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Kinetic Studies of Adenylyl Cyclase of Fat Cell Membranes

II. Comparison of Activities Measured in the Presence of Mn^{++}-AMP-P(NH)P and Mg^{++}-AMP-P(NH)P Effects of Insulin, Fluoride, Isoproterenol, and GMP-P(NH)P

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Summary. The kinetics of fat cell adenylyl cyclase were studied, with AMP-P(NH)P and Mn^{++} or Mg^{++} as the divalent cation. In general, the reaction times were not linear. In the presence of fluoride or GMP-P(NH)P, the time curves were concave upwards; in other cases (i.e., basal activity, insulin, or isoproterenol), transient rates tended to decrease with time during the assay. Kinetic data were analyzed according to a previously described procedure (Torres *et al.*, 1978*b*) which isolates two kinetic components: initial and final.

With AMP-P(NH)P, kinetic activities were about ten times lower than those for ATP. With Mn^{++}, activities were at least two-times higher than for Mg^{++}.

Spontaneous inactivation of adenylyl cyclase was higher in assays containing Mg^{++} than in those supplemented with Mn^{++}. In the latter case, insulin was able to increase the inactivation rate. Fluoride and isoproterenol both activated adenylyl cyclase in both the initial and final kinetic components; under most of the conditions explored, their effects on the final component appeared to be more dramatic. Assays with GMP-P(NH)P showed inhibited activity in the initial component and increased activity in the final one.

When the results obtained with AMP-P(NH)P are compared with those of ATP (Torres *et al.*, 1978*b*. *J. Membrane Biol.* **43**:000), the following differences were found: (i) in the presence of insulin and Mn^{++}, cyclase inactivation was higher with AMP-P(NH)P than with ATP; (ii) fluoride stimulation of the final component was more marked with ATP than with AMP-P(NH)P; (iii) cyclase stimulation by isoproterenol was slightly higher with the nucleotide analog; and (iv) GMP-P(NH)P stimulation of the final component resulted in higher activity with ATP than with AMP-P(NH)P.

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A detailed study of the kinetic behavior of the adenylyl cyclase activity in pure fat cell membrane preparations has been presented in the preceding papers (Torres *et al.*, 1978*a–b*). One of the most important conclusions of this work is that adenylyl cyclase interconverts between active and inactive forms under enzyme assay conditions. The resultant conversions were slow enough to influence the shape of time courses. That is, in the absence of modifiers or in the presence of insulin, the activity rate tended to decrease with time; on the other hand, in the presence of isoproterenol, fluoride or GMP-P(NH)P, the activity rate increased with time.

Of the several factors that could affect such interconversions of active-inactive adenylyl cyclase forms, ATP and divalent cations are the two most important. Several studies point to the fact that ATP-dependent enzyme phosphorylation and its counterpart reaction, a divalent cation-modulated dephosphorylation, participate in the regulatory schemes of several enzymes. For example, this regulatory mechanism has been proposed for peritoneal granulocytes as well as platelet systems (Constantopoulos & Najjar, 1973; Najjar & Constantopoulos, 1973). Helmreich *et al.* (1976) concluded in a recent study that an ATP-dependent phosphorylation reaction was not involved in fluoride-stimulated rabbit myocardial cyclase. In addition, from the meticulous studies done by Birnbaumer *et al.* on the gonadotrophin-stimulated ovarian systems (Birnbaumer *et al.*, 1976; Hunzicker-Dunn & Birnbaumer, 1976*a–c*), it appears that an ATP-dependent phosphorylation is involved in a reaction leading to desensitization of adenylyl cyclase activity to lutenizing hormone (Bockaert, Hunzicker-Dunn & Birnbaumer, 1976).

Whatever the implications of putative covalent modifications of membrane-associated proteins on the control of adenylyl cyclase, it is evident that at least fluoride induces a permanent modification of this enzyme. As shown first by Perkins and Moore in rat brain membranes (Perkins & Moore, 1971), and more recently by Manganiello & Vaughan (1976) in membranes from fat cells, the anion induces a permanent activation of adenylyl cyclase.

Since the discovery that the nucleotide AMP-P(NH)P can serve as a substrate for adenylyl cyclase (Rodbell *et al.*, 1971), it has been used in incubation mixtures not supplemented with an ATP-generating system (Birnbaumer & Yang, 1974; Rodbell, Lin & Salomon, 1974; Salomon *et al.*, 1975). Its use has been based on its ability to resist the action of mammalian phosphohydrolase activities at the level of the β, γ-pyrophosphate bond (Rodbell *et al.*, 1971; Yount, Ojala & Babcock, 1971*b*).

However, the stability of AMP-P(NH)P in incubation mixtures for adenylyl cyclase has been questioned, since its α-β-pyrophosphate bond might be susceptible to cleavage by hydrolases (Johnson, 1977).

Another interesting property of this compound is its inability to serve as phosphate donor in protein phosphorylation reactions (Bockaert *et al.*, 1976; Yount *et al.*, 1971*a*). The use of AMP-P(NH)P instead of ATP as substrate for adenylyl cyclase could be an excellent tool to determine whether or not ATP is playing some role in the interconversions between active and inactive adenylyl cyclase forms. The purpose of this work is to discover precisely the important differences between these two states as they relate to the kinetic behavior of adenylyl cyclase. Using the same approach as in our preceding paper (Torres *et al.*, 1978*b*), we evaluated changes of transient kinetic components of the fat cell adenylyl cyclase system using AMP-P(NH)P under varying conditions. The magnitude of AMP-P(NH)P-related changes compared with those found in ATP-containing assays (Torres *et al.*, 1978*a–b*) could indicate, in principle, that a phosphate donor may produce relatively slow control reactions.

Materials and Methods

Unless otherwise indicated, details of experimental conditions are given in the preceding papers (Torres *et al.*, 1978*a–b*). Studies were done on a fat cell membrane preparation purified by centrifugation on sucrose gradients.

Standard components of adenylyl cyclase assay mixture were: 50 mM Tris-HCl buffer, pH 7.4; 0.2 mM 3-isobutyl-1-methyl xanthine; 1 mM cyclic AMP; 0.1 mg per ml albumin and membranes (10 to 20 µg of total protein). Concentrations of ^{32}P-α-labeled AMP-P(NH)P (about 1×10^7 cpm per assay), divalent cation (Mn^{++} or Mg^{++}) and other additions are as indicated in the legend of each figure.

Incubations were done at 37 °C for 1.5, 3 and 5 min. The total volume of each sample was 0.1 ml. Reactions were stopped by the addition of 0.1 ml of a solution containing 40 mM ATP, 12.5 mM[^{3}H]cAMP (sp act about 3000 cpm per µmole) and 1% sodium dodecylsulfate. The boiling step was omitted.

Concentrations of free Mn^{++}, free Mg^{++}, HAMP-P(NH)P^{3-} and AMP-P(NH)P^{4-} as well as the corresponding MeHAMP-P(NH)P^{1-} and MeAMP-P(NH)P^{2-} complexed species, were calculated as previously indicated (Torres *et al.*, 1978*a–b*). The values assigned to the association constants were as follows:

$$K_1 = \frac{[\text{MeHAMP} - \text{P(HN)}\,\text{P}^{1-}]}{[\text{HAMP} - \text{P(NH)}\,\text{P}^{3-}]\,[\text{free Me}^{++}]} \quad (K_{1(\text{Mn})} = 0.2\ \text{mM}^{-1};\quad K_{1(\text{Mg})} = 0.2\ \text{mM}^{-1})$$

(Khan & Martell, 1966)

$$K_2 = \frac{[\text{MeAMP} - \text{P(NH)}\,\text{P}^{2-}]}{[\text{ATP}^{4-}]\,[\text{free Me}^{++}]} \quad (K_{2(\text{Mn})} = 85\ \text{mM}^{-1};\quad K_{2(\text{Mg})} = 38.2\ \text{mM}^{-1})$$

(Yount *et al.*, 1971)

$$K_3 = \frac{[\text{HAMP} - \text{P(NH)P}^{3-}]}{[\text{AMP} - \text{P(NH)P}^{4-}][\text{H}^+]} = 50118 \text{ mM}^{-1} \text{ (Khan \& Martell, 1966)}$$

$$[\text{H}^+] = 3.9811 \times 10^{-5} \text{ mM}.$$

Since a value for K_1 was not available in the literature, it was assumed to have a value similar to the one reported for ATP (Khan & Martell, 1966).

Analysis of transient kinetic components (initial rates and final rates) was done as previously described (Torres *et al.*, 1978*b*).

Results

The kinetic behavior of the adenylyl cyclase-catalyzed reaction was studied under three conditions. First, the total concentration of divalent cation (Mg^{++} or Mn^{++}) was fixed, and AMP-P(NH)P was varied; next, the concentration of AMP-P(NH)P was fixed, and the divalent cation was varied. Third, the effect of different modifiers (insulin, isoproterenol, fluoride, and GMP-P(NH)P) was studied. Time courses of the cyclase reaction corresponding to the three studies are shown in Figs. 1 to 4. As occurs with ATP (Torres *et al.*, 1978*b*), time courses were generally not linear. In some cases (fluoride or GMP-P(NH)P-modified), the curves were concave upwards; but in other cases (basal activity, insulin, or isoproterenol), transient rates tended to decrease with the assay time. Analysis of kinetic data was done in accordance to criteria used in studies performed with ATP as substrate (Torres *et al.*, 1978*b*); that is, two kinetic components, initial and final, were defined.

Independent of the conditions selected for the assays, specific activities of adenylyl cyclase measured with AMP-P(NH)P resulted in much lower values than those observed with ATP (*see* Figs. 1 to 4 in Torres *et al.*, 1978*b*). Under optimal conditions (0.1 mM AMP-P(NH)P; 2 mM Mn^{++}, and 2.5 mM F^-), the specific activity of the initial component was 36 pmoles/min per mg protein. In the case of assays done with ATP under conditions similar but not identical, specific activities were at least 10-fold higher. A similar observation was reported for the beef renal medullary system studied by Birmbaumer and Yang (1974). A practical consequence of these facts is that it is very difficult to determine cyclase activities for short incubation periods with AMP-P(NH)P concentrations higher than 0.1 mM. (sp act lower than 1000 cpm per pmole).

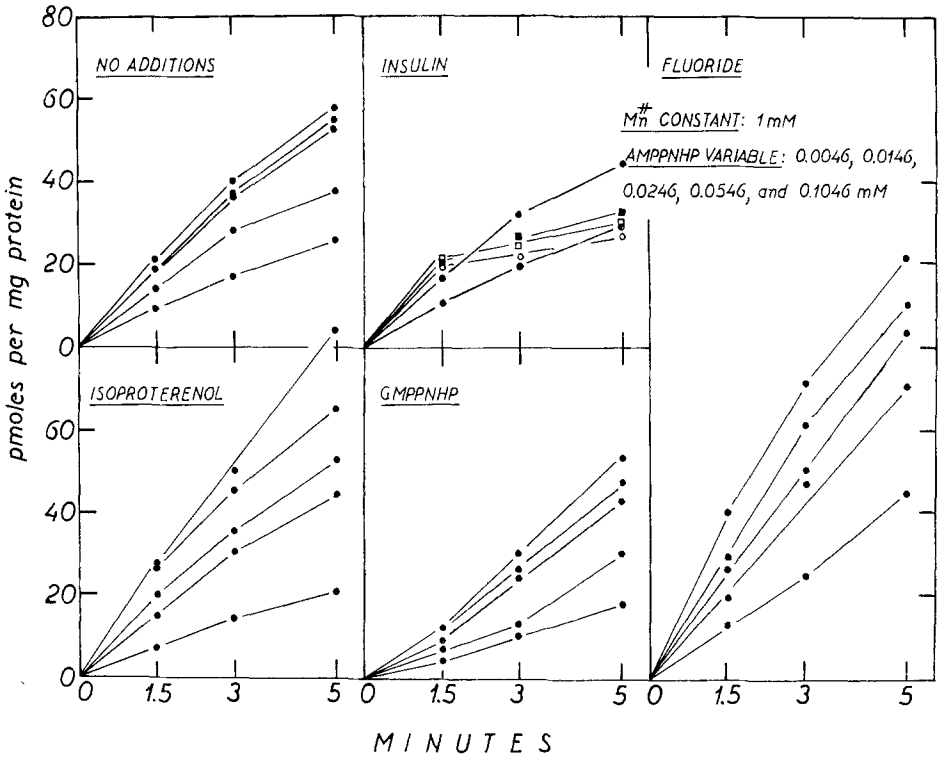


Fig. 1

Figs. 1–4. Time courses of adenylyl cyclase reaction catalyzed by pure fat cell membranes. In each set of curves, increasing enzyme activities correspond to increasing concentrations of total AMP-P(NH)P or divalent cation in the assay mixtures. Concentration of the different additions in these mixtures was 1000 μU insulin per ml, 2.5 mM fluoride, 10^{-5} M isoproterenol, and 10^{-5} M GMP-P(NH)P. AMP-P(NH)P and divalent cation concentrations are indicated in each figure

Influence of Variable Concentrations of AMP-P(NH)P

Basal activity. Figure 5 shows the relationship between initial and final activity rates using varying AMP-P(NH)P concentration levels and a fixed divalent cation concentration. Several facts were evident. First, activities measured with Mn^{++} were two to three times higher than those measured with Mg^{++}. Second, in all cases initial rates were higher than final rates; such differences were higher with Mg^{++} than with Mn^{++}. Third, inactivation tended to increase with AMP-P(NH)P concentration, particularly in the case of Mg^{++}.

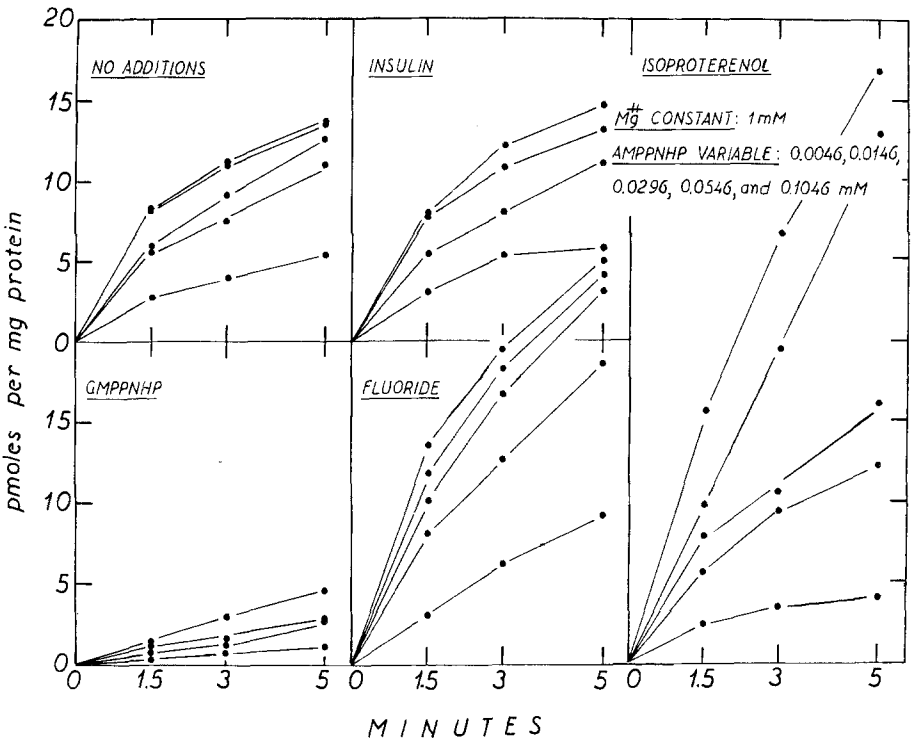


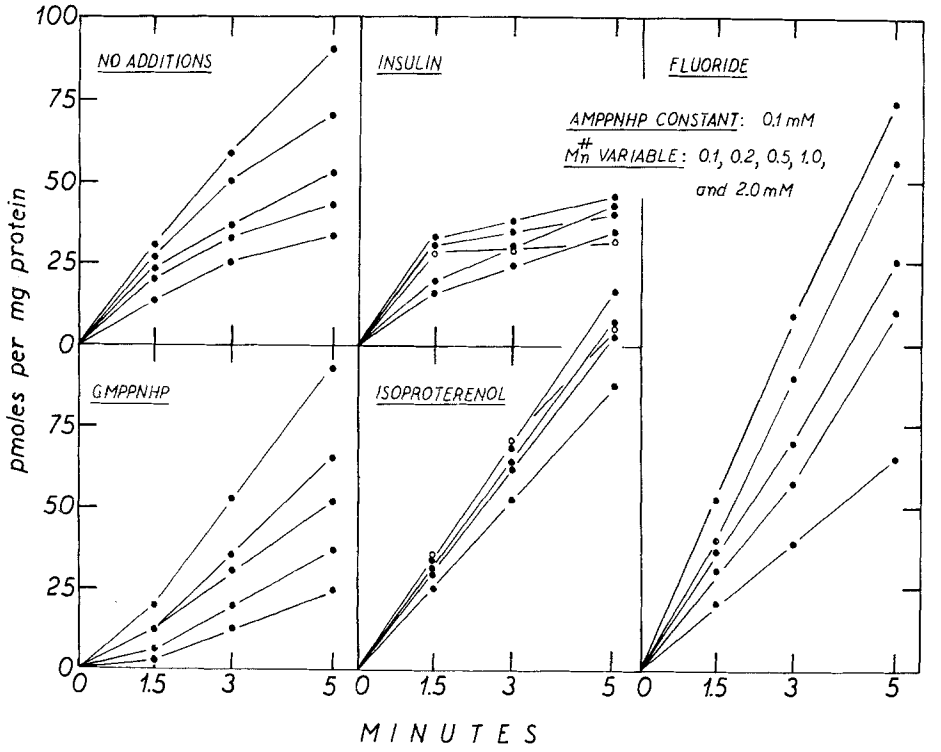
Fig. 2

Insulin. Figures 6 and 7 show the behavior of the cyclase system in the presence of insulin. The hormone decreased the final activity rates. With Mn^{++}, the inactivation was strongly affected by the concentration of AMP-P(NH)P. This result suggests that the transition from active to inactive cyclase forms operates at the level of substrate-enzyme complexes. This explanation seems to be the more probable since changes in the concentration of free Mn^{++} were negligible compared with those corresponding to [MnAMP-P(NH)P^{2-}] in the whole interval of AMP-P(NH)P concentrations (*see* Table 1).

In the case of Mg^{++} (Fig. 7), a 60% inactivation of the final component was observed only at the lowest AMP-P(NH)P concentration tested. As shown in Fig. 5, this same concentration was the one which led to the lowest inactivation in the absence of insulin.

When the results obtained with AMP-P(NH)P are compared with those described for ATP, cyclase inactivation appeared to be much more efficient with the former nucleotide than with the latter.

Fluoride. Fluoride stimulation of cyclase observed with AMP-P(NH)P (Figs. 8 and 9) resulted in relatively modest changes when compared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INSULIN
FLUORIDE
AMPPNHP CONSTANT: 0.1 mM
$M_n^{\#}$ VARIABLE: 0.1, 0.2, 0.5, 1.0, and 2.0 mM
GMPPNHP
ISOPROTERENOL
pmoles per mg protein
MINUTES

Fig. 3

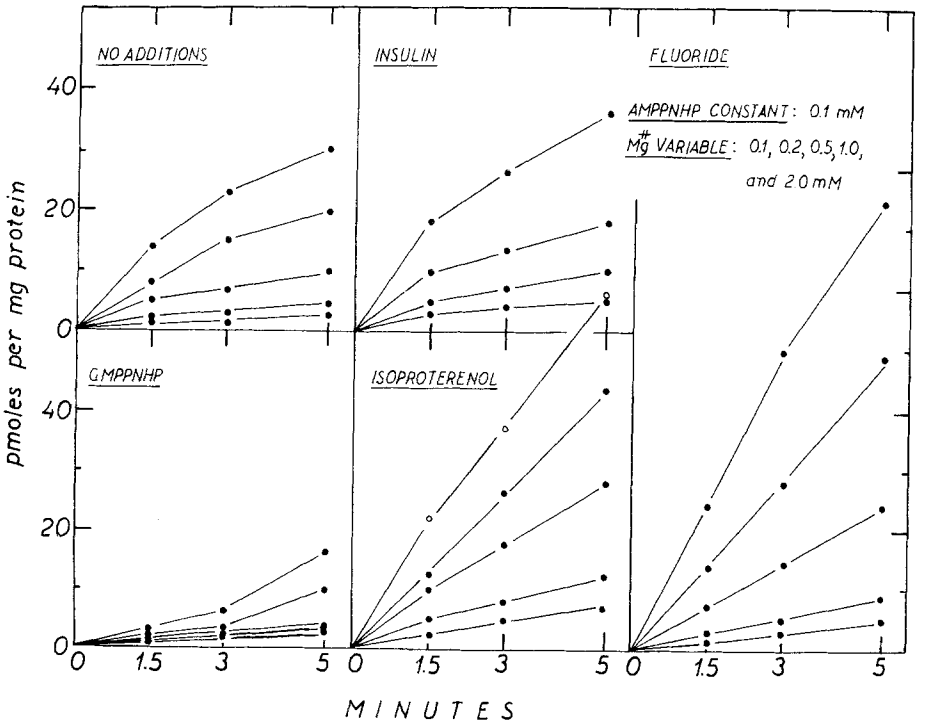

NO ADDITIONS
INSULIN
FLUORIDE
AMPPNHP CONSTANT: 0.1 mM
$M_g^{\#}$ VARIABLE: 0.1, 0.2, 0.5, 1.0, and 2.0 mM
GMPPNHP
ISOPROTERENOL
pmoles per mg protein
MINUTES

Fig. 4

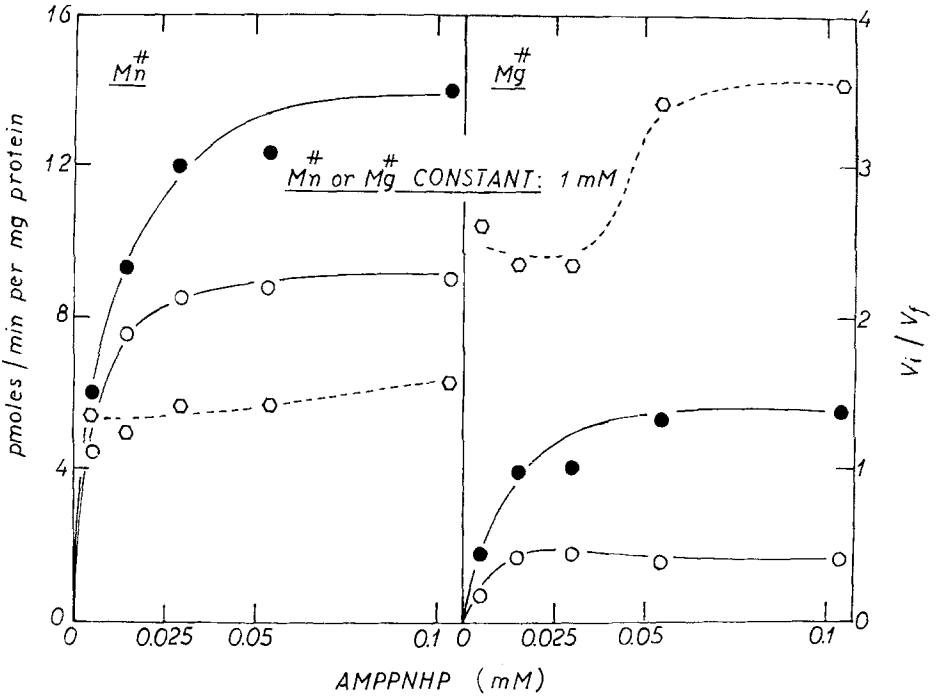


Fig. 5. Basal adenylyl cyclase activity measured in the presence of total 1 mM Mn^{++} or Mg^{++}, as a function of total AMP-P(NH)P concentration. ●, initial rates; ○, final rates; ○, ratio between initial and final rates (*Vi*/*Vf*). Other conditions are the same as in Figs. 1–4

with those for ATP (Torres *et al.*, 1978*b*). Maximal stimulations observed with Mg^{++} or Mn^{++} were in the order of onefold at the level of the final component. Under most conditions, *Vi*/*Vf* ratios were equal to or higher than one, indicating that in the presence of AMP-P(NH)P, cyclase inactivation was operative in assays supplemented with fluoride. On the other hand, the concentration of AMP-P(NH)P influenced the extent of the activation mainly in the initial component.

Isoproterenol. In the presence of Mn^{++}, stimulation by isoproterenol was evident only in the final component at the highest concentration of AMP-P(NH)P (Fig. 10). With Mg^{++}, activation of the final component was also most preponderant at the highest concentration of the analog. Effects on initial rates were less dramatic. A dependency on substrate concentration (Fig. 11) for the stimulation of the final component has already been shown with ATP (Torres *et al.*, 1978*a–b*).

GMP-P(NH)P. Figures 12 and 13 show the inhibition of the initial component, particularly in assays containing Mg^{++}. When these

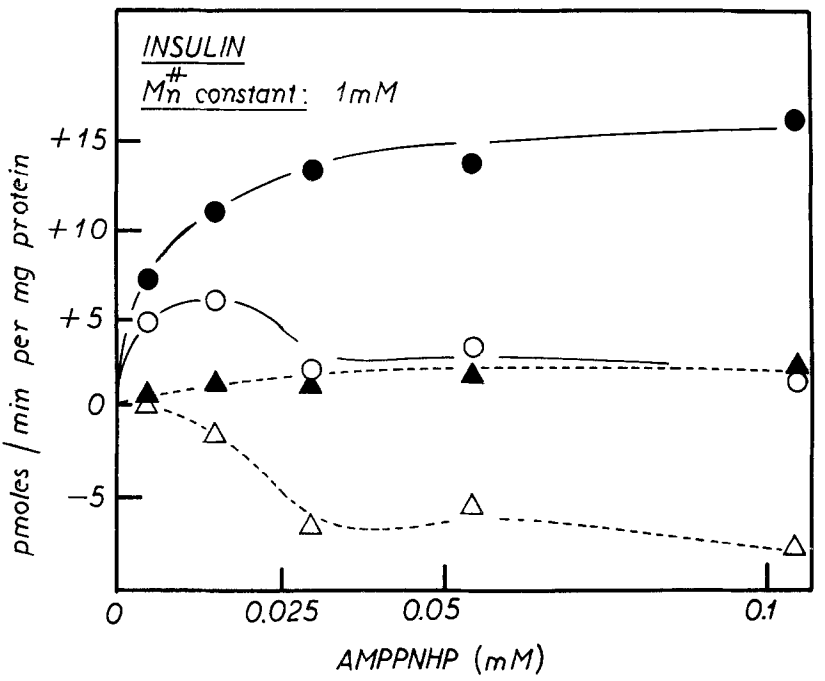


Fig. 6

Figs. 6–13. Effect of different modifiers (insulin, fluoride, isoproterenol, and GMP-P(NH)P on adenylyl cyclase activity measured at constant total divalent cation concentration (1 mM Mn^{++} or Mg^{++}) and varying AMP-P(NH)P concentrations. ●, initial rates; ○, final rates; ▲, difference between initial rates shown in each figure and those corresponding to basal activities (Fig. 5): $Vi_{(modifier)} - Vi_{(basal)}$; △, difference between final rates shown in each figure and those corresponding to basal activities (Fig. 5): $Vf_{(modifier)} - Vf_{(basal)}$. Conditions are the same as indicated in Figs. 1–5

results are compared with those for ATP, it appears that the AMP-P(NH)P recovery took more time. In any case, the effects of AMP-P(NH)P appeared to have little dependence on the concentration of this analog.

Influence of Variable Concentrations of Divalent Cation

Basal activity. Figure 14 shows plots of initial and final activity rates as a function of total Mn^{++} or Mg^{++} concentration. In this case, total concentration of AMP-P(NH)P was fixed at 0.1 mM. In the presence of Mn^{++}, the enzyme system showed a clear inactivation at the lowest divalent cation concentrations. With Mg^{++}, the inactivation increased

Table 1. Kinetic parameters of adenylyl cyclase reaction catalyzed by fat cell pure mem-

Ligand	Condition	$K_{m\text{app}}$ (mM)	Vmx_i (pmoles/min/mg (prot)	Total ligand (mM)	
				Constant	Variable
Free Mn^{++}	Basal	0.166	24.4	AMP-P(NH)P:	Mn^{++}:
	Insulin	0.166	24.4	0.10	0.1 to 2.0
	Isoproterenol	0.029	23.8		
	Fluoride	0.215	37.0		
Free Mg^{++}	Basal	2.500	23.2	AMP-P(NH)P:	Mg^{++}:
	Insulin	2.500	23.2	0.10	0.2 to 2.0
	Isoproterenol	0.830	21.4		
	Fluoride	2.500	38.5		
$MnAMP\text{-}P(NH)P^{2-}$	Basal	0.007	14.5	Mn^{++}: 1.0	AMP-P(NH)P:
	Insulin	0.007	16.7		0.0046 to
	Isoproterenol	0.014	20.8		0.1046
	Fluoride	0.014	26.3		
$MgAMP\text{-}P(NH)P^{2-}$	Basal	0.012	7.5	Mg^{++}: 1.0	AMP-P(NH)P:
	Insulin	0.009	7.5		0.0046 to
	Isoproterenol	0.024	10.0		0.1046
	Fluoride	0.013	10.0		

[a] Initial velocities were plotted as a function of free Me^{++} or $MeAMP\text{-}P(NH)P^{2-}$.

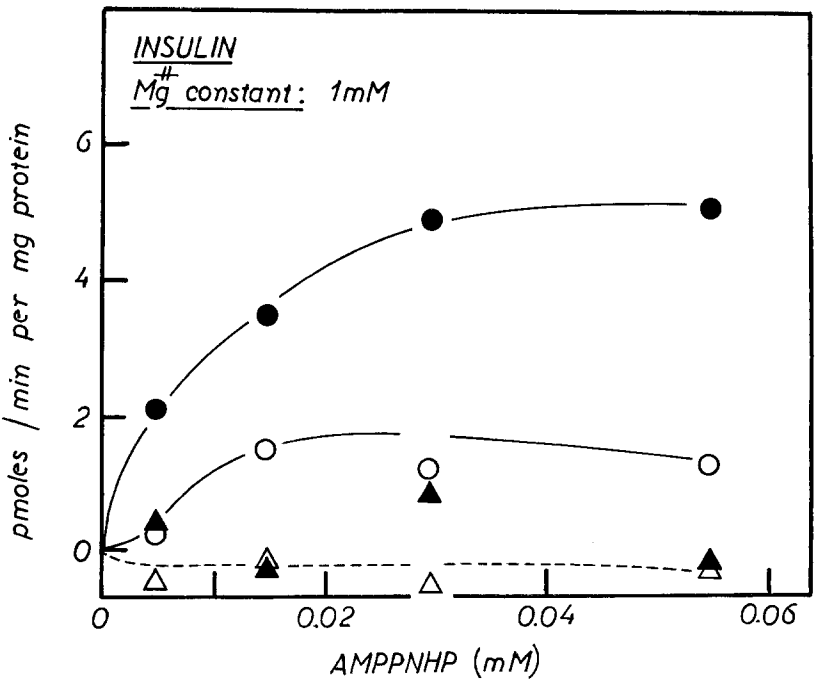


Fig. 7

branes, and concentration of Me^{++} and AMP-P(NH)P species in the assay mixtures[a]

Concentration range of AMP-P(NH)P or Me^{++} species in the interval of variable ligand (mM)				
Free Me^{++}	MeHAMP-P(NH)P^{1-}	HAMP-P(NH)P^{3-}	MeAMP-P(NH)P^{2-}	AMP-P(NH)P^{4-}
4.42×10^{-2} to 1.90×10^{-2}	2.61×10^{-4} to 4.59×10^{-4}	2.95×10^{-2} to 1.21×10^{-3}	5.55×10^{-2} to 9.77×10^{-2}	1.48×10^{-2} to 6.04×10^{-4}
0.14×10^{-2} to 1.90×10^{-2}	6.59×10^{-4} to 9.93×10^{-4}	2.42×10^{-2} to 2.61×10^{-3}	6.31×10^{-2} to 9.51×10^{-2}	1.21×10^{-2} to 1.31×10^{-3}
0.99×10^{-2} to 0.90×10^{-2}	2.08×10^{-5} to 4.70×10^{-4}	1.04×10^{-4} to 2.61×10^{-3}	4.42×10^{-3} to 1.00×10^{-1}	5.23×10^{-5} to 1.31×10^{-3}
0.99×10^{-2} to 0.90×10^{-2}	4.41×10^{-5} to 9.96×10^{-4}	2.21×10^{-4} to 5.51×10^{-3}	4.22×10^{-3} to 9.53×10^{-2}	1.11×10^{-4} to 2.76×10^{-3}

Other conditions were given under *Materials and Methods* and in Fig. 23.

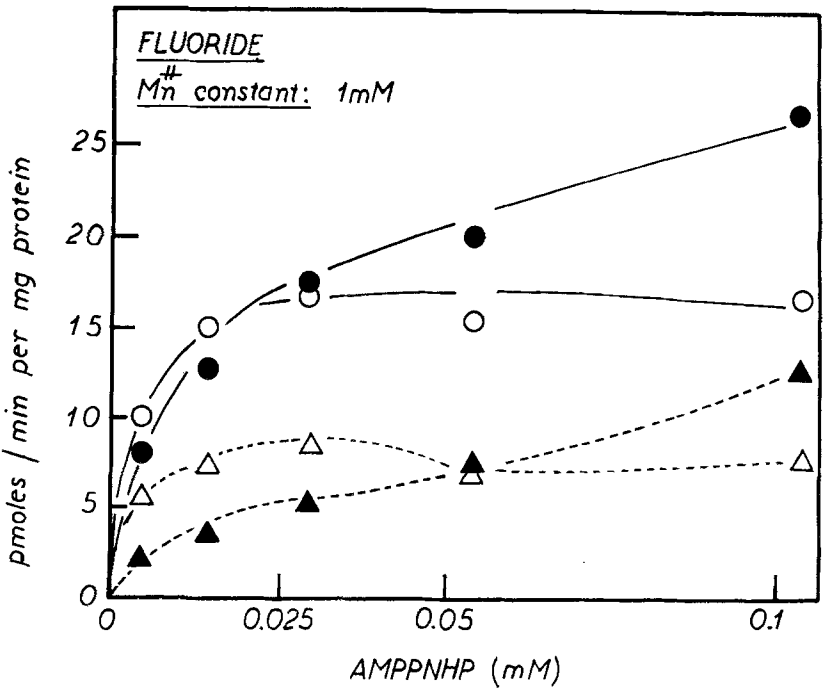
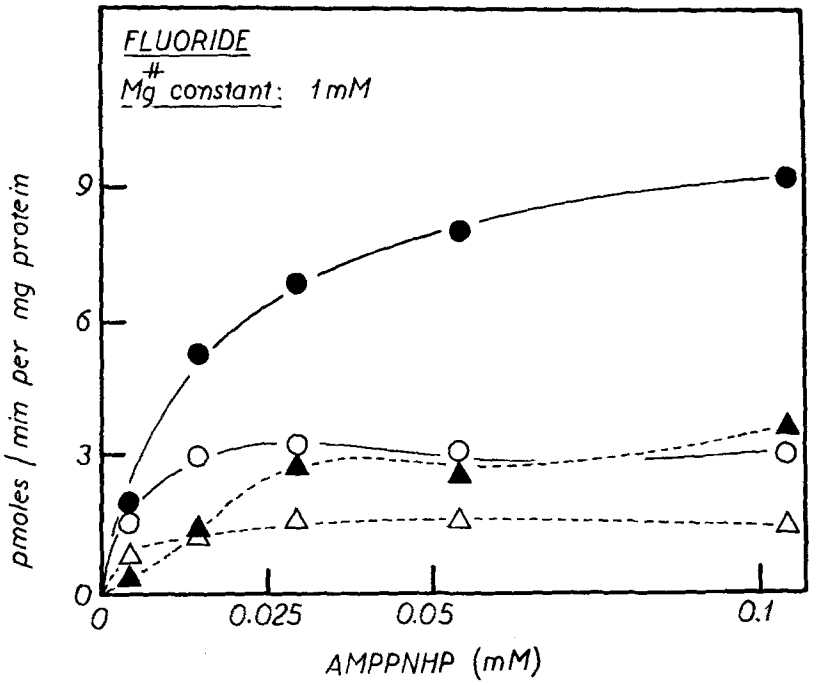


Fig. 8


FLUORIDE
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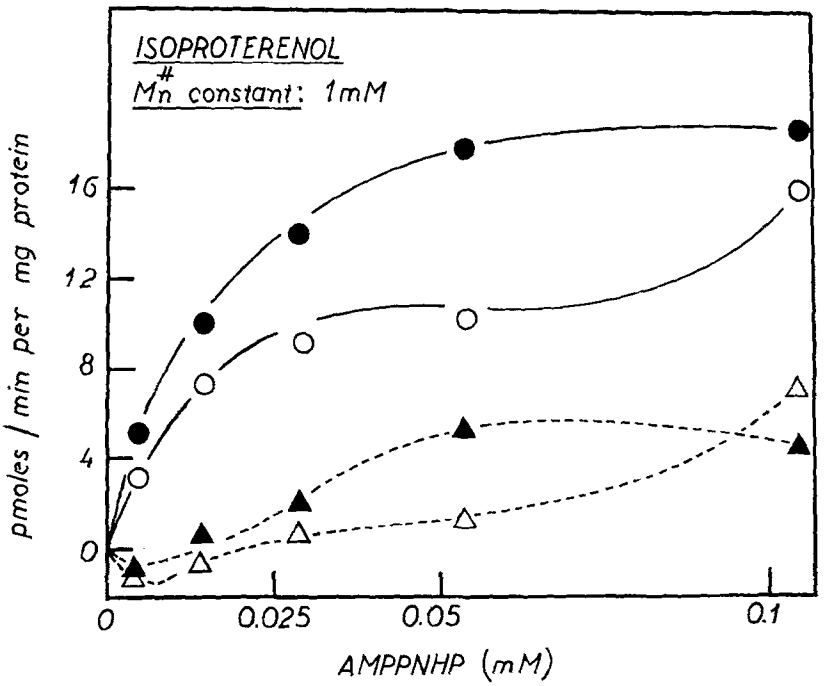
pmoles / min per mg protein
9
6
3
0
0
0.025
0.05
0.1
AMPPNHP (mM)

Fig. 9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Mn# constant: 1mM
pmoles / min per mg protein
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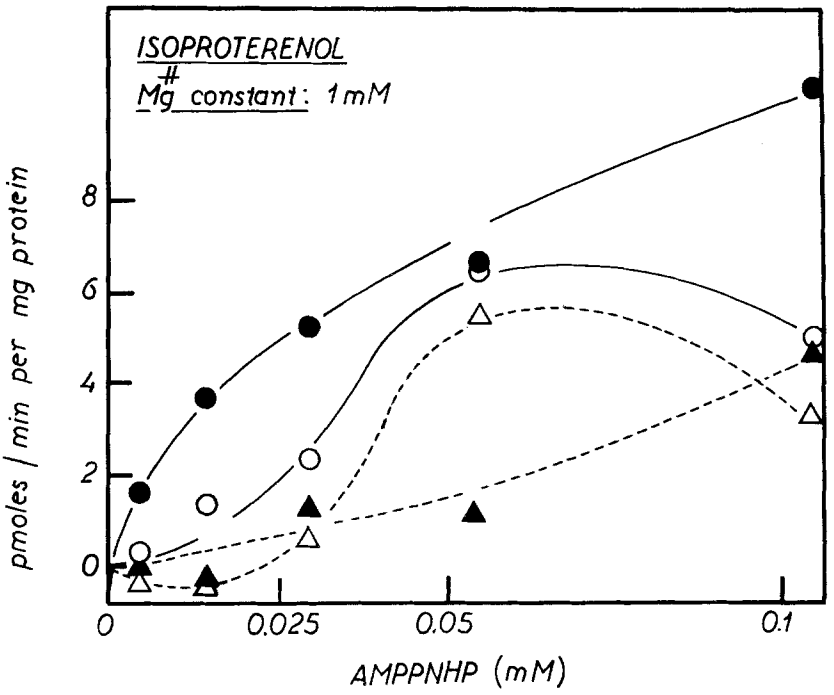
8
4
0
0
0.025
0.05
0.1
AMPPNHP (mM)

Fig. 10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Mg# constant: 1mM
pmoles / min per mg protein
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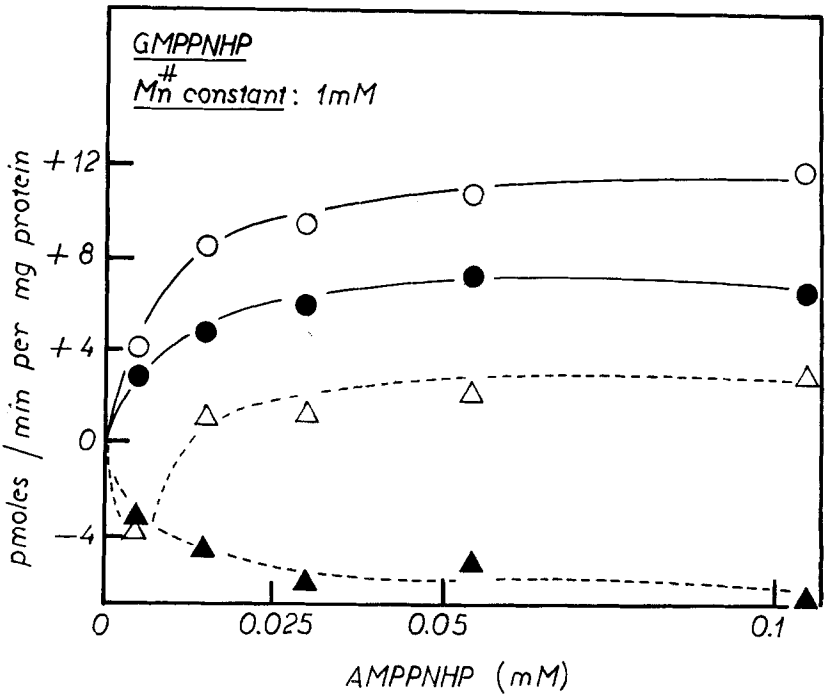
6
4
2
0
0
0.025
0.05
0.1
AMPPNHP (mM)

Fig. 11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+8
+4
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−4
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0.1
AMPPNHP (mM)

Fig. 12

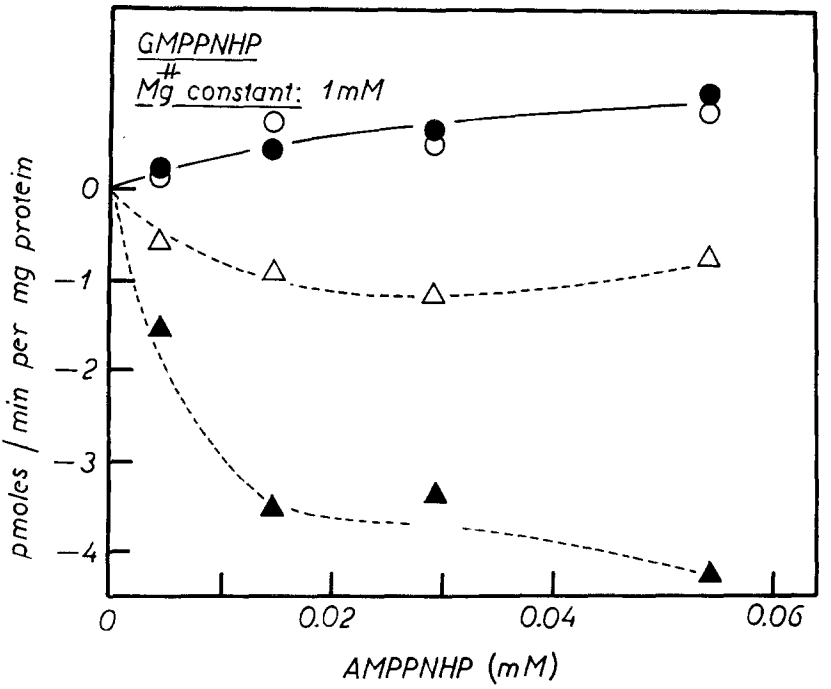

Fig. 13

with increasing concentrations of the cation. Interpretation of these results is difficult. In most of the divalent cation concentrations tested it was evident that cyclase inactivation operated more efficiently with Mg^{++} than with Mn^{++}. However, when the results are compared with those previously described (Torres *et al.*, 1978*b*), it appears that AMP-P(NH)P decreases the ability of Mn^{++} to maintain the activity of the cyclase system as compared with ATP.

Insulin. In the presence of insulin, increasing concentrations of Mn^{++} led to an increased degree of cyclase inactivation (Fig. 15). The hormone did not modify initial velocities. In terms of the concentration range of Mn^{++} leading to cyclase inactivation, the effect of insulin was observed under conditions that in the absence of hormone did not produce *per se* a high inactivation of adenylyl cyclase.

On the other hand, when the results are compared with those obtained using ATP, it is clear that the extent of cyclase inactivation observed with AMP-P(NH)P is higher than that elicited by ATP.

In the case of assays performed in the presence of Mg^{++}, insulin did not increase the inactivation of the cyclase system (Fig. 16). On the contrary, the hormone tended to increase both initial and final rates.

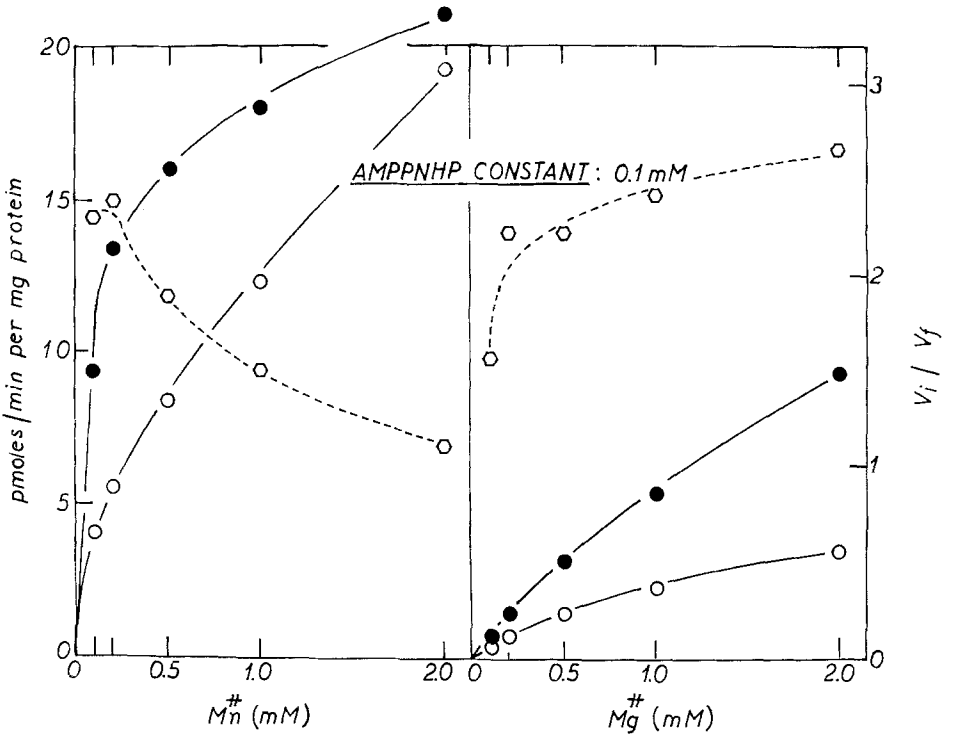

Fig. 14. Basal adenylyl cyclase activity measured as a function of total divalent cation concentration in the presence of 0.1 mM AMP-P(NH)P. Symbols and conditions are the same as those as indicated in Figs. 1–5

Fluoride. Activations observed in the presence of fluoride were more evident at the level of final velocities (Figs. 17 and 18). With Mn^{++} the effect tended to decrease with increasing concentrations of the divalent cation; however, an increase was noted in the extent of the activation with increasing concentrations of Mg^{++}. On the other hand, under optimal conditions the relative magnitude of activation was equivalent with both divalent cations (three- to fourfold).

When the results are compared with those obtained in the absence of fluoride, it is evident that activations might be a simple consequence of the reversion of cyclase inactivation observed under basal conditions. In addition, activation observed in the presence of the analog were smaller than those found with ATP (Torres *et al.*, 1978*b*). Cyclase forms with the same level of activity in assays performed with Mn^{++} or Mg^{++} were not generated in the presence of the AMP-P(NH)P.

Isoproterenol. Varying Mn^{++}, kinetic behavior observed in the presence of isoproterenol (Fig. 19) resembled that found for fluoride (Fig. 17).

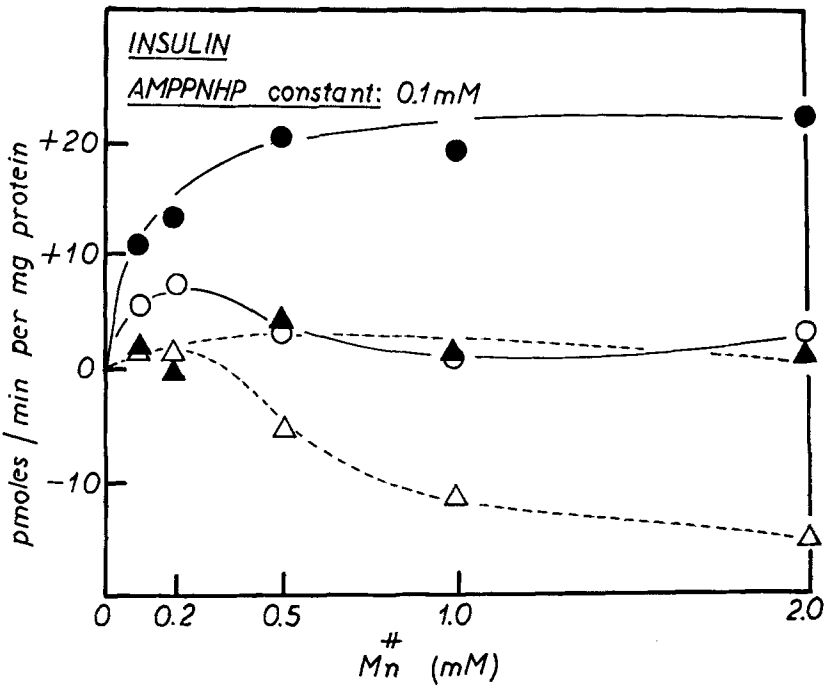

Fig. 15

Figs. 15–22. Effect of different modifiers on adenylyl cyclase activity measured at constant total AMP-P(NH)P concentration (0.1 mM) and varying the total divalent cation concentration. Symbols and conditions are the same as indicated in Figs. 6–13

That is, activations became negligible at the highest divalent cation concentration tested. However, activation of the final component was higher than that found at the initial level.

With Mg^{++}, activations were also preponderant at the level of final component (Fig. 20); but in this case they were evident in the whole range of divalent cation concentration.

Under optimal conditions, activation with both divalent cations was equivalent (about three-fold). Comparison of these results with those found with ATP indicates that in both cases activation decreased with increasing concentration of divalent cation.

GMP-P(NH)P. In the presence of Mn^{++}, inhibition of the initial kinetic component was only slightly reverted by increasing concentrations of divalent cation (Fig. 21). Under the same condition, activation of final velocities was relatively small.

With Mg^{++} (Fig. 22), no inhibition by GMP-P(NH)P is evident at a divalent cation concentration level equivalent to that of AMP-P(NH)P.

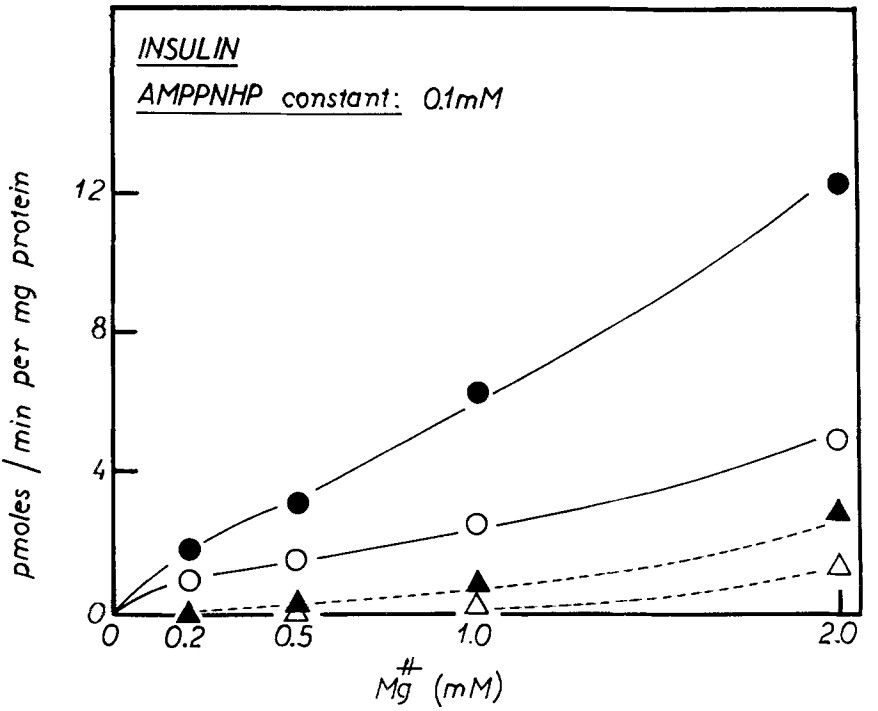
INSULIN
AMPPNHP constant: 0.1mM
pmoles / min per mg protein
12
8
4
0
0
0.2
0.5
1.0
2.0
Mg# (mM)

Fig. 16

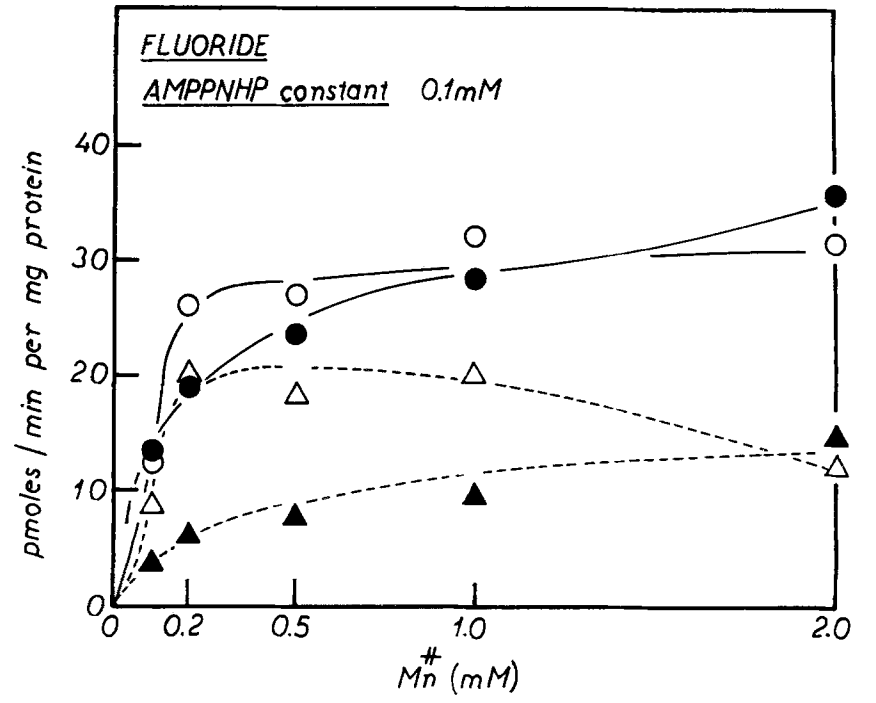
FLUORIDE
AMPPNHP constant 0.1mM
pmoles / min per mg protein
40
30
20
10
0
0
0.2
0.5
1.0
2.0
Mn# (mM)

Fig. 17

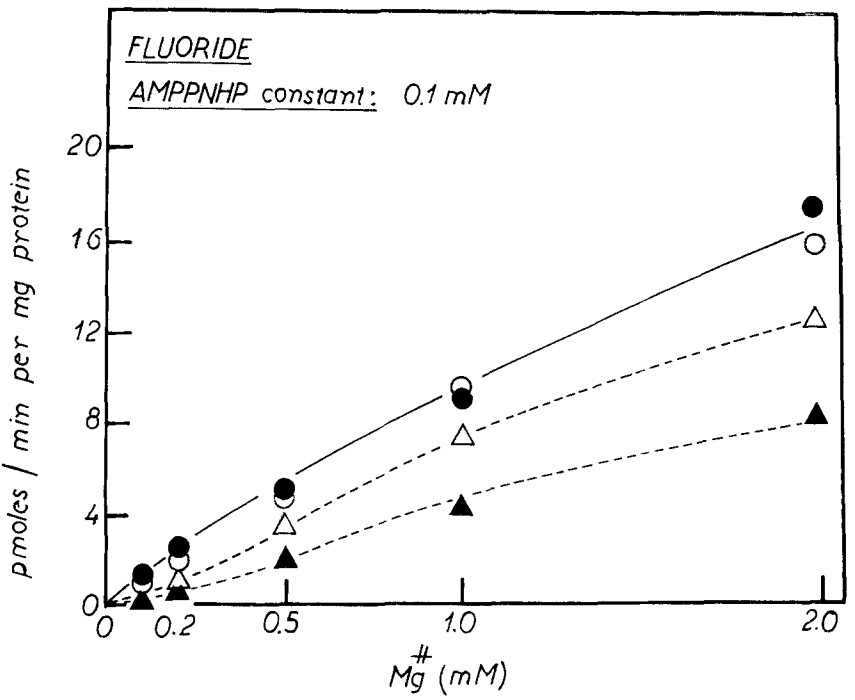
FLUORIDE
AMPPNHP constant: 0.1 mM
pmoles / min per mg protein
20
16
12
8
4
0
0
0.2
0.5
1.0
2.0
Mg# (mM)

Fig. 18

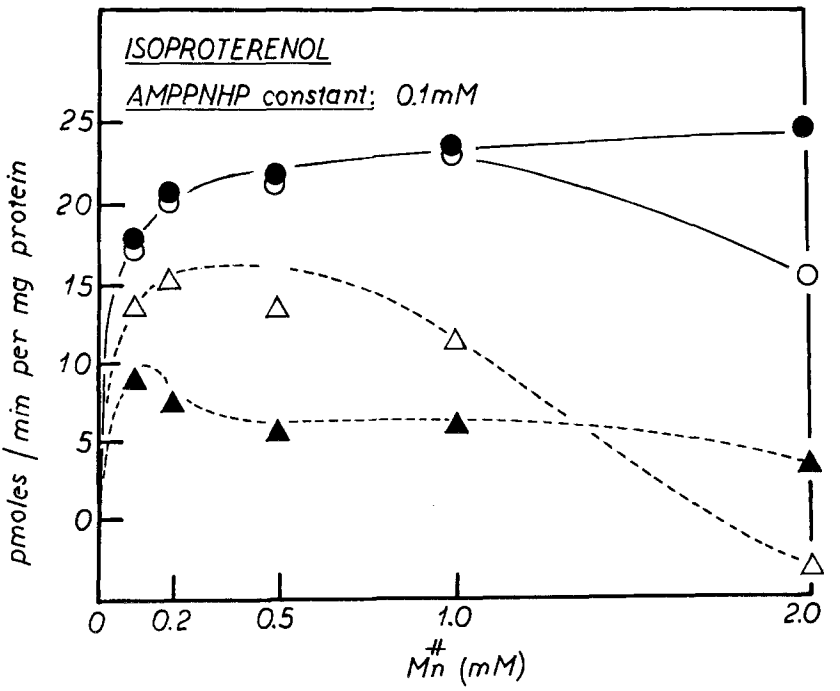
ISOPROTERENOL
AMPPNHP constant: 0.1mM
pmoles / min per mg protein
25
20
15
10
5
0
0
0.2
0.5
1.0
2.0
Mn# (mM)

Fig. 19

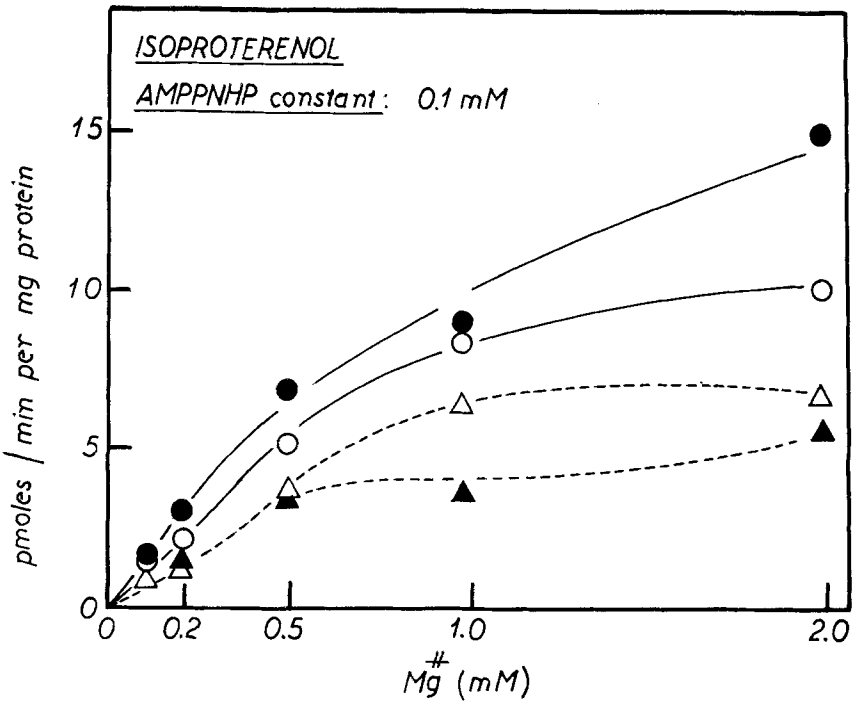
ISOPROTERENOL
AMPPNHP constant: 0.1 mM
pmoles / min per mg protein
15
10
5
0
0
0.2
0.5
1.0
2.0
Mg# (mM)

Fig. 20

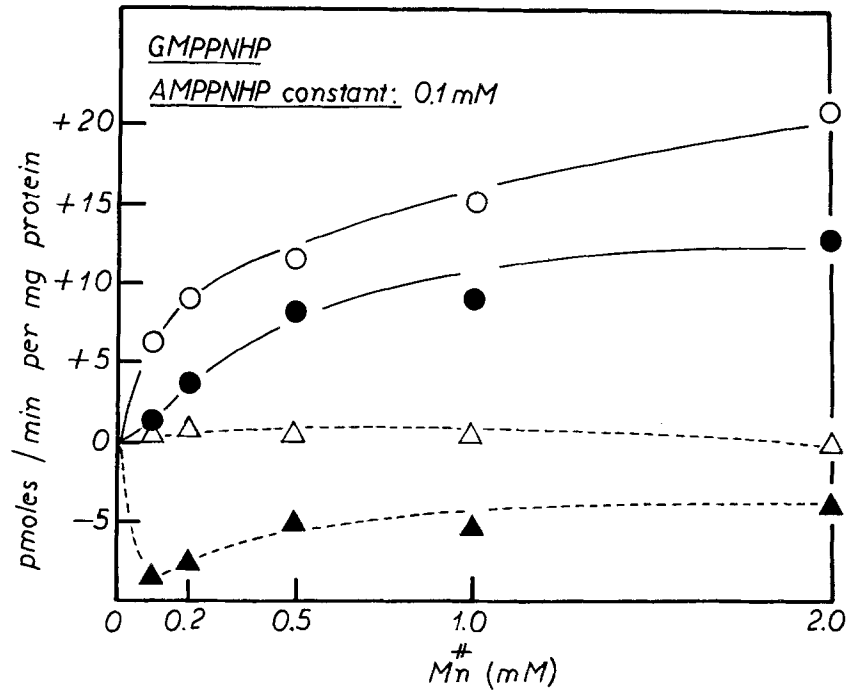
GMPPNHP
AMPPNHP constant: 0.1 mM
pmoles / min per mg protein
+20
+15
+10
+5
0
−5
0
0.2
0.5
1.0
2.0
Mn# (mM)

Fig. 21

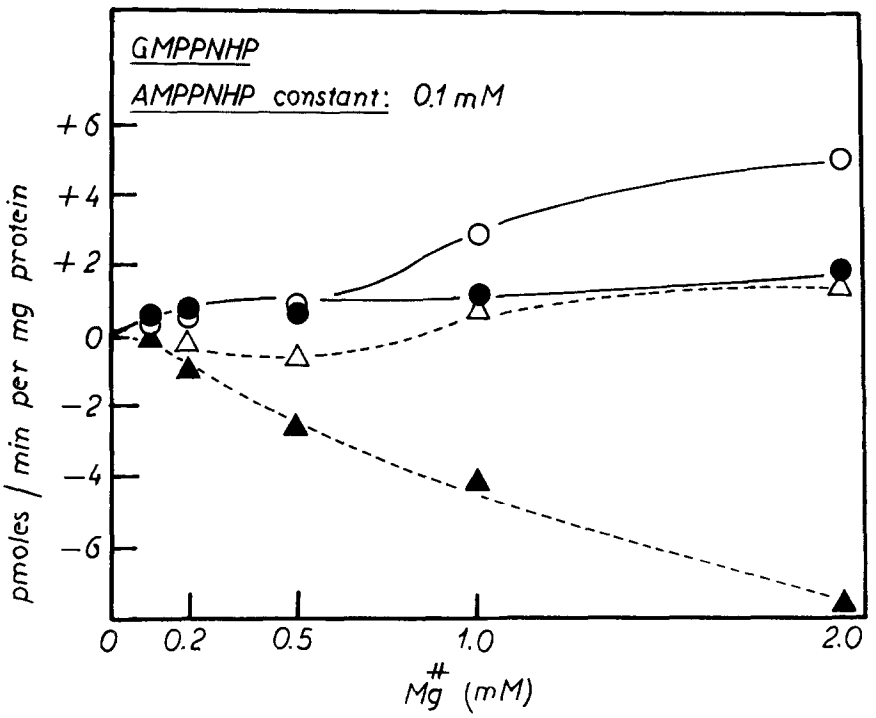

Fig. 22

At higher concentrations, the inhibition increased to reach a plateau of about 80% inhibition.

According to the results previously shown for ATP (Torres *et al.*, 1978*b*), under some conditions it was possible to demonstrate a substrate dependency for the inhibition elicited by GMP-P(NH)P. At the lowest Mg^{++} concentration tested, less than half of the total AMP-P(NH)P was used in forming the substrate complex Mg-AMP-P(NH)P^{2-} (4.2×10^{-2} mM). Such a value is well below the expected K_m for such a complex (Table 1). Thus, it is possible that the behavior of the initial component as a function of Mg^{++} might reflect such a dependency.

When the results are compared with those found for ATP, the activation observed in the final component with AMP-P(NH)P was less marked.

Kinetic Parameters

The influence of divalent cations or of substrate concentration on initial rates is shown in Fig. 23 and Table 1. For reasons previously

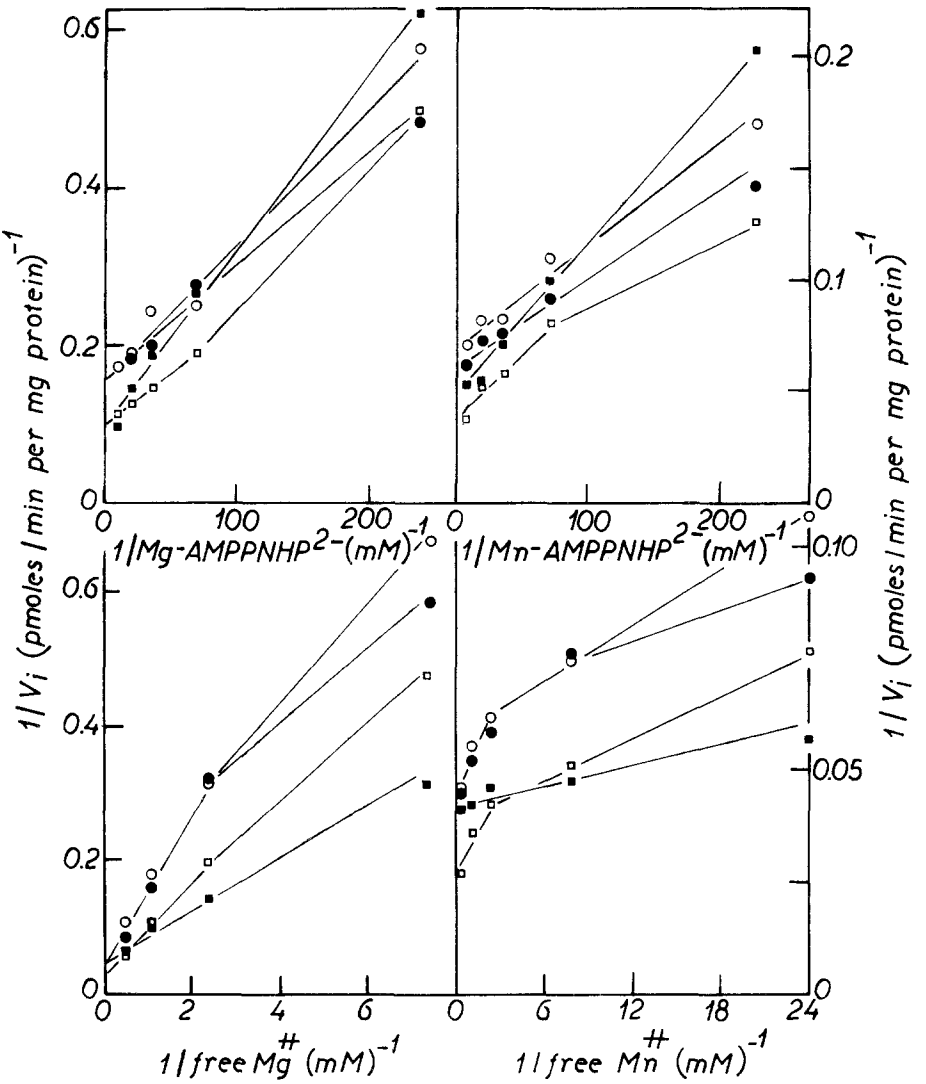

Fig. 23. Reciprocal plots of adenylyl cyclase initial rates measured against varying total divalent cations or total AMP-P(NH)P. Results were plotted as a function of reciprocal concentrations of free divalent cation and $[MeAMP\text{-}P(NH)P]^{2-}$, respectively. ○, no additions (basal activity); ● insulin; ■ isoproterenol; □ fluoride. Other conditions are the same as indicated in Table 1

discussed (Torres *et al.*, 1978*b*), in most cases the extent of the effects elicited by different modifiers was underestimated.

Although the data presented are not readily amenable to a detailed analysis, some facts are evident: (i) in some cases nonlinear Lineweaver-Burk plots were obtained which could indicate that reaction rates were

the expression of more than one enzyme form; (ii) fat cell adenylyl cyclase assayed with AMP-P(NH)P showed much less affinity for free Mg^{++} than for Mn^{++}; (iii) isoproterenol elicited a marked increase in the affinity for the divalent cation (free Mg^{++} or Mn^{++}), indicating that changes induced by the catecholamine analog were faster than those of other modifiers studied (i.e., insulin and fluoride); (iv) insulin did not affect any initial rates; (v) surprisingly, at saturating concentrations, free Mg^{++} and Mn^{++} gave similar initial velocities; (vi) apparent affinities for Mn AMP-P(NH)P^{2-} and Mg AMP-P(NH)P^{2-} were nearly equivalent.

Kinetic data obtained with AMP-P(NH)P differed from those found with ATP in the following respects: (i) with the analog, maximum velocities were one order of magnitude lower than those of ATP; (ii) apparent affinities for AMP-P(NH)P metal complexes (Mn^{++} or Mg^{++}) were higher than with ATP, particularly in the case of [Mn-AMP-P(NH)P]$^{2-}$; and (iii) affinities for free Mn^{++} were higher with AMP-P(NH)P than with ATP, and the opposite effect was found with Mg^{++}.

Discussion

One of the clearest kinetic differences between ATP and AMP-P(NH)P as substrates for fat cell adenylyl cyclase is in the initial maximal rates. Since apparent affinities for metal-nucleotide complexes were higher for AMP-P(NH)P than for ATP, it is possible that the nature of the bridge between β- and γ-phosphate might influence the rate of the cyclase reaction in the formation of the substrate-enzyme complex. Considering the different alternatives for the regulation of the adenylyl cyclase system, analysis of the reaction itself should precede other considerations. Although knowledge on this point is scarce, it may be accepted that the enzyme reaction cycle involves at least three steps, including formation of the substrate-enzyme complex and the sequential liberation of the two reaction products (cyclic AMP and pyrophosphate or imidodiphosphate). Each of these steps could be a limiting step when AMP-P(NH)P is used as a substrate for adenylyl cyclase.

Figure 24 summarizes the behavior of the cyclase system in terms of the transient changes of reaction rates that operate with ATP or AMP-P(NH)P. The following facts are of interest: (i) cyclase inactivation in the presence of Mg^{++} is not affected by the type of nucleoside triphosphate. In the presence of insulin and Mn^{++}, however, the conversion

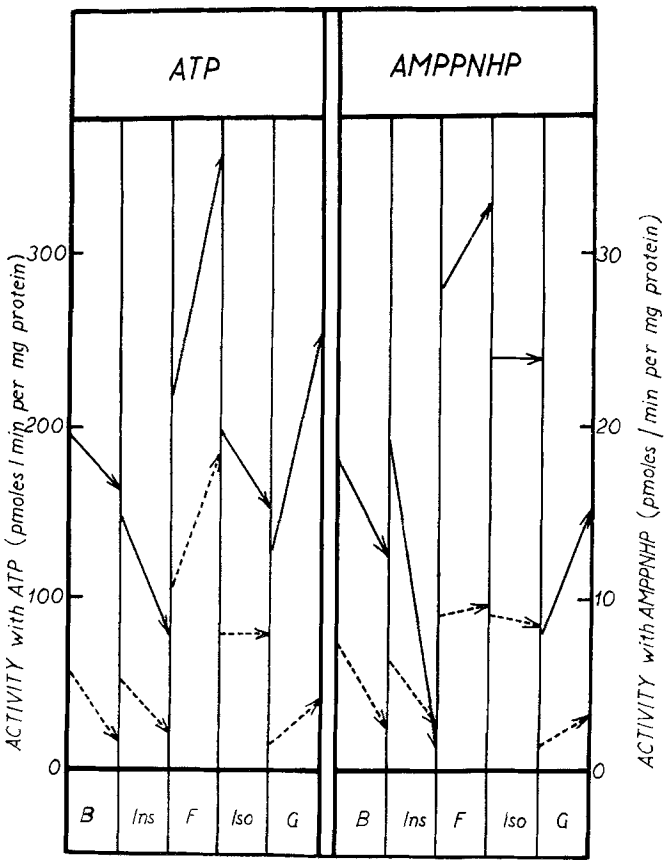

Fig. 24. Transient changes of reaction rates of adenylyl cyclase measured with ATP or AMP-P(NH)P. Each arrow represents the transition between initial and final rates (left and right extremes, respectively). Conditions correspond to assays containing 0.5 mM ATP and 1.5 mM divalent cation or 0.1 mM AMP-P(NH)P and 1 mM divalent cation. Full arrows, Mn^{++}-containing assays; broken arrows, Mg^{++}-containing assays. *B*, basal activity; *Ins*, insulin; *F*, fluoride; *Iso*, isoproterenol; and *G*, GMP-P(NH)P. Other conditions were as given in Figs. 5–22 in this paper and in the preceding one (Torres *et al.*, 1978*b*)

was higher with AMP-P(NH)P; (ii) at the level of the initial kinetic component, fluoride was not affected by the type of nucleoside triphosphate. On the other hand, stimulation of the final component was more evident with ATP. The extent of isoproterenol stimulation was slightly higher with AMP-P(NH)P; and (iv) inhibition of the initial kinetic component by GMP-P(NH)P was roughly the same with both substrates. Stimulation of the final component, however, was higher with ATP than with AMP-P(NH)P.

Data presented in this and the preceding papers (Torres *et al.*, 1978*a–b*) could be amenable to more rigorous analysis, but not until a better

study is made of the kinetic behavior of a basic cyclase system. In theory, the response of such a system to a variety of modifiers (isoproterenol, fluoride, GMP-P(NH)P, insulin, etc.) should approach the one described for the initial kinetic component. That is, in this system most of the time-dependent changes in activity should be minimal. Until further chemical characterization of the cyclase system occurs, interpretations of kinetic behavior will be of limited value.

References

Birnbaumer, L., Yang, P.C. 1974. Studies on receptor mediated activation of adenylyl cyclase. I. Preparation and description of general properties of an adenylyl cyclase system in beef renal medullary membranes sensitive to neurohypophyseal hormones. *J. Biol. Chem.* **249**:7848

Birnbaumer, L., Yang, P.C., Hunzicker-Dunn, M., Bockaert, J., Duran, J.M. 1976. Adenylyl cyclase activities in ovarian tissues. I. Homogenization and conditions of assay in graafian follicles and corpora lutea of rabbits, rats and pigs; Regulation by ATP and some comparative properties. *Endocrinology* **99**:163

Bockaert, J., Hunzicker-Dunn, M., Birnbaumer, L. 1976. Hormone-stimulated desensitization of hormone-dependent adenylyl cyclase. *J. Biol. Chem.* **251**:2653

Constantopoulos, A., Najjar, V.A. 1973. The activation of adenylate cyclase. II. The postulated presence of (A) adenylate cyclase in a phospho (inhibited) form, (B) a dephospho (activated) form with a cyclic adenylate stimulated membrane protein kinase. *Biochem. Biophys. Res. Commun.* **53**:794

Helmreich, E.J.M., Zenner, H.P., Pfeuffer, T., Cori, C.F. 1976. Signal transfer from hormone receptor to adenylate cyclase. *In:* Current Topics in Cellular Regulation. B.L. Horecker and E.R. Stadtman, editors. Vol. 10, pp. 41–87. Academic Press, New York

Hunzicker-Dunn, M., Birnbaumer, L. 1976*a*. Adenylyl cyclase activities in ovarian tissues. II. Regulation of responsiveness to LH, FSH, and PGE_1 in the rabbit. *Endocrinology* **99**:185

Hunzicker-Dunn, M., Birnbaumer, L. 1976*b*. Adenylyl cyclase activities in ovarian tissues. III. Regulation of responsiveness to LH, FSH and PGE_1 in the prepuberal, pregnant and pseudopregnant rat. *Endocrinology* **99**:198

Hunzicker-Dunn, M., Birnbaumer, L. 1976*c*. Adenylyl cyclase activities in ovarian tissues. IV. Gonadotrophin induced desensitization of the luteal adenylyl cyclase throughout pregnancy and pseudopregnancy in the rabbit and the rat. *Endocrinology* **99**:211

Johnson, R.A. 1977. Hydrolysis of 5′ adenylylimidodiphosphate [AMP-P(NH)P]; Implication for the study of adenylate cyclase (AC). *Fed. Proc.* **36**:736

Khan, J., Martell, A.E. 1966. Thermodynamic quantities associated with the interaction of adenosine triphosphate with metal ions. *J. Am. Chem. Soc.* **88**:668

Manganiello, V.C., Vaughan, M. 1976. Activation and inhibition of fat cell adenylate cyclase by fluoride. *J. Biol. Chem.* **251**:6205

Najjar, V.A., Constantopoulos, A. 1973. The activation of adenylate cyclase. I. A postulated mechanism for fluoride and hormone activation of adenylate cyclase. *Mol. Cel. Biochem.* **2**:87

Perkins, J.P., Moore, M.M. 1971. Activation by sodium, fluoride and detergents. *J. Biol. Chem.* **246**:62

Rodbell, M., Birnbaumer, L., Pohl, S.L., Krans, H.N.L. 1971. Cyclase system in plasma membrane of rat liver. V. An obligatory role of guanyl nucleotides in glucagon activation. *J. Biol. Chem.* **246**:1877

Rodbell, M., Lin, M.C., Salomon, Y. 1974. Evidence for interdependent action of glucagon and nucleotides on the hepatic adenylate cyclase system. *J. Biol. Chem.* **249**:59

Salomon, Y., Lin, M.C., Lodos, C., Rendell, M., Rodbell, M. 1975. The hepatic adenylate cyclase system. I. Evidence for transition states and structural requirements for guanine nucleotide activation. *J. Biol. Chem.* **250**:4239

Torres, H.N., Flawiá, M.M., Hernaez, L., Cuatrecasas, P. 1978*a*. Effects of insulin on the adenylyl cyclase activity of isolated fat cell membranes. *J. Membrane Biol.* **43**:1

Torres, H.N., Flawiá, M.M., Medrano, J.A., Cuatrecasas, P. 1978*b*. Kinetic studies of adenylyl cyclase of fat cell membranes. I. Comparison of activities measured in the presence of Mg^{++}-ATP and Mn^{++}-ATP. Effects of insulin, GMP-P(NH)P, isoproterenol, and fluoride. *J. Membrane Biol.* **43**:19

Yount, R.G., Babcock, D., Ballantyne, W., Ojala, D. 1971*a*. Adenylyl imidodiphosphate, an adenosine triphosphate analog containing a P-N-P linkage. *Biochemistry* **10**:2484

Yount, R.G., Ojala, D., Babcock, D. 1971*b*. Interaction of P-N-P and P-C-P analogs of adenosine triphosphate with heavy meromyosin, myosin and actomyosin. *Biochemistry* **10**:2490