Biochem. J. (1978) **171**, 357–366
Printed in Great Britain

Ultraviolet Difference-Spectroscopic Studies of Substrate and Inhibitor Binding to *Lactobacillus casei* Dihydrofolate Reductase

By KEVIN HOOD and GORDON C. K. ROBERTS*
Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

(*Received* 10 *August* 1977)

The u.v. difference spectra generated when methotrexate, trimethoprim or folate bind to *Lactobacillus casei* dihydrofolate reductase were analysed. The difference spectrum produced by methotrexate binding is shown to consist of three components: (*a*) one closely resembling that observed on protonation of methotrexate, reflecting an increased degree of protonation on binding; (*b*) a pH-independent contribution corresponding to a 40nm shift to longer wavelengths of a single absorption band of methotrexate; (*c*) a component arising from perturbation of tryptophan residue(s) of the enzyme. Quantitative analysis of the pH-dependence of component (*a*) shows that the p*K* of methotrexate is increased from 5.35 to 8.55 (±0.10) on binding. In contrast, folate is not protonated when bound to the enzyme at neutral pH. At pH 7.5, where methotrexate is bound 2000 times more tightly than folate, one-third of the difference in binding energy between the two compounds arises from the difference in charge state. A similar analysis of the difference spectra generated on trimethoprim binding demonstrates that this compound, too, shows an increase in p*K* on binding, but only from 7.22 to 7.90 (±0.10), suggesting that its 2,4-diaminopyrimidine ring does not bind to the enzyme in precisely the same way as the corresponding moiety of methotrexate.

The inhibition of dihydrofolate reductase (5,6,7,8-tetrahydrofolate–$NADP^+$ oxidoreductase, EC 1.5.1.3) by 'anti-folate' drugs such as methotrexate and trimethoprim is of considerable therapeutic importance, but the molecular details of the interactions of these compounds with the enzyme remain incompletely understood. The substrates folate and dihydrofolate are 2-amino-4-oxopteridines, whereas all the most effective inhibitors have 2,4-diamino substituents on a pteridine, pyrimidine or related ring system (Hitchings & Burchall, 1965; Baker, 1967; Blakley, 1969; Roberts, 1977). Changing the 4-oxo group to a 4-amino group leads to an increase in the affinity for the enzyme of as much as 10000-fold (Baker, 1967; Blakley, 1969), and a number of proposals have been advanced to explain this dramatic effect.

In particular, Baker (1959, 1967) and Pullman (Perault & Pullman, 1961; Collin & Pullman, 1964) have drawn attention to the increased basicity of N-1 and of the 2-amino group in 2,4-diaminopteridines as compared with 2-amino-4-oxopteridines. Baker (1959, 1967) has suggested that the former compounds bind to the enzyme in the protonated (positively charged) form, and that an additional coulombic interaction is the basis of their substantially greater affinity for the enzyme. Some support for this proposal has come from studies of the u.v. difference spectra generated when methotrexate binds to dihydrofolate reductase from bacteriophage T_4 (Erickson & Mathews, 1972), *Escherichia coli* (Poe *et al.*, 1974) or pig liver (Poe *et al.*, 1975), where a resemblance to the difference spectrum between neutral and protonated methotrexate was noted.

We now report a detailed analysis of the difference spectra generated by the binding of methotrexate and trimethoprim to *Lactobacillus casei* dihydrofolate reductase, and a comparison of these spectra with those accompanying folate binding. This allows us to determine the ionization states of the bound ligands unambiguously, and to assess the contribution of protonation to the binding of the inhibitors. A preliminary report of part of this work has been presented (Hood & Roberts, 1977).

Abbreviation used: Bistris, 2-[bis-(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol.

* To whom reprint requests should be addressed.

Experimental

Materials

Methotrexate was obtained from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A., trimethoprim from Wellcome Research Laboratories, Beckenham, Kent, U.K., and folate and dihydrofolate were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. All other chemicals were of the highest purity commercially available and used without further purification.

Dihydrofolate reductase was purified from *Lactobacillus casei* MTX/R as described by Dann *et al.* (1976).

Ligand concentrations were determined spectrophotometrically by using the following absorption coefficients: methotrexate, ε_{302} (pH13)$=22.0\times10^3$ litre$\cdot$mol$^{-1}\cdot$cm^{-1} (Seeger *et al.*, 1949); trimethoprim, ε_{271} (pH1)$=6.06\times10^3$ litre $\cdot$ mol^{-1} $\cdot$ cm^{-1} (Roth & Strelitz, 1969); folate, ε_{282} (pH7)$=27.6\times10^3$ litre$\cdot$mol$^{-1}\cdot$cm^{-1} or ε_{365} (pH13)$=8.6\times10^3$ litre$\cdot$mol$^{-1}\cdot$cm^{-1} (Rabinowitz, 1960). The enzyme concentration was determined from its A_{280} ($\varepsilon_{280}=27.2\times10^3$ litre$\cdot$mol$^{-1}\cdot$cm^{-1}; Dann *et al.*, 1976), by assaying its catalytic activity, and by fluorimetric titration with methotrexate (by procedures described by Dann *et al.*, 1976).

The buffers used were 100mM-KCl/50mM-Tris/HCl (for pH7.0–8.5) and 100mM-KCl/50mM-Bistris/HCl (for pH5.3–7.0). All experiments were conducted at 25°C.

Optical absorption spectroscopy

Absorption spectra and difference spectra were measured with a Cary 118 spectrophotometer. All difference spectra were obtained by using matched quartz split-compartment cells with each compartment of path-length 0.4375cm. For each difference spectrum, a baseline was run first (sample and reference cells unmixed), then the difference spectrum (sample cell mixed), then a second baseline (sample and reference cells mixed). The two baselines differed by less than 0.002A unit, and their average was used to correct the difference spectrum. Enzyme concentrations of 10–20μM (after mixing) were used, with a sufficient excess of ligand to ensure complete formation of the complex (except for folate at high pH, where the affinity was low; in this case the observed difference-spectra amplitudes were corrected for incomplete complex-formation by using the measured binding constant). Spectra were obtained over the range 250–450nm, with a bandwidth of 0.5–1.5nm and a scan rate of 0.2 or 0.5nm/s.

For a compound that has different absorption characteristics in two ionization states, and whose pK changes on binding to an enzyme, the difference spectrum generated on binding will contain a component arising from the change in the degree of protonation of the ligand. The pH-dependence of the amplitude of this component of the difference spectrum is given by:

$$\Delta\varepsilon=\varepsilon_B^0-\varepsilon_F^0+(\varepsilon_B^+-\varepsilon_B^0)\left(\frac{[H^+]}{[H^+]+K_B}\right)-(\varepsilon_F^+-\varepsilon_F^0)\left(\frac{[H^+]}{[H^+]+K_F}\right) \quad (1)$$

where ε^0 and ε^+ are the absorption coefficients of the neutral and protonated forms of the ligand, K is its acid dissociation constant, and the subscripts B and F refer to the bound and free states of the ligand respectively. This equation predicts that the pH-dependence of the amplitude of the difference spectrum will have the form of a bell-shaped curve (as expected, since it is essentially the difference between two titration curves). The three unknown parameters, ε_B^0, ε_B^+ and K_B, were determined by non-linear least-squares analysis. The analysis was performed on a Hewlett–Packard 3000 computer, with a non-linear regression program package, written by Dr. John G. Batchelor, which employs the minimization algorithm of Marquardt (1963).

Determination of binding constants

Equilibrium constants for the binding of ligands to the enzyme were determined by measuring the quenching of the tryptophan fluorescence of the enzyme that accompanies complex-formation. The procedures used are described by Dann *et al.* (1976) and in more detail by Birdsall *et al.* (1978). Enzyme concentrations of 0.02–5.0μM were used, and between 15 and 40 additions were made for each equilibrium-constant determination. The data were analysed by non-linear least-squares methods as outlined above, and the standard deviations quoted are those obtained from this analysis.

Results

Methotrexate

The binding of methotrexate (4-amino-4-deoxy-10-methylfolate) to *L. casei* dihydrofolate reductase produces a large-amplitude difference spectrum in the range 250–450nm (Fig. 1). The main features are positive bands at 362 and 345nm, and negative bands at 380, 320 and 260nm. Fig. 1 also shows the differ-

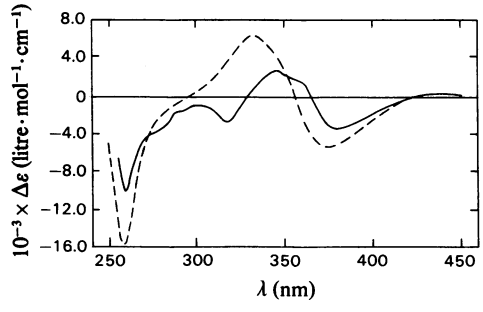


Fig. 1. *Difference spectrum generated on methotrexate binding to L. casei dihydrofolate reductase at pH*7.30 (*solid line*)

For comparison, the difference spectrum between methotrexate at pH1.0 and 7.0 is shown as the broken line.

ence spectrum between methotrexate at pH1 and pH7, that is, between the protonated and neutral forms. (The ionizations of the glutamate carboxy groups of methotrexate and folate have no effect on the spectrum in the wavelength region of interest and will be ignored. For simplicity, the designations 'neutral', 'protonated' and 'anionic' will be used for these compounds; these refer only to the charge state of the pteridine ring.) As noted for dihydrofolate reductase from other sources (Erickson & Mathews, 1972; Poe *et al.*, 1974, 1975), there is some resemblance between the two spectra. Both spectra show large negative bands at approx. 380 and 260nm, but the 300–365nm region of the difference spectrum generated on binding to the enzyme is clearly more complex than that generated on protonation.

Perhaps the simplest interpretation of the difference spectrum associated with methotrexate binding to the enzyme would be that it is the sum of a protonation difference spectrum and other contribution(s) caused by the altered environment of methotrexate when bound to the enzyme. If there is a contribution to the spectrum from the protonation of methotrexate, then its amplitude should be pH-dependent (cf. eqn. 1). Fig. 2(*a*) shows the difference spectra generated on methotrexate binding to the enzyme at a series of pH values in the range 5.0–8.5 (limited by the stability of the enzyme). The difference spectra of Fig. 2(*a*) do not return completely to the baseline at 450nm; this is due to a weak long-wavelength $n \rightarrow \pi^*$ band of methotrexate, which is red-shifted and somewhat increased in intensity when methotrexate binds to the enzyme. The form of the difference spectrum and the wavelengths of the band maxima do not change with pH in this range, but the amplitudes of the bands clearly do. This is most marked for the bands at 380 and 260nm, which correspond to features in the protonation difference spectrum. If this pH-dependence of the difference spectrum arises solely from the pH-dependence of the amplitude of the protonation contribution, the difference between the difference spectra obtained at two pH values should have the form of the protonation difference spectrum. In Fig. 2(*b*) we compare the difference between the spectra at pH5.3 and 7.3 with the protonation difference spectrum of free methotrexate. Clearly the two spectra are closely similar; in particular the wavelengths of the band maxima (260, 334 and 375nm) are identical. The weak feature at 290–310nm in the enzyme difference spectrum reflects a pH-dependent contribution other than that from protonation of the methotrexate, most probably a perturbation of aromatic residue(s) of the protein, which differs slightly at the two pH values (see below).

This comparison shows clearly that there is a component in the difference spectrum generated on methotrexate binding to the enzyme that corresponds to the ligand's protonation difference spectrum, and

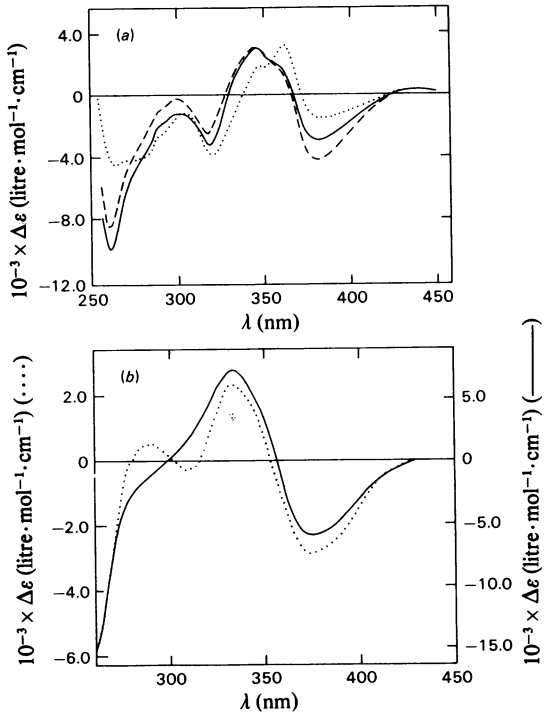


Fig. 2. *pH-dependence of the difference spectra generated on methotrexate binding to L. casei dihydrofolate reductase*
(*a*) Difference spectra at pH5.30 (dotted line), pH7.30 (broken line) and pH8.2 (solid line). (*b*) The difference between the difference spectra for methotrexate binding at pH5.30 and 7.30 (dotted line), compared with the protonation difference spectrum of free methotrexate (solid line).

that thus reflects a greater degree of protonation (a higher pK) of methotrexate in its complex with dihydrofolate reductase than in free solution.

A quantitative estimate of the pK of methotrexate in the complex can now be obtained by fitting the amplitude of the difference spectrum as a function of pH to eqn. (1). The data for 400nm are shown in Fig. 3, together with the best-fit curve from eqn. (1); the parameters used for this curve are given in the legend to Fig. 3.

The absorption coefficient at this wavelength of protonated methotrexate appears to increase substantially on binding to the enzyme; however, this parameter is not well determined by the curve-fitting analysis, and if the data are analysed with the constraint that $\varepsilon_B^+ = \varepsilon_F^+$, the value of p$K_B$ obtained changes by less than one standard deviation (±0.1 pH unit). The wavelength of 400nm was chosen for this analysis, since the only contribution to the difference spectrum at this wavelength is that from protonation

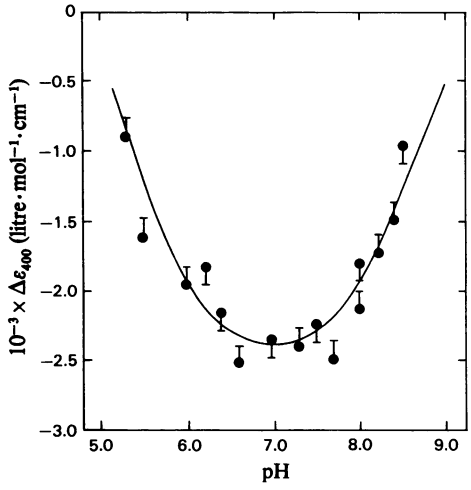


Fig. 3. *pH-dependence of $\Delta\varepsilon_{400}$ of the difference spectrum generated on methotrexate binding to L. casei dihydrofolate reductase*

The points are experimental values, the bars indicating the range of experimental error and the line is the 'best-fit' curve calculated from eqn. (1) by using the following parameter values: $pK_F = 5.35$; $pK_B = 8.55$; $\varepsilon_F^0 = 3.8 \times 10^3$ litre · mol^{-1} · cm^{-1}; $\varepsilon_B^0 = 4.0 \times 10^3$ litre · mol^{-1} · cm^{-1}; $\varepsilon_F^+ = 0.6 \times 10^3$ litre · mol^{-1} · cm^{-1}; $\varepsilon_B^+ = 1.2 \times 10^3$ litre · mol^{-1} · cm^{-1}. The values of pK_F, ε_F^0 and ε_F^+ were fixed at the experimentally determined values, and the remaining parameters were then determined by non-linear regression.

of the ligand. The pH-dependence of the amplitude at 260, 335 and 380nm is closely similar and can be described adequately by eqn. (1) by using the value for pK_B obtained from the data at 400nm; as there are contributions from other sources at the shorter wavelengths, the curve-fitting is less precise. From this analysis we find that the pK of methotrexate bound to the enzyme, pK_B, is 8.55 (±0.10); the main factor limiting the accuracy of this value is the instability of the enzyme at pH values above 8.5. The pK of methotrexate in the free state is 5.35; thus its pK is increased by 3.2 units on binding, showing that the protonated form of methotrexate binds 1600 (±300) times more tightly to the enzyme than does the neutral form. This obviously implies that methotrexate should bind more tightly to the enzyme at low pH than at high pH. The measured binding constants at pH7.5 and 8.5 are shown in Table 1, which shows that the binding is some 20-fold tighter at the lower pH value. As the pH is further lowered, binding becomes still tighter, but accurate values cannot be obtained below pH7.5 by the fluorescence-quenching method, which is limited to dihydrofolate reductase concentrations of 20nM or more.

The knowledge of the relevant absorption co-

Table 1. *Binding constants of methotrexate, folate and trimethoprim to L. casei dihydrofolate reductase*

Values are measured at 25°C in 100mM-KCl/50mM-Tris/HCl. Errors are standard deviations.

	K' (M^{-1})	
	pH7.5	pH8.5
Methotrexate	$2.1(\pm0.3) \times 10^8$	$1.1(\pm0.1) \times 10^7$
Folate	$9.8(\pm2.0) \times 10^4$	$3.3(\pm0.8) \times 10^4$
Trimethoprim	$7.1(\pm0.4) \times 10^6$	$6.3(\pm0.3) \times 10^5$

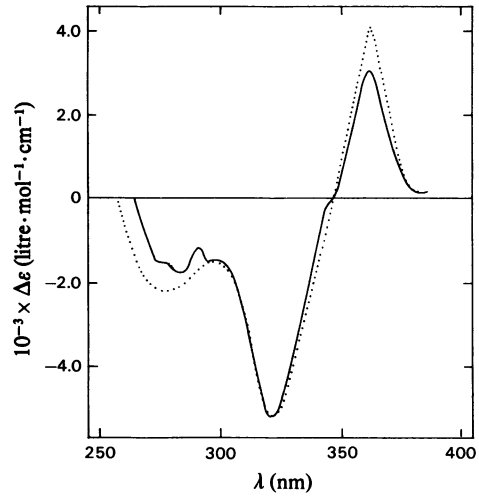


Fig. 4. *The 'non-protonation' component of the difference spectrum generated on methotrexate binding to dihydrofolate reductase*

The broken line shows the calculated 'non-protonation' component, obtained by subtraction of the protonation component from the experimental difference spectrum at pH6.0. This is compared with the (experimental) difference spectrum between the enzyme–methotrexate complex at pH6.0 and free methotrexate at pH1.0 (solid line).

efficients and pK values allows us to calculate the contribution of the protonation difference spectrum to the observed difference spectrum at each individual pH value. Subtraction of this contribution then reveals the component(s) of the observed difference spectrum arising from sources other than protonation of the methotrexate. The results of such a subtraction are shown in Fig. 4, where they are compared with an experimental determination of this 'non-protonation' component, that is the difference spectrum between the enzyme–methotrexate complex and protonated methotrexate. The very close similarity of the two spectra confirms that our analysis is essentially correct. The main features of the spectra are a posi-

tive band at 362nm and a negative band at 321nm. A careful comparison of the absorption spectra of protonated methotrexate and the enzyme–methotrexate complex (and of their first derivatives) confirmed that a band at 321nm in methotrexate was replaced by one at 362nm in the complex. Thus the major contribution to the difference spectra of Fig. 2(*b*), other than that caused by protonation, arises from a single absorption band of methotrexate that shifts about 40nm to longer wavelengths when the ligand binds to the enzyme. Within the accuracy of the subtraction procedure (±10% in amplitude, ±2nm in wavelength), this component of the difference spectrum is independent of pH in the range 5.0–8.5. Also shown in Fig. 4 is a weaker negative band at 275nm. These latter peaks most probably arise from perturbation of one or more aromatic amino acid residues of the protein when the ligand binds, a third contribution to the total difference spectrum. Similar features are seen more clearly in the trimethoprim difference spectra (below).

Trimethoprim

The difference spectrum generated when trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] binds to dihydrofolate reductase at pH8.0 is compared with that caused by protonation of the ligand in Fig. 5. In both cases there is a negative band at 292nm and a positive band at about 255nm; however, in the difference spectrum associated with binding to the enzyme the isosbestic point is at 266nm (rather than 275nm) and there is obvious fine structure on the 292nm band. As with methotrexate, it seems likely that this difference spectrum is the sum of a contribution from protonation of the ligand and a contribution from other sources.

The difference spectra generated on trimethoprim binding to the enzyme at various pH values in the range 5.3–8.2 are shown in Fig. 6(*a*); there is a marked pH-dependence of the amplitude of the difference spectrum, both at 292nm and at shorter wavelengths. The difference between the difference spectra obtained at pH5.3 and 8.0 is shown in Fig. 6(*b*), where it is compared with the protonation difference spectrum. The agreement is excellent for the 292nm band, and reasonable at shorter wavelengths (see below), confirming the existence of a protonation component. Thus for trimethoprim, as for methotrexate, binding to the enzyme is associated with an increased degree of protonation of the ligand.

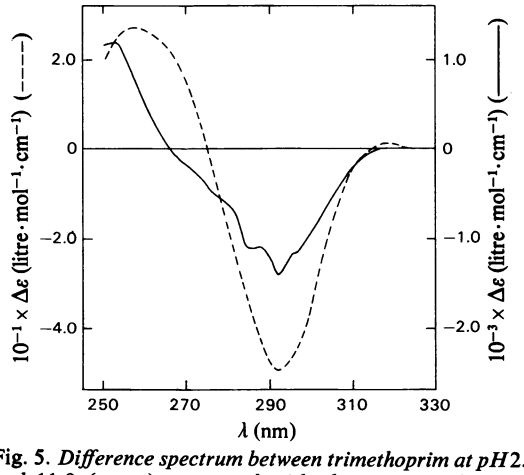


Fig. 5. *Difference spectrum between trimethoprim at pH*2.0 *and* 11.0 (----) *compared with that generated on trimethoprim binding to dihydrofolate reductase at pH*8.0 (——)

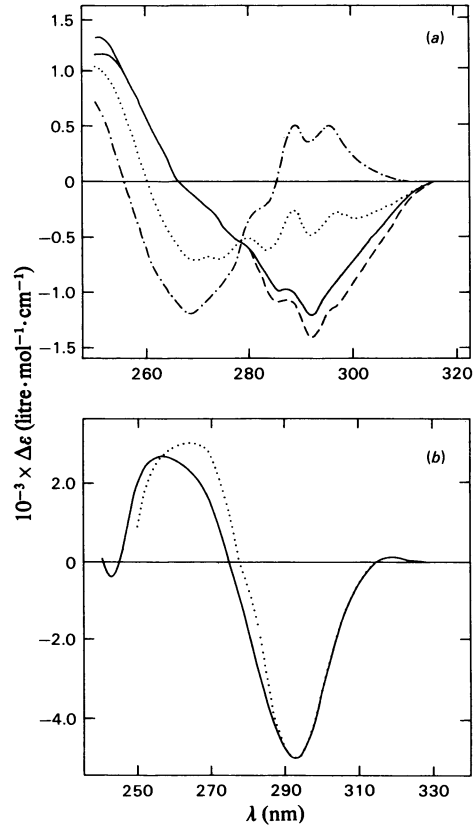


Fig. 6. *pH-dependence of difference spectra generated on trimethoprim binding to dihydrofolate reductase*
(*a*) Difference spectra at pH5.30 (·–·–·–), pH7.00 (····), pH8.00 (----) and pH8.20 (——). (*b*) The difference between the difference spectra for trimethoprim binding at pH5.30 and 8.00 (····), compared with the protonation difference spectrum of free trimethoprim (——).

The spectra in Fig. 6 show that for trimethoprim (unlike methotrexate) none of the bands in the difference spectrum can be said to arise solely from protonation. However, since the pK of trimethoprim is 7.22 in the free state, and apparently significantly higher in the bound state, at pH 5.3 both free and bound trimethoprim will be essentially fully protonated. The difference spectrum obtained at this pH (Fig. 6*a*) thus contains no protonation contribution. The two small positive bands at 289 and 296nm (responsible for the fine structure on the 292nm band at higher pH values) closely resemble those seen with methotrexate (see above) and most probably arise from perturbation of aromatic amino acid residues on ligand binding. In addition, there is a larger, negative, band at 268nm, arising from perturbation of an absorption band of the ligand. The spectra in Fig. 6 show that the 289 and 296nm bands do not change significantly with pH, so that the pH-dependence of the amplitude of the protonation contribution to the 292nm band can be obtained by subtracting the $\Delta\varepsilon_{292}$ at pH 5.3 from the corresponding values at higher pH.

By fitting this pH-dependence to eqn. (1), we obtain an estimate for the pK of trimethoprim in the complex of 7.9 (±0.1), a difference of 0.68 unit from the value in free solution, indicating that protonated trimethoprim binds 4.8 (±1.1) times more tightly than the neutral molecule.

The pH-dependence of the amplitude of the difference spectrum at 268nm is more complicated, and is not adequately described by eqn. (1). As noted above, there is a negative band at this wavelength that arises from sources other than protonation of the ligand, and it appears that the amplitude of this band decreases markedly as the pH is increased above about pH 8.0. This implies an additional pH-dependent change in the environment of the bound ligand at high pH, and this is also indicated by the pH-dependence of the binding constant of trimethoprim. This is shown in the form of a Dixon (1953) plot in Fig. 7, and the values measured at pH 7.5 and 8.5 are included in Table 1. The dotted curve in Fig. 7 shows the pH-dependence expected simply from the measured change in pK of the ligand on binding, and it is clear that ionization of a group or groups on the enzyme has the effect of further decreasing the binding constant at pH values above 8.0.

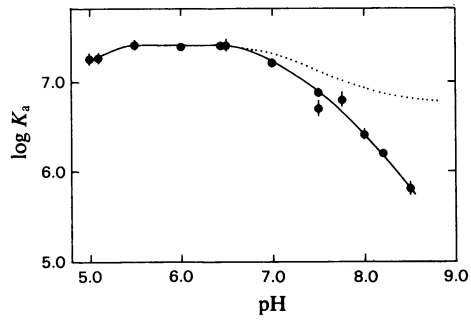


Fig. 7. *pH-dependence of the equilibrium constant for trimethoprim binding to dihydrofolate reductase plotted by the method of Dixon* (1953)

····, The pH-dependence expected from the measured change in the pK of trimethoprim on binding. The bars on the experimental points indicate the standard deviation. K_a represents the association constant.

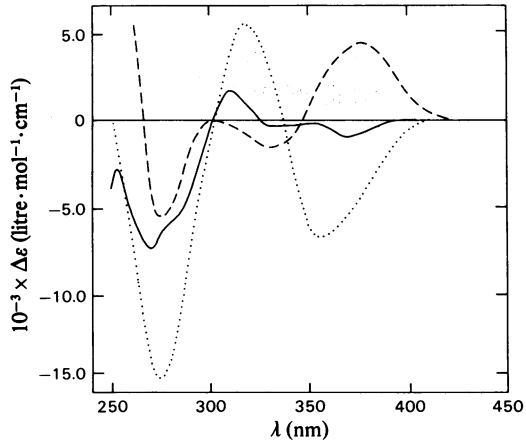


Fig. 8. *Difference spectrum generated on folate binding to dihydrofolate reductase at pH* 5.30 (——), *compared with the difference spectra between folate at pH* 12.0 *and* 7.0 (----), *and pH* 1.0 *and* 7.0 (····)

Folate

For this 2-amino-4-oxopteridine there are two ionizations in the pH range of interest that affect the absorption spectrum: protonation (pK 2.0) and anion formation (pK 8.06).

The difference spectrum generated on folate binding to the enzyme at pH 5.3 is shown in Fig. 8, along with the difference spectra between neutral folate and its protonated and anionic forms respectively. All three spectra show large negative bands in the region of 275nm, but at longer wavelengths there is very little resemblance between the spectrum produced by binding to the enzyme and either of the other spectra. In particular, although the change in ionization state of free folate leads to large bands in the difference spectrum at 356nm ($\Delta\varepsilon = -6 \times 10^3$ litre · mol^{-1} · cm^{-1}) for protonation or at 377nm ($\Delta\varepsilon = +4 \times 10^3$ litre · mol^{-1} · cm^{-1}) for anion formation, only a small band at 370nm ($\Delta\varepsilon = -900$ litre · mol^{-1} · cm^{-1}) is seen

when folate binds to the enzyme. The difference spectrum produced when folate binds to the enzyme is independent of pH over the range 5.3–8.5, in terms of both the wavelengths of the band maxima and their amplitudes. This shows clearly that over the whole of this pH range the ionic form of folate bound to the enzyme is the same as that of free folate. At pH 7 and below, this is the neutral molecule. It is clear from the spectra in Fig. 8 that a pH-dependent contribution amounting to 10% of the protonation difference spectrum would be detectable. Since no such contribution is detectable at pH 5.3, we can set an upper limit of 4.3 to the pK for protonation of folate in the complex. Above pH 7, the anionic form of folate becomes important; since the accessible pH range includes the pK of this ionization (8.06) and yet no component corresponding to the anion difference spectrum is discernible, it appears that the pK for this ionization is not significantly perturbed when folate binds to the enzyme.

The binding constants of folate at pH 7.5 and 8.5 are given in Table 1. Since the ionic form of the ligand does not change significantly on binding, the decrease in binding constant at the higher pH, which is less than that with trimethoprim, must be ascribed to the ionization of a group or groups on the enzyme.

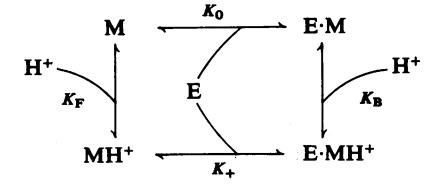
Discussion

Methotrexate and folate binding

There is clearly a general, though far from complete, resemblance between the difference spectrum generated by protonation of methotrexate and that observed when this inhibitor binds to *L. casei* dihydrofolate reductase. The quantitative analysis of the difference spectrum described here shows unambiguously that the pK of methotrexate is substantially increased when it binds to the enzyme, so that at pH 7.0, more than 97% of the methotrexate bound to the enzyme is in the protonated form. In contrast, a similar analysis shows that folate is not protonated when bound to the enzyme at neutral pH. In addition, the pK for anion formation is not significantly perturbed on binding. Zakrzewski (1963) has proposed that folate and dihydrofolate bind to the enzyme as the enol tautomers (with a hydroxy group at the 4-position), whereas the keto form is known to be favoured in solution [see, e.g., Pfleiderer *et al.* (1960) and Brown & Jacobsen (1961)]; this change in tautomeric form, if it occurs, should be reflected in the difference spectrum. As a model for the enol form of folate, we take 2-amino-4-methoxypteridine (Pfleiderer *et al.*, 1960); in its neutral form, this molecule shows a strong absorption band at 358nm (ε=6500litre·mol^{-1}·cm^{-1}; Pfleiderer *et al.*, 1960). This is very similar to the long-wavelength absorption band of the anionic forms of 2-amino-4-oxopteridine (360nm; ε=7000litre·mol^{-1}·cm^{-1}; Pfleiderer *et al.*, 1960) and folate (366nm, ε=7200litre·mol^{-1}·cm^{-1}; Rabinowitz, 1960). The difference spectrum between the enol and keto tautomers ought therefore to resemble that between the anionic and neutral forms. There is no indication of such a component in the difference spectrum generated when folate binds to the enzyme. We conclude that bound folate is in the normal keto tautomeric form, and at pH 7.0 is more than 90% in the neutral ionization state.

The substrate and inhibitor are thus shown to be in different ionization states when bound to the enzyme at neutral pH, as first postulated by Baker (1959, 1967). We can now assess quantitatively the contribution that this difference makes to the large difference in binding constant between methotrexate and folate. The measured pK values of methotrexate in the free and bound states show that the protonated form binds 1600 (±300) times more tightly to the enzyme than does the neutral form, a Gibbs free-energy difference of 18.18 (±0.84)kJ/mol. This must presumably be due to an interaction with a negatively charged carboxylate group on the enzyme. Both 2,4-diamino- and 2-amino-4-oxopteridines are protonated on N-1, with the formation of an amidinium structure involving N-1, C-2 and the 2-amino group (Pfleiderer *et al.*, 1960; Dieffenbacher & von Philipsborn, 1969; Konrad & Pfleiderer, 1970; Ewers *et al.*, 1973, 1974). Such a structure would be able to form two hydrogen bonds as well as a charge–charge interaction with a carboxylate group. The recently reported crystal structure of the *E. coli* dihydrofolate reductase–methotrexate complex (Matthews *et al.*, 1977) shows that the carboxylate group of Asp-27 is indeed in close proximity to N-1 of bound methotrexate.

If we represent the binding of methotrexate to the enzyme at a particular pH value by the equilibria shown in Scheme 1, then the observed binding constant, $K_{app.}$, can be expressed in terms of the binding constant of protonated methotrexate, K_+, and the



Scheme 1. *Equilibria describing the binding of neutral and protonated methotrexate to the enzyme*

K_F=[M][H$^+$]/[MH$^+$]; K_B=[E·M][H$^+$]/[E·MH$^+$]; K_0=[E·M]/[E][M]; K_+=[E·MH$^+$]/[E][MH$^+$] where MH$^+$ represents protonated methotrexate and M represents neutral methotrexate.

ionization constants of methotrexate in the free and bound states, K_F and K_B:

$$K_{app.} = K_+ \frac{([H^+]+K_B)}{([H^+]+K_F)} \quad (2)$$

In addition we have, for the binding constant of neutral methotrexate:

$$K_0 = \frac{K_+ K_B}{K_F} \quad (3)$$

The values of K_+ and K_0, calculated from the measured binding constants (Table 1) and pK values, are given in Table 2. Since no account has been taken of the ionization of groups on the enzyme, the values of K_+ and K_0 apply only to the pH at which $K_{app.}$ was measured. The ratio of the values obtained at pH7.5 and 8.5 (approx. 3) is the same as the ratio of the binding constants of folate at these two pH values, suggesting that the decrease in binding constant with increasing pH is governed by the same group(s) on the enzyme for both ligands. The 6.4-fold increase in the ratio of the measured binding constant ($K_{app.}$) for methotrexate to that for folate on going from pH8.5 to 7.5 can be accounted for quantitatively by the larger proportion of bound methotrexate that is protonated at the lower pH value. An increase in the ratio of the binding constants of these two ligands with decreasing pH has been noted qualitatively for dihydrofolate reductase from a number of sources (see, e.g., Blakley, 1969). Table 2 shows that protonated methotrexate binds very tightly to the enzyme: a binding constant of 2.7×10^{10} M^{-1} corresponds to a Gibbs free-energy change of −59.36kJ/mol. Furthermore, even the neutral form of methotrexate binds 170 times more tightly (12.5kJ/mol) to the enzyme than does folate. This shows clearly that the protonation of methotrexate, although it contributes substantially to the overall binding energy, cannot be solely responsible for the increased affinity of methotrexate compared with folate. In fact, of the 18.8kJ/mol difference in binding energy between folate and methotrexate at pH7.5, two-thirds arise from the difference between neutral methotrexate and neutral folate.

Table 2. *Calculated binding constants for the two ionic forms of methotrexate, compared with that of folate*
Values are measured at 25°C in 100mM-KCl/50mM-Tris/HCl. For methotrexate, K_0 and K_+ are defined in Scheme 1, and calculated as described in the text; the errors given are derived from the standard deviations of $K_{app.}$ and pK_B. The binding constants for folate are taken from Table 1.

	Methotrexate		Folate
	K_+ (M^{-1})	K_0 (M^{-1})	K (M^{-1})
pH7.5	$2.75(\pm0.4)\times10^{10}$	$1.75(\pm0.6)\times10^{7}$	9.8×10^{4}
pH8.5	$8.2\ (\pm1.6)\times10^{9}$	$5.2(\pm1.2)\times10^{6}$	3.3×10^{4}

Part of the difference in binding energy between the two neutral molecules may arise from the difference in hydrogen-bonding ability of the two different 4-substituents. However, there is increasing evidence for a conformational difference between the folate and methotrexate complexes. The pH-independence of the difference spectrum generated on folate binding, down to pH5.3, allows us to place an upper limit of 4.3 on the pK for protonation of bound folate. This implies that protonated folate binds at most 200 times more tightly than neutral folate, compared with the 1600-fold difference seen with methotrexate. Since, as noted above, both folate and methotrexate are protonated on N-1, this shows that folate binds as the neutral molecule at pH5–7 not simply because its pK is substantially lower than that of methotrexate, but because it interacts in a significantly different way with the enzyme. We have studied the effects of folate and methotrexate binding on individual amino acid residues of the enzyme by ^{1}H and ^{19}F n.m.r. spectroscopy (Birdsall *et al.*, 1977; Feeney *et al.*, 1977; Kimber *et al.*, 1977; Roberts, 1977; Roberts *et al.*, 1977). Of the 16 residues studied in detail thus far (six histidine, five tyrosine and five tryptophan), nine are affected by the binding of these ligands. Of these nine, only three are affected in a similar way by both folate and methotrexate, and there is good (though indirect) evidence that many of the differences seen by n.m.r. reflect conformational differences between the two complexes. Direct evidence for a conformational change accompanying the binding of folate and methotrexate has been obtained from stopped-flow studies of the binding reaction (R. W. King, J. G. Batchelor, K. Hood & A. S. V. Burgen, unpublished work), and the equilibrium constant for this conformational change is substantially greater for methotrexate than for folate. It seems likely that the conformational differences between the folate and methotrexate complexes are responsible for a large part of the difference in binding energy of these two compounds.

Comparison with enzyme from other sources

The difference spectra generated on methotrexate binding to dihydrofolate reductase from bacteriophage T$_4$, *E. coli* and pig liver (Erickson & Mathews, 1972; Poe *et al.*, 1974, 1975) are similar to that observed with the *L. casei* enzyme. The maxima occur at the same wavelengths, and the amplitudes of the bands at 260 and 380nm, which arise predominantly from the protonation contribution, are very similar, indicating that the degree of protonation of the bound methotrexate is similar in each case. The major difference in the spectra observed with the enzyme from different sources is in the amplitude of the negative band at 320nm. The analysis of the

difference spectrum into its components shows that this band arises from the shift of an absorption band of methotrexate at 320nm to longer wavelengths when the inhibitor binds to the enzyme. The varying amplitude of the 320nm band is presumably due to differences in the magnitude of this red shift. For the *L. casei* enzyme, this band shows a red shift of some 40nm, a strikingly large value. The extinction coefficient of this band indicates that it is most probably a $\pi \xrightarrow{L} \pi^*$ rather than an $n \rightarrow \pi^*$ band, and yet changes in solvent polarity generally lead to shifts of only 2–5nm in the $\pi \rightarrow \pi^*$ bands of simple nitrogen heterocycles (see, e.g., Jaffé & Orchin, 1962). This suggests that a more specific interaction with the enzyme may be involved, but the absorption spectrum of methotrexate is not yet sufficiently well understood for us to suggest any particular model.

In contrast with the difference spectra generated by methotrexate binding, those observed when folate binds to the enzymes from *L. casei*, bacteriophage T_4 (Erickson & Mathews, 1972) and *E. coli* (Poe *et al.*, 1974) are markedly different. Although folate is a substrate for all these enzymes, its activity relative to dihydrofolate does vary markedly from one enzyme to another.

Trimethoprim binding

Like methotrexate, trimethoprim binds preferentially to the enzyme in the protonated form, but in this case the preference amounts only to a factor of 4.8 (3.8kJ/mol). 2,4-Diaminopyrimidines are protonated on N-1 (Brown & Teitei, 1965), and it is generally assumed that the diaminopyrimidine ring of trimethoprim and related compounds binds to the enzyme in the same site as that occupied by the corresponding part of the pteridine ring of methotrexate. The effects of trimethoprim on the histidine and tyrosine resonances in the n.m.r. spectrum of *L. casei* dihydrofolate reductase are very similar to, though not identical with, those of methotrexate (Birdsall *et al.*, 1977; Feeney *et al.*, 1977). However, the much smaller effect of protonation on trimethoprim binding than on methotrexate binding implies a significantly different interaction with the negatively charged carboxylate group on the enzyme in the two cases. The trimethoxybenzyl moiety of trimethoprim makes a major contribution to its binding energy: at pH6.5 the binding constant for trimethoprim is $2.6 \times 10^7 M^{-1}$, whereas that for 2,4-diaminopyrimidine is $1.28 \times 10^3 M^{-1}$ (Birdsall *et al.*, 1978). It is possible that optimal interaction of the trimethoxybenzyl group with the enzyme requires a somewhat different orientation of the trimethoprim molecule in the binding site from that for optimal interaction with the carboxylate group. In this connection it is interesting that the band at 260nm in the trimethoprim difference spectrum, which arises from sources other than protonation, is clearly pH-dependent above pH8, in contrast with the 'non-protonation' contribution to the methotrexate spectrum, which is independent of pH. Although the origin of this difference is unknown, it does further imply a difference in the interactions of pyrimidines and pteridines with the enzyme.

Both methotrexate and trimethoprim appear to perturb the spectrum of one or more aromatic amino acid residues in the protein when they bind. The wavelength maxima in the trimethoprim difference spectrum, where they can be measured fairly precisely, are 289 and 296nm. The solvent perturbation difference spectrum of tryptophan has bands at 284 and 294nm, whereas that of tyrosine has bands at 278 and 287nm (Herskovits, 1967). It seems likely therefore that a tryptophan residue is perturbed when these ligands bind. ^{19}F n.m.r. studies of 6-fluorotryptophan-labelled enzyme (Kimber *et al.*, 1977) show that methotrexate binding causes a substantial shift in the resonance position of a single tryptophan residue. Treatment of dihydrofolate reductase from *E. coli* or *L. casei* with *N*-bromosuccinimide leads to complete loss of activity concomitant with the oxidation of a single tryptophan residue (Liu & Dunlap, 1974; Williams, 1975; Freisheim *et al.*, 1977; K. Hood & G. C. K. Roberts, unpublished work), whereas with the enzyme from *Streptococcus faecium*, the loss of activity correlates with modification of two tryptophan residues (Warwick *et al.*, 1972).

We are grateful to Mrs. G. Ostler and Mr. P. Turner for their skilled technical assistance, and to Dr. J. G. Batchelor for help with programming.

References

Baker, B. R. (1959) *Cancer Chemother. Rep.* **4**, 1–10

Baker, B. R. (1967) *Design of Active-site Directed Irreversible Enzyme Inhibitors*, Ch. 10, Wiley, New York

Birdsall, B., Griffiths, D. V., Roberts, G. C. K., Feeney, J. & Burgen, A. S. V. (1977) *Proc. R. Soc. London Ser. B* **196**, 251–265

Birdsall, B., de Miranda, J. R., Burgen, A. S. V. & Roberts, G. C. K. (1978) *Biochemistry* in the press

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, Ch. 5, North-Holland, Amsterdam

Brown, D. J. & Jacobsen, N. W. (1961) *J. Chem. Soc.* 4413–4420

Brown, D. J. & Teitei, T. (1965) *J. Chem. Soc.* 755–761

Collin, R. & Pullman, B. (1964) *Biochim. Biophys. Acta* **89**, 232–241

Dann, J. G., Ostler, G., Bjur, R. A., King, R. W., Scudder, P., Turner, P. C., Roberts, G. C. K., Burgen, A. S. V. & Harding, N. G. L. (1976) *Biochem. J.* **157**, 559–571

Dieffenbacher, A. & von Philipsborn, W. (1969) *Helv. Chim. Acta* **52**, 743–763

Dixon, M. (1953) *Biochem. J.* **55**, 161–171
Erickson, J. S. & Mathews, C. K. (1972) *J. Biol. Chem.* **247**, 5661–5667
Ewers, U., Günther, H. & Jaenicke, L. (1973) *Chem. Ber.* **106**, 3951–3961
Ewers, U., Günther, H. & Jaenicke, L. (1974) *Chem. Ber.* **107**, 3275–3286
Feeney, J., Roberts, G. C. K., Birdsall, B., Griffiths, D. V., King, R. W., Scudder, P. & Burgen, A. S. V. (1977) *Proc. R. Soc. London Ser. B* **196**, 267–290
Freisheim, J. H., Ericsson, L. H., Bitar, K. G., Dunlap, R. B. & Reddy, A. V. (1977) *Arch. Biochem. Biophys.* **180**, 310–317
Herskovits, T. T. (1967) *Methods Enzymol.* **11**, 748–775
Hitchings, G. H. & Burchall, J. J. (1965) *Adv. Enzymol. Relat. Areas Mol. Biol.* **27**, 417–483
Hood, K. L. & Roberts, G. C. K. (1977) *Biochem. Soc. Trans.* **5**, 771–772
Jaffé, H. H. & Orchin, M. (1962) *Theory and Applications of Ultraviolet Spectroscopy*, pp. 361–383, Wiley, New York
Kimber, B. J., Griffiths, D. V., Birdsall, B., King, R. W., Scudder, P., Feeney, J., Roberts, G. C. K. & Burgen, A. S. V. (1977) *Biochemistry* **16**, 3492–3500
Konrad, G. & Pfleiderer, W. (1970) *Chem. Ber.* **103**, 722–734
Liu, J.-K. & Dunlap, R. B. (1974) *Biochemistry* **13**, 1807–1814
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* **11**, 431–441
Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N., Kraut, J., Poe, M., Williams, M. & Hoogsteen, K. (1977) *Science* **197**, 452–455
Perault, A. M. & Pullman, B. (1961) *Biochim. Biophys. Acta* **52**, 266–280
Pfleiderer, W., Liedek, E., Lohrmann, R. & Rukwied, M. (1960) *Chem. Ber.* **93**, 2015–2024
Poe, M., Greenfield, N. J., Hirshfield, J. M. & Hoogsteen, K. (1974) *Cancer Biochem. Biophys.* **1**, 7–11
Poe, M., Bennett, C. D., Donoghue, D., Hirshfield, J. M., Williams, M. N. & Hoogsteen, K. (1975) in *Chemistry and Biology of Pteridines* (W. Pfleiderer, ed.), pp. 51–58, de Gruyter, Berlin
Rabinowitz, J. C. (1960) *Enzymes 2nd Ed.* **2**, 185–190
Roberts, G. C. K. (1977) in *Drug Action at the Molecular Level* (G. C. K. Roberts, ed.), pp. 127–150, Macmillan, London
Roberts, G. C. K., Feeney, J., Birdsall, B., Kimber, B., Griffiths, D. V., King, R. W. & Burgen, A. S. V. (1977) in *NMR in Biology* (R. A. Dwek, I. D. Campbell, R. E. Richards and R. J. P. Williams, eds.), pp. 95–109, Academic Press, London
Roth, B. & Strelitz, J. Z. (1969) *J. Org. Chem.* **34**, 821–836
Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr. & Hultquist, M. E. (1949) *J. Am. Chem. Soc.* **71**, 1753–1758
Warwick, P. E., D'Souza, L. & Freisheim, J. H. (1972) *Biochemistry* **11**, 3775–3779
Williams, M. N. (1975) *J. Biol. Chem.* **250**, 322–330
Zakrzewski, S. F. (1963) *J. Biol. Chem.* **238**, 1485–1490