occurring during malignant transformation.

Primary chick skin fibroblasts, grown to confluence in F-12 medium containing serum (0.5 percent) on 60-mm tissue culture plates (Falcon) (*3*), were incubated with $2 \times 10^{-5}M$ cycloheximide, a concentration which inhibits 95 percent of the incorporation of [^{3}H]leucine into material that is precipitable by trichloroacetic acid. The cells were suspended after a 10-minute incubation at 37°C with 5 ml of 0.02 percent ethylenediaminetetraacetic acid in Puck's saline G, by gentle pipetting, and then centrifuged in the cold for 5 minutes at 1000 rev/min. The pellet of cells was suspended in saline G at a concentration of 4×10^6 cells per milliliter. A portion of the suspension (0.1 ml) was added to 0.1 ml of serial dilutions from 50 to 2000 μg of Con A per milliliter (Calbiochem) in saline G and shaken for 10 minutes at 20°C (*4*). Cells shaken by this procedure do not form excessively large clumps and, although the amount of agglutination varies, agglutination can be quantified under a microscope as the percentage of cells present in clumps of two or more cells. Agglutination increased from 30 percent in untreated cells to more than 75 percent in treated cells (Fig. 1). This increase occurred linearly over the first 4 to 6 hours of cycloheximide treatment and persisted for at least 20 hours of incubation with cycloheximide. Agglutination after 6 hours was about the same as that obtained from a standard procedure of trypsin treatment (*5*). The agglutination by Con A of the untreated cells may reflect their embryonic origin (*6*) but it was similar in degree over a concentration range of 100 to 2000 μg of Con A per milliliter of solution.

The increased agglutinability resulted from inhibition of cellular protein synthesis rather than from some other effect of cycloheximide, because inhibition of more than 95 percent of protein synthesis by $10^{-6}M$ emetine (*7*) or $10^{-7}M$ pactamycin (*8*) increased the agglutinability of cells by Con A just as cycloheximide did (Fig. 1).

The increased agglutination of cells treated with cycloheximide cannot be attributed to any irreversible damage to the cells; normal (low level) agglutination reappears within 6 hours after the removal of cycloheximide from cells previously treated for 5 hours. This reversal of agglutinability follows the same time course as did the original increase in agglutinability brought about by inhibiting protein synthesis.

The results were obtained with confluent, contact-inhibited cell cultures. Since there is evidence that the glycoproteins of the cell surface turn over rapidly in contact-inhibited cells but not in growing cells (*9*), we examined the Con A agglutination of growing cells treated with cycloheximide. Growing cells did not have an increase in agglutinability even after a 20-hour incubation in cycloheximide (Fig. 1), suggesting that the components of the cell surface are more stable in growing cells.

The increase in Con A agglutination of contact-inhibited cells that were exposed to cycloheximide probably indicates the loss of cell surface molecules containing protein. This may be a result of either the turnover or the release of these molecules by contact-inhibited cells.

There are a number of observations which relate the increase in Con A agglutinability to the failure of cells to respond to contact inhibition (*2*, *10*). Thus, the molecules which turn over in cells that are contact inhibited may be intimately involved in the maintenance of the cell surface structure necessary for contact inhibition. The cells treated with cycloheximide may be released from contact inhibition as they become agglutinable, but it is difficult to test this possibility because of the absence of growth during inhibition of protein synthesis.

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Modulation of Adenylate Cyclase Activity in Liver and Fat Cell Membranes by Insulin

Abstract. *Insulin depresses both the activity of adenylate cyclase stimulated by glucagon, epinephrine, and sodium fluoride in liver cell membranes and the activity of adenylate cyclase stimulated by epinephrine and adrenocorticotropin in particulate preparations from homogenates of isolated fat cells. Significant inhibition is detected with very low concentrations (10^{-11} molar) of insulin but not with unphysiologically high ($> 10^{-9}$ molar) concentrations of the hormone. These direct effects of insulin on an enzymatic system in broken-cell preparations suggest a fundamental role of adenylate cyclase activity and of cyclic adenosine monophosphate in the mechanism of action of insulin.*

It has not been possible to explain, by a single common mechanism, the effects of insulin on such diverse activities as membrane transport, lipolysis, glycogen synthesis, and protein metabolism. In that most of these can be classified as anabolic processes and are thus the reverse of processes mediated by adenosine 3′,5′-monophosphate (cyclic AMP), a fundamental effect of insulin may be to modulate the intracellular concentration of this nucleotide.

Under certain conditions insulin can decrease the concentration of cyclic AMP in adipose tissue cells (*1*) and in liver cells (*2*), and these changes appear to correlate with at least some of the biological effects of insulin in these tissues (*1–3*). The manner by which insulin lowers cyclic AMP concentrations, however, is unknown. Senft *et al.* (*4*) have reported a decrease in activity of cyclic AMP phosphodiesterase which can be reversed by insulin administration in rats made diabetic by injection of alloxan, but others (*5*) have failed to detect effects on this enzyme by administration in vivo or in vitro of insulin. Furthermore, the inability to demonstrate effects of insulin on adenyl-

ate cyclase activity in preparation of broken fat cells (*1*, *6*) has failed to support the view that insulin may act directly by modulating the synthesis of this cyclic nucleotide. It has therefore been necessary to invoke indirect mechanisms that are dependent on cell integrity to explain the effects of insulin on cyclic AMP metabolism (*1*, *7*).

We have demonstrated that, under certain conditions, physiological concentrations of insulin of 5 microunits per milliliter (5 μU/ml) can directly inhibit adenylate cyclase activity that had been stimulated in partially purified liver cell membranes and in particulate preparations of homogenates of isolated adipose tissue cells. Effects of physiologic concentrations of insulin on enzymic or other biochemical processes have not been shown, up to now, in subcellular systems.

Liver cell membranes from Sprague-Dawley rats (80 to 140 g) were prepared (*8*) by differential centrifugation of homogenates (in 0.25*M* sucrose) prepared with a Polytron PT-10 (Brinkmann) at 21,000 rev/min for 90 seconds. After centrifugation for 10 minutes at 600*g*, the supernatant was centrifuged at 12,000*g* for 30 minutes. This supernatant was adjusted with NaCl (0.1*M*) and $MgSO_4$ (0.2 m*M*) and centrifuged at 40,000*g* for 40 minutes. The pellet was then suspended in 0.05*M* tris(hydroxymethyl)aminomethane-HCl buffer (*p*H 7.4), homogenized, and centrifuged at 40,000*g* for 40 minutes; these steps were repeated three times. The membranes obtained in this way exhibit adenylate cyclase activity that is sensitive to NaF, epinephrine, and glucagon (*8*), and the membranes contain specific receptors for glucagon (*8*) and insulin (*9*). They rapidly degrade glucagon (*8*), as was reported for liver cell membranes prepared by different procedures (*10*); but no significant inactivation of [^{125}I]insulin can be detected at concentrations lower than $10^{-9}M$ (*9*). Isolated fat cells, obtained from rats (*11*), were homogenized (Polytron) and centrifuged at 23,000*g* to obtain a particulate fraction (referred to as "membrane fraction") which contains virtually all of the specific insulin-binding capacity detectable in intact cells (*12*). We measured adenylate cyclase activity (*13*) with [α-^{32}P]adenosine triphosphate (ATP) (*13a*).

Although insulin does not clearly inhibit the baseline activity (*14*) of adenylate cyclase in liver cell membranes, the enzyme activity stimulated by epinephrine or glucagon is significantly depressed by 5 to 50 μU of insulin (Table 1). The adenylate cyclase activity stimulated by low concentrations of NaF is also inhibited by 5 μU of insulin per milliliter. As the concentration of insulin is raised to unphysiologic levels (200 μU/ml and above) the inhibitory effects are abolished and, instead, stimulation of activity occurs.

Table 1. Effect of insulin on adenylate cyclase activity in isolated liver cell membranes. Incubation mixtures contain 50 m*M* tris-HCl buffer, *p*H 7.6, 5 m*M* $MgCl_2$, 1 m*M* ethylenediaminetetraacetic acid, 2.5 m*M* theophylline, 0.1 percent albumin (weight/volume), 5 m*M* [α-^{32}P]ATP (1.5 μc), 5 m*M* phosphoenolpyruvate, 6 μg of pyruvate kinase (E.C. 2.7.1.40), 40 μg of membrane protein, and the compounds listed in the table in a total volume of 0.1 ml. After incubation for 10 minutes at 30°C the reaction is stopped by adding excess cyclic AMP (containing [^{3}H]cyclic AMP for determination of recovered compounds) and boiling for 3 minutes. Cyclic AMP is isolated by chromatography on AG-50 WX2, 100 to 200 mesh, followed by precipitation with barium hydroxide and zinc sulfate (*13*). All incubations are performed in triplicate. Adenylate cyclase activity is expressed in nanomoles of cyclic AMP produced per minute per milligram of membrane protein. Results are given as averages ± standard error of the mean.

Addition	Adenylate cyclase activity at insulin concentrations (μU/ml) of:					
	0	5	50	200	500	1000
None	30 ± 2	27 ± 1.5	30 ± 2	37 ± 3		37 ± 3
Epinephrine, 0.1 m*M*	66 ± 7	40 ± 5		57 ± 5	76 ± 6	
Glucagon, $10^{-10}M$	30 ± 2	28 ± 2				
$10^{-9}M$	50 ± 4	50 ± 5	33 ± 3	41 ± 3	66 ± 7	70 ± 8
$10^{-8}M$	83 ± 8	77 ± 6	66 ± 7	73 ± 7	88 ± 8	90 ± 10
NaF, 0.2 m*M*	85 ± 5	70 ± 3	65 ± 4			

A complicating factor in the study of glucagon-stimulated adenylate cyclase activity in liver cell membranes is the rapid degradation of glucagon by these membranes (*8*, *10*). Inactivation of glucagon can be blocked by the addition of certain peptides, such as bacitracin, which appear to compete for the degradative enzymes of the membrane (*8*). After such additions, it is possible to detect activation of adenylate cyclase with concentrations of glucagon in the physiological range (10^{-10} to $10^{-11}M$), which have not previously been used successfully to activate the enzyme in vitro. Under these conditions, glucagon-stimulated adenylate cyclase activity is definitely inhibited (Table 2) with a concentration of insulin of 5 μU/ml ($3 \times 10^{-11}M$). Desoctapeptide insulin, which is biologically inactive, does not depress or stimulate adenylate cyclase activity in concentrations from 5 to 1000 μU/ml.

In isolated fat cell membranes, insulin also inhibits the activity of adenylate cyclase when the enzyme is stimulated by epinephrine or adrenocorticotropic hormone (Table 3) (*14*). Significant effects are seen in this preparation with 5 μU of insulin per milliliter. The inhibitory effects of insulin in this tissue also disappear as the concentration of the hormone is greatly increased.

The effects of insulin on adenylate cyclase activity cannot be explained by changes in the rate of degradation of cyclic AMP since these effects of insulin are not altered by addition of large concentrations (0.1 m*M*) of unlabeled nucleotide to the liver or fat cell membrane assay mixture before initiating the enzymatic reaction. This is an important control since, despite the presence of theophylline in the incubation medium, low but significant phosphodiesterase (E.C. 3.1.4.1) activity is detectable in the liver cell membrane preparations. However, no effect of insulin can be detected on cyclic AMP breakdown or recovery (concentration range tested, 5 m*M* to 0.1 m*M* cyclic AMP) under conditions similar to those used in the assay for adenylate cyclase.

The search for a common mechanism to explain the various actions of insulin has been spurred by (i) the demonstration in fat cells (*12*), fat cell membranes (*15*), and liver cell membranes (*9*, *16*) of insulin receptor structures, which are kinetically homogeneous, and (ii) the increasingly convincing evi-

Table 2. Effect of insulin on glucagon-stimulated adenylate cyclase activity of liver membranes in the presence of bacitracin. Incubations were performed as in Table 1 except that all samples contain bacitracin (0.5 mg/ml). The latter does not by itself alter the basal activity of adenylate cyclase. Adenylate cyclase activity is expressed as nanomoles of cyclic AMP produced per minute per milligram of membrane protein. All results are given as averages ± S.E.M.; Gl, glucagon.

Addition	Adenylate cyclase activity at insulin concentrations (μU/ml) of:		
	0	5	50
None	30 ± 2	29 ± 2	30 ± 3
Gl, $10^{-10}M$	105 ± 3	70 ± 6	70 ± 7
$10^{-9}M$	130 ± 5	75 ± 8	65 ± 7
$10^{-8}M$	156 ± 10	90 ± 9	65 ± 7

Table 3. Effect of insulin on adenylate cyclase activity of membrane fraction from isolated fat cells; incubations as described in Table 1; ACTH, adrenocorticotropin. Adenylate cyclase activity is expressed as nanomoles of cyclic AMP produced per minute per milligram of membrane protein. All results are given as averages ± standard error of the mean.

Addition	Cyclic AMP (nmole min^{-1} mg^{-1}) at insulin concentrations (μU/ml) of:				
	0	50	200	500	1000
None	9 ± 1		12 ± 2		
Epinephrine, 1 μM	37 ± 3	27 ± 2	27 ± 2	39 ± 4	42 ± 4
Epinephrine, 10 μM	50 ± 4	26 ± 2	27 ± 3	50 ± 5	55 ± 6
ACTH, 0.2 μg/ml	18 ± 2	9 ± 1		20 ± 2	

dence for the localization of these receptors in the cell membrane (*15, 17*). Indeed the unifying process may be the modulation of adenylate cyclase activity in the cell membrane. Insulin can significantly affect adenylate cyclase activity in simple cell-free systems at concentrations as low as can be detected by the most sensitive biological assays ($10^{-11}M$), and these concentrations are related to the affinity of insulin for its receptor (about $5 \times 10^{-11}M$) in the same membrane preparations (*9, 12, 15*). Furthermore, insulin can decrease cyclic AMP in intact systems such as fat cells (*1*) and liver (*2*), and many of the biological effects of the hormone (effects on synthesis of glycogen and protein, on lipolysis, and on ketogenesis and ureogenesis) are opposite to those of cyclic AMP.

It is not possible, at the present time, to relate satisfactorily the modulation of adenylate cyclase activity to certain of the biological effects of insulin, such as its effects on transport processes. However, the known effects of cyclic AMP, dibutyryl cyclic AMP, and theophylline on glucose transport in fat cells (*18*), as well as the evidence that cyclic AMP is involved in the permeability of membranes to water, potassium, sodium, and calcium (*19*), suggest that there may indeed be a link between cyclic AMP and insulin-regulated transport processes. There is also no immediate explanation for the well-established fact that certain metabolic effects of insulin, such as its transport effects, occur under conditions where there is no apparent stimulation of baseline adenylate cyclase activity or elevation of intracellular cyclic AMP; furthermore, insulin does not decrease the total intracellular cyclic AMP concentration under these conditions. It must be stressed, however, that the in vivo activity of adenylate cyclase in complex metabolic states may not be reflected by the total intracellular content of the nucleotide (*1*). The inability to consistently detect effects of insulin on the baseline activity (*14*) of adenylate cyclase in the subcellular preparations may result from the lack of sensitivity of the available techniques, or it may result from uncertainties in experimentally reproducing the in vivo baseline activity of this enzyme. At the present time it is not possible to say whether the effects of insulin on adenylate cyclase explain all of the metabolic effects of insulin, or whether insulin causes major conformational changes in the membrane which in turn alter, independently, various transport and enzymic processes located in the cell membrane.

The reason for the stimulatory effects on adenylate cyclase activity observed with very high insulin concentrations is not yet known, but it does not appear to be simply a result of contamination with glucagon. The ability of insulin to depress the stimulated adenylate cyclase activity only at the lowest concentrations of the hormone probably explains the failure of others (*1, 6*) to demonstrate the inhibition since only very high concentrations appear to have been tested. Solomon *et al.* (*21*) have described similar paradoxical effects of insulin in lipolytic processes of fat cells. Recent reports (*22*) have indicated effects on liver cell membrane adenylate cyclase activity by extremely high concentrations of insulin and glucagon.

Despite the current inadequacies in trying to relate all the metabolic effects of insulin to the activity of adenylate cyclase, the effects of physiologic concentrations of the hormone on isolated preparations of the enzyme forms a basis for designing experiments to test the possible fundamental role of this enzyme in the mechanism of action of insulin.

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