The purification and properties of hydropyrimidine dehydrogenase

This note presents some properties and a method for the partial purification from beef liver acetone powder, of an enzyme catalyzing the reaction:

$$\text{Hydropyrimidine} + \text{TPN}^{\S} \rightleftharpoons \text{Pyrimidine} + \text{TPNH} + \text{H}^+$$

All centrifugations were at 5000 × *g*. The ammonium sulfate solutions were saturated and adjusted to pH 7.4 at 0° C. All the steps of the fractionation procedure were carried out at 0° C. The volume of the reagent added during fractionation is referred to the volume at the beginning of the particular step.

It is important to carry out the fractionation procedure and any additional studies in a single day, since the enzyme is highly unstable.

50 g acetone powder[1,2] were extracted with 500 ml deionized water for 20 min and then centrifuged for 15 min. The supernatant fluid (crude extract in Table I) was mixed with one vol. ammonium sulfate solution and centrifuged for 15 min. The precipitate was dissolved in water to a concentration of 20 ± 1 mg protein/ml (1st ammonium sulphate in Table I). The enzyme was further fractionated by the addition of 0.59 vol. ammonium sulphate solution, followed by centrifugation. The precipitate was discarded and the enzyme was precipitated by the addition of 0.465 vol. ammonium sulphate to the supernatant fluid. The mixture was centrifuged for 20 min and the precipitate taken up in water (2nd ammonium sulphate in Table I). The enzyme was further fractionated by adjusting the pH to 4.1 (pH measured at about 5° C) with 0.1 *N* acetic acid (about 15 ml were required) and then adding 0.1 vol. 0.37 *M* sulphosalicylic solution adjusted to pH 4.1. The precipitate was centrifuged off and discarded. The supernatant fluid, containing the enzyme, was mixed with 1 vol. calcium phosphate gel[3] (30 mg dry weight/ml), centrifuged for 5 min and the supernatant discarded. The gel was eluted first with 40 ml 0.2 *M* phosphate buffer, pH 7.35, and then with 50 ml 0.5 *M* phosphate buffer, pH 7.35. The second eluate containing the enzyme with higher specific activity was retained (see Table I, gel eluate). The enzyme can be concentrated at this stage with 3 vol. ammonium sulphate. Table I shows the summary of the purification procedure.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

Fraction	*Volume ml*	*Units**	*Protein mg*	*Specific* activity*	*Activation** %*
Crude extract	420	1,260	11,700	0.11	0
1st Amm. sulfate	180	1,230	3,600	0.34	40
2nd Amm. sulfate	80	800	1,600	0.50	50
Gel eluate	50	375	168	2.2	50

The purification procedure reported here has been repeated more than 10 times with maximum differences of 20% in yields and in specific activity.

* With hydrothymine as a substrate.

** The enzyme is activated by sulphosalicylate and salicylate in a wide range of concentrations. About maximum activation is seen at 0.02 *M*.

Routinely the enzyme was mixed with the following components in a 1 cm Beckman cuvette in a final volume of 3 ml: 200 μmoles phosphate buffer pH 7.35, 25 μmoles hydrothymine or other substrates, and 0.5 μmole TPN. When measuring pyrimidine reduction the hydropyrimidines were replaced by pyrimidines and the TPN was replaced by 0.3 μmole TPNH. One enzyme unit causes a change of optical density at 340 mμ and 30° C of 0.001/min. Specific activity is defined as the number of enzyme units/mg protein estimated by the method of MOKRASCH *et al.*[4]. Blank values (without substrate) are nil with the 1st ammonium sulphate fraction or purer fractions when studying the reaction from the hydropyrimidine side, but they still show considerable "blank" when TPNH is used (about 10% of maximal rate with substrate).

Under the conditions of assay indicated in the legend of Table I there is proportionality between enzyme concentration and rate and also between time and effect. This is particularly apparent when using pyrimidines as substrates. Linear kinetics can be observed at low levels of conversion from the hydropyrimidine side, as shown in Fig. 1, with hydrothymine and hydro-

§ The following abbreviations are used: TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

uracil as substrates; however, at higher levels of conversion, there is no proportionality between effect and time due either to an unfavorable equilibrium for pyrimidine formation or to the presence of the hydropyrimidine hydrase[1,2] in the preparations of hydropyrimidine dehydrogenase used, or both. The effect of pH on the rate of conversion of pyrimidines into hydropyrimidines and of hydropyrimidines into pyrimidines is illustrated in Fig. 2. The optimum pH is about 7.4 in either case.

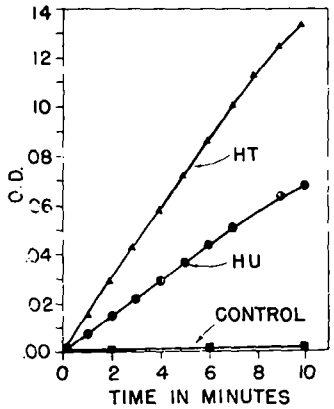

Fig. 1. The effect of hydrouracil and of hydrothymine upon TPN reduction. Experiments were carried out under the conditions of assay indicated in the text using 14 units of the gel eluate fraction (concentrated by ammonium sulphate precipitation). O.D. changes shown were corrected for initial O.D. values.

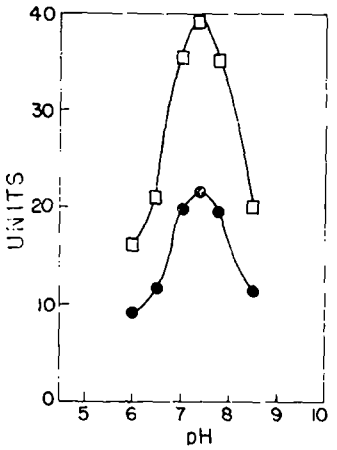

Fig. 2. Effect of pH upon the enzymic formation of pyrimidine and hydropyrimidines. Conditions of assay were as described in the text except that the pH was changed as indicated in the figure. The following buffers were used: phosphate buffer from pH 6 to 7.8 and tris-(hydroxymethyl)aminommethane for pH 8.5.

Relative rates expressed as % of values obtained with uracil as a substrate were 130, 57, 56, 55, 28 and 5 with 5-bromouracil, 5-iodouracil, thymine, hydrothymine, hydrouracil and hydrouridylic acid. Cytosine, uridine, 5-bromohydrouracil and 6-uracil methyl sulphone were inactive. Also the latter was not an inhibitor when uracil was used as a substrate.

In confirmation of the work of CANELLAKIS[5] we found that DPN cannot replace TPN. Mg^{++}, Mn^{++}, Zn^{++}, Fe^{++}, Co^{++}, ethylenediaminetetraacetate and cysteine up to $1 \cdot 10^{-3} M$ had no effect on the enzyme when tested with hydrothymine as substrate.

The Michaelis constant (K_m) calculated graphically[6] is $6 \cdot 10^{-4} M$ for hydrothymine and for hydrouracil. It has not been possible, due to the lack of a more sensitive assay, to measure the K_m from the pyrimidine side. However, from the data obtained with the spectrophotometric method the K_m values are less than $3 \cdot 10^{-6}$ with either thymine or uracil.

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