J. Membrane Biol. 43, 19–44 (1978)

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

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Received 30 January 1978

Summary. The kinetic behavior of the adenylyl cyclase activity associated with fat cell membranes purified by centrifugation on sucrose gradients was studied. Under most of the conditions explored, with either Mn^{++} or Mg^{++} as the divalent cation in the assay mixtures, the time courses of the reaction were not linear. In the absence of modifiers (i.e., basal activity) or in the presence of insulin, the rate tended to decrease with time; on the other hand, with fluoride or GMP-P(NH)P the curves were concave upwards. To simplify analysis of the results, two kinetic components were defined: an "initial component" corresponding to the transient rate measured between zero time and 1.5 min of assay and a "final component" corresponding to the transient rate determined between 3 and 5 min.

Over the entire range of Mn^{++} concentration explored (0.5 to 6.0 mM), the basal initial rates were slightly higher than the final ones. With Mg^{++} in the range between 1.5 and 2.5 mM, the final rates were fourfold lower than the initial ones. Higher or lower Mg^{++} concentrations gave velocity ratios equivalent to those observed with Mn^{++}.

Insulin clearly decreased the final rates at Mn^{++} concentrations up to 2.5 mM. With higher concentrations the effects were completely reversed. The effects of insulin on initial rates measured with Mn^{++}, or the initial or final rates measured with Mg^{++}, were less evident.

Stimulation of adenylyl cyclase activity by fluoride was most pronounced on the final rates. In addition, this stimulation was higher with Mg^{++} than with Mn^{++}.

Isoproterenol stimulation of adenylyl cyclase was negligible in the presence of Mn^{++} (0.5 to 6.0 mM). With Mg^{++} (0.5 to 6.0 mM), stimulation was more evident on the final rates.

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0022-2631/78/0043-0045 $05.00

GMP-P(NH)P inhibited the initial but activated the final components of the reaction. The extent of this inhibition or activation was more pronounced with Mg^{++} than with Mn^{++}.

Under conditions which lead to maximal inactivation of the final component, adenylyl cyclase activity was tenfold or more higher with Mn^{++} than with Mg^{++}. Similar effects were observed with GMP-P(NH)P on the initial component. However, insulin, isoproterenol and fluoride decreased the Mn^{++} dependence of the final component. With fluoride, the final rates measured with Mg^{++} were almost equivalent to those found in assays containing Mn^{++}.

Under the conditions used for measurements of adenylyl cyclase activity, the enzyme system slowly interconverts between active and inactive forms.

Evidence presented in the preceding paper (Torres *et al.* 1978) indicates that insulin is able to decrease adenylyl cyclase activity associated with membrane preparations from rat fat cells. The hormone leads to a time-dependent inactivation of this enzyme activity in assay mixtures containing Mn^{++} but not Mg^{++} (Torres *et al.*, 1978).

Detailed kinetic studies on mammalian adenylyl cyclases assayed in the presence of Mn^{++} are not available in the literature. Besides the demonstration that Mg^{++} can be replaced by Mn^{++} in the reaction catalyzed by the brain enzyme (Sutherland, Rall & Menon, 1962), there is a report showing that activation of liver adenylyl cyclase by fluoride or glucagon is somewhat different from that observed with Mg^{++} (Birnbaumer, Pohl & Rodbell 1971).

Some eucaryotic adenylyl cyclases have been shown to specifically require Mn^{++} for activity. One of these is a membrane-bound enzyme found in the ascomycete fungus, *Neurospora crassa* (Flawiá & Torres, 1972*a*). The other is a soluble form identified in rat testis (Braun & Richard, 1975). Both of these enzymes share the unique property of being insensitive to fluoride.

This paper principally reports the kinetic parameters of the insulin effect on adenylyl cyclase activity. In addition, the kinetic behavior of this enzyme in the presence of Mg^{++} or Mn^{++} is analyzed in some detail.

Materials and Methods

Unless otherwise indicated, details of the experimental conditions were as described in the preceding paper (Torres *et al.*, 1978). All of the kinetic studies were done on fat cell membranes purified by centrifugation on sucrose gradients.

Standard components of adenylyl cyclase mixture were: 50 mM Tris-HCl buffer, pH 7.5; 0.2 mM 3-isobutyl-l-methyl xanthine; 1 mM cyclic AMP, 2 mM phosphocreatine, 0.2 mg

per ml creatine kinase; 0.1 mg per ml albumin and membrane (10 to 20 µg of total protein). Concentrations of α-^{32}P-labeled ATP (about 2×10^7 cpm per assay), divalent cation (Mg^{++} or Mn^{++}) and other additions were as indicated in the legends to the figures. Incubations were done at 37° for 1, 3 and 5 min. The total volume was 0.1 ml.

Concentrations of free Mn^{++}, free Mg^{++}, $MnATP^{2-}$ and $MgATP^{2-}$ in the incubation mixtures were calculated according to expressions of the type:

$$K_4 X^3 + [K_4(p-q)+(K_4 r+1)]\, X^2 - \left[\frac{K_4}{K_2}(1+K_3 h)\, c-(p-q)\, X - \frac{1}{K_2}(1+K_3 H)\, c = 0\right.$$

with:

$X = [\text{free } Me^{++}]$ and Me^{++}: Mn^{++} or Mg^{++}

$p = [{}^{n}ATP] = [MeHATP^{1-}] + [HATP^{3-}] + [MeATP^{2-}] + [ATP^{4-}]$

$q = [Me^{++}] = [MeHATP^{1-}] + [MeATP^{2-}] + [MeCP^{1-}] + [\text{free } Me^{++}]$

$r = [\text{creatine phosphate}] = [MeCP^{1-}] + [CP^{3-}]$

$H = [H^+]$

$c = [MeATP^{2-}]$

and

$$K_1 = \frac{[MeHATP^{1-}]}{[HATP^{3-}]\,[\text{free } Me^{++}]}$$

$$K_2 = \frac{[MeATP^{2-}]}{[ATP^{4-}]\,[\text{free } Me^{++}]}$$

$$K_3 = \frac{[HATP^{3-}]}{[ATP^{4-}]\,[H^+]}$$

$$K_4 = \frac{[MeCP^{1-}]}{[CP^{3-}]\,[\text{free } Me^{++}]}.$$

The values assigned to the association constants were determined according to Khan and Martell (1966) as follows:

$$K_{1(Mn)} = 0.19953 \text{ mM}^{-1}$$

$$K_{1(Mg)} = 0.19498 \text{ mM}^{-1}$$

$$K_{2(Mn)} = 42.66 \text{ mM}^{-1}$$

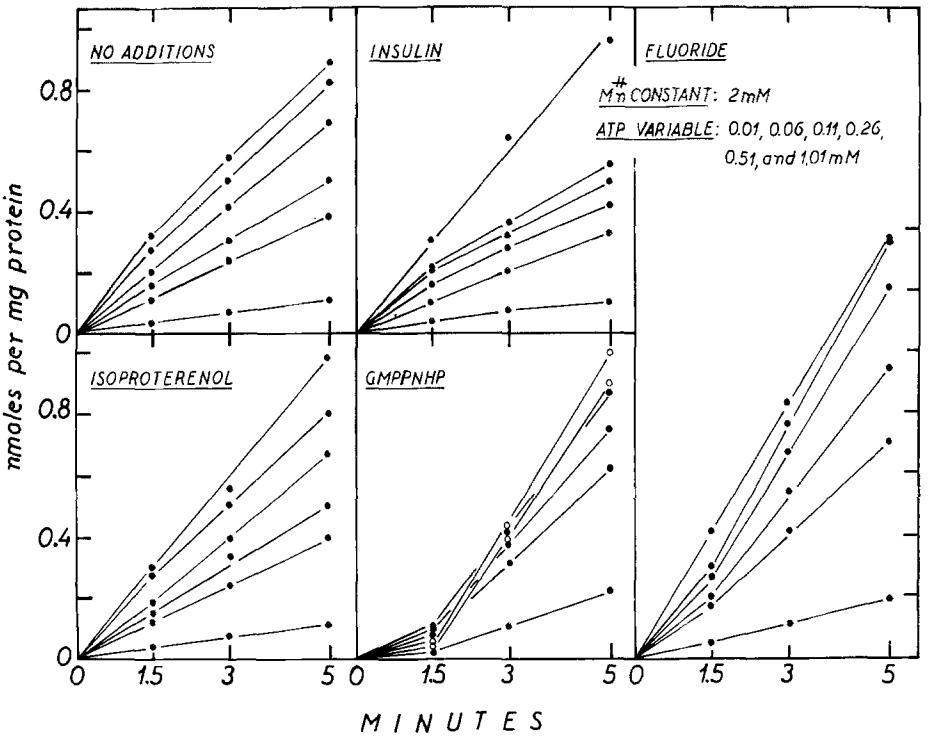
$$K_{2(Mg)} = 19.055 \text{ mM}^{-1}$$

$$K_3 = 3311.3 \text{ mM}^{-1}.$$

The association constants for the metal-creatinephosphate complex were taken from Smith and Alberty (1956). The assigned values were as follows:

$$K_{4(Mn)} = 0.11 \text{ mM}^{-1}$$

$$K_{4(Mg)} = 0.02 \text{ mM}^{-1}.$$



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

Results

Kinetic analysis of the adenylyl cyclase reaction catalyzed by fat cell membranes was complicated by the fact that under most conditions used the time course was not linear. For example, with Mg^{++} or Mn^{++} and in the absence of modifiers (i.e., basal activity), or in the presence of insulin, the rate tended to decrease with time (Torres *et al.*, 1978). With fluoride or GMP-P(NH)P, however, the curves were generally concave upwards (Figs. 1–4). These basic properties, which have been observed previously under a variety of conditions (Rodbell, 1975) might indicate that this enzyme is able to interconvert between active and inactive (or less active) forms. Reaction rates leading to such conversions appear to be sufficiently slow to influence the shape of time curves of adenylyl cyclase catalyzed reactions.

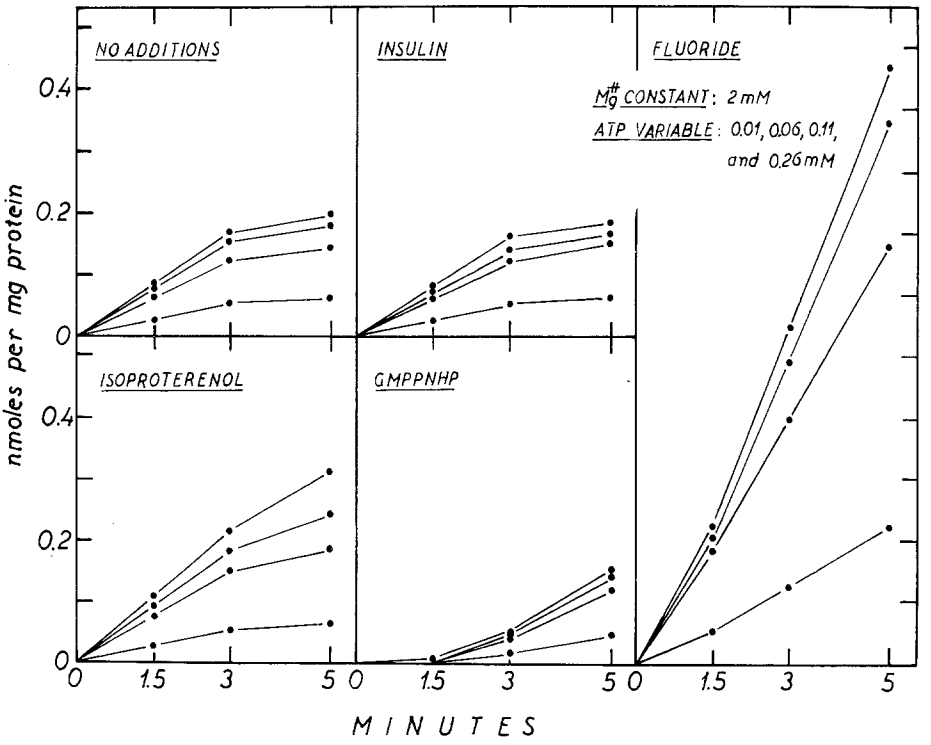

NO ADDITIONS
INSULIN
FLUORIDE
Mg# CONSTANT: 2 mM
ATP VARIABLE: 0.01, 0.06, 0.11, and 0.26 mM
ISOPROTERENOL
GMPPNHP
nmoles per mg protein
0.4
0.2
0
0.4
0.2
0
0
1.5
3
5
MINUTES

Fig. 2

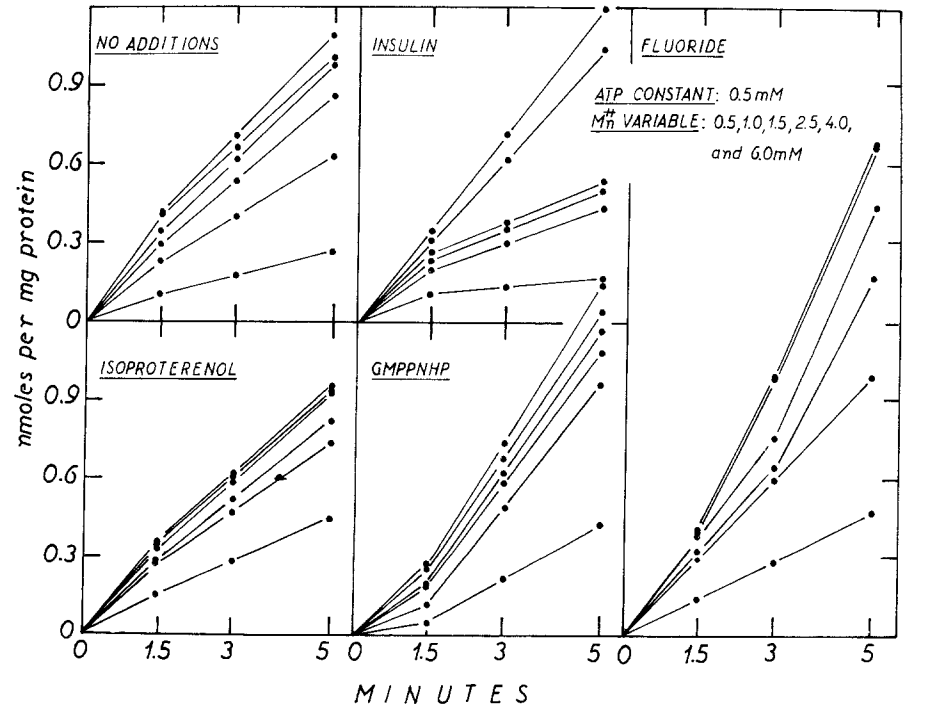

NO ADDITIONS
INSULIN
FLUORIDE
ATP CONSTANT: 0.5 mM
Mn# VARIABLE: 0.5, 1.0, 1.5, 2.5, 4.0, and 6.0 mM
ISOPROTERENOL
GMPPNHP
nmoles per mg protein
0.9
0.6
0.3
0
0.9
0.6
0.3
0
0
1.5
3
5
MINUTES

Fig. 3

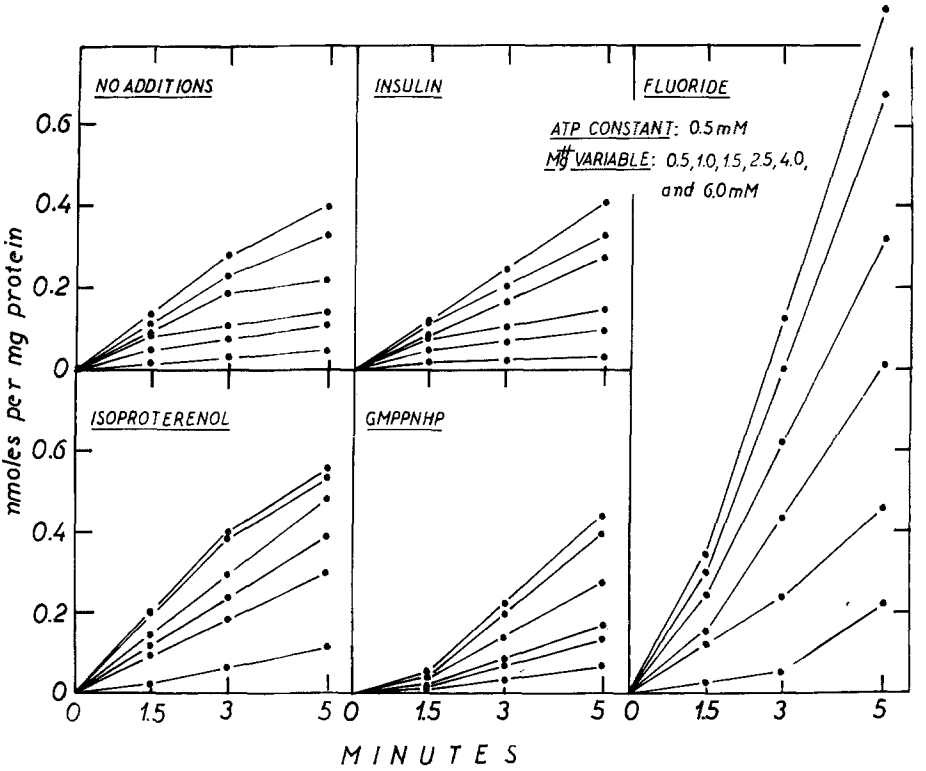


Fig. 4

In order to analyze such curves two different kinetic components were defined arbitrarily on the basis of careful examination of much data. The first one, termed "initial", might indicate the influence of factors in rapid equilibrium with the enzyme system. The second, termed "final", could reflect the effects of factors affecting the relatively slow interconversions of the adenylyl cyclase system. Consequently, "initial" and "final" rates will correspond to the transient rates measured between the origin and 1.5 min, and between 3 and 5 min, respectively.

The absolute magnitude of the influence of the different factors studied is expressed as the difference between rates (initial or final) measured in the presence and absence of that factor. Thus, a positive value indicates activation and a negative one inhibition. In addition, the ratio between the initial and final rates defines the shape of the time course.

Influence of Variable Concentrations of ATP

Basal activity. Figure 5 shows plots of enzyme activity measured at a fixed concentration (2 mM) of Mn^{++} or Mg^{++} as a function of

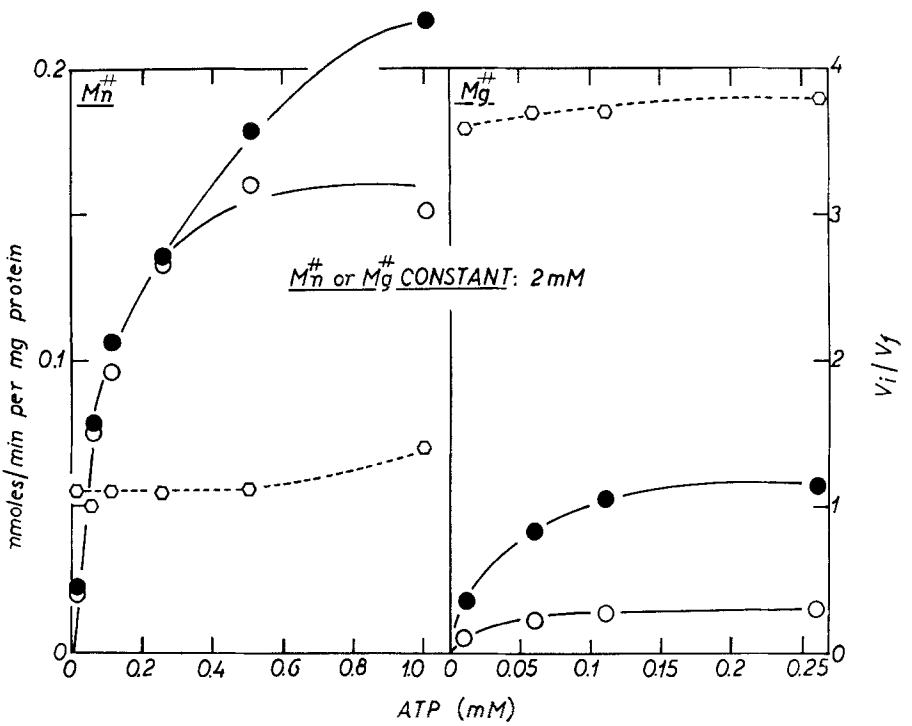
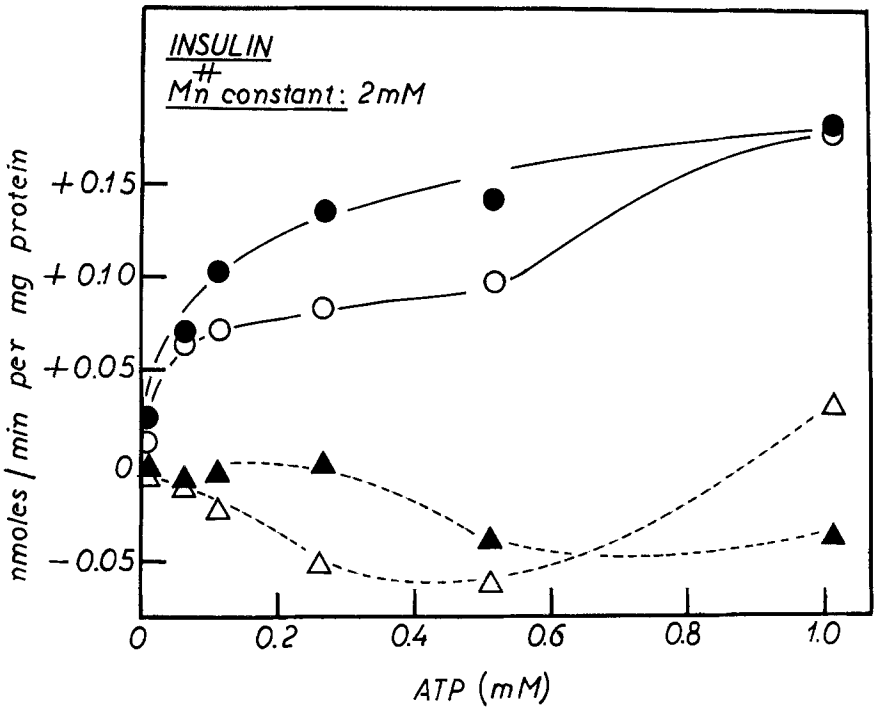


Fig. 5. Basal adenylyl cyclase activity measured in the presence of 2 mM Mn^{++} or Mg^{++} as a function of total ATP concentration. ●, initial rates; ○, final rates; ○, ratio between initial and final rates (V_i/V_f). Other conditions were as in Figs. 1–4

varying ATP concentration. In the presence of Mn^{++}, initial and final rates were nearly equivalent in a range between 0.01 and 0.26 mM ATP; at higher concentrations, final rates were slightly lower than the initial ones. On the contrary, in the presence of Mg^{++} the final rates were uniformly much lower than the initial ones, without any significant change of the corresponding V_i/V_f ratio between them. These findings might indicate that under these assay conditions adenylyl cyclase inactivation is relatively independent of ATP concentration but may be significantly affected by the type of divalent cation used. Inactivation may be strikingly enhanced by Mg^{++}.

In addition, it is evident that throughout the whole range of ATP concentration explored, activities measured with Mn^{++} were substantially higher than with Mg^{++}.

Insulin. As shown in Fig. 6, in the presence of 2 mM Mn^{++} the effects of insulin were strikingly influenced by the concentration of ATP. Maxi-



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 (2 mM Mn^{++} or Mg^{++}) and varying the total ATP concentration. ●, 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 were as indicated in Figs. 1 to 5

mal inhibition was observed on the final rates in the range between 0.26 and 0.5 mM ATP, and the effect was completely abolished at 1.0 mM ATP. On the other hand, the effect of insulin on the initial rates was much less evident over the entire ATP concentration range.

As occurs with basal activity, the final rates in the presence of Mg^{++} were clearly below the initial ones, and both rates were at least three-times lower than with Mn^{++} (Fig. 7). In addition, the inhibition of activity was less marked throughout the range of ATP concentration examined (i.e., 0.01 to 0.26 mM ATP). It is important that the data corresponding to the highest ATP concentrations are not included in the figures because of the small differences obtained between incubated and control (blank) samples. However, the data indicated that in the presence of Mg^{++}

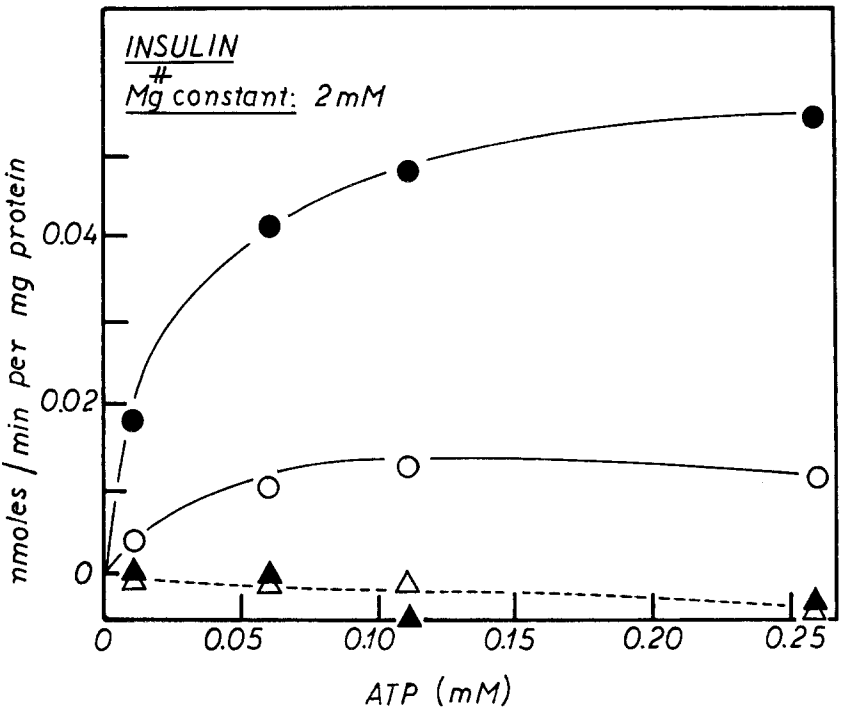


Fig. 7

the insulin effects at these high ATP concentrations were also much less evident than with Mn^{++}.

These experiments suggest that inactivation of adenylyl cyclase in the presence of insulin is influenced by ATP concentrations and the type of divalent cation used in the assay. It seemed that a negative correlation existed between the ability of the divalent cation to affect inactivation of the cyclase system and the existence of a clear insulin effect.

Fluoride. With fluoride the rates measured with Mg^{++} were only slightly lower than those found with Mn^{++} (Figs. 8 and 9). However, it is evident that fluoride caused very large increases of the final velocities. In fact, the latter were higher than the corresponding initial velocities irrespective of the ATP concentration or type of divalent cation used.

The extent of the activation by fluoride was relatively insensitive to changes in the ATP concentration and much more evident with Mg^{++} than with Mn^{++}. In this case a 12-fold stimulation of the final velocity was found with Mg^{++} and only a onefold stimulation with Mn^{++}.

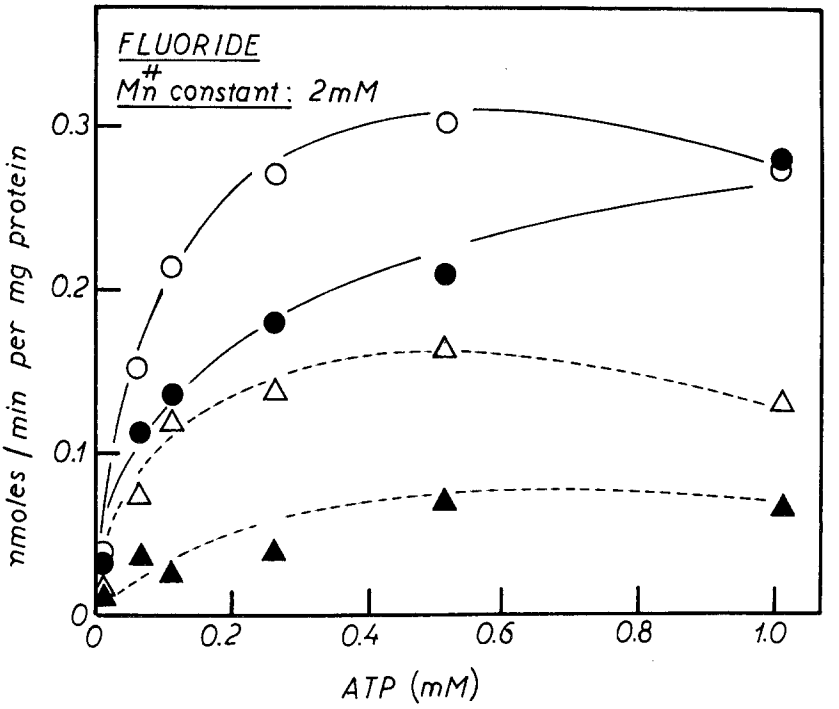

FLUORIDE
Mn constant: 2mM
nmoles / min per mg protein
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1.0
ATP (mM)

Fig. 8

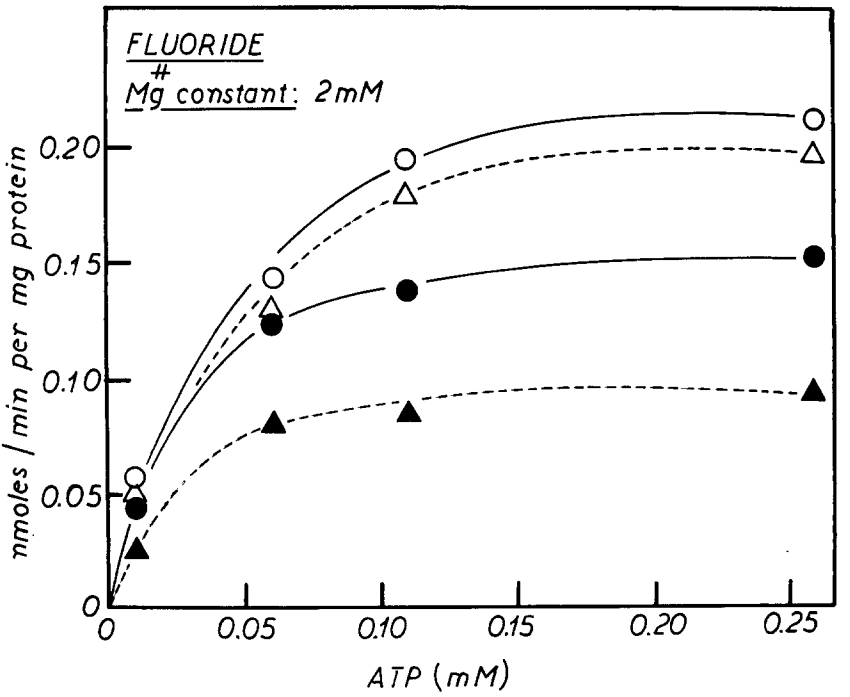

FLUORIDE
Mg constant: 2mM
nmoles / min per mg protein
0.20
0.15
0.10
0.05
0
0
0.05
0.10
0.15
0.20
0.25
ATP (mM)

Fig. 9

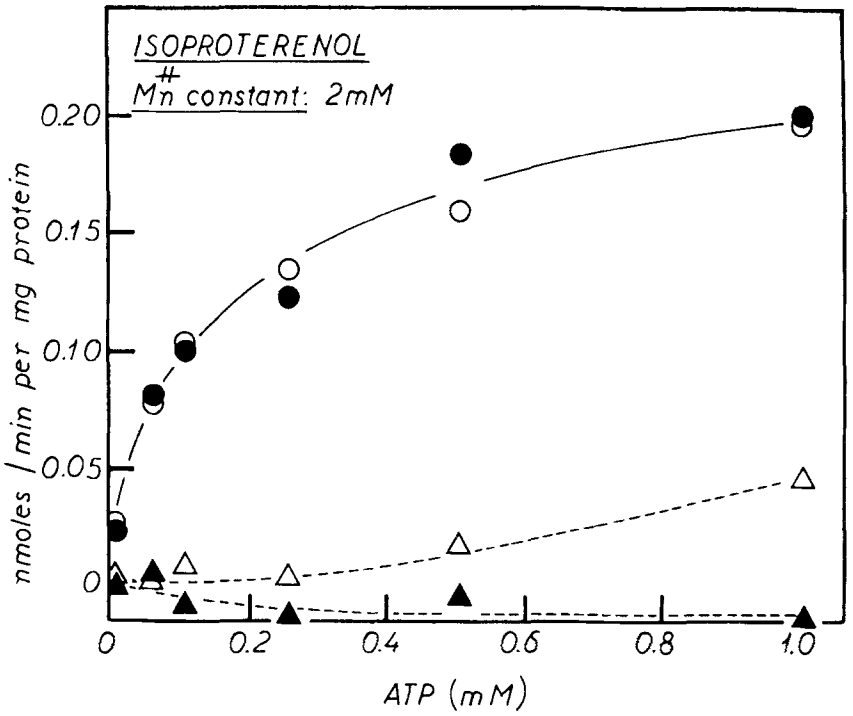


Fig. 10

This suggests that fluoride may have counteracted the inactivation observed with Mg^{++}, and that it may have converted the cyclase to a form having activity equivalent with Mg^{++} and Mn^{++}.

Isoproterenol. In the presence of Mn^{++} the magnitude of the isoproterenol effect was relatively small (Fig. 10). There was a modest increase in the final velocity only at the highest ATP concentrations tested. The time courses were linear in the whole range of substrate concentration.

With Mg^{++} the effects of isoproterenol were exerted at the level of the final velocity (Fig. 11). The hormonal activation was clearly dependent on the ATP concentration, increasing with increasing substrate concentration. Isoproterenol may thus in some way block inactivation of cyclase at the highest ATP concentration tested. Since this inactivation is much more pronounced with Mg^{++}, only with this cation is the isoproterenol effect evident.

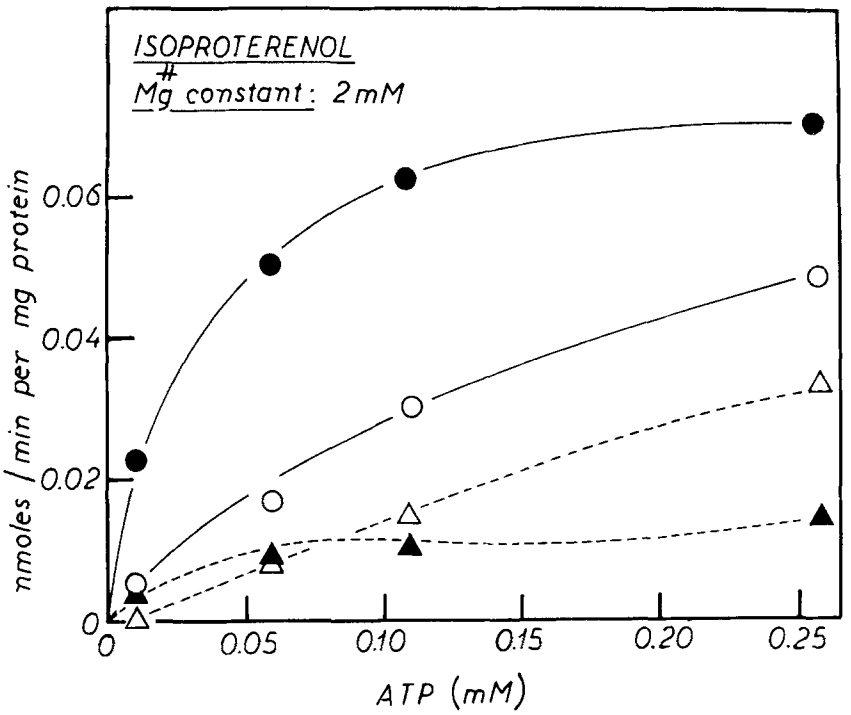


Fig. 11

GMP-P(NH)P. This guanylyl analog resulted in a clear inhibition of initial rates of adenylyl cyclase reaction either in the presence of Mn^{++} or Mg^{++} (Figs. 12 and 13).

In addition, this compound was also an activator at the level of the final velocities. Consequently, the final velocities were much higher than the initial ones through the whole range of substrate concentration examined.

The inhibitory effect of GMP-P(NH)P was complete in the presence of Mg^{++} over the whole range of ATP concentration, but in the presence of Mn^{++} such inhibition clearly increased with increasing concentrations of ATP. This could be taken as an example of substrate-promoted inhibition. It is possible that this type of inhibition is also operative with Mg^{++}, but in this case it may not be detected since the K_m for $MgATP^{2-}$ is more than two times lower than that for Mn^{++}-ATP (Fig. 23 and Table 1).

The relative extent of GMP-P(NH)P activation of the final rates was nearly insensitive to changes in ATP concentration and was two to three times higher with Mg^{++} than with Mn^{++}. In this regard, the

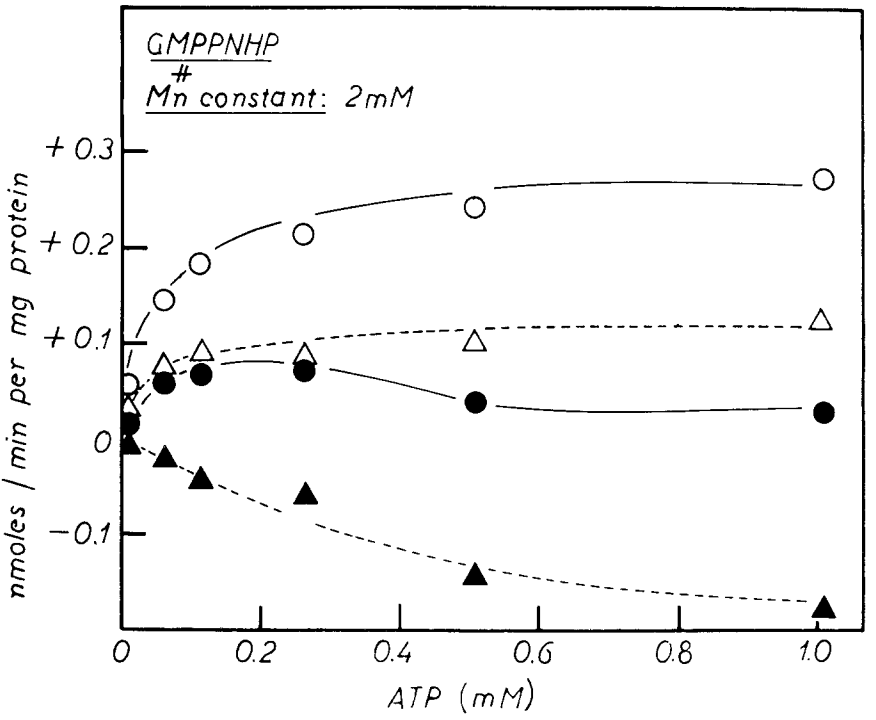

GMPPNHP
Mn constant: 2mM
+0.3
+0.2
+0.1
0
−0.1
nmoles / min per mg protein
0
0.2
0.4
0.6
0.8
1.0
ATP (mM)

Fig. 12

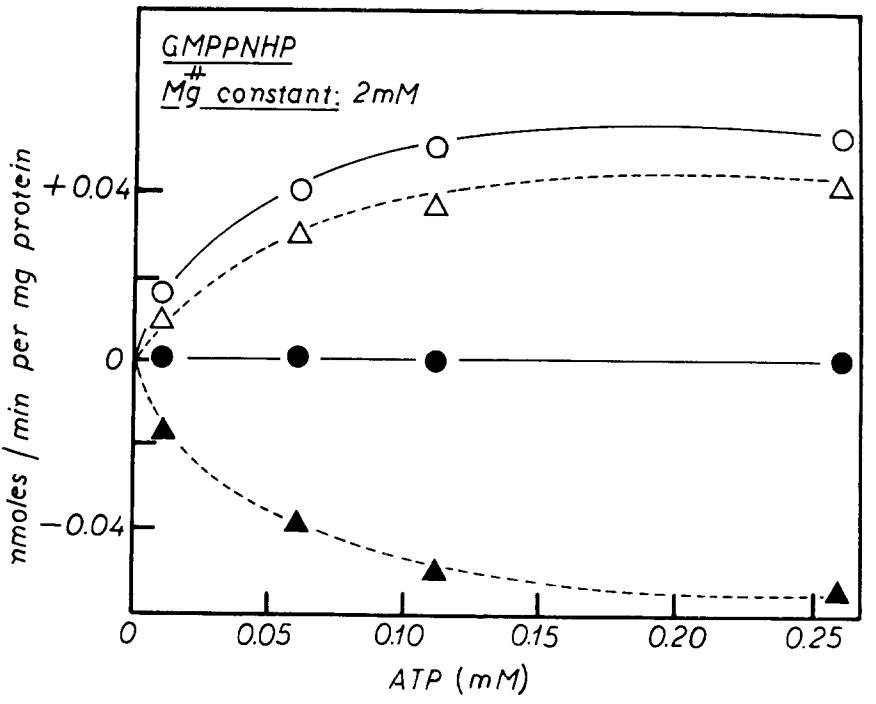

GMPPNHP
Mg constant: 2mM
+0.04
0
−0.04
nmoles / min per mg protein
0
0.05
0.10
0.15
0.20
0.25
ATP (mM)

Fig. 13

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

Ligand	Condition	$K_{m_{app}}$ (mM)	Vmx_i (nmoles/min/mg prot.)	Total ligand (mM)	
				Constant	Variable
Mn^{++}	Basal	0.410	0.300	ATP: 0.5	Mn^{++}: 0.5 to 6.0
	Insulin	0.460	0.230		
	Isoproterenol	0.150	0.240		
	Fluoride	0.320	0.280		
Mg^{++}	Basal	0.910	0.086	ATP: 0.5	Mg^{++}: 0.5 to 6.0
	Insulin	1.000	0.086		
	Isoproterenol	0.800	0.143		
	Fluoride	1.330	0.250		
Mn^{++}-ATP	Basal	0.123	0.230	Mn^{++}:2.0	ATP: 0.06 to 1.01
	Insulin	0.114	0.200		
	Isoproterenol	0.114	0.200		
	Fluoride	0.080	0.230		
Mg^{++}-ATP	Basal	0.024	0.061	Mg^{++}:2.0	ATP: 0.01 to 0.26
	Insulin	0.024	0.061		
	Isoproterenol	0.035	0.083		
	Fluoride	0.028	0.173		

[a] Initial velocities were plotted as a function of free Me^{++} or $MeATP^{2-}$. Other conditions were given under *Materials and Methods* and in Fig. 23.

fluoride and the guanyl analog effects showed qualitatively similar properties.

Influence of Variable Concentrations of Divalent Cation

Basal activity. Figure 14 shows plots of adenylyl cyclase activity as a function of divalent cation (Mn^{++} or Mg^{++}) concentration. In this experiment the ATP concentration was fixed at 0.5 mM. With Mn^{++} and in the presence of relatively high substrate concentrations (*see* for comparison Fig. 5), the initial rates were slightly higher than the final ones. In addition, the ratio between initial and final velocities was insensitive to the divalent cation concentration.

With Mg^{++}, the results were strikingly different. In the range between 1.5 and 2.5 mM Mg^{++} there is a striking preponderance of the initial over the final velocities. Higher or lower Mg^{++} concentrations gave velocity ratios equivalent to those observed with Mn^{++}. It appears that in the range of 1.5 to 2.5 mM Mg^{++} concentration, enzyme inactivation

branes and the concentration of Me^{++} and ATP species in the assay mixtures[a]

Concentration range of ATP CR Me^{++} species in the interval of variable ligand (mM)						
Free Me^{++}	$MeHATP^{1-}$	$HATP^{3-}$	$MeATP^{2-}$	ATP^{4-}	$MeCP^{1-}$	CP^{3-}
9.1921×10^{-2} to 4.8106	2.3913×10^{-4} to 3.642×10^{-4}	1.3038×10^{-2} to 3.1924×10^{-4}	3.8782×10^{-1} to 4.9695×10^{-1}	9.8904×10^{-2} to 2.4217×10^{-3}	2.0020×10^{-2} to 6.9210×10^{-1}	1.9800 to 1.3079
0.1418 to 5.3134	1.7492×10^{-4} to 6.6619×10^{-4}	1.7176×10^{-2} to 6.4302×10^{-4}	3.5206×10^{-1} to 4.9385×10^{-1}	1.3029×10^{-1} to 4.8778×10^{-3}	5.6564×10^{-3} to 1.9212×10^{-1}	1.9943 to 1.8079
1.6359 to 0.8496	3.6383×10^{-5} to 6.0354×10^{-4}	1.1146×10^{-4} to 3.5602×10^{-3}	5.9006×10^{-2} to 9.7883×10^{-1}	8.4554×10^{-4} to 2.7007×10^{-2}	3.0502×10^{-1} to 1.7094×10^{-1}	1.6950 to 1.8291
1.9165 to 1.6837	1.3058×10^{-5} to 3.3833×10^{-4}	3.4943×10^{-5} to 1.0306×10^{-3}	9.6798×10^{-3} to 2.5081×10^{-1}	2.6507×10^{-4} to 7.8177×10^{-3}	7.3829×10^{-2} to 6.5154×10^{-2}	1.9662 to 1.9348

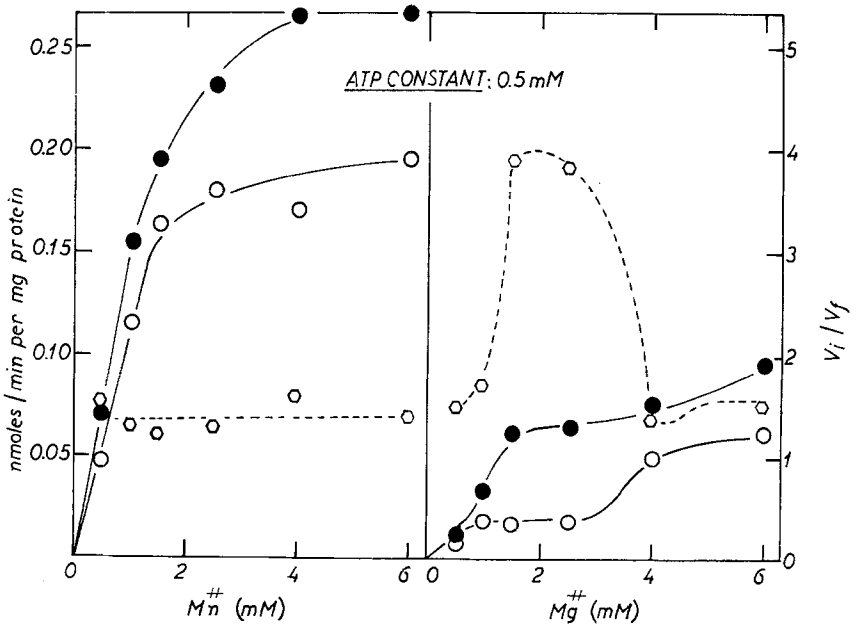
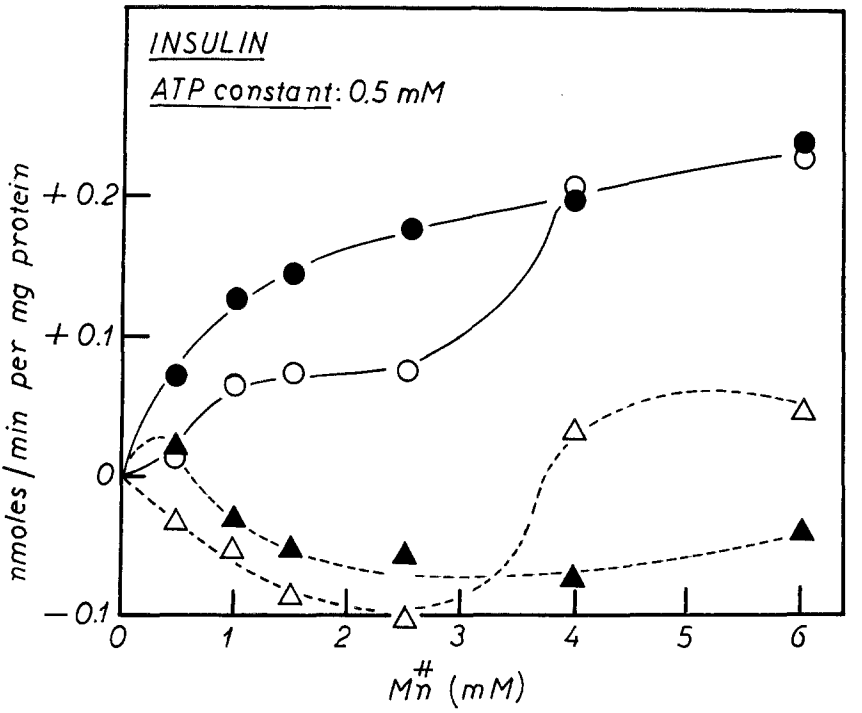


Fig. 14. Basal adenylyl cyclase activity measured in the presence of 0.5 mM ATP as a function of total divalent cation concentration. Symbols and conditions were as indicated in Figs. 1–5



Figs. 15–22. Effect of different modifiers on adenylyl cyclase activity measured at constant total ATP concentration (0.5 mM) and varying the total divalent cation concentration. Symbols and conditions were as indicated in Figs. 6–13

is more evident than with Mn^{++}, indicating that Mg^{++} is more efficient than Mn^{++} in bringing about inactivation of the cyclase. However, the clear enhancement of final velocities observed at 4 and 6 mM Mg^{++} could suggest that this cation might play a dual role: inactivation of adenylyl cyclase, leading to the formation of a putative inactive form, and, at high concentrations, allowing the catalytic expression of the less active form. However, as discussed above, initial and final velocities are much higher with Mn^{++} than with Mg^{++}.

Insulin. Additional evidence for a dual role of divalent cations on the control of adenylyl cyclase was observed in assays containing insulin. As shown in Fig. 15, insulin leads to a clear decrease in the final velocity at Mn^{++} concentrations up to 2.5 mM. At higher cation concentrations the effect was completely reversed. The effects of insulin on initial rates were always much less evident, as described earlier (Torres *et al.*, 1978).

In the presence of Mg^{++}, inactivation of adenylyl cyclase by insulin was small and only evident at the two lowest cation concentrations

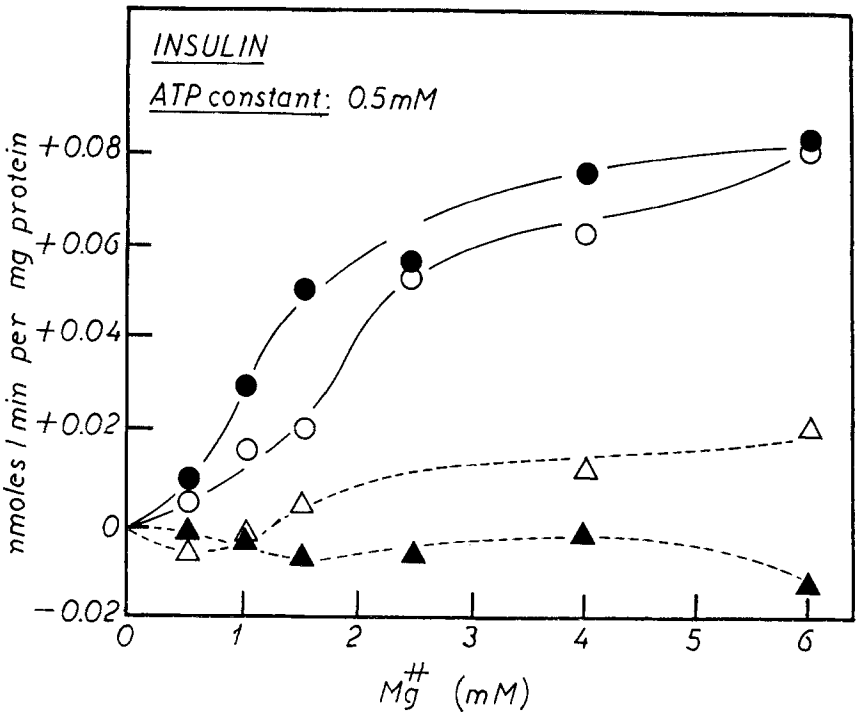


Fig. 16

tested (Fig. 16). Higher Mg^{++} concentrations provided a recovery of the final velocities to values slightly above the control assays not supplemented with the hormone.

The data may suggest that in some way insulin is able to accelerate cyclase inactivation. Obviously, such hormone effects can be observed only under conditions that do not by themselves induce a high inactivation of the enzyme. On the other hand, a high divalent cation (Figs. 14–16) or a high ATP (Fig. 6) concentration might allow expression of the activity of this relatively "inactive" form of the enzyme.

Fluoride. Figures 17 and 18 show the effects of fluoride as a function of the divalent cation concentration. As discussed earlier, the enhancement of the final rates appears again to be the most relevant effect. In addition, the extent of the activation is negatively correlated with the ability of the divalent cation to inactivate the enzyme in the absence of fluoride. Since the highest rates attained were similar with Mg^{++} or Mn^{++}, it is evident that fluoride elicited the conversion of cyclase to a form equally active with both divalent cations.

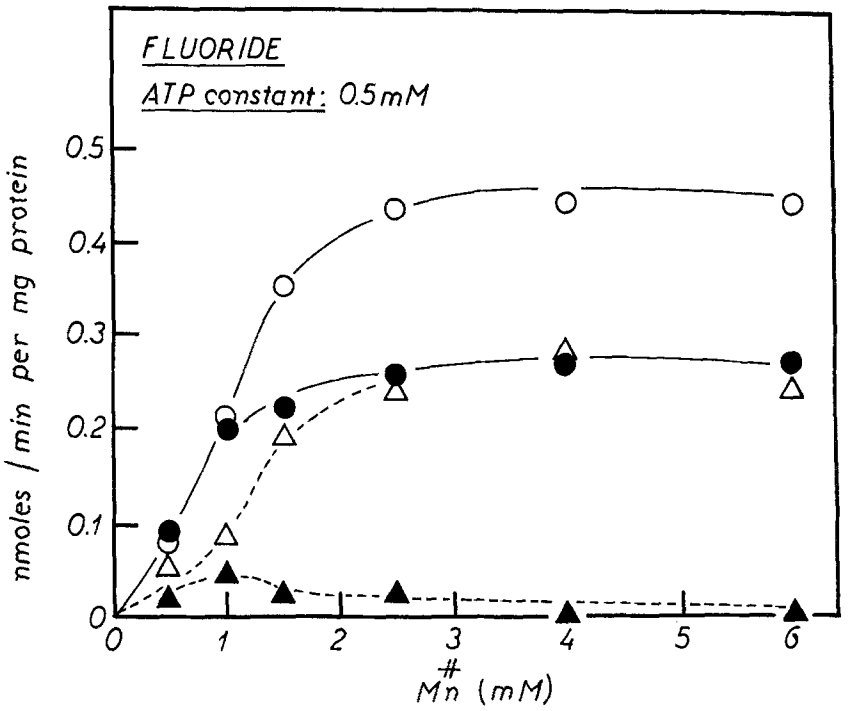

FLUORIDE
ATP constant: 0.5mM
nmoles / min per mg protein
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6
Mn (mM)

Fig. 17

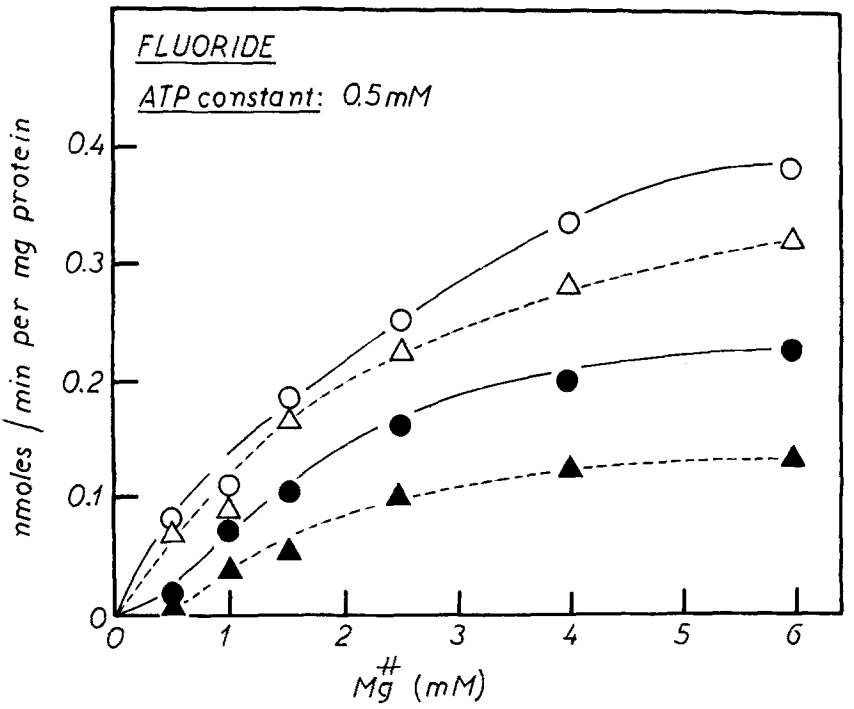

FLUORIDE
ATP constant: 0.5mM
nmoles / min per mg protein
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6
Mg (mM)

Fig. 18

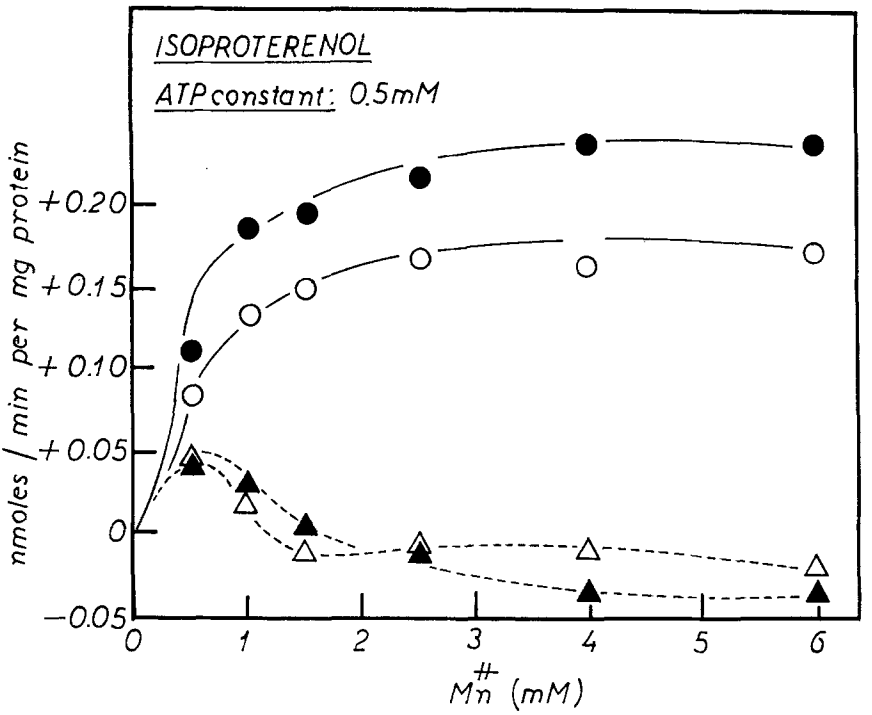


Fig. 19

Isoproterenol. As indicated in previous paragraphs, the effects of isoproterenol were very much less evident in the presence of Mn^{++}. As shown in Fig. 19 a small activation was observed, but only at low concentrations of this divalent cation. However, in the presence of Mg^{++} there was a 2- to 5-fold stimulation of the final rates in the range between 0.5 and 2.5 mM. Above such levels the stimulation disappeared (Fig. 20). The fact that the highest degree of isoproterenol stimulation was found between 1.5 and 2.5 mM Mg^{++} may suggest that there may be some correlation between such stimulation and the ability of this cation to induce inactivation of adenylyl cyclase. As shown in Fig. 14, the highest inactivation was observed in this range of concentration. The putative ability of high Mg^{++} concentrations (above 2.5 mM) to allow the expression of the activity of the inactive cyclase form(s) could explain the negligible effect of isoproterenol at those concentrations.

GMP-P(NH)P. Figures 21 and 22 show that this GTP analog inhibited the initial and activated the final rates. The inhibition did not seem to be significantly affected by the type or concentration of the divalent cation used.

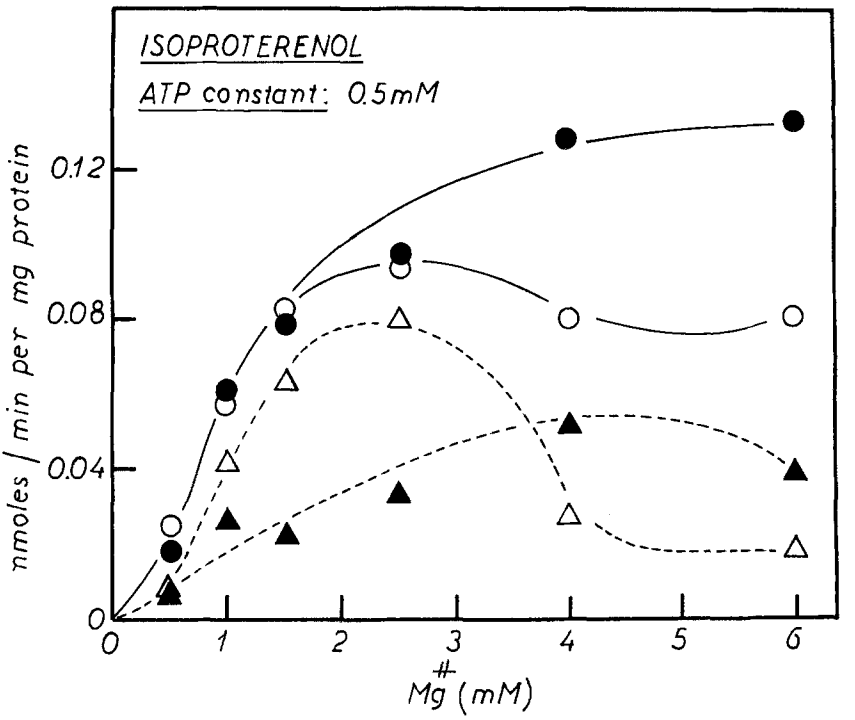


Fig. 20

The extent of activation of the final rates was more pronounced with Mg^{++} than with Mn^{++}. In the former case, the maximal stimulation was coincident with the Mg^{++} concentration giving the maximal inactivation of the basal enzyme activity. Except for the magnitude of the activation, this behavior resembles qualitatively that in the presence of fluoride (*see* Fig. 18).

Kinetic Parameters

Information about the influence of modifiers on the apparent affinities and maximal initial rates for the fat cell adenylyl cyclase is provided in Fig. 23 as well as in Table 1. Since the calculations were done only in terms of initial velocities, the data presented will describe only the extent of modification induced by factors during the first 90 sec of incubation. This means that in most cases the ability of different modifiers to affect adenylyl cyclase activity will be underestimated.

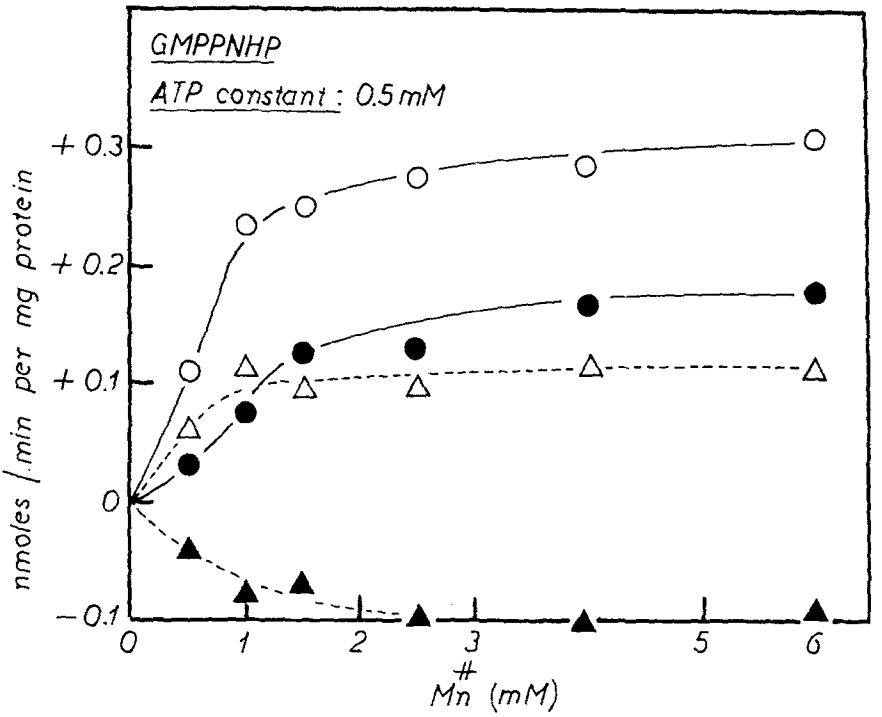

GMPPNHP
ATP constant : 0.5 mM
+ 0.3
+ 0.2
+ 0.1
0
− 0.1
nmoles / min per mg protein
0
1
2
3
5
6
Mn (mM)

Fig. 21

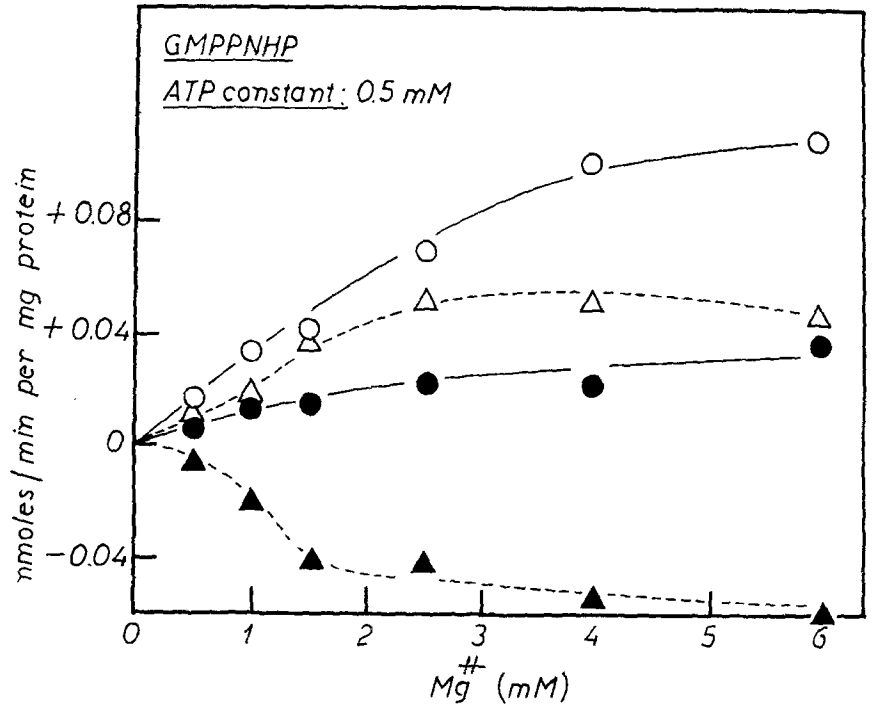

GMPPNHP
ATP constant : 0.5 mM
+ 0.08
+ 0.04
0
− 0.04
nmoles / min per mg protein
0
1
2
3
4
5
6
Mg (mM)

Fig. 22

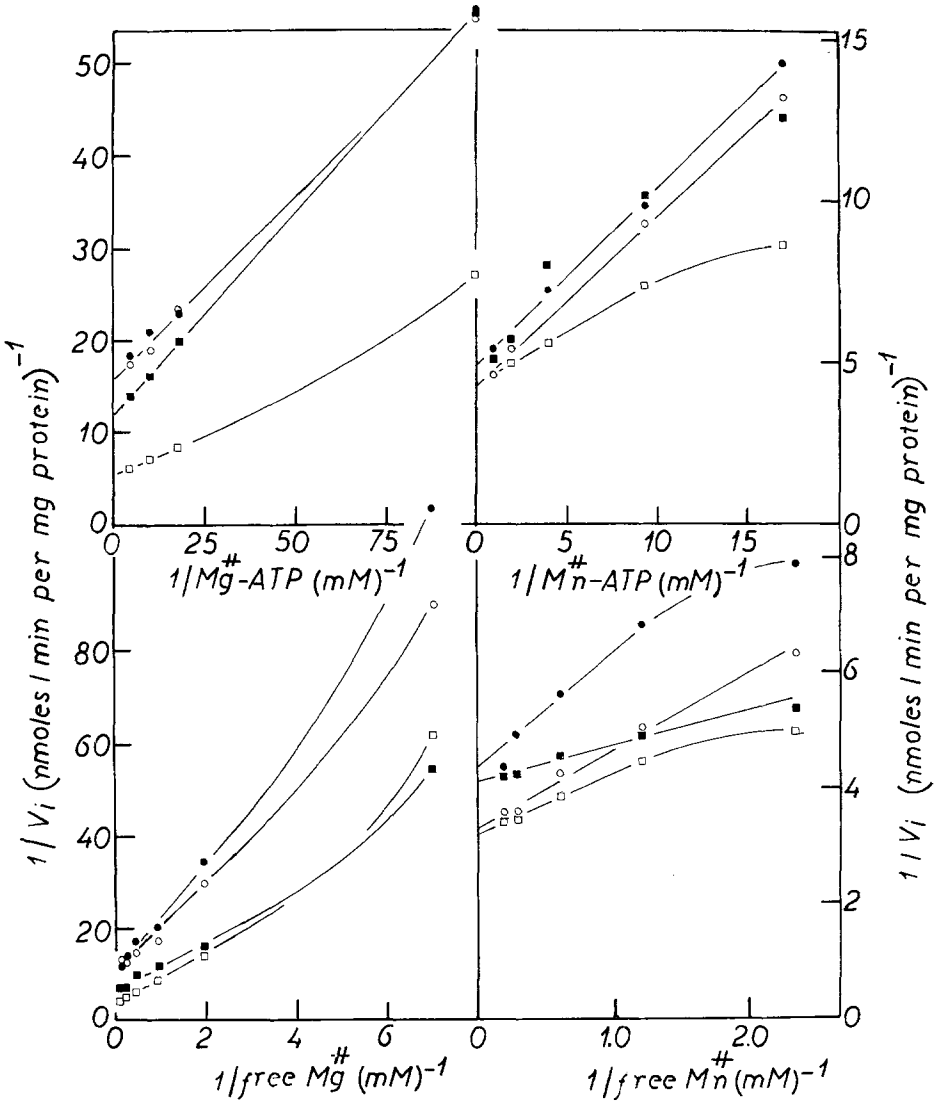


Fig. 23. Reciprocal plots of adenylyl cyclase initial rates measured at varying concentrations of total divalent cation or total ATP. The results are plotted as a function of reciprocal concentrations of free divalent cation or $MeATP^{2-}$, respectively. ○, no additions (basal activity); ●, insulin; ■, isoproterenol; □, fluoride. Other conditions were as indicated in Table 1

Measuring the cyclase activity as a function of either free divalent cation or divalent cation-ATP complex concentration, maximal velocities resulted greater with Mn^{++} than with Mg^{++}. On the other hand, apparent affinities for Mn^{++} or $MgATP^{2-}$ were higher than those found for Mg^{++} or $MnATP^{2-}$, respectively.

The influence of insulin, fluoride, and isoproterenol on the kinetic behavior of adenylyl cyclase is also shown in Fig. 23 and Table 1. It is evident that under conditions leading to maximal changes in enzyme activity, these factors affected the maximal velocity. In the case of isoproterenol, changes in the apparent affinities were also observed.

Discussion

There are certain discrepancies between the results of the present studies and those reported by others. For example, in the case of activation of fat cell adenylyl cyclase by isoproterenol, Harwood, Löw & Rodbell (1973) found a clear effect at ATP and Mg^{++} concentrations of 0.5 and 5 mM, respectively, (*see* Fig. 2 of this reference). As shown in Fig. 20, the activation found here at similar concentrations of ATP and Mg^{++} was negligible. Discrepancies of this type can be explained by the fact that the concentrations of some of the components in the reaction mixtures used in this work and that by others are often quite different. This is particularly important for phosphocreatine; in the present work the concentration of this compound was 2 mM, whereas that used by Harwood *et al.* was 25 mM (Harwood *et al.*, 1973). Under these conditions chelation of divalent cation by phosphocreatine (Smith & Alberty, 1956) should decrease the amount of available free cation.

Evidence obtained from these studies clearly indicates that adenylyl cyclase becomes inactivated under some of the conditions selected for its assay. This is clearly evident over a given range of Mg^{++} concentration (Fig. 14), and a similar effect is evident with Mn^{++} in the presence of insulin. These experiments indicate that in some way divalent cations are playing a dual role in the control of adenylyl cyclase activity. At some critical concentration they can lead to the inactivation of cyclase, whereas at higher concentrations they enable the expression of such intrinsically diminished activity.

Inactivation of the fat cell cyclase system during assay has already been recognized by Rodbell and colleagues (Rodbell, 1975). Results not shown in this paper indicate that such inactivation increases with the degree of membrane purity, and that it can be nearly completely reverted by the addition of fluoride. It is evident that further studies on the mechanism leading to such inactivation might clarify important aspects regarding the regulatory properties of this important enzyme system.

In addition to the differential inactivation of adenylyl cyclase in assays

containing Mg^{++} or Mn^{++}, at least two other aspects of the enzyme response to divalent cations might be of importance. One of these is the increase in enzyme activity as a function of the concentration of the cation. The second is the absolute magnitude of the enzyme activity capable of being expressed by each of these divalent cations. The first point introduces various highly controversial concepts. Since the first report by Birnbaumer *et al.* (1969) indicating that fat cell adenylyl cyclase might have a site for Mg^{++} "that is distinct from the catalytic site reacting with Mg^{++}-ATP", evidence favoring or challenging such a possibility has been broadly discussed and debated. De Haën, for example, challenged the existence of such a divalent cation site (De Haën, 1974). According to De Haën, free ATP as a competitive inhibitor for the catalytic site should have an affinity constant two orders of magnitude higher than the substrate Mg^{++}-ATP. Thus, activation by Mg^{++} under such hypothetical conditions could be explained simply in terms of a decrease in the concentration of the inhibitor. By similar reasoning, evidence was given by Rodbell's group indicating that the protonated form of ATP ($HATP^{3-}$) plays an important role as an inhibitor in the regulation of adenylyl cyclase (Rendell *et al.*, 1975). Such models have been extended to include the suppositions that hormones in some way change the enzyme system towards states with lower affinities for such inhibitors.

Evidence obtained with the *Neurospora crassa* enzyme supports the existence of a Mn^{++} site interacting with the catalytic one (Flawiá & Torres, 1972*b*). With this enzyme, it is clear that Mn^{++} activates at low but not at high Mn^{++}-ATP concentrations, indicating that the divalent cation might modulate the apparent affinity for the substrate. More recently, Garbers & Johnson (1975) obtained evidence with the brain enzyme that supported the existence of an independent site for Mg^{++}. The same concept is also supported by a simple model analyzed by Hammes and Rodbell (1976). Under our experimental conditions it is difficult to discriminate between these two possibilities, since free divalent cation concentrations as well as $[MeATP^{2-}]/[ATP^{4-}]$ and $[MeHATP^{1-}]/[HATP^{3-}]$ ratios were changed simultaneously.

In reference to the difference in the absolute magnitude of enzyme activity that can be measured with Mn^{++} or Mg^{++}, it is evident that all the maximal velocities measured in the absence of stimulants are higher with Mn^{++} than with Mg^{++}. However, the affinities for the Mn^{++}-ATP complex are lower than those for the Mg^{++}-complex (Table 1). This behavior is not compatible with the supposition that the

Table 2. Ratios of adenylyl cyclase activities measured with Mg^{++} and Mn^{++} under certain conditions selected from time courses shown in Figs. 1 to 4

	Condition (mM)		Modifier	$V_{(Mg^{++})}/V_{(Mn^{++})}$	
	ATP	Me^{++}		V_i	V_f
1	0.26	2.0	Basal	0.419	0.113
			Insulin	0.397	0.132
			Fluoride	0.847	0.787
			Isoproterenol	0.577	0.358
			GMP-P(NH)P	0.014	0.239
2	0.50	1.5	Basal	0.292	0.098
			Insulin	0.337	0.256
			Fluoride	0.477	0.523
			Isoproterenol	0.396	0.516
			GMP-P(NH)P	0.118	0.157
3	0.50	6.0	Basal	0.348	0.318
			Insulin	0.346	0.346
			Fluoride	0.831	0.865
			Isoproterenol	0.487	0.462
			GMP-P(NH)P	0.198	0.354

differences in affinities could be a consequence of a competitive inhibition by higher concentrations of an ATP species different from $MnATP^{2-}$. In fact, concentrations of ATP^{4-}, $MeHATP^{1-}$ and $HATP^{3-}$ are lower with Mn^{++} than with Mg^{++}.

It is notable that the ratios of enzyme activities measured with Mg^{++} and Mn^{++} varied with the time of incubation (initial or final rates), concentration of ATP and divalent cation, as well as with the presence or absence of modifiers such as insulin, isoproterenol, fluoride or GMP-P(NH)P. This is depicted in Table 2. It is evident that under conditions leading to maximal cyclase inactivation in assays containing Mg^{++} or Mn^{++} plus insulin (conditions *1* and *2*), basal enzyme activity was converted to a form much more dependent on Mn^{++}. A similar effect was elicited by GMP-P(NH)P during the initial period. On the other hand, insulin, isoproterenol, and fluoride decreased the Mn^{++} dependence. In this regard, fluoride seems to be the most effective since in its presence an enzyme form was detected which exhibited activities with Mg^{++} similar to those found with Mn^{++}.

However, at high divalent cation concentrations (condition *3*, Table 2) the changes elicited by these modifiers were less evident, indicating that

the expression of activity by the putative, inactive adenylyl cyclase form(s) improves at high Mg^{++} concentrations. The results obtained with insulin are rather difficult to understand since the cyclase inactivation observed did not appear to increase the enzyme dependence on Mn^{++}.

The results reported in this paper appear to indicate that adenylyl cyclase can interconvert between active and inactive forms under conditions used for its assay. Such conversions seem to be rather slow and are influenced by factors such as insulin, isoproterenol, fluoride and GMP-P(NH)P. It is evident that the complexity of the observed changes will demand much more work in order to develop a more coherent basic model for understanding the control of this activity.

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