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Adenyl Cyclase Activity in Particles from Fat Cells*

Martha Vaughan and Ferid Murad

ABSTRACT: Adenyl cyclase activity has been studied in a particle fraction prepared from fat cells which consists in the main of smooth membrane fragments. These preparations can be stored for at least 2 weeks at −80° without significant loss of cyclase activity or diminution of responsiveness to epinephrine. Cyclase activity was assayed as described by Chase and Aurbach using α-^{32}P- or ^{3}H-labeled adenosine triphosphate. Maximal activity (basal and hormone or NaF stimulated) was observed at pH 8.0 which was used in all assays. Values for cyclase activity assayed by this procedure agreed closely with those obtained when cyclic 3′,5′adenosine monophosphate accumulation was quantified in a bioassay. Under the conditions of the assay, about 10% of 0.5 mμmole of ^{3}H-labeled 3′,5′-adenosine monophosphate was degraded. Maximal adenyl cyclase activities were observed in the presence of 3 mM NaF. Activity was less with higher concentrations. Adenyl cyclase activity was enhanced, usually to different maximal levels, by epinephrine, adrenocorticotropin or glucagon. The relative effectiveness of the three hormones varied in different preparations. The effect of epinephrine was prevented by the β-adrenergic blocking agents, dichloroisoproterenol and pronethalol, which themselves enhanced cyclase activity. No effects of insulin or prostaglandin E_1 on basal or epinephrine-stimulated cyclase activity could be demonstrated.

It is generally believed that epinephrine and other so-called fat-mobilizing hormones stimulate lipolysis in fat cells by enhancing the activity of adenyl cyclase, thus producing an increase in the intracellular concentration of 3′,5′-AMP which nucleotide in turn brings about activation of a specific lipase that controls the rate of triglyceride breakdown. Several years ago it was reported that epinephrine increased cyclase activity in a particulate fraction prepared from homogenized adipose tissue (Klainer *et al.*, 1962). More recently, Rodbell (1967) has demonstrated stimulation of cyclase activity by ACTH in "ghosts" prepared from fat cells. It has been difficult, however, to obtain cell-free preparations with which large hormone effects can be consistently demonstrated and which can be stored without loss of responsiveness to hormones. A procedure has been devised for preparing a particulate fraction from fat cells which can be stored at −80° for at least 2 weeks without significant loss of cyclase activity or diminution of responsiveness to epinephrine. Some characteristics of the adenyl cyclase activity in such preparations are reported below.

Materials and Methods

Epididymal fat pads were obtained from Osborne–Mendel rats (120–170 g) that were permitted free access to food until they were decapitated. Fat cells were prepared as described by Rodbell (1966). Incubations with collagenase were carried out in Krebs–Ringer phosphate medium containing bovine serum albumin, 30 mg/ml, for 1 hr. After passage through a silk screen, the cells were washed three times with Krebs–Ringer phosphate medium from which Ca^{2+} and Mg^{2+} were omitted (albumin, 30 mg/ml). Cells were then washed once with a solution containing 2 mM glycylglycine buffer (pH 7.5) and 1 mM magnesium sulfate. Incubations and washes were carried out at 37°. The cells were dispersed again in glycylglycine–$MgSO_4$ solution (approximately 10 ml/5 g of fat pad) and stirred constantly while freezing in a bath of ethanol and Dry Ice. When the cell suspension was solidified it was removed from the freezing bath and permitted to thaw at room temperature with stirring. As soon as it was completely thawed, a large portion of the lard-like fat was rapidly removed and the fluid was centrifuged for 10 min at *ca.* 12,000*g* (4°). The supernatant fluid was discarded; small clumps of fat were removed from the walls of the tubes; 0.5 ml of cold glycylglycine–$MgSO_4$ solution was added to each tube and the sediment was dispersed using a Vortex mixer. The contents of all the tubes were pooled; each tube was washed again with 0.5 ml of cold medium and the washings were added to the pooled sediments. The latter was mixed again with a Vortex mixer to obtain an apparently homogeneous suspension, then diluted with cold medium (total volume *ca.* 10 ml/5 g of fat pad) and replaced in centrifuge tubes. After centrifugation for 10 min at 2000–3000*g* (usually *ca.* 2800*g*), the sediment was suspended in a small volume of the same medium (*ca.* 1 ml/5 g of fat pad) using the Vortex mixer. The protein content (Lowry *et al.*, 1951) of the final suspension was usually about 2 mg/ml (range 1.5–4).

Each preparation was divided into several portions which were immediately frozen in a Dry-Ice–ethanol bath and stored

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TABLE I: Adenyl Cyclase Activity in Particle Fractions Separated by Centrifugation.[a]

Expt	Sediment (*g* for 10 min)	Protein (Total mg)	3′,5′-AMP Accumulated (mμmoles/mg of Protein 10 min)				
			C	E	A	G	F
1	800	1.6	0		0.45	0.48	1.64
	2,800	1.5	0.10	0.78	0.79	0.48	2.01
	15,000	0.6	0.24	0.34	0.29	0.18	1.78
2	2,200	8.1	0.36	2.84	0.55	1.32	5.03
	12,000	6.1	0.47	2.16	0.51	1.59	5.86

[a] The sediment obtained from centrifugation at 15,000*g* was suspended as described in Methods. After centrifugation at the lowest speed indicated, the resulting supernatant fluid was centrifuged again to collect another fraction of particles, and in expt 1 this supernatant fluid was centrifuged again to yield a third fraction of particles. C = without hormone or NaF; in expt 1, E = epinephrine, 4.2×10^{-5} M; A = ACTH, 0.77 U/ml; G = glucagon, 7.7 μg/ml; F = NaF, 7.7 mM. In expt 2, E, 8.4×10^{-5} M; A, 1.5 U/ml; G, 15.4 μg/ml; F, 3.1 mM. [^{3}H]ATP was used in assays.

at −80°. Particles stored at −80° for up to 2 weeks and assayed repeatedly during that time have exhibited no significant change in basal activity or response to ephinephrine. The stability of the responsiveness to glucagon and ACTH has not been systematically investigated. In a few experiments it has appeared that the magnitude of the response to ACTH was diminished in stored preparations in which the response to epinephrine and glucagon was unimpaired, but further studies will be necessary to establish the reproducibility and significance of these observations.

Several experiments were carried out to compare the basal activity and effects of hormones in particulate fractions separated by differential centrifugation. In expt 1, of Table I, three fractions of particles were separated by centrifugation from the initial 15,000*g* sediment. Although there were no large or consistent differences in the specific activity of the three fractions when assayed in the presence of NaF, large effects of hormones were observed only with the two heavier fractions. In a few more recent experiments, *e.g.*, expt 2 of Table I, we have found little difference in the basal activity or hormone effects with different fractions of particles. In addition, the specific activity of these preparations was, in general, distinctly higher than that of the majority of earlier preparations. The reasons for these differences are not readily apparent. We have not compared the morphology of various preparations with high or low specific activity or with wide differences in patterns of hormone response. One preparation of particles, collected at 2200*g* and examined by electron microscopy, consisted predominantly of smooth membrane fragments but included some rough endoplasmic reticulum, mitochondria, and dense granules about 0.2 μ in diameter.

Adenyl cyclase was assayed using [α-^{32}P]ATP essentially as described by Chase and Aurbach (1968), except that recovery of 3′5′-AMP was estimated from the optical density at 260 mμ of the purified nucleotide fraction in which ^{32}P was assayed (Weiss and Costa, 1967). The final reaction mixture for the assay contained usually 38.5 mM Tris-HCl buffer (pH 8.0), 7.7 mM caffeine, 2.3 mM ATP (10–20 cpm/μμmole), 4.6 mM $MgCl_2$, 0.77 mM glycylglycine buffer, and 0.38 mM $MgSO_4$. The total volume of the assay mixture was 65 μl containing usually 25 μl of particle suspension (40–80 μg of protein). Incubations were carried out at 37° for 10 min unless otherwise stated, after which 0.5 ml of a solution containing 7 mM ATP, 2.5 mM 3′5′-AMP, and 10 mM Tris-HCl buffer (pH 7.5) was added and the tubes were placed in boiling water for 3 min. Labeled 3′,5′-AMP was separated from other ^{32}P-labeled compounds and counted, as described by Chase and Aurbach (1968) and by Krishna *et al.* (1968). In a few experiments as indicated below, [^{3}H]ATP was used instead of [^{32}P]ATP. Except in Figures 1, 2, and 6, in which each point represents the result of a single assay, all values presented in figures and tables are the mean of duplicate (occasionally triplicate) assays. Values usually agreed within 5–10%.

[^{3}H]3′5′-AMP was used to investigate the degradation of 3′,5′-AMP under the conditions used for assay of adenyl cyclase (but without radioactive ATP). The incubation period was terminated in these experiments by the addition of 100 μl of a mixture of ATP, 5′-AMP, 3′,5′-AMP, and adenosine (in some cases also inosine or ADP). After heating for 3 min in a boiling-water bath, the tubes were cooled and centrifuged. A 50-μl sample of each supernatant fluid was placed directly in a counting vial and another 50-μl sample was applied to Whatman 1 paper which was then developed overnight using usually a solvent mixture of 95% ethanol and 1 M ammonium acetate (pH 5.0) with 3 mM EDTA (70:30, v/v). After the papers were dried, the spots were outlined under ultraviolet light, cut out, and divided into small pieces which were placed in a glass counting vial; 1 ml of 0.1 N HCl was added. After 30 min at room temperature 50 μl of 2 N NaOH was added followed by 15 ml of the scintillation counting solution described by Bray (1960). Similar additions were made to the vials which contained samples of the total supernatant fluid.

L-Epinephrine bitartrate was purchased from Nutritional Biochemical Co. Crystalline glucagon (lot 258-234B-167-1) and glucagon-free amorphous insulin (20 U/mg) were obtained from the Eli Lilly Co. through the courtesy of Dr. O. K. Behrens. Porcine ACTH was purchased from Calbiochem. Prostaglandin E_1 was obtained from the Upjohn Co. Propanolol and pronethalol (Ayerst Laboratories, Inc., New York) were diluted for use from ampoules prepared for parenteral use. [α-^{32}P]ATP and [^{3}H]ATP were purchased from International

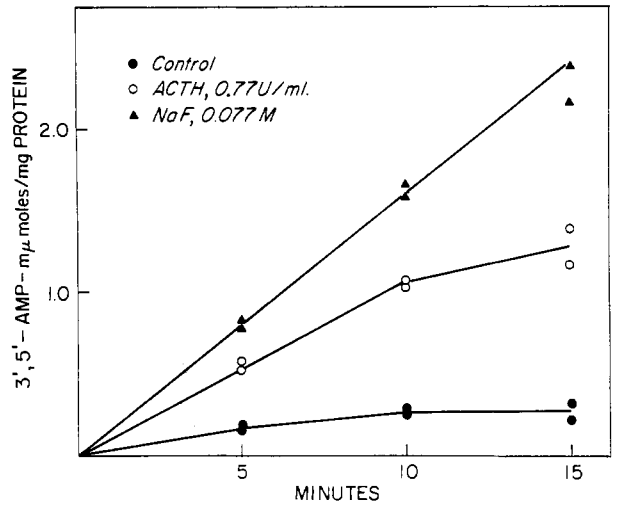

FIGURE 1: Accumulation of [^{3}H]3',5'-AMP as a function of time. Each assay contained 65 μg of sediment protein.

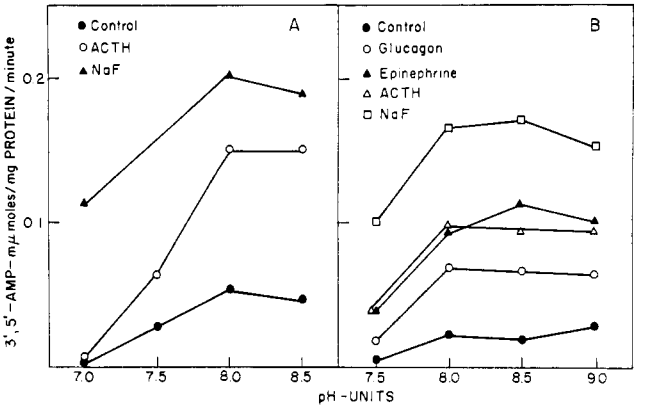

FIGURE 3: Effect of pH on adenyl cyclase activity. (A) Each assay contained 92 μg of protein incubated for 5 min with ACTH, 0.77 U/ml, or NaF, 77 mM, as indicated. (B) Each assay contained 79 μg of protein incubated for 10 min with glucagon, 7.7 μg/ml; epinephrine, 4.2 × 10^{-5} M; ACTH, 0.77 U/ml; or NaF, 77 mM, as indicated.

Chemical and Nuclear Corp., [^{3}H]3',5'-AMP from Schwartz Bioresearch Inc.

Results

The Assay. In the experiment summarized in Figure 1, the rate of accumulation of 3',5'-AMP was apparently constant for 10 min in the presence of NaF or ACTH and was almost so under control conditions. As shown in Figure 2, adenyl cyclase activity expressed as micromicromoles of 3',5'-AMP accumulated in 10 min in the presence of epinephrine or NaF was proportional to the amount of enzyme added up to about 80 μg of protein/assay. With these amounts of protein, cyclase activity in the absence of hormone or NaF was not measurable with any accuracy in this experiment.

The effect of pH on adenyl cyclase activity is shown in Figure 3. The activity under all conditions increased markedly between pH 7.5 and 8.0 and changed little between 8.0 and 9.0. Since the magnitude of the hormone effects relative to control activity as well as the absolute levels of cyclase activity were essentially maximal at pH 8.0, all of the experiments reported here were carried out at this pH. No significant difference in the effects of pH on the action of the three hormones, epinephrine, ACTH, and glucagon, was demonstrated.

As shown in Figure 4, increasing the ATP concentration from 2.3 to 3.4 mM increased 3',5'-AMP production in the presence of NaF or epinephrine by about 10% and increased the basal production by a somewhat larger percentage. Since, with lower concentration of ATP, 3',5'-AMP production was essentially constant for 10 min, and 3',5'-AMP production was directly proportional to enzyme concentration over the range used in these studies, it was used in the standard assay system despite the fact that it was not optimal. It will be noted that al-

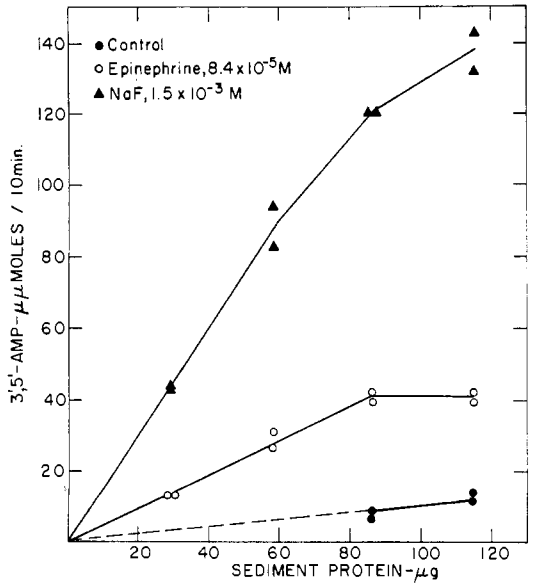

FIGURE 2: Accumulation of [^{3}H]3',5'-AMP as a function of the amount of sediment protein. Incubation for 10 min, pH 8.0.

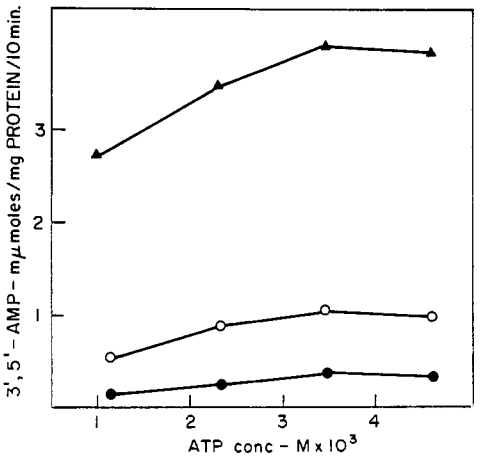

FIGURE 4: Effect of ATP concentration on adenyl cyclase activity. Each assay contained 41 μg of protein incubated for 10 min. (●) Control; (○) epinephrine, 8.4 × 10^{-5} M; (▲) NaF, 3.08 × 10^{-3} M. The concentration of $MgCl_2$ was varied along with that of ATP so that the ratio of $MgCl_2$ to ATP was 2.0 in all incubations.

TABLE II: Comparison of Adenyl Cyclase Activity Assayed by Two Methods.[a]

Additions	3′,5′-AMP Produced (mμmoles/mg of Protein 10 min)	
	From [^{3}H]ATP	Bioassay
None	0.21	0.30
Epinephrine, 1.68×10^{-4} M	1.32	1.3
ACTH, 3.1 U/ml	0.77	0.80
Glucagon, 30.8 μg/ml	1.07	1.14
NaF, 3.1×10^{-3} M	4.53	4.25

[a] Incubations for bioassay were carried out with ten times as much of each of the solutions used in the radioactive assay except that [^{3}H]ATP was omitted. After termination of the incubation by heating, appropriate dilutions of the supernatant fluid were assayed for 3′,5′-AMP by a slight modification of the method of Butcher *et al.* (1965) which is based on the acceleration of the activation of liver phosphorylase by 3′,5′-AMP.

though the ratio of $MgCl_2$ to ATP was 2 in all incubations, in fact, due to the $MgSO_4$ contained in the enzyme preparation, the ratio of Mg^{2+} to ATP varied from 2.08 at the highest ATP concentration to 2.33 at the lowest. The effect of varying the ratio of Mg^{2+} to ATP has not been systematically investigated. It may be pointed out that, although the apparent K_m for ATP in the presence of NaF is less than half of that in the absence of NaF, the amount of ATP remaining at the end of the assay period was much less in the absence of fluoride than in its presence.

Adenyl cyclase activity assayed as described above using [^{3}H]ATP was compared with that estimated using he bioassay method of Butcher *et al.* (1965). As shown in Table II, the values for cyclase activity assayed by the two procedures are in good agreement except in the case of the basal levels. In those incubations the amount of cyclic [^{3}H]AMP formed was at the lower limits of the assayable range and the duplicates deviated 20% from the mean. In all other radioactive assays, the duplicate values were within 6% of the mean. In the bioassay, duplicates varied no more than 10% from the mean.

Effect of NaF. Data from two experiments showing the effect of NaF concentration on accumulation of 3′,5′-AMP are summarized in Figure 5. Maximal cyclase activity was observed with a concentration of *ca.* 3 mM NaF. At 1.5 or 7.7 mM, activity was about 85% of the maximal. ACTH added in the presence of 7.7 mM NaF was without effect. At a concentration of 0.3 mM (not shown) NaF had little or no effect, nor did it alter the amount of 3′,5′-AMP accumulated in the presence of maximal stimulation by ACTH or glucagon. It should be noted that in the experiments presented in Figures 1 and 3, the concentration of NaF employed was 77 mM. As shown in Figure 5, the adenyl cyclase activity with this concentration of NaF is only about 50% of that assayed in the presence of 3 mM NaF.

The inhibitory effect of higher than optimal concentrations

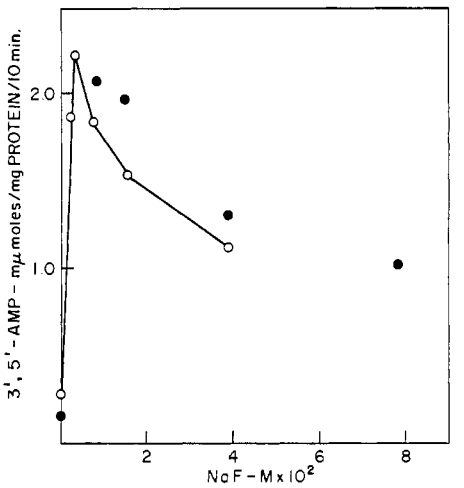

FIGURE 5: Effect of NaF concentration on adenyl cyclase activity. Data from two experiments carried out at different times with different particle preparations. (●) 86 μg of sediment protein per assay; (○) 49 μg of sediment protein per assay.

of NaF is apparently not due to the sodium ion since the effect of 38.5 mM NaF was not reproduced by a similar concentration of NaCl (Table III). The addition of 38.5 mM NaCl to assays under control conditions or in the presence of NaF, 3.8 or 7.7 mM, was associated with an approximately 30% increase in the amount of 3′,5′-AMP accumulated but in expt 3 of Table III, no stimulatory effect of 38.5 mM NaCl was observed in assays containing epinephrine or ACTH.

Hormone Effects. The effects of several concentrations of epinephrine, ACTH, or glucagon are shown in Figure 6. With the preparation of particles used in this experiment, the maximal effect observed with epinephrine was greater than that with glucagon which was in turn greater than that with ACTH. With eight different preparations of particles assayed with all three hormones at concentrations demonstrated in each experiment to be producing a maximal stimulation of cyclase activ-

TABLE III: Effect of NaCl on Adenyl Cyclase Activity.

Expt	Additions (concn)	3′,5′-AMP Produced (mμmoles/mg of Protein 10 min)	
		No NaCl	NaCl, 38.5 mM
1	None	0.30	0.39
	NaF, 3.85 mM	1.72	2.32
	NaF, 38.5 mM	1.00	
2	None	0.28	
	NaF, 7.7 mM	1.84	2.29
	NaF, 38.5 mM	1.12	
3	None	0.12	0.16
	Epinephrine, 8.4×10^{-5} M	0.74	0.71
	ACTH, 1.5 U/ml	0.26	0.23

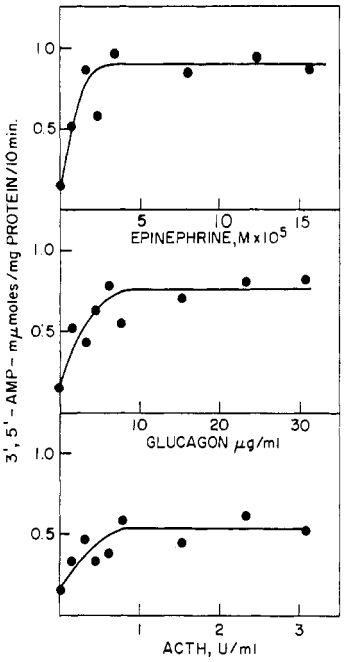

FIGURE 6: Effect of hormone concentration on adenyl cyclase activity. Each assay contained 86 μg of sediment protein.

TABLE IV: Maximal Effect of ACTH and Glucagon on Cyclase Activity Relative to that of Epinephrine.[a]

Sediment Prepn	Max Effect as % of Epinephrine Effect	
	With ACTH	With Glucagon
1	89	54
2	86	66
3	80	49
4	56	75
5	45	21
6	34	100
7	28	61
8	23	65

[a] The maximal increase over basal activity due to epinephrine ranged from 0.62 to 1.65 mμmoles of 3',5'-AMP/mg of protein 10 min for these 8 particle preparations. (Mean $\pm$ SEM = 0.87 $\pm$ 0.22.) The basal activity was 0.19 $\pm$ 0.025. The data included here are only for sediment fractions assayed immediately after preparation since with some preparations the magnitude of the ACTH effect appeared to decrease with storage at $-80°$ while the effects of the other two hormones were unchanged.

ity, the effect of epinephrine was always greatest, whereas the magnitude of the effects of the peptide hormones relative to that of epinephrine varied widely. Data obtained with these preparations are summarized in Table IV. There was no apparent correlation between the magnitude of the epinephrine effect and the relative activity of the other hormones.

The effects of combinations of hormones are summarized in Table V. In expt 1, the addition of ACTH in the presence of epinephrine at a concentration sufficient to produce maximal stimulation of 3',5'-AMP accumulation was without effect. In two out of three experiments, however, the effect of epinephrine plus glucagon was greater than the effect of either alone. Glucagon and ACTH together in two experiments produced an effect greater than that of either hormone alone. In fact, the effects appeared to be additive.

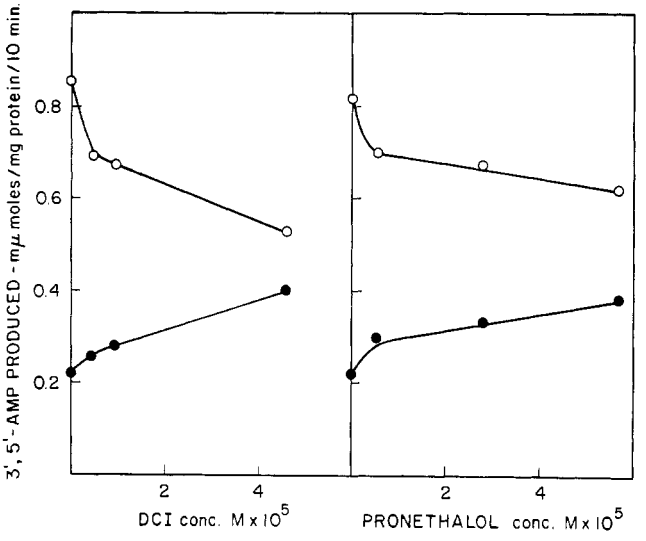

FIGURE 7: Effect of dichloroisoproterenol and pronethalol on adenyl cyclase activity. Each assay contained 72 μg of sediment protein. (●) No epinephrine; (○) plus epinephrine, 4.2×10^{-5} M. (The experiments with dichloroisoproterenol and pronethalol were carried out on successive days using samples of the same preparation of particles.)

Stimulation of cyclase activity by epinephrine was inhibited by the β-adrenergic blocking agents, dichloroisoproterenol and pronethalol, which themselves induced an increase in 3',5'-AMP accumulation. As shown in Figure 7, the effect of 4.2×10^{-5} M epinephrine was decreased 50% by an equimolar concentration of pronethalol and almost 80% by a similar concentration of dichloroisoproterenol. In another experiment, the effect of the same concentration of epinephrine was decreased by 90% in the presence of four times that concentration of dichloroisoproterenol and was completely blocked by propanolol at a molar concentration seven times that of the epinephrine. In the latter experiment propanolol (3×10^{-4} M) had no effect in the absence of epinephrine. It has been reported earlier that dichloroisoproterenol (Butcher *et al.*, 1965) and pronethalol (Butcher and Sutherland, 1967) both slightly increased the concentration of 3',5'-AMP in fat pads when added alone to the incubation medium. When either was added in the presence of epinephrine, the level of 3',5'-AMP was lower than it was in the presence of epinephrine alone. Murad *et al.* (1962) had previously found that the effect of epinephrine on cyclase activity in particle fractions from dog heart and liver was inhibited by dichloroisoproterenol which alone had a slight stimulatory effect on the preparation from heart but not on that from liver.

Insulin, prostaglandin E_1, and nicotinic acid all have been shown to decrease the concentration of 3',5'-AMP in fat cells incubated in medium containing caffeine and epinephrine (Butcher *et al.*, 1968; Butcher and Baird, 1968). Insulin (0.015-

TABLE V: Effect of Combinations of Hormones on Accumulation of 3′,5′-AMP.[a]

Hormone(s) Added	3′,5′-AMP (mμmoles/mg of Protein 10 min) Δ Due to Hormone		
	Expt 1	Expt 2	Expt 3
Epinephrine	0.79 ± 0.143 (5)	0.70 ± 0.052 (4)	1.01 ± 0.078 (4)
ACTH	0.36 ± 0.079 (6)	0.20 ± 0.029 (4)	
Glucagon	0.37 ± 0.091 (6)	0.66 ± 0.067 (4)	0.93 ± 0.057 (4)
Epinephrine + ACTH	0.91 ± 0.317 (3)		
Epinephrine + glucagon	0.88 ± 0.115 (2)	1.25 ± 0.145 (2)	1.40 ± 0.008 (2)
ACTH + glucagon	0.87 ± 0.117 (4)	0.82 ± 0.040 (2)	

[a] Data for effects of each hormone alone are based on assays at each of two concentrations, *i.e.*, epinephrine, 8.4×10^{-5} and 16.8×10^{-5} M; ACTH, 1.5 and 3.0 U/ml; and glucagon 15.4 and 30.8 μg/ml. In each case the lower concentration of the hormone was apparently producing a maximal effect for that hormone. Each hormone was used at the lower concentration in the assays containing two hormones in expt 1, and at the higher concentrations in expt 2 and 3. [^{3}H]ATP was used in expt 2 and 3. The values in the table are the mean plus or minus standard error of the mean for the number of assays indicated in parentheses.

30.8 mU/ml) had no effect on cyclase activity in our experiments when added alone or in the presence of epinephrine (4.2 or 8.5×10^{-5} M). Prostaglandin E_1 (7.7–38.5 μg/ml) and carbachol (0.015–1.5 mM) were also without effect in similar experiments. Accumulation of 3′,5′-AMP in the presence of epinephrine was not altered by the addition of 10^{-3} M nicotinic acid. Nor was there any effect of nicotinic acid on the degradation of 3′,5′-AMP in the assay system, whether or not caffeine was present.

Since the presence of Ca^{2+} ion in the incubation medium influences greatly the sensitivity of fat cells to the lipolytic action of ACTH, and to a lesser extent that of epinephrine (Mosinger and Vaughan, 1967), the effect of $CaCl_2$ on fat cell cyclase activity was investigated. $CaCl_2$ (8.5×10^{-5} M) inhibited both basal and epinephrine-stimulated activity by 40–45%, and 8.5×10^{-6} M inhibited about 20%. Neither basal nor ACTH-stimulated activity was influenced by 4.2×10^{-6} M $CaCl_2$. Øye and Sutherland (1966) reported that the epinephrine responsiveness of cyclase in erythrocyte membranes decreased during storage in the cold and could be restored by treatment with mercaptoethanol. With several fat cell cyclase preparations, dithiothreitol (7.7×10^{-4} M), enhanced basal, epinephrine-, and ACTH-stimulated activity by about 30%, but with other preparations no effect was observed. There was no apparent correlation between the age of the particles and the presence or absence of stimulation by dithiothreitol.

Metabolism of 3′,5′-AMP in the Assay. As shown in Table VI, under the conditions of the assay, about 10% of 0.5 mμmole of ^{3}H-labeled 3′,5′-AMP was degraded. (The amount of 3′,5′-AMP formed during an assay ranged from 0.005 to 0.20 mμmoles, occasionally more in the presence of NaF.) When caffeine was omitted, approximately 40% of the 3′,5′-AMP was degraded in 10 min. ATP did not significantly influence the disappearance of 3′,5′-AMP, but markedly affected the distribution of ^{3}H in the products. When both ATP and caffeine were omitted, adenosine was the major product of 3′,5′-AMP degradation. In the presence of caffeine, only about 25% of the ^{3}H that disappeared from 3′,5′-AMP appeared in adenosine. Most of the remainder was cochromatographed with 5′-AMP. When ATP was present, whether or not caffeine was added, essentially no ^{3}H accumulated in adenosine and only 25–35% of the ^{3}H that disappeared from 3′,5′-AMP could be recovered as 5′-AMP. Most of the remainder was recovered from areas of the chromatograms occupied by ATP and ADP.

Several attempts were made to demonstrate synthesis of ATP from 3′,5′-AMP, *i.e.*, reversal of the cyclase reaction. Even in the absence of added ATP and pyrophosphate, a small amount of ^{3}H, 0.1–0.2% of [^{3}H]3′,5′-AMP, was incorporated into ATP and ADP in a system containing Tris-HCl buffer (pH 7.4), caffeine, and $MgCl_2$. In a few experiments this was approximately doubled by the addition of 2.3 mM sodium pyrophosphate, but in this crude particle system it seems unlikely that one can establish with any certainty the pathway by which label from 3′,5′-AMP is incorporated into ATP and ADP.

TABLE VI: Degradation of 3′,5′-AMP under Conditions of Adenyl Cyclase Assay.[a]

Caffeine (7.7 mM)	ATP (2.3 mM)	3′,5′-AMP Disappearance Percentage/10 min	$\frac{\Delta^3H \text{ in Product}}{\Delta^3H \text{ in 3′,5′-AMP}} \times 100$	
			Adenosine	5′-AMP
+	+	8.8	0	35
0	+	40.0	0	25
+	0	9.4	25	65
0	0	41.2	>90	0

[a] The reaction mixture was made up exactly as for the cyclase assays except that no labeled ATP was added and each tube contained 0.5 mμmole [^{3}H]3′,5′-AMP. Incubation was carried out for 10 min at 37°. The mean of results from two experiments with different sediment preparations is presented. In both, each incubation contained 70 μg of sediment protein and the findings were essentially identical.

Discussion

Before the method of preparing particles described above was devised, numerous attempts were made to obtain a fraction from homogenized fat pads in which the effects of hormones on adenyl cyclase activity could be studied. Various methods of homogenization in different media yielded homogenates and fractions thereof that contained cyclase activity which was markedly enhanced by NaF, but only small effects of hormones could be demonstrated. The procedure described here has invariably yielded preparations that respond well to hormones. With 17 different particle preparations, the maximal activity attained with epinephrine was 4.8 ± 0.38 (mean plus or minus standard error of the mean) times the basal level. The epinephrine maximum was 39.6 ± 5.04% of the activity assayed in the presence of NaF with ten preparations. There were, however, obvious differences between preparations both in specific activity and in the extent of activation by individual hormones. The specific activity of our preparations was of the same order of magnitude as that reported by Rodbell (1967) for fat cell "ghosts." The two kinds of preparations are probably rather similar in composition although the particles obtained after freezing the cells in hypotonic medium are apparently considerably smaller than "ghosts."

Assays like the one used in these studies have recently been employed in several laboratories, but no comparison has been reported between the apparent adenyl cyclase activities obtained with this assay and those based on bioassay of 3′,5′-AMP which have been in use for considerably longer. Often the extracts in which 3′,5′-AMP is measured contain inhibitors of the bioassay, the presence of which may necessitate correction of the assayed values for cyclic AMP (Rall and Sutherland, 1958; Murad *et al.*, 1962, 1969; Murad, 1965). The inhibitor described by Murad (1965) is apparently very similar in structure to 3′,5′-AMP and is not separated from it by most chromatographic procedures. If it were synthesized from ATP in the radioactive assay system, this method could yield falsely high values for adenyl cyclase activity. In the experiment reported above, samples used in the bioassay were tested for inhibitory activity and none was detected. It is possible that under conditions in which inhibitors are produced (or are present), the results of assays by the two methods would not agree as well as they did in this instance.

The concentration of epinephrine required to produce a maximal effect on cyclase activity (4×10^{-5} M) was approximately 100-fold that which we have found produces maximal stimulation of lipolysis in fat cells. Maximal effects of ACTH and glucagon on cyclase activity were obtained with concentrations of 0.8 U/ml and 8 μg/ml, respectively. These concentrations are about 20 times greater than those that induce maximal rates of lipolysis in fat cells. As has been observed in most other studies of adenyl cyclase from various sources, the maximal level of activity attainable with hormone stimulation was never as great as that assayed in the presence of NaF.

It has also been previously noted that two different hormones may produce different maximal levels of cyclase activity in the same tissue preparation. This is not difficult to understand in particle fractions prepared from kidney, for example, since it seems quite clear that vasopressin and parathyroid hormone act on different populations of cells and in broken cell preparations on different cyclases derived therefrom (Chase and Aurbach, 1968). Similarly in homogenates of testis, it seems probable that epinephrine and interstitial cell stimulating hormone enhance the activity of cyclase derived from different cell types. In these preparations the effect of epinephrine can be observed in the presence of maximal stimulation by interstitial cell stimulating hormone or follicle-stimulating hormone (Murad *et al.*, 1969). It has often been assumed (implicitly if not explicitly) that epinephrine and glucagon act on the same cells in liver, although this is by no means established and may in fact be incorrect. Bitensky *et al.* (1968) have recently reported that in washed particles prepared from homogenates of liver, the maximal cyclase activity attainable with glucagon is considerably higher than that produced by epinephrine and the effects of the two hormones are additive. Similarly, in particles from fat cells that we have studied, although the relative effectiveness of the three hormones varies widely from one preparation to another, the maximal effects of epinephrine, ACTH, and glucagon are usually different, and in some instances the effect of two hormones is greater than that of either alone.

There is reason to believe, however, that adenyl cyclase activity in an intact fat cell can be increased to essentially the same maximal level by all three hormones. We had found earlier (M. Vaughan, unpublished data) that although the maximal rate of glycerol production induced by glucagon was somewhat less than that attainable with epinephrine or ACTH, combinations of any two or three hormones never produced an effect greater than that of epinephrine or ACTH alone. It was quite possible, however, that the concentration of intracellular 3′,5′-AMP attained with epinephrine or ACTH alone was sufficient to maximally activate lipolysis so that any further increase would not have been reflected in an enhanced rate of glycerol production.[1] Using concentrations of the hormones that had previously been demonstrated to induce maximal effects of glycerol production, we measured the concentration of 3′,5′-AMP in fat cells (plus medium) that had been exposed to epinephrine and/or ACTH or glucagon (F. Murad, V. Manganiello, and M. Vaughan, unpublished data). The concentration of 3′,5′-AMP in cells (plus medium) incubated with glucagon was somewhat less than that in cells exposed to epinephrine, but addition of either ACTH or glucagon in the presence of epinephrine produced no further elevation. Similar results were obtained by Butcher *et al.* (1968) in experiments in which caffeine was present in the incubation medium so that the concentrations of 3′,5′-AMP were several times higher than those attained in our studies. Thus, there is no evidence that there are different populations of fat cells in terms of hormone responsiveness.

It appears that a fat cell contains receptors[2] for each of the

[1] Inferences from these experiments are based on the assumption that 3′,5′-AMP influences lipolysis only in the cell in which it is formed. At least under the conditions of these experiments, this assumption appears to be valid since it has been shown that 3′,5′-AMP added to Krebs–Ringer phosphate medium usually inhibits lipolysis (in concentrations between 10^{-6} and 10^{-3} M).

[2] The question of whether or not there are separate receptors for each of the hormones that act on cyclase seemingly must be answered in the affirmative in the sense that it would be expected that such different molecules as, for example, epinephrine and glucagon, would require chemically different structures with which to interact, given the demonstrated specificity of hormone action. The implications of this discussion are similar whether one considers that the hormone-receptor is a "regulatory" portion of the cyclase molecule or a regulatory subunit of a cyclase system, *i.e.*, not covalently bonded to the catalytic subunit, as discussed by Robison *et al.* (1967).

three hormones (and probably for others also). But is a single adenyl cyclase molecule associated with "receptors" for each of the hormones? Or does a cyclase molecule respond to only one receptor or regulatory site, *i.e.*, each cell contains several populations of cyclase molecules distinguishable at least in terms of the nature of the receptor site with which they are associated? If the latter were the case, one might expect that even after all of the "epinephrine cyclase" molecules were activated, the ACTH and glucagon cyclases would remain available for activation. Perhaps they do, and the concentration of 3′,5′-AMP is not an adequate index of cyclase activity in the fat cell experiments or perhaps there is some factor(s) that limits cyclase activity in the cell. The observations on intact fat cells, however, seem more readily compatible with the first alternative, since a cyclase molecule already "activated" by the interaction of a hormone with its receptor site presumably would not be further activated by binding of a different hormone at its receptor site.

In contrast to the findings with intact fat cells, adenyl cyclase activity of particle preparations in the presence of two hormones appeared at times to be greater than the maximal level attainable with either hormone alone. The effects of two hormones were not, however, invariably and strictly additive as might be expected if each cyclase molecule could respond to only one of the hormones. (Of course, if there were some mechanism that limits cyclase activity in the intact cell, it could be operative, perhaps to a limited or variable extent in the particle preparations.) If, in the intact cell, every cyclase molecule were associated with receptors for several hormones and could be maximally activated by interaction of a hormone at any one site, it would be necessary to postulate that in the process of preparations of the particle fractions, receptor sites for one or more hormones have been separated from the cyclase molecules or altered so that they can no longer influence enzyme activity. Receptor sites may not in fact be part of adenyl cyclase itself, but rather integrated into the organized structure of the cell membrane in such a way that formation of a hormone–receptor complex produces alterations in the configuration of the enzyme and thereby its activity. In this case extensive fragmentation of the cell membrane or alterations in the configuration of other components of the structure might lead to destruction of the capacity to respond to hormonal activation without loss of or damage to either the hormone–receptor site or the cyclase enzyme. The observed variability in responsiveness to the three hormones and in the effects of combinations of two would be compatible with this possibility.

We have not discussed the possibility that activation of adenyl cyclase does not result directly from interaction of a hormone with its receptor site, but rather that such interaction might lead through one or more steps to formation of an intermediate which could increase the activity of adenyl cyclase, either by a direct effect (*e.g.*, allosteric) or by favoring the conversion of cyclase from an inactive or less active form into a more active form. We considered that if this hypothesis were correct it might be possible to demonstrate hormonal stimulation of cyclase activity in a fraction apparently unresponsive to hormone by incubating it with a responsive fraction. Accordingly, samples of the 15,000*g* and the 2800*g* sediments from expt 1 of Table I, were assayed separately and together with and without ACTH. As shown in Table I, the activity of the 15,000*g* sediment was affected little if at all by ACTH, whereas the activity of the 2800*g* fraction was increased 7-fold. The activity of the two fractions assayed together was equal to the sum of the individual activities with and without ACTH, thus providing no evidence in support of this hypothesis. It remains, nevertheless, attractive in many ways, not the least of which is that it could readily provide an explanation for the mechanism of the stimulatory action of NaF. The presence of a cyclase-activating intermediate at the beginning of an assay period or its formation in relatively small amount (in the absence of added hormone) might, if its degradation were prevented by NaF, be sufficient to provide for apparently maximal activity of adenyl cyclase throughout the incubation period. Alternatively, inhibition by NaF of the conversion of cyclase from an active to a less active form could lead, possibly very rapidly, to accumulation of all of the cyclase in an active form.

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