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Binding of Coenzyme Analogues to *Lactobacillus casei* Dihydrofolate Reductase: Binary and Ternary Complexes†

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ABSTRACT: The binding, or association, constants of $NADP^+$, NADPH, and a series of structural analogues to dihydrofolate reductase from *Lactobacillus casei* MTX/R have been determined fluorometrically. Modification of the adenine or nicotinamide rings has little effect on the binding of the oxidized coenzyme, but the thionicotinamide and acetylpyridine analogues of the reduced coenzyme bind much more weakly than NADPH itself. In the presence of the substrate, folate, or the inhibitors methotrexate or trimethoprim, the oxidized coenzymes bind appreciably more tightly to the enzyme. The magnitude of this "cooperativity", which covers a range of 1–37-fold, depends markedly on the structure of both the coenzyme and the substrate or substrate analogue; the nicotinamide ring of the coenzymes is clearly important in these effects. The binding constants of the reduced coenzymes in the presence of methotrexate or trimethoprim were too high to be measured fluorometrically. The dissociation rate constants of the coenzymes from their ternary complexes were therefore measured and compared with the values for the binary complexes reported by Dunn and co-workers [Dunn, S. M. J., Batchelor, J. G., & King, R. W. (1978) *Biochemistry 17*, 2356]. The presence of the inhibitors leads to very substantial decreases in the coenzyme dissociation rate constant—by factors of 300–2200. The binding constant of methotrexate in the ternary complex is calculated to be $\sim 1.3 \times 10^{12}\ M^{-1}$. The structural origins of the differences in binding constant and cooperative behavior of the various coenzymes and coenzyme analogues are discussed in the light of information from crystallography and NMR spectroscopy.

Dihydrofolate reductase is the target for the "antifolate" drugs such as methotrexate and trimethoprim (Hitchings & Burchall, 1965; Blakley, 1969). It is also one of the smallest known pyridine nucleotide dependent dehydrogenases, the enzyme from *Lactobacillus casei* having a molecular weight of 18 300 (Dann et al., 1976; Bitar et al., 1977). In the better known dehydrogenases, it is frequently observed that inhibitors bind substantially more tightly in the presence of coenzyme (either oxidized or reduced). Among the many examples of this are the binding of pyrazole (Theorell & Yonetani, 1963) or of fatty acids and their amides (Winer & Theorell, 1959; Woronick, 1963) to liver alcohol dehydrogenase, and the binding of carboxylic acids such as oxalate and oxamate to lactate dehydrogenase (Winer & Schwert, 1959; Kolb & Weber, 1975; Holbrook et al., 1975). Similar observations have been reported with dihydrofolate reductase (Perkins & Bertino, 1966; Williams et al., 1973a,b; Roberts et al., 1974; Birdsall et al., 1978), and this cooperativity between coenzyme and inhibitor binding may be of importance in the therapeutic action of the inhibitors.

In this and the following paper (Birdsall et al., 1980) we report measurements of the binding of the natural coenzymes and a series of structural analogues to *L. casei* dihydrofolate reductase, alone or in the presence of inhibitors. Coenzyme analogues have of course been widely employed in studies of other dehydrogenases since the early work of Kaplan and colleagues (Kaplan et al., 1956; Anderson & Kaplan, 1959), but their use with dihydrofolate reductase has been more limited. Recently Neef & Huennekens (1976) have studied the binding of etheno-$NADP^+$ and etheno-NADPH to the reductase from L1210 cells. Williams et al. (1977) have reported the activity of a number of coenzyme analogues as substrates of the enzyme, and a report from our laboratory has described the kinetics of formation of the binary complexes of various coenzyme analogues with the *L. casei* enzyme (Dunn et al., 1978). In order to interpret the differences in behavior of these coenzyme analogues, it is important to establish whether they bind to the enzyme in the same way; ^1H and ^{31}P

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NMR experiments to this end are described in the following papers (Hyde et al., 1980a,b).

Experimental Section

Materials

Dihydrofolate reductase was isolated and purified from *L. casei* MTX/R as described by Dann et al. (1976); its concentration was determined from its absorbance at 280 nm, by assaying its catalytic activity, and by fluorometric titration with methotrexate (Dann et al., 1976). $NADP^+$, NADPH, and the coenzyme analogues $NHDP^+$,[1] NHDPH, $APADP^+$, $\epsilon NADP^+$, and $TNADP^+$ were obtained from Sigma (London) Chemical Co. Ltd. $APADP^+$ and $TNADP^+$ were converted to their reduced forms by using isocitrate and isocitrate dehydrogenase as outlined by Dunn et al. (1978), and $\epsilon NADP^+$ was reduced by using glucose 6-phosphate and glucose-6-phosphate dehydrogenase as described by Neef & Huennekens (1976). PADPR-OMe was prepared by the calf spleen NAD^+ glycohydrolase catalyzed methanolysis of $NADP^+$ (Pascal & Schuber, 1976) and purified on a column of diethylaminoethylcellulose (Whatman DE-52).

Methods

Determination of Equilibrium Constants. The equilibrium constants for the formation of the binary complexes of the oxidized and reduced coenzymes and of the ternary complexes of the oxidized coenzymes were measured fluorometrically. A Perkin-Elmer MPF-44A fluorescence spectrometer was used, with 1-cm path length quartz cells; ligands were added as microliter volumes of concentrated stock solutions. All measurements were made at 25 °C, in a buffer of 15 mM Bistris[2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol] hydrochloride, pH 6.0, containing 0.5 M KCl. Depending on the magnitude of the binding constant to be determined, enzyme concentrations between 0.1 and 25 μM were used; no dependence of binding constant on enzyme concentration was observed. All measurements were corrected for dilution and for self-absorption (see below).

The formation of the binary complexes of most of the coenzymes was followed by measuring the quenching of the tryptophan fluorescence of the protein (excitation, 290–310 nm; emission, 330–350 nm; wavelengths chosen for maximal change in fluorescence and minimal self-absorption). The binding of $\epsilon NADP^+$ was followed by measuring the increase in its fluorescence [excitation, 325–350 nm; emission, 395–400 nm; see Neef & Huennekens (1976)], and the binding constants of the oxidized coenzymes, in both the binary and ternary complexes, were measured by competition with $\epsilon NADP^+$. Competition experiments were performed in two ways, either by titration with $\epsilon NADP^+$ in the presence of a fixed concentration of competing coenzyme (sufficient to decrease the apparent binding constant of $\epsilon NADP^+$ by 4–11-fold) or by titration with the competing coenzyme in the presence of a fixed concentration of $\epsilon NADP^+$. In the case of APADPH, formation of the binary complex was followed by measuring the energy-transfer fluorescence (excitation of tryptophan residues at 285 nm; emission from the dihydropyridine ring at 440 nm).

Determination of Dissociation Rate Constants. The rate constants for dissociation of coenzyme or inhibitor from the ternary enzyme-reduced coenzyme–inhibitor complexes were sufficiently slow to be determined conveniently by manual mixing. A competition method was used: to a solution of the ternary complex of interest was added an excess of competing ligand (methotrexate when trimethoprim dissociation was being measured; NADPH or APADPH for measurements of coenzyme dissociation), and the formation of the new complex was followed fluorometrically. The rate-limiting step in the process is the dissociation of the original complex, provided that a sufficient excess of the competing ligand has been added (see eq 9 below); this was checked by measurements at two or more concentrations of competing ligand. The measurements were performed at 25 °C, in a buffer containing 15 mM Bistris hydrochloride and 0.5 M KCl, pH 6.0 or 7.0.

Data Analysis. The measured fluorescence intensity in many of the experiments involving quenching of protein fluorescence was decreased owing to absorption of the exciting light by the added ligand. This effect must be corrected far before the change in fluorescence intensity can be used as a measure of complex formation. The observed fluorescence, F_{obsd}, is related to the "true" fluorescence, F, by (Parker, 1968; Holland et al., 1977)

$$F_{obsd} = F\left[\frac{\exp(-\epsilon[L]_T d) - \exp(-\epsilon[L]_T)}{\epsilon[L]_T(1-d)}\right] + F_{blank} \quad (1)$$

where $[L]_T$ is the total ligand concentration, ϵ is its extinction coefficient at the excitation wavelength, d is a parameter related to the cuvette geometry ($d < 1$), and F_{blank} is the fluorescence of the buffer. The fluorescence of a *p*-aminobenzoyl glutamate solution was measured (using the same excitation and emission wavelengths as those used for the binding measurements) as a function of added ligand concentration, and the data were fitted to eq 1 by nonlinear regression so as to obtain estimates of the parameters ϵ, d, and F_{blank}. These parameters were then used in eq 1 to correct the measured fluorescence intensities of the enzyme solution.

The corrected fluorescence intensity is simply related to the fractional saturation of the enzyme with ligand. For measurements of protein fluorescence

$$[EL]/[E]_T = (F_0 - F)/(F_0 - F_\infty) \quad (2)$$

where [EL] is the concentration of the complex at a ligand concentration giving a fluorescence intensity F, $[E]_T$ is the total enzyme concentration, and F_0 and F_∞ are the fluorescence intensities at zero and saturating ligand concentrations, respectively. Similarly, when the ligand fluorescence is measured

$$[EL]/[E]_T = (F - F_0)/(F_\infty - F_L) \quad (3)$$

where F_L is the fluorescence of an equal concentration of ligand in the absence of enzyme. (Here, the measurements in the absence of enzyme are used to calculate the parameters in eq 1 and thus to correct for self-absorption.) In addition, from the mass action equation

$$K' = [EL]/[E][L] \quad (4)$$

we have

$$[EL]/[E]_T = [1/K' + [E]_T + [L]_T - [(1/K' + [E]_T + [L]_T)^2 - 4[E]_T[L]_T]^{1/2}]/2[E]_T \quad (5)$$

where K' is the binding constant. By combination of eq 5 with either eq 2 or 3, the corrected fluorescence intensity can be described in terms of two unknowns, K' and F_∞. These were estimated by a nonlinear least-squares fit of at least 15 values

[1] Abbreviations used: $NHDP^+$, nicotinamide hypoxanthine dinucleotide phosphate; $\epsilon NADP^+$, nicotinamide 1,N^6-ethenoadenine dinucleotide phosphate; $APADP^+$, 3-acetylpyridine adenine dinucleotide phosphate; $TNADP^+$, thionicotinamide adenine dinucleotide phosphate; with corresponding abbreviations (NHDPH, etc.) for the reduced forms; PADPR-OMe, the methyl β-riboside of 2′-phosphoadenosine-5′-diphosphoribose.

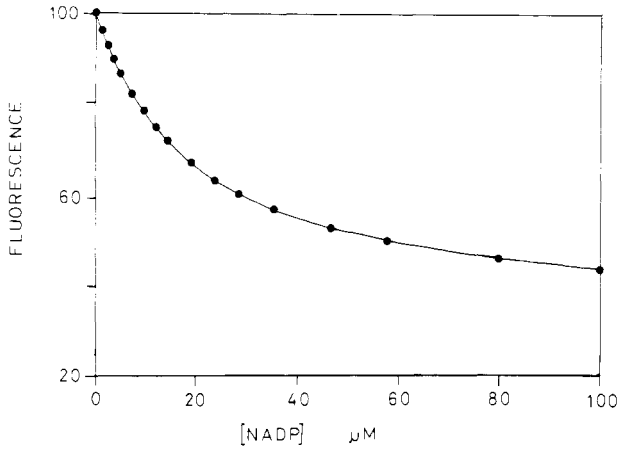

FIGURE 1: Effect of $NADP^+$ on the fluorescence of 3 μM dihydrofolate reductase. The curve is the best-fit curve, calculated with a binding constant of 6.1×10^4 M^{-1}.

of F and $[L]_T$ to the above equations. The standard errors quoted for K' are those obtained from the nonlinear regression analysis; as noted previously (Birdsall et al., 1978), these are similar to those estimated from replicate determinations.

In measurements of binding constants by competition with $\epsilon NADP^+$, the binding constant of the competing ligand is given by

$$K_C = (1/[C])(K_E/K_{app}) - 1 \quad (6)$$

where K_E is the true binding constant of $\epsilon NADP^+$ and K_{app} is its apparent binding constant in the presence of a free concentration [C] of competing ligand. [C] was approximated as $[C]_T - [E]_T$, which gives an error of less than 2% for $K_C[C] \geq 3$; the experiments were conducted, as noted above, at concentrations such that $K_E/K_{app} > 4$, so that $K_C[C] > 3$.

For measurements of the dissociation rate constants, the time dependence of the fluorescence intensity was fitted by nonlinear regression to a single exponential

$$F(t) = A \exp(-kt) + B$$

treating A, B, and k as unknown parameters.

Results

Binary Complexes. The tryptophan fluorescence of *L. casei* dihydrofolate reductase is markedly quenched on the binding of either oxidized or reduced coenzymes (Dann et al., 1976; Birdsall et al., 1978; Dunn et al., 1978), providing a convenient method for the determination of equilibrium constants for formation of the binary enzyme–coenzyme complexes. A typical binding curve, for $NADP^+$, is shown in Figure 1. For $TNADP^+$ and $APADP^+$, self-absorption limits the precision with which the binding constant can be determined by this approach, while the coenzyme analogue 2′-phosphoadenosine-5′-diphosphoribose methyl β-riboside (PADPR-OMe), in which the nicotinamide ring has been replaced with a methoxy group, does not produce more than ~5% quenching of the protein fluorescence. The binding constants of these compounds are better determined by competition with $\epsilon NADP^+$; binding curves for $\epsilon NADP^+$, alone and in the presence of $TNADP^+$, are shown in Figure 2.

The equilibrium constants obtained in this way are presented in Table I. The values for all the oxidized coenzymes, together with that for PADPR-OMe, fall within a sevenfold range (a variation in binding energy of only 1.1 kcal/mol), suggesting at first sight that neither the adenine nor the nicotinamide subsite is particularly specific. However, reduction of the nicotinamide ring leads to much tighter binding for all the

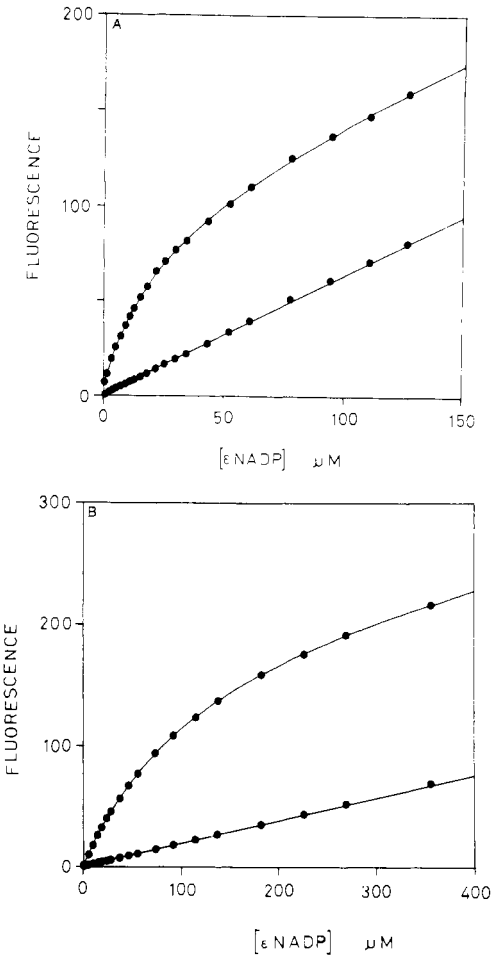

FIGURE 2: (A) Fluorescence of $\epsilon NADP^+$ in the presence of 24.9 μM dihydrofolate reductase (upper curve) and in the absence of enzyme (lower curve). (B) The same experiment, but in the presence of 286 μM $TNADP^+$. Note the different scales of the abscissas of (A) and (B).

Table I: Equilibrium Constants and Gibbs Energy Changes for the Binding of Coenzymes to Dihydrofolate Reductase

coenzyme	K' (M^{-1})	ΔG° (kcal/mol)
$NADP^+$	$6.1 (\pm 0.6) \times 10^4$	6.50
$NHDP^+$	$9.4 (\pm 1.4) \times 10^3$	5.40
$\epsilon NADP^+$	$4.9 (\pm 0.7) \times 10^4$	6.37
$TNADP^+$	$1.4 (\pm 0.2) \times 10^4$	5.62
$APADP^+$	$8.9 (\pm 1.3) \times 10^3$	5.36
PADPR-OMe	$5.2 (\pm 0.8) \times 10^4$	6.41
NADPH[a]	$1.0 (\pm 0.1) \times 10^8$	10.87
NHDPH	$3.1 (\pm 0.1) \times 10^7$	10.18
$\epsilon NADPH$	$3.1 (\pm 0.3) \times 10^7$	10.18
TNADPH	$2.0 (\pm 0.3) \times 10^5$	7.20
APADPH	$2.1 (\pm 0.1) \times 10^6$	8.60

[a] Value taken from Dunn et al. (1978).

coenzymes, by a factor ranging from 14 for the thionicotinamide analogue to 1600 for NADPH itself. There is thus a much greater variation in binding constant among the reduced than among the oxidized coenzymes; in particular, TNADPH and APADPH, in which the nicotinamide ring has been modified, bind much more weakly than NADPH.

Ternary Complexes: Oxidized Coenzymes. Under the conditions of these experiments, the inhibitors methotrexate (4-amino-4-deoxy-N^{10}-methylfolate) and trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] bind tightly to the enzyme [$K' = 2 \times 10^9$ M^{-1} and 2×10^7 M^{-1}, respec-

tively; calculated from the results of Hood & Roberts (1978)]. Effectively complete saturation with these compounds could therefore be maintained at the enzyme concentrations used, 1–5 μM, by adding them in 1.5–3-fold excess over the enzyme. The substrate folate binds more weakly ($K' = 1 \times 10^5$ M^{-1}; Hood & Roberts, 1978), so that a larger excess had to be used. Inhibitor binding leads to almost complete (~90%) quenching of the protein fluorescence [e.g., Dann et al. (1976) and Hood & Roberts (1978)], and the slight further quenching accompanying coenzyme binding was insufficient to use to measure the binding constant. Although folate binding produces less quenching of the protein fluorescence, the relatively high concentration which had to be used to saturate the enzyme led to substantial self-absorption, and again the protein fluorescence could not be used to measure coenzyme binding. However, the fluorescence of ϵNADP$^+$ was still enhanced on binding to the enzyme–substrate or enzyme–inhibitor complexes, so that its binding could be followed in this way and the binding constants of the other coenzymes could be determined by competition.

The measured binding constants and the corresponding Gibbs energy changes are given in Table II, together with a comparison of these values to those for the binary complexes. With one exception all the coenzymes bind significantly more tightly to the enzyme–inhibitor and enzyme–substrate complexes than to the enzyme alone. In the case of methotrexate, the coenzymes with a normal nicotinamide ring (NADP$^+$, NHDP$^+$, and ϵNADP$^+$) bind ~11 times more tightly, while those with a modified (or missing) nicotinamide ring show a smaller effect. The opposite trend is seen with trimethoprim, TNADP$^+$, APADP$^+$, and PADPR-OMe showing approximately the same effect as with methotrexate, while for the coenzymes with a normal nicotinamide ring the difference between ternary and binary complexes is smaller (two- to threefold). NADP$^+$ itself and its analogues NHDP$^+$ and ϵNADP$^+$ all bind rather more tightly to the folate complex than to the methotrexate complex. Two other analogues behave quite differently in these two complexes, APADP$^+$ showing much more cooperativity with the substrate than with the inhibitor and PADPR-OMe showing much less.

It is clear that the effect of a substrate or inhibitor on the binding of the coenzyme depends significantly on the structure of both ligands, so that the relation between structure and binding constant for the coenzyme analogues is significantly different in the different complexes. For example, the ratio of the binding constant of NADP$^+$ to that of APADP$^+$ is 6.9, 23, 6.0, and 2.2 for binding to the enzyme, the enzyme–methotrexate complex, the enzyme–folate complex, and the enzyme–trimethoprim complex, respectively.

Ternary Complexes: Reduced Coenzymes. As noted above, the reduced coenzymes bind much more tightly to the enzyme than do their oxidized counterparts; in addition, they seem to show a much larger increase in binding constant in the presence of inhibitors. The combination of these two facts meant that we were unable to determine the equilibrium constants for the binding of the reduced coenzymes to the enzyme–inhibitor complexes. In order to measure a binding constant with reasonable precision, the enzyme concentration must be less than $\sim 1/(2K')$; we estimate (see below) that for these complexes $K' \geq 10^{10}$ M^{-1}, and at enzyme concentrations below 10^{-10} M the fluorescence signals are too weak to be useful.

In order to obtain an estimate of the stability of these ternary complexes, we have measured the rate constant for dissociation of the coenzyme from them. This can be done at any enzyme

Table II: Equilibrium Constants[a] and Gibbs Energy Changes for the Binding of Oxidized Coenzymes to Dihydrofolate Reductase in the Presence of Methotrexate or Trimethoprim or Folate

coenzyme	methotrexate			trimethoprim			folate		
	$K_{E\text{-}MTX}$ (M^{-1})	$\Delta G^{\circ}_{E\text{-}MTX}$ (kcal/mol)	$K_{E\text{-}MTX}/K_E$	$K_{E\text{-}TMP}$ (M^{-1})	$\Delta G^{\circ}_{E\text{-}TMP}$ (kcal/mol)	$K_{E\text{-}TMP}/K_E$	$K_{E\text{-}FOL}$ (M^{-1})	$\Delta G^{\circ}_{E\text{-}FOL}$ (kcal/mol)	$K_{E\text{-}FOL}/K_E$
NADP$^+$	7.6 (±1.1) × 10^5	8.0	12.5	1.2 (±0.2) × 10^5	6.91	2.0	1.2 (±0.2) × 10^6	8.3	20.0
NHDP$^+$	1.1 (±0.2) × 10^5	6.85	11.7	2.7 (±0.4) × 10^4	6.01	2.9	1.4 (±0.2) × 10^5	7.0	14.9
ϵNADP$^+$	5.0 (±0.7) × 10^5	7.74	10.2	1.2 (±0.2) × 10^5	6.91	2.5	1.8 (±0.3) × 10^6	8.5	36.7
TNADP$^+$	6.4 (±1.0) × 10^4	6.53	4.6	6.6 (±1.0) × 10^4	6.55	4.7	4.2 (±0.6) × 10^4	6.3	3.0
APADP$^+$	3.3 (±0.5) × 10^4	6.14	3.7	5.4 (±0.8) × 10^4	6.43	6.1	2.0 (±0.3) × 10^5	7.2	22.5
PADPR-OMe	2.5 (±0.3) × 10^5	7.33	4.8	2.5 (±0.4) × 10^5	7.32	4.8	4.4 (±0.7) × 10^4	6.3	0.84

[a] K_E is the equilibrium constant for formation of the binary enzyme–coenzyme complexes (Table I); $K_{E\text{-}MTX}$, $K_{E\text{-}TMP}$, and $K_{E\text{-}FOL}$ are the equilibrium constants for the binding of coenzyme to the complexes of enzyme with methotrexate, trimethoprim, and folate, respectively; the same subscripts are used for the corresponding ΔG° values.

Table III: Dissociation Rate Constants for Reduced Coenzymes from Their Binary and Ternary Complexes with Dihydrofolate Reductase and Calculated Equilibrium Constants for Formation of the Ternary Complexes

	enzyme alone[a]	+methotrexate			+trimethoprim		
coenzyme	$k_{off,E}$ (s^{-1})[b]	$k_{off,E-MTX}$[b] (s^{-1})	$k_{off,E}/k_{off,E-MTX}$	estimated[b,c] K_{E-MTX} (M^{-1})	$k_{off,E-TMP}$[b] (s^{-1})	$k_{off,E}/k_{off,E-TMP}$	estimated[b,c] K_{E-TMP} (M^{-1})
NADPH	0.27	4.0×10^{-4}	675	6.8×10^{10}	2.0×10^{-3}	135	1.35×10^{10}
NHDPH	0.19	5.7×10^{-4}	333	1.0×10^{10}	*d*	*d*	*d*
TNADPH	13.9	9.3×10^{-3}	1490	3.0×10^{8}	1.6×10^{-2}	870	1.74×10^{8}
APADPH	3.1	1.4×10^{-3}	2210	4.6×10^{9}	1.6×10^{-2}	195	4.10×10^{8}

[a] From Dunn et al. (1978); determined by stopped-flow methods. [b] $k_{off,E}$, $k_{off,E-MTX}$, and $k_{off,E-TMP}$ are the rate constants for dissociation of the coenzyme from the enzyme, the enzyme–methotrexate complex, and the enzyme–trimethoprim complex, respectively; a corresponding notation is used for the equilibrium constants, K_{E-MTX} and K_{E-TMP}. [c] By use of eq 10; see the text. [d] Not determined.

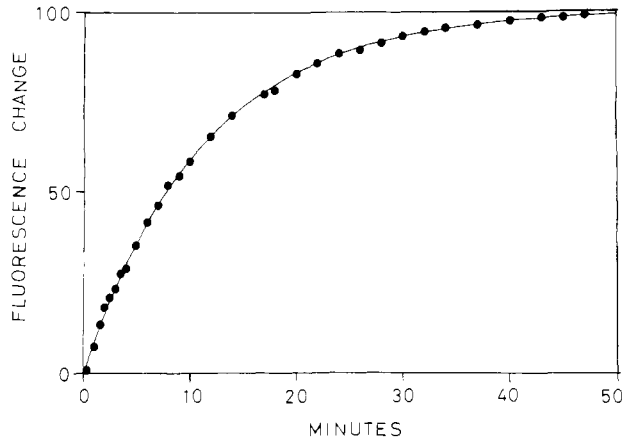

FIGURE 3: Time course of the fluorescence changes on addition of 8 μM NADPH to a solution containing 1 μM enzyme, 2 μM methotrexate, and 1 μM APADPH. The curve is the best-fit single exponential.

concentration by an "exchange" experiment in which the bound coenzyme is displaced by another coenzyme of appreciably different fluorescence characteristics so that their exchange is accompanied by a net change in fluorescence. Figure 3 shows the time course of the change in fluorescence after adding NADPH to a solution containing the enzyme–APADPH–methotrexate complex. Given the two equilibria

$$E + L_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL_1 \qquad (7)$$

and

$$E + L_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EL_2 \qquad (8)$$

for the binding of the two competing ligands (L_2 being the ligand added to initiate the reaction), then provided

$$k_2[L_2] \gg k_{-1}, k_1[L_1] \qquad (9)$$

the observed rate of ligand exchange is a measure of k_{-1}, the dissociation rate constant of the leaving ligand. As shown in Figure 3, the observed change in fluorescence with time was described well by a single exponential; together with the fact that the apparent rate constant was independent of the concentration of L_2 in the range used, this demonstrates that the conditions of eq 9 were satisfied.

The rate constants measured in this way are presented in Table III, along with the dissociation rate constants of the corresponding binary complexes determined by Dunn et al. (1978) in analogous experiments using rapid-mixing methods. The dissociation rate of the coenzymes from the enzyme–coenzyme–methotrexate complex was so slow that appreciable decomposition of the free reduced coenzyme occurred in the course of the experiment at pH 6.0 (the bound coenzyme and the enzyme itself were completely stable over the time periods involved). These experiments were therefore carried out at pH 7.0 to ensure the stability of the coenzyme. There is only a slight difference in binding constant of the coenzymes between pH 6.0 and pH 7.0 (Dunn et al., 1978), and for the ternary complexes containing trimethoprim the coenzyme dissociation rate constants were identical within experimental error at both pH values. We have therefore assumed that the dissociation rate constants measured at pH 7.0 will also hold at pH 6.0.

The precise relationship between these dissociation rate constants and the equilibrium constants for coenzyme binding will of course depend upon the detailed mechanism of coenzyme binding. This is discussed by Hyde et al. (1980b); the essential conclusion is that the equation

$$k_{off,ternary}/k_{off,binary} = K'_{binary}/K'_{ternary} \qquad (10)$$

which is valid for a simple one-step binding process, is unlikely to be in error here by more than about a factor of 2. We have therefore used eq 10 to estimate the equilibrium constants for coenzyme binding to the methotrexate and trimethoprim complexes, and these estimates are included in Table III.

It is apparent that the presence of the inhibitors has a much larger effect on the binding of the reduced coenzymes than on that of the oxidized coenzymes. Thus methotrexate increases the binding of $NADP^+$ 12-fold but that of NADPH almost 700-fold; the corresponding factors for the acetylpyridine analogue are 3.7 and 2200. In the binary complexes NADPH already binds some 1600 times more tightly than $NADP^+$, but in the ternary complex this ratio has increased to the striking value of 89 000 (6.7 kcal/mol). It is also notable that, although TNADPH and APADPH bind much less well than the natural coenzyme, their cooperativity with methotrexate is significantly greater, indicating that the two effects have a different structural specificity.

Discussion

The results presented here show clearly that, as for other dehydrogenases [e.g., Theorell & Yonetani (1963) and Kolb & Weber (1975)], coenzymes and substrates or substrate analogues enhance one another's binding to the enzyme. Since the size of this mutual effect on the binding of the two ligands depends strongly upon their structure (factors between 1 and 2200 being observed for the complexes studied here), the specificity of the enzyme for one group of ligands is affected in an important way by the presence of members of the other group. We can now attempt to understand this specificity in terms of the three-dimensional structure of the enzyme [determined for the enzyme–methotrexate–NADPH ternary complex by Matthews et al. (1978)] and the nuclear magnetic resonance studies of some of the coenzyme complexes reported in the following papers (Hyde et al., 1980a,b).

Binary Complexes. The most striking feature of the binding constants for the binary complexes is the marked increase

observed on reduction of the nicotinamide ring. Substantially tighter binding of reduced than of oxidized coenzyme has been observed with many other dehydrogenases; for example, Jaenicke and colleagues report $K_{NADH}/K_{NAD^+} = 550$ for liver alcohol dehydrogenase (Schmid et al., 1978), 57 for M_4, and 88 for H_4 lactate dehydrogenase (Hinz et al., 1978). In dihydrofolate reductase from other species, the difference in binding constant between NADPH and $NADP^+$ is a factor of 10–20, much smaller than that reported here for the *L. casei* enzyme (Perkins & Bertino, 1966; D'Souza & Freisheim, 1972; Erickson & Mathews, 1973; Poe et al., 1974; Subramanian & Kaufman, 1978; Williams et al., 1979). Although, because of differences in solution conditions, a detailed comparison is not possible, this difference in behavior seems to arise because the *L. casei* enzyme binds $NADP^+$ rather more weakly and NADPH rather more strongly than the other enzymes. Inspection of the environment of the nicotinamide ring throws little light on the origins of these differences, since the majority of the amino acid residues in contact with it are highly conserved (Matthews et al., 1979).

Recently Danenberg et al. (1978) showed that the coenzyme analogue having a phenyl ring in place of the nicotinamide ring behaves like NADH rather than NAD^+ in its interaction with lactate dehydrogenase. They proposed that the crucial factor determining the much weaker binding of oxidized compared to reduced coenzymes is the positive charge on the nicotinamide ring in the former case. The nicotinamide binding site of dihydrofolate reductase is largely hydrophobic and binding an isolated positive charge in such a situation would clearly be unfavorable (Matthews et al., 1979). The coenzyme "fragment" 2′-phosphoadenosine-5′-diphosphoribose methyl β-riboside binds very nearly as tightly to the enzyme as does $NADP^+$; similarly, adenosine-5′-diphosphoribose binds as well as NAD^+ to alcohol and lactate dehydrogenases (Schmid et al., 1978; Hinz et al., 1978). The presence of an oxidized nicotinamide ring thus has no effect on the binding. However, the large changes in chemical shift of the nicotinamide proton resonances of $NADP^+$ when it binds to dihydrofolate reductase (Hyde et al., 1980a) show quite clearly that the oxidized nicotinamide ring does interact with the enzyme in a highly specific manner. It may be that the energetically unfavorable effects of the positive charge balance out the favorable hydrophobic and hydrogen-bonding interactions of the ring.

There are, however, a number of features of coenzyme binding to dihydrofolate reductase which suggest strongly that the simple disappearance of the positive charge (and thus of its unfavorable interactions) is not sufficient to explain the tighter binding of the reduced coenzymes. First, the increase in binding energy which follows reduction of the nicotinamide ring varies from 1.6 kcal/mol for TNADP(H) to 4.4 kcal/mol for NADP(H) and 4.8 kcal/mol for NHDP(H). This clearly cannot, in every case, represent the effect of removing a charge from an otherwise identically situated nicotinamide ring. Indeed, the chemical shifts of the nicotinamide ring protons show clearly that the environment of the bound nicotinamide ring is in fact noticeably different in NADPH as compared to that in $NADP^+$ (Hyde et al., 1980a) so that its interactions with the protein must also be different. There are also indications (Feeney et al., 1977; Kimber et al., 1977) of conformational differences, involving tyrosine and tryptophan residues, between the $NADP^+$ and NADPH complexes. For example, one of the tyrosine 2,6-proton resonances in the spectrum of the selectively deuterated enzyme (Feeney et al., 1977) shifts in opposite directions on the binding of $NADP^+$ or NADPH. This signal is unaffected by the binding of PADPR-OMe (B. Birdsall, unpublished work), suggesting that the nicotinamide ring is responsible for these shifts. The crystal structure (Matthews et al., 1978) shows that there is no tyrosine residue in the immediate vicinity of the nicotinamide ring, so that these shifts must be conformational in origin, and binding of $NADP^+$ or NADPH must have different conformational effects. Markedly different chemical shift effects of $NADP^+$ and NADPH binding to *Streptococcus faecium* dihydrofolate reductase have been observed by Blakley and co-workers (1978; Cocco et al., 1978) in their ^{13}C NMR studies of enzyme containing ^{13}C-enriched methionine or arginine, and it seems likely that at least some of these effects are conformational in origin (Cocco et al., 1978).

The crystal structure of the enzyme–NADPH–methotrexate complex (Matthews et al., 1978, 1979) shows that the carboxamide group of the nicotinamide ring forms two hydrogen bonds with the enzyme. The bond involving the carboxamido nitrogen could not form in APADP(H), while that to the oxygen would be at least very much weaker when this is replaced by a sulfur in the thionicotinamide analogues. These analogues are indeed observed to bind more weakly than the natural coenzyme. The oxidized forms, $APADP^+$ and $TNADP^+$, in fact bind less well than PADPR-OMe; one possibility is that the less favorable interactions of the carboxamide group are no longer sufficient to offset the unfavorable charge effect. The effects of the acetyl and thioamide modifications are much larger in the reduced than in the oxidized state (factors of 500 and 50, compared to 7 and 4). Patrick et al. (1974) have concluded, from Raman and infrared spectroscopy, that reduction of the ring substantially alters the electron distribution in the carboxamide group and thus its hydrogen-bonding ability. However, it is also clear from the chemical shifts of the nicotinamide ring protons (Hyde et al., 1980a) that the environment of this ring in $TNADP^+$ is quite different from that in $NADP^+$. This could, for example, be a difference in orientation of the ring arising from the weakening of the hydrogen bonds; in any case, it would contribute to the very different effect of reduction of $TNADP^+$ and $NADP^+$.

In contrast, the environment of bound $NHDP^+$ is very similar to that of $NADP^+$ (Hyde et al., 1980a,b), so that the decrease of 1.1 kcal/mol in binding energy can reasonably be attributed to the differences in the interactions of the adenine or hypoxanthine rings themselves with the enzyme. The binding site for the adenine ring is essentially hydrophobic in nature, with no hydrogen bonds, and the 6-amino group appears to interact only with the solvent (Matthews et al., 1979). Thus, the weaker binding of $NHDP^+$ cannot be explained by differences in hydrogen-bonding ability. Hypoxanthine is significantly more polar than adenine (Tinker & Brown, 1948; Bergmann & Weiler-Felchenfeld, 1973) and, if the interaction with the enzyme is "hydrophobic", the weaker binding of $NHDP^+$ compared to $NADP^+$ may arise from its more favorable interaction with water rather than from any specific change in its interactions with the enzyme. Indeed, the ratio of the butanol/water partition coefficients of adenine and hypoxanthine (Tinker & Brown, 1948) is equivalent to a Gibbs energy difference of 0.95 kcal/mol, very similar to the difference in $\Delta G°$ for binding to the enzyme.

Ternary Complexes: Oxidized Coenzymes. When considering the changes in coenzyme binding produced by methotrexate or trimethoprim, it is immediately apparent that the coenzymes fall into two groups. Coenzyme analogues having modified or missing nicotinamide rings (i.e., $TNADP^+$,

APADP$^+$, or PADPR-OMe) bind ~5 times more tightly to both the enzyme–methotrexate and enzyme–trimethoprim complexes than to the enzyme alone. In contrast, coenzymes with a normal nicotinamide ring bind ~11 times better to the methotrexate complex but only 2–3 times better to the trimethoprim complex than to the enzyme alone. Increases of a similar order in the binding of inhibitors in the presence of oxidized coenzyme (or vice versa) have been reported for dihydrofolate reductase from L1210 cells (Perkins & Bertino, 1966), *Escherichia coli* (Poe et al., 1974), and *S. faecium* (Williams et al., 1979).

This division of the coenzyme analogues according to their binding behavior has a parallel in the ^{31}P NMR studies (Hyde et al., 1980b). Addition of methotrexate to the enzyme–NADP$^+$ or –NHDP$^+$ complexes produces small upfield shifts of the ^{31}P resonances of the coenzyme but with PADPR-OMe, TNADP$^+$, or APADP$^+$ formation of the ternary complexes leads to substantial *downfield* shifts of at least one of the pyrophosphate ^{31}P resonances.

For NADP$^+$ and NHDP$^+$ the changes in environment accompanying the order of magnitude increase in binding constant produced by methotrexate are minimal (Hyde et al., 1980a,b). In the ternary complex with the *reduced* coenzyme (Matthews et al., 1978), the nicotinamide and pteridine rings are in contact. If a similar direct interaction occurred with the oxidized coenzyme [which would be quite consistent with the NMR data of Hyde et al. (1980a)], this might contribute to the observed increase of 1.47 kcal/mol in binding energy. Some indication that there may also be a conformational contribution comes from measurements of the accessibility of aromatic amino acid residues by the photo-CIDNP method of Kaptein (Kaptein, 1978; Kaptein et al., 1978). One histidine residue shows an increase in accessibility on addition of methotrexate to the enzyme–NADP$^+$ complex (Feeney et al., 1980), demonstrating that some degree of conformational change accompanies formation of the ternary complex. This change must be largely localized to the nicotinamide end of the coenzyme, since no effects on the adenine end are observed (Hyde et al., 1980a,b).

The ^{31}P chemical shift changes (Hyde et al., 1980b) indicate that methotrexate binding produces larger changes in the environment of TNADP$^+$, APADP$^+$, and PADPR-OMe than in that of NADP$^+$. The evidence for a difference in coenzyme environment between binary and ternary complexes is particularly clear for the trimethoprim complexes. We have shown by ^{1}H and ^{31}P NMR (A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) that these complexes exist in two interconverting conformational states [see also Hyde et al. (1980b)] which differ in the conformation of the bound coenzyme and whose relative population varies from one coenzyme analogue to another. It is clear from these experiments that TNADP$^+$, for example, has a quite different conformation in its ternary complex with trimethoprim than in its binary complex. The 4.8-fold increase in its binding constant produced by trimethoprim thus originates, at least in part, from a conformational change. This change in coenzyme conformation most probably involves a significant change in the position of the nicotinamide ring (A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments) which would certainly be consistent with the fact that TNADP$^+$ and APADP$^+$ behave, as far as binding constants are concerned, more like PADPR-OMe than like NADP$^+$. This similarity extends to the methotrexate complexes, and similar conformational effects may be involved here. However, trimethoprim and methotrexate have clearly distinct effects on the ^{1}H and ^{31}P resonances of NADP$^+$ (Hyde et al., 1980a,b; A. Gronenborn, B. Birdsall, E. I. Hyde, G. C. K. Roberts, J. Feeney, and A. S. V. Burgen, unpublished experiments), and it is possible that they affect coenzyme binding by qualitatively different mechanisms.

As with methotrexate, the tighter binding of NADP$^+$ and NHDP$^+$ in the presence of folate is accompanied by minimal changes in the coenzyme environment (Hyde et al., 1980a,b); the three coenzymes with unmodified nicotinamide rings bind 1.3–3.5 times more tightly in the presence of the substrate than in the presence of the inhibitor. APADP$^+$ shows substantial cooperativity with folate, behaving much more like NADP$^+$ than like TNADP$^+$ (in contrast to the observations in the presence of methotrexate). However, although APADP$^+$ and NADP$^+$ show a similar degree of cooperativity with folate, they show very different changes in environment on folate binding. In contrast to NADP$^+$, both the nicotinamide protons and the pyrophosphate ^{31}P signals of APADP$^+$ show substantial shifts on addition of folate (Hyde et al., 1980b; E. I. Hyde, unpublished work). The cooperativity seen with these two coenzymes must thus arise by different mechanisms (Hyde et al., 1980b). In both cases it seems to be associated with the nicotinamide ring, since PADPR-OMe shows no cooperativity. Folate has recently been shown (Charlton et al., 1979; Hitchings, 1980) to bind to the enzyme in quite a different orientation to that observed for methotrexate in the crystal (Matthews et al., 1978), and these observations suggest that its relationship to and effects on the binding of coenzyme analogues are consequently altered.

Ternary Complexes: Reduced Coenzymes. The presence of methotrexate or trimethoprim leads to a very striking decrease in the rate of dissociation of the coenzyme from the complex. What evidence there is on the detailed mechanism of coenzyme binding [discussed by Hyde et al. (1980b)] suggests that, although a two-step mechanism is most probably involved, the change in dissociation rate constant is likely to be a good indication of the change in binding constant. The inhibitors thus increase the binding energy of the reduced coenzymes by between 2.9 and 4.5 kcal/mol, a considerably larger effect than that seen with the oxidized coenzymes. The origin of this further substantial difference between oxidized and reduced coenzymes (in addition to the large difference in binding constant in the binary complexes) is as yet unknown. Perkins & Bertino (1966) observed a 60-fold increase in the binding constant of NADPH to L1210 dihydrofolate reductase in the presence of triamterene, while very recently Williams et al. (1979) have estimated, using steady-state kinetic methods, that methotrexate binds as much as 6000 times more tightly to the *S. faecium* enzyme in the presence of NADPH. In both cases, the "cooperativity" seen with the reduced coenzyme was greater than with the oxidized coenzyme.

As noted above, the nicotinamide ring of NADPH makes contact with the pteridine ring of methotrexate in the ternary complex with the *L. casei* enzyme (Matthews et al., 1978). However, the overlap of the two rings is not extensive and their planes are not parallel, so that it is unlikely that this interaction could account for all the 3.8 kcal/mol additional binding energy in the ternary complex. It is probable that some at least of the cooperativity between NADPH and methotrexate arises from a conformational change. This is particularly likely in the case of trimethoprim, for which (at least in the conformation we have proposed: Cayley et al., 1979) a favorable direct interaction such as that seen with methotrexate is difficult to envisage. The cooperativity with trimethoprim is

uniformly less than that with methotrexate. Comparison of the NMR spectra of selectively deuterated and fluorine-labeled analogues of dihydrofolate reductase (Feeney et al., 1977; Kimber et al., 1977) in the presence of methotrexate with those obtained in the presence of methotrexate and NADPH shows a number of shifts in the resonances of specific residues which might reflect such a conformational change. [One fluorotryptophan signal shows a very large change in chemical shift on addition of NADPH, but this probably arises from Trp-21 which is in contact with the nicotinamide ring (Matthews et al., 1978, 1979; Matthews, 1979).] These possible conformational shifts are limited in number, however, and it is clear that any conformational change accompanying formation of the ternary complex must be much less extensive than those which occur on formation of the binary complexes (Feeney et al., 1977; Roberts et al., 1977; Kimber et al., 1977). Further understanding of the mechanisms underlying these striking cooperative effects must thus await a more detailed structural comparison of the binary and ternary complexes.

Even with the limited information presently available, the behavior of the thionicotinamide and acetylpyridine analogues is particularly striking. As noted above, in their oxidized form these analogues show only as much cooperativity as PADPR-OMe, and there is evidence that they may bind in a different way from $NADP^+$. On reduction, however, the cooperativity they show with methotrexate or trimethoprim increases 30–600-fold, becoming 1.4–6 times greater than that shown by NADPH. Clearly, the reduced nicotinamide ring play a major part in this large cooperativity, and it seems unlikely that TNADPH and APADPH would behave in this way unless their pyridine rings bound in at least approximately the same way as that of NADPH. Indeed, circular dichroism experiments (Hood et al., 1979) strongly suggest that the nicotinamide rings of TNADPH and NADPH have rather similar orientations with respect to the pteridine ring of methotrexate, since in both cases a "couplet" CD spectrum is observed, suggestive of an exciton interaction between the two chromophores. Both APADPH and TNADPH do have some activity as coenzymes [about 10 and 1%, respectively, of that of NADPH (Williams et al., 1977; Dunn et al., 1978; E. I. Hyde, unpublished work)], so their nicotinamide rings must clearly bind "correctly" for at least part of the time. The evidence thus strongly suggests that the thioamide and acetyl analogues bind to the enzyme in a very different way—at least in the ternary complex with methotrexate—in the oxidized and reduced forms. The nature of this change in mode of binding on reduction remains to be established. It may be related to the postulated difference between $NADP^+$ and NADPH or, perhaps more probably in view of the good evidence for differences in binding between $TNADP^+$ and $NADP^+$, it may be a quite distinct conformational change, specific to the modified coenzymes.

Implications for Inhibitor Binding. Since cooperativity in ligand binding must be reciprocal [see, e.g., Weber (1975) and Birdsall et al. (1978)], these large effects of inhibitors on the binding of reduced coenzymes must be accompanied by equal effects of reduced coenzymes on the binding of inhibitors. At pH 6.0, the equilibrium constant for formation of the binary complex of methotrexate with the *L. casei* reductase is $\sim 2 \times 10^9\ M^{-1}$ [calculated from the results of Hood & Roberts (1978)], so that the binding constant to the enzyme–NADPH complex will be $\sim 1.3 \times 10^{12}\ M^{-1}$. This value is consistent with measurements of the dissociation rate constant of methotrexate from the ternary complex ($\sim 3 \times 10^{-5}\ s^{-1}$; A. S. V. Burgen, unpublished work). There have been a number of qualitative reports of much stronger binding of methotrexate to dihydrofolate reductase from various sources in the presence of NADPH (Perkins & Bertino, 1966; Blakley, 1969; Williams et al., 1973a,b), but this is the first time a quantitative estimate of this effect has been possible. Although the binding constant is based in part on kinetic rather than equilibrium measurements, it is probably within a factor of 2 of the true thermodynamic equilibrium constant. This equilibrium constant is the relevant one for most studies of the inhibition of the enzyme by methotrexate, since the enzyme is commonly assayed in the presence of a large excess of NADPH, and is consistent with earlier upper limits to the K_i of methotrexate of 10^{-10}–10^{-11} M (Werkheiser, 1961; Bertino et al., 1964; Williams et al., 1973a). Williams et al. (1979) have estimated the K_i of methotrexate for the *S. faecium* enzyme as 5.8×10^{-11} M, and Jackson et al. (1976) have reported values ranging from 5.3×10^{-12} M to 1.35×10^{-10} M for the reductases from four mammalian cell lines. It is likely that this equilibrium constant (rather than that for the formation of the enzyme–methotrexate binary complex) is the therapeutically relevant one. In this context it is interesting, and potentially important, that the effects of coenzyme on inhibitor binding are dependent on the structure of the inhibitor. Thus, NADPH increases the binding of methotrexate, trimethoprim, and 2,4-diaminopyrimidine by factors of 675, 135, and 8, respectively (this work; Birdsall et al., 1978). Pattishall et al. (1976) have shown for the reductase from *E. coli* RT500 that NADPH markedly increases the affinity of the enzyme for trimethoprim but has no effect on that for pyrimethamine. We have earlier reported that NADPH changes the specificity of the *L. casei* enzyme for *N*-(*p*-(alkylamino)benzoyl)-L-glutamates (Birdsall et al., 1978). It is clear that the cooperativity with NADPH is an important consideration in the design of inhibitors of this enzyme. In an attempt to understand this cooperativity better, we have studied the effects of coenzymes on the binding of *p*-aminobenzoyl-L-glutamate and 2,4-diaminopyrimidine. These can be regarded as fragments of methotrexate, and the diaminopyrimidine is clearly also a fragment of trimethoprim. The results of these experiments are described in the following paper (Birdsall et al., 1980).

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