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# A NEW ENDOGENOUS INHIBITOR OF CELL DIVISION IN THE CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

BY

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ABSTRACT

A NEW ENDOGENOUS INHIBITOR OF CELL DIVISION IN THE CELLULAR SLIME MOLD Dictyostelium discoideum

BY

Margaret D'Addone Hanish

Advisor: Professor Costante Ceccarini

An endogenous macromolecule which inhibited cellular division in Dictyostelium discoideum was partially purified by Sephadex chromatography. The Division Inhibitor (DI) was inactivated by heat and by trypsin, but not by nucleases, indicating protein nature.

It was found to be present even during rapid growth, but more activity was present at onset of stationary phase. Therefore, it was suggested that this division inhibitor (DI) may be responsible for the onset of stationary phase since removal prolonged the growth phase. Also it was proposed that DI may promote hypertrophy of the amoebae, which gradually undergo a 150% increase in protein content between exponential growth and stationary phase.

Effects of purified DI on incorporation of radioactive precursors into nucleic acids were investigated. Incorporation of uridine and of thymidine was not significantly inhibited at least for one generation. This fact, together with the finding that DNA content of stationary cells is almost

double that of rapidly growing cells, supported the previously drawn conclusion that DI arrests amoebae in G2 phase. Removal of DI from stationary phase amoebae by washing resulted in a subsequent simultaneous division in axenic medium. However this division was not preceded by a peak in $^{3}$H-methly-Thymidine incorporation. This finding may support the above conclusion.

Addition of partially purified DI to rapidly growing cells prevented any further increase in cell number. Experiments with trypsin and with Concanavalin A revealed that the added DI seemed to exert its effect through the cell surface, at least initially.

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# INTRODUCTION

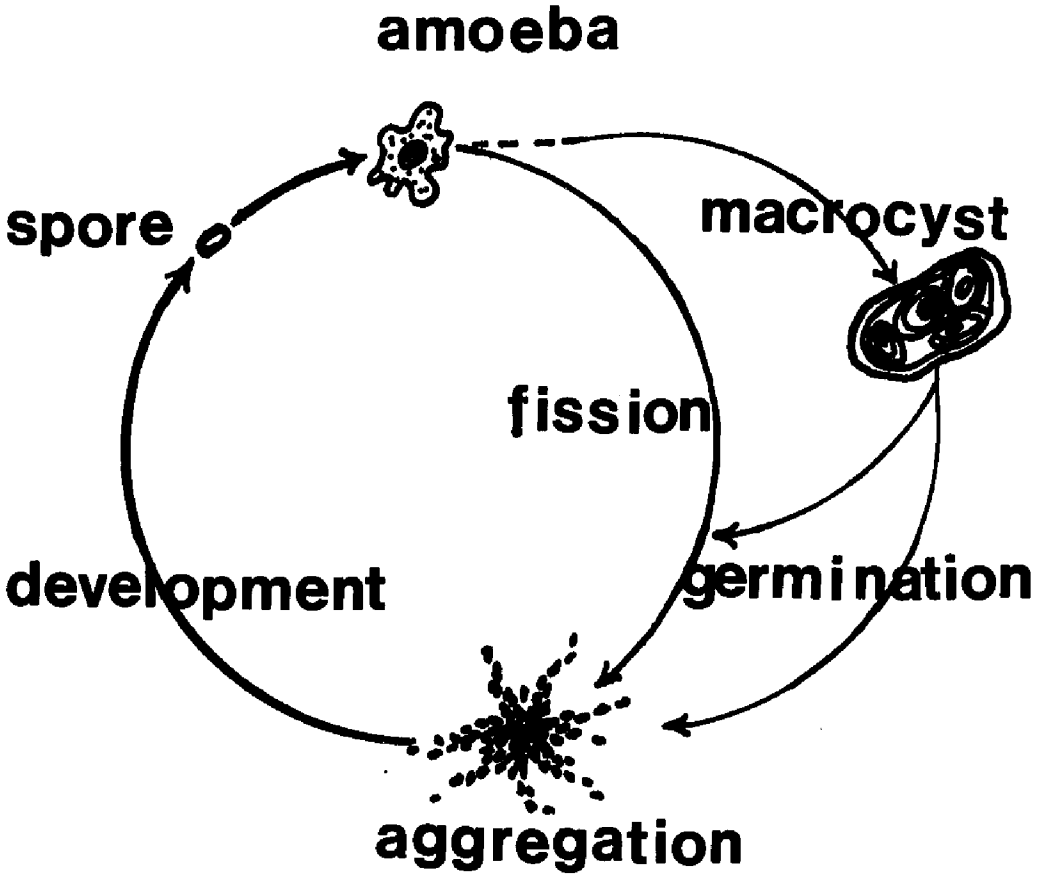
## Life Cycle

Dictyostelium discoideum, a species of the Acrasiales (Mycetozoa) which was isolated in 1935 by K.B. Raper, has a life cycle with three distinct phases: vegetative growth, spore and macrocyst formation (Figure 1). Vegetative amoebae arise by germination of a spore. Amoebae feed on soil bacteria and divide by fission (Bonner, 1967). In some rare cases they also feed on each other, by anastomosis or engulfment (Huffman and Olive, 1964). When environmental conditions become unfavorable for growth, the amoebae may enter either of two developmental pathways, macrocyst or sorocarp formation. The formation of macrocysts is favored by darkness, excess moisture and low phosphate ion concentration in the environment (Erdos et al., 1973). This process begins with fusion of two amoebae to form a giant diploid cell which will engulf others and ultimately undergo meiosis. Amoebae liberated by macrocyst germination may undergo vegetative growth or proceed into the other developmental pathway (Blaskovics and Raper, 1957; Nickerson and Raper, 1973; Machac and Bonner, 1975).

The process of sorocarp formation begins with the aggregation of unicellular amoebae into a multicellular organism. Amoebae become aggregation-competent several hours after feeding ceases (Bonner, 1967). During the interphase, a variable percentage of the population undergoes cell fusion (Huffman

Figure 1. Life Cycle of Dictyostelium discoideum.

Spores germinate into vegetative amoebae which feed and divide by mitosis. Macrocysts are formed under certain conditions (low phosphate ion concentration, high humidity and darkness). Upon macrocyst germination, the released cells can either aggregate immediately or return to vegetative growth. Under favorable environmental conditions, aggregation of amoebae occurs, forming a multicellular organism (a pseudoplasmodium). The cells of the pseudoplasmodium differentiate into dead stalk cells and spores.


amoeba
spore
macrocyst
fission
development
germination
aggregation

and Olive, 1964). Syngamy may occur in some of these cases, with or without haploidization by chromosome loss. This parasexual process may therefore result in recombinant formation through mitotic chiasmata (Sussman and Sussman, 1963).

## Aggregation

Orientation of amoebae towards an aggregation center requires the presence of an initiator cell. Any amoeba may become an initiator. Generally, these become larger than other cells, have enlarged nuclei and higher rates of contractile vacuole formation and cyclosis. Although they are capable of rapid movement, they remain within a small perimeter. However, diploidy is not necessary as was initially thought (Ennis and Sussman, 1958). The totipotency of either haploid or diploid single cell has been repeatedly demonstrated (Konijn and Raper, 1961; Kahn, 1962; Huffman and Olive, 1964). As many as one hundred thousand cells can respond to the presence of an initiator cell (Ennis and Sussman, 1958; Newell, 1971). The initiator cells are believed to be the first in the population to begin secreting a chemical attractant, formerly called acrasin, to which other amoebae respond by first orienting and then by migrating towards it (Bonner, 1967). This response depends upon "a great increase in their orientation sensitivity" (Bonner et al., 1966).

The responding cells, once stimulated by acrasin, also

begin secreting the attractant (Bonner, 1967).

Acrasin has been identified as cyclic Adenosine 3', 5' - Monophosphate (cAMP) in Dictyostelium (Konijn et al., 1967; Konijn et al., 1969; Barkley, 1969; Konijn et al., 1969). Other genera of Acrasiales apparently have other molecules serving as attractants for aggregation, since a mixture of cells of two different genera will result in each aggregating to its own center, and aggregating streams of cells will even cross over each other (Bonner et al., 1972). The presence of cAMP also induces the differentiation of amoebae into stalk cells in the absence of aggregation (Bonner, 1970).

Before the attractant had been identified, it was reported that a spacing factor existed. Work in J.T. Bonner's laboratory showed that the number of aggregating units per unit of substratum remained constant despite great variations in cell density, and it was suggested that ammonia might be the spacing substance (Bonner, 1967). Recently, however, other investigators have suggested that possibly territory size may be a complicated function of initial cell density and that an acrasin gradient may be sufficient to explain spacing (Hashimoto et al., 1975).

Centers of D. discoideum show rhythmic periodicity in chemotactic activity (Bonner, 1944; Shaffer, 1962, 1964; Gerisch, 1968; Durston, 1974; Robertson et al., 1972) with a periodicity of 2.5 to 5 minutes (Gerisch et al., 1974). Signal

propagation by cells stimulated by cyclic AMP was proposed and it was shown that cells respond first by chemotactic orientation and then by releasing attractant themselves (Robertson et al., 1972). It has been suggested that the gradient of cAMP may be due to a phosphodiesterase activity, both extracellular and membrane-associated (Pannbacker and Bravard, 1972; Chassy, 1972). A specific inhibitor of the extra-cellular phosphodiesterase also exists. It is released at the end of growth, only in species which respond chemotactically to cAMP (Riedel et al., 1973). Therefore it is now believed that the function of extracellular phosphodiesterase is the degradation of extracellular cAMP during the growth phase (Gerisch et al., 1974), and not the creation of a cAMP gradient during aggregation. The generation of signal pulses in D. discoideum seems to be coupled to an oscillator which is independent of pulse generation. However, the possible coupling of the electron transport system to concentration of ATP, the substrate of both pyrophosphohydrolase and adenyl cyclase, has been discussed and rejected as not consistent with all the data (Gerisch et al., 1974). These authors also mention that there is no available data on the transport of cAMP across the plasma membrane, which might increase understanding of the chemotactic mechanism. For example, why do amoebae actually move toward higher concentrations of this nucleotide, forming streams in the case of D. discoideum, while in D. mucoroides each cell migrates indepen-

dently? Part of the answer may lie in differences in timing of acquisition of adhesivity. In leucocytes, changes in substrate adhesion caused by local elevations in cAMP concentration have been postulated as the cause of cell migration (Willingham, 1976). In addition, consideration should be given to the possibility that aggregation competent plasma membranes in slime molds may have adenyl cyclase oriented so as to release its product extracellularly, instead of intracellularly, as is the case in mammalian systems which have been studied (Rubin, 1973; Cuatrecasas, 1974).

After aggregation, the amoebae rearrange to form a sombrero-shaped mass. This mass may form an elongate slug (also called the pseudoplasmodium, grex or conus; cf. Gerisch, 1968) which will either migrate towards light and warmth, or alternatively develop directly into a sorocarp consisting of vacuolated dead cells and mature spores.

The migratory stage therefore is not essential to development, but may serve as an adaptive mechanism (Bonner, 1967). Loomis has discussed this optional period of the cellular slime mold's development: the polarity of migration is fixed early in morphogenesis and under natural conditions is never reversed or extinguished (Loomis, 1972). As the center of the aggregate rises to form a conus, glycoprotein surface coat forms and surrounds the entire cell mass in the so-called slime sheath. This sheath is continually synthesized as the

older sheath layers are left behind during migration, forming a trail. When most of the aggregate is in the sheath, the conus, formed from the center of the aggregate, bends over onto the substratum support and becomes the migrating slug. Once the position of cells in the pseudoplasmodium is set, no rearrangement occurs in subsequent migration. Loomis continues, "It seems likely that the anterior cells are free to control the direction of migration because they can deform the surface sheath newly formed around them." Moreover, the sheath forms not only at the anterior tip, but instead synthesis occurs uniformly over the entire axis as indicated by the uniform incorporation of Acetylglucosamine along the slug (Loomis, 1972). Since the sheath is left behind as the slug migrates, anterior cells are covered only by the thin layer of sheath which they have just synthesized while posterior cells are covered by the discarded anterior sheath, plus their own recently synthesized sheath. Thus there is a gradient of sheath thickness and this may be the factor which limits the posterior cells' movement (Loomis, 1972). In support of this theory is the fact that cutting the slug at the posterior end results in a smaller migrating slug, but only as long as the cut is left open; then reversal of polarity can be experimentally induced, and normal development into mature sorocarps follows. Loomis also suggested that some induction of spore-specific differentiation may depend upon properties of the surface slime sheath, which may

modify the concentrations of low molecular weight diffusible effectors. Furthermore, accumulation of prespore vacuoles and synthesis of developmentally regulated enzymes such as UDP-Galactose Polysaccharide Transferase may occur only where the thickness of the sheath would allow accumulation of hypothetical low molecular weight effectors of spore differentiation. However, the logical flaw in this hypothesis is the fact that the migratory slug stage is not required and differentiation proceeds normally in its absence. Also, other investigators believe that preexisting differentiation occurs as early as the late aggregation stage and that a process of sorting out occurs in the formation of the slug (Takeuchi, 1963; 1976).

Sorting Out

It has been reported that preaggregation cells show considerable variation in the amount of antigenic constituents which can react with an antispore antiserum but the differentiated cells are collected randomly in aggregation. Therefore, the cells in the slug undergo a process of sorting out (Takeuchi, 1966). This variation must then develop during vegetative growth and it has been suggested that by undergoing cell divisions during the vegetative stage, a population of amoebae, all derived from spores, will gradually become heterogenous as regards developmental potential. However, Takeuchi has written

that he does not believe the antigenic difference is due to a difference in the timing of the cessation of growth. This conclusion was reached by allowing reaggregation of cells from the posterior third of Thymidine labeled slugs with cells from the anterior third of unlabeled slugs. More silver grains were then found in autoradiograms in the posterior part of the reconstituted slug. This evidence corroborated Bonner's suggestion that cells "know" their position in the slug and will return to it even when grafted to another position. This was shown by the use of vital stains to uniformly dye vegetative amoebae. Sorting out has also been reported by Müller and Hohl, 1973, who presented evidence that the distribution of prespore and prestalk cells takes place within the first two hours of grex formation. As a marker, they used the prespore specific vacuole which appears in cells during late aggregation. While presumptive prestalk areas are totally free of cells containing prespore vacuoles, the posterior region does contain a few prespore vacuole-less cells. The authors then concluded that cell commitment and determination, cell differentiation and multicellular pattern formation overlap in time, since prespore vacuole number per cell increases over a considerable period of time. This may explain how regulation is possible despite the apparent prior commitment of cells in different regions of the grex to form only one cell type of the mature sorocarp. Müller and Hohl (1973) also hypothesize

that only the very tip of the anterior which is totally devoid of prespore vacuoles may organize and guide grex motility instead of the entire anterior region. In addition to the overlap in time differentiation, these authors claim that inclusion of a few cells of the other type in either region may be a cause of regulation. They also criticize Gregg and Badman's (1970) use of "spore vacuole" (a tight association of an electron-transparent vacuole with a cisterna of the rough endoplasmic reticulum), because it does not occur frequently in early stages of slug formation, and it is found in both cell types.

Mechanism Of Sorting Out

It is now generally accepted that differentiation occurs to some extent even before aggregation and that two cell types must sort out during later phases. Bonner, Sieja and Hall (1971) demonstrated that the same chemotactic response occurs in both cell types. It was therefore concluded that differential rates of movement were not responsible for sorting out, but that differential adhesion may be the cause of this phenomenon (Bonner, Sieja and Hall, 1971). This conclusion is in agreement with what has been found in embryonic systems and with the currently accepted view of aggregation; that metabolic and biosynthetic activities are required for aggregation to occur and that the histogenic attachment of cells may be

mediated by specific macromolecular products functioning at the cell surface (Moscona, 1971). That regulation may occur indicates that the primary differentiation is reversible. A slug may be cut transversely and the result will be two stalks and two fruiting bodies (Raper, 1940, as cited by Bonner et al., 1971). This is another indication that no commitment occurs in the slime molds even after aggregation.

Cellular Adhesiveness

The new adhesiveness rapidly acquired by preaggregating amoebae (Bonner, 1967) has been tentatively attributed to lipoproteins (Yabuno, 1971), carbohydrate-associated proteins (Gerisch, 1969; Beug, et al., 1970; Siu et al., 1976) and to new surface antigens (Takeuchi, 1963). It has also been suggested that the increase in adhesiveness might be attributed to structural changes in integral components of the plasma membrane following aggregation (Aldrich and Gregg, 1973). However no causative effect was actually shown in the paper just cited: the reasoning followed in this work was of the post hoc, ergo proter hoc variety. The fact that freezefracture studies show structural changes have occurred in the matrix of the plasma membrane does not reveal anything about either aggregation or adhesivity.

It has been shown that cAMP addition causes clustering of cells and also induces filopodia (Kobilinsky et al., 1976).

This work cites the earlier theoretical work of Pethica who suggested that the first event in cell adhesion may involve fine processes extending from cell to cell. In fact, it has been shown that slime mold amoebae with cAMP-induced filopods are more easily agglutinated by Concanavalin A. (Kobilinsky et al., 1976).

Summary On Sorting Out And Aggregation

Aggregation in the cellular slime molds occurs in response to cAMP. The onset of the cAMP secretion has long been considered to be the result of the beginning of starvation (Bonner, 1967) however another view will be presented below, with the evidence which prompted its formulation.

After its role in aggregation has occurred, cAMP hinders development. Both slug migration and fruiting body construction temporarily halt when the cyclic nucleotide is added in high concentration early in development. But if added late, cAMP causes abnormalities in sorocarps, probably as a result of its known repression of developmental enzyme synthesis (Chassy, 1972; Nestle and Sussman, 1972).

The amoeba's developmental fate is a consequence of its position in the grex and position, in turn, depends on its previously differentiated properties (Newell, 1971). A continuous regulation among cells which have not irreversibly differentiated, by incessant conversion from one cell type to

another until the time of spore formation has been proposed (Hayashi and Takeuchi, 1976). The same type of regulation had already been proposed by Bonner in the migrating slug of D. mucoroides which continually forms mature stalk cells while it migrates, so that ratio of stalk to spore cells in a fruiting body varies with length of migration (Bonner, 1967). The synthesis of a developmentally regulated enzyme, UDP-galactose transferase, is necessary for the construction of the prespore specific vacuole. Sussman's laboratory has studied the developmental kinetics of the antigenic polysaccharide and of the enzyme and showed evidence that both appear first after aggregation is almost completed, just before the migrating slug stage (Ellingson et al., 1971). However, the more sensitive immunocytochemical technique reveals the presence of the prespore vacuole in early aggregation (Hayashi and Takeuchi, 1976).

## Developmentally Regulated Enzyme Synthesis

Differential gene activity occurs after cells enter the developmental cycle. A different set of formerly inactive genes will then be transcribed and new protein syntheses will occur to form the following developmental-program enzymes: UDP-Glucose-Pyrophosphorylase, UDP-Galactose-Transferase (involved in building spore wall), UDP-Galactose-Epimerase, Trehalose-Phosphate-Synthetase (which will form the spore's energy

reserves), β-N-Acetylglucosaminidase, d-Mannosidase, Alkaline Phosphatase, β-Glucosidase, Threonine Dehydratase, Cellulase, Glycogen Phosphophorylase. (Newell, 1971).

As reviewed by Newell (1971), the best evidence that these enzymes require new protein synthesis and not activation, comes not from incorporation studies, but from the use of antisera to the isolated developmental enzyme, UDP-Glucose Pyrophosphorylase. An increase in activity of the enzyme coincides with increase in serological activity (Frank and Sussman, 1973). This opposes the notion of substrate-availability regulating differentiation, which implies that enzymes may be reformed and preexisting, (Wright and Anderson, 1960; Gustafson and Wright, 1972).

## The Cellular Slime Molds As A Model System Of Development

As a model system of eukaryotic development, the cellular slime molds have stimulated the interest of biologists of diverse disciplines. A great deal is known about their life cycle, yet many unanswered questions and problems still remain (for further discussion, see Conclusion). As a system in which the formation of a multicellular structure by a homogeneous population of individual cells may be examined, the cellular slime molds are without equal. No hormonal, serum, or other extracellular growth factors need be considered, and the contribution of morphogenetic information preformed and stored in

inactive form, as in embryogenesis, may be eliminated.

## Introduction To Experiments

The cell surface glycoproteins of cultured mammalian cells have been shown to differ in growing and nongrowing states (Oseroff, et al., 1973; Winzler, 1970; Martinez-Palomo, 1971).

When a study was undertaken to examine whether turnover of prelabeled glycoproteins differs in D. discoideum, under growing and nongrowing conditions, a peculiar phenomenon was witnessed. After several washes to eliminate radioactive precursors from the medium, and addition of fresh medium, it was impossible to have a "nongrowing" culture. Cell division occurred despite the very high initial cell density. It was then believed that the nutrients of the fresh medium were the cause, and that this problem could be circumvented by resuspending the washed prelabeled cells in medium which had already supported the growth of a culture of slime molds. To prevent any possible contamination, and also to caramelize any dextrose remaining in the medium so as to deprive the prelabeled cells of any Carbon source, the spent (or conditioned) medium was reautoclaved. Surprisingly, cell division occurred under these conditions, to the extent that the population doubled within sixteen hours. Next, nonnutrient phosphate buffer was used to resuspend prelabeled cells. Again, a round of cell

division occurred. It was then decided that these observations should be further investigated. It was observed that the washing served as stimulus for cell division. Reconstitution, i.e., addition of the cell wash back to the washed cells, resulted in no increase in cell number. From this, it has been concluded that some substance hereafter referred to as Division Inhibitor (DI) was removed from the cells by washing.

The aim of this work was to partially purify the DI, thus confirming its existence, to begin its characterization, and to find its site of action. The results are presented here.

## MATERIAL AND METHODS

### Cell Culture And Buffers

Two strains of Dictyostelium discoideum were used. NC4, a haploid strain obtained from Dr. J.T. Bonner (Princeton University), was grown in liquid culture in the presence of live nondividing Escherichia coli B/r. The bacteria were grown overnight in nutrient broth. Washed by centrifugation (5000 g, 10 minutes) three times with 0.016 M Sorensen's buffer, pH 6.0 (Bonner, 1967), bacteria were resuspended to a concentration of $10^{10}$ cells/ml. Spores from several sori were added to the bacterial suspension (50 mls per 125 ml Erlenmeyer flask). Under these conditions, spores germinate into amoebae, and these divide with a generation time of about 3 hr, as reported by others (Hohl and Raper, 1963). Ax-3, obtained from Dr. B. Weinstein (Mount Sinai Medical College), was grown in axenic medium (Loomis, 1971; Sussman and Sussman, 1967). The amoebae used in these experiments divided with an average generation time of 12 to 15 hr, unless otherwise stated.

Washing and counting of amoebae: The cells were washed by centrifugation. Early stationary phase cultures of NC4 amoebae, which were visibly free of bacteria in a hemacytometer, were centrifuged at 1100g for 2 min. (International centriguge PRJ). The resulting pellet was suspended in Lower Pad Solution (LPS) buffer and recentrifuged. Low-

er pad solution consists of 0.04 M $KH_2PO_4$-$Na_2HPO_4$, pH 6.4, containing per liter: 1.5 g of KCL, 0.5 g of $MgCl_2.6H_20$, and 0.5 g of streptomycin sulfate (Telser and Sussman, 1971). The resuspended cells were again pelleted twice. Ax-3 amoebae were also washed twice by centrifugation. Consistent results were obtained with a ratio of 5 X $10^7$ cells to 35 ml of LPS. Counting of cells was done with an hemacytometer; at least three samples with a minimum of 100 cells each were counted and the average taken.

In all cases in which no cell number increase is reported, cells were monitored for at least 4 hours after division occurred in control cultures. This was done to eliminate the possibility that the difference was due to the timing of division. In some cases, the cell number of unwashed cells resuspended in LPS was monitored for 2 days after washed cells divided. No cell division was observed.

## Partial Purification Of Division Inhibitor

The Division Inhibitor was released from the amoebae by washing them with LPS as described above. Cell wash from approximately $10^{11}$ stationary Ax-3 cells was collected, sterilized by passage through Morton filters, and concentrated by an Amicon filtration apparatus with PM10 filters. This filter does not retain molecules with molecular weight

less than 10,000 daltons. The high molecular weight fraction was then lyophilized and dialyzed against thirty volumes of 50 mM ammonium acetate and aliquots passed through a Sephadex G-200 column, 93 x 2.5 cm, equilibrated with 50 mM ammonium acetate. Elution rate was 20 ml/hour and 3.5 ml fractions were collected. Inhibitory activity was assayed (see below) by adding 0.1 ml of each fraction to 5 ml growing cell cultures in 50 ml Erlenmayer flasks.

The fractions were not sterilized. However, the cultures did not become contaminated within 16h, as seen by microscopic examination. Fractions with 30% or more inhibitory activity were pooled; lyophilized, resuspended in LPS buffer, dialyzed against 30 volumes LPS, sterilized and frozen in small aliquots.

Assay For Division Inhibition

A biological assay was used to detect the presence of the Division Inhibitor. To cultures of exponentially growing cells, preparations of DI were added and cell number increase was monitored with the hemacytometer (see above). With phase microscopy where cell number increase failed to occur the subsequent cell growth was monitored for 16 hours after DI addition. As results show, cell number in controls doubled during this period.

Characterization And Cell Surface Effects:

Trypsin in varying concentrations, dissolved in 10 mM Tris-HCL, 50 mM in $CaCl_2$, pH 7.9, was used at 23°C, (see tables). Ribonuclease A, 100 µg per ml, and $T_1$, 2 µg per ml, dissolved together in 10 mM Tris chloride, 5 mM in $MgCl_2$, pH 7.4 and DNAse I, 10 µg per ml in 100 mM Tris chloride, 50 mM in $MgCl_2$, pH 7.4, were both incubated at 23°C (Mushynski and Spencer, 1957). All the above chemicals were obtained from Calbiochem. Concanavalin A, from Sigma, was dissolved in LPS at varying concentrations, see Tables 8 and 9.

Studies On Nutrient Qualities Of Conditioned Medium:

Conditioned medium is medium in which a culture of cells has grown to stationary phase. It was collected by pelleting the cells at 1100 g for 2 minutes, decanting the medium and repeating the process to insure removal of all cells. In this work, conditioned medium was collected within 10 hours of the onset of stationary phase.

Autoclaving of conditioned medium was done by incubating 10-ml aliquots in 50-ml Erlenmeyer flasks at 121°C, and 18-20 psi for the time described in test and figures. Other heat treatments of conditioned medium were also done with 10-ml aliquots. Dialysis was performed at 4°C over-

night, against 100 volumes of fresh medium. After dialysis of conditioned medium it is sterilized by filtration.

## Protein Determinations

Protein determinations were done by a modification of Lowry's method (Oyama and Eagle, 1956) using bovine serum albumin as standard. See Appendix for detailed description.

## Radioactive Incorporation Studies

Cells which had grown in axenic medium to a density of $10^6$ cells per ml. were used for incorporation studies. $^3$H-methyl-thymidine (specific activity 3 C/m Mole) was obtained from New England Nuclear. Thymidine (A grade) was obtained from Calbiochem and Adenosine-5'-Monophosphate was obtained from Schwarz/Mann. Uridine-2-$^{14}$C (specific activity 50 mCi/mmol.) was obtained from Amersham/Searle. Millipore filters were immersed in Aquasol (New England Nuclear) for liquid scintillation counting.

## DNA Determination

Total cellular DNA was extracted by a modification of the Schmidt-Thannhauser Procedure: (Schmidt-Thannhauser, 1945) alkali hydrolysis was performed at 37°C with 3 volumes

of 0.3 N KOH for 36 hours. DNA determination was by the Diphenylamine Method (Giles and Myers, 1965). Detailed descriptions of methods may be found in Appendix.

# RESULTS

## Existence Of A Division Inhibitor

When stationary phase amoebae of either strain $NC_4$ or Ax-3 were resuspended in LPS, no division occurred. However, if they were first washed as described in Materials and Methods, the amoebae underwent a round of cell division at about 10 to 12 hours after resuspension in fresh LPS. Cell doubling occurred within one hour, suggesting some degree of synchrony. Table 1 shows the results of washing exponentially growing cells of Ax-3, and stationary cells of Ax-3 and $NC_4$.

This cell division occurred in the absence of cell growth, since LPS contains no nutrients, and the division results in visibly smaller amoebae. For this division to occur, the number of washings is critical, as is the ratio of cells to buffer volume. One round of division occurred when $5 \times 10^7$ cells were washed twice in 35 mls LPS. More cells, less volume or only one washing was not sufficient to stimulate the entire cell population to divide. One wash resulted only in 4-26% cell number increase within a

TABLE 1

THE EFFECT OF WASHING AMOEBAE ON CELL DIVISION

| Cell stage and strain | Number of washes | Resuspension fluid | Cell number increase (%) per 10-14 hr | Number of trials |
|---|---|---|---|---|
| Stationary Ax-3 | 0 | LPS | 0 | 5 |
| | 1 | LPS | 26 | 3 |
| | 2 | LPS | 94.4 ± 14(SD) | 20 |
| | 2 | Cell wash | 0 | 4 |
| | 2 | Dialyzed cell wash | 0 | 6 |
| Exponential Ax-3 | 0 | LPS | 0 | 4 |
| | 1 | LPS | 4 | 2 |
| | 2 | LPS | 89 ± 12(SD) | 7 |
| | 2 | Cell wash | 0 | 4 |
| Stationary NC-4 | 0 | LPS | 4 | 4 |
| | 1 | LPS | 13 | 4 |
| | 2 | LPS | 110 ± 20(SD) | 6 |
| | 2 | Dialyzed axenic cell wash | 4 | 5 |

[a]Amoebae (Ax-3 and NC-4) were washed as described in Materials and Methods. They were resuspended in either LPS, cell wash or dialyzed cell wash and cell number was monitored periodically for the next 16 to 18 hours. Cell division occurred between 10 and 14 hours, or not at all.

Standard deviation (SD) $=\sqrt{\Sigma(X-M)^2/N-1}$, where N is the number of samples; X, an individual sample; and M, the arithmetic mean.

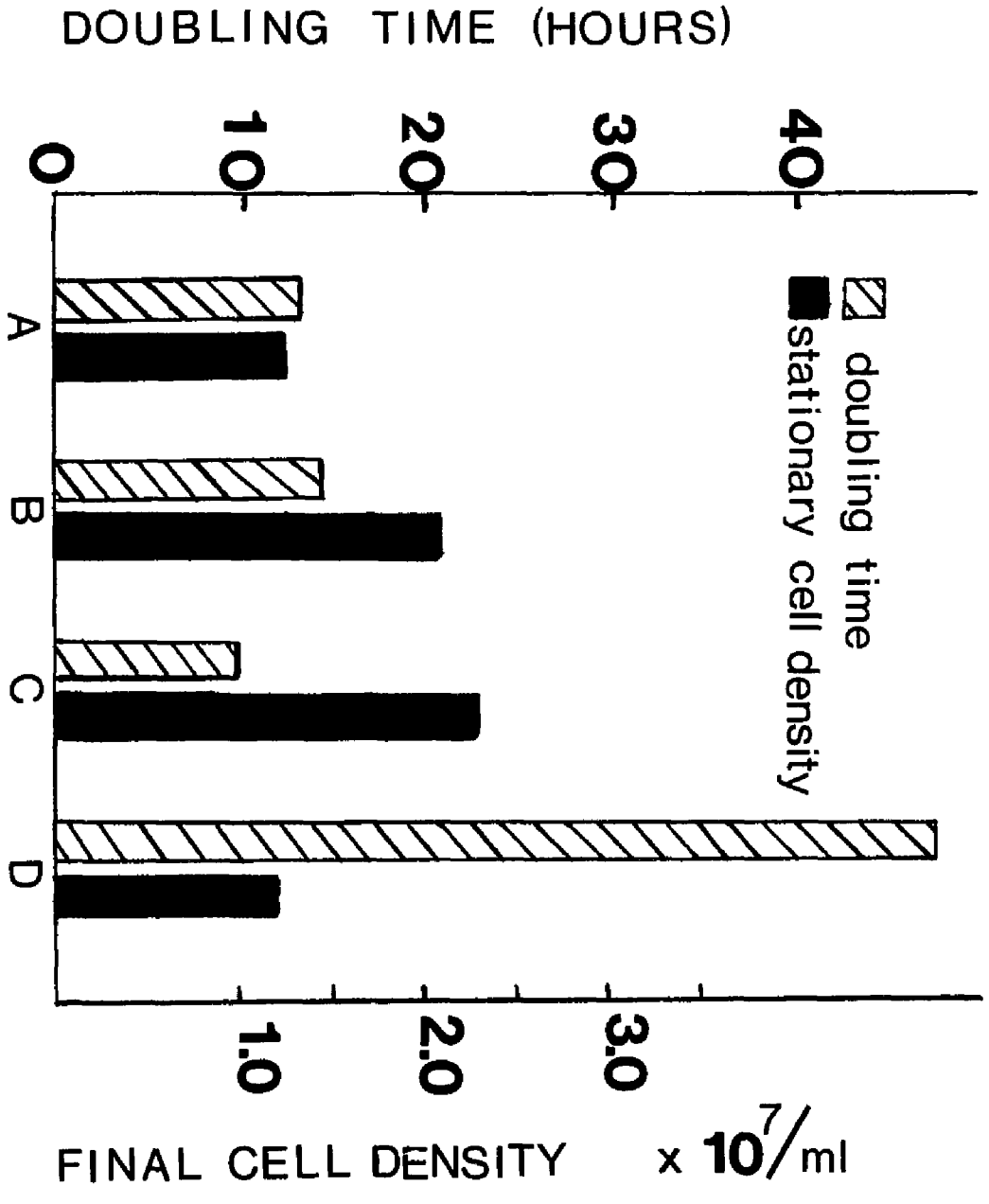
12 hour period, depending on strain and stage, Table 1. Note that this level was higher than that of the controls, while the twicewashed cells underwent an average of one round of cell division (94% increase) within the same period. No significant difference was seen in the extent of division for washed stationary Ax-3 cells resuspended in LPS from 5 X $10^5$/ml through 1.9 X $10^7$/ml. That is, the initial cell density of washed amoebae after resuspension does not seem to affect extent of division, within this range. Exponentially growing cultures of Ax-3 were also induced to divide by washing; Table 1. Although the generation time of $NC_4$ cells had been less than 3 hours, while that of Ax-3 cells was four times longer, this division after washing occurred at about 10-12 hour in both strains.

When twice-washed cells were resuspended in the combined buffers in which they had been washed, no cell division occurred. Furthermore, dialysis of cell wash does not remove the inhibitory activity, Table 1. It is suggested that washing amoebae with LPS buffer seems to remove a non-dialyzable Division Inhibitor (DI).

One might expect that removal of a mitotic inhibitor from a growing culture would either stimulate growth rate or prolong the growth phase, resulting in elevated cell density at stationary phase. To verify the existence of a division inhibitor in exponential cultures, Ax-3 cells which

had grown to 3 X $10^6$ to 5 X $10^6$/ml were washed as described above, resuspended in their original growth medium, in heat-treated growth medium, or in fresh medium. Subsequent growth under these conditions supported the suggestion that a mitotic inhibitor can be washed off growing cells. Washing cells, and returning them to their original medium prolongs their growth phase, since they attain a stationary cell density nearly twice that of controls, Fig. 2, compare sets A and B. Heat-treating the growth medium before returning washed cells to it had no significant effect on the final cell density, thus it appears that only small amounts of DI existed in the medium at that point, Figure 2, set C.

When the doubling times of washed cells and of controls were compared, it seemed that washing the cells had no significant effect, Fig. 2, sets A-C. However, we found that mock-washed amoebae (cells pelletted and resuspended in their own supernatant growth medium, twice) had a greatly decreased growth rate for the first day after treatment. Thereafter the growth rate approached that of controls, as monitored by direct cell counts (data not shown). The doubling time of mock-washed cells was more than triple that of washed cells (Fig. 2, compare A and D). The mock-washed cells exhibited a slow but continuous increase in cell number throughout the time before their first doubling (data


DOUBLING TIME (HOURS)
0
10
20
30
40
A
B
C
D
doubling time
stationary cell density
1.0
2.0
3.0
FINAL CELL DENSITY x 10^7/ml

## LEGEND TO FIGURE 2

The effect of washing growing cells upon doubling time and extent of growth period.

Ax-3 cells which had grown to 3-5 X $10^6$/ml were harvested, washed with LPS as described and resuspended in their original growth medium. In some cases, growth medium was immersed in boiling water bath for ten minutes, before washed cells were returned to it. Set A. Control cultures (average of 7). Set B. Washed cells, returned to same medium in which they had been growing (average of 3). Set C. Washed cells, resuspended in heat-treated original medium, (average of 4). Set D. Mock-washed cells: Cells were pelleted, then resuspended in their own supernatant growth medium, instead of in LPS. This mock-wash procedure was repeated twice, so that conditions duplicated those of washing the cells (average of 2).

not shown). After recovery, these cells reached the same cell density as controls.

The results presented above suggest that decanting, pipetting, and centrifugation have a transitory deleterious effect upon cell growth and that this effect is counterbalanced by some beneficial effect of washing. The only difference between mock-washed and washed cells has been exposure to LPS, and removal of whatever dissolved in LPS. This supports the hypothesis that washing removes an inhibitor of cell division, even from exponentially growing amoebae.

To test whether the amount of DI per cell increases during growth, the following experiment was performed. Cell washes were prepared in an identical manner from exponetial cultures (2-4 X $10^6$/ml) and from cultures about to become stationary (circa $10^7$/ml). That is, 5 x $10^7$ cells from each culture were washed twice in a total volume of 70 mls LPS. This was done on three separate occasions for both stages. The wash thus obtained was filter-sterilized and tested for the ability to inhibit the cell division of washed cells. Since the cell number per volume was identical, any difference in inhibitory activity may be due to a difference in DI concentration or activity between cells of the two stages. Results are shown in Table 2. Undiluted,

both washes fully inhibited cell division. However when exponential wash was diluted ten times with fresh LPS inhibition of division almost entirely disappeared while the ten time diluted stationary wash contained considerable inhibitory activity. Even diluted 20 times, stationary wash inhibited cell division to a greater extent than did ten times diluted exponential wash (Table 2). Therefore, it is suggested that the same washing procedure removes either more molecules of DI or a more active form of DI from stationary than from exponential Ax-3 cells.

Removal of more DI activity from stationary phase amoebae may be due to differential release or to their larger size (cf Table 4). The possibility that two different inhibitory macromolecules exist at the two stages of growth cannot be excluded at this time. Notwithstanding, we favor the hypothesis that more DI molecules exist in stationary phase cells than in exponential phase.

An important point to be considered is whether the DI can inhibit normal growth divisions as well as the division in nonnutrient buffer. Results shown in Table 3 indicate that DI does indeed stop all further mitoses when added to exponential cultures. Cyclic AMP has been shown to block cell division in animal cells (Abell and Monahan, 1973). When added to an exponentially growing culture of Ax-3 cells,

TABLE 2

INHIBITORY ACTIVITY IN EXPONENTIAL AND STATIONARY CELL WASH[a]

| Resuspension fluid | Cell Number increase (%) | Inhibition of division (%)[b] | Number of trials |
|---|---|---|---|
| LPS (Control) | 76 | 0 | 3 |
| Exponential Cell Wash | | | |
| 1X | 0 | 100 ± 0.0 (SD) | 6 |
| 0.1X | 61 | 20 ± 3.8 | 3 |
| Stationary Cell Wash | | | |
| 1X | 0 | 100 ± 0.0 (SD) | 6 |
| 0.1X | 11 | 83 ± 3.5 | 6 |
| 0.05X | 50 | 34 | 2 |

[a]Growing and nongrowing Ax-3 amoebae were washed twice and resuspended in cell wash derived from either exponential or stationary cells and cell number was monitored with the hemacytometer. Cell washes were prepared on three separated occasions for each type of wash, by washing 5 X $10^7$ cells per 35 ml LPS, twice. Cell wash was diluted with LPS.

[b]These values were calculated from the observed increase in cell number achieved by controls.

$10^{-4}$ M cAMP blocked cell number increase, Table 3. In all cases, the arrested cells appeared normal, although somewhat larger than controls, as seen by contrast phase microscopy. When washed and resuspended in fresh medium, the cells resumed dividing and appeared normal. Addition of cAMP to LPS also inhibited mitosis of washed cells in non-nutrient buffer (Table 3). Thus normal mitosis was inhibited by the same agents which inhibit the mitosis uncoupled from cell growth, indicating that the latter phenomenon may be subject to the same controls as are normal mitoses, and therefore, a suitable handle for the study of control of division.

It was also noted that as amoebae approached stationary phase, the cell size increased, as judged by the protein content per cell, Table 4. The increase is by a factor of 2.5 in strain Ax-3 during the period from log phase to stationary phase. The increase occurred in both strains. We have not yet determined whether this increase also occurs when log phase cells are arrested by DI or cAMP.

## Biological Assay For Division Inhibitor

The assay for the presence of active DI during partial purification and characterization was to add varying amounts of the fraction being tested to a culture of rapidly growing

TABLE 3

EFFECT OF cAMP AND OF DI ON CELL DIVISION IN STRAIN Ax-3[a]

| Experiment | Growing cultures | Washed cells in LPS |
|---|---|---|
| | Cell number increase after 24 hr (%) | Cell number increase after 12 hr (%) |
| Control | 180 | 93 |
| $10^{-5}$M cAMP | 200 | 102 |
| $10^{-4}$M cAMP | 0 | 5 |
| 2-5 X $10^{-4}$M cAMP | --- | 3 |
| DI[b] | 0 | 0 |

[a]Growing cultures of Ax-3 at cell densities of 0.5 X $10^6$ to 2.0 X $10^6$/ml were used to test the effects on mitosis of cAMP and of partially purified DI. Washed cells were resuspended in fresh LPS, to which cAMP had been added. Cell counts were done at intervals and amount of increase calculated. All results shown are the average of three trials.

[b]Cell wash from 2 X $10^9$ stationary cells was concenstrated and partially purified by an Amicon ultrafiltration apparatus filter $PM_{10}$, dialyzed, lyophilized and resuspended in 5 ml of 5 mM ammonium acetate. After sterilization by Sweenex filter, 50 λ was added to 50 ml of growing culture.

TABLE 4

PROTEIN CONTENT PER CELL DURING GROWTH AND STATIONARY PHASE

| | Cell density | Protein/cell picograms | Number of determinations |
|---|---|---|---|
| STRAIN NC4 | | | |
| Exponential growth | $3 \times 10^6$ to $8 \times 10^6$/ml | 37 ± 5 | 4 |
| Stationary phase | $2.3 \times 10^7$ to $3.0 \times 10^7$/ml | 69 ± 8 (SD) | 8 |
| STRAIN AX-3 | | | |
| Early log phase | $10^6$/ml | 46 ± 6.0 (SD) | 4 |
| Log phase | $3 \times 10^6$ to $5 \times 10^6$/ml | 54 ± 6.8 | 4 |
| Stationary phase | $6 \times 10^6$ to $9 \times 10^6$/ml | 81 ± 15 | 14 |
| | $1.2 \times 10^7$/ml | 116 ± 17 | 16 |

Cells that had grown to the densities shown above were washed as described in Materials and Methods. Protein determinations were performed by a modification of the Lowry method, using bovine serum albumen as standard (Oyama and Eagle, 1956).

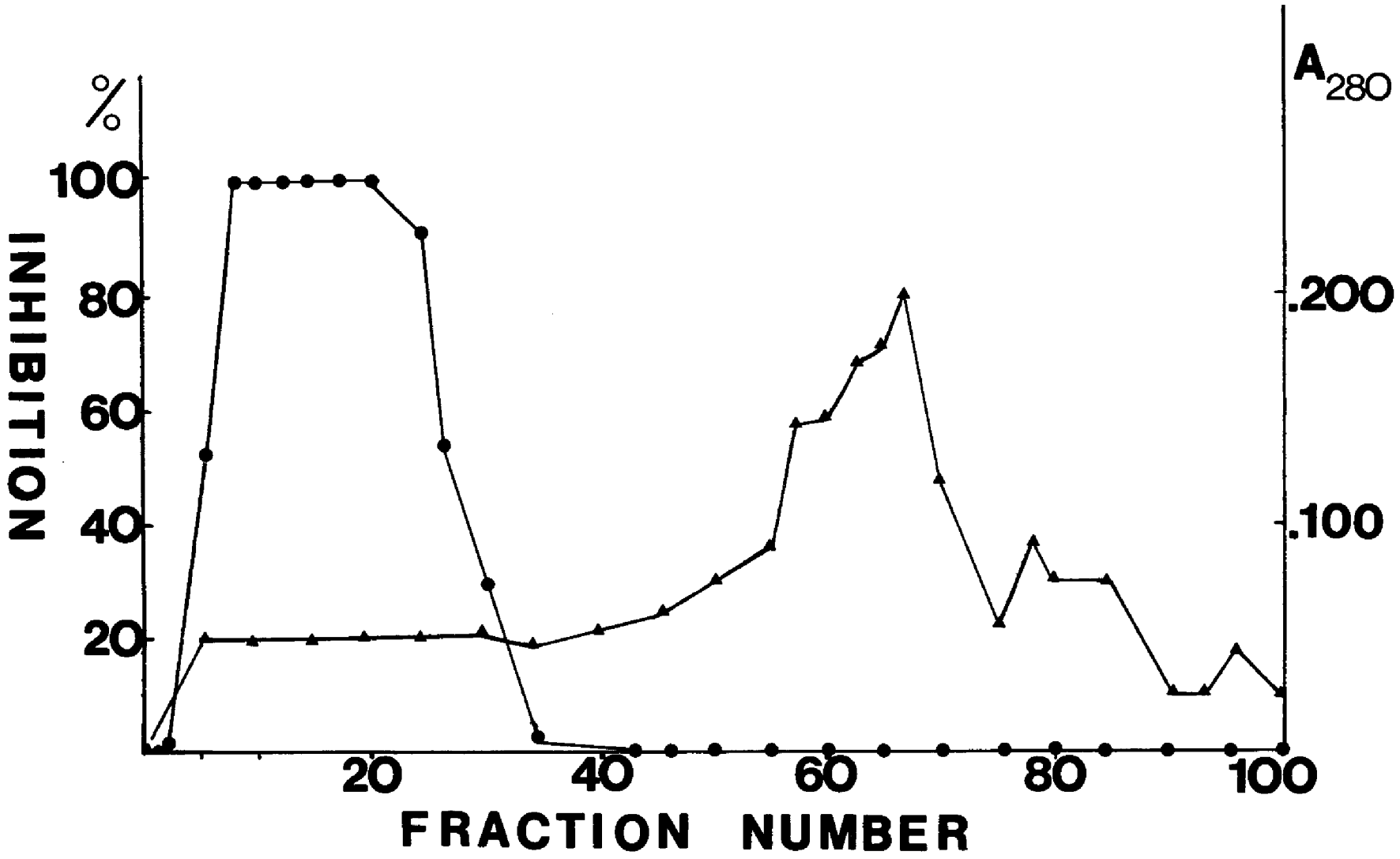
Ax-3 amoebae. After mixing by swirling the culture flask, cell counts were taken and averaged, and cell growth was monitored by sampling cell counts at intervals after the addition. (See Materials and Methods).

## Partial Purification And Characterization Of Division Inhibitor

Figure 3 shows the optical density profile of the high molecular weight fraction of concentrated cell wash eluted from Sephadex G-200, and the cell division-inhibiting activity of the fractions. Division Inhibitor thus prepared remained active when stored at -20°C for at least eighteen months. No loss of activity occurred during this time. The amount required per ml growing cells to achieve 100% inhibition of cell number increase remained the same for one and one-half years. However, the activity is heat-labile. Immersion in a boiling water bath for twenty minutes results in loss of seventy per cent of the inhibitory activity of one milliliter sample of DI. One hundred per cent loss occurred after sterile incubation at 37°C for ten hours.

## Concentration And Complete Inhibition

Figure 4 shows the relationship between micrograms of protein in the partially purified DI concentrate, added to growing cell culture, and the amount of inhibition obtained.


%
INHIBITION
100
80
60
40
20
A280
.200
.100
20
40
60
80
100
FRACTION NUMBER

## LEGEND TO FIGURE 3

Elution Pattern of High Molecular Weight Fraction of Stationary Cell Wash From Sephadex G-200.

Fraction numbers are marked along abscissa. Ordinate represents optical density measurements at 280 mμ, and the percentage of cell number increase inhibition obtained by testing an aliquot of the fraction on a rapidly growing Ax-3 culture (0.05 ml of fraction was added to a 5 ml culture, cell density circa 5 X $10^5$ cells per ml).

Blue dextran was eluted at fraction number one.

3 ml fractions were collected at flow rate of 15 ml/hour.

Seven and one-half micrograms of protein per milliliter of growing culture results in no cell number increase.[1] The graph shows that percent inhibition is directly proportional to protein concentration.

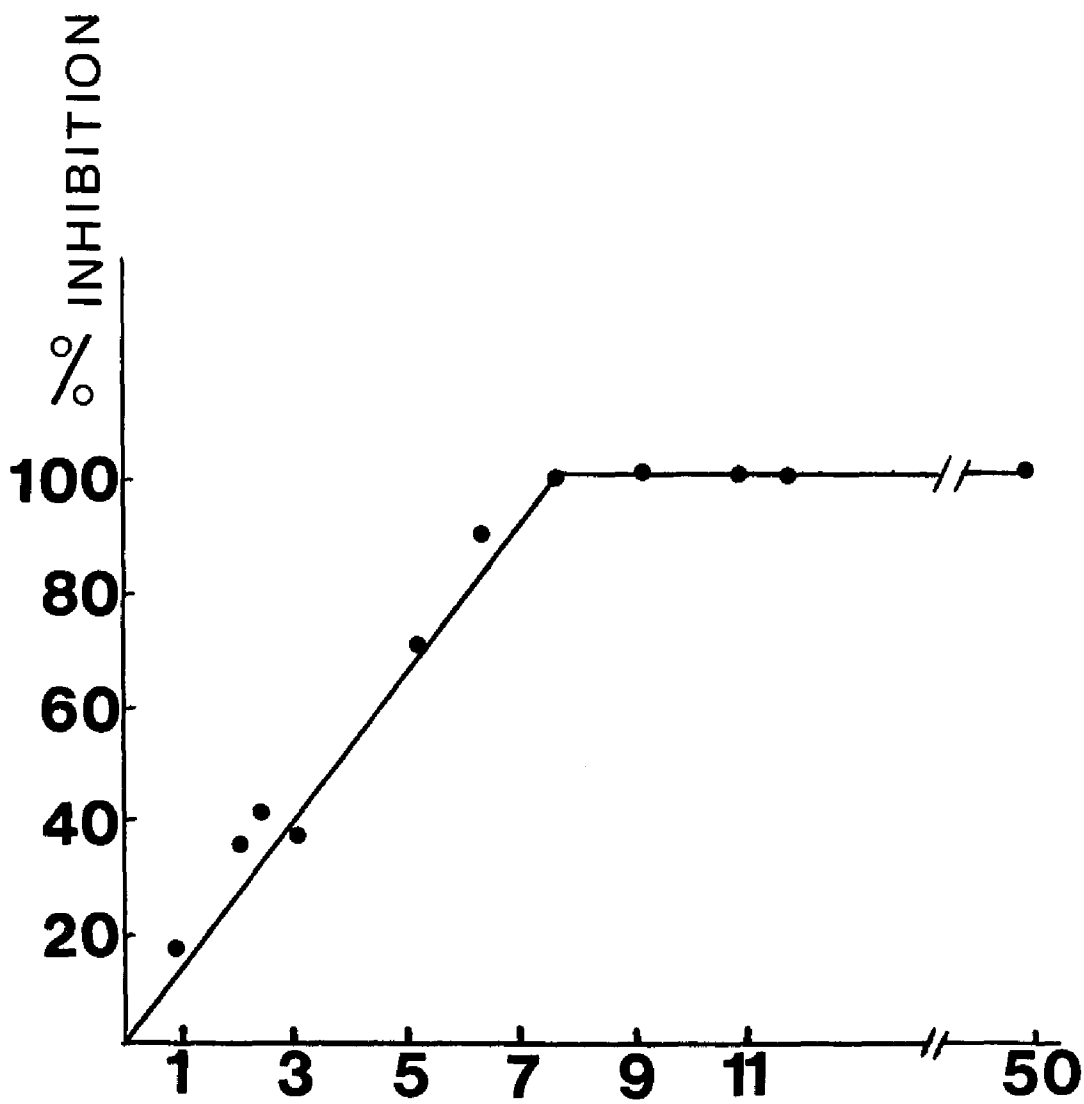
Phase microscopy of the inhibited cells showed they appeared similar to normal stationary cells but slightly smaller. The amoebae were rounded and not lysed. Cells removed from inhibited cultures, after sterile centrifugation to remove DI-containing medium, were capable of growing normally when inoculated into fresh medium. However, a large excess of DI (for example, 50 or more µg/ml cell culture) seemed cytotoxic; many cells appeared abnormally enlarged, extensively vacuolated and considerable lysis occurred.

Effect Of Trypsin On DI Activity

Preincubation of DI with 10 micrograms and with 100 micrograms of trypsin per milliliter was performed at room temperature for one hour and for six hours. The activity of DI remaining after this treatment was subsequently assayed.

The trypsin was not removed from the digested DI before addition to the cell suspension. Controls show trypsin had

[1]Therefore, this was the amount used subsequently, unless otherwise indicated.


% INHIBITION
100
80
60
40
20
1
3
5
7
9
11
50
DI CONCENTRATION
µg protein/ml

## LEGEND TO FIGURE 4

Percent Inhibition of Increase in Cell Number as a Function of Protein Concentration of Partially Purified Division Inhibitor.

Results shown are from twelve trials. Initial cell density was 0.5 to 1.0 X $10^6$ amoebae per ml; cell counts were performed at 16 and at 24 hours after addition of the purified DI.

no effect on cell division at these concentrations (Table 5). Incubation of purified DI with 100 µg trypsin/ml destroyed division-inhibiting activity while preincubation with the lower enzyme concentration resulted in loss of two-thirds of DI activity. This loss is not due to the length of time DI remained at room temperature in Tris Buffer, since that alone resulted in negligible loss of DI activity (Table 5).

Incubation with papain also seemed to destroy the inhibitory activity, although here the results were somewhat difficult to interpret, because papain itself had some inhibitory effect on cellular slime mold cell division at the concentration used (results not shown).

Deoxyribonuclease and ribonucleases had no effect on DI's inhibition of cell number increase (Table 5). Therefore, it may be concluded that activity of DI is protein in nature.

Cell Surface Binding

Since trypsin may be assumed to affect only the cell surface, and since it does destroy DI activity, it was decided to trypsinize DI-treated cells. Elimination of inhibition would show that Division Inhibitor binds to the amoebal cell surface, since trypsin is known to remove the gly-

TABLE 5

EFFECT OF TRYPSIN AND OF NUCLEASES ON PARTIALLY PURIFIED DI ACTIVITY

| | % CELL NUMBER INCREASE AFTER 16 HRS. |
|---|---|
| CONTROLS | 108% |
| CELLS WITH UNTREATED DI | 2% |
| CELLS WITH TRYPSIN (10 µg/ml) | 106% |
| CELLS WITH ROOM TEMP. INCUBATED DI (6 hours) | 6% |
| CELLS WITH TRYPSINIZED DI | |
| 1 hour$_A$ | 60% |
| 1 hour$_B$ | 100% |
| 6 hour$_A$ | 66% |
| 6 hour$_B$ | 136% |
| CELLS WITH DNAse & RNAse | 100% |
| RNAse (2 µg/ml) | 113% |
| DNAse (.4µg/ml) | 105% |
| CELLS WITH DNAse & RNAse-treated DI | 0% |
| DNAse-treated DI | 0% |
| RNAse-treated DI | 5% |

LEGEND TO TABLE 5

Cells were grown to a density of $10^6$/ml, then DI which was untreated, or which had been pretreated with the enzymes(s) to be tested was added to the culture and subsequent cell number increase monitored by counting in a hemacytometer.

Trypsin: A, DI incubated with trypsin at 10 µg/ml.

B, DI incubated with trypsin at 100 µg/ml.

Incubation of the above was at room temperature for one or six hours.

Nucleases: 2 µg DNAse and 40 µg DI and/or 10 µg RNAse and 40 µg DI were indubated at room temperature for one hour.

% cell number increase for controls of all experiments were averaged at top of table. All other figures are averages of at least two trials.

cocalyx from the cell surface (Oseroff, et. al. 1973; Martinez - Palomo, 1971). Results shown in Table 6A indicate that this was indeed true. Adding trypsin at ten micrograms per milliliter to a cell suspension up to one hour after DI addition resulted in normal levels of cell division. An equivalent amount of DI without the enzyme prevented cell number increase (see control, Table 6A). However, when the same amount of trypsin was added two hours or more after the addition of DI to the growing suspension, negligible cell number increase occurred. That is, after two hours, DI activity is not interfered with or destroyed by the presence of the enzyme. This seems to indicate that by two hours after its addition, most of the DI has either left the cell surface, perhaps entering the cell, or possibly becomes sequestered on the cell surface, so that it is no longer susceptible to proteolytic attack. These data, although surprising, are in agreement with the previous finding, shown in Table 6B, that higher levels of trypsin, when added to cultures approaching stationary phase, failed to stimulate cell division. Table 6C shows that addition of trypsin to unwashed cells suspended in phosphate buffer failed to stimulate division. However, it was shown above that washing the cells resulted in their subsequent division (Table 1).

The effects of added DI are reversible at any time, by

TABLE 6

EFFECT OF TRYPSIN ON CELL DIVISION

| | % CELL NUMBER INCREASE AFTER 16 HRS. |
|---|---|
| **A. IN DI-TREATED CELLS** | |
| CONTROL (NO TRYPSIN) | 2% |
| TRYPSIN ADDED 0.5 H AFTER DI | 100%* |
| 1 H AFTER DI | 96%* |
| 2 H AFTER DI | 8%* |
| 5 H AFTER DI | 3% |
| **B. ON ONSET OF STATIONARY PHASE** | |
| CONTROL | 26% |
| 70 µg trypsin / ml | 24% |
| 100 µg trypsin / ml | 30% |
| **C. ON UNWASHED CELLS IN NONNUTRIENT PHOSPHATE BUFFER** | |
| CONTROL | 0% |
| 5 µg trypsin / ml | 0% |
| 10 | 0% |
| 30 | 0% |

LEGEND TO TABLE 6

All results shown are averages of two trials, except those marked with an asterisk (*) which are averages of 5 trials.

A. Cells had grown to $10^6$/ml, were incubated with 7.5 µg DI/ml and after intervals, 10 µg trypsin /ml was added.

B. Cells had grown to 9 X $10^6$/ml and the effect of varying amounts of trypsin on cell number increase measured.

C. Cells which had grown to 3 X $10^6$/ml were pelleted, resuspended in LPS at a cell density of $10^6$/ml, and cell number monitored.

washing the treated cells and returning them to fresh medium (Table 7). Even after 24 hours of total inhibition, the amoebae were able to undergo division upon removal of the inhibitor by washing (Table 7). Therefore, the temporary binding of DI by cells does not initiate an irreversible chain of events, leading to static cell number even after removal of DI. In summary, these results indicate that endogenous and/or added partially purified DI can be removed by washing at any time (Table 7). However, DI is accessible to trypsin only shortly after it has been added to the amoebae; subsequently it becomes impervious to attack by trypsin (Table 6).

## Concanavalin A And Division Inhibition

Since DI appears to interact with the cell surface, at least initially, it was decided to examine the effects of Concanavalin A on division-inhibited cultures. Concanavalin A (Con A) is a plant lectin which seems to interact with carbohydrate determinants on the cell surface (Inbar and Sachs, 1969; Edelman, et. al. 1973). Others working with cellular slime molds have also made the assumption that "since it has been well established that Con A interacts with cell surfaces, for other organisms, it is possible that this lectin exerts its effects on slime molds in the same

TABLE 7

EFFECT OF WASHING ON GROWTH OF EXPONENTIAL CELLS TREATED WITH PARTIALLY PURIFIED DI

| | % CELL NUMBER INCREASE 16 HRS. AFTER WASH |
|---|---|
| CONTROL | 100% |
| DI | 0% |
| DI ADDED, 5 MINUTES BEFORE WASH | 82% |
| 60 | 88% |
| 90 | 83% |
| 120 | 92% |
| 24 HOURS | 110% |
| NOT WASHED* | 4% |

Cells which had grown to $10^6$ml were incubated with 7.5 µg DI/ml and washed after intervals shown, and resuspended in fresh medium. Note that the 24 hour sample remained constant in cell number until after washing.

*Cells incubated with DI for 30 minutes to two hours were collected by centrifugation and resuspended at the same cell density in fresh medium, results shown are the average of seven experiments. Above results are averages of two trials.

manner" (Gillette and Filosa, 1973). It has been shown that this lectin agglutinates Dictyostelium discoideum amoebae (Weeks, 1973). In this study (2 µg Concanavalin A /ml), caused a one hundred percent increase in the number of doublet cells as compared to controls after only ten minutes of shaking the culture to which the lectin has been added (data not shown). This indicates that the lectin-cell surface interaction occurs rapidly. Further, at all concentrations of Con A below 100 µg/ml there was no appreciable interference with cell number increase (data not shown).

The results summarized in Tables 8 and 9 demonstrate the effect of Con A on the activity of the Division Inhibitor. In the first set of experiments shown in Table 8, DI was added to rapidly growing cells and at varying lengths of time afterward the lectin was also added. The effect of Con A binding reversed the effect of the DI until three hours after addition of the Inhibitor, at which time the division inhibition seems irreversible. This is in agreement with the proteolysis data presented above: (Trypsin added within 2 hours after DI prevented inhibition). When Con A was added to the culture the ability of subsequently added DI to block cell division is greater the sooner it is added (Table 8). When DI is added one hour after Con A, only 29% of cells divided but when added after three hours, 48% of

TABLE 8

EFFECT OF CONCANAVALIN A ON CELL DIVISION INHIBITION

| ADDITIONS TO CULTURE | % CELL NUMBER INCREASE AFTER 16 HOURS |
|---|---|
| DI | 2% |
| DI: AFTER 0.5 HOUR, CON A | 57% |
| 1 HOUR | 63% |
| 3 HOURS | 5% |
| CON A: NO DI | 98% |
| DI ADDED AFTER ONE HOUR | 29% |
| TWO | 36% |
| THREE | 48% |

Results shown are averaged from four separated experiments. Cultures utilized had grown to cell density of $10^6$ amoebae/ml. Concentration of Con A was 4 µg / ml.

TABLE 9

EFFECT OF HAPTEN INHIBITION ON CONCANAVALIN A

| SIMULTANEOUS ADDITIONS TO CULTURE | | | % CELL NUMBER INCREASE AFTER 16 HRS. |
|---|---|---|---|
| DI & CON A | | | 16% |
| DI & $\alpha$-METHYL-MANNOSIDE | ( $10^{-8}$M) | & CON A(4 µg/ml) | 17% |
| DI & $\alpha$-METHYL-MANNOSIDE | ( $10^{-6}$M) | | 46% |
| DI & $\alpha$-METHYL-MANNOSIDE | ( $10^{-8}$M) | | 43% |
| $\alpha$-METHYL-MANNOSIDE | ( $10^{-6}$M) | | 50% |
| | ( $10^{-8}$M) | | 59% |

Cells employed had grown to an initial cell density of $10^6$ amoebae / ml. Results shown are the averages of two experiments.

the cells divided. Note that Con A partially protects the cells from the effects of the inhibitor. See also first line of Table 9. This may indicate that DI and Concanavalin A may bind to the same site on the cell surface, a site which became somehow protected several hours after DI binding occurred. Many other possibilities also exist and some will be discussed below.

When DI and 4 µg Con A/ ml are added simultaneously to growing culture only 16% of the cells divided within the next 16 hours (Table 9). If DI is added one or two hours after the lectin, twice as many mitoses ultimately occurred (Table 8). Gillette and Filosa (1973) found that Con A induced membranebound phosphodiesterase to appear on the cell surface after circa one hour. However, this enzyme induction was blocked by α-methyl-glucoside (Gillette and Filosa, 1973). It has been suggested that DI acts through the elevation of cAMP levels, (Hanish, 1975) which is lowered by the Con A induced enzyme. Therefore we examined the effect of the simultaneous addition of the hapten inhibitor, α-methyl-D-man noside, with DI and Con A. Table 9 shows that the same degree of inhibition was obtained with the DI, glycoside and lectin as with the lectin and DI. In other words, the glycoside had no effect. The glycoside was itself inhibitory of cell division. These data indicate that

that the Con A interference with the effect of DI is not due to its activation of the membrane bound phosphodiesterase. Some other mechanism of action of Con A is therefore responsible for inactivation of DI: perhaps Con A's restructuring of the cell surface by altering the positions of various receptors in the fluid mosaic membrane (Edelman, 1976).

## Adsorption To Cells

DI seemed to be rapidly adsorbed or absorbed by exponentially growing cells, as may be inferred from the indication that cell division was blocked immediately upon its addition. A small increase in cell number (less than 5 percent) cannot be excluded due to sampling error. (Table 10). When cell wash was preincubated with washed cells, it lost its cell division inhibiting activity, Table 10, compare line 1 and line 2. That is DI was removed from cell wash by the brief presence of washed cells. Forty-four percent of the second batch of cells divided in the DI-depleted cell wash (Table 10, line 2). The inhibition was transferred from the cell wash to the washed cells (Table 10, line 3). Instead of undergoing one round of cytokinesis as controls do (Table 10, line 4), the washed cells transiently placed in cell wash, underwent no increase in cell number when placed in fresh phosphate buffer. This leads to the

TABLE 10

REMOVAL OF INHIBITORY ACTIVITY FROM CELL WASH BY PASSAGE OF CELLS THROUGH IT (BY CELLULAR "AFFINITY" CHROMATOGRAPHY)

| PROCEDURE | % CELL NUMBER INCREASE AFTER 12 HRS. |
|---|---|
| 1. WASHED CELLS RESUSPENDED IN CELL WASH | 1 % |
| 2. WASHED CELLS RESUSPENDED IN TREATED CELL WASH | 44 % |
| 3. WASHED CELLS INCUBATED WITH CELL WASH 15', RESUSPENDED IN FRESH LPS | 0 % |
| 4. WASHED CELLS, RESUSPENDED IN FRESH LPS | 93 % |

---

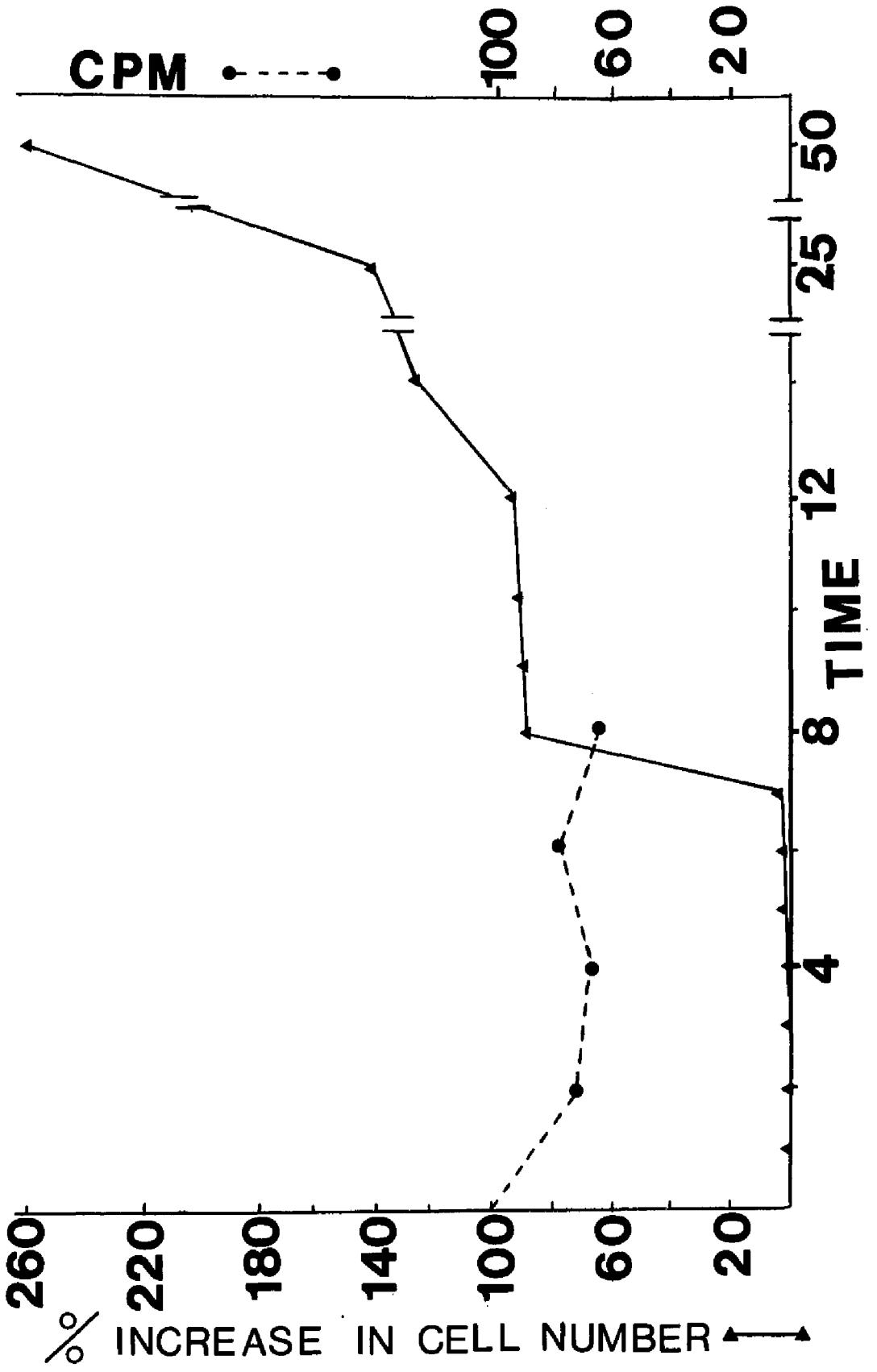
Cells which had grown to 2 x $10^6$/ml were washed as previously described. Cell wash was then divided into two aliquots; one was untreated and immediately tested for its division inhibition while the other was incubated with washed amoebae for 15 minutes, and then tested for its division inhibiting activity with another batch of freshly washed amoebae.

conclusion that DI, the active agent of the cell wash is "picked up" by the amoebae very rapidly, yet adhered to the cells during centrifugation and resuspension, unless washing occurred.

## Cell Division Synchrony

Since synchronization of cell division occurs after washed cells were resuspended in nonnutrient buffer, we examined the timing of cytokinesis subsequent to washing stationary axenic cells and resuspending them in fresh growth medium. The first cell division in fresh growth medium did indeed occur synchronously as shown in Figure 5. For the first six hours after resuspension, less than 1% cell number increase occurred, while during the seventh to eighth hour, 90.4% of the cells divided. Little cell number increase was observed for the next fifteen hours after which the cells grew asynchronously. Note that the first cell division in growth medium occurred several hours prior to those occurring after resuspension in LPS (see Table 1). This may indicate that nutrient concentration may play a role in cell division.

Stationary cells appeared to be blocked at one phase of the cell cycle since after being washed, they divided synchronously (Figure 5). At what point in the cell cycle


CPM
100
60
20
50
25
12
8
4
TIME
260
220
180
140
100
60
20
% INCREASE IN CELL NUMBER

LEGEND TO FIGURE 5

CELL DIVISION SYNCHRONIZATION AND DNA SYNTHESIS IN WASHED STATIONARY AXENIC CELLS

▲———▲% cell number increase plotted against time (average of 10). ●– – –●Incorporation of $^{3}$H-methyl-Thymidine into TCA-precipitable material in two hour pulses as percentage of that incorporated into asynchronous rapidly growing cells in two hours (average of 3 trials).

Stationary cells were twice washed and resuspended in fresh growth medium. Cell counts were taken hourly, at least, up to 10 hours. Radioactive precursor incorporation: 1 μC $^{3}$H-methyl-Thymidine/ml was incubated for a two hour period, either with washed cells or with controls (rapidly growing cells). Results were expressed as CPM per million cells, and plotted as percentage of control.

the block occurred is not known. However, labeling washed stationary cells for two hour periods with $^{3}$H-methyl-Thymidine prior to their synchronous division resulted in a contant level of incorporation (Figure 5). A peak in incorporation would have indicated the occurrence of S phase, the period of nuclear DNA synthesis. Lack of a peak suggests the block occurred later in the cell cycle, possibly in G2. This suggestion is corroborated by the data in Table 13, which shows that stationary cells have almost twice the DNA content of growing cells. We note that there was considerable thymidine incorporation, although levels of incorporation were lower than in asynchronous rapidly growing cultures (Figure 5). This may possibly be due to continued mitochondrial DNA synthesis only, since mitochondrial DNA has been estimated to be thirty percent of the total DNA in Dictyostelium (Firtel and Bonner, 1972).

## Effects On Nucleic Acid Synthesis

DI had no inhibitory effect on incorporation of $^{14}$C-Uridine into acid-precipitable material, Table 11. In fact there seemed to be a slight increase in incorporation several hours after the addition of the DI. DI also seemed to have no significant effect on degradation of total prelabeled $^{14}$C-Uridine-containing acid precipitates (data not shown).

TABLE 11

EFFECT OF PARTIALLY PURIFIED DI ON $^{14}$C-URIDINE INCORPORATION

| | CPM/$10^6$ CELLS | |
|---|---|---|
| | CONTROL | DI |
| 7½h | 788 | 974 |
| 20h | 2,461 | 2,555 |

---

Uridine-2-$^{14}$C /Amersham/Searle, 60 mCi/m mole, lot number 315 B31/ was added to cells in early exponential growth to a final concentration of 0.25 uCi/ml. The cell suspension was then divided into two aliquots: one served as control; Division Inhibitor was added to the other (7.5 µg/ml). Cell density of each was measured by use of a hemacytometer. At intervals, samples were withdrawn and precipitated with cold trichloroacetic acid. Simultaneously, cell density was again measured. Results are expressed as CPM per million cells.

Data shown was the average of 2 experiments. Similar results were obtained with $^{3}$H-Uridine /New England Nuclear 3.66 Ci/mmol./ By 24 hours incorporation diverged from linear.

Table 12 shows DI does not inhibit $^{3}$H-methyl-thymidine incorporation for the 1st several hours after its addition. There seems to be some stimulation at first. However, towards the end of 1 generation, incorporation into DNA in mitotic inhibited cells seems to have been arrested, although incorporation continues to increase in the controls. This may be interpreted as follows: DI does not primarily interfere with DNA synthesis but is a specific noncytotoxic inhibitor of mitosis in the G2 phase of the cell cycle.

If such is the case and if it is also indeed true that DI is a causative agent in the onset of stationary phase, one must conclude that stationary Dictyostelium discoideum cells are in the G2 phase of the life cycle. This was suggested by the work of Katz and Bourguignon in 1974, in a study of synchronized temperature-sensitive Ax-3 mutants, in which they showed that aggregation occurs in mid G-2. Also the early work of Bonner and Frascella, 1952, which showed a round of mitosis occurred after aggregation, indicates that stationary cells may be in G2. We have measured the DNA content of growing and of stationary populations of amoebae using the diphenylamine method of Giles and Meyers after extracting DNA according to the Schmidt-Thannhauser procedure. The results are shown in Table 13. One would ex-

TABLE 12

EFFECT OF PARTIALLY PURIFIED DI ON $^{3}$H-METHYL-THYMIDINE INCORPORATION

| TIME | CPM PER $10^6$ CELLS CONTROL | CPM PER $10^6$ CELLS EXPERIMENTAL |
|---|---|---|
| 1½ h | 32 | 39 |
| 3 h | 41 | 55 |
| 4 h | 51 | 76 |
| 5½ h | 72 | 64 |
| 11 h | 91 | 55 |

---

$^{3}$H-Methyl-Thymidine (Schwarz/Mann lot #ZR-1393) was added to cells in early exponential growth to a final concentration of 0.25 µCi/ml. Purified DI (7.5%/ml) and 2 mM 5'AMP is added simultaneously with the precursor. Four ml samples were withdrawn at intervals and precipitated with cold acid and radioactivity collected on membranes for counting by liquid scintillation.

Data shown is the average of 2 experiments, each done in duplicate.

Total counts per sample averaged 300 and over.

pect if stationary cells are indeed in G2 while the growing cells are asynchronous, that the stationary population would have at least fifty percent more DNA as the asynchronous population. There is indeed an increase in DNA content at stationary phase.

An interesting feature of DI's effect on rapidly growing cells is that its activity was destroyed by simultaneous addition of nucleosides to the culture. Although at least 95% mitotic inhibition was expected in uridine or thymidine incorporation experiments. Only circa 50% inhibition of cell division resulted in the former, while in the latter, no cell division whatever occurs in the first 5 hours after DI treatment has begun, but by the end of 1 generation time, no inhibition still existed: the cells had resumed normal growth rates.

In an attempt to circumvent this nucleoside-abolition of mitotic inhibition, Adenosine-5'-Monophosphate was added at different concentrations to rapidly growing cells with and without added partially purified DI. 5'AMP alone, and thymidine alone had no inhibitory effect on cell division at the concentration used. When thymidine and 5'AMP are added simultaneously, there is still no mitotic inhibition. Yet when a rapidly growing culture was made 2mM in 5'AMP, thymidine no longer abolishes the effect of the DI and 95%

TABLE 13

DNA CONTENT OF GROWING AND STATIONARY Ax-3 *DICTYOSTELIUM discoideum*

| | $\mu g$ DNA PER $10^8$ CELLS | | |
|---|---|---|---|
| Growing Cells | 10.5 | Stationary | 20.0 |
| | 11.0 | | 22.0 |
| | 17.9 | | 26.2 |
| | 14.0 | | 29.8 |
| | 17.8 | | 25.6 |
| | ——— | | 33.2 |
| M = | 14.2 ± 4.3(S.D.) | | 30.5 |
| | | | ——— |
| M = | | | 26.9 ± 7.5(S.D.) |

DNA isolation was performed according to the Schmidt-Thannhauser Procedure. A diphenylamine test was then performed, according to the method of Giles and Meyers. The results shown are form individual experiments done over a period of time. (S.D. is the standard deviation)

mitotic inhibition was seen. Therefore, our thymidine incorporation experiments were performed in the presence of 2mM 5'AMP. Lower concentrations of 5'AMP innitially had the same effect of restoring DI activity. However the restored inhibition disappeared after several hours and 2mM 5'AMP was the lowest concentration capable of insuring no cell number increase for approximately one generation time. Incorporation of $^{3}$H-methyl-Thymidine was not affected by the addition of 2mM 5'AMP in both control and DI-treated flasks.

The rationale which led to the investigation of the effect of 5'AMP on thymidine's interference with DI was as follows:

1) It has been suggested previously that DI may work by stimulating adenyl cyclase activity (Hanish, 1975). Rossomando and Sussman (1973) have reported that 5'AMP stimulates the activity of adenyl cyclase in Dictyostelium.

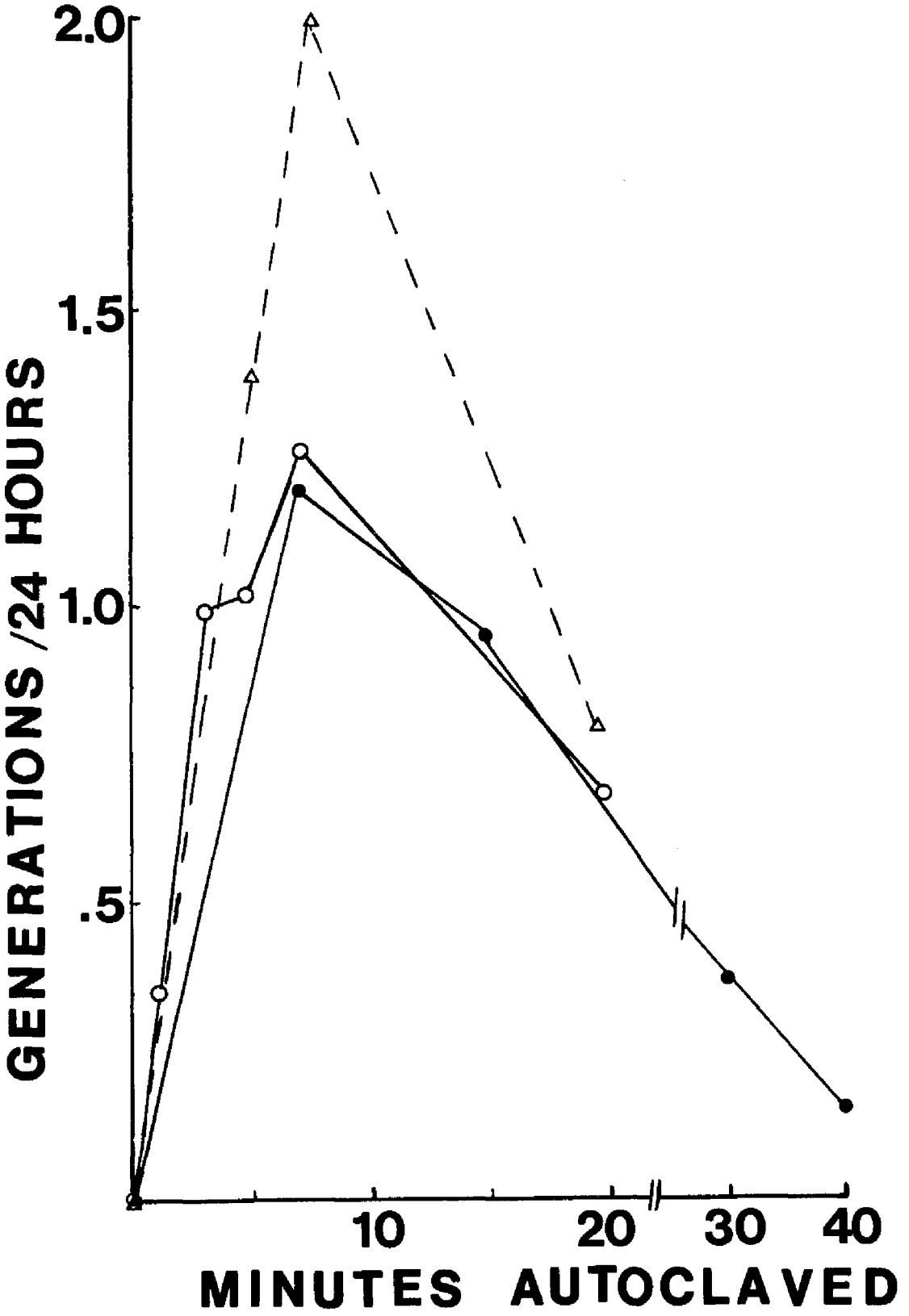
2) In the regulation of deoxyribonucleotide biosynthesis in E. coli, deoxythymidine triphosphate stimulates the reduction of ATP to deoxyadenosine triphosphate, thus driving the equilibrium of the reaction ATP→ADP→dADP→dATP to the right. (Lehninger, A.L. 1970) If this phenomenon exists in the Acrasiales also, adding thymidine would eventually deplete the pool of ATP (adenyl cyclase's substrate). As dTTP accumulates so would dATP.

The fact that adding 5'AMP does counteract the effect of thymidine on DI does reinforce the hypothesis that DI's effect may be on the adenylcyclase. (see also discussion on Con A effects, of phosphodiesterase activity, below.)

Addition of up to 2mM 5'AMP did not alter 3H-methyl-thymidine incorporation: virtually identical counts were obtained in its presence as in its absence (results not shown). However, the decreased incorporation found in the later intervals was found only in the inhibited cultures, i.e. in cultures to which both 5'AMP and DI were added. Cultures to which DI alone was added exhibited no alteration in either label incorporation or extent of division 6-22 hours after DI addition.

Depletion Of Nutrients And Termination Of Cell Division

Potts (1902) attributed the end of growth phase to depletion of the nutrient source (Bonner, 1967). This has remained the accepted cause of cessation of growth until the present. Recently, this conclusion has been accepted even for amoebae (Ax-3) grown in axenic media (Yarger et. al. 1974). Yarger, Stults and Soll, have claimed that autoclaving conditioned medium does not affect its incapacity to support cell growth (Yarger, et. al.) This is contray to our findings. Figure 6 shows that washed cells did increase


2.0
1.5
1.0
.5
GENERATIONS /24 HOURS
10
20
30
40
MINUTES AUTOCLAVED

LEGEND TO FIGURE 6

Cell growth in autoclaved conditioned medium. Conditioned medium was obtained by pelleting stationary cells (strain Ax-3). Rates of growth (ordinate) for three cultures, at different cell densities, after resuspension in conditioned medium, which had been autoclaved for varying lengths of time, are shown. Autoclaving is performed at 120°C, 18-22 lb/in.$^2$ Δ- - - - - -Δ , cells which had brown to $10^6$/ml were pelleted and resuspended at 4 X $10^5$/ml. o_____o, cells which had grown to 6 X $10^6$/ml; were resuspended at 7 X $10^5$/ml. ●_____●, cells were not washed.

in number in autoclaved conditioned medium. However, since washed cells divide in nonnutrient buffer as well as in autoclaved conditioned medium, we repeated that work using unwashed log phase cells. Again, we found that cell number increase did indeed occur (Figure 6). Furthermore, this increase in numbers was accompanied by an increase in cell mass, as measured by protein content (data not shown). Thus the cessation of growth in axenic medium cannot be due to exhaustion of nutrients, as others have claimed (Yarger et. al., 1974). However, insight into this problem has been obtained from the data described herein.

We conclude that nutrients are present in conditioned medium, and are not made available to amoebae as a result of the autoclaving. We have found that initial autoclaving of fresh medium as opposed to its sterilization by filtration makes no difference to the number of cells which can be grown in the medium subsequently. Neither does multiple autoclaving of fresh medium augment growth. Since autoclaving makes no nutritional addition before growth, it is unlikely that it would do so after the medium has supported the growth of a culture. Three possible indirect effects of heating conditioned medium have been considered: (1) We exclude the possibility that autoclaving conditioned medium restored its original pH. Fresh medium had an average pH of

6.5, while that of stationary medium was 6.9, and autoclaving further increased the pH to 7.1 (data not shown). (2) Aerating conditioned medium for 76 hours had no effect upon its incapacity to support cell growth. This indicates that heating did not effect the change in stationary medium by expulsion of trapped gases. Similar results on these two points have been reported by Yarger et. al. A final possibility was that heating released additional nutrients. Cultures grown in axenic medium attained a stationary cell density of $1.3 \times 10^7$/ml, whether the medium has been sterilized by autoclaving or by filtration (data not shown).

Therefore, it may be concluded that conditioned medium contains nutrients and a heat-labile inhibitor of cell division. Thus, the question of why growth ceases cannot be answered by the traditional explanation.

Summary

The data presented above show that a heat-labile macromolecular inhibitor of cell division was present in the cellular slime molds, during rapid growth as well as at the end of growth. It was easily removed from the cells by washing and was reconstituted with them after removal. It was partially purified by chromatography. It was protein in nature. It bound to the cell surface during reconstitution, and remained there available for proteolytic attack or lectin-induced exchange or removal for several hours after initial addition. Subsequently the inhibitor, although still active, becomes protected from those agents. It seemed to arrest amoebae in the G2 phase of the cell cycle, since its removal stimulates synchronous division without any apparent peak in thymidine incorporation.

Stationary cells had almost double the DNA content per cell, than rapidly growing amoebae, possibly indicating the latter were in G2.

Protein content of stationary amoebae is two - to three-fold that of growing amoebae. More DI activity is removeable from stationary cells than from growing cells. Therefore it is suggested that the increasing protein concentration may be due to the increasing DI concentration or activity during growth, before the DI activity rises enough to in-

hibit growth phase entirely. DI had no marked effect on either $^{14}$C-Uridine incorporation or turnover, nor did it inhibit DNA synthesis for some ten hours after its addition (by which time all cells had presumably completed S and entered G2 phase).

Cells from which DI had been removed underwent synchronous cell division. Finally, conditioned medium seemed to contain nutrients and supported normal growth (increase in cell mass as well as in numbers) of amoebae, upon heat-inactivation of the DI.

# DISCUSSION

## Cell Surface Interactions Of Division Inhibitor

Evidence has been presented which is consistent with the thesis that a cellular division inhibitor exists and is a heat-labile protein. It seemed to exert its effect on cell division through the cell surface, at least initially, because although its inhibition of cell number increase occurred simultaneously with its addition to a growing culture, DI may be destroyed by trypsin, or displaced or inactivated by Con A for several hours after addition. Con A added to a culture up to one hour after DI restored 60% of the expected cell number increase (Table 9); but three hours after the addition of DI, the lectin no longer interfered with the division inhibitory effect. However, Con A interference with DI is not abolished by the addition of ∝-methyl-mannoside. In addition, this glycoside itself abolished DI's effect. (Table 10).

There are several possible explanations for these data: I. Perhaps the most probable effect of Con A on division inhibition is due to its widely documented effect of anchorage modulation of components of the cell surface. This is described as a propagated phenomenon restricting movement of various receptor sites and other cell surface features, concomitant with cross-linkage of certain glycoproteins of

of the cell surface, which presumeably restricts or alters their normal reactivity (Edelman, et. al., 1973). This "global anchorage modulation" which involves changes in anchorage and motility of all surface receptors also seems involved in the regulation of cell movement and growth, as Edelman has indicated. Submembranous structures, including microfilaments and microtubules, seem involved in the cell surface-regulated control of cellular motility and replication (Edelman, 1976). (Note that microtubules, which also play direct roles in mitosis are influenced by cAMP concentrations, (Marx, 1973) which may be affected by DI (Hanish, 1975). Therefore Con A may act through surface modulation on DI's receptor site and/or may affect submembranous components of the surface Modulating Assembly (described by Edelman) interfering with the communication of the state of the cell surface with other cell elements involved in control of cell replication.

II. There is a possibility that Con A and DI may bind to the same carbohydrate moieties of the cell surface; however, this is untestable at present especially in the absence of the isolated receptor sites from the surface, if such do indeed exist.

III. Another possibility is that Con A may counteract the DI's hypothetical mode of action, which we have suggested to be the activation of adenyl cyclase (Hanish, 1975) by

stimulating the activity of the membrane-bound phosphodiesterase of the cellular slime molds (Gillette and Filosa, 1973). They found that approximately one hour was needed for the Con A - induced membrane-bound phosphodiesterase to appear on the cell surface. The timing coincides with our results: lectin added before DI resulted in many more mitoses than if additions were simultaneous (Tables 5 & 6). However, this effect is blocked by ∝-methyl-glucoside (Gillette and Filosa, 1973). Our results show that the same degree of inhibition was obtained with the glycoside and lectin as with the lectin alone (Table 9). Therefore this indicates that the Con A interference with DI activity is probably not due to its effects on the phosphodiesterase.

The concentrations of Con A used by Gillete and Filosa (1973) was approximately one-hundred-fold that used in this study, but since their use of Con A was on agar plates, and not in solution, as was ours, the actual concentration at the cell surface may have been much lower. Although we used the same range of concentration used by Weeks (1973) with the same organism, we did not find 90% of the cells clumped together, as he did. This would have seriously interfered with cell counts. Instead we observed a 150% increase in the number of doublet cells after ten minutes in the presence of the lectin). The difference may be due to the speed of shaking of the cultures.

It may be relevant that in mouse fibroblasts, low levels of cAMP are correlated with increased Con A agglutinability while high cyclic nucleotide levels correlate with decreased lectin-induced agglutinability (Willingham and Pastan, 1974). Their findings led the authors to the conclusion that in normal and transformed cells the level of cAMP regulates the agglutinability. The mechanism through which the cyclic nuleotide operates is unknown; however, it has been shown that changes in the gross structure of surface membrane (e.g., appearance of microvilli) during mitosis and transformation as well as after trypsin proteolysis, are associated with agglutinability (Rutter et. al. 1973). This may indicate cAMP plays a regulatory role in the state of the cell surface. There seem to be two alternative states. One is associated with cell division, surface proteolysis, low cAMP concentration, surface microvilli, high agglutinability, and transformation. The other state of the cell surface is associated with the opposites, including low rate of division, and is linked with high cAMP levels (or in the cellular slime molds, presence of DI). These have been termed P and Q, respectively, by Pardee et. al., 1974.

From the evidence and conclusions presented above, we have formed the following speculative model of the cell sur-

face action of the DI:

I. DI only accumulates to effective levels in scarcity of bacteria. Incubation of partially purified DI with washed non-growing bacteria destroyed all DI activity (data not shown).

II. DI binds to a surface site which activates adenyl cyclase.

III. This site is linked to internal metabolic machinery regulating growth--both increase in cell mass as well as in numbers.

IV. After two to three hours of the binding of the DI to the cell surface, some alteration of the receptor-inhibitor configuration occurs, such that the inhibitor is no longer susceptible to surface-acting agents.

V. This alteration may in turn induce the synthesis of the membrane-bound phosphodiesterase. On a solid substrate, a gradient of cyclic AMP concentration would thus be formed. This has been suggested by the data of Gillette and Filosa, who wrote that Con A might be "mimicking a factor produced during normal development which enhances membrane-bound phosphodiesterase (synthesis and) activity at aggregation." Data suggested above indicated there is some possibility that the lectin may bind to the same site as DI. Perhaps DI may be the factor hypothesized by Gillette and Filosa.

Recently, Ferguson and Soll (1976) have reported that

washing amoebae in LPS did not result in cell number increase. We believe their contrary findings were due to difference in methods of washing the cells. Ferguson and Soll used LPS at pH 5.4, while we had used that buffer at pH 6.4. More importantly, they washed $10^8$ cells in 5 mls of buffer, which was one-fourteenth the volume recommended in our published report (Hanish, 1975). We can only reiterate that we found the ratio of cells to buffer volume was critical in eliciting the cell division in buffer (Hanish, 1975).

The Presence Of Nutrients In Conditioned Medium

The question of whether nutrients remain available when a culture of cellular slime mold amoebae becomes stationary is a crucial one, because if they are, the traditional view on the cause of termination of growth must be reevaluated. The idea that depletion of nutrients causes cessation of growth of amoebae has recently been reaffirmed by Yarger et. al. (1974). These authors presented evidence that conditioned medium contained a heat-stable dialyzable substance(s) which prevented cell growth. However, in this report we have shown that autoclaving conditioned medium restored its capacity to support cell growth, Fig. 6. We have confirmed that dialysis of conditioned medium against fresh medium restored its growth capacity (Hanish, 1975) while addition of nutrients did not (Yarger et. al., 1974). A possible expla-

nation for the difference in results may lie in the autoclaving conditions: In this study autoclaving of conditioned medium was performed at a temperature 30°C higher than that used by the pervious authors.

It seems possible that the dialyzable inhibitor of division described by Yarger et. al. (1974) may be a breakdown product on the macro-molecular DI described in this thesis. An example of how breakdown of DI and release of degradation products into the medium may possibly occur was furnished by the work of Parish and Muller (1976). They have reported that phagocytic vacuoles fuse with lysosomes in amoebae of cellular slime molds and the contents are excreted during interphase.

Recently, protein content, cell diameter and mass, and DNA content of stationary phase amoebae have been published (Soll et. al., 1976). Although they did not measure DNA content during rapid growth, their report of that at stationary phase (24.3 µg DNA/$10^8$ cells) is close to our finding (26.9 µg DNA/$10^8$ cells), Table 13. Their measurement is the only one published dealing with this particular part of the life cycle of cellular slime molds. Our findings are comparable to previous estimates of DNA content (Sussman and Rayner, 1971; Leach and Ashworth, 1972; Firtel and Bonner, 1972). Soll et. al. also reported a 24% increase in protein content from the onset of stationary phase to 36 hours later;

in general, this agrees with our finding, Table 4.

As to cell synchrony after washing and phase of the cell cycle of stationary amoebae, Soll et. al. have reported similar findings to those shown in Fig. 4. However, these authors did not perform any incorporation studies with the synchronized amoebae. They reported that cell number was constant for four hours and then increased "synchronously" for the next two hours. We found there was a longer lag before the division, followed by a synchronous division occurring over a much shorter time span. The difference may be due to difference in washing procedures. Soll et. al. attributed this phenomenon to the accumulation of stationary amoebae "at a stage late in the cell cycle." Although we agree stationary cells may be in G2, we attribute the synchronous division to the removal of the high MW DI described above. The "synchrony" of the division, per se, is not evidence of cells being in G2. The time lag before the first division, which has been reported as $T_{50}$ 5.3 hours (Soll et. al. 1976) or $T_{50}$ = 7.5 (Fig. 4) may be considered as time needed to traverse S and G2. According to Katz and Bourguignon (1973) the time required for S and G2 is 5 to 6 hours. We consider the best evidence that stationary cells are in the G2 phase of the cell cycle (or completed DNA replication) is the fact that they contain almost double the total cellular DNA of rapidly growing cells (Table 13). Furthermore stationary amoebae contain twice the protein per cell

than growing cells (Table 4). Finally, lack of a peak in thymidine incorporation before the synchronous division (Fig. 6) supports the suggestion that stationary cells are blocked late in the cell cycle.

## Growth Regulation

Diffusible factors which regulate cell division in metazoans were postulated before any evidence for their existence was collected, purely on logical grounds (Weiss and Kavanau, 1957). The presence of such a factor, termed chalone, from the Greek "to trim sail," was deduced in studies of proliferation of mouse epidermis (Bullough, 1973). Such activity has been found since in a variety of other mammalian tissues, including liver, (Verly, 1973) and granulocytes (Paukovits, 1971; Rytomaa, 1973). Chalone is currently defined as a diffusible, noncytotoxic inhibitor of cell proliferation, reversible, in effect and tissue-specific but not species-specific (Bullough, 1973). The epidermal chalone has been identified as a glycoprotein (Laurence, 1973) which indicated it may be a cell surface-interacting molecule.

Inverson (1969) has suggested that the mechanism of action of chalones may involve stimulation of cAMP production. However, the chalone concept is still somewhat controversial and not universally accepted, possible due to the inconclusive data in some reports. Many experiments were done with

crude fractions of cell extracts and interpretation was complicated by the possibility of interaction or antagonism by hormones, the presence of postulated antichalones, and the need for a better assay of chalone activity than fewer mitotic figures.

The data presented on the DI are clear-cut and without all the complications of mammalian cell proliferation control and hence may offer a better system for elucidation of mechanisms involved in control of cell replication.

Several cases of interactions between the surface membrane and cell genome, especially with regard to proliferation, have been studied (Oseroff, et. al. 1973; Wolpert and Gingell, 1969; Houck, 1973; Cooper, 1969; Rutter et. al. 1968, 1973; Crandall, 1974). These include egg activation; agglutination of opposite mating type by cell surface glycoproteins; lectin mitogenesis; contact inhibition of growth factors; peptide hormones; the immune response and mesenchyme factor. In egg activation, a membrane-associated glycoprotein, which is removeable by trypsin and binds Concanavalin A in affinity chromatography, has been identified as a specific sperm receptor in sea urchins (Schmell et. al. 1977). A high molecular weight glycoprotein which is released from the surface of sea urchin eggs at fertilization seems to be involved in maintainance of the low metabolic state of the ova, particularly as involves protein synthesis, with suppression of DNA synthesis

also a possibility (Johnson and Epel, 1975).

Nerve Growth Factor, which is structurally analogous to insulin (Frazier et. al. 1972) can induce temporal changes in embryonic optic tectal cell surface adhesive specificity (Merrell et. al. 1975). Mesenchyme-specific macromolecules interact with epithelial receptors on the cell surface to induce morphogenetic differentiation (Levine et. al. 1973; Ronzio and Rutter, 1974).

The mechanism of action of at least some of these may involve stimulation of adenyl cyclase. For instance, epinephrine has long been known as a reversible G2 inhibitor of mitosis in epidermis (Voorhees et. al. 1973) and cAMP, which is the intracellular mediator of many peptide hormones, is at high concentrations, an inhibitor of mitosis, as well as of DNA synthesis (Froehlich and Rachmeler, 1974).

It appears that the Division Inhibitor of the cellular slime molds must be added to this list. Results presented above show DI is reversible, noncytotoxic and probably acts at a specific phase of the cell cycle.

## Similarities To Other Systems

The restoration of DI activity upon its addition to cells, is reminiscent of reconstitution experiments performed in cell adhesivity studies, in which it has been found that removal of one or more macromolecular components from the cell ligand

system can be reversed by addition of the removed substance to the cells, effecting a functional ligand once more (Balsamo and Lilien, 1974; Weinbaum and Burger, 1973). However, the system most closely resembling the findings herein reported is the large, external (cell surface), transformation sensitive (LETS) protein of mammalian cells independently found and named by six different laboratories (Hynes, 1974). LETS is found on normal cell surfaces, but is absent from, and not merely masked in viraltransformed cells; it is released into serum and is removeable from the cell surface by trypsin; it is absent (possibly released) in mitosis; rapidly growing cells have less of it per weight cell membrane protein than do cells inhibited in confluency (Hynes, 1974). LETS mediates adhesion of fibroblasts to collagen and has been found to span the cell membrane, which has led to the suggestion it may be involved in modulation of receptor mobility and in growth control (Perlstein, 1976). It has been suggested to directly bind lectins (Hynes, 1974).

It therefore appears that DI is an example in a very simple experimental system of the same phenomenon occurring in higher organisms, and is hence, an approach to investigations which may prove too complicated by serum growth factors, hormones and the many other factors in mammalian systems.

Phases Affected By DI:

The Division Inhibitor seemed to affect only vegetative growth phase, since washing cells before allowing them to develop on Millipore filters had no significant effect on the timing of aggregation. Since a round of cytokinesis occurred after aggregation on filters (unpublished observation) as well as on agar (Bonner and Frascella, 1952), it is possible that cell contact abolished the activity of DI endogenously present at stationary phase.

## The Value Of The Cellular Slime Molds To Biology

Biologists are interested in the Acrasiales because of the three distinct phases of their life cycle and the ease with which they are induced to enter a different stage, their short generation times and the simplicity of growing very large numbers easily and inexpensively. There are now several axenic strains, use of which eliminates possible sources of error in biochemical studies from the contribution of semi-digested or undigested bacterial molecules.

Also of interest is the fact that cells acquire new biochemical constituents on schedule, under defined conditions. This led to the conclusion that the cellular slime molds have three sets of genetic information; a "housekeeping" set, a vegetative genome, controlling vegetative stage syntheses and a developmental set, controlling only development stage structural protein and enzyme sythesis (Ashworth, 1971) and this has been shown to be the case:30% of the genome is involved only in gene products of the vegetative stage, and 50% is the genetic information necessary only in development (Firtel, 1972). However, exactly how the switch from one genetic set to another is effected is totally unknown, as is the case for most eukaryotic regulation, except for the case of steroid hormones. Therefore, the Acrasiales, being such a simple systen apparently without hormones, and growth factors, tissue interactions (although multicellular organization is required

for normal development of spores) and other complicating factors, will prove to be fruitful field of investigation; and may even elucidate the underlying regulatory mechanisms of the more complex metazoan development. Here is a model system of eukaryote development in the simplest possible form, with only two developmental possibilities, depending on environmental conditions, instead of the numerous canalizations and cascade effects possible in metazoan differentiation. Among the features yet to be elucidated are commitment, when cells become no longer capable of vegetative growth even when dispersed in a nutrient environment; the cell surface changes involved in adhesiveness, and sensitivity to acrasin; engulfment in macrocyst formation, and the acquisition of sensitivity to warmth and light.

Mandelstam has pointed out that the response to starvation in yeast, bacteria and in the cellular slime molds is to change the protein synthetic pattern. In bacteria, this involves a genetic regulatory protein which binds cAMP; it will be interesting to find whether a similar mechanism operates in the Acrasiales. And even " . . . higher in the evolutionary scale, protein turnover in the starving organism occurs as a major part of the mechanism in the conversion of a pupa into a moth or of a tadpole into a frog" (Mandelstam, 1971). The changes which occur in Dictyostelium, without starvation (Hanish, 1975) are changes in transcription of genetic mate-

rial; breakdown of existing ribosomes (Cocucci and Sussman, 1970) degradation of existing protein (Wright and Anderson, 1960); changes in the DNA-like RNA transcribed (Firtel, 1972); and the appearance of developmentally regulated proteins by de novo synthesis are similar to those reported to occur in amphibial development as a result of several days' continuous exposure to Thyroxin, secreted in response to changes in amount of sunlight and/or heat (Tata, 1971). Therefore, it is not unreasonable to expect that the "phenomena of growth and intercellular organization leading to differentiation presented in a simple form" (K.B. Raper, 1956) in the cellular slime molds will indeed be applicable to other systems.

It is interesting to note that much of the development-associated genetic program in Dictyostelium becomes transcribed prior to the cAMP-dependent aggregation, although the translation of these transcripts occurs afterwards (Sussman and Sussman, 1969). One laboratory suggests that all of the mRNA necessary for differentiation may preexist (Wright, 1967; Gustafson and Wright, 1972). Therefore, the nature of the stimulus causing the change in the genetic program is still unknown. Despite the popularity of investigation of this organism, one may still conclude that " . . . the cellular slime molds have much to teach us, if we could understand what we . . . . observe" (Raper, 1956).

## CONCLUSION

Among the objectives in future work with the DI will be: purification by exchange chromatography; investigation of homogeneity by polyacrylamide gel electrophoresis; investigation into whether DI is glycoprotein in nature; examination of the possibility that mitochondrial DNA synthesis may proceed in the absence of nuclear DNA synthesis due to DI's arresting cell in G2 (see page 84 for discussion); effect of purified DI on protein synthesis; and confirmation of the data on DI's effects on nucleic acid synthesis.

# APPENDIX

BUFFERS:

Sorensen's Buffer, 1/60 M, pH 6.0 : 3.95 gms $NaH_2PO_4$ and o.675 gms $Na_2HPO_4$ per liter.

LPS , pH6.4: 0.275 gm $Na_2HPO_4$; 0.725 gm $KH_2PO_4$; 1.5 gm Mg $Cl_2$ . $6H_2O$; and 0.5 gm Streptomycin Sulphate per liter.

Elution buffer for Sephadex chromatography: 50 mM Ammonium Acetate, pH 5. O .

GROWTH MEDIA:

Axenic Medium: 10 gm Proteose Peptone; 10 gm Dextrose; 5 gm Yeast Extract; 0.35 gm each of $Na_2HPO_4$ and $KH_2PO_4$; and 0.2 gm Streptomycin Sulphate per liter.

Liquid medium for E. coli: 0.5 gm Yeast Extract; 5 gm each Dextrose and Bacto Peptone; 2.25 gm $KH_2PO_4$; 1.5 gm $K_2HPO_4$; 0.5 gm Mg $SO_4$.7 $H_2O$ per liter.

Solid Medium: 10 gm each Bacto-Peptone and Dextrose; 0.96 gm $Na_2HPO_4$ and 1.45 gm $KH_2PO_4$ and 20 gm agar per liter to make 32 100 mm Petri dishes.

## MODIFICATION OF SCHMIDT-THANNHAUSER PROCEDURE FOR DNA EXTRACTION

Resuspend pelleted cells in cold 10% trichloroacetic acid (TCA). Leave in cold overnight. Ethanol-ether washes are omitted since identical results are obtained without lipid extraction. Pellet TCA - precipitable material. Resuspend in smallest possible volume of 0.3 N KOH. Incubate at 37°C overnight or until lucent. Neutralize with equal volume N HCL. Bring to 5% TCA environment. Chill overnight and pellet precipitate. The supernatant may be collected for RNA measurement with Orcinol. The precipitate normally is then made 5% with regard to Perchloric acid PCA) and boiled for twenty minutes at 100°C to achieve acid hydrolysis of DNA. However, this step has been changed to 10% PCA for fifteen minutes at 70°C, which gives greater recovery. Leach and Ashworth (1972) also found that less gentle hydrolysis normally used results in decreased diphenylamine-staining material in the case of DNA from the cellular slime molds. After pelleting, DNA fragments are in the supernatant, and the pellet is protein.

## GILES-MYERS DIPHENYLAMINE METHOD

Two mls of the test solution DNA, in 10% PCA is added to an equal volume of 4% recrystallized Diphenylamine in glacial acetic acid. After addition of 0.1 ml of 1.6 mg/ml acetaldehyde, the solutions are incubated at 30°C overnight. Optical

density at 700 mμ is subtracted from that at 595 mμ, and the result compared with standard curve and standards of calf thymus DNA.

MODIFIED LOWRY PROCEDURE

Cells are dissolved in Lowry's solution C, which consists of 50 parts solution A per 1 part solution B and must be made fresh daily.

Color Development: Add sample to fresh solution C to total 5 ml. Add to this 1 ml of distilled water. With each determination, duplicates of 50 micromoles and 100 micromoles of Bovine Serum Albumin are used as standards. Ten minutes minimum is allowed to elapse after addition of albumin to solution C before addition of Folin-Ciocalteau reagent. One-half ml of diluted Folin-Ciocalteau reagent is jetted into tubes rapidly and the tubes are immediately shaken very hard. After 40 minutes incubation at room temperature, color has developed and may be read for absorption at 660 mμ. One set of standards should be read at the end of the determination, to insure no wavering of the spectrophotometer has occurred during the procedure.

Color Development Solution A is prepared by dissolving 200 gm $Na_2CO_3$ and 40 gm NaOH while stirring rapidly in 1 liter of distilled water, then adding 2 grams of Sodium Potassium Tartrate and bringing total volume up to 10 liters. Solution

B is 5 gm $CuSO_4.5H_2O$ per liter water.

Dilution of Folin-Ciocalteau Reagent: one part of 100γ BSA/ml is mixed thoroughly with 5 parts solution C. 6 ml aliquots are distributed into test tubes into which are jetted 0.5 ml of various dilutions of the reagent prepared by adding graduated amounts of water to 5 ml of reagent.

The absorption at 660 m μ of these are read in the spectrophotometer after 15, 30, 60, 90, 120 and 240 minutes. The dilution of Folin-Ciocalteau reagent which reaches maximal color development in thirty minutes and remains relatively stable for 2 hours is selected.

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