THE COMPLEXING BEHAVIOUR OF BISHYDROXYCOUMARIN WITH MACROMOLECULES

by

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This thesis, in the opinion of the examiners, exceeds the usual standards considered necessary for research at the M. Sc. level. It is, in many respects, equal to investigations carried out at the Ph. D. level and reflects the student's ability to carry out research at these more advanced levels.

ABSTRACT

The strong binding of bishydroxycoumarin to serum albumin was first reported about 20 years ago. However, the mechanism of binding has not been studied. In this investigation, attempts have been made to reveal the mechanism. The work was extended to some other synthetic macromolecules including polyvinylpyrrolidone.

The literature survey covers the physicochemical properties and the complexing behaviours in aqueous solution of the individual substances examined. The theory of multiple equilibria, which is fundamental to an understanding of the binding process, has been summarized. Spectrophotometric, solubility, dynamic and equilibrium dialyses, and viscometric methods were used and their theoretical back-ground has been reviewed and discussed.

Some physicochemical properties of BHC, necessary for the interpretation of binding data, were estimated. Maximum binding capacities of macromolecules and association constant of each binding site were obtained from the binding data. The nature of the site and intermolecular forces were characterized from thermodynamic analysis.

This abstract represents the true contents of the thesis submitted.

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I. INTRODUCTION

Many investigators have studied the interactions between drugs and a wide variety of organic and inorganic molecules which are associated with a therapeutically active substance in either an *in vitro* or *in vivo* system. It is known that drugs form 'complexes' with plasma proteins, enzymes, other drugs and many of the adjuvants which are added to dosage forms. The physicochemical properties of these complexes differ significantly, in many instances, from those observed for the interacting drug. Although these properties can be determined, they are not based, in general, on the complex itself but on studies in which the complex, the drug, and the interacting molecule are associated with each other in some *in vitro* system.

A complex is a co-ordination compound which arises from a Lewis acid-base reaction. This classical definition includes those complexes which are formed by reacting the drug with a metallic ion or an organic molecule. For the purposes of this thesis, the latter definition is broader than necessary. The word 'complex', as used herein, is defined as that substance formed by a reversible chemical reaction in which equilibrium rates are much higher than any of the rates associated with the measuring process. Chemical (covalent) bonds are not formed and the long range forces which hold the interacting molecules together are much weaker than those found in most chemical compounds.

Bishydroxycoumarin (BHC) is strongly bound to plasma proteins. It has been suggested that the interaction leads to erratic therapeutic results and that binding of drugs to macromolecules may affect the in vivo activity of the substance. The object of this study is, therefore, to investigate the mechanism of interaction between this drug and human serum albumin (HSA), starch sol, polyvinylpyrrolidone (PVP), dextran, and hydroxyethyl starch (HES). The latter three substances have been used as plasma expanders.

Complex formation may be studied by utilizing a wide variety of methods. However, only the equilibrium and dynamic dialyses, solubility, spectrophotometric, and viscometric methods will be used in this investigation. Quantitative information on the interactions may be obtained from equations which are based on the law of mass action. The nature of the intermolecular forces between the molecules is derived from thermodynamic data obtained during the investigation.

Although studies of this type have been carried out by many investigators, the significance of their observations has not always been evident. The bulk of the papers on the subject appeared in the literature during the 1950's but, in recent years, many investigators have again begun to study complex formation. This is due, in part, to the possibility that both the stability of the drug and its in vivo activity may be affected by other drugs or adjuvants in the dosage form. The results in this thesis are not directly related to the latter problem but do offer additional proof that therapeutically significant drugs bind easily to macromolecules.

II. THEORY

A general discussion of the principles and concepts fundamental to the binding capacity of proteins with various substances may be found in the papers by Scatchard (1949; and others, 1954), Klotz (1946a; 1949a; 1953a), Edsall and Wyman (1958c), Foster (1960), Tanford (1965), and Weber (1965). The mathematical theory associated with such studies is discussed in detail by Krüger-Thiemer, et al. (1964), Hart (1965), Sandberg, et al. (1966), and Rosenthal (1967).

Scatchard (1949; and others, 1954) stated that four questions should be answered at the conclusion of any study on the interaction between a protein and a small molecule. These are: "How many molecules are bound to the protein?" "How tightly are the molecules bound to the protein?" "Where does the binding occur?" "Why does the binding occur?" The answers to these questions deal, therefore, with the number of binding site, the equilibrium constant at the site, and the type of interaction between the molecule and the functional group or groups on the protein.

If the functional groups on the large molecule act independently, the law of mass action may be used to explain the interaction with a small molecule and the binding strength can be expressed as a constant. If the protein (M) combines with a molecule (D) to form a single complex (MD), then

$$M + D = MD \qquad \text{(Eq. 1)}$$

The association constant (K) is defined by the following equation.

$$K = \frac{(D_b)}{(M_f)\ (D_f)} \qquad \text{(Eq. 2)}$$

The quantities in parentheses represent the concentrations of the respective species and the subscripts (b and f) indicate bound or complexed and free or unbound species, respectively. However,

$$(M_t) = (D_b) + (M_f) \qquad \text{(Eq. 3)}$$

(M_t), in Eq. 3, indicates the total concentration of macromolecule. Rearrange Eq. 2 and substitute $(M_t) - (D_b)$ for (M_f).

$$(D_b) = K(D_f)\left[(M_t) - (D_b)\right] \qquad \text{(Eq. 4)}$$

Divide both sides of Eq. 4 by $K(D_f)(D_b)$ and rearrange.

$$\frac{(D_b)}{(M_t)} = \frac{1}{1 + \frac{1}{K(D_f)}} \qquad \text{(Eq. 5)}$$

The molar ratio of bound drug to total macromolecule is equal to r. This ratio indicates the extent of binding.

$$r = \frac{(D_b)}{(M_t)} \qquad \text{(Eq. 6)}$$

Substitute the above into Eq. 5 and rearrange.

$$r = \frac{K\ (D_f)}{1 + k\ (D_f)} \qquad \text{(Eq. 7)}$$

Interactions between macro and simple molecules are usually more complex than that indicated above. If there are n binding sites, if each site is not influenced by its neighbor, and if each has the same intrinsic affinity for D, then the successive interactions and their corresponding constants may be represented in the following way.

$$\begin{aligned}
M + D_f &= MD & k_1 &= \frac{(MD)}{(M)(D_f)} \\
MD + D_f &= MD_2 & k_2 &= \frac{(MD_2)}{(MD)(D_f)} \\
MD_2 + D_f &= MD_3 & k_3 &= \frac{(MD_3)}{(MD_2)(D_f)} \\
&\vdots & &\vdots \\
MD_{i-1} + D_f &= MD_i & k_i &= \frac{(MD_i)}{(MD_{i-1})(D_f)} \\
&\vdots & &\vdots \\
MD_{n-1} + D_f &= MD_n & k_n &= \frac{(MD_n)}{(MD_{n-1})(D_f)}
\end{aligned} \qquad \text{(Eq. 8)}$$

Furthermore, the step association constants are not independent of each other. However, the relationships between constants can be stated mathematically by applying the rules of combination and permutations. A schematic diagram of a macromolecule is shown in Figure 1. The equilibrium constant for the association between D and site 1 on M is the same as that for the reaction between D and any other position on the molecule. Therefore:

$$\left.\begin{aligned} M + D_f &= {}_1MD \\ M + D_f &= {}_2MD \\ &\vdots \\ M + D_f &= {}_iMD \end{aligned}\right\} \qquad \text{(Eq. 9)}$$

$$K = \frac{({}_1MD)}{(M)(D_f)} = \frac{({}_2MD)}{(M)(D_f)} = \cdots\cdots = \frac{({}_nMD)}{(M)(D_f)} \qquad \text{(Eq. 10)}$$

Therefore: $$k_1 = nK \qquad \text{(Eq. 11)}$$

since

$$(MD) = ({}_1MD) + ({}_2MD) + \cdots\cdots + ({}_nMD) \qquad \text{(Eq. 12)}$$

Similarly,

$$k_2 = \left(\frac{n-1}{2}\right) K \qquad \text{(Eq. 13)}$$

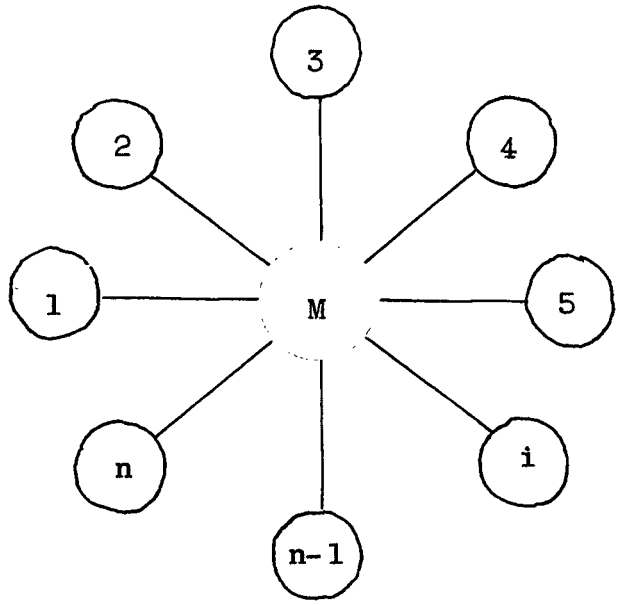

Figure 1. Schematic diagram of a macromolecule with n sites for the attachment of a simple molecule (D).

and $$k_i = \left[\frac{n-i+1}{i}\right] K \qquad \text{(Eq. 14)}$$

This, then, is the general relationship between the step association constant (k_i) for the formation of the MD_i complex and the intrinsic association constant (K). The coefficient of K in Eq. 14 is frequently called the 'statistical factor' since this may also be derived on the basis of statistical considerations. The value of r may now re-defined.

$$r = \frac{(MD)+2(MD_2)+3(MD_3)+ \ \ldots \ +i(MD_i)+ \ \ldots \ +n(MD_n)}{(M)+(MD)+(MD_2)+ \ \ldots \ +(MD_i)+ \ \ldots \ +(MD_n)} \qquad \text{(Eq. 15)}$$

The (MD) terms in Eq. 15 may now be appropriately expressed by K, (M) and (D_f) terms in Eq. 8.

$$r = \frac{(D_f)\ f'}{f} \qquad \text{(Eq. 16)}$$

The f and f' represent the denominator of Eq. 15 and its first derivative with respect to (D_f). Substitute the appropriate terms of the left side of Eq. 14 into the corresponding step association constant terms involved in Eq. 16 and rearrange on the basis of the binomial theorem.* Thus r can be expressed without using step association constant terms.

$$r = \frac{n\ K\ (D_f)}{1 + K\ (D_f)} \qquad \text{(Eq. 17)}$$

Therefore, if there are n independent binding sites, the extent of binding is n times that for a single site and the intrinsic association constant is the same as that in Eq. 7.

Rearrange Eq. 17.

$$1/r = 1/n + 1/nK(D_f) \qquad \text{(Eq. 18)}$$

Therefore, a plot of 1/r versus $1/(D_f)$ is a straight line with a slope value of 1/nK and an intercept value of 1/n. Both the binding constant and the number of binding sites can, therefore, be determined. Enzymologists refer to this method of presenting

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* Eq. 16 and 17 are not derived herein. The equations leading to these may be found in the papers published by Klotz (1946a; 1953a) or by Edsall and Wyman (1958c).

data graphically as the Lineweaver - Burk plot (Lineweaver and Burk, 1943). The main problem with this double reciprocal plot is that a few low solute concentrations will outweigh many high solute concentrations. Small extrapolating errors at high (D_f) will result in large errors in the n value (Goldstein, 1949; Dowd and Riggs, 1965).

Klotz, Walker, and Pivan (1946b) used this method of plotting in their investigations into the binding of azosulfonic acids with bovine serum albumin. Scatchard (1949), on the other hand, plotted his data in a different manner. The 'Scatchard equation', which may be derived from Eq. 17, is

$$r/(D_f) = Kn - Kr \qquad \text{(Eq. 19)}$$

A plot of $r/(D_f)$ versus r is, therefore, a straight line. The intercept value on the abscissa yields n; the intercept value on the ordinate is equal to Kn. This equation lays less stress on the values of r at very low (D_f) values than does the double reciprocal plot (Eq. 18). In addition, it gives a more even relative weight to the different points on the curve.

Eq. 17 is similar to the equation that was derived by Langmuir (1918) to describe certain adsorption isotherms. The equation is

$$m = \frac{bkC}{1 + kC} \qquad \text{(Eq. 20)}$$

where m is the number of grams of solute adsorbed by one gram of adsorbent; C is the total concentration of solute

in solution. The constants in this equation (b and k) arises from the mathematical derivation of the isotherm. Although Eq. 17 and 20 are similar, it is not correct to assume that binding and adsorption are identical processes. The equations are similar because both have been derived from the law of mass action. The subject is discussed in detail by Goldstein (1949) and Klotz (1953a).

The Freundlich isotherm is one of the first equations proposed to explain adsorption phenomena.

$$m = k\, C^{1/n} \qquad \text{(Eq. 21)}$$

This isotherm can not be used if the concentration of adsorbate with respect to adsorbent is too high. The isotherm itself is empirical. Patel and Foss (1965) described the binding of benzoic acids by polysorbate 80 and cetomacrogol 1000 in terms of a Freundlich-type adsorption relationship.

Many binding systems cannot be described mathematically by using the simple mass action equation based on the assumption that there is but one intrinsic association constant. Attempts have been made to correct for electrostatic interactions between the binding sites on M. If charged ions are bound to M, the first ion tends to reduce the affinity of M for the second oncoming ion because of electrostatic repulsion between species of like charge. Eq. 17 is, therefore, no longer valid even if the intrinsic affinity of each site on M is the same for the small ion.

If electrostatic forces are significant, there is a relationship between two successive binding constants (k_{i-1} and k_i). The correction procedure described by Klotz, Walker, and Pivan (1946b) is similar to that used by Kirkwood and Westheimer (1938) in their study of the first and second ionization constants of a dibasic acid.

The equation for the free energy changes for the reaction

$$MD_{i-2} + 2\ MD_i = 2\ MD_{i-1} \qquad \text{(Eq. 22)*}$$

consists of two terms. It takes into consideration the free energy change of the interaction in the absence of electrostatic effect and the electrostatic free energy change (ΔG_{el}) which can be estimated from the Born and Debye-Hückel Theory.

$$-\Delta G^\circ = RT\ \ln\frac{k_{i-1}}{k_i} = RT\ \ln\left[\frac{n-(i-2)i}{n-(i-1)i-1}\right] - \Delta G_{el} \qquad \text{(Eq. 23)}$$

In order to calculate any constant k_i, k_1 must first be obtained from a suitable extrapolation of experimental data. The k_2 value may be calculated from Eq. 23. By using a similar procedure, k_3 may be calculated from the k_2 value. Other constants are obtained in a similar manner. If successive binding constants are known, r values can be estimated by using Eq. 16.

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* Eq. 22 is the summation of two successive reactions to produce the complexes MD_{i-1} and MD_i.

Scatchard (1949) corrected for the electrostatic effect by using the following equation.

$$\frac{r}{(D_f)} e^{2wr} = Kn - Kr \qquad \text{(Eq. 24)}$$

The w term may be calculated from theory or an approximate value may be determined empirically. The Debye-Hückel equation for a charge spread uniformly over the surface of a sphere of radius b which excludes small ions to a radius a is given below.

$$w = \frac{\epsilon^2 z^2}{2Dk_BT}\left[1/b - \frac{\mathbb{k}}{1 + \mathbb{k}a}\right] \qquad \text{(Eq. 25)}$$

D is the dielectric constant of the medium, k_B is the Boltzmann constant, T is the absolute temperature, ϵ is the electronic charge, z is the valence of the small molecule, and $\mathbb{k}$ is defined by the Debye-Hückel Theory. Tanford, Swanson, and Shore (1955a) reported that, in the hydrogen ion titration of bovine serum albumin, empirical values of w are independent of pH between pH values of 4.3 and 10.5 but change drastically when pH values are outside the latter limits.

Karush and Sonenberg (1949) found that the binding of bovine serum albumin with three alkyl sulfates could not be described mathematically by using the mass action equation even if a correction is made for electrostatic interaction. They assumed that the free energies of binding at the various sites obeyed a Gaussian distribution and from this deduced a theoretical expression which adequately described their data.

In a second study on the interaction between bovine serum albumin and an anionic azo dye, Karush (1950) found that the data failed to fit the Gaussian distribution hypothesis. Experimental results could, on the other hand, be explained by assuming the existence of two different groups of binding sites. Interactions between macro and simple molecules are probably more complex than that indicated in Eq. 17. Most macromolecules probably contain several sets or groups of sites with different affinities for the simple molecule. If the macromolecule contains m different sets of sites, the first set with n_1 equivalent and independent binding sites, each with intrinsic association constant K_1, the second set with n_2 such sites, each with an intrinsic association constant K_2, and so forth, then the mean number of sites occupied by D is

$$r = \sum_{i=1}^{m} \frac{n_i K_i (D_f)}{1 + K_i (D_f)} \qquad \text{(Eq. 26)}$$

The K_i in this equation is an intrinsic association constant and is different from the step equilibrium constant (designated as k_i) in Eq. 8. The K values have the following order; $K_1 > K_2 > \ldots > K_m$. The total number of binding sites are defined by Eq. 27.

$$n = \sum_{i=1}^{m} n_i \qquad \text{(Eq. 27)}$$

In order to further illustrate the effect of more than one set of binding sites, a schematic diagram of a macromolecule with two sets of sites is shown in Figure 2.

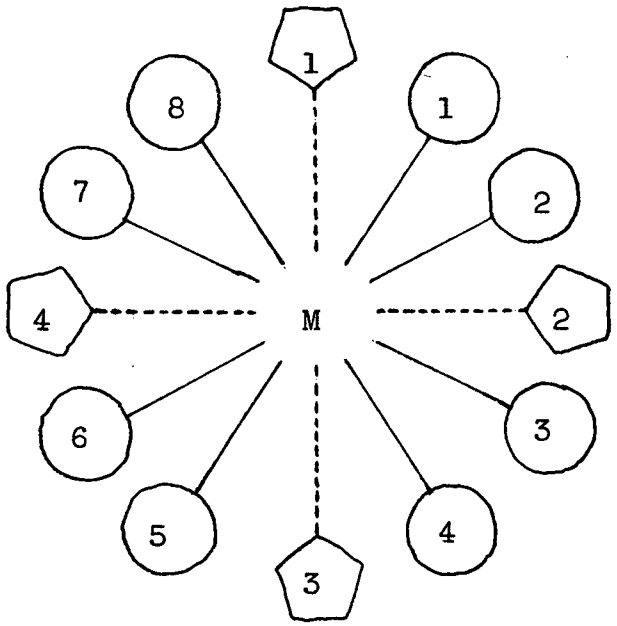

Figure 2. Schematic diagram of a macromolecule with two sets of binding sites. The molecule contains four sites with an association constant equal to K_1 and eight sites with an association constant equal to K_2.

Eq. 26 may now be re-written on the basis of two sets of binding sites illustrated in Figure 2.

$$r = \frac{4K_1 (D_f)}{1 + K_1 (D_f)} + \frac{8K_2 (D_f)}{1 + K_2 (D_f)} \qquad \text{(Eq. 28)}$$

The m term, for the example cited, is equal to two. There are twelve sites on the macromolecule.

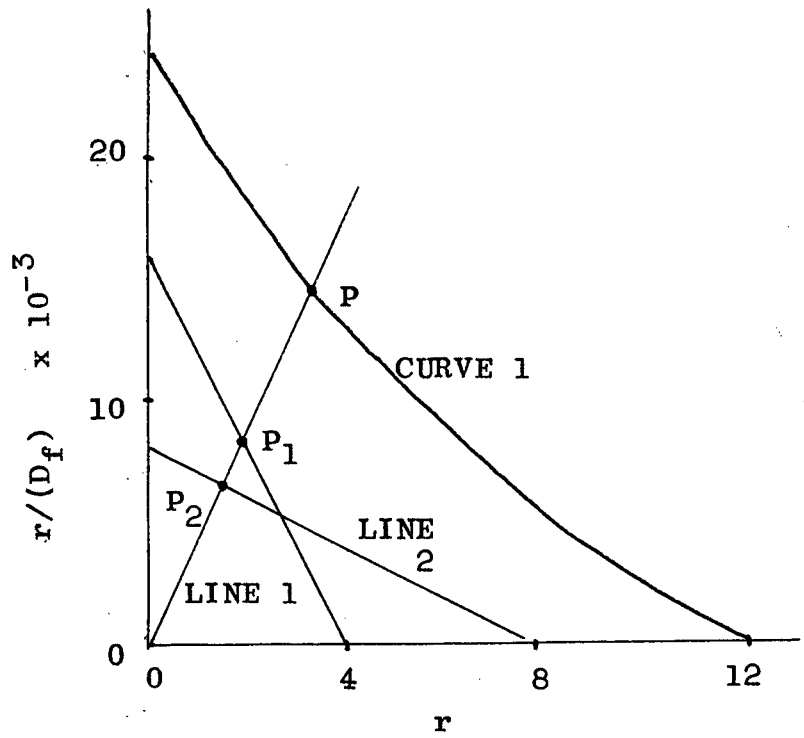

Figure 3. Hypothetical binding curve for a macromolecule with two groups of binding sites. $n_1 = 4$, $K_1 = 4000$; $n_2 = 8$, $K_2 = 1000$.

Sandberg, et al. (1966) and Rosenthal (1967) showed how an experimental curve obtained from a Scatchard equation can be resolved into two or more straight lines, each of which represents a different set of binding sites. This graphical approach to the treatment of binding data is illustrated in Figure 3. The K_1 and K_2 values for this hypothetical system are 4000 and 1000 respectively. If line 1 represents the first binding system and line 2 the second system, then curve 1 illustrates experimental data. Any point P on curve 1 is the sum of the binding coordinates of system 1 at point P_1 and of system 2 at point P_2. Points P_1, P_2, and P are so chosen

that they lie on a straight line which passes through the origin. Consequently, the geometric relationship

$$\overline{OP} = \overline{OP_1} + \overline{OP_2} \qquad \text{(Eq. 29)}$$

is obtained. Experimental data is resolved by drawing straight lines under the curve so as to satisfy the relationship shown in Eq. 29.

For systems with more than two sets of binding sites, the mathematical procedures for calculating K have been worked out by Hart (1965). Scatchard et al. (1950), in their study on the interaction between the thiocyanate ion and human serum albumin (HSA), obtained a binding curve which when resolved indicated the presence of two sets of binding sites with n values of 10 and 30 and K values of 1000 and 250. Karush (1950), in his study on the interaction between bovine serum albumin (BSA) and an anionic azo dye, used the mathematical approach described by Scatchard et al. (1950) but did not correct for electrostatic effects between binding sites.

Binding studies may also be carried out in the presence of two or more substances, both of which can attach themselves to the same site on M. If the two reacting species C and D have corresponding association constants K_C and K_D, then the binding of D in the presence of C is explained mathematically by the following equation.

$$r_D = \frac{n\, K_D\, (D_f)}{1 + K_D(D_f) + K_C(C_f)} \qquad \text{(Eq. 30)}$$

If the n sites are equivalent and independent, then

$$r_D = \frac{n\ K_D'\ (D_f)}{1 + K_D'\ (D_f)} \qquad \text{(Eq. 31)}$$

K_D' is defined by Eq. 32.

$$K_D' = \frac{K_D}{1 + K_C\ (C_f)} \qquad \text{(Eq. 32)}$$

The above equations imply that, in the presence of a constant concentration of C, the binding of D to M follows the same pattern as that indicated in Eq. 17, except that the K_D' value will be lower than that observed for K_D and is a function of (C). If K_D, or K in Eq. 17, is known, and K_D' is determined for a known value of (C), then K_C can be calculated from an equation which is derived from Eq. 32.

$$K_C = \frac{1}{(C_f)} \left(\frac{K_D}{K_D'} - 1 \right) \qquad \text{(Eq. 33)}$$

Klotz et al. (1948) studied the effects of salicylate, dodecylsulfate, and other anions on the binding of methyl orange by serum albumin and found that their results could be explained by using Eq. 33. Cogin and Davis (1951) studied, by use of Eq. 33, the competition in the binding of long chain fatty acids and methyl orange to BSA.

It has also been observed that, in many instances, the binding of anions by albumin does not decrease over the pH range of 6 to 9 to the extent that would be expected from the increased negative charge on the protein. Karush (1951) observed an increase in binding ability of albumin for methyl orange when the pH was increased from 6.4 to 7.6. The net charge changes, under these conditions, from -8 to -16. These discrepancies have been attributed to a failure of the Debye-Hückel Theory when applied to complex protein molecules or to the possible configurational changes which may occur in the albumin molecule over the 6 to 9 pH range. Such configurational rearrangements could change n and K values in a direction which could compensate for the repulsive electrostatic effects of an increasing pH.

Binding studies at more than one temperature have resulted in a thermodynamic evaluation of complexes. Enthalpy of binding, ΔH°, can be computed from the temperature depencence of association constant.

$$\left(\frac{\partial \ln K}{\partial (1/T)}\right)_p = \frac{-\Delta H^\circ}{R} \qquad \text{(Eq. 34)}$$

The standard free energy of binding, ΔG°, at equilibrium can be estimated from a knowledge of binding constants at various temperatures.

$$\Delta G^\circ = -RT \ln K \qquad \text{(Eq. 35)}$$

For isothermal changes in a system, the variation of free energy with temperature is expressed by the Gibbs-Helmholtz equation.

$$\left(\frac{\partial \Delta G^\circ}{\partial T}\right)_p = -\Delta S^\circ = \frac{\Delta G^\circ - \Delta H^\circ}{T} \qquad \text{(Eq. 36)}$$

Since association constants are dependent on the composition of the buffer, the standard state includes the buffer employed in the experiment. Thermodynamic parameters are, therefore, subject to possible error which would arise if the buffer ion binds significantly to M and varies considerably with temperature.

Thermodynamic data helps to explain the nature of the intermolecular forces responsible for binding (Karush, 1950; Klotz and others, 1949a; 1949b; 1953b). Temperature changes do not appear to affect greatly the extent of binding of ions with serum albumin. However, Klotz and Ayers (1952) have shown that there is a marked temperature-dependent binding between p-aminoazobenzene and bovine serum albumin.

For any equilibrium reaction which is not affected significantly by temperature, the heat of reaction is small. It follows from Eq. 36 that, if ΔH° is small, the magnitude of ΔG° at any fixed temperature is determined primarily by the value of ΔS°, the entropy change in the reaction. The favorable free energies of binding (i.e., negative ΔG° value) which have been observed for many ion-albumin complexes seem, therefore, to be a result of a favorable entropy change (i.e., positive ΔS° value) during binding rather than to be any favorable heat effect (i.e., negative ΔH° value).

The positive and relatively high values of ΔS° are in themselves unique because the reactions as written in Eq. 8 and 9 are association reactions for which one would expect unfavorable entropy changes. One explanation for this phenomenon is given below. Although an anion is usually written as D^{-}, it has been claimed that this ion has several polarized water molecules 'frozen' to it in aqueous solution. The subject has been extensively discussed by various investigators: Frank and Evans (1945), Claussen and Polglase (1952), Masterton (1954), Buswell and Rhodebush (1956), Feates and Ives (1956), Frank and Wen (1957), Klotz (1958), Nemethy and Scheraga (1962a; 1962b), Nemethy, Steinberg, and Scheraga (1963), Mohammad (1965), and Bernal (1965). Similarly, the protein molecule is highly hydrated.* This probably occurs around the charged loci of the cationic nitrogen atoms which seem to be directly involved in the binding process. Consequently, the formation of a bond between these two oppositely charged species would release some of the 'frozen' water molecules. The system, therefore, becomes more randomized and it becomes reasonable to expect an increase in the entropy of the system. This implies that, at the molecular level, there would be an increase in the number of molecule species upon formation of the anion-protein complex rather than a decrease as indicated in the M + D = MD equation. A schematic diagram of the changes in water structure around M and D in the M + D = MD reaction is shown in Figure 4.

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* It is well known that, in aqueous solution, macro and small molecules are hydrated. However, Frank and Evans (1945) were probably the first researchers to emphasize the importance of 'iceberg' around solute molecules in water.

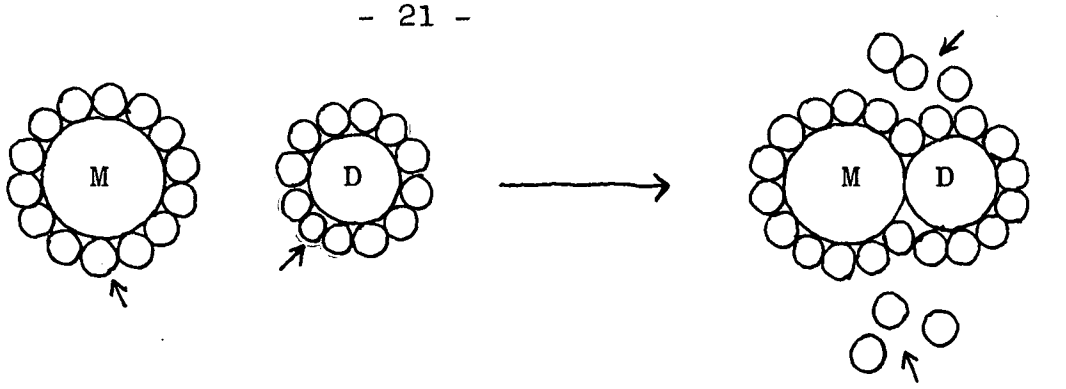

Figure 4. Schematic diagram of changes in water structure accompanied by an interaction between M and D.

Karush (1950), in his study on the interaction between an anionic azo dye and BSA, reported ΔS° values of 8.7 e.u. and 3.3 e.u. for the first and second groups of binding sites, respectively. He attributed the differences in ΔS° values to the structural differences between the two binding groups. It was suggested that the cationic group 1 sites are not bonded intramolecularly. Group 2 sites, on the other hand, are linked to nearby anionic carboxyl groups. Therefore, the binding of the anionic dye by group 2 sites would require the breaking of these bonds and would be accompanied by the release of an equal number of carboxyl groups. Binding on group 1 sites would involve a net neutralization of charge and this would result in a positive ΔS° value because water molecules are liberated from the ions. Such an entropy increase would not be observed at group 2 sites.

III. METHODOLOGY

Goldstein (1949), in his paper on the interaction between drugs and plasma proteins, has reviewed the methodology associated with binding studies. Similar papers have been published by Klotz (1953a). More recently, Meyer and Guttman (1968a) have reviewed those methods (e.g., kinetic, or dynamic, dialysis, partitioning, gel filtration, utilization of isotopes, nuclear magnetic resonance, and fluorescence quenching techniques) which have been developed during the past several years.

These methods fall into one of two categories. The first group depends upon the properties of the interacting molecule; the second, on the behavior of the macromolecule. Quantitative investigations must be based, therefore, on a method which will yield numerical values for two of the three unknowns, (D_f), (D_b), and (D_t) and for (M_t). These symbols are an inherent part of Eq. 17. Of the many methods described in the literature, only those based on spectrophotometry, solubility analysis, equilibrium and dynamic dialysis methods, and viscometry will be discussed here. Except viscometry, these methods measure changes in the properties of D, the interacting molecule.

1. Spectrophotometry

The spectrum of D is frequently changed by the macromolecule, M. These spectral changes have been used by many investigators to determine the extent of binding of a wide variety of substances with macromolecules (Job, 1926; Robinson and Hogden, 1941; Klotz, 1946c; 1947; Benesi and Hilderbrand, 1949; Oster and Immergut, 1954; Worley and Klotz, 1966; Connors and Mollica, 1966).

If the concentration of M is low, the total absorbance at a specified wavelength of free and bound D is defined by Eq. 37.

$$A = \epsilon_f b(D_f) + \epsilon_b b(D_b) \qquad \text{(Eq. 37)}$$

A is the absorbance; b is the cell length; and ϵ is the molar absorptivity of the specified forms of D. The term α is defined by Eq. 38.

$$\epsilon_b = \alpha \epsilon_f \qquad \text{(Eq. 38)}$$

The fraction of D_f, F_f, is expressed by the following equations.

$$F_f = \frac{\alpha \epsilon_f(D_t) - A}{\epsilon_f(D_t) - \alpha \epsilon_f(D_t)}$$

or

$$F_f = \frac{\epsilon_b - \epsilon_{app}}{\epsilon_f - \epsilon_b} \qquad \text{(Eq. 39)}$$

The apparent molar absorptivity in Eq. 39 is defined by Eq. 40.

$$A = \epsilon_{app} (D_t) \qquad \text{(Eq. 40)}$$

Molar absorptivity values for the bound drug may be determined by extrapolating absorbance values for D in the presence of increasing quantities of M. At high M concentrations, it is assumed that D is completely bound to M. The hypothetical curves in Figure 5 illustrate absorbance changes as a function of M concentration.

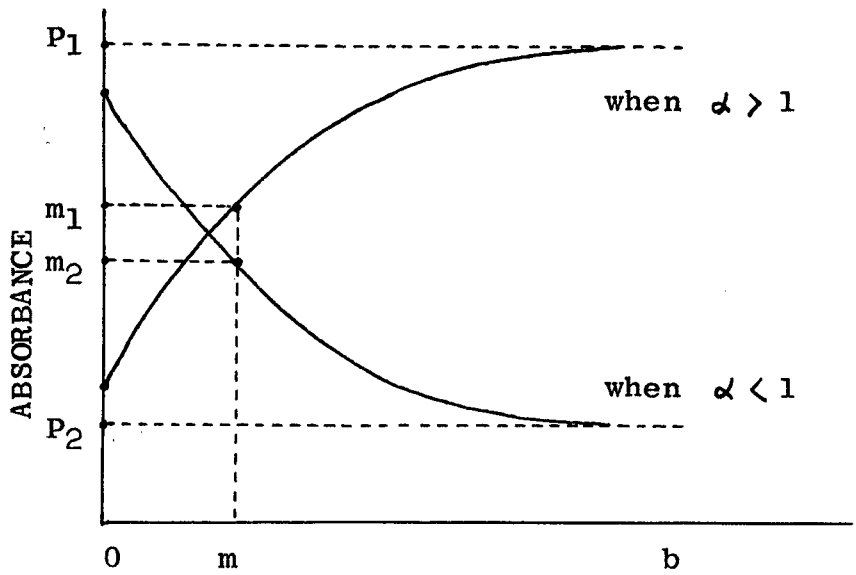

Figure 5. Hypothetical curves for changes in absorbance as a function of (M). ϵ_b values can be estimated from asymptotic values. At (M) = 0, m, b; F_f = 1, $\frac{1}{2}$, 0, respectively.

The precision of this method depends on the magnitude of the difference in absorption produced by the presence of M. Binding data obtained in this way must be complimented by data obtained in other ways because D concentrations are restricted by Beer's Law (i.e., they are too low and too narrow). Furthermore, M should not absorb energy at the wavelength at which the absorbance change for D due to the presence of M is maximum. In spite of these disadvantages, this method is important because small quantities of D can be determined with accuracy. Moreover, it is not necessary to separate artificially D_f and D_b as in the equilibrium dialysis technique.

Klotz (1946c), in his paper on the interaction between an azo dye and BSA, reported good agreement between the spectrophotometric method and the equilibrium dialysis technique in the region in which the two methods overlap. Oster and Immergut (1954) found that the absorbance of iodine at 290 mμ changed drastically in the presence of polyvinylpyrrolidone (PVP). This increase in absorbance in the presence of increasing PVP concentrations resulted in a sigmoidal curve similar to that in the upper part of Figure 5. However, at lower PVP concentrations, the curves changed slowly. This appears to indicate that the first few molecules of iodine are bound to PVP with difficulty but that further molecules are more easily taken up by the polymer.

2. Phase Solubility

This technique has been discussed in detail by Higuchi and Connors (1965). It involves the addition of an equal quantity (in excess of its normal solubility) of D into each of several solutions containing successively increasing amount of M. The solutions are brought to equilibrium at a constant temperature and then analyzed for D_t. A phase diagram is constructed by plotting the amount of D in solution versus (M_t). If there is no interaction between D and M, there will be no changes in (D) in the presence of M. If a soluble complex is formed, (D_t) will increase as (M_t) increases within a range of concentrations which is a characteristic of both the small and macro molecules. Increased quantities of D in the presence of M represent D_b since (D_f) is a fixed constant under specified conditions and is in equilibrium with (D_b) throughout the (M) range.

When the theory of multiple equilibria is applied to the data, the method becomes a 'spot' analysis because only one value for (D_f), or the solubility of D in the absence of M, is used throughout the experiment. Furthermore, this method is suitable only for substances of relatively low solubility. Certain aspects of the binding process can, however, be easily studied by utilizing phase solubility analysis. For example, the method has been used to investigate the effects of various solvents, pH, ionic strength, and temperature on the extent of binding. The value of r (see Eq. 6) is a constant under controlled

conditions and therefore a comparison of r values, under different conditions, gives information about the binding mechanism.

The thermodynamic parameters obtained from solubility analysis need not be corrected for disorientation entropy, because the interacting molecules possess no rotational freedom in the crystalline or bound state (Sahyun, 1964). In some methods (e.g., dialysis method), however, binding occurs in an unsaturated system, which implies that the bound molecules possess fewer degrees of freedom (Tanford, 1950; McMenamy and Seder, 1963). Therefore comparison of thermodynamic data from different methods should be carried out after corrections have been made for differences in the standard state.

Many investigators and in particular Higuchi and his co-workers have used this technique to study a wide variety of intermolecular reactions. The papers covering these interactions are not specifically referenced here but are listed in detail in the 'REFERENCES' section of this thesis (Higuchi and others, 1953a; 1953b; 1954a; 1954d; 1954e; 1954f; 1959; 1961; 1964;1965; Mader, 1954; Kostenbauder and Higuchi, 1956; Poole and Higuchi, 1959; Dittert and others, 1961; Breuninger and Goettsch, 1965; Wadke and Guttman, 1965; Wolfson and Banker, 1965; Singh and others, 1967). Most of these papers have no direct bearing on this study. However, several will be briefly reviewed in order to illustrate the applicability of the method.

Higuchi and Lach (1954d) reported that an insoluble complex was formed between phenobarbital and polyethylene glycol. Their results indicated that a 2:1 complex was formed (i.e., two

ethylene oxide units reacted with one phenobarbital molecule). Mansour and Guth (1968) studied the complexing behaviour of starch and starch fractions with benzoic acid, some of its derivatives, sorbic acid, and other selected molecules. Breuning and Goettsch (1965) studied the interactions between p-chlorometaxylenol and various synthetic polymers.

The method has not been used extensively to study the interaction between macromolecules. Laurent (1963) reported a relative decrease in the solubility of human serum albumin, γ-globulin, and fibrinogen in the presence of various types of dextran. He studied the effect of ionic strength and pH and showed that the solubility of proteins in the presence of dextran increased with an increase in the size of the protein.

3. Equilibrium Dialysis

Interactions between small and macro molecules may be studied quantitatively by utilizing the equilibrium dialysis technique.* A container is divided into two compartments by a semi-permeable membrane. A macromolecule solution is placed in one compartment; a solution containing the small molecule is placed in the second compartment. The small molecule passes through the membrane but the macromolecule is retained in its own compartment. At equilibrium, the total number of small

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* The kinetic or dynamic dialysis technique (Andreoli and others, 1965, Stein, 1965; Agran and Elofsson, 1967; Reuning and Levy, 1968; Meyer and Guttman, 1968b; 1970a; 1970b) will be briefly discussed in the 'RESULTS AND DISCUSSION' section of this thesis.

molecules in the M compartment will exceed that in the M-free compartment. The difference between these two concentrations is a measure of (D_b). Two possible sources of error, the Donnan effect, and membrane binding of small molecules must, however, be taken into consideration before applying this technique.

When a charged macromolecule is retained in one of the two compartments, at equilibrium, the concentration of diffusible ions is no longer identical across the membrane. This phenomenon has been described as the Donnan equilibrium (Overbeek, 1956). The ion ratio characterizing the distribution of diffusible ions across the membrane (R) is expressed by Eq. 41 when both anion and cation are univalent.

$$R = \frac{(C^+)_M}{(C^+)_F} = \frac{(C^-)_F}{(C^-)_M} \qquad \text{(Eq. 41)}$$

The subscripts, F and M, represent the M-free and M compartments, and the parentheses represent the concentrations of the specified ion species.

Values of R may be expressed as a function of the concentration of the neutral salt in the M-free compartment, $(C^+)_F$, the valence on the macromolecule, Z_M, and (M) in molality (Bull, 1964a).

$$R = \frac{Z_M\,(M)}{2(C^+)_F} + \sqrt{1 + \frac{Z_M{}^2(M)^2}{4(C^+)_F{}^2}} \qquad \text{(Eq. 42)}$$

Figure 6 illustrates the relationship between R and $(C^+)_F$ when the M-compartment contains 10 grams of the macromolecule per 1000 grams of solvent. The molecular weight of M is 40,000; the valence,

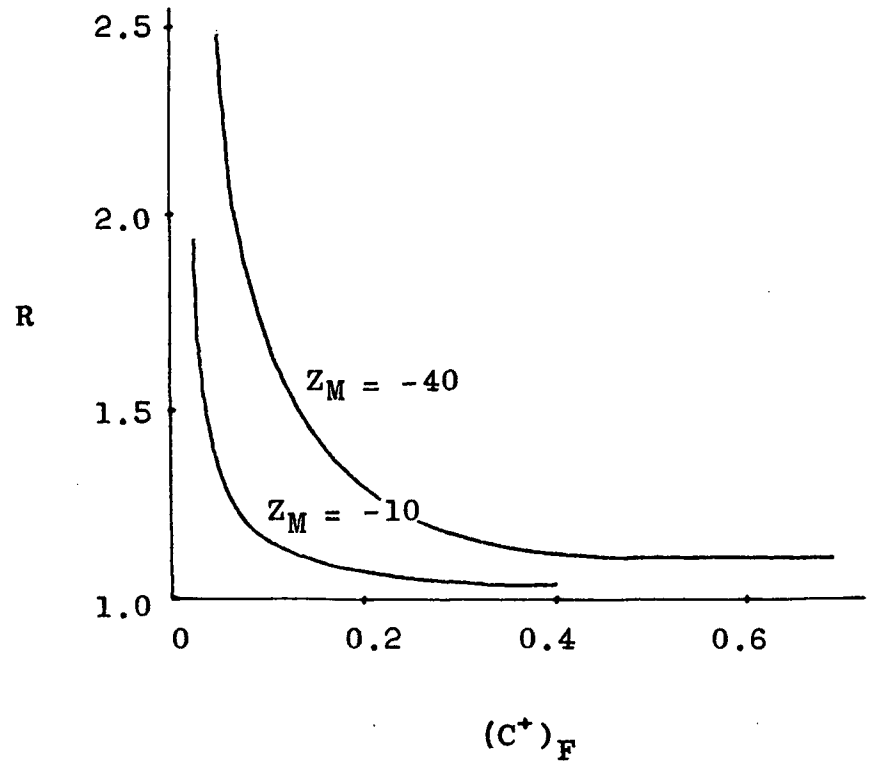

Figure 6. Calculated values of R as a function of salt concentration in the M-free compartment. See Bull (1964a)

in one series of experiments, is -10 and in the other is -40.

In a dilute solution of M, the Donnan effect can be neglected only if the concentration of the diffusible ion is reasonably high and the valence of M is fairly low. In a solvent system of high ionic strength and pH at which the macromolecule has a small valence charge, the abnormal distribution of small molecules across the membrane due to the Donnan equilibrium can be neglected.

The dialysis membrane may act as a binding site for the small molecule and a correction must be made for this interaction. Most corrections are made by using a control in which no macromolecule is present in the apparatus. It is then possible to

measure the 'loss' of small molecule from the solution. It has been either observed or assumed that the extent of membrane binding is proportional to the amount of small molecule added to the system.

Osborne (1906), at the turn of the century, studied the interaction between salt and proteins by utilizing the dialysis method. The procedure was refined by Klotz, Walker, and Pivan (1946b) and, of approximately 400 papers on protein binding reviewed by Meyer and Guttman (1968a), more than 130 papers reported the use of dialysis thechnique.

Its main advantage is that an interaction can be studied through a range of small molecule concentrations. Nearly complete saturation of the macromolecule with a given D can often be achieved. It is one of the few methods which are conducive to quantitative work and is thermodynamically sound. By using this method, it is possible to cover a wider range of r value (see Eq. 6) and thus obtain more information about the interaction. Karush and Sonenberg (1949) covered an r range from 0 to 10 in a study of the interaction between alkyl sulfates and bovine serum albumin. In a similar study, Pollansch and Briggs (1954) studied r values up to 40. They used volume ratios of protein compartment to protein-free compartment of 1 to 79 and 1 to 9. By using the former ratio, they were able to utilize a large quantity of detergent in their study. The maximum amount of detergent which could be used was limited only by the solubility of the detergent in the protein-free compartment.

Equilibrium dialysis would be carried out by equilibrating the macromolecule solution in a cellophane bag with an external solution containing the small molecule. However, the concentration of the small molecule is frequently limited by its solubility. Consequently, a large volume of solution containing a small quantity of the small molecule is required to cover the whole range of interaction. Under these conditions, a long period of time is required to equilibrate the system. Yang and Foster (1953) used a 1 to 100 volume ratio of protein compartment to protein-free compartment in their study on the interaction between dodecylbenzenesulfonate (SDBG) and bovine plasma albumin. However, even after a one-month time interval, dialysis equilibrium was not attained. They, therefore, modified their procedure by storing the mixed solutions for at least two days at 1 to 3° C and then dialyzing against an equal volume of the buffer used for an additional two days. The amount of free SDBG was then determined in the dialyzate.

Patel and Foss (1964) used a dialysis cell consisting of two plexiglas blocks separated by a semi-permeable membrane. Eide and Speiser (1967a) used a similar apparatus but stirred the solutions in the chambers with a magnetic stirrer. By using these approaches, a better control of membrane binding was attained because the surface area of the membrane is more or less constant throughout the study.*

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* Goldstein (1949) reported that loss of small molecules due to membrane binding is not only large (for example, to 20 per cent of total methylene blue in his study) but also variable from bag to bag.

A dialysis membrane is a very thin layer of a specified substance or mixture of substances. Various types of membranes are described by Craig (1965). Kostenbauder et al. (1969) discussed the use of nylon membranes. Nylon reacts with phenolic compounds, however, and Mitchell and Brown (1966) used rubber latex membranes in a study of the interaction between p-chloro-metaxylenol and a non-ionic surfactant. Most investigators, however, use cellophane membranes. These membranes are usually marketed in rolls and are stored in plastic bags to prevent drying. They contain glycerin and small amounts of other solutes but these can be easily removed by washing. When stored in a refrigerator, their porosity remains fairly constant over long periods of time. The main advantage of this type of membrane is that it is relatively free of fixed charges which would be ion selective (Craig, 1965).

4. Viscometry

A dilute solution of concentration C will have a slightly higher viscosity (η) than the solvent itself (η_o). The relative viscosity is defined by Eq. 43.

$$\eta_{rel} = \eta / \eta_o \qquad \text{(Eq. 43)}$$

The relative viscosity is, therefore, slightly more than one and includes the effect of solvent (unity) and solute. Specific viscosity, which isolates the solute effect, is defined by Eq. 44.

$$\eta_{sp} = \eta_{rel} - 1 \qquad \text{(Eq. 44)}$$

Specific viscosity depends on concentration, is small number, and is related the reduced viscosity.

$$\eta_{red} = \eta_{sp} / C \qquad \text{(Eq. 45)}$$

The reduced viscosity is a large number, does not change much with concentration in dilute solutions, and measures the increase in viscosity per unit concentration in a slotuion of concentration C.

If the above value is determined at several low concentrations, an extrapolation to zero concentration will yield a value which is due to the solute at infinite dilution per unit concentration. This value is called the intrinsic viscosity and is the value which is quantitatively important in solute-solvent interactions.

$$[\eta] = \lim_{C \to 0} \eta_{sp} / C \qquad \text{(Eq. 46)}$$

Huggins (1942) related concentration to reduced viscosity by using Eq. 47.

$$\eta_{sp} / C = [\eta] + K_H [\eta]^2 C \qquad \text{(Eq. 47)}$$

K_H is a constant, is known as Huggins parameter, and is determined experimentally.* Intrinsic viscosity depends on hydration and molecular shape. The relationship between particle assymetry and viscosity are complex but Mysels (1959), Yang (1961), and Flory (1953) have discussed the changes in intrinsic viscosity due to structural changes in macromolecules.

Miller and Hamm (1953) studied the properties of polyvinyl-pyrrolidone (PVP) by measuring viscosity, sedimentation velocity, and diffusion. Configurational changes in bovine serum albumin have been investigated by Yang and Foster (1954) by measuring intrinsic viscosity and specific rotation over a 1.3 to 7.0 pH range. They concluded that viscosity changes are due to swelling rather than coulombic repulsion and suggested that the expansion reaction is an all-or-none rather than a stepwise phenomenon which is fast and completely reversible. A similar study was carried out by Tanford and Buzzell (1956). They concluded that the expansion process was much more complicated than that suggested by Yang and Foster (1954). Doty et al. (1957) measured the intrinsic viscosity of poly-L-glutamic acid as a function of pH in 0.2 M sodium chloride-dioxane (2:1). They concluded that the polypeptide exists in an α-helix below a pH of 5.5 and as a random coil at pH values in excess of 6.5.

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* Kraemer (1938) proposed a similar equation; $\ln \eta_{rel}/C = [\eta] - K'[\eta]^2 C$ (Eq. 48). K_H and K' are related by each other in the following way; $K_H + K' = 0.5$ (Eq. 49). Both Eq. 47 and 48 indicate that a plot of η_{sp}/C and $\ln \eta_{rel}/C$ versus C should yield the same intercept, $[\eta]$, and the limiting slopes at C = 0 should satisfy the Eq. 49 relationship. By using both equations, intrinsic viscosity can be determined with some confidence.

Changes in viscosity have been used to detect interactions between small and macro molecules. Frank et al. (1957) measured the reduced viscosity of PVP in the presence of dye. In the absence of salt, the reduced viscosity was increased by the dyes and a sharp viscosity maximum was observed. The reduced viscosity increased with a decrease in PVP concentration. This appears to be typical polyelectrolytes because electrostatic repulsion of identical charges will lead to an unfolding of the polymer molecule. As the PVP concentration was decreased in the presence of a constant quantity of dye, the concentration of unbound 'gegenions' (counterions) increased. This increase will tend to suppress the electroviscous effect. A viscosity increase in the presence of salt was explained by assuming a cross-linking effect due to aggregation of dye ions.

A similar study was carried out by Molyneux et al. (1961b). Their results indicated that, in general, the polymer expands in the presence of anionic cosolutes. With non-ionic cosolutes, the polymer contracts and, in the presence of cationic cosolutes, no appreciable viscosity effects were observed. The influence of buffers and complexing substances on the rheological properties of PVP has been studied by Eide and Speiser (1967b).

The properties of PVP, in water and in salt solution, were determined by Goldfarb and Rodriguez (1968) by measuring heat capacities, specific volumes, and reduced viscosities. They concluded that the decrease in intrinsic viscosity of aqueous PVP with increased temperature was due to the progressive coiling of the molecule. Interactions between dodecyl sulfate

anions and BSA at high pH have been studied by Lovrien (1963). He maintained pH at a value at which the protein has a large negative charge. Under such conditions, an increase in hydrodynamic volume would be expected. However his viscometric data indicated that the detergent reduces expansion. The hydrocarbon portion of the molecule appears to induce conformational changes which counter unfavorable electrostatic energy changes.

Complexation has been studied by using capillary viscometers. The operating characteristics of these viscometers have been described by Van Wazer et al. (1963). Specifications may be found in documents issued by the American Society for Testing and Materials (A.S.T.M., 1966a; 1966b). The viscosity equation applicable to capillary viscometers is based on Poiseuille's Law.

$$\eta / \rho = Ct - B/t \qquad \text{(Eq. 50)}$$

ρ is the density of the liquid; t is the flow time in seconds; the constant C and B have been characterized by Cannon et al. (1960); the quantity B/t is called the kinetic energy correction factor. In a well designed viscometer, B/t is usually a small per cent of the Ct term. When the correction factor can be neglected, the calibration constant C is determined by measuring the flow time of a standard liquid of known kinematic viscosity. It may also be determined by comparing the flow time of a liquid in an uncalibrated viscometer with that observed in a master viscometer with a known C value.

IV. THE CHEMICAL AND BIOLOGICAL CHARACTERISTICS OF THE SUBSTANCES USED IN THIS INVESTIGATION

1. Bishydroxycoumarin

Bishydroxycoumarin (BHC), was first synthesized by Link (1943-1944), and is official in the U.S.P. It is also described as dicoumarin or dicumarol. Its chemical name is 3,3'-methylene-bis-(4-hydroxycoumarin) or 3,3'-methylene-bis-(4-hydroxy-1,2-benzopyrone).

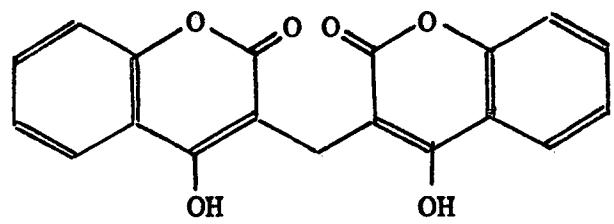

Figure 7. Chemical structure of BHC.

BHC is a white crystalline or amorphous powder practically insoluble in water. It is a weak dibasic acid and soluble in alkaline solutions. Burns, Wexler, and Brodie (1953) reported a pKa value of 5.7. This value was obtained by titrating 10 ml. of a 90% ethanolic solution (containing 20 mg. of BHC) with 0.025N sodium hydroxide solution. Nagashima, Levy and Nelson (1968a) obtained a value of 6.5 by using a partitioning technique.

In alkaline solution, BHC absorbs a maximum of radiant energy at 314 mμ. The pH of the solution affects the spectrum and isosbestic points are observed at 254 and 286 mμ (Findlay

and others, 1965). The molecular weight of BHC is 336.29. Melting points of 287-293°C (Merck Index, 1968) and 288-290°C (Nagashima, Levy, and Nelson, 1968a) have been reported. French and Wehrli (1965) published the infrared spectra of some of the coumarin anticoagulants, including BHC.

The first clinical trials utilizing BHC were carried out in the early 1940's. Ingram (1961) and Douglas (1962) reviewed the use of BHC in the treatment of myocardial infarction, angina pectoris, rheumatic heart disease, cerebrovascular disease, venous thrombosis, and pulmonary embolism. The pharmacological properties of coumarin anticoagulants have been reviewed by Levine (1967). Owren (1963a; 1963b) discussed the use of anticoagulant medication.

The importance of dissolution rate on clinical effect was first reported by Lozinski (1960). Findlay et al. (1965) reported that the particle size distribution is a major factor governing dissolution rate. O'Reilly, Aggeler, and Leong (1964) observed that the absorption of BHC from solution (or when administered as a powder) was rapid but that absorption was slow when whole tablets were administered to the patient.

Weiner et al. (1950) studied the physiological disposition of BHC in man. They reported a strong interaction between the drug and plasma albumin. A similar study was initiated by Lee et al. (1950) but the test animals in this instance were mice and rabbits. O'Reilly et al. (1964) carried out a pharmacodynamic study of BHC and warfarin in man. Other investigations utilizing different animal species were carried out by Jaques et al. (1957),

Christensen (1964), Solomon et al. (1967), and Nagashima et al. (1968b; 1968c; 1968d). The metabolism of BHC was investigated by Christensen (1966).

The in vitro binding of warfarin to albumin was extensively studied by O'Reilly et al. (1966; 1967; 1968; 1969). In addition to equilibrium dialysis, they used a heat burst micro-calorimeter to measure the heat evolved in the interaction (O'Reilly and others, 1968). The exothermic and non-ionic nature of the interaction was observed. The introduction of a polar hydroxy group on the coumarin ring during metabolism reduced its hydrophobic binding surface and thus decreases albumin binding (O'Reilly and others, 1969).

During their investigation of the analysis of BHC in biological fluids, Nagashima et al. (1968a) observed a decrease in plasma albumin binding at pH 4. It is at this pH that the plasma albumin undergoes a largely reversible structural alteration from compact to expanded form. The configurational expansion results in a disruption of non-polar clusters located in the interior of the albumin molecule and thus causes a decrease in binding strength.

2. Human Serum Albumin

Human serum albumin (HSA) is characterized by its solubility in water or by its electrophoretic behaviour. Its physicochemical properties may be explained in terms of an elongated ellipsoid with a molecular weight of 69,000, a length of 150 Å, and a diameter of 38 Å. Certain investigators report a molecular weight of 65,000. The arguments for or against these values are given by Putnam (1965). Utilizing intrinsic viscosity measurements, Tanford and Buzzell (1956) obtained an axial ratio of about 3 to 1.

HSA appears to be made up of amino acid residues but their primary sequence has not been established. The residues are joined together by a single long peptide chain which is internally cross-linked by 17-18 disulfide bridges. These disulfide linkages contribute greatly to the stability of the configuration. The importance of such cross-links in protein structures has been discussed by Edsall and Wyman (1958a).

A fractionation procedure for the isolation of albumin has been worked out by Cohn et al. (1950). This 'Cohn Fraction V' is mainly albumin and is prepared by precipitation at pH of 4.8 and an ethanol concentration of 40%. Spectrophotometric and turbidimetric (Layne, 1956), Kjeldahl and isotopic techniques (Haurowitz, 1963) have been used to analyze proteins. Although serum albumin is available in crystalline form, the substance itself is micro-heterogeneous. This heterogeneity may be established by examining the substance electrophoretically at low ionic strength, chromatographically, interferometrically, or serologically. Foster (1968) has written a review on this subject.

Because of its availability, HSA is one of the most widely investigated proteins. Its ion binding behaviour, its amphoteric heterogeneity at low pH, and its denaturation have been investigated. Foster (1960) reviewed these characteristics. Titration curves have been evaluated by Tanford et al. (1950; 1955a; 1955b). They estimated the number of dissociable groups and calculated their intrinsic dissociation constants. A satisfactory agreement was obtained with regard to the amino acid contents. They and Foster et al. (1956), Aoki et al. (1957) and Clark et al. (1962) observed that both HSA and bovine serum albumin exhibited an anomalous titration behaviour below the isoelectric point, 4.7, and beginning at about a pH of 4. Yang and Foster (1954), on the basis of a study of the effect of pH on optical rotation and viscosity, concluded that an isotropic expansion of the albumin molecule ocurred in acid solution. They attributed the molecular expansion to a mutual repulsion of the positively charged ammonium groups.

This phenomenon was confirmed by Tanford et al. (1955b; 1956). They proposed that the expansion takes place through an intermediate expandable form - the so-called 'F form'. Foster and Clark (1962) presented evidence that the native form (the 'N form') has a large number of carboxylate groups that are masked and that isomerization to the F form leads to the normalization of all carboxylate sites.

Because it has a strong affinity for ions and other substances, albumin has been used to elucidate protein-ion interactions. Klotz (1953a) was the first investigator to review this subject. Karush (1950) attributed the high reactivity of albumin with a

variety of anions to the particular configurational adaptability of the molecule. Serum albumin interacts readily with detergents ions such as dodecyl sulfate. Interaction of this type have been investigated in various laboratories (Putnam, 1945; Karush, 1949; Pollansch and Briggs, 1954; Lovrien, 1963) and reviews on the subject have been published by Foster (1960) and Ray (1968).

The classical review on drug-albumin interactions was published by Goldstein (1949). More recently, Meyer and Guttman (1968a) have re-reviewed this particular aspect of serum albumin binding. The physilogical consequences of ion binding and the affinity of serum albumin for dyes, drugs, and similar molecules have been emphasized by Bennhold (1961). The effect of drug-albumin interactions on the absorption, distribution, and excretion of the drug has been discussed by Martin (1965).

Wishnia and Pinder (1964) observed that the extent of binding of alkanes (C_2 to C_5) to the F form of bovine serum albumin is much lower than that obserbed for the N form. They concluded that the large hydrophobic clusters in the interior of the protein are responsible for the interaction. Nagashima et al. (1968a) observed a similar decrease in binding capacity of BHC to HSA in the acid range. The role of hydrophobic bonding in relation to physicochemical behaviour of protein solutions has been discussed by Kauzmann (1959), Klotz (1958; 1960), Scheraga (1961; 1963), Nemethy et al. (1962c; 1963), and Cecil (1967). In this context, studies on water structure have been made by various investigators (see p. 20). Solvent effects on the binding or organic ions by proteins have been discussed by Klotz and Luborsky (1959).

3. Polyvinylpyrrolidone

Polyvinylpyrrolidone (PVP) is a water soluble, high molecular weight polymer. It may be represented structurally in the following way.

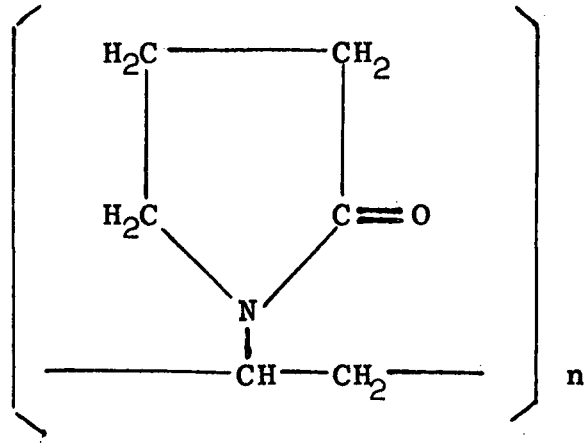

Figure 8. Chemical structure of monomer of PVP, N-vinylpyrrolidone (C_6H_9ON - 111.14).

Because of its chemical and physical properties, the polymer has been used as a plasma extender. The molecular weight of PVP is defined in terms of the viscosity of a dilute solution. For this purpose, the Fikentscher K_F value is commonly used (G.A.F., 1957). The average molecular weight of the PVP which is used as a plasma extender is approximately 40,000 (K_F = 30). This value is derived from ultracentrifugation and osmosis measurements. Its physicochemical properties were investigated by May et al. (1954). An aqueous solution of PVP is slightly acidic (pH of 4) but has no buffering action.

Using light scattering measurements and assuming that the PVP molecule is a random coil, Hengstenberg et al. (1952) reported a mean square molecular diameter of 360 Å corresponding

to a molecular weight of 249,000. A similar study was carried out by Miller and Hamm (1953). Assuming an elongated ellipsoid shaped model, they obtained an axial ratio of 22 to 1 and a root mean square distance of 169 Å for PVP of molecular weight of 41,500. At a lower temperature, PVP was believed to be more tightly coiled.*

Drugs, dyes, and toxins bind with PVP. References on this particular subject and to its physicochemical and physiological properties are available from the manufacturer of the polymer (G.A.F., 1967). Oster and Immergut (1954) studied the iodine-PVP complex and this complexation process has been used quantitatively to determine PVP (Campbell, 1953). Scholtan (1953) expressed the binding process of PVP with dyes by use of Langmuir adsorption isotherm. His thermodynamic data indicated that in one case the affinity of the binding process was determined preferably by the entropy, in the other (meta-benzopurpurin 4B) preferably by the heat of reaction. In solutions containing PVP, albumin and dyes simultaneously, a complexation balance was formed. Theoretical relations were given between these three components. The calculated and experimental values showed a close agreement.

Spitzer and McDonald (1956) studied the interactions between bovine serum albumin and PVP with bromophenol blue (BPB). There was no evidence of an electrostatic factor with respect to the exothermic PVP-BPB binding. Frank et al. (1957) studied the interaction between PVP and azo dyes. Orange II and benzopurpurin 4B appear to be bound to the chain segment of seven

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* This statement is in contradiction to that appeared on p. 36 (Goldfarb and Rodriguez, 1968). See 'RESULTS AND DISCUSSION' section of this thesis for more detail.

or ten monomer units. Molyneux and Frank (1961a; 1961b) studied the interaction of PVP with a large number of aromatic compounds. They observed entropy gains in almost all systems and concluded that hydrophobic bonding ocurred. Infrared spectra of dry PVP films containing varying concentrations of cosolutes indicated the presence of polymer-cosolute hydrogen bonds (1961a). Using light scattering and viscometric methods, they concluded that anionic cosolutes expand the polymer, nonionic cosolutes contract the molecule, and cationic cosolutes had no effect on molecular size (1961b).

Worley and Klotz (1966) studied the effect of PVP on the near infrared spectra of H_2O - D_2O solutions. They suggested that PVP exhibits a structure making character. Goldfarb and Rodriguez (1968), however, found no evidence for the existence of more 'structural water' in the vicinity of the PVP molecule.

PVP has several properties which makes it of particular interest to the pharmaceutical scientist. PVP forms water soluble or water dispersible complexes with a wide variety of water insoluble drugs (G.A.F., 1967). References covering these complexation studies are given in Table 1. Binding depends not only on the nature of the simple molecule but also on the concentration of the macromolecule (Edsall and Wyman, 1958c). In most of the investigations cited in Table 1, at a fixed total drug concentration, concentration ratio of total to free (or vice versa) is a linear function of macromolecule concentration. The ratio can, therefore, be an approximation of the extent of binding. Ratios at 1% PVP concentration are shown in Table 1.

Table 1. The Complexing Behaviour of PVP with Various Types of Drugs.

Drug	°C	Solvent System	$(D_t)x10^3$ mole/L.	%PVP	$\frac{(D_t)}{(D_f)}$	Methodology and Reference
Sulfathiazole	0	Water	.393	1.0	1.42	Equilibrium Dialysis, Higuchi and Kuramoto (1954b).
Sodium Salicylate	0	Water	12.49	1.0	1.1	
Procaine HCl	0	Water	5.56	1.0	1.00	
Chloramphenicol	0	Water	1.547	1.0	1.05	
	30	Water	1.547	1.0	1.05	
	30	Water	3.095	1.0	1.05	
Benzyl Penicilin	0	Water	2.68	1.0	1.00	
Mandelic acid	0	Water	6.619	1.0	1.08	
Caffeine Theophylline Cortisone	No Evidence of Complex Formation					
Benzoic Acid	0	Water	5.74	1.0	1.13	Equilibrium Dialysis and Solubility, Higuchi and Kuramoto (1954c), Guttman and Higuchi (1956).
Salicylic Acid	0	Water	3.62	1.0	1.22	
m-Hydroxy Benzoic Acid	0	Water	1.81	1.0	1.43	
	0	Water	7.25	1.0	1.47	
p-Hydroxy Benzoic Acid	0	Water	1.82	1.0	1.40	
	0	Water	7.25	1.0	1.40	
p-Amino Benzoic Acid	0	Water	?	1.0	1.23	
Phenobarbital	30	Water	2.58	1.0	1.19	
	30	Water	4.31	1.0	1.19	
Citric Acid Aminopyrine	No Evidence of Complex Formation					
Phenol	0	Water	200.0	.37	1.19	
	0	Water	160.0	.12	1.23	
Chlorobutanol	15	Water	28.5	1.0	1.06	Equilibrium Dialysis, Bahal and Kostenbauder (1964).
	30	Water	28.7	1.0	1.08	
	30	Water	57.7	1.0	1.08	
	45	Water	28.8	1.0	1.11	
Benzyl Alcohol Phenylethyl Alcohol	No Evidence of Complex Formation					

(Continued on next page.)

(Table 1. Continued)

Drug	°C	Solvent System	$(D_t)x10^3$ mole/L.	%PVP	$\frac{(D_t)}{(D_f)}$	Methodology and Reference
p-Chlorometa-xylenol *a	30	Water	.113	2.0	1.13	Equilibrium Dialysis and Solubility, Breuninger et al. (1965).
	30	Water	.140	2.0	1.17	
	30	Water	.197	2.0	1.19	
	30	Water	.225	2.0	1.18	
	30	Water	.283	2.0	1.18	
	30	Water	.401	2.0	1.15	
Methylparaben	27	Water	6.12	1.0	1.15	Eqm. Dialysis, Miyawaki et al. (1959), Polli et al. (1969).
Propylparaben	30	Water	1.23	1.0	1.30	
Hexylresor-sinol	23	Water	.858	.05	3.00	
Tannic Acid *b	Soluble Complex Formed				—	Kabadi et al. (1966).
Benzoic Acid	22	Ionized	33.0	1.0	1.02	Equilibrium Dialysis, Eide and Speiser (1967a).
	22	pKa	13.7	1.0	1.03	
	22	pKa	36.3	1.0	1.03	
	22	Union.	10.1	1.0	1.11	
Phenol	22	pKa	22.5	1.0	1.02	
	22	pKa	45.1	1.0	1.05	
	22	Union.	32.4	1.0	1.07	
Aniline	22	Ionized	35.8	1.0	1.00	
	22	pKa	38.5	1.0	1.02	
	22	pKa	74.3	1.0	1.02	
	22	Union.	33.9	1.0	1.04	
Nitrobenzene	22	Union.	3.52	1.0	1.03	
p-Nitrophenol	22	Union.	13.3	1.0	1.11	
p-Nitrobenzo-ic Acid	22	Ionized	4.49	1.0	1.02	
	22	pKa	1.32	1.0	1.02	
	22	pKa	3.82	1.0	1.03	
	22	Union.	1.02	1.0	1.06	
p-Hydroxy Benzoic Acid	22	Ionized	13.5	1.0	1.08	
	22	pKa of COO^-	12.5	1.0	1.11	
	22	pKa of COO^-	33.7	1.0	1.13	
	22	Union.	1.51	1.0	1.25	
p-Amino Benzoic Acid	22	$-COO^-$	12.1	1.0	1.08	
	22	pKa of COO^-	12.3	1.0	1.09	
	22	pKa of COO^-	21.5	1.0	1.11	
	22	pKa of $-NH_3^+$	11.9	1.0	1.11	

(Continued on next page.)

(Table 1. Continued)

Drug	°C	Solvent System	(Dt)x10^3 mole/L.	%PVP	$\frac{(D_t)}{(D_f)}$	Methodology and Reference
Bezocaine	22	pKa of	1.09	1.0	1.11	Equilibrium Dialysis, Eide et al. (1967a).
		$-NH_3^-$	2.25	1.0	1.12	
		Union.	1.13	1.0	1.13	
Methylparaben	22	pKa	.74	1.0	1.09	
	22	pKa	2.66	1.0	1.11	
	22	Union.	1.32	1.0	1.10	
Butylparaben	22	Union.	.517	1.0	1.20	
Propylparaben	22	pKa	1.06	1.0	1.13	
	22	pKa	1.59	1.0	1.15	
	22	Union.	.875	1.0	1.18	
Methyl-benzoate	22	Union.	?	1.0	1.02	
Ethylbanzoate	22	Union.	4.1	1.0	1.03	
Procaine HCl	22	Ionized	8.16	1.0	1.02	

*a - Insoluble complex is formed up to PVP concentrations of 0.4%, after which solubility increases linearly with regard to PVP concentration.

*b - The presence of dextrose slightly enhances the complexation.

Some investigators presented their results in graphical form and it was necessary, therefore, to make approximations directly from these graphs. Similarly, the different concentration terms in the various publications necessitated a conversion to an identical concentration term. Several of the factors affecting binding are illustrated by the results in Table 1. Bezoic acid, for example, is more weakly bound to PVP than its derivatives. Orthohydroxy benzoic acid (Salicylic acid) has a lower binding tendency than does the meta or para forms. The former substance has a higher internal coordination or chelation between -OH and -COOH groups.

The lesser complexing tendency of the corresponding para-amino compound is probably due to the weaker electrophilic nature of amino hydrogen as compared with the hydroxyl hydrogen. This results in weaker hydrogen bond formation (Higuchi and Kuramoto, 1954c).

The effect of temperature is illustrated by the studies of the PVP-chlorobutanol complex. Binding strength increases with temperature. This indicates an endothermic reaction. Bahal et al. (1964) concluded that the large positive entropy change accompanying the binding was due to the formation of hydrophobic bonds. If the PVP concentration is fixed, the extent of interaction is often enhanced by increasing total drug concentration (Breuninger and Goettsch, 1965; Eide and Speiser, 1967a). This concentration dependency is not, however, observed in most investigations.

The results obtained by Eide and Speiser (1967a) strongly suggest that the nonionic species react more readily with PVP. The low interaction tendency of the ionic compounds is most likely due to the hydrophobic nature of the substances. The increased complexing tendency of ionic compounds containing -OH, $-NH_2$, or -COOH groups indicates that hydrogen bonding also plays a significant role in reactions of this type. Buffer substances (Eide and Speiser, 1967b) and third components in the solvent system (Kabadi and Hammarlund, 1966) will also alter PVP-drug binding. Simonelli et al. (1969) reported that the apparent solubility and rate of solution of sulfathiazole

from compressed tablets containing PVP is greatly increased if the drug is first coprecipitated with the polymer. These investigators developed methods for the preparation of these coprecipitates in water and in 95% alcoholic solution.

4. Dextran, Starch, and Hydroxyethyl Starch

Dextrans are polysaccharides and consist of a variety of α-polyglucosans produced by Leuconostoc mesenteroides and closely related bacteria under suitable environmental conditions. Synthetic procedures have been developed by Ruckel and Schuerch (1966). The concentration of dextran in solution may be determined by heating the carbohydrate with anthrone in sulfuric acid (Scott and Melvin, 1953). Arond and Frank (1954) studied the molecular weight distribution of native dextran by utilizing light scattering techniques and the intrinsic viscosity in aqueous solution. Granath (1958) studied the properties of branched dextran in solution. By measuring viscosity, light scattering properties, and sedimentation rates, Granath was able to obtain a more complete picture of the hydrodynamic behaviour of dextran molecule.

Grönwall (1957) discussed the use of dextran solutions as plasma extenders. It is generally recognized that as the molecular weight of dextran increases its interaction with proteins increases. In this context, Ricketts (1966) published data on the molecular composition of dextran solutions which are currently used as plasma extenders. Reese et al. (1966) compared the extent of

branching of the synthetic dextran with that of the natural product. Enzymatic analysis confirmed the basic similarity of the two forms. More recently, Laurent and Granath (1967) fractionated dextran by using Sephadex G-200 packed into chromatographic columns.

Greenwood (1956) reviewed the physicochemical properties of starch. BeMiller (1965) briefly discussed complexation of carbohydrates with organic substances. Analytical procedures for determining starch sol have been described by Launer (1963) and Whistler et al. (1965). Saito (1957) reported that anionic surfactants are readily adsorbed on various nonionic polymers including starch and PVP, especially above the critical micelle concentration. They suggested that the polar part of the anion is probably adsorbed on the oxygen atom in the polymer. However, little attention has been given to this complexation phenomenon. Gray and Schoch (1962) studied the influence of various fatty adjuvants on the swelling behavior of several starches. Goudah et al. (1965) and Mansour et al. (1968) studied the solubility characteristics of benzoic acid derivatives in the presence of various starch sols. They concluded that amylose is the main complexing component in starch.

Hydroxyethyl starch (HES) is a waxy or branched starch which has been hydroxyethylated to retard intravascular hydrolysis. The substance is being promoted as a plasma substitute. However, not much is known about the physicochemical properties of HES solutions. Most publications deal with the in vivo behaviour in animals and have been collected by the National Academy of Sciences (N.A.S. - N.R.C., 1965).

V. EXPERIMENTAL

1. Apparatus

(a) Spectrophotometers

Beckman DU
Beckman DU-2
Bausch & Lomb Spectronic 505
Beckman IR-10

(b) Fisher Accumet 310 pH Meter

(c) International Equipment Company HN Centrifuge

(d) American Laboratory Sterilizer

(e) Westphal Balance

(f) Blue M Electric Company Refrigerated Bath

(g) Cannon-Fensk Viscometer (Size 50)

(h) Haake Thermoregulator (Type FE)

(i) B-D Cornwall Continuous Pipetting Outfit with Swinny Filter Adopter. MF-Millipore (WP) Filter Paper (pore size, 0.65 ± 0.30 μ) was used with the apparatus.

2. Chemicals and Reagents

(a) Bishydroxycoumarin, U.S.P., (BHC). The melting point of the substance was 287-288°C. The drug was obtained from Abbott Laboratories Limited, Montreal, Quebec and was identified by infrared spectrophotometry.

(b) Polyvinylpyrrolidone (PVP). Plasdon C was purchased from the General Aniline Corporation, New York, N.Y. The K_F value range is 28 to 32 (upper 15% not higher than K_F, 41; lower 25% not less than K_F, 16) and the molecular weight is 40,000. To remove monomer, the PVP was extracted with

anhydrous ether in a soxhlet apparatus for 24 hours. The PVP was then dried in an oven, under vacuum and at a temperature of 35-40°C for 12 hours. May (1954) reported that the monomer could be extracted with methylene chloride. It was found, however, that this solvent dissolved the polymer and for this reason, anhydrous ether was used to extract the monomer (see Higuchi et al., 1954b). The water content of PVP was determined from the loss in weight after drying in an oven at 110-115°C for 24 hours. Five such determinations were carried out. The average value was 3.79% (range, 0.28%).

(c) Dextran-75. The sample was obtained from Abbott Lab., Montreal, Quebec. Its molecular weight was 75,000 ± 15,000. Its water content was determined in the manner indicated above. The average value was 2.24% (range 0.6%).

(d) Human Serum Albumin (Cohn Fraction V, HSA). The albumin was obtained from Pentex Inc., Kankakee, Ill. No loss in weight on drying was detected.

(e) Hydroxyethyl Starch (HES). The starch was obtained from McGaw Lab., Glendale, Calif. The company also supplied a 6% solution containing 0.9% sodium chloride.

(f) Potato Starch. The starch was purchased from Baker and Adamson Products, New York, N.Y.

(g) Tris(hydroxymethyl)aminomethane (Tris). Reagent Grade.

(h) 0.1N I_2 T.S., 1N HCl, 1N NaOH, U.S.P.

(i) Buffer Components. Reagent Grade.

(j) Dimethylformamide(DMF). Reagent Grade.

3. Determination of Apparent pKa Values for BHC

(a) Potentiometric Titration.

Dissolve an accurately weighed sample of BHC (approximately 140 mg.) in 200 ml. of DMF. To a 10.0 ml. aliquot of this solution, add 2.5 ml. of 0.01N HCl, 30.0 ml. of DMF, and sufficient water to make 100.0 ml. of solution. Titrate the solution with 0.01N NaOH, using a glass-calomel combination electrode to follow pH changes. Perform a blank titration.

(b) Spectrophotometric Determination.

Add 400 ml. of 0.01N NaOH to an accurately weighed sample of BHC (approximately 50 mg.). Shake until dissolved (approximately 2 hours) and dilute to 500.0 ml. with 0.01N NaOH. Dilute this solution to give a final concentration of approximately 10 mg./L.

A series of buffer solutions were prepared (pH range of 2.5 to 10.5) by utilizing Perrin's buffer tables (Perrin, 1963). The ionic strength of these solutions was 0.01. The pH difference between buffer solutions was 0.5 units except in the case of the buffers in the 4.0 to 7.0 range. For these solutions, the pH interval between solutions was of the order of 0.2 units. Chloroacetic acid - KOH gave a 2.5 to 3.0 range; formic acid - KOH, a 3.2 to 4.2 range; acetic acid - KOH, a 4.4 to 6.2 range; phosphates, a 6.4 to 7.6 range; Tris - HCl, an 8.0 to 8.8 range; borates, a 9.0 to 9.7 range; and carbonates, a 10.0 to 10.5 range. A 2.5 ml. aliquot of the BHC stock solution (BHC concentration of approximately 10 mg./L.) was diluted to 50.0 ml. with buffer. Visual observation indicated the BHC remained in solution over the entire pH range. Actual pH values were determined by using a pH meter.

Absorbance values were determined by the trace analysis technique (Reilley and Crawford, 1955; Pernarowski, 1969) at 276.5 and 315 mμ. For convenience, a BHC solution of 0.01N NaOH (BHC concentration of approximately 3 mg./L.) was used to adjust zero per cent transmittance (0% T) at both wavelengths. Absorbance values were determined in the following manner.

> Select wavelength and adjust instrument to read 0% T. Place reference solution in the light beam. Turn the selector switch on the Beckman DU spectrophotometer to the one position and zero the instrument using the dark current control knob. Return selector switch to the check position. Place buffer solution in the beam and set the instrument to 100% T. Determine the absorbance of a solution containing the drug.

Absorbance was plotted versus pH. The pH values at the inflection points corresponds to the apparent pKa values. The mathematical basis for the calculation is given in Eq. 51.

$$pKa = pH - \log \frac{(\text{ionized form})}{(\text{unionized form})} \qquad \text{(Eq. 51)}$$

The solubility of BHC in buffer solutions of low pH is the limiting factor in the determination of pKa_1 value. However, the second pKa value can be more easily determined since the drug is more soluble at higher pH values. In determining pKa_1 value, BHC concentration of approximately 0.5 mg./L. was used to a pH of 7. The absorbance values of such solution were determined at 276.5 mμ by the trace analysis method. Two more runs were made in buffer-DMF systems which containing 1.0 and 0.8 mg. drug per liter of solution containing 2.5 and 10.0% v/v DMF, respectively.

The pKa_2 value was determined by using solutions containing 10 mg. drug per liter. The spectrum-wavelength curves of these solutions (pH range of 6.0 to 10.5) were recorded by using a B & L Spectronic 505 spectrophotometer. The procedure was repeated with solutions containing 5 and 20% v/v DMF.

4. Solubility Measurements on BHC

(a) Effect of pH on the Solubility of BHC. Buffer solutions (pH range of 6.4 to 8.0) were prepared by using Tris and hydrochloric acid. The buffers were prepared in the following manner.

> Dilute 50.0 ml. of 1N HCl with water. Insert glass-calomel electrodes into the solution and titrate with 0.5M Tris solution to the desired pH. Dilute with water to make 250 ml. and redetermine the pH. The ionic strength of these buffers was 0.2.

Solubility measurements were carried out in the following manner.

> Transfer 50 mg. of BHC to a 125-ml. glass bottle and add 100 ml. of buffer. Tumble in a water bath at 30°C at a rotational speed of approximately 30 rpm. After 24 and 48 hours, withdraw aliquots filtered through a Swinny filter adaptor and determine the absorbance at 286 mμ, the isosbestic point for BHC.

The solutions must be diluted prior to measurement, the extent of dilution depending on the pH of the buffer. The filtering device must be maintained at 30°C in order to prevent drug from precipitating from solution. The pH values of the solutions were determined both before and after solubilization.

(b) The Effect of Ionic Strength on the Solubility of BHC. Hydrochloric acid solutions were prepared from 1N HCl. The pH of each of these solutions was adjusted to 7.2 with either 0.1 or 0.5M Tris solution. The ionic strength of each of the solutions was calculated from the amount of hydrochloric acid used to prepare the buffer. The solubility of BHC in these buffer solutions was determined in the manner described in the previous section.

5. The Solubility of BHC as a Function of Macromolecule Concentration

(a) Starch Sol. A starch sol was prepared by a procedure similar to that described by Goudah and Guth (1965).

> Prepare a slurry, in a 100-ml. beaker, containing 25 Gm. of potato starch with Tris buffer (pH of 7.2; ionic strength, 0.2). Slowly add the slurry to 350 ml. of hot buffer at 90-95°C. Agitate continuously for five minutes in a boiling water-bath. Transfer the starch sol to an autoclave and heat for three hours at approximately 125°C. Remove the sol from the autoclave, cool, and adjust the pH, if necessary, to 7.2 with 0.1M Tris. Make volume to 500 ml. with Tris buffer. Calculate the starch concentration on the basis of the amount of starch weighed initially.

Solubility measurements were carried out in the following manner.

> Prepare starch solutions by diluting the sol with Tris buffer. To each solution, add excess BHC. Tumble these preparations in closed containers in a 30°C water-bath for 40 hours. Filter the solutions through a coarse sintered glass filter. Maintain the filtering apparatus at 30°C prior to use. Dilute the samples, if necessary, and determine the absorbance of the solution at 286 mμ. Place a starch solution of the same concentration as that in the test sol in the reference beam of the B & L Spectronic 505 spectrophotometer.

(b) HES. Solubility measurements were carried out in the following manner.

Prepare a 3% HES solution by diluting a 6% solution containing 0.9% sodium chloride with Tris buffer. Add hydrochloric acid solution to obtain an ionic strength of 0.2 and a pH of 7.2. Typical quantities are listed below.

6% HES in 0.9% NaCl		500.0 ml.
1N HCl		123.0 ml.
1M Tris		134.6 ml.
Water	q.s. to	1000.0 ml.

Calculate the ionic strength from the quantity of hydrochloric acid and sodium chloride in the solution.

Prepare a series of HES solutions containing various concentrations of HES by diluting with Tris buffer. Add an excess of BHC to each solution and determine the solubility in the manner described in the previous section.

(c) PVP. Solubility measurements were carried out in the following manner.

Prepare a 4% PVP solution by dissolving the substance in Tris buffer (pH of 7.4; ionic strength, 0.15). Dilute this stock solution with Tris buffer to prepare solutions containing various concentrations of PVP. Add excess BHC to 25.0 ml. of each of these solutions. Transfer the sealed 30-ml. centrifuge tubes to a 20°C water-bath and tumble for 40 hours. Transfer aliquots to a second series of centrifuge tubes. Centrifuge for 20 minutes at 2,500 rpm. Withdraw aliquots, dilute with Tris buffer and determine the absorbance at 304 mμ using Beckman DU-2 spectrophotometer. Use Tris buffer as the blank solution. Correct measured absorbance for PVP content and absorbance depression due to complex formation (a typical calibration curve will be shown later).

(d) HSA. The procedures for determining solubility were the same as that given above (a typical calibration curve will be shown later).

6. Spectrophotometric Analysis

(a) Infrared Spectrum of BHC.

Prepare a KBr pellet and record the spectrum on a Beckman IR-10 spectrophotometer.

(b) The Absorptivity Value of BHC.

Weigh accurately 100.0 mg. of BHC and dissolve in 500.0 ml. of 0.01N NaOH. Dilute aliquots with 0.01N NaOH to give final concentrations of 2 to 18 mg./L. Prepare five such solutions from three different stock solutions. Record spectrum on a B & L Spectronic 505 spectrophotometer and calculate absorptivity values at 286 mμ.

Repeat the procedure but dilute the stock solution with Tris buffer of pH 7.4. Prepare ten solutions from each of four different stock solutions in such a way that the final concentration varies from 4 to 12 mg./L. Check the pH of the solutions both before and after reading the absorbance on a Beckman DU and DU-2 spectrophotometers set at 304 mμ. Calculate the absorptivity value at 304 mμ.

(c) Absorbance Values of HSA and PVP Solutions.

Prepare 4% stock solutions using Tris buffer. Prepare a series of solutions by diluting the stock solution with Tris buffer. Determine the absorbance on a Beckman DU-2 spectrophotometer set at 304 mμ. Prepare calibration curves by plotting absorbance versus the per cent concentration of macromolecule in solution.

(d) Determination of Changes in Absorbance at a Fixed BHC Concentration as a Function of Macromolecule Concentration.

Shake an excess of BHC with Tris buffer for one hour. Filter through a Milipore filter paper. To each of a series of 50-ml. volumetric flasks, add different amounts of 4% macromolecule solution and a constant amount of the BHC stock solution. To two flasks, add only BHC stock solution. Dilute to volume with Tris buffer.

(e) Determination of the Changes in Absorbance at a Fixed Macromolecule Concentration as a Function of BHC Concentration.

Add varying quantities of the BHC stock solution (see previous section) to fixed amounts of the macromolecule. Dilute and record absorbance as indicated above. Calculate the theoretical absorbance value of each solution from the amount of BHC in solution. Subtract the observed value from the theoretical value and plot the difference against the observed value.

Prepare solutions to contain 0.02% PVP and use different BHC concentrations.

Data from (d) above and that obtained here is used to prepare the calibration curves.

7. Equilibrium Dialysis

(a) Dialysis Cell. A diagram of a dialysis cell is shown in in Figure 9. Cells of this type are described in detail by Patel and Foss (1964). Total volumes in the dialysis cell may be changed by adding additional spacers on either side of the membrane.

(b) Preparation of the Cellophane Membrane.

Immerse dialyzer tubing (flat width is equal to 1.735 inches) in distilled water for several minutes. Unfold, cut to size, and transfer to 1.5 liters of water. Shake the container for approximately ten hours and, at hourly intervals, replace the water with fresh water. Store in a refrigerator at 0-5°C and use within two weeks. Prior to use, immerse the membrane in Tris buffer. Remove the membrane from the buffer, drain but do not allow drying to occur, and attach the membrane to the cell.

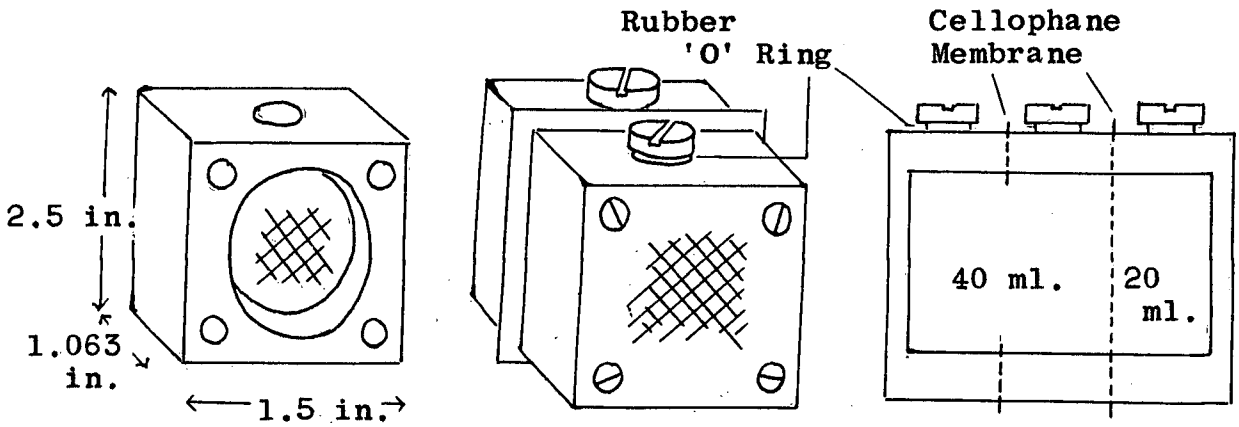

Figure 9. Diagram of a plexiglas block and an assembled dialysis cell.

(c) Dialysis Equilibrium and Membrane Binding of BHC.

Prepare three solutions containing varying concentrations of BHC. Transfer a portion of the solution to one side of the dialysis cell and dialyze against Tris buffer in the other compartment. Remove aliquots and analyze for BHC. Continue the process until the BHC concentration in both compartments is the same for at least two sampling periods. On the basis of the data obtained, determine the time required for equilibration in the cell.

Membrane binding of BHC was determined in the following manner.

Transfer a solution containing a known quantity of BHC to the cell. Dialyze until both compartments contain the same amount of drug. Calculate the total amount of drug in solution. Subtract this value from the amount of drug added to the cell. Calculate the per cent recovery and estimate membrane binding.

In experiments involving macromolecules, a control cell containing no macromolecule was maintained under the conditions specified for that study. Thus additional information on membrane binding was obtained throughout the investigation.

(d) Dynamic Dialysis. A diagram of the apparatus used is shown in Figure 10. The procedure has been used by Meyer and Guttman (1968b) to study protein binding with drugs.

> Add excess of BHC to a solution containing a macromolecule. Shake for four to five hours. Filter the solution through Millipore filter paper. Analyze for BHC in a similar manner described previously (see p. 59).
>
> To the cellophane bag, transfer 50.0 ml. of the macromolecule solution nearly saturated with BHC. Add 500.0 ml. of Tris buffer to the apparatus. Stir this external solution with a magnetic stirrer. Stir the macromolecule solution within the bag in the manner indicated in Figure 10. At specified times, remove 50.0 ml. of solution from the main part of the apparatus. Immediately replace this solution with the same volume of Tris buffer. Calculate the amount of drug in the cellophane bag by subtracting the amount of drug removed from initial amount added to the system.

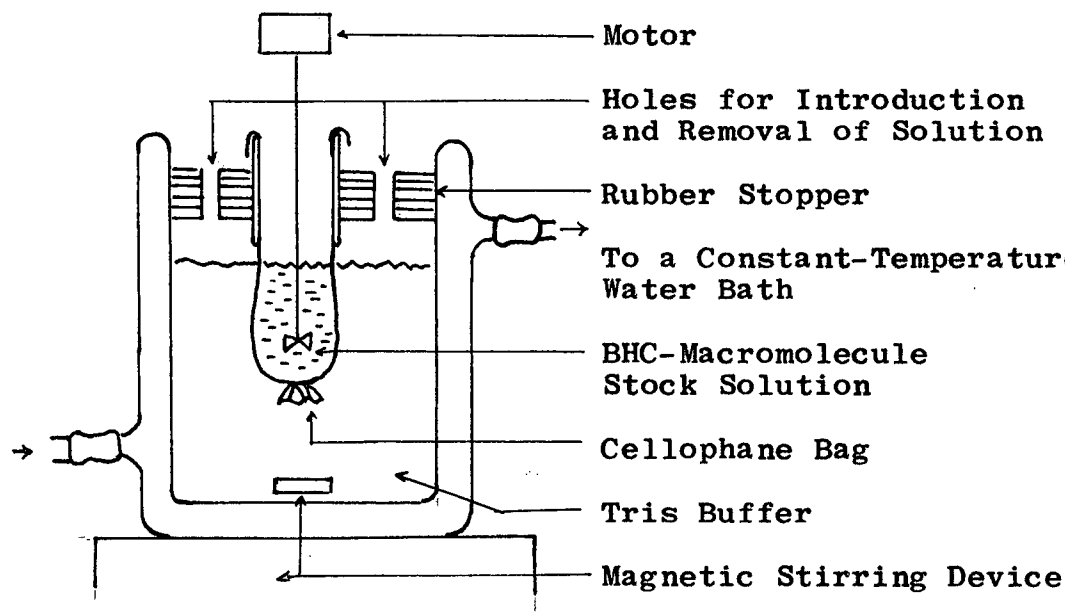

Figure 10. A set-up for dynamic dialysis method.

(e) Permeability of PVP through the Cellophane Membrane.

Prepare PVP solutions in Tris buffer (0.1 to 0.4%). Transfer aliquots to one of the compartments in the dialysis cell and dialyze against Tris buffer for 40 hours. Analyze the Tris buffer compartment for PVP, following the method of Campbell et al. (1953).

To a 10.0 ml. of sample of the solution, add 0.1 ml. of 0.1N iodine in 0.1M potassium iodide solution. After 12 to 15 minutes, read the absorbance of the solution on a Beckman DU-2 spectrophotometer set at 500 mμ. Dilute the iodine test solution to 10.0 ml. of Tris buffer and use as the blank solution. Calculate concentration from a calibration curve based on solutions containing known amount of PVP.

(f) Equilibrium Dialysis Studies.

Prepare a series of BHC and BHC-macromolecule solutions (see p. 63). Determine BHC concentration in these solutions (see p. 59). Transfer 20.0 ml. (or 40.0 ml., depending on compartment volume) of the BHC solution to the dialysis cell and dialyze versus the BHC-macromolecule solution or macromolecule solution containing no BHC. Also dialyze BHC-macromolecule solution against Tris buffer.

In one run of experiment, use six to twelve cells depending on the experiment and the number of control cells required. Attach the cells to the shaft of the water bath and tumble at 30 rpm for 40 hours (a diagram of the apparatus is shown in Figure 11). Remove aliquots from the BHC compartment, store in a water bath at 20°C, and analyze for BHC content. Dilute the aliquots, if necessary (dilution factors of from 1:1 to 1:10), with Tris buffer. Carry out at least two analyses on each aliquot taken from the BHC compartment.

PVP studies were carried out at 10, 20, 30, and 40°C and at PVP concentrations of 0.1, 0.2, and 0.4%. Similar HSA concentrations were used but studies were carried out at 20 and 40°C only. HES studies were carried out at 30°C and at a concentration of 0.5% HES. Since HES does not significantly increase BHC solubility, BHC-HES stock solutions were not used in the

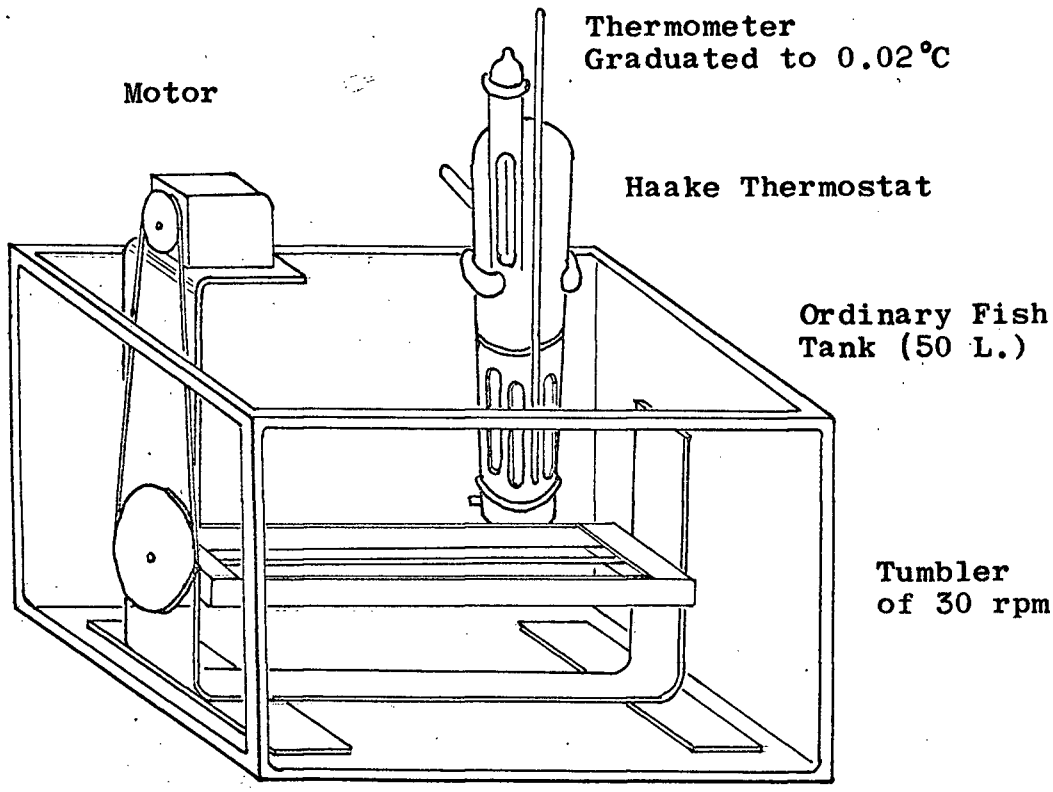

Figure 11. A water bath with a tumbler used in solubility and equilibrium dialysis studies.

investigation. Dialyses were based on BHC and HES solutions only. Temperature fluctuations during dialysis were less than 0.1°C.

8. Viscometric Analysis

(a) Calibration of the Viscometer.

Calibrate seven Cannon-Fenske Viscometers in the manner described in an A.S.T.M. bulletin (1966b). Use freshly distilled water as the reference standard.

Viscosity and density values for water at various temperatures are given in the literature (Bull, 1964b; Weast, 1969). With respect to the viscosity values for water, the values from Bull's textbook were used in the calculation.

The viscometers were filled and allowed to stand in a water bath for at least two hours. Flow time was determined to 0.1 seconds. At least five determinations were made for each viscometer. The average flow time for each viscometer was substituted into Eq. 50 along with the two water constants and the instrument constant at the specified temperature was calculated. Temperature variation in any experiment was less than 0.1°C.

(b) Density Measurement.

Calibrate a Westphal balance at each temperature with distilled water. Maintain the specified temperature by immersing the glass cylinder of the balance in a water bath (800 ml.) set to the desired temperature. Make calibration both before and after measurement of PVP solutions. The reproducibility was 0.0002 density units. Prepare a series of PVP solutions and determine density. Note that the apparatus measures apparent specific density but, for the purposes of the experiment, the two terms were considered to be synonymous.

(c) Viscosity Measurements of PVP Solutions in the Presence of BHC.

Dissolve PVP in a BHC stock solution to yield a final PVP concentration of 4%. Dilute with BHC stock solution to yield solutions which contain the same quantity of BHC and from 0.2 to 3.0% PVP.

Prepare seven such solutions. Determine flow-times in the manner described in (a) above.

Substitute the mean flow-time, the density value, and the instrument constant into Eq. 50 and calculate the viscosity. Calculate relative viscosity by dividing each viscosity value by that of the BHC solution without PVP.* Calculate reduced and specific viscosities by using Eq. 44 and 45.

Determine intrinsic viscosity and Huggins parameter from the intercept and slope of the graph obtained by plotting reduced viscosity versus PVP concentration.

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* This implies that the relative viscosity of the BHC solution was considered to be unity (see Eq. 43).

VI. RESULTS AND DISCUSSION

1. Intramolecular Hydrogen Bonding in BHC

The infrared spectrum of BHC is shown in Figure 12 and is similar to that published by French and Wehrli (1965). The vibrational frequency corresponding to an -OH stretch occurs at approximately 3500 $Cm.^{-1}$ The first overtone is usually observed at 7000 $Cm.^{-1}$ If chelation occurs with the carbonyl group in phenols through the formation of an intramolecular hydrogen bond, the former frequency value becomes less but the extent of change depends on the strength of the hydrogen bond. The band is broad and, at times, very weak (Nakanish, 1964; Dyer, 1965). With BHC a broad absorption band occurs at approximately 3100 $Cm.^{-1}$ and this may indicate an intramolecular hydrogen bond.

The molecular model for BHC suggests the possible formation of two intramolecular hydrogen bonds between the carbonyl group at C_2 and the -OH group at C_4, (or vice versa). Solubility data (see later) appear to support this conclusion. These two internal eight-membered chelations (see Figure 13) appear to restrict the rotation of the one-sided moiety of the methylene bridge and fix its configuration. The most probable three dimensional structure of BHC is shown in Figure 14.

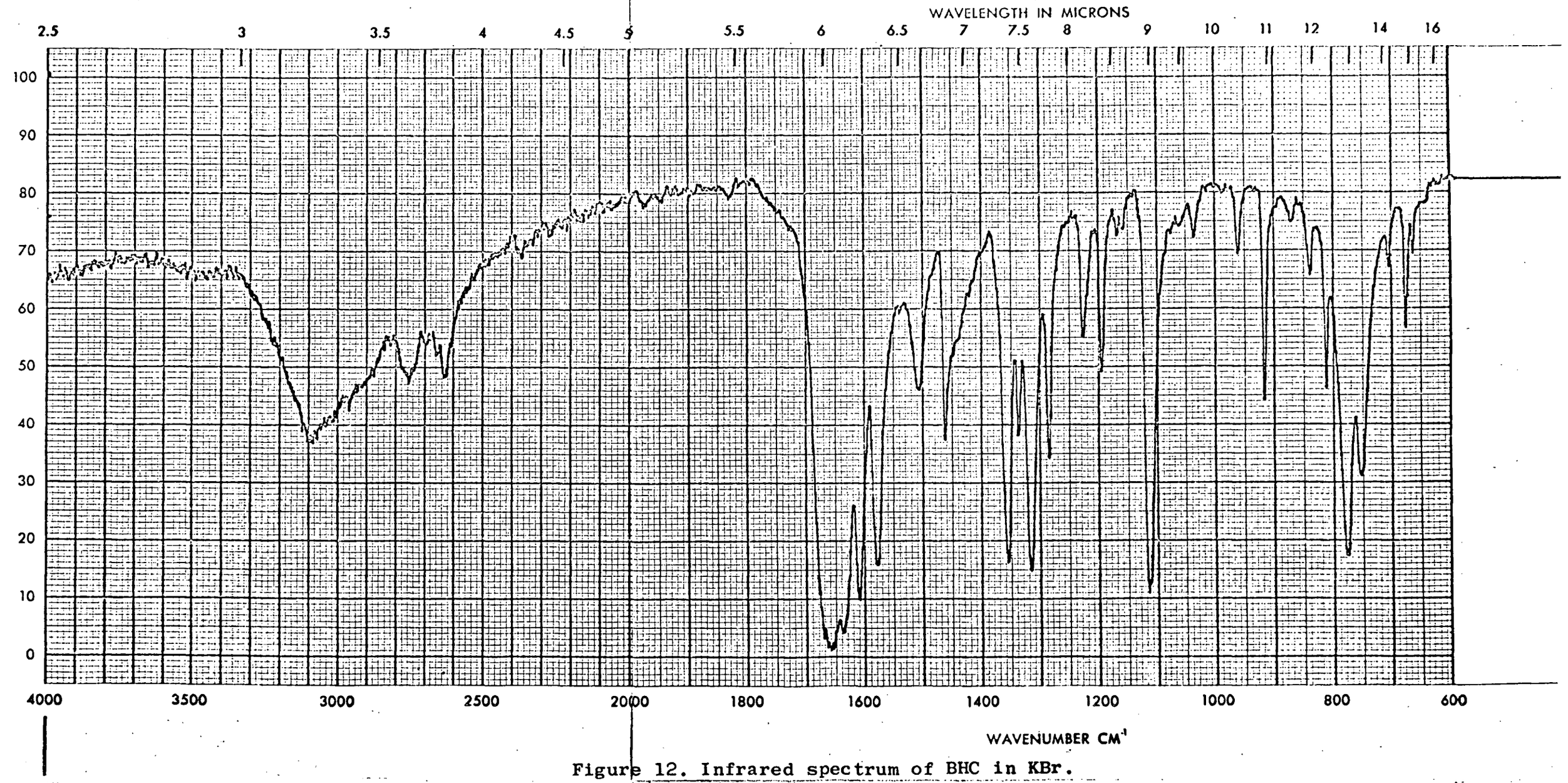

Figure 12. Infrared spectrum of BHC in KBr.

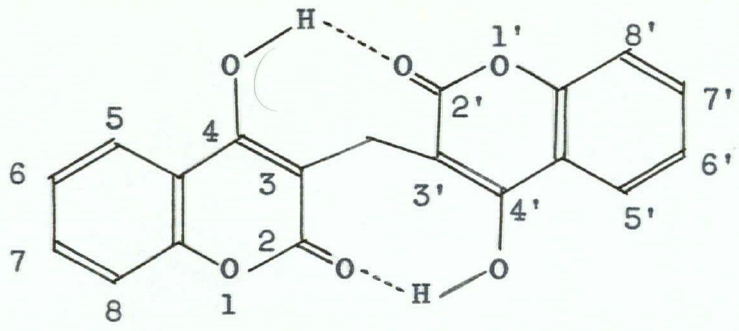

Figure 13. Chemical structure of BHC showing two intramolecular eight-membered chelations.

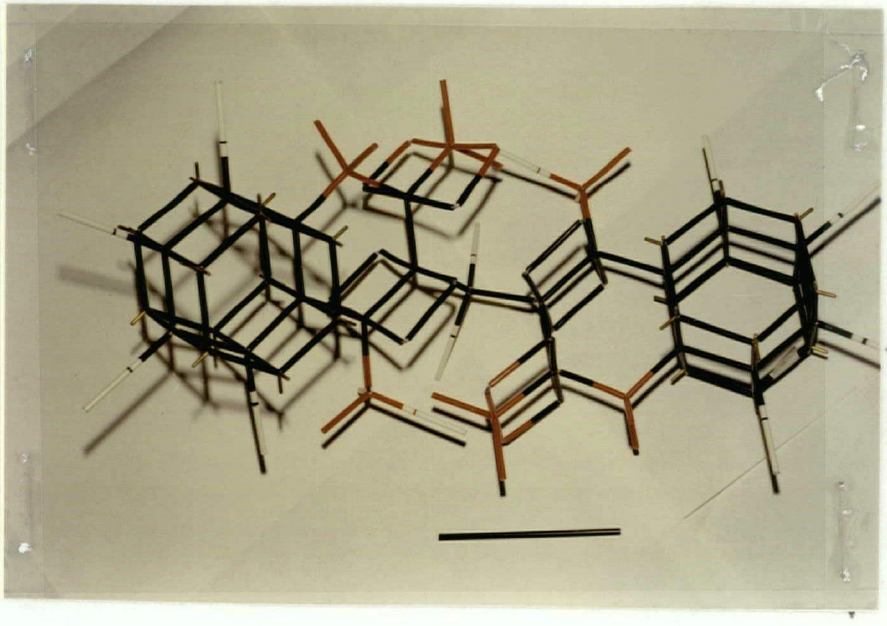

Figure 14. Three dimensional structure of BHC. Black, red, and white bars represent carbon, oxygen, and hydrogen atoms, respectively. The black bar below the model has a length of 4 Å.

2. Apparent pKa Values of BHC

If a dibasic acid is symmetrical with respect to the two ionizable hydrogen atoms and if the negative charge on the first ionizable group is sufficiently removed from the remaining hydrogen atom, then the effect on the second group is negligible and the first ionization constant is approximately four times as large as the second constant (Robinson and Stokes, 1968). In general, however, the effect of the negative charge does make it more difficult for the second hydrogen to ionize. For example, for oxalic acid, the first constant is approximately 1000 larger than the second. On the other hand, there is only a six fold difference for azelaic acid ($COOH(CH_2)_7COOH$). Kirkwood and Westheimer (1938), under reasonable assumptions with respect to the size and configuration of the molecules, discussed the effect of electrostatic interaction between ionizable groups on the cosecutive ionization constants.*

Although pKa values have been reported for BHC (Burns and others, 1953; Nagashima and others, 1968a), the uncertainties associated with the determination and the absence of values for the total ionization process made it necessary to study the ionization behaviour of this drug. The molecule has two ionizable hydrogen atoms and both are involved in the internal hydrogen bond. As shown in Figure 15, the negative charge on the oxygen

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* This study formed the basis for the theory of multiple equilibria in the complexation process in the presence of electrostatic interactions between binding sites (see p. 11).

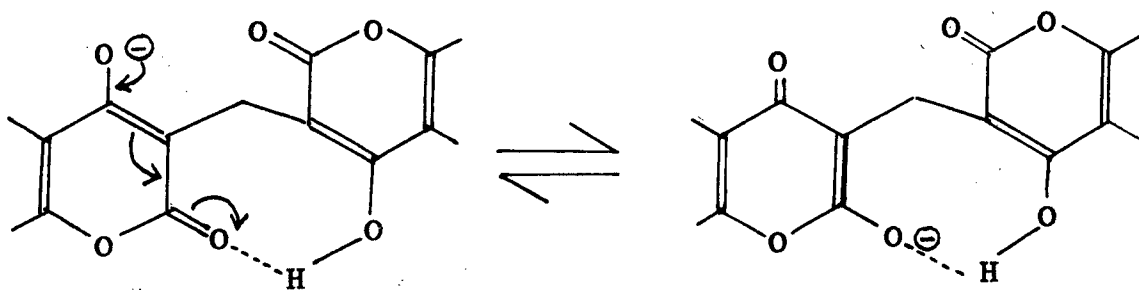

Figure 15. Resonance structure of BHC after the first ionization.

atom produced by the first ionization is expected to make the oxygen atom in the carbonyl group more negative by an electronic shift through the conjugated chain. This increase in electronegativity apparently increases its ability to form hydrogen bond with the remaining hydrogen atom (Pauling, 1967). The strengthened hydrogen bond will, therefore, make it more difficult for the second hydrogen atom to ionize. For this reason, and the fact that the statistical considerations of two successive ionization constants would appear to be sound when applied to long and thin molecules but not to shorter and more spherical molecules (Robinson and Stokes, 1968) like BHC, the ratio of the first to the second ionization constants for BHC should be at least 1000:1. It is rather surprising, therefore, that only single ionization constants for BHC have been reported in the literature.

The pK values of acids and bases may be determined in many different ways (Albert and Serjeant, 1962). BHC is, however, very insoluble in acidic solution and the basic titrimetric approach

in aqueous media cannot be utilized. However, solubility can be increased by utilizing an organic solvent - water mixture and potentiometric and spectrophotometric titrations can be carried out in such systems. Alcohol, dioxane, and DMF have been recommended in such systems (Parke and Davis, 1954).

Attempts were made, therefore, to measure the apparent pKa values of BHC in DMF-water systems containing varying quantities of DMF. These values may be extrapolated to zero per cent DMF in order to determine the value in water alone. Garrett (1963) studied the variation of pKa values of tetracyclines in a DMF-water system in a similar manner.

A solution containing 706.0 mg. BHC per liter of DMF was prepared. A 10.0 ml. aliquot of this solution was transferred to a 100 ml. volumetric flask. When 2.5 ml. of 0.01N HCl were added to the flask, the solution became turbid but the turbidity disappeared when 30.0 ml. of DMF was added. Addition of water to 100.0 ml. produced no further turbidity (see p. 55). It became necessary to consider the following factors when preparing BHC sample solutions for titration; pH of the solution before titration, strength of titrant, concentration of BHC, size of sample, and per cent DMF.

The results in Figure 16 were obtained when a 100.0 ml. sample of BHC (70.6 mg. per liter of a 40% solution of DMF in water) solution containing 2.5 ml. of 0.01N HCl was titrated with 0.01N NaOH. The blank titration curve was obtained by titrating 100 ml. of 40% DMF solution containing 2.5 ml. of 0.01N HCl with 0.01N NaOH. The blank titration curve was subtracted

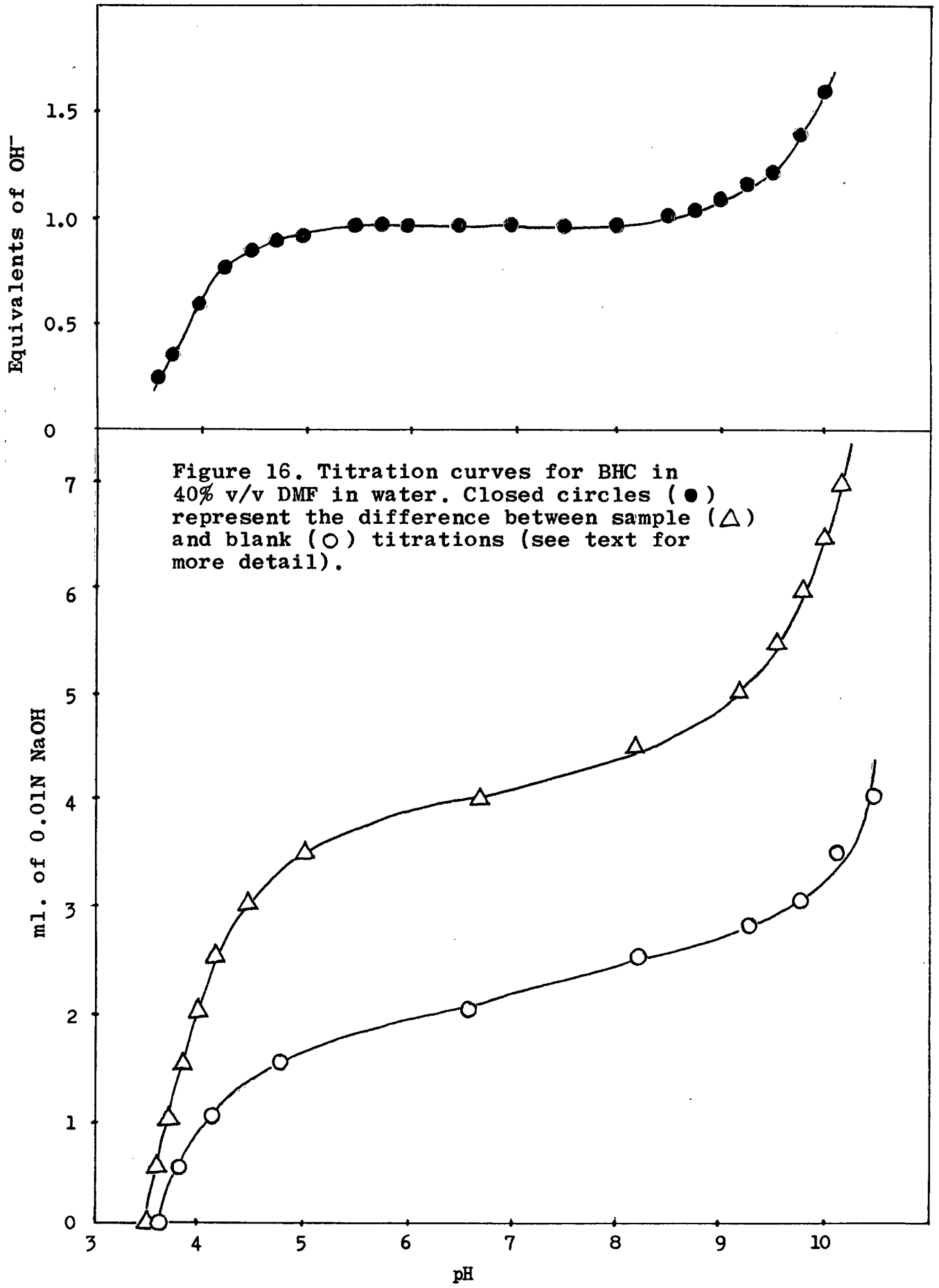

Figure 16. Titration curves for BHC in 40% v/v DMF in water. Closed circles (●) represent the difference between sample (△) and blank (○) titrations (see text for more detail).

volumewise from the BHC curve and the difference converted to equivalents of OH ion per mole of BHC and plotted as a function of pH.

The pH range covered in these titrations was from 3.5 to 10. Because BHC is insoluble in acidic media, titrations could not be carried out below pH 3.5 and no potentiometric break is observed. One endpont region (pH 5 to 8) is present; the second region fails to appear on the curve. It is impossible, therefore, to report quantitative pKa values for BHC in this system. However, the difference curve does show the presence of two pKa values, the one below pH 4, the other, above pH 9. No further attempts were made to determine pKa values in this way because it would have been necessary to increase DMF concentrations and hence operate much further from the zero per cent DMF point.

The low solubility of BHC in aqueous media caused similar difficulties during the spectrophotometric determination of pKa values. The solubility of the unionized BHC (AH_2 form) is approximately 0.5 mg./L. (1.5×10^{-6} mole/L.). At these concentrations, the spectral characteristics of the unionized species cannot be obtained. However, the change from the AH^- to the A^{--} form can be followed. These spectrophotometric curves are shown in Figure 17. Each solution contained 10.0 mg. BHC per liter of the specified buffer. As the pH decreases, an absorption maximum appears at 276.5 mμ; with increasing pH, a peak is observed at 315 mμ.

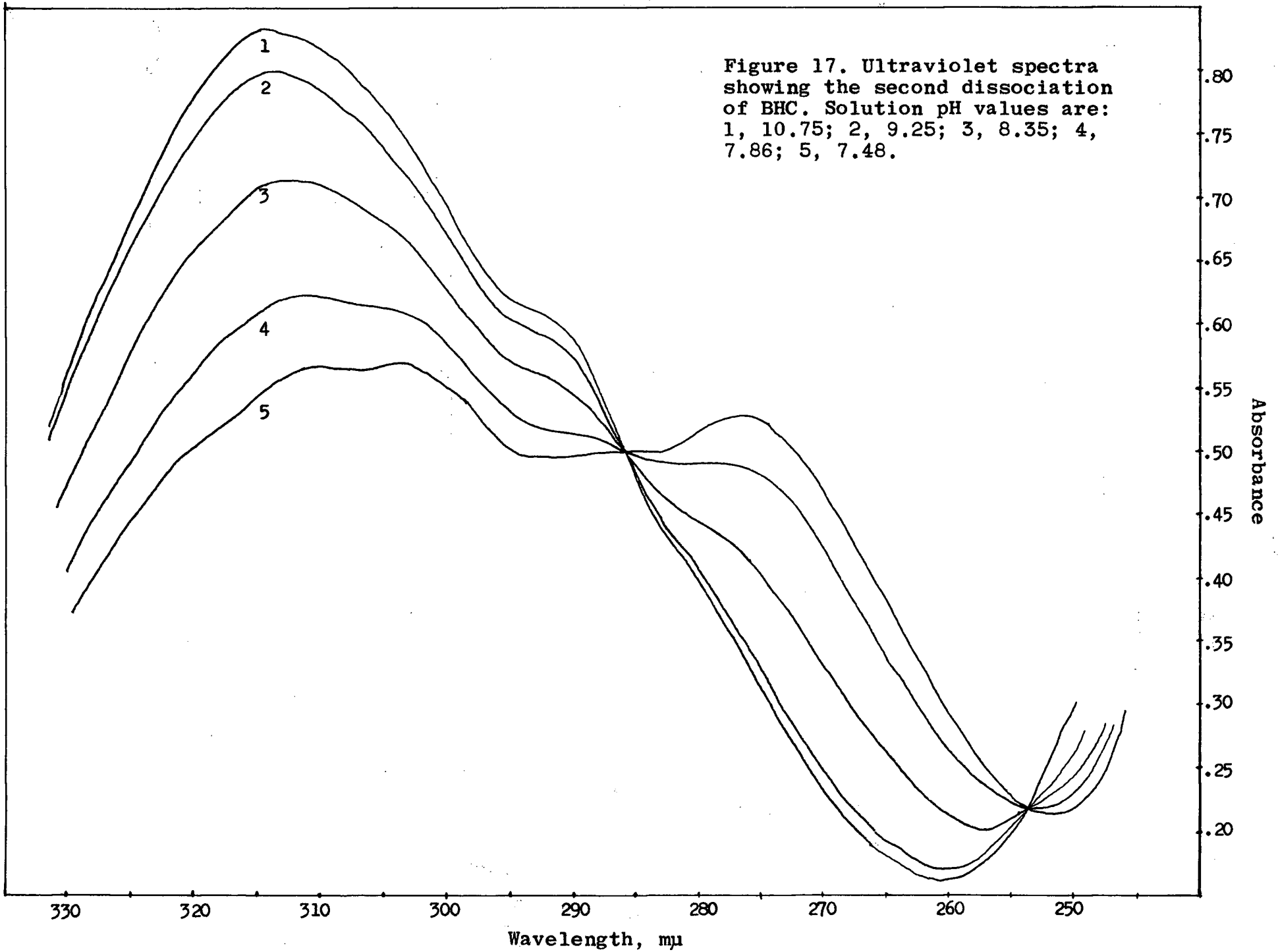

Figure 17. Ultraviolet spectra showing the second dissociation of BHC. Solution pH values are: 1, 10.75; 2, 9.25; 3, 8.35; 4, 7.86; 5, 7.48.

If a similar spectral change occurs when AH_2 is converted to AH^- and H^-, the most suitable wavelengths for analysis would appear to be 276.5 or 315 mμ. A series of solutions were prepared to contain 0.5 mg. BHC per liter of buffer. To prevent spectral distortion, the ionic strength of each solution was kept as low as possible (Sager and others, 1945). Perrin (1963) describes buffers of this type. They have a low ionic strength (0.01), cover the 2.2 to 11.6 pH range, and absorb very little energy in the ultraviolet region of the electromagnetic spectrum.

The absorbance of a solution containing small quantities of drug can be determined in one of two ways. First, a long spectrophotometric cell may be used in the determination and, secondly, precision spectrophotometry (trace analysis) may be used to analyze the solutions. Sager et al. (1945) used a 5-Cm. cell to determine the pKa values of some esters of p-hydroxybenzoic acid. The solubilities of these esters ranged from 10^{-4} to 10^{-5} molar. Precision spectrophotometry (Reilley and Crawford, 1955; Pernarowski, 1969) has not been used to determine such values.

By using the principles inherent in precision spectrophotometry, the absorbance values of a series of BHC solutions were determined at 276.5 and 315 mμ and plotted versus pH values. These plots are shown in Figure 18. Absorbance values did not exceed 0.1 absorbance unit even though the absorbance scale was expanded to a maximum by appropriate use of reference standards. A slight turbidity was observed in those BHC solutions in 0.01N HCl. No turbidity was observed in the solutions with higher pH

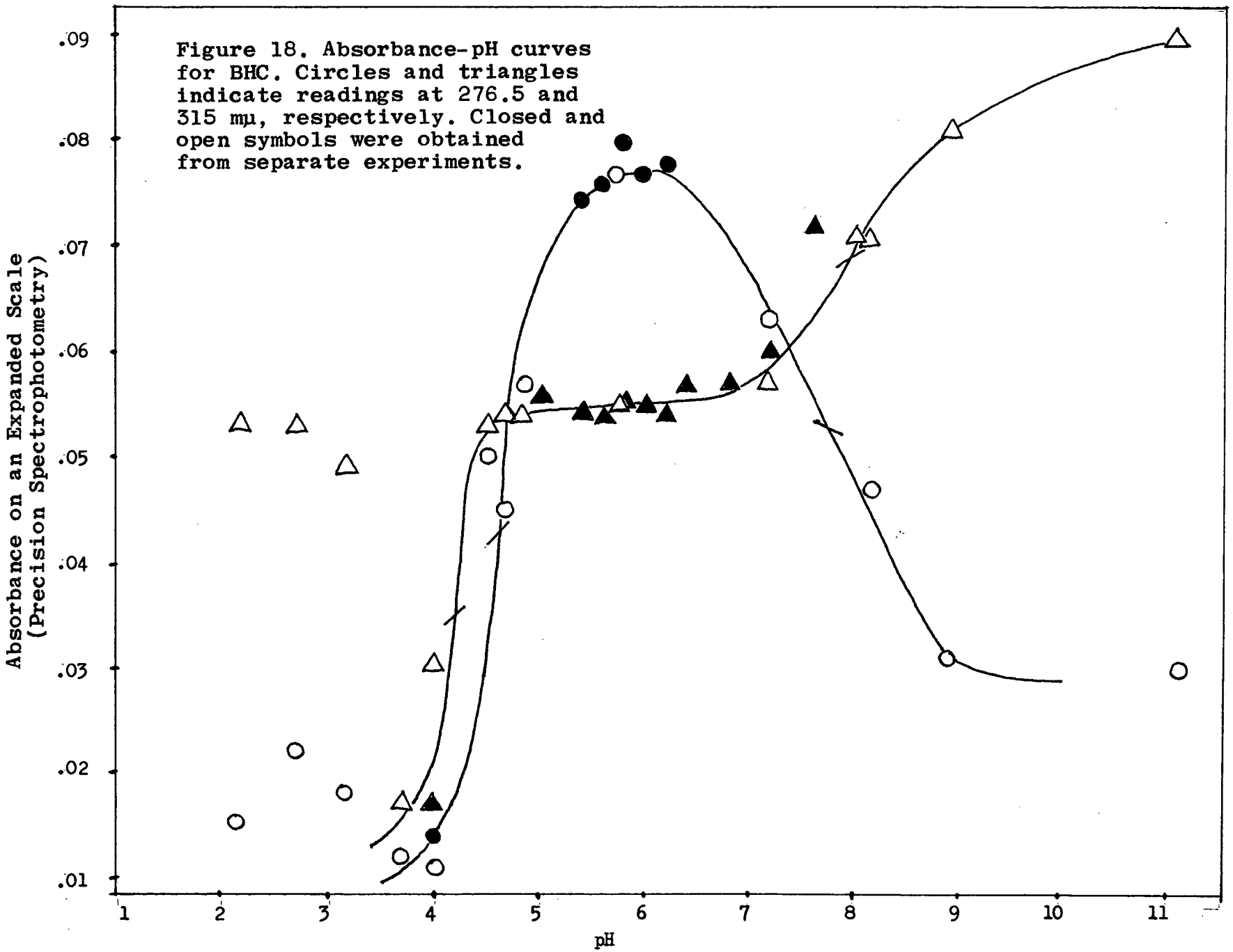
Figure 18. Absorbance-pH curves for BHC. Circles and triangles indicate readings at 276.5 and 315 mμ, respectively. Closed and open symbols were obtained from separate experiments.
Absorbance on an Expanded Scale (Precision Spectrophotometry)
.09
.08
.07
.06
.05
.04
.03
.02
.01
1
2
3
4
5
6
7
8
9
10
11
pH

values but undetected over-saturation may be the cause of the scattered values below pH 4 in Figure 18. Absorbance changes show clearly the two ionization steps. The calculated values from the curves in Figure 18 are 4.6 and 4.2 for pKa_1 and 7.75 and 7.9 for pKa_2. The former values were obtained from absorbance readings at 276.5 mμ; the latter, from readings at 315 mμ. Average values are 4.44 and 7.83.*

Figure 18 also shows that the spectral changes due to the first ionization is apparently different from those associated with the second ionization. At both 276.5 and 315 mμ, absorbance increases as pH increases from 3 to 5 (compare with Figure 17). This implies that no isosbestic point occurs between these two wavelengths on the first ionization process.

The pKa_2 value may be determined directly by measuring absorbance values of buffered solutions of BHC, because BHC has a reasonable solubility at that pH range (see later). Solutions containing 10.0 mg. of drug per liter of buffer, 5% DMF in buffer, and 20% DMF in buffer were prepared. Absorbance-wavelength curves were recorded on B & L Spectronic 505 spectrophotometer. Figure 19 shows spectral changes occurred in the presence of 20% DMF.

However, at low pH values, drug solubility must be increased by the addition of DMF. This solvent does not affect the pH of the buffer and absorbs little energy at the two analytical wavelengths. Solutions containing 0.5 mg. of drug per liter of buffer, 1.0 mg. of drug per liter of 2.5% DMF in buffer, and 0.8 mg.

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* The mean of 4.2 and 4.4 is 4.44 because a log scale is being used. Antilog values for the pair are added. An average value is the log of the value obtained divided by two. Similarly the average of 7.75 and 7.9 is 7.83.

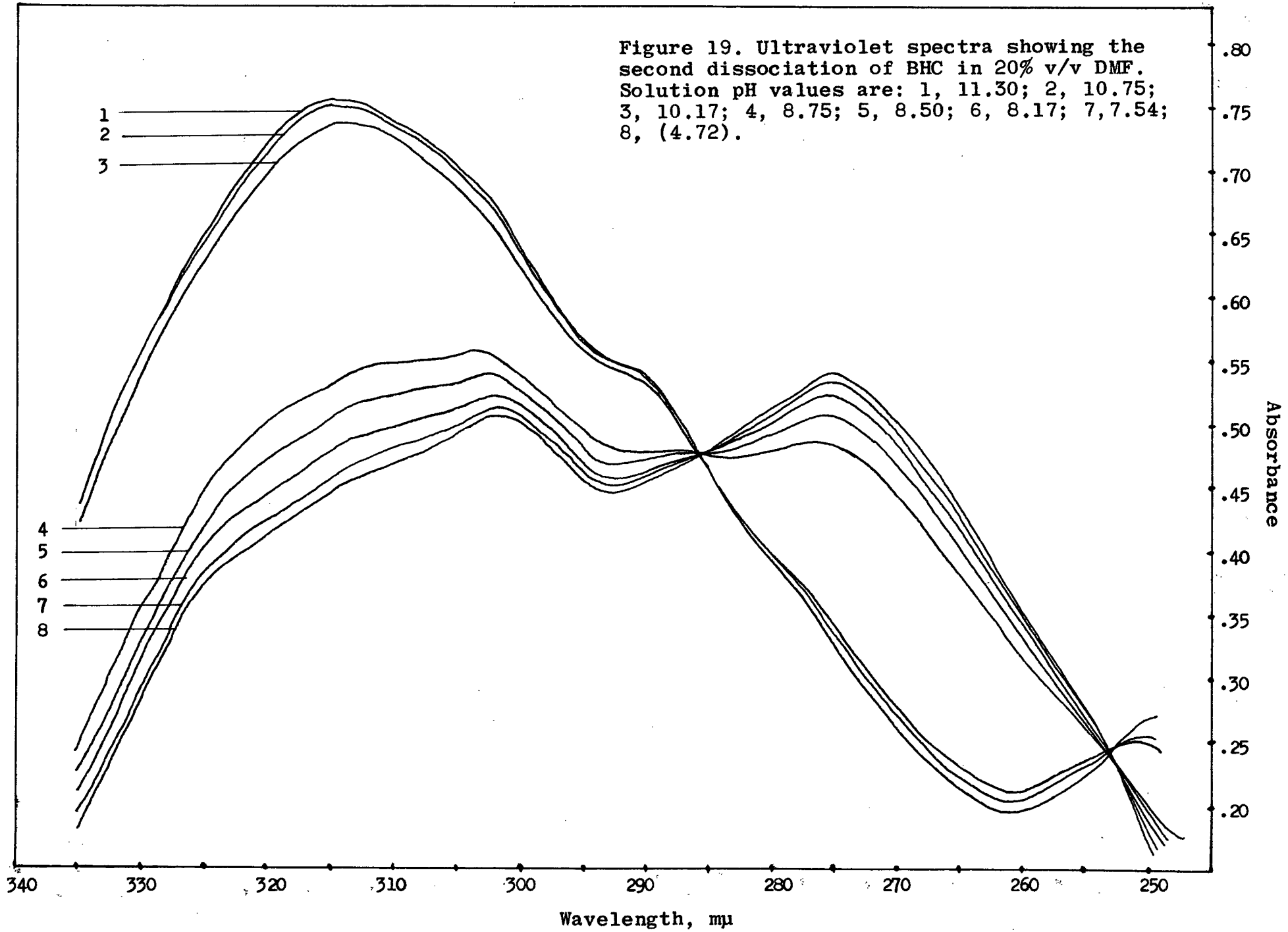

Figure 19. Ultraviolet spectra showing the second dissociation of BHC in 20% v/v DMF. Solution pH values are: 1, 11.30; 2, 10.75; 3, 10.17; 4, 8.75; 5, 8.50; 6, 8.17; 7, 7.54; 8, (4.72).

of drug per liter of 10% DMF in buffer were used in determining pKa_1 value. Absorbance values of these solutions were measured at 276.5 mμ by means of precision spectrophotometry (see p. 56).

Results are shown in Figure 20. The relationship between pKa values and per cent DMF is shown in Figure 21. Values obtained from this data are 4.4 for pKa_1 and 8.2 for pKa_2. The dielectric constant for DMF is reported to be 36.7 (Leader, 1951). As the solvent becomes less polar by addition of DMF the first ionization occurs more easily and the weak acid behaves as a stronger acid in this system. In aqueous media, the BHC molecule is believed to exist in the enol form which is more acidic than keto form (see Figure 22).* The enol-keto ratio would be greater when the polarity of the environment decreases (Gould, 1963). The increase in the mole fraction of enol form may be partly responsible for the increased acidity of BHC in the DMF system. The extent of solvation around the monoionized BHC molecule would be expected to decrease as the polarity of the solvent decreases (i.e., removal of charges become more difficult). As a result, the second ionization constant increases slightly when DMF content is increased. These results and those obtained by potentiometric titration (see p. 75) indicate pKa values of the same magnitude.

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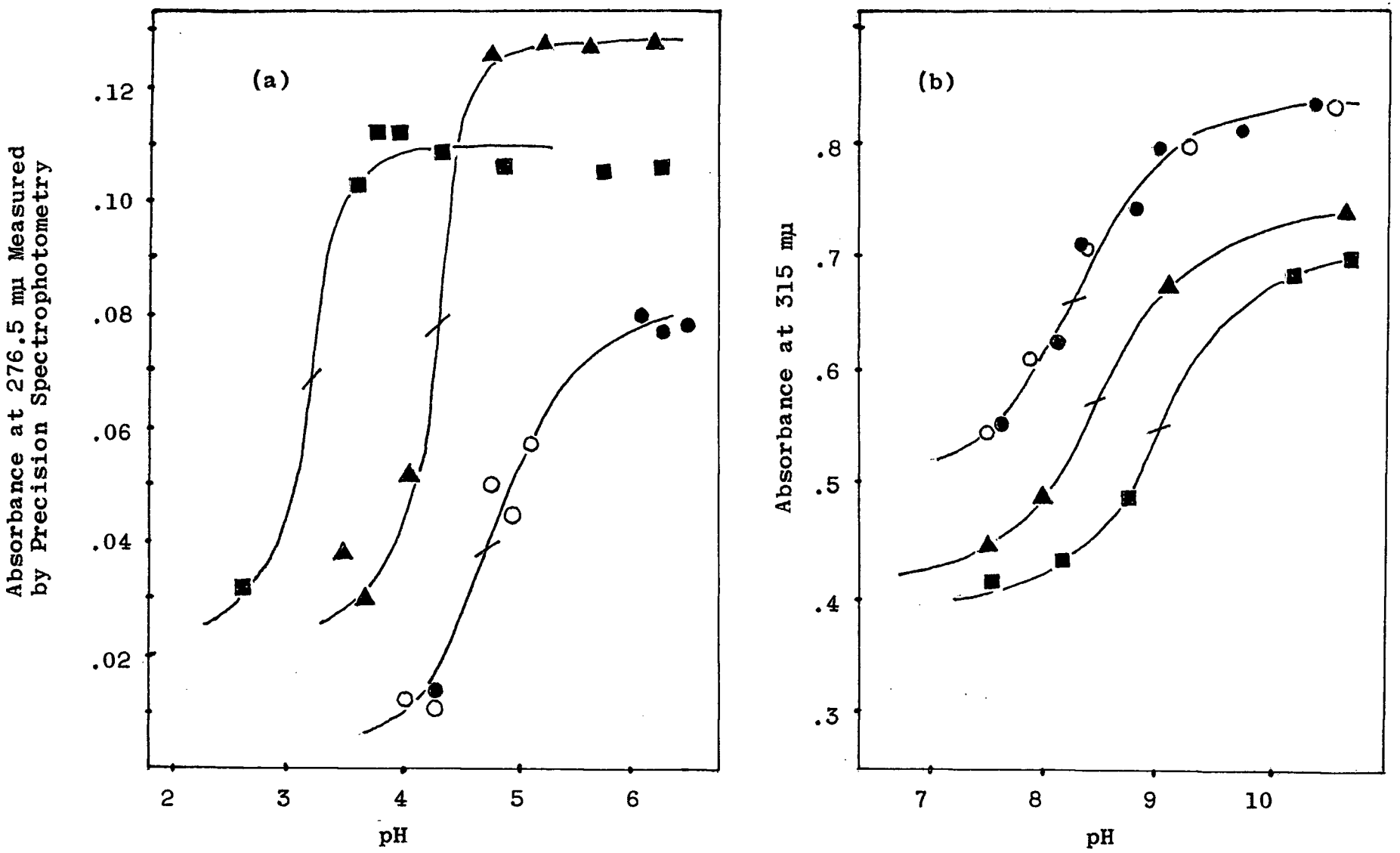
* After this work had been completed, a study on the structure of BHC and a related compounds appeared in the literature. Hutchinson and Tomlinson (1969) proposed a hydrogen-bonded structure for BHC, identical to ours (Figure 13), from a study of nuclear magnetic resonance and infrared spectra. They also made the conclusion that in tautomeric structures similar to Figure 22 4-hydroxycoumarin is preponderantly in the enolic form both in the solid state and in solution in polar solvent. We had further inferred that enol:keto ratio increases with decrease in polarity of solvent. We are grateful to Mr. J. Coates for bringing this paper to our attention.

Figure 20. Absrobance-pH curves for BHC at 276.5 mμ (a) and at 315 mμ (b). In Figure (a): circles, 0 and 0.5; triangles, 2.5 and 1.0; squares, 10% v/v DMF and 0.8 mg./L. BHC. In Figure (b): circles, 0; triangles, 5; squares, 20% v/v DMF. For all three plots, (BHC) = 10 mg./L. Closed and open symbols were obtained from different experiments.



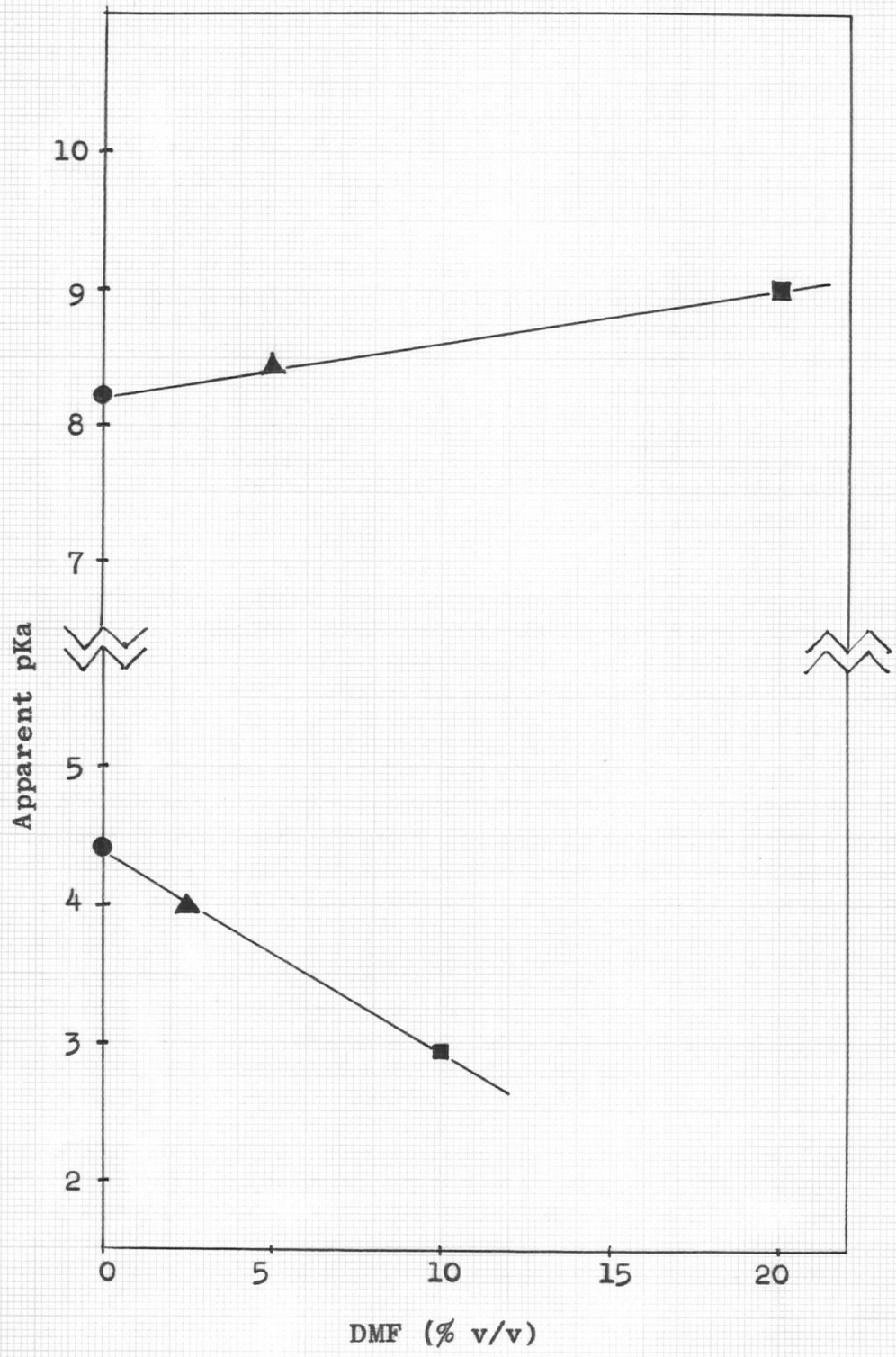
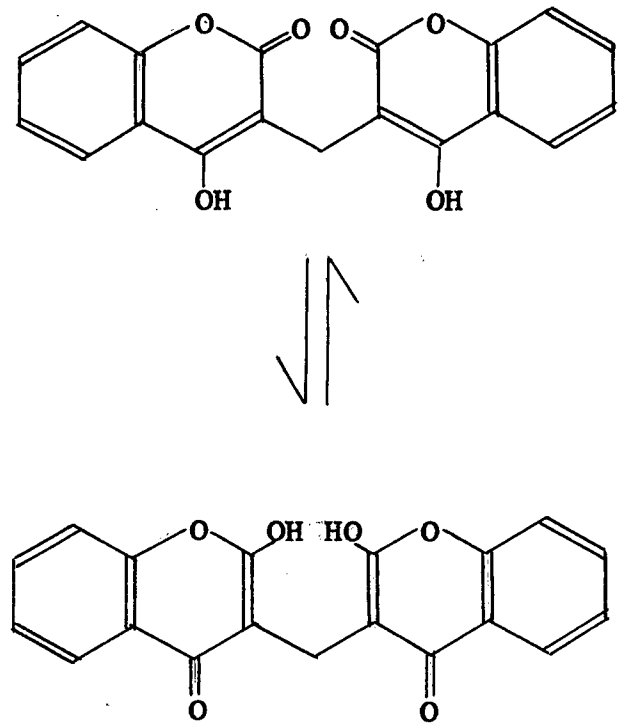


Figure 21. Apparent pKa values of BHC as a function of per cent DMF. Values were obtained spectrophotometrically.

Enol Form
(more acidic)



Keto Form

Figure 22. Tautomerism of a BHC molecule.

From all of the data obtained herein, it is reasonable to assign a pKa_1 value of 4.4 and a pKa_2 value of 8.0 to BHC. By means of non-logarithmic linear titration curves, Levy (1969) obtained values of 4.6 and 7.7. However, in order to obtain these values, he assumed a solubility of 0.2 mg./L. for the unionized BHC.

3. Solubility

(a) Effects of pH, Ionic Strength and Buffer Components. The effect of pH on the apparent solubility of BHC at 30°C is shown in Figure 23. A Tris-HCl buffer system (ionic strength, 0.2) was used in the determinations. Solubility increases rapidly with increase in pH from 7.6 to 8.0. BHC appears to be unstable in 0.1N NaOH solutions, since they turn yellow after standing at room temperature for three days.

Low aqueous solubility strongly indicates that the hydroxyl groups at C_4 and $C_{4'}$ do not contribute to solubility by forming hydrogen bonds with water. A parallel situation exists with salicylic acid in which the -OH group is involved in an intramolecular hydrogen bond (Martin, 1968b). Solubility is expected to increase after the first ionization since an intramolecular hydrogen bond, in which the ionized hydroxyl group has been involved, is no longer present. However, a marked change in solubility (such as observed around pH 8) is not expected, because the molecule is still more or less rigidly fixed by one remaining internal hydrogen bond to allow little interaction with water. After complete ionization, a significant ion-dipole interaction between BHC and water can be expected and this will result in a marked increase in solubility.

The effect of ionic strength on the apparent solubility of BHC is shown in Figure 24. The pH of each solution was maintained at 7.2. If neutral salts are used to alter ionic strength, organic molecules will, in general, show a decreased water solubility

Figure 23. Effect of pH on the apparent solubility of BHC at 30°C (ionic strength, 0.2). Solubility was determined after 24 (⊙) and 48 (●) hours.

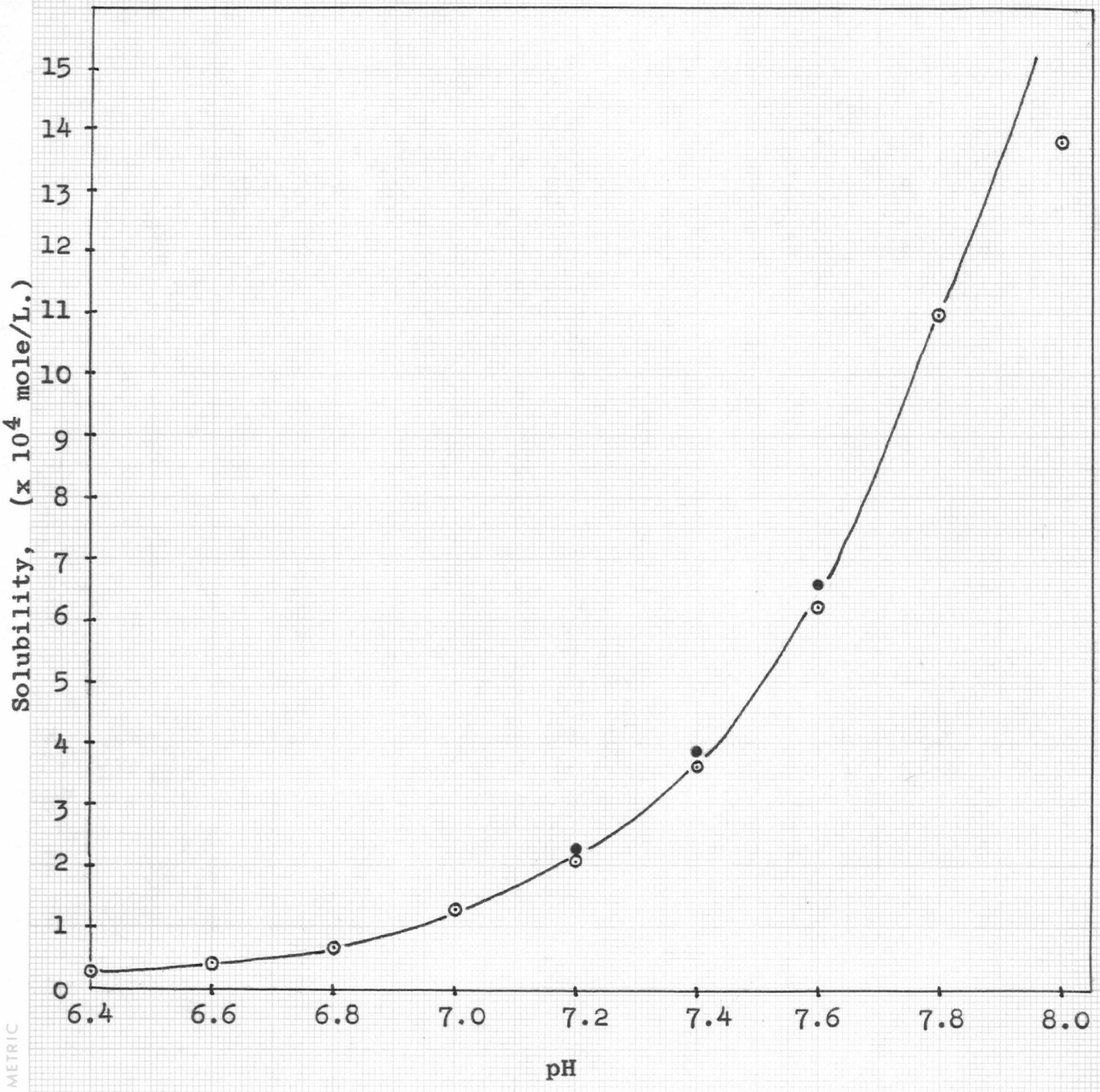
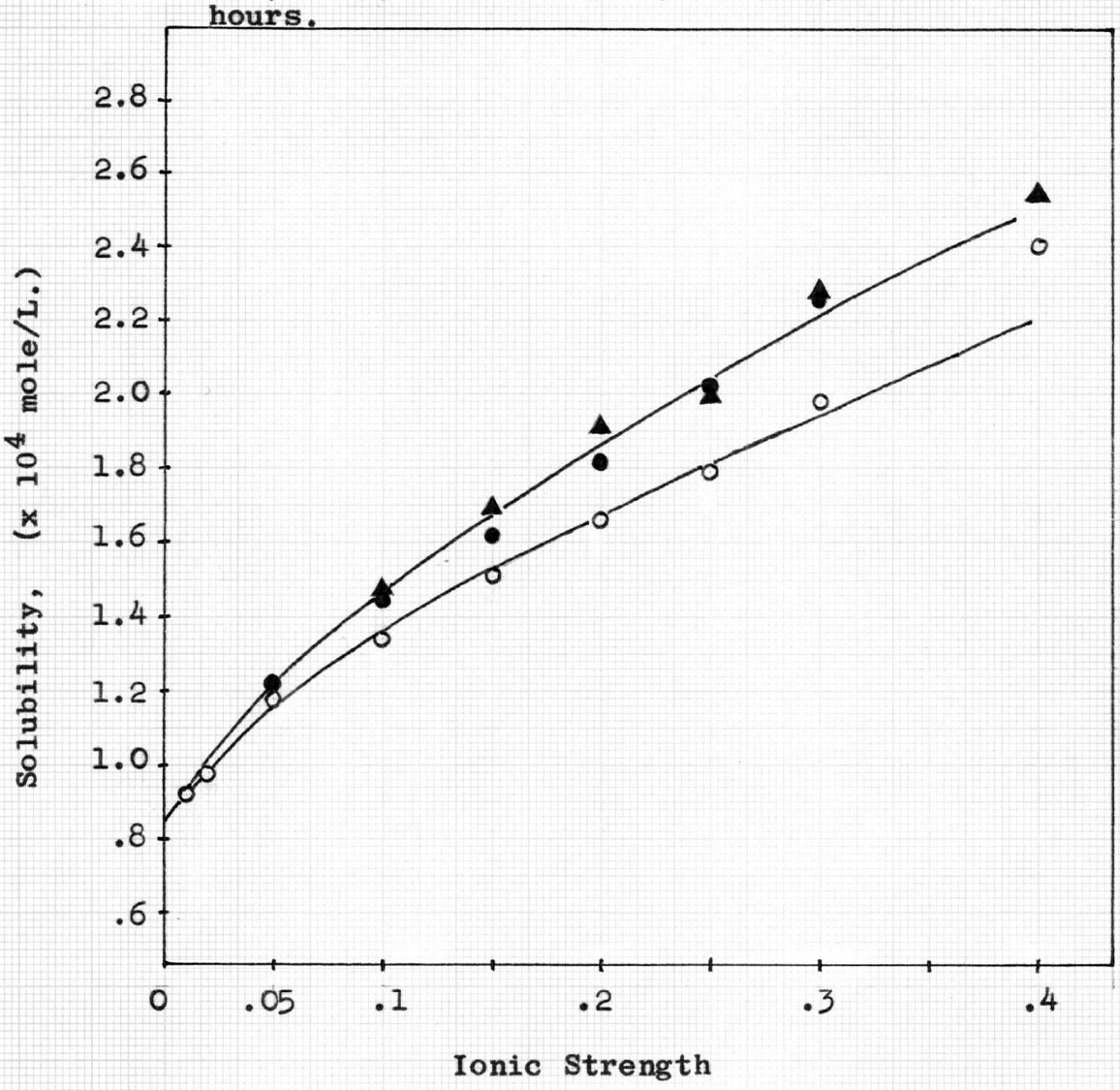


Figure 24. Effect of ionic strength (chloride ion) on the apparent solubility of BHC at 30°C (pH, 7.2), after 24 (○), 48 (●), and 72 (▲) hours.



because of the 'salting out' effect.* The results for BHC do not follow this pattern. However, the ionic strength of these solutions was governed by HCl content and the study may, therefore, be nothing more than the specific effect of chloride ion on the solubility of BHC. According to Frank and Wen (1957), halide ions except fluoride ion are water structure breakers. Fewer 'icebergs' would be expected around the chloride ion and this should enhance BHC-water interaction, i.e., the number of unbound water molecules is greater in the presence of chloride ion and this provides more cavities for the solution of the hydrocarbon (Nemethy and Scheraga, 1962b; Mohammad, 1965).

All drug-macromolecule interactions were studied in buffer systems. Buffer composition is, therefore, an important part of the investigation. For example, the ionic strength of the buffer arises from the hydrochloric acid added to the buffer. This implies that buffer with a low ionic strength contains less buffer component. Consequently, the buffer capacity of the Tris buffer (β) is lower at low ionic strength.

$$\beta = \frac{d(b)}{d(pH)} \approx 2.303 \left[\frac{Ka\ C\ (H^+)}{\left[Ka + (H^+)\right]^2} + (H^+) + (OH^-) \right] \qquad \text{(Eq. 52)}$$

* Hydrocyanic acid and glycine, which are alike in that their aqueous solution have higher dielectric constants than pure water, become more soluble in the presence of salts. The data herein may reflect such a 'salting in' effect (Edsall and Wyman, 1958b), but evidence for this is lacking because the dielectric constant of the BHC solution was not determined.

In Eq. 52, Ka and C are the ionization constant of the buffer component and its concentration, respectively, and b is the number of gram equivalents of alkali added to one liter of buffer solution (Bates, 1961). As shown in Eq. 52, buffer capacity is a function of pH and the concentration of the buffer component. These relationships between buffer capacity and pH for Tris buffer is shown in Figure 25. As expected, maximum buffer capacity is obtained at a pH equal to the pKa of Tris. The value reported in the literature is 8.075 (Bates, 1961). The Tris buffer used in this investigation is 0.063M with respect to Tris and 0.15N with respect to hydrochloric acid. The buffer capacity is 0.021.

Buffer components may interact with macromolecules. Tris, however, has an intramolecular hydrogen bond as shown in Figure 26 (Benesch and others, 1955) and should not react significantly with macromolecules. For example, Higuchi and Kuramoto (1954c) claimed that complexation of salicylic acid with PVP is less than observed between p-hydroxy-benzoic acid because a strong internal hydrogen bond exists in the former drug (see pp. 49-50). Chloride ions, on the other hand, may also interfere with BHC-macromolecule interaction. Klotz (1953a) briefly reviewed the binding of chloride ion to serum albumin. However, these effects were neglected in this investigation because competitive interaction of chloride ion with the macromolecule would be far less than that usually observed drug-macromolecule interactions. A typical example of correction for competitive effect of buffer components may be found in the paper published by Klotz and Urquhart (1949c). Using Eq. 33, they corrected for the contribution of phosphate to the interaction between methyl orange and albumin.

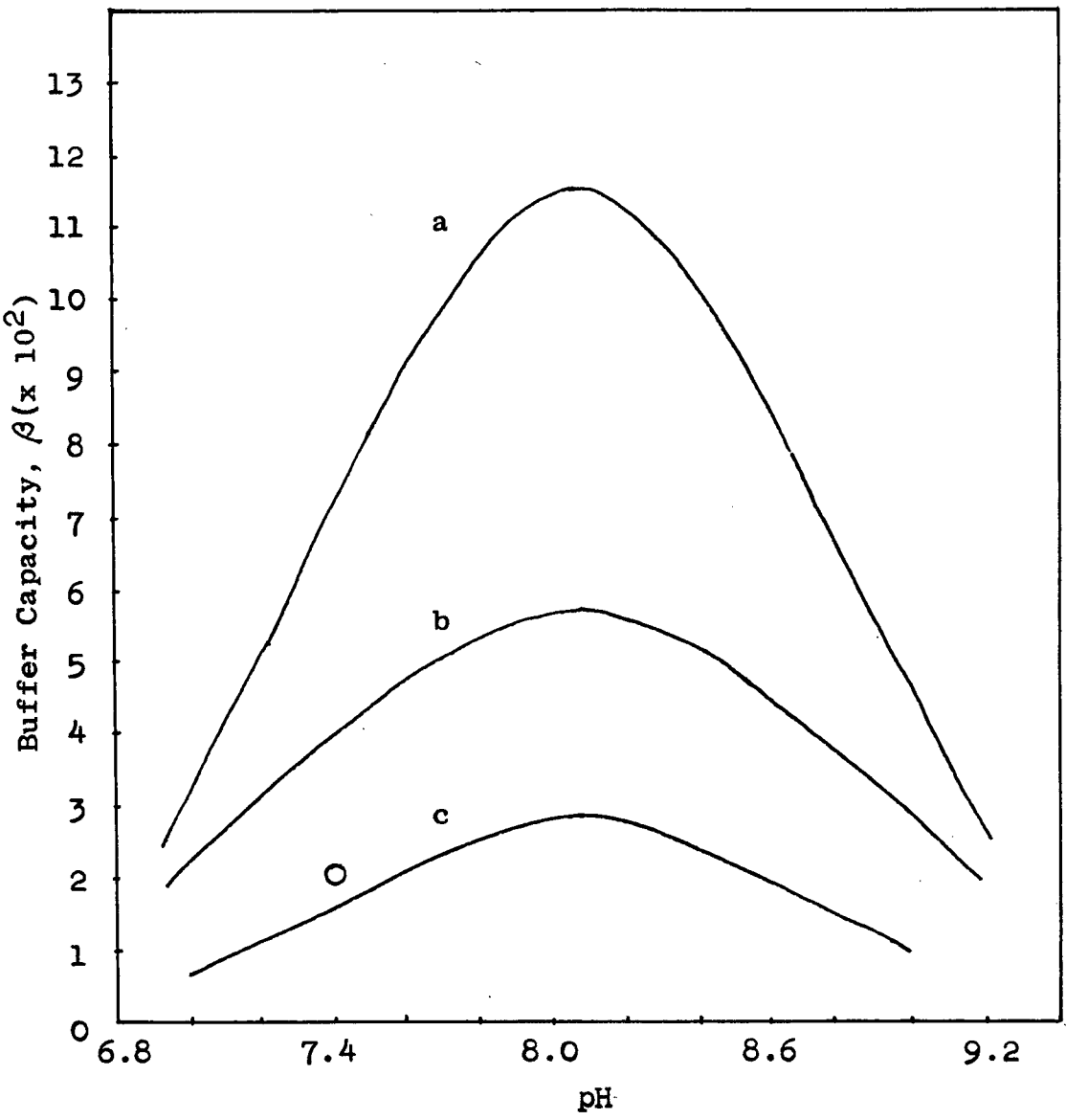


Figure 25. Buffer value of Tris buffer solutions in the pH range from pKa - 1.3 to pKa + 1.1. Total Tris concentrations are 0.2M (a), 0.1M (b), and 0.05M (c). See Bates (1961). Open circle represents the buffer capacity of Tris buffer used in this investigation.

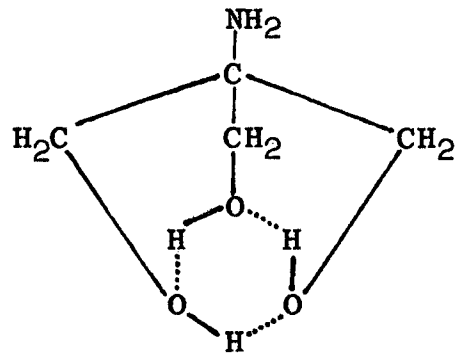
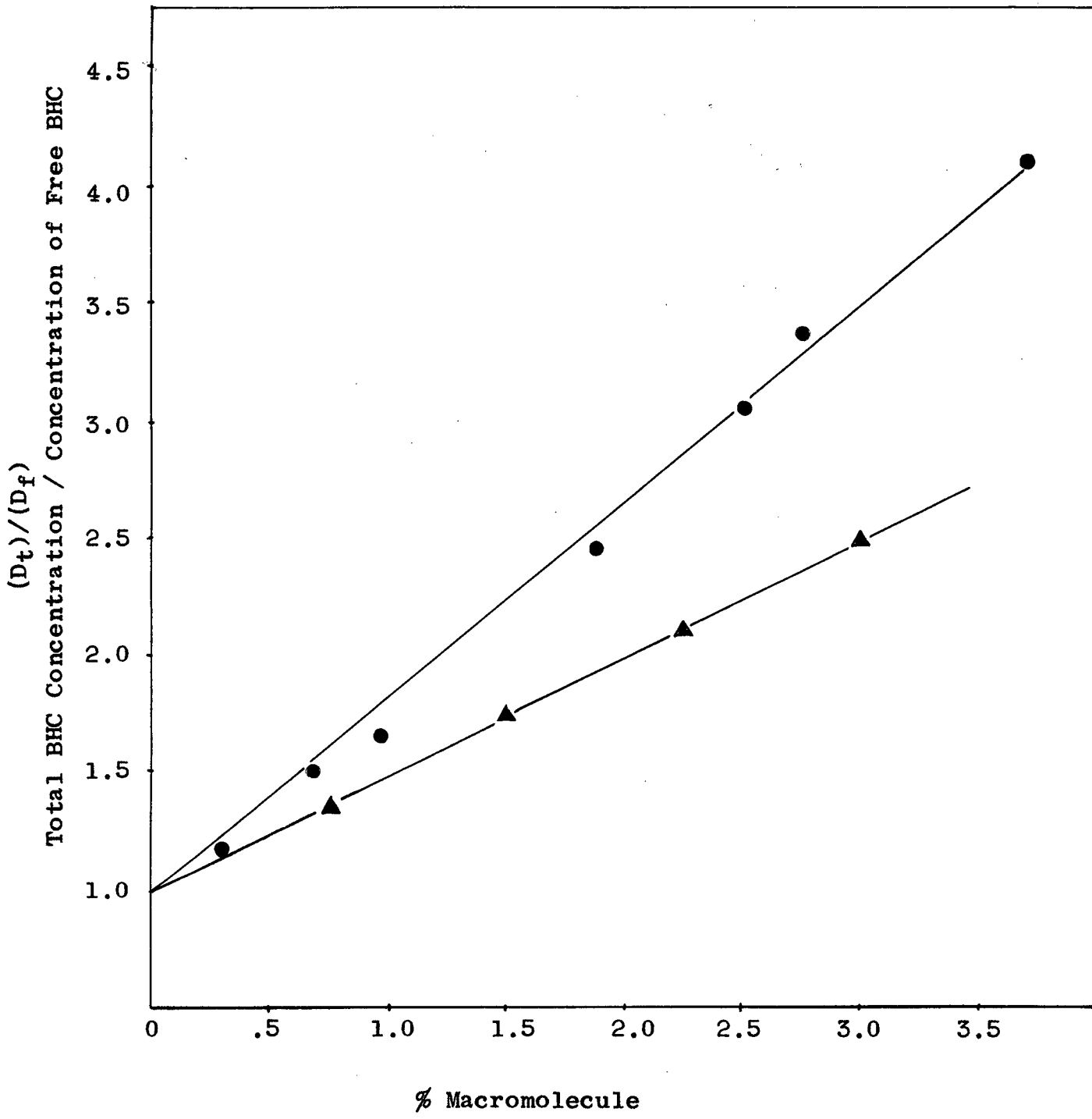


Figure 26. Two dimensional structure of Tris showing intramolecular hydrogen bonds. See Benesch and Benesch (1955).

(b) Effect of Macromolecule - i) Starch Sol and HES.

The binding tendency of BHC to potato starch sol and HES in Tris buffer (pH, 7.2; ionic strength, 0.2) is illustrated in Figure 27. All solutions were euilibrated for at least 40 hours at 30°C. Concentration ratio of total to free BHC is plotted against macromolecule concentration. When linear relationship is observed the slope (r value in Eq. 6) can be an approximation of binding capacity (see p. 4 and 46). If the drug and macromolecule do not interact, a straight line with zero slope passing through the unity should be obtained. This type of presenting solubility and equilibrium dialysis data has been frequently used in the literature (see, for example, Higuchi and Kuramoto, 1954b). Results in Figure 27 show that approximately 60 and 72% of the total BHC exist in the complexed form when the drug is equilibrated with 3% starch sol and HES solutions, respectively.

Figure 27. Effect of various concentrations of HES (▲) and potato starch sol (●) on the apparent solubility of BHC at 30°C in Tris buffer (pH, 7.2; ionic strength, 0.2).



(a) (b)

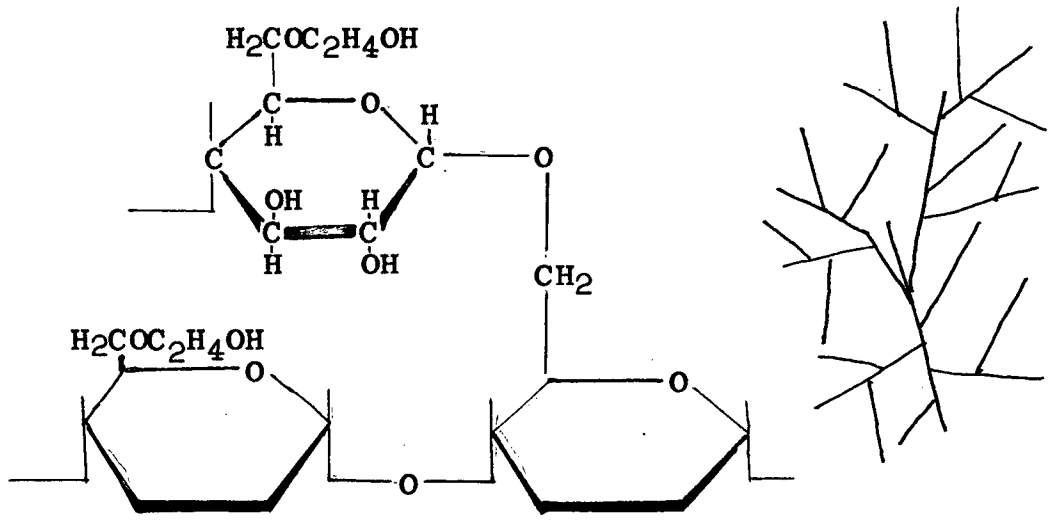


Figure 28. Chemical structure (a) and molecular configuration (b) of HES.

The chemical structure of HES is shown in Figure 28 (a). Its molecular configuration, illustrated in Figure 28 (b), is believed to be similar to that of amylopectin in that both molecules have many dilated branches. The average number of hydroxyethyl groups per glucose unit has been reported to be 0.7 to 0.9 (N.A.S. - N.R.C., 1965).

Potato starch consists of 20% amylose and 80% amylopectin (Greenwood, 1956) whereas HES has no amylose-type configuration. It has been reported that amylose is more likely to undergo a side-by-side association with organic molecules than amylopectin (BeMiller, 1965; Goudah and Guth, 1965; Mansour and Guth, 1968). The starch sol should, therefore, have a slightly higher affinity

for BHC than HES and this is confirmed by the results shown in Figure 27. Unfortunately, the limited results obtained do not lend themselves to an interpretation of mechanisms. Mansour and Guth (1968) assumed that benzoic acid, derivatives of benzoic acid, and sorbic acid form complexes with starches in a manner similar to that observed for the starch-iodine and starch-n-butanol systems. There appears to be an entrapment of the 'guest' molecule in the α-helical structure of amylose with a supplimentary stabilization of dipole-dipole interactions (Stein, 1948). A similar mechanism might explain BHC-starch and BHC-HES interactions.

(b) Effect of Macromolecule - ii) HSA and PVP. The apparent solubility of BHC in Tris buffer (pH, 7.4; ionic strength, 0.15) at 20°C as a function of either HSA or PVP concentration is illustrated in Figure 29. Solutions were analyzed for BHC spectrophotometrically. Corrections were made for depression of BHC absorbance due to complex formation in a manner described elsewhere in this thesis (see pp. 59-60 and 104-105).

In 1.0% HSA or PVP solution, apparent solubility is enhanced by approximately 5.4 and 21.5 times, respectively. In these solutions, approximately 84.4 and 95.6% of total BHC exist in the complexed form. In low macromolecule concentration range (up to 1.0% for HSA and 0.4% for PVP), linear relationship is observed between change in solubility and macromolecule concentration. When BHC-PVP binding affinity is compared with that for other drugs (see Table 1), this interaction is unusually strong. Interaction mechanisms will be extensively discussed later.

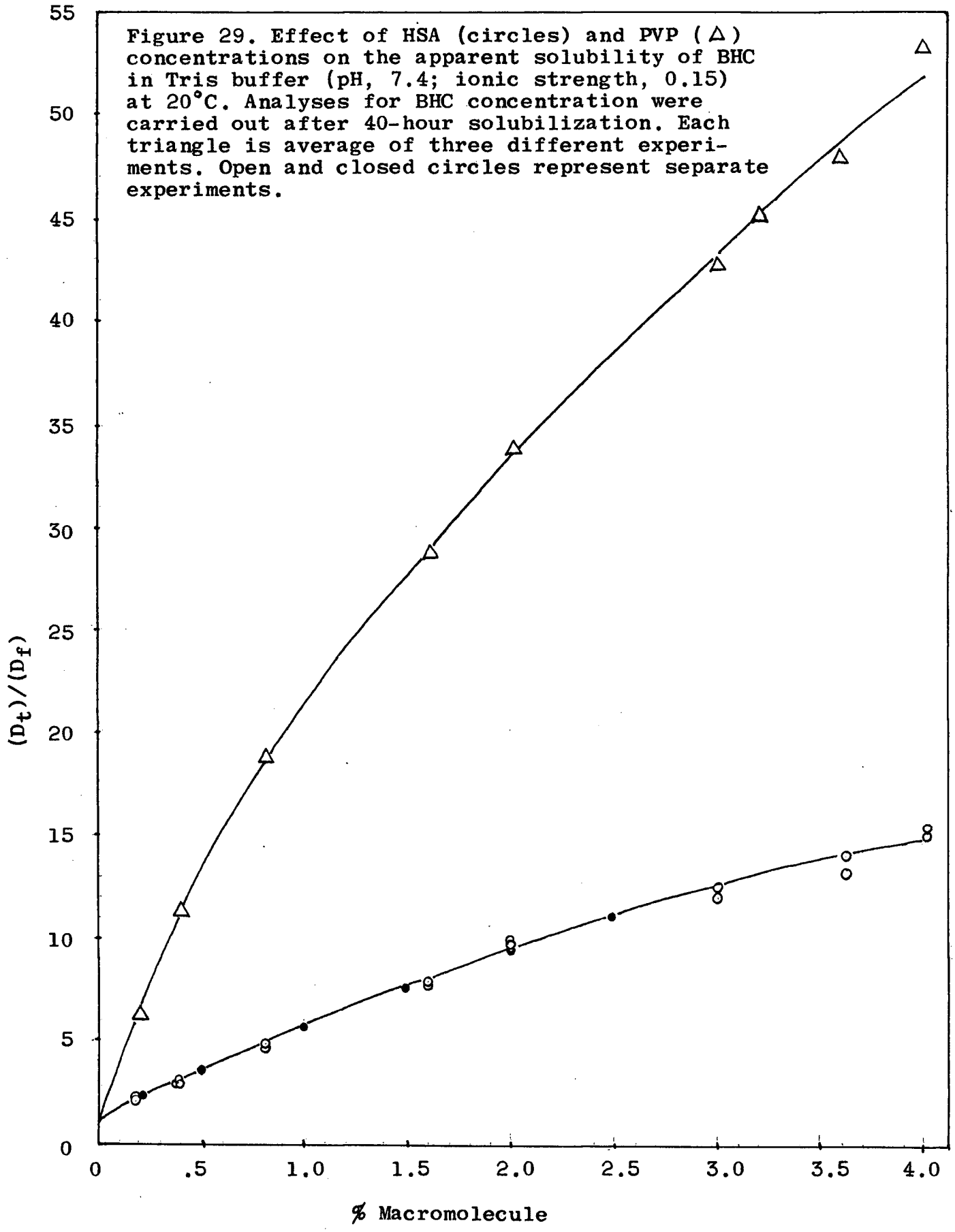


Figure 29. Effect of HSA (circles) and PVP (△) concentrations on the apparent solubility of BHC in Tris buffer (pH, 7.4; ionic strength, 0.15) at 20°C. Analyses for BHC concentration were carried out after 40-hour solubilization. Each triangle is average of three different experiments. Open and closed circles represent separate experiments.

4. Spectrophotometry

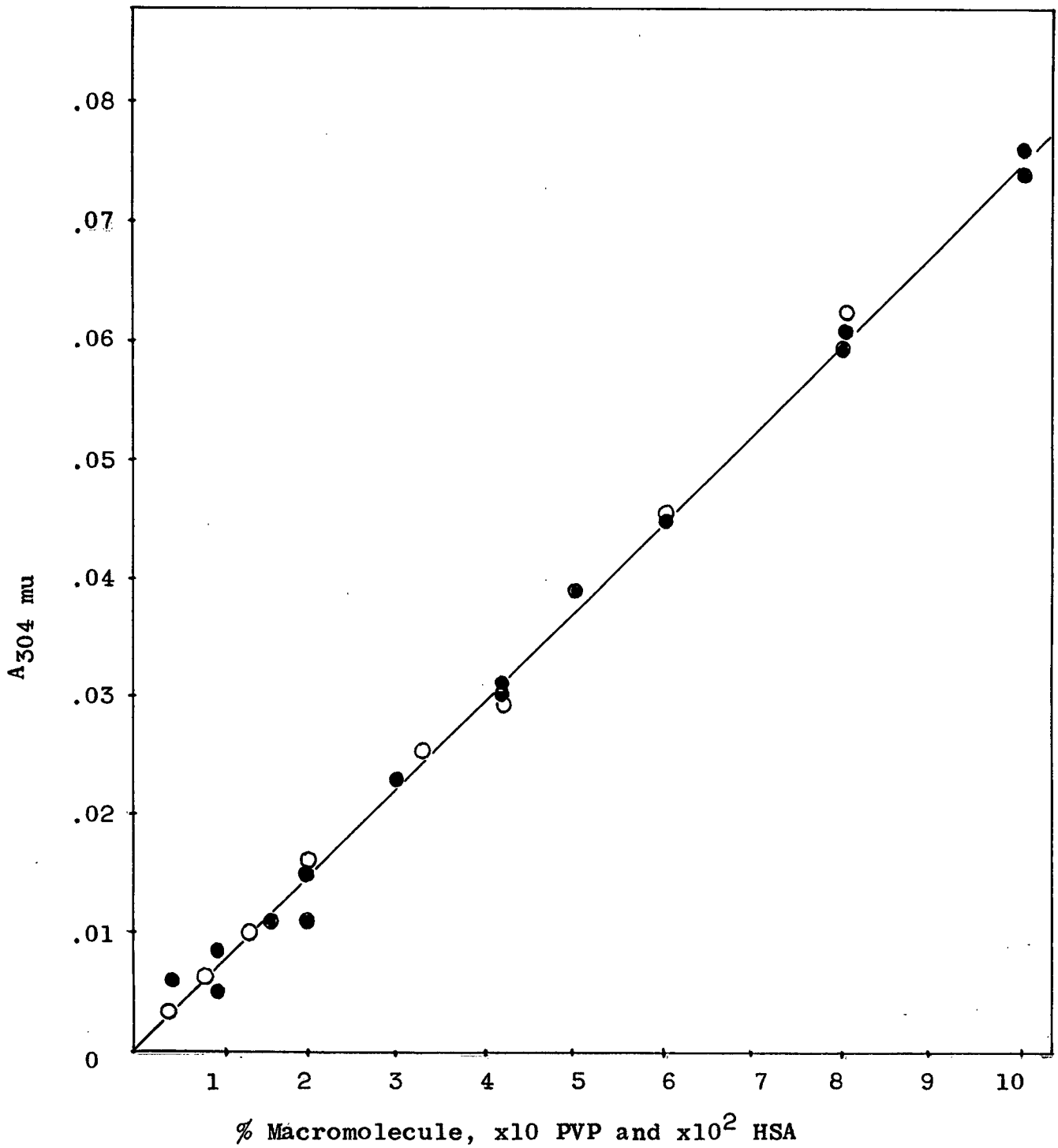
(a) Absorptivity Values of BHC. BHC concentrations were determined spectrophotometrically. Absorbance readings were carried out at either 304 mμ, the wavelength at which BHC exhibits maximum absorption in pH 7.4 buffer, or at 286 mμ, an isosbestic point. Absorptivity values (a_s) are reported in Table 2. Molar absorptivity values may be obtained by multiplying a_s values by 336.29, the molecular weight of BHC.

Wavelength / Instrument	286 mμ	304 mμ
B & L 505	47.35 ± 0.26	-
Beckman DU	48.38 ± 0.48	-
Beckman DU-2	-	55.5 ± 0.57

Table 2. Absorptivity Values for BHC in a pH-7.4 Buffer.

(b) Absorbance Contribution of HSA and PVP. HSA and PVP absorb some radiant energy at the above wavelengths. It is necessary to correct for this absorption by using appropriate calibration curves, before calculating BHC concentrations in the presence of these macromolecules. Such curve is shown in Figure 30. Both HSA and PVP obey Beer's Law over the concentration range investigated. HSA appears to absorb 10 time more energy than PVP, when both macromolecule solutions are expressed on a w/v percentage basis. Under experimental conditions,

Figure 30. Absorbance measurements at 304 mμ for HSA (●) and PVP (○) solutions in Tris buffer (pH, 7.4). Each point represents the average of at least two determinations on Beckman DU-2.



absorbance values corresponding to the macromolecule concentration in the solution, were determined from this curve and subtracted from the values obtained for the BHC-macromolecule solution.

(c) Depression of BHC Absorbance in the Presence of HSA and PVP. The spectrophotometric characteristics of bound BHC differ from those observed for the free drug. Spectral changes in the presence of HSA and PVP are illustrated in Figures 31 and 32. The reference solution, in both instances, contained the same quantity of macromolecule as the BHC solution. The spectra are, therefore, due to BHC in the solution. For comparison, spectra of BHC in the absence of macromolecule are also shown in Figures 31 and 32.

Because the absorption maximum at 304 mμ is depressed, it is probable that the functional group producing this absorption is involved in the complexation process. It is difficult to relate ultraviolet maximum to configuration. However, the chromophore in BHC is probably the α,β-unsaturated lactone structure (see Figure 33). As the pH of the solvent changes, the -OH group in the β-carbon position has a tendency to ionize and cause the type of electronic shift shown in Figure 15. This implies that the energy repuired for the elctronic transition will be altered resulting in a shift of the spectrum.

(d) Spectrophotometric Analysis of Complex Formation. Since absorbance is depressed, attempts were made to evaluate these spectral changes quantitatively. The procedures used are similar to those described by Klotz (1946c) and Oster and Immergut (1954). A series of BHC solutions containing identical

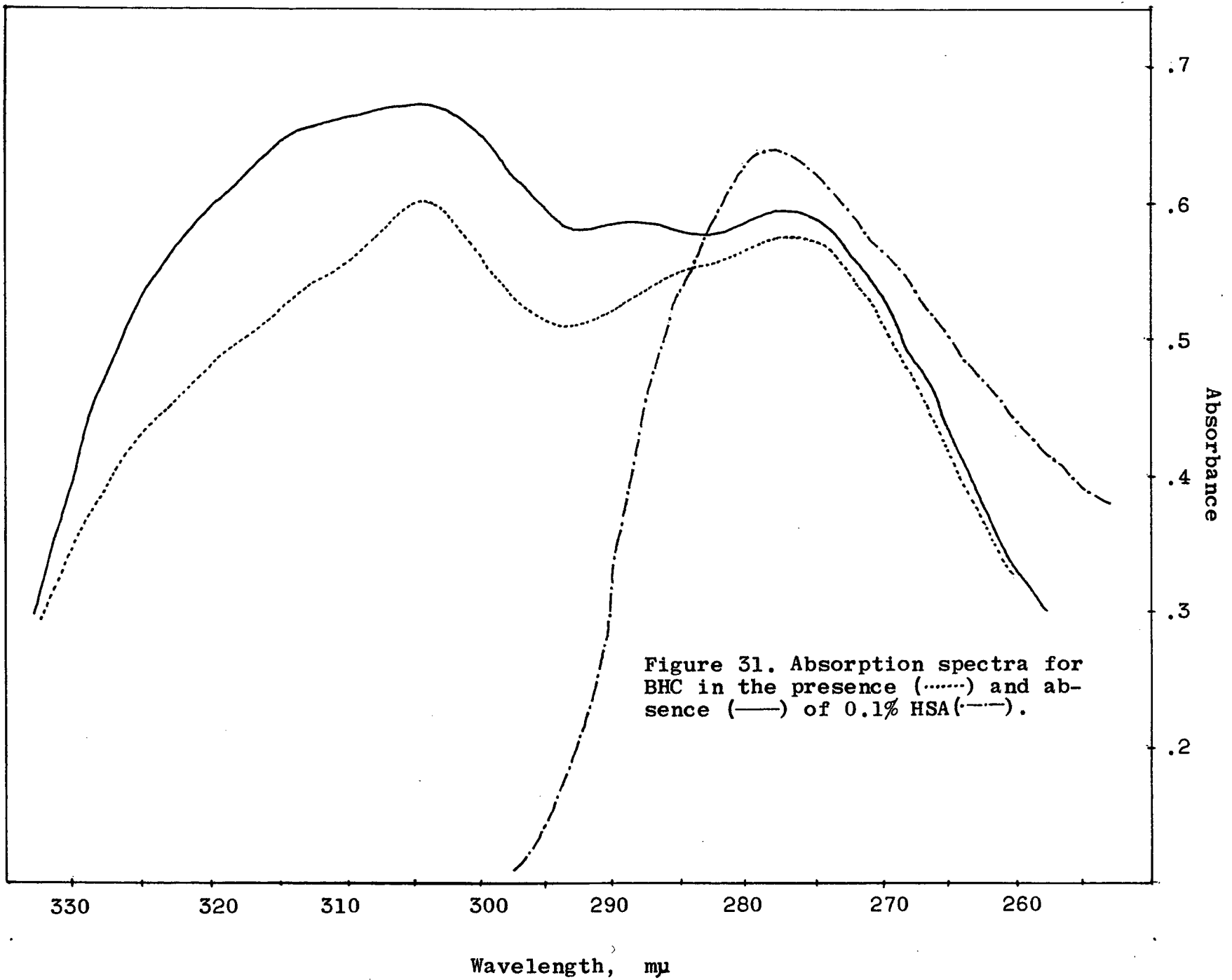
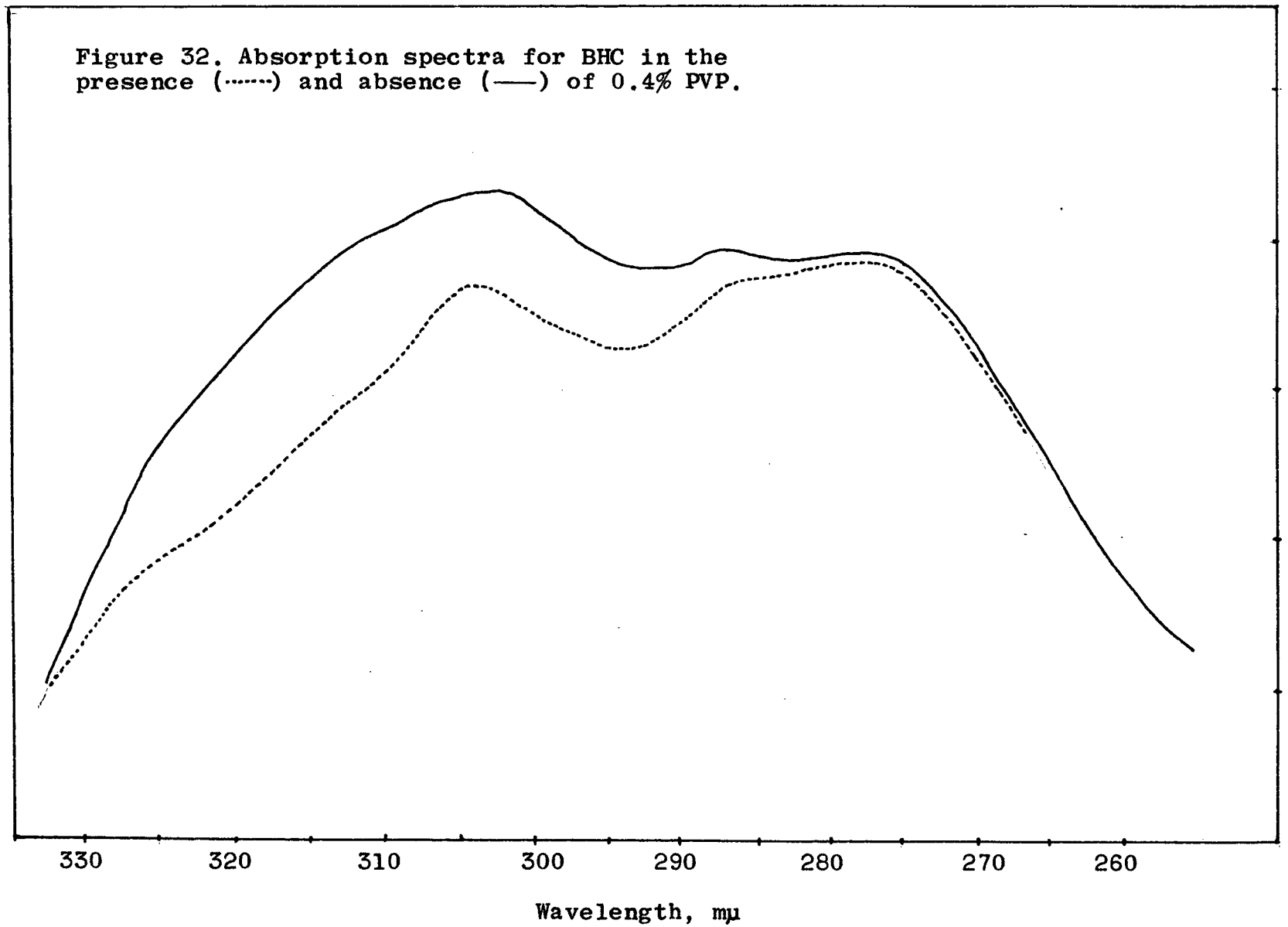


Figure 31. Absorption spectra for BHC in the presence (·····) and absence (——) of 0.1% HSA(·—·—).

Figure 32. Absorption spectra for BHC in the presence (·····) and absence (——) of 0.4% PVP.



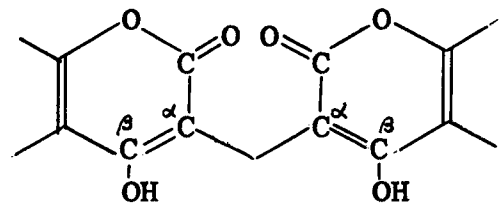


Figure 33. Predominating chromophore (α,β - unsaturated lactone) in the BHC molecule.

quantities of BHC but varying amount of HSA and PVP were prepared and absorbance values were determined at 304 mμ. The depressions in absorbance values, plotted as a function of macromolecule concentration, are illustrated in Figures 34 and 35.

Beyond a certain macromolecule concentration, there is no further depression, which implies that all BHC molecules are in the bound form. Molar absorptivity values of bound BHC were estimated from the known per cent depression of absorbance readings. The results are summarized in Table 3. The ϵ_f and ϵ_b values represent molar absorptivities for the free and bound species, respectively. The per cent depression is equal to α value in Eq. 38.

In Figure 36, absorbance depression at a constant macromolecule concentration was plotted as a function of BHC concentration. Most of the results illustrated are from the experiments used to produce the plots in Figures 34 and 35. A linear relationship was observed between the difference in absorbance in the absence and presence of macromolecule and the observed absorbance. From this, it is possible to obtain information which can be used

Figure 34. Absorbance depression of BHC as a function of HSA concentration. BHC concentrations are 26.7 (a), 23.7 (b), 15.4 (c), and 9.1 (d) x10^{-6} mole/L.

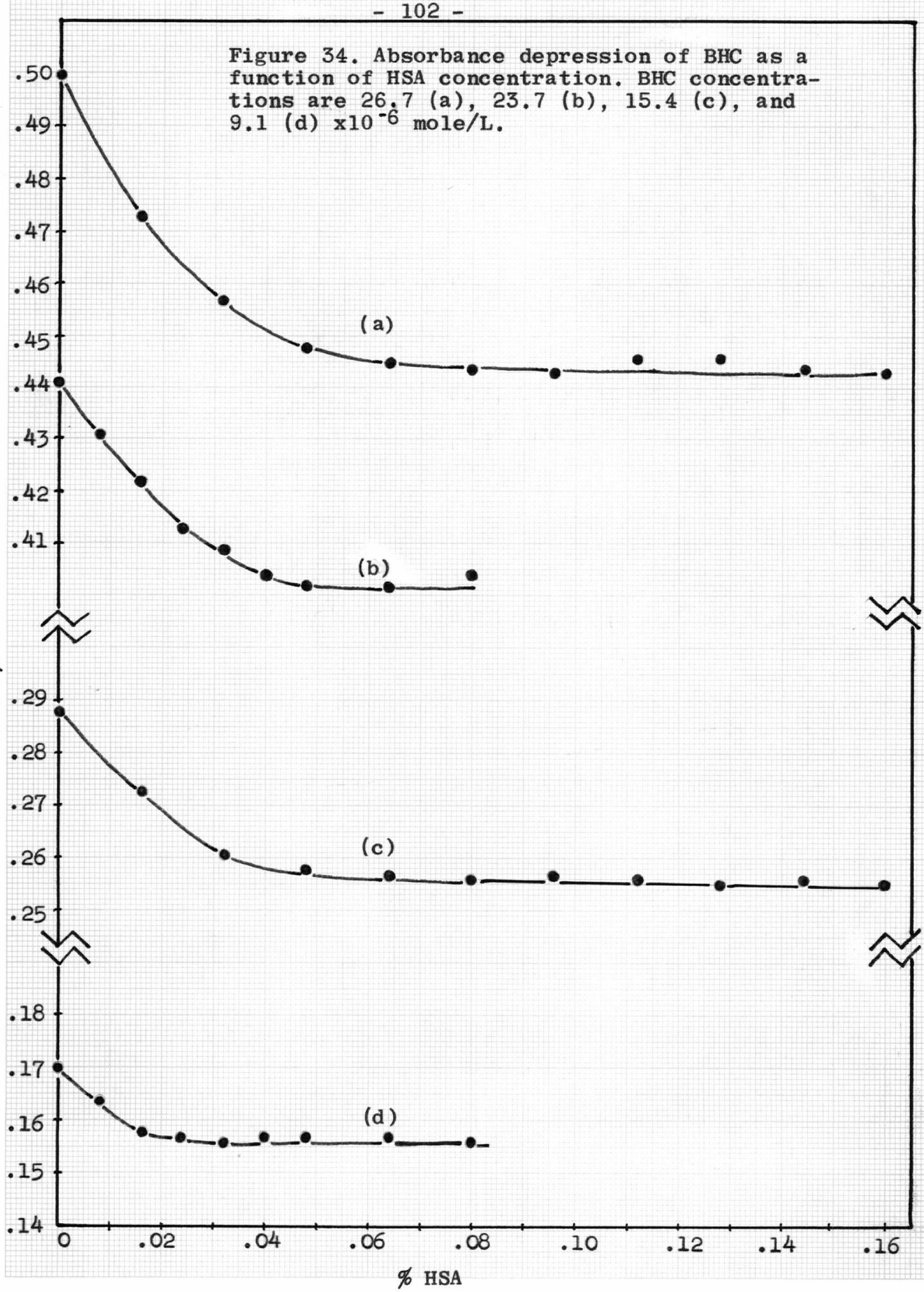
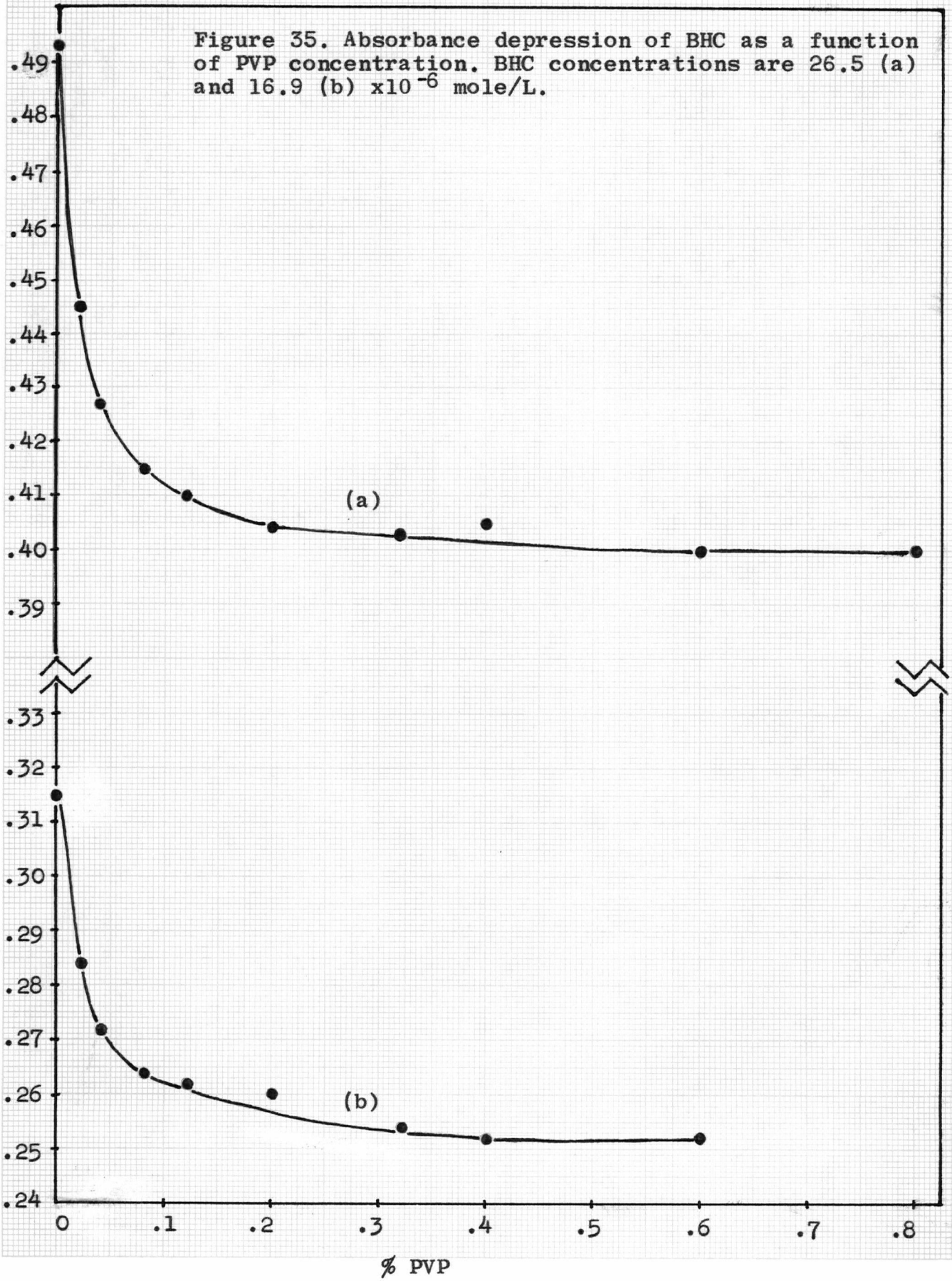


Figure 35. Absorbance depression of BHC as a function of PVP concentration. BHC concentrations are 26.5 (a) and 16.9 (b) x10^{-6} mole/L.



in the law of mass action. From Eq. 37 to 40, (D_f) and r values can be estimated. The calculation procedure is illustrated in Table 4, when PVP concentration is equal to 0.02% (i.e., $5x10^{-6}$ mole/L.).

Experiment	$\epsilon_f(D_t)$	$\epsilon_b(D_t)$	% Depression	Average
HSA-BHC (a)	.499	.443	88.8	89.9
(b)	.442	.402	90.0	
(c)	.288	.256	88.9	
(d)	.170	.155	91.1	
PVP-BHC (a)	.493	.400	81.1	80.5
(b)	.315	.252	80.0	

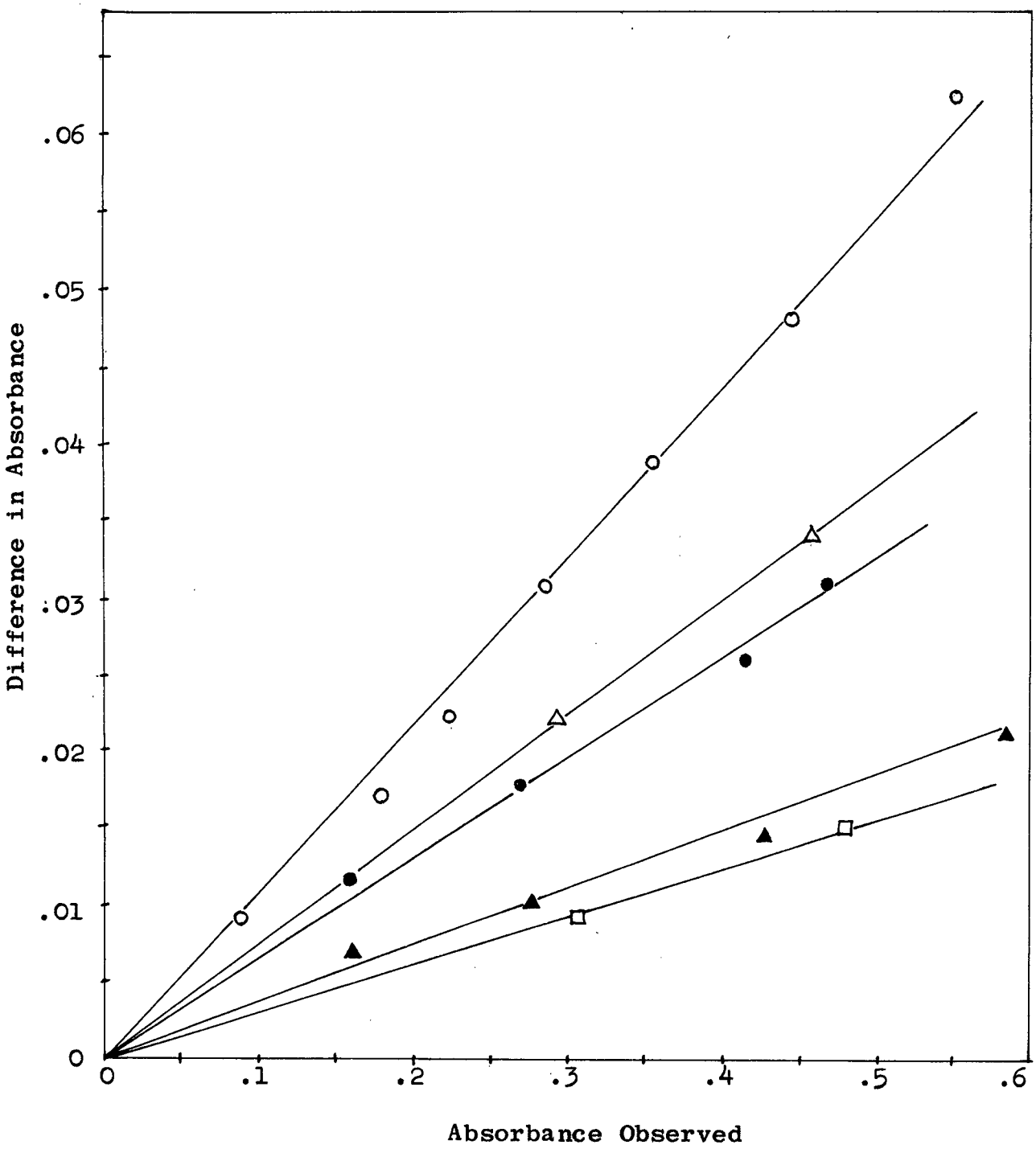
Table 3. Estimate of Molar Absorptivity Ratio of Bound BHC to Free BHC.

$\epsilon_f(D_t)$	$A_{304\ m\mu}$ Observed	ΔA	(D_t)	(D_b)	(D_f)	r
.0985	.0895	.009	5.28	2.47	2.81	0.494
.197	.180	.017	10.56	4.67	5.89	0.934
.246	.224	.022	13.18	6.04	7.14	1.21
.315	.284	.031	16.88	8.52	8.36	1.70
.394	.355	.039	21.11	10.72	10.39	2.14
.493	.445	.048	26.41	13.19	13.22	2.64
.616	.553	.063	33.00	17.31	15.69	3.46

Table 4. Analysis of Spectrophotometric Data for PVP-BHC Interaction in 0.02% PVP (5 µmole/L.). Absorbance observed is average of a pair of separate measurements on sample solutions separately prepared. The concentration terms are in µmole/L. $\epsilon_b = 0.805\ \epsilon_f = 0.805 \times 18{,}660$ (see Table 3).

Figure 36 was also used to correct for the presence of macromolecule in BHC analyses. For example, if a BHC solution in 0.02% PVP has an absorbance value of 0.400, the correction factor (0.043, read directly from the ordinate in Figure 36) is added, and the new value is divided by ϵ_f to yield the molar concentration of BHC in the solution.

Figure 36. Absorbance differences for BHC solutions in the absence and presence of constant amounts of macromolecules as a function of total BHC concentration. Closed symbols: HSA-BHC; open symbols: PVP-BHC. Macromolecule concentrations are: squares, 0.004%; triangles, 0.01%; circles, 0.02%.



5. Dynamic Dialysis

The results of the dynamic dialysis experiments are illustrated in Figures 37 and 38. On the basis of molecular weights of 75,000 and 450,000, the systems contained 53.3 and 8.89 micromoles dextran and HES per liter of Tris buffer, respectively. In studies of the PVP-BHC interaction, two different PVP concentrations (50 and 100 µmole/L.) were used.

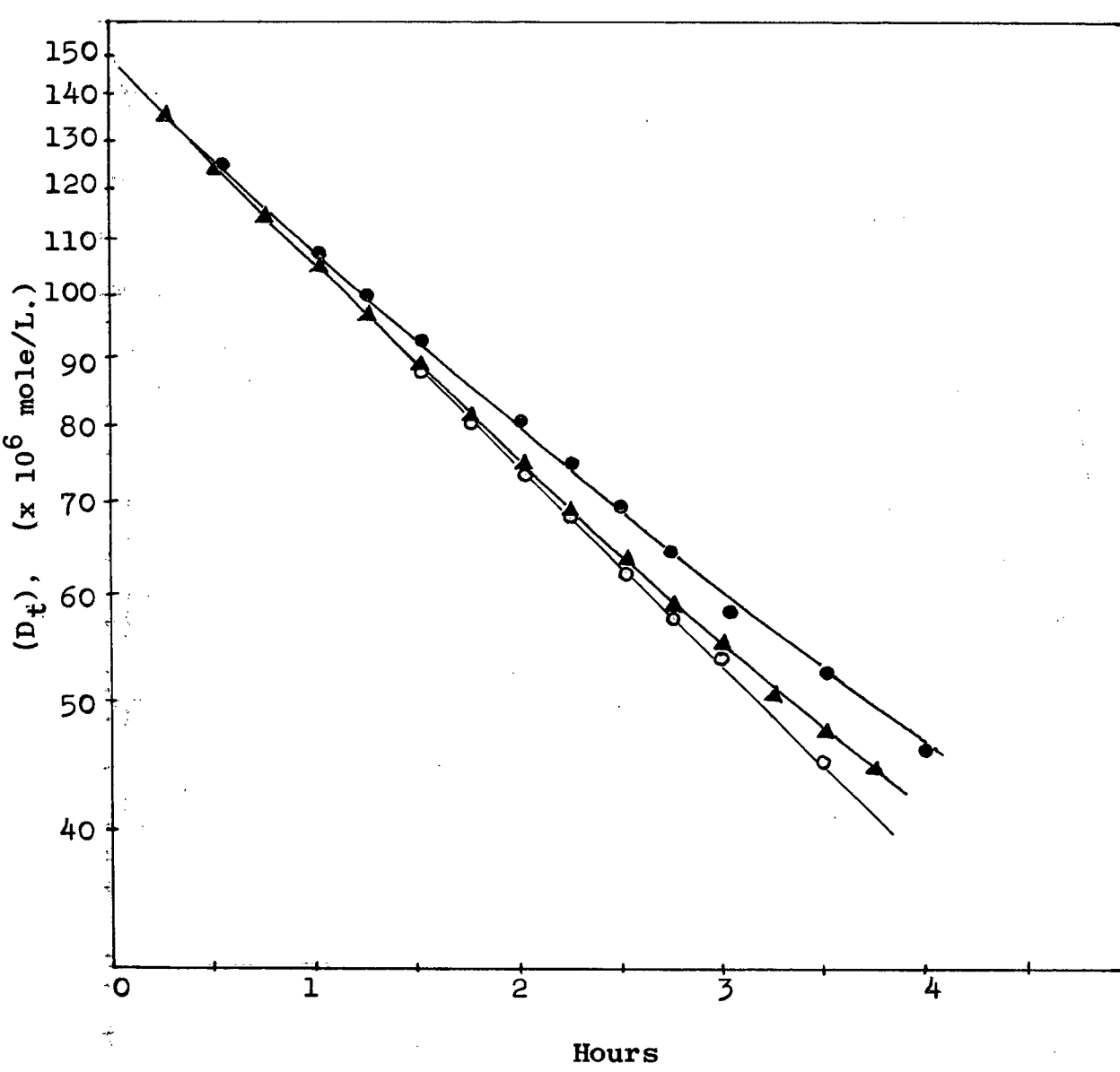
In the absence of macromolecule, dialysis of BHC follows Fick's law of first order kinetics. This is indicated by the straight line obtained when log concentration is plotted versus time. The interactions of BHC with dextran and HES appear to be weak. On the other hand, the dialysis rate of BHC in the presence of PVP is significantly different from that observed in the absence of the macromolecule.

Quantitative analysis of this data can be carried out by utilizing Eq. 21 and Eq. 53 given below. Dialysis rate is proportional to the concentration of free BHC inside the dialysis bag.

$$- \frac{d(D_t)}{dt} = k_d \, (D_f) \qquad \text{(Eq. 53)}$$

The concentration of unbound (i.e., free) BHC in the macromolecule compartment at any total BHC concentration can be calculated from Eq. 53. The k_d value is obtained from the slope of the semilog plot of (D_t) versus time in the absence of macromolecule. The instantaneous rate at a value of (D_t) in the presence of macromolecule can be estimated from a plot of (D_t) versus time on linear graph paper.

Figure 37. Loss of free BHC from inside a dialysis bag in the absence (o) and presence of 0.4% HES (●) and dextran (▲) in Tris buffer (pH, 7.4; ionic strength, 0.15) at 30°C.



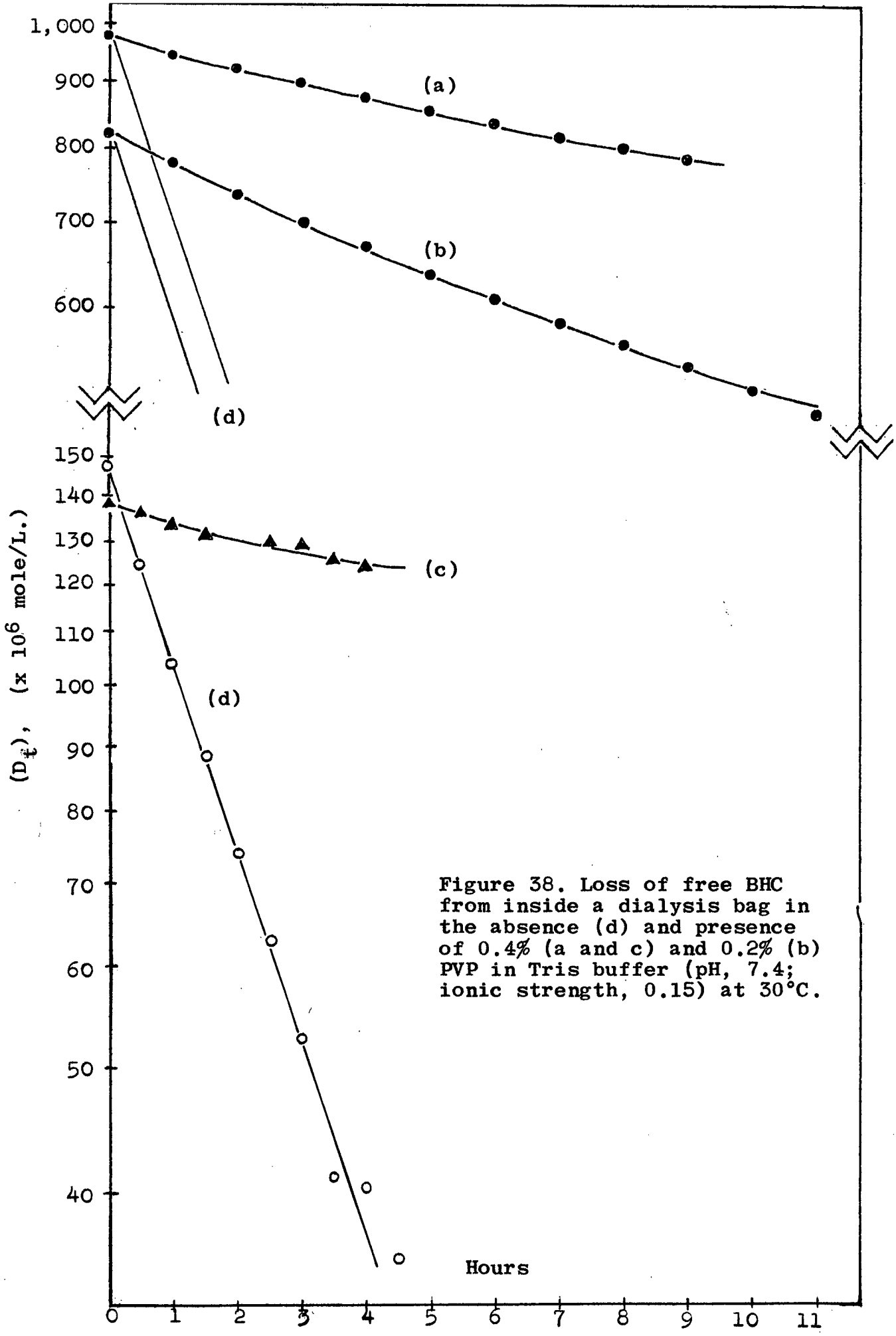


Figure 38. Loss of free BHC from inside a dialysis bag in the absence (d) and presence of 0.4% (a and c) and 0.2% (b) PVP in Tris buffer (pH, 7.4; ionic strength, 0.15) at 30°C.

The first-order rate constant (k_d), which characterizes the diffusion process and incorporates the area and thickness of the membrane, was found to be 5.71 x 10^{-3} $Cm.^2$/min. Calculations of (D_f) and r values for the experiment (b) in Figure 38 are illustrated in Table 5. The initial BHC concentration was 828x10^{-6} mole/L. and PVP concentration was 0.2% (i.e., 50x10^{-6} mole/L.). The second colume shows BHC concentrations at a given time in the PVP-free compartment which contains 500 ml. of Tris buffer. These values were converted to amount terms and subtracted from the total amount of drug in the PVP compartment which contains 50 ml. of PVP-BHC solution. The amount of BHC remaining inside the bag was re-converted to a concentration term and this value is shown in column three. The fourth column is the slope of a tangent line at a given time obtained from a plot of (D_t) versus time.

Time (Hour)	(C)	(Dt)	$k_d(D_f)$	(D_f)	(D_b)	r
0	0	828	-	-	-	-
1	4.7	781	.756	132	649	13.0
2	8.8	735	.631	111	624	12.5
3	11.5	700	.578	101	599	12.0
4	13.6	667	.554	97	570	11.4
5	15.5	635	.475	83.2	552	11.0
6	16.6	608	.460	80.5	527	10.5
7	17.4	583	.413	72.5	510	10.2
8	18.2	558	.387	67.8	490	9.8
9	18.4	537	.363	63.5	473	9.5
10	18.8	515	.350	61.3	454	9.1

Table 5. Calculated Data for the Dynamic Dialysis of BHC in the Presence of PVP. The concentration terms are in µmole/L. PVP concentration was 50 µmole/L. See the text for more detail.

A single dynamic dislysis experiment would fail to cover a wide range of r values or (D_f) (especially when the binding affinity is remarkably high as in the case of PVP-BHC interaction). It is, therefore, necessary to repeat the procedure several times with different experimental conditions (e.g., use of much lower macromolecule concentration) in order to obtain data covering a wider range of r values or (D_f).

For example, the results illustrated in curves (a) to (c) in Figure 38 cover a (D_f) range from 36.8 to 68.9, from 61.3 to 132.0, and from 6.3 to 14.7 μmole/L., respectively. In terms of r value, they are from 7.4 to 8.8, from 9.1 to 13.0 (see Table 5), and from 1.19 to 1.22. These ranges could be extended by dialyzing for longer periods of time but analytical errors will increase with time and result in unreliable data. These difficulties are mentioned by Stein (1965) but not by Meyer and Guttman (1968b; 1970a; 1970b).

For this reason, the experimental design (i.e., volume ratio of the two compartments, concentration of drug and macromolecule, size of sample, duration of dialysis, etc.) is critical when this technique is used to study completely unknown drug-macromolecule interactions. However, this method can be efficiently used for a study of well known binding systems such as albumin-dye complexations (Meyer and Guttman, 1970b). Data obtained for HES-BHC and dextran-BHC interactions failed to cover a wide range enough (D_f) range to permit an evaluation of the mechanism of the interaction. Because of this, the data obtained for these macromolecules is not reported here.

Dynamic dialysis experiments confirm the reversibility of binding of BHC with the macromolecules investigated. Continuous dialysis appeared to remove a portion of the BHC which had been bound to the macromolecule within the dialysis bag. For example, in 0.2% PVP solution, solubility data indicates that approximately 84% of the total BHC exists in the bound form (see Figure 29). This value is not necessarily equal to that in the initial stages of the dynamic dialysis experiment (b) in Figure 38 because extent of binding depends on BHC concentration. However, if it is assumed that they are approximately equal,* then 133 (i.e., 828-828x0.84) μmole/L. exists in the unbound form at time zero. After ten hours of continuous dialysis, the BHC concentration inside the bag was reduced to 515 μmole/L. (see Table 5). This means that 313 μmole/L. have been removed from the bag in this period of time. This, of course, is much greater than the value of 133 μmole/L. and indicates dissociation of the PVP-BHC complex during the experiment.

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* This assumption seems to be valid, since the PVP solution for the dynamic dialysis experiment (b) was nearly saturated with BHC (see p. 63).

6. Equilibrium Dialysis

(a) Binding of BHC to Cellophane Membrane. One of the main sources of error in the dialysis method is the irregular adsorption of small molecules by the membrane (see pp. 30-32). It was necessary, therefore, to correct for this binding on an experimental basis.* Extent of binding of BHC to membrane (and also possibly to the plexiglas cell) was studied at three different temperatures. Results are shown in Figure 39. No significant differences in binding were observed at the three temperatures. Although the same procedure for membrane preparation was used throughout the study, the extent of binding varied. However, on the basis of all the data, a correction factor of four per cent of the total BHC was used throughout this investigation.

(b) Permeability of Membrane to PVP. Hengstenberg and Schucht (1952) reported that the permeability of PVP molecule through the membrane can be neglected only if its molecular weight is greater than 10,000. However, Spitzer and McDonald (1956) found that some PVP molecules cross the membrane even if the molecular weight is higher than 40,000. By using differential titration, they confirmed that the dialyzable PVP is titratable species which is either a sub-fraction of the PVP or an impurity (or impurities) arisen during synthesis of the polymer.

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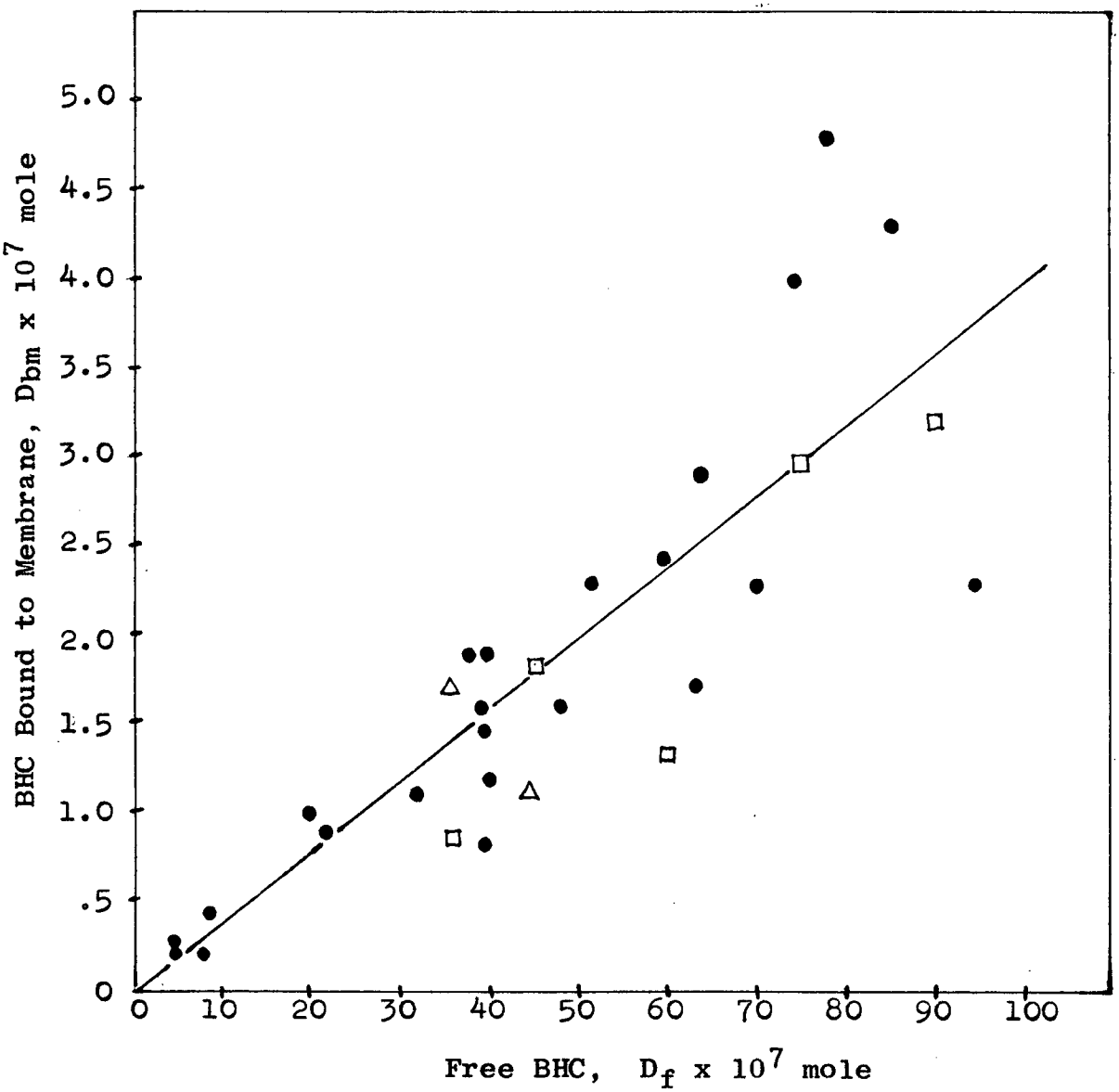
* When the concentration of small molecules is analyzed on both compartments, the correction for membrane binding is unnecessary. However in this work only macromolecule-free compartment was analyzed for BHC concentration.

Figure 39. Estimate of the extent of adsorption of BHC to the cellophane membrane at three different temperatures: 10 (△), 20 (●), and 40 (□) C.



Although the PVP used in this investigation was purified by extraction with anhydrous ether, experiments were carried out to determine if the polymer passed through the membrane. Analysis of the PVP-free compartment was carried out spectrophotometrically using the iodine-PVP reaction. A calibration curve is shown in Figure 40. When 20 ml. of a 0.4% PVP solution was added to one of the compartments and dialyzed for 40 hours against Tris buffer, approximately 10 mg./L. PVP was detected in the PVP-free compartment. This implies that approximately 0.25% of the total amount of PVP used can pass through the membrane. This value is so small that it can be neglected.

(c) Donnan Effect. In equilibrium dialysis, an appropriate ionic strngth must be maintained in order to prevent Donnan equilibrium across the membrane (see p. 30). The alternative is to use low macromolecule concentrations. HSA has a net negative charge of 18 at pH 7.4 (White and others, 1968). The ionic strength in the Tris buffer used is 0.15 and the highest HSA concentration used in the investigation was 0.4% (5.8×10^{-5} mole/L.). When these values are substituted into Eq. 42, the distribution ratio of univalent anions across the membrane (R value in Eq. 42) is equal to 1.0035. It is possible, therefore, to neglect the abnormal distribution of BHC anion due to the Donnan effect. PVP has no electrical charge at pH 7.4 (May and others, 1954). The Donnan effect can, therefore, be neglected.*

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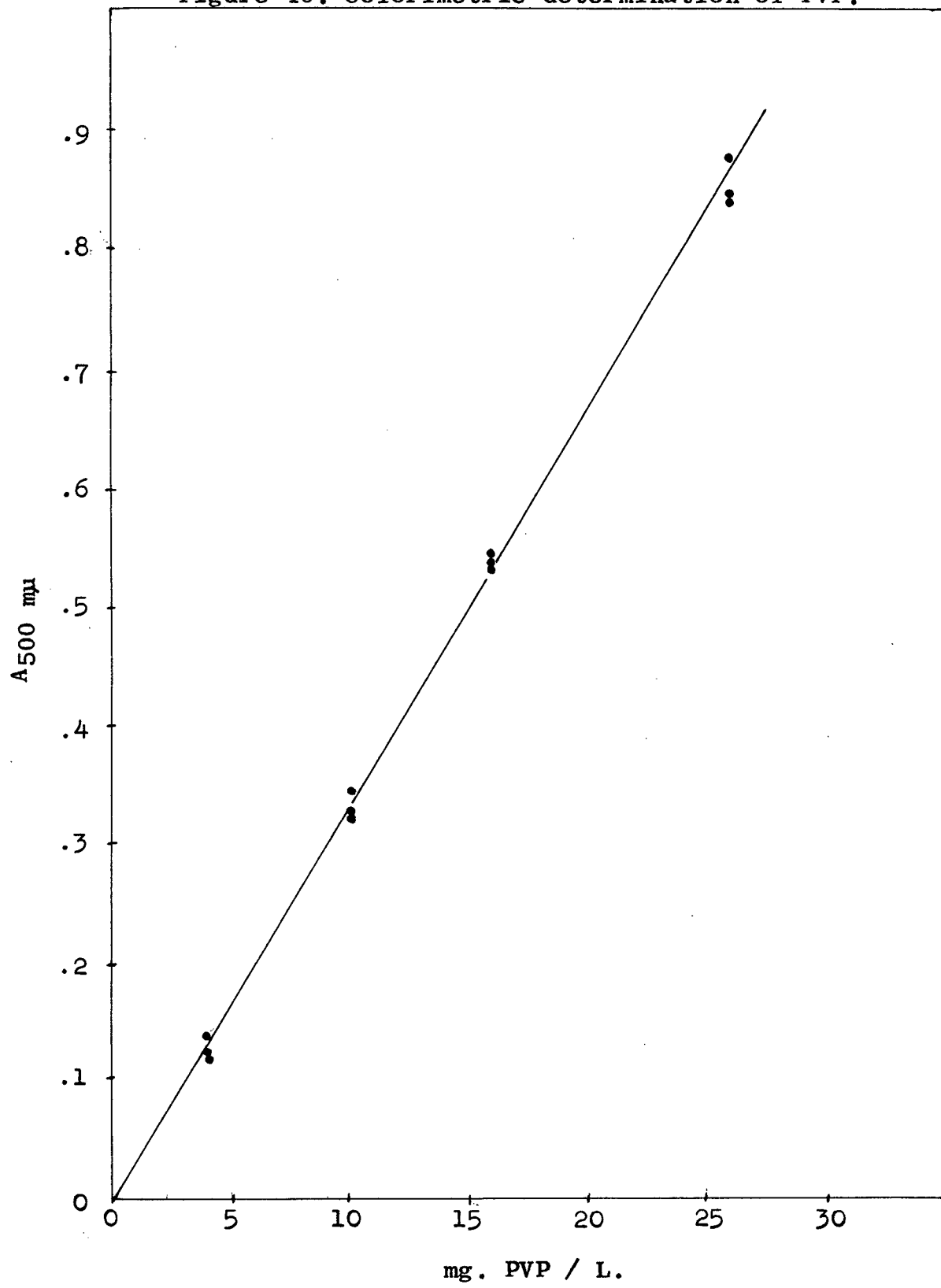
* PVP will also behave as a polyelectrolyte after binding with the BHC anion. However this characteristic will not be remarkable in a solution of high ionic strength (see Frank and others, 1957, for example).

Figure 40. Colorimetric determination of PVP.



(d) Free Drug Concentration and Volume Ratio. In order to study binding mechanisms, it is necessary to cover a wide range of (D_f) or r values. Pollansch and Briggs (1954) claimed that maximum efficiency is obtained by assigning appropriate volume ratios to the macromolecule to macromolecule-free compartments. Furthermore, the total amount of BHC that can be handled under given conditions can be enhanced by dialyzing BHC solutions against macromolecule solutions which have been nearly saturated with BHC. Yang and Foster (1953) used this approach in their binding studies (see pp. 31-32).

Typical design and results of a dialysis experiment are shown in Tables 6 and 7. Calculations can be carried out on the basis of either concentration or amount term. If 1:1 volume ratio is used, it is more convenient to use concentration term. However, in this investigation at least one of the dialysis cells had a 2:1 volume ratio. It is, therefore, more convenient to use amount term.* The corrected total amount of BHC (D_t in the fifth column of the tables) is obtained by multiplying D_i, the uncorrected initial amount of BHC, by 0.96, the correction factor for membrane binding. The BHC content of the macromolecule-free compartment is determined spectrophotometrically and converted to the amount present in either 20 or 40 ml. The initial BHC concentration in the macromolecule compartment was determined from the correction curves illustrated in Figure 36.

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* Attention must be paid to term used in the calculation procedure. In the literature, some investigators failed to convert concentration term to amount term, although they used volume ratio rather than 1:1. For example, O'Reilly and Kowitz (1967) showed a typical miscalculation of r values. It was pointed out by Meyer and Guttman (1970b).

Designing				Results					
No.	ml.of Stock Solutions	D_i	D_t	D.F.	A_{304}	(D_f)	D_f	D_b	r
1A	40(BHC/1)	7.65	11.58	10	.241	129.1	7.75	3.83	6.60
1B	20(H-B)	4.41							
2A	20(BHC/1)	3.83	7.91	5	.427	114.4	4.58	3.33	5.74
2B	20(H-B)	4.14							
3A	20(BHC/2)	1.91	1.85	1	.198	10.6	0.42	1.42	2.45
3B	20(HSA)	0							
4A	20(BHC/4)	0.96	0.92	1	.039	2.1	0.084	0.834	1.44
4B	20(HSA)	0							
5A	20(BHC/5)	0.765	0.734	1	.031	1.67	0.064	0.67	1.16
5B	20(HSA)	0							
6A	40(BHC/10)	0.765	0.734	1	.047	2.52	0.15	0.58	1.01
6B	20(HSA)	0							

Table 6. Calculation Procedures for (D_f) and r Value. Compartment B contains HSA. The BHC concentration of the stock solution (BHC/1) was 191.3 µmole/L. Denominator represents the dilution factor (D.F.) of the stock solution. HSA stock solution nearly saturated with BHC is designated by H-B, for which the BHC concentration is 220.7 µmole/L. HSA concentration is 0.2% (28.98 µmole/L. = 0.58 µmole/20 ml.). Temperature was maintained at 20°C. The concentration terms are in µmole/L. The amount terms are in µmole.

Designing				Results					
No.	ml.of Stock Solutions	D_i	D_t	D.F.	A_{304}	(D_f)	D_f	D_b	r
1A	40(BHC/1)	7.87	16.77	10	.243	130.0	7.80	8.97	17.94
1B	20(P-B)	9.60							
2A	40(3BHC/4)	5.90	14.88	10	.216	115.5	6.93	7.95	15.9
2B	20(P-B)	9.60							
3A	20(3BHC/4)	2.95	12.05	10	.206	110.4	4.42	7.64	15.27
3B	20(P-B)	9.60							
4A	20(BHC/2)	1.97	11.10	10	.189	100.2	4.01	7.10	14.19
4B	20(P-B)	9.60		5	.370				
5A	20(Tris)	0	9.22	5	.300	80.4	3.22	6.00	12.00
5B	20(P-B)	9.60							
6A	20(3BHC/4)	2.95	2.83	1	.429	23.0	0.92	1.91	3.82
6B	20(PVP)	0							

Table 7. Calculation Procedures for (D_f) and r Value. Compartment B contains PVP. The BHC concentration of the stock solution (BHC/1) was 196.6 µmole/L. The fractional expression gives the dilution factor for the stock solution. PVP stock solution nearly saturated with BHC is designated by P-B, for which the BHC concentration is 480.1 µmole/L. PVP concentration is 0.1% (25 µmole/L. = 0.5 µmole/20 ml.). Temperature was maintained at 20°C.

7. Interpretation of Binding Data

(a) Langmuir-Type Plot. The simplest way to handle binding data is to plot r value versus (D_f). The curve obtained from Eq. 17, which is applicable only to a binding system containing a single set of binding sites, is a segment of a rectangular hyperbola passing through the origin. If (D_f) in Eq. 17 becomes infinite, the r value approaches n as a limit,

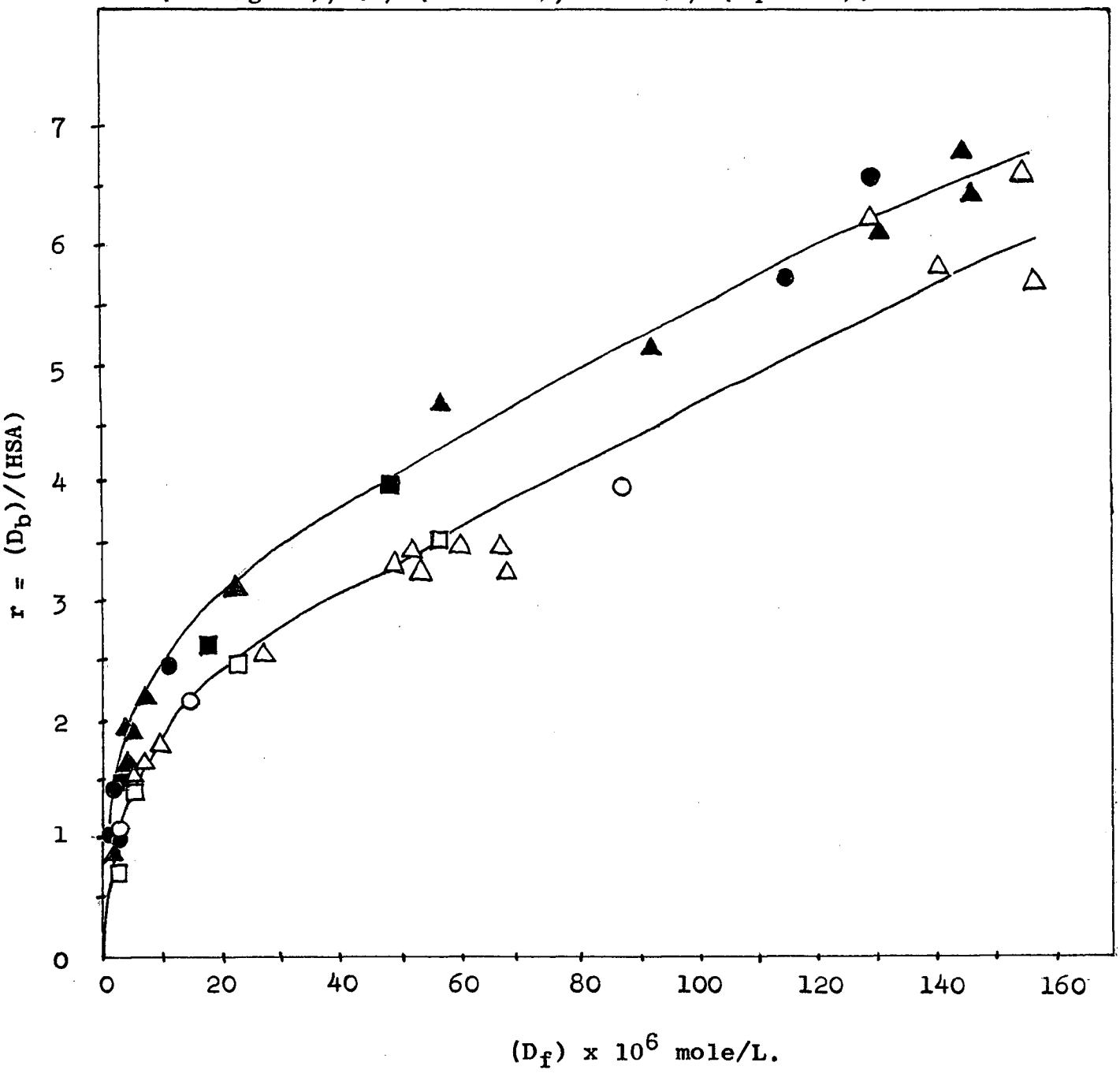
$$\lim_{(D_f)\to\infty} r = n \qquad \text{(Eq. 54)}$$

and at $r = n/2$, $\qquad (D_f) = K^{-1} \qquad$ (Eq. 55)

In Eq. 55, K^{-1} represnts the intrinsic 'dissociation' constant. These two equations indicates the importance of a wide range of (D_f) values. For example, Dowd and Riggs (1965) pointed out that many investigators chose unsuitable substrate concentration ranges in studies of enzyme-substrate complexation. They discussed the significance of the Michaelis-Menton equation which is similar to Eq. 17 or 20.

Binding data for BHC to HSA and PVP is illustrated in Figures 41 and 42. For the (D_f) range studied, both macromolecules failed to show saturation of binding sites. As illustrated in Figure 41, at lower BHC concentrations the drug is more easily bound to HSA than at high concentrations. However, with PVP the curve does not change as (D_f) increases. In other words, the (D_f) range investigated is too narrow to permit an evaluation

Figure 41. Plot of r values versus the concentration of free BHC for HSA-BHC binding at two temperatures; 20°C (closed symbols) and 40°C (open symbols). Values were obtained from three different HSA concentrations; 0.1% (triangles), 0.2% (circles), and 0.4% (squares).



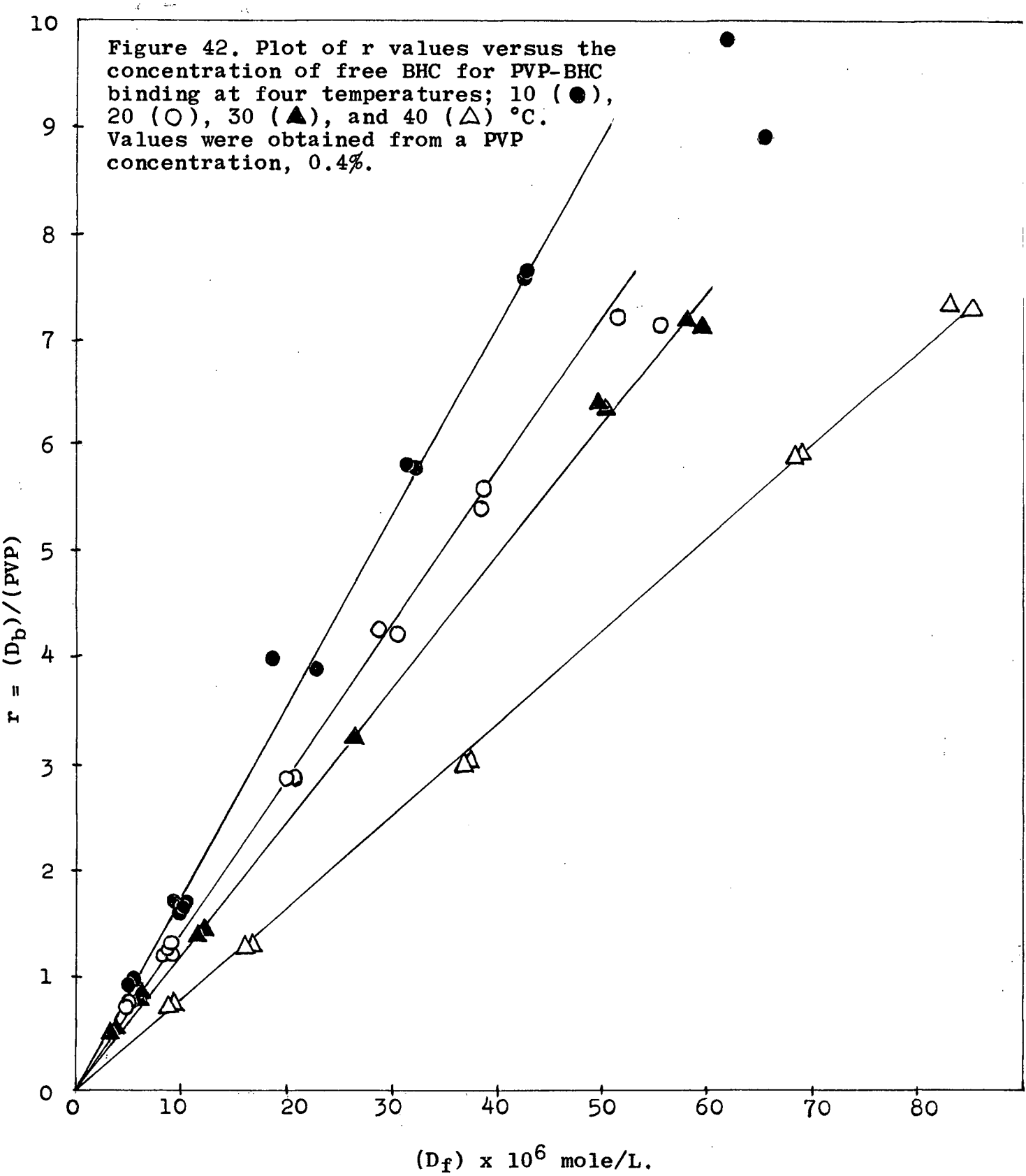


Figure 42. Plot of r values versus the concentration of free BHC for PVP-BHC binding at four temperatures; 10 (●), 20 (○), 30 (▲), and 40 (△) °C. Values were obtained from a PVP concentration, 0.4%.

of binding mechanisms. It was impossible, from equilibrium dialysis method, to obtain data at a higher (D_f) range, even though attempts were made to saturate binding sites with BHC prior to dialysis experiments.

However, solubility method can give information at high (D_f). The r values calculated from solubility data at 20°C are 6.8 for HSA-BHC and 20.2 for PVP-BHC interactions.* For PVP-BHC interaction, a further equilibrium dialysis experiment was carried out in which the PVP concentration was reduced from 0.4% to 0.1% to get additional data at a high (D_f) range. These results at 20°C and results from solubility data are shown in Figure 43. For HSA-BHC interaction, complete saturation of the binding sites was reached. The curve shows that about six or seven binding sites occur on the HSA molecule (see Eq. 54). The intrinsic association constant is approximately 3.5×10^4 L./mole (see Eq. 55). However these results are valid only if there is a single set of binding sites for BHC (for further discussion, see p.123). In case of PVP-BHC interaction, even the data obtained from solubility method failed to show complete saturation of binding sites. It was, therefore, impossible to interprete the binding curve quantitatively.

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* From Figure 29, it can be seen that up to 1% HSA and 0.4% PVP, the apparent solubility of BHC changes linearly with respect to macromolecule concentration. Therefore in this range of macromolecule concentrations, the r value (slope of a line) is a constant and independent of macromolecule concentration. The r values reported was obtained in this range of macromolecule concentrations. The fact also explains why data obtained from dialysis studies in which 0.1, 0.2, and 0.4% HSA were used, produced identical binding curves as shown in Figure 41.

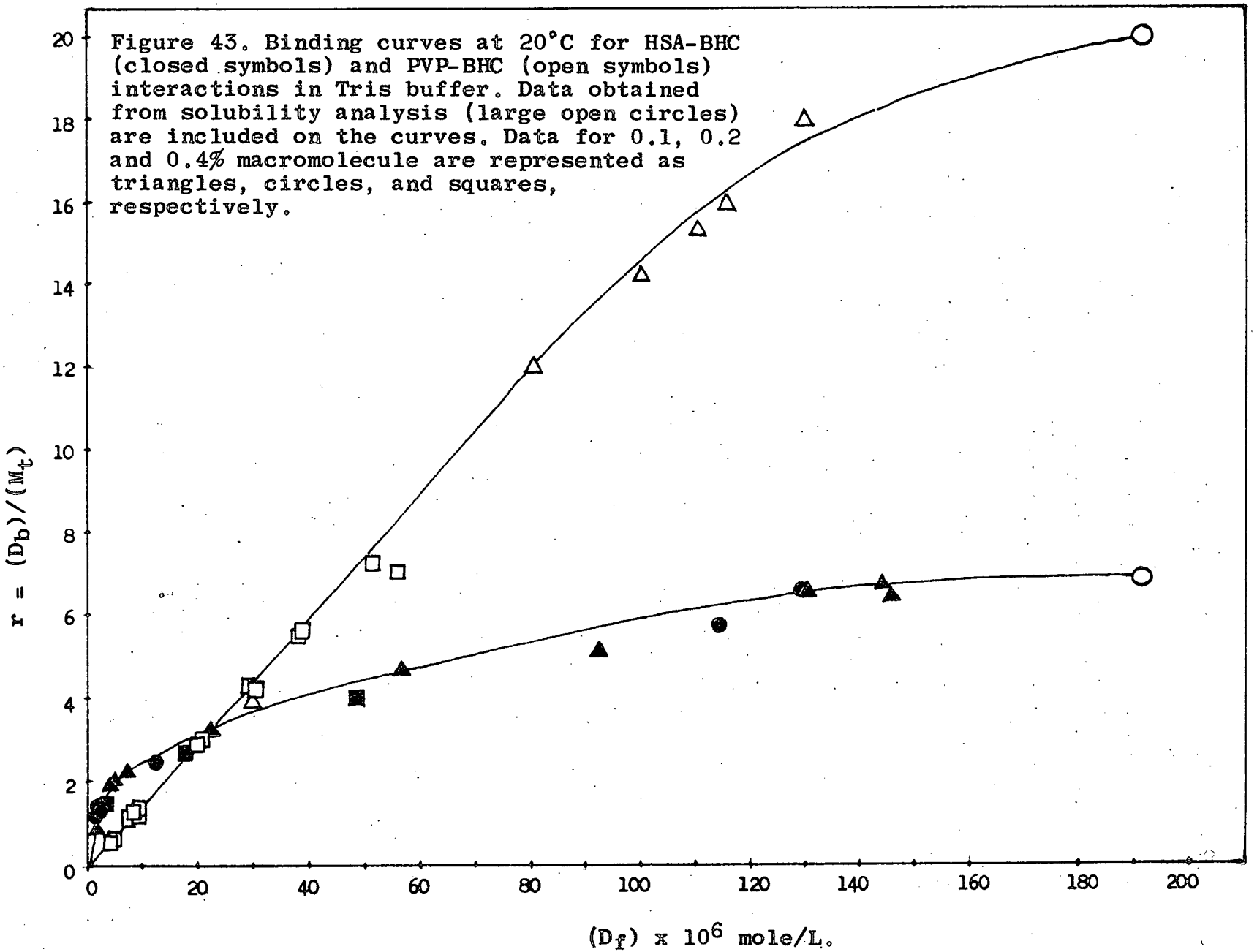

Figure 43. Binding curves at 20°C for HSA-BHC (closed symbols) and PVP-BHC (open symbols) interactions in Tris buffer. Data obtained from solubility analysis (large open circles) are included on the curves. Data for 0.1, 0.2 and 0.4% macromolecule are represented as triangles, circles, and squares, respectively.

(b) Scatchard Plot. The binding curves for HSA-BHC interaction illustrated in Figures 41 and 43 are replotted on the basis of Eq. 21 in Figure 44. At both 40 and 20°C, the binding data follows a curvilinear course that bends sharply near the abscissa. This indicates that more than one set of binding sites is present in the HSA molecule. The values of n, the average number of the first set of binding sites, 2.8 at 40°C and 3.0 at 20°C, was rounded off to the nearest whole number. This rounding-off procedure has been used by Scatchard et al. (1957). The intercepts of the curves on the ordinate, nK, are 51 x 10^4 and 105 x 10^4, which yield intrinsic association constants of 17 x 10^4 and 35 x 10^4 (L./mole) at 40 and 20°C, respectively. The association constant at 20°C is nearly twice that at 40°C. The second set of binding sites will not be considered in this discussion because the intrinsic affinity of the BHC molecule for these sites is far smaller than that for the first set.

Binding data for the PVP-BHC interaction at various temperatures plotted according to the Scatchard equation (Eq. 21) is illustrated in Figure 45. Although the data is scattered, differences in binding strength at various temperatures can be seen. Straight lines were drawn on the assumption that there are 50 identical binding sites on the PVP molecule irrespective of temperature (for further discussion, see p.126). The point marked with an arrow is the value obtained from the solubility analysis.

(c) Double Reciprocal Plot. Because of error inherent in the double reciprocal plot, it is preferable to use the Scatchard plot for quantitative analysis of binding whenever possible. However in the case of PVP-BHC interaction it was difficult to

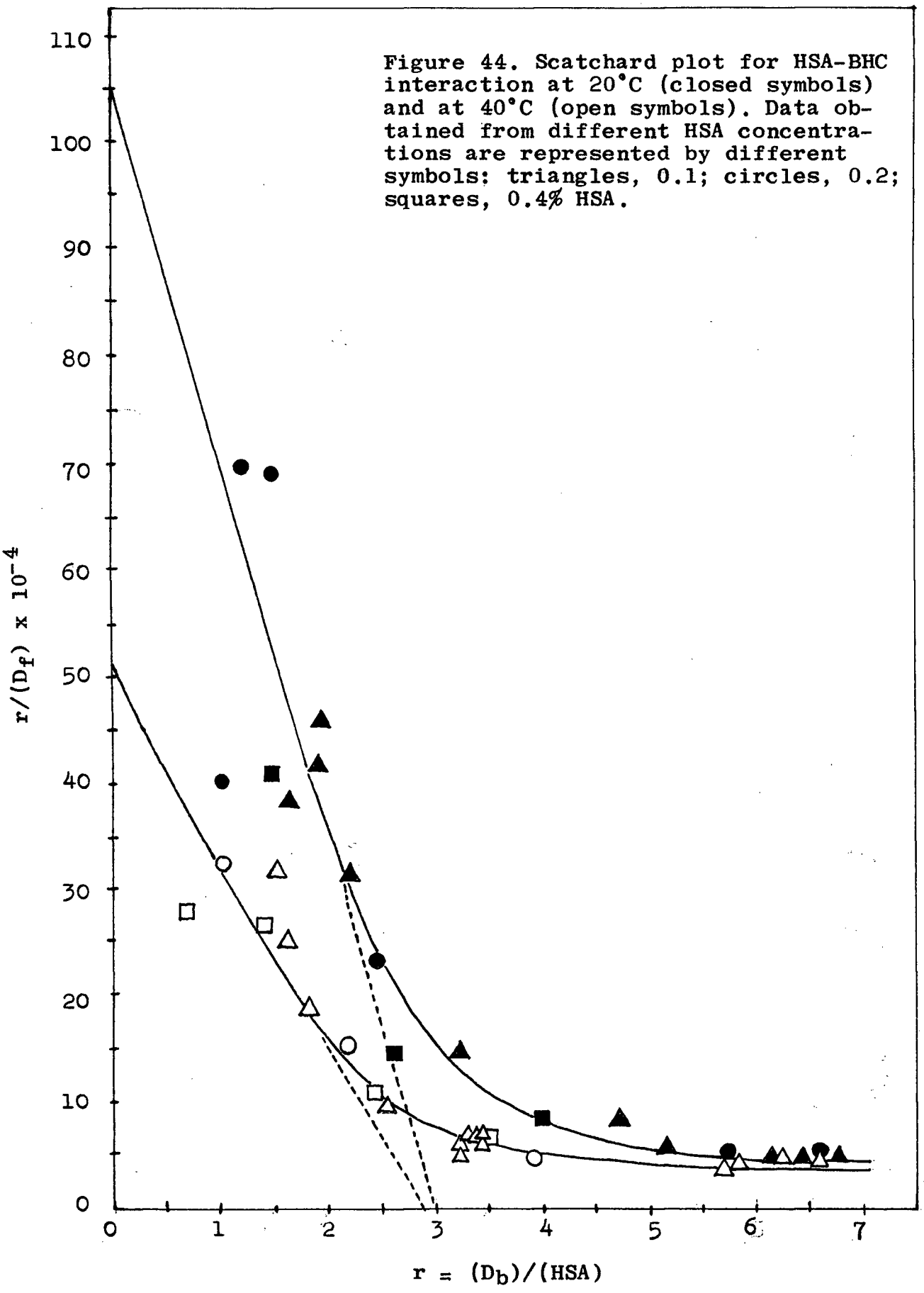

Figure 44. Scatchard plot for HSA-BHC interaction at 20°C (closed symbols) and at 40°C (open symbols). Data obtained from different HSA concentrations are represented by different symbols: triangles, 0.1; circles, 0.2; squares, 0.4% HSA.

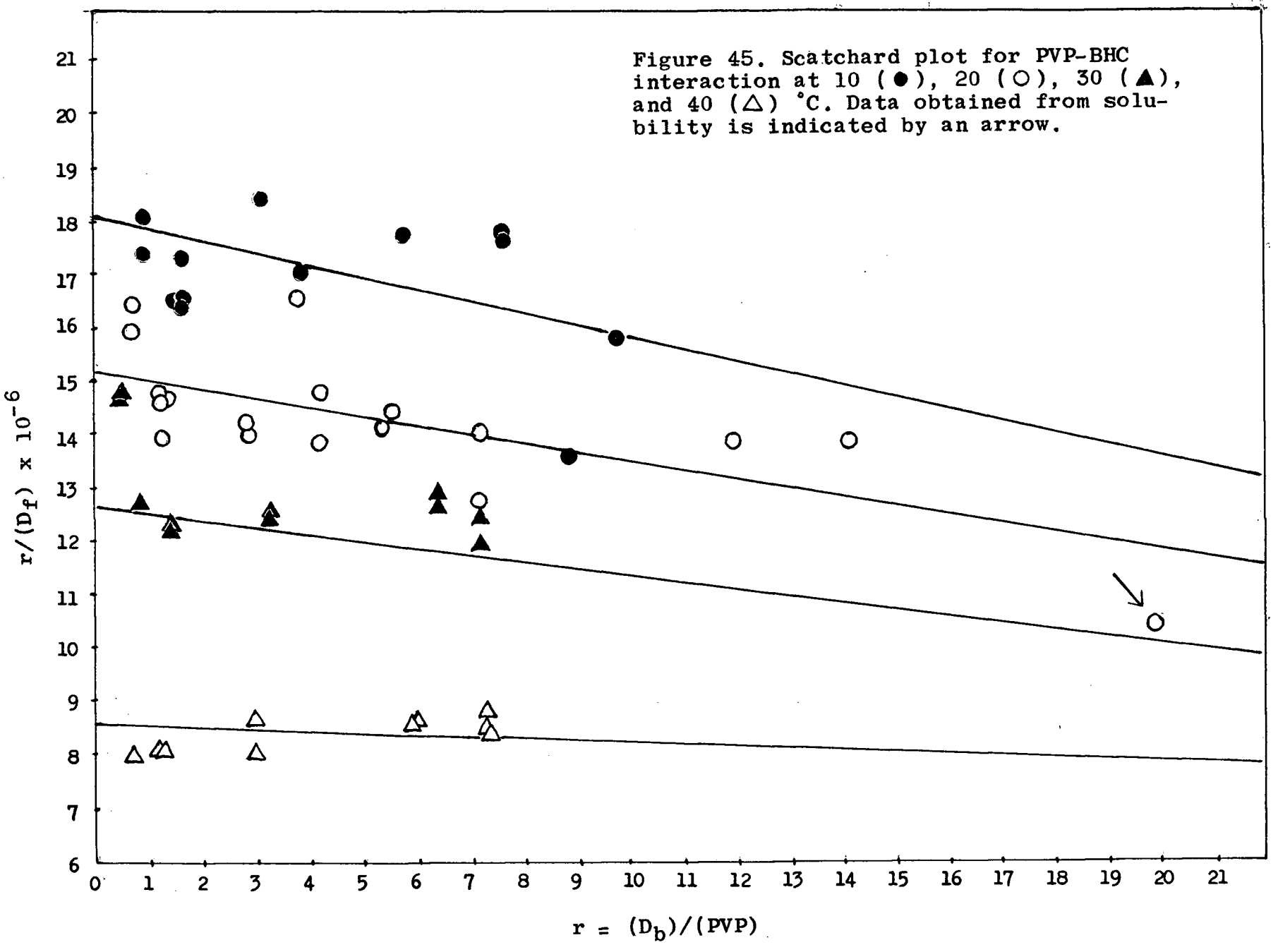

Figure 45. Scatchard plot for PVP-BHC interaction at 10 (●), 20 (○), 30 (▲), and 40 (△) °C. Data obtained from solubility is indicated by an arrow.

derive accurate binding parameters from the Scatchard plot. Hence a double reciprocal plot (see Eq. 20) was used, Figure 46. These curves pass through approximately the same point on the ordinate (i.e., 0.02). The reciprocal of the intercept (i.e., 50) was taken as the average number of binding sites on the PVP molecule and used in the extrapolation of the Scatchard plot (see Figure 45). Intrinsic association constant at various temperatures was calculated from the slopes of the curves.

8. Thermodynamic Analysis and Mechanism of Interaction

(a) HSA-BHC Interaction.

i) Enthalpy, Entropy, and Free Energy Changes. Assuming that there is no significant temperature dependence of enthalpy change within the temperature range in which the interaction was carried out, it is possible to estimate the standard enthalpy change (ΔH°) for the association of one mole of BHC to one mole of the binding sites of the first set on the HSA molecule. The calculation is shown below.

$$\log\left(\frac{K \text{ at } 20^\circ C}{K \text{ at } 40^\circ C}\right) = \frac{-\Delta H^\circ}{2.303R}\left(\frac{1}{293} - \frac{1}{313}\right) \qquad \text{(Eq. 56)}$$

Standard free energy (ΔG°) and entropy (ΔS°) changes were calculated using Eq. 35 and 36, and are reported in Table 8. Because of possible competitive role of the buffer ions during binding, the K values must be regarded as being dependent on buffer composition. With respect to the thermodynamic quantities, this implies that the standard state includes the Tris buffer

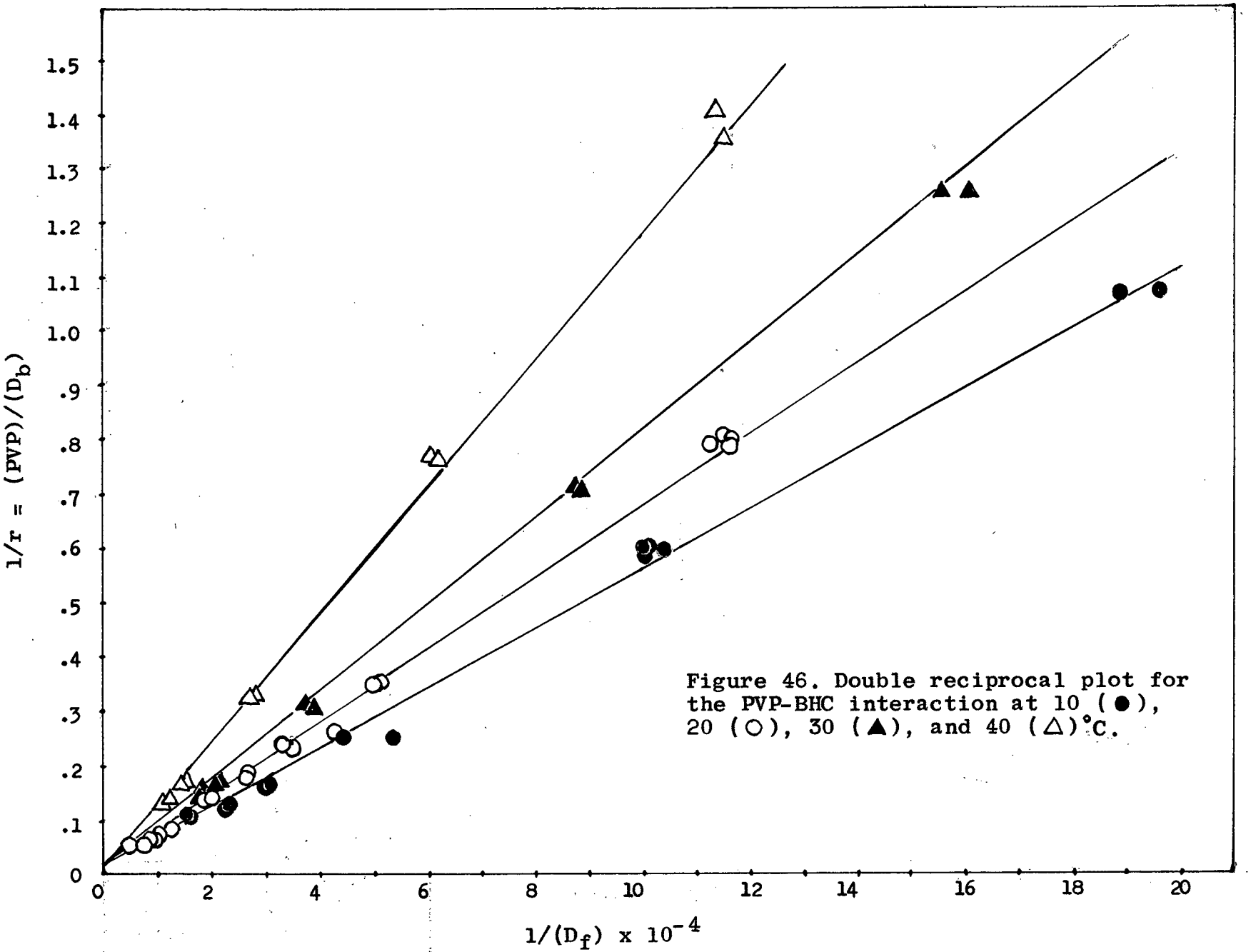

Figure 46. Double reciprocal plot for the PVP-BHC interaction at 10 (●), 20 (○), 30 (▲), and 40 (△)°C.

°C	K x 10^{-5} (L./mole)	ΔH° (Kcal/mole)	ΔG°	ΔS° (e.u.)
20	3.5	- 6.58	- 7.43	+2.90
40	1.7	- 6.58	- 7.49	+2.91

Table 8. Effect of Changes in Temperature on the Binding of BHC to HSA.

used in the investigation. This approach to calculation is that used by Karush (1950).

The decrease in binding strength of HSA for BHC with increasing temperature is characteristic of exothermic reaction. Similar decreases have been observed for many protein interactions with a wide variety of substances (see, for example, O'Reilly and Kowitz, 1967). The negative sign for ΔG° means that the binding process is spontaneous. The ΔS° value, the disorder factor of thermodynamic changes, is positive. This is in agreement with observations for most of albumin-anion interactions. However, the magnitude of ΔS° is small compared with that observed for the interaction between albumin and azo dye anions.

ii) Possibility of Ionic Interaction. At pH 7.4, the HSA molecule has a net negative charge of 18 (White and others, 1968). On the basis of the determined pKa values (see p. 84), 80 and 20% of total BHC exists as the monoionized and di-ionized species, respectively. This does not mean that the possibility of ionic interaction between these substances can be ruled out. A net negative charge on the protein molecule merely implies an excess of negative charges over positive residues. There is evidence in the literature to substantiate the view that cationic centers on the protein at a pH 7 are intimately involved in the binding

with anionic molecules (Klotz, 1949a).

Thermodynamic changes for the HSA-BHC interaction indicate an unusually large contribution to the $-\Delta G^{\circ}$ value by the $-\Delta H^{\circ}$ value. In general, anion-protein interactions have shown little temperature dependence (Klotz and others, 1949b). A small temperature coefficient is a characteristic of an interaction between oppositely charged species (Klotz and others, 1952). If the nature of the HSA-BHC interaction were largely electrostatic, i.e., if the ionic part of the BHC molecule combined with the cationic parts of the HSA molecule, the main source of the $-\Delta G^{\circ}$ value would be a large positive ΔS° value with little contribution from the ΔH° factor. It is very unlikely, therefore, that the HSA-BHC interaction is ionic in nature. O'Reilly et al. (1967;1969) proposed similar suggestions for the interaction between HSA and warfarin.

iii) Possibility of Pre-Existing Binding Site. In the absence of information about attendant conformational changes, most interpretations of binding processes make the assumption that pre-existing binding sites are involved (Lovrien, 1963). On the other hand, Karush (1950) postulated that serum albumin possesses 'configurational adaptability' for a variety of small molecules. It is clear from Karush's discussion that there is a strong possibility that some proteins might form binding sites during the binding process. Using this hypothesis, Karush (1956; 1957) satisfactorily interpreted the differences in thermodynamic parameters usually observed in non-specific anion-albumin and specific hapten-antibody interactions.

The positive ΔS° associated with many reaction involving proteins are usually attributed to disorientation and unfolding of the protein molecule. This does not appear to be a satisfactory explanation for the HSA-BHC interaction because the enthalpy changes observed are very negative whereas a process of unfolding presumably requires the breaking of several bonds and should, therefore, result in an endothermic reaction of appreciable magnitude (Klotz and others, 1949b). It is, therefore, preferable to postulate that the HSA molecule may have some kind of pre-existing site for the BHC molecule. This site may be more or less rigid as the binding sites of antibody for haptens. Thermodynamic data obtained for the HSA-BHC interaction is compared, in Table 9, with that obtained by Karush (1956) for antibody-anionic hapten binding.

°C	K x 10^{-5} (L./mole)	ΔH° (Kcal/mole)	ΔG°	ΔS° (e.u.)
7.1	4.4 - 6.7	-(7.1 - 7.3)	-(7.24 - 7.48)	+(0.3 - 0.7)
25	2.1 - 3.1	-(7.1 - 7.3)	-(7.25 - 7.50)	+(0.3 - 0.7)

Table 9. Thermodynamic data for the binding of D-phenyl-(p-azobenzoylamino)-acetate by purified antibody specific for the compound, in 0.02M phosphate buffer of pH 7.4 containing 0.15M NaCl (from Karush, 1956).

Free energy changes, for both interactions, are of the same magnitude. In the specific binding between antibody and haptens, the free energy change is due almost entirely to the enthalpy. The contribution of the entropy term is negligible.

iv) Nature of Binding Site and Effect of Binding on Water Structure. The BHC combining region on the HSA molecule probably consists of an inter-helical cavity whose van der Waals contour is closely complementary to, and therefore selective for the BHC molecule. In this respect, the interaction may be considered specific, and is explained by a large contribution of enthalpy to free energy changes and by a relatively sharp change in slope of the binding curves near the abscissa (see Figure 44). The change in slope is similar at both 20 and 40°C which implies that there is no significant change in the average number of binding sites at the two temperatures. Presumably the cavity is not completely rigid, as in case of an antibody for haptens, and cannot resist the disruptive tendencies of intra-molecular electrostatic repulsions to which the protein is subjected at extreme values of pH, e.g., a pH value of 3. Therefore, as Nagashima and others (1968a) have observed, the HSA-BHC interaction would be much less at pH ranges where N to F conversion of the protein takes place (see p. 42). The cavity can, therefore, be easily disrupted by expansion of the albumin molecule.

The HSA-BHC binding involves a transfer of a hydrophobic molecule from an aqueous environment to a region with a lower dielectric constant. After binding occurs, a hypothetical hole, previously occupied by the BHC molecule, remains and this will then be filled with an equal volume of hydrogen-bonded water molecules (Karush, 1956). The number of hydrogen bonds formed in this way will exceed the number of hydrogen bonds which

the free BHC molecule previously formed with its neighboring water molecule.* On the basis of this explanation, complex formation would be exothermic and should be associated with a substantial decrease in the enthalpy of the system, which will exceed the endothermic nature of the heat of fusion of icebergs around the BHC and HSA molecules.

The melting of iceberg-structured water around BHC and HSA molecules will result in an increase in randomness (i.e., positive ΔS°). The BHC molecule will lose rotational and transitional degrees of freedom after binding to yield a higher ordering (i.e., negative ΔS°). Formation of a bonded-water cluster at the hypothetical hole left by the BHC molecule will give an ordering effect (Nemethy and Scheraga, 1962a). The two disordering effects mentioned first probably exceed the last two ordering effects and the result is a net entropy change of $\pm$ 2.9. In more specific interactions (such as antibody-hapten interactions), the loss of degrees of freedom would be highly significant and could result in a net negative change (Karush, 1957).

(b) PVP-BHC Interaction.

i) Enthalpy, Entropy, and Free Energy Changes. Intrinsic association constants are shown in Figure 47, a van't Hoff type plot, as a function of temperature. The enthalpy change appears to be temperature dependent. A similar temperature dependence has been observed in other studies (see, for example, Hymes and others, 1969). From the slope of a tangent line at a given temperature the standard enthalpy change was estimated. Thermodynamic parameters were calculated by utilizing Eq. 35 and 36.

* From low aqueous solubility of BHC, it was assumed that BHC molecule has little hydrogen bonds with neighboring water molecules (see p. 85).

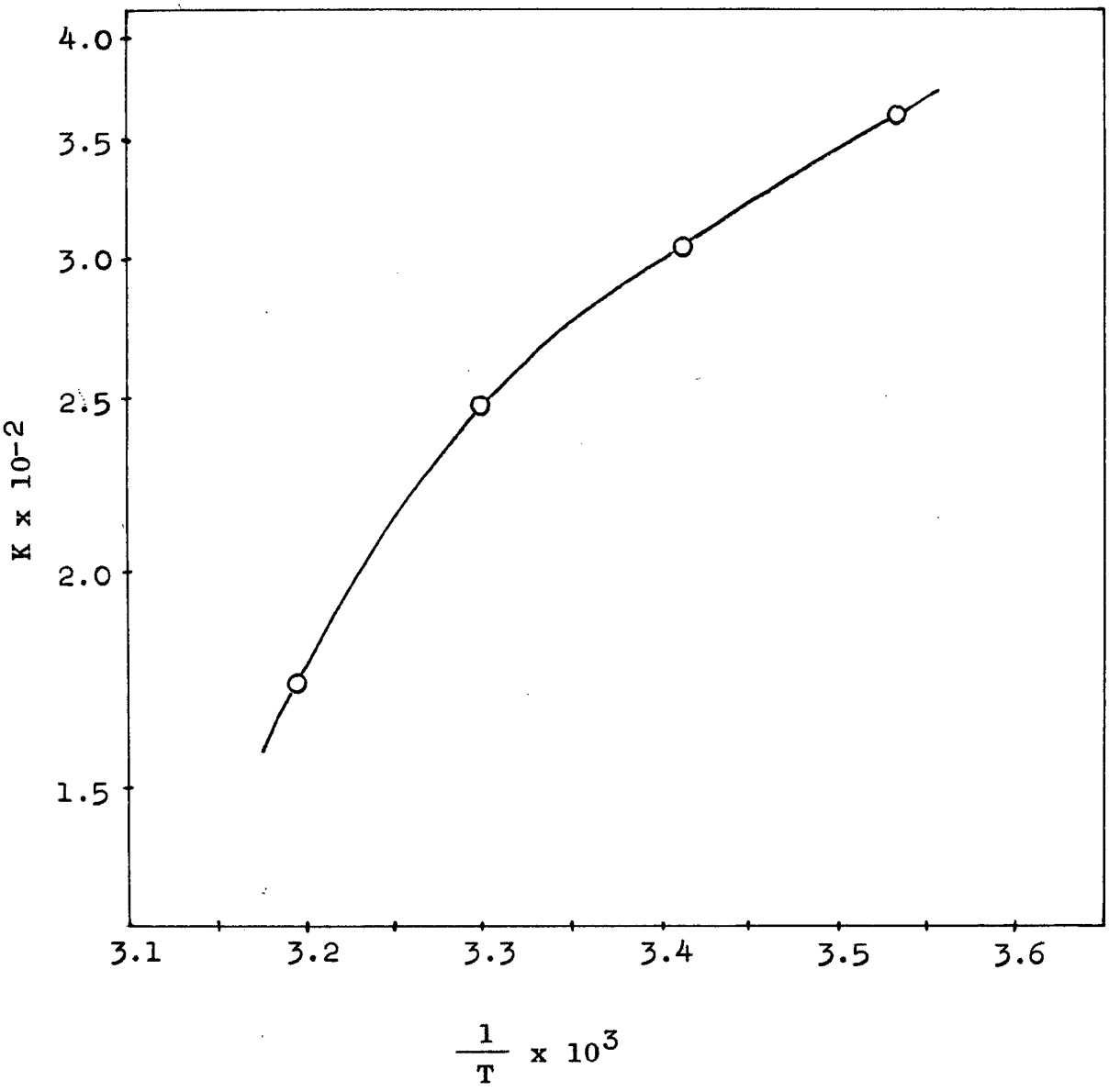

Figure 47. Van't Hoff plot (log K versus 1/T) for the PVP-BHC interaction.

Results are listed in Table 10. If the concentration used in calculating the association constant are expressed in moles per liter, ΔS° is the entropy change when one mole of BHC and one mole of a binding site on a PVP molecule, each at a concentration of one mole per liter, react to give one mole of the complex, again at concentration of one mole per liter. If different concentration units are used, ΔS° will have a different value. It is desirable to eliminate this rather arbitrary factor before trying to interpret the magnitude of ΔS° in terms of the molecular structures present in the solution. If we assume that the ΔS° values listed in Table 10 are determined from measurements at sufficiently high dilution, the so-called 'unitary (or contact)' entropy change (ΔSu) for the reaction can be calculated from Eq. 57 (Gurney, 1953; Kauzmann, 1959).

$$\Delta Su = \Delta S^\circ + 7.98 \qquad \text{(Eq. 57)}$$

The unitary entropy change depends, therefore, only on those factors which involve the interaction of the BHC molecule and the binding site on a PVP molecule with the solvent and with each other and not on the contribution due to randomness of the mixing with the solvent. The 'cratic' term (7.98 e.u.) originates from the expression, - R ln (1/55.6), where 55.6 (mole/L.) is the concentration of water in a highly diluted aqueous solution. This term takes into account the reduction in number of independent solute species by one on the combination of a BHC molecule with a PVP molecule (Molyneux and Frank, 1961a). In order to compare this data with that for the inter-

actions between PVP and a variety of organic substances (Molyneux and Frank, 1961a), the unitary values for PVP-BHC interaction are also listed in Table 10.

°C	K x 10^{-2} (L./mole)	ΔH° (Kcal/mole)	ΔG°	ΔS° (e.u.)	ΔSu
10	3.64	-2.74	-3.32	+2.05	+10.03
20	3.05	-2.96	-3.33	+1.28	+ 9.26
30	2.48	-4.69	-3.32	-4.53	+ 3.45
40	1.72	-8.57	-3.20	-17.14	- 9.16

Table 10. Thermodynamic functions for the binding of one mole of BHC by one mole of vacant binding site on PVP.

ii) Analysis of Enthalpy of Binding. Emphasizing the 'iceberg' concept of water structure (Frank and Evans, 1945) around the BHC and PVP molecules, it is possible to divide the binding enthalpy into the following five contributions (Molyneux and Frank, 1961a). The corresponding binding processes are schematically illustrated in Figure 48. (1) Heat is needed to break (or bend) hydrogen bonds in the icebergs neighboring the polymer and BHC molecules. (2) Enthalpy will also have to be provided to overcome any specific interactions (e.g., true hydration) between the water and PVP and between the water and BHC. (3) The actual binding process between the 'dehydrated' entities will be exothermic. (4) The interaction between the complex formed and the neighboring water (i.e., reformation of true hydration) will be exothermic. (5) Enthalpy will, finally, also be gained by the reforming of hydrogen bonds in the icebergs associated with the complex.

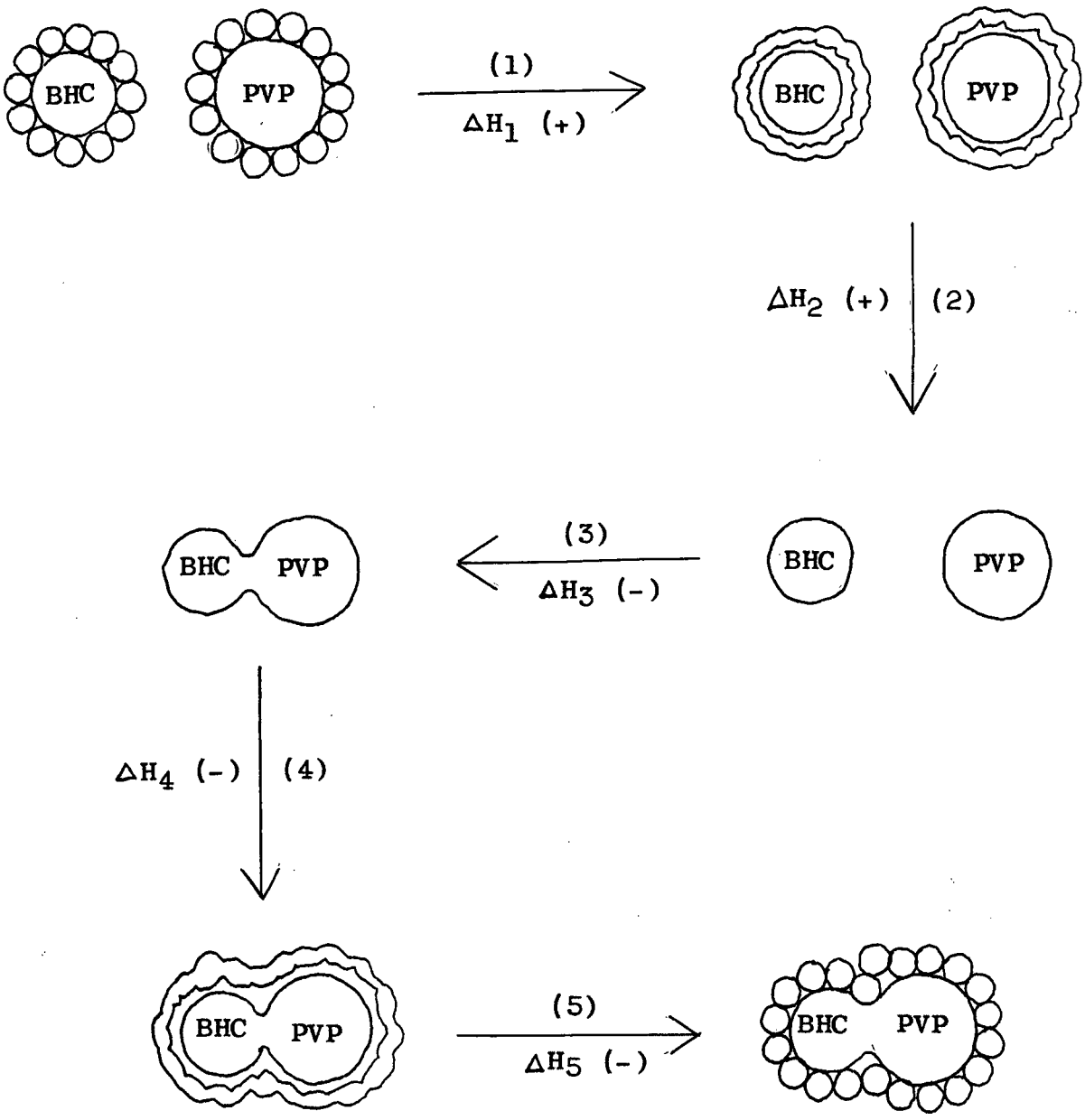

Figure 48. Schematic illustration of the binding processes between BHC and PVP molecules. Small circles represent a hydrogen bonded water cluster indicating the formation of a partial cage around a solute.

The net enthalpy change associated with the purely hypothetical binding processes illustrated in steps two to four in Figure 48 was reported to be essentially a constant (-5 Kcal/mole) for interactions of PVP with a variety of substances (Molyneux and Frank, 1961a). If this value is applied to the data herein, the net enthalpy changes associated with the first and fifth steps would be approximately +2.3, +2.0, +0.3, and -3.6 Kcal/mole at 10, 20, 30, and 40°C, respectively. Total changes are shown in Table 10. As temperature increases, the enthalpy change decreases to a negative value at 40°C.

The mole fractions of various water species in the first layer around an aromatic hydrocarbon were estimated as a function of temperature by Nemethy and Scheraga (1962b) on the basis of their theory of water structure (Nemethy and Scheraga, 1962a). For example, the fraction of unbroken hydrogen bonds changes from 59.3% at 10°C to 49.0% at 40°C. On this basis, the decreasing tendency of the enthalpy changes, associated with the first and fifth binding processes, with increasing temperature can be easily explained. At a given temperature, the absolute value of the enthalpy change associated with the breaking hydrogen bonds in the icebergs around the solutes, ΔH_1, will exceed that for the formation of hydrogen bonds in the icebergs around the complex, ΔH_5, since the number of hydrogen bonds around the solutes before binding would exceed the number of hydrogen bonds around the complex. However, the difference becomes less important as temperature increases. The value approaches zero and finally becomes negative at 40°C.

iii) Possibility of Hydrophobic Bonding. The so-called 'iceberg' concept (Frank and Evans, 1945) assumes that hydrocarbon groups, such as those present both in the polymer and in the BHC molecules, are surrounded in aqueous solution with one or more layers of water molecules which are more highly ordered than the molecules in ordinary liquid water. Entropy gains in protein (Klotz, 1958; Kauzmann, 1959; Nemethy and Scheraga, 1962c; Nemethy and others, 1963; Cecil, 1967; Hymes and others, 1969) and PVP (Molyneux and Frank, 1961a; Bahal and Kostenbauder, 1964; Eide and Speiser, 1967a) interactions with a variety of small molecules or ions have been attributed to increased disorderness of the iceberg structure due to complex formation.

The positive unitary entropy change observed over the room temperature range (see Table 10) can be explained in a manner similar to that above. It is postulated, therefore, that the PVP-BHC complex is accompanied by either a less ordered iceberg or by an iceberg containing a smaller number of water molecules as compared with the iceberg of the two separate entities. The release of water molecules from the ordered structure should produce a proportional gain in entropy. The free energy change associated with PVP-BHC interaction shows no significant temperature dependence. The contribution to the free energy of entropy term becomes more important as temperature increases from 17.5% at 10°C to 38.5% at 40°C. A similar trend has been observed for the formation of a typical hydrophobic bond between leucine and isoleucine (Nemethy and Scheraga, 1962c) although, in the case, the magnitude of its contribution is approximately

eighty per cent. At a high temperature (e.g., 40°C), hydrophobic bonding seems to play a lesser role in the PVP-BHC interaction. The exothermic reaction here is enhanced by temperature whereas the formation of hydrophobic bonds is essentially endothermic in nature (Nemethy and others, 1963). This inference is also supported by the decrease in the favorable entropy change with increase in temperature.

iv) Nature of the Intermolecular Forces and of the Binding Site. The PVP molecule has no ionizable groups (May and others, 1954) and it is, therefore, unlikely that electrostatic interactions would play an important role in its binding with BHC anion. The high temperature dependence of binding strength also rules out the possibility of significant electrostatic interactions (see p. 129). However, the lactam bond in the pyrrolidone ring represents a dipole, which is likely to undergo ion-dipole interaction with BHC anion. It will aid the binding of the anion in such a way as to supply the necessary attraction force to bring the two components into close contact (Frank and others, 1957). After the close contact of the two entities by ion-dipole force, which varies with the inverse fifth power of the distance, van der Waals forces* will stabilize the complex.

The photograph in Figure 49 shows a molecular model of a PVP chain segment with eight repeating units and is similar to that proposed by Frank et al. (1957). To avoid unnecessary complications, all hydrogen atoms are omitted. Pyrrolidone

* The term 'van der Waals forces' is often used without explanation. In this context, the dipolar forces of Keesom (dipole-dipole), Debye (dipole-induced dipole), and London (induced dipole-induced dipole) are called van der Waals forces. The forces vary inversely with the seventh power of the distance (Martin, 1968a).

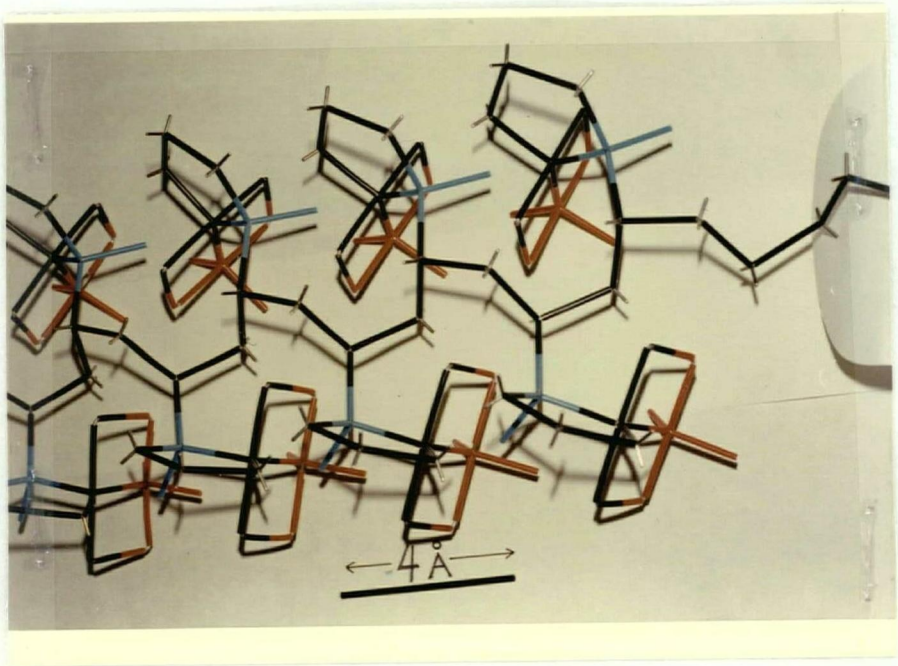

Figure 49. Molecular model of PVP chain segment with eight monomer units. Black, blue, and red colours represent carbon, nitrogen, and oxygen atoms, respectively.

rings appear to make a channel-type of cavity in both sides of the paraffin backbone which is apparently accessable for complexation with BHC. A PVP molecule of molecular weight, 40,000, has approximately 360 monomer units, since the molecular weight of the latter is about 111. The binding data shown in Figure 46 indicates an average number of binding sites on a PVP molecule of approximately 50, irrespective of temperature. This implies that on the average, 7.2 repeating units provide a binding site for BHC molecule. As shown in Figure 50, a BHC molecule fits quite well on approximately eight pyrrolidone rings.

As pointed out earlier (p. 85), a monoionized BHC molecule is expected to have less rotational degrees of freedom around the methylene bridge than a fully ionized molecule. A model

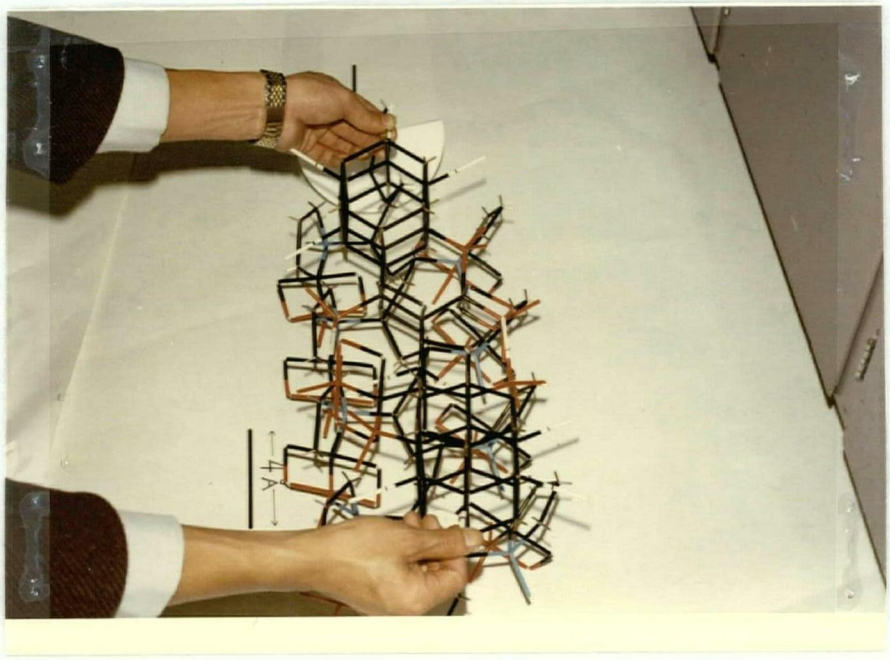

Figure 50. Proposed Configuration of PVP-BHC complex.

of the PVP-BHC complex indicated that a better fit of monoionized BHC molecule may require a slight bending, or folding, of the PVP molecule toward the BHC anion. However, no such requirement is necessary in case of binding of the di-ionized BHC species, since the high degrees of freedom would allow the anion to have a suitable form for fitting to PVP. The possibility of binding of another BHC anion on the other side of the PVP molecule (below the plane of the paper in Figure 50) can be ruled out by an expected repulsive forces between BHC anion bound to the polymer and an oncoming BHC anion.

9. Viscometry

Any changes in hydrodynamic volume as a result of configurational changes in the macromolecule during the binding process should alter the reduced viscosity. The reduced viscosity was calculated using Eq. 43 to 45 in the presence and absence of BHC. The BHC concentration was 185 mg./L. (550 x 10^{-6} mole/L.). Results of the density measurements for PVP solution necessary for the calculation of reduced viscosity are shown in Figure 51.

Table 11 shows the calculation procedure for specific and reduced viscosities from measurements of flow time and density. In Figure 52, the reduced viscosity was plotted against PVP concentration. Slopes and intercepts were calculated using the method of least squares. Table 12 shows slope and intercept (i.e., intrinsic viscosity) values for each line. The correlation coefficient, Rc, was calculated between the two variables, reduced viscosity and PVP concentration. From the slope, the Huggins constant, K_H, was also estimated (Eq. 47).

%PVP	Ax10^5	ρ (Gm./ml.)	t (sec.)	η	η_{sp}	η_{sp}/C
4.0	2.72	1.0184	1274.6	.03531	1.5312	38.28
3.0	2.71	1.0161	1034.7	.02849	1.0423	34.74
2.0	2.64	1.0138	847.9	.02269	.6265	31.33
1.5	2.48	1.0127	804.6	.02021	.4487	29.91
1.0	2.47	1.0115	716.3	.01790	.2832	28.32
0.4	2.46	1.0102	620.8	.01543	.1061	26.53
0	2.42	1.0092	571.3	.01395	0	-

Table 11(a). Calculation procedure for estimating specific and reduced viscosities of PVP solution at 10°C in the absence of BHC. See pp. 33-37 for definition of terms used.

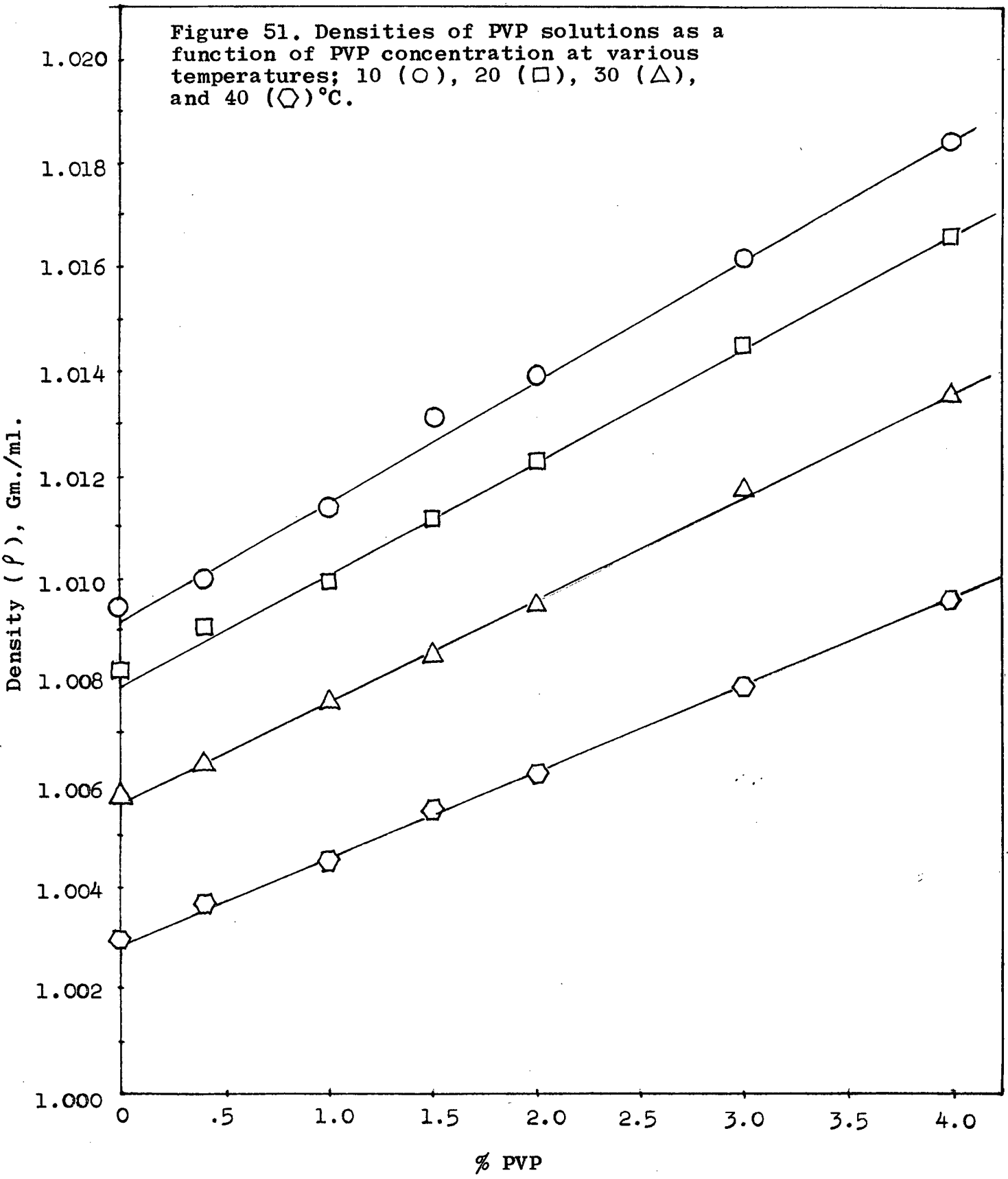

Figure 51. Densities of PVP solutions as a function of PVP concentration at various temperatures; 10 (○), 20 (□), 30 (△), and 40 (⬡)°C.

%PVP	$Ax10^5$	ρ (Gm./ml.)	t (sec.)	η	η_{sp}	η_{sp}/C
4.0	2.72	1.0184	1260.9	.03493	1.5220	38.05
2.0	2.71	1.0183	820.9	.02255	.6282	31.41
1.5	2.64	1.0127	747.9	.02000	.4440	29.60
1.0	2.48	1.0115	705.5	.01770	.2780	27.80
0.4	2.47	1.0102	611.3	.01525	.1011	25.28
0	2.42	1.0092	566.9	.01385	0	-

Table 11(b). Calculation procedure for estimating specific and reduced viscosities of PVP solution at 10°C in the presence of 550 umole/L. BHC. See pp. 33-37 for definition of terms used.

°C	In the Absence of BHC				In the Presence of BHC (550 umole/L.)				V
	Slope	K_H	$[\eta]$	Rc	Slope	K_H	$[\eta]$	Rc	
10	339.0	.55	24.81	1.048	343.5	.58	24.32	.980	.98
20	305.1	.56	23.35	1.004	330.3	.65	22.54	1.001	.97
30	271.2	.54	22.38	1.003	303.8	.67	21.28	.995	.95
40	248.6	.55	21.22	.991	290.6	.73	20.02	1.015	.94

Table 12. Influence of BHC binding on the rheological properties of PVP at various temperatures. Intrinsic viscosity ($[\eta]$) units are ml./Gm. Rc is the correlation coefficient. V is the intrinsic viscosity ratio (Eq. 58). See pp. 33-37 for definition of other terms.

In the absence of BHC, the intrinsic viscosity of PVP decreases with increase in temperature (Table 12). The same temperature effect was reported by Goldfarb and Rodriguez (1968). This finding may be interpreted in terms of a progressive coiling of the polymer with an increase in temperature.* It may be postulated that the van der Waals contour of the binding site is changed by the configurational change in an unfavorable way for BHC binding which results in lower binding affinity at higher temperature.

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* Miller and Hamm (1953) found that the diffusion coefficient of PVP is higher at 5°C than at 21.4°C. From this they postulated that the PVP molecule is more tightly coiled at the lower temperature. In this respect, our data and that obtained by Goldfarb et al. (1968) contradict that of Miller et al. The Fikentscher's K_F value was 30 in each case.

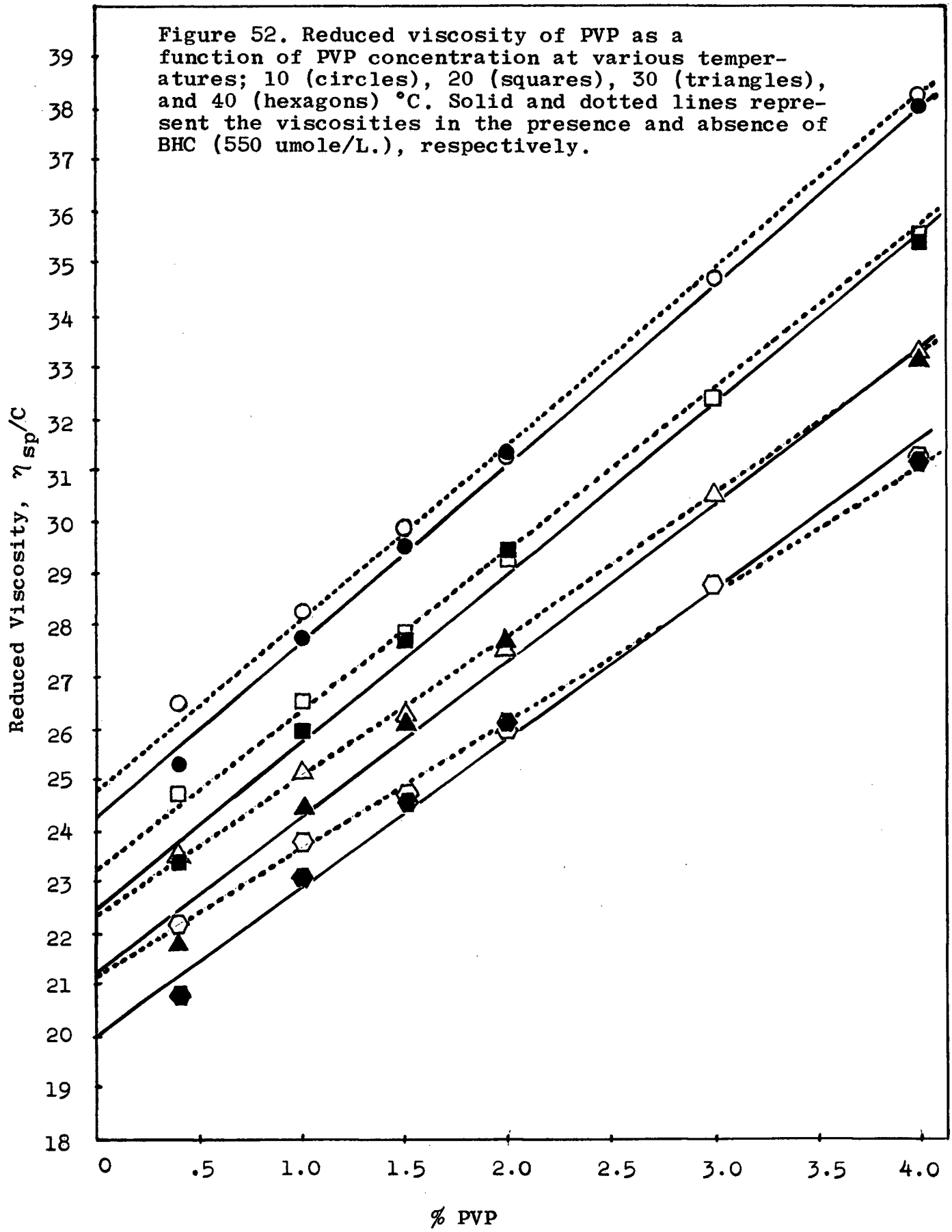

Figure 52. Reduced viscosity of PVP as a function of PVP concentration at various temperatures; 10 (circles), 20 (squares), 30 (triangles), and 40 (hexagons) °C. Solid and dotted lines represent the viscosities in the presence and absence of BHC (550 umole/L.), respectively.

At all temperatures studied, binding of BHC is accompanied by a slight but significant decrease in intrinsic viscosity. The shrinking of the polymer coil, indicated by the reduction in intrinsic viscosity, supports the postulate previously made (see p. 141). Molyneux and Frank (1961b) employed the 'intrinsic viscosity ratio' (V), defined in Eq.58, as a direct indication of the effect of cosolute on the size of the polymer molecule in solution.

$$V = \frac{[\eta] \text{ in the Presence of Cosolute}}{[\eta] \text{ in the Absence of Cosolute}} \qquad \text{(Eq. 58)}$$

The ratio for BHC-PVP binding at 30°C (see Table 12) is in the same order as that for the interactions of sodium hydrogen phthalate and sodium-p-hydroxy benzoate with PVP studied by Molyneux and Frank (1961b). In solutions of high ionic strength, expansion due to the coulombic repulsion between the BHC anions bound to the polymer coil is believed to be greatly reduced by the 'screening' effect of the free counterions, Tris of RH_3^+ form, within the Tris buffer solution encompassed by the polymer molecule. In other words, van der Waals forces stabilizing the PVP-BHC complex probably exceed repulsive forces and hence a slight degree of coiling can be maintained. Molyneux and Frank (1961b) also observed shrinking of the polymer coil in the presence of nonionic cosolutes such as phthalic acid and benzoic acid. The bulky nonionic part of BHC molecule may also have such an effect and cooperate to the shrinking of PVP.

Changes in intrinsic viscosity caused by the cosolute are generally not a linear function of the concentration of the latter (Eliassaf and others, 1960). In the present study, measurements were made at only one BHC concentration, a concentration which is near the solubility limit. Therefore, the changes measured only serve to ascertain the existence of an effect at that concentration and the general nature of this effect.

As shown in the last column in Table 12, the extent of coiling of PVP due to complexation with BHC becomes greater as the temperature increases. The variation of intrinsic viscosity with temperature is more clearly demonstrated in Figure 53, in which Eq. 59 analogous to the Arrhenius equation of kinetics (Martin, 1968c) is used to illustrate the data.

$$\eta = A\, e^{Ev/RT} \qquad \text{(Eq. 59)}$$

A is a constant and depends on the solution being studied. Ev is the 'activation energy' required to initiate flow of the solution. As shown in Figure 53, the data obtained fits the equation well. From the slope, the activation energy was calculated to be 0.92 and 1.13 Kcal/mole in the absence and presence of BHC, respectively.

The Huggins constant is some function of the solute-solvent interaction (Huggins, 1942; Yang, 1961). As the temperature increases, the parameter for the polymer in the presence of BHC anion shows a gradual but significant deviation from the average value of 0.55 for the polymer in the absence of BHC. No attempt has been made to interpret these findings, since the deviations are, in general too irregular for them to be correlated with any definite molecular effect (Molyneux and Frank, 1961b).

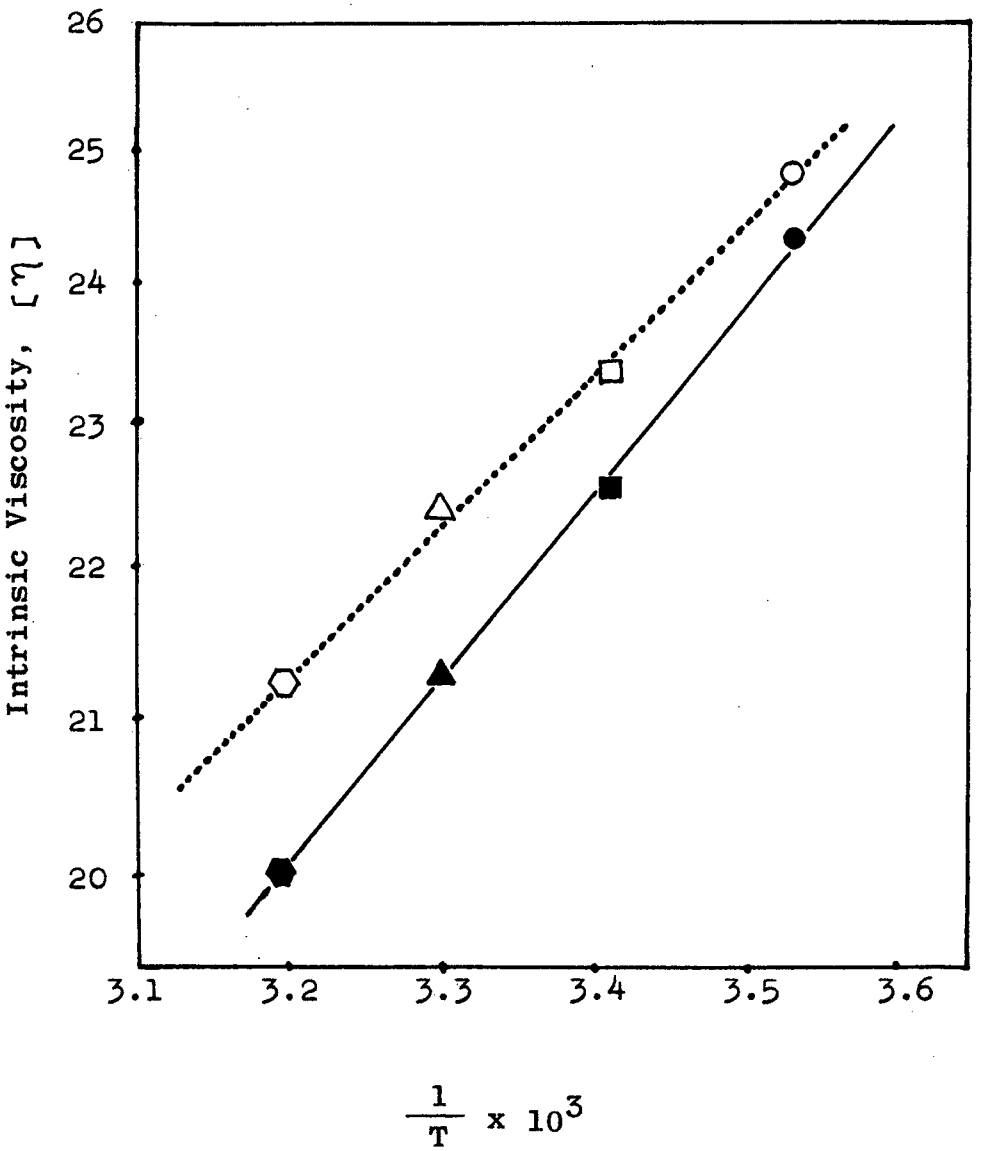

Figure 53. Effect of temperature on the intrinsic viscosities of PVP in the presence and absence of BHC. Symbols have same meanings as those in Figure 52.

10. Comparison of Methods Used to Evaluate Binding

In the present work, five different methods have been used to investigate the mechanism of binding of BHC to various macromolecules; spectrophotometric, dynamic and equilibrium dialyses, solubility, and viscometric methods. The first four methods are based on the analysis of changes in properties of BHC due to complex formation. Viscometry measures changes in properties occurring in the macromolecule.

The data from the spectrophotometric method is, in general, of doubtful value. The main difficulty is estimation of absorptivity value of bound drug (Klotz, 1953a). As shown in Table 3, the per cent depression of BHC absorbance is reproducible in the presence of excess amount of macromolecules. However, it can be expected that bound BHC will have different molar absorptivity values if binding sites on a macromolecule shows heterogeneity (e.g., HSA) because the actual binding environment will be different from one set of sites to another set of sites.

Figure 54 compares the spectrophotometric data with that obtained from the equilibrium dialysis method for the HSA-BHC interaction. All points are from the absorbance depression measured in 0.04% HSA (see Figure 34 for data and Table 4 for calculation procedure). Different values are assigned for molar absorptivity of bound drug. It is surprising to note that the largest deviations occur when the experimentally determined absorptivity value (closed circles in Figure 54) is used.

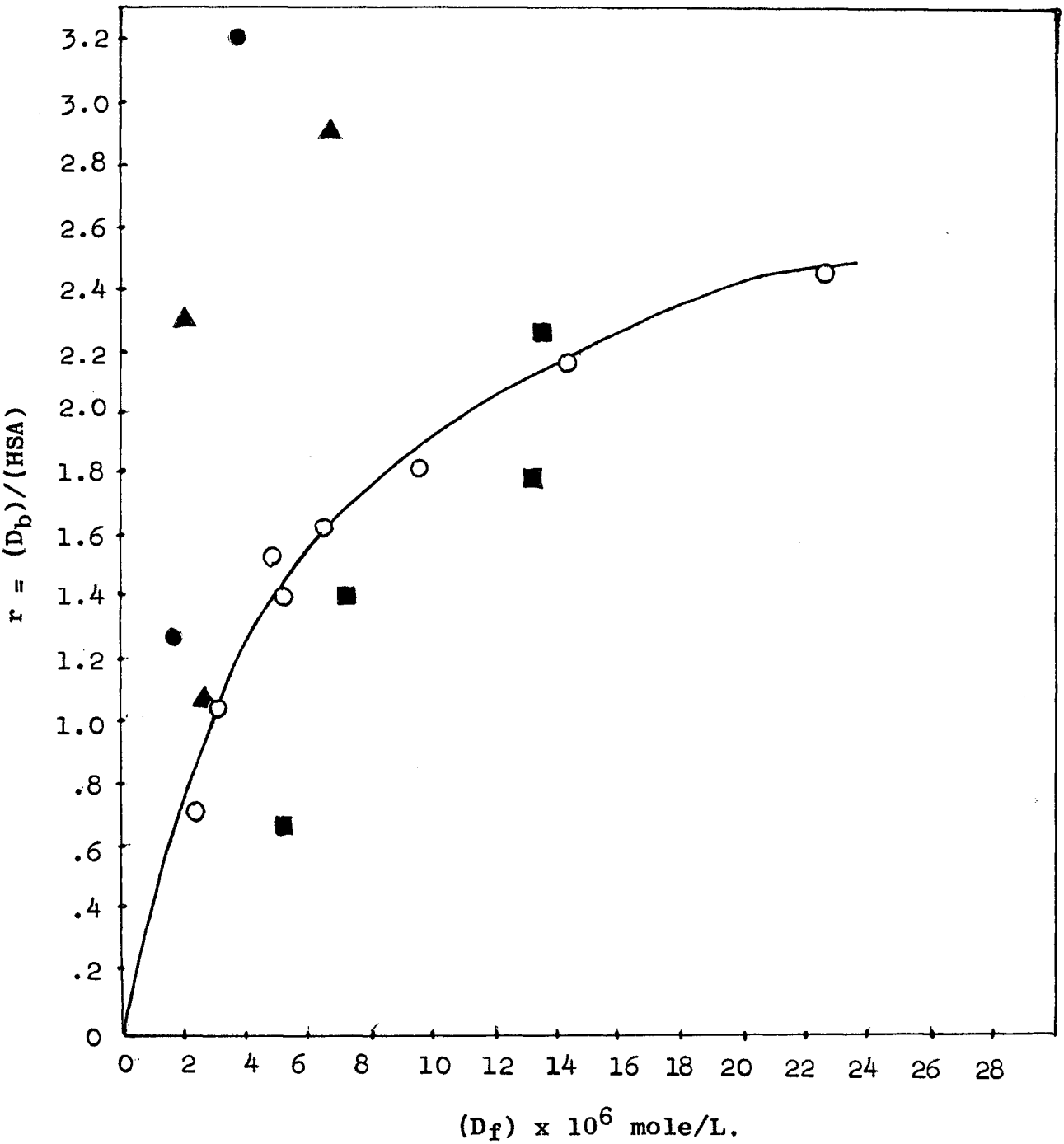

Figure 54. Comparison of binding data obtained from spectrophotometric analysis (closed symbols) with those from the equilibrium dialysis method (open circles) for the HSA-BHC interaction at 20°C. Various values were given to α in Eq. 38; 0.899 (●, experimentally determined), 0.880 (▲, arbitrarily chosen), and 0.805 (■, value for PVP-BHC interaction). See Table 3 for more detail.

Figure 55 shows the comparison for the PVP-BHC interaction. At extremely low concentration range of free drug, data from both methods agree. The agreement may be due to the fact that the PVP molecule has only one set of binding sites for BHC. However, the precision of the spectrophotometric method falls off rapidly at higher concentrations of free drug and the data cannot be used by itself. Klotz (1946c) arrived at a similar conclusion.

The dynamic dialysis method is based on the fact that non-diffusible macromolecule-drug complexes are 'reversibly' formed in the macromolecule compartment and that the rate of loss of drug molecule from that compartment is directly proportional to the free drug concentration, provided that care is taken to ensure that 'sink' conditions are maintained for the diffusing species. The main advantage of this method is that it is less tedious and time-consuming and requires fewer individual measurements to define binding behaviour. However, in order to prevent back diffusion of drug molecule into the macromolecule compartment, large amounts of buffer are required. In addition, it is difficult to obtain the experimental conditions for an unkown binding system over a wide range of free drug concentration. Numerical comparison of the data from this method under a specified condition (see p. 63) has been made already (see p. 110).

Equilibrium dialysis is one of most suitable methods for binding studies. The volume ratio of the two compartments must be appropriately assigned in order to cover a wide range of free drug concentration (or to improve precision of analysis). A dialysis cell which has a fixed volume (e.g., plexiglas block used by Patel and Foss, 1964) is not completely satisfactory for the study of very insoluble compounds.

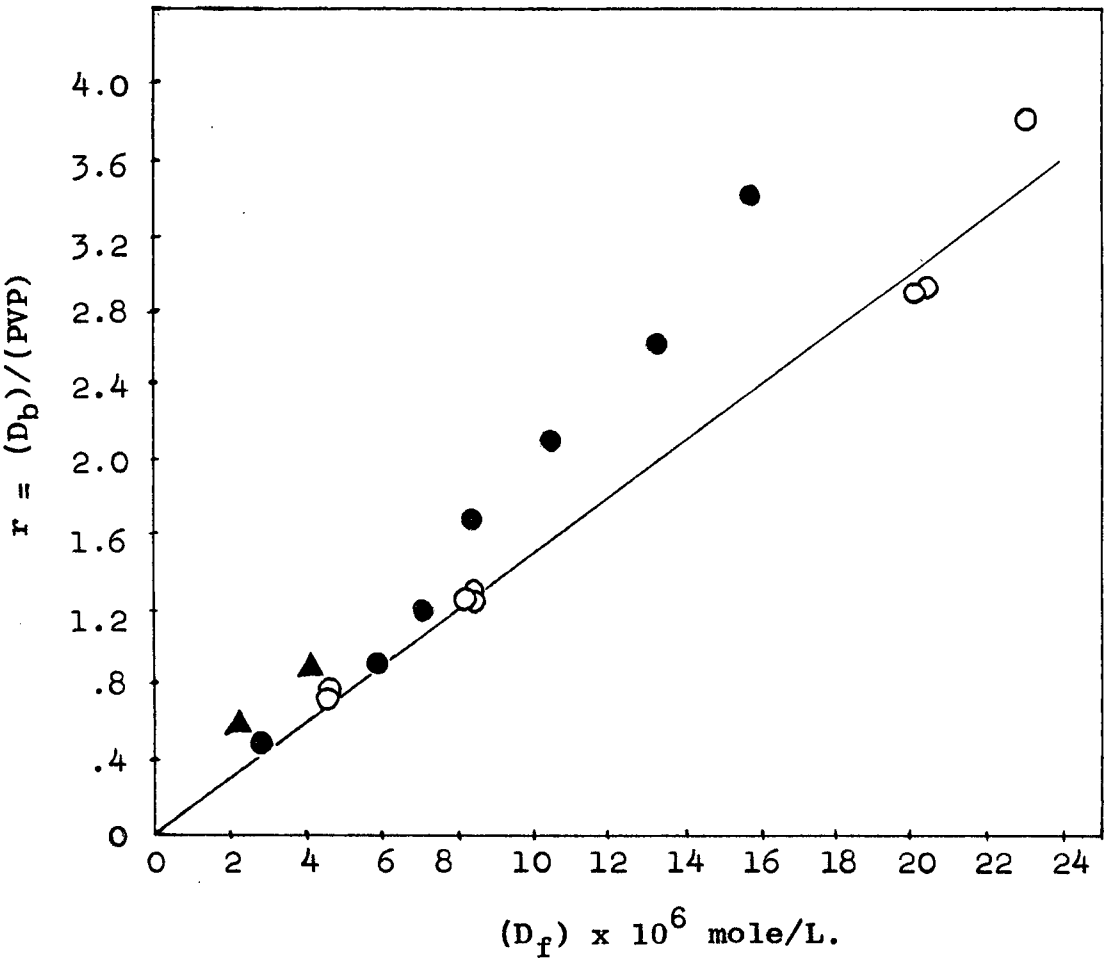

Figure 55. Comparison of binding data obtained from spectrophotometric analysis (closed symbols) with those obtained from the equilibrium dialysis method (open circles) for the PVP-BHC interaction at 20°C. Data are from absorbance depression measured in 0.02% (●) and 0.1% (▲) PVP.

Solubility analysis has been widely used to study molecular interaction in solution. If there is a sharp break in the solubility curve, the overall step stability constant can be estimated (Higuchi and Connors, 1965; Connors and Mollica, 1966).* However, in the present work, the macromolecules studied have appreciable aqueous solubility and formed soluble complexes with BHC; the solubility curves were non-linear and failed to show a break. The r value obtained from the linear portion is a constant (see p. 121). Hence the method gives only one point on any type of binding curve (i.e., Langmuir, Scatchard, and double reciprocal plots).

For the PVP-BHC interaction, the r value was approximately 20 compared with a value of 50 obtained from equilibrium dialysis (see p. 121 and p. 126). The molecular model of the PVP-BHC complex supports the value of 50 identical binding sites per PVP molecule (see pp. 140-141). It is apparent, therefore, that all the binding sites are not accessible to BHC molecules and that uptake of BHC

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* The overall step stability constant (k_{ov}) is defined by

$$k_{ov} = \frac{(MD_n)}{(M)(D)^n} \qquad \text{(Eq. 60)}$$

for the reaction, $$nD + M = MD_n \qquad \text{(Eq. 61)}$$

The right-hand side of Eq. 60 can be obtained by multiplying Eq. 14 by itself from i=1 to n. Eq. 62 then shows the relationship between overall step stability constant (k_{ov}), step stability constant (k_i), and intrinsic association constant (K).

$$k_{ov} = k_1 \cdot k_2 \cdot k_3 \cdots k_n = K^n \left[n \cdot \left(\frac{n-1}{2}\right) \cdot \left(\frac{n-2}{3}\right) \cdots \left(\frac{1}{n}\right) \right] \qquad \text{(Eq. 62)}$$

interferes with the interaction at other sites. Interference due to repulsion of bound BHC anion for unbound BHC anion is unlikely because of the screening effect of high ionic strength (see p. 146).

Viscometry does not provide quantitative information about binding and in the present study its applicability was limited by the low solubility of BHC. However, a configurational change in PVP was detected from measurements made at one BHC concentration. Coiling of the polymer, as a result of binding (see p. 146), is a possible explanation of the inaccessibility of all binding sites to BHC molecules.

VII. SUMMARY AND CONCLUSION

1. Physicochemical Properties of BHC in Aqueous Solutions

Potentiometric and spectrophotometric methods were used to determine the apparent pKa values of the weak dibasic acid, BHC. BHC is very insoluble in acidic solutions and it was necessary to modify the usual titration approach to pKa determinations. Values obtained were 4.4 and 8.0 for pKa_1 and pKa_2, respectively.

The effect of ionic strength (chloride ion) and pH on the apparent solubility of BHC was investigated. Increase in solubility of BHC with increase in chloride ion concentration was explained in terms of water structure. The effect of pH on the BHC solubility was interpreted on the basis of intramolecular hydrogen bonding. This view was supported by the infrared spectrum of the drug in KBr disc.

2. Binding Studies

Data obtained by utilizing the solubility analysis and dynamic dialysis methods indicated that binding strength of BHC increases in the order dextran, HES and potato starch sol. HSA and PVP possess much greater affinity for BHC than the others and for these two macromolecules the mechanism of interaction with BHC was investigated in detail. The following conclusions were reached.

(a) With respect to both HSA-BHC and PVP-BHC interactions: i) Both interactions are exothermic and occur spontaneously under the experimental conditions. ii) The α,β -unsaturated lactone structure in BHC is involved in the complexations.

(b) With respect to HSA-BHC interaction: i) A heterogeneity of binding sites was observed. The average number of binding sites of the first set was approximately three. ii) The intrinsic association constant is equal to 17×10^4 and 35×10^4 L./mole at 40 and 20°C, respectively. iii) The binding sites are believed to be inter-helical cavities whose van der Waals contour approximates, and is thus in a sense selective for, the BHC molecule. iv) The selectivity of the binding sites was supported by the large contribution of ΔH° to the ΔG° value. v) The main binding energy is derived from non-ionic sources.

(c) With respect to PVP-BHC interaction: i) A PVP molecule with a molecular weight of 40,000 provides approximately 50 identical binding sites for BHC molecules. This implies that one binding site consists of seven or eight monomer units of PVP. ii) The intrinsic association constant is in the order of 10^2 L./mole. iii) Ion-dipole and van der Waals forces play an important role in the binding process. iv) At low temperatures, the thermodynamic data indicates that hydrophobic bond formation plays a significant part in the process. v) Intrinsic viscosity data indicates that coiling of the polymer takes place during the binding process.

VIII. REFERENCES

Agran, A. and Elofsson, R. (1967). Acta Pharm. Suecica, 4, 281. Complex Formation Between Macromolecules and Drugs. I. Dialysis Studies of Phenols and Polyethylene Glycol.

Albert, A. and Serjeant, E.P. (1962). "Ionization Constants of Acids and Bases," John Wiley and Sons, New York.

Andreoli, M., Robbins, J., Rall, J.E., and Berman, M. (1965). in "Current Topic in Thyroid Research," Proceedings of the Fifth International Thyroid Conference (Rome), Cassano, C. and Andreoli, M. ed., Academic Press, New York and London, pp. 635-647. Methods for Measuring Association-Dissociation Rates of Thyroxine-Serum Protein Complexes.

Aoki, K. and Foster, J.F. (1957). J. Am. Chem. Soc., 79, 3385. Electrophoretic Behaviour of Bovine Plasma Albumin at Low pH.

Arond, L.H. and Frank, H.P. (1954). J. Phys. Chem., 58, 953. Molecular Weight, Molecular Weight Distribution and Molecular Size of a Native Dextran.

A.S.T.M. Standard (1966a). Designation D445-65 (IP Designation 71/66), pp. 184-191. Standard Method of Test for Viscosity of Transparent and Opaque Liquids (Kinematic and Dynamic Viscosities).

A.S.T.M. Standard (1966b). Designation D2515-66, pp. 912-925. Standard Specification for Kinematic Glass Viscometers.

Bahal, C.K. and Kostenbauder, H.B. (1964). J. Pharm. Sci., 53, 1027. Interaction of Preservatives with Macromolecules. V. Binding of Chlorobutanol, Benzyl Alcohol, and Phenylethyl Alcohol by Nonionic Agents.

Bates, R.G. (1961). Ann. N. Y. Acad. Sci., 92, 341. Physicochemical Properties of Amine Buffers. Amine Buffers for pH Control.

BeMiller, J.N. (1965). in "Starch," Whistler, R.L. and Paschall, E.F. ed., Academic Press, New York and London, vol. 1, chap. XIII, pp. 309-329. Organic Complexes and Coordination Compounds of Carbohydrates.

Benesch, R.E. and Benesch, R. (1955). J. Am. Chem. Soc., 77, 2749. The Stability of the Silver Complex of Tris-(hydroxymethyl)-aminomethane.

Benesi, H.A. and Hilderbrand, J.H. (1949). Ibid., 71, 2703. A Spectrophotometric Investigation of the Interaction of Iodine with Aromatic Hydrocarbons.

Bennhold, H. (1962). in "Protides of the Biological Fluids," Proceedings of the Ninth Colloquim (Bruges, Belgium, 1961), Peeters, H. ed., Elsevier Publishing, Amsterdam and New York, pp. 58-69. Überblick über die Transportfunktion der Serumeiweisskörper.

Bernal, J.D. (1965). in "The State and Movement of Water in Living Organisms," Symposia of the Society for Experimental Biology, No. XIX, Cambridge University Press, pp. 17-32. The Structure of Water and Its Biological Implications.

Breuninger, W.B. and Goettsch, R.W. (1965). J. Pharm. Sci., 54, 1487. Interaction of p-chlorometaxylenol with Macromolecules.

Bull, H.B. (1964a). "An Introduction to Physical Biochemistry," F.A. Davis Co., Philadelphia, chap. 7, pp. 142-164. Osmotic Pressure and Related Topics.

Bull, H.B. (1964b). Ibid., chap. 10, pp. 232-244. Viscosity and the Flow of Liquids.

Burns, J.J., Wexler, S., and Brodie, B.B. (1953). J. Am. Chem. Soc., 75, 2345. The Isolation and Characterization of a Metabolic Product of 3,3'-Carboxymethylenebis-(4-hydroxy-coumarin) Ethyl Ester (Tromexan) from Human Urine.

Buswell, A.M. and Rhodebush, W.H. (1956). Sci. Am., 194(4), 77. Water.

Campbell, H. and Hunter, G. (1953). Lancet, 264, 197. Colorimetric Estimation of Polyvinylpyrrolidone by Iodine.

Cannon. R.M., Manning, R.E., and Bell, J.D. (1960). Anal. Chem., 32, 355. Viscosity Measurement. The Kinetic Energy Correction and a New Viscometer.

Cecil, R. (1967). Nature, 214, 369. Model System for Hydrophobic Interactions.

Christensen, F. (1964). Acta Pharmacol Toxicol., 21, 307. Studies on the Fate of Intravenous Dicoumarol in the Rat.

Christensen, F. (1966). Ibid., 24, 232. Crystallization and Preliminary Characterization of a Dicoumarol Metabolite in the Faeces of Dicoumarol-Treated Rats.

Clark, P., Rachinsky, M., and Foster, J.F. (1962). J. Biol. Chem., 237, 2509. Moving Boundary Electrophoresis Behaviour and Acid Isomerization of Human Mercaptalbumin.

Claussen, W.F. and Polglase, M.F. (1952). J. Am. Chem. Soc., 74, 4817. Solubilities and Structures in Aqueous Aliphatic Hydrocarbon Solutions.

Cogin, G.E. and Davis, B.D. (1951). J. Am. Chem. Soc., 73, 3135. Competition in the Binding of Long Chain Fatty Acids and Methyl Orange to Bovine Serum Albumin.

Cohn, E.J., Gurd, F.R.N., Surgenor, D.M., Barnes, B.A., Brown, R.K., Derouaux, G., Gillespie, J.M., Kahnt, F.W., Lever, W.F., Liu, C.H., Mittelman, D., Mouton, R.F., Schmid, K., and Uroma, E. (1950). Ibid., 72, 465. A System for the Separation of the Components of Human Blood. Quantitative Procedures for the Separation of the Protein Components of Human Plasma.

Connors, K.A. and Mollica, J.A., Jr. (1966). J. Pharm. Sci., 55, 772. Theoretical Analysis of Comparative Studies of Complex Formation. Solubility, Spectral, and Kinetic Techniques.

Craig, L.C. (1965). in "Advances in Analytical Chemistry and Instrumentation," Reilley, C.N. ed., Interscience Publishers, New York, vol. 4, pp. 35-74. Differential Dialysis.

Dittert, L.W., Higuchi, T., and Reese, D.R. (1964). J. Pharm. Sci., 53, 1325. Phase Solubility Technique in Studying the Formation of Complex Salts of Triamterene.

Doty, P., Wada, A., Yang, J.T., and Blout, E.R. (1957). J. Polymer Sci., 23, 851. Polypeptides. VIII. Molecular Configurations of Poly-L-Glutamic Acid in Water-Dioxane Solution.

Douglas, A.S. (1962). "Anticoagulant Therapy," Blackwell Scientific Publications, Oxford.

Dowd, J.E. and Riggs, D.S. (1965). J. Biol. Chem., 240, 863. A Comparison of Estimates of Michaelis-Menton Kinetic Constant from Various Transformation.

Dyer, J.R. (1965). "Applications of Absorption Spectroscopy of Organic Compounds," Prentice-Hall, Englewood Cliffs, N.J., p. 36. Alcohols and Phenols.

Edsall, J.T. and Wyman, J. (1958a). "Biophysical Chemistry," Academic Press, New York, vol. I, chap. 3, pp. 47-135. Problems of Protein Structure.

Edsall, J.T. and Wyman, J. (1958b). Ibid., pp. 263-278. The Salting-Out Effect.

Edsall, J.T. and Wyman, J. (1958c). Ibid., chap. 11, pp. 591-662. Some General Aspects of Molecular Interactions.

Eide, G.J. and Speiser, P. (1967a). Acta Pharm. Suecica, 4, 185. Interaction Between Drugs and Nonionic Macromolecules. (I).

Eide, G.J. and Speiser, P. (1967b). Ibid., 4, 201. Interaction Between Drugs and Nonionic Macromolecules. II. Influence of Buffer Substances and Ionic Strength on Complex Formation. Influence of Buffer and Complexing Compounds on the Rheological Properties of Polyvinylpyrrolidone.

Eliassaf, J., Eriksson, F., and Eirich, F.R. (1960). J. Polymer Sci., 47, 193. The Interaction of Polyvinylpyrrolidone with Cosolutes.

Feates, F.S. and Ives, D.J.G. (1956). J. Chem. Soc., 2798. The Ionization Function of Cyanoacetic Acid in Relation to the Structure of Water and Hydration of Ions and Molecules.

Findlay, L.E., Lothing, H., Ackers, S., and Miller, J.F. (1965). Paper Presented at the Twelfth Canadian Conference on Pharmaceutical Research (University of Alberta). Factors Affecting the in vitro Dissolution Rate of Dicoumarol from Tablets.

Flory, P.J. (1953). "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N.Y., chap. X, pp. 399-431. Configuration of Polymer Chains.

Foster, J.F. and Sterman, M.D. (1956). J. Am. Chem. Soc., 78, 3656. Conformation Changes in Bovine Plasma Albumin Associated with Hydrogen Ion and Urea Binding. II. Hydrogen Ion Titration Curves.

Foster, J.F. (1960). in "The Plasma Proteins," Putnam, F.W. ed., Academic Press, New York, vol. 1, pp. 179-239. Plasma Albumin.

Foster, J.F. and Clark, K.P. (1962). J. Biol. Chem., 237, 3163. A Re-examination of the Acid Titration Behaviour of Human Mercaptalbumin. Changes in Amphoteric Properties Associated with the N-F Transformation.

Foster, J.F. (1968). Chem. Soc. (London), Spec. Public., 23, 25. On the Microheterogeneity of Proteins.

Frank, H.P., Barkin, S., and Eirich, F.R. (1957). J. Phys. Chem., 61, 1375. The Interaction of Polyvinylpyrrolidone with Some Azo Dyes.

Frank, H.S. and Evans, M.W. (1945). J. Chem. Phys., 13, 507. Free Volume and Entropy in Condensed Systems. III. Entropy in Binary Liquid Mixtures; Partial Molal Entropy in Dilute Solutions; Structure and Thermodynamics in Aqueous Electrolytes.

Frank, H.S. and Wen, W. (1957). Disc. Farad. Soc., 24, 133. III. Ion-Solvent Interaction. Structural Aspects of Ion-Solvent Interaction in Aqueous Solutions: A Suggested Picture of Water Structure.

French, W.N. and Wehrli, A. (1965). Can. Pharm. J., Sci. Sec., 98, 174. Identification and Assay of Some Coumarin Anticoagulants.

G.A.F. (1957). "PVP (Polyvinylpyrrolidone)," General Aniline and Film Corporation, 15M-8-57, p. 2. Molecular Weight.

G.A.F. (1967). "PVP - Annotated Bibliography: 1951-1966," General Aniline and Film Corporation, vol. 1. Chemical Physical, and Physiological Properties.

Garrett, E. (1963). J. Pharm. Sci., 52, 797. Variation of pKa' Values of Tetracyclines in Dimethylformamide-Water Solvents.

Goldfarb, J. and Rodriguez, S. (1968). Makromol. Chem., 116, 96. Aqueous Solution of Polyvinylpyrrolidone.

Goldstein, A. (1949). Pharmacol. Rev., 1, 102. The Interactions of Drugs and Plasma Proteins.

Goudah, W. and Guth, E.P. (1965). J. Pharm. Sci., 54, 298. Complex Interaction of Starches with Certain Drug Pharmaceuticals.

Gould, E.S. (1963). "Mechanism and Structure in Organic Chemistry," Holt, Reinhart and Winston, New York, chap. 10, pp. 365-411. Carbanions and Enolization.

Granath, K.A. (1958). J. Colloid Sci., 13, 308. Solution Properties of Branched Dextrans.

Gray, V.M. and Schoch, T.J. (1962). Staerke, 14, 239. Effects of Surfactants and Fatty Adjuncts on the Swelling and Solubilization of Granular Starches.

Greenwood, C.T. (1956). Adv. Carbohyd. Chem., 11, 336. Aspects of the Physical Chemistry of Starch.

Grönwall, A. (1957). "Dextran and Its Use in Colloidal Infusion Solutions," Academic Press, New York.

Gurney, R.N. (1953). "Ionic Process in Solution," McGraw-Hill, New York, Toronto, and London, pp. 100-103. The Unitary Part of a Free Energy Change.

Guttman, D. and Higuchi, T. (1956). J. Am. Pharm. Assoc., Sci. Ed., 45, 659. Possible Complex Formation Between Macromolecules and Certain Pharmaceuticals. X. The Interaction of Some Phenol Compounds with Polyethylene Glycol, Polypropylene Glycol, and Polyvinylpyrrolidone.

Hart, H.E. (1965). Bull. Math. Biophys., 27, 87. Determination of Equilibrium Constants and Maximum Binding Capacities in Complex *in vitro* System. I. The Mammillary System.

Haurowitz, F. (1963). "The Chemistry and Function of Proteins," Academic Press, New York and London, pp. 20-22. Quantitative Determination of Proteins.

Hengstenberg, J. and Schucht, E. (1952). Makromol. Chem., 7, 236. Molekulargewichtsbetimmung von PVP mittels des osmotischen Drucks und der Lichtstreuung ihrer Losungen.

Higuchi, T. and Zuck, D.A. (1953a). J. Am. Pharm. Assoc., Sci. Ed., 42, 132. Investigation of Some Complexes Formed in Solution by Caffeine. II. Interactions with Benzoic Acid and Benzoate Ion.

Higuchi, T. and Zuck, D.A. (1953b). Ibid., 42, 138. Investigation of Some Complexes Formed in Solution by Caffeine. III. Interactions Between Caffeine and Aspirin, p-Hydroxybenzoic Acid, m-Hydroxybenzoic Acid, Salicylic Acid, Salicylate Ion, and Butyl Paraben.

Higuchi, T. and Lach, J.L. (1954a). Ibid., 43, 349. Invetigation of Some Complexes Formed in Solution by Caffeine. IV. Interactions Between Caffeine and Sulfathiazole, Sulfadiazine, p-Aminobenzoic Acid, Benzocaine, Phenobarbital, and Barbital.

Higuchi, T. and Kuramoto, R. (1954b). Ibid., 43, 393. Study of Possible Complex Formation Between Macromolecules and Certain Pharmaceuticals. I. Polyvinylpyrrolidone with Sulfathiazole, Procaine Hydrochloride, Sodium Salicylate, Benzyl Penicillin, Chloramphenicol, and Cortisone.

Higuchi, T. and Kuramoto, R. (1954c). Ibid., 43, 398. Study of Possible Complex Formation Between Macromolecules and Certain Pharmaceuticals. II. Polyvinylpyrrolidone with p-Aminobenzoic Acid, Aminopyrine, Benzoic Acid, Salicylic Acid, p-Hydroxybenzoic Acid, m-Hydroxybenzoic Acid, Citric Acid and Phenobarbital.

Higuchi, T. and Lach, J.L. (1954d). Ibid., 43, 465. Study of Possible Complex Formation Between Macromolecules and Certain Pharmaceuticals. III. Interaction of Polyethylene Glycol with Several Organic Acids.

Higuchi, T. and Lach, J.L. (1954e).Ibid., 43, 525. Investigation of Complexes Formed in Solution by Caffeine. V. Interactions Between Caffeine and p-Aminobenzoic Acid, m-Hydroxybenzoic Acid, Picric Acid, o-Phthalic Acid, Suberic Acid, and Valeric Acid.

Higuchi, T. and Lach, J.L. (1954f). Ibid., 43, 527. Investigation of Complexes Formed in Solution by Caffeine. VI. Comparison of Complexing Behaviors of Methylated Xanthines with p-Aminobenzoic Acid, Salicylic Acid, Acetylsalicylic Acid, and p-Hydroxybenzoic Acid.

Higuchi, T. and Bolton, S. (1959). ibid., 48, 557. The Solubility and Complexing Properties of Oxytetracycline and Tetracycline. III. Interactions in Aqueous Solution with Model Compounds, Biochemicals, Metals, Chelates, and Hexametaphosphate.

Higuchi, T. and Drubulis, A. (1961). J. Pharm. Sci., 50, 905. Complexation of Organic Substances in Aqueous Solution by Hydroxyaromatic Acids and Their Salts. Relative Contribution of Several Factors to the Overall Effect.

Higuchi, T. and Pisano, F.D. (1964). Ibid., 53, 644. Complexation of Organic Substances in Aqueous Solution by Hydroxyaromatic Acids and Their Salts. II. Influence of Halogen, Nitro, and Other Substituents and Correlation of Binding Tendencies Toward Prednisolone, Theophylline and Phenacetin.

Higuchi, T. and Connors, K.A. (1965). in "Advances in Analytical Chemistry and Instrumentation," Reilley, C.N. ed., Interscience Publishers, New York, vol. 4, pp. 117-212. Phase Solubility Techniques.

Huggins, M.L. (1942). J. Am. Chem. Soc., 64, 2716. The Viscocity of Dilute Solutions of Long-Chain Molecules. IV. Dependence on Concentration.

Hutchinson, D.W. and Tomlinson, J.A. (1969). Tetrah., 25, 2531. The Structure of Dicoumarol and Related Compounds.

Hymes, A.J., Cuppett, C.C., and Canady, W.J. (1969). J. Biol. Chem., 244, 637. Thermodynamics of α-Chymotrypsin-Inhibitor Complex Formation.

Ingram, G.I.C. (1961). Pharmacol. Rev., 13, 279. Anticoagulant Therapy.

Jaques, L.B., Froese, E.L., O'Toole, R., and Spinks, J.T.W. (1957). Arch. Intern. Pharmacodyn., 111, 478. Relation Between Duration of Hypoprothrombinemia with Dicoumarol and the Level of the Drug in the Liver.

Job, P. (1928). Ann. Chim., 9, 113. Recherches sur la formation de complexes minéraux en solution et sur leur stabilité.

Kabadi, B.N. and Hammarlund, E.R. (1966). J. Pharm. Sci., 55, 1069. Interaction of Nonionic Hydrophillic Polymers with Phenols. I. Interaction of Phenol and Hydroxylphenols with Certain Macromolecules.

Karush, F. and Sonenberg, M. (1949). J. Am. Chem. Soc., 71, 1369. Interaction of Homologous Alkyl Sulfates with Bovine Serum Albumin.

Karush, F. (1950). Ibid., 72, 2705. Heterogeneity of the Binding Sites of Bovine Serum Albumin.

Karush, F. (1951). Ibid., 73, 1246. The Study of Protein Binding by Partition Analysis. The Effect of Protein Charge.

Karush, F. (1956). Ibid., 78, 5519. The Interaction of Purified Antibody with Optically Isomeric Haptens.

Karush, F. (1957). J. Am. Chem. Soc., 79, 3380. The Interaction of Purified Anti-β-lactoside Antibody with Haptens.

Kauzmann, W. (1959). Adv. Prot. Chem., 14, 1. Some Factors in the Interpretation of Protein Denaturation.

Kayushin, L.P. (1969). ed., "Water in Biological System (translated from Russian)," Consultants Bureau, New York.

Kirkwood, J.G. and Westheimer, F.H. (1938). J. Chem. Phys., 6, 506. The Electrostatic Influence of Substituents on the Dissociation Constants of Organic Acids.

Klotz, I.M. (1946a). Arch. Biochem., 9, 109. The Application of the Law of Mass Action to Binding by Protein. Interaction with Calcium.

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

Klotz, I.M. (1946c). Ibid., 68, 2299. Spectrophotometric Investigations of the Interactions of Proteins with Organic Anions.

Klotz, I.M. (1947). Chem. Rev., 41, 373. The Effects of Salts and Proteins on the Spectra of Some Dyes and Indicators.

Klotz, I.M., Triwush, H., and Walker, F.M. (1948). J. Am. Chem. Soc., 70, 2935. The Binding of Organic Ions by Proteins. Competition Penomena and Denaturation Effects.

Klotz, I.M. (1949a). Cold Spr. Harb. Sympo. Quant. Biol., 14, 97. The Nature of Some Ion-Protein Complexes.

Klotz, I.M. and Urquhart, J.M. (1949b). J. Am. Chem. Soc., 71, 847. The Binding of Organic Ions by Proteins. Effect of Temperature.

Klotz, I.M. and Urquhart, J.M. (1949c). J. Phys. Coll. Chem., 53, 100. The Binding of Organic Ions by Proteins. Buffer Effects.

Klotz, I.M. and Ayers, J. (1952). J. Am. Chem. Soc., 74, 6178. Interactions of Some Neutral Organic Molecules with Proteins.

Klotz, I.M. (1953a). in "The Protein," Neurath, H. and Bailey, K. ed., Academic Press, New York, vol. 1, pt. B, chap. 8, pp. 727-806. Protein Interaction.

Klotz, I.M. and Ayers, J. (1953b). Disc. Farad. Soc., 13, 189. Protein Interactions with Organic Molecules.

Klotz, I.M. (1958). Science, 128, 815. Protein Hydration and Behaviour.

Klotz, I.M. and Luborsky, S.W. (1959). J. Am. Chem. Soc., 81, 5119. Effects of Changes in Solvent and Their Implications as to the Nature of the Complexes.

Klotz, I.M. (1960). Broohaven Sympo. Biol., 13, 25. Non-Covalent Bonds in Protein Structure.

Kostenbauder, H.B. and Higuchi, T. (1956). J. Am. Pharm. Assoc., Sci. Ed., 45, 518. Formation of Molecular Complexes by Some Water Soluble Amide. I. Interaction of Several Amides with p-Hydroxybenzoic Acid, Salicylic Acid, Chloramphenicol, and Phenols.

Kostenbauder, H.B., Boxnbaum, H.G., and Deluca, P.P. (1969). J. Pharm. Sci., 58, 753. Nylon as a Dialysis Membrane.

Kraemer, E.O. (1938). Ind. Eng. Chem., 30, 1200. Molecular Weights of Celluloses and Cellulose Derivatives.

Krüger-Thimer, E., Dettli, L., Spring, P., and Diller, W. (1964). Estratto dal Vol. degli Atti del 3° Congr. Intern. di Medicina Cibernetica (Napoli, 1964, 1967), pp. 249-256. Mathematical Problems in the Calculation of Protein Binding of Drugs with Digital Computers.

Langmuir, I. (1918). J. Am. Chem. Soc., 40, 1361. The Adsorption of Gases on Plane Surfaces of Glass, Mica and Platinium.

Launer, H.F. (1963). in "Methods in Carbohydrate Chemistry," Whistler, R.L. ed., Academic Press, New York and London, vol. III (Cellulose), pp. 29-31. Determination of Cellulose. Hear-of-Dilution Dichromate Method.

Laurent, T.C. (1963). Biochem. J., 89, 253. The Interaction Between Polysaccharides and Other Macromolecules.

Laurent, T.C. and Granath, K.A. (1967). Biochem. Biophys. Acta, 136, 191. Fractionation of Dextran and Ficoll by Chromatography on Sephadex G-200.

Layne, E. (1956). in "Methods in Enzymology," Colowick, S.P. and Kaplan, N.O. ed., Academic Press, New York, vol. III, sec. IV, pp. 447-528. Quantitative Determination of Proteins.

Leader, G.R. and Gormley, J.F. (1951). J. Am. Chem. Soc., 73, 5731. The Dielectric Constant of N-Methylamides.

Lee, C.C., Trevoy, L.W., Spinks, J.W.T., and Jaques, L.B. (1950). Proc. Soc. Exptl. Biol. Med., 74, 151. Dicoumarol Labelled with C-14.

Levine, W.C. (1967). in "The Pharmacological Basis of Therapeutics," Goodman, L.S. and Gilman, A. ed., Macmillan, New York, Collier-Macmillan, London and Toronto, chap. 65, pp. 1443-1464. Anticoagulants.

Levy, R.H. (1969). Graduate Student under Dr. Rawland, School of Pharmacy, University of California, San Francisco, Calif. Personal Communication.

Lineweaver, H. and Burk, D. (1943). J. Am. Chem. Soc., 56, 658. The Determination of Enzyme Dissociation Constants.

Link, K.P. (1943-1944). Harvey Lecture, 39, 162. The Anticoagulant from Spoiled Sweet Clover Hay.

Lovrien, R. (1963). J. Am. Chem. Soc., 85, 3677. Interaction of Dodecyl Sulfate Anions of Low Concentration with Alkaline Bovine Serum Albumin.

Lozinski, E. (1960). Can. Med. Assoc. J., 83, 177. Physiological Availability of Dicoumarol.

Mader, W.J. (1954). in "Organic Analysis," Mitchell, J., Jr., Kolthoff, I.M., Proskauer, E.S., and Weissberger, A., ed., Interscience Publishers, New York, vol. II, pp. 253-275.

Mansour, Z. and Guth, E.P. (1968). J. Pharm. Sci., 57, 404. Complexing Behavior of Starches with Certain Pharmaceuticals.

Martin, A.N. (1968a). "Physical Pharmacy," 7th prin., Lea and Febiger, Philadelphia, pp. 62-66. Binding Forces Between Molecules.

Martin, A.N. (1968b). Ibid., chap. 14, pp. 339-394. Solubility and Related Phenomena.

Martin, A.N. (1968c). Ibid., pp. 534-535. Temperature Dependence and the Theory of Viscosity.

Martin, B.K. (1965). Nature, 207, 274. Potential Effect of the Plasma Proteins on Drug Distribution.

Masterton, W.L. (1954). J. Chem. Phys., 22, 1830. Partial Molal Volumes of Hydrocarbons in Water Solution.

May, L., Hines, M., Weintraub, L., Scudder, J., and Graff, S. (1954). Surg., 35, 191. Some Physico-Chemical Properties of Polyvinylpyrrolidone.

McMenamy, R.H. and Seder, R.H. (1963). J. Biol. Chem., 238, 3241. Thermodynamic Values Related to the Association of L-Tryptophan Analogoues to Human Serum Albumin.

"The Merck Index," (1968). Eighth ed., Merck and Co., Inc., Rahway, N.J., p. 153. Bishydroxycoumarin.

Meyer, M.C. and Guttman, D.E. (1968a). J. Pharm. Sci., 57, 895. The Binding of Drugs by Plasma Proteins.

Meyer, M.C. and Guttman, D.E. (1968b). J. Pharm. Sci., 57, 1672. Novel Method for Studying Protein Binding.

Meyer, M.C. and Guttman, D.E. (1970a). Ibid., 59, 33. Dynamic Dialysis as a Method for Studying Protein Binding. I. Factors Affecting the Kinetics of Dialysis Through a Cellophane Membrane.

Meyer, M.C. and Guttman, D.E. (1970b). Ibid., 59, 39. Dynamic Dialysis as a Method for Studying Protein Binding. II. Evaluation of the Method with a Number of Binding Systems.

Miller, L.E. and Hamm, H.A. (1953). J. Phys. Chem., 57, 110. Macromolecular Properties of Polyvinylpyrrolidone. Molecular Weight Distribution.

Mitchell, A.G. and Brown, K.F. (1966). J. Pharm. Pharmacol., 18, 115. The Interaction of Benzoic Acid and Chloroxylenol with Cetomacrogol.

Miyawaki, G.M., Patel, N.K., and Kostanbauder, H.B. (1959). J. Am. Pharm. Assoc., Sci. Ed., 48, 315. Interaction of Preservatives with Macromolecules. III. p-Hydroxybenzoic Acid Esters in the Presence of Some Hydrophyllic Polymers.

Mohammad, A.-H. (1965). J. Phys. Chem., 69, 2720. Effect of Urea on the Structure of Water and Hydrophobic Bonding.

Molyneux, P. and Frank, H.P. (1961a). J. Am. Chem. Soc., 83, 3169. The Interaction of Polyvinylpyrrolidone with Aromatic Compounds in Aqueous Solution. I. Thermodynamics of the Binding Equilibria and Interaction Forces.

Molyneux, P. and Frank, H.P. (1961b). Ibid., 83, 3179. The Interaction of Polyvinylpyrrolidone with Aromatic Compounds in Aqueous Solution. II. The Effect of the Interaction on the Molecular Size of the Polymer.

Mysels, K.J. (1959). "Introduction to Colloid Chemistry," Interscience Publishers, New York, chap. XIII, pp. 275-294. Structural Rheology.

Nagashima, R., Levy, G., and Nelson, E. (1968a). J. Pharm. Sci., 57, 58. Comparative Pharmacokinetics of Coumarin Anticoagulants. I. Unusual Interaction of Bishydroxycoumarin with Plasma Proteins - Development of a New Assay.

Nagashima, R., Levy, G., and Back, N. (1968b). Ibid., 57, 68. Comparative Pharmacokinetics of Coumarin Anticoagulants. II. Pharmacokinetics of Bishydroxycoumarin Elimination in the Rat, Guinea Pig, Dog, and Rhesus Monkey.

Nagashima, R., Levy, G., and Sarcione, E.J. (1968c). J. Pharm. Sci., 57, 1881. Comparative Pharmacokinetics of Coumarin Anticoagulants. III. Factors Affecting the Distribution of Bishydroxycoumarin in Isolated Liver Perfusion Studies.

Nagashima, R., Levy, G., and O'Reilly, R.A. (1968d). Ibid., 57, 1888. Comparative Pharmacokinetics of Coumarin Anticoagulants. IV. Application of a Three-Compartmental Model to the Analysis of the Dose-Dependent Kinetics of Bishydroxycoumarin Elimination.

Nakanish, K. (1964). "Infrared Absorption Spectroscopy: Practical," Holden-Day, San Francisco, p. 30. Table 5. Alcohols and Phenols, O-H Stretching Vibration (Intramol. H-bond).

N.A.S. - N.R.C. (1965). "Third Conference on Artificial Colloidal Agents," National Academy of Science - National Research Council.

Nemethy, G. and Scheraga, H.A. (1962a). J. Chem Phys., 36, 3382. Structure of Water and Hydrophobic Bonding in Proteins. I. A Model for Thermodynamic Properties of Liquid Water.

Nemethy, G. and Scheraga, H.A. (1962b). Ibid., 36, 3401. Structure of Water and Hydrophobic Bonding in Proteins. II. Model for the Thermodynamic Properties of Aqueous Solutions of Hydrocarbons.

Nemethy, G. and Scheraga, H.A. (1962c). J. Phys. Chem., 66, 1773. Structure of Water and Hydrophobic Bonding in Proteins. III. The Thermodynamic Properties of Hydrophobic Bonds in Proteins.

Nemethy, G., Steinberg, I.Z., and Scheraga, H.A. (1963). Biopolymer, 1, 43. Influence of Water Structure and of Hydrophobic Interactions on the Strength of Side-Chain Hydrogen Bonds in Proteins.

O'Reilly, R.A., Aggeler, P.M., and Leong, L.S. (1964). Thromb. Diath. Haemorrhag., 11, 1. Studies on the Coumarin Anticoagulant Drugs: A Comparison of the Pharmacodynamics of Dicoumarol and Warfarin in Man.

O'Reilly, R.A. and Kowitz, P.E. (1966). Clin. Res., 14, 324. Wararin-Albumin Binding: Correlation of Physicochemical and Metabolic Characteristic in Man.

O'Reilly. R.A. and Kowitz, P.E. (1967). Clin. Invest., 46, 829. Studies on the Coumarin Anticoagulant Drugs: Interaction of Human Plasma Albumin and Warfarin Sodium.

O'Reilly, R.A., Ohms, J.I., and Motley, C.M. (1968). J. Biol. Chem., 244, 1303. Studies on Coumarin Anticoagulant Drug. Heat of Interaction of Sodium Warfarin and Human Plasma Albumin by Heatburst Microcalorimetry.

O'Reilly, R.A. (1969). J. Clin. Invest., 48, 193. Interaction of the Anticoagulant Drug Warfarin and Its Metabolites with Human Serum Albumin.

Osborne, W.A. (1906). J. Physiol., 34, 84. Intracellular Colloidal Salts.

Oster, G. and Immergut, E.H. (1954). J. Am. Chem. Soc., 76, 1392. Ultaviolet and Infrared Spectral Studies of Polyvinylpyrrolidone.

Overbeek, J. TH. (1965). in "Progress in Biophysics and Biophysical Chemistry," Butler, J.A.V. ed., Pergamon, London and New York, vol. 6, chap. 3, pp. 67-84. The Donnan Equilibrium.

Owren, P.A. (1963a). Arch. Intern. Med., 111, 248. Control of Anticoagulant Therapy.

Owren, P.A. (1963b). New Engl. J. Med., 268, 1173. Indications for Anticoagulant Therapy.

Parke, T.V. and Davis, W.W. (1954). Anal. Chem., 26, 642. Use of Apparent Dissociation Constants in Qualitative Organic Analysis.

Patel, N.K. and Foss, N.E. (1964). J. Pharm. Sci., 53, 94. Interaction of Some Pharmaceuticals with Macromolecules. I. Effect of Temperature on the Binding of Parabens and Phenols by Polysorbate 80 and Polyethylene Glycol 4000.

Patel, N.K. and Foss, N.E. (1965). Ibid., 54, 1495. Interaction of Some Pharmaceuticals with Macromolecules. II. Binding of Certain Benzoic Acid Derivatives by Polysorbate 80 and Cetomacrogol 1000.

Pauling, L. (1967). "The Nature of the Chemical Bond," Third ed., Cornell University Press, Ithaca, N.Y., chap. 12, pp. 449-504. The Hydrogen Bond.

Pernarowski, M. (1969). in "Pharmaceutical Chemistry," Chatten, L.G. ed., Marcel Dekker, New York and London, vol. 2, chap. 1, pp. 1-58. Absorption Spectrophotometry.

Perrin, D.D. (1963). Austr. J. Chem., 16, 572. Buffers of Low Ionic Strength for Spectrophotometric pK Determination.

Pollansch, M.J. and Briggs, D.R. (1954). J. Am. Chem. Soc., 76, 1396. A Study of the Interaction of Dodecyl Sulfate with Bovine Serum Albumin.

Polly, G.P. and Frost, B.M. (1969). J. Pharm. Sci., 58, 1543. Complexation Between Hexylresorcinol and Polyvinylpyrrolidone and Its Microbiological Evaluation.

Poole, J.W. and Higuchi, T. (1959). J. Am. Pharm. Assoc., Sci. Ed., 48, 592. Complexes Formed in Aqueous Solutions by Sarcosine Anhydride: Interactions with Organic Acids, Phenols, and Aromatic Alcohols.

Putnam, F.W. and Neurath, H. (1945). J. Biol. Chem., 159, 195. Interaction Between Proteins and Synthetic Detergents. II. Electrophoretic Analysis of Serum Albumin - Sodium Dodecyl Sulfate Mixtures.

Putnam, F.W. (1965). in "The Protein," Second ed., Neurath, H. ed., vol. III, pp. 187-199. Structure and Function of the Plasma Proteins: Serum Albumin.

Ray, A. (1968). Chem. Soc. (London), Spec. Public., 23, 49. Association of Proteins Induced by Co-operative Binding of Sodium Dodecyl Sulfate.

Reese, E.T. and Parrish, F.M. (1966). Biopolymer, 4 , 1043. A Comparison of Synthetic Dextran with a Natural Product by Enzyme Methods.

Reilley, C.N. and Crawford, C.M. (1955). Anal. Chem., 27, 716. Principles of Precision Colorimetry. A General Approach to Photoelectric Spectrophotometry.

Reuning, R.H. and Levy, G. (1968). J. Pharm. Sci., 57, 1556. Characterization of Complex Formation Between Small Molecules by Membrane Permeation Measurement.

Ricketts, C.R. (1966). Brit. Med. J., 5527. Molecular Composition of Transfusion Dextran.

Robinson, H.W. and Hogden, C.G. (1941). J. Biol. Chem., 137, 239. The Influence of Serum Proteins on the Spectrophotometric Absorption Curve of Phenol Red in a Phosphate Buffer Mixture.

Robinson, R.A. and Stokes, R.H. (1968). "Electrolyte Solutions," Second ed., Butterworths, London, pp. 346-351. Dibasic Acids.

Rosenthal, H.E. (1967). Anal. Biochem., 20, 525. A Graphical Method for the Determination and Presentation of Binding Parameters in a Complex System.

Ruckel, E.R. and Schuerch, C. (1966). J. Am. Chem. Soc., 88, 2605. Chemical Synthesis of a Stereoregular Linear Polysaccharide.

Sager, E.E., Schooley, M.R., Carr, A.S., and Acree, S.F. (1945). J. Res. Nat. Bur. Stand., 35, 521. Ultraviolet Spectra and Dissociation Constants of p-Hydroxybenzoic Acid, Methyl, Ethyl, n-Butyl, and Benzyl p-Hydroxybenzoate and Potassium p-Phenolsulfonate.

Sahyun, M.R.V. (1964). Nature, 203, 1045. Enthalpy of Binding of Aromatic Amines to Bovine Serum Albumin.

Saito, S. (1957). Kolloid-Z., 154, 19. Die Untersuchung der Adsorptionskomplexe von Polymeren mit Netzmittelionen.

Sandber, A.A., Rosenthal, H., Schneider, S.L., and Slaunwhite, W.R., Jr. (1966). in "Steroid Dynamics," Nakao, T., Pincus, G., and Tait, J.F. ed., Academic Press, New York, pp. 33-41. Theoretical and Practical Considerations in Transcortin Determination.

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

Scatchard, G., Scheinberg, I.H., and Armstrong, H. (1950). J. Am. Chem. Soc., 72, 540. Physical Chemistry of Protein Solutions. V. The Combination of Human Serum Albumin with Thiocyanate Ion.

Scatchard, G., Hughes, W.L., Jr., Gurd, F.R.N., and Wilcox, P.E. (1954). in "Chemical Specificity in Biological Interactions," Gurd, F.R.N. ed., Academic Press, New York, chap. XI, pp. 193-219. The Interaction of Protein with Small Molecules and Ions.

Scatchard, G., Coleman, J.S., and Shen, A.L. (1957). J. Am. Chem. Soc., 79, 12. Physical Chemistry of Protein Solutions. VII. The Binding of Some Small Anions to Serum Albumin.

Scheraga, H.A. (1961). J. Phys. Chem., 65, 1071. Effect of Hydrophobic Bonding on Protein Reactions.

Scharaga, H.A. (1963). in "The Protein," Second ed., Neurath, H. ed., Academic Press, New York and London, chap. 6, pp. 477-594. Intrmolecular Bonds in Proteins. II. Noncovalent Bonds.

Schotan, W. (1953). Makromol. Chem., 11, 131. Über die Adsorptionsfähigkeit wasserlöslicher polymerer Verbindungen, insbesondere von Polyvinylpyrrolidone.

Scott, T.A. and Melvin, E.H. (1953). Anal. Chem., 25, 1656. Determination of Dextran with Anthrone.

Silverstein, R.M. and Bassler, G.C. (1967). "Spectrometric Identification of Organic Compounds," Second ed., John Wiley and Sons, New York, London and Sydney, pp. 158-162. Carbonyl Chromophore.

Simonelli, A.P., Mehta, S.C., and Higuchi, W.I. (1969). J. Pharm. Sci., 58, 538. Dissolution Rates of High Energy Polyvinylpyrrolidone (PVP) - Sulfathiazole Coprecipitates.

Singh, P., Desai, S.J., Simonelli, A.P., and Higuchi, W.I. (1967). J. Pharm. Sci., 56, 1548. Release Rates of Solid Drug Mixtures Dispersed in Inert Matrices. II. Mutually Interacting Drug Mixtures.

Solomon, H.M. and Schrogie, J.J. (1967). Clin. Pharmacol. Therap., 8, 65. The Anticoagulant Response to Bishydroxycoumarin. I. The Role of Individual Variation.

Spitzer, R.H. and McDonald, H.J. (1956). Clin. Chim. Acta, 1, 545. Comparison of the Binding Properties of Bovine Plasma Albumin and Polyvinylpyrrolidone. Equilibrium Dialysis and Titrimetry.

Stein, H.H. (1965). Anal. Biochem., 13, 305. Studies of Binding by Macromolecules: A New Dialysis Technique for Obtaining Quantitative Data.

Stein, R.S. and Rundle, R.E. (1948). J. Chem. Phys., 16, 195. On the Nature of the Interaction Between Starch and Iodine.

Tanford, C. (1950). J. Am. Chem. Soc., 72, 441. Preparation and Properties of Serum and Plasma Proteins. XXIII. Hydrogen Ion Equilibria in Native and Modified Human Serum Albumins.

Tanford, C., Swanson, S.A., and Shore, W.S. (1955a). Ibid., 77, 6414. Hydrogen Ion Equilibria of Bovine Serum Albumin.

Tanford, C., Buzzell, J.G., Rands, D.G., and Swanson, S.A. (1955b). Ibid., 77, 6421. The Reversible Expansion of Bovine Serum Albumin in Acid Solutions.

Tanford, C. and Buzzell, J.G. (1956). J. Phys. Chem., 60, 225. The Viscosity of Aqueous Solutions of Bovine Serum Albumin Between pH 4.3 and 10.5.

Tanford, C. (1965). "Physical Chemistry of Macromolecules," John Wiley and Sons, New York, London and Sydney, chap. 8, pp. 526-586. Multiple Equilibria.

Van Wazer, J.R., Lyons, J.W., Kim, K.Y., and Colwell, R.E. (1963). "Viscosity and Flow Measurement: A Laboratory Handbook of Rheology," Interscience Publishers, New York and London, pp. 224-230. Commercial Glass Capillary Viscometers.

Wadke, D.A. and Guttman, D.E. (1965). J. Pharm. Sci., 54, 1239. Complex Formation Influence on Reaction Rate. III. Interaction of Some Isoalloxazines with 8-Chlorotheophylline as Determined by Spectral, Solubility, and Kinetic Methods.

Weast, R.C. (1969). ed., "Handbook of Chemistry and Physics (College Edition)," 49th ed., The Chemical Rubber Co., Cleveland, Ohio, p. F-5. Volume Properties of Ordinary Water.

Weber, G. (1965). in "Molecular Biophysics," Pullman, B. and Weissbluth, M. ed., Academic Press, New York and London, pp. 369-396. The Binding of Small Molecules to Proteins.

Weiner, M., Shapiro, S., Axelrod, J., Cooper, J.R., and Brodie, B.B. (1950). J. Pharmacol. Exptl. Therap., 99, 409. The Physiological Disposition of Dicoumarol in Man.

Whistler, R.L. and Medcalf, D.G. (1965). in "Methods in Carbohydrate Chemistry," Whistler,R.L. ed., vol. IV, Academic Press, New York, p. 147. Determination of Solids in Starch Dispersions.

White, A., Handler, P., and Smith, E.L. (1968). "Principles of Biochemistry," McGraw-Hill, New York, Sydney, Toronto, and London, p. 709.

Wishnia, A. and Pinder, T. (1964). Biochem., 3, 1377. Hydrophobic Interactions in Proteins: Conformation Changes in Bovine Serum Albumin Below pH 5.

Worley, J.D. and Klotz, I.M. (1966). J. Chem. Phys., 45, 2868. Near Infrared Spectra of H_2O-D_2O Solutions.

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

Yang, J.T. and Foster, J.F. (1954). Ibid., 76, 1588. Changes in the Intrinsic Viscosity and Optical Rotation of Bovine Plasma Albumin Associated with Acid Binding.

Yang, J.T. (1961). Adv. Prot. Chem., 16, 323. The Viscosity of Macromolecules in Relation to Molecular Conformation.