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# INORGANIC PYROPHOSPHATE HYDROLYSIS BY RAT-LIVER MICROSOMES

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## SUMMARY

1. Inorganic pyrophosphate hydrolysis by rat-liver microsomes has been studied.

2. On the basis of activity–pH studies, it appears that such microsomes contain two and possibly more enzymes capable of hydrolyzing inorganic pyrophosphate.

3. Michaelis constants for added pyrophosphate have been calculated as $1 \cdot 10^{-4}$ M at pH 8.1 in the presence of $Mg^{2+}$ concentrations initially three times those of pyrophosphate, and as $6.1 \cdot 10^{-4}$ M at pH 5.0 without added $Mg^{2+}$.

4. Unlike its mitochondrial counterpart, microsomal inorganic pyrophosphatase (Pyrophosphate phosphohydrolase, EC 3.6.1.1) inhibition by nucleotides appears to be due entirely to chelation of $Mg^{2+}$, since for the latter activity, (a) relatively high concentrations of nucleotides are required for inhibition, (b) ATP inhibition is reversed by elevated $Mg^{2+}$ concentrations, (c) GTP inhibits to an even slightly greater extent than does ATP, (d) AMP, is without effect.

5. While $F^-$ inhibits the microsomal activity just as it did that in mitochondria, ATP does not partially reverse this inhibition with the former preparation.

## INTRODUCTION

Inorganic pyrophosphatases (Pyrophosphate phosphohydrolase, EC 3.6.1.1) play an important role in the overall metabolism of living organisms, since pyrophosphate is a reaction product of many synthetase-catalyzed reactions involved in the formation of such complex, physiologically significant compounds as proteins, glycogen, phospholipids, cholesterol, chondroitin sulfate, and others. The hydrolysis of $PP_i$ produced in some of the individual steps in such processes prevents reversal by pyrophosphorolysis[1], and, coupled with synthetase reactions, makes the overall thermodynamics more favorable for the synthetic processes[2,3]. A number of synthetase reactions for example, cytidine-5-diphosphorylcholine synthesis[4], cholyl-coenzyme A formation[5], activation of some long-chain fatty acids[6], and several reactions involved in the rather complicated synthesis of cholesterol from CoASAc[7,8], in which $PP_i$ is liberated, are known to occur in the endoplasmic reticulum (microsomes).

Inorganic pyrophosphatase has been detected in guinea-pig-liver microsomes[5].

NORDLIE AND LARDY[9] observed that microsomes, mitochondria, and nuclei, as well as soluble portion of rat liver, contain appreciable amounts of such activities. Some properties of two inorganic pyrophosphatase activities present in mitochondria recently have been considered[10]. The results of studies of the hydrolysis of $PP_i$ by rat-liver microsomal enzymes are reported in this paper; a comparison is made of the properties of enzymic activities associated with rat-liver microsomal and mitochondrial fractions.

## MATERIALS AND METHODS

All nuclotides were supplied by Pabst Laboratories, Milwaukee, Wisc. (U.S.A.) $Na_4P_2O_7 \cdot 10\ H_2O$ was obtained from Fisher Scientific Co., Fair Lawn, N.J. $P_i$ and $PP_i$ were assayed according to FLYNN *et al.*[11] and protein according to NORDLIE AND LARDY[10] and LAYNE[12]. The procedure for measuring inorganic pyrophosphatase activity has been described previously[10]. Enzyme concentrations and incubation periods were so adjusted that enzymic activities were in all instances based on measurements of initial reaction velocities. Microsomes were isolated by conventional differential centrifugation methods[13] from livers of male, young adult, albino rats (Badger Research Corp., Madison, Wisc. (U.S.A.)) The washed microsomes were suspended in 0.25 M sucrose solution (1 ml/g wet liver), and were maintained frozen (−15°) until used.

## RESULTS

### *Effects of pH on microsomal inorganic pyrophosphatase activity*

In the absence of added $Mg^{2+}$ a pyrophosphatase activity maximum was observed at pH 4.3, while in the presence of initially equimolar $Mg^{2+}$ and $PP_i$, two activity peaks were apparent, at pH 5.1 and 8.15 (Fig. 1). While the data presented are for a representative experiment, these same pH optima, as well as the shoulders

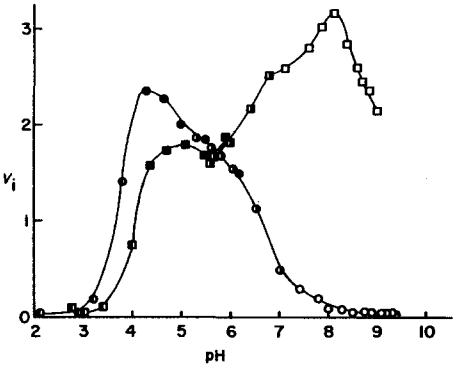


Fig. 1. Variation of rat-liver microsomal inorganic pyrophosphatase activity with pH. Assay mixtures contained 40 mM buffer (◐—◐ and ◧—◧, glycylclycine; ●—● and ■—■, acetate; ◑—◑ and ◨—◨ histidine; or ○—○ and □—□, Tris–HCl), 42 mM sucrose, 5 mM sodium pyrophosphate, 3.54 mg microsomal protein and 5 mM (squares) or no (circles) added magnesium sulfate in a total volume of 3.0 ml. Incubation was for 5 min at 30°. Enzymic activity ($v_i$) is expressed as the decrease in $\mu$moles of $PP_i$ per 3.0 ml reaction mixture per 5 min incubation.

in the range pH 5–7 (no added $Mg^{2+}$) and pH 6.5–7.5 ($Mg^{2+}$ initially equimolar with $PP_i$), were observed in repeated experiments. In some studies enzymic activity at pH 4.3 without added $Mg^{2+}$ was greater that that at pH 8.15 in the presence of $Mg^{2+}$; however, in no instance did the non-$Mg^{2+}$-requiring activity at pH 5.55 exceed that of the $Mg^{2+}$-stimulated activity at pH 7.25 as previously observed[9].

*Effect of $Mg^{2+}$ concentration on pyrophosphatase activity*

A study of the effects of variation of $Mg^{2+}$ concentration in the presence of 0.6, 2.5, and 5.0 mM $PP_i$ (Fig. 2) indicated that in all three instances maximal enzymic activity was obtained when the initial $Mg^{2+}/PP_i$ ratio was between 2:1

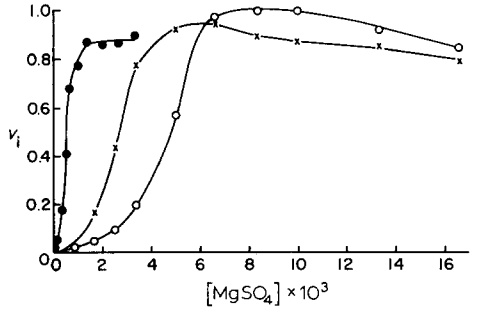


Fig. 2. Effect of magnesium ion concentration on microsomal inorganic pyrophosphatase activity at pH 8.1. Assay mixtures contained 40 mM Tris–HCl, 42 mM sucrose, 0.70 mg microsomal protein, indicated amounts of magnesium sulfate (parentheses indicate molar concentrations in all figures), and: ○—○, 5 mM; ×—×, 2.5 mM; or ●—●, 0.6 mM sodium pyrophosphate in a total volume of 3.0 ml. Incubation time and temperature, and definition of enzymic activity ($v_i$), are as in Fig. 1.

and 3:1. Supraoptimal $Mg^{2+}$ concentrations were slightly inhibitory. While the inclusion of $Mg^{2+}$ initially equimolar with $PP_i$ stimulated guinea-pig-liver microsomal inorganic pyrophosphatase activity but 22% at pH 7.4 (see ref. 5), this same amount of $Mg^{2+}$ caused an 8-fold increase in the rat-liver enzyme activity at this pH (Fig. 1).

*Effects of $PP_i$ concentration on enzymic activity*

Variations in enzymic activity with $PP_i$ concentration (a) at pH 5.0 without $Mg^{2+}$, (b) at pH 8.1 with initial $Mg^{2+}/PP_i$ ratio of 3:1, are depicted in Figs. 3 and 4, respectively. In the latter instance, half-maximal initial reaction velocity was obtained with an initial $PP_i$ concentration of approx. $1 \cdot 10^{-4}$ M (Fig. 4), while in the former experiment $K_m$ for $PP_i$ was calculated[14] as $6.1 \cdot 10^{-4}$ M.

*Effects of $F^-$ and EDTA on enzymic activity*

All KF concentrations tested (0.33, 3.33 and 15 mM) markedly inhibited the $Mg^{2+}$-dependent activity at pH 8.4, while only the latter two concentrations were effective inhibitors at pH 5.0 without added $Mg^{2+}$ (Table I). The stimulation of

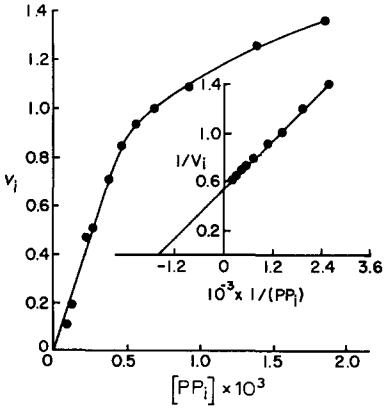


Fig. 3. Effect of $PP_i$ concentration on microsomal inorganic pyrophosphatase activity at pH 5.0 in the absence of added magnesium. Assay mixtures contained 40 mM acetate buffer, 42 mM sucrose, indicated sodium pyrophosphate concentrations, and 2.38 mg microsomal protein per 3.0 ml. Incubation time and temperature, and definition of enzymic activity ($v_i$), are as in Fig. 1.

the former activity by 0.33 mM EDTA, and inhibition by higher concentrations of this compound (Table I), are similar to observations by SWANSON[15] and NORDLIE AND MIDBOE[16] with $PP_i$ activity of soluble fraction of rat liver. Stimulation of this enzyme was shown[15] to be due to removal of endogenous, highly inhibitory $Ca^{2+}$ by the chelating agent. Interpretation of the data as they bear on the possible

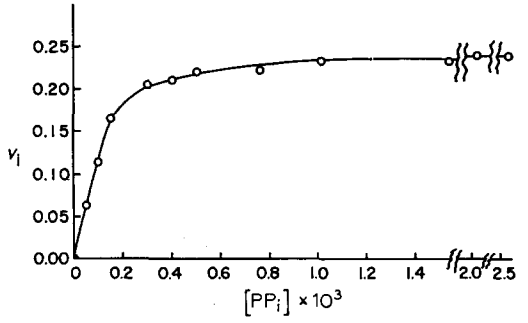


Fig. 4. Effect of $PP_i$ concentration on microsomal inorganic pyrophosphatase activity at pH 8.1 in the presence of magnesium. Assay mixtures contained 40 mM Tris–HCl, 42 mM sucrose, sodium pyrophosphate as indicated, magnesium sulfate at concentrations initially 3 times those of sodium pyrophosphate, and 0.67 mg microsomal protein per 3.0 ml. Incubation time and temperature, and definition of enzymic activity ($v_i$), are as in Fig. 1.

involvement of a $Mg^{2+}$-containing enzyme, active at pH 5, is complicated by (a) the relatively low affinity of EDTA for divalent cation at low pH (see ref. 17), (b) the fact that $F^-$ inhibits certain enzyme systems not involving $Mg^{2+}$ activation[18,19] as well as a large number of $Mg^{2+}$-stimulated enzymes[20].

*Effects of nucleotides on enzymic activity*

On the basis of a specific adenine nucleotide inhibition, independent of $Mg^{2+}$-

TABLE I

EFFECTS OF FLUORIDE ION AND EDTA ON HYDROLYSIS OF $PP_i$ BY RAT-LIVER MICROSOMES

Assay mixtures (pH 8.4) contained 40 mM Tris–HCl, 42 mM sucrose, 1 mM magnesium sulfate, 0.60 mM sodium pyrophosphate, and 1.50 mg microsomal protein in a total volume of 3.0 ml. Assay mixtures (pH 5.0) were identical with the pH-8.4 mixtures, except that acetate rather than Tris buffer was used and magnesium sulfate was omitted. Incubations were carried out for 10 min at 30°. Enzymic activity is expressed as the μmoles of $PP_i$ hydrolyzed per 3.0 ml reaction mixture per 10 min.

| *Addition* | *pH 8.4* | | *pH 5.0* | |
|---|---|---|---|---|
| | *Δ μmoles $PP_i$ (−)* | *Inhibition (%)* | *Δ μmoles $PP_i$ (−)* | *Inhibition (%)* |
| None | 0.79 | — | 0.92 | — |
| KF (0.33 mM) | 0.24 | 70 | 0.88 | 4.5 |
| KF (3.33 mM) | 0.04 | 95 | 0.16 | 83 |
| KF (15 mM) | 0.02 | 97 | 0.04 | 95 |
| EDTA (0.33 mM) | 1.34 | −69 | 0.98 | −6.5 |
| EDTA (3.33 mM) | 0.05 | 93 | 0.95 | −3.2 |
| EDTA (15 mM) | 0.01 | 99 | 0.88 | 4.5 |

binding, of a rat-liver mitochondrial inorganic pyrophosphatase, and partial reversal of $F^-$ inhibition of this activity by these same nucleotides, it has been suggested[10] that there may be some relationship between a mitochondrial inorganic pyrophosphatase and some of those enzymes involved in the process of oxidative phosphorylation accompanying electron transport. To determine whether these properties are unique to a mitochondrial inorganic pyrophosphatase, or whether they are more general properties of mammalian-liver pyrophosphatases, some of the experiments originally performed with rat-liver mitochondria[10] were repeated with microsomes from this same source.

ATP inhibited microsomal pyrophosphatase (Fig. 5); however, $8 \cdot 10^{-4}$ M nucleotide was required to produce 50% inhibition. This is equivalent to an initial ATP/$PP_i$ ratio of 1.33, while 50% inhibition of rat-liver mitochondrial pyrophos-

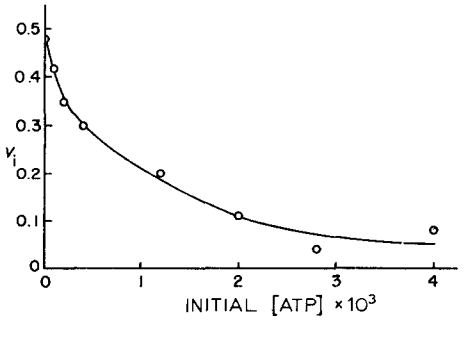


Fig. 5. Effects of ATP concentration on microsomal inorganic pyrophosphatase activity. Assay mixtures contained 40 mM Tris–HCl, 42 mM sucrose, 0.6 mM sodium pyrophosphate, 1.0 M magnesium sulfate, indicated amounts of ATP, and 2.56 mg microsomal protein per 3.0 ml at pH 8.4. Incubation time and temperature, and definition of enzymic activity ($v_i$), are as in Fig. 1.

phatase activity was obtained with an initial adenine nucleotide/$PP_i$ ratio of only 0.006 (see ref. 10). With both microsomal and mitochondrial pyrophosphatases, the initial $PP_i$ concentrations were in the range where initial reaction velocity was proportional to substrate concentration (*i.e.*, the enzyme was in neither instance fully saturated with substrate, and the possibility of obscuring competitive inhibition by a large excess of substrate was avoided). Calculations of the sort previously employed by ROBBINS AND BOYER[21] with the hexokinase system, in which we used binding and acid dissociation constants tabulated in ref. 22 and assumed that the ionic species present were $Mg^{2+}$, $HPP_i^{3-}$, $PP_i^{4-}$, $HATP^{3-}$, $ATP^{4-}$, $MgPP_i^{2-}$ and $MgATP^{2-}$ indicated that an appreciable amount of $Mg^{2+}$ was made unavailable to $PP_i$ by the inclusion in reaction mixtures of concentrations of ATP found effective as inhibitor (Fig. 5). (For example, it was calculated that when $2.5 \cdot 10^{-3}$ M ATP was included, approx. 65% of added $PP_i$ was in the form of $MgPP_i^{2-}$, 31.5% as $HPP_i^{3-}$, and 3.5% as $PP_i^{4-}$. In the absence of ATP, 98.8% of the added $PP_i$ was calculated to be present as $MgPP_i^{2-}$).

However, the assumptions made in these calculations necessarily involved certain oversimplifications (*e.g.*, it has been shown[22] that as much as 2/3rds of added $PP_i$ may be present as the $Mg_2PP_i$ complex, a species not considered, in the presence of excess $Mg^{2+}$ at pH 8); in addition, the nature of the true substrate for this microsomal pyrophosphatase is unknown. For these reasons, additional proof for the nature of the ATP inhibition of the activity was sought by direct experimentation. Nucleotide inhibition of microsomal inorganic pyrophosphatase appears to be due only to $Mg^{2+}$-binding, since GTP inhibits the microsomal enzyme as well as does ATP (Table II, Series II), AMP does not inhibit (Table II, Series II), and the inhibition due to ATP can be reversed completely by elevation of $Mg^{2+}$ concentration (Table II, Series I). Fluoride inhibition of the microsomal enzyme is unaffected by nucleotides (Table II, Series III), while inhibition of the mitochondrial enzyme by this ion is partially reversed by adenine nucleotides[10].

## DISCUSSION

Rat-liver microsomes and mitochondria[10] are similar in that they both contain at least two inorganic pyrophosphatase activities, one of which has no requirement for added $Mg^{2+}$, is optimally active in the acidic pH range, and is inhibited by $F^-$; and a second which is markedly stimulated by $Mg^{2+}$, is optimally active near neutrality (mitochondria) or in the alkaline pH range, and is highly fluoride- and EDTA-sensitive. The presence of a third pyrophosphatase activity is suggested by the shoulders on the pH profile (Fig. 1); experimental evidence also indicates a third pyrophosphatase in mitochondria[10]. Activity maxima (at pH 4.3 without added $Mg^{2+}$, and at pH 5.1 and 8.15 with $Mg^{2+}$ and $PP_i$ initially equimolar, for microsomes; at pH 5.7 without added $Mg^{2+}$, and at pH 5.45 and 6.7 with initial $Mg^{2+}/PP_i = 1$, for mitochondria[10]), which were determined in essentially identical reaction mixtures with both particulate preparations, suggest that $PP_i$ hydrolysis is not due principally to identical enzymes present in both particulate preparations. The differences in optimal $Mg^{2+}/PP_i$ ratios (Fig. 2 and ref. 10), studied at pH's found optimal when this ratio was 1:1 (Fig. 1), also suggest that the $Mg^{2+}$-stimulated enzymes in these two particles are dissimilar. While this ratio might vary with $PP_i$ concentration

TABLE II

EFFECTS OF NUCLEOTIDES ON $PP_i$ HYDROLYSIS BY RAT-LIVER MICROSOMES

Assay mixture composition and experimental conditions were identical with the pH-8.4 reaction mixture described in Table I, except that 2.56 mg microsomal protein and indicated amounts of magnesium sulfate, nucleotides, and potassium fluoride were included per 3.0 ml reaction mixture. $PP_i$ disappearance was measured directly. Enzymic activities are expressed as in Table I.

| *Addition* | *$MgSO_4$ (mM)* | *$\Delta$ µmoles $PP_i$ (−)* | *Inhibition (%)* |
|---|---|---|---|
| | *Series I* | | |
| None | 1 | 0.91 | — |
| None | 3 | 1.07 | — |
| None | 10 | 0.85 | — |
| ATP (2 mM) | 1 | 0.07 | 92 |
| ATP (2 mM) | 3 | 1.10 | −2.8 |
| ATP (2 mM) | 10 | 0.81 | 4.7 |
| | *Series II* | | |
| None | 1 | 0.90 | — |
| ATP (1 mM) | 1 | 0.52 | 42 |
| GTP (1 mM) | 1 | 0.41 | 54 |
| AMP (1 mM) | 1 | 0.87 | 3.3 |
| | *Series III* | | |
| None | 1 | 0.91 | — |
| KF (3.33 mM) | 1 | 0.07 | 92 |
| KF (3.33 mM) plus ATP (1 mM) | 1 | 0.06— | 93 |
| KF (3.33 mM) plus ATP (3 mM) | 1 | 0.07 | 92 |

employed if, for example, a $MgPP_i^{2-}$ or $Mg_2PP_i$ complex were to serve as substrate, effects of variation of $Mg^{2+}$ concentration were studied in each instance in the presence of two or more $PP_i$ concentrations which were known to be in the range where enzymic activity was dependent upon $PP_i$ concentration (see Fig. 4 and ref. 10). Apparent Michaelis constants for added $PP_i$ ($9.5 \cdot 10^{-3}$ M and $2.9 \cdot 10^{-4}$ M for mitochondria[10], $1 \cdot 10^{-4}$ M for microsomes), determined for $Mg^{2+}$-stimulated activities at their pH optima, also vary for the two particulate preparations, although the significance of the difference between the last-mentioned value for mitochondrial enzyme and that for microsomal pyrophosphatase is left open to question due to the fact that these parameters were based on measurement made in crude preparations in the presence of other pyrophosphatases. $Mg^{2+}$-stimulated inorganic pyrophosphatase of rat-liver microsomes also differs from this enzyme in mitochondria[10] since nucleotide inhibition of the former appears to be due solely to nonspecific chelation of $Mg^{2+}$ by nucleotides, as discussed in the RESULTS section.

In addition to variations in degree of $Mg^{2+}$-stimulation discussed in the RESULTS section, rat-liver microsomal inorganic pyrophosphatase differs from its guinea-pig-liver counterpart[5] in that, while we obtained 95% or greater inhibition of the rat-liver activity both at pH 5.0 and 8.4 by inclusion of 15 mM KF in reaction mixtures

(Table I), ELLIOTT[5] observed appreciable activity with guinea-pig-liver preparations even in the presence of 25 mM KF. Further, while no reversal of $F^-$ inhibition of the rat-liver-enzymic activity (pH 8.4, $Mg^{2+}$ added) was obtained by addition of ATP (Table II, Series III), this nucleotide reversed $F^-$ inhibition of the guinea-pig-liver microsomal enzymic activity (assayed at pH 7.4 with $Mg^{2+}$ added).

NORBERG[23] has observed activity peaks for $PP_i$ hydrolysis by rat-liver homogenates at pH's 4–4.2, 5, 5.8–7, and 8–8.4. While pH optima determined in crude mixtures of enzymes are not necessarily the same as those which would be observed with the individually purified enzymes due to possible overlapping of activities, it is none-the-less interesting to note that microsomal pyrophosphatase pH optima, pH 4.3 and 8.1, correspond closely to the two extreme values of NORBERG[23]. And one pH optimum for mitochondrial enzyme[10] falls within the pH 5.8–7 range while the second optimum at pH 5.45 is not too far removed from the pH 5 value reported by NORBERG[23]. It must be pointed out, however, that the very active, highly specific inorganic pyrophosphatase present in soluble portion of rat liver[15] exhibits a sharp activity maximum, when assayed under the conditions used for study of microsomal and mitochondrial activities, at pH 7.5 which is between two of NORBERG's[23] activity peaks[16].

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