

The Fat Cell Adenylate Cyclase System

CHARACTERIZATION AND MANIPULATION OF ITS BIMODAL REGULATION BY GTP*

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GTP evokes both an activatory and an inhibitory response from adipocyte adenylate cyclase. This paper describes the persistence of the bimodal response under a variety of assay conditions. Additionally, manipulations are described which eliminate one or other of these actions. Treatment of adipocyte plasma membranes with cholera toxin A₁ peptide and NAD⁺ abolishes the inhibitory phase of GTP action while preserving the activating phase. Treatment of the membranes with *p*-hydroxymercuriphenylsulfonic acid eliminates the activatory phase while maintaining the inhibitory action of the nucleotide. Thus it appears that the two processes mediated by GTP in adipocytes normally coexist and operate through different pathways since either phase can be abolished leaving the other intact. Adenosine and its purine-modified analogs inhibit fat cell adenylate cyclase in the GTP inhibitory phase (Londos, C., Cooper, D. M. F., Schlegel, W., and Rodbell, M. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 5362-5366). When this effect of GTP is abolished by either cholera toxin or Gpp(NH)p pretreatment, the inhibitory action of adenosine analogs is also lost. These data suggest a central role for GTP in mediating both activation and inhibition of adenylate cyclase by agents which act through cell surface receptors.

The enhancement of hormonal stimulation and the stimulatory effects of GTP on many adenylate cyclase systems have received widespread attention (see Ref. 1 and references therein). The adenylate cyclase of rat adipocyte plasma membranes is unusual in that GTP not only enhances activity but also causes inhibition of the enzyme (2-6). The potential regulatory significance of this latter, seemingly paradoxical behavior of GTP has become apparent from recent findings in this laboratory (7). These studies showed that the potent inhibitory actions of adenosine and its purine-modified analogs on cyclic AMP¹ production in intact adipocytes could be explained by inhibition of adenylate cyclase in the GTP inhibitory phase.² Yamamura *et al.* (6) have suggested that

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¹ The abbreviations used are: cyclic AMP, adenosine 3':5'-monophosphate; ACTH, adrenocorticotrophic hormone; Gpp(NH)p, 5'-guanylylimidodiphosphate; $K_{0.5}$, the concentration of an agent evoking half of its maximal effect.

² Throughout this paper, the terms "activatory" and "inhibitory" GTP phases are used as follows: the activatory phase refers to the progressive increase in activity seen with increasing GTP concentrations from zero GTP to the peak activity. The decline in activity from this peak with increased GTP levels is referred to as the GTP inhibitory phase.

the two effects of GTP are mediated by separate proteins since trypsin treatment of fat cell membranes abolished the inhibitory effect of GTP.

It is not clear from the literature whether the biphasic effects of GTP on adipocyte adenylate cyclase are always encountered or to what extent assay conditions modify either phase. It has been suggested that at low temperatures or low magnesium concentrations GTP selectively inhibits fat cell cyclase activity (4, 5). Furthermore, Yamamura *et al.* (6) recently showed that chelators and sulfhydryl reagents, commonly included in the assay of adenylate cyclase, modify the actions of GTP on the fat cell enzyme. A further complication arises from the contamination of many commercial ATP preparations with GTP (8) such that the effects of low GTP concentrations are obscured.

In the present study using a simplified incubation medium and purified ATP we have shown that the biphasic effects of GTP are present under a wide variety of conditions and should be considered to represent the normal response of the fat cell enzyme to the nucleotide. In addition, we have established procedures whereby either the activating or inhibitory phase can be examined separately. When the inhibitory process is absent, the enzyme is no longer sensitive to inhibition by purine-modified adenosine analogs. The regulatory importance and the molecular basis for this behavior is discussed.

MATERIALS AND METHODS

The sources of materials used in the assay of adenylate cyclase have been reported (9). L-isoproterenol-D-bitartrate and calf intestinal adenosine deaminase (230 units/ml) were purchased from Sigma. ACTH¹⁻²⁴ (Synacthen) was a gift of Ciba-Geigy. *N*⁶-Phenylisopropyladenosine was from Dr. J. N. Fain, Brown University. 3-ethyl-3-isobutyl xanthine was bought from Aldrich Chemical Co. The ATP used in these studies was either purified according to the method of Kimura *et al.* (8) or was the Sigma product (A-2383) prepared by phosphorylation of adenosine.

Preparation of Fat Cell Membranes—Plasma membranes were isolated by a simplification of the method of Avruch and Wallach (10), suspended in 1 mM EDTA containing 10 mM Tris-HCl, pH 7.5, and stored in liquid N₂ as described by Harwood *et al.* (3).

Adenylate Cyclase Assay—Adenylate cyclase activity was assayed by the method of Salomon *et al.* (9) in a medium containing 0.1 mM ATP, 1 μCi of [α -³²P]ATP, 4 mM MgCl₂, 0.1 mM cyclic AMP, 2 mM creatine phosphate, creatine phosphokinase at 25 units/ml, 30 mM Tris-HCl, pH 7.5, and 0.1% crystalline bovine serum albumin. Reactions were initiated by the addition of approximately 1 μg of membrane protein to give a total volume of 0.1 ml. Incubations were carried out for 30 min at 24°C, apart from the exceptions noted in the text. Experiments were performed in duplicate or triplicate and three or more batches of fat cell plasma membranes. Replicates agreed to within 5%; intraexperimental variation was within 80 to 120% of the values shown.

Treatment with Cholera Toxin—The A₁ peptide of cholera toxin was prepared as described previously (11). Fat cell membranes (200 μg) were incubated with 40 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mg/ml of bovine serum albumin, 2 mM NAD⁺, and 20 μg of A₁

peptide of cholera toxin in a final volume of 100 μ l. After 5 min at 30°C, 2 ml of 10 mM Tris-HCl (pH 7.5) was added. The sample was then centrifuged at 30,000 \times g (15 min, 4°C) and the pellet was resuspended in 20 mM Tris-HCl containing 1 mg/ml of bovine serum albumin.

Treatment with Mercurial—Fat cell membranes (200 μ g) were incubated with 40 mM Tris-HCl, pH 7.5, and 0.3 mM *p*-hydroxymercuriphenylsulfonic acid (Sigma) in a final volume of 100 μ l. After 10 min at 0°C, 2 ml of Tris-HCl, pH 7.5 containing 0.2 mM dithiothreitol was added; the membranes were sedimented (30,000 \times g, 15 min, 4°C) in 20 mM Tris-HCl containing 1 mg/ml of bovine serum albumin and resuspended in the same medium prior to adenylate cyclase assay.

RESULTS

Effects of GTP, ITP, and 2'-Deoxy-GTP—A relatively symmetrical biphasic (activating and inhibitory) relationship is apparent between the concentration of the three nucleotides and hormone-stimulated activity (Fig. 1). GTP is the most potent compound in terms of both its maximum activation and its effective concentration range. Stimulation of activity can be obtained by 2 nM GTP while maximal activity is evoked by 30 nM GTP. The relative steepness of both sides of these curves should be noted, the concentration range in going from 10 to 90% (or 90 to 10%) of the maximal effect is only approximately 10-fold in both cases. Such behavior is strongly suggestive of positive cooperative interactions (12).

Effect of Temperature—The effect of increasing GTP concentration on basal, ACTH, and isoproterenol-stimulated adenylate cyclase activity is compared at 24°C and 36°C in Fig. 2.³ Previous studies (3, 5) had shown that the inhibition caused by GTP at 25°C could be either reduced or altered to activation by raising the temperature to 37°C. Indeed Pairault (5) had suggested that the only action of GTP was inhibition at low temperatures which was changed to activation at high temperatures. The basis for these observations can be appreciated by reference to Fig. 2, where it is apparent that the entire biphasic curve is present at both temperatures but is shifted 3- to 4-fold to the right on elevating the temperature. The total dependence of ACTH and the relative independence of isoproterenol on GTP for stimulation of activity, which had been previously pointed out by Yamamura *et al.* (6), is maintained at both temperatures.

Effects of $[Mg^{2+}]$ and $[Mn^{2+}]$ on GTP Inhibition—Rodbell (4) previously showed that at high magnesium concentrations the inhibition caused by 0.1 mM GTP (3) was changed to activation. Fig. 3 demonstrates that raising $[Mg^{2+}]$ from 4 to 20 mM caused no selective effect on either the inhibitory or the activatory GTP phase. Londos and Preston (13) have shown Mn^{2+} to be 50 to 100 times more potent than Mg^{2+} in activating hepatic adenylate cyclase. When 2 mM Mn^{2+} was included with 20 mM Mg^{2+} in the incubation medium, the inhibitory phase of GTP action on the fat cell cyclase system was eliminated and activation by GTP was reduced to the extent that ACTH stimulation was at most 10% above basal activity (Fig. 3). These results suggest that full activation of adenylate cyclase at a putative metal ion site (13) renders the enzyme relatively insensitive to regulation by GTP, as has been noted previously for hormonal activation of the adipocyte adenylate cyclase system (14).

Effects of *p*-Hydroxymercuriphenylsulfonate—Generally, mercurials inhibit adenylate cyclase in a manner that can be reversed by thiol reagents (15-18). However, pretreatment of fat cell membranes with *p*-hydroxymercuriphenylsulfonate

³The GTP curve obtained at 36°C in the present study is considerably more sensitive than that previously reported (6). We attribute this difference to the use of purified ATP and to the exclusion of ascorbate from the assay. We find that ascorbate can diminish the GTP inhibitory phase under some circumstances.

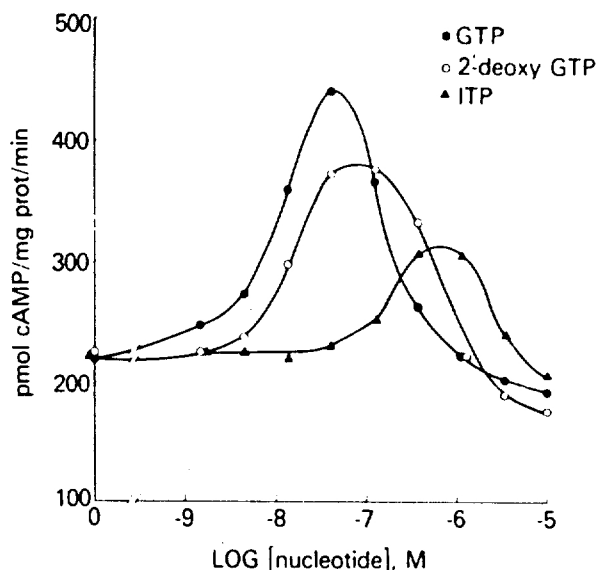


FIG. 1. Effects of nucleotide triphosphates on isoproterenol-stimulated fat cell adenylate cyclase activity. Adenylate cyclase activity was assayed in the presence of 1 μ M isoproterenol under standard conditions described under "Materials and Methods."

followed by neutralization with dithiothreitol resulted in a 3-fold stimulation of basal adenylate cyclase activity (Fig. 4). Accompanying the stimulation of basal activity by the mercurial was complete loss of the stimulatory effects of GTP on the enzyme system. Instead, GTP caused frank inhibition of enzyme activity even at concentrations as low as 2 nM (Fig. 4); note that the apparent $K_{0.5}$ for GTP was decreased by mercurial treatment relative to that seen in nontreated membranes (Fig. 1). The unique effects of *p*-hydroxymercuriphenylsulfonic acid pretreatment will be dealt with in detail in a future report.

Cholera Toxin Treatment—Following pretreatment of fat cell membranes with cholera toxin (the A-1 subunit) and NAD^+ , the typical biphasic effects of GTP were converted largely to a monophasic activating relationship both in the absence and presence of hormones (Fig. 5). NAD^+ was required for this effect of the A₁ subunit. It can be seen that the stimulatory effect of GTP was enhanced by toxin treatment as has been reported for other cyclase systems (11, 19-21). It has been reported that the toxin inhibits the breakdown of GTP at a specific GTPase associated with the nucleotide activation process and that this effect of the toxin explains its activating effects (21). The finding that the toxin abolishes the GTP inhibitory effect on the fat cell cyclase system raises the possibility that the toxin also may influence cyclase activity by eliminating a competing GTP-dependent inhibitory process.

Adenosine Action—Adenosine and analogs such as *N*⁶-phenylisopropyladenosine inhibit fat cell adenylate cyclase through a receptor that reacts competitively with methylxanthines; with isolated membrane preparations, inhibition by *N*⁶-phenylisopropyladenosine is observed only in the presence of inhibitory concentrations of GTP (Ref. 7 and Table I).⁴ Mercurial treatment, shown above to convert GTP action to a purely inhibitory mode, does not affect the ability of the adenosine analog to inhibit cyclase activity (Table I). How-

⁴No effect of *N*⁶-phenylisopropyladenosine was observed at low (activatory range; 6×10^{-9} M) GTP concentrations following any of the treatments described in Table I.

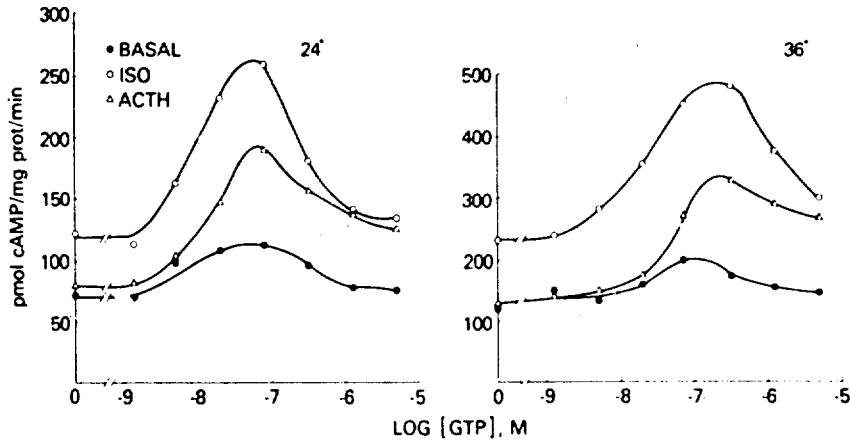


FIG. 2. Effect of temperature on the GTP dependency of basal and hormone-stimulated activity. Standard assays were performed for 30 min at 24°C (left panel) and for 7½ min at 36°C (right panel) in the absence (●) or presence of either 5 μM isoproterenol (ISO) (○) or 1 μM ACTH (Δ).

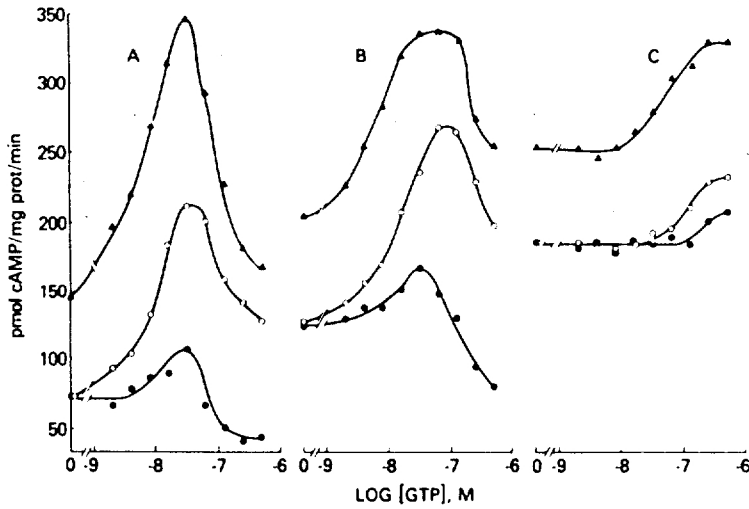


FIG. 3. Effects of metal ion concentration on the GTP titration. Adenylate cyclase activity was determined in the absence (●) or presence of either 10 μM isoproterenol (Δ) or 2 μM ACTH (○). Standard conditions were employed using A, 4 mM Mg²⁺; B, 20 mM Mg²⁺; C, 20 mM Mg²⁺ plus 4 mM Mn²⁺.

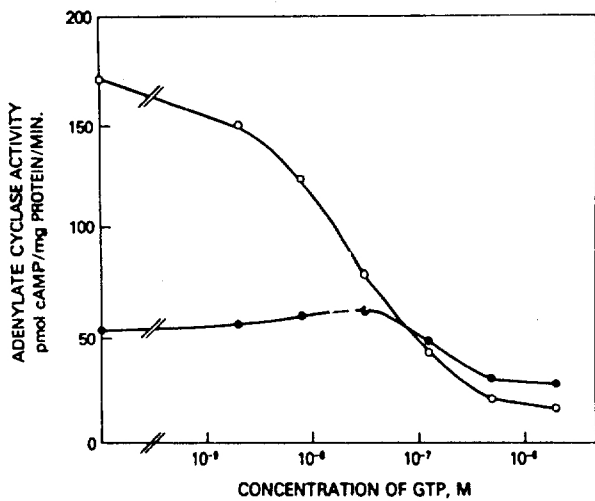


FIG. 4. Effects of mercurial pretreatment on the GTP titration of basal adenylate cyclase activity. Cyclase activity was determined under standard conditions with membranes which had been pretreated with *p*-hydroxymercuriphenyl sulfonate (○) or not (●) under conditions described under "Materials and Methods."

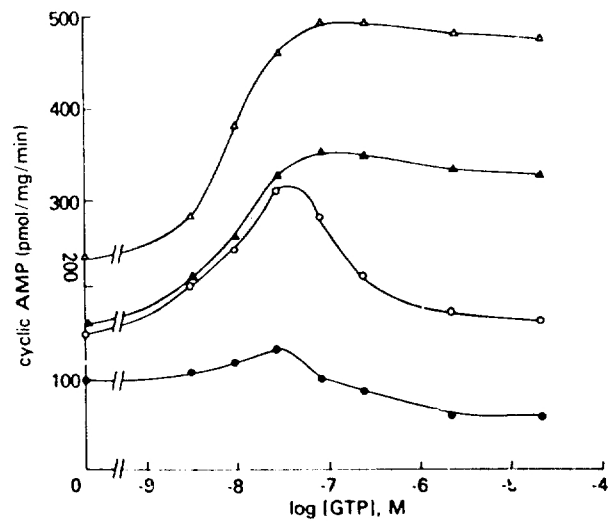


FIG. 5. Effects of cholera toxin pretreatment on the bimodal actions of GTP. Aliquots (1 μg) of membranes which had been pretreated with either cholera toxin and NAD⁺ (▲, Δ) or NAD⁺ alone (●, ○) were assayed under standard conditions in the absence (●, ▲) or presence (○, Δ) of 0.4 μM isoproterenol. Pretreatment with NAD⁺ alone had no effect on control activity (results not shown).

TABLE I

Modification of *N*⁶-phenylisopropyladenosine inhibition of fat cell adenylate cyclase activity by various procedures

The adenylate cyclase activity of 0.5- to 1.5- μ g aliquots of variously treated membranes was determined under standard assay conditions in the presence of 2.5 units/ml of adenosine deaminase to reduce endogenous adenosine levels. Where indicated, GTP and *N*⁶-phenylisopropyladenosine (PIA) were present at final concentrations of 9 μ M and 0.4 μ M, respectively.

Pretreatment or condition of assay	Isoproterenol μ M	-GTP -PIA	+GTP -PIA	+GTP +PIA
Normal	None	1.75	1.55	0.39 (25) ^a
	0.4	2.67	4.60	2.52 (48)
Cholera toxin pre-treated ^b	None	1.70	4.51	3.97 (88)
	0.4	3.73	7.33	6.36 (86)
GppNHp preactivated ^c	0.4	24.57	25.80	23.49 (92)
Mn ²⁺ (2 mM), Mg ²⁺ (20 mM) in assay ^d	0.4	10.49	14.34	14.09 (98)
Mercurial treatment ^e	None	2.01	0.39	0.18 (48)

^a Values in brackets express the percentage of activity in the presence of PIA compared to that in its absence. The inhibition caused by PIA could in all cases be reversed by 250 μ M 1-methyl-3-isobutyl xanthine (results not shown).

^b Pretreatment of membranes with cholera toxin A₁ peptide plus NAD and the mercurial MPS as described under "Materials and Methods."

^c Membranes (56 μ g) were incubated for 60 min at 24°C in the presence of 10 μ M Gpp(NH)p and all adenylate cyclase assay components except [α -³²P]ATP. The activity of aliquots (1.4 μ g) of this material was then determined over 10 min by its addition to a mixture containing [α -³²P]ATP, GTP, isoproterenol, and PIA as indicated.

^d Activities were determined in the presence of 20 mM Mg²⁺ containing 2 mM Mn²⁺ under the conditions indicated in the table.

ever, any treatment which caused loss of GTP inhibition, including cholera toxin treatment, incubation with 2 mM Mg²⁺ and 20 mM Mn²⁺ or preincubation with Gpp(NH)p,⁵ resulted in loss of the inhibitory effects of both GTP and *N*⁶-phenylisopropyladenosine. These findings indicate an intimate linkage between the GTP inhibitory process and the process through which adenosine inhibits adenylate cyclase.

DISCUSSION

The activating and inhibiting phases of GTP regulation of fat cell adenylate cyclase, which are particularly pronounced in the presence of hormones and adenosine, have been clearly established. A striking feature of the GTP titration curves is that in most instances each phase occurs over a narrow concentration range (cf. Fig. 1), which might indicate positive cooperative interactions (12). However, such indications disappear when one process is abolished or masked. Thus, when the activating phase is abolished by mercurial treatment, the inhibitory effects of GTP occur over a broader concentration range and the sensitivity of GTP action is enhanced (cf. Fig. 4). Conversely, when the inhibitory phase is obscured by cholera toxin treatment, the stimulatory effect of GTP is enhanced. Such findings lead us to conclude that, in unperturbed conditions, both processes are active and mutually repressing, possibly through competition for some limited catalytic activity. Thus, although assay conditions (temperature, divalent metal ions, and type of hormone) may modify

⁵ Gpp(NH)p inhibits basal adenylate cyclase in short incubations; however with longer incubations (>10 min), Gpp(NH)p activates at all concentrations (4, 22). Following a 60-min preincubation of fat cell membranes with 10 μ M Gpp(NH)p, adenylate cyclase becomes persistently activated and insensitive to GTP inhibition (unpublished results).

the sensitivity, or the amplitude of the GTP effects, or both, both phases normally co-exist.

Although the precise mechanism remains obscure, the importance of the bimodal behavior of GTP action is becoming increasingly apparent in terms of the regulatory flexibility it provides. The activatory phase amplifies hormonal stimulation (Figs. 2 and 3). In the inhibitory phase, adenosine and purine-modified adenosine analogs, acting through so-called R-type receptors inhibit both cyclic AMP production and lipolysis in the fat cell (7). Here we have shown that the inhibitory actions of a potent R-site adenosine analog are abolished when the GTP inhibitory phase is abolished or obscured by pretreatment with Gpp(NH)p, cholera toxin, or by incubating in the presence of high concentrations of divalent cations. Such total dependence of the analog's action on the integrity of the GTP inhibitory phase underlines the regulatory importance of the latter and elevates it to the same degree of importance as the role of GTP in the stimulatory actions of hormones such as catecholamines, ACTH, and glucagon on the fat cell system. The facility of GTP to regulate an enzyme system such that in one mode it is insensitive to regulation by adenosine and in another mode extremely sensitive to such regulation hints that the bimodal effects of the nucleotide are mediated by separate proteins. Consistent with this notion is the finding of Yamamura *et al.* (6) that trypsin treatment of fat cell membranes abolished the GTP inhibitory process, leaving the GTP stimulatory process intact.

A similar bimodal regulation of adenylate cyclase by GTP may exist in other cells. In human platelets, prostaglandins stimulate adenylate cyclase activity whereas catecholamines, acting through an α -type receptor, inhibit cyclase activity. Both actions require GTP in the incubation medium when membrane fragments containing cyclase activity are examined for the effects of prostaglandins and catecholamines (23). Thus, it is possible that those extracellular agents which inhibit the production of cyclic AMP in cells may do so, in part, through a GTP-dependent process that inhibits adenylate cyclase. As a model, the apparent paradoxical bimodal responses of the fat cell adenylate cyclase system to GTP may yet prove to be the key for understanding how hormones or other extracellular agents can promote either increased or decreased levels of cyclic AMP in their target cells.

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REFERENCES

- Rodbell, M., Lin, M. C., Salmon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M., and Berman, M. (1975) *Adv. Cyclic Nucleotide Res.* 5, 3-29
- Cryer, P. E., Jarrett, L., and Kipnis, D. M. (1969) *Biochim. Biophys. Acta* 177, 586-590
- Harwood, J. P., Löw, H., and Rodbell, M. (1973) *J. Biol. Chem.* 248, 6239-6245
- Rodbell, M. (1975) *J. Biol. Chem.* 250, 5826-5830
- Pairault, J. (1976) *Eur. J. Biochem.* 62, 323-334
- Yamamura, H., Lad, P. M., and Rodbell, M. (1977) *J. Biol. Chem.* 252, 7964-7966
- Londos, C., Cooper, D. M. F., Schlegel, W., and Rodbell, M. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 5362-5366
- Kimura, N., and Negata, N. (1977) *J. Biol. Chem.* 252, 3823-3835
- Selomon, Y., Londos, C., and Rodbell, M. (1974) *Anal. Biochem.* 58, 541-548
- Avruch, J., and Wallach, D. F. M. (1971) *Biochim. Biophys. Acta* 233, 334-337
- Lin, M. C., Welton, A. F., and Berman, M. F. (1978) *J. Cyclic Nucleotide Res.* 4, 159-168
- Newsholme, E. A., and Start, C. (1973) *Regulation in Metabolism*, pp. 34-87, John Wiley and Sons, London
- Londos, C., and Preston, M. S. (1977) *J. Biol. Chem.* 252, 5957-5961

14. Pohl, S. L., Birnbaumer, L., and Rodbell, M. (1971) *J. Biol. Chem.* **246**, 1849-1856
15. Schramm, M., and Naim, E. (1970) *J. Biol. Chem.* **245**, 3225-3231
16. Storm, D. R., and Chase, R. A. (1975) *J. Biol. Chem.* **250**, 2539-2545
17. Spiegel, A. M., Brown, E. M., and Aurbach, G. D. (1976) *J. Cyclic Nucleotide Res.* **2**, 393-404
18. Kather, H., Geiger, M., and Simon, B. (1976) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **292**, 177-182
19. Bennett, V., and Cuatrecasas, P. (1975) *J. Membr. Biol.* **22**, 1-28
20. Levinson, S. L., and Blume, A. J. (1977) *J. Biol. Chem.* **252**, 3766-3774
21. Cassel, D., and Selinger, Z. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 3307-3311
22. Ebert, R., and Schwabe, U. (1974) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **286**, 297-313
23. Jakobs, K. H., Saur, W., and Schultz, G. (1978) *FEBS Lett.* **85**, 167-170