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Factors Influencing the Activity and the Stability of IMP Dehydrogenase of Rat Liver

Yong-Sung Juhnn

Department of Biochemistry, College of Medicine, Seoul National Universty, 28 Yeunkeun-dong, Chongro-ku, 110-460 Seoul, Korea

=Abstract=IMP dehydrogenase is the key enzyme in the biosynthesis of guanine nucleotides, and is known to have close relationship with many biological phenomena including malignant transformation. Its inhibitors have been utilized in attempt to interrupt the growth of cancer cells and viruses. This experiment was performed to define the conditions for assay of IMPD activity and purification of the enzyme by elucidating the effect of various factors on its activity and stability. The enzyme was prepared from partially hepatectomized rat liver by treatment with ammonium sulfate and DEAE-cellulose chromatography. The enzyme exhibited maxium activity at pH 7.0 and sharp decrease in its activity below and above pH 7.0. The enzyme required free sulfhydryl groups such as mercaptoethanol and dithioerythritol for its full activity, and mercaptoethanol inhibited IMPD activity at the concentration above 10 mM. Among the monovalent cations tested, both K^+ and Na^+ manifested activating effect in contrast with the inhibitory effect of Li^+, and the activating effect of sodium was only about half the effect of potassium. All the divalent cations such as Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+} exhibited strong inhibitory effect on IMPD activity. EDTA was added to the buffers to inhibit various phosphatases and also to remove any trace divalent actions, and did not exert any inhibitory effect on IMPD activity. Glycerol added to purification buffers to mimic intracellular environment manifested slightly inhibitory effect at 10% concentration, but the ethanol showed relatively strong inhibition. As the duration of dialysis increased, the IMPD activity was increased proportionally until 24 hours. And ammonium sulfate used for salting out IMPD also showed strong inhibitory effect, therefore complete removal of the salt are required before assaying the activity. When IMPD preparation was stored at −60°C, −20°C, 4°C, and 25°C for 7 days, only the preparation stored at −60°C retained most of the initial activity, and that stored at 25°C lost most of its activity in 24 hours. Thiols such as dithioerythritol and mercaptoethanol exhibited stabilizing effect on IMPD stored at 4°C, and K^+, glycerol, and EDTA did not show any protective effect. Change of Tris buffer (pH 7.4) buffer neither to phosphate nor to pH 8.0 improved the stability of IMPD.

Key words: ***IMP dehydrogenase, Rat liver, Activity and stability***

INTRODUCTION

IMP dehydrogenase (IMP:NAD^+ oxidoreductase, EC 1.2.1.14. IMPD) catalyzes the oxidation of inosine 5′-monophosphate (IMP) into xanthosine 5′-monophosphate (XMP), and this reaction is the rate-limiting step in the biosynthesis of guanine nucleotides. The IMPD activity is regulated by both the concentrations of adenine nucleotides and guanine nucleotides so that the cell can maintain the proper balance in the concentrations of the two purine nucleotides. The

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alteration of IMPD activity was observed to be linked to cell differentiation, maturation (Knight *et al.* 1987), regeneration, and malignant transformation (Jackson *et al.* 1977). Since IMPD is considered to be a promising target enzyme for cancer chemotherapy (Weber 1983), many inhibitors have been under investigation to develop better anticancer and antiviral durgs (O'Dwyer *et al.* 1986).

All the studies on IMPD and its inhibitors necessitate an assay method to measure the IMPD activity with sufficient sensitivity and reproducibility, and a purification method efficient enough to supply pure enzyme in a sutiable amount. IMPD activity is usually determined by radiometry (Proffitt *et al.* 1983), spectrophotometry and high performance liquid chromatography, but each method, however, leaves something to be improved (Juhnn and Kimm 1988). In addition, IMPD has the lowest activity among all the enzymes involving purine matabolism in normal tissues (Cooney *et al.* 1983). Purification by the conventional methods such as precipitation, gel filtration, and ion exchange chromatography has not been successful in producing the enzyme with sufficient purity or quantity (Okada *et al.* 1983). Thus better assay and purification methods need to be developed to promote research on the IMPD and its inhibitors. For the improvement of assay and purification methods it is indispensable to know the factors that can influence the activity and the stability of IMPD. Thus, in this experiment, effect of some factors on the activity and the stability of the enzyme obatined from rat liver was studied.

MATERIALS AND METHODS

Materials

Allopurinol, IMP, XMP, NAD^+, ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (Tris), Sephadex G-50, dithioerythritol (DTE), were obtained from the Sigma Chemical Company (MO, U.S.A.). Mercaptoethanol (ME) and ethanol were purchased from the Merck Company (Darmstadt, West Germany), and DEAE-cellulose (DE 52) and DEAE-cellulose paper (DE 81) from the Whatman Ltd. (Maidstone, England). Ammonium salt of [8-^{14}C]IMP was the product of Amersham Company (Buckinghamshire, England), and liquid scintillation solution was obtained from Beckman Instrument Inc (CA, U.S.A.). All other reagents were of analytical grade.

Radiochemical assay of IMPD activity

The activity of IMPD was assayed as described previously (Juhnn and Kimm, 1988). The standard assay mixture was composed of 100 mM Tris-Cl, pH 8.0, 100 mM KCl, 5 mM EDTA, 0.2 mM allopurinol, 0.25 mM NAD^+, 0.25 mM [8-^{14}C]IMP (4 mCi/mmol), and enzyme solution to the make a final volume of 50 ul in a microfuge tube. The assay mixture was incubated at 37°C for 1 hour.

One unit of IMPD activity was defined as the amount of the enzyme that could turnover 1.0 nanomole of IMP into XMP in 60 minutes at 37°C.

The protein content was measured by the method of Lowry *et al.* (1951).

Quantitation of ammonium sulfate

The concentration of ammonium sulfate was determined by measuring free ammonia colorimetrically (Chaney and Marbach 1962).

Preparation of IMPD from rat liver

Preparation of cytosol. About half of the liver of Sprague-Dawley rat was surgically removed to induce IMPD, and the animal was sacrificed after 24 hours. The regenerating liver was excised out and washed with a buffer mixture (GTEM), which was composed of 40 mM Tris-Cl, pH 7.4, 10% (w/v) glycerol, 1 mM EDTA, and 10 mM mercaptoethanol. After mincing, the tissue was homogenized with a blender to make 20%(w/v) homogenate. The homogenate was spun down at 10,000 *g* for 30 minutes followed by cenbtrifugation at 20,000 *g* for 90 minutes.

Salting out with ammonium sulfate. Solid ammonium sulfate was added to the 20,000 *g* supernatant to make 30% saturation, and the mixture was centrifuged to remove the precipitate. The concentration of ammonium sulfate in the supernatant was increased to 70% saturation and the protein precipitate was suspended in a buffer mixture (TEM) composed of 40 mM Tris-Cl, pH 7.4, 1 mM EDTA and 10 mM mercaptoethanol. The protein solution was dialyzed against TEM for 5 hours, and denatured proteins were removed by centrifugation.

DEAE-cellulose column chromatography. The dialysed enzyme solution was applied to DEAE-cellulose column equilibrated with GTEM.

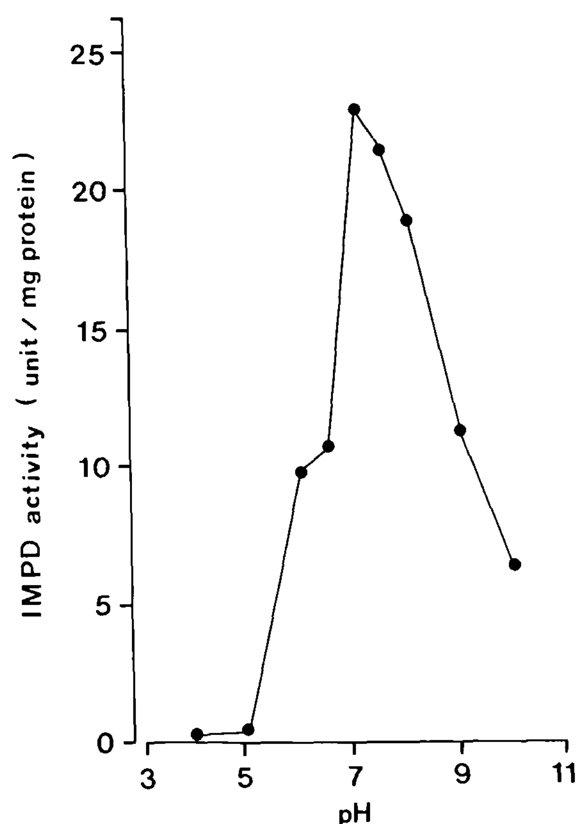

Fig. 1. pH-activity profile for IMPD of rat liver. The IMPD preparation was concentrated, and then diluted with the buffer mixtures, composed of 0.1 M KH_2PO_4, 0.1 M Tris and 0.1 M glycine at various pH. The enzyme activity was determined in the standard assay mixture containing the same buffer used for dilution.

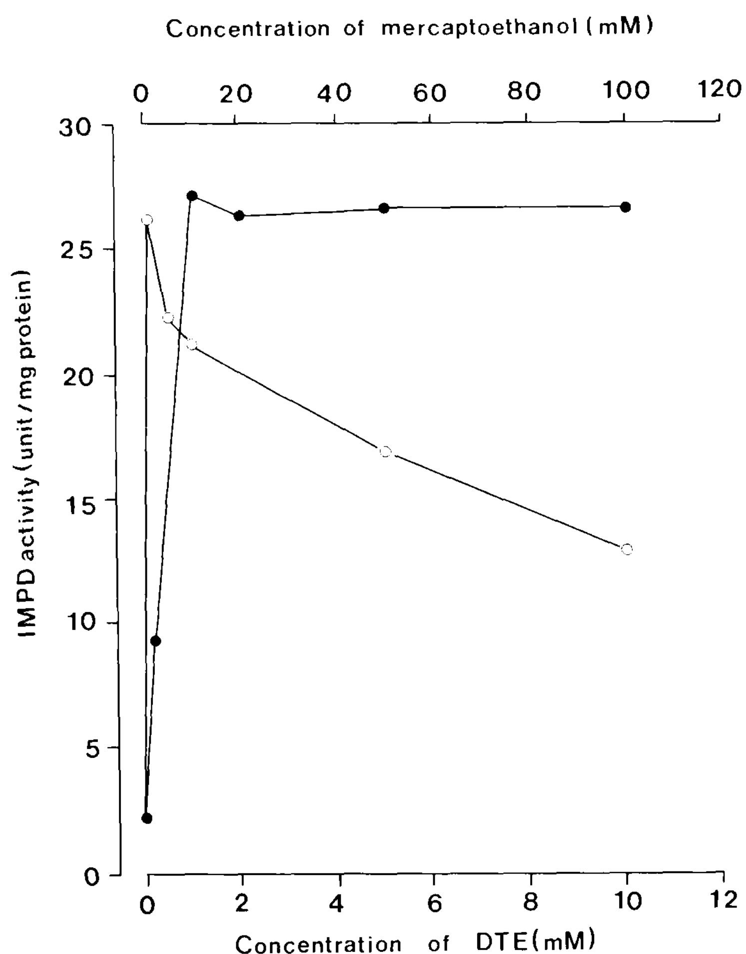

Fig. 2. The effect of thiols on IMPD activity of rat liver. ●——●; dithioerythritol(DTE), ○——○, mercaptoethanol. IMPD activity in the preparation obtained by gel filtration was measured in the presence of the various concentrations of dithioerythritol or mercaptoethanol.

After washing with GTEM, IMPD was eluted with 0.2 M KCl in GTEM. The eluate was concentrated by ultrafiltration, and the preparation was used in this study.

RESULTS

Preparation of IMPD from rat liver

The IMPD of regenerating rat liver was prepared by salting out and DEAE-cellulose chromatography, and had a specific activity of 11.4 unit/mg protein.

Effect of pH on the activity of IMPD of rat liver

The IMPD preparation was further concentrated with molecular cutting cones, and the concentrated enzyme was diluted with the buffer mixture, which was composed of 0.1 M KH_2PO_4, 0.1 M Tris and 0.1 M glycine and adjusted to each pH. The enzyme activity of the diluted preparations was determined in the assay mixture, of which pH was the same as that used for the dilution. The IMPD was observed to have maximum activity at pH 7.0 and there was marked decrease in its activity as the pH changed to more acidic side (Fig. 1).

Effect of thiols on the IMPD activity of rat liver

The buffer of IMPD preparation was replaced with 40 mM Tris-Cl, pH 7.4 by gel filtration on Sephadex G-50 column (26 mm × 15 cm). Mercaptoethanol and dithioerythritol were added to the mixture to determine their effect on the IMPD activity in the gel filtrate. Dithioerythritol exhibited full activating effect at 1.0 mM, and no significant change in the activity was observed at the higher concentrations. Mercaptoethanol also caused full activity of IMPD at 1.0 mM, but further increase in its concentration resulted in decrease of the enzyme activity (Fig. 2).

Table 1. Effect of monovalent cations on IMPD activity

Concentration (mM)	IMPD activity (unit/mg protein)		
	Na^+	K^+	Li^+
0	1.3	1.3	1.3
10	2.0	6.0	1.3
50	3.3	9.7	1.2
100	4.4	11.4	1.0
500	5.9	10.8	0.2
1000	6.7	10.4	0.2

Each cation was added to the enzyme assay mixture composed of 0.1 M Tris-Cl, pH 7.4, 1 mM EDTA 0.25 mM NAD^+ 0.25 mM [^{14}C] IMP, and enzyme prepared by filtration on a Sephadex G-50 gel column.

Table 2. Effect of divalent cations on IMPD activity

Concentration (mM)	IMPD activity (unit/mg protein)			
	Mg^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}
0	1.3	1.3	1.3	1.3
0.1	1.4	0.9	0.8	1.3
1	1.2	0.8	0	0.1
10	0.8	0	0	0
50	0.1	0	0	0

See the legend of Table 1.

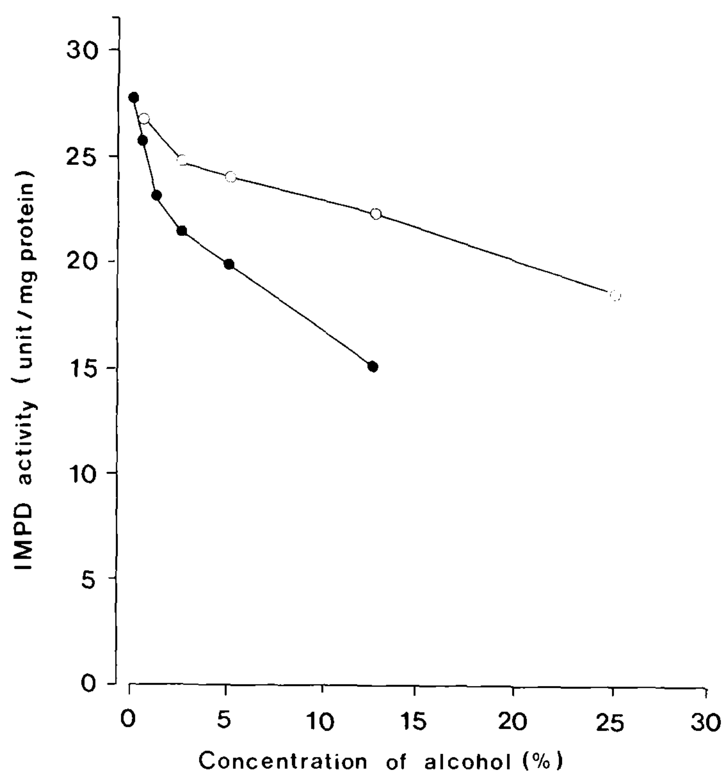

Fig. 3. Effect of ethanol and glycerol on IMPD activity of rat liver. ●——●; ethanol, ○——○, glycerol.

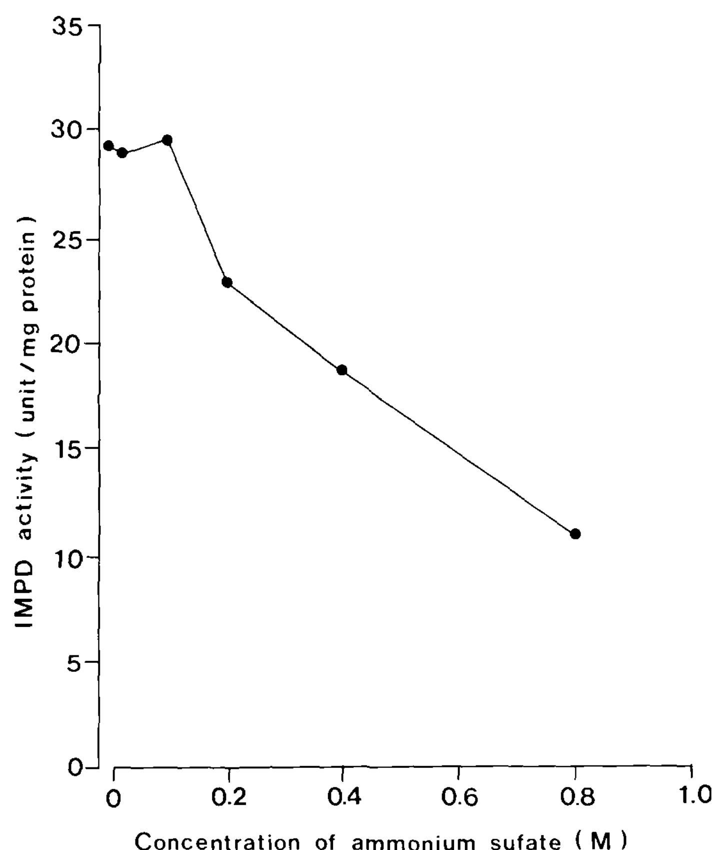

Fig. 4. Effect of ammonium sulfate on IMPD activity of rat liver. IMPD activity was measured in the standard assay mixture, which contained of the respective final concentration ammonium sulfate.

Effect of EDTA

The assay mixture for IMPD activity was composed of 0.1 M Tris-Cl, pH 8.0, 0.1 M KCl, 0.2 mM allopurinol, 0.25 mM [8-^{14}C] IMP, 0.25 mM NAD^+, EDTA, and the enzyme prepared by gel filtration on Sephadex G-50 column. The EDTA added to the assay mixture in the final concentration of 1 mM, 2 mM, 5 mM, 10 mM, and 50 mM, respectively did not cause inhibitory effect on IMPD activity.

Effect of monovalent cations

Potassium chloride, NaCl and LiCl were added to the assay system which was devoid of 0.1 M KCl. Potassium ion caused about 8-fold increase in IMPD activity at 100 mM, and Na^+ did about 5-fold increase at 1.0 M. In contrast, Li^+ inhibited IMPD activity (Table 1).

Effect of divalent cations

The divalent cation such as Mg^{2+}, Ca^{2+}, Mn^{2+}, and Zn^{2+} were added to the assay system

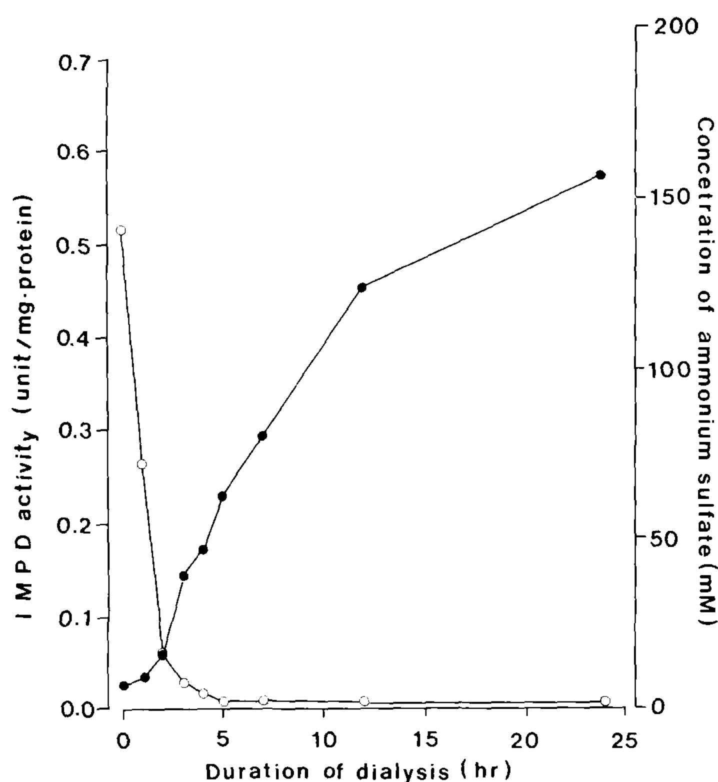

Fig. 5. Effect of duration of dialysis on IMPD activity and concentraion of ammonium sulfate. ●——●; IMPD activity, ○——○; concentration of ammonium sulfate. The precipitate from rat liver cytosol obtained by ammonium sulfate was suspended in a minimum volume of TEM buffer, and dialyzed against it. The specific activity of the enzyme was determined at various time intervals.

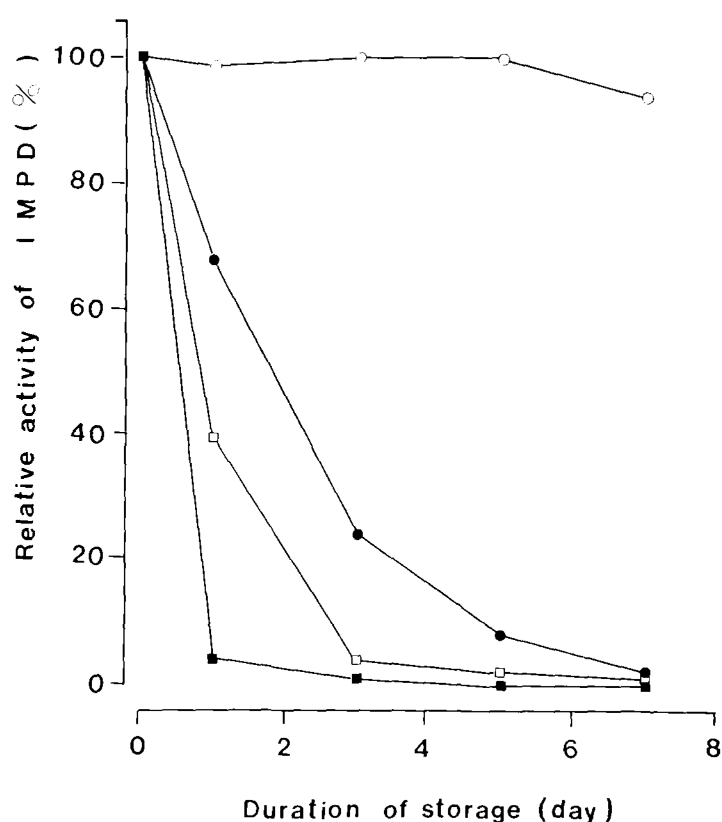

Fig. 6. Effect of storage temperature on the stability of IMPD from rat liver. ○——○; −60°C, ●——●; −20°C, □——□; 4°C, ■——■; 25°C. IMPD preparations were stored at each temperature, and their activity were determined at various time intervals. IMPD activity was presented as relative activity that was the percentage of its activity to the initial activity.

which was devoid of EDTA and KCl. All of the divalent cation tested resulted in strong inhibition of IMPD activity (Table 2).

Effect of glycerol and ethanol

When glycerol and ethanol were added to the standard assay mixture, glycerol resulted in about 20 percent decrease in IMPD activity at 12.5 mM, and ethanol caused so strong inhibition that about 40 percent decrease was observed at 12.5 mM (Fig. 3).

Effect of ammonium sulfate

When ammonium sulfate was added to the standard assay system, a sharp decrease in IMPD activity was resulted in above 0.2 M concentration so that about 40 percent of the activity was expressed at 0.8 M (Fig. 4).

Effect of duration of dialysis

The salting out fraction from the rat liver cytosol was suspended in minimum volume of TEM buffer and dialyzed against 200 volume of TEM. The specific activity of IMPD was determined in each aliquot obtained at one hour intervals for 24 hours. The specific activity increased continuously as the duration prolonged until 24 hours, but the concentration of ammonium sulfate decreased sharply to 2 mM during first 5 hours (Fig. 5).

Effect of storage temperature

IMPD preparation was stored at −60°C, −20°C, and 25°C, respectively, and the enzyme activity was determined at 0, 1st, 3rd, 5th, and 7th day. IMPD activity was expressed as the relative activity, which was the percentage of the remaining activity to the initial activity. The only preparation stroed at −60°C, retained most of the IMPD activity, but those stored at −20°C, 4°C, and 25°C lost most of their activity (Fig. 6). The preparation stored at 25°C lost most of its activity in 24 hours.

Table 3. Effect of some factors on the stability of IMPD from rat liver

Factors	IMPD activity (unit/mg protein)			
	0	1st	4th	7th day
Control	1.7	1.0	0.9	0.5
10 mM Mercaptoethanol	10.	7.6	7.0	5.3
1 mM Dithioerythritol	11.4	8.7	8.3	7.3
1 mM EDTA	10.6	1.1	0.9	0.7
10% Glycerol	11.1	0.7	0.5	0.4
0.1 M KCl	2.3	1.0	0.7	0.6

Controls was IMPD preparation which was eluted from a Sephadex G-50 column equilibrated with 40 mM Tris-Cl, pH 7.4. Each factor was added to the control IMPD preparation, and IMPD activity was determined in a standard assay mixture at each interval during its storage at 4°C.

Table 4. Effect of buffers on the stability of IMPD from rat liver

Buffer	IMPD activity (unit/mg protein)			
	0	3rd	5th	7th day
A	2.9	1.6	0.9	0.8
B	2.6	1.4	0.9	0.6
C	3.3	1.7	1.1	1.0
D	10.9	9.9	9.1	6.7
E	11.4	11.4	9.2	6.9

Concentrated IMPD was diluted with various buffers and the activity was determined in the standard assay mixture at each intervals. A; 40 mM Tris-Cl, pH 7.4, B; 40 mM Tris-Cl, pH 8.0, C; 40 mM KH_2PO_4, pH 7.4, D; 40 mM Tris-Cl, pH 7.4 containing 1 mM EDTA and 10 mM mercaptoethanol, E; 40 mM Tris-Cl, pH 7.4 containing 1 mM EDTA, 10 mM mercaptoethanol and 10% glycerol.

Effect of some factors on the stability of IMPD

IMPD preparation was enriched with 10 mM mercaptoethanol, 1 mM dithioerythritol, 1 mM EDTA, 10% glycerol, and 0.1 M KCl, respectively, and the enzyme activity in each preparation was determined 0, 4th, and 7th day. The preparation containing 10 mM mercaptoethanol or 1 mM dithioerythritol retained about half of its initial activity at 7th day (Table 3).

Effect of buffers on the stability of IMPD

Concentrated IMPD from rat liver was diluted, respectively, with 40 mM Tris-Cl, pH 7.4, 40 mM Tris-Cl, pH 8.0, 40 mM KH_2PO_4, pH 7.4. TEM, and GTEM, and activity in each preparation was determined 0, 1st, 4th, and 7th day. IMPD preparation in TEM and GTEM exhibited about 60% of its initial activity at 7th day, but others lost most of their activity (Table 4).

DISCUSSION

IMPD is the key enzyme in the biosynthesis of guanine nucleotides, and its activity was observed to be altered in many biological phenomena such as malignant transformation, tissue regeneration, and maturation (Weber *et al.* 1975). Inhibition of IMPD activity with various inhibitors could result in the impairment of the nucleotide matabolism including DNA replication and transcription (Lowe *et al.* 1977), which could cause inhibition of the growth of rapidy dividing cells such as cancer cells (Weber 1983). Thus many inhibitors of IMPD were tried to interfere the growth of various cancer cells (Lee *et al.* 1985), and viruses (Robins *et al.* 1985). All the researches for IMPD and its inhibitors must have both the method which can measure the enzyme activity with sensitivity, reproducibility and conuenience and the method which can supply the IMPD preparation with proper purity and amount. Though many assay systems have been reported (Ikegami *et al.* 1980; Cooney *et al.* 1983; Juhnn and Kimm 1988), no method fulfills the requirements of the sensitivity and convenience. IMPD was reported to be purified from many sources by various methods (Jackson *et al.* 1973; Shimura *et al.* 1983; Ikegami *et al.* 1987), but it is still difficult to purify the IMPD with good recovery and purity from the tissues reproducibly. To define the conditions for the assay of IMPD activity and for its purification in details, some factors were studied on their effect on the activity and stability of IMPD in this experiments.

The IMPD was prepared from the regenerating rat liver, and was partially purified by salting out and DEAE-cellulose chromatography to have the proper activity for the analyses and to remove interfering substances. Some of the preparation was further concentrated and some was filtered on a Sephadex G-50 column to change the buffer contents.

Since the radiochemical assay to IMPD activity used in this study exhibited linear response to both the concentration of the enzyme and incubation time (data were not shown), it was suitable to analysis the effects of the factors.

To determine the pH where the maximum activity of IMPD was expressed, a buffer mixture composed of Tris, phosphate, and glycine was used in order to equalize the effect of the buffer at each pH. The IMPD exhibited sharp peak of activity at pH 7.0 (Fig 3), which decreased abruptly below pH 7.0 and less steeply above pH 7.0. Maximum activity of IMPD at pH 8.1 was reported previously (Jackson *et al.* 1977), but in this experiment only 80% of the activity at 7.0 was observed at pH 8.0.

When requirement of free sulfhydryl group in IMPD activity was tested with mercaptoethanol and dithioerythriol, full activity was observed above 1.0 mM dithioerythritol and at 1.0 mM mercaptoethanol. Jackson *et al.* (1977) reported inhibitory effect of thiols, that is, above 1 mM dithioerythhritol and 2 mM mercaptoethanol, but no significant inhibition was observed above 1 mM dithioerythritol in contrast to the similar inhibitory effect of mercaptoethanol in this experiment.

The effect of cations on IMPD activity was tested, and 100 mM potassium ion increased the activity about 8-fold. The maximum activation by sodium ion was only two-thirds of that by potassium ion, and lithium ion, on the other hand, exhibited inhibitory effect above 100 mM. All the divalent cations tested in this experiment showed strong inhibitory effect 1 a mM concentrations, so EDTA must be added to remove these inhibitory divalent cations in assay systems.

EDTA has been added to the buffers used for assay of the activity and for purification of IMPD to inhibit various phosphatases which can break down both the substrate, IMP, and the product, XMP. In addition, EDTA removes contaminated trace divalent cations, which have strong inhibitory effect on IMPD activity. In the presence of KCl, EDTA displayed no significant inhibition on IMPD activity, and thus 1.0 mM EDTA is recommended to be added to the buffers.

Glycerol was added to the buffer to mimic the condition of relatively low water activity (Gekko and Timasheff 1981), and showed slight inhibitory effect on IMPD activity near 10% at the concentration of the usual use (Fig. 6). Ethanol showed strong inhibitory effect on the IMPD, which exclude its use in purifying IMPD.

The effect of ammonium sulfate was not significant until 0.1 mM concentration, but marked decrease of IMPD activity was observed above it. Thus complete dialysis must be followed to the salting-out procedure to restore all the IMPD activity. However, Jackson *et al.* (1977) reported the inactivation of IMPD by dialysis for more than 4 hours, but continuous increase without such inactivation was observed until 24 hours in this experiment. Thus complete dialysis is recommended before assaying IMPD activity in the samples prepared by precipitation with ammonium sulfate.

Storage temperature exhibited profound effect on IMPD stability, and less than 10 percent of the initial activity remained at 25°C in 24 hours. Only the preparation stored in deep freezer maintained most of its IMPD activity in one week, therefore tissues and IMPD preparation must be kept in deep freezer to store for a long time, and the least steps in the procedures as possible must he performed in a cold chamber to purify the enzyme in a short time.

Among the factors tested thiol reagents such as dithioerythritol and mercaptoethanol were indispensable to maintain IMPD activity, which was also confirmed by the results that only the enzyme preparation in buffers containing mercaptoethanol maintained their activity. Other factors that increased IMPD activity in its assay system did not protect the enzyme from inactivation. Neither change of buffer component from Tris to phosphate nor change of pH from 7.4 to 8.0 caused any improvement of stability of the enzyme.

The stability of enzymes depends on many factors including the concentration and the purity of the enzyme. Thus the buffers used to purify IMPD is recommended to contain 1 mM EDTA, 1 mM dithioerythritol, and 10% glycerol to stabilize the enzyme, and the assay of IMPD activity might be performed in a reaction mixture of pH 7.4 containing 1 mM EDTA, 1 mM mercaptoethanol, and 0.1 M KCl.

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= 국문초록 =

흰쥐 간 IMP 탈수소 효소의 활성과 안정도에 영향을 주는 인자

서울대학교 의과대학 생화학교실

전 용 성

Guanine nucleotides의 합성 과정에서 중요한 조절작용을 수행하는 IMP 탈수소 효소와 항암제나 항 바이러스 제제로 각광을 받고 있는 이 효소의 저해제에 대한 연구에는 이 효소의 활성을 정확하고 민감하게 측정하는 간편한 방법이 필요하고, 또한 연구에 필요한 만큼 효소를 충분히 공급하는 정제방법이 필수적이나 현재 이용되는 두 방법은 모두 여러가지 문제점을 갖고 있다. 기존의 효소 활성측정법과 정제법을 개선하기 위한 연구의 준비단계로 이 효소의 활성과 안정성에 영향을 주는 여러 요소들의 효과를 구명하였다. IMP 탈수소효소는 부분 간 절제한 쥐의 재생 조직의 세포질에서 황산 암모늄에 의한 염석과 DEAE-cellulose chromatography로 부분 정제하여 본 실험에 사용하였다. IMP 탈수소효소는 pH 7.0에서 최대 활성을 보였으며, pH가 증가하거나 감소함에 따라 효소활성이 급격히 감소하였다. Mercaptoethanol이나 dithioerythritol 등 thiol은 IMPD가 최대 활성을 나타내는 데에 필수적이었으며, mercaptoethanol은 고농도에서 이 효소의 활성을 저해하였다. 정제되지 않은 시료에 존재하는 여러 phosphatase의 활성을 억제하고 미량으로 존재하는 2가 양이온을 제거하기 위하여 EDTA를 활성측정계와 정제에 사용하는 완충액에 첨가 하는 바, 이는 IMPD활성에 저해효과를 나타내지 않았다. 일가 양이온 중에서 K^+과 Na^+이 효소의 활성을 증대시켰고 Na^+에 의한 증가폭은 K^+에 의한 것의 절반 정도였으며, Li^+은 오히려 활성을 저해하였다. 이가 양이온, 즉 Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}은 모두 IMPD 활성을 강력히 저해하는 효과를 보였다. 정제에 사용하는 완충액에 첨가되는 glycerol은 10% 농도에서 20%의 저해효과를 보였으나 ethanol은 더 큰 저해 작용을 나타냈다. 황산 암모늄은 비교적 낮은 농도에서 저해효과를 보였고 염석한 시료를 dialysis하면 24시간이 경과할때 까지 활성이 계속 증가하였으므로, 염석법으로 준비한 시료는 활성을 측정하기 전에 투석을 충분하게 시행해야 한다. 효소 시료를 −60°C, −20°C, 4°C, 25°C에서 보관한 결과, −60°C에 보관한 시료만이 1주일이 경과한 후에도 대부분의 활성을 유지하였으며, 25°C에 보관한 시료는 24시간만에 대부분의 활성을 소실하였다. 효소활성을 증대시킨 인자중에서 dithioerythritol과 mercaptoethanol만이 IMPD의 안정도를 증가시켰으며, 그외에 K^+, glycerol, EDTA는 안정화 효과를 나타내지 않았다. 효소시료의 완충액을 Tris, pH 8.0이나 potassium phosphate, pH 7.4로 바꾸어도 안정도가 증가되지 않았다.