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THE RECOVERY OF LIVER TETRAHYDROFOLATE DEHYDROGENASE ACTIVITY FROM INHIBITION BY METHOTREXATE

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Abstract—Rat liver tetrahydrofolate dehydrogenase activity *in vivo* recovers rapidly from inhibition by methotrexate, despite the known stability of the enzyme-inhibitor complex. Control levels of activity are usually regained within 24 hr following a single injection of methotrexate. A similar pattern of recovery of activity has been found using the Guerin T8 epithelioma. Evidence that the enzyme is actually inhibited *in situ* has been obtained using a tetrazolium assay system. No evidence could be found for metabolism of the methotrexate to a form inactive with respect to tetrahydrofolate dehydrogenase. Synthesis of new enzyme with retention of the inhibited enzyme did not appear to play a major role in recovery of activity. Experiments using tritiated methotrexate indicated that both *in vivo* and *in vitro*, recovery of enzymic activity is probably associated with a dissociation of the enzyme-inhibitor complex.

ANTI-FOLATE drugs, such as methotrexate, are widely used in the chemotherapy of acute leukaemia. It appears possible that their clinical usefulness is related to their powerful ability to inhibit tetrahydrofolate dehydrogenase, a key enzyme in the synthesis of DNA. Recovery of enzymic activity from inhibition by antifolates could be a factor limiting the drug's effectiveness. Despite the apparent stability of anti-folate-tetrahydrofolate dehydrogenase complexes *in vitro*, dissociation of the complex in resistant S-180 cells *in vivo* has been found to play an important role in determining the degree of resistance of the cells to inhibition by anti-folate drugs.[1] The present study was directed towards an examination of the recovery of rat liver tetrahydrofolate dehydrogenase activity both *in vivo* and *in vitro* from inhibition by methotrexate, and also a preliminary study was made of the biochemical processes which may be involved.

MATERIALS AND METHODS

Animals

Fresh livers from adult Sprague–Dawley rats weighing 200–250 g were used. The tumour used in some of the experiments was the Guerin T8 epithelioma, transplanted in rat spleen.

Chemicals

Methotrexate (MTX) and calciumle ucovorin were kindly donated by Lederle Laboratories. MTX was dissolved at pH 8·0 by dropwise addition of NaOH solution, and subsequently adjusted to pH 7·0 with 0·1 N HCl. Injections were carried out i.p. in 0·5 ml physiological saline. Radioactive MTX (3′ 5′ ^{3}[H], specific activity

5·3 c/mM) was obtained from the Radiochemical Centre, Amersham. Reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) was obtained from C. F. Boehringer and Son. Fresh solutions of FH_2 were prepared immediately before use according to the method of Futterman.[2] MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) and PCMB (*p*-chloromercuribenzoic acid) were obtained from Koch-Light. The PCMB was dissolved at pH 8–10 and subsequently adjusted to pH 7·4.

Preparation of partially purified FH_4 dehydrogenase

Weighed samples (5 g unless otherwise specified) of liver were homogenised in 10 ml 0·1 M Tris-HCl buffer pH 7·4 using a Teflon–Potter homogeniser. Equal amounts of tissue from each liver in a group were pooled. The homogenate was centrifuged at 100,000 *g* for 1 hr. Supernatant protein was precipitated between 40 and 90% saturation using solid ammonium sulphate and was dissolved in 2 ml (unless otherwise specified) of 0·1 M Tris-HCl pH 7·4. This was then dialysed overnight against 1l, 0·01 M Tris pH 7·4. The whole procedure was carried out in the cold. The specific activity of the preparation was in the order of 10 enzyme units/mg protein. (1 unit of activity is defined as that amount of enzyme which catalyses a Δ O.D. of 0·010/min).

Occasionally a highly purified FH_4 dehydrogenase preparation was used with a specific activity of 300 units/mg protein. This was prepared according to the method of Mathews, Scrimgeour and Huennekens.[3]

It has been suggested that certain conditions of dialysis can cause reactivation of an FH_4 dehydrogenase-anti-folate complex.[4] Consequently, it was considered necessary during these studies to check that the overnight dialysis conditions used in enzyme preparation did not cause any reactivation of an enzyme-MTX complex by removal of MTX. No significant reactivation was found to occur under the experimental conditions used.

Assays of FH_4 dehydrogenase activity

Two techniques for assaying FH_4 dehydrogenase activity were used. An MTT assay was used with rat liver sections 15 μ thick, cut from cylindrical blocks (6 mm dia.) of fresh frozen liver using a Bright's cryostat.[5] Diaphorases present in the system were inhibited by prior incubation of tissue with 4 mM PCMB in 0·1 M Tris pH 7·4. After addition of substrates and MTT, activity was estimated by measuring the increase in extinction at 560 mμ due to reduction of MTT by FH_4 produced.

FH_4 dehydrogenase activity was more routinely assayed by measuring the decrease in extinction at 340 mμ due to disappearance of FH_2 and $NADPH_2$. A typical assay cuvette contained 20μM $NADPH_2$, 20 μM FH_2, an aliquot of enzyme, and 0·05 M Tris-HCl pH 7·4 in a total volume of 2 ml.

Determinations of radioactivity

Enzyme preparations or the homogenised whole tissues were boiled for 5–10 min. The supernatants, containing the MTX, obtained after centrifuging for 30 min at 100,000 *g* were used for radioactive determinations. seven ml scintillator[6] was added to 0·5 ml of the samples. When larger volumes of sample were used, they were first reduced to 0·5 ml or less by evaporation. Disintegrations per min were measured using a Beckman L.S. 100 liquid scintillation system.

Reactivation of inhibited enzymes

Enzyme preparations, which were inhibited due to the presence of methotrexate, were reactivated by dissociation of the enzyme-inhibitor complex on DEAE-cellulose columns, by a modification of the method of Mathews.[7] Inhibited enzyme preparations, containing approx 10 mg protein, were run on to 5 × 1 cm. DEAE-cellulose columns, previously equilibrated with 20 ml 0·005 M phosphate buffer pH 6·5. Samples were run on to the column in a total volume of 1 ml. Columns were eluted with 0·05 M phosphate buffer, 5 ml fractions of eluate being collected. Enzymic assays were carried out using 0·2 ml samples of the eluted fractions. In the test system, 1 ml of enzyme preparation was placed on one column, 1 ml of the same enzyme preparation, completely inactivated by addition of MTX at a final concentration of 10^{-7} M was placed on the second column. Elution was carried out as above. Eluted activities were:

Fraction No.	1	2	3	4
Initially active	0·052	0·454	0·082	0·018
Initially inactivated	0·056	0·430	0·046	0·010

activity μmoles FH_2/hr/0·2 ml preparation.

It appeared that almost all the activity was eluted in the first 3 fractions, and that recovery of 90 per cent of the previously inhibited activity was obtained. Higher activities were obtained in the eluates of the control solutions than those applied to the columns (approx. 3-fold increases) presumably due to retention of inhibitory substances by the columns.* 70–80 per cent of the protein applied to the column was recovered in the eluted fractions.

Protein determinations were carried out according to the method of Lowry, Rosebrough, Farr and Randall.[8]

Investigations of ^{3}*[H] MTX levels and* FH_4 *dehydrogenase recovery* in vitro

Two rats were each injected i.p. with 5 mg. ^{3}[H] MTX (10μc). The livers were removed ½ hr later, together with two livers from control rats. They were perfused with physiological saline and used immediately. All weighings, washings and enzyme preparations were carried out at 0–4° (loss of ^{3}[H] MTX during incubations at this temperature was found to be negligible in agreement with findings using human leukocytes).[9] The lobes of the livers from the treated groups were combined and chopped finely with scissors; the control group was treated in a similar manner. Each sample was then washed twice in a large volume (approx. 200 ml) of cold sterile Tris medium (consisting of 62 mM Tris-HCl pH 7·2, 65 mM NaCl, 15 mM KCl, 8 mM $CaCl_2$ and 1 g glucose/l), and centrifuged at 500 *g* for 2 min. The chopped tissue was then blotted gently. Duplicate samples of 0·6 g tissue were used.

(a) *Change in* ^{3}*[H] MTX content of whole tissue* in vitro. The zero time samples were rapidly frozen immediately after washing and stored. All other samples were suspended in 10 ml sterile Tris incubation medium and incubated at 30° with shaking for the time indicated (¼ hr, ½ hr, ¾ hr or 1 hr). After incubation, the tissue was cooled immediately, washed in ice-cold medium and rapidly frozen and stored. Subsequently, samples were thawed and their radioactive content determined.

* See Table 2.

(b) *Recovery of enzyme activity* in vitro. Both the livers from the MTX injected rats and the control livers were used. The zero time samples were suspended in 10 ml 0·1 M Tris-HCl pH 7·4 and rapidly frozen and stored. The remaining samples were suspended in 10 ml sterile Tris medium and incubated at 30° with shaking (for $\frac{1}{4}$ hr, $\frac{1}{2}$ hr, $\frac{3}{4}$ hr or 1 hr). After incubation the tissue was cooled immediately, washed in ice-cold medium, then resuspended in 10 ml cold 0·1 M Tris pH 7·4 and quickly frozen and stored. The samples were subsequently thawed and FH_4 dehydrogenase enzyme preparations obtained, the final preparations each being made up to 1·5 ml. The enzymic activity and radioactive content of these preparations was determined.

Investigation of ^{3}[*H*] *MTX levels and* FH_4 *dehydrogenase recovery* in vivo

Livers were removed at various time intervals after ^{3}[H] MTX injection, (together with uninjected control livers) and perfused. Washing procedures were used similar to those given to the liver tissue, subsequent to the *in vitro* incubations described above.

(a) *Change in* ^{3}[*H*] *MTX content of whole tissue* in vivo. 0·6 g of each liver was chopped finely with scissors and washed twice in 10 ml cold Tris medium (by centrifugation at 500 *g* for 2 min). The tissue was rapidly frozen and stored. Their radioactive content was subsequently determined.

(b) *Recovery of enzyme activity* in vivo. six g of each liver was chopped finely and washed twice in 10 ml cold Tris medium, the tissue was then resuspended in 10 ml cold 0·1 M Tris-HCl pH 7·4, rapidly frozen and stored.

Subsequently, FH_4 dehydrogenase was prepared, the final enzyme preparations each being made up to 6 ml. FH_4 dehydrogenase activities and radioactive contents of the preparations were determined. In some experiments, the effects of i.p. injection of inhibitors such as cycloheximide and Actinomycin D on the recovery of the liver enzyme activity from methotrexate inhibition were determined. It has been reported that cycloheximide inhibits protein synthesis selectively, and Trakatellis, Montjar and Axelrod,[10] have reported that a single i.p. injection of 8 mg/100 g body wt. into a mouse inhibits liver protein synthesis virtually completely for more than 24 hr following the injection. In the present experiments however, it was found that the rats did not survive for more than 4–5 hr following a dose at that level. It was found that the highest dose which the rats would tolerate with 100 per cent survival after 24 hr was 0·1 mg/100 g body wt. Animals were therefore injected at this level with cycloheximide, and methotrexate was injected 1 hr later. Subsequent recovery of enzyme activity was compared with control rats receiving methotrexate or cycloheximide alone.

The effect of Actinomycin D on recovery of liver enzyme from inhibition by methotrexate *in vivo* was also studied. This antibiotic has been widely used in biochemical studies because of its ability to inhibit RNA synthesis, although its ability to affect processes other than DNA-directed RNA synthesis has been pointed out.[11] Animals received an i.p. injection of 0·1 mg/100 g body weight, 1 hr before methotrexate injection, and subsequent recovery of enzyme activity was compared with controls not receiving Actinomycin D.

Titration of FH_4 *dehydrogenase extracted at different times after MTX injection*

FH_4 dehydrogenase was extracted from 5 g liver pooled from 3 rats per group. The groups had been injected either $\frac{1}{2}$ hr, $1\frac{1}{2}$ hr, or 24 hr before death with 5 mg MTX, except for an uninjected control group. Enzyme activity was assayed spectro-

photometrically at 340 mμ using 0·05 ml each enzyme preparation to show the degree of recovery at the different times after injection. Each enzyme was then titrated by addition of varying amounts of 10^{-7} M MTX to the assays.

Attempted fractionation of inhibited FH_4 dehydrogenase into fractions with different MTX titration properties

FH_4 dehydrogenase was prepared from 5 g normal rat liver. Sufficient MTX was added to 0·5 ml enzyme preparation to produce approximately 90 per cent inhibition (0·6 ml 10^{-6} M MTX was required). The added MTX was labelled with 1 μc ^{3}[H] MTX. The inhibited enzyme was then run on to a DEAE-cellulose (10 × 1 cm) column and gradient elution with PO_4 buffer carried out as described by Mathews[7]. Two ml fractions were collected. Certain fractions were selected, their radioactivity determined and their FH_4-dehydrogenase activity titrated against 10^{-7} M MTX.[4]

Attempts to demonstrate metabolic inactivation of MTX with respect to its effect on FH_4 dehydrogenase activity

An attempt to inactivate MTX by incubation with chopped liver was carried out according to the method of Jacobson and Cathie.[12] All processes were carried out aseptically. 0·1 g finely chopped normal liver was washed three times in 10 ml sterile Tyrode solution. It was then resuspended in 10 ml Tyrode solution containing 10^{-3} M MTX, and incubated at 37° for 22 hr with shaking. The tissue was then spun off. The effect of 0·1 ml of the supernatant on a standard FH_4 dehydrogenase assay system containing 0·1 ml highly purified FH_4 dehydrogenase was examined at 340 mμ. The final concentration of the supernatant MTX when added to the assay cuvette was 5×10^{-5} M. The experiment was repeated using 1 g liver per 10 ml Tyrode containing 10^{-3} M MTX. It was then repeated a third time using 1 g liver per 10 ml Tyrode containing 10^{-5} M MTX. The final concentration of this supernatant MTX when added to the assay cuvette was 5×10^{-7} M.

RESULTS AND DISCUSSION

Recovery of FH_4 dehydrogenase activity following MTX injection

It has been shown that the large inhibition of mouse liver FH_4 dehydrogenase activity observed shortly after MTX injection is followed by a rapid recovery of

TABLE 1. FH_4 DEHYDROGENASE ACTIVITY IN RAT LIVER FOLLOWING MTX INJECTION

Time after MTX injection	Control Activity	½ hr		17 hr	
		Activity	% of control	Activity	% of control
Δ 340 mμ Assay	*0·241	*0·019	8	*0·163	67
MTT Assay	†0·054	†0·006	11	†0·034	63

Rats were killed ½ hr or 17 hr after injection of 5 mg MTX/0·5 ml physiological saline.

* Δ E_{340}/hr/mg protein.

† E_{560}minus FH_2 and $NADPH_2$ blanks/3½ hr/mg protein. Assay system contained 50 μM FH_2, 80 μM $NADPH_2$, 200 μg MTT, 2 mM PCMB, 20 sections (15 μ thickness) cut from fresh frozen liver, and Tris pH 7·4 in a total vol. of 2 ml. The sections were preincubated for ½ hr in 1·0 ml buffered 4 mM PCMB. After adding the other reagents the sections were incubated 3½ hr at room temperature. Tissue was then spun down and formazan extracted into 3 ml absolute methanol. Colour was determined at 560 mμ.

activity, the control level often being regained within 24 hr.[13] The apparent inability of MTX to suppress hepatic regeneration in rats might also be attributable to a rapid recovery of enzymic activity.[14] It appeared possible that rapid recovery of enzymic activity observed using homogenates, might in fact be due to loss of non-enzyme bound methotrexate from the tissue, and that the large initial inhibitions observed using isolated enzyme fractions might be in part at least, artifacts induced by free methotrexate coming into contact with the enzyme during homogenisation. However, using a colorimetric assay system[5] it was possible to compare the inhibition of the enzyme activity *in situ*, with the inhibition observed using the isolated enzyme fraction. The results given in Table 1 show that the inhibition and subsequent recovery of the enzyme activity observed using isolated enzyme fractions, appear to reflect the situation *in situ*. Although one cannot entirely rule out the possiblity that redistribution of MTX occurred during the MTT assay. It was also observed that the addition of calcium leucovorin to the assays *in vitro* (10^{-3} M) or injected i.p. into the animals *in vivo* (10 mg) had no significant effect on the degree of inhibition of enzymic activity observed at various time intervals after *in vivo* injection of methotrexate. Sartorelli Booth and Bertino[13] have also shown that a similar pattern of recovery of FH_4 dehydrogenase from MTX inhibition occurs in mouse ascites cells. It was considered to be of interest to examine the effect of MTX (5 mg i.p.) on FH_4 dehydrogenase activity in a solid tumour. Using an enzyme extract from the Guerin T8 tumour grown in rats, and assaying the FH_4 dehydrogenase at 340 mμ a similar pattern of results was obtained to that observed using liver. A large inhibition was followed by a rapid recovery of activity. Using the MTT colorimetric assay system and sections cut from the outer edges of a fresh frozen tumour, it was found that $\frac{1}{2}$ hr after injection of MTX, the FH_4 dehydrogenase activity was only 29 per cent of that of a tumour excised from an uninjected rat, clearly demonstrating that *in situ* inhibition had occurred. A similar degree of inhibition was observed in sections cut from non-necrotic inner areas of the tumour. It thus appeared that despite poor vascularisation of the tumour tissue,considerable inhibition of FH_4 dehydrogenase occurred throughout the tumour within $\frac{1}{2}$ hr after injection of MTX.

In view of the rapidity of the recovery of enzyme activity, both in the liver and solid tumour, and the possible relationship between duration of inhibition and clinical effectiveness of the drug, it was decided to carry out a preliminary examination of the possible mechanisms of recovery of enzyme activity. It appeared that three main possibilities existed:

(1) Metabolism of the methotrexate to an inactive form.
(2) Synthesis of new FH_4 dehydrogenase enzyme.
(3) Dissociation of the methotrexate—FH_4 dehydrogenase complex.

Experiments were therefore carried out examining each of these possibilities.

(1) *Metabolism of MTX to an inactive form*

Evidence has been produced that aminopterin causes "metaphase arrest" in dividing cells, and that certain cell types, including mouse liver, are able to overcome this inhibition by inactivating the inhibitor.[12] Attempts were therefore made to metabolise MTX to an inactive form, according to the method of Jacobson and Cathie[12] and, subsequently, to study the effect of the "metabolised" MTX on highly-purified FH_4 dehydrogenase. Initial activity of the enzyme was 0·15 ΔOD/hr/0·1 ml enzyme.

Addition of 0·1 ml of each supernatant fraction, following incubations as detailed in the methods section caused total inhibition of the dehydrogenase activity. Thus even using ten times the amount of tissue and 1/100 of the concentration of anti-folate originally used by Jacobson and Cathie,[12] no inactivation of MTX *with respect to its inhibitory power of FH_4 dehydrogenase* was detected *in vitro*. It appeared possible however that inactivation might occur *in vivo*. Jacobson and Cathie[12] have suggested that *in vivo* inactivation of methotrexate might occur as a result of ring closure of the terminal glutamate, and have demonstrated that aminopterin is not inactivated *in vitro* in the presence of high concentrations of glutamate. Experiments were therefore carried out in the present study in which recovery of FH_4 dehydrogenase activity from MTX inhibition was compared between animals receiving hourly i.p. injections of 1 mg glutamate and other animals which did not. No significant differences between the rates of recovery of enzyme activities was observed.

(2) *Synthesis of new FH_4 dehydrogenase enzyme*

In view of the reports of prolonged retention of unmetabolised methotrexate in man, suggested as being bound to tetrahydrofolate dehydrogenase,[15] it appeared possible that the recovery of enzymic activity represented synthesis of new enzyme, and that the original enzyme-inhibitor complex still remained within the cells. Two experimental lines of approach were adopted examining this possibility.

(a) *Reactivation of inhibited enzyme.* As reported in the methods section, the MTX-enzyme complex can be dissociated chromatographically on DEAE-cellulose columns. It would appear that if enzyme recovery was due to synthesis of new enzyme, dissociation of enzyme-inhibitor complex should increase the activity of a "recovered" enzyme preparation above that of a similarly treated control extract. It can be seen from the results of a typical experiment given in Table 2 that no such discrepancy in activities between control and formerly inhibited preparations was observed after passage through the columns.

TABLE 2. REACTIVATION OF INHIBITED FRACTIONS ON DEAE-CELLULOSE

Enzyme preparation	Total activity placed on column	% of Control	Total activity eluted from column	% of control
Control	2·15	100	7·7	100
½ Hr MTX	0·60	28	5·9	77
48 Hr MTX	1·80	84	7·2	94

2 rats per group. 1 mg MTX injected i.p. Enzyme extracted from 5 g of pooled liver. Activities μmole FH_2/hr. For details of chromatography see Methods section.

(b) *Effect of cycloheximide or Actinomycin D on recovery from MTX inhibition.* Animals were injected i.p. with 0·1 mg cycloheximide or 0·1 mg Actinomycin D, and 1 hr later received an i.p. injection of 1 mg MTX. There were 2 rats per group. 20 hr after the injections of MTX, the rats were killed, livers excised and enzymes prepared and assayed. Previous results had established that addition of 100 μg of cycloheximide to the *in vitro* assay system did not reduce the activity by > 10 per cent, and that Actinomycin D at this level was without effect on the activity. In a typical experiment, the specific activity of the enzyme prepared from animals receiving methotrexate

alone was 0·09 μmole FH_2/hr/mg protein, that prepared from animals receiving cycloheximide and methotrexate 0·08 μmole FH_2/hr/mg protein, and that of the Actinomycin D—methotrexate injected group, 0·12 μmole FH_2/hr/mg protein. In some experiments higher concentrations of cycloheximide were used for shorter time experiments, for reasons given in the Methods section. In no cases were cycloheximide or Actinomycin D injections found to inhibit significantly the recovery of enzyme activity. The elevation of activity often observed following Actinomycin D injections was not particularly investigated.

(3) *Dissociation of enzyme-inhibitor complex.*

Werkheiser[4] originally suggested that the interaction of FH_4 dehydrogenase with folate antagonists is characterised by the fact that, at levels of the inhibitor which are inadequate to cause complete inhibition, all the inhibitor is enzyme bound. He also suggested that the binding of the enzyme by the inhibitor is sufficiently "tight" to preclude any possibility of dissociation. However, titration data obtained by Mathews[7] has indicated that stoichiometric binding is obtained only at lower pH values, and that at pH 7·5, diversion from the stoichiometric relationship is observed. Hakala[1] using Sarcoma 180 cells observed that the development of resistance to methotrexate in these cells is not accompanied by changes in permeability to the drug, and has suggested rather that dissociation of the enzyme-methotrexate complex within the cells plays an important role in determining the degree of resistance. The possibility therefore exists that at the pH of the enzyme location *in situ*, dissociation of the enzyme-inhibitor complex might take place in the liver.

(a) *Experiments on recovery of activity* in vivo. These experiments were carried out as detailed in the Methods section, with the result given in Fig. 1. It appears that recovery of enzymatic activity is accompanied by a decrease in tritiation of the total homogenate and also in the partially purified enzyme fraction itself. It was found during the course of these experiments that recovery of enzymatic activity 24 hr after MTX injection varied between 30 and 100 per cent. It appears however, that the recovery of enzyme activity is related to a fall in MTX content of the extracts. This data would appear to be consistent with a dissociation of the enzyme-inhibitor complex.

(b) *Experiments on recovery of activity* in vitro. Experiments were carried out in an attempt to reactivate inhibited enzymes *in situ*, by *in vitro* incubations of liver tissue removed from rats injected with MTX. The details of the incubation technique used were based on those described by Kessel and Hall.[9] As can be seen from Fig. 2 when liver was taken from rats ½ hr after injection of MTX, finely chopped and incubated at 30°, a two-phase curve of decrease in ^{3}[H] MTX content of the tissues was obtained, similar to that observed by Kessel and Hall.[9] Although levels of FH_4 dehydrogenase of control liver tended to decrease with increasing time of incubation at 30°, the enzyme level of the inhibited livers increased in activity. In Fig. 2 therefore, the activities of the inhibited tissues after various periods of incubation are expressed as percentages of the control tissues incubated for similar periods of time. It can be seen that the increase in enzymatic activity is accompanied by a loss of tritium from both the total homogenate of the tissue, and also from the enzyme fraction. The results given in Fig. 2 are expressed per unit weight of original tissue. It can be seen from Fig. 3 that in a separate experiment when the results are

expressed per mg of protein of the enzyme fractions, a similar rise in enzyme activity accompanying loss of ^{3}[H] MTX could be demonstrated. It therefore appeared that dissociation of enzyme-inhibitor complex could play a major role in the recovery of the enzymic activity *in vivo*, and that *in vitro* systems could be used to examine this process.

(c) *Titration of FH_3 dehydrogenase preparations.* Further evidence supporting the theory that dissociation of the enzyme-inhibitor complex was a major factor in the recovery of enzyme activity, was obtained from experiments in which the activity of of enzyme fractions obtained from control or inhibited livers, removed at various times after MTX injections, were titrated against MTX *in vitro*. As already referred to (see Methods section) different shaped titration curves are obtained depending on the pH buffering medium. Preliminary tests showed that whereas a more nearly linear relationship was obtained between enzyme activity and MTX added at pH 5·0, using

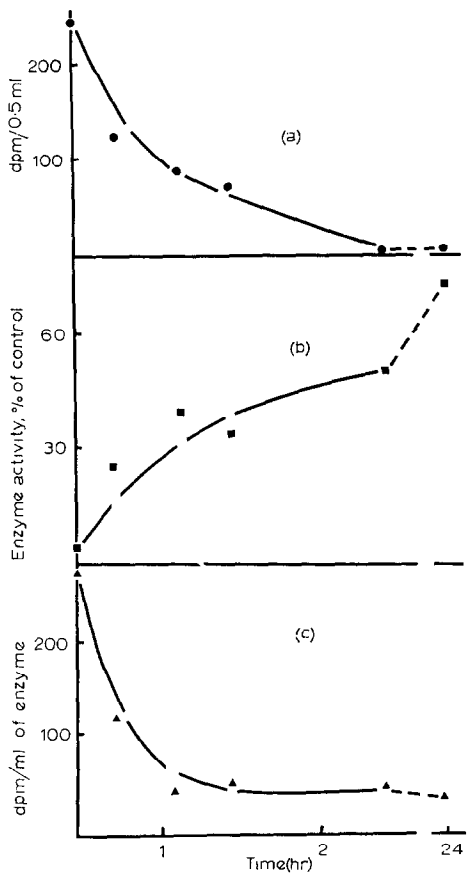

FIG. 1. ^{3}[H]-MTX content and FH_4 dehydrogenase recovery *in vivo*.

Two livers were removed at each of the specified times after injection of 5 mg ^{3}[H]-MTX (sp. act. 1 μc/5 mg MTX), together with uninjected control livers. (a) change in ^{3}[H]-MTX content of whole tissue *in vivo*, dpm per 0·5 ml whole homogenate were counted. (b) recovery of enzyme activity *in vivo*, the change in extinction at 340 mμ/0·05 ml enzyme preparation was measured and the results expressed as percentages of the control values; (c) loss of ^{3}[H]-MTX from the enzyme fraction, dpm/1 ml enzyme fractions were counted.

citrate buffer and the highly purified enzyme preparation, when it was attempted to assay the less highly purified preparations at this pH, considerable precipitation occurred. Since it was thought to be inadvisable to attempt to purify the preparations too vigorously, in case the enzyme-inhibitor complex was partially disrupted, the titrations were routinely carried out at pH 7·4 and the results of a typical experiment are given in Fig. 4 The initial rates of enzyme activities for the preparations used in Fig. 4 were as follows: ½ hr extract—31 per cent of the control level, 71 per cent for the 1½-hr sample and 90 per cent for the 24-hr sample. It can be seen from Fig. 4, that recovery of the enzyme activity is accompanied by a recovery of the original titration curve. It appears therefore, that at this pH value, in contrast to the situation at pH 5·0, increasing amounts of inhibitor-bound activity cause the shape of the titration curve to change indicating apparently, increasing resistance to inhibition by the further

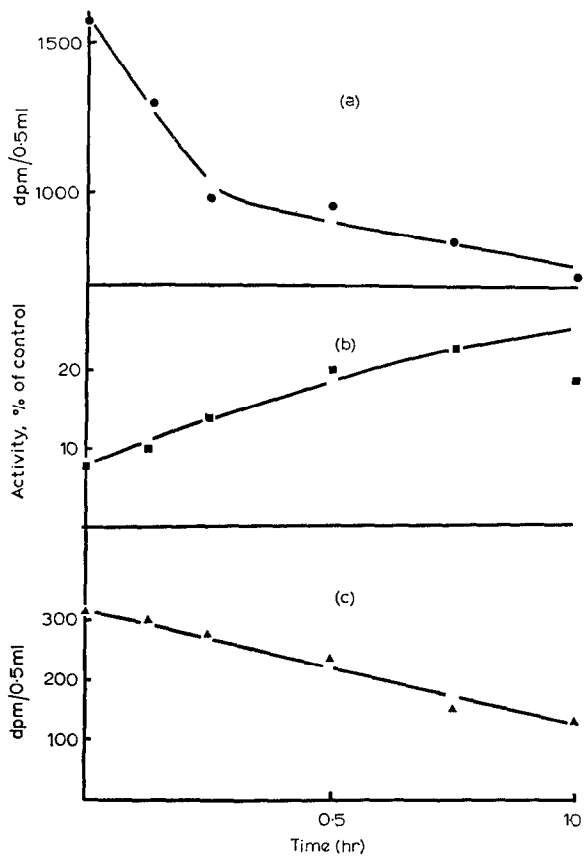

FIG. 2. ^{3}[H]-MTX content and FH_4 dehydrogenase recovery *in vitro*.
(a) Change in ^{3}[H]-MTX content of whole tissue *in vitro*, dpm/0·5 ml total homogenate were measured and are plotted against time of incubation at 30°; (b) Recovery of enzyme activity *in vitro*, the enzyme rates/0·1 ml enzyme preparations were measured and are expressed as percentages of the values of control livers at the various times of incubation; (c) change in ^{3}[H]-MTX content of the enzyme fraction *in vitro*, dpm/0·5 ml enzyme extract after the various periods of incubation were measured.

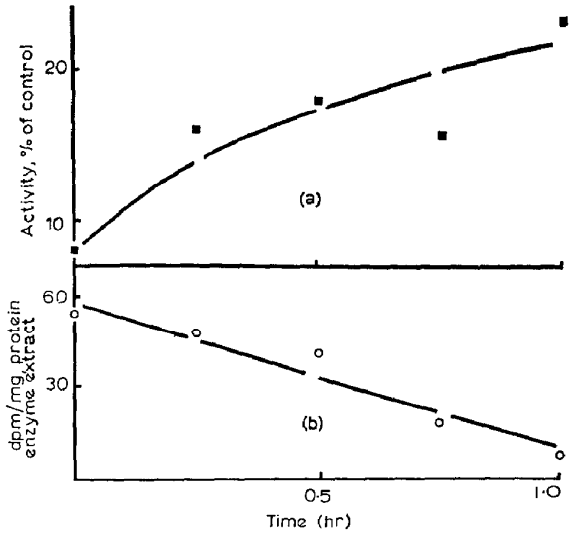

FIG 3. Recovery of enzyme activity and loss of ^{3}[H]-MTX from a partially-purified enzyme fraction on incubation *in vitro*.
(a) Enzyme activities. Specific activities of enzyme fractions expressed as percentages of controls; (b) Radioactivity per mg protein. Results are means of duplicate determinations (± 5%).

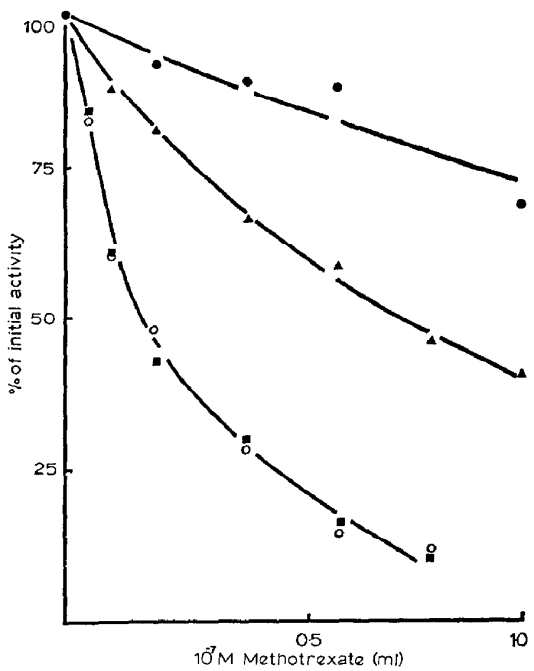

FIG. 4. Effect of addition of MTX *in vitro* on FH_4 dehydrogenase extracted from liver at various times after MTX injection *in vivo*. Enzyme activities on addition of 10^{-7} M MTX expressed as percentages of the initial activities. ○—○, FH_4 dehydrogenase extracted from control animals; ■—■, FH_4 dehydrogenase extracted 24 hr after i.p. injection of 5 mg MTX; ▲—▲, FH_4 dehydrogenase extracted 1½ hr after i.p. injection of 5 mg MTX; ●—●, FH_4 dehydrogenase extracted ½ hr after i.p. injection of 5 mg MTX.

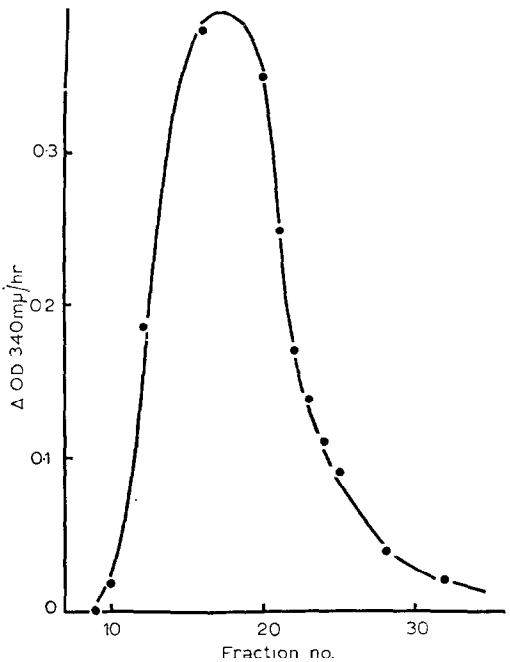

FIG. 5a. Gradient elution of FH_4 dehydrogenase from DEAE-cellulose. A DEAE-cellulose column (10 × 1 cm) was used, and inhibited enzyme placed on it as described in the text. The constant volume mixing chamber contained 100 ml distilled water initially, and the reservoir contained 0·075 M phosphate buffer pH 6·9. 2-Ml fractions were collected (at 0–4°). Activities of the eluted fractions were assayed at 340 mμ using 0·5 ml each fraction.

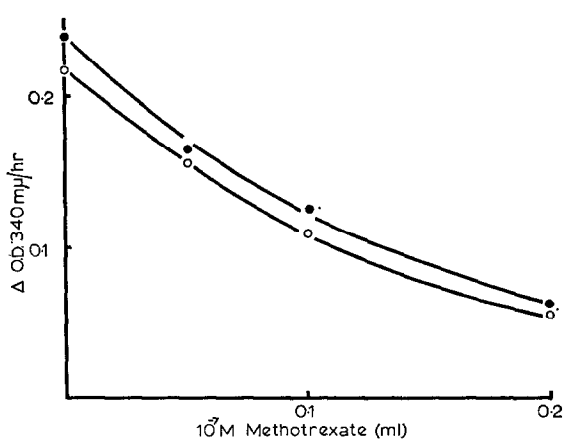

FIG. 5b. Titration of FH_4 dehydrogenase in the early and late fractions with MTX. The activities of 0·75 ml of a combined early fraction (11 + 12 + 13) and a combined late fraction (21 + 22 + 23) were titrated with various amounts of 10^{-7} M MTX. ●—●, Early fraction; ○—○, Late fraction.

addition of methotrexate. These results would therefore appear to indicate that recovery of enzymic activity is accompanied by a loss of bound enzyme activity.

An examination of enzyme fractions for the possible presence of different forms of FH_4 dehydrogenase

The rapid recovery of activity of FH_4 dehydrogenase could possibly be related to the presence of an isomeric form of the enzyme, with greater resistance to MTX inhibition than the bulk of the enzyme. The possibility that the dehydrogenase enzyme can exist in isomeric form has been reported.[16] A higher percentage of the activity of such a fraction would be retained following the injection of MTX, and the subsequent activity of this enzyme fraction could be involved in the displacement of MTX from the more susceptible enzyme fraction. It was thought that the shape of the titration curves, referred to in the previous section, although clearly indicating the amount of bound-enzyme present, could also, by reason of increasing resistance to MTX inhibition (Fig. 4) indicate the presence of such a species of enzyme with higher resistance to MTX inhibition. Experiments were carried out therefore, as detailed in the Methods section, in which partially-purified enzyme was inhibited by addition of labelled MTX, and subsequently reactivated by progressive elution from a DEAE-cellulose column. Fig. 5(a) shows the elution pattern of the enzyme activity, and radioactive determinations showed that all the active fractions were free of methotrexate. It was thought that ease of dissociation of the enzyme-inhibitor complex might be related to order of elution of the enzyme. An early fraction and a late fraction were therefore titrated against methotrexate, with the result given in Fig. 5(b). It therefore appears that, using this technique no evidence can be obtained for the presence of more than one species of FH_4 dehydrogenase, differing in their susceptibilities to inhibition by MTX.

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