Biochemical Pharmacology, Vol. 20, pp. 2033–2041. Pergamon Press, 1971. Printed in Great Britain

EFFECTS OF METHOTREXATE ON THE SYNTHESIS AND PROPERTIES OF DIHYDROFOLATE REDUCTASES IN RAT LIVER

ANDREW C. LOVESEY

Research Department, Marie Curie Memorial Foundation, Limpsfield Chart, Surrey

(*Received* 27 *August* 1970; *accepted* 28 *November* 1970)

Abstract—Two distinct species of hepatic dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NAD(P) oxidoreductase EC 1.5.1.3) have been isolated from rats injected with the antimetabolite methotrexate. The reductase of lower molecular weight was also isolated from rats lacking the methotrexate treatment. Kinetic studies have shown that the inhibition of one enzyme (molecular weight 24,800) is of a non-competitive nature, whereas the other (molecular weight 20,200) gives rise to competitive inhibition. Differences between the properties of these enzymes were also noted in electrophoresis experiments. The relevance of these results to the problem of methotrexate resistance in mammalian cells is discussed.

A MAJOR problem in the chemotherapeutic treatment of neoplastic diseases is due to the development of drug resistance. Various mechanisms have been proposed[1] to explain the causes of this phenomenon in antifolate therapy. Recently, Ferone[2] has shown that a dihydrofolate reductase isolated from a bacterial strain sensitive to methotrexate displays competitive drug inhibition, whereas a reductase from a resistant strain acts as a non-competitive inhibitor. The possibility that a similar situation operates in mammalian cells has therefore been investigated.

As Reizenstein[3] has pointed out, it is necessary to distinguish between resistance due to genetic changes of the tumour, and that due to physiological adaptation of the host. In this paper, resistance due to physiological adaptation has been studied using non-tumour bearing rats, and the effects of methotrexate administration upon the content and properties of dihydrofolate reductases in rat liver has been examined.

MATERIALS AND METHODS

Chemicals

Sephadex dextran G-100 was purchased from Pharmacia; Folic acid from British Drug Houses; NADPH and MTT [3-(4,5-dimethyl thiazolyl-2-)-2,5-diphenyl tetrazolium bromide] from Sigma, and ribonuclease, γ-globulin, ovalbumin and bovine serum albumin from Koch Light. Methotrexate was generously donated by Lederle Laboratories, and a solution of 5 mg sodium intra-parenteral form in 125 ml H_2O was used for the injections. Folic acid was reduced to the dihydro form with sodium dithionite.[4]

Preparation of dihydrofolate reductases

Male Sprague–Dawley rats were anaesthetised using ether and the livers were excised. The animals were killed by exsanguination before they regained consciousness.

In each preparation, 10 g of rat liver were homogenized with a Potter homogeniser in 20 ml of ice-cold tris–HCl buffer (0·1 M, pH 7·4). The homogenate was centrifuged at 130,000 *g* for 70 min. Then 4·6 g of ammonium sulphate (40 per cent saturation) was dissolved in the decanted supernatant and the solution was centrifuged at 130,000 *g* for 20 min. To the decanted supernatant was added 6·9 g of ammonium sulphate (82 per cent saturation) and the solution was centrifuged at 130,000 *g* for 30 min. The precipitate was dissolved in 4 ml tris–HCl buffer (0·1 M, pH 7·4), and the solution was dialysed overnight against 1 l. of tris–HCl buffer (0·01 M, pH 7·4). The solution was centrifuged at 130,000 *g* for 20 min and the liquid was applied to a glass chromatography column containing Sephadex dextran G-100 (1·8 cm dia. and 148 cm long), pre-equilibrated with tris–HCl buffer (0·05 M, containing 0·1 M KCl) which was also used as the eluting solvent. Chromatography was carried out at 18° whereupon the enzymes and other protein were resolved into separate fractions (as detailed in Fig. 1). Where enzymes of high purity were required the active fractions were reduced in volume by freeze-drying and re-cycled through the Sephadex column.

The enzymatic activity at 340 mμ and the u.v. absorption at 280 mμ of each fraction were recorded on a Unicam SP 800A spectrophotometer linked to a SP 20 slave-recorder. Fractions were stored at −15°.

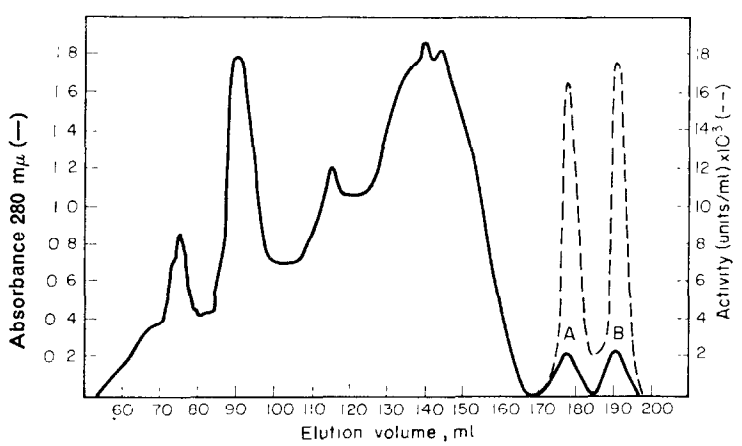

FIG. 1. Dihydrofolate reductase fractionation. Chromatography of 197 mg of protein isolated from the liver of a rat given 18 injections of 0·01 mg methotrexate over 43 days. Eluted from a Sephadex G-100 column (1·8 × 148 cm) at 18° with tris–HCl buffer (0·05 M, containing 0·1 M KCl); 1·0 ml fractions collected, the flow rate being 0·5 ml/min.

Protein and enzymatic activity determinations

Protein was determined by the method of Lowry *et al.*[5] Each cuvette contained 0·9 ml of 0·05 M tris–HCl buffer, pH 7·3 (containing 0·01 M 2-mercaptoethanol, 0·001 M EDTA and 0·6 M NaCl), 0·9 ml of 0·00033 M NADPH, 0·9 ml of H_2O and 0·15 ml of 0·002 M dihydrofolic acid suspension in H_2O. The assay mixture was incubated for 15 min at 25° and the reaction was started by addition of 0·6 ml (about 0·2 mg of protein) of enzyme solution. After a further 1·5 min the enzymatic activity was measured by observing the decrease in absorbance at 340 mμ for 2·5 min at 25°. One unit of enzyme activity is defined as the number of μmoles of dihydrofolate

reduced per minute per millilitre of enzyme solution, under the conditions described, assuming extinction coefficients at 340 mμ of 6200 $mole^{-1}cm^{-1}$ for NADPH and 5800 $mole^{-1}cm^{-1}$ for dihydrofolate.[6]

Electrophoresis determinations

High voltage electrophoresis (30 V/cm) was carried out on a Shandon Universal Electrophoresis apparatus with Oxoid cellulose acetate membranes as the supporting medium. Determinations were effected using 0·16 M tris–borate buffer pH 7·5, for 4 hr at 5°. Ion-agar gels were prepared from addition of a solution of 6·2 g MTT in 2·5 ml 0·1 M tris–HCl buffer, pH 7·4, to a freshly boiled solution of 0·08 g Oxoid no. 2 ion-agar gel in 5 ml of 0·1 M tris–HCl buffer, pH 7·4, followed by addition of 0·25 ml of 0·002 M dihydrofolate suspension and 0·25 ml of 0·00033 M NADPH solution. Gel slides were prepared from 1·8 ml aliquots of this mixture and were incubated in the presence of cellulose acetate membranes at 37° for 30 min.

Molecular weight determinations

Molecular weights were calculated by the method of Whitaker[7] using a Sephadex G-100 column (1·8 cm diameter and 148 cm long) and protein standards of γ-globulin, bovine serum albumin, ovalbumin and ribonuclease. Elution was carried out at 18° using 0·05 M tris–HCl buffer, pH 7·4, containing 0·1 M KCl.

RESULTS AND DISCUSSION

Purification of dihydrofolate reductases

The procedure is exemplified in Table 1 which relates to the isolation of dihydrofolate reductase enzymes from the liver of a rat given 18 injections of 0·01 mg methotrexate over a 43 day period.

The data presented in Table 1 give an indication of the effectiveness of various stages in the purification of the enzymes. The ammonium sulphate precipitates provided material with an increase in specific activity from 33·4 to 91·0 units/mg for a loss in yield of 29 per cent. Dialysis resulted in a slight lowering of the specific activity and a 3 per cent loss in yield from the preceding stage. The inactive protein was separated from the enzymatic protein in the next stage (see Fig. 1), where complete resolution of the active material into two clearly defined peaks was attained by Sephadex chromatography, using a column 1·8 cm in diameter and 148·0 cm long. Shorter columns adversely affected the separation, as did the use of dextrans of larger particle size than G-100.

Prior to chromatography, electrophoresis of the dialysed enzyme preparation showed the presence of three bands which produced formazan dyes with MTT in the presence of NADPH, indicating that there were three systems present which were each capable of initiating a NADPH-mediated hydrogen transfer reaction. The bands appeared from the origin at 1·6 cm towards the cathode (not inhibited by methotrexate), 0·5 cm towards the cathode (completely inhibited by methotrexate) and 0·8 cm towards the anode (also completely inhibited by methotrexate). The band insensitive to methotrexate (probably a diaphorase system) was separated from the enzymically active fractions during the chromatography with Sephadex G-100 and electrophoresis experiments with lyophilized protein from enzymatically active chromatography

TABLE 1. PURIFICATION OF RAT LIVER DIHYDROFOLATE REDUCTASES*

Stage	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Total activity (units) $\times 10^3$	Specific activity (units/mg protein) $\times 10^3$	Yield (%)
Supernatant from homogenate	20·1	0·0136	0·407	272·6	33·4	100
Supernatant from $(NH)_2SO_4$ stage (40% saturation)	13·2	0·0173	0·298	229·0	58·0	84
$(NH_4)_2SO_4$ precipitate (82% saturation)	4·0	0·0484	0·527	193·5	91·0	71
Dialysis	5·1	0·0363	0·415	185·4	87·4	68
First Sephadex chromatography A:	11·0	0·0077	0·013	84·5	581·2	31
B:	10·1	0·0083	0 014	84·0	611·1	31
Second Sephadex chromatography A:	10·5	0·0075	0·009	79·0	804·3	29
B:	9·0	0·0088	0·009	79·0	1000·3	29

* The above Table represents results obtained from the liver of a rat given 18 injections of 0·01 mg methotrexate over 43 days. The two rows of figures for the chromatography stages relate to two enzymes, dihydrofolate reductase A and dihydrofolate reductase B, as indicated by the letters to the left of each row.

fractions showed that the electrophoresis band which moved 0·5 cm towards the cathode arose from material with elution volume centred at 191 ml, whilst protein from the chromatography peak at 178 ml gave rise to the electrophoresis band which moved 0·8 cm towards the anode. The latter band was not detected in control experiments using livers from rats lacking methotrexate administration.

These results provide evidence that two species of dihydrofolate reductase were present in the active protein. The species were denoted A and B, corresponding to the protein in the peaks at 178 and 191 ml respectively. Both gave only one absorption peak in the u.v. region, at 280 mμ, and both failed to reduce dihydrofolate when no NADPH was present.

The specific activity of each enzyme was about seven times that of the mixture prior to chromatography, and only 6 per cent of the total activity was lost during the elution. Further increases in the specific activity of the enzymes were induced by lyphilization and repeating the chromatography, when enzymes in a very high state of purity were obtained (see later), in a satisfactory total yield of 58 per cent based on the first stage in the purification.

From the elution volumes of the peaks, the molecular weights of enzymes A and B were estimated by Whitaker's method,[7] using the elution volumes of proteins of known molecular weight. The void volume V_0 of the column was given by the elution volume of γ-globulin,[8] and from the standard curve (Fig. 2) the molecular weights of species A and B were calculated to be 24,800 $\pm$ 700 and 20,000 $\pm$ 700 respectively. Bertino and co-workers[9] have estimated the molecular weight of the reductase derived from Ehrlich ascites cells to be 20,200 and a molecular weight of 21,000 has been given for the reductase from a methotrexate-resistant subline of cultured Sarcoma-180 cells.[8]

Molecular weight values in the range 20,000–30,000 have been recorded in the literature.[10]

At very low concentrations of inhibitor (less than 5×10^{-12} M in these experiments), the reaction of methotrexate with the reductases may be used to evaluate the enzymatic purity. Under these conditions the inhibition is stoichiometric[11] and the enzyme may be titrated with the inhibitor. The results of these experiments are shown in Fig. 3. Assuming that 1 mole of methotrexate reacts with 1 mole of enzyme, the specific activity of pure A should be $4{\cdot}00 \times 10^{-8}$ moles/mg of protein and of pure B, $4{\cdot}95 \times 10^{-8}$ moles/mg of protein, based on the calculated molecular weights. From Fig. 3 the specific activity of A is $3{\cdot}63 \times 10^{-8}$ moles/mg and that of B is $4{\cdot}65 \times 10^{-8}$ moles/mg. The purities of A and B are therefore 91 and 94 per cent respectively. However, attempts to crystallise the enzymes have so far been unsuccessful.

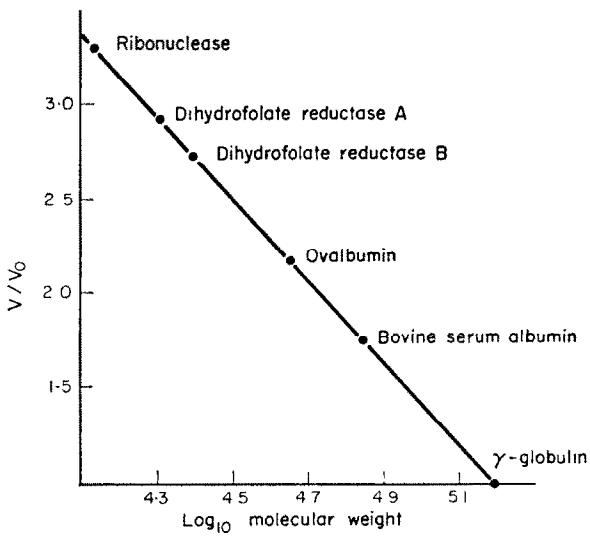

FIG. 2. Standard protein curve for molecular weight determination. Method according to Whitaker,[7] 5 mg of each protein being applied separately to a Sephadex column of G-100 dextran ($1{\cdot}8 \times 148$ cm). Elution volumes were determined as described for Fig. 1. The void volume V_0 was given by γ-globulin. Molecular weights of standards were according to Zakrzewski *et al.*[8]

Effects of methotrexate administration on rat hepatic dihydrofolate reductases

The effects of intra-peritoneal injections of methotrexate upon the dihydrofolate reductase content of rat liver can be seen from Fig. 4 which shows the elution profiles of active protein obtained from rats given courses of methotrexate (three injections/week) over various time periods. Control experiments showed that in the absence of the antimetabolite the entire dihydrofolate reductase content consisted of species B. As the total amount of administered drug increased, the activity of B increased initially, but later (72 and 78 injections) less change in activity was recorded (see Fig. 5) and after the maximum point was reached (at 78 injections) there appeared to be a falling-off in the cellular synthesis of species B. At the same time, whilst these changes took place, the methotrexate treatment appeared to induce formation of a new species of dihydrofolate reductase (A), not described before in the literature, the activity of

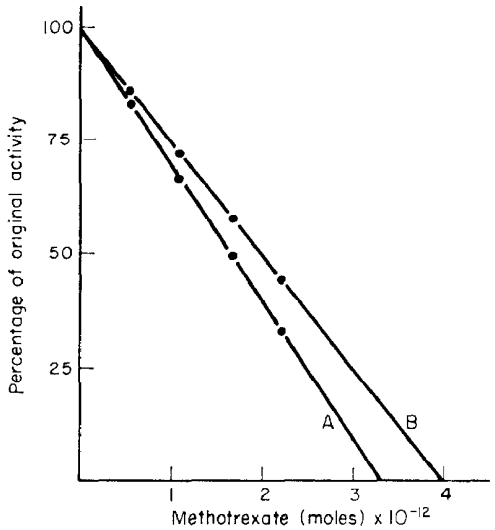

FIG. 3. Methotrexate titration of dihydrofolate reductases A and B. In a total volume of 3·6 ml, 0·9 ml of 0·05 M tris–HCl buffer, pH 7·4 (containing 0·01 M 2-mercaptoethanol, 0·6 M NaCl and 0·001 M EDTA), 0·9 ml of 0·00033 M NADPH, and 0·086–0·088 μg of enzyme were incubated for 1 min at 25° with varying quantities of a 10^{-12} M methotrexate solution. The reaction was initiated by addition of 0·15 ml of 0·002 M dihydrofolate suspension in H_2O, and the decrease in absorption at 340 mμ was followed for 2·5 min.

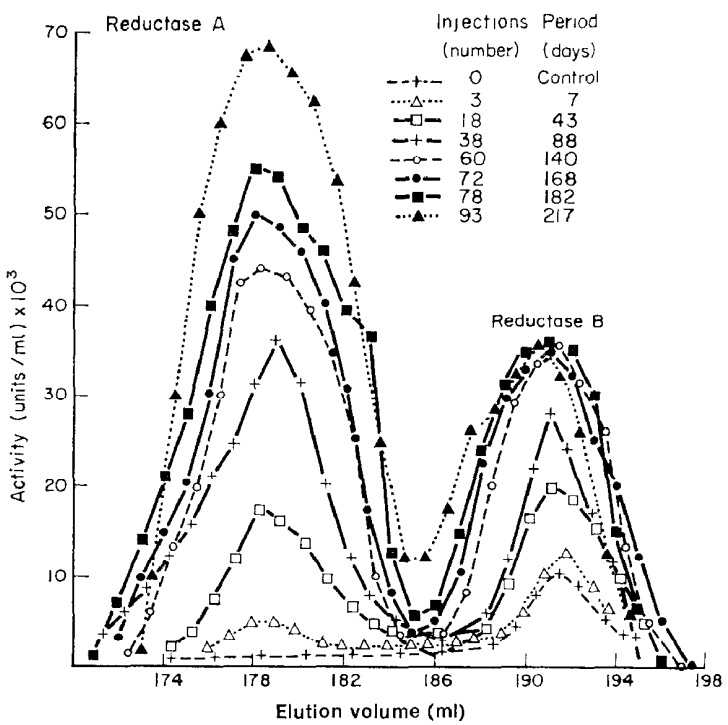

FIG. 4. Activity–elution profiles of dihydrofolate reductases isolated from rats given intra-peritoneal injections of 0·01 mg methotrexate three times a week for various time periods. Sephadex G-100 column chromatography as described for Fig. 1.

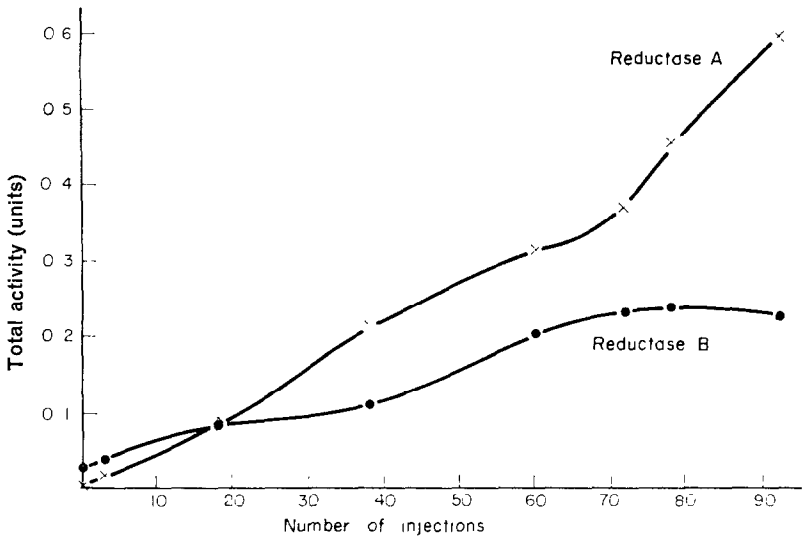

FIG. 5. Growth of dihydrofolate reductase activity with number of injections of methotrexate.

which increased with the number of injections. This is clearly illustrated in Fig. 5 which represents the growth in activity with respect to the number of injections given.

After 43 days (18 injections), the activity of species A became greater than that of B, and the cellular synthesis of A continued at about the same rate until the production of B slowed down. At this point the rate of growth of reductase A noticeably accelerated. It seems likely therefore that the presence of one species affected the growth of the other. It may be that A inhibited the growth of B. In relation to this, it is well known that resistance to methotrexate in mammalian cells can be accompanied by a pronounced increase in dihydrofolate reductase activity,[12] and differences in properties of reductases derived from bacterial strains sensitive and resistant to methotrexate have been recorded.[12] Ferone[2] has shown that reductase species isolated from some bacterial systems react with methotrexate in a non-competitive manner, in contrast with the competitive inhibition with the drug-sensitive species. Since it was possible that a similar situation could exist for dihydrofolate reductases isolated from mammalian cells, the inhibition of enzymatic species A and B was studied.

Ackermann–Potter plots[13] such as those shown in Fig. 6 were performed in order to test the range of inhibitor concentrations over which the reactions were reversible. It was found that the reactions for both species were reversible at concentrations of methotrexate greater than 8×10^{-12} M. The effects of inhibitor concentration upon the reversibility of the reaction are well known[10] and conventional Michaelis–Menten kinetics may be applied to determine whether the inhibition is competitive or non-competitive. Dixon's method[14] was used to estimate the inhibitor constants, and the results (see Fig. 7) showed that the drug inhibition of reductase B was competitive ($K_i = 1{\cdot}5 \times 10^{-11}$ M), whereas that of A was non-competitive ($K_i = 9{\cdot}3 \times 10^{-10}$ M). There was no change in the values of these constants when the pH of the assay medium was altered from 7·4 to 6·5, similar to results obtained by Bertino and co-workers.[9] The relative affinity of B for methotrexate was therefore about sixty times that of A.

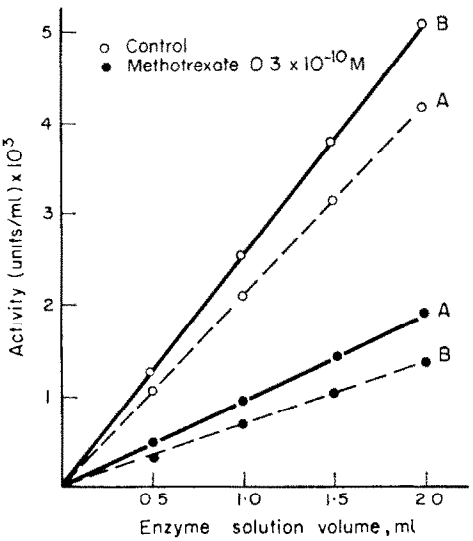

FIG. 6. Ackermann–Potter plots. Conditions as described for Fig. 3, except that methotrexate solution was $0{\cdot}3 \times 10^{-10}$ M and assay cuvette contained 0·01–0·04 mg enzyme.

The Michaelis constant for B was found to be $4{\cdot}1 \times 10^{-7}$ M and that for A (calculated from a Lineweaver-Burk double reciprocal plot) was $4{\cdot}3 \times 10^{-7}$ M.

In conclusion, the experiments described show that, (1) when a course of methotrexate is administered to Sprague–Dawley rats, the total cellular dihydrofolate reductase content increases considerably. In addition to the normal reductase (B) which may be isolated from rats lacking any drug treatment, livers from drug-treated animals also contain a dihydrofolate reductase species A. (2) Reductases A and B differ in their response to inhibition by methotrexate. The drug binding affinity of A (which gives rise to non-competitive inhibition) is considerably weaker

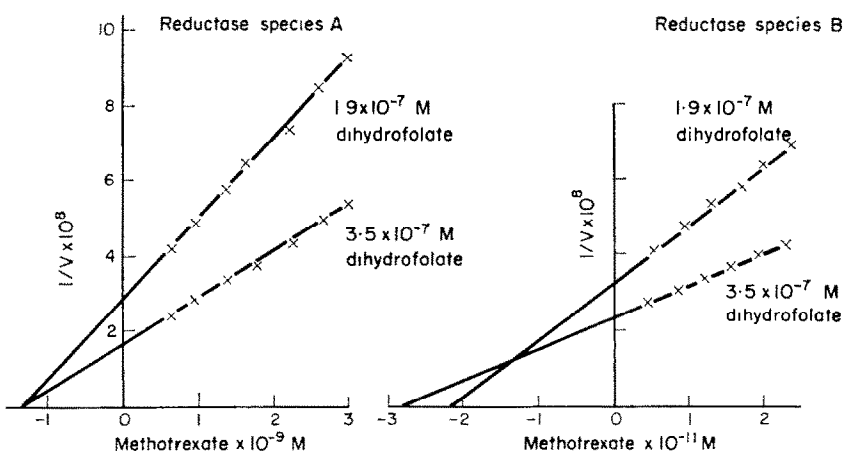

FIG. 7. Methotrexate inhibition of dihydrofolate reductase species A and B. Method according to Dixon.[14] Assay mixtures as described for Fig. 3, except that volumes of dihydrofolate used were 0·1 and 0·3 ml, and various amounts of 10^{-9} to 10^{-11} M methotrexate were used as shown above.

than that of B (which gives competitive inhibition). (3) Differences in molecular weight are also evident, A having a molecular weight of 24,800 whereas B has a molecular weight of 20,200, and the enzymes migrate in opposite directions under electrophoresis. (4) The combined action of effects 1 and 2 is to gradually weaken the response of the cell to the inhibitor, and these biochemical changes in the cell give rise to methotrexate resistance due to physiological adaptation.

Acknowledgements—The author is indebted to Dr. D. C. Williams for many helpful discussions and is grateful to the Lederle Laboratories for the generous gift of methotrexate. The author also wishes to thank all those who have contributed to support research work at the Marie Curie Memorial Foundation.

REFERENCES

1. H. G. MANDEL, *Pharmac. Rev.* **11,** 794 (1959).
2. R. FERONE, *J. biol. Chem.* **245,** 850 (1970).
3. P. G. REIZENSTEIN, *Acta haemet.* **25,** 184 (1961).
4. C. K. MATHEWS, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), Vol. 6, p. 801, Academic Press, London (1962).
5. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
6. C. K. MATHEWS, Thesis, Univ. of Washington, p. 32 (1962).
7. J. R. WHITAKER, *Anal. Chem.* **35,** 1950 (1963).
8. S. F. ZAKRZEWSKI, M. T. HAKALA and C. A. NICHOL, *Molec. Pharmac.* **2,** 423 (1966).
9. J. R. BERTINO, J. P. PERKINS and D. G. JOHNS, *Biochemistry* **4,** 839 (1965).
10. P. REYES and F. M. HUENNEKENS, *Biochemistry* **6,** 3519 (1967).
11. W. C. WERKHEISER, *J. biol. Chem.* **236,** 888 (1961).
12. R. L. BLAKLEY, *The Biochemistry of Folic Acid and Related Pteridines*, Ch. 14, p. 501, North Holland, London (1969).
13. W. W. ACKERMANN and V. R. POTTER, *Proc. Soc. exp. Biol. Med.* **72,** 1 (1949).
14. M. DIXON, *Biochem. J.* **55,** 180 (1953).