11
Complexation and Protein Binding

Metal Complexes
Organic Molecular Complexes
Inclusion Compounds
Methods of Analysis

Protein Binding
Thermodynamic Treatment of
Stability Constants

Complexes or coordination compounds, according to the classic definition, result from a donor-acceptor mechanism or Lewis acid–base reaction (p. 144) between two or more different chemical constituents. Any nonmetallic atom or ion, whether free or contained in a neutral molecule or in an ionic compound, that can donate an electron pair may serve as the donor. The acceptor, or constituent that accepts a share in the pair of electrons, is frequently a metallic ion, although it can be a neutral atom. Complexes may be divided broadly into two classes depending on whether the acceptor component is a metal ion or an organic molecule; these are classified according to one possible arrangement in Table 11–1. A third class, the inclusion/occlusion compounds, involving the entrapment of one compound in the molecular framework of another, is also included in the table.

Intermolecular forces involved in the formation of complexes are the van der Waals forces of dispersion, dipolar, and induced dipolar types. Hydrogen bonding provides a significant force in some molecular complexes, and coordinate covalence is important in metal complexes. Charge transfer and hydrophobic interaction are introduced later in the chapter.

METAL COMPLEXES

A satisfactory understanding of metal ion complexation is based upon a familiarity with atomic structure and molecular forces, and the reader would do well to go to texts on inorganic and organic chemistry to study those sections dealing with electronic structure and hybridization before proceeding.

Inorganic Complexes. This group constitutes the simple inorganic complexes first described by Werner in 1891. The ammonia molecules in hexamminecobalt III chloride, as the compound $[Co(NH_3)_6]^{3+}Cl_3^-$ is called, are known as the *ligands* and are said to be

**TABLE 11–1. *Classification of Complexes****

I. Metal Ion Complexes
  A. Inorganic type
  B. Chelates
  C. Olefin type
  D. Aromatic type
    1. Pi (π) complexes
    2. Sigma (σ) complexes
    3. "Sandwich" compounds
II. Organic Molecular Complexes
  A. Quinhydrone type
  B. Picric acid type
  C. Caffeine and other drug complexes
  D. Polymer type
III. Inclusion/Occlusion Compounds
  A. Channel lattice type
  B. Layer type
  C. Clathrates
 D. Monomolecular type
  E. Macromolecular type

*This classification does not pretend to describe the mechanism or the type of chemical bonds involved in complexation. It is meant simply to separate out the various types of complexes that are discussed in the literature. A highly systematized classification of electron donor-acceptor interactions is given by R. S. Mulliken, J. Phys. Chem. **56**, 801, 1952.

coordinated to the cobalt ion. The *coordination number* of the cobalt ion, or number of ammonia groups coordinated to the metal ions is six. Other complex ions belonging to the inorganic group include $[Ag(NH_3)_2]^+$, $[Fe(CN)_6]^{4-}$, and $[Cr(H_2O)_6]^{3+}$.

Each ligand donates a pair of electrons to form a coordinate covalent link between itself and the central ion having an incomplete electron shell. For example,

$$Co^{3+} + 6:NH_3 = [Co(NH_3)_6]^{3+}$$
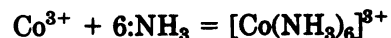
Hybridization plays an important part in coordination compounds in which sufficient bonding orbitals are not ordinarily available in the metal ion. The reader's understanding of hybridization will be refreshed by a brief review of the argument advanced for the quadrivalence of carbon. It will be recalled that the ground state configuration of carbon is

1s   2s   2p

⊕ ⊕   ⊕⊕◯
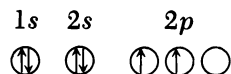
This cannot be the bonding configuration of carbon, however, since it normally has four rather than two valence electrons. Pauling[1] suggested the possibility of *hybridization* to account for the quadrivalence. According to this mixing process, one of the 2s electrons is promoted to the available 2p orbital to yield four equivalent bonding orbitals:

1s   2s   2p

⊕ ⊕   ⊕⊕⊕
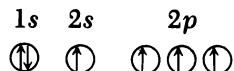
These are directed toward the corners of a tetrahedron, and the structure is known as an sp^3 hybrid because it involves one s and three p orbitals. In a double bond, the carbon atom is considered to be sp^2 hybridized, and the bonds are directed toward the corners of a triangle.

Orbitals other than the 2s and 2p orbitals can become involved in hybridization. The transition elements, such as iron, copper, nickel, cobalt, and zinc, seem to make use of their 3d, 4s, and 4p orbitals in forming hybrids. These hybrids account for the differing geometries often found for the complexes of the transition metal ions. Table 11–2 shows some compounds in which the central atom or metal ion is hybridized differently and it shows the geometry that results.

Ligands such as H_2O:, H_3N:, NC:$^-$, or Cl:$^-$ donate a pair of electrons in forming a complex with a metal ion, and the electron pair enters one of the unfilled orbitals on the metal ion. A useful but not inviolate rule to follow in estimating the type of hybridization in a metal ion complex is to select that complex in which the metal ion has its 3d levels filled or that can use the lower-energy 3d and 4s orbitals primarily in the hybridization. For example, the ground-state electronic configuration of Ni^{2+} may be given by
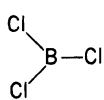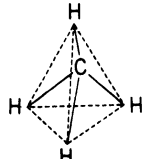
3d   4s   4p

⊕⊕⊕⊕⊕   ◯   ◯◯◯
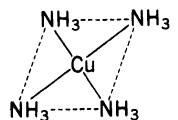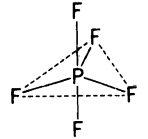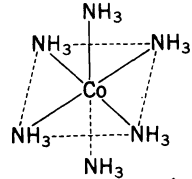
In combining with 4CN:$^-$ ligands to form $[Ni(CN)_4]^{2-}$, the electronic configuration of the nickel ion may become either

3d   4s   4p

⊕⊕⊕⊕⊕   ⊙   ⊙⊙⊙

sp^3 tetrahedral structure
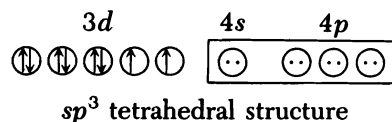
or

TABLE 11–2. *Bond Types of Representative Compounds*

Coordination Number	Orbital Configuration	Bond Type	Examples	
			Formula	Structure
2	sp	linear	O_2	O—O
3	sp^2	trigonal	BCl_3	
4	sp^3	tetrahedral	CH_4	
4	dsp^2	square planar	$Cu(NH_3)_4^{2+}$	
5	dsp^3	bipyramidal	PF_5	
6	d^2sp^3	octahedral	$Co(NH_3)_6^{3+}$	

$$3d \qquad\qquad 4s \qquad 4p$$

dsp^2 planar structure
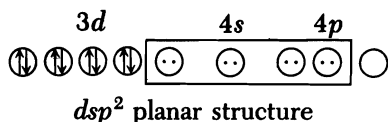
in which the electrons donated by the ligand are shown as dots. The dsp^2 or square planar structure is predicted to be the complex formed since it uses the lower-energy $3d$ orbital. By the preparation and study of a number of complexes, Werner deduced many years ago that this is indeed the structure of the complex.

Similarly, the trivalent cobalt ion Co(III) has the ground state electronic configuration

$$3d \qquad\qquad 4s \qquad 4p$$

and one may inquire into the possible geometry of the complex $[Co(NH_3)_6]^{3+}$. The electronic configuration of the metal ion leading to filled $3d$ levels is
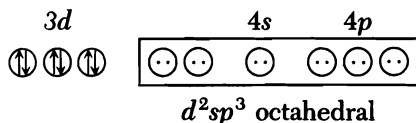
$$3d \qquad\qquad 4s \qquad 4p$$

d^2sp^3 octahedral

and thus the d^2sp^3 or octahedral structure is predicted as the structure of this complex. Chelates (see following section) of octahedral structure can be resolved into optical isomers, and in an elegant study, Werner used this technique to prove that cobalt complexes are octahedral.

In the case of divalent copper Cu(II), which has the electronic configuration

$$3d \qquad\qquad 4s \qquad 4p$$
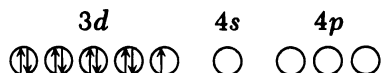
the formation of the complex $[Cu(NH_3)_4]^{2+}$ requires the promotion of one d electron of Cu^{2+} to a $4p$ level to obtain a filled $3d$ configuration in the complexed metal ion, and a dsp^2 or planar structure is obtained
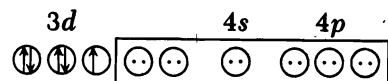
$$3d \qquad\qquad 4s \qquad 4p$$
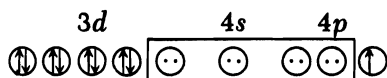
Although the energy required to elevate the d electron to the $4p$ level is considerable, the formation of a planar complex having the $3d$ levels filled entirely more than "pays" for the expended energy.

The metal ion Fe(III) has the ground-state configuration

$$3d \qquad\qquad 4s \qquad 4p$$
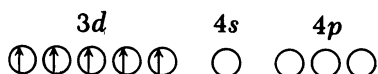
and in forming the complex $[Fe(CN)_6]^{3-}$, no electron promotion takes place

$$3d \qquad\qquad 4s \qquad 4p$$

since no stabilization is gained over that which the d^2sp^3 configuration already possesses. Compounds of this type, in which the ligands lie "above" a partially filled orbital, are termed *outer-sphere complexes;* when the ligands lie "below" a partially filled orbital, as in the previous example, the compound is termed an *inner-sphere complex*. The presence of unpaired electrons in a metal ion complex can be detected by *electron spin resonance spectroscopy* (pp. 90, 91).

Chelates. A substance containing two or more donor groups may combine with a metal to form a special type of complex known as a *chelate* (Greek: kelos, claw). Some of the bonds in a chelate may be ionic or of the primary covalent type, while others are coordinate covalent links. When the ligand provides one group for attachment to the central ion, the chelate is called *monodentate*. Pilocarpine behaves as a monodentate ligand toward Co(II), Ni(II), and Zn(II) to form chelates of pseudotetrahedral geometry. The donor atom of the ligand is the pyridine-type nitrogen of the imidazole ring of pilocarpine (Fig. 3–4, p. 72, shows the pyridine-like nitrogen of pilocarpine). Molecules with two and three donor groups are called *bidentate* and *tridentate*, respectively.[2] Ethylenediaminetetraacetic acid (EDTA) has six points for attachment to the metal ion and is accordingly *hexadentate;* however, in some complexes, only four or five of the groups are coordinated.

Chelation places stringent steric requirements on both metal and ligands. Ions such as Cu(II) and Ni(II), which form square planar complexes, and Fe(III) and Co(III), which form octahedral complexes, can exist in either of two geometric forms. As a consequence of this isomerism only *cis-coordinated ligands*—ligands adjacent on a molecule—will be readily replaced by reaction with a chelating agent. Vitamin B_{12} and the hemoproteins are incapable of reacting with chelating agents, because their metal is already coordinated in such a way that only the *trans*-coordination positions of the metal are available for complexation. In contrast, the metal ion in certain enzymes, such as alcohol dehydrogenase, which contains zinc, can undergo chelation, suggesting that the metal is bound in such a way as to leave two *cis* positions available for chelation.

Chlorophyll and hemoglobin, two extremely important compounds, are naturally occurring chelates involved in the life processes of plants and animals. Albumin is the main carrier of various metal ions and small molecules in the blood serum. The N-terminal portion of human serum albumin binds Cu(II) and Ni(II) with higher affinity than that of dog serum albumin. This fact partly explains why humans are less susceptible to copper poisoning than are dogs. The binding of copper to serum albumin is important since this metal is possibly involved in several pathologic conditions.[3] The synthetic chelating agent, ethylenediaminetetraacetic acid (Fig. 11–1), has been used to tie up or *sequester* iron and copper ions so that they cannot
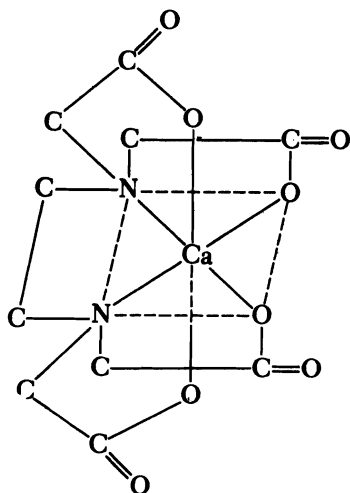
Fig. 11–1. Calcium ions sequestered by ethylenediaminetetraacetic acid (EDTA).

catalyze the oxidative degradation of ascorbic acid in fruit juices and in drug preparations. In the process of sequestration, the chelating agent and metal ion form a water-soluble compound. EDTA is widely used to sequester and remove calcium ions from hard water.

Chelation can be applied to the assay of drugs. A calorimetric method to assay procainamide in injectable solutions is based on the formation of a 1:1 complex of procainamide with cupric ion at pH 4 to 4.5. The complex absorbs visible radiation at a maximum wavelength of 380 nm.[4] The many uses to which metal complexes and chelating agents can be put are discussed by Martell and Calvin.[5]

ORGANIC MOLECULAR COMPLEXES

An organic coordination compound or molecular complex consists of constituents held together by weak forces of the donor–acceptor type or by hydrogen bonds.

The difference between complexation and the formation of organic compounds has been shown by Clapp.[6] The compounds, dimethylaniline and 2,4,6-trinitroanisole, react in the cold to give a molecular complex:

(11–1)
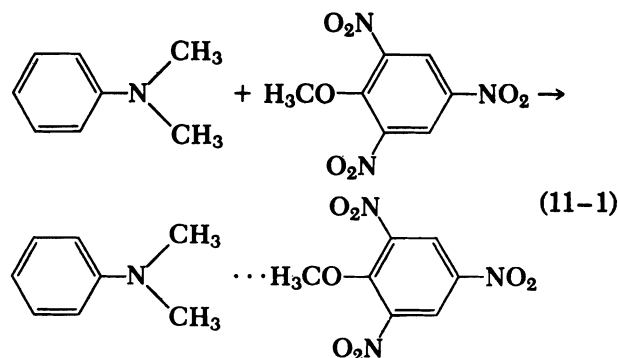
On the other hand, these two compounds react at an elevated temperature to yield a salt, the constituent

molecules of which are held together by primary valence bonds.

(11–2)
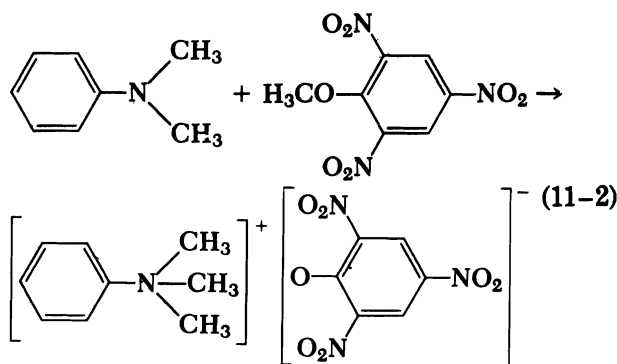
The dotted line in the complex of equation (11–1) indicates that the two molecules are held together by a weak secondary valence force. It is not to be considered as a clearly defined bond but rather as an overall attraction between the two aromatic molecules.

The type of bonding existing in molecular complexes in which hydrogen bonding plays no part is not fully understood, but it may be considered for the present as involving an electron donor–acceptor mechanism corresponding to that in metal complexes but ordinarily much weaker.

Many organic complexes are so weak that they cannot be separated from their solutions as definite compounds, and they are often difficult to detect by chemical and physical means. The energy of attraction between the constituents is probably less than 5 kcal/mole for most organic complexes. Since the bond distance between the components of the complex is usually greater than 3 Å, a covalent link is not involved. Instead, one molecule polarizes the other, resulting in a type of ionic interaction or charge transfer, and these molecular complexes are often referred to as *charge transfer complexes*. For example, the polar nitro groups of trinitrobenzene induce a dipole in the readily polarizable benzene molecule, and the electrostatic interaction that results leads to complex formation.

(donor)   (acceptor)

Electron drift or partial electron
transfer by polarization (π bonding)
⟶
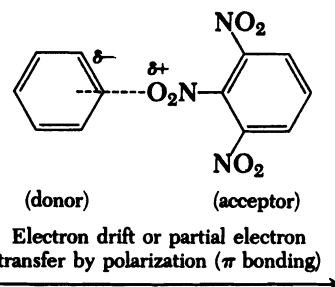
X-ray diffraction studies of complexes formed between trinitrobenzene and aniline derivatives have shown that one of the nitro groups of trinitrobenzene lies over the benzene ring of the aniline molecule, the intermolecular distance between the two molecules being about 3.3 Å. This result strongly suggests that the interaction involves π bonding between the π

electrons of the benzene ring and the electron-accepting nitro group.

A factor of some importance in the formation of molecular complexes is the steric requirement. If the approach and close association of the donor and acceptor molecules are hindered by steric factors, the complex is not likely to form. Hydrogen bonding and other effects must also be considered, and these are discussed in connection with the specific complexes considered on the following pages.

The difference between a *donor–acceptor* and a *charge-transfer* complex is that in the latter type resonance makes the main contribution to complexation, while in the former, London dispersion forces and dipole–dipole interactions contribute more to the stability of the complex. Resonance interaction is shown in Figure 11–2 as depicted by Bullock.[7] Trinitrobenzene is the acceptor, A, molecule and hexamethyl benzene is the donor, D. On the left side of the figure weak dispersion and dipolar forces contribute to the interaction of A and D; on the right side of the figure the interaction of A and D results from a significant transfer of charge, making the electron acceptor trinitrobenzene negatively charged (A⁻) and leaving the donor hexamethylbenzene positively charged (D⁺). The overall complex, Donor–Acceptor, is shown by the double-headed arrow to resonate between the uncharged D . . . A and the charged D⁺ . . . A⁻ moieties. If, as in the case of hexamethylbenzene-trinitrobenzene, the resonance is fairly weak, having an intermolecular binding energy ΔH of about -4700 calories, the complex is referred to as a *donor–acceptor complex*. If, on the other hand, resonance between the charge-transfer structure (D⁺ . . . A⁻) and the uncharged species (D . . . A) contributes greatly to the binding of the donor and acceptor molecule, the complex is called a *charge-transfer complex*. Finally, those complexes bound together by van der Waals forces, dipole–dipole interactions, and hydrogen bonding but lacking charge transfer are known simply as *molecular complexes*. In both charge-transfer and donor–acceptor complexes, new absorption bands occur in the spectra, as shown in Figure 11–13, p. 266. In this book we shall not attempt to separate the first two classes but rather refer to all interactions that produce absorption bands as charge-transfer or as electron donor–acceptor complexes without distinction. Those com-
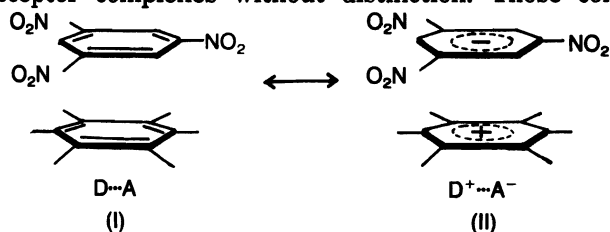
plexes that do not show new bands are called molecular complexes.

Charge-transfer complexes are of importance in pharmacy. Iodine forms 1:1 charge-transfer complexes with the drugs disulfiram, chlomethiazole, and tolnaftate. These drugs have recognized pharmacologic actions of their own: disulfiram is used against alcohol addiction, clomethiazole is a sedative-hypnotic and anticonvulsant, and tolnaftate is an antifungal agent. Each of these drugs possesses a nitrogen–carbon-sulfur moiety (see the structure of tolnaftate below), and a complex may result from the transfer of charge from the pair of free electrons on the nitrogen and/or sulfur atoms of these drugs to the antibonding orbital of the iodine atom. Thus, by tying up iodine, molecules containing the N – C = S moiety inhibit thyroid action in the body.[8]
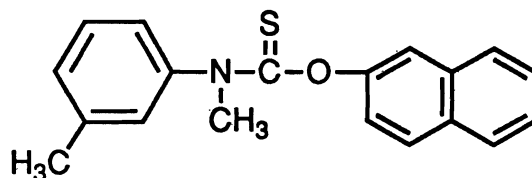
Tolnaftate (Tinactin)

Quinhydrone Complexes. The molecular complex that was referred to in Chapter 9 as quinhydrone is formed by mixing alcoholic solutions of equimolar quantities of benzoquinone and hydroquinone. The complex settles as green crystals. When an aqueous solution is saturated with quinhydrone, the complex dissociates into equivalent amounts of quinone and hydroquinone and is used as an electrode in pH determinations.

The 1:1 complex formed between benzoquinone and hydroquinone may be said to result from the overlap of the pi-framework of the electron-deficient quinone molecule with the pi-framework of the electron-rich hydroquinone molecule. Maximum overlap between the pi-frameworks is expected if the aromatic rings are parallel and are oriented in such a way as to have their centers directly over one another. Hydrogen bonding may contribute in stabilizing this complex, but it is not the sole means of association, since hydroquinone dimethyl ether also forms a colored adduct with quinone.

An interesting quinone is obtained from salicylic acid. This compound is readily oxidized, yielding blue-black quinhydrone compounds of the type
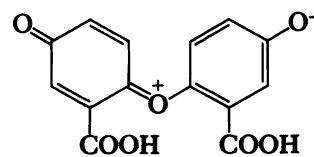
Quinhydrone of salicylic acid

Picric Acid Complexes. Picric acid, 2,4,6-trinitrophenol, $pK_a = 0.38$, reacts with strong bases to form salts

Fig. 11–2. Resonance in a donor–acceptor complex of trinitrobenzene and hexamethylbenzene. (From F. Y. Bullock, Charge transfer in biology, Chapter 3 in *Comprehensive Biochemistry*, M. Florkin and E. H. Stotz, Eds., Vol. 22 of *Bioenergetics*, Elsevier, N.Y., 1967, pp. 82–85, reproduced with permission of the copyright owner.)

and with weak bases to form molecular complexes. Butesin picrate (Abbott Laboratories), presumably a 2:1 complex, may be represented by the formula
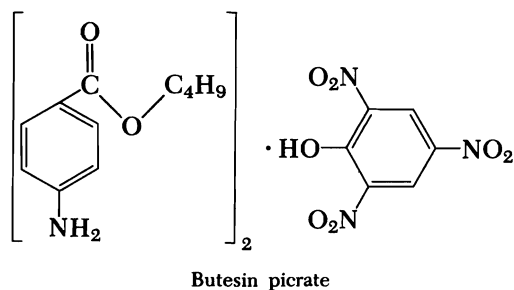
Butesin picrate

It is a yellow powder, insoluble in water but soluble in organic solvents. Butesin picrate is used as a 1% ointment for burns and painful skin abrasions. It combines the antiseptic property of picric acid and the anesthetic property of butesin.

It has been suggested that the stability of the complexes formed between carcinogenic agents and picric acid is related to carcinogenic activity, and any substitution on the carcinogen molecule that hinders picrate complexation also reduces carcinogenicity. Symmetric trinitrobenzene forms more complexes than does picric acid, and perhaps trinitrobenzene may also be used to provide a test for carcinogenicity.

Drug Complexes. Higuchi and his associates[9] have investigated the complexing of caffeine with a number of acidic drugs. They attribute the interaction between caffeine and a drug such as a sulfonamide or a barbiturate to a dipole–dipole force or hydrogen bonding between the polarized carbonyl groups of caffeine and the hydrogen atom of the acid. A secondary interaction probably occurs between the nonpolar parts of the molecules, and the resultant complex is "squeezed out" of the aqueous phase owing to the great internal pressure of water. These two effects lead to a high degree of interaction.

The complexation of esters is of particular concern to the pharmacist, since many important drugs belong to this class. The complexes formed between esters and amines, phenols, ethers and ketones, have been attributed to the hydrogen bonding between a nucleophilic carbonyl oxygen and an active hydrogen. This, however, does not explain the complexation of esters such as benzocaine, procaine, and tetracaine with caffeine, as reported by Higuchi et al.[10] There are no activated hydrogens on caffeine; the hydrogen in the number 8 position (formula I) is very weak ($K_a = 1 \times 10^{-14}$) and is not likely to enter into complexation. It might be suggested that, in the caffeine molecule, a relatively positive center exists that serves as a likely site of complexation. The caffeine molecule is numbered in I for convenience in the discussion. As observed in formula II, the nitrogen at the 2 position presumably can become strongly electrophilic or acidic just as it is in an imide, owing to the withdrawal of electrons by the

oxygens at position 1 and 3. An ester such as benzocaine also becomes polarized (formula III) in such a way that the carboxyl oxygen is nucleophilic or basic. The complexation can thus occur as a result of a dipole–dipole interaction between the nucleophilic carboxyl oxygen of benzocaine and the electrophilic nitrogen of caffeine.
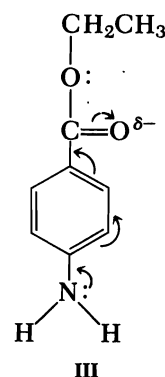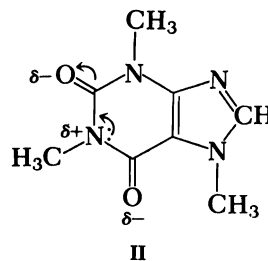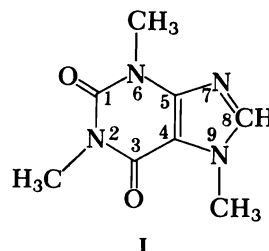
Caffeine forms complexes with organic acid *anions* that are more soluble than the pure xanthine, but the complexes formed with organic acids, such as gentisic acid, are less soluble than caffeine alone. Such insoluble complexes provide caffeine in a form that masks its normally bitter taste and should serve as a suitable state for chewable tablets. Higuchi and Pitman[11] synthesized 1:1 and 1:2 caffeine–gentisic acid complexes and measured their equilibrium solubility and rates of dissolution. Both the 1:1 and 1:2 complexes were less soluble in water than caffeine, and their dissolution rates were also less than that of caffeine. Chewable tablets formulated from these complexes should provide an extended-release form of the drug with improved taste.

York and Saleh[12] studied the effect of sodium salicylate on the release of benzocaine from topical vehicles, it being recognized that salicylates form molecular complexes with benzocaine. Complexation between drug and complexing agents can improve or impair drug absorption and bioavailability; the authors found that

the presence of sodium salicylate significantly influenced the release of benzocaine, depending on the type of vehicle involved. The largest increase in absorption was observed for a water-miscible polyethylene glycol base.

Polymer Complexes. Polyethylene glycols, polystyrene, carboxymethylcellulose, and similar polymers containing nucleophilic oxygens can form complexes with various drugs. The incompatibilities of certain polyethers, such as the Carbowaxes®, Pluronics®, and Tweens® with tannic acid, salicylic acid, and phenol, can be attributed to these interactions. Marcus[13] has reviewed some of the interactions that may occur in suspensions, emulsions, ointments, and suppositories. The incompatibility may be manifested as a precipitate, flocculate, delayed biologic absorption, loss of preservative action, or other undesirable physical, chemical, and pharmacologic effects.

Plaizier-Vercammen et al.[14] have studied the interaction of povidone (PVP) with ionic and neutral aromatic compounds. Several factors affect the binding to PVP of substituted benzoic acid and nicotine derivatives. While ionic strength has no influence, the binding increases in phosphate buffer solutions and decreases as the temperature is raised.

Crosspovidone, a cross-linked insoluble PVP, is able to bind drugs owing to its dipolar character and porous structure. Frömming et al.[15] studied the interaction of crosspovidone with acetaminophen, benzocaine, benzoic acid, caffeine, tannic acid, and papaverine hydrochloride, among other drugs. The interaction is mainly due to any phenolic groups on the drug. Hexylresorcinol shows exceptionally strong binding, but the interaction is less than 5% for most drugs studied (32 drugs). Crosspovidone is a disintegrant in pharmaceutical granules and tablets. It does not interfere with gastrointestinal absorption because the binding to drugs is reversible.

Solutes in parenteral formulations may migrate from the solution and interact with the wall of a polymeric container. Hayward et al.[16] showed that the ability of a polyolefin container to interact with drugs depends linearly on the octanol–water partition coefficient of the drug. For parabens and drugs that exhibit fairly significant hydrogen bond donor properties, a correction term related to hydrogen-bonding formation is needed. Polymer–drug container interactions may result in loss of the active component in liquid dosage forms.

Polymer–drug complexes are used to modify biopharmaceutical parameters of drugs; the dissolution rate of ajmaline is enhanced by complexation with PVP. The interaction is due to the aromatic ring of ajmaline and the amide groups of PVP to yield a dipole–dipole induced complex.[17]

Some molecular organic complexes of interest to the pharmacist are found in Table 11–3. (Complexes involving caffeine are listed in Table 11–6.)

INCLUSION COMPOUNDS

The class of addition compounds known as *inclusion* or *occlusion* compounds results more from the architecture of molecules than from their chemical affinity. One of the constituents of the complex is trapped in the open lattice or cage-like crystal structure of the other to yield a stable arrangement.

Channel Lattice Type. The *choleic acids* (bile acids) can form a group of complexes principally involving deoxycholic acid in combination with paraffins, organic acids, esters, ketones, and aromatic compounds and with solvents such as ether, alcohol, and dioxane. The crystals of deoxycholic acid are arranged to form a channel into which the complexing molecule can fit (cf. Fig. 11–3). Such stereospecificity should permit the resolution of optical isomers. In fact, camphor has been partially resolved by complexation with deoxycholic

TABLE 11–3. *Some Molecular Organic Complexes of Pharmaceutical Interest**

Agent	Compounds That Form Complexes with the Agent Listed in the First Column
Polyethylene glycols	*m*-Hydroxybenzoic acid, *p*-hydroxybenzoic acid, salicylic acid, *o*-phthalic acid, acetylsalicylic acid, resorcinol, catechol, phenol, phenobarbital, iodine (in I_2 • KI solutions), bromine (in presence of HBr).
Povidone (polyvinyl-pyrrolidone, PVP)	Benzoic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, salicylic acid, sodium salicylate, *p*-aminobenzoic acid, mandelic acid, sulfathiazole, chloramphenicol, phenobarbital.
Sodium carboxymethylcellulose	Quinine, benadryl, procaine, pyribenzamine.
Oxytetracycline and tetracycline	*N*-methylpyrrolidone, *N,N*-dimethylacetamide, γ-valerolactone, γ-butyrolactone, sodium *p*-aminobenzoate, sodium salicylate, sodium *p*-hydroxybenzoate, sodium saccharin, caffeine.

*Compiled from the results of T. Higuchi et al., J. Am. Pharm. Assoc., Sci. Ed. **43**, 393, 398, 456, 1954; ibid. **44**, 668, 1955, ibid. **45**, 157, 1956; ibid. **46**, 458, 587, 1957 and from J. L. Lach et al., Drug Standards **24**, 11, 1956. An extensive table of acceptor and donor molecules that form aromatic molecular complexes has been compiled by L. J. Andrews, Chem. Revs. **54**, 713, 1954. Also refer to T. Higuchi and K. A. Connors, Phase solubility techniques. *Advances in Analytical Chemistry and Instrumentation*, C. N. Reilley, Ed., New York, Wiley, 1965, pp. 117–212.
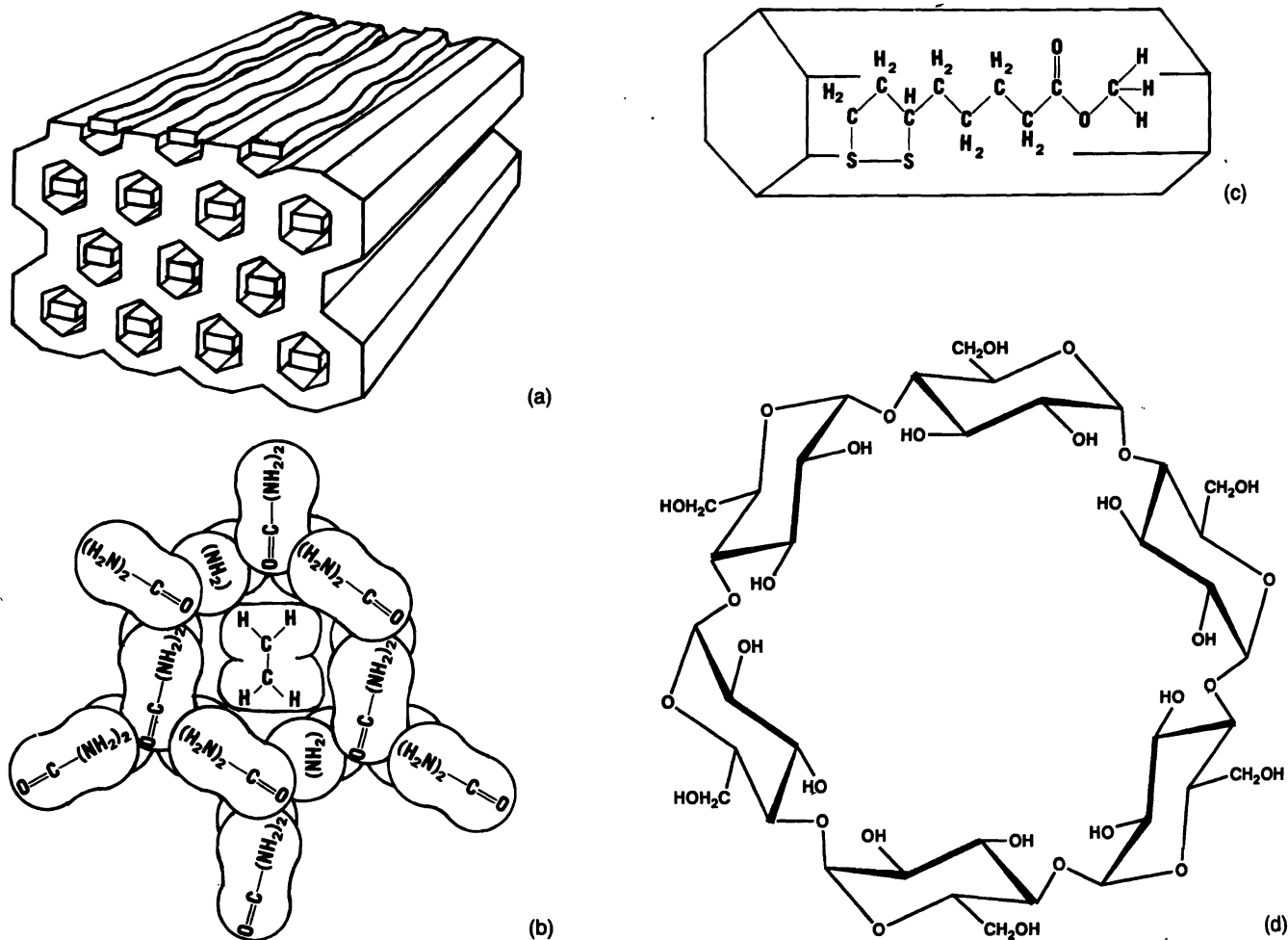
Fig. 11–3. (*a*) A channel complex formed with urea molecules as the host. As the lower sketch (*b*) shows, these molecules are packed in an orderly manner and held together by hydrogen bonds between nitrogen and oxygen atoms. The hexagonal channels, approximately 5 Å in diameter, provide room for guest molecules such as long chain hydrocarbons, as shown here. (From J. F. Brown, Jr., Sci. Am. **207**, 82, 1962. Copyright © 1962 by Scientific American, Inc. All rights reserved.) (*c*) A hexagonal channel complex (adduct) of methyl α-lipoate and 15 g of urea in methanol prepared with gentle heating. Needle crystals of the adduct separated overnight at room temperature. This inclusion compound or adduct begins to decompose at 63° C and melts at 163° C. Thiourea may also be used to form the channel complex. (From H. Mima and M. Nishikawa, J. Pharm. Sci. **53**, 931, 1964, reproduced with permission of the copyright owner.) (*d*) Cyclodextrin (cycloamylose, Schardinger dextrin). See *Merck Index*, Edition 11, Rahway, N.J., 1989, p. 425.

acid, and *dl*-terpineol has been resolved by the use of digitonin, which occludes certain molecules in a manner similar to that of deoxycholic acid.

Deoxycholic acid
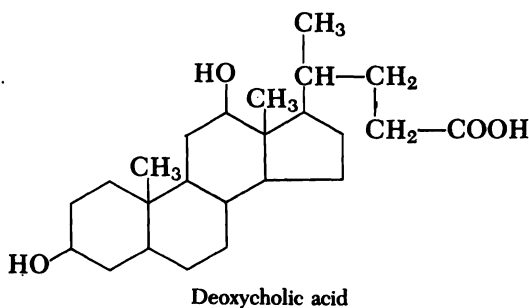
Urea and thiourea also crystallize in a channel-like structure permitting enclosure of unbranched paraffins, alcohols, ketones, organic acids, and other compounds, as shown in Figure 11–3*a* and *b*. The well-known starch-iodine solution is a channel-type complex consist-

ing of iodine molecules entrapped within spirals of the glucose residues.

Forman and Grady[18] found that monostearin, an interfering substance in the assay of dienestrol, could be extracted easily from dermatologic creams by channel-type inclusion in urea. They felt that urea inclusion might become a general approach for separation of long-chain compounds in assay methods. The authors reviewed the earlier literature on urea inclusion of straight-chain hydrocarbons and fatty acids.

Layer Type. Some compounds such as the clay montmorillonite, the principal constituent of bentonite, can trap hydrocarbons, alcohols, and glycols between the layers of their lattices.[19] Graphite can also intercalate compounds between its layers.

Clathrates.[20] The clathrates crystallize in the form of a cage-like lattice in which the coordinating compound is entrapped. Chemical bonds are not involved in these complexes, and only the molecular size of the encaged

component is of importance. Ketelaar[21] observed that the stability of a clathrate may be related to the confinement of a prisoner. The stability of a clathrate is due to the strength of the structure, that is, to the high energy that must be expended to decompose the compound, just as a prisoner is confined by the bars that prevent his escape.

Powell and Palin[22] have made a detailed study of clathrate compounds and have shown that the highly toxic agent hydroquinone (quinol) crystallizes in a cage-like hydrogen-bonded structure, as seen in Figure 11–4. The holes have a diameter of 4.? Å and permit the entrapment of one small molecule to about every two quinol molecules. Small molecules such as methyl alcohol, CO_2, and HCl may be trapped in these cages, but smaller molecules such as H_2 and larger molecules such as ethanol cannot be accommodated. It is possible that clathrates may be used to resolve optical isomers and to bring about other processes of molecular separation.

One official drug, warfarin sodium USP, is a clathrate of water, isopropyl alcohol, and sodium warfarin in the form of a white crystalline powder.

Monomolecular Inclusion Compounds. Cyclodextrins. Inclusion compounds are reviewed by Frank.[23] In addition to channel- and cage-type (clathrate) compounds, Frank adds classes of *mono-* and *macromolecular* inclusion compounds. Monomolecular inclusion compounds involve the entrapment of a single guest molecule in the cavity of one host molecule. Monomolecular host structures are represented by the cyclodextrins. These compounds are cyclic oligosaccharides containing a minimum of six D-(+)-glucopyranose units attached by α-1,4 linkages produced by the action on starch of *Bacillus macerans* amylase. The natural α, β, and γ cyclodextrins (α-CD, β-CD, and γ-CD) consist of 6, 7, and 8 units of glucose, respectively.
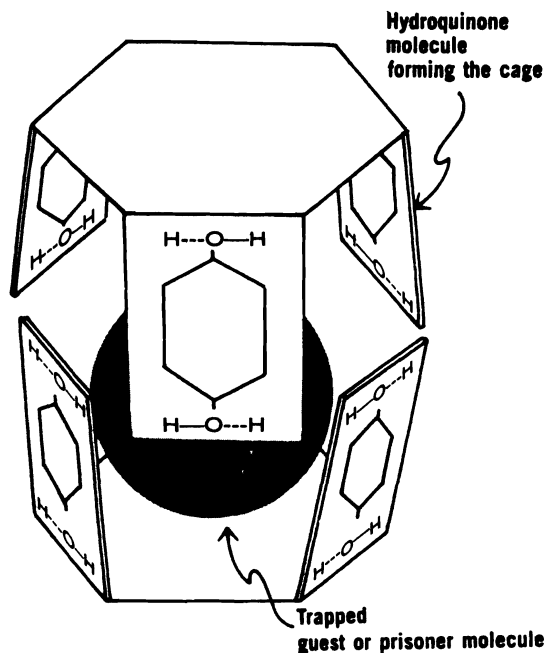
Their ability to form inclusion compounds in aqueous solution is due to the typical arrangement of the glucose units (see Fig. 11–3d). As observed in cross-section in the figure, the cyclodextrin structure forms a torus or doughnut ring. The molecule actually exists as a truncated cone, which is seen in Figure 11–5a; it can accommodate molecules such as mitomycin C to form inclusion compounds (Fig. 11–5b). The interior of the cavity is relatively hydrophobic because of the CH_2 groups, whereas the cavity entrances are hydrophilic owing to the presence of the primary and secondary hydroxyl groups.[24,25] α-CD has the smallest cavity (internal diameter almost 5 Å). β-CD and γ-CD are the most useful for pharmaceutical technology owing to their larger cavity size (internal diameter almost 6 Å and 8 Å, respectively). Water inside the cavity tends to be squeezed out and to be replaced by more hydrophobic species. Thus, molecules of appropriate size and stereochemistry can be included in the cyclodextrin cavity by hydrophobic interactions. (See pp. 272–273). Complexation does not ordinarily involve the formation of covalent bonds. Some drugs may be too large to be accommodated totally in the cavity. As

Fig. 11–4. Cage-like structure formed through hydrogen bonding of hydroquinone molecules. Small molecules such as methanol are trapped in the cages to form the clathrate. (Modified from J. F. Brown, Jr., Sci. Am. **207**, 82, 1962. Copyright © 1962 by Scientific American, Inc. All rights reserved.)

Fig. 11–5. (a) Representation of cyclodextrin as a truncated cone. (b) Mitomycin C partly enclosed in cyclodextrin to form an inclusion complex. (From O. Beckers, Int. J. Pharm. **52**, 240, 247, 1989, reproduced with permission of the copyright owner.)

shown in Figure 11–5*b*, mitomycin C interacts with γ-CD at one side of the torus. Thus, the aziridine ring

$$\left\langle \begin{array}{c} | \\ C \\ | \\ C \\ | \end{array} \right\rangle N{-}R$$
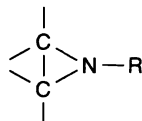
of mitomycin C is protected from degradation in acidic solution.[26] Bakensfield et al.[27] studied the inclusion of indomethacin with β-CD using an [1](H-N)MR technique. The *p*-chlorobenzoyl part of indomethacin (shaded part of Fig. 11–6) enters the β-CD ring, whereas the substituted indol moiety (the remainder of the molecule) is too large for inclusion and rests against the entrance of the CD cavity.

Cyclodextrins are studied as solubilizing and stabilizing agents in pharmaceutical dosage forms. Lach and associates[28] used cyclodextrins to trap, stabilize, and solubilize sulfonamides, tetracyclines, morphine, aspirin, benzocaine, ephedrine, reserpine, and testosterone. The aqueous solubility of retinoic acid (0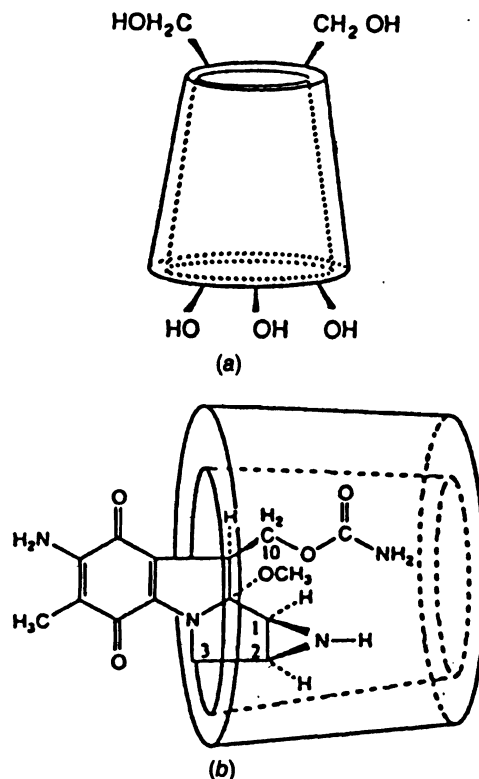.5 mg/liter), a drug used topically in the treatment of acne,[29] is increased to 160 mg/liter by complexation with β-CD. Dissolution rate plays an important role in bioavailability of drugs, fast dissolution usually favoring absorption. Thus, the dissolution rate of famotidine,[30] a potent drug in the treatment of gastric and duodenal ulcers, and tolbutamide, an oral antidiabetic drug, are both increased by complexation with β-cyclodextrin.[31]

Cyclodextrins may increase or decrease the reactivity of the guest molecule depending on the nature of the reaction and the orientation of the molecule within the CD cavity. Thus, α-cyclodextrin tends to favor pH-dependent hydrolysis of indomethacin in aqueous solution, whereas β-cyclodextrin inhibits it.[27] Unfortunately, the water solubility of β-CD (1.8 g/100 mL at 25° C) is often insufficient to stabilize drugs at therapeutic doses, and is also associated with nephrotoxicity when CD is administered by parenteral routes.[32] The relatively low aqueous solubility of the cyclodextrins may be due to the formation of intramolecular hydrogen bonds between the hydroxyl groups (see Fig. 11–3*d*), which prevent their interaction with water molecules.[33]

Derivatives of the natural crystalline CD have been developed to improve aqueous solubility and to avoid

toxicity. Partial methylation (alkylation) of some of the OH groups in CD reduces the intermolecular hydrogen bonding, leaving some OH groups free to interact with water, thus increasing the aqueous solubility of CD.[33] According to Müller and Brauns,[34] a low degree of alkyl substitution is preferable. Derivatives with a high degree of substitution lower the surface tension of water, and this has been correlated with the hemolytic activity observed in some CD derivatives. Amorphous derivatives of β-CD and γ-CD are more effective as solubilizing agents for sex hormones than the parent cyclodextrins. Complexes of testosterone with amorphous hydroxypropyl β-CD allow an efficient transport of hormone into the circulation when given sublingually.[35] This route avoids both metabolism of the drug in the intestines and rapid *first-pass* decomposition in the liver (see Chapter 19), thus improving bioavailability.

In addition to hydrophilic derivatives, hydrophobic forms of β-CD have been found useful as sustained-release drug carriers. Thus, the release rate of the water-soluble calcium antagonist diltiazem was significantly decreased by complexation with ethylated β-CD. The release rate was controlled by mixing hydrophobic and hydrophilic derivatives of cyclodextrins at several ratios.[36] Ethylated β-CD has also been used to retard the delivery of isosorbide dinitrate, a vasodilator.[37]

Cyclodextrins may improve the organoleptic characteristics of oral liquid formulations. The bitter taste of suspensions of femoxetine, an antidepressant, is greatly suppressed by complexation of the drug with β-cyclodextrin.[38]

Molecular Sieves. Macromolecular inclusion compounds, or *molecular sieves* as they are commonly called, include zeolites, dextrins, silica gels, and related substances. The atoms are arranged in three dimensions to produce cages and channels. Synthetic zeolites may be made to a definite pore size so as to separate molecules of different dimensions, and they are also capable of ion exchange. See the review article by Frank[23] for a detailed discussion of inclusion compounds.

METHODS OF ANALYSIS[39]

A determination of the *stoichiometric ratio* of ligand-to-metal or donor-to-acceptor and a quantitative expression of the *stability constant* for complex formation are important in the study and application of coordination compounds. A limited number of the more important methods for obtaining these two quantities are presented here.

Method of Continuous Variation. Job[40] suggested the use of an additive property such as the spectrophotometric extinction coefficient (dielectric constant or the square of the refractive index may also be used) for the measurement of complexation. If the property for two
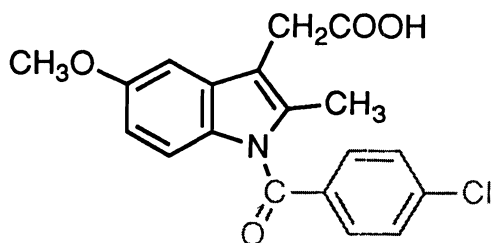

Fig. 11–6. Indomethacin (Indocin).

species is sufficiently different and if no interaction occurs when the components are mixed, then the value of the property is the weighted mean of the values of the separate species in the mixture. This means that if the additive property, say dielectric constant, is plotted against the mole fraction from 0 to 1 for one of the components of a mixture where no complexation occurs, a linear relationship is observed, as shown by the dotted line in Figure 11–7. If solutions of two species A and B of equal molar concentration (and hence of a fixed total concentration of the species) are mixed and if a complex forms between the two species, the value of the additive property will pass through a maximum (or minimum), as shown by the upper curve in Figure 11–7. For a constant total concentration of A and B, the complex is at its greatest concentration at a point where the species A and B are combined in the ratio in which they occur in the complex. The line therefore shows a break or a change in slope at the mole fraction corresponding to the complex. The change in slope occurs at a mole fraction of 0.5 in Figure 11–7, indicating a complex of the 1:1 type.

When spectrophotometric absorbance is used as the physical property, the observed values, obtained at various mole fractions when complexation occurs, are usually subtracted from the corresponding values that would have been expected had no complex resulted. This difference D is then plotted against mole fraction, as shown in Figure 11–8. From such a curve, the molar ratio of the complex is readily obtained. By means of a calculation involving the concentration, and the property being measured, the stability constant of the formation may be determined by a method described by Martell and Calvin.[41] Another method, suggested by Bent and French,[42] is given here.

If the magnitude of the measured property, such as absorbance, is proportional only to the concentration of the complex MA_n, the molar ratio of ligand A to metal
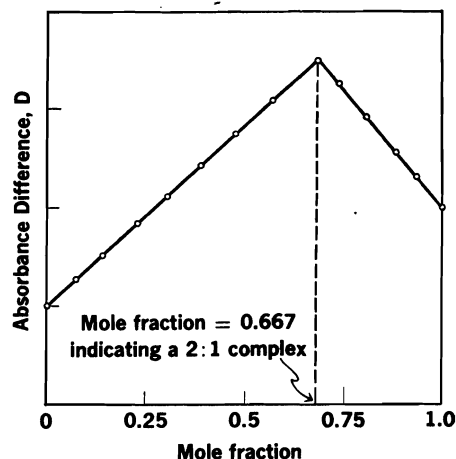
Fig. 11–8. A plot of absorbance difference against mole fraction showing the result of complexation.

M and the stability constant may be readily determined. The equation for complexation can be written as

$$M + nA = MA_n \qquad (11\text{–}3)$$

and the stability constant as

$$K = \frac{[MA_n]}{[M][A]^n} \qquad (11\text{–}4)$$

or in logarithmic form

$$\log [MA_n] = \log K + \log [M] + n \log [A] \qquad (11\text{–}5)$$

in which $[MA_n]$ is the concentration of the complex, $[M]$ the concentration of the uncomplexed metal, $[A]$ the concentration of the uncomplexed ligand, n the number of moles of ligand combined with one mole of metal ion, and K the equilibrium or *stability* constant for the complex. The concentration of a metal ion is held constant while the concentration of ligand is varied, and the corresponding concentration $[MA_n]$ of complex formed is obtained from the spectrophotometric analysis.[40] Now, according to equation (11–5), if $\log [MA_n]$ is plotted against $\log [A]$, the slope of the line yields the stoichiometric ratio or the number n of ligand molecules coordinated to the metal ion, and the intercept on the vertical axis allows one to obtain the stability constant, K, since $[M]$ is a known quantity.

Job restricted his method to the formation of a single complex; however, Vosburgh et al.[43] modified it so as to treat the formation of higher complexes in solution. Osman and Abu-Eittah[44] used spectrophotometric techniques to investigate 1:2 metal–ligand complexes of copper and barbiturates. A greenish-yellow complex is formed by mixing a blue solution of copper (II) with thiobarbiturates (colorless). By using the Job method, an apparent stability constant as well as the composition of the 1:2 complex was obtained.

pH Titration Method. This is one of the most reliable methods and can be used whenever the complexation is attended by a change in pH. The chelation of the cupric ion by glycine, for example, may be represented as
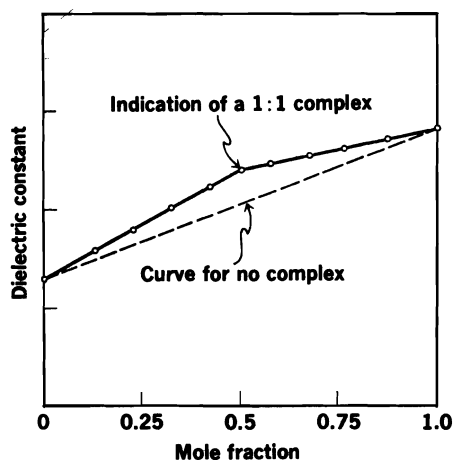

Fig. 11–7. A plot of an additive property against mole fraction of one of the species in which complexation between the species has occurred. The dotted line is that expected if no complex had formed. (C. H. Giles et al., J. Chem. Soc., 1952, 3799, should be referred to for similar figures.)
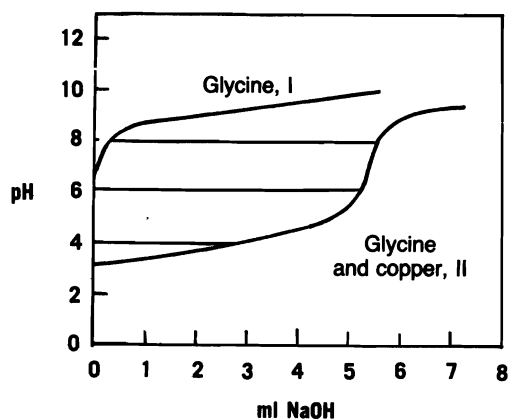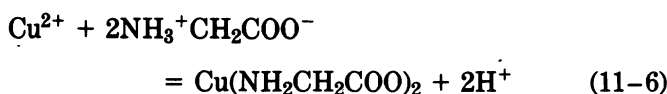
Fig. 11–9. Titration of glycine and of glycine in the presence of cupric ions. The difference in pH for a given quantity of base added indicates the occurrence of a complex.

$$Cu^{2+} + 2NH_3^+CH_2COO^-$$

$$= Cu(NH_2CH_2COO)_2 + 2H^+ \qquad (11-6)$$

Since two protons are formed in the reaction of equation (11–6), the addition of glycine to a solution containing cupric ions should result in a decrease in pH.

Titration curves can be obtained by adding a strong base to a solution of glycine, and to another solution containing glycine and a copper salt, and plotting the pH against the equivalents of base added. The results of such a potentiometric titration are shown in Figure 11–9. The curve for the metal–glycine mixture is well below that for the glycine alone, and the decrease in pH shows that complexation is occurring throughout most of the neutralization range. Similar results are obtained with other zwitterions and weak acids (or bases), such as N,N'-diacetylethylenediamine diacetic acid, which has been studied for its complexing action with copper and calcium ions.

The results can be treated quantitatively in the following manner to obtain stability constants for the complex. The two successive or stepwise equilibria between the copper ion or metal M and glycine or the ligand A may be written in general as

$$M + A = MA; \quad K_1 = \frac{[MA]}{[M][A]} \qquad (11-7)$$

$$MA + A = MA_2; \quad K_2 = \frac{[MA_2]}{[MA][A]} \qquad (11-8)$$
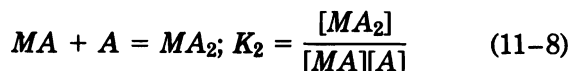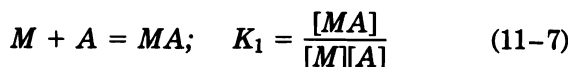
and the overall reaction (11–7 and 11–8) is

$$M + 2A = MA_2; \quad \beta = K_1K_2 = \frac{[MA_2]}{[M][A]^2} \quad (11-9)$$
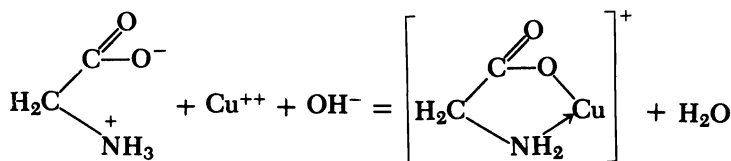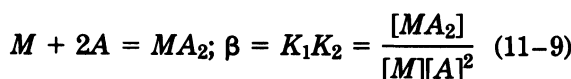
Bjerrum[45] called K_1 and K_2 the *formation constants*, while the equilibrium constant β for the overall reaction is known as the *stability constant*. A quantity n may now be defined. It is the number of ligand molecules bound to a metal ion. The *average* number of ligand groups bound per metal ion present is therefore designated $\bar{n}$ (n bar) and is written

$$\bar{n} = \frac{\text{(total concentration of ligand bound)}}{\text{(total concentration of metal ion)}} \qquad (11-10)$$

or

$$\bar{n} = \frac{[MA] + 2[MA_2]}{[M] + [MA] + [MA_2]} \qquad (11-11)$$

While n has a definite value for each species of complex (1 or 2 in this case), it may have any value between 0 and the largest number of ligand molecules bound, 2 in this case. The numerator of equation (11–11) gives the total concentration of ligand species bound. The second term in the numerator is multiplied by 2 since two molecules of ligand are contained in each molecule of the species, MA_2. The denominator gives the total concentration of metal present in all forms, both bound and free. For the special case in which $\bar{n} = 1$, equation (11–11) becomes

$$[MA] + 2[MA_2] = [M] + [MA] + [MA_2]$$

$$[MA_2] = [M] \qquad (11-12)$$

Employing the results in equations (11–9) and (11–12), we obtain the following relation:

$$\beta = K_1K_2 = \frac{1}{[A]^2} \text{ or } \log \beta = -2 \log [A]$$

and finally

$$p[A] = \tfrac{1}{2} \log \beta \text{ at } \bar{n} = 1 \qquad (11-13)$$

in which p[A] is written for $-\log [A]$. Bjerrum has also shown that, to a first approximation,

$$p[A] = \log K_1 \text{ at } \bar{n} = \tfrac{1}{2} \qquad (11-14)$$

$$p[A] = \log K_2 \text{ at } \bar{n} = \tfrac{3}{2} \qquad (11-15)$$

It should now be possible to obtain the individual complex formation constants K_1 and K_2 and the overall stability constant β if one knows two values: $\bar{n}$ and p[A].

Equation (11–10) shows that the concentration of bound ligand must be determined before $\bar{n}$ can be evaluated. The horizontal distances represented by the lines in Figure 11–9 between the titration curve for glycine alone (curve I) and for glycine in the presence of Cu^{2+} (curve II) give the amount of alkali used up in the reactions (equations 11–16 and 11–17):

$$(11-16)$$

$$\left[\begin{array}{c} O \\ \| \\ C-O \\ H_2C \quad \quad Cu \\ NH_2 \end{array}\right]^+ + H_2C\begin{array}{c} O \\ \| \\ C-O^- \\ \overset{+}{NH_3} \end{array} + OH^- = \left[\begin{array}{c} O \quad \quad NH_2 \\ \| \quad \quad \quad | \\ C-O \quad \quad \quad CH_2 \\ H_2C \quad \quad Cu \\ NH_2 \quad O-C \\ \| \\ O \end{array}\right] + H_2O \quad (11-17)$$
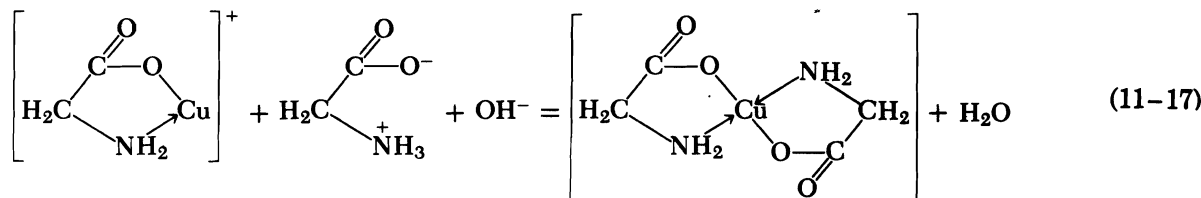
This quantity of alkali is exactly equal to the concentration of ligand bound at any pH, and, according to equation (11–10), when divided by the total concentration of metal ion, gives the value of $\bar{n}$.

The concentration of free glycine $[A]$ as the "base," $NH_2CH_2COO^-$, at any pH is obtained from the acid dissociation expression for glycine:

$$NH_3^+CH_2COO^- + H_2O = H_3O^+ + NH_2CH_2COO^-$$

$$K_a = \frac{[H_3O^+][NH_2CH_2COO^-]}{[NH_3^+CH_2COO^-]} \quad (11-18)$$
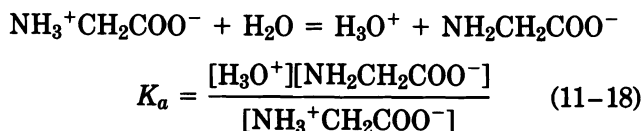
or

$$[NH_2CH_2COO^-] = [A] = \frac{K_a[HA]}{[H_3O^+]} \quad (11-19)$$

The concentration $[NH_3^+CH_2COO^-]$ or $[HA]$ of the acid species at any pH is taken as the difference between the initial concentration $[HA]_{init}$ of glycine and the concentration $[NaOH]$ of alkali added. Then

$$[A] = K_a\frac{([HA]_{init} - [NaOH])}{[H_3O^+]} \quad (11-20)$$

or

$$-\log[A] = p[A] = pK_a - pH$$
$$- \log([HA]_{init} - [NaOH]) \quad (11-21)$$

in which $[A]$ is the concentration of the ligand, glycine.

Example 11–1. If 75-mL samples containing 3.34×10^{-2} mole/liter of glycine hydrochloride alone and in combination with 9.45×10^{-3} mole/liter of cupric ion are titrated with 0.259 N NaOH, the two curves I and II respectively, in Figure 11–9 are obtained. Compute $\bar{n}$ and p[A] at pH 3.50 and pH 8.00. The pK_a of glycine is 9.69 at 30° C.

(a) From Figure 11–9, the horizontal distance at pH 3.50 for the 75-mL sample is 1.60 mL NaOH, or 2.59×10^{-4} mole/mL $\times 1.60 = 4.15 \times 10^{-4}$ mole. For a 1-liter sample, the value would be 5.54×10^{-3} mole. The total concentration of copper ion per liter is 9.45×10^{-3} mole, and $\bar{n}$ from equation (11–10) is

$$\bar{n} = \frac{5.54 \times 10^{-3}}{9.45 \times 10^{-3}} = 0.59$$

From equation (11–21),

p[A] = 9.69 − 3.50 − log [(3.34 × 10⁻²) − (5.54 × 10⁻³)] = 7.66

(b) At pH 8.00, the horizontal distance between the two curves I and II in Figure 11–9 is equivalent to 5.50 mL of NaOH in the 75-mL sample or $2.59 \times 10^{-4} \times 5.50 \times 1000/75 = 19.0 \times 10^{-3}$ mole/liter.

$$\bar{n} = \frac{19.0 \times 10^{-3}}{9.45 \times 10^{-3}} = 2.01$$

p[A] = 9.69 − 8.00 − log [(3.34 × 10⁻²) − (1.90 × 10⁻²)] = 3.15
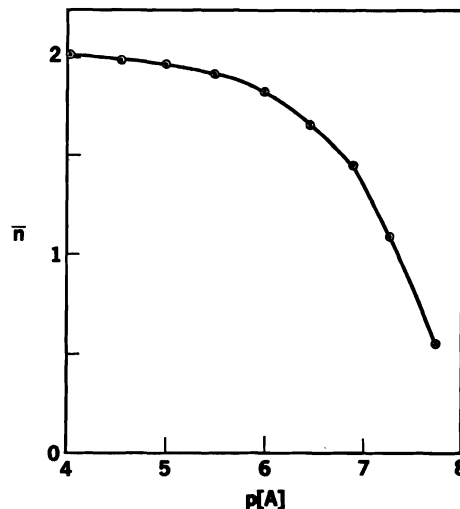
Fig. 11–10. Formation curve for the copper–glycine complex.

The values of $\bar{n}$ and p[A] at various pH values are then plotted as shown in Figure 11–10. The curve that is obtained is known as a *formation curve*. It is seen to reach a limit at $\bar{n} = 2$, signifying that the maximum number of glycine molecules that can combine with one atom of copper is two. From this curve at $\bar{n} = 0.5$, at $\bar{n} = \frac{3}{2}$, and at $\bar{n} = 1.0$, the approximate values for $\log K_1$, $\log K_2$, and $\log \beta$ respectively are obtained. A typical set of data for the complexation of glycine by copper is shown in Table 11–4. $\log K_1$, $\log K_2$, and $\log \beta$ values for some metal complexes of pharmaceutical interest are given in Table 11–5.

TABLE 11–4. *Potentiometric Titration of Glycine Hydrochloride (3.34×10^{-2} mole/liter, pK_a 9.69) and Cupric Chloride (9.45×10^{-3} mole/liter) in 75 mL Samples Using 0.259 N NaOH at 30° C*

pH	Δ mL NaOH (per 75-mL sample)	Moles OH⁻, MA Complexed (mole/liter)	$\bar{n}$	p[A]
3.50	1.60	5.54 × 10⁻³	0.59	7.66
4.00	2.90	10.1 × 10⁻³	1.07	7.32
4.50	3.80	13.1 × 10⁻³	1.39	6.85
5.00	4.50	15.5 × 10⁻³	1.64	6.44
5.50	5.00	17.3 × 10⁻³	1.83	5.98
6.00	5.20	18.0 × 10⁻³	1.91	5.50
6.50	5.35	18.5 × 10⁻³	1.96	5.02
7.00	5.45	18.8 × 10⁻³	1.99	4.53
7.50	5.50	19.0 × 10⁻³	2.03	4.03
8.00	5.50	19.0 × 10⁻³	2.01	3.15

From the data in the last two columns of Table 11–4, the formation curve, Figure 11–10, is plotted, and the following results are obtained from the curve: $\log K_1 = 7.9$, $\log K_2 = 6.9$, and $\log \beta = 14.8$ (average $\log \beta$ from the literature at 25° C is about 15.3).

TABLE 11–5.　*Selected Constants for Complexes between Metal Ions and Organic Ligands**

Organic Ligand	Metal Ion	$\log K_1$	$\log K_2$	$\log \beta = \log K_1 K_2$
Ascorbic acid	Ca^{2+}	0.19	—	—
Nicotinamide	Ag^+	—	—	3.2
Glycine (aminoacetic acid)	Cu^{2+}	8.3	7.0	15.3
Salicylaldehyde	Fe^{2+}	4.2	3.4	7.6
Salicylic acid	Cu^{2+}	10.6	6.3	16.9
p-Hydroxybenzoic acid	Fe^{3+}	15.2	—	—
Methyl salicylate	Fe^{3+}	9.7	—	—
Diethylbarbituric acid (barbital)	Ca^{2+}	0.66	—	—
8-Hydroxyquinoline	Cu^{2+}	15	14	29
Pteroylglutamic acid (folic acid)	Cu^{2+}	—	—	7.8
Oxytetracycline	Ni^{2+}	5.8	4.8	10.6
Chlortetracycline	Fe^{3+}	8.8	7.2	16.0

*From J. Bjerrum, G. Schwarzenback, and L. G. Sillen, *Stability Constants,* Part I, Organic Ligands, The Chemical Society, London, 1957.

Pecar et al.[46] described the tendency of pyrrolidone 5-hydroxamic acid to bind the ferric ion to form mono, bis, and tris chelates. These workers later studied the thermodynamics of these chelates using a potentiometric method to determine stability constants. The method employed by Pecar et al. is known as the *Schwarzenbach method* and may be used, instead of the potentiometric method described here, when complexes are unusually stable. Sandman and Luk[47] measured the stability constants for lithium catecholamine complexes by potentiometric titration of the free lithium ion. The results demonstrated that lithium forms complexes with the zwitterionic species of catecholamines at pH 9 to 10 and with deprotonated forms at pH values above 10. The interaction with lithium depends on the dissociation of the phenolic oxygen of catecholamines. At physiologic pH, the protonated species show no significant complexation. Some lithium salts such as lithium carbonate, lithium chloride, and lithium citrate are used in psychiatry.

Agrawal et al.[48] applied a pH titration method to estimate the average number of ligand groups per metal ion, $\bar{n}$, for several metal–sulfonamide chelates in dioxane–water. The maximum $\bar{n}$ values obtained indicate 1:1 and 1:2 complexes. The linear relationship between the pK_a of the drugs and the log of the stability constants of their corresponding metal ion complexes shows that the more basic ligands (drugs) give the more stable chelates with cerium IV, palladium II, and copper II. A potentiometric method was described in detail by Connors et al.[49] for the inclusion-type complexes formed between α-cyclodextrin and substituted benzoic acids.

Distribution Method. The method of distributing a solute between two immiscible solvents (p. 237) can be used to determine the stability constant for certain complexes. The complexation of iodine by potassium iodide may be used as an example to illustrate the method. The equilibrium reaction in its simplest form is

$$I_2 + I^- = I_3^- \qquad (11-22)$$

Addition steps also occur in polyiodide formation; for example, $2I^- + 2I_2 = I_6^{2-}$ may occur at higher concentrations, but it need not be considered here.

Example 11–2. When iodine is distributed between water (w) at 25°C and carbon disulfide as the organic phase (o), as depicted in Figure 11–11, the distribution constant $K(o/w) = C_o/C_w$ is found to be 625. When it is distributed between a 0.1250-M solution of potassium iodide and carbon disulfide, the concentration of iodine in the organic solvent is found to be 0.1896 mole/liter. The aqueous KI solution is analyzed, and the concentration of iodine is found to be 0.02832 mole/liter.

In summary, the results are:

Total concentration of I_2 in the aqueous
　　　　layer (free + complexed iodine):

　　　　　　　　　　　　　　　　0.02832 mole/liter

Total concentration of KI in the aqueous
　　　　layer (free + complexed KI):

　　　　　　　　　　　　　　　　0.1250 mole/liter

Concentration of I_2 in the CS_2 layer (free): 0.1896 mole/liter

Distribution coefficient, $K(o/w) = [I_2]_o / [I_2]_w = 625$

The species common to both phases is the free or uncomplexed iodine; the distribution law expresses only the concentration of *free* iodine, whereas a chemical analysis yields the *total* concentration of iodine in the aqueous phase. The concentration of free iodine in the aqueous phase is obtained as follows:

$$[I_2]_w = \frac{[I_2]_o}{K(o/w)} = \frac{0.1896}{625} = 3.034 \times 10^{-4} \text{ mole/liter}$$
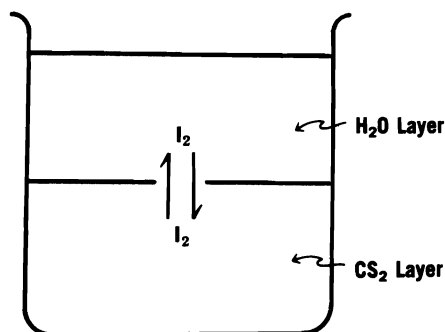

Fig. 11–11. The distribution of iodine between water and carbon disulfide.

To obtain the concentration of iodine in the complex and hence the concentration of the complex $[I_3^-]$, one subtracts the free iodine from the total iodine of the aqueous phase:

$$[I_2]_{complexed} = [I_2]_{w, total} - [I_2]_{w, free}$$
$$= 0.02832 - 0.000303$$
$$= 0.02802 \text{ mole/liter}$$

According to equation (11–22), I_2 and KI combine in equimolar concentrations to form the complex. Therefore,

$$[KI]_{complexed} = [I_2]_{complexed} = 0.02802 \text{ mole/liter}$$

KI is insoluble in carbon disulfide and remains entirely in the aqueous phase. The concentration of *free* KI is thus

$$[KI]_{free} = [KI]_{total} - [KI]_{complexed}$$
$$= 0.1250 - 0.02802$$
$$= 0.09698 \text{ mole/liter}$$

and finally

$$K = \frac{[\text{complex}]}{[I_2]_{free}\,[KI]_{free}}$$
$$= \frac{0.02802}{0.000303 \times 0.09698} = 954$$

Higuchi and his associates investigated the complexing action of caffeine, polyvinylpyrrolidone, and polyethylene glycols on a number of acidic drugs using the partition or distribution method. According to Higuchi and Zuck,[50] the reaction between caffeine and benzoic acid to form the benzoic acid–caffeine complex is

Benzoic acid + caffeine = (benzoic acid – caffeine)

$$(11-23)$$
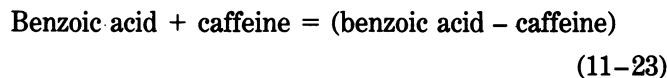
and the stability constant for the reactions at 0° C is

$$K = \frac{[\text{benzoic acid} - \text{caffeine}]}{[\text{benzoic acid}][\text{caffeine}]} = 37.5 \quad (11-24)$$

The results varied somewhat, the value 37.5 being an average stability constant. Guttman and Higuchi[51] later showed that caffeine exists in aqueous solution primarily as a monomer, dimer, and tetramer, which would account in part for the variation in K as observed by Higuchi and Zuck.

Solubility Method. According to the solubility method, excess quantities of the drug are placed in well-stoppered containers, together with a solution of the complexing agent in various concentrations, and the bottles are agitated in a constant-temperature bath until equilibrium is attained. Aliquot portions of the supernatant liquid are removed and analyzed.

Higuchi and Lach[52] used the solubility method to investigate the complexation of *p*-aminobenzoic acid (PABA) by caffeine. The results are plotted as shown in Figure 11–12, and the graph is explained as follows. The point *A* at which the line crosses the vertical axis is the solubility of the drug in water. With the addition of caffeine, the solubility of *p*-aminobenzoic acid rises linearly owing to complexation. At point *B*, the solution is saturated with respect to the complex and to the drug itself. The complex continues to form and to precipitate
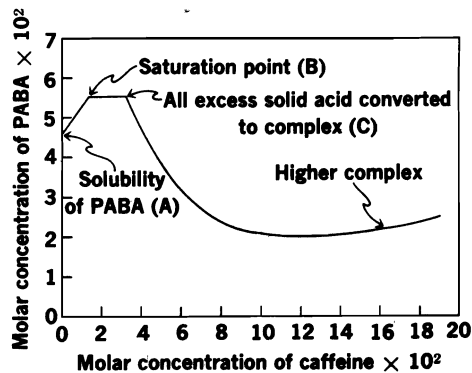
Fig. 11–12. The solubility of *para*-aminobenzoic acid in the presence of caffeine. (After T. Higuchi and J. L. Lach, J. Am. Pharm. Assoc., Sci. Ed. 43, 525, 1954).

from the saturated system as more caffeine is added. At point *C*, all the excess solid PABA has passed into solution and has been converted to the complex. Although the solid drug is exhausted and the solution is no longer saturated, some of the PABA remains uncomplexed in solution, and it combines further with caffeine to form higher complexes such as (PABA–2 caffeine) as shown by the curve at the right of the diagram.

Example 11–3. The following calculations are made to obtain the stoichiometric ratio of the complex. The concentration of caffeine, corresponding to the plateau *BC*, equals the concentration of caffeine entering the complex over this range, and the quantity of *p*-aminobenzoic acid entering the complex is obtained from the undissolved solid remaining at point *B*. It is computed by subtracting the acid in solution at the saturation point *B* from the total acid initially added to the mixture, since this is the amount yet undissolved that can form the complex.

The concentration of caffeine in the plateau region is found from Figure 11–12 to be 1.8×10^{-2} mole/liter. The free undissolved solid PABA is equal to the total acid minus the acid in solution at point *B*, namely, $7.3 \times 10^{-2} - 5.5 \times 10^{-2}$ or 1.8×10^{-2} mole/liter, and the stoichiometric ratio is

$$\frac{\text{Caffeine in complex}}{\text{PABA in complex}} = \frac{1.8 \times 10^{-2}}{1.8 \times 10^{-2}} = 1$$

The complex formation is therefore written

$$\text{PABA} + \text{caffeine} \rightleftharpoons \text{PABA–caffeine} \quad (11-25)$$

and the stability constant for this 1:1 complex is

$$K = \frac{[\text{PABA–caffeine}]}{[\text{PABA}][\text{caffeine}]} \quad (11-26)$$

K may be computed as follows. The concentration of the complex [PABA–caffeine] is equal to the total acid concentration at saturation less the solubility [PABA] of the acid in water. The concentration [caffeine] in the solution at equilibrium is equal to the caffeine added to the system less the concentration that has been converted to the complex. The total acid concentration of saturation is 4.58×10^{-2} mole/liter when no caffeine is added (solubility of PABA), and is 5.312×10^{-2} mole/liter when 1.00×10^{-2} mole/liter of caffeine is added.

$$[\text{PABA–caffeine}] = (5.31 \times 10^{-2}) - (4.58 \times 10^{-2}) = 0.73 \times 10^{-2}$$
$$[\text{PABA}] = 4.58 \times 10^{-2}$$
$$[\text{caffeine}] = (1.00 \times 10^{-2}) - (0.73 \times 10^{-2}) = 0.27 \times 10^{-2}$$

therefore

$$K = \frac{[PABA-caffeine]}{[PABA][caffeine]} = \frac{0.73 \times 10^{-2}}{(4.58 \times 10^{-2})(0.27 \times 10^{-2})} = 59$$

The stability constants for a number of caffeine complexes obtained principally by the distribution and the solubility methods are found in Table 11–6. Stability constants for a number of other drug complexes have been compiled by Higuchi and Connors.[53] Kenley et al.[54] studied water-soluble complexes of various ligands with the antiviral drug acyclovir using the solubility method.

Spectroscopy and Change Transfer Complexation. Absorption spectroscopy in the visible and ultraviolet regions of the spectrum is commonly used to investigate electron donor–acceptor or charge-transfer complexation.[55,56] When iodine is analyzed in a noncomplexing solvent such as CCl_4, a curve is obtained with a single peak at about 520 nm. The solution is violet in color. A solution of iodine in benzene exhibits a maximum shift to 475 nm, and a new peak of considerably higher intensity for the charge-shifted band appears at 300 nm. A solution of iodine in diethyl ether shows a still greater shift to lower wavelength and the appearance of a new maximum. These solutions are red to brown in color. Their curves are observed in Figure 11–13. In benzene and ether, iodine is the electron acceptor and the organic solvent is the donor; in CCl_4, no complex is formed. The shift towards the ultraviolet region becomes greater as the electron donor solvent becomes a stronger electron-releasing agent. These spectra arise from the transfer of an electron from the donor to the acceptor in close contact in the excited state of the complex. The more easily a donor such as benzene or diethyl ether releases its electron, as measured by its ionization potential, the stronger it is as a donor. Ionization potentials of a series of donors produce a straight line when plotted against the frequency maxi-
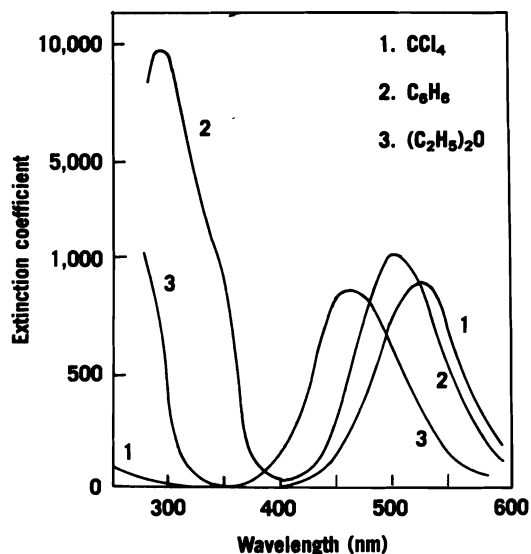
Fig. 11–13. Absorption curve of iodine in the noncomplexing solvent, (1) CCl_4, and the complexing solvents, (2) benzene, and (3) diethyl ether. (From H. A. Benesi and J. A. Hildebrand, J. Am. Chem. Soc. **70,** 2832, 1948.)

mum or charge-transfer energies (1 nm = 18.63 cal/mole) for solutions of iodine in the donor solvents.[55,56]

The complexation constant, K, may be obtained by use of visible and ultraviolet spectroscopy. The association between the donor D and acceptor A is represented as

$$D + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} DA \qquad (11-27)$$
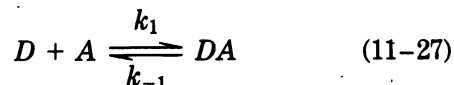
in which $K = \dfrac{k_1}{k_{-1}}$ is the equilibrium constant for complexation (stability constant), and k_1 and k_{-1} are the interaction rate constants. When two molecules associate according to this scheme and the absorbance A of the charge transfer band is measured at a definite wavelength, K is readily obtained from the *Benesi-Hildebrand equation*[57]:

$$\frac{A_0}{A} = \frac{1}{\epsilon} + \frac{1}{K\epsilon}\frac{1}{D_0} \qquad (11-28)$$

A_0 and D_0 are initial concentrations of the acceptor and donor species, respectively, in mole/liter, ϵ is the molar absorptivity of the charge-transfer complex at its particular wavelength, and K, the stability constant, is given in liter/mole or M^{-1}. A plot of A_0/A versus $1/D_0$ results in a straight line with a slope of $1/(K\epsilon)$ and an intercept of $1/\epsilon$, as observed in Figure 11–14.

Borazan et al.[58] investigated the interaction of nucleic acid bases (electron acceptors) with catechol, epinephrine, and isoproterenol (electron donors). Catechols have low ionization potentials and hence a tendency to donate electrons. Charge-transfer complexation was evident as demonstrated by ultraviolet absorption measurements. Assuming 1:1 complexes, the equilibrium constants K for charge-transfer inter-

TABLE 11–6. *Approximate Stability Constants of Some Caffeine Complexes in Water at 30° C**

Compound Complexed with Caffeine	Approximate Stability Constant
Suberic acid	3
Sulfadiazine	7
Picric acid	8
Sulfathiazole	11
o-Phthalic acid	14
Acetylsalicylic acid	15
Benzoic acid (monomer)	18
Salicylic acid	40
p-Aminobenzoic acid	48
Butylparaben	50
Benzocaine	59
p-Hydroxybenzoic acid	>100

*Compiled from T. Higuchi et al., J. Am. Pharm. Assoc., Sci. Ed., **42,** 138, 1953; ibid. **43,** 349, 524, 527, 1954; ibid. **45,** 290, 1956; ibid. **46,** 32, 1957. Over 500 such complexes with other drugs are recorded by Higuchi and Connors, Phase solubility techniques, in *Advances in Analytical Chemistry and Instrumentation,* C. N. Reilley, Ed., Wiley, Vol. 4, 1965, pp. 117–212.
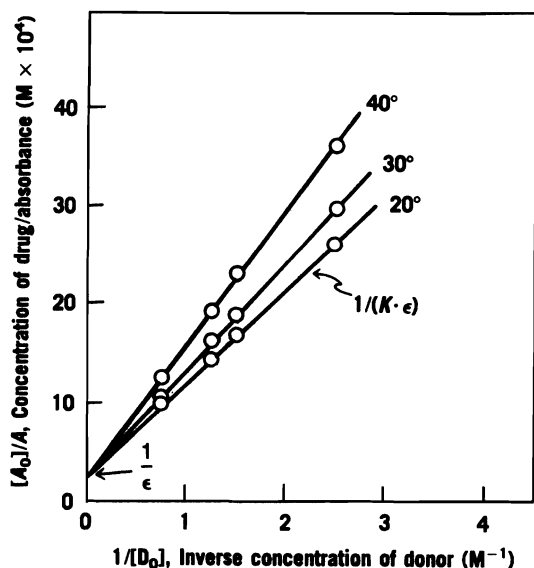
Fig. 11–14. A Benesi–Hildebrand plot to obtain the stability constant, K, from equation (11–28) for charge transfer complexation. (From M. A. Slifkin, Biochim. Biophys. Acta 109, 617, 1965.)

action were obtained from Benesi–Hildebrand plots, Figure 11–14, at three or four temperatures, and $\Delta H°$ was obtained at these same temperatures from the slope of the line as plotted in Figure 11–15. The values of K and the thermodynamic parameters $\Delta H°$, $\Delta G°$, and $\Delta S°$ are found in Table 11–7. The thermodynamic values are calculated according to methods described on pages 274 to 277.

Example 11–4. When A_0/A is plotted against $1/D_0$ for catechol (electron-donor) solutions containing uracil (electron acceptor) in 0.1 N HCl at 6°, 18°, 25°, and 37° C, the four lines were observed to intersect the vertical axis at 0.01041. Total concentration, A_0, for uracil was $2 \times 10^{-2}\ M$, and D_0 for catechol ranged from 0.3 to 0.8 M. The slopes of the lines determined by the least-squares method, were

| 6° C	18° C	25° C	37° C
0.02125	0.02738	0.03252	0.04002
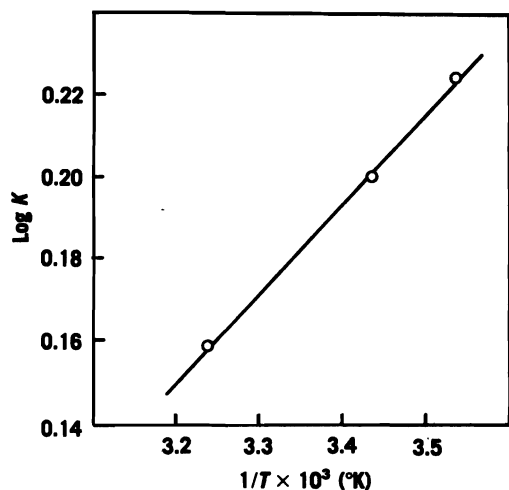
Fig. 11–15. Adenine–catechol stability constant for charge-transfer complexation measured at various temperatures at a wavelength of 340 nm. (From F. A. Al-Obeidi and H. N. Borazan, J. Pharm. Sci. 65, 892, 1976, reproduced with permission of the copyright owner.)

TABLE 11–7. *Stability Constant, K, and Thermodynamic Parameters for Charge-Transfer Interaction of Nucleic Acid Bases with Catechol in Aqueous Solution.*[*]

Temperature (°C)	K (M^{-1})	$\Delta G°$ (cal/mole)	$\Delta H°$ (cal/mole)	$\Delta S°$ (cal/(deg mole))
Adenine–Catechol				
9	1.69	−294		
18	1.59	−264	−1015	−2.6
37	1.44	−226		
Uracil–Catechol				
6	0.49	394		
18	0.38	533	−3564	−14
25	0.32	625		
37	0.26	745		

Calculate the molar absorptivity and the stability constants, K. Knowing K at these four temperatures, how does one proceed to obtain $\Delta H°$, $\Delta G°$, and $\Delta S°$?

The intercept, from the Benesi–Hildebrand equation, is the reciprocal of the molar absorptivity, or $1/(0.01041) = 96.1$. The molar absorptivity, ϵ, is a constant for a compound or a complex, independent of temperature or concentration. K is obtained from the slope of the four curves:

$$(1)\ 0.02125 = 1/(K \times 96.1);\ K = 0.49\ M^{-1}$$
$$(2)\ 0.02738 = 1/(K \times 96.1);\ K = 0.38\ M^{-1}$$
$$(3)\ 0.03252 = 1/(K \times 96.1);\ K = 0.32\ M^{-1}$$
$$(4)\ 0.04002 = 1/(K \times 96.1);\ K = 0.26\ M^{-1}$$

These K values are then plotted as their logarithms on the vertical axis of a graph against the reciprocal of the four temperatures, converted to degrees Kelvin. This is a plot of equation (11–49), and yields $\Delta H°$ from the slope of the line. $\Delta G°$ is calculated from log K at each of the four temperatures using equation (11–48), in which the temperature, T, is expressed in degrees Kelvin. $\Delta S°$ is finally obtained using equation (11–51), $\Delta G° = \Delta H° - T \Delta S°$. The answers to this sample problem are found in Table 11–7. The details of the calculation are explained in *Example 11–8*.

Webb and Thompson[59] studied the possible role of electron donor–acceptor complexes in drug receptor binding using quinoline and naphthalene derivatives as model electron donors and a trinitrofluorene derivative as the electron acceptor. The most favorable arrangement for the donor 8-aminoquinoline (heavy lines) and the acceptor 9-dicyanomethylene trinitrofluorene (light lines), as calculated by a quantum chemical method, is the arrangement:
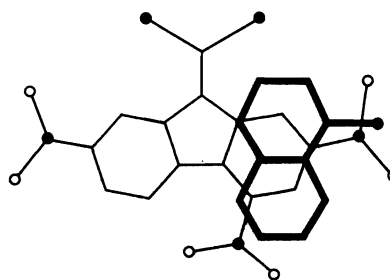
Filled circles are nitrogen and open circles oxygen atoms. The donor lies above the acceptor molecule at an intermolecular distance of about 3.35 Å and is attached by a binding energy of -5.7 kcal/mole. The negative sign signifies a positive binding force.

Other Methods. A number of other methods are available for studying the complexation of metal and organic molecular complexes. They include NMR and infrared spectroscopy, polarography, circular dichroism, kinetics, x-ray diffraction, and electron diffraction. Several of these will be discussed briefly in this section.

Complexation of caffeine with L-tryptophan in aqueous solution was investigated by Nishijo et al.[60] using ^{1}H–NMR spectroscopy. Caffeine interacts with L-tryptophan at a molar ratio of 1:1 by parallel stacking. Complexation is a result of polarization and π–π interactions of the aromatic rings. A possible mode of parallel stacking is shown in Figure 11–16. This study demonstrates that tryptophan, which is presumed to be the binding site in serum albumin for certain drugs, can interact with caffeine even as free amino acid. However, caffeine does not interact with other aromatic amino acids such as L-valine or L-leucine.

Borazan and Koumriqian[61] studied the coil–helix transition of polyadenylic acid induced by the binding of the catecholamines norepinephrine and isoproterenol using circular dichroism (see p. 98). Most mRNA molecules contain regions of polyadenylic acid, which are thought to increase the stability of mRNA and to favor genetic code translation. The change of the circular dichroism spectrum (see Chapter 4, p. 98) of polyadenylic acid was interpreted as being due to intercalative binding of catecholamines between the stacked adenine bases. These researchers suggested that catecholamines may exert a control mechanism through induction of the coil to helix transition of polyadenylic acid which influences genetic code translation.

De Taeye and Zeegers-Huyskens[62] used infrared spectroscopy to investigate the hydrogen bonded complexes involving polyfunctional bases such as proton donors. This is a very precise technique to determine the thermodynamic parameters involved in the hydrogen bond formation and to characterize the interaction sites when the molecule has several groups available to form hydrogen bonds. Caffeine forms hydrogen bonded complexes with various proton donors: phenol, phenol derivatives, aliphatic alcohols, and water. From the infrared technique, the preferred hydrogen bonding sites are the carbonyl functions of caffeine. Seventy percent of the complexes is formed at the C=O(6) group and thirty percent of the complexes at the C=O(2) function of caffeine (see structure I, p. 256, for numbering of the atoms of caffeine). El Said et al.[63] used conductometric and infrared methods to characterize 1:1 complexes between uranyl acetate and tetracycline. The structure suggested for the uranyl-tetracycline complex is
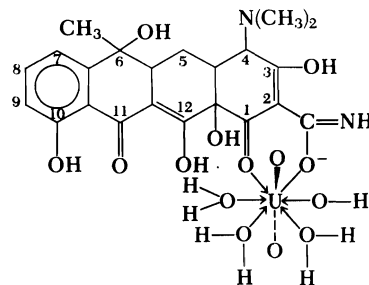
PROTEIN BINDING

The binding of drugs to proteins contained in the body can influence their action in a number of ways. Proteins may (a) facilitate the distribution of drugs throughout the body, (b) inactivate the drug by not enabling a sufficient concentration of free drug to develop at the receptor site, or (c) retard the excretion of a drug. The interaction of a drug with proteins may cause (a) the displacement of body hormones or a coadministered agent, (b) a configurational change in the protein, the structurally altered form of which is capable of binding a coadministered agent, or (c) the formation of a drug–protein complex that itself is biologically active. These topics are discussed in a number of reviews.[64,65] Among the plasma proteins, albumin is the most important owing to its high concentration relative to the other proteins and owing also to its ability to bind both acidic and basic drugs. Another plasma protein, α_1-acid glycoprotein, has been shown to bind numerous drugs; this protein appears to have greater affinity for basic than for acidic drug molecules.

A complete analysis of protein binding, including the multiple equilibria that are involved, would go beyond
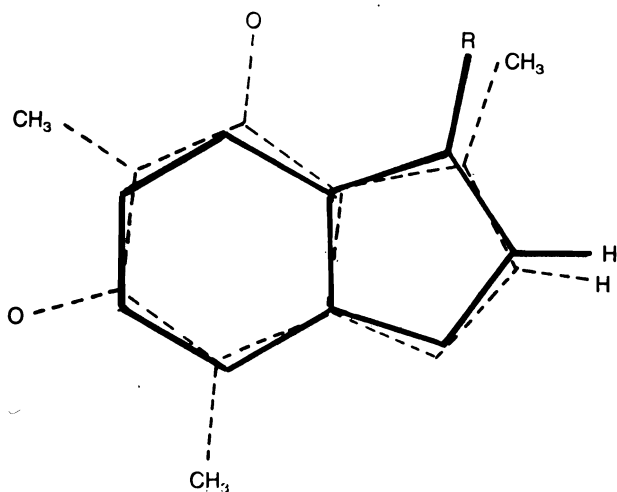

Fig. 11–16. Stacking of L-tryptophan (solid line) overlying caffeine (dashed line). The benzene ring of tryptophan is located above the pyrimidine ring of caffeine, and the pyrrole ring of L-tryptophan above the imidazole ring of caffeine. (From J. Nishijo, I. Yonetami, E. Iwamoto, et al., J. Pharm. Sci. **79,** 18, 1990, reproduced with permission of the copyright owner.)

our immediate needs. Therefore, only an abbreviated treatment is given here.

Binding Equilibria. The interaction between a group or free receptor P in a protein and a drug molecule D is written

$$P + D \rightleftharpoons PD \qquad (11-29)$$

The equilibrium constant, disregarding the difference between activities and concentrations, is

$$K = \frac{[PD]}{[P][D_f]} \qquad (11-30a)$$

or

$$K[P][D_f] = [PD] \qquad (11-30b)$$

in which K is the association constant, $[P]$ is the concentration of the protein in terms of free binding sites, $[D_f]$ is the concentration, usually given in moles, of free drug, sometimes called the ligand, and $[PD]$ is the concentration of the protein–drug complex. K varies with temperature and would be better represented as $K(T)$, $[PD]$, the symbol for bound drug is sometimes written as $[D_b]$ and $[D]$, the free drug, as $[D_f]$.

If the total protein concentration is designated as $[P_t]$, we can write

$$[P_t] = [P] + [PD]$$

or

$$[P] = [P_t] - [PD] \qquad (11-31)$$

Substituting the expression for $[P]$ from (11–31) into (11–30b) gives

$$[PD] = K[D_f]([P_t] - [PD]) \qquad (11-32)$$

$$[PD] + K[D_f][PD] = K[D_f][P_t] \qquad (11-33)$$

$$\frac{[PD]}{[P_t]} = \frac{K[D_f]}{1 + K[D_f]} \qquad (11-34)$$

Let r be the number of moles of drug bound $[PD]$ per mole of total protein $[P_t]$; then $r = [PD]/[P_t]$ or

$$r = \frac{K[D_f]}{1 + K[D_f]} \qquad (11-35)$$

The ratio r may also be expressed in other dimensions, such as milligrams of drug bound x per gram of protein m. Equation (11–35) is one form of the Langmuir adsorption isotherm to be found on page 381. Although it is quite useful for expressing protein binding data, it must not be concluded that obedience to this formula necessarily requires that protein binding be an adsorption phenomenon. Expression (11–35) can be converted to a linear form, convenient for plotting, by inverting it:

$$\frac{1}{r} = \frac{1}{K[D_f]} + 1 \qquad (11-36)$$

If ν independent binding sites are available, the expression for r, equation (11–35), is simply ν times that for a single site, or

$$r = \nu \frac{K[D_f]}{1 + K[D_f]} \qquad (11-37)$$

and equation (11–36) becomes

$$\frac{1}{r} = \frac{1}{\nu K} \frac{1}{[D_f]} + \frac{1}{\nu} \qquad (11-38)$$

Equation (11–38) produces what is called a *Klotz reciprocal plot*.[66]

An alternative manner of writing equation (11–37) is to rearrange it first to

$$r + rK[D_f] = \nu K[D_f] \qquad (11-39)$$

and subsequently to

$$\frac{r}{[D_f]} = \nu K - rK \qquad (11-40)$$

Data presented according to equation (11–40) are known as a *Scatchard plot*.[66,67] The binding of bis-hydroxycoumarin to human serum albumin is shown as a Scatchard plot in Figure 11–17.

Graphical treatment of data using equation (11–38) heavily weights those experimental points obtained at low concentrations of free drug D and may therefore lead to misinterpretations regarding the protein binding behavior at high concentrations of free drug. Equation (11–40) does not have this disadvantage and is the method of choice for plotting data. Curvature in these plots usually indicates the existence of more than one type of binding site.
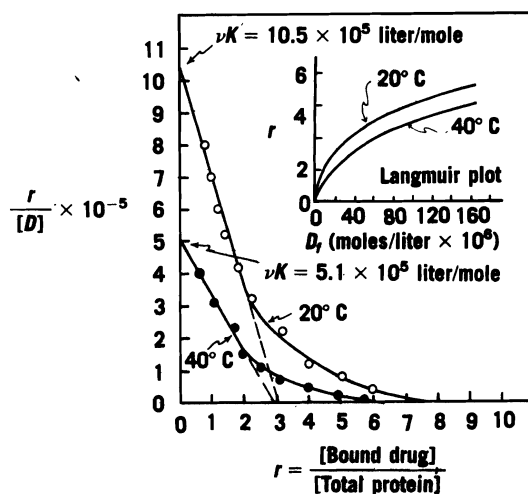
Fig. 11–17. A Scatchard plot showing the binding of bis-hydroxy-coumarin to human serum albumin at 20° and 40° C plotted according to equation (11–40). Extrapolation of the two lines to the horizontal axis, assuming a single class of sites with no electrostatic interaction, gives an approximate value of 3 for ν. (From M. J. Cho, A. G. Mitchell and M. Pernarowski, J. Pharm. Sci. **60**, 196, 1971; **60**, 720, 1971, reproduced with permission of the copyright owner.) The insert is a Langmuir adsorption isotherm of the binding data plotted according to equation (11–35).

Equations (11–38) and (11–40) cannot be used for the analysis of data if the nature and the amount of protein in the experimental system is unknown. In these situations, Sandberg et al.[68] recommend the use of a slightly modified form of equation (11–40):

$$\frac{[D_b]}{[D_f]} = -K[D_b] + \nu K[P_t] \qquad (11\text{–}41)$$

in which $[D_b]$ is the concentration of bound drug. Equation (11–41) is plotted as the ratio $[D_b]/[D_f]$ versus $[D_b]$, and in this way K is determined from the slope while $\nu K[P_t]$ is determined from the intercept.

The Scatchard plot yields a straight line when only one class of binding sites is present. Frequently in drug binding studies, n classes of sites exist, each class i having ν_i sites with a unique association constant K_i. In such a case, the plot of $r/[D_f]$ vs. r is not linear but exhibits a curvature that suggests the presence of more than one class of binding sites. The data in Figure 11–17 were analyzed in terms of one class of sites for simplification. The plots at 20° and 40° C clearly show that multiple sites are involved. Blanchard et al.[69] reviewed the case of multiple classes of sites. Equation (11–37) is then written

$$r = \frac{\nu_1 K_1 [D_f]}{1 + K_1 [D_f]}$$
$$+ \frac{\nu_2 K_2 [D_f]}{1 + K_2 [D_f]} + \cdots \frac{\nu_n K_n [D_f]}{1 + K_n [D_f]} \qquad (11\text{–}42a)$$

or

$$r = \sum_{i=1}^{n} \frac{\nu_i K_i [D_f]}{1 + K_i [D_f]} \qquad (11\text{–}42b)$$

As previously noted, only ν and K need be evaluated when the site are all of one class. When n classes of sites exist, equation (11–42) may be written as

$$r = \sum_{i=1}^{n-1} \frac{\nu_i K_i [D_f]}{1 + K_i [D_f]} + \nu_n K_n [D_f] \qquad (11\text{–}43)$$

The binding constant K_n in the term on the right is small, indicating extremely weak affinity of the drug for the sites, but this class may have a large number of sites so as to be considered unsaturable.

Equilibrium Dialysis and Ultrafiltration. A number of methods are used to determine the amount of drug bound to a protein. Equilibrium dialysis, ultrafiltration, and electrophoresis are the classic techniques used, and in recent years other methods, such as gel filtration and nuclear magnetic resonance, have been used with satisfactory results. We shall discuss the equilibrium dialysis, ultrafiltration, and kinetic methods.

The equilibrium dialysis procedure was refined by Klotz et al.[70] for studying the complexation between metal ions or small molecules and macromolecules that cannot pass through a semipermeable membrane.

According to the equilibrium dialysis method, the serum albumin (or other protein under investigation) is placed in a Visking cellulose tubing (Visking Corporation, Chicago) or similar dialyzing membrane. The tubes are tied securely and suspended in vessels containing the drug in various concentrations. Ionic strength and sometimes hydrogen ion concentration are adjusted to definite values, and controls and blanks are run to account for the adsorption of the drug and the protein on the membrane.

If binding occurs, the drug concentration in the sac containing the protein is greater at equilibrium than the concentration of drug in the vessel outside the sac. Samples are removed and analyzed to obtain the concentrations of free and complexed drug.

Equilibrium dialysis is the classic technique for protein binding and remains the most popular method. Some potential errors associated with this technique are the possible binding of drug to the membrane, transfer of substantial amounts of drug from the plasma to the buffer side of the membrane, and osmotic volume shifts of fluid to the plasma side. Tozer et al.[71] developed mathematical equations to calculate and correct for the magnitude of fluid shifts. Briggs et al.[72] proposed a modified equilibrium dialysis technique to minimize experimental errors for the determination of low levels of ligand or small molecules.

Ultrafiltration methods are perhaps more convenient for the routine determination because they are less time-consuming. The ultrafiltration method is similar to equilibrium dialysis in that macromolecules such as serum albumin are separated from small drug molecules. Hydraulic pressure or centrifugation is used in ultrafiltration to force the solvent and the small molecules, unbound drug, through the membrane while preventing the passage of the drug bound to the protein. This ultrafiltrate is then analyzed by spectrophotometry or other suitable technique.

The concentration of the drug D_f that is free and unbound is obtained by use of the Beer's law equation (equation 4–9) and *Example 4–4*.

$$A = \epsilon bc \qquad (11\text{–}44)$$

in which A is the spectrophotometric absorbance (dimensionless), ϵ is the molar absorptivity, determined independently for each drug (see Table 4–4, p. 82), c (D_f in binding studies) is the concentration of the free drug in the ultrafiltrate in moles per liter, and b is the optical path length of the spectrophotometer cell, ordinarily 1 cm. The following example outlines the steps involved in calculating the Scatchard r value and the percent drug bound.

Example 11–5. The binding of sulfamethoxypyridazine to human serum albumin was studied at 25° C, pH 7.4, using the ultrafiltration technique. The concentration of the drug under study $[D_t]$ is 3.24×10^{-5} mole/liter and the human serum albumin concentration $[P_t]$ is 1.0×10^{-4} mole/liter. After equilibration the ultrafiltrate has an

absorbance (A) of 0.559 at 540 nm in a 1-cm cell (b). The molar absorptivity (ϵ) of the drug is 5.6×10^4 liter mole^{-1} cm^{-1}. Calculate the Scatchard r value and the percent drug bound.

The concentration of free (unbound drug), $[D_f]$ is

$$[D_f] = \frac{A}{b\epsilon} = \frac{0.559}{(5.6 \times 10^4)1} = 0.99 \times 10^{-5} \text{ mole/liter}$$

The concentration of bound drug $[D_b]$ is

$$[D_b] = [D_t] - [D_f] = (3.24 \times 10^{-5}) -$$
$$(0.99 \times 10^{-5}) = 2.25 \times 10^{-5} \text{ mole/liter}$$

The r value is

$$r = \frac{[D_b]}{[P_t]} = \frac{2.25 \times 10^{-5}}{1.0 \times 10^{-4}} = 0.225$$

The percent of bound drug is $[D_b]/[D_t] \times 100 = 69\%$

A potential error in ultrafiltration techniques may result from the drug binding to the membrane. The choice between ultrafiltration and equilibrium dialysis methods depends on the characteristics of the drug. The two techniques have been compared in several protein binding studies.[73-75]

Dynamic Dialysis. Meyer and Guttman[76] developed a kinetic method for determining the concentrations of bound drug in a protein solution. The method has found favor in recent years because it is relatively rapid, economical in terms of the amount of protein required, and readily applied to the study of competitive inhibition of protein binding. It is discussed here in some detail. The method, known as *dynamic dialysis*, is based on the rate of disappearance of drug from a dialysis cell that is proportional to the concentration of unbound drug. The apparatus consists of a 400-mL jacketed (temperature-controlled) beaker into which 200 mL of buffer solution are placed. A cellophane dialysis bag containing 7 mL of drug or drug–protein solution is suspended in the buffer solution. Both solutions are stirred continuously. Samples of solution external to the dialysis sac are removed periodically and analyzed spectrophotometrically, and an equivalent amount of buffer solution is returned to the external solution. The dialysis process follows the rate law:

$$\frac{-d[D_t]}{dt} = k[D_f] \qquad (11\text{–}45)$$

in which $[D_t]$ is the total drug concentration, $[D_f]$ the concentration of free or unbound drug in the dialysis sac, $-d[D_t]/dt$ the rate of loss of drug from the sac, and k the first-order rate constant (see Chapter 12) representative of the diffusion process. The factor k may also be referred to as the apparent permeability rate constant for the escape of drug from the sac. The concentration of unbound drug, $[D_f]$, in the sac (protein compartment) at a total drug concentration, $[D_t]$, is calculated using equation (11–45), knowing k and the rate $-d[D_t]/dt$ at a particular drug concentration, $[D_t]$. The rate constant k is obtained from the slope of a semilogarithmic plot of $[D_t]$ versus time when the experiment is conducted in the absence of the protein.
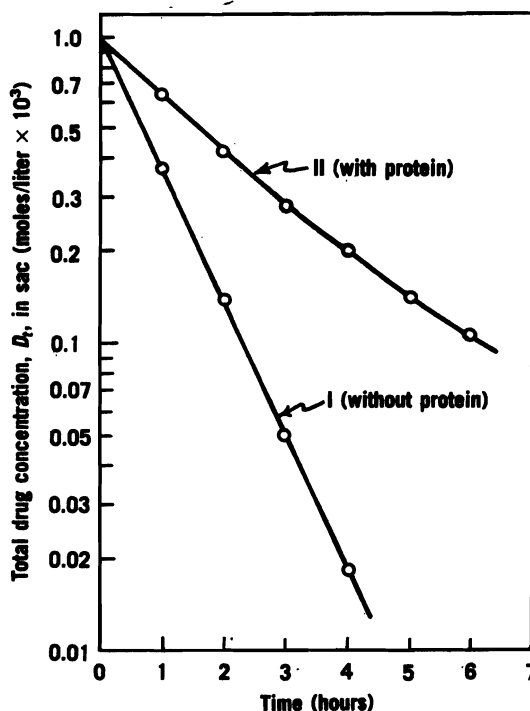
Fig. 11–18. The dynamic dialysis plot of Meyer and Guttman[76] for determining the concentration of bound drug in a protein solution.

Figure 11–18 illustrates the type of kinetic plot that can be obtained with this system. Note that in the presence of protein, Curve II, the rate of loss of drug from the dialysis sac, is slowed compared with the rate in the absence of protein, Curve I. In order to solve equation (11–45) for free drug concentration $[D_f]$, it is necessary to determine the slope of Curve II at various points in time. This is not done graphically but, rather, it is accurately accomplished by first fitting the time-course data to a suitable empiric equation, such as that given as equation (11–46), using a computer.

$$[D_t] = C_1 e^{-C_2 t} + C_3 e^{-C_4 t} + C_5 e^{-C_6 t} \qquad (11\text{–}46)$$

The computer fitting provides estimates of C_1 through C_6. The values for $d[D_t]/dt$ may then be computed from equation (11–47), which represents the first derivative of equation (11–46):

$$-\frac{d[D_t]}{dt} = C_1 C_2 e^{-C_2 t} + C_3 C_4 e^{-C_4 t} + C_5 C_6 e^{-C_6 t} \qquad (11\text{–}47)$$

Finally, once we have a series of $[D_f]$ values, computed from equations (11–47) and (11–45), corresponding to experimentally determined values of $[D_t]$ at each time t, we can proceed to calculate the various terms for the Scatchard plot.

*Example 11–6.** Assume that the kinetic data illustrated in Figure 11–18 were obtained under the following conditions: Initial drug

*Example 11–6 was prepared by Professor M. Meyer of the University of Tennessee.

concentration $[D_{t_0}] = 1 \times 10^{-3}$ mole/liter; protein concentration = 1×10^{-3} mole/liter. Assume also that the first-order rate constant (k) for the control (Curve I) was determined to be 1.0 hr^{-1} and that fitting of Curve II to equation (11–46) resulted in the following empiric constants: $C_1 = 5 \times 10^{-4}$ mole/liter, $C_2 = 0.6$ hr^{-1}, $C_3 = 3 \times 10^{-4}$ mole/liter, $C_4 = 0.4$ hr^{-1}, $C_5 = 2 \times 10^{-4}$ mole/liter, and $C_6 = 0.2$ hr^{-1}.

Calculate the Scatchard values (the Scatchard plot was discussed in the previous section) for r and $r/[D_f]$ if, during the dialysis in the presence of protein, the experimentally determined value for $[D_t]$ was 4.2×10^{-4} mole/liter at 2 hours. $r = [D_b]/P_t$, in which $[D_b]$ is drug bound and P_t is total protein concentration.

Using equation (11–47),

$$-\frac{d[D_t]}{dt} = k[D_f] = (5 \times 10^{-4})(0.6)e^{-0.6(2)}$$

$+ (3 \times 10^{-4})(0.4)e^{-0.4(2)} + (2 \times 10^{-4})(0.2)e^{-0.2(2)}$, where the (2) in the exponent stands for 2 hr.

Thus,

$$[D_f]_{2 \text{ hr}} = \frac{1.7 \times 10^{-4} \text{ mole/liter hr}^{-1}}{1.0 \text{ hr}^{-1}} = 1.7 \times 10^{-4} \text{ mole/liter}$$

It follows that at 2 hours,

$$[D_b] = [D_t] - [D_f] = 4.2 \times 10^{-4} \text{ mole/liter}$$
$$-1.7 \times 10^{-4} \text{ mole/liter} = 2.5 \times 10^{-4} \text{ mole/liter}$$

$$r = [D_b]/[P_t] = (2.5 \times 10^{-4})/(1 \times 10^{-3}) = 0.25$$

$$(r)/[D_f] = (0.25)/(1.7 \times 10^{-4}) = 1.47 \times 10^{-3} \text{ liter/mole}$$

Additional points for the Scatchard plot would be obtained in a similar fashion, using the data obtained at various points throughout the dialysis. Accordingly, this series of calculations permits one to prepare a Scatchard plot (see Fig. 11–17).

Judis[77] investigated the binding of phenol and phenol derivatives by whole human serum using the dynamic dialysis technique and presented the results in the form of Scatchard plots.

Hydrophobic Interaction. Hydrophobic "bonding," first proposed by Kauzmann,[78] is actually not bond forma-

tion at all, but rather the tendency of hydrophobic molecules or hydrophobic parts of molecules to avoid water because they are not readily accommodated in the hydrogen-bonding structure of water. Large hydrophobic species such as proteins avoid the water molecules in an aqueous solution insofar as possible by associating into micelle-like structures (Chapter 14) with the nonpolar portions in contact in the inner regions of the "micelles," the polar ends facing the water molecules. This attraction of hydrophobic species, resulting from their unwelcome reception in water, is known as hydrophobic bonding, or better, *hydrophobic interaction*. It involves van der Waals forces, hydrogen bonding of water molecules in a three-dimensional structure, and other interactions. Hydrophobic interaction is favored thermodynamically because of an increased disorder or entropy of the water molecules that accompanies the association of the nonpolar molecules, which squeeze out the water. Globular proteins are thought to maintain their ball-like structure in water because of the hydrophobic effect. Hydrophobic interaction is depicted in Figure 11–19.

Nagwekar and Kostenbauder[79] studied hydrophobic effects in drug binding using as a model of the protein a copolymer of vinylpyridine and vinylpyrrolidone. Kristiansen et al.[80] studied the effects of organic solvents in decreasing complex formation between small organic molecules in aqueous solution. They attributed the interactions of the organic species to a significant contribution by both hydrophobic bonding and the unique effects of the water structure. They suggested

(a) ordered water structure surrounding two large nonpolar molecules.

(b) Hydrophobic interaction of the nonpolar molecules with the "squeezing out" of water molecules into a more randomized structure.
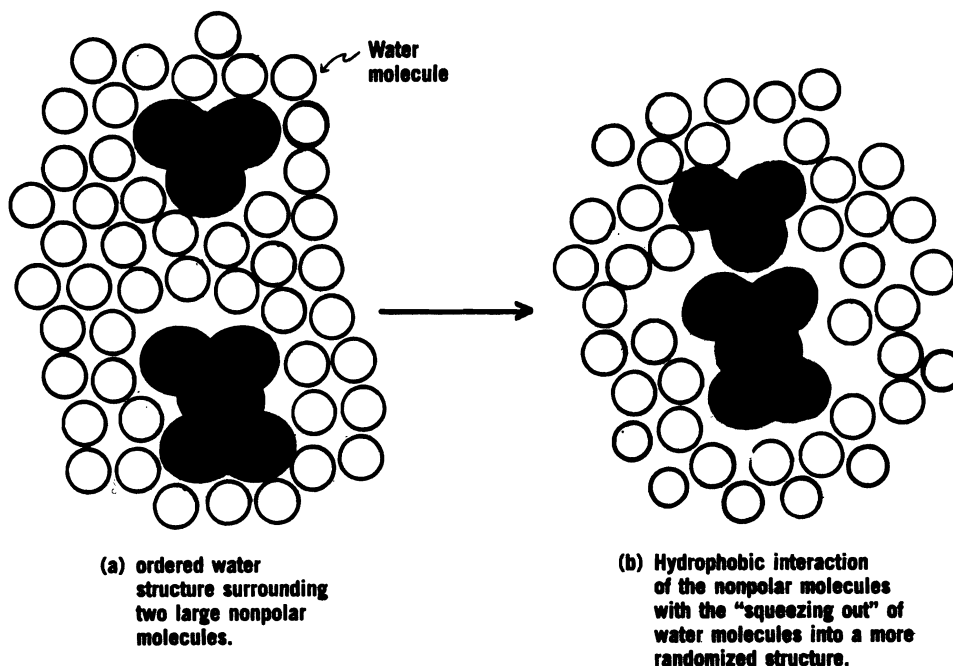
Fig. 11–19. Schematic view of hydrophobic interaction. In (a), two hydrophobic molecules are separately enclosed in cages, surrounded in an orderly fashion by hydrogen-bonded molecules of water, $\bigcirc$. The state at (b) is somewhat favored by breaking of the water cages of (a) to yield a less ordered arrangement and an overall entropy increase of the system. Van der Waals attraction of the two hydrophobic species also contributes to the hydrophobic interaction.

that some nonclassic "donor–acceptor" mechanism may be operating to lend stability to the complexes formed.

Feldman and Gibaldi[81] studied the effects of urea, methylurea, and 1,3-dimethylurea on the solubility of benzoic and salicylic acids in aqueous solution. They concluded that the enhancement of solubility by urea and its derivatives was a result of hydrophobic bonding rather than complexation. Urea broke up the hydrogen-bonded water clusters surrounding the nonpolar solute molecules, increasing the entropy of the system and producing a driving force for solubilization of benzoic and salicylic acids. It may be possible that the ureas formed channel complexes with these aromatic acids as shown in Figure 11–3a, b, and c.

The interaction of drugs with proteins in the body may involve hydrophobic bonding at least in part, and this force in turn may affect the metabolism, excretion, and biologic activity of a drug.

Self-Association. Some drug molecules may self-associate to form dimers, trimers, or aggregates of larger sizes. A high degree of association may lead to formation of micelles, depending on the nature of the molecule (see Chapter 15). Doxorubicin forms dimers, the process being influenced by buffer composition and ionic strength. The formation of tetramers is favored by hydrophobic stacking aggregation.[82] Self-association may affect solubility, diffusion, transport through membranes, and therapeutic action. Insulin shows concentration-dependent self-association, which leads to complications in the treatment of diabetes. Aggregation is of particular importance in long-term insulin devices, where insulin crystals have been observed. The initial step of insulin self-association is a hydrophobic interaction of the monomers to form dimers, which further associate into larger aggregates. The process is favored at higher concentrations of insulin.[83] Addition of urea at nontoxic concentrations (1.0–3 mg/mL) has been shown to inhibit the self-association of insulin. Urea breaks up the "icebergs" in liquid water and associates with structured water by hydrogen bonding, taking an active part in the formation of a more open "lattice" structure.[84]

Sodium salicylate improves the rectal absorption of a number of drugs, all of them exhibiting self-association. Touitou and Fisher[85] chose methylene blue as a model for studying the effect of sodium salicylate on molecules that self-associate by a process of stacking. Methylene blue is a planar aromatic dye that forms dimers, trimers, and higher aggregates in aqueous solution. The workers found that sodium salicylate prevents the self-association of methylene blue. The inhibition of aggregation of porcine insulin by sodium salicylate results in a 7875-fold increase in solubility.[86] Commercial heparin samples tend to aggregate in storage depending on factors such as temperature and time in storage.[87]

Factors Affecting Complexation and Protein Binding. Kenley et al.[54] investigated the role of hydrophobicity

in the formation of water-soluble complexes. The logarithm of the ligand partition coefficient between octanol and water was chosen as a measure of hydrophobicity of the ligand. The authors found a significant correlation between the stability constant of the complexes and the hydrophobicity of the ligands. Electrostatic forces were not considered as an important factor since all compounds studied were uncharged under the conditions investigated. Donor–acceptor properties expressed in terms of orbital energies (from quantum chemical calculations) and relative donor–acceptor strengths correlated poorly with the formation constants of the complex. It was suggested that ligand hydrophobicity is the main contribution to the formation of water-soluble complexes. Coulson and Smith[88] found that the more hydrophobic chlorobiocin analogs showed the highest percent of drug bound to human serum albumin. These workers suggested that chlorobiocin analogs bind to human albumin at the same site as warfarin. This site consists of two non-coplanar hydrophobic areas and a cationic group. Warfarin, an anticoagulant, serves as a model drug in protein binding studies because it is extensively but weakly bound. Thus, many drugs are able to compete with and displace warfarin from its binding sites. The displacement may result in a sudden increase of the free (unbound) fraction in plasma, leading to toxicity, since only the free fraction of a drug is pharmacologically active. Diana et al.[89] investigated the displacement of warfarin by nonsteroidal antiinflammatory drugs. Table 11–8 shows the variation of the stability constant K and the number of binding sites n of the complex albumin–warfarin after addition of competing drugs. Azapropazone decreases markedly the K value, suggesting that both drugs, warfarin and azapropazone, compete for the same binding site on albumin. Phenylbutazone also competes strongly for the binding site on albumin. Conversely, tolmetin may increase K, as suggested by the authors, by a conformational change in the albumin molecule which favors warfarin binding. The other drugs (see Table 11–8) decrease the K value of warfarin to a lesser extent, indicating that they do not share exclusively the same binding site as that of warfarin.

TABLE 11–8. *Binding Parameters (± S.D.) for Warfarin in the Presence of Displacing Drugs**

| Competing Drug | Racemic Warfarin | |
	n	$K \times 10^{-5}\,M^{-1}$
None	1.1 ± 0.0	6.1 ± 0.2
Azapropazone	1.4 ± 0.1	0.19 ± 0.02
Phenylbutazone	1.3 ± 0.2	0.33 ± 0.06
Naproxen	0.7 ± 0.0	2.4 ± 0.2
Ibuprofen	1.2 ± 0.2	3.1 ± 0.4
Mefenamic acid	0.9 ± 0.0	3.4 ± 0.2
Tolmetin	0.8 ± 0.0	12.6 ± 0.6

*F. J. Diana, K. Veronich and A. L. Kapoor, J. Pharm. Sci. **78**, 195, 1989.

Plaizier-Vercammen[90] studied the effect of polar organic solvents on the binding of salicylic acid to povidone. He found that in water–ethanol and water–propylene glycol mixtures, the stability constant of the complex decreased as the dielectric constant of the medium was lowered. Such a dependence was attributed to hydrophobic interaction and may be explained as follows. Lowering the dielectric constant decreases polarity of the aqueous medium. Since most drugs are less polar than water, their *affinity to the medium increases* when the dielectric constant decreases. As a result, the binding to the macromolecule is reduced.

Protein binding has been related to the solubility parameter δ of drugs (solubility parameter is defined on p. 224). Bustamante and Selles[91] found that the percent of drug bound to albumin in a series of sulfonamides showed a maximum at $\delta = 12.33$ cal$^{1/2}$ cm$^{-3/2}$. This value closely corresponds to the δ-value of the postulated binding site on albumin for sulfonamides and suggests that the closer the solubility parameter of a drug to the δ-value of its binding site, the greater the binding.

THERMODYNAMIC TREATMENT OF STABILITY CONSTANTS

The standard free energy change of complexation is related to the overall stability constant K (or any of the formation constants) by the relationship (pp. 70, 161).

$$\Delta G^\circ = -2.303RT \log K \qquad (11\text{–}48)$$

The standard enthalpy change ΔH° may be obtained from the slope of a plot of log K versus $1/T$, following the expression

$$\log K = -\frac{\Delta H^\circ}{2.303\,R}\frac{1}{T} + \text{constant} \qquad (11\text{–}49)$$

When the values of K at two temperatures are known, the following equation may be used:

$$\log(K_2/K_1) = \frac{\Delta H^\circ}{2.303R}\left(\frac{T_2 - T_1}{T_1 T_2}\right) \qquad (11\text{–}50)$$

The standard entropy change ΔS° is obtained from the expression

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \qquad (11\text{–}51)$$

Andrews and Keefer[92] demonstrated that ΔH° and ΔS° generally become more negative as the stability constant for molecular complexation increases. As the binding between donor and acceptor becomes stronger, ΔH° would be expected to have a larger negative value. Apparently, the specificity of interacting sites or structural restraint also becomes greater, leading to a larger negative ΔS° value. Although the negative ΔS° value disfavors complexation, the negative ΔH° is large enough to overcome the unfavorable entropy contribution, leading to a negative ΔG°. See Table 11–11, row 4.

The results of Borazan et al.[58] in the charge-transfer complexation of nucleic acid bases with catechol are given in Table 11–7. These results run counter to the generalization just given. It is observed that the uracil–catechol complex exhibited both larger negative ΔH° and ΔS° than the adenine–catechol interaction, yet complexation constants for the uracil–catechol complex were much smaller than for the adenine interaction with catechol.

Nagwekar and Kostenbauder[79] used alkyl vinylpyridine–vinylpyrrolidone copolymers to test the strength of binding to a model drug, *p*-toluene sulfonic acid sodium (PTSAS), and to calculate thermodynamic parameters. The binding constants, K(liter/mole) and the thermodynamic values for interaction of PTSAS with various alkyl copolymers at 15° to 37° C are found in Table 11–9. In ascending the homologous series of alkyl copolymers, the binding constants increased in a sawtooth or zigzag manner, the K for a copolymer of an odd-numbered alkyl carbon chain being higher than for the next member of even carbon number. The K values and the thermodynamic functions (negative ΔG° and positive ΔH° and ΔS°), however, increased with increasing alkyl chain length for a series of odd or even alkyl copolymers. The binding process is endothermic (positive ΔH°), but the large increase in entropy on complexation resulted in a free energy that was negative.

The binding in these molecular complexes may be considered as a kind of hydrophobic interaction, the *p*-toluene sulfonic acid anion interacting with the positively charged vinylpyridine units to form a hydrophobic compound that squeezes out the water molecules that originally surrounded both the copolymer and PTSAS in an orderly iceberg-like structure (Fig. 11–20). When binding occurs between the copolymer molecules and PTSAS, the iceberg structure of water is partly destroyed and becomes less ordered. Presumably, this is the reason for the increase in entropy on complexation (positive ΔS°) as observed in Table 11–9.

Example 11–7. Basolo[83] obtained the following results for the complexation between ethylenediamine and the cupric ion: log K = 21.3 at 0° C and log K = 20.1 at 25° C. Compute ΔG°, ΔH°, and ΔS° at 25° C.

$$\Delta G^\circ = -2.303RT \log K = -2.303 \times 1.987 \times 298 \times 20.1 =$$

$$-27.4 \text{ kcal/mole} \qquad \log K_2 - \log K_1 = \frac{\Delta H^\circ}{2.303\,R}\left(\frac{298 - 273}{298 \times 273}\right)$$

$$\Delta H^\circ = \frac{(20.1 - 21.3)2.303 \times 1.987 \times 298 \times 273}{25} = -17.9 \text{ kcal/mole}$$

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} = \frac{-17.9 + 27.4}{298} = +32 \text{ cal/(deg mole)}$$

The positive entropy change in *Example* 11–7 is characteristic of chelation. It occurs because the water molecules that are normally arranged in an orderly fashion around the ligand* and metal ion have acquired a more random configuration as a result of chelation, as in hydrophobic binding. This is referred to as a gain of

TABLE 11–9. *Binding Constants and Thermodynamic Functions for the Interaction of PTSAS with Various Alkyl Copolymers Over a Temperature Range of 15° to 37° C**

Temperature (°C)	K (liter/mole)	$\Delta G°$ (cal/mole)	$\Delta H°$ (cal/mole)	$\Delta S°$ (cal/(mole deg))
Ethyl Copolymer—PTSAS				
15	46.00	−2193		
30	46.66	−2316	458	9.21
37	50.00	−2414		
Propyl Copolymer—PTSAS				
15	61.00	−2354		
30	85.70	−2683	4068	22.32
37	102.14	−2853		
Butyl Copolymer—PTSAS				
15	52.84	−2272		
30	55.00	−2430	1373	12.64
37	63.28	−2558		
Pentyl Copolymer—PTSAS				
15	62.80	−2372		
30	111.00	−2837	5611	27.77
37	127.20	−2987		
Hexyl Copolymer—PTSAS				
15	63.36	−2377		
30	94.80	−2743	4728	24.41
37	108.08	−2888		

*From J. B. Nagwekar and H. B. Kostenbauder, J. Pharm. Sci. **59**, 751, 1970, reproduced by permission of the copyright owner.

configurational entropy. The effect is shown clearly by Calvin and Melchior,[94] who complexed the salicylaldehyde-5-sulfonate ion (A) with the cupric ion. The ions are normally hydrated with a certain number of water molecules in aqueous solution, and these molecules are "squeezed out" when the complex is formed. Thus, the ordered arrangement of the solvent around the ions is lost and the entropy of the system increases. The process is represented as

$$Cu^{2+} \cdot (H_2O)_x + 2A \cdot (H_2O)_y = CuA_2 + z(H_2O);$$
$$\Delta S \cong + 100 \text{ cal/deg}$$

in which x and y are the number of water molecules bound and z is the number free in solution.

The decrease in ionic charge that usually accompanies complexation of polydentate ligands* (chelation) also decreases the possibility of hydration and leads to an additional increase in entropy. The entropy change involved in complexation of monodentate ligands and in electron donor–acceptor interactions (molecular complexation), on the other hand, usually is attended by a negative $\Delta S°$. This effect is due to an increased ordering of the species by complexation. These complexes are not ordinarily as stable as the chelates, and their formation is not attended by the same loss of solvent around the ions. Anthralin, an antipsoriatic drug, rapidly decomposes in aqueous solution near neutral pH to give principally dantron (Fig. 11–21). The thermodynamic parameters of the binding of dantron to bovine serum albumin at 25° C are $\Delta G° = -8.03$ kcal/mole, $\Delta H° = -11.8$ kcal/mole, and $\Delta S° = -12.6$ u.e.[95] The negative $\Delta S°$ value indicates that electrostatic forces are not important. (Electrostatic forces lead to positive entropies, which favor the binding process.) The large negative $\Delta H°$ as well as the negative $\Delta S°$ suggest

*The term *ligand* is from "ligate"—to bind—and is a general term meaning the agent that binds. The ligand referred to here is the large molecule attached to the central metal. Conversely, in molecular complexes the ligand is the drug (the small molecule) and the protein, polypeptide, and so on, constitutes the large molecule.

Water molecules (O) less well ordered than in (a). Increased entropy results.

(a)　　　　(b)

Fig. 11–20. Interaction of a *p*-toluene sulfonic acid anion with a positively charged vinylpyridine unit of a copolymer chain, with a squeezing out of water molecules. The ice-like cages of water molecules around the two ionic species (a) are disrupted on complexation of the anion and cation and in (b) the entropy of the system is increased, which favors the process. The open circles, O, represent partly hydrogen-bonded water molecules (see Fig. 11–19).
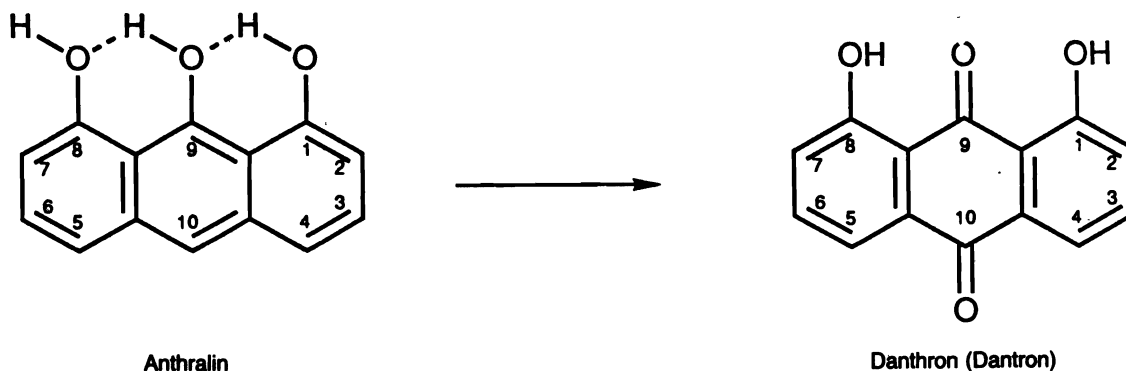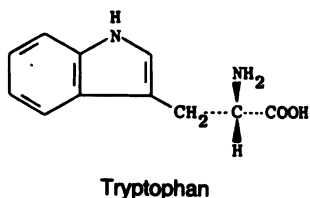
Fig. 11–21. Decomposition of anthralin to yield dantron.

hydrogen bonding between dantron and its binding site in albumin. The carbonyl group in the 10-position of dantron (see Fig. 11–21) is able to hydrogen-bond to the tryptophan residue in bovine serum albumin, which

Tryptophan

could serve as a proton donor. The negative entropy does not favor complexation but, owing to hydrogen bonding, the large negative enthalpy is the driving force that leads to a negative free energy change (see Table 11–11, row 4).

Plaizier-Vercammen and De Nève[96] studied the interaction forces for the binding of several ligands to povidone and interpreted the thermodynamics of the binding. The influence of dissociation of the ligand on $\Delta G°$, $\Delta H°$, and $\Delta S°$ and on the equilibrium constant K is shown in Table 11–10. $\Delta H°$ becomes more negative and $\Delta S°$ decreases as the degree of dissociation increases. If the binding were exclusively due to hydrogen bonding, both $\Delta H°$ and $\Delta S°$ would be negative (see Table 11–11). However, $\Delta S°$ is positive, which can be due to either electrostatic or hydrophobic interactions (see Table 11–11). The fact that the dielectric constant of the medium has a positive influence on the binding (see

TABLE 11–11. *Positive and Negative Thermodynamic Functions Resulting from Several Kinds of Interactions*

Type of Interaction	Sign on $\Delta H°$	Sign on $\Delta S°$	$-\Delta G°$ is Favored By
1. Electrostatic	~0	+	$+\Delta S°$
2. Hydrophobic	+	+	large $+\Delta S°$
3. Chelation (polydentate ligand)	–	+	$+\Delta S°$ and/or $-\Delta H°$
4. Donor–acceptor (hydrogen bonding and chelation [monodentate ligand])	–	–	$-\Delta H°$
5. Unfolding of proteins	+	+	$+\Delta S°$

problem 11–14) and that povidone has no ionizable groups suggest that hydrophobic rather than electro-static interactions influence the binding. The positive $\Delta S°$ value is due to the disordering of the iceberg structure of water surrounding both the polymer and the drug. The negative $\Delta H°$ values can be due to van der Waals or hydrogen bonding interactions that together with the hydrophobic interaction lead to complex formation.

The value of $\Delta H°$ may be obtained from equation (11–49) by plotting log K versus $1/T$. The slope is $-\Delta H°/(2.303R)$ and is ordinarily calculated in analytic geometry by use of the two-point formula: slope = $\dfrac{Y_2 - Y_1}{X_2 - X_1}$. It is more correct, however, to linearly regress log K versus $1/T$ using the method of least squares. A number of inexpensive hand calculators available today do the linear least-squares method automatically, providing the slope and intercept of the line and statistical quantities such as the linear correlation coefficient, r.

Example 11–8. The association constants K at the temperatures, 6°, 18°, 25°, and 37° C for the interaction between uracil and catechol are found in Table 11–7. Calculate $\Delta H°$, $\Delta G°$, and $\Delta S°$ for this complexation reaction over the temperature range 6° to 37° C.

When the four log K values and the corresponding $1/T$ (in which T is in Kelvin degrees) are run on a hand calculator using equation (11–49) and a linear regression program, the slope obtained is −776.65, and $\Delta H° = -776.65 \times 1.987 \times 2.303 = -3554$ cal/mole.

$\Delta H°$ is a constant over the temperature range 6° to 37° C for this complexation reaction. $\Delta G°$, on the other hand, (in which $\Delta G° =$

TABLE 11–10. *Binding Constants, K, and Thermodynamic Functions for the Interaction of Salicylamide (pK_a = 8.2) with Povidone at 25° C*

pH	Degree of Dissociation	$K(M^{-1})$	$\Delta G°$ (kcal/mole)	$\Delta H°$ (kcal/mole)	$\Delta S°$ (kcal/mole)
5.0	0.00063	9.3	−5.4	−0.5	16.5
7.2	0.091	8.6	−5.4	−2.1	10.9
9.2	0.909	2.1	−4.5	−3.2	4.6

*From J. A. Plaizier-Vercammen and R. E. De Nève, J. Pharm. Sci. 71, 552, 1982. The degree of dissociation is calculated using equation (13–77), page 342.

$-2.303RT \log K$) has a different value for each temperature. Now $\Delta S° = -(\Delta G° - \Delta H°)/T$; therefore, $\Delta S°$ also might be expected to have different values at each temperature. Using these equations, $\Delta G°$ and $\Delta S°$ are calculated, and the results are

Thermodynamic Function	Temperature (°C)			
	6	18	25	37
$\Delta G°$ cal/mole	396	560	675	830
$\Delta S°$ cal/(mole deg)	−14	−14	−14	−14

From the values obtained, we learn that although $\Delta G°$ varies considerably for the four temperatures, $\Delta S°$, like $\Delta H°$, is reasonably constant. In fact, the constant in equation (11–49),

$$\log K = -\frac{\Delta H°}{2.303R} \frac{1}{T} + \text{constant}$$

may actually be written as $\Delta S°/2.303R$. In other words,

$$\log K = -\frac{\Delta H°}{2.303R} \frac{1}{T} + \frac{\Delta S°}{2.303R} \qquad (11-52)$$

By comparing this equation with (11–48) and (11–51), we can verify that the constant of equation (11–49) is in fact $\Delta S°/2.303R$. Therefore, $\Delta S°$ remains essentially constant at about −14 cal/mole deg over this temperature range. From equation (11–52), it should be possible to calculate $\Delta S°$ from the intercept on the vertical axis on a plot of $\log K$ versus $(1/T)$. This is a long extrapolation from ambient temperatures to the vertical axis where $1/T = 0$ or $T = \infty$ and can be done only by least-squares analysis and a computer or hand calculator. A line estimated by eye and drawn with a ruler through the experimental points would create a large error in the extrapolated intercept. The least-square value obtained from equation (11–52) gives $\Delta S° = -14.4$ cal/mole deg, which agrees with the average value of −14 obtained in *Example 11–8*.

Table 11–11 summarizes, in a qualitative way, the values of $\Delta H°$ and $\Delta S°$ that would be expected depending on the kind of interaction occurring in the complex. Column 4 shows the main contribution, either $\Delta H°$ and/or $\Delta S°$, that is needed to get a negative (favorable) $\Delta G°$ value. For example, for donor–acceptor and hydrogen bonding interactions, a large negative $\Delta H°$ value overcomes the unfavorable (negative) entropy change, leading to a favorable negative $\Delta G°$ value. On the other hand, the positive entropy change is the main factor in the unfolding of proteins that yields a negative $\Delta G°$ in spite of the positive (unfavorable) $\Delta H°$ value.

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Problems

11–1. Albert[97] studied the chelation of cadmium ion by asparagine. Potentiometric titration of 0.01 M asparagine, $pK_a = 8.85$, and 0.005 M cadmium sulfate was conducted in 50-mL samples by adding successive quantities of 0.1 N KOH. Plot the data of $\bar{n}$ versus p[A] and compute $\log K_1$, $\log K_2$, and $\log \beta$. The data table is on p. 279. ($\bar{n}$, p[A], and β are defined on pp. 262–263).

Answer: $\log K_1 = 3.9$, $\log K_2 = 2.97$, $\log \beta = 6.87$

11–2. Calvin and Melchior[94] investigated the chelation between the 5-salicylaldehydesulfonate ion and the cupric ion and obtained the following results; $\log K = 9.79$ at 40° C and $\log K = 9.27$ at 25° C. Calculate $\Delta H°$, $\Delta G°$, and $\Delta S°$ for the chelation process at 40° C. Give possible reasons for the entropy change $\Delta S°$ obtained by these investigators.

Answer: $\Delta H° = 14.8$ kcal/mole, $\Delta G° = -14$ kcal/mole, $\Delta S° = +92$ cal/mole deg

11–3. The following results were obtained by Higuchi and Zuck[98] for the complex formed between caffeine and benzoic acid. In the analytic procedure, benzoic acid was distributed between water and a hydrocarbon solvent, Skellysolve-C.

Molar concentration of *free* benzoic acid in aqueous solution of caffeine obtained by partition study 11.94×10^{-3} mole/liter

Data for *Problem 11-1*

mL of 0.1 N NaOH	pH	$\bar{n}$	p[A]
0	4.81	—	—
0.25	6.12	0.10	4.75
0.50	6.50	0.20	4.40
1.0	6.85	0.40	4.10
1.5	7.20	0.57	3.80
2.0	7.45	0.74	3.62
2.5	7.70	0.93	3.45
3.0	7.95	1.11	3.30
3.5	8.21	1.26	3.16
4.0	8.50	1.42	3.05
4.5	8.93	1.56	2.92

Experimentally determined molar concentration of *total* undissociated benzoic acid in the aqueous phase, corrected for partial dissociation (free + complexed benzoic acid) 20.4×10^{-3} mole/liter

Original concentration of caffeine added (free + complexed caffeine) 2.69×10^{-2} mole/liter

Assuming that the stoichiometric ratio of the two species in the complex is 1:1, compute the association constant, K.
Answer: K = 38.5

11-4. Using the solubility method, Higuchi and Lach[99] studied the complexation between a polyethylene glycol and phenobarbital. The findings obtained at 30° C are given as follows:

Polyethylene glycol content of the complex formed in the plateau region of the solubility diagram 30×10^{-3} mole/liter

Total phenobarbital added 21.5×10^{-3} mole/liter

Phenobarbital dissolved at point B in the solubility diagram, Fig. 11–12 6.5×10^{-3} mole/liter

Compute the stoichiometric ratio [PGE]/[phenobarbital].
Answer: 2:1 complex

11-5. According to Higuchi and Lach,[100] the following results are obtained for the interaction of caffeine and sulfathiazole at 30° C.

Total sulfathiazole concentration at saturation when no caffeine is present (cf. Point A in Fig. 11–12, p. 265) 2.27×10^{-3} mole/liter

Total sulfathiazole concentration at saturation when 3.944×10^{-2} mole/liter of caffeine is added to the system 3.27×10^{-3} mole/liter

Compute the stability constant, assuming a 1:1 complex.
Answer: 11.5.

11-6. Higuchi and Zuck[101] investigated the complex formation betwene caffeine and butyl paraben by the solubility method. The results at 15° C are

Solubility of butyl paraben when no caffeine is present 0.58×10^{-3} M

Concentration of added caffeine 6.25×10^{-2} M

Solubility of butyl paraben when above amount of caffeine is present 3.72×10^{-3} M

Assuming that the complex has a stoichiometric ratio of 1:1, compute the stability constant.
Answer: K = 91

11-7. The formation of an inclusion complex of 1,8 dihydroxyanthraquinone with γ-cyclodextrin in aqueous solution was studied using the solubility technique[102] (see p. 265). The concentrations of anthraquinone derivative found after addition of several increments of γ-cyclodextrin to 10 mL of buffer containing an excess of the anthraquinone (1×10^{-3} M) are

Data for *Problem 11-7*

Cyclodextrin added ($\times 10^3$ M)	Anthraquinone found ($\times 10^6$ M)
2.37	2.56
7.89	8.72
11.58	12.56
15.79	15.60
18.95	15.81
22.63	16.41
30.0	16.41
38.0	13.84

(a) Obtain the phase diagram by plotting the concentration of the anthraquinone found (vertical axis) against the concentration of γ-cyclodextrin added (see Fig. 11–12 for a similar diagram).
(b) Compute the solubility of 1,8 dihydroxyanthraquinone.
(c) Compute the apparent stability constant K of the complex from the slope of the initial linear portion of the plot obtained in part (a). (Use the first five points.) K is obtained from the expression,[53] K = slope/[intercept $(1 - $ slope)].
Answers: (a) The phase diagram should look similar to Figure 11–12 on p. 265; (b) $S_0 = 1.7 \times 10^{-6}$ M (the solubility in water reported by the authors is about 1×10^{-6} M); (c) $K = 479$ M^{-1}

11-8. Griseofulvin contains two keto groups, four ether oxygen atoms, and an aromatic ring, all capable of accepting protons to form hydrogen bonds. Griseofulvin has no proton donating groups so it acts only as a proton acceptor, A. The molar solubility of griseofulvin in isooctane, $[A_o] = 0.9358 \times 10^{-5}$ mole/liter, increases rapidly with increasing molar concentrations of hexanoic acid, $[D_t]$, an acidic donor, owing to the formation of a donor–acceptor complex, AD_m:

$$A + m D \rightleftharpoons AD_m$$
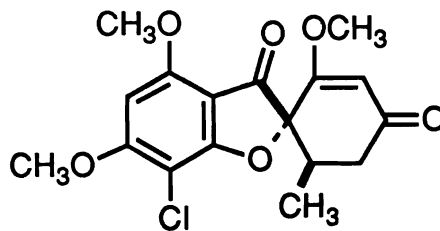
Griseofulvin

where m is the stoichiometric number of D molecules interacting with one A molecule. Mehdizadeh and Grant[103] determined the experimen-

tal solubilities of griseofulvin in isooctane with increasing concentrations of hexanoic acid, $[D_t]$; the data are shown in the following table:

Data for *Problem 11–8**

$[D_t]$, molar concentration of hexanoic acid (donor)	$[A_t]$ ($M \times 10^5$), concentration of griseofulvin (acceptor)
0.1632	2.317
0.465	4.178
0.784	7.762
1.560	20.902
3.118	77.581
4.693	207.16
6.316	435.18
7.855	858.98

*The concentrations given here are selected from among the 15 concentrations each of hexanoic acid and griseofulvin given in the original article.

The authors show that if only one complex species, AD_m, is considered, say $m = 2$, the increase in solubility $[A_t] - [A_o]$ of the acceptor (griseofulvin) in isooctane is proportional to the $m/2$ power of the total concentration of the donor (hexanoic acid), $[D_t]^{m/2}$, according to the following expression:

$$[A_t] - [A_o] = [AD_m] = K [D_t]^{m/2} \qquad (11-53)$$

where K includes K_m, the stability constant of the complex, K_d, the dimerization constant of hexanoic acid raised to the power $(-m/2)$, and an additional term, $2^{-m/2}$:

$$K = K_m K_d^{-m/2} 2^{-m/2} \qquad (11-54)$$

(a) Take the log of both sides of equation (11–53) and regress log $([A_t] - [A_o])$, the dependent variable, against log $[D_t]$, the independent variable. Compute the stoichiometric number m of the complex from the slope.

(b) Obtain the stability constant of the complex, K_m, using the intercept you got in part (a) and equation (11–54). The dimerization constant of hexanoic acid from a separate experiment is $K_d = 6000$ M^{-1}

Answers: (a) $m = 3.39$ (number of hexanoic acid molecules per griseofulvin molecule in the complex); (b) $K_m = 1248$ M^{-1}, the stability constant of the complex of the formula, AD_3. The number 3.39 is obtained by regression analysis and is therefore an average. It is assumed to be an integer value, $m = 3$, for the complex.

11–9. Al-Obeidi and Borazan[104] investigated the charge transfer complex formation between epinephrine and the nucleic acid bases adenine, thymine, and uracil by ultraviolet absorption spectrometry. Epinephrine is an electron donor, and the nucleic acid bases are assumed to act as electron acceptors.

Obtain the molar absorptivity ϵ and the equilibrium constant K (1/molarity) of the Benesi–Hildebrand equation (equation (11–28),

Data for *Problem 11–9*

$1/D_o$ (liter/mole)	1.0	2.0	3.0
A_o/A at 2° C	0.022	0.034	0.047
A_o/A at 18° C	0.029	0.047	0.066
A_o/A at 25° C	0.031	0.053	0.075
A_o/A at 37° C	0.037	0.065	0.093

p. 266) by plotting A_o/A versus $1/D_o$. A_o and D_o are the total concentrations of adenine and epinephrine, respectively. A is the absorbance of the complex at a definite wavelength. It is assumed that epinephrine forms 1:1 charge transfer complexes with these nucleic acid bases in acidified aqueous solution.

The accompanying table shows the values for A_o/A and $1/D_o$ for the adenine–epinephrine complex at four temperatures, as back-calculated from the K and ϵ values, Table 1, of the paper.[104]

Answer: See Table 1 in the paper, J. Pharm. Sci. 65, 982, 1976.

11–10. Assuming 1:1 complexes and using the Benesi–Hildebrand equation, Al-Obeidi and Borazan[104] obtained the following stability constants, K (1/molarity) for thymine–epinephrine at three temperatures, at a wavelength of 314 nm:

Data for *Problem 11–10*

Temperature (°C)	2	18	37
K (M^{-1})	0.97	0.73	0.57

Calculate the standard free energy ($\Delta G°$), standard enthalpy ($\Delta H°$), and standard entropy ($\Delta S°$) changes for the complexation. Give an explanation for the magnitude and the arithmetic sign of these thermodynamic quantities.

Answer: Check your results against those in Table 1 of the paper in J. Pharm. Sci. 65, 982, 1976. The discussion section of the paper will assist you in explaining the meaning of the $\Delta G°$, $\Delta H°$, and $\Delta S°$ values.

11–11. Al-Obeidi and Borazan[104] investigated the charge transfer complex formation between epinephrine and the nucleic acid bases adenine, thymine, and uracil by ultraviolet absorption spectrometry. Epinephrine is an electron donor, and the nucleic acid bases are assumed to act as electron acceptors.

Assuming 1:1 complexes and using the Benesi–Hildebrand equation, these workers obtained the following stability constants, K (M^{-1}), for adenine–epinephrine at four temperatures at a wavelength of 326 nm:

Data for *Problem 11–11*

T (°C)	2	18	25	37
K (M^{-1})	0.79	0.52	0.45	0.35

Calculate the standard free energy ($\Delta G°$), standard enthalpy ($\Delta H°$), and standard entropy ($\Delta S°$) changes for the complexation.

Answer: Compare your results with those in Table 1 of Al-Obeidi and Borazan, J. Pharm. Sci., 65, 982, 1976.

11–12. The charge transfer complex between tryptamine and isoproterenol was studied in aqueous solution containing 0.1 M HCl at several temperatures.[105] The equilibrium constants K were obtained using the Benesi–Hildebrand equation

Data for *Problem 11–12*

T (°C)	5.0	15.0	25.0
K (M^{-1})	3.50	2.30	1.42

(a) Compute $\Delta G°$, $\Delta H°$, and $\Delta S°$. (b) Compute the absorbance of the complex A at 5° C from the Benesi–Hildebrand equation. The molar absorptivity ϵ of the complex is 66.0, the initial concentration of the acceptor (tryptamine) is 0.02 M, and the concentration of the donor (isoproterenol) is 0.5 M.

Answers: (a) $\Delta H° = -7.4$ kcal/mole, $\Delta S° = -24.2$ cal/(mole deg), $\Delta G°$ at 5° C $= -0.69$ kcal/mole; (b) the absorbance A of the charge-transfer band was calculated from the Benesi–Hildebrand equation and is 0.833 at 5° C

11-13. Hanna and Askbaugh[106] derived an expression to compute the apparent equilibrium constant of 1:1 π-molecular complexes from nuclear magnetic resonance data:

$$\frac{1}{\Delta_{obs}^A} = \frac{1}{K(\delta_c^A - \delta_m^A)} \frac{1}{C_D} + \frac{1}{\delta_c^A - \delta_m^A} \qquad (11\text{-}55)$$

where C_D is the concentration of the donor on the molality scale, δ_c^A is the chemical shift (see Chapter 4, p. 92) of the acceptor in the pure complex form, and δ_m^A is the chemical shift of the acceptor in the uncomplexed form. Therefore, $\delta_c^A - \delta_m^A$ is the shift due to complexation. Δ_{obs}^A is the difference between the observed chemical shift and δ_m^A.

The equation requires that the concentration of the donor be much larger than that of the acceptor, and is analogous to the Benesi–Hildebrand equation (p. 266) except that the shift of acceptor protons on the pure complex replaces the molar absorptivity of the complex, and the concentration of acceptor does not appear.

Nishijo et al.[107] studied the complexation of theophylline with an aromatic aminoacid, L-tryptophan, in aqueous solution using proton nuclear magnetic resonance. L-Tryptophan is a constituent of serum albumin and was suggested to be the binding site on serum albumin for certain drugs. The authors added L-tryptophan to a fixed concentration of theophylline at 25° C.

Data for Problem 11-13

(Tryp), $1/C_D$ (M^{-1})	25	50	75	100
$1/\Delta_{obs}^A$	5.9	9.8	13.7	17.6

Compute the apparent equilibrium constant K and the complexation shift, $(\delta_c^A - \delta_m^A)$ using equation (11-55).

Answer: $K = 12.8$, $(\delta_c^A - \delta_m^A) = 0.50$

11-14. The binding or association constant K for the complex povidone-5-hydroxysalicylic acid was determined by equilibrium dialysis at 25° C and 35° C in solvent mixtures of ethanol and water. Using the *Klotz reciprocal plot* (equation (11-38), p. 269),

$$\frac{1}{r} = \frac{1}{\nu K'} \frac{1}{[D_f]} + \frac{1}{\nu}$$

Plaizier-Vercammen and De Nève[108] obtained $\nu K'$ as the slope of the line, plotting $1/r$ against $1/[D_f]$. K' is the binding constant in liters/mole and ν is the binding capacity, i.e, the number of binding sites per mole of the macromolecule, povidone. $[D_f]$ is the molar concentration of free ligand or drug and r is the moles of ligand bound per mole of povidone. The ν and K' are combined in this work to give $\nu K' = K$ as an association constant that measures the strength of binding of 5-hydroxysalicylic acid to povidone. The K values at various percent concentrations of ethanol in water and corresponding dielectric constants, D, at 25° C and 35° C, are given in the following table:

Data for Problem 11-14

% Ethanol in water	Dielectric Constant, D		$K \times 10^{-3}$ liter/mole	
	25° C	35° C	25° C	35° C
2.5	75.2	72.1	20.5	18.0
5.0	73.9	70.9	19.4	17.1
10.0	71.5	68.6	18.0	16.0
20.0	66.6	63.8	15.9	14.1

(a) Compute $\Delta H°$ and $\Delta S°$ over this temperature range, and $\Delta G°$ at both 25° C and 35° C for each mixture.

(b) On the same graph, plot $\Delta G°$ and $\Delta H°$ obtained in (a) as a function of dielectric constant. Two lines will be obtained for $\Delta G°$ versus dielectric constant, D; one for 25° C and a second for 35° C. The values of $\Delta H°$ at various D values are the same at 25° C and 35° C and yield a single line on this plot, as will become evident from the results of the calculations made.

(c) Give a plausible explanation in terms of intermolecular interaction for the binding of 5-hydroxysalicylic acid to povidone, using the magnitude and the signs of the thermodynamic quantities. Compare your answers with the information given on pp. 272–273 and 275–277, Table 11–11, and in J. Pharm. Sci. 71, 552, 1982.

Partial Answer: (a) The thermodynamic values at 2.5% ethanol are:

Partial Answer for Problem 11-14

% Ethanol in water	$\Delta H°$ kcal/mole	$\Delta S°$ cal/(mole deg)	$\Delta G°$ kcal/mole	
			25° C	35° C
2.5	−2.38	+11.8	−5.88	−6.00

11-15. The binding of warfarin to human serum albumin was studied at pH 6, ionic strength 0.170. The following values were found by O'Reilly.[109] For a definition of symbols, see page 269 of this book.

Data for Problem 11-15

[PD] μmole/L	$[D_f]$ μmole/L	$r/[D_f]$ L/μmole	r
9.1	3.0	0.13	0.40
17.8	6.4	0.11	0.72
30.2	17.2	0.08	1.35
46.1	50.8	0.04	2.00

(a) Obtain the Scatchard plot, equation (11-40), using this data and compute K and ν using linear regression; it is the number of independent binding sites. Express K in L/mole, were L stands for liters.

(b) Assume that the concentration of protein is unknown, and compute K from $[PD]$ and $[D_f]$ equation (11-41). Compare the constant K obtained in (a) and (b). Compute $[P_t]$ (total concentration of protein) using the number of binding sites obtained in (a). See equation (11-41) on page 270.

Answers: (a) $K = 0.0552$ L/μmole $= 55,200$ L/mole; $\nu = 2.75$; (b) $K = 0.0602$ L/μmole $= 60,200$ L/mole; using $\nu = 2.75$ from (a), $[P_t] = 22.2$ μmole/L

11-16. In a study by Meyer and Guttman[110] of the binding of caffeine to bovine serum albumin by the equilibrium dialysis method, 2.8×10^{-4} M of albumin was allowed to equilibrate with 1×10^{-4} M of caffeine. After equilibrium was established, 0.7×10^{-4} M of caffeine was contained in the dialysis bag, while 0.3×10^{-4} M of caffeine was found in the external solution. Calculate r, the ratio of bound to total protein. What is the fraction bound, β, of caffeine?

Answer: $r = 0.14$; $β = 0.571$

11.17. Chan and his associates[111] investigated the in vitro protein binding of diclofenac sodium, a nonsteroidal antiinflammatory drug, by equilibrium dialysis and plotted the results according to the Scatchard equation (equation (11-42b)) used to describe two classes of sites:

$$r = \frac{\nu_1 K_1 [D_f]}{1 + K_1 [D_f]} + \frac{\nu_2 K_2 [D_f]}{1 + K_2 [D_f]}$$

These workers used a statistical method known as *nonlinear regression* on the data given below to calculate the parameters ν_1, ν_2, K_1, and K_2. The number of binding sites ν_1 and ν_2 found for the two classes of sites are 2.26 and 10.20, respectively. The corresponding association constants are $K_1 = 1.32 \times 10^5$ M^{-1} and $K_2 = 3.71 \times 10^3$ M^{-1}

Using the equation given above, calculate the values of r (dimensionless) for the following free drug concentrations: $[D_f]$ in millimole/liter ($\times 10^3$) = 1.43, 4.7, 16, 63, 132.4, 303.4, and 533.2.

Plot $r/[D_f]$ (liter/millimole) versus r to obtain what is called a *Scatchard plot*. Compare your results with those of Chan et al. To obtain an answer to this problem, the student may compare his or her calculated $r/[D_f]$ values with the $r/[D_f]$ abscissa values read from the graph of Chan et al.

Hint: Use the same units on K_1, K_2, and $[D_f]$ to calculate r.

Partial Answer: for $[D_f] = 1.43 \times 10^{-3}$ millimole/liter (1.43×10^{-6} mole/liter), $r = 0.41$; $r/[D_f] = 289$ (liter/millimole)

11–18. The number of binding sites and the association constant for the binding of sulfamethoxypyridazine to albumin at pH 8 can be obtained from the data given as follows:

Data for *Problem 11–18*

$r = [D_b/P_t]$	0.23	0.46	0.66	0.78
$[D_f] \times 10^4$ (mole/liter)	0.10	0.29	0.56	1.00

where $[D_b]$ is the concentration of drug bound, also referred to as $[PD]$, and $[P_t]$ is the total protein concentration. What values are obtained for the number of binding sites ν and for the association constant K?

Answer: $\nu = 1$; $K = 26,821$

11–19. The effect of phenylbutazone in displacing acetaminophen from its binding sites on human serum albumin (HSA) was studied by the ultrafiltration method (p. 270) at 37° C and pH 7.4, with a constant concentration of acetaminophen, $[D_t] = 3.97 \times 10^{-4}$ mole/liter, and with increasing concentrations of phenylbutazone $[D'_t]$. After ultrafiltration the absorbance A of the free fraction of acetaminophen, corresponding to several concentrations of phenylbutazone, is

Data for Problem 11–19

Case	I	II	III	IV
$D'_t \times 10^4$ mole/liter	0	0.65	3.89	6.48
A	0.683	0.782	0.809	0.814

The table also shows the absorbance of acetaminophen in the absence of phenylbutazone, $D'_t = 0$. The molar absorptivity, ϵ, of acetaminophen at 420-nm wavelength in a cell of path length $b = 1$ cm is 2.3×10^3 liter mole^{-1} cm^{-1}. The HSA concentration $[P_t]$ is 5.8×10^{-4} mole/liter.

Calculate the percent decrease in the Scatchard r values for acetaminophen and the percent bound at different concentrations of phenylbutazone, D'_t, shown in the table.

Partial Answer: In case I (see the table above), the concentration, $[D_f] = A/\epsilon b$, of unbound acetaminophen in the absence of phenylbutazone, $[D'_t] = 0$, is 2.97×10^{-4} mole/liter. The concentration $[D_b]$ of bound acetaminophen is 1.00×10^{-4} mole/liter. The r value, $[D_b]/[P_t] = 0.17$, and the percent bound ($[D_b]/[D_t]) \times 100 = 25\%$.

In case II (in the presence of phenylbutazone, $[D_t] = 0.65 \times 10^{-4}$ mole/liter), the concentration of unbound acetaminophen is $[D_f] = A/(\epsilon b) = 0.782/(2.03 \times 10^3)(1) = 3.4 \times 10^{-4}$ mole/liter. $[D_b]$ is $(3.97 \times 10^{-4}) - (3.4 \times 10^{-4}) = 0.57 \times 10^{-4}$ mole/liter and $r = (0.57 \times 10^{-4})/(5.8 \times 10^{-4}) = 0.10$.

11–20.* In a study of protein binding, using the dynamic dialysis method of Meyer and Guttman,[76] 2×10^{-3} mole/liter of drug was placed in a dialysis sac. In the absence of protein, the following values for $[D_t]$ were determined:

Data (a) for *Problem 11–20*

Time (hr)	2.0	4.0
$[D_t]$ mole/liter $\times 10^3$	0.74	0.27

Equation (11–45) may be written as

$$\ln [D_t] = \ln [D_f] - kt$$

Compute k, the slope, from the two-point formula using the data given in the table above.

When the dialysis study was repeated in the presence of 5×10^{-4} mole/liter of protein, the rate of loss of drug from the dialysis sac was again determined. The resulting data were fit by computer to equation (11–46) and the following empiric constants were obtained:

$$C_1 = 1 \times 10^{-3} \text{ mole/liter, } C_2 = 0.2 \text{ hr}^{-1},$$
$$C_3 = 6 \times 10^{-4} \text{ mole/liter, } C_4 = 0.1 \text{ hr}^{-1},$$
$$C_5 = 4 \times 10^{-4} \text{ mole/liter, } C_6 = 0.05 \text{ hr}^{-1}$$

The experimentally determined values for $[D_t]$ in the presence of protein were as follows at 1, 3, and 5 hours:

Data (b) for *Problem 11–20*

Time (hr)	1	3	5
$[D_t]$ mole/liter $\times 10^3$	1.74	1.34	1.04

Calculate the three values of the Scatchard terms r and $r/[D_f]$, which can be determined from these data. Although many more points than three are required to prepare a satisfactory Scatchard plot, sketch these three points on a plot of $r/[D_f]$ versus r to obtain a rough idea of the curve that would result. See Figure 11–17, page 269, for the general shape of a Scatchard plot.

Answer: At 1 hr, $r = 2.53$, $r/[D_f] = 5.35 \times 10^3$ liter/mole; at 3 hr, $r = 1.99$, $r/[D_f] = 5.80 \times 10^3$ liter/mole; at 5 hr, $r = 1.58$, $r/[D_f] = 6.31 \times 10^3$ liter/mole

11–21. Higuchi and Zuck[112] investigated the complex formed between caffeine and benzoic acid and obtained the following results: $K = 29$ at 0° C and $K = 18$ at 30° C. Compute $\Delta H°$, $\Delta G°$, and $\Delta S°$ at 30° C. What significance can be attributed to each of these values? See Table 11–11.

Answer: $\Delta H° = -2.62$ kcal/mole, $\Delta G° = -1.74$ kcal/mole, $\Delta S° = -2.9$ cal/mole deg.

11–22. The data in Table 11–12 are similar to those obtained by Cho et al.[113] using equilibrium dialysis for the binding of bishydroxycoumarin to human serum albumin (HSA). The concentration of HSA was 0.20% (2.90×10^{-5} mole/liter). The pH was held at 7.4 by the use of a tris (hydroxymethyl) aminomethane–hydrochloric acid buffer, and ionic strength was maintained at 0.15. Using the data of Table 11–12 at 20° C and 40° C,

(a) plot $r = $ [Drug bound]/[Total HSA] against free drug concentration, $[D_f]$, to obtain what is known as a *Langmuir isotherm*. The HSA concentration is 2.9×10^{-5} mole/liter.

(b) Plot $r/[D_f]$ on the vertical axis of a graph and r on the horizontal axis to obtain a Scatchard plot as represented by equation (11–40). You will obtain a curve rather than a straight line.

Problem 11–20 was prepared by Professor M. Meyer of the University of Tennessee.

TABLE 11–12. *Bishydroxycoumarin Interaction with Human Serum Albumin* at 20° C and 40° C (Data for Problem 11–22)*

[D_b]	[D_f]		20° C		40° C
moles/liter × 10^6	moles/liter × 10^6	r †	$\frac{r}{[D_f]} \times 10^{-5}$	r	$\frac{r}{[D_f]} \times 10^{-5}$
23.20	1.000	0.8	8.0	0.6	4.0
29.00	1.430	1.0	7.0	1.1	3.1
34.80	2.000	1.2	6.0	1.7	2.3
40.60	2.690	1.4	5.2	1.9	1.5
52.20	4.290	1.8	4.2	2.5	1.1
63.80	6.880	2.2	3.2	3.1	0.7
92.80	14.55	3.2	2.2	3.9	0.5
116.0	33.33	4.0	1.2	4.9	0.3
145.0	62.50	5.0	0.8	5.7	0.1
174.0	150.00	6.0	0.4		

*Serum albumin concentration, 2.9×10^{-5} mole/liter.
†$r = D_b$/albumin concentration.

(c) Determine ν, the average number of the first type of binding sites at 20° C and 40° C, and round off the values of ν to obtain integer numbers. Use the roughly linear part of the Scatchard plot, i.e., the first five points given in Table 11–12. The intercept on the ordinate is νK, from which K may be obtained at the two temperatures.

(d) Using the first five values of Table 11–12, compute the association constant, K, for the first type of binding sites of bishydroxycoumarin on human serum albumin, at both 20° C and 40° C. You may use either the two-point formula or regression analysis.

(e) The authors obtained $K = 3.5 \times 10^5$ liter/mole and 1.7×10^5 for the binding constants at 20° C and 40° C, respectively. Using these values, estimate the standard free energy changes, $\Delta G°$, for the interactions at 20° C and 40° C from the expression, $\Delta G° = -RT \ln K$.

(f) Calculate the standard enthalpy change, $\Delta H°$, using the association constants at the two temperatures, and the equation

$$\ln \frac{K_{20°}}{K_{40°}} = -\frac{\Delta H°}{R} \left(\frac{1}{293.15} - \frac{1}{313.15} \right)$$

(g) Obtain the standard entropy change $\Delta S°$ for the complexation using $\Delta G° = \Delta H° - T \, \Delta S°$.

(h) Give a plausible explanation for the magnitude and sign of the thermodynamic quantities obtained.

Answers: Compare your results with those obtained by Cho et al.[113] Table I. For example, the authors obtain $\Delta G° = -7.43$ kcal/mole at 20°C. See Table 11–11, p. 276, to help you explain the results obtained.