Analogs of Tetrahydrofolic Acid XXVIII

Mode of Pyrimidine Binding to Dihydrofolic Reductase pH Profile Studies

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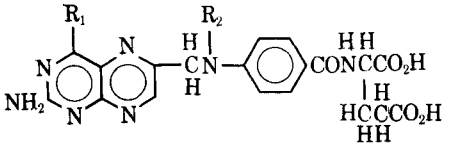
Three 2,4-diamino heterocycles, which were closely related structurally, were chosen for the study of the effect of pH on inhibition of dihydrofolic reductase. 1-(*p*-Chlorophenyl)-4,6-diamino-2,2,-dimethyl-1,2-dihydro-*s*-triazine (VI) (pKa 11.2) was chosen since it remained fully protonated throughout the pH range of 5–9. In contrast, 5-(*p*-chlorophenyl)-2,4-diamino-6-(trifluoromethyl)pyrimidine (V) (pKa 2.8) was chosen since it remained fully unprotonated throughout the pH range of 5–9. These two pH profiles were then compared with a pyrimidine of intermediate pKa (7.7), namely, 5-(*p*-chlorophenyl)-2,4-diamino-6-methylpyrimidine (IV) which is 99 per cent protonated at pH 5 and 5 per cent protonated at pH 9. The fully protonated heterocycle (VI) showed strongest inhibition at pH 9 and weakest inhibition at pH 5; in contrast, the unprotonated pyrimidine (V) showed weakest inhibition at pH 9 and strongest inhibition at pH 5. The intermediate basic-strength pyrimidine (IV) showed weakest inhibition at both pH 5 and 9 and strongest inhibition at intermediate pH's. These pH profiles support the concept that strongly basic 2,4-diamino heterocycles complex best at pH 9 to dihydrofolic reductase when the heterocycle is protonated and the complexing region of the enzyme is an anionic form; in contrast, at pH 5 the complexing region of the enzyme is believed to be protonated which would repel a protonated heterocycle. Furthermore, a weakly basic heterocycle unprotonated at pH 5 binds best at pH 5 where the enzymic binding site is presumably protonated. Both aminopterin (pKa 5.5) and folic acid (pKa 2.5) had pH inhibition profiles in agreement with this concept.

THE POTENT folic acid (I) antagonists, aminopterin (II) and amethopterin (III), have been known for more than 15 years (1). Since that time these two antagonists have been found to be extremely potent inhibitors of the enzyme,



I, R_1 = OH, R_2 = H; folic acid
II, R_1 = NH_2, R = H; aminopterin
III, R_1 = NH_2, R_2 = CH_3; amethopterin

dihydrofolic reductase (1). Under the non-physiological conditions of pH 6.1 with folic acid as a substrate, amethopterin has been found to bind 100,000 times stronger to the enzyme from rat liver than the substrate (2). However, when folic acid (I) is compared to aminopterin (II) as an inhibitor of dihydrofolic reductase from pigeon liver at pH 7.4 with dihydrofolate as a substrate, the difference is only 3000-fold (3, 4); similarly, amethopterin (III) is a 1400-fold better inhibitor of dihydrofolic reductase from Ehrlich ascites cells at pH 7.4 than is folic acid when dihydrofolate is used as the substrate (5).

Two schools of thought have arisen to rationalize the high potency of aminopterin and amethopterin compared to folic acid in their relative ability to bind to dihydrofolic reductase. Since a 2,4-diamino heterocycle such as II is a stronger base than a 2-amino-4-hydroxy heterocycle such as folic acid (I), Baker (6) proposed that the increase in binding of II was due to a protonated species of aminopterin complexing with an anionic site on the enzyme; this proposal was amplified further by Perault and Pullman (7) on a mathematical basis.

A second rationalization by Zakrzewski (8) was based on thermodynamic studies on the binding of amethopterin (III) to folic reductase. As a result of these experiments, Zakrzewski (8) proposed that four hydrogen bonds between the enzyme and the four nitrogen functions of the 2,4-diaminopyrimidine moiety of amethopterin (III) could account for the strong binding of III; folic acid (I) would then have to isomerize from the more stable 4-oxo form with a resultant loss of energy. These thermodynamic studies had several tenuous assumptions and admittedly were difficult to perform due to the small change in K_i or K_m with temperature. Furthermore, on the basis of an analysis of the relationship to basicity of ten purines, pyrimidines, and pteri-

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Previous paper: Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).
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TABLE I.—EFFECT OF pH ON PROTONATION OF INHIBITORS AND THEIR INHIBITION OF DIHYDROFOLIC REDUCTASE

Compd.	pKa of Heterocyclic Ring	μM Concn. for 50% Inhibition at pH 7.4	% Protonation at pH 5	% Protonation at pH 5.9	% Protonation at pH 7.4	% Protonation at pH 8	% Protonation at pH 9	V_0/V_I μM Concn.	V_0/V_I at pH[a] 5	V_0/V_I at pH[a] 5.9	V_0/V_I μM Concn.	V_0/V_I at pH[a] 8	V_0/V_I at pH[a] 9
IV[e]	11.2[b]	0.050	99.9+	99.9+	99.9+	99.9	99.4	0.050	1.05	1.76	0.10	2.13	3.21
VI[e]	7.7[c]	0.20	99.8	98.0	66.6	33.4	4.8	0.20	1.30	2.00	1.0	2.22	1.52
V[e]	2.8[d]	50	0.63	0.079	0.001	0.000	0.000	50	3.80	2.95	300	2.00	1.54
Folic acid I	~2.5[e]	3.0	0.31	0.031	0.001	0.000	0.000	3.0	2.04	2.02	3.0	1.60	1.04
Aminopterin II	~5.5[e]	0.001	76	24	1.2	0.32	0.032	0.002	1.56	2.86	0.001	1.90	1.00

[a] V_0 = velocity without inhibitor and V_I = velocity with inhibitor. With no inhibition, $V_0/V_I = 1$; with 50% inhibition, $V_0/V_I = 2$. [b] Data from *Reference 18*. [c] Data from *Reference 9*. [d] Determined spectrophotometrically by variation of absorbance at 302 mμ with pH. [e] See text.

dines to binding with folic reductase, Zakrzewski (9) concluded that "it appears that the ionic binding between folate reductase and its substrates or inhibitors is unlikely." His data suffer from the difficulty that proper compounds were not available for a realistic comparison (4), particularly the use of a control compound that would be fully protonated at both the pH's used, 5.2 and 6, to show what changes may have taken place on the enzyme, such as protonation, that could be either favorable or detrimental to binding. Huennekens and Scrimgeour (10) have proposed that an additional hydrogen bond between the H of the 4-amino group of amethopterin and the enzyme that cannot exist between the 4-oxo form of dihydrofolate and the enzyme could account for the observed difference in free energy of binding of the two compounds (3.7 Kcal./mole) in their system.

Two more recent studies shed further light on these two different rationalizations. Bertino *et al.* (5) performed a pH profile study on dihydrofolic reductase with folic acid (I) and amethopterin (III) as inhibitors; they noted that the relative inhibition by amethopterin (III) was more pH dependent than folic acid, but preferred not to draw any conclusion about the protonation of amethopterin (III) since "the ionization of the active center of the enzyme or the enzyme–inhibitor complex may be of importance."

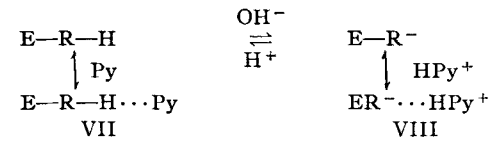
Baker and Jordaan (4) noted that conversion of the 6-methyl group of 5-(*p*-chlorophenyl)-2,4-diamino-6-methylpyrimidine (IV) to a trifluoro-

IV, R=CH_3
V, R=CF_3

VI

methyl group (V) caused a 250-fold reduction in the binding of V to dihydrofolic reductase compared to IV; furthermore, due to the electron-withdrawing properties of the trifluoromethyl group, V was a weak base (pKa 2.8) unprotonated at pH 7.4, whereas IV was mainly protonated (pKa 7.7), which presumably accounts for IV being a better inhibitor than V. They proposed (4) that one of the binding points of the pyrimidines, IV and V, to dihydrofolic reductase involved complexing to a weakly acidic group on the enzyme that is only partially ionized at pH 7.4. This group on the enzyme (E) was represented by RH. If Py is a nonprotonated pyrimidine and HPy^+ is a protonated pyrimidine,

then two types of complexes, VII and VIII, respectively, could be formed. The only difference between VII and VIII is whether the proton in question is firmly associated with the enzymic acidic group as in VII, or is firmly associated with the strongly basic pyrimidine as in VIII, or in between. The more associated the proton is with the pyrimidine, the more salt-like would be the bond and more energy would be involved in the bonding. Conversely, the weaker Py is as a base, the weaker would be the bodinng energy which could approach the strength of a hydrogen bond, or even weaker.



To gain evidence for or against the binding mechanism depicted with VII and VIII, inhibition pH profiles with the three structurally related heterocycles (IV–VI) were performed; these three compounds were further selected since V is unprotonated through the pH range of 5–9, VI is fully protonated, and IV varies from 99.8% protonated at pH 5 to 4.8% protonated at pH 9 (Table I). The results are the subject of this paper.

EXPERIMENTAL

Materials.—Folic acid (Nutritional Biochemicals Co.) was reduced with sodium dithionite to dihydrofolic acid as described by Futterman (11) and stored as a 1.86 m*M* homogenized suspension in 0.005 *N* hydrochloric acid containing 0.1 *M* mercaptoethanol. Aminopterin and TPNH were purchased from Sigma Chemical Co. 1-(*p*-Chlorophenyl) - 4,6 - diamino - 2,2 - dimethyl - 1,2 - dihydro-*s*-triazine hydrochloride (VI) (NSC-3074) was a gift from Dr. Harry B. Wood, Jr., Cancer Chemotherapy National Service Center. 5-(*p*-Chlorophenyl) - 2,4 - diamino - 6 - methylpyrimidine (IV) was prepared in this laboratory by W. H. Myers according to the literature procedure (12). The synthesis of 5-(*p*-chlorophenyl)-2,4-diamino-6-(trifluoromethyl)pyrimidine (V) has been described previously (4). The infrared spectra of IV and V were almost identical in the 2.8–6.8 μ region of NH_2, C=C, and C=N, thus showing that IV and V had the same 2,4-diaminopyrimidine character in the solid state.

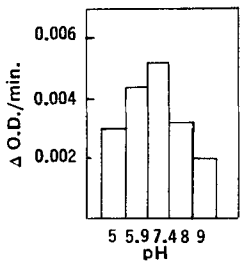


Fig. 1.—The effect of pH on the velocity of reduction of 6 μM dihydrofolate by 12 μM TPNH in 10 m*M* mercaptoethanol and 1 m*M* NaEDTA; pH 7.4–9 in 0.05 *M* Tris buffer and pH 5–5.9 in 0.05 *M* citrate buffer.

Enzyme Source and Assay.—Pigeon liver acetone powder was purchased from General Biochemicals; the dihydrofolic reductase used was a 45–90% saturated ammonium sulfate fraction prepared as previously described (13).

The enzyme assays at pH 7.4, 8, and 9 were performed in 0.05 *M* Tris buffer [containing 10 m*M* mercaptoethanol and 1 m*M* tetrasodium ethylenediaminetetraacetate (NaEDTA)] with 6 μM dihydrofolate and 12 μM TPNH (13); for pH 5.9 and 5.0, 0.05 *M* citrate buffer, containing 10 m*M* mercaptoethanol and 1 m*M* NaEDTA was employed. The rates were measured by the change in absorbance per minute at 340 mμ on a single-beam Gilford 2000 spectrophotometric system for all runs except at pH 5; negligible blanks were obtained without dihydrofolate or without TPNH. At pH 5, the extraneous TPNH oxidation was appreciable; the assays were therefore run with a double-beam Cary model 11 spectrophotometer with balanced cells except for the dihydrofolate in the upper cell. All points in Table I and the figures are the average of four determinations.

RESULTS

The effect of pH on inhibition of dihydrofolic reductase by the various inhibitors is shown in Figs.

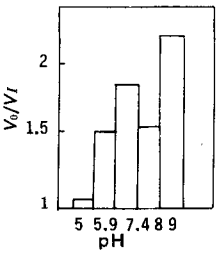


Fig. 2.—The effect of pH on the inhibition of reduction of 6 μM dihydrofolate with 12 μM TPNH in the presence of 0.05 μM 1-(*p*-chlorophenyl) - 4,6 - diamino - 2,2 - dimethyl-1,2-dihydro-*s*-triazine (VI); pH 7.4–9 in 0.05 *M* Tris buffer and pH 5–5.9 in 0.05 *M* citrate buffer, both containing 10 m*M* mercaptoethanol and 1 m*M* NaEDTA. V_0 = velocity without inhibitor, and V_I = velocity with inhibitor.

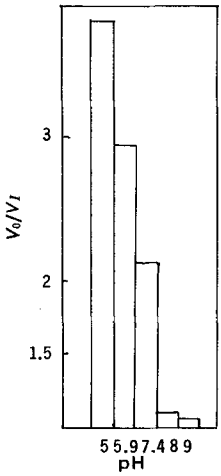


Fig. 3.—The effect of pH on the inhibition of reduction of 6 μM dihydrofolate with 12 μM TPNH in the presence of 50 μM 5-(*p*-chlorophenyl) - 2,4 - diamino - 6 - (trifluoromethyl)pyrimidine (V); pH 7.4–9 in 0.05 *M* Tris buffer and pH 5–5.9 in 0.05 *M* citrate buffer, both containing 10 m*M* mercaptoethanol and 1 m*M* NaEDTA. V_0 = velocity without inhibitor, and V_I = velocity with inhibition.

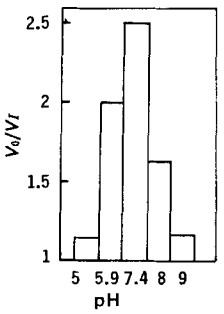


Fig. 4.—The effect of pH on the inhibition of reduction of 6 μM dihydrofolate with 12 μM TPNH in the presence of 0.40 μM 5-(*p*-chlorophenyl)-2,4-diamino-6-methylpyrimidine (IV); pH 7.4–9 in 0.05 M Tris buffer and pH 5–5.9 in 0.05 M citrate buffer, both containing 10 mM mercaptoethanol and 1 mM NaEDTA. V_0 = velocity without inhibitor, and V_I = velocity with inhibitor.

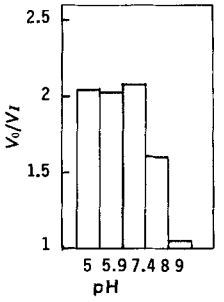


Fig. 5.—The effect of pH on the inhibition of reduction of 6 μM dihydrofolate with 12 μM TPNH in the presence of 3 μM folic acid (I); pH 7.4–9 in 0.05 M Tris buffer and pH 5–5.9 in 0.05 M citrate buffer, both containing 10 mM mercaptoethanol and 1 mM NaEDTA. V_0 = velocity without inhibitor and V_I = velocity with inhibitor.

2–5; this inhibition is recorded as the ratio of the velocity without inhibitor (V_0) to the velocity with inhibitor (V_I). Note that V_0 varies with the pH as shown in Fig. 1.

The first compound examined was the dihydro-*s*-triazine (VI) which has pKa 11.2 and is therefore greater than 99% protonated over the pH range of 5–9 (Table I). Therefore, this heterocycle most probably has to bind to the enzyme as a protonated species; if an unprotonated species were essential for binding, then VI should have been a poorer inhibitor than the unprotonated 6-trifluoromethyl pyrimidine (V) rather than a better inhibitor (Table I). The proposal of two possible enzyme–inhibitor species VII and VIII, for binding a 2,4-diamino heterocycle predicts that at pH 5 where the enzyme binding site is more protonated, VI should be a poorer inhibitor than at higher pH's. Due to both the enzyme binding site and the inhibitor being protonated, a repulsion should be noted. Furthermore, since VI is still protonated at pH 9 and the enzymic binding site is at the pH of least protonation in the range studied, the system predicts that VI should bind best at pH 9 as species VIII. That this was the case is shown in Fig. 2 and Table I. The drop in inhibition between pH 7.4 and 8 is probably due to other ionizations of the enzyme or substrate since all of the compounds (Figs. 2–6) except aminopterin show this drop.

The 6-trifluoromethyl pyrimidine (V) is unprotonated throughout the pH range 5–9 (Table I) and should therefore complex best with a more protonated binding site of the enzyme at pH 5, as in species VII, and poorest to the less protonated enzymic binding site at pH 9. That such is the case is seen clearly in Fig. 3.

The 6-methyl pyrimidine (IV) is intermediate between V and VI in basicity, being 99% protonated at pH 5 and 5% protonated at pH 9 (Table I). Therefore, IV should bind poorly at pH 5 with a protonated form of enzyme binding site (VII) and poorly with the ionized form of the enzyme binding site at pH 9 (VIII) for the same reasons cited for V and VI. Therefore, IV should bind best at intermediate pH. These predictions were born out as shown in Fig. 4, the maximum inhibitor effect being observed at pH 7.4 with poorer binding at the pH extremes.

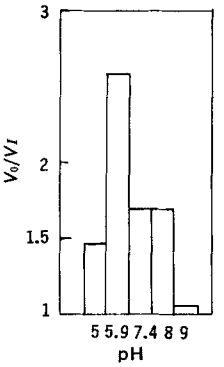


Fig. 6.—The effect of pH on the inhibition of reduction of 6 μM dihydrofolate with 12 μM TPNH in the presence of 0.001 μM aminopterin; pH 7.4–9 in 0.05 M Tris buffer and pH 5–5.9 in 0.05 M citrate buffer, both containing 10 mM mercaptoethanol and 1 mM NaEDTA. V_0 = velocity without inhibitor and V_I = velocity with inhibitor.

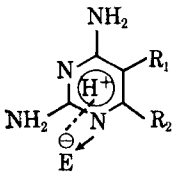
It should be noted (Table I) that the dihydro-*s*-triazine (VI) was a fourfold better inhibitor than the 6-methylpyrimidine (IV) at pH 7.4, and IV was in turn a 250-fold better inhibitor than the 6-trifluoromethyl pyrimidine (V). Corrected for per cent protonation at pH 7.4, IV and VI have the same magnitude of binding of their protonated species, whereas V is a much poorer inhibitor presumably because it is unprotonated. It should also be noted that since IV and V had almost identical bands in the NH, NH_2, and double bond regions, the difference in activity between IV and V was unlikely to be due to some unusual tautomeric form of V.

Due to the complexity of their titration curves, the pKa's for the pteridine ring of aminopterin (II) and folic acid (I) are difficult to determine; however, it can be estimated (8, 9) that the basicity of the pteridine of folic acid should be about the same as 2-amino-6-methyl-4-pteridinol (pKa 2.5) and aminopterin about the same as 2,4-diaminopteridine (pKa 5.5). On this basis, aminopterin (II) should have an inhibition pH profile similar to the 6-methyl pyrimidine (IV), that is, maximum inhibition at pH 5.9–7.4 and minimum inhibition at pH 5 and 9; such a profile was observed experimentally as shown in Fig. 6. Note that aminopterin has a lower pH maximum than the diaminopyrimidine (IV) since the latter is a 100-fold stronger base.

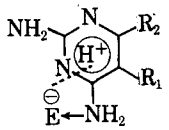
On the other hand, folic acid (I) should have a pH profile resembling the nonprotonated 6-trifluoromethyl pyrimidine (V); in Fig. 5 it can be seen that folic acid has best inhibition at pH 5–7.4, then drops off rapidly at pH 8 and 9. These results infer that the substrate, dihydrofolate, is a stronger base than folate, perhaps being intermediate between the more basic pyrimidine and the less basic pteridine (7, 19).

DISCUSSION

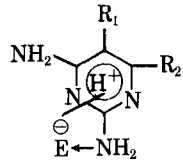
With the not too unlikely assumption that these inhibitors are complexed with the active site, the results in Figs. 2–6 and Table I support the concept



IX*A*



IX*B*



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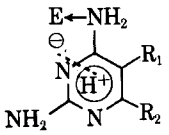
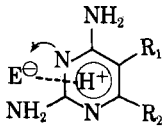
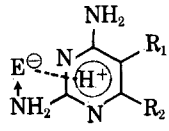


X



XI



XII

that there are two possible species for the complex between dihydrofolic reductase and a 2,4-diamino heterocycle, as depicted in structures VII and VIII; that is, a protonated combining site of the enzyme can complex the free base (as in VII) and the unprotonated combining site of the enzyme can complex with the protonated base (as in VIII). This concept still fully agrees with the data obtained by Zakrzewski (9). There were two factors which unfortunately he was unable to consider in his experimental design: (*a*) he did not have candidate inhibitors available that stayed either fully unprotonated or fully protonated throughout the pH range, and (*b*) he was unable to do the pH profile much above pH 6 with the use of folic acid as a substrate since folic acid shows about one-quarter the velocity at pH 6.5 and about one-tenth the velocity at pH 7.0 that it shows at pH 5 (5).

Based on the relative inhibition of dihydrofolic reductase by 2,4-diamino-6-methylpyrimidine *versus* 2-amino-6-methyl-4-pyrimidinol and IV *versus* V, we proposed (4) that a two point attachment of a 2,4-diamino heterocycle is sufficient to account for both the magnitude and specificity of binding of this type of heterocycle; the current pH profile study lends further support to this concept. Although Perault and Pullman (7) have performed calculations to determine which is the most basic nitrogen of a 2,4-diaminopteridine, it is not necessarily the most basic nitrogen which will protonate when complexed to the enzyme. More probable is the concept of an interaction between the delocalized charge on a 2,4-diamino heterocycle such as IX*A* and the ionized combining site of the enzyme; a further single hydrogen bond with the enzyme containing the electron-accepting group would complete the binding. Such a type of binding depicted in IX*A* would also allow binding of the pyrimidine in other conformations, for example IX*B* or IX*C*, depending upon the substituents, R_1 and R_2. Similarly, the enzyme could be placed to hydrogen bond with one of several alternate positions such as X–XII. The four-hydrogen bond theory of Zakrzewski (9) would allow only one conformation of binding; recent results on studies from this laboratory on hydrophobic bonding to dihydrofolic reductase (14) could not be rationalized by the conformationally-fixed four-hydrogen bond theory, but could be rationalized by a variety of conformations, such as IX*A*, IX*B*, and IX*C*. Such a rationalization with two conformations has recently been invoked by Leonard and Laursen (15) to explain the binding properties of isoadenosine and its nucleotide derivatives to several enzyme systems. The different types of conformations have also been useful for a better understanding (16) of the heretofore unrationalizable binding of a series of 6-phenyl pyrimidines (17).

A more recent paper by the Pullman group (19) also can explain the results of Zakrzewski (9) by the increase in basicity of the 2,4-diamino heterocycles leading to stronger inhibition of dihydrofolic reductase. They proposed that the most likely attachment of pyrimidines and aminopterin was through the 2-amino group and the N_1-nitrogen since these are the two most basic nitrogens (19). As pointed out previously, it is not necessarily the most basic nitrogens which will complex with the enzyme. The Pullman group (7) has calculated that N_5 is the most basic nitrogen in dihydrofolate, N_8 in folate, and N_1 in aminopterin. It would be difficult to imagine that either N_1, N_5, or N_8 will be complexed depending upon the substrate or inhibitor. It could be expected that the same nitrogens of all three closely related structures would be bound to the enzyme.

The Pullman group (19) stated that it would be theoretically possible for either a protonated or unprotonated form of a diamino heterocycle to bind to the enzyme even when the heterocycle was a strong enough base to be fully protonated at the pH of the inhibition assay. The current pH profile studies reported here give experimental support to the hypothesis that a species protonated at the pH of the assay—such as IV or VI—can bind to an ionized, but weakly acidic site on the enzyme and that an unprotonated compound at the pH of the assay such as V can bind to the protonated (unionized) form of this acidic site on the enzyme.

In conclusion, it is not our intent to infer that the stronger basicity of aminopterin (II) compared to folic acid is the only factor in the 1000–3000-fold difference in their binding to dihydrofolic reductase, but to state that we are in agreement with the Pullman group (7, 19) that relative basicity is one of the most important factors in this difference, if not the most important. It is also not our intent to infer that close analogs of dihydrofolate, such as folate and aminopterin, can have more than one conformation for binding that is different than the mode of binding of the substrate, but only that pyrimidine-type inhibitors can if there are other strong binding forces such as hydrophobic bonding (14).

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Interaction of Pharmaceuticals with Schardinger Dextrins V

Interaction with a Series of Phenyl-Substituted Carboxylic Acids

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Interactions were observed between beta-cyclodextrin and a series of phenyl-substituted carboxylic acids in aqueous solution. By use of the solubility method of analysis, definite interactions were found to occur with each of the acids. When phase diagrams allowed, stoichiometric ratios were calculated for the complexes and the corresponding K_f and ΔF^0 values determined. Stoichiometries from the analysis of various isolated complexes agreed quite closely with those obtained from the phase diagrams. Several of the inclusion complexes exhibit extremely high formation constants indicative of thermodynamically favorable interactions. Even though a complex mechanism consisting of pure inclusion and other attractive forces is expected for these interactions, the experimental data indicate the relative importance of the separation between the carboxyl and phenyl groups in the net interactions observed. Saturated acids were found to be far more reactive with beta-cyclodextrin than were the corresponding unsaturated acids. This finding could have important pharmacological and biochemical implications, as beta-cyclodextrin has been used as an enzyme model by numerous investigators.

COMPLEX FORMATION, by means of molecular inclusion formation, has only recently been recognized as a promising area in the field of pharmacy. Schlenk (1) defines inclusion compounds as addition products in which one of the components fits into and is surrounded by the crystal lattice of a second. They are probably best described in a negative way, as they do not form by means of ionic, covalent, or coordinate covalent bonds, and are thus often referred to as "no-bond" interactions. Actually inclusion is believed to be the result of the ability of one compound, because of its peculiar stereochemical properties and possibly its polarity, to enclose a second compound spatially. The terms "guest" and "host" have been applied to the enclosed molecule and the enclosing molecular network, respectively. For inclusion formation to occur, the host must be capable of forming a solid structure containing hollow spaces large enough to accommodate a prospective guest species.

An inclusion compound will have a stability largely dependent on the spatial arrangement and fit between the guest and the host. Powell (2) notes that the important factor in inclusion formation is geometry rather than chemistry, and therefore the geometrical features of the interacting species are more critical than are their chemical characteristics. Pauling (3) has pointed out that often the host network is formed through the intermolecular hydrogen bonding of the individual host units.

The cyclodextrins, frequently called the Schardinger dextrins because they were originally prepared by him in 1903 (4), are macrocyclic nonreducing glucosyl polymers produced by the

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