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Effects of Insulin on the Adenylyl Cyclase Activity of Isolated Fat Cell Membranes

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Summary. Insulin decreased markedly the adenylyl cyclase activity associated with fat cell membranes purified by centrifugation in sucrose gradients. The hormone effect was not readily evident in crude membrane preparations.

The kinetics of this effect indicate that some time was required for the onset of the insulin-induced inactivation. This lag period decreased when the insulin concentration was increased. The hormone dose dependence for adenylyl cyclase inactivation measured at a fixed time (3 min) showed a 10 to 15% decrease in activity at 1 to 30 μU per ml insulin; 30 to 40% at 100 to 1000 μU per ml; and 75% at 0.1 U per ml.

The insulin effect was completely abolished by 0.1 mM GMP-P(NH)P, 10 mM fluoride, or 50 ng per ml glucagon, or by increasing the Mn^{++} concentration to 4 mM. In addition, it was partially reversed by the addition of a fraction from the sucrose gradient, which contained soluble factors.

The kinetics of the adenylyl cyclase-catalyzed reaction were studied using ATP or AMP-P(NH)P as adenylyl donor, and Mn^{++} or Mg^{++} as divalent cation, in the absence or presence of insulin. With ATP and Mg^{++} there was a striking reduction of the transient reaction rates after 1.5 min of incubation. Under these conditions the insulin effect was not evident. On the contrary, with ATP and Mn^{++} this spontaneous reduction of activity was less evident; however, in the presence of insulin there was a clear and marked reduction of the transient reaction rate measured after 1.5 min of incubation. With AMP-P(NH)P the kinetic data were qualitatively similar to those observed with ATP.

It is concluded that under certain assay conditions adenylyl cyclase may be converted to an inactive enzyme form, and that such a conversion is more evident in the presence of Mg^{++} than with Mn^{++}. In the latter case, insulin appears to enhance the rate of this conversion.

Some evidence indicates that physiological concentrations of insulin decrease adenylyl cyclase activity associated with isolated fat cell and liver (Hepp, 1971; Hepp & Renner, 1972; Illiano & Cuatrecasas, 1972) fibroblast (Jiménex de Asúa *et al.*, 1973), and *Neurospora* (Flawiá &

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Torres, 1973*a*, *b*) membranes. The presence of insulin in the assay mixture is the only apparent requirement for decreasing basal and hormone- or fluoride-stimulated activities. These observations are particularly relevant for understanding the molecular basis of insulin action since they constitute the only evidence that insulin might affect the rate of cyclic AMP synthesis by cell-free preparations.

In this report further evidence is presented, indicating that insulin decreases adenylyl cyclase activity associated with fat cell membranes, and some of the properties of this effect are described. The conditions under which this hormonal effect can be reliably reproduced are described in detail.

Materials and Methods

Fat Cell Membrane Preparations

Epidydimal fat pads were obtained from about 50 Sprague-Dawley rats, weighing 125 to 150 g and fed *ad libitum* with laboratory chow, by a modification of the Rodbell procedure (Rodbell, 1967*a*). The tissue was collected in a 250 ml polyethylene bottle containing 50 ml of a warm 0.5% albumin solution in Krebs-Ringer bicarbonate buffer, pH 7.4. The time for killing the rats and extracting the fat pads was about 15 min. Collagenase (75 mg) was immediately added to the tissue suspension, and it was shaken at 37° for 30 min. A piece of silk mesh was attached to the mouth of the bottle, which was then squeezed, the filtrate being recovered in a 50-ml plastic conical centrifuge tube. Usually it was necessary to change the silk mesh twice to complete the filtration of the digested tissue. The floating cells were separated from the infranatant by centrifugation at $200 \times g$ for 15 sec (room temperature). The red infranatant was then aspirated and discarded. Using the same procedure, the cells were washed three times with warm 1% albumin solution in Krebs-Ringer bicarbonate buffer (40 ml each time). The white, washed cell suspension was then cooled, mixed with two volumes of ice-cold 1-mM sodium bicarbonate solution, and homogenized with an all-glass 50-ml Dounce-type homogenizer (Kontex; loose fitted pestle); five strokes were enough to obtain complete cell lysis. Washings and homogenization took about 10 min.

The cell homogenate was centrifuged at $46{,}000 \times g$ for 5 min. The solid, fat material, the upper layer oil, and the supernatant fluid were then removed by aspiration, and the membrane pellet was resuspended in 2 ml of ice-cold 1-mM sodium bicarbonate solution with a 7-ml Dounce homogenizer (tight fitted pestle; five strokes). This suspension is referred to as "crude membrane preparation". Aspiration and homogenization took about 5 min.

The "crude membrane preparation" was purified further using centrifugation on a discontinuous sucrose density gradient. Plastic tubes ($^7/_{16} \times 2^3/_8''$) were successively filled with 0.8-ml aliquots of the following sucrose solutions: 1.2 M, 0.86 M, 0.52 M and 0.26 M. The gradients were allowed to stand in the cold for about 2 hr. Aliquots of 0.6 ml of the "crude membrane preparation" were loaded on the top of the gradients, which were subjected to centrifugation in a SW60 rotor at 58,000 rpm for 15 min. Thus, the gradients were exposed (Sorvall, OTD-2) to the top speed for only 5 min. This centrifugation step consumed about 25 min.

Table 1. Adenylyl cyclase activity in different fractions from a sucrose gradient[a]

Fraction	Total protein		Adenylyl cyclase activity			
			Total activity (Without insulin)		Specific activity	
					Without insulin	With insulin
	mg	%	pmoles/min	%	pmoles/min / mg protein	
Interphase I	0.23	4.2	1.8	1.3	7.8	10.6
Interphase II	0.11	2.0	2.1	1.5	19.1	7.9
Interphase III	0.10	1.8	6.3	4.5	63.0	39.9
Interphase IV	0.30	5.5	65.1	46.4	217.0	121.0
Pellet	4.68	86.5	64.9	46.3	13.9	8.4
Total	5.42	100.0	140.2	100.0	–	–

[a] The opalescent material contained in the interphases between the supernatant and 0.26 M sucrose (*I*), 0.26 and 0.52 M sucrose (*II*), 0.52 and 0.86 M sucrose (*III*), and 0.86 and 1.2 M sucrose (*IV*) were aspirated and diluted in 1 mM sodium bicarbonate; the pellet was homogenized and also diluted in the bicarbonate solution. Assays were done for 2.5 min in the presence or absence of 1250 μU of insulin per ml. Other conditions were as indicated under *Materials and Methods*.

As can be seen in Table 1, four membrane bands were located in the corresponding density gradient interphases; some material was also found on the bottom of the tubes. The band found between 1.2 and 0.86 M sucrose contained the highest specific activity of adenylyl cyclase. Consequently, the material above this band was discarded (by aspiration). This step was followed by careful aspiration of the membranous material in this interphase, which was further diluted with ice-cold 1-mM sodium bicarbonate buffer and used as the enzyme source ("pure membrane preparation"). All the steps following cell lysis were done at 2 to 4°. Starting from the fat pads, the entire preparation of "pure membranes" took about 90 min.

More recent evidence indicates that pure membranes prepared from cells isolated and washed in a hypotonic medium exhibited increased responses to insulin. The method employed for the preparation of these cells was the same (*see Materials and Methods*), except that the composition of the medium was as follows (in mM): 15.4, NaCl; 6, KCl; 0.15, KH_2PO_4; 0.15, $MgSO_4$; 0.33, $CaCl_2$; and 3, $NaHCO_3$, plus albumin as indicated above. The solution was gassed in an O_2-CO_2 mixture (95:5, v/v) and adjusted to pH 7.4 by the dropwise addition of 1 N NaOH.

Adenylyl Cyclase Assay

The standard incubation mixture contained 50 mM Tris-HCl buffer, pH 7.5, 0.2 mM 3-isobutyl-1-methyl xanthine, 1 mM cyclic AMP, 2.5 mM $MnCl_2$, 0.5 mM [α-^{32}P]ATP (specific activity, 100 to 400 cpm per pmole), 2 mM phosphocreatine, 0.2 mg per ml creatine kinase,

0.1 mg per ml albumin and membranes (10 to 20 μg of protein). The volume was 0.1 ml. Incubations were performed at 37° for 1 to 5 min. Reactions were stopped by the addition of 0.1 ml of a solution containing 12.5 mM [^{3}H]cAMP (specific activity, 3,800 cpm per μmole) plus 40 mM ATP, and heating in a boiling water bath for 3 min (Rodbell, 1967*a*). The volume of each sample was adjusted to 1 ml with water.

Some experiments were performed using [α-^{32}P] adenyl-5′yl imidodiphosphate [AMP-P(NH)P] as substrate. In this case the incubation mixture contained 50 mM Tris-HCl buffer, pH 7.4, 0.2 mM 3-isobutyl-l-methyl xanthine, 1 mM cyclic AMP, 1 mM $MnCl_2$ (or $MgCl_2$), 0.1 mM [α-^{32}P]AMP-P(NH)P (specific activity, 100 to 400 cpm per pmole), 0.1 mg per ml albumin and membranes. The total volume was 0.1 ml. Reactions were stopped by the addition of 0.1 ml of a mixture containing 1% sodium dodecylsulfate plus ATP and [^{3}H]cyclic AMP as described above. The boiling step was omitted (Harwood, Löw & Rodbell, 1973).

The cyclic AMP in the samples was purified according to the sequential column procedure (Dowex 50 and alumina) described by Salomon, Londos & Rodbell (1974). The blanks of the reaction (nonincubated samples, or incubated without enzyme) were in the range of 5 to 8 cpm above the background per 10^6 cpm of [α-^{32}P]ATP or [α-^{32}P]AMP-P(NH)P added.

Synthesis of [α-^{32}P]ATP

The synthesis was performed using previously described procedures (Flawiá & Torres, 1972), with several modifications. Starting with radioactive inorganic phosphate, the synthesis was performed in two steps. The first was the chemical coupling of phosphate to 2′,3′-isopropylideneadenosine (Symons, 1968), followed by an acid hydrolysis of the isopropylidene group. The product of this reaction, ^{32}P-labeled 5′-adenosine monophosphate, was then used as the substrate for the enzymatic synthesis of [α-^{32}P]ATP. The exact procedures used are described below.

The radioactive phosphate (50 to 80 mCi, carrier-free, hydrochloric solution; Union Carbide) was mixed with a solution of phosphoric acid (1 μmole H_3PO_4 for each 10 mCi ^{32}P) and evaporated in a reaction flask (bulb 3 cm in diameter, neck 10 cm long and 1 cm in diameter). The residue was dissolved in 2 ml water and evaporated again. This evaporation was repeated two more times. Before the last evaporation a drop of triethylamine was added. Isopropylidene adenosine (60 mg) was added to the reaction flask, and its content was brought to dryness by three successive evaporations with dry acetonitrile, using a vacuum manifold as described by Greenlees and Symons (1966). The following reagents were then added rapidly: dimethylsulfoxide (3.2 ml), triethylamine (24 μl), and trichloroacetonitrile (20 μl). The flask was immediately plugged with a glass stopper, and after mixing it was incubated at 37° for 20 min. The reaction was stopped by the addition of 6 ml of 5 N acetic acid. The mixture was then heated for 60 min at 100° (boiling water bath) in order to hydrolyze the isopropylidene group. After hydrolysis, the contents of the flask were evaporated to dryness by heating the sample at about 70°. The sample was dissolved in 3 ml of water and evaporated again. These evaporations were repeated two more times. Before the last evaporation the contents of the flask were transferred to an 18 × 150 mm test tube. For the enzymatic synthesis of α-labeled ATP the following components were added successively: 1 M Tris-HCl buffer, pH 7.4, 0.32 ml; 20 mM ATP, 0.02 ml; 0.3 M $MgCl_2$, 0.16 ml; 2 M KCl, 0.16 ml; 100 mM sodium phosphoenolpyruvate, 0.32 ml; H_2O, 1.8 ml; myokinase (5 mg protein per ml), 0.02 ml and pyruvate kinase (10 mg protein per ml), 0.02 ml. The incubation was performed at 37° for 15 min, and the reaction was stopped by the addition of methanol (8 ml) and heating in a boiling water bath

for 3 min, with precaution to avoid fast bubbling. Under these conditions most of the methanol was evaporated.

The ^{32}P-labeled ATP was purified on a DEAE Sephadex A-25 column (0.8 × 19 cm, bicarbonate form), which was washed and equilibrated with water. After applying the sample and washing with about 10 ml of water, elution was performed with a linear gradient of triethylammonium bicarbonate, pH 7.5, from 0 to 0.8 M (200 ml total volume). The elution rate was 3 ml per min. The radioactive ATP was eluted near the middle of the gradient. The yield of [α-^{32}P]ATP from radioactive inorganic phosphate was generally about 50%.

The radioactive ATP was characterized by thin layer chromatography on polyethyleneimino cellulose, using 1.5 M lithium chloride as the solvent system (Randerath & Randerath, 1967). This system enables good separation of ATP from fast moving compounds (AMP or P_i), as well as from other compounds with slow chromatographic mobility which are generated during the chemical synthesis.

Analytical Procedures and Reagents

Protein was determined by the procedure of Lowry *et al.* (1951) using crystalline human serum albumin as standard.

ATP, cyclic AMP, creatine phosphate, neutral alumina and phosphoenolpyruvate were purchased from Sigma. Rabbit muscle myokinase, pyruvate kinase, and creatine kinase were from Boheringer. AG 50W-X4, 200–400, mesh, and sodium dodecylsulfate were from Bio-Rad, DEAE Sephadex A-25 from Pharmacia, and sucrose, human crystalline albumin, tris-(hydroxymethyl)-aminomethane (Tris) from Schwartz-Mann, and collagenase (CLS 130 units per mg) from Worthington. Guanyl-5′-yl imidodiphosphate [GMP-P(NH)P] was purchased from P-L Biochemicals and AMP-P(NH)P from ICN Pharmaceuticals Inc. [^{32}P]phosphate was from Union Carbide, [^{3}H]cyclic AMP from New England Nuclear and [α-^{32}P]AMP-P(NH)P from ICN Pharmaceuticals Inc. Bovine albumin (fraction *V* from plasma) was from Reheis Chemical Company. Trichloroacetonitrile, 2′,3′-isopropilidene adenosine and 3-isobutyl-1-methyl-xanthine were purchased from Aldrich and acetonitrile, dimethylsulfoxide and triethylamine from Mallinckrodt; these three solvents were distilled and stored over calcium hydride. The latter was from Matheson, Coleman and Bell. Polyethyleneimino cellulose sheets (PEI-F) were obtained from J.T. Baker Chemical Company. Crystalline beef zinc-insulin (25.1 units per mg) and glucagon were from Elanco.

Results

Attempts to detect effects of insulin on adenylyl cyclase in "crude membrane preparations" of fat cells were unsuccessful. Figure 1*A* shows a representative experiment using such a preparation. Such negative experiments might be explained by several possibilities: (i) the membrane preparation used in these particular experiments does not respond to insulin under the conditions used; (ii) "crude membrane preparations" contain some factor(s) that impairs the action of insulin at the level of adenylate cyclase; and (iii) only a small fraction of adenylyl cyclase molecules per cell are under the control of insulin receptors. The first

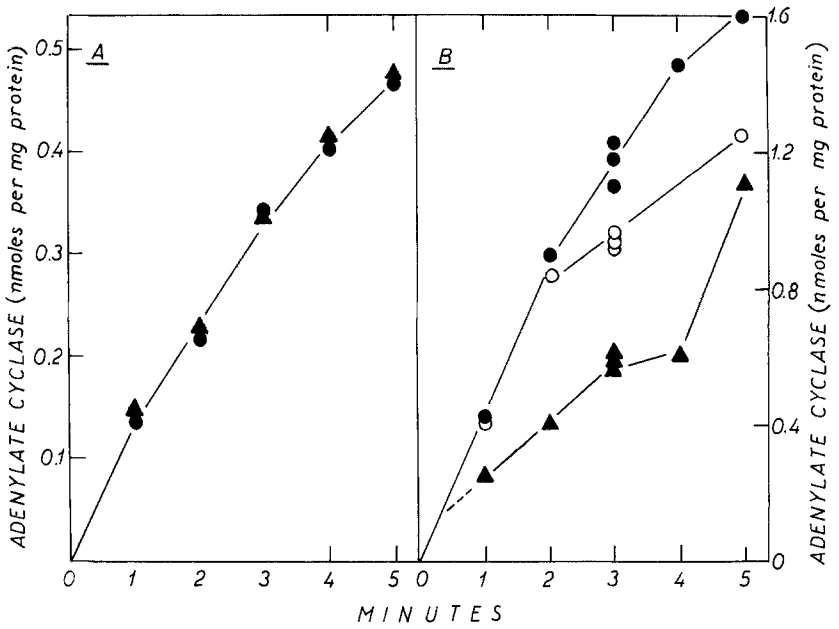

Fig. 1. Effect of insulin on adenylyl cyclase activity from a fat cell "crude"(*A*) or "pure membrane preparation" (*B*). Assays were done in the absence of insulin (●) or in the presence of 125 μU per ml (○) or 1250 μU per ml (▲) insulin. All the components of the reaction mixture (except membranes), including insulin, were premixed in the assay tube and left to stand at 37° for 2 min. Reaction was started by the addition of membranes

possibility was considered unlikely on the basis of previous evidence indicating that "crude" fat cell membrane preparations can exhibit a response to insulin at the level of this enzyme (Hepp, 1971; Hepp & Renner, 1972; Illiano & Cuatrecasas, 1972) and on sugar transport (Rodbell, 1967*b*; Illiano & Cuatrecasas, 1971).

In order to check the second and third possibilities, the heterogenous preparation of "crude membranes" was fractionated by discontinuous sucrose gradients, using short periods of centrifugation. The results of such an experiment are shown in Table 1. It is clear that a large proportion of adenylyl cyclase activity is distributed in the pellet and in the interphase between 1.2 and 0.83 M sucrose; the latter shows the highest specific activity. Nevertheless, all but one of the collected membrane fractions exhibit a clear inhibitory effect of insulin on adenylyl cyclase. The fraction which does not show such inhibition is located at the top of the gradient and surely contains salts and soluble factors.

Figure 1*B* shows the kinetics of the insulin inhibition of adenylyl cyclase from the membrane fraction purified in the sucrose gradient

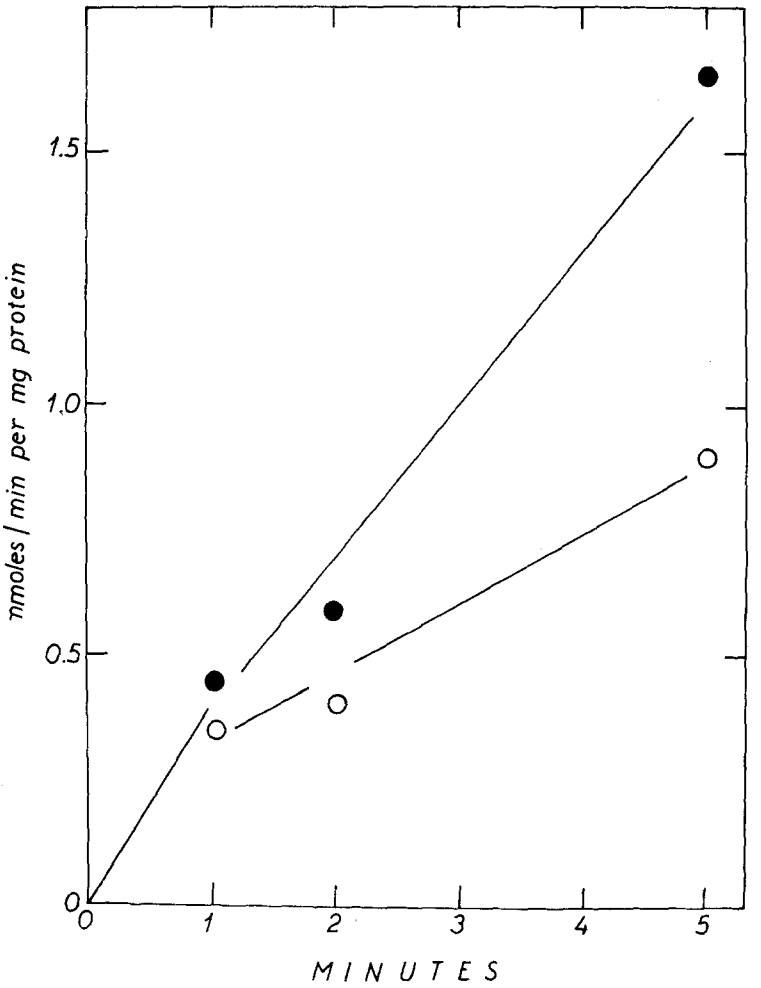

Fig. 2. Effects of insulin on adenylyl cyclase activity from a fat cell "pure membrane preparation". Conditions for the assays were as indicated under *Materials and Methods*, except that incubations were supplemented with [^{3}H]cyclic AMP (31,500 cpm per assay). The radioactive cyclic AMP was omitted in the stopping mixture. In addition, the assays were supplemented (○) or not (●) with 1,250 μU insulin per ml

and exhibiting the highest specific activity (Interphase *IV*; Table 1). In the absence of hormone, the enzyme activity tends to decline with increasing incubation time up to 5 min. In the presence of two different concentrations of insulin (120 and 1200 μU per ml) it is evident that after some period (i.e., latency phase) the hormone increases the rate of cyclase "inactivation". The time of onset of the hormonal effect tends to decrease when the hormone concentration is increased, but this latency phase

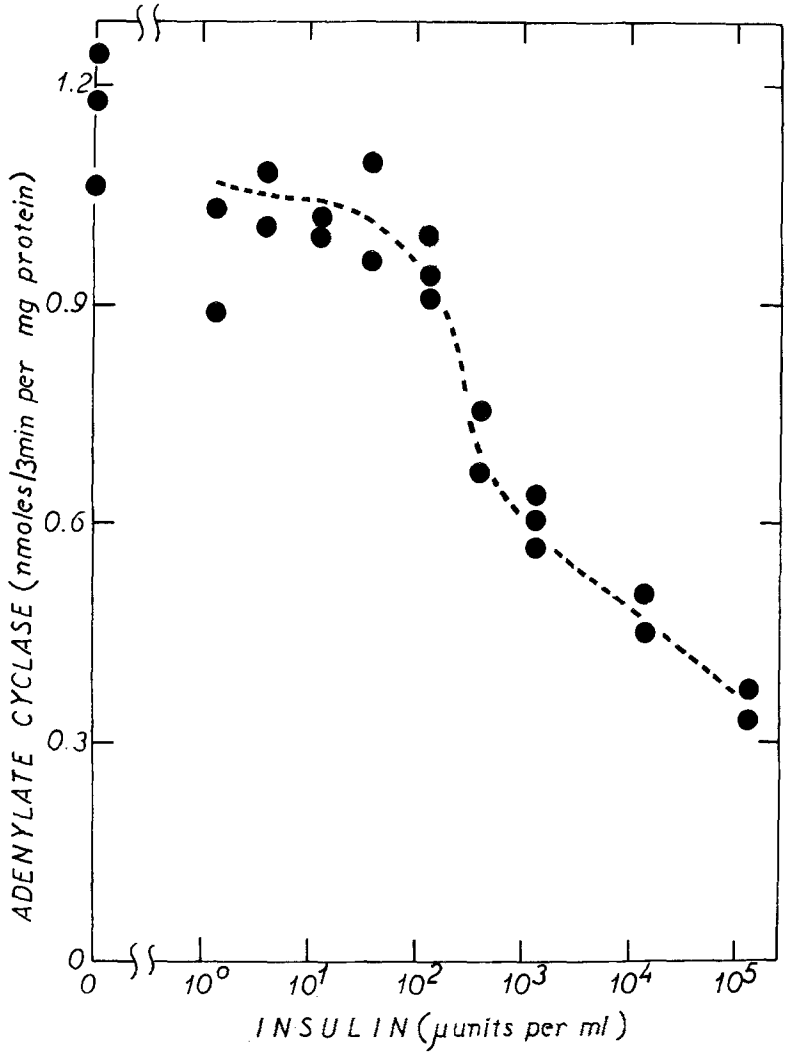

Fig. 3. Dose-response relationship between insulin concentration and adenylyl cyclase activity from fat cell "pure membranes". Assays were done for 3 min. Other conditions were as indicated under *Materials and Methods* and in Fig. 1

cannot be completely obliterated even at the highest hormone concentrations.

The insulin effect is not a result of increased rates of ATP or cyclic AMP-degradation. Standard assay mixtures of adenylyl cyclase activity included an ATP-generating system as well as high concentrations of cyclic AMP and 3-isobutyl-l-methyl xanthine; the latter compound is a potent inhibitor of cyclic nucleotide phosphodiesterase activities. The possible interference by phosphodiesterases was also checked by measur-

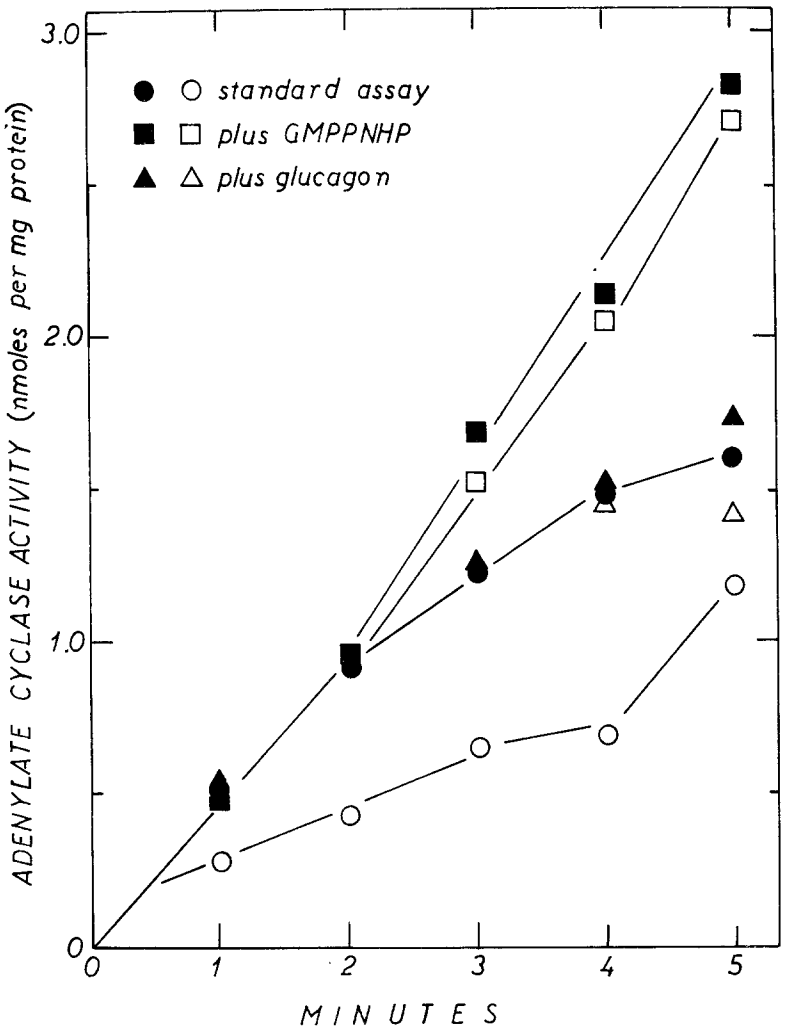

Fig. 4. Effect of insulin, glucagon and GMP-P(NH)P on adenylyl cyclase associated with fat cell "pure membranes". The standard assay mixture was supplemented (open symbols) or not (closed symbols) with 1,250 μU per ml insulin, plus the following additions: none (● ○); 5 μM GMP-P(NH)P (■ □); or 50 ng per ml glucagon (▲ △). Other conditions were as indicated under *Materials and Methods* and in Fig. 1

ing the recoveries in assay mixtures containing [^{3}H]cyclic AMP *during* the assay. Using such a procedure to measure recoveries, results similar to those shown in Fig. 1*B* were obtained (Fig. 2). In the latter case (Fig. 1*B*), measurements were made in samples supplemented with [^{3}H]cyclic AMP *after* the assay. In addition, interference by membrane-bound ATPase activities can also be discounted from the results of ex-

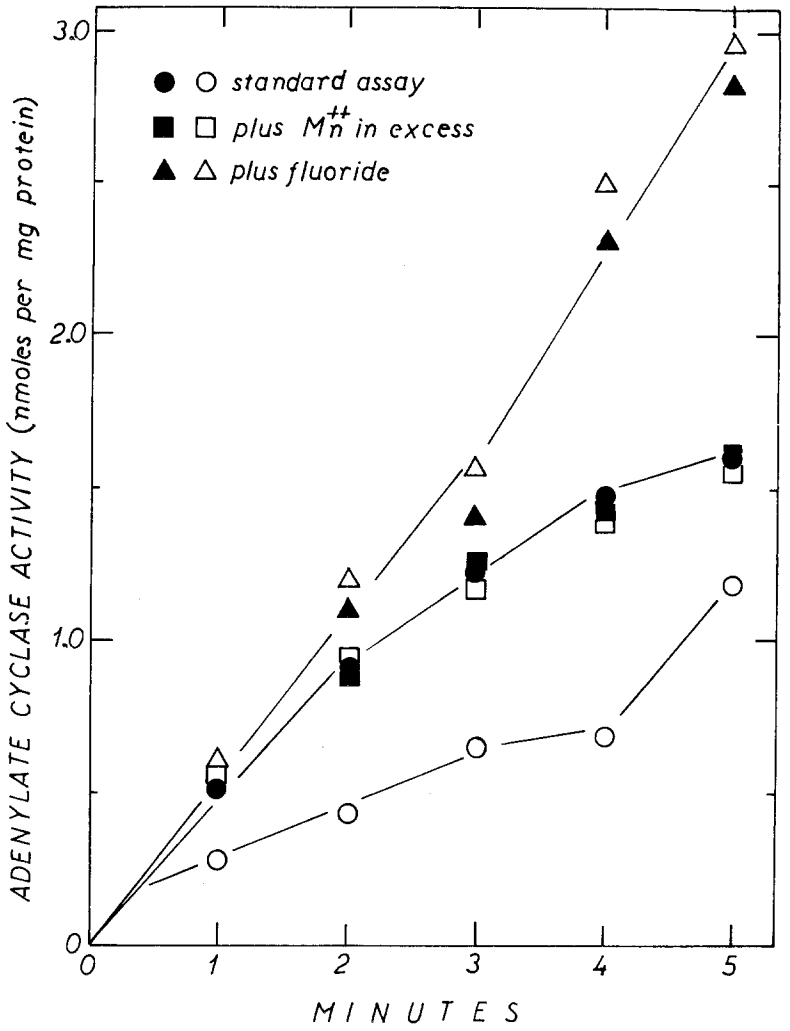

Fig. 5. Effect of insulin, fluoride and Mn^{++} in excess on adenylate cyclase associated with fat cell "pure membranes". The standard assay mixture was supplemented (open symbols) or not (closed symbols) with 1,250 μU insulin per ml plus the following additions: none (●○); or 2.5 M Na F (▲△); or 1.5 mM $MnCl_2$ (■□). In the latter case the total Mn^{++} concentration was 4 mM. Other conditions were as indicated under *Materials and Methods* and in Fig. 1

periments using [α-^{32}P]AMP-P(NH)P as substrate. With this ATP analog, which is resistant to phosphohydrolases (Yount *et al.*, 1971), the insulin-dependent inactivation of adenylyl cyclase is also quite clearly observed.

The dose dependence of the hormonal effect was studied by selecting a fixed time (3 min) for the assay (Fig. 3). It is clear that insulin concentrations as low as 1 to 30 μU per ml decrease the adenylyl cyclase activity

by 10 to 15%, and concentrations near 10,000 μU per ml lead to a 70% inactivation. Because the insulin effect is increasingly evident with time, dose-response curves at later time points would presumably be shifted to the left.

Other factors which affect adenylyl cyclase were also studied in assay mixtures in the presence and absence of insulin. Glucagon (Fig. 4) as well as an excess of manganous ions (Fig. 5) do not *per se* affect the basal activity of the enzyme under the conditions of the assay. However, both factors completely reverse the insulin-dependent inactivation of the cyclase system.

Under the conditions used for adenylyl cyclase assay, GMP-P(NH)P and fluoride cause a small time-dependent stimulation which is negligible during the first 2 min of incubation (Figs. 4 and 5). In the presence of these compounds the insulin effect is abolished. It is also clear that additional Mn^{++}, at concentrations that do not change basal enzyme activity, also causes an abolition of the insulin effect (Fig. 5).

As discussed earlier, "crude membrane preparations" appear to contain some factor(s) that impairs the insulin effect. According to the data presented in Table 1, this factor seems to be located in the upper fraction of the sucrose gradient. This possibility was confirmed in the experiment shown in Fig. 6. Addition of this fraction to the insulin-sensitive membrane fraction clearly reduces the insulin effect observed during the second and third minute of incubation; after this period the insulin-dependent inactivation is completely reversed. In addition, it is clear that this fraction also inhibits adenylyl cyclase activity measured in the absence of insulin.

The past difficulties of some laboratories in observing reproducible insulin effects on adenylyl cyclase activity could be explained, at least in part, by the generalized use of magnesium ions as a standard component of the assay mixtures. As shown in Fig. 7, in the presence of ATP as substrate and using magnesium instead of maganese, there is a striking reduction in the transient reaction rates after 1.5 min incubation. Furthermore, under these conditions the system is unaffected by insulin.

It is well known that AMP-P(NH)P is a substrate for adenylyl cyclase (Rodbell, *et al.*, 1971). In addition, this nucleotide does not appear to participate in phosphorylation reactions as a phosphate donor, and it is resistant to nucleotide phosphohydrolases (Yount *et al.*, 1971). Consequently, the use of AMP-P(NH)P as substrate for adenylyl cyclase could be important for ascertaining whether the hydrolysis or transfer of the

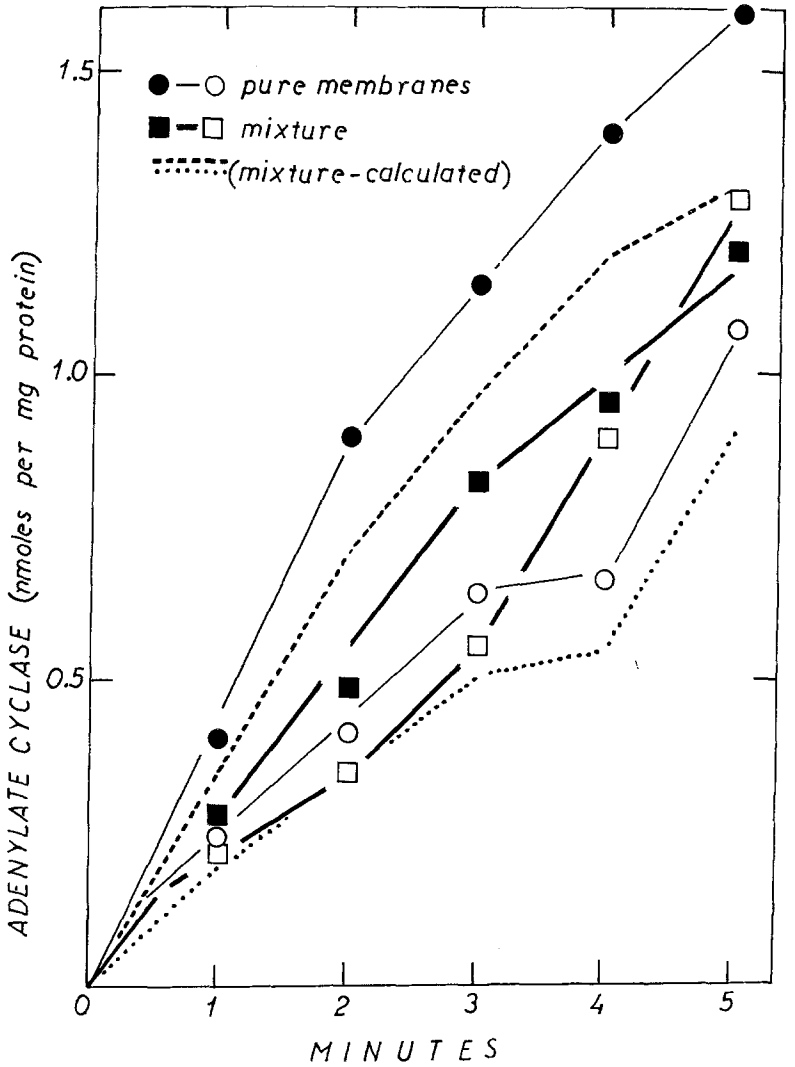

Fig. 6. Effect of insulin on adenylyl cyclase activity from fat cell "pure membranes" or from a mixture of these membranes and the fraction remaining above interphase *I* after centrifugation of sucrose gradients. The assays were done in the absence (closed symbols) or in the presence (open symbols) of 1,250 μU insulin per ml. Protein was 4.8 μg and 4.4 μg per assay containing "pure membranes" alone or the mixture, respectively. This mixture contained 1 vol of the upper fraction plus 3 vol of "pure membrane" preparation. Dashed and pointed lines indicate the theoretical values which would correspond to the summation of enzyme activities of both fractions assayed separately in the absence (——) or in the presence (.....) of insulin. Other conditions were given under *Materials and Methods* and in Fig. 1 and Table 1

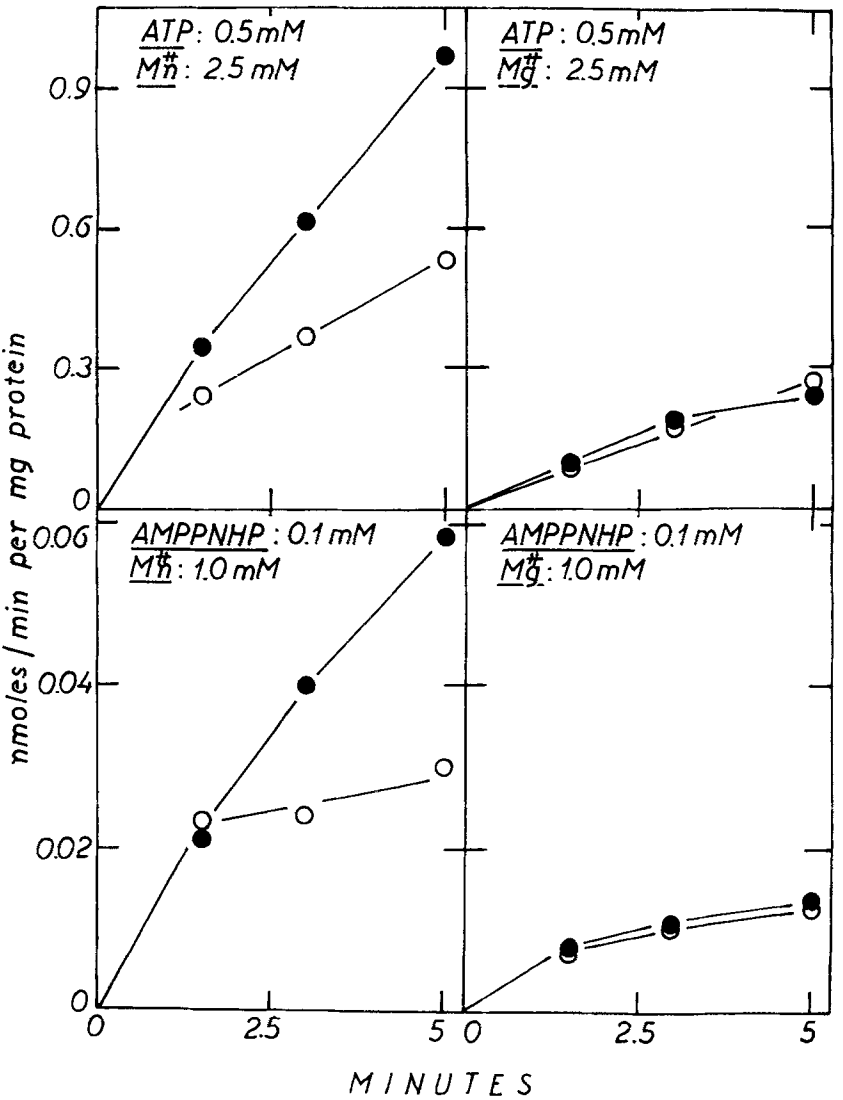

Fig. 7. Effect of insulin on adenylyl cyclase activity from fat cell "pure membranes" assayed with ATP or AMP-P(NH)P as adenylyl donor, and Mn^{++} or Mg^{++} as divalent cation. Conditions were given under *Materials and Methods*

terminal phosphate group of ATP (e.g., by protein phosphorylation) could be a requirement for the effect of insulin on cyclase. As shown in Fig. 7, this does not appear to be the case since in the presence of Mn^{++} and using the ATP analog as substrate insulin elicits a drastic reduction of the reaction rate. With Mg^{++}, however, there is again a clear reduction of the transient rates after 1.5 min, and the insulin effect is not observed.

Table 2. Effect of insulin on transient rates of adenylyl cyclase reaction catalyzed by a "pure membrane" preparation

Conditions	Transient rates		Inhibition by insulin %
	1st to 2nd min	2nd to 4th min	
	(nmoles/min per mg protein)		
Basal activity	0.44	0.30	–
125 μU/ml insulin	–	0.14	53.3
1250 μU/ml insulin	0.17	–	61.4

[a] Rates were calculated from the curves shown in Fig. 1 *B*. Other conditions are given in the text.

Discussion

A slow, spontaneous decay in the adenylyl cyclase reaction rate as a function of the incubation time has been demonstrated for fat cell membranes assayed in the absence of hormones, fluoride, or guanyl nucleotides. This decay is probably not the consequence of an irreversible denaturation of the enzyme since at least GMP-P(NH)P has been reported to reverse such "inactivation" (Rodbell, 1975).

Insulin markedly increases the rate of "inactivation" of adenylate cyclase. The characteristics of this inhibition of enzyme activity could suggest that the hormone is affecting the conversion of the enzyme system to a putative inactive form rather than directly suppressing the enzymatic activity itself. It is particularly pertinent that some period of time is required for the onset of the hormonal effect and that an approximately inverse relationship exists between the length of this period and the hormone concentration. In addition, after the onset of the insulin effect, the magnitude of the inhibition is relatively independent of the insulin concentration in a range between 100 and 1000 μU per ml. This is shown in Table 2, which compares the transient reaction rates and the corresponding values for the inhibitions observed at these two concentrations of insulin. It is evident that the kinetics of the insulin inhibition of enzyme activity are complex and not subject to meaningful quantitative interpretations without a better mechanistic understanding.

The effect of insulin on adenylate cyclase is strikingly dependent on the composition of the reaction mixture as well as on the type of membrane preparation used. The inactivation is only observed clearly and reproducibly when Mn^{++} instead of Mg^{++} is included as the standard

divalent cation. Although Mn^{++} has been used only rarely in studies on adenylate cyclase from mammalian cells (Birnbaumer, Pohl & Rodbell, 1971), the available evidence indicates that the enzyme activity measured in its presence could possibly reflect some important physiological functions. In the case of the ascomycete fungus, *Neurospora crassa*, the membrane-associated adenylyl cyclase displays an almost absolute requirement for Mn^{++} (Flawiá & Torres, 1972) and a Mn^{++}-dependent adenylyl cyclase has also been described recently in rat seminiferous tubules (Braun & Richard, 1975). These observations might indicate that Mn^{++}-ATP complexes could be *in vivo* substrates for this enzyme. In this regard it is important to emphasize that the association constant of ATP and Mn^{++} to form the Mn-ATP^2-complex is almost ten-times higher than that corresponding for Mg^{++} (Izatt, Christensen & Rytting, 1971). Such differences might enable the *in vivo* formation of Mn^{++}-complexes in the μM range, even in the presence of Mg^{++} concentrations 10- to 100-times higher than those of Mn^{++}. It is also important that the fat cell enzyme activity in the presence of Mn^{++} is substantially greater than that observed with Mg^{++} (Fig. 7).

Whatever the exact role of Mn^{++} as a component of the substrate complex for adenylyl cyclase, the possible involvement of a metal-enzyme complex in the inactivation of the enzyme cannot be overlooked. In this regard there appears to be a negative correlation between the magnitude of the insulin effect in the presence of Mn^{++} or Mg^{++} and the ability of the divalent cation to affect spontaneous inactivation of the cyclase (Fig. 7).

Two types of evidence suggest that the insulin-dependent inactivation of adenylyl cyclase is a reversible phenomenon. Firstly, in some experiments, such as in that shown in Fig. 1*B*, the insulin effect tends to revert spontaneously at the longest incubation periods examined. Also, the reversion of the insulin effect is complete in assays to which the upper fraction of the sucrose gradient has been added (Fig. 6).

The detailed kinetic properties of these effects of insulin on adenylate cyclase are presented elsewhere along with a more detailed description of the general behavior of this enzyme (Torres *et al.*, 1978*a*, *b*).

In considering the molecular basis of insulin action, the possible effects of this hormone on other enzymes must be taken into consideration. In the case of the low K_m cyclic AMP phosphodiesterase, insulin treatment of intact isolated fat cells leads to an increase in the enzyme activity when subsequently measured in broken cell preparations, (Loten & Sneyd, 1970; Manganiello & Vaughan, 1973; Zinman & Hollenberg,

1974). A similar effect has also been reported for a membrane-bound phosphodiesterase in livers from diabetic rats treated with the hormone (Thompson, Little & Williams 1973). In addition, the hormone also slightly stimulates an ATPase system of the adipocyte plasma membrane (Jarrett & Smith, 1974).

The evidence discussed in this report indicates that some of the insulin effects could be mediated through a depression of the rate of cyclic AMP synthesis. The ability of insulin to decrease intracellular cyclic AMP levels is well documented in the literature, although several investigators have not been able to demonstrate such effects. Under certain conditions the hormone leads to such a decrease in adipose tissue cells (Butcher *et al.*, 1966; Manganiello, Murad & Vaughan, 1971), liver (Jefferson *et al.*, 1968) and cultured cells (Otten, Johnson & Pastan, 1972; Sheppard, 1972; Jiménex de Asúa, *et al.*, 1973). In the latter system the insulin effect could also be explained in terms of a decrease of adenylyl cyclase activity measured in the presence of Mn^{++} (Jiménex de Asúa *et al.*, 1973). However, certain metabolic parameters affected by this hormone, such as those related to membrane transport, cannot yet be adequately explained on the basis of changes in the concentration of this cyclic nucleotide. In addition, it has been difficult to quantitatively correlate the cellular content of cyclic AMP with physiological responses to the hormone (Fain, 1973; Kono & Barham, 1973; Lavis & Williams, 1973; Trueheart, Herrera & Jungas, 1973). It is possible that insulin-receptor complexes might interact simultaneously with several membrane-associated systems [e.g., as allowed by the mobile receptor hypothesis (Cuatrecasas, 1974; Cuatrecasas & Hollenberg, 1976)], or that a yet unrecognized biochemical consequence might precede and secondarily modify several independent membrane-associated processes.

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