Biochem. J. (1973) **134**, 311–319
Printed in Great Britain

Adenosine 3′:5′-Cyclic Monophosphate Concentrations and Phosphodiesterase Activities during Axenic Growth and Differentiation of Cells of the Cellular Slime Mould *Dictyostelium discoideum*

By A. M. MALKINSON* and J. M. ASHWORTH
Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, U.K.

(*Received* 27 *November* 1972)

During growth of myxamoebae of *Dictyostelium discoideum* (strain Ax-2) in axenic medium, the myxamoebae secrete cyclic AMP. As the cells leave the exponential phase of growth and enter the stationary phase, there is an approximate doubling of the intracellular cyclic AMP content, but the amount of extracellular cyclic AMP remains proportional, at all times, to the number of myxamoebae present. During development of axenically grown myxamoebae, there is first a rise in the intracellular concentration of cyclic AMP, followed by a rise in the amount of extracellular cyclic AMP, which reaches a peak at the time of aggregation and then declines. There is a second peak in the amount of extracellular cyclic AMP found at the time of fruiting-body formation, but this second peak is not associated with a rise in the intracellular cyclic AMP concentration. Controls thus exist over the synthesis and secretion of cyclic AMP. Evidence is presented for the belief that the activity of the adenylate cyclase enzyme controls the amount of cyclic AMP synthesized rather than the activity or amount of cyclic AMP phosphodiesterase present. Similar changes occur in extracellular cyclic AMP and phosphodiesterase concentrations during incubation of myxamoebae in buffered suspensions to those occuring during the first few hours of development of such cells on solid media, but the timing of these changes is different.

The life cycle of the cellular slime mould *Dictyostelium discoideum* is divided into two mutually exclusive phases, the growth phase, during which all DNA synthesis and most cell division occurs, and the differentiation phase, during which the hitherto solitary myxamoebae construct a multicellular fruiting body (Bonner, 1967). These two phases are connected by the aggregation stage of the life cycle when the non-growing myxamoebae gather about a central point by a process of chemotaxis and form a multicellular aggregate, the grex. The chemotactic gradient is composed of a signal [identified by Konijn *et al.* (1968) as 3′:5′-cyclic AMP] and an enzyme that destroys the signalling molecule (Shaffer, 1956), identified as a phosphodiesterase by Goidl *et al.* (1972).

In addition to its role in chemotaxis, high concentrations of extracellular cyclic AMP have been shown by Bonner (1970) to convert isolated myxamoebae into cells resembling those normally found in the stalk of the fruiting body.

Bonner *et al.* (1969), using a bioassay, showed that there was an increase in the extracellular cyclic AMP concentration before aggregation of the myxamoebae and that after aggregation was completed there was a decrease in the extracellular cyclic AMP concentration. Malkinson & Ashworth (1972), using a protein-binding assay, showed that as axenically grown myxamoebae leave true exponential growth and enter the stationary phase there is a dramatic increase in the extracellular cyclic AMP concentration. The mechanism whereby these changes in cyclic AMP concentration are achieved is unknown.

Cyclic AMP is synthesized by the reaction catalysed by adenylate cyclase:

$$\text{ATP} = \text{cyclic AMP} + \text{PP}_i$$

and is hydrolysed by the reaction catalysed by cyclic AMP phosphodiesterase:

$$\text{Cyclic AMP} = 5'\text{-AMP}$$

In principle, an alteration in the amount or activity of either of these two enzymes or an alteration in the rate, or extent, of excretion could affect the extracellular cyclic AMP concentration.

Rossomando & Sussman (1972) measured the specific activity of adenylate cyclase throughout the growth and differentiation phases of the life cycle and

* Present address: Department of Pharmacology, Yale School of Medicine, Yale University, New Haven, Conn. 06510, U.S.A.

found that the specific activity remains constant at 3.4nmol of cyclic AMP formed/min per mg of protein at 37°C. Two extracellular phosphodiesterase activities have been reported. One of these has a high K_m value (2×10^{-3}M; Chang, 1968) and the other a low K_m value (4×10^{-6}M; Reidel & Gerisch, 1971) for cyclic AMP. It is possible that these two enzymes are alternative forms of the same protein species since Chassy (1972) reported the spontaneous conversion of the low-K_m enzyme into the high-K_m one during incubation in cell-free systems. The extracellular cyclic AMP concentration is such that only the low-K_m enzyme could be significant in regulating the cyclic AMP concentration. Reidel & Gerisch (1971) have found that in addition to an extracellular phosphodiesterase activty, the myxamoebae excrete a proteinaceous inhibitor of the low-K_m enzyme. This inhibitor is excreted specifically at the end of the growth phase (when the myxamoebae were fed on bacteria) and causes a marked decrease in the extracellular phosphodiesterase activity. Gerisch *et al.* (1972) have correlated the changes in phosphodiesterase activity caused by the secretion of this inhibitor with the development by the cells of 'aggregation competence' when they are shaken in buffered suspensions.

In the present paper we report measurements of the concentration of cyclic AMP and of low-K_m phosphodiesterase activity inside and outside the cells during their growth and subsequent development.

Materials and Methods

Materials

Cyclic AMP, cyclic AMP phosphodiesterase from ox heart, and snake venom 5′-nucleotidase were obtained from Sigma (London) Ltd., London S.W.6, U.K., and all other chemicals were of the highest purity available commercially and were obtained either from BDH Chemicals Ltd., Poole, Dorset, U.K., or from Fisons, Loughborough, Leics., U.K. Cyclic [^{3}H]AMP was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Cycloheximide was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Methods

Preparation of samples during the growth and differentiation of myxamoebae. Dictyostelium discoideum (strain Ax-2) was grown on axenic medium containing 86mM-glucose as described by Watts & Ashworth (1970). Samples of growth medium were obtained and assayed for cyclic AMP as described previously (Malkinson & Ashworth, 1972). These samples were also used for phosphodiesterase assays. Myxamoebal pellets were washed by centrifugation in 5mM-Tris–HCl buffer, pH7.5, and finally resuspended in this buffer at approx. 10^8 cells/ml before being stored frozen. Cell-free extracts for assay of intracellular cyclic AMP and of phosphodiesterase were obtained by exposing thawed cell suspensions to the output of an MSE ultrasonic disintegrator (peak-to-peak amplitude 8μm) for 30s at 0°C.

Differentiation was initiated by harvesting myxamoebae in the exponential phase of growth (2×10^6–5×10^6 cells/ml), washing them with ice-cold 0.9% NaCl by centrifugation and resuspending them in cold water. Myxamoebae were deposited in 0.5ml of water on to Millipore filter supports at 2.5×10^7–3.5×10^7 cells per filter (Sussman, 1966). Cells were harvested from these filters into 5mM-Tris–HCl buffer, pH7.5, as described above and cell-free extracts were obtained by using total ultrasonication times of 30s for samples collected during the 0–16h stages of development, 60s for samples collected during the 17–20h stages and 90s for samples collected during the last stages of development. The extracellular cyclic AMP concentration and phosphodiesterase activity were assayed by taking the cellulose pad that underlies the Millipore filter (Sussman, 1966) and squeezing it with hands protected with plastic gloves. One support pad yields about 1.5ml of liquid, which was centrifuged to remove residual cellulose fibres and then stored frozen.

Myxamoebae harvested when in the exponential phase of growth were also suspended in 16.7mM-sodium phosphate buffer, pH6.0, at a density of 3×10^6 cells/ml and shaken at 22°C. Such suspensions are analogous to those first used by Gerisch (1959). We find that for the first 6h of incubation in such suspensions events seem to occur similar to those occurring to cells on Millipore filters. Thus cells placed on Millipore filters after 6h incubation in 16.7mM-phosphate buffer, pH6.0, form fruiting bodies after 18h further incubation at 22°C instead of the 24h required by cells freshly harvested from growth medium. However, cells incubated in 16.7mM-phosphate buffer, pH6.0, for times longer than 6h seem to suffer damage, and their ability to form fruiting bodies deteriorates and the time taken to form fruiting bodies also increases. Similar observations have been made by Lee (1972).

Assay of cyclic AMP. Cyclic AMP was assayed as described previously (Malkinson & Ashworth, 1972). In this assay incubation mixtures contain no $MgCl_2$ and are at pH4.0, i.e. conditions which inhibit phosphodiesterase activity. The material assayed as cyclic AMP was shown to be cyclic AMP by (1) showing that it was destroyed by purified cyclic AMP phosphodiesterase from ox muscle and (2) by direct chromatographic analysis. Material from 50 Millipore-filter support pads was freeze-dried and resuspended in 1.5ml of water. Cyclic [^{3}H]AMP was added and the

mixture chromatographed on Dowex-50 (H^+ form) columns. The radioactivity and cyclic-AMP-assayable material were eluted identically.

Phosphodiesterase activity was determined in samples that had been dialysed against 200vol. of 5mM-Tris–HCl buffer, pH7.5, for 4–5h at 0°C by a modification of the method of Brooker *et al.* (1968). Reaction mixtures contained, in 0.5ml; 26nmol of cyclic [^{3}H]AMP (4×10^4–5×10^4 d.p.m.), 25μmol of Tris–HCl buffer, pH7.5, 1.25μmol of $MgSO_4$ and 40μg of 5′-nucleotidase from snake venom. Such mixtures were incubated at 35°C for 20min, during which time the concerted action of the phosphodiesterase and nucleotidase converted cyclic [^{3}H]-AMP into [^{3}H]adenosine. The reaction was stopped by addition of 1.5ml of an ethanolic slurry of Dowex-1 (X8; 200–400 mesh; Cl^- form) containing 6g of resin in 40ml of ethanol. This resin binds cyclic AMP but not adenosine. The mixture was well shaken, left for 15min and then centrifuged. A portion (0.5ml) of the resulting supernatant was added to 8ml of Bray's (1960) scintillation fluid. A blank correction (which amounts to some 6–8% of the added label) was made on the basis of an incubation mixture that contained no phosphodiesterase, but was otherwise complete. The release of label was linear with respect to enzyme concentration and with respect to time of incubation provided that not more than 75% of the added cyclic [^{3}H]AMP was converted into adenosine. A unit of activity catalyses the hydrolysis of 1nmol of cyclic AMP/min under the conditions described above. Results are expressed as means ±S.E.M. with the number of determinations in parentheses where appropriate.

Results

Extracellular concentration of cyclic AMP and phosphodiesterase during the growth of myxamoebae

The concentration of extracellular cyclic AMP increases from about 70 to 1000pmol/ml during the growth of myxamoebae in axenic culture (Malkinson & Ashworth, 1972). The rate of increase appears fastest just before the cells enter the stationary phase of growth. This increase could be due to an increased rate of synthesis of cyclic AMP by all the cells or it could be a consequence of the rapid absolute increase in cell number which also occurs at this time. If it is assumed that the rate of synthesis and of secretion of cyclic AMP is constant at all times, then the amount of cyclic AMP present at any moment should be proportional to the number of myxamoebae present at that time. As shown in Fig. 1, this is so both for cells grown in medium containing 86mM-glucose and for cells grown in medium containing different amounts of glucose.

The activity of the extracellular phosphodiesterase is constant at 1.42±0.2 (10) units/ml throughout the growth phase.

Intracellular concentration of cyclic AMP and phosphodiesterase during the growth of myxamoebae

The intracellular concentration of cyclic AMP is constant throughout the exponential phase of growth, but as the cells leave the exponential phase there is a doubling in the steady-state concentration (Table 1). Since 10^7 myxamoebae contain approx. 1 mg of protein (Ashworth & Watts, 1970) and 0.005ml of water, the concentration of cyclic AMP in the cells (assuming no intracellular compartmentation) is 1500–2000pmol/ml during exponential growth and 3000–4000pmol/ml during the stationary phase of growth. There is no need, therefore, to postulate any active secretion process to account for the extracellular concentrations of cyclic AMP (Fig. 1).

The intracellular activity of phosphodiesterase is constant at 0.27±0.02 (10) unit/mg of protein throughout the exponential and stationary phases of growth.

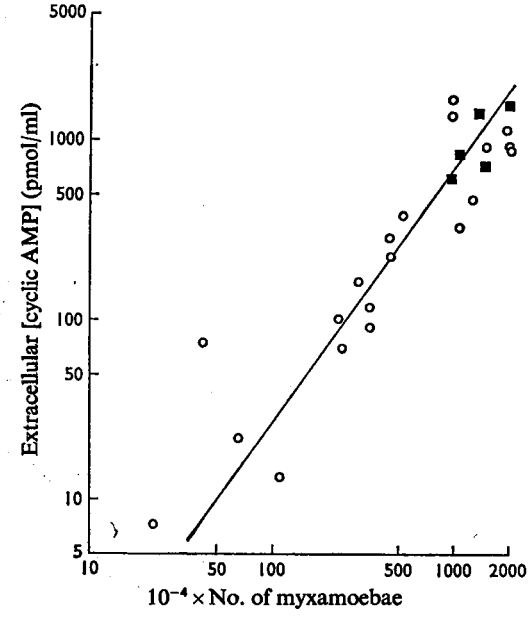

Fig. 1. *Extracellular cyclic AMP concentrations during the exponential and stationary phases of growth of D. discoideum Ax-2 in axenic medium*

Media containing 86mM-glucose (○; the results of three separate experiments are shown) or various other concentrations of glucose (■; data from Table 1 of Malkinson & Ashworth, 1972) were used. For details see the Materials and Methods section.

Changes in the intracellular and extracellular concentrations of cyclic AMP during the differentiation of axenically grown myxamoebae

Fig. 2. summarizes the results from six independent determinations of the cyclic AMP concentration of the fluid squeezed out of the cellulose support pads after various times of development. Fruiting-body formation was completed within 24±2h and aggregation was completed by 10±2h. The rise in extracellular cyclic AMP concentration that occurs during the first 12h of development has been observed in every experiment that we have carried out and seems to be independent of the growth medium used for production of the myxamoebae. Thus myxamoebae harvested in the exponential or in the stationary phases of growth from media containing or lacking added carbohydrate all produced curves of the type shown in Fig. 2. This is in marked contrast to some other changes (Quance & Ashworth, 1972) which have been assumed to be part of the developmental programme.

Newell *et al.* (1969) showed that if the phosphate-buffered salts solution used as a routine to keep the cellulose support pad moist is replaced by water then development of the myxamoebae stops at the motile grex (16h) stage. When axenically grown *D. discoideum* Ax-2 myxamoebae are allowed to differentiate in the presence of water the aggregation stage is much delayed, but eventually motile grexes are formed and, in agreement with the findings of Newell *et al.* (1969), we find that these slugs migrate indefinitely without further development. During this developmental sequence there is a slow continuous increase in the concentration of extracellular cyclic AMP to a

Table 1. *Intracellular concentration of cyclic AMP during growth of myxamoebae in axenic medium + 86 mM-glucose*

The doubling time of the cells is 8h and growth is exponential up to approx. 8×10^6 myxamoebae/ml. For other details see the text.

$10^{-6}\times$Cell no.	[Cyclic AMP] (pmol/mg of protein)
1.5	7.1
3.1	7.6
4.7	8.4
6.5	5.7
7.5	6.4
	(mean = 7.04)
10.5	14.9
11.3	12.5
15	14.5
16.3	18.6
	(mean = 15.1)

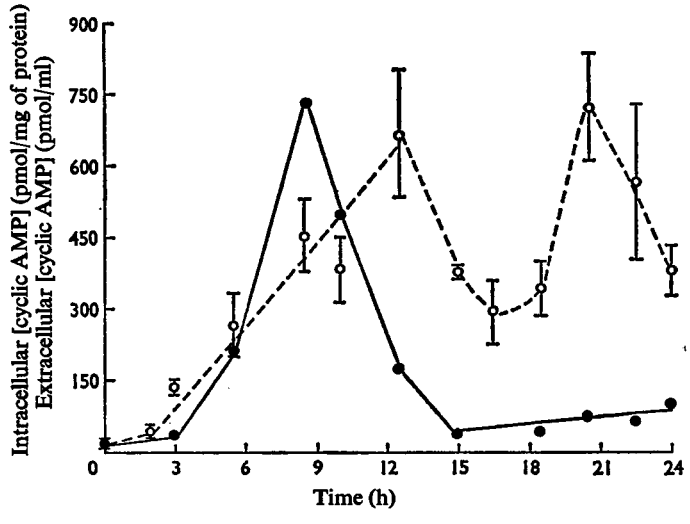

Fig. 2. *Changes in intracellular* (●) *and extracellular* (○) *cyclic AMP concentrations during development of myxamoebae of D. discoideum Ax-2 on Millipore filters*

The results for the extracellular cyclic AMP concentration represent the mean ±S.E.M for six separate experiments and the results for the intracellular cyclic AMP concentration represent the mean for three separate experiments. For details see the Materials and Methods section.

final value of 120pmol/ml attained after 32h of incubation at 22°C (Fig. 3).

The second peak of extracellular cyclic AMP (Fig. 2) is rather more variable than the first (note the large standard error of the 22h value) and we have occasionally failed to observe it at all.

The intracellular cyclic AMP concentration (Fig. 2) increases during development to many times the value seen during the growth phase and reaches a peak at the time aggregation is completed. No second peak at the 20–22h stage has been observed, even in experiments where the peak in extracellular cyclic AMP concentration at this time (Fig. 2) has been particularly marked.

Changes in the intracellular and extracellular concentrations of cyclic AMP phosphodiesterase during the differentiation of axenically grown myxamoebae

Fig. 4 summarizes the changes in activity of the low-K_m cyclic AMP phosphodiesterase in the cells and in the fluid squeezed out of the cellulose support pads during the development of myxamoebae. The extracellular phosphodiesterase that we have assayed here appears to be identical with that described by Chassy (1972). It is inhibited by dithiothreitol (as also reported by Pannbacker & Bravard, 1972) and has a K_m in the range 4×10^{-6}–10×10^{-6}M for cyclic AMP. The intracellular cyclic AMP phosphodiesterase activity that we have assayed has a similar low K_m and is probably largely membrane bound, since most of the activity is pelleted by centrifugation of crude extracts at 12000*g* for 15min. In these respects this activity resembles that described by Malchow *et al.* (1972), who also reported that a rise in the intracellular phosphodiesterase activity occurred during the development of aggregation competence of myxamoebae shaken in buffer.

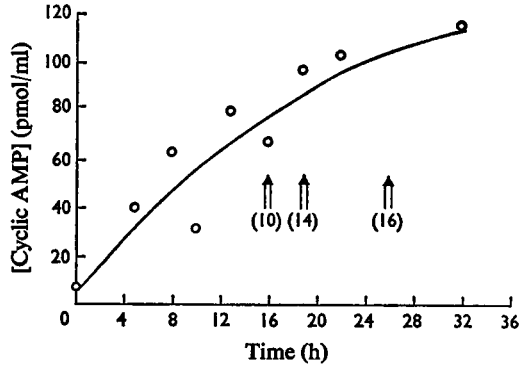

Fig. 3. *Changes in extracellular cyclic AMP concentrations during development of myxamoebae of D. discoideum Ax-2 on Millipore filters in the absence of any added salts*

The arrows show the time (h) taken to reach a comparable developmental stage in the presence of the usual salts solution (cf. Fig. 2). For details see the Materials and Methods section.

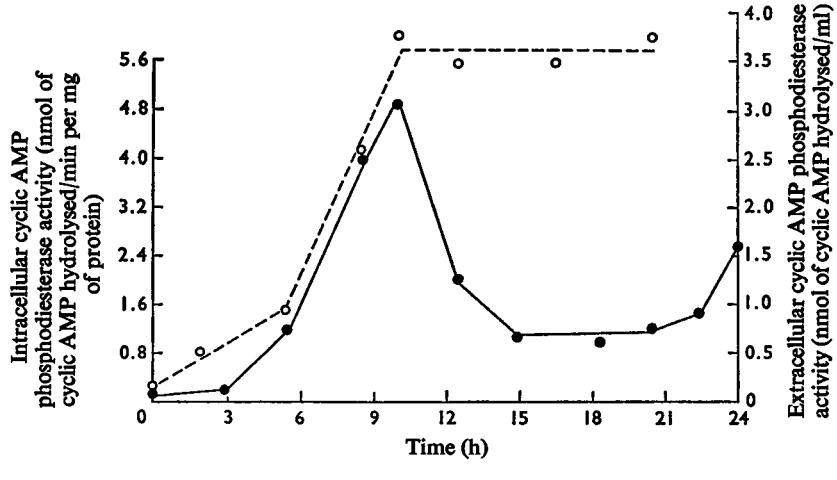

Fig. 4. *Changes in intracellular* (●) *and extracellular* (○) *cyclic AMP phosphodiesterase activity during development of myxamoebae of D. discoideum Ax-2 on Millipore filters*

For details see the Materials and Methods section.

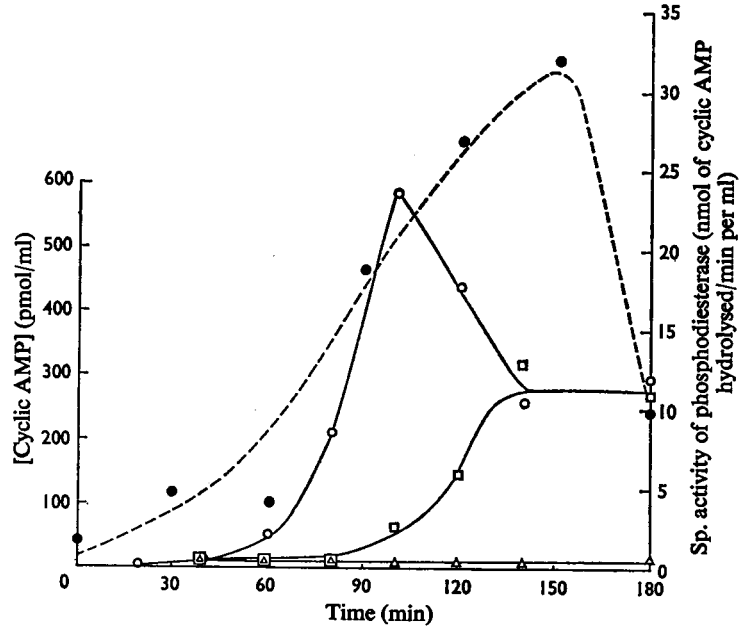

Fig. 5. *Changes in extracellular cyclic AMP phosphodiesterase activity* (●) *and extracellular cyclic AMP concentration* (○) *during the incubation of myxamoebae* (3×10^6 *cells/ml*) *of D. discoideum Ax-2 in* 16.7 mM-*phosphate buffer, pH* 6.0 *at* 22°C

Cycloheximide (□, 50 μg/ml; △, 150 μg/ml) was added to two similar suspensions and the extracellular cyclic AMP concentration was determined. For details see the Materials and Methods section.

Table 2. *Inhibition of protein synthesis by cycloheximide*

Myxamoebae grown in axenic medium + 86mM-glucose were harvested when in the exponential phase of growth and resuspended at 3×10^6 myxamoebae/ml in 16.7mM-sodium phosphate buffer, pH 6.5 (150ml), containing 10mM-[^{14}C]leucine (10 μCi total) and various concentrations of cycloheximide. Such suspensions were then shaken at 22°C and the amount of radioactivity precipitated by 5% trichloroacetic acid was determined at 20min intervals. Incorporation of radioactivity was linear with time after the first 60min of incubation in all cases and 100 is equivalent to the incorporation of 12pmol of leucine/h by 10^7 cells.

Concn. of cycloheximide (μg/ml)	Relative rate of incorporation of [^{14}C]leucine into protein
0	100
50	90
100	82
150	34
200	5

Changes in the intracellular and extracellular concentrations of cyclic AMP and its phosphodiesterase during incubation of myxamoebae in buffered suspensions

For many purposes it is inconvenient that development of the myxamoebae requires that the cells be placed on a solid surface. However, it seems that many of the initial steps characteristic of the first 6h of development on Millipore filters will occur in myxamoebae shaken at 22°C in 16.7mM-sodium phosphate buffer, pH 6.0. It is noteworthy, and probably very significant, that myxamoebae harvested when in the stationary phase of growth from axenic media take as long to form fruiting bodies when placed on Millipore filters as do myxamoebae harvested when in the exponential phase of growth. In this sense myxamoebae shaken in buffer are clearly different from myxamoebae harvested from stationary-phase cultures and it is as if there were in the spent growth media an inhibitor(s) of development. The nature of the inhibitory substance(s) (if they exist) is unknown (Lee, 1972).

In Fig. 5, the change in extracellular cyclic AMP concentration is shown for myxamoebae grown in axenic medium + 86mM-glucose, harvested when in

the exponential phase of growth and shaken in phosphate buffer in the presence and absence of cycloheximide. Cycloheximide causes a marked inhibition of protein synthesis (Table 2) as measured by [^{14}C]-leucine incorporation into trichloroacetic acid-precipitable material, but cyclic AMP synthesis and excretion (Fig. 5) appears to be more sensitive to the drug than does protein synthesis (Table 2). Fig. 5 also shows the changes in activity of extracellular low-K_m cyclic AMP phosphodiesterase during incubation of myxamoebae in phosphate buffer.

Discussion

We have studied the changes in concentration of cyclic AMP, and in activity of cyclic AMP phosphodiesterase, during the exponential and stationary phases of axenic growth of the myxamoebae (Table 1 and Fig. 1) and also during the subsequent differentiation of such myxamoebae (Table 2 and Figs. 2–5).

The increase in extracellular cyclic AMP found during the growth of myxamoebae in axenic medium (Malkinson & Ashworth, 1972) does not appear to be due to any marked change in the rate of synthesis of cyclic AMP (Fig. 1) nor, since the amount of extracellular phosphodiesterase is constant and small throughout the growth phase, is it due to any marked change in the rate of removal of cyclic AMP. It thus seems likely that this change reflects the change in cell density that occurs during growth and that, contrary to our previous ideas (Malkinson & Ashworth, 1972), the extracellular cyclic AMP concentration does not correlate well with the process of starvation. In agreement with this, we find that addition of cyclic AMP to growth media has no effect on the growth rate of the myxamoebae, on the cell yield or on the capacity of the myxamoebae to differentiate when subsequently placed on Millipore filters.

In contrast, the intracellular cyclic AMP concentration (Table 1) seems to change abruptly as the myxamoebae leave the exponential and enter the stationary phase of growth, and this might therefore initiate a number of events known to occur at this time (Quance & Ashworth, 1972). Interestingly, there appears to be an approximate doubling in the cellular cyclic AMP content and this might therefore represent another instance of 'quantal control' (Sussman & Newell, 1972). Froehlich & Rachmeler (1972) have reported that when mammalian cells cease to grow in culture as a consequence of contact inhibition, their cyclic AMP content rises from 4.2 to 9.8 pmol/mg of protein. These values are very close to those that we observe (Table 1) and suggest that this might be a general phenomenon characteristic of cells that have ceased growth, whether because of contact inhibition or, as presumably in our case, because of the absence of an essential nutrient.

Myxamoebae differentiate and form fruiting bodies when placed on a solid, but moist, surface. During this time, there are marked changes in the extracellular and intracellular cyclic AMP concentrations (Fig. 2). In these experiments there is, of course, no cell growth or division and thus no alteration in the number of cells. The changes found must therefore represent changes in the amount, or activity, of adenylate cyclase or cyclic AMP phosphodiesterase. Cyclic AMP is known to act as a chemotactic signalling molecule during aggregation, and Bonner *et al.* (1969) have reported that at aggregation there is an increase in the extracellular concentration of cyclic AMP. However, the second peak at 20–22 h (when the fruiting bodies are being formed and differentiation into spore and stalk cells occurs) has not been reported before. Bonner (1970) reported that high (1 mM) concentrations of cyclic AMP induce isolated amoebae to form cells which resemble stalk cells in appearance, and it is possible that this second peak reflects this second role of cyclic AMP in inducing the final differentiation of the stalk cells.

When myxamoebae are incubated in the absence of any salts (Fig. 3), they will aggregate and form grexes, but they take much longer to carry out these stages. The concentration of extracellular cyclic AMP attained during development in these conditions is very much less than is normally found (Fig. 2). Thus it is not necessary for the cells to secrete sufficient cyclic AMP to achieve an extracellular concentration of 500–1000 pmol/ml to aggregate successfully, although such high concentrations clearly expedite this process. The decrease in extracellular cyclic AMP content found after aggregation has been completed (Fig. 2, 13–19 h) is not found when development occurs in the absence of salts (Fig. 3) and when development is thus prevented from proceeding past the migrating-grex stage. It is thus conceivable that decreases in the extracellular cyclic AMP content are significant for regulating development as well as are increases. Nestle & Sussman (1972) have reported that addition of large concentrations of cyclic AMP (3 mM) at 16–18 h to the Millipore filter support pads causes marked abnormalities in the fruiting bodies finally found but that addition of cyclic AMP at times outside this 'sensitive period' causes little effect. It is noteworthy that the sensitive period defined by Nestle & Sussman (1972) coincides with the period of minimum extracellular cyclic AMP concentration (Fig. 2). The marked changes in phosphodiesterase activity (Fig. 4) also found during normal development agree with this idea. It is also clear that there must, as has been suggested by Mason *et al.* (1971), be some close connexion between cyclic AMP metabolism and ionic environment.

Fig. 2 shows that, preceding the first peak in the extracellular cyclic AMP concentration, there is a peak in the intracellular concentration of cyclic AMP. There is no such peak in the intracellular cyclic AMP

concentration, however, associated with the second peak of extracellular cyclic AMP (Fig. 2, 20–22h). Thus not only are there controls over the rate of synthesis and/or destruction of cyclic AMP but there must also be changes during development in the control of the secretion of cyclic AMP.

Rossomando & Sussman (1972) have measured the specific activity of adenylate cyclase during growth and development of axenically grown myxamoebae and have reported that there is no change in the specific activity of 3.4nmol/min per mg of protein at 37°C. This amount of enzyme would appear to be several orders of magnitude greater than that needed to account for the rate of increase in the intracellular cyclic AMP that we have observed (Fig. 2). Measurement of the activity of the intracellular cyclic AMP phosphodiesterase (Fig. 4) shows that, far from there being a decrease in the activity of this enzyme as the amount of cyclic AMP rises, there is an increase. In fact, the ratio of intracellular cyclic AMP to intracellular phosphodiesterase activity remains approximately constant throughout development. Thus it is most unlikely that the intracellular concentration of cyclic AMP is regulated by the rate of its removal by phosphodiesterase, as might be inferred from the work of Gerisch *et al.* (1972). Indeed, since the peak in cyclic AMP concentration precedes the peak in phosphodiesterase activity, it is more logical to argue, like Konijn (1972), that cyclic AMP induces phosphodiesterase activity. Since the specific activity of adenylate cyclase does not change during development it would seem likely that the intracellular concentration of cyclic AMP is regulated by alterations in the activity of adenylate cyclase. It is, however, difficult to measure the effect of effectors on this enzyme, since it is membrane bound and can be reliably assayed only in the presence of detergents such as Triton X-100 (Rossomando & Sussman, 1972). It is probable that the consequent destruction of the structural integrity of the enzyme–membrane complex will alter the kinetic properties of the molecule. This certainly would be a reasonable explanation for the discrepancy between the specific activity of solubilized preparations of adenylate cyclase (Rossomando & Sussman, 1972) and our determinations of the rates of cyclic AMP synthesis *in vivo* (Fig. 2). We have thus looked for other ways to investigate the action of effectors on this enzyme.

When myxamoebae are shaken in buffer they make and secrete cyclic AMP and phosphodiesterase (Fig. 5). Preceding the peaks of extracellular enzyme activity and cyclic AMP concentration are peaks in the concentration of intracellular cyclic AMP and phosphodiesterase. Comparison of Figs. 5 and 2 suggests that the peak in cyclic AMP concentration found in buffered suspensions at 90min is analogous to the peak found at 12h during development on Millipore filters. Similarly, comparison of Figs. 5 and 4 suggests that the peak in phosphodiesterase concentration found in buffered suspensions at 150min is analogous to the plateau in phosphodiesterase activity reached at 12h during development on Millipore filters. It is noteworthy that the amounts of phosphodiesterase activity which we detect in buffered cell suspensions are comparable with those reported by Gerisch *et al.* (1972) to be found in suspensions of bacterially grown cells and are much greater than the amounts found when development proceeds on a solid surface (Fig. 4). Fig. 5 shows that inhibition of protein synthesis causes a complete inhibition of secretion of cyclic AMP. Simultaneous measurement of the intracellular cyclic AMP has shown that this is not a specific effect on the secretory process but is an inhibition of cyclic AMP synthesis. Since the cells have at all times such a considerable excess of adenylate cyclase it seems likely that this effect of cycloheximide is not a direct one on the synthesis of adenylate cyclase itself. Alteration of the ionic composition of the buffer and addition of metabolites have marked effects on the rate and extent of cyclic AMP synthesis and secretion, so this explanation is not unreasonable (J. M. Ashworth & J. Kwasniak, unpublished work).

The second peak in the concentration of extracellular cyclic AMP (Fig. 2) is also not due to a change in amount of assayable adenylate cyclase (Rossomando & Sussman, 1972) nor to a change in the activity of cyclic AMP phosphodiesterase (Fig. 4). However, since it has not yet been possible to persuade cells to construct fruiting bodies in homogeneous suspensions, it will clearly be more difficult to investigate the factors that lead to this enhanced rate of synthesis than those that lead to the first peak.

We thank Dr. U. Sinha for help with the phosphodiesterase assays, and Mrs. J. Kwasniak and Miss J. Johnson for technical, and the Science Research Council for financial, assistance.

References

Ashworth, J. M. & Watts, D. J. (1970) *Biochem. J.* **119**, 175–182

Bonner, J. T. (1967) *The Cellular Slime Molds*, 2nd edn., Princeton University Press, Princeton

Bonner, J. T. (1970) *Proc. Nat. Acad. Sci. U.S.* **65**, 110–113

Bonner, J. T., Barkley, E. M., Hall, T. M., Konijn, T. M., Mason, J. W., O'Keefe, G., III & Wolfe, P. B. (1969) *Develop. Biol.* **20**, 72–87

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285

Brooker, G., Thomas, C. J. & Appleman, M. M. (1968) *Biochemistry* **7**, 4177–4181

Chang, Y. Y. (1968) *Science* **160**, 57–59

Chassy, B. M. (1972) *Science* **175**, 1016–1018

Froehlich, J. E. & Rachmaler, M. (1972) *J. Cell Biol.* **55**, 19–31

Gerisch, G. (1959) *Naturwissenschaften* **46**, 654–655

Gerisch, G., Malchow, D., Riedel, V., Muller, E. & Every, M. (1972) *Nature (London) New Biol.* **235**, 90–92

Goidl, E. A., Chassy, B. M., Love, L. L. & Krichevsky, M. I. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 1128–1130

Konijn, T. M. (1972) in *Advances in Cyclic Nucleotide Research* (Greengard, P., Robinson, G. A. & Paoletti, R., eds.), vol. 1, pp. 17–31, Raven Press, New York

Konijn, T. M., Barkley, D. S., Chang, Y. Y. & Bonner, J. T. (1968) *Amer. Natur.* **102**, 225–233

Lee, K.-C. (1972) *J. Gen. Microbiol.* **72**, 457–484

Malchow, D., Nagele, B., Schwarz, H. & Gerisch, G. (1972) *Eur. J. Biochem.* **28**, 136–142

Malkinson, A. M. & Ashworth, J. M. (1972) *Biochem. J.* **127**, 611–612

Mason, J. W., Rasmussen, H. & Dibella, F. (1971) *Exp. Cell Res.* **67**, 156–160

Nestle, M. & Sussman, M. (1972) *Develop. Biol.* **28**, 545–554

Newell, P. C., Telser, A. & Sussman, M. (1969) *J. Bacteriol.* **100**, 763–768

Pannbacker, R. G. & Bravard, L. J. (1972) *Science* **175**, 1014–1015

Quance, J. & Ashworth, J. M. (1972) *Biochem. J.* **126**, 609–616

Reidel, V. & Gerisch, G. (1971) *Biochem. Biophys. Res. Commun.* **42**, 119–124

Rossomando, E. F. & Sussman, M. (1972) *Biochem. Biophys. Res. Commun.* **47**, 604–610

Shaffer, B. M. (1956) *Science* **123**, 1172–1173

Sussman, M. (1966) *Methods Cell Physiol.* **2**, 397–408

Sussman, M. & Newell, P. C. (1972) in *Molecular Genetics and Developmental Biology* (Sussman, M., ed.), pp. 275–302, Prentice-Hall, London and New York

Watts, D. J. & Ashworth, J. M. (1970) *Biochem. J.* **119**, 171–174