*ROLE OF CYCLIC-3′,5′-AMP IN THE RESPONSE OF ADIPOSE TISSUE TO INSULIN**

BY ROBERT L. JUNGAS

DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, BOSTON, MASSACHUSETTS

Communicated by Eric G. Ball, June 22, 1966

The ability of insulin to accelerate the translocation of sugars and amino acids across cellular membranes is generally considered to be primarily responsible for its biological effects.[1] A variety of actions of this hormone have been documented, however, which do not appear to be dependent upon alterations in transport processes. One of these is the antilipolytic action of insulin on adipose tissue.[2, 3] This effect of insulin can be observed when rat epididymal adipose tissue is incubated *in vitro* in a medium containing no added substrate and is reflected in a decreased rate of release into the medium of glycerol derived from tissue triglyceride stores. Since this tissue cannot utilize glycerol owing to the absence of glycerol kinase,[4] the decreased release of glycerol must result from an action of insulin on some process involved in the regulation of lipolysis in this tissue.

The lipolytic process in adipose tissue is enhanced by a variety of hormones, including epinephrine.[5] Almost immediately following exposure of the tissue to epinephrine, a large increase in the formation of glycerol and free fatty acids is observed.[6] This observation suggested that cyclic-3′, 5′-AMP (cAMP) might be involved in the regulation of lipase activity, and evidence for this view has recently been obtained in several laboratories. It has been shown that epinephrine increases the concentration of cAMP in adipose tissue[7] and that the lipase activity of homogenates of this tissue may be increased by cAMP under certain conditions.[8] These findings have raised the question as to whether the inhibitory influence of insulin on the lipolytic activity of this tissue may result from an effect on the tissue concentration of cAMP. This paper will present evidence in support of this possibility by showing that insulin inhibits the activity of adenyl cyclase in adipose tissue. Moreover, in addition to the lipase the activity of two other enzymes known to be influenced by cAMP, glycogen phosphorylase and glycogen synthetase, are also affected by insulin.

Materials and Methods.—Rats were obtained from the Charles River Breeding Labs, Inc., North Wilmington, Mass., and had been maintained on Purina laboratory chow *ad libitum* for 1–2 weeks at the time of their use. Rats in the weight range 150–250 gm were selected for most experiments. In experiments in which tissue from fasted-refed animals was employed, the animals were fasted 3 days and then given free access for 2 days to the high-carbohydrate fat-free diet of Wooley and Sebrell[9] obtained in pelleted form from General Biochemicals, Inc. Fragments of epididymal adipose tissue obtained from rats sacrificed by decapitation were incubated in 4 ml of Krebs-Ringer phosphate medium, pH 7.4, containing half the recommended amount of calcium.[10] Air served as the gas phase. Each vessel contained tissue from 2–4 rats pooled as described previously.[2]

Assays for phosphorylase were carried out essentially as described by Sutherland and Wosilait.[11] The concentrations of the components in the assay mixture were as follows: glycogen, 6.1 mg per ml; glucose-1-phosphate, 40 mM, pH 6.4; 5′-AMP, 1 mM; and extract prepared as described under *Results* from 20–30 mg of tissue in a total volume of 0.65 ml. The mixture was incubated 15 min at 37°, then 3 min in a boiling water bath. Four ml of 0.125 *M* sodium acetate, pH 4.0, were then added and inorganic phosphate was assayed by the procedure of Lowry and Lopez.[12]

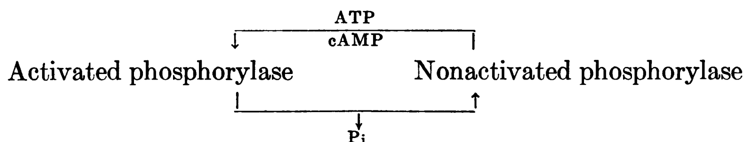
The glycogen employed was obtained from Fisher Scientific Co. and was treated with charcoal or passed over Dowex 1 resin to remove contaminating nucleotides. Inorganic phosphate was removed from the glucose-1-phosphate by precipitation with the triethylamine-ammonium molybdate reagent described by Sugino and Miyoshi.[13]

Glycogen synthetase was assayed by following the transfer of radioactivity from UDPG containing uniformly labeled glucose-C^{14} to glycogen.[14] The assay tubes contained in 0.50 ml: glycogen, 10 mg per ml; tris-Cl, pH 7.8, 50 mM; $MgCl_2$, 8 mM; EDTA, 2 mM; UDPG, 2 mM and 0.01 μc; glucose-6-phosphate, 5 mM; and extract from 30–50 mg of tissue. The tubes were incubated 15–30 min at 37°, 0.5 ml 20% KOH was added, and the mixture placed in a boiling water bath for 10 min. Glycogen was precipitated by the addition of 2 ml ethanol, recrystallized twice, and counted in a liquid scintillation instrument using Bray's fluid.[15]

Adenyl cyclase was assayed by incubating extracts of adipose tissue with ATP-U-C^{14} and following the production of labeled cAMP. Extracts were incubated 30 min at 37° in a centrifuge tube containing the following reagents: ATP-U-C^{14}, 0.1 μc and 1.4 mM; cAMP-H^3, 0.04 μc and 0.36 mM; $MgSO_4$, 2.1 mM; theophylline, 3.2 mM; and extract from 100–300 mg tissue in a total volume of 0.67 ml. The tritiated cAMP served as a measure of recovery of cAMP during its exposure to the tissue extracts and subsequent isolation. The reaction was stopped by boiling for 3 min and the mixture was then centrifuged and decanted through a millipore filter onto a column of Dowex 2-formate 4 cm by 4 mm. After washing the column with 2 ml water, 5′-AMP was removed with 10 ml 0.12 *M* formic acid. The cAMP was then eluted with 15 ml of 0.20 *M* formic acid. This fraction was lyophilized and subjected to thin-layer chromatography on silica gel using as developing solvent acetone:isopropanol:n-butanol:0.03 *M* NH_4HCO_3, pH 8.6 (2:2:3:2). cAMP was eluted from the gel with 50% ethanol and counted in Bray's solution.

The beef zinc insulin employed was kindly supplied by Dr. O. K. Behrens of Eli Lilly and Co. and assayed 25.6 units per mg. L-epinephrine bitartrate was obtained from Sigma Chemical Co. Stock solutions of both hormones were prepared in 0.004 *N* HCl and diluted daily with 0.15 *M* NaCl. ATP-U-C^{14} was a product of New England Nuclear Corp. and cAMP-H^3 was obtained from Schwarz BioResearch, Inc.

Results.—It is well known that the phosphorylase of muscle, liver, and other organs exists in two forms differing in their requirement for 5′-AMP[16] and that the interconversion of these forms is influenced by cAMP:



Accordingly, if the effect of insulin on the lipase activity of adipose tissue is a consequence of a reduction in the tissue concentration of cAMP, the phosphorylase activity should also be affected by insulin. This possibility was examined in the following manner. Paired tissues were placed into four vessels containing the phosphate-buffered medium without added substrate and incubated at 37°. After 25 min insulin was added to two of the vessels and at 30 min epinephrine was added to one control vessel and to one vessel containing insulin. Ten minutes later the tissues were removed and placed in ice-cold 0.15 *M* NaCl. The chilled tissue fragments were then quickly blotted and homogenized by hand in 2 ml 0.1 *M* NaF at room temperature using Ten Broeck all-glass homogenizers. The homogenate was centrifuged for 10 min at 600 *g* at 4° and the aqueous supernate assayed for phosphorylase activity. The results of experiments using tissue from normally fed rats are shown in Figure 1. The extracts prepared from insulin-treated tissue exhibited about 40 per cent less phosphorylase activity when assayed in the absence of added 5′-AMP. Epinephrine, on the other hand, exerted the expected activat-

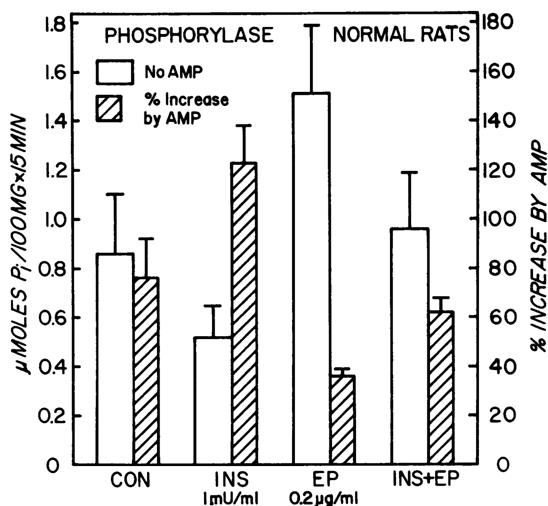


FIG. 1.—The effects of insulin and epinephrine on the phosphorylase activity of adipose tissue removed from normally fed rats. Tissue weighing 100–300 mg was employed in each vessel. The concentration of epinephrine shown refers to that of the free base. The results are the averages of five experiments. The standard errors shown in the figure reflect primarily variations in the level of phosphorylase between rats. The effects of insulin are significant at a P value of 0.02 or less both in the presence and absence of epinephrine.

ing effect[17] under these conditions, and this effect was counteracted by the simultaneous presence of insulin. The addition of 5′-AMP during the assay incubation increased the activity of the control tissue by about 75 per cent, but it had a considerably greater effect on the insulin-treated tissue. This indicates that a larger fraction of the enzyme was present in the nonactivated 5′-AMP-dependent form in the tissue exposed to insulin. The opposite was true in the presence of epinephrine and again an intermediate result was obtained when both hormones were present. Because quantitative data concerning the dependence of the several forms of adipose tissue phosphorylase on 5′-AMP are not available, it is not possible to provide a quantitative picture as to the amounts of each form of the enzyme present at any time. However, it is clear that insulin has exerted the effects to be expected, were it to lower the tissue concentration of cAMP.

Similar experiments have also been performed using tissue removed from fasted-refed rats. The results, summarized in Figure 2, revealed a qualitatively similar response to the hormones. The phosphorylase activity in the refed tissue is several-fold higher than in the normal tissue, and this would be true even if the data were expressed on a nitrogen basis.[18] In addition, a larger fraction of the enzyme is present in the activated form as shown by the relatively slight effect of adding 5′-AMP. Thus the conditions in this tissue are more favorable for demonstrating the inhibitory action of insulin than they are for showing the activating effect of epinephrine, and only a small effect of the latter hormone could be seen in this tissue. However, epinephrine did overcome the inhibitory influence of insulin when both hormones were present.

The finding that both lipase and phosphorylase activity are lowered by insulin supports the view that both actions of the hormone result from an effect on cAMP

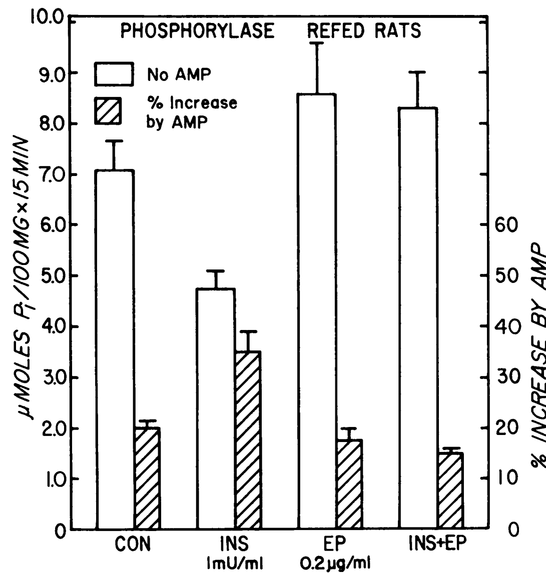
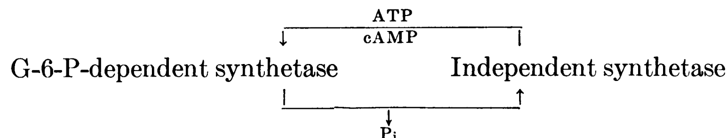


FIG. 2.—The effects of insulin and epinephrine on the phosphorylase activity of adipose tissue removed from fasted-refed rats. Tissue weighing 100–170 mg was present in each vessel. The averages of five experiments are shown. The effects of insulin on the control tissue are significant at a P value of 0.005.

production. A third enzyme known to be influenced by this nucleotide is glycogen synthetase.[19] This enzyme exists in two forms, one of which is dependent on glucose-6-phosphate for activity. These forms may be interconverted in the following manner:



Although it is clear that insulin influences this enzyme in muscle,[14, 20] previous workers were unable to show this effect in adipose tissue.[21] It was therefore necessary to clarify this point and studies were undertaken of the action of insulin on this enzyme. The experiments were carried out in the same manner as those just described except that no epinephrine was employed and the tissue was homogenized in 2 ml of 0.01 M NaF–0.005 M tris-Cl, pH 7.2. The results of an experiment using tissue from normally fed rats are shown in Table 1. When glucose-6-phosphate was omitted from the assay incubation so that only the independent form of the enzyme was measured, the extract prepared from insulin-treated tissue showed a considerably enhanced activity. This difference was abolished when glucose-6-phosphate was added. Thus insulin had no effect on the total synthetase activity but it did increase the fraction of the enzyme in the form independent of glucose-6-phosphate. This again is the result to be expected were insulin to lower the tissue levels of cAMP and is very similar to the results previously described with muscle tissue.[14, 20]

TABLE 1

EFFECT OF INSULIN ON GLYCOGEN SYNTHETASE IN ADIPOSE TISSUE

Source of supernate	Assay Conditions: Glucose-6-PO_4	Assay Conditions: Time (min)	Synthetase Activity: μMoles/100 mg	Synthetase Activity: Increase (%)
Control	—	15	0.089	—
Insulin	—	"	0.171	+93
Control	5 mM	"	0.388	—
Insulin	5 mM	"	0.395	+ 2
Control	—	30	0.243	—
Insulin	—	"	0.365	+50
Control	5 mM	"	0.716	—
Insulin	5 mM	"	0.715	0

Insulin concentration 1 milliunit per ml.

TABLE 2

EFFECT OF INSULIN ON ADENYL CYCLASE OF ADIPOSE TISSUE

Expt.	Separation method	Formation of cyclic-AMP-C^{14}, insulin/control
1	DEAE paper + silica gel TLC	0.73
2	DEAE paper + silica gel TLC	0.73
3*	Dowex 2 + silica gel TLC	0.66
4*	Dowex 2 + silica gel TLC	0.76
5*	Dowex 2 + silica gel TLC	0.76
6*, †	Dowex 2 + silica gel TLC	0.58
	Average	0.70 ± 0.03
		$P < 0.001$

* H^3-cyclic-AMP added.
† Fasted-refed rats.
Insulin concentration 1 milliunit per ml. Average of last four control values = 0.078 ± 0.013 μmoles cyclic-AMP per gram tissue in 30 min.

The tissue level of cAMP should represent the balance between the rates of its synthesis by adenyl cyclase and of its hydrolysis by the specific cAMP diesterase.

$$\text{ATP} \xrightarrow{Mg^{++}} \text{cAMP} \xrightarrow{H_2O} 5'\text{-AMP}$$

The cyclase appears to be subject to activation by a number of hormones in various tissues, including epinephrine and glucagon.[22, 23] The data presented here prompted a direct assessment of the possible influence of insulin on this enzyme. The incubation of the intact tissue, its exposure to insulin, and the preparation of 600 *g* supernates were performed in the manner just described for the assay of glycogen synthetase. These extracts were then assayed for adenyl cyclase with the results summarized in Table 2. In every case the extracts of the insulin-treated tissues showed a lower cyclase activity. On the average, insulin inhibited the cyclase by 30 per cent. More than a dozen experiments of this type have now been performed and in every case the inhibitory action of insulin has been evident. In two of the experiments included in Table 2 the preliminary separation of cAMP was achieved using DEAE cellulose paper rather than Dowex 2 columns, but this procedure has proved to be less advantageous.

An attempt was next made to influence the cyclase activity of adipose tissue extracts by adding the insulin directly to the extracts rather than to the incubation medium containing the tissue fragments. These attempts have so far proved unsuccessful, as shown in Table 3. Thus it appears that insulin affects the cyclase in some indirect manner which remains to be clarified but which may require the presence of intact fat cells.

TABLE 3

ADENYL CYCLASE—INSULIN ADDED FOLLOWING HOMOGENIZATION

Expt.	Formation of Cyclic-AMP-C^{14} (μmoles/gm in 30 min) Control	Insulin
1	0.094	0.093
2	0.050	0.033
3	0.040	0.054
4	0.026	0.024
Average	0.064 ± 0.010	0.064 ± 0.014

Insulin concentration 10 milliunits per ml.

Discussion.—The hypothesis that insulin reduces the concentration of cAMP in adipose tissue is of course amenable to direct test. However, the assay for cAMP at levels such as may be anticipated in fat cells is difficult and has not been attempted in this laboratory. Hence, the recent report of Butcher *et al.*[24] is of great interest. These workers were able to show that insulin does reduce cAMP levels in this tissue under certain conditions, namely, when epinephrine is also present. These findings coupled with the data presented here provide solid support for the premise that the effects of insulin on glycogen synthetase, glycogen phosphorylase, and triglyceride lipase are mediated by its action on adenyl cyclase. The important question remains as to whether the effects of insulin on glucose transport are also related to cAMP. No decisive information is presently available to clarify this point. In this connection it is of interest that caffeine, which is thought to elevate cAMP levels in adipose tissue by inhibiting the cAMP diesterase, has been reported to inhibit glucose utilization by this tissue.[25] Conversely, nicotinic acid, which is reported to activate the diesterase,[26] increases glucose utilization.[27]

The data presented here confirm an earlier inference that insulin acts to diminish glycogen breakdown in adipose tissue. In the previous work[28] it was noted that insulin reduced the rate at which glycogen was lost from fasted-refed tissue incubated aerobically without added substrate, and that it inhibited lactic acid production under these conditions. Moreover, the ability of epinephrine to activate glycogenolysis under anaerobic conditions was inhibited when insulin was present. Thus these observations made with intact cells confirm the physiological relevance of the present findings using adipose tissue homogenates.

In addition to the large variety of hormones and other agents which activate both the lipase and phosphorylase of adipose tissue,[29] we may now add a common inhibitor, insulin. Previous workers[30] have been unable to observe the effects of insulin on adipose tissue phosphorylase reported here. It is important to emphasize the conditions under which the present experiments were performed. In particular, note that the tissue was not exposed to insulin until 25 min after the incubation period started. When the insulin was added at the start of the incubation period, less consistent responses have been obtained, in agreement with the earlier observations.[30] The explanation for this requirement is presently unclear, although it does not appear to be important when tissue from fasted-refed animals is employed.

Summary.—The activity of adenyl cyclase in extracts of rat epididymal adipose tissue is reduced when the tissue is exposed to insulin prior to homogenization. Concomitantly, glycogen synthetase is activated and glycogen phosphorylase is inactivated. It is suggested that the effects of insulin on the latter two enzymes, as well as its inhibitory influence on lipolysis, may be secondary to a fall in the tissue levels of cAMP caused by the reduction in cyclase activity.

The expert technical assistance of Hans Schären and Charles Brendler is gratefully acknowledged. The C^{14}-labeled UDPG was kindly donated by Dr. J. B. Alpers.

* This work was supported by grant no. AM 08076 from the USPHS.

[1] Levine, R., *Diabetes*, **10**, 421 (1961).
[2] Jungas, R. L., and E. G. Ball, *Biochemistry*, **2**, 383 (1963).
[3] Mahler, R., W. S. Stafford, M. E. Tarrant, and J. Ashmore, *Diabetes*, **13**, 297 (1964).
[4] Wieland, O., and M. Suyter, *Biochem. Z.*, **329**, 320 (1957).
[5] Gordon, R. S., and A. Cherkes, *Proc. Soc. Exptl. Biol. Med.*, **97**, 150 (1958).
[6] Vaughan, M., J. E. Berger, and D. Steinberg, *J. Biol. Chem.*, **239**, 401 (1964).
[7] Butcher, R. W., R. J. Ho, H. C. Meng, and E. W. Sutherland, *J. Biol. Chem.*, **240**, 4515 (1965).
[8] Rizack, M. A., *J. Biol. Chem.*, **239**, 392 (1964).
[9] Wooley, J. G., and W. H. Sebrell, *J. Nutrition*, **29**, 191 (1945).
[10] Umbreit, W. W., R. H. Burris, and S. F. Stauffer, *Manometric Techniques* (Minneapolis: Burgess Publishing Co.,), 3rd ed., p. 149.
[11] Sutherland, E. W., and W. D. Wosilait, *J. Biol. Chem.*, **218**, 459 (1956).
[12] Lowry, O. H., and J. A. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).
[13] Sugino, Y., and Y. Miyoshi, *J. Biol. Chem.*, **239**, 2360 (1964).
[14] Villar-Palasi, C., and J. Larner, *Arch. Biochem. Biophys.*, **94**, 436 (1961).
[15] Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).
[16] Krebs, E. G., and E. H. Fischer, *Advan. Enzymol.*, **24**, 263 (1962).
[17] Vaughan, M., D. Steinberg, and E. Shafrir, *J. Clin. Invest.*, **38**, 1051 (1959).
[18] Ball, E. G., and R. L. Jungas, *Biochemistry*, **2**, 586 (1963).
[19] Rosell-Perez, M., and J. Larner, *Biochemistry*, **3**, 81 (1964).
[20] Danforth, W. H., *J. Biol. Chem.*, **240**, 588 (1965).
[21] Gutman, A., and E. Shafrir, *Am. J. Physiol.*, **207**, 1215 (1964).
[22] Rall, T. W., and E. W. Sutherland, *J. Biol. Chem.*, **232**, 1065 (1958).
[23] Sutherland, E. W., I. Oye, and R. W. Butcher, *Recent Progr. Hormone Res.*, **21**, 623 (1965).
[24] Butcher, R. W., J. G. T. Sneyd, C. R. Park, and E. W. Sutherland, *J. Biol. Chem.*, **241**, 1651 (1966).
[25] Vaughan, M., *J. Biol. Chem.*, **236**, 2196 (1961).
[26] Krishna, G., B. Weiss, J. I. Davies, and S. Hynie, *Federation Proc.*, **25**, 719 (1966).
[27] Krahl, M. E., *Am. J. Physiol.*, **207**, 1169 (1964).
[28] Jungas, R. L., and E. G. Ball, *Biochemistry*, **3**, 1696 (1964).
[29] Ball, E. G., and R. L. Jungas, these PROCEEDINGS, **47**, 932 (1961).
[30] Vaughan, M., *J. Biol. Chem.*, **235**, 3049 (1960).