FLUCTUATIONS IN CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE AND CYCLIC GUANOSINE 3':5'-MONOPHOSPHATE DURING THE MITOTIC CYCLE OF THE ACELLULAR SLIME MOULD *PHYSARUM POLYCEPHALUM*

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SUMMARY Cyclic adenosine 3':5'-monophosphate (cyclic AMP) and cyclic guanosine 3':5'-monophosphate (cyclic GMP) have been determined at half-hourly intervals throughout the mitotic cycle of *Physarum polycephalum*. Cyclic AMP was constant at 1pmole/mg protein throughout except for a transient peak of 17pmoles/mg protein in the last quarter of G2. Cyclic GMP was more variable (2-4pmole/mg protein) rising to 9.5pmole/mg protein during the 3 hour S period and to 7pmole/mg protein during the last hour of G2. The significance of these changes is discussed.

INTRODUCTION Variations in the intracellular levels of cyclic AMP during the cell cycle of animal and human cells *in vitro* have been described (1-3) and reviewed (4-6). These changes reflect the regulatory function of cyclic AMP on cell growth (6) and morphology (7,8) and, more specifically, on several processes with distinct temporal patterns of activity in synchronously dividing cell populations. Recent reports suggest an equally important role for cyclic GMP in the cell cycle (9,10). One of the important functions of cyclic AMP and cyclic GMP is the activation (or inhibition) of protein kinases and thus the control of phosphorylation of F1 histone (11-13), nuclear acidic (14), membrane (15,16), microtubule (17-19) and ribosomal proteins (20). The natural mitotic synchrony of plasmodia of the acellular slime mould *Physarum polycephalum* provide excellent material for studies of the mitotic cycle.

MATERIALS AND METHODS (a) Culture of Physarum. Strain M_3c was a kind gift of Dr. Joyce Mohberg. Microplasmodia were grown in a partially defined medium with hemin (21). Surface plasmodia were established by inoculating a washed suspension of microplasmodia on to the centre of a filter paper disk supported on a stainless steel mesh in a 9cm petri dish. After 70 minutes the microplasmodia had fused and medium was added to wet the paper from below. The first post-fusion mitosis (M1) occurred $4\frac{3}{4}$ hours after feeding, the second (M2) 8 hours later and the third (M3) $8\frac{1}{4}$ hours after the second. Half-an-hour before M2 the medium was discarded and cultures refed with fresh medium. The time of metaphase was determined by phase contrast observation of ethanol fixed smears of small pieces of plasmodia mounted in glycerol-ethanol (1:1 v/v). Measurement of cyclic AMP and cyclic GMP was made on a single batch of cultures; determination of the duration of S period was made on a similar batch on another occasion.

(b) Extraction of cyclic AMP and cyclic GMP. Samples were taken at thirty minute intervals from one hour before M2 to one hour after M3. Four plasmodia on filter paper were frozen for five minutes in isopentane-methylcyclohexane (92:8 v/v) at the temperature of liquid nitrogen. The filter papers were withdrawn and the inoculum discarded. The remaining material was scraped off and plunged in 10ml ice-cold 5% (w/v) trichloroacetic acid (TCA) and mechanically homogenised at 0°C for 30 seconds using an ILA x-1020 mixer. 50nCi (^{3}H)cyclic AMP was added to all samples in order to monitor recovery and allowed for in the subsequent assay. To one sample only 8nCi(^{3}H) cyclic GMP was added for the same reason. The extracts were centrifuged at 17,000g for 10 minutes at 4°C and the supernatant carefully removed. The pellet was allowed to dissolve in 4ml 0.4N NaOH at room temperature for 48 hours. Protein was determined by the method of Lowry *et al.* (22) using bovine serum albumin as standard. TCA was removed from the supernatant by five extractions with an equal volume of water saturated diethyl ether and the ether layer discarded. Polysaccharides were removed by precipitation with 10ml ice-cold 95% (v/v) ethanol followed by centrifugation at 2500g for 5 minutes at room temperature. The supernatant was removed and taken to dryness in a rotary evaporator. The residue was dissolved in 1ml 50% (v/v) ethanol and loaded on to a 1x2.5cm column of Dowex AG50 X4 100-200 mesh resin ammonium form.

The tube containing the extract was carefully washed out with another 1ml 50% ethanol and this added to the column. After the sample had drained into the resin it was washed with 10ml sterile distilled water and the eluate allowed to drip into a 1.5x2cm column of QAE Sephadex A25 formate form. This column was washed with 20ml sterile distilled water and the eluate discarded. Cyclic AMP was eluted with 18ml 0.1N ammonium formate pH 9.0. Cyclic GMP was then eluted from the column with 18ml 0.1N ammonium formate pH 6.0. Each fraction was collected on a separate 1.5x5cm column of Dowex AG50 X4 200-400 mesh H^+ form. Cyclic AMP (or cyclic GMP) was eluted with 12ml 0.1N HCl. The eluate was lyophilized and stored at -20°C until assayed.

(c) Assay of cyclic AMP and cyclic GMP. Cyclic AMP was measured using a binding protein obtained from beef adrenal glands (23). Zero dose binding was 27% and the assay linear

over the range 0.3 to 8pmole cAMP per assay tube. Cyclic AMP in each extract was also measured using a commercially available kit; zero dose binding was 57% and the assay linear over the range 0.25 to 16pmole cAMP per tube. With both methods the standard curve was fitted by the method of least squares, r=0.996 and r=0.994 for the adrenal protein and kit assay respectively.
The extract was taken up in 200µl assay buffer (4mM EDTA 0.05M Tris-HCl pH 7.5) and two 50µl aliquots removed. From each 5µl was removed in order to monitor recovery. 100µl buffer was added to the remaining extract and again two 50µl aliquots removed for assay. This was repeated until four dilutions of each sample were obtained.
For the assay of cyclic GMP the extract was taken up in 400µl assay buffer and two 100µl portions removed for assay. 200µl buffer was added and again two 100µl portions removed. Cyclic GMP was measured using a commercially available kit. Zero dose binding was 37%. The assay was linear over the range 0.5 to 8pmole cGMP per assay tube; the standard curve was fitted as described above (r=0.993).
Radioactive samples were added to 8ml scintillation fluid (Sulphur-free toluene 1000ml, Triton X-100 500ml, PPO 4.4g, POPOP 0.1g) in plastic vials to which 0.8ml distilled water was then added. Radioactivity was measured to better than 2% SD in a Packard Tri-Carb Model C2425 scintillation spectrometer at 30.5% efficiency.
(d) Determination of S period. This was with the following minor modifications based on the method of Braun and Wili (24). A single synchronous plasmodium was transferred to a medium containing 5µCi (^{3}H)thymidine/ml at intervals of 20 minutes. After 15 minutes the plasmodium was plunged in 30ml ice-cold TCA-acetone (TCA 4g, acetone 50ml, distilled water 50ml). After mechanical homogenisation duplicate 5ml samples were filtered through 2.5cm glass-fibre disks GF/C(Whatman) washed twice with 10ml TCA-acetone and finally with 10ml ethanol. After drying each disk was placed in 10ml scintillation fluid (Sulphur-free toluene 100ml, PPO 4g, POPOP 50mg) contained in a plastic vial and radioactivity determined as above.
(e) Chemicals. Cyclic AMP assay kit (TRK 432), cyclic GMP assay kit (TRK 500), (^{3}H) cyclic AMP (27.5Ci/mmol), (^{3}H) cyclic GMP (19Ci/mmol) and (^{3}H) thymidine (5Ci/mmol) were purchased from the Radiochemical Centre, Amersham, U.K. Bovine adrenal cyclic AMP binding protein was purchased from B.D.H., Poole, Dorset, U.K. Other chemicals were of reagent grade and obtained from B.D.H. or Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K.

RESULTS AND DISCUSSION Recovery of cyclic GMP was 56%, that of cyclic AMP 75% to 79% (mean 78% SD 0.87). The results in Table 1 demonstrate the good agreement between results obtained with the two methods of analysis. Purity of the extracts and absence of interfering compounds is shown in Table 2 by the agreement between dilution and cyclic nucleotide content.

Table 1. Determination of cyclic AMP by two Methods

Sample Time (hr) M2=O	Beef Adrenal Binding protein assay (pmole)		Commercial Kit assay (pmole)	
-1	20.4	20.3	21.1	19.8
-½	9.5	9.1	10.0	8.8
+7	73.6	77.1	77.8	72.1
+7½	61.0	61.5	61.9	60.4

Duplicate samples not corrected for recovery.

Table 2. Effect of Dilution on Cyclic Nucleotide Content

Dilution	Cyclic AMP* (pmole)	Cyclic GMP (pmole)
1/1	61.3	1.9
1/2	34.1	0.9
1/4	16.5	-
1/8	8.0	-

Each value an average of two determinations on +7½ hr. sample not corrected for recovery.

*beef adrenal binding protein assay.

Figure 1 shows that during mitosis, the S period and the first three-quarters of G2 cyclic AMP remained constant at less than 1 pmole cyclic AMP per mg protein. Within the last quarter of G2 cyclic AMP rose rapidly to 17pmole per mg protein to return to a basal level by mitosis 3. Two samples taken before M2 showed a decrease from a high level; this suggests that a similar peak occurred before M2. Two clear peaks of cyclic GMP were detected. The duration of the larger coincided with

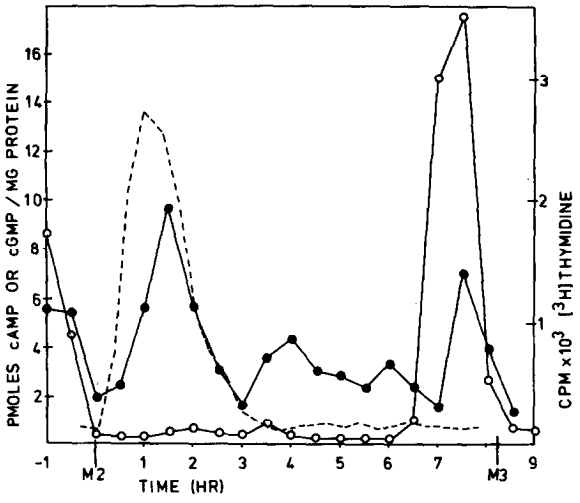

FIGURE 1. Cyclic AMP (open circles) and cyclic GMP (solid circles) content of synchronous plasmodia of the slime mould *Physarum polycephalum* from 1 hour before M2 to 1 hour after M3 (M2 = 0 hr.). Uptake of tritiated thymidine (dotted line) by synchronous plasmodia (See methods). Duration of S period 3 hours, G2 period 5 1/4 hours.

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the S period rising to 9pmole cyclic GMP per mg protein at the time of maximum incorporation of labelled thymidine. The smaller peak (7pmole cyclic GMP per mg protein) coincided with that of cyclic AMP late in G2 but showed a thirty minute lag in its onset. Again results for the hour before M3 parallel those for the same period before M2.

Bradbury *et al.* (11) have shown nuclear histone phosphorylating activity in *Physarum polycephalum* increases from a minimum at metaphase to a maximum late in G2. The peak of cyclic AMP and cyclic GMP is at a time when F1 histone phosphorylation is maximal and probably betrays the involvement of these cyclic nucleotides in the regulation of distinct protein kinases.

The time of mitosis in a plasmodium early in G2 can be accelerated by the application of homogenised material; that from a plasmodium late in G2 is more effective than that from a plasmodium early in G2 (25). This may result from the

addition in the homogenate of cyclic AMP and cyclic GMP and (or) enzymes capable of their net synthesis. This hypothesis may also explain why, when halves of two plasmodia each in a different phase of the mitotic cycle are fused, all the nuclei divide synchronously at a time intermediate between the time of mitosis in the unfused halves (26). Clearly the regulation of cyclic AMP and cyclic GMP will depend not only on the kinetics of the regulatory systems and their distribution within the cell but also on how they interact when two plasmodia are fused.

Recently cyclic GMP has been implicated in the initiation of cell proliferation (9,10). In Physarum the peak of cyclic GMP during the S period suggests that it is tied in some way to DNA synthesis. One way in which it might act is to stimulate DNA dependent RNA polymerase and RNA synthesis as has been shown to occur in lymphocytes (10,27). The RNA so formed could act as a primer of DNA synthesis. Evidence for this in Physarum is provided by the observation that pulse labelled DNA is attached to RNA (28). The role of cyclic GMP during the S period is likely to be quite distinct from the part it plays late in G2.

Some of these problems mentioned above are under further investigation in our laboratory.

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REFERENCES

1 Zeilig,C.E., Johnson,R.A., Friedman, D.L. and Sutherland,E.W. (1972) *J.Cell Bio., 55,* 296a.
2 Sheppard,J.R. and Prescott,D.M.(1972) *Exp.Cell Res., 75,* 293-296.
3 Millis,A.J.T., Forrest,G. and Pious,D.A.(1973) *Biochem. Biophys. Res.Commun., 49,* 1645-1649.
4 Abell, C.W. and Monahan, T.M.(1973) *J.Cell Biol., 59,* 549-558.

5 Ryan, W.L. and Heidrick,M.L.(1974) *Adv.Cyclic Nucleotide Res.*(Greengard,P. and Robinson, G.A.,eds.) *4*, 81-116.
6 Pastan,I.H., Johnson,G.S. and Anderson,W.B.(1975)*Ann. Rev. Biochem.*,*44*,491-522.
7 Korinek,J. Spelsberg,T.C. and Mitchell,W.M.(1973) *Nature*, *246*, 455-458.
8 Ortiz,J.R., Yamada,T. and Hsie,A.W.(1973) *Proc.Nat.Acad. Sci.U.S.A.*,*70*, 2286-2290.
9 Hadden,J.W., Hadden,E.M., Haddox,M.K. and Goldberg,N.D. (1972) *Proc.Nat.Acad.Sci.U.S.A.*,*69*,3024-3027.
10 Goldberg,N.D.,Haddox,M.K., Dunham,E.,Lopez,C. and Hadden,J.W. (1974) *Cold Spring Harbor Conf.Cell Proliferation* (Clarkson,B. and Baserga, R., eds.) *1*,609-625.
11 Bradbury,E.M.,Inglis,R.J. and Matthews,H.R.(1974) *Nature*, *246*, 455-458.
12 Comber,H.J. and Taylor,D.M.(1974) *Biochem.Soc.Trans.*, *2*, 74-76.
13 Bradbury,E.M., Langan,T. and Matthews,H.R.(1973) *Eur.J. Biochem.*, *33*,131-139.
14 Allfrey,V.G., Inous,A.,Kern,J., Johnson,E.M. and Vidali,G. (1974) *Cold Spr.Hrb.Symp.Quant.Biol.*,*38*,785-801.
15 Weller,M. and Rodnight,R. (1970) *Nature*,*225*, 187-188.
16 Krause,E.-G.,Will,H., Schirpke,B. and Wollenberger,A.(1975) *Adv.Cyclic Nucleotide Res.* (Drummond,G.I., Greengard,P. and Robinson,G.A.,eds.) *5*,473-490.
17 Gillespie,E.(1975) *Ann.N.Y.Acad.Sci.*,*253*,771-779.
18 Kram,R. and Tomkins,G.M.(1973) *Proc.Nat.Acad.Sci.U.S.A.*,*70*, 1659-1663.
19 Soifer,D.Laszlo,A., Mack,K., Scotto,J. and Siconolfi,L. (1975) *Ann.N.Y.Acad.Sci.*, *253*,598-610.
20 Jungman,,R.A., Lee,S-g. and DeAngelo,A.B.(1975) *Adv.Cyclic Nucleotide Res.* (Drummond,G.I., Greengard,P. and Robinson, G.A., eds.) *5*,281-360.
21 Daniel,J.W. and Baldwin,H.H.(1964) *Methods in Cell Physiology* (Prescott,D.M.,ed.) *1*,9-41.
22 Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J.Biol.Chem.*, *193*, 265-275.
23 Brown,B.L., Albano,D.M., Ekins, R.P., Sgherzi, A.M. and Tampion, W. (1971) *Biochem.J.*, *121*, 561-562.
24 Braun,R. and Wili,H.(1969) *Biochem.Biophys.Acta*, *174*, 246-252.
25 Oppenheim,A. and Katzir,N.(1971) *Exp.Cell Res.*, *68*, 224-226.
26 Rusch,H.P., Sachsenmaier,W., Behrens,K. and Gruter,V. (1966) *J.Cell Biol.*, *31*,204-209.
27 Johnson,L.D., Goldberg,N.D. and Hadden,J.W.(1974) *Adv. Cyclic Nucleotide Res.* Drummon,G.I., Greengard,P. and Robison,G.A., eds.) *5*, 821a.
28 Waqar, M.A. and Huberman,J.A.(1973) *Biochem.Biophys.Res. Commun.*, *51*, 174-180.