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# An immunological study of an enzyme made by phage containing 5-iodo-2′-deoxyuridine-substituted deoxyribonucleic acid

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T4 PHAGE in which 5-iodo-2′-deoxyuridine (IUdR) has replaced more than 60 per cent of DNA-thymidine (60-heavy phage) are unable to induce normal levels of the enzyme dCMP hydroxymethylase in *E. coli*.[1] Since IUdR in DNA may alter the codon for dCMP hydroxymethylase, an attempt was made to determine if an altered enzyme fraction exists that is biologically inactive but immunologically related to the normal dCMP hydroxymethylase molecules. For this purpose the precipitin reaction between control and anti-dCMP hydroxymethylase mouse ascitic fluids and preparations of dCMP hydroxymethylase from *E. coli* B3 cells infected with normal or 60-heavy T4td8 phage was investigated.

## MATERIALS AND METHODS

The dCMP hydroxymethylase for eliciting antibody production was prepared from *E. coli* B cells infected with the amber mutant T4N82 and purified 270-fold, according to the method of Mathews *et al.*[2] The purified enzyme (774 units/ml) in 0·05 ml portions was injected with 0·1 ml Freund's incomplete adjuvant intraperitoneally into mice weekly for 5 weeks, and Sarcoma 180 TG cells were injected simultaneously at the fifth week.[3] The immune ascitic fluid, collected 9 to 16 days later, was centrifuged (2000 *g* for 20 min) and the supernatant fraction was stored at − 20°.

The normal and 60-heavy phage were prepared and used for infection of *E. coli* B3 cells as described previously.[1] The dCMP hydroxymethylase preparations used in the precipitin reaction were only partially purified from the phage-infected cells by the method of Mathews *et al.*[2] This included treatment with streptomycin sulfate, ammonium sulfate fractionation, and treatment with Sephadex G25. This gave a 2- to 3-fold purification.

The general character of the precipitin reaction used in these experiments is demonstrated in the antibody titration. A series of dilutions of antibody was made. A solution of 3% gelatin (0·02 ml) and an appropriate volume of potassium phosphate buffer (50 mM, pH 8·0) were added such that the final volume, after addition of enzyme, was 0·6 ml. The reaction was initiated by the addition of a constant volume of enzyme to each of the tubes and mixing. The mixtures were incubated at 37° for 2 hr, at 4° for 3 days, and then centrifuged. The enzymic activity in the supernatant fluid and in the precipitate was determined.

The dCMP hydroxymethylase assay was performed on 0·2 ml of the supernatant fluid and 0·2 ml of a solution of the precipitate (dissolved in 0·01 ml 3% gelatin and 0·29 ml potassium phosphate buffer). The reaction mixture contained 1 μmole of tetrahydrofolic acid, 20·3 μmoles of β-mercapto-ethanol, 2 μmoles of $^3$H-dCMP (50 mμc/μmole), and 120 μmoles of potassium phosphate, pH 8·0, in a total volume of 0·2 ml. The reaction vessel was incubated 30 min at 37° and terminated by the

addition of 0·5 ml 10% activated charcoal (in 1 mM potassium phosphate buffer, pH 7·0). This solution was passed through a Millipore filter, and the radioactivity of a 0·2-ml portion of the filtrate was determined by liquid scintillation. A unit of enzyme is defined as the release of that amount of tritium present in 0·01 μmole of $^3$H-dCMP (50 mμc/μmole) in 30 min at 37°.[4]

## RESULTS AND DISCUSSION

A typical antibody titration with about 60 units of normal enzyme is shown in Fig. 1. As expected, with control ascitic fluid (collected from mice injected with buffer) all of the enzyme remains in the supernatant fluid. At high concentrations of immune ascitic fluid, all of the enzyme is precipitated and is inactive. At the equivalance point the optimal ratio of enzyme and antibody are combined, and there is neither excess of enzyme in the supernatant fluid, nor, presumably, of antibody in the precipitated

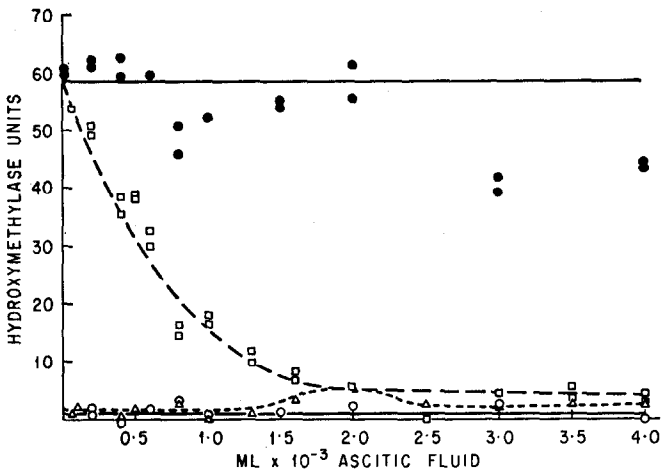


FIG. 1. Antibody titration with normal dCMP hydroxymethylase. Symbols: solid lines, control ascitic fluid; dashed lines, immune ascitic fluid; △, ○, precipitated enzymes; □, ●, supernatant enzyme.

complexes. At lower antibody concentrations, less of the enzyme can be precipitated and more activity remains in the supernatant fluid. These characteristics of the antibody titration are also found for the antibody titration with the 60-heavy enzyme. Furthermore, the shape and position of the precipitin curves for the normal and 60-heavy enzymes do not differ in any significant manner to suggest molecules grossly different immunologically.

Although it appears from the antibody titration curves that the optimal combining ratio of the immune ascitic fluid is the same with both enzyme preparations, a more precise comparison is desirable. Therefore, these curves were used to determine appropriate antibody concentrations for enzyme titrations. In such titrations, if two enzymes differ in their ratio of immunological binding sites/enzymatic activity, then for a given antibody concentration the equivalence points for the two enzymes should differ.

The results of a typical experiment from a series of enzyme titrations are presented in Fig. 2. The nonprecipitable enzyme units in the supernatant fluid after the precipitin reaction are graphed versus the milligram protein in the enzyme sample. The 7-fold difference in slope between the normal and 60-heavy enzymes is commensurate with a 7-fold difference in specific activities of these preparations. For a given enzyme, the data for control and immune ascitic fluid fall along two parallel lines separated by a distance dependent on the equivalence point. This point is the greatest quantity of

enzyme which, in the presence of immune ascitic fluid, does not allow excess enzyme in the supernatant fluid and occurs at the intersect of the graph of the immune ascitic fluid titration with the abscissa.

From these graphs, the number of units of each of the enzyme preparations which is bound by a given amount of antibody at the equivalance point was determined and compared (Table 1). There

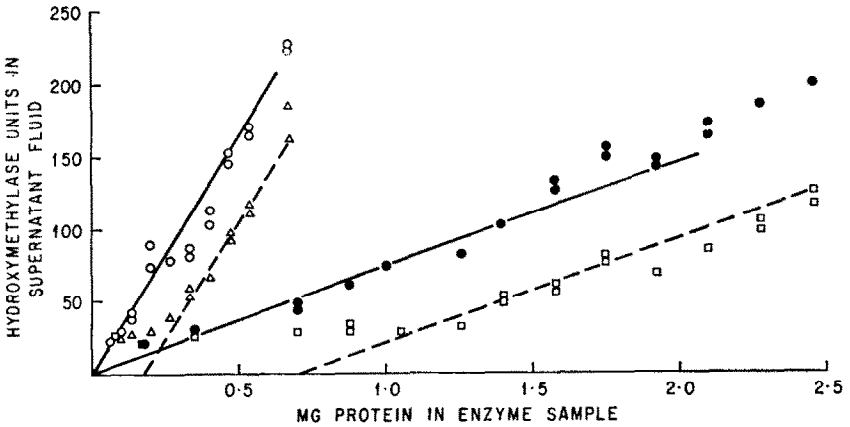


FIG. 2. Enzyme titration with 0·0012 ml mouse ascitic fluid. Symbols: solid lines, control ascitic fluid; dashed lines, immune ascitic fluid; ○, △, normal enzyme; ●, □, 60-heavy enzyme.

TABLE 1. UNITS OF NORMAL AND 60-HEAVY ENZYME BOUND AT THE EQUIVALENCE POINT

| Volume immune ascitic fluid ($\mu$l) | Units of enzyme bound at the equivalence point | |
|---|---|---|
| | Normal | 60-heavy |
| 0·3 | 35 | 20 |
| 0·5 | 30 | 44 |
| 0·5 | 29 | 38 |
| 0·8 | 45 | 41 |
| 1·2 | 56 | 53 |

does not appear to be a significant difference between the two enzyme preparations. The ratio of antigen combined with antibody, expressed as units of enzyme per 0·1 $\mu$l of immune ascitic fluid, has an average value of 7 for both the normal and 60-heavy preparations.

In conclusion, the above experiments do not demonstrate a significant difference between the dCMP hydroxymethylase enzyme induced by normal or 60-heavy phage. The similarity of the data suggests two explanations: (1) The 60-heavy phage only produces dCMP hydroxymethylase molecules which are identical to the normal enzyme; (2) The 60-heavy phage produces both enzymatically normal and inactive dCMP hydroxymethylase molecules. The inactive molecules are immunologically unreactive with the dCMP hydroxymethylase antibody, and thus the precipitin reaction would appear as in (1).

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