Biochem. J. (1971) **124**, 1–12
Printed in Great Britain

Affinity Chromatography of Dihydrofolate Reductase

BY P. C. H. NEWBOLD
Division of Dermatology, Scripps Clinic and Research Foundation, La Jolla, Calif. 92037, U.S.A.
AND N. G. L. HARDING
Postgraduate Medical School, Hills Road, Cambridge CB2 1QT, U.K.

(*Received* 13 *January* 1971)

1. Dihydrofolate reductase was purified from *Lactobacillus casei* MTX/R, and studied on affinity columns containing folic acid and methotrexate. Two forms of the enzyme were interconverted by incubation with substrates. 2. Affinity columns were prepared from agarose activated with cyanogen bromide and coupled with 1,6-diaminohexane. Stable folate derivatives were covalently attached by using a carbodi-imide condensation. 3. Columns containing folic acid retarded but did not retain the enzyme. 4. Methotrexate at pH 6.0 was particularly effective for retention of the enzyme. 5. There is selective loss of one form of the enzyme during affinity chromatography in the absence of added NADPH. This loss is due to conversion into a single enzyme form on the column. 6. NADPH has a dual effect in stabilizing the enzyme and in sensitizing it to inactivation by methotrexate, particularly in the presence of glycine. 7. Protein with affinity for methotrexate, but without dihydrofolate reductase activity, may also be eluted from the columns. 8. In a single-step procedure the enzyme was purified nearly 4000-fold from mammalian skin.

When cells of the mouse leukaemia L1210 become resistant to methotrexate (2,4-diamino-10-methylpteroyl-L-glutamate) there occurs increased activity of each of two forms of intracellular dihydrofolate reductase (Harding, Martelli & Huennekens, 1970). Both enzyme forms are powerfully inhibited by methotrexate (K_i about 10^{-8} M). In *Lactobacillus casei* increased activity of thymidylate synthetase is also seen (Dunlap, Harding & Huennekens, 1971), and this enzyme functions in concert with dihydrofolate reductase. In view of the ninefold increase in turnover time in active psoriatic plaques, and the enhanced thymidine incorporation in these lesions (Van Scott & Ekel, 1963), the observed increase in dihydrofolate reductase activity in these plaques (Grignani, Martelli, Tonato & Finzi, 1967) is apparently integrated with the alterations in cell kinetics and DNA turnover. Inhibition of the reductase provides a rationale for the effective use of methotrexate in psoriasis (Rees, Bennett, Maibach & Arnold, 1967) and in certain acute leukaemias (reviewed by Martelli, Tonato, Harding, Larizza & Grignani, 1969), although the properties of pure dihydrofolate reductase from human skin and leucocytes have not yet been reported.

It is therefore important to determine whether the increased activity of the enzyme in psoriatic lesions, and in leukaemic cells both sensitive and resistant to methotrexate, is due to an increase in extant enzyme, or to the emergence of catalytic forms specific for an abnormal cell clone. Alternative forms of the enzyme might be expected if viral transformation were a significant factor, and this proposal can be tested, with adequate amounts of enzyme, since Mathews (1967) has shown with *Escherichia coli* that bacteriophage-specific dihydrofolate reductases may be distinguished from those of the host.

A second question concerns the site of action of methotrexate, which has been thought to have exclusive avidity for intracellular dihydrofolate reductase (Werkheiser, 1961; Schrecker & Huennekens, 1964). There is increasing evidence that other sites are also involved (Roberts & Wodinsky, 1968; Borsa & Whitmore, 1969; Yoshino & Condit, 1969). To aid study of these questions we now describe a rapid method for retrieval of small amounts of catalytically active dihydrofolate reductase and other pteridine-binding proteins on columns of agarose containing covalently linked folate derivatives.

EXPERIMENTAL

Chemicals. A major portion of the methotrexate used was a gift from Dr J. C. Smith of Lederle Laboratories, New York, N.Y., U.S.A., and was of more than 95% purity when checked by chromatography as described

below. The 5-formyltetrahydrofolate (leucovorin) and 10-methylfolate were also gifts from Dr J. C. Smith of Lederle Laboratories and were used as supplied. Dihydrofolate was prepared by the method of Blakley (1960) and was stored freeze-dried in vacuum-sealed ampoules at −20°C.

NADPH, tris and 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide were obtained both from Sigma Chemical Co., St Louis, Mo., U.S.A., and from British Drug Houses Ltd., Poole, Dorset, U.K. Hydroxyapatite was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Sepharose-4B, CM-Sephadex C-50 and SE-Sephadex S-50 were obtained from Pharmacia, Uppsala, Sweden. They were washed and used as the H^+ form (S-50) or K^+ form (C-50). Cyanogen bromide was obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A., and from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. The 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride was from the Ott Chemical Co., Muskegon, Mich., U.S.A., or from Cambrian Chemicals Ltd., Croydon, U.K., and was used without further preparation. Norit A (Matheson, Coleman & Bell, Los Angeles, Calif., U.S.A.) and Norit OL (L. Light and Co. Ltd., Colnbrook, Bucks., U.K.) charcoals were used interchangeably. They were cleaned in several changes of 2M-HCl, dried at 100°C and stored at room temperature.

Eastman Kodak Co. supplied the 1,6-hexanediamine dihydrochloride. This material, deep brown in colour, was purified as follows. A 10g portion of impure compound was stirred in 50ml of water, adjusted to pH4.0 with HCl and treated batchwise with 2g of SE-Sephadex S-50 (H^+ form). The product was obtained from the supernatant, after the solution had cleared. The mixture was centrifuged and the pad discarded, and the cleaned hexanediamine recovered as an off-white solid by freeze-drying. It was stored desiccated at 0–5°C.

All materials for microbial growth were obtained from sources used previously (Harding, 1966). Glass-distilled water was used throughout, and pH values were obtained with a Beckman glass electrode at solution temperatures of 0–5°C. Centrifugation and chromatography procedures were executed at 0–10°C.

Biological material. *L. casei* (strain N.C.B. 6375) was obtained from the Torrey Research Station, Aberdeen, U.K., and was grown as described previously (Harding, 1966; Dunlap *et al.* 1971). Methotrexate-resistant strains (*L. casei* MTX/R) were selected by growth of the wild-type on folate-depleted medium solidified with agar (1%, w/v) and supplemented with folic acid (1.0ng/ml) and with methotrexate (0.1ng/ml). Colonies possessing increased dihydrofolate reductase activity were selected and maintained in liquid culture in the presence of methotrexate as described by Dunlap *et al.* (1971).

Dihydrofolate reductase was purified from the cell-free extracts by precipitation with $(NH_4)_2SO_4$ and filtration through Sephadex G-100 (Dunlap *et al.* 1971) and chromatography on hydroxyapatite (Kaufman & Gardiner, 1966). A major portion of the enzyme was eluted as a single peak during development of the gradient from 5mM to 1.0M-potassium phosphate buffer, pH7.0. Two forms of the enzyme were separated on CM-Sephadex (Harding *et al.* 1970) except that in the present study the columns of CM-Sephadex were eluted with a more shallow gradient (750ml of 5mM-potassium phosphate buffer, pH6.0, and 750ml of 0.15M-potassium phosphate buffer, pH8.0) than used previously.

Since other enzymes were obtained from these preparations, 2-mercaptoethanol was usually present at a final concentration of 10mM except in the last two chromatographic steps. As with the mouse dihydrofolate reductase (Harding *et al.* 1970) the presence of the 2-mercaptoethanol in these preparations was shown not to be an important determinant of interconversion of the two major forms of the enzyme separated on CM-Sephadex. Pooled fractions of each form of the enzyme were stored frozen at −20°C under argon and transported in solid CO_2, or maintained in solution at 0°C for short periods. The enzyme was also obtained from the skin of 3-day-old Sprague–Dawley rats killed by cervical dislocation. The fresh skin was sliced, kept on ice and homogenized in 6vol. (w/v) of ice-cold 50mM-tris–HCl buffer, pH7.5, in a VirTis 45 homogenizer. Blending was performed at low speeds for 10s and the velocity was then increased to maximum for 30s. Samples were cooled on ice and the blending cycle was repeated. As the enzyme at this stage is sensitive to thermal denaturation, care was taken to maintain the samples close to 0°C. The homogenized sample was centrifuged at 30000rev./min for 6h in a Beckman model L2 refrigerated ultracentrifuge with rotor type 30. The supernatant was decanted through several layers of cheesecloth and kept at 0°C until use.

Methods. Assay of dihydrofolate reductase was performed by measuring the decrease in E_{340} with a multiple-sample absorption recorder, either a Gilford model 2000 or a Unicam SP.1800B. The method of Mathews & Huennekens (1963) was used, except that the buffer was 50mM-tris–HCl, pH7.5, and no mercaptoethanol was present. Incubation for both assay and stability studies was at 37°C. One unit of enzyme activity will reduce 1μmol of dihydrofolate/min under these conditions. Specific activity of the enzyme is expressed in units/mg of protein. Protein was determined by the biuret method (Gornall, Bardawill & David, 1949). Kinetic analyses were performed by using the spectrophotometric assay method, except that the buffer was 50mM-tris–HCl, pH7.5, containing 50mM-KCl, and the concentration of either NADPH or dihydrofolate was varied. Values for K_m and K_i were obtained by the method of Freudenthal (1970).

Methotrexate was assayed in diffusates and column eluates by a modification of the standard assay procedure by using the principle of Werkheiser, Zakrzewski & Nichol (1962). The stock *L. casei* dihydrofolate reductase solution consisted of fractions from the Sephadex G-100 step adjusted to 0.1unit/ml and stabilized with albumin (0.1 mg/ml). The standard assay contained, in 1.0ml, 50μmol of tris–HCl buffer, pH7.5, 0.01unit of enzyme, the sample and the usual amount of dihydrofolate. The cuvette was preincubated for 1min at 37°C and the residual activity assayed after the addition of the standard amount of NADPH. Methotrexate was deemed to be absent from a solution when the E_{340} changes to the control and sample cuvettes were similar.

Dialysis tubing was obtained from the Union Carbide Corp., Chicago, Ill., U.S.A. and was prepared for use by the method of Kaufman & Gardiner (1966), and stored in 5mM-EDTA, pH7.0, at 4°C. It was well washed with water before use. Diffusion of folate derivatives is usually slow, but was facilitated by addition of acid-washed

Norit (1g/l) to the solution external to the sac. In the standard procedure preparations were dialysed against 2litres of 50mM-tris–HCl buffer, pH7.5, at 5°C. The dialysis time was varied according to the folate derivative present. Times varied from 24h for 5-formyltetrahydrofolate up to 60h for methotrexate, but in the latter case some activity inhibitory in the dihydrofolate reductase assay remained in the sac. In all cases the dialysing fluid was changed every 8h. Dialysis was stopped when enzyme activity ceased to increase within the sac and the E_{280} value reached a minimum. Samples were concentrated by using an Amicon concentrator with a UM10 membrane (Amicon Corp., Cambridge, Mass., U.S.A.).

Electrophoresis was performed in 7.5% (w/v) polyacrylamide gel at 5°C (Davis, 1964). The Bromophenol Blue front was marked with a bristle, and protein was detected by staining for 10h with Coomassie Blue [0.25% (w/v) in 50% (v/v) methanol containing 5% (v/v) of acetic acid] followed by electrophoretic destaining in 5% (v/v) acetic acid. Dihydrofolate reductase was detected by a modification of the method of Nixon & Blakley (1968), in which the freshly extruded gel was incubated in the dark at 37°C in a solution containing 1.5mM-NADPH, 3.0mM-dihydrofolate, 0.2M-tris–HCl buffer, pH7.6, and 0.25mg of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide/ml of solution. All solutions were gassed with argon before use. After about 30min, or when the tetrazolium band had adequately developed, the gel was placed in several changes of ice-cold water to permit diffusion of the excess of reagents and to stop the reaction. When the yellow background colour had leached out, the gel was preserved in 7% (v/v) acetic acid. It is important that enzyme activity should be developed in the dark to minimize photocatalytic production of tetrazolium, which would obscure trace amounts of enzyme activity. Electrophoresis in sodium dodecyl sulphate–polyacrylamide gel was performed by the method of Weber & Osborn (1969). R_F values used in the text are the ratios of the distance moved by either a band of protein or of enzyme activity to the distance migrated by the Bromophenol Blue marker. Some slight variations in R_F values noted between experiments were ascribed to variations in development temperature and conditions in the gel.

Folate derivatives were chromatographed, at room temperature and in the dark, on cellulose sheets (MN CEL 300) obtained from Macherey Nagel, Düren, Germany. The solvent used was aq. ammonia (sp. gr. 0.88)–2-methylpropan-2-ol–water (1:1:8, by vol.); it was allowed to ascend for 15cm, and the strips were dried and the bands were detected under u.v. light. Preparative chromatograms were performed in the same manner on sheets (20cm × 20cm) on which the derivatives had been streaked at the origin. Zones of separated compounds were sectioned from the sheet, and the compounds were eluted by a descending method with 5mM-$(NH_4)_2CO_3$ as solvent.

Preparation of substituted agarose. The principle of the method is to activate agarose with cyanogen bromide (Porath, Axen & Ernback, 1967), to couple this with one of the terminal amino groups of 1,6-hexanediamine and finally to couple pteroylglutamate derivatives via their carboxyl groups to the free amino groups on the hexamethylene-substituted agarose by means of a carbodiimide condensation (Mell, Whiteley & Huennekens, 1968).

Agarose was thoroughly washed with water to remove free azide and to obtain uniform particle size. When it had settled, 20g was made up to 40ml with water at room temperature. In a fume cupboard 1g of cyanogen bromide was dissolved, with warming, in 5ml of water, and added to the agarose. The pH was continuously monitored with a glass electrode, and adjusted to 11.0 with 1M-NaOH. After 5min the pH had fallen to 10.0, and after 9min the reaction vessel was cooled on ice for 3min. The cold mixture was rapidly filtered on a Buchner funnel and washed with 250ml of ice-cold 0.125M-potassium borate buffer, pH10.0. The activated agarose was quickly transferred to an ice-cold solution containing 10g of 1,6-hexanediamine dihydrochloride in 60ml of 0.125M-potassium borate buffer, pH10.0, and stirred for 20h at 0–5°C. The product, essentially 1-aminohexamethylene-6-aminoagarose, was harvested by low-speed centrifugation at 5°C and exhaustively washed with water. A brownish-pink product was obtained, and this was stored at 0–5°C until use.

Folate compounds were coupled in the following manner. A 50mg portion of the selected compound was suspended in 20ml of water and adjusted to pH6.5 with 0.1mM-NaOH. Then 4.5g of the substituted agarose was added and the mixture stirred at 0–5°C for 1h. When thoroughly equilibrated, 500mg of the carbodi-imide was added in 100mg portions at 2h intervals and the reaction allowed to proceed for 24h at 0–5°C. A bright-yellow insoluble product was obtained by using methotrexate or folic acid in this procedure. It was carefully washed by centrifugation with ice-cold water, and the washings were retained for estimation of the pteridine content. By altering the proportions of cyanogen bromide, agarose and added folate, products with a higher degree of substitution could be prepared. The product could be stabilized for transport without refrigeration, or for prolonged storage at 0–5°C, by suspension in 1M-NaCl containing 5mM-$NaHCO_3$, which was removed before use.

The pteridine content of the substituted aminohexamethylene-agarose was obtained as follows. The washed product was placed in a pre-weighed silicone-treated column, which was equilibrated with 25 bed volumes of buffer 2 (0.1M-potassium borate, adjusted to pH10.0 with KOH), then with 75 bed volumes of buffer 1 (citric acid, 30mmol; KCl, 0.12mol; EDTA, 2mmol; adjusted to pH6.0 with KOH). The total eluate was pooled with the washings previously retained, and the free pteridine determined spectrophotometrically. Calculations were based on ϵ_{370} 7400$M^{-1}\cdot cm^{-1}$ in 0.1M-NaOH for methotrexate and ϵ_{360} 7500$M^{-1}\cdot cm^{-1}$ for folic acid under the same conditions. Excess of buffer was removed from the column, and the wet weight of the substituted agarose obtained. The retained pteridine is expressed in μmol/g wet wt. of substituted agarose.

Absorption spectra were obtained with a Unicam SP.1800B recording spectrophotometer, with as blanks the solutions described in the text.

RESULTS

Binding of methotrexate by aminohexamethylene-agarose. Complete elution of non-covalently bound methotrexate from the substituted agarose is obligatory for satisfactory retention of binding

Table 1. *Release of methotrexate during washing of methotrexate–agarose gels*

Methotrexate was removed from preparations A and B by decanting the supernatant from the reaction (wash 0). The agarose was then resuspended in successive portions of ice-cold water as described in the text (washes 1–4). Preparation C was washed free of methotrexate with ice-cold water (wash 0). It was then packed to yield a column (5 cm × 1 cm) and eluted at 0.5 ml/min with 0.5 M-NaCl containing 50 mM-$NaHCO_3$. Flow was stopped for 24 h, and then 2 column volumes were collected for methotrexate assay (wash 1) and elution was continued for 24 h. Flow was again stopped for 24 h, and this elution procedure was repeated three times (washes 2–4). All experiments with *L. casei* MTX/R were performed with preparation A, and rat skin experiments were solely with preparation B.

	Methotrexate eluted (μmol)		
	A	B	C
Final methotrexate content ...	(6.0 μmol/g)	(20.0 μmol/g)	(18.0 μmol/g)
Wash			
0	68	660	Nil
1	11	95	0.8
2	2.5	17	0.2
3	0.4	8.5	0.2
4	0.07	4.7	0.06
Methotrexate recovery in the washes	74%	70%	1.2%

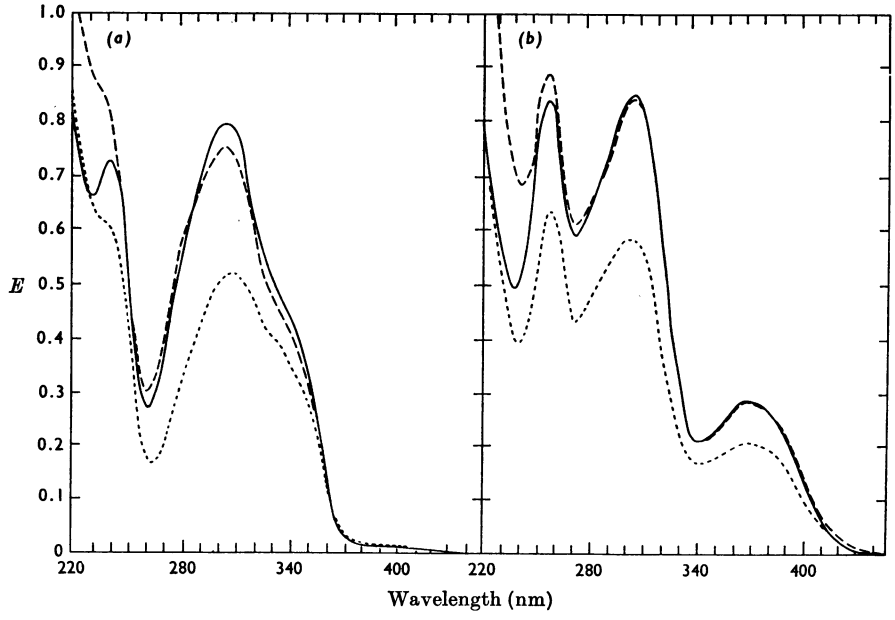


Fig. 1. U.v.-absorption spectra of methotrexate and reaction products obtained from t.l.c. (*a*) Spectra in 0.1 M-HCl; (*b*) spectra in 0.1 M-NaOH. ——, Methotrexate (19.20 μg/ml); – – – –, derivative eluted from the sheet at R_F 0.78 (see the text); -----, derivative eluted from the sheet at R_F 0.70.

protein by the column. Free methotrexate can bind to methotrexate coupled with agarose either by mechanisms such as hydrogen-bonding in relatively hydrophobic domains created in the affinity matrix, or by ionic mechanisms involving the carboxyl groups of methotrexate and unsubstituted amino groups of the matrix. Release of methotrexate was always followed during washing steps, and it was rapid at both levels of substitution during the first five washes (Table 1, preparations A and B), but thereafter a slow release occurs, which soon becomes undetectable. The remaining methotrexate could not be displaced with 8 M-urea at pH 6–10. However, if flow was stopped for 24 h and then restarted,

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

Peaks on the Sephadex G-100, hydroxyapatite and CM-Sephadex profiles refer to a pooled sequence of fractions each containing enzyme activity. Peak 1 is the first peak to emerge from the column.

	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)
Cell-free extract	373	480	0.77
Ammonium sulphate	245	164	1.49
Sephadex G-100, pooled peak	347	86	4.03
Hydroxyapatite			
Peak 1	165	9.5	17.37
Peak 2	237	12.4	19.15
CM-Sephadex			
Peak 1 (CM-A)	50	2.5	20.0
Peak 2 (CM-B)	345	16.0	21.56

about 1% of the total methotrexate could be eluted (Table 1, preparation C). An alternative explanation could be that there is slow hydrolysis of a covalent structure created during the preparation of the methotrexate–agarose complex. Portions of the supernatant remaining after the carbodiimide steps were analysed by t.l.c. as described above. Two major u.v.-absorbing zones were found at R_F 0.70 (zone X) and R_F 0.78 (zone Y). Methotrexate co-chromatographed with the latter zone, which was in threefold molar excess over the former. On preparative-scale t.l.c., these appeared as bright-yellow zones in visible light with u.v.-absorption spectra very similar to but not identical with those of the parent methotrexate (Fig. 1). When folic acid was substituted for methotrexate in these preparations two u.v.-absorbing zones were again observed in the chromatograms, the compound in the major zone at R_F 0.84 being in about a fourfold molar excess over that located at R_F 0.73 (folic acid has R_F 0.84).

Dihydrofolate reductase from L. casei *purified by conventional procedures.* To evaluate the affinity compounds dihydrofolate reductase was purified from *L. casei* MTX/R by conventional procedures. The enzyme appears as two components during chromatography on both hydroxyapatite and CM-Sephadex (Table 2). Usually, 20–35% of the enzyme applied to the column emerged in the first peak on the hydroxyapatite profile eluted during passage of the gradient (approx. 40mM-potassium phosphate) with the remainder being eluted at about 85mM-potassium phosphate. Polyacrylamide-gel analysis for enzyme activity showed that the first peak contained two active components with R_F 0.54 and R_F 0.82 respectively. However, in the second peak there was a single active component located in the gel at R_F 0.54. The enzyme in both peaks was about 80% pure, estimated from identical polyacrylamide gels which had been stained for protein. Since the major components of the total reductase activity were not clearly separated on hydroxyapatite, further purification and separation of the two forms of the active enzyme was achieved on CM-Sephadex (Harding *et al.* 1970). Enzyme in the first peak of activity (CM-A) moved at R_F 0.83, and that from the second peak (CM-B) appeared at R_F 0.54 (Plate 1*a*). The purity of the enzyme form persisted when electrophoresis was performed after several cycles of freezing and thawing (Plate 1*a*), so the separated forms were stored and transported as described.

The difference in charge shown by each form on CM-Sephadex and in polyacrylamide gel was examined to determine the possible binding of either NADPH or dihydrofolate. This was important as we had observed selective loss of the form with R_F 0.83 (or apparent interconversion of the two forms) during preliminary studies with the affinity column. The relative purity of the enzyme from each peak of the CM-Sephadex column made it unlikely that these observations could be due to aggregation, differences in molecular weight, or the presence of a carrier protein. The forms had similar molecular properties (Table 3), and co-purified until separated on hydroxyapatite or CM-Sephadex. Both forms were therefore studied for substrate-dependent interconversion (Plate 1*b*).

Each form of the enzyme remained electrophoretically stable after incubation at 37°C (Plate 1*b*, gel 0) or on ice (Plate 1*b*, gel A1). Form CM-A remained unchanged after incubation with NADPH (Plate 1*b*, gel A2), but on incubation with dihydrofolate it was largely converted into a form (Plate 1*b*, gel A3) with mobility R_F 0.57, which is identical with that of form CM-B incubated alone (Plate 1*b*, gel B1). This mode of interconversion was reversible, for form CM-B when incubated with NADPH was converted into a form with mobility identical with that of form CM-A (Plate 1*b*, gel B2). Similarly when form CM-B was incubated with dihydrofolate there appeared a small amount of a form corresponding to form CM-A (Plate 1*b*, gel B3), but the mobility of the bulk of the enzyme remained

unchanged. These findings, in conjunction with the molecular properties of each form of the enzyme (Table 3), offer evidence that the two enzyme forms are derived from a single protein whose electrophoretic mobility is considerably increased when it is incubated with NADPH.

Enzyme stability and affinity chromatography. There is no information relating the K_m of a folate derivative to its performance as a reagent in affinity chromatography. The profile of the folate–agarose compound (Fig. 2) showed that some enzyme was obtained in the buffer-1 wash, and that further activity was removed by the pH gradient, in a peak with a sharp trailing edge. About 20% of the added enzyme activity was recovered, and polyacrylamide gels showed that only form CM-B was present. Further studies with this column showed that the enzyme was consistently retarded but only minimally retained, hence methotrexate–agarose complexes were used instead to see if a compound of K_i about 10^{-8} M (Table 3) would improve retention of the enzyme.

As the interaction between dihydrofolate reductase and methotrexate is essentially stoicheiometric (Werkheiser, 1961) at pH values below 7.0, columns of methotrexate–aminohexamethylene-agarose were equilibrated and loaded with buffer 1 as solvent. The stability of the *L. casei* enzyme at pH 6.0 at 37°C in buffer 1 is summarized in Fig. 3. Citrate improved the stability of the enzyme in the presence of potassium chloride, which was included since the affinity of methotrexate for the enzyme was thereby increased (P. C. H. Newbold, unpublished work). The harmful effects of mercaptoethanol, reduced by the presence of NADPH, were observed in a variety of buffer systems.

Dissolved methotrexate may be separated from dihydrofolate reductase above pH 7.5 (Mathews & Huennekens, 1963). Our preliminary studies showed that effective elution from these agarose columns could be obtained at basic pH values. However, the enzyme was unstable, and there was

Table 3. *Comparison of some properties of each form of dihydrofolate reductase from* L. casei *MTX/R*

Molecular weights were estimated (*a*) on a calibrated column of Sephadex G-100 eluted at pH 6.0 with 50 mM-potassium phosphate and (*b*) in sodium dodecyl sulphate–polyacrylamide gel. Other analyses are described in the Experimental section.

	Form CM-A	Form CM-B
Mol.wt. (*a*)	16000	16800
Mol.wt (*b*)	15450	15600
K_m (NADPH)	4.2×10^{-5} M	4.1×10^{-5} M
K_m (dihydrofolate)	4.8×10^{-6} M	5.2×10^{-6} M
K_i (methotrexate)	7.1×10^{-9} M	6.7×10^{-9} M

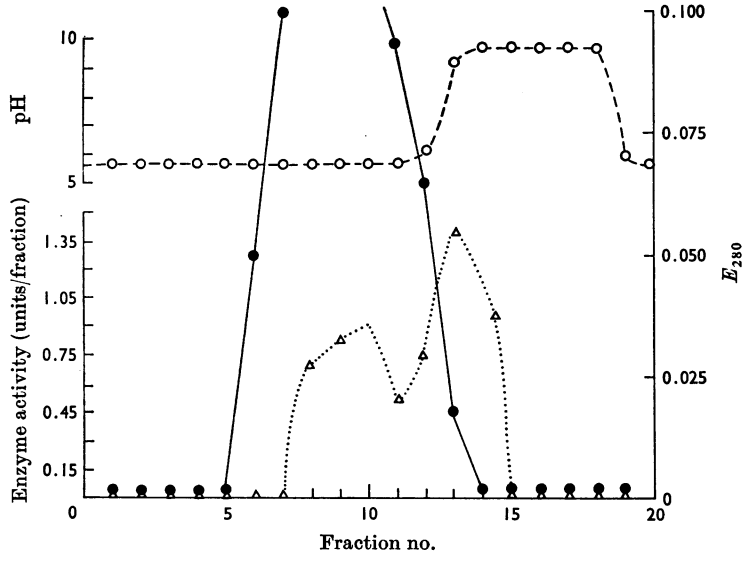
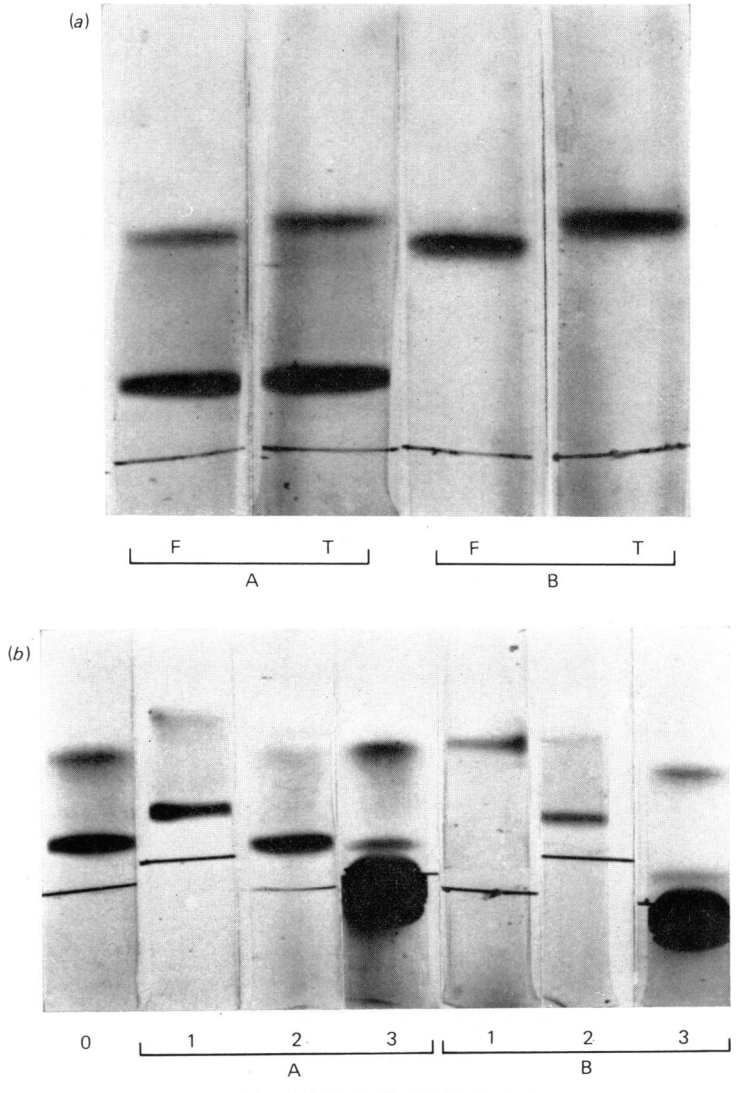
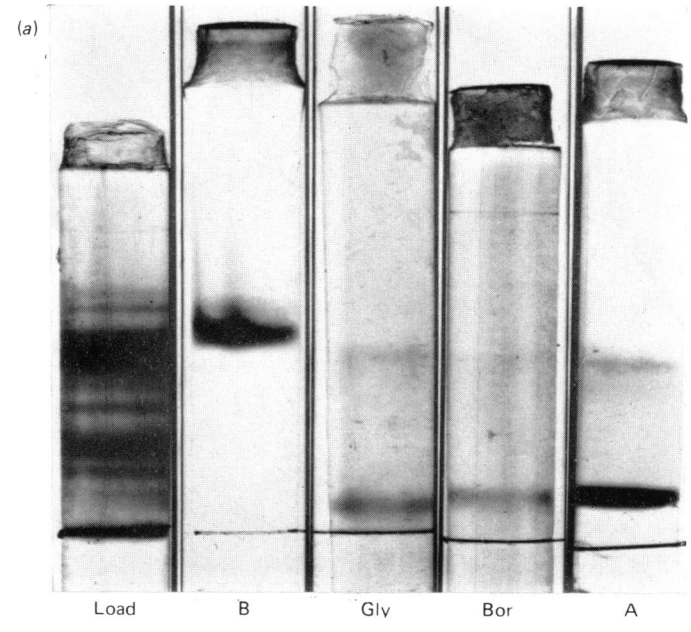


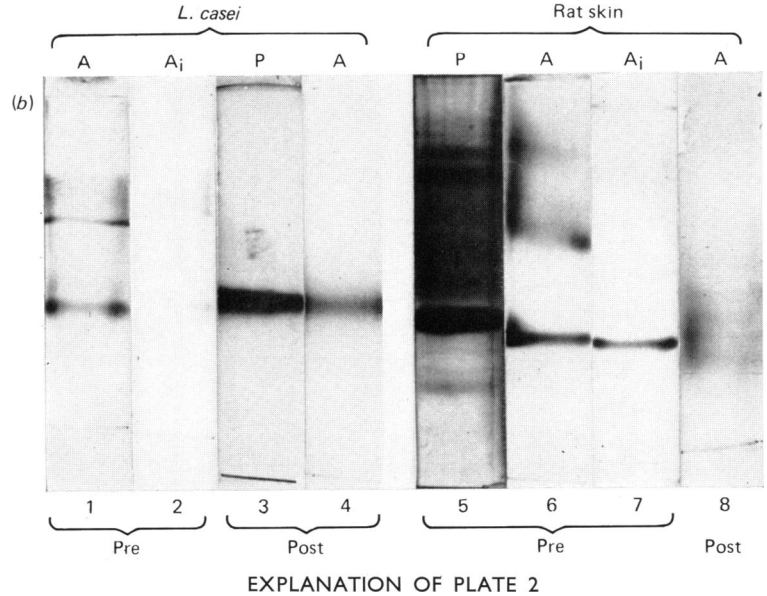
Fig. 2. Chromatography of dihydrofolate reductase on a folate–agarose column. Folate–agarose (containing 15 μmol of folic acid/g) was packed to 6.5 cm × 1.0 cm. This was equilibrated with buffer 1, adjusted to pH 5.7 and loaded with 40 units of enzyme from the Sephadex G-100 stage, in 4.0 ml. A further 10 ml of buffer 1 at pH 5.7 was run in. Then 15 ml of buffer was added, containing 3 mmol of glycine and 30 μmol of EDTA and adjusted to pH 10.0 with KOH. Buffer 1 followed, and the 1.5 ml fractions were monitored for enzyme activity (△), pH (○), and E_{280} (●).



EXPLANATION OF PLATE 1

(*a*) Stability of forms CM-A and CM-B of dihydrofolate reductase. Enzyme forms CM-A and CM-B in 50mM-potassium phosphate buffer, pH 6.5, were stored at −70°C under argon (gels AF and BF) or were frozen at −20°C and thawed five times (gels AT and BT) before electrophoresis. Gels AF and AT were loaded with 50μg of protein, comprised of 40μg of form CM-A and 10μg of form CM-B. Gels BF and BT were loaded with 30μg of protein of form CM-B. Gels AF and BF were stained for protein, and gels AT and BT for enzyme activity. On similar gels, not shown, the enzyme bands failed to show activity after preincubation for 5min with 1mmol of methotrexate. (*b*) Substrate-dependent interconversion of both forms of the enzyme. Gel 0 contained 40μg of protein of form CM-A and 15μg of protein of form CM-B that had been incubated at 37°C for 90min. The protein in gels A1 and B1 was maintained on ice, and that in gels A2 and B2 was incubated in 0.15ml containing 20μmol of tris–HCl buffer, pH 7.6, and 3μmol of NADPH. Protein in gels A3 and B3 was incubated with 3μmol of 7,8-dihydrofolate and 20μmol of tris–HCl buffer, pH 7.6, in a volume of 0.15ml. Samples were incubated at 37°C for 90min under argon, subjected to electrophoresis and the gels stained for protein. Although not shown, enzyme stain corresponded to the protein. In gels A3 and B3 dihydrofolate appears as a dense mass just ahead of the bristle at the gel front.





EXPLANATION OF PLATE 2

(*a*) Elution of bound protein from a methotrexate–agarose column by glycine or borate. Protein (100 μg) of the Sephadex G-100 fraction loaded on to the column is shown (Load), with purified forms CM-A (A) and CM-B (B) for comparison. Methotrexate–agarose columns were eluted with leucovorin and NADPH in either glycine (Gly) or borate (Bor) as described in the text. (*b*) Purification of dihydrofolate reductase by affinity chromatography. Gels show enzyme activity developed with (A_i) and without (A) methotrexate, or protein stain (P). The cell-free extract (Pre) from *L. casei* MTX/R or rat skin was purified on columns of methotrexate–agarose as described in the text. Enzyme was eluted with the leucovorin–borate system, concentrated and analysed (Post). Gels 1, 2, 6 and 7 contained 150 μg protein, gels 3 and 4 25 μg of protein and gel 5 50 μg of protein. Gel 8 was loaded with 9 μg of protein in 400 μl (hence the increased size of the spacer gel). Corresponding gels stained for protein did not show discernible bands.

P. C. H. NEWBOLD AND N. G. L. HARDING

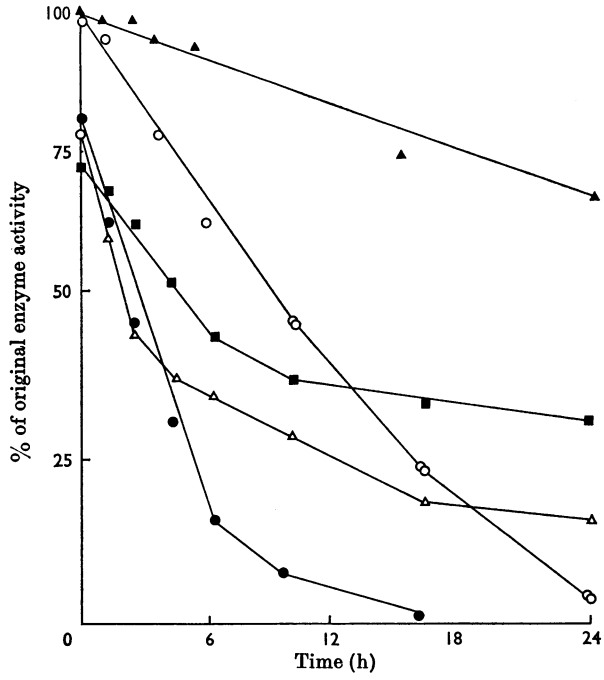


Fig. 3. Stability of dihydrofolate reductase at pH 6.0. All samples contained 5.0 units of dihydrofolate reductase made up to a total volume of 1.5 ml with buffer 1, except for 'no citrate', where water was used instead. NADPH (0.25 μmol) and mercaptoethanol (20.0 μmol) were added as indicated, and the pH was adjusted to 6.0. ■, No addition; △, no citrate; ▲, +NADPH; ●, +mercaptoethanol; ○, +NADPH+mercaptoethanol.

poor recovery of catalytic activity. Fig. 4 summarizes the data at pH 10.0 and shows the stimulation of activity in glycine–KOH buffer before its later decline. Further studies indicated that NADPH is effective in maintaining stability of the enzyme in lysine or glycine, although it did not prevent the initial loss of activity due to the high pH, especially in the presence of mercaptoethanol (Fig. 5). Rapid loss of enzyme activity occurred in the presence of dihydrofolate, even when mercaptoethanol was added to stabilize the reduced folate derivative (Fig. 5).

Since the loss of activity on methotrexate–agarose columns could be ascribed either to retention of enzyme or to increased loss of a catalytic form particularly sensitive to inactivation in the presence of methotrexate, further studies of both anionic and zwitterionic buffers at pH 10.0 were carried out. Table 4 shows the stabilizing effect of NADPH, and also that prior activation by salt or NADPH was obligatory for inactivation in glycine buffer but not in borate buffer. In borate buffer the effect of potassium chloride and NADPH, alone or in combination, was to increase inhibition of the enzyme by methotrexate (Table 5). Hence, NADPH acts both to stabilize the enzyme at pH 10.0 and also to increase the sensitivity of the enzyme to methotrexate. Other studies suggested that some of the loss of catalytic activity was due to separation from the methotrexate, so we added folate derivatives to the buffer as counter-substrates for more efficient elution of active enzyme from affinity columns.

Elution of methotrexate–agarose columns by different folate derivatives. Standard columns were packed and washed with buffer 1, then loaded with 20 units of enzyme in 2.0 ml. The flow rate was 0.5 ml/min, and fractions of volume 1.5 ml were monitored for pH, E_{280} and enzyme activity. No detectable reductase activity was eluted from the column by passage of 200 ml of buffer, which was adequate to remove all protein not specifically adsorbed. For elution, 15 ml of a buffer containing the desired folate derivative at pH 10.0 was freshly prepared and de-gassed on ice. Usually all fractions with pH above 6.5 were visibly yellow owing to the presence of the added derivative, and were dialysed before assay for enzyme activity. A typical elution profile is shown in Fig. 6.

All the folate derivatives tested (Table 6) were

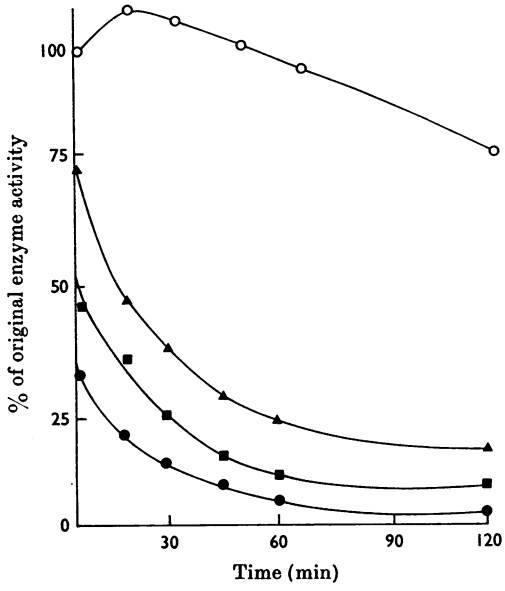


Fig. 4. Stability of dihydrofolate reductase in different buffers at pH 10.0. Samples contained, in 1.0 ml, 3 units of dihydrofolate reductase, 100 μmol of K_2CO_3 (■) or glycine (○), or 50 μmol of potassium borate (●), or water alone (▲). The pH in each case was adjusted to 10.0 with KOH, and incubation and assays were performed at 37°C. Samples were withdrawn for assay at the indicated times.

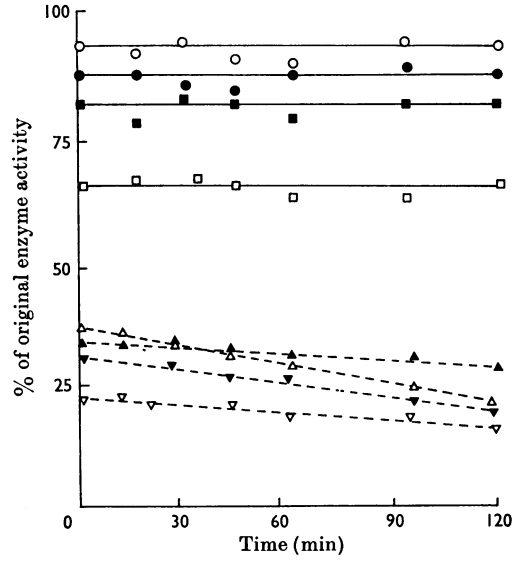


Fig. 5. Stability of dihydrofolate reductase in zwitterionic buffers at pH 10.0. Each sample contained 3 units of dihydrofolate reductase in a volume of 1.2 ml, incubated at 37°C, and the pH was adjusted to 10.0 with KOH. Glycine or lysine (50 μmol), NADPH (0.25 μmol), mercaptoethanol (20.0 μmol) and dihydrofolate (0.50 μmol) were added as indicated. In each case the decrease in activity occurred so rapidly that it could not be followed in the first minute. The first point in each experiment represents the first assayable value, and for clarity these have not been shown emerging from the 100% value on the ordinate. ●, Glycine+NADPH; ■, glycine+NADPH+mercaptoethanol; ▲, glycine+dihydrofolate; ▼, glycine+dihydrofolate+mercaptoethanol; ○, lysine+NADPH; □, lysine+NADPH+mercaptoethanol; △, lysine+dihydrofolate; ▽, lysine+dihydrofolate+mercaptoethanol.

present at a concentration of 2.0 mM and NADPH at a concentration of 0.35 mM. Table 6 also shows the effect of added NADPH on the enzyme activity eluted, with poor total yields although both form CM-A and form CM-B were obtained. Since 10-methylfolate effectively eluted active enzyme, the loss of activity seen with methotrexate elution was due either to retention of methotrexate by the enzyme or to specific inactivation during removal from the agarose matrix by free methotrexate. Form CM-B is predominantly eluted either because form CM-A is retained or because it is converted into form CM-B. When 10 units of each form were passed separately into a methotrexate–agarose column, and eluted with borate and folic acid alone, without added NADPH, 45% yield of enzyme activity was obtained as form CM-B exclusively.

During these experiments we observed that other proteins were eluted from the methotrexate–agarose columns, distinguished from dihydrofolate reductase on polyacrylamide gels. Plate 2(*a*) shows that enzyme fractions eluted with borate show an additional small protein band at R_F 0.164, whereas this was not seen with a glycine-based eluent. This band is similar to one seen in the column load, so it is unlikely to be due to an artifact during adsorption on or separation from the column. This band did not stain for enzyme activity.

Purification of dihydrofolate reductase from mammalian skin. A standard column of methotrexate–agarose was loaded with a rat skin extract over 12 h and then washed with 500 ml of buffer 1. No enzyme activity was detected in the run-through. Elution was with 20 ml of buffer containing 40 μmol of leucovorin, 20 μmol of EDTA and 1 mmol of potassium borate, adjusted to pH 10.0 with KOH. A typical profile was obtained (Fig. 6), and the fractions with pH above 6.5 were pooled and dialysed for 48 h. Multiple bands of enzyme activity occurred (Plate 2*b*) but the protein concentration was too low to stain. This represents a purification of 3700-fold (Table 7).

Prolonged use of methotrexate–agarose columns. The gel was stable in the presence of both trypsin

Table 4. *Stability of dihydrofolate reductase in borate and glycine at* pH 10.0 *with potassium chloride and NADPH added*

Each sample contained in 1.0 ml: 0.85 unit of enzyme, 50 μmol of borate or glycine buffer, pH 10.0, 0.15 μmol of NADPH and 250 μmol of KCl. Incubation and assays were performed at 37°C.

Time of incubation (min)	Activity (unit)			
	Glycine buffer		Borate buffer	
	−NADPH	+NADPH	−NADPH	+NADPH
2	0.063	0.079	0.059	0.056
10	0.050	0.092	0.049	0.048
20	0.035	0.100	0.032	0.039
40	0.025	0.098	0.025	0.037
60	0.018	0.085	0.018	0.023
90	0.012	0.068	0.011	0.014
120	0.006	0.067	0.005	0.007

Table 5. *Inhibition of dihydrofolate reductase by methotrexate after incubation at* pH 10.0 *in borate or glycine buffer*

All samples were incubated, in 1.0 ml, at 37°C at pH 10.0. Borate or glycine buffer, pH 10.0 (50 μmol), NADPH (0.15 μmol) and KCl (250 μmol) were added, as indicated, to 0.085 unit of dihydrofolate reductase. Samples were removed for assay at 2 min. One series was assayed with methotrexate (10 μM) present in the cuvette. In samples withdrawn at 10 min methotrexate completely inhibited the activity.

Reagent present	Activity (unit)		Inhibition at 2 min (%)
	Enzyme alone	Enzyme + methotrexate	
Borate	0.054	0.038	30
Borate + NADPH + KCl	0.069	0.035	49
Borate + NADPH	0.064	0.027	58
Borate + KCl	0.046	0.015	67
Glycine	0.059	0.022	63
Glycine + NADPH + KCl	0.063	0.018	71
Glycine + NADPH	0.070	0.033	53
Glycine + KCl	0.059	0.010	83

and chymotrypsin for 10 h at 37°C. It could also be stored at room temperature in the dark for at least 1 month. Columns can be cycled at least 50 times with partially purified enzyme fractions. With tissue extracts, however, there is progressive discoloration of the column, and the flow rate falls. There was, however, no apparent loss of affinity.

DISCUSSION

Conventional enzyme purification is slow and gives poor yields. If a specific competitive inhibitor of the enzyme is covalently attached to a cross-linked gel or polymer, an enzyme will be preferentially retarded in proportion to its affinity constant for the inhibitor (Cuatrecasas, Wilchek & Anfinsen, 1968). The relatively low yields of dihydrofolate reductase obtained from methotrexate coupled to soluble starch may have been partly due to steric interference (Mell *et al.* 1968). The hexamethylene chain is apparently effective as a spacer in these gels, as shown by our higher yields. Variations in the spacer group could yield information on the structure of the active site of the enzyme, and could indicate modifications directed towards the extraction of other enzymes. The comparatively poor performance of the folic acid–agarose columns indicates that the K_m and K_i values of a derivative are predictive of its value as an agent in affinity chromatography, provided that it does not undergo undersirable modifications during the coupling reactions.

The carboxyl groups of methotrexate are used as points of attachment in the present method, and conditions are designed to suppress the side reactions leading to formation of folate homopolymers (Harding, 1971). The t.l.c. results indicate that although side reactions do occur, the absorption

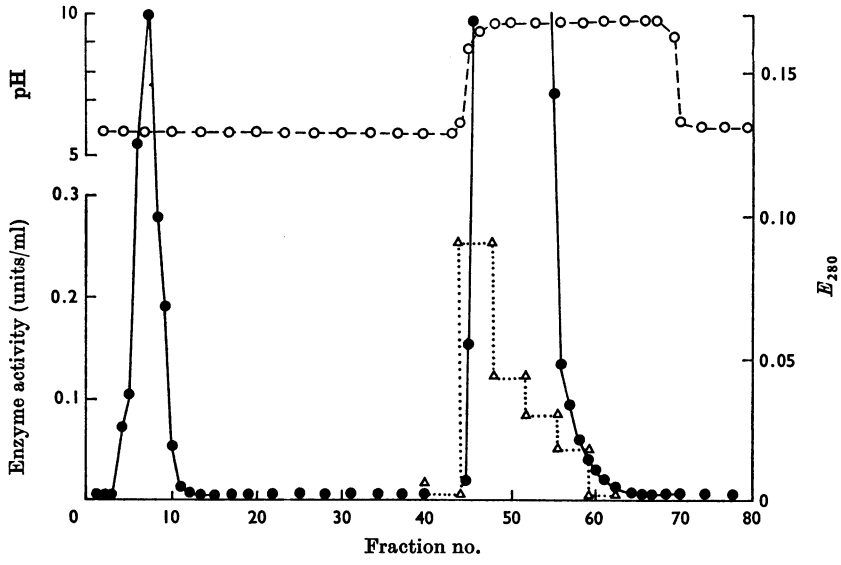


Fig. 6. Chromatography of dihydrofolate reductase on a methotrexate–agarose column. Methotrexate–agarose (containing 6.0 μmol/g) was packed to 6.5 cm × 1.0 cm. This was equilibrated with buffer 1, at pH 6.0, and was loaded with 20 units of enzyme from the Sephadex G-100 stage, in 2.0 ml. A further 40 ml of buffer 1 was run in. Then 15 ml of buffer was added, containing 3 mmol of glycine, 30 μmol of EDTA, 20 μmol of NADPH and 160 μmol of dihydrofolate, mixed on ice and adjusted to pH 10.0 with KOH. Buffer 1 followed, and the 1.5 ml fractions were monitored for enzyme activity (△), pH (○) and E_{280} (●).

Table 6. *Dihydrofolate reductase recovered from the affinity column under different elution conditions*

The conditions are given in the text. (A) implies the presence of form CM-A, but in small amounts relative to form CM-B. — implies that the combination has not been used.

	Glycine buffer				Borate buffer	
	+NADPH		−NADPH		−NADPH	
Folate derivative in eluent	% of active enzyme recovered	Enzyme forms present	% of active enzyme recovered	Enzyme forms present	% of active enzyme recovered	Enzyme forms present
None	5	(A), B	0	0	0	0
Folate	0	0	10	B	54	B
Dihydrofolate	15	A, B	22	B	42	B
Leucovorin	7	A, B	36	B	56	B
Methotrexate	0	A, B	0	(A), B	—	—
10-Methylfolate	—	—	16	B	34	B

spectra of these aberrant compounds are so similar to those of the parent compound that modification must have occurred away from the chromophore. During coupling these products are either not bound, or if bound to the hexamethylene chain, they are also effective in retaining the enzyme. Tetrahydromethotrexate and tetrahydroaminopterin are very unstable (Zakrzewski, Hakala & Nichol, 1962; Kisliuk & Levine, 1964), and hence methotrexate appears to bind the enzyme so that it cannot accept a hydride ion from the NADPH donor (Huennekens, Mell, Harding, Gundersen & Freisheim, 1970) since the matrix of enzyme-charged affinity columns is stable when eluted with buffers containing NADPH. Zakrezwski (1963) found that folate and methotrexate have similar high binding energies, but the change of entropy is +13 cal/degree per mol on dissociation of folic acid from dihydrofolic reductase, and is −7 cal/degree per mol for methotrexate at pH 5.2.

Table 7. *Single-step purification of dihydrofolate reductase on methotrexate–agarose columns*

	Total protein (mg)	Total enzyme (units)	Specific activity (units/mg of protein)	Purification
Rat skin				
Cell-free extract	2200	45	0.049	
Pooled eluate from column	0.071	14	183	3700
L. casei MTX/R				
Cell-free extract	1750	500	0.28	
Pooled eluate from column	2.8	150	20	71

This suggests that, although structural rearrangement of the pteridine ring occurs during passage of the pH gradient, reversible disorder of the protein structure may also be an important factor in elution of the enzyme. In view of the high recovery of activity under optimum conditions, the enzyme seems to be removed from its binding site on the affinity matrix and does not rebind in the presence of excess of folate derivative in the eluent.

Loss of one form, or interconversion of two forms, during purification of the *L. casei* enzyme upon methotrexate–agarose columns is relevant to appearance of two forms of the reductase during the emergence of methotrexate-resistant cells with the L 1210 (Harding *et al.* 1970) and L 4946 (Blumenthal & Greenberg, 1970) leukaemias. Interconversion of the two forms has shown that the crucial factor is the presence or absence of NADPH bound to a common protein. Chromatographic and fluorescence studies (R. B. Dunlap & L. Gundersen, personal communication) have also confirmed this. A similar phenomenon may account for the biphasic profiles on hydroxyapatite (Perkins, Hillcoat & Bertino, 1967) and CM-Sephadex (Harding *et al.* 1970) of the L 1210 enzyme. An analogy exists with alcohol dehydrogenase (Jacobson, Murphy & Hartman, 1970), where forms of the enzyme, interrelated by incubation with NAD, may be separated on both hydroxyapatite and DEAE-cellulose. Our data indicate that bound NADPH may be lost on the methotrexate–agarose columns, as both forms of the enzyme are eluted when NADPH is present. Kaufman & Gardiner (1966) showed that chicken liver dihydrofolate reductase is stabilized by added NADPH in the absence of methotrexate, and they also found that leucovorin was effective as a stabilizing agent without affecting the activity of the enzyme. Leucovorin also gives much sharper bands on electrophoresis of the mouse enzyme than any other folate analogue (N. G. L. Harding, unpublished work). Hence our findings of the effectiveness of leucovorin as a counter-substrate are further evidence of a specific interaction of this compound with the enzyme.

After elution from the affinity column, many bands of enzyme activity were seen on gels of dihydrofolate reductase purified from rat skin (Plate 2*b*). The intensity of activity increased with the R_F value, and all were inhibited by methotrexate. However, of the three bands in the skin homogenate, only two were inhibited by methotrexate, so the third appears to be an NADP-tetrazolium reductase. By analogy with albumin (Marinis & Ott, 1964) the bands obtained with the skin enzyme from the affinity column are due to self-association. This contrasts with the behaviour of the purified *L. casei* enzyme, where single protein bands are consistently seen.

The profound inhibitory effect of mercaptoethanol at pH 10.0 has not been explained. At this pH mercaptoethanol is a potent nucleophile and might therefore be expected to inactivate a protein catalysing proton transfer through a susceptible group at the active site. Alternatively, it could combine with a thiol group. However, Kessel & Roberts (1965) argue that dihydrofolate reductase from *Lactobacillus leichmannii* behaves like an activated mammalian reductase, but it is not inactivated by mercurials. If the *L. casei* enzyme is similar then the mercaptoethanol effect must be on a group which is not thiol in nature.

The gels shown in Plate 2(*a*) provide further evidence that there is another binding protein for methotrexate besides dihydrofolate reductase. Our current studies with other mammalian tissues indicate that reductase-independent proteins that can bind methotrexate are widely distributed. These may be of importance in the known long persistence of the drug in the tissues, but it is not yet known if they play any part in the transport or mode of action of the drug in the host.

Aspects of this work were performed by N.G.L.H. at the Postgraduate Medical School, Cambridge, during leave from the Department of Biochemistry, Scripps Clinic and

Research Foundation. Support from the Medical Research Council and the American Cancer Society is gratefully acknowledged. P.C.H.N. was supported by Research Grant AM 11649 and Training Grant AM 05566 from the National Institutes of Health, U.S. Public Health Service, and a travel grant from the Wellcome Foundation. We thank Dr John Whiteley, Dr Frank Hayhoe and Dr Richard Stoughton for helpful discussions, and Dr Frank Huennekens and Dr Bruce Dunlap for encouragement. We also gratefully acknowledge the technical assistance of Mrs Barbara Melkerson and Miss Susan Parker.

REFERENCES

Blakley, R. L. (1960). *Nature, Lond.*, **188**, 231.

Blumenthal, G. & Greenberg, D. M. (1970). *Oncology*, **24**, 223.

Borsa, J. & Whitmore, G. F. (1969). *Molec. Pharmac.* **5**, 303.

Cuatrecasas, P., Wilchek, M. & Anfinsen, C. B. (1968). *Proc. natn. Acad. Sci. U.S.A.* **61**, 636.

Davis, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 321.

Dunlap, R. B., Harding, N. G. L. & Huennekens, F. M. (1971). *Biochemistry, Easton*, **10**, 88.

Freudenthal, R. (1970). *Analyt. Biochem.* **35**, 346.

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

Grignani, F., Martelli, M. F., Tonato, M. & Finzi, A. (1967). *Archs Derm.* **96**, 577.

Harding, N. G. L. (1966). Ph.D. Thesis: University of Oxford.

Harding, N. G. L. (1971). *Ann. N.Y. Acad. Sci.* (in the Press).

Harding, N. G. L., Martelli, M. F. & Huennekens, F. M. (1970). *Archs Biochem. Biophys.* **137**, 296.

Huennekens, F. M., Mell, G. P., Harding, N. G. L., Gundersen, L. E. & Freisheim, J. H. (1970). *Proc. int. Cong. Pteridine, Toba*, p. 329.

Jacobson, K. B., Murphy, J. B. & Hartman, F. C. (1970). *J. biol. Chem.* **245**, 1075.

Kaufman, B. T. & Gardiner, R. C. (1966). *J. biol. Chem.* **241**, 1319.

Kessel, D. & Roberts, D. (1965). *Biochemistry, Easton*, **4**, 2631.

Kisliuk, R. L. & Levine, M. D. (1964). *J. biol. Chem.* **239**, 1900.

Marinis, S. & Ott, H. (1964). In *Protides of the Biological Fluids*, vol. 12, p. 420. Ed. by Peeters, H. Amsterdam: Elsevier Publishing Co.

Martelli, M. F., Tonato, M., Harding, N., Larizza, L. & Grignani, F. (1969). *Revue roum. Méd. intern.* **6**, 179.

Mathews, C. K. (1967). *J. biol. Chem.* **242**, 4083.

Mathews, C. K. & Huennekens, F. M. (1963). *J. biol. Chem.* **283**, 3436.

Mell, G. P., Whiteley, J. M. & Huennekens, F. M. (1968). *J. biol. Chem.* **243**, 6074.

Nixon, P. F. & Blakley, R. L. (1968). *J. biol. Chem.* **243**, 4722.

Perkins, J. P., Hillcoat, B. L. & Bertino, J. R. (1967). *J. biol. Chem.* **242**, 4771.

Porath, J., Axen, R. & Ernback, S. (1967). *Nature, Lond.*, **215**, 1491.

Rees, R. B., Bennett, J. H., Maibach, H. I. & Arnold, H. L. (1967). *Archs Derm.* **95**, 2.

Roberts, D. & Wodinsky, I. (1968). *Cancer Res.* **28**, 1955.

Schrecker, A. W. & Huennekens, F. M. (1964). *Biochem. Pharmac.* **13**, 731.

Van Scott, E. J. & Ekel, T. M. (1963). *Archs Derm.* **88**, 373.

Weber, K. & Osborn, M. (1969). *J. biol. Chem.* **244**, 4406.

Werkheiser, W. C. (1961). *J. biol. Chem.* **236**, 888.

Werkheiser, W. C., Zakrzewski, S. F. & Nichol, C. A. (1962). *J. Pharmac. exp. Ther.* **137**, 162.

Yoshino, T. & Condit, P. T. (1969). *Abstr. 60th Meet. Am. Ass. Cancer Research, San Francisco*, p. 15.

Zakrzewski, S. F. (1963). *J. biol. Chem.* **238**, 1485.

Zakrzewski, S. F., Hakala, M. T. & Nichol, C. A. (1962). *Biochemistry, Easton*, **1**, 842.