FLUORESCENT PRODUCTS SECRETED BY *DICTYOSTELIUM DISCOÏDEUM* CELLS WHICH ARE ABLE TO AGGREGATE

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1. Introduction

As unicellular myxamoebae, which are easily triggered into an aggregation process and further differentiate into a multicellular organism, consisting of only 2 major cell types, the widely studied *Dictyostelium discoïdeum* [1] is a good candidate for becoming the '*E. coli*' of eukaryotes.

To find out the key to cell-to-cell communication during development is the goal of a great many approaches, but very few use dynamic physical techniques. We built up a fully automated set up, taking advantage of the fluorescence technique, to try to detect lipid–protein interactions and assess the importance of the so-called 'membrane fluidity' during aggregation. However, in a control experiment, performed to look at the contribution of secreted proteins to the tryptophan fluorescence of the whole cellular suspension, we did observe an extraneous unexpected fluorescence in the extracellular medium. It turned out that the *Dictyostelium discoïdeum* axenic strain AX-2 secretes some low M_r fluorescent compounds, starting early in starvation-induced development. Cells physiologically unable to aggregate do not secrete those compounds. Thus the mere observation of the fluorescence of the extracellular medium 4 h after the starvation shock gives a straightforward means of appreciating the cells ability to aggregate.

2. Experimental

The cells, axenic strain AX-2, are grown at 22°C, in HL5 suspension, and shaken at 175 rev./min. They are harvested in an exponential phase of growth, at 2–6 × 10^6 cells/ml as counted by an hemocytometer, then centrifuged at 700 × *g* for 2 min and washed twice in potassium phosphate buffer (17 mM, pH 6.2). Resuspension of the cells at 1–4 × 10^7/ml in the same buffer starts starvation-induced development. The amoebae, kept at 22°C, are shaken at 175 rev./min and at t_x (i.e., *x* hours after the start of starvation) the cells are separated from their extracellular medium by mild centrifugation (700 × *g*) for 2 min. Then, 0.6–0.8 ml of the supernatant is observed in a 10 × 2 mm fluorescence quartz cell. In these experiments, as we are only interested in relative changes, the given fluorescence spectra (emission or excitation) are uncorrected. In this wavelength range, the experimental correction shifts the emission ~10 nm towards the red. Parallel to the spectroscopic measurements, the aggregation and differentiation abilities of the cells are respectively assayed, by observing under phase-contrast and stereomicroscope the submerged monolayer cells and the development on filters.

3. Results

Fig.1a shows the appearance of an extraneous fluorescence around 440 nm in addition to the expected protein fluorescence peaking at 330 nm, when exciting a t_4 supernatant at 280 nm. The corresponding cells were taken in an exponential growth phase and had been starved for 4 h; they became later fully able to aggregate as shown by their acquisition of characteristic elongated shapes and further aggregation patterns [2]. In contrast, fig.1b points out

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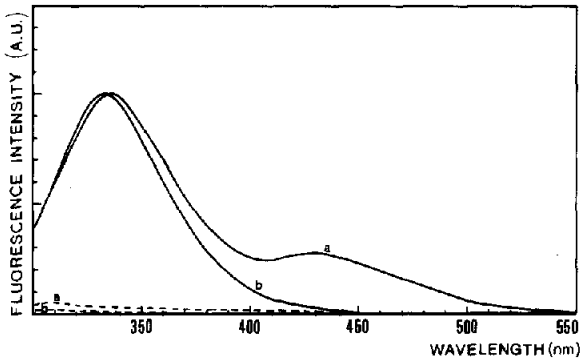

Fig.1. Aggregation correlated fluorescence. Cells taken from: (a) an exponential growth phase, *able* to aggregate; (b) a stationary growth phase, *unable* to aggregate. Fluorescence excited at 280 nm in the supernatants of *Dictyostelium* AX-2, starved 4 h at 10^7 cell/ml (a), or 2×10^7 cell/ml (b) in a potassium phosphate buffer, 17 mM, (pH 6.2); the spectra are normalized on the protein fluorescence with maximum intensity of 14.1 (a) and 35.0 (b) in arbitrary units. The dashed curves correspond to background for buffer alone.

the absence of such an emission from a t_4 supernatant originating from cells taken from a stationary growth phase, and also starved for 4 h; they kept their original rounded morphology and did not achieve any aggregation competence. The important point is that at the end of the interphase period, before the involvement of all the complex pulsatile cAMP machinery orchestrating the aggregation [2], the fate of the cell population can be predicted merely by observing the fluorescence of their extracellular medium excited at 280 nm. Fig.2 depicts the influence of starvation time from 0.3–22 h on the weight of this extraneous fluorescence in supernatants relative to their maximum normalized tryptophan emission. By using a higher excitation wavelength to get rid of the protein fluorescence, the extraneous fluorescence is better described as shown in fig.3. Furthermore, the observed red-shift between $t_{1.25}$ (fig.3a) and t_4 (fig.3b) reveals

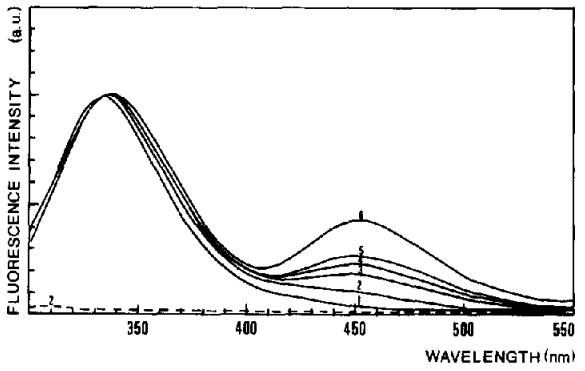

Fig.2. Influence of starvation time on the aggregation correlated fluorescence.

· Spectrum	1	2	3	4	5	6
· Starvation time (h)	0.3	1.25	4	5	6	22
· Maximum intensity of protein fluorescence	8.5	21	21.9	22.5	22.4	36.7

Fluorescence excited at 280 nm in the supernatants of AX-2, starved at 4×10^7 cells/ml.

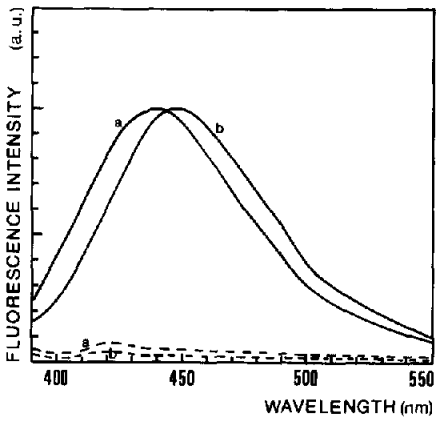

Fig.3. Aggregation correlated fluorescence excited at 370 nm in supernatants of AX-2 starved at 4×10^7 cells/ml for: (a) 1.25 h (maximum intensity 1.65); (b) 4 h (maximum intensity 3.47).

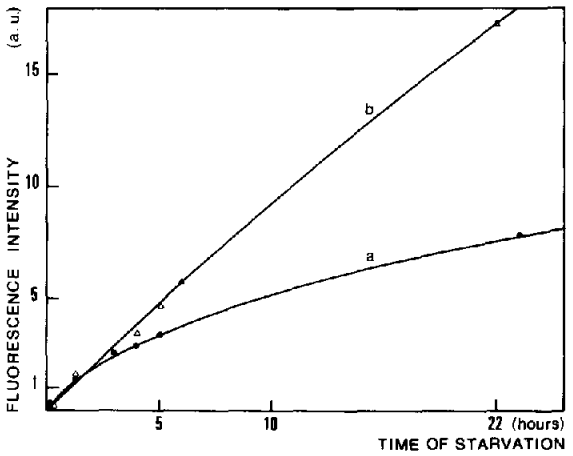

Fig.4. Influence of cell density in the appearance of the fluorescent secreted products in supernatants of AX-2 starved at: (a) 2×10^7 cells/ml; (b) 4×10^7 cells/ml.

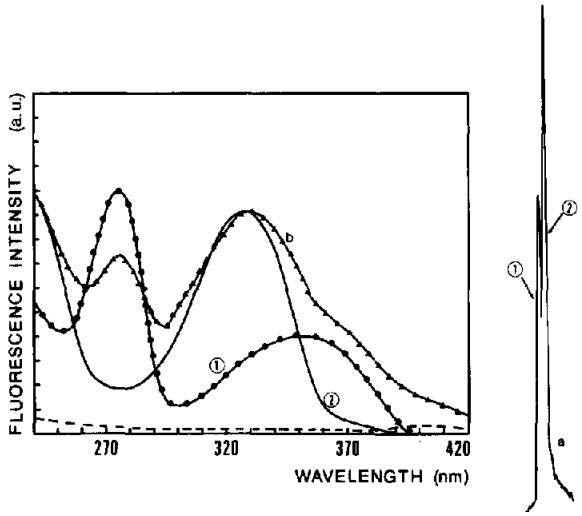

Fig.5. Preliminary isolation of 2 fluorescent emitters as checked by their fluorescence excitation spectra observed at 450 nm and normalized at their highest intensities given in brackets: (a) isolation of compounds (1) and (2) by HPLC; (b) excitation spectrum in the t_4 supernatant (10^7 cells/ml) before HPLC (5.23); (1) excitation spectrum of compound (1) after HPLC (0.90); (2) excitation spectrum of compound (2) after HPLC (3.34).

that at least 2 emitters are involved. By further increasing the starvation time up to 22 h a much smaller red shift occurs. No appreciable fluorescence was observed in the t_4 supernatant from the stationary cells. Fig.4 shows the kinetics of appearance of the fluorescent extracellular secreted compounds as a function of starvation time for 2 cellular densities: as expected their concentration depends on cellular density. Fig.5 gives the very first insight in identification of the compounds involved as fluorescent emitters. Both dialysis and fluorescence polarization measurement gave evidence that we are dealing with low M_r compounds. We did succeed in isolating them by reverse-phase, high-pressure liquid chromatography (HPLC) with fluorescence detection. They were eluted as 2 major compounds in a KH_2PO_4–methanol gradient, with ~20% CH_3OH (fig.5a). The fluorescence excitation spectrum at 450 nm of each HPLC fraction (fig.5 1,2) is compared to the one of the total t_4 supernatant (fig.5b) showing that both compounds, depicting quite different excitation spectra contribute to the fluorescence observed in supernatants of starving cells. Further purification and studies on those molecules are under investigation and will form the basis of forthcoming papers.

4. Discussion

Although the influence of low M_r factors on *Dictyostelium* development has been acknowledged since the mysterious 'acrasine' turned out to be cAMP [3], experimental evidence suggests that the popularity of cAMP may have eclipsed the influence of other low M_r factors, whose diffusibility is also in favour of their acting as intercellular 'communication' molecules.

Many such factors have been shown for *Dictyostelium*, as the effector of *N*-acetylglucosaminidase biosynthesis [4], the factor necessary for differentiation in submerged clumps [5], the DIF (differentiation inducing factor) [16], which now turns out to be essential for stalk cell differentiation [7], that AIF (aggregation inhibiting factor) [8], existing in growing cells and developmentally regulated and the STF (slug turning factor) [9,10], involved in phototaxis and thermotaxis. None of these factors has been identified up to now. In view of our finding, it would be worth checking for any common spectroscopic properties, which could help identification.

In a preliminary attempt at identification, we compared the fluorescence and the HPLC of supernatants with those of molecules possibly involved in cellular fluorescence emission, including tryptophan derivatives (kynurenin, kynurenic acid, *N*-acetyltryptophan, *N*-acetyltryptophanamide, tryptamine, tryptophol, melatonin), adrenergic derivatives (serotonin, L-epinephrine, tyramine), coenzyme derivatives (NADH, NADPH, FAD, riboflavine) and some folic acid derivatives (folic acid, pterin, *N*-5-methyltetrahydrofolic acid). None of these molecules, nor, obviously, cAMP which is non-fluorescent, were either of the 2 fluorescent molecules present in the supernatants after starvation. However, it turned out from preliminary results obtained by fluorescence, Raman resonance, HPLC and mass spectrometric studies that some folic acid derivatives, which remain to be identified, could be the best candidates, as will be argued in the future. Furthermore, we did find the same fluorescence in the cells and in cytosolic extracts of cells, with the important difference that, in the cells, the fluorescence emission is polarized, i.e., originates from bound emitters. We now work with the assumption that the amoebae, which need folic acid for growth [11], are metabolizing folic acid, derivatives of which remain fixed on some macromolecules or cellular structures until the starvation

shock, then they use these excreted derivatives as density dependent information.

This putative scheme could fit with other evidence [7]. Two enzymes acting on folic acid [1] were evidenced in the extracellular medium of *Dictyostelium* cells, a 2-folate deaminase [12] and a C_9–N_{10} splitting enzyme [13], supposed to inactivate folic acid as chemio-attractant. However, the 2-folate deaminase activity and location have been followed during development: it mimics cAMP phosphodiesterase in its regulatory function [14,15]. Folate receptors have also been shown on the cell surface [16]. Furthermore, amino and amethopterin [2] interact with the same folate receptors [16,17], but are not degraded by the 2-folate deaminase [17]. Folic acid, long considered as a mere chemio-attractant, is now suggested as being somewhat interchangeable with the other chemio-attractant cAMP, an important mediator of the aggregation competence, and both extracellular signals could share a common cellular pathway of signal transduction [18,19]. In addition, there is a need for a density-dependent 'first messenger' [7,20] acting during the first 3 h of starvation, contrary to the widely accepted view of cAMP being the first messenger. Based on ionic strength experiments, a 2 'timers' model has been advanced for the onset of aggregation [21], whereas on a theoretical basis, pattern formation, was explained [7] by the mere balance of 1 activator and 1 low M_r inhibitor.

Many puzzling points remain to be understood before controlling the differentiation of such a 'simple' eukaryote as *Dictyostelium* (e.g., the influence of cell density, ionic environment, ammonia and oxygen). Because of their sensitivity to pH, their oxido-reduction and metal-chelating properties and also their potency as methyl-group donors, pterin derivatives could well have a major influence on cellular differentiation. In view of their wide occurrence [22–24] and the recent evidence of different pterin secretions from normal and malignant transformed cells [23,25], we feel that this deserves further investigation. Quite unexpectedly, *Dictyostelium discoïdeum* may prove useful for that purpose, if our assumption that the secreted fluorescent products correlated with the aggregation ability are folic acid derivatives is right.

Acknowledgements

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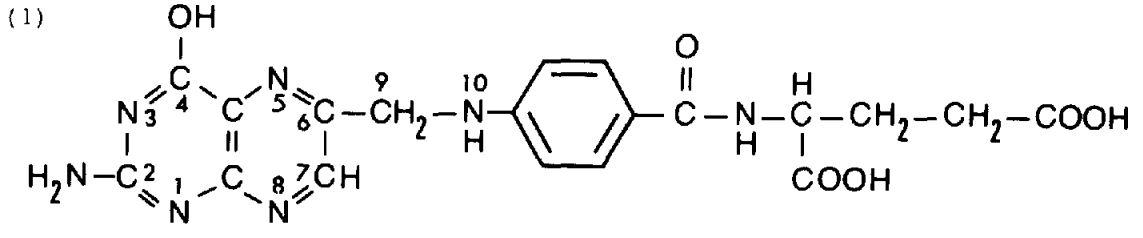
(1)



2-Amino-4-hydroxy-6-methylpterin — p-Aminobenzoic acid — Glutamic acid

Pteroic acid

Pteroylglutamic acid (folic acid)

(2) Aminopterin = 4-NH_2 instead of 4-OH folic acid.

Amethopterin = N 10 methylated aminopterin

Paris. We are also indebted to H. Strapelias for performing the HPLC measurements and to P. Janiaud for the gift of the model molecules used for comparison.

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Note added

M. Schweiger, Inst. für Biochemie, Universität Innsbrück, working in collaboration with G. Gerisch, Max-Planck Institüt für Biochemie, Martinsried, also showed such fluorescence in starved *Dictyostelium* supernatants and they agree with the experimental results presented here (personal communication at Tützing [7], with G. Gerisch and M. Schweiger).