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Large-Scale Purification and Characterization of Dihydrofolate Reductase from a Methotrexate-Resistant Strain of *Lactobacillus casei*

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Dihydrofolate reductase has been purified from a methotrexate-resistant strain of *Lactobacillus casei* NCB 6375. By careful attention to growth conditions, up to 2.5g of enzyme is obtained from a 400 litre culture. The purification procedure, involving polyethyleneimine treatment, DEAE-cellulose chromatography and affinity chromatography on methotrexate-aminohexyl-Sepharose, operates on the gram scale, with overall yields of 50–60%. Elution of the affinity column by reverse (upward) flow was used, as it led to recovery of the enzyme in a much smaller volume. The enzyme obtained appears to be more than 98% pure, as judged by gel electrophoresis, isoelectric focusing, and gel filtration. It has a mol.wt. of approx. 17900 and a turnover number of $4\,s^{-1}$ (50mM-triethanolamine/400mM-KCl, pH7.2, 25°C) with dihydrofolate and NADPH as substrates. The turnover number for folate is $0.02\,s^{-1}$. Michaelis constants for a variety of substrates have been measured by using a new fluorimetric assay (0.36μM-dihydrofolate; 0.78μM-NADPH), and binding constants determined by using the quenching of protein fluorescence (dihydrofolate, $2.25\times10^{6}\,M^{-1}$; NADPH, $>10^{8}\,M^{-1}$). The pH/activity profile shows a single maximum at pH 7.3; at this pH, marked activation by 0.5M-NaCl is observed.

Dihydrofolate reductase (tetrahydrofolate–$NADP^+$ oxidoreductase, EC 1.5.1.3) is responsible for maintaining the intracellular pool of tetrahydrofolate by reducing dihydrofolate (arising either by biosynthesis *de novo* or by the action of thymidylate synthetase on 5,10-methylenetetrahydrofolate) to tetrahydrofolate (Blakley, 1969). This enzyme is of considerable pharmacological interest as the site of action of a group of powerful chemotherapeutic agents, the 'anti-folates', which includes methotrexate, trimethoprim and pyrimethamine (Blakley, 1969; Baker, 1967).

We are undertaking a detailed study of the binding of substrates, coenzymes and inhibitors to the dihydrofolate reductase from a methotrexate-resistant strain of *Lactobacillus casei*, principally by high-resolution n.m.r. (nuclear-magnetic-resonance) spectroscopy (Roberts *et al.*, 1974; Way *et al.*, 1975; Feeney *et al.*, 1975; Roberts, 1975). In common with a number of other bacterial and mammalian cells (see Blakley, 1969), resistance to methotrexate in *L. casei* is often accompanied by a marked increase in the production of dihydrofolate reductase, providing a convenient source of the large quantities of enzyme required for a detailed study by n.m.r. spectroscopy. As a consequence, an efficient purification procedure must be capable of operation on a large scale. In addition, for studies of selectively ^2H- and fluorine-labelled dihydrofolate reductase (J. Feeney, G. C. K. Roberts, B. Birdsall, D. V. Griffiths, B. Kimber, R. W. King, P. Scudder & A. S. V. Burgen, unpublished work), a good recovery of enzyme is essential, in view of the high cost of the labelled amino acids. In the present paper we describe a simple purification procedure which meets these criteria, and report some of the properties of the purified enzyme.

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Experimental

Materials

Sepharose 4B, Sephadex G-25 and Sephadex G-75 were products of Pharmacia Fine Chemicals Ltd., Uppsala, Sweden, and DE-23 and DE-52 diethyl-

aminoethylcellulose of Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Methotrexate (amethopterin) and aminopterin were obtained from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.; folate, H_2folate (dihydrofolate), NADPH, $NADP^+$ and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide from Sigma Chemical Co., St. Louis, MO, U.S.A.; 1,6-hexanediamine and polyethyleneimine from BDH Chemicals Ltd., Poole, Dorset, U.K., and trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] from Wellcome Research Laboratories, Beckenham, Kent, U.K. Other chemicals were of the highest purity commercially available and used without further purification.

[3′,5′,9(n)-^{3}H]Methotrexate was obtained from the Radiochemical Centre, Amersham, Bucks., U.K.

N^{10}-Formylfolate and N^{10}-formylaminopterin were synthesized from folate and aminopterin respectively by treatment with 98% formic acid, essentially by the method of Blakley (1959), and purified by chromatography on DEAE-cellulose DE-52, by using a gradient of 50mM–2M-ammonium bicarbonate, pH 8.0. The products were characterized by t.l.c. [cellulose; ammonia (sp.gr. 0.880)/t-butyl alcohol/water (1:1:8, by vol.)], n.m.r. and u.v. spectroscopy.

Synthesis of methotrexate-aminohexyl-Sepharose

Approximately 225g of Sepharose 4B was treated with 56g of CNBr in a total volume of 600ml of water maintained at pH 11.0±0.2 and temperatures below 20°C. After 10 min the activated Sepharose was filtered off, washed rapidly with 3 litres of ice-cold 0.1M-$NaHCO_3$, pH 10.0, and mixed immediately with a solution of 60g of 1,6-hexanediamine in 75ml of water, pH 10. The suspension was shaken at 4°C for 12h and then left for a further 24h at 4°C before washing with several litres of water, followed by dilute HCl (pH 3), until the washings were at pH 3. The aminohexyl content of the substituted Sepharose was estimated by potentiometric titration with KOH; various preparations had aminohexyl contents in the range 12–20μmol/g.

Coupling of methotrexate was carried out in a solution containing 110ml (settled volume) of aminohexyl-Sepharose, 827.5mg of methotrexate (representing approx. 50% excess over aminohexyl groups) and 2.5g of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide in a total volume of 260ml of water, pH 5.8. The suspension was protected from light, and shaken at 20°C for 7h, then left for a further 36h. Extensive washing was required to remove uncoupled methotrexate (see also Newbold & Harding, 1971; Kaufman, 1974); several washes with each of water, 1M-$NaHCO_3$, pH 8.5, and 4M-NaCl were used until the E_{302} of the supernatant was negligible.

The extent of coupling with methotrexate was estimated by a one-tenth scale reaction incorporating 50μCi of [^{3}H]methotrexate. Portions of all washings were counted for radioactivity, and the [^{3}H]methotrexate content of the resin determined by solubilizing 0.5ml samples in 10ml of 1M-HCl at 60°C for 24h, followed by liquid-scintillation counting in a standard Triton X-100 scintillation fluid. The average methotrexate content was 9μmol/ml of settled Sepharose. The [^{3}H]methotrexate column was also used to determine the rate of leakage of ligand from the column; a rate of 1–2% per month was measured at pH 6.5, though an appreciable increase was noted at pH 8.

Organism and culture conditions

The organism used was a methotrexate-resistant strain of *Lactobacillus casei* N.C.B. 6375 (Torrey Research Centre, Aberdeen, Scotland, U.K.), designated *L. casei* MTX/R, selected by growing the wild type on successively increasing concentrations of methotrexate (Newbold & Harding, 1971). The resistant strain is maintained in gelled medium in the presence of methotrexate.

The composition of the semi-defined medium used for growth on the 400 litre scale is shown in Table 1. The folate content of Difco 'vitamin-free' casein

Table 1. *Composition of semi-defined medium for the growth of Lactobacillus casei MTX/R*

Component	Composition (g/380 litres of medium)
Casein hydrolysate (charcoal-treated)	2000
K_2HPO_4	1000
KH_2PO_4	1000
Sodium acetate	2400
Glucose	8000
L-Asparagine	120
L-Cysteine	40
L-Tryptophan	30
Ascorbic acid	400
Guanine	4
Adenine sulphate	4
Xanthine	4
Uracil	4
$MgSO_4,7H_2O$	400
Ferrous ammonium sulphate	25
$MnSO_4,H_2O$	60
Thiamin hydrochloride	0.08
Biotin	0.004
Nicotinic acid	0.16
p-Aminobenzoic acid	0.4
Pyridoxine hydrochloride	0.8
Calcium pantothenate	0.16
Riboflavin	0.2
Folate	0.0024

hydrolysate was decreased to a low value (equivalent to <0.6ng/ml of medium) by autoclaving [30min, 103.35kPa (15lb/in²)] in the presence of activated wood-charcoal (40g of charcoal, 2kg of casein hydrolysate, 1kg of KH_2PO_4 in 40 litres of water, adjusted to pH4.9 with acetic acid).

Growth was initiated by adding 16 litres of inoculum to 380 litres of medium, adjusted to pH7.2, 37°C. The growth was monitored by turbidity (E_{600}), pH and enzyme assay (on sonicated samples). The pH was allowed to fall to 5.0, and then maintained at this value by automatic addition of 10M-KOH. After approximately 18h, when the turbidity and enzyme content showed signs of levelling off, the tank was cooled as rapidly as possible to 10°C (using coils circulated with a $CaCl_2$ solution at −18°C), and simultaneously a stabilizing solution, consisting of 200g of glucose, 0.5g of nicotinic acid and 0.5g of adenine sulphate in 2 litres of water, was added.

Cells were harvested by using a continuous-flow (1–2litres/min) Sharples centrifuge fitted with cooling coils circulating $CaCl_2$ solution at −18°C. Between 2.5 and 3.5kg of cells were obtained. The cells were resuspended in 6 litres of 50mM-Tris/HCl, pH7.4, 4°C, and passed six times through a Manton–Gaulin homogenizer at 51.7MPa (7500lb/in²). Immediately after each pass, the suspension was cooled (from about 20° to 4°C) and diluted with the 2 litres of cold Tris buffer used to wash through the homogenizer. The cell debris was removed by using the Sharples centrifuge.

Removal of DNA with polyethyleneimine

Polyethyleneimine was dialysed for 2 weeks against daily changes of water. The flocculent precipitate which formed in the bag was removed by vacuum filtration through a layer of Hyflo Supercel on Whatman no. 1 paper. The polyethyleneimine in the filtrate had an exchange capacity of 320 mequiv./litre.

The amount of this polyethyleneimine solution required to precipitate the nucleic acid (and some protein) without precipitating dihydrofolate reductase (approximately 80ml/litre of extract) was determined empirically on portions of each batch of cell extract. The required amount was diluted to 3 litres with water and added to the stirred ice-cold cell extract at a rate of 500ml/h. The dense precipitate formed was readily removed with the Sharples centrifuge, passing the slurry through at a rate of 20 litres/h.

$(NH_4)_2SO_4$ precipitation

$(NH_4)_2SO_4$ was added to a final concentration of 600g/litre and the suspension stirred overnight at 4°C to ensure complete precipitation. The precipitate was harvested by using the Sharples centrifuge and stored at −20°C.

DEAE-cellulose column chromatography

Whatman DE-23 DEAE-cellulose was pre-cycled with 0.5M-NaOH/0.25M-phosphoric acid, poured into a column (47cm×8cm; bed vol. 2100ml) and equilibrated with 25mM-potassium phosphate, pH 6.5, the column being operated at 4°C. Approximately half the $(NH_4)_2SO_4$ slurry from one fermentation (about 470g) was dissolved in 650ml of 200mM-potassium phosphate, pH6.5, centrifuged if necessary, and dialysed against five 4 litre batches of 25mM-potassium phosphate, pH6.5. The dialysed solution (about 1600ml) was pumped on to the column at 200ml/h, and the column eluted with a gradient formed from 2 litres of 25mM-potassium phosphate, pH6.5, and 2 litres of 100mM-NaCl/25mM-potassium phosphate, pH6.5, followed by the high-salt buffer alone. Fractions (23ml) were collected and monitored for conductivity, pH, E_{280} and enzyme activity. Enzyme-rich fractions were pooled, freeze-dried and stored at −20°C. After each run, the column was unpacked, recycled and re-poured.

Affinity chromatography

Methotrexate-aminohexyl-Sepharose (bed-vol. 100ml) in a column (60cm×1.6cm) with sinters at top and bottom, maintained at 4°C, was washed with 50mM-potassium phosphate/100mM-KCl, pH6.5, until the effluent E_{302} was less than 0.02. The enzyme from the DEAE-cellulose column was dissolved in about one-fifth of its original volume of water and pumped on to the affinity column at 80ml/h (downwards flow), followed by 2 bed-vol. of equilibrating buffer. The column was washed with about 4 bed-vol. of 50mM-potassium phosphate/2M-KCl, pH6.5, at 40ml/h. The flow was then reversed (to upward flow), and after about one-third of a bed volume of high-salt buffer had passed through, the enzyme was eluted with 50mM-Tris/HCl/1M-KCl, 2mM-folic acid, pH8.5. During elution of the enzyme, the column effluent was passed through the fibres of a hollow-fibre dialysis unit (Biofibre 50 Beaker, Bio-Rad Laboratories, Richmond, CA, U.S.A.). Around the outside of the fibres, two 4 litre batches of 10mM-Tris/HCl, pH8.5, were circulated at a rate of 1 litre/h. Fractions (23ml) of the column effluent were collected, and elution continued until the enzyme concentration fell below 0.5mg/ml, at which point the column was immediately re-equilibrated with 50mM-potassium phosphate/100mM-KCl, pH6.5. Enzyme-rich fractions were pooled and freeze-dried.

Sephadex G-25 chromatography

The remaining folate was removed, and the buffer changed to 10mM-potassium phosphate/100mM-

KCl, pH6.5, on a column (37cm×4.4cm) of Sephadex G-25. The enzyme from the affinity column was re-dissolved in 10–15% of its original volume (enzyme concn. <12mg/ml), pumped on to the column and eluted at 60ml/h. The enzyme was pooled in two fractions: (*a*) all fractions with E_{280} >2.0, and (*b*), all remaining fractions with E_{280} >0.1. The pools were freeze-dried and stored at −20°C.

Bio-Rex 9 ion-exchange chromatography

A column of Bio-Rex 9 (Bio-Rad) was equilibrated with 20mM-Bis-Tris [bis-(2-hydroxyethyl)iminotris-(hydroxymethyl)methane], 50mM-KCl, pH5.5. The freeze-dried enzyme from the Sephadex G-25 column was re-dissolved to a concentration of up to 0.5mM and the pH of the solution carefully adjusted to 5.5 with 0.1M-HCl. This solution was pumped on to the column, which was then eluted with the Bis-Tris buffer. The eluate was passed through a hollow-fibre dialyser circulated with either Tris or phosphate buffer (50mM, 500mM-KCl) to restore the pH to 6.5, and the salt concentration to 500mM-KCl. The low-molecular-weight impurity was subsequently eluted with Bis-Tris buffer, pH5.5, containing 1.0M-KCl.

Analytical gel filtration

Sephadex G-75 (superfine grade) was swollen in 10mM-potassium phosphate/100mM-NaCl, pH6.5, packed into a column (80cm×1.6cm; bed vol. 140ml) and pumped with several bed-vol. of the same buffer at 6ml/h, with a pump between the column and the fraction collector (dead vol. 1.0ml). Fractions were collected and weighed (average 1.5g). The void volume of the column, determined with Blue Dextran, was 35.9ml. Molecular-weight markers used were ovalbumin (mol.wt. 43000), human carbonic anhydrase C (30000), α-chymotrypsinogen (25666), horse heart myoglobin (16951), and pancreatic ribonuclease (13684). These, and dihydrofolate reductase, were applied as 1.0ml of a 2–3mg/ml solution in the above buffer.

Gel electrophoresis

Polyacrylamide gels (7.5 or 10%) were run in Tris/glycine, pH8.3, at 3mA/tube. Sodium dodecyl sulphate gels contained 0.1% sodium dodecyl sulphate and were run in 0.2M-sodium phosphate (pH7.0)/0.2% sodium dodecyl sulphate at 6mA/tube. In both cases staining was with Coomassie Brilliant Blue in acetic acid/methanol/water (1:6:6, by vol.), and de-staining with acetic acid/methanol/water (1.0:1.5:17.5, by vol.). Samples contained 2.5–10μg of protein in 5–10μl, together with 5μl of 0.05% Bromophenol Blue as tracking dye. For the sodium dodecyl sulphate gels, bovine serum albumin (mol.wt. 67000), myoglobin (16951) and lysozyme (14314) were used as molecular-weight markers, and were incubated in 10mM-sodium phosphate (pH7.0)/1% sodium dodecyl sulphate/1% 2-mercaptoethanol at 37°C for 2h before use. Dihydrofolate reductase was dialysed against 10mM-sodium phosphate (pH7.0)/0.1% sodium dodecyl sulphate. Separations were recorded photographically, or by scanning at 600nm (Pye Unicam SP.1800).

Isoelectric focusing

This was carried out with the LKB 8101 ampholine column (LKB-Producter A.B., Stockholm, Sweden), operated at 4°C. LKB Ampholine solutions giving pH ranges of 3–10, 4–8 and 5–7 were used, adding the enzyme (1.5ml of a 5mg/ml solution in 10mM-potassium phosphate/100mM-NaCl, pH6.5) at the middle of the gradient. Focusing started at 300V and without exceeding 5mA, this was increased to 600V over 12–18h, and left at 600V for a further 24–48h. The focused gradient was collected in 2ml fractions (at 0°C) and monitored for pH (within 30min), E_{280} and enzyme activity.

Fluorescence-quenching experiments

Protein fluorescence was measured with a Farrand spectrophotofluorimeter by using an excitation wavelength of 290nm, and detecting emission at 340 or 350nm.

A standard tryptophan solution was used to correct for light absorption by added ligands. Exposure of the enzyme to light was kept to a minimum. The maximal quenching of the enzyme fluorescence (after correction for absorption) at high ligand concentrations varied from 40% to 95% for different ligands. Given this value for the fluorescence at high ligand concentrations, F_∞, and the fluorescence of the enzyme alone, F_0, the concentration of the complex, [EL], at any ligand concentration could be calculated from

$$[EL]/E_T = (F_0 - F)/(F_0 - F_\infty)$$

where E_T is the total enzyme concentration. The binding constant was then calculated from the dependence of [EL] on ligand concentration, usually by the method of Scatchard (1949).

Since methotrexate binding is stoicheiometric under the conditions used (see Table 5), titration of the protein fluorescence with methotrexate provided an accurate measure of the protein concentration. These experiments were carried out either with the Farrand fluorimeter or with a Turner model 111 filter fluorimeter (with a 110-855 lamp and filters 7-54 and 110-815 to select an excitation band centred on 290nm, and a 110-811 filter to select an emission band centred on 360nm). The concentration of the

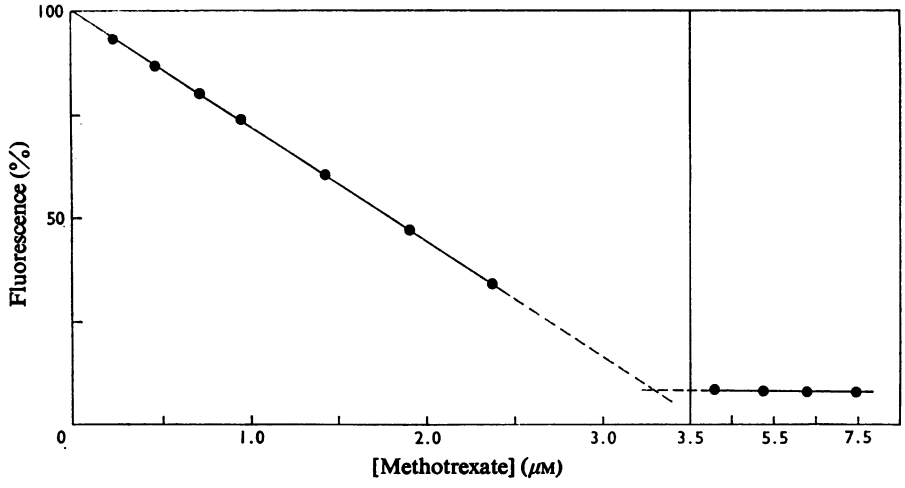

Fig. 1. *Determination of dihydrofolate reductase concentration by titration with methotrexate*

The fluorescence (excitation 290nm, emission 340nm) of a solution of dihydrofolate reductase in 50mM-bis-(2-hydroxyethyl)-iminotris(hydroxymethyl)methane/500mM-KCl, pH6.5, 25°C, is shown as a function of the concentration of methotrexate added. The point of intersection of the two straight lines gives the enzyme concentration.

methotrexate solution was determined from its absorbance at 302nm in 0.1M-KOH, by using an extinction coefficient of 22000 litre·mol^{-1}·cm^{-1} (Blakley, 1969). An example of such a methotrexate titration is shown in Fig. 1. Similar titrations could be performed following enzyme activity rather than protein fluorescence.

Enzyme assay

The standard assay used during the purification procedure was a spectrophotometric one in which the decrease in E_{340} accompanying the conversion of H_2folate+NADPH into H_4folate (tetrahydrofolate) +$NADP^+$ was followed. The assay was run at 25.0° ±0.5°C, in 3.15ml of 50mM-Tris/HCl/500mM-KCl, pH7.5, with 50μM-H_2folate and 50μM-NADPH and the reaction followed by using a Pye–Unicam SP. 1800 spectrophotometer. The difference extinction coefficient for the reaction was taken as 12300 litre·mol^{-1}·cm^{-1} (Hillcoat *et al.*, 1967), and the unit of enzyme activity was defined as the amount required to reduce 1μmol of H_2folate/min under the specified conditions.

For the kinetic studies a variety of buffers was used, details of which are given in the Results and Discussion section; all assays were carried out at 25°C. Measurements of maximal velocity were conveniently made with the spectrophotometric assay, but to measure K_m values (0.3–1μM; see Table 4) a recording fluorimetric assay was developed. This was performed with a modified Turner model 111 filter fluorimeter, with a 110-850 lamp and 7-54 filter to give an excitation band centred on 360nm, and a 110-816 filter (giving a sharp cut-off below 415 nm) on the emission side. Under these conditions, both the oxidation of NADPH and the reduction of H_2folate contribute to the observed fluorescence change; the assay was calibrated by determining the total change in fluorescence on completion of the reaction at various concentrations of NADPH and H_2folate. In a typical assay mixture, with [NADPH]/[H_2folate] = 3, the fluorescence was linear with concentration up to 12μM-NADPH+4μM-H_2folate (approx. ten times their respective K_m values). Measurements were possible down to substrate concentrations of 0.1μM.

For K_m determinations, the velocity was measured (at least in triplicate) at five to six concentrations of one substrate, while the concentration of the other substrate was held at a value at least four and usually ten times its K_m value (the concentrations used are given in Table 4). The K_m values were obtained by a linear least-square fit to the plot of [S]/v against [S].

Results and Discussion

Isolation and purification of the enzyme

The large increase (approx. 100-fold) in the production of dihydrofolate reductase which accompanies methotrexate resistance in *L. casei* allows up to 2.5g of enzyme to be obtained from a 400 litre culture (2.5–3.5kg wet wt. of cells). For maximum

Table 2. *Purification of dihydrofolate reductase from extracts of Lactobacillus casei MTX/R*

	Volume (ml)	Total protein (E_{280}^{1cm} × vol.)	Total activity (units)	Total activity (mg)	Specific activity (units/E_{280})	Cumulative yield (%)
Polyethyleneimine supernatant	14 000	—	27 000	2570	—	(100)
$(NH_4)_2SO_4$ precipitate (re-dissolved and dialysed)	2060	40 330	20 000	1900	0.50	74
DEAE-cellulose chromatography*	2900	4390	18 270	1740	4.16	68
Affinity chromatography*	1140	—†	14 630†	—	—†	—†
Sephadex G-25 chromatography*	288	1746	16 485	1570	9.44	61

* Sum of two runs [the $(NH_4)_2SO_4$ precipitate was divided into two for further purification].

† Owing to the presence of folate, the E_{280} is not a useful measure of protein concentration, and the measured activity is somewhat low.

yield of enzyme the folate concentration in the medium must be carefully controlled (Ohara & Silber, 1969) so that folate-depleted casein hydrolysate must be used, and the pH must not be allowed to fall below 5. As the growth curve reaches the end of the exponential phase, rapid cooling and the addition of the 'enzyme-stabilizing solution' are essential to avoid a loss of up to 90% of the dihydrofolate reductase activity over a period of 2–3h. Similar, though less precipitous, decreases in dihydrofolate reductase activity in the stationary phase have been noted in another strain of *L. casei* (Ohara & Silber, 1969), and in *Streptococcus faecalis* (Nixon & Blakley, 1968), *Streptococcus thermophilus* and *Lactobacillus plantarum* (Nurmikko *et al.*, 1965) and *Escherichia coli* (Poe *et al.*, 1972).

The use of affinity chromatography for the purification of dihydrofolate reductase has become widespread; detailed descriptions have been given by Pastore *et al.* (1974) and by Kaufman (1974), who also listed references to the use of affinity chromatography for the purification of this enzyme.

The extract obtained after breaking the cells has a high viscosity, owing to the presence of large quantities of nucleic acids. In early experiments, the viscosity was decreased by addition of deoxyribonuclease, but the dihydrofolate reductase obtained from these experiments showed an absorbance maximum at 260nm, suggesting the presence of significant amounts of bound oligonucleotides. This problem was overcome by the use of polyethyleneimine to precipitate the nucleic acid (Atkinson & Jack, 1973), though careful control of the amount of polyethyleneimine added is necessary to avoid the precipitation of too much of the dihydrofolate reductase. The subsequent precipitation with $(NH_4)_2SO_4$ afforded some purification, but served primarily as a convenient means of concentrating the enzyme from the large volume of cell extract. The data for this and the subsequent steps in the purification are given in Table 2. The chromatographic steps were carried out with half the ammonium sulphate precipitate from one fermentation (i.e. about 0.8g of enzyme) at one time; the quantities given in Table 2 are thus the sum of two chromatographic runs.

The elution profile of the DEAE-cellulose column is shown in Fig. 2. For the experiment shown in Fig. 2, a somewhat smaller DEAE-cellulose column was used and elution was continued to 500mM-NaCl/25mM-potassium phosphate, to elute essentially all the protein. For routine large-scale purification the gradient was stopped at approximately 100mM-NaCl/25mM-potassium phosphate, after the dihydrofolate reductase had been eluted, and the column material was recycled and repacked as described in the Experimental section. Fig. 2 shows that dihydrofolate reductase elutes before the majority of the other proteins, and seven- to eight-fold purification is achieved with excellent (~90%) recovery. The purification achieved at this stage was somewhat variable, depending on the amount of protein which had been removed by polyethyleneimine precipitation. This purification step markedly increases the efficiency of the subsequent affinity-chromatography step. Removal of a large proportion of the unwanted protein allows the use of considerably larger loads on the methotrexate column, and proteins other than dihydrofolate reductase which bind to the methotrexate column (Newbold & Harding, 1971; Poe *et al.*, 1972; Kaufman, 1974) seem to be largely removed on the DEAE-cellulose column. For example, thymidylate synthetase and methylenetetrahydrofolate dehydrogenase are separated from dihydrofolate reductase on this column (N. G. L. Harding R. W. King & P. C. Turner, unpublished results).

The concentrated enzyme-rich fractions from the DEAE-cellulose column were purified to homogeneity with a methotrexate-aminohexyl-Sepharose column; the elution profile is shown in Fig. 3. Since 50–80% of the amino groups on the column were substituted with methotrexate (see under 'Methods'), the ion-exchange behaviour of the column was

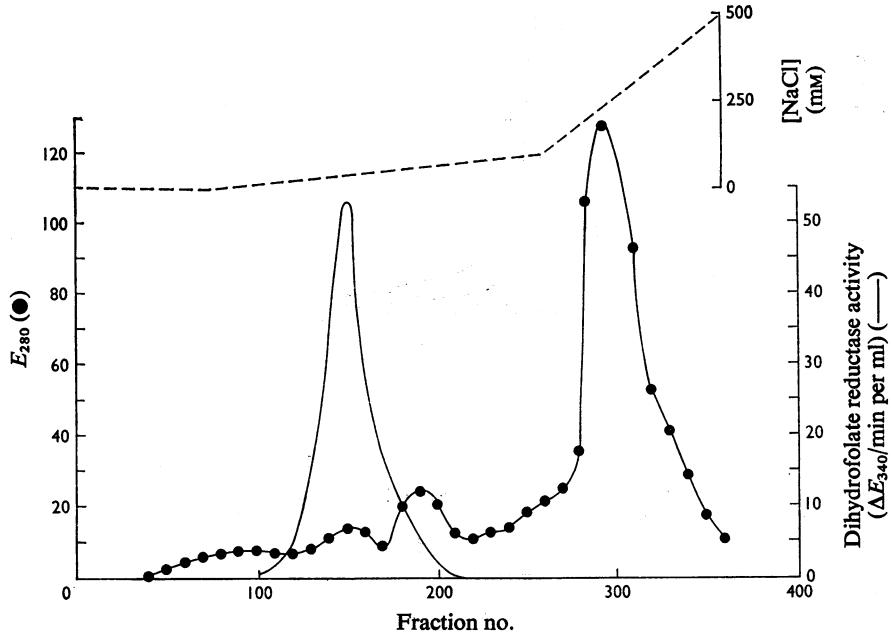

Fig. 2. *DEAE-cellulose chromatography of L.casei dihydrofolate reductase*

The $(NH_4)_2SO_4$ precipitate was re-dissolved and dialysed against 25mM-sodium phosphate, pH6.5, as described in the Experimental section. After pumping on the load, the column was eluted with a two-stage gradient of increasing salt concentration. The first stage was formed from 2 litres of 25mM-potassium phosphate, pH6.5, and 2 litres of 100mM-NaCl/25mM-phosphate, pH 6.5, whereas the second stage was formed from 1 litre of 100mM-NaCl and 1 litre of 500mM-NaCl in the same buffer. Fractions were monitored for E_{280} (●), dihydrofolate reductase activity (——) and conductivity (----) (expressed as NaCl concentration).

minimal, and contaminating proteins were readily removed by washing with a high-salt buffer. Dihydrofolate reductase was then eluted by a high-salt buffer, pH8.5, containing the competing ligand folate. Once it had been established that the enzyme obtained in this way is essentially pure (see below), the protocol for the affinity column was modified so that the enzyme was eluted by reverse (upward) flow. Since the affinity column is being used at much less than its theoretical capacity, this procedure has the advantage that the enzyme is obtained in a volume of buffer less than half of that obtained with downward-flow elution, as illustrated in the inset of Fig. 3. Reverse-flow elution may be a generally useful procedure for minimizing the pronounced tailing of the eluted enzyme peak which is often observed in affinity chromatography with mild elution conditions.

The in-line hollow-fibre dialysis of the eluate from the affinity column was found to remove 94% of the salt and up to 80% of the folate. The remainder of the folate was removed on a column of Sephadex G-25. Folate itself was completely removed in this way (see below), since it tends to bind to Sephadex and elutes at 2–2.5 column-volumes, being well separated from the enzyme peak. However, all batches of folate tested contain small amounts (~2%) of u.v.-absorbing impurities which elute much closer to the enzyme peak, and the load volume of the Sephadex G-25 column must be kept fairly small (~60ml for the size of column used here) if contamination with these impurities is to be avoided. Despite these precautions, some batches of enzyme have been found to be contaminated to the extent of up to 0.4mol/mol of enzyme with a folate-like compound which is reducible by NADPH. This can readily be removed by passing the enzyme solution at pH5.5 down a column of Bio-Rex 9 anion-exchange resin. The of enzyme passes straight through the column and is recovered quantitatively, whereas the inhibitor is adsorbed to the column and can be eluted by 1.0M-KCl. This treatment leads to complete removal of the NADPH-reducible impurity.

The purified enzyme is freeze-dried and stored at −20°C; in this state it is stable for several months. It is also stable in solution (at pH6.5) at 4°C for a few

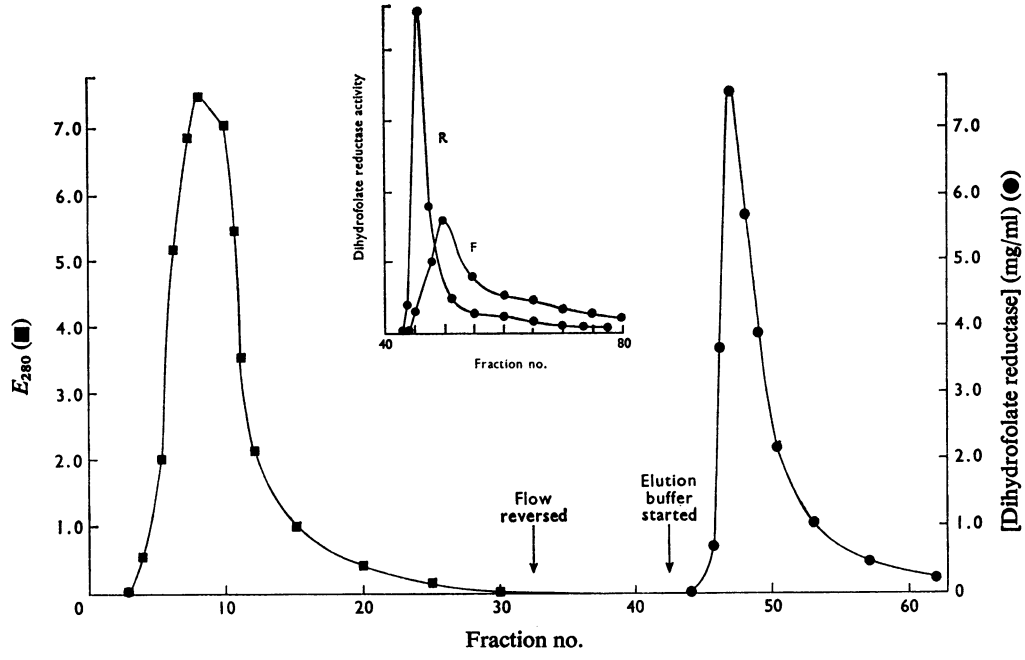

Fig. 3. *Affinity chromatography of L. casei dihydrofolate reductase*

The active fractions from the DEAE-cellulose column were applied to a methotrexate-aminohexyl-Sepharose column (see the Experimental section). The column was washed with 50mM-potassium phosphate/2M-KCl, pH6.5, fractions being monitored for E_{280} (■) and activity (undetectable). The flow was then reversed and the enzyme eluted with 50mM-Tris/1M-KCl, pH8.5, containing 2mM-folate. Fractions were monitored for activity, expressed as enzyme concentration (●). (Owing to the presence of folate, all elution fractions had $E_{280} > 10$.) The inset compares the elution of enzyme by forward (F) or reverse (R) flow.

days, but frozen solutions are not stable for long periods.

In many of the previous reports of purification of dihydrofolate reductase by affinity chromatography, the column material used was methotrexate-aminoethyl-Sepharose (Nakamura & Littlefield, 1972; Poe *et al.*, 1972; Chello *et al.*, 1972; Gauldie & Hillcoat, 1972; Gauldie *et al.*, 1973). In common with Whiteley *et al.* (1972) and Kaufman (1974), we have used an aminohexyl 'spacer', since we find that this gives significantly better recoveries of the *L. casei* enzyme (Dann *et al.*, 1972). Pastore *et al.*, (1974) used a pteroyl-lysine-Sepharose, and Erickson & Mathews (1971) an N^{10}-formylaminopterin-aminoethyl-Bio-Gel P-10 column, since the weaker binding of the enzyme by these ligands makes for easier elution. In earlier attempts to devise an affinity column from which the enzyme could be eluted without the use of a competing ligand, we prepared N^{10}-formylaminopterin-aminohexyl-Sepharose. *L. casei* dihydrofolate reductase can be eluted from this column by a high-salt buffer at pH10. However, the column lost a large fraction of its capacity to bind the enzyme over a period of several weeks, apparently owing to release of the pteridine from the matrix. Further, the *L. casei* enzyme is rather unstable at high pH (Newbold & Harding, 1971; Dann, 1975).

By contrast, the methotrexate column has been in routine use for over 2 years with no decrease in performance. The very slow leakage of methotrexate from the column does not affect its performance (at the low load/capacity ratio used) provided that the column is thoroughly washed immediately before each use. Elution with folate and subsequent removal of the folate by in-line dialysis and gel filtration gives a recovery of 90% for the affinity-chromatography and gel-filtration steps together. The procedures described yield pure enzyme (see below) in quantities of 800–1000mg per run, with overall yields of 50–60%. This is a considerable improvement on many of the previously published methods of purification of dihydrofolate reductase, and provides ample material for a detailed study of the properties of this important enzyme.

Purity and molecular weight

A densitometric scan of a sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of 10 μg of the purified dihydrofolate reductase is shown

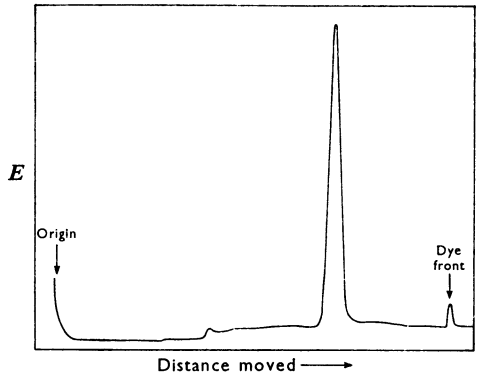

Fig. 4. *Spectrophotometric scan of a sodium dodecyl sulphate/polyacrylamide-gel electrophoretogram of purified dihydrofolate reductase* (10μg), *stained with Coomassie Blue*

Details are given in the Experimental section.

in Fig. 4. Only a single sharp band of R_F 0.72±0.02 ($n = 5$) is observed, indicating the high degree of purity of the preparation. Calibration with molecular-weight markers gave an excellent linear relationship between R_F and log (molecular weight), and a mol.wt. of 18000±800 was calculated for the dihydrofolate reductase. Measurements with a calibrated Sephadex G-75 column gave a closely similar value, namely 17900±400. On the Sephadex column a single symmetrical peak was observed, with constant specific activity across the peak. Samples of enzyme which were left for several days showed up to 10% of the absorbance eluting at the void volume, with about 1% of the activity eluting at a position corresponding to the dimer. Evidence for the aggregation of up to 10% of the material was also obtained in a sedimentation-equilibrium experiment (kindly performed by Dr. P. A. Charlwood) at a protein concentration of 3mg/ml. This experiment gave a mol.wt. of 17800 for the major component; a value of 0.73 ml/g for the partial specific volume was used.

Thus we conclude that the dihydrofolate reductase isolated from our strain of methotrexate-resistant *L. casei* has a mol.wt. of approximately 18000. Gundersen *et al.* (1972) have reported the purification of dihydrofolate reductase from a methotrexate-resistant strain of *L. casei* A.T.C.C. 7469 (Dunlap *et al.*, 1971*a*), which has a mol.wt. of approx. 15000, determined by three methods. This difference, though relatively small, is probably real; on both sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel filtration, the enzyme described here reproducibly showed a molecular weight somewhat greater than that of myoglobin. In addition, the two enzymes differ in their activity towards folate (see below). It seems likely that these differences can be attributed to the different strains of bacteria used; similar differences have been noted for the dihydrofolate reductases from different strains of *E. coli* (Mathews & Sutherland, 1965; Burchall & Hitchings, 1965; Burchall & Chan, 1969; Poe *et al.*, 1972; Erickson & Mathews, 1973) and *S. faecalis* (see, for example, Albrecht & Hutchinson, 1969; D'Souza *et al.*, 1972).

Polyacrylamide-gel electrophoresis (in the absence of sodium dodecyl sulphate) of 20μg of the purified enzyme gave a single major, rather diffuse, band (R_F~0.46). Addition of methotrexate and NADPH converted this into a sharp band at $R_F = 0.83$. In both cases, very faint bands were observed at R_F values of 0.32 and 0.37; densitometric scanning indicated that together they constituted less than 1% of the total protein. Both Gundersen *et al.* (1972) and Newbold & Harding (1971) noted that *L. casei* dihydrofolate reductase was obtained in two forms of different electrophoretic mobility which were separable by ion-exchange chromatography. Dunlap *et al.* (1971*b*) showed that these 'two forms' corresponded to the enzyme with and without one equivalent of tightly bound NADPH. Since no such separation into 'two forms' has been observed in the present work, it seems likely that the bound NADPH was removed during the purification, most probably on the DEAE-cellulose column. D'Souza *et al.* (1972) have noted the conversion of 'form 1' to 'form 2' on hydroxyapatite chromatography. Whiteley *et al.* (1972) suggested that the enzyme–NADPH complex bound too tightly to the affinity column to be eluted with folate. In view of the virtually quantitative yields obtained from the methotrexate column, it appears that this is not the case in the present work.

The results of an isoelectric-focusing experiment with a pH gradient from 4–8 are shown in Fig. 5. A symmetrical peak of E_{280} and activity is obtained with indications of a very small amount (<1%) of an impurity at fraction 38. Similar results were obtained with pH gradients of 3–10 and 5–7; the isoelectric point is calculated to be 6.25±0.1 (2°C). This can be compared with the values for dihydrofolate reductase from other sources measured by Kaufman (1971), who noted that the enzyme from bacterial sources had markedly lower isoelectric points than that from eukaryotic organisms (about 5.5 as against 8.3–9.3). The present value for *L. casei*, although significantly higher than those reported by Kaufman (1971) for other bacteria, is still much lower than the values he gives for eukaryotic organisms, though Baumann & Wilson (1975) have reported an isoelectric point of 5.94 for the minor component of the bovine liver enzyme.

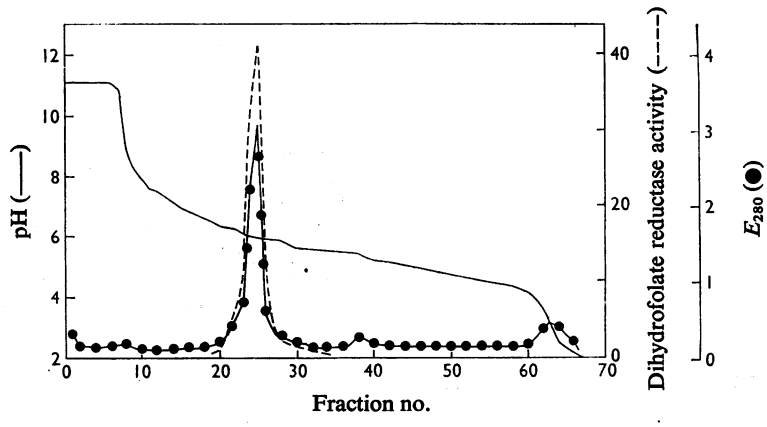

Fig. 5. *Isoelectric focusing of purified L. casei dihydrofolate reductase*

A pH 4–8 gradient was used as described in the Experimental section; fractions were monitored for E_{280} (●), pH (——) and enzyme activity (----).

Table 3. *Amino acid composition of L. casei dihydrofolate reductase*

Compositions were calculated from analyses of 12, 24 and 72 h hydrolyses (extrapolating to zero time where appropriate), assuming a mol.wt. of approx. 18000 [indicated by the method of Delaage (1968)].

Amino acid	Composition (residues/mol)
Ala	14
Arg	8
Asp+Asn	16
Cys	0
Glu+Gln	16
Gly	9
His	6
Ile	5
Leu	13
Lys	8
Met	2
Phe	8
Pro	7
Ser	4
Thr	12
Trp*	5
Tyr	5
Val	15
	Total 153

* Determined by the method of Goodwin & Morton (1946). Five peptides staining for Trp are observed on a tryptic 'map' (K. Hood, unpublished work).

Amino acid analysis

The amino acid composition of the purified dihydrofolate reductase is given in Table 3. A notable feature is the absence of cysteine, which is also absent from the *L. casei* dihydrofolate reductase of Gundersen *et al.* (1972). The presence of six histidine and five tyrosine residues per mol has been confirmed by n.m.r. spectroscopy (Roberts *et al.*, 1974; J. Feeney, G. C. K. Roberts, B. Birdsall, D. V. Griffiths, R. W. King, P. Scudder & A. S. V. Burgen, unpublished work). The mol.wt. calculated from the amino acid composition is 17448, consistent with the values obtained from the measurements described above.

U.v. spectrum

In 50mM-Tris/HCl/100mM-KCl/1% lactose, pH 7.0, 25°C, the u.v. spectrum shows a maximum at 280.5nm, with a pronounced shoulder at 290nm, and essentially zero absorbance above 310nm. From this spectrum we can place an upper limit of 1% on the relative concentration of two of the most likely low molecular-weight impurities, folate and NADPH. By using methotrexate titration to determine the protein concentration, a molar extinction coefficient of 27200 litre·mol^{-1}·cm^{-1} at 280.5nm is obtained.

Kinetic properties

A detailed analysis of the steady-state kinetics of *L. casei* dihydrofolate reductase is hampered by the non-linear progress curves for the reaction observed under some conditions. These effects, whose origin is not yet fully understood, are described in detail by Dann (1975). Only the general kinetic properties of the enzyme will be described here.

The K_m values for NADPH and H_2folate did not vary appreciably in a variety of buffers {Tris, triethanolamine, Mes [2-(*N*-morpholino)ethanesulphonic acid]} with salt concentrations of 100–500mM-KCl or NaCl and pH7.0–7.5; representative

Table 4. *Steady-state kinetic parameters of L. casei dihydrofolate reductase*

Abbreviations: TEA, triethanolamine; Mes, 2-(*N*-morpholino)ethanesulphonic acid.

Substrate	Second substrate (concentration*, μM)	K_m(μM)	$k_{cat.}$(s^{-1})	Conditions
H_2folate	NADPH(3.2)	0.36±0.08	4.0±0.5	50mM-TEA, 400mM KCl, pH 7.2
		0.5		20mM-Tris, 2M-NaCl, pH 7.3
NADPH	H_2folate(6.0)	0.78±0.08		50mM-TEA, 100mM-KCl, pH 7.0
		16.6		20mM-Tris, 2M-NaCl, pH 7.3
Folate	NADPH(3.7)	6.3 ±0.5	0.02	50mM-TEA, 100mM-KCl, pH 7.0
NADH	H_2folate(50)	136 ±12	0.3	50mM-TEA, 400mM-KCl, pH 7.2
N^{10}-Formylfolate	NADPH(50)	4.1 ±0.9	0.07	50mM-Mes, 100mM-KCl, pH 7.2

* Concentration used in determination of K_m for substrate in column 1.

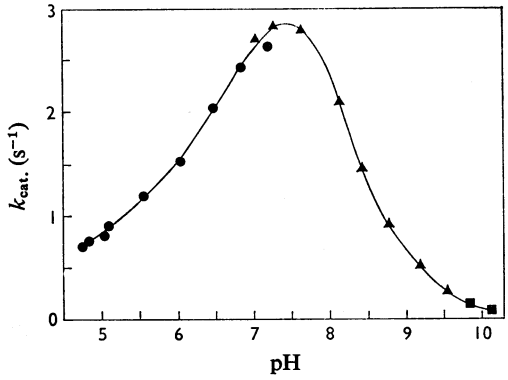

Fig. 6. *pH-dependence of the activity of L. casei dihydrofolate reductase*

Enzyme activity was measured by using the standard spectrophotometric assay, at 25°C, in buffer (20mM) 100mM-NaCl. Buffers used were 2-(*N*-morpholino)-ethanesulphonic acid (●); Tris/HCl (▲); and glycine (■).

values are given in Table 4, together with those for a number of alternative substrates. As has been observed for dihydrofolate reductase from other sources (see Blakley, 1969), the K_m values for the natural substrates are very low [Gundersen *et al.* (1972) report values of <1 μM for both NADPH and H_2folate]. It is noteworthy that the K_m for NADH is more than 100-fold greater than for NADPH, suggesting a marked effect of the 2′-phosphate group, for which there is other evidence [see below, and Feeney *et al.* (1975)]. Under optimal conditions, a $k_{cat.}$ (turnover number) of $4s^{-1}$ is obtained (Table 4), by using methotrexate titration to determine the enzyme concentration. The other substrates have markedly lower values of $k_{cat.}$ under comparable conditions, that for folate being approximately 1% of that for H_2folate. Gundersen *et al.* (1972) could detect no reduction of folate by their *L. casei* enzyme at neutral pH, although they found about 1% activity at low pH. N^{10}-Formylfolate, usually regarded as an inhibitor of dihydrofolate reductase (d'Urso-Scott *et al.*, 1974) is in fact a better substrate than folate for the *L. casei* enzyme; it is also a substrate for the chicken liver enzyme (Mathews & Huennekens, 1963).

Fig. 6 shows the pH-dependence of $k_{cat.}$ at a salt concentration of 0.1M; a single maximum is observed at about pH 7.3. Other bacterial dihydrofolate reductases also show a single pH optimum (Blakley, 1969; D'Souza *et al.*, 1972; Gundersen *et al.*, 1972; Poe *et al.*, 1972), though usually in the range pH 6.0–6.5. By contrast, the enzyme from mammalian sources, when examined at moderate ionic strength, usually shows optimum activity at pH 4.5–5.5, with a shoulder or subsidiary peak in the activity curve at neutral pH (Blakley, 1969; Rowe & Russel, 1973; Nakamura & Littlefield, 1972; Jarabak & Bachur, 1971). The alkaline limb of the pH/activity curve shown in Fig. 6 approximates to the shape expected for a single titrating group of pK~8.5. The acid side of the pH/activity curve is more complex; it appears that the maximal velocity may reach a plateau at low pH. This could not be confirmed directly, since the instability of the enzyme and, particularly, of NADPH made it impossible to obtain reliable initial velocity measurements below pH 4.75.

The maximal velocity with the natural substrates (NADPH and H_2folate) is markedly dependent on salt concentration; data for two pH values are shown in Fig. 7. A marked activation by salt, reaching a maximum at about 500mM-NaCl, is observed at pH 7.35, but not at pH 8.7; although detailed studies have not been made, it is apparent from Fig. 6 and Fig. 7 that the form of the pH/activity curve will be markedly dependent on salt concentration. No activation by salt was observed with the substrate pairs NADPH/folate or NADH/H_2folate. Activation by salt at 0.4–0.6M, accompanied by an alteration in the

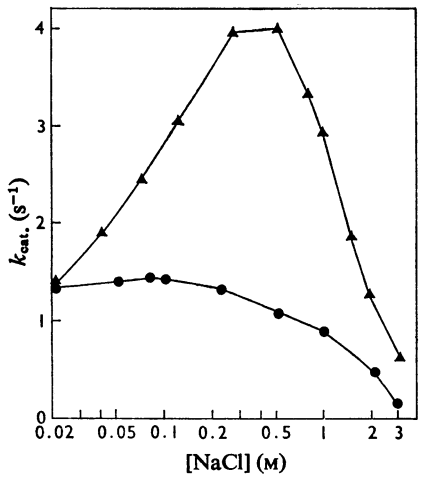

Fig. 7. *Dependence of the activity of L. casei dihydrofolate reductase on salt concentration at pH* 7.35 (▲) *and pH* 8.7 (●)

The assay buffer was 20mM-Tris/HCl, with the indicated concentration of NaCl.

pH/activity curve to a single optimum at pH 6.0–6.5, is generally observed for dihydrofolate reductase from mammalian sources (Blakley, 1969; Gauldie *et al.*, 1973; Rowe & Russel, 1973), but not for the bacterial enzyme. Thus the *E. coli* enzyme, for example, shows only a decrease in activity on increasing the salt concentration above 50mM (Poe *et al.*, 1972; Erickson & Mathews, 1973), although Nixon & Blakley (1968) reported a slight activation by 0.2M-KCl for the enzyme from *S. faecium*. Dihydrofolate reductase from mammalian sources is also activated by urea (2–4M) and by thiol reagents, notably mercurials (Blakley, 1969; Perkins & Bertino, 1965; Kaufman, 1968; Rowe & Russel, 1973). The absence of cysteine presumably precludes any effects of thiol reagents on the *L. casei* enzyme (though this has not yet been tested), and any activation by urea is very small [~10%; comparable to the results of Gundersen *et al.* (1972)]. It is noteworthy that Kaufman (1968) reported that activation by urea and related compounds was much less marked with the substrate pairs NADPH/folate and NADH/H_2folate. It is possible that the activation, at least by salt, is in part due to relief of substrate inhibition (Rowe & Russel, 1973; Dann, 1975).

Thus with the possible exception of its activation by salt, the kinetic properties of the dihydrofolate reductase from *L. casei* are typical of the enzyme isolated from other bacterial species.

Binding of substrates and inhibitors

The formation of binary complexes between the enzyme and a variety of substrates and inhibitors can readily be followed by the accompanying quenching of the protein fluorescence. Table 5 gives values for the equilibrium constants for some of the binary complexes determined in this way.

Table 5. *Binding constants for ligands to L. casei dihydrofolate reductase* (50 *mM-triethanolamine*/100 *mM-KCl, pH* 7.0, 25°*C*)

Values in parentheses are $1/K_i$ from steady-state kinetics.

Ligand	K_a (M^{-1})
NADPH	$>10^8$
$NADP^+$	2.1×10^5 (5.5×10^5)
NAD^+	(~20)
H_2folate	2.25×10^6
Folate	5.1×10^4
Methotrexate	$>10^8$
N^{10}-Formylaminopterin	1.4×10^7
Trimethoprim	3.2×10^6

The lowest concentration of enzyme at which measurements could conveniently be made was 10^{-7}M, and at this concentration the binding of methotrexate and of NADPH was stoicheiometric, so that only lower limits to their binding constants could be obtained. Measurements of the dissociation rate constant of the enzyme–methotrexate complex (A. S. V. Burgen, unpublished results), combined with an assumed value for the diffusion-limited association rate constant of $10^8 M^{-1}\cdot s^{-1}$, lead to an estimate of the equilibrium constant of the order of $10^{11} M^{-1}$. Methotrexate thus binds to the enzyme at least three and probably six orders of magnitude more tightly than does folate.

The K_i values for the products $NADP^+$ and NAD^+ (shown in Table 5 as $1/K_i$) were determined with H_2folate and NADH as substrates. The initial velocity was measured at three different NADH concentrations for each of four $NADP^+$ concentrations (0–6.4μM), the concentration of H_2folate being held at 48μM. The observed pattern was characteristic of competitive inhibition by $NADP^+$ with respect to NADH, and an apparent K_i value of $1.8\pm0.2\mu$M was calculated. Only about 20% inhibition was observed even at 5mM-NAD^+, and an approximate K_i value of 50mM was obtained, assuming competitive inhibition. The marked difference in the K_i values for $NADP^+$ and NAD^+, more than four orders of magnitude, further illustrates the important role of the 2′-phosphate group in coenzyme binding.

The binding constant for H_2folate to the *L. casei* dihydrofolate reductase given in Table 5 is comparable with the values found for the enzyme from *E. coli* ($2.4\times10^6 M^{-1}$, Erickson & Mathews, 1973; $9\times10^5 M^{-1}$, Greenfield *et al.*, 1972), T_4 phage ($2.0\times10^6 M^{-1}$; Erickson & Mathews, 1973) and L1210 lymphoma ($5.0\times10^6 M^{-1}$; Perkins & Bertino, 1966). NADPH

appears to bind appreciably more tightly to the *L. casei* enzyme than to these other enzymes, for which binding constants in the range 1.3×10^{6}–2×10^{7} M^{-1} were reported. Williams *et al.* (1973) and Poe *et al.* (1974) reported the existence of two binding sites for coenzyme on the enzyme from their strain of *E. coli*; no evidence for such a phenomenon in the *L. casei* enzyme has been found, either in the fluorescence-quenching experiments or by n.m.r. spectroscopy (Roberts *et al.*, 1974; Way *et al.*, 1975; Feeney *et al.*, 1975).

Some of the material in this paper is taken from a thesis submitted to the University of Cambridge in partial fulfilment of the requirements for the Ph.D. degree by J. G. D., who was supported by a scholarship from the Medical Research Council. We are most grateful to Mr. A. C. Young and Mr. W. A. Marshment for their invaluable assistance with the large-scale fermentations.

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