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THE CONTROL OF HEPATIC PHOSPHORYLASE PHOSPHATASE BY ATP, ADP and Mg^{2+}

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Summary

1. The effects of ATP, ADP, Mg^{2+} and pH on three different preparations of hepatic phosphorylase phosphatase (phosphorylase *a* phosphohydrolase, EC 3.1.3.17) were investigated.

2. The phosphorylase phosphatase activity in these preparations was decreased by ATP, ADP, adenylyl imidodiphosphate and by Mg^{2+}. The effect produced by the adenine nucleotides was less pronounced at high pH than at low pH.

3. Increasing concentrations of Mg^{2+}, Mn^{2+} and Ca^{2+} in the presence of a constant concentration of ATP prevented the effect of ATP. At high concentrations the divalent cations decreased the reaction rate of the phosphatase in the presence of ATP as in its absence. At pH 7.5 and 8.5, ATP and Mg^{2+} in equimolar concentrations decreased the phosphatase activity to a smaller extent than ATP alone.

4. The activity of phosphorylase phosphatase was progressively less affected if $ATPMg^{2-}$ and free Mg^{2+} were increased at constant concentrations of uncomplexed ATP. In the concentration range expected to occur in the intact cell an increasing degree of complexation of ATP with Mg^{2+} diminished the inhibitory effect of the nucleotide. Since addition of Mg^{2+} after the preincubation with free ATP did not reverse the loss of phosphatase activity we concluded that the mechanism of action of ATP and ADP is not a simple inhibition as defined by an immediate and reversible loss of activity after the binding of a ligand.

Introduction

Phosphorylation and dephosphorylation of hepatic phosphorylase (EC 2.4.1.1) plays a central role in the control of liver glycogen metabolism. Regu-

latory signals modulating the activity of phosphorylase *b* kinase (EC 2.7.1.38) and of phosphorylase phosphatase (EC 3.1.3.17) determine the rate of the interconversion of phosphorylase and thus its activation and inactivation. Phosphorylase *b* kinase is thought to be activated (*b* to *a* conversion) in response to glucagon and cyclic AMP and stimulated (without interconversion) by increased intracellular Ca^{2+} concentrations [1]. The phosphorylase phosphatase reaction is stimulated in the presence of high glucose concentrations [2]. Additional control of the phosphatase activity by protein inhibitors (one of them activated in response to cyclic AMP) and by a deinhibitor have been found in vitro [3,4]. Whether these proteins play a regulatory role in the intact cell remains unknown. An inhibition of hepatic phosphorylase phosphatase by adenine and uridine nucleotides as well as by divalent cations has been observed in some enzyme preparations and $ATPMg^{2-}$ has been reported to activate another form of the enzyme [2,5—7]. Some effects of the interaction between divalent cations and nucleotides on phosphoprotein phosphatase from canine heart have been reported recently [8]. Half maximal inhibition and half maximal activation of the hepatic enzyme by ATP has been observed by Merlevede et al. in two different enzyme preparations at concentrations of 7 μM and 0.3 μM respectively. Because ATP levels in the intact cell are above these values, these authors have considered it unlikely that phosphorylase phosphatase is regulated by ATP in the intact liver cell [6]. In experiments with perfused rat livers we have obtserved a negative correlation between tissue ATP/AMP ratios and phosphorylase *a* activities under different metabolic conditions [9,10]. We have also shown that the activation of glycogenolysis by α-adrenergic stimulation parallels an enhanced cellular energy turnover and therefore seems to occur by a mechanism different from that triggered by cyclic AMP [11]. These observations have led to our decision to further investigate the properties of hepatic phosphorylase phosphatase in an attempt to answer the question whether a cyclic AMP independent activation of hepatic glycogenolysis occurs via control of the activity of this enzyme by the cellular energy needs. Preliminary results of these experiments have been reported [12, 13].

Materials and Methods

Enzyme preparations

To obtain phosphorylase phosphatase, livers from male rats (Z b Z cara formely Osborne Mendel, 200—250 g) were rapidly excised and homogenized. Rabbit livers were frozen in liquid N_2 and kept at —20°C until homogenization. Preparation 1 was obtained from rat liver homogenates by following the procedures described by Stalmans et al. [2]. For preparation 2 rat liver homogenates were precipitated with ethanol at room temperature as described by Brandt et al. [14] the last steps (DEAE-cellulose and Sephadex G 25) were the same as for preparation 1. Preparation 3 was obtained from rabbit livers by following the procedure described by Brandt et al. [7]. For further details on the purifications see Table I.

For the preparation of phosphorylase *a* the procedure used by Stalmans [2] was adopted. The enzyme preparations contained negligible ATP hydrolysing

TABLE I

PURIFICATION OF PHOSPHORYLASE PHOSPHATASE

The values are means of two separate preparations obtained by each procedure. P 1 and P 2 were from rat livers, P 3 from rabbit livers. (*) In early preparations the enzyme activites measured in homogenates varied widely because the dilution was not always identical. Therefore the mean values of six other appropriately diluted rat liver preparations are shown.

Enzyme preparation	Procedure	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
P 1	Homogenate (40 g liver)	104 *	2 800 *	0.037 *	100	—
	2nd high speed supernatant	123	735	0.17	118	5
	$CaCl_2$ supernatant	95	105	0.91	91	25
	DEAE-cellulose and Sephadex G 25	4.2	2.6	1.6	4	43
P 2	Homogenate (40 g liver)	104 *	2 800 *	0.037 *	100	—
	Ethanol precipitate	48	118	0.41	46	11
	DEAE-cellulose	44	24	1.8	42	49
P 3	Homogenate (360 g liver)	4451	70 000	0.063	100	—
	acid precipitate	1780	33 980	0.052	40	0.8
	$(NH_4)_2SO_4$, 70%	1414	34 400	0.041	32	0.7
	Ethanol precipitate	1087	1 447	0.75	24	12
	$(NH_4)_2SO_4$. 75%	851	1 582	0.54	19	9
	DEAE-Sephadex (3X)	115	5.33	22	2.6	343
	Sephadex G75	57.3	0.89	64	1.3	1020
	Sepharose (2X)	24.3	0.22	110	0.5	1750

activity in the presence and absence of Mg^{2+}. Protein was determined by the method of Bensadoun and Weinstein [15].

Preincubation and enzyme assays

Preparations 1 and 2 (usually 2—6 mU of phosphorylase phosphatase in 65 μl) were preincubated for 20—30 min at 30°C in 100 mM Tris-HCl at pH 6.5, 7.5 and 8.5. Preparation 3 was preincubated in 50 mM imidazol or 100 mM Tris-HCl containing 0.5 mM dithioerythritol and 1 mg/ml of human serum albumin. 1 mM EDTA was added unless Mg^{2+} or other divalent cations were present. Phosphorylase phosphatase activity was determined by measuring the rate of conversion of phosphorylase *a* to *b*. Purified phosphorylase *a* (1—3 U/ml) and 5 mM caffeine were added to the preincubation mixture. In each experimental group the amount of added phosphorylase (taken as 100%) was determined in vials containing 50 mM NaF in addition to the preincubation mixture. Phosphorylase phosphatase activity was expressed as percentage of phosphorylase *a* converted to *b* after 20 min at 30°C. Apparent K_m values for phosphorylase *a* were determined in the homogenate of rabbit liver and with preparation 3. They were 1.8 and 1.0 U/ml respectively. Assays were thus

usually performed with a substrate concentration close to the K_m value. If the percentage of conversion of phosphorylase *a* to *b* was 20—40% the assay was linear with time and enzyme concentration.

Phosphorylase *a* activity was determined by measuring the rate of incorporation of radioactivity from [U-^{14}C]glucose 1-phosphate into glycogen according to Gilboe [17]. The composition of the incubation mixture proposed for the measurement of phosphorylase *a* activity [16] was used. Incubation proceeded for 20 min at pH 6.1 and 30°C. The assay was linear with time and enzyme concentration. ATP (disodium salt) was obtained from Boehringer, Mannheim and used without further purification. Adenylyl imidodiphosphate (tetralithium salt) was from Sigma, [U-^{14}C]glucose 1-phosphate was from the Radiochemical Centre, Amersham.

Calculations

The inhibition constants (K_i) for the different forms of ATP and for Mg^{2+} were obtained with the method of Dixon [18] assuming non-competitive inhibition. The concentrations of free and complexed nucleotide species and free Mg^{2+} at equilibrium were calculated from the dissociation constant of the protonated form of ATP ($ATPH^{3-}$) and from the formation constants of the metal complexes ($ATPMg^{2-}$ and $ATPHMg^{-}$). $(Mg)_2ATP$, $Mg(ATP)_2$, $ATP(H)_2$ and $ATP(H)_2Mg$ were neglected since they were considered to occur only at negligibly low concentrations. The following dissociation constants of the protonated species of ATP and adenylyl imidodiphosphate were used [19]: $K_{a(ATP)} = 7.94 \cdot 10^{-8}$ M and $K_{a(adenylyl\ imidodiphosphate)} = 2.00 \cdot 10^{-8}$ M. The formation constants for $ATPHMg^{-}$ (K_1) and $ATPMg^{2-}$ (K_2) at 25°C were [20]: $K_1 = 1.74 \cdot 10^2\ M^{-1}$ and $K_2 = 1.66 \cdot 10^4\ M^{-1}$. All the computations were performed on a Hewlett-Packard 9810 A calculator.

Results

ATP decreased the activity of phosphorylase phosphatase. During preincubation for 15 min with 1 mM ATP the activity of preparation 3 was further diminished by 40%. The decrease during preincubation was faster and larger (over 90%) if an impure preparation was assayed. No significant loss of activity occurred in the absence of ATP. The pH optimum of phosphorylase phosphatase was around pH 7.7. At low pH the loss of activity following the addition of ATP was more pronounced than at higher pH. ADP and AMP had a similar effect on the reaction rate, but only ADP exhibited a pH dependence comparable with that of ATP. Cyclic AMP was inactive, even at a high concentration (Table II).

In order to compare the effects of different adenine nucleotides the influence of pH on apparent K_i values was determined. ADP was possibly somewhat less active than ATP (Table III). The response to ATP was essentially the same with the three enzyme preparations. To test the possibility that only the protonated form of the nucleotide was effective, which could explain the pH dependence of the inhibition, the response to ATP was compared with that of adenylyl imidodiphosphate. The protonation of these two nucleotides at the same pH is different because their pK_a differs [19]. If only the protonated

TABLE II

pH-DEPENDENCE OF THE EFFECTS OF VARIOUS ADENINE NUCLEOTIDES ON PHOSPHORYLASE PHOSPHATASE ACTIVITY

Phosphorylase phosphatase (preparation 1) was preincubated with the additions shown. Its activity was expressed as percentage of phosphorylase *a* converted to *b* after 20 min. Values are means ± S.E.M. of 3 experiments.

Additions	Phosphorylase phosphatase activity (%) at		
	pH 6.5	pH 7.5	pH 8.5
none	47.1 ± 4.6	54.2 ± 4.8	32.6 ± 2.0 *
ATP (1 mM)	9.6 ± 3.5	14.3 ± 6.8	22.5 ± 3.0 *
ADP (1 mM)	8.1 ± 2.8	24.1 2 3.6	29.7 ± 3.4 *
AMP (1 mM)	8.1 ± 3.5	3.0 ± 3.2	9.8 ± 2.7
cyclic AMP (1 mM)	39.2 ± 6.3	51.9 ± 4.5	31.9 ± 3.4

* By Student's *t*-test versus value at pH 6.5, $P < 0.05$.

species were effective a difference of the K_i values for total nucleotides and the same K_i for the protonated forms would be expected at the same pH. For all the experimental incubation conditions that were used, the concentrations of the protonated form of each nucleotide were calculated and the K_i values were determined for the total nucleotides and the protonated species. At pH 6.5, 7.5 and 8.5 the K_i for protonated adenylyl imidodiphosphate was higher than for $ATPH^{3-}$. The K_i values for protonated adenylyl imidodiphosphate were 0.08 mM, 0.2 mM and 0.2 mM at pH 6.5, 7.5 8.5 respectively, for $ATPH^{3-}$ they were 0.03 mM, 0.04 mM and 0.06 mM respectively. In contrast, the K_i values for total nucleotides were not different (Table III). The hypothesis that only protonated nucleotides have an effect was thus invalidated. Free Mg^{2+} at high concentrations (>10 mM) also resulted in a loss of phosphatase activity but not in a pH dependent manner (Table III).

Since in the intact cell ATP and Mg^{2+} occur predominantly in complexed form the effects of equimolar concentrations of ATP and Mg^{2+} were investigated. The comparison of the results with those obtained with ATP alone

TABLE III

INFLUENCE OF pH ON THE EFFECTS OF ADENINE NUCLEOTIDES AND Mg^{2+}

K_i values were obtained by the method of Dixon [18], mean values of 2 or 3 experiments are shown.

Additions	Enzyme preparations	Apparent K_i (mM) at		
		pH 6.5	pH 7.5	pH 8.5
ATP	1	0.04	0.2	1.0
	2	0.06	0.5	1.6
	3	0.02	—	4.0
ADP	3	0.09	1.0	5.6
Adenylyl imidodiphosphate	2	0.07	0.3	1.5
Mg^{2+}	2	24	25	28
	3	12	—	6.5

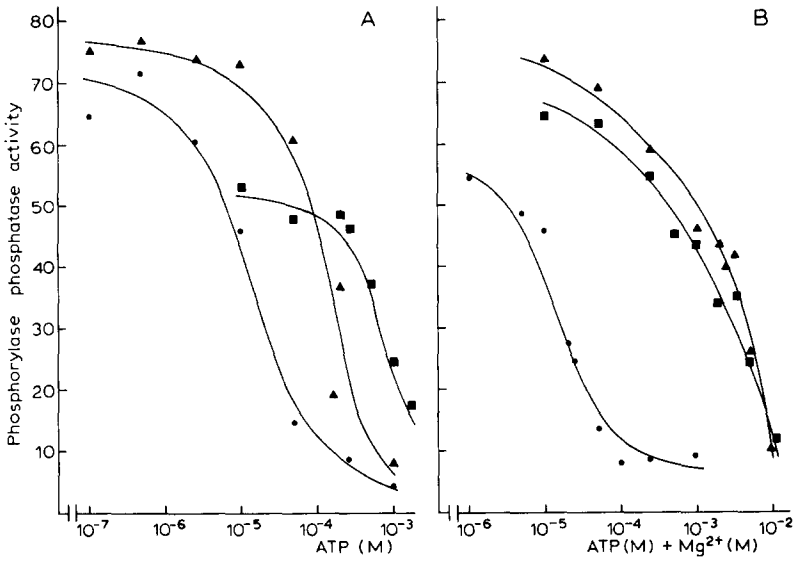

Fig. 1. Modification of ATP effects by equimolar concentrations of Mg^{2+}. Phosphorylase phosphatase (preparation 1) was preincubated and assayed at pH 6.5 (●——●), pH 7.5 (▲——▲) and pH 8.5 (■——■). Panel A: with ATP and EDTA (1 mM). Panel B: with equimolar concentrations of total ATP and total Mg^{2+}.

showed that at pH 7.5 and 8.5 the curves were shifted to the right by the addition of Mg^{2+} (Fig. 1). Since it seemed possible that the effect of ATP was partially suppressed by Mg^{2+}, additional experiments were performed with increasing concentrations of total Mg^{2+} in the presence of a fixed amount of total ATP. The incubation of the enzyme with 0.1 to 1 mM Mg^{2+} in the presence of 0.2 mM ATP prevented the loss of phosphatase activity produced by the nucleotide alone. At higher concentrations of Mg^{2+} the effect of the ion itself became apparent (Fig. 2A). If ATP was replaced by ADP, Mg^{2+} was less potent in preventing the decrease of the reaction rate elicited by the nucleotide probably because in comparison with ATP, ADP has a markedly lower affinity for Mg^{2+} [21]. Mn^{2+} and Ca^{2+} had an effect comparable with that of Mg^{2+} (Fig. 2B). At the same concentration Mn^{2+} was however more and Ca^{2+} was less active than Mg^{2+}. This may be explained by a difference of affinity of the cations for ATP which increases in the sequence $Mn^{2+} > Mg^{2+} > Ca^{2+}$ [20]. As shown in Table IV, Mg^{2+} was only capable of preventing the ATP effect if the ion was added simultaneously with the nucleotide. Addition of Mg^{2+} to the enzyme after preincubation with ATP was ineffective. Thus under our in vitro conditions the loss of phosphatase activity produced by ATP was not reversible.

In order to further elucidate the interaction between phosphorylase phosphatase and Mg^{2+}-complexed or uncomplexed ATP, additional experiments were performed. The concentrations of the uncomplexed forms of the nucleotide ($ATPH^{3-}$ + ATP^{4-}) were kept constant at three different levels while the concentrations of free Mg^{2+} and therefore also of $ATPMg^{2-}$ were increased. $ATPHMg^{-}$ was neglected because it was present only at comparatively low concentrations. Total amounts of ATP and Mg^{2+} that had to be added to the

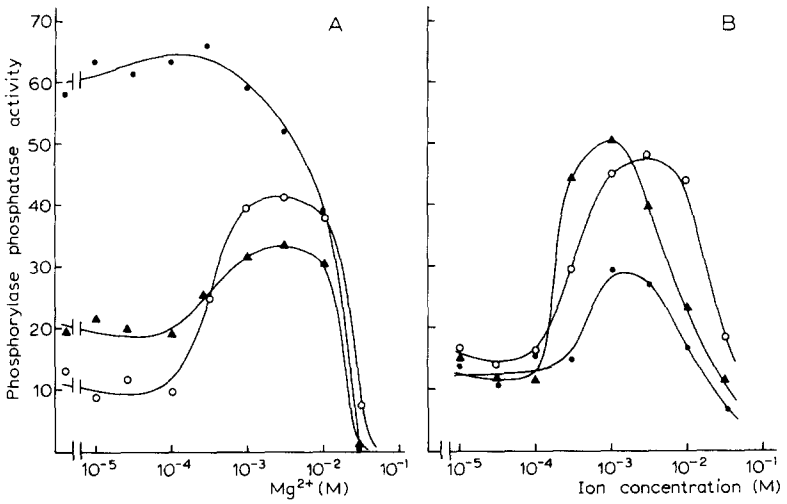

Fig. 2. Effects of increasing concentrations of Mg^{2+}, Ca^{2+} and Mn^{2+} on phosphorylase phosphatase (preparation 3) and interactions with ATP or ADP. Panel A: Increasing concentrations of Mg^{2+} were added to the enzyme in the presence of 0.2 mM ATP (○———○) or 0.2 mM ADP (▲———▲). Effect of Mg^{2+} in the absence of nucleotides (●———●). Preincubation and assay at pH 6.5. Panel B: Effect of Mn^{2+} (▲———▲) and Ca^{2+} (●———●) as compared with those of Mg^{2+} (○———○). Preincubation and assay in the presence of 0.2 mM ATP at pH 6.5.

incubation mixture were calculated as indicated in the section on methods. The concentrations necessary to obtain the two extreme levels of uncomplexed ATP and increasing concentrations of $ATPMg^{2-}$ are shown in Fig. 3A. The activity of phosphorylase phosphatase measured under these conditions was correlated with the concentrations of free Mg^{2+} (not shown) and with those of $ATPMg^{2-}$ (Fig. 3B). Concentrations of Mg^{2+}-complexed nucleotide above 10^{-4} M diminished the loss of phosphatase activity at the three levels. As it appeared that the ratio between complexed and uncomplexed ATP played a role in determining the activity of phosphorylase phosphatase the data from

TABLE IV

DIFFERENCE BETWEEN THE EFFECTS OF Mg^{2+} PRESENT DURING PREINCUBATION AND ADDED AFTER PREINCUBATION

Phosphorylase phosphatase (preparation 2) was preincubated and assayed at pH 6.5. Values are means ± range of two experiments.

ATP (0.2 mM)	Mg^{2+} (3.8 mM)		Phosphorylase phosphatase activity (%)
	Present during preincubation	Added after preincubation	
—	—	—	71.5 ± 9.9
+	—	—	13.3 ± 2.1
—	+	—	59.6 ± 5.4
+	+	—	48.5 ± 6.8 *
—	—	+	57.6 ± 3.8
+	—	+	14.2 ± 2.4 *

* Difference between the values by Student's t-test $P < 0.05$.

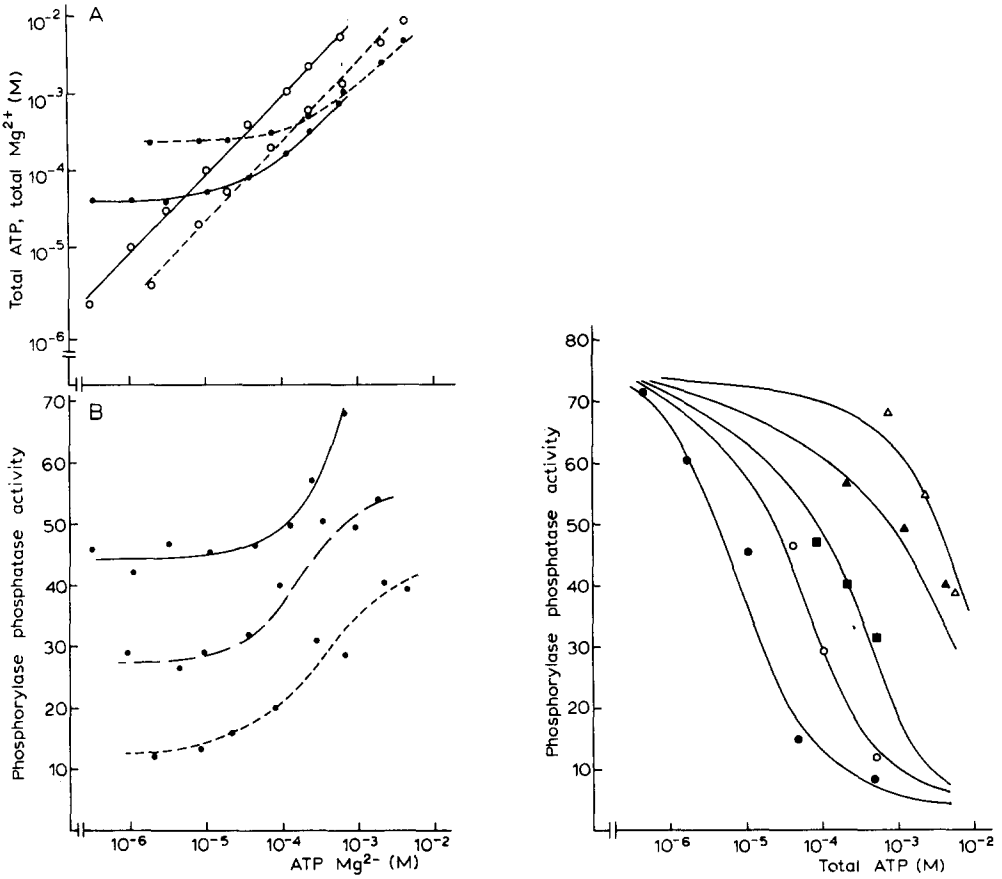

Fig. 3. Correlation of the activity of phosphorylase phosphatase (preparation 2) with increasing concentrations of $ATPMg^{2-}$ at constant concentrations of uncomplexed ATP and pH 6.5. Uncomplexed ATP was kept constant at three different levels while Mg^{2+}-complexed ATP and free Mg^{2+} progressively increased. The amounts of total ATP and total Mg^{2+} necessary to produce these conditions were calculated for pH 6.5 from the pK_a of ATP and the formation constants of the Mg^{2+} complexes. Panel A: Concentrations of total ATP (●) and total Mg^{2+} (○) necessary to obtains a rise of $MgATP^{2-}$ at a constant level of 40 μM uncomplexed ATP (———) and at 250 μM uncomplexed ATP (- - - - - -). Panel B: Activity of phosphorylase phosphatase preincubated and assayed in the presence of three constant concentrations of uncomplexed ATP and increasing concentrations of $MgATP^{2-}$. The mean concentrations (±S.E.M.) of uncomplexed ATP were (μM) 38.6 ± 0.6 ●———●, 98.6 ± 2.4 ●— — —●), 248 ± 8 ●- - - - -●.

Fig. 4. Dependence of the loss of activity of phosphorylase phosphatase on ATP and the ratio between complexed and uncomplexed nucleotide. Part of the data from experiments shown in Figs. 1 and 3 were replotted. ●———● 0%, ○———○ 0.9%, ■———■ 50%, ▲———▲ 91%, △———△ 94% of total ATP was complexed with Mg^{2+}.

experiments shown in Figs. 1 and 3 were rearranged as shown in Fig. 4. The sigmoidicity of the curves showing the ATP mediated decrease of enzyme activity changed according to the extent of complexation of ATP with Mg^{2+}. At concentrations of ATP close to those expected to occur in the intact cell ($\approx 10^{-3}$ M) less phosphorylase phosphatase activity was lost as the fraction of Mg^{2+}-complexed nucleotide increased.

Discussion

Our preparation 3 of hepatic phosphorylase phosphatase used to study the effects of ATP, ADP and metal ions has a lower specific activity than expected from reports in the literature. Brandt et al. [7] have obtained the enzyme in homogeneous form from liver homogenates after a 25 000-fold enrichment and with a specific activity of 2000 units per mg protein. In contrast, our preparation 3 has been purified 2000-fold and has a specific activity of 100 units per mg protein. These discrepancies which remain basically unreconsiled have resulted from differences at the first steps of the procedure, at later steps the values have been similar.

Khandelwal et al. obtained two homogeneous forms of hepatic phosphorylase after a purification of 500 and 6000-fold respectively [22]. A phosphoprotein phosphatase from rat liver catalyzing the dephosphorylation of pyruvate kinase has been purified 1000-fold [23]. The molecular weights reported for these enzymes are between 30 000 and 35 000 and correspond to the estimated molecular weight of our own preparation from rabbit liver. In crude extracts hepatic phosphorylase phosphatase activity has previously been found to exist in multiple forms of higher molecular weight [24] and it has been suggested that the high molecular weight forms may result from the association of catalytic subunits of the enzyme with a protein inhibitor [14]. Whether the low molecular weight form corresponds to catalytic subunits or to split products of limited, regulatory importance is still unknown. It is possible that purification procedures favouring the recovery of a homogeneous, unspecific phosphoprotein phosphatase of low molecular weight may enhance the liberation of identical enzymatically active subunits from high molecular weight holoenzymes. The specificity of high molecular weight enzymes for different substrates such as phosphorylase *a*, glycogen synthase *b* and phosphohistone may thus be lost after ethanol treatment [25], while the susceptibility to inhibition by nucleotides and cations remains unchanged. The latter conclusion is supported by our observation that ethanol treatment of phosphorylase phosphatase left the K_m for phosphorylase *a* and the effects of ATP and Mg^{2+} unaltered. Varying responses of hepatic phosphorylase phosphatase to ATP and divalent cations ranging from inhibition to activation [6] may not only be explained by the demonstrations of different molecular weight forms of the enzyme, but also by the hypothesis proposing that ATP chelates bound Mg^{2+} off the enzyme and thereby decreases its activity [26]. Our results are compatible with the proposal that the phosphatase is a metalloenzyme that is 'reactivated' by Mn^{2+} after treatment with pyrophosphate [27].

The question whether ATP and ADP interact with phosphorylase *a* rather than with the phosphatase cannot be answered conclusively until their effects have been checked with an other substrate such as histone. Indirect evidence obtained by comparison of the pH dependence of the inhibition by nucleotides (Table II) indicates however, that the mechanism of action of ATP and ADP is different from that of AMP. The latter is known to exert its effect by interacting with phosphorylase [2].

The lack of reactivation by Mg^{2+} of phosphorylase phosphatase preincubated with ATP (Table IV) suggests that the mechanism of action of the nucleotide is

more complex than that of an inhibitor reversibly binding to an enzyme and causing an immediate and reversible loss of activity. In our experiments components lost during the purification of the enzyme may have been missing for reactivation, or enzyme concentrations may have been too low.

Whether ATP, ADP and Mg^{2+} play a role in the control of phosphorylase phosphatase in intact hepatic cells, remains unknown at present. However, the finding that apparent K_i values for uncomplexed ATP and ADP at physiological pH are in the region of calculated cytosolic concentrations of these nucleotides in liver cells [28] suggests that regulation of the enzyme in vivo by ATP, ADP and Mg^{2+} has to be considered.

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