Inosinic Acid Dehydrogenase in Mammalian Tissues

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SUMMARY

The enzyme inosinic acid dehydrogenase (IMP:NAD oxidoreductase, EC 1.2.1.14), which catalyzes the reaction

$$IMP + NAD^+ + H_2O \rightarrow XMP + NADH + H^+$$

has been demonstrated and quantitatively determined for the first time in crude extracts of spleen, lung, and brain. This was achieved by means of a new radiochemical assay for this enzyme. Reasons for the previous inability to measure this enzyme in extracts of mammalian tissues with spectrophotometric techniques are discussed. Potential uses of this new IMP dehydrogenase assay in investigating the mechanism of action of several cytolytic purine analogues *in vivo* are suggested.

INTRODUCTION

Inosinic acid dehydrogenase (IMP:NAD oxidoreductase, EC 1.2.1.14) catalyzes the reaction.

$$IMP + NAD^+ + H_2O \rightarrow XMP + NADH + H^+$$

and is essential for the biosynthesis of guanine nucleotides. Metabolic control of this enzyme activity is achieved via feedback inhibition by GMP (1, 2). The carcinostatic effects of several purine analogues have been attributed in part to the inhibition of IMP dehydrogenase by the nucleoside 5′-monophosphates of 6-chloropurine, 6-mercaptopurine, and 6-thioguanine (3–5). Yet the presence of this enzyme has been reported only in extracts of *Aerobacter aerogenes*, pigeon liver, rabbit bone marrow, and tumor cells (4, 6–8). The ability to measure this enzyme activity in crude extracts by following spectrophotometrically the appearance of the absorption of NADH at 340 mμ or of xanthylic acid at 290 mμ has been reported only for bacterial extracts (2, 6). Using these techniques, we could not demonstrate IMP dehydrogenase activity in the supernatant fractions of homogenates of rat brain even though brain contains the highest tissue levels of guanine nucleotides (9). The incorporation of ^{14}C-labeled adenine, adenosine, hypoxanthine, and inosine into the guanine bases of RNA synthesized by slices of cerebral cortex indicated that rat brain contains IMP dehydrogenase activity (10). The inability to detect and measure IMP dehydrogenase in extracts of mammalian tissues has hampered the isolation and study of the properties of this important enzyme. This report concerns a new radiochemical assay for IMP dehydrogenase activity and the demonstration and assay of this enzyme in supernatant fluids obtained from

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homogenates of mammalian tissues. The level of IMP dehydrogenase in rat brain was reported in an earlier work from this laboratory (10).

MATERIALS AND METHODS

Inosinic acid-8-^{14}C (33 μC/μmole) was purchased from Schwarz BioResearch, Inc., and NAD was a product of Sigma Chemical Company.

Samples of brain, heart, lung, liver, spleen, and kidney from two male Sprague-Dawley rats (180 g) were homogenized in 0.15 M KCl (4 ml of 0.15 M KCl per gram of tissue) and centrifuged at 37,000 $\times$ g for 30 min at 0°.

Radiochemical assay for IMP dehydrogenase. The reaction mixture (125 μl) contained 200 mM Tris-acetate (pH 7.5), 1.3 mM $MgCl_2$, 2 mM KCl, 0.25 mM NAD, 0.03 mM IMP-8-^{14}C, and enzyme. The reaction was started by the addition of enzyme (25 μl of the 37,000 $\times$ g supernatant fraction) and incubated at 25°. Aliquots (20 μl) were removed at ½, 18, 36, and 54 min, spotted on a sheet of Whatman No. 3MM paper, dried, and spotted with IMP, XMP, inosine, and hypoxanthine as carriers to aid detection by ultraviolet light of the substrate and products after chromatographic separation. Ascending chromatography was carried out with a mixture of 95% ethanol and ammonium acetate solution (70:30, v/v) as the solvent system. The ammonium acetate solution was 1 M with respect to ammonium acetate, 3.3 mM with respect to EDTA, and adjusted to pH 5.0 with glacial acetic acid. XMP and IMP are completely separated (R_F values of 0.11 and 0.24, respectively), whereas inosine and hypoxanthine migrate together with an R_F value of 0.56. The ultraviolet-absorbing spots corresponding to XMP, IMP, and the mixture of inosine and hypoxanthine were cut out and the radioactivity on the paper was measured without elution as previously described (10).

RESULTS

The radioactivity associated with the carrier XMP, used to detect XMP on the chromatograms, was characterized as XMP-8-^{14}C from reaction products obtained from the supernatant fractions of brain homogenates. The radioactivity was eluted from the chromatograms with a minimum amount of a 1:10 dilution of concentrated NH_4OH. One portion of the eluate was rechromatographed in boric acid–95% ethanol–H_2O–concentrated NH_4OH (1.5 g:67 ml:158 ml:25 ml). The radioactivity co-chromatographed with carrier XMP as a single, symmetrical peak. This solvent system separates 5′-nucleotides, nucleosides, and bases (10). A second portion of the eluate was hydrolyzed in 1 N HCl in boiling water for 1 hr. The radioactivity in the hydrolysate co-chromatographed as a single, symmetrical peak with xanthine as the carrier in water-saturated *n*-butyl alcohol–concentrated NH_4OH (100:1, v/v) as the solvent system. Separation of xanthine, guanine, hypoxanthine, and adenine is achieved by descending chromatography in this solvent system.

IMP dehydrogenase activity was demonstrable in the supernatant fractions of homogenates of spleen (1.0×10^{-3} μM unit/ml), lung (2.6×10^{-4} μM unit/ml), and brain (2.5×10^{-4} μM unit/ml) (Fig. 1). The rate of formation of XMP was linear for the first 30 min with extracts of lung and brain. In other experiments in our laboratory, the rate of formation of XMP was found to be proportional to the amount of supernatant from brain homogenates, provided that the reaction time did not exceed 30 min. The progressive decrease in the rate of XMP formation, especially in the case of the spleen extract, is presumably due to the rapid cleavage of IMP to inosine.

With supernatant fluids obtained from homogenates of liver, kidney, and heart, no significant increase in radioactivity associated with the carrier XMP occurred with time (Fig. 1). In these extracts over 50% of the IMP-8-^{14}C was degraded to inosine during the first few minutes of the reaction. Although the present radiochemical method greatly increases the sensitivity of detecting IMP dehydrogenase activity, it is still

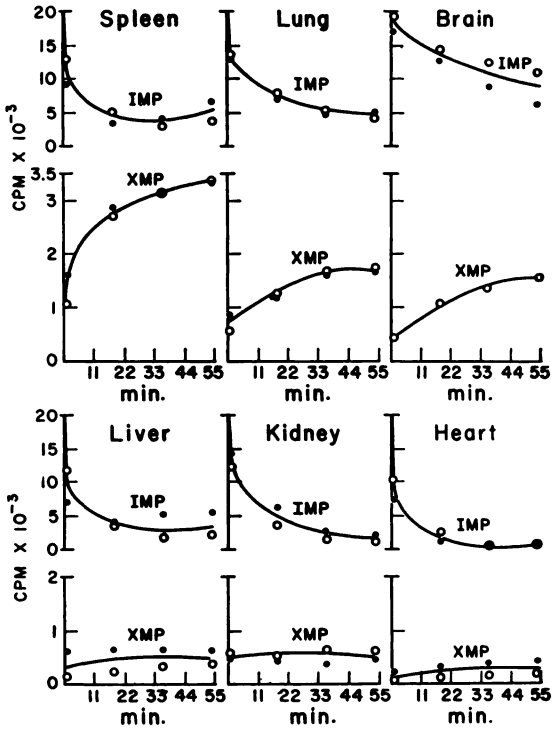

FIG. 1. *Radioactivities of IMP and XMP spots from the chromatograms plotted against reaction time*

The enzyme sources were the supernatant fractions of tissue homogenates from two rats, represented by ● and ○. The disappearance of radioactivity associated with the IMP standard and the appearance of radioactivity associated with the XMP standard were determined from each reaction sample. The rapid disappearance of IMP-8-^{14}C was accompanied by the appearance of radioactivity which chromatographed with the inosine and hypoxanthine standards. Known amounts of ^{14}C-labeled compounds, spotted on similar pieces of Whatman No. 3MM paper, gave 3.67×10^4 cpm/mμmole.

limited in tissues such as liver, kidney, and heart by the rapid degradation of IMP.

DISCUSSION

The previous inability to detect and measure IMP dehydrogenase activity in crude extracts of mammalian tissues may be attributable to the following reasons. The spectrophotometric assays based on the change in absorbance at 340 mμ (NADH) or at 290 mμ (XMP) were incapable of detecting the rapid degradation of IMP by IMP phosphatase activity. The spectrophotometric methods for assaying this enzyme are relatively low in sensitivity in comparison to the radiochemical method, and the presence of "NADH oxidase" activity or xanthine oxidase activity interferes with the measurement of IMP dehydrogenase activity. Tissue levels of IMP dehydrogenase are exceedingly low in relation to other enzymes involved in nucleotide metabolism (10). Finally, mammalian tissues are relatively rich in IMP phosphatase in comparison to IMP dehydrogenase activity. The biochemical significance of the relatively high activity of IMP phosphatase in mammalian tissues remains to be determined.

IMP dehydrogenase isolated from *A. aerogenes* is irreversibly inactivated by 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphate (2, 3). Inactivation of this enzyme by 6-thio-9-β-D-ribofuranosylpurine 5′-phosphate and 6-thio-2-amino-9-β-D-ribofuranosylpurine 5′-phosphate can be reversed by the addition of glutathione (2, 3). The formation of a covalent bond between these nucleotide analogues and a reactive sulfhydryl group on the enzyme

has been proposed (3). Similar findings were observed with 6-thio-2-amino-9-β-D-ribofuranosylpurine 5'-phosphate in the inhibition of IMP dehydrogenase from Sarcoma 180 cells (4). Because of the irreversible nature of this titrating inhibition and the sensitivity of the radiochemical method for assaying IMP dehydrogenase activity, it may be possible to demonstrate lower levels of this important enzyme in tumor tissue and/or various organs from tumor-bearing animals treated with therapeutic doses of these purine analogues. Experiments of this nature would permit the testing of the hypothesis that the cytolytic action of these purine analogues *in vivo* is due in part to the inactivation of IMP dehydrogenase.

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