

**MULTIPLE FORMS OF DIHYDROFOLATE REDUCTASE**

**MULTIPLE FORMS OF DIHYDROFOLATE  
REDUCTASE FROM CULTURED MAMMALIAN CELLS**

By

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SCOPE AND CONTENTS:

Dihydrofolate reductase from a subline of the L1210 lymphoma was purified by affinity chromatography using substituted Sepharose -4B to which was coupled methotrexate, a specific, tight binding inhibitor of the enzyme. The purified enzyme was subjected to disc gel electrophoresis at pH 8.5. At least two bands of activity were detected on the gel by the formation of a reduced formazan. Their ratios were dependent on enzyme concentration. Similar bands were found in the presence of EDTA ( $10^{-6}M$ ), 4M and 8M urea. When a substrate, NADPH ( $5 \times 10^{-5}M$ ), was added to the buffers used in electrophoresis, three bands of enzyme activity were present in a fixed ratio which was independent of enzyme concentration. Protein bands showed a different but constant ratio. When folate replaced dihydrofolate as substrate in the assay mixture, the bands of activity corresponded at high concentrations of the enzyme. When activity was detected in the presence of an increasing concentration of methotrexate, different inhibition of the bands resulted. Preliminary experiments with crude extracts of the same subline gave activity profiles with multiple peaks.

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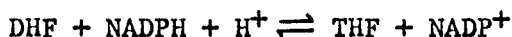
## CHAPTER 1

## Introduction

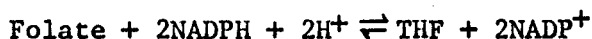
### 1.1 Dihydrofolate reductase

#### 1.1.1 Role in metabolism

The enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NAD(P)<sup>+</sup> oxidoreductase (EC 1.5.1.3)) catalyses the reduction of dihydrofolate (DHF) (fig. 1b) by reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to 5,6,7,8-tetrahydrofolate (THF) (fig. 1d) as shown by the equation:



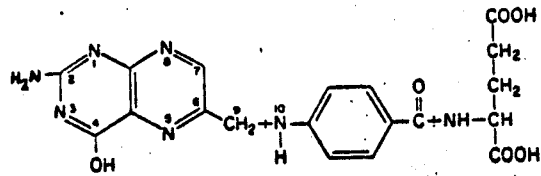
Most dihydrofolate reductase enzymes from vertebrate sources catalyze the reduction at a much slower rate with NADH (reduced nicotinamide adenine dinucleotide) as reductant than with NADPH. As well, the reduction of folate (fig. 1a) to THF occurs at a much slower rate than with DHF (1,2) according to the equation:



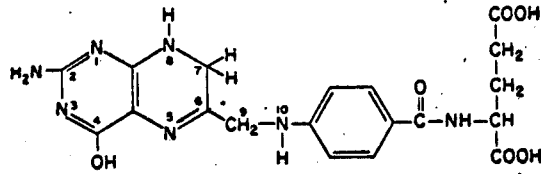
The enzyme is of importance since it forms part of a cyclic reaction in which THF receives a one carbon unit which is then transferred to deoxyuridylate to form thymidylate and DHF (see fig. 2).

#### 1.1.2 Distribution

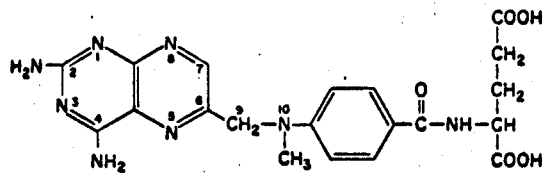
The enzyme occurs in the liver of chicken (3,4), the tissues of various vertebrates (5) including the leucocytes of patients with leukemia (6), and in many micro-organisms (2,1). In rat, the highest activities are found in the liver and kidney (7). Developing tissues also have a high content of the reductase while the activity in many tumors is comparable



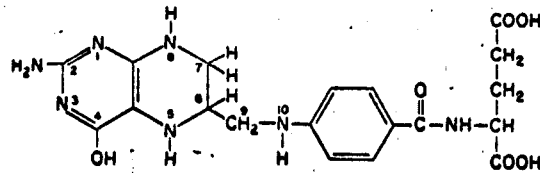
a) folic acid



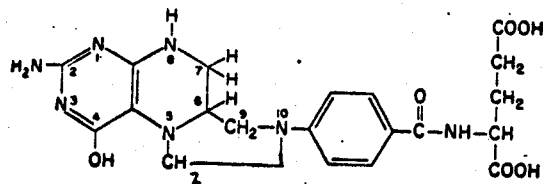
b) dihydrofolic acid (DHF)



c) methotrexate (MTX)



d) 5,6,7,8 - tetrahydrofolic acid (THF)



e) 5,10 - methylene tetrahydrofolic acid

Fig. 1-- Folic acid and derivatives

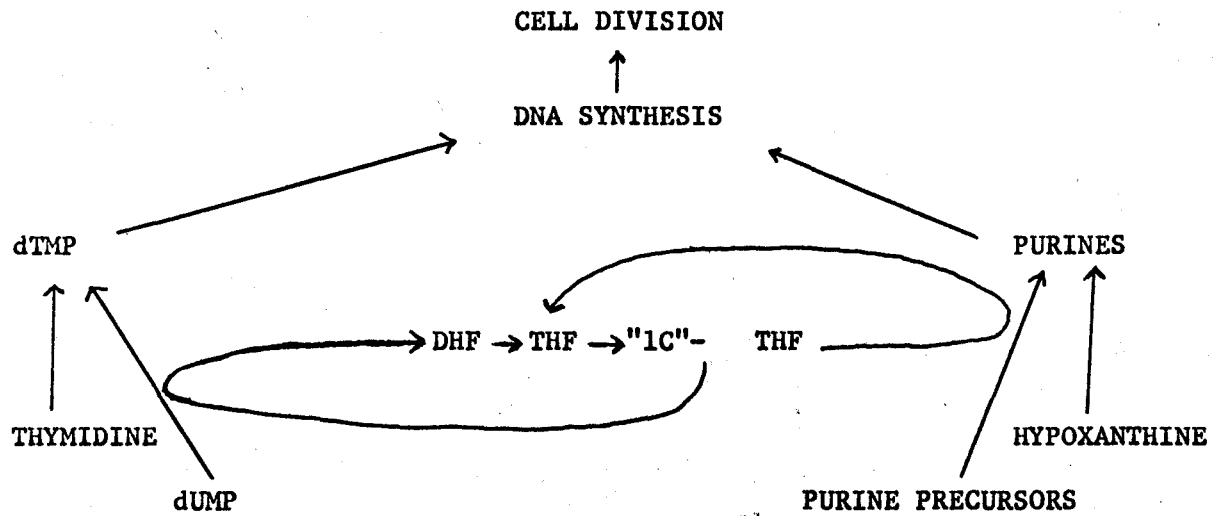


Fig. 2 Metabolic interrelationships of folate derivatives. The abbreviations are: dTMP, thymidylate; dUMP, deoxyuridylate; DHF, dihydrofolate; THF, 5,6,7,8 - tetrahydrofolate; "1C"- THF, various tetrahydrofolates carrying a one carbon group.

with the highest level in the host tissues. The enzyme is mainly present in the cytoplasmic and microsomal fractions of rat liver cells (8). The source of the enzyme for this project was a mouse lymphoma with high enzyme activity.

### 1.1.3 Purification and properties

The enzyme has been purified from a subline of L1210 murine lymphoma (9) by ammonium sulfate and acid precipitation, gel filtration on Sephadex G-100 and chromatography on hydroxylapatite. The resulting purification was 1000-fold with a 49% yield and purity nearing 100%. The specific activity of the enzyme was 44.5  $\mu$ moles of substrate per minute per mg. of protein and the turnover number 1190 moles of substrate per minute per mole of enzyme near the optimum pH. The enzyme had a high affinity for DHF and NADPH, the  $K_m$  for both being in the order of  $10^{-6}$  M. Recent evidence suggests that 2 molecules of NADPH can bind to the chicken liver and Lactobacillus casei enzymes since they can catalyze the transhydrogenation of NADPH and acetylpyridine NADP (10). The reaction mechanism from kinetic data for L1210 enzyme is rapid equilibrium, random (11). This enzyme has a cysteine residue which reacts very rapidly with paramercuribenzoate and has a molecular weight of approximately 20,000 as determined by gel filtration on Sephadex (9). Similar values have been obtained for chicken liver enzyme by sucrose gradient ultracentrifugation and sedimentation equilibrium studies (12). When NADPH and DHF are used as substrates, the purified enzyme from most mammalian cells has double pH optima (9,12) but where folate or NADH is substituted, a single pH optimum is observed.

A simpler purification procedure than that described above has been developed in this laboratory (13) and will be described in section 3.2.

#### 1.1.4 Activation by salts, mercurials and urea

Dihydrofolate reductase is stimulated in the presence of reagents which fall into the following groups (1,4,9):

1. Salts, urea and guanidine which can alter the tertiary structure of the enzyme by changing hydrophobic, hydrogen and ionic bonds.

2. Reagents which react with sulfhydryl groups such as mercurials, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and iodine.

Such stimulation can be as great as 5-fold (14). Various salts cause maximum activation at different molarities. Such an effect is very rapid, in the order of 15 seconds, and is quickly reversed on the removal of the salt (14). That a conformational change in the enzyme may be responsible for the increased activity is consistent with the following observations:

1. There is an alteration in the relative substrate specificity (15).

2. The double pH optima curve changes to a single bell-shaped curve on the addition of activators (14).

3. There is an increased  $K_m$  for NADPH as well as an increased  $V_m$  (14).

The mercurials most often used are methylmercuric bromide and p-mercuribenzoate (PMB). They apparently combine with the free sulfhydryl group of a cysteine residue in the protein (16) and as expected, the resulting stimulation is reversed by excess thiol. Substrate both stabilizes the more labile mercurial-enzyme complex and prevents deactivation by excess thiol (16) which again is evidence for a conformational change.

Some reductases from mammalian sources such as calf thymus (17) and Sarcoma 180 (18) are not activated by urea and mercurials nor are the enzymes from a variety of bacterial strains.

## 1.2 Inhibitors of dihydrofolate reductase

Many analogs of folic acid strongly inhibit dihydrofolate reductase. The most widely used of these is methotrexate (MTX, amethopterin, 4-amino-10-methyl-4-deoxyfolic acid) (fig. 1c).

At pH 5.5, the L1210 lymphoma enzyme is stoichiometrically inhibited by methotrexate (9). The dissociation constant for the enzyme-inhibitor complex is so low (in the order of  $10^{-10}$  to  $10^{-11}$  M) that although the binding is reversible, in practice the enzyme can be titrated with this inhibitor (19). This type of binding represents a special case for which the kinetics have been formulated (20). In a higher pH range and in the presence of KCl, the binding becomes more reversible and the inhibition resembles normal competitive inhibition (21).

Some sublines of L1210 lymphoma cells are more resistant to methotrexate in cell culture than the parent strain, while others are more sensitive (22). The sublines used in this project have resistance proportional to the amount of enzyme per cell although in other cases resistance is due to impaired membrane transport of the drug (23). The variation in enzyme activity in the L1210 cells is believed due to a genetic mutation since the enzyme level stays constant during many generations of culture without the inhibitor. This is not the case with a human hematoblastoid line RPM1 4265 which produces a greater reductase activity (3.7 fold) when the cells are cultured with sublethal levels of MTX (24). There is only a slight increase in the enzyme in the L1210 sublines under similar conditions.

Extensive investigations have been carried out on the design of dihydrofolate reductase inhibitors by systematically varying the substituent groups of folate and analogs in order to determine the stereochemistry of

the binding sites on the enzyme and with the hope of obtaining inhibitors more useful in chemotherapy (25).

### 1.3 Assay

The usual assay for the enzyme measures the decrease in absorbance at 340 m $\mu$  caused by the oxidation of NADPH and reduction of DHF (22). The change in molar extinction coefficient for DHF and NADPH to THF and NADP<sup>+</sup> has been determined as 12,300 (26). The reduction of folate can be assayed in a similar manner with the equivalent coefficient of 18,480 (27).

A colorimetric method to detect enzyme activity is based on the formation of a colored formazan which results from the chemical reduction of the compound 3-(4,5-dimethylthiozoly-2)-2, 5-diphenyl tetrazolium bromide (MTT) by THF (28). This method can be used to detect enzyme activity after electrophoresis (29) or in tissue sections (28). Less widely used assay procedures include fluorometry (30) and estimation of the product THF by oxidative degradation (31).

### 1.4 Multiple forms of dihydrofolate reductase

#### 1.4.1 Chromatography

Two forms of enzyme from a L1210 subline have been isolated by column chromatography on hydroxylapatite (9). About 20% of the total activity was recovered at low specific activity following washing of the column by a 0.05M potassium phosphate buffer while the remaining amount, at high specific activity was eluted with 0.075M buffer of similar composition. Both fractions were inhibited to the same degree by methotrexate and showed similar maximum velocities in the presence of urea, KCl, guanidine-HCl and PMB. Dialysis and rechromatography on hydroxylapatite resulted in the same distribution. When chromatography of either fraction



was performed in the presence of  $10^{-5}$  M NADPH, 90% of the activity was found in the low specific activity peak and the remainder as before.

Chicken liver enzyme has also been separated into two forms, in this case on DEAE cellulose and CM-Sephadex column chromatography (4). In the later case, only one peak was found if NADPH was included in the elution buffer. Two forms have also been reported for the enzyme from a methotrexate-sensitive bacterium Streptococcus faecium var. durans strain A(32,33). Preliminary evidence indicates that the dihydrofolate reductase activity is separable into two enzymes, one of which also reduces folate.

#### 1.4.2 Electrophoresis

Three enzyme forms have been reported in the case of the chicken liver enzyme after electrophoresis on cellulose acetate membrane at pH 8.5 (29). The activity was detected on the membrane by a modification of the MTT assay described in section 1.1.3. The band which moved slowest toward the positive electrode contained 55% of the activity and was called peak I; the fastest moving band (peak III) contained 7%; peak II, the intermediate band, contained 38%. The amounts varied somewhat from preparation to preparation. Sometimes, peak II appeared as a doublet, II'. This peak was found to result from dialysis or treatment with PMB. Preparative electrophoresis showed the forms were not interconvertible and only small differences were found in their properties.

#### 1.4 Purpose of the project

- 1) To separate the forms of dihydrofolate reductase, in crude and purified preparations from various sources.

- 2) To study the properties of these forms, in more detail, from the LM4 subline.

## 2.1 Reagents and materials

Reagents and materials were obtained from the following sources: NADPH and MTT, Sigma; Methotrexate, Lederle; acrylamide electrophoresis grade and other gel reagents, Eastman Organic Chemicals; Ampholine, LKB Produkter AB; cell culture materials, Grand Island Biological Company. All other materials were of the highest grade available. We prepared dihydrofolate by the method of Blakley (34). Materials for the preparation of columns were obtained from Pharmacia Fine Chemicals except for the Bio-Gel HT (Hydroxylapatite) which was obtained from Bio-Rad Laboratories.

## 2.2 Cell sources and extracts

The enzyme was purified from an L1210 lymphoma subline, LM4 (35), which initially grew in suspension culture in which it was maintained and used as a source of small quantities of cells. When larger quantities of cells were required, we grew  $1$  to  $2 \times 10^8$  cells in culture, injected BDF<sub>1</sub> mice intraperitoneally with  $5 \times 10^6$  cells per mouse, and after 6 days, when the specific activity of the enzyme was highest (35), killed the mice by ether inhalation and washed the cells from the peritoneal cavity. The cells were concentrated by centrifugation and frozen for future use.

In contrast to the LM4 subline, with high resistance to MTX and enzyme of high specific activity, another subline, LS2 (35), has low resistance and specific activity. This subline did not grow satisfactorily in the mouse and so we were limited to smaller quantities of cells from

culture.

The third cell type used was a human hematoblastoid line, RPM1 4265. This cell line responds to sublethal levels of MTX with increased specific activity of dihydrofolate reductase as described in section 1.2.

Crude extracts were made at 4° in 0.05 M Tris-HCl pH 7.5 by suspending LM4 cells at 10<sup>6</sup> per ml and LS2 or 4265 cells at 10<sup>7</sup> per ml and disrupting them by sonic oscillation for 20 seconds at a setting of 30 (microprobe tip) on a Bronwill Biosonik III generator. After centrifugation, the supernatant was assayed and used within 48 hours.

### 2.3 Assay procedure

The assay procedure has been described (36). The assay mixture consisted of 100 µmoles of Tris-HCl pH 7.5 (or 8.5 in some instances noted in the results), 150 µmoles KCl, 1 µmole 2-mercaptoethanol, 0.1 µmole NADPH, 0.05 µmoles dihydrofolate, with water and enzyme added to one milliliter. The enzyme was added to a mix composed of all ingredients except DHF and reductase and incubated for four minutes at 37°C. DHF was then added, the contents of the cuvette mixed and the change in absorbance at 340 mµ recorded on a Gilford 2400 recording spectrophotometer. Initial slopes were measured on the chart paper, corrected for a blank consisting of the above mix without enzyme and converted into µmoles of substrate reacted per minute per ml of enzyme, using the change in millimolar extinction coefficient of 12 (26).

A unit of activity is defined as 1 µmole of substrate reacted per minute. A specific activity of 1 refers to 1 unit of activity per mg of protein.

A modification of the assay procedure was used to "titrate" the

enzyme. If the pH was lowered to 6.0 and the KCl omitted, the binding of MTX to the enzyme became stoichiometric (8). We used 0.1 M Tris-maleate buffer pH 6.0 instead of the usual 0.1 M citrate-phosphate buffer pH 6.0 since the enzyme appeared to be more stable in the presence of the former. Since NADPH is labile at pH 6.0, it was not added to the mix until just prior to the enzyme addition. We plotted enzyme activity against increasing volume of enzyme in the presence of a constant concentration ( $10^{-9}$  M) of MTX and obtained a graph typical of a tight binding inhibitor (20). The points on the linear section were fitted by the least squares method using the computer program LINE (37). We assumed a 1:1 binding between MTX and enzyme (30) and thus the intercept on the enzyme volume axis can be used to calculate the number of moles of enzyme present in that volume. By comparing this with a known activity for the same sample, we calculated a turnover number in moles of substrate per min per mole of enzyme.

#### 2.4 Protein determination

We determined protein by the method of Lowry et al (38). Standard curves were made with bovine serum albumin. Some buffers contained 2-mercaptoethanol which was found to interfere with the color development. We dialyzed such samples for 18 hours against 500 ml of the same buffer without 2-mercaptoethanol.

#### 2.5 Electrophoresis

##### 2.5.1 Disc gel method

Electrophoresis was performed at 4°C in a Canalco apparatus, model 1200, using 7.0% polyacrylamide with riboflavin as a catalyst (39). We layered the enzyme on the gels in a volume of 0.2 ml of 12% sucrose

and bromophenol blue. The tank buffer was 0.038 M Tris-glycine pH 8.5 and the gel buffer 0.4 M Tris-HCl pH 8.9. A current of 1 mA per tube was applied for 30 minutes, then increased to 2 mA per tube for a further hour and one-half. The addition of other substances to the solutions will be described in the results.

### 2.5.2 Activity stain

The gels were incubated at 25°C in 2 ml of reaction mixture to localize the enzyme by the MTT method outlined in sec 1.1.3. The incubation mixture contained 0.1 M Tris-HCl buffer pH 7.5; KCl, 0.15 M; MTT, 0.4 mg; dihydrofolate,  $5 \times 10^{-5}$  M; NADPH,  $4 \times 10^{-4}$  M. When the color was sufficient (dependent on many factors outlined in the results), we scanned the washed gels at 600 or 560 m $\mu$  in a Gilford model 2410 gel scanner and recorded the absorbance. In some instances other substances were added to the mix. We ran control gels by omitting DHF from or adding  $5 \times 10^{-5}$  M MTX to the reaction mixture. When folate activity was determined, we substituted 0.1 M phosphate-citrate buffer, pH 6.0 and folate,  $1.5 \times 10^{-4}$  M, for the corresponding reagents described above. When the time of reaction was important, the DHF was omitted from the mix and added to individual tubes to start the reaction.

### 2.5.3 Protein stain

Gels were stained in an amido-black solution (5 parts methanol, 1 part acetic acid, 5 parts water and 0.1% amido black) for 1 hour, washed twice with 5% trichloroacetic acid solution and allowed to stand in this solution for 48 hours or more in the presence of a small amount of Dowex 1-X8. The gels were washed and scanned at 600 m $\mu$  as described in the previous section.

## 2.6 Isoelectric focusing

### 2.6.1 Isoelectric focusing on polyacrylamide gels

We followed the method of Wrigley (40) and used the electrophoresis apparatus described in the previous section. The 10% acrylamide solution containing the enzyme sample and 0.9% carrier ampholytes was photopolymerized at 25°C. The enzyme sample, dialyzed against 500 ml of 0.005 M Tris-HCl pH 7.5 for 18 hours to remove excess salt and buffer, was assayed and 0.1 units of activity mixed with the gelling solution. In some instances when the protein concentration was low, gelling did not occur and we added 100 µg of bovine serum albumin to the mix. A constant current of 1 mA per tube was applied up to a maximum voltage of 350 volts which was maintained for 3 hours. The enzyme activity was visualized as described in section 2.6.2, and the protein by suspending the gels for approximately 10 minutes in an 8-anilino-1-naphthalene-sulfonic acid (ANS) solution (41), and washed with water before viewing under an ultraviolet light. Slices (0.5 cm) of gel were crushed in 0.5 ml water and the pH determined.

### 2.6.2 Isoelectric focusing in a sucrose gradient

Small scale isoelectric focusing was attempted on a sucrose gradient. An acrylamide solution in 40% sucrose was prepared as in section 2.6.1 and 0.3 ml photopolymerized in the narrow end of a Canalco gel destaining tube. Dense (40%) and light (10%) sucrose solutions were prepared with dialyzed enzyme and 0.9% in carrier ampholytes. The bottom buffer, 0.4% triethanolamine contained 40% sucrose to prevent disintegration of the gel plug and the upper buffer, 0.2% H<sub>2</sub>SO<sub>4</sub>, contained 7% sucrose to reduce diffusion. The 10% to 40% sucrose gradient was prepared in the tube by

adding 0.2 ml aliquots of solutions of decreasing sucrose concentration. When the 7% buffer was layered on top of the 2.8 ml gradient, a current of 0.25 mA was applied for 2 hours at 4°C and then increased to 0.50 mA for 6 hours. Fractions (0.1 ml) were collected by lowering a capillary tube down the center of the tube and pumping out the gradient from the bottom. These were assayed as in section 2.3 and some fractions were diluted to 0.5 ml with distilled water to obtain the pH profile.

## 3.1 Method 1

We purified dihydrofolate reductase from LM4 cells for the electrofocusing and preliminary electrophoresis experiments by a multi-step process similar to that described by Perkins et al (9). All purification steps were done at 4°. The yield from 26 mice was  $1.96 \times 10^9$  cells in a packed volume of 16 ml. The cells were lysed in 32 ml of hypotonic solution (0.05 M Tris-HCl pH 7.5) by stirring for 30 minutes and then 2-mercaptoethanol (2ME) was added to give a concentration of 0.1 M. The suspension was centrifuged in an International B-20 centrifuge at 20,000 x g (A211 rotor; 16,000 rpm) for 15 minutes and a sample of the supernatant, called the crude extract, was put aside for assay and protein determination.

Three successive ammonium sulfate fractions (0-25, 25-50, 50-95%) were obtained by slowly adding the solid salt to the stirred solution. After 30 minutes, the fractions were centrifuged as before and the pellets redissolved in the minimum amount of standard buffer: 0.05 M Tris-HCl pH 7.5, 0.1 M KCl, 0.1 M 2ME. Each fraction was assayed for enzyme activity and protein concentration.

We found 84% of the activity in the 50-95% fraction which was then divided into two parts for successive gel filtrations on a Sephadex G-100 column (2.5 x 80 cm) equilibrated with standard buffer. The void volume was found using blue dextran. The absorbance at 280 m $\mu$  was recorded by an ISCO model UA-2 Ultraviolet Analyzer (10 mm flow cells) and 2.9 ml fractions were collected and assayed for enzyme activity (see fig 3).



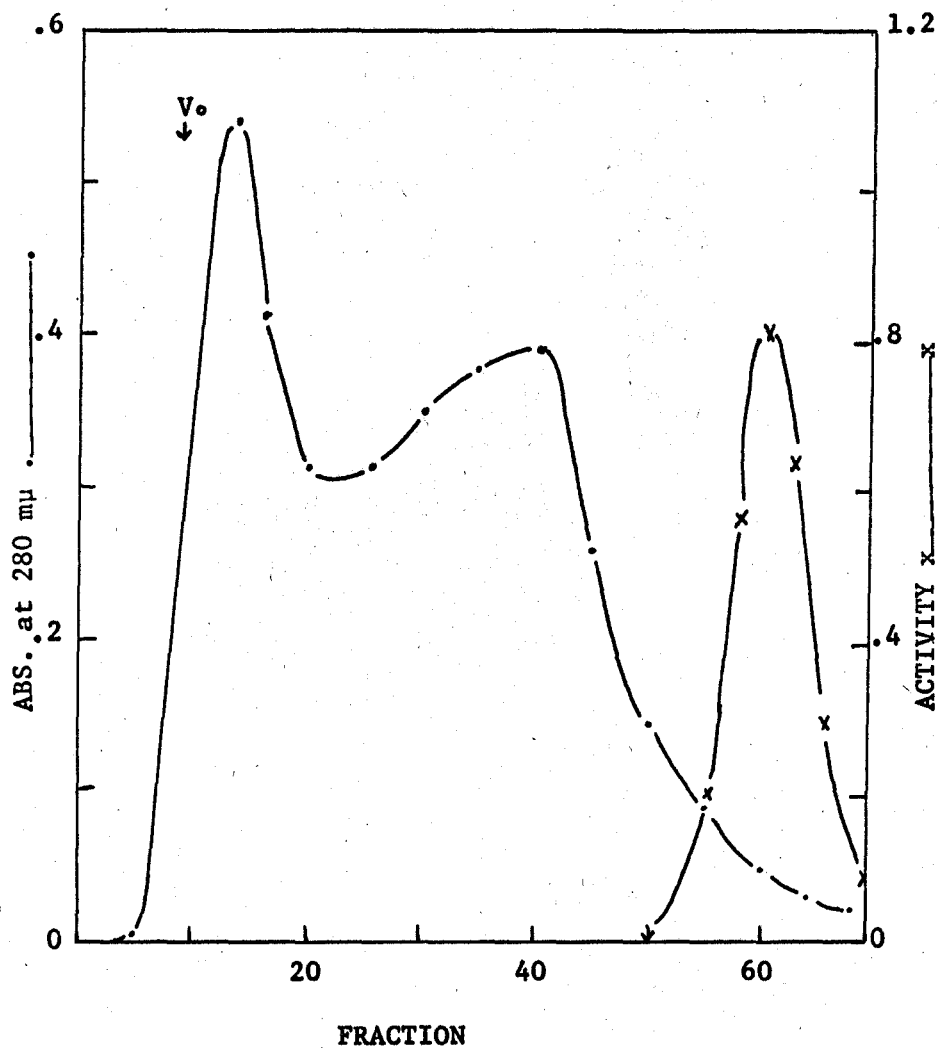


Fig.3 Purification of dihydrofolate reductase by gel filtration on Sephadex G-100. Fractions were in a 2.9 ml volume and  $V_0$  represents the void volume of the column. The measurement of enzyme activity and absorbance at 280 mμ are described in the text.

Those fractions with an activity: absorbance ratio greater than 4 were pooled. This partially purified enzyme was used in many of the experiments.

The results of the purification procedures are summarized in Table 1. The Sephadex G-100 values refer to pooled material from 2 similar runs. Aliquots of this were further purified on a hydroxylapatite column (1 x 1 cm). Purification of large amounts of enzyme was not attempted since the enzyme is labile at this stage and only small amounts were required. About 10-20 units were absorbed on a column which had been equilibrated with 0.05 M potassium phosphate buffer pH 7.0 and 0.1 M KCl. The protein was eluted by a two step gradient of 0.05 M and 0.075 M phosphate buffer both pH 7.0 and 0.1 M in KCl. The absorbance at 280 m $\mu$  was recorded as before and fractions of 3 ml were collected under positive pressure to increase the flow rate. A typical elution profile is shown in figure 4. Only twice did we obtain a double peak activity profile similar to Perkins et al (9). The cause of this is unknown.

The yields and specific activity of the enzyme compared favourably with those of Perkins et al (9), except for the hydroxalapatite step. In our hands, the highest specific activity of fractions from the hydroxylapatite column was 20 units per absorbance unit (280 m $\mu$ ) compared to 45 reported by Perkins et al (9).

We attempted a number of variations on this purification procedure. The pellet after cell lysis and centrifugation was suspended in buffer and sonically disrupted to determine how much activity remained in the cell debris. It was found to be about 15%. Much of this could be recovered by washing the pellet with the standard buffer and adding the supernatant,

Step	Volume (ml)	Total Activity <sup>1</sup>	Total Protein (mg)	Specific Activity <sup>2</sup>	% Recovery
Crude extract	38	80	413.2	0.19	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (50-95%)	13	67.7	130.2	0.51	85
Sephadex G-100	58	59.2	6.0	9.9	74

Table 1 - Purification of dihydrofolate reductase from LM4 lymphoma

1 Units of  $\mu$ moles of substrate per min.

2 Units per mg protein

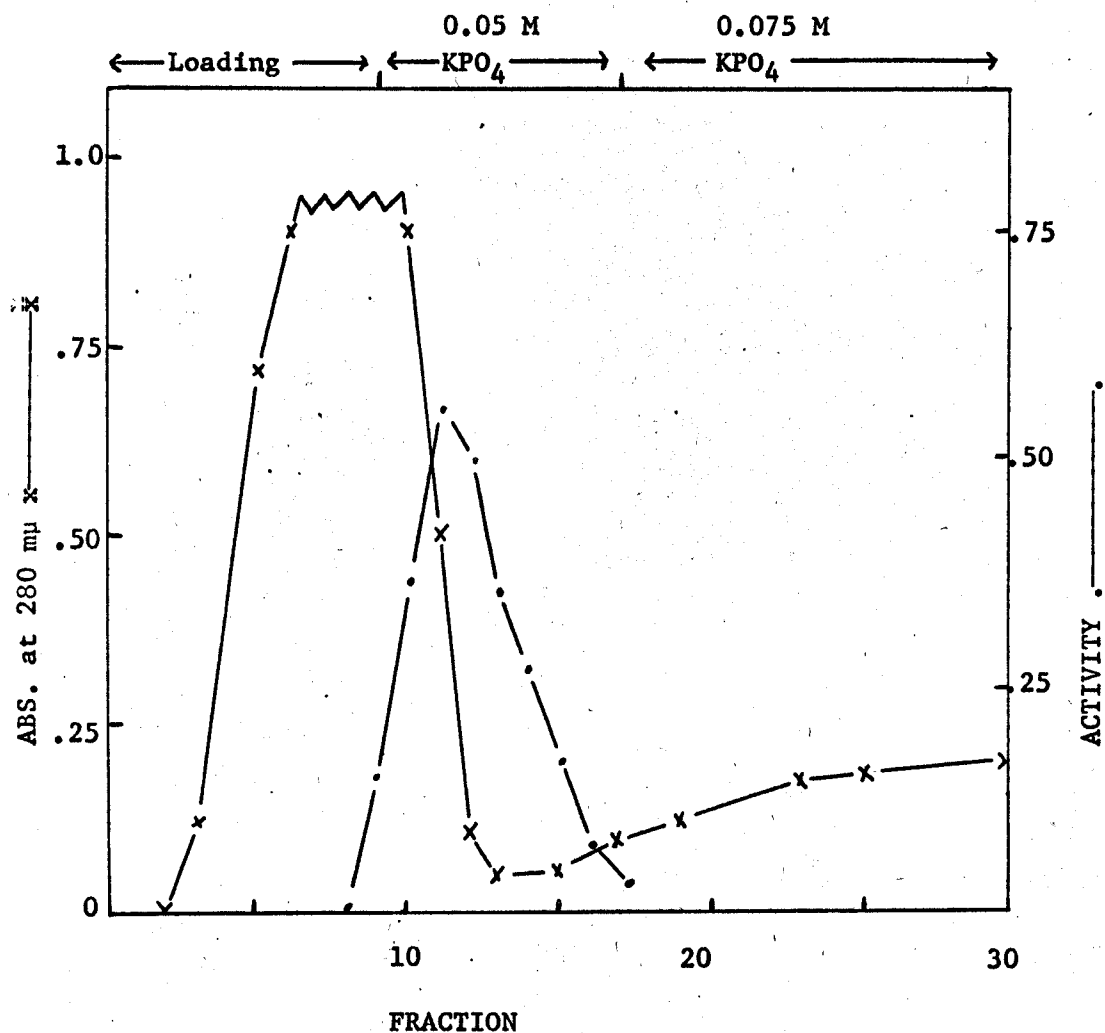


Fig. 4 Purification of dihydrofolate reductase by column chromatography on hydroxylapatite. The details are described in the text. KPO<sub>4</sub> indicates potassium phosphate buffer pH 7.0, 0.1 M KCl.

after centrifugation, to the crude extract. This procedure was carried out in later preparations.

The 2ME in the standard buffer did not have an appreciable effect on the activity or stability of the enzyme over 60 days compared with a similar enzyme sample alone. Since 2ME interfered with the Lowry protein determination (section 2.4) and the absorbance at 280 m $\mu$  by causing an increasing absorbance with time, we discontinued its use in subsequent preparations.

Methotrexate titrations were attempted on samples of all three fractions. We experienced considerable difficulty because the blank was much higher than usual due to the decomposition of NADPH at the acid pH and it was difficult to obtain consistent duplicate values. As noted in section 2.3, these difficulties were partially overcome by using 0.05 M Tris-maleate buffer pH 6.0, adding the NADPH to the mix just prior to the enzyme and using samples of high activity. Our best plot, shown in figure 5, was obtained using a sample of enzyme eluted from Sephadex G-100. The horizontal intercept calculated by LINE (38) was  $3.94 \times 10^{-3}$  ml (standard error  $1.22 \times 10^{-3}$ , 31%) which corresponds to  $1 \times 10^{-12}$  moles of MTX or enzyme. The turnover number for this enzyme sample at pH 7.0 could thus be calculated to be 2100. The values obtained for other samples were in the same order but with much higher standard errors.

### 3.2 Method II - affinity chromatography

Affinity chromatography is a technique whereby a specific binding agent for an enzyme, coupled to a column, retards the passage of that enzyme while contaminating proteins are eluted (52). The method for purification of dihydrofolate reductase is still being developed at this

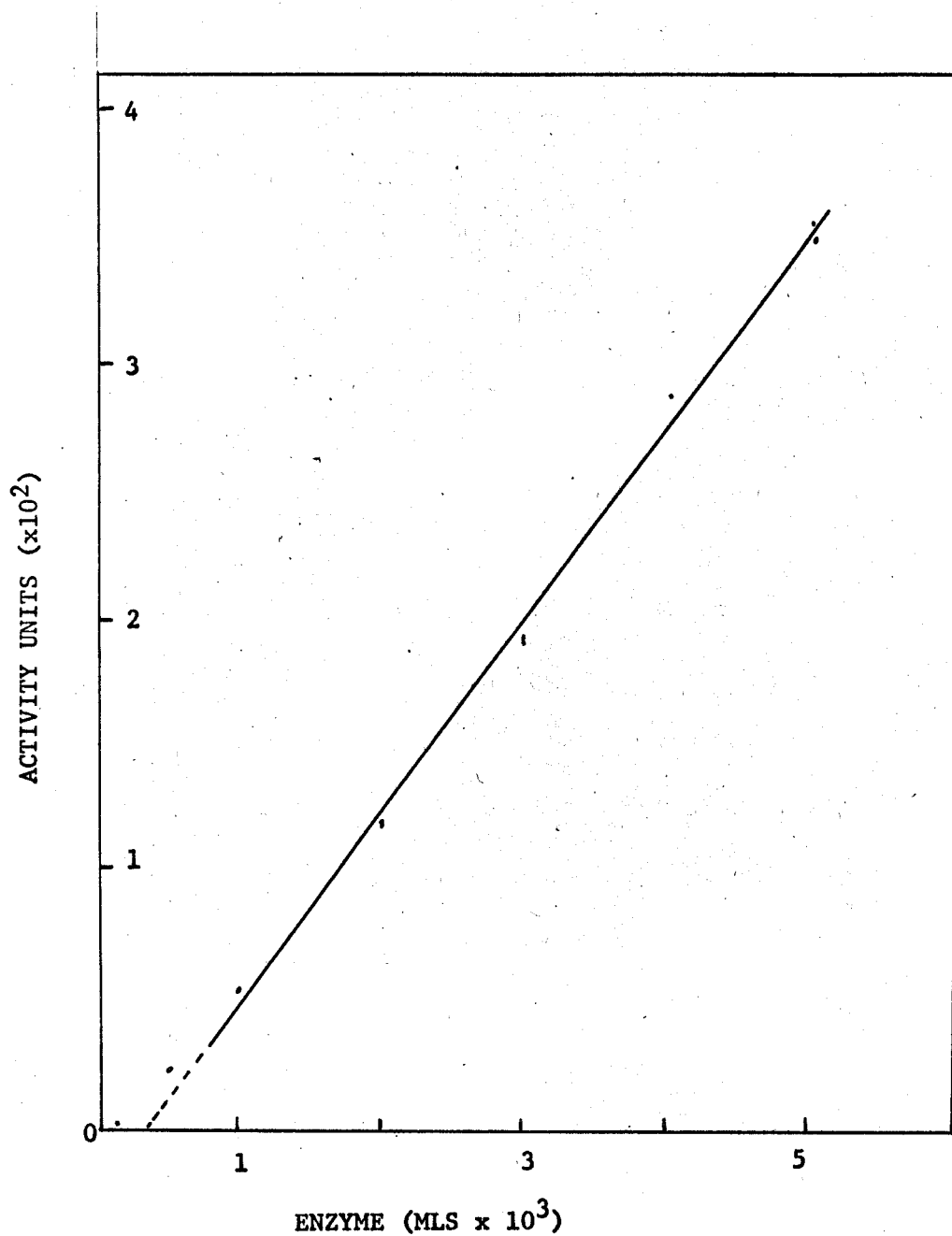


Fig. 5 - Titration of dihydrofolate reductase purified by SG 100 chromatography with MTX. Increasing amounts of enzyme were added to assay mixture which contained a constant amount ( $1 \times 10^{-9}$  M) of MTX as described in the text.

time and details will be published later (13). The cells are lysed as before and a 40-90% ammonium sulfate fraction was prepared. The precipitate was dissolved in a small amount of 0.1 M Tris-HCl pH 7.5 and the solution was applied to a Sephadex G-10 column (2.5 x 12 cm) to remove excess salt and small molecules. The eluted enzyme was then applied to a column of Sepharose 4B to which MTX had been coupled. The column was prepared by stirring aminoethyl Sepharose with 40% dimethylformamide, 1-ethyl-3-(3-dimethylamino propyl) carbodiimide and 10.0 mg of MTX at pH 4.8 for 16 hours, to couple the carboxylic groups of the inhibitor to the aminoethyl Sepharose (42). One liter of 0.1 M Tris-HCl pH 8.5, containing 0.1 M KCl, washed the column (1 x 5 cm) of Sepharose-MTX free of unreacted MTX. When the G-10 material was passed through the column, equilibrated with 0.1 M Tris-HCl pH 7.5, dihydrofolate reductase was retained while most other material was not. The eluting buffer was changed to 0.1 M Tris-HCl pH 7.5, with 1 M and 2 M KCl in succession, in order to remove nonspecific absorbing material. A wash with 0.2 M Tris-HCl pH 8.5 and 2 M KCl then eluted the enzyme. If the enzyme was too dilute after the Sepharose-MTX chromatography, it was concentrated by vacuum dialysis against 0.1 M Tris-HCl pH 7.8 and 0.1 M KCl. An ultraviolet spectrum showed the presence of 260 m $\mu$  absorbing material which could be removed by filtering the sample through a Sephadex G-100 column equilibrated with 0.05 M Tris-HCl pH 7.8 and 0.1 M in KCl.

When this enzyme sample was scanned in the ultraviolet range a typical protein spectrum was obtained. If a turnover number of 2000 is assumed (sec 3.1), and if the enzyme has an absorbance of 1 O.D. unit at 280 m $\mu$  at a concentration of 1 mg per ml, then the enzyme purity nears

100%. There were no other bands of contaminating protein when the concentrated material was subjected to electrophoresis (see section 4.4.7).



#### 4.1 Detection of enzyme activity by formation of formazan

The reduction of the tetrazolium salt, MTT, to a colored formazan to indicate enzyme activity was investigated initially by determining the spectrum, in the visible region, of the complete assay mixture. The formazan produced showed a broad absorbance peak in the 560 to 600 m $\mu$  region compared to a control with MTT absent. We therefore scanned the gels at 600 m $\mu$ .

Numerous blue-purple bands were produced in a set of gels accidentally left near a fluorescent light source for approximately 30 minutes. This observation prompted an investigation of the specificity of formazan formation and a series of experiments were performed involving the various reagents of the gelling and assay mixtures. As expected a solution of MTT, NADPH, DHF and enzyme rapidly yielded a solution of a deep purple color under either light or dark conditions while the omission of any one reagent gave a negative result. Solutions with MTT, riboflavin and a protein (e.g. bovine serum albumin) gave a blue purple color in the presence of light but not in the dark. The polyacrylamide in the gels may also have contributed to the color change but this was not investigated further. Subsequently, all gels were incubated in the absence of light to prevent non-specific color formation from these sources.

#### 4.2 Isoelectric focusing on polyacrylamide gels

Although much time was spent trying to separate the enzyme forms by isoelectric focusing on a polyacrylamide gel, the results were un-

satisfactory. Enzyme prepared by method I, gave two main bands of activity in the basic region of a pH 3 to 10 gel and sometimes bands of low intensity in the neutral region. The ANS stain (section 2.6.1) indicated that the bulk of the protein was effectively removed by the hydroxylapatite step. No change in the band pattern could be observed during enzyme purification (method I).

The main difficulties with the technique were these:

1. Gelling did not take place with samples of low protein concentration. If we added bovine serum albumin (which migrated to the pH 3 to 4 region) gelling did occur. This indicated that protein either was required as a catalyst or was chemically altered during the polymerization process. If a high protein concentration was used, it tended to precipitate in the gel, severely weakening the gel structure. Such gels were difficult to remove and to assay for activity.

2. The enzyme sample required a low concentration of buffer and salt since high concentrations caused distortion in the pH profile. Dialysis against a 0.005 M Tris-HCl pH 7.5 caused 30 to 40% enzyme inactivation.

3. Profiles of the pH were linear over the range of 2.5 to 9.0 when the carrier ampholytes had a range of 3 to 10. This discrepancy will be explained in section 4.3.

4. When enzyme recovery was attempted by slicing and crushing the gels in a small amount of 0.5 M Tris-HCl pH 7.5 and 0.1 M KCl, very little activity was found in any of the solutions. This was probably due, in part, to the small amount of enzyme activity present on the gel.

5. The gels yielded poor activity profiles after assaying and

scanning as described in section 2.5.2, due to discontinuities in the gel itself, precipitated proteins and the low intensity of the formazan stain. A record of the bands was kept by marking their relative positions on a paper facsimile.

#### 4.3 Isoelectric focusing in a sucrose gradient

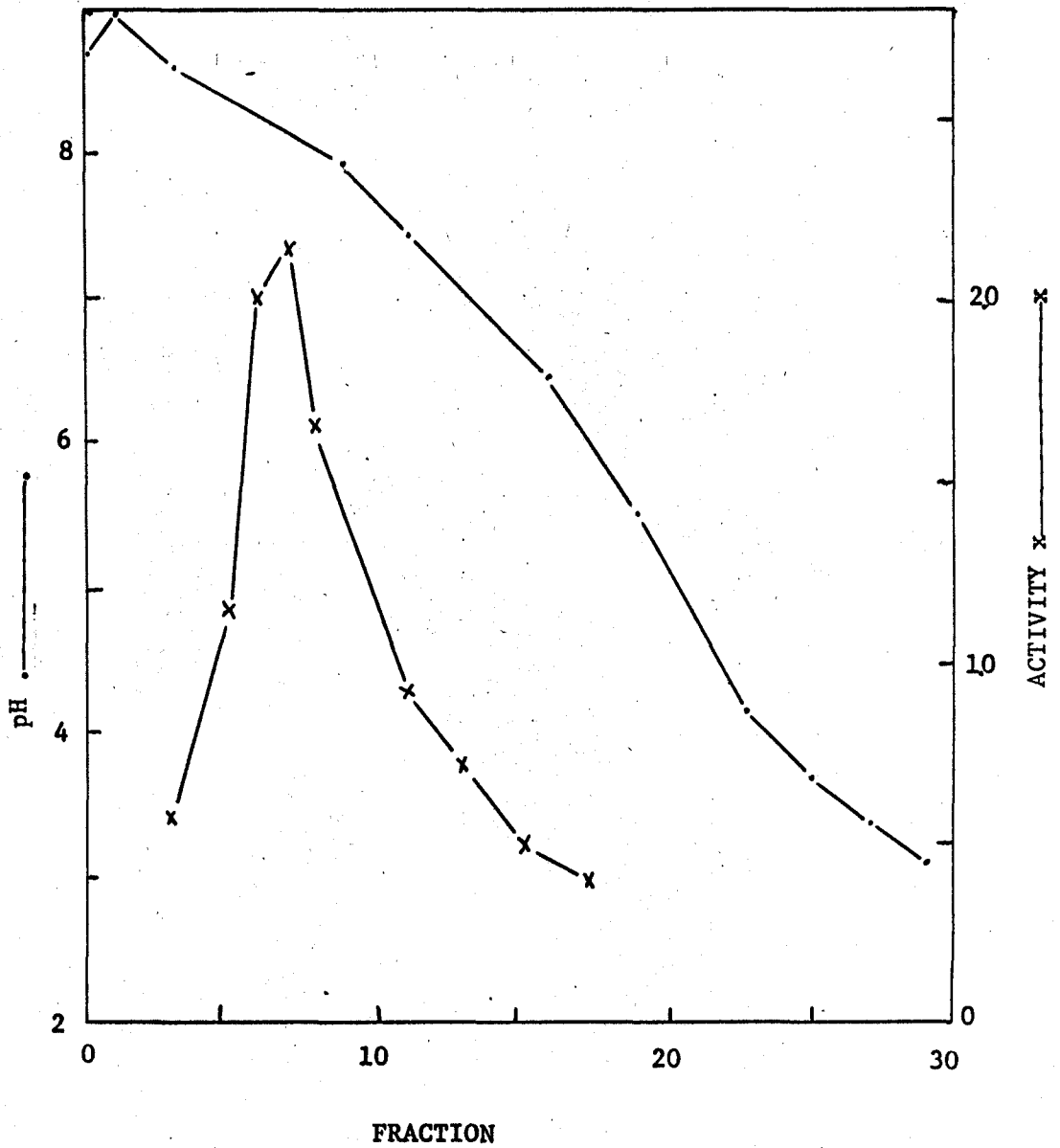
Sucrose gradient isoelectric focusing was somewhat more successful and the formation of two bands in the basic region was observed visually on the intact gradient. The best profile from a number of attempts is shown in figure 6. Multiple bands of activity are not evident on the graph but there is a peak near pH 8.2 and this would be the average isoelectric point for these conditions.

A number of problems were encountered:

1. As in section 4.2, precipitating protein appeared after a few hours and in this case it tended to disturb the gradient by settling to the bottom.

2. The pH profile moved on the gradient. As the bands were forming they moved down toward the negative electrode which resulted in the loss of the basic end of the pH profile as mentioned in section 4.2. The effect was not due to gravity and/or seepage through the polyacrylamide plug since the bands migrated upward when the buffers and polarity were reversed. This migration was faster when a short range pH carrier ampholyte was used in an attempt to get better separation of the enzyme bands.

3. The procedure for pumping out the gradient was not completely satisfactory since the void volume of the tubing was large compared to the sample volume so that considerable mixing may have occurred.



**Fig. 6** Electrofocusing of dihydrofolate reductase on a sucrose gradient. Activity was measured in  $\mu\text{moles}/\text{min}/\text{ml}$  of enzyme. Fractions were of 0.1 ml volume.

In view of these problems, we returned to disc gel electrophoresis as a method of separation and obtained better results.

#### 4.4. Disc gel electrophoresis with purified enzyme

##### 4.4.1 Preliminary experiments

A typical profile produced using 0.06 units of enzyme activity is shown in figure 7. The dye band also absorbed at 600 m $\mu$  but was a useful marker for determining when to terminate the electrophoresis and for the calculation of the  $R_f$  values. These were calculated as the ratio of the migration of the peak to the migration of the dye band both measured from the end of the gel on which the sample had been placed. Various kinetic experiments were done to test whether the profiles have a quantitative significance. Previous studies (43) had shown that the enzyme activity increased linearly with increasing enzyme concentration and, as shown in figure 8, the peak height of the band with the lower  $R_f$  value increased in the same manner, except at low concentrations. A plot of peak area (weight of paper facsimiles of the area of activity) against enzyme volume was also linear. If one substrate concentration is held constant and if the reciprocal of the peak height versus the reciprocal of the concentration of the other is linear, we can calculate the apparent substrate concentration for half-maximal velocity ( $K_m$ ). From figure 9, this was found to be  $1 \times 10^{-4}$  M for NADPH and  $5 \times 10^{-5}$  M for DHF compared with  $10^{-6}$  to  $10^{-7}$  M in the normal assay. A slow rate of diffusion of these substrates into the gel where it reacts with localized enzyme would account for the differences. The specificity of the formazan formation was tested by electrophoresis of identical samples and subsequently omitting DHF from or adding  $5 \times 10^{-5}$  M MTX to the assay mix. Significant

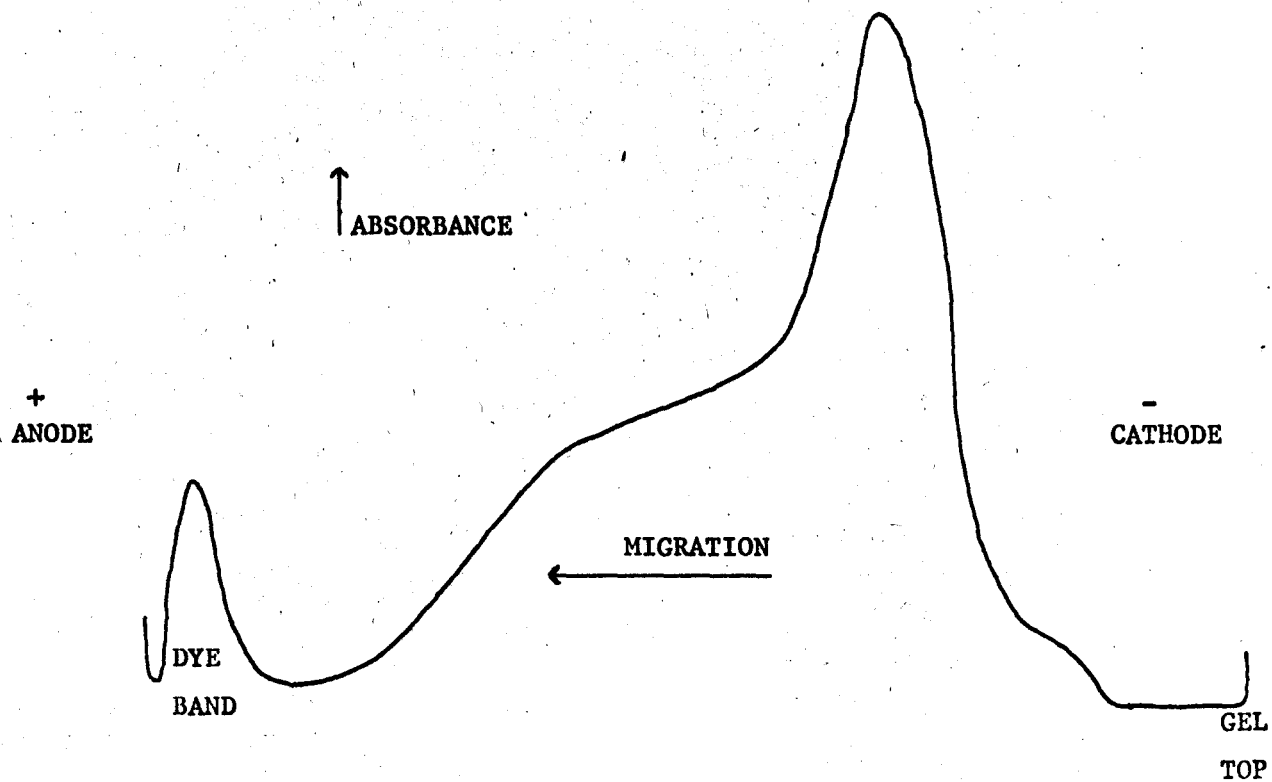


Fig. 7 - A typical activity profile of dihydrofolate reductase after electrophoresis on a polyacrylamide gel. The gels were scanned for formazan at 600 m $\mu$ . Details are described in the text.

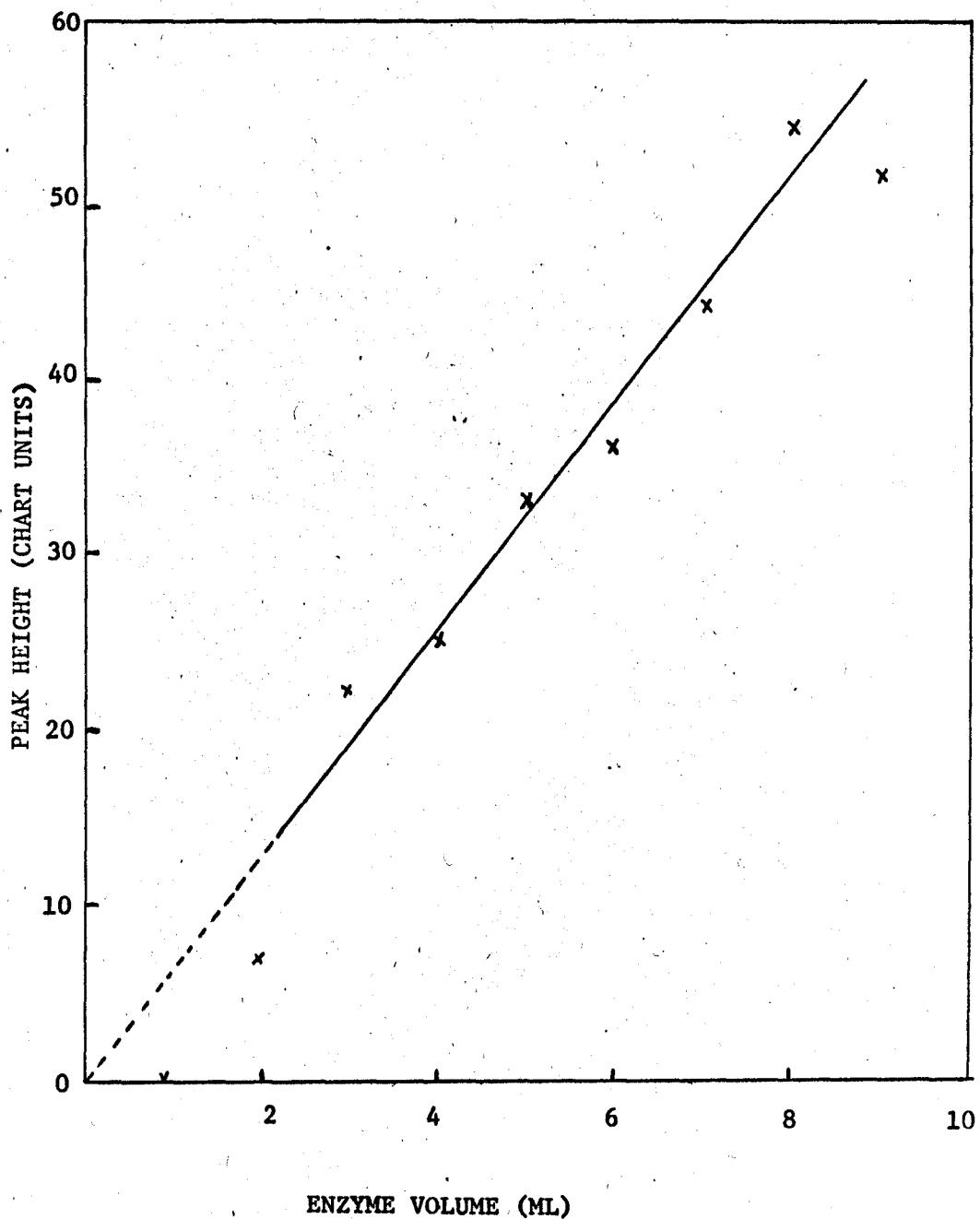


Fig. 8 - A plot of enzyme activity measured by absorbance (peak height) against volume of enzyme applied to a polyacrylamide gel.

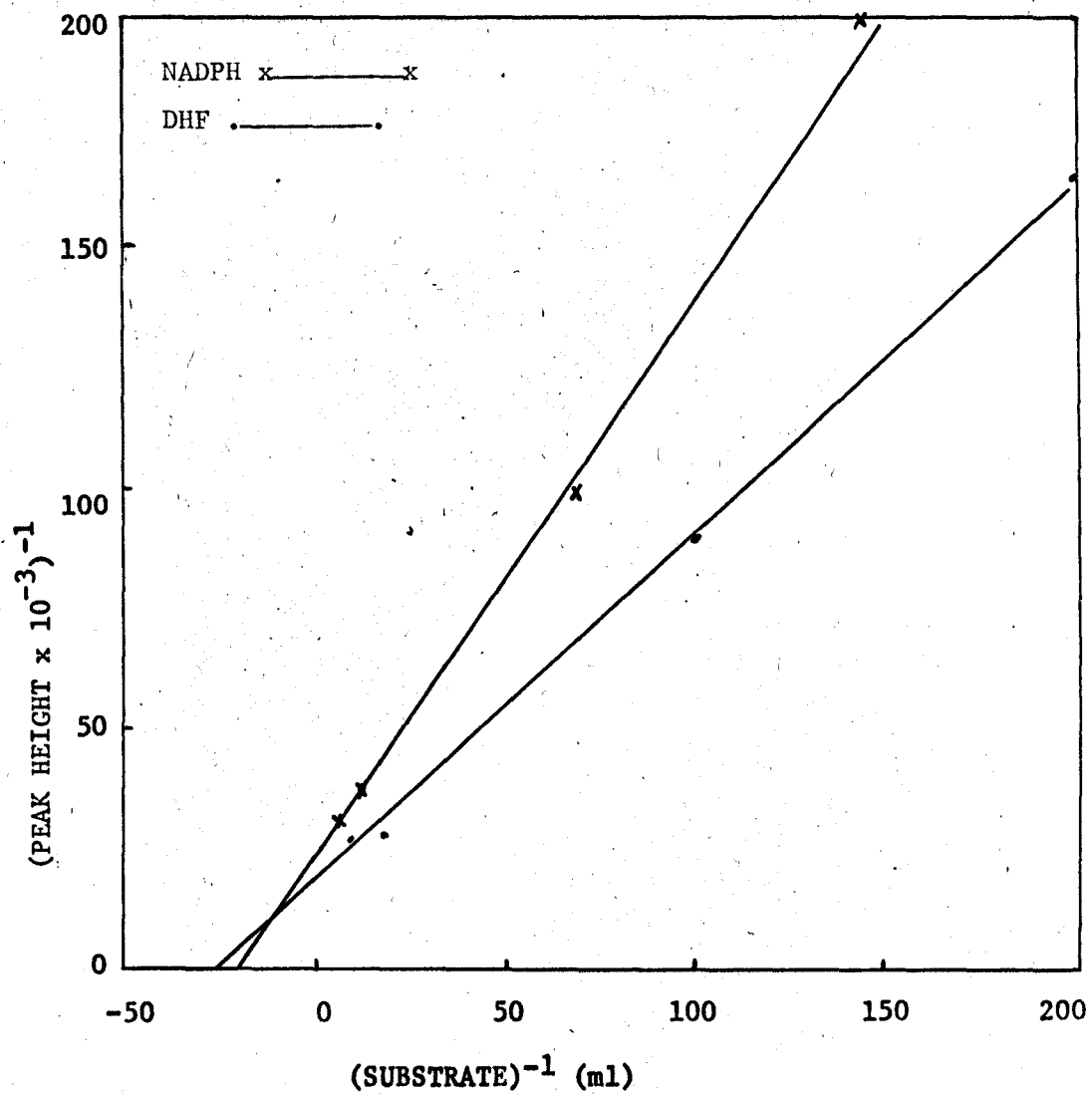


Fig. 9 - Double reciprocal plot of activity as measured by absorbance (peak height) as a function of NADPH or DHF concentration.



non-specific formazan formation occurred only when either crude enzyme was used or when the electrophoresis took place in the presence of NADPH (section 4.4.6).

#### 4.4.2 Dependence of the enzyme profile on enzyme concentration

It was noted in the last section that low concentrations of enzyme did not fit the linear pattern of increasing peak height with volume. At low concentrations ( $1.5 \times 10^{-2}$  units), the left hand peak ( $R_f = 0.64$ ) became the major peak in contrast to higher concentrations ( $>3.0 \times 10^{-2}$  units) where the right hand peak ( $R_f = 0.37$ ) is greater (see fig. 10). Thus it appeared the pattern of enzyme activity was dependent on the concentration of the enzyme since all samples were taken from the same solution of enzyme. The following four sections describe experiments which attempt to explain these results.

#### 4.4.3 Variation of buffer and salt concentration

Since the stock enzyme was dissolved in a buffer solution of Tris-HCl and KCl, the concentration effect of the previous section may have been a function of the buffer-salt concentration. A series of gels was run with increasing volume of enzyme and with the buffer-salt concentrations increased to give the same buffer-salt concentration in all samples (section 2.5.1). The results were identical to those of section 4.4.2. At very high concentrations of salt and buffer, the hyperfine trailing edge was absent and the dye band was more diffuse. For this reason, when a large volume of enzyme was used, as in section 4.4.7, we filtered the sample on a Sephadex G-10 column equilibrated and eluted with 0.1 M Tris-HCl pH 7.8 to rid the sample of its salt content.

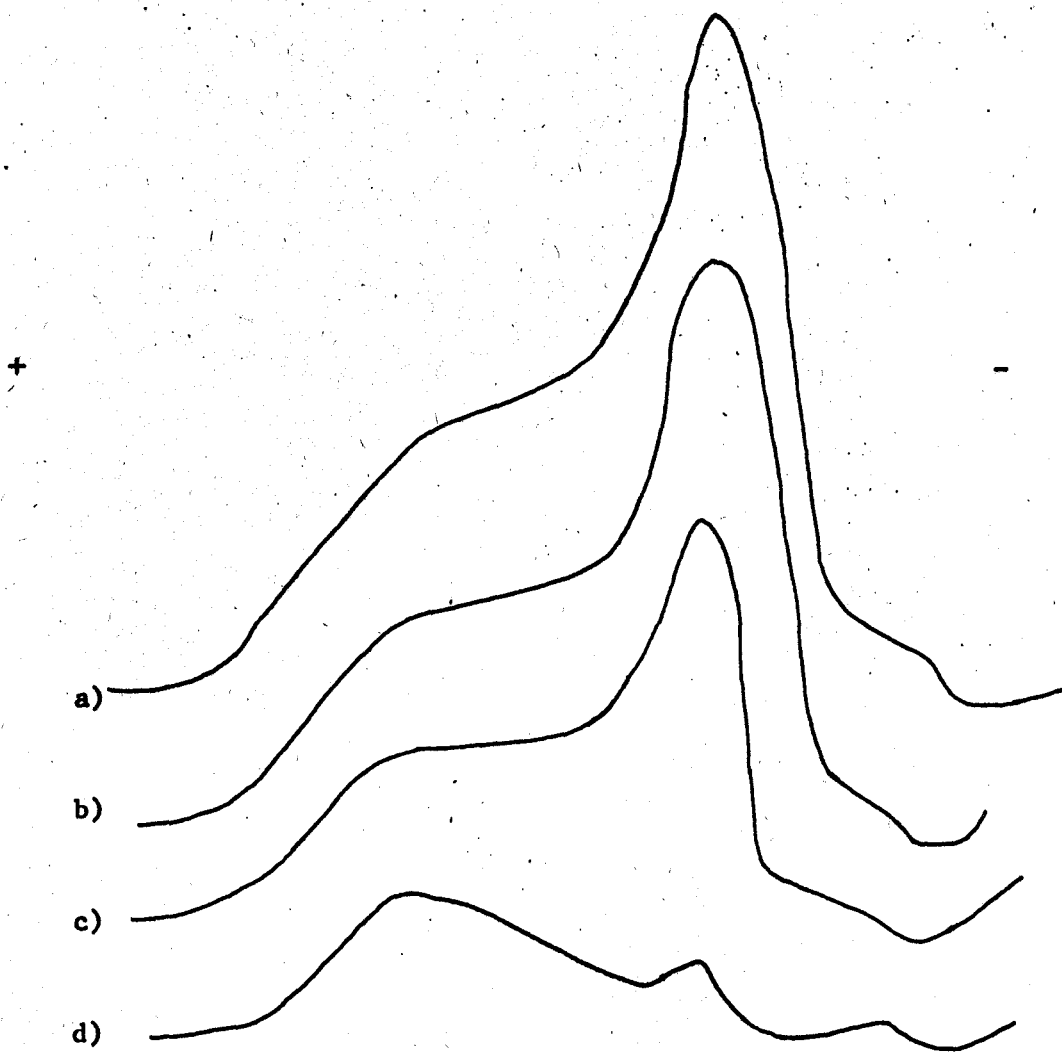


Fig. 10 - Activity profiles of 4 gels containing various concentrations of dihydrofolate reductase; units ( $\times 10^2$ ): a, 6.0; b, 4.5; c, 3.0; d, 1.5. Migration was towards the positive electrode.

#### 4.4.4 Electrophoresis in the presence of EDTA

The results in section 4.4.2 could be explained by the enzyme reacting with residual heavy metals in the buffers (section 1.1.5) and producing the minor fast-moving peak. In order to complex these metals, all buffers were made  $10^{-5}$  M in EDTA. The profiles of a concentration series were as in figure 10.

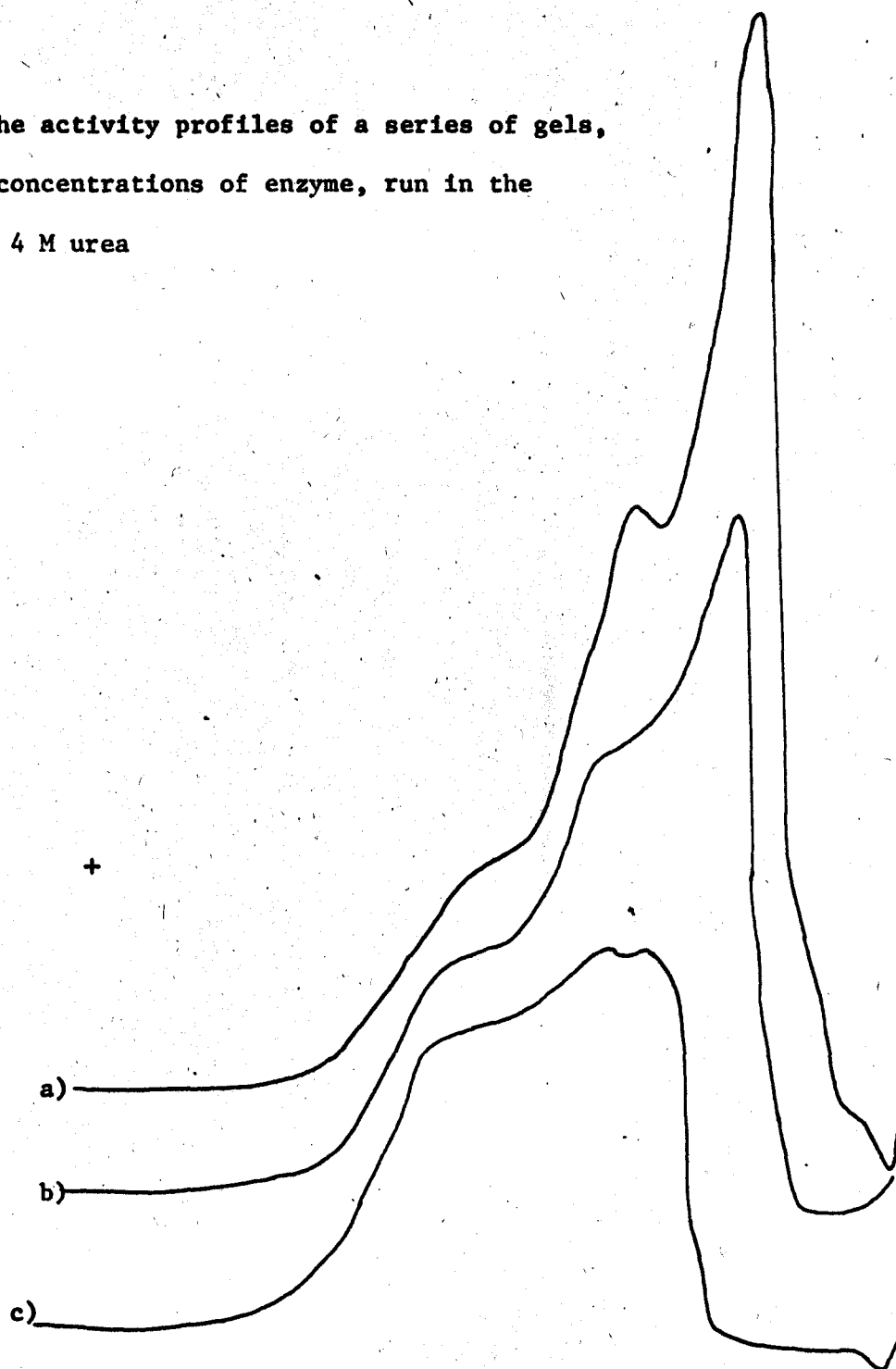
#### 4.4.5 Electrophoresis in the presence of urea

Another explanation of the enzyme patterns observed could be an association of protein units to polymeric complexes. To investigate this possibility, a series of gels was run with 4 or 8 M urea in the buffers. Three peaks were observed (figure 11) in the presence of 4 M urea, the major having an  $R_f$  value of 0.15 compared to that of 0.37 for the normal gel indicating that the enzyme migrated more slowly in the presence of urea. There was still a concentration dependence. There also appeared to be an overall stimulation of activity although this effect was not measured with precision. The enzyme migrated even more slowly in the presence of 8 M urea and had just entered the gel when the dye neared the opposite end. The activity profile was not as well defined in this case but there was still a definite hyperfine trailing edge and a sloping leading edge. The interpretation of these gels was more difficult since the activity was low compared to that in the experiment with 4 M urea. The experiment was repeated, this time allowing the dye to run off the gel and thirty minutes later the current was stopped. The pattern of activity was similar to the previous profile run for a shorter time.

#### 4.4.6 Electrophoresis in the presence of 2-mercaptoethanol

Since the enzyme has an easily accessible sulfhydryl group (section 1.1.5),

Fig. 11 - The activity profiles of a series of gels, at various concentrations of enzyme, run in the presence of 4 M urea

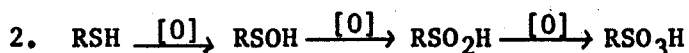


Units ( $\times 10^2$ ): a, 7; b, 4; c, 1. Migration towards the positive electrode.

differences in mobility on the gel may have been due to the oxidation state of the SH group. Two types of oxidation are possible (44):



This type of oxidation can occur under mild conditions e.g. in the presence of molecular oxygen.



Strong oxidants are necessary to promote this type of oxidation and the resulting derivatives are relatively stable.

Three samples were subjected to electrophoresis: first, enzyme from the stock solution; second, enzyme with 0.01 M 2 ME added just prior to the start of electrophoresis, and third, enzyme incubated at 4° in 0.01 M 2 ME for 60 hours. The resulting profiles were very similar for all three gels except for a small increase in the minor peak (compared to figure 7) for gel 2 and slightly more for gel 3. The overall effect was small.

#### 4.4.7 Electrophoresis in the presence of NADPH

In this series, the upper tank, sample and gel buffers contained  $5 \times 10^{-5}$  M NADPH while the lower buffer remained unchanged. Activity profiles are shown in figure 12, where there are narrow distinct peaks with a constant area ratio of 9.3 : 3.9 : 1, independent of enzyme concentration. This is in contrast to the previous experiments without NADPH, where the activity profile changed with concentration. The significance of this observation will be discussed later. The bands of activity migrated further into the gel than usual ( $R_f$  of the major peak is 0.56), possibly due to the binding of the negatively charged NADPH. The activity stain appeared more rapidly, in the order of 15 minutes, compared to 1 hour or more for gels run in the absence of NADPH. For this reason we

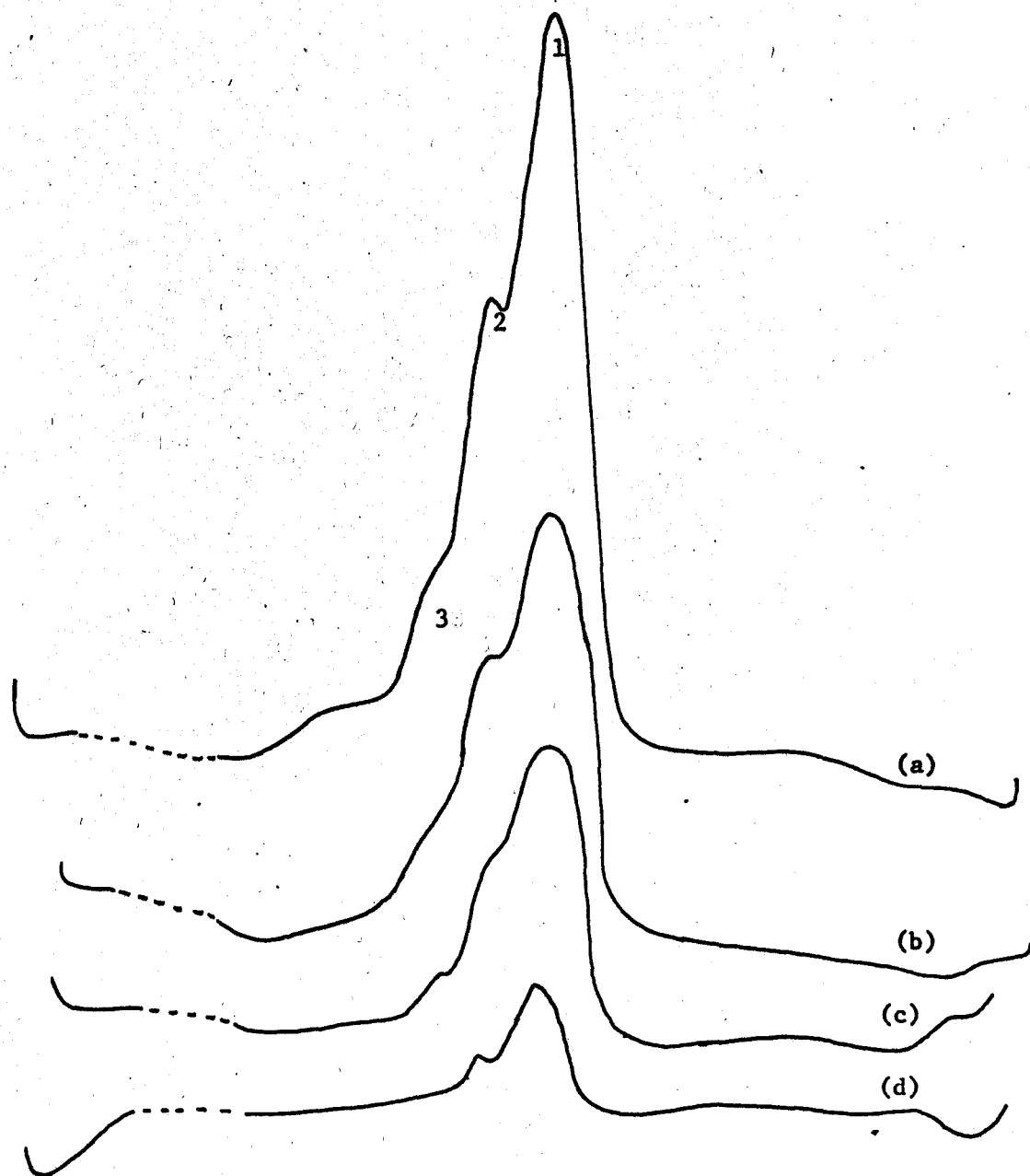


Fig. 12 - The activity profiles of a series of gels run in the presence of  $5 \times 10^{-5}M$  NADPH. The units of activity applied ( $\times 10^2$ ) and time of incubation (min) were: a) 1.5, 40 b) 4.5, 15 c) 3,10 d) 1.5, 5. Migration was toward the positive electrode. There was non-specific staining in the dashed area.

added DHF to the assay mix at intervals so that all gels were scanned at equivalent times. Additional blue-purple bands appeared near the dye marker, and controls, without DHF, proved these to be non-specific. To avoid unnecessary absorbance in this area, the dye was omitted from the sample in later experiments and the progress of the buffer front was followed by a fast-moving refractive band in the gel. Folate reductase activity was detected on gels run with enzyme of high activity and these results will be dealt with in section 4.4.11.

#### 4.4.8 Effect of PCMB

Two series of gels were subjected to electrophoresis. In the first, 5 identical gels were run, and incubated without DHF but with PCMB for not less than twenty minutes, in amounts varying from five times less to twenty times more than the concentration of the enzyme. DHF was added at timed intervals and each gel was scanned 30 minutes later. The profiles were nearly identical to that of figure 7 except for slight increases in the minor peak area. In the second series, the PCMB was added to the enzyme 15 minutes prior to electrophoresis in amounts similar to those above. When the gels were assayed for activity, definite increases in absorbance were noted in the region with an  $R_f$  of 0.70 (compare to an  $R_f$  of 0.64 for the minor peak of section 4.4.2) while the remainder of the profile was typical. The increased velocity of migration of enzyme to which PCMB has been coupled has been noted after electrophoresis on a cellulose acetate support (29). This change was attributed to the extra charge on the benzoic acid residue of PCMB since there was no change in migration with methylmercury bromide.

#### 4.4.9 Effect of methotrexate

Equal amounts of enzyme were subjected to electrophoresis in six identical gels and assayed for activity in the following manner: the first acted as a control; the second was assayed in the presence of MTX at a concentration 1/10 that of the enzyme; the third at 1/5 the concentration; the fourth at equimolar concentration; the fifth at five times the concentration and the sixth at 10 times the concentration (figure 13). The overall effect was a rapid decrease in the height of the minor peak compared to the major. The apparent stimulation of the major peak at low concentrations of MTX was perplexing. The fact that the minor peak decreases more rapidly than the major may be a concentration effect since there is a constant concentration of MTX diffusing into areas of various enzyme concentrations, and since at pH 7.5 the binding is relatively "tight", the small concentration of enzyme could be inhibited totally before there is a large effect on the major concentration of enzyme. This experiment was repeated with gels run in the presence of NADPH and it was found that the peak corresponding to number 2 of figure 12 was inhibited more rapidly than peak 1 in a manner similar to the above. We had difficulty with the high blanks on these gels and the inhibition of peak 3 in figure 12 could not be determined with certainty. The significance of the above observations will be discussed in the light of other results in Chapter 5.

#### 4.4.10 Heat inactivation studies

At an early stage of this project, we attempted to determine the relative stability of the enzyme forms by incubating a sample at 37°C. At time intervals of 0, 20, 40 and 60 minutes, aliquots were withdrawn,



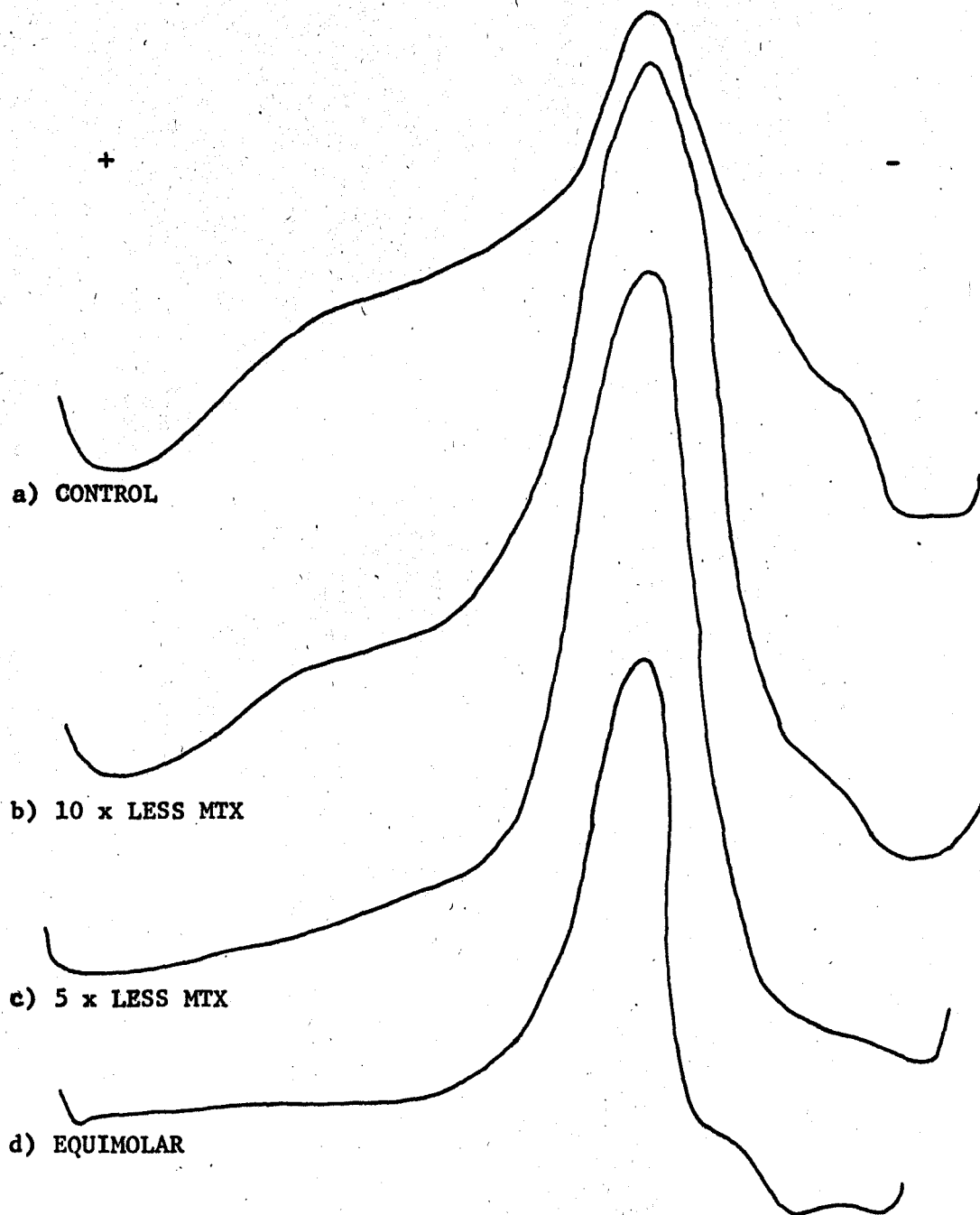
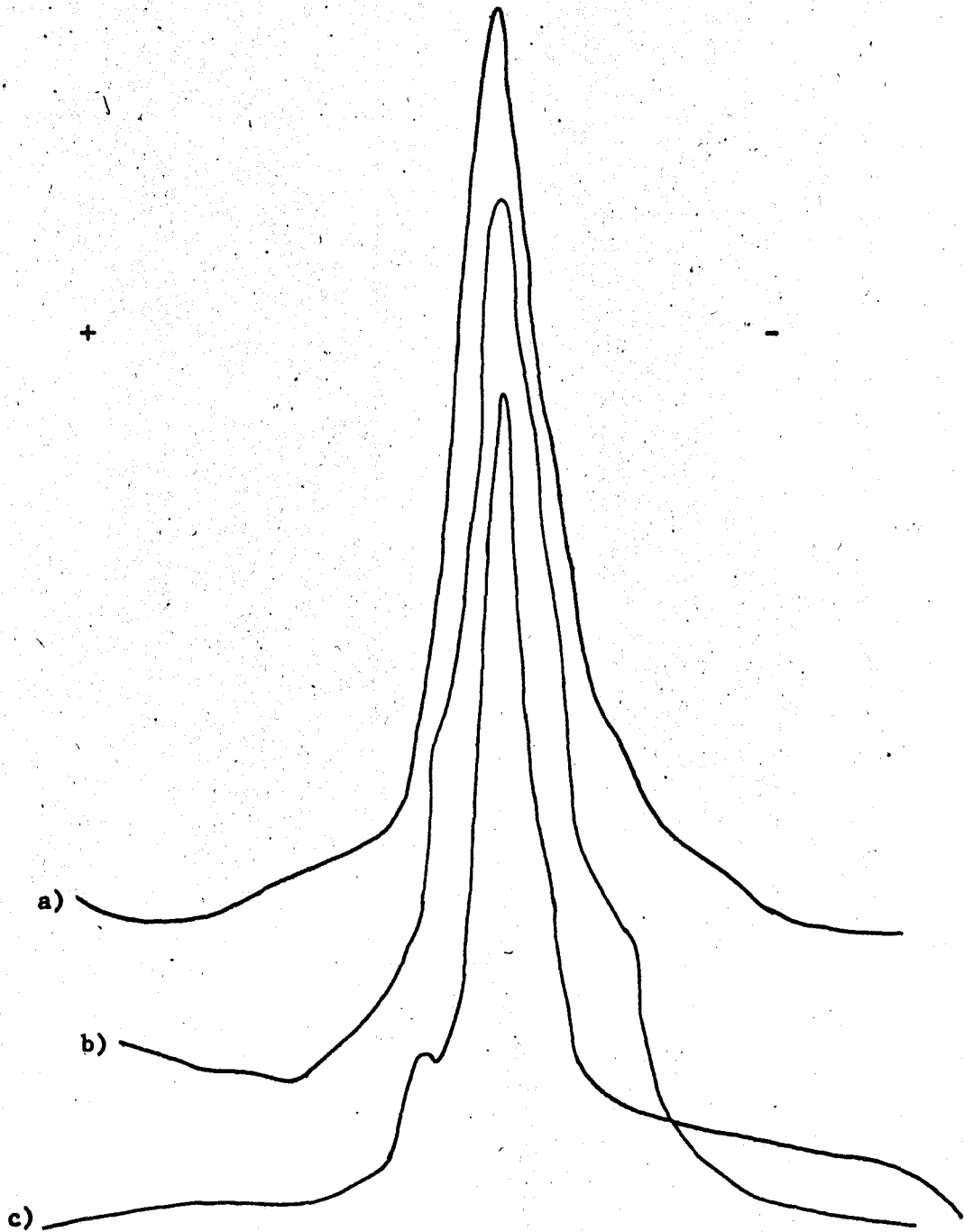


Fig. 13 - A series of similar gels was assayed for enzyme activity in the presence of various concentrations of MTX, shown above in relation to the concentration of enzyme used.

kept at 4° until all samples were ready and then subjected to electrophoresis. Since NADPH stabilizes the enzyme against degradation at 37°C (24), we did not use it during this experiment. In the activity profiles, the major peak tended to decrease in area with increasing time while the minor peak area stayed constant. These results could be interpreted in two ways; first, that the enzyme form represented by the major peak is being inactivated more rapidly than the minor, or second and more probably, that one or both of the enzyme forms are being inactivated but the equilibrium is shifting to the enzyme form represented by the minor peak because of the reduced enzyme concentration as in section 4.4.2.

#### 4.4.11 Detection of enzyme protein

Attempts to stain the usual amount of enzyme protein on the gel (with NADPH absent) using amido-black were not successful. However, enzyme bands of narrower width were obtained by using NADPH in the buffers (section 4.4.7) and these gels gave adequate protein stain. Pooled enzyme was concentrated by vacuum dialysis, desalted as described (section 4.4.3) and run at a high concentration of 2.5 units/gel or about 50 times the usual activity. Figure 14 shows the profiles of duplicate gels stained for protein, folate and dihydrofolate reductase activity. Some difficulty was encountered with the dihydrofolate reductase activity stain because the unusually high amount of enzyme produced rapid reduction of the tetrazolium salt (MTT) at the surface of the gel resulting in a blue assay mixture with little banding in the gel. The banding was improved when the assay mix was changed three times before scanning. This difficulty may also account for the lack of multiple activity bands such as



**Fig. 14 - Three similar gels run with 2.5 units of enzyme activity per gel and stained for a) dihydrofolate reductase activity, b) folate reductase activity and c) protein by amido black. Details in text.**

were seen at a lower enzyme concentration in figure 12. The folate reduction was slow and this gel was scanned after 18 hours. The results indicated that:

1. The protein stain and activity stains were in the same area of the gel.
2. The amount of contaminating protein either was less than 20% or ran with the dihydrofolate reductase activity band, confirming the high purity noted (section 3.2).

A series of gels containing 0.5 to 2.5 units of enzyme was subjected to electrophoresis in the presence of NADPH and stained for protein. It was found that there were two absorbance peaks and that the ratio of the area of the minor to major (figure 14) was constant at 1 to 5.6. This evidence is in agreement with the concentration independence of the enzyme activity in the presence of NADPH (section 4.4.7). A third peak of protein, which would correspond to the smallest peak of figure 6, was absent probably indicating the concentration of protein was below the sensitivity of the staining procedure.

#### 4.5 Disc gel electrophoresis with crude enzyme.

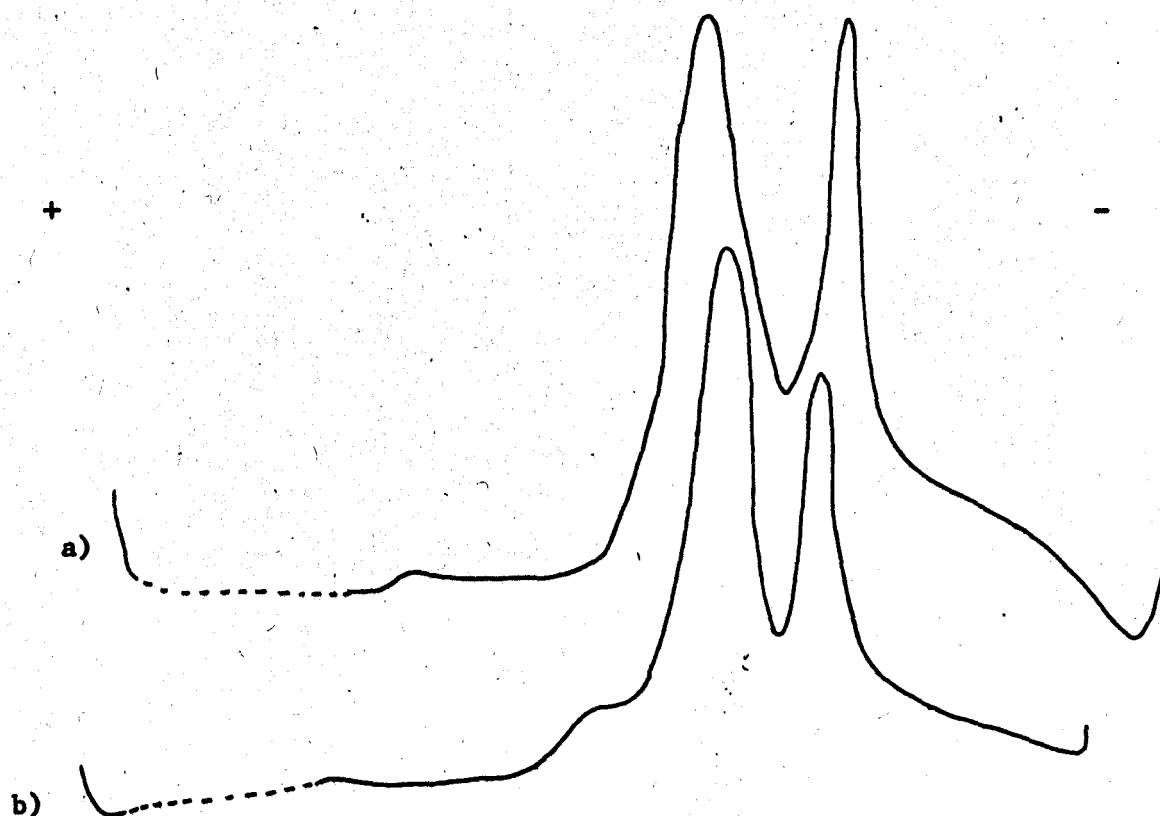
##### 4.5.1 Dihydrofolate reductase from LM4 cells

We have performed preliminary experiments with crude extracts of cells obtained from cell culture (see section 2.2). The results are difficult to correlate with those from purified enzyme or other cell line extracts since the patterns probably change with protein concentration. In the absence of NADPH in the buffers, there is one relatively narrow peak of activity with an  $R_f$  of 0.36 (compare to 0.37 with purified enzyme) and a slight shoulder at a higher  $R_f$ . This type of profile appears to be

constant for samples taken at various periods of cell growth. The presence of NADPH in the buffer (as in section 4.4.6) altered the activity pattern and produced the result shown in figure 15. The three peaks have  $R_f$  values of 0.31, 0.42 and 0.54 and area ratios of 3.2 : 5.7 : 1 compared to  $R_f$  values of 0.56, 0.63 and 0.70 and area ratios of 9.4 : 3.9 : 1 for the purified enzyme. Other experiments showed the ratios and  $R_f$  values obtained with crude enzyme changed on storage of the samples.

#### 4.5.2 Dihydrofolate reductase from LS2 cells

The experiments in this section were done mainly to compare the enzyme with that from the LM4 cells. Since the LS2 cells have a specific activity ten times less than that of LM4 cells and since only limited numbers of cells were available, only a small amount of enzyme could be used for electrophoresis. In the absence of NADPH, activity profiles with two or three variable peaks were obtained which had  $R_f$  values of 0.40, 0.32 and 0.55 (approximately) compared with 0.37 for LM4 extracts under similar conditions. As noted before, the correlation with the activity of LM4 extracts is difficult but it would appear that the LM4 peak with a  $R_f$  of 0.37 corresponds to at least one of the LS2 peaks (see the following section for further discussion). The areas of the peaks of activity did not appear to be constant except that the one with an  $R_f$  of 0.55 was always much smaller than the other two. In the presence of NADPH, a profile very similar to the LM4 profile was obtained (figure 15). Upon purification by affinity chromatography (similar to section 3.2), a similar single asymmetrical activity peak was obtained from LS2 and LM4 enzyme after electrophoresis.



Incubation times: LS2, 28 min; LM4, 17 min.

Fig. 15 - Activity profile of two gels run with crude extracts of dihydrofolate reductase from (a) LS2 cells and (b) LM4 cells. Gels were run in the presence of  $5 \times 10^{-5}$  M NADPH. Migration was toward the positive electrode. There was non-specific stain in the dashed area.

#### 4.5.3 Addition of LM4 and LS2

The differences in the profiles of the enzyme from the LM4 and LS2 sources in the absence of NADPH have been described. In an attempt to elucidate these differences, two samples containing 0.063 ml of enzyme from each source and a third sample containing a mixture 0.063 ml of each were subjected to electrophoresis. As can be seen in figure 16, these profiles appear to be additive. This apparent lack of dependence on concentration could be explained if there were distinct enzymes or enzyme forms in the two lines, but this argument is not supported by the close resemblance of the profiles in the presence of NADPH and the similarity of the purified activity profiles; or if the presence of non enzyme protein interfered with the concentration equilibrium.

#### 4.5.4 Dihydrofolate reductase from other cell lines

One purpose of these experiments was to compare the enzyme from RPM1 4625 cells grown under normal conditions of culture to those grown in presence of sublethal levels of MTX (see section 1.2). Localization of the enzyme in the gel was more difficult in this case due to the formation of several colored bands in the absence of DHF and in the presence of  $5 \times 10^{-6}$  M MTX. When these peaks were subtracted from the electrophoretic profile obtained from cells grown with and without MTX, similar asymmetrical peaks were obtained. Since only small quantities of these cells are available, no attempt was made at purification of the enzyme.

Bands of enzyme activity were produced on gels using extracts of various other cell lines. It appears that the techniques described can be used for separation of the forms of dihydrofolate reductase from a variety of sources.

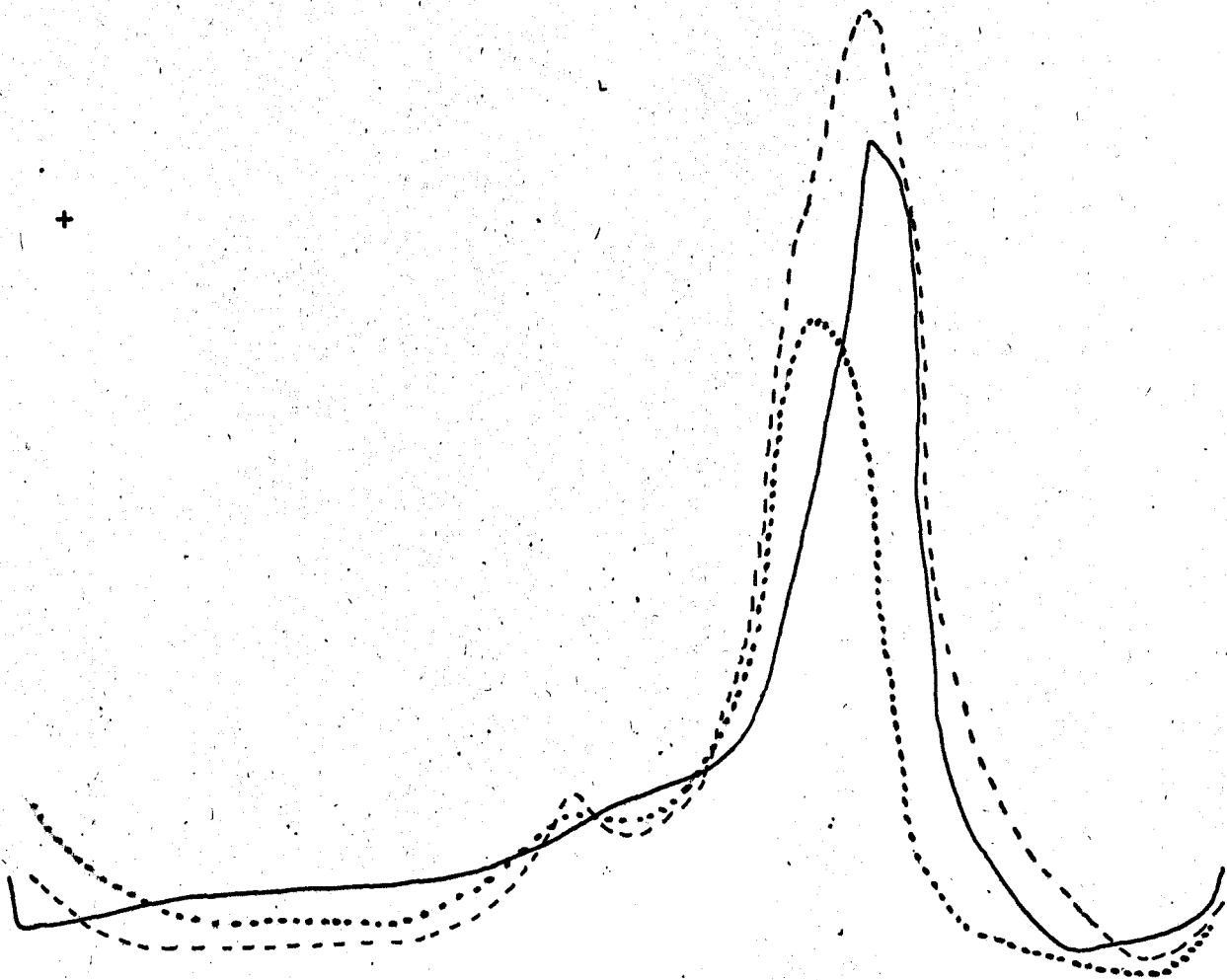


Fig. 16 - The profile of a mixture of LS2 and LM4 extracts after electrophoresis is compared to that of extracts of LS2 and LM4 cells run separately. LS2, .....; LM4, ———; LS2 + LM4-----. The migration was toward the positive electrode.



The hypothesis of "conformers", which are thought to be conformational variations of covalently identical enzymes, has been used to explain the existence of multiple forms of malate dehydrogenase (47), beef heart cytochrome c (48), human hemoglobin H (49) and other proteins. Some such "conformers" however have been shown to have differing amino acid compositions (50) and the structural basis of such forms is still under investigation (51). It would appear likely that the forms observed on electrophoresis are "conformers" of the same enzyme. In the absence of NADPH, the more mobile form can exist as the only band whereas at higher concentrations, an additional slower moving form is present, indicating that the forms are interconvertible since the same enzyme solution was used in both cases. In the presence of NADPH, the forms are stabilized into three "conformers". As noted before, since we did not find a single band in the presence of urea, aggregation is not likely. Recent independent work states, but does not give supporting evidence for this view (46).

We can determine some properties of the two major forms in the presence of NADPH (figures 12 and 14) by comparing the protein and activity stains, with the following assumptions:

- 1) The minor, fast moving peak at low concentrations and the lack of multiple bands of enzyme activity at high concentration can be disregarded for the reasons stated before.

- 2) The major peak of protein stain (figure 14) at high activity and the major peak of activity (figure 12) at low activity are comparable as are the minor peak of protein stain and the intermediate peak of activity

stain.

3) The absorbance of the peaks are a true indication of the relative enzymes activities and protein concentrations of the two bands. If these assumptions are true, then the enzyme in the intermediate peak has approximately three times the turnover number of the enzyme in the slowest moving peak. The more rapid inhibition of this intermediate peak by MTX is consistent with there being enzyme of higher specific activity in this peak, assuming that the binding constant for MTX is similar for both forms of the enzyme and that the MTX binding to enzyme is one to one.

It is difficult to compare these enzyme forms with those reported elsewhere because:

1) Such enzymes are from various sources and of different purities and are used at different concentrations.

2) The methods used to separate the forms vary from column chromatography (9,12,46) to electrophoresis on starch gel and cellulose acetate membrane in different buffer systems which themselves alter the electrophoresis mobility (29).

Nevertheless there appear to be two major forms of the enzyme and, in many cases, an additional minor form. For example, chicken liver enzyme has been separated into three such forms (29) in the ratio of 7.7 : 5.4 : 1 compared with a ratio of 9.3 : 3.9 : 1 in our case, for peaks in the same relative position.

It has been reported (45) that the two forms of dihydrofolate reductase from an MTX-resistant subline of Lactobacillus casei can be converted from one form to another by the addition of NADPH:



It is difficult to reconcile this scheme with our system where three forms were noted in the presence of NADPH at concentrations 50 (or more) times greater than the  $K_m$  for this substrate. The evidence given for the above interconversion was based on electrophoretic banding which showed that the protein and activity stains coincided for Form I (slow-moving form). As we have noted, at the high concentrations which are necessary for protein staining by amido-black, the enzyme would be mainly in the slow moving form and it may be difficult to resolve other peaks under these conditions (section 4.4.7). When this enzyme was treated "with an excess of TPNH" (45), there was one fast moving main band of activity and protein stain. We have also noted that the  $R_f$  of the enzyme increased when NADPH was added to the electrophoresis buffer, but we have resolved enzyme forms under these conditions at low concentrations of enzyme. Again, higher concentrations would be necessary in order to obtain an adequate protein stain, in which case, resolution becomes poor.

In summary:

- 1) Several forms of dihydrofolate reductase have been separated by electrophoresis under the conditions described.
- 2) In the absence of NADPH, the intensity of two or more of these forms was concentration dependent. Since a similar dependence was observed in the presence of urea, aggregation of small units to larger units appeared unlikely.
- 3) In the presence of NADPH, the ratio of the activity of three

distinct forms and of two forms of enzyme protein was independent of concentration.

4) MTX affected these forms differently under the conditions described.

5) Electrophoresis indicated the enzyme used was of high purity (at least 80%).

6) The purified enzyme appeared to reduce folate as well as dihydrofolate but at a much slower rate.

7) The enzyme activity profiles of crude extracts differed from those of the purified enzyme.

8) The reductase from LS2 and LM4 sublines appeared similar but this evidence is not conclusive.

9) The isoelectric point of the enzyme purified from LM4 cells was approximately 8.2.

10) The turnover number of the partially purified LM4 enzyme was approximately 2,100.

11) The reductase from the RPM1 4265 subline cultured in the absence of MTX was similar to that grown in the presence of sublethal levels of MTX.

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