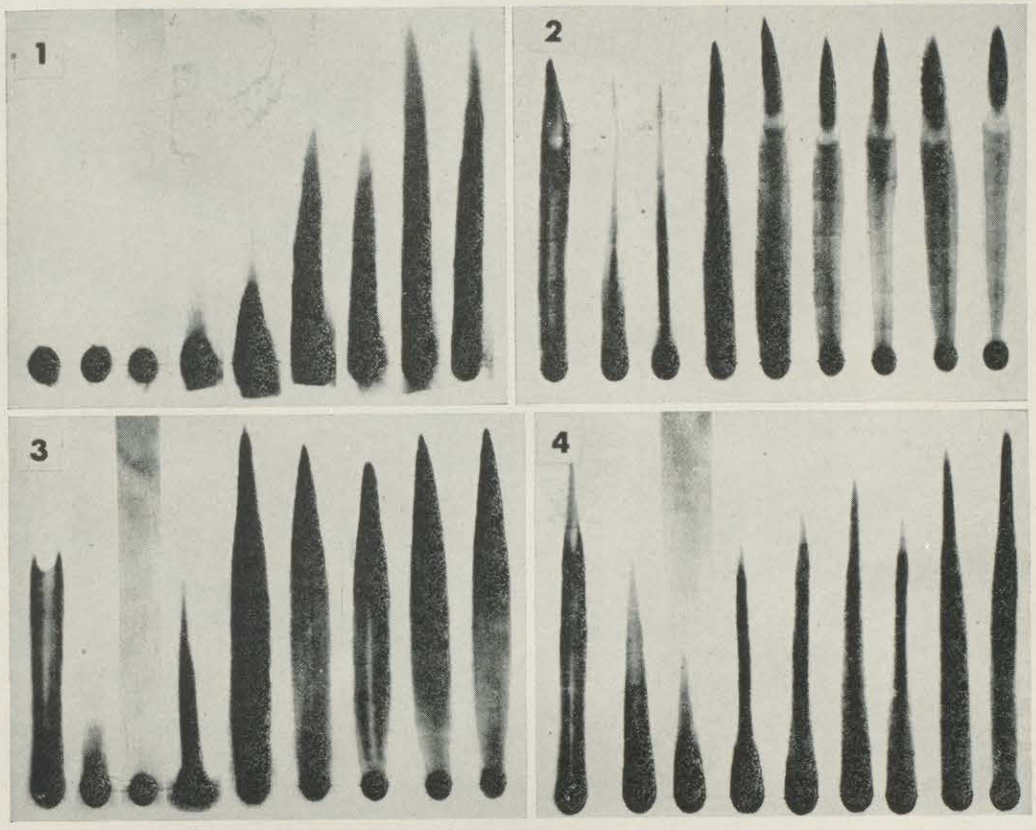
Color development with protein-hemin complexes is generally blue, but may be purple, brown, or green. Some salts, e.g., magnesium sulfate and sodium phosphate, interfere with color development. Citrate, glucose, trisodium phosphate, and potassium hydrogen phthalate at some pH values give rise to a deep purple color.

Background color development varies considerably, depending on the ease with which the benzidine reagent is oxidized. Acid pH values tend to retard background color development, while alkaline pH values give rise to a deeply colored background. Photographs are made of the chromatograms at the optimal development of color. It is necessary to photograph the chromatogram while the paper is still damp.

Hemin. A stock solution of 2% hemin in 3% sodium bicarbonate solution was prepared and 0.02 ml was added to 1 ml of the protein solution. In the proteins studied,

[1] Aided by a grant from the National Cancer Institute of Canada.

[2] Holder of a Canada Packers Research Fellowship of McGill University.



FIGS. 1–4. Composite photographs of unidimensional chromatograms of hemin and protein-hemin mixtures, showing the effects of changes of pH from 2 to 10 at 1-pH-unit intervals, from left to right. 1. Hemin control. 2. Bovine serum albumin-hemin. 3. β-Lactoglobulin-hemin. 4. γ-Globulin-hemin.

it has been observed that the presence of hemin does not alter the R_f value of the protein.

A control hemin chromatogram, in which 0.02 ml is diluted to 1 ml with distilled water, shows that movement is nil up to pH 5 and very slight at pH 6, and that it increases considerably above this pH (Fig. 1). Some developing solutions, among them those containing bicarbonate, glucose, sucrose, glycine, methionine, alanine, and urea, allow maximum or near maximum movement.

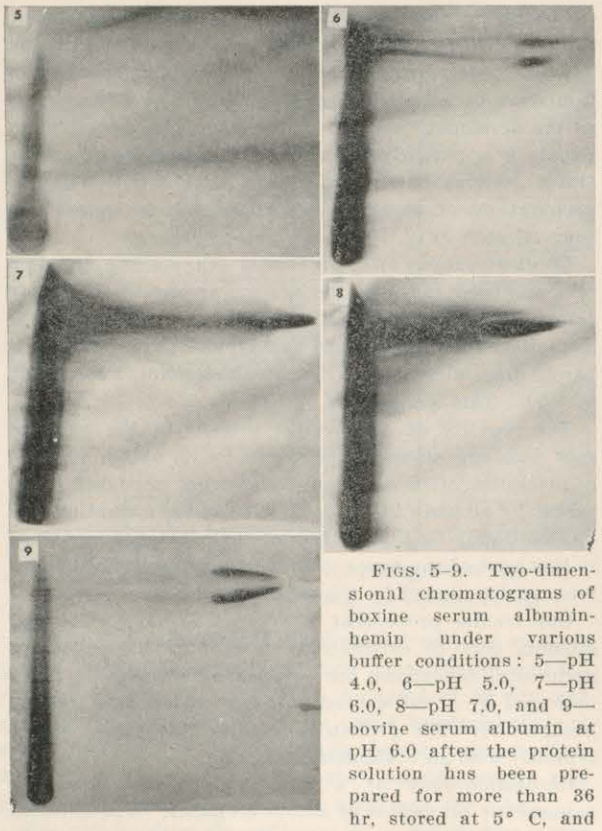
It has been found that some proteins and protein mixtures such as egg albumin, pepsin, papain, diastase, and urease will not combine with hemin. However, if hemin is added to a solution of these proteins, chromatographed, and streaked with the benzidine reagent, it is noticed that hemin remains at or near the point of origin (below pH 7). If the paper is allowed to stand for some time at room temperature, it is found that the background will color (to varying degree, depending on the nature of the salt in the developing solution), and a colorless spot will appear. It will be shown later that this colorless region actually contains the protein. Presumably the protein retards background color development and so produces a colorless "spot." Casein and β-lactoglobulin give rise to colorless spots or fractions up to about pH 5, and to deep blue fractions at higher pH values. γ-Globulin and bovine albumin give rise to colored fractions over the entire pH range (see Figs. 2, 4).

Time of development. It has not been found necessary to allow the solution front to advance beyond 20–25 cm, which requires about 90 min. The composition and shape of the chromatogram is determined within the first 20 min, and further development results in a more easily evaluated fraction. A complete two-dimensional chromatogram may be obtained within 5 hr.

The protein chromatogram. A long, rather narrow finger is formed, provided that the protein combines with hemin. The color intensity varies along with the finger, depending on the pH with which it is developed. Bovine albumin-hemin at pH 2 shows two intensely colored fractions inside the finger (Fig. 2). It may be shown that the greatest portion of the protein is concentrated in the leading fraction, and only traces remain in the finger.

One-dimensional chromatography results in the formation of a yellow-colored solution front, which fluoresces in ultraviolet light, and at pH 2, two separated bands appear at the leading edge of the chromatogram. Two-dimensional chromatograms result in a series of villus-like structures appearing in the second dimension. The presence of proteins on the paper tends to repress the full movement of those structures that are in the immediate vicinity of the protein.

Effect of pH. The variation of protein movement with change of pH seems to be much the same for all the proteins studied. At very acid pH values, e.g., pH 2, there



FIGS. 5–9. Two-dimensional chromatograms of boxine serum albumin-hemin under various buffer conditions: 5—pH 4.0, 6—pH 5.0, 7—pH 6.0, 8—pH 7.0, and 9—bovine serum albumin at pH 6.0 after the protein solution has been prepared for more than 36 hr, stored at 5° C, and then chromatographed with citrate in the first dimension and fumarate in the second dimension.

usually appears a well-defined leading section. In the region of pH 4–5 there is often little or no movement, and where movement does occur, no well-defined leading fraction occurs. Above this pH, a large, well-defined spot appears, which is quite constant in size. The R_f values depend upon pH in a similar way, being least around pH 4, which is near the isoelectric point of some of the proteins. R_f values and sizes of the spot are remarkably constant from pH 6 upwards.

Chromatography of bovine serum albumin-hemin complex. The R_f value of a bovine albumin-hemin complex is least in the region of pH 4. At pH 2, there appear two separated fractions inside the finger. This is also true at pH 3 but the spots are smaller and less intensely colored. From pH 5–12, there is a well-defined leading fraction, quite constant in size and position (Fig. 2).

In two-dimensional chromatograms, at pH 4, there is movement in the first dimension only (Fig. 5). At pH 5, with citrate-NaOH in the first dimension, and phthalate-NaOH in the second dimension, two separate fractions are obtained (Fig. 6). At pH 5.5 with citrate-NaOH in the first dimension and acetate-HCl in the second dimension, there appear two main fractions and a minor fraction. At pH 6 and 7 there is a single fraction appearing in the second dimension (Figs. 7 and 8).

It has been observed that if bovine serum albumin is allowed to remain in solution for a period of more than 36–48 hr, multiple fractions, sometimes as many as four, will appear. Various combinations of buffers have shown the separation of these fractions at pH 6 (Fig. 9).

It is apparent from these results that the crystalline bovine albumin which we have used behaves, when a solution is freshly prepared, as a homogeneous protein at pH 6–7, whereas two components are present at pH 5–5.5.

A private communication from Dr. G. Perlmann, to whom we are indebted for our supply of crystalline bovine serum albumin, states that this protein is homogeneous electrophoretically from pH 3.0 to 3.8 and pH 5.0 to 10.0. In the region of the isoelectric point the protein separates into two distinct electrophoretic components. This phenomenon seems to be a property of most serum albumins and indicates that crystalline albumin preparations contain several closely related proteins. Paper chromatography appears to confirm and may extend the results of electrophoretic analysis.

Chromatography of β-lactoglobulin-hemin complex. It is found that the union between β-lactoglobulin and hemin is not complete if the pH is 5 or less, i.e., the leading fraction is colorless. From pH 6 upwards, the complex shows as an intense blue. Thus the formation of a complex between this protein and hemin is dependent upon pH. R_f values show much less variation than those of bovine albumin. The R_f value is least at pH 4 and greatest at pH 6, where it is 0.86. All other values lie very close to 0.80 (Fig. 3).

Two-dimensional chromatograms between pH 6 and 7 demonstrate that this crystalline protein is not homogeneous. The use of citrate-NaOH in the first dimension and tartrate-NaOH, phthalate-NaOH, or acetate-HCl in the second, shows that there are a number of fractions in this sample. However, their complete separation under these conditions has not yet been achieved (Fig. 10).

We are again indebted for this sample of protein to Dr. Perlmann, who states that in 1945 this protein was found to be a mixture, there being two distinct electrophoretic components in the pH range 3.7–5.0, and also between pH 6.0 and 7.0.

Chromatography of human γ-globulin-hemin complex. It appears that γ-globulin, at the concentrations used, does not combine completely with hemin over the entire pH range. At each pH value, some combination takes place, but it is evident that from pH 3 to pH 8, there is a portion that does not combine, the amount of this portion decreasing with an increase in pH.

At pH 2, γ-globulin gives rise to two separate fractions inside the finger, in much the same manner as that shown by bovine albumin, but the fractions are smaller in size. Movement is least at pH 4. R_f values are 0.78 at pH 2.0 and 7.0, and increase to 0.81 at pH 10 (Fig. 4).

A two-dimensional analysis shows an excellent separation of two components of γ-globulin, the separation occurring at right angles. The first developing solution may be a phthalate-NaOH buffer at pH 6.0, 5% ammonium sulfate or 2% oxalic acid, followed by 2% tartaric acid in the second dimension (Fig. 11).

This protein, according to Dr. G. Perlmann, who kindly provided the sample, consists of one main electrophoretic component (96% of the total protein content), and it shows a marked boundary spreading, which indicates lack of homogeneity.

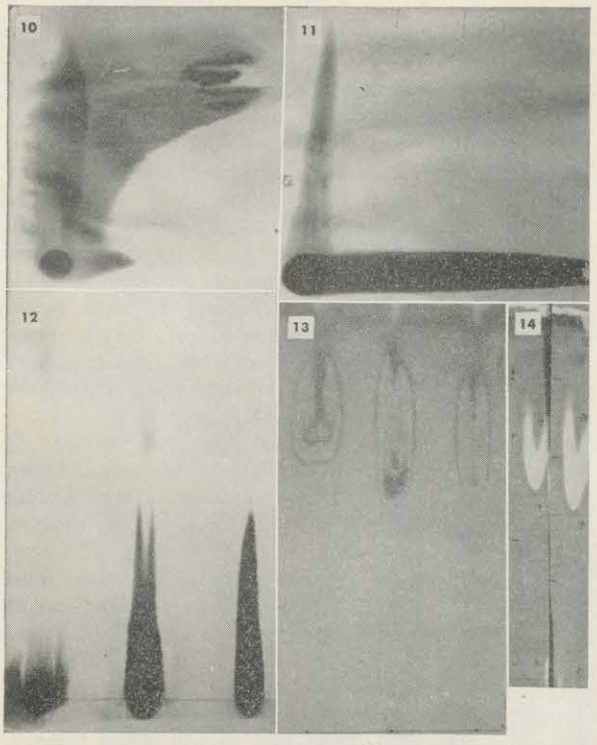


FIG. 10. Two-dimensional chromatograph of β-lactobulin-hemin at pH 6.5.

FIG. 11. Two- dimensional chromatogram of γ-globulin-hemin, using a phthalate buffer at pH 6.0 in the first dimension, followed by a 2% tartaric acid in the second dimension.

FIG. 12. Papain, papain-casein, and casein, in that order, developed with 2% succinic acid as the developing solution.

FIG. 13. Papain-bovine serum albumin, diastase-β-lacto-globulin, and β-lactoglobulin developed in a phthalate buffer at pH 5.0. There is no separation.

FIG. 14. Egg albumin developed in distilled water at pH 6.4. The resulting white spot was eluted, and identified by serological means.

Chromatography of enzyme preparations. Some preliminary work has been done on the movement of enzyme solutions. Preparations of pepsin, papain, malt diastase (commercial preparations from British Drug Houses, Ltd.) and urease (25-mg Urease-Dunning tablets) were used, and it was found that hemin will not combine with any of them. After streaking with the benzidine reagent and allowing the paper to stand for a time, it is observed that the malt diastase and papain preparations give rise to very well-defined, colorless oval spots. The urease preparation produces a vertical streak, but the pepsin preparation fails to show any definable movement. The papain preparation shows R_f values of 0.81 up to pH 3, which increase to 0.90 at pH 8.0–12.0. The malt diastase preparation shows a very constant R_f value over the entire pH range, varying only between 0.90 and 0.95, whereas the urease has an R_f value of 0.78 at pH 2, which increases to 0.85 at pH 3 and then gradually increases to 0.92 at pH 12. It seems likely that paper chromatography will be of service in the evaluation and examination of commercial enzyme preparations.

Chromatography of mixtures of proteins. One-dimensional chromatograms rarely separate a pair of proteins. We have succeeded, however, in separating components of a mixture of papain and casein, using 2% succinic acid as the developing solution (Fig. 12). The movement of papain is apparently retarded by the presence of casein. Other proteins have been run in combination with the preparations of papain and diastase, but no separations were effected (Fig. 13).

Chromatography of egg albumin. Crystalline egg albumin was used and, like the enzyme preparations already referred to, it will not unite with hemin. It appears as a colorless spot on a colored background (Fig. 14). It is barely detectable below pH 4, at which point it has an R_f of 0.60. This increases to 0.75 at pH 9.

The identity of the protein present in the colorless spot with egg albumin was shown by its ability to form a precipitin with a specific antiserum produced in a rabbit by albumin injections.[3] Precipitin formation was produced only by the solution of the substance contained in that part of the paper strip believed to contain the protein, as determined by the streaked control.

Our results prove that a solution of crystalline egg albumin may be chromatographed under the given experimental conditions, the position of the protein being indicated by a well-defined colorless region on a colored background. The protein present in this region reacts typically with an ovalbumin antiserum.

Chromatography of a urease preparation and the manometric estimation of urease on paper. The question arose as to whether enzymes will retain their catalytic activities if they are subjected to our chromatographic technique. A series of chromatograms was therefore set up for this purpose, the enzyme selected being urease.

The enzyme solution was prepared by grinding twenty 25-mg urease-Dunning tablets in a small mortar in 2 ml of a 2% cystein-glycine solution that had been adjusted to pH 6. It was then centrifuged for 15 min, and the supernatant solution was used for chromatography. Aliquots of 0.04 ml were applied to the paper by placing 0.02 ml on the paper, allowing it to dry, and adding another 0.02 ml. The developing solution was prepared by dissolving 2 g cystein hydrochloride in water and adjusting the pH to 6 with caustic soda solution, making the volume to 100 ml, and then adding 1 g glycine. This reagent should be prepared just prior to use. The solution was allowed to advance about 20–22 cm. The paper was then removed and allowed to stand at room temperature for a few min. While the paper was still damp, it was cut into 3-cm sections, starting 0.5 cm from the bottom of the paper. The boundaries were marked 7 mm on each side of the point of application of the aliquot.

Each section of filter paper was then cut into four parts, all of which were placed immediately in a Warburg manometric vessel with 3 ml acetate buffer at pH 5, and 0.2 ml 10% urea was placed in the side arm. As a control, a ten times dilution of the original supernatant was

[3] This antiserum was kindly prepared for us by Prof. E. G. D. Murray, of the Department of Bacteriology, McGill University.

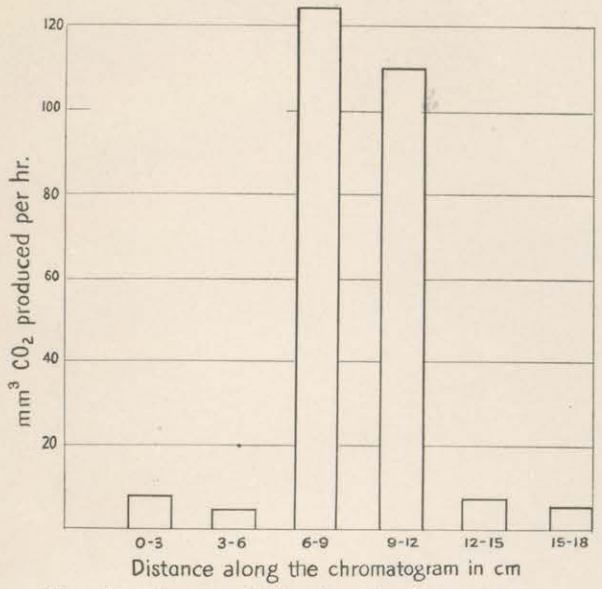


FIG. 15. Urease activity along the chromatogram.

used (diluted with physiological saline) and 0.4 ml of it placed in the Warburg vessel with 2.6 ml buffer and 0.2 ml 10% urea in the side arm. A period of 30 min was allowed for the contents of the vessel to attain thermal equilibrium (37° C). The urea in the side tube was then tipped into the vessels, and carbon dioxide output rate measured manometrically in the usual way for 1 hr.

The results show the existence of a distribution curve of urease on the paper strip. Traces of enzyme are left at the point of origin, but a fair proportion ascends the paper, whose maximum urease activity lies between 6 and 12 cm from the origin. On either side of the maximum there is a drop in enzymic activity. These results are shown in Fig. 15. If a parallel chromatogram is streaked with the freshly prepared benzidine reagent, it is found that the greatest extent of the colorless region on the colored background is 6–12 cm from the origin.

On assessing the activity of the urease over the entire strip, by adding together the activities of the various parts of the strip it is found that the total activity amounted to 85% of that expected from the amount of urease placed on the paper. Another assessment of the total urease activity over the entire strip showed a recovery of 110%, the assessment being made from a calibration curve previously prepared, relating activity (rates of CO_2 production) to the quantity of urease. These results show that urease activity is not diminished within experimental error by our chromatographic technique, and that movement of the urease molecule on filter paper can be followed. It is evident, however, from our preliminary results that metallic constituents of the filter paper may appreciably affect the rate of movement of urease and possibly other proteins. This needs more study.

Chromatography of human serum. A few experiments have been carried out on the chromatography of human serum. The results show the presence, at pH 6, of a complex mixture of hemin-reacting proteins. Three of the fractions appear to give R_f values identical with those found with a preparation of crystalline human serum albumin. Human serum globulins do not seem to move appreciably in the second dimension. We estimate that, with our technique, between 6 and 10 protein fractions appear. This work is now being extended with a view to discovering whether paper chromatography of blood serum may be used for diagnostic purposes.

References

1. CONSDEN, R., GORDON, A. H., and MARTIN, A. J. P. *Biochem. J.*, 1944, **38**, 224.
2. WILLIAMS, R. J. and KIRBY, H. *Science*, 1948, **107**, 481.

Reprinted from SCIENCE, June 2, 1950, Vol. 111, No. 2892, pages 605–608.
"PRINTED IN U. S. A."

Presence of Iodinated Amino Acids in Unhydrolyzed Thyroid and Plasma

J. Gross, C. P. Leblond, A. E. Franklin, and J. H. Quastel
Department of Anatomy, McGill University, and
Research Institute, Montreal General Hospital

Reprinted from SCIENCE, June 2, 1950, Vol. 111, No. 2892, pages 605–608.
"PRINTED IN U. S. A."

Presence of Iodinated Amino Acids in Unhydrolyzed Thyroid and Plasma[1]

J. Gross, C. P. Leblond, A. E. Franklin,[2] and J. H. Quastel

Department of Anatomy, McGill University, and Research Institute, Montreal General Hospital

De Robertis (*1*) demonstrated the presence of a proteolytic enzyme in the colloid of the thyroid follicle. He postulated that this enzyme causes the breakdown of thyroglobulin with the release of biologically active fragments which are of low enough molecular weight to diffuse out of the follicle, and which presumably constitute the thyroid hormone. The only compound of small molecular weight found so far in unhydrolyzed thyroid is thyroxine (*4*). In the present work, the technique of paper chromatography has been used to confirm this result, and to study the production of other iodinated amino acids which might be formed during the breakdown of thyroglobulin. The appearance of these substances in the circulation has been studied by observing their presence in *n*-butanol extracts of plasma. In addition, chromatographic analysis of whole plasma has supplied evidence as to the nature of the circulating thyroid hormone.

Methods. Female albino rats weighing 150–200 g and maintained on Remington's low iodine diet No. 342 (*5*), to which 10% brewers' yeast had been added, were injected subcutaneously with about 100 μc of carrier-free radioactive iodide (NaI^{131}). After 48 hours the animals were anesthetized with ether and exsanguinated with a heparinized syringe via the inferior vena cava.

Chromatography of n-butanol extracts of thyroid and plasma. The thyroids were removed immediately, ground in 1 ml of ice-cold saline in a chilled mortar, and extracted three times with an equal volume of *n*-butanol. Similarly, the plasma was extracted three times, first with a double volume and then twice with an equal volume of butanol. In both cases, the combined butanol extracts were reduced to dryness *in vacuo* at room temperature. The dry residue was taken up in distilled water, 0.1 ml and 0.2 ml being used for the thyroid and plasma residues, respectively.

Aliquots of 0.02 ml of this solution were then chromatographed by the capillary ascent method of Williams and Kirby (*10*), with or without the addition of 20 μg of each of the following carriers: DL-thyroxine, DL-diiodothyronine, DL-diiodotyrosine, and DL-monoiodotyrosine. The aliquots were placed in the lower left-hand corner of 10 in. × 12 in. Whatman No. 1 filter paper sheets and dried. The developing solvent used for the first dimension was the upper layer obtained after shaking *n*-butanol with 2*N* formic acid in a separatory funnel. For the second dimension, the upper layer of a mixture of 4 parts *n*-butanol and 1 part dioxane shaken with 5 parts of 2*N* NH_4OH was used. After overnight development for each dimension, the papers were removed and dried in a current of air. They were then applied to Eastman No-Screen x-ray film, with a sheet of cellophane intervening, and thus exposed for varying periods of time. The duration of exposure depended on the activity present, as determined with a Geiger probe monitor. After autography (Figs. 1–3) the papers were dried for 30 min at 100° C, sprayed with ninhydrin (0.1% solution in $CHCl_3$ with the addition of 0.1% collidine), and dried at 100° C again. The colors obtained with ninhydrin were rather pale, but were sufficiently clear to locate the added carrier amino acids. By this technique it was possible to obtain a complete separation of the four iodinated amino acids and of iodide as well.

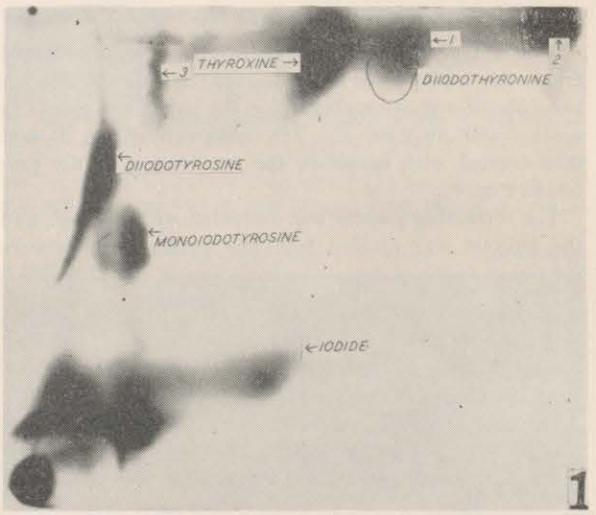


FIG. 1. An autograph of a two-dimensional chromatogram of the entire butanol extract of the fresh thyroid taken from an adult rat given 100 μc of carrier-free NaI^{131} subcutaneously, 48 hr previously. Twenty μg each of nonradioactive DL-monoiodotyrosine, DL-diiodotyrosine, DL-thyroxine, and DL-diiodothyronine were added to the butanol extract. The paper was developed vertically with a butanol-formic acid mixture and horizontally with a butanol-dioxane-ammonia mixture. The material was deposited on the left lower corner. Some spots are due to thyroxine, diiodotyrosine, monoiodotyrosine, and iodide; others are due to unidentified substances indicated by the numbers 1, 2, and 3.

Chromatography of the plasma proteins. The plasma proteins, combined with hemin in the manner described by Franklin and Quastel (*2*), were chromatographed at pH 7 using M/20 citrate buffer in the first dimension and M/20 phthalate buffer in the second dimension. Two hours were allowed for a satisfactory development in each dimension. After the paper had dried, the benzidine reagent was applied with a small paint brush, resulting in the formation of a blue color at the site of the plasma protein. The results were photographed immediately.

[1] Carried out in part with the aid of a grant from the National Research Council of Canada to C. P. Leblond, and in part with the help of a grant from the National Cancer Institute of Canada to J. H. Quastel. The help of Mrs. J. Dougherty and Miss J. Cambron is acknowledged. We are indebted to Dr. R. Pitt Rivers for the monoiodotyrosine and to Hoffman-LaRoche, Ltd. for the other iodinated amino acids used in this study.

[2] Canada Packers Research Fellow, McGill University.

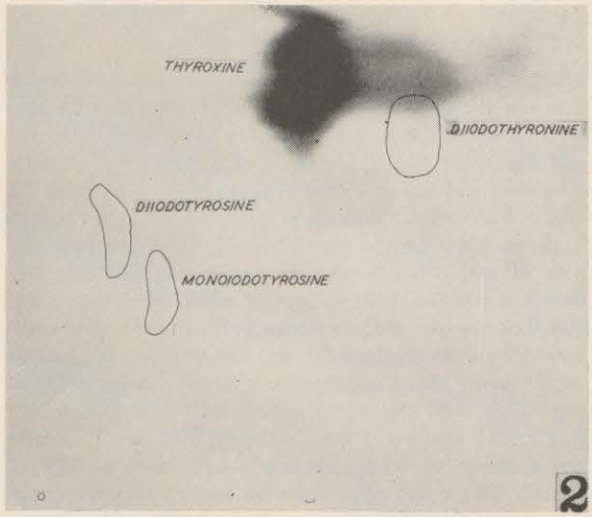


FIG. 2. An autograph of the entire butanol extract of 2.5 ml of the plasma of the same animal as in Fig. 1; the same carriers were added. An intense thyroxine spot is visible. The much less intense spot next to it is probably due to the substances causing spot 1 in Fig. 1.

(0.02 ml) was applied directly to the paper. After the

This method was used with radioactive plasma obtained from the radioiodide-treated rats described. Plasma two-dimensional chromatography, the paper was placed in contact with an x-ray film for autography (Fig. 5) and then treated with benzidine for localization of the proteins (Fig. 4).

The remaining plasma was extracted with butanol, and the butanol was divided into two portions which were

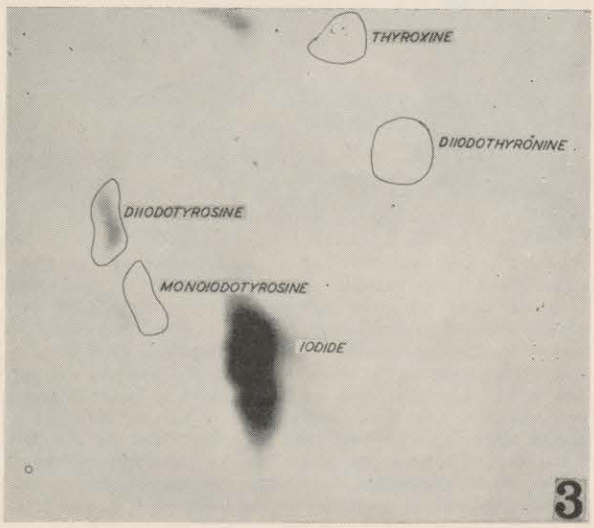


FIG. 3. An autograph of a control chromatogram of a mixture containing carrier-free NaI^{131}, and 20 μg each of nonradioactive DL-monoiodotyrosine, DL-diiodotyrosine, DL-thyroxine, and DL-diiodothyronine. Besides an intense spot due to iodide, a faint spot is visible in the location of diiodotyrosine. Two spots due to unidentified substances are also present. When the marked intensity of the iodide spot is compared with the low intensity of all other spots, it is apparent that only a very small percentage of the iodide present exchanged with diiodotyrosine and none at all with thyroxine.

dried in the usual manner. The first portion was dissolved in 0.1 ml of 4% NaCl, of which 0.01 ml was chromatographed with the butanol developers and autographed. Another 0.01-ml aliquot was chromatographed with buffers and autographed (Fig. 6). Finally, the second portion of dried butanol extract was dissolved in nonradioactive plasma obtained from another rat, mixed with hemin, chromatographed with buffers (Fig. 7), and autographed (Fig. 8).

Confirmatory experiments with plasma proteins not containing hemin were carried by the addition of nonradioactive DL-thyroxine. Thyroxine (100 μg) was mixed with 0.02 ml radioactive plasma, chromatographed with buffers and the location of thyroxine determined by spraying with ninhydrin.

Identification of components. Thyroid and plasma fractions of ten animals were chromatographed and autographed. The autographs (Fig. 1) of the butanol extract of thyroid, with added carriers, showed the presence of spots corresponding to monoiodotyrosine, diiodotyrosine, thyroxine, and iodide; all of which were found in the ten animals investigated. These spots were similar in location and intensity when no carrier was used, and therefore the presence of these substances cannot be due to an exchange between the labeled iodide and the carriers. This was borne out by control experiments in which tracer iodide was chromatographed with carriers (Fig. 3). In addition, several compounds, whose identities are unknown, were found more or less regularly throughout the series of thyroid chromatograms. Thus, of the unknowns marked 1, 2, and 3 (Fig. 1), compound 1 was found in ten and the other two compounds in seven of the preparations.

Thyroxine made up the greater part of the radioactivity of the butanol extract of plasma (Fig. 2). By using the entire plasma extract and exposing for a long period of time, it was possible to distinguish faint spots corresponding to iodide in five plasma samples and to diiodotyrosine in six. Both substances were found together in extracts of five samples of plasma. The results were identical whether or not carriers were added to the plasma extract.

A possible source of error lay in the production of artifacts by the radiochemical effect of the large doses of radioactivity used. However, it could be demonstrated that the same amino acids were present in thyroid and plasma of two rats in which radioactivity of the injected radioiodide was only 1 μc, a dose which has been shown to have no effect on the physiological processes of the thyroid (7). A very long exposure had to be used to detect the minute amounts of radioactivity present in the chromatographic spots obtained under these conditions.

Nature of the circulating thyroid hormone. When an aliquot of whole plasma from an animal given radioactive iodide was mixed with hemin and chromatographed using buffer solutions for development, the distribution of the proteins, as shown by the benzidine reagent (Fig. 4), corresponded to the location of the plasma radioactivity (Fig. 5), indicating that the radioactivity had moved with the plasma proteins. When the radioactivity ex-

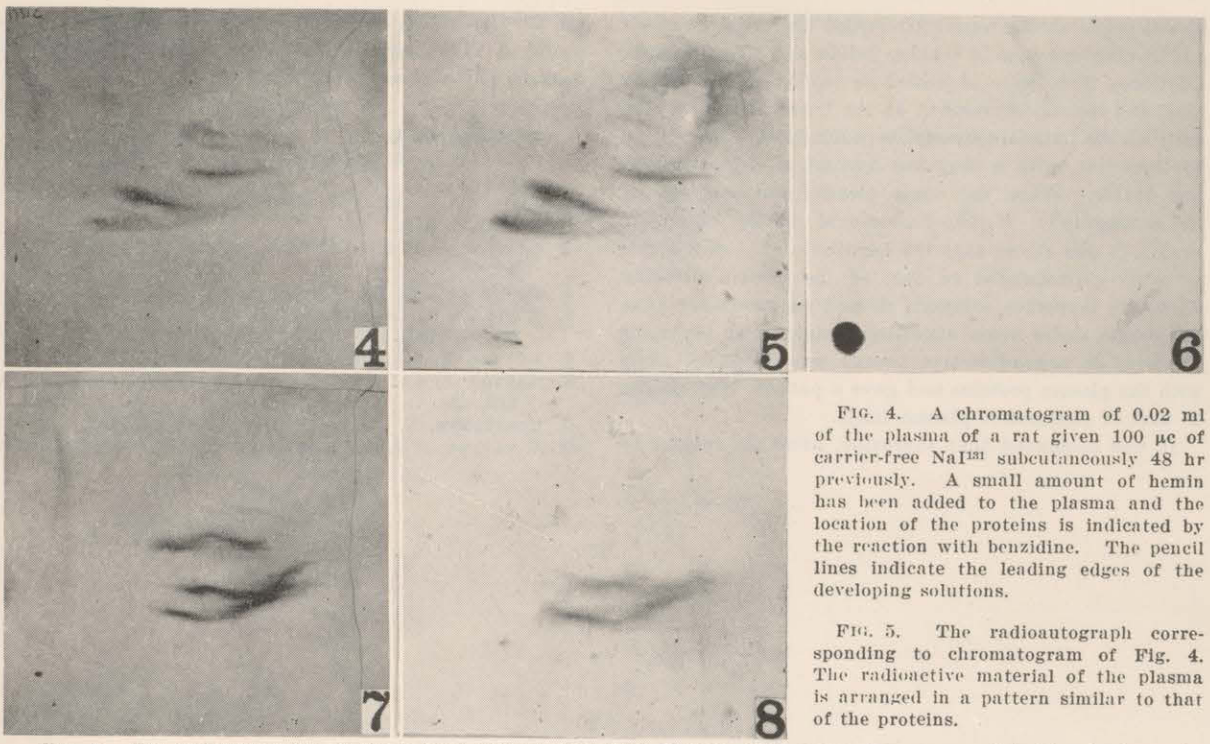


FIG. 4. A chromatogram of 0.02 ml of the plasma of a rat given 100 μc of carrier-free NaI^{131} subcutaneously 48 hr previously. A small amount of hemin has been added to the plasma and the location of the proteins is indicated by the reaction with benzidine. The pencil lines indicate the leading edges of the developing solutions.

FIG. 5. The radioautograph corresponding to chromatogram of Fig. 4. The radioactive material of the plasma is arranged in a pattern similar to that of the proteins.

FIG. 6. The radioautograph of the butanol extract of another aliquot of the same plasma as used in Fig. 4, chromatographed with buffers as in Fig. 4. By the method used in ig 2, this extract was found to consist wholly of radioactive thyroxine. The radioactive thyroxine did not move from the origin.

FIG. 7. A chromatogram of 0.02 ml of nonradioactive plasma, to which hemin had been added and combined with an aliquot of the butanol extract used in Fig. 6, chromatographed with buffers as in Fig. 4, and sprayed with benzidine. The protein pattern is apparent.

FIG. 8. The radioautograph corresponding to chromatogram of Fig. 7. The radioactive thyroxine added to nonradioactive plasma has moved with the proteins, giving a picture similar to that obtained with radioactive plasma (see Figs. 4 and 5).

tracted by butanol was chromatographed with butanol developers, it proved to consist almost exclusively of radiothyroxine. However, when chromatographed with buffers, this radiothyroxine remained at the origin (Fig. 6). Nevertheless, if the radiothyroxine was added to nonradioactive plasma, it was displaced (Fig. 8) along with the plasma proteins (Fig. 7), in a manner similar to that of the original radioactive plasma (Figs. 4 and 5).

The same results were obtained when nonradioactive thyroxine was chromatographed with plasma that did not contain hemin, indicating that presence of hemin was not necessary for plasma-thyroxine combination.

Interpretation. These results demonstrate that, besides thyroglobulin and iodide, the thyroid gland contains small amounts of monoiodotyrosine, diiodotyrosine, and thyroxine (Fig. 1). It has been shown that the presence of these amino acids is not due to exchange phenomena (Fig. 3) or to radiochemical action. The presence in the thyroid of these amino acids, which are known to be components of thyroglobulin, lends support to the hypothesis that thyroglobulin undergoes proteolysis by the enzyme described by De Robertis (*1*).

The thyroxine found in plasma is most likely of thyroid origin. This is indicated by experiments showing that the butanol-soluble radioactivity of plasma is extremely low in thyroidectomized rats (*3*). The passage of thyroxine into the circulation has been previously explained by a gradient of thyroxine concentration in thyroid tissue and plasma (*4*). It may be pointed out further that the diffusion of thyroxine from the thyroid would tend to correct the increase in osmotic tension due to proteolysis in the colloid. The failure to find significant amounts of the other iodinated amino acids in plasma may be explained by their destruction within the follicle and a reutilization of their iodine by the gland.

In the plasma, thyroxine has been found combined with proteins. This combination may be severed easily by butanol, but is reconstituted when thyroxine is placed in contact with plasma proteins (Figs. 7 and 8). Furthermore, since plasma proteins may combine with amounts of thyroxine well above the physiological doses (100 μg in 0.01 ml), it may be assumed that under physiological conditions the thyroxine secreted from the thyroid combines with plasma proteins as it is released.

The nature of the thyroxine complex in plasma is obscure. However, iodine has been found in the albumin fraction of plasma (*6, 8, 9*), and to a somewhat lesser extent in the globulin fraction (*6, 8*). Attempts to determine the plasma constituent which is the thyroxine carrier are currently under way.

In summary, when adult female rats on a low iodine intake were sacrificed 48 hr after an injection of carrier-free radioiodide, butanol extracts of unhydrolyzed thyroid and plasma analyzed by radioautography of two-dimen-

sional paper chromatograms revealed the presence of six radioactive compounds besides iodide and thyroglobulin. Of these, three were identified as thyroxine, diiodotyrosine, and monoiodotyrosine. In the blood plasma, practically all the butanol-extractable radioactivity was present as thyroxine, with a very low amount of diiodotyrosine and iodide. When the whole plasma was analyzed by radioautography of two-dimensional buffer chromatograms, it was shown that the location of the radioactive material corresponded to that of the plasma proteins. Although thyroxine solutions showed no movement from the origin under these conditions, radioactive thyroxine dissolved in nonradioactive plasma was displaced along with the plasma proteins and gave a pattern very similar to that of radioactive plasma itself.

It was concluded that thyroxine, after its release by the thyroid gland, circulates in combination with plasma proteins. The complex thus formed can be split with butanol and reconstituted *in vitro*.

References

1. De Robertis, E. *N. Y. Acad. Sci.*, 1949, **50**, 279.
2. Franklin, A. E. and Quastel, J. H. *Science*, 1949, **110**, 447.
3. Gross, J. Doctoral dissertation, McGill University, 1949, p. 174.
4. Leblond, C. P. and Gross, J. *J. clin. Endocrinol.*, 1949, **9**, 149.
5. Remington, R. E. *J. Nutrition*, 1937, **13**, 223.
6. Riggs, D. S., Lavietes, P. H., and Man, E. B. *J. biol. Chem.*, 1942, **143**, 363.
7. Skanse, B. *J. clin. Endocrinol.*, 1948, **8**, 707.
8. Taurog, A. and Chaikoff, I. L. *J. biol. Chem.*, 1948, **176**, 639.
9. Treverrow, V. *J. biol. Chem.*, 1939, **127**, 737.
10. Williams, R. J. and Kirby, H. *Science*, 1948, **107**, 481.

Reprinted from PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE,
1950, v74, 803-808

Paper Chromatography of Protein Mixtures and Blood Plasmas. (18055)

A. E. FRANKLIN* AND J. H. QUASTEL. (Introduced by C. P. Leblond)
From the Montreal General Hospital Research Institute, Montreal

It has been shown by Franklin and Quastel (1) that it is possible, by means of paper chromatography, to effect separations of proteins on filter paper, using the capillary ascent technic (Williams & Kirby)(2) and hemin as a 'marker' for the proteins with which it combines. Buffer and aqueous salt solutions, instead of nonaqueous solvents, are employed as developing agents. Working with solutions of pure (crystallized) proteins these workers have shown that the results of two dimensional paper chromatography are in accordance with the findings of electrophoretic analyses, those proteins exhibiting homogeneity or heterogeneity under the conditions of electrophoresis giving substantially the same patterns on filter paper. Moreover, they have shown that a protein such as egg albumin, does not lose its serological properties (precipitin formation with the specific antiserum) on its ascent on filter paper, and that an enzyme such as urease, may ascend filter paper to a particular position without loss of its enzyme properties. It is evident, therefore, that the technic may be applied to the separation, and possible identification, of proteins and enzymes. It is intended, in a series of forthcoming papers, to show how the technic, modified from that originally described by Franklin and Quastel, may be applied to the study of animal sera and plasmas under different pathological conditions and to the investigations of various problems relating to the interaction of proteins, enzymes and a variety of organic substances.

It has already been demonstrated by Gross, Leblond, Franklin and Quastel(3) that the technic of paper chromatography may be used for showing the combinations between thyroxine and plasma proteins.

It is of interest, in connection with this work, that Cochran(4) has already shown that the protein of tobacco mosaic virus will ascend filter paper and still retain its infectivity; incidentally, its position on paper was marked by the application of the Sakaguchi test for arginine. More recently, Mitchell, Gordon and Haskins(5) have succeeded in separating constituents of takadiastase by the use of a chromatopile.

It is intended, in this paper, to give a brief account of the chromatography of protein mixtures with particular reference to animal plasmas.

Procedure. Whatman No. 1 filter paper is used almost exclusively throughout this work, although other filter papers have been tried. The paper is cut into 8″ x 8″ squares, and the aliquot of protein solution is placed in the lower left hand corner. Plasmas are taken with a heparinized syringe (1/10 ml of 1% heparin-Na salt for 10 ml blood), and centrifuged for about 20 minutes. The plasma is placed in culture tubes in 0.5 ml quantities, and 0.02 ml 0.3% hemin is added. If a surface active agent is to be used, it is generally added to the tube first to facilitate its solution. The contents of the tube are now well mixed, and 0.02 ml aliquots are applied to the paper, each sample usually being tested in duplicate. After the aliquot has dried at room temperature, the paper is formed into a cylinder in such a manner that the edges do not touch. The cylinders are placed upright in a pyrex plate, which contains 100 ml of the developing solution. It is covered with a suitable container (a large

* Canada Packers Research Fellow, McGill University, Montreal.

1. Franklin, A. E., and Quastel, J. H., *Science*, 1949, v110, 447.

2. Williams, R. J., and Kirby, H., *Science*, 1948, v107, 481.

3. Gross, J., Leblond, C. P., Franklin, A. E., and Quastel, J. H., *Science*, 1950, v111, 605.

4. Cochran, G. S., *J. Phytopathology*, 1947, v37, 850.

5. Mitchell, H. K., Gordon, M., and Haskins, F. A., *J. Biol. Chem.*, 1949, v180, 1071.

Printed in U. S. A.

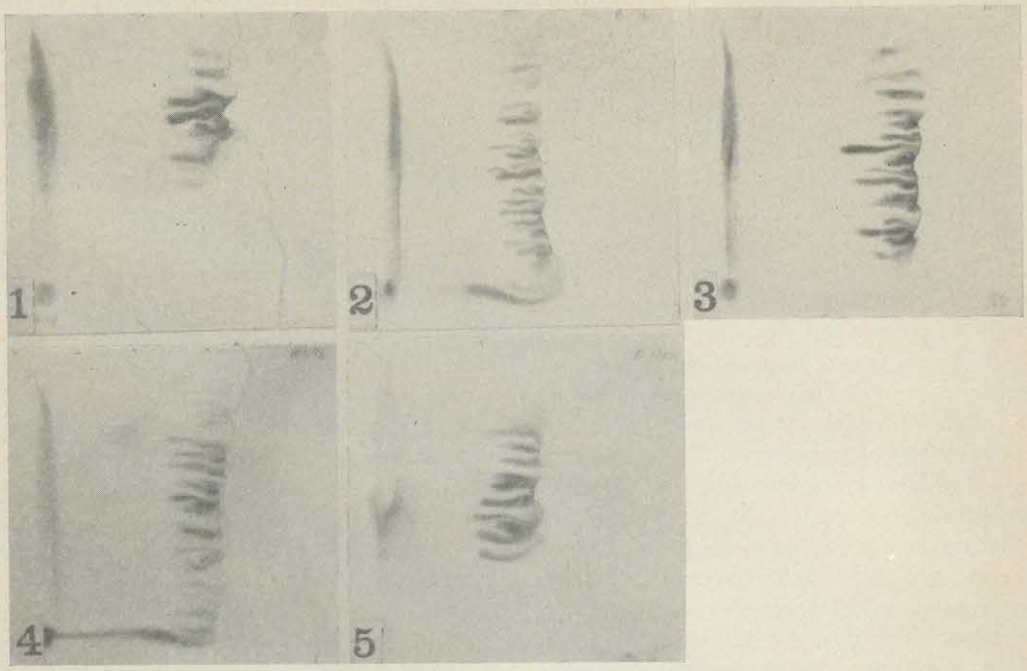


FIG. 1. Human plasma.
FIG. 2. Human plasma with 0.03 ml "Span 20" added per 0.5 ml plasma.
FIG. 3. Human plasma with 0.03 ml "NNO" added per 0.5 ml plasma.
FIG. 4. Human plasma with 0.05 ml "BRIJ-30" per 0.5 ml plasma.
FIG. 5. Human plasma with 0.04 ml "Tween 20" per 0.5 ml plasma.

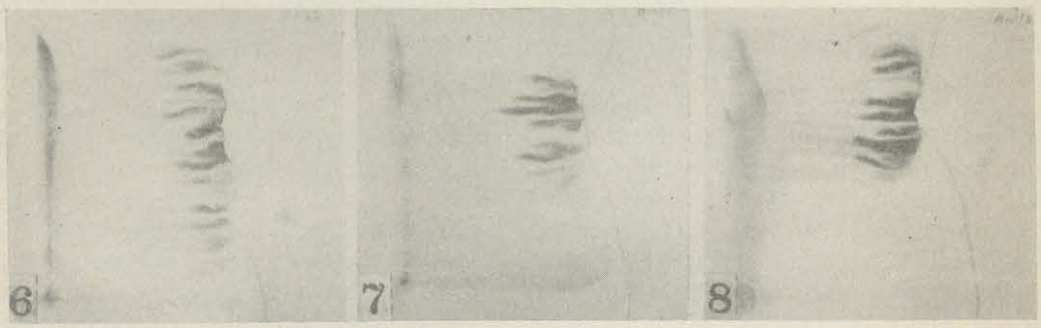


FIG. 6. Human plasma with 5 mg "Elvanol 31-31" added per 0.5 ml plasma.
FIG. 7. Rat plasma with 5 mg "Elvanol 31-31" added per 0.5 ml plasma.
FIG. 8. Guinea pig plasma with 5 mg "Elvanol 31-31" added per 0.5 ml plasma.

cylinder) to maintain constant humidity. After the solution has reached the top of the cylinder, which usually requires about 90 minutes, the cylinder is taken out and allowed to dry at room temperature. After thorough drying, the cylinder is re-formed and stapled again at right angles to the original direction and placed in the pyrex dish which contains the developing solution required for the second dimension. It is not necessary to allow the solution to reach the top of the filter paper in the second dimension, but it may be stopped about two thirds of the way up. The cylinder is again dried, and the filter paper is streaked with freshly prepared benzidine reagent. The chromatogram is photographed immediately due to the rather rapid background color development. The entire process may be easily carried out in 5-6 hours.

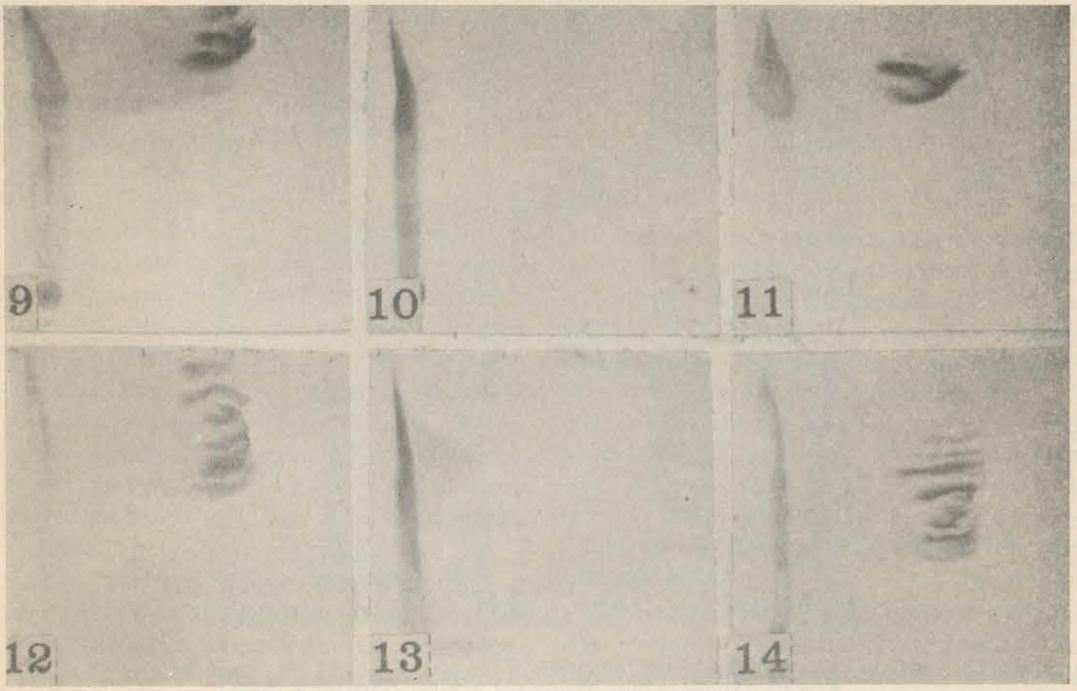


FIG. 9. Protein (Cohn) Fraction V (16 mg) dissolved in 0.5 ml 0.85% saline.
FIG. 10. Protein (Cohn) Fraction III (8 mg) dissolved in 1.0 ml 0.85% saline.
FIG. 11. Mixture of Protein (Cohn) Fractions; 22 mg V + 4 mg II + 5 mg IV—3,4, dissolved in 0.5 ml 0.85% saline.
FIG. 12. As in Fig. 9 with 0.01 ml "Tween 85" per 0.5 ml solution.
FIG. 13. As in Fig. 10 with 0.01 ml "Tween 85" per 0.5 ml solution.
Fig. 14. As in Fig. 11 with 0.01 ml "Tween 85" per 0.5 ml solution.

Hemin. The hemin solution is prepared by dissolving 30 mg crystalline hemin in 10 ml of distilled water, to which has been added a small quantity of sodium bicarbonate. Hemin solution is generally added in the ratio of 0.02 ml hemin to 0.5 ml of the protein solution.

Benzidine solution. This solution must be prepared daily, by mixing equal volumes of saturated alcoholic benzidine solution and 3% hydrogen peroxide. It is made acid with glacial acetic acid. The solution is applied with a paint brush, and the chromatogram is photographed immediately.

Developing solutions. A large number of solutions of organic salts and buffers at different concentrations have been investigated for their suitability as developing agents. It has been found that M/10 sucrose solution is very efficient for use in the first dimension, when this is followed by M/10 sodium potassium tartrate solution in the second dimension. This combination is used in all the chromatograms given in this paper (Fig. 1-18). Other combinations also give very good results, as for example, dextrose, lactose or maltose in the first dimension, and nicotinate, malate, succinate, fumarate, mandelate, acetate or citrate in the second dimension. Certain amino-acids also give good results when employed in developing solutions, for example, M/10 methionine solution followed by M/10 acetyl glycine solution, or M/10 cysteine solution followed by M/10 sodium glutamate solution.

Effects of various filter papers. The choice of filter papers seems to be very important, as it appears that not all animal plasmas give the best results with the same filter paper. Human and rat plasmas give the best chromatograms with Whatman No. 1 but guinea pig plasma gives the best results when

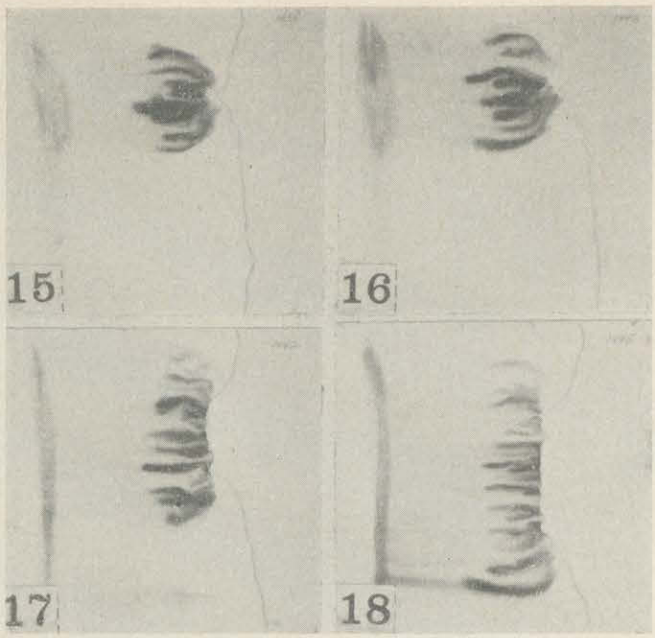


FIG. 15. Normal horse serum.
FIG. 16. Horse serum immunized against diphtheria toxin.
FIG. 17. As in Fig. 15 with 0.01 ml "Tween 85" per 0.5 ml serum.
FIG. 18. As in Fig. 16 with 0.01 ml "Tween 85" per 0.5 ml serum.

using S and S 589. When comparing the plasmas of various species, however, Whatman No. 1 has been consistently used.

Effects of the addition of surface active substances. When a plasma is chromatographed in the manner that has been described above, the various protein components do not separate very well and tend to remain bunched together. This is illustrated in Fig. 1. It is found that if certain surface active substances are added to the plasma before chromatography, good separations of the protein fractions take place. The following substances yield excellent results: sodium alginate, the "Elvanols," "Spans," "Tweens," "BRIJ" and "NNO."* Results with sodium tauroglycholate are relatively poor. The various surface active substances do not give identical results, both their structures and their concentrations being important in determining the pattern of the protein chromatogram. It has been made a matter of practice to make chromatograms of a blood plasma, or a protein mixture, in presence of different concentrations of a selected surface active substance. Usually 0.01 ml to 0.06 ml of the solution of the surface active agent is added to 0.5 ml plasma before addition of the hemin, and subsequent chromatography. The separation of the plasma constituents, as facilitated by the presence of surface active agents, are shown in Fig. 2, 3, 4 and 5. High concentrations of surface active agents must not be added to the plasma; in their presence, the movement of the proteins on filter paper appears to be greatly suppressed.

Effects of dialysis of blood plasma. Dialysis of blood plasma against running water seems not to disturb the pattern of the

* "Elvanols": A group of polyvinyl alcohols produced by hydrolysis of polyvinyl acetates of varying degrees of polymerization (Dupont).
Span 20: Sorbitan monolaurate.
NNO: Glycerol mannitan laurate.
BRIJ 30: Polyoxyethylene lauryl alcohol.
Tween 20: Polyoxyethylene sorbitan monolaurate.
Tween 85: Polyoxyethylene sorbitan trioleate.

plasma chromatogram. It is concluded, therefore, that the dialysable material of plasma has little or no influence on the movement of the proteins on filter paper.

Consistency of results. With any given plasma, or protein mixture, excellent duplication of results has been obtained. It is necessary to be strictly accurate in the measurement of the surface active material added to the plasma, but if due care is taken, there is no difficulty in obtaining good duplication with any sample of a protein mixture.

Applications of the technic. Comparison of human, rat and guinea pig plasmas. Heparinized plasmas of different animals show different patterns on the chromatogram when they are chromatographed under similar conditions.

With rat and guinea pig, the blood is removed with a heparinized syringe from the ether anaesthetized animal, and centrifuged for about 20 minutes. 0.5 ml of plasma is mixed with 0.02 ml 0.3% hemin solution, and various amounts of a surface active agent is added to a number of such samples. Using 5 mg "Elvanol 31-31" as surface active agent and two dimensional chromatography with M/10 sucrose solution in the first dimension followed by M/10 sodium potassium tartrate solution in the second, evident differences between the plasma chromatograms appear (Fig. 6, 7, and 8). On the other hand, the presence of "Elvanol 51-05" fails to show any obvious differences. "Elvanol 54-22" yields a much better separation of constituents of human plasma than is obtained with either rat or guinea pig plasma. "Span 20" gives excellent separations of the protein components, but, whilst failing to show a marked difference between human and rat plasma, it gives a distinctive pattern with guinea pig plasma. "NNO" gives a good separation of the constituents of each of the plasmas, but differences between the plasma patterns are not very evident. "Tween 20," "Tween 81" and "Tween 85" are the best surface active agents to use when examining various human plasmas.

As a matter of practice when chromatographing human plasmas, from different pathological conditions, "Tween 81" and "Tween 85" are being used regularly. They seem to give the most satisfactory separations so far. It is obvious, however, that there is great room for improvement, and new surface active agents are being continually tested.

It has already been found that the pattern of the chromatogram of a plasma, depends on the nutrition of the animal and the results of our investigation into this matter will form the subject of a separate communication. It is desirable, in comparing the plasmas of patients, to keep dietary conditions as constant as possible. Preliminary work with the plasmas of dogs, maintained on a selected diet, has shown little individual variation, so long as a proper control of the diet is maintained.

Chromatography of pure proteins and protein mixtures. A number of chromatograms has been made of preparations of purified proteins and protein fractions isolated from blood. These preparations have been kindly presented by the courtesy of the Cutter Laboratories and consist of the fractions of blood plasma isolated according to the procedures of Dr. Cohn and his colleagues(6). Electrophoretic analyses have shown that fraction III consists of a mixture of α, β, and γ globulins and that of fraction V is largely albumin. Chromatograms of fraction V are shown in Fig. 9 (without Tween) and Fig. 12 (with Tween) and those of fraction of III are shown in Fig. 10 (without Tween) and in Fig. 13 (with Tween). It will be noted that the chromatograms of fraction V indicate the presence of more than one protein, whilst those of fraction III show but little movement in the second dimension. The chromatograms of the separate fractions do not resemble that of blood plasma. When mixtures of the fractions are taken in such proportions and in such quantities as to constitute the proportions and amounts of albumin and globulin in blood plasma, chromatograms are obtained which resemble those of normal blood plasma.

6. Cohn, E. J., Strong, L. E., Hughes, W. L.,, Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

This is shown in Fig. 11 (without Tween) and Fig. 14 (with Tween). The evidence from a number of chromatograms of protein mixtures indicates that an interaction between proteins occurs, whereby the chromatogram of a mixture of proteins is not necessarily that to be expected from a simple addition of the chromatograms of the proteins taken separately.

This fact is also illustrated by the results of some preliminary experiments carried out with mixtures of proteins with hemoglobin and with cytochrome *C*. Chromatograms indicate that both these substances may form associations with other proteins, but further experiments are required to decide whether the conditions of the chromatographic technic may result in sufficient dissociation of the hemin from the hemoglobin or from the cytochrome to produce hemin complexes with the other proteins present.

Comparison of normal and hyper-immunized horse serum. Through the courtesy of the Department of Microbiology and Hygiene of the University of Montreal, specimens of normal horse sera and the sera of hyperimmunized horses (immunized against diphtheria toxin) have been obtained. Chromatograms of the two sera both with and without the addition of a surface active agent are shown in Fig. 15, 16, 17, and 18. For experiment, 0.01 ml "Tween 85" was added to a mixture of 0.02 ml 0.3% hemin solution and 0.5 ml serum. Chromatography was carried out on Whatman No. 1 filter paper using M/10 sucrose solution in the first dimension and M/10 sodium potassium tartrate solution in the second dimension. It will be seen that the chromatograms of the sera (both with and without the addition of the Tween) show differences from each other. That of the hyperimmunized serum indicates the existence of fractions not present in the normal serum.

Summary. A technic for the two dimensional chromatography of blood plasma and protein mixtures on filter paper is described. Separations of protein constituents are greatly facilitated by the addition of surface active substances such as the "Tweens" or "Spans," etc. The technic at present adopted is to add "Tween 85" or "Tween 81" and, using hemin as protein marker, to employ M/10 sucrose solution in the first dimension and M/10 sodium potassium tartrate solution in the second dimension. Differences between the protein patterns of chromatograms of human, rat and guinea pig plasma are noted. With protein mixtures, associations of proteins may occur that may be detected by chromatography. The sera of hyperimmunized horses (as against diphtheria toxin) yield chromatograms which differ from, and exhibit fractions not present in, chromatograms of normal horse sera.

Grateful acknowledgment is made to the National Cancer Institute of Canada for a grant in aid of this work, to the Department of Microbiology and Hygiene of the University of Montreal for gifts of normal and immune sera, to Dr. J. Gross for the protein fractions obtained from the Cutter Laboratories, Berkeley, Calif., to the Atlas Powder Co. (per G. F. Sterne & Son, Brantford, Ont.), for gifts of "Spans," "Tweens," "NNO," and "BRIJ-30," to the Canadian Industries, Ltd., Montreal, for gifts of "Elvanols," and to Mrs. Mary Hersey for her able assistance in this work.

Received July 5, 1950. P.S.E.B.M., 1950, v74.