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LEAD-ADENOSINE TRIPHOSPHATE COMPLEXES IN ADENOSINE TRIPHOSPHATASE HISTOCHEMISTRY

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Chelation of lead by adenosine triphosphate (ATP) and its consequences for adenosine triphosphatase histochemistry were examined. The formation constant of lead-ATP chelates was found by two methods to be 4.6–4.7 × 10^4. The characteristics of enzyme inhibition by lead were consistent with the predicted effects of lead-ATP chelation. Inhibition was overcome by increasing ATP concentrations. With the adenosine triphosphatase from liver microsomes, which retained some activity in the presence of 4*mM* $Pb(NO_3)_2$, substrate inhibition disappeared and increased $MgCl_2$ was required for optimal activity. Increased solubility of lead phosphate was observed in the presence of increasing quantities of ATP in a manner predictable from lead-ATP chelation.

For more than a decade, lead precipitation methods for demonstrating ATPase[1] activity have enjoyed wide popularity in histochemistry. In their earliest formulation, such reactions were simply described (where E is enzyme)

$$Mg + ATP \rightleftharpoons Mg\text{-}ATP \xrightarrow{E} Mg\text{-}ADP + P_i$$

$$Pb + P_i \rightarrow PbP_i$$

Over the years this simple scheme has had to be modified. Loss of ATPase activity in the presence of lead has repeatedly been described—in tissue sections (20, 22), in whole homogenates (20, 22) and in subcellular fractions (5, 14, 20, 26). No studies of the mechanism of lead inhibition have been reported. However, Novikoff, Hausman and Podber (20) observed that, in media containing lead, ATPase activity could be partly restored by the use of higher ATP concentrations. Recently, Moses and Rosenthal (24) have observed that lead also is effective in catalyzing nonenzymatic hydrolysis of ATP and that this nonenzymatic hydrolysis may account for a substantial fraction of phosphate released into histochemical ATPase media (18).

Another effect of lead in systems containing ATP has seldom been considered. Binding of divalent cations by ATP is widely appreciated, but formation of lead-ATP complexes is only occasionally mentioned in the histochemical literature. Deane, Barrnett and Seligman (4) suggested that, when the pH is raised in media containing ATP and lead, the formation of lead-ATP salts might decrease the effective ATP concentration. Pratt (22) observed that the solubility of lead phosphate was greater in media containing ATP than in media containing β-glycerophosphate but did not investigate the phenomenon further. Berg (1) attempted to use formation of lead-polyphosphate chelates to construct histochemical methods for polyphosphatase demonstration. He suggested that, when the soluble lead-polyphosphate chelate was enzymatically hydrolyzed, the chelate dissociated, and that lead so released then precipitated. He further suggested that similar mechanisms operated in methods for the demonstration of ATPase activity, evidently assuming that Pb-ATP chelates were hydrolyzed by enzymes hydrolyzing ATP.

Most studies of ATP chelation have dealt with its complexes with Ca^{++} and Mg^{++} (2, 15, 19), and the formation constants of Pb-ATP chelates have apparently not been obtained. If such complexes are formed to any great extent, then in histochemical media containing Pb^{++}, ATP and Mg^{++} as activating ion:

$$Mg^{++} + Pb\text{-}ATP \rightleftharpoons Mg^{++} + ATP + Pb^{++} \rightleftharpoons Mg\text{-}ATP + Pb^{++}$$

At equilibrium, the quantity of free ATP or of

The abbreviations used are: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; ITP, inosine triphosphate; CTP, cytidine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5-monophosphate; k_f, formation constant; K_m, Michaelis constant.

Mg-ATP will be reduced in the presence of added lead. Conversely, free Pb^{++} available to inhibit enzyme activity (or to precipitate released phosphate) will be affected by the amount of ATP present. The magnitude of these effects depends on an important but unknown factor, the formation constant of Pb-ATP.

In the experiments reported here, the formation constant of Pb-ATP was determined, and its variation with pH and temperature was examined. Some possible consequences of Pb-ATP binding were explored using the ATPase activity associated with two microsomal fractions (liver and kidney). Precipitation of lead phosphate was studied under conditions which might be used in histochemistry, and the effects of lead-ATP complexation on the solubility of lead phosphate were investigated. In a subsequent paper, the application of these results to *in situ* studies will be examined.

MATERIALS AND METHODS

Deionized distilled water was used in all experiments. Lead nitrate (Specpure) was obtained from the Jarrell-Ash Company (Waltham, Mass.). In most experiments, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (9) was used. Although Tris-maleate buffers are widely used in histochemistry, preliminary experiments, using a divalent cation electrode, showed that maleate binds lead weakly ($k_f = 4.3 \times 10^2$). This limited the usefulness of Tris-maleate buffers in these experiments. HEPES did not appear to bind lead to any significant extent, as shown by titrations in the presence and absence of lead (9).

Formation constants of Pb-ATP complexes were obtained using two techniques. With the first method, reviewed by Schubert (25), and used by Nanninga (19) in a study of Ca- and Mg-ATP chelates, a cationic resin was allowed to compete with lead for ATP. A weighed quantity of Dowex 1 resin, averaging 100 mg, previously prepared by cycling with NaOH and NaCl, was equilibrated on a metabolic shaker with 10 ml 0.1 *M* NaCl and 0.1 *M* HEPES buffer containing ATP and 0–1 *mM* $Pb(NO_3)_2$. For each determination of k_f 10 flasks were used, containing 0–1 *mM* $Pb(NO_3)_2$, and values of k_f were calculated for each flask containing lead. In preliminary experiments it was found that lead did not alter the extinction of ATP at 260 mμ. Equilibration was found to be relatively rapid when incubations were at temperatures above 20°C, and measurements were made after 15 min to minimize lead-catalyzed hydrolysis of ATP. At lower temperatures longer equilibration times were required. Assay of parallel media to which resin had not been added revealed no significant nonenzymatic hydrolysis of ATP with these low lead concentrations. After equilibration the resin was removed by centrifugation and the ATP remaining in the supernatant was determined by its extinction at 260 mμ. From this, the distribution coefficient could be calculated.

The distribution coefficient was found to be independent of the weight of resin added over a 4-fold range (25–100 mg) and of the ATP concentration. Because plots of the reciprocal of the apparent distribution coefficient[2] in the presence of lead against total lead were linear, it could be assumed that lead binds ATP in a 1:1 ratio; *i.e.*, $k_f = (\text{Pb-ATP}^{-2})/(\text{Pb}^{++})\,(\text{ATP}^{-4})$. Because these plots extrapolated to the distribution coefficient that was actually observed in the absence of lead, it appeared that Pb-ATP was not significantly bound to resin.

The formation constant (k_f) was calculated as follows: AR, ATP bound to resin in moles; A, free ATP in moles; AM, lead-ATP complex in moles; v, volume of solution in milliliters; m, mass of resin in grams. 1 and 2 are subscripts indicating the absence and presence of added metal.

Q and P, determined from measured quantities, are defined as follows. Q is the ratio of ATP bound to resin to ATP in the supernatant in the absence of metal, *i.e.*, $Q = AR_1/A_1$, where A_1 is measured directly and AR_1 = total ATP $- A_1$. P is the ratio of ATP bound to resin to ATP in the supernatant in the presence of metal. Since Pb-ATP is not significantly bound to resin, $P = AR_2/(AM_2 + A_2)$, where $(A_2 + AM_2)$ is measured directly and AR_2 = total ATP $- (AM_2 + A_2)$.

As indicated above, the distribution coefficient, under the conditions of the experiments, was constant over a wide ATP range; *i.e.*, $(AR_1/m_1)/(A_1/v_1) = (AR_2/m_2)/(A_2/v_2)$. Since the volume was kept the same, $v_1 = v_2$, and $(AR_1m_2)/(A_1m_1) = (AR_2)/(A_2)$ or $Qm_2/m_1 = (AR_2)/(A_2)$. Multiplying by $(1/P + 1)$:

$$(1/P + 1)(Qm_2/m_1) = \left[\frac{A_2 + AM_2}{AR_2} + 1\right]\left[\frac{AR_2}{A_2}\right] = \frac{A_2 + AM_2 + AR_2}{A_2}$$

Thus A_2 = total ATP$/(1/P + 1)(Qm_2/m_1)$. A_2 can now be calculated, since P and Q can be deter-

[2] The apparent distribution coefficient refers to the distribution coefficient observed in the presence of metal: $(AR_2/m_2)/(A_2 + AM_2)/v_2$. A more detailed exposition of this point is given by Nanninga (19).

mined from measured quantities and total ATP, m_1 and m_2 are set by the experimenter.

Next we calculate AM = total ATP − A_2 − AR_2 and, from this, free Pb^{++} = total lead − AM.

Finally, we obtain $k_f = (AM)/(\text{free } Pb^{++})(A_2)$ or k_f = (Pb-ATP)/(free Pb^{++})(free ATP).

In other experiments, a divalent cation electrode[3] (Orion), calibrated against solutions of known lead concentration, was used to measure free Pb^{++} in solutions containing 0.1 *M* HEPES, 0–1 *mM* $Pb(NO_3)_2$ and 0.5–1.0 *mM* ATP. Knowing free Pb^{++}, total ATP and total $Pb(NO_3)_2$ added, k_f could be calculated, again assuming that lead was bound to ATP in a 1:1 ratio. In both calculations, corrections were made for formation of $PbOH^+$ (pK = 6.2 (23)) and for the dissociation of ATP with varying pH, taking pK_4 as 6.92 (17).[4]

Liver microsomes were prepared in 0.25 *M* sucrose, using the method described by Ernster, Siekevitz and Palade (7). Kidney microsomes from rat and guinea pig were prepared using the method of Post and Sen (21).

ATPase activity was assayed in a 1.0-ml test system containing 0.1 *M* Tris-maleate or HEPES buffer at pH 7.0, at 24°C. ATP, $MgCl_2$ and $Pb(NO_3)_2$, were added in the concentrations indicated. Incubations were begun by the addition of enzyme and were concluded by the addition of 0.5 ml 1 *M* perchloric acid, if HEPES buffer were used, or of 0.02 *M* silicotungstic acid. All determinations were made in duplicate. Incubation times were purposely kept short (8–10 min) to minimize nonenzymatic release of phosphate by lead. Parallel blanks were included in all experiments in which ATP was varied and in experiments where lead concentrations of 1 *mM* or more were used. Preliminary experiments showed that, with lead concentrations of less than 1 *mM*, altered blank values attributable to nonenzymatic ATP hydrolysis could not be detected.

[3] The two methods differ in that ATP is measured in the first and free Pb^{++} in the second. However, because the divalent cation electrode uses an exchange resin to detect changes in free Pb^{++}, the two methods are basically similar.

[4] As used in this report, the apparent values of distribution coefficient, formation constant and solubility product are those values calculated under the assumption that, for example, the measured total lead concentration is the same as the actual concentration of free lead ion (23). These may then be corrected, for $PbOH^+$ formation, for example, as follows. The apparent formation constant (k_f^*) = Pb-ATP/(total Pb)(ATP). Total Pb = free Pb^{++} + $PbOH^+$. $PbOH^+ = (OH^-)(10^{6.2})$ (free Pb^{++}). At pH 7.0, $PbOH^- = 10^{-7} \times 10^{6.2} \times$ free Pb^{++} = 0.158 (free Pb^{++}). Total Pb = (1 + 0.158) free Pb^{++}. Pb-ATP/(ATP) = k_f(free Pb^{++}) = k_f^* (total Pb). k_f(free Pb^{++}) = k_f^* (1.158) (free Pb^{++}). Corrections for ATP dissociation are made in a similar manner.

Inorganic phosphate was assayed with the method of Fiske and SubbaRow (8) or the modified method of Martin and Doty (16). Protein was measured by the method of Lowry *et al.* (13).

In experiments studying precipitation of lead phosphate, tracer amounts of ^{32}P in 0.1 *M* sodium phosphate buffer as carrier, pH 7.0, were added to solutions containing 0.1 *M* Tris-maleate or Hepes buffer (pH 7.0), lead, ATP and other cations as indicated. The solutions were centrifuged for 15 min at 1000 × *g* to remove precipitate, and an aliquot of the supernate was taken for counting. In preliminary experiments using higher *g* forces, it was found that the dense lead phosphate precipitate was readily sedimented at these relatively low speeds. In other experiments precipitate was collected on Millipore filters, and it was found that precipitation was essentially complete within 1 min after mixing. Scintillation counting was carried out in a Packard model 3375. A Triton-toluene counting fluid (200 ml and 800 ml respectively) containing 64 ml Liquifluor per liter was used. Since counting conditions were constant and experiments contained external controls, quench corrections for ^{32}P were not required.

RESULTS

Pb-ATP binding: The apparent formation constants (k_f) of Pb-ATP complexes, determined from pH 6.5–7.8, with a cationic resin, are shown in Table I. The mean k_f was 4.6×10^4 after correction for ATP dissociation and for Na-ATP binding (k_f = 10) (11). In common with other ATP metal complexes (19), the stability of the complex increased with increasing temperature (Fig. 1). From measurements of free Pb^{++} with the divalent cation electrode, k_f, corrected for ATP dissociation and Na-ATP binding, was 4.7×10^4. The k_f of lead complexes of other nucleoside triphosphates were not extensively investigated but appeared to be of the same order of magnitude as the k_f of Pf-ATP.

From the k_f of Pb-ATP and literature values for the k_f of Mg-ATP, it was possible to calculate the

TABLE I

Variation in Apparent Formation Constants (k_f) of Pb-ATP Complexes with Varying pH[a]

pH	k_f (S.E.)	Corrected k_f
	× 10^4	× 10^4
6.5	1.32 (0.13)	6.11
6.9	1.33 (0.06)	4.04
7.2	1.57 (0.07)	3.96
7.5	1.35 (0.19)	3.45
7.7	2.54 (0.12)	5.51

[a] Mean corrected $k_f = 4.61 \times 10^4$.

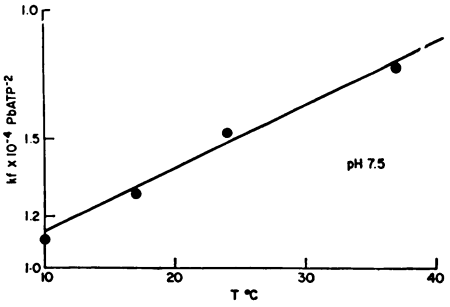

FIG. 1. Effects of temperature on apparent formation constant (k_f) of Pb-ATP chelates; 0.1 *M* HEPES buffer, 0.1 *M* NaCl, pH 7.5.

free Pb^{++} and free ATP, or Mg-ATP, present in mixtures containing varying quantities of total ATP, lead and magnesium. It was assumed in these calculations that lead is not bound by other constituents of the medum, *e.g.*, buffers. However, many buffers commonly used in enzyme studies bind lead to a significant extent, including maleate (see above), glycine, glycylglycine and histidine (3).[5] Although both ATP and lead would be expected to be bound by tissue proteins, this would not significantly affect free Pb^{++} or ATP levels so long as the tissue specimen was equilibrated with a large volume of solution, so that lead and ATP were present in large molar excess of tissue-binding sites.

Figure 2 shows a family of curves relating Mg-ATP to total ATP added, at pH 7.0, with 4 *mM* $MgCl_2$ and from 0–4 *mM* $Pb(NO_3)_2$. As more lead is added, more ATP is required for the same level of Mg-ATP to be attained. For example, in the absence of lead, to achieve an effective concentration of 1 *mM* Mg-ATP, 1.15 *mM* ATP is required, but, if 1 *mM* lead is added to the system, 1.95 *mM* ATP is needed. With 4 *mM* lead, the same level of Mg-ATP is reached only when 3.9 *mM* ATP has been added. The mutual buffering of lead and ATP is also seen when free Pb^{++} is plotted against ATP added (Fig. 3). As ATP is increased, free Pb^{++} progressively falls. With 4 *mM* lead, free Pb^{++} (total Pb − PbOH − Pb-ATP) is about 2.5×10^{-3} *M* when 1 *mM* ATP is added. With 4 *mM* ATP, free Pb^{++} decreases to 6.5×10^{-4} *M* and approaches 1×10^{-4} *M* with 8 *mM* added ATP.

These results indicated that the interaction of lead and ATP would be of practical importance in dealing with histochemical media for the demonstration of ATPase activity. When ATP was pre-

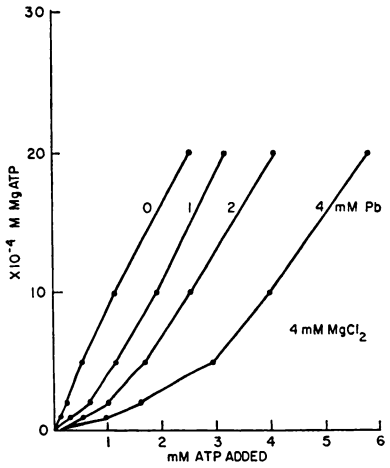

FIG. 2. Calculated effects of increasing lead concentrations on Mg-ATP formed in the presence of varying quantities of ATP and 4 *mM* $MgCl_2$. The apparent k_f of Mg-ATP is taken as 2×10^3 and that of Pb-ATP as 1.3×10^4 (pH 7.0).

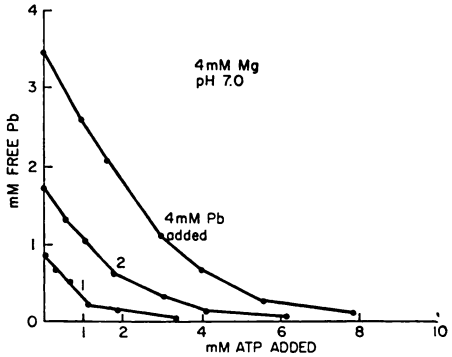

FIG. 3. Effects of increasing ATP on calculated free Pb^{++} (total Pb − $PbOH^+$ − Pb-ATP) with varying total lead; 4 *mM* $MgCl_2$, pH 7.0.

[5] Exact numerical solutions for the concentrations of species present in mixtures containing Mg^{++}, ATP, Pb^{++} and relatively low concentrations of buffer can be obtained only by solving the appropriate cubic, or quartic, equations. The effects of buffers which bind lead may be estimated from the relation:

$$\text{free } Pb^{++}/\text{total Pb} = 1/1 + (OH^-)\,(10^{6.2}) + (\text{free ATP})\,(4.6 \times 10^4) + (\text{free buffer})\,(\text{lead-buffer } k_f)$$

From this it is apparent that the relative influence of buffer and ATP on free Pb^{++} levels is affected by the k_f of Pb-buffer complex relative to that of Pb-ATP, and by the concentration of unbound buffer and of free ATP. The effects of buffer may vary even when nominally the same buffer system is used, since different laboratories prepare the same buffer in different ways. For example, 0.1 *M* Tris-maleate may be prepared by titrating 0.1 *M* maleate with Tris, by titrating a 0.1 *M* Tris solution with maleate or by titrating a 0.1 *M* Tris-maleate solution with NaOH.

sent in excess, free Pb^{++} would be maintained at a relatively low level and, in effect, ATP would behave as a metal buffer. When lead was present in excess, ATP or Mg-ATP available as substrate for enzyme activity would be similarly reduced.

Enzyme effects: In subsequent experiments the effects of lead on ATPase activity were examined to determine whether the characteristics of lead inhibition were consistent with lead-ATP chelation. Two microsomal ATPases from liver and kidney were employed. Since both of these enzymes have been extensively characterized (6, 11, 12), these studies were not repeated to any great extent, except as they related specifically to the effects of lead.

The relation of ATP concentration to lead inhibition of liver microsomal ATPase activity is shown in Figure 4. In all preparations, the degree to which lead inhibited phosphate release was determined not only by the amount of lead added but also by ATP present in the medium. At low ATP concentrations (1 *mM*), 4 *mM* $Pb(NO_3)_2$ inhibited activity by 50–60%. With 4 *mM* ATP the same lead concentration produced only 10–20% loss in activity and, with 10 *mM* ATP, virtually no inhibition was observed.

In these preparations, maximal release of phosphate was obtained with 2–4 *mM* ATP, regardless of whether $MgCl_2$ was held constant at 4 or 10 *mM* or was added in quantities equimolar with ATP (Fig. 5). Further addition of ATP resulted in loss of activity. In the presence of 4 *mM* $Pb(NO_3)_2$, substrate inhibition was not observed.

Altered Mg^{++} activation was also observed in the presence of 4 *mM* $Pb(NO_3)_2$. With 4 *mM* ATP, maximal activity was obtained with 4 *mM* $MgCl_2$, and with higher ATP concentrations the $MgCl_2$ concentration needed for maximal activity was correspondingly increased. However, when 4 *mM* $Pb(NO_3)_2$ was added to media containing 4 *mM* ATP, progressive activation by Mg^{++} was observed up to the highest concentrations tested (Fig. 6). With 4 *mM* ATP and 4 *mM* $Pb(NO_3)_2$, activity with 16 *mM* $MgCl_2$ was increased by about 50% over that observed in the presence of 4 *mM* $MgCl_2$. Similar effects on Mg^{++} activation were observed with 1^0 *mM* ATP in the presence of 4 *mM* $Pb(NO_3)_2$.

In microsomes from rat kidney, the Na^+-K^+-activated component of the ATPase was, of course, of primary interest. This component of activity was always relatively small, both because of the high Mg-activated background found in microsomes from rat kidney and because low incubation temperatures were used to minimize nonenzymatic hydrolysis of ATP by lead.

In these preparations ATPase activity was far more sensitive to lead inhibition, and Na^+-K^+

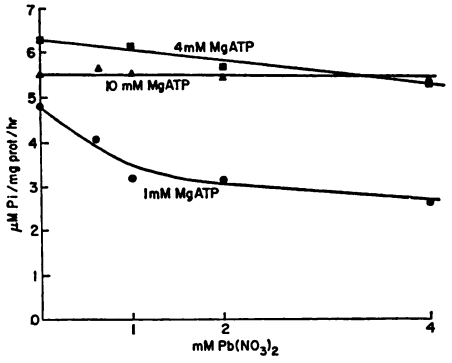

FIG. 4. Effects of lead on ATPase activity of liver microsomes with varying quantities of Mg-ATP. The reaction mixture contained 0.1 *M* HEPES buffer, PH 7.0. Incubation time, 10 min; temperature, 24°C. Similar results were obtained when Tris-maleate was substituted for Hepes.

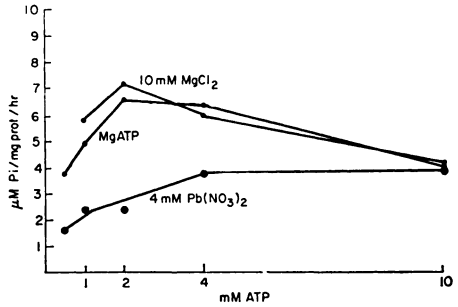

FIG. 5. Effects of ATP concentration on ATPase activity in the presence and absence of 4 *mM* $Pb(NO_3)_2$; 0.1 *M* HEPES buffer, pH 7.0. Similar results were obtained when 0.1 *M* Tris-maleate was substituted for Hepes.

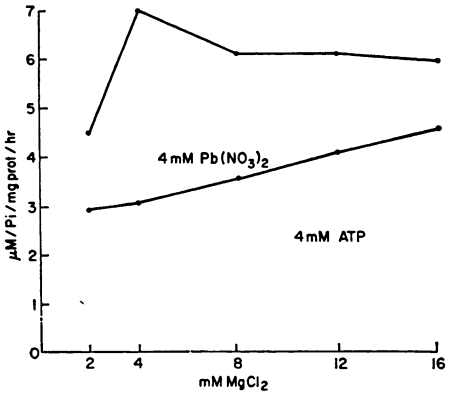

FIG. 6. Effects of $MgCl_2$ on ATPase activity in the presence and absence of 4 *mM* $Pb(NO_3)_2$. Reaction mixture contained 4 *mM* ATP and 0.1 *M* HEPES, pH 7.0.

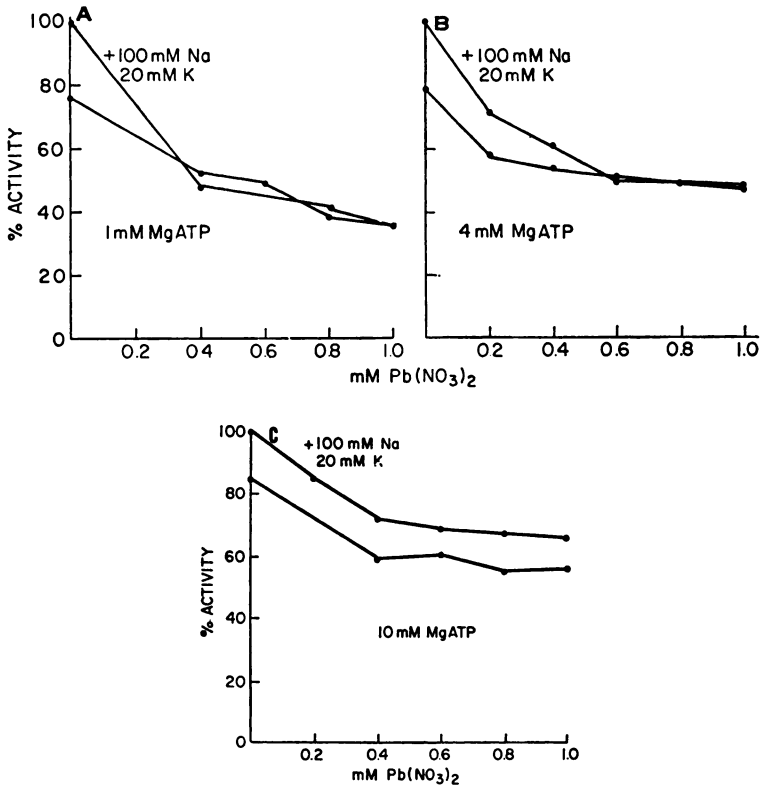

FIG. 7. Effects of $Pb(NO_3)_2$ on ATPase activity of kidney microsomes in the presence of varying amounts of Mg-ATP. A, 1 *mM* Mg-ATP; B, 4 *mM* Mg-ATP; C, 10 *mM* Mg-ATP. 0.1 *M* Hepes buffer, pH 7.0.

activation was always abolished by lead in concentrations exceeding 1 *mM* with 1–10 *mM* ATP. However, in the range of 0–1 *mM* $Pb(NO_3)_2$, the effects of ATP were similar to those observed with liver microsomes (Fig. 7). With 1 *mM* Mg-ATP, Na^+-K^+ activation was lost with concentrations of $Pb(NO_3)_2$ above 0.4 *mM*; with 4 *mM* Mg-ATP, activation was retained up to 0.6 *mM* $Pb(NO_3)_2$; and with 10 *mM* Mg-ATP, activation was retained even with 1 *mM* $Pb(NO_3)_2$ added. Similar findings were obtained when guinea pig kidney was used as a source of enzyme, although the enzyme from guinea pig had lower Mg-activated background and was somewhat more resistant to inhibition by lead.

Lead phosphate precipitation: In other experiments the effects of ATP on lead phosphate precipitation were examined. If ATP chelates lead, then

$$P_i + \text{Pb-ATP} \rightleftharpoons \text{ATP} + \text{Pb} + P_i \rightarrow PbP_i$$

and, in the presence of ATP, an increase in the apparent solubility product of lead phosphate may be expected. With either 1 or 4 *mM* $Pb(NO_3)_2$, increased solubility of lead phosphate was observed as increasing quantities of ATP were added to the reaction mixture (Fig. 8). With less than 1 *mM* lead, and 4 or 10 *mM* ATP, loss of ^{32}P counts to precipitate could not be reliably detected even if 20 *mM* phosphate were added, since losses to precipitate were frequently less than 1% and thus well within the range of pipetting error. Other nucleoside triphosphates had effects similar to those of ATP (Table II). AMP and β-glycerophosphate had no detectable effect, an observation consistent with the relative affinities of these substrates for divalent cations (3).

In either the presence or absence of ATP, precipitation increased moderately as the pH was increased from 6.0–8.0. Phosphate precipitation was unaffected by addition of $MgCl_2$ in the absence of ATP. In its presence, Mg^{++} markedly potentiated precipitation (Fig. 9). With 4 *mM* ATP, the addition of 20 *mM* Mg^{++} resulted in a nearly 3-fold increase in phosphate precipitated. Significant quantities of phosphate were precipitated in the presence of Ca^{++} alone (Fig. 10), and precipitation was further increased when lead was added to the

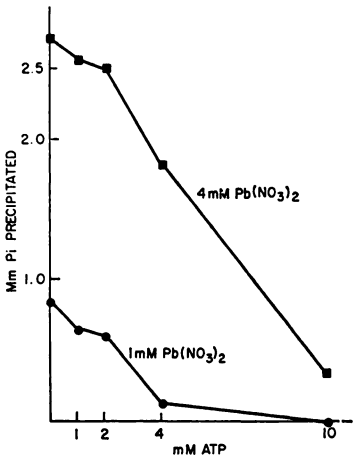

FIG. 8. Effects of ATP on precipitation of lead phosphate in the presence of 1 and 4 *mM* $Pb(NO_3)_2$. System contained 0.1 *M* Tris-maleate buffer, pH 7.0, and 10 *mM* sodium phosphate buffer, pH 7.0.

TABLE II

Precipitation of Lead Phosphate in the Presence of Various Phosphatase Substrates[a]

Substrate	P_i Precipitated
	mM
None	2.8
β-Glycerophosphate	2.8
AMP	2.8
ADP	2.6
ATP	2.1
ITP	2.5
CTP	2.4

[a] The reaction mixture contained 0.1 *M* Tris-maleate buffer (pH 7.0), 4 *mM* of substrate indicated, 4 *mM* $Pb(NO_3)_2$ and 10 *mM* sodium phosphate buffer (pH 7.0). The differences observed were significant.

medium. Although the net effect of adding 4 *mM* ATP was to decrease the phosphate precipitated, the addition of 10 or 20 *mM* $CaCl_2$ to a system containing 4 *mM* ATP and 1 *mM* lead resulted in far more efficient precipitation than when Ca^{++} was omitted.

DISCUSSION

The question initially asked in this study was whether the k_f of Pb-ATP was sufficiently large to be of practical importance in ATPase histochemistry. If k_f could be shown to be of the same order of magnitude as the k_f of Mg-ATP,

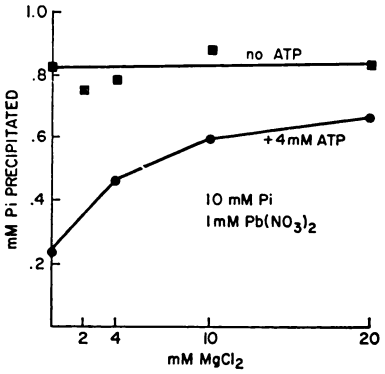

FIG. 9. Effects of $MgCl_2$ on precipitation of lead phosphate in the presence and absence of 4 *mM* ATP. System contained 0.1 *M* Tris-maleate buffer (pH 7.0), 1 *mM* $Pb(NO_3)_2$, and 10 *mM* sodium phosphate buffer (pH 7.0).

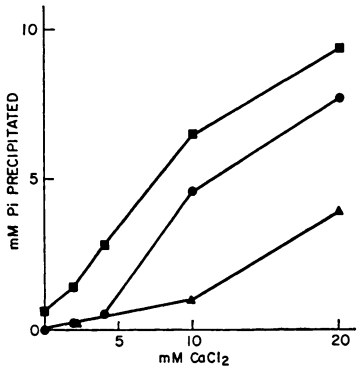

FIG. 10. Effects of $CaCl_2$ on phosphate precipitation in the presence and absence of 1 *mM* $Pb(NO_3)_2$ and 4 *mM* ATP. ●——●, $CaCl_2$, no $Pb(NO_3)_2$, no ATP; ■——■, $CaCl_2$ and 1 *mM* $Pb(NO_3)_2$, no ATP; ▲——▲, $CaCl_2$, 1 *mM* $Pb(NO_3)_2$ and 4 *mM* ATP. Reaction mixture contained 0.1 *M* Tris-maleate buffer (pH 7.0) and 10 *mM* sodium phosphate buffer (pH 7.0).

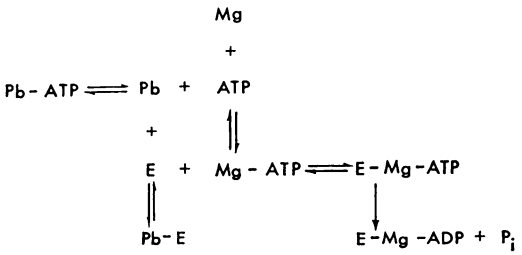

FIG. 11. A partial schematization of the interaction of lead, ATP and enzymes splitting ATP. As drawn, lead is acting as a purely competitive inhibitor and competes with ATP for the active site of the enzyme. For the sake of simplicity, the interactions of lead with released phosphate have been omitted from the diagram.

then chelation effects would have to be taken into account in any systematic analysis of histochemical ATPase media.

The situation which results from lead-ATP chelation is partly schematized in Figure 11. Because of Pb-ATP formation, lead competes with enzyme for Mg-ATP or ATP (as substrate), and ATP competes with enzyme (and with released phosphate) for lead (as inhibitor or as precipitating cation). As a result of Pb-ATP formation, unless Pb-ATP is enzymatically hydrolyzed at nearly the same rate as free ATP or Mg-ATP, some loss of ATPase activity will always be seen if sufficient lead is present. Inhibition of this type, in which the inhibitor acts by combining with substrate, mimics competitive inhibition in its effects (28), and indeed the two types of inhibition may be difficult to distinguish. While the effect of competitive inhibition is to increase K_m relative to substrate concentration, the effect of inhibition due to combination of lead with the substrate, ATP, is to decrease the substrate concentration relative to K_m. The effect of removing substrate will be most obvious when lead is present in excess of ATP and when ATP is present in less than saturating concentration (as in the case of liver microsomes incubated in the Wachstein-Meisel medium (27)).

When lead reacts directly with enzyme protein to produce inhibition, chelation of lead by ATP may also be important. In such a case ATP competes with enzyme for lead, and the extent to which inhibition occurs, *i.e.*, Pb-*E* is formed, again depends not only on Pb^{++} and on the characteristics of the enzyme, but also on the quantity of ATP added.

The aim of these experiments was not to distinguish the effects of lead-ATP interactions from lead-enzyme interactions, but rather to establish whether the effects of lead on ATPase activity were predictable from lead-ATP chelation. If chelation were significantly involved in loss of ATPase activity in the presence of lead, activity would be restored by further addition of ATP, as it would if lead were acting as a competitive inhibitor. If substrate inhibition occurred, it might be lost or reduced in the presence of high lead concentrations. Finally, if activity were lost because of removal of substrate as Pb-ATP, and if Mg-ATP, rather than ATP, were the true substrate of the enzyme, then in the presence of lead the concentration of Mg^{++} necessary for maximal activity would be increased. Alternate explanations could be given for all of these effects, but they all would be predicted from Pb-ATP binding.

The enzymes studied were chosen because they represent two different classes of ATPase. The ATPase associated with liver microsomes is relatively nonspecific in its substrate requirements (6) and appears to be relatively insensitive to lead inhibition. The ATPase from kidney was chosen because of its physiologic interest and because such Na^+-K^+-stimulated enzymes are relatively specific ATPases, highly sensitive to inhibition by a variety of agents, including lead (5, 14, 18). Although the two enzymes differed considerably in their sensitivity to lead inhibition, in both cases inhibition was overcome by increasing ATP.

With liver microsomes, significant loss of activity occurred when lead was present in excess of ATP. Even in the presence of 4 *mM* lead, activity was inhibited only slightly, or was not inhibited at all, when 4 and 10 *mM* ATP was present. As noted earlier, although substrate inhibition was normally observed with more than 4 *mM* ATP, this was not apparent with 4 *mM* $Pb(NO_3)_2$ and up to 10 *mM* ATP. Finally, a marked increase in optimal Mg^{++} concentration was observed when 4 *mM* $Pb(NO_3)_2$ was added to the reaction mixture.

The ATPase from kidney differed in several respects from that in liver. The Na^+-K^+-stimulated component of activity from kidney was far more sensitive to lead inhibition and was lost even when small (1 *mM*) amounts of lead were added and when ATP was present in excess (4 *mM*). If ATP was increased to 10 *mM*, activation by Na^+-K^+ reappeared. With liver microsomes, reduction in substrate concentration could account for much of the loss of activity which occurred. With kidney microsomes this effect could not account for loss of ATPase activity for, even if lead were completely bound to ATP, ATP or Mg-ATP would still be present at saturating concentrations. Therefore, some direct enzyme inhibition by lead was occurring. With the low lead concentrations required to retain any detectable Na^+-K^+ activation, no measurably large effects on Mg^{++} activation curves could be expected, nor were they observed. In kidney, substrate inhibition was not observed in the ATP range studied.

Although the interactions of lead, ATP and magnesium with enzyme systems splitting ATP are subject to several interpretations, the effects of ATP on precipitation of lead phosphate are less ambiguous. The results again suggest that the concentration of free Pb^{++} is determined not only by the quantity of lead added, but also by ATP present in the medium. The effects of Mg^{++} can be simply explained from the competition of Mg^{++} with Pb^{++} for ATP. As Mg^{++} is increased, the equilibrium $Mg^{++} + Pb\text{-}ATP \rightleftharpoons Pb^{++} + ATP + Mg^{++} \rightleftharpoons Mg\text{-}ATP + Pb^{++}$ shifts to the right, with an increase in lead available to precipitate phosphate. The effects of Ca^{++} are more complex, since both Ca^{++} and Pb^{++} form insoluble phosphates under the experimental conditions used. Because both Ca^{++} and Pb^{++} are complexed by ATP, the addition of ATP to mixtures containing Ca^{++} and Pb^{++} results in a net decrease in phosphate precipitated. However, phosphate is still more efficiently precipitated in the presence of Ca^{++} than under any other conditions tested.

In the course of these experiments an attempt was made to determine the form in which phosphate was precipitated by lead. The apparent solubility product was calculated over a range of lead and phosphate concentrations in the absence of ATP, assuming various formulae for the precipitate. $PbHPO_4$, $Pb_3(PO_4)_2$ and $Pb_5(PO_4)_3Cl$. By far the best fit of solubility product data was obtained when the form of the precipitate was assumed to be $PbHPO_4$. Although Pratt has suggested (22) that the precipitate was pyromorphite, $Pb_5(PO_4)_3Cl$, this suggestion was based on determinations of precipitate composition after prolonged equilibration in strongly acid solutions (10). These conditions are not equivalent to those used in enzyme histochemistry.

Although the use of high ATP concentrations may permit the demonstration of even rather sensitive ATPases in the presence of lead, the usefulness of this approach is limited by the increased solubility of lead phosphate which occurs under these conditions. With high ATP and low lead concentrations, the threshold for visible precipitation may exceed 10 *mM* phosphate in the absence of other added cations, and addition of Ca^{++} or Mg^{++} may result in large changes in lead phosphate solubility. These alterations in lead phosphate solubility raise unexpected and fundamental questions about the interpretation of many histochemical results. Basic to all such studies are the assumptions that alterations in staining with altered staining conditions are due only to altered enzyme activity and that the solubility of lead phosphate is relatively constant, regardless of staining conditions. These assumptions are not supported by the experimental results, which show that large changes in the apparent solubility of lead phosphate can occur with changes in substrate and substrate concentration, as well as activating ion concentration.

The results of this study suggest that, in media containing nucleoside triphosphate substrates, Mg^{++} or other activating divalent cations, and lead to precipitate released phosphate, the constituents of the medium interact. Because of this interaction, variation of one constituent of the medium may result in large and unexpected changes in the effective concentration of other species present. For this reason, the effects of lead depend not only on its concentration, but also on that of ATP and, at times, Mg^{++}.

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