MOLECULAR PHARMACOLOGY, 11, 485-491

Desensitization of *Beta* Adrenergic Receptors in Human Fibroblasts in Tissue Culture

T. J. FRANKLIN, W. P. MORRIS, AND P. A. TWOSE

Department of Biochemistry, Pharmaceuticals Division, Imperial Chemical Industries, Ltd., Alderley Park, Macclesfield, Cheshire, England

(Received February 5, 1975)

SUMMARY

FRANKLIN, T. J., MORRIS, W. P. & TWOSE, P. A. (1975) Desensitization of *beta* adrenergic receptors in human fibroblasts in tissue culture. *Mol. Pharmacol.*, **11**, 485-491.

The ability of isoproterenol, epinephrine, norepinephrine, and salbutamol to desensitize the *beta* receptor-coupled cyclic 3',5'-AMP response of human diploid fibroblasts in tissue culture correlates well with their activity as *beta* stimulants. Partial desensitization of the cells results in a marked fall in the maximal cyclic AMP response to isoproterenol; fully desensitized cells are totally unresponsive to isoproterenol. Recovery of *beta* adrenergic sensitivity is a slow process and is delayed by extremely low concentrations of isoproterenol (picomolar). Recovery is also inhibited by concentrations of puromycin, cycloheximide, and actinomycin D that inhibit protein or RNA synthesis.

INTRODUCTION

A previous report from our laboratory (1) described the loss of the cyclic 3',5'-AMP response to isoproterenol in human diploid fibroblasts, derived from embryonic lung, after these cells had been incubated with isoproterenol. The desensitization was apparently specific, as the cells retained full responsiveness to prostaglandin E_1. Furthermore, there was no evidence for increased phosphodiesterase activity in the desensitized cells (1). Reports from other laboratories have described the hormone-induced loss of catecholamine responsiveness of the adenylate cyclase activity of several different types of cells, including fibroblasts (2), adipocytes (3), macrophages (4), leukocytes (5), and glioma cells (6, 7). We have been concerned to establish whether activation of the *beta* receptor system is essential for the development of desensitization. The abilities of several *beta* adrenergic agents to desensitize fibroblasts have therefore been compared.

We have also examined the effects of inhibitors of protein and nucleic acid synthesis on the slow recovery of hormonal sensitivity.

MATERIALS AND METHODS

Fibroblast cultures. Diploid fibroblasts from 20-week human fetal lung were grown on the inside flat surface of liquid scintillation vials in Eagle's minimal essential medium (1 ml) buffered to pH 7.4 with 19 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and supplemented with 8% heat-inactivated calf serum, 4.1 mM glutamine, 500 units/ml of penicillin G, and 100 μg/ml of streptomycin (medium 1). The cells, usually in the 10th to 20th passage, appeared confluent after 7 days with medium changes on alternate days. An atmosphere of air was used throughout.

Experimental conditions. Before all experiments the cultures were equilibrated with fresh medium 1 at 37° for 1 hr. Whenever possible the drugs were dissolved in water and added to the cultures in a volume not exceeding 1% of that of the culture medium. It was necessary to dissolve cycloheximide in dimethyl sulfoxide, and the final concentration of this solvent in the culture medium was adjusted to 0.5%. At this concentration dimethyl sulfoxide did not exert any detectable effects of its own on the cells.

Assay of cyclic AMP. At the end of challenge incubations at 37° with *beta* adrenergic agents, the culture vials were plunged into a boiling water bath for 10 min. The precipitated proteins were dispersed in the medium by ultrasonic irradiation for 30 sec and removed by centrifugation. The cyclic AMP content of the supernatant liquid was determined by the method of Brown *et al.* (8). All incubations were carried out in triplicate, and duplicate cyclic AMP assays were performed on the contents of each vial. The procedure described above measures the cellular cyclic AMP and also that released from the cells into the medium during the incubation period. Previous work has demonstrated the necessity of measuring this cyclic AMP released into the medium to avoid a gross underestimate of cellular cyclic AMP production (9). Because this procedure precluded the measurement of the cell protein content of the vials, it was necessary to carry out parallel cell protein determinations (10) on batches of vials after careful rinsing of the cell sheets with 0.9% sodium chloride solution to remove traces of culture medium. With careful inoculation procedures the coefficient of variability for the cell protein content of identically treated cultures was 5%. The cyclic AMP response of the fibroblasts used in this work to *beta* adrenergic agents and also basal cyclic AMP levels varied significantly from one batch of cultures to another, whereas variations within a particular batch, i.e., cultures prepared from the same inoculum, were generally insignificant. Individual experiments, therefore, were always performed using cultures prepared from the same inoculum.

Chemicals. DL-Isoproterenol was purchased from Sigma Chemical Company, and L-epinephrine and L-norepinephrine, from Koch-Light Laboratories. DL-Salbutamol was a generous gift of Allen and Hanburys, Ltd., Ware, U. K. L-Propranolol was a product of Imperial Chemical Industries. In all challenge incubations in which cyclic AMP production was to be subsequently determined, we routinely added the potent phosphodiesterase inhibitor 2-amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*s*-triazolo[1,5-α]pyrimidine (I.C.I. 63, 197) at a concentration of 25 μM. We have previously found this inhibitor to be nontoxic to our line of cells at this concentration (1). I.C.I. 63, 197 is a product of Imperial Chemical Industries. Cycloheximide was purchased from BDH Chemicals, Ltd., and puromycin and actinomycin D, from Sigma Chemical Company.

RESULTS

Cyclic AMP response of fibroblasts to beta adrenergic agonists. Following equilibration with fresh medium 1 for 1 hr, confluent cultures derived from the same

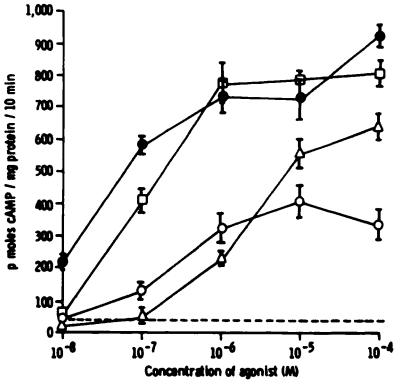

FIG. 1. *Stimulation of cyclic AMP (cAMP) response of human diploid fibroblasts by beta adrenergic agents*

Confluent cultures of human diploid fibroblasts were challenged for 10 min with graded concentrations of DL-isoproterenol (●), L-epinephrine (□), L-norepinephrine (Δ), or DL-salbutamol (○) in the presence of the phosphodiesterase inhibitor I.C.I. 63,197 (25 μM). The total cyclic AMP in the cells and supernatant growth medium was determined at the end of the incubation. Each point represents the mean and standard error of duplicate determinations on each of three cultures.

inoculum were challenged for 10 min with various *beta* stimulants. Figure 1 shows that isoproterenol and epinephrine were considerably more potent agonists than norepinephrine and salbutamol. The maximal response elicited by salbutamol was less than 50% of the maximal responses produced by isoproterenol and epinephrine.

Desensitization of fibroblasts by beta adrenergic agonists. Confluent fibroblast cultures were incubated individually with isoproterenol, epinephrine, norepinephrine, and salbutamol (1 μM in each case). After the initial incubation periods the cell sheets were rinsed three times with drug-free medium 1 at 37° and challenged for 10 min in fresh medium 1 containing 1 μM isoproterenol, and the cyclic AMP accumulated at the end of the 10-min period was determined. It is clear from Fig. 2 that the rates at which agonists desensitized fibroblasts to a subsequent isoproterenol challenge correlate reasonably well with their

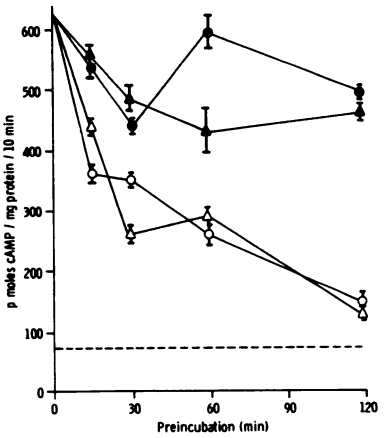

FIG. 2. *Desensitization of cyclic AMP (cAMP) response to isoproterenol induced by incubation with beta adrenergic agents*

Confluent cultures of human diploid fibroblasts were incubated separately with DL-isoproterenol (○), L-epinephrine (△), L-norepinephrine (●), or DL-salbutamol (▲) for the indicated periods. In each case the amine concentration was 1 μM. The cells were rinsed three times with fresh medium 1 and then incubated for 10 min with DL-isoproterenol (1 μM), and the cyclic AMP accumulated in the cells and medium was determined. Each point represents the mean and standard error of duplicate determinations on each of three cultures. - - -, concentration of cyclic AMP in fresh, unstimulated cultures, i.e., cells plus medium.

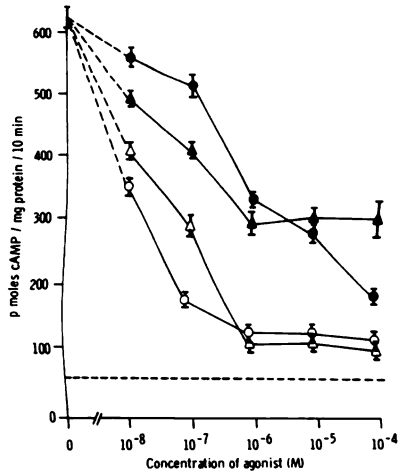

FIG. 3. *Desensitization of cyclic AMP (cAMP) response to isoproterenol induced by incubation with beta adrenergic agents*

Cultures of human diploid fibroblasts were incubated for 3 hr with graded concentrations of DL-isoproterenol (○), L-epinephrine (△), L-norepinephrine (●), or DL-salbutamol (▲). The cells were rinsed three times with fresh medium 1 and then incubated with DL-isoproterenol (1 μM) for 10 min, and the total cyclic AMP in cells and medium was determined. Each point represents the mean and standard error of duplicate determinations on each of three cultures. - - -, total concentration of cyclic AMP in fresh, unstimulated cultures.

agonistic activities as revealed in Fig. 1. Thus isoproterenol and epinephrine desensitized the fibroblasts significantly more rapidly than the less effective agonists, norepinephrine and salbutamol. In another experiment the cells were incubated for 3 hr with graded concentrations of the four agonists and then challenged as before with 1 μM isoproterenol (Fig. 3). Again there was a clear correlation between the potency of the agonists and their ability to desensitize the cells.

The experiments indicated in Figs. 2 and 3 involved preliminary incubation with *beta* adrenergic stimulants in the absence of the phosphodiesterase inhibitor I.C.I. 63,197. The inclusion of this agent in the preliminary incubations had no significant effect on the development of desensitization (not shown).

Effects of the beta adrenergic antagonist propranolol on response of fibroblasts to isoproterenol. It was of interest to determine whether *beta* receptor activation was

necessary to induce loss of the cyclic AMP response to a subsequent isoproterenol challenge. The potent *beta* blocking drug L-propranolol is devoid of intrinsic sympathomimetic activity and does not elicit a cyclic AMP response from the fibroblasts. Table 1 shows that simultaneous addition of propranolol to fibroblast cultures with isoproterenol suppressed the cyclic AMP response. Replicate cultures were incubated for 2.5 hr with graded concentrations of propranolol, washed nine times with 3-ml aliquots of medium 1 at 37°, and then challenged for 10 min with graded concentrations of isoproterenol. Table 1 indicates that incubation with propranolol resulted in some loss of cyclic AMP response to the subsequent challenge with isoproterenol (1 μM). A normal response was elicited with 100 μM isoproterenol, whereas even this concentration of agonist failed to produce a response from cells incubated for 2 hr with isoproterenol. It is conceivable that trace amounts of propranolol left after the washing procedure may have hindered the access of isoproterenol to the receptor sites, although further work would be necessary to establish this point.

TABLE 1

Effect of preliminary incubation with propranolol on subsequent cyclic AMP response of human fibroblasts to isoproterenol

Confluent cultures of fibroblasts were incubated for 2.5 hr as indicated. The cultures were then rinsed nine times with 3-ml aliquots of medium 1 at 37° and incubated for a further 10 min ("challenge"). The total cyclic AMP in cells plus medium was then determined as described in the text. Note that all challenge incubations contained the phosphodiesterase inhibitor I.C.I. 63,197 at 25 μM. DL-Isoproterenol and L-propranolol were used. Values are means ± standard errors.

Additions to		Cyclic AMP in challenge incubation
Preliminary incubation	Challenge	
		pmoles/mg protein
None	None	16.5 ± 0.8
None	Isoproterenol, 10 μM	214.5 ± 6.4
None	Isoproterenol, 100 μM	169.0 ± 3.1
None	Propranolol, 1 μM	14.5 ± 1.7
None	Isoproterenol, 10 μM, + propranolol, 1 μM	58.0 ± 6.7
Propranolol, 1 μM	Isoproterenol, 10 μM	60.5 ± 4.3
Propranolol, 1 μM	Isoproterenol, 100 μM	184.5 ± 4.5
Isoproterenol, 1 μM	Isoproterenol, 100 μM	6.5 ± 0.4

Effect of isoproterenol on recovery of fibroblasts from desensitization. The recovery of *beta* adrenergic sensitivity following isoproterenol-induced desensitization of fibroblasts in culture is a slow process, taking at least 20–24 hr (1), and we had observed that recovery was frequently erratic unless the cells were repeatedly rinsed after incubation with isoproterenol. We suspected that the erratic recovery of hormonal sensitivity following inadequate rinsing of desensitized cells might be attributable to trace amounts of isoproterenol, or possibly one of its breakdown products, interfering with the recovery process. We therefore tested the effects of graded concentrations of isoproterenol on the recovery of 9-fold washed cells desensitized by incubation for 2.5 hr. It is clear from Table 2 that isoproterenol suppressed the recovery of *beta* adrenergic sensitivity at remarkably low concentrations, only partial recovery being observed in the presence of 1 pM isoproterenol.

Effects of inhibitors of protein and nucleic acid synthesis on recovery of cyclic AMP response by desensitized fibroblasts. The slowness of the recovery of *beta* adrenergic sensitivity led us to consider whether a biosynthetic process might be involved. We therefore decided to test the effects of inhibitors of protein and nucleic acid synthesis on recovery. Puromycin and cycloheximide were chosen as inhibitors of protein synthesis; at the concentrations employed, these compounds completely suppressed the incorporation of [^{14}C]leucine into cellular protein.[1] However, incubation of confluent cell sheets for 24 hr with these concentrations of puromycin and cycloheximide produced no obvious

[1] Unpublished observations.

TABLE 2

Effect of low concentrations of isoproterenol on recovery of human fibroblasts from isoproterenol-induced densensitization

Confluent cultures were incubated for 2.5 hr with DL-isoproterenol (1 μM) to induce loss of the cyclic AMP response to isoproterenol. The cell sheets were rinsed nine times with 3-ml aliquots of medium 1 at 37° and then incubated with graded concentrations of isoproterenol for 24 hr ("recovery"). The cells were again rinsed nine times with 3-ml aliquots of warm medium 1 and then challenged for 10 min with isoproterenol (10 μM) in the presence of I.C.I. 63,197 (25 μM). Values are means ± standard errors.

Preliminary treatment		Cyclic AMP in challenge incubation
Desensitization	Recovery	
		pmoles/mg protein
None	None	87.0 ± 5.5[a]
None	None	349.5 ± 2.4
+	No recovery period	125.5 ± 3.5
+	No additions	290.0 ± 10.4
+	+ Isoproterenol, 100 pM	100.0 ± 4.5
+	+ Isoproterenol, 10 pM	110.0 ± 2.6
+	+ Isoproterenol, 1 pM	146.0 ± 12.7

[a] No isoproterenol in challenge medium.

changes in the gross morphology of the fibroblasts and there was no measurable loss of protein from the cell sheets compared with controls in drug-free medium. Actinomycin D served as an inhibitor of RNA synthesis; at 16 μM it effectively inhibits the incorporation of [^{14}C]uridine into cellular RNA. In typical experiments (Table 3) puromycin, cycloheximide, and actinomycin D, when added to the challenge incubations, had no effect on the cyclic AMP response to isoproterenol. Incubation of the cells for 24 hr with these drugs, however, had a depressing effect on the subsequent response to isoproterenol. In experiments with desensitized cells, each inhibitor markedly inhibited the recovery of the cyclic AMP response to isoproterenol.

Does desensitization of fibroblasts induced by isoproterenol result in right shift of dose-response curve to isoproterenol? It was possible that desensitization of the cyclic AMP response to isoproterenol involved a decreased affinity of the *beta* receptor for isoproterenol. If this were the sole mechanism causing desensitization, a parallel right shift in the dose-response curve for isoproterenol might be expected. We therefore obtained isoproterenol dose-response curves for fresh fibroblasts and for cultures partially and fully desensitized by incubation with 1 μM isoproterenol for 15 min and 120 min, respectively (Fig. 4). The response of the partially desensitized cells was clearly depressed throughout the concentration range of isoproterenol, and the maximal response was markedly less than that of fresh cells at the highest concentration (1 mM) of isoproterenol used. No response at all could be elicited from the cells desensitized for 120 min. It is unlikely, therefore, that a diminished affinity of the receptor for isoproterenol can account for the desensitization phenomenon.

DISCUSSION

We have established that the rate and extent of the desensitization by *beta* adrenergic agonists of the *beta* receptor-coupled cyclic AMP response of cultured human fibroblasts depends on the concentration and potency of the agonists. Thus isoproterenol and epinephrine, which are excellent stimulants of the cyclic AMP response in fibroblasts, provoked a more rapid and profound desensitization than the inferior *beta* agonists, salbutamol and norepinephrine. We suspect that the desensitization produced by the *beta* adrenergic antagonist propranolol was more apparent than real and it seems likely that difficulty in displacing propranolol from the specific *beta* adrenergic recognition sites may account for the depressed response of the cells to a subsequent isoproterenol challenge as the residual effect of propranolol was overcome by a high concentation of isproterenol. We suggest, therefore, that the loss of the cyclic AMP response to isoproterenol results directly or indirectly from prior activation of the receptor and that interaction with the receptor without activation, such as occurs with propranolol, does not desensitize the system. The recovery of the cyclic AMP response to isoproterenol was prevented by

TABLE 3

Effect of inhibitors of protein and nucleic acid synthesis on recovery of human fibroblasts from isoproterenol-induced desensitization

Confluent cultures were desensitized during the 2.5-hr preliminary incubation period with DL-isoproterenol (1 μM). The cell sheets were then rinsed nine times with 3-ml aliquots of medium 1 at 37° and incubated in fresh medium 1 with various additions for 24 hr ("recovery"). The cells were again rinsed with nine 3-ml aliquots of medium 1 and then challenged for 10 min with isoproterenol (10 μM) in the presence of I.C.I. 63,197 (25 μM). Values are means ± standard errors.

Addition to		Challenge	Cyclic AMP in challenge
Preliminary incubation	Recovery		
			pmoles/mg protein
None	None	None	12.5 ± 2.6
None	None	Isoproterenol, 10 μM	254.0 ± 8.9
Isoproterenol, 1 μM	No recovery period	Isoproterenol, 10 μM	15.0 ± 2.6
Isoproterenol, 1 μM	None	Isoproterenol, 10 μM	298.0 ± 19.9
Isoproterenol, 1 μM	Cycloheximide, 0.18 mM	Isoproterenol, 10 μM	17.5 ± 4.2
Isoproterenol, 1 μM	Cycloheximide, 0.35 mM	Isoproterenol, 10 μM	18.5 ± 3.8
None	Cycloheximide, 0.35 mM	Isoproterenol, 10 μM	183.5 ± 8.4
None	None	Isoproterenol, 10 μM, + cycloheximide, 0.35 mM	280.0 ± 9.0
None	None	None	68.0 ± 3.4
None	None	Isoproterenol, 10 μM	302.5 ± 17.7
Isoproterenol, 1 μM	No recovery period	Isoproterenol, 10 μM	136.0 ± 7.5
Isoproterenol, 1 μM	None	Isoproterenol, 10 μM	273.5 ± 28.0
Isoproterenol, 1 μM	Puromycin, 0.11 mM	Isoproterenol, 10 μM	78.5 ± 9.4
None	Puromycin, 0.11 mM	Isoproterenol, 10 μM	225.0 ± 6.2
None	None	Isoproterenol, 10 μM, + puromycin, 0.11 mM	310.5 ± 14.1
None	None	None	2.5 ± 0.3
None	None	Isoproterenol, 10 μM	163.5 ± 16.5
Isoproterenol, 1 μM	No recovery period	Isoproterenol, 10 μM	0
Isoproterenol, 1 μM	None	Isoproterenol, 10 μM	95.0 ± 6.5
Isoproterenol, 1 μM	Actinomycin D, 16 μM	Isoproterenol, 10 μM	17.0 ± 2.3
Isoproterenol, 1 μM	Actinomycin D, 8 μM	Isoproterenol, 10 μM	39.0 ± 3.6
None	Actinomycin D, 16 μM	Isoproterenol, 10 μM	118.0 ± 6.7
None	None	Isoproterenol, 10 μM, + actinomycin D, 16 μM	153.0 ± 4.6

inhibitors of protein and nucleic acid synthesis, suggesting perhaps that new receptors or some other component involved in controlling cyclic AMP levels must be synthesized to permit recovery. Our results with nonmalignant fibroblasts are in marked contrast to those obtained with rat glioma cells by DeVellis and Brooker (7). They found that the addition of cycloheximide to the 2B subclone of RGC6 cells, previously desensitized to noreprinephrine, strikingly restored the cyclic AMP response of the cells to norepinephrine.

The ability of very low concentrations of isoproterenol to suppress the recovery of desensitized diploid fibroblasts may be explicable if newly generated, sensitized *beta* receptors are "picked off" by the agonist as they appear, thus delaying the generation of a significant pool of active receptors.

The results of the present investigation

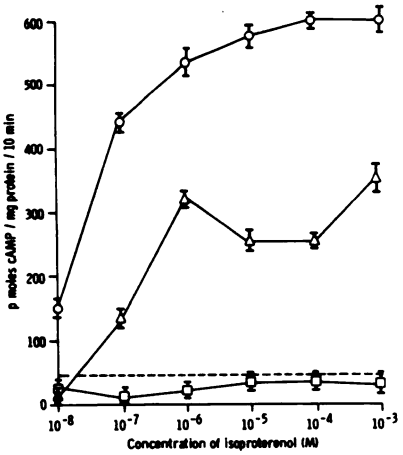

FIG. 4. *Displacement of isoproterenol dose-cyclic AMP (cAMP) response curve after partial and complete desensitization of fibroblasts*

Confluent cultures of human diploid fibroblasts were incubated for 0 (○), 15 (△), or 120 min (□) with DL-isoproterenol (1 μM) to induce partial and complete desensitization, respectively. The cells were rinsed three times with fresh medium 1 and then incubated for 10 min with graded concentrations of isoproterenol, and the total cyclic AMP appearing in cells and medium was determined. Each point represents the mean and standard error of duplicate determinations on each of three cultures. - - -, total concentration of cyclic AMP in fresh, unstimulated cultures.

have been discussed in terms of desensitization of the *beta* adrenergic receptor, which might involve changes in the receptor, in the coupling of the receptor to adenylate cyclase, or in the enzyme itself. Since our criterion for activation of the system is the change in level of cyclic AMP in response to an isoproterenol challenge, the possible contribution of increased phosphodiesterase activity to a loss of the cyclic AMP response cannot be ignored. A recent study (6) with a glioma cell line indicated that prolonged incubation of these cells with norepinephrine resulted in increased phosphodiesterase activity. Addition of cycloheximide prevented the elevation of phosphodiesterase activity and also partially protected the cells against desensitization to norepinephrine. DeVellis and Brooker (7), on the other hand, who also found that the presence of cycloheximide during preliminary incubation of the 2B subclone of RGC6 glioma cells with norepinephrine protected the cells against *beta* adrenergic desensitization, were unable to detect any increase in phosphodiesterase activity in desensitized cells. Furthermore, a previous study (1) failed to reveal an increase in phosphodiesterase activity in desensitized human diploid fibroblasts. The inhibition of recovery of isoproterenol sensitivity by inhibitors of protein and nucleic acid synthesis reported in this paper is difficulat to explain in terms of a model involving increased phosphodiesterase activity.

REFERENCES

1. Franklin, T. J. & Foster, S. J. (1973) *Nat. New Biol.*, **246**, 146-148.
2. Makman, M. H. (1971) *Fed. Proc.*, **30**, 458.
3. Ho, R. J. & Sutherland, E. W. (1971) *J. Biol. Chem.*, **246**, 6822-6827.
4. Remold-O'Donnell, E. (1974) *J. Biol. Chem.*, **249**, 3615-3621.
5. Morriss, H. G., DeRoche, G. B. & Caro, C. M. (1974) *2nd Int. Conf. Cyclic AMP*, p. 42.
6. Schwartz, J. P. & Passoneau, J. V. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 3844-3848.
7. DeVellis, J. & Brooker, G. (1974) *Science*, **186**, 1221-1223.
8. Brown, B. L., Albano, J. D. M., Ekins, R. P., Sgherzi, A. M. & Tampion, W. (1971) *Biochem. J.*, **121**, 561-562.
9. Franklin, T. J. & Foster, S. J. (1973) *Nat. New Biol.* **246**, 119-120.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.