Proc. Natl. Acad. Sci. USA
Vol. 73, No. 9, pp. 3183–3187, September 1976
Cell Biology

Effect of sugars on early biochemical events in development of *Dictyostelium discoideum*

(adenosine 3′:5′-cyclic monophosphate/cyclic AMP phosphodiesterase/receptors/aggregation competence)

H. J. RAHMSDORF*§, HÉLÈNE L. CAILLA‡, EVA SPITZ*, MARY J. MORAN*, AND H. V. RICKENBERG*†¶

*Division of Molecular and Cellular Biology, National Jewish Hospital and Research Center; †Department of Biophysics and Genetics, University of Colorado School of Medicine, Denver, Colo. 80206; and ‡Centre National de la Recherche Scientifique, Centre de Biochimie et de Biologie Moléculaire, Marseille, France

Communicated by J. T. Bonner, June 9, 1976

ABSTRACT **Metabolizable sugars blocked development of the slime mold *Dictyostelium discoideum*; the same sugars also inhibited the formation of contact sites "A", of membranal 3′:5′-cyclic adenosine monophosphate (cAMP)-binding sites, and of total cAMP phosphodiesterase (3′:5′-cyclic-nucleotide phosphodiesterase; EC 3.1.4.17; 3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase). These inhibitory effects of the sugars on the synthesis of cellular components, required for the aggregation of developing amebae, were paralleled by an inhibition of the accumulation of cAMP which normally accompanies development. The inhibition by sugars could be overcome partially by pulsing the amebae with nanomolar concentrations of cAMP only after the amebae had acquired cAMP-binding sites.**

The findings suggest that metabolizable sugars inhibit development by blocking the formation of cAMP and, conversely, that development in *D. discoideum* may be related to the energetic state of the cell.

Exhaustion of nutrients is the signal for the transition from vegetative growth, as single cells, to pseudoplasmodial development in the cellular slime mold, *Dictyostelium discoideum* (1, 2). Whether or not the exhaustion of any one required nutrient, or class of nutrients, suffices to trigger development is not clear; it has been claimed recently (3) that the omission of essential amino acids from a partially defined medium initiates development. Our earlier work (4) showed that development is blocked by the addition of metabolizable sugars (glucose, maltose, trehalose) to a buffer which, if unsupplemented, permits development.

It is known that one consequence of starvation is the pulsatile release of adenosine 3′:5′-cyclic monophosphate (cAMP) by the amebae (5, 6) and that the extracellular cAMP acts as a chemotactic agent that brings about the formation of multicellular aggregates (7–9). Aggregation is preceded, paralleled, and followed by profound changes in the structural and enzymic constitution of the amebae (for review, see refs. 2 and 10), and it is tempting to speculate that cAMP may be the effector of these cellular transformations as well as the agent of multicellularity. We showed earlier (4) that the sugars that inhibited development also blocked certain catabolic reactions that normally accompany development; we extend these observations in the present communication by the demonstration that the sugars also interfere with synthetic reactions required for development. This interference with development by the sugars is paralleled by an inhibition of the increase in cAMP which occurs in the absence of the sugars. It therefore appeared likely that normally cAMP mediates the effects of starvation by causing the catabolic events [breakdown of pre-existing RNA, protein, and glycogen (4)] as well as the biosynthetic events (synthesis of cAMP phosphodiesterase, of contact sites "A", of cAMP binding sites, etc.) typical of early stages of development. The observation, presented here, that under appropriate conditions the pulsing of the amebae with exogenous cAMP partially overcame the inhibition by the sugars strengthens our hypothesis.

Abbreviation: cAMP, adenosine 3′:5′-cyclic monophosphate.

§ Permanent address: Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, West Germany.
¶ To whom reprint requests should be addressed.

MATERIALS AND METHODS

Strain AX-2 (ATCC 24379) was used. Conditions of growth and development have been described (4, 11–13). The presence of contact sites "A" was assayed indirectly on the basis of the ability of the amebae to agglutinate in the presence of 10 mM EDTA (14, 15). The presence of cAMP-binding sites was tested by the procedure of Malchow and Gerisch (16), modified as indicated in the legend to Fig. 2. Resuspension of the amebae in the imidazole buffer used in the cAMP-binding test after prior, prolonged incubation in the presence of 0.1 M sugar, caused osmotic fragility of the cells. Lysis of the amebae during the binding test was minimized by transferring them from the buffer containing the sugar to unsupplemented 0.017 M phosphate buffer and incubating them in it for 10 min. The amebae were then resedimented and suspended in the buffer used in the cAMP-binding test. Residual lysis during the test is not excluded, but control experiments indicated that amebae preincubated with maltose were no more fragile than amebae preincubated with α-methylglucoside. cAMP phosphodiesterase (3′:5′-cyclic-nucleotide phosphodiesterase; EC 3.1.4.17; 3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase) was measured by the method of Thompson and Appleman (17) as modified by Nielsen et al. (18); cAMP was determined by the method of Cailla *et al.* (19) as modified by Harper and Brooker (20). For pulsing with cAMP, amebae were incubated at 22° with shaking in 0.017 M sodium phosphate buffer in the presence of sugars, as specified. At different times after the start of incubation, the amebal suspensions were pulsed at 7-min intervals with cAMP at 75 pmol per pulse in a volume of 10 μl/15 ml of suspension (i.e., 5 nM final); the suspension contained 10^7 amebae/ml. The developmental stage of the amebae was tested at the end of the 8-hr incubation period by plating them on Millipore filters and on petri dishes and observing them at frequent intervals after plating.

RESULTS

Effects of Sugars on Formation of Contact Sites "A" (Agglutinability of Amebae in the Presence of EDTA). The appearance of the immunologically defined contact sites "A" is an early indication that the amebae have entered the preaggregative stage of development (14); it seems that the contact sites "A" are required for the end-to-end concatenation of the amebae typical of streaming and that the cohesion between the

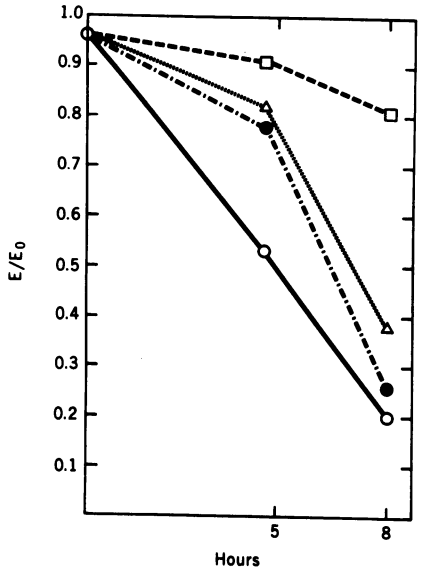

FIG. 1. Effect of preincubation with sugars on agglutinability of amebae in presence of EDTA. Amebae were grown to a density of 6 × 10^6 amebae per ml, washed three times with cold 0.075 M sodium phosphate buffer, pH 6, resuspended to a density of 10^7 amebae per ml in the same buffer (○) and in buffer supplemented with 0.05 M maltose (□) or 0.05 M α-methylglucoside (△) or 0.05 M D-xylose (●), and incubated with shaking at 22°. The suspensions were sampled at 0, 5, and 8 hr. The amebae were washed twice in unsupplemented buffer and resuspended to a density of 10^7 cells per ml in 0.075 M sodium phosphate buffer containing 10^{-2} M EDTA. The agglutinability of the amebae was then tested as described (15). The quotient of the optical density of the sample (E) and that of a reference sample (E_0), consisting of the same number of aggregation-noncompetent, single cells, is plotted. The decrease in optical density is a measure of the formation of amebal aggregates.

cells, based on the interaction of these sites, is resistant to EDTA. The occurrence of contact sites "A" can therefore be measured indirectly by the decrease in optical density of a suspension of amebae: amebae with contact sites "A" agglutinate in the presence of 10^{-2} M EDTA (decrease in optical density of suspension), whereas amebae devoid of the sites do not agglutinate under these conditions. Fig. 1 shows that amebae incubated for 8 hr in unsupplemented buffer or in buffer supplemented with either α-methylglucoside or xylose (not utilized as sources of carbon) agglutinated and, presumably, had acquired contact sites "A", whereas amebae incubated in buffer containing maltose did not agglutinate and, presumably, lacked the sites. Xylose and α-methylglucoside served as controls for nonspecific, possibly osmotic, effects of the sugars on the development of amebae (4).

Effect of Sugars on the Appearance of cAMP-Binding Sites. Malchow and Gerisch (16) showed that the ability of *D. discoideum* to bind exogenously added cAMP increased during the starvation of the amebae in dilute phosphate buffer and reflected the formation of specific binding sites. In our experiments the appearance of cAMP-binding sites could be detected after 3–5 hr of starvation (Fig. 2). Maltose at 0.1 M (or 0.05 M, not shown) severely inhibited the formation of the sites; α-methylglucoside at 0.1 M inhibited slightly.

Effect of Sugars on the Synthesis of cAMP Phosphodiesterase. The synthesis of cAMP phosphodiesterase in *D. discoideum* is developmentally regulated. At least two forms of the enzyme occur; one is membrane-bound and the other exerts its activity in the medium. The presumptive roles of the two cAMP phosphodiesterases in signalling and aggregation have been discussed (see refs. 9, 6, and 21 for reviews).

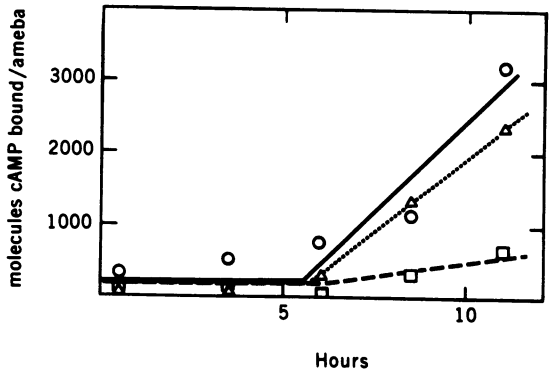

FIG. 2. Effect of sugars on formation of cAMP-binding sites. Amebae were incubated with shaking at 22° in either 0.017 M sodium phosphate buffer, pH 6, (○) or in the same buffer supplemented with 0.1 M maltose (□) or with 0.1 M α-methylglucoside (△). At the indicated times 10 ml of the suspensions were harvested by centrifugation, resuspended in 10 ml of unsupplemented buffer, and incubated for 10 min at 22°. The amebae were then harvested again, washed twice with 15 mM imidazole buffer, pH 6, and resuspended in 0.4 ml of the same buffer. Two 200-μl samples of the suspension were kept on ice for at least 5 min, and 50 μl of the assay mixture (13 mM imidazole, pH 6; 2.5 mM cGMP; 45 nM ^{3}H-labeled cAMP, 35.6 Ci/mmol) were added to each of the suspensions. One of the two suspensions received, in addition to the radioactive cAMP, nonradioactive cAMP to a final concentration of 10^{-4} M. The amebae, now in the assay mixture, were kept on ice for another 30 sec. Then two 90-μl portions of each mixture were transferred to 1.5-ml conical polyethylene microsample tubes (Bel-Art Products) and centrifuged for 10 sec in an Eppendorf Micro model 3200 centrifuge at 12,000 × *g*. The supernatant fluids were removed as completely as possible. The pellets were dissolved in 1 ml of Cellosolve and their radioactivity was determined by liquid scintillation counting; Bray (26) solution was used as scintillant. The radioactivity found in the samples to which the large excess of nonradioactive cAMP had been added was considered to represent contamination of the pellets by residual supernatant fluid; the results were corrected for this (approximately 30% of the radioactivity found in amebae preincubated in the absence of a sugar for 11 hr corresponded to this contamination and was the same in terms of *counts* for the three sets of amebae). The total number of the amebae in the assay mixture was approximately 5 × 10^7 and was determined accurately for individual experiments by cell counts of a dilution of the suspension.

Our preliminary experiments indicated that whereas metabolizable sugars blocked the appearance of both cellular and extracellular cAMP phosphodiesterase, nonmetabolizable sugars occasionally brought about a decrease in cellular cAMP phosphodiesterase activity with a concomitant increase in the activity of extracellular phosphodiesterase. We decided therefore to assay total cAMP phosphodiesterase activity in the absence of any sugar, in the presence of the metabolizable sugar maltose, and in the presence of the nonmetabolizable sugars, α-methylglucoside and arabinose. Fig. 3 shows that maltose completely blocked the increase in cAMP phosphodiesterase activity which occurred in the absence of the sugar and that α-methylglucoside and arabinose delayed the increase in enzyme activity. The fact that cycloheximide blocked the increase in cAMP phosphodiesterase activity, confirms the earlier observation (22) that the increase in activity requires protein synthesis.

Effect of Pulses of cAMP on Starving Amebae and on Amebae Incubated with Sugars. Gerisch *et al.* (23) and Darmon *et al.* (24) showed that exposure of amebae to pulses of cAMP at nanomolar concentrations accelerated the formation of contact sites "A" and of fruiting bodies, respectively. We confirmed this observation; the fruiting bodies formed by the pulsed amebae were smaller than those formed by nonpulsed

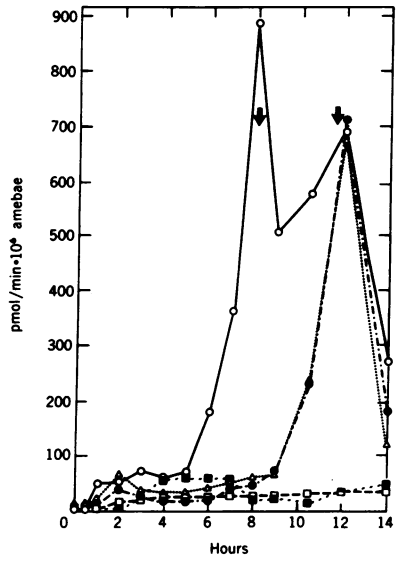

FIG. 3. Effects of sugars on cAMP phosphodiesterase. Amebae were incubated with shaking at 22° in either 0.017 M sodium phosphate buffer, pH 6 (○), or in the same buffer supplemented with 0.1 M maltose (□), or with 0.1 M α-methylglucoside (△), or with 0.1 M L-arabinose (●), or 250 μg/ml of cycloheximide (■). At the indicated times the suspensions were sampled and the samples were frozen rapidly in dry ice–alcohol. The amebae were broken by freeze-thawing. The uncentrifuged preparation was assayed, after appropriate dilution with the phosphate buffer, for cAMP phosphodiesterase activity as follows: the homogenate was brought to a volume of 7 ml with Tris·HCl buffer. The assay (17, 18) was started after a 5-min preincubation at 30° by adding to four parts of the homogenate, one part of the assay mixture containing 0.1 M $MgCl_2$, 5′-nucleotidase (0.5 mg/ml; Sigma *Crotalus adamanteus*), ^{3}H-labeled cAMP (2.5×10^{-4} M, 15 Ci/mol) and 600 mM Tris·HCl, pH 7.5. Samples of 0.5 ml volume were taken in duplicate after 20, 40, and 60 min of incubation at 30° and mixed with 2.2 ml of a Dowex slurry [25 g of Dowex-2 (200–400 mesh) in the formate form per 100 ml of absolute ethanol]. The slurry was kept at room temperature for 30 min and shaken occasionally. It was then centrifuged and the radioactivity of 500 μl of the supernatant fluid was measured by liquid scintillation counting in a toluene scintillator containing 30% absolute ethanol. The cAMP phosphodiesterase activity was determined from computer-calculated regression curves. Activity is expressed as pmol of cAMP hydrolyzed per min in a volume corresponding to 10^6 amebae of the original suspension. The arrows mark the times at which the amebae in the unsupplemented suspension and in the suspensions containing α-methylglucoside or arabinose had become aggregation-competent.

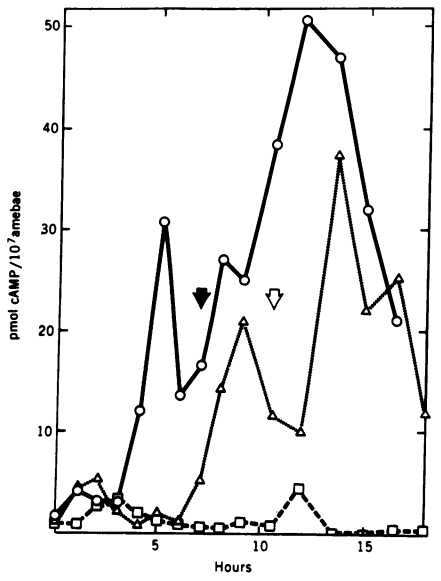

FIG. 4. Effect of sugars on total cAMP formed by amebae aggregating on petri dishes. 10^7 Amebae were plated in 55-mm plastic petri dishes in a volume of 3 ml of unsupplemented buffer M (13) (○), the same buffer containing 0.1 M maltose (□) or 0.1 M α-methylglucoside (△). Incubation was in the dark at 22°. At the times indicated three plates of every series were picked at random, at 4-min intervals, and 1 ml of 20% trichloroacetic acid was added to each plate. The contents of the three plates of the same series were pooled and the plates were rinsed with 3 ml of 5% trichloroacetic acid. The homogenates were then boiled for 10 min, the precipitates sedimented, and 5 ml of the supernatant fractions (this volume corrresponded to 10^7 cells) extracted six times with 25 ml of water-saturated ether. The aqueous phase was dried down and resuspended in 1 ml of water. Acetylation of the samples and the assay for cAMP were performed as described (20). The cAMP in the standard curve was made up in an extract of amebae corresponding in content to that of the samples; the cAMP of this extract had been hydrolyzed by digestion of the extract with extracellular amebal cAMP phosphodiesterase. The use of amebal extract in the construction of the standard curve was necessary, because preliminary experiments showed that cAMP-free extracts interfered occasionally with the binding of radioactive antigen by the antibody. The treated cell extract was precipitated with trichloroacetic acid and boiled for 10 min. The trichloroacetic acid precipitate was removed by centrifugation, and known amounts of cAMP were added to the supernatant fraction. These standards were then treated in the same manner as the samples. The treatment of samples with cAMP phosphodiesterase prior to acetylation abolished the ability of these samples to reduce the maximal binding of ^{125}I-labeled 2′-*O*-succinyl-cAMP tyrosine methyl ester; this indicates that indeed cAMP was measured in the assay. The black arrow marks the onset of streaming in the control preparation and the open arrow the onset of streaming in the preparation containing α-methylglucoside.

amebae. In our experiments the acceleration of development occurred, irrespective of whether the pulses of cAMP were administered immediately upon suspension of the amebae in the starvation buffer or only 3–5 hr after the start of starvation. Presumably the amebae respond to extracellular cAMP only after the appearance of the cAMP-binding sites (see Fig. 2), and thus the administration of pulses of cAMP during the first few hours of starvation, prior to the formation of cAMP-binding sites, is apparently ineffective.

Maltose inhibited completely the stimulation of development by pulses of cAMP in the nanomolar concentration range; amebae incubated with α-methylglucoside responded to pulses of cAMP in a manner identical with that of the control amebae. An attempt to overcome the inhibition by maltose by pulsing the amebae with high concentrations of cAMP (2.25 μmol/pulse per 15 ml) gave variable results: occasionally a very slight acceleration of development was observed. If, however, the amebae were starved for 3–5 hr in unsupplemented buffer prior to the addition of maltose, and maltose was then added and the amebae pulsed with 5 nM cAMP (75 pmol/pulse per 15 ml of suspension), subsequent development on filters was accelerated by 2–3 hr. The addition of maltose at this time, in the absence of pulses of cAMP, still severely inhibited development and showed that even after 3–5 hr of starvation the amebae were still sensitive to inhibition by the sugar. The effectiveness of pulses of 4 nM cAMP in partially overcoming the inhibition by maltose in amebae that had been starved underlines the role of cAMP-binding sites in mediating the effects of extracellular cAMP.

Effect of Sugars on cAMP Concentrations in Starving Amebae. Sugars metabolized by *D. discoideum* either inhibit completely, or retard severely, the formation of aggregates by amebae plated in plastic petri dishes. Nonmetabolizable sugars have a relatively slight inhibitory effect (4). Fig. 4 shows that the inhibition of aggregation by maltose is paralleled by an

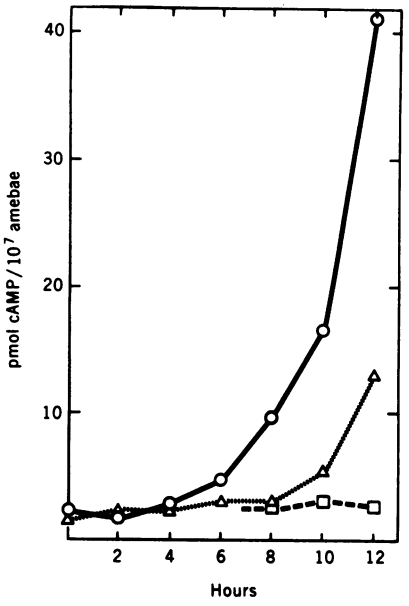

FIG. 5. Cellular cAMP in suspensions of amebae incubated in phosphate buffer. Amebae at a density of 10^7 cells per ml were incubated with shaking at 22° in 0.017 M sodium phosphate buffer (○), the same buffer supplemented with 0.1 M maltose (□) or with 0.1 M arabinose (△). At the times indicated, eight 1-ml samples were harvested by filtration and processed for the determination of cAMP as described in the legend to Table 1 and Fig. 4.

Table 1. Cellular cAMP after 9 hr of incubation in phosphate buffer

Addition	Cellular cAMP (pmol/10^7 amebae) Exp. 1	Exp. 2
—	14	16
Maltose	1	1
Glucose	1	1
α-Methylglucoside	3	5
Ribose	13	3
Arabinose	12	7

Effects of sugars on cellular concentration of cAMP in amebae incubated with shaking in 0.017 M phosphate buffer. Amebae were incubated at a density of 10^7 cells per ml, and sugars were added to a concentration of 0.1 M. A series of eight 1-ml samples was harvested at 1.5-min intervals by gentle suction (vacuum of 18 cm H_2O) through Millipore SSWP025 (pore size 3 μm) filters. The filtration took about 30 sec; the filters were immersed in 1 ml of 5% trichloroacetic acid containing 25 mM Tris·HCl and 10 mM $MgCl_2$. Samples were boiled for 10 min, the filters removed, the trichloroacetic acid precipitates sedimented, and equal volumes of the eight supernatant fractions combined. Extraction of the trichloroacetic acid, assay for cAMP, and preparation of the samples for the standard curve are described in the legend to Fig. 4. Exp. 1 was done with strain AX-2; Exp. 2, with strain AX-3 furnished by Dr. D. R. Soll, University of Iowa.

inhibition of the formation of cAMP; α-methylglucoside retarded slightly both the aggregation of the amebae and the formation of cAMP. These findings suggest that metabolizable sugars inhibit aggregation of the amebae by interfering with the synthesis or accumulation of cAMP.

It is possible to separate the acquisition of competence for aggregation from aggregation itself by incubation of the amebae as a shaken suspension in 0.017 M phosphate buffer; under these conditions the amebae will acquire aggregation competence but will not aggregate. Table 1 shows that levels of cAMP in amebae incubated in buffer supplemented with the metabolizable sugars glucose and maltose are much lower than those of amebae incubated with the nonmetabolizable sugars or without any sugar. We observed, in agreement with the findings of Gerisch *et al.* (5), that the cellular concentration of cAMP in amebae incubated for 9 hr in either unsupplemented buffer or in buffer supplemented with arabinose oscillated with a periodicity of 5–7 min and an amplitude that peaked at 10 to 20 times the basal level (basal level = 0.3–1.0 pmol per 10^7 amebae) found in unstarved amebae. Oscillations with the same periodicity, but reaching only about twice the basal level, first appeared 3–4 hr after starvation. No oscillations were observed in amebae incubated with maltose or glucose; α-methylglucoside and ribose gave highly variable results (unpublished observations). The possibility that cells starved in the presence of α-methylglucoside or ribose synthesized cAMP but released it into the medium could not be tested meaningfully, since suspensions of amebae incubated with these sugars, unlike amebae incubated with maltose or glucose, had high levels of cAMP phosphodiesterase (Fig. 3).

Fig. 5 shows that the level of cellular cAMP in amebae starved in phosphate buffer increased 4–6 hr after the onset of starvation, i.e., sufficiently early to play a role in the synthesis of the "early" developmental proteins. Maltose completely inhibited, whereas arabinose (not a source of carbon) delayed, but did not block, this increase in the cellular cAMP.

DISCUSSION

We demonstrated earlier that sugars that are metabolized by *D. discoideum* and that stimulate vegetative growth inhibit the development of the slime mold (4). Our findings also showed that the sugars blocked the breakdown of RNA, protein, and glycogen, which normally accompanies development in this organism; *D. discoideum* uses cellular macromolecules formed during vegetative growth as a source of building blocks for the anabolic processes which culminate in the formation of the fruiting body. These findings suggest that starvation for carbon acts as trigger for development; a recent report (3), however, indicates that starvation for a required amino acid may also serve as stimulus for development. Clearly, a dual mechanism of the nutritional control of development in which starvation for either carbon or nitrogen triggers development is not excluded.

In the present report we show that the availability of a source of carbon controls, in addition to catabolism, also biosynthetic reactions that occur early in development: metabolizable sugars inhibit the formation of the contact sites "A" (as defined by the ability of the amebae to agglutinate in the presence of EDTA), of the specific cAMP-binding sites, and of cAMP phosphodiesterase. The sugars also block the accumulation and release of cAMP, which parallels the acquisition of aggregation competence in starving amebae (8, 25). Our preliminary observations (unpublished results) indicate that the cellular concentration of cGMP in *D. discoideum* is approximately 0.5–1.0 pmol per 10^7 amebae and does not change significantly during development.

It is tempting to conclude that there is a causal relationship between the increase in cellular cAMP and those biosynthetic and catabolic reactions that constitute development. Our findings are clearly compatible with a role of cAMP in the synthesis of contact sites "A", the cAMP-binding sites, as well as the increase in cAMP phosphodiesterase activity in that the synthesis of these entities occurs at about the same time as the increase in cellular cAMP. A role of cAMP in the catabolic re-

actions, which are triggered by starvation, however, remains to be established; we found (4) that the breakdown of RNA, protein, and glycogen starts immediately upon resuspension of the vegetative amebae in the starvation buffer. It appears then that either our methodology of sampling for, and measuring, cAMP immediately after the removal of the amebae from the growth medium is inadequate for the detection of the, conceivably slight and transient, increases in cAMP required for the initiation of catabolic reactions or, alternatively, that cAMP plays no role in these reactions.

The ability of pulses of cAMP, in the nanomolar concentration range, to accelerate development has been demonstrated earlier (23, 24). The fact that pulses of the cyclic nucleotide partially overcame the inhibition by maltose, *provided* that the amebae were starved for about 3 hr prior to the addition of the inhibitory sugar, suggests that amebae respond to extracellular cAMP only after the formation of the membranal cAMP-binding sites. That exogenous cAMP overcomes, at least partially, the inhibitory effects of sugars is also compatible with our hypothesis of a causal relationship between the inhibition by sugars of the accumulation of cAMP and the inhibition of development. The experiments reported in this paper have no bearing on the mechanism by which metabolizable sugars inhibit the accumulation of cAMP; recent work by Rossomando (*J. Biol. Chem.*, in press), however, shows that ATP at millimolar concentrations can bring about the loss of adenylate cyclase activity and that 5′-AMP can prevent this loss. This finding suggests that the energy charge of the cell regulates the cellular cAMP concentration and that it may thus be the ultimate effector of development.

This research was supported by Grant no. AM 11046 from the National Institutes of Arthritis, Metabolism, and Digestive Diseases, U.S. Department of Health, Education and Welfare. H.J.R. was supported by the Deutsche Forschungsgemeinschaft; H.V.R. is the Ida and Cecil Green Investigator in Developmental Biochemistry. H.V.R. thanks Prof. G. Gerisch of the Biozentrum, Basel, for the hospitality of his laboratory in which the assay for contact sites "A" was performed, and for helpful discussions.

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