

STUDIES ON THE NUCLEIC ACID ASSOCIATED PROTEINS

OF THE

CELLULAR SLIME MOULD DICTYOSTELIUM DISCOIDEUM

by

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Submitted in partial fulfilment of the requirements
for the degree of Ph.D.

University of Leicester

LEICESTER, England

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ABBREVIATIONS

The following buffers have been abbreviated in the text,

- TSS : 0.07% (v/v) Triton X-100, 0.004% (w/v) spermidine,
0.25 M sucrose, pH 7.5
- SSK : 0.25 M sucrose, 0.004% (w/v) spermidine, 0.05 M KCl, pH 7.5
- HMK : 0.05 M HEPES buffer (N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid) adjusted to pH 7.5 with NH_4OH ,
0.04 M MgCl_2 , 0.02 M KCl
- TKMM : 0.01 M Tris/Cl buffer pH 7.6, 0.025 M KCl, 0.01 M MgCl_2 ,
0.006 M 2-mercaptoethanol
- SSC : 0.15 M NaCl, 0.015 M sodium citrate, pH 7.5
- 0.1 x SSC : 0.015 M NaCl, 0.0015 M sodium citrate, pH 7.5
- 0.01 x SSC : 0.0015 M NaCl, 0.00015 M sodium citrate, pH 7.5

Saturated Phenol Solution :

Saturated phenol solution was prepared by the addition of 55 ml distilled water to 500 g of Phenol (detached crystals) and 0.5 g 8-hydroxyquinoline.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The process by which a single, unicellular fertilized egg is able to transform itself into the numerous structurally and functionally diverse cell types that comprise an adult organism is a problem that has commanded investigation and demanded explanation for centuries in both scientific and theistic terms (Morgan, 1934). Much time and effort has been expended over recent years in an attempt to elucidate the mechanism whereby identical total genetic information is differentially expressed in functionally different cells and, whereas it is true to say that our knowledge is now considerable, the complete understanding of the control of developmental processes is still many years away.

Because of the intrinsic complexity of the problem, it has become necessary for it to be divided into parts that can feasibly be studied. In general, development, although one concerted process, can be considered to comprise growth, polarity, determination, pattern formation, regulation and differentiation, and it important that these terms be accurately defined :

Growth is that stage which includes increase in cell mass, DNA replication and cell division. Polarity is the structural and functional asymmetry within individual cells. Determination is that stage when the developmental identity of a cell is established (but not necessarily expressed). Pattern formation is the process which orders requisite determinations both temporally and spatially. Regulation is the process of adaptation of the pattern formation mechanisms in response to interference or loss of parts so as to re-construct the whole pattern from the available material. Differentiation

is the stage when a determined cell constructs the specialized organelles and synthesizes specific biochemical products that characterize its structural and functional integrity.

Differentiation differs from the other aspects of development in that it is an intracellular process whereas polarity, determination, pattern formation and regulation are processes mediated by cell-cell interactions.

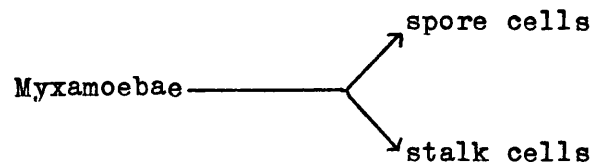
To study the control mechanisms of developmental processes we thus need an organism that displays all the above characteristics. The Cellular Slime Moulds - so named by Shaffer in 1953 after their original discovery by Brefeld in 1869 - are one of the simplest eukaryotic organisms to satisfy the above criterion. They comprise a group of soil protists that contain many different species of which the most commonly studied is Dictyostelium discoideum¹ which was discovered by Raper in 1935. As a model system for development, Dictyostelium discoideum has many advantages. Firstly the two phases of its life cycle (Plate 1, Chapter 1), namely vegetative growth and development, are mutually exclusive and can be examined separately (Bonner & Frascella, 1952; Sussman & Sussman, 1960). Secondly the homogeneous vegetative myxamoebae differentiate into one of only

¹This species has recently been reclassified (Olive, 1970)

Phylum	Myxamycophyta
Class	Mycetozoa
Order	Dictyosteliida
Genus	<u>Dictyostelium</u>
Species	<u>discoideum</u>

Previously the Order was designated 'Acrasia'.

two main cell types¹;



Thirdly, by utilizing rigorously defined conditions, the developmental phase can be highly synchronized and fourthly, the relative ease with which mutants can be isolated (Sussman, 1955 ; Loomis & Ashworth, 1968) and their capability of genetic recombination (Sussman & Sussman, 1963) by a parasexual process (Sinha & Ashworth, 1969 ; Loomis, 1969) provides a good basis for genetic analysis. Because of these numerous advantages, Dictyostelium discoideum has been extensively studied with the result that our understanding of some of the regulatory processes of development, although a long way from being complete, is now becoming a little clearer, and current knowledge has been well reviewed by many authors (Sussman & Sussman, 1969 ; Newell, 1971 ; Ashworth, 1971 ; Gustafson & Wright, 1972 ; Garrod & Ashworth, 1973).

We thus have a model system and can now consider ways in which the differentiation process may be studied. Now, differentiation is the process whereby the right amount of the right material is manufactured at the right time and in the right place. The problem then, is to determine how this highly ordered process takes place. What are the control mechanisms leading to such quantitative, qualitative, temporal and spatial specificity ?

¹At the base of the stalk there is a region of cells known as the Basal Disc cells, which are different, structurally at least, from the remainder of the stalk cells, but they comprise only a very small proportion of the total number of cells in the fruiting body.

The control mechanisms must surely be at different levels. A first distinction would be between control at a cellular level - how cell-cell interactions may affect differentiation - and control at a sub-cellular level - the intracellular mechanism of differentiation itself. Both types of control are evident in the development of the cellular slime mould Dictyostelium discoideum. The sorting out of developing cells to become either spore cells or stalk cells in a definite ratio (Bonner, 1957 ; Garrod & Ashworth, 1972) indicates the presence of control at a cellular level. At the sub-cellular level, we can make a further division, between nuclear and cytoplasmic control. Nuclear, or genetic control is clearly demonstrated by the aberrant development of many mutants (Loomis & Ashworth, 1968 ; Sussman, 1955) and cytoplasmic, or epigenetic control, by the action of inhibitors of cytoplasmic functions - such as cycloheximide on protein synthesis - which inhibit normal development (Mizukami & Iwabuchi, 1970 ; Hirschberg et al, 1968).

Such studies have clearly shown the involvement of genetic and epigenetic controls, but it is important to know how these controls operate, so that their functioning can be satisfactorily explained. But how can function best be investigated ? F.H.C. Crick's famous axiom, " If you don't understand function, look at structure ", forms the basis of this thesis. If the function of the basic nuclear proteins (or ribosomes) changes during development, it would be reasonable, therefore, to expect to find changes in the structure of these materials from different stages. The structure of the basic nuclear proteins - as a possible mediator of genetic control - and the structure of ribosomes - as a possible mediator of epigenetic control - will be investigated. Justification for this resides in previous studies. For control at a genetic level, there is some

evidence to suggest that the modification of histones may be involved in the control of template activity of DNA (Kleinsmith et al, 1966 ; Ord & Stocken, 1968 ; Stevely & Stocken, 1968). For control at an epigenetic level, there is evidence to suggest that ribosomes are capable of distinguishing between different mRNA molecules (Ilan & Ilan, 1971).

However, it is not clear that the converse of the above argument is necessarily true. It is important to be able to show that any changes observed in the structure of the basic nuclear proteins (or ribosomes) are causally, and not consequentially, related to the developmental programme. A number of biochemical parameters change during development, for example, the spore and stalk cells become surrounded by a rigid cell wall. In this instance the biochemical change (cell wall synthesis) is clearly a consequence of the developmental programme and is not causally significant. In the same way, changes in the structure of the basic nuclear proteins (or ribosomes) may be consequences of the altered metabolism of the differentiating cells rather than themselves causing these alterations. It is very difficult to distinguish causally and consequentially related structural changes by mere analysis of the changes themselves. However, in the case of a microbial developmental system, such as the cellular slime mould D.discoideum, it is, in principle, possible to distinguish between changes of these two types, since microorganisms may be grown under differing growth conditions and thus be obtained with differing characteristic chemical and biochemical properties. If, despite these differences, the cells carry out the same developmental programme, one can thus ask whether any differences observed during the developmental sequence are a function of the growth conditions

of the cells initially. If they are, then by this token, those differences are unlikely to be causally related to the developmental programme.

Formally, this is equivalent to the study of changes

$$\begin{array}{l} A \longrightarrow B \\ A' \longrightarrow B' \\ A'' \longrightarrow B'' \quad \text{etc.} \end{array}$$

where A, A' and A'' represent the structures of a certain cell component in metabolically distinct cells during one stage of development, and B, B' and B'' represent the altered structures of these components at a different developmental stage. Only if the differences (B-A, B'-A' and B''-A'') are the same, can the alteration in that cellular component be regarded as developmentally significant.

The great advantage of the cellular slime mould is that it is possible to do just this kind of study. It has been shown that myxamoebae of a mutant strain, Ax-2, can be grown in media which differ in carbohydrate content (Watts & Ashworth, 1970) and that changes in chemical composition (Weeks & Ashworth, 1972), enzyme composition (Ashworth & Quance, 1972) and physiological behaviour (Ashworth & Watts, 1970) can be thereby induced. This fact has been utilized by Hames & Ashworth (1974) to establish the developmental significance of changes in the carbohydrate metabolism during the developmental programme.

In this thesis, I intend to develop this approach and apply it to the study of the basic nuclear proteins and ribosomes of D.discoideum during its developmental programme.

CHAPTER 1

THE GROWTH AND DEVELOPMENT OF THE CELLULAR SLIME MOULD DICTYOSTELIUM DISCOIDEUM

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THE GROWTH AND DEVELOPMENT OF THE CELLULAR SLIME MOULD DICTYOSTELIUM DISCOIDEUM

INTRODUCTION

Until recently, Dictyostelium discoideum myxamoebae could only be grown in association with bacteria - usually Escherichia coli or Aerobacter aerogenes - on solid agar medium as detailed by Raper (1937) and later refined by Sussman (1966). The inherent disadvantage of this method for biochemical studies, namely that the myxamoebae were in different physiological states, was overcome by growth in shaking suspensions of either live or dead bacteria (Gerisch, 1959, 1960; Hohl & Raper, 1963). Although this was an improvement, the continued presence of bacteria and bacterial products severely hampered biochemical studies and the advantages of the use of axenic media were readily apparent. A number of strains of Dictyostelium discoideum have now been developed that are capable of growth in media which although chemically complex and non-defined, is free of bacteria (Sussman & Sussman, 1967; Watts & Ashworth, 1970; Schwalb & Roth, 1970; Coccuci & Sussman, 1970).

The method used for studying development has been considerably improved since first described by Raper (1935), the most important modification being the complete removal of any food source from the vegetative myxamoebae and depositing the cells on a solid substratum which results in synchronous and highly reproducible development (Bonner, 1947; Sussman, 1966; Newell et al, 1969).

One of the fundamental problems in the study of developing systems is the distinction between biochemical changes that are a function of development - differentiation - and changes that are merely a function of growth. The use of strain Ax-2, developed by Watts and Ashworth, goes some way in making this distinction possible as it is capable of growth in a variety of different media. The changes in the cellular components caused by the alteration of the growth conditions can thus be ascertained so that changes occurring during development may be identified unambiguously.

The first chapter of this thesis describes the procedures followed for the growth and development of Dictyostelium discoideum and the methods used in the estimation of the main macromolecular cell constituents ; DNA, RNA and Protein.

MATERIALS

Bacteriological peptone, yeast extract, nutrient broth and agar were purchased from Oxoid Ltd, London, U.K. All other chemicals were of the highest purity commercially available and were the products of BDH Chemicals Ltd, Poole, Dorset, U.K., Fisons Scientific Apparatus Ltd, Loughborough, Leicester, U.K., or Sigma (London) Ltd, London, U.K.

METHODS

(i) Growth of Myxamoebae Axenically

Myxamoebae of strain Ax-2 (ATCC 24397) were grown axenically as described by Watts & Ashworth (1970). The composition of the basic axenic medium - hereafter referred to as NS (no sugar) medium - was,

Oxoid bacteriological peptone	(14.3g)	} pH 6.7
Oxoid yeast extract	(7.15g)	
$\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$	(1.28g)	
KH_2PO_4	(0.486g)	
distilled water to 1 litre		

Glucose, fructose or glycerol may be added to this NS medium as described under RESULTS. In addition, the quantity of bacteriological peptone and yeast extract may be altered to produce NS media of varying strengths, e.g. 2 NS medium contains twice as much peptone and yeast extract as NS medium.

The myxamoebae were grown either in 65-70 ml of medium contained in a 250 ml Erlenmeyer flask or in 700 ml of

medium contained in a 2 litre Erlenmeyer flask, shaken on a rotary shaker with an eccentric rotation of 3 cm at 130 - 150 rpm. at 22 - 23°C air temperature. Stocks were maintained as unshaken 60 ml cultures.

Cell densities were measured either by counting with a haemocytometer or, when more precise determinations were required, with the Coulter Counter.

Cells were harvested from growth medium by centrifugation at 1000g for 15 min at 4°C, washed in an equal volume of ice-cold distilled water and centrifuged as above. For developmental experiments cells were suspended in ice-cold distilled water at a density of approximately 5×10^7 cells/ml. Alternatively cells were suspended at approximately 10^8 cells/ml, accurately counted and then stored in 4 ml aliquots at -70°C for subsequent analysis of DNA, RNA and protein.

(ii) Growth of Myxamoebae on Bacteria

Myxamoebae of strain Ax-2 were grown in suspension with Escherichia coli B/r as a food source as described by Gerisch (1960). Bacteria grown to early stationary phase on 2.5% nutrient broth were harvested, washed and resuspended in sterile 0.0167 M sodium phosphate buffer, pH 6.0, at a density of $5 - 10 \times 10^9$ cells/ml. Vegetatively growing axenic myxamoebae were then inoculated into either 60 ml of the bacterial suspension contained in a 250 ml Erlenmeyer flask or 1 litre of the bacterial suspension contained in a 2 litre Erlenmeyer flask and shaken on a rotary shaker as described above.

Myxamoebae were also grown in association with

Aerobacter aerogenes on solid agar medium as described by Sussman (1966).

Myxamoebae growing in association with bacteria were harvested by repeated centrifugation (400g, 5 - 10 min) and resuspended in ice-cold distilled water until the myxamoebae appeared microscopically free of bacteria and were then stored at -70°C for subsequent analysis.

(iii) Development of Myxamoebae

Washed myxamoebae were resuspended in ice-cold distilled water at a density of approximately 5×10^7 cells/ml. 0.5 ml aliquots were placed on Millipore filters (Cat. No. AABP 04700) resting on Millipore absorbant pads (Cat. No. AP100 4700) saturated with 1.6 ml of pad diluting fluid, PDF, as described by Sussman (1966). The composition of the PDF used throughout this thesis was,

KCl	1.5 g
MgCl ₂	0.5 g
Streptomycin sulphate	0.5 g

In 1 litre of 50 mM phosphate buffer, pH 6.5

Development was then allowed to take place at $22 - 23^{\circ}\text{C}$ in a dark and humid environment.

(iv) Determination of DNA, RNA and Protein

Frozen 4 ml aliquots of cells were thawed, made up to 5 ml with water and sonicated using a 100W MSE Ultrasonic Disintegrator (1 cm probe tip, peak to peak amplitude $11 \mu\text{m}$) for four 15 second periods, with

continuous cooling in an ice bath to prevent excessive heating. A sample of this extract was assayed for total cellular protein by the method of Lowry et al (1951).

The DNA content of such sonicated cell suspensions was determined by a modification of the procedure of Giles & Myers (1965) as described by Hames et al (1972). 1 ml of cold 50% (w/v) trichloroacetic acid was added to 4 ml of extract and the mixture incubated at 0°C for 1 hour. The supernatant from a 20 min. centrifugation at 1000g was discarded and the pellet washed twice in 2 ml lots of cold 10% (w/v) trichloroacetic acid. The final pellet was resuspended in 2.5 ml of 5% (w/v) trichloroacetic acid and heated at 90°C for 30 min. to hydrolyse the DNA and RNA. The hydrolysate was centrifuged (1000g, 20 min.) and the pellet discarded. To 1.6 ml of the supernatant were added 0.4 ml of 50% (w/v) HClO_4 , 2 ml of 4% (w/v) recrystallized diphenylamine in glacial acetic acid and 0.2 ml of aqueous acetaldehyde (1.6 mg/ml). The reaction mixture was incubated at 30°C for 22 hours and the difference in extinction at 595 nm and 700 nm was determined.

A further sample of the supernatant was assayed for ribose by the orcinol reaction (Mejbaum, 1939).

For samples containing less than 10^8 cells, a more sensitive DNA assay was used. This was a modification of the fluorometric procedure of Kissane & Robins (1958) developed by Dr. U. Yalofsky (personal communication). 1 ml of cold 10% (w/v) trichloroacetic acid was added to 1 ml of a sonicated cell suspension and the mixture incubated at 0°C for 20 min. The supernatant from a 5 min. centrifugation at 1000g was discarded and the pellet dried in a vacuum desiccator overnight. The dry pellet was resuspended

in 30 μ l of 30% (w/v) 3'5' diaminobenzoic acid in 4 N HCl (decolourized with activated charcoal) and incubated at 60°C for 1 hour. 75 μ l of 0.6 N HClO₄ were added and the mixture centrifuged at 1000g for 10 min. 50 μ l of the supernatant were added to 2 ml of 0.6 N HClO₄ and the fluorescence measured in an Eppendorf Fluorimeter with an excitation wavelength of 405 nm and an emission wavelength of 530 - 3000 nm.

The standards used for comparison in the determinations of DNA, RNA and protein were calf thymus DNA, ribose and bovine serum albumin.

RESULTS AND DISCUSSION

(i) Growth of D.discoideum

In the vegetative phase of their life cycle, myxamoebae of D.discoideum grow and divide by binary fission (PLATE 1). In NS medium, myxamoebae of strain Ax-2 grow with a mean generation time of approximately 9 hours to a cell yield of $4 - 6 \times 10^6$ cells/ml (FIGURE 1). In live bacterial suspensions, myxamoebae of strain Ax-2 grow with a mean generation time of approximately 4 hours to a cell yield of $1 - 1.5 \times 10^7$ cells/ml (FIGURE 1). The mean generation time of myxamoebae of strain Ax-2 grown on solid agar medium in association with bacteria is also approximately 4 hours.

(ii) Development of D.discoideum on Solid Substratum

When myxamoebae of D.discoideum are washed free of all exogenous nutrient and placed on a solid substratum, the cells embark upon a developmental program characterized by distinct morphological stages (PLATE 1, FIGURE 2) which have been described by many authors (Raper, 1937; Ashworth, 1971). The cells, which during the vegetative phase are all but mutually repulsive, are now chemotactically attracted to central points (PLATE 1, Aggregation) to form a tissue-like assembly of $10^4 - 10^5$ cells. This aggregate rises up to produce a finger-like column of cells known as the pseudoplasmodium grex or slug. The slug is motile, capable of moving along temperature, light and humidity gradients - a function which, in its natural environment of the soil, presumably enables it to reach an advantageous

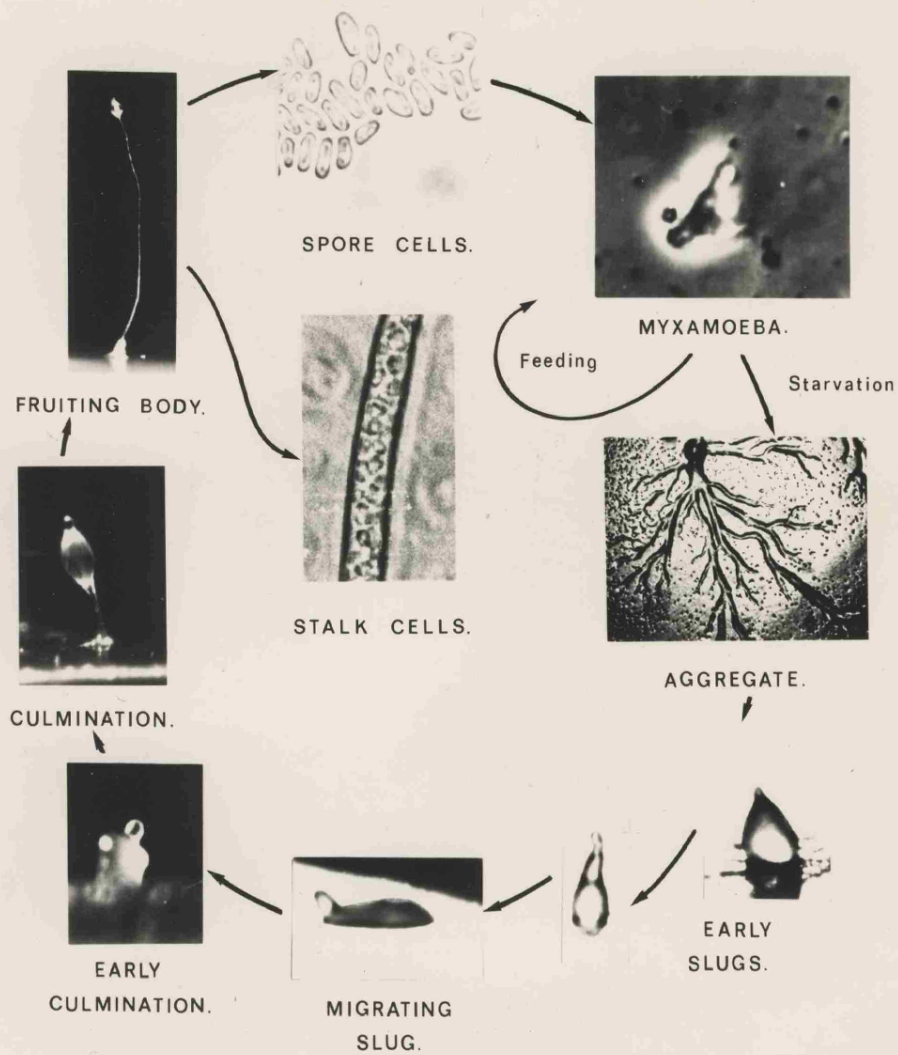


PLATE 1. The Life Cycle of Dictyostelium discoideum

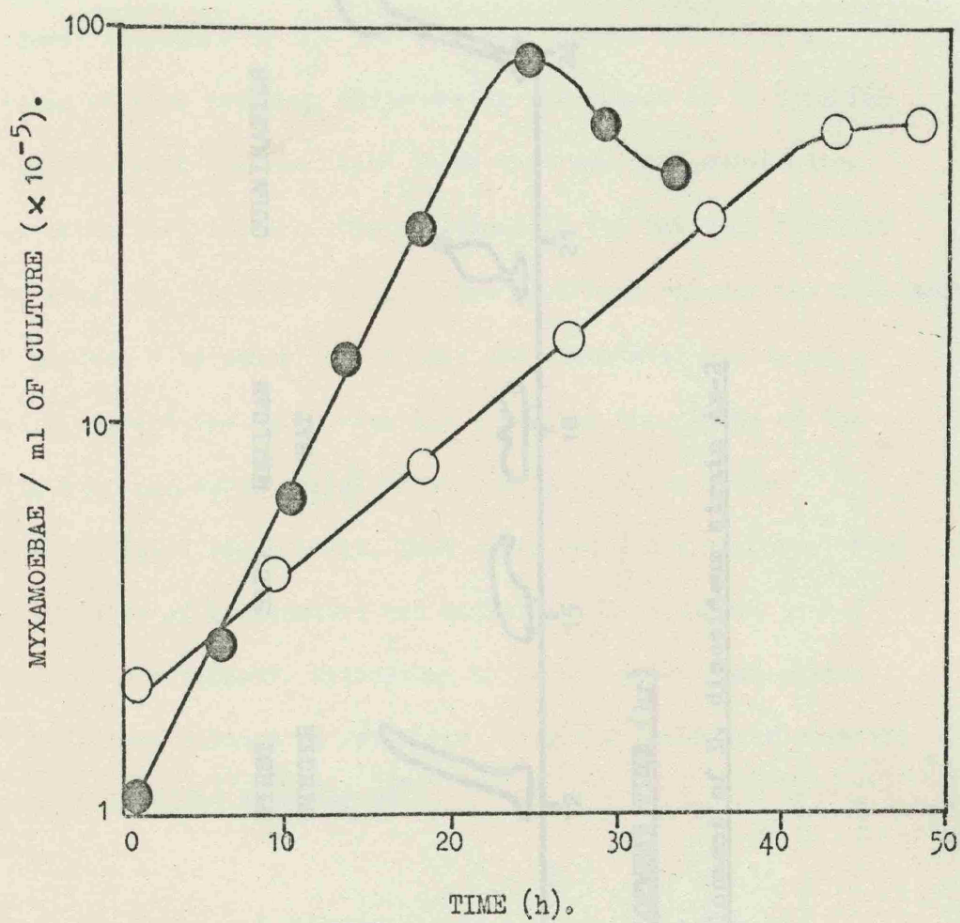


FIGURE 1. Growth of *D. discoideum* myxamoebae in suspension culture

- Growth in Axenic medium
- Growth in living bacterial suspension

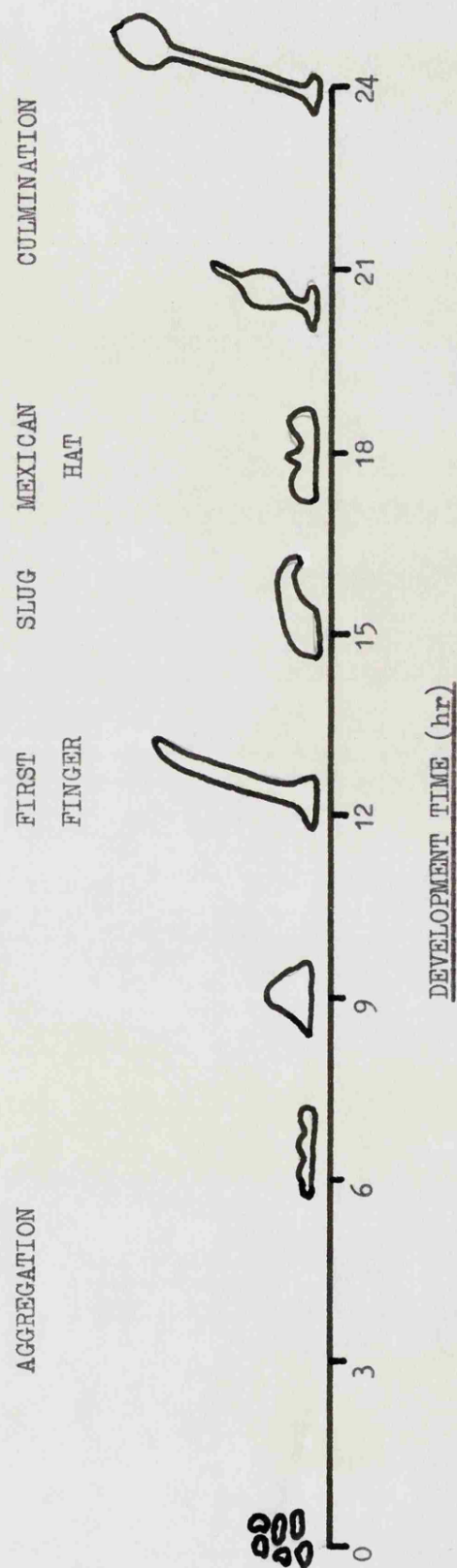


FIGURE 2. Chronology of Development of D. discoideum strain Ax-2

location for spore dispersal. This migration period is not however essential to the production of spores and after a period of time the slug stops moving and rounds up to form the characteristic "mexican hat" stage that precedes culmination. The cells that occupied the positions in the anterior third of the slug (the pre-stalk cells) then move down through the cell mass to produce a column of cells that are vacuolated and dying, which support the cells from the posterior two-thirds of the slug (the pre-spore cells) which mature into the fully differentiated spore cells. Such spore cells are very resistant to extremes of temperature and humidity and germinate in a suitable environment. Myxamoebae of strain Ax-2, when plated on Millipore filters as described, complete their developmental program in 24 ± 2 hours at 22°C .

(iii) The Effect of Varying the Growth Conditions on the Levels of DNA, RNA and Protein

The inclusion of glucose, fructose or glycerol in the basic NS medium or variation in the concentration of peptone and yeast extract, produces cultures of vegetative myxamoebae differing markedly in mean generation time and cell yield. Generally the addition of an extra carbon source produces a higher cell yield whereas a decrease in the peptone and yeast extract concentration results, not suprisingly, in the opposite effect (see TABLE 1).

However, differences in mean generation time and cell yield are not reflected in any significant changes in the levels of DNA, RNA and protein, with the exception that myxamoebae grown in association with bacteria have approximately twice the DNA content

Medium	Doubling Time (h)	Cell yield cells/ml $\times 10^{-6}$	DNA $\mu\text{g}/10^8$ cells	RNA(ribose) $\mu\text{g}/10^8$ cells	Protein mg/ 10^8 cells
NS + 86mM glucose	7.3 $\pm$ 0.9 (4)	12 $\pm$ 2 (4)	18.1 $\pm$ 1.9 (8)	280 $\pm$ 28 (8)	9.1 $\pm$ 1.0 (8)
NS + 43mM glucose	8.5 $\pm$ 1.5 (4)	11 $\pm$ 2 (4)	17.6 $\pm$ 1.6 (8)	308 $\pm$ 24 (8)	9.0 $\pm$ 0.4 (8)
NS + 86mM fructose	9.6 $\pm$ 1.4 (5)	10 $\pm$ 1 (5)	18.4 $\pm$ 2.1 (8)	279 $\pm$ 27 (8)	8.5 $\pm$ 1.1 (8)
NS + 43mM fructose	9.2 $\pm$ 0.4 (4)	10 $\pm$ 2 (4)	19.1 $\pm$ 1.0 (8)	304 $\pm$ 21 (8)	8.1 $\pm$ 1.0 (8)
NS + 168mM glycerol	9.8 $\pm$ 2.0 (5)	9 $\pm$ 1 (5)	18.6 $\pm$ 2.2 (8)	301 $\pm$ 31 (8)	7.8 $\pm$ 1.4 (8)
NS + 84mM glycerol	10.4 $\pm$ 2.0 (4)	10 $\pm$ 2 (4)	17.5 $\pm$ 1.5 (8)	284 $\pm$ 24 (8)	8.2 $\pm$ 1.3 (8)
NS	8.7 $\pm$ 1.2 (4)	5 $\pm$ 1 (4)	17.5 $\pm$ 1.5 (8)	278 $\pm$ 29 (8)	8.5 $\pm$ 0.5 (8)
2 NS	10.4 $\pm$ 0.6 (2)	4 $\pm$ 1 (2)	17.9 $\pm$ 1.7 (4)	269 $\pm$ 19 (4)	8.1 $\pm$ 1.2 (4)
0.75 NS	10.5 $\pm$ 1.5 (2)	2 $\pm$ 0.5 (2)	18.3 $\pm$ 2.1 (4)	281 $\pm$ 31 (4)	8.7 $\pm$ 0.9 (4)
0.5 NS	9.5 $\pm$ 1.5 (3)	1 $\pm$ 0.5 (3)	17.4 $\pm$ 2.0 (6)	307 $\pm$ 26 (6)	7.9 $\pm$ 0.8 (6)
0.1 NS	16.3 $\pm$ 0.7 (2)	0.2 $\pm$ 0.1 (2)	18.8 $\pm$ 1.9 (4)	292 $\pm$ 21 (4)	8.9 $\pm$ 1.2 (4)
<u>A.aerogenes</u> on agar	4.0 $\pm$ 0.9 (2)	-	42.0 $\pm$ 3.5 (4)	320 $\pm$ 18 (4)	8.4 $\pm$ 0.6 (4)
<u>E.coli</u> in suspension	4.0 $\pm$ 0.2 (4)	10 $\pm$ 1 (4)	43.2 $\pm$ 4.1 (8)	290 $\pm$ 11 (8)	7.8 $\pm$ 0.5 (8)

TABLE 1 The Growth of Dictyostelium discoideum in Different Media

Results are given as Means $\pm$ the standard error of the mean (S.E.M.)

Figures in parentheses refer to the number of determinations

of axenically grown cells. The DNA content thus remains constant at approximately $18 \mu\text{g DNA}/10^8$ cells for axenic myxamoebae with mean generation times of between 7.3 hours (glucose medium) and 16.3 hours (0.1 NS medium), and at approximately $42 \mu\text{g DNA}/10^8$ cells for the bacterially grown myxamoebae. This extra DNA has been shown to consist largely of D.discoideum DNA and that endogenous bacterial DNA constitutes only a small proportion (Leach & Ashworth, 1972).

The RNA content remains constant at approximately $300 \mu\text{g ribose}/10^8$ cells for all growth conditions, and the protein content also remains constant at approximately 8.5 mg BSA equivalents/ 10^8 cells. These results are in agreement with those of Ashworth & Watts, 1970).

It is apparent from TABLE 1 that there is a considerable variation in the mean generation time for myxamoebae grown in any particular axenic medium and this merits comment. It appears that prolonged maintenance of the standing cultures (more than 3 months) results in increased mean generation times, lower cell yields and, eventually, aberrant development. The reason for this is not clear but it could be due to the lack of aeration in such cultures as stocks subsequently maintained as shaking cultures did not deteriorate as rapidly and hence this modification to stock maintenance was used throughout this thesis.

SUMMARY

1. The growth of myxamoebae of D.discoideum strain Ax-2 in axenic medium and in association with bacteria is described. Axenically growing myxamoebae divide at half the rate of cells growing on bacteria, but in suspension culture both reach approximately the same cell density.
2. The sequence of morphogenetic stages that characterize the development of D.discoideum myxamoebae is described. The axenically grown cells take 24 ± 2 hours to produce mature fruiting bodies.
3. The DNA, RNA and protein content of myxamoebae grown in a variety of growth conditions is relatively constant at approximately 18 μg DNA, 300 μg ribose equivalents and 8.5 mg BSA equivalents per 10^8 cells. The only exception is in the case of bacterially grown myxamoebae whose DNA content is approximately twice that of the axenically grown cells.

CHAPTER 2

THE BASIC NUCLEAR PROTEINS OF THE CELLULAR SLIME MOULD DICTYOSTELIUM DISCOIDEUM

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THE BASIC NUCLEAR PROTEINS OF THE CELLULAR SLIME MOULD DICTYOSTELIUM DISCOIDEUM

INTRODUCTION

The regulation of protein synthesis is a fundamental problem in the understanding of the mechanism of cellular development. Protein synthesis may be regulated at a variety of steps which, in the introduction to this thesis, were conveniently classified into transcriptional regulation and translational regulation. However, the dividing line is not clear - if indeed it exists at all - for all the participating molecules involved in the translation of messenger RNA molecules, such as polymerizing enzymes, transfer RNA, initiation, elongation and termination factors etc., are themselves directly or indirectly the products of genes and are therefore subject to regulation either at transcription or at later stages. Whether transcriptional and translational regulation can be separated with any degree of validity is (largely) theoretical. However, for practical purposes it is necessary to subdivide the overall process of regulation and the division between transcriptional and translational regulation is a convenient, if arbitrary distinction to draw.

At the transcriptional level there are many ways by which protein synthesis may be regulated. Unfortunately, at the present time the mechanism whereby specific regions of the DNA are made suitably available for the synthesis of complementary RNA (or DNA) molecules is largely unknown. However one aspect of this complex process that has been extensively studied is that of the role of the proteins associated with the DNA, the basic nuclear proteins or

histones.

Histones have been studied for almost 100 years (Kossel, 1884) and work on them has recently been reviewed by many authors (Georgiev, 1969 ; DeLange & Smith, 1971). Before considering the roles that histones may play in the regulation of protein synthesis and the specific expression of genetic information, it is important to establish what is meant by the term ' histone '.

Histones are basic proteins which are major components of chromosomes in most eukaryotic organisms. Moreover, all higher animal and plant cells, regardless of source, contain essentially the same types of histones and approximately the same number of distinguishable components (DeLange et al, 1969a ; Fambrough & Bonner, 1966). Further confirmation of this generalization has been presented by Kedes & Birnsteil (1971) who reported that 9S messenger RNA, believed to code for histones in sea urchin embryos, can hybridize to the DNA of other eukaryotes, notably Xenopus laevis and Rhynchosciara angelae.

Histones have been grouped into three main classes -

- (i) lysine-rich (sometimes called very lysine-rich) histones,
- (ii) intermediate or slightly lysine-rich histones and
- (iii) arginine-rich histones.

Within these classes, histones can generally be separated into 5 major fractions and with more modern high resolution techniques, subfractions can be obtained. Unfortunately, there is a certain diversity of nomenclature for these fractions and subfractions due to the different criteria used in formulating them. Some authors have assigned symbols based on extraction procedures whilst others base their nomenclature on physicochemical properties such as electrophoretic mobility. It is thus useful to compare the more

common systems, which have been developed in relation to calf thymus histones -

<u>Class</u>	<u>Fraction</u> (Rasmussen <u>et al</u> , 1962)	<u>Fraction</u> (Johns & Butler, 1962)	<u>Fraction</u> (Panyim & Chalkley, 1969)
lysine-rich	I	f1	1
slightly	IIb2	f2b	3
lysine-rich	IIb1	f2a2	4
arginine-rich	III	f3	2
	IV	f2a1	5

The number of subfractions comprising any one histone fraction varies from species to species, but is usually at least 2, and improved techniques will probably reveal additional subfractions so that the most likely number of histones will be between 10 and 20. Despite this large number, there is great similarity between the subfractions of any particular histone, which appear to be produced by side-chain modifications of amino acid residues (DeLange et al, 1968, 1969b, 1970) or by microheterogeneity in certain regions of the sequence (Kinkade & Cole, 1966 ; Bustin & Cole, 1969). Although most tissues contain only the 5 major histone fractions, avian erythrocytes (Johns & Diggle, 1969), fish and frog erythrocytes (Edwards & Hnilica, 1968) and frog liver (Nelson & Yunis, 1969) contain a specific, slightly lysine-rich histone, designated histone V (f2c), which is not found in other cells.

Great difficulty was experienced by early workers in the determination of the molecular weights of histones. In retrospect, it seems this was caused by the use of experimental procedures which allowed proteolytic degradation of the histones during isolation, and also by a lack of understanding of the properties of the histones

themselves. Some histones aggregate at neutral and alkaline pH (Fambrough & Bonner, 1968 ; Philips, 1967) and all, by virtue of their high positive charge, behave anomalously in the usual systems of gel filtration and gel electrophoresis used to determine molecular weights (Philips & Clarke, 1970). More recent studies, including the complete sequencing of some of the histone fractions (Iwai et al, 1970 ; Hnilica et al, 1970 ; DeLange et al, 1968, 1969a, 1969b ; Ogawa et al, 1969) have placed the molecular weights between 11,000 and 20,000. In general then, histones are relatively small proteins, containing approximately 25 mole% lysine plus arginine, and they lack the amino acid residues cysteine (except histone III) and tryptophan.

Taken altogether, the results of the detailed studies on histones from many different sources suggest that, since histones seem to be remarkably homogeneous and to have conserved their amino acid sequences to a large extent during evolution, they probably fulfil a very basic function(s) common to all cells. In considering the nature of this function(s), much emphasis has been placed on the various reversible side-chain modifications known to be inherent in the histones, for this may produce evidence to suggest the involvement of histones with the cyclical changes which occur during replication of DNA during the cell cycle.

In general, lysyl, histidyl, arginyl and seryl residues have been found to be modified in various ways (e.g. acetylation, methylation, phosphorylation) and we can consider each process, and its implications, in turn.

Acetylation of lysyl residues was first discovered in calf thymus histones by DeLange (1968) and has since been reported in several other tissues including trout testis (Sung & Dixon, 1970),

pea seedlings (DeLange et al, 1969a) and HeLa cells (Wilhelm & McCarty, 1970). The ϵ -N-acetyl derivative has so far been found only in the arginine-rich histones (fractions III and IV) and it appears to turn over at an appreciable rate (Vidali et al, 1968). The precise role of acetylation is so far unknown, but studies relating the time of histone acetylation to other cellular events (Pogo et al, 1968) have led to the suggestion that there may be a correlation between histone acetylation and DNA-dependent RNA synthesis and that it may be an early event in the process of de-repression.

The methylation of lysyl residues was first described by Murray (1964). Since then, not only the monomethyl derivative but also the dimethyl and possibly trimethyl derivatives have been found (Paik & Kim, 1967 ; Hempel et al, 1968). Like acetylation, methylation occurs mainly in arginine-rich histones, but it does not appear to have any temporal correlation to DNA, RNA or histone synthesis and it has been suggested (DeLange & Smith, 1971) that methylation may play some role at the time of chromosome condensation.

The methylation of histidyl and arginyl residues has been reported by Gershey et al (1969) and Paik & Kim (1969) respectively, but their precise roles have yet to be elucidated.

Probably the most extensively studied histone modification is the phosphorylation of seryl residues. First reported by Ord & Stocken (1966a) and Kleinsmith et al (1966), there have since been many reports of the presence of phosphoserine in most histone fractions from many species, the phosphorylation of histone I undergoing by far the most extensive investigation (Langan, 1969 ; Cross & Ord, 1970 ; Ord & Stocken, 1967, 1968 ; Stevely & Stocken, 1968a, 1968b). The role that histone phosphorylation may play in the control of genetic activity appears complex. It has been shown that cyclic-AMP stimulates histone

phosphorylation (Langan, 1969) and this may provide a link in the stimulation of histone phosphorylation, RNA synthesis and protein synthesis by certain hormones. It also appears that whereas other histones are phosphorylated at a time concomitant with new RNA synthesis (Buckingham & Stocken, 1970), histone I phosphorylation occurs later, during new DNA synthesis (Stocken & Ord, 1969). Sung & Dixon (1970) showed that phosphorylation of histones in trout testis occurred mainly during the displacement of histones from the DNA by protamine, a somewhat specialized process not found in other tissues. More recently, Bradbury et al (1973) have shown that late in the G2 phase of the cell cycle, where chromosome condensation is seen to be occurring, the phosphate content of very lysine-rich histone (f1) of Physarum polycephalum is dramatically increased. The same workers have since proposed a model for the molecular basis of the control of mitotic cell division which suggests that an enzyme that phosphorylates histone f1 in Physarum polycephalum nuclei - f1 phosphokinase - " triggers " an increase in f1 phosphate content and this is the initiation step in mitosis (Bradbury et al, 1974a, 1974b).

A last modification of histones is that of the oxidation-reduction of cysteinyl-cystinyl residues. Only histone III (f3) contains this amino acid residue and its presence results in the appearance of multimers. Ord & Stocken (1966b) showed that the ratio of thiol to thiol plus disulphide is low in metaphase chromatin where genetic activity is low, whereas in interphase chromatin, where genetic activity is increased, it is high (Sadgopal & Bonner, 1970). This has led to the suggestion that histone III, in its oxidized state, protects the DNA during mitosis, and reduction of the disulphide bonds results in increased template activity of the DNA.

There has been much speculation about the role of histones

as structural components of chromatin. On the one hand, Richards & Pardon (1970) suggest histones IIb1 and III (f2a2 and f3) are primarily involved in maintaining the supercoiled structure of DNA in nucleohistone, while on the other, Smart & Bonner (1971) report that specific changes in chemical and physical characteristics of nucleoprotein complexes are not dependent upon the presence of any one particular histone fraction. The situation is thus contentious, but it does seem likely that histones are structurally important although their precise roles have yet to be firmly established.

From the above, it seems probable that histones are involved in the regulation of transcription and replication and are also important structural elements, serving to protect and maintain the structure of the chromatin. More conclusive evidence has been obtained and it can be briefly outlined.

(a) Histones are generally synthesized during or just before DNA replication (Sadgopal & Bonner, 1969). This could imply that they are required to prevent replication (as well as transcription) of newly synthesized DNA. Wang (1969) has suggested that the suppression of this inhibitory action when the DNA is to be replicated could be by interaction with non-histone proteins.

(b) Any histone fraction added to DNA in vitro reduces DNA-dependent RNA synthesis (Shih & Bonner, 1970) and removal of histones from chromatin results in moderately increased template activity of the DNA (Spelsburg & Hnilica, 1971).

(c) The relatively small number of histones (less than 20) have led to the hypothesis that they are non-specific repressors, interacting with any region of DNA to reduce template activity. Marushige & Bonner (1966) demonstrated that when histones are removed from chromatin so as to leave most of the non-histone protein still

attached, the template activity becomes essentially the same as that of the DNA itself, suggesting that some specificity may exist by the interaction of histones with other chromosomal components.

(d) Apart from the supercoiling involvement of histones IIb1 and III already described, it has been reported (Littau et al, 1965) that histone I is necessary for the conversion of diffuse chromatin into condensed chromatin.

It is important to remember that most of the research on histone structure and function has been carried out on histones isolated from higher plants and animals, especially calf thymus and pea seedlings. Investigation of the histone content of primitive eukaryotes is essential when one considers that the appearance of histones in evolution is roughly coincidental with the appearance of a well defined nucleus and with differentiation (Georgiev, 1969). Several reports have been published to show that histones, similar to those of higher organisms, are present in simple eukaryotes such as Chlorella ellipsoidea (Iwai, 1964), Polysphondylium pallidum (Horgen & O'Day, 1973), Physarum polycephalum (Mohberg & Rusch, 1969) and Tetrahymena pyriformis (Iwai et al, 1965). On the other hand, several organisms have been reported as lacking typical histones. These include the Chytridiomycetes (Stumm & Van Went, 1968), the Zygomycetes, Ascomycetes and Deuteromycetes (Leighton et al, 1971) and the dinoflagellate alga Gyrodinium cohnii (Rizzo & Nooden, 1972).

The relationship between histone content and development has also been considerably investigated. Stedman & Stedman (1950) observed some tissue specificity in relation to histone content and first proposed the hypothesis that basic nuclear proteins inhibited genetic activity. More recently, Hnilica & Johnson (1970), showed that histones were not synthesized before the blastula stage in sea

urchin development and that the lysine-rich histones of sea urchin sperm were different from those of the embryo. Sung & Dixon (1970) showed it was the modification of histones during spermiogenesis in trout testis that facilitates sperm development and Bradbury et al, (1974) report quantitative differences in the histone content of Volvox during its development. On the other hand, Spiker & Krishna (1972) have reported the constancy of wheat seedling histones during development.

The majority of these studies however, have involved investigation of histone fractions from organisms that are not only going through processes of development into many different cell types, but which are also growing. This makes accurate deductions about observed changes in histone content impossible as such changes may be a function of either growth or development. The developmental system of the cellular slime mould Dictyostelium discoideum notably lacks these disadvantages, for the development of the vegetative myxamoebae proceeds in the complete absence of DNA synthesis and cell mass increase, and the fully differentiated fruiting bodies comprise only two cell types. There has been only one report concerning the basic nuclear proteins of D.discoideum (Coukell & Walker, 1973) and it suggests the presence of fractions that roughly comigrate with calf thymus histone fractions on gel electrophoresis. It is the purpose of this chapter to investigate the basic nuclear proteins of D.discoideum to confirm, or otherwise, the previous finding and to determine whether any gross changes in the basic nuclear protein content occur during development that may suggest possible control mechanisms for this process in this organism.

MATERIALS

Cemulsol NPT 12 was obtained from Tar Residuals Ltd, London, U.K. and acrylamide was obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks, U.K. All other chemicals were of the highest purity commercially available and were the products of BDH Chemicals Ltd, Poole, Dorset, U.K., Fisons Scientific Apparatus Ltd, Loughborough, Leicester, U.K. or Sigma (London) Ltd, London, U.K.

METHODS

(1) Growth and Development of Myxamoebae

Vegetative myxamoebae of D.discoideum, strain Ax-2, were obtained by growth in NS medium as described in Chapter 1. Cells were harvested when they were approaching the end of their exponential growth phase (this corresponds to a cell density of approximately 4×10^6 cells / ml).

Cells representative of the developmental phase were obtained by harvesting vegetative myxamoebae and allowing them to develop on Millipore filter supports as previously described until they had reached the slug stage - at approximately 16 hours after deposition of the cells on the Millipores - at which time they were harvested.

The slug stage was chosen to be representative of the developmental phase for two reasons. Firstly, by this stage, numerous other changes in the cells have occurred so that any changes in the basic nuclear protein content should also be evident, and secondly, the technical difficulty of isolating nuclei from cells which have been developing for longer than this period are overwhelming. It must thus

be made clear that differences between the basic nuclear proteins in vegetative and slug stage cells may only be part of more substantial differences that would be evident at a later stage in development, but which, unfortunately, cannot be investigated using present techniques.

(ii) Isolation of Nuclei

Myxamoebae were harvested from either growth medium or Millipore filter supports and washed twice in ice-cold water as described in Chapter 1. Two methods were then used to isolate the nuclei.

Method A : This procedure is a modification of the method of Sussman & Rayner (1971) as described by Coukell & Walker (1973), and is based on the fact that the detergent, Triton X-100, solubilizes all cellular membranous material with the exception of the inner nuclear membrane. All operations were performed at 0 - 2°C unless otherwise indicated.

Washed myxamoebae were resuspended in distilled water at a cell density of approximately 3×10^8 cells / ml. Five volumes of TSS (see page iv) were added and the suspension shaken vigorously in a 28°C water bath for 8 min. The lysate was centrifuged at 400g for 5 min. to sediment unbroken cells and the supernatant then centrifuged (2000g, 20 min.) to pellet the nuclei. The crude nuclear pellet was resuspended in approximately 50 volumes of SSK (see page iv) and differentially centrifuged as described above. The nuclear pellet was then washed twice by resuspension in 0.01 M Tris/Cl buffer, pH 7.5, containing 0.15 M NaCl, followed by centrifugation (2000g, 10 min.).

Method B : This procedure is a combination of the methods of Coccuci & Sussman (1970) and Coukell & Walker (1973). All operations were

performed at 0 - 2°C unless otherwise indicated.

Washed myxamoebae were resuspended in ice-cold water at a cell density of approximately 2×10^8 cells / ml. 40 ml aliquots were dispensed into 50 ml polycarbonate centrifuge tubes and the cells pelleted at 1000g for 5 min. The pellet was then vortexed for 1 min. with 20 ml of HMK (see page iv), containing 4% (v/v) of the detergent Cemulsol NPT 12 and 5% (w/v) sucrose and the suspension incubated on ice for 9 min. 20 ml of HMK, containing 4% (v/v) Cemulsol NPT 12 and 22% (w/v) sucrose were added. Microscopic examination of the lysate at this stage showed the incidence of whole cells to be less than 1×10^{-4} . The nuclei were collected by centrifugation (1000g, 10 min.) and washed twice by gentle resuspension in (i) 40 ml SSK followed by centrifugation at 1000g for 10 min. and (ii) 40 ml 0.01 M Tris/Cl buffer, pH 7.5, containing 0.15 M NaCl, again followed by centrifugation at 1000g for 10 min.

(iii) Photography of Nuclei

Nuclei resuspended in 0.01 M Tris/Cl buffer, pH 7.5, containing 0.15 M NaCl, were photographed with a Leica M2 camera coupled to a Leitz Ortholux microscope using an oil immersion x90 objective for a final magnification of x 1350.

(iv) Extraction of Basic Nuclear Proteins

Because of the high acid protease activity present in nuclear and cytoplasmic fractions of D.discoideum myxamoebae (Sussman & Sussman, 1969 ; Weiner & Ashworth, 1970 ; Braun et al, 1972), the procedure for the extraction of the basic nuclear proteins is a modification of the method of Mohberg & Rusch (1969) as described by Coukell & Walker (1973).

Washed nuclei, containing 1 - 2.4 mg of DNA were resuspended in 6 ml of 1 M CaCl_2 and immediately placed in a boiling water bath for 15 min. After cooling, the nuclei were diluted with 3 ml of 1 M CaCl_2 and sonicated for 1 min. using a 100W MSE Ultrasonic Disintegrator (1 cm tip probe, peak to peak amplitude 12 μm). The mixture was then stirred at 4°C for 16 - 18 hours. The CaCl_2 - insoluble material was removed by centrifugation (25,000g, 15 min.), washed in 3 ml of 1 M CaCl_2 and centrifuged as before. The combined supernatants were transferred to an ice bath and 0.33 volumes of cold, 100% (w/v) trichloroacetic acid (TCA) were added and the mixture incubated on ice for 2 hours. The TCA - precipitable material was pelleted by centrifugation (100,000g, 45 min.), scraped from the walls of the centrifuge tube, gently resuspended in 0.02 N H_2SO_4 and dialysed against three changes, each of 500 volumes, of 0.02 N H_2SO_4 for 24 hours at 4°C. The material sedimenting in the dialysis tubing during this period was resuspended several times. The non-diffusible material was centrifuged (500g, 5 min.) to remove the acid - insoluble protein and any remaining DNA, the supernatant constituting the clarified basic nuclear protein suspension which was stored at -20°C until analysis.

(v) Estimation of Tryptophan

Tryptophan was estimated by a modification of the methods of Shaw & McFarlane (1938) and Fischl (1960) as described by Opienska-Blauth et al, (1963). To 1 ml of sample were added 2 ml of glacial acetic acid containing 56 mg Fe^{3+} / litre, and 2 ml of concentrated sulphuric acid. The solution was thoroughly mixed and incubated at room temperature for 15 min. The extinction at 545 nm was measured and the amount of

tryptophan present determined by reference to a standard curve of L-tryptophan.

(vi) Estimation of DNA, RNA and Protein

At various stages during the isolation procedure, samples were taken and analysed for DNA, RNA and protein by the methods described in Chapter 1.

(vii) Isolation of Ribosomal Proteins

Ribosomes were isolated from vegetative myxamoebae as described in Chapter 3, and the basic proteins extracted therefrom with 1 M CaCl_2 as described for the basic nuclear proteins.

(viii) Polyacrylamide Gel Electrophoresis

The polyacrylamide gel electrophoresis of the basic nuclear proteins of D.discoideum was based on the modifications of the methods of Reisfeld et al (1962) and Clarke (1964) as described by Panyim & Chalkley (1969).

Preparation of Gels :

The following solutions were employed -

Solution A : 60% (w/v) acrylamide, 0.4% (w/v) methylene bisacrylamide in water.

Solution B : 4% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) in 43.2% (v/v) glacial acetic acid.

Solution C : Aqueous urea (for concentration see Results).

Solution D : Freshly prepared 10% (w/v) ammonium persulphate in water.

Solutions A, B and C were stored at 0°C and warmed to room temperature

before being mixed in the ratio of 2 parts A : 1 part B : 5 parts C and the mixture was de-aerated using a vacuum line. 0.1 ml of solution D were added per 8 ml of gel solution and the mixture transferred to 10 cm x 0.6 cm (I.D.) Pyrex tubes sealed at the bottom with Parafilm and held vertical by means of a clamp. The gel solution was carefully overlaid with water and polymerization was allowed to take place for approximately 2 hours, depending on the urea concentration (the presence of urea reduces the polymerization time by approximately 50%). Electrophoresis was performed at room temperature using a Shandon vertical gel system set to deliver constant current.

Pre-electrophoresis :

Gels were routinely pre-electrophoresed to remove all charged species other than protons and acetate anions. Pre-electrophoresis was performed by electrophoresing the gels at a constant current of 2 mA / gel for approximately 6 hours using a tray buffer of 0.9 N acetic acid. Pre-electrophoresis was adjudged complete when the voltage stabilized.

Sample Preparation :

0.2 ml of the basic nuclear protein solution were added to 145 mg of urea. This gives a final volume of 0.3 ml and a final urea concentration of 8 M. The samples were incubated in 8 M urea with 0.5 M 2-mercaptoethanol for approximately 12 hours before 0.1 ml was applied to the top of the gels for electrophoresis.

Electrophoresis

Electrophoresis was performed for 4 hours in a cathodic direction at 2 mA / gel (constant current) using a tray buffer of 0.9 N acetic acid. Gels were removed from the tubes by first immersing them in water at 4°C for 5 min., carefully rimming

the gels with a blunted 26-gauge hypodermic needle connected to a water-filled syringe and, when loosened, by applying gentle water pressure to one end.

Staining :

Gels were stained for at least 8 hours in 0.1% (w/v) Napthalene Black 12 B dissolved in 7% (v/v) acetic acid.

Destaining :

Gels were destained electrolytically in 7% acetic acid for 2 hours at 4 volts and 2 amps. Any background stain remaining was then removed by diffusion against 7% acetic acid.

Measurement :

Destained gels were photographed and scanned using a Unicam 1809 gel scanning attachment for a Unicam SP1800 spectrophotometer at a wavelength of 620 nm.

(ix) Hybridization of Sea Urchin 9S Histone mRNA to *D.discoideum* DNA

DNA isolated from myxamoebae of *D.discoideum* by the method of Leach & Ashworth (1972) was sheared by passage through a 21 - gauge hypodermic needle and banded on a CsCl equilibrium gradient. Fractions were collected and the DNA loaded onto Sartorius membrane filters as described by Birnsteil et al (1968). The hybridization with radioactively labelled 9S messenger RNA from sea urchins was kindly performed by Professor M. Birnsteil using techniques previously described (Kedes & Birnsteil, 1971).

RESULTS

(i) Isolation of Nuclei

Method A : The procedure for the isolation of nuclei described by Coukell & Walker (1973) was found to be unsatisfactory. Incubation of the cell suspension with 0.06% (v/v) Triton X-100 only resulted in approximately 70% cell lysis. Moreover, even after the two differential centrifugations, the purified nuclei still contained many unbroken cells.

Method B : The combination of the methods of Coccuci & Sussman (1970) and Coukell & Walker (1973) was considerably more successful in the preparation of purified nuclei than Method A. Microscopic examination of the Cemulsol-treated cells routinely showed the incidence of whole cells to be very low (less than 10^{-4}). Phase contrast microscopy of the purified nuclear suspension showed the nuclei to be intact (although perhaps slightly swollen) and free of unruptured cells, mitochondria and membranous debris (PLATE 2).

(ii) Component Analysis During Nuclei Preparation

TABLE 2 shows the DNA, RNA and protein content of various fractions obtained during the isolation of the purified nuclei from both vegetative and developing cells of D.discoideum. It can be seen that although there is considerably less RNA and protein present in developing cells, the macromolecular composition of the nuclei isolated from the cells of both phases are almost identical, both containing approximately 75% of the total cellular DNA. This approximate figure of 25% for the mitochondrial DNA content is in agreement with that of Leach & Ashworth (1972). The precise DNA : RNA : protein ratios for the

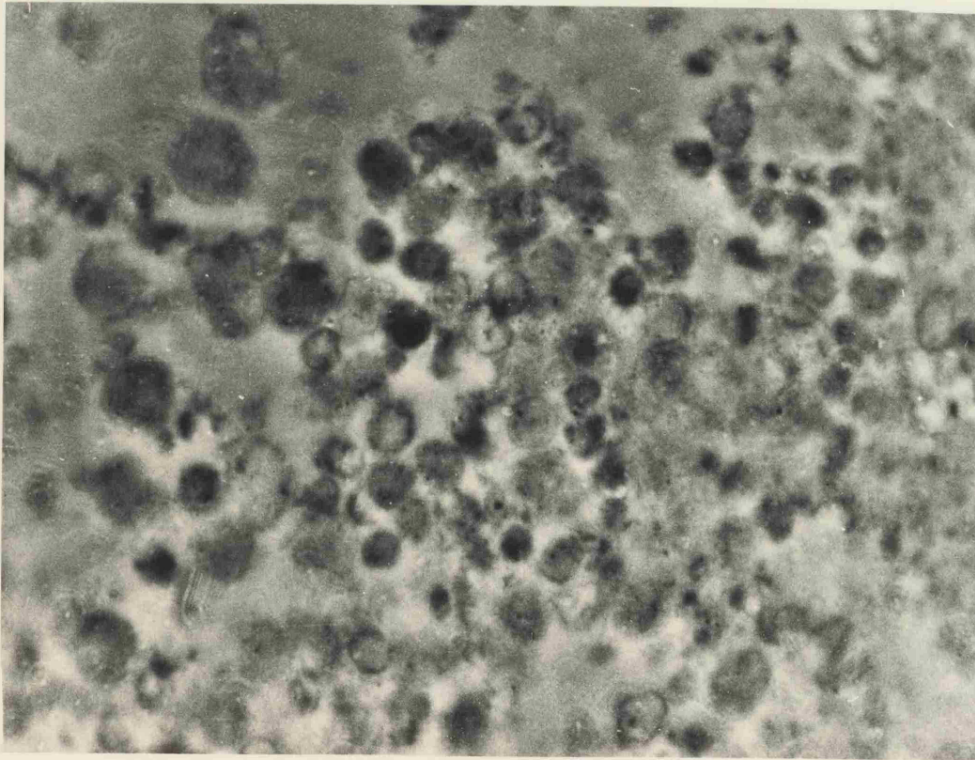


PLATE 2. Phase Contrast Micrograph of a Preparation of
Nuclei from D.discoideum

The nuclei were resuspended in 0.01 M Tris/Cl buffer,
pH 7.5, containing 0.15 M NaCl.

Magnification x 1350, the bar represents 2 μ m.

nuclei were determined as:

Nucleol (vegetative), 1: 5.3 ± 1.2 : 30.0 ± 4.6 (5 determinations)

Nucleol (developing), 1: 9.3 ± 2.1 : 28.1 ± 2.7 (4 determinations)

(iii) Basic Nucleol Protein Extraction

The ratios of DNA : RNA : soluble protein present in the nucleol were determined as:

FRACTION	VEGETATIVE CELLS			DEVELOPING CELLS		
	DNA (μ g)	RNA (mg)	PROTEIN (mg)	DNA (μ g)	RNA (mg)	PROTEIN (mg)
Whole Cell Lysate	2070	161	977	1344	41	382
Supernatant 1	507	94	864	300	21	310
Supernatant 2	51	41	58	40	8	38
Supernatant 3	7	14	13	12	4	5
Nuclear Pellet	1480	12	44	992	8	28

mg of protein and whole cells 28 μ g tryptophan/mg of protein.

(v) Polyacrylamide Gel Electrophoresis

The electrophoreses described below are represented in the figures by a photograph and a drawing.

TABLE 2. Localization of Major Cell Constituents During the Isolation of Nuclei from D. discoideum

Perlin & Chalkley (1969) have reported that, in the absence of pre-electrophoresis (Numbers shown are typical of several (4) experiments performed and refer to approximately 1.1×10^{10} cells in the vegetative phase and to approximately 7.5×10^9 cells in the developmental phase). Figs 4 and 5 show the banding patterns obtained in the absence of pre-electrophoresis. Only 2 major and 3 relatively minor bands are discernible and a considerable amount of the protein appears not to have entered the gel. It has been suggested (Perlin & Chalkley, 1969) that before electrophoresis the

nuclei were determined as ;

Nuclei (vegetative), 1 : 8.3 ± 1.2 : 30.0 ± 4.6 (6 determinations)

Nuclei (developing), 1 : 8.3 ± 2.1 : 28.1 ± 3.7 (4 determinations)

(iii) Basic Nuclear Protein Extraction

The ratios of DNA : acid-soluble protein present in the nuclei were determined as

Nuclei (vegetative), 1 : 0.96 ± 0.04 (6 determinations)

Nuclei (developing), 1 : 0.90 ± 0.05 (4 determinations)

(iv) Tryptophan analysis

The highly sensitive assay procedure for the estimation of tryptophan enabled very small quantities ($1\mu\text{g}$) to be detected (FIGURE 3). The acid-soluble nuclear proteins of D.discoideum contained less than this minimum level of $1\mu\text{g}$ tryptophan /mg of protein. In contrast, whole nuclei contained $15\mu\text{g}$ tryptophan /mg of protein and whole cells $28\mu\text{g}$ tryptophan /mg of protein.

(v) Polyacrylamide Gel Electrophoresis

The electrophoreses described below are represented in the figures by a photograph and a drawing. Some of the minor bands, clearly visible to the eye, are not easily discernible on the photographs. These are included in the drawings.

Panyim & Chalkley (1969) have reported that, in the absence of pre-electrophoresis, calf thymus histone fractions were both slow moving and badly resolved on urea-polyacrylamide gel electrophoresis. This was also found to be the case when the basic nuclear proteins of D.discoideum were similarly fractionated. FIGURES 4 and 5 show the banding patterns obtained in the absence of pre-electrophoresis. Only 2 major and 5 relatively minor bands are discernible and a considerable amount of the protein appears not to have entered the gel. It has been suggested (Panyim & Chalkley, 1969) that before electrophoresis the

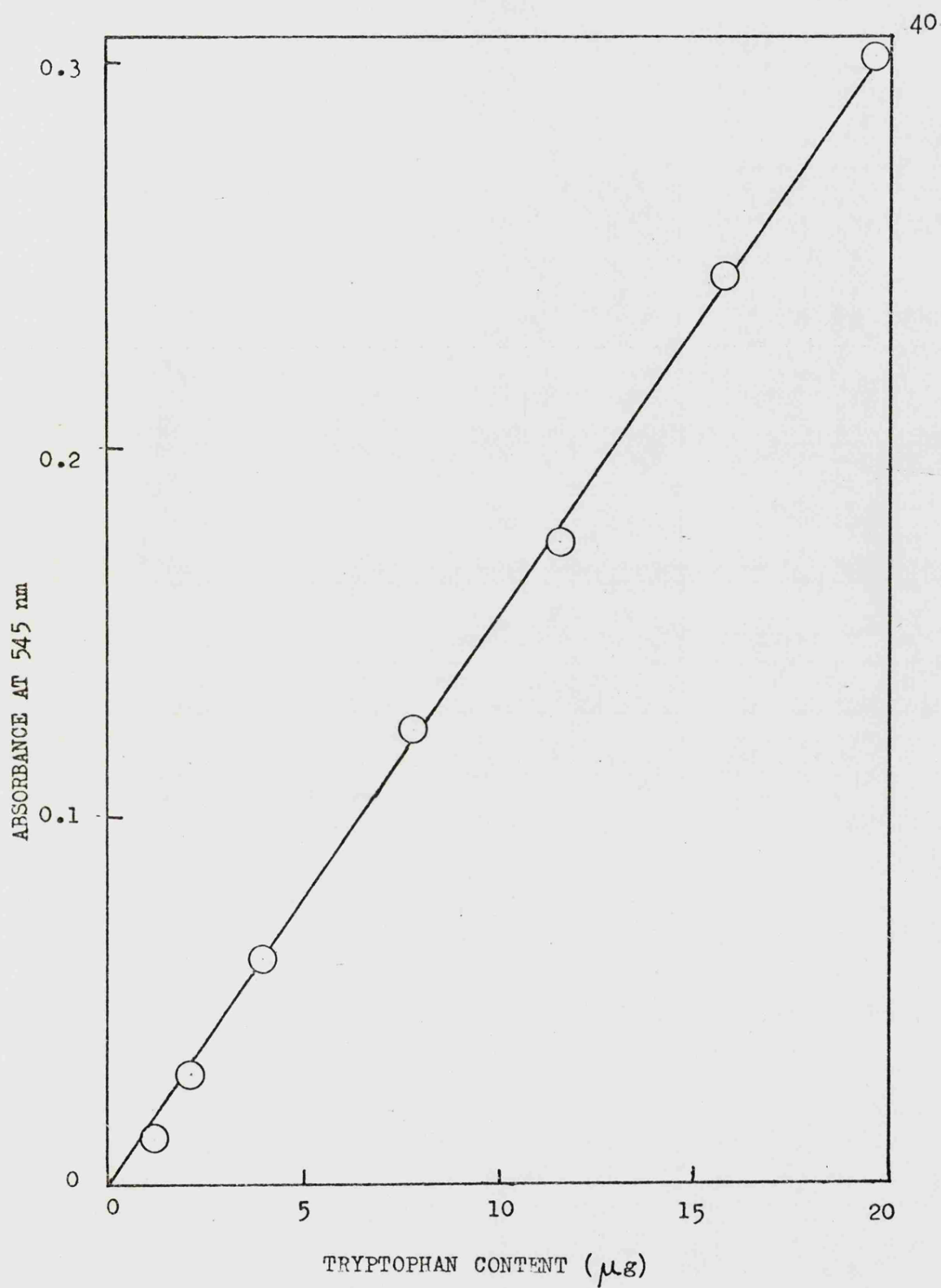


FIGURE 3. Standard Curve for the Estimation of Tryptophan

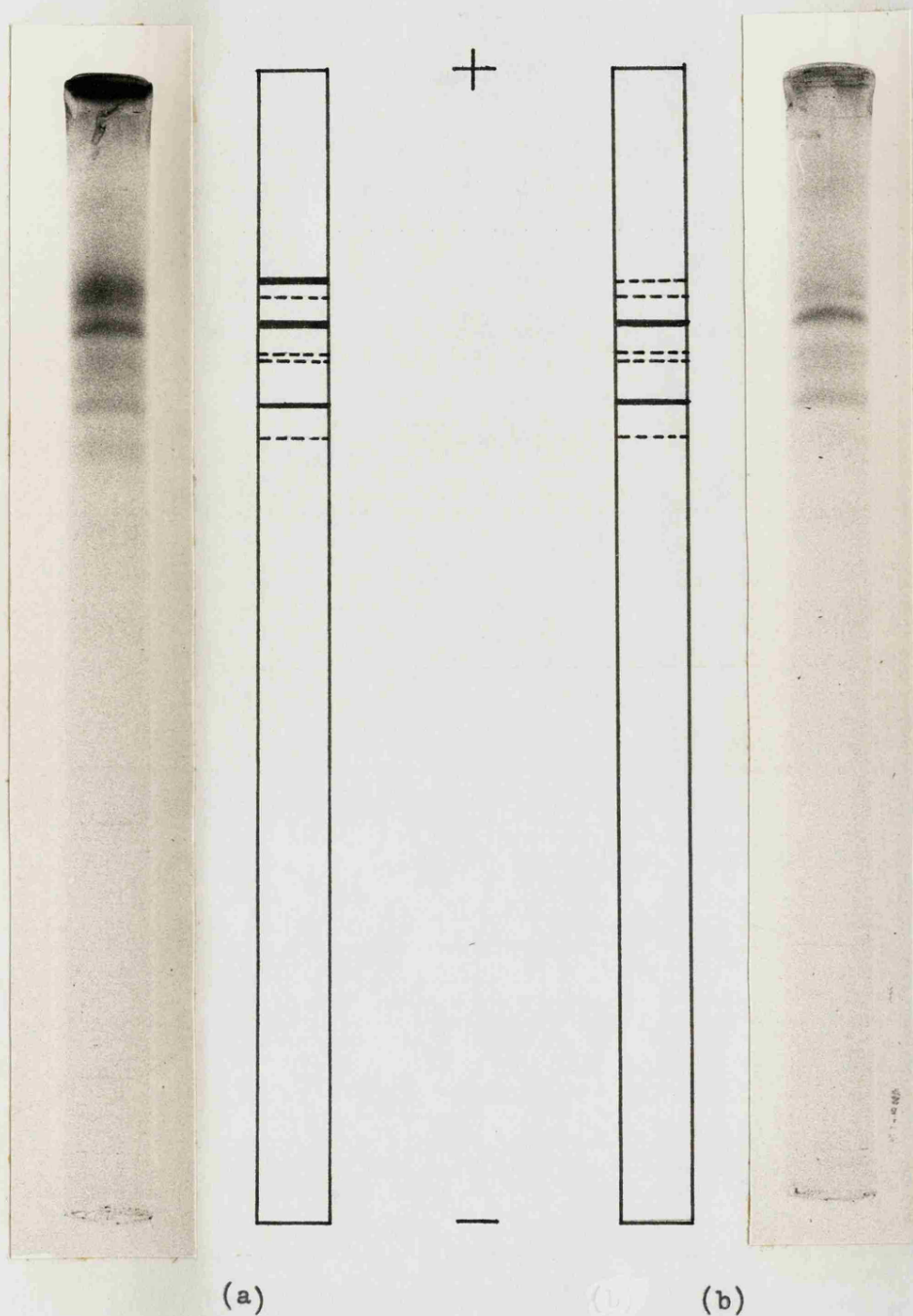


FIGURE 4. Electrophoresis of the Basic Nuclear Proteins of *D. discoideum* on Gels which had not been pre-electrophoresed. Electrophoresis was performed in gels containing 15% acrylamide, 2.5 M urea and 0.9 N acetic acid for 4 hours at 2 mA/gel constant current.

(a) 40 μ g basic nuclear proteins from vegetative cells,
 (b) 25 μ g basic nuclear proteins from developing cells.

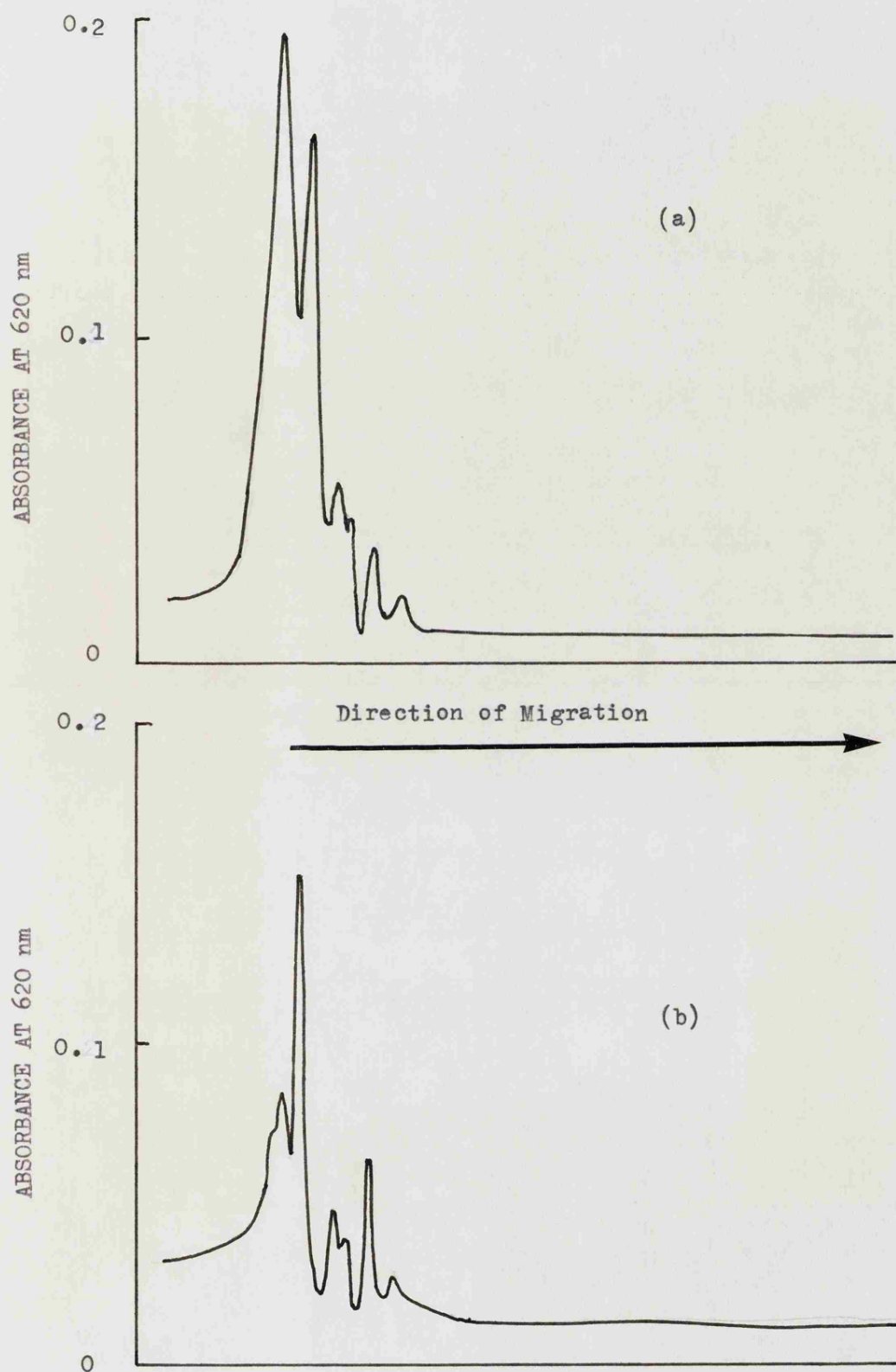


FIGURE 5. Densitometric Tracings of the electrophoresis of the basic nuclear proteins of D.discoideum

(a) $40\mu\text{g}$ basic nuclear protein from vegetative cells

(b) $25\mu\text{g}$ basic nuclear protein from developing cells.

gels contain ions required for the polymerization of the acrylamide (e.g. TEMED²⁺ and S₂O₈²⁻) as well as 0.9 N acetic acid. Pre-electrophoresis thus causes a drop in the pH due to the removal of the basic TEMED²⁺ ions and to a replacement of all ions by protons and acetate anions that associate and decrease the ionic strength. This is reflected by a rise in the voltage required to maintain the constant current of 2 mA / gel. Indeed, in the electrophoresis shown in FIGURES 4 and 5, the initial voltage of 31 volts increased to a final value of 75 volts after the 4 hour period. To overcome this problem, gels were routinely pre-electrophoresed to remove all charged species except protons and acetate anions. Pre-electrophoresis was monitored by recording the change in voltage across the gels and was adjudged complete when the voltage stabilized - usually after approximately 6 hours (FIGURE 6).

Electrophoresis of the basic nuclear proteins of D.discoideum produced different banding patterns depending on the urea concentration of the gels. In the absence of urea (FIGURES 7 and 8) 7 bands were discernible. Those of vegetative cells comprised 3 major bands, 3 minor bands and a fast-moving, very diffuse band, whilst those of developing cells comprised 2 major bands (both of which comigrated with 2 of the major bands of vegetative cells), 4 minor bands (3 of which corresponded to the 3 minor bands, and 1 to the remaining major band of vegetative cells) and a similar fast-moving, very diffuse band. In addition, the appearance of the gels suggested that only part of the sample loaded had actually entered the gel.

When urea is included as a solubilizer in the gels, quite different banding patterns are obtained. In gels containing 6 M urea (FIGURES 9 and 10), 6 bands were produced from both vegetative and developing basic nuclear proteins. The electrophoretic mobilities of

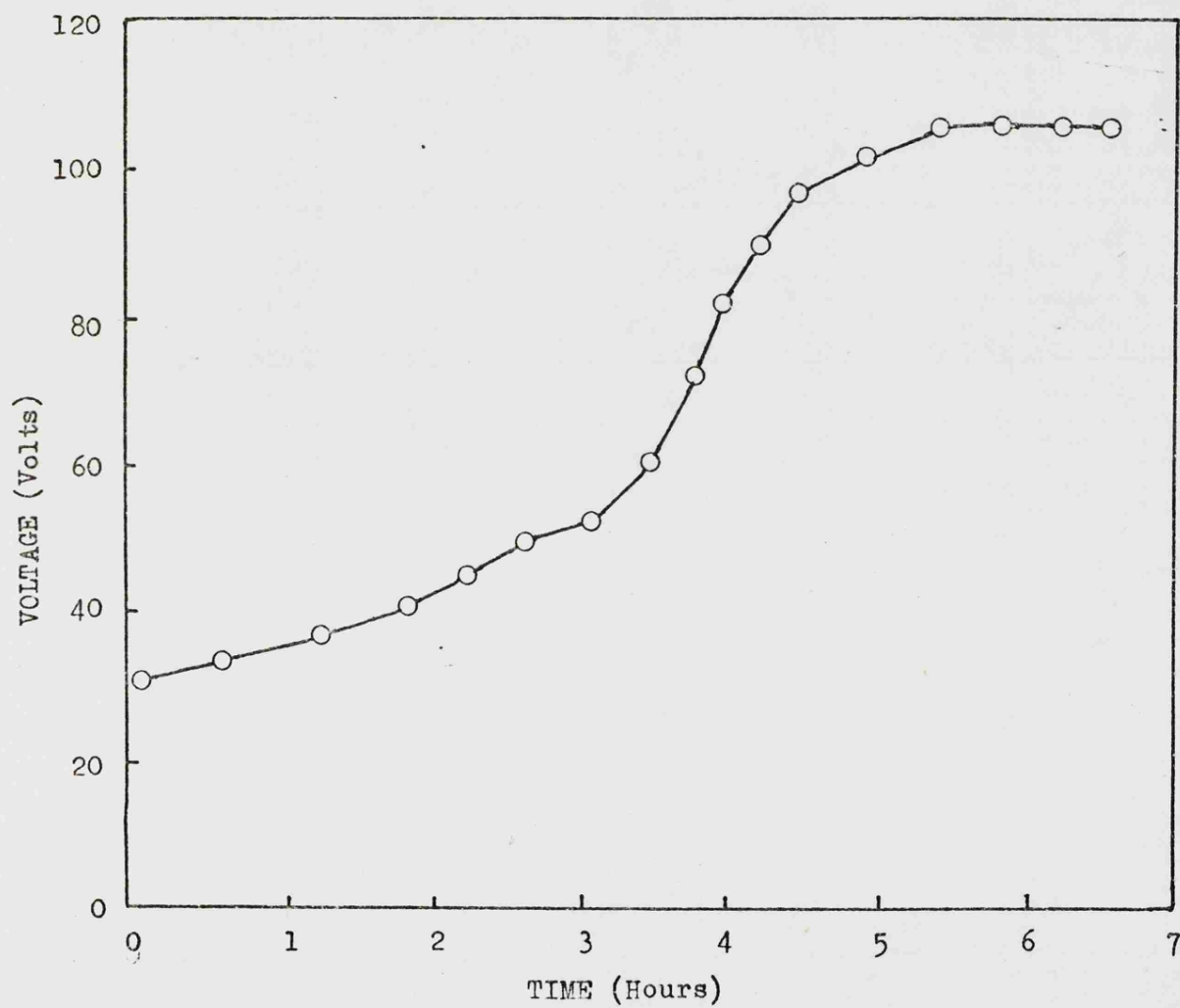


FIGURE 6. Voltage change during pre-electrophoresis at
constant current

Pre-electrophoresis of gels containing 15% acrylamide,
2.5 M urea and 0.9 N acetic acid was performed at 2 mA
per gel at constant current



FIGURE 7. Electrophoresis of the Basic Nuclear Proteins of
D. discoideum on gels containing 15 % acrylamide,
and 0.9 N acetic acid.

(a) 30 μg basic nuclear proteins from vegetative cells

(b) 20 μg basic nuclear proteins from developing cells

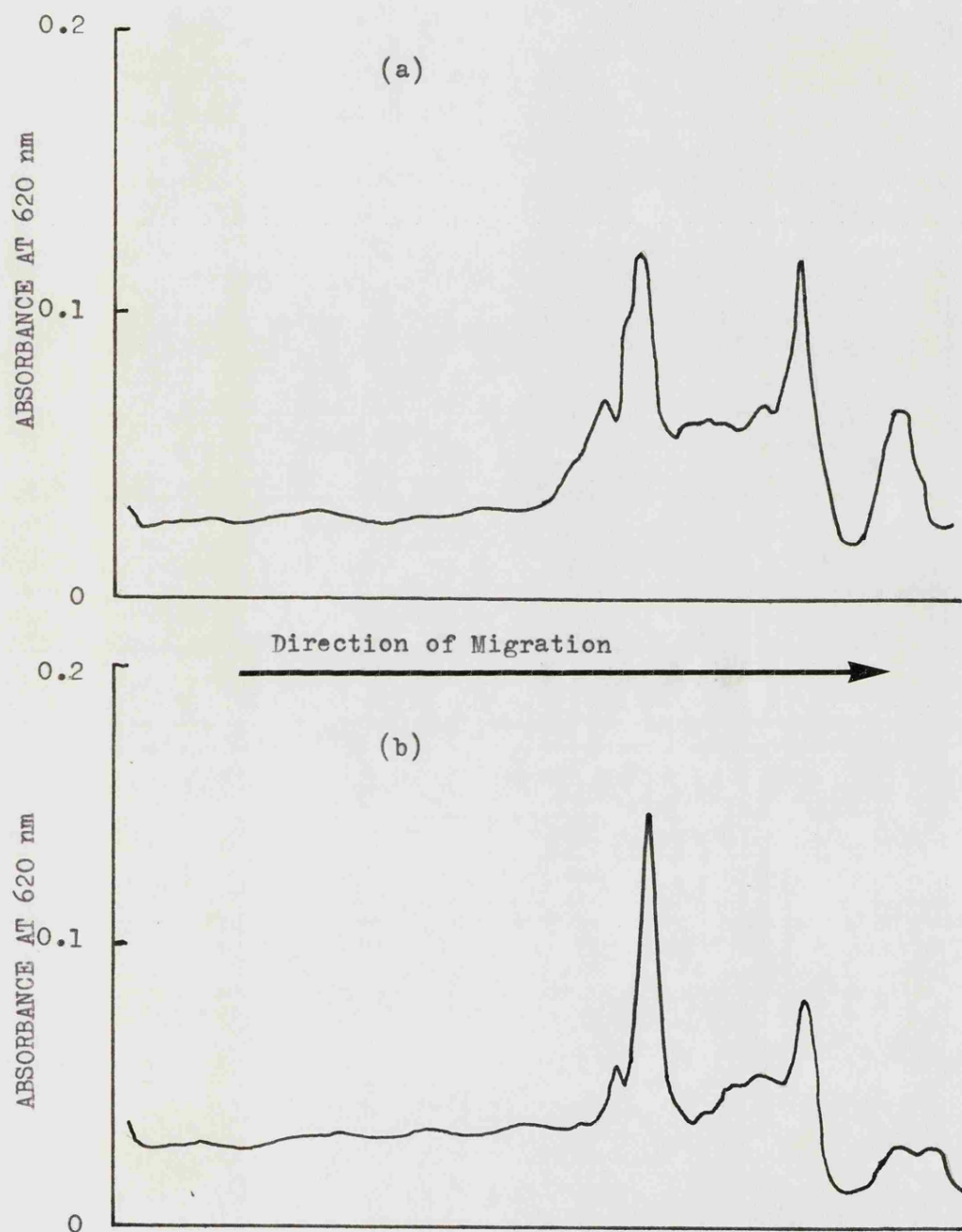


FIGURE 8. Densitometric Tracings of the Electrophoresis of the Basic Nuclear Proteins of *D. discoideum* on gels containing 15% acrylamide and 0.9 N acetic acid.

(a) 30 μ g basic nuclear proteins from vegetative cells

(b) 20 μ g basic nuclear proteins from developing cells



FIGURE 9. Electrophoresis of the Basic Nuclear Proteins of
D. discoideum on gels containing 15% acrylamide,
6.25 M urea and 0.9 N acetic acid.

(a) 30 μ g basic nuclear proteins from vegetative cells

(b) 20 μ g basic nuclear proteins from developing cells

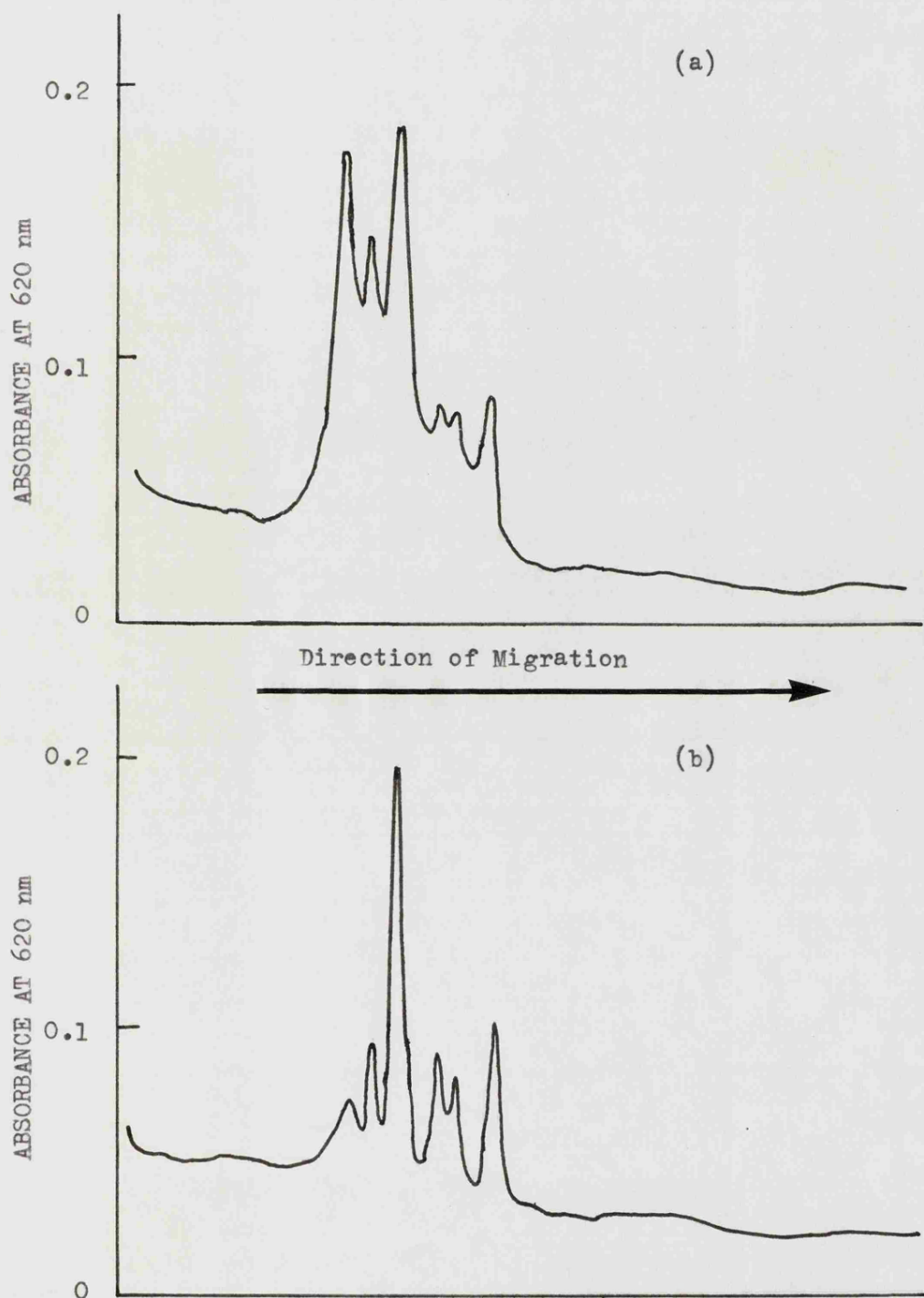


FIGURE 10. Densitometric Tracings of the Electrophoresis of the Basic Nuclear Proteins of *D. discoideum* on gels containing 15% acrylamide, 6.25 M urea and 0.9 N acetic acid.

(a) 30 μ g basic nuclear proteins from vegetative cells

(b) 20 μ g basic nuclear proteins from developing cells

the bands were approximately identical and the only difference was a quantitative one - one of the major bands of the vegetative basic nuclear proteins was represented by only a minor band in the basic nuclear proteins of developing cells.

Lowering the urea concentration to 4 M (FIGURES 11 and 12) results in the production of 10 bands from both vegetative and developing basic nuclear proteins. Similarly, in 2.5 M urea (conditions which Panyim & Chalkley (1969) report as resolving most bands from calf thymus histones), both vegetative and developing basic nuclear proteins produce 10 bands (FIGURES 13 and 14). These appear to correspond with the 10 bands obtained in 4 M urea but are slightly better resolved and can now be discussed.

It will be convenient, at this stage, to introduce a nomenclature for these bands, and in the hope of assigning the accepted histone nomenclature to the basic nuclear proteins of D.discoideum, a sample of calf thymus histone was similarly electrophoresed in gels containing 2.5 M urea (FIGURE 15).

Unfortunately, a comparison between the electrophoretic pattern of calf thymus histone fractions, named according to the system of Johns & Butler (1962), and the pattern of the basic nuclear proteins of D.discoideum shows very little similarity whatsoever (FIGURE 16). Consequently the accepted histone nomenclatures cannot be meaningfully applied to the basic nuclear proteins of D.discoideum. Instead, the bands have been grouped, somewhat arbitrarily, into 5 main fractions.

Fraction 1 consists of 4 bands (a, b, c and d) of which only lc has an electrophoretic mobility similar to the lysine-rich histone (fl) of calf thymus. Bands lc and ld (of vegetative cells) are major bands, band lb a medium band and la a minor band. In developing

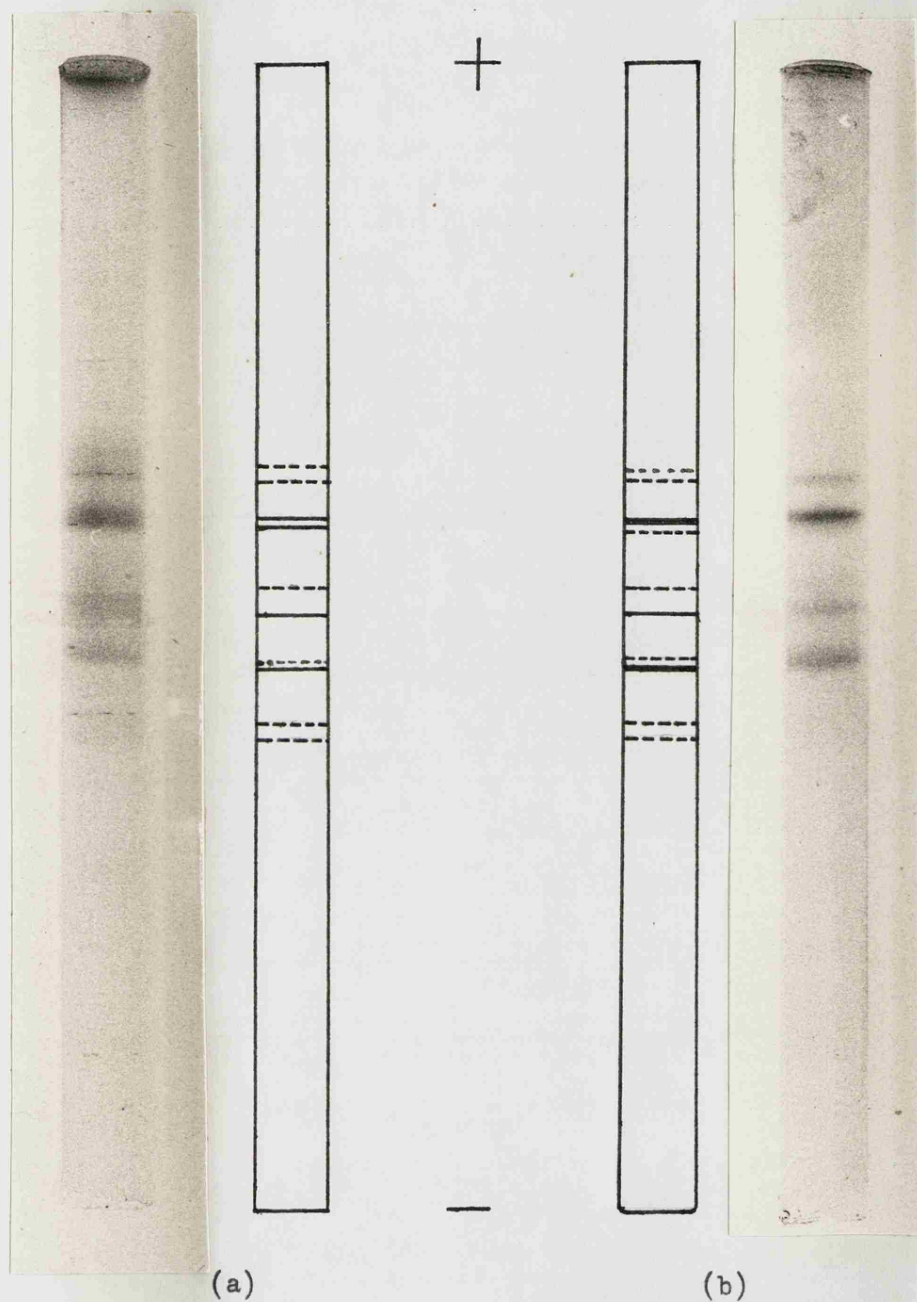


FIGURE 11. Electrophoresis of the Basic Nuclear Proteins of *D. discoideum* on gels containing 15% acrylamide, 4 M urea and 0.9 N acetic acid.

(a) 30 μ g basic nuclear proteins from vegetative cells

(b) 20 μ g basic nuclear proteins from developing cells

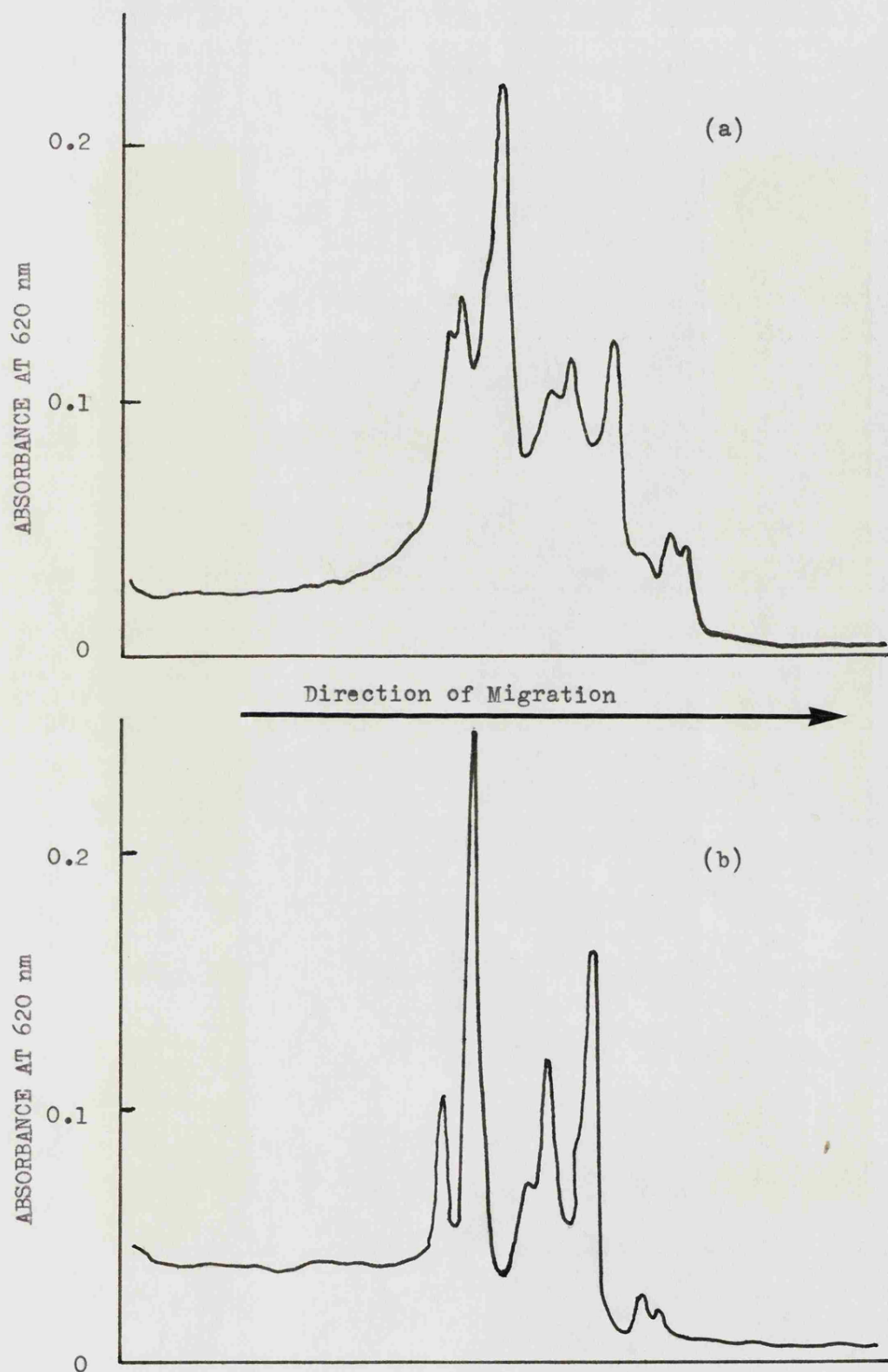


FIGURE 12. Densitometric Tracings of the Electrophoresis of the Basic Nuclear Proteins of *D. discoideum* on gels containing 15% acrylamide, 4 M urea and 0.9 N acetic acid.

(a) 30 μ g basic nuclear proteins from vegetative cells

(b) 20 μ g basic nuclear proteins from developing cells

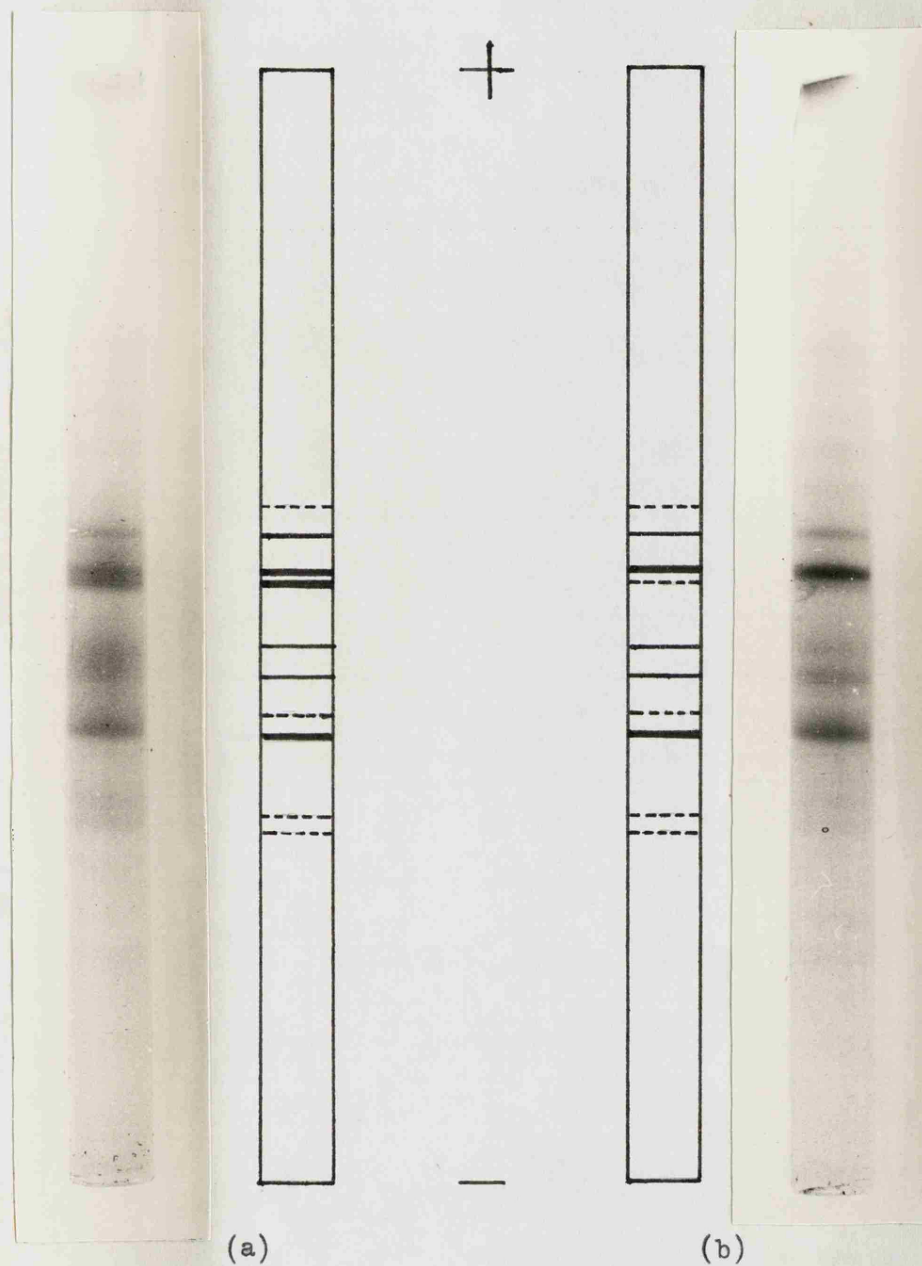


FIGURE 13. Electrophoresis of the Basic Nuclear Proteins of *D. discoideum* on gels containing 15% acrylamide, 2.5 M urea and 0.9 N acetic acid.

(a) 30 μ g basic nuclear proteins from vegetative cells

(b) 20 μ g basic nuclear proteins from developing cells

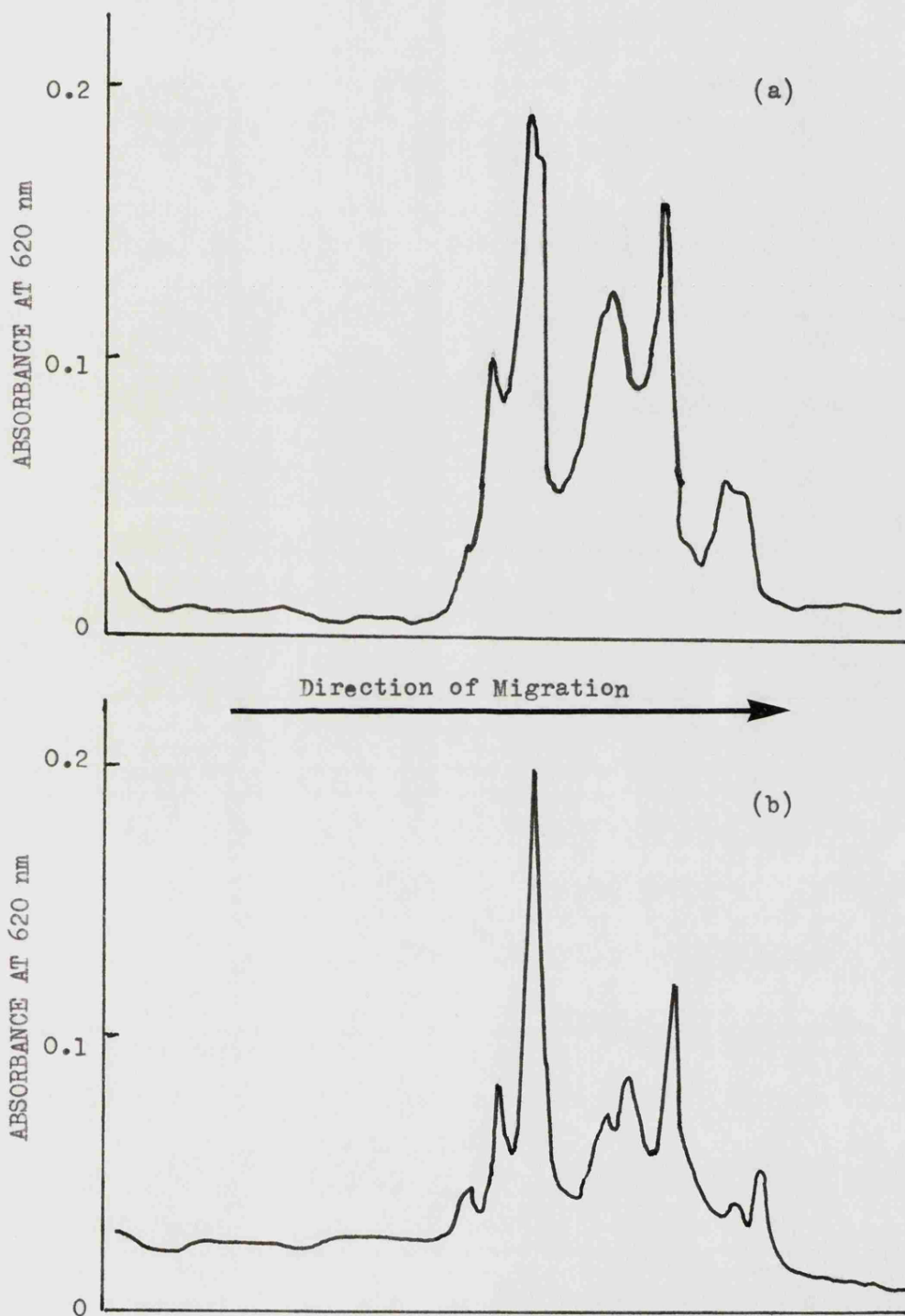


FIGURE 14. Densitometric Tracings of the Electrophoresis of the Basic Nuclear Proteins of *D. discoideum* on gels containing 15% acrylamide, 2.5M urea and 0.9 N acetic acid.

(a) 30 μ g basic nuclear proteins from vegetative cells

(b) 20 μ g basic nuclear proteins from developing cells

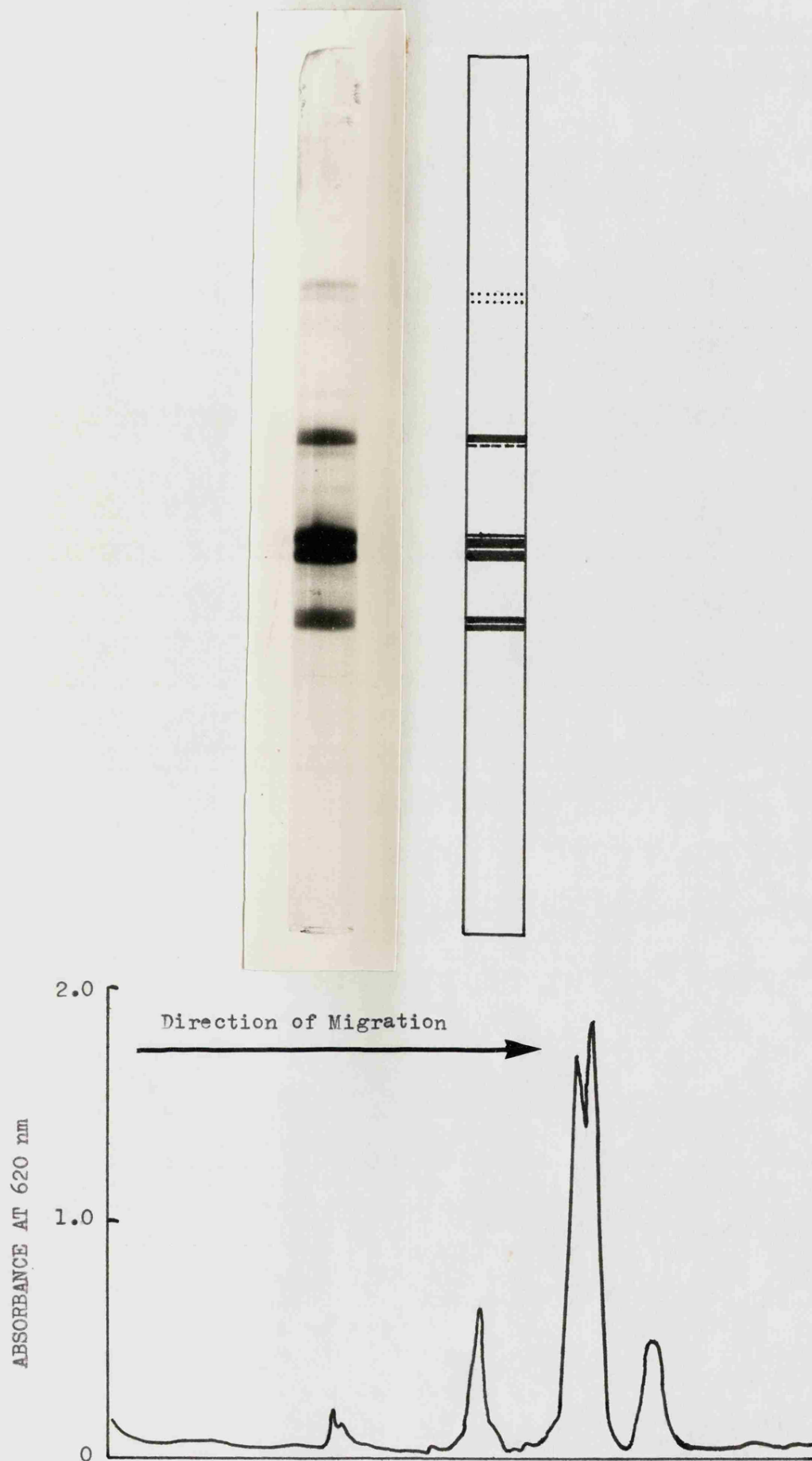


FIGURE 15. Electrophoresis and Densitometric Tracing of Calf Thymus Histones (50 μ g) in gels containing 15% acrylamide, 2.5 M urea and 0.9 N acetic acid.

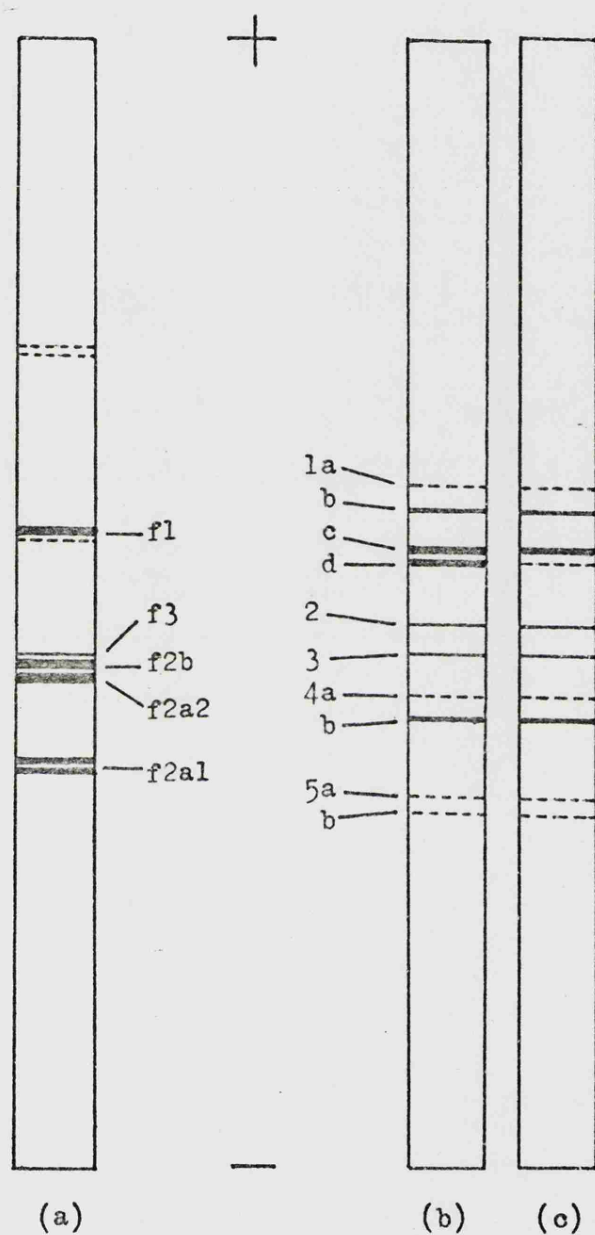


FIGURE 16. Comparison of the Basic Nuclear Proteins of *D. discoideum* with Calf Thymus Histones fractionated on gels containing 15% acrylamide, 2.5 M urea and 0.9 N acetic acid.

(a) 50 μ g calf thymus histones

(b) 30 μ g basic nuclear proteins from vegetative cells

(c) 20 μ g basic nuclear proteins from developing cells

basic nuclear proteins, band 1d is considerably reduced in its intensity, but bands 1a, 1b and 1c are present in the same proportion as they were in vegetative cells.

Fraction 2, a single medium intensity band present in the same quantity in both vegetative and developing basic nuclear proteins, appears to migrate slightly slower than the arginine-rich histone (f3) of calf thymus.

Fraction 3, another single, medium intensity band which is found to the same degree in vegetative and developing samples, does however appear to resemble the arginine-rich histone (f3) of calf thymus in its electrophoretic mobility.

Fraction 4, which comprises 2 relatively major bands (4a and 4b), migrates faster than either of the two slightly lysine-rich histones (f2b and f2a2) of calf thymus. The doublet is present in similar quantities in both vegetative and developing samples.

Fraction 5, which is also a doublet but this time composed of two relatively minor bands (5a and 5b) appears to migrate slightly faster than the arginine-rich histone (f2a1) of calf thymus. Again, this doublet is present in the basic nuclear proteins of both vegetative and developing cells.

The only difference, then, between the basic nuclear proteins of vegetative and developing cells appears to be a quantitative one, fraction 1d being a major band in vegetative cells and only a minor component of developing cells.

In any study of basic nuclear proteins isolated by the extraction of whole nuclei, one has to guard against the possibility of contamination with ribosomal proteins as these are transiently associated with the nucleus (see Chapter 3). FIGURE 17 shows the electrophoresis of 100 μ g of the basic ribosomal proteins of

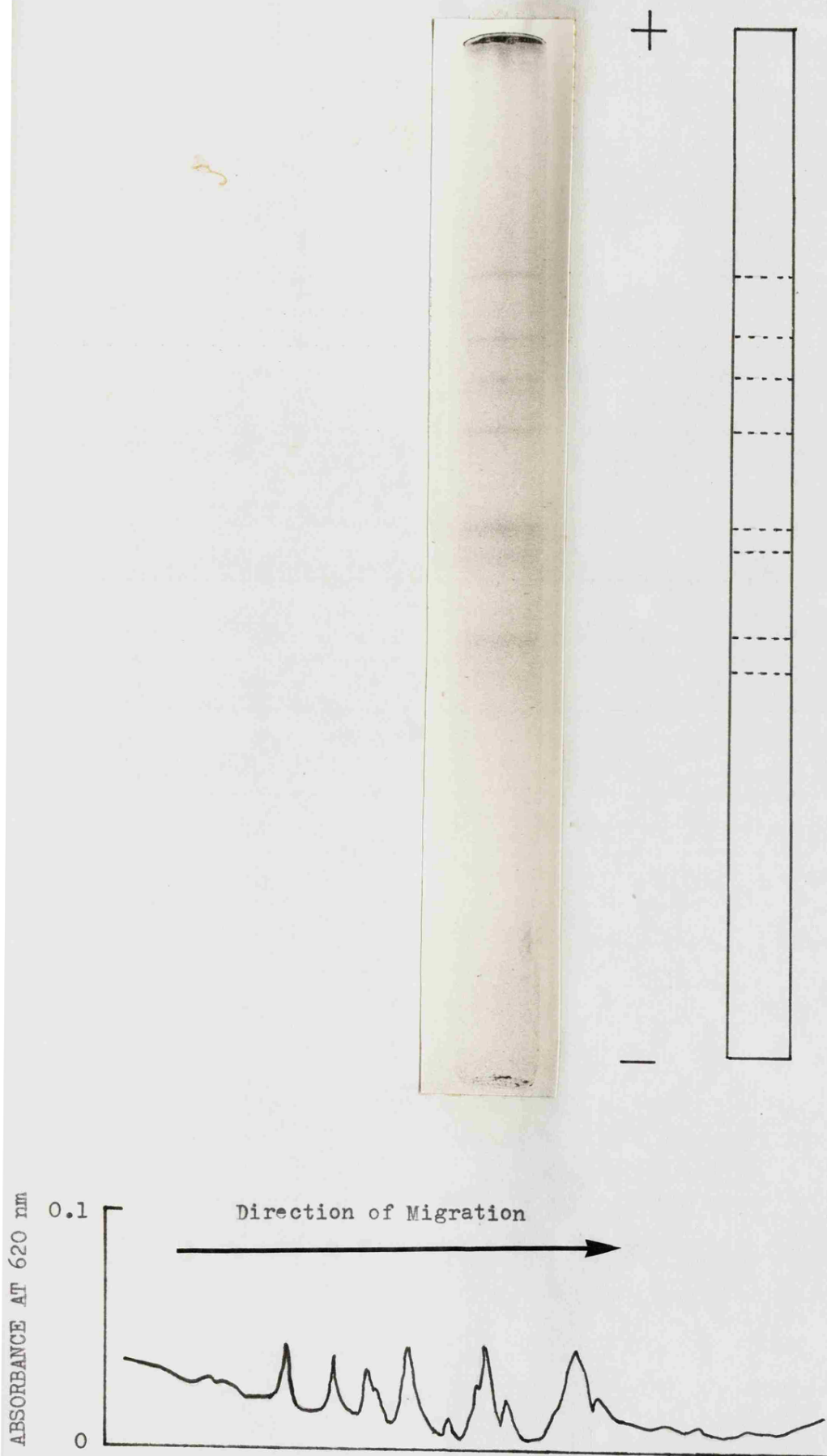


FIGURE 17. Electrophoresis and Densitometric Tracing of the Basic Ribosomal Proteins(100 μ g) from *D.discoideum* in gels containing 15% acrylamide, 2.5 M urea and 0.9 N acetic acid.

D.discoideum on gels containing 15% acrylamide, 2.5 M urea and 0.9 N acetic acid. It is evident that much of the protein does not enter the gel for only a few very faint bands are observed. Moreover, a comparison with the bands obtained from the electrophoresis of the basic nuclear proteins (FIGURE 18) shows there to be no electrophoretically identical bands.

(vi) Hybridization of Sea Urchin 9S histone mRNA to D.discoideum DNA

The results of the hybridization of sea urchin 9S histone mRNA to fractions of sheared D.discoideum DNA banded on a CsCl equilibrium gradient are shown in FIGURE 19. In assessing the results, the following two points must be considered -

- (i) The counts were obtained without the filter being treated with RNAase and
- (ii) A similar experiment using sea urchin DNA gave 5000 cpm in the peak fraction after being treated with RNAase.

It is thus quite clear that sea urchin histone messenger RNA does not hybridize to any significant degree to D.discoideum DNA.

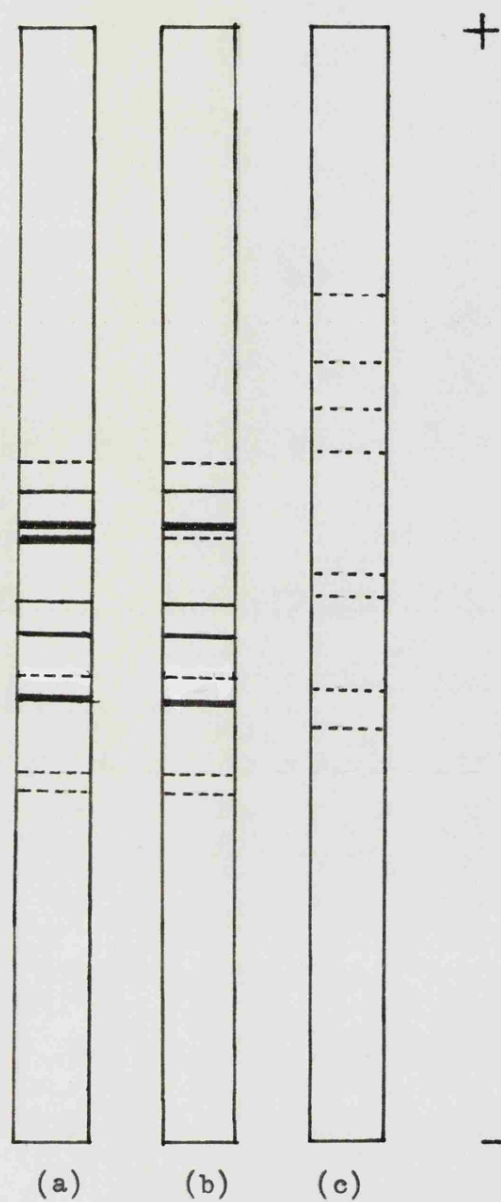


FIGURE 18. Comparison of the Basic Nuclear Proteins of *D.discoideum* with the Basic Ribosomal Proteins of *D.discoideum* fractionated on gels containing 15% acrylamide, 2.5 M urea and 0.9 N acetic acid.

(a) 30 μ g basic nuclear proteins from vegetative cells

(b) 20 μ g basic nuclear proteins from developing cells

(c) 100 μ g basic ribosomal proteins from vegetative cells

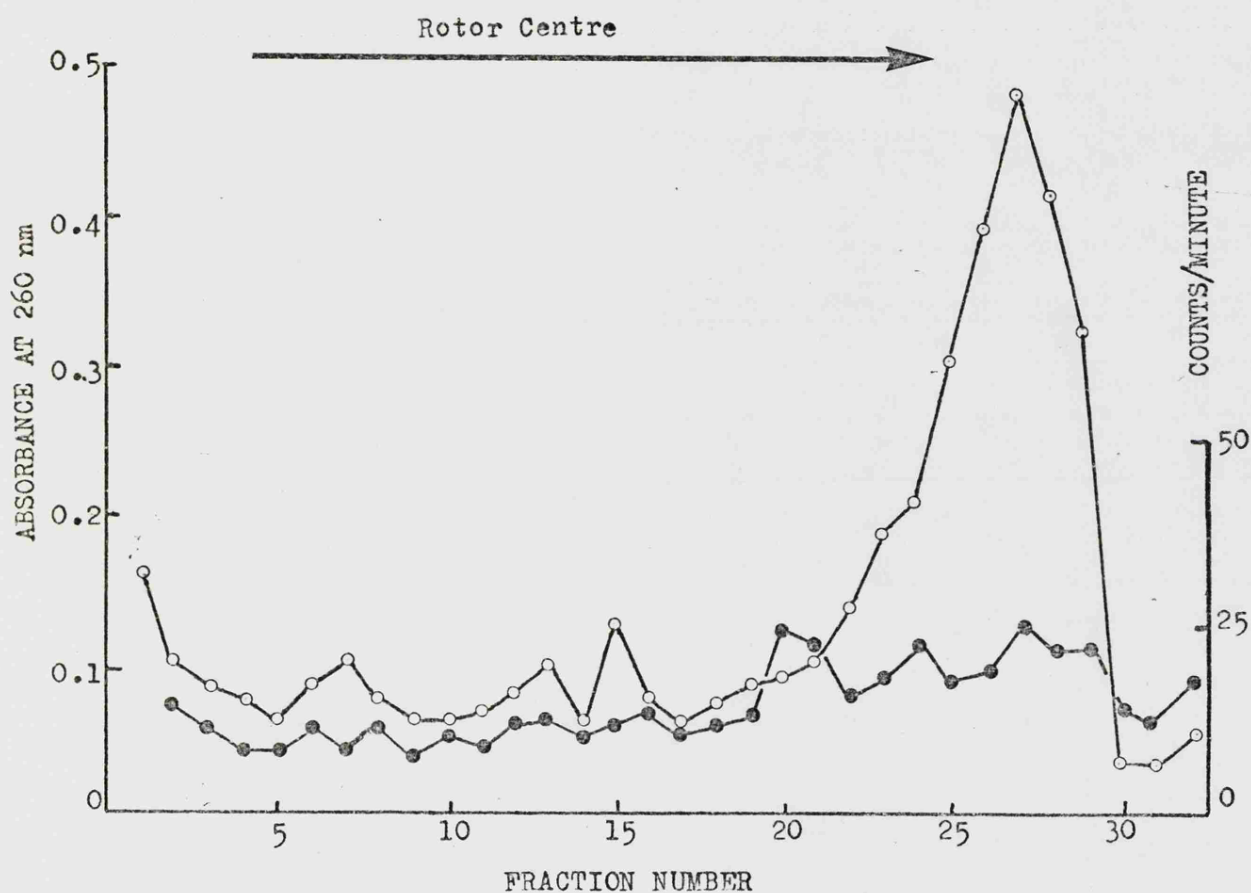


FIGURE 19. Hybridization of sea urchin 9S Histone mRNA to fractions of *D. discoideum* DNA banded on CsCl equilibrium gradient.

- : Absorbance of fractions at 260 nm before hybridization
 ● : Counts per minute of sea urchin 9S mRNA adhering to filters after hybridization.

DISCUSSION

Before the results of the electrophoresis of the basic nuclear proteins of D.discoideum can be meaningfully discussed, it is necessary to assess critically the procedures used in their isolation. Although the method used is similar to that described for Physarum polycephalum (Mohberg & Rusch, 1969), the initial step - the boiling of the nuclear suspension - is worth considering in more detail. Coukell & Walker (1973) have shown that this heat treatment is necessary to inactivate various acid proteases present in the nuclear suspension which rapidly degrade the basic nuclear proteins during their isolation. Indeed, this satisfactorily explains the disappointing results of experiments performed at the beginning of this work, when chromatin, prepared by sucrose density centrifugation as described by Bonner et al (1968), and extracted with 0.4 N sulphuric acid, failed to produce bands on polyacrylamide gel electrophoresis, usually only a blue smear was observed. However, one could question the validity of a comparison between the electrophoretic mobilities of the basic nuclear proteins of D.discoideum, which have been isolated from boiled nuclei, and the histones of calf thymus, which have been isolated by less extreme techniques. This criticism has been resolved by Coukell & Walker (1973) who demonstrated that the electrophoretic mobilities of calf thymus histones were unaffected by the heat treatment used in the preparation of the basic nuclear proteins of D.discoideum. This fact is comforting, as attempts to inhibit the proteolytic degradation by other means - namely by the presence of 0.05 M sodium bisulphite, which has been reported (Panyim & Chalkley, 1969) to inhibit proteolysis of calf thymus histones during isolation - were unsuccessful, with again, only a blue smear being observed on the gels. This demonstrates the

extremely high content and activity of proteases in D.discoideum which can only be effectively inhibited by such drastic treatment as boiling. This is, perhaps, not too suprising when one considers that, in their natural environment of the soil, the myxamoebae grow by the digestion of many different bacteria - a process that clearly requires the presence of a comprehensive array of highly active degradative enzymes.

Since the starting point for the isolation of the basic nuclear proteins has been the nuclei, we can consider the major components of the nuclei with respect to those of other organisms. The DNA : RNA : protein ratio of D.discoideum nuclei has been determined as approximately 1 : 8 : 30. In this respect, the nuclei of D.discoideum resemble more closely the nuclei of the yeast, Saccharomyces cerevisiae, which have a DNA : RNA : protein ratio of 1 : 3.7 : 35 (Rozijn & Tonino, 1964) than they do the nuclei of the true slime mould Physarum polycephalum with a ratio of 1 : 1 : 5 (Mohberg & Rusch, 1971). The high ratios of D.discoideum and yeast are probably a reflection of the extremely low DNA content of these simple eukaryotic organisms (Ogur et al, 1952 ; Sussman & Rayner, 1971). However, this resemblance of D.discoideum nuclei to those of yeast and several other fungi is not continued in the content of their basic nuclear proteins. In marked contrast to several fungi, the nuclei of D.discoideum contain many basic proteins, in a quantity similar to that found in higher eukaryotes (the ratio of DNA : acid soluble protein is approximately 1 : 1), a fact which is certainly reassuring when one considers the extensive use of this organism as a model system for development.

So far we have discussed the major components of D.discoideum nuclei and one point is clear, namely that since the acid soluble

proteins are present in an approximate 1 : 1 ratio with the DNA, they comprise only a small proportion (3%) of the total nuclear protein, which is present in an approximate 30 : 1 ratio with the DNA. This fact can be utilized for the estimation of the purity of the isolated basic nuclear proteins. One first has to make the assumption that, like the histones of all other organisms, the basic nuclear proteins of D.discoideum lack the amino acid residue tryptophan. Since the results of the tryptophan analyses showed that the total nuclear proteins contained $15\mu\text{g}$ of tryptophan / mg of protein, and the basic nuclear proteins contained less than $1\mu\text{g}$ of tryptophan / mg of protein, then the contamination of the preparation with 'non-histone' protein is less than 7%. Exactly what constitutes non-histone protein is hard to ascertain. Many papers concerned with histones that have been isolated from whole nuclei have discussed the possibility of contamination with basic ribosomal proteins, and indeed such contamination is difficult to assess on the basis of tryptophan content alone, since it appears that these too have low levels of this amino acid residue ($2 - 3\mu\text{g}$ / mg of protein). However, contamination with ribosomal proteins is not considered significant for two reasons. Firstly, CaCl_2 is very inefficient in extracting proteins from ribosomes, especially in the absence of a denaturing agent such as urea or acetic acid (see Chapter 3) and secondly, electrophoresis of the ribosomal proteins that are extracted produces no bands electrophoretically similar to the basic nuclear proteins.

There does remain the possibility that those proteins, transiently associated with the maturing ribosome, which remain in the nucleolus when the mature ribosomal sub-units migrate to the cytoplasm (see Chapter 3) are a potential source of contamination. It is difficult

to resolve this further, as fractionation of the nuclei (for example to produce chromatin) allows proteolytic degradation of the basic nuclear proteins to occur.

Having established that the methods used for the isolation of the basic nuclear proteins are appropriate, we can now consider the results of the polyacrylamide gel electrophoresis. The ten bands resolved upon the electrophoresis of the basic nuclear proteins of D.discoideum showed very little similarity to the pattern obtained from calf thymus histones. Only the electrophoretic mobilities of D.discoideum fraction 3 and the arginine-rich histone (f3) of calf thymus appeared to be identical. Although slightly faster moving than the f2a1 fraction of calf thymus histones, D.discoideum fraction 5 could be tentatively correlated with this calf thymus fraction since it too is present as a doublet and in consideration of the fact that of all the histones, the structure of f2a1 is the most highly conserved amongst widely different species (DeLange, 1969a).

However, the implied assumption that a similarity in electrophoretic mobility is synonymous with identical amino acid sequences is far from valid. Chemical studies on the histones of Physarum polycephalum (Mohberg & Rusch, 1971) and Tetrahymena (Hamana & Iwai, 1971) have shown that electrophoretically similar histones can vary significantly in amino acid composition. It thus appears that identical electrophoretic mobilities only suggest homology between proteins, and that differences in mobilities must be regarded as significant, even if they are only slight. From this viewpoint, the results suggest quite considerable differences between D.discoideum basic nuclear proteins and calf thymus histones. This is supported by the results of the hybridization studies. The 9S mRNA of sea urchins that is believed to code for histones, can

hybridize with the DNA of several other eukaryotes (Kedes & Birnstiel, 1971). However, it does not appear to hybridize at all to D.discoideum DNA, which implies that the genes coding for the basic nuclear proteins in D.discoideum are really very different from those coding for histones in other eukaryotes.

The comparison between the basic nuclear proteins of vegetative cells and those isolated from developing cells revealed only one slight difference. All of the fractions present in the vegetative basic nuclear proteins were also found in the basic nuclear proteins of developing cells. The one difference was that fraction 1d, a relatively major component of the basic nuclear proteins of vegetative cells, was apparently only a minor component of the basic nuclear proteins of developing cells. The implications of this finding are not clear. Whilst it is apparent that most of the basic proteins of D.discoideum undergo no change (either qualitatively or quantitatively, as detected by polyacrylamide gel electrophoresis) during the passage from vegetative growth to development, fraction 1d is present to a greater extent in vegetative cells. Although the basic nuclear proteins of D.discoideum are electrophoretically dissimilar to the histones of calf thymus, this does not necessarily mean that the functions of the various fractions in the two organisms are different. The observed electrophoretic differences may simply reflect the different degree of complexity of the genomes. This functional similarity may be especially true of D.discoideum fraction 1 proteins whose multiplicity corresponds to the observed multiplicity of fraction f1 proteins in the histones of higher eukaryotes (Bustin & Cole, 1969). If this similarity is true, then we can speculate further.

Since cell division during vegetative growth of D.discoideum is asynchronous, nuclei isolated from such cells will contain

chromatin in different states, depending on whether the cell has just divided, is just about to divide or is in the interphase period. Thus analysis of the basic nuclear proteins will give an average distribution of histones present throughout all the stages of the cell cycle. However, development of D.discoideum myxamoebae proceeds in the absence of DNA synthesis and cell mass increase and it is evident that since development takes approximately 24 hours (or three times as long as the cell cycle for axenic cells and six times as long as the cell cycle for bacterially grown cells) the normal cyclical changes are interrupted and suspended. The important question is whether these cyclical changes are suspended at a definite point in the cell cycle or whether the cells merely decelerate (drastically) their growth rate. Since histone f1 is known to be involved in the control of cell division in Physarum polycephalum (Bradbury et al, 1974a, 1974b), it is interesting that the (presumed) corresponding histone fraction in D.discoideum is the only basic nuclear protein to exhibit changes on passage of the cells from the vegetative phase to the developmental phase. Further analysis of the way in which histone f1 is involved in the control of cell division has shown that its phosphorylation is the trigger for mitosis to begin (Bradbury et al, 1974a, 1974b). Thus, in the absence of mitosis, histone f1/D.discoideum fraction 1 may not be phosphorylated. The hypothesis would then be that fraction 1d of the basic nuclear proteins of D.discoideum constitutes the phosphorylated form of the fraction and is thus present to only a small (residual ?) level during development. However, no evidence could be obtained to support this hypothesis - incubation of the basic nuclear proteins with alkaline phosphatase had no effect on their subsequent electrophoretic mobility or concentration.

Before concluding this chapter, one point must be re-emphasized. Although not identical to calf thymus histones from a structural viewpoint (as determined by electrophoretic mobility), the similar low number of different fractions may imply a degree of functional similarity. The ^{small} ~~few~~ number of basic nuclear proteins also implies that they are unlikely candidates for the direct control of specific genetic expression. It is more likely that most of the histone fractions act as non-specific repressors of transcriptional activity, specific genes perhaps being derepressed by the removal of the histone fraction, possibly by interaction with non-histone proteins (present in 30 times the concentration that histones are) or nuclear RNA, but by virtue of their close association with DNA, it is highly improbable that they are not involved at all with genetic activity and its specific regulation.

SUMMARY

1. Nuclei isolated from D.discoideum contain DNA, RNA and protein in a ratio of 1 : 8.3 : 30.
2. Basic nuclear proteins, approximately equal in weight to the nuclear DNA, can be extracted from the nuclei with 1 M CaCl_2 .
3. On urea-polyacrylamide gel electrophoresis, these basic nuclear proteins are resolved into 10 bands which have been grouped into 5 fractions. A comparison with the histones of calf thymus reveals very little similarity with respect to electrophoretic mobilities.
4. 9S histone mRNA from sea urchins does not hybridize to D.discoideum DNA.
5. A comparison between the basic nuclear proteins of vegetative and developing cells reveals only a small quantitative difference in one of the fractions. This difference is discussed with respect to the possible role of histones in the nuclear control of development and their involvement in the cyclical changes that occur during replication of the DNA during the cell cycle.

CHAPTER 3.

THE RIBOSOMAL PROTEINS OF THE CELLULAR
SLIME MOULD DICTYOSTELIUM DISCOIDEUM

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THE RIBOSOMAL PROTEINS OF THE CELLULAR

SLIME MOULD DICTYOSTELIUM DISCOIDEUM

INTRODUCTION

In the introduction to Chapter 2 a convenient, if somewhat arbitrary distinction was made between transcriptional and translational control of the regulation of protein synthesis - a fundamental problem in the study of differential gene expression. One of the many factors that may be involved in the transcriptional control of protein synthesis, that of the involvement of histones, has already been discussed and while it is clear that basic nuclear proteins are probably involved in the expression of genetic activity, the precise mechanism of their regulatory action is still far from being completely understood.

We can now turn to study the concept of translational control, bearing in mind however, that it cannot be divorced from transcriptional control because, as stated in Chapter 2, all of the components involved in the complex process of protein synthesis are themselves the products of genes and may hence be subject to transcriptional (and other) regulatory processes.

An appropriate starting point in the discussion is a definition of the term "translational control". In its broadest sense, translational control includes any situation where the rate at which proteins are synthesized from cytoplasmic mRNA is different for (i) dissimilar messages in the same cell, (ii) identical messages in different cells and (iii) identical messages at different times during the life of the cell. It is important to make a

distinction between control by factors which are specific to messages and control by unspecific components. In the former case, the absence of such message-specific components would affect the kinds of proteins synthesized and would thus profoundly affect the differentiation of the cell whereas in the latter case, the absence of an unspecific component such as ATP would not alter the kinds of proteins synthesized but would have the same quantitative effect on all proteins synthesized and would thus not be, developmentally, so interesting. An understanding of the mechanism, as opposed to the control, of protein synthesis is perhaps a prerequisite of the latter and we can consider the mechanism of translation by looking first at the structure and formation of the site of protein synthesis, the ribosome.

Although ribosomes have been studied in detail for only the relatively short period of about 20 years, so much information has been accumulated that a comprehensive review of ribosome structure, formation and function is quite beyond the scope of this thesis. It is necessary therefore to limit our discussion in many ways. The first limitation is an evolutionary one. Georgiev (1969) states that, in evolution, the appearance of a well-defined nucleus is roughly coincidental with the appearance of the phenomenon of differentiation or development. As the main aim of this thesis is the understanding of some of the mechanisms involved in development, the discussion will therefore be limited to ribosomes of eukaryotic organisms. 'Limited' is perhaps the wrong word as it implies some sort of simplification whereas quite the contrary appears to be the case. In comparison to prokaryotic systems, the process of translation in eukaryotes is far more complex. From a functional viewpoint, the most obvious difference is that all the components

for protein synthesis in eukaryotes are formed in the nucleus and as such are spatially and temporally separated from the actual process of protein synthesis in the cytoplasm by the presence of the nuclear membrane. From a structural viewpoint also, eukaryotic ribosomes are more complex than their prokaryotic counterparts. As well as containing an extra species of RNA, the major RNA species are larger and highly methylated. The ribosomal proteins of eukaryotes also appear more numerous, larger and comprise a higher proportion of the ribosome than do the proteins of prokaryotic ribosomes. We can look at the structure of eukaryotic ribosomes in more detail.

Ribosomes of eukaryotic organisms are characterized by a sedimentation coefficient of approximately 80S. The two sub-units that comprise the intact ribosome are also characterized by sedimentation coefficients of approximately 60S and 40S. The ribosome consists only of RNA and protein (Petermann, 1964) in an approximate ratio of 1 : 1 (Martini et al, 1973). The large (60S) sub-unit contains two species of RNA having sedimentation coefficients of approximately 28S and 5S and approximately 50 proteins. In addition, a low molecular weight species of RNA has been found to associated with the 28S RNA. This has an approximate sedimentation coefficient of 5 - 7 S [Udem and Warner (1972), showed the presence of a 5.8S RNA species associated with the 26S RNA in the large sub-unit of yeast ribosomes] and is believed to be formed during the maturation of the large RNA species (see later). The small (40S) sub-unit contains an 18S RNA species and approximately 30 proteins.

The process of ribosome formation in eukaryotic cells has been reviewed by many authors (e.g. Maden, 1971) and only a brief outline is given here.

Ribosomes are formed in the nucleolus of eukaryotic cells (Sillevis-

involving cleavage of the ribosomal precursor RNA to its RNA species, degradation of the non-ribosomal polynucleotide material and concomitant assembly of the ribosomal proteins to produce ribosomal sub-units that are substantially complete. The sub-units then pass across the nuclear membrane to the cytoplasm where, possibly after final maturation processes, they function as ribosomes. The maturation process in the nucleolus appears to be similar in a wide variety of eukaryotes ranging from HeLa cells (Weinberg and Penman, 1970) where the ribosomal RNA species are derived from a single 45S ribosomal precursor RNA molecule to yeast cells (Udem and Warner, 1972) where the RNA species are derived from a single 35S precursor.

One interesting aspect of this process is that the ribosomal proteins are attached to the ribosomal precursor RNA before, during and after its maturation so that the process is more rigourously defined as a maturation of ribonucleoprotein rather than ribosomal precursor RNA (Kumar and Warner, 1972). In addition a significantly greater proportion of protein is associated in the ribonucleoprotein particles than is found in mature ribosomes, implying the association of ribosomal proteins with non-ribosomal polynucleotide material. Kumar and Warner(1972) have presented evidence that the proteins that are associated with non-ribosomal RNA are released during degradation of the latter and re-utilized.

It is evident from the foregoing that the structure and formation of ribosomes in eukaryotes are complex processes that are only just beginning to be understood. The same is true for the actual process of protein synthesis performed by these ribosomes. In bacteria, the process of translation has been extensively studied and the functioning of the ribosome described in considerable

detail (e.g. Wittmann et al, 1973) with specific functions being ascribed to the various initiation, elongation and termination factors etc. In eukaryotes however no such wealth of information exists, but it does appear likely that the process is similar to that in prokaryotes. The process of initiation, for example, has been shown to depend on the presence of methionine AUG near the beginning of the messenger RNA species (Ilan, 1969; Ilan and Ilan, 1970; Smith and Marcker, 1970) and also upon the presence of three initiation factors (Prichard et al, 1970), the same number as in prokaryotes. It can therefore be seen that ribosome structure, formation and function are all extremely complex processes and there is very little reason to suppose that the mechanisms which regulate these processes are any less labrynthine.

We can now turn to study the control of the translation process and it is here that a major problem arises - how does one investigate translation ? Many approaches to this problem have been tried and indeed much valuable information has been obtained, although it is perhaps true to say that most experiments have shown only that translational control may be involved and very few have proved the fact. Greininger and Zetsche (1972) determined that the rates of synthesis of UDPG-pyrophosphorylase and phosphoglucose-isomerase during differentiation of the unicellular alga Acetabularia are different and since the experimental conditions precluded the possibilities of differential transcription and differential enzyme stability, they concluded that the control was at the level of translation.

Gross et al (1964) showed that the maternal RNA in developing sea urchins is translated on maternal ribosomes whereas the translation of newly-synthesized messenger RNA coincides with a burst of new

ribosome synthesis.

The use of cell-free protein synthesizing systems in studies of this kind have been widespread. Heywood (1969) found that chick myosin mRNA was only poorly translated in a chick erythroblast cell-free system unless accompanied by initiation factors from muscle cell ribosomes, so indicating the presence of message - specific initiation factors. Ilan and Ilan (1971) showed the presence of stage-specific initiation factors associated with the ribosome during insect development.

However studies of this type, although producing much information on the mechanism of translation may fail to provide a true test of the normal, in vivo control of translation. Gurdon (1974) states,

"First, it is possible that the specificity of cell components may be altered when they are extracted and purified. Second, components extracted from a cell and mixed together in a cell-free system may not have been free to interact in the living cell, since the distribution of some cell components is certainly compartmentalized. Lastly, it is impossible to know at exactly what concentrations each component should be supplied in a cell-free system to simulate in vivo conditions. This is a problem which affects not only ribosomes, initiation factors etc, but also the inorganic ingredients of the medium. For example, a doubling of the Mg^{2+} concentration in a reticulocyte cell-free system can alter by a factor of two the ratio of $\alpha:\beta$ globin synthesized from added mRNA. Even if the overall ionic composition of a cell is known, it is possible that local differences exist within the cell and in this case it would be impossible to be sure of reproducing in vitro the normal ionic conditions."

While these problems have not been overcome in studies on most developing systems, Gurdon (1971) has described a technique that can be used in maturing amphibian oocytes with meaningful results. By injecting haemoglobin mRNA from different species into oocytes of Xenopus laevis it was shown that translation was not restricted by absolute cell type specificity. However such a technique is not directly applicable to the study of the model system of development used in this thesis - that of the development of the cellular slime mould D.discoideum and so it appears that other methods must be used, even though such methods may only indicate the involvement of translational control and not actually prove the case. In this context the structure of the site of protein synthesis has been extensively investigated, the hypothesis being that structural changes in the ribosome may well give rise to the functional distinctions characteristic of translational control. It must however be emphasized that this is a 'second best' approach as widely applicable techniques for the direct study of translational control are, as yet, unavailable.

There is good evidence to suggest that a functional distinction between ribosomes in different developmental stages in D.discoideum does exist. In 1970, Coccuci and Sussman showed that during vegetative growth there was no appreciable turnover of ribosomal RNA. This persistence however was not maintained when the myxamoebae entered stationary phase and were allowed to proceed through their developmental programme. Ribosomal RNA turnover during this period was such that at least 75% of the ribosomes fabricated during exponential growth were degraded and replaced by ribosomes synthesized during the developmental phase. Moreover it was the newly-synthesized ribosomes that appeared exclusively

in the polysomal fractions of whole cell lysates and did not appear in the monosomal contingent for at least 6 hours. Since development of the myxamoebae proceeds in the complete absence of exogenous nutrient, it is clear that cells would have a selective advantage if they were able to utilize pre-formed ribosomes during the developmental phase and avoid, as much as possible, the considerably energetic process of manufacturing new ribosomes.

It has also recently been shown (Ishida et al, 1973) that cells of a mutant of D.discoideum, whose nucleoli seem to disappear sometime after aggregation, are unable to complete their developmental programme, but which, if supplied with exogenous nutrient, revert to their original vegetative state.

These two reports clearly demonstrate that an ability to synthesize new ribosomes during development is an absolute requirement for successful development and suggest very strongly that there is some form of functional distinction between ribosomes formed during the vegetative phase and those formed during the developmental phase. The question one can ask is whether these (presumed) functionally distinct ribosomes are structurally different. If they are structurally identical, then this still does not preclude the involvement of translational control during development, but merely indicates that one part of the translation machinery remains the same. On the other hand, if they are structurally different, then this does not prove the involvement of translational control but it may suggest ways in which it may be functioning.

Because one can separate the two types of molecules - RNA and proteins - from ribosomes fairly readily, it is convenient to examine them independently. An investigation into the question

of whether the ribosomal RNA synthesized during the developmental phase is the same as that synthesized in the vegetative phase is described in Chapter 4 of this thesis. For the present chapter, we are concerned with the possibility that the ribosomal proteins synthesized during development are different from those synthesized during vegetative growth.

Studies of this kind are not without their precedents. However, most of the earlier work suffered from the use of techniques that were - in retrospect - unable to resolve the many proteins associated with the ribosome. Thus Low and Wool (1966) found no differences in the ribosomal protein content of ribosomes isolated from 5 different mammalian tissues but they could resolve only 24 bands on polyacrylamide gel electrophoresis of the ribosomal proteins - approximately one third of the number that are now known to be present. Similarly Rothschild et al (1967) detected no differences in ribosomal proteins isolated from various stages of development in Neurospora crassa. With the introduction of more sophisticated techniques, especially the two-dimensional polyacrylamide gel electrophoresis apparatus described by Kaltschmidt and Wittmann (1970) and the use of sub-units rather than intact ribosomes, more meaningful results have been obtained. On the one hand, there appear to be differences in the ribosomal proteins isolated from various stages of development in Drosophila melanogaster (Lambertsson, 1972) while on the other, Delaunay and Schapira (1972) have shown no major differences in the ribosomal proteins isolated from normal and hepatoma rat liver. It is the purpose of this chapter to characterize the ribosomal proteins of D.discoideum by two-dimensional polyacrylamide gel electrophoresis and to compare the ribosomal protein complement of cells during the vegetative and developmental phases of the life cycle.

MATERIALS

Acrylamide was obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks, U.K. All other chemicals were of the highest purity commercially available and were the products of BDH Chemicals Ltd, Poole, Dorset, U.K., Fisons Scientific Apparatus Ltd, Loughborough, Leicester, U.K. or Sigma (London) Ltd, London, U.K.

METHODS

(i) Growth and Development of Myxamoebae

Axenically grown vegetative myxamoebae of D.discoideum were obtained by growth on NS medium as described in Chapter 1. Cells representative of the developmental phase were obtained by harvesting the axenically grown vegetative myxamoebae and allowing them to develop on Millipore filter supports, as previously described, until the Mexican hat stage had been reached - at approximately 18 hours after deposition of the cells on the Millipores - at which point they were harvested as before.

Bacterially grown myxamoebae, representative of the vegetative phase were obtained by growth in association with Aerobacter aerogenes on solid agar medium as described in Chapter 1. By allowing the myxamoebae to completely digest all the bacteria on the agar medium and allowing them to form fruiting bodies, cells representative of the developmental phase (spores) of bacterially grown myxamoebae were obtained by then harvesting the plates.

(ii) Isolation of Ribosomes

Ribosomes were isolated from vegetative

and developing cells by the same procedure. All operations were performed at $0 - 2^{\circ}\text{C}$ except where otherwise indicated.

Cells were harvested either from growth medium or from Millipore filter supports and washed twice in distilled water as described in Chapter 1. The cells were resuspended in TKMM buffer (see page iv) at a cell density of approximately 5×10^8 cells / ml and disrupted by two passages through a French pressure cell at $14,000 \text{ lb/in}^2$. The disrupted cell suspension was then centrifuged at $30,000 \text{ g}$ for 30 min. In the case of spore cells, it was necessary to first sonicate the cell suspension using a 100W MSE Ultrasonic Disintegrator (1 cm tip probe, peak to peak amplitude $12 \mu\text{m}$) for 12×30 second periods, with continuous cooling in an ice bath to prevent overheating, before it was passed through the French pressure cell. The supernatant from the 30 min. centrifugation was carefully removed and centrifuged at $140,000 \text{ g}$ for 2 hours. The resulting crude ribosomal pellets were resuspended, with the aid of a glass rod, in approximately 10 ml of TKMM buffer and clarified by centrifugation at $30,000 \text{ g}$ for 15 min. to remove aggregates and polysaccharide contamination. The clear supernatant constituted the clarified ribosome suspension.

Ribosomes that had been washed in high salt were prepared by resuspension of the crude ribosomal pellet in TKMM buffer, containing 0.5 M ammonium chloride, followed by centrifugation at $140,000 \text{ g}$ for 2 hours. Such ammonium chloride - washed ribosomes were similarly resuspended and clarified as described above.

There is the possibility that ribosomes, during centrifugation, will adsorb in a non-specific fashion, cytoplasmic proteins and so cause ambiguities in the interpretation of the results of protein analyses. In order to test this hypothesis, the ribosomes prepared from cells of the vegetative stage were

resuspended in the 140,000 g supernatant remaining after the ribosomes had been prepared from cells of the developmental stage, re-pelleted, resuspended and clarified as described above.

(iii) Purity of Ribosomes

The RNA and protein content of the clarified ribosome suspension was determined using the methods described in Chapter 1, using yeast RNA and bovine serum albumin as standards. The level of polysaccharide contamination was also determined using the anthrone assay of Hassid & Abraham (1957).

A sample of the clarified ribosome suspension was also analysed by sucrose density centrifugation. 0.05 ml of a 1/100 dilution of the clarified ribosome suspension was carefully layered on to the top of a linear 5 - 20% (w/v) sucrose gradient, prepared in TKMM buffer, contained in a 15 ml polypropylene centrifuge tube. Centrifugation was performed in the 6 x 15 SW rotor of the MSE Superspeed 65 centrifuge, at 25,000 rpm for 2 hours at 15°C. The gradient was analysed using an Isco Model 180 density gradient fractionator coupled to an Isco Model UA-2 ultraviolet analyser and Vitatron UR 400 linear recorder. The peaks obtained were compared to those obtained from a ~~post-mitochondrial~~ supernatant of a crude extract of Escherichia coli B/r which had been centrifuged for the same period on an identical gradient.

(iv) Extraction of Ribosomal Proteins

Two methods were used to extract the proteins from the ribosome suspensions and their efficiencies are compared. In both methods, the starting material was the ribosome suspension diluted, to give a concentration of 15 mg of

ribosomes / ml¹.

Method 1 ; This procedure is described by Ochiai & Iwabuchi (1971).

To one volume of the ribosome suspension are added 2 volumes of 3 M CaCl₂ containing 4 M urea. The mixture is then stirred at 4°C for 8 hours and the precipitated RNA removed by centrifugation (20,000 g, 15 min.). The supernatant comprising the ribosomal proteins was dialyzed exhaustively against distilled water at 4°C and lyophilized.

Method 2 : This procedure is described by Hardy et al (1969). To one volume of the ribosome suspension stirred vigorously at 0°C were added, in rapid succession, 0.1 volumes of 1 M MgCl₂ and 2 volumes of glacial acetic acid. The mixture was stirred for 45 min. and the precipitated RNA removed by centrifugation (20,000 g, 15 min), washed in a small volume of 67% acetic acid and re-centrifuged. The combined supernatants were dialyzed exhaustively against distilled water at 4°C and lyophilized.

The efficiencies of the two methods were compared by determining the RNA and protein content of both the RNA precipitate and the protein supernatant.

(v) Two-Dimensional Gel Electrophoresis of Ribosomal Proteins

The technique of two-dimensional electrophoresis for the separation and characterization of ribosomal proteins was comprehensively described by Kaltschmidt & Wittmann (1970). Despite numerous reports of results using this general technique, there is no

¹A ribosome suspension of 66 µg / ml has an extinction of 1.0 at 260 nm in a 1 cm path length silica cell.

suitable apparatus currently being marketed and thus reliance must be placed in one's own ingenuity of design. Because of this, the technique and the apparatus used will be described in some detail.

The principle of two-dimensional gel electrophoresis is that the proteins are first separated on the basis of charge difference by electrophoresis in the usual Pyrex tubes containing a low (4%) acrylamide concentration and a high pH (8.6). The gel is then placed on the top of a vertical gel slab of 18% acrylamide and low pH (4.6) which maximises separations based on molecular size. The technique now described is a modification of that described by Kaltschmidt & Wittmann (1970).

First Dimension

The following gel solution was employed as the separation gel in the first dimension -

boric acid	3.2 g	} pH 8.6 de-aerated and stored at 0°C
EDTA Na ₂ .2H ₂ O	0.8 g	
Tris	4.87 g	
urea	36.0 g	
acrylamide	4.0 g	
methylenebisacrylamide	0.133 g	
TEMED	0.3 ml	
distilled water to	99 ml	

Pyrex tubes, 10 cm x 0.6 cm (I.D.) were sealed at one end with Parafilm and a mark made on the tube 9 cm from this end. 0.01 volumes of freshly prepared 7% (w/v) ammonium persulphate solution were added to the separation gel and the mixture transferred to the 9 cm mark on the gel tubes. The gel was carefully overlaid with water and allowed to polymerize for 3 hours. The gels were then placed in a Shandon vertical gel system and pre-electrophoresed at 3 mA / gel

(constant current) at 4°C until the voltage stabilized (after approximately 6 hours) using a tray buffer containing 14.55 g Tris ; 2.4 g EDTA Na₂·2H₂O ; 9.6 g boric acid / litre.

Lyophilized samples of ribosomal proteins (100 - 500 µg / 0.1 ml) were allowed to dissolve in 8 M urea containing 0.5 M 2-mercapto-ethanol at room temperature.

After pre-electrophoresis, the tray buffer was renewed and 0.1 ml of the ribosomal protein solution layered on the top of the gel. Electrophoresis was carried out in a cathodic direction for 12.5 hours at 3 mA / gel (constant current) at 4°C, the voltage remaining constant at 90 volts throughout this period.

Second Dimension

The first dimension gels were removed from their tubes by careful rimming with a blunted 26 gauge hypodermic needle connected to a water-filled syringe, and were adapted to the buffer of the second dimension by dialysis against 500 ml of equilibration buffer containing 480 g urea, 0.74 ml glacial acetic acid and 2.4 ml 5 N KOH / litre, for 1 hour at 4°C.

The composition of the separation gel employed in the second dimension was -

urea	180 g	} pH 4.6 de-aerated and stored at 0°C.
acrylamide	90 g	
methylenebisacrylamide	2.5 g	
glacial acetic acid	26.15 ml	
5 N KOH	4.8 ml	
TEMED	2.9 ml	
distilled water to	483.5 ml	

An apparatus was constructed for the second dimension permitting two electrophoreses to be performed simultaneously and hence under

identical conditions. The apparatus (see PLATE 3 and FIGURE 20) was constructed of $\frac{1}{4}$ " thick Perspex sheets and consists of two identical gel plate assemblies, each consisting of two 12 cm x 12 cm Perspex sheets separated by a gap of 5 mm by means of two Perspex spacers firmly attached to one sheet. The two sheets are held together by means of two stainless steel screws positioned 8 cm from the top. The top of each gel plate assembly was grooved to accommodate the first dimension gel.

The gel plate assemblies are positioned in slots in the base of the anode reservoir such that they project about 6 cm below the base. They are held firmly in place by tightening the screws securing the pressure plate to the bottom of the upper electrode compartment and forcing the rubber 'O'-ring, running round the slots, to press tightly against the sides of the gel plate assemblies so as to effect a water-tight seal. The bottoms of the gel plate assemblies were sealed with strips of Plasticine and the apparatus was ready for use.

4 ml of freshly prepared 10% (w/v) ammonium persulphate were added to 116 ml of the second dimension separation gel and the mixture transferred to the gel plate assemblies, to a level just above the bottom of the V-shaped groove. Each gel slab required approximately 55 ml of gel solution and the excess was kept on ice. The first dimension gels were carefully drained of excess equilibration buffer and transferred to the V-shaped grooves in the gel plate assemblies, taking care that no air bubbles were trapped underneath. The level of separation gel was then adjusted so that the first dimension gels were two-thirds covered. At room temperature, the polymerization process takes about 1 hour. Towards the end of this period, the heat generated during the polymerization causes the

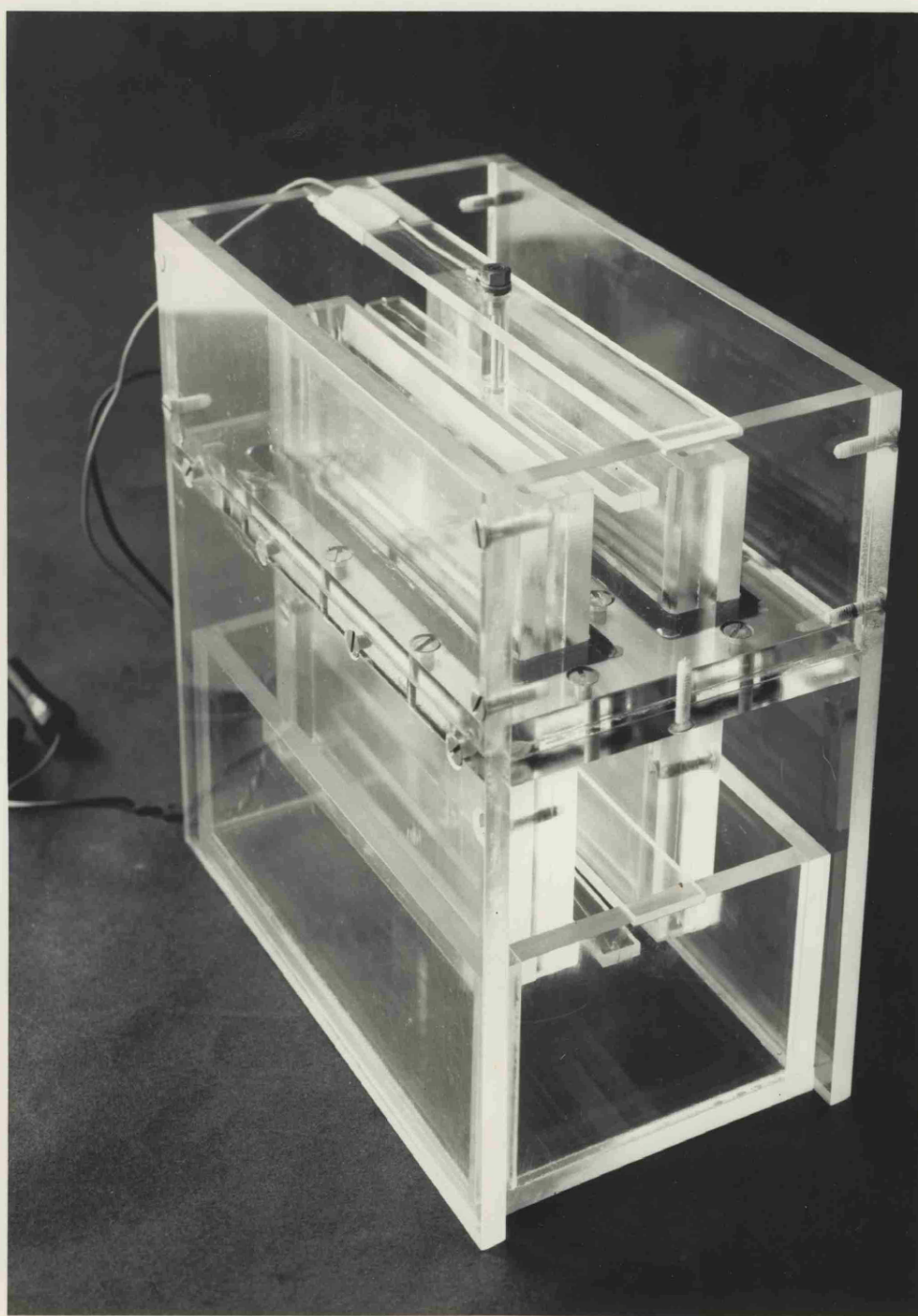
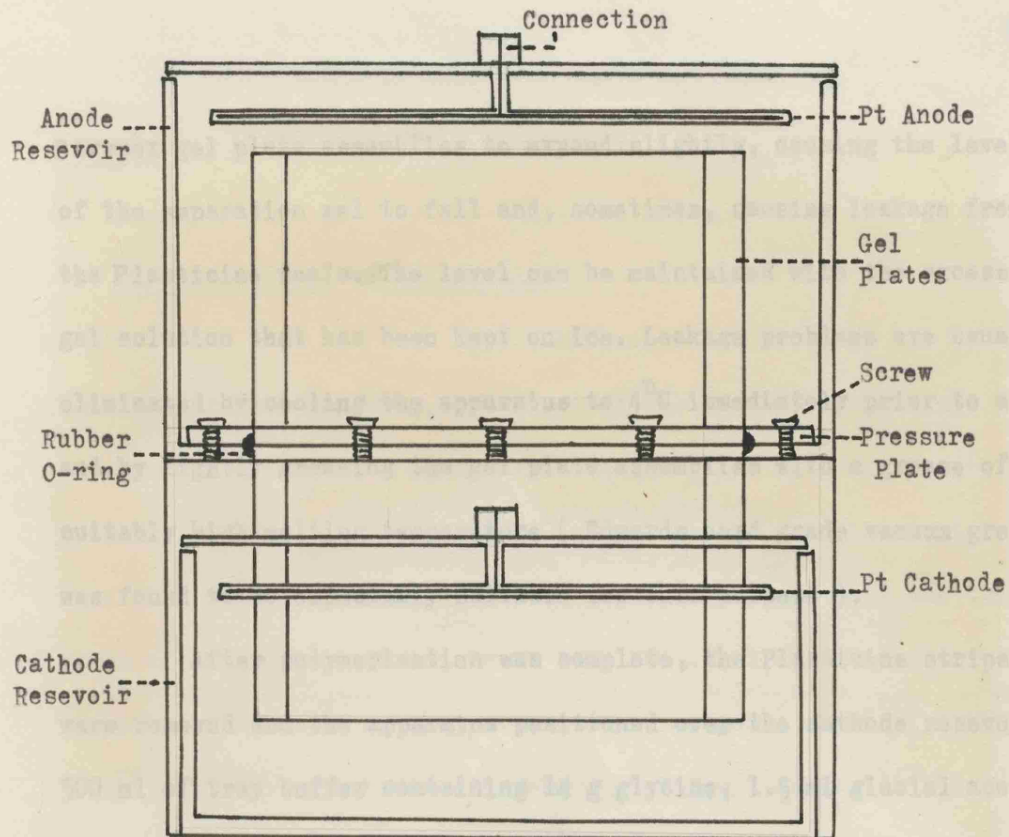
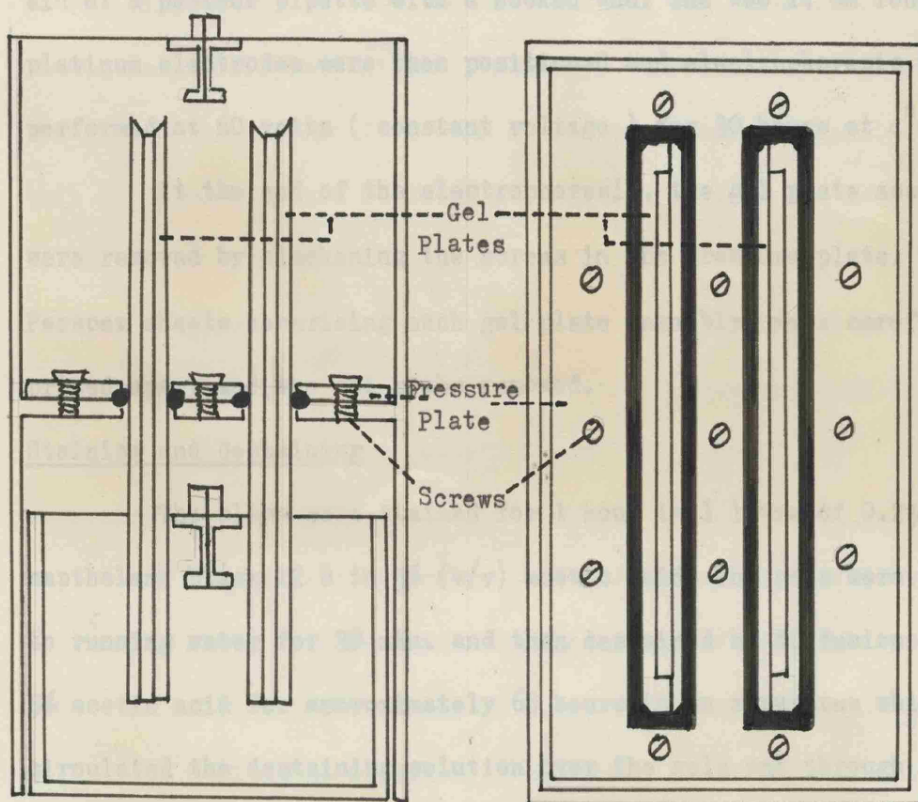


PLATE 3. Apparatus used for Electrophoresis
in the Second Dimension

Front ElevationSide ElevationTop Elevation (Anode removed)FIGURE 20. Electrophoresis Apparatus used in theSecond Dimension

Perspex gel plate assemblies to expand slightly, causing the level of the separation gel to fall and, sometimes, causing leakage from the Plasticine seals. The level can be maintained with the excess gel solution that has been kept on ice. Leakage problems are usually eliminated by cooling the apparatus to 4°C immediately prior to use and by lightly greasing the gel plate assemblies with a grease of suitably high melting temperature (Edwards hard grade vacuum grease was found to be especially suitable for this purpose).

After polymerization was complete, the Plasticine strips were removed and the apparatus positioned over the cathode reservoir. 500 ml of tray buffer containing 14 g glycine, 1.5 ml glacial acetic acid and 360 g urea / litre, were placed in each electrode reservoir and the air bubbles trapped beneath the gel slabs removed with the aid of a pasteur pipette with a hooked end. The two 14 cm long platinum electrodes were then positioned and electrophoresis was performed at 60 volts (constant voltage) for 30 hours at 4°C .

At the end of the electrophoresis, the gel plate assemblies were removed by slackening the screws in the pressure plate. The two Perspex sheets comprising each gel plate assembly, were carefully prised apart and the gel slabs removed.

Staining and Destaining

The slabs were stained for 1 hour in 1 litre of 0.25% (w/v) naphthalene black 12 B in 5% (v/v) acetic acid. The gels were exposed to running water for 30 min. and then destained by diffusion against 5% acetic acid for approximately 60 hours in an apparatus which circulated the destaining solution over the gels and through a compartment containing absorbent wool pads (Gradipore Ltd) which absorbed the excess dye. The destained gels were photographed and stored in 5% acetic acid.

RESULTS

(i) Purity of Ribosome Preparation

Ribosomes collected by

centrifugation (140,000g, 2 hours) of the post-mitochondrial supernatant contained a considerable quantity of anthrone-positive material (TABLE 3). Attempts to extract the ribosomal proteins from such crude preparations were unsuccessful - not only was the yield of extractable protein very low, but it also contained a very significant amount (approximately 40% by weight) of RNA (TABLE 4). This difficulty was resolved however, by 'clarification' of the crude ribosome suspension by centrifugation at 30,000g for 15 min. Although this procedure resulted in the loss of approximately 40% of the ribosomes, the resulting supernatant was essentially free of contaminating polysaccharide (TABLE 3). Moreover, extraction of the ribosomal proteins from such clarified ribosomes resulted in both an improved yield of total protein extractable and in negligible contamination with RNA (TABLE 4).

The relative amounts of RNA and protein present in the clarified ribosomes from both vegetative and developing cells (shown in TABLE 3) give a composition of the ribosome of approximately 56% RNA and 44% protein.

When analysed by sedimentation velocity centrifugation on a linear 5 - 20% sucrose gradient, the clarified ribosome suspension exhibited 1 major peak, which, by comparison with the Escherichia coli crude cell extract similarly analysed, had a sedimentation coefficient of approximately 80S (FIGURE 21). There was also a small, fairly broad peak sedimenting slightly slower than the Escherichia coli 70S ribosome, which probably represents a slight

<u>RIBOSOME SUSPENSION</u> <u>IN TKMM BUFFER</u>	<u>RNA(mg/ml)</u>	<u>PROTEIN</u> <u>(mg/ml)</u>	<u>TOTAL HEXOSE</u> <u>(mg/ml)</u>
Crude (vegetative)	26.4 ± 1.1(8)	22.5 ± 0.9(8)	13.4 ± 1.7(8)
Clarified (vegetative)	14.2 ± 0.4(8)	11.1 ± 0.3(8)	0.05 ± 0.01(8)
Crude (developing)	24.5 ± 1.3(8)	20.9 ± 1.0(8)	12.4 ± 1.6 (8)
Clarified (developing)	13.4 ± 0.3(8)	10.5 ± 0.2(8)	0.04 ± 0.01(8)

TABLE 3. The Relative RNA, Protein and Total Hexose Content of Crude and Clarified Ribosomes Isolated from Vegetative and Developing Cells of D.discoideum

Results are given as Means ± the standard error of the mean
 Figures in parentheses refer to the number of determinations

<u>RIBOSOME SUSPENSION</u>	<u>RNA (mg)</u>	<u>PROTEIN (mg)</u>	<u>PROTEIN YIELD</u>	<u>RNA CONTAMINATION</u>
Crude Ribosome Suspension				
3M CaCl ₂ /4M urea precipitate	42.4 ± 2.1(2)	35.5 ± 1.9(2)		
3M CaCl ₂ /4M urea supernatant	26.4 ± 1.6(2)	16.3 ± 1.0(2)		
67% acetic acid/0.1M MgCl ₂ precipitate	16.7 ± 0.9(2)	18.9 ± 1.1(2)	50%	47%
67% acetic acid/0.1M MgCl ₂ supernatant	32.2 ± 1.9(2)	12.4 ± 0.8(2)		
	10.8 ± 0.7(2)	24.9 ± 1.2(2)	68%	29%
Clarified Ribosome Suspension				
3M CaCl ₂ /4M urea precipitate	41.9 ± 1.7(4)	33.0 ± 1.3(4)		
3M CaCl ₂ /4M urea supernatant	40.0 ± 1.3(4)	7.8 ± 0.6(4)		
67% acetic acid/0.1M MgCl ₂ precipitate	1.54 ± 0.14(4)	25.7 ± 1.2(4)	78%	6%
67% acetic acid/0.1M MgCl ₂ supernatant	42.0 ± 0.9(4)	1.5 ± 0.2(4)		
	0.09 ± 0.02(4)	31.7 ± 1.1(4)	96%	0.3%

TABLE 4 RNA and Protein Content of Fractions obtained during the Extraction of the Ribosomal Proteins from D.discoideum

Results are given as Means ± the standard error of the mean. Figures in parentheses refer to the number of dets.

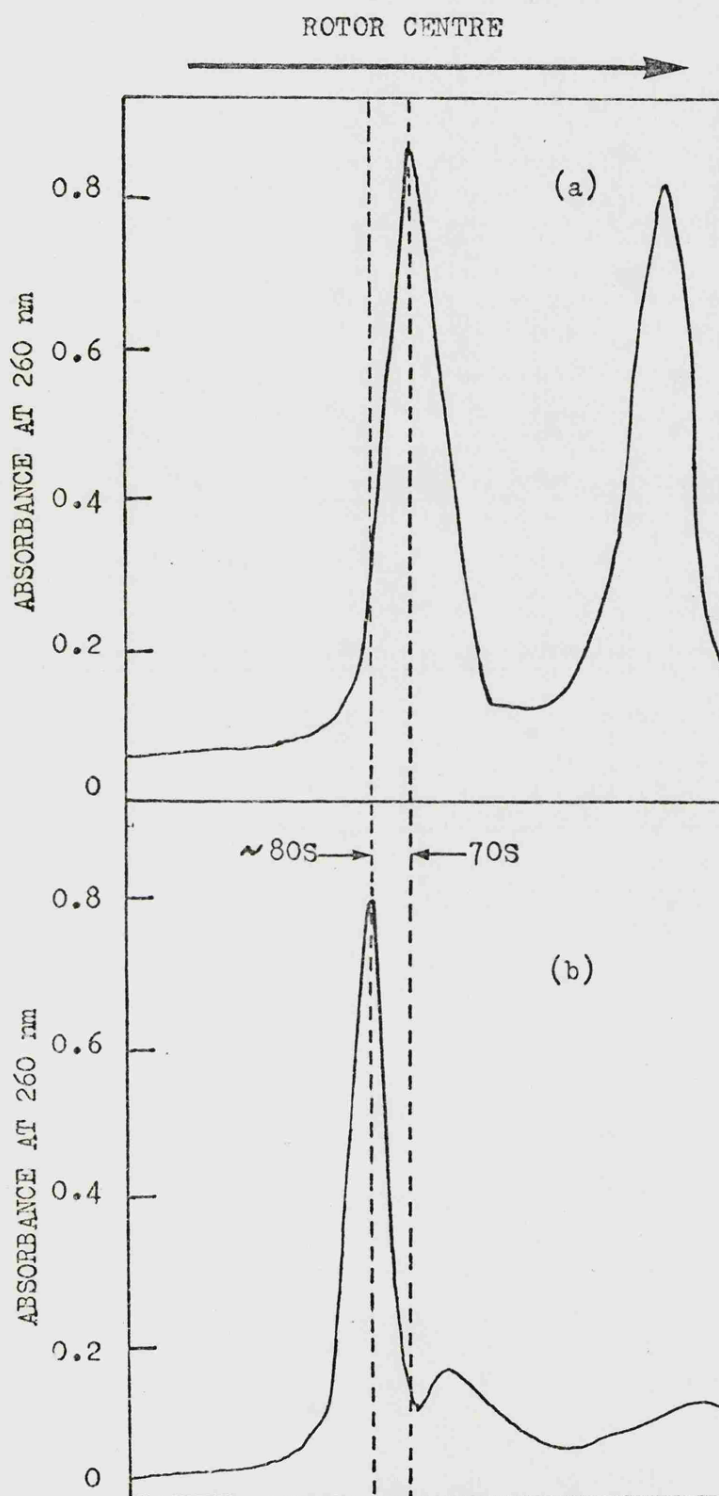


FIGURE 21. Sedimentation Velocity Centrifugation on Linear
5 - 20% Sucrose Gradients

(a) Escherichia coli crude cell extract

(b) Clarified ribosomes from D. discoideum

degree of dissociation of the monomeric 80S ribosome into its component sub-units which are reported to have sedimentation coefficients of approximately 60S and 40S (Iwabuchi et al, 1970)

(ii) Extraction of the Ribosomal Proteins

TABLE 4 shows the efficiency of the two methods used for the extraction of the ribosomal proteins from crude and clarified ribosome suspensions. It has already been made clear that the extraction of proteins from crude ribosome suspensions was unsatisfactory with regard to both the yield and purity for both methods used. With the clarified ribosomes, the method of Hardy et al (1969) is evidently the most efficient in the extraction of ribosomal proteins, both in high yield and in apparant freedom from RNA contamination. This method was therefore used throughout.

(iii) The Two-dimensional Polyacrylamide Gel Electrophoresis

Before considering the results of the two-dimensional electrophoresis of the ribosomal proteins, it is important to verify that the modifications made to the 'standard' procedure of Kaltschmidt & Wittmann (1970) are justified.

The only major modification, apart from size, in the technique is that in the first dimension, the protein sample is placed at the top of the acrylamide gel (instead of being polymerized in a sample gel in the middle of the first dimension gel) and is run in only one (cathodic) direction (instead of being run in both anodic and cathodic directions). Since preliminary experiments that utilized, ^{precisely} ~~verbally~~, the procedure of Kaltschmidt & Wittmann (1970) showed that no proteins appeared to migrate in an anodic direction, it is felt that this modification is justified.

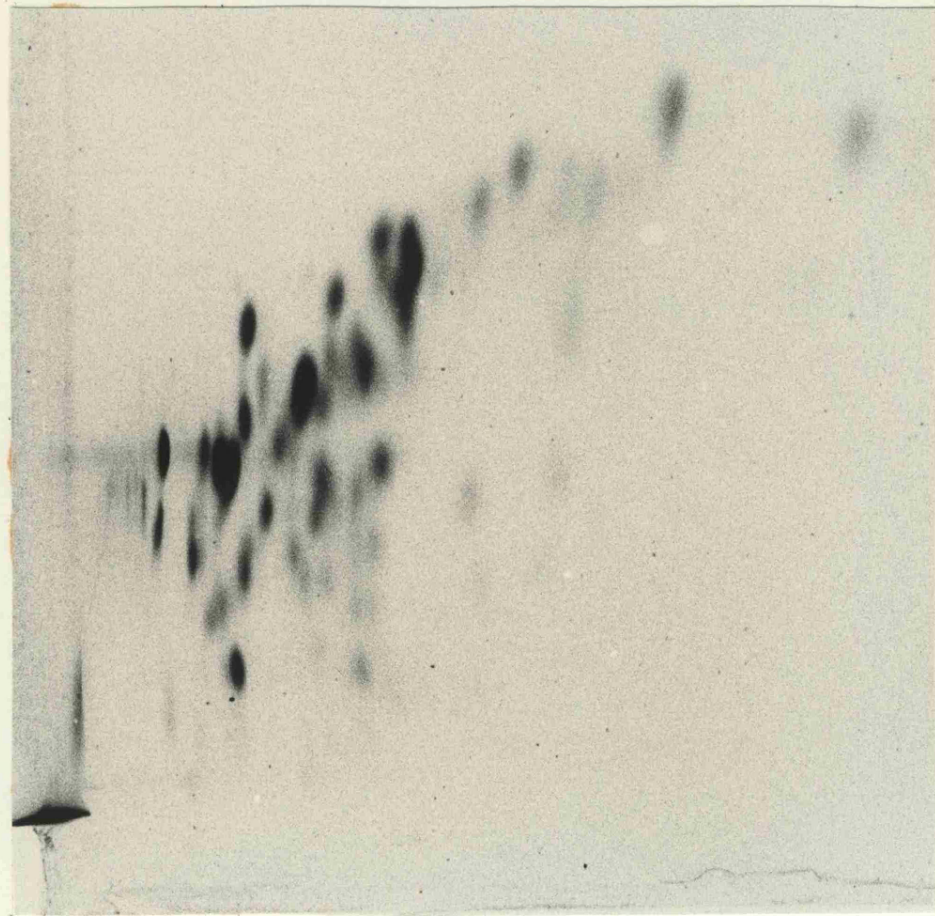
This modification also meant that the first dimension gels could be pre-electrophoresed before the protein sample was applied. This resulted in most of the protein entering the gel - a situation in contrast to that in the 'standard' system where protein precipitation in the sample gel is often considerable (Dr. S. Hardy, personal communication). The advantages of pre-electrophoresis are discussed in Chapter 2 and will not be repeated here.

The only other modification is the inclusion of urea (6 M) in the second dimension electrode buffer. When urea is omitted from the electrode buffer, the urea present in the first dimension gel diffuses out during the 30-hour electrophoresis, causing it to swell and buckle, which results in considerable distortion of the final protein spots on the gel. When the urea concentration in the electrode buffer is the same as that in the first dimension gel, this problem does not arise. Diffusion of urea was not significant in the first dimension electrophoresis despite the fact that the electrode buffer did not contain urea. This is presumably because of the very small surface area of the gels that are in contact with the electrode buffer, and also the shorter period (12.5 hours) of electrophoresis.

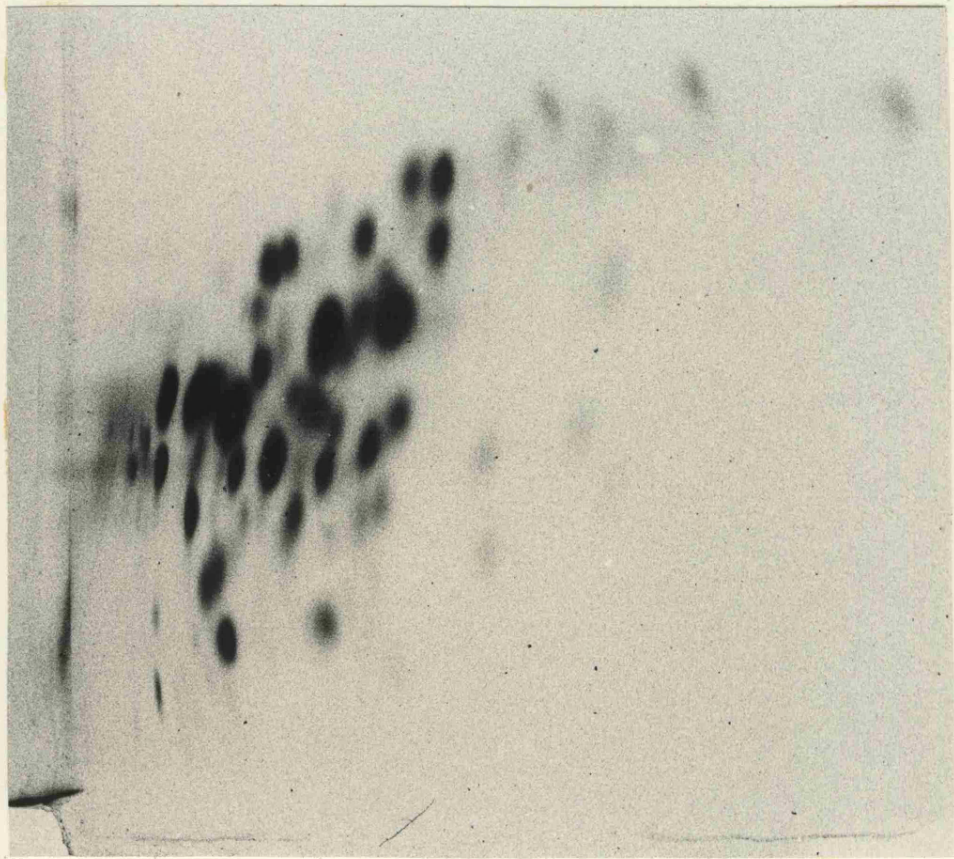
We can now consider the two-dimensional electrophoretograms of the ribosomal proteins. The protein spots in the following electrophoretograms are numbered separately along horizontal lines beginning at the upper left. Some of the spots on the gels are clearly visible to the eye, but are barely discernible or cannot be seen at all in the photographs. Such spots are included in the schematic drawings of the gels which therefore do not represent with fidelity the intensity of staining of individual spots. In addition, the drawings are composites from at least two gels and as such they may differ in detail from individual gels photographed.

The two-dimensional electrophoresis of the ribosomal proteins isolated from vegetative and developing cells of D.discoideum are shown in PLATE 4. Approximately 69 spots were resolved from both vegetative and developing ribosomal proteins and the two patterns are schematically compared in FIGURE 22, from which it can be seen that there is great similarity between them. However, there are several differences. Proteins 56, 60, 61 and 67 appear to be present only in ribosomes isolated from vegetative cells, whereas proteins 3, 24, 31 and 38 appear to be present only in ribosomes isolated from developing cells. These apparently stage-specific proteins are mostly only lightly stained and are presumably fairly minor components of the ribosome. Whilst this is true for proteins 56, 61, 67, 3 and 24, proteins 31, 38 and 60 do appear to be stained more heavily. Such stoichiometric considerations are, however, difficult to determine precisely. Although an equal amount of ribosomal protein (as determined by Folin-positive material) was applied to each gel, it is evident from PLATE 4 that there is a considerable difference in the amount of protein that actually entered the gel, even with pre-electrophoresis. This partial insolubility of the lyophilized ribosomal proteins is thought to be a function of the high pH (8.6) at which they are dissolved and/or the presence of contaminating RNA and polysaccharide material. Some evidence for the latter hypothesis comes attempts to isolate ribosomal proteins from cells that had been grown in NS + Glucose medium (see Chapter 1). Such preparations contained considerable quantities (approximately 5% by weight) of anthrone-positive material and were largely insoluble, producing very few spots on two-dimensional electrophoresis.

The two-dimensional electrophoresis of the ribosomal



(a)



(b)

PLATE 4. The Two-dimensional Electrophoresis of the Ribosomal Proteins of Axenically grown *D. discoideum*
(a) The ribosomal proteins of vegetative cells. (b) The ribosomal proteins of developing cells.

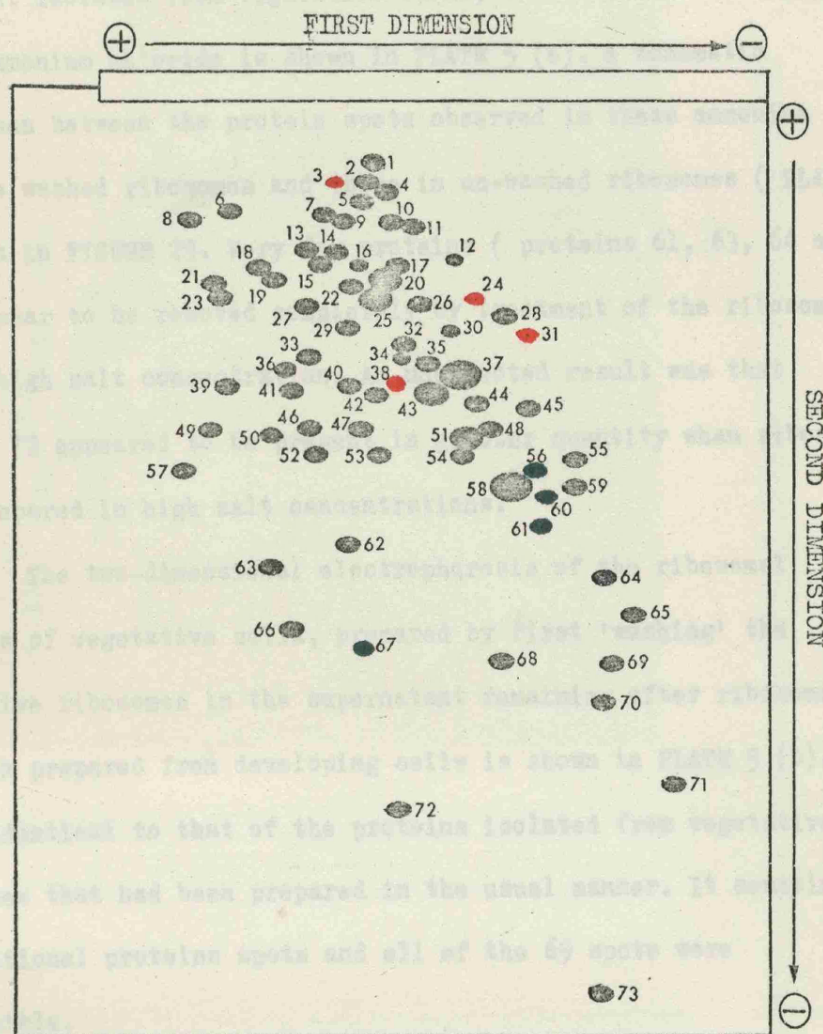


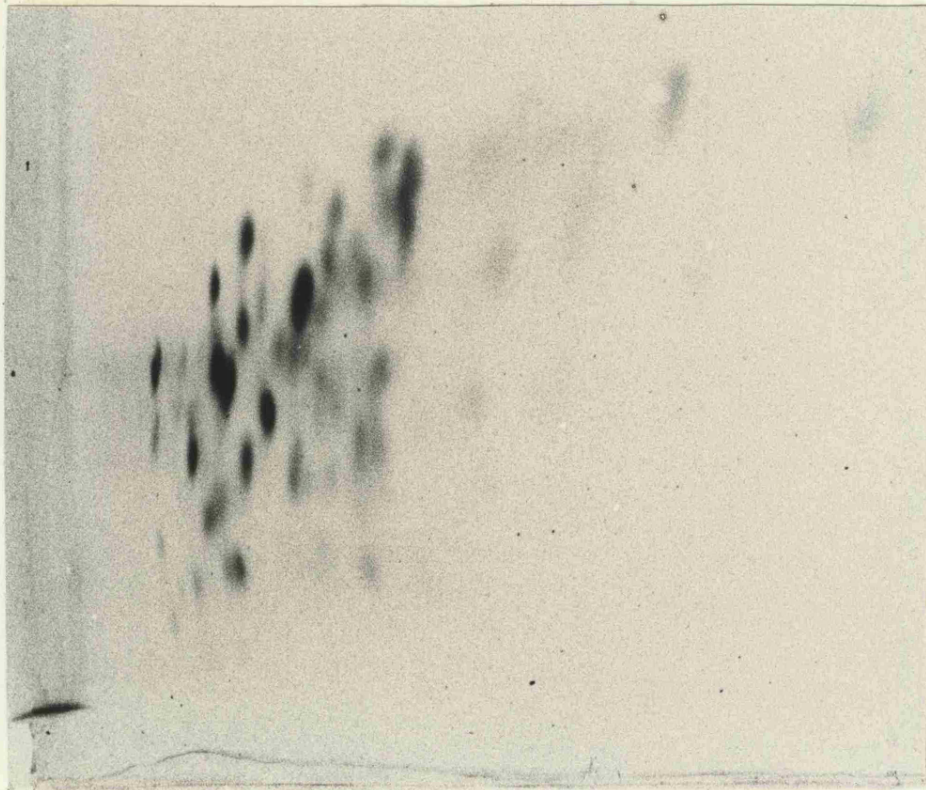
FIGURE 22. Schematic Comparison of the Ribosomal Proteins of Vegetative and Developing Cells of *D. discoideum*

- : Proteins common to both developing and vegetative cells
- : Proteins present only in vegetative cells
- : Proteins present only in developing cells

proteins, isolated from vegetative cells, that had been washed in 0.5 M ammonium chloride is shown in PLATE 5 (a). A schematic comparison between the protein spots observed in these ammonium chloride washed ribosomes and those in un-washed ribosomes (PLATE 4a) is shown in FIGURE 23. Very few proteins (proteins 61, 63, 66 and 67) appear to be removed completely by treatment of the ribosomes with a high salt concentration. An unexpected result was that protein 12 appeared to be present in greater quantity when ribosomes were prepared in high salt concentrations.

The two-dimensional electrophoresis of the ribosomal proteins of vegetative cells, prepared by first 'washing' the vegetative ribosomes in the supernatant remaining after ribosomes had been prepared from developing cells is shown in PLATE 5 (b). It is identical to that of the proteins isolated from vegetative ribosomes that had been prepared in the usual manner. It contains no additional proteins spots and all of the 69 spots were discernible.

PLATE 6 shows the two-dimensional electrophoresis of the ribosomal proteins isolated from vegetative cells and mature spore cells of D.discoideum that had been grown in association with Aerobacter aerogenes on solid agar medium. The proteins from the two stages are schematically compared with each other and with the electrophoretograms of axenically grown cells in FIGURE 24. Although the electrophoretic patterns obtained from bacterially grown cells are not exactly superimposable with those from axenically grown cells, the general similarity is remarkable and the spots have been appropriately numbered to facilitate comparisons. Proteins 1 and 2 - which are observed in vegetative and developing cells of axenically grown myxamoebae - appear to be absent from



(a)



(b)

PLATE 5. The Two-dimensional Electrophoresis of the Ribosomal Proteins of Asexually grown *D. discoideum*

(a) Proteins extracted from ammonium chloride washed ribosomes.

(b) Proteins extracted from the ribosomes of vegetative cells that had been washed in the supernatant remaining after ribosomes had been prepared from developing cells.

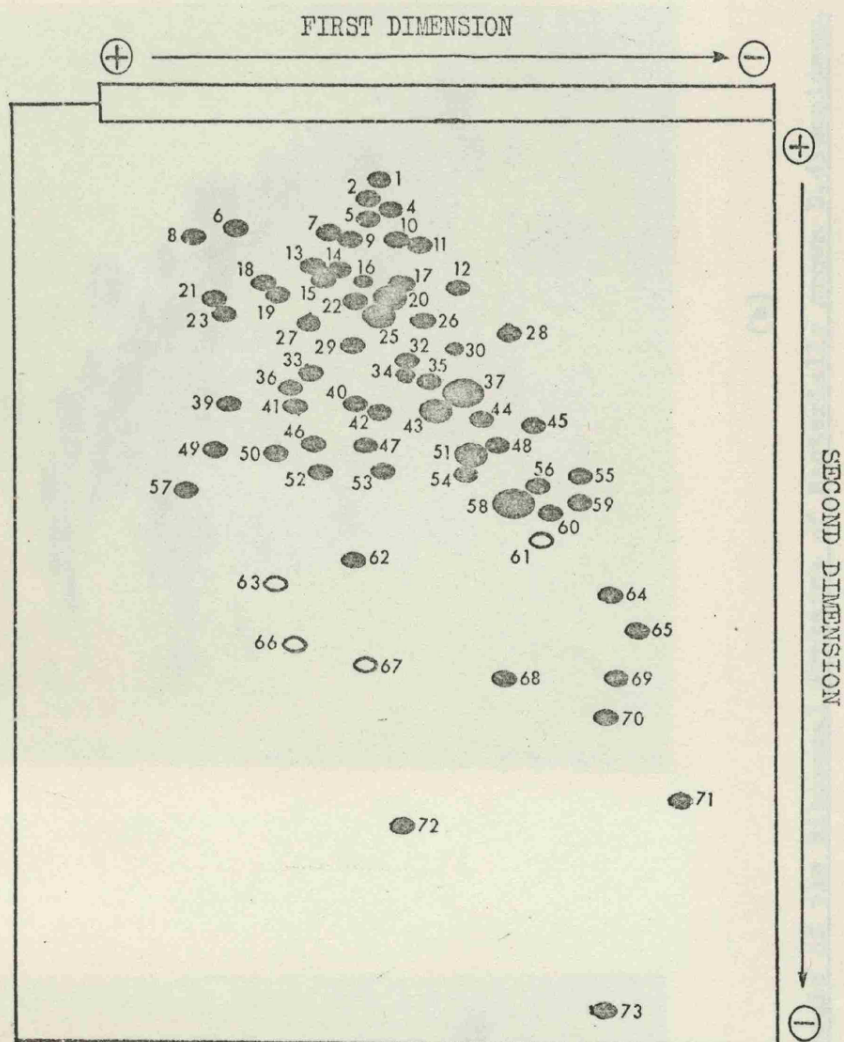
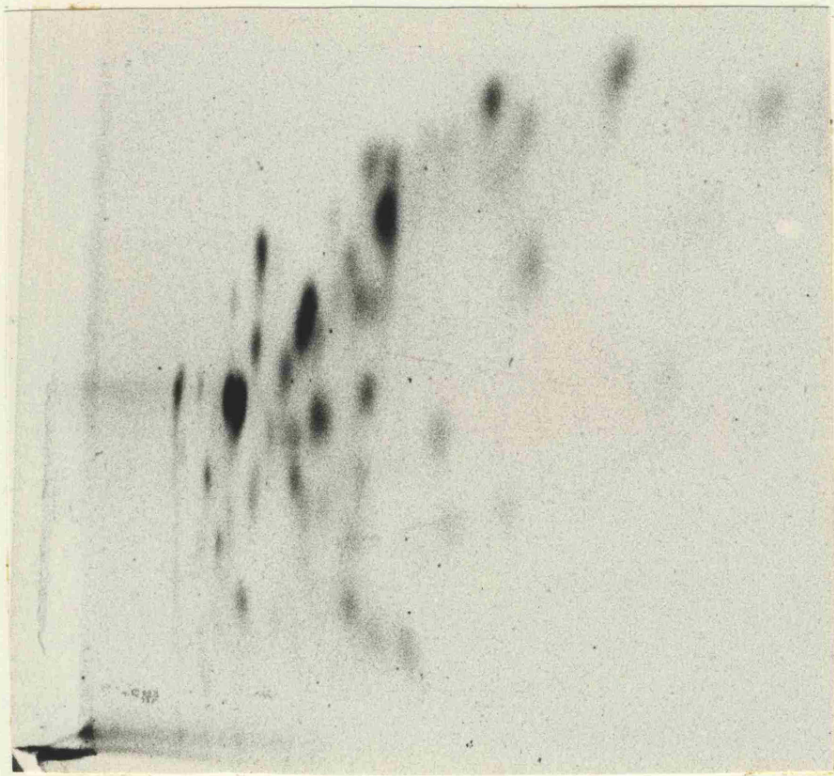
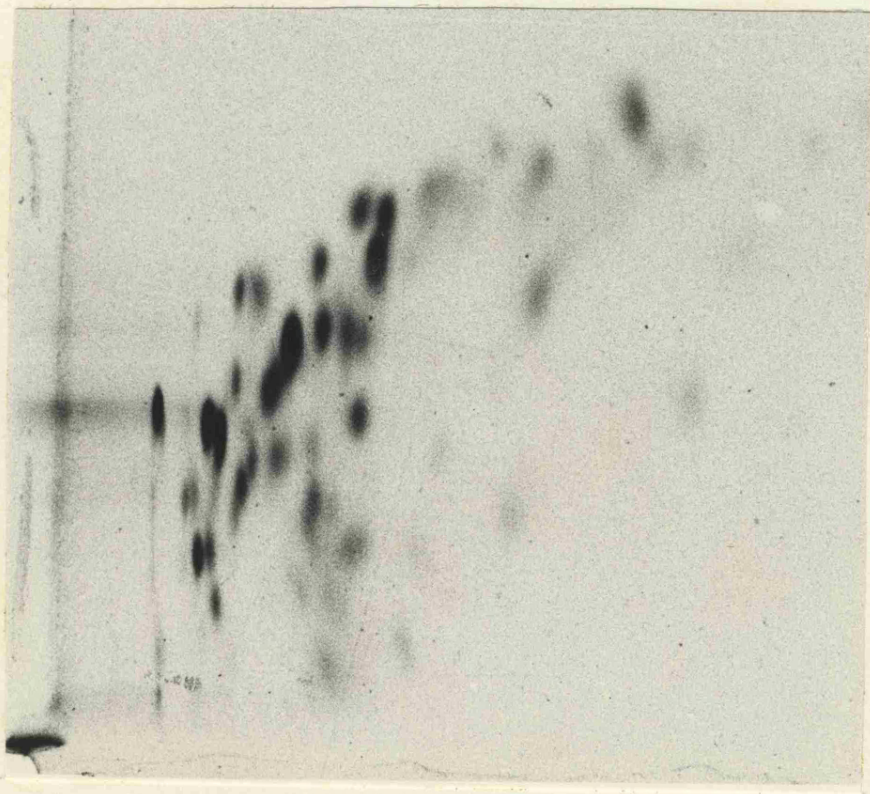


FIGURE23 Schematic Comparison between the Ribosomal Proteins of
'Unwashed' and 'Ammonium Chloride washed' Ribosomes of
D. discoideum

- : Proteins present in both washed and unwashed ribosomes
- : Proteins present only in unwashed ribosomes



(a)



(b)

PLATE 6. The Two-dimensional Electrophoresis of the Ribosomal Proteins of Bacterially grown *D. discoideum*.

(a) The ribosomal proteins of vegetative cells. (b) The ribosomal proteins of developing cells.

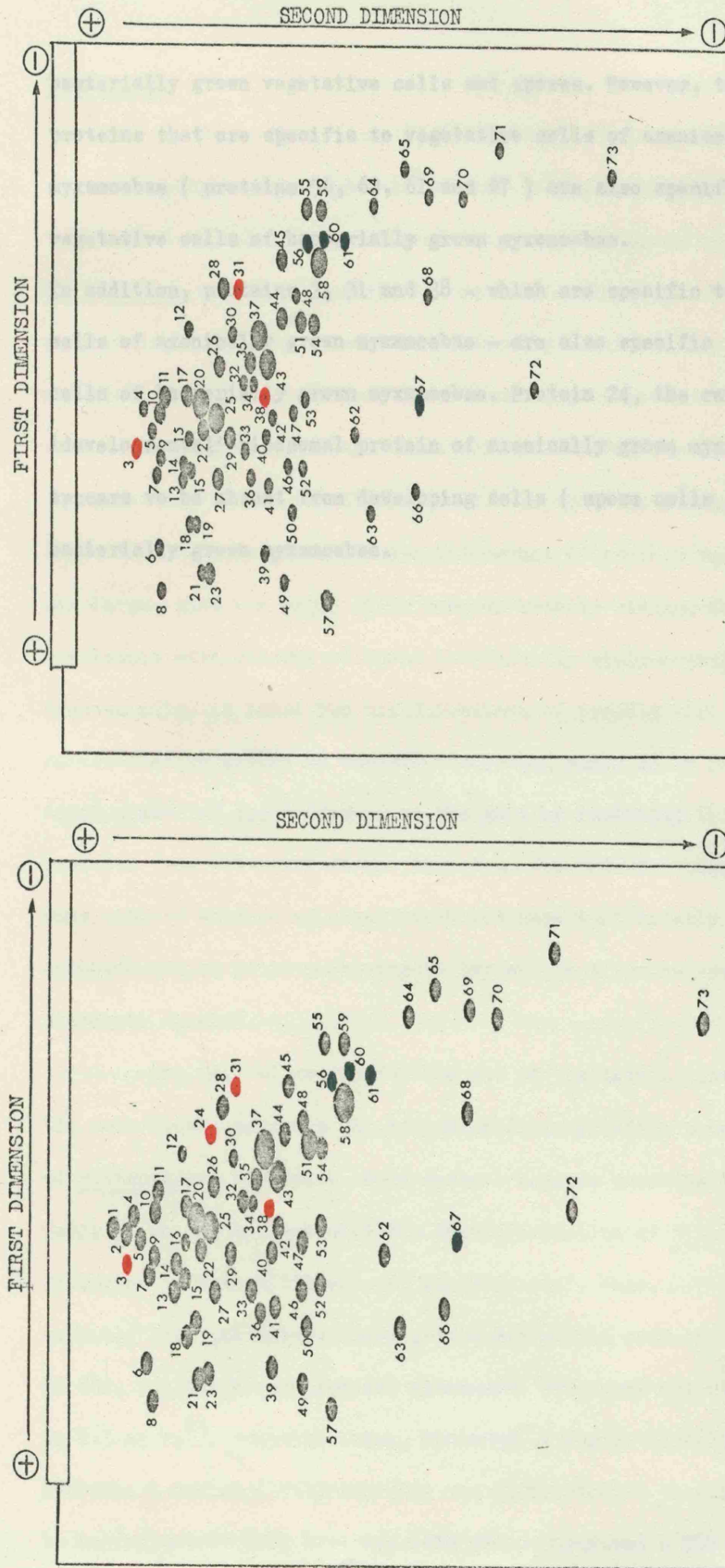


FIGURE 24. Schematic Comparison Between the Two-Dimensional Electrophoretograms of Ribosomal Proteins Isolated From Axenic and Bacterially Grown *D. discoideum*

- (a) Axenic. ●: 'common' proteins, ●: vegetative proteins, ●: developmental proteins
 (b) Bacterial. ●: 'common' proteins, ●: vegetative proteins, ●: developmental proteins

bacterially grown vegetative cells and spores. However, those proteins that are specific to vegetative cells of axenically grown myxamoebae (proteins 56, 60, 61 and 67) are also specific to vegetative cells of bacterially grown myxamoebae.

In addition, proteins 3, 31 and 38 - which are specific to developing cells of axenically grown myxamoebae - are also specific to spore cells of bacterially grown myxamoebae. Protein 24, the remaining 'developmental' ribosomal protein of axenically grown myxamoebae, appears to be absent from developing cells (spore cells) of bacterially grown myxamoebae.

DISCUSSION

Before a meaningful discussion of the results of the two-dimensional electrophoresis of the ribosomal proteins of D.discoideum can be attempted, the methods employed for their preparation and analysis must be critically assessed. The starting material for the isolation of the ribosomal proteins has been the monomeric ribosome. Although reasonable resolution was achieved, the detection of all the different protein spots on a single gel was often difficult, especially when the amount of protein analysed was large, when the major spots were so heavily stained that they overlapped with several of their less-heavily stained neighbours. Consequently, at least two concentrations of protein were analysed. An alternative method to improve resolution would be to reduce the total number of spots present on the gels by isolating the ribosomal proteins from sub-units rather than from the intact ribosome. Indeed, many similar studies on other organisms have successfully used this approach and so it is necessary to try and justify the use of the monomeric species.

One trivial reason for the use of the intact ribosome was the notoriously aberrant and ill-defined dissociation properties of D.discoideum ribosomes. From several reports over the last ten years or so, it appears that the characterization of D.discoideum ribosomes is both difficult and controversial. Thus, Ashworth (1966) reported the ribosomes to have a prokaryote-like sedimentation coefficient of 68S, but unlike prokaryotic ribosomes, they were not dissociated in 1.5 mM Mg^{2+} . Somewhat later, Ceccarini & Maggio (1968) and Iwabuchi & Ochiai (1969) reported the sedimentation coefficient to be approximately 80S, however, both groups observed a 70S particle

which they suggested was an intermediate in the dissociation of the 80S monomer. One is very tempted to correlate this species with the 68S partical found in such apparent abundance in the earlier study of Ashworth (1966). Dissociation studies have revealed quite a considerable complexity of the ribosome. Whilst all groups agree that monomers exist in the presence of 1.5 mM Mg^{2+} , Ceccarini & Maggio (1968) report that the lowering of the Mg^{2+} concentration to 0.25 mM produced "intermediate particles" (68S and 56S) and in 10^{-6} M Mg^{2+} , the final dissociated species had sedimentation coefficients of approximately 50S and 30S. On the other hand, Iwabuchi & Ochiai (1969) and Iwabuchi et al (1970) reported that the dissociated species in 0.1 mM Mg^{2+} had sedimentation coefficients of 61S and 42S. However, the same group (Kanda et al, 1974 ; Ochiai et al, 1973) have recently employed a concentration of 50 mM Mg^{2+} for the isolation of ribosomal sub-units - conditions which. in their previous paper, were reported to cause precipitation of the ribosomes. Preliminary experiments were made to isolate ribosomal sub-units at the beginning of this work, but these were unsuccessful and served only to emphasize the current lack of detailed information on the properties of D.discoideum ribosomes.

However, it was not only more convenient to use the monomeric ribosome as the starting material for the isolation of ribosomal proteins but also, perhaps, more appropriate. Martini et al (1973) state,

"...we also find that the intact 80S ribosome (from rabbit reticulocytes) contains several additional proteins of relatively high molecular weight that are lost when the particle is dissociated into sub-units."

This really brings us to a very important question : what is a

ribosomal protein ? Unfortunately, rigorous criteria for classifying the proteins into truly ribosomal, additional functional (such as translation and initiation factors) and contaminant do not yet exist, primarily because the ribosome is a non-covalent assembly of RNA and protein. Warner et al (1973) have suggested that only those proteins that are associated permanently with the ribosome deserve the name ribosomal proteins. From a structural and formational point of view this may be an adequate definition, but from a functional point of view, it is insufficient. It has recently been reported that several of the proteins associated with the 80S ribosome are not essential for the in vitro translation of poly(U) messages (McConkey, 1974). However, for the reasons described in the introduction, this may bear little relationship to the in vivo situation. What does seem to be clear is that most of the ribosomal proteins are permanently associated with the ribosome and this unit can, in vitro, effect the formation of peptide bonds. But several proteins are not permanently associated with the ribosome and these may well be involved in the in vivo translation of natural messenger RNA. Since the present studies are concerned with ribosome structure and how it may affect ribosome function it was felt that ribosomes containing as many proteins as possible should be used in the experiments, i.e. the monomeric species, prepared in a medium which did not contain a high salt concentration as this may remove certain loosely-bound ribosomal proteins. One preparation of ribosomes however was washed in 0.5M ammonium chloride to determine which, if any, proteins are removed.

Whilst the use of the monomeric ribosome has been at least partially justified, the actual procedure used for its isolation contained one possible source of error. The fact that, during the isolation of the ribosomes, the removal of contaminating polysaccharide

material was achieved only at the expense of approximately 40 % of the ribosomes raises the possibility that the final clarified ribosome suspension may not be representative of the total ribosome content. Two factors are worth considering here. The first is whether there is any interaction between the polysaccharide material and the ribosome which causes the loss of ribosomes during clarification and the second is whether the ribosomes that are lost constitute a distinct class of ribosomes and that their loss during clarification merely accompanies the removal of polysaccharide material. Although not studied in detail, some evidence has been obtained concerning the interaction between the polysaccharide material and the ribosomes from the results of attempts to prepare ribosomes from myxamoebae grown in different media. Cells grown in NS medium and in NS+glucose medium have the same RNA content and fairly similar growth rates and hence probably contain similar ribosome complements. If the 40 % of the ribosomes that are lost in the clarification of crude ribosome suspensions from NS grown cells constituted a distinct class of ribosomes, then it would be expected that the same proportion should be lost in the clarification of ribosomes from NS+glucose grown cells. However, in the latter case, clarification results in the loss of approximately 70 % of the ribosomes (as well as producing a clarified suspension that still contained significant quantities of polysaccharide material). Since the polysaccharide content of NS+glucose cells is much greater than that of NS cells (Weeks and Ashworth, 1972) it appears the more polysaccharide present, then the greater the loss of ribosomes. This suggests that there is some causal interaction between ribosomes and polysaccharide which results in the loss of the ribosomes and thus it seems unlikely that the lost ribosomes constitute a special class.

What then, are the characteristics of the clarified ribosome suspension that has been used for the isolation of the ribosomal proteins ? Sucrose density centrifugation has shown that the ribosomes have a sedimentation coefficient of approximately 80S, a value in agreement with the more recent studies by other workers previously described. The protein content of these ribosomes has been determined as approximately 44%. This figure is less than that reported by Ceccarini & Maggio (1968) who suggested a 50% protein content but more than that of Ochiai & Iwabuchi (1971) who reported a 41% protein content. In this context, the ribosomes of D.discoideum appear to resemble more closely the ribosomes of yeast, which contain 40-44% protein (Warner, 1971), than they do those of higher eukaryotes in which a 50% protein content is usually found (Martini et al, 1973).

The results of the two-dimensional electrophoresis of the ribosomal proteins of D.discoideum are summarized in TABLE 5 which shows the extent to which the various proteins are present in the ribosomes of axenic cells (both vegetative and developing), bacterially-grown cells (both vegetative and developing) and also from ribosomes that had been washed in 0.5 M ammonium chloride or in the supernatant remaining after ribosomes had been prepared from developing cells (referred to as 'dev.-washed ribosomes').

Two limitations of the technique must be made explicit. The first is that two proteins having different amino acid sequences may migrate together on two-dimensional gel electrophoresis provided that they have the same net charge and the same molecular size. The second limitation is that the stoichiometric estimations are only very approximate, having been made by a simple visual inspection of the gels. The problem of devising a suitable densitometric analysis technique for two-dimensional gels is, I think, nicely illustrated

RIBOSOMAL PROTEINS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Vegetative(axenic)	L	L	-	L	L	L	M	L	M	M	M	M	L	L	L	L	M	M	M	H	L	L	M	-	H
Developing(axenic)	L	L	M	L	L	L	M	L	M	M	M	L	M	L	L	L	H	M	M	H	L	L	M	M	H
NH ₄ Cl - washed	L	L	-	L	L	L	M	L	M	M	M	M	M	L	L	L	M	M	M	H	L	L	M	-	H
Dev. s/n - washed	L	L	-	L	L	L	M	L	M	M	M	M	M	L	L	L	M	M	M	H	L	L	M	-	H
Vegetative(bacterial)	-	-	-	L	L	L	L	L	L	M	M	M	M	L	L	L	M	M	M	H	L	L	M	-	H
Developing(bacterial)	-	-	L	L	L	L	L	L	L	M	M	M	M	L	L	L	M	M	M	H	L	L	M	-	H
RIBOSOMAL PROTEINS	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Vegetative(axenic)	M	M	M	M	L	-	M	L	L	L	L	L	H	-	L	M	L	M	M	M	L	L	M	L	L
Developing(axenic)	M	L	M	M	L	M	M	L	L	L	L	L	H	M	L	M	L	M	M	M	L	M	M	L	L
NH ₄ Cl - washed	M	M	M	M	L	-	M	L	L	L	L	L	H	-	L	M	L	M	M	M	L	M	M	L	L
Dev. s/n - washed	M	M	M	M	L	-	M	L	L	L	L	L	H	-	L	M	L	M	M	M	L	M	M	L	L
Vegetative(bacterial)	M	M	M	M	L	-	M	L	L	L	L	L	H	-	L	M	L	M	M	M	L	M	M	L	L
Developing(bacterial)	M	M	M	M	L	M	M	L	L	L	L	L	H	L	L	L	L	M	M	M	L	M	M	L	L
RIBOSOMAL PROTEINS	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73		
Vegetative(axenic)	M	L	M	L	M	L	L	M	M	M	L	L	L	L	L	L	L	L	L	L	M	L	L		
Developing(axenic)	M	L	M	M	M	-	L	M	M	-	-	L	L	L	L	L	-	L	L	L	M	L	L		
NH ₄ Cl - washed	L	L	M	L	M	L	L	M	M	M	-	L	-	L	L	-	-	L	L	L	M	L	L		
Dev. s/n - washed	M	L	M	L	M	L	L	M	M	M	L	L	L	L	L	L	L	L	L	L	M	L	L		
Vegetative(bacterial)	M	L	M	L	M	L	L	M	M	M	L	L	L	L	L	L	L	L	L	L	M	L	L		
Developing(bacterial)	M	L	M	M	M	-	L	M	M	-	-	L	L	L	L	L	-	L	L	L	M	L	L		

TABLE 5. Approximate Estimation of the Presence and Stoichiometry of the Ribosomal Proteins of *D. discoideum*

'H' = heavily stained proteins. 'M' = medium stained proteins. 'L' = lightly stained proteins

'-' = proteins absent.

by the words of Martini et al, (1973) ;

" We are now attempting to adapt a densitometric system, used by the X-ray crystallography group at Kings College for automatic scanning and computerized integration of densities on X-ray diagrams, for our purpose. "

The results of the two-dimensional electrophoresis have shown that there are 69 proteins associated with the ribosomes of axenically grown vegetative myxamoebae. This number compares favourably with a recent paper by Ochiai et al (1973) who isolated ribosomal proteins from sub-units and separated them using the high resolution, one-dimensional polyacrylamide gel electrophoresis technique described by Panyim & Chalkley (1969). This suggests that whether proteins are isolated from intact ribosomes or from sub-units the same number are distinguishable, although it is impossible to say whether they are qualitatively identical.

Of these 69 proteins, four (proteins 61, 63, 66 and 67) appear to be 'loosely-bound' since they can be removed by washing the ribosomes in 0.5 M ammonium chloride - a process which also appears to partially remove several other proteins (proteins 40, 42 and 51). But in the absence of a working definition of a 'ribosomal protein' these loosely bound proteins will be considered as ribosomal. The case of protein 12, which appears in greater quantities in ammonium chloride washed ribosomes, is difficult to explain satisfactorily. It may be that this is a membrane protein which is mostly removed during the normal clarification process, but which is rendered more soluble by treatment of the crude ribosomal pellet with high salt concentrations.

The two-dimensional electrophoresis of the ribosomal proteins from developing cells (initially grown axenically) also reveals 69 proteins, the vast majority of which correspond well

with those isolated from vegetative cells. However, there are small differences between the two and these are thought to be significant. Proteins 56, 60, 61 and 67 are found to be present only in the ribosomes of vegetative cells, whereas proteins 3, 24, 31 and 38 are present only in the ribosomes of developing cells. In addition to these qualitative differences, there may also be small quantitative differences. Those ribosomal proteins which appear to be more heavily stained when isolated from developing cells include proteins 17, 29, 47 and 54. On the other hand, protein 27 appears to be more heavily stained when it is isolated from vegetative cells. But in the absence of precise stoichiometric analysis, these quantitative differences must be considered trivial and will not be discussed further.

It must now be established that these qualitative differences between the ribosomal proteins of axenically grown vegetative and developing cells are real and not artefactual.

It could be argued that since many novel proteins are present in the cytoplasm of developing cells, proteins 3, 24, 31 and 38 represent the non-specific adsorption by the ribosomes of cytoplasmic proteins during the isolation procedure. That this is not the case has been demonstrated by washing the ribosomes isolated from vegetative cells in the supernatant remaining after ribosomes had been prepared from developing cells. The two-dimensional electrophoresis of the ribosomal proteins prepared in such a way reveals exactly the same number of proteins and in the same positions as those from vegetative ribosomal proteins prepared in the normal way.

We have established that the differences between the ribosomal proteins of vegetative and developing cells of axenically

grown myxamoebae are real, but the critical question is whether they are developmentally important. The developmental significance of these changes has been unequivocally demonstrated by the analysis of the ribosomal proteins from vegetative and developing cells of bacterially grown myxamoebae. Such cells are metabolically quite distinct from axenically grown cells - they have twice the DNA content and a mean generation time approximately half that of axenically grown cells (see Chapter 1). This is reflected, to a small degree, in their complement of ribosomal proteins. Proteins 1 and 2, present in axenically grown cells, are absent from bacterially grown cells. In addition, the two-dimensional electrophoretograms are not precisely superimposable although the general patterns are much the same. But the changes between vegetative and developing ribosomal proteins are the same. Proteins 3, 31 and 38 are specific to developing cells that have been grown either axenically or in association with bacteria, and proteins 56, 60, 61 and 67 are specific to vegetative cells that have been grown either axenically or in association with bacteria. Protein 24, which is specific to axenically grown developing cells, is not present in bacterially grown cells, either vegetative or developing. This fact must cast serious doubt on the developmental significance of protein 24.

Within the limitations of this technique, it has therefore been shown that the ribosomes formed during development are structurally distinct from those formed during the vegetative phase. The implication of this is that these structural differences may reflect some kind of functional distinction between the ribosomes formed during the two phases of the life cycle that may mediate the cytoplasmic control of development.

SUMMARY

1. A procedure and apparatus for the analysis of the ribosomal proteins of D.discoideum by two-dimensional polyacrylamide gel electrophoresis has been described.

2. Analysis of the ribosomal proteins of axenically grown vegetative and developing cells has revealed the presence of 4 proteins that are specific to vegetative cells and 4 proteins that are specific to developing cells. The developmental significance of these differences has been established by similar analyses of the ribosomal proteins of metabolically distinct cells, and is discussed in relation to the role of the ribosomes in the cytoplasmic control of development.

CHAPTER 4

RIBOSOMAL RNA FROM THE CELLULAR
SLIME MOULD DICTYOSTELIUM DISCOIDEUM

CHAPTER 4

RIBOSOMAL RNA FROM THE CELLULAR

SLIME MOULD DICTYOSTELIUM DISCOIDEUM

INTRODUCTION

For the reasons outlined in the introduction to Chapter 3, the involvement of control at a ribosomal level in developmental processes may be indicated by structural analysis of the ribosomal RNA at different times during the growth and development of the cellular slime mould D. discoideum. Such studies are not without their precedents. Sussman (1967) showed that during the development of another cellular slime mould Polysphondelium pallidum, pre-formed ribosomal RNA was degraded and replaced by newly-synthesized ribosomal RNA. However the ribosomal RNA species appeared identical with regard to base composition and hybridization ability. Earlier studies examining the base composition and sedimentation coefficients of ribosomal RNA isolated from conidia and mature hyphae of Neurospora crassa (Henney and Storck, 1963) also failed to reveal significant changes in ribosomal RNA associated with a particular stage of development.

Since a common technique used to determine whether two species of RNA are identical involves the hybridization of the species to DNA, it is worthwhile to consider briefly what is known about the genes which code for the ribosomal RNA.

In eukaryotes there are four ribosomal RNA species; the two large RNA species, the 28S (which has one of the small RNA species, the 5 - 7S RNA associated with it) and the 18S are derived from a single ribosomal precursor RNA (see previous chapter) and the remaining small RNA species, the 5S RNA is believed to be

genetically separate from the genes coding for the large RNA species (see Ford, 1973). The most noteworthy fact concerning the genes that code for ribosomal RNA is that they almost universally exist as multiple copy genes, comprising approximately 0.03% of the genome in animals and approximately 0.5% of the genome in plants (Birnsteil et al, 1971). Despite the large number of genes for the large RNA species, it appears that they are situated on very few chromosomal loci (Pardue et al, 1970) and these are correlated with the nucleolar organizers (Birnsteil et al, 1966 ; Ritossa and Spiegelman, 1965). The ribosomal RNA genes in Xenopus - about which probably more is known than any other plant or animal gene - consists of two parts, the transcription unit and the spacer DNA (Birnsteil et al, 1968 ; Dawid et al, 1970 ; Brown et al, 1972). The transcription unit contains the coding sequence for the 18S and 28S ribosomal RNA species and a portion of the spacer DNA which is transcribed. The 5S ribosomal RNA genes are also believed to include regions of spacer DNA, although in contrast to that of the large ribosomal RNA genes, the spacer DNA of the 5S gene appears to have a low (G+C) content (Brown et al, 1971).

In view of the large number of ribosomal RNA genes present in all organisms, it seems important to know whether the genes are identical within any one species. Unfortunately, despite numerous experiments, there is no unequivocal report of sequence heterogeneity in an RNA species from a eukaryote, although such heterogeneity has been demonstrated in Escherichia coli 5S RNA (Brownlee et al, 1968).

We can now consider some examples of development where the genes for ribosomal RNA appear to be a potential source of control. During the early stages of oogenesis in Xenopus laevis,

it has been shown (Ford, 1971) that the 5S ribosomal RNA is synthesized in great excess of the large RNA species. It is also known (Ford & Southern, 1973) that the 5S ribosomal RNA species in kidney cells and ovaries of Xenopus laevis display some sequence heterogeneity.

In developing oocytes of Xenopus laevis, the ribosomal RNA genes are selectively replicated (about 1000-fold) in order to furnish the oocytes with a sufficient number of ribosomal RNA genes to support the intense synthesis of ribosomal RNA that occurs at a particular stage in their maturation (Gall, 1969). However, this is not an example of control at a ribosomal level, but is perhaps the opposite, as it seems likely that the amplification of the ribosomal RNA genes makes the number of ribosomes available for protein synthesis strictly non-limiting. Indeed, by injecting messenger RNA into such developing oocytes, Gurdon (1974) shows that it is possible to increase the net rate of protein synthesis by approximately 50%, indicating that in such cells, saturation of the ribosomes is never reached.

A useful technique in the comparison of RNA species is that of competition hybridization to DNA. By observing how the presence of one species of RNA affects the hybridizing ability of a second RNA species, it can be determined whether the two species have a common origin on the DNA and thus identical base sequences. It is the purpose of this chapter to use this technique to determine whether the ribosomal RNA synthesized during the developmental phase of the life cycle of D.discoideum is the same as that synthesized during the vegetative phase

MATERIALS

Enzymes were obtained from Sigma (London) Ltd, London, U.K., inorganic $^{32}\text{[P]}$ -phosphate was obtained from the Radiochemical Centre, Amersham, Bucks, U.K., hydroxyl apatite BioGel HT was obtained from Biorad Laboratories, Richmond, California, U.S.A. and caesium chloride, 'suprapur' grade, was obtained from Merck, Darmstadt, West Germany. All other materials were of the highest purity commercially available and were the products of either BDH Chemicals Ltd, Poole, Dorset, U.K. or Fisons Scientific Apparatus Ltd, Loughborough, Leicester, U.K.

METHODS

(i) Growth and Development of Myxamoebae

Myxamoebae representative of the vegetative phase were obtained by growth in NS medium to a cell density of approximately 4×10^6 cells / ml as described in Chapter 1.

Cells representative of the developmental phase were obtained by harvesting cells grown in NS medium and allowing them to develop on Millipore filter supports as previously described, until they reached the Mexican hat stage, (after approximately 18 hours) at which point they were harvested.

(ii) Preparation of DNA

The high level of RNA and low DNA content of myxamoebae of D.discoideum makes the extraction of DNA from this organism difficult. The method used is a modification of the procedure of Sussman & Rayner (1971) as described by Leach & Ashworth (1972). All operations were performed at $0 - 2^{\circ}\text{C}$ unless otherwise stated, except for the centrifugations which were performed

at 4°C.

Myxamoebae grown in NS medium were harvested, washed and resuspended in cold SSC (see page iv) containing 0.1 M EDTA, pH 8.0, at a cell density of approximately 3×10^8 cells / ml. An equal volume of saturated phenol solution (see page iv) was added and the mixture gently stirred for 5 min. The resulting emulsion was clarified by centrifugation (5000g, 10 min.) and the interphase material was resuspended in half the original volume of SSC containing 0.02 M EDTA, pH 8.0. This process is believed to remove much of the cellular RNA and makes the subsequent extraction of the DNA easier.

The interphase material was dispersed by the addition of 20% sodium dodecylsulphate to a final concentration of 0.5% and the solution warmed to 37°C until dispersal was complete (usually after approximately 20 min.). The clear viscous solution was cooled and an equal volume of saturated phenol solution was added and the mixture was stirred at room temperature for 30 min. Centrifugation (10,000g, 15 min.) separated the two phases. The upper aqueous phase was removed with a wide-tipped pipette taking great care to ensure very little contamination with the lower phenol phase, and the DNA precipitated with an equal volume of 2-ethoxyethanol. The DNA was collected by 'spooling' and dissolved in 2 - 3 ml of 0.1 x SSC (see page iv) and dialysed against 500 volumes of 0.1 x SSC overnight at 4°C.

The dialysed DNA solution was incubated at 37°C for 90 min. with ribonuclease (10 µg/ml, previously heated to 100°C for 10 min. to destroy any contaminating deoxyribonuclease activity) and α-amylase (500 µg/ml). An equal volume of saturated phenol solution was added and the mixture was stirred at room temperature for 30 min. After centrifugation (10,000g, 15 min.), the upper aqueous

phase was carefully removed and the DNA precipitated with 0.54 volumes of isopropanol. The DNA was collected by spooling, dissolved in 1 - 2 ml of 0.1 x SSC and dialysed against 500 volumes of 0.1 x SSC overnight at 4°C. The DNA was reprecipitated with an equal volume of 2-ethoxyethanol, collected by spooling and dissolved in 1 - 2 ml of SSC and dialysed exhaustively against 0.01 x SSC at 4°C. The final preparation was observed to be free of protein, RNA and polysaccharide material as determined by the methods previously described.

(iii) Preparation of Unlabelled Ribosomal RNA

Ribosomes were prepared

as described in Chapter 3, except that polyvinylsulphate (10 µg/ml) was included in the TKMM buffer to inhibit ribonuclease activity. The ribosomal RNA was extracted using the procedure described by Leach & Ashworth, (1972). 20% sodium dodecylsulphate was added to the clarified ribosome suspension to a final concentration of 0.5% and an equal volume of saturated phenol solution was added. The mixture was stirred at room temperature for 30 min. and then clarified by centrifugation (10,000g, 15 min.). The upper aqueous phase was carefully removed and extracted once more with an equal volume of saturated phenol solution. The final aqueous phase was carefully removed and the RNA precipitated with 2 volumes of ethanol at -17°C for 16 hours.

The crude RNA precipitate was collected by centrifugation (20,000g, 10 min.) and resuspended in 0.05 M sodium acetate buffer, pH 4.6, and incubated at 37°C for 2 hours with deoxyribonuclease (5 µg/ml) and α-amylase (500 µg/ml) and then for a further 1 hour with pronase (50 µg/ml). The mixture was

then de-proteinized by two extractions with saturated phenol and the RNA precipitated from the final aqueous phase with 2 volumes of ethanol as before. The resulting RNA precipitate was collected by centrifugation (20,000g, 15 min.), dissolved in 0.1 x SSC and dialysed exhaustively against 0.01 x SSC at 4°C. The final preparation was free of DNA, protein and polysaccharide material as detected by the methods previously described.

(iv) Preparation of ^{32}P - labelled ribosomal RNA

^{32}P - labelled

ribosomal RNA was prepared in the same manner as unlabelled ribosomal RNA, from cells that had been grown for 7 generations in a modified NS medium in which the phosphate buffer had been replaced with ^{32}P - labelled inorganic phosphate (specific radioactivity 1 mCi / mmole).

(^{32}P -labelled inorganic phosphate is supplied in dilute HCl, pH 2 - 3, but the small volume (1 ml) added to the medium (660 ml) did not change the pH of the medium, which remained at 6.7.

(v) Analytical Ultracentrifugation of DNA

Isopycnic centrifugation

of whole cell DNA was performed on a CsCl (suprapur grade) density gradient (average density 1.700 g cm^{-3}) as described by Leach & Ashworth (1972), using Proteus mirabilis DNA (bouyant density 1.698 g cm^{-3}) as a standard. Centrifugation was performed using an MSE analytical ultracentrifuge equipped with an ultra-violet scanning system for 20 hours at 47,900 rpm at 25°C.

The bouyant densities were calculated using the equation

$$P = P_0 - 4.2 \omega^2 (r^2 - r_0^2) \times 10^{-10} \text{ g cm}^{-3}$$

where,

P is the bouyant density of *D.discoideum* DNA in g cm^{-3}

P_0 is the bouyant density of the standard DNA in $g\ cm^{-3}$

ω is the angular velocity in radians / sec

r is the distance of D.discoideum DNA peak from rotor centre

r_0 is the distance of standard DNA peak from rotor centre

(Professor J. Gall, personal communication)

(vi) Competition Hybridization

Principle :

By observing how the presence of one species of RNA affects the annealing ability of a second RNA species, it can be decided if the two RNA species have a common origin on the DNA. If the two RNA species do have a common origin on the DNA, then the competing RNA (non-radioactive) will take up sites on the DNA that would ordinarily be taken up by the testor RNA (radioactive). One infers that RNA species having a common origin on the DNA have an identical base sequence. In this present study, two such experiments are performed. The first experiment uses unlabelled ribosomal RNA isolated from developing cells as the competing RNA and $^{32}[P]$ -labelled ribosomal RNA isolated from vegetative cells as the testor RNA. The second experiment, to which the first can be compared, uses unlabelled ribosomal RNA isolated from vegetative cells as the competing RNA and $^{32}[P]$ -labelled ribosomal RNA isolated from vegetative cells as the testor RNA. In this latter experiment, there should be 100% competition for the sites on the DNA between labelled and unlabelled ribosomal RNA species since they were isolated from similar (vegetative) cells.

Hybridization :

The conditions used are those described by Firtel & Bonner (1972). The concentrations of all RNA and DNA solutions

were determined spectrophotometrically and the DNA sheared by passage through a 21 - gauge hypodermic needle and denatured by placing it in a boiling water bath for 10 min. and then immediately cooling it to 0°C. Denaturation was monitored by measuring the increase in absorbance at 260 nm, the accepted value for the hyperchromicity being 1.4 (Leach & Ashworth, 1972 ; Firtel & Bonner, 1972).

Reaction vials were set up at 0°C. Each hybridization mixture contained 35.5 µg DNA and 50 µg total ribosomal RNA in 2 ml of 0.24 M phosphate buffer (equimolar with respect to Na_2HPO_4 and NaH_2PO_4). The ratio of competing RNA : testor RNA was varied as shown in Results. Control vials were also set up which included all of the components of the hybridization vials except the DNA. Hybridization was carried out at 66°C for 20 hours and the vials then rapidly cooled to 0°C.

Hybrid Purification

The raw hybrid was purified by incubating each hybridization mixture with ribonuclease A (20 µg/ml) and ribonuclease T₁ (50 units/ml) for 1 hour at 25°C. Both enzymes had previously been heat treated to remove contaminating deoxyribonuclease activity.

Hybrid Detection :

The method used for the separation of the real hybrid from the other components of the hybridization mixture is based on the ability of double-stranded nucleic acids to bind hydroxy apatite, and the procedure has been described by Firtel & Bonner (1972). Hydroxy apatite columns were prepared on sintered glass filters contained in 1 cm (I.D.) glass tubes immersed in a 60°C water bath and the material incubated with 0.12 M phosphate buffer (equimolar with respect to Na_2HPO_4 and NaH_2PO_4) containing 0.2%

sodium dodecylsulphate (SDS). The contents of each hybridization vial were applied to the hydroxyl apatite and the column washed with 0.12 M phosphate buffer containing 0.2% SDS, until all the unhybridized RNA was eluted. The real hybrid was then eluted with 0.48 M phosphate buffer (equimolar with respect to Na_2HPO_4 and NaH_2PO_4), containing 0.2% SDS. 2 ml aliquots of the eluent were assayed for radioactivity using the scintillation system of Hall & Cocking (1965).

RESULTS

(i) Analytical Ultracentrifugation of DNA

DNA prepared from vegetative myxamoebae of D.discoideum formed a complex band after isopycnic centrifugation in a CsCl density gradient, (FIGURE 25), with peaks corresponding to bouyant densities of 1.682 g cm^{-3} and 1.688 g cm^{-3} . These values are in good agreement with those obtained by other workers (Leach & Ashworth, 1972) and are believed to represent nuclear and mitochondrial DNA respectively.

(ii) Competition Hybridization

Since only comparative results were required, the radioactivity was determined as counts / min rather than disintegrations / min. The specific radioactivity of the ^{32}P -labelled ribosomal RNA isolated from vegetative cells was determined as 10,004 cpm / μg RNA. The amount of ^{32}P -labelled ribosomal RNA present in the hybrid was calculated from the cpm contained in each hybrid formed (after subtraction of the cpm due to RNA 'noise' which was provided by the control vials that lacked the DNA). The maximum amount of ribosomal RNA hybridized to $35.5 \mu\text{g}$ of DNA under the conditions employed was $0.1055 \mu\text{g}$. This corresponds to 0.3% of the genome coding for ribosomal RNA, a value in good agreement with that of Leach & Ashworth (1972) and hence validates the assumption that the concentrations of RNA and DNA employed in the hybridization were sufficient to saturate all the available sites on the DNA, the ratio of rRNA : rDNA being approximately 500 : 1.

When unlabelled ribosomal RNA isolated from either

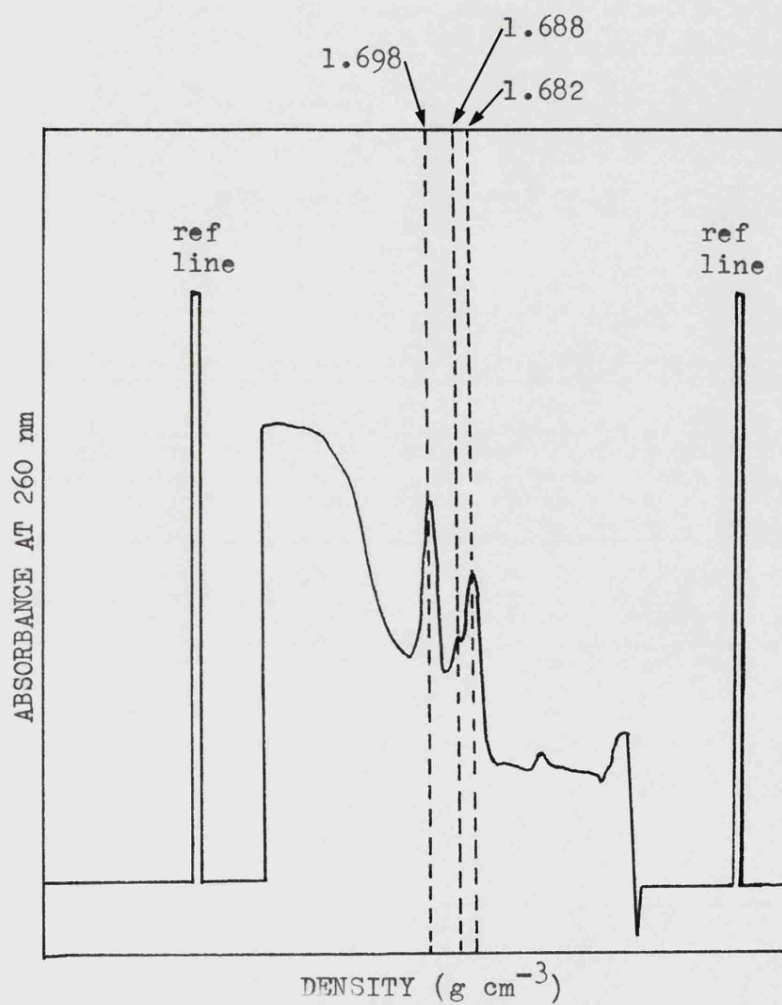


FIGURE 25. Density Gradient Centrifugation Analysis of DNA Prepared from Axenically Grown Myxamoebae of *D. discoideum*

Proteus mirabilis DNA (bouyant density 1.698 g cm⁻³) was added as an internal density standard

vegetative cells or developing cells is used as the competing RNA in a competition hybridization experiment, the effect on the annealing ability of ^{32}P -labelled ribosomal RNA isolated from vegetative cells is exactly the same, and the extent of competition is very close to the theoretical curve for 100% homology between the RNA species (FIGURE 26).

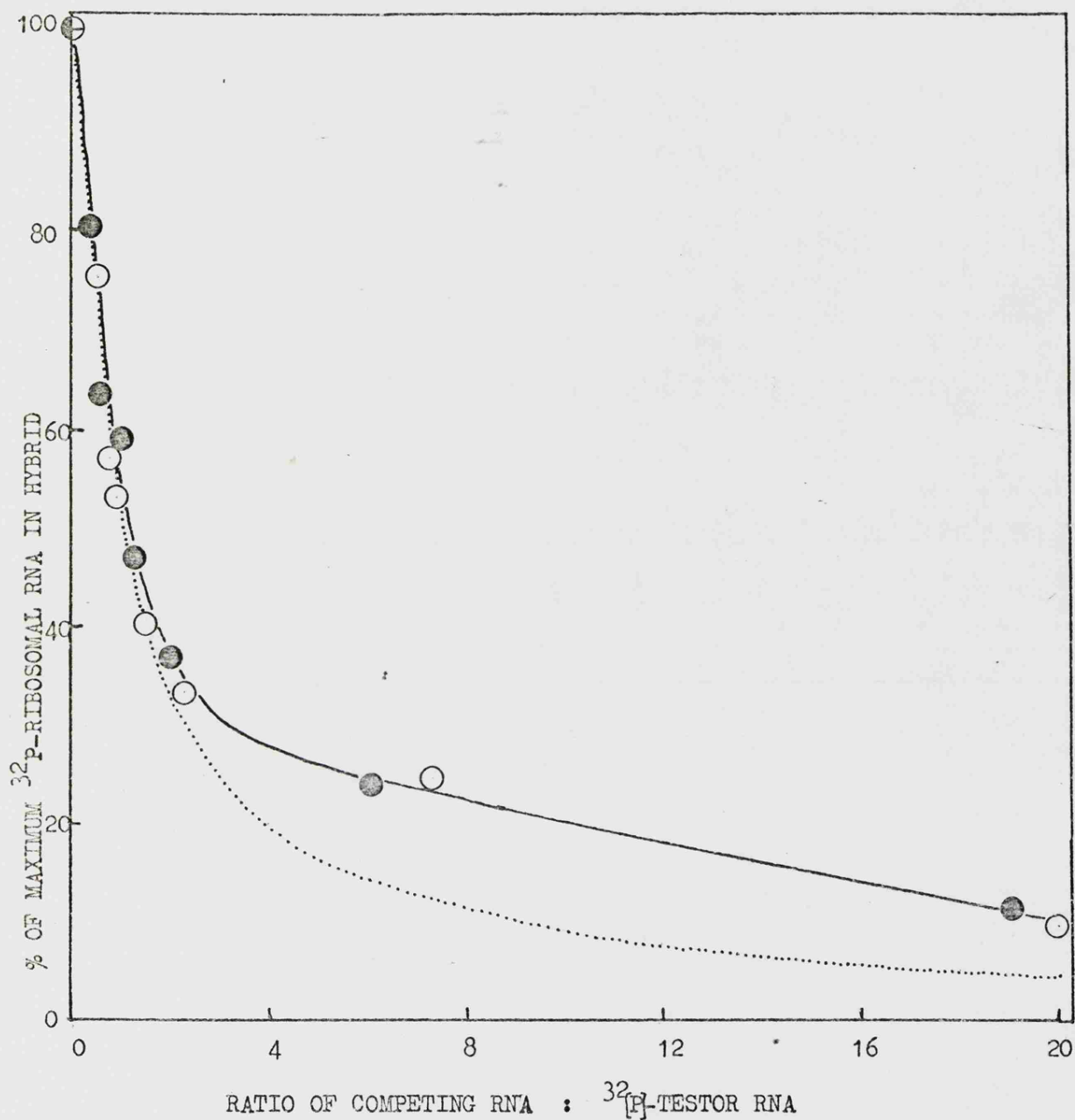


FIGURE 26. The Competition Hybridization of ^{32}P -Ribosomal RNA isolated from Vegetative cells of *D. discoideum* with unlabelled Ribosomal RNA isolated from vegetative and developing cells.

The competing RNA species are

○ : Unlabelled vegetative ribosomal RNA

● : Unlabelled developmental ribosomal RNA

The dotted line (.....) is the theoretical 100% competition curve

DISCUSSION

The purpose of this chapter has been to determine whether the ribosomal RNA synthesized during the developmental phase of the life cycle of D.discoideum is the same as that synthesized during the vegetative phase. Within the limits of the technique, it has been shown that ribosomal RNA from vegetative and developing cells appears to have the same origin on the DNA and thus, by inference, the same base sequence. There is, however, one disadvantage inherent in the technique of competition hybridization when performed in solution as described. This is the fact that sensitive complexes (hybrids that are only partially complementary, or complementary in a non Watson-Crick sense and which are removed by subsequent purification of the raw hybrid with ribonuclease) formed by the competing RNA may exclude the formation of the real hybrid that would ordinarily be formed by the testor RNA and would therefore result in an artefactually high degree of competition. This possible source of error is not considered significant in the present study because the important result is arrived at by a comparison between the competing abilities of ribosomal RNA isolated from two different phases of the life cycle. When the competing ribosomal RNA and the testor RNA are isolated from similar (vegetative) cells, the competition curve is the same as that obtained when the competing ribosomal RNA is isolated from developing cells, and both are close to the theoretical 100% competition curve.

The deviation observed from the theoretical 100% competition curve at high ratios of competing RNA : testor RNA is not considered significant since it is present in both experiments and at these high ratios, one is trying to measure small amounts of radioactivity and thus the numbers are statistically less meaningful.

One must be careful however, not to assume too much from these results. Whilst it appears that vegetative ribosomal RNA and developing ribosomal RNA are formed on the same region of DNA, this does not necessarily mean that they are structurally identical. In the introduction to Chapter 3, it has been said that ribosomal RNA is highly methylated, presumably to facilitate the assembly, with ribosomal proteins, of the ribosomal sub-units. Differences in the extent of methylation in vegetative and developing ribosomal RNA may not be detected by the technique of competition hybridization. Therefore we can only say that their structures are similar.

Another point relating to the formation of ribosomal sub-units, concerns the processing of the ribosomal precursor RNA, consideration of which (see introduction to Chapter 3) show that the RNA species observed in the mature ribosomes constitute only a part of the total ribosomal precursor RNA. Thus the observed similarity between the ribosomal RNA of vegetative and developing cells reflects only a partial homology in the genes which code for the ribosomal precursor RNA. This may be significant since that portion of the ribosomal precursor RNA that is not destined to appear in the mature ribosome is, none the less, essential for the in vivo assembly of the sub-units, and thus heterogeneity in this region may result in the assembly of a different class of ribosomes - perhaps with respect to the ribosomal protein complement as this has been shown to be different between vegetative and developing ribosomes.

This chapter can thus be concluded by saying that although it has been shown that the ribosomal RNA of vegetative cells and developing cells are structurally similar they may not, by virtue of their highly methylated state, have exactly the same base sequence. Moreover, there remains the possibility that developmentally

important heterogeneities in ribosomal precursor RNA may reside in that portion of it which does not appear in the mature ribosome.

SUMMARY

1. The technique of competition hybridization is described.
2. Within the limits of this technique, it has been shown that the ribosomal RNA synthesized during the developmental phase of the life cycle of D.discoideum has a similar base sequence to the ribosomal RNA formed during the vegetative phase.
3. The shortcomings of the technique of competition hybridization as a precise analytical tool in this study, are discussed with respect to the modifications of ribosomal precursor RNA during maturation and assembly of the ribosomes in the nucleolus.

CONCLUDING REMARKS

CONCLUDING REMARKS

In this thesis, I have elected to study the changes in the structure of the basic nuclear proteins and ribosomes between vegetative and developing cells of D.discoideum in the hope that it could be established how controls at nuclear and cytoplasmic levels may effect differentiation. The degree of success can now be discussed.

To take first the nuclear control. The differences between the basic nuclear proteins of vegetative (A) and developing (B) cells can be represented in the form described in the introduction to this thesis,

A—————→B

However, the differences, B - A , are very slight. No qualitative differences were observed between the basic nuclear proteins of vegetative and developing cells, the only difference was, in fact, a relatively minor quantitative one, developing cells containing less of fraction 1d than vegetative cells. But is this small change developmentally significant ? At the moment, this question cannot be resolved with certainty. The criteria that has been used in establishing the developmental significance of changes in the ribosomal proteins - by the use of metabolically distinct cells - cannot, practicably, be utilized in the study of the basic nuclear proteins. The reasons are two-fold. Firstly, the isolation of large numbers (more than 10^{11}) of clean nuclei is technically very difficult from bacterially grown cells since the exhaustive washing procedure used to remove exogenous bacteria results in the loss of a significant proportion of the cells, and secondly, the presence of endogenous bacteria and bacterial products are likely to cause ambiguities in the results.

From a general viewpoint, the basic nuclear proteins of D.discoideum - if they may be functionally correlated with the histones of higher organisms - are unlikely candidates for the specific expression of genetic activity. Rather, they may be involved in changes in the DNA during the cell cycle. In this context, the small quantitative difference between the basic nuclear proteins of vegetative and developing cells may well be a reflection of the deceleration / suspension of the cell cycle since development proceeds in the absence of DNA synthesis, and this idea has already been discussed.

Thus the question as to whether the changes in the basic nuclear proteins are developmentally significant remains open. It is hoped that future studies, including the preparation of basic nuclear proteins from metabolically distinct cells and also the production of synchronized cell cultures will help resolve this question further.

Cytoplasmic control has been investigated by structural analysis of the ribosomes formed during growth and development. The results of the ribosomal RNA analysis showed that, within the limits of the technique, there were no differences in the ribosomal RNA of vegetative and developing cells,

i.e. $A \longrightarrow B$

but $B - A \neq 0$

It can thus be said that the cytoplasmic control of development is not mediated by the formation of a novel class of ribosomal RNA.

However, it does appear to be mediated by the formation of different ribosomal proteins. The results of the two-dimensional electrophoresis of the ribosomal proteins of vegetative and developing cells, grown either axenically or in association with bacteria can be illustrated as follows,

A $\longrightarrow$ B (axenically grown)
 A' $\longrightarrow$ B' (bacterially grown)

Both axenically grown and bacterially grown vegetative myxamoebae contain proteins 56, 60, 61 and 67, and these are specific to cells in the vegetative phase. Axenically grown, developing cells contain 4 novel ribosomal proteins (proteins 3, 24, 31 and 38) and bacterially grown, developing cells contain 3 novel ribosomal proteins (proteins 3, 31 and 38). Since proteins 3, 31 and 38 are specific to development in these metabolically distinct cell populations, then this conclusively demonstrates their developmental significance. It implies a functional distinction between ribosomes formed at different stages during development, but precisely how these changes mediate cytoplasmic control is, as yet, unknown.

But what of protein 24 ? This protein is only found in developing cells that had been grown, initially, in axenic medium, and is definitely not present in developing cells of bacterially grown cultures. The presence of this protein in just one of the metabolically distinct cell populations used in the study justifies the criterion we have used to establish the developmental significance of observed changes in cell components during development. If we had elected to study solely axenically grown cells, then we would have found 4 proteins that were apparently specific to developing cells, and all these proteins would have assumed equivalent developmental significance. But in bacterially grown cells, protein 24 is not present during development whereas the other 3 proteins (3, 31 and 38) are found. Since the development of axenically grown cells and bacterially grown cells is identical, this means that protein 24 cannot be developmentally

significant, but that it is a consequential change of growing the cells in axenic medium which is merely expressed during development.

In this thesis, I have attempted to utilize the advantages of a microbial system of development to determine developmentally significant changes in the structure of the basic nuclear proteins and ribosomes in an attempt to discover possible mechanisms by which nuclear and cytoplasmic control may regulate differentiation. Whilst the developmental significance of changes in the basic nuclear proteins remains, as yet, unresolved, it has been shown that developmentally significant changes do occur in the structure of the ribosomes and this strongly suggests the involvement of cytoplasmic control during the development of Dictyostelium discoideum.

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STUDIES ON THE NUCLEIC ACID ASSOCIATED PROTEINS OF THE CELLULAR
SLIME MOULD DICTYOSTELIUM DISCOIDEUM

SUMMARY

The work presented in this thesis has utilized the advantages of a microbial system of development in order to determine possible ways in which nuclear and cytoplasmic controls may regulate cellular differentiation. The studies that have been carried out have been concerned with the structure of the basic nuclear proteins and ribosomes of Dictyostelium discoideum. If the process of development is, in part, regulated by functional changes in these components, then it is reasonable to expect to find structural changes in these components when isolated from cells in different developmental stages.

The differences between the basic nuclear protein content of vegetative and developing cells (as determined by urea-polyacrylamide gel electrophoresis) were very slight. No stage-specific basic nuclear proteins were observed, and the only reproducible difference in the electrophoretic patterns was a quantitative one with respect to one subfraction that was present in less quantity in developing cells than it was in vegetative cells. This small difference may thus be due to the differing requirements of cells actively growing and cells not committed to a growth orientated cell cycle, further, from this evidence, it cannot be said with certainty that the basic nuclear proteins are involved in the nuclear control of development.

Analysis of the ribosomes of D. discoideum did however reveal stage-specific differences. Within the limits of the technique of competition hybridization, the rRNA moieties of ribosomes



isolated from vegetative and developing cells were observed to be identical. The ribosomal proteins however did display a small, but reproducible degree of stage specificity. During development, ribosomes that were formed contained three ribosomal proteins that were not found to be associated with the ribosomes of vegetative cells. The developmental significance of these changes was established by the use of metabolically distinct cell populations that proceeded through the same developmental programme and were observed to exhibit the same changes in the ribosomal protein content between vegetative and developing cells. Further, the use of this approach has suggested that the presence of one other apparently stage-specific ribosomal protein is a function of the initial growth conditions of the cells and not of their subsequent differentiation.

In conclusion, the advantages of a microbial system of development, such as the cellular slime mould D.discoideum, have enabled developmentally significant changes in the ribosomes to be observed which may be involved in the cytoplasmic control of the developmental programme. At the moment, the involvement of the basic nuclear proteins in the nuclear control of development is not firmly established, but it is hoped that in the future, this approach and this developmental system will provide answers to this and many other questions.