Proc. Nat. Acad. Sci. USA
Vol. 70, No. 6, pp. 1659–1663, June 1973

Pleiotypic Control by Cyclic AMP: Interaction with Cyclic GMP and Possible Role of Microtubules

(prostaglandins/colcemid/vinblastine/membrane transport)

RAPHAEL KRAM AND GORDON M. TOMKINS

Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, Calif. 94122

Communicated by J. Brachet, February 15, 1973

ABSTRACT **Previous studies have shown that exogenous dibutyryl cyclic AMP inhibits the uptake of uridine, leucine, and 2-deoxyglucose by cultured mouse fibroblasts. 3′:5′-cyclic GMP is shown here to counteract these inhibitory effects as well as the inhibition of precursor transport and leucine incorporation into proteins produced by prostaglandin E_1. We conclude, therefore, that cyclic GMP antagonizes the "pleiotypic" effects of cyclic AMP in these cells.**

Colcemid and vinblastine, but not cytochalasin B, reverse the transport inhibition caused by cyclic AMP without affecting the intracellular concentrations of cyclic AMP. These results suggest the possibility that cyclic AMP regulates the membrane transport of certain substrates by influencing the organization of microtubules.

Recent work from various laboratories has implicated cAMP in the regulation of cellular growth. In a preceding communication (1) we presented evidence that cAMP exerts this control by coordinately influencing several biochemical processes related to growth. This set of reactions was defined as the "pleiotypic" program, and its regulation as pleiotypic control (2). Earlier we had singled out this coordinated control as being of general significance since the same set of reactions is affected in various cell types by specific hormones and growth-promoting factors (2). The fact that cAMP controls the pleiotypic reactions provides a basis for relating differentiation, malignant transformation, and hormonal control of growth (1). We report here some observations pertaining to the mechanisms of cAMP control of the pleiotypic parameters.

cGMP antagonizes the cAMP stimulated synthesis of β-galactosidase in cell-free bacterial extracts (3, 4). In certain animal cells as well, cGMP appears as if it might also directly or indirectly counteract the effects of cAMP (5–9). We have, therefore, investigated the interaction between these cyclic nucleotides on the pleiotypic reactions and conclude that here, too, cGMP overcomes the actions of cAMP.

We also suggest, on the basis of studies with inhibitors of microtubule assembly, that the cyclic nucleotides may influence some membrane transport processes by their effects on colcemid- and vinblastine-sensitive structures, possibly the microtubular apparatus itself.

MATERIALS AND METHODS

The Balb/c 3T3 mouse fibroblasts and their SV 40-transformed derivatives were grown in MEM plus 10% calf serum.

Abbreviations: cGMP, 3′:5′-cyclic GMP; PBS, phosphate buffered saline, 25 mM potassium phosphate (pH 7.4)–0.1 M NaCl; PGE_1, prostaglandin E_1; $(Bu)_2$cAMP, dibutyryl cAMP; MEM, Dulbecco's modification of Eagle's minimal essential medium.

The rates of precursor transport and incorporation into macromolecules were measured as described (2), with pulse periods of 1 hr. 2-Deoxyglucose uptake was measured according to Martin *et al.* (10). after preincubation for 45 min in glucose-free medium.

Intracellular cAMP concentrations were assayed by Gilman's method on samples prepared as described (1). N^6, O^2–dibutyryl cAMP [$(Bu)_2$cAMP] and guanosine cyclic 3′:5′-monophosphate were purchased from Calbiochem, theophylline and cycloheximide from Sigma, colcemid from Ciba, and vinblastine sulfate from Eli Lilly and Co. Prostaglandin E_1 (PGE_1) was a gift from the Upjohn Co.

RESULTS

Reversal by cGMP of the pleiotypic inhibition of 3T3 cells treated with $(Bu)_2$cAMP or PGE_1

In 3T3 cells, the rates of transport of uridine, leucine, and 2-deoxyglucose are depressed during serum deprivation and stimulated by the addition of serum again. We have reported (1) that $(Bu)_2$cAMP + theophylline or PGE_1 added to cells maintained in serum-containing medium mimic the response to serum starvation. These effectors also inhibit serum-stimulated uptake of precursor by previously starved cells (1). We now show that cGMP antagonizes the effects of both $(Bu)_2$cAMP + theophylline and PGE_1 on these biochemical processes (Figs. 1–4). Figs. 1 and 2 show the rates of uridine, leucine, and 2-deoxyglucose transport in serum-starved cells and in comparable cells to which serum has again been added. The presence of $(Bu)_2$cAMP + theophylline partially blocks the stimulation by serum of precursor uptake, while the inclusion of cGMP tends to restore the inhibited rates toward those of the control. Figs. 3 and 4 compare the decline in the rates of uridine and leucine transport when serum is removed to the rates when PGE_1 is added either alone or with cGMP. These experiments illustrate that PGE_1 inhibits precursor uptake and that cGMP completely reverses this inhibition. The results suggest that cGMP antagonizes the inhibition of transport produced by cAMP.

5′-AMP and 2′:3′-cyclic AMP also inhibit the transport of uridine by 3T3 cells. Adenosine decreases not only uridine, but leucine and 2-deoxyglucose, uptake as well (1). In the present series of experiments we tested the ability of 1 mM cGMP to reverse the inhibitory action of these adenine derivatives (at 0.5 mM). The experimental design was similar to that described under Figs. 1 and 2. Serum was readded to previously starved cells, and inhibition of precursor uptake by the adenine derivatives was measured in the absence or presence of cGMP. This cyclic nucleotide slightly increases the inhibition of

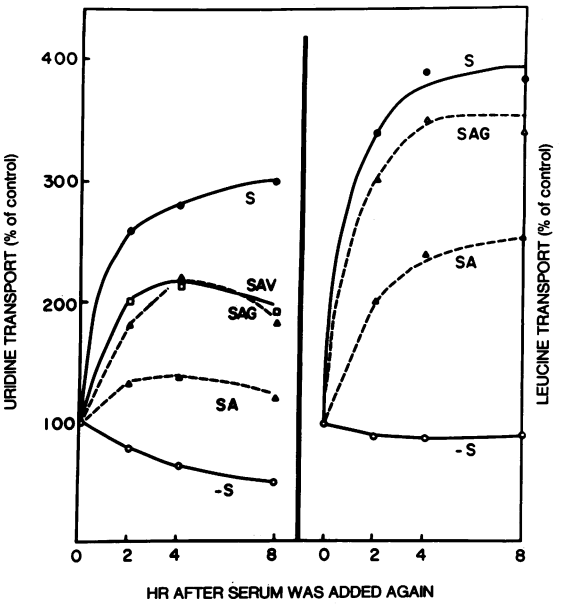

FIG. 1. Effect of $(Bu)_2$cAMP, cGMP, and vinblastine on the serum-stimulated rates of uridine and leucine transport in 3T3 cells. After serum deprivation for 16 hr, the cells were, from time 0, further incubated in either fresh MEM ($-S$) or MEM plus 10% calf serum (S; control), with or without the following additions symbolized in the figures as follows: A = $(Bu)_2$cAMP (0.2 mM) + theophylline (1 mm); G = cGMP (1 mM); V = vinblastine (10 pg/ml).

uridine transport by adenosine, 5′-AMP, and 2′:3′-cyclic AMP. The stimulation of leucine and 2-deoxyglucose uptake upon serum readdition was inhibited by adenosine, whether or not cGMP was present. Thus, in contrast to the transport inhibition caused by cAMP, the inhibition of these processes by other adenine derivatives is not antagonized by cGMP. These results imply that different mechanisms underlie the transport inhibition by cAMP and by other adenine derivatives.

PGE_1, in addition to mimicking the inhibitory effects of $(Bu)_2$cAMP on membrane transport, also depresses other pleiotypic parameters, such as protein and RNA synthesis

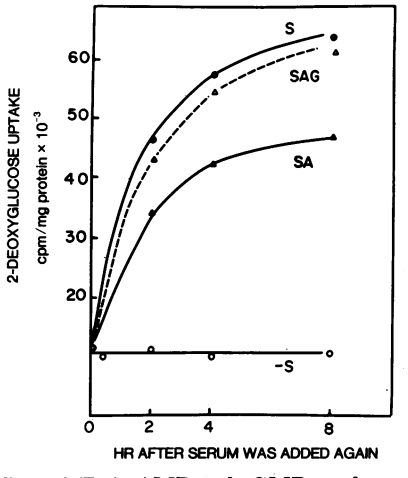

FIG. 2. Effect of $(Bu)_2$cAMP and cGMP on the serum-stimulated uptake of 2-deoxyglucose by 3T3 cells. Experimental conditions were as in Fig. 1.

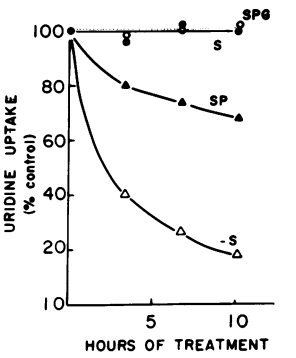

FIG. 3. Effect of cGMP on inhibition by PGE_1 of uridine transport in 3T3 cells. At time 0, the media were removed and the cells were further incubated in MEM ($-S$) or MEM plus serum (S) with, as indicated, the addition of 5 μg/ml of PGE_1 (P) alone or in combination with 1 mM cGMP (G).

(1). Fig. 4 shows that cGMP also overcomes the inhibition by PGE_1 of leucine incorporation into proteins. We have not been able to assess the effect of cGMP on RNA synthesis because this cyclic nucleotide interferes with the transport of adenosine, the precursor that gives the most valid measurement of this process (2).

Effects of colcemid and vinblastine on membrane transport

Serum deprivation and other treatments, which increase the intracellular cAMP concentrations, alter the morphology of various cell types (11–17) as well as the biochemical reactions under pleiotypic control (1). Microtubules determine cell shape, and it has been proposed that cAMP affects cell morphology by promoting the aggregation and organization of microtubules (11). To explore the possible relationship between structural and biochemical changes induced by cAMP, we investigated the effects of inhibitors of microtubule assembly on the pleiotypic reactions.

When 3T3 cells starved overnight for serum are treated with either colcemid or vinblastine sulfate, a significant stimulation of uridine transport is observed (Fig. 5). Transport of leucine and to a somewhat lesser extent of 2-deoxyglucose, is similarly enhanced under these conditions (not shown). An indication of

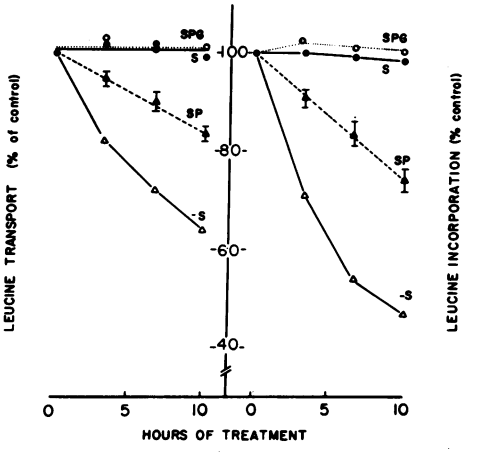

FIG. 4. Effect of cGMP on inhibition by PGE_1 of leucine transport and incorporation. Experimental conditions were as described under Fig. 3.

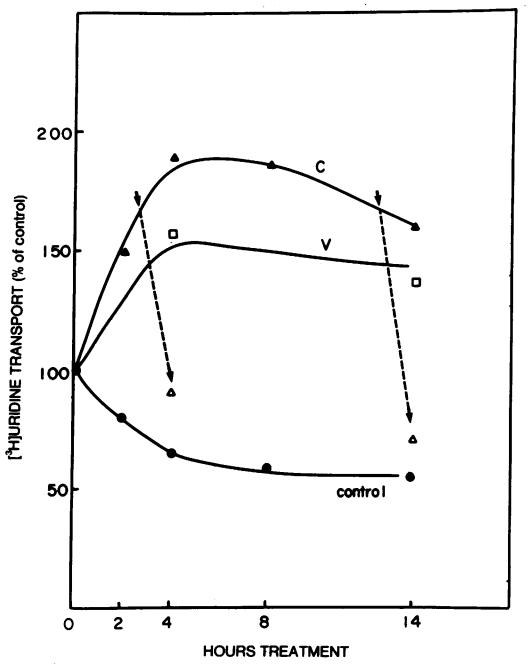

FIG. 5. Stimulation by colcemid and vinblastine of the rates of uridine transport in starved 3T3 cells. After serum deprivation for 16 hr, the cells were, from time 0, further incubated in fresh MEM without serum (*control*) or in MEM plus, as indicated, either colcemid (*C*) or vinblastine (*V*) at respective concentrations of 0.2 and 0.01 μg/ml. At the times indicated by the *arrows*, the media were removed in all dishes and the cells were washed and further incubated in fresh media with or without (↓) the inhibitors.

the specificity of these drugs in stimulating membrane transport reactions inhibited by cAMP is that they do not increase adenosine transport. Colcemid and vinblastine also overcome the $(Bu)_2$cAMP + theophylline inhibition of serum-stimulated transport of uridine (Fig. 1) and leucine (not shown). As illustrated in Fig. 5, the stimulatory effects of colcemid and vinblastine on transport are readily reversible. Within 2 hr after removal of either of these drugs the stimulated transport of

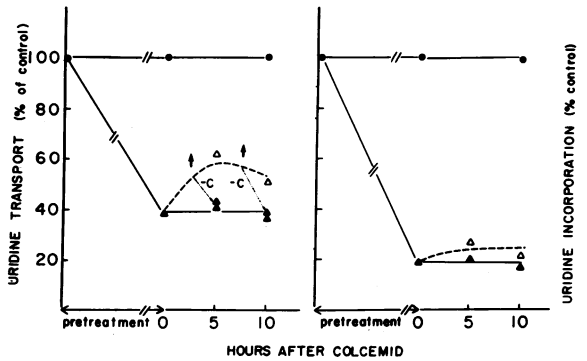

FIG. 6. Effect of colcemid on the rates of uridine transport and incorporation in 3T3 cells treated with $(Bu)_2$cAMP. The cells were first treated for 16 hr in MEM plus serum alone (●——●) or with the addition of 0.2 mM $(Bu)_2$cAMP + 1 mM theophylline (▲——▲). At time 0, some of the cells treated with $(Bu)_2$cAMP + theophylline received colcemid (0.2 μg/ml) (△- - -△). Later, the media containing colcemid were removed from some of the dishes at times indicated by the *arrows*, and the cells were further incubated in the presence of $(Bu)_2$cAMP + theophylline (△).

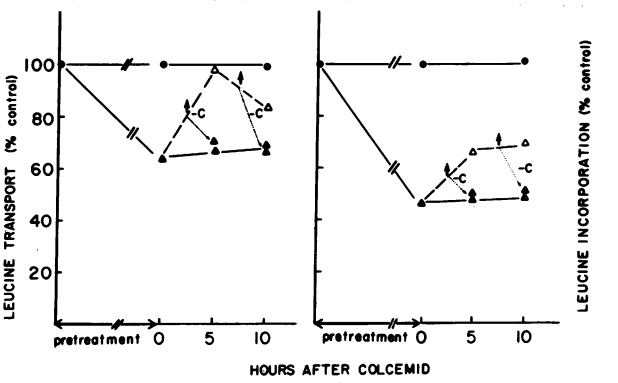

FIG. 7. Effect of colcemid on the rates of leucine transport and incorporation in 3T3 cells treated with $(Bu)_2$cAMP. Experimental conditions were as described under Fig. 6.

both uridine and leucine (not shown) has returned nearly to the inhibited level seen in serum-starved cells. On the contrary, cytochalasine B at 4 μg/ml, an inhibitor of microfilament assembly, depressed the rates of transport below those in serum-deprived 3T3 cells; this effect was also reversible (data not shown).

cGMP did not enhance further the stimulation produced by colcemid and vinblastine. Moreover, in cells treated with these drugs, the transport processes became insensitive to $(Bu)_2$cAMP. Thus, the antagonism between the two cyclic nucleotides could no longer be demonstrated in the presence of inhibitors of microtubule assembly. It should also be pointed out that these drugs caused 15–20% inhibition of uridine and leucine transport in both 3T3 and SV 40-3T3 fibroblasts maintained in serum-complemented medium.

The effect of colcemid on membrane transport in 3T3 cells treated with $(Bu)_2$cAMP + theophylline in the presence of serum is illustrated in Figs. 6 and 7. As shown previously (1) these effectors reduce the rates of uridine and leucine uptake. Under these conditions as well, colcemid partially reactivates these processes, and its effect is reversible. Indeed, removal of the drug restores the degree of transport inhibition observed in cells not treated with colcemid. The experiments illustrated in Figs. 6 and 7 indicate, in addition, that colcemid increases the uptake, but not the incorporation, of leucine and uridine into macromolecules.

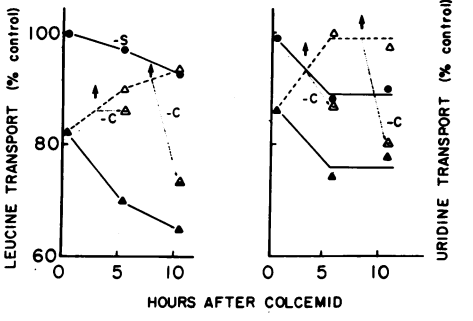

FIG. 8. Effect of colcemid on the rates of leucine and uridine transport in SV 40-3T3 fibroblasts. Experimental conditions were as in Figs. 6 and 7, except for the omission of serum in all incubation media.

TABLE 1. *cAMP concentrations in 3T3 cells under conditions affecting membrane transport*

Additions to serum-free MEM	cAMP (pmol/mg of protein)
None	18.6 ± 0.8
Serum	12.8 ± 0.5
Colcemid	18.3 ± 0.5
Cycloheximide	13.6 ± 0.3

Cells were seeded 3 days before the beginning of the experiment and then maintained in the absence of serum for 18 hr. Thereafter, they were incubated for 2 hr in fresh MEM alone or with the addition of calf serum (10%), colcemid (0.2 μg/ml), or cycloheximide (0.1 mM). Each value is the mean of four experiments with the corresponding standard deviation shown. Some of the data were taken from a previous communication, where the procedure and controls for the assays are described (1).

Despite the lack of significant inhibition of membrane transport during serum starvation of SV 40-transformed 3T3 fibroblasts, exogenous $(Bu)_2$cAMP + theophylline can decrease uridine and leucine uptake by these cells, and as shown in Fig. 8, colcemid reverses this inhibition.

We investigated whether the transport stimulation by inhibitors of microtubule assembly could have involved a decrease in cellular cAMP concentration. Such a mechanism was invoked to explain apparently similar effects of cycloheximide (1). The data in Table 1 show that colcemid does not affect the intracellular concentrations of cAMP in serum-starved cells. In an additional experiment, vinblastine likewise did not change the cAMP concentration. On the contrary, cAMP decreases under two other conditions that also enhance precursor uptake in starved 3T3 cells: serum readdition and cycloheximide treatment.

DISCUSSION

cAMP inhibits diverse membrane transport processes, slows the rates of RNA and protein synthesis, and stimulates protein degradation in cultured mouse fibroblasts (1). The present experiments suggest that cGMP antagonizes these pleiotypic effects of cAMP. Thus cGMP overcomes the cAMP-inhibited uptake of uridine, leucine, and 2-deoxyglucose, whether this inhibition is caused by $(Bu)_2$cAMP + theophylline or PGE_1. cGMP also restores to normal the rate of leucine incorporation into protein decreased by PGE_1. These results imply that the same mechanisms underlie the pleiotypic effects of PGE_1 and $(Bu)_2$cAMP and support the conclusion that cAMP itself is a pleiotypic mediator (1).

The mechanism of the antagonism between the cyclic nucleotides remains to be investigated. Several reports indicate that cAMP and cGMP compete for binding to a regulatory protein controlling gene expression in bacteria (3–4). On the other hand, in animal-cell extracts, cGMP stimulates hydrolysis of cAMP (7, 8). Whether either of these mechanisms or others, as yet undiscovered, operate in the present case is an open question.

Since the concentrations of cGMP used in the present study are much higher than the physiological concentration (18), judgment of the biological significance of the antagonism between cAMP and cGMP must be withheld until their respective intracellular concentrations and subcellular distribution are better known. However, the results suggest that pleiotypic control might be modulated not only by cAMP, but also by cGMP. If this were the case, fluctuations of cGMP concentration could influence cAMP-controlled processes even in the absence of any change in cAMP concentrations. A recent report (19) attributing the mitogenic activities of phytohemagglutinin and concanavalin A on lymphocytes to increases in cGMP concentration lends support to the physiological relevance of our observations.

As reported in the second section of the results, inhibitors of microtubule assembly rather surprisingly increase the rates of uridine and leucine transport depressed by serum starvation or treatment of 3T3 cells with $(Bu)_2$cAMP + theophylline. A similar effect was noted in SV 40-transformed fibroblasts treated with $(Bu)_2$cAMP. In unperturbed cells, we observe instead that both colcemid and vinblastine slightly inhibit (15–20%) the uptake of uridine and leucine. This last result is consistent with a recent detailed study (20) that has concluded that colchicine and some related compounds inhibit nucleoside transport in several mammalian cell lines by a mechanism not involving microtubules. Thus, whatever the mechanism of the transport stimulation we observe, its magnitude might even have been underestimated.

The site of action of colcemid and vinblastine in the present experiments is unknown. These agents could, in principle, have acted to decrease the intracellular cAMP concentration, thus stimulating membrane transport. However, direct measurements indicate that cAMP concentrations are unaffected by colcemid or vinblastine and, furthermore, these drugs are effective in the presence of added $(Bu)_2$cAMP. We are unable to rule out the possibility that these drugs might have increased the cGMP concentration. Colcemid and vinblastine affect microtubules, although it might be that the stimulatory actions reported here result from their interaction with other cellular structures. In the absence of further information, the microtubules seem a likely target, especially in view of the fact that the effective concentrations for transport stimulation and for microtubular disaggregation are similar.

If microtubules are indeed the structures through which the stimulatory effects of colcemid and vinblastine are exerted, the question arises as to how these compounds control transport. It seems improbable that the accumulation of mitotic cells is the basis for the observed effects, since maximal stimulation occurs well before a significant proportion of the cell population would be arrested. Secondly, the drugs accelerate membrane transport in serum-starved cells, which are blocked in G_1. We are thus left with the conclusion that microtubules themselves, either directly or indirectly, control these specific membrane transport processes involved in the pleiotypic control. If this were the case, the effects of cAMP, and possibly the antagonistic action of cGMP as well, might be mediated by a modulation of the state of aggregation and/or organization of the microtubular apparatus.

This conclusion would seem to correlate well with the numerous reports that cAMP induces changes in the morphology of malignant ovary cells (11), virally transformed fibroblasts (12, 13), and neuroblastoma cells by promoting microtubular assembly (14–17). An early event in cAMP-treated cells is the "quieting" of membrane activity (21). Puck and coworkers have also found that cAMP causes the disappearance of knob-like processes during the conversion of epithelial malignant ovary cells to fibroblast-like morphology. This disappearance is prevented by colcemid which,

in addition, elicits knobbed structures on the smooth surface of other cultured cell lines (22, 23). Other morphological studies have shown a similar increase in plasma membrane activity promoted by colcemid or vinblastine treatment of contact-inhibited mouse embryo fibroblasts. These changes were correlated with subsequent resumption of thymidine incorporation in DNA (24).

These and earlier considerations lead to the conclusions that microtubular organization determines both cell morphology and membrane transport. cAMP, the concentration of which varies inversely with the growth-promoting properties of the cellular environment, both promotes microtubular aggregation and depresses certain uptake processes. Malignant transformation leads to decreased concentrations of, and/or diminished cellular responsiveness to, cAMP with corresponding changes in cell shape and increases in precursor uptake. cGMP antagonizes at least certain aspects of cAMP action and may, therefore, participate in the pleiotypic regulation of cell function and growth.

This study was supported by Contract no. 72-3236 within the Special-Virus Cancer Program of the National Cancer Institute, NIH–PHS and Grant no. GM 17239 from the National Institute of General Medical Sciences of the National Institutes of Health. The work reported in this paper was undertaken during the tenure of a Research Training Fellowship no. R-305 awarded by the International Agency for Research on Cancer to R.K. and completed during the tenure of a Dernham Senior Fellowship no. D-194 of the American Cancer Society, California Division.

1. Kram, R., Mamont, P. & Tomkins, G. M. (1973) *Proc Nat. Acad. Sci. USA* **70**, 1432–1436.
2. Hershko, A., Mamont, P., Shields, R. & Tomkins, G. M. (1971) *Nature New Biol.* **232**, 206–211.
3. Zubay, G., Schwartz, D. & Beckwith, J. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 104–110.
4. Emmer, M., de Crombrugghe, B., Pastan, I. & Perlman, R. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 480–487.
5. Pagliara, A. S. & Goodman, A. D. (1969) *J. Clin. Invest.* **48**, 1408–1412.
6. Pagliara, A. S. & Goodman, A. D. (1970) *Amer. J. Physiol.* **218**, 1301–1306.
7. Beavo, J. A., Hardman J. G. & Sutherland, E. W. (1970) *J. Biol. Chem.* **245**, 5649–5655.
8. Beavo, J. A., Hardman, J. G. & Sutherland, E. W. (1971) *J. Biol. Chem.* **246**, 3841–3846.
9. Franks, D. J. & MacManus, J. P. (1971) *Biochem. Biophys. Res. Commun.* **42**, 844–849.
10. Martin, G. S., Venuta, S., Weber, M. & Rubin, H. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2739–2741.
11. Hsie, A. W. & Puck, T. T. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 358–361.
12. Johnson, G. S., Friedman, R. M. & Pastan, I. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 425–429.
13. Sheppard, J. R. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1316–1320.
14. Seeds, N. W., Gilman, A. G., Amano, T. & Nirenberg, M. W. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 160–167.
15. Furmanski, P., Silverman, D. J. & Lubin, M. (1971) *Nature* **233**, 413–415.
16. Prasad, R. N. & Hsie, A. W. (1971) *Nature New Biol.* **233**, 141–142.
17. Schubert, D. & Jacob, F. (1970) *Proc. Nat. Acad. Sci.* **67**, 247–254.
18. Ishikawa, E., Ishikawa, S., Davis, J. W. & Sutherland, E. W. (1969) *J. Biol. Chem.* **244**, 6371–6376.
19. Hadden, J. W., Hadden, E. M., Haddox, M. K. & Goldberg, N. D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3024–3027.
20. Mizel, S. B. & Wilson, L. (1972) *Biochemistry* **11**, 2573–2578.
21. Johnson, G. S., Morgan, W. D. & Pastan, I. (1972) *Nature* **245**, 54–56.
22. Hsie, A. W., Jones, C. & Puck, T. T. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1648–1652.
23. Puck, T. T., Waldren, C. A. & Hsie, A. W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1943–1947.
24. Vasiliev, J. M., Gelfand, I. M. & Guelstein, V. I. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 977–979.