Enzymic formation of 6-mercaptopurine ribotide§

Inosinic acid pyrophosphorylase, discovered independently by KORNBERG, LIEBERMAN AND SIMMS[1] and KORN *et al.*[2], has been purified approximately 35-fold from extracts of beef liver acetone powder. A study of the specificity of this enzyme has now shown that it catalyzes a reaction between 5-phosphoribosylpyrophosphate (PRPP) and each of the three purines, hypoxanthine, guanine and 6-mercaptopurine (6-MP) to form the corresponding ribotides. The enzyme is devoid of activity toward adenine, 5-amino-4-imidazolecarboxamide, 5-formamido-4-imidazolecarboxamide, xanthine, uric acid, 8-azaguanine, 2,6-diaminopurine, and orotic acid. A separate enzyme has been found in beef liver which acts on adenine and 5-amino-4-imidazolecarboxamide. Its purification will be described elsewhere. The enzyme described in this note affords a convenient method for preparing the ribotide of 6-MP, a compound of unusual interest because of the relative effectiveness of 6-mercaptopurine in the treatment of neoplastic disease.

The assay for the enzyme was based on the disappearance of hypoxanthine, the concentration of which was determined at the end of the reaction by the spectrophotometric method of KALCKAR[3]. The test system contained 0.45 μmole hypoxanthine, 2 μmoles PRPP, 10 μmoles $MgCl_2$, 25 μmoles potassium phosphate buffer, pH 7.4, and enzyme in a final volume of 2.5 ml. After a 15-min incubation at 38°, protein was removed by the addition of 0.09 ml 70 % perchloric acid followed by centrifugation. Hypoxanthine was determined in the protein-free neutralized supernatant solutions. The disappearance of hypoxanthine was proportional to the amount of enzyme present. A unit of enzyme was defined as the amount causing the disappearance of 0.1 μmole hypoxanthine under the assay conditions. The specific activity was defined as the units of enzyme per mg of protein. Protein concentration was determined by measuring optical density at 280 mμ in a Beckman DU spectrophotometer (1 mg protein/ml in a light path of 1 cm has an optical density of 1.6[4]).

Table I reports the results of the purification procedure. The preparation of the acetone powder was as described previously[4] except that the liver was blended in an equal volume of water and added to 3 vol. acetone. All operations except the heating steps were performed at + 3°. The extract (Fraction I) was made by stirring the powder with potassium phosphate buffer (0.033 *M*, pH 7.4), 10 ml/g of powder, for 1 h and then removing the insoluble material by centrifugation. In the first heating step the extract was placed in a water bath at 74° and kept there for 3 min after the temperature of the extract reached 56°. The final temperature of the extract was approximately 62°. The extract was then cooled rapidly in an ice bath and the denatured protein removed by centrifugation. The heated extract (Fraction II) was then fractionated by the addition of solid ammonium sulfate. The precipitate (Fraction III) which formed between 0.50 and 0.65 saturation with salt was dissolved in a minimal volume of potassium phosphate buffer (0.003 *M*, pH 7.4) and dialyzed against several changes of the same buffer. The dialyzed solution, containing about 16 mg protein/ml, was heated as before except that the solution was kept in the 74° bath for 5 min after the temperature of the solution had reached 56°. The final temperature of the enzyme solution was about 70°. After removal of the denatured protein, the supernatant solution (Fraction IV) was treated with solid ammonium sulfate to obtain a precipitate between 0.45 and 0.65 saturation with salt. After dialysis against potassium phosphate buffer (0.003 *M*, pH 7.4) this enzyme fraction (Fraction V) was kept frozen for 4 months without loss of activity.

TABLE I

PURIFICATION OF ENZYME

Fraction	*Specific activity*	*Yield*	*Fold purification*
I. Acetone powder extract	1.6	100*	1
II. Heated acetone powder extract	3.2	100**	2
III. First $(NH_4)_2SO_4$ precipitate	19	76	12
IV. First $(NH_4)_2SO_4$ precipitate after heating	32	76**	20
V. Second $(NH_4)_2SO_4$ precipitate	58	59	36

* The extract contained about 38 units/ml.

** This figure refers to recovery of units/unit volume of solution; mechanical losses are not taken into account.

§ This work was supported by a grant-in-aid from the Damon Runyon Memorial Fund for Cancer Research.

For the preparation of 6-mercaptopurine ribotide, equal amounts of PRPP and 6-MP, at a concentration of approximately 0.30 μmole/ml, were incubated with an excess of inosinic acid pyrophosphorylase (approximately 3 units/μmole 6-MP) for 1 h at 37° in the presence of tris-(hydroxymethyl)aminomethane (Tris) buffer (0.003 *M*, pH 8) and 0.004 *M* $MgCl_2$. Acetone powder extracts which had been subjected to one heating step and one ammonium sulfate fractionation could be used satisfactorily for such preparations. The yield of ribotide, based on either the 6-MP or PRPP, was around 40 %. PRPP could be replaced, with some improvement of yield, by a PRPP generating system containing, for every micromole of PRPP desired, 3 μmoles ATP, 4 μmoles ribose-5-phosphate and 1.3 mg lyophilized 0–15 % ethanol fraction from pigeon liver extract[5]. At the end of the incubation, the solution was placed in a boiling water bath for approximately 3 min in order to denature the proteins which were then removed by centrifugation. The supernatant solution was chromatographed on a column of Dowex-1 bromide form (200 to 400 mesh, 10 % cross linked) containing 1.4 ml of resin bed volume for every 100 ml of incubation mixture. The elution of 6-MP and 6-MP ribotide was followed by reading the optical densities of the eluate fractions in a Beckman D.U. spectrophotometer at 323 mμ, at which wavelength the two compounds exhibit very high ultraviolet absorption. 6-MP was detected in fractions eluted with 30 to 60 resin bed volumes of 0.006 *N* HBr. When 6-MP had been eluted, the elutriant was changed to 0.012 *N* HBr. The elution of the 6-MP ribotide began immediately and was completed after approximately 70 resin bed volumes. The tubes containing the ribotide were then pooled and the solution evaporated on a Flash Evaporator* to give a concentration of ribotide of at least 2 μmoles/ml. The solution was adjusted to pH 7 with KOH and the ribotide precipitated as the barium salt by the addition of a 2-fold excess of $BaBr_2$ and 3 volumes of ethanol. This solution was allowed to stand for at least 2 h at —18° before collecting the precipitate by centrifugation. The barium salt could be further purified by dissolving in a minimal amount of water and reprecipitating with ethanol.

A sample of ribotide approximately 90 % pure by dry weight and giving a single ultraviolet-absorbing spot on paper chromatography, contained 6-mercaptopurine**, pentose[7], total phosphate[8] and –SH groups[9] in the molar ratio 1.0:1.0:1.2:1.0. The ultraviolet absorption spectrum of the ribotide resembled that of the base at pH 1. The absorption maximum for the ribotide was at 323 mμ.

The possibility exists that the biological activity of 6-mercaptopurine requires its prior conversion to the ribotide. Both 6-MP and its ribotide, however, failed to inhibit the *de novo* synthesis of inosinic acid from glycine by soluble enzymes of avian liver.

We should like to thank Dr. J. M. BUCHANAN for his advice and encouragement.

Division of Biochemistry,
Department of Biology, Massachusetts Institute of Technology,
Cambridge, Mass. (U.S.A.)

L. N. LUKENS***
KATHERINE A. HERRINGTON

1 A. KORNBERG, I. LIEBERMAN AND E. S. SIMMS, *J. Biol. Chem.*, 215 (1955) 417.
2 E. D. KORN, C. N. REMY, H. C. WASILEJKO AND J. M. BUCHANAN, *J. Biol. Chem.*, 217 (1955) 875.
3 H. M. KALCKAR, *J. Biol. Chem.*, 167 (1947) 429.
4 E. D. KORN AND J. M. BUCHANAN, *J. Biol. Chem.*, 217 (1955) 183.
5 W. J. WILLIAMS AND J. M. BUCHANAN, *J. Biol. Chem.*, 203 (1953) 583.
6 G. H. HITCHINGS, *J. Am. Chem. Soc.*, 74 (1952) 441.
7 W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques and Tissue Metabolism*, Burgess Publishing Co., Minneapolis, 1949, p. 191.
8 G. GOMORI, *J. Lab. Clin. Med.*, 27 (1941–42) 955.
9 P. D. BOYER, *J. Am. Chem. Soc.*, 76 (1954) 4331.

Received February 9th, 1957

* Made by Laboratory Glass Supply Company, New York 31, N.Y.

** The concentration of 6-mercaptopurine bound as the ribotide was measured spectrophotometrically at 323 mμ with the assumption that the extinction coefficient of the ribotide is the same as the value reported[6] for the base, $2.1 \cdot 10^4$.

*** Predoctoral Research Fellow of the National Science Foundation 1954–56.