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Biochemical Pharmacology, Vol. 17, pp. 484–487. Pergamon Press 1968. Printed in Great Britain

A colorimetric assay system for tetrahydrofolate dehydrogenase

(*Received* 19 *January* 1967: *accepted* 17 *July* 1967)

IT APPEARS that no chromogenic assay method for the demonstration of FH_4 dehydrogenase (EC 1.5.1.3) activity has so far been devised. Frequent reference has been made to the need for such a method which could possibly be applied to the demonstration of enzymic activity *in situ*.[1] This need arises because of the importance of the enzyme in cell replication and its association with the antifolate therapy of leukaemia.[2]

This study was carried out using the following systems:—

A. *A partially purified FH_4 dehydrogenase preparation, obtained from rat liver.* 5 g. Liver was homogenised in 0·1M Tris -HCl buffer, pH 7·4. This was then centrifuged at 100,000 *g* for 1 hr. Supernatant protein which precipitated at 40–80% saturation using solid ammonium sulphate was dissolved in 0·1M Tris-HCl buffer, pH 7·4, and then dialysed at 4° overnight. This preparation contained $NADPH_2$ diaphorase as well as FH_4 dehydrogenase activity. The sp. act. of the FH_4 dehydrogenase was 10 enzyme units/mg protein.[3]

B. *A highly purified FH_4 dehydrogenase preparation.* The partially purified FH_4 dehydrogenase preparation was further purified, according to the method of Mathews *et al.*[3] yielding a preparation with a sp. act. of 300 units/mg protein.

C. *Rat liver sections.* Tissue sections (15 μ in thickness) were cut from cylindrical blocks (6 mm dia.) of fresh frozen liver, using a Bright's Cryostat.

D. Mouse ascites cells. Mouse Ehrlich-Landschütz ascites cells were isolated according to the method of Lord.[4] After removal of the clots formed by incubation at 37°, cells were collected by centrifugation, washed twice in physiological saline, and finally resuspended in approximately 10 vol. of saline.

Preliminary experiments showed the impracticability of demonstrating colorimetrically the enzyme activity by reversing the normal direction of the reaction i.e. attempting to demonstrate the dehydrogenation of FH_4. It was, however, observed that under the assay conditions usually employed, FH_4 reduced MTT (3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide) (from Koch-Light) non-enzymically. The results given in Fig. 1. show the successful application of this observation to assaying the activity of the highly purified FH_4 dehydrogenase preparation and the inhibition of the

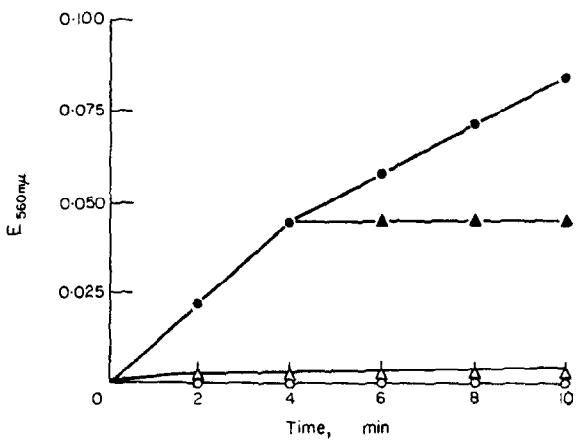

FIG. 1. Assay of FH_4 dehydrogenase using MTT.
● — ● complete system:— 20μM FH_2, 20μM $NADPH_2$, 0·05 M Tris (pH 7·5), 100μg MTT, 0·01 ml. highly purified FH_4 dehydrogenase, total vol. = 2 ml.
△ — △ minus $NADPH_2$.
○ — ○ minus FH_2.
▲ — ▲ complete system, 10^{-5} M MTX added after 4 min.

reaction by 10^{-5}M MTX (methotrexate). The results also show that the direct reduction of MTT by FH_2 (dihydrofolate) at the concentration of FH_2 used in this assay system is negligible. However, higher concentrations of FH_2 yield correspondingly higher blanks. Experiments showed the feasibility of using this chromogenic reaction for the location of FH_4 dehydrogenase activity on agar gel electrophoresis plates. The method has also been applied successfully to the location of enzymic activity in polyacrylamide gel electrophoresis studies.[5]

Although it is clearly possible to assay highly purified systems using MTT (see Fig. 1.), attempts to assay activity in less highly purified enzyme preparations or in intact tissues would be complicated by the simultaneous reduction of MTT by $NADPH_2$ diaphorase (EC 1.6.99.1). If this MTT assay system is to be applied to intact tissues or less highly purified isolated enzyme systems, it would appear desirable to reduce the very high rates of formazan production from MTT by diaphorase activity (diaphorases being of widespread distribution). Experiments were therefore carried out using the partially purified enzyme preparation in an attempt to inhibit selectively the diaphorase activity in the presence of FH_4 dehydrogenase. PCMB (*p*-chloromercuribenzoic acid) (Koch-Light) proved to be the most suitable inhibitor of those examined. The effect of various concentrations of this inhibitor on $NADPH_2$ diaphorase and FH_4 dehydrogenase activity in the partially purified enzyme system is shown in Table 1. Other experiments have shown that usually more than half the FH_4 dehydrogenase activity of a highly purified enzyme system remains even in the presence of 3mM PCMB.

FH_4 dehydrogenase activity present in fresh frozen liver sections was assayed using this technique and the results are given in Table 2. When using tissue slices or whole cells, preincubation in 4 mM

PCMB was found to be desirable in order to reduce formazan production due to diaphorase activity to a minimum, presumably due to slow penetration of the inhibitor to the active sites. Dicoumarol,

TABLE 1. EFFECT OF VARIOUS CONCENTRATIONS OF PCMB ON $NADPH_2$ DIAPHORASE AND FH_4 DEHYDROGENASE ACTIVITIES

	Enzyme activity in partially purified rat liver system		
PCMB conc. (mM)	0·5	1·0	1·5
* $NADPH_2$ diaphorase	35	20	10
† FH_4 dehydrogenase	100	124	72

Results are expressed as percentages of the control activity (no PCMB present).

* Experimental assay contained:— 20μM $NADPH_2$, 100μg MTT, 0·05 M Tris (pH 7·5), 0·05 mlp artially purified enzyme fraction. Total vol. = 2 ml. The rate of increase in extinction at 560 mμ/hr/mg protein was measured.

† Experimental assay contained:— 20μM $NADPH_2$, 20μM FH_2, 0·05 M Tris (pH 7·5), and 0·05 ml partially purified enzyme fraction. Total vol. = 2 ml. The rate of decrease in extinction at 340 mμ/hr/mg protein was measured.

TABLE 2. ASSAY OF FH_4 DEHYDROGENASE IN RAT LIVER SECTIONS USING MTT

	E_{560} mμ/mg. protein
* Complete system	0·131
Minus $NADPH_2$	0·030
Minus FH_2	0·025
† Complete system (boiled sections)	0·056
Complete system + 10^{-5} M MTX	0·064

* Complete system contained:—50μM FH_2, 80μM $NADPH_2$, 0·05 M Tris (pH 7·4), 200μg MTT, 2 mM PCMB and 20 sections (15μ thickness) containing 2·84 mg protein, cut from fresh frozen liver. Total vol. = 2 ml. Other systems varied with reference to complete system as indicated. Sections preincubated ½ hr in 1·0 ml buffered 4 mM PCMB, together with 2×10^{-5} M MTX where indicated. Further reagents added and sections incubated 3 hr at room temperature. Tissue was then spun down and formazan extracted into 3 ml absolute methanol. Colour was determined at 560 mμ.

† Sections (in 0·05 M Tris (pH 7·4)) boiled for 5 min prior to preincubation and assay.

which is as effective as PCMB in the selective inhibition of $NADPH_2$ diaphorase in the presence of FH_4 dehydrogenase when isolated enzyme systems were used, was found to be incapable of penetrating to the sites of diaphorase activity in intact cells. From Table 2. it can be seen that the reaction is capable of assaying FH_4 dehydrogenase *in situ*. 10^{-5} M MTX reduced formazan production to a level similar to that of the sum of the amounts of colour formed in the absence of either $NADPH_2$ or FH_2.

Table 3. shows that the assay system can be successfully applied to intact ascites cells and also demonstrates the feasibility of using this reaction to demonstrate the inhibition of FH_4 dehydrogenase in intact cells following *in vivo* antifolate injections.

It thus appears that using this technique, FH_4 dehydrogenase activity can be demonstrated and also assayed colorimetrically using isolated enzyme systems, small quantities of intact ascites cells, or fresh frozen tissue sections.

TABLE 3. ASSAY OF FH_4 DEHYDROGENASE IN INTACT ASCITES CELLS USING MTT

	E_{560} mμ/mg protein	
	*Ascites cells	†Ascites cells harvested ½ hr after MTX injection
Complete system	0·165	0·094
Complete system + 10^{-5} M MTX	0·098	0·096

* Complete system and incubation times as for liver (see Table 2) but using 0·1 ml dilute ascites cell suspension (containing 0·75 mg protein) in place of the liver sections. The cells were maintained in an isotonic medium.

† Complete system as for liver (see Table 2.) but using 0·1 ml ascites cell suspension, the cells having been harvested ½ hr after i.p. injection of 1 mg MTX dissolved in 0·5 ml physiological saline.

Acknowledgements—The Authors would like to thank Miss G. M. Baker for carrying out the animal experiments, and Miss C. Stevens and Miss L. Goldsmith for technical assistance. We also wish to thank Lederle Co., Great Britain, for the generous gift of Methotrexate.

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