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AN INHIBITOR OF CELL COHESION FROM AXENICALLY GROWN CELLS OF THE SLIME MOULD, *DICTYOSTELIUM DISCOIDEUM*

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SUMMARY

Medium from a stationary phase culture of axenically grown *D. discoideum* cells contains an inhibitor of cohesion of log phase cells. The inhibitor is a heat-stable, low molecular weight substance. Its biological effects include inhibition of cohesion of aggregation-competent cells, of cells of other slime mould species, the blocking of development on Millipore filters and a reduction in adhesiveness of slime mould cells to glass. Present evidence suggests that the inhibitor may bind to the cell surface.

INTRODUCTION

We have previously described a difference in properties between log and stationary phase populations of *D. discoideum* cells grown axenically (Swan & Garrod, 1975). Early evidence suggested that the medium from stationary phase cultures contains a substance(s) which inhibits the cohesion of normally cohesive log phase cells. In addition stationary phase medium has been shown by other workers to contain one or more substances which inhibit division (Yarger, Stults & Soll, 1974; Hanish, 1975) and transcription (Yarger & Soll, 1975). This paper describes some important biological effects of the cohesion inhibitor and our progress towards biochemical characterization of the molecule.

MATERIALS AND METHODS

Growth of slime mould cells

Axenic strain (Ax-2) cells of *D. discoideum* were grown in shaking culture (as described in Swan & Garrod, 1975) in axenic medium supplemented with 86 mM glucose (Watts & Ashworth, 1970). Log phase cells were harvested when at a density of 2–3 × 10^6 cells/ml; stationary phase cells were harvested when the cell count had remained constant (within 10 %) for 24 h (approx. 1–2 × 10^7 cells/ml). The cells were washed once in cold distilled water and twice in cold 17 mM phosphate buffer (pH 6·0) unless otherwise stated.

Agar plate cultures. Spores of the species *Polysphondylium violaceum*, *Dictyostelium purpureum*, *Dictyostelium mucoroides* and *Dictyostelium discoideum* (NC4) were inoculated on to nutrient agar plates (containing 10·0 g glucose; 10·0 g bacteriological peptone; 1·0 g yeast extract; 2·06 g $MgSO_4.7H_2O$; 1·5 g KH_2PO_4; 1·0 g K_2HPO_4; and 20 g agar per litre (Sussman, 1966)

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and grown at 22 °C in association with *Escherichia coli* B/r. Feeding stage cells were harvested by gently rubbing the agar surface with a glass rod in the presence of about 10 ml cold distilled water, and were washed by centrifugation in cold distilled water between 3 and 6 times until contaminating bacteria had been removed.

Development on Millipore filters

Log phase Ax-2 cells were harvested, washed and suspended in cold distilled water at 1×10^8 cells/ml. 0·5 ml of this suspension was pipetted on to each Millipore filter (black, average pore size 0·8 μm; diameter 4·7 cm) supported in a 55-mm plastic Petri dish on an absorbent cellulose pad saturated with 1·6 ml Millipore salt solution (containing 1·5 g KCl, 1·0 g $MgCl_2.6H_2O$, 0·5 g streptomycin sulphate, 0·3 g Na_2HPO_4, 1·2 g KH_2PO_4 in 1 litre; pH 6·1). To test the effect of inhibitor on development, freeze-dried inhibitory extract was dissolved in the Millipore salt solution to a concentration equivalent, per cell, to that in stationary phase medium, and the solution used to saturate the support pads in the usual way. Filters were incubated at 22 °C under humid conditions.

Cohesion assay

Washed cells were dissociated into single cells by repeated gentle pipetting through a fine-tipped Pasteur pipette in a small volume of cold distilled water, and suspended in a test solution at 1×10^6 cells/ml. Four-millilitre volumes of test suspension were shaken in 25-ml Erlenmeyer flasks on a New Brunswick G86 rotary shaker at 140 rev/min (22 °C, radius of rotation 0·7 cm) and sampled at intervals. Cohesion was assessed using a haemocytometer to determine the number of particles per ml. The cohesion of bacterially grown cells was assayed in the same way.

To assay chromatography column fractions, a Perspex titre plate drilled with a series of wells (3 mm deep and 7 mm in diameter) was used; 0·08 ml test solution and 0·02 ml cell suspension were added to each well, giving a final cell concentration of 1×10^6 per ml. The plate was placed on the rotary shaker for 20 min and then cohesion in each well assessed visually.

Isolation of inhibitor for tests of biological activity

It was found that inhibitory activity could be completely removed from stationary phase medium by dialysis against 4 changes of distilled water over 36 h at 4 °C. To obtain the low molecular weight fraction containing inhibitory activity 50 ml of stationary phase medium was dialysed against 500 ml of distilled water for 15 h at 4 °C. The dialysate containing the inhibitory activity was then reduced to 5 ml by evaporation under vacuum.

This concentrated dialysis product was then applied to a 100 × 2·6 cm column packed with Sephadex G50, using distilled water as eluant. Resulting fractions containing inhibitory activity were pooled, concentrated to 3 ml as described above and run through a 60 × 1·5 cm column packed with Sephadex G10 to reduce salt content and to remove other contaminating small molecules. Active fractions were again pooled and reduced in volume to give a concentration of inhibitory activity equivalent to that in the original stationary phase medium.

Molecular-weight estimation

The method was essentially that of Andrews (1964). A 40 × 2·6 cm column packed with Sephadex G50 was calibrated using a range of water soluble substances of differing molecular weights. These were: Blue dextran (mol. wt. 2000000), bovine serum albumin complexed with Evan's blue (60000), myoglobin (17000), cytochrome c (13000), vitamin B_{12} (1350), flavin monophosphate (514) and phenol red (354), eluted with 10 mM phosphate buffer, pH 7·0. The void volume, V_o, was determined using blue dextran. The elution volumes of the standards, $V_e - V_o$, were then plotted against the log molecular weight to give a calibration curve (Fig. 2, p. 110). Fifty millilitres of stationary phase medium (SPM) were dialysed for 18 h against distilled water, and the dialysate concentrated to 2 ml before being applied to the column. The fraction containing maximum activity when assayed was used to determine the V_e of the inhibitor. $V_e - V_o$ was then calculated and the molecular weight derived from the calibration curve.

RESULTS

Properties of the inhibitor

Dialysed stationary phase medium showed no inhibitory effect on the cohesion of log phase cells (Fig. 1). Inhibitory activity was recovered by dialysing the medium against one volume of distilled water overnight and evaporating the dialysate to the

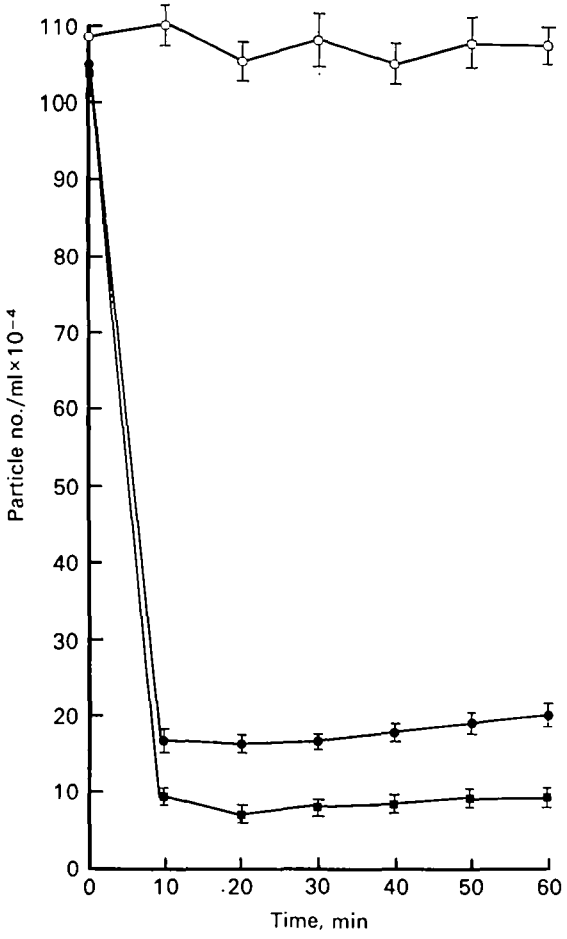

Fig. 1. Cohesion of washed Ax-2 log cells in 17 mM phosphate buffer (■), stationary phase medium (○), and dialysed stationary phase medium (●); points are shown ± 1 standard error.

appropriate volume. Also inhibitory activity was unaffected by heating at 90 °C for 2 h, immersion in boiling water for 10 min, brief direct boiling followed by aeration, freezing and thawing, and the inhibitor was not soluble in organic solvents to any detectable degree.

The molecular weight of the inhibitor, determined by column chromatography on Sephadex G50 was estimated to be 500–700 (Fig. 2). The activity was eluted at the

lower end of the resolution range for this gel, but with a similar elution volume to flavin monophosphate (which has a mol. wt of 514).

Biological effects

Stationary phase medium completely inhibited the cohesion of log phase cells in shaking suspension (Swan & Garrod, 1975). It seems important to determine the specificity of this effect. The activity of the inhibitor on the cohesion of other species of slime moulds, on adhesiveness of cells to glass and on the cohesiveness of aggregation-competent cells of *D. discoideum* was therefore tested.

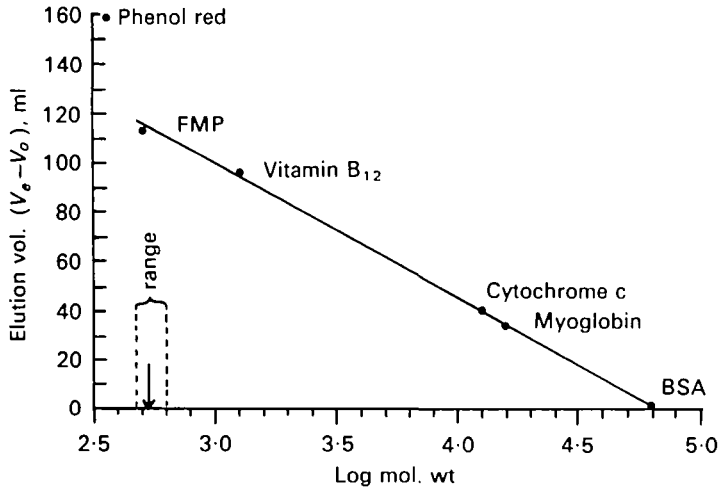

Fig. 2. Sephadex G50 calibration curve for molecular weight determination. (BSA, bovine serum albumin; FMP, flavin monophosphate). The mean (arrowed) and range of values obtained for the molecular weight of the inhibitor are indicated.

Cohesion of the log phase cells of *D. mucoroides* and *D. purpureum* was completely inhibited: cells of *Polysphondylium violaceum* and of the bacterially grown wild-type strain, NC-4, of *D. discoideum* were likewise inhibited, but to a lesser extent (Fig. 3).

The inhibitor also affected the adhesion of slime mould cells to glass. Feeding cells of different species were suspended in buffer (control) and in inhibitor solution, and allowed to settle on a glass coverslip held in a Perspex slide. A chamber was formed by insertion of another coverslip above the first. After 30 min the cells were counted. The slide was then inverted, and the number of cells remaining attached to the glass after 30 min was assessed. Almost 100% of the cells in buffer remained attached to the glass after inversion. By contrast, a significant proportion of cells in inhibitor detached from the glass after inversion (see Table 1). For a given concentration of inhibitor, the degree of inhibition of adhesion to glass was less complete than that of cohesion in shaking suspension.

Aggregation-competent cells of *D. discoideum* Ax-2, obtained by shaking washed log cells in phosphate buffer for 8 h, were partially inhibited from cohering by a

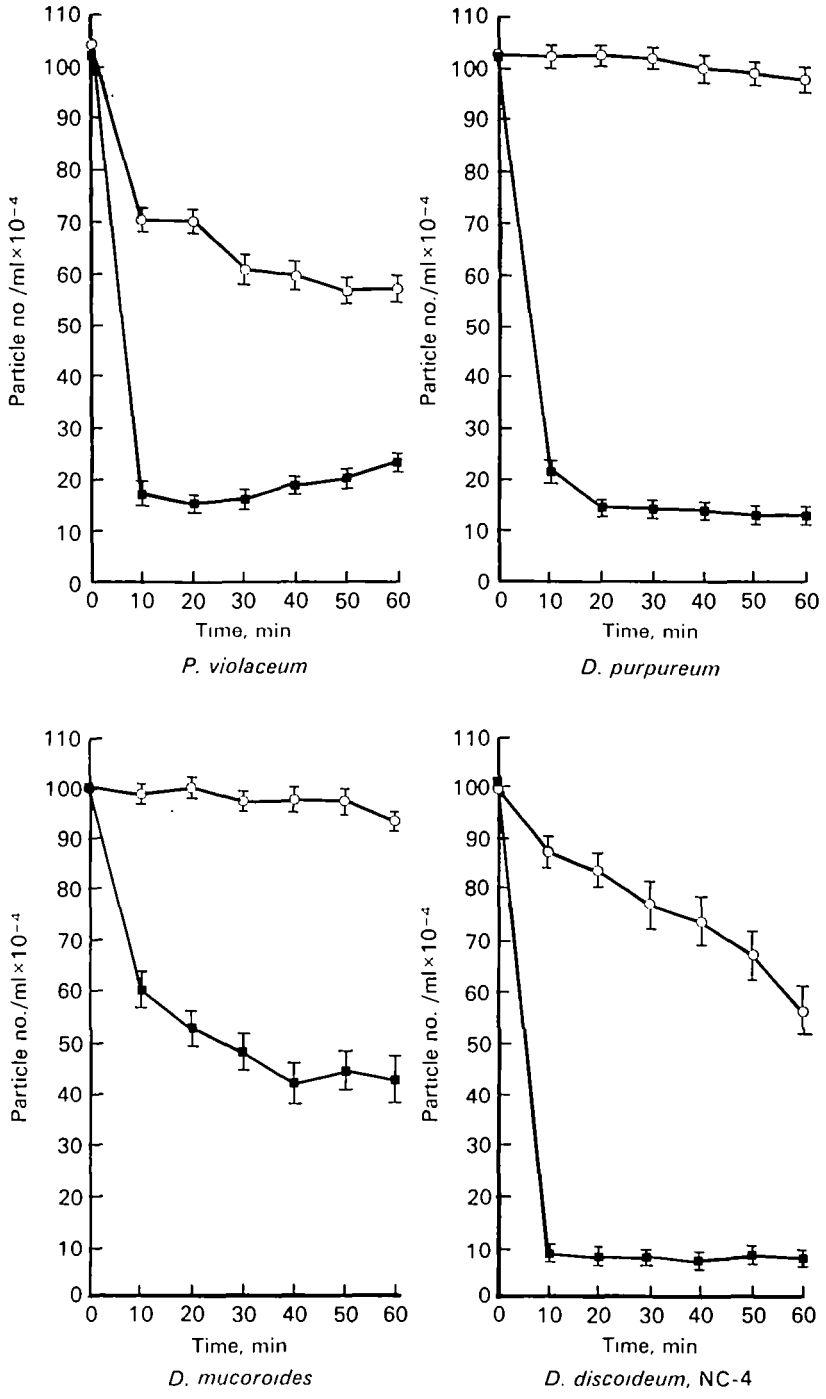

Fig. 3. Cohesion of slime mould cells of different species in 17 mM phosphate buffer (■) and in inhibitor (○). Points are shown ± 1 standard error.

concentration of inhibitor sufficient to completely prevent cohesion of log phase cells (Fig. 4).

Because of these effects on cell cohesion and adhesion, it was interesting to determine whether the inhibitor had a direct effect on development of cells on Millipore filters.

Table 1. *Effect of inhibitor on adhesion of slime mould cells of different species on glass*

Species	Test solution	Mean % decrease in adhering cells	S.D.	*t*	Probability, *P*
D. discoideum (NC-4)	Buffer	2·58	±2·01	3·94	0·02
	Inhibitor	19·34	±6·90		
D. discoideum (NC-4) (aggregation-competent)	Buffer	−1·03	±7·34	8·73	0·02
	Inhibitor	45·44	±1·12		
P. violaceum	Buffer	2·28	±1·05	7·66	0·001
	Inhibitor	22·72	±5·24		
D. purpureum	Buffer	2·75	±0·60	2·81	0·05
	Inhibitor	16·89	±10·06		
D. mucoroides	Buffer	2·85	±4·04	3·37	0·02
	Inhibitor	22·52	±10·97		

The mean % decrease of adhering cells after 30 min is shown (see text for details). The 2 treatments (buffer and inhibitor) for each species were compared by Student's *t* test. Differences with $P \leqslant 0{\cdot}05$ are considered significant.

Log cells allowed to develop on Millipore filters began to aggregate after about 8 h, and subsequently underwent development through the stages of aggregation, standing grex, migrating grex and fruiting body in 24 h. If the cells were exposed to inhibitor before the aggregation stage development did not occur.

Inhibition of development was reversible up to at least 24 h of exposure to inhibitor. Development, however, could only be inhibited prior to aggregation. If cells which had developed beyond aggregation stage were exposed to inhibitor their development continued normally.

Preliminary evidence suggests that the inhibitor may bind to the cell surface. Log cells were fixed in 2% glutaraldehyde for 1·5 h to prevent them producing inhibitor, washed 3 times in a large volume of cold distilled water, suspended at 1×10^7 cells/ml in stationary phase medium and shaken under normal conditions. After 1 h the suspension was centrifuged and a sample of the cell-free stationary phase medium was then assayed for its ability to inhibit cohesion of fresh living log cells. The remainder of the stationary phase medium was incubated for another hour with a new batch of fixed cells. At each stage the degree of inhibitory activity was diminished, and the normal degree of cohesion for fresh axenic medium plus glucose was reached after 5 incubations. Since the cells were fixed, it is most likely that the disappearance of inhibitor from the medium was due to adsorption to the cells. However, it should be noted that as certain enzymes remain active after glutaraldehyde fixation this does not

preclude the possibility that the inhibitor was being metabolized. Dilution effects were avoided by using extremely dense cell suspension for inoculation into the stationary phase medium and a comparatively large volume of medium.

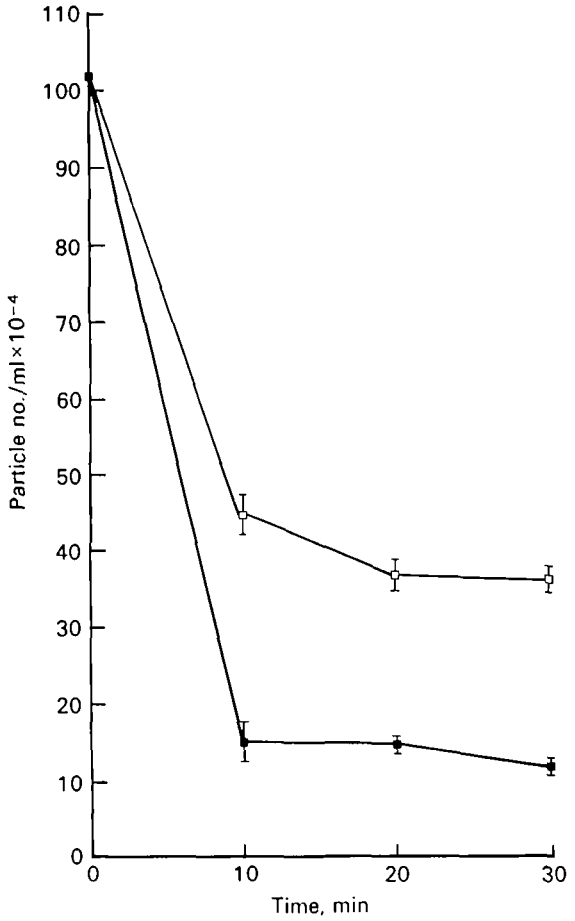

Fig. 4. Cohesion of aggregation-competent Ax-2 cells in 17 mM phosphate buffer (■) and in inhibitor (□). Points are shown ± 1 standard error.

DISCUSSION

The evidence presented indicates that stationary phase medium from cultures of *D. discoideum* Ax-2 contains a low molecular weight fraction which possesses a number of inhibitory effects on cell adhesion and development. These effects are (i) complete inhibition of the cohesion of log phase Ax-2 cells, (ii) partial inhibition of cohesion of aggregation-competent Ax-2 cells, (iii) partial inhibition of cohesion of cells of other slime mould species, (iv) reduction of adhesion of cells of *D. discoideum* and other species to glass, and (v) reversible inhibition of development of *D. discoideum* prior to aggregation. Since the inhibitor has not been purified it is possible that these effects are caused by more than one substance in the low molecular weight fraction. For the

same reason we cannot tell yet whether the inhibitor(s) we have described is distinct from the low molecular weight growth inhibitor and transcripton inhibitor (Yarger *et al.* 1974; Yarger & Soll, 1975). Positive identification of these substances would seem therefore to be of prime importance.

Our initial interest lay in the cohesive properties of different axenic cell populations. Our results show that the cohesion inhibitor probably adsorbs to fixed cells, possibly to the cell surface. One interpretation would be that it binds to the adhesive contact sites described by Beug *et al.* (1970). Adhesion of cells to glass was blocked by our inhibitory material as it was by anti-contact site Fab fragments (Beug *et al.* 1970). There is evidence for 2 types of contact sites, A and B, the former being specific to aggregation-competent cells and the latter being present on both preaggregation and aggregation-competent cells. It is interesting therefore that the cohesion of preaggregation cells was more sensitive to the cohesion inhibitor than that of aggregation-competent cells.

The fact that the inhibitor was effective against the cohesion and adhesion of other species could indicate a lack of specificity. However, it seems probable that cohesion of different slime mould species is not highly specific in any case. It has been shown that cells of *D. discoideum*, *D. mucoroides*, *D. purpureum*, and *P. violaceum* readily mutually cohere in shaken suspension (Nicol & Garrod, 1977). Also lectin-like substances which may be involved in cell adhesion have been extracted from 6 different species of slime moulds and found to be fairly similar with regard to inhibition by a series of sugars (Rosen, Reitherman & Barondes, 1975).

We cannot be certain that the inhibitory effect of the low molecular weight material on development is solely due to the inhibition of cell cohesion. However, there is considerable evidence implicating cell contact in several aspects of slime mould development (Newell, Longlands & Sussman, 1971; Newell, Franke & Sussman, 1972; Gregg & Badman, 1970; Gregg, 1971; Aldrich & Gregg, 1973; Yu & Gregg, 1975; Sakai & Takeuchi, 1971; Garrod & Forman, 1977; Forman & Garrod, 1977). It therefore might be expected that a cohesion inhibitor would also inhibit development.

We are puzzled about the biological significance of a cohesion inhibitor in stationary phase but we feel it is worth investigating for what it may tell us about the mechanism of cell adhesion in slime moulds. Our present research is therefore directed towards trying to identify positively the substance involved. We have already eliminated a number of possibilities suggested by previous work, as follows.

Cyclic AMP plays a major role in the life-cycle of *D. discoideum*, where it functions as a chemical attractant during aggregation (Konijn, Barkley, Chang & Bonner, 1968). It has also been suggested to be important in cell adhesion in mammals (Johnson & Pastan, 1972; Willingham & Pastan, 1975; Storrie, 1975). Furthermore it accumulates in the medium during growth of Ax-2 cells to a final concentration of 1 mM (Malkinson & Ashworth, 1972). Log cell cohesion is not, however, inhibited to any detectable extent by cyclic AMP concentrations from 10^{-3} M to 10^{-9} M. Nor is there any inhibition over the same concentration range by cyclic GMP, or by the breakdown products 5'AMP and 5'GMP (unpublished observations). It has been reported that incubation of log cells in 3 mM glucose for 10 min prior to assay will inhibit subsequent

cohesion in buffer (Weeks & Weeks, 1975). Glucose and other sugars which enhance vegetative growth have also been shown to block slime mould development on Millipore filters, and prevent the acquisition of aggregation competence (Rickenberg *et al.* 1975). When log cells were assayed for cohesion in the standard way in 10 mM glucose in phosphate buffer, no inhibition could be detected. Although cohesion was reduced in axenic medium plus glucose in comparison with axenic medium alone, the degree of inhibition was low compared with the complete inhibition afforded by stationary medium. Cohesion was also unaffected by a range of simple sugars including maltose, lactose, sucrose, fructose and ribose. Development on Millipore filters in the presence of 86 mM glucose was only slightly delayed in comparison with control filters, although filters with inhibitor incubated concurrently showing no development. It seems, therefore, that these simple sugars are not responsible for the effects described in this paper.

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