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REDUCTION OF OXIDISED FOLATES BY DIHYDROFOLATE REDUCTASE FROM METHOTREXATE-RESISTANT *LACTOBACILLUS CASEI*

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Summary

The use of alternative substrates by dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) was investigated as a possible mechanism for the resistance of *Lactobacillus casei* to the cytotoxic drug methotrexate. The reduction of folic acid and 10-formylfolic acid by homogeneous enzyme was compared to that of the normal substrate, dihydrofolic acid. The three substrates have different pH optima and K_m values. In addition, it was found that the reduction of 10-formylfolic acid was markedly stimulated by the presence of ions. Although the reduction was sensitive to methotrexate in all cases, the ion activation may be of importance in partially inhibited systems.

Introduction

A major problem in the use of cytotoxic drugs is the unwanted selection of drug-resistant cells. Methotrexate is a commonly used cytotoxic pteridine derivative which is thought to prevent tetrahydrofolate production by inhibiting dihydrofolate reductase (5,6,7,8,-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3). Subsequent arrest of cell division is generally ascribed to a failure of tetrahydrofolate-dependent thymidylate biosynthesis. A route of methotrexate-insensitive tetrahydrofolate biosynthesis would therefore be expected to confer methotrexate resistance upon the cell.

In the presence of intracellular methotrexate, tetrahydrofolate could be pro-

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duced in several ways: by expression of alternative folate biosynthetic routes, e.g. from purines [1,2]; by an alteration in the interaction between methotrexate and dihydrofolate reductase [3]; by an increase in the amount of reductase [4–6], or by use of routes involving the reductase and an alternative folate substrate [7].

As part of a program of structural and functional study directed at these broad questions, the present work was undertaken to clarify whether the reductase from methotrexate-resistant cells is able to use an alternative substrate. The substrate must be reduced to the corresponding tetrahydrofolate which then enters the tetrahydrofolate pool, perhaps involving a one-carbon transformation in doing so.

The most likely naturally occurring oxidised alternative substrates are folic acid and 10-formylfolic acid. As the 10-methyltetrahydrofolate derivative is not reported as a one-carbon donor and inhibits cell growth [8,9], it seems that the formation of tetrahydrofolates by methylation, reduction, and subsequent demethylation is an unlikely pathway. In contrast, the formyl derivative is a precursor of 10-formyltetrahydrofolic acid, an important one-carbon donor [10,11].

The present study was therefore directed at characterising the reduction of folic acid and 10-formylfolic acid by dihydrofolate reductase. At the outset it seemed probable that if the enzyme was capable of reducing these compounds to the 7,8-dihydro derivatives then, in the presence of excess NADPH, further reduction to the corresponding 5,6,7,8-tetrahydrofolate would follow rapidly. The potential biological usefulness of such reactions would depend upon their sensitivity to methotrexate. For this study, dihydrofolate reductase was isolated from a methotrexate-resistant mutant of *Lactobacillus casei* (MTX/R-(DHFR+)) [12]. This organism metabolises folate in a manner similar to that of man [13,14] and, like man, is free from the complication of purine-based dihydrofolate synthesis.

Materials and Methods

Materials

Folic acid was supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and was purified by ion-exchange chromatography using a column of DE-52 cellulose (W. and R. Balston Ltd., Maidstone, Kent, U.K.). NADPH was obtained from Sigma Chemical Co., Surrey, KT2 7BH, UK. 7,8-Dihydrofolic acid was prepared using a modification of the method of Blakley [15] and stored under vacuum at −20°C until used. Methotrexate was a gift from Dr. H. Worth, National Cancer Institute, Bethesda, Md., U.S.A. 10-Formyltetrahydrofolic acid, chemically prepared, was a gift from Dr. J.W. Dow, Biochemistry Department, Glasgow University, Glasgow, U.K., Polygram Cel 300 precoated thin-layer chromatography plates were supplied by Camlab Ltd., Cambridge, U.K. Generally labeled [^{3}H]folic acid was supplied by the Radiochemical Centre, Amersham, U.K., at a specific activity of 5 Ci/mmol. It was then isotopically diluted to give a standard solution containing 5 $\mu Ci \cdot \mu mol^{-1} \cdot ml^{-1}$ which was stored frozen. Dihydrofolate reductase was obtained as a homogeneous protein

after purification from methotrexate-resistant *L. casei* [12]. Mutant MTX/R-(DHFR+) was used. Glass-distilled deionised water was used in all experiments.

Methods

Preparation of 10-formylfolic acid. Folic acid was dissolved in 98% formic acid (10 mol/100 l) and heated at 60°C. Formation of 10-formylfolic acid was followed chromatographically on cellulose thin-layers using as a solvent, ethanol/1 M ammonium acetate/water (40 : 8 : 52, by vol.). On completion of the reaction, formic acid was removed by rotary evaporation and freeze-drying. The 10-formylfolic acid was purified by ion-exchange chromatography on DE-52 cellulose using a gradient of ammonium acetate from 10 mM to 0.5 M. The fractions containing 10-formylfolic acid were pooled and freeze-dried.

Spectrophotometric assay of enzyme activity. Spectrophometric assays of dihydrofolate reductase rely upon a decrease in absorbance at 340 nm due to both reduction of dihydrofolic acid and oxidation of NADPH. Reduction of the oxidised folate derivatives can be followed in the same way. In measuring the reduction of dihydrofolic acid to tetrahydrofolic acid, the method of Mathews and Huennekens [16] was used, except that the buffer was 50 mM Tris · HCl, pH 7.5, no mercaptoethanol was present and the reaction mixture contained 1 M KCl except in the salt studies. An enzyme-free blank was used in all assays. The reaction mixture used to measure reduction of folic acid and 10-formylfolic acid was 100 µl 1 M potassium acetate, pH 5.0, 50 µl folic acid or 10-formylfolic acid (1 mg/ml), 50 µl NADPH solution (2.5 mg/ml) and 10 µl of enzyme solution in a total volume of 1 ml.

At pH 7.5 the value calculated for the molar absorbance change upon reduction of dihydrofolic acid to tetrahydrofolic acid was 11 960. Values measured for the molar absorbance change on the reduction of folic acid and 10-formylfolic acid were 19 010 and 18 320, respectively. All reactions were carried out at 37°C.

Radioassay of enzyme activity. Dihydrofolate reductase was also measured using [^{3}H]folic acid as described by Nakamura and Littlefield [17]. In this case the specific activity of the [^{3}H]folic acid was increased to 5 Ci/mol. Samples for radioassay were counted at ambient temperature using a Nuclear Enterprises 8312 scintillation counter and scintillation fluid consisting of two parts toluene, containing 4 g/l PPO and 0.1 g/l POPOP, with one part Triton X-100 by volume.

Effect of pH on substrate utilisation. The standard assay system was used except that 1 M potassium acetate buffer was replaced by 1 M Tris/phosphate or sodium acetate buffers at the different pH values indicated in the relevant legends.

Inhibition by methotrexate. Effects of methotrexate upon the three reactions were studied using the appropriate amounts of a 10^{-5} M solution of the inhibitor added to the assay system, the total volume remaining constant.

Ion-dependent alterations of reductase activity. In the case of dihydrofolic acid, 100 µl of a 2 M salt solution was added to the normal system, maintaining a constant volume. For folic acid and 10-formylfolic acid assays, the concentration of potassium acetate in the mixture was reduced to 50 mM and 100 µl 2 M salt solution was added. Where necessary, the pH of the assay mixture was corrected after addition of the salt, prior to adjustment to standard volume.

Results

Effect of pH on substrate utilisation. The rate at which dihydrofolate reductase reduced dihydrofolic acid, folic acid and 10-formylfolic acid was measured over a range of pH values from 3.8 to 8.7, using the buffers described previously (Fig. 1). Dihydrofolic acid was reduced with a single wide pH optimum at 6.5—7.3 (Fig. 1a). Folic acid is optimally reduced at pH 4.5 (Fig. 1c), while reduction of 10-formylfolic acid is optimal below pH 4.0 (Fig. 1b), To minimise problems arising from the acid lability of NADPH and the relative insolubility of the folates at low pH, further investigations of the oxidised substrates were performed at pH 5.0.

Relative substrate activities and identity of reaction product. Table I shows the relative rates of reduction of folic acid, dihydrofolic acid and 10-formylfolic acid, using equivalent amounts of substrate and the same enzyme prepara-

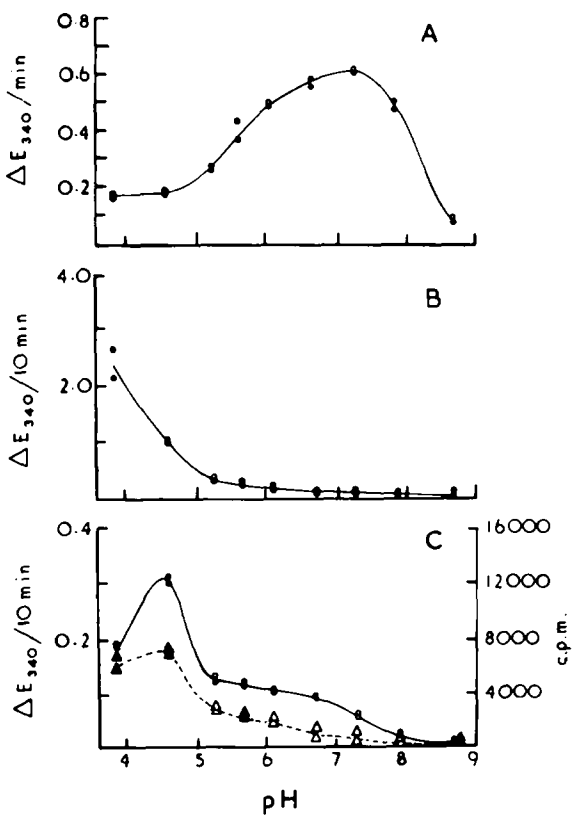

Fig. 1. Effect of pH on the reduction of folic, 10-formylfolic and 7,8-dihydrofolic acid. The rate of reduction of the three substrates at different pH values was measured using the following modification of the second system of assay described in Methods: 100 µl 1 M Tris/phosphate (○) or 1 M sodium acetate (●) buffer, 50 µl NADPH solution, 50 µl pteridine substrate solution (1 mg/ml) and 10 µl of enzyme in a total volume of 1 ml. The change in E_{340} was followed as a measure of the reduction of the three substrates; A, dihydrofolic acid; B, 10-formylfolic acid and C, folic acid. The reduction of folic acid was also followed using the radioassay (△).

TABLE I

RELATIVE RATES OF REDUCTION OF THE THREE FOLATE SUBSTRATES BY DIHYDROFOLATE REDUCTASE

The rates of reduction of the three substrates were measured as described in the text. Values quoted are the average of duplicate estimations carried out with the same enzyme preparation.

Substrate	Rate of reduction (mol pteridine/mol enzyme per min)
Dihydrofolic acid	218.0
10-Formylfolic acid	12.1
Folic acid	1.93

tion. Similar data are obtained using both radioassay and spectrophotometric assay.

Using the conditions described in the legend to Table I, dihydrofolic acid is the best substrate for the enzyme.

Inhibition by methotrexate. Inhibition by methotrexate of the reduction of dihydrofolic, folic and 10-formylfolic acids was examined by monitoring the three reactions independently. Fig. 2 shows that inhibition profiles are very similar but dihydrofolate reductase activity is slightly less sensitive to methotrexate than are the folate or 10-formylfolate reductase activities. In the case of dihydrofolic acid, there seems to be a residual activity over the concentration range used. This effect disappeared with increasing concentrations of methotrexate.

Comparison of radio- and spectrophotometric assays. As an independent check of the reaction products, specifically as a means for detecting intermediates, the radioactive and spectrophotometric assays were initially used in parallel. The only practical oxidised substrate for the radioassay was folic acid.

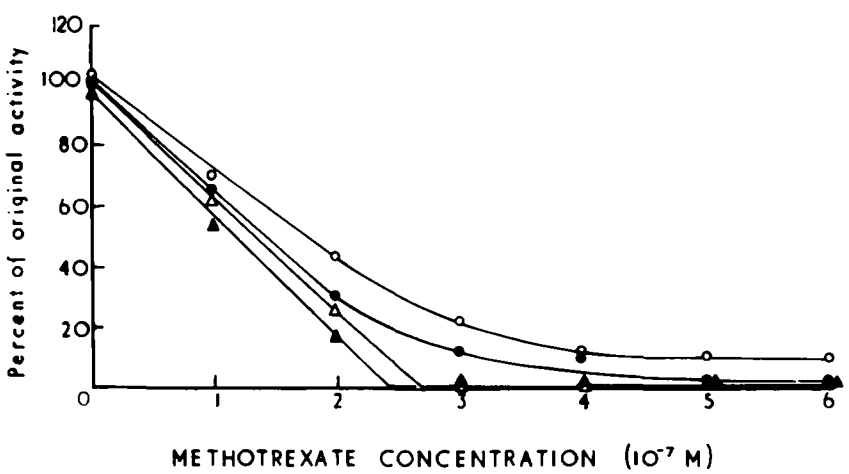

Fig. 2. Inhibition by Methotrexate of reduction of the different folates. Increasing amounts of a 10^{-5} M solution of methotrexate were added to the assay systems for dihydrofolic acid (○), 10-formylfolic acid (●) and folic acid, spectrophotometric assay (△) and radioassay (▲). The same amount of enzyme was used in all cases.

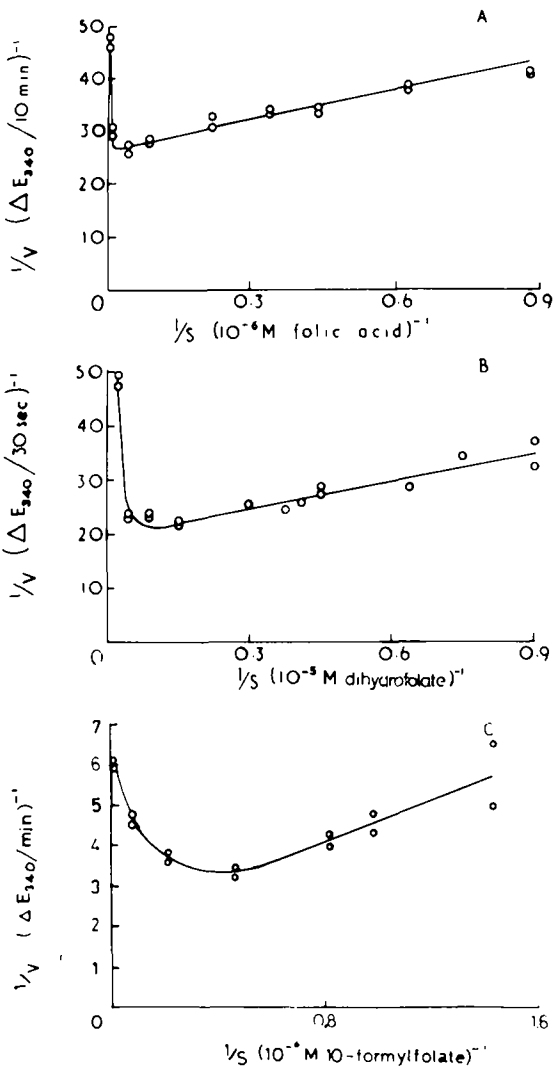

Fig. 3. Determination of K_m values for the three folate substrates. Standard assay systems were used to obtain double-reciprocal plots for the reduction of folic (A), dihydrofolic (B) and 10-formylfolic (C) acids.

10-Formylfolic acid was found to be unsuitable for this purpose due to the similarity of the solubility characteristics of reduced and oxidised forms. Figs. 1 and 2 indicate that the two assay profiles were identical under a wide variety of conditions. The spectrophotometric assay was therefore used in all further experiments.

Determination of K_m values. Double-reciprocal plots for utilisation of dihydrofolic, folic and 10-formylfolic acid by dihydrofolate reductase are shown in Fig. 3. The respective K_m values are $8.7 \cdot 10^{-6}$, $0.7 \cdot 10^{-6}$ and $2.7 \cdot 10^{-6}$ M. Substrate inhibition of the reaction was observed at high substrate: enzyme ratios. This effect cannot be explained by substrate insolubility at high concentra-

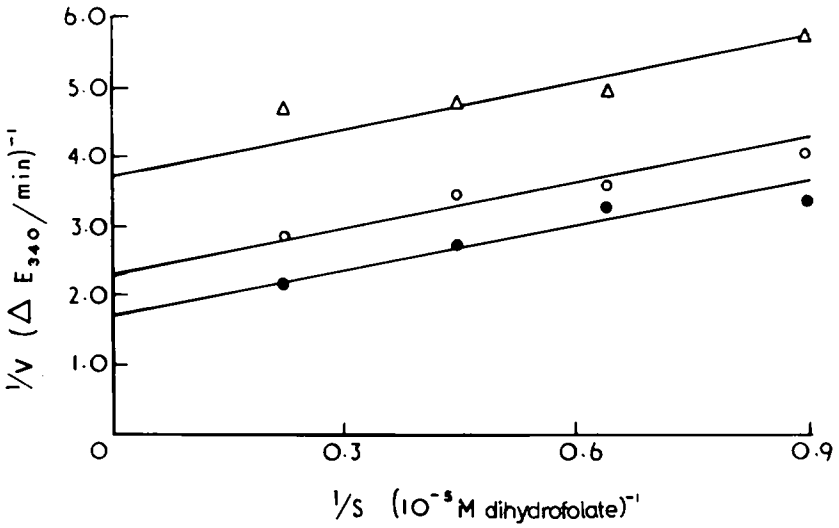

Fig. 4. Initial velocity pattern. In the standard assay the effect on the rate of reaction of varying the concentration of dihydrofolic acid was examined at several different concentrations of NADPH: 28 μM (△), 56 μM (○) and 140 μM (●).

tions, as control studies indicated that all substrates were soluble throughout the concentration range used.

Competition between dihydrofolic acid and NADPH. The observed substrate inhibition was investigated further to clarify the mechanism of reaction. A series of double reciprocal plots was produced [18] by varying the concentration of dihydrofolic acid at several different concentrations of NADPH. In Fig. 4 the parallel pattern of lines indicates that dihydrofolic acid is not competing with NADPH at the same binding site on the enzyme. This implies that the substrate inhibition observed previously occurs at the dihydrofolic acid binding site. This experiment could not be repeated with folic or 10-formylfolic acid as substrate because the assay system was not sensitive enough over the required concentration range necessary. This problem may be overcome by the development of more sensitive assay procedures, such as fluorimetry.

Effect of ions upon reductase activity. Table II shows the effect of different ions upon the three activities of this reductase. At 0.2 M, different salts influence the reduction of folic acid in a similar manner to that of dihydrofolic acid, in most cases. Magnesium and calcium inhibit both folate and dihydrofolate reductase activity, the effect on dihydrofolic acid reduction being the more marked. The other ions tested inhibit both activities equally, but to a lesser extent than observed with divalent cations.

A very different effect was observed in the case of 10-formylfolic acid. Both anions and cations markedly increased the rate of reduction of this substrate. Chloride has the greatest effect, resulting in three times the control activity. This was not offset by varying the cation, and indeed NH_4^+ and Ca^{2+} gave some further increase. The other anions studied also raised the original rate, but to a lesser extent.

Inhibition of salt-activated reduction of 10-formylfolic acid. The marked acti-

TABLE II

EFFECT OF IONS ON THE ACTIVITY OF DIHYDROFOLATE REDUCTASE

The rates of reduction of folic, 7,8-dihydrofolic and 10-formylfolic acid by dihydrofolate reductase were measured in the presence of 0.2 M salt as described in the text.

Salt in assay mixture	Percent of normal activity		
	7,8-Dihydrofolic acid	Folic acid	10-Formylfolic acid
NaCl	84.6	85.0	290.4
Sodium acetate	80.8	69.2	123.5
Sodium phosphate	77.7	84.3	161.5
KCl	87.7	89.2	303.8
$MgCl_2$	58.5	96.1	306.9
$CaCl_2$	19.6	37.7	367.3
NH_4Cl	95.8	99.6	336.5

vation of reduction of this substrate by a wide range of ions offers a mechanism for by-passing methotrexate block particularly if the activated enzyme has reduced sensitivity to methotrexate. Fig. 5 shows that activation produces a pH vs. activity profile similar in shape to the non-activated enzyme but about one unit more basic. At the pH at which *L. casei* grows, around pH 5.0, identical inhibition by methotrexate was observed for both activated and non-activated enzyme. It is important to note, however, that in the partially inhibited system the salt-activated enzyme has three times the activity of the normal assay.

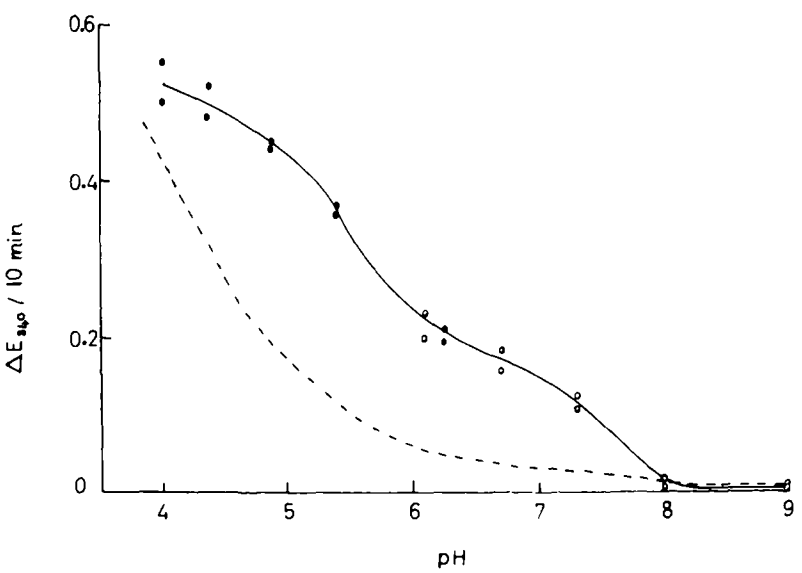

Fig. 5. Effect of pH on rate of reaction of salt-activated reductase. The conditions were as described in Fig. 1, the substrate being 10-formylfolic acid. 0.2 M KCl was present, and the buffers used were 1 M Tris · HCl/potassium phosphate (○) and 1 M sodium acetate (●). The non-activated profile is shown for comparison (— — —).

Discussion

Present evidence from inhibitory [19], titration [20] and protein binding studies [12] favours dihydrofolate reductase as a principal site of action of the cytotoxic pteridine methotrexate. In refining the possible mechanisms of resistance to methotrexate, using *L. casei* MTX/R(DHFR+), an alteration in the reaction between the drug and dihydrofolate reductase has been excluded [21]. This has focussed attention on other possibilities, including the use of alternative substrates. Two possible oxidised substrates, folic acid and 10-formylfolic acid, were compared with the normal substrate, dihydrofolic acid.

The pH vs. activity profiles emphasise that relatively small changes in substrate markedly influence reduction. In the absence of KCl, dihydrofolate reductase from methotrexate-resistant *L. casei* MTX/R(DHFR+) has a broad pH optimum in the range 6.5—7.3 for reduction of dihydrofolic acid. An approximately neutral pH optimum is in agreement with results obtained using enzyme prepared from a wide variety of cells, tabulated by Blakley [11]. Dihydrofolate reductase from eukaryotic cells tends to have two pH optima, around 5.0 and 7.5, while that of prokaryotes tends to have a single pH optimum [11]. Gundersen et al. [22] found a single pH optimum for an *L. casei* reductase, the maximum occurring at pH 6.5. When folic acid is used as a substrate, the present enzyme has a pH optimum of 4.5. This result is in agreement with the work of Zakrzewski [23] and Mathews and Huennekens [16] who recorded pH optima of 4.8 and 4.2 respectively, for reductase from chicken liver.

The pH vs. activity profile for 10-formylfolic acid yielded no clear pH optimum in the range studied, an optimum apparently being below pH 4.0. There are no published details with which this might be compared.

In cellular terms, the observed range of pH optima are not only compatible with cell growth but could accomodate an altering folate metabolism of benefit to *L. casei.* This organism commences growth at around pH 6.8, consumption of the buffering capacity of the medium being associated with log-phase and termination of growth occurring at around pH 3.5.

Reaction rates and studies of substrate binding yield evidence supporting a possible utilisation of 10-formylfolic acid at the pteridine site of the enzyme. The relative reaction rates show that, even at pH 5.0, 10-formylfolic acid is reduced several times faster than folic acid (Table I). A consideration of the order of K_m values (Fig. 3a—3c) relates inversely the relative binding affinity of the substrates and their rates of reduction. In addition, during determination of K_m values for the three substrates, substrate inhibition was observed in all cases. Dihydrofolic acid was used to test whether this was due to competition between the folate derivative and NADPH (Fig. 4). It was found that dihydrofolic acid was uncompetitive with respect to NADPH, thereby extending the previous observation [12] that dihydrofolic acid and oxidised folate derivatives compete with methotrexate. Studies of inhibition of reduction of the three folate substrates by methotrexate (Fig. 2) confirm that they all share the common pteridine-binding site [24]. The present results can be interpreted to show that the dihydrofolate reductase from methotrexate-resistant *L. casei* has a non-sequential "ping-pong" reaction mechanism in which one substrate is

bound to, and one product released from, the enzyme before the second substrate is bound [18].

The use of oxidised folate compounds in mechanisms of resistance to methotrexate is possible only if their reduction is much less sensitive to inhibition by the drug. The present study clearly shows that reduction of the oxidised substrates is as susceptible to methotrexate as is reduction of dihydrofolic acid. It seems unlikely therefore that the cell overcomes the inhibiting effects of methotrexate simply by altering the pteridine substrate specificity of the wild-type enzyme.

Despite this, the surprising finding of a unique activation of 10-formylfolic acid reduction (Table II) enables an alternative substrate to underlie a mechanism of resistance. Activation is a complex phenomenon as dihydrofolate reductases are activated by salts [25], mercurials [26] and other thiol reagents [27]. Dihydrofolate reductase from *L. casei* MTX/R(DHFR+) has the advantage of being cysteine free [21], so the effect of different salts on the three reactions of the enzyme was investigated. In all the studies, 0.2 M salt was present in the assay mixture, as previous work has shown that alterations in the rate of reaction produced by salt will be apparent at this concentration [25, 28].

In the case of 10-formylfolic the pattern obtained is very similar to that described for reduction of dihydrofolic acid by the reductase from eukaryotic cells [11], implying that the cysteine-less enzyme is capable of ionic activation. As reduction of 10-formylfolic acid is markedly stimulated whilst that of folic and dihydrofolic acids is not, it seems likely that activation is due to an increased affinity of the enzyme for 10-formylfolic acid rather than an ion-induced facilitation of hydride transfer.

In terms of methotrexate resistance, however, the activated enzyme was as sensitive to the drug as the normal reductase. Despite this, activation of 10-formylfolic acid reduction provides a possible route of tetrahydrofolate production in resistant cells where inhibition of the reductase is incomplete. Such a model requires that the wild-type dihydrofolate reductase is unable to be stimulated by ions when reducing 10-formyfolic acid. This will be investigated when the wild-type enzyme has been purified to homogeneity.

Activation of reduction of 10-formylfolic acid is of interest not only as a potential mechanism of drug resistance but also from the point of view of an understanding of the catalytic action of the enzyme. Therefore a complete understanding of the significance of the activated enzyme requires both an investigation of the cellular pools of folate derivatives, and a more detailed kinetic study of the activated enzyme itself.

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