MULTIPLE FORMS OF DIHYDROFOLATE REDUCTASE[1]

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Dihydrofolate reductase, which catalyzes the TPNH-dependent reduction of dihydrofolate, has been purified from a variety of sources (reviewed by Huennekens, 1968). In some instances, multiple forms of the enzyme have been detected by column chromatography (Kaufman and Gardiner, 1966; Sirotnak and Hutchison, 1967; Perkins, et al, 1967), by filtration through Sephadex (Hillcoat and Blakley, 1966; Sirotnak and Hutchison, 1966), and by electrophoresis on starch gel (Mell, et al, 1968). The present communication provides further information about the properties and interrelationship of the electrophoretically separable forms of the chicken liver dihydrofolate reductase.

Fig. 1 illustrates the pattern of enzyme activity obtained when a partially purified preparation (sp. act. = 1.4 μmole of dihydrofolate reduced/min/mg protein) of the chicken liver enzyme dihydrofolate reductase was subjected to electrophoresis on a cellulose acetate membrane at pH 8.5 (Tris-borate-EDTA). Three separate enzymatic components were present, all of which migrated toward the positive electrode. Enzyme activity was detected by placing the membrane against an agar plate containing dihydrofolate, TPNH and MTT[3], a tetrazolium

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[3] 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide.

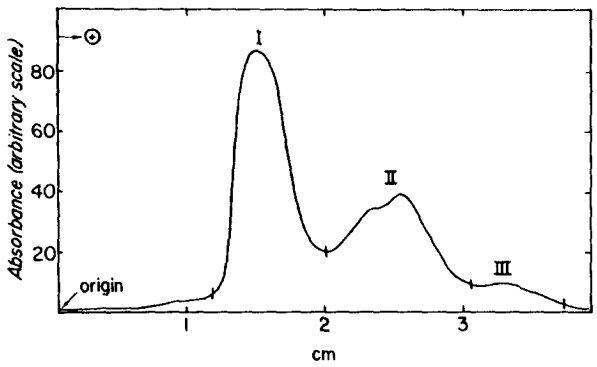

Figure 1. Electrophoresis of chicken liver dihydrofolate reductase on cellulose acetate.

The enzyme (sp. act. = 1.4; 3.3×10^{-4} units of activity in 0.75 μliters) was applied to the origin of a 5.7 x 14.3 cm sheet of cellulose acetate and subjected to electrophoresis at 5° using the Beckman Microzone apparatus, Model R-100 (450 volts; 70 minutes). The buffer was 0.13 M Tris-0.071 M borate, pH 8.5, containing 3×10^{-3} M EDTA. Protein was located with Beckman Fixative-Dye (0.2% Ponceau-S stain). Enzyme activity was detected by the following procedure: Special Agar-Noble (135 mg) was dissolved with heating in 5 ml of 0.1 M Tris buffer, pH 7.5. When the agar had cooled to about 70°, MTT (2.5 mg in 2.5 ml of the same buffer), TPNH (2.5 mg in 0.5 ml of buffer) and dihydrofolate (1.5 mg in 0.5 ml of buffer) were added. The resulting mixture was poured quickly into a shallow, rectangular tray and allowed to solidify. The cellulose acetate membrane was placed on the agar and allowed to stand at 37° in an incubator. Quantitation was obtained by scanning the membranes with a Beckman Microzone densitometer, Model R-110.

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dye that reacts non-enzymatically with tetrahydrofolate (Gunlack, et al, 1968) Omission of dihydrofolate, or addition of Aminopterin (10^{-5} M) to the agar, completely suppressed the appearance of the purple bands, thereby ruling out artifacts. Quantitation of enzyme activity was obtained by passing the membrane through a densitometer. By integration of the areas under the peaks in Fig. 1, bands I, II and III were found to contain 55, 38 and 7%, respectively, of the total activity. The protein profile (not shown in the Fig.) contained components in addition to those associated with the enzymes.

Examination of a number of electrophoretograms revealed that the amount of II, relative to I, varied from sample to sample of enzyme, and that II occasionally appeared as a doublet (cf. Fig. 1). It was then observed that dialysis of the enzyme increased the amount of II, but this effect was found

to be due to some factor associated with the dialysis tubing (sizes 20, 27 and 36, obtained from Union Carbide Corporation), rather than to dialysis itself. This conversion of I into II'(the designation for a component whose mobility is similar to naturally-occurring II) was not observed with tubing that had been pretreated with 10^{-2} M mercaptoethanol for 1 hour at 60 - 70°.

The conversion of I to a II' form could also be achieved in a more definite manner (Fig. 2) by treatment of the enzyme with p-chloromercuribenzoate (pCMB)[4], an agent known to stimulate the activity of dihydrofolate reductase (Kaufman, 1964; Perkins and Bertino, 1964). For comparison, the effect of pCMB upon enzyme activity is also shown in the Figure. Since only one cysteine residue in this enzyme is accessible to mercurials (Mell, et al, in preparation), it is likely that one additional negative charge is introduced via pCMB. This appears to account for the difference in mobility between II' and I. Support for this hypothesis is provided by the fact that methylmercury bromide, which lacks a negative group, was unable to convert I to II', even though it, too, stimulated enzyme activity about 5-fold.

In light of these results, the purification procedure was reinvestigated using only dialysis tubing that had been washed with mercaptoethanol. Under these conditions, enzyme at a stage of purification comparable to that in Fig. 1 showed about 85 to 95% of the total enzymatic activity in peak I and only about 5 to 15% in the peak II region. That the latter is the "natural" II was further shown by the fact that it was not converted to I by treatment with mercaptoethanol or dithiothreitol. By contrast, II' forms (whether arising from treatment with pCMB or the dialysis tubing factor) were completely transformed into I by exposure to thiols. The existence of a naturally-occurring II is also supported by electrophoretic analysis of a dihydrofolate reductase from Amethopterin-sensitive L1210 cells. This enzyme, purified by a modification of the method

[4] A faster moving component (III') was also detected in some pCMB-treated enzymes.

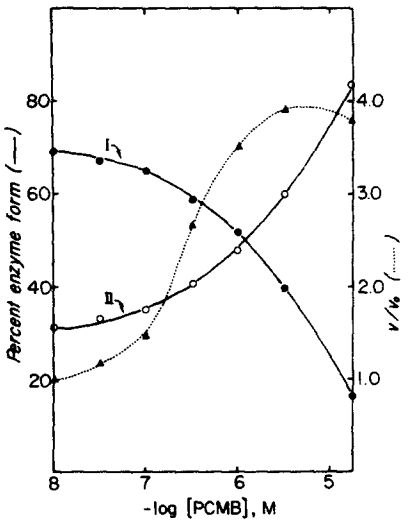

Figure 2. Effect of pCMB concentration on the electrophoretic mobility and activity of chicken liver dihydrofolate reductase.

The preincubation mixture contained the following components in a total volume of 0.45 ml: enzyme (sp. act. = 2.1, 30 μg protein); potassium phosphate buffer, pH 7.5, 25 μmoles; and pCMB at the indicated final concentrations. After preincubation at room temperature for 5 min, a 20 μl aliquot was removed and assayed for enzyme activity(-----). Activity was measured in the presence (v) and absence (v_o) of the mercurial and the ratio v/v_o plotted against negative log of pCMB concentration. The samples were placed on ice and after one hour subjected to electrophoresis on cellulose acetate, as in Fig. 1. The percent of total activity represented by I and II (----) was obtained from densitometer tracings.

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of Reyes and Huennekens (1967) in which dialysis was not involved, has a 3-banded pattern similar to that in Fig. 1 except that II was more intense than I. In order to prevent streaking of the L1210 bands, it was necessary to prewash the membrane by electrophoresis or to include TPNH (10^{-5} M) in the electrophoresis buffer.

I and II have been separated on a preparative scale by means of vertical electrophoresis on starch gel (Mell et al, 1968) or chromatography on DEAE-Sephadex. In the former method, zones of enzyme activity were located as dark quenching bands against a fluorescent background by examining the gel surface under ultraviolet light after treatment with TPNH and dihydrofolate

(each at 10^{-3} M). Three bands[5], comparable to those in Fig. 1, were seen on the starch gel. These were excised, macerated with 0.5 M phosphate buffer, pH 7.5, frozen and thawed, and centrifuged; about 25% of the original enzyme activity was recovered by this procedure. The second method, i.e. chromatography on DEAE-Sephadex, gave two peaks (Fig. 3) which were found to correspond, respectively, to electrophoretic forms I and II. When I or II was subjected a second time to electrophoresis or chromatography, there was no interconversion to the other form, thereby providing evidence that I and II are not in equilibrium with each other.

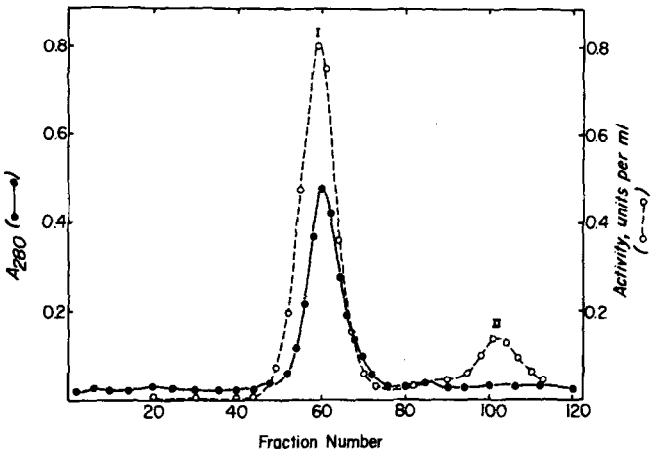

Figure 3. Chromatography of chicken liver dihydrofolate reductase on DEAE-Sephadex.

Enzyme (sp. act. = 2.2, 41 mg protein) in 48 ml of 0.5 M Tris buffer, pH 8.5, was chromatographed on 2.5 x 30 cm column of DEAE-Sephadex (A-50) that had been equilibrated previously with 0.05 M Tris, pH 8.5. Gradient elution was carried out with 500 ml of 0.05 M Tris, pH 8.5, in the mixing chamber and 500 ml of 0.15 M Tris, pH 7.5, in the reservoir, Fractions (8 ml) were collected automatically.

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[5] When the Tris-borate-EDTA buffer at pH 8.5 was replaced by a phosphate buffer at the same pH, migration of all proteins in the sample was greatly reduced and only one zone of enzymatic activity was seen. Furthermore, when EDTA was omitted from the Tris-borate buffer, the bands of either the chicken liver or L1210 enzyme were poorly resolved. This could be overcome, however, by adding TPNH (10^{-5} M) to the system. TPNH effects on the enzyme are treated in detail elsewhere (Mell, et al, in preparation).

Preliminary experiments (Mell, et al, 1968) failed to disclose any difference in physical or catalytic properties of I and II that might be correlated with the observed differences in their electrophoretic mobilities. However, these results were obtained with preparations of II that included considerable amounts of II' (produced by dialysis). More recent experiments have shown that "natural" II is activated by pCMB to a lesser degree than I, and there may be small differences in the sensitivity of I and II to Amethopterin.

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