Intracellular Adenosine 3′,5′-Phosphate Binding Proteins in *Dictyostelium discoideum*: Partial Purification and Characterization in Aggregation Competent Cells†

Jean de Gunzburg‡ and Michel Veron*

ABSTRACT: Three adenosine 3′,5′-phosphate (cAMP) binding proteins were separated and partially purified from cytoplasmic extracts of *Dictyostelium discoideum* cells developed to aggregation competence. Two species, A and B, representing respectively 50% and 20% of the total activity, bind cAMP with very rapid kinetics and high specificity. Species A (K_d = 7.5 nM) is a monomeric protein of 36 000 daltons with a sedimentation coefficient of 2.3 S. Species B, which binds cAMP with positive cooperativity, also displays a high affinity for the ligand (K_d = 3.2 nM). This protein is present in the extracts as an equilibrium between monomeric, dimeric, and tetrameric forms with respective sedimentation coefficients of 2.4, 4.5, and 6.5 S; binding of cAMP to the monomer induces the appearance of the multimeric forms. A third cAMP binding protein (species C, K_d = 9.5 nM) was characterized as a larger protein (M_r 190 000, sedimentation coefficient of 9.2 S) which also binds adenosine and adenosyl derivatives. Species C represents 30% of the activity in the extracts and resembles the "adenosine analogue binding proteins" described in mammalian cells. The relevance of the properties of these proteins to the developmental process of *D. discoideum* amoebas is discussed.

Upon food deprivation, *Dictyostelium discoideum* amoebas enter a developmental cycle in which cells aggregate toward specific centers. Multicellular aggregates are formed that eventually differentiate into stalk and spore tissues (Loomis, 1975). Adenosine 3′,5′-phosphate (cAMP)[1] plays a dual role in the developmental program. During the aggregation phase, amoebas secrete cAMP, the chemotactic mediator through which they communicate, in a pulsatile manner (Shaffer, 1957, 1975; Konijn et al., 1967). cAMP signals, which trigger the chemotactic responses of amoebas (Robertson et al., 1972), are relayed from cell to cell throughout the aggregation territory in a rhythmic process that involves an intracellular accumulation of cAMP and its subsequent release into the extracellular medium (Gerisch & Wick, 1975; Roos et al., 1975). Specific cell surface cAMP receptors that appear after a few hours of starvation have been described (Malchow & Gerisch, 1974; Green & Newell, 1975; Wallace & Frazier, 1979).

Aside from its role in chemotaxis, cAMP appears to induce differentiation. The rhythmic signals stimulate cell differentiation from the growth phase to the aggregation competent stage (Darmon et al., 1975; Gerisch et al., 1975). High exogenous cAMP concentrations were shown to induce specifically stalk cell formation (Bonner, 1970). More recent work has extended these findings in demonstrating that cAMP was required for the differentiation of both stalk and spore cells (Town et al., 1976; Kay et al., 1978; Town & Stanford, 1979). The involvement of cAMP as an effector of differentiation is further supported by the recent evidence that treatment of cells with cAMP directly affects the expression of developmentally regulated genes (Landfear et al., 1980); in some specific cases, the effect is likely to be at the transcriptional level (Williams et al., 1980).

Soluble cAMP binding proteins have already been reported in *D. discoideum* cells (Malkinson et al., 1973; Veron & Patte, 1978; Rahmsdorf & Gerisch, 1978; Wallace & Frazier, 1979; Cooper et al., 1980; Leichtling et al., 1980) which could constitute intracellular targets for the regulatory activity of cAMP. These proteins were found in vegetative and aggregation competent cells as well as in culminating aggregates and mature fruiting bodies. Differences were reported among cAMP binding proteins relative to the stages of development (Veron & Patte, 1978; Cooper et al., 1980). In the present report, we have investigated in greater detail the characteristics of intracellular cAMP binding proteins in aggregation competent cells of *D. discoideum*.

Materials and Methods

Chemicals and Buffers. Buffer KPB was 17 mM potassium phosphate buffer, pH 6.2, in mineral water from Volvic (France). All the other buffers were in distilled water. Buffer A was 25 mM sodium phosphate buffer, pH 6.5, containing 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM PMSF (phenylmethanesulfonyl fluoride) freshly prepared at 0.1 M in dimethyl sulfoxide. Buffers A_2 and A_5 were respectively 2- and 5-fold dilutions of buffer A. Buffer C was 10 mM Tris-HCl buffer, pH 7.0, containing 5 mM $MgCl_2$, 2 mM MgEDTA, and 1 mM dithiothreitol.

[5′,8-$^3H_2$]cAMP (45 Ci/mmol) and [2,5′,8-$^3H_3$]adenosine (40 Ci/mmol) were from the Radiochemical Centre (Amersham, U.K.). Selectron filters (0.45 μm, type BA85, 25-mm diameter) were purchased from Schleicher & Schüll (West Germany). DEAE-Sephacel was obtained from Pharmacia (Uppsala, Sweden) and Bio-Gel HTP hydroxylapatite from Bio-Rad (Richmond, CA). All chemicals were of analytical grade.

Strains, Conditions for Growth, and Differentiation. *Dictyostelium discoideum* amoebas strain AX2 (Watts & Ashworth, 1970) were grown at 22 °C in HL5 broth. Cells

† From the Unité de Differenciation Cellulaire, Institut Pasteur, 75724 Paris Cedex 15, France. *Received January 2, 1981.* This work was supported by grants from the Centre National de la Recherche Scientifique (ATP A15020).

* Address correspondence to this author at the Unité de Biochimie Cellulaire, Institut Pasteur, 75015 Paris, France.

‡ Recipient of a scholarship from the Délégation Générale à la Recherche Scientifique et Technique.

[1] Abbreviations used: cAMP, adenosine 3′,5′-phosphate; cGMP, guanosine 3′,5′-phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; PEI, poly(ethylenimine); NaDodSO$_4$, sodium dodecyl sulfate.

0006-2960/81/0420-4547$01.25/0

were collected when they reached a density of (4–7 × 10^6 cells/mL and washed free of the nutrient medium in KPB. For differentiation to aggregation competence, cells were resuspended in that buffer at a density of 10^7 cells/mL and shaken at 22 °C for 7 h. Aggregation competence was evaluated as described (Darmon & Klein, 1978). Cells were usually collected and used immediately. In some cases, the pellet of cells was frozen in dry ice/ethanol and stored at −70 °C.

cAMP Binding Assay. cAMP binding was measured with a filter assay by a modification of the procedure of Gilman (1970). Incubations were carried out at 0 °C for 30 min in a final volume of 100 μL containing the same buffer as the sample and [^{3}H]cAMP as indicated. The reaction was stopped by the addition of 3 mL of ice-cold washing buffer (20 mM sodium phosphate buffer, pH 6.5, containing 0.5 M NaCl when the sample was in buffer A or B, or buffer C containing 0.5 M NaCl when the sample was in buffer C) and immediate filtration through Selectron filters. The filters were rapidly washed 3 times with 3 mL of washing buffer and removed from the suction apparatus. The entire filtration procedure took no more than 10–15 s. Filters were then dried and counted in 4 mL of toluene–POPOP scintillant. Binding assays were always performed in duplicates; the standard error on the binding value usually did not exceed 10%. The background consisting of [^{3}H]cAMP retained by the filters in the absence of protein was subtracted from the cAMP binding values. Nonspecific binding of cAMP to extracts was measured in the presence of 1 mM cold cAMP; it was within background. It is worth noting that such an assay does not really provide a measure of cAMP binding at equilibrium; yet values obtained for the partially purified extracts with this procedure were always more than 80% of those determined by equilibrium dialysis (see further in the text). The filter assay thus provides a satisfactory approximation of the measure of cAMP binding at equilibrium.

The same filtration procedure was used for the determination of adenosine binding, using a final concentration of 1 μM [^{3}H]adenosine (9 Ci/mmol) in the assay.

Determination of cAMP Binding by Equilibrium Dialysis. At low cAMP concentrations (0.1–10 nM), the dialysis technique at "constant free" was used in order to maintain a large excess of free ligand; thus the sample volume was 0.2 mL when cAMP was 0.1 nM and 15 mL for concentrations in the 0.25–10 nM range. At higher ligand concentrations (up to 0.1 μM cAMP), experiments were performed in 0.5-mL dialysis cells (Blangy, 1970) with 0.4 mL in each compartment. In both cases, [^{3}H]cAMP (45 Ci/mmol) was introduced in the "free" compartment only, and equilibrium was reached after 16 h at 4 °C. At that time, two 90-μL samples were collected from each compartment and counted. Nonspecific cAMP binding was looked for in the presence of 0.1 mM cold cAMP, but it was not detectable.

Thin-Layer Chromatography. Samples to be analyzed (5–10 μL) were deposited on a sheet impregnated with cellulose MN 300 poly(ethylenimine) (Macherey-Nägel and Co., Düren, West Germany). The chromatogram was developed with 1-butanol/acetone/acetic acid/5% NH_4OH/water (7:5:3:3:2 v/v) at room temperature and air-dried. Spots from cAMP, 5′-AMP, adenosine, and adenine markers were visualized under a UV light. The corresponding fractions were cut out and counted in toluene–POPOP scintillant.

Gel Electrophoresis. Nondenaturing gel electrophoresis was carried out in 6% polyacrylamide slab gels according to Davis (1964). It was performed at 15 mA/gel in a Bio-Rad cell (Model 220) thermostated at 4 °C by a Lauda XR2 circulating cooling bath. When the tracking dye (bromophenol blue) neared the bottom, the gel was removed and sliced down its long axis into two halves, one of which was stained with Coomassie brilliant blue. The other half of the gel was sliced in 3-mm fractions, and each slice was eluted overnight in 1.1 mL of 10-fold concentrated buffer C containing 15 mM additional HCl in order to neutralize the alkalinity due to the gel. cAMP binding was determined in the eluates as described, except that the incubation volume of the test was 1 mL at a final concentration of 10 nM [^{3}H]cAMP (45 Ci/mmol).

Sucrose Gradient Centrifugation. Samples in buffer C (0.2 mL) were layered on top of a 12.6-mL linear sucrose density gradient (5–20%) prepared in the same buffer. After centrifugation in a Beckman SW41 rotor at 40000 rpm for 18–20 h at 4 °C, 0.5-mL fractions were collected from the bottom of the tube with a peristaltic pump and assayed for cAMP binding activity with 50 nM [^{3}H]cAMP (45 Ci/mmol). Prior to centrifugation, 0.15 mg of catalase (bovine liver), 0.1 mg of lactic dehydrogenase (rabbit muscle), and 0.3 mg of hemoglobin (human) were added to the samples as markers.

Enzyme Assays. cAMP phosphodiesterase was assayed as described (Dicou & Brachet, 1979). Hemoglobin was measured by its absorbancy at 415 nm; catalase and lactic dehydrogenase were assayed according to Bergmayer (1955), Bonnicksen & Brink (1955), and Kornberg (1955), respectively.

Results

Separation and Partial Purification of cAMP Binding Proteins. (A) Preparation of Crude Soluble Extracts. Frozen pellets amounting to 1.5 × 10^{11} aggregation competent cells were adjusted to a density of 3 × 10^8 cells/mL in buffer A and thawed; the suspension was homogenized by five gentle strokes of a tight-fitted pestle in a Dounce homogenizer at 0 °C. This procedure resulted in the disruption of more than 99% of the cells. All of the following procedures were performed in the cold. The few unbroken cells and large debris were removed by a 15-min centrifugation at 3000*g*, particulate material was pelleted at 30000*g* for 20 min, and the supernatant was finally cleared at 100000*g* for 60 min, yielding the crude soluble extract.

(B) Chromatography. Figure 1 shows the elution profile from chromatography on DEAE-Sephacel of the crude soluble extract. The first peak of cAMP binding activity (peak I) was not adsorbed by the resin; it was not saturable by cAMP and thus appeared to represent nonspecific binding. This activity was not further studied. The bulk of the cAMP binding activity was separated into two peaks; a major one, peak II, was eluted at an ionic strength of 116 mM and a minor one, peak III, at 176 mM. They represented respectively 80% and 20% of the activity adsorbed on the column; this pattern was reproducible in several experiments.

As soon as possible after elution of the DEAE column, the pooled fractions of peak II were diluted and loaded onto a hydroxylapatite column. Figure 2 shows that the activity was eluted into two peaks; the major one (A) represented 64% of the activity in peak II, and the minor one (C), which was eluted at a higher ionic strength, represented 36% of the activity in peak II. Figure 3 shows an analogous experiment performed with fractions of peak III. All the activity was eluted from the column as a single peak (B).

The purification procedure, from disruption of the cells to completion of the hydroxylapatite chromatography, took no more than 5 days. Addition of 0.1 mM PMSF to the buffers and rapidity of the procedure were designed to minimize damage to the cAMP binding proteins.

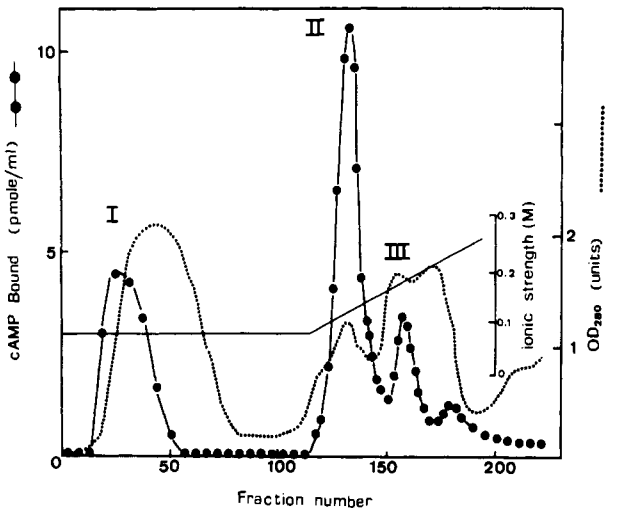

FIGURE 1: DEAE-Sephacel chromatography of the crude soluble extract. The crude soluble extract (470 mL in buffer A) was loaded onto a DEAE-Sephacel column (4.5 × 32 cm) previously equilibrated in buffer A. It was washed with the same buffer and eluted with a linear 0–300 mM NaCl gradient in buffer A (—); salt concentrations are expressed in ionic strength. The absorbance at 280 nm was recorded (dotted line). Fractions (20 mL) were analyzed for cAMP binding with 0.25 μM [^{3}H]cAMP (5 Ci/mmol) (●). Those representing peak II (137–152) and peak III (162–173) were pooled.

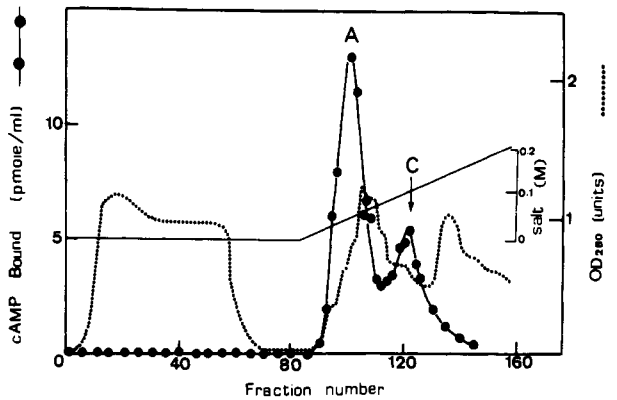

FIGURE 2: Hydroxylapatite chromatography of peak II from the DEAE column. The pooled fractions of peak II were diluted 2-fold with water, made up to 1000 mL with buffer A_2, and loaded onto a Bio-Gel HTP hydroxylapatite column (2.8 × 30 cm) equilibrated in the same buffer. It was eluted by a gradient of 0–200 mM sodium phosphate buffer, pH 6.5, in buffer A_2 (—). The absorbance at 280 nm was recorded (dotted line), and fractions (10 mL) were assayed for cAMP binding as described in the legend to Figure 1 (●). Fractions of peaks A (94–104) and C (111–118) were pooled.

(*C*) *Polyacrylamide Gel Electrophoresis.* Figure 4 shows the migration patterns of extracts from peaks A, B, and C described in Figures 2 and 3 in 6% polyacrylamide gels under nondenaturing conditions. Although staining of the gels showed that the extracts were only partially purified, each one exhibited a single peak of cAMP binding activity. The activity in B migrated somewhat closer to the tracking dye than that in A whereas the activity in C was much more retarded. This criterion shows that extracts A, B, and C contain homogeneous cAMP binding activities which will be referred to as species A, B, and C. All of the following experiments were performed with these partially purified extracts.

(*D*) *Notes on Purification and Distribution of Species A, B, and C in the Extracts.* The partially purified fractions from the hydroxylapatite columns contained no detectable cAMP phosphodiesterase activity. They were also free of any other cAMP-degrading activity: analysis by thin-layer chromatography on PEI-cellulose sheets showed that all of the radiolabel remaining in the assay mixture just prior to filtration migrated like authentic cAMP. Incubation of the extracts with DNase I or RNase A did not affect the cAMP binding activities which, in contrast, were destroyed after treatment by trypsin or Pronase.

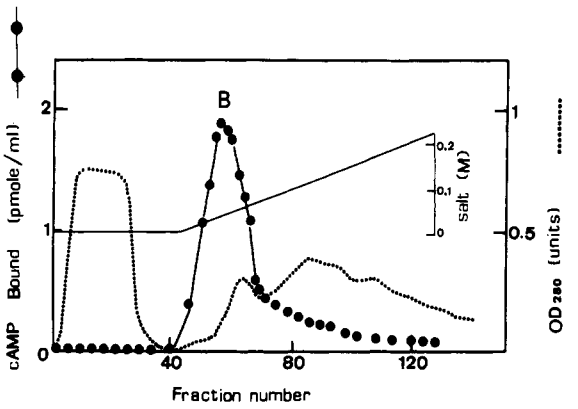

FIGURE 3: Hydroxylapatite chromatography of peak III from the DEAE column. The pooled fractions of peak III were diluted 5-fold with water and loaded onto a Bio-Gel HTP column (2.8 × 15 cm) equilibrated in buffer A_5. The procedure was the same as described in the legend to Figure 2 except that all solutions were in buffer A_5. (—) Concentration of sodium phosphate buffer, pH 6.5. (···) Absorbance at 280 nm. (●) cAMP binding activity. Fractions 48–66 (peak B) were pooled.

The partially purified extracts were stable for periods of up to 3 months when stored at 4 °C with 0.02% sodium azide. However, prolongated storage of species C led to a limited decrease in its sedimentation coefficient and affinity for cAMP, while other properties remained unchanged; species A and B were not affected. Prior to use, the extracts were dialyzed overnight against 100 volumes of buffer C.

The contributions of species A, B, and C to the cAMP binding activity present in the crude soluble extract were calculated from the data obtained after the hydroxylapatite step, assuming a 100% recovery. When only the "specific" cAMP binding activity adsorbed on the DEAE-Sephacel column (i.e., that of peaks I and II in Figure 1) was taken into account, species A, B, and C, respectively, represented 50%, 20%, and 30% of the total activity. The latter amounted to 0.15 pmol of cAMP bound per 10^7 cells.

Properties of Species A, B, and C. (*A*) *Sedimentation in Sucrose Gradients.* Figure 5 shows the sedimentation patterns of species A and C in 5–20% linear sucrose gradients. They sedimented as single peaks of 2.3 ± 0.1 S and 9.2 ± 0.1 S, respectively, in four independent experiments. The presence of 10 nM cAMP in the gradients did not affect the sedimentation profile; no dissociation was observed in the presence of 2 M urea or 0.1% Triton X-100. With the assumption that they are globular proteins, the sedimentation coefficients of species A and C correspond to molecular masses of 35 000–40 000 and 180 000–200 000 daltons, respectively. Those values were consistent with those obtained by gel filtration through a calibrated Sephacryl S 300 column (data not shown); there was no evidence in several experiments for heterogeneity or dissociation of species A and C.

Figure 6a shows the sedimentation pattern in sucrose gradients of species B: it was separated into three cAMP binding activity peaks of respective sedimentation coefficients of 2.4, 4.5, and 6.5 S. Under mild dissociating conditions (2 M urea and 0.1% Triton X-100; Figure 6b), all the activity was recovered in a single peak of 2.4 ± 0.2 S (calculated from six independent experiments). This suggests that the 4.5- and 6.5S peaks were due to multimeric forms of the 2.4S protein; indeed,

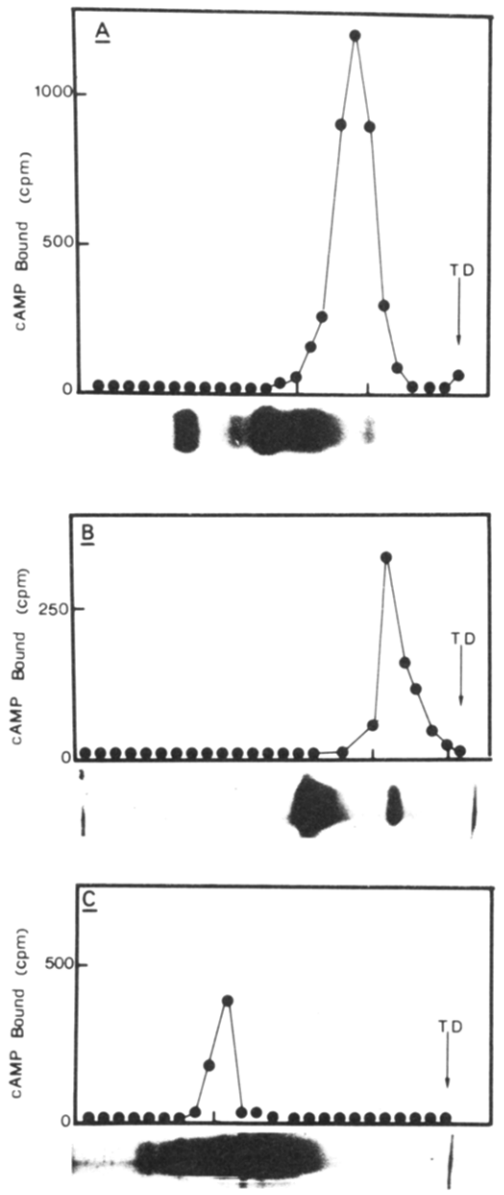

FIGURE 4: Polyacrylamide gel electrophoresis of partially purified cAMP binding proteins. Samples (0.15 mL) from peaks A, B, and C of the hydroxylapatite columns were layered onto a 6% polyacrylamide gel and submitted to electrophoresis under nondenaturating conditions at 4 °C. After migration, the stacking gel was discarded; one part of the separating gel was stained with Coomassie blue. The other was sliced and eluted, and cAMP binding activity of the fractions was determined in the eluates (●). The position of the tracking dye (TD) is shown.

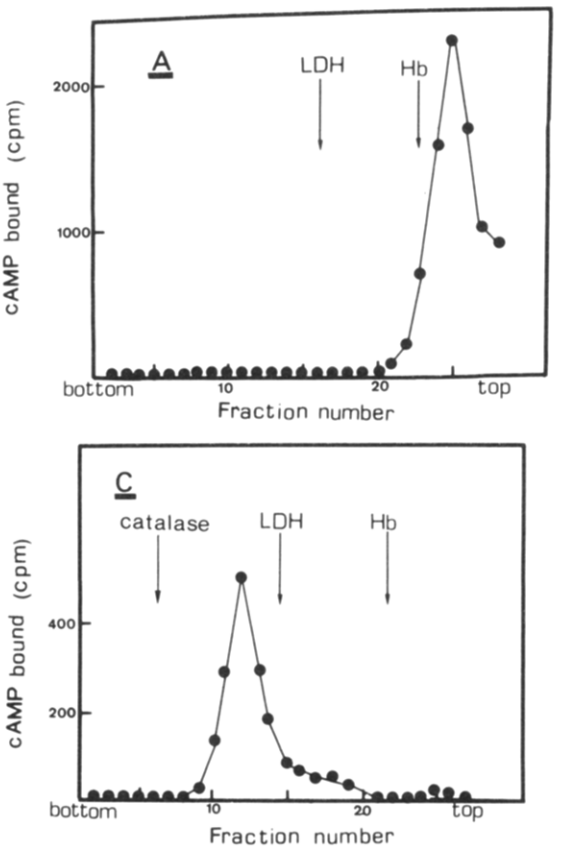

FIGURE 5: Sedimentation of species A and C in sucrose gradients. The experiments were performed as described under Materials and Methods. A typical one is shown. The gradients were calibrated with internal markers: catalase (11.3 S); lactic dehydrogenase (7.3 S); hemoglobin (4.2 S). (Top) Sedimentation of species A; (bottom) sedimentation of species C.

these values would be consistent with dimeric and tetrameric forms of a 2.4S monomer, respectively. The same shift in pattern was obtained with a sample containing species B that had been stored for more than 3 months at 4 °C and had retained full cAMP binding activity. Figure 6 shows that preincubation of this "spontaneously dissociated" extract with 10 nM cAMP and sedimentation in a gradient containing the ligand at the same concentration resulted in restoration of the original pattern, which strongly suggests that species B is an equilibrium between a 2.4S monomer, its dimer, and tetramer.

(*B*) *Characteristics of the Binding of cAMP to Species A, B, and C.* As shown in Figure 7a, the binding of cAMP to species A was fast: at a concentration of 5 nM cAMP, half-maximal binding occurred in 12 s, and saturation was obtained in less than 5 min. The reaction followed a single exponential, as expected for a simple process since the experiment was performed with a large excess of cAMP over binding sites. The association time constant ($k_{+1} = 1.2 \times 10^7$ M^{-1} s^{-1}) was calculated from the semilogarithmic plot (inset of Figure 7a). Figure 7b shows the dissociation of cAMP from species A; half of the cAMP initially bound was released 35 s after dilution. The process is a first-order reaction, which enables the calculation of the dissociation time constant $k_{-1} = 2.7 \times 10^{-2}$ s^{-1}. Very similar results were obtained with species B (results not shown). In contrast, species C displayed much slower association and dissociation kinetics for cAMP; in the presence of 50 nM cAMP, saturation was only achieved in 1–2 h. Half of the cAMP bound at equilibrium was dissociated in 65 min (results not shown).

A comparison between the respective dissociation rates of cAMP from the binding proteins and the time required to perform the filtration procedure of the cAMP binding assay (10–15 s) lead to the following remarks. The assay can only be considered to be a valid measure of cAMP binding at equilibrium if a negligible amount of the bound ligand is dissociated during filtration. This is the case for species C. With species A and B, the filter assay yields an underestimate of cAMP binding; consequently, the values of k_{-1} and k_{+1} are respectively over- and underestimated. Nevertheless, measures of cAMP binding by means of equilibrium dialysis were no more than 20% higher than that with the filter assay, showing that the latter was satisfactory for routine determinations.

For true measures of cAMP binding at equilibrium to species A and B over a wide range of cAMP concentrations, the experiments depicted in Figures 8 and 9 were performed by means of equilibrium dialysis. Figure 8 shows a Scatchard plot (1949) of the data obtained with species A. It exhibited a single "high affinity" for cAMP of 7.5 nM, and saturation

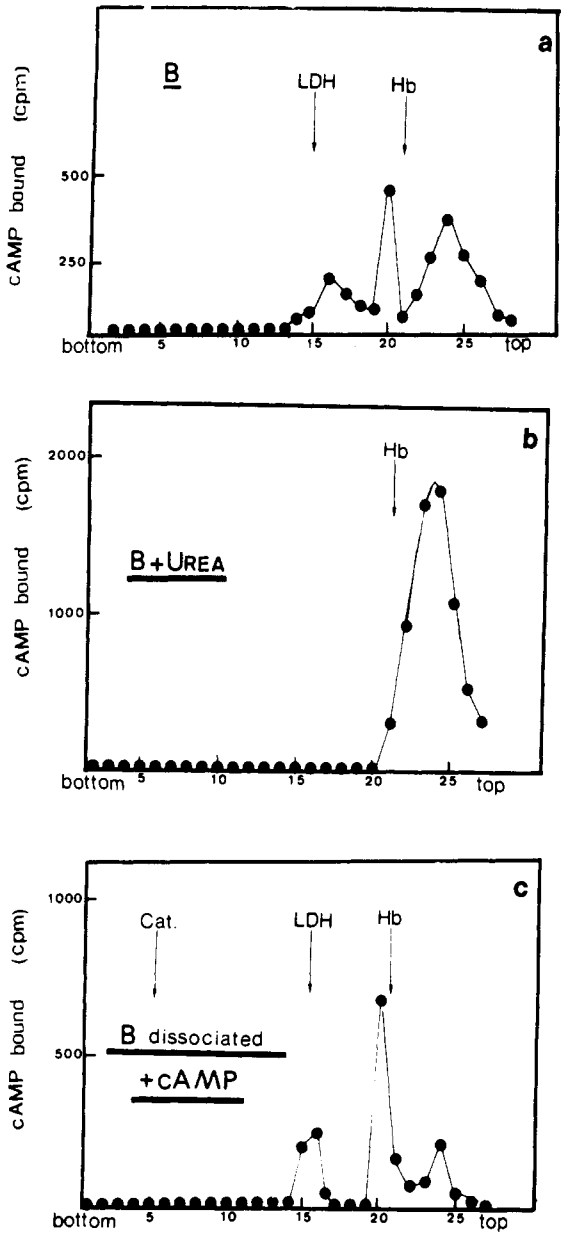

FIGURE 6: Sedimentation pattern of species B in surcose gradients. The experiments were performed as previously described. (a) No addition. (b) The sample was preincubated for 30 min at 0 °C with 2 M urea and sedimented in a urea-containing gradient. After their collection, fractions were diluted 4-fold with buffer C. Urea did not affect the determination of cAMP binding activity, but catalase and lactic dehydrogenase could not be assayed. (c) A sample of "spontaneously dissociated" species B (see text) was treated for 30 min at 0 °C with 10 nM cAMP and sedimented in a gradient containing the nucleotide at the same concentration. All further steps remained unchanged.

was reached with 50 nM cAMP. Binding of cAMP to species B displays positive cooperativity (Figure 9); the linear part of the curve gave a dissociation constant of 3.2 nM cAMP, and binding was maximal at 50 nM cAMP. The Hill plot of the data (not shown) leads to a value of $n = 1.7$; the positive cooperativity effect was seen for cAMP concentrations lower than 0.5 nM.

Dissociation constants of species A and B for cAMP were also obtained by measuring cAMP binding at various ligand concentrations with the filter assay; alternatively, the K_d was directly calculated from the kinetic experiments as the ratio of the dissociation time constant to the association time constant obtained from the data of Figure 7. All three values of the dissociation constant agreed closely.

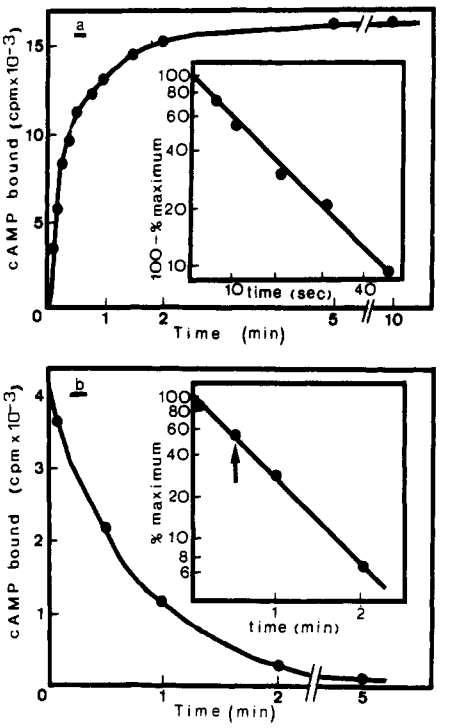

FIGURE 7: Kinetics of cAMP binding and dissociation with species A. The experiments were performed at 0 °C in buffer C. (a) Association kinetics of cAMP to species A. Samples were incubated for increasing periods of time with 5 nM [^{3}H]cAMP (45 Ci/mmol) in a final volume of 1 mL. The amount of [^{3}H]cAMP bound was determined by the filtration procedure. The inset shows the semilogarithmic plot of the data. (b) Dissociation kinetics of cAMP bound to species A. Association was performed with 5 nM [^{3}H]cAMP (45 Ci/mmol) in a final volume of 0.1 mL until equilibrium was reached. The samples were then diluted 30-fold with buffer C, and the remaining [^{3}H]cAMP bound after increasing periods of time was determined by the filtration procedure. Rebinding of cAMP after dilution was less than 10% of the total amount of cAMP bound at equilibrium. The inset provides a semilogarithmic plot of the data; the arrow indicates the time of half-dissociation.

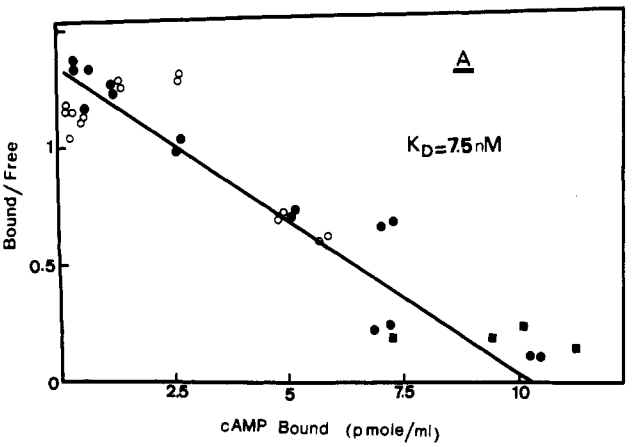

FIGURE 8: Scatchard plot for the binding of [^{3}H]cAMP to species A; 0.2–0.4-mL samples containing species A in buffer C were analyzed for cAMP binding at various cAMP concentrations (0.1–100 nM) by equilibrium dialysis as described under Materials and Methods. The data result from three independent experiments (●, ○, ■).

Affinity of species C for cAMP was determined by using the filtration assay only. A value of $K_d = 9.5$ nM was found (results not shown). There was also an indication of lower affinity sites with dissociation constants in the micromolar range, but the latter were not fully competed by the addition of 1 mM cold cAMP.

(C) Specificity of Ligand Binding. Table I summarizes the effect of various purine derivatives on the binding of [^{3}H]-

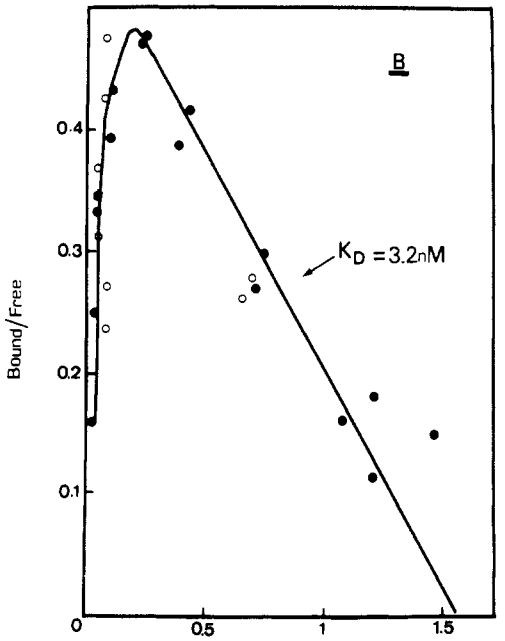

FIGURE 9: Scatchard plot for the binding of [^{3}H]cAMP to species B. The results of two experiments are shown (●, ○).

Table I: Inhibition of cAMP Binding to Species A and B by Various Purine Derivatives[a]

competing ligand	10^{-6} M (×20)	5×10^{-5} M (×1000)	10^{-3} M (×20000)
ATP	100	95	100
		100	100
ADP	100	95	90
		100	100
5′-AMP	100	100	95
	100	100	90
adenosine	100	90	90
		100	95
adenine	95	90	90
		100	95
8-azido-cAMP	12	0	0
	12	0	0
2′,3′-cAMP	100	90	95
		100	100
GTP	100	95	75
		100	100
5′-GMP	100	90	85
		95	90
2′,3′-cGMP	100	85	90
		90	65
3′,5′-cGMP	95	20	0
	85	15	0

[a] Samples containing species A or B were incubated in a final volume of 0.1 mL with 50 nM [^{3}H]cAMP (45 Ci/mmol) in the presence of various purine derivatives at the concentrations indicated in the table. The incubation (30 min, 0 °C) was initiated by the addition of the extract; cAMP binding was determined by the filter assay. Results are expressed as percent of the control containing only 50 nM [^{3}H]cAMP, buffer, and the extract. The upper and lower numbers, respectively, correspond to species A and B.

cAMP to species A and B. 8-Azido-cAMP, a photoreactive cAMP analogue, was a good competitor whereas cGMP was a poor one; 150- and 100-fold excesses, respectively, over cAMP were required to prevent half of the [^{3}H]cAMP binding to species A and B. None among the other purine derivatives investigated produced a significant inhibition or competition of cAMP binding to species A and B, even at a 20000-fold excess. In contrast, ligand binding to species C exhibited a much broader specificity. As shown in Figure 10, cold cAMP and adenosine had similar effects on the inhibition of [^{3}H]-cAMP binding. The inhibition curves were not strictly parallel,

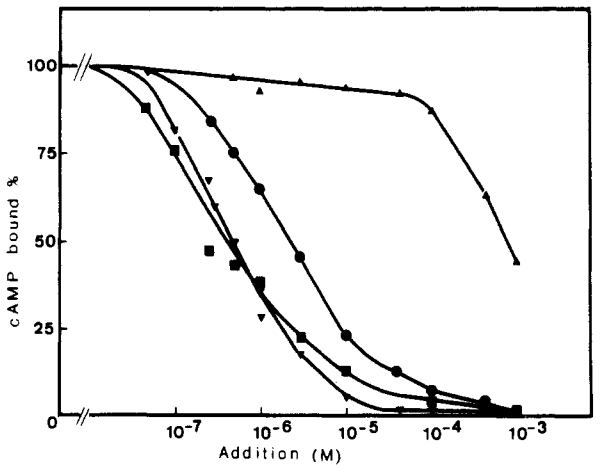

FIGURE 10: Inhibition of [^{3}H]cAMP binding to species C by cold cAMP, adenosine, 5′-AMP, and cGMP. The experiment was performed as described under Table I with 0.5 μM [^{3}H]cAMP (9 Ci/mmol) and the following additions: (▼) cAMP; (■) adenosine; (●) 5′-AMP; (▲) cGMP. The curves result from two independent experiments.

Table II: Inhibition of cAMP Binding to Species C by Various Purine Derivatives[a]

competing ligand	10^{-6} M (×20)	10^{-5} M (×200)	10^{-3} M (×20000)
ATP	32	15	5
ADP	28	5	0
5′-AMP	23	7	0
adenosine	13	5	0
adenine	91	50	20
8-azido-cAMP	20	0	0
2′,3′-cAMP	100	90	65
GTP	100	100	100
5′-GMP	90	90	80
2′,3′-cGMP	100	100	100
3′,5′-cGMP	95	90	45
3′,5′-cIMP	100	95	75

[a] The experiment was performed as described under Table I with 0.5 μM [^{3}H]cAMP (9 Ci/mmol).

but they intersected at 50% inhibition; this could result from different binding kinetics of the two compounds. 5′-AMP was a potent competitor, and 50% inhibition required a 5-fold excess over cAMP. cGMP only exerted an effect at very high concentrations. None of the guanosyl derivatives, even at millimolar concentrations, were inhibitors of [^{3}H]cAMP binding to species C. In contrast, adenosine and all of the adenosyl derivatives investigated were good competitors (Table II). Their order of potency was cAMP = adenosine > 5′-AMP > ADP > ATP > adenine. We have also investigated the ability of species C to bind [^{3}H]adenosine; the protein bound 2–3-fold more adenosine than [^{3}H]cAMP, but with much slower kinetics, and part of the adenosine binding could only be inhibited by 0.1 mM cold cAMP (data not shown). These results probably indicate heterogeneity of binding sites; alternatively, since the extracts containing species C are not pure, it could be due to the presence of other proteins carrying adenosine binding activity.

Discussion

Three distinct species of cAMP binding proteins were resolved and partially purified in extracts from aggregation competent *Dictyostelium discoideum* cells. These proteins were found in high-speed supernatants (100000*g*, 1 h) of cell lysates and thus are assumed to be of cytosolic origin.

Differential elution from DEAE-Sephacel and hydroxylapatite columns resulted in the separation of three cAMP

binding proteins (species A, B, and C) and elimination of nonspecific cAMP binding activity. Although purification was only partial, it was sufficient to completly resolve the three cAMP binding proteins studied and separate them from any cAMP-degrading activity.

Species A and B share many common features. They both exhibit fast binding kinetics and affinity for cAMP in the nanomolar range. Both proteins bind cAMP with extreme specificity; except for cGMP, which competes cAMP binding at 100-fold higher concentrations, a number of other purine derivatives had no inhibitory effect on cAMP binding, even at a 20 000-fold excess. Aside from their elution from the DEAE column at different ionic strengths, species A and B differ in their sedimentation coefficients: species A is a small protein of 2.3 S whereas species B seems to be in equilibrium between a 2.4S monomer, its dimer, and tetramer. Binding of cAMP to the dissociated protein induces the appearance of the multimeric forms. This could generate the positive cooperativity shown for cAMP binding, which is indeed the major difference between species A and B, and may be the basis for their hypothetically distinct physiological actions.

The kinetics of cAMP binding to species A and B deserve comments. The binding is rapid since at a cAMP concentration as low as 5 nM, half-maximal binding was reached in 12–15 s; the half-life of the cAMP–protein complex is also relatively short (35–40 s). These kinetic parameters have important physiological implications. In aggregating *D. discoideum* cells, the cAMP concentration oscillates every 5–10 min (Gerisch & Wick, 1975); the cGMP concentration, which also oscillates, remains 10-fold lower (Wurster et al., 1977; Mato et al., 1977) whereas levels of other purine derivatives such as ATP are steady and 100-fold higher (Roos et al., 1977). When their very high affinity and specificity are considered, species A and B are likely to bind cAMP in the living cells. Furthermore, the rapid kinetics of cAMP binding and dissociation could enable these proteins to "sense" oscillations of the cAMP concentration. Several authors have shown that the rhythmic emissions of cAMP by the cells, which result in temporary elevations of the intracellular cAMP concentration (Gerisch & Wick, 1975), control the differentiation of amoebas to aggregation competence (Darmon et al., 1975; Gerisch et al., 1975) and the transcription rate of some developmentally regulated genes (Williams et al., 1980); cAMP binding proteins of species A and B could be relevant to this process.

In contrast, species C is a larger protein exhibiting a sedimentation coefficient of 9.2 S. Although sedimentation in the presence of 2 M urea or 0.1% Triton X-100 did not cause any dissociation, preliminary results of polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (not shown) suggest that the protein is a tetramer. Species C also binds cAMP with a high affinity (K_d = 9.5 nM), but its kinetics of cAMP binding and dissociation are much slower. It exhibits a broad specificity of binding toward adenine derivatives with an order of potency of cAMP = adenosine > 5′-AMP > ATP > adenine. Moreover, species C binds more adenosine than cAMP, and a fraction of the adenosine binding is not inhibited by cold cAMP. Since adenosine, 5′-AMP, ADP, and ATP are present at high concentrations in the living cells, species C is much more likely to bind adenosine or any of its derivatives rather than cAMP under physiological conditions. Several authors have described cAMP–adenosine binding proteins, also termed "adenosine analogue binding proteins", in mammalian tissues such as mouse liver (Ueland & Doskeland, 1977), rabbit erythrocytes (Yuh & Tao, 1974), and rat and bovine liver (Sugden & Corbin, 1976). These proteins are tetra- or pentameric with molecular masses in the range 180 000–240 000 daltons. They are distinct from regulatory subunits of cAMP-dependent protein kinases and share many common features with species C.

Previous studies by Veron & Patte (1978) have analyzed some characteristics of cAMP binding proteins in *D. discoideum* amoebas. Cytosolic extracts from vegetative cells contained a cAMP binding activity that was inhibited by 5′-AMP and eluted from a DEAE-cellulose column similarly to species C. A recent report (Leichtling et al., 1980) seems to describe an analogous protein. In contrast, the activity described by Veron & Patte (1978) from differentiated cells resembles species B. Rahmsdorf & Gerisch (1978) have also reported on soluble cAMP binding proteins in vegetative and aggregation competent *D. discoideum* cells. They were of two types: one had a molecular mass of 160 000 daltons, and the other exhibited two peaks corresponding to 40 000 and 180 000 daltons upon gel filtration. In all cases, cAMP binding activity was inhibited by cGMP and not by 5′-AMP. Other authors have analyzed cAMP binding activities by covalent photoaffinity labeling with 8-azido-[^{32}P]cAMP and subsequent electrophoresis in polyacrylamide gels in the presence of $NaDodSO_4$. Wallace & Frazier (1979) reported the specific labeling in aggregation competent cells (strains A3 and M3) of a 36 000-dalton protein; cGMP was inhibitory whereas 5′-AMP was not. Cooper et al. (1980) have investigated other strains with a similar method. Two labeled bands of 38 000 and 39 000 daltons in strain V12/M2 and 39 000 and 42 000 daltons in strain NC4 appeared at the culmination stage. They had the same specificity of labeling as the proteins from strains A3 and M3. These results along with others (A. Tsang, personal communication) are compatible with our data on species A and B extracted from aggregation competent cells of strain AX2.

No function has yet been attributed to cAMP binding proteins in *D. discoideum*. The report of a cAMP-dependent protein kinase activity present during early differentiation (Sampson, 1977) has not been confirmed by others (Veron & Patte, 1978; Rahmsdorf & Gerisch, 1978). Yet this attractive possibility cannot be excluded since we could be dealing with an activity that only phosphorylates specific *D. discoideum* proteins. Although regulatory subunits of mammalian cAMP-dependent protein kinase types I and II have similar affinities for cAMP and specificity of ligand binding as species A and B, it is worth noting that dissociation of the bound nucleotide is much slower (Gilman, 1970). Moreover, the regulatory subunits have somewhat higher molecular weights (50 000 and 54 000 daltons, respectively, for types I and II).

Although elementary precautions were taken, we cannot exclude that we are dealing with proteolyzed products. Partial proteolysis, due to endogenous degradating activities, has been shown to occur in *D. discoideum* extracts and seems to affect a cAMP binding protein (S. Cooper, personal communication). We are currently investigating the possibility that such a phenomenon would promote the conversion of species B to species A.

Acknowledgments

We are indebted to Dr. Philippe Brachet for stimulating discussions and for providing working facilities and to Professor Georges N. Cohen for his critical reading of the manuscript.

References

Bergmayer, H. U. (1955) *Biochem. Z. 327*, 255.
Blangy, D. (1970) *Biochimie 53*, 135–144.

Bonner, J. T. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 110–113.
Bonnicksen, R. K., & Brink, N. G. (1955) *Methods Enzymol. 1*, 495–500.
Cooper, S., Chambers, D. A., & Scanlon, S. (1980) *Biochim. Biophys. Acta 629*, 235–242.
Darmon, M., & Klein, C. (1978) *Dev. Biol. 63*, 377–389.
Darmon, M., Brachet, P., & Pereira da Silva, L. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3163–3166.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.
Dicou, E. L., & Brachet, P. (1979) *Biochim. Biophys. Acta 548*, 232–242.
Gerisch, G., & Wick, U. (1975) *Biochem. Biophys. Res. Commun. 65*, 364–370.
Gerisch, G., Fromm, H., Huesgen, A., & Wick, U. (1975) *Nature (London) 255*, 547–549.
Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 305–312.
Green, A. A., & Newell, P. C. (1975) *Cell (Cambridge, Mass.) 6*, 129–136.
Kay, K. K., Garrod, D., & Tilly, R. (1978) *Nature (London) 271*, 58–60.
Konijn, T. M., Van de Meene, J. G. C., Bonner, J. T., & Barkley, D. S. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1152–1154.
Kornberg, A. (1955) *Methods Enzymol. 1*, 441–444.
Landfear, S. M., & Lodish, H. F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1044–1048.
Leichtling, B. H., Tihon, C., Spitz, E., & Rickenberg, H. V. (1980) *Dev. Biol. 82*, 150–157.
Loomis, W. F. (1975) in *Dictyostelium discoideum, A Developmental System*, Academic Press, New York.
Malchow, D., & Gerisch, G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2423–2427.
Malkinson, A. M., Kwasniak, J., & Ashworth, J. M. (1973) *Biochem. J. 133*, 601–603.
Mato, J. M., Krens, F. A., Van Haastert, P. J., & Konijn, T. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2348–2351.
Rahmsdorf, H. J., & Gerisch, G. (1978) *Cell Differ. 7*, 249–257.
Robertson, A., Drage, D. J., & Cohen, M. H. (1972) *Science (Washington, D.C.) 175*, 333–343.
Roos, W., Nanjundiah, V., Malchow, D., & Gerisch, G. (1975) *FEBS Lett. 53*, 139–142.
Roos, W., Scheidegger, C., & Gerisch, G. (1977) *Nature (London) 266*, 259–261.
Sampson, J. (1977) *Cell (Cambridge, Mass.) 11*, 173–180.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Shaffer, B. M. (1957) *Am. Nat. 91*, 19–35.
Shaffer, B. M. (1975) *Nature (London) 255*, 549–552.
Sugden, P. H., & Corbin, J. D. (1976) *Biochem. J. 159*, 423–437.
Town, C., & Stanford, E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 308–312.
Town, C., Gross, J., & Kay, R. R. (1976) *Nature (London) 262*, 717–719.
Ueland, P. M., & Doskeland, S. O. (1977) *J. Biol. Chem. 252*, 677–686.
Veron, M., & Patte, J. C. (1978) *Dev. Biol. 63*, 370–376.
Wallace, L. J., & Frazier, W. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4250–4254.
Watts, D. J., & Ashworth, J. M. (1970) *Biochem. J. 119*, 171–174.
Williams, J. G., Tsang, A. S., & Mahbubani, H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7171–7175.
Wurster, B., Schubiger, K., Wick, U., & Gerisch, G. (1977) *FEBS Lett. 76*, 141–144.
Yuh, K. C., & Tao, M. (1974) *Biochemistry 13*, 5220–5226.