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Cyclic AMP as a First Messenger

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I. INTRODUCTION

Cyclic AMP is of prime importance not only for intracellular mechanisms in higher organisms (Hechter, 1972) and bacteria (Pastan and Perlman, 1972) but also for extracellular communication in the cellular slime molds, where this nucleotide plays the role of a hormone or first messenger.

Myxamoebae of the cellular slime molds feed on bacteria during the vegetative phase, and may be oriented and attracted towards their food source by extracellular bacterial cyclic AMP. Upon starvation the cells enter the social phase. Amoebae of several *Dictyostelium* species secrete cyclic AMP themselves in sufficiently large quantities to attract each other and to form aggregates, which then differentiate into spores supported by vacuolated stalk cells. During this last step in their life cycle, the ratio of spores and stalk cells is affected by cyclic AMP (Bonner, 1970).

The cellular slime molds are a favorite object for the study of cell differentiation. The various levels at which cyclic AMP plays an essential role in these simple organisms deserve the attention of investigators interested in the various different actions of cyclic AMP.

II. MICROBIOLOGICAL ASSAY OF CYCLIC AMP

Several biochemical assays for cyclic AMP are available (Gilman, 1972). In addition, myxamoebae of four species of *Dictyostelium* can be used to detect cyclic AMP in tissues and to assess its concentration.

The small population assay that will be described here requires a hydrophobic agar surface of a specific rigidity. After agar has been washed frequently with deionized water, dissolved by boiling, and allowed to gelate, the agar surface is hydrophobic (Ennis and Sussman, 1958).

The lack of salts on such an agar surface results in hypotonicity which is harmful to cells. When salts are added back before gelation of the washed agar, the agar surface remains hydrophobic but becomes more suitable as a medium for myxamoebae (Konijn and Raper, 1961). Populations of myxamoebae can either be grown in situ together with bacteria on such agar, or pregrown amoebae can be placed on it as small droplets of a cell suspension. The amoebae do not cross the boundaries of the droplets, in contrast to their behavior on a normal hydrophilic agar or on a hydrophobic agar surface of too low rigidity.

An essential condition for the small population bioassay is that the hydrophobic agar surface be sufficiently rigid to keep the cells within the boundaries of the droplet, yet soft enough to allow the cells to cross the margins of the drop when attracted by an external stimulus. The attractant outside the drop can be another population of aggregating myxamoebae (Fig. 1), a bacterial population, or an extract containing chemotactic compounds.

Purification of active bacterial extracts and testing with the small population assay led to the discovery of cyclic AMP as the chemotactic agent for amoebae of *D. discoideum* (Konijn, van de Meene, Bonner, and Barkley, 1967). At cyclic AMP concentrations in the attracting drops of 10^{-5} to 10^{-6} M, chemotactic activity is expressed as a distinct attraction (Fig. 2). At higher cyclic AMP concentrations (10^{-3} to 10^{-4} M) the amoebae in the responding populations cross the boundaries of the drop on all sides (Fig. 3). It may be that phosphodiesterase, the enzyme which inactivates cyclic AMP (Chang, 1968), is responsible for this crawling of amoebae in all directions away from the original amoeboid population (Konijn, 1969). Cyclic AMP normally initiates the social phase, but at these concentrations it prevents aggregation. At still higher concentrations aggregates are dispersed completely, even when nearly all "streams" of amoebae have entered the aggregate (Konijn, 1969).

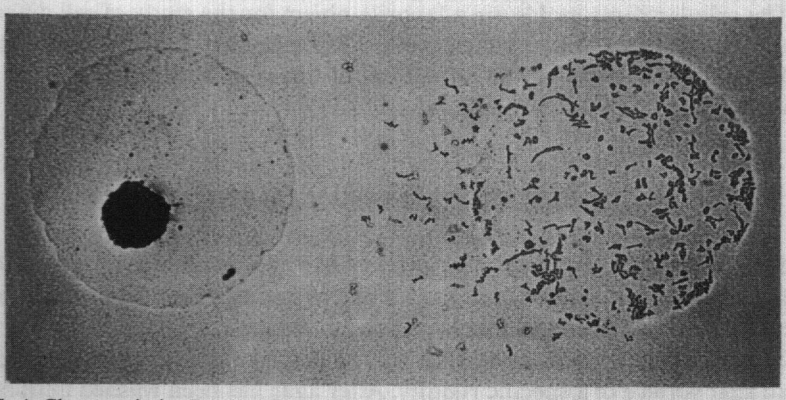

FIG. 1 Chemotaxis in *Dictyostelium discoideum*. The aggregation in the drop on the left attracts myxamoebae outside the boundary of the drop on the right. Cells inside the drop move on the agar surface. Cells outside the drop crawl through the agar (Konijn, 1970). (× 60)

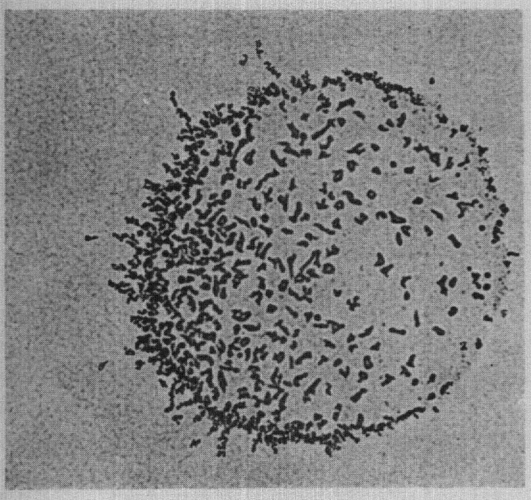

FIG. 2 Drops (0.1 μl) containing 3×10^{-8} g cyclic AMP were deposited three times at 5-min intervals to the left of the responding drop. ($\times$ 80)

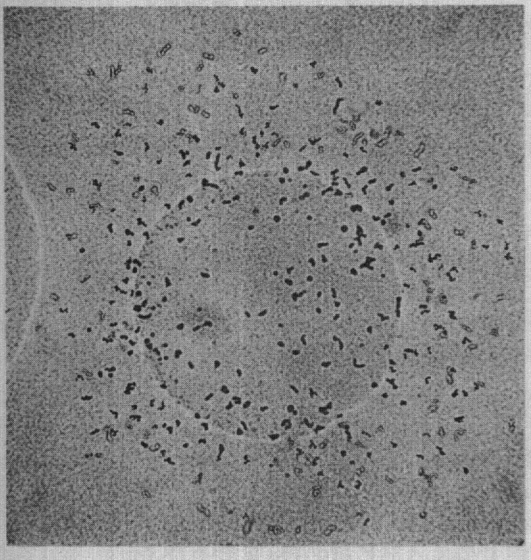

FIG. 3. As in Fig. 2., except that the drops contain 3×10^{-6} g cvclic AMP (Konijn, 1970). ($\times$ 60)

Above a concentration of 10^{-2} to 10^{-3} M the amoebae clump together in small groups (Fig. 4); apparently the receptor mechanism of the amoebae becomes overloaded. These clumps of cells move seemingly at random over the surface inside the drop.

All these different reactions can occur when extracts are tested for the presence of cyclic AMP. The assay becomes more quantitative by measuring the distance over which the attracting drop can induce myxamoebae in the responding drop to cross its boundary (Konijn, 1970).

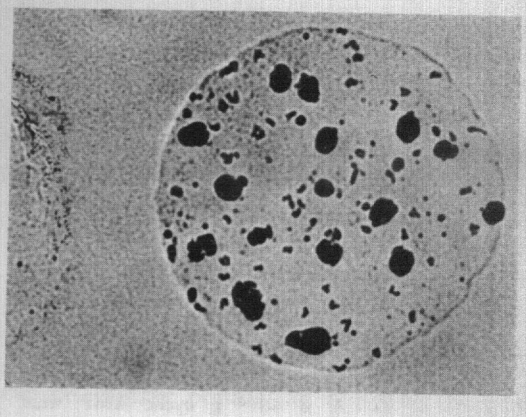

FIG. 4 As in Fig. 2., except that the drops contain 3×10^{-3} g cyclic AMP. (× 80)

Another way to quantify the method is the dilution of an active extract until only a certain percentage of the responding populations react positively. This percentage can then be used as a criterion for activity. The response to extracts with weak activity can be amplified by placing drops of the extract near the sensitive myxamoebae 3 to 5 times at 5 min intervals. At very low concentrations of cyclic AMP (10^{-8} to 10^{-9} M) the amoebae are not attracted outside the responding drop but become pressed against the margin. Such a response is considered positive if at least twice as many cells are pressed against the margin closest to the attracting drop as on the opposite side (Fig. 5). Since the volume of the attracting drops is only 0.1 μl, less than 10^{-12} g of cyclic AMP can induce a positive response, as shown in Fig. 5. When 10^{-12} g of cyclic AMP diffuses into the agar at a distance of, say, 300 μ from the responding drop, it is evident that the number of cyclic AMP molecules that ultimately activate a single cell must be extremely small.

The cyclic AMP content in an extract can be estimated by diluting a commercial cyclic AMP solution to a known concentration which will evoke a similar percentage of positively responding populations as does the extract. Cyclic AMP concentration differences of 2-fold can be shown in this way (Konijn, 1970).

The myxamoebae of *D. discoideum* react specifically to cyclic AMP. Other cyclic nucleotides or analogues of cyclic AMP are less active (Konijn, van de Meene, Chang, Barkley, and Bonner, 1969). Purification of the extract which is being tested by column and paper chromatography makes it possible to confirm that the observed activity is due to cyclic AMP and not to its analogues or to other cyclic nucleotides.

The high sensitivity and specificity of this small population assay made it suitable for demonstrating the presence of cyclic AMP in various bacteria, in yeast, and in extracts of various animal organs and tissues such as spleen, liver, kidney, and heart, and in milk and urine. Purification of the extracts

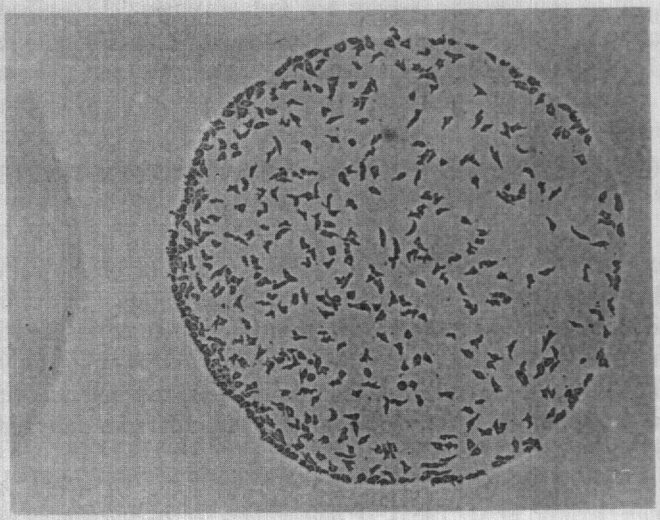

FIG. 5 Drops (0.1 μl) containing 3×10^{-13} g cyclic AMP were deposited three times at 5-min intervals to the left of the responding drop (Konijn, 1970). ($\times$ 95)

showed that cyclic AMP was the chemotactic agent. Purification was not required for the assay itself, because the activity in crude extracts could also be assessed. A typical example of cyclic AMP determination is shown in Table 1. The concentration of cyclic AMP found in urine was similar to those recorded in the literature (Butcher and Sutherland, 1962).

The small population assay has been used in a variety of projects, e.g., (1) to obtain conclusive evidence that cyclic AMP is involved in the physiological melanophore reaction in *Xenopus laevis* (van de Veerdonk and Konijn, 1970), (2) to show the effect of parathyroid extract on the cyclic AMP content

TABLE 1. *Comparison of the chemotactic response induced by different concentrations of urine and cyclic AMP (Konijn, 1970)*

	Urine		Cyclic AMP		Urine	
	(dil. $\times$ 500)		(0.01 μM)		(dil. $\times$ 2000)	
	Total Positive	Percent Positive	Total Positive	Percent Positive	Total Positive	Percent Positive
test 1	25	83	32	73	14	48
test 2	22	88	25	73	20	59
test 3	18	60	22	47	7	23

The drops containing the attractant were placed near (less than 0.45 mm) the responding drops three times with 5 min intervals. Observation took place 5 min after the last deposition.

of embryonic mouse calvaria (Herrmann-Erlee and Konijn, 1970), and (3) to measure cyclic AMP levels in yeast under various conditions of catabolite repression (van Wijk and Konijn, 1971).

III. CYCLIC AMP AND CHEMOTAXIS IN DICTYOSTELIUM AND POLYSPHONDYLIUM

Cyclic AMP is synthesized in at least two genera of the cellular slime molds. It has been isolated from *Polysphondylium pallidum* (Konijn, Barkley, Chang, and Bonner, 1968) and has also been identified as the acrasin synthesized by amoebae of *D. discoideum* (Konijn, Chang, and Bonner, 1969). Barkley (1969), using the same purification techniques, isolated and identified cyclic AMP as the attractant in *D. discoideum* by starting out from dialyzed products of the amoebae.

The species of *Dictyostelium* which are attracted by cyclic AMP are: *D. discoideum* (Konijn et al., 1967), *D. mucoroides*, *D. purpureum* (Konijn et al., 1968) and *D. rosarium*. Myxamoebae of these species are attracted when drops of a solution of 10^{-6} to 10^{-8} M cyclic AMP are placed close to the amoebae. At higher concentrations of cyclic AMP the cells cross the boundaries of the drops on all sides (Fig. 3). If the assumption is correct that this is a consequence of the inactivation of cyclic AMP by phosphodiesterase produced by the cells, resulting in a concentration gradient of cyclic AMP (Konijn, 1969), the centrifugal movement of amoebae in all directions indicates that all these species produce phosphodiesterase. Bonner (*personal communication*) confirmed this assumption by demonstrating both extracellular and intracellular phosphodiesterase activity in these species.

A regulatory role for phosphodiesterase during aggregation was suggested by Riedel and Gerisch (1971); they observed a decrease in phosphodiesterase activity during the transition from growth to cell aggregation in suspension cultures. The reduction in activity to 2% of the original activity was due to the appearance of an inhibitor of phosphodiesterase after the supply of bacteria became exhausted.

Incubation of myxamoebae with 10^{-5} M cyclic AMP results in a minimal activity of phosphodiesterase, while both higher and lower concentrations result in an increase of the phosphodiesterase activity (*unpublished observations* in collaboration with G. Speziali).

Another effect of the addition of cyclic AMP to amoebae is an increased calcium outflow from the cells (Chi and Francis, 1971). Mason, Rasmussen, and Dibella (1971) found that exogenous cyclic AMP inhibited the endogenous production of cyclic AMP; moreover, aggregation was completely inhibited at calcium concentrations below 10^{-6} M. They tested tetracaine, a local anesthetic, which alters the binding and transport of calcium. Tetracaine did not

influence cyclic AMP secretion but did inhibit aggregation of the amoebae.

The smaller species, *D. minutum*, *D. lacteum*, *D. aureum*, *D. polycephalum*, and *D. vinaceo-fuscum* (all generously provided by Professor Kenneth B. Raper) are not attracted by cyclic AMP, not even after frequent application. A variety of other substances do attract these species, but their exact nature is still under investigation. Biological materials which attract nearly all these species are: bacteria, milk, urine, and liver homogenates. The attractants are dialyzable. Purification results in a partial loss of activity.

The main attractant(s) of the genus *Polysphondylium* is a small molecule, just as cyclic AMP is, which is also present in bacteria, urine, and milk. It is unlikely that cyclic AMP itself is the attractant, since it does not have a chemotactic effect at concentrations ranging from 10^{-2} M to 10^{-8} M, nor upon frequent (8–16) application at intervals of 5, 2, or 1 min. Only with high concentrations of cyclic AMP (10^{-2} M) in the attracting drops are the finished aggregates of *Polysphondylium* flattened and more aggregates (4–10) formed than normally (1–3) in populations. Again, a complication during the purification of the bacterial fractions active in *Polysphondylium* is the reduction in activity found with increasing purity of the extracts.

The possibility that *D. discoideum* may secrete a cofactor which is necessary for *Polysphondylium* to be activated by cyclic AMP was studied in mixtures of *D. discoideum* with *P. pallidum* or *P. violaceum*. Amoebae were mixed in various ratios. The extracellular metabolites secreted by *D. discoideum* had no noticeable effect on the sensitivity of amoebae of *P. violaceum* for cyclic AMP. The chemotactic response of *D. discoideum* was reduced in these mixed populations when cyclic AMP was applied nearby. Despite the fact that some cell division must have taken place, the total number of cells dwindled to 1/3 or 1/4 of the original population, and cellular debris was spread all over the drop. A time-lapse film of a mixed population of *D. discoideum* and *P. pallidum* (1 : 1) revealed that, instead of cooperating, the amoebae of *D. discoideum* attack the smaller *P. pallidum* amoebae; after most *P. pallidum* amoebae have been destroyed, amoebae of *D. discoideum* even start to engulf other cells of their own species.

The effect of other cyclic nucleotides and analogues of cyclic AMP on species that are not attracted by cyclic AMP was examined. Some cyclic nucleotides, e.g., thymidine 3′,5′-monophosphate, show minor activity if applied near amoebae of *D. lacteum*. On the other hand, an extract of homogenized axolotl embryos contained a strong chemotactic agent for amoebae of *P. pallidum*.

There are indications that the species which do not react to cyclic AMP are sensitive to attractant(s) that act at very low concentrations. Small populations (*c.* 1000 cells) of *D. aureum* and *P. violaceum* attract amoebae of the same species in responding drops 1500 μ away. The chemotactic agent secreted by *D. vinaceo-fuscum* activates cells of the same species over a distance up to 2200 μ. If the attractant diffuses through the agar in all directions its concentration must be extremely low by the time it reaches an amoeba in the responding population.

Size alone does not determine whether a species will be attracted by cyclic AMP. A small mutant of *D. mucoroides* (generously supplied by Dr. Erik Bille-Hansen) with sorocarps of similar size as those of *D. minutum* is attracted by cyclic AMP, whereas *D. minutum* is insensitive.

Sensitivity to cyclic AMP often goes together with pulsations during aggregation which are clearly observable in time-lapse films. Amoebae of *D. purpureum*, *D. mucoroides*, *D. discoideum*, and *D. rosarium*, which are all sensitive to cyclic AMP, move towards the center of the aggregate in rhythmic waves. Most large *Dictyostelium* species pulsate at 5-min intervals. The rhythmic waves in *D. rosarium* appear at 20-min intervals.

It is obvious from time-lapse films that a longer interval goes with a stronger pulse. Early aggregates of *D. rosarium* may disintegrate completely before they come together again with the next pulsation, and several amoebae in advanced aggregates become free again between pulsations.

However, distinct pulsations are not a prerequisite for attraction by cyclic AMP in *D. discoideum*. Strain 66 of this species (kindly supplied by Dr. G. Gerisch) does not show clear pulsations (Gerisch, Normann, and Beug, 1966), although it is sensitive to cyclic AMP.

A third characteristic often found in species that are insensitive to cyclic AMP is the presence of founder cells. Certain cells in a population may round off and attract other cells that are nearby. These founder cells, first described by Shaffer, have been shown to be active in *P. violaceum* (Shaffer, 1961), *P. pallidum* (Francis, 1965), and *D. minutum* (Gerisch, 1964)—species which are not sensitive to cyclic AMP. No evidence is yet available for the presence of founder cells in species that are sensitive to cyclic AMP.

IV. CHEMOTACTIC ATTRACTION OF MYXAMOEBAE BY BACTERIAL CYCLIC AMP

Arndt (1937) was the first to observe the tendency of myxamoebae to move to bacteria. Renewed interest in this kind of chemotaxis started in the early sixties when Samuel (1961) demonstrated the attraction of amoebae of *D. mucoroides* by a dab of *Escherichia coli*. Bacteria attract amoebae of *D. discoideum* over large distances, without intervening amoebae, and the attraction was studied with the small population assay (Konijn, 1961).

A dialyzed aqueous bacterial extract was chromatographed on Whatman no. 1 paper in butyl alcohol-acetic acid-water (4:1:5). The paper was cut into 1-cm wide strips. Each strip was eluted with water and the eluate was tested with the assay; the active fractions were pooled and concentrated, and it was subsequently shown by paper electrophoresis at pH 3.9 that the active molecule was negatively charged. The active fractions deposited close to sensitive amoebae delayed aggregation, disturbed aggregation patterns in drops where

amoebae were coming together, and attracted cells outside the boundaries of the small populations.

It was possible to obtain a large increase in activity by using a concentrated aqueous bacterial extract, which was collected by growing *E. coli* on large trays, washing off the bacteria, and centrifugation (Konijn et al., 1969). The supernatant was concentrated and fractionated by gel-filtration, paper chromatography, and paper electrophoresis at pH 3.9. After each fractionation, the fractions were tested with the assay and the active fractions collected, concentrated, and further purified.

The purified product had an UV absorption spectrum which strongly suggested that the attracting compound was a derivative of adenine. The characteristics mentioned above, together with the fact that the attracting compound is present in bacteria and urine, both known to contain cyclic AMP, led to the testing of commercial cyclic AMP; this proved to be chemotactically active at amounts of 0.1 ng (Konijn et al., 1967). Further purification of the highly purified active bacterial extract on a DEAE-A-25 Sephadex column and with active charcoal resulted in a product that migrated identically to cyclic AMP in three different solvents (Konijn et al., 1969).

Secretion of cyclic AMP by bacteria appears to be less dependent on environmental conditions than its secretion by amoebae. The chemotactic activity of amoebae is reduced by light (Konijn and Raper, 1966) or by an increase in temperature (Konijn, 1965); light and temperature do not affect the chemotactic activity of bacteria or bacterial extracts. Whereas the attraction by aggregating amoebae is largely independent of the size of the aggregate (Konijn, 1968), bacterial populations are more active at higher cell densities. At high densities, *E. coli* attract cells of amoebae populations that are deposited as far as 5 mm away (Konijn, 1969). Bacterial drops near amoebae delay aggregation in the responding drop or inhibit aggregation completely.

All species of *Dictyostelium* and *Polysphondylium* are attracted by *E. coli* and other gram-negative and gram-positive bacteria (Konijn, 1969). There may be some difference in chemotactic activity even within the same bacterial species. The attraction sphere of *E. coli*, B/r, extends over a larger distance than that of *E. coli*, 281.

Since cyclic AMP is the only natural attractant isolated from *E. coli*, and no other chemotactic agents are found that are active at such low concentrations, it is suggested that all bacteria secrete cyclic AMP, and that this constitutes an effective food-seeking mechanism for myxamoebae.

Recently Bonner, Hall, Sachsenmaier, and Walker (1970) have suggested that a second chemotactic factor may be of great importance during the vegetative phase of myxamoebae. This chemotactic agent is a nondialyzable molecule which is present in bacterial extracts and can be demonstrated by means of an assay (Bonner, Kelso, and Gillmor, 1966) in which an active compound is mixed with the agar before gelation. A square of cellophane

covered with amoebae is transferred to the agar and the distance over which cells move away from the cellophane square in a given period of time is used as a criterion for the activity of the compound added to the agar.

This second chemotactic compound is not active in the small population assay. Bonner et al. (1970) found that it does not diffuse through agar, although the diffusion of molecules as large as haemocyanin (molecular weight 6,600,000) is hardly affected in a 0.5% agar-gel and only slightly in a 1% agar-gel (Polsen, 1958). Several acid and basic dyes, when tested on a hydrophobic agar surface, diffuse through the agar independent of the charge of the molecule.

The further purification of this second chemotactic system is of great importance since, should it serve as activator of the membrane of the cell, it could indicate the presence of different types of chemoreceptor.

V. CHEMOTAXIS BY ANALOGUES OF CYCLIC AMP AND OTHER CYCLIC NUCLEOTIDES

Before and after the discovery of cyclic AMP as the main attractant for myxamoebae, several other compounds were tested for chemotactic activity. Amino acids, sugars, and several nucleotides were found to be not active.

The only compounds besides cyclic AMP which attracted myxamoebae in the small population test were its analogues and other cyclic nucleotides (Konijn et al., 1969). Cyclic AMP is 100 to 10,000 times more active than the other cyclic nucleotides (Table 2). The cyclic nucleotides containing a purine

TABLE 2. *Chemotactic activities of cyclic nucleotides*

Cyclic Nucleotide	50% of Populations React Positively Between:
cyclic AMP	10^{-8}–10^{-9} M
cyclic UMP	10^{-6}–10^{-7} M
cyclic CMP	10^{-5}–10^{-6} M
cyclic GMP	10^{-5}–10^{-6} M
cyclic IMP	10^{-4}–10^{-5} M
cyclic TMP	10^{-4}–10^{-5} M

base, such as cyclic AMP, are not always stronger chemotactic agents than those containing a pyrimidine base.

All analogues of cyclic AMP are less active than cyclic AMP itself (Table 3). Again, there is a large variation in the chemotactic activity of the various derivatives, which may differ from cyclic AMP in the base, the phosphate, or

TABLE 3. *Chemotactic activities of analogues of cyclic AMP*

Analogue of Cyclic AMP	50% of Populations React Positively Between:
3′-cyclic ester of 9-[5′-deoxy-5′-dihydroxyphosphinylmethyl-β-D-ribofuranosyl]-adenine	10^{-8} 10^{-9} M
desoxy-cyclic AMP	10^{-7} 10^{-8} M
adenosine 3′,5′-cyclic phosphorothioate	10^{-6}–10^{-7} M
N^6-2′-O-dibutyryl cyclic AMP	10^{-4}–10^{-6} M
tubercidin 3′,5′-cyclic monophosphate	10^{-5}–10^{-6} M
N^6-2′-O-dibutyryl cyclic AMP	10^{-4} 10^{-6} M
iso-cyclic AMP	10^{-3} 10^{-4} M
5′-cyclic ester of 9-[3′-deoxy-3′-dihydroxyphosphinylmethyl-β-D-ribofuranosyl]-adenine	10^{-2}–10^{-3} M

the ribose moiety. All these moieties can undergo modifications without leading to complete loss of activity. Tubercidin 3′,5′-monophosphate and N^6-2′-O-dibutyryladenosine 3′,5′-monophosphate, both containing a modified base moiety, are 100 to 10,000 times less active than cyclic AMP.

The site in the phosphate moiety where an oxygen atom has been replaced by a methyl group is extremely important for the chemotactic activity of the analogue: the 3′-cyclic ester of 9-[5′-deoxy-5′-dihydroxyphosphinyl-methyl-β-D-ribofuranosyl]-adenine is about 10^5 times more active than the 5′-cyclic ester of 9-[3′-deoxy-3′-dihydroxyphosphinylmethyl-β-D-ribofuranosyl]-adenine (Table 3).

The configurational binding of the base moiety to the ribose moiety is also of great significance for chemotactic activity. Iso-cyclic AMP (kindly supplied by Professor Th. Posternak) which has its adenine moiety bound to the sugar moiety at N^3 is about 10^5 times less active compared with cyclic AMP. Small changes in the base moiety may result in a strongly reduced activity, as is shown in the case of tubercidin 3′,5′-monophosphate, which differs from cyclic AMP by the replacement of N^7 by a carbon atom.

Cyclic TMP, which shows low activity (Table 2), is only available as deoxythymidine 3′,5′-monophosphate. In cyclic AMP itself, the deoxy configuration does not result in low activity. Deoxyadenosine 3′,5′-monophosphate (kindly supplied by Drs. Nelboeck and Weiman, Boehringer Co.) attracts myxamoebae at only slightly higher concentrations than cyclic AMP itself (Table 3).

The 100 to 10,000 times lower activity of N^6-2′-O-dibutyryl-adenosine 3′,5′-monophosphate is apparently due to the presence of two butyryl groups;

the activity of the dibutyryl analogues of guanosine 3',5'-monophosphate and inosine 3',5'-monophosphate is lower by about a factor 10 to 1000 as compared with cyclic GMP or cyclic IMP. The activity of the monobutyryl analogue of cyclic AMP is in between the activities of the dibutyryl analogue and cyclic AMP itself.

In higher organisms, dibutyryl cyclic AMP often has the same or even higher activity than cyclic AMP. The concentration used in higher organisms far exceeds the active concentration of these cyclic nucleotides in *D. discoideum*. If in the cellular slime molds cyclic AMP would not penetrate into the cell, as seems to be the case in cells of higher organisms, but would only activate the cell membrane, its action would be independent of the permeability of the membrane for cyclic nucleotides. In higher organisms the dibutyryl analogue may derive its higher activity from its faster penetration into the cell.

If analogues have only a slight chemotactic effect on myxamoebae, their activity may partly be due to contamination with trace amounts of cyclic AMP. Dibutyryl cyclic AMP obtained from two different sources had a 10- to 100-fold difference in activity. Dibutyryl cyclic AMP may contain up to 5% monobutyryl cyclic AMP. Levine and Lewis (1967) showed that the monobutyryl cyclic AMP which they used contained 4% cyclic AMP.

To explain the large variation in activity among the different cyclic nucleotides and their analogues, further investigation is required. Steep gradients of an attractant may facilitate the movement of amoebae towards the source of attraction. Phosphodiesterase secreted by the amoebae inactivates nearby acrasin molecules and thus steepens the gradient. If the cyclic nucleotides and analogues of cyclic AMP were differentially inactivated by phosphodiesterase, the molecules more resistant to phosphodiesterase would be chemotactically less active. Bonner and co-workers (*personal communication*) have found evidence that a lower chemotactic activity of cyclic nucleotides may be related to a higher resistance to inactivation by phosphodiesterase. Cehovic, Marcus, Vengadabady, and Posternak (1968), however, noted that phosphodiesterase inactivates iso-cyclic AMP and cyclic AMP to the same extent, although the chemotactic activity of iso-cyclic AMP is 10^5 times less than that of cyclic AMP.

The differential activities of the various cyclic nucleotides and their analogues, shown by means of the small population test, are in disagreement with the observations made by Chassy, Love, and Krichevsky (1969*a*), who found the same chemotactic activity for different 3',5'-cyclic nucleotides, and no activity for dibutyryl cyclic AMP. However, they used a different test in which the activity was measured by depositing myxamoebae on agar which had been mixed with the compound to be tested before gelation, or by placing a streak of amoebae opposite to a streak of the compound being tested. Observations were made after 18 hr or later. In the small population test, chemotactic activity is assayed 5 min after the last deposition of the substance being tested.

Chassy, Love, and Krichevsky (1969*b*) also found that phosphodiesterase hydrolyzed cAMP, cUMP, cCMP, and cTMP with similar V_m and K_m values. The dibutyryl analogue of cyclic AMP was not hydrolyzed. However, dibutyryl cyclic AMP does attract amoebae in the small population test (Table 3).

VI. CONCLUSION

By using a microbiological assay it was possible to show that cyclic AMP is the main, if not the only, attractant during aggregation of myxamoebae of the cellular slime molds. Cyclic AMP initiates aggregation at low concentrations, but at higher concentrations it prevents the myxamoebae from coming together. Concentrated solutions of cyclic AMP disperse aggregates, even if they are already in an advanced stage.

The specific response to cyclic AMP allows the use of myxamoebae in a bioassay to assess the levels of this nucleotide in extracts of various biological materials.

Minor chemotactic activity has been shown for other cyclic nucleotides and analogues of cyclic AMP. Differences in their effectiveness illustrate the importance of the configurational features of cyclic nucleotides for their chemotactic activity. Changes in the base, sugar, or phosphate moiety of cyclic AMP reduce its chemotactic activity to various degrees.

The chemotactic activity of cyclic AMP is limited to the large *Dictyostelium* species. The presence of this nucleotide in other species which are not attracted by it indicates that cyclic AMP functions intracellularly in all species, but has become an extracellular attractant only in those species in which aggregation has to cover large territories.

The use of extracellular cyclic AMP as an attractant is not related to the complexity of the fruiting structure. Species with small, simple fruiting structures consisting of spores, supported by stalk cells, e.g., *D. minutum*, and species which have complex fruiting structures with several whorls, e.g., *P. pallidum*, are both insensitive to cyclic AMP.

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