Loyola University Chicago
Loyola eCommons

Dissertations   Theses and Dissertations

1955

Comparison of the Binding Mechanisms of the Plasma Proteins and Polyvinylpyrrolidone

Robert Harry Spitzer
Loyola University Chicago

LIFE

Robert Harry Spitzer was born in Chicago, Illinois, On July 25, 1929.

He was graduated from Steinmetz High School, Chicago, in June 1947. From September, 1947, to June, 1951, with the exception of the period September, 1949 to January, 1950, he attended Valparaiso University in Valparaiso, Indiana from which he graduatedin June, 1951, with the degree of Bachelor of Arts. During the period he did not spend at this University he enrolled at Wright Junior College, Chicago, and at Roosevelt College, Chicago, He began work for his Master of Science degree in the Graduate School of Loyola University in September, 1951, and obtained this degree in June 1953. M.S. Thesis, Biochemistry, 1953, Loyola University, Chicago; "The Ionographic Properties of Polyvinylpyrrolidone".

From September, 1951 to June, 1955, he served as a research assistant in the Department of Biochemistry.

He is co-author of the following publications:

"Polyvinylpyrrolidone: The Electromigration Characteristics of the Blood Plasma Expander", Circulation Research I, 396-404 (1953);

"Electromigration in Stabilized Electrolytes, Part II, Factors Influencing Mobility" Clin. Chem. 5, 35-40 (1953);

"Electromigration in Stabilized Electrolytes, Part III, Application of the Technique", Clin. Chem. 5, 51-59 (1953);

"Equilibrium Dialysis and Ionographic Studies of Protein and Polyvinylpyrrolidone Interactions with Bromphenol blue", Fed. Proc. 14, (1955), 285.

He is a collaborator of the book "Ionography: Electrophoresis in Stabilized Media", Year Book Publishers, Chicago, 1955.

ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Biological Importance of Protein Interaction Studies

The plasma proteins have numerous functions in the circulatory system. Perhaps, from a chemical standpoint, their most interesting action is associated with their role as transporting agents for steroids (Bischoff and Pilhorn, 1948; Boettiger, 1946), glycosides (Goldstein, 1949), urea (Bresler, 1949), mercuric ion (Hughes, 1950), zinc (Gurd and Goodman, 1952), iodide ion and chloride ion (Putman and Neurath, 1944), and many other physiologic and non-physiologic products.

A large number of studies have been initiated to elucidate the properties of these complexes and to relate the structural characteristics of both interacting species with the thermodynamic quantities of the binding process. It is probable that the results will yield pertinent data to aid in the development of a mechanism of enzymatic catalysis. Also, these investigations will contribute to the description of more complicated associations such as those between large molecules of the following types: antigen-antibody, insulin-insulin, protein-nucleic acids, and hemocyanin-hemocyanin.

In pharmacological investigations there is current interest in the ramifications of protein interaction studies, since an important consideration is the estimation of active drug concentration in the presence of serum proteins (Bennhold et al., 1938; Davis and Dubos, 1947; and Tempsett et al., 1947). Presumably, the biological activity of a drug is dependent, at least in many

1

cases, on uncomplexed drug molecules.

Evidently, as more biological processes begin to be viewed on a molecular level, the value of interaction studies will be extended.

Chemical and Mechanistic Aims

There are, however, more immediate chemical and mechanistic consequences to be gathered from the study of the protein complex. Klotz (1949) has enumerated the important purposes of interaction studies. These may be stated as follows:

(1). A determination of the number of interacting molecules that are held by a given protein molecule, under specified environmental conditions.

(2). A determination of the maximum number of sites on a protein molecule available to a given interacting species.

(3). A thermodynamic consideration of the strength of the bond between the protein and any given interacting species.

(4). A quantitative statement as to the effect of the environment on the energy of combination.

(5). Quantitative information on the structural characteristics of the interacting molecules which favor combination with the protein.

(6). A quantitative elucidation of the molecular and configurational nature of the site on the protein at which a given species is bound.

It should be emphasized that, in general, a thermodynamic treatment has been applied to the protein complex for the purpose of acquiring the aforementioned information. Recently, as a result of moving-boundary electrophoretic investigations, interest has evolved in relation to the rates of complex formation and degradation (Longsworth and MacInnes, 1942; Alberty and Marvin, 1950;

Smith and Briggs, 1950; and Yang and Foster, 1953). Though the amount of work possessing kinetic significance is, at present, meager, the results have an obvious importance in that they aid in constructing a mechanism of protein interactions.

Binding Properties of Polyvinylpyrrolidone

The problems surrounding and related to protein complexes have been under scrutiny for many years, but it has been only in recent times (1943-55) that the synthetic polymer polyvinylpyrrolidone (PVP) has created a good deal of interest with regard to its binding ability. PVP was first synthesized in Germany by Reppe (1938, 1939), in the late nineteen thirties, during the course of investigating techniques for safely handling acetylene under conditions of high temperature and high pressure. It was produced commercially in time to be used by the Germans at the battle front in World War II as a blood plasma expander.

It soon became evident that the polymer exhibited a number of important properties aside from its use in shock and related therapy. Up to 1951 about one-third of the literature references relating to PVP reported on studies relative to its efficacy in extending the effective period of drug action (General Aniline and Film Corp. 1951). An early paper described a retardent action on pantocaine when used in peridural anesthesia (Düttmann, 1941). When a $2\frac{1}{2}$ per-cent pantocaine solution was "fortified" with PVP, a longer anesthetic action was observed. Since that time, a vast array of experimental work has been presented to illustrate an action on anesthetics, hypnotics, antibiotics, anticoagulants, antihistamines, diuretics, hormones, and other compounds. These have been summarized in Table I.. The pharmacological or physiological

TABLE I
LITERATURE SUMMARY OF INTERACTIONS BETWEEN PVP
AND DRUGS

Drug Affected	Literature Reference	Comments
Procaine Hydrochloride	Bourdreaux and Bourdine (1945)	anesthetic affect prolonged, drug associated with 20% PVP solution
	Choffel and Amoudrux (1947) Starlinger (1945)	
Hexobarbital Soluble	Quevauviller (1947a)	hypertonic PVP solution, duration of anesthesia was doubled.
Terracaine Hydrochloride	Goepel (1947)	in peridural anesthesia, best results with 5% drug-6% PVP to which adrenalin was added
	Mennenga (1949)	
Dibucaine Hydrochloride	Leger (1947)	action attributed to prevention of drug diffusion
Tetraethylammonium bromide	Lian and Bergamo (1948)	i.m. injection associated with 25% PVP
Opiates	Siguier et al. (1948)	prolongs analgesic action of opium and morphine.
p-Aminosalicyclic acid	Brun et al. (1950)	drug blood levels maintained for longer time
ACTH, pituitary extracts	Murat (1949)	considers the retardent mechanism to involve two factors: physical effect in drug diffusion and intervention in drug metabolism

3A

TABLE I (cont.)

Drug Affected	Literature reference	Comments
Insulin	Levrat et al. (1945a) (1945b)	prolongs hypoglycemic effect, insulin-PVP effects better than those of insulin-portamine-zinc, considers that PVP delays absorption of drug (s.c. injection) and renders physicochemical protective action or a neutralizing effect on the proteolytic enzymes that destroy insulin.
	Loubatieres (1946)	postulates PVP-insulin complex from which insulin is slowly liberated, also restricted diffusion of drug due to absorption of fluid at point of injection by PVP.
Sulfonamides (dimethylbenzolysolfanilamide, sulfaethiodiazole, Sulfa-2-4-dimethylpyrimidine)	Schubert and Greser (1950)	urinary excretion studies sulfonamides shown to be bound by PVP (electrophoresis), no accelerating effect on excretion.
Quinine	Quevauviller (1947b)	retards excretion of Quinine, 20% PVP
Thrombin	Lian et al. (1947)	coagulation time of a hemophiliac was reduced by i.v. injections of thrombin-PVP solutions more than thrombin alone
Neptal	Perrault and Rousseau (1947)	patients who did not respond to neptal injections alone were affected by PVP-Neptal preparations
Heparin	Gumy and Quivy (1948)	negative results, no effect on the rate of elimination of heparin injected intravenously in the dog.

3B

Table I (cont.)

Drug Affected	Literature reference	Comments
Estrogenic hormone (Follioulin)	Lavedan (1948)	estrus initiated by 5 units follioulin remained only half as long as when 30% PVP was injected with Hormone
Penicillin	Cosar (1945)	drug effectivness increased as indicated by the average life of animals and percentage survival of staphylococci or pneumococci infected mice.

action appears to be associated with the formation of a drug-PVP complex which maintains drug levels by limiting or minimizing excretion, diffusion, or metabolism of the drug (see "comments" Table I.)

Polyvinylpyrrolidone has also been demonstrated to combine with various dyes. A summary of these experiments has been assembled in Table II. Compounds other than dyes which happened to be studied during the course of these investigations are also included.

Contemporary Status of the Binding Mechanism of PVP

Most of the descriptions of the binding characteristics of PVP are of a strictly qualitative nature, and at most, indicate that a complex has been formed. In semiquantitative work, Franke (1951) used a modified equilibrium dialysis procedure to determine the overall equilibrium constant and the enthalpy of the reaction between PVP and eosin.

The work of Oster (1952) and Oster and Immergut (1954) is significant in any assay of the contemporary status of the polymer's binding mechanism. They found PVP to bind molecular iodine. Interaction was demonstrated to occur by observing an increase in the absorption spectra of iodine in potassium iodide at 290 mu. as PVP was added. A Langmuir isotherm was constructed from the data, and the equilibrium constant for the complex was calculated to be 1.32. However, it was notable that if iodine was added to a PVP solutions, a different binding curve was found. Speculation on the mechanism was attempted. The latter curve indicated that the initial iodine molecules are bound to PVP with difficulty, but once some iodine molecules are bound to the polymer the succeeding iodine molecules are bound with greater ease. They suggest that mutual polarizability of the molecules aids in the binding of the succeeding

TABLE II
LITERATURE SUMMARY OF INTERACTIONS BETWEEN PVP
AND DYES

Compound Bound to PVP	Literature Reference	Comments
Congo red, Eosin, Prontosil Methylene blue, Methyl violet, Bilirubin, Atabrine	Bennhold and Schubert (1943)	Electrophoresis and Diffusion Studies
Bilirubin, Indigo, Carmine	Schubert (1945)	In vivo excretion experiments
Azorubin, Bromphenol blue, Phenol red, Indigo Carmine, Diamine red 3B	Bennhold et al. (1950)	electrophoresis and in vivo excretion studies
Diamine Red 3B, Trypan Red M	Schubert (1950o)	in vivo excretion studies
Trypan Red Stand	Schubert (1950b)	in vivo excretion studies
veronal, Diamine Red 3B, Diamine Pure Blue, Trypan Red	Schubert (1950a)	in vivo excretion studies, electrophoresis
Diamine Pure Blue FF	Schubert and Werner (1950)	in viter excretion studies
Neutral Red	Masoré (1950)	10 to 20% PVP solution prevents or retards the diffusion of dilute solutions of dye into the leaves of Elodea canadensis
Caffeine, antipyrine	Masoré (1950)	20% PVP hinders penetration of these compounds into spirogyra. Evidence is also presented that PVP combines with water and renders it unavailable for plants.

Table II (cont.)

Compound Bound to PVP	Literature Reference	Comments
Rosin	Franke (1951)	Equilibrium dialysis $\Delta H = 3200$ cal/mol (from $\frac{d\ln K}{dT} = \frac{\Delta H}{RT^2}$), binding decreased as temperature increased
Iodine, Rose Bengal, Benzo Purpurine, Congo Red	Oster (1952)	Spectrophotometry
Iodine	Oster (1954)	Spectrophotometry
Evan's Blue	Wunderly (1950)	compared elution of stained membranes by albumin and PVP
Congo Red	Meidinger (1947)	in vitro, protective colloid studies; in vivo, toxicity
Brilliant Vital Red	Chinard (1952)	a colorimetric method for the estimation of PVP in whole blood, plasma, and urine

molecules. It was possible to experimentally reach a point at which the binding sites (one per monomer unit) were saturated.

Prior to the work of Oster, the reaction of iodine was extensively used as an analytical procedure for the detection of PVP (Ammon and Braunschmidt, 1949; Korth and Heinlein, 1943; Lespagnol, 1948; Murat, 1949; and Poullain and Piette, 1948). None of these workers made an attempt to unravel the underlying nature of the reaction.

Doebbler (1953) questioned the accuracy of this method. Rather, he found it convenient and accurate to determine PVP during paperchromatographic studies with a bismuth iodide reagent or a potassium iodoplatinate reagent. No mention was made regarding the site or sites on PVP that are responsible for these color tests.

In summary, it has become evident that the polymer, PVP, has many properties of biological importance that warrant its use beyond that of a blood plasma expander. It may be anticipated that its value will be extended in the near future due to an intense current interest in these properties. These considerations were prime instigators in the development of the investigation that constitutes the bulk of this dissertation.

Theoretical Treatment of Systems Exhibiting Multiple Equilibria

The earliest experiments that were devised in order to investigate protein complexes, indicated that more than one interacting species could be bound on a given protein molecule. As a result of this, mathematical treatments involving multiple equilibria were developed to treat the resultant data. The development of pertinent equations was hastened by previous and contemporary workers whose interest was not necessarily directed to protein interactions, but

to adsorption and electrostatic processes. The theoretical treatment of systems exhibiting multiple equilibria is much too complex to be considered here in its entirety, or even to be adequately summarized. Klotz (1953) has reviewed the treatments in a most concise form and has fortified the review with a thorough bibliography.

Ana analysis of the general mathematical formulations for the calculations of association constants of complex ion systems has been considered by Sullivan and Hindman (1952). The calculation of the successive constants of the uranium (IV) sulfate complex system was attained by three methods, namely, that of Leden, of Bjerrum, and of Freneaus.

Steiner (1954) has presented a theoretical study of protein associations when investigated by light scattering, osmotic pressure, diffusion, electrophoresis, and sedimentation studies. With the aid of the equations therein developed it was possible, by means of light scattering and osmotic pressure, to obtain the consecutive association constants. If these are known it is possible to obtain the diffusion coefficients, electrophoretic mobilities, and sedimentation constants of the consecutive polymer species.

Certain aspects of these theoretical treatments will be included in later chapters of this dissertation.

Methods for Studying Interactions.

Summary of Procedures

Klotz (1953) has enumerated the many methods which are available for investigating protein interactions. He has found it convenient to divide the procedures into two groups, (1) processes exemplified by changes in the interacting molecules and (2), changes in the behavior of the protein. In the former

category was included the following schemes: solubility, reduction in thermodynamic activity (equilibrium dialysis, ultrafiltration, ultracentrifugation, distribution between phases, electromotive force measurements), migration in an electric field, polarographic reduction, diffusion, changes in spectra, and biological activity. Methods which are dependent upon changes in the properties of proteins include: changes in spectra (titration curves, shifts in isoionic pH), optical measurements (spectrophotometry, refractometry, light scattering, optical rotation), osmotic pressure, sedimentation, electrophoresis, precipitation, viscosity, surface tension, magnetic properties, and biological activity.

During the course of proceeding to make a functional literature survey to aid in the investigation of protein and PVP binding, it became apparent that much more pertinent data could be gathered if more than one method of studying binding could be initiated. This was in line with the thinking of Yang and Foster (1955) who supplemented a proposed binding mechanism for alkylbenzenesulfonate and albumins by including equilibrium dialysis and moving boundary data.

After some deliberation, it was decided to proceed with the protein and PVP investigations in a somewhat similar fashion. It seemed that the studies would lend themselves conveniently to investigation with both the equilibrium dialysis and ionographic procedures. Therefore, only these two procedures will be considered in detail in this chapter. Since ionography shares in many of the principles of classical electrophoresis, the latter technique will also be considered.

a.) Equilibrium Dialysis --

As Klotz has pointed out, this technique is dependent on a reduction in the thermodynamic activity of the interacting substance. The apparatus, in its simplest form, consists of two compartments separated by a membrane. This dialysis membrane is prepared so as to allow free penetration of small interacting molecules only.

Initially, a colloidal solution is placed in one compartment and the solution of small molecules in the other. At equilibrium, the total number of small molecules per unit volume in the colloid compartment will be greater than the total number of small molecules per unit volume in the original small molecule compartment. This is a result of an interaction occurring between colloid and small molecules. The binding data is assembled into appropriate graphic representations and the equations of multiple equilibria are applied to calculate the equilibrium constants and thermodynamic data. There are a number of errors associated with the procedure, some of which are so serious as to eliminate the method in certain studies. Adsorption of the small molecules on the membrane renders a percentage of these molecules unable to enter in the equilibrium system. Under certain conditions, if the quantity is small, appropriate correction may be applied to eliminate the error. The Donnan effect may also interfrere and cause the binding data to be in error. Again, corrections may be applied, or else the error minimised by the utilization of low colloid concentrations at a pH near the isoelectric point of that specific colloid.

b.) Electrophoresis -- Free Solution -- Moving Boundary Technique

The proteins, being composed of amino acids and in some instances a

small percentage of phosphates or other ionizable groups other than amino acid and groups, exist in aqueous solutions as electrically charged entities. The specific amino acids in their groups responsible for ionization include: arginine, the guanidinium group; aspartic acid, the beta carboxyl group; glutamic acid, the gamma carboxyl group; histidine, the imidazole group; lysine, the epsilon-amino group; tyrosine, the phenolic group; and iodinated tyrosine derivatives, also the phenolic groups. The magnitude of the electric charge on the protein is related to the number of these ionizable groups and to the environment of the protein. The degree of ionization is dependent on the hydrogen ion concentration, the ionic strength of the medium, the temperature, and on the presence of non-ionic organic compounds. Besides affecting the ionization of the aforementioned groups, the buffer ions of the solution are capable of binding with the protein; for example, the isoelectric point of a given protein is dependent upon the buffer system employed and also upon the buffer concentration. This is attributable to the binding of buffer ions with the protein.

The electrophoretic mobility of a given protein is a function of the net electric charge and hence of the number of ionogenic groups, molecular weight, shape, and the environmental factors. By the intelligent control of the variables, primarily pH, a mixture of proteins usually may be resolved with any of the electrophoretic methods. An invaluable treatment of the electrochemical properties of the proteins is given by Alberty (1953). Included in his discussion are the ionization of amino acids, peptides, and proteins; moving-boundary apparatus; electrophoretic mobility; isoelectric points; electrphoresis in packed columns; and electrophoresis convection.

The quantitative study of protein-ion interactions by means of electromigration in free solution involves the use of complicated equations. Qualitatively, interactions may be demonstrated by a change in protein isoelectric point accompanying a change in buffer type or a change in buffer concentration. Likewise, certain asymmetries in the ascending and descending boundary patterns may be indicative of interaction phenomena.

Possibly, before the concept of the protein complex is applied to electrophoresis studies, it is appropriate to define a term that is used continually in related mathematical derivations. It is known that a given protein may exist in a sequence of molecular forms in solution. The electrophoretic mobility u_x of protein x is a reflection of the various protein forms in this solution. In essence, at a given pH, the protein may be said to migrate with a constituent mobility $\bar{U}_x$ in a given ionic environment. This term is only used when the different protein forms are in rapid equilibrium with each other, that is, the equilibrium is established rapidly as compared to the rate of electrophoretic separation. It may be defined as follows:

$$\bar{u}_x = a_1 u_{x_1} + a_2 u_{x_2} + \cdots a_n u_{x_n}$$

$\bar{u}_x$, the constituent mobility of protein

a_1, the fraction of molecules of x in form 1, having mobility u_{x_1}

a_2, the fraction of molecules of x in form 2, having mobility u_{x_2}

a_n, the fraction of molecules of x in form n, having mobility u_{x_n}

The term "constituent mobility" will be used extensively in later chapters of this dissertation.

Longsworth and MacInnes (1942) appear to have initiated the necessary impetus to demonstrate the potentialities of electrophoresis in the study of

interactions. In the course of their investigation of the reaction between ovalbumin and yeast nucleic acid, consideration was given to the probable nature of the interaction. If the constituents combine reversibly to form a complex, i.e.:

$$P + \nu N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} PN_\nu$$

where P is a polymer with more than one site capable of interacting with ν molecules of N, at least five cases might be distinguished on an electrophoretic analysis of the mixture.

(1). If k_1 is small and k_2 large, K (i.e. $\frac{k_2}{k_1}$) will be also large. Under these conditions the complex is essentially completely dissociated and the mixture will behave as a normal mixture of P and N.

(2). If k_1 is large and k_2 small, then K will be small. This system will behave like a mixture of the complex and either P or N, depending upon which is in excess.

(3). If k_1 and k_2 are both small and of similar magnitudes then K will be near unity. Finite concentrations of P, N, and PN_ν will exist at equilibrium. The mixture will behave like a normal mixture of the three components since the adjustment of the equilibrium is slow in comparison to the rate of electrophoretic separation.

(4). If the rate of adjustment of the equilibrium is comparable with the rate of electrophoretic separation the pattern will depend upon the rate of separation of the constituents.

(5). If k_1 and k_2 are both large and of the same order of magnitude, the equilibrium is adjusted as rapidly as required by the electrophoretic separation of the components. It was believed that the ovalbumin-nucleic acid

system was an example in this case.

Subsequent to this latter study, it has become evident that a quantitative treatment may be applied to systems possessing characteristics of case(5). Smith and Briggs (1950) studied the interaction between bovine serum albumin and methyl orange in solution at pH 5.5 in acetate buffer of 0.05 ionic strength. Methods were devised to obtain the interaction constants from the boundary patterns after electrophoresis of the solution. The results agreed satisfactorily with those obtained from equilibrium dialysis studies on the same system. Alberty and Marvin (1950) have applied similar concepts to the bovine serum albuminchloride ion system. The theory of moving-boundary systems for weak electrolytes is the basis for the mathematical treatment. (Svensson, 1948; Alberty and Nichol, 1948; Alberty, 1950; and Nichol, 1950).

Wang and Foster (1953) have utilized both equilibrium dialysis and electrophoretic methods in a study of binding between bovine plasma albumin and sodium dodecylbenzenesulfonate. Three regions could be distinguished in the binding curve. In the first region, the binding is statistical with a limit of about twelve ions per protein molecule. In region 2 the reaction is all-or-none giving rise to a complex containing about 48 ions. In region 3 the binding was found again to be statistical with no upper limit being attained under the conditions employed.

   c.) Ionograph - Comparison with Chromatography and Free Solution
       Electrophoresis

A large fraction of the papers concerned with the technique of electromigration in stabilized electrolytes involves the use of proteins, protein derivatives, and protein hydrolytic products as the migrating species. Included in those studies will be found procedures for the fractionation,

identification, and analysis of normal and pathological blood serum proteins, normal and abnormal hemoglobins, urinary proteins, cerebrospinal fluid proteins, iodinated proteins, gastric juice proteins, serum proteins of various animals, aqueous humor proteins, proteins of beef and human lens, muscle proteins, antibodies, antigens, hemolysins, agglutinins, milk proteins, enzymes, protein hormones, peptides, and amino acids. This is in contrast to the literature of chromatography wherein it is evident that only limited success has attended protein investigations. Impediments associated with the chromatographic process include slow resolution, incomplete resolution, difficult elution of proteins from columns, and variable pH and salt effects that complicate the resolution. A method more applicable than chromatography for the separation and quantitative estimation of the proteins is free solution electrophoresis. That this technique has proven to be extremely valuable is evident from a persual of any contemporary scientific journal. There are a number of factors, however, that limit a greater use of the instrument. For example, the apparatus is not inexpensive, relatively large quantities of proteins are required, time-consuming manipulations are associated with the preparation of the electrophoresis cell, and the analytical manipulations are not simple. These considerations have definitely limited the clinical use of the instrument. It is appropriate, here, to stress a point of optimism with regard to the ionographic method; for some of the difficulties and impracticalities of the chromatographic and free solution methods are eliminated, and protein resolution is accomplished, in most cases with ease and simplicity. It is also pertinent to mention that a number of protein studies employing the stabilized electrolyte method have resulted in conclusions very similar to those

elaborated from investigations involving classical electrophoresis procedures. This is especially evident in view of the contents of the book by Abramson, Moyer, and Gorin (1942), and the electrophoresis bibliography assembled by Henley and Schuettler (1953). However, these efforts are not in vain since they serve to place ionography on a firm basis and initiate its use as an analytical and research tool. An evaulation to determine the order of error and reproducibility of the ionographic method with the ultimate aim of comparing the data with that derived from free solution techniques has been undertaken by various workers. However, different analytical schemes were employed and it becomes difficult to make a rigorous comparison of the two methods.

Levin and Oberholser (1952) (1953) have compared the paper electrophoretic technique with the moving boundary method of Tiselius and with salt fractionation methods. The protein fractions on the paper were localized with the bromphenol blue reagent (1% tetrabromo-phenolsulfonphthalein in 95% ethyl alcohol saturated with mercuric chloride). The protein fractions, the boundaries of which were determined by the midline of the troughs between the peaks, were cut out and a micro-kjeldahl analysis was made of each section. After a reagent and paper blank are obtained, the protein quantity is determined. The results, using various types of sera, indicated a good agreement with those obtained by free solution electrophoresis and by salt fractionation. With paper electrophoretic analyses of 17 sera, the reproducibility of duplicate determinations was such that a mean percentage difference between the duplicates was, for albumin, 0.65 per cent, with a range of 0.0-2.2 per cent; for alpha-1-globulin, it was 0.46 per cent, with a range of 0.0-1.5 per cent; for alpha-2-globulin, it was 0.8 per cent, with a range of 0.1-2.0 per cent; for beta-globulin, it was 0.65 per cent, with a range of 0.0-1.7 per cent; and

for gamma globulin, it was 0.43 per cent with a range of 0.0-1.5 per cent.
(Expressed as percentages of total protein).

Köiw and coworkers (1951) (1952) have performed similar studies.
After staining with bromphenol blue, the filter paper was segmented into 5 mm.
sections and the dye was eluted from each strip with a 5 per cent solution of
sodium carbonate in 50 per cent methanol. The solutions were analyzed at a
wavelength of 595 mu and a plot was obtained of protein quantity as a function
of migration distance. There was general agreement between the Tiselius method
and that of paper electrophoresis. A qualitative agreement was also observed
between the electrophoretic patterns and ionograms of abnormal sera, thus en-
abling the latter procedure to be used for clinical and diagnostic purposes.
The investigations of Barbagallo-Sangiorgi (1952) and of Riva and Martini (1952)
are in general accord with that of the above workers, but the latter group
considers that great divergencies from normal patterns are necessary before
abnormalities can be demonstrated on the ionograms.

Previous Ionographic Interaction Studies

Electromigration in stabilized electrolytes has not yet yielded
binding data of the caliber obtained by other methods. Qualitative evidence
of protein interactions has been shown but a number of factors have thus far
precluded the obtaining of quantitative data. Foremost among these factors is
the presence of the stabilizer which might be thought of as inhibiting the bind-
ing between the protein and the interacting species. With filter paper, the
cellulose and other components may combine with some of the sites on the pro-
tein and decrease the number of sites available to combine with the interacting
species. The inexact methods by which the protein migrant is at present added

to the stabilizer is an additional cause for concern, for the protein concentration is difficult to evaluate.

Since the migrant generally is added to a previously moistened stabilizer by means of a micropipette, the volume through which the protein penetrates on a mechanical basis will be dependent on the evenness with which the migrant solution had been applied. Although the protein quantity is known, the concentration will vary as a result of this dilution effect. The calculation of equilibrium constants, and subsequently the related thermodynamic quantities, is dependent on the knowledge of the various component concentrations and, hence, it is evident that a more exact method of migrant application must be instituted. With the sandwich technique, for example, as with other ionographic procedures, the mode of migrant addition is not at present satisfactory for interaction studies. The paper is dipped into the buffer and then placed on another sheet of filter paper to remove most of the liquid. The moist paper is placed on one glass plate and the protein (bromphenol blue added) is applied. A second glass plate is placed over the paper and an even pressure is applied. The ends of the filter paper are put in the electrode vessels and a quantity of buffer moves into the filter paper and the system becomes equilibrated. The protein is located by means of the blue spot. Thus, prior to electromigration, the protein concentration is difficult to evaluate due to the unknown dilution and chromatographic effects.

Procedures that allow evaporation of water from the buffer in the stabilizer may, under a given set of experimental conditions, result in a continually changing medium and therefore also complicate interaction studies.

Spitzer and McDonald have been able to obtain qualitative evidence of

interaction using filter paper as the stabilizer (1955). Equilibrium dialysis experiments were carried out using bovine serum albumin (BSA) and bromphenol blue (BPB) in both a phosphate buffer (0.05M pH 8.6, 4°C) and a veronal buffer (0.05M pH 8.6, 4°C). Similar experiments were conducted using polyvinylpyrrolidone (PVP) and bromphenol blue. The results of this work were used to calibrate the ionographic method. Two ionographic procedures were used to study these systems. The first method used 2-15 lamda migrants of either the BSA-BPB or PVP-BPB systems. After the passage of current for a period of time long enough to separate some of the faster moving BPB but short enough to retain some of the equilibrium mixture, the strips were dried and subjected to a direct spectrophotometric analysis (585 mu). An asymmetric pattern was found representing separated bromphenol blue and the bromphenol blue in BSA-BPB or PVP-BPB equilibrium mixtures. Two blue zones of slightly different hue could be distinguished visually on the filter paper. It was interesting to note that on prolonged passage of the current, the BPB could be completely separated from the albumin. This is of interest since many of the workers have used this compound in a mistaken attempt to "label" the albumin of serum and to follow its migration during the course of the experiment. The second method consisted in introducing BPB into the buffer solution and then adding BSA or PVP as migrant. The mobility of both migrants have been previously determined in the same buffer solution with the exception that the dye had been omitted. The compounds, in the presence of the dye, possessed mobilities characteristic of the complexes. The PVP had its direction of movement reversed when the dye was introduced into the buffer solutions, thus moving to the anode. These results will be considered in greater detail in this dissertation.

Roth and Kallee have reported similar interaction experiments involving a "continuous desorption" process (1953) (1953). Amido black 10B, azorubin S, or naphthol yellow were added to the buffer solution and the mixture was used to moisten the filter paper strips. Albumin was placed on the strips and allowed to migrate. A white zone on the strip was found in front of or behind the albumin zone, depending on the mobility of the dye. The interpretation of these occurrences was suggested as being dependent on the formation of albumin-dye complexes.

Statement of the Problem

The possible biological significance and immediate chemical aims of protein binding studies have been previously considered. PVP and its intriguing properties have also been discussed. The pharmacological importance of PVP's properties have been reviewed in detail. In view of the similarities between some of the proteins and PVP in their ability to bind various physiological and non-physiologic compounds, an investigation was instituted to compare their binding mechanisms. Methods employed to accomplish this, and the results subsequently derived, constitute the remaining portion of this dissertation.

CHAPTER II

EXPERIMENTAL: MATERIALS, APPARATUS, AND PROCEDURE

A chronological discussion of the events comprising a given investigation has sometimes a tendency to appear illogical when formulated into a written description. This is mainly due to the investigator's lack of foresight and to the critic's overabundance of hindsight. The experimental description that follows in this chapter makes no pretense to any exceptional thread of continuity. The reasons for choosing an equilibrium dialysis and an ionographic method to facilitate an elucidation of the binding mechanisms of the proteins and PVP have been previously summarized in Chapter I. In subsequent chapters this choice of study will have been at least partially justified. A description of the materials, apparatus, and procedures that were utilized in these binding studies is included here to centralize such related information and to minimize extensive repetition during the course of the dissertation.

MATERIALS

Polyvinylpyrrolidone - Three samples of PVP powder were obtained from the General Aniline and Film Corporation. Actually, one of the samples was received from the Abbott Laboratories, who had previously obtained the material from this corporation.

The manufacturer has found it convenient to describe these preparations in terms of their viscometric properties, i.e., K-30, K-60, and K-90; where K has been related to relative viscosity in aqueous solutions, and to the concentration of the polymer in the following equation:

$$\frac{\log (\eta_{rel})}{C} = \frac{75K^2}{1 + 1.5 (K)(C)} + K$$

(η_{rel}) is the relative viscosity, that is, the ratio of the solution viscosity to the viscosity of the pure solvent.

(C) is the concentration of the PVP in grams per 100 cc. of solution.

(k) is termed a viscosity coefficient. It is usually reported in the literature as 1000 times the calculated value to avoid the use of decimals.

The temperature at which the viscosity measurements are made is usually 25°C. It should be emphasized that the equation has been derived to describe dilute soltuions. The relation between K and molecular weight is not a simple one. Suffice it to state at this point that the molecular weight increases as the K-value increases. This relationship will be considered in finer detail in subsequent chapters.

The manufacture's specifications of the three samples are found in Table III.

TABLE III

Description of PVP Samples — Witwer (1954)

K-value	K-30 (K-4605)	K-60 (Type NP)	K-90 (Type NP)
Approximate Molecular Weight	50,000	150,000	350,000
Moisture Content	<5%	6.1 (Karl Fischer)	6.5 (Karl Fischer)
Unsaturation (Calculated as Monomer)	<1%	0.7%	0.8%
Ash–	<0.02%	0.03%	0.04%
Heavy Metals	< 20 PPM	< 20PPM	<20 PPM
Arsenic	< 2 PPM	Neg. (Gutzeit)	Neg. (Gutzeit)
Nitrogen	12.6 ± 0.4%	12.3%	12.4%

To prepare the various PVP solutions during the course of titrations and interactions studies, standard PVP stock solutions were formulated and appropriate aliquots were diluted to the desired concentration. This was necessary since the powder was extremely hygroscopic and definite quantities were difficult to weigh. Hence it was also imperative that micro-Kjeldahl nitrogen analyses be performed on the standard stock solutions to enable the aforementioned aliquots to be conveniently and correctly utilisable.

Prior to the development of this necessary procedure for the compounding of PVP solutions, attempts were made to calculate the water content of the powder samples by oven-drying at 105°C. It was interesting to note that there was at first a decrease in weight, followed by a weight increase with a concomitant change in color from white to yellow.

Certain parts of the investigations utilised both undialysed material and solutions which had been exhaustively dialysed against distilled water (4°C) for approximately one and one-half weeks. The water was frequently changed during that period to insure the obtaining of a final characteristic spectrum of PVP molecules. This procedure was performed prior to some (but not all) titration and equilibrium dialysis experiments. As a result of this, a number of abbreviations and symbols have been instituted to denote the specific preparations utilised in a given experiment. These conventions will aid in constructing a lucid discussion surrounding the investigation and will be maintained henceforth in this dissertation.

PVP, general abbreviation to represent polyvinylpyrrolidone in each and every preparation. The statements surrounding the symbol will refer to every preparation and will be a characteristic of the polymer in general.

PVP-U30, PVP-U60, PVP-U90, undialyzed polyvinylpyrrolidone samples in

specified aqueous solutions, containing polymers characteristic of

K-30, K-60, and K-90 samples, respectively.

PVP-D30, PVP-D60, PVP-D90, exhaustively dialyzed (vs. distilled water)

polyvinylpyrrolidone samples in specified aqueous solutions, con-

taining polymers originally characteristic of K-30, K-60, and K-90

samples, respectively.

Bovine Plasma Gamma Globulin -- A bovine gamma globulin sample repre-

senting fraction II from bovine plasma (Lot BP. 201-204) was obtained from

Armour and Company, Chicago. No attempt was made to further purify this ma-

terial prior to its utilization in equilibrium dialysis experiments. Electro-

phobetic data obtained from Armour indicated better than 98% purity of the

material. In a veronal buffer at pH 8.6, ionic strength 0.1, the gamma glo-

bulin had a mobility of $-1.78 \times 10^{-5} \frac{cm^2}{volt,sec.}$ A small amount of a faster

moving component ($-3.22 \times 10^{-5} \frac{cm^2}{volt,sec.}$) was also present.

BPGG will be used to represent this protein in subsequent discussions.

Binding data and solutions were assembled on a weight basis rather than on a

molar basis. Moisture content determinations have been assembled in Table IV.

TABLE IV

DETERMINATION OF BPGG MOISTURE CONTENT

Determination	Weight	Weight Tare	Final Weight Tare and Gamma Glob. (Dry)	Weight Water Lost	Weight Wet Gamma Globulin	%water
I	18.48902	18.40374	18.48170	0.00732	0.08533	8.56
II	17.03980	16.94896	17.03238	0.00742	0.09084	8.17

A water content of 8.37% was used in later calculations involving gamma globulin.

Crystallized Bovine Plasma Albumin -- Two different Armour albumin samples were used: List 2266, Lot N66706; and List 2267, Lot M66909. BPA will be used to represent this protein in subsequent discussion. The molecular weight of albumin has been given various values in similar studies i.e. 70,000, Klotz (1946); 69,000, Yang and Foster, (1953); and 67,000, Smith and Briggs (1950), An intermediate value of 69,000 has been used in this investigation. Armour has given the following electrophoretic data to describe both albumin samples: 97% albumin (u = -6.86 x 10^{-5} $\frac{cm^2}{volt,sec.}$), 3% alpha globulin (u = -5.57 x 10^5 $\frac{cm^2}{volt,sec.}$). All data was derived from a veronal buffer of pH 8.6, and ionic strength 0.1.

The following data (Table V) was used to calculate the water content of the two powders. (Oven drying 110°C. 2 days; Sartorius Balance, I - II; Grammatic Balance, III - IV).

TABLE V

DETERMINATION OF BPA MOISTURE CONTENT

Determination	Wt.Tare and Albumin	Wt. Tare	Final Wt. Tare and Albumin (Dry)	Wt. Water Lost	Wt.Wet Albumin	% Water
I. List 2267	18.60264	18.38069	18.59240	0.01024	0.22195	4.61
II. List 2267	18.67289	18.38901	18.65944	0.01345	0.28388	4.73
III. List 2766	19.6298	18.5057	18.6245	0.0053	0.1241	4.27
IV. List 2766	19.2808	19.1473	19.2750	0.0058	0.1335	4.34

The average water content of List 2267 was, therefore, 4.67%, and of List 2766 was 4.31%. There is, of course, a valid criticism of any protein

water content data, for it is known that while a protein will come to a constant weight at a given temperature, an increase in temperature will result in the loss of more moisture until the sample again comes to constant weight (Haurowitz, 1951).

Buffers - All components of the veronal and phosphate buffer were reagent grade chemicals. It should be stated, also, that while the buffers are reported in terms of ionic strengths which assumes explicitly that molal quantities were used, all solutions were prepared on a molar basis.

Interacting Species

A.) Methyl Orange - Since only exploratory experiments were devised to utilize methyl orange, p-(p-dimethyl-aminophenylazo) benzenesulfonic acid, sodium salt, no attempts were made to determine or improve the purity of this compound. However, it was reported in the literature that the only impurity to be expected in the dye sample would be a small amount of sodium chloride as a result of the "salting out" procedure accompanying the precipitation of the dye (Klotz, 1946). The symbol MO will be used to designate this dye in later discussion.

B.) Bromphenol Blue - This dye (tetrabromophenolsulfonphthalein) was employed in most of the equilibrium dialysis and ionographic experiments. The dye was twice recrystallized from glass-distilled water. Such a preparation appeared homogenous by both chromatography (aqueous solvents, various pH's and buffer solutions, visible and ultraviolet "Mineralight" models SL2537 and SL3660, light sources). Water content determinations subsequent to recrystallizations indicated that less than 0.3% water was present in the samples. Since the crystals were not appreciably hygroscopic and were resistant to de-

composition associated with oven drying at 110°C. for two days (identical spectrophotometric patterns and same ionographic mobility), the anhydrous crystals were weighed directly to prepare the necessary solutions. To eliminate the possibility that the manufacturer had distributed the sodium salt rather than the free acid form of bromphenol blue, a saturated aqueous dye solution was prepared and subjected to a flame photometric analysis. A negligible quantity of sodium was found. BPB will be used to represent this dye during subsequent discussions.

APPARATUS AND PROCEDURE

Titrations - The PVP solutions were titrated in a water-jacketed Pyrex vessel. All experiments were performed at a constant temperature of 25°C. by means of water circulation from a thermostat. A Beckman model G pH-meter with a standard No. 1190-80 glass electrode was employed for all potentiometric measurements. The action of the meter was standardized and checked at appropriate intervals against freshly prepared 0.05 molal potassium acid phthalate solution to give a pH of 4.01 at 25°C.

In all experiments 50 ml. of PVP solutions (58 mg % N, 0.05 M with respect to NaCl) were added to the titration vessel. Quantities of approximately 0.01 N HCl, but of known normality (also 0.05 M with respect to NaCl), were then added to bring the pH to about 3 to 3.6. Helium was then bubbled through this resultant solution for twenty to thirty minutes prior to titration with a 0.01 N NaOH (0.05 M with respect to NaCl).

A 5 ml. buret graduated into 0.01 ml. divisions was used for both the addition of the acid and titrations with the base. Base was usually added at 2 minute intervals. It had been previously determined that the time inter-

val was not important, insofar as a minimum time was needed to perform the associated mechanical manipulations and to obtain a stable pH reading.

Equilibrium Dialysis Studies - Equilibrium dialysis experiments were devised to yield quantitative evidence of colloid-small molecule interaction. The results were found to aid in the development of two ionographic procedures to demonstrate and study binding phenomena. The basic constituents of a dialysis cell are easily acquired and assembled to form a functional unit.

Pyrex culture tubes, 8" x $\frac{3}{4}$", are rendered immaculately clean with conventional chromic acid-sulfuric acid cleaning solution followed by free use of distilled water to remove all traces of this reagent. Twenty milliliters of a buffered solution of small-molecule interaction species (BPB, MO, etc., as the case may be), at known concentration were pipeted into the tubes. Various initial dye concentrations were used to insure the production of enough data to construct the classical interaction graphs.

The breadth of the concentration spectrum was limited at the highest level to about 0.01 M BPB by virtue of the dye's solubility characteristics in aqueous buffer solutions. A dialysis bag was then assembled to contain 20 ml. of PVP, HPGG, or HPA solutions. These colloids existed in identical buffer solutions as those in which the dyes resided. The bag was fastened at each end with glass thread and suspended in the dye solution.

In all cases an attempt was made to reduce the air space in the sack to a minimum and to perform the dialysis at constant volume. The test-tube was stoppered and placed into a shaking device. The entire apparatus was then put into constant temperature rooms ($4^{\circ}C$ or $37^{\circ}C$) and the dialysis cells were gently agitated for a period of time to allow an equilibrium to occur.

After equilibrium has been attained (as determined by using a series of identical vessels and removing and analyzing one at intervals to find the time required until the concentration of dye outside the bag does not change), an aliquot of dye solution outside the bag is subjected to a spectrophotometric analysis to estimate the free dye. The aliquot is either analyzed as is, or else if it is too concentrated, it is diluted with the same buffer until it may be analyzed within the constructed concentration - optical density graph.

Following the calculation of free dye in equilibrium with the colloid-dye complex, the amount of bound dye is easily estimated, and binding data is then assembled. Usually two independent sets of identical dialysis cells were constructed to formulate a given binding curve.

A detailed critique of the dialysis - equilibrium technique has been assembled in the following paragraphs.

1. The dialyzer tubing was a seamless product made of regenerated cellulose tubing by the viscose process, and obtained through the Arthur H. Thomas Co., Phil. (No. 4465-A2, width flat 1 1/16", diameter inflated 3/4"). The manufacturer had treated the tubing with glycerine to prevent brittleness, and consequently the tubing was exhaustively washed in distilled water prior to all dialysis studies. A micro-Kjeldahl analysis indicated that a small percentage of nitrogenous material was lost from the tubing during the equilibrium experiments. Yang and Foster (1953) have observed random errors in the second decimal of the optical densities which they attribute partly to impurities in the dialyzer tubing.

2. Glass thread - Neoprene treated glass tying cord EC-2N, 1/32" was obtained

from Insulation Manufacturers Corporation, Chicago. This was used to tie the majority of the dialysis sacks.

3. Adsorption of dye on membranes - To eliminate any errors due to adsorption of dye on the cellulose tubing, a control unit was prepared for each dye concentration. These differed from the aforementioned units in that the control contained buffer, but no protein or PVP, inside the sack. At equilibrium the amount of dye inside and outside was found to be equivalent to the total amount of dye put initially on the outside of the bag. Thus, it was indicated that very little or no dye was absorbed or bound to the dialyzer tubing. This was interesting in light of the work of Bulgakova and Shaferehtein (1951). Using collodion tubing, large quantities of BPB were found to be adsorbed at low pH values. Increasing the pH decreased the amount of bound dye. Denitration of collodion or the utilization of cellophane membranes decreased the adsorption of BPB.

A question arises as to the amount of protein or PVP that was adsorbed on the cellulose surface. For the former, at least, it can be stated the interaction studies with bovine serum albumin and methyl orange using equilibrium dialysis, electrophoresis, and spectrophotometry have yielded similar data indicating that albumin adsorption may be a negligible factor (Smith and Briggs, 1950).

4. Spectrophotometry - a) Solution Analysis

A Coleman Universal Spectrophotometer Model 14 was used for most dye analyses. Standard curves were obtained by constructing a plot of optical density as a function of dye molarity in a buffer of specified pH and ionic strength. The Beckman Spectrophotometer Model DU was also employed in certain

isolated experiments. EPB was analyzed at its absorption maximum 585 mu. In all cases, it was ascertained that the different dye molecules were not modified in the buffer solutions from the time of recrystallization to that of analysis.

b) Analysis of Material on Filter Paper Strips

For the analysis of dye on filter paper strips obtained as a result of ionographic studies associated with the investigation of binding mechanisms, an automatic scanning recorder was used. The specific scanner employed was that manufactured by W. M. Welch Co., Chicago, Illinois. A photograph of this instrument is shown by Urbin (1954). The description of the apparatus that follows, is essentially that as given by Urbin (1954) and McDonald et. al. (1955). The instrument is composed of six main components: (1) a Bausch and Lomb grating monochromator; (2) the Welch blue sensitive photoelectric probe; (3) a Welch engineered motor driven feed system made to accommodate filter paper strips; (4) a Welch Densichron amplifying unit; (5) a logarithmic amplifier; (6) a Brown Electronik strip chart recorder.

A description of the events occurring in the utilization of the apparatus is as follows: When a paper strip containing a light-absorbing material is presented to the exit slit of the monochromator, the amount of light transmitted to the photoelectric tube through the paper is a logarithmic function of the concentration of light absorbing material on the paper, all other variables being held constant. This current, varying as an exponential function of the amount of material on the paper, is amplified with fidelity and fed to an electrical circuit, the logarithmic converter, which in essence takes the logarithmic value of the light entering the phototube and feeds the

"linearized" signal into 10 millivolt range Minneapolis-Honeywell Brown Electronik strip chart recorder. Thus, a tracing can be obtained such that the area under the various portions of the curve is directly proportional both to the quantity of material on the corresponding section of the ionogram and to the area over which the material is spread. Hence, regardless of the geometry of the tracing from the strip chart recorder, by simply ascertaining the area under the curve (compensating polar planimeter Keuffel and Esser Co. No. 803, was found appropriate) a direct measure of the absolute amount of material on the paper is obtained. This holds provided the results can be standardized in terms of a known quantity of the material under investigation.

Ionography

a) General details of the apparatus

The main features of the ionographic technique which was employed for all mobility and fractionation determinations has been described in various papers (McDonald, 1952; Marbach, 1952; Urbin, 1952). However, for purposes of clarity in this dissertation, it is appropriate to describe the instrument in some detail here. The particular ionograph used in this investigation was manufactured by the Precision Scientific Company, Chicago.

The principal components of the instrument include:

1. A removable frame constructed to hold up to seven filter paper strips under the proper tension and permitting variation of the strip length. The frame is fitted with a bull's eye spirit level and leveling screws. This along with the leveling screws in the base of the apparatus aid in obtaining horizontality of the strips. This is an important manipulation to be considered when mobility determinations are attempted.

2. Two buffer vessels into which the filter paper strips dip.

3. Two electrode vessels containing the electrodes and connected by agar-salt bridges to the buffer vessels.

4. A line-operated transformer-rectifier able to produce a direct current with any potential up to 2000 volts and equipped with a built-in milliammeter and voltmeter.

5. At the rear of the instrument an inlet and outlet to allow circulation of a fluid through the double-walled chambers and cover enabling a constant temperature to be maintained throughout the course of an experiment.

6. Hose connections to the chamber so that a water-saturated atmosphere may be introduced. Due to the low molecular weight of helium, it is an excellent conductor of heat and therefore aids in dissipating the heat developed in the strips. Evaporation of water from the buffer is thereby minimized.

b) General Procedure

A few statements on the general procedure will be made before the specific modifications utilized with PVP will be discussed. The buffer and electrode vessels are filled with the buffer solution.

The filter paper strips in the frame are saturated with the buffer by means of a pipette and the excess solution is allowed to drain off by holding the frame at an angle. The strips which in all cases are forty-eight to fifty centimeters in length are pulled taut and made to approach the horizontal. Water at a definite temperature is circulated through the double-walled cover and chamber by means of a motor-driven pump.

A small bore siphon tube was maintained in position during the

experiment to hold constant the liquid level in the buffer trough thus obviating differences in hydrostatic pressure that could cause chromatographic movement of liquid through the strips.

No equilibrium vessels, i.e., the so called "feeders" were used to add buffer to various points on the length of the strips (McDonald, 1952). It was found that under the experimental conditions used, the water evaporation was actually small and the addition of these feeders was unnecessary.

After the filter paper strips were saturated with buffer solution, the helium was introduced and the desired potential gradient was applied. These conditions were maintained for one to two hours before the migrant was added to the strips. These factors were observed to develop equilibrium conditions in the chamber and aided in obtaining mobilities that were duplicatable.

The migrant was then applied by means of a micropipet as a thin streak across each strip on a previously penciled-in line midway between the ends of the strip. The ionograph was then allowed to run for an appropriate length of time to obtain satisfactory distance of migrant movement. The ionograms, that is, the filter paper strips containing the migrant, are removed from the ionograph and dried either in the oven at 100 degrees Centigrade for five minutes or in a jet of warm air.

The color test used to locate the PVP on the strip involved the use of Lugol's solution (five grams iodine and ten grams potassium iodide in one hundred milliliters of water). The strips were dipped into this reagent and subsequently washed with tap water until the migrant appeared as a yellow-brown field against the bluish background of the rest of the strip. These

ionograms were then dried at room temperature to prolong the color retention of the PVP iodine complex, and the migrant movement was obtained directly.

The proteins were identified with the conventional bromphenol blue reagent (1% dye dissolved in 95% ethanol which is saturated with mercuric chloride).

c) Specific Procedure for Interaction Studies

Subsequent to the equilibrium dialysis studies two ionographic methods have been developed for obtaining qualitative and quantitative evidence of interactions. These procedures have been described previously by Spitzer and McDonald (1954) (1955). The first ionographic process used a 2-15 lambda portion of migrants of either BPA-BPB or PVP-BPB systems obtained during the course of equilibrium dialysis studies. Other mixtures of known compositions were likewise utilized. The migrants were placed on filter paper strips which had been previously moistened with the buffer solution. After passage of current for a period long enough to separate some of the faster-moving BPB but short enough to retain some of the equilibrium mixture, the strips were dried and subjected to a direct spectrophotometric analysis in the previously described Welch Automatic scanning recorder.

The second method consisted of introducing BPB into the buffer solution and then adding BPA or PVP as migrant. The mobility of both migrants had been previously determined in the same buffer solution with the exception that the dye had been omitted. The compounds, in the presence of the dye, possessed mobilities characteristic of the complexes.

d) Acid-Base Equilibria

In conjunction with the constituent mobility determinations described

in the previous section, it seemed appropriate to investigate further the possibility of acid-base reactions occurring, following application of the migrant to the moistened filter paper strip. Two experiments were devised to clarify the situation.

    1. A buffer of sodium veronal (either Mallinckrodt U.S.P. powder or Mallinckrodt U.S.P. XIV powder) and acid diethylbarbituric (Merck U.S.P.) were formulated into a $\Gamma = 0.02$, pH 8.6 buffer which was 5×10^{-4}M with respect to bromphenol blue. Three migrant solutions were made (0.1 M HCl, 0.1 M KOH, 0.1 HCl) and added to the moistened (with the veronal buffer with BPB) filter paper strips (E & D #613). The migration was carried out for 40 minutes at a potential gradient of 5 volts/cm. at a temperature of 4°C.

    2. A similar veronal buffer was prepared but metacresol purple (m-cresolsulfonphthalein) was substituted for BPB. The dye concentration was 0.5g per liter in veronal buffer of pH 8.6 and ionic strength of 0.02. Hereafter, the symbol MCP will be used to represent this dye. The following migrants were added to the strips following equilibration with ionograph.

        veronal buffer, pH 8.6, 0.02M

        Potassium chloride, 0.5N

        Hydrochloric acid, 0.5N

        Sodium chloride, 0.5N

        Nitric acid, 0.5N

        Sodium nitrate, 0,5N

        Sodium hydroxide, 0.2N

        Potassium hydroxide, 0.65N

        Sulfuric acid, 0.5N

Phosphoric acid, 0.5N

Sodium veronal, 0.5N

Lactic acid, 0.5N

Disodium phosphate (heptahydrate), 0.5M

Monosodium phosphate (monohydrate), 0.5M

Usually, 2-4 lamdas of the solutions were employed.

SUMMARY OF EXPERIMENTAL

A description has been given of the materials, apparatus, and pro-
cedures employed in this investigation. Certain aspects will be discussed in
finer detail in later chapters and in the tables found at the end of the
dissertation.

CHAPTER III

TITRATIONS OF DIALYZED AND UNDIALYZED PVP PREPARATIONS

Brief Recapitulation of Experimental Conditions

Six different PVP solutions (PVP-U30, PVP-U60, PVP-U90, PVP-D30, PVP-D60, and PVP-D90) were titrated under conditions described previously in the experimental section of this dissertation. Each PVP preparation was compounded to contain 58 mg % of PVP nitrogen. The titrations were carried out to maintain an approximately constant ionic strength (0.05). Aqueous solutions were prepared from water which had been redistilled in an all-Pyrex still (PVP-U30, PVP-U60, PVP-U90, and 0.05 M sodium chloride solutions). The PVP dialyzed samples, of practical necessity, contained approximately 25% non-Pyrex distilled water.

A point by point discussion of the results is included as follows:

(1) Typical initial pH measurements after prolonged (30 min.) passage of helium through 50 ml. of undialyzed PVP samples are

PVP-U30	pH 4.2-4.4
PVP-U60	pH 4.6
PVP-U90	pH 4.8-4.9

Apparently there were more acidic components and/or components of stronger acidity in the lower molecular weight preparations.

(2) Similar measurements on dialyzed PVP preparations indicated that they behaved as did the aqueous 0.05 M sodium chloride solutions. That is, prior to the addition of acid or base, the pH rose from about 6.6 to higher than 7 during the passage of helium.
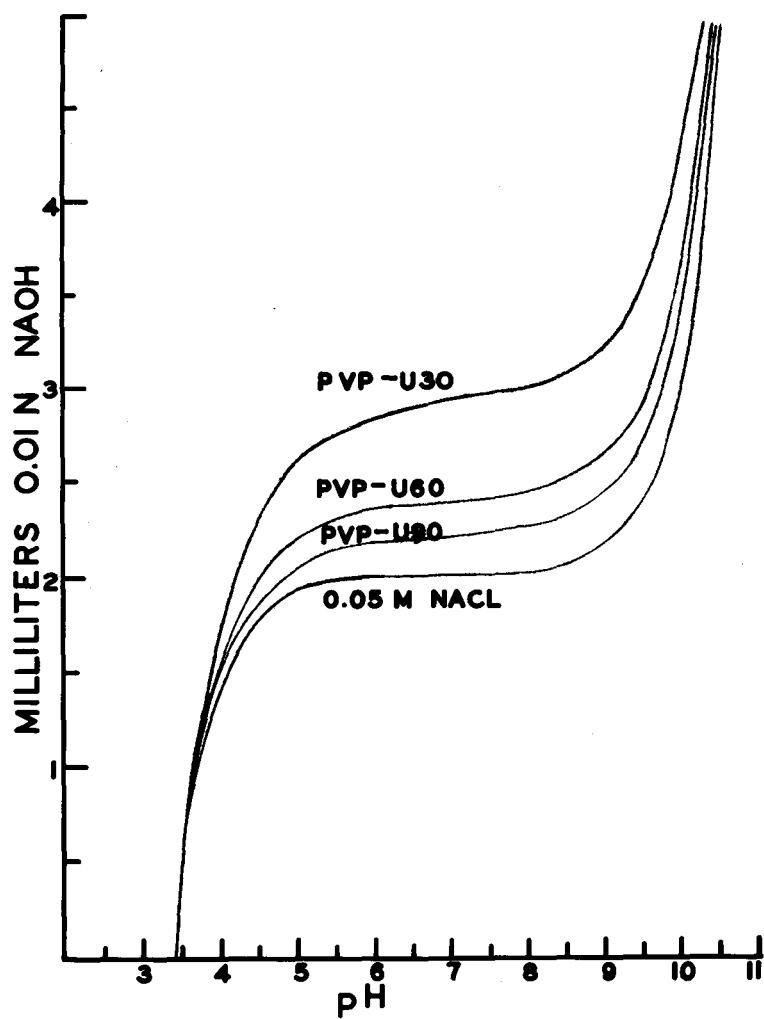
FIGURE 1

RAW TITRATION CURVES OF UNDIALYZED POLYVINYLPYRROL-

IDONE AND 0.05M SODIUM CHLORIDE SOLUTIONS

(3) Figure 1 represents raw titration curves constructed from typical titration data (Tables IX, X, XI, and XII) using undialyzed preparations. While the largest difference between a given PVP sample (PVP-U30) and the 0.05 M sodium chloride curve amounts only to about 1.5 ml. of 0.01 N base, the difference is real and reproducible.

(4) To demonstrate more clearly the effect of the PVP solutions on the hydrogen ion concentration of the medium, differential titration curves have been assembled in Figure 2. The curves were prepared by subtracting the number of ml. of 0.01 N base required to reach a given pH in the absence of PVP from the ml. of base required in the presence of PVP. Thus, a plot of ml. of base as a function of pH was prepared for the three undialyzed PVP preparations.

(5) The shape of the curves in Figure 2 suggests that there are at least two different ionizable groups in each PVP preparation. From a visual inspection of the PVP-U30 titration curve it appears that there exists a pK near 4.3 and another near 10. The latter value was not exactly located due to the basic pH limits of the titration studies. Because of the small quantities of base that were used, it was more difficult to estimate the pK's of the other two PVP preparations. However, analagous trends were evident from inspection of their increment curves.

(6) From a pH of about 6.0 to 8.5 there exists a minimum of groups that ionize in the three preparations. (In subsequent equilibrium dialysis studies a pH of 7.5 was utilized.) The possible presence of dissolved carbon dioxide to cause this small progressive rise in the amount of bound base must be considered. The solutions were prepared and titrated under conditions to
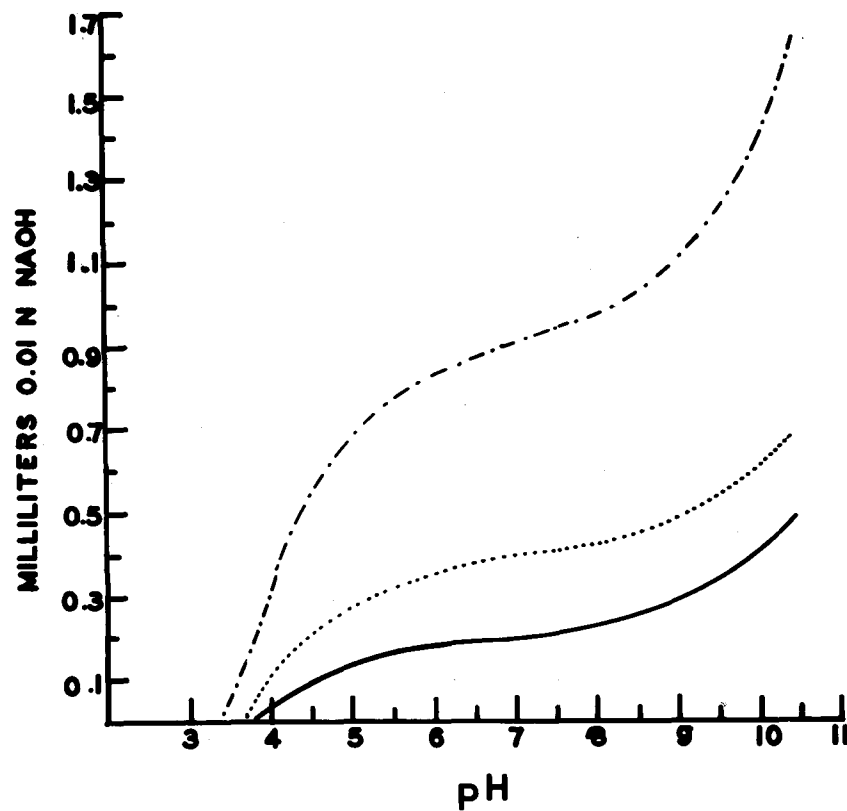
FIGURE 2

DIFFERENTIAL TITRATION CURVES OF UNDIALYZED POLYVINYLPYRROLIDONE SOLUTIONS

- — - — - — PVP-U30
- PVP-U60
- ————— PVP-U90

maintain all conditions identical. There would be expected to be similar
quantities of carbon dioxide in all samples. It must be emphasized that
the greatest slope, in the aforementioned pH range, was always associated
with the lower molecular weight species when the three differential titration
curves were compared. It is probable, therefore, that the curves are truly
illustrative and characteristic of the three (PVP-U30, PVP-U60, PVP-U90)
preparations whether a function of the polymer, impurities, or both. (Mechan-
ical difficulties were found in attempts to construct the differential titra-
tion curves through the extreme basic range of the studies, especially for
the PVP-U90 curve. This was mainly a result of the few ionizable groups that
were present in this sample.)

(7) The three dialyzed samples (PVP-D30, PVP-D60, PVP-D90) were
also titrated and compared to a titration of a 0.05 M sodium chloride solu-
tion. (See data in Tables XIII, XIV, XV, and XVI). A basic shift in the raw
titration curve of one sample (PVP-D30) from that of the salt solution is
exhibited in Figure 5. (This is the maximum shift to be expected.) While
this basic shift was always evident, the amount was not reproducible and no
order sequence seemed to occur on comparison of the three samples. The pres-
ence of small amounts of impurities from the cellulose dialyzer tubing or the
existence of PVP decomposition products may be the cause of this shift. In
any event, it was definitely demonstrable that the titration curves charac-
teristic of the undialyzed material were eliminated.

It was subsequently demonstrated that exhaustive dialysis did not
abolish the affinity of PVP for bromphenol blue. In different experiments,
a micro-Kjeldahl analysis of various dialyzates indicated the passage of
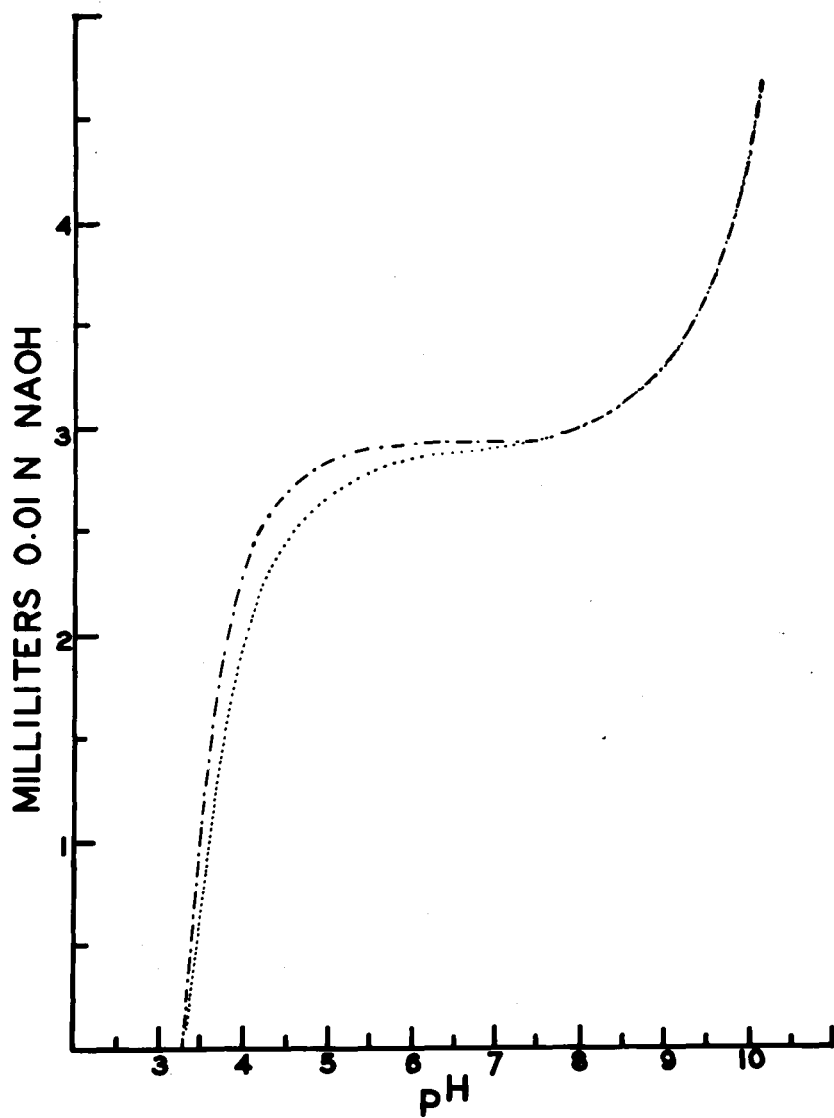
FIGURE 3

RAW TITRATION CURVE OF A DIALYZED POLYVINYL-

PYRROLIDONE SAMPLE

-..- 0.05M SODIUM CHLORIDE

..... PVP-D30

40

nitrogenous components through the membrane. Lugol's reagent was used to demonstrate the probable existence of dialyzable PVP.

(8) Because of the molecular heterogeneity of the undialyzed polymers and of the unknown heterogeneity of the resultant residual molecules after dialysis, it seemed illogical to present a typical graph representing a plot of moles of base or hydrogen ions combined with one mole of PVP, versus pH. However, at a pH of about 9.5 (PVP-U30), about 0.01 meq. of base was bound as a result of 29 mg PVP nitrogen, or about 0.005 eq. of base per mole PVP nitrogen. It must be emphasized that this is not a characteristic description of a property of PVP, since the ionizable species appears to be associated with the small molecular PVP species, a dialyzable impurity, or both.

A discussion on the relation of the titration curves to the binding ability of PVP will be considered in Chapter V.

CHAPTER IV

IONOGRAPHIC INTERACTION STUDIES

Acid-Base Equilibria

The experiments of Roth and Kallee (1953)(1953) have already been considered. In essence, they studied the constituent mobility of albumin in the presence of amido black 10B, azorubin S, and naphthol yellow. Their observation of a white zone which preceded or followed the movement of albumin, was interpreted on the basis of the formation of an albumin-dye complex.

However, the possibility appeared to exist that the explanation was dependent or partly associated with the effects of other phenomena, namely, that of acid-base equilibria and/or dilution processes, and/or buffer-ion equilibria. For these reasons, two experiments were devised to elucidate the mechanisms of similar interaction studies. In the first experiment, three migrant solutions were prepared (0.1 M potassium chloride, 0.1 M potassium hydroxide, 0.1 M hydrochloric acid) and added to the moistened (with veronal buffer, ionic strength 0.02, pH 8.6, and 5×10^{-4} M, with respect of BPB) filter paper strips. After migration, various zones were observed on the strips. (See Figure 4 for a schematic representation of typical ionograms). It was evident that electromigration of acids, bases, and salts in this dye-buffer medium was not a simple process.

The second experiment utilized similar experimental conditions with the exception that metacresol purple was substituted for BPB (0.5q MCP/L buffer). A photograph of typical ionograms is demonstrated in Figure 5. It is with this second case that the discussion will begin. Metacresol purple
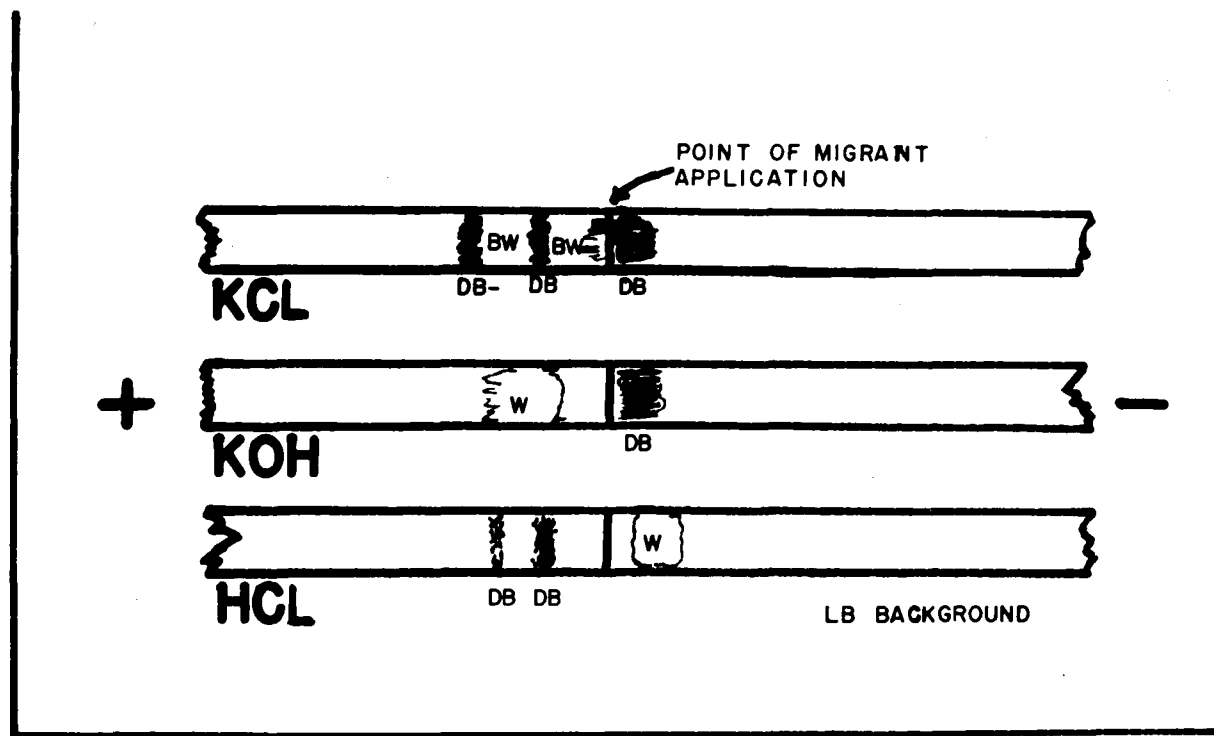
FIGURE 4

SCHEMATIC REPRESENTATION OF IONOGRAMS SUBSEQUENT TO THE MIGRATION

OF INORGANIC IONS IN BUFFERED BPB SOLUTION

KEY
db   dark blue
bw   bluish white
w   white
lb   light blue

43

(A). WY DY Y W

(B). WY DY Y W

(C). WY Y DY W

(D). Y P DR P RY Y

(E). Y WY DY Y W

(F). WY P

(G). BW DB

(H). WY P

KEY

wy whitish yellow
dy dark yellow
y   yellow
w   white
p   purple
dr dark red
ry reddish yellow
bw bluish white
db dark blue
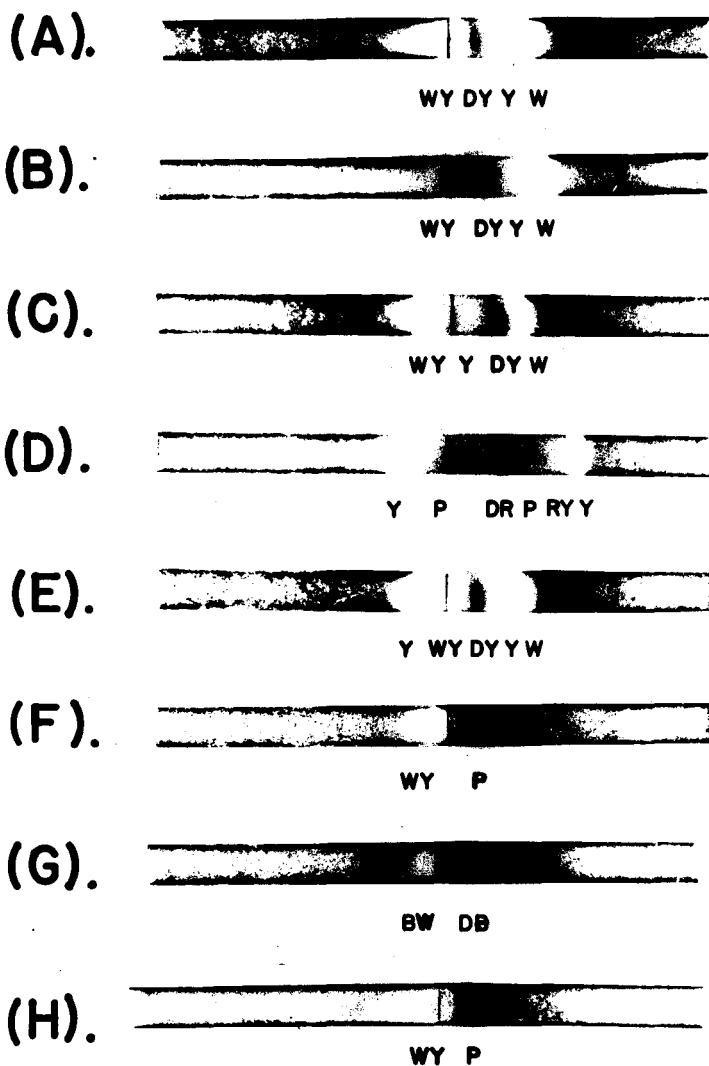
FIGURE 5

PHOTOGRAPHS OF IONOGRAMS SUBSEQUENT TO THE MIGRATION OF ACIDS, BASES, AND SALTS IN BUFFERED META CRESOL PURPLE SOLUTION. POSITIVE POLE IS AT LEFT.

(A) sulfuric acid	(E) phosphoric acid
(B) hydrochloric acid	(F) potassium chloride
(C) lactic acid	(G) sodium hydroxide
(D) nitric acid	(H) sodium nitrate

44

exhibits two color changes: acid range, 1.2-2.8, red-yellow; alkaline range, 7.6-9.2, yellow purple. Prior to migration but subsequent to application, the migrants exhibited characteristic colors on the purple colored strips: hydrochloric, sulfuric, nitric, and phosphoric acids created red zones; lactic acid and sodium dihydrogen phosphate possessed yellow zones; water, veronal buffer, sodium chloride, potassium chloride, and sodium nitrate had greyish zones; sodium hydroxide, potassium hydroxide, sodium veronal, and disodium hydrogen phosphate exhibited dark blue or dark purple zones. In view of the approximate pK's of MGP, and of concentrations and of the migrants, these colored zones are to be expected. The appearance of a colorless zone on application of neutral salts, veronal buffer, and water to the strips was probably indicative of a simple dilution effect by a mechanical displacement of the dye solution by these migrants. At the perimeters of the strongly acidic zones (i.e., hydrochloric, sulfuric, nitric and phosphoric acid spots) a yellow color was seen to develop from the red nucleus. This "alkaline shift" may undoubtedly be attributed to the buffer action of the veronal-sodium veronal system and aided by diffusion processes within the migrant volume.

During the first few minutes of electromigration, there were color changes that may be described as being both rapid and pronounced. After approximately 10-15 minutes of migration at 5 volts/cm, the visual changes became less rapid and each strip achieved a characteristic pattern.

Basic zones (pH 9 or thereabouts, identified by a dark purple or dark blue band) were found to migrate to the negative pole when sodium hydroxide, potassium hydroxide, sodium acetate, sodium chloride, sodium veronal, sodium nitrate, potassium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate, and veronal buffer were used as the migrant. The neutral

salts, acid salts, and basic salts (except for sodium veronal) had a yellow band that migrated to the positive pole. The bases and sodium veronal had a white zone that migrated to the positive pole.

All the acidic migrants possessed a yellow band that migrated to the positive pole.

A discussion of these observations at the molecular level would be extremely complicated because of the necessity of introducing rates and affinities of all the species involved in the various equilibria. An enumeration of the associated processes must include:

1.) the relation of the pK or pK's, concentration and type of indicator to the type, quantity, concentration, and acidity of the migrant.

2.) the relation of the indicator and migrant to the buffer system with regard to the pK, concentration, quantity, and color of all species.

3.) a consideration of the rates and direction of migration of all species.

4.) a consideration of the rates of diffusion of the migrating species in a specified medium.

5.) a consideration of the rates of all reactions that occur on the strip both preceding and following electromigration and diffusion.

6.) the possibility of concentration gradients being the cause of a given color zone or the lack of one.

7.) the possibility of an acidic zone (on the basis of indicator color in that zone) in reality being a basic zone that is temporarily "confined" in exhibiting its properties by the buffering action in the strip.

It was not the purpose of this dissertation to thoroughly investigate these phenomena. Rather, it was necessary to demonstrate, experimentally, the existence of these complex equilibria and their possible relation to ionographic interaction studies. That these factors may influence mobility determinations and separatory processes must be considered during the course of any ionographic interaction investigation.

Should a protein solution having a different ionic environment than the dye-buffer on the strip be utilized as a migrant, it is evident that the presence of a colored or colorless band must not necessarily be considered to be a result of dye-protein interaction.

Resolution Analysis of Bromphenol Blue-Bovine Plasma
Albumin Complex Systems and Similar Systems

Smith and Briggs (1950) chose the (methyl-orange)(bovine serum albumin) system for use in their free solution electrophoretic investigations. Primarily this was to compare their data with that of Klotz (1946) who studied the same system using an equilibrium dialysis technique. The former group used an acetate buffer of pH 5.5 of ionic strength 0.05. Klotz used a 0.1 M phosphate buffer at pH 5.67. The n-value and dissociation constant K in the Langmuir adsorption isotherm of the form:

$$\frac{1}{r} = \frac{K}{n(A)} + \frac{1}{n}$$, where n is the maximum number of sites available for binding; r is the average number of bound molecules per colloid molecule; and (A) is the concentration of interacting species, were found to be in fair agreement. Smith and Briggs also performed equilibrium dialysis experiments and found the aforementioned constants to agree quite satisfactorily with those calculated from electrophoretic data.

In the present study to compare the binding mechanisms of PVP and some plasma proteins it seemed logical to begin by using methyl-orange as the small molecule interacting species, and subsequently to compare the constants with Smith, Briggs, and Klotz. Since data was to be gathered from both equilibrium dialysis and ionographic methods, (studies were not limited to only these methods), an attempt was made to initiate the work by analyzing "known" BPA-MO complex systems with the latter technique.

S & D #613 filter paper strips were moistened with acetate buffer at pH 5.5 of ionic strength 0.05. Two to five lambdas of BPA-MO migrant mixture (3.0×10^{-4} M with crystallized BPA in the acetate buffer) were added as a streak across the stabilizer. Also, pure MO in acetate buffer was used to obtain mobility data on this compound.

However, it was at once evident that MO adsorbed on the filter paper. Its movement was so hindered that the calculation of a mobility was not possible. The dye migrated to the positive pole but an appreciable quantity remained at the origin.

It was also apparent that when the complex mixture was utilized as the migrant, the methyl-orange moved a greated distance than when only the dye was used. (The protein also moved to the positive pole as determined by differential staining with the bromphenol blue reagent). Thus, there was a qualitative indication that a complex was formed. It might be considered that there was competition for MO between albumin molecules and sites on the filter paper. Smith and Briggs found the albumin to migrate with a mobility (approximately -2.7×10^{-5} cm.^{2}/volt.sec.) slower than that of methyl-orange (-10.50×10^{-5} cm.^{2}/volt.sec.).

The ionographic studies indicated that the albumin moved faster than the dye; probably as a result of filter paper – MO interaction. Consequently, it was decided to discontinue ionographic studies with this system.

Certainly all interaction data obtained from ionographic experiments is suspect, and subject to criticism on the basis of this dye-filter paper adsorption factor. Possibly the BPA-MO system could be more accurately studied using a different stabilizer.

THE BROMPHENOL BLUE-BOVINE PLASMA ALBUMIN SYSTEM

Similar experiments were assembled to investigate the BPA-BPB system. The buffer utilized was the sodium veronal-veronal buffer of pH 8.6 (25°C) and ionic strength 0.02. The temperature was maintained at 5°C during electromigration. Helium was used to minimize evaporation from the E & D #615 filter paper strips. A potential gradient between 2.4 to 5.8 volts/cm. was maintained during the electromigration time of 6 to 12 hours. Preparation of a complex system was carried out to create a solution 2.73×10^{-5} M with respect to albumin and 13.65×10^{-5} M with respect to BPB in the buffer. It was found that the system was quickly separable. Using small migrant volumes (of the order of 3.5 lambdas) the entire complex dissociated into pure BPB and BPA. Possibly it is superfluous to indicate that there exists an inverse relationship between the rate of entire complex resolution and the migrant volume employed or the area on the filter paper which it spread subsequent to application but prior to migration.

A point by point critique of the experiment follows:

(1.) The BPB-BPA system was not a simple physical mixture. Definite interaction had been demonstrated to occur on the basis of two experiments:

equilibrium dialysis studies on the same system under similar conditions (Chapter V), and a spectrophotometric study demonstrating a shift to a higher absorption maximum on the addition of protein to the dye solution (Figure 6).

(2.) the reaction:

$$BPA + nBPB \underset{k_r}{\overset{k_f}{\rightleftharpoons}} (BPA \cdot BPB_n)$$
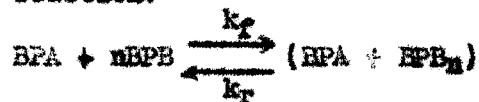
most closely resembles case 5 (Longsworth and MacInnes, 1942) (also see Chapter II, Experimental) in which k_f and k_r are both large and of the same order of magnitude and both greater than the rate of ionographic separation. The equilibrium is adjusted as rapidly as required by the electrophoretic separation of the constituents. The interaction of bovine serum albumin and methyl-orange (Smith and Briggs 1950) and bovine serum albumin and chloride ion (Alberty and Marvin, 1950) also are representative of case 5. Should k_r be less than the rate of electrophoretic separation, it would be possible to isolate the complex assuming its mobility would be different enough to separate out the reactants. This was found not to be the case with the BPA-BPB system.

(3.) There appeared to be no absorption problem between the dye and filter paper. However, a slight trailing of albumin was noticed.

(4.) Both BPA and BPB possess net negative charges in this buffer system.

(5.) The mobilities of the dye obtained from the dissociation of the complex and that of "pure" dye appeared to be identical within experimental error. This is also the case for the protein mobility obtained from dissociated protein from the complex when compared to the mobility of "pure" protein. (See Table VI). This is to be expected since almost immediately the
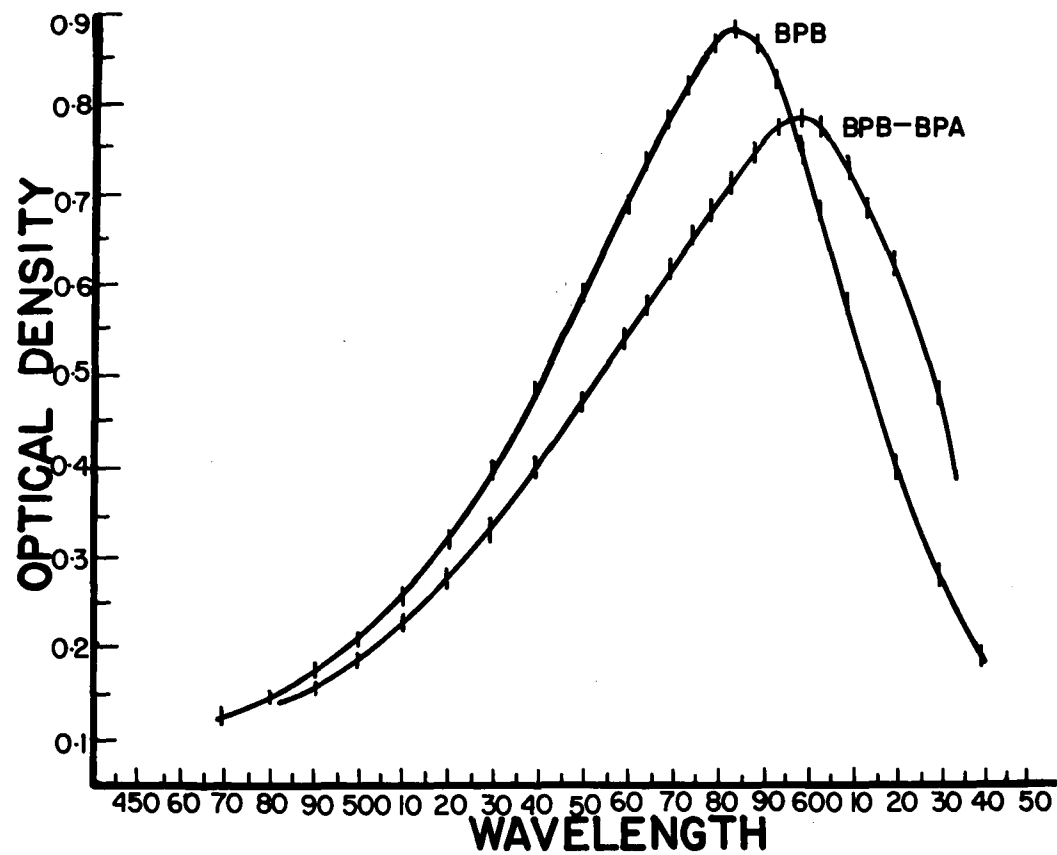
FIGURE 6

ABSORPTION SPECTRUM OF BPB AND BPB-BPA SYSTEMS

TABLE VI

MOBILITY OF PURE AND SEPARATED BROMPHENOL
BLUE AND BOVINE PLASMA ALBUMIN

$$(cm^2/V.Sec. \times 10^5)$$

	BOVINE PLASMA ALBUMIN	BROMPHENOL BLUE
"pure" migrants	-3.9 ± 0.3	-6.3 ± 0.3
migrants dissociated from complex	-4.3 ± 0.1	-6.8 ± 0.5

reactants began to separate with their "pure" mobility.

(6.) In Figure 7 is found a plot of migrant movement (mm./volt/cm.) as a function of migration time. The values (Table VI) that represent the BPB curve have been obtained from "pure" and "separated" BPB determinations. The BPA curve has been constructed from data obtained in a similar fashion using albumin as the migrant. The two linear relationships justify the utilization of the term mobility to describe the electromigration velocities of BPB and BPA under the specified conditions.

(7.) Equilibrium dialysis experiments were devised to prepare and analyze the BPB-BPA system. the r-values were calculated (i.e., the average number of BPB molecules bound to one BPA molecule) and the various preparations (r = 3.00, 2.82 etc., see Table XIII) were used as 2 lambda migrants. All preparations were found to be quickly dissociable on the basis of ionographic resolution accompanied by visual inspection and recorder analysis of the ionograms.

(8.) Similar equilibrium dialysis studies on the PVP-BPB system were used to calculate r-values (i.e., total moles of PVP nitrogen, see Table XXX). The components of all samples of this system were found to be completely separable. Only pure BPB and pure PVP were found after resolution (phosphate buffer, pH 7.4, 0.05 M with respect to phosphates). The system behaved very similarly to the BPB-BPA system. PVP moved slightly to the negative pole, probably by electroosmosis. (Spitzer, 1953).

The complex system was not a simple mixture. Both equilibrium dialysis studies (Chapter V) and spectrophotometry (Figure 8, Table XVIII) indicated a definite association. In the latter case there was evident a
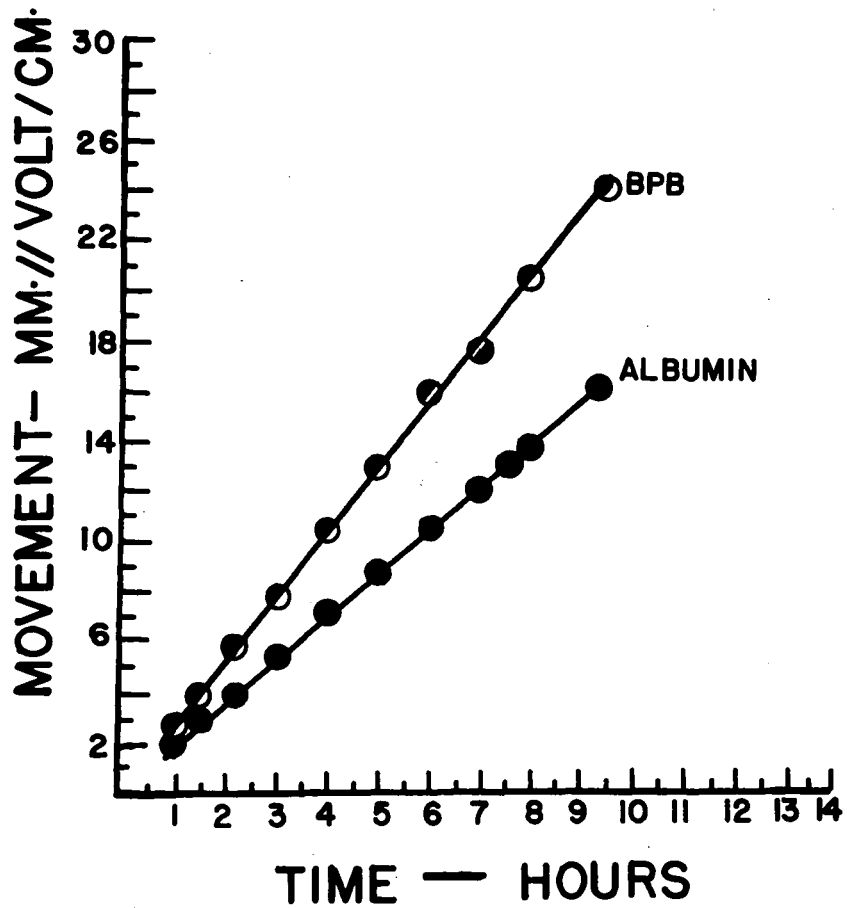
FIGURE 7

IONOGRAPHIC LINEARITY CURVES OF BPB

AND BPA: MOVEMENT VS. TIME

54
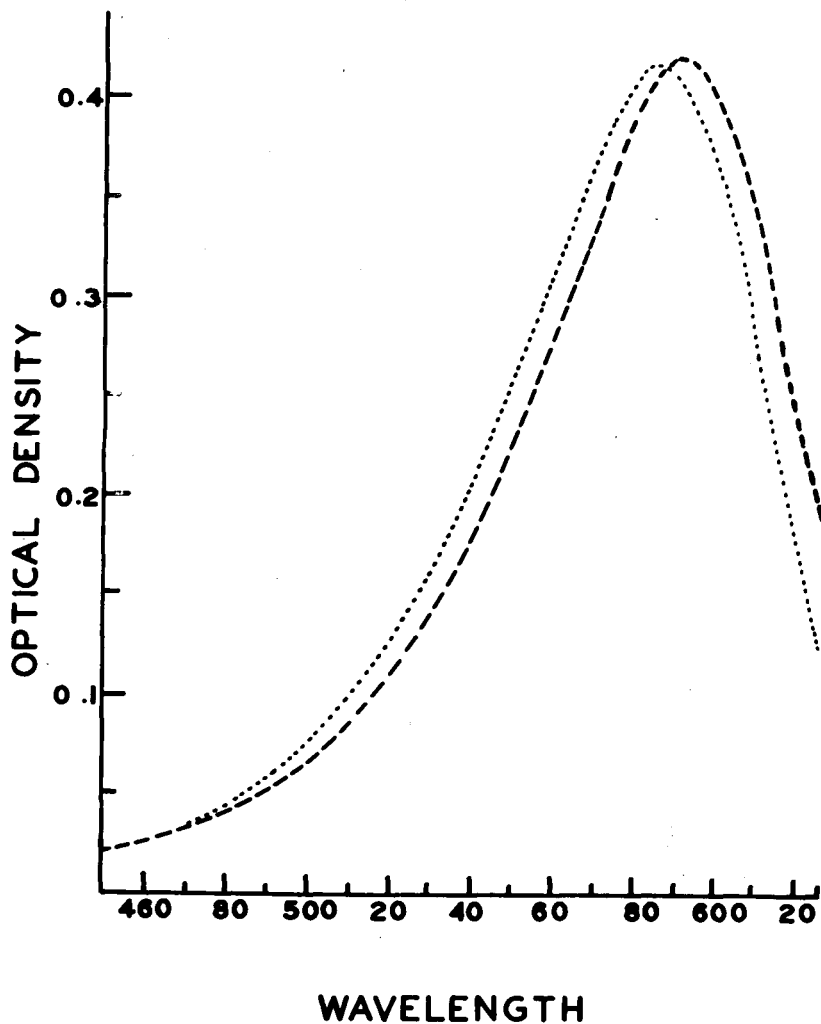
FIGURE 8

ABSORPTION SPECTRUM OF BPB AND BPB-PVP

SYSTEMS (SEE TABLE XVIII)

.... BPB

————— (BPB)-(PVP-D30)

shift to a higher absorption maximum as PVP was added to a buffered BPB solution. This was demonstrable with PVP-U30, PVP-U60, PVP-U90, PVP-D30, PVP-D60, and PVP-D90 polymer preparations.

Ionographic Demonstration and Characterization
of BPA-BPB and PVP-BPB Interaction

Asymmetric Automatic Scanner Pattern

It has been stated that the BPA-BPB system may be quickly and completely dissociated by means of ionography. Rate of resolution of complexes of this type is dependent on a number of factors:

1.) the net charge on BPA, BPB and the various complexes.

2.) the quantity and concentration of the complexes.

3.) the rates of dissociation of the complexes.

4.) the potential gradient across the equilibrium system.

5.) the initial migrant volume and the volume through which the migrant had spread on application to the moistened filter paper.

Since small migrant volumes had been previously used (2 to 3.5 lambdas) to obtain a swift dissociation of the complex system, it was decided to employ large migrant volumes (5 to 15 lambdas) to further study the BPA-BPB system. There are two immediate results of this manipulation: first, the total quantity of complex on the filter paper is increased, and second, the time needed for complete resolution of all components would be increased.

A current was passed for a period sufficient to separate some but not all BPB from BPA. At an appropriate time, the strips were quickly dried and placed in completely closed vessels prior to subsequent analysis with the automatic scanner. An asymmetric pattern could be demonstrated (Figure 9A),
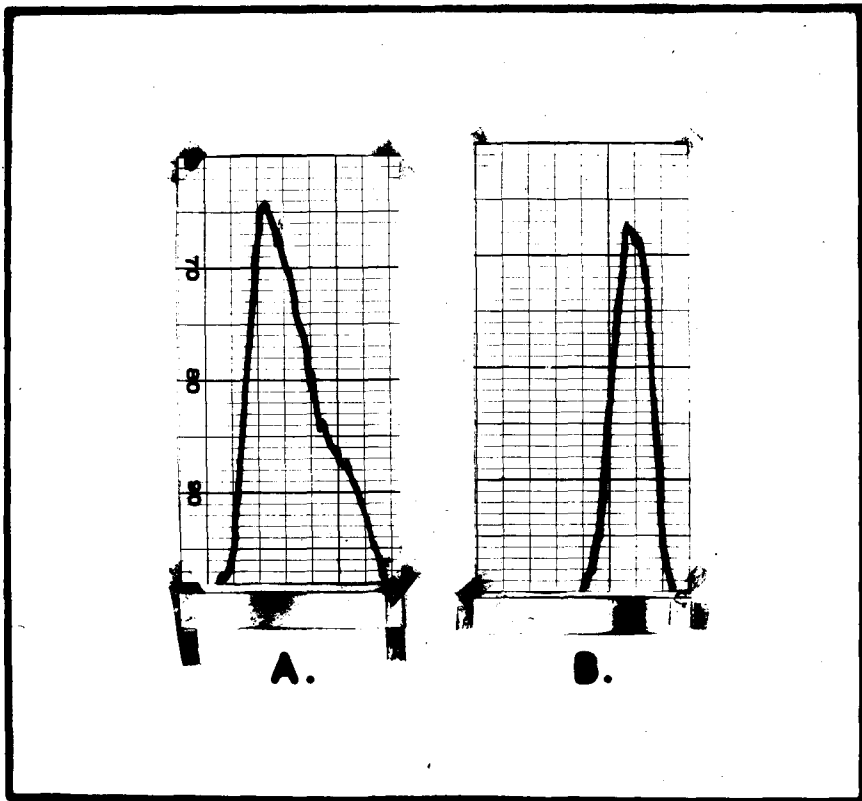
FIGURE 9

PHOTOGRAPHS OF AUTOMATIC SCANNER PATTERNS SUBSEQUENT TO THE MIGRATION OF THE BPB-BPA SYSTEM (A).

AND PURE BPB (B). THE RESPECTIVE IONOGRAMS ARE BENEATH THE PATTERNS.

POSITIVE POLE IS AT LEFT.

(A light blue zone to the right of pronounced zone in ionogram (A.) is not discernible).

after analysis at 585 mu. By a differential staining technique, localization of the protein zone was accomplished (zone was composed of separated protein and protein that was previously in equilibrium with BPB). A 1% BPB in ethanol, saturated with mercuric chloride, was used to stain the proteins. (Any protein staining procedure would suffice.)

The same strip was again subjected to analysis (585 mu) in the recorder and the resultant curve was superimposed against the initial asymmetric patterns.

A point by point critique on the experimental procedure and the significance of the experiment follows:

1.) Within limits, the variable wavelength automatic scanner can be used to obtain a plot of optical density as a function of migration distance (i.e., a plot of optical density as a function of filter paper length, or a plot of migrant quantity as a function of migration distance) (Urbin, 1954). When the quantity of migrant per unit area of filter paper exceeds a quantity (dependent on migrant type, filter paper, etc.) the observed optical density is related to migrant quantity by a non-linear function.

2.) Optical density measurements as a function of wavelength have been assembled in Table XIX, for both "pure dye" and dye in the environment of BPA. The absorption maxima appear to be both near 580 mu using the automatic scanner. As mentioned previously, the addition of protein to a BPB solution caused a shift in the absorption maximum from about 585 to 605 mu. The adsorption of dye on the filter paper precludes an absolute comparison of the two spectrophotometric techniques. Nevertheless, all ionograms were analyzed at 585 mu.

3.) Using various volumes (1 to 10 lambdas) of 5×10^{-4} M BPB in veronal buffer, as the migrant, there were obtained relatively symmetrical patterns when the ionograms were analyzed with the recorder (Figure 9B). This was also true of patterns obtained after migration of BPB alone (neglecting the quantity of protein that trailed behind the mass of the material).

4.) Figure 10 shows an asymmetric pattern obtained after the passage of the current for a period sufficient to separate some but not all BPB from BPA. The same figure shows a typical pattern obtained after differential staining of the protein. The two patterns have been superimposed to illustrate the location of BPB (separated, free in presence of albumin, and bound to albumin with respect to BPA. The ionogram has been placed below the pattern to further clarify the pertinent relationships.

5.) The relation of these ionographic studies to analagous interaction studies using the moving boundary method is illustrated schematically in Figure 11.

a.) Representation of either Tiselius Cell (U-tube visualized as horizontal) or of ionogram prior to migration of equilibrium components. The migrant zone is composed of buffer; free anion, (BPB), represented as A; and a series of protein complexes PA; from i = 0 to i = n, all in equilibrium with one another. At pH 8.6 the equilibrium components are all negatively charged.

b.) A graphic representation of the concentration of any interaction species (except buffer ions) as a function of location in U-tube or in filter paper (differential diffusion or chromatographic effects assumed to be non-existent). Components are visualized prior to migration.

c.) Initial schlieren pattern demonstrating a plot of refractive
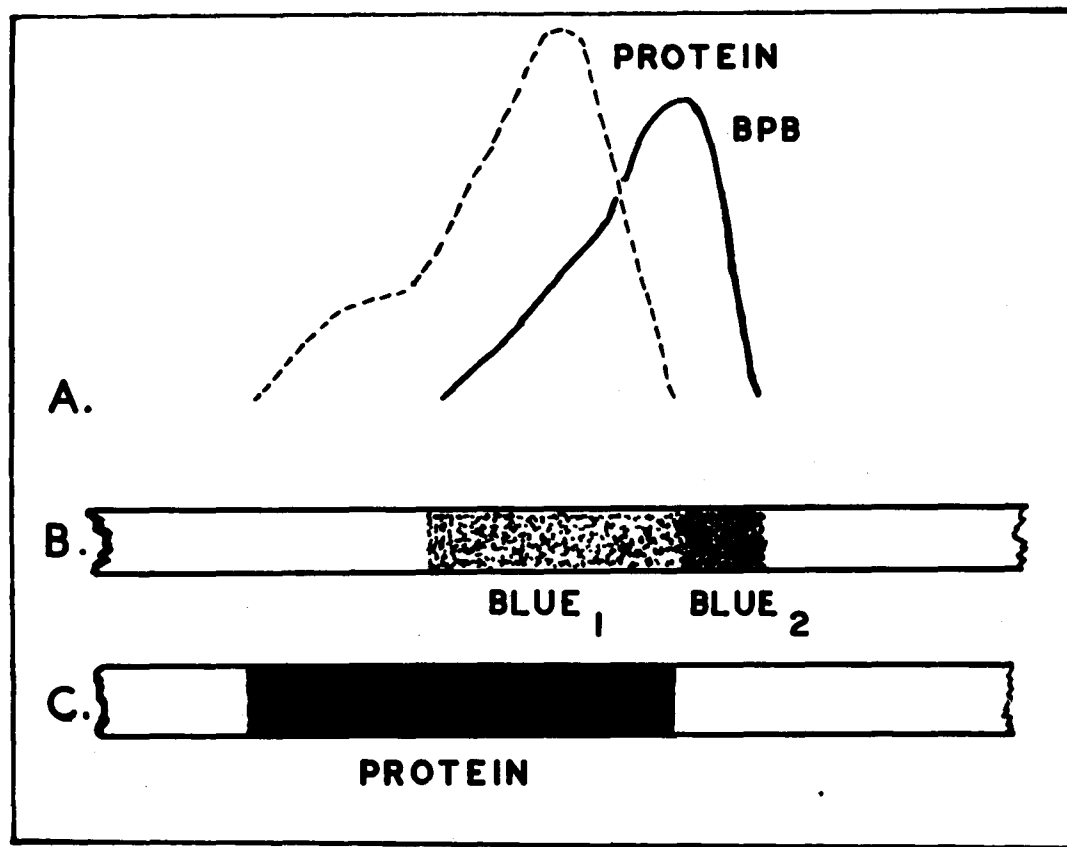
FIGURE 10

SCHEMATIC REPRESENTATION OF SCANNER PATTERNS (A.) AND IONOGRAMS (B.) AND (C.) USING THE DIF-
FERENTIAL STAINING TECHNIQUE. BPB CURVE IN (A) WAS OBTAINED FROM (B). BLUE$_2$ CORRESPONDS TO
SEPARATED BPB. BLUE$_1$ CORRESPONDS TO BPB IN THE EQUILIBRIUM SYSTEM. PROTEIN CURVE IN (A) WAS
OBTAINED FROM IONOGRAM (C) AFTER STAINING FOR PROTEIN. POSITIVE POLE IS AT RIGHT.
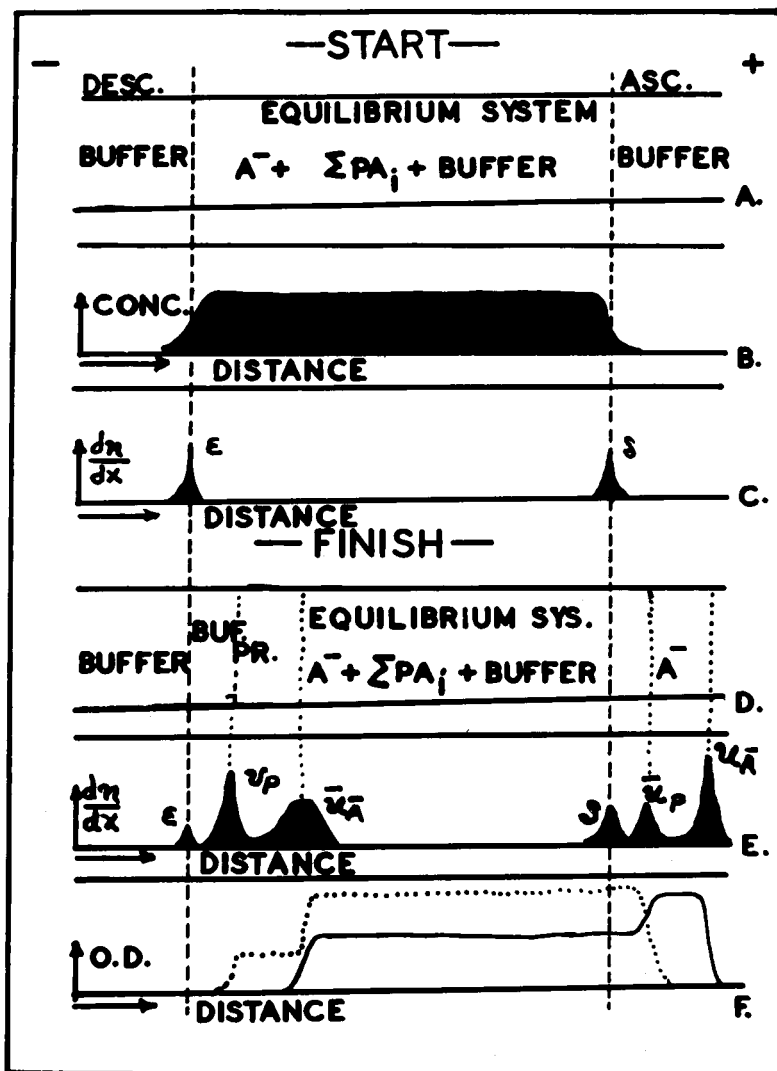
FIGURE 11

RELATION OF IONOGRAPHIC INTERACTION

STUDIES TO THOSE OF FREE

SOLUTION ELECTROPHORESIS

index gradient as a function of location in the U-tube.

d.) representation of either Tiselius cell or of ionogram following electromigration for a period sufficient to separate some of the anion from the equilibrium system but not all anion.

e.) Final schlieren pattern (Smith and Briggs, 1950).

f.) Schematic final recorder patterns.

Smith and Briggs (1950) and Colvin and Briggs (1952) have discussed and described moving boundary experiments and equations relating to the bovine serum albumin-methyl orange equilibria. Reference to Figure 11E will indicate that the fastest moving boundary (ascending arm) corresponds to the migration of the separated free dyestuff. That is, when the current is allowed to flow through the cell, the free dye in this arm moves out ahead of the slower moving complexes and travels up the tube with a characteristic mobility. This boundary is followed by one which migrates with the constituent mobility of the protein. The next boundary proceeding from right to left is the stationary delta salt boundary. The fourth boundary, $\bar{u}_A$, conforms to the constituent mobility of the anion dye. The mobility of albumin may be calculated by using the fifth boundary epsilon salt boundary. With certain assumptions and equations these workers were able to calculate r, n, and k in the Langmuir adsorption isotherm.

6.) These equations were applied to data obtained from ionography. It was apparent that the imperfections intrinsic in the moving-boundary equations were additionally complicated by certain factors inherent in ionography. Foremost, was the method of migrant application. In free solution, the electrophoresis cell is constructed in three sections which may be slid relative

to one another, so that with adequate precautions, sharp boundaries are created and maintained prior to electromigration. The situation is not as simple and accurate with existing ionographic techniques; for the method of migrant application necessitates dilution of the complex mixture and erratic spreading of the migrant solution. Basically, these factors did not permit quantification of this ionographic procedure.

7.) Qualitatively, this procedure associated with the demonstration of an asymmetric recorder pattern and the aforementioned differential staining technique was adequate to indicate:

a.) an interaction between BPA and BPB. Also between PVP and BPB.

b.) that the reaction between BPA and BPB produced a complex possessing a greater net negative charge than the protein; likewise the reaction between PVP and BPB produced a complex posessing a greater net negative charge than PVP.

Constituent Mobility Determinations

Another ionographic procedure to demonstrate the formation of complexes involves the calculation of the constituent mobility of a given compound. (See Experimental, Chapter II). BPA, PVP or BPB complexes of these compounds were employed as migrants in BPB-buffer solutions. Electromigration of PVP or BPA in this medium will be characteristic of their complexes. Should their mobilities be different from those in non-BPB buffers, an indication of complexing is evident.

Mobility measurements were obtained in phosphate buffer (pH 7.4, 0.05 M) and in phosphate buffer (pH 7.4, 0.05 M, 1.785×10^{-3} M with respect to BPB). A summary of some values appears in Table VII.

TABLE VII

MOBILITIES OF PVP AND BPA IN PRESENCE AND

ABSENCE OF BROMPHENOL BLUE

phosphate buffer without dye

(Mobilities in $Cm^2/V.Sec. X10^5$)

BPA	-3.5
PVP	+2.6
BPB	-5.8

phosphate buffer with dye

BPA	-4.7
PVP	-3.8
White Band	-8.4

It appears evident that the BPB complexes of PVP and BPA have a greater net negative charge than the non-complexed species. That is, the mechanism of binding of BPB to either PVP or BPA is such that an increased negative charge is added to the colloidal species. (The convention Utilized here in reporting mobilities is such that the prefix; i.e. + or -, referes to the apparent charge on the molecule. Caution is necessary when obtaining the charge by ionography due gainly to the results of electroosmosis, (See Spitser 1953, and McDonald and Spitzer 1953).

It was interesting to note the appearance of a white band that moved with a mobility greater than PVP, BPA, and BPB. This zone had a similar mobility regardless of the large molecular species that was used. The possible explanations for this process have been considered priviously.

CHAPTER V

EQUILIBRIUM DIALYSIS

General Structural Comparisons Between Proteins and Polyvinylpyrrolidone

There are a number of obvious structural differences between the proteins and PVP which must be considered in a discussion of their comparative binding abilities. Polyvinylpyrrolidone is a synthetic polymer, possibly of identical repeating units. (It must be emphasized that the ionizable groups as exhibited in the differential titration curves have not been exactly located on the PVP molecules.) The molecular weight description of PVP molecules in a specified sample is dependent on the methods employed in the polymerization process. A given PVP preparation consists of a spectrum of molecular sizes and, probably, molecular shapes.

From osmotic-pressure measurements on aqueous PVP solutions, number average molecular weights (M_n) have been calculated,

$$\text{I.} \quad M_n = \frac{\sum (M)(N_m)}{\sum (N_m)}$$

where N_m molecules of actual molecular weight M are present in the sample.

Another method to describe the molecular heterogeneity involves the use of viscosity to enable the weight average molecular weight (M_w) to be calculated.

$$\text{II.} \quad M_w = \frac{\sum (M^2)(N_m)}{\sum (M)(N_m)}$$

where N_m molecules of actual molecular weight M are present.

The intrinsic viscosity (n) is proportional to the weight average

molecular weight.

$$\text{III.} \quad (n) = \underset{c \longrightarrow 0}{\text{Lt.}} \quad N_{sp}/C$$

where N_{sp}, is the specific viscosity of a solution of concentration C grams per 100 ml. of solution.

Thrower and Campbell (1951) have used the ratio of number average molecular weights to weight average molecular weights (more specifically, $\frac{M_n}{(n)}$ to obtain an indication of the distribution of molecular weights in various PVP preparations prior to blood plasma expander experiments. It was stated that the number of average molecular weights are more sensitive to the low molecular weight molecules, and the weight average description is more sensitive to the high molecular weight molecules.

As a result of ultra-centrifugal and viscometric molecular weight measurements, Scholtan (1952) has concluded that the molecules of PVP exist as randomly linked chains encompassing "immobilized solvent".

Hengstenberg and Schuch (1952) have studied the molecular weight distribution of PVP samples by means of osmotic pressure and light scattering techniques. With the data of the former method in conjunction with the assumption that the molecules exist in a random coiled form, the mean molecular diameters were calculated to be 560A for a molecular weight of 249,000 and 930A for 1,116,000. It was indicated that these values correspond to those calculated for tightly coiled molecules.

Miller and Hamm (1955) have applied the Svedberg equation to PVP to describe the molecular weight distribution. The inaccuracies inherent in the characterization of the average molecular weights by means of a single relative viscosity coefficient of the Fikentscher K-value type was emphasized.

(See Chapter II for the K-values ascribed to the three PVP samples used in this dissertation).

Studies of these types generally need not be considered in protein interaction investigations since a given protein may be theoretically assigned a definite molecular weight. (Obviously, this is dependent on the procedure devised to determine the molecular weight. The purity of the protein extract is an important point. In these studies the purity of BPA and BPGO was based on electrophoretic studies. Also, the molecular weight description of a BPGO sample is not a simple matter).

It is evident that protein interaction studies are dependent on a knowledge of primary structures (amino acid configuration and sequences), secondary structures (intramolecular associations), and tertiary structures (intermolecular associations). Admittedly, the effects of the latter two types of structures on binding, are, at present, unclear.

PVP interaction studies must be interpreted on a description of molecular heterogeneity and the repeating unit, vinylpyrrolidone. The importance of other groups not represented in the assumed structure must also be reconciled with binding data.

Results of Equilibrium Dialysis Studied

The time necessary to attain equilibrium in the dialysis cells, for a definite system, was dependent on a number of factors (rate of shaking, length of time required to prepare the cells at room temperature, etc.) that need not be considered here. Suffice it to state that all systems were in equilibrium prior to analysis. (see Chapter II for details).

Adsorption of BPB on the membranes was found not to occur throughout

all equilibrium dialysis experiments. (See Tables XXXI and XXIV for typical data).

A system by system discussion of all studies is included as follows:

I) BPA-BPB; Veronal buffer, pH 8.6, ionic strength 0.05; 4°C; 0.7% BPA.

Binding appeared to be statistical throughout the entire (BPB) free limits. That is, the sites (probably similar groups) possessed similar intrinsic affinities for BPB. An electrostatic factor was not evident.

The data (Table XXII) is represented in a typical binding curve (Figure 12) in which the r-values ($\frac{\text{MOLES BOUND BPB}}{\text{MOLES TOTAL ALBUMIN}}$) are plotted as a function of the logarithm of free (BPB) in equilibrium with the complexes. A sigmoid curve is noted with an indication that the protein has its sites saturated (3 sites) when (BPB) free is near 1×10^{-3} M. It should be stated that any r-value (exclusive of the saturation value) is an average value, and may have a fractional unit. This is an implicit concept in multiple equilibria equations that utilize r-values in such a manner.

The logarithm function of (BPB) free is not important. Rather, it enables the binding data to be presented over a large concentration range.

Binding curves of this and similar forms do not usually indicate a saturation point. Generally, recourse to some extrapolation technique is needed. Figure 13 illustrates one of the most common methods to obtain pertinent parameters should the binding be statistical. (See Table XXIII). When $\frac{1}{r}$ is utilized as a function of $\frac{1}{(A)\text{free}}$, where (A) free represents the concentration of free interacting species in equilibrium with protein complexes
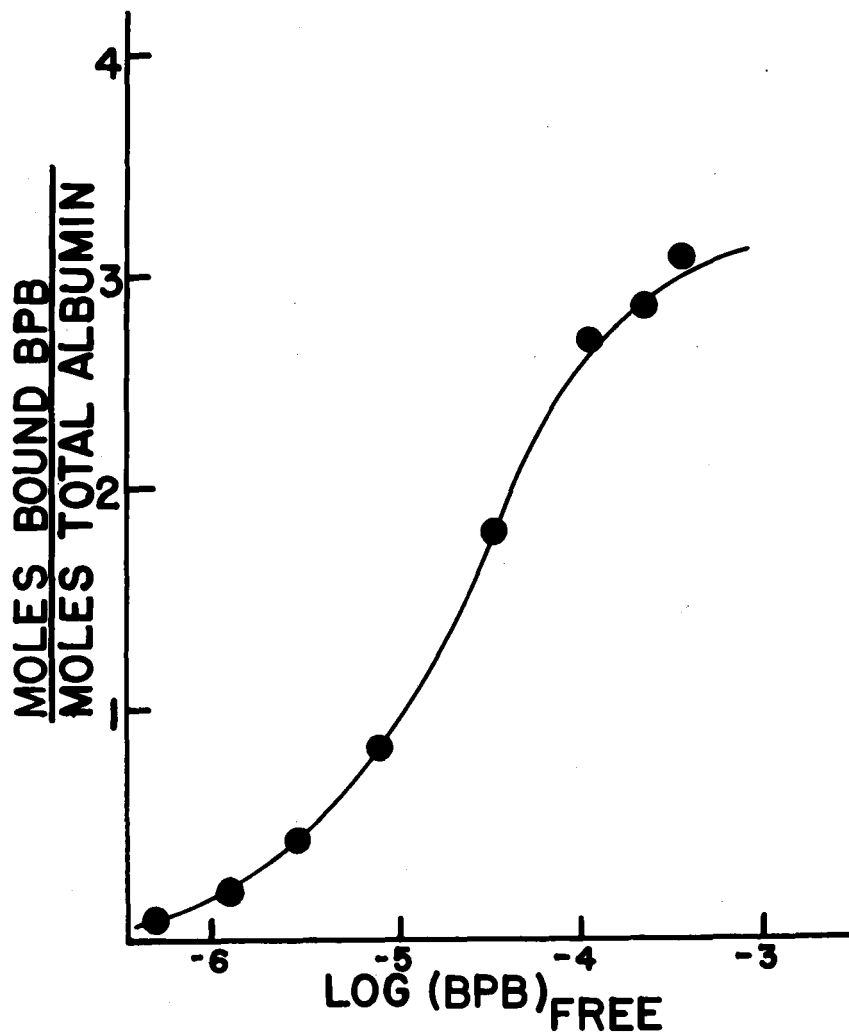
FIGURE 12

BINDING OF BROMPHENOL BLUE BY BOVINE

PLASMA ALBUMIN (VERONAL BUFFER,

0.05M, pH 8.6, 4°C.) See Table XXII
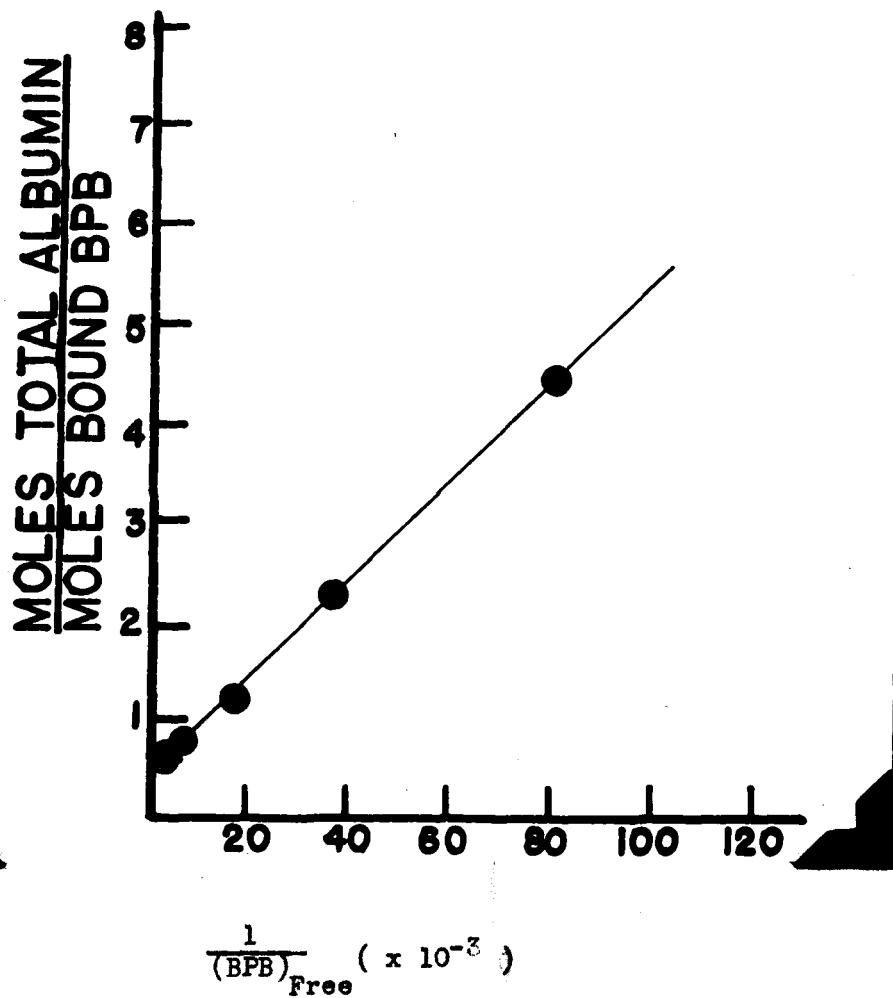
FIGURE 13

VALIDITY OF STATISTICAL BINDING OF THE

BPB-BPA SYSTEM. (SEE TABLES XXII and XXIII.)

of the type PA_i ($i = 1$ to n), a straight line would result if the binding is statistical. The intercept on the ordinate is $\frac{1}{n}$. The intrinsic binding constant, k, may be calculated from a knowledge of both n and the slope. The value $\frac{1}{kn}$ is equivalent to the slope of the straight line. Klotz (1953) has extensively used this method.

It should be stated that this relationship has been derived from general theories of multiple equilibria for the case in which there are n sites available for association on P, and the equations are presented in the form:

$$\frac{1}{r} = \frac{1}{nk} \cdot \frac{1}{(A)} + \frac{1}{n}$$

Scatchard (1949) has suggested the utilization of an essentially similar equation to obtain the parameters:

$$\frac{r}{(A)} = kn - kr.$$

In this case $\frac{r}{(A)}$ is plotted as a function of r. The intercept on the ordinate is kn and on the abscissa is n.

Both extrapolation procedures were applied to the data derived from this BPA-BPB system. A summary of the pertinent values is found in Table VIII.

<div align="center">

TABLE VIII

BINDING CONSTANTS OF BPA-BPB SYSTEM

</div>

	Method of Klotz	Method of Scatchard
n	5.05	3.18
k	6.20 x 10^4	6.06 x 10^4
k_1	18.60 x 10^4	18.18 x 10^4

TABLE VIII (cont.)

	Method of Klotz	Method of Scatchard
k_2	6.20×10^4	6.06×10^4
k_3	2.07×10^4	2.02×10^4
ΔF_1^0	-6723 cal/mole	-6701 cal/mole
ΔF_2^0	-6111 cal/mole	-6094 cal/mole
ΔF_3^0	-5499 cal/mole	-5485 cal/mole

It was interesting to note that a value of -6510 cal/mole (ΔF_1^0) was found by Klotz and Urquhart (1949) in their studies on the interaction between methyl-orange and bovine plasma albumin. In this case, the $(-SO_3^-)$ group of the dyestuff was visualized as binding on albumin cationic nitrogen sites. A similar situation may exist within the BPA-BPB system.

Brode (1924) and Haring and Heller (1941), quoted by Colichman (1951) have indicated that two and only two colored forms of BPB are responsible for the tautomeric equilibrium between a pH of 1 and physiological pH's. The yellow acidic form (I) is present at pH 1.

$$(\text{I}) \quad C_6H_4 \, (SO_3^-) \overset{+}{\underset{C}{}} \begin{cases} C_6H_3Br_2OH \\ C_6H_3Br_2O^- \end{cases}$$
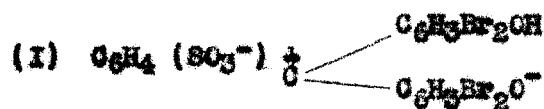
in aqueous solutions, while the purple or basic form II exists at higher pH

$$(\text{II}) \quad C_6H_4 \, (SO_3^-) \overset{+}{\underset{C}{}} \begin{cases} C_6H_3Br_2O^- \\ C_6H_3Br_2O^- \end{cases}$$
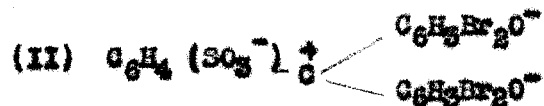
values in aqueous solutions. In all equilibrium dialysis studies an attempt was made to utilize structure II of BPB. Table XX demonstrates the effect of pH on the BPB absorption spectrum. Essentially, the curves are superimposable, probably indicating that similar quantities of BPB (II) exist in each solution.

A further study of the BPA-BPB system at pH 8.6, was not attempted, primarily because of the relatively low average number of dye molecules bound to one protein molecule. It was thought that the charge change on the protein in the complex form would not be greatly different from that of the pure protein, and, hence, would not lend itself to accurate ionographic investigation.

II and III.) BPA-BPB; Phosphate buffer, pH 7.4, 0.05 M with respect to phosphates; 4° and $36.5^{\circ}C$.; 0.7% BPA.

Figure 14 represents data (from Tables XXV and XXVI) in a graphic form demonstrating the small effect of temperature on the binding between BPA and BPB under the described experimental conditions.

A cursory comparison with Figure 12 will indicate that at a given (BPB) free value, r has a greater value in the phosphate buffer at pH 7.5. Presumably, this is mainly due to the increased number of cationic nitrogen groups as a result of an increase in the hydrogen ion concentration. The buffer system components must be considered as inhibiting dye-binding and, hence, it is not justifiable to attribute protein-dye interaction a function only of the medium pH. However, this trend (increased anion binding of proteins by decreasing the pH) has been observed by other workers; Karush, 1951; Klotz and Urquhart, 1949; and Boyer et al., 1947.

From the extrapolation procedures it was not possible to state that the binding was statistical. It is the author's observation and opinion that there exists a misleading contemporary tendency to make the data fit one of the linear parametric formulas. This can be accomplished by a biased selection of a distorted plot of the data.
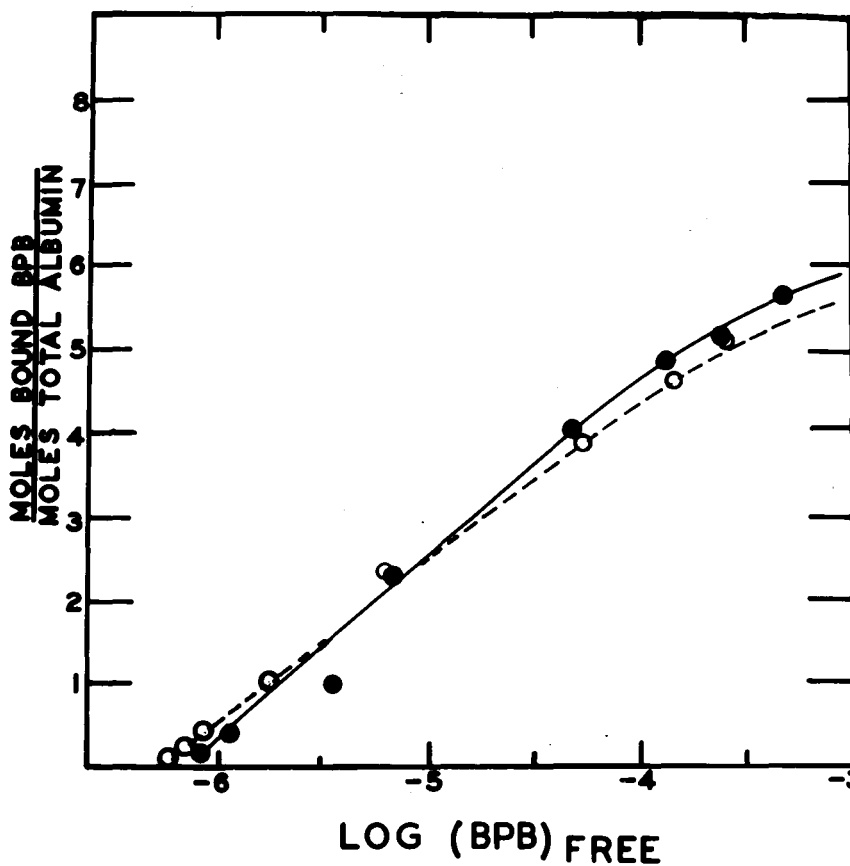
FIGURE 14

EFFECT OF TEMPERATURE ON THE BINDING BETWEEN BPB AND BPA.

(Phosphate Buffer, pH 7.4, 0.7% BPA)

o-----o 4°C.
•-----• 36.5°C.

(See Tables XXV and XXVI)

75

Smith and Briggs (1950) have used an alternate linear form of the Langmuir adsorption isotherm to plot the results of bovine serum albumin and methyl-orange interaction studies. Two straight lines of differing slope were drawn through two different groups of data. It was interpreted that two different types of sites were available on the protein for binding the dye-stuff. While these conclusions seem to be borne out in a second paper (Colvin and Briggs, 1952) there appear to be definite dangers in such a practice. Electrostatic phenomena may be obliterated.

The best straight line through the data of the BPA-BPB system at $36.5^{o}C.$ would indicate an n-value near seven. At $4^{o}C.$ n values of 5.1 to 6.5 may be calculated. The data, however, is probably indicative of electrostatic processes.

IV-V.) BPA-BPB; Phosphate buffer, pH 7.4, 0.05 M with respect to phosphates; 4° and $36.5^{o}C.$, 0.2% BPA.

The results of the studies on this system are presented in Figure 15 and Tables XXVII and XXVIII. Inspection of the figure reveals two important points. First, there is a definite decrease in the amount of binding as the temperature was increased; second, there appears to be a linear relationship between r and log (BPB) free. Comparison with the data of Figure 14 (Tables XXV and XXVI) reveals that the binding has generally decreased as a result of using a lower concentration of BPA.

Extrapolation techniques demonstrate that the binding fits into statistical forms better than does the 0.7% BPA-BPB system in the phosphate buffer. Assuming the binding is statistical, at $4^{o}C.$ the n value is 5.9 and at $36.5^{o}C.$ the n value is very near 5. However, the binding is probably
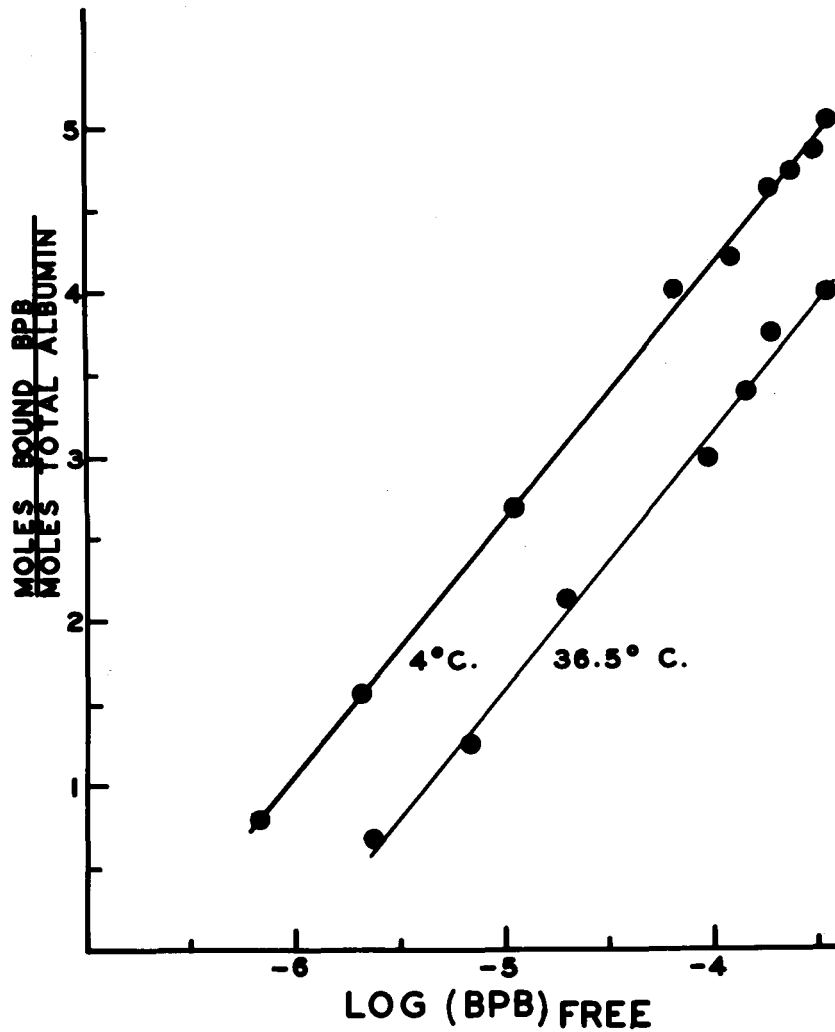
FIGURE 15

EFFECT OF TEMPERATURE ON THE BINDING BETWEEN BPB AND BPA

(Phosphate Buffer, pH 7.5, 0.2% BPA)

(See Tables XXVII and XXVIII)

electrostatic since definite deviation from a strict linear form does occur.

Klotz (1953) has already pointed out the significance of the effect of temperature on binding. Generally, there is little temperature effect on protein - ion interactions. It was indicated that for any equilibrium reaction which is not sensitive to temperature, the enthalpy is small. And if ΔH is small, the numerical value of ΔF at a given temperature is dependent on ΔS, the change in entropy.

It has been found that the entropies for such a reaction are of the order of $+5$ to $+25$ cal./mol/deg. The positive entropy values for such an association reaction have been interpreted as being mainly due to the release of water molecules from the binding groups on both the protein and ion. Positive entropies also exist for the association of BPB with BPA. Absolute values are not easily calculated since the binding is not statistical.

The linear relationships observed in Figure 15 may be only limit values. That is, should (BPB) free be varied higher or lower than the existing limits, possible deviations from a strict adherence to a linear form could result.

In the variations of the Langmuir adsorption isotherms that were discussed, it was not evident that the concentration of the protein was relevant. Rather, binding values, r, encompass only the concept of total protein. As demonstrated by the present studies, protein concentration definitely affects the r-value. Whether this reflects a change in protein structure, or the equilibrium medium, or both, is not clear.

A paper (Kusunoki, 1953) has come to the author's attention in which the interaction of bovine serum albumin (purity said to be better than

90%) with BPB has been studied by the spectrophotometric method of Shimao (1952). Apparently, the binding was assumed to be statistical and ΔF_1^o's were calculated on a conventional statistical basis. (Kusnoki refers only to ΔF's, but probably means ΔF_1^o values.) It was found that the n-values decreased both on alkali and formaldehyde denaturation. The trends are probably significant but all absolute values are questionable due to the omission of an electrostatic consideration of the results.

BFGG-BPB; Phosphate buffer, pH 7.4, 0.05 M with respect to phosphates; 4°C; 0.2% BFGG (See Table XXIX).

Within experimental error and limits of (BPB) free (1.29 x 10^{-5} to 2.67 x 10^{-3} M) there was no interaction between the protein and the dyestuff.

The relatively meager binding ability of the gamma globulins for a given anion is typical and is certainly of second-order importance when compared to the affinity of BPA for the same anion. The basis for this difference in proteins and the mechanisms describing similar reactions has caused some interest (Klotz, 1949). Klotz uses the ratio $\dfrac{\Sigma(\equiv NH^-)}{\Sigma(-OH) - \Sigma(-COO^-)}$

as a binding index. For a specified protein molecule, $\Sigma(\equiv NH^-)$ is equivalent to the sum of the cationic nitrogen groups; $\Sigma(-OH)$ is the sum of all hydroxy groups; $\Sigma(COO^-)$ is the sum of all carboxyl side chains. Generally, there is a direct relation between the binding index and binding affinity for a given anion when different proteins are compared.

The reasoning surrounding the utilization of this ratio involves a knowledge of hydrogen bond strengths and of the juxtaposition of the three groups. The application of the ratio seems adequate in some cases, but is

only a preliminary attempt to obtain a molecular viewpoint of the binding process.

Polyvinylpyrrolidone systems

Initial interaction studies were attempted with undialyzed K-30 preparations and BPB. It was evident that a measurable amount of PVP appeared in the dialyzate (of the order of 7-8% total PVP nitrogen when the previously described equilibrium dialysis cells were utilized). Obviously, the presence of PVP in the outer compartment would lead to misleading values after spectrophotometric-analyses of the quantity of BPB therein. Nevertheless, an attempt was made to correct the derived values and the data was plotted by a variation in conventional procedure. The data is not presented in this dissertation since the experiment was of preliminary importance only. It did serve to emphasize the importance of using pre-dialyzed PVP samples.

Subsequent equilibrium dialysis studies were performed on exhaustively pre-dialyzed polymer preparations of which titration data was available. (Chapter III).

Typical data has been plotted in Figure 16. In this case, the r-value is defined as $\left(\dfrac{\text{MOLES BOUND BPB}}{\text{MOLES TOTAL PVP NITROGEN}} \right)$. (A molecular weight of 14.0 was used for nitrogen). Since the molecular weight distribution was not characterized after the pre-dialysis procedure, it was not accurate to describe r in terms of $\left(\dfrac{\text{MOLES BOUND BPB}}{\text{MOLES TOTAL PVP}} \right)$. The dialysis sacs contained 58 mgs. % PVP nitrogen and presumably a similar number of repeating units. (A PVP activity difference existed in the PVP-D30, PVP-D60, PVP-D90 samples).

The following points, concepts, and trends seem evident from the
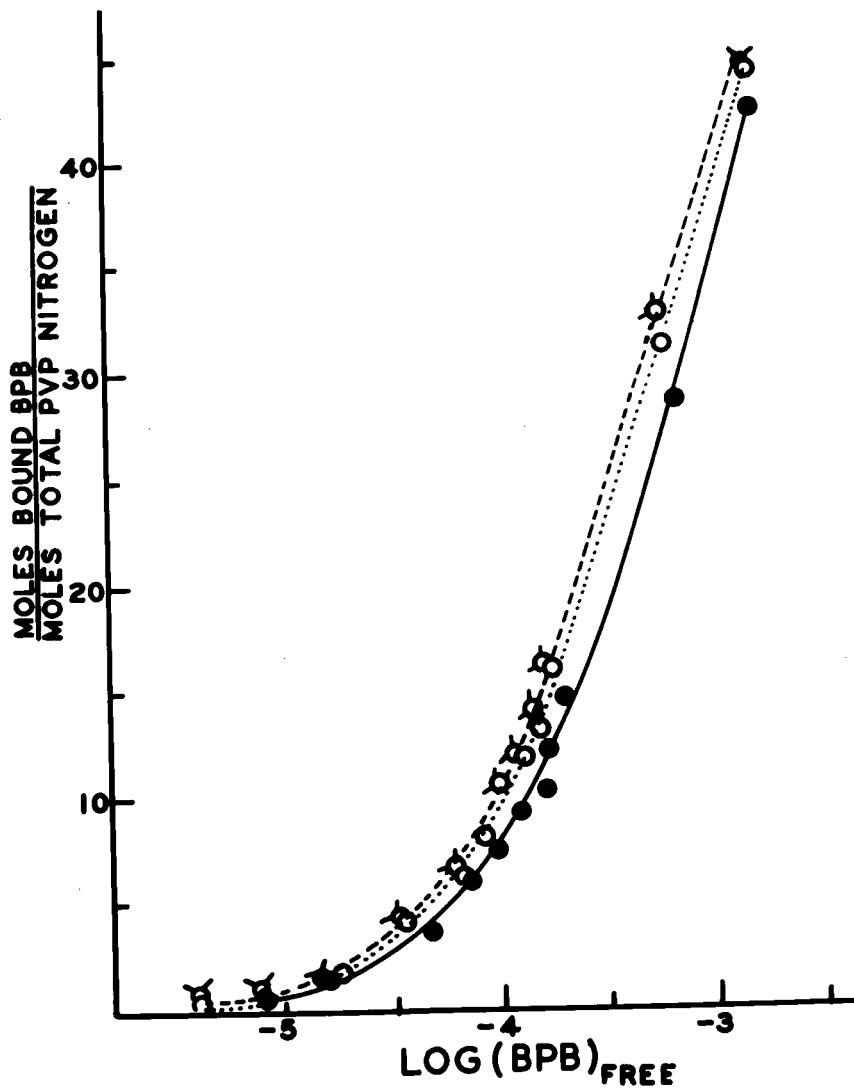
FIGURE 16

BINDING OF BROMPHENOL BLUE BY PVP-D30,

PVP-D60, and PVP-D90 PREPARATIONS;

●——● PVP-D30   ⊅——⊅ PVP-D90
o...o PVP-D60   ordinate (r x 10^3)
(See tables XXX, XXXII, and XXXIV)

data:

1.) At 4°C. the binding at high (BPB) free values was seen to decrease in the order: PVP-D90, PVP-D60, PVP-D50. (See Figure 16, Tables XXX, XXXII, XXXIV). Possibly this may indicate an activity difference.)

2.) At 36.5°C. no analagous trends appeared. Essentially, the three samples exhibited similar affinities for BPB at all (BPB) free values. (Results not shown in graphic form but may be substantiated by perusal of Tables XXXI, XXXIII, and XXXV).

3.) Binding was decreased by increasing the temperature. This was characteristic of all polymer preparations. (As an example, see Figure 17 for the effect of a 22.5°C. change in temperature on the binding between PVP-D90 and BPB.)

4.) At the highest levels of (BPB) free, r had values near 0.045. This indicated that about 4.5% of the repeating units were associated with the dye. (One atom of nitrogen was assumed to be associated with one PVP repeating unit. It is not necessarily evident that BPB combines with the nitrogen atom.) Using the data obtained with the PVP-D90 sample and assuming a molecular weight of 350,000, it can be calculated that the greatest binding (experimental) conforms to a value of 143 molecules of dye bound with one molecule of PVP.

5.) The results of extrapolation procedures using the (PVP-D90)-(BPB) system are found in Figure 18, (after Klotz), and Figure 19, (after Scatchard). Typically linear relations were found for other PVP-D systems. On this basis, the binding between BPB and PVP appeared to be statistical throughout the entire (BPB) free range that was investigated. Apparently
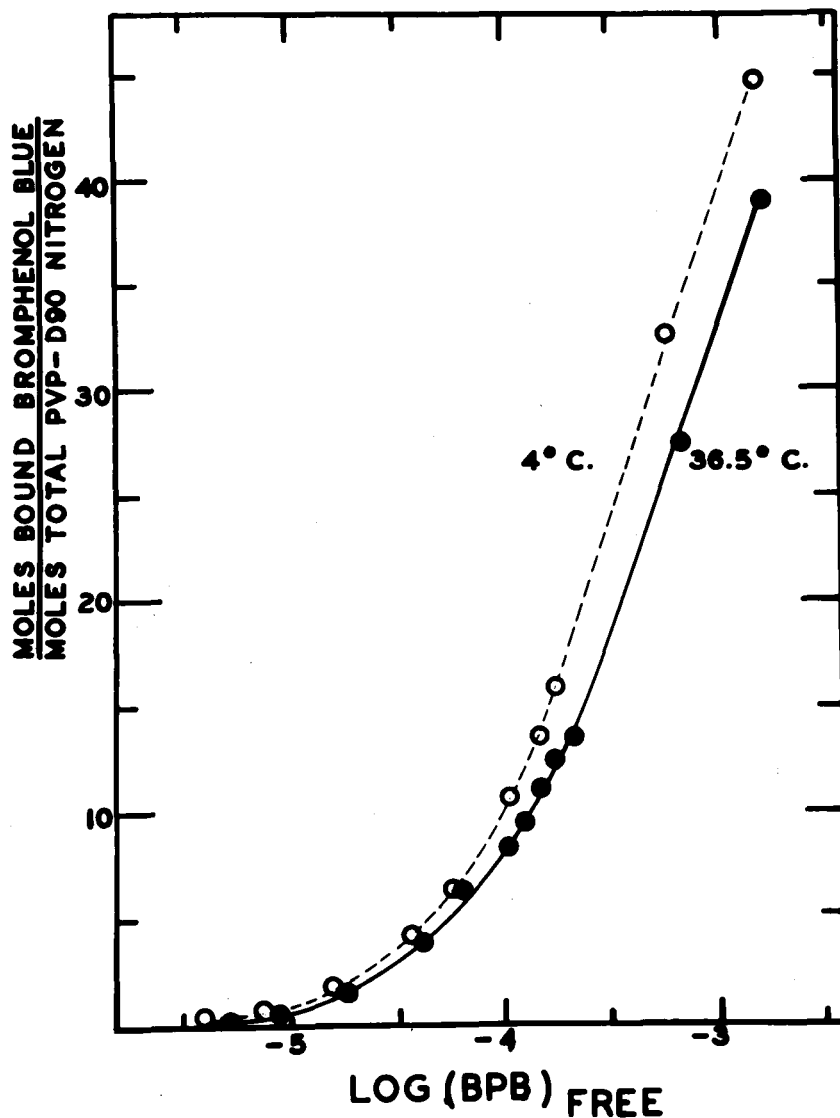
FIGURE 17

EFFECT OF TEMPERATURE ON THE BINDING OF BPB BY PVP-D90. Ordinate ($r \times 10^3$)

PHOSPHATE BUFFER, pH 7.4, 4°C.

(See Tables XXXIV and XXXV.)

83
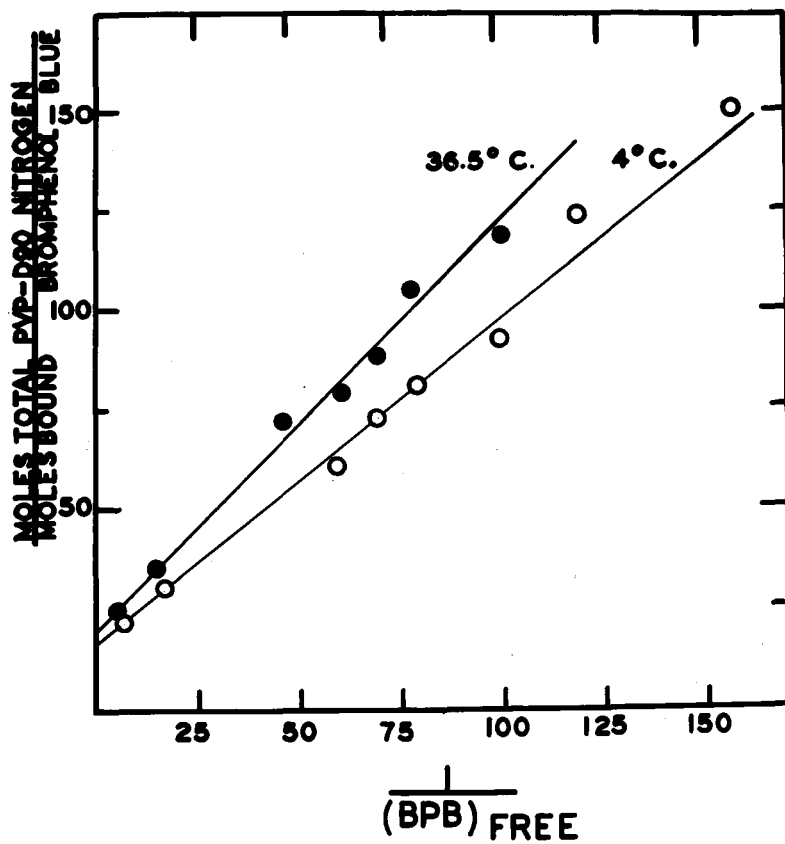
FIGURE 18

VALIDITY OF STATISTICAL BINDING BETWEEN

BPB AND PVP-D90. ABSCISSA IS $\frac{1}{\overline{(BPB)free}}$ x 10^{-2}

(SEE TABLES XXXIV AND XXXV.)
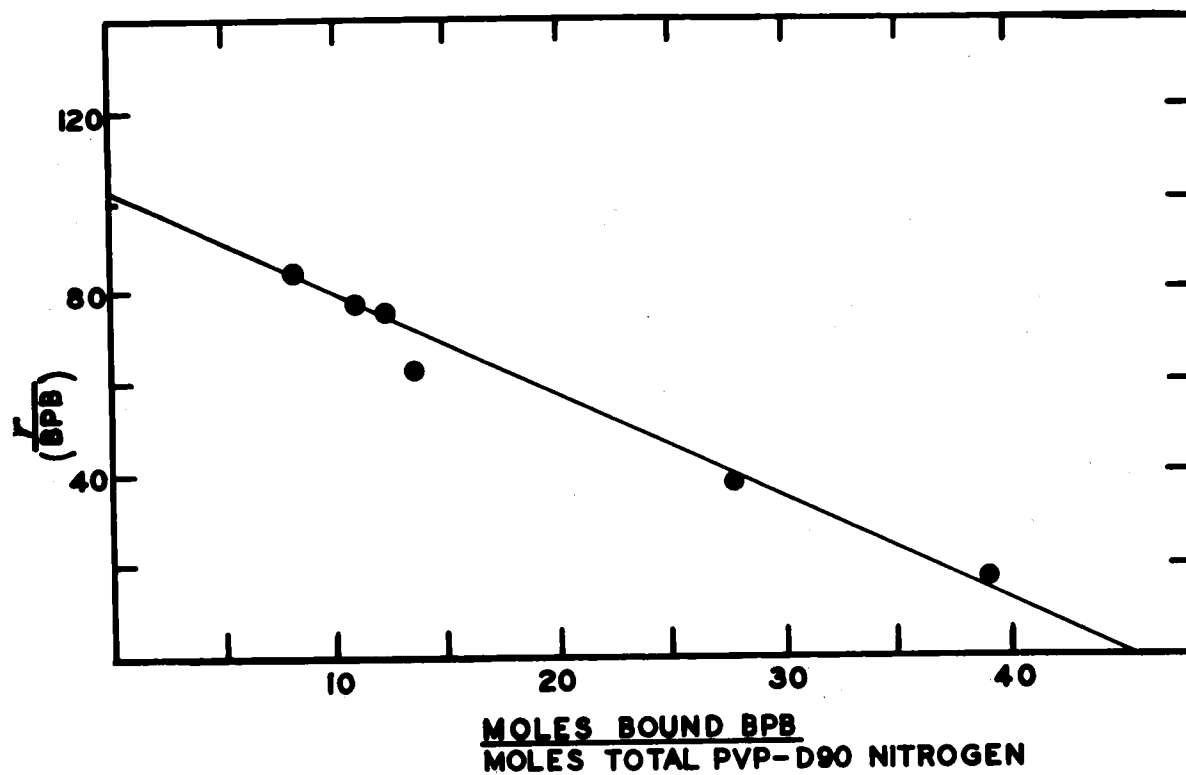
FIGURE 19

VALIDITY OF STATISTICAL BINDING BETWEEN BPB AND PVP-D90 (After Scatchard).

Abscissa is $(r \times 10^2)$, ordinate is $\left(\dfrac{r}{BPB}\right) \times 10^6$

one type of site was available or else a spectrum of nearly identical sites were present. That these sites must be predominantly on the repeating units is evident from a knowledge of the r-values and with the assumption of a non-branched polymer.

The question must arise as to whether the small number of titratible groups in the PVP samples could be responsible for binding in conjuction with non-ionizable sites on the repeating units. If only a small number of such sites occur and possess a small affinity for dye (ΔF_1^0 a small negative number), a deviation in the two linear parametric extrapolation techniques would not be expected to occur.

6.) It will be observed that both the intercept and slope are increased in Figure 17 as a result of an increase in temperature. The data corresponds to that of the (PVP-D90)-(BPB) system, but similar trends were observed with the other two PVP-D systems. Since the intercept corresponds to the reciprocal of the maximum number of sites available to the BPB, it is apparent that more sites are open to BPB on PVP as a result of a decrease in temperature. (Caution must be exerted with respect to a strict definition of n and the values obtained as a result of this extrapolation. Should the binding occur on the repeating unit, n would be expected to be maximally a small whole number.) In this case $n_{36.5^\circ C.} = 0.050$, and $n_{4^\circ C.}$ is equivalent to 0.059. This may be interpreted to mean that 5.0% and 5.9%, respectively, of the repeating units may be occupied by BPB at a maximum.

Calculation of k, the intrinsic binding constant, reveals that its value is a function of the temperature. k was found to decrease slightly as the temperature was increased.

A few comments should be made to describe the type of structures on PVP that may be responsible for binding the BPB. Oster and Immergut (1954) have found the ultraviolet spectrum of PVP to be sensibly independent of pH except at extremes of pH (0.8 and 12). A similar dependence on pH was exhibited by N-ethylpyrrolidone. The tentative polymer structure of PVP is shown in Figure 20 (I). (After Thrower and Campbell, 1951). Accordingly, Weese (1948) has suggested that the molecule should be neutral. That the polymer is essentially non-ionic and only weakly amphoteric (Miller and Hamm, 1953) may be substantiated by the free solution electrophoretic work of Sullivan et al. (1952). PVP at pH 7 was found to have almost a zero mobility. At a low pH, the polymer migrated to the negative pole. At high pH values, the direction of movement was reversed. Oster and Immergut would like to base these observations on the structure in Figure 20. The repeating unit II would represent the electrically neutral form. At a pH value below 1, the polymer is positively charged and possesses repeating units of the form III. At high pH's a proton was visualized as being abstracted from the carbon atom adjacent to the carbonyl group. The extra pair of electrons then shifts into the ring to give the enolic structure. Further study is necessary to localize the binding site or sites involved in the interaction with the compounds listed in Tables I and II.
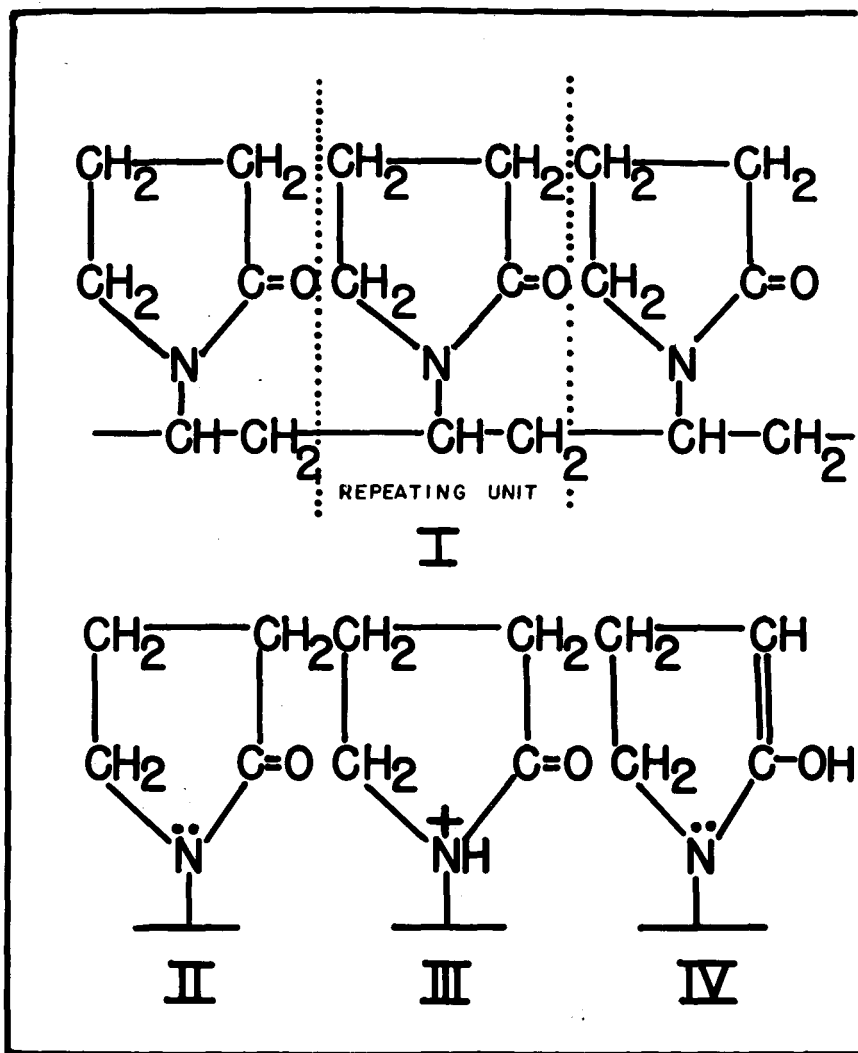
FIGURE 20

STRUCTURES OF POLYVINYLPYRROLIDONE

I. TENTATIVE POLYMER STRUCTURE

II, III, AND IV, SEE TEXT.

CHAPTER VI

SUMMARY

Studies were initiated to compare the binding mechanisms of certain plasma proteins and those of three polyvinylpyrrolidone samples. The resultant interpretations were clarified by correlating the data obtained by the technique of titrimetry, ionography, equilibrium dialysis, and spectrophotometry. As a result of the investigation, a number of concepts have taken shape and several analytical procedures were developed which appear to possess valuable potentialities when used in interaction studies.

Statements of the more pertinent points are as follows:

1.) Two ionographic analytical procedures have been developed to study the properties of protein-dye complexes and PVP-dye complexes. These techniques enable the rate of complex dissociation to be compared with the rate of ionographic resolution. However, their primary value appears to be in the determination of the type of charge change as a result of complexing.

The interference and significance during interaction studies of acid-base-salt equilibria, and dilution and diffusion processes have been discussed.

2.) Undialyzed PVP samples seem to possess at least two different types of ionizable groups; one having a pK near 4.3; the other near 10.

3.) Dialyzed PVP samples have titration curves which are distinctly different from the undialyzed analogues. There are less titratable groups present in dialyzed preparations. The dialyzed differential titration curves

89

closely resemble the curve obtained from the titration of an aqueous neutral salt solution.

4.) Exhaustively dialyzed PVP samples and undialyzed PVP samples all combined with the organic anion BPB. An exact comparison of the affinities of these PVP preparations was not made, but quantitative binding data was assembled for three dialyzed PVP samples (PVP-D50, PVP-D60, PVP-D90).

5.) Dialyzed PVP preparations combine with bromphenol blue to form complexes which:

    a.) have a greater net negative charge than PVP.

    b.) are quickly dissociable on the basis of ionographic resolution.

    c.) have the BPB bound on the repeating unit of the polymer rather than only on acidic end groups as postulated by other workers.

6.) The association reaction between dialyzed PVP preparations and BPB has certain characteristics:

    a.) the binding may be described with statistical terminology using the concepts of multiple equilibria in conjunction with several forms of the Langmuir isotherm. No evidence of an electrostatic factor could be demonstrated. Only one type of site appeared to be involved in the binding process. Should the binding occur on the repeating units, only a small percentage can be involved (of the order of 5%).

    b.) binding decreased with an increase in temperature. With certain assumptions, the entropy may be calculated to have a positive value.

7.) Bovine plasma albumin combines with bromphenol blue in such a manner as to indicate that:

a.) the binding is statistical at a pH of 8.6 under specified experimental conditions. By decreasing the pH to 7.4, the binding is increased but is no longer statistical. Thermodynamic quantities have been calculated where possible.

b.) the $(-SO_3^-)$ group of BPB is probably attached to a protein cationic nitrogen group.

c.) the complexes have a greater net negative charge than does the uncomplexed albumin. All complexes were quickly dissociable on the basis of ionographic resolution.

d.) secondary and tertiary albumin structures affect the amount of dye interaction.

8.) Bovine plasma gamma globulin does not combine with BPB (phosphate buffer, 0.05 M, pH 7.4, 4°C). The basis of comparative protein binding abilities has been discussed in conventional terminology.

APPENDIX

TABLE IX

TITRATION OF 0.05 M SODIUM CHLORIDE SOLUTION
(glass distilled water)

Experimental Conditions:

        Temperature: 25°C

        Titrated with: 0.01 N sodium hydroxide

        Titration of: 50 ml 0.05 M sodium chloride solution plus 2 ml 0.0099

                N hydrochloric acid - 0.05 M sodium chloride solution.

pH before addition of acid: 6.5 to 7.5

pH after addition of acid: 3.45

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
0.00	3.45	1.40	3.99	2.05	8.17
0.10	3.47	1.45	4.02	2.065	8.24
0.20	3.48	1.50	4.06	2.085	8.47
0.30	3.51	1.55	4.12	2.105	8.64
0.40	3.53	1.60	4.17	2.125	8.75
0.50	3.58	1.65	4.22	2.145	8.86
0.60	3.60	1.70	4.29	2.165	8.92
0.70	3.63	1.75	4.38	2.180	9.01
0.80	3.68	1.80	4.49	2.200	9.08
0.90	3.71	1.85	4.62	2.220	9.13
1.00	3.75	1.90	4.78	2.25	9.20
1.10	3.80	1.98	5.28	2.30	9.32
1.20	3.86	2.00	5.70	2.35	9.41
1.30	3.91	2.025	6.42	2.40	9.49

TABLE IX (cont.)

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
2.45	9.55	3.20	10.07	5.00	10.49
2.50	9.61	3.40	10.14	5.40	10.52
2.60	9.71	3.60	10.20	6.00	10.61
2.70	9.79	3.85	10.26	7.00	10.70
2.90	9.92	4.00	10.30	8.00	10.78
3.00	9.98	4.25	10.37	9.00	19.82
3.10	10.02	4.55	10.42	10.00	10.88

TABLE X

TITRATION OF PVP-U30 SOLUTION

Experimental Conditions:

Temperature: 25° C.

Titration of: 50 ml PVP-U30 (58 mg % N) - 0.05 M sodium chloride

solution plus 2 ml 0.0099 N Hydrochloric acid - 0.05

M sodium chloride solution

pH before addition of acid: 4.19

pH after addition of acid: 3.43

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
0.00	3.43	1.80	4.04	2.74	5.35
0.10	3.44	1.90	4.10	2.76	5.60
0.20	3.45	2.00	4.18	2.83	5.86
0.30	3.48	2.05	4.20	2.85	6.02
0.40	3.50	2.10	4.24	2.87	6.21
0.60	3.56	2.25	4.38	2.89	6.43
0.80	3.62	2.30	4.44	2.90	6.54
1.00	3.68	2.35	4.50	2.91	6.68
1.10	3.72	2.40	4.57	2.92	6.80
1.20	3.75	2.45	4.64	2.94	6.98
1.30	3.78	2.50	4.71	2.96	7.25
1.40	3.83	2.60	4.92	2.98	7.51
1.51	3.88	2.65	5.04	3.00	7.71
1.60	3.92	2.70	5.18	3.02	7.98
1.70	3.99	2.72	5.27	3.05	8.23

TABLE X (cont.)

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
3.085	8.38	3.60	9.45	6.20	10.42
3.10	8.45	3.70	9.63	6.80	10.51
3.135	8.58	4.00	9.78	7.20	10.55
3.15	8.65	4.20	9.88	7.90	10.63
3.20	8.81	4.40	9.98	9.00	10.72
3.30	9.04	4.70	10.09	10.00	10.78
3.35	9.13	5.00	10.17	11.00	10.84
3.40	9.21	5.40	10.26	12.20	10.89
3.50	9.34	5.80	10.34	13.00	10.92

TABLE XI

TITRATION OF PVP-U60 SOLUTION

Experimental Conditions:

Temperature: 25° C.

Titration of: 50 ml PVP-U60 (58 mg % N) - 0.05 M sodium chloride

solution plus 2 ml 0.0099 N hydrochloric acid - 0.05

M sodium chloride solution

pH before addition of acid: 4.63

pH after addition of acid: 3.47

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
0.00	3.47	1.40	3.92	2.32	5.53
0.10	3.48	1.50	3.98	2.345	5.78
0.20	3.51	1.60	4.06	2.365	6.05
0.30	3.52	1.70	4.12	2.39	6.53
0.40	3.55	1.80	4.22	2.40	6.72
0.50	3.58	1.90	4.32	2.415	7.06
0.60	3.61	2.00	4.48	2.44	7.58
0.70	3.64	2.05	4.55	2.45	7.76
0.80	3.68	2.15	4.77	2.46	7.92
0.90	3.70	2.20	4.91	2.47	7.98
1.00	3.74	2.22	4.98	2.48	8.08
1.10	3.78	2.25	5.09	2.50	8.28
1.20	3.82	2.27	5.21	2.52	8.42
1.30	3.88	2.29	5.32	2.54	8.52

TABLE XI (cont.)

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
2.56	8.67	2.95	9.48	4.50	10.25
2.59	8.76	3.00	9.53	4.70	10.29
2.60	8.78	3.05	9.57	5.00	10.36
2.65	8.92	3.10	9.62	5.50	10.44
2.70	9.04	3.20	9.71	6.00	10.51
2.75	9.17	3.40	9.82	7.00	10.62
2.80	9.26	3.50	9.93	8.00	10.71
2.85	9.34	4.00	10.10	9.00	10.78
2.90	9.41	4.20	10.17	10.00	10.83

TABLE XII

TITRATION OF PVP-U90 SOLUTION

Experimental Conditions:

        Temperature: 25° C.

        Titration of: 50 ml PVP-U90 (58 mg % N) - 0.05 M Sodium chloride

                       solution plus 2 ml 0.0099 N hydrochloric acid - 0.05

                       M sodium chloride solution

pH before addition of acid: 4.84

pH after addition of acid: 3.44

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
0.00	3.44	1.40	3.97	2.155	5.52
0.11	3.48	1.50	4.03	2.170	5.67
0.20	3.49	1.60	4.12	2.19	5.91
0.30	3.52	1.70	4.21	2.20	6.08
0.40	3.55	1.85	4.41	2.215	6.33
0.50	3.58	1.90	4.49	2.23	6.69
0.60	3.60	1.95	4.59	2.24	6.89
0.70	3.63	2.00	4.72	2.25	7.21
0.80	3.68	2.03	4.82	2.26	7.43
0.90	3.71	2.05	4.88	2.275	7.73
1.00	3.75	2.085	5.02	2.285	7.90
1.10	3.79	2.10	5.11	2.29	8.03
1.20	3.84	2.125	5.24	2.30	8.13
1.30	3.90	2.14	5.37	2.31	8.23

TABLE XII (cont.)

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
2.32	8.32	2.70	9.45	4.25	10.24
2.33	8.38	2.75	9.51	4.50	10.29
2.34	8.48	2.80	9.56	4.75	10.35
2.35	8.56	2.90	9.66	5.00	10.40
2.37	8.67	3.00	9.74	5.50	10.48
2.39	8.72	3.10	9.80	6.00	10.53
2.45	8.96	3.20	9.87	6.50	10.59
2.50	9.10	3.30	9.92	7.00	10.64
2.55	9.21	3.40	9.97	8.00	10.72
2.60	9.29	3.50	10.01	9.00	10.79
2.65	9.38	3.70	10.09	10.00	10.83
		4.00	10.18		

TABLE XIII

TITRATION OF 0.05 M SODIUM CHLORIDE SOLUTION

Experimental Conditions:

     Temperature: 25° C.

     Titration of: 50 ml 0.05 M sodium chloride solution plus 3 ml 0.094

               N hydrochloric acid - 0.05 M sodium chloride solution.

pH before addition of acid: 6.5 to 7.5

pH after addition of acid: 3.29

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
0.00	3.29	1.60	3.67	2.79	4.72
0.10	3.31	1.70	3.71	2.803	4.77
0.20	3.33	1.80	3.74	2.81	4.79
0.30	3.35	1.90	3.79	2.82	4.84
0.40	3.37	2.00	3.86	2.83	4.88
0.50	3.39	2.10	3.89	2.84	4.96
0.60	3.42	2.20	3.94	2.85	4.98
0.70	3.44	2.40	4.09	2.86	5.05
0.80	3.48	2.50	4.19	2.87	5.13
0.90	3.50	2.60	4.32	2.88	5.22
1.00	3.51	2.65	4.38	2.89	5.32
1.10	3.52	2.70	4.49	2.90	5.45
1.20	3.55	2.72	4.53	2.91	5.58
1.30	3.58	2.75	4.62	2.92	5.83
1.40	3.61	2.77	4.67	2.93	6.22

TABLE XIII (cont.)

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
2.94	6.70	3.25	9.23	4.30	10.08
2.95	7.22	3.30	9.32	4.50	10.14
2.96	7.78	3.35	9.39	4.70	10.19
2.97	7.94	3.40	9.46	5.00	10.28
2.98	8.09	3.45	9.52	5.50	10.39
2.99	8.22	3.50	9.58	6.00	10.47
3.00	8.34	3.60	9.68	6.50	10.53
3.02	8.53	3.70	9.76	7.00	10.59
3.04	8.67	3.80	9.82	8.00	10.68
3.10	8.75	3.90	9.89	8.50	10.72
3.15	8.94	4.00	9.95	9.00	10.76
3.20	9.08	4.10	9.99		

TABLE XIV

TITRATION OF PVP-D30 SOLUTION

Experimental Conditions:

Temperature: 25° C.

Titration of: 50 ml PVP-D30 (58 mg % N) - 0.05 M sodium chloride

solution plus 3 ml 0.0094 N hydrochloric acid - 0.05

M sodium chloride solution.

pH before addition of acid: 7 -

pH after addition of acid: 3.37

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
0.00	3.37	1.50	3.77	2.76	5.32
0.10	3.38	1.60	3.80	2.78	5.45
0.20	3.40	1.70	3.84	2.80	5.58
0.30	3.41	1.80	3.90	2.82	5.77
0.40	3.44	1.90	3.96	2.84	6.01
0.50	3.47	2.00	4.02	2.86	6.25
0.60	3.49	2.10	4.10	2.87	6.38
0.70	3.51	2.30	4.19	2.88-	6.52
0.80	3.53	2.40	4.42	2.89	6.70
0.90	3.57	2.50	2.57	2.905	6.82
1.00	3.60	2.55	4.65	2.915	7.02
1.10	3.62	2.60	4.77	2.925	7.17
1.20	3.65	2.70	5.05	2.93	7.28
1.30	3.68	2.72	5.13	2.94	7.43
1.40	3.72	2.74	5.22	2.95	7.58

TABLE XIV (cont.)

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
2.96	7.69	3.20	8.98	4.40	10.05
2.97	7.77	3.25	9.11	4.60	10.13
2.98	7.90	3.30	9.21	4.70	10.17
2.99	7.99	3.35	9.29	5.00	10.24
3.00	8.09	3.40	9.34	5.50	10.36
3.02	8.26	3.45	9.42	6.00	10.36
3.04	8.39	3.50	9.48	6.50	10.51
3.06	8.50	3.70	9.68	7.00	10.58
3.08	8.59	3.80	9.74	7.50	10.61
3.10	8.68	3.90	9.82	8.00	10.68
3.12	8.74	4.00	9.87	8.50	10.70
3.14	8.81	4.10	9.93	9.00	10.74
3.16	8.88	4.20	9.98	10.00	10.81

TABLE XV

TITRATION OF PVP-D60 SOLUTION

Experimental Conditions:

        Temperature: 25°C.

        Titration of: 50 ml PVP-D60 - 0.05 M sodium chloride solution plus

                3 ml 0.0094 N hydrochloric acid - 0.05 M sodium chlor-

                ide solution.

pH before addition of acid: 6.12-

pH after addition of acid: 3.32

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
0.00	3.32	1.50	3.67	2.77	4.93
0.10	3.34	1.60	3.72	2.80	5.08
0.20	3.36	1.70	3.75	2.82	5.23
0.30	3.38	1.80	3.79	2.85	5.46
0.40	3.39	1.90	3.85	2.86	5.57
0.50	3.42	2.00	3.89	2.87	5.69
0.60	3.43	2.10	3.96	2.88	5.88
0.70	3.46	2.20	4.02	2.89	6.08
0.80	3.48	2.30	4.10	2.90	6.30
0.90	3.51	2.40	4.19	2.91	6.61
1.00	3.53	2.50	4.32	2.92	6.94
1.10	3.55	2.60	4.48	2.93	7.26
1.20	3.58	2.65	4.58	2.94	7.49
1.30	3.61	2.70	4.72	2.95	7.67
1.40	3.64	2.75	4.87	2.96	7.84

TABLE XV (cont.)

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
2.97	7.96	3.20	9.12	4.00	9.92
2.98	8.09	3.25	9.22	4.20	10.02
2.99	8.20	3.30	9.30	4.40	10.10
3.00	8.30	3.45	9.52	4.60	10.17
3.02	8.48	3.50	9.57	5.00	10.27
3.04	8.58	3.60	9.67	6.00	10.46
3.06	8.68	3.70	9.75	7.00	10.58
3.10	8.84	3.80	9.82	8.00	10.68
3.15	9.02	3.90	9.87	9.00	10.75
				10.00	10.82

TABLE XVI

TITRATION OF PVP-D90 SOLUTION

Experimental Conditions:

Temperature: 25° C.

Titration of: 50 ml PVP-D90 - 0.05 M sodium chloride solution plus

3 ml 0.01 N hydrochloric acid - 0.05 M sodium chloride

solution.

pH before addition of acid: 6.5 -

pH after addition of acid: 3.33

CC. BASE ADDED (TOTAL)	ph	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
0.00	3.33	1.60	3.75	2.74	5.49
0.10	3.36	1.70	3.79	2.75	5.58
0.20	3.38	1.80	3.84	2.76	5.68
0.30	3.39	1.90	3.89	2.77	5.83
0.40	3.42	2.00	3.96	2.78	6.04
0.50	3.44	2.10	4.03	2.79	6.26
0.60	3.46	2.20	4.10	2.80	6.49
0.70	3.48	2.30	4.21	2.81	6.85
0.90	3.53	2.40	4.32	2.82	7.13
1.00	3.56	2.50	4.50	2.83	7.42
1.10	3.58	2.60	4.91	2.84	7.63
1.20	3.62	2.70	5.13	2.85	7.82
1.30	3.65	2.71	5.22	2.86	7.99
1.40	3.68	2.72	5.27	2.87	8.13
1.50	3.72	2.73	5.33	2.89	8.31

TABLE XVI (cont.)

CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH	CC. BASE ADDED (TOTAL)	pH
2.90	8.35	3.30	9.43	4.20	10.04
2.95	8.67	3.40	9.56	4.40	10.12
3.00	8.87	3.50	9.63	4.60	10.18
3.05	9.00	3.60	9.73	5.00	10.29
3.10	9.13	3.70	9.80	6.00	10.46
3.15	9.21	3.80	9.86	7.00	10.58
3.20	9.32	3.90	9.92	8.00	10.68
3.25	9.38	4.00	9.96	9.00	10.75
				10.00	10.81

TABLE XVII

LINEARITY CURVE — MOVEMENT VS. TIME

Experimental Conditions:

    general ionographic procedure (see text).

    Sodium veronal — veronal buffer, ph 8.6, ionic strength 0.02;

    temperature of 2-3°C.; E & D #613 filter paper; helium atmosphere.

TIME (hrs.)	MOVEMENT: mm. // V. / cm	
	BPB	BPA
1.00	3.08	2.42
1.50	4.40	3.21
2.26	5.92	4.90
3.00	8.34	5.40
4.00	10.30	7.50
5.00	12.66	9.18
6.00	15.63	9.94
7.00	17.40	12.00
8.00	20.24	13.54
9.50	24.40	16.34

TABLE XVIII

PVP-BPB ABSORPTION SPECTRA

Experimental Conditions:

5×10^{-6} M with respect to BPB, 11.6 mg% PVP nitrogen, 0.01 M with respect to sodium chloride, 0.01 M with respect to phosphates in phosphate buffer of pH 7.4; Coleman Spectrophotometer with PC-4 filter

WAVELENGTH (mµ)	BPB	OPTICAL DENSITY PVP-U30	PVP-D30	PVP-U90	PVP-D90
400	0.041	0.045	0.047	0.048	0.048
410	0.033	0.037	0.033	0.039	0.038
420	0.024	0.026	0.027	0.029	0.027
430	0.023	0.023	0.023	0.027	0.026
440	0.019	0.021	0.027	0.023	0.023
450	0.023	0.023	0.023	0.027	0.024
460	0.029	0.028	0.027	0.030	0.030
470	0.037	0.033	0.033	0.034	0.034
480	0.049	0.042	0.041	0.043	0.044
490	0.063	0.055	0.052	0.055	0.053
500	0.083	0.068	0.068	0.068	0.068
510	0.107	0.089	0.087	0.088	0.088
520	0.138	0.117	0.113	0.114	0.113
530	0.174	0.150	0.147	0.147	0.147

TABLE XVIII (cont.)

WAVELENGTH (mu)	BPB	PVP-U30	OPTICAL DENSITY PVP-D30	PVP-U90	PVP-D90
540	0.216	0.189	0.188	0.186	0.187
550	0.264	0.234	0.231	0.228	0.232
560	0.314	0.284	0.278	0.283	0.279
570	0.368	0.340	0.332	0.333	0.332
575	0.393	0.370	0.358	0.363	0.359
580	0.411	0.395	0.384	0.388	0.383
585	0.420	0.418	0.404	0.409	0.403
590	0.413	0.423	0.415	0.420	0.414
595	0.398	0.419	0.412	0.419	0.410
600	0.362	0.400	0.394	0.403	0.393
605		0.380		0.371	0.367
610	0.270	0.331	0.327	0.333	0.328
620	0.182	0.244	0.237	0.243	0.242
630	0.112	0.164	0.163	0.163	0.158

TABLE XIX

BPB AND PBP-BPA ABSORPTION SPECTRA

Experimental Conditions:

Variable wavelength automatic scanner.

WAVELENGTH	OPTICAL DENSITY	
(mu)	BPB	PBP-BPA
400	0.040	0.040
410	0.040	0.075
420	0.055	0.065
430	0.063	0.078
440	0.040	0.060
450	0.055	0.065
460	0.64	0.100
470	0.085	0.100
480	0.115	0.120
490	0.125	0.125
500	0.178	0.188
510	0.220	0.203
520	0.265	0.240
530	0.270	0.273
540	0.308	0.265
550	0.338	0.330
560	0.379	0.360
575	0.388	0.385

TABLE XIX (cont.)

WAVELENGTH (mu)	OPTICAL DENSITY	
	BPB	BPB-BPA
580	0.405	0.380
585	0.417	0.360
590	0.405	0.306
595	0.330	0.338
600	0.298	0.320
610	0.214	0.242
620	0.135	0.163

TABLE XX

EFFECT OF pH ON BPB ABSORPTION SPECTRUM

Experimental Conditions:

Coleman Spectrophotometer with PC-4 filter;

Solutions were 5×10^{-6} M with respect to BPB,

Phosphate buffers of pH 6.95 and pH 7.52 were 0.05 M with respect to phosphates;

Veronal buffer of pH 8.65 was of approximately 0.05 ionic strength.

WAVELENGTH (mu)	OPTICAL DENSITY		
	pH 6.95	pH 7.52	pH 8.65
400	0.046	0.042	0.042
410	0.034	0.032	0.032
420	0.026	0.023	0.024
430	0.025	0.021	0.022
440	0.021	0.018	0.022
450	0.026	0.022	0.022
460	0.031	0.028	0.029
470	0.038	0.035	0.038
480	0.049	0.047	0.050
490	0.066	0.062	0.064
500	0.084	0.081	0.083
510	0.110	0.108	0.110
520	0.139	0.138	0.140

TABLE XX (cont.)

WAVELENGTH (mu)	OPTICAL DENSITY		
	pH 6.95	pH 7.52	pH 8.65
530	0.179	0.178	0.180
540	0.223	0.222	0.225
550	0.270	0.270	0.272
560	0.320	0.318	0.322
570	0.378	0.375	0.381
580	0.421	0.420	0.426
585	0.432	0.430	0.436
590	0.428	0.425	0.432
600	0.370	0.369	0.376
610	0.278	0.278	0.285
620	0.188	0.187	0.192
630	0.113	0.112	0.116
640	0.067	0.064	0.069
650	0.039	0.039	0.042

TABLE XXI

DETERMINATION OF DYE-MEMBRANE ADSORPTION (I)

Experimental Conditions:

Veronal buffer, pH 8.6, ionic strength 0.05, 4°C;

Inside: 20 ml. buffer; Outside: 20 ml. dye-buffer solution.

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION ($\times 10^6$)	DILUTION AFTER EQUILIBRATION	OPTICAL DENSITY	CORRESPONDING MOLARITY ($\times 10^6$)	CORRECTED MOLARITY ($\times 10^6$)
50	1:10 (in)	0.216	2.52	25.2
	1:10 (out)	0.218	2.57	25.7
100	1:10 (in)	0.416	4.95	49.5
	1:10 (out)	0.412	4.93	49.3
500	1:50 (in)	0.413	4.94	247
	1:50 (out)	0.422	5.07	254
1000	1:100 (in)	0.434	5.22	522
	1:1000 (out)	0.436	5.27	527

TABLE XXII

EQUILIBRIUM DIALYSIS STUDIES ON THE BPA-BPB SYSTEM

Experimental Conditions:

Veronal buffer, pH 8.6, ionic strength 0.05, 4°C.; 8 days equilibration time;

Inside: 20 ml. 0.7% BPA in buffer; Outside:: 20 ml. BPB-buffer solution;

Each of the subsequent values has been derived from two cells.

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION $(X\ 10^6)$	$(BPB)_{free}$ $(X\ 10^6)$	$\dfrac{MOLES\ BOUND\ BPB}{MOLES\ TOTAL\ ALBUMIN}$
1000	348	3.00
750	232	2.82
500	115	2.66
250	33.9	1.79
100	7.65	0.83
50	2.70	0.44
25	1.25	0.22
10	0.45	0.09

TABLE XXIII

VALIDITY OF STATISTICAL BINDING BETWEEN BPA AND BPB (I)

Data obtained from XXII

$\dfrac{1}{r}$	$\dfrac{1}{(\overline{BPB})_{free}}$ $(X\ 10^{-4})$	$\dfrac{r}{(\overline{BPB})_{free}}(X\ 10^{-4})$
0.33	0.287	0.66
0.36	0.431	1.22
0.38	0.870	2.31
0.56	2.950	6.06
1.20	13.07	10.35
2.28	37.04	16.30
4.55	80.00	17.00
11.11	222.20	20.00

TABLE XXIV

DETERMINATION OF DYE-MEMBRANE ADSORPTION

Experimental Conditions:

Phosphate buffer, pH 7.4, 0.05 M with respect to phosphates; $4^\circ C$;

Inside: 20 ml, buffer; Outside: 20 ml. dye-buffer solution

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION $(X\ 10^6)$	DILUTION AFTER EQUILIBRATION	OPTICAL DENSITY	CORRESPONDING MOLARITY $(X\ 10^6)$	CORRECTED MOLARITY $(X\ 10^6)$
5000	1:500 (in)	0.436	5.07	2540
	1:500 (out)	0.451	5.21	2610
100	1:25 (in)	0.183	2.02	50.5
	1:25 (out)	0.183	2.02	50.5
100	1:25 (in)	0.186	2.04	51.0
	1:25 (out)	0.182	2.01	50.3

TABLE XXV

EQUILIBRIUM DIALYSIS STUDIES ON THE BPA-BPB SYSTEM (II)

Experimental Conditions:

Phosphate buffer, pH 7.4, 0.05 M with respect to phosphates; 4°C.;

inside: 20 ml. 0.7% BPA in buffer; outside: 20 ml. BPB-buffer solution;

each of the subsequent values has been derived from two cells. Only the final data

is presented here.

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION ($X\ 10^6$)	r (see text)	$\frac{1}{r}$	$\frac{1}{(BPB)_{free}}$ ($X\ 10^{-4}$)
7500	6.40	0.156	0.02915
5000	6.30	0.169	0.04566
1000	5.02	0.199	0.4000
750	4.55	0.220	0.6779
500	3.86	0.259	1.7543
250	2.37	0.422	15.873
100	0.96	1.038	54.050
50	0.48	2.065	126.58
10	0.09	11.709	136.99
6	0.05	20.833	166.66

TABLE XXVI

EQUILIBRIUM DIALYSIS STUDIES ON THE BPA-BPB SYSTEM (III)

Experimental Conditions:

Identical with Table XXV except a temperature of 36.5°C. was maintained.

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION (X 10^6)	r (see text)	$\frac{1}{r}$	$\frac{1}{(BPB)_{free}}$ (X 10^{-4})
1500	5.70	0.175	0.21505
1000	5.10	0.196	0.40816
750	4.89	0.205	0.76628
500	4.05	0.247	2.1052
250	2.37	0.422	15.150
100	0.93	1.081	26.809
50	0.48	2.092	88.495
25	0.23	4.292	111.76

TABLE XXVII

EQUILIBRIUM DIALYSIS STUDIES ON THE BPA-BPB SYSTEM (IV)

Experimental Conditions:

Identical with Table XXV except that a 0.2% BPA-buffer solutions was used in the dialysis sac. (4°C.)

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION ($\times 10^3$)	r (see text)	$\frac{1}{r}$	$\frac{1}{(\overline{BPB})_{free}}$ ($\times 10^{-4}$)	$\frac{r}{(\overline{BPB})_{free}}$ ($\times 10^{-4}$)
0.85	5.07	0.197	0.2845	1.4424
0.75	4.86	0.205	0.3284	1.5960
0.65	4.74	0.216	0.3902	1.8496
0.50	4.62	0.216	0.5464	2.5244
0.40	4.20	0.238	0.7181	3.0160
0.25	4.02	0.249	1.501	6.0332
0.10	2.67	0.375	8.811	23.524
0.05	1.58	0.633	48.07	75.951
0.025	0.82	1.220	149.3	122.39

TABLE XXVIII

EQUILIBRIUM DIALYSIS STUDIES ON THE BPA-BPB SYSTEM (V)

Experimental Conditions:

Identical with Table XXV except that a 0.2% BPA-buffer solution was used in the dialysis

sac. (36.5°C.)

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION (X 10^5)	r (See text)	$\frac{1}{r}$	$\frac{1}{(\overline{BPB})_{free}}$ (X 10^{-4})	$\frac{r}{(\overline{BPB})_{free}}$ (X 10^{-4})
0.85	4.00	0.250	0.2724	1.089
0.75	4.07	0.246	0.3170	1.290
0.50	3.74	0.267	0.5110	1.911
0,40	3.40	0.294	0.6630	2.25
0.25	2.99	0.334	1.225	3.66
0.10	2.13	0.469	4.235	11.16
0.05	1.24	0.805	14.33	17.81
0.025	0.70	1.422	43.48	30.57

TABLE XXIX

EQUILIBRIUM DIALYSIS STUDIES ON THE BPGG-BPB SYSTEM

Experimental Conditions:

Phosphate buffer, pH 7.4, 0.05 M with respect to phosphates; $4^{\circ}C$.; 10 days equilibration time;

inside: 20 ml. 0.2% BPGG-buffer solution; outside: 20 ml. BPB-buffer solution;

each of the subsequent values has been derived from two cells.

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION $(\times 10^6)$	DILUTION AFTER EQUILIBRATION	OPTICAL DENSITY	CORRESPONDING MOLARITY $(\times 10^6)$	CORRECTED MOLARITY $(\times 10^6)$
5000	1:500	0.461	5.33	2670
2500	1:200	0.536	6.27	1250
1000	1:100	0.428	4.92	492
750	1:100	0.338	3.83	383
500	1:25	0.805	9.90	248
250	1:50	0.224	2.48	124
100	1:5	0.805	9.90	49.5
50	1:5	0.433	5.00	25
25	1:5	0.234	2.58	12.9

TABLE XXX

EQUILIBRIUM DIALYSIS STUDIES ON THE (PVP-D30)-(BPB) SYSTEM

Experimental Conditions:

phosphate buffer, pH 7.4, 0.05 M with respect to phosphates; 4°C.; inside: 20 ml of 58 mg %

PVP-D30 nitrogen in buffer; outside: 20 ml BPB-buffer solution.

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION ($\times 10^3$)	r ($\times 10^2$) (see text)	$\frac{1}{r}$	$\frac{1}{(BPB)_{free}}$ ($\times 10^{-4}$)
5.0	4.248	23.54	0.062
2.5	2.848	35.11	0.152
1.0	1.479	67.61	0.517
0.85	1.235	80.97	0.592
0.75	1.043	95.88	0.629
0.65	0.956	104.61	0.787
0.50	0.754	132.57	1.067
0.40	0.605	165.28	1.340
0.25	0.369	270.78	2.067
0.10	0.165	604.96	6.349
0.05	0.082	1219.51	12.58

TABLE XXXI

EQUILIBRIUM DIALYSIS STUDIES ON THE (PVP-DSO)-(BPB) SYSTEM

Experimental Conditions:

Identical with Table XXX, except a temperature of 36.5°C. was used.

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION $(X10^3)$	r $(X\ 10^2)$ (see text)	$\frac{1}{r}$	$\frac{1}{(BPB)}_{free}$ $(X\ 10^{-4})$
5.0	3.862	25.69	0.068
2.5	2.448	40.85	0.135
1.0	12248	80.13	0.414
0.75	1.033	96.79	0.621
0.65	0.997	100.30	0.844
0.50	0.739	135.37	1.309
0.40	0.653	153.13	1.547
0.25	0.379	263.85	2.151
0.10	0.148	675.67	5.168
0.05	0.076	1315.7	10.893
0.025	0.038	2631.5	21.598

TABLE XXXII

EQUILIBRIUM DIALYSIS STUDIES ON THE (PVP-D60)-(BPB) SYSTEM

Experimental Conditions:

Identical with Table XXX except that PVP-D60 was utilized (4°C.).

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION ($\times 10^3$)	r ($\times 10^2$) (see text)	$\frac{1}{r}$	$\frac{1}{(BPB)_{free}}$ ($\times 10^{-4}$)
5.0	4.49	22.27	0.064
2.5	3.12	32.05	0.166
1.0	1.59	62.89	0.583
0.85	1.31	76.34	0.649
0.75	1.20	83.33	0.791
0.65	0.932	107.29	0.758
0.50	0.810	123.47	1.214
0.40	0.618	161.89	1.386
0.25	0.432	231.48	2.826
0.10	0.155	645.16	5.540
0.05	0.086	1162.7	13.642
0.025	0.042	2380.9	26.666

TABLE XXXIII

EQUILIBRIUM DIALYSIS STUDIES ON THE (PVP-D60)-(BPB)SYSTEM

Experimental Conditions:

Identical with Table XXX except that PVP-D60 was utilized ($36.5^\circ C$.).

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION ($\times 10^3$)	r ($\times 10^2$) (see text)	$\frac{1}{r}$	$\frac{1}{(BPB)_{free}}$ ($\times 10^{-4}$)
5.0	3.402	29.39	0.056
2.5	2.453	40.77	0.135
1.0	1.376	72.67	0.455
0.85	1.289	77.58	0.633
0.75	1.112	89.66	0.694
0.65	0.970	103.06	0.807
0.50	0.723	138.31	0.998
0.40	0.652	153.42	1.538
0.25	0.348	287.68	1.887
0.10	0.143	699.79	4.902
0.05	0.067	1484.7	9.050
0.025	0.037	2711.1	20.576

TABLE XXXIV

EQUILIBRIUM DIALYSIS STUDIES ON THE (PVP-D90)-(BPB) SYSTEM

Experimental Conditions:

Identical with Table XXX except that PVP-D90 was utilized ($4^{\circ}C.$).

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION ($X\ 10^3$)	$r\ (X\ 10^2)$	$\frac{1}{r}$	$\frac{1}{(\overline{BPB})_{free}}\ (X\ 10^{-4})$
5.0	4.490	22.23	0.064
2.5	3.269	30.60	0.175
1.0	1.596	62.66	0.590
0.85	1.359	73.58	0.697
0.75	1.207	82.85	0.800
0.65	1.081	92.51	0.990
0.50	0.800	125.00	1.187
0.40	0.659	151.74	1.578
0.25	0.430	232.72	2.787
0.10	0.165	604.96	6.349
0.05	0.085	1183.4	13.458
0.025	0.058	1712.3	24.875

TABLE XXXV

EQUILIBRIUM DIALYSIS STUDIES ON THE (PVP-D90)-(BPB) SYSTEM

Experimental Conditions:

Identical with Table XXX except that PVP-D90 was utilized (36.5°C.).

INITIAL DYE CONCENTRATION IN OUTSIDE SECTION $(\times 10^3)$	$r \; (\times 10^2)$ (see text)	$\frac{1}{r}$	$\frac{1}{(BPB)_{free}} \; (\times 10^{-4})$
5.0	3.910	25.58	0.059
2.5	2.740	36.50	0.146
1.0	1.361	73.48	0.457
0.85	1.260	79.36	0.610
0.75	1.112	89.28	0.699
0.65	0.946	105.67	0.775
0.50	0.849	117.86	1.008
0.40	0.655	152.57	1.567
0.25	0.398	251.06	2.360
0.10	0.154	649.35	5.526
0.05	0.077	1305.4	10.958
0.025	0.038	2645.5	21.367

BIBLIOGRAPHY

Abramson, H., Moyer, L., and Gorin, M., (1942) Electrophoresis of Proteins and the Chemistry of Cell Surfaces, Reinhold Publishing Co., New York.

Alberty, R., (1950) "Moving-Boundary Systems Formed by Weak Electrolytes: Theory of Simple Systems Formed by Weak Acids and Bases", J. Am. Chem. Soc. 72, 2361-7.

Alberty, R. A., (1953) The Proteins, Chemistry, Biological Activity, and Methods, edited by Neurath, H., and Bailey, K., Academic Press, Inc., New York, I. A., 461-548.

Alberty, R. A., and Marvin, H. H., (1950) "Study of Protein-Ion Interaction by the Moving Boundary Method", J. Phys. and Colloid, Chem. 54, 47-52.

Alberty, R., and Nichol, J., (1948) "Chemical Reactions in Moving-Boundary Systems of Weak Electrolytes", J. Am. Chem. Soc. 70, 2297.

Ammon, R., and Braunschmidt, G., (1949) "The Fate of Periston in the Organism" Biochem. Z. 319, 370-77.

Barbagallo-Sangiorgi, O., (1952) "Correspondence of the Results on Paper with those Obtained by the Classical Nernst Electrophoresis", Boll. Soc. ital. biol. sper. 28, 1956-8.

Bennhold, H., Kylin, E. and Rusnyak, (1938) Die Eiweisskörper des Blutplasmas T. Steinkopff, Dresden.

Bennhold, H., Ott, H., and Wiech, M., (1950) "The Difference in the Manner in which Substances Which are Excreted through the Liver and those Excreted Thorough the Kidneys are Bound to Serum Proteins", Deut. med. Wochschr. 75, 11-15.

Bennhold, H., and Schubert, R., (1943) "Investigation of the Possible Vehicle Function of the Plasma Substitute Periston", Z. ges. exptl. Med. 113. 722-36.

Bischoff, F., and Pilhorn, H., (1948) "The State and Distribution of Steroid Hormones in Biologic Systems", J. Biol. Chem. 174, 663-82.

Boettiger, E., (1946) "The Adsorption of Estriol by the Plasma Proteins", J. Cellular Comp. Physiol. 28, 139.

Bourdreaux, J., and Bourdin, J., (1945) "Delayed Action Local Anesthesia", Le medecin franc. 5, 6-11.

Boyer, P., Ballou, G., and Luck, M., (1947) "The Combination of Fatty Acids and Related Compounds with Serum Albumin. III. The Nature and the Extent of the Combination", J. Biol. Chem. 167, 407-24.

Bresler, S., (1949) "Structure of Globulin Proteins and their Interaction with the External Medium", Biokhimiya 14, 180-9.

Brode, W., (1924), "The Determination of Hydrogen-Ion Concentration by a Spectrophotometric Method and the Absorption Spectra of Certain Indicators", J. Am. Chem. Soc. 46, 581-596.

Brun, J., Pellerat, J., and Kalb, J. C., (1950) "P.A.S. (para-aminosalicyclic acid) Associated the Hypertonic Subtosan for Intravenous Injection", Lyon. Med. 183, No. 18, 285-6.

Bulgakova, A. M., and Shafershtein, I. Y., (1951) "Membranes for Equilibrium Dialysis", Kolloid Zhur, 13, 76-80.

Chinard, F., (1952) "A Colorimetric Method for the Estimation of Polyvinylpyrrolidone (PVP) in Whole Blood Plasma and Urine", J. Lab. Clin. Med. 39, 666-72.

Choffel, Cl., and Amoudrux, J., (1947) "Treatment of Raynaud's Disease", Le medecin franc. 227-9.

Colichman, E. L., (1951) "Spectral Study of Long Chain Quaternary Ammonium Salts in Bromphenol blue Solutions", J. Am. Chem. Soc. 73, 3385-88.

Colvin, J. and Briggs, D., (1952) "Electrophoretic Analysis of Protein Interaction. II. Explanation of a Boundary Anomaly in the System Bovine Serum Albumin-Methyl Orange", J. Phys. Chem. 56, 717-722.

Cosar, C., (1945), "Increasing the Therapeutic Action of Penicillin in Various Experimental Injections of Mice," Compt. rend. soc. biol. 139, 388-91.

Cuny, L. and Quivy, D., (1948) "Total Lack of Influence of Polyvinylpyrrolidone on the Rate of Elimination of Heparin Injected Intravenously in the Dog", Compt. rend. soc. biol. 142, 1399-1402.

Davis, B., and Dubos, R., (1947) "Binding of Fatty Acids by Serum Albumin, A Protective Growth Factor in Bacteriological Media", J. Exptl. Med. 86, 215-28.

Doebbler, G. F., (1953) "Blood Plasma Substitutes: II. A Paper Chromatographic Method for the Determination of Polyvinylpyrrolidone", Tex. J. Sci. 5, 443-448.

Düttmann, G., (1941) "Peridural, Segmental Anesthesia", Zentr. Chir. 68, 530-35.

133

Foster, J. F., and Yang, J. T., (1953b) "Competitive Interaction between Proteins and Surface Active Anions Demonstrated by Electrophoresis", J. Am. Chem. Soc. 75, 5743-44.

Franke, H., (1951) "Experimentelle Untersuchungen zur Frage des Arzneimitteltransportes", Z. ges. Exptl. Med. 118, 31-54.

General Aniline and Film Corporation, PVP Polyvinylpyrrolidone (1951), New York.

Goepel, H., (1947) "Peridural Anesthesia, Use of Pantocaine-Periston Mixture in Segmental Anesthesia", Zentr. Chir. 72, 467-73.

Goldstein, A., (1949) "Interactions of Drugs and Plasma Proteins", Pharm. Rev. 1-26.

Gurd, F., and Goodman, D., (1952) "Preparation and Properties of Serum and Plasma Proteins. XXXII. The Interaction of Human Serum Albumin with Zinc Ions", J. Am. Chem. Soc. 74, 670-5.

Haring, M., and Heller, H., (1941) "A Spectrophotometric Study of the Characteristics of Some Halogen Substituted Sulfonphthalein Indicators", J. Am. Chem. Soc. 46, 1024-27.

Haurowitz, F., (1950) Chemistry and Biology of Proteins, Academic Press, New York.

Hengstenberg, J., and Schuch, E., (1952) "Molecular-Weight Determinations of Polyvinylpyrrolidone by Means of Osmotic Pressure and Light Scattering Measurements", Makromol. chem. 7, 236-58.

Henley, A., and Schuettler, C., (1953) Electrophoresis Bibliography, American Instrument Co., Silver Springs, Md.

Hughes, W., (1950) "Protein Mercaptides", Cold Spring Harbor Symposia Quant. Biol. 14, 79-84.

Kallee, E., and Roth, E., (1953) "The Paper Electrophoretic Detection of Dye-Binding Ability", Ztschr. Naturforsch. 8b, 34-5.

Karush, F., (1951) "The Study of Protein Binding by Partition Analysis. The Effect of Protein Charge", J. Am. Chem. Soc. 73, 1246-52.

Klotz, I. M., (1949), "The Nature of Some Ion-Protein Complexes", Symposia Quant. Biol. Cold Spring Harbor, XIV, 97-112.

Klotz, I. M., (1953) The Proteins, Chemistry, Biological Activity, and Methods edited by Neurath, H., and Bailey, K., Academic Press Inc., New York, I.B., 727-806.

Klotz, I. M., and Urquhart, J. M., (1949), "The Binding of Organic Ions by Proteins, Buffer Effects", *J. Phys. and Colloid Chem.* 53, 100-14.

Klotz, I. M., Walker, F. M., and Pivan, R. B., (1946) "The Binding of Organic Ions by Proteins", J. Am. Chem. Soc. 68, 1486-90.

Köiw, E., Wallenius, G., and Grönwall, A., (1951) "Clinical Utilizationoof Electrophoresis in Filter-Paper Strips: Comparison with the U-tube Electrophoresis Method of Tiselius", *Bull. Soc. chim. biol.* 33, 1940-50.

Köiw, E., Wallenius, G., and Grönwall, A., (1952) "Paper Electrophoresis in Clinical Chemistry: A Comparison with the Original Tiselius Method", *Scandinav. J. Clin. and Lab. Invest.* 4, 47-54.

Korth, J., and Heinlein, H., (1943) "Functional and Morphological Investigations of the Action of Colloidal Blood Substitutes with Special Attention to Periston", *Arch. Klin. Chir.* 205, 230-86.

Kusumoki, (1953) "Further Studies on the Binding of Dyes by Proteins", *J. Biochem. (Japan)*, 40, 227-85.

Lavedan, J. P., (1948) "Influence of Subtosan given with Estrogenic Hormone on Duration of Provoked Estrus in C$_n$strated Mice", *Compt. rend. soc. biol.* 142, 1481-2.

Levin, B., and Oberholzer, V., (1952) "Paper Electrophoresis of Serum Proteins" *Nature*, 170, 123.

Levin, G., and Oberholzer, V., (1953) "Paper Electrophoresis of Serum Proteins" with a Micro-Kjeldahl Nitrogen Analysis of the Protein Fractions", *Am. J. Clin. Path.* 23, 205-17.

Leger, L., (1947) "Subtosan as a Retarding Medium in Rachianesthesia", *Presse méd.* No. 58, 670.

Lespagnol, L., (1948) "Urinary Albumin and PVP", *Bull. Soc. pharm. Lille*, *No.2*, 26-8.

Levrat, M., Pellerat, J., Moindrot, R., and Murat, M., (1945a) "Therapeutic Use of Insulin-Subtosan in Diabetes", *Bull. Mem. soc. méd. hop. Paris*, 61 196-8.

Levrat, M., Pellerat, J., Moindrot, R., and Murat, M., (1945b) "Effect of the New Retarded Insulin Preparation - Insulin-Subtosan-on Normal and Diabetic Humans", *Bull. Mem. soc. med. hop. Paris*, 61, 198-200.

Lian, G., and Bergamo, G., (1948) "Tetraethylammonium Bromide (TEA) in the Treatment of Permanent Arterial Hypertension", *Bull. mem. soc. med. hop. Paris*, No. 7-8, 239-44.

Lian, C., Siquier, F., Piette, Poulain, and Sarrazin, (1947) "Retarded Thrombin in the Treatment of Hemophilia", Bull. Acad. nat. med. 512-3.

Longsworth, L. G., and MacInnes, D. A., (1942) "An Electrophoretic Study of Mixtures of Ovalbumin and Yeast Nucleic Acid", J. Gen. Physiol. 25, 507-516.

Loubatieres, A., (1946) "PVP, a Retarding Agent for Insulin and Protamine Zinc Insulin: Experimental Demonstration in the Depancreatized Dog", Annales endocrinol. 7, 173-8.

Marbach, E. P., (1952) Ionographic Studies of Simple and Complex Proteins", M. S. Thesis, Loyola University, Chicago.

Marmont, A., and Palmieri, Jr., A., (1949) "Prolongation of Heparin Action by Association with Hypertonic Polyvinylpyrrolidone", Boll. soc. ital. dio. sper. 25, 1341-3.

Mascré, M., (1950) "Retardant Effect of PVP on Some vegetable Organs", Ann. pharm. franc. 8, 388-91.

McDonald, H. J., (1952) "Ionography: A New Frontier in Electrophoresis", J. Chem. Educ. 29, 428-37.

McDonald, H. J., Lappe, R. J., Marbach, E. P., Spitzer, R. H., and Urbin, M.C. (1953a) "Electromigration in Stabilized Electrolytes, Part III, Applications of the Technique", Clin. Chem. 5, 51-59.

McDonald, H. J., Lappe, R. J. Marbach, E. P., Spitzer, R. H., and Urbin, M.C., (1955) Ionography: Electromigration in Stabilized Media, Year Book Publishers, Chicago.

McDonald, H. J. Marbach, E. P., and Urbin, M. C., (1953b) "Electromigration in Stabilized Electrolytes, Part I, Development of the Technique", Clin. Chem. 5, 17-23.

McDonald, H. J., and Spitzer, B., (1953) "Polyvinylpyrrolidone: The Electromigration Characteristics of the Blood Plasma Expander", Circ. Res. I, 396-404.

Meidinger, F., (1947) "Polyvinylpyrrolidone (PVP) as a Synthetic Protective Colloid in Vitro and in Vivo", Bull. soc. chim. biol. 29, 411-16.

Mennenga, (1949) "Peridural Anesthesia", Deut. Gesundheitsw. 4, 362.

Miller, L. E., and Hamm, F. A., (1953) "Macromolecular Properties of Polyvinylpyrrolidone Molecular Weight Distribution", J. Physic. Chem. 57, 110-112.

Murat, M., (1949) "Investigations of Certain Chemical and Pharmacodynamic Properties of Polyvinylpyrrolidone", Products, Pharm. 4, 350-6.

Nichol, J., (1950) "Moving Boundary Systems Formed By Weak Acids and Bases:
  An Experimental Study", J. Am. Chem. Soc. 72, 2367-74.

Oster, G., (1952) "Spectral Studies of Polyvinylpyrrolidone (PVP)", J. Polymer
  Sci. 9, 553-55.

Oster, G., and Immergutt, E. H., (1954) "Ultraviolet and Infrared Studies of
  Polyvinylpyrrolidone", J. Am. Chem. Soc. 76, 1393-96.

Perrault, M., and Rousseau, P., (1947) "Hypertonic Subtosan Associated with the
  Diuretic, Neptal", Progress. med. 75, 323-4.

Poullain, P. and Piette, M., (1948) "Determination of the Blood Volume by
  means of Polyvinylpyrrolidone", Bull. soc. chim. biol. 30, 496-500.

Putnam, F., and Neurath, H., (1944) "The Precipitation of Proteins by Synthe-
  tic Detergents", J. Am. Chem. Soc. 66, 692-7.

Quevauviller, A., (1947a) "Hypertonic Subtosan Prolongs the Anesthetic Action
  of i.v. Injected Evipan in the Mouse", Presse med. #19, 219.

Quevauviller, A., (1947b) "Subtosan Retards the Urinary Excretion of i.v. In-
  jected Quinine in the Rabbit," Ann. pharm. franc. 5, 93-8.

Reppe, W., Kralkalla, H., Dorhheim, O., and Sauerbier, R., (1938), "N-Vinyl
  Lactams", German Patent 744, 414.

Reppe, W., Schuster, C., and Hartmann, A., (1939), "Polymeric N-Vinyl Lactams
  and Process of Producing Same", German Patents 737, 663 and 738, 753.

Riva, G., and Martini, V., (1953), "Experience with Electrophoresis on Filter
  Paper," Schweiz, Med. Wehrschr. 83, 73-7.

Roth, E., and Kallee, E., (1953) "Paper Electrophoresis of Dye-Binding Ability",
  Ztschr. Naturforsch. 8, 614-5.

Scatchard, G., (1949) "The Attraction of Proteins for Small Molecules and Ions",
  Ann. N. Y. Acad. Sci. 51, 660.

Scholtan, W. (1952) "Molecular-Weight Determinations of Polyvinylpyrrolidones
  by means of the Ultracentrifuge and Viscometry", Makromol. Chem. 7,
  209-35.

Schubert, R., (1945) "Excretion of Periston and of Periston-Bound Substances
  (Bilirubin-Indigo Carmine)", Z. gesam. exp. Med. 114, 634-55.

Schubert, R., (1950a), "Mechanism of Serum and Tissue Cleaning by Kollidon",
  Arztl. Forschg, 4, 42-6.

Schubert, R., (1950b), "Liberation from the Serum of Substances not Eliminated Through the Kidneys by Kollidon of Various Particle Sizes", Deut. Z. Verdauungs-u. Stoffwechselkrankh, 10, 79-86.

Schubert, R., (1950c) "A New Means of Detoxicating the Body by replacing the Bile-Liver System by the Kidneys as Organs of Excretion of Substances Bound to Artificial Colloids", Rev. Gastreenterol. 17, 165-79.

Schubert, R., and Graser, V., (1950) "Is the Excretion of Sulfonamides Affected by i.v. Injection of Lower Molecular Kollidon", Arch. inn. med. I, 515-36.

Schubert, R., and Werner, H., (1950), "Serum and Tissue Washing with Kollidon Experiments with Diamine Pure Blue FF on Rabbits", Z. ges. inn. med. 5, 298-308.

Shimma, K., (1952) "Spectrophotometric Determination of Binding of Dye by Proteins", J. Biochem. (Japan), 39, 239-43.

Siguier, F., Guidicelli, R., and Walter, C., (1948) "The Use of a Retardent in Opiate Therapy", Progres med. No. 9, 223-4.

Smith, R. F., and Briggs, D. R., (1950) "Electrophoretic Analysis of Protein Interaction, I. The Interaction of Bovine Serum Albumin and Methyl Orange", J. Phys. and Colloid Chem. 54, 33-47.

Spitzer, R. H., (1953) The Ionographic Properties of Polyvinylpyrrolidone, M.S. Thesis, Loyola University, Chicago.

Spitzer, R. H., and McDonald, H. J., (1955) "Equilibrium Dialysis and Ionographic Studies of Protein and Polyvinylpyrrolidone Interactions with Bromphenol Blue", Federation Proc. 14, 285.

Starlinger, (1943) "Delayed Action Anesthesia", Zentr. Chir. No. 48, 1726.

Steiner, R., (1954), "Reversible Association Process of Globular Proteins, V. The Study of Associating Systems by the Methods of Macromolecular Physics" Arch. Biochem. Biophysics. 49, 400-16.

Sullivan, J. C., and Hindman, J. C., (1952), "An Analysis of the General Mathematical Formulations for the Calculation of Association Constants of Complex Ion Systems", J. Am. Chem. Soc. 74, 6091-96.

Sullivan, R., Palermiti, F., and Armino, R., (1952), Meeting-in-Miniature, New York Section of A.C.S., Feb. 8.

Svensson, H., (1948) "The Behavior of Weak Electrolytes in Moving Boundary Systems: I. Moving Boundary Equation", Acta chem. Scandinav. 2, 841-55.

Thrower, W., and Campbell, H. (1951) "Plasmosan-A Synthetic Substitute for Plasma", Lancet, 260, 1096.

Tompsett, R., Shults, S., and McDermott, W., (1947) "The Relation of Protein Binding to the Pharmacology and Antibacterial Activity of Penicillins X, O, Dihydro F, and K", J. Bact. 53, 581-95.

Urbin, M. C., (1952) Ionography: Electromigration on Wet Surfaces, M.S. Thesis, Loyola University, Chicago.

Urbin, M. C., (1954) An Experimental Approach to the Quantification of Ionographic Values. Ph. D. Dissertation, Loyola University, Chicago.

Weese, H., (1948) "Blood Substitutes", Die Pharmazie 3, 337-40.

Witwer, D., (1954) Personal Communication.

Wunderly, C., (1950) "Model Experiments with Plasma Proteins as Elution Agents", Arztl. Forschg. 4, 29-35.

Yang, J. T., and Foster, J. F., (1953) "Statistical and All-or-None Binding of Alkylbenzene-sulfonate by Albumins," J. Am. Chem. Soc. 75, 5560-67.

APPROVAL SHEET

The dissertation submitted by Robert H. Spitzer has been read and approved by five members of the faculty of the Stritch School of Medicine, Loyola University.

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

The dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

May 25, 1955
_____
Date

Hugh J. McDonald
_____
Signature of Adviser