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Cyclic AMP, folic acid and pterin-mediated protein carboxymethylation in cellular slime molds

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In aggregative cells of *Dictyostelium discoideum*, extracellular cAMP induces transient methylation of a M_r 46000 protein. Starvation induces a 10–100-fold increase in the number of cAMP-receptors, but no change in the amount of the methyl accepting protein. In vegetative amoebae, a temporal increase of methylation of the protein is induced by stimulation with folic acid. Aggregative amoebae of *Dictyostelium lacteum* also contain a M_r 46000 protein, which is methylated after addition of the attractant monapterin. Therefore, protein carboxymethylation seems to be a general phenomenon during chemotaxis of the cellular slime molds.

Protein carboxymethylation *Chemotaxis* *Folic acid* *Monapterin* Dictyostelium discoideum Dictyostelium lacteum

1. INTRODUCTION

Starved amoebae of the cellular slime mold *Dictyostelium discoideum* respond chemotactically to cyclic AMP [1], which is detected by cell surface receptors [2–5] and induces cell aggregation. Addition of cAMP to aggregative cells causes a rapid increase of methylation of a protein with apparent M_r 46000, methylation being maintained above the control level from 1–15 s [6]. It is not known whether this response occurs only upon signals mediating cell aggregation, or also during the vegetative stage as a component of the mechanism for food detection. Vegetative amoebae of *D. discoideum* are able to find their bacterial food by positive chemotaxis to folic acid [7], which is detected by specific receptors [8]. During development from the vegetative stage to the onset of cell aggregation, the number of cAMP-binding sites on the cell surface of *D. discoideum* shows a 10–100-fold increase [2–5]. Chemotactic sensitivity to cAMP is acquired during the same period, while chemotaxis to folic acid disappears [9].

The cellular slime mold *Dictyostelium lacteum* is a relatively well-studied species in which cell aggregation is not mediated by cAMP, but by a yet unknown pterin derivative [10]. Monapterin is a commercially available compound with equipotent chemotactic activity as the natural attractant [10]. Monapterin has been reported to induce a similar transient increase in intracellular cGMP of *D. lacteum* as is observed in *D. discoideum* upon stimulation with cAMP [11].

Here, I show that there is no developmental increase in the amount of the M_r 46000 methyl acceptor of *D. discoideum*. Vegetative amoebae contain a similar protein as aggregative cells, and during the vegetative stage its methylation is sensitive to the level of the attractant folic acid. Aggregative cells of *D. lacteum* contain a similar methyl acceptor as *D. discoideum*, which is transiently methylated upon addition of the acrasin analogue monapterin. Therefore, protein carboxymethylation seems to be a general phenomenon in slime mold chemotaxis, like cGMP-accumulation [11,12].

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2. MATERIALS AND METHODS

2.1. *Materials*

L-[*methyl*-^{3}H]Methionine (3.15 TBq/mmol) was obtained from Amersham International. cAMP was from Boehringer Mannheim. Folic acid and monapterin were purchased from Fluka AG.

2.2. *Organisms*

Dictyostelium discoideum NC-4(H) was grown on a solid medium (3.3 g peptone, 3.3 g glucose, 4.5 g KH_2PO_4, 1.5 g $Na_2HPO_4.H_2O$ and 15 g agar/l) in association with *Escherichia coli* '281' and harvested as in [13]. After the cells had been harvested, they were starved for different periods of time by shaking in 10 mM Na_2HPO_4–KH_2PO_4-buffer (pH 6.5) at a density of 10^7/ml and a temperature of 20–22°C. *Dictyostelium lacteum* was grown on a solid medium (1.0 g peptone, 1.0 g lactose, 0.27 g KH_2PO_4, 0.72 g $Na_2HPO_4.12H_2O$ and 15 g agar/l) in association with *Escherichia coli* '281'. Cells were harvested as in [13] and starved on buffered, non-nutrient agar plates at a density of 2.5×10^6/cm^2 for 6 h at 20°C.

2.3. *Methylation*

Methylation assays were performed as in [6,14,15]. Briefly, cells were conditioned at a density of 10^8/ml in siliconized glass flasks which were continually shaken (130 rev./min). After 45 min of preincubation with 250 μg cycloheximide/ml, [^{3}H-*methyl*]methionine was added at a spec. act. of 2 μCi/ml. After 30 min, 100-μl samples of labeled cells were stimulated with a 10^{-6} M cAMP, 10^{-5} M folic acid or 10^{-5} M monapterin pulse as indicated in section 3. The reaction was stopped by addition of 0.1 vol. 70% $HClO_4$. Electrophoresis of proteins was performed as in [16].

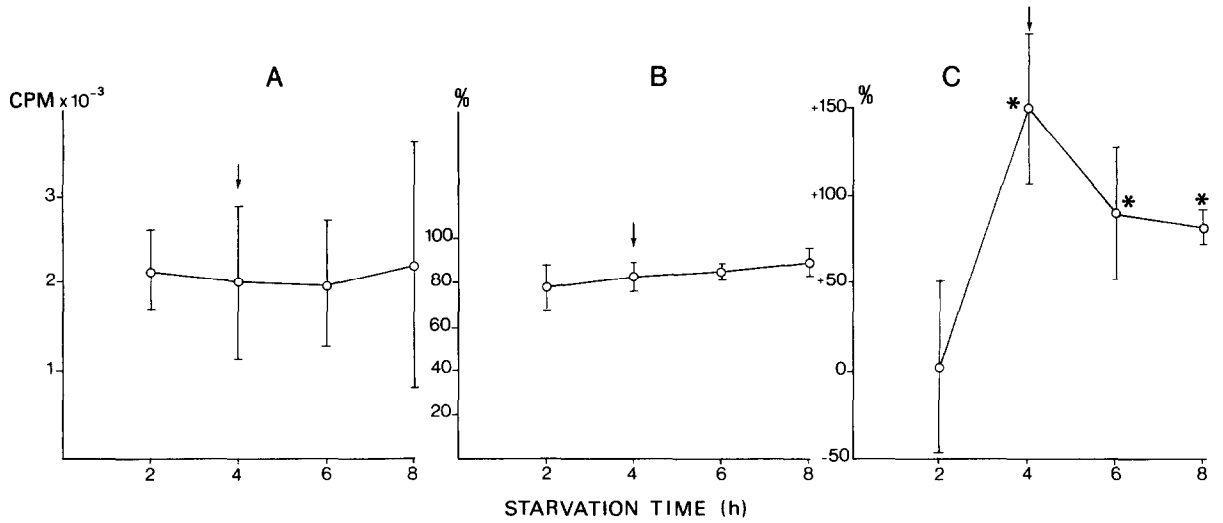

Fig.1. Changes in the electrophoretic pattern of *D. discoideum* homogenates during starvation. (A) Total incorporation of radioactivity from [^{3}H-*methyl*]methionine into the M_r 46000 region (cpm). Values are means ± SD of 5–8 independent experiments. (B) Percentage of total protein radioactivity present in the M_r 46000 region. Values are means ± SD of 5–8 independent experiments. (C) Response of the methylation state of the M_r 46000 protein upon stimulation with 10^{-6} M cAMP, expressed as the observed maximal percentual increase with respect to an unstimulated control. Values are the means ± SD of 4 independent experiments. Differences between the unstimulated control and experimental values were tested by Wilcoxon's Q-test; significant differences (at the 5% level) are indicated by asterisks. The onset of cell aggregation (as shown by the formation of cell clumps in the suspension and the acquirement of 'stickiness' by the cells to glass surface) is indicated by an arrow. The R_f-value of the major methyl acceptor, as established in all experiments, is 0.44 ± 0.06 (n = 16).

3. RESULTS

As shown in fig.1, no significant changes can be observed in the methyl accepting protein pattern of unstimulated amoebae of *D. discoideum* during development. Both vegetative and aggregative cells contain a M_r 46000 protein, and neither the absolute amount (total incorporation of radioactive methyl groups in the M_r 46000 region) nor its relative abundance (incorporation of methyl groups in the M_r 46000 region/incorporation of methyl groups in total protein) is altered by starvation (fig.1A,B). Results obtained in this study differ somewhat from those reported previously [6]. In my first article, 50% of all methyl groups were incorporated into the M_r 46000 protein, the remainder being distributed over 3 other methyl accepting compounds. Under the present conditions, however, 80–95% of all methyl groups are incorporated in the M_r 46000 protein, which now shows a triphasic and no longer a monophasic response on stimulation with cAMP. The 3 other methyl accepting compounds have very low activity and are usually only visible after stimulus administration. These differences have arisen since we started to cultivate our amoebae on a different strain of *E. coli*. Because we no longer possess the former strain, it is not possible to make a direct comparison between the former and present growth conditions.

Although changes in the basal methylation level during development are lacking, the transient effect of cAMP on methylation shows a strong and significant increase. Sensitivity of methylation to cAMP is low in vegetative amoebae, but it rises 30-fold during starvation, reaching peak levels at the onset of aggregation, which in our hands occurred after 4 h of shaking in suspension (fig.1C).

In vegetative amoebae, methylation of the protein responds to stimulation with folic acid.

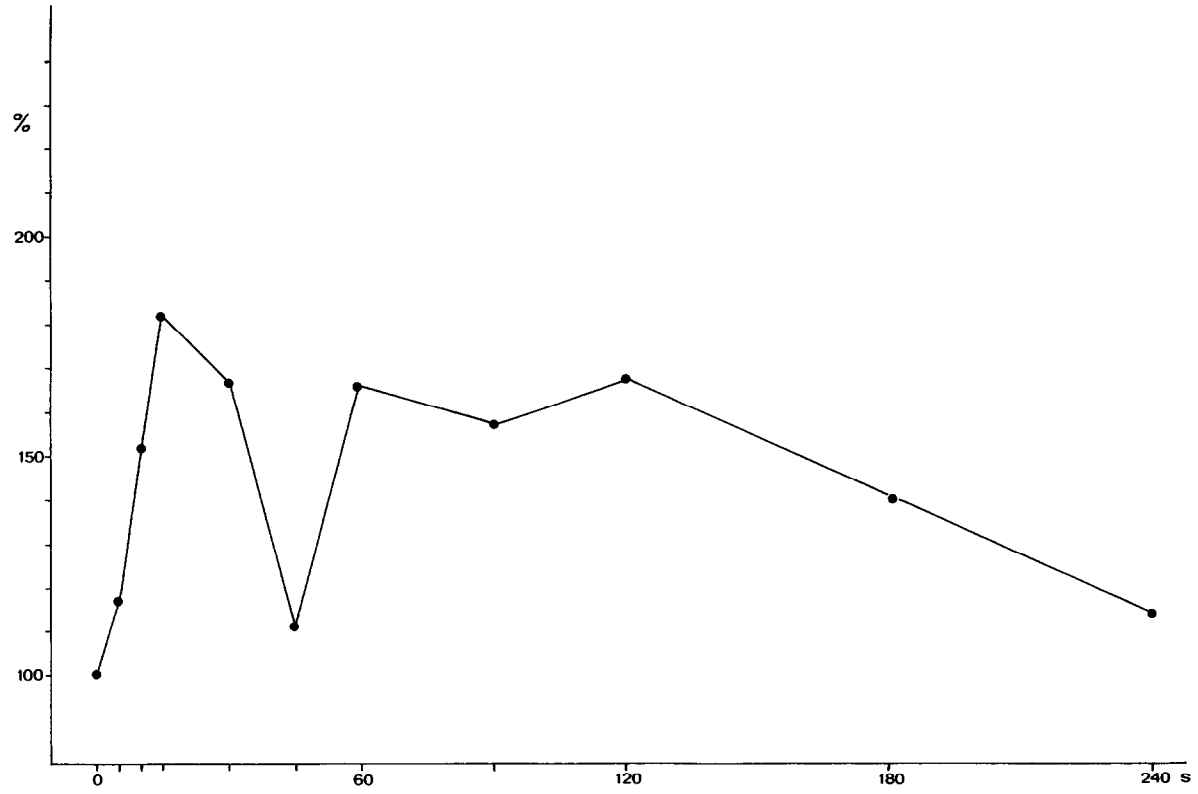

Fig.2. Time course of protein carboxymethylation in vegetative cells of *D. discoideum* upon stimulation with 10^{-5} M folic acid. Each experimental point is a mean of 4 independent observations on total protein radioactivity. Levels are presented as percentages of an unstimulated control.

10^{-5} M folic acid induces a rapid increase of carboxymethylation, maxima being observed 15 and 60–120 s after stimulus administration (fig.2).

The electrophoretic pattern of homogenates of *D. lacteum* is quite similar to that of *D. discoideum* (fig.3). Both species contain a M_r 46000 protein as the major methyl acceptor. When aggregative cells of *D. lacteum* are stimulated with a 10^{-5} M pulse of their attractant monapterin, a significant increase of carboxymethylation is observed with maxima occurring 10 and 90 s after stimulus administration (fig.4). Under identical conditions, aggregative cells of *D. discoideum* react to a 10^{-6} M cAMP-pulse with maxima after 15, 60 and 180 s (fig.5).

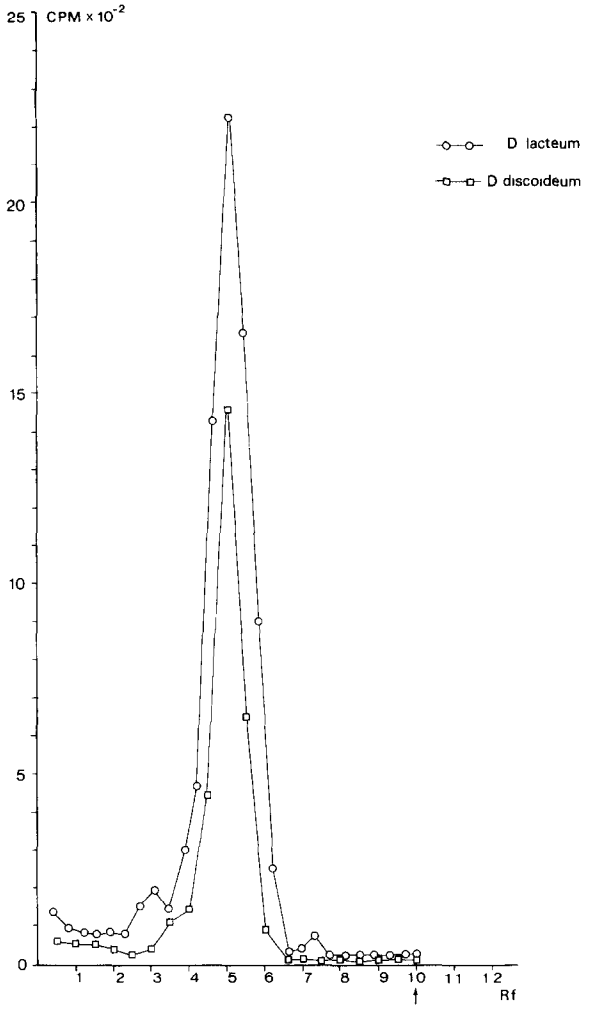

Fig.3. Electrophoretic pattern of homogenates of unstimulated amoebae of *D. discoideum* and *D. lacteum*. The relative mobility of the marker, cytochrome *c*, is taken as 1.0. Similar patterns are observed in homogenates of aggregative amoebae of *D. discoideum* upon stimulation with cAMP, in vegetative amoebae of *D. discoideum* with and without stimulation with folic acid, and in aggregative amoebae of *D. lacteum* after stimulation with monapterin (values not shown, only peak heights are increased by stimulation).

4. DISCUSSION

Vegetative and aggregative amoebae of *D. discoideum* contain about the same amount of a M_r 46000 methyl accepting protein (fig.1A,B). The 10–100-fold increase in the number of cAMP-receptors, which is known to occur during development [2–5] and is accompanied by a corresponding increase of chemotactic sensitivity [9], is reflected by an increasing effect of cAMP on protein methylation. Sensitivity of methylation to cAMP is low in vegetative cells, but it reaches peak levels at the onset of aggregation (fig.1C). The relatively simple slime mold *D. lacteum* contains a similar M_r 46000 methyl accepting protein as *D. discoideum* (fig.3). In all cell types studied thus far, the methylation state of this compound is sensitive to addition of the appropriate chemoattractant (folic acid, cAMP, or monapterin, fig.2,4,5).

These results can be interpreted in at least two ways. Firstly, the methylated proteins could be the chemotactic receptors for the respective chemoattractants. An analogy would then be known to exist in the membrane-bound acetylcholine receptor, which is carboxymethylated to be kept in its 'active state' [17,18]. Another analogy is provided by the chemotactic aspartate receptor of the bacterium *Salmonella typhimurium*, which is methylated at a specific locus (different from the attractant-binding site) as a mechanism for adaptation to continuous stimuli [19].

During development of *D. discoideum*, changes in the amount of the M_r 46000 protein would not be observed because the decline of folic acid receptors is balanced by an increase in the number of binding sites for cAMP. All apparent M_r's of the receptors would be in the 42000–48000 range, making them indistinguishable in the present electrophoretic system. Only when these conditions are

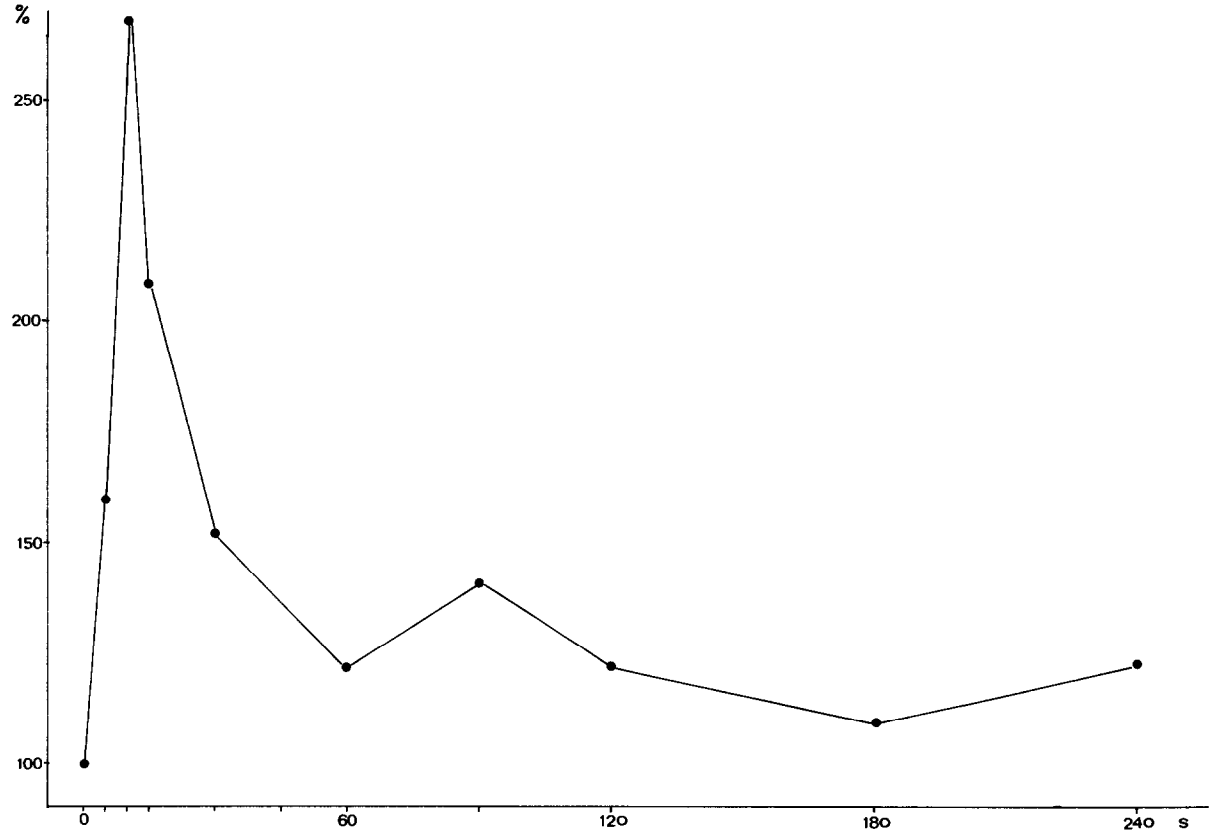

Fig.4. Time course of protein methylation in aggregative amoebae of *D. lacteum* upon stimulation with 10^{-5} M monapterin. Each experimental point is a mean of 2 independent observations on total protein radioactivity. Levels are presented as percentages of an unstimulated control.

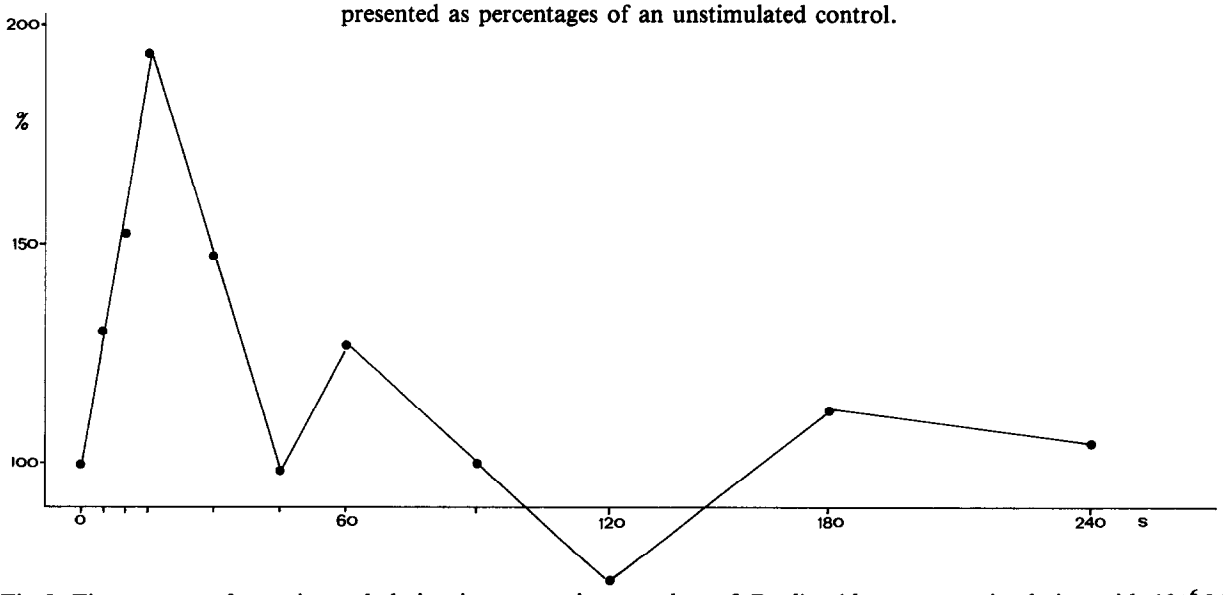

Fig.5. Time course of protein methylation in aggregative amoebae of *D. discoideum* upon stimulation with 10^{-6} M cAMP. Each experimental point is a mean of 2 independent observations on total protein radioactivity. Levels are presented as percentages of an unstimulated control.

met, can the hypothesis of receptor methylation explain the available data.

Secondly, the methyl acceptor could be a transducer protein located distally from the chemotactic receptor in the chemosensory transduction chain. This transducer protein would be similar in vegetative and aggregative amoebae of *D. discoideum* and *D. lacteum*. Transduction of all chemotactic signals would be accompanied by methylation of this protein, and coupling of the protein to the cAMP-receptor would increase in *D. discoideum* during development.

At present, it is not possible to choose between these two alternative interpretations of the available data. It can be positively stated, however, that protein carboxymethylation is a general phenomenon during slime mold chemotaxis, like the transient accumulation of cGMP [11,12].

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REFERENCES

[1] Konijn, T.M., Van de Meene, J.G.C., Bonner, J.T. and Barkley, D.S. (1967) Proc. Natl. Acad. Sci. USA 58, 1152–1154.
[2] Malchow, D. and Gerisch, G. (1974) Proc. Natl. Acad. Sci. USA 71, 2423–2427.
[3] Green, A.A. and Newell, P.C. (1975) Cell 6, 129–136.
[4] Henderson, E.J. (1975) J. Biol. Chem. 250, 4730–4736.
[5] Mato, J.M. and Konijn, T.M. (1975) Biochim. Biophys. Acta 385, 173–179.
[6] Van Waarde, A. (1982) FEBS Lett. 149, 266–270.
[7] Pan, P., Hall, E.M. and Bonner, J.T. (1972) Nature New Biol. 237, 181–182.
[8] De Wit, R.J.W. (1982) FEBS Lett. 150, 445–448.
[9] Varnum, B. and Soll, D.R. (1981) Differentiation 18, 151–160.
[10] Van Haastert, P.J.M., De Wit, R.J.W., Grijpma, Y. and Konijn, T.M. (1982) Proc. Natl. Acad. Sci. USA 79, 6270–6274.
[11] Van Haastert, P.J.M., Van Lookeren Campagne, M.M. and Kesbeke, F. (1983) Biochim. Biophys. Acta 756, 67–71.
[12] Mato, J.M., Van Haastert, P.J.M., Krens, F.A., Rhijnsburger, E.H., Dobbe, F.C.P.M. and Konijn, T.M. (1977) FEBS Lett. 79, 331–336.
[13] Konijn, T.M. and Raper, K.B. (1961) Dev. Biol. 3, 725–756.
[14] Mato, J.M. and Marin-Cao, D. (1979) Proc. Natl. Acad. Sci. USA 76, 6106–6109.
[15] Van Waarde, A. and Van Haastert, P.J.M. (1983) submitted.
[16] Gagnon, C., Viveros, O.H., Diliberto, E.J. and Axelrod, J. (1978) J. Biol. Chem. 253, 3778–3781.
[17] Kloog, Y., Flynn, D., Hoffman, A.R. and Axelrod, J. (1980) Biochem. Biophys. Res. Commun. 97, 1474–1480.
[18] Flynn, D., Kloog, Y., Potter, L.T. and Axelrod, J. (1982) J. Biol. Chem. 257, 9513–9517.
[19] Russo, A.F. and Koshland, D.E. (1983) Science 220, 1016–1020.