PART I

REGULATION OF DEVELOPMENT IN THE CELLULAR SLIME MOLD *DICTYOSTELIUM discoideum*

PART II

POLYSOMES AND RNA SYNTHESIS DURING EARLY DEVELOPMENT OF THE SURF CLAM *SPISULA solidissima*

Thesis by

Richard Alan Firtel

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1972

(Submitted July 12, 1971)

To Judy

ACKNOWLEDGEMENTS

I entered Dartmouth as a pre-med student and it was not until my sophomore year when I took Introductory Biology that I became aware of and interested in molecular biology and research. The turning point was just before Christmas vacation of that year when Dr. Thomas Roos discussed Spemann's Primary Organizer experiments in lecture. The experiments caught my imagination and since then I have been fascinated with problems in developmental biology. I would like to thank all those at Dartmouth College for stimulating my interests in science and directing me to Caltech and for their confidence in me. I would especially like to thank Dr. Roos for following-up his lecture with many hours of discussion with me and for his help and friendship during my stay at Dartmouth and Dr. George Saul, my undergraduate advisor, who introduced me to independent research and to Caltech.

I would like to thank my parents for their help and confidence throughout my academic career and my life.

I thank Sally Elgin, Steve Beckendorf, Mike Dahmus, Stan Froehner, Steve Harris, David Holmes, Dan McMahon, John Smart, John Wilson, and Bill Wood for their friendship, help, and constructuve criticism which helped me become a better scientist as well as for many,many hours of bull sessions in and out of the lab. My special thanks

go to John Mayfield and Eric Davidson whose discussions and criticisms have been extremely helpful.

Outside of Caltech, I would like to thank Bill Loomis for all his help and discussions and for his instructions on the hows and whys of slime molds, Alberto Monroy for an enjoyable, interesting, and stimulating summer at Woods Hole, and Roy Britten for the helpful discussions we had together.

I would like to thank Ken Pischel, Ratchford Higgins and Bob Brackenbury for much assistance during the course of my research. I hope that I have helped them become more familiar with science and that I taught them something.

There are many other people who have made Caltech an enjoyable place to live and work. I would especially like to thank Ingelore Bonner who kept me supplied with endless amounts of chemicals and supplies and who always managed to get rush orders to arrive on time. It would be impossible to keep the lab going without her.

I thank Ben Fischer who fixed anything and everything and Sue Finch and Ludia Brown for keeping me supplied with an almost infinite amount of clean glassware and pipettes.

I am greatly indebted to my advisor, James Bonner, whose flexibility, generousity and freedom he permits in the lab has enabled me to actively pursue my individual scientific interests. His infectious enthusiasm for

science has made these years that much more enjoyable. Beyond this, I would like to thank him for his helpful counselling and his availability and willingness to talk at any time on any matter.

I especially appreciate and thank David McConnell and Jessica Tuchman for their warm, sincere, and understanding friendship and for many long hours of discussion on non-academic matters. I would also like to thank and acknowledge Jessica for all the time and effort she spent getting my papers and thesis into understandable and interpretable English.

Most important I would like to thank my wife Judy for her constant loving companionship, for sharing with me all the times things did not go well as well as all the happiness and excitement when they did, for all her understanding of the long nights and weekends in the lab, and for all the love and happiness which keeps me going. I would also like to mention our son Andrew who brings us so much pleasure and happiness and who always manages to find my data sheets and destroy them.

I would also like to acknowledge the National Science Foundation and National Institute of Health for financial support.

ABSTRACT

Part I. The cellular slime mold Dictyostelium discoideum is a simple eukaryote which undergoes a multicellular developmental process. Single cell myxamoebae divide vegetatively in the presence of a food source. When the food is depleted or removed, the cells aggregate, forming a migrating pseudoplasmodium which differentiates into a fruiting body containing stalk and spore cells. I have shown that during the developmental cycle glycogen phosphorylase, aminopeptidase, and alanine transaminase are developmentally regulated, that is their specific activities increased at a specific time in the developmental cycle. Phosphorylase activity is undetectable in developing cells until mid-aggregation whereupon it increases and reaches a maximum at mid-culmination. Thereafter the enzyme disappears. Actinomycin D and cycloheximide studies as well as studies with morphologically aberrant and temporally deranged mutants indicate that prior RNA and concomitant protein synthesis are necessary for the rise and decrease in activity and support the view that the appearance of the enzyme is regulated at the transcriptional level. Aminopeptidase and alanine transaminase increase 3 fold starting at starvation and reach maximum activity at 18 and 5 hours respectively.

The cellular DNAs of D. discoideum were characterized by CsCl buoyant density gradient centrifugation and by renaturation kinetics. Whole cell DNA exhibits three bands

in CsCl: ρ = 1.676 g/cc (nuclear main band), 1.687 (nuclear satellite), and 1.682 (mitochondrial). Reassociation kinetics at a criterion of Tm -23°C indicates that the nuclear reiterated sequences make up 30% of the genome ($Cot_{\frac{1}{2}}$ (pure) 0.28) and the single-copy DNA 70% ($Cot_{\frac{1}{2}}$(pure) 70). The complexity of the nuclear genome is 30 x 10^9 daltons and that of the mitochondrial DNA is 35-40 x 10^6 daltons ($Cot_{\frac{1}{2}}$ 0.15). rRNA cistrons constitute 2.2% of nuclear DNA and have a ρ = 1.682.

RNA extracted from 4 stages during developmental cycle of Dictyostelium was hybridized with purified single-copy nuclear DNA. The hybrids had properties indicative of single-copy DNA-RNA hybrids. These studies indicate that there are, during development, qualitative and quantitative changes in the portion of the single-copy of the genome transcribed. Overall, 56% of the genome is represented by transcripts between the amoeba and mid-culmination stages. Some 19% are sequences which are represented at all stages while 37% of the genome consists of stage specific sequences.

Part II. RNA and protein synthesis and polysome formation were studied during early development of the surf clam Spisula solidissima embryos. The oocyte has a small number of polysomes and a low but measurable rate of protein synthesis (leucine-^{3}H incorporation). After fertilization, there is a continual increase in the percentage of ribosomes sedimenting in the polysome region. Newly synthesized RNA (uridine-5-^{3}H incorporation) was found in polysomes as early as the 2-cell stage. During cleavage, the newly formed RNA is associated mainly with the light polysomes.

RNA extracted from polysomes labeled at the 4-cell stage is polydisperse, nonribosomal, and non-4 S. Actinomycin D causes a reduction of about 30% of the polysomes formed between fertilization and the 16-cell stage.

In the early cleavage stages the light polysomes are mostly affected by actinomycin.

TABLE OF CONTENTS

PART I

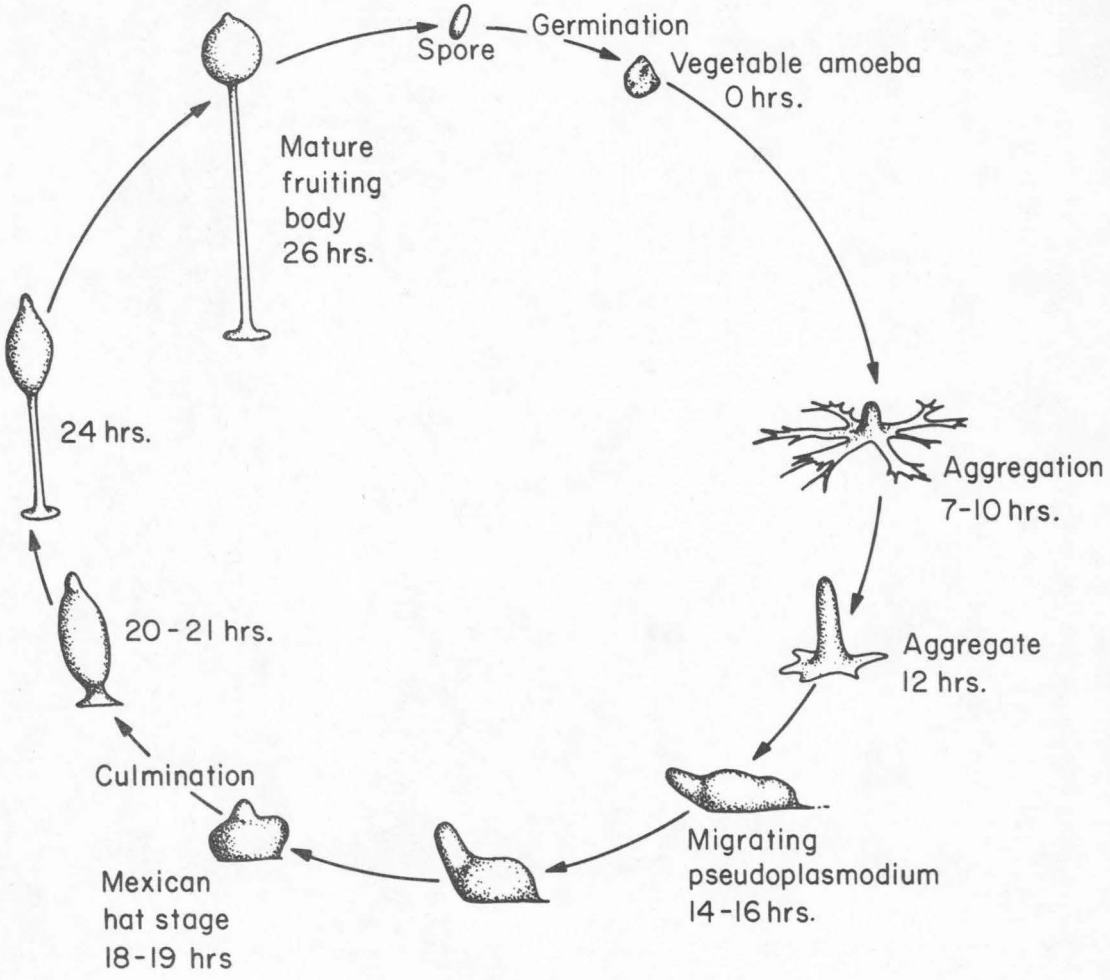
REGULATION OF DEVELOPMENT IN THE CELLULAR SLIME MOLD *DICTYOSTELIUM discoideum*

General Introduction:
A short analysis of *Dictyostelium discoideum* as a developmental system.

The choice of organism is critical to the successful study of development in eukaryotes. The perfect organism, so far undiscovered, would be a very simple eukaryote containing few cell types, easily cultured in the laboratory with a visible and short developmental cycle, accessible to various drugs and biochemicals, and amenable to genetic analysis. With such a creature there should be a method of synchronizing a large population so that any desired amount of material at a given developmental stage could be obtained. To support the assumption that analysis of such a simple organism would lead to an understanding of regulatory mechanisms in higher eukaryotes, one would hope to find that the organization of its genome resembled that of higher organisms. At the present time, the only available clues to this organization are the presence of histones (Elgin, et al., 1971) and possibly the presence of repetitive DNA (Britten and Kohne, 1968; Britten and Davidson, 1969; 1971). It is my feeling that the cellular slime molds, especially Dictyostelium discoideum, fulfill many of these criteria. This introduction is an analysis of Dictyostelium as such an organism.

Dictyostelium can be grown vegetatively as an apparently homogeneous group of cells (see footnote at end) on live or dead non-mucoid bacteria, especially E. coli and Aerobacter aerogenes (Raper, 1935; 1940a; Bonner, 1967; Sussman, 1966). In the presence of food the cells

DEVELOPMENTAL CYCLE OF
DICTYOSTELIUM DISCOIDEUM



continue to divide. When the food is depleted the cells stop dividing (Bonner and Frascella, 1952; Sussman and Sussman, 1960) and undergo a developmental process which under proper laboratory conditions is synchronous between large groups of cells. In this developmental process the cells first aggregate into groups of approximately 10^5 cells. This aggregation is mediated by a chemotactic substance known to be 3'-5'-cyclic AMP, a phosphodiesterase and phosphodiesterase inhibitor (Bonner et al. 1966;Konijn et al.,1967; 1969; Chang, 1968; Konijn, 1969; Riedel and Gerish, 1971; Bonner 1971). The cells then form a migrating pseudoplasmodium which is phototactic, thermotactic, chemotactic, and attracted towards higher humidity (see Bonner, 1967; Bonner, 1971 for a discussion of this). The slug will continue to migrate if it is cultured on a non-buffered surface in the absence of overhead light (Newell et al., 1969b). However, if it is briefly illuminated or transferred to a buffered medium, it will culminate immediately, forming a fruiting body containing two main cell types: vacuolated stalk cells, which die, and spores, in a ratio of approximately 1:2.

During the period between aggregation and culmination two intermediate cell types, pre-stalk cells which are present in the front 1/3 of the slug and pre-spore cells in the back 2/3 of the slug, can be distinguished histologically, morphologically and biochemically (see

footnote at end). The very back tip of the slug represents cells which will differentiate into the base of the stalk and stain like stalk cells (Bonner, 1958; 1967). Pre-spore cells contain cell specific vacuoles which apparently fuse with the pre-spore cell membrane to form the spore cell wall (Hohl and Hamamotot, 1969; Maeda and Takeuchi, 1969). It has recently become possible to isolate, presently only on a small scale, the pre-stalk and pre-spore cells from the pseudoplasmodium and it has also been shown that certain enzyme activities are higher in one of the cell types than in the other (Miller et al., 1969; Newell et al., 1969a).[1] Although the differences between these two intermediate cell types are already pronounced, their developmental fate is not irrevocably fixed. If the pseudoplasmodia are disaggregated and placed in the presence of a food source, the cells will dedifferentiate and develop into vegetative amoebae. If the pseudoplasmodia are cut between the pre-spore and pre-stalk regions and allowed to culminate immediately, the front half develops into a long stalk with very few spores and the back half differentiates into a large sorocarp with very few supporting stalk cells (Raper, 1940b; Bonner, 1967). However, if culmination is prevented and the slugs are allowed to migrate some of the cells will dedifferentiate and form the missing cell type. If subsequently allowed to culminate, these slugs will form

[1]Some of the enzymes are developmentally regulated (see below), indicating a preferential synthesis in one of the cell types.

a normally proportioned fruiting body.

The entire developmental cycle proceeds essentially in the absence of cell division (Bonner and Frascella, 1952; Sussman and Sussman, 1960) eliminating the usual background of metabolic events concerned with DNA replication and cell division. The recent discovery of an axenic medium (Sussman and Sussman, 1967; Ashworth and Watts, 1970; Schwalb and Roth, 1970), in which very large numbers of cells can be grown without bacterial food source, has therefore made it possible to study developmentally controlled changes in nucleic acid metabolism. Prior to this development it was difficult to do molecular biology on RNA and DNA components of Dictyostelium due to constant bacterial contamination. Axenic strains of wild type NC-4 have been obtained which have a division time of 10 hours, grow to a concentration of better than 1.5×10^7 cells/ml, and develop as synchronously as cells grown on bacteria. Recently a more defined media which uses tryptone and a vitamin and mineral mixture instead of protease peptone and yeast extract has been worked out (Ashworth, personal communication). Large numbers of axenically grown cells can be made to develop synchronously on cellulose filters placed on an absorbant pads saturated with buffer. By simply transferring the filters to fresh pads, any desired substance can be added to, or removed from, the system. Developing cells are

permeable to many of the commonly used precursors as well as inhibitors of protein and nucleic and metabolism.

The ability to achieve synchronous development of any desired number of cells allows the study of developmentally regulated changes in the synthesis of a wide variety of soluble proteins, polysaccharides and mucopolysaccharides (see Sussman and Sussman, 1969). Fifteen enzymes are now known to be developmentally regulated in Discoideum discoideum that is they increase in specific activity at a specific stage in the developmental cycle (see Part I, Chapter III, this Thesis). Although open to some criticism, inhibitor studies with actinomycin D and cycloheximide suggest that for many of the enzymes prior RNA and concomitant protein synthesis are necessary for the rise in activity. For two enzymes, N-acetyl glucosaminidase (Loomis, personal communication) and UDPG pyrophosphorylase (M. Sussman, personal communication) radioactive amino acid incorporation studies indicate that the enzymes are being synthesized coincidentally with the period of appearance of enzyme activity. In the case of cellulase (Rosness, 1968) however, the increase in activity of the enzyme in crude extracts of culminating cells appears to be due to the loss of an inhibitor and not to the de novo synthesis of enzymes at that developmental period. For several of the enzymes especially UDPG pyrophosphorylase and alkaline phosphatase, actinomycin D and cycloheximide

studies strongly suggest translational regulation of enzyme synthesis once the putative message has been transcribed (Ashworth and Sussman, 1967; Roth et al., 1968; Loomis, 1969).

The slime mold developmental cycle displays a surprising degree of flexibility. The sequence of events at both the morphological and biochemical levels is normally strictly ordered, giving the impression of a program being read off in a linear, sequential fashion. However, if the program is experimentally interrupted, the cells appear to be able to switch to a different stage of the program and then to proceed without ill effects. As mentioned above, both pre-spore and pre-stalk cells, though well on the way to becoming fully differentiated, can dedifferentiate to amoebae if they are placed in the presence of food or to the other cell type if the slug is cut or dissociated and a delay is imposed before culmination. The homogenous group of cells in a slug fraction will dedifferentiate, redifferentiate and resort themselves eventually forming a normal slug and fruiting body (Raper, 1940b; Bonner, 1967; Francis and O'Day, 1971). It has also recently been shown by Newell and Sussman (1970) that an unusual sequence of molecular events occurs when slugs are transferred from an environment where migration will continue indefinitely to one where culmination is induced. In the former environment, the specific activity of the

developmentally regulated enzyme UDPG pyrophosphorylase slowly rises to the maximum level reached in normal culminating cells while the developmentally controlled synthesis of UDPGal-4-epimerase never occurs. If the cells are then shifted to a culminating environment, the cells synthesize epimerase and initiate a second round of pyrophosphorylase synthesis. They have also shown that these events require RNA synthesis (actinomycin D studies), indicating that slime molds are capable of altering the prescribed developmental program by regulating gene activity depending upon the environmental conditions. It is known that if pseudoplasmodia at the "Mexican hat" stage (18 hrs.) are dissociated into single cells or small clumps of cells, the cells will reassociate and culminate normally with a short lag (Loomis and Sussman, 1966). During the course of the reassociation and subsequent development, at least four enzymes (trehalose-6-phosphate synthetase, UDPGal polysaccharide transferase, the two mentioned above and probably glycogen phosphorylase) undergo a second round of synthesis for which both RNA and protein synthesis is required (Newall and Sussman, personal communication; Part I, Chapter III, this Thesis). Although these phenomena are not yet well understood, it seems highly possible that they will provide an opportunity to study the nature of the control mechanisms which regulate progress through a developmental program.

As Max Delbruck has stated many times and has shown with T4, genetic analysis can be of enormous value in the study of development. The lack of a sexual cycle in the cellular slime mold represents a serious genetic drawback. It has been found that Dictyostelium does have a parasexual cycle (see Pontecorvo for a discussion of parasexual cycles). During aggregation a small fraction of the cells fuse, forming a diploid cell which then can remain stable or lose chromosomes going through aneuploid stages to form haploid cells (Sussman, 1961; Sussman and Sussman, 1962; Shina and Ashworth, 1968; Yanagisawa, Yamada and Hashimoto, 1969; Yamada et al., 1970; Sackin and Ashworth, 1969). Gene Katz (Sussman, personal communication; Lodish, personal communication) has recently been able to demonstrate the existence of at least 5 linkage groups and has found evidence of crossing over between markers in the same linkage groups. During the past several years,3 types of slime mold mutants have become available. The most numerous class consists of mutants which stop development at various times during the developmental cycle. Temporally deranged mutants, which develop at a faster or slower rate than the wild type, (Loomis,1970;Sonneborn et al.,1963) and temperature sensitive mutants in growth and development (Loomis and Ashworth, 1969) now are also available. The existence of a semi-defined medium may enable us to obtain a fourth class of mutants, biochemical auxotrophs, which should

greatly help in the analysis of slime mold genetics. Although the genetic analysis of this organism is only just beginning, the results so far are promising.

I have tried to take advantage of several of the experimental attributes of the cellular slime mold to examine some of the problems related to development. Several new developmentally regulated enzymes involved in polysaccharide and amino acid metabolism were examined, and their relationship to the physiology and developmental changes occurring in slime mold differentiation were analyzed. The appearance and disappearance of glycogen phosphorylase was found to be developmentally regulated. The enzyme reaches maximum activity during midculmination during the period of soluble glycogen degradation (Wright et al., 1968; Sussman and Sussman, 1969; Marshall et al., 1970) and the synthesis of spore and stalk cell polysaccharides (see Sussman and Sussman, 1969). Aminopeptidase and alanine transaminase were also found to be developmentally regulated and reach maximum activity at approximately 18 and 5 hours respectively. These enzymes are thought to be involved with protein degradation and amino acid catabolism during differentiation. The breakdown of amino acids probably provides the major source of energy in developing Dictyostelium cells (Gregg et al., 1954; Gregg and Bronsweig, 1956; Wright, 1964; Sussman and Sussman, 1969).

The availability of axenic strains made it possible to investigate the molecular organization of the slime mold genome, and the changes in its pattern of transcription during development. It has been shown that the genome of Dictyostelium discoideum displays an organization similar to other higher eukaryotes (see Britten and Cohen, 1968; Britten, 1970; Britten and Davidson, 1971 for a review). The nuclear DNA contains,at a criterion of 23^{o} below the Tm, nonribosomal repetitive DNA components which may be analogous to those found in metazoans as well as a large nonrepetitive DNA component. The genome was found to be only 11 times as large as that of E. coli -- extremely small for a eukaryote. The small genome greatly simplifies specific molecular hybridization studies since each nonrepetitive DNA sequence is approximately 100 times more concentrated in Dictyostelium DNA than in mammalian DNA. Of the whole cell DNA, approximately 25-30% was found to be mitochondrial DNA with a molecular complexity of approximately $35\text{-}40 \times 10^{6}$, similar to that found in Paramecium and Tetrahymena (Flavell and Jones, 1970; 1971) and Drosophila, (Botcham, et al., 1971). RNAs from various stages of the developmental cycle were extracted and hybridized with a purified single-copy DNA fraction to analyze the qualitative and quantitative changes in the nonrepetitive portion of the nuclear genome which was being transcribed during the developmental cycle. The

results indicate that at least 56% of the single-copy portion of the genome is being used during the stages of development examined. Of this, approximately 19% of the genome represents sequences which are common to all the stages examined, while approximately 37% represent stage specific sequences which presumably are involved in the differentiation of various intermediate stages. These studies hopefully open the way to further experiments which will bring us closer to an understanding of how a relatively simple eukaryote regulates its differentiation from a single cell type to two different cell types.

Although the results are not discussed in this thesis, it might be interesting to point out some of the work being done by Ken Pischel and myself on the chromatin of Dictyostelium discoideum. We have found that the chromatin has properties very similar to the chromatin of higher eukaryotes. It contains histone-like basic proteins complexed with the DNA, as well as a large variety of nonhistone chromosomal proteins. The melting profile of Dictyostelium chromatin in 0.25 mM EDTA, pH 8, shows a triphasic melting profile with Tms of approximately 51,65, 79°C while native DNA has a Tm of 41°C. under these conditions. These results are similar to those that are found in higher eukaryotes (Li and Bonner, 1971) while yeast chromatin has been found to have only a biphasic melting profile (van der Vliet et al., 1969). These results

suggest that the genome Dictyostelium discoideum may have a very similar organization to that of metazoans and supports the hypothesis that regulatory mechanisms may be very similar.

Footnote

One investigator (Takeuchi, 1969), as yet unconfirmed by others, has reported that preaggregation cells and even logarithmically growing amoebae can be distinguished with respect to whether they will develop into stalk or spore cells. Labeled amoebae from germinated spores when mixed with marked dividing amoebae tend to sort out in the back 2/3 of the slug. If, however, these cells are allowed to divide, they can be separated into two groups of cells: cells which float or sink in a 1.061 gr/cc dextrin solution (150g, 20 min). Takeuchi found that the heavier cells tended to differentiate into spores while the lighter cells tended to develop into stalks. It was shown that the two apparently separable cells types can be derived from a single cell clone. Larger amoebae tend to sort into the anterior of the slug (Bonner, 1959). The apparent difference between the two types may come from unequal cell division (Takeuchi, 1969). There is also some indication that pre-aggregating cells contain variable amounts of cytoplasmic inclusions capable of cross-reacting with

pre-spore antigens (Takeuchi,1963). It is not clear what these results mean with respect to some pre-programming of the development of individual cells. However, it is known that every cell at all stages of development is totipotent and that after cell division any individual amoeba may ultimately be found in either the posterior or anterior portion of the slug. (Bonner, 1971, Takeuchi, 1969). As discussed in the text, it is also clear that individual cells in a slug are capable of redifferentiation without cell division (Gregg, 1965; 1968; Bonner, 1971; Francis and O'Day, 1971).

REFERENCES

Ashworth, J.M. & Sussman, M. (1967). J. Biol. Chem. 242, 1696.

Ashworth, J.M. & Watts, D.J. (1970. Biochem. J. 119,175.

Bonner, J.T. (1958). In The Evolution of Development, Cambridge University Press.

Bonner, J.T. (1959). Proc. Nat. Acad. Sci., Wash. 45, 379.

Bonner, J.T. (1967). The Cellular Slime Molds, 2nd Edition, Princeton University Press.

Bonner, J.T. (1971). Ann. Rev. Microbiol. 25, in press.

Bonner, J.T. & Frascella, E.B. (1952). J. Exptl. Zool. 121, 561.

Bonner, J.T., Kelso, A. & Gillmor, R.G. (1966). Biol. Bull. 130, 28.

Botcham, M., Kram, R., Schmid, C.N. & Hearst, J.E. (1971). In press.

Britten, R.J. (1970). In Problems in Biology: RNA in Development, p. 187, E.W. Hanly, editor.

Britten, R.J. & Davidson, E.H. (1969). Science 165, 349.

Britten, R.J. & Davidson, E.H. (1971). Quart. Rev. Biol. in press.

Britten, R.J. & Kohne, D.E. (1968). Science 161, 529.

Elgin, S.C.R., Froehner, S.C., Smart, J.E. & Bonner, J. (1971). Adv. Cell Mol. Biol. 1, 1, E.J. Du Praw, editor, Academic Press, N.Y.

Flavell, R.A. & Jones, I.G. (1970). Biochem. J. 116, 811.

Flavell, R.A. & Jones, I.G. (1971). Biochim. Biophys. Acta 232, 255.

Francis, D. & O'Day, D.H. (1971). J. Expt. Zool. 176, 265.

Gregg, J.H. (1965). Develop. Biol. 12, 377.

Gregg, J.H. (1968). Exp. Cell Res. 51, 633.

Gregg, J.H. & Bronsweig, R.D. (1956). J. Cell. Comp. Physiol. 48, 293.

Gregg, J.H., Hackney, A.L. & Krivanek, J.O. (1954). Biol. Bull. 107, 226.

Hohl, H.R. & Hamamoto, S.T. (1969). J. Ultrastruct. Res. 26, 442.

Konijn, T.M. (1969). J. Bacteriol. 99, 503.

Konijn, T.M., Chany, Y.Y. & Bonner, J.T. (1969). Nature 224, 1121.

Konijn, T.M., van de Meere, J.G.C., Bonner, J.T. & Barkley, D.S. (1967). Proc. Nat. Acad. Sci., Wash. 58, 1152.

Li, H.J. & Bonner, J. (1971). Biochemistry 10, 1461.

Loomis, W.F., Jr. (1969). J. Bacteriol. 100, 417.

Loomis, W.F., Jr. & Ashworth, J.M. (1969). J. Gen. Microbiol. 53, 181.

Loomis, W.F., Jr. (1970). Exp. Cell Res. 60, 285.

Loomis, W.F., Jr. & Sussman, M. (1966). J. Mol. Biol. 22, 401.

Maeda, Y. & Takeuchi, I. (1969). Devel. Growth Diff. 11,232.

Marshall, R., Sargent, D. & Wright, B.E. (1970). Biochemistry 9, 3087.

Miller, Z.I., Quance, J. & Ashworth, J.M. (1969). Biochem. J. 114, 815.

Newell, P.C., Ellingson, J. & Sussman, M. (1969a). Biochim. Biophys. Acta 177, 610.

Newell, P.C. & Sussman, M. (1970). J. Mol. Biol. 49, 627.

Newell, P.C., Telser, A. & Sussman, M. (1969b). J. Bacteriol. 100, 763.

Pontecorvo, G. (1956). Ann. Rev. Microbiol. 10, 393.

Raper, K.B. (1935). J. Agr. Res. 50, 135.

Raper, K.B. (1940a). Am.J. Botan. 27, 436.

Raper, K.B. (1940b). J. Elisha Mitchell Sci. Soc. 56, 241.

Riedel, V. & Gerish, G. (1971). Biochem. Biophys. Res. Commun. 42, 119.

Rosness, P. (1968). J. Bacteriol. 96, 639.

Roth, R., Ashworth, J.W. & Sussman, J.M. (1968). Proc. Nat. Acad. Sci. Wash. 59, 1235.

Sackin, M.J. & Ashworth, J.M. (1969). J. Gen. Microbiol. 59, 275.

Schwalb, M. & Roth, R. (1970). J. Gen. Microbiol. 60,283.

Sihba, U. & Ashworth, J.M. (1968). Proc. Roy. Soc. London B 173, 531.

Sonneborn, D., White, G. & Sussman, M. (1963). Devel. Biol. 7, 79.

Sussman, M. (1961). In Growth in Living Systems, M.X. Zarrow, editor, Basic Books, Inc.

Sussman, M. (1966). Methods in Cell Physiology, vol 2, p. 397, D. Prescot, editor, Academic Press, Inc. N.Y.

Sussman, M. & Sussman, R.R. (1962). J.Gen. Microbiol. 28, 417.

Sussman, M. & Sussman, R. (1967). Biochem. Biophys. Res. Commun. 29, 34.

Sussman, M. & Sussman, R. (1969). Symp. Soc. Gen. Microbiol. XIX, 403.

Sussman, R.R. & Sussman, M. (1960). J. Gen Microbiol. 23, 287.

Takeuchi, I. (1963). Devel. Biol. 8, 1.

Takeuchi, I. (1969). In Nucleic Acid Metabolism, Cell Differentiation and Cancer Growth. E.V. Cowdry and S. Seno, editors, Pergamon Press, N.Y.

van der Vliet, P. Ch., Tonino, G.J.M. & Rozijn, Th. H. (1969). Biochim. Biophys. Acta. 195, 473.

Wright, B.E. (1964). In Biochemistry and Physiology of Protozoa III, p.341. S.H. Hunter, editor, Academic Press, N.Y.

Wright, B.E., Dahlberg, D. & Ward, C. (1968). Arch. Biochem. Biophys. 124, 380.

Yamada, T., Yanigasawa, K. & Ono, H. (1970). Cell. Slime Mold Bull. No. 3, p.6.

Yanagisawa, K., Yamada, T. & Hashimoto, Y. (1969). Cell. Slime Mold Bull. No. 2, p.3.

CHAPTER I

CHARACTERIZATION OF THE GENOME OF THE CELLULAR SLIME MOLD *DICTYOSTELIUM discoideum*

CHARACTERIZATION OF THE GENOME OF THE CELLULAR SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

Richard A. Firtel* and James Bonner

Division of Biology, California Institute of Technology

Pasadena, California 91109

Running Title: *Dictyostelium* DNA

*Present address: Department of Biology, Massachusetts of Technology, Cambridge, Massachusetts 02139

ABSTRACT

The cellular DNAs of Dictyostelium discoideum have been characterized by their behavior in CsCl buoyant density gradient centrifugation and by their renaturation kinetics. Whole cell DNA exhibited three bands on CsCl density gradient centrifugation. Two of these, of ρ = 1.676 g/cc (main band) and 1.687 (satellite) are nuclear. The third, of ρ=1.682 is mitochondrial and represents approximately 28% of whole cell DNA.

Appropriate hybridization experiments with cytoplasmic rRNA show that rRNA cistrons constitute 2.2% of nuclear DNA. This portion of the nuclear DNA possesses a ρ of approximately 1.682 and bands in the mitochondrial DNA region.

Non-reiterated sequences make up approximately 70% of the Dictyostelium genome (criterion of Tm -23°C) and are characterized by a $Cot_{\frac{1}{2}}$ (pure) of 70. Reiterated sequences make up 30% of the genome and are characterized by a $Cot_{\frac{1}{2}}$ (pure) of 0.28. The complexity of the nuclear DNA of Dictyostelium is but 11 times that of E. coli (approximately 30 x 10^9 daltons). The mitochondrial DNA of Dictyostelium exhibits a $Cot_{\frac{1}{2}}$ of 0.15 and a calculated complexity of 35-40 x 10^6 daltons.

1. Introduction

Dictyostelium discoideum is a useful organism for analysis of the biochemical changes which occur during differentiation. It grows as a homogeneous, vegetative cell population which, upon removal of the food source, differentiates synchronously into two non-dividing cell types, stalk and spore cells. Present evidence suggests that at least 15 enzymes are genetically regulated during the 26 hr of differentiation (see Telser & Sussman, 1971; Loomis, 1970; Firtel & Bonner, 1971, for more on this subject). These enzymes are involved in many biochemical pathways which involve both synthetic and catabolic processes. Developmental time courses have been worked out for the enzymes involved in the pathways for polysaccharide metabolism during culmination and something is now known about the regulation of these enzymes (Newell & Sussman, 1970; Telser & Sussman, 1971; Firtel & Bonner, 1971).

To better understand how the Dictyostelium genome controls development and differentiation it is necessary to analyze the molecular composition of this genome, to discover what extent it is transcribed in vivo, and to elucidate the quantitative and qualitative changes in the sequences used during differentiation. This paper concerns the structure of the Dictyostelium genome. It shows

below that the DNA of the cellular slime mold Dictyostelium discoideum contains single-copy (unique, nonrepetitive) DNA and repetitive nuclear DNA components (by the renaturation criterion used), as well as mitochondrial DNA. The complexities of these components have also been determined. The total amount of nuclear DNA per genome in Dictyostelium as determined by renaturation experiments is approximately 11 times that present in E. coli.

In another paper (Firtel, 1971), the amount of, and changes in, the nonrepetitive DNA present as transcripts in the various stages of the developmental cycle of Dictyostelium has been measured.

2. Materials and Methods

(a) Culturing of cells

The axenic strain of Dictyostelium discoideum (AX-3) used for the experiments was derived from Dictyostelium discoideum NC4 (Raper, 1935, 1940) by Dr. William F. Loomis, Jr. (University of California, San Diego). The development of this strain of Dictyostelium is similar to that of wild type NC-4 (see Bonner, 1967). AX-3 was grown on HL-5 media (Cocucci & Sussman, 1970; Watts and Ashworth, 1970) containing per liter: 10 g glucose, 10 g proteose peptone, 5 g yeast extract, 0.25 g Na_2HPO_4, and 0.4 g KH_2PO_4, adjusted to pH 6.5 with H_3PO_4. The cultures were

grown at 22°C on an obliquely rotating shaker at 150 rev./min. During logarithmic growth the generation time of the cells is 8-12 hr. Cells are capable of growing in log phase to approximately 1-1.7 x 10^7 cells/ml. and reach concentrations of 1.5-2.5 x 10^7 cells/ml. at stationary phase.

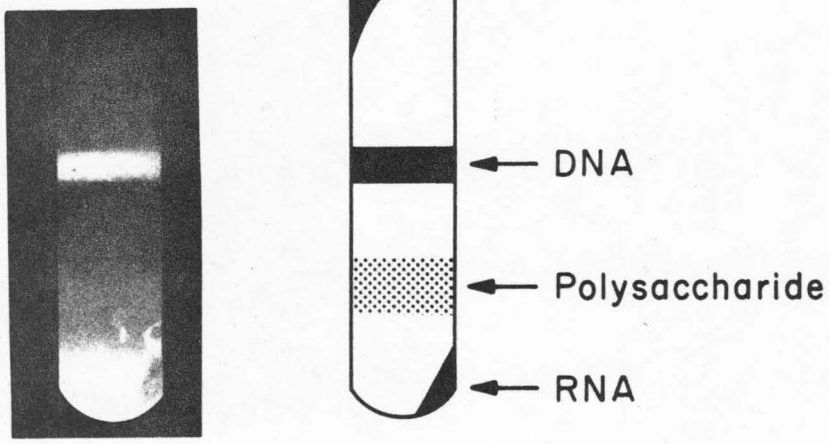
(b) Isolation of whole cell DNA

Cells were harvested by centrifugation at 3000 rev./min in a Servall SS-34 rotor and washed 2-3 times in ice-cold 7% sucrose. 50 ml. of 0.1 M EDTA pH 8 was added per 2.5 x 10^{10} cells and 15 ml. of 20% sodium N-lauryl sarcosine (Sigma Chemical Co.) was added and the solution stirred at room temperature until all cells were lysed. Solid CsCl (Rare Earth Div., American Potash & Chemical Corp.) was added to 0.27 g/ml. and the mixture heated at 60° C for 5-10 min. The heat treatment is necessary for high yields and probably helps both by destroying nucleases and by liberating the DNA from proteins. Solid CsCl (0.7 g/ml. of original volume) was then added to final concentration of 0.97 g/ml. ($\rho \approx 1.55$). After further cooling, 1/20th volumes of a 10 mg/ml. solution of ethidium bromide (Calbiochem) in water was added. The preparation was then centrifuged at 40,000 rev./min for 48 hr in a Spinco Ti 50 rotor. Figure 1 shows a picture of the resulting CsCl gradient; a red protein skin, a red DNA band, a white opalescent polysaccharide band, and

Fig. 1. Preparative CsCl gradient of lysed Dictyostelium cells in the presence of ethidium bromide.

Left: Photograph of gradient in near UV light showing fluorescent DNA band and RNA pellet.

Right: Drawing of all visible components and their location in the gradient.


Protein skin
DNA
Polysaccharide
RNA

a red RNA pellet. In this procedure, the ethidium serves two purposes. The first is that it causes the DNA to band at a position separate from that of the polysaccharide which has a density of approximately 1.63 g/ml. The DNA can therefore be recovered free of polysaccharide. The second is that since the DNA is red it can be visualized and removed easily with a Pasteur pipette. This eliminates the need of determining the position of the DNA by determination of the optical density of individual fractions. The DNA band is removed with a Pasteur pipette and rebanded at a concentration of approximately 0.5 mg/10 ml. tube. After the second centrifugation, the band is removed, diluted with 3 volumes of water, and extracted 2 times with an equal volume of isopentyl alcohol to extract the bound ethidium bromide from the DNA. Two volumes of 2-ethoxyethanol are added and the DNA is wound out, dissolved, and dialyzed against 1 M NaCl, 0.01 M EDTA, pH 8, for several changes over a time period of approximately a day and a half. It is then dialyzed against 0.12 M phosphate buffer (equimolar in N_2HPO_4 and NaH_2PO_4) for at least two changes. The DNA is then dialyzed against the storage buffer, 0.0012 M PB. The DNA has been found to be free of RNA, polysaccharide and protein. The yield is greater than 95% of the DNA in the cell as determined by the amount of DNA label extracted from ^{3}H-thymidine-labeled cells. The total amount of DNA label in the cells was determined by a modification of

Ts'o & Sato's (1959) modifications of the Schmidt & Tannhauser (1943) procedure.

(c) Chemical analysis of Dictyostelium DNA

The optical spectrum of the DNA in 0.01 M equimolar Na_2H:NaH_2 phosphate buffer (PB) shows an A_{260}:A_{280} ratio of 1.90-1.95 and an A_{260}:A_{230} ratio of 2.35-2.5. By chemical analysis it contains less than 1% protein (Lowry positive material) and no detectable RNA by orcinol reaction. No polysaccharide was detectable as material banding at 1.63 to 1.65 g/cc in CsCl. The hyperchromicity upon heating to 95°C is 41-42% in 0.12 M phosphate buffer.

(d) Purification of mitochondria and extraction of DNA from purified mitochondria

Washed Dictyostelium discoideum cells were homogenized in 5 volumes of 0.1 M sucrose, 0.2 M mannitol, 0.001 M NaEDTA, 0.01 M Tris, pH 8,(SMET), in a Brinkman Polytron homogenizer at a power of 1 for 1-5 min, depending upon the sensitivity of the cells to lysis. The cells were examined by phase microscopy and were rehomogenized if over 90% of the cells were not broken. The homogenate was then centrifuged at 2500 rev./min for 10 min in an International centrifuge, supernatant removed and recentrifuged at 3000 rev./min for 10 min. The supernatant was then centrifuged at 12,000 rev./min for 15 min in a GSA head of a Servall centrifuge, suspended, homogenized and recentrifuged 2 times in the SMET buffer at 17,000 rev./min

in a Servall SS-34 rotor. The resulting mitochondrial pellet was suspended, homogenized gently, and centrifuged at 3000 rev./min for 10 min. The pellet was discarded and the supernatant then centrifuged at 15,000 rev./min for 15 min. The pellet was suspended in TNM buffer (0.01 M MgCl, 0.01 NaCl, 0.01 M Tris, pH 7.6) containing 0.2 M mannitol. DNase I (Worthington, RNase free, electrophoretically purified) was added (20 µg/ml.) and allowed to incubate for 30 min at 4°C. The material was then centrifuged at 15,000 rev./min for 15 min. The pellet was suspended in SMET buffer and layered over 30 ml. of a 1.0 M sucrose solution in SMET buffer which was in turn layered over 20 ml. of 1.7 M sucrose solution in the same buffer. The top interface was stirred slightly and the whole then placed in a Spinco SW-25.2 rotor and centrifuged for 2 hr at 25,000 rev./min. The mitochondrial band was found at the 1.0-1.7 M sucrose interface. The band was then extracted for DNA by the method described for whole cell DNA.

(e) Nuclear purification

Nuclei were purified by a modification of the method of Cocucci & Sussman (1970). Cells were suspended in modified HKM buffer (0.02 M MgCl, 0.01 M KCl, 0.02 M HEPES, 5% sucrose, pH 7.5, adjusted with ammonium hydroxide) to a concentration of 2×10^8 cells/ml. One tenth volume of 15% Nonidet P-40 (Shell Chemical Co.), a non-ionic detergent, was added with shaking. The cells were then passed

twice through a 20 gauge hypodermic needle, and then centrifuged at 3000 rev./min for 10 min in a Servall centrifuge (SS-34 rotor). The small white pellet was resuspended in homogenizing media containing 12% sucrose and made 1% with Nonidet, vortexed, and recentrifuged. The crude nuclear pellet contained no whole cells (<0.1%), little cell debris or cell ghosts, and consisted principally of nuclei. For purer nuclei, the pellet was suspended in HKM buffer and layered over 2.3 M sucrose containing 0.04 M MgCl, 0.02 M KCl, 0.03 M HEPES, pH 7.5. The interface was stirred to approximately one-half way down the tube and the material centrifuged for 2.5 hr at 25,000 rev./min in an SW-25.1 rotor. The nuclei pelleted through the 2.3 M sucrose and was termed purified nuclei. Under oil immersion phase optics nuclei only could be detected. They appeared free of mitochondrial contamination.

(f) Analytical CsCl centrifugation

Isopycnic CsCl centrifugation was performed by standard techniques in the Spinco Model E ultracentrifuge equipped with a scanner. The densities were determined using M. lysodeikticus as a marker at a density of 1.725 g/cc. Optical grade CsCl was used. Most of the runs were performed by Mr. Cameron Schlehuber.

(g) RNA-DNA hybridization

Filter RNA-DNA hybridization of ribosomal RNA (rRNA)

was performed in 50% formamide, 5X SSC (SSC is 0.15 M NaCl, 0.015 M Na_3 citrate) at 40°C (Gillespie & Spiegelman, 1965; McConaughy et al., 1969). Heat denatured DNA was applied to nitrocellulose filters (Schleicher & Schuell, Keene , New Hampshire, B6 variety) in 6X SSC. At the end of the hybridization reaction the filters were washed and treated with ribonuclease (RNase) A (Sigma) at 50 ug/ml. for 30 min at room temperature in 2X SSC. The RNase had been pretreated at 85°C for 20 min. The filters were then washed to remove any unbound RNA and counted in a liquid scintillation system. The DNA retention was monitored using labeled DNA. It was always greater than 70%.

rRNA-DNA hybridization kinetics were carried out in solution using 50,000 lb/sq in sheared DNA (see next section) in 0.24 M phosphate buffer at 66°C. The reaction was stopped by plunging the reacting solution into ice water. The reaction mixture was then treated with 20 ug RNase A/ml. and 50 units/ml. of T1 RNase for approximately 20 min. The material was next applied to hydroxyapatite (HAP), (Bio-Gel HT hydroxyapatite in 0.001 M phosphate buffer, Bio-Rad, equilibrated in 0.12 M phosphate buffer at 60°C), washed with 0.12 M phosphate buffer until all unhybridized RNA was removed and then eluted with 0.48 M phosphate buffer to remove the hybrid. In all cases the phosphate buffer was equimolar mono- and dibasic sodium

phosphate and contained 0.2% SDS. It has also been shown by other workers that well-matched RNA-DNA hybrids can be analyzed just as can DNA-DNA duplexes on HAP (Kohne, 1968; Gelderman et al., 1969, 1971; Davidson & Hough, 1969, 1971).

(h) DNA-DNA optical melting and reassociation profiles

DNA melting profiles and DNA-DNA renaturation kinetics were done in a continuous recording Gilford 2000 or 2400 spectrophotometer with an automatic zero to correct for drift. At all times a cell containing material of known optical density or a metal screen optical density standard was used to test for machine drift. The melting profiles were performed in various concentrations of equimolar (Na_2HPO_4:NaH_2PO_4) phosphate buffer (PB), (pH 6.8) as indicated for each experiment. Standard phosphate buffer is 0.12 M phosphate buffer and represents approximately the same sodium ion concentration (0.18 M Na^+) as 1X SSC. The Gilford spectrophotometer was set to continuously record the change in optical density as well as change in temperature. The rate of temperature increase can be programmed and was linear over the period examined.

DNA was sheared at 50,000 lb/sq in. either in a Servall Ribi Refrigerated French press to a weight average size of about 300-400 nucleotides or at the same pressure to 400-500 nucleotides by Dr. Roy Britten. The molecular

weight of the sheared DNA was determined both by alkaline sedimentation velocity centrifugation and by its contour length by electron microscopy (samples mounted from formation). After shearing, the DNA was filtered, dialysed extensively against 1 M NaCl in 0.01 M EDTA, pH 8, and precipitated with 2 volumes of ethanol. It was dissolved in 0.03 M PB and passed through a HAP column at 60°C and washed. The DNA was passed through Chelex 100 (Bio-Rad) (equilibrated in 0.12 M PB) (Davidson & Hough, 1971), dialyzed against water and lyophilized.

Cuvettes containing DNA samples for melting and renaturation studies were placed under a partial vacuum to remove air dissolved in the sample. Each sample was then covered with silicone oil and a glass-stopper placed on the cuvette to prevent evaporation.

DNA melting profiles of native and renatured DNA samples were made with a linear temperature programmer which increases the temperature of the cuvette at a constant rate. The Gilford spectrophotometer automatically records change in hyperchromicity and temperature of the sample with time. For renaturation profiles the DNA sample was placed in the Gilford and heated to 98° and this value marked as the initial value A_{260} at zero time. The sample was either maintained at 98°C while the Gilford was rapidly cooled to the renaturation temperature or placed at 60°C. In this case samples were then heated to

98°C for approximately 5 to 10 min and then rapidly placed into the constant temperature chamber of the Gilford spectrophotometer (kept at the appropriate renaturation temperature by Haake water bath). All samples were melted after renaturation to measure the T_m and to assure that the Gilford reading of the denatured DNA was the same as the initial measurement.

Renaturation profiles were obtained at various phosphate buffer concentrations as described in the Results section.

The fraction of DNA renatured at time t, is plotted vs. the log of the equivalent Cot of the DNA solution. Cot is an abbreviation for initial DNA concentration (in moles nucleotide/liter) x time of annealing in seconds (Britten and Kohne, 1967). The equivalent Cot is the Cot value multiplied by a salt correction factor (Britten & Kohne, 1967; Britten & Smith, 1970) which corrects the rate of reassociation at a given Na^+ concentration to that of the standard condition 0.18 M Na^+ (0.12 M PB). The amount of DNA renatured at any particular time is calculated from the hyperchromicity determined for each fraction by melting it when it was at least 97% (calculated) renatured (further incubation showed no further decrease in optical density) rather than by comparison with the hyperchromicity of unsheared native DNA. The value for the hyperchromicity agreed with the value calculated from

computer analysis of the renaturation profile (see Results section). In all cases the hyperchromicity of renatured DNA was less than that of native unsheared DNA.

(i) Hydroxyapatite renaturation profile

In addition to optical measurements, DNA-DNA renaturation was also analyzed by hydroxyapatite chromatography as described by Britten & Kohne (1967, 1968a). DNA samples in phosphate buffer were placed in conical Reactivial glass vials (Pierce Chemical Co.) which can be tightly sealed by a Teflon lined screw cap top. Samples were heated at 98°C for 5-10 min and immediately placed in a water bath 22-25° below T_m depending upon the experiment. After the appropriate renaturation time the sample vial was removed and plunged immediately into ice water to stop the reaction. The sample was then diluted to 0.12 M PB and applied to a hydroxyapatite column jacketed at 60°C and previously equilibrated with 0.12 M PB. The column was washed with 0.12 M PB to remove unrenatured DNA. The renatured DNA was then eluted with 0.48 M PB. To do a melting profile on hydroxyapatite, the sample was applied and washed with 0.12 M PB. The temperature was then raised in increments of 4 or 5°, and after about 5 min equilibration at each temperature the column was washed with 2-3 volumes of 0.12 M PB. The column was then raised another temperature increment and rewashed. The washing in 0.12 M PB after the temperature

rise removed DNA which had become single stranded due to the rise in temperature. In all cases hydroxyapatite renaturation experiments were done using DNA labeled with ^{32}P. Thus optical densities did not have to be measured and the samples could be counted directly in a liquid scintillation system to determine the amounts of denatured and of renatured DNA. In all cases the PB used contained 0.2% SDS.

To determine the background binding of the hydroxyapatite was, a dilute DNA solution, approximately 2 µg/ml. in 0.12 M PB, was heated for 10 min at 95°C in a water bath, and immediately plunged into an ethanol-dry ice mixture and slowly brought up to room temperature. It was then diluted, applied to a HAP column at 60°C, and immediately washed with 0.12 M PB. The amount of DNA bound is taken as that nonspecifically bound at zero Cot. Under these conditions approximately 0.5% of the total DNA bound to the column.

(j) Labeling of cells

To label cells with $^{32}PO_4$, they were grown to mid-log phase in HL5 medium lacking added phosphate buffer, adjusted to pH 6.4 with HCl. Approximately 80 mC of carrier-free $^{32}PO_4$ per liter of medium were then added and the cells were allowed to grow to late log phase. The DNA was then extracted as described above. Purified DNA extracted from cells labeled with 80 mC/L had a specific

activity of approximately 100,000-120,000 cts/µg. Specific activity was measured after all purification procedures had been completed.

(k) Ethidium bromide treatment

Ethidium bromide (Calbiochem) was added to an axenic culture at various concentrations during different stages of cell growth as described in the text. All experiments in which ethidium bromide was used were performed in darkness.

(l) Labeling and extraction of cytoplasmic (nonmitochondrial) ribosomal RNA

One liter of HL5 medium containing no added phosphate was inoculated with a 1/200th volume of a late log phase culture. To label ribosomal RNA, 40 µC/ml. carrier-free $^{32}PO_4$ was added for three generations. Cells were then harvested, washed once with HL5 containing unlabeled phosphate and then cultured in such medium for 1.5 generations. In other experiments the cells were labeled in the presence of 50 µg/ml. of ethidium bromide which inhibits most of the mitochondrial RNA synthesis (Firtel, unpublished results).

Cells were harvested, washed 3 times in 7% sucrose, and lysed with a Dounce homogenizer in HKM buffer containing 50 µg/ml. polyvinyl sulfate. The 25,000 g supernatant was treated with 0.5% Triton X-100 for 5 min at 4°C and layered over 5 ml of a 1 M sucrose solution (HKM buffer) which was underlayered with 2 ml. of 1.7 M

sucrose (HKM buffer). It was next centrifuged for 3 hr at 40,000 rev./min in a Spinco SW-41 rotor. The material present at the 1.7 M sucrose cushion was then collected, made 0.4 M in Na acetate (NaAc) and precipitated with 3 volumes of ethanol. The pellet was collected by centrifugation and suspended in 0.4 M NaAc containing 1% SDS and extracted 3 times at 4°C with redistilled phenol saturated with 0.2 M sodium acetate, pH 5. The supernatant was then precipitated for 8 hr at 4°C with an equal volume of Millipore filtered 4 M lithium chloride. The pellet was collected by centrifugation and dissolved in 0.01 M EDTA, pH 8, and reprecipitated with 2 M lithium chloride. The pellet was redissolved in 0.4 M KAc, pH 6, and precipitated 2 times with 2.5 volumes of 100% ethanol. The pellet was resuspended in 1 mM Na_2 EDTA and centrifuged on a 5-20% sucrose gradient (0.1 M NaCl, 0.01 NaAc, pH 6). Both peaks (17 and 26 S) were collected and precipitated with 2.5 volumes of 100% ethanol. The material was then used directly or dialyzed against water and lyophilized.

3. Results

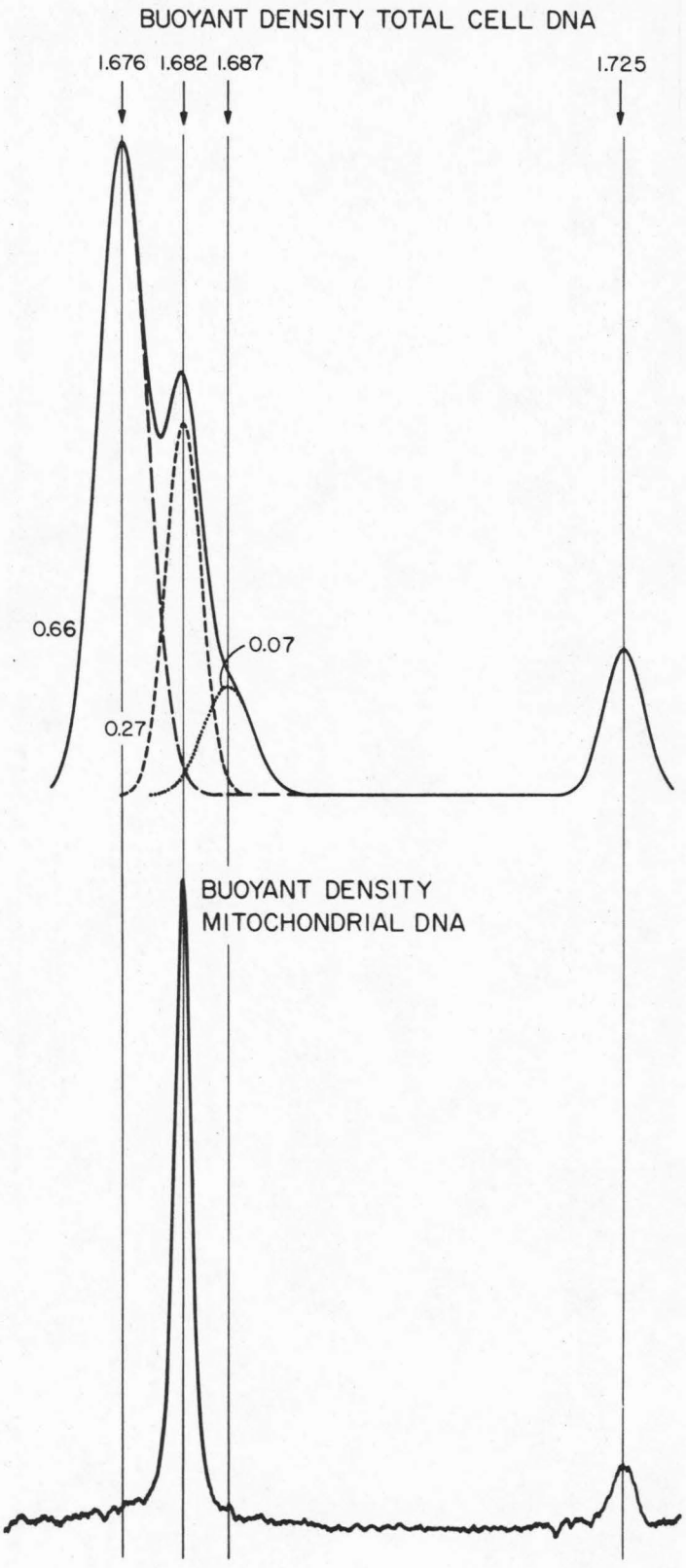
(a) Buoyant density analysis of cellular DNA components

DNA isolated from various cell fractions was analyzed by analytical ultracentrifugation with the results shown in Figure 2. Analysis of the number of components which

Fig. 2. Analytical buoyant density analysis of whole cell and mitochondrial DNA.

(Upper). Computer analysis of X-Y plotter scan of analytical centrifugation run of total cell DNA. Solid line represents optical density profile. Open lines represent location and size of individual components suggested by computer analysis.

(Lower). X-Y plotter scan of analytical run of mitochondrial DNA. The band at ρ = 1.725 g/cc is the marker DNA, M. lysodeiktious.


BUOYANT DENSITY TOTAL CELL DNA
1.676
1.682
1.687
1.725
0.66
0.07
0.27
BUOYANT DENSITY
MITOCHONDRIAL DNA

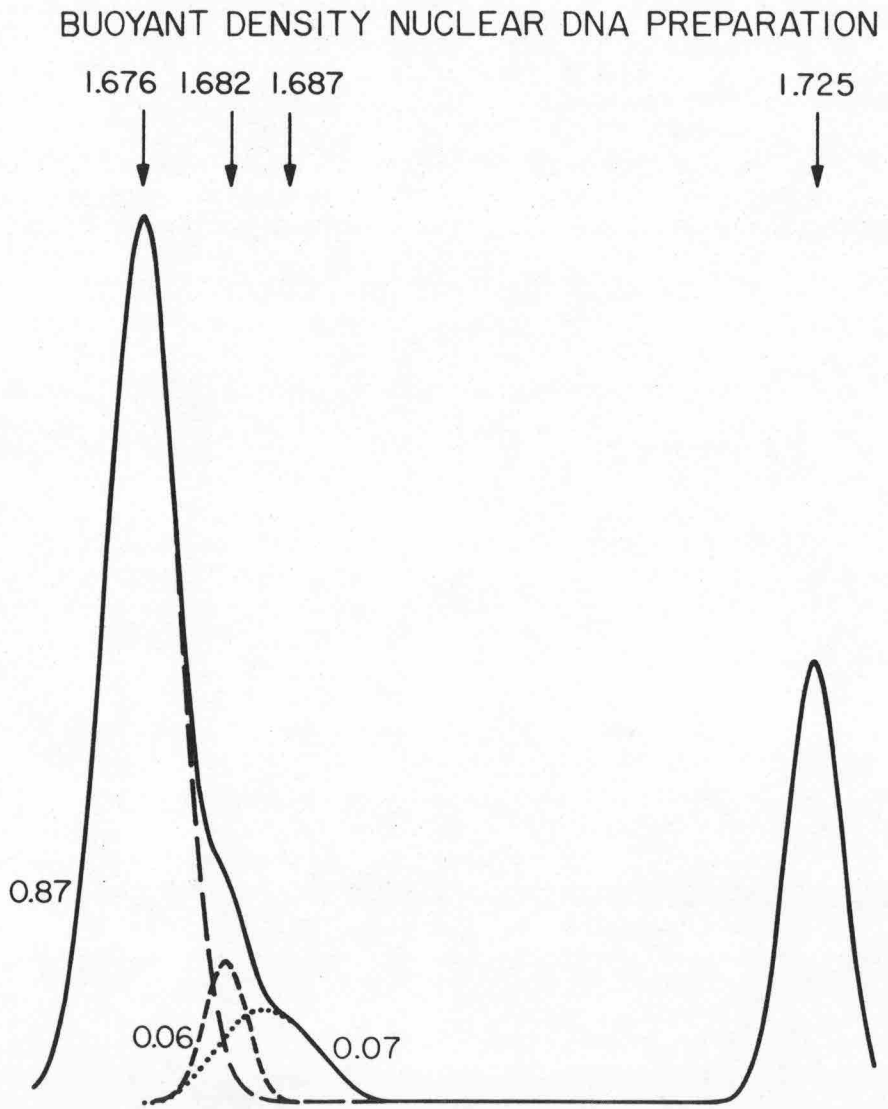
make up the total curve and their buoyant densities was done by a computer Gaussian curve fitting program. In over 90% of all runs, the whole cell DNA exhibited three detectable peaks with densities of 1.676, 1.682, and 1.687, corresponding to 0.23, 0.28, 0.33 Mole fraction G + C (Schildkraut et al., 1962). Under the conditions used, the polysaccharide has a density of approximately 1.63 to 1.64.

Figure 3 shows buoyant density analysis of the DNA of purified nuclei. The majority of the second peak (ρ = 1.682) is removed by nuclear purification, thus suggesting that it is cytoplasmic. To further study this matter, DNA was isolated from mitochondria and analyzed by analytical centrifugation. As shown in Figure 2, mitochondria DNA bands as a single sharp peak with a density of 1.682, identical to the density of peak 2 of whole cell DNA.

It is clear in Fig 2 that the band width of the GC rich 1.687 g/cc peak is greater than that of the other two bands. One would suppose that if this material is nuclear in origin that it should possess approximately the same molecular weight as the main nuclear component. The increased band width may be due to: (1) heterogeneity in GC content of the component(s) in the peak, or (2) to the addition by the computer of low molecular weight fragments of the whole DNA preparation to the high weight

Fig. 3. Computer analysis of analytical buoyant density centrifugation of nuclear DNA.

Growth conditions: 1 liter of media in 2.8 l Fernback flasks shaking at 150 rev./min, 22°C. Under these conditions the division time is approximately 9 hr.


BUOYANT DENSITY NUCLEAR DNA PREPARATION
1.676
1.682
1.687
1.725
0.87
0.06
0.07

GC rich component which bands in the same region. We feel that the second possibility is the correct one and may account also for some of the variability in the quantity of material found in the heavy band. These considerations may lead to overestimation of the amount of the heavy satellite. In fact in a few preparations which contained little low molecular weight material, the proportion of DNA in the GC rich peak was approximately 5% of the total.

(b) Thermal denaturation profiles

Melting profiles were made on whole cell DNA, nuclear DNA and mitochondrial DNA (see Fig. 15). Nuclear DNA has a Tm of approximately 78.5°C, mitochondrial DNA a Tm of 80°C, while total cell DNA has a Tm of approximately 79°C. This corresponds to G + C content of 23% for nuclear DNA and 26% for the mitochondrial DNA (Marmur & Doty, 1962). These values agree well with those obtained from buoyant density analysis.

The hyperchromicity of nuclear DNA was 43-44%, of whole cell DNA 41-42%, and of mitochondrial DNA 35%. The dispersion for the DNA's at $\sigma_{2/3}$ (Doty et al., 1959; Mahler & Dutton, 1964) was 6°, 7°, and 4°C respectively. The lower hyperchromicity and higher dispersion of whole cell DNA is expected since it consists of a mixture of nuclear and mitochondrial components. A disperion of 6°C indicates some heterogeneity, part of which may be due to the two denser satellites found in the nuclear

preparations (see Fig. 3).

(c) Dictyostelium mitochondrial DNA

Under the growth conditions employed for the experiments described in Figures 2 and 3 (division time 10-12 hr), mitochondrial DNA constitutes a relatively constant fraction of whole cell DNA. If the cells are grown with more aeration (faster shaking speed), a faster growing strain is selected with a generation time of 8 hr. After two-three weeks of culturing under these conditions, mitochondrial DNA makes up 40-50% of whole cell DNA. Such cells normally do not undergo synchronous development and often do not culminate.

It is apparently not readily possible to separate the two complementary strands of Dictyostelium mitochondrial DNA. Mitochondrial DNA was denatured in K_3PO_4-KOH buffer, pH 12.8, and examined by analytical CsCl equilibrium centrifugation. The denatured DNA is more dense and yields a broader band than does the native. There is no separation observed with respect to buoyant density of the two strands of mitochondrial DNA.

To try to discover whether or not Dictyostelium mitochondria have closed circular DNA, experiments were performed in which total cells were extracted for DNA using the method described with and without heat treatment. Experiments were also performed in which a crude 20,000 g pellet of the 1000 g supernatant of homogenized cells was

extracted for DNA. In all cases tested there was no evidence of bands of lower density in cesium chloride gradients saturated at 30 ug/ml. with ethidium bromide such as would indicate that closed circular DNA was present (Radloff, et al., 1967). Such circular DNA may be present but extensively nicked by endogenous nucleases during preparation.

Since ethidium bromide is known to inhibit mitochondrial DNA synthesis (as well as cell growth) in mammalian cells (Smith et al., 1971) and in the acellular slime mold Physarum polycephalum (Horwitz & Holt, 1971), the effect of ethidium bromide on cell growth and mitochondrial DNA synthesis of Dictyostelium was examined. The data of Figure 4 show the effect on cell growth of various concentrations of ethidium bromide added in early log phase. Even at the lowest concentration used (5 ug/ml) ethidium bromide drastically reduced the rate of cell division. Figure 5 shows the buoyant density analysis of whole cell DNA after treatment with ethidium bromide. It is clear that the amount of mitochondrial DNA per nuclear genome is decreased from 28% to approximately 7-8%. Mitochondrial DNA synthesis is probably being inhibited by ethidium bromide (see below). As the cells divide there is presumably a dilution of mitochondria until only a small number are present in each cell. Since the cells are obligatory aerobes (Gregg, 1950; Gerish, 1962), growth probably stops

Fig. 4. Growth curves of AX-3 in presence of various concentrations of ethidium bromide.

500 ml. flasks containing 125 ml. media were shaken at 150 rev./min, 22°C. Various concentrations of ethidium bromide were added in early log phase. Division time of the control cells during logarithmic growth was approximately 12 hr.

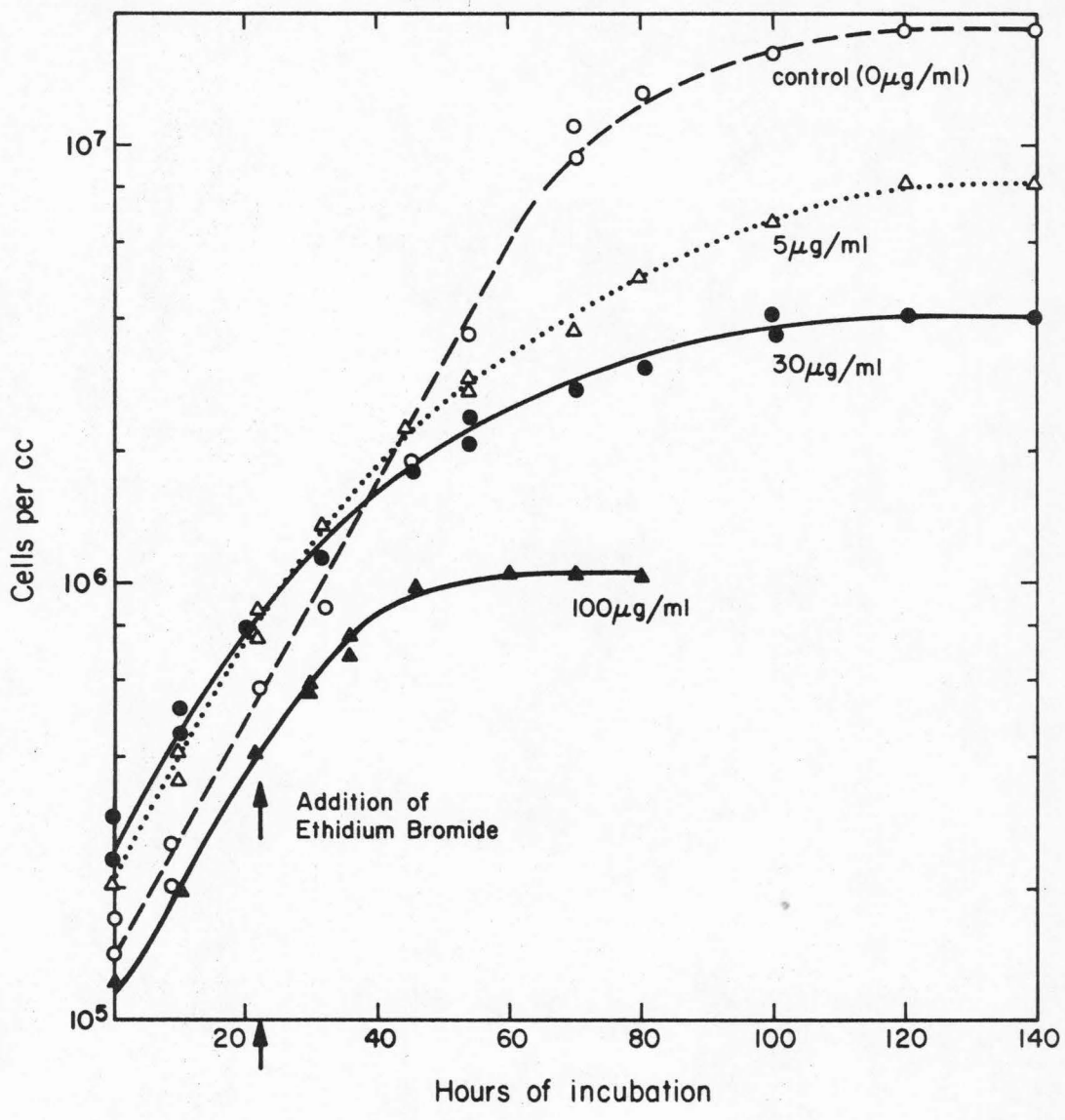
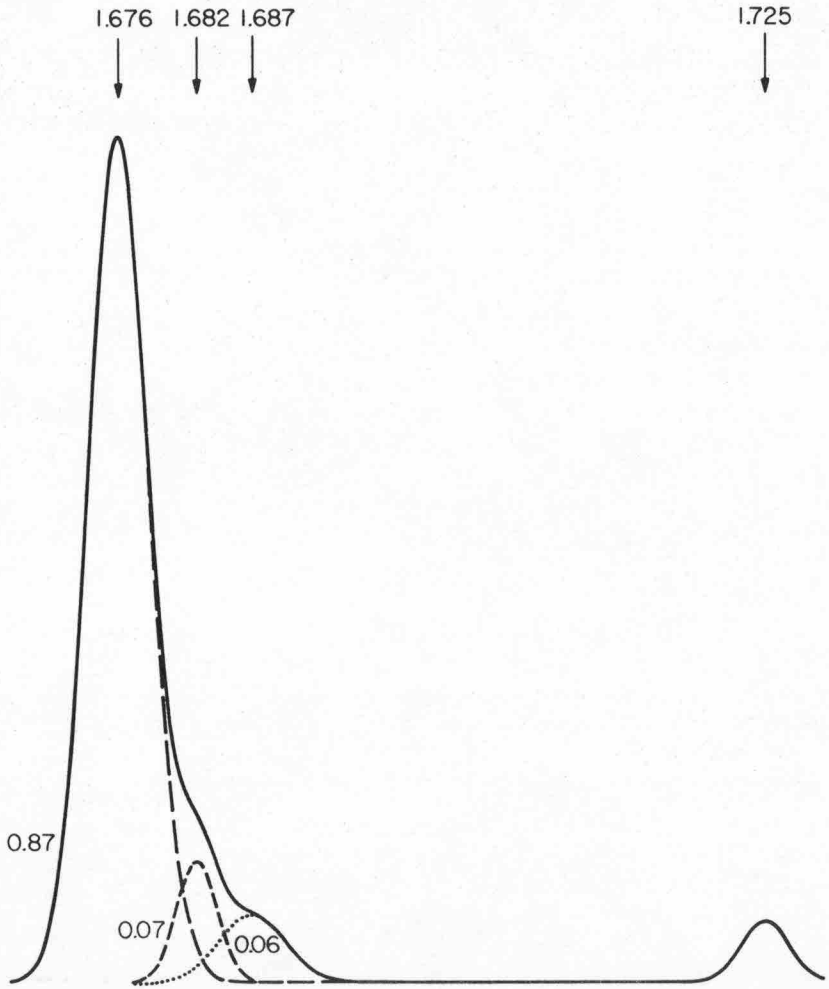

control (0μg/ml)
5μg/ml
30μg/ml
100μg/ml
Addition of
Ethidium Bromide
Cells per cc
10⁷
10⁶
10⁵
20
40
60
80
100
120
140
Hours of incubation

Fig. 5. Computer analysis of DNA from cells treated with ethidium bromide.

Early log phase cells were treated for 3 days with 70 µg/ml ethidium bromide. Growth conditions were same as for Fig. 3.

BUOYANT DENSITY DNA FROM
ETHIDIUM BROMIDE TREATED CELLS



when the number of mitochondria per cell reaches the minimum capable of sustaining cell metabolism.

The proportion of mitochondrial DNA (DNA banding in peak 2) per nuclear genome (DNA banding in peak 1) depends on the time during the growth cycle the drug is given, on the length of the culture period, and on the concentration of the drug. The value 7-8% of total DNA in band 2 in the experiment of Figure 5 is the smallest value obtained but was reproducible for these conditions. Cells treated for the same period of time during late log phase showed an average of 13-15% of total DNA in peak II.

As can be seen by comparison of the DNA extracted from purified nuclei (see Fig. 3) with that of DNA from ethidium bromide-treated cells, the amount of the denser "GC rich" satellite (33% GC) remains constant relative to the main nuclear band. We therefore assume that the heavy satellite is a nuclear component.

To determine the effect of ethidium bromide directly on mitochondrial DNA synthesis, the following experiments were performed. First cells labeled with ^{3}H-thymidine, (5μC/ml.) for several generations were divided into two aliquots. One aliquot was allowed to continue growing undisturbed while 70 μg/ml. of ethidium bromide was added to the other. After 5 hr, $^{32}PO_4$ (5 μC/ml.) was added to each flask and the cells harvested after 6-8 hr. Preparative cesium chloride buoyant density centrifugation runs

were made of the purified DNA of each aliquot and the ratios of $^{32}P:^{3}H$ across the DNA band calculated. As shown in Figure 6, the ratio of $^{32}P:^{3}H$ is constant for cells grown in the absence of ethidium bromide. The DNA of cells treated with ethidium bromide show a lower $^{32}P:^{3}H$ ratio on the dense side of the main band, the region in which mitochondrial DNA bands. This experiment suggests directly that mitochondria cannot efficiently incorporate $^{32}PO_4$ into DNA in the presence of ethidium bromide.

In a second experiment (Fig. 7), the cells were labeled and treated as just described, but in this case, a cell brei was fractionated into a crude nuclear pellet and a supernatant fraction containing the mitochondria (see legend to Fig. 7). Preparative CsCl gradients were run on DNA extracted from the two cell fractions from both ethidium bromide treated and untreated cells. In the controls (not shown) the $^{3}H:^{32}P$ ratio was identical in nuclear and mitochondrial fractions. In the case of the DNA from treated cells an increased $^{3}H:^{32}P$ ratio was found in the supernatant fraction and the ^{32}P peak was skewed to the light side of the gradient as would be expected if nuclear main band DNA was preferentially labeled. Analytical centrifugation of the supernatant DNA indicated it contained ca. 70-80% mitochondrial DNA and 20-30% nuclear contaminants, owing to nuclear breakage in the homogenization procedure. In summary, these experiments

Fig. 6. Effect of ethidium bromide on synthesis of mitochondrial DNA.

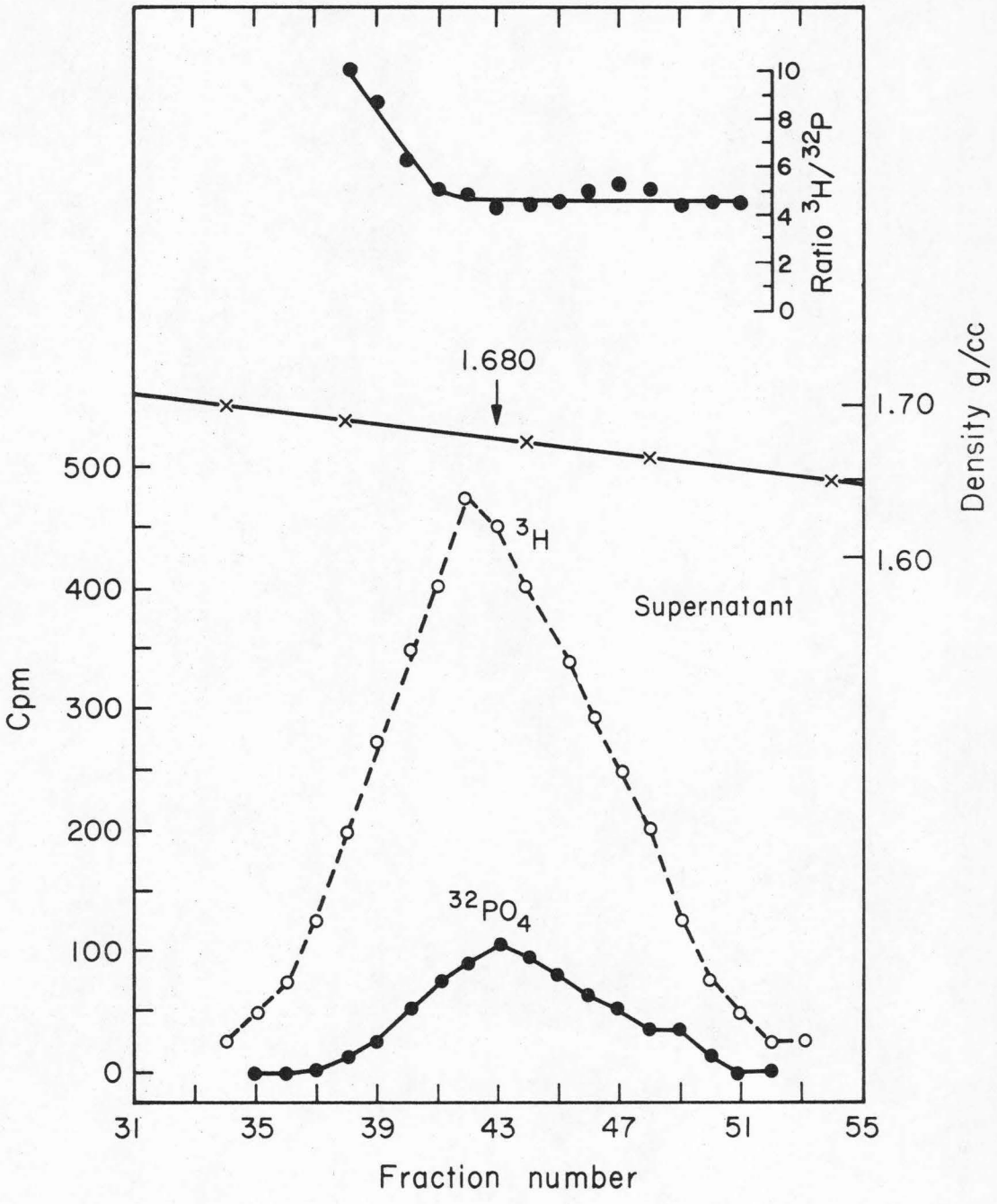
Cells were uniformly labeled for several generations with ^{3}H-thymidine (5μC/ml.) and then divided into two aliquots. The first aliquot was allowed to continue to grow (Control). The second aliquot had ethidium bromide added to a concentration of 70 μg/ml. After 5 hr, 5μC/ml. of $^{32}PO_4$ was added to each flask and growth was allowed to continue for 6 hr. DNA was extracted from each and run on preparative CsCl gradients (9 ml. gradients centrifuged 40 hr at 40,000 rev./min and then 36 hr at 32,000 rev/min, 20°C, Spinco Ti 50 rotor). 60 0.15 ml. fractions were collected and total ^{32}P and ^{3}H measured. Only that portion of the gradient containing labeled DNA is shown. Also plotted is the $^{32}P/^{3}H$ ratio for each fraction. Densities were calculated from the refractive indexes of the fractions.

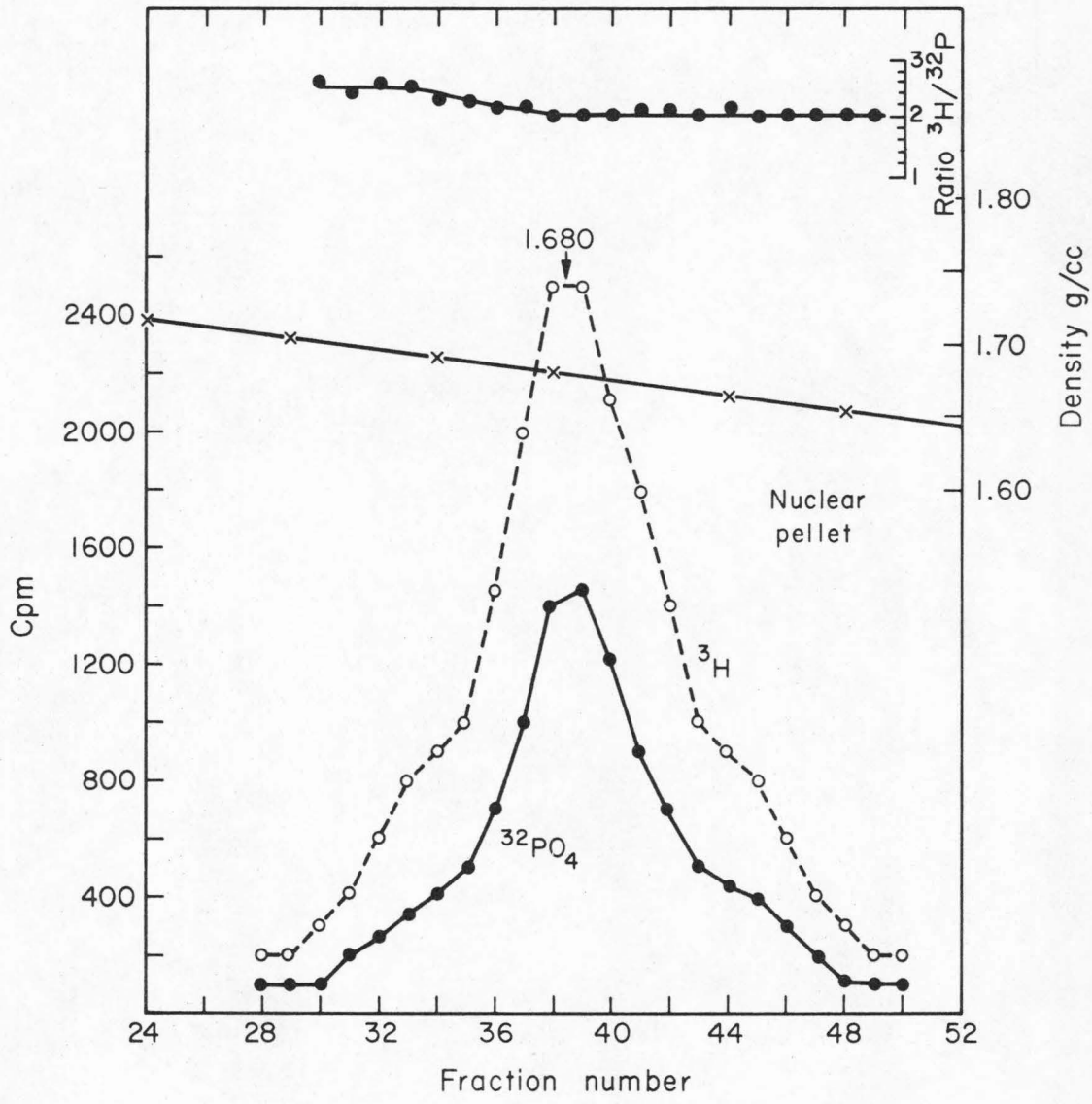
(a)
1.69
1.68
1.67
ρ(gr/cc)
2.0
1.5
Ratio 32P/3H
7000
6000
5000
4000
3000
2000
1000
Control
cpm 3H-Thymidine
cpm 32PO4
10
20
30
Fraction number

(b)
Experimental
3500
3000
2500
2000
1500
1000
500
2.3
2.0
1.5
1.68
1.67
1.69
ρ(gr/cc)
Ratio 32P/3H
7000
6000
5000
4000
3000
2000
1000
Ethidium
Bromide
Treated
cpm 3H-Thymidine
cpm 32PO4
10
20
30
Fraction number

Fig. 7. Effect of ethidium bromide on DNA synthesis.

Cells were treated as described in legend to Fig. 6. The cells were then fractionated into a crude nuclear pellet and cytoplasmic fraction. The cells were homogenized in a tight fitting Dounce homogenizer in 10 volumes of HKM buffer and centrifuged at 3,000 rev./min for 10 min in a Sorval SS34 rotor. The top two-third supernatant was collected as the supernatant fraction. The pellet was then treated with 1% Nonidet P-40 in HKM and homogenized and recentrifuged. This was repelleted and the pellet used as a crude nuclear preparation. DNA was extracted from both fractions and run on CsCl gradient and analyzed as described in the legend to Fig. 6.


Ratio $^{3}H/^{32}P$
0
2
4
6
8
10
1.680
1.70
1.60
Density g/cc
500
400
300
200
100
0
Cpm
^{3}H
Supernatant
$^{32}PO_4$
31
35
39
43
47
51
55
Fraction number


Ratio $^3H/^{32}P$
1
2
3
1.80
1.70
1.60
Density g/cc
1.680
2400
2000
1600
1200
800
400
Cpm
Nuclear
pellet
^3H
$^{32}PO_4$
24
28
32
36
40
44
48
52
Fraction number

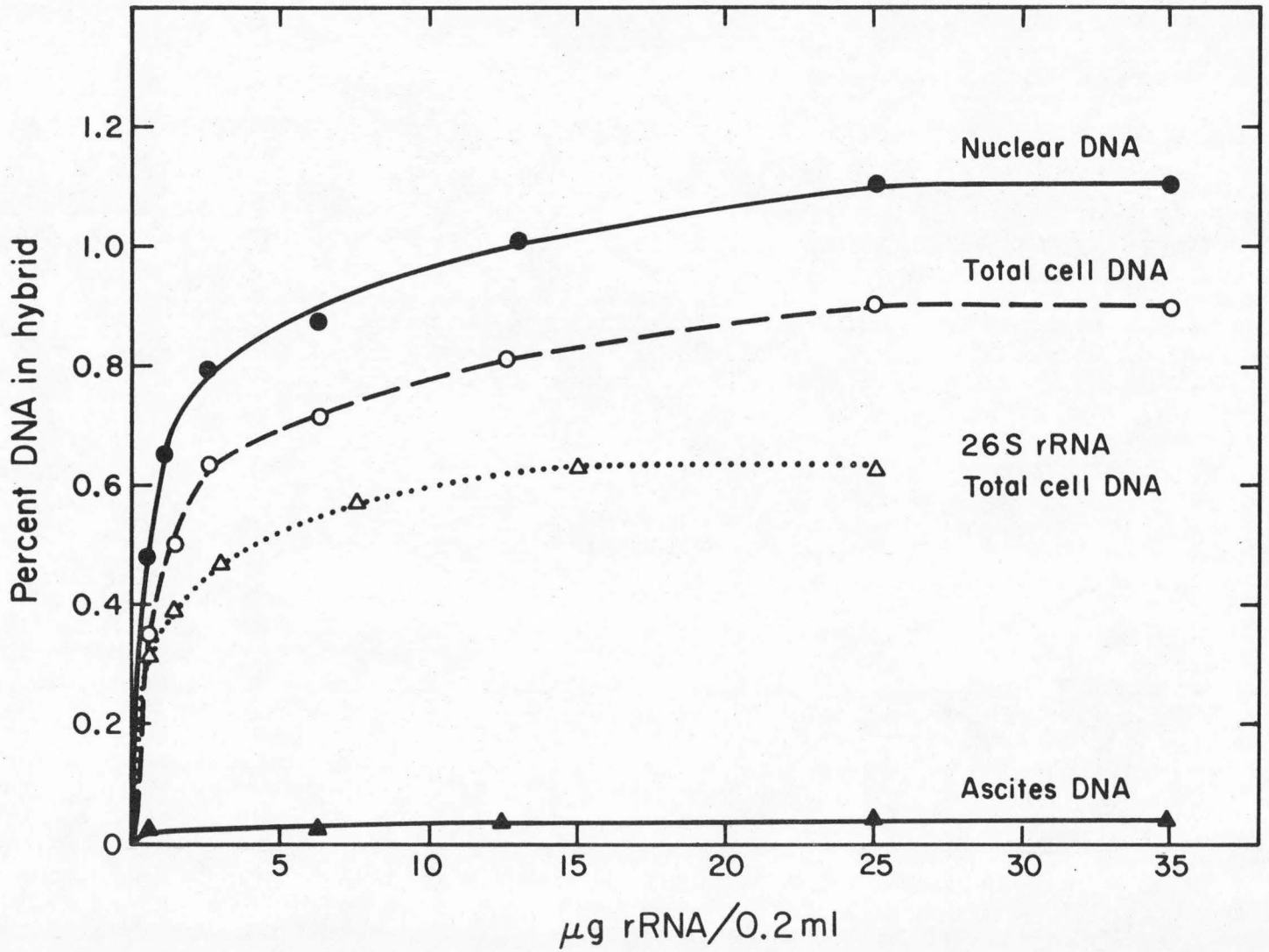
again indicate that mitochondrial DNA synthesis is preferentially inhibited by the presence of ethidium bromide.

(d) Hybridization of rRNA

To determine which portions of the cellular DNA was homologous to ribosomal RNA, RNA-DNA hybridization experiments were performed on filters with DNA extracted both from nuclei and from whole cells. Hybridization was carried out under stringent conditions, 50% formamide and 5X SSC at 40°C (McConaughy et al., 1969). The results of hybridization saturation experiments are shown in Figure 8. Ribosomal RNA hybridizes to approximately 0.85- 0.90% of the whole cell DNA and to 1.1% of nuclear DNA. This ratio (0.9:1.1) agrees well with the relative amount of whole cell DNA which is nuclear. Assuming a nuclear haploid genome size of 3.0×10^{10} daltons (duplex DNA) (see later in paper) and assuming the sum of the molecular weights of 17 and 26 S Dictyostelium ribosomal RNA to be approximately 2×10^6 daltons (estimated, from Attardi & Amaldi, 1970), the multiplicity of ribosomal RNA-DNA cistrons is approximately 150. Hybridization of the purified 26 S ribosomal RNA yields a saturation value approximately two-thirds of that for both ribosomal RNA, agreeing with what is known about the transcription, processing and molecular weight ratio of the two major ribosomal RNAs in eukaryote ribosomes. Preliminary results of RNA extracted from $^{32}PO_4$ pulse-labeled whole cells and

Fig. 8. Filter hybridization saturation curve of nuclear and total cell DNA with purified cytoplasmic ribosomal RNA.

Purified 17 and 26 S or 26 S rRNA was hybridized according to the conditions described in the Materials and Methods. 10 ug of labeled nuclear or total cell DNA were used on 11 mm filters. The amount of non-specific background hybridization was determined by hybridizing the Dictyostelium rRNA with 10 ug/ml of rat ascites DNA.


Percent DNA in hybrid
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
35
μg rRNA/0.2 ml
Nuclear DNA
Total cell DNA
26S rRNA
Total cell DNA
Ascites DNA

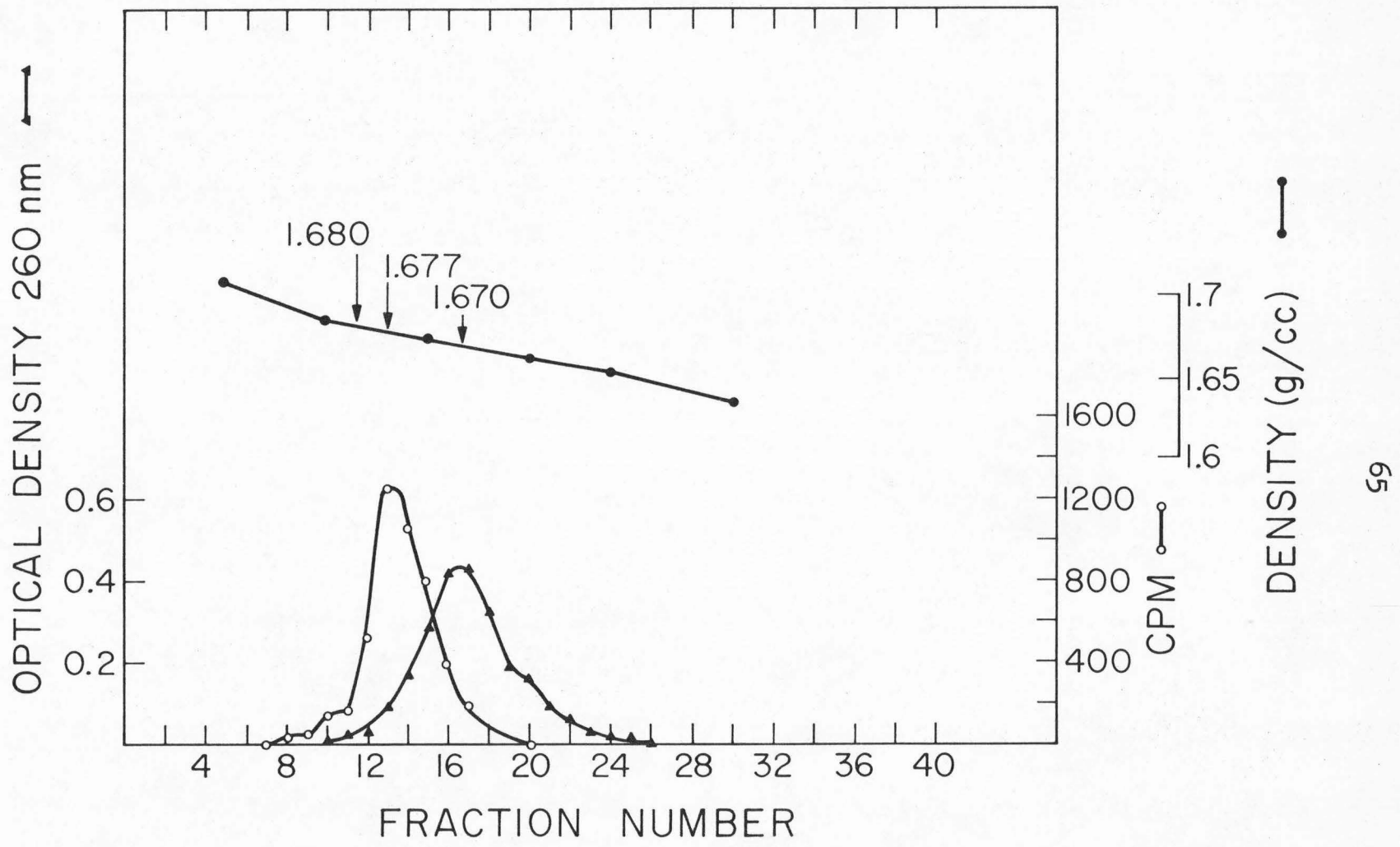
partially purified nuclei show the presence of a 36-38 S RNA on sucrose gradients. This peak is absent from labeled cells chased with excess cold PO_4 or actinomycin D and may represent the ribosomal RNA precursor as shown by Iwabuchi et al. (1971).

In solution RNA-DNA hybridization experiments using very large excesses of rRNA to rDNA (1000:1) yielded results which agree with those of the DNA filter method as to the number of ribosomal sequences present in the genome. These experiments were done at 66°C in 0.24 M PB and with sheared DNA.

The buoyant density of the rRNA cistrons was studied as outlined below. Nuclear DNA was centrifuged in a preparative CsCl buoyant density gradient. Each successive fraction collected was quantified by O.D. 260 measurement, diluted with water, denatured at 100°C, rapidly cooled, made up to 6X SSC, and collected on a nitrocellulose membrane. Ribosomal RNA was then hybridized to each DNA fraction and the amount of ribosomal RNA which hybridized to it was determined as described in Materials and Methods. Figure 9 shows the peak of ribosomal RNA hybridization is skewed towards the heavy side of the main nuclear band and is centered at approximately 0.007 of a density unit greater than the main nuclear band. This region is the same (approximately) as that in which the mitochondrial DNA bands. For comparison, the arrow at p= 1.680 shows the approximate region in

Fig. 9. Analysis of buoyant density of rRNA cistrons.

Nuclear DNA was centrifuged in a preparative CsCl gradient as described in the legend to Fig. 6. Fractions were collected and optical density at 260 nm was measured. 75 µl aliquots from the fractions were diluted to 5 ml. with H_2O, heat denatured, applied to 11 mm nitrocellulose membranes, and hybridized in 0.2 ml. with 25 µg/ml. of ^{32}P labeled purified cytoplasmic rRNA for 8 hr. The cts/min profile represents the cts/min of rRNA hybridized by that DNA fraction. See Materials and Methods for details.


OPTICAL DENSITY 260 nm
0.6
0.4
0.2
1.680
1.677
1.670
4
8
12
16
20
24
28
32
36
40
FRACTION NUMBER
1600
1200
800
400
CPM
1.7
1.65
1.6
DENSITY (g/cc)

which the nuclear satellite is expected to band. In other experiments the range varied from 0.005-0.007 density units greater than the main peak, including one experiment in which labeled total cell DNA and purified 26 S rRNA were used. Control experiments have shown that the 26 S rRNA does not hybridize detectably to purified Dictyostelium mitochondrial DNA. Although the resolution of the experiment is not high (it was repeated three times with different batches of ribosomal RNA and DNA), the results suggest that the ribosomal RNA cistrons may band in the same region as does the mitochondrial DNA. It cannot, however, be excluded that they may band under the heavy satellite and that the satellite represents the rRNA cistrons. If they do band in the same region as does the mitochondrial DNA there is reason to assume that at least 2.2% (probably more due to processing of 36-38 S precursor and possible presence of nontranscribed spacer regions) of the nuclear DNA (or DNA from cells treated with ethidium bromide) which bands in the mitochondrial region may be ribosomal cistrons. Thus some of the 5-7% of nuclear DNA which bands at p= 1.682 gr/cc is probably nuclear in origin.

(e) DNA-DNA reassociation experiments

Several factors affect rate of renaturation of denatured DNA include the complexity and the amount of repetitive DNA, length of DNA pieces used, salt concentration, GC content, and temperature (Britten and Kohne,

1967; Wetmur & Davidson, 1968; Gillis et al., 1970). Dictyostelium discoideum DNA has a Tm of 78.5°C in 0.12 M PB and a Tm of 82.5°C in 0.24 M phosphate buffer. In all cases the DNA used in renaturation experiment was sheared at 50,000 lb/sq in. and renaturation conditions were 23° below melting temperature unless otherwise stated. Dictyostelium discoideum DNA has an average GC content of 23% for the main nuclear band which makes it difficult to compare with other organisms. Rate of renaturation for a given complexity under the same conditions has been shown to decrease by a factor of approximately 2 with a reduction of GC content from 72% to 34% (Wetmur & Davidson, 1968; Gillis et al., 1970). Extrapolation of these data yields a factor which is used to correct renaturation rate of Dictyostelium DNA for its GC as compared to that of E. coli (50% GC) (Wetmur & Davidson, 1968; Gillis et al., 1970). It is expected that DNA of the same GC content as Dictyostelium DNA should renature at a rate 2/3 that of E. coli DNA if they were of the same complexity.

Figure 10 shows the renaturation profile (followed optically) of whole cell DNA of Dictyostelium. The renaturation kinetics were followed at several concentrations of DNA and at various PB salt concentrations. The rates obtained have all been corrected to equivalent phosphate buffer (0.12 M phosphate buffer) according to the

Fig. 10. Computer analysis of optical Cot plot of whole cell DNA. The computer program was kindly supplied by Dr. Roy Britten.

A value of 40 µg DNA was used as a conversion factor for a 1 ml. solution of denatured DNA having an A_{260} of 1.00 in a 1 cm path length cell. Shown are the fraction of the total hypochromicity for each component and its observed $Cot_{\frac{1}{2}}$.

Conditions:

o 40.8 µg/ml., 0.12 M PB, 56°C.

Δ 100 µg/ml., 0.24 M PB, 60°C.

● 1000 µg/ml., 0.24 M PB, 60°C.

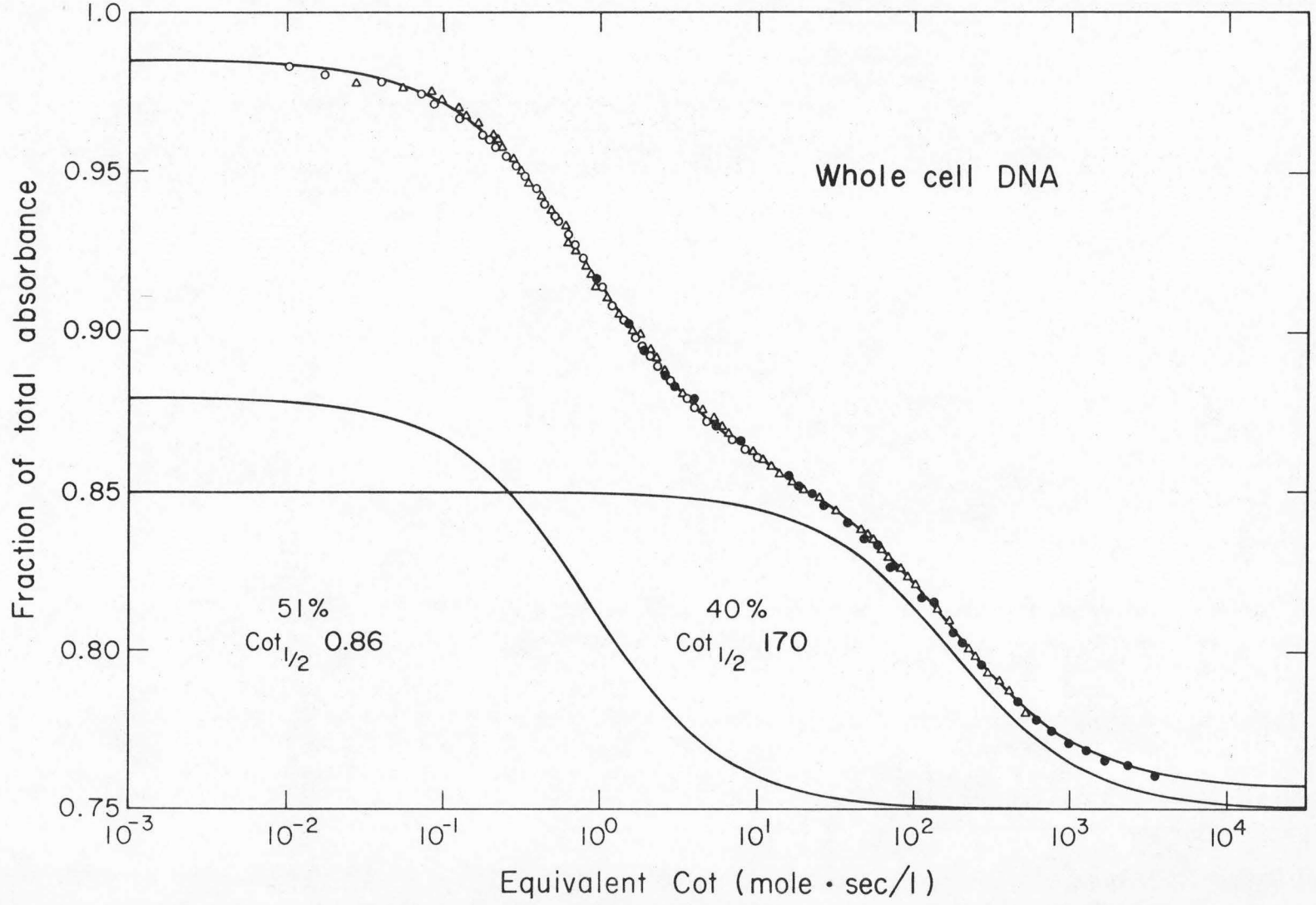

Whole cell DNA
51%
Cot1/2 0.86
40%
Cot1/2 170
Fraction of total absorbance
1.0
0.95
0.90
0.85
0.80
0.75
10-3
10-2
10-1
100
101
102
103
104
Equivalent Cot (mole • sec/l)

table of Britten & Smith (1970). In each case the first point was taken after the cuvette had cooled to within several degrees of the temperature at which the cuvette holder was maintained. The time at which this temperature was reached was determined by use of a cuvette containing EDTA which has a high thermal hyperchromicity (Britten, personal communication). In 0.12 M PB the hyperchromicity of the DNA sample due to this temperature drop is approximately 2% of the A_{260} at 95°C.

The data of the optical renaturation profile (Fig. 10) were subjected to computer analysis which fits possible second order reaction components to the curve by a least square fitting program (supplied by Roy Britten, Carnegie Institution of Washington and modified by C. Schlehuber). The best fit was obtained by fitting two components to the curve. The first is a repetitive one and represents approximately 51% of the hypochromicity. This DNA renatures with an average $Cot_{\frac{1}{2}}$ (pure);i.e. $Cot_{\frac{1}{2}}$ if the DNA consisted solely of this fraction, calculated by multiplying the observed $Cot_{\frac{1}{2}}$ by the fraction the component is of the total DNA) of approximately 0.44. The second component (40% of the hypochromicity) (the single-copy DNA, see below) renatures with a $Cot_{\frac{1}{2}}$ (pure) of approximately 70. From the results below, it is evident that whole cell repetitive DNA fraction consists of two main fractions: the mitochondrial DNA which is

shown above to be approximately 28% of the whole cell DNA by buoyant density analysis and nuclear repetitive components.

(f) Reassociation of nuclear DNA

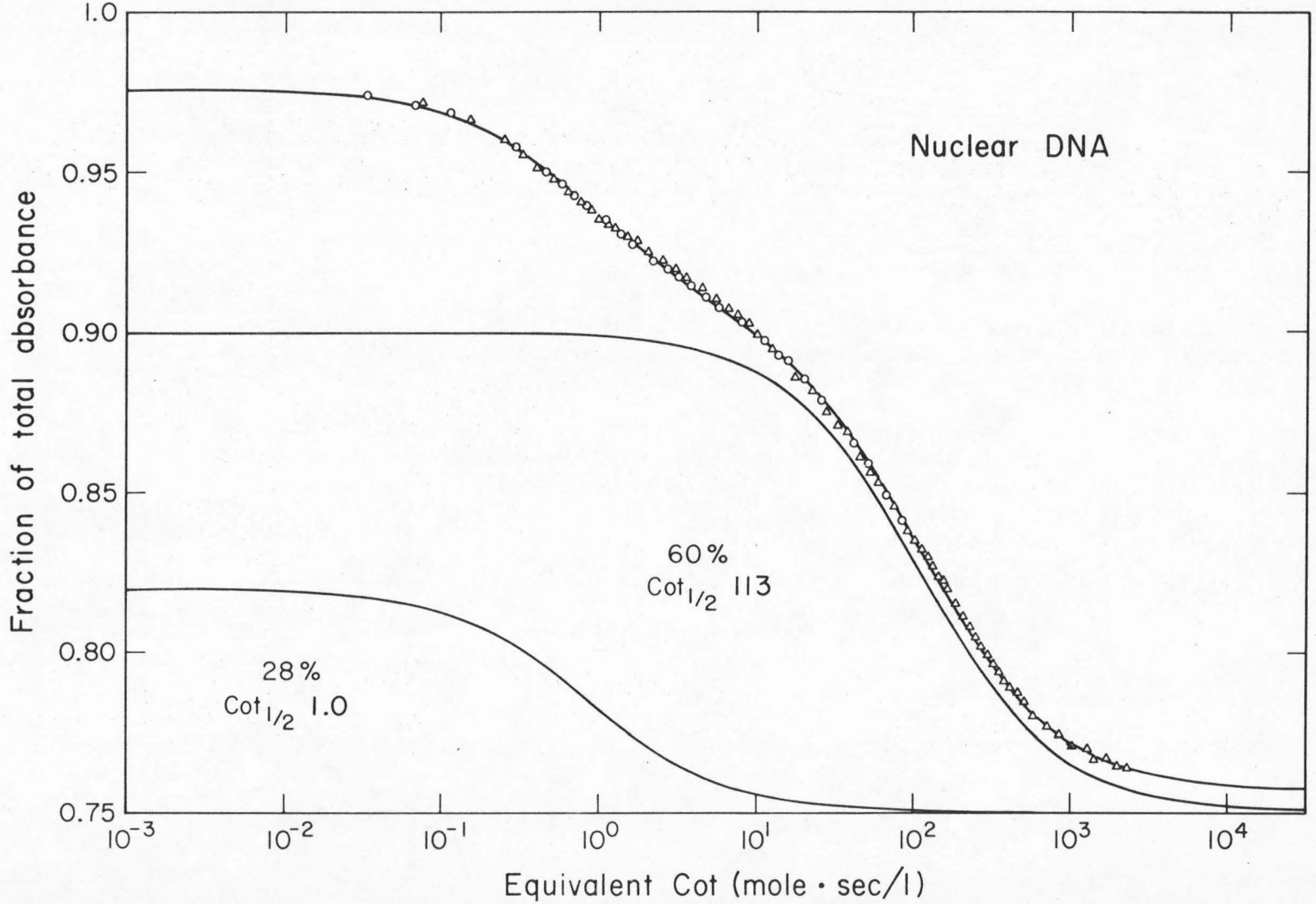
Figure 11 shows the optical renaturation profile of nuclear DNA. The least squares computer fit suggests that the repetitive DNA sequences constitute 28% of the hypochromicity. This fraction exhibits a $Cot_{\frac{1}{2}}$ (pure) of 0.28. The slower renaturing DNA component represents approximately 60% of the hypochromicity and exhibits a $Cot_{\frac{1}{2}}$ (pure) of 68. The $Cot_{\frac{1}{2}}$ of E. coli DNA under the same conditions is 6.5 (see Fig. 17). After correcting for the GC effect (Wetmur & Davidson, 1968; Gillis et al., 1970), this portion of the genome has a calculated complexity of 7 times greater than E.coli or approximately 20×10^9 daltons (E. coli taken to be 2.8×10^9 daltons, Cairns, 1963). The average complexity of the repetitive fraction is 8×10^7 daltons, and the average repetition number relative to the slow component is approximately 120. From the calculated complexity of these components and from the size of the Dictyostelium nuclear genome determined by chemical methods (Sussman and Rayner, 1971), we assume that the slower reassociating component is single-copy DNA. The repetitive fraction consists of four different DNA fractions: rRNA cistrons; mitochondrial DNA; main band nuclear repetitive DNA containing possibly

Fig. 11. Computer analysis of optical Cot plot of nuclear DNA.

Conditions:

○ 105 µg/ml., 0.12 M PB, 56°C.

△ 1150 µg/ml., 0.24 M PB, 60°C.


Nuclear DNA
60%
Cot1/2 113
28%
Cot1/2 1.0
Fraction of total absorbance
Equivalent Cot (mole • sec/l)
1.0
0.95
0.90
0.85
0.80
0.75
10-3
10-2
10-1
100
101
102
103
104

fractions of various complexity; and possibly the heavy GC buoyant density satellite. The repetition number of 120 must therefore be interpreted as an average over all the fractions.

DNA-DNA reassociation profiles were also made for DNA from cells treated with ethidium bromide (see Fig.5 for buoyant density analysis of DNA). The computer analysis of the renaturation profile (Fig. 12),suggests the presence of a repetitive and a unique component. The proportion of repetitive DNA is greatly reduced as compared to whole cell DNA. This is as expected since as shown above, ethidium bromide causes a reduction of approximately 70-80% of mitochondrial DNA. The repetitive region as indicated by the computer analysis suggests that the repetitive sequences make up only 22% of the total instead of 51% and exhibit a $Cot_{\frac{1}{2}}$ of 0.26 instead of 0.44. The observed equivalent $Cot_{\frac{1}{2}}$ (pure) of the non-repetitive fraction is the same as that determined for nuclear DNA.

To further analyze these components, the repetitive and single-copy DNAs were purified on hydroxyapatite (see figure legends for details). Figure 13 shows a renaturation profile of purified single-copy DNA by optical and by HAP (hydroxyapatite) measurements. Also shown is a HAP renaturation profile of whole cell DNA. In optical measurements the A_{260} of the DNA drops by 2% due to

Fig. 12. Computer analysis of optical Cot plot of DNA from cells treated with ethidium bromide as described in the legend to Fig. 5.

● 97 µg/ml., 0.12 M PB, 56°C.

o 1150 µg/ml., 0.24 M PB, 60°C.

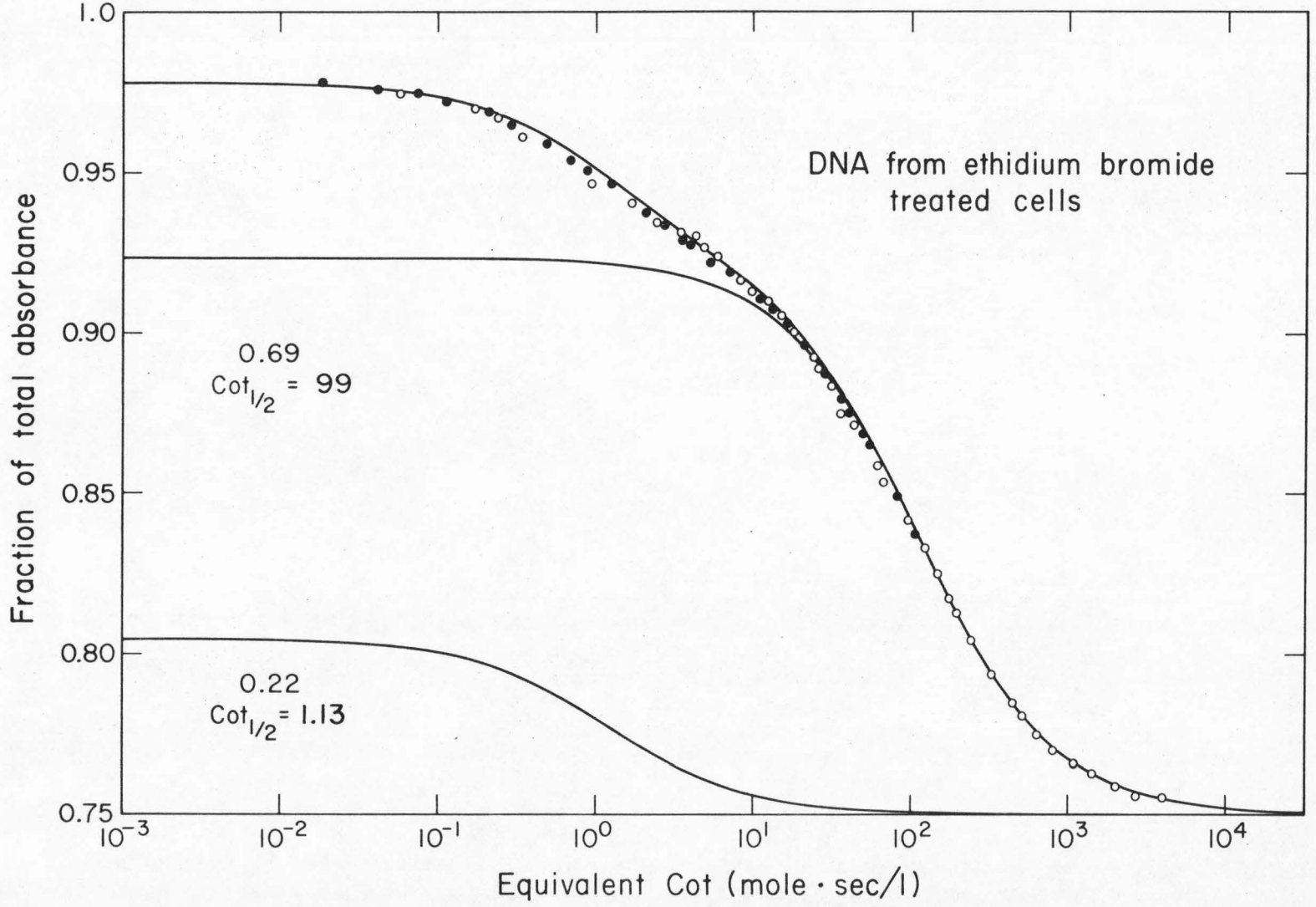

1.0
0.95
0.90
0.85
0.80
0.75
Fraction of total absorbance
DNA from ethidium bromide
treated cells
0.69
Cot1/2 = 99
0.22
Cot1/2 = 1.13
10-3
10-2
10-1
100
101
102
103
104
Equivalent Cot (mole · sec/l)

Fig. 13. Optical and HAP Cot plots of single-copy DNA and HAP Cot plot of total cell DNA.

o HAP curve of total cell DNA.

Δ····Δ HAP curve of tracer amounts of single copy DNA reassociated with total cell DNA. A ratio of 1000-1, total cell DNA:single-copy (100,000 cts/min/μg) was used.

● Data from single copy DNA tracer curve plotted as a fraction (45%) of total cell DNA.

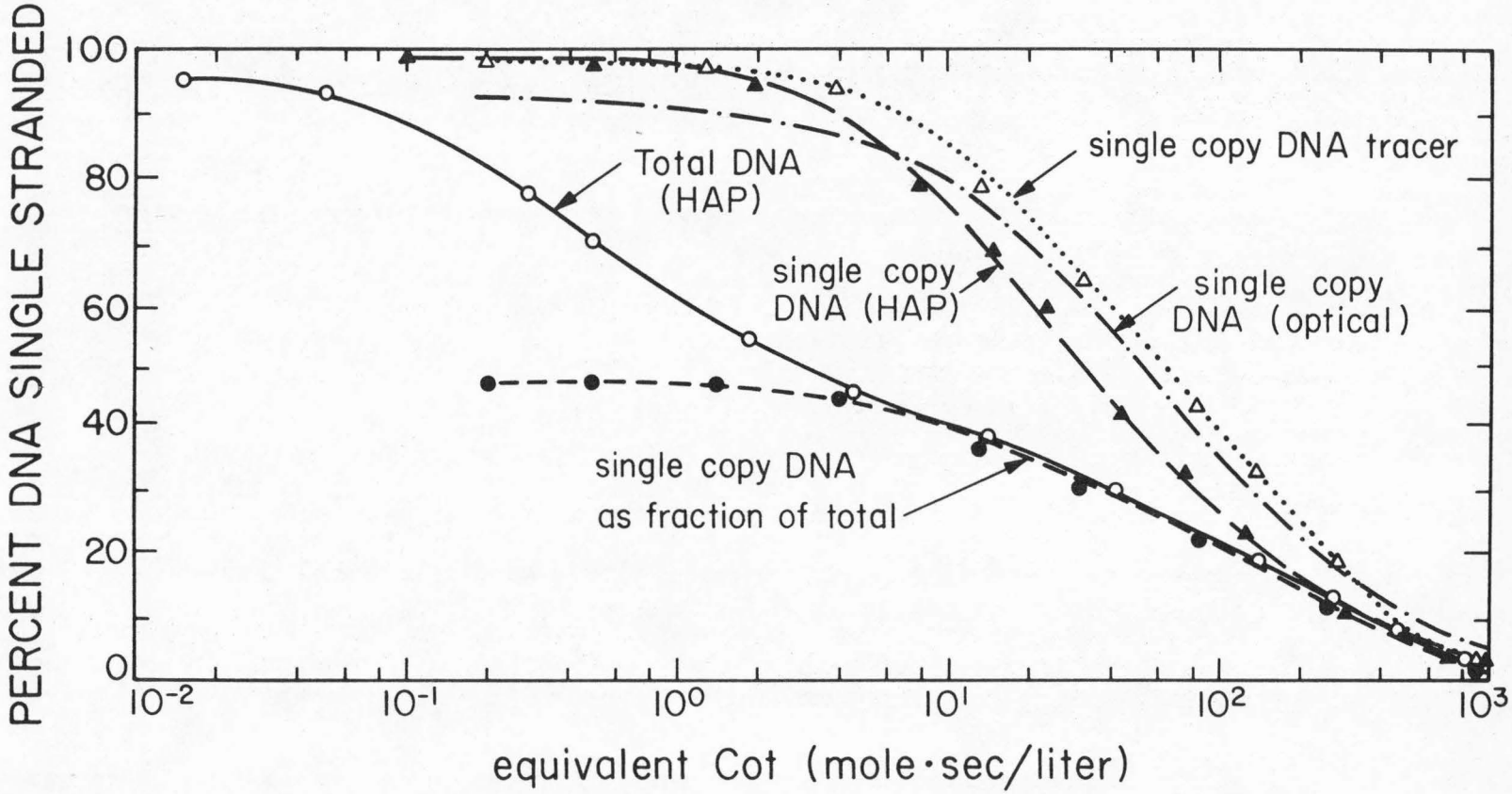
▲—-▲ HAP curve of single copy DNA.

-·-· Optical curve of single copy DNA. This curve is a combination of curves seen at 112 μg/ml., 0.12 M PB, 56°C, and 1160 μg/ml., 0.24 M PB, 60°C.

Conditions for HAP curves: For points below Cot 0.4, 50 μg DNA (25 μg/ml.) were used in 0.12 M PB at 56°C. For points between Cot 0.4-10, 100 μg at 100 μg/ml. were used. For points above Cot 10, 100 μg DNA at 500 μg/ml. in 0.24 M PB at 60°C. HAP Cot curve of renaturation of total cell DNA was done in parallel experiments using labeled total cell DNA (2,000 cts/min/μg).

Purification of single-copy DNA (Fraction II). Sheared DNA was adsorbed to HAP at 60°C in 0.03 M PB, washed, eluted at 0.48 M PB, and reassociated to an equivalent Cot 150 (0.48 M PB, 62°C). The DNA was chromatographed on HAP, and the single-stranded DNA eluting at 0.12 M PB was reassociated to an equivalent Cot 75. The single-stranded

material eluted from the HAP column at 0.12 M PB was used as Fraction II.


PERCENT DNA SINGLE STRANDED
100
80
60
40
20
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
equivalent Cot (mole·sec/liter)
Total DNA
(HAP)
single copy
DNA (HAP)
single copy DNA tracer
single copy
DNA (optical)
single copy DNA
as fraction of total

reduction of sample temperature from 95° to 55°C. The $Cot_{\frac{1}{2}}$ is 70 by optical measurements and 30-35 by hydroxyapatite, these values agree with other estimations given above. A Wetmur & Davidson plot (1968) of the data shows that it behaves as a single second order reaction component. The $Cot_{\frac{1}{2}}$ from hydroxyapatite curve is approximately $\frac{1}{2}$ times that of the optical curve in agreement with what has been found by other investigators in other systems and it seems to be a general property of the hydroxyapatite renaturation profile (Britten & Kohne, 1967). Also shown in Figure 13 is a renaturation profile of tracer amounts of ^{32}P-labeled nonrepetitive DNA hybridized with excess total cell DNA. The kinetics are also plotted assuming that single-cell DNA represents 45% of the whole cell DNA. It is clear that there is little contamination of the unique DNA fraction with repetitive DNA. If the average repetition number of the repetitive DNA is 200, then a minimum 0.6% (0.55 x 2/200) of the nonrepetitive DNA represents repetitive sequences.

Figure 14 shows a Cot plot of the purified nuclear repetitive DNA (fraction I). This material renatures over 4-5 decades and is therefore heterogeneous and composed of several components. Computer analysis of the profile is compatable with three fractions: an extremely rapidly renaturing fraction making up approximately 7% of the hypochromicity, $Cot_{\frac{1}{2}}$ (pure) 4×10^{-4}; a fraction making up

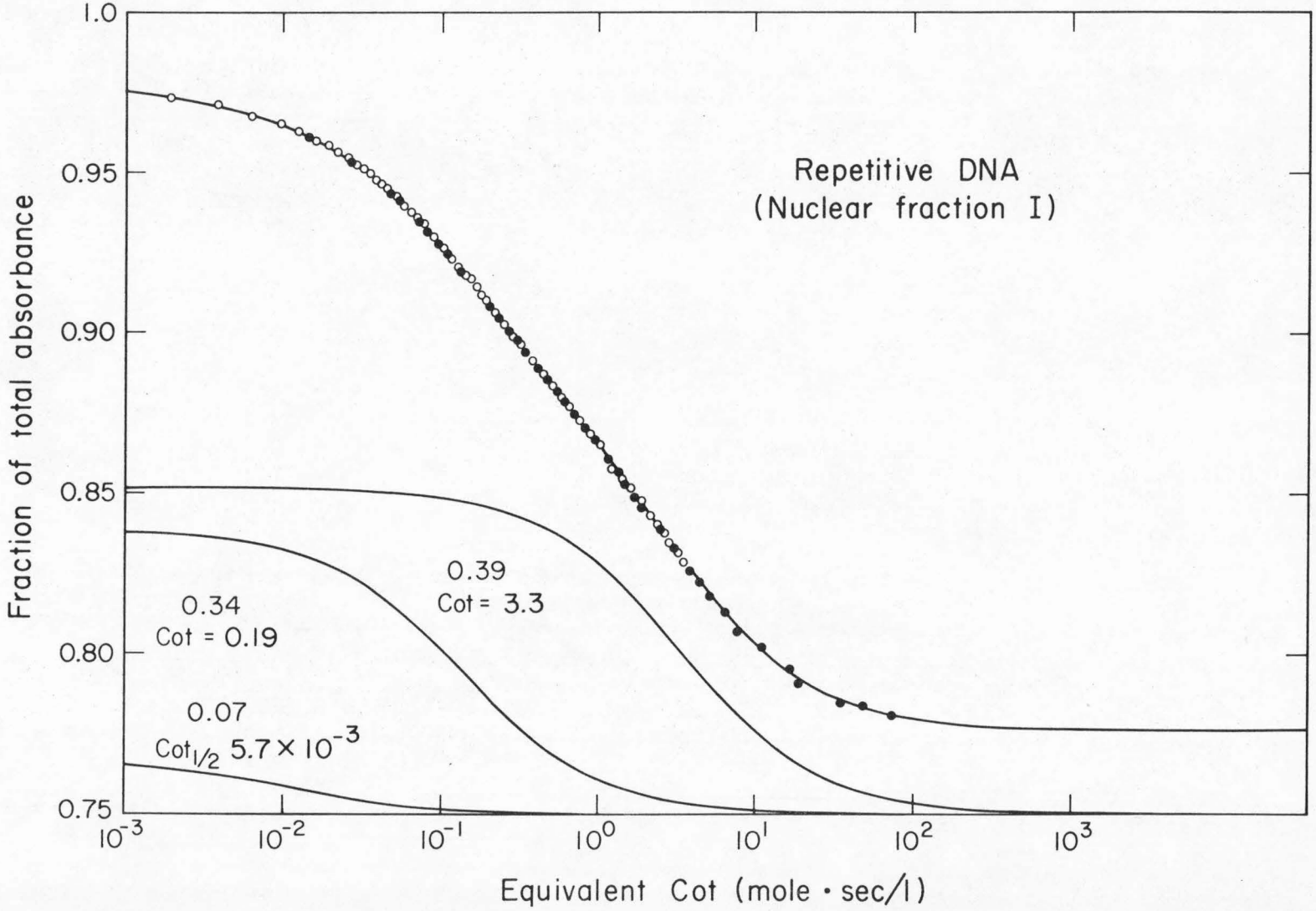
Fig. 14. Computer analysis of optical Cot plot of nuclear repetitive DNA.

Conditions:

o 11.4 µg/ml., 0.12 M PB, 56°C.

● 80 µg/ml., 0.12 M PB, 56°C.

Purification of nuclear repetitive DNA (Fraction I). Sheared DNA was adsorbed to HAP in 0.03 M PB, 60°C and eluted at 0.48 M PB. The DNA was renatured in 0.12 M PB, 56°C to a Cot of 0.75 and chromatographed on HAP. The double-stranded material eluting in the 0.48 M PB wash after the column had been washed in 0.12 M PB was used as Fraction I.


1.0
0.95
0.90
0.85
0.80
0.75
Fraction of total absorbance
Repetitive DNA
(Nuclear fraction I)
0.34
Cot = 0.19
0.39
Cot = 3.3
0.07
Cot1/2 5.7 × 10-3
10-3
10-2
10-1
100
101
102
103
Equivalent Cot (mole • sec/l)

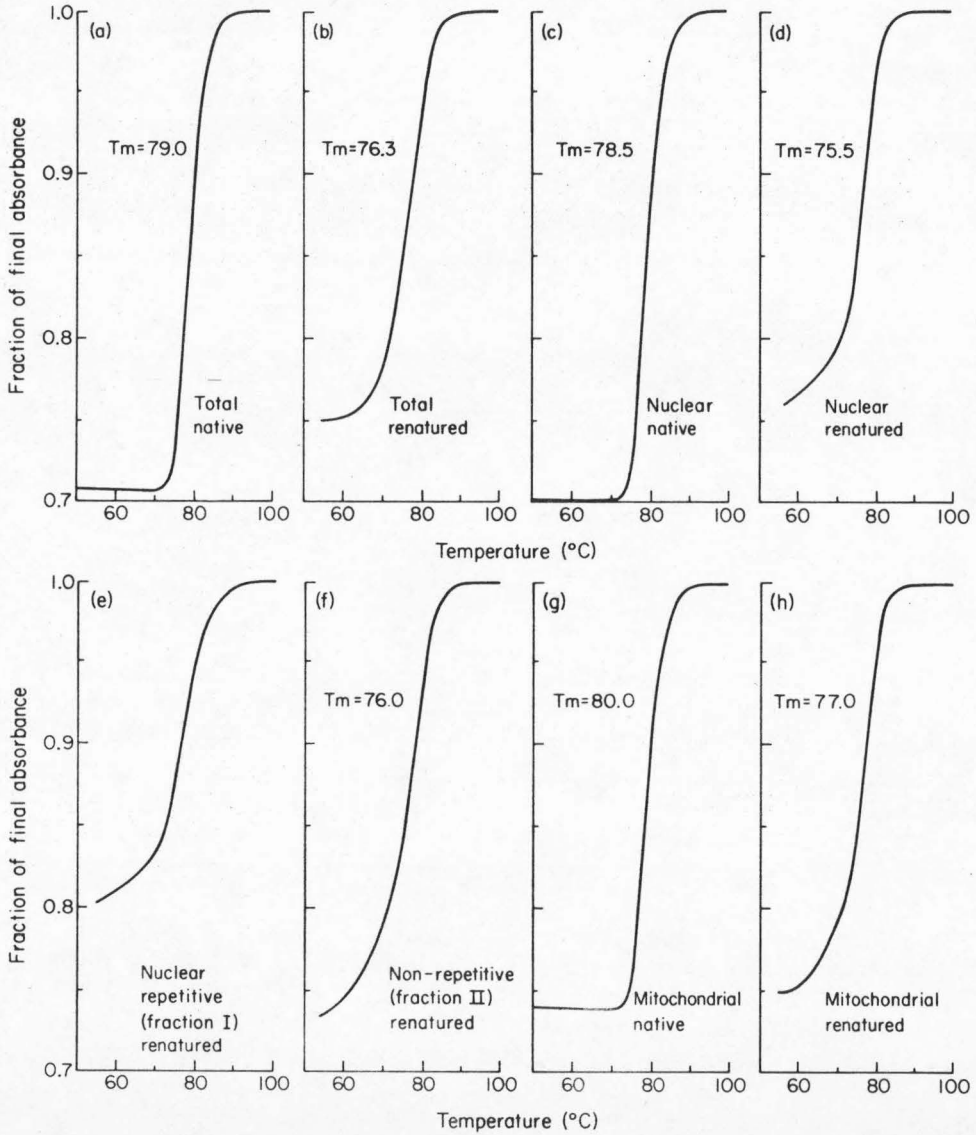
34% of the hypochromicity with a $Cot_{\frac{1}{2}}$ (pure) of 0.065; and a fraction making up 39% of the hypochromicity with a $Cot_{\frac{1}{2}}$ (pure) of 1.3. It is possible that the rapidly renaturing component, which may represent a larger fraction of the DNA than the value obtained by optical measurement, is a DNA fraction similar to mouse satellite (Waring & Britten, 1966), Drosophila satellite (Botcham et al., 1971), or to the extremely rapidly renaturing component of Illyanassa DNA (Davidson et al., 1971). This DNA may represent a fraction of the DNA which shows internal strand homology. It is also possible that this DNA is the heavy nuclear GC satellite seen in buoyant density analysis. Unfortunately neither Ag^{+} nor Hg^{++} - C_2SO_4 preparative centrifugation (Nandi et al., 1965; Jensen & Davidson, 1966) separated this component from the remainder of the DNA. Because of this it has not been possible to obtain a renaturation analysis of the GC rich satellite and accurately determine its $Cot_{\frac{1}{2}}$.

There are two principle sources of uncertainty in the analysis of repetitive DNA-DNA reassociation kinetics. The first is a small effect (approximately a factor of 2) on the second order rate constant due to mismatch if sequences which hybridize are not exact compliments. The second is a general lower hyperchromicity for isolated repetitive DNA duplexes per unit DNA. The observed hypochromicity of the repetitive fraction is 20%, less

than the average hypochromicity of the total nuclear or cell DNA. This can be explained if the repetitive DNA sequences are linked to single-copy sequences (see Britten and Kohne, 1969). At the low Cot values at which the repetitive sequences reassociate, the single-copy portions of the segment will not reassociate. Since these regions are not base paired the hybrid will have an overall lower hyperchromicity when melted. It may also be due to sequences which have a high degree of base mismatch. There may also be a fraction of the DNA segments isolated by HAP chromatography which will not reassociate under the conditions used. These problems were encountered with isolated fractions of Illyanassa DNA (Davidson et al., 1971). If there are components of lower hypochromicity they will represent a greater fraction of the total DNA than measured optically. Thus, the amount of nuclear repetitive DNA may be greater than the 32% (0.28/0.88 total hypochromicity due to DNA-DNA renaturation) observed by optical renaturation analysis.

Figure 15 shows melting profiles of renatured whole cell DNA, nuclear DNA, nuclear repetitive DNA, and single-copy DNA. Hyperchromicities of the renatured DNAs were obtained from samples renatured to a Cot of 3-4 x 10^3 for nuclear, whole cell, and single-copy DNA and to a Cot of 75 for the isolated repetitive fraction. In all cases the Cot selected is one at which over 97% of the DNA is renatured. The hyperchromicity as a per cent of the A_{260} (at 95°C) is 25% for whole cell DNA, 26-27% for unique DNA, 25% for nuclear

Fig. 15. Optical melting profiles of native and reassociated DNA fractions in 0.12 M PB.


(a)
Tm=79.0
Total native
(b)
Tm=76.3
Total renatured
(c)
Tm=78.5
Nuclear native
(d)
Tm=75.5
Nuclear renatured
(e)
Nuclear repetitive (fraction I) renatured
(f)
Tm=76.0
Non-repetitive (fraction II) renatured
(g)
Tm=80.0
Mitochondrial native
(h)
Tm=77.0
Mitochondrial renatured
Fraction of final absorbance
1.0
0.9
0.8
0.7
60
80
100
Temperature (°C)

DNA, and 18-19% for the nuclear repetitive fraction. The repetitive fraction profile exhibits an early melting foot and the lowered hyperchromicity typical of some eukaryote repetitive DNA. The Tm of well base-paired reassociated DNA is expected to be about three degrees below that of native DNA due to the effects of shearing (Britten & Kohne, 1967). As is expected for well base paired hybrids, the melting profile of the single-copy DNA shows cooperative melting and is of high thermal stability, Tm = 76°C in 0.12 M PB and 80-81°C in 0.24 M PB. The repetitive DNA fractions show a broader early melting region and a sharper later melting region. The overall Tm is approximately 74-75°C, higher than that expected from the results found with some repetitive DNA from other eukaryotes (Britten and Kohne, 1968 a,b). However, the fact that it exhibits both lowered hyperchromicity and a slightly lowered Tm may indicate that a portion of the repetitive DNA does consist of short reassociated sequences or sequences with base mismatching. Other fractions (as residual mitochondrial DNA, ribosomal cistrons, or other nuclear components) may have little base mismatching and probably exhibit a higher hyperchromicity. From the known Tm of the mitochondrial fraction and the estimate of the Tm of the rRNA cistrons from buoyant density analysis, these components are expected to raise the Tm of the whole fraction. As expected, the

melting profile of the renatured total nuclear DNA showed a combination of the melting found in both fraction I and fraction II. Whole cell DNA exhibits a Tm of 81°C in 0.24 M PB and 76-77°C in 0.12 M PB. This slight increase in Tm is presumably due to the presence of mitochondrial DNA which has a higher Tm than does the nuclear material. A melting profile (not shown) of whole cell DNA only 40% renatured again indicates a higher Tm than that of a nuclear preparation also renatured to the same degree. This again is due to the presence of mitochondrial DNA in the former one.

(g) Renaturation of mitochondrial DNA

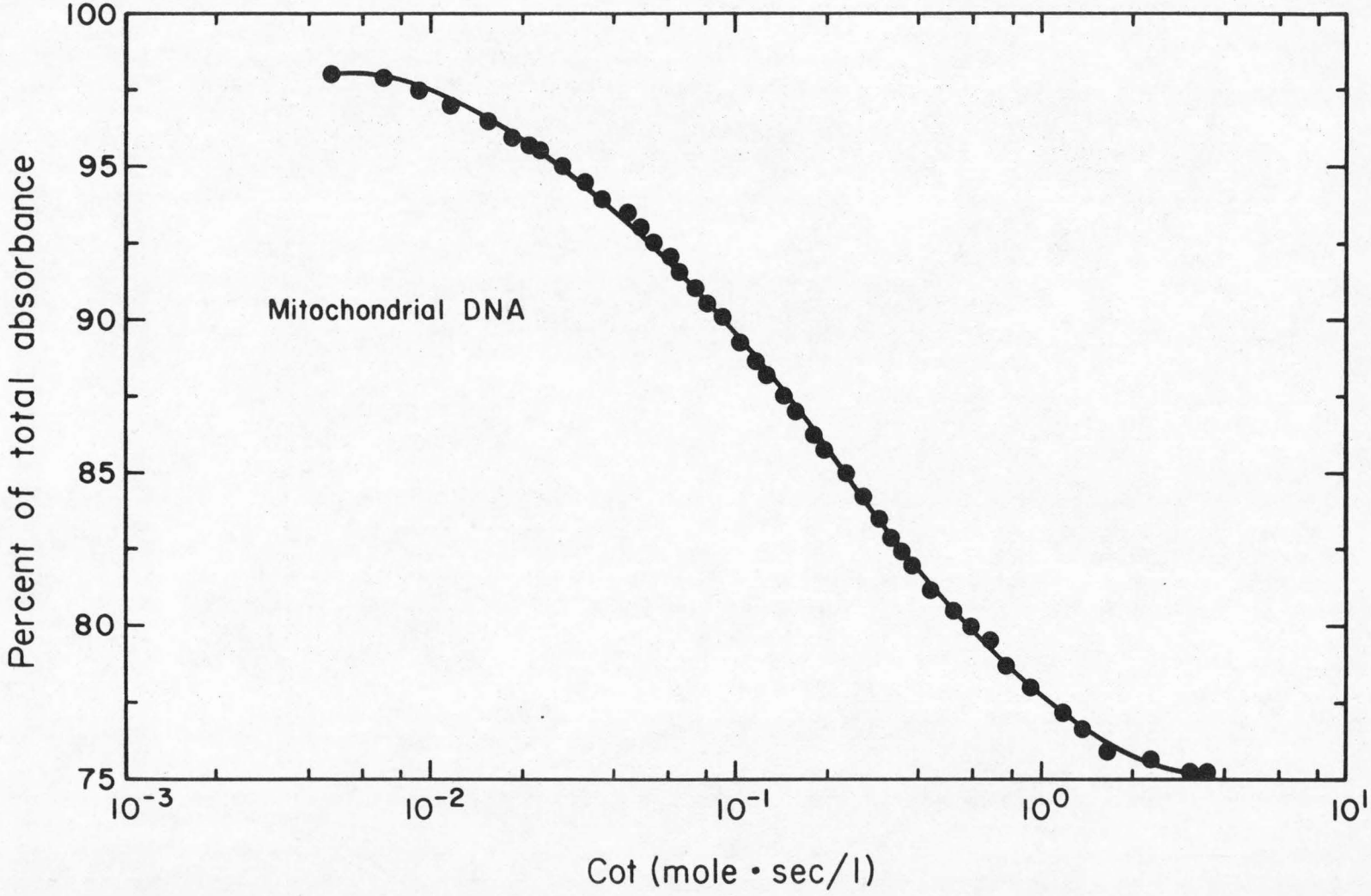
Figures 16 a and 16 b show respectively a Cot plot and a Wetmur-Davidson plot for reassociation of sheared mitochondrial DNA. Due to its larger second order rate constant, the mitochondrial DNA was renatured at low concentration (approximately 0.2-0.4 O.D.) and renatured in 0.12 PB or 0.09 M PB. Its $Cot_{\frac{1}{2}}$ is 0.15 $\pm$ 0.015 (7 renaturations and 2 batches of DNA). The complexity of the mitochondrial DNA calculated from its renaturation kinetics is approximately 35-40 x 10^6 daltons. The value for mammalian and higher animal eukaryotes and mitochondrial DNAs of the 5u closed circular type is approximately 10 x 10^6 daltons (see Borst et al., 1968). The linear Wetmur & Davidson plot of mitochondrial DNA reassociated in 0.09 M PB extrapolates to an intercept of approximately

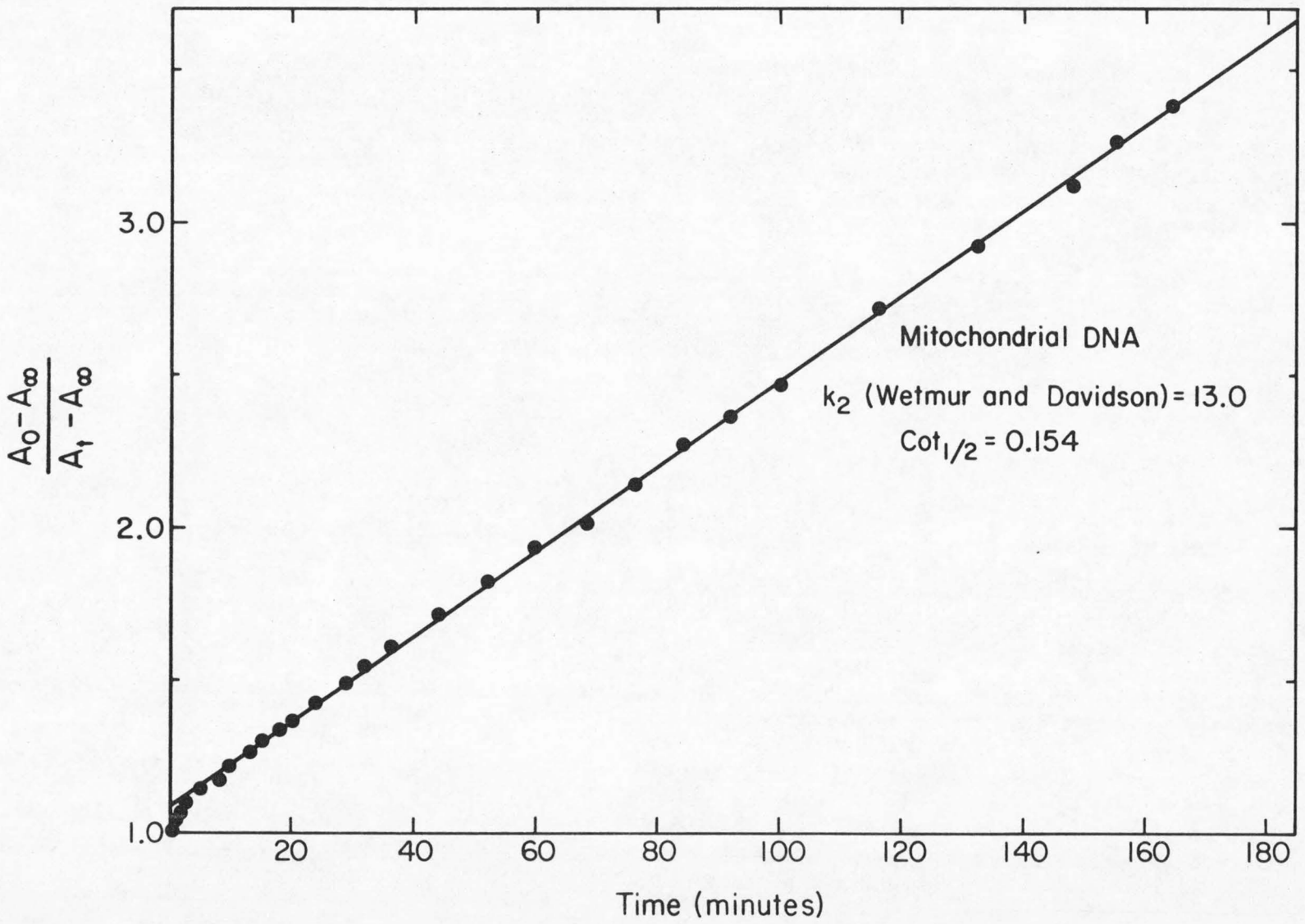
Fig. 16. Reassociation kinetics of mitochondrial DNA.

(a) Optical Cot plot.

(b) Wetmur & Davidson plot.

Conditions: 12 µg/ml., 0.12 M PB, 57°C.


100
95
90
85
80
75
Percent of total absorbance
Mitochondrial DNA
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
Cot (mole • sec/l)


$\frac{A_0 - A_\infty}{A_t - A_\infty}$
3.0
2.0
1.0
20
40
60
80
100
120
140
160
180
Time (minutes)
Mitochondrial DNA
k_2 (Wetmur and Davidson) = 13.0
$Cot_{1/2}$ = 0.154

1.1 indicating that about 90% of the hypochromicity represents a single second order component. The initial 10% drop in absorbance probably represents the approximately 2% of A_{260} (at 95°C) hypochromicity drop due to temperature shift. These results indicate that mitochondrial DNA has less than 10%, if any, repetitive sequences relative to the remainder of the mitochondrial genome.

Renaturation kinetics of Drosophila (Botcham et al., 1971), Tetrahymena (Flavell & Jones, 1970), and Paramecium (Flavell & Jones, 1971) mitochondrial DNAs have shown that these species also exhibit a complexity of 30-40 x 10^6 daltons. There may therefore be a large group of organisms which possess mitochondrial DNA of complexity between the 10 x 10^6 dalton variety found in many higher animals and the 50 x 10^6 dalton and even higher mitochondrial DNA found in many fungi and higher plants (see Ashwell & Work, 1970; Hollenberg et al., 1970; Wood & Luck, 1969; Wolstenholme & Gross, 1968).

There are approximately 200-250 mitochondrial genomes per nuclear genome as calculated from its $Cot_{\frac{1}{2}}$ (pure) and its fraction of total cell DNA.

Figure 15 shows the thermal melting profile of renatured mitochondrial DNA. As expected for simple DNAs, it shows cooperative melting and a hyperchromicity of 53% as compared to 26% for the native mitochondrial DNA. Its Tm is 77°C, 3° below that of native, unsheared DNA.

4. Discussion

In the developmental cycle of Dictyostelium discoideum there are only three main cell types: vegetative amoebae which differentiate to stalk cells and spores. During the developmental process, there are intermediate stages in which prestalk and prespore cells can be distinguished cytologically and biochemically from the mature stalk and spore cell and from the amoeba (Takeuchi, 1963; Sussman, 1966; Bonner, 1967; Hall & Hamamoto, 1969; Maeda and Takeuchi, 1969; Gregg & Badman, 1970; Bonner, 1971).

The present analysis concerns the various component DNA species present in the whole cell DNA of Dictyostelium. Since the spore germinates into a vegetative amoeba, the spore cell must contain all of the genetic material required for the subsequent development stages. Since differentiation of both prespore and prestalk cells is reversible and since the cells can not only dedifferentiate into amoebae but also redifferentiate into either spore or stalk cells (Raper, 1940; Bonner, 1967; Bonner, 1971), it is apparent that all the cell types contain a complete set of genes. Analytical buoyant density analysis and Cot curves of nuclear DNA extracted from 14 hr slugs are not detectably different from those of DNA extracted from amoebae.

Table I summarizes the physical properties of the

Table I

Physical Properties of Dictyostelium DNA Components

DNA Component	Density	Tm	% GC	% GC	Fraction of DNA in density region		
	gr/cc	0.12 M PB	from density	from Tm	Total DNA	Nuclear prep.	Ethidium bromide treated cells
Nuclear, main band	1.676	78.5	23	23	~70	~87	~87
Mitochondrial	1.682	80.0	28	26	24-28	6-8	7-9*
Nuclear satellite	1.687		33		5-7	5-7	5-7

*Under the growth conditions described in legend to Figure 5.

various DNA components of Dictyostelium. The GC content of the principle nuclear band both from buoyant density analysis and from Tm is approximately 23%. The GC content of the mitochondrial DNA by buoyant density analysis is 28% and approximately 26% from the thermal denaturation melting profile. The GC content of the nuclear satellite is 33% by buoyant density analysis. The ratio of the amount of nuclear satellite relative to amount of nuclear main band is approximately the same for whole cell, nuclear preparation, and whole cell DNA from cells treated with ethidium bromide. It appears likely from the hybridization results with rRNA that a part of the material which bands at p= 1.682 gr/cc may be nuclear in origin. It has been impossible to obtain nuclear fractions free of this component even from nuclei which appear pure under phase microscopy. DNA purified from chromatin isolated from purified nuclei according to modifications of the method of Bonner et al., (1968) also exhibit this peak. It is also possible that some of this fraction may represent a second nuclear satellite. While this paper was being written a preprint by R. Sussman and E. Rayner (1971) was made available to me. Their results agree with those presented here. They also found that nuclear DNA synthesis is preferentially inhibited with cycloheximide and that the second peak (p= 1.682 g/cc) is

cytoplasmic in origin and represents DNA extracted from mitochondria. They do not, however, find a peak at p= 1.687 and it is possible that in their experiments it is partially obscured by the E. coli DNA marker used (p= 1.704 g/cc).

Table II summarizes the reassociation kinetics of the various cell DNA fractions. Figure 17 shows a comparison of the reassociation kinetics of Dictyostelium mitochondria and nuclear non-repetitive DNA and E. coli DNA. In all cases, the criterion used in analyzing the DNA for repetitive and nonrepetitive classes was 23° below the Tm of native DNA. It has been shown with other eukaryotic DNAs that as the stringency of the criterion is reduced, to say 35° below Tm, the amount of sequences showing similarity increases (Martin & Hoyer, 1966;McCarthy & McConaughy,1968; Church & McCarthy, 1968; Davidson et al., 1971). The lower temperature allows less stringent base pair matching and allows hybrids to form with more mismatch of base pairing. In the analysis of the nuclear repetitive DNA, it becomes clear that there are several factors which are important for Dictyostelium but which are not critical in the analysis of the kinetics of repetitive DNA in higher eukaryotes. Because of its small genome size (approximately 11 times E. coli) the rRNA cistrons of Dictyostelium comprise at least 10% of the nuclear repetitive sequences. The repetitive fraction may contain some mitochondrial contamination, and it is possible that the nuclear heavy satellite renatures in the repetitive region. As a result it is impossible to

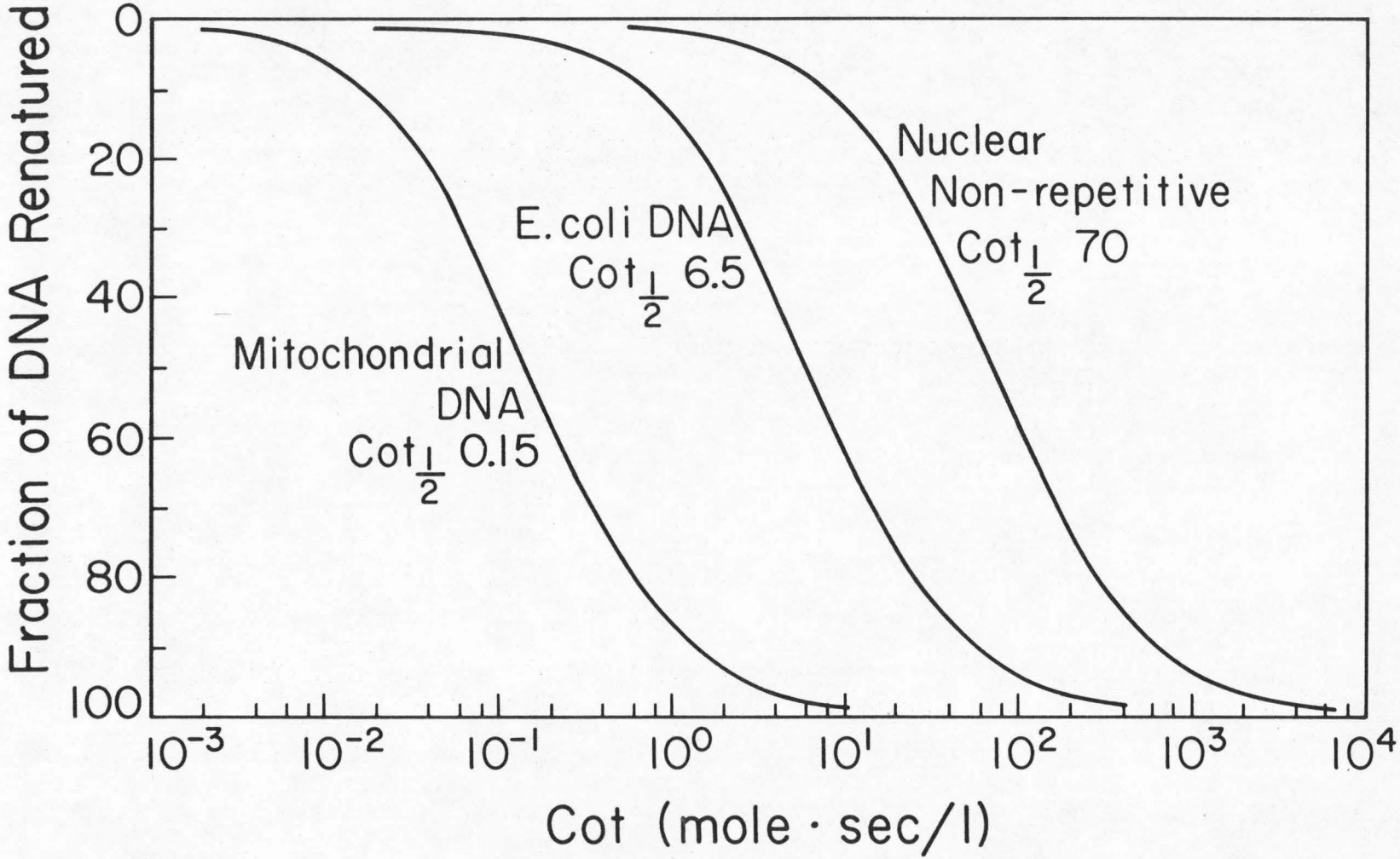
Table II

Observed Reassociation Components of DNA Fractions of Dictyostelium

DNA fraction	Repetitive Component				Non-Repetitive Component			
	$Cot_{1/2}$ observed	% of total hypochromicity	Average $Cot_{1/2}$ pure (est)	Average complexity (daltons)	$Cot_{1/2}$ observed	% of total hypochromicity	Average $Cot_{1/2}$ pure(est)	Average complexity (daltons)
Total cell	0.86	51	0.44	1.3×10^8	170	40	69	20×10^9
Nuclear	1.0	28	0.28	8.1×10^7	113	60	68	20×10^9
Ethidium bromide treated cells	1.13	22	0.25	7.2×10^7	99	69	68	20×10^9
Mitochondrial					0.15 ± 0.015[†]	~92	0.15 ± 0.015	$35\text{-}40 \times 10^6$
Single copy (Fraction II)					70 ± 3[†]	~92	70 ± 3	20×10^9
Repetitive (nuclear Fraction I)								
Component I	0.0057	7	4×10^{-4}	$\sim 10^5$				
Component II	0.19	34	0.065	1.2×10^7				
Component III	3.3	39	1.3	3.8×10^8				

[†]The errors shown represent the range of half Cots observed from Wetmur & Davidson plots.

Fig. 17. Comparative optical Cot plots of mitochondrial, nuclear nonrepetitive, and E. coli DNA. All curves have been normalized and reassociation is plotted 0 to 100.


Fraction of DNA Renatured
0
20
40
60
80
100
Mitochondrial DNA Cot 1/2 0.15
E. coli DNA Cot 1/2 6.5
Nuclear Non-repetitive Cot 1/2 70
10^-3
10^-2
10^-1
10^0
10^1
10^2
10^3
10^4
Cot (mole · sec/l)

estimate the amount of homology between members of a family or to estimate the diversity in family sizes. A very rapidly renaturing component of low complexity and repetition number appears to be present in the nuclear repetitive fraction.

The complexity of Dictyostelium nuclear DNA, 30×10^9 daltons (This number is slightly below the number obtained by Sussman and Rayner (1971) using chemical analysis.), is very low compared to most other eukaryotes. Drosophila melanogaster DNA has complexity approximately 2-3 times greater than Dictyostelium (Laird & McCarthy, 1969). Drosophila DNA also contains a highly repetitive fraction and a middle repetitive fraction comprising approximately 5-15% of the nuclear DNA. The repetition number of the middle repetitive DNA is approximately 50-100 (Laird & McCarthy, 1969; Rae, 1970; Botcham et al., 1971). In other eukaryotes the repetition number of the repetitive DNA varies from a few to many thousand copies (see Britten & Kohne, 1968a; Britten & Davidson, 1971). In Dictyostelium there is probably a small range in the diversity of family sizes. The average repetition number of the middle repetitive DNA is approximately 150.

The renaturation data on Dictyostelium DNA now allow us to study the biological properties of the different components. In another paper (Firtel, 1971) RNA-DNA hybridization studies are described. These analyze the

changes in amount of unique DNA transcript present at various stages of development by hybridization of cell RNA to purified unique DNA. The small genome size of Dictyostelium and, therefore, the low complexity of its single-copy DNA makes DNA-RNA hybridization experiments in solution much more practical than are the equivalent experiments with mammalian or amphibian DNA. The small genome size may also make it easier to analyze and study the mechanisms controlling gene regulation.

Acknowledgements

We would like to thank Drs. Eric Davidson and John Mayfield, Mr. David Holmes, and Miss Jessica Tuchman, California Institute of Technology for their good advice and helpful suggestions during the time the work was being done and for their critical evaluations of the manuscript. We would like to thank Dr. Roy Britten, Carnegie Institution of Washington, for his advice and helpful suggestions, for his generosity in shearing some of the DNA samples, and for supplying the computer program for analysing the reassociation kinetics. We also thank Dr. Jung-Rung Wu , California Instutute of Technology, for the EM analysis of the sheared DNA, Cameron Schlehuber for his help in running the analytical centrifuge and for his assistance with the computer analyses, and Ratchford Higgins for technical assistance on some of the experiments.

During the period this work was done one of us (RAF) was a NSF predoctoral fellow and was also supported by USPHS training grant GM00086. The work was supported in part by USPHS grant GM13762 (to Dr. James Bonner).

REFERENCES

Ashwell, M. & Work, T.S. (1970). Ann. Rev. Biochem. 39, 251.

Attardi, G. & Amaldi, F. (1970). Ann Rev. Biochem. 39, 183.

Bolton, E.T., Britten, R.J., Cowie, D.B., Roberts, R.B., Szahanski, P. & Waring, M.G. (1966). Carnegie Inst. Wash. Yearbk. 64, 313.

Bonner, J.T. (1967). The Cellular Slime Molds. 2nd ed., Princeton University Press.

Bonner, J.T. (1971) Ann. Rev. Microbiol. 25, in press.

Bonner, J., Chalkley, G.R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R.C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B. & Widholm, J. (1968) In Methods in Enzymology XII B, p.3. New York: Academic Press.

Borst, P., Van Bruggen, E.F.J. & Ruttenberg, G.J.C.M. (1968). In Biochemcial Aspect of the Biogenesis of Mitochondria, ed. by E.C. Slater, J.M. Tager, S. Papa & E. Quagliariello, p. 51. Bari: Adriatica Editrice.

Botcham, M., Kram, R., Schmid, C.W. & Hearst, J.E. (1971). Submitted for publication.

Britten, R.J. (1969) In Problems in Biology: RNA in E.W. Hanley, p. 187.

Britten, R.J. & Davidson, E.H. (1971). Quart. Rev. Biol., in press.

Britten, R.J. & Kohne, D.E. (1967). Carnegie Inst. Wash. Yearbk. 65, 78.

Britten,R.J. & Kohne, D.E. (1968a). Science 161, 529.

Britten, R.J. & Kohne, D.E. (1968b). Careg. Inst. Wash. Yearbk. 66, 73.

Britten, R.J. & Smith, J.C. (1970). Carneg. Inst. Wash. Yearbk. 68, 378.

Church, R.B. & McCarthy, B.J. (1968). Biochem. Gen. 2, 55.

Cocucci, S.M. & Sussman, M. (1970). J. Cell Biol. 45, 399.

Davidson, E.H. & Hough, B.R. (1969). Proc. Nat. Acad. Sci. Wash. 63, 342.

Davidson E.H. & Hough, B.R. (1971). J. Mol. Biol. 56, 491.

Davidson, E.H., Hough, B.R., Chamberlin, M.E. & Britten, R.J. (1971). Develop. Biol., in press.

Doty, P., Marmur, J. & Sueoka, W. (1959). Brookhaven Symp. Biol. 12, 1.

Firtel, R.A. (1971). Submitted for publication.

Firtel, R.A. & Bonner, J. (1971). Submitted for publication.

Flavell, R.A. & Jones, I.G. (1970). Biochem. J. 116, 811.

Flavell, R.A. & Jones, I.G. (1971). Biochem. Biophys. Acta 232, 255.

Gelderman, A.H., Rake, A.V. & Britten, R.J. (1969) Carnegie Inst. Wash. Yearbk. 67, 320.

Gelderman, A.H., Rake, A.V. & Britten, R.J. (1971) Proc. Nat. Acad. Sci. Wash. 68, 172.

Gerish, G. (1962). Exptl. Cell Res. 26, 462.

Gillespie, D. & Spiegelman, S.(1965). J.Mol.Biol. 8, 29.

Gillis, M., De Ley, J. & De Cleene, M. (1970). European J. Biochem. 12, 143.

Gregg, J.H. (1950). J. Exptl Zool. 114, 173.

Gregg, J.H. & Badman, N.S. (1970). Devel Biol. 22, 96.

Hohl, H.R. & Hamamoto, S.T. (1969). J. Ultrastruct. Res. 26, 442.

Hollenberg, C.P., Borst, P. & Van Bruggen, E.F.J. (1970). Biochem Biophys. Acta 209, 1.

Horwitz, H.B. & Holt, C.E. (1971).J.Cell Biol. 49, 546.

Jensen, R.H. & Davidson, N.(1966). Biopolymers, 4, 17.

Iwabuchi,M., Mizukami, G.& Sameshima, M.(1971). Biochim. Biophys. Acta 228, 693.

Kohne, D.E. (1968). Biophys. J. 8, 1104.

Laird, C.D. & McCarthy, B.J. (1969). Genetics 63, 865.

Loomis, W.F., Jr. (1970). J. Bacteriol. 103, 375.

McCarthy, B.J. & McConaughy, B.L.(1968). Biochem. Gen. 2,37.

McConaughy, B.L.,Laird, C.D. & McCarthy, B.J. (1969). Biochemistry, 8, 3289.

Maeda, Y. & Takeuchi, I. (1969). Devel. Growth Diff. 4, 232.

Mahler, H.R. & Dutton, G.J. (1964). J. Mol. Biol. 10, 157.

Marmur, J. & Doty, P. (1962). J. Mol. Biol. 5, 109.

Martin, M.A. & Hoyer, B.H. (1966). Biochemistry 5, 2706.

Nandi, M.S., Wang, J.C. & Davidson, N. (1965). Biochemistry, 4, 1687.

Newell, P.C. & Sussman, M.(1970). J. Mol. Biol. 49, 627.

Rae, P.M.M. (1970). Proc. Nat. Acad. Sci. Wash. 67, 1018.

Raper, K.B. (1935). J. Agr. Res. 50, 135.

Raper, K.B. (1940). J. Elisha Mitchell Sci.Soc. 56,241.

Radloff, R., Bauer, W. & Vinograd, J. (1967). Proc. Nat. Acad. Sci. Wash. 57, 1514.

Roth, R.M., Ashworth, J.M. & Sussman, M. (1968). Proc. Nat. Acad. Sci. Wash. 59, 1235.

Schildkraut, C.L., Marmur, J. & Doty, P. (1962) J. Mol. Biol. 4, 430.

Schmidt, G. & Tannhauser, S.J. (1945). J. Biol. Chem. 161, 83.

Smith, C.A., Jordan, J.M. & Vinograd, J. (1971).In press.

Sussman, M. (1966). Methods in Cell Physiology, ed. by D. Prescott, vol. 2, p. 397. New York: Academic Press, Inc.

Sussman, R. & Rayner, E.P. (1971). In press.

Takeuchi, I. (1963). Develop. Biol. 8, 1.

Telser, A. & Sussman, M. (1971). J. Biol. Chem. 246, 2252.

Ts'o, P.O.P. & Sato, C. (1959). Exptl. Cell Res. 17, 227.

Waring M. & Britten, R.J. (1966). Science 154, 791.

Watts, D.J. & Ashworth, J.M. (1970). Bioche . J. 119, 171.

Wetmur, J.B. & Davidson, N. (1968). J. Mol. Biol. 31, 349.

Wolstenholme, D.R. & Gross, N.J. (1968). Proc. Nat. Acad. Sci. Wash. 61, 245.

Wood, D.D. & Luck, D.J.L. (1969). J. Mol. Biol. 41, 211.

Appendix

Short review on DNA-DNA reassociation kinetics.

The reassociation of simple DNAs such as T4 and *E. coli* has been shown by Marmur and Doty (1961) and more recently by Bolton *et al.* (1965), Britten and Kohne (1967; 1968a,b), and Wetmur and Davidson (1968) to follow second order reaction kinetics. Since the reassociation reaction is second order and since the concentrations of the complementary strands are equal the rate equation for renaturation is $dC/dt = -k_2C^2$. Integration yields $C/C_o = \frac{1}{1 + k_2\ Cot}$ where C is the concentration of single strand DNA at time t (Britten and Kohne, 1967; 1968a,b). A double reciprocal plot of this equation yields a straight line with the slope proportional to the second order rate constant k_2 (Wetmur and Davidson, 1968). When half of the DNA is renatured, i.e., $C = \frac{1}{2} C_o$ then $Cot_{\frac{1}{2}}$ (the Cot at half renaturation)is equal to $1/k_2$. Thus k_2 is inversely proportional to the complexity of the specific portion of the DNA which renatures with a given $Cot_{\frac{1}{2}}$.

For simple DNAs composed of a single second order reaction component, in which each nucleotide sequence is present only once, the complexity or genome size relative to a standard genome (*E. coli* for example) can be obtained directly from a second order renaturation constant since

complexity of the DNA is inversely proportional to the second order rate constant (Wetmur and Davidson, 1968; Britten and Kohne, 1968a). DNA-DNA reassociation kinetics of the DNA of all higher eukaryotes studied thus far indicate that at least two or more components of different degrees of complexity are always present (see Britten and Kohne, 1967, 1968a,b). Thus eukaryotic genomes include both a repetitive component and a single copy (nonrepetitive or unique) component. Repetitive DNA is composed of DNA sequences, either isologous or homologous (very similar), which are present in more than one copy per genome. Each such group of isologous or homologous DNA copies constitute a family of sequences (Britten and Kohne, 1968a). A homologous DNA sequence is one in which the majority of the bases pair at a given criterion (temperature and salt conditions of the reannealing) such that under the conditions used a stable hybrid is formed. These homologous sequences can be differentiated from isologous ones (exact copies a multiple mitochondrial and ribosomal cistrons) by virtue of this lower efficiency of base pairing and the resulting base mismatching causes them to form duplexes of lower Tm than the native DNA. In addition they exhibit decreased hyperchromicity and increased noncooperativity of melting profile (Britten and Waring, 1965; Britten and Kohne, 1967, 1968a,b). The proportion of redundant or reiterated DNA can also vary

appreciably between species in the same genus (Laird and McCarthy, 1969).

It should be noted that the apparent amount of homologous reiterated DNA varies depending upon the criterion used. Normally the reaction is run approximately 25° below the melting temperature of the DNA. It has been shown that as the stringency is reduced, to say 35° below Tm, the amount of sequences showing similarity increases (Martin and Hoyer, 1966; McCarthy and McConaughy, 1968; Church and McCarthy, 1968; Davidson et al., 1971). This is due to the fact that the lower temperature allows less accurate base pair matching and hybrids form with more mismatched base pairs. The repetitive sequences also may be short (on the order of 50 base pairs) as is indicated in rat , and lower Tm observed for repetitive sequences may be due to the short length (Holmes et al., 1971). As shown by Wetmur and Davidson (1968) the rate of reassociation of a single copy DNA, i.e. T4 or E. coli DNA is approximately constant between 16 and 32° below the melting temperature.

Therefore from renaturation kinetics at a specific criterion one obtains an estimation of the number of second order components (families of nucleotide sequences which can be distinguished from each other by the number of copies in a family), the complexity of the components, and from the melting profile of isolated fractions the

size and/or homology (amount of base mismatching in the hybrid)(see Britten, 1970; McCarthy and Church, 1970).

Extra References

Britten, R.J. & Waring, M.C. (1965). Carneg. Inst. Wash. Yearbk. 64, 316.

Holmes, D., Mayfield, J., Murthy, L. & Bonner, J. (1971). Submitted for publication.

McCarthy B.J. & Church, R.B. (1970). Ann. Rev. Biochem. 39, 131.

Marmur, J. & Doty, P. (1961). J. Mol. Biol. 3, 585.

CHAPTER II

CHANGES IN THE EXPRESSION OF SINGLE-COPY DNA DURING DEVELOPMENT OF THE CELLULAR SLIME MOLD *DICTYOSTELIUM discoideum*

CHANGES IN THE EXPRESSION OF SINGLE-COPY DNA DURING DEVELOPMENT OF THE CELLULAR SLIME MOLD *Dictyostelium discoideum*

Richard A. Firtel*

Division of Biology, California Institute of Technology

Pasadena, California 91109

Running Title: Changes in single-copy DNA transcripts

*Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

SUMMARY

RNA extracted from four stages during the developmental cycle of the cellular slime mold Dictyostelium discoideum was hybridized with purified single-copy nuclear DNA. The hybrids formed were shown to have the high thermal stability indicative of single-copy DNA-RNA hybrids and the DNA extracted from them reassociates with single-copy DNA.

The hybridization studies indicate that there are, during development, qualitative and quantitative changes in the portion of the single-copy portion of the genome transcribed. Overall, 56% of the genome (approximately 16-17 x 10^6 nucleotide pairs) are represented by transcripts between the amoeba and midculmination stages. Some 18-20% of the genome consist of sequences which are represented at all stages while approximately 36-38% of the genome consist of sequences which are represented only at particular developmental stages. The possible uncertainties of these numbers are discussed.

INTRODUCTION

The cellular slime mold Dictyostelium discoideum is a simple eukaryote which undergoes development. In the developmental cycle, one cell type, a vegetative amoeba, differentiates into two new, non-dividing cell types, stalk and spore cells. Developmental is initiated by exhaustion of the food supply. Under laboratory conditions this process occurs synchronously between individual aggregates, and mature fruits are formed in 26 hours at 22°C (see Bonner, 1967).

Dictyostelium regulates the appearance and disappearance of at least 15 enzymes during development (see Loomis, 1970; Telser & Sussman, 1971; Firtel & Bonner, 1971). Studies with cycloheximide and actinomycin D suggest that the genes coding for these enzymes are turned on and off during development. Changes in these enzymes' activities are not restricted to any particular stage of development but occur throughout. Several developmentally regulated enzymes begin to increase in amount as soon as food is removed, and one (alanine transaminase, Firtel & Brackenbury, 1971) reaches peak activity within four hours. Several others do not begin to increase in amount until 18 hours after the food has been removed, and reach maximum activities between 24 and 26 hours (Loomis, 1969). The decrease as well as the increase in the activities of several enzymes require prior RNA synthesis and is inhibited by

cycloheximide. The RNA synthesis necessary for this decrease in activity takes place quite late in the developmental process (Roth et al., 1968; Firtel & Bonner, 1971).

Differentiation is reversible until quite late in developmental cycle until the stalk cells become vacuolated and die. To that point, prestalk and prespore cells can "dedifferentiate" into vegetative amoebae if given a food source. Prespore cells can also be made to develop into stalk cells and vice versa (see Bonner, 1967). These facts and those outlined above concerning enzyme studies indicate that Dictyostelium regulates its development and indicate that most probably new sequences of DNA are successively transcribed as development progresses.

This paper concerns an analysis of changes during development in the RNA transcripts of the unique portion of the Dictyostelium genome. To this end, RNA was extracted from various developmental stages of Dictyostelium and hybridized in solution to the purified non-repetitive DNA of the Dictyostelium genome . The amount of DNA which hybridizes to such RNA at high RNA:DNA ratio is an indication of the portion of the genome which is represented as RNA transcript at that particular stage of development. I have analysed the changes in these transcripts at each successive stage. There are changes in the transcripts of the unique portion of the genome at

each developmental stage and, further, that Dictyostelium uses an estimated 50-60% of its single-copy DNA during the first 20 hours of development.

MATERIALS AND METHODS

Culturing of cells

Strain AX-3 (Dictyostelium discoideum) was derived by William F. Loomis, Jr. (University of California, San Diego, La Jolla, California), from wild type Dictyostelium discoideum NC-4, first described by Raper (1935, 1940). Strain AX-3 can grow in a semi-defined medium, HL-5 (Cocucci & Sussman, 1970; Watts & Ashworth, 1970), with a generation time of 8 to 12 hours. For further analysis of the growth conditions of this strain, see Firtel (1971).

To obtain cells at a given developmental stage, late logarithmically-growing cells were harvested by centrifugation at 2,000 rev./min in a Servall SS-34 rotor for 3 minutes and washed twice with 0.2% NaCl. The cells were then suspended in PDF (0.04 M sodium phosphate buffer, pH 6.5, 1 g/l. KCl, 0.61 g/ml. $MgSO_4$, 0.5 g/l. streptomycin sulfate, Sussman, 1966), placed in a nutrient-deficient environment to initiate development. Three Whatman #3 filter papers, 12.5 cm in diameter, were placed in a 15 cm diameter plastic petri dish. One 12.5 cm Whatman #50 filter paper was placed on top of these and all were saturated with PDF solution. Excess PDF solution was removed and approximately 1×10^9 cells (4 ml. of 2.5×10^8 cells/ml.) were applied evenly to the Whatman #50 filter paper. Cells were allowed to settle and extra

PDF was added until the proper saturation of the pads was achieved. Each petri dish was covered with Saran Wrap and its top then pressed down upon it to obtain a high humidity chamber. The petri dishes were incubated at 22^{o} until the desired developmental stage was reached. These incubation conditions are similar to those previously described by Newell & Sussman (1969) and Sussman (1966). This method has the advantage that a large number of cells can be developed synchronously to any required stage. Normally, 50 such petri dishes were used with a total of about 5×10^{10} cells. On these large pads development is synchronous.

Extraction of the RNA

Logarithmically growing cells in axenic culture are collected by centrifuging the cells at 3,000 rev./min for 5 min in a Servall SS-34 rotor. The pellets are then washed twice by repelleting with 7% sucrose. Cells developing on Whatman #50 filters were harvested by washing the cells off of the filters using a 10 ml. medium bore pipette. Cells were harvested in 7% sucrose, 1 mM Na-EDTA, 0.01 M Tris, pH 8. The harvested cells were centrifuged at 3,000 rev./min for 5 min and the pellet washed twice with the sucrose-EDTA-Tris buffer. RNA was extracted from the cells by first dissociating the pellet in ice-cold 0.025 M Tris buffer, pH 8 (100 ml./5×10^{10} cells). One-tenth volume 20% SDS was added and the cells homogenized at 4^{o}C with a motor driven glass-Teflon homogenizer.

After the cells appeared to be lysed and homogenized, the mixture was made 2-3% (v/v) with respect to diethyl pyrocarbonate (Eastman Kodak) and rehomogenized (Solymosy et al., 1968). The homogenization mixture was allowed to stand at 0°C for approximately 5 min, and an equal volume of water-saturated, redistilled phenol containing 0.1% 8-hydroxyquinoline added. The mixture was extracted for 10 min at 60°C (swirled with intermittent shaking), and centrifuged at 15,000 rev./min for 10 min. The upper, aqueous phase was removed, added to an equal volume of phenol, and reextracted at 60°C. The interphase and lower phenol phase were added to an equal volume of 0.025 M Tris buffer and extracted at 75° for 10 min. After the two solutions were centrifuged at 15,000 rev./min for 10 min, the upper aqueous phases were combined and made 0.5 M with respect to sodium acetate (NaAc), pH 5. The interphases and phenol phases were combined, an equal volume of 0.025 M Tris added, and the solution made 0.5 M with respect to NaAc, pH 5. The aqueous phase mixture was reextracted at 60°C and the interphase and phenol mixture again extracted at 75° for 10 min. After extractions and centrifugation at 15,000 rev./min for 15 min, the aqueous phases were combined, an equal volume of phenol added, and the mixture was extracted twice at 4°C for 20 min. The phenol phase and interphase were discarded. After centrifugation, 2-1/2 volumes of absolute ethanol were added to the aqueous phase.

This was allowed to precipitate at -20°C for at least 6 hours. A dense granular precipitate containing the RNA and polysaccharide was obtained. The precipitate was collected by centrifugation and redissolved at a 0.01 M EDTA, pH 5, made 0.5 M with respect to NaAc and reprecipitated with 2-1/2 volumes of absolute ethanol. The precipitate was again collected by centrifugation and dissolved in TNM buffer (0.01 M Tris, 0.01 M NaCl, 0.01 M $MgCl_2$, pH 8). Approximately 40 ml. of TNM buffer are used for RNA (approximately 200-250 mg) extracted from approximately 5×10^{10} cells. 100 ug/ml. of electrophoretically purified DNase I (ribonuclease-free, Worthington Chem. Co. Freehold, N.J.) was added and the solution incubated at 37°C for 60-90 min. 1 mg/ml. pronase (Calbiochem) (predigested 30 min, 4 mg/ml. TNM buffer, 37°C) and 1/20 vol. of 0.25 M EDTA, pH 8, were added and the solution incubated for 1½ hours at 37°C. The mixture was made 0.5 M with respect to KAc, pH 6, and extracted 2 or 3 times with redistilled phenol at 4°C. After centrifugation, the aqueous phase was removed and 2-1/2 volumes of absolute ethanol were added. The precipitate was collected as previously described, dissolved in 0.01 M EDTA, pH 8, made 0.5 M with respect to KAc, and reprecipitated. The RNA-polysaccharide pellet was dissolved in 0.025 M Tris buffer, pH 8, at a concentration of approximately 5 mg/ml. RNA, 1/3 volume of 5 M potassium phosphate, pH 8, was added and the solution allowed to stand for several minutes at 0°C. A ½ volume of ice

cold 2-methoxyethanol was added dropwise with vigorous stirring. After vortexing or shaking vigorously for several minutes the mixture was centrifuged at 15,000 rev./min for 10 min. The upper phase was removed leaving behind the interphase and the lower phase which contain much of the polysaccharide contamination. The aqueous upper phase was diluted with an equal volume of water and made 0.1 M with respect to KAc. Approximately 1/10 volume of 1% CTAB (cetyltrimethyl ammonium bromide) in 0.05 M KAc was added with stirring. After standing for approximately 5 min, approximately ½ volume of CTAB was added with stirring. The white precipitate was allowed to form, and after standing for 30 min in ice the mixture was centrifuged at 15,000 rev./min for 15 min. The upper phase was discarded and the pellet retained. This procedure has been shown by Bellamy & Ralph (1968) to remove polysaccharide from nucleic acid preparations. Controls using labeled Dictyostelium rRNA and high molecular weight nuclear RNA obtained from rat ascites tumor (supplied by David Holmes) have shown that the RNA is quantitatively recovered. The pellet was suspended in 0.01 M EDTA, pH 8, made 1 M with respect to KAc, and 2-1/2 volumes of ethanol were added. The RNA was precipitated at -20°C for at least 6 hours. The RNA precipitate, free of much of the polysaccharide, was collected by centrifugation, redissolved in 0.01 M EDTA and 1 M KAc and reprecipitated with ethanol. This step was

repeated 3 additional times, except that during the last step it was made 0.5 M with respect to NaAc. The RNA pellet, obtained by centrifugation, was allowed to drain thoroughly, dissolved in water, and lyophilized to dryness. The RNA was dissolved by heating in 0.48 M phosphate buffer containing 2 mM TES (Calbiochem) buffer, pH 6.8. The phosphate buffer (PB), equimolar disodium and monosodium phosphate, had previously been passed through a column of Chelex 100 (Bio-Rad) to remove divalent cations (Davidson & Hough, 1971). The RNA was normally stored at -20°C, or for longer periods of time at -70°C. The yield of RNA as extracted by this method is over 95% of the total RNA determined by Ts'o and Sato's (1959) modification of the Schmidt and Tannhauser (1948) procedure.

Labeling of RNA

Axenic cells were labeled with carrier-free $^{32}PO_4$ in PO_4-free HL5 medium. The cultures were inoculated with a 1:250 dilution from a normal HL5 stock culture.

Purification of DNA

Axenically growing cells in PO_4-free HL5 medium were labeled midway through logarithmic growth with 80 mC/liter of carrier-free $^{32}PO_4$. The cells were labeled for 1 to 2 days after which the DNA was extracted according to the method of Firtel (1971). The DNA was sheared at 50,000 lbs/sq.in. in a French press. This was done either in a Sorvall Ribi refrigerated cell fractionator (French Press) or, generously by Dr. Roy Britten

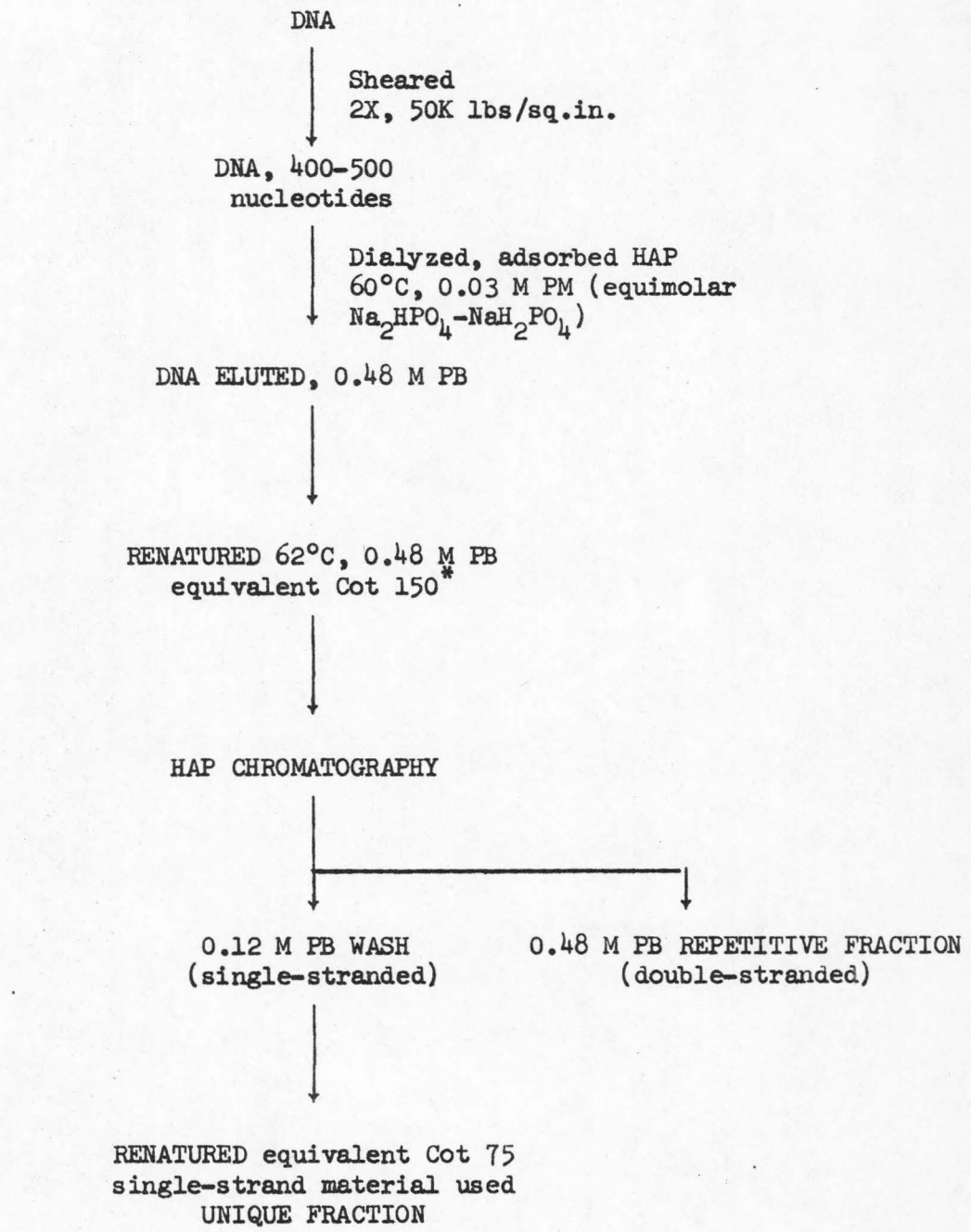
(Department of Terrestrial Magnetism, Carnegie Institution of Washington) in a specially built French press. The press produces DNA pieces of uniform size, weight average of approximately 300-400 nucleotides (see Firtel, 1971), as determined by alkaline sedimentation and electron microscopy of denatured DNA. The electron microscopy was performed by Jung-rung Wu. The sheared DNA was filtered and then exhaustively dialyzed against 0.01 M EDTA, 1 M NaCl, pH 8, dialyzed against 0.12 M PB, water, and lyophilized. It was dissolved in 0.03 M PB, adsorbed to hydroxyapatite at 60°C, and the DNA eluted with 0.48 M PB. The purification of the single-copy DNA of the nuclear genome has been previously described (Firtel, 1971). Figure 1 summarizes the method.

In solution RNA-DNA hybridization procedure

Purified unique DNA with a specific activity approximately 80,000 to 120,000 cts/min/ug was added to a known quantity of purified RNA from all of the various stages. The reaction mixture was sealed in a Reacti-Vial (Pearce Chemical Co.). These vials have a small conical chamber of known volume. The vials were sealed tightly with a Teflon-lined screw top and wrapped in plastic electrical tape which is waterproof at the temperatures used. The small volume of the vials eliminates the problem of evaporation and condensation of water on the sides of the vial. The reaction mixture was heated at 98°C for 4 min and placed

Fig. 1. Flow sheet for purification of unique DNA.

FLOW SHEET FOR PURIFICATION OF UNIQUE DNA



*Cot has the dimensions of moles nucleotides/liter x time of annealing in seconds. Equivalent Cot is a Cot value corrected for the renaturation rate differences at salt concentrations other than 0.12 M PB according to Britten and Smith (1970).

in a water bath at 62°C for incubation. After incubation for a length of time to achieve the desired Cot the vial was removed and placed in an ice-water solution for immediate cooling to stop the reaction. The hybridization mixture was then purified as described in the figure legends and Results section.

Hydroxyapatite chromatography

Hydroxyapatite (HAP) chromatography was performed according to the method described by Firtel (1971). Bio-Gel HT hydroxyapatite (Bio-Rad) in 0.001 M phosphate buffer was used. The HAP was thoroughly suspended and added to a water-jacketed column maintained at 60°C. The column was washed with the appropriate concentration of PB (see Results). Normally the column was reused for several experiments if these were done over a 3 or 4 hour period. Otherwise the columns were discarded. To regenerate a column after use, it was thoroughly washed in 0.48 M PB, 0.2% SDS and the HAP resuspended within the column. The column was washed exhaustively with 0.12 M PB, 0.2% SDS and again suspended.

RESULTS

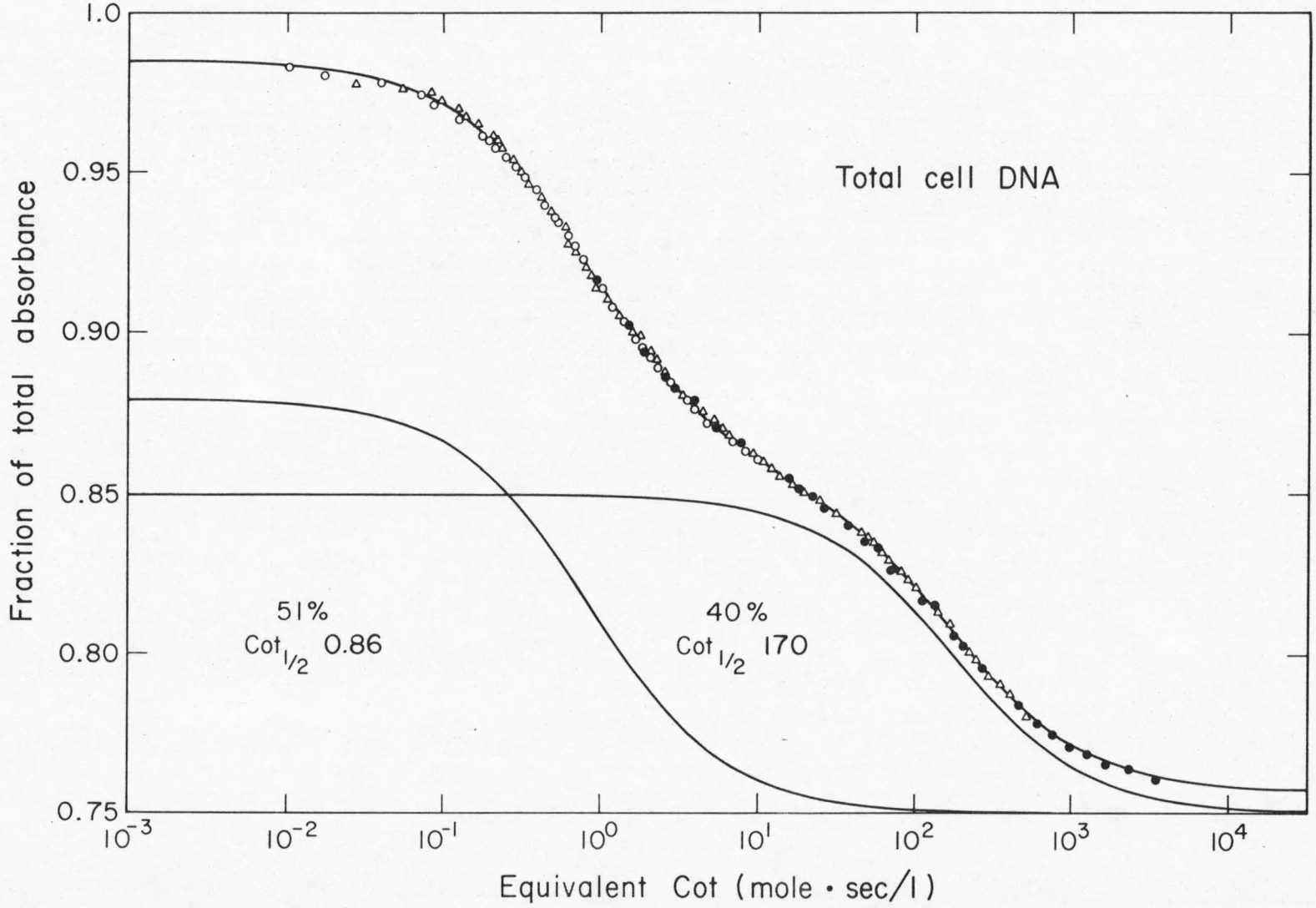
Figure 2 shows an optical DNA-DNA renaturation profile of Dictyostelium discoideum total cell DNA resolved into second order reaction components by at least square fitted computer program supplied by Dr. Roy J. Britten, Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D.C. As has been shown for other eukaryotes, the genome of Dictyostelium can be divided into repetitive and unique DNA fractions according to its reassociation kinetics. The first fraction is a repetitive or reiterated fraction in which sequences renature at an average rate approximately 200 times faster than would be the case if they were present in only one copy per genome. The second fraction is the unique (single-copy or non-repetitive)DNA. This DNA renatures at a rate which indicated that each sequence is present only once per genome. The values given are the fraction of the total hypochromicity for each individual component and the observed equivalent Cot (mole nucleotide·seconds/liter) for $\frac{1}{2}$ renaturation of each individual fraction suggested by the computer analysis (see Firtel, 1971, for a further description of these results). The analysis suggests that the Dictyostelium non-repetitive DNA represents approximately 40% of the hypochromicity of the total cell DNA and exhibits a $Cot_{\frac{1}{2}}$(pure) of 70, as determined by optical

Figure 2. Optical renaturation profile of total cell DNA. The curve has been analyzed by a least square curve fitting computer program (Britten and Kohne, 1968). The values given are the fraction of the hypochromicity in the two components and the estimated $Cot_{\frac{1}{2}}$ (pure) suggested by computer analysis. See Firtel (1971) for details on the method.

o 40.8 ug/ml/. 0.12 M PB, 55°C

Δ 100 ug/ml., 0.24 M PB, 59°C

● 1000 ug/ml., 0.24 M PB, 59°C


1.0
0.95
0.90
0.85
0.80
0.75
Fraction of total absorbance
Total cell DNA
51%
Cot1/2 0.86
40%
Cot1/2 170
10-3
10-2
10-1
100
101
102
103
104
Equivalent Cot (mole • sec/l)

techniques. This represents a genetic complexity 7 times greater than E. coli, i.e. 2.0×10^{10} daltons or 2.9×10^{7} nucleotide pairs (Cairns, 1963).

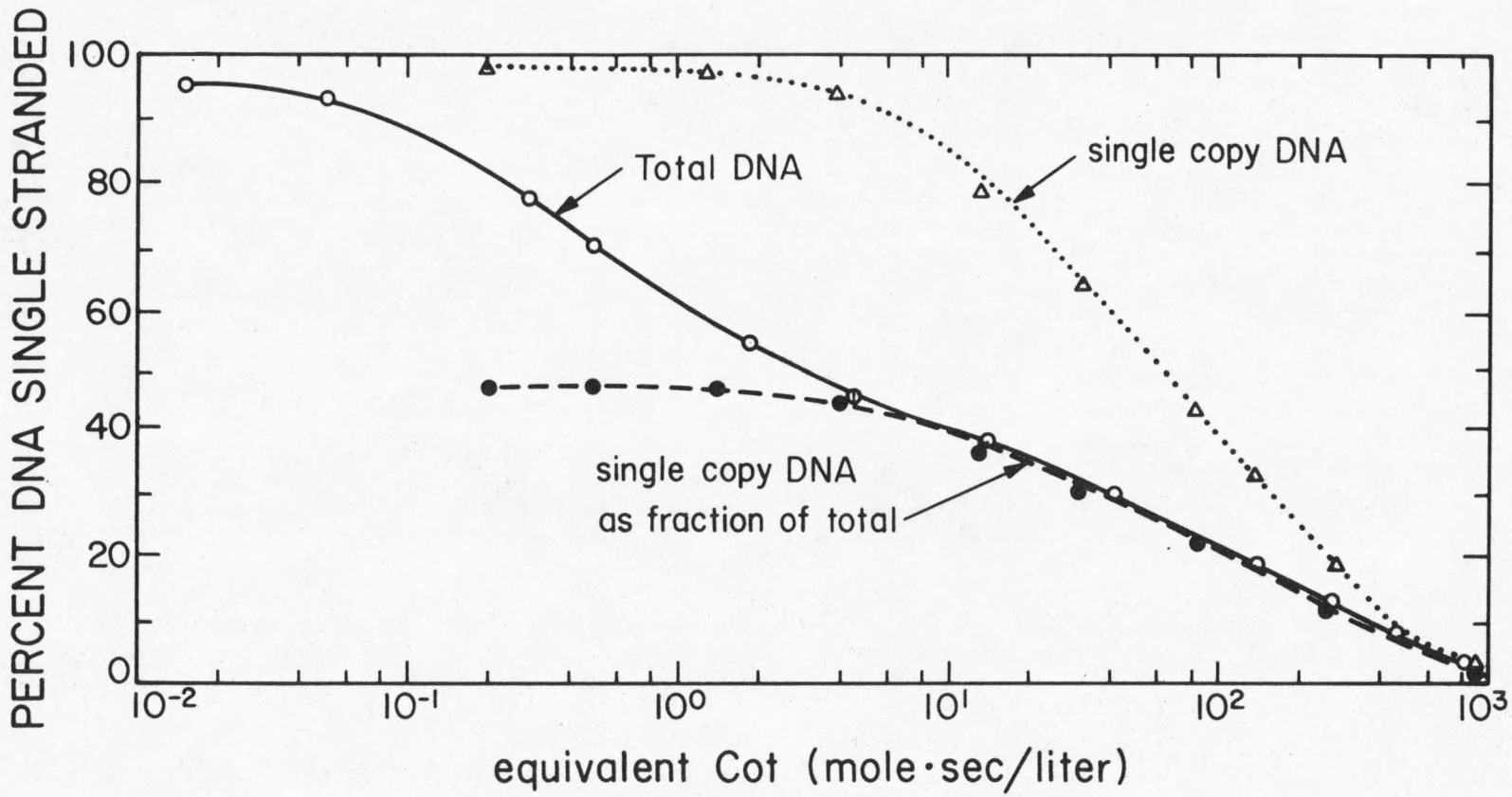
Figure 3 shows the renaturation kinetics of $^{32}PO_4$-labelled purified unique DNA, hybridized to an excess of total cell DNA. By adding a small amount of labeled non-repetitive DNA to a large amount of carrier total cell DNA (ratio of 1:1000) one can determine whether or not some amount of the non-repetitive DNA fraction hybridizes with the repetitive DNA. As can be seen in Figure 3, the unique DNA fraction behaves as a single second-order reaction component. Computer analysis suggests that the single-copy DNA represents approximately 44% of the total cell DNA. It exhibits a $Cot_{\frac{1}{2}}$ (pure) of 30-35 on HAP which indicates that most likely the purified unique DNA fraction is essentially free of repetitive sequences. It should be remembered that the non-repetitive fraction does contain in the limit at least one copy each of each sequence of the repetitive DNA. Thus one expects a very small amount of the DNA of the non-repetitive fraction to hybridize to repetitive sequences. Since approximately 56% of the total cell DNA represents repetitive sequences, and since these sequences are present at an average repetition of approximately 200, at least 0.8% of the total unique DNA must consist of repetitive sequences.

Figure 3. Hybridization of tracer amounts of ^{32}P-labeled single-copy DNA to total cell DNA. A ratio of 1000-1, total cell DNA:single-copy DNA (100,000 cts/min/µg) was used. For points below Cot 0.4, 50 ug DNA (25 ug/ml.) were used in 0.12 M PB at 56°C. For points between Cot 0.4-10, 100 µg at 100 µg/ml. were used. For points above Cot 10, 100 µg DNA at 500 µg/ml. in 0.24 M PB at 59°C. HAP Cot curve of renaturation of total cell DNA was done in parallel experiments using labeled total cell DNA (2,000 cts/min/µg).

o——o Total cell DNA

Δ···Δ Single-copy DNA (tracer)

●——● Single-copy DNA (tracer) data plotted as fraction of total DNA assuming that it represents 45% of the cell DNA.


PERCENT DNA SINGLE STRANDED
100
80
60
40
20
0
Total DNA
single copy DNA
single copy DNA
as fraction of total
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
equivalent Cot (mole·sec/liter)

Estimation of fraction of RNA hybridizing to various DNA fractions

We next consider what fraction of RNA hybridizes to the repetitive and to the unique fraction of total cell DNA. A large excess of DNA was renatured in the presence of trace amounts of highly radioactive RNA. In this experiment, total cell RNA labeled for two days was used (approximately four generations). 100 ug of DNA were renatured in solution with 0.1 ug of labeled RNA. A thousandfold excess of DNA to RNA was chosen to insure an excess of ribosomal DNA cistrons over ribosomal RNA sequences. Hybridization saturation experiments indicate that approximately 2% of the genome consists of ribosomal RNA cistrons (Firtel, 1971), and assuming that the majority of labeled RNA is ribosomal RNA, the excess of DNA complementary to rRNA over the total RNA is 10:1 (0.02 x 100/2:0.1). The effective excess of DNA over RNA for other sequences including mitochondrial is probably much greater. The DNA was allowed to reassociate to various Cots at 23° below T_m. The incubation mixture was then treated as described in the legend of Figure 4 (see Fig. 4 and Table I), and the RNA-DNA hybrids and DNA-DNA duplexes separated from unhybridized material by hydroxyapatite chromatography. Experiments by Gelderman et al. (1969, 1971) and Davidson and Hough (1969, 1971) have shown that RNA-DNA hybrid duplexes behave like DNA-DNA duplexes on hydroxyapatite. Table I and Figure 4 show the results of

Figure 4. Hybridization of tracer amounts of ^{32}P whole cell RNA to total cell DNA. The conditions are given in Table I.

Methods:

After cooling the sample was treated with ribonuclease (20 ug/ml. of RNase A, Worthington Biochemical Corp., Freehold, N.J., in phosphate buffer) for 30 minutes in 0.24 M PB. The material was diluted to 0.12 M PB, made 0.5% SDS and applied to a HAP column. The column was washed with 0.12 M PB and hybrid eluted with 0.48 M PB. Controls showed that if RNA is treated with ribonuclease and then applied to hydroxyapatite all of it is eluted in the 0.12 M PB wash.

o——o HAP Cot curve total cell DNA

Δ --- Δ Fraction of input RNA adsorbed to HAP at a particular DNA Cot.

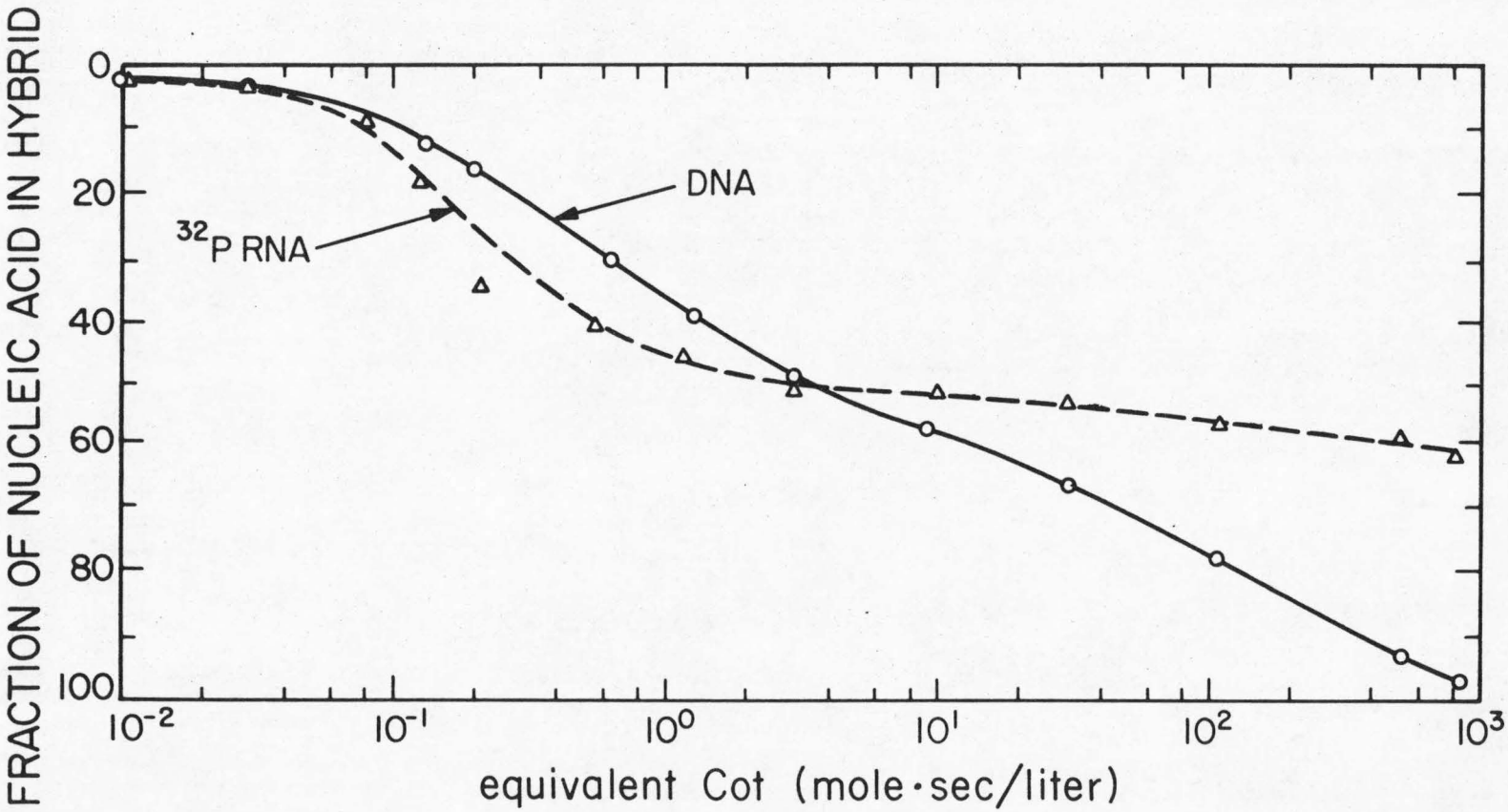

FRACTION OF NUCLEIC ACID IN HYBRID
0
20
40
60
80
100
DNA
32P RNA
10-2
10-1
100
101
102
103
equivalent Cot (mole·sec/liter)

Table I

Reaction conditions for tracer RNA hybridization curve shown in Figure 4

Equivalent Cot	Concentration DNA (μg/ml.)	Concentration PB	Hours Incubated	Fraction DNA Annealed	Fraction DNA in Hybrid
0[1]	20	0.09	0	2	2
.029[1]	20	0.12	0.12	5	3
0.83[1]	20	0.12	0.33	9	11
0.14[1]	20	0.12	0.75	16.5	38
0.6[2]	40	0.12	1.2	30	45
3.0[2]	80	0.24	1.0	48	51
10.0[2]	80	0.24	3.3	58	53
30.0[2]	80	0.24	10	66	54
108.0[2]	160	0.36	12	77	58
540.0[2]	400	0.36	24	93	60
800.0[2]	400	0.48	28.8	98	61

Conditions: 23-25°C below T_m of DNA. Ratio DNA:RNA constant 1000:1.

[1] 50 μg DNA:0.05 μg RNA

[2] 100 μg DNA: 0.1 μg RNA

experiments such as outlined above. Approximately 90% of the RNA which hybridized to DNA reassociated at DNA Cots less than 10. It is unclear why only 60% of the RNA was retained as hybrid. The results suggest that some 5-10% of RNA hybridizes to DNA reassociating in the single-copy region. Most of the hybridized RNA renatures to the repetitive DNA fraction. This includes mitochondrial, nuclear reiterated, and ribosomal DNA.

Analysis of RNA-DNA hybrids

To determine the amount of non-repetitive DNA which is homologous to the RNA sequences present during logarithmic growth (called "0-hour cells" in this paper), a RNA-driven saturation experiment was carried out. In this experiment increasing amounts of RNA were reacted with small amounts of single-copy DNA under conditions such that the rate of hybrid formation is controlled by the concentration of the RNA sequences homologous to the DNA. The results of such an experiment are shown in Figure 5. The percent DNA in DNA-RNA hybrid is plotted vs. the equivalent Rot. (Rot is an abbreviation standing for RNA concentration in moles of nucleotide per liter x time of annealing in seconds.) The equivalent Rot was not corrected for viscosity which is appreciable at higher RNA concentrations. Table II shows the conditions of the reaction indicating the total RNA to unique DNA ratios used. In these experiments, the DNA is labeled and the RNA is unlabeled, and one measures total amount of DNA

present in duplex form as determined by HAP chromatography. Saturation is reached when the concentration of all the RNA molecules present complementary to the single-copy DNA are in sufficient concentration to hybridize to all the complementary DNA sequences during the reaction period. Saturation approached only at higher Rots.

Because this technique measures total duplex DNA it was necessary to determine how much is DNA-DNA duplex and how much RNA-DNA hybrid. This was done in two ways. First, if DNA is present as a contaminant in the RNA preparation, it would increase the DNA Cot and result in increased amount of DNA-DNA duplexes. A sample of RNA was treated with RNase A prior to incubation with labeled single-copy DNA. As shown in Table III, approximately 4% of the DNA is present in duplex form. If DNA is incubated without RNA approximately 5% is present as duplex as is expected for single-copy DNA having a $Cot_{\frac{1}{2}}$ or 30-35. Most probably the discrepancy results from an increase in the viscosity of the RNA samples due to polysaccharide contamination which reduces the rate of the reaction (Wetmur & Davidson, 1968). These results indicate that the RNA preparations are not contaminated with DNA. To determine the exact amount of DNA-DNA duplex in total hybrid a second method was used. The hybrid was eluted from

Table II

Reaction conditions for 0 hour RNA-DNA hybridization saturation curve shown in Figure 5

RNA Concentration mg/ml.	Hours Incubation	DNA (µg)	RNA:DNA	Volume ml.	Equivalent Rot (x 10^{-3})	% DNA in DNA-RNA Hybrid
2	9	0.6	670:1	0.2	0.9	3.6
4	9	0.6	1300:1	0.2	1.8	5.5
8	9	0.6	2700:1	0.2	3.7	9.7
8	13	0.6	4000:1	0.3	6.0	12.3
16	13	0.6	8000:1	0.3	12	13.6
16	24	0.3	16000:1	0.3	22	14.4
16	48	0.3	32000:1	0.6	45	15.1
16	96	0.3	64000:1	1.2	90	15.4

Conditions: 0.48 M PB, 0.002 M TES, 0.1% SDS, 62°C.

Figure 5. Hybridization saturation curve of labeled nonrepetitive DNA with 0 hour (logarithmically growing cells) RNA. Conditions for each point are given in Table III.

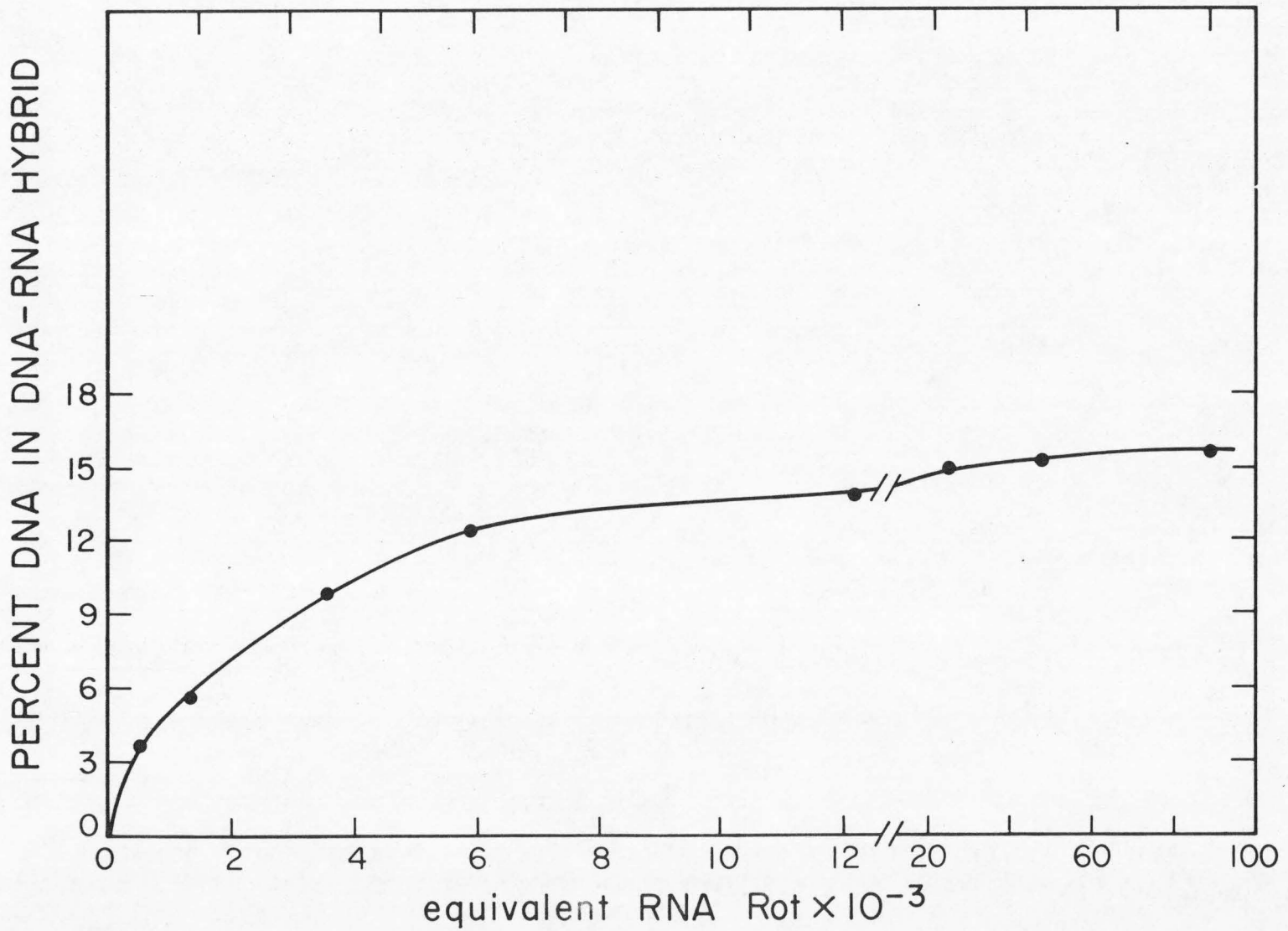

PERCENT DNA IN DNA-RNA HYBRID
0
3
6
9
12
15
18
0
2
4
6
8
10
12
20
60
100
equivalent RNA Rot × 10⁻³

Table III

Hybridization of RNA from Several Developmental Stages with Unique DNA

Developmental Stage of RNA	Conc. Mg/ml.	Hours Incubation	% DNA in Hybrid	% DNA in DNA-RNA Hybrid	% DNA in DNA-DNA Hybrid
Log phase cells (0 hrs)	16	24	17.3 ± .2	14.4 ± .2	2.9 ± .1
		48	18.0 ± .2	15.1 ± .2	2.9 ± .1
Pre-aggregation (6 hrs)	16	24	15.1 ± .3	12.6 ± .3	2.5 ± .2
		48	16.2 ± .3	13.7 ± .3	2.5 ± .2
Late-aggregate Early slug (12 hrs)	16	24	18.3 ± .3	15.8 ± .3	2.5 ± .2
		48	19.6 ± .3	17.1 ± .3	2.5 ± .2
Early culmination (20 hrs)	16	24	21.5 ± .2	18.9 ± .2	2.6 ± .1
		48	21.9 ± .2	19.3 ± .2	2.6 ± .1
0 hr - RNased[1] before incubation	16	24	4.0 ± .2		
None		24	5.0 ± .2		
Ascites RNA	15	24	4.0		

Conditions: 0.48 M PB, 0.002 M TES, 0.1% SDS, 0.3 µg $^{32}PO_4$-DNA, 80-120,000 cts/min/µg, 62°C. Volume for 24 hr incubation 0.3 ml., 0.6 ml. for 48 hr incubation. Duplicate points were run with each batch of RNA and DNA. Two batches of RNA and 3 batches of DNA were used to determine average for 6 and 12 hour RNA; 4 batches of RNA and DNA were used for 0 and 20 hour. The errors shown represent the ranges of the data used.

[1]Experiment was performed with all RNA samples with the same results ± 0.5%.

hydroxyapatite with 0.48 M PB and 50 ug/ml. RNase A and 100 units/ml. of T1 RNase (both preheated at 80°C, 20 min) added to it. The solution was then dialyzed against 0.06 M PB at 37°C for 12 hours. Under these conditions the RNA-DNA hybrid is sensitive to RNase and is degraded but the DNA-DNA hybrid is stable (checked by incubating a known amount of DNA-DNA duplex under these conditions). Similar conditions were used by Gelderman et al. (1969, 1971) and Davidson and Hough (1971) to digest RNA-DNA hybrids. After the extensive treatment with RNase, the mixture was reapplied to HAP. The DNA which had been hybridized to RNA is now single-stranded and therefore elutes in the 0.12 M wash,while the DNA present in DNA-DNA duplexes sticks to the hydroxyapatite and is eluted by 0.48 M PB. As shown in Table III, after incubation for 48 hours at an RNA concentration of 16 mg/ml. in a volume of 0.5 ml., 0.48 M PB (an equivalent Rot of 45,000), the double-stranded DNA represents approximately 16% of the total DNA hybrid present, or approximately 2.9% of the initial input DNA. The value for the reaction run at a Rot of 90,000 was 2.8. The actual values for the amount of DNA-DNA duplex at the lower Rot values were not determined but were estimated by interpolation from a linear plot of the amount of DNA-DNA duplex with no RNA present to amount of DNA-DNA hybrid at a Rot of approximately 12,000.

As can be seen from Figure 5, approximately 15.5% of the DNA is present as RNA-DNA hybrid at an equivalent Rot

of approximately 90,000. If we assume that only one strand of the DNA duplex is transcribed into RNA, then approximately 31% or 1/3 of the total unique DNA duplex material is present as transcript in logarithmically growing cells. This represents approximately 2.2 times the total genomic information in an E. coli. Doubling the Rot from 45,000 to 90,000 (maintaining a constant DNA Cot) increases the amount of RNA-DNA hybrid by less than 3%. This would indicate, although saturation has not been reached, I may be very close to it.

Complete saturation is difficult to obtain because the RNA sequences present at low concentrations hybridize slowly. 15.5% of the DNA is in DNA-RNA hybrids, but this number actually represents a lower limit and the final saturation value may be somewhat higher. However, the values do provide an estimate of the complexity of the RNA sequences which are present at a high enough concentration to hybridize at these Rots.

Analysis of DNA in RNA-DNA hybrids

We next consider whether the DNA present in a DNA-RNA hybrid is actually non-repetitive DNA or whether it contains contaminating repetitive sequences. The hybrid

(including 16% DNA-DNA duplex) eluted from HAP was dialyzed against 0.3 N NaOH and then KOH for 18 hours at 37°C to destroy the RNA. The material was then dialyzed against water, lyophilized, and tracer amounts were added to cold carrier total cell DNA. Figure 6 shows renaturation kinetics of this DNA as compared to that of total cell DNA. Also shown is a theoretical second-order reaction curve of a single-copy DNA assuming that it has a $Cot_{\frac{1}{2}}$ of 35 on HAP, and represents 45% of the total cell DNA. As can be seen, the labeled DNA follows the theoretical curve and indicates that little, if any, of the DNA present in the DNA-RNA hybrid renatures with the repetitive sequences. This indicates that the DNA present in the hybrid consists in fact of non-repetitive sequences.

Thermal stability of RNA-DNA hybrids

In the analysis of RNA-unique DNA hybrids, it is more important to know their degree of thermal stability. This is a means of determining whether or not they possess the degree of base pairing expected of good hybrids. It has been shown (Davidson & Hough, 1969; Davidson & Hough, 1971; Brown & Church, 1971; Gelderman et al., 1971) that non-repetitive RNA-DNA hybrids exhibit T_m's close to those of native DNA-DNA duplexes and that they melt cooperatively. Purified repetitive DNA-RNA hybrids, on the contrary, exhibit lowering of their T_m's and melt from hydroxyapatite less cooperatively (Davidson & Hough, personal

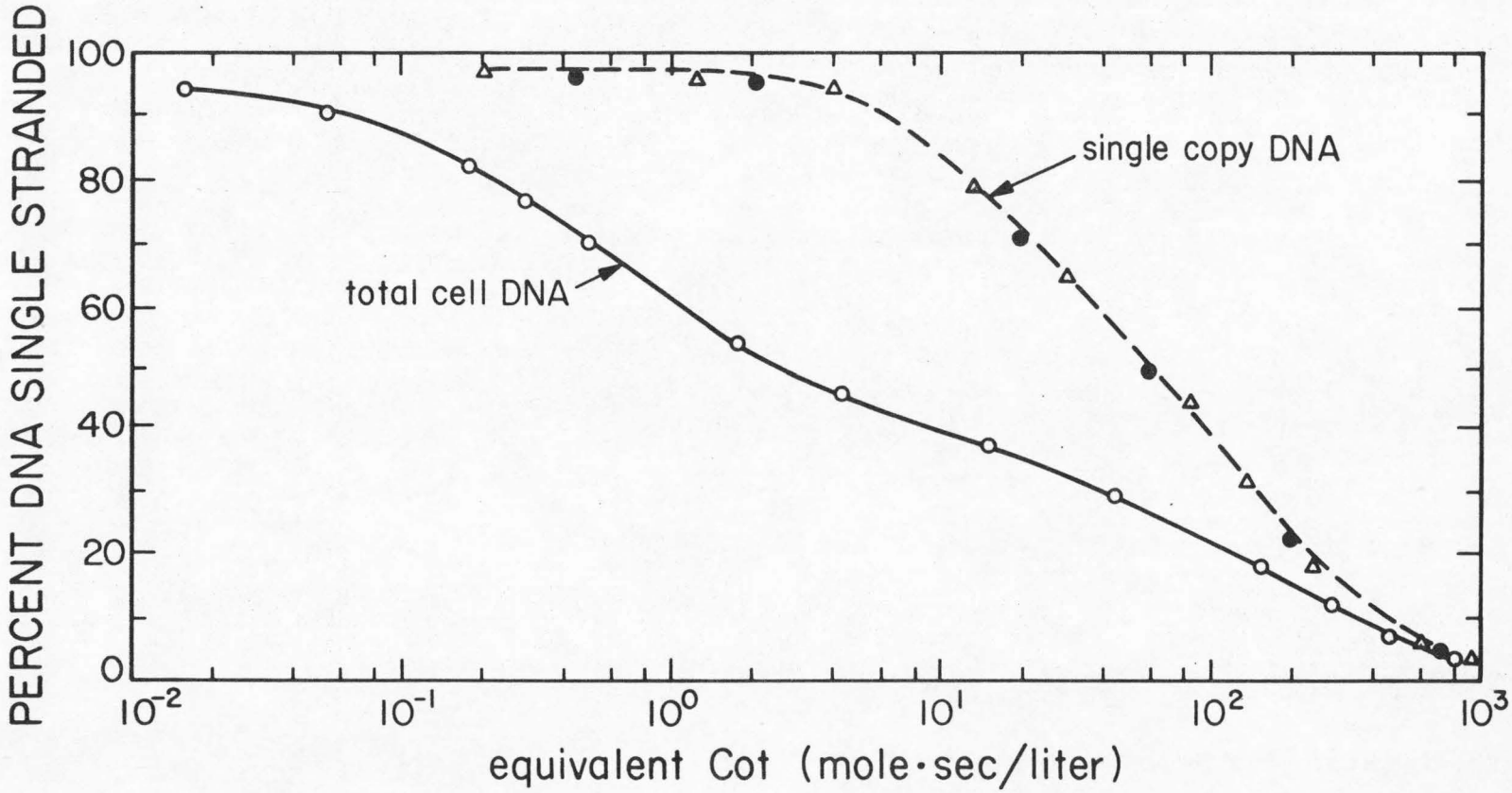
Figure 6. Renaturation of tracer amounts of DNA from RNA-DNA hybrids with total cell DNA. DNA present in hybrid after hybridizing to a Rot of 45×10^3 (see text for method) was annealed to total cell DNA according to the protocol in legend of Figure 3. Approximately 36,000 cts/min of hybrid DNA was used for each point on curve.

Data for total cell DNA curve and single copy DNA curve from Figure 3.

o—o HAP Cot curve total cell DNA

Δ——Δ HAP Cot curve of tracer amounts of single copy DNA with total cell DNA

• Renaturation of DNA from hybrids


PERCENT DNA SINGLE STRANDED
100
80
60
40
20
0
single copy DNA
total cell DNA
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
equivalent Cot (mole·sec/liter)

communication).

The hydroxyapatite melting profiles of renatured unique DNA duplexes and of RNA-unique DNA hybrids are shown in Figure 7. Renatured RNA-DNA hybrids or unique DNA-DNA duplexes were adsorbed on HAP in 0.12 M PB and washed in 0.12 M PB at 55°C. 55° was used to determine if there was any material melting below the 60° criterion used when doing the hydroxyapatite chromatography to measure amount of hybrid. The temperature of the column was then raised in increments, allowed to reach temperature equilibrium for 5 min and washed with 0.12 M PB. The 0.12 M PB elutes DNA which has become single-stranded with the rising temperature. As can be seen, the RNA-DNA hybrid melting profile shows a small amount of early melting material. The T_m of unique DNA-DNA duplexes is 76°C; this compares to the T_m of 78.5°C for native, unsheared DNA. The decrease of 3° is about that which has been observed in other systems in which the DNA was sheared to small pieces (Britten & Kohne, 1967). The T_m of the RNA-DNA hybrid is 73°C. The melting appears to be cooperative as expected for well matched RNA-DNA hybrids. The T_m of 73°C is 3° below the T_m of reassociated non-repetitive DNA. This difference in T_m compares well with the ΔT_m observed for bacterial systems which do not contain repetitive DNA and for RNA-driven single-copy DNA hybridization reactions with mammalian systems. Kohne

(1968) found a ΔT_m of 8° between E. coli rRNA-DNA hybrids and reassociated rRNA cistron duplexes using 50 K sheared DNA. A 4° difference was found by Bolton and McCarthy (1964) and Moore and McCarthy (1967) for E. coli mRNA-DNA hybrids on filters. Gelderman et al. (1969, 1971) found a 5-7° difference for RNA-driven mouse unique DNA hybrids; a 5° difference was found by Brown and Church (1971), also using mouse RNA-single-copy DNA reactions. Davidson and Hough (1971) found only a 1-2° difference in T_m using single-copy DNA-driven hybrids.

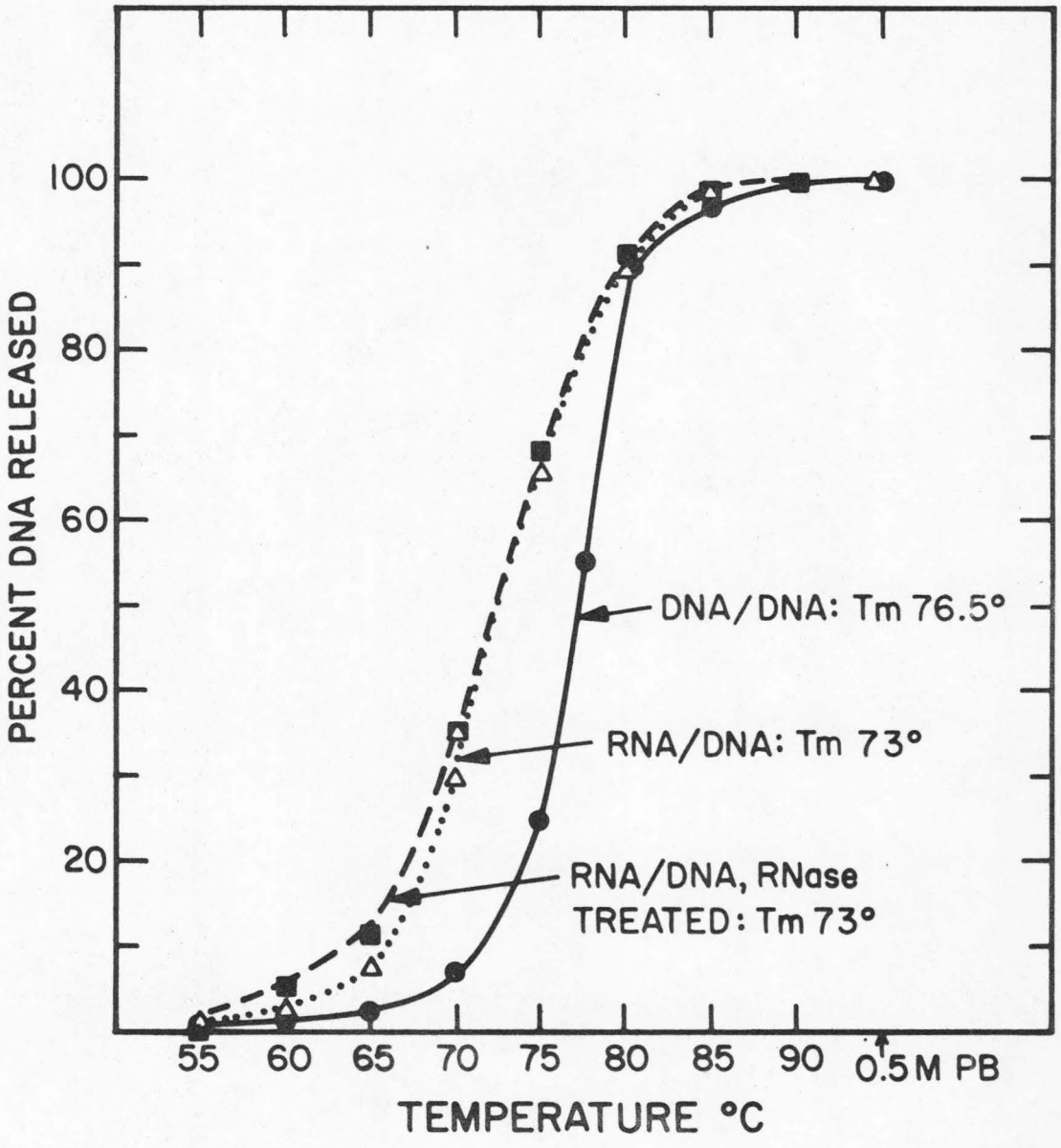
The effect of RNase on the amount of DNA in RNA-DNA hybrids and on the T_m of the hybrids was also determined. Single-copy DNA-RNA hybrids are known to be stable to mild RNase treatment (Davidson & Hough, 1969, 1971). A hybridization reaction mix which had been incubated at a Rot of 45×10^3 was split into two fractions. One was applied directly to HAP at 55°C and washed with 0.12 M PB. The material remaining was then melted as described earlier. The other sample was treated with 10 ug/ml. of RNase A for 30-45 min in 0.24 M PB, and was made 1% in SDS and applied to a Sephadex G-100 column, and run in 0.12 M PB, 0.2% SDS. The first peak containing $^{32}PO_4$-DNA was clearly separated from ribonucleotides and small RNA polynucleotides which were eluted in the second peak. The fractions containing DNA-RNA hybrids were then applied to HAP at 55°C and melted as described above. Figure 7 shows the melting profile of RNase-treated hybrids. After treatments the

Figure 7. HAP melting curve of renatured ^{32}P-single copy DNA and 0 hour DNA-RNA hybrid. Methods are given in text.

o——o DNA-DNA

■ ---■ RNA-DNA hybrid

Δ···Δ RNA-DNA hybrid, RNase treated and chromatographed on G-100.


PERCENT DNA RELEASED
100
80
60
40
20
55
60
65
70
75
80
85
90
0.5 M PB
TEMPERATURE °C
DNA/DNA: Tm 76.5°
RNA/DNA: Tm 73°
RNA/DNA, RNase
TREATED: Tm 73°

hybrids showed the same amount of DNA in hybrid melting at 60°C and over, same T_m, and very little material denaturing between 55 and 60°C. These results indicate that the DNA which is bound to RNA adsorbed to hydroxyapatite is a well-matched hybrid. Poorly base paired hybrids would show a large amount of early melting material.

Hybridization with RNA from various stages of development

RNA-driven hybridization experiments were performed to determine the complexity of the RNA transcribed from the non-repetitive DNA at various stages of the developmental cycle of Dictyostelium discoideum. Three stages were chosen: 6 hours after the food source had been removed, just prior to aggregation; 12 hours after removal of food, postaggregation-early pseudoplasmodium (slug); and 20 hours of development, early culmination (formation of fruiting body). These stages represent times during the development cycle at which major biochemical changes occur. By 6 hours of development the cells are probably still of the single cell type but have acquired new cell antigens (see Gerish, 1968) and are becoming sensitive to cyclic-AMP, to which the amoebae are attracted (Bonner et al., 1969). 12 hours of development is the time of the first cytological signs of the appearance of prespore and prestalk cells (Hohl & Hamamoto, 1969; Takeuchi , 1963, 1969; Miller et al.,

1969; Gregg, 1966). At this point, most of the enzymes necessary for the synthesis of the polysaccharides for stalk cell wall and spore coat have become sensitive to actinomycin D (see Roth et al., 1968; Firtel & Bonner, 1971; Telser & Sussman, 1971). At 20 hours, the stalk cells and spores are in the final stages of differentiation and the cells are actively synthesizing the enzymes necessary for the new polysaccharides as well as enzymes which may be involved with other processes in spore cell differentiation (see Loomis, 1968, 1969).

Table III shows the hybridization values for an RNA-driven reaction using RNA from the various developmental stages. To determine an approximate saturation value, the RNA Rot was increased while the DNA Cot was kept constant. If saturation were being approached the amount of DNA in RNA-DNA hybrids should increase very little when the Rot is doubled. This is what was found as has been previously described for RNA extracted from log phase cells. The data indicate that these hybridizations are most probably close to saturation with respect to the species of RNA that are present in high enough concentration to hybridize effectively.

When the DNA is present at low concentration, a RNA-DNA hybridization reaction driven by the RNA concentration is a pseudo first order reaction and basically a function of the concentration of the sequences of RNA present.

$$\frac{d[C]}{dt} = -k_2[C]^2 - k_2'[C][R]$$

where [C] = concentration of unrenatured or unhybridized DNA at any time t

[R] = concentration of RNA sequences homologous to the DNA

k_2 is the second order rate constant for DNA renaturation

k_2' is the second order rate constant for RNA-DNA hybrid formation.

If one assumes that

$k_2 \approx k_2'$ and $[R] >> [C_o]$, the initial DNA concentration, at all times during the reaction, then

$[R] \approx [R_o]$, the initial RNA concentration and

$$\frac{d[C]}{dt} \approx -k_2'[C][R_o]$$

The results of integration, with respect to the DNA able to hybridize, is:

$$\ln \frac{[C]}{[C_o]} = -k_2' R_o t \quad \text{or} \quad \frac{[C]}{[C_o]} = e^{(-k_2[R_o]t)}$$

where $[C_o]$ is the initial DNA concentration homologous to the RNA input. Therefore, sequences of RNA which are present at very low concentration should not effectively hybridize. Unfortunately, for these experiments the time period of hybridization is close to the maximum possible for technical reasons. Longer time periods result in a

decrease in amount of RNA-DNA hybrid possibly due to degradation of the RNA. The true saturation values cannot therefore be readily obtained with RNA-driven reactions, and the values obtained represent a minimum for the amount of RNA sequences complementary to unique DNA, thus giving us a minimum value for the amount of unique DNA whose transcripts are present in a particular stage of development. It should also be noted that the RNA present in each hybridization reaction is the RNA which was present in a particular cell at the time it was harvested. As a result, some of the RNA species present at 12 hours of development may be stable species which were actually synthesized during logarithmic growth. Therefore, we are looking at the total accumulation of sequences, stable and unstable, which are present in the cells at each particular stage of development.

Table III indicates that there are changes in the amounts of DNA which hybridize as development proceeds. There is a slight decrease between 0 and 6 hours, an increase at 12 hours , and another increase at 20 hours. Thus it appears that there is an increase in diversity of RNA sequences at later stages of development.

Control experiments determined that none of the RNA samples used contained contaminating DNA. Hydroxyapatite melting profiles of the RNA-DNA hybrids from the various stages showed the same T_m's as found for 0 hour RNA. Experiments with rat ascites RNA showed that the rat RNA

does not detectably hybridize to Dictyostelium DNA. This stresses the fact that the hybridization conditions are stringent and that the hybrids formed are specific for their homologous DNA sequences.

To measure the changes in the transcript of the non-repetitive DNA present at various developmental stages, combination hybridization experiments were done. In these, RNA from two stages was combined in the same vial and hybridized with DNA for 48 hours. Since the concentration of the RNA specific for an individual stage is diluted by a factor of 2 by adding RNA of the other stage, hybridization for 48 hours should yield a Rot equivalent to hybridization for 24 hours for the individual RNA if it were not diluted. However, the Rot for the RNA sequences which are the same for the two stages will be increased. I compare the extent of hybridization of RNA from each individual stage hybridized for 24 hours with that achieved by combinations of RNA hybridized for 48 hours. Control experiments indicate that this comparison is valid. If 0 hour RNA is diluted twofold with an equal concentration of rat ascites RNA and hybridized for twice the period of time, the extent of hybridization is the same as if the 0 hour RNA had been hybridized alone. This would be expected since the equivalent Dictyostelium RNA Rot is unchanged. The main difficulty in this type of analysis is that the RNA sequences which are the same in the two stages will probably hybridize at slightly higher values

since their Rots have been doubled. This is shown in Table III.

The histogram of Figure 8 shows hybridization values for combination experiments using 0, 6, 12, and 20 hour RNA. All combinations were done. As can be seen, there appeared to be differences in the RNA sequences present at the various stages. The unmarked regions of the bar represent an estimate of the fraction of sequences present in both stages. The striped region estimates the sequences from the earlier stage which are no longer present at the later stage. The stippled region estimates the sequences present at the later stage which are new, i.e., which have been synthesized and accumulated during the relevant period of development. As development proceeds, there is a trend toward increasing diversity of sequences present, as well as toward accumulation of new sequences and loss of others previously present. The change is most evident between 0 and 20 hour RNA samples. The hybridization values at a Rot of 45,000 for 0 and 20 hour RNAs are 15.1 and 19% of DNA, respectively. 0 and 20 hour combination experiments run for 96 hours give a value of 24.7 (see Table IV). The complexity of the RNA sequences in common between these stages is 9.4% of the non-repetitive DNA. There is also a loss of RNA sequences homologous to 5.7% of the non-repetitive DNA between 0 and 20 hours development, and a gain of 9.6% (5.7 + 9.6 +

Table IV

Hybridization of Recycled Hybridized and Unhybridized DNA

RNA source	DNA	% DNA in hybrid	% DNA in RNA-DNA hybrid	% DNA in DNA-DNA hybrid
0 hr (amoebae)	unique 0.3 µg	18.0 ± .2	15.1 ± .3	2.9 ± .1
20 hr (culminating fruits)	unique 0.3 µg	21.6 ± .2	19.0 ± .3	2.6 ± .1
0 + 20 hr[1]	unique 0.3 µg	27.0 ± .2	24.7 ± .3	2.3 ± .1
0 hr	0.12 M PB 0 hr DNA 0.22 µg	5 ± .5	2	3 est.
0 hr	0.5 M PB 0 hr DNA 0.08 µg	80 ± .2	80	0 est.
20 hr	0.12 M PB 0 hr DNA 0.22 µg	14.5 ± .5	12	2.5 est.
20 hr	0.5 M PB 0 hr DNA 0.08 µg	40 ± .2	40	0 est.

Conditioning: RNA concentration 16 mg/ml. hybridized 48 hr in 0.48 M PB, 0.5 ml. DNA specific activity 96,000 cts./min/γ. Averages given from two separate experiments. Est. means values were estimated. DNA in DNA-DNA hybrid determined from extensive RNase treatment of hybrid. See text for further details.

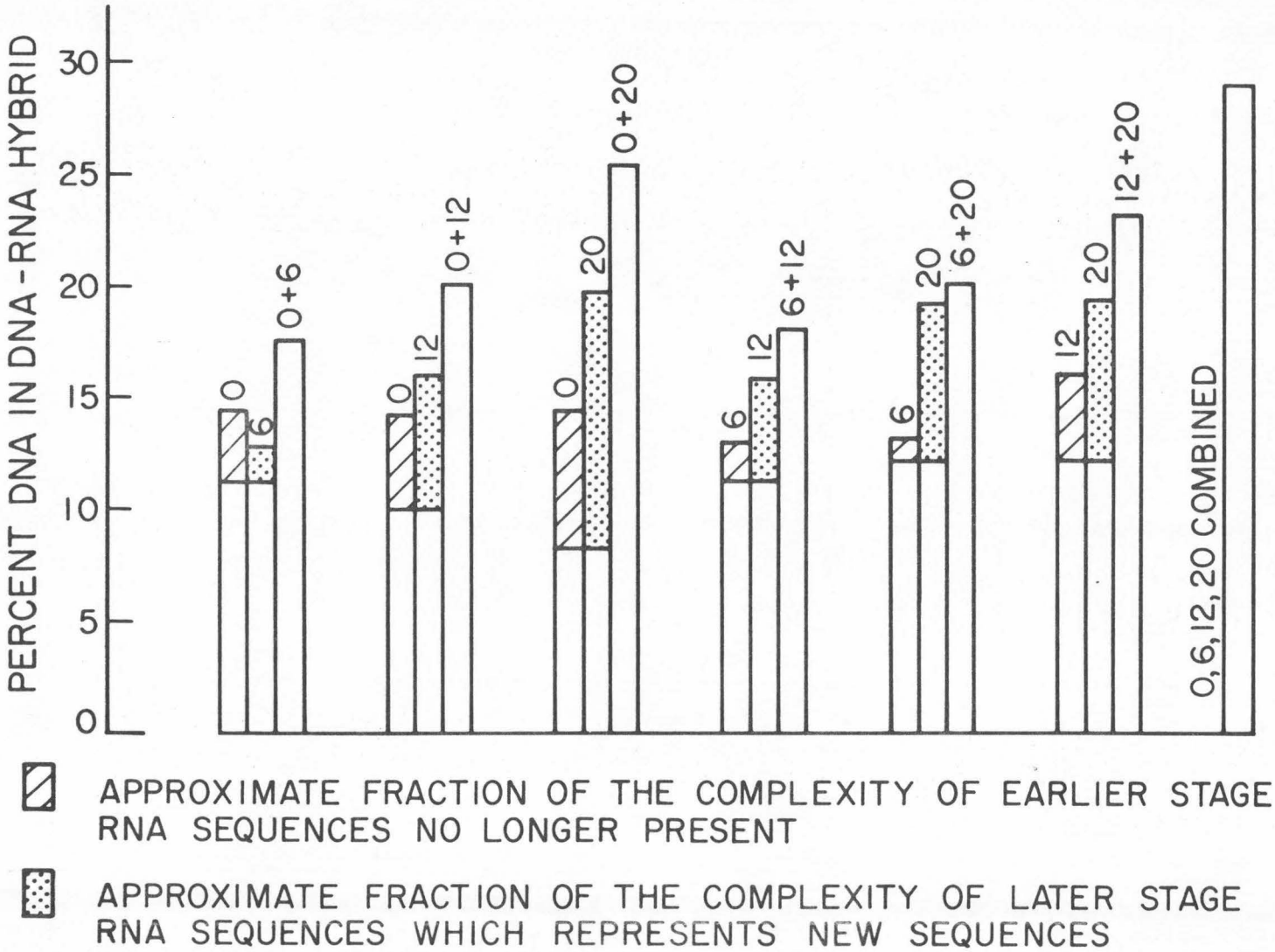
[1]Hybridized for 96 hr, 1.2 ml., using 0.55 ml. of each 0 and 20 hr RNA.

Figure 8. Histograms of amount of DNA hybridized to RNA at or near saturation. Histogram shows percent of DNA in hybrid for RNA from individual development of stages and from two or four stages combined. Rot of the RNA from each stage was maintained at 22×10^3. 4.8 mg of RNA from each stage were incubated at 62°C, 0.48 M PB in 0.3 ml. for 24 hours. For experiments in which RNA from two stages were combined, the incubation was in 0.6 ml. for 48 hours and for RNA from four stages 1.2 ml. and 96 hours were used.

Estimation of fraction of complexity of DNA sequences present as transcripts in both stages. (Refers only to histograms of hybridization of RNA from a single stage.)

Estimates of fraction of complexity of the RNA sequences from the earlier of the two stages no longer present in later stage.

Estimates of fraction of complexity of RNA from later stage which represents transcripts of new sequences.


PERCENT DNA IN DNA-RNA HYBRID
30
25
20
15
10
5
0
0
6
0+6
0
12
0+12
0
20
0+20
6
12
6+12
6
20
6+20
12
20
12+20
0,6,12,20 COMBINED
APPROXIMATE FRACTION OF THE COMPLEXITY OF EARLIER STAGE RNA SEQUENCES NO LONGER PRESENT
APPROXIMATE FRACTION OF THE COMPLEXITY OF LATER STAGE RNA SEQUENCES WHICH REPRESENTS NEW SEQUENCES

9.4 = 24.7). The last bar on the histogram represents a hybridization experiment in which all four species, 0, 6, 12, and 20 hour RNA, were hybridized together in the same vial for 96 hours. Data from hybridization experiments incubated for this length of time show more scatter than those run for shorter periods. We assume that the increase in scatter is due to variability in the breakdown of RNA when incubated over long time periods. A value of approximately 28% is obtained. Assuming asymmetrical transcription, this would represent 56% of the non-repetitive DNA of the genome at the criterion used. Given that the amount of non-repetitive DNA present in the nucleus is approximately 7 times that of E. coli (Firtel, 1971), this indicates that the amount of DNA represented in RNA transcript during the developmental period between 0 hour logarithmically growing cells and 20 hour culminating cells is approximately three times the complexity of and E. coli genome. This is approximately 11 x 10^9 daltons or approximately 1.6 x 10^7 nucleotide pairs.

Recycling of hybridized and unhybridized DNA

In order to check the interpretations of the previous section, the following experiments were performed. 0 hour RNA was hybridized to non-repetitive DNA (Rot = approximately 45 x 10^3, Cot = 0.3). The reaction mix was passed through HAP and 0.12 M PB run-off and 0.48 M PB eluted peak were collected separately. These were dialyzed against 200 volumes of 0.3 N NaOH, 25°C, for 4 hours, and

then dialyzed against 0.3 N KOH for 12 hours at 37°C. The material was neutralized by dialyzing against 0.01 M Na_2-EDTA, 0.12 M PB for two changes and then against H_2O for two additional changes. The recovery of DNA in both fractions was about 75-80%. This yielded two fractions, "0.12 M PB, 0 hour DNA" which did not hybridize to 0 hour RNA, and "0.48 M PB, 0 hour DNA" which did hybridize. This latter fraction also included some DNA which had been present in DNA-DNA hybrid, most of which will not be homologous to the 0 hour RNA. Table IV shows the results of rehybridizing these DNA fractions to 0 hour and 20 hour RNA. The 0.12 M 0 hour DNA, as expected, hybridized only slightly while the 0.48 M 0 hour DNA hybridized to an extent of 80%. In the initial hybridization reaction with the 0 hour RNA at a Rot of 45,000, 2.9% of the input DNA or 16% of the DNA in duplex form was present as DNA-DNA duplex rather than RNA-DNA hybrid. Therefore, even at saturation only 84% of the DNA eluted at 0.48 M PB should react, i.e., the fraction of the 0.48 M eluate which was present in an RNA-DNA hybrid. This experiment indicates that at Rot of 45×10^3 saturation is approached.

To determine the fraction of DNA transcripts which are the same in 0 and 20 hour RNA, the 0.12 M and 0.48 M 0 hour DNA were hybridized to 20 hour RNA to a Rot of 45×10^3. Under these conditions (see Table IV) 14-15% (2% in DNA-DNA) of the 0.12 M 0 hour DNA reacted with 20 hour RNA,

while only 40% reacted with 0.48 M 0 hour DNA. These results indicate that about 12% of unique DNA is present as transcripts in the 20 hour cells, but not in the amoebae, and that ½ of the unique DNA present as transcripts in amoebae (8% of unique DNA) are no longer present. These results agree well with the results shown in Figure 8 and Table III for the 0 and 20 hour combination experiments. It should be pointed out that this experimental method allows the isolation of DNA whose transcripts are either distinct or common to two different RNA populations. It may be possible with this technique to isolate specific species of DNA or RNA in eukaryotes which may be activated as a result of hormone stimulation or other inducing stimuli.

DISCUSSION

As shown in Figure 1, the genome of Dictyostelium consists of repetitive and non-repetitive fractions as determined by DNA-DNA reassociation kinetics. Although each fraction represents approximately 50% of the DNA nucleotides in the cell (at a criterion of 55°C, 0.12 M PB), the single-copy DNA possesses over 95% of the gentic complexity of the organism. It has been postulated (Britten & Davidson, 1969) that the non-repetitive DNA represents primarily the structural genes. Recently it has been found that the majority of the different mRNAs

synthesized during early sea urchin embryogenesis are complementary to the non-repetitive DNA, although it does appear that what is possibly the message for a specific class of proteins, the histones, reassociate with a repetitive fraction (Kedes & Birnstiel, 1971).

For the most part, hybridization experiments on eukaryotes have been limited to filter hybridization techniques which have been shown to measure mostly RNA complementary to the repetitive DNA sequences (see McCarthy & Church, 1970). This is partially due to the fact that, in at least one case which has been studied, the number of copies of RNA per cell of sequences homologous to the repetitive DNA is much greater than the number of those homologous to the non-repetitive DNA (Davidson & Hough, 1971, for discussion; Davidson et al., 1968; Crippa et al., 1967). In order to examine transcripts from single-copy DNA, two main approaches have been taken: one using RNA-driven reactions and the other, DNA-driven. These make use of hybridization in solution with single-copy DNA purified from total DNA by DNA-DNA reassociation. Gelderman et al., (1969, 1971) have used RNA-driven reactions (high RNA:DNA ratios) to estimate the sequence complexity of unique DNA transcripts present in mouse embryos. In these experiments a ratio of approximately 460,000 unlabeled total cell RNA to 1 (labeled DNA) was incubated for up to 50 hours in 0.24 M PB. They obtained a minimum estimate of approximately 8% of nonrepetitive DNA

hybridized to RNA. They showed in these experiments that the RNA-DNA hybrids, monitored as are the DNA-DNA duplexes, adsorb to hydroxyapatite in 0.12 M PB. In a brief report Brown & Church (1971) indicate that the complexity of transcripts from brain appeared to be much greater than those from kidney, liver or spleen. Although the experiments of Brown and Church (1971) examine single tissues, these experiments do not answer the question of the complexity of the DNA which is transcribed in a single cell type.

Davidson and Hough (1969, 1971) used a combination of RNA and DNA-driven reactions to estimate the complexity of the RNA transcribed from unique DNA at the lampbrush stage in Xenopus oogenesis and the RNA stored in mature Xenopus oocytes, a single cell type destined to develop into a large number of cell types. To estimate saturation with the DNA-driven reaction the authors increased the labeled RNA:DNA ratios while they maintained a high DNA Cot using purified single copy DNA. The amount of hybrid was determined by the amount of radioactive RNA which eluted with the DNA in the exclusion volume of a G-200 Sephadex column after mild ribonuclease treatment. The authors also showed that, like DNA-DNA duplexes, the hybrids can be purified by hydroxyapatite chromatography. As expected for single copy DNA hybrids and as determined for bacteria, phage, and eukaryotic ribosomal RNA-DNA

hybrids (see McCarthy & Church, 1970, for review), the hybrids showed a high thermal stability indicative of a low degree of base mismatching.

In this paper I have used similar techniques to analyze the amount and changes in the RNA transcripts complementary to single-copy DNA during the development of Dictyostelium. These experiments were done with purified single-copy DNA. The renaturation kinetics of this DNA in the presence of excess total cell DNA show that there is little contamination by repetitive sequences. The hybrids were examined by hydroxyapatite chromatography under stringent conditions for low GC DNA (60°C, 0.12 M PB) where poorly base paired hybrids would not be stable. Melting profiles showed a high thermal stability, with only small amounts of hybrid melting below the 60°C criterion used. Kinetic experiments showed that the DNA in RNA-DNA hybrids was non-repetitive DNA and was not contaminated by repetitive sequences. The high fraction of the input DNA present in a hybrid (up to 28%) also indicate that the majority of the hybrid DNA must be non-repetitive. Rehybridization of hybridized DNA suggests that the hybridization values obtained are very close to saturation for those RNA species which are effectively hybridized. It should be mentioned that the low complexity of the single-copy portion of the genome of Dictyostelium as compared to vertebrates reduces the technical difficulties in doing saturation experiments.

The values obtained from these RNA-driven reactions represent a minimum estimate of the number of nucleotides transcribed *in vivo*. It should be mentioned that in a RNA-driven reaction using labeled DNA, the value obtained is the amount of DNA which binds to hydroxyapatite at the specific criterion used due to the presence of RNA-DNA hybrid on some portion of the DNA molecule.

Estimates can be made as to the effective ratio of RNA transcribed from unique DNA to the DNA which is being used in the experiment. At the highest Rot used for 0 hour RNA, the total RNA:DNA ratio was 60,000:1. Assuming that 1% of the RNA is unique transcripts, it can be calculated that saturation should be reached by Rot = 45,000[1]. Even if one assumes that the lowest frequency for an RNA species is one molecule/cell and given that there are approximately 50 times more RNA than DNA per cell, calculations still indicate that saturation should have been reached. This indicates that if saturation was not reached for certain RNA species, they must be present in less than one copy per cell or else the efficiency of extraction of these species is low compared to the total. Experimental results show that at a Rot of 45,000, saturation has not been reached. Since approximately 0.3% more of the non-repetitive DNA is found in hybrids if the Rot is doubled, it must be concluded that although over 95% of the bulk RNA is extracted, not all the species of RNA were extracted with the same efficiency. Another possibility

is that a small percent of the cells may have transcripts not present in the general population. This would lead to a class of RNAs present at an average frequency of much less than one per cell.

Overall it has been shown that assuming one strand of DNA is transcribed, 56% of the unique portion of the genome is transcribed during this developmental period. Dictyostelium discoideum single copy DNA has a complexity of 7 times that of E. coli (Firtel, 1971). The complexity of the transcribed portion of the genome is 16-17 x 10^6 nucleotide pairs (7 x 0.56 x 4.2 x 10^6 nucleotide pairs per E. coli, Cairns, 1963). This represents enough information for 16,000 genes of 1,000 nucleotides each. Although the represents a large number of proteins, it is quite small compared to the complexity of the DNA transcribed in fetal mouse (Gelderman et al., 1969, 1971). In the developmental cycle of Dictyostelium discoideum one cell type, a vegetative amoeba, differentiates into two cell types, stalk and spore cells. The change in the non-repetitive DNA transcripts present from 0 hour to 20 hour cells represents a loss of transcripts complimentary to 11-12% of the non-repetitive portion of the genome and accumulation of approximately 20-22%. Thus close to half the sequences present in the logarithmically growing cells are still present at 20 hours. Again it is not clear if these represent stable sequences or whether they represent continual synthesis of the same

sequence. Assuming that not all of these are stable, many of the functions in 0 hour logarithmically growing cells may represent so-called "housekeeping functions" which are necessary for the general maintenance of the cell and which are used at all stages of the developmental process. Assuming this it would mean that approximately 19% of the non-repetitive portion of the genome or approximately 5.7×10^6 nucleotide pairs of DNA are used for these housekeeping functions or approximately 5,700 genes of 1,000 nucelotides each.

Although it should be stressed that the numbers obtained in these hybridization experiments are not absolute, one does obtain a general indication of the changes which are taking place. Biochemical experiments on the enzymes and proteins present during development have shown that the regulation of differentiation at the transcriptional level appears to be a continuous process rather than a series of abrupt changes in the pattern of RNA sequences synthesized during a particular stage. In the biochemical experiments one is examining changes in a small number of enzymes, while in the hybridization experiments one is examining changes in the non-repetitive DNA transcript, whose complexity is large enough to code for over 8,000 proteins of the size of globin chains. This indicates the enormous diversity of sequences required to regulate differentiation of a simple eukaryote.

Unlike many developmental systems, the developmental fate of the cells of Dictyostelium at any stage of development until midculmination,when stalk cells become vacuolated and die, is quite plastic. If food is given to developing Dictyostelium, the cells respond by dedifferentiating into vegetative amoebae (Bonner, 1967). We might expect that differentiation (spore formation) is necessary only when food is scarce or other harsh conditions prevail. The organism then responds by producing spores which are stable until these conditions are relieved. If food is present before completion of sporulation the cells would differentiate into amoebae and resume vegetative growth. It is also interesting to note that when slugs are dissociated and allowed to reassociate or are artificially cut in half, the prestalk and prespore cells dedifferentiate and then are capable of subsequently differentiating into the other cell type (Raper, 1940; Bonner et al., 1955; Gregg, 1965, 1968; Francis & O'Day, 1971). The cells must therefore retain the ability not only to sense changes in the environment, but to sense and respond to physiological and biochemical regulatory factors produced by the organism itself. It appears that unlike many embryonic induction systems of higher organisms, the developmental fate of a Dictyostelium cell is never fixed irreversibly until the final stage in stalk or spore formation.

The experiments presented in this paper give one an

estimation of the genetic requirement for the differentiation of a very simple eukaryote and of the changes which take place during the differentiation process of Dictyostelium discoideum. The amount of genetic information this represents is approximately the same as is present in a mature Xenopus oocyte (Davidson & Hough, 1971) or approximately $\frac{1}{4}$ the complexity of mouse non-repetitive DNA present as transcript in a liver or kidney (Brown & Church, 1971).

ACKNOWLEDGEMENTS

I would like to thank Drs. James Bonner, Eric Davidson, and John Mayfield, Mr. David Holmes, and Miss Jessica Tuchman, California Institute of Technology, for their good advice and helpful suggestions during the time the work was being done and for their critical evaluations of the manuscript. I would also like to thank Drs. Roy Britten and David Kohne, Carnegie Institution of Washington, for their advice and helpful suggestions and Ken Pischel and Ratchford Higgins, California Institute of Technology, for technical assistance on some of the experiments.

During the period this work was done the author was a NSF predoctoral fellow and was also supported by USPHS training grant GM00086. The work was supported in part by USPHS grant (to Dr. James Bonner) GM13762.

FOOTNOTE

If 1% of the RNA represents unique transcripts, then the effective ratio would be 600:1. This ratio is actually higher (4200:1) if one takes into account that only 1/7 ($\sim$ 15%) of the DNA is complementary to this RNA. If an equal frequency for all the RNA sequences is assumed, then we see that saturation will be reached.

$$C/C_o = e\ (-k_2' \text{ Rot})$$

$$C/C_o = e^{-90}$$

where:

$$k_2' = (1/35)(7)$$

$$\text{Rot} = 450$$

REFERENCES

Bellamy, A.R. & Ralph, R.K. (1968). Methods in Enzymology 12B, 156.

Bolton, E.J. & McCarthy, B.J. (1964). J. Mol. Biol. 8, 201.

Bonner, J.T. (1967). The Cellular Slime Molds, 2nd edition. Princeton University Press, Princeton, N.J.

Bonner, J.T., Barkley, D.S.,Hall, E.M., Konijn, T.M., Mason, J.W., O'Keefe, G., III., and Wolfe, P.B.(1969). Develop. Biol. 20. 72.

Bonner, J.T., Chiquoine, A.D. & Koldeire, M.Q. (1955). J. Exp. Zool. 130, 133.

Britten, R.J. & Davidson, E.H. (1969). Science 165, 349.

Britten, R.J. & Kohne, D.E. (1967). Carneg. Inst. Wash. Yearbk. 65, 78.

Britten, R.J. & Smith, J. (1970). Carneg. Inst. of Wash. Yearbk. 68, 378.

Brown, I.R. & Church, R.B. (1971). Biochem. Biophys. Res. Comm. 42, 805.

Cairns, J. (1963). Cold Spring Harb. Symp. Quant. Biol. 28, 43.

Cocucci, S.M. & Sussman, M. (1970). J. Cell Biol. 45,399.

Coston, M.B. & Loomis, W.F., Jr.(1969).J. Bacteriol.100,1208.

Crippa, M., Davidson, E.H. & Mirsky, A.E. (1967). Proc. Nat. Acad. Sci., Wash. 57, 885.

Davidson, E.H., Crippa, M., Kramer, F.R. & Mrsky, A.E. (1966). Proc. Nat. Acad. Sci., Wash. 56, 856.

Davidson, E.H., Crippa, M. & Mirsky, A.E. (1968). Proc. Nat. Acad. Sci., Wash. 60, 152.

Davidson, E.H. & Hough,B. R. (1969). Proc. Nat. Acad. Sci., Wash. 63, 342.

Davidson, E.H. & Hough, B.R. (1971). J.Mol.Biol. 56, 491.

Firtel, R.A. (1971), submitted for publication.

Firtel, R.A. & Bonner, J. (1971), submitted for publication.

Firtel, R.A. & Brackenbury, R.(1971),submitted for publication.

Francis, D. & O'Day, D.H. (1971). J. Exp. Zool. 176,265.

Gelderman, A.H., Rake, A.V. & Britten, R.J. (1969). Carnegie Inst. of Wash. Yearbk. 67, 320.

Gelderman, A.H., Rake, A.V. & Britten, R.J. (1971). Proc. Nat. Acad. Sci., Wash. 68, 172.

Gerish, G. (1968). Curr. Topics Develop. Biol. 3, 157.

Gregg, J.H. (1965). Devel. Biol. 12, 377.

Gregg, J.H. (1966). In: The Fungi, Vol II (G.C. Ainsworth & A.S. Sussman eds.) New York, Academic Press, p. 235.

Gregg, J.H. (1968). Exp. Cell Res. 51, 633.

Hohl, H.R. & Hamamoto, S.T. (1969). J. Ultrastruct. Res. 26, 442.

Kedes, L.H. & Birnstiel, M.L. (1971). Nature New Biol. 230, 165.

Kohne, D.E. (1968). Biophys.J. 8, 1104.

Loomis, W.F., Jr. (1969). J. Bacteriol. 100, 417.

Loomis, W.F., Jr. (1970). J. Bacteriol. 103, 375.

McCarthy, B.J. & Church, R.B. (1970). Ann. Rev. Biochem. 39, 131.

Miller, Z.I., Quance, J. & Ashworth, J.M. (1969). Biochem. J. 114, 815.

Moore, R.L. & McCarthy, B.J. (1967). J. Bacteriol. 94,1066.

Newell, P.C. & Sussman, M. (1969). J. Biol. Chem. 244, 2990.

Raper, K.B. (1935). J. Agr. Res. 50, 135.

Raper, K.B. (1940). J. Elisha Mitchell Sci. Soc. 56, 241.

Roth, R.M., Ashworth, J.M. & Sussman, M. (1968). Proc. Nat. Acad. Sci., Wash. 59, 1235.

Schmidt, G. & Tannhauser, S.J. (1945). J. Biol. Chem. 161, 83.

Solymosy, F., Fedorcsak, I., Julyas, A., Farkas, G.L. & Ehrenberg, L. (1968). Europ. J. Biochem. 5, 520.

Sussman, M. (1966). Methods in Cell Physiology, vol.2, p. 397. New York: Academic Press.

Sussman, R. & Rayner, E. (1971).In press.

Takeuchi,I. (1963). Devel. Biol. 8, 1.

Takeuchi, I. (1969). In: Nucleic Acid Metabolism, Cell Differentiation and Cancer Growth. New York: Academic Press.

Telser, A. & Sussman, M. (1971). J. Biol. Chem. 246, 2252.

Ts'o, P.O.P. & Sato, C. (1959). Exptl. Cell Res. 17, 227.

Watts, D.J. & Ashworth, J.B. (1970).Biochem. J. 119,171.

Wetmur, J.G. & Davidson, N. (1968). J. Mol. Biol. 31, 349.

CHAPTER III

DEVELOPMENTAL CONTROL OF α 1-4 GLUCAN PHOSPHORYLASE IN THE CELLULAR SLIME MOLD *DICTYOSTELIUM discoideum*

DEVELOPMENTAL CONTROL OF α-1-4 GLUCAN PHOSPHORYLASE IN THE CELLULAR SLIME MOLD *DICTYOSTELIUM discoideum*[1]

Richard A. Firtel[2] and James Bonner

Division of Biology
California Institute of Technology
Pasadena, California 91109

Running Title: *Dictyostelium* phosphorylase

SUMMARY

Glycogen phosporylase activity is not found in vegetative, multiplying cells of wild type and mutant strains of Dictyostelium discoideum but appears during development. The specific activity of this enzyme is absent from developing cells until mid-aggregation and reaches a maximum at mid-culmination. Thereafter the enzyme activity begins to decrease and eventually disappears.

Appropriate experiments with actinomycin D and cycloheximide suggest that both the appearance and disappearance of glycogen phosphorylase require prior RNA and concomitant protein synthesis. The activity of this enzyme in cells of Dictyostelium discoideum appears therefore to be regulated at the level of transcription. Further studies with mutants of D. discoideum support this view.

The enzyme was purified 100-fold from crude extracts and its properties were examined.

These results are discussed in the light of what is known about the regulation of polysaccharide metabolism during culmination.

INTRODUCTION

The cellular slime mold Dictyostelium discoideum grows vegetatively as unicellular myxamoebae in the presence of a food source. When the food source is depleted or removed, the amoebae stream into aggregates containing approximately 10^5 cells each. After extensive cell sorting the aggregate forms a migrating cell mass termed a pseudoplasmodium. This in turn differentiates into a fruiting body containing two non-dividing cell types: stalk and spore. During development major changes occur in cell metabolism. These include the synthesis of cell wall material, accumulation of trehalose, decrease of soluble glycogen and nonpolysaccharide glucose, and decrease in total cell protein (Gregg et al., 1954; Gregg and Brőnsweig, 1956; White and Sussman, 1961, 1963a,b; Ceccarini and Filosa, 1965; Wright et al., 1968; Sussman and Sussman, 1969).

One approach to the study of developmental events in Dictyostelium is to examine the enzymes presumably involved in these events. The specific activities of several enzymes of carbohydrate and protein metabolism have already been found to change during Dictyostelium differentiation. Such an enzyme is said to be developmentally controlled, because the observed changes in its specific activity always occur at particular stages in the developmental cycle. Such enzymes include uridine diphosphoglucose pyrophosphorylase (Ashworth and Sussman, 1967), uridine disphosphogalactose

polysaccharide transferase (Sussman and Osborn, 1964), uridine disphosphate glactose-4-epimerase (Telser and Sussman, 1971), trehalose-6-phosphate synthetase (Roth and Sussman, 1966; Roth et al., 1968), N-acetylglucosaminidase (Loomis, 1969a), β-glucosidase (Coston and Loomis, 1969), α-mannosidase (Loomis, 1970a), α-fucosidase (Firtel, unpublished observations; Loomis, unpublished observation), glycogen synthetase (Wright and Dahlberg, 1967; Pischel and Firtel, in preparation), aminopeptidase and alanine transaminase (Firtel and Brackenbury, 1971), tyrosine transaminase (Pong and Loomis, in preparation), and threonine dehydrolase (Pong and Loomis, in preparation). Appropriate studies with inhibitors of RNA and protein synthesis (actinomycin D and cycloheximide) suggest that prior RNA and concomitant protein synthesis is necessary for the increase in specific activity of all of these enzymes. Studies with morphological and temporally deranged mutants derived from the wild type haploid strain NC-4 indicated that morphological differentiation and changes in specific enzyme activity are linked. The results, taken together, suggest that the appearance of any particular enzyme may be due to regulation of the activity of the relevant gene.

It is known that there are changes during development in the amount of soluble glycogen in Dictyostelium cells. The glycogen content increases early in development, remains constant until culmination, and then decreases (White and

Sussman, 1963a; Wright et al., 1968; Sussman and Sussman, 1969). The decrease is coincident with the synthesis of cell wall polysaccharide. Glucose-1-phosphate appears to be the precursor of much of this polysaccharide (Newall and Sussman, 1969). We have therefore asked whether glycogen phosphorylase (α-1-4 glucan: phosphate-glucosyl transferase, E.C. 2.4.1.1) is also developmentally regulated.

This paper examines the developmental regulation of glycogen phosphorylase in wild type and mutant strains of Dictyostelium discoideum. We have found that glycogen phosphorylase is first detectable at late aggregation (9-10 hours). Its specific activity rises rapidly and reaches a maximum during culmination (22 hours). It then decreases and is not detectable 8 hours after spore formation (33-34 hours). The increase in specific activity (between 14 hours and 22 hours) is approximately 40-fold. The peak activity of the enzyme corresponds with the period in development during which there is maximum rate of decrease in soluble glycogen (Marshall et al., 1970) and in which increasing amounts of cell wall polysaccharides are synthesized. Our studies suggest that both the increase and the decrease in the activity of glycogen phosphorylase require prior RNA synthesis and concomitant protein synthesis. Studies with developmental mutants of Dictyostelium discoideum also suggest that the appearance of glycogen phosphorylase is tightly linked to the whole developmental cycle of the organism.

MATERIALS AND METHODS

Organisms. Dictylostelium discoideum NC-4 (haploid) was first isolated by Raper (1935, 1940). All mutants used in this work were derived from this strain and were kindly supplied by Dr. William F. Loomis, Jr., University of California, San Diego. Mutant KY-19 forms spores with few stalk cells (Ashworth and Sussman, 1967); KY-3 ceases development at the pseudoplasmodium stage (Yanagisawa et al., 1967); VA-4 (Loomis, 1969a), DA-2 (Loomis, 1969b), and Agg-206 (Loomis, 1970a) fail to aggregate. Two temporally deranged strains, FR-17 (Sonneborn, et al., 1963) and GN-3 (Loomis, 1970b) were also used. FR-17 completes development in 16 rather than the usual 26 hours, has stalk and spore cells, but does not form a fruiting body. GN-3 undergoes normal morphogenesis but develops in 60 to 70 hours.

Conditions of Culture and Development. 10^5 spores (1 sorocarp) in 0.2 ml H_2O were inoculated with 0.2 ml of a stationary culture of Aerobacter aerogenes on SM agar plates containing 1% dextrose, 1% Difco proteose peptone, 0.1% Difco yeast extract, 5 mM magnesium sulfate, and 10^{-2} M potassium phosphate, pH 6.4, and 2% Difco agar (Sussman, 1966). (Periodically, wild type and all mutants were grown on NZCase plates containing 1% NSC peptones (Sheffield Chemical Co.), and 0.1% BBL yeast extract (M. Sussman, personal communication)). A prototrophic

Aerobacter aerogenes strain, generously supplied by M. Sussman (Brandeis University) is used as food source with this medium. After approximately 40 hours at 22°C the plates were washed and bacteria removed by centrifugation. 1 x 10^8 cells were deposited on 47.5 mm Whatman No. 50 filter paper, supported by Millipore pads saturated in PDF solution containing 0.04 M phosphate buffer, 1.5 g/liter KCl, 0.5 g/liter Mg_2SO_4, 0.5 g/liter streptomycin sulfate, pH 6.5 (Sussman, 1966; Newell and Sussman, 1969).

Glycogen Phosphorylase Assay Conditions. The cells from one filter containing approximately 1 x 10^8 cells were collected in water or in 5 mM potassium phosphate, 5 mM α-sodium glycerol phosphate, pH 6.8, 5% glycerol, 0.2 mM dithiothreitol (DTT) and used immediately, or stored at -70°C until used. Activity was not affected by freezing the cells and was the same whether they were collected in water or in the buffer solution. Crude extracts were prepared by slowly thawing cells followed by sonication for 2 x 20 sec (with a 45 sec. rest period) at 2 ma with a Branson sonifier at 0°C. Assays were performed within 30 min following sonication. One enzyme unit is defined as the amount of enzyme required to produce 1 mumole of product/minute.

The reaction catalyzed by glycogen phosphorylase is reversible:

$$1,4 \text{ glucan polymer } (G_x) + PO_{4i} \underset{II}{\overset{I}{\longleftrightarrow}} \text{glucose-1-}PO_4 \text{ (G-1-P)} + G_{x-1},$$

and has been assayed in both directions.

Assay I (Forward direction): The amount of glucose-1-PO_4 produced from glycogen or starch and inorganic phosphate (Pi) was assayed using a linked enzyme system with phosphoglucomutase (PGM), glucose-6-phosphate (G-6-P) dehydrogenase (G-6-PDH), and 6-phosphogluconic acid (6-P Gate) dehydrogenase-decarboxylating (6-P Gate DH).

$$G_x + P_i \xrightarrow{\text{(phosphorylase)}} G_{x-1} + \text{G-1-P} \qquad (1)$$

$$\text{G-1-P} \xrightarrow{\text{(PGM)}} \text{G-6-P} \qquad (2)$$

$$\text{G-6-P} + NADP^+ \xrightarrow{\text{(G-6-PDH)}} \text{6-PGate} + \text{NADPH} \qquad (3)$$

$$\text{6-PGate} + NADP^+ \xrightarrow{\text{(6-PGate DH)}} \text{ribulose-5-}PO_4^+ + \text{NADPH} + HCO_3^- \qquad (4)$$

Net change

$$G_x + P_i + 2\ NADP^+ \longrightarrow G_{x-1} + \text{ribulose-5-}PO_4 + HCO_3^- + 2\ \text{NADPH} \qquad (5)$$

The production of NADPH was measured by the increase in absorbance at 340 nm on a Beckman Kintrac VII recording spectrophotometer. The final reaction mixture contained per ml: 20 µmoles Na glycerol phosphate and 45 µmoles potassium phosphate, pH 6.8; 6 mg soluble starch or 6 mg

repurified glycogen; 5 µmoles $MgCl_2$; 1.6 µmoles $NADP^+$; 1 µg glucose-6-phosphate dehydrogenase; 10 µg phosphoglucomutase; 100-240 µg protein from the crude extract. The reaction was run at 35°C. A molar extinction coefficient of 6.22×10^3 was used for NADPH at 340 nm.

Assay II (Reverse direction): The synthesis of α 1, 4 glucan was assayed by measuring the incorporation of ^{14}C-glucose-1-phosphate into ethanol precipitable polysaccharide. The reaction mixture contained, in 125 µl: 2 µmoles ^{14}C-glucose-1-phosphate (0.5-1.0 µC) pH 6.8, 1 mg soluble starch or glycogen; 5 µmoles sodium glycerol phosphate and 0.5 µmoles sodium EDTA, pH 6.8; 25-50 µl of extract (12-60 µg protein). The reaction was incubated (normally 30 min) at 35°C. It was stopped by adding 1 ml of 1 N KOH and 50 µl of 5% glycogen solution. The vials were placed in boiling water bath for 5 min. 1.5 ml of ethanol was added. The tube was cooled at 0°C for 30 min to precipitate the glycogen. The precipitate was collected on GFA filters and washed with 60% ethanol. The filters were counted in a toluene based scintillation fluid in a Beckman LS-200B counter. Efficienty of counting was 75%. Protein was determined by the method of Lowry et al.,(1951) using crystalline bovine serum albumin (BSA) as a standard.

Chromatography. The sugar product of the assay was verified using a slightly different reaction mix. The reaction mixture contained per 0.2 ml: 4 µmole sodium glycerol phosphate, 8 µmoles potassium phosphate, and

1 umole EDTA, pH 6.8, 1.4 mg of soluble starch or repurified glycogen, in the presence of $^{32}PO_4$ (25-100 μc) and 25 μg of crude extract or purified enzyme. The reaction was stopped by boiling for 3 min and part of the supernatant was then chromatographed on Whatman No. 52 chromatography paper with a descending system of ethylene glycol-dimethyl-ether-methylethyl ketone-0.5 M morpholinium tetraborate, pH 8.6, and 0.01 M EDTA (70:20:30) and developed for 6 to 10 days according to Carminatti et al. (1965). ^{14}C-glucose-1-phosphate was used as a marker. The distribution of radioactivity was determined with a paper chromatograph scanner.

Materials. ^{14}C-glucose-1-phosphate (277 mC/mM) was obtained from Amersham-Searle. Glycogen was obtained from Calbiochem and soluble starch from Mallinckrodt. Cycloheximide was purchased from Calbiochem and actinomycin D was a gift from Dr. W.B. Gall of Merck, Sharp & Dohme. UDPG (uridine diphosphoglucose) was obtained from Schwarz BioResearch Labs, $NADP^+$ from Boehringer Mannheim or Calbiochem, and glucose-6-phosphate dehydrogenase, phosphoglucomutase, and 6-phosphogluconic acid dehydrogenase from Boehringer Mannheim. α-glycerol phosphate and MOPS buffer were also purchased from Calbiochem. All other reagents were reagent grade.

RESULTS

Characterization of the Enzymatic Reaction of Glycogen

Phosphorylase. The data of Table 1 show the requirements for assay I. The reaction is dependent on added glycogen, phosphate and phosphoglucomutase. There is no reduction of $NADP^+$ if Mg^{++} is replaced with EDTA. However, the addition of glucose-1 6-diphosphate is not necessary. This cofactor is probably associated with the phosphoglucomutase. There is no reduction of $NADP^+$ if glucose is substituted for glycogen. The presence of glucose, in the reaction mixture together with glucogen, is not inhibitory. 5'AMP or a combination of cyclic 3'-5' AMP and ATP which affect mammalian phosphorylase b, has no effect on the present enzyme. Newell and Sussman (1969) have shown that there is negligible NADPH oxidase activity in extracts of Dictyostelium discoideum.

The reverse reaction (assay II) is dependent upon added glucose-1-phosphate and glycogen and does not require Mg^{++} since the reaction proceeds in the presence of EDTA. The product formed is resistant to alkali, precipitated by ethanol, and sensitive to acid at 100°C and to α - and β -amylase. The rate of the reaction of assay I is constant for approximately 30-45 min; that of assay II is constant for over 60 min at 35°C.

The enzyme was also assayed using 0.02 M MOPS or imidizole hydrochloride buffer, from 6.4-7.2. The enzyme is about 80 to 90% as active in MOPS or imidizole buffer, as in sodium glycerol phosphate buffer. In sodium glycerol phosphate the enzyme has a pH optimum of 6.6 to 6.8.

Table 1

Requirements for Glycogen Phosphorylase (Assay I)

System	% Maximum Activity
Complete	100%
- phosphate	< 1%
- $MgCl_2$	85%
- $MgCl_2$ + 5 mM EDTA	< 1%
- $NADP^+$	< 1%
- G-6-PO_4DH	40%
- PGM	< 1%
- glycogen	< 1%
- glycogen + glucose	< 1%
- dithiothreotol	100%
+ 5'-AMP (4 mM)	100%
+ 0.5 mM 3'-5' cyclic AMP + 2 mM ATP	100%
+ glucose	100%

Assay I was performed under conditions described in Materials and Methods. 200 µg of protein from the crude extract of cells which had been starved for 20 hrs was used per ml of assay mixture.

Purification of Glycogen Phosphorylase. Glycogen phosphorylase was purified 100 to 150-fold from crude extracts of 20-22 hour developing slime mold cells harvested from Whatman No. 50 filter papers in 0.02 M sodium glycerol phosphate buffer, 5% glycerol and 0.2 mM dithiothreitol (DTT), pH 6.8. The cells were sonicated and centrifuged at 45,000 rpm in SW-50 rotor for 3 hours. The supernatant contained all the enzyme activity. This step gave a 2.5-fold purification. The supernatant was then treated with streptomycin sulfate to a final concentration of 1%. After standing at 0°C for 30 min the mixture was centrifuged at 45,000 rpm for 1 hour in the SW-50 rotor. The supernatant was next applied to a BioGel P-150 column. The enzyme eluted near the void volume. The enzyme was then precipitated by 65% saturated ammonium sulfate. The precipitate was collected by centrifugation and redissolved in 0.02 M glycerol phosphate buffer containing 5% glycerol and 0.2 mM DTT, pH 6.8. At this point the enzyme is purified approximately 25 to 100-fold over the crude extract and 75-85% of the initial activity is recovered. The enzyme is stable when stored at -70°C. Further purification was achieved by chromatographing the enzyme on DEAE (diethylaminoethyl)-cellulose resin. The ammonium sulfate precipitate was dialyzed in 0.0025 M Tris HCl buffer, pH 7.2, 0.01 M KCl, 0.001 M sodium-DETA, pH 7.2, 5% glycerol and 0.2 mM DTT. The enzyme was then applied to a DEAE cellulose column equilibrated with the

same buffer and the enzyme was eluted with a linear gradient of KCl (0.01 M to 1.0 M) in the dialysis buffer. The enzyme elutes as a single peak at approximately 0.22 M KCl (see Figure 1). This results in a further 2 to 4-fold purification.

Sucrose Gradient Sedimentation of Partially Purified Enzyme. The sedimentation coeficient of partially purified glycogen phosphorylase was determined by layering 200 ul of the initial 100,000 g supernatant or BioGel P-150 purified enzyme over a linear 5-20% sucrose gradient (0.05 M sodium glycerol phosphate, 0.05 M potassium phosphate, 0.2 mM DTT, pH 6.8) and centrifuging for 5 hours at 60,000 rpm in a Spinco SW-65 rotor. Fractions were collected from the bottom of the tube and assayed for glycogen phosphorylase activity. Catalase (11.2S) and bovine serum albumin (4.3S) were used as markers. The markers were run in another tube and were measured by absorbance at 230 nm. Glycogen phosphorylase exhibited the same sedimentation velocity relative to the BSA and catalase in four separate experiments. As shown in Fig. 2, phosphorylase sediments as a single peak with a sedimentation coeficient of approximately 10S.

Evidence that the Primary Product is Glucose-1-Phosphate. Chromatography of the reaction mixture indicates that the primary product of the reaction is glucose-1-phosphate. Partially purified enzyme and crude extracts were incubated in the presence of $^{32}PO_4$, soluble starch, and EDTA to

Figure 1. DEAE cellulose chromatography of BioGel P150 purified glycogen phosphorylase. Sample was applied in 0.0025 M Tris HCl, 0.01 M KCl, 0.001 M sodium EDTA, 5% glycerol, and 0.2 mM DTT, pH 7.6. A linear gradient of 0.01-1.0 M KCl in the above buffer was run. The fractions were assayed for phosphorylase activity using Assay I.

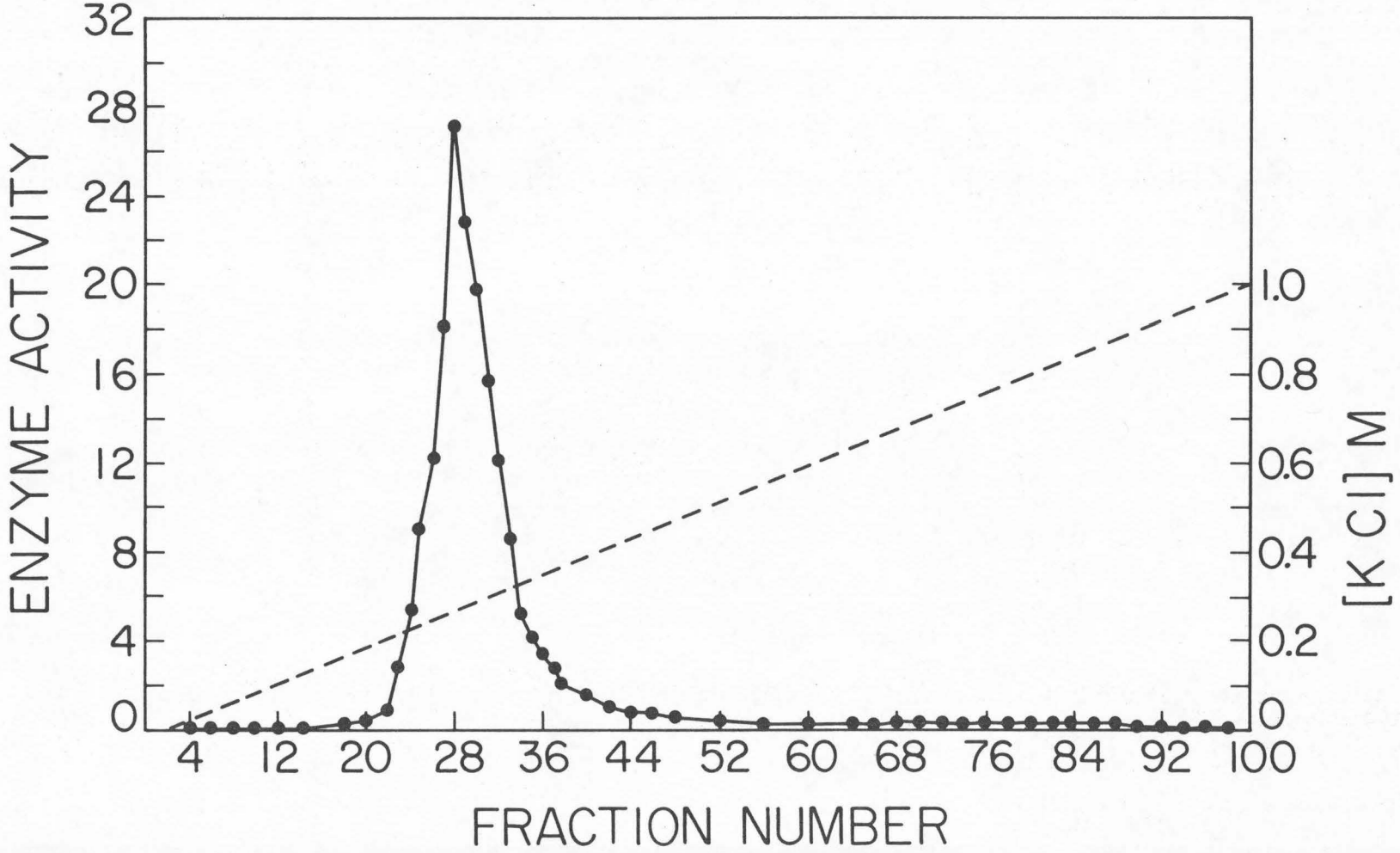
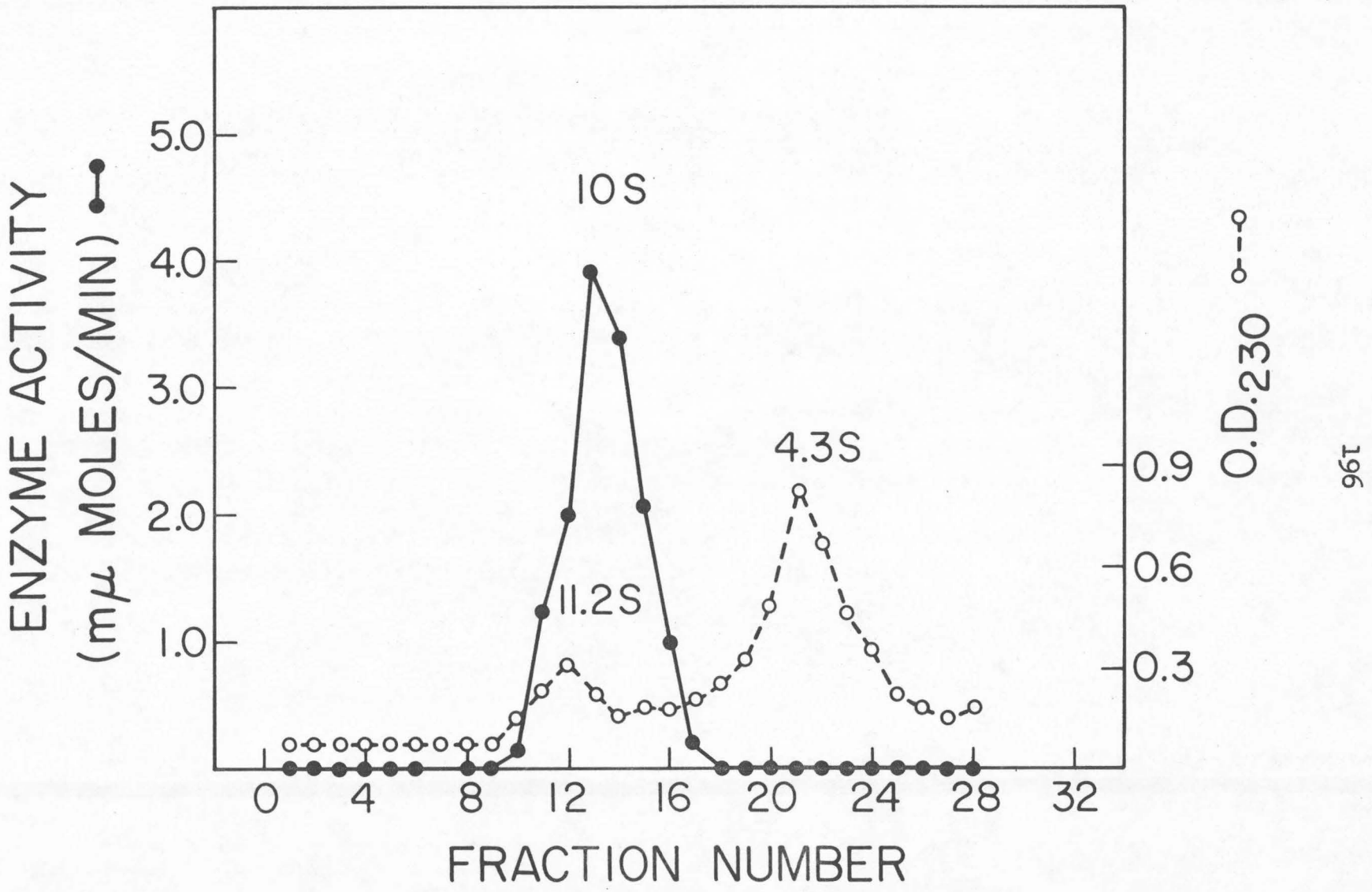

ENZYME ACTIVITY
32
28
24
20
16
12
8
4
0
4
12
20
28
36
44
52
60
68
76
84
92
100
FRACTION NUMBER
1.0
0.8
0.6
0.4
0.2
0
[K Cl] M

Figure 2. Sucrose gradient centrifugation of BioGel P150 purified enzyme. 5-20% sucrose gradient containing 0.05 M Na glycerol phosphate, 0.05 M potassium phosphate, 0.2 mM DTT, pH 6.8. Centrifuged 5 hours, 60,000 rpm in Spinco SW65 rotor at 4°C. Markers: BSA, 4.3S; catalase, 11.2S.


ENZYME ACTIVITY
(mμ MOLES/MIN)
5.0
4.0
3.0
2.0
1.0
10S
4.3S
11.2S
0.9
0.6
0.3
O.D.230
0
4
8
12
16
20
24
28
32
FRACTION NUMBER

inhibit the PGM reaction (see Materials and Methods). After 120 min, the reaction mixture was boiled for 3 min and the protein removed by centrifugation in a clinical centrifuge. The supernatant was then chromatographed. Only two radioactive peaks results. The first contained the majority of the radioactivity and chromatographed with a $^{32}PO_4$ marker. A second small peak co-chromatographed with authentic ^{14}C-glucose-1-phosphate. If PGM and Mg^{++} were added to the reaction mixture, then the second radioactive peak no longer co-chromatographed with the ^{14}C-glucose-1-phosphate, but rather, with an authentic glucose-6-phosphate marker.

Kinetic Properties of the Partially Purified Enzyme.

Glycogen phosphorylase in crude extracts was determined to have a Q_{10} of 1.4 to 1.5 at temperatures ranging between 20 and 42°C. Above 42°C the activity declines. It is not known whether this is due to an inactivation of phosphorylase or to increased inactivity of other enzymes present in assay system I. The Michaelis constants (K_ms) of all reactants were obtained for BioGel P-150 purified glycogen phosphorylase. K_ms of 0.67 mg/ml and 2.2 mg/ml were obtained for soluble starch (Fig. 3) and purified glycogen, respectively. The K_m for P_i (Assay I) is 3.4 mM (see Fig. 4) and 1.2 mM for glucose-1-phosphate (Assay II) (Fig. 5).

Uridine diphosphoglucose (UDPG) is a competitive inhibitor of both glycogen and inorganic phosphate in

Figure 3. Lineweaver and Burk plot (1934) for determination of Km for soluble starch (Assay I). Concentration of all other reactants given in Materials and Methods.

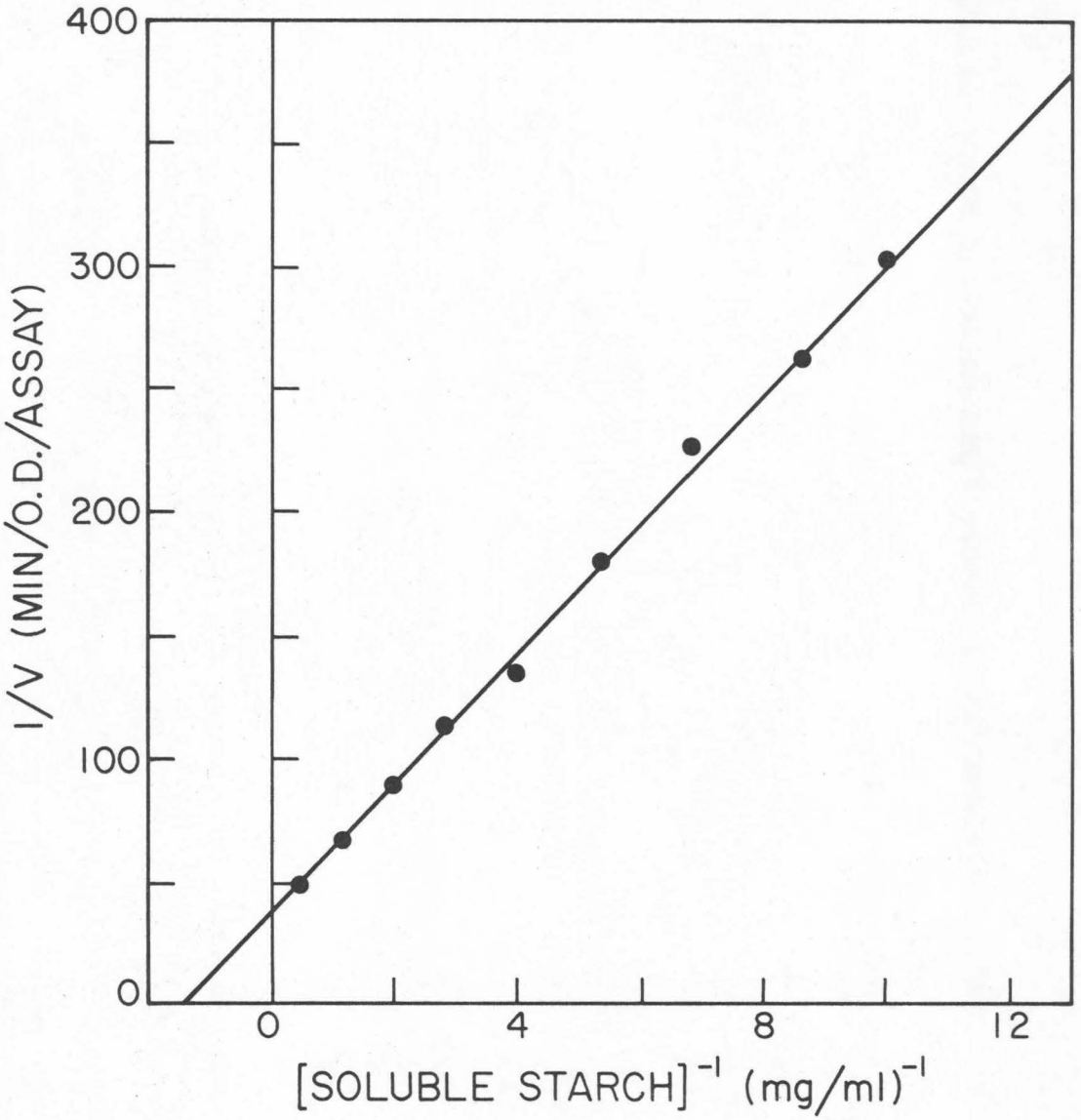

400
300
200
100
0
1/V (MIN/O.D./ASSAY)
0
4
8
12
[SOLUBLE STARCH]$^{-1}$ (mg/ml)$^{-1}$

Figure 4. Lineweaver and Burk plot for determination of the competition between UDPG and PO_4 (Assay I) according to Dixon and Webb (1964). Concentration of all other reactants given in Materials and Methods.

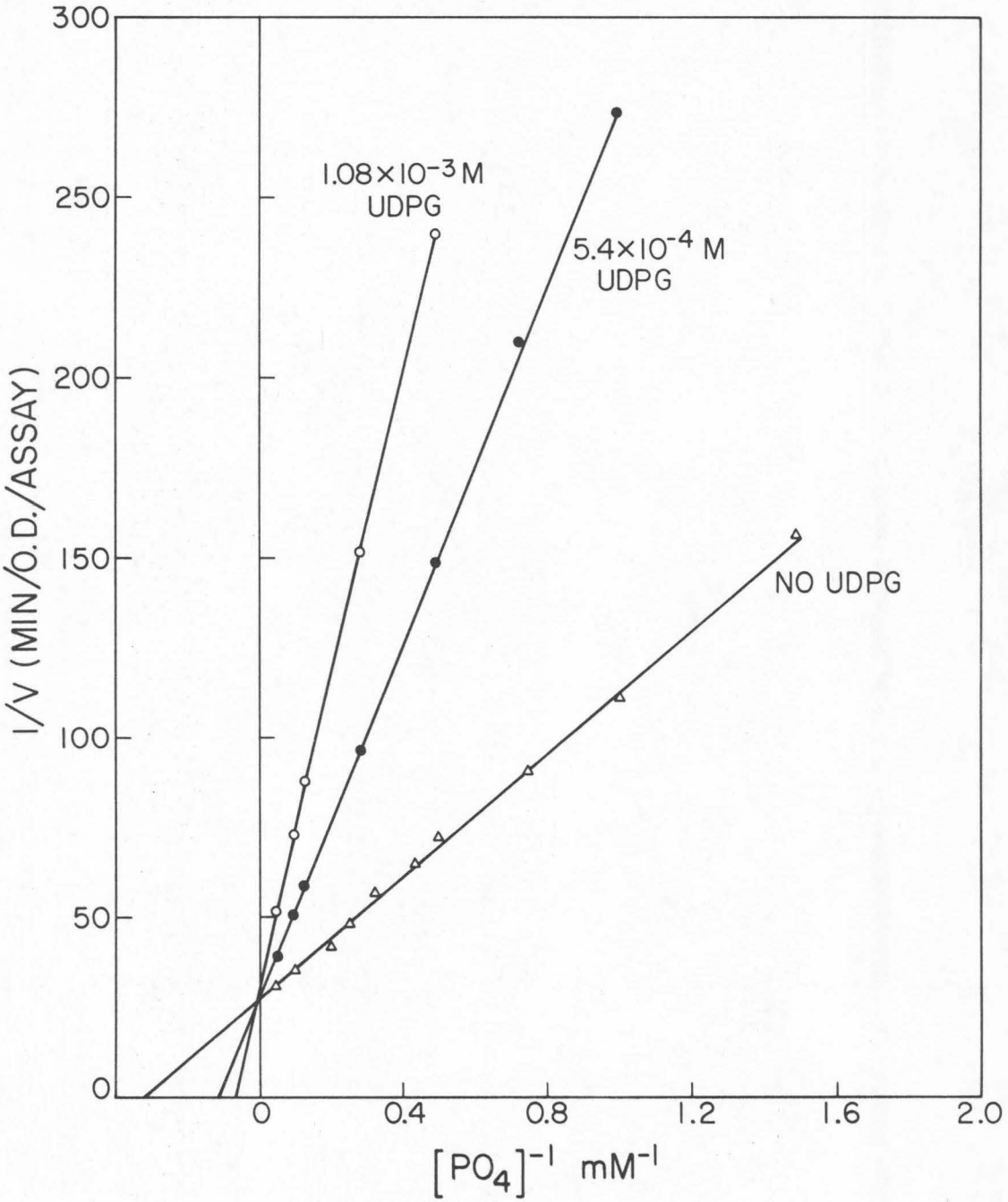
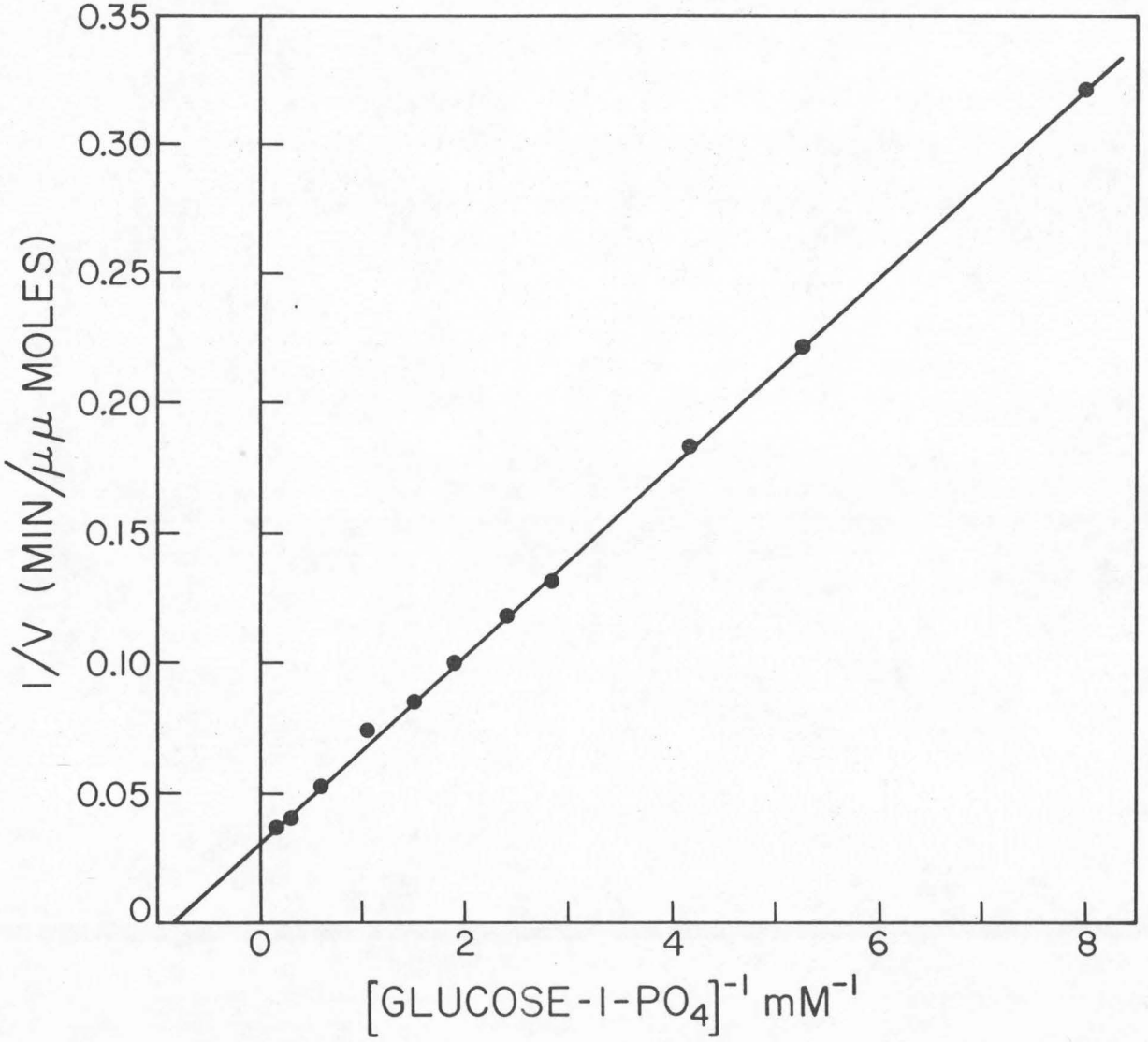

300
250
200
150
100
50
0
1/V (MIN/O.D./ASSAY)
1.08×10⁻³ M
UDPG
5.4×10⁻⁴ M
UDPG
NO UDPG
0
0.4
0.8
1.2
1.6
2.0
[PO4]⁻¹ mM⁻¹

Figure 5. Lineweaver and Burk plot (1934) for determination of Km for glucose-1-PO_4 (Assay II). Concentration of all other reactants given in Materials and Methods.


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1/V (MIN/μμ MOLES)
0
2
4
6
8
[GLUCOSE-1-PO4]-1 mM-1

the forward reaction. Fig. 4 and Fig. 6 show kinetic plots of the effect of UDPG on enzyme activity. In Fig. 4 the K_p for 5.4×10^{-4} UDPG with respect to P_i was found to be 9.8 mM, and the K_p for 1.08×10^{-3} M UDPG, 17 mM. The K_i is calculated to be 2.93×10^{-4} M. This K_i agrees with that obtained by varying UDPG concentrations and keeping the phosphate concentration constant (Fig. 6). Jones and Wright (1970) have shown that adenosine diphosphoglucose, thymidine diphosphoglucose, and guanosine diphosphoglucose are much better inhibitors of glycogen phosphorylase than in UDPG and that cytosine diphosphoglucose inhibits phosphorylase to approximately the same extent as UDPG. UDPG also inhibits the reverse reaction. UDPG, 0.44 mM, inhibits the reverse reaction by 27% and UDPG, 4.4 mM by 54%.

Developmental Kinetics of Glycogen Phosphorylase.

Figure 7 shows the kinetics of glycogen phosphorylase appearance and disappearance during development of wild type NC-4. Activity first appears 11 to 12 hours after the initiation of starvation. Enzyme activity rises quickly to reach a maximum during culmination, at approximately 22 hours. It then rapidly decreases and disappears by 32 to 34 hours. The sensitivity of the forward assay is great enough to detect 0.2 to 0.3 units of enzyme activity at a protein concentration of 200 µg per ml of assay. When the reaction is measured in the reverse direction, the sensitivity is much greater due to the low

Figure 6. Determination of Ki of UDPG with respect to PO_4 according to the method of Dixon (Dixon and Webb, 1964). The effect of (UDPG) on the rate of phosphorylase is plotted for 3 concentrations of PO_4: 0.01, 0.02, 0.04 M. Concentration of all other reactants given in Materials and Methods.

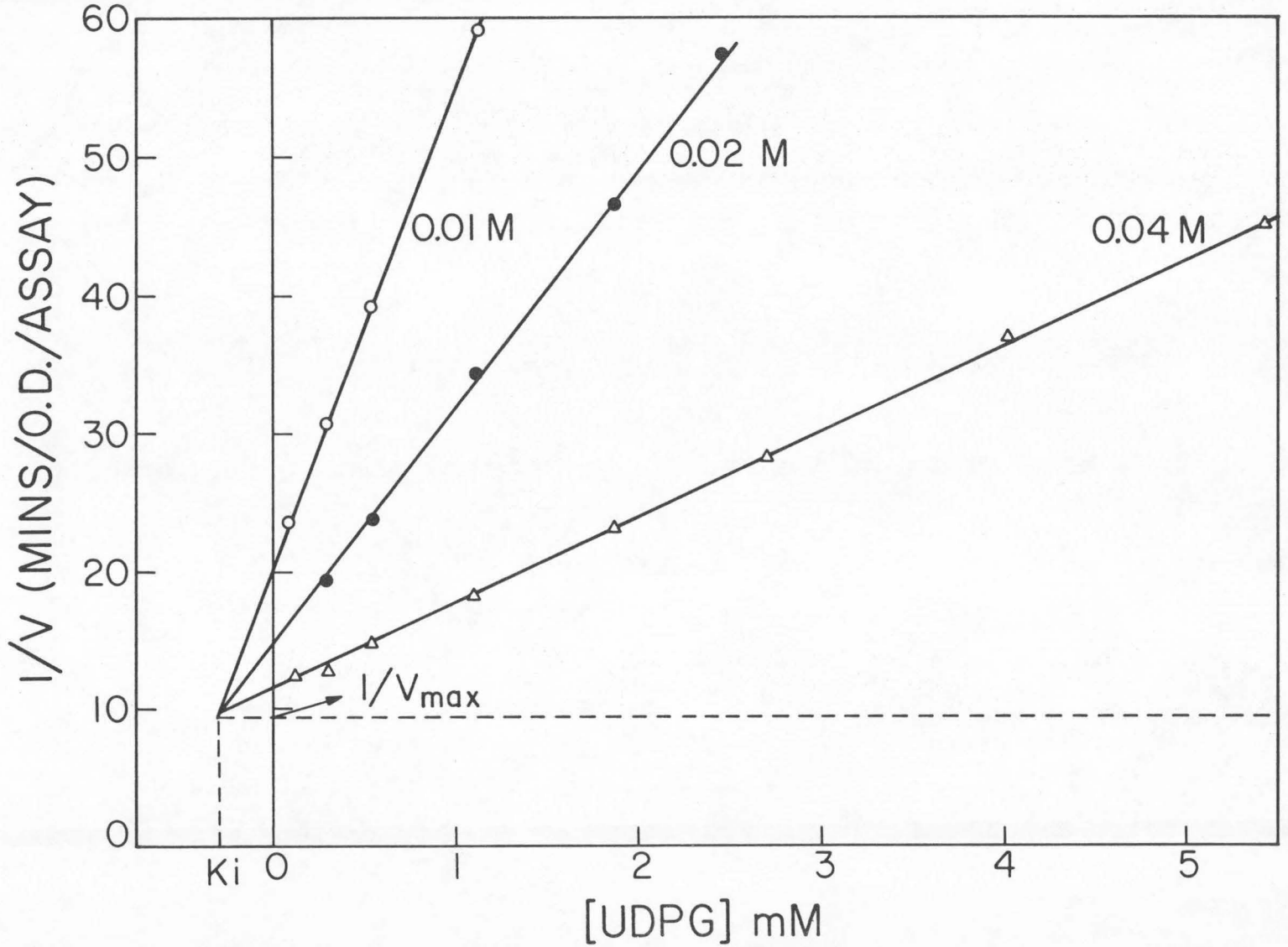
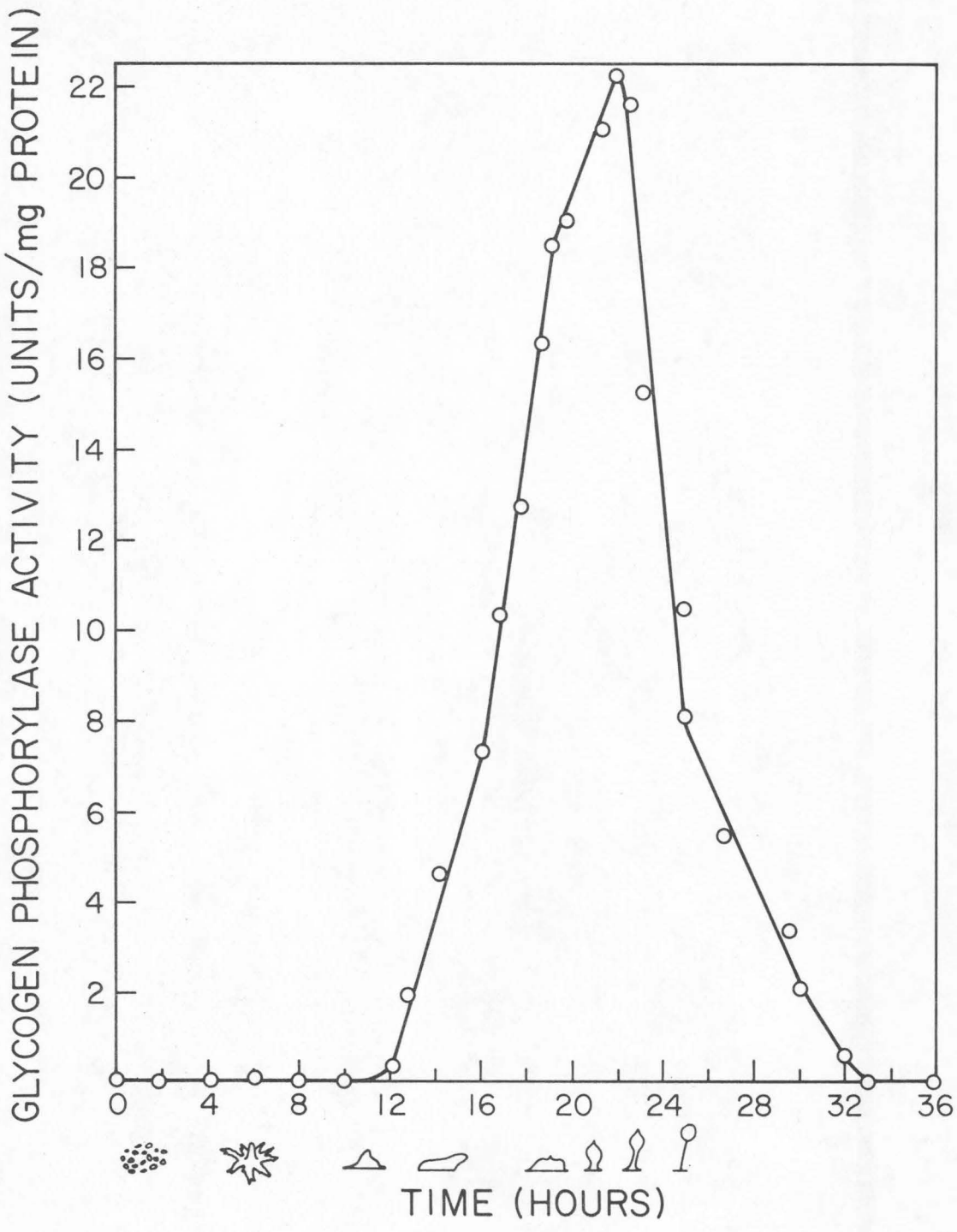

60
50
40
30
20
10
0
1/V (MINS/O.D./ASSAY)
0.01 M
0.02 M
0.04 M
1/Vmax
Ki
0
1
2
3
4
5
[UDPG] mM

Figure 7. Developmental kinetics of glycogen phosphorylase (Assay I). 1 unit represents the reduction of 1 mμ mole $NADP^+$/min. The developmental stages are given on the abscissa.


GLYCOGEN PHOSPHORYLASE ACTIVITY (UNITS/mg PROTEIN)
22
20
18
16
14
12
10
8
6
4
2
0
4
8
12
16
20
24
28
32
36
TIME (HOURS)

background of the counting technique and to the fact that high specific activity substrate can be used and 0.005 units of activity can be detected. Under these conditions the first appearance of enzyme activity is at 9 hours. No activity can be measured before 8.5 hours even if the specific activity of the substrate is increased to 1.6 μC/mole.

If the crude extract contained no 6-phosphogluconic acid dehydrogenase, 1 mole of NADPH should be produced per mole of glucose-1-phosphate. In the presence of excess 6-phosphogluconic acid dehydrogenase (6-PGate DH), 2 moles of NADH are produced per mole of glucose-1-phosphate. Therefore without 6-PGate DH in the crude extract the ratio of assay I with and without added enzyme (B/A in Table 2) should be 2. Since the observed ratio is only 1.3-1.4 the extract contains 6-PGate DH, enough to produce 1.5-1.6 moles of NADPH per mole of glucose-1-phosphate. The activity of 6-PGate DH during development of strain NC-4 is shown in Table 3. Assay I was used except that glycogen was omitted and 0.035 M 6-phosphogluconic acid was substituted as substrate. The data of Table 3 show that the activity of 6-PGate DH remains almost constant during the portion of the developmental cycle examined. Since the ratio of phosphorylase activity with and without added 6-PGate DH is essentially constant throughout development, either assay I as described in the Methods can be used to determine the specific activity of phosphorylase.

Table 2

Glycogen Phosphorylase Activity in Extracts of NC-4

Stage (hours)	Assay 1 (A) ($NADP^+$ reduced)**	Assay 1 plus (B) *6 phosphogluconic acid dehydrogenase ($NADP^+$ reduced)**	Ratio B/A	Assay 2 (C) (Glucose transferred)**	Ratio B/C	Assay (Glucose-1-P produced from Column B)**
6	0	0	-	0	-	-
9	0	0	-	0.014	-	-
13	3.4	4.4	1.3	0.29	15	2.2
15.5	8.7	11.4	1.3	0.75	15	5.7
18.8	18.0	23.7	1.33	1.70	14.0	11.9
21.5	22.2	29.7	1.33	2.11	14.1	14.9
24.5	7.6	10.6	1.4	0.77	14	5.3
30	3.5	5.1	1.4	0.40	13	2.5
34	0	0	-	0	-	-

*Amount of 6 phosphogluconic acid dehydrogenase added was 0.5 International Units (μmole/min at 25°C). No difference was found if 0.25 of 1.0 I.U. were added.

**mμmoles product produced/min/mg protein.

Table 3

Activity of 6 phosphogluconic acid dehydrogenase in NC-4

STAGE (hours)	ACTIVITY (mμm NADPH produced/min/mg/protein)
6	33.0
9	37.3
13	42.1
15.5	46.8
18.75	48.0
21.5	49.7
24.5	49.3
30	31.6

Assay conditions were the same as glycogen phosphorylase assay I except that no glycogen was present and a concentration of 0.035 M 6-phosphogluconic acid was used. In the absence of substrate there was no reduction of $NADP^+$.

Table 2 also shows the levels of glycogen phosphoylase activity as measured in the reverse direction. The ratios of phosphorylase activity as determined by the forward and the reverse reaction are constant throughout development. This suggests that it is unlikely that there is an artifact in either assay.

The BioGel P-150 purified enzyme exhibits the same activity with or without added 6-phosphogluconic acid dehydrogenase (Table 2). This is because the purified enzyme also contains a high level of the 6-phosphoglucinic acid dehydrogenase. Ratios of the phosphorylase activities of the purified BioGel P-150 enzyme in the forward and reverse directions are the same as those of crude extracts with added 6-PGate DH. This also strongly suggests that the crude extracts yield accurate measures of enzyme activity.

Fig. 8 shows that enzyme activity is linear with amount of added crude extract at three different stages of development for both the forward and the reverse direction.

The rise and subsequent fall of enzyme activity during development might be due to the presence or synthesis of soluble activator and/or inhibitor molecules. To examine these possibilities, extracts of different developmental stages were combined and the total amount of glycogen phosphorylase activity measured (see Table 4). Extracts from early developmental stages before enzyme activity appears, or from stages from which enzyme has

Figure 8. Linearity of product formation with increasing amounts of protein from crude extracts from 16, 21, and 23.5 hour cells (Assays I and II). Assay I had a volume of 1.0 ml, assay II 0.75 ml.

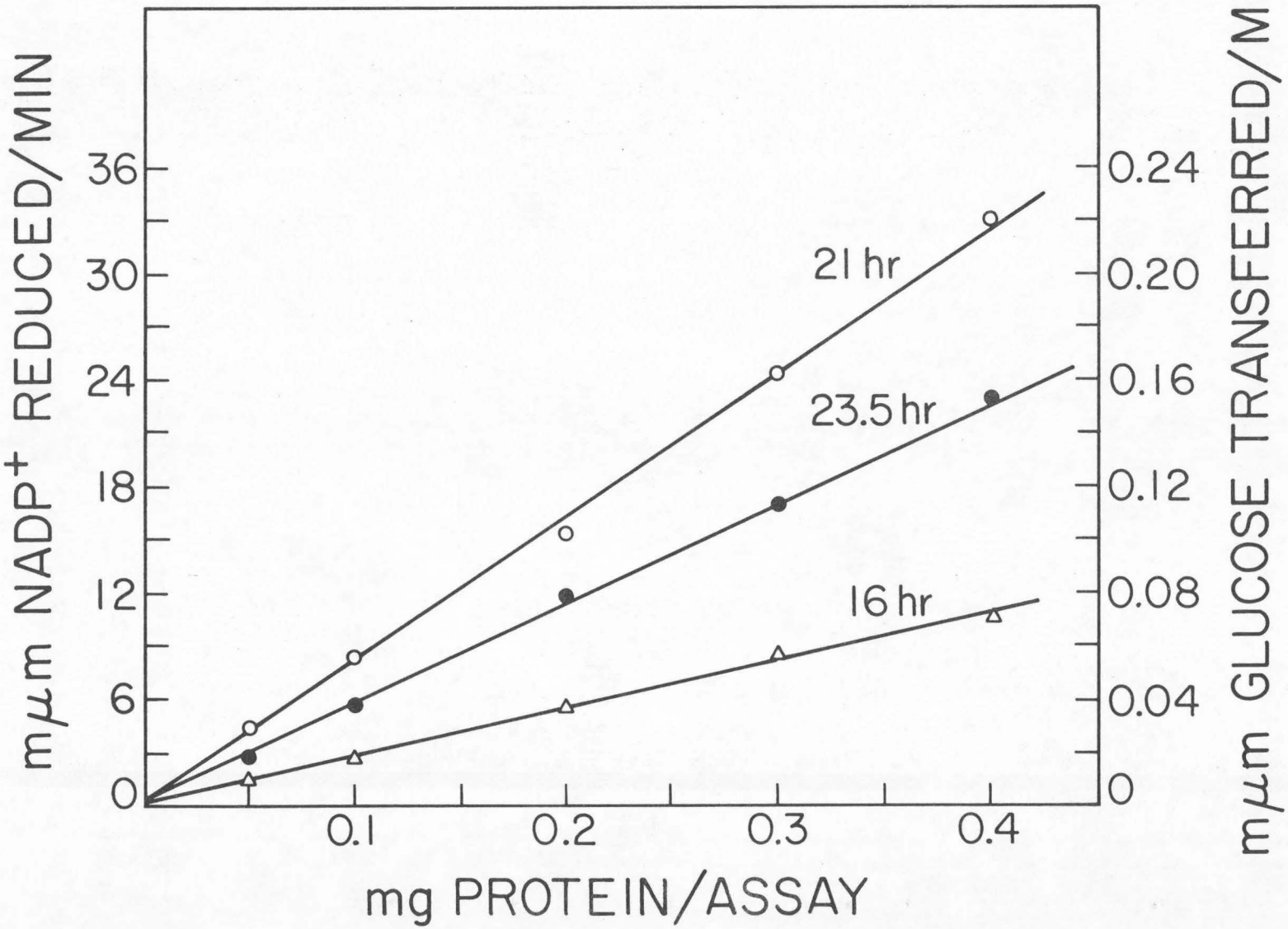

mμm NADP+ REDUCED/MIN
36
30
24
18
12
6
0
0.1
0.2
0.3
0.4
mg PROTEIN/ASSAY
21 hr
23.5 hr
16 hr
0.24
0.20
0.16
0.12
0.08
0.04
0
mμm GLUCOSE TRANSFERRED/MIN

Table 4

Additivity of Enzyme Activity in Crude Extracts

Stage (hour)	Measured Activity (OD/min)	Expected Activity (OD/min)
0	0	-
6	0	-
17	0.00609	-
22.2	0.0186	-
24.5	0.00541	-
34	0	-
0 + 2.22	0.0180	0.0186
6 + 22.2	0.0178	0.0186
17 + 22.2	0.0265	0.0248
22.2 + 22.2	0.0370	0.0372
22.2 + 24.5	0.0241	0.0240
22.2 + 34	0.0188	0.0186
17 + 24.5	0.0113	0.0115
8 + 17	0.00623	0.0609
17 + 34	0.00601	0.0609
Purified enzyme	0.0279	-
Purified + 34	0.0271	0.0279

totally disappeared, had no effect on the activity of extracts from the intermediate stages. This result indicates that there is either no soluble inhibitor or activator of enzyme activity at these stages or at least that there is no excess inhibitor or activator. It is also possible that inhibition or activation of preformed enzyme might require an active, energy-requiring process which cannot take place in crude extracts. When crude extracts are preincubated at 35°C for 20 min prior to adding substrates, a 10 to 15% loss of activity in all fractions is obtained; however, all combinations yield total additivity. Purified enzyme was assayed in the reverse direction (assay II) in the presence of extracts of 34 and 36 hour cultures. The expected amount of ^{14}C-glucose incorporation was obtained. In the case of one enzyme of Dictyostelium, cellulase, it is known that a specific inhibitor of the enzyme is present during early stages of development and that it inactivates the enzyme (Rosness, 1968). At later stages of development the inhibitor is apparently inactivated and enzyme activity appears and increases.

Sussman and Lodgren (1965) and Telser and Sussman (1971) have demonstrated that accumulated uridine diphosphogalactose polysaccharide transferase and uridine diphosphate galactose-4-epimerase activity are both excreted from maturing spore cells. It has been hypothesized that these two enzymes are present inside the

pre-spore vesicles and that when these vesicles fuse with the outer cell membrane of the pre-spore cells, forming a mature outer spore coat, the enzymes are released into the medium. To test whether glycogen phosphorylase activity is excreted in this fashion, cells were collected in buffer containing 5% glycerol buffer (containing 0.2 mM DTT) and centrifuged for 5 min at 3,000 rpm in a Sorvall SS-34 rotor. Both pellet and supernatant were assayed for enzyme activity. Essentially all enzyme activity was found in the pellet at all stages of development. In the few cases in which a small amount of enzyme activity was found in the supernatant, its specific activity was the same as that of the whole cell pellet. Any small amount of enzyme activity found in the supernatant probably results from a small amount of cell damage during the harvesting procedure.

Kinetics of Glycogen Phosphorylase in Developmental Mutants. Many developmental mutants of Dictyostelium discoideum have been isolated independently from the wild type NC-4. These mutants fall into two main categories, morphologically aberrant mutants (aggregation-minus, mutants which do not culminate, stalkless mutants, etc.) and temporally deranged mutants (mutants which take a longer or shorter time than the wild type to complete morphogenesis). Glycogen phosphorylase activity was studied in mutants of both classes (Figs. 9 and 10). Three mutants which show no signs of any morphogenetic

Figure 9. Developmental kinetics of glycogen phosphorylase in various morphologically aberrant and temporally deranged mutants.

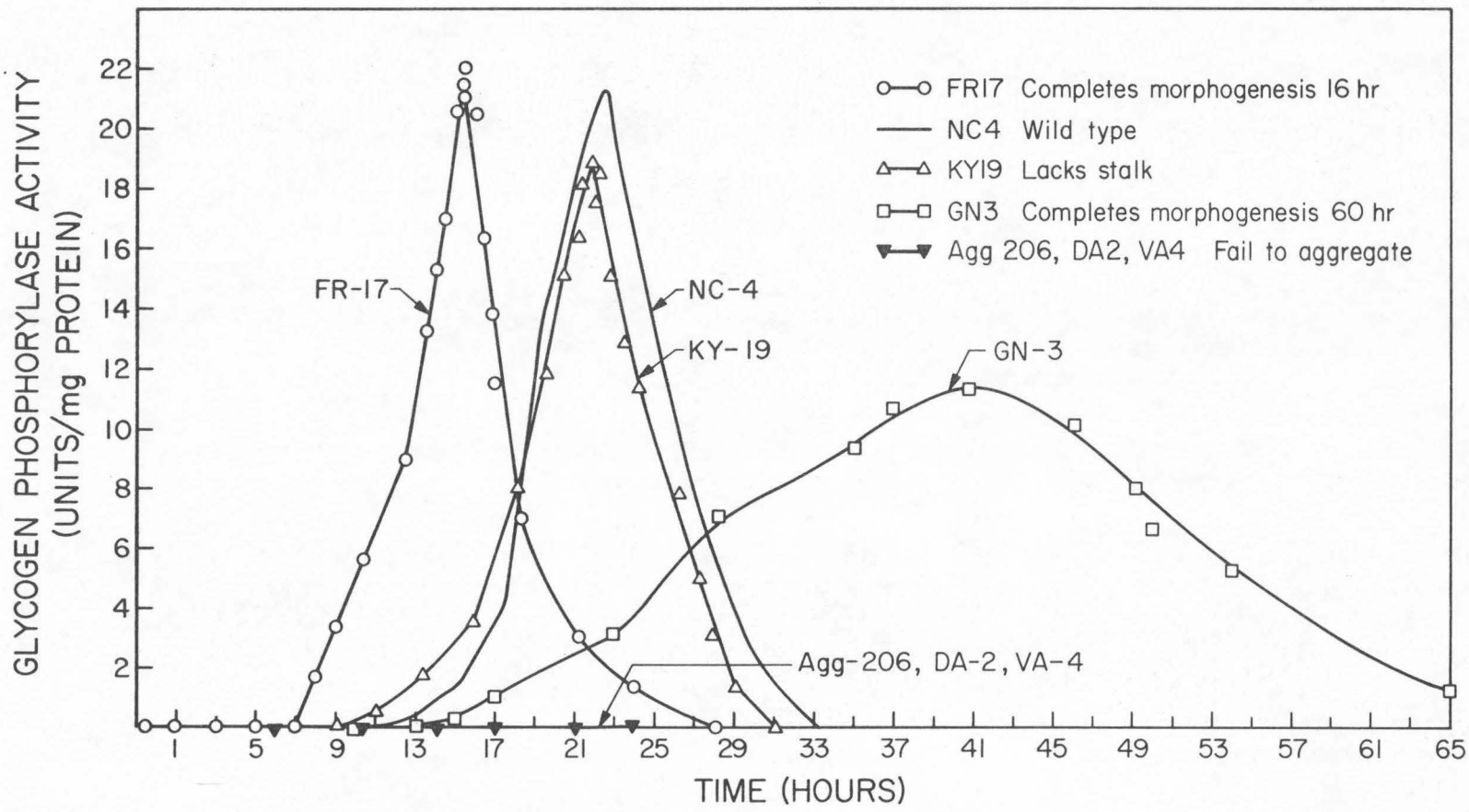
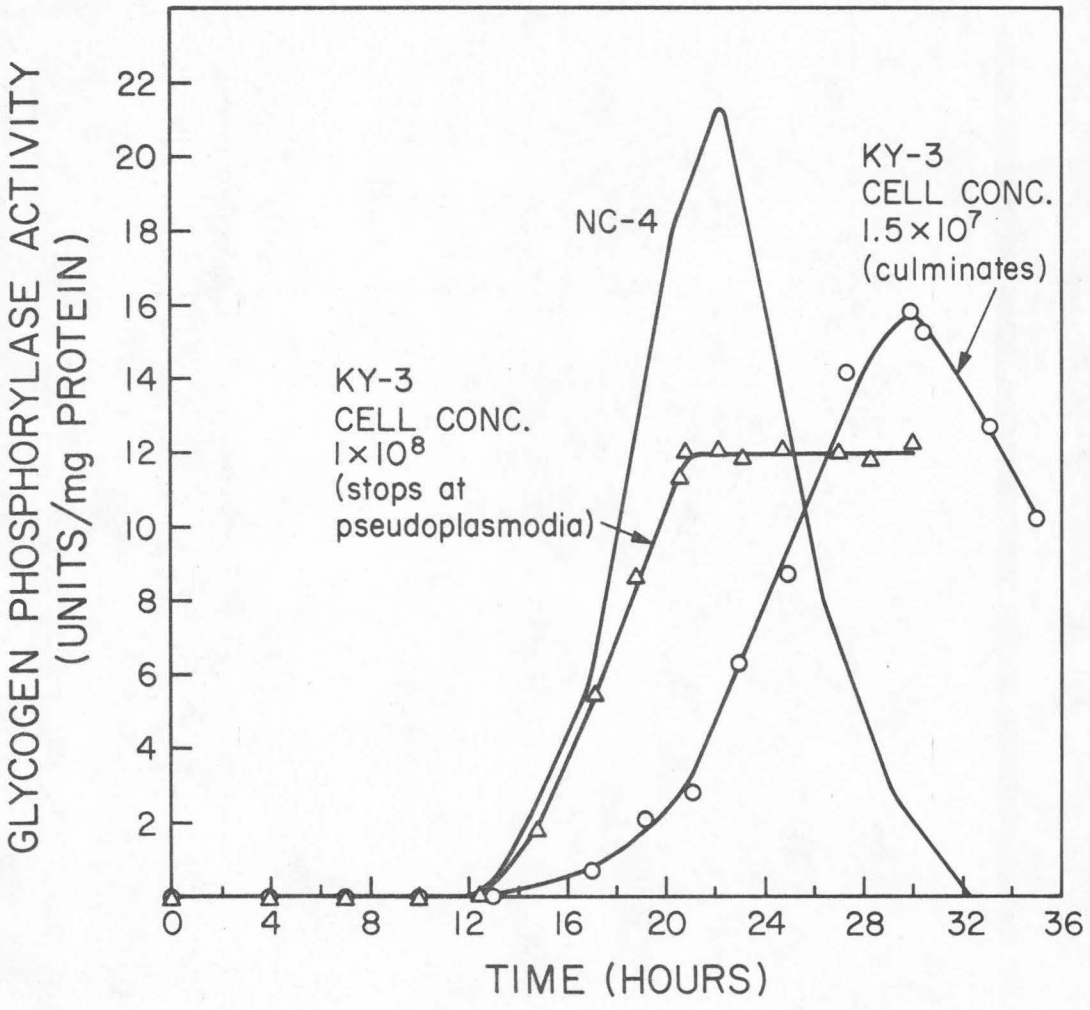

GLYCOGEN PHOSPHORYLASE ACTIVITY
(UNITS/mg PROTEIN)
22
20
18
16
14
12
10
8
6
4
2
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
61
65
TIME (HOURS)
FR-17
NC-4
KY-19
GN-3
Agg-206, DA-2, VA-4
FRI7 Completes morphogenesis 16 hr
NC4 Wild type
KYI9 Lacks stalk
GN3 Completes morphogenesis 60 hr
Agg 206, DA2, VA4 Fail to aggregate

Figure 10. Development of kinetics of KY-3 allowed to develop under varying conditions.


GLYCOGEN PHOSPHORYLASE ACTIVITY
(UNITS/mg PROTEIN)
22
20
18
16
14
12
10
8
6
4
2
0
4
8
12
16
20
24
28
32
36
TIME (HOURS)
NC-4
KY-3
CELL CONC.
1.5×10^7
(culminates)
KY-3
CELL CONC.
1×10^8
(stops at
pseudoplasmodia)

differentiation (aggregation-less mutants, DA-2, VA-4, Agg-206) yield no glycogen phosphorylase activity even 30 hours after the removal of the food source. Mutant KY-3 forms a pseudoplasmodium under normal culture conditions (cell concentration 7-10 x 10^7 cell/filter) but develops no further. In this mutant, glycogen phosphorylase accumulates to approximately one-half maximum activity, remains constant, and does not decline. However, if the mutant is cultured at a lower concentration (1-3 x 10^7 cells/filter), it does culminate and forms fruits (Yanagasawa, et al., 1967), although the morphological events occur more slowly than in the wild type strain. Under these conditions glycogen phosphorylase reaches a specific activity approximately 3/4 that of NC-4, and the activity declines normally. It can be seen in Fig. 10 that under these conditions the enzyme peaks at a later time after removal of the food source than in the wild type. Nevertheless, the maximum activity occurs at the same time relative to morphological development as it does in the wild type. Mutant KY-19 forms normal spore cells but forms a much smaller number of stalk cells than does NC-4. In this mutant enzyme activity reaches approximately the same level as is found in the wild type and declines over the same period of time. Thus it appears that the morpholigical differentiation of prestalk into stalk cells is not necessary for full enzyme activity in KY-19.

Mutants FR-17 and GN-3 are temporally deranged. Mutant FR-17 undergoes development in 16 hours but does not form normal fruiting bodies. Morphologically normal spore and stalk cells are mixed in an amorphous mound of cells (Sonneborn et al., 1963). Mutant GN-3 develops normally but requires 60 to 70 hours to produce mature fruits (Loomis, 1970b). In FR-17, maximum phosphorylase activity equals or is slightly greater than in wild type. GN-3, however, produces only one-half of the maximum phosphorylase activity of wild type. In addition the peak is broad and develops over a much longer period of time. The low, broad peak may be due in part to some asynchrony in the population, which is apparent in GN-3 cells. It is also possible that during the longer than normal period of phosphorylase increase and decrease, a normal number of phosphorylase molecules are produced but that some are inactivated while others are still being synthesized. The sharp peak in phosphorylase activity in FR-17 precludes the possibility of such asynchrony in this mutant. The developmental time course of phosphorylase activity in FR-17 was also assayed in the reverse direction (Assay II) and the data are shown in Table 5. As with NC-4, the ratio of the activity in the forward to that in the reverse direction is constant throughout development.

Table 5

Glycogen Phosphorylase Activity in FR-17

STAGE (hours)	ACTIVITY (mμm/min/mg protein) Assay I (A) ($NADP^+$ reduced)	Assay II (B) (Glucose transfered)	B/A
4	0	0	-
10	1.6	0.11	14
13.5	10.1	0.73	14
14.8	11.3	1.36	14.0
15.5	21.5	1.54	14.0
16.3	18.0	1.28	14.0
18	8.0	0.60	13
21	5.0	0.38	13
24.5	2.5	0.19	13

These data strongly suggest that the appearance and disappearance of glycogen phosphorylase is developmentally regulated and that the appearance of phosphorylase and morphogenesis in Dictyostelium are linked. The results with mutants FR-17 and GN-3 indicate a temporal control while the data from KY-3 and other mutants indicate that the enzyme reaches a level of activity equivalent to that reached by NC-4 at the same morphological stage in development.

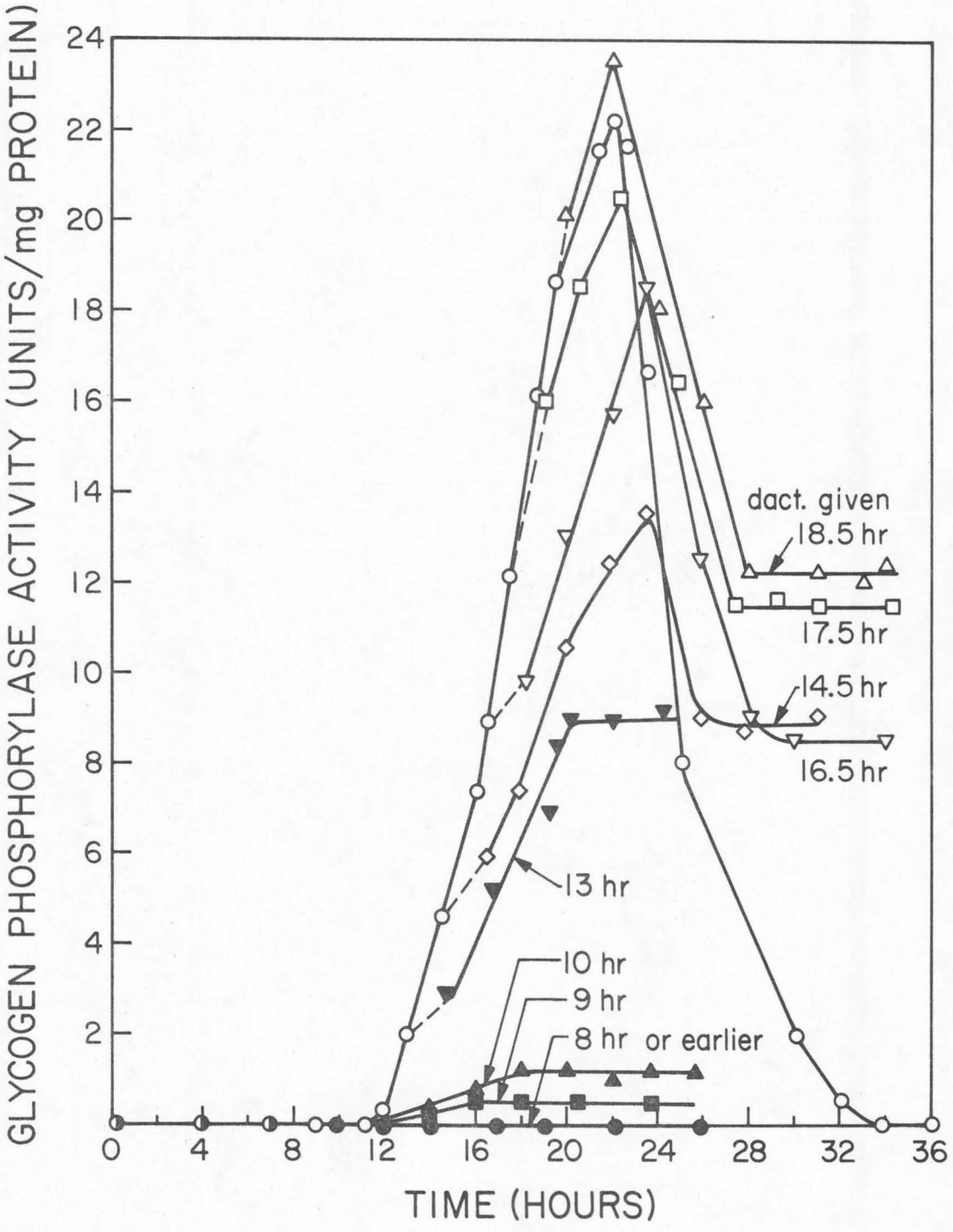
Effects of Actinomycin D, Cycloheximide, and L-Canavanine. To determine if RNA synthesis is necessary for the increase in enzyme activity, developing slime molds were treated at various stages during the developmental cycle with actinomycin D. The amount of enzyme which subsequently accumulated was then measured. Actinomycin D, under the conditions used, is known to inhibit more than 95% of Dictyostelium RNA synthesis within 30 min (Sussman et al., 1967; Sussman and Sussman, 1965, Firtel, unpublished observations). In our hands, actinomycin D (125 ug/ml) causes more than 95% inhibition of incorporation of ^{3}H uridine into RNA at all stages of development tested up to the 18 hour Mexican hat stage. Sussman et al., (1967) showed that the RNA which is labeled in the presence of actinomycin D sediments at 4S and may represent turn over of the terminal-CCA group of t RNA. Mizukami and Iwabuchi (1970) have

reported that actinomycin D inhibits ^{3}H-uridine incorporation into RNA by 46% during aggregation, and by 26% during late pseudoplasmodium formation for cells developed on Millipore filters. The work of these authors, however, agrees with the work of Sussman et al., (1967) in showing that actinomycin D had no effect on ^{14}C amino acid incorporation into protein for 7 hours.

The effect of actinomycin D, added at various times during the developmental cycle, on the subsequent accumulation of glycogen phosphorylase is shown in Fig. 11. When actinomycin D is added at 8 hours or earlier no enzyme activity detectable by assay I appears. A small amount is detected by assay II. No enzyme activity can be detected by either assay I or assay II if actinomycin D is given prior to 6.5 hours. As the time at which actinomycin D is added is delayed from 9 to 18.5 hours increasing amounts of enzyme activity accumulate. Actinomycin D given at 18.5 hours causes cells to accumulate slightly more than the normal maximum amount of enzyme activity. Addition of actinomycin D after 18.5 hours exerts no effect on enzyme accumulation (see results with KY-19).

The simplest hypothesis to account for the effects of actinomycin D is that the drug inhibits transcription of the phosphorylase messenger RNA. The transcription period (the developmental period in which the messenger

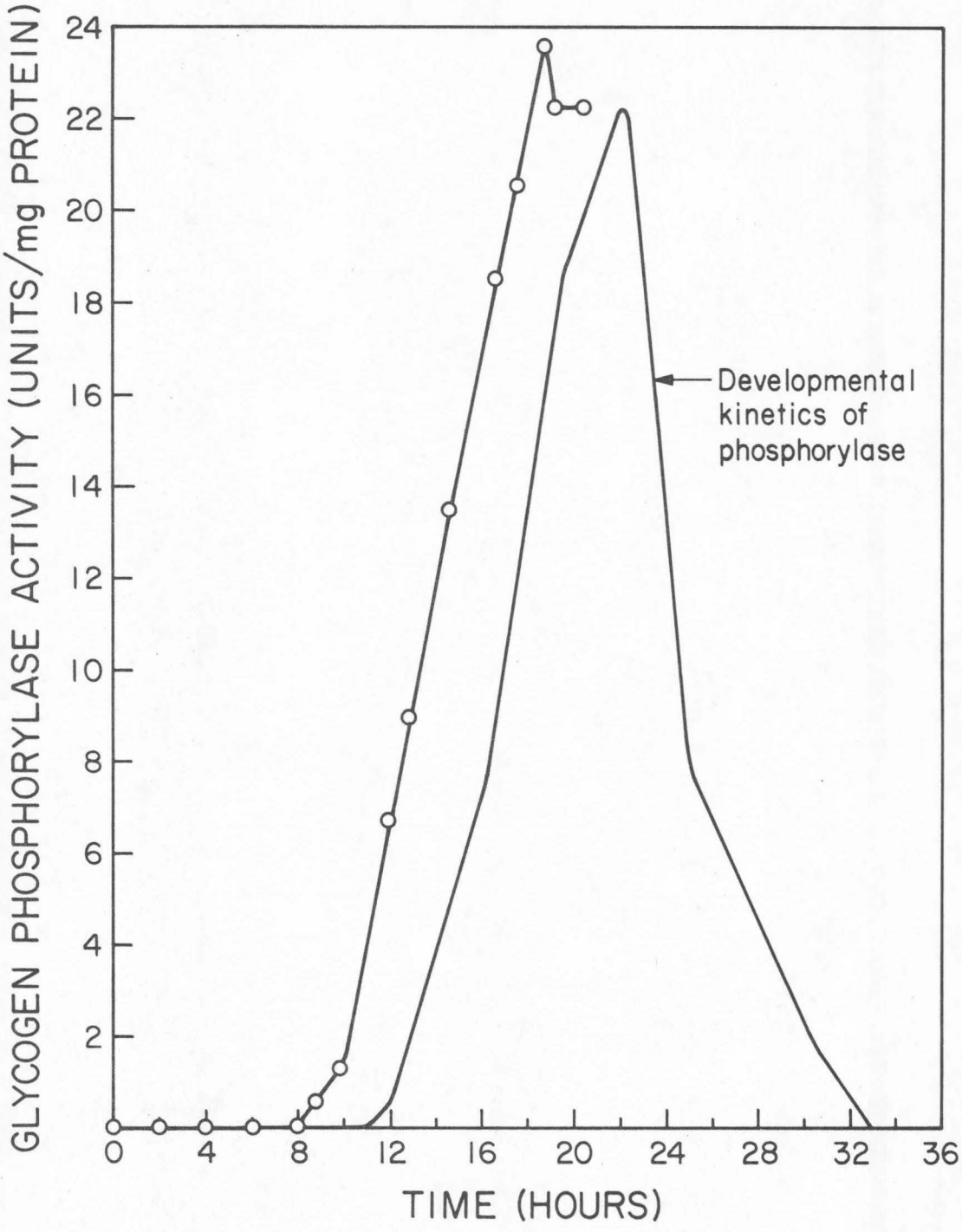
Figure 11. Effect of actinomycin D on increase in specific activity of glycogen phosphorylase in NC-4. Whatman #50 filters with developing cells were transferred to Millipore pads saturated with PDF containing 125 µg/ml actinomycin D (dact.) at the times indicated. Cells were then harvested at various times after that and phosphorylase activity measured. All culturing in the presence of actinomycin D was done in darkness.


GLYCOGEN PHOSPHORYLASE ACTIVITY (UNITS/mg PROTEIN)
TIME (HOURS)
dact. given
18.5 hr
17.5 hr
14.5 hr
16.5 hr
13 hr
10 hr
9 hr
8 hr or earlier

for glycogen phosphorylase appears to be synthesized) would then be between approximately 7 and 18.5 hours. It may be noted that, although RNA synthesis is inhibited within 30 min after treatment with actinomycin D, enzyme continues to accumulate for approximately 3.5 hours. Fig. 12 shows that there is a sigmoidal relationship between the time at which actinomycin D is added and the maximum level of enzyme activity attained. It may be noted that there is a 2 to 3 hour lag between the beginning of the putative transcription period and the first appearance of glycogen phosphorylase. Part of this time probably represents time required for the actinomycin D to inhibit RNA synthesis (30 min) while part may represent some post-transcriptional processing of mRNA. In studies with other developmentally regulated enzymes in Dictyostelium discoideum, this lag period ranges, depending upon the enzyme studied, from 2 to 12 hours (see Loomis, 1969b).

To determine whether de novo protein synthesis is required for the appearance of glycogen phosphorylase activity, the inhibitor cycloheximide was used. Before examining these results it is important to look at the effects of cycloheximide on Dictyostelium discoideum. Cycloheximide inhibits the incorporation of labeled amino acid into hot TCA precipitable material in NC-4 within 15-30 min (Firtel, unpublished observations). Inhibition is greater than 90% with the concentration used (500 µg/ml).

Figure 12. Relationship between maximum specific activity of phosphorylase reached in the presence of actinomycin D *vs.* time the drug is given. The level of phosphorylase accumulated is plotted against the time during development when the cells are transferred to pads containing actinomycin D.


GLYCOGEN PHOSPHORYLASE ACTIVITY (UNITS/mg PROTEIN)
24
22
20
18
16
14
12
10
8
6
4
2
0
4
8
12
16
20
24
28
32
36
TIME (HOURS)
Developmental kinetics of phosphorylase

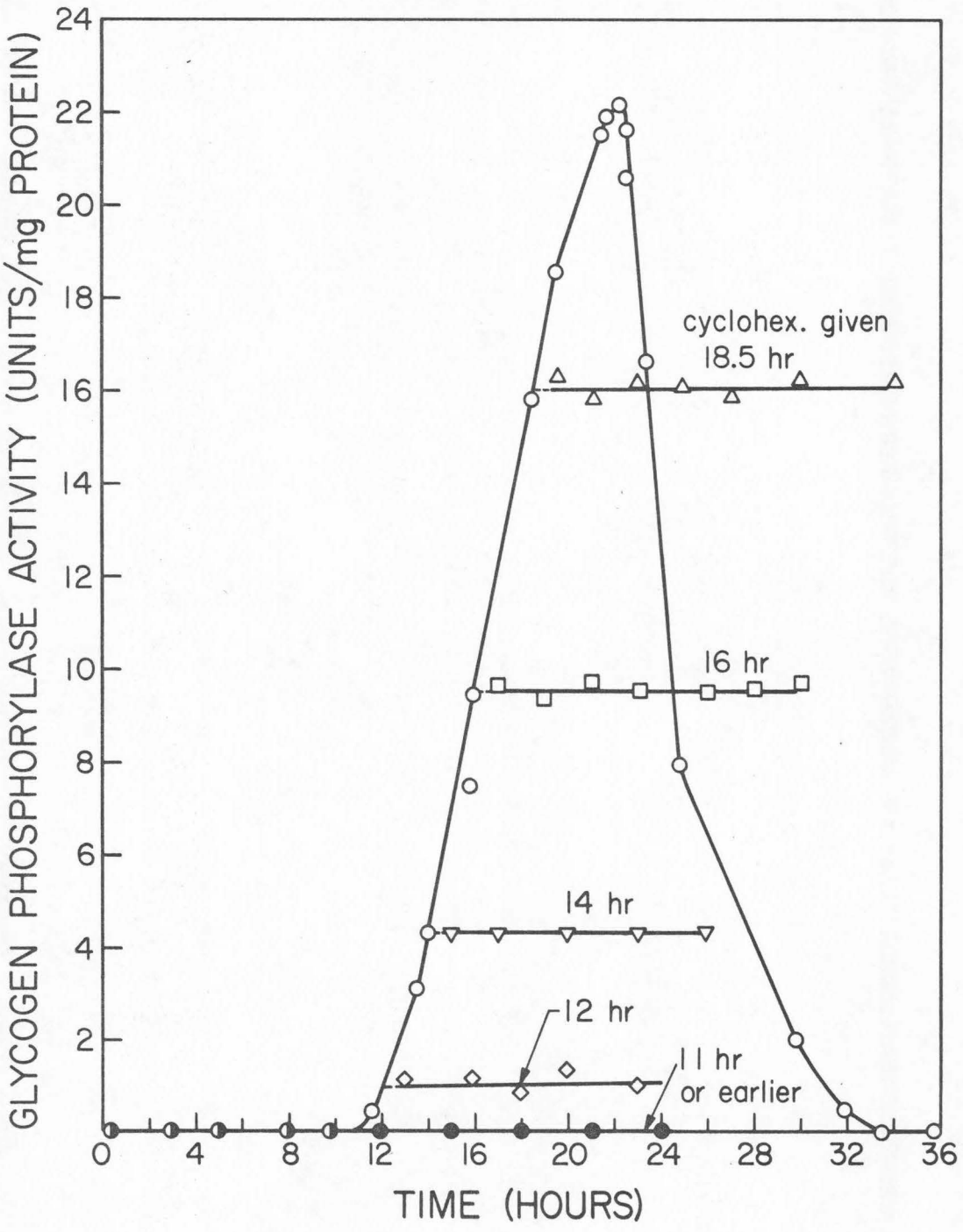
This inhibition is found for all stages of development up through late Mexican hat stage (approximately 18-20 hours). In mutants KY-19 and FR-17 which do not form completed fruits, cycloheximide inhibits protein synthesis at all times during the developmental cycle. If cells which have been incubated in the presence of the drug for up to 6 hours are transferred to PDF solution without cycloheximide they will form normal fruiting bodies with mature spores. This result indicate that the drug does not kill the cells and that the inhibition of protein synthesis is reversible. Although earlier results indicated that the incorporation of precursors into RNA is not inhibited by cycloheximide (Sussman, 1965), more recent experiments show that during development this drug inhibits RNA synthesis by approximately 50% within 30 to 60 min (Firtel, unpublished observations; Mizukami and Iwabuchi, 1970). It is not known if this early inhibition represents a general inhibition of the synthesis of all RNA species, or if only certain species are affected. By 90 min after addition of the drug during development all RNA synthesis ceases. This is also true in axenic, logarithmically growing Dictyostelium discoideum (J. Tuchman, personal communication). In a recent study of the effects of cycloheximide on logarithmically growing Chlamydomonas reinhardi, McMahon (in preparation) shows that cycloheximide inhibits rapidly labeled RNA synthesis by 50% in 1 to 2 hours and almost completely inhibits the accumulation of total RNA.

The ATP pools decrease by 50% and the cells are killed with exponential kinetics. Such killing starts within 30 min after cycloheximide is given. It is clear then that any conclusion obtained from experiments in which cycloheximide is used to inhibit protein synthesis must be interpreted with caution.

The effects of cycloheximide on the accumulation of glycogen phosphorylase in NC-4 are shown in Fig. 13. When cycloheximide is added before glycogen phosphorylase can be measured, no phosphorylase activity appears. If it is added after enzyme accumulation has begun, subsequent accumulation is inhibited and enzyme activity remains constant for over 10 hours. By adding cycloheximide at different times during development, the translational period of the enzyme can be estimated. The results suggest that the enzyme is synthesized de novo and concomitantly with the actual appearance of enzyme activity.

The accumulation of enzyme activity stops within 15 to 30 min after the drug is given and remains constant thereafter. Since the enzyme continues to accumulate in the presence of actinomycin D for several hours even though RNA synthesis is inhibited within 30-45 min, it is reasonable to conclude that the immediate effect of cycloheximide on enzyme accumulation is the result of the inhibition of enzyme synthesis. One possible alternative view might be that the appearance of glycogen phosphorylase

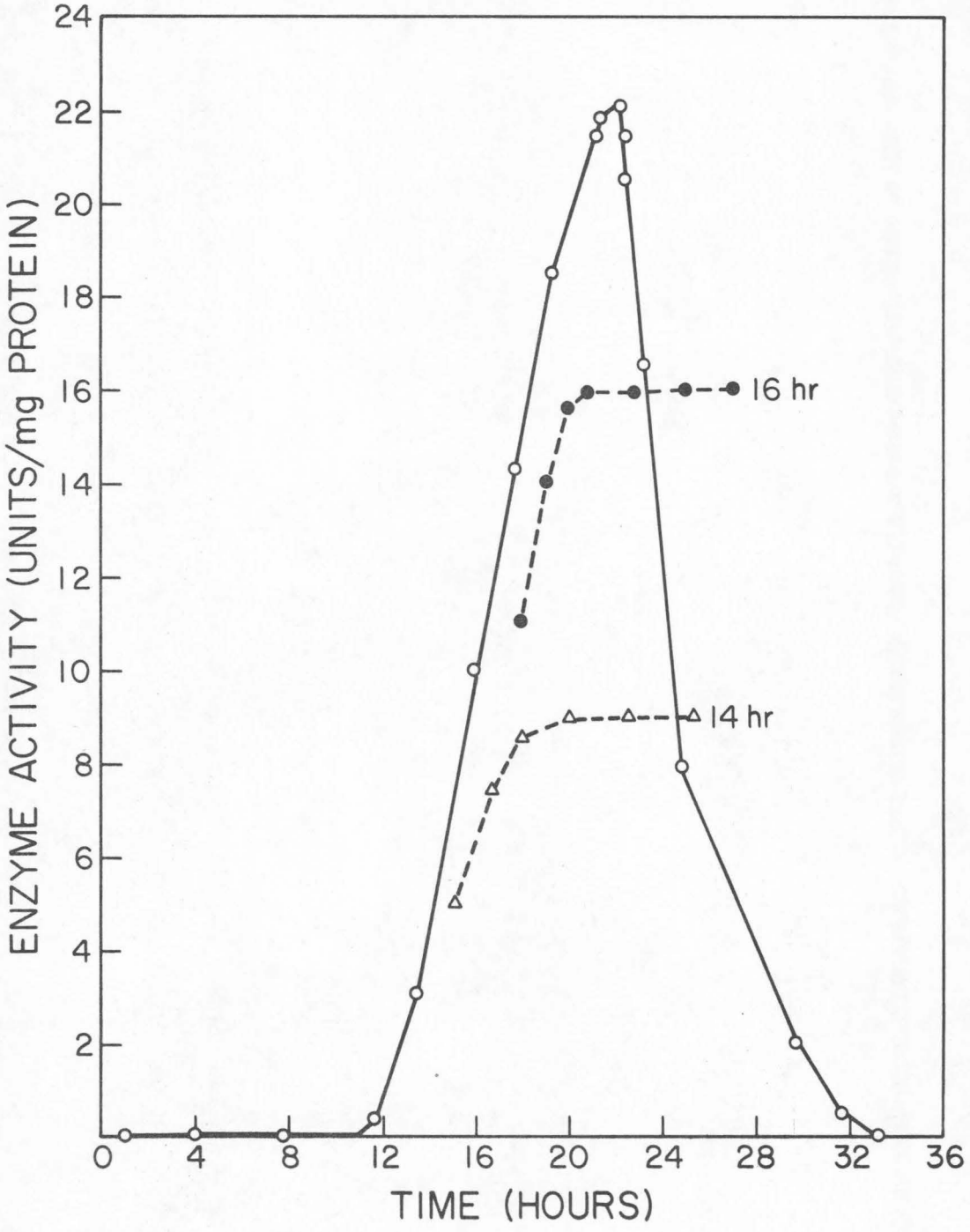
Figure 13. Effect of cycloheximide on increase in specific activity of glycogen phosphorylase in NC-4. Filters were transferred to pads saturated with PDF containing 500 µg/ml cycloheximide.


GLYCOGEN PHOSPHORYLASE ACTIVITY (UNITS/mg PROTEIN)
TIME (HOURS)
cyclohex. given
18.5 hr
16 hr
14 hr
12 hr
11 hr
or earlier
0
4
8
12
16
20
24
28
32
36
2
4
6
8
10
12
14
16
18
20
22
24

activity is concomitant with the synthesis of an activator for already present but inactive enzyme. However, as shown in Table 4, when extracts of NC-4 of various stages earlier than 8 hours are incubated together with extracts of later stages, no changes in activity result. This suggests that at least in vitro no activation of preformed enzyme occurs. Although the cycloheximide results strongly suggest de novo synthesis of glycogen phosphorylase at the time of increased enzyme activity, final proof must await labeling studies in which the enzyme is labeled during the developmental cycle and then purified.

The effect of L-canavanine, an arginine analog, on accumulation of glycogen phosphorylase was also studied. Loomis (1968) has shown that L-canavanine, given at the pseudoplasmodium stage, inhibits fruiting body formation. Cotter and Raper (1970) have shown that L-canavanine inhibits spore germination in Dictyostelium and that this inhibition can be overcome by arginine. If the analog is given at 14 or 16 hours of development, phosphorylase activity continues to accumulate for approximately 3 hours but at a decreased rate (Figure 14). In Chlamydomonas reinhardi (McMahon and Langstroth, submitted for publication) canavanine is known to be incorporated into protein and to inhibit RNA synthesis and subsequently

Figure 14. Effect of L-canavanine on increase in specific activity of phosphorylase in NC-4. Filters were transferred to pads saturated with PDF containing 4 mM L-canavanine SO_4.


ENZYME ACTIVITY (UNITS/mg PROTEIN)
TIME (HOURS)
16 hr
14 hr
0
4
8
12
16
20
24
28
32
36
2
4
6
8
10
12
14
16
18
20
22
24

protein synthesis. It is not known for Dictyostelium whether the effects of L-canavanine result from its being incorporated into protein (with consequent formation of inactive phosphorylase), to competition with arginine for the relevant tRNA and activating enzymes, or to inhibition of RNA synthesis.

The Effects of cycloheximide and Actinomycin D on Decrease in Enzyme Activity. Since only a small fraction of the cells of the fruiting body are in contact with the buffered saturated filter, it is difficult to obtain quantitative inhibition of macromolecular synthesis by actinomycin D or cycloheximide in NC-4 after 20 hours of development. Therefore, strains KY-19 and FR-17 which do not make vertical stalks were used to test the effects of these inhibitors on the final disappearance of enzyme activity. Figures 15 and 16 show the effects of these drugs on mutants FR-17 and KY-19. As already shown for NC-4, cycloheximide inhibits accumulation of enzyme activity within 15-30 minutes after it is supplied. As also shown for NC-4, the specific activity of glycogen phosphorylase in the presence of cycloheximide remains constant for over 10 hours. The actinomycin D results with KY-19 are essentially the same as those with NC-4. As is expected, the transcriptional and translational periods for glycogen phosphorylase in mutant FR-17 occur at earlier times than with NC-4. Addition of actinomycin

D at 11 hours of development results in an enzyme level which is approximately 15% greater than that reached without inhibitor. If the inhibitor is given at 13 hours, the level is approximately 10% higher. These numbers are quite reproducible from experiment to experiment. The higher level of enzyme accumulations may be due to an inhibition of the decrease in enzyme activity which appears to begin before enzyme accumulation is finished.

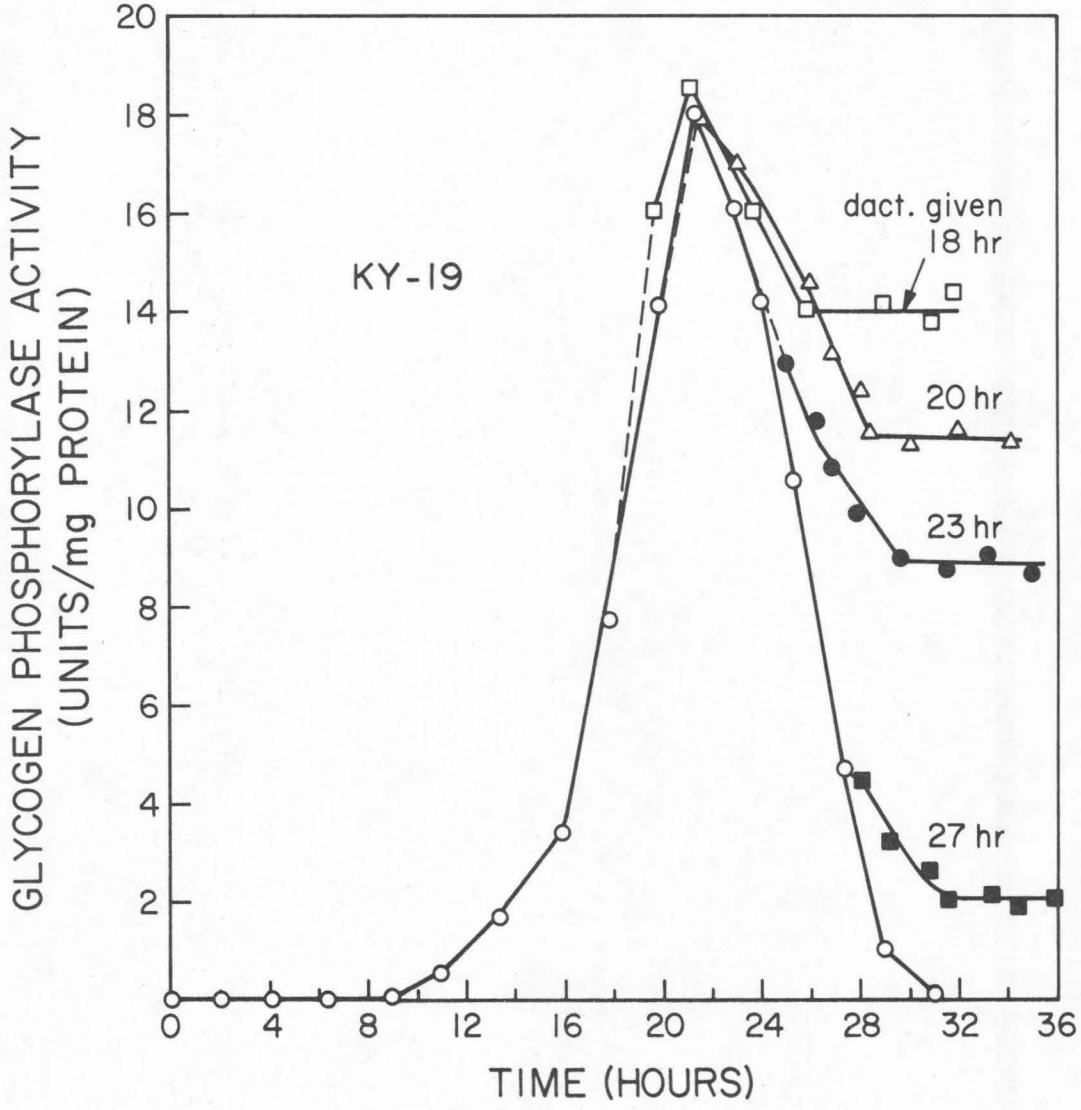
Cycloheximide also inhibits the final decrease of phosphorylase activity in both KY-19 and FR-17. In both mutants the decrease in phosphorylase activity is inhibited within 30 minutes after the drug is given, although at very late stages there is a lag of approximately 1-2 hours.

The results of Fig. 11 show that if actinomycin D is given to NC-4 before 14.5 hours, enzyme activity increases for approximately 3.5 hours and subsequently remains constant. If the drug is given after 14.5 hours, the enzyme continues to increase, then decreases, and levels off at an intermediate level. The amount of enzyme that is inhibited or destroyed in the presence of actinomycin D appears to be related to the time at which actinomycin D is given. Figure 15 and 16 show similar results with FR-17 and KY-19. When actinomycin D is given to FR-17 at 8.5 to 13 hours enzyme activity increases and then decreases to an intermediate level. When it is given at

Figure 15. Effect of actinomycin D on phosphorylase activity in mutants KY-19 and FR-17. Methods same as in Figure 11.

15a. Effect of actinomycin D on decrease in specific activity in KY-19.

15b. Effect of actinomycin D and rise and fall of specific activity in mutant FR-17.


GLYCOGEN PHOSPHORYLASE ACTIVITY
(UNITS/mg PROTEIN)
TIME (HOURS)
KY-19
dact. given
18 hr
20 hr
23 hr
27 hr
0
4
8
12
16
20
24
28
32
36
2
4
6
8
10
12
14
16
18
20

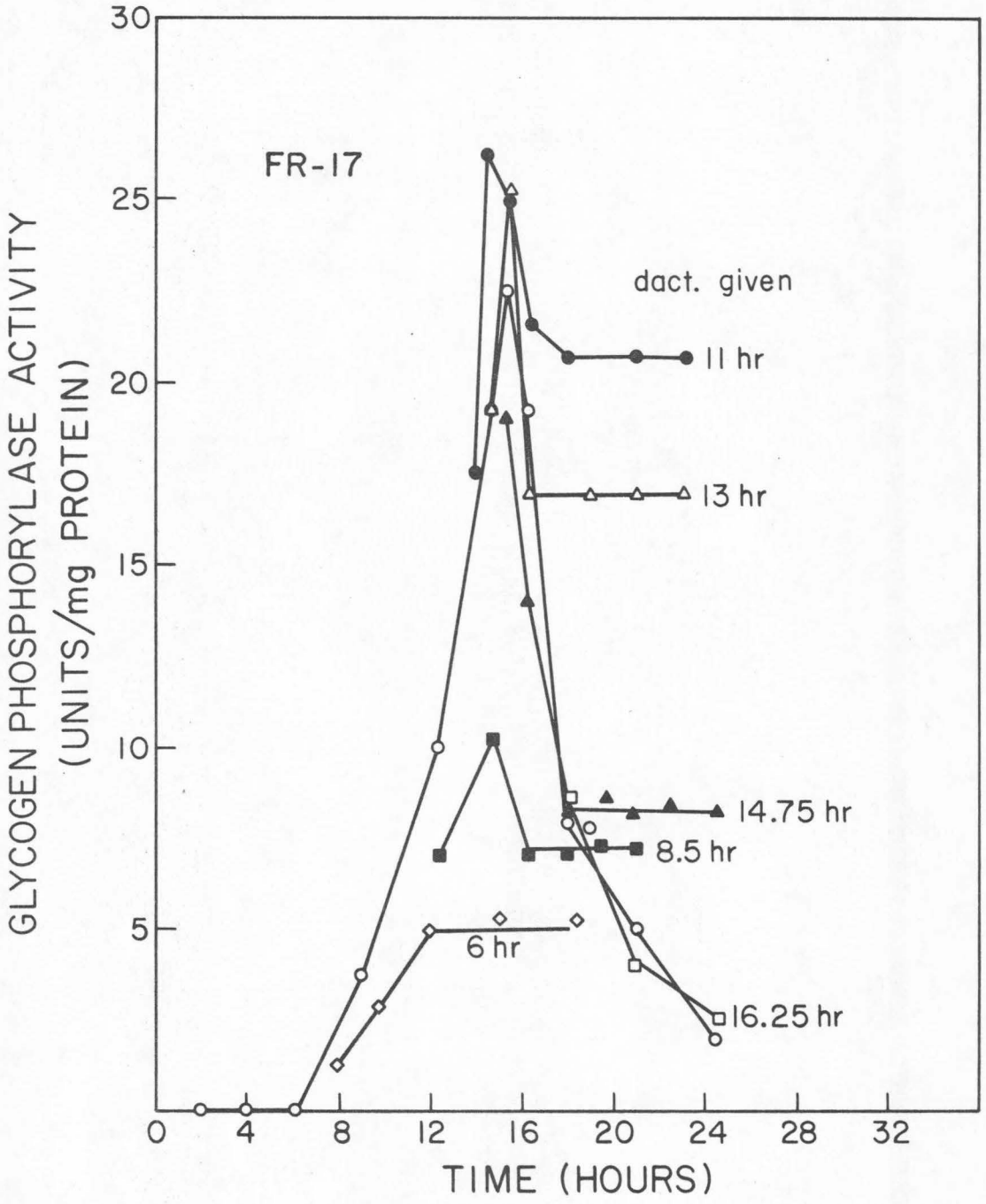
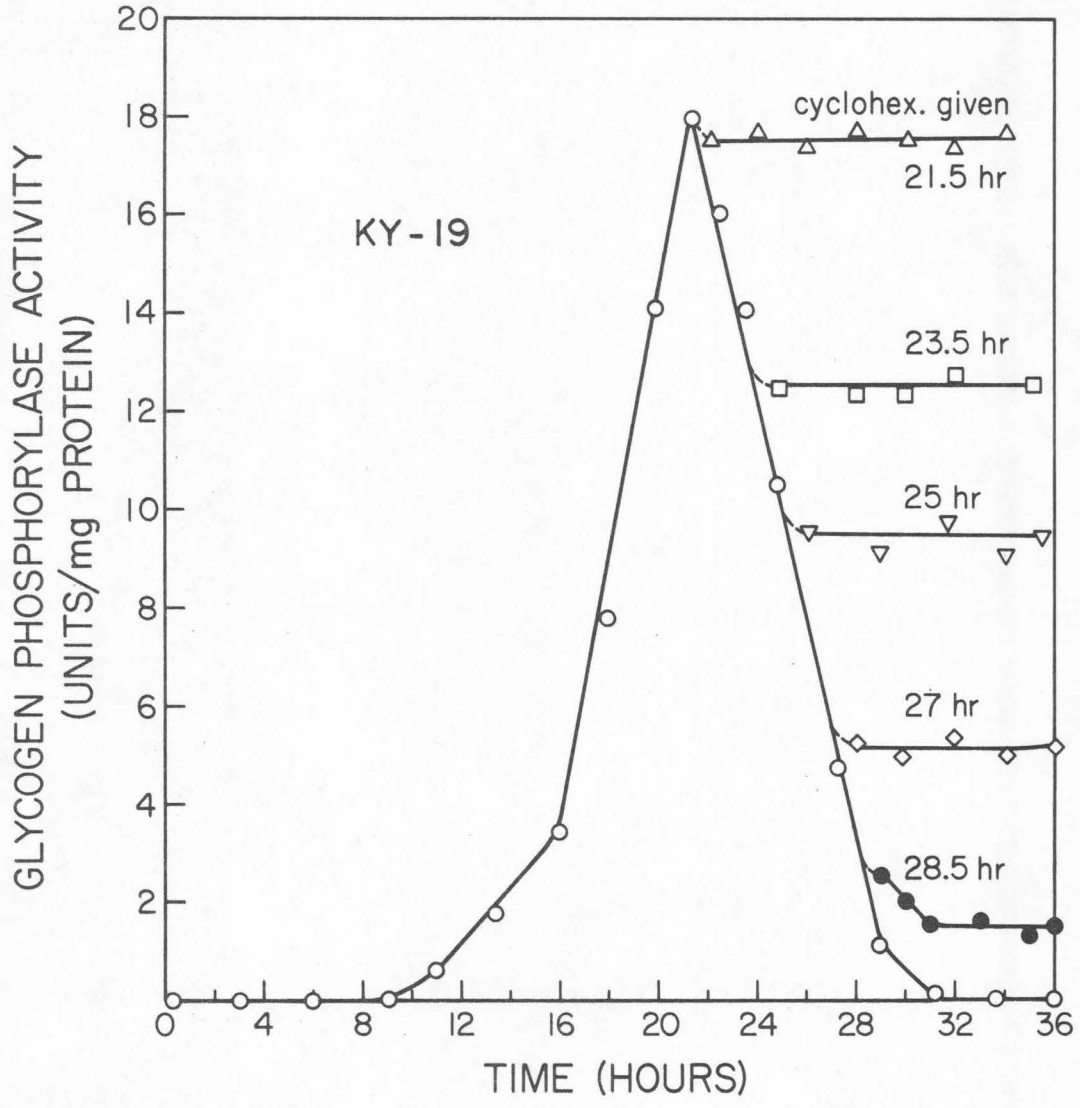
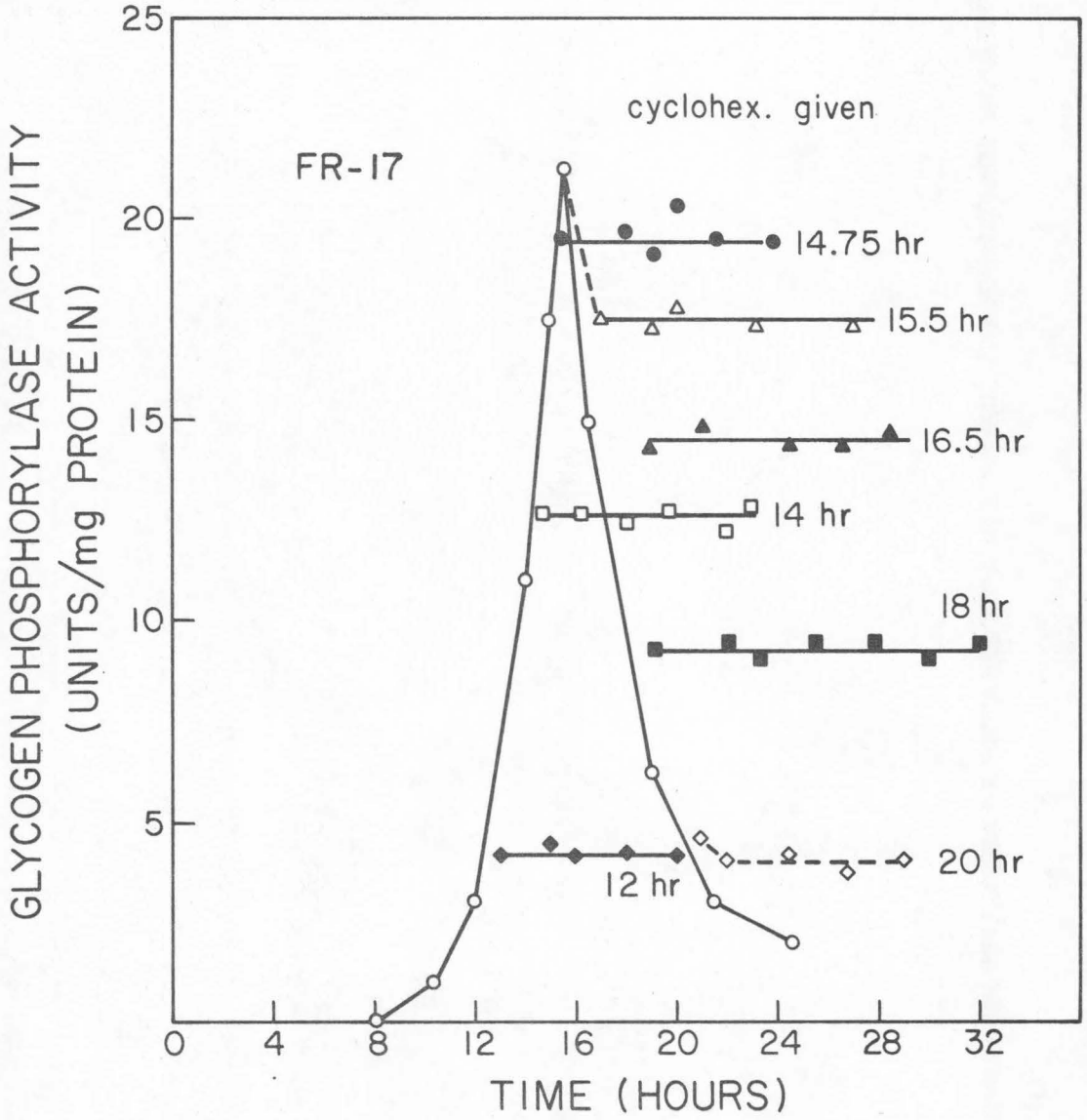

FR-17
dact. given
11 hr
13 hr
14.75 hr
8.5 hr
6 hr
16.25 hr
GLYCOGEN PHOSPHORYLASE ACTIVITY
(UNITS/mg PROTEIN)
TIME (HOURS)
0
4
8
12
16
20
24
28
32
5
10
15
20
25
30

Figure 16. Effect of cycloheximide on phosphorylase activity in mutants KY-19 and FR-17. Methods same as in Figure 13.

16a. Effect of cycloheximide on decrease in activity in KY-19.

16b. Effect of actinomycin D on increase and decrease in activity in FR-17.


GLYCOGEN PHOSPHORYLASE ACTIVITY
(UNITS/mg PROTEIN)
TIME (HOURS)
KY-19
cyclohex. given
21.5 hr
23.5 hr
25 hr
27 hr
28.5 hr
0
4
8
12
16
20
24
28
32
36
2
4
6
8
10
12
14
16
18
20


GLYCOGEN PHOSPHORYLASE ACTIVITY
(UNITS/mg PROTEIN)
25
20
15
10
5
0
4
8
12
16
20
24
28
32
TIME (HOURS)
FR-17
cyclohex. given
14.75 hr
15.5 hr
16.5 hr
14 hr
18 hr
12 hr
20 hr

16-16.5 hours the decrease in enzyme activity follows normal kinetics. When actinomycin D is given to KY-19 during the period of decrease, the enzyme activity continues to decrease for a short period of time and then levels off.

The results presented above suggest that protein and RNA synthesis are required for the decrease in phosphorylase activity. It is not clear whether Dictyostelium synthesizes a specific inhibitor of phosphorylase or, more probably, a protease. In any case the decrease in enzyme activity appears to be specific for phosphorylase. Other enzymes, for example 6-phosphogluconic acid dehydrogenase, exhibit a slow decrease in activity during culmination which might be due to the general protein catabolism which is known to occur throughout development. In one case, uridine diphosphoglucose pyrophosphorylase is destroyed in the stalk cells as these die but remains stable in spore cells (Ashworth and Sussman, 1967). As noted above, uridine diphosphogalactose polysaccharide transferase (Sussman and Longren, 1965) and uridine diphosphate galactose-4-epimerase (Telser and Sussman, 1971) are excreted from maturing spores. Such excretion also appears to require RNA and protein synthesis. The differences in kinetics of decay of the various enzymes does not support a hypothesis of a general control over degradation of developmentally regulated proteins.

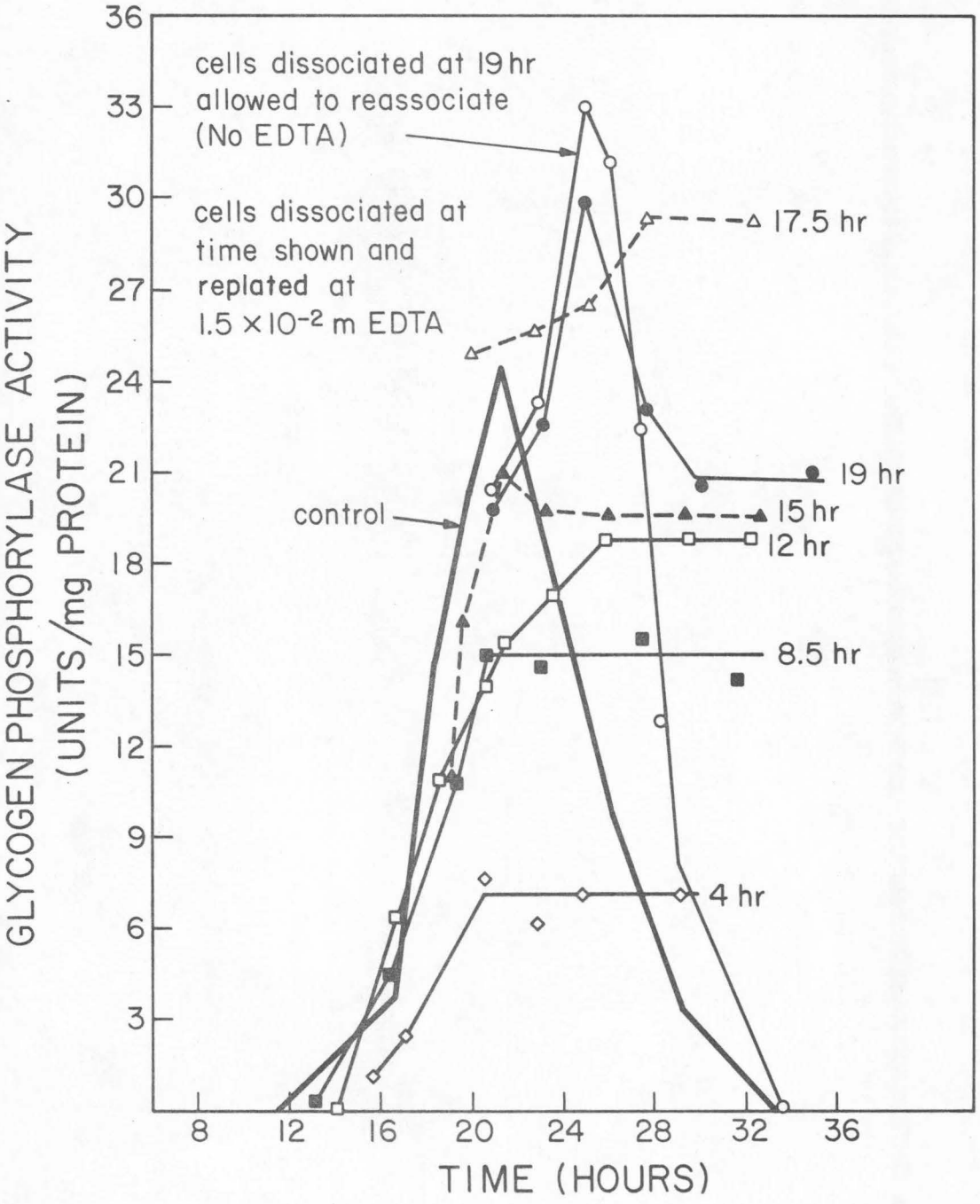
Effect of Cell Dissociation upon the Accumulation of Glucogen Phosphorylase. Aggregates of Dictyostelium discoideum up to the 19th hour of development can be dissociated into individual cells or clumps of several cells by triturating in H_2O or 5 x 10^{-3}M EDTA. When the cells are washed and replaced on filters in normal buffer solution (PDF), the cells aggregate and form small clumps within two hours and then continue their development (Loomis and Sussman, 1966). Normal fruiting bodies develop with a lag of less than 2 hours. If the cells are replaced on filters in buffered salt containing 1-3 x 10^{-2}M EDTA (pH 6.5) but no divalent cation, the cells either fail to aggregate or form a rippled lawn of nondicrete amorphous mounds. No morphological development occurs. EDTA (1 x 10^{-2}M) does not affect either cell viability of ability of cells to incorporate ^{14}C amino acid (Loomis and Sussman, 1966).

The effect of dissociation with 5 x 10^{-3} M EDTA on the appearance of glycogen phosphorylase was tested. When Dictyostelium discoideum aggregates are dissociated and redepositied on filter pads without EDTA at any stage prior to culmination, the enzyme continues to accumulate although with a slight lag (not shown). If the cells are dissociated at 19 hours and then allowed to reassociate, the specific enzyme activity reaches approximately

1.5 times greater than normal, before it subservently decreases (Figure 7). Cells were also dissociated and replated on filters in the presence of 1.5×10^{-2}M EDTA, conditions under which the cells form a rippled lawn. The effect of such replating is also shown in Fig. 17. The amount of phosphorylase accumulated is a function of the time at which cells are dissociated. If this is done after 17 hours of development, a higher than normal activity is accumulated.

Loomis and Sussman (1966) studied the effect of dissociation on accumulation of uridine diphosphogalactose polysaccharide transferase. Their studies indicate that if cells are dissociated after a specific period of development but prior to the initiation of the transcriptive period as determined by actinomycin D, a full complement of enzyme appears in the presence of EDTA. If such cells are dissociated and replated in EDTA before this time, little or no enzyme is accumulated. They conclude that after a specific time period the cells commit themselves to synthesize uridine diphosphogalactose polysaccharide transferase mRNA and that defined morphological structures are not necessary. The effect of EDTA on the appearance of enzymes is quite different from one enzyme to the next. Loomis (1969b) found that dissociation and replating of Dictyostelium discoideum in normal

Figure 17. Effect of EDTA on kinetics of phosphorylase in NC-4. Cells were dissociated in 5 mM EDTA at various times during the developmental cycle and allowed to reassociate or replated in Mg free PDF containing 1.5×10^{-2} M EDTA.


GLYCOGEN PHOSPHORYLASE ACTIVITY
(UNITS/mg PROTEIN)
TIME (HOURS)
cells dissociated at 19 hr
allowed to reassociate
(No EDTA)
cells dissociated at
time shown and
replated at
1.5 × 10⁻² m EDTA
17.5 hr
19 hr
15 hr
12 hr
8.5 hr
4 hr
control
36
33
30
27
24
21
18
15
12
9
6
3
8
12
16
20
24
28
32
36

normal buffered salt after the transcriptive period of alkaline phosphatase inhibits the appearance of the enzyme. With the enzyme β -glucosidase, the appearance was inhibited only if the cells were replated in the presence of EDTA (Coston and Loomis, 1969). Newell and Sussman (personal communication, M. Sussman) have found that if cells are dissociated by titurating in H_2O late in the pseudoplasmodium stage a full, second complement of uridine diphosphoglucose pyrophosphorylase, trehalose-6-phosphate synthetase, uridine diphosphate galactose-4-epimerase and uridine diphosphogalactose polysaccharide transferase is synthesized. Our results with glycogen phosphorylase appear to agree with those of Newell and Sussman and suggest that a second complement of phosphorylase may also be synthesized. In the case of glycogen phosphorylase, it is not known if the larger amount of enzyme that accumulates requires a second round of mRNA transcription.

DISCUSSION

There are at least 15 enzymes of Dictyostelium discoideum whose specific activities increase by at least several fold during the developmental cycle. Studies with inhibitors of RNA and protein synthesis strongly suggest that most of the increases require prior RNA synthesis and de novo synthesis of the enzymes (see Introduction for

references). The presumptive periods of transcription and translation have been delineated by the same procedures described in this paper. It is also known from hybridization studies with purified nonrepetitive DNA that during the developmental cycle of Dictyostelium there are specific qualitative changes in the sequences of the nonrepetitive DNA which are transcribed (Firtel, 1971). These facts, combined with our detailed knowledge of metabolic control systems in prokaryotes, lead us to assume that Dictyostelium has a genetic program which calls for the synthesis of certain enzymes at specific times during the developmental cycle. These enzymes are said to be developmentally regulated. Studies with actinomycin D suggest that the program calls for a sequential turning on and off of certain genes at specific times. In some cases, regulation at the translational level is also indicated (see specifically Loomis, 1969b). In many cases the biochemical changes which are observed can be associated with, and occasionally causally related to, specific morphological changes (for example, synthesis of spore coat mucopolysaccharide, Telser and Sussman, 1971).

The 15 developmentally regulated enzymes which have been studied in this organism can be ordered as to their relative time of appearance in the wild-type strain NC-4. In the morphologically aberrant and temporally deranged mutants tested, the order of

appearance of each enzyme has never been found to be altered relative to its appearance in NC-4. In the temporally deranged mutants FR-17 and GN-3, the periods of transcription and translation of must enzymes tested are temporally shifted in relation to overall development. This is reasonable: assuming that many of the enzymes are probably essential for differentiation, their regulation would be expected to be directly linked to the controls regulating overall timing of development. In the temporally deranged mutants, the increase in specific activity of the developmentally regulated enzymes always coincides with the same morphological changes as observed in NC-4. Thus the development of Dictyostelium appears to run on a sequential, regulated pathway with certain options depending upon the prevailing environmental, physiological, and biological factors. For example, in the absence of light on a poorly buffered medium, the pseudoplasmodia migrate indefinitely and do not synthesize certain of the developmentally regulated enzymes necessary for maturation of the fruiting body. However, if the organisms are transferred to a buffered environment or illuminated with overhead light, the pseudoplasmodia cease migrating, synthesize the enzymes necessary to complete the required biochemical pathways and produce mature fruiting bodies (Newell et al., 1969; Newell and Sussman, 1970).

The developmental kinetics of the enzyme α1-4 glucan phosphorylase has been characterized in crude extracts of Dictyostelium discoideum. The enzyme is not detectable in vegetative cells and cannot be detected in developing cells until mid-aggregation (9 hours). After this stage the enzyme rapidly increases in specific activity and reaches a level of approximately 15 nanomoles of glucose-1-phosphate produced/min/milligram crude extract protein. The activity then falls rapidly and cannot be detected in matured fruits (after 32 hours). Studies with actinomycin D and cycloheximide strongly suggest that prior RNA and concomitant protein synthesis are necessary for both the rise and fall in enzyme activity. The period of RNA transcription for the rise in enzyme activity is between approximately 7-18.5 hours and it is approximately 14.5-27 hours for the factor involved in the decrease in activity. The period of cycloheximide sensitivity for both the rise and fall in activity occurs coincidentally with the period of the rise and fall of specific activity measured in crude extracts.

During the 12 to 13 hour period of rise of glycogen phosphorylase specific activity there is a decrease in the amount of total cell protein of approximately one-third (Gregg et al., 1954; Gregg and Brönsweig, 1956; Wright and Anderson, 1960; White and Sussman, 1961; Mizukami and Iwabuchi, 1970). It is generally assumed

that the number of moles of product produced at a specific substrate concentration in an enzymatic reaction is directly proportional to the number of enzyme molecules present in the reaction mixture. Product formation has been shown to be proportional to the amount of crude extract added to the assay. If glycogen phosphorylase is nonspecifically degraded to the same degree as total cell protein during its period of accumulation, then there is a direct proportion between the increase in specific activity and the number of enzyme molecules synthesized. If, on the other hand, phosphorylase is for some reason exempt from the general protein degradation, this proportionality no longer holds, and the number of new enzyme molecules synthesized is actually proportionally less than the observed increase in the specific activity. It should be noted that the rate of decrease of glycogen phosphorylase activity after peak activity is reached is much greater than the rate of decrease in overall cell proteins.

As has been shown in Fig. 12, there is a sigmoidal relationship between maximum enzyme activity accumulated in the presence of actinomycin D and the time that the drug is given. The simplest explanation for this relation is that the primary site of regulation of glycogen phosphorylase is at the level of transcription. According to this view, the gene for the enzyme is turned on at a specific time in development, RNA is transcribed and translated at a specific rate, and finally the gene is turned

off at a specific later stage in the developmental cycle. The data indicate that there is a 3 hour lag between the onset of transcription and the beginning of translation. This lag period might be interpreted as the result of specific translational control for this enzyme. However, for several reasons it seems more likely to be an artifact of the procedures used. For example, one can easily account for the 3 hour lag period by considering that there is (1) an approximately 30-45 min lag between administration of actinomycin D and shutdown of RNA synthesis, (2) an unknown time period required for maturation of mRNAs in the nucleus and their transport to the cytoplasm, and (3) an unknown period of enzyme synthesis before enough accumulates to be detectable by our assay in vitro. This explanation for the observed lag is strengthened by the fact that 3 hours is close to the minimum found in similar actinomycin D studies with other developmentally controlled enzymes in this organism (Loomis, 1969a; Firtel and Brackenbury, 1971; see also Loomis, 1969b).

The developmental cycle of Dictyostelium is marked by four main stages: vegetative growth, aggregation of free moving amoebae, formation and migration of the pseudoplasmodium in a polysaccharide slime sheath, and when conditions are proper, culmination into a fruiting body containing resistant spores on top of a cellulose-glycogen

sheath of vacuolated stalk cells (Bonner, 1967; Newell et al., 1969). During vegetative growth and early development, sugar moieties are accumulated as soluble glycogen and other polysaccharides and in either macromolecules or small diffusible molecules containing acid-labile sugar residues (White and Sussman, 1961; White and Sussman, 1963a; Sussman and Sussman, 1969; Ashworth and Watts, 1970). During the developmental cycle there are two main periods of carbohydrate synthesis requiring large amounts of sugar residues. The first is slime sheath synthesis during late aggregation and pseudoplasmodium stage. The second is during culmination when trehalose, a storage disaccharide, is accumulated, presumably to serve as a food source during germination (Ceccarini and Filosa, 1965; Cotter and Raper, 1970). At this time stalk-cell cellulose and glycogen and spore coat mucopolysaccharides and cellulose are also synthesized.

The total amount of anthrone positive material (D-glucose used as standard) per cell is approximately 5.5 picograms in bacterially fed amoebae. This decreases by only 24% to 4.2 pg/cell in mature fruits, indicating that most of the polysaccharide is not used as a food source but is reconverted from a storage form during early development, to the polysaccharide present in mature fruits (Wright, 1964; Sussman and Sussman, 1969). During this time period

there is a decrease in approximately 50% of the total cell protein and 60-70% of total cell RNA per cell coupled with a large output of ammonia (Gregg et al., 1954; Gregg and Bronsweig, 1956; Wright and Anderson, 1960; Sussman and Sussman, 1969; Mizukami and Iwabuchi, 1970). The level of glycolytic enzymes in culminating fruits suggests that little of the fruiting body polysaccharide sugar comes from amino acids via gluconeogenesis (Cleland and Coe, 1968). Additional support for this belief comes from studies using ^{14}C-aspartic acid which indicate that there is very little conversion of aspartate to glucose via the gluconeogenesis pathways (Cleland and Coe, 1969).

White and Sussman (1963a) found that approximately 15% of the storage carbohydrate was present as glycogen polysaccharide when amoebae were grown on Aerobacter aerogenes. Ashworth and Watts (1970) however, using the axenic strain AX-2, found that the amount of total carbohydrate and the fraction which was represented by glycogen polysaccharide depends on the growth media. In glucose-grown cells, the amount of carbohydrate is approximately 4-5 times that found in bacterial-grown cells and approximately 94% of this carbohydrate is present as glycogen. On the other hand, if the cells are grown in the absence of added sugar the level of carbohydrates is approximately 60% of the bacterial-grown cells and approximately 30% of the total cell carbohydrate is found as glycogen. In nature, it is not

known what the actual composition of the stored carbohydrate is, and it is quite conceivable that it would be a function of the chemical composition of the specific bacterium the Dictyostelium cells were feeding on. In any case it is reasonable to assume that glycogen storage could represent a major source of glucose moieties for use during culmination and that its mobilization may be necessary under certain conditions to supply polysaccharide building blocks for spore and stalk cell walls. Under other conditions, as when cells are grown on Aerobacter, glycogen represents a minor component of the storage carbohydrates. Under these conditions, glycogen phosphorylase may not be essential to culmination although there is, nevertheless, a 50-70% decrease in the amount of glycogen during culmination (Wright et al., 1968; Sussman and Sussman, 1969).

In considering the developmental significance of glycogen phosphorylase it is assumed that the direction of enzyme activity in vivo is toward the breakdown of the stored soluble glycogen. The partially purified glycogen phosphorylase has been shown to be competitively inhibited by UDPG. Results of Jones and Wright (1970) also indicate that all nucleotide sugars tested act as competitive inhibitors. It is not clear if nucleotide sugar inhibition is significant in vivo. It is conceivable that during culmination if there is a large intracellular pool of

UDPG, or other nucleotide sugars which may be precursors of cellulose of mucopolysaccharides, the glycogen phosphorylase activity may be inhibited, reducing the rate of glycogen breakdown and glucose-1-phosphate production. The UDPG pool size determined for total cells by Pannbacker (1967) is between 1-3 x 10^{-4} M. If UDPG is freely accessible to glycogen phosphorylase, then at the concentration of UDPG determined by Pannbacker (1967), glycogen phosphorylase activity would be severely inhibited *in vivo*. Unfortunately nothing is known about the compartmentalization of UDPG with respect to glycogen phosphorylase. Telser and Sussman (1971) present evidence suggesting that UDPG polysaccharide transferase and UDPGal-4-epimerase are compartmentalized in pre-spore vesicles and it is conceivable that UDPG, respectively a substrate precursor and substrate for these enzymes, may also be compartmentalized to some degree. It has been found that during culmination there is a 3-fold increase in the amount of free inorganic phosphate (Jones and Wright, 1970). If compartmentalization is not a factor, such an increase would increase the *in vivo* rate of glycogen phosphorylase.

During culmination there is a transient increase followed by a decrease in the free glucose and dialyzable, acid-labile glucose moieties. These changes are presumably the result of storage carbohydrate breakdown followed by utilization of the glucose moieties in polysaccharide

synthesis. Seventy-five percent of the anthrone positive material found in the mature fruit represent these newly synthesized polysaccharides, consisting mainly of trehalose, cell-wall cellulose and glycogen, and mucopolysaccharides (Sussman and Sussman, 1969). The above facts and considerations have been incorporated into a possible scheme of polysaccharide degradation and synthetic pathways shown in Figure 18. All enzymes known to be developmentally regulated in Dictyostelium are starred. It might also be mentioned that the breakdown of glycogen could be catalyzed either by the α-amylase or by glycogen phosphorylase. However, since glycogen phosphorylase is developmentally regulated and α-amylase is not (Jones and Wright, 1970; Wiener and Ashworth, 1970) and since glycogen phosphorylase reaches maximum specific activity within 1 hour of UDPG phosphorylase, UDPGal polysaccharide transferase, and UDPGal-4-epimerase (see Figure 19), it is reasonable to assume that the glycogen phosphorylase is actually used to break down the soluble glycogen in vivo.

The data presented in this paper indicate that the appearance and subsequent disappearance of glycogen phosphorylase is controlled by a developmental program which can be monitored both at the biochemical and morphological levels. It is reasonable to postulate, therefore, that the period of transcription, and possibly of translation is controlled independently for each enzyme. Actinomycin

Figure 18. Partial scheme of polysaccharide pathways in Dictyostelium discoideum.

* Developmentally regulated enzymes.

** Inhibitor present at early developmental stages. Measurable activity increases with loss of inhibitor.

*** Since galactosamine and galactouronic acid are also present in the spore cell wall mucopolysaccharide (White and Sussman, 1963b), other enzymes must be present. The following have been tested but no activity can be detected in crude extracts: UDPG dehydrogenase[1], UDPGal hydrogenase[1], galactokinase[2], UDPG-Gal-1-P uridylyltransferase[2], glutamine-fructose-6-PO_4 aminotransferase[1], glucosamine-6-P-acetyl-CoA N-acetyltransferase[2]. 1: tested in our laboratory and in Dr. M. Sussman's; 2: tested in laboratory of Dr. M Sussman (Telser and Sussman, 1971).

The numbers in parenthese next to the enzyme refer to the references below:

(1) Ashworth and Sussman (1967);

(2) Wright and Dahlberg (1967);
Pischel and Firtel (in preparation);

(3) This paper; Jones and Wright (1970);

(4) Telser and Sussman (1971);

(5) Sussman (1967);

(6) Roth and Sussman (1968);

(7) Ceccarini (1967);

Figure 18. (cont'd)

(8) Rosness (1968);

(9) Costen and Loomis (1969).

PARTIAL SCHEME OF POLYSACCHARIDE PATHWAYS

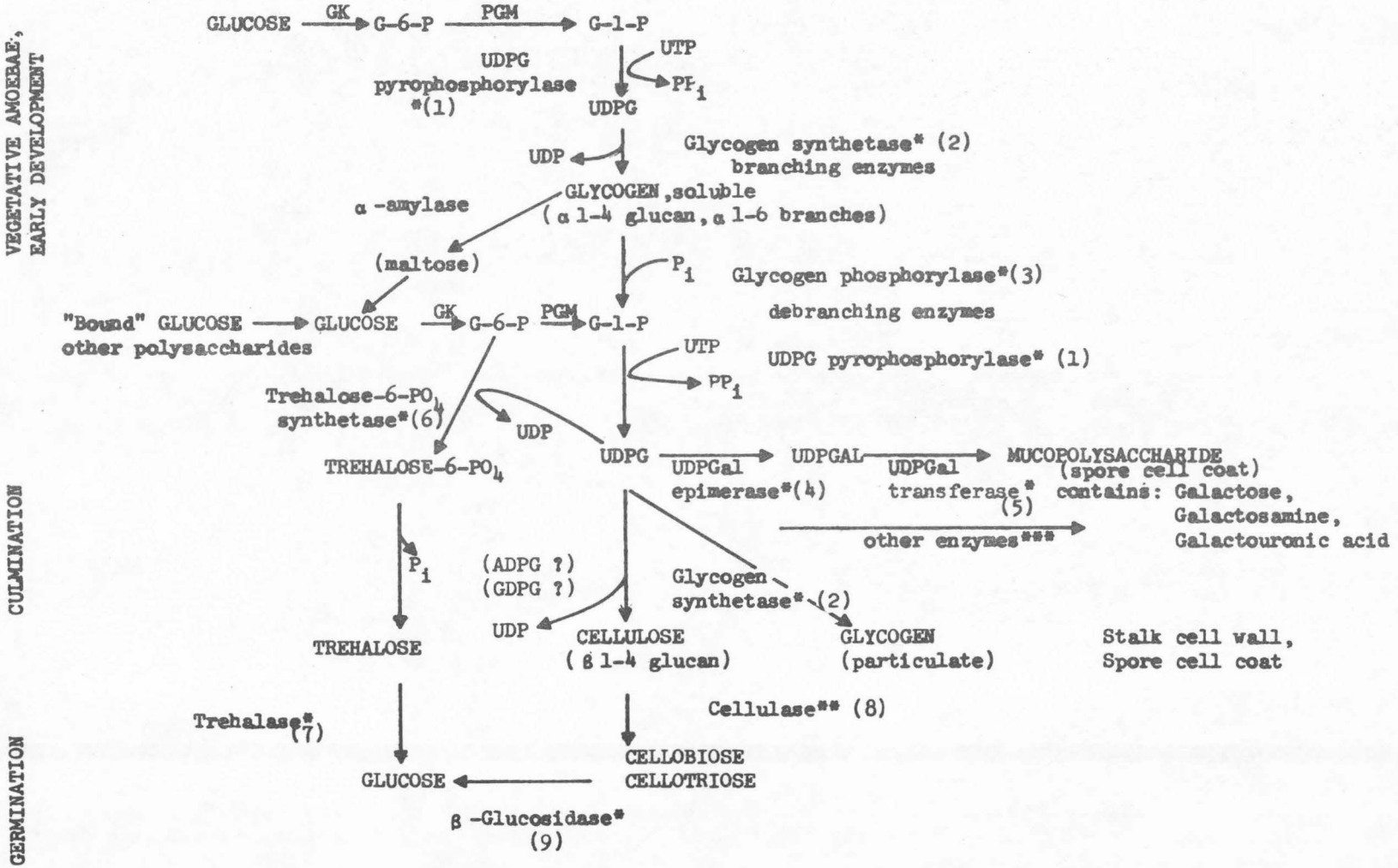


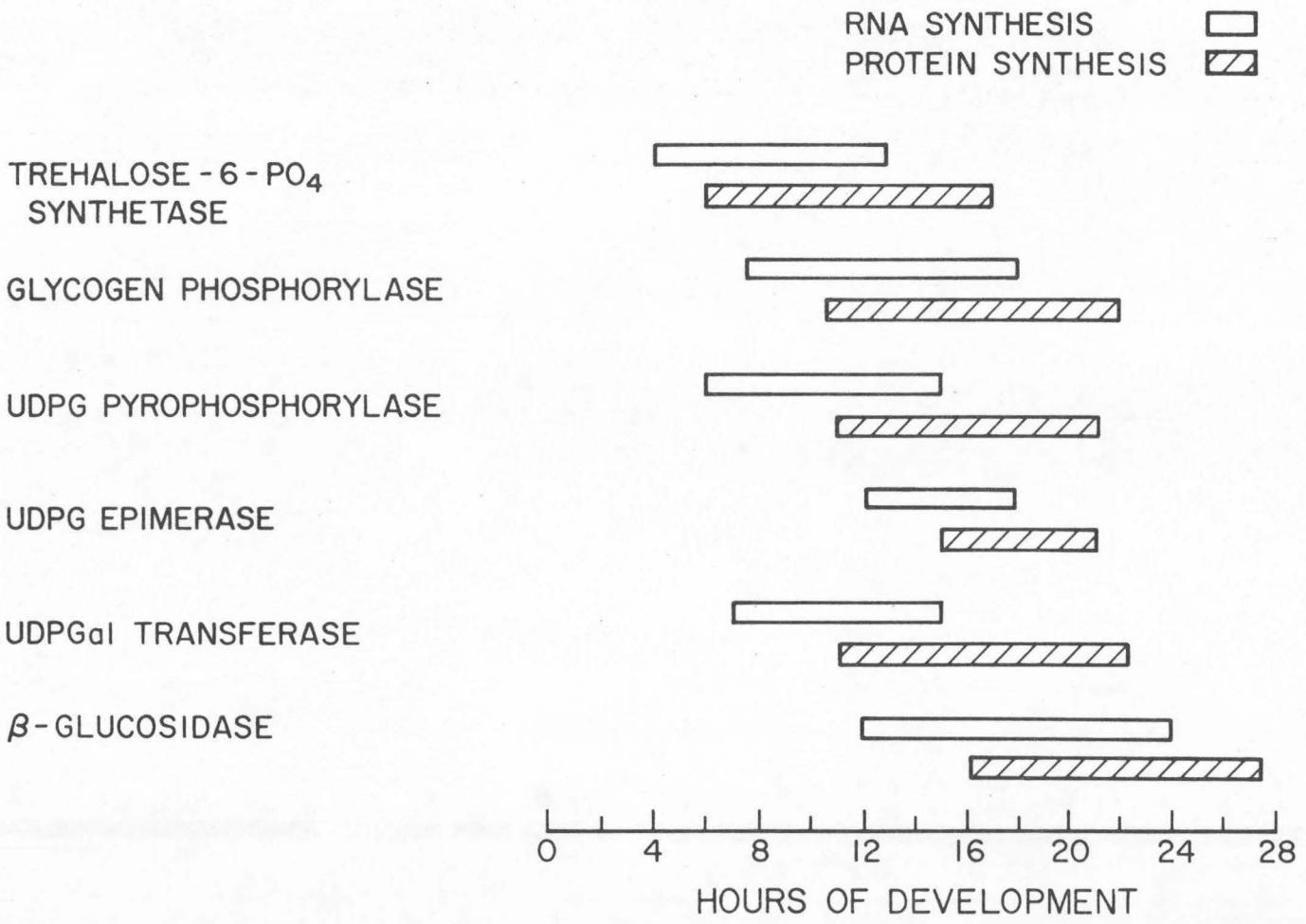
Figure 19. Periods of required RNA and protein synthesis from inhibitor studies.

Developmental period during which the RNA synthesis necessary for enzyme accumulation occurs as determined by actinomycin D studies.

Developmental period during which protein synthesis is necessary for enzyme accumulation as determined by cycloheximide studies. This period is concomitant with the rise in enzyme activity.

For references, see legend to Figure 18.

PERIODS OF REQUIRED RNA AND PROTEIN SYNTHESIS
FROM INHIBITOR STUDIES



D studies which indicate different periods of transcription for each of these polysaccharide enzymes (Fig. 19) strongly suggest that their messenger RNAs are not associated in a single operon and are regulated independently. Although we have no direct evidence as yet, the available data suggest that although they may be transcribed independently, the developmentally controlled synthesis of these enzymes is, in some unknown fashion, tightly coordinated.

FOOTNOTES

1. A preliminary report of some of this work was presented at the 54th Annual Meeting of the Federation of American Societies for Experimental Biology (Fed. Proc., abs. 2384, p. 669, 1970).

2. Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

ACKNOWLEDGEMENTS

We would like to thank Dr. Daniel McMahon and Miss Jessica Tuchman, California Institute of Technology, for their good advice and helpful suggestions during the time the work was being done and for their critical evaluation of the manuscript. We would also like to thank Dr. William F. Loomis, Jr., University of California, San Diego, for many helpful discussions.

During the period this work was done, one of us (RAF) was a NSF predoctoral fellow. The work was supported in part by USPHS grant GM13762 (to Dr. James Bonner).

REFERENCES

Ashworth, J.M., and Sussman, M. (1967). Appearance and disappearance of UDPG pyrophosphorylase activity during the differentiation of D. discoideum. J. Biol. Chem. 242, 1696.

Ashworth, J.M., and Watts, D.J. (1970). Metabolism of the cellular slime mold Dictyostelium discoideum grown in axenic culture. Biochim. J. 119, 175.

Carminatii, H., Passeron, S., Dankert, M., and Recondo, E. (1965). Separation of sugar nucleotides, phosphoric esters and free sugars by paper chromatography with solvents containing borates of organic bases. J. Chromatog. 18, 342.

Ceccarini, C., and Filosa, M. (1965). Carbohydrate content during development of the slime mold D. discoideum. J. Cell. Comp. Physiol. 66, 135.

Cleland, S.V., and Coe., E.L. (1968). Activities of glycolytic enzymes during early stages of differentiation of Dictyostelium discoideum. Biochim. Biophys. Acta 156,44.

Cleland, S.V., and Coe, E.L. (1969). Conversion of aspartic acid to glucose during culmination in Dictyostelium discoideum. Biochim. Biophys. Acta 192, 446.

Coston, M.B., and Loomis, W.F., Jr. (1969). Isozymes of β -glucosidase in Dictyostelium discoideum. J. Bacteriol. 100, 1208.

Cotter, D.A. and Raper, K.B. (1970). Spore germination in Dictyostelium discoideum: Trehalose and requirement for protein synthesis. Develop. Biol. 22, 112.

Dixon, M., and Webb, E.C. (1964). The Enzymes, 2nd ed., Academic Press, New York.

Firtel, R.A. (1971). Changes in the expression of single-copy DNA during development of the cellular slime mold Dictyostelium discoideum. Submitted for publication.

Firtel, R.A., and Brackenbury, R.W. (1971).Partial characterization of several enzymes of protein and amino acid metabolism in the cellular slime mold Dictyostelium discoideum. In preparation.

Gregg, J.H., Hackney, A.L., and Krivanek, J.O. (1954). Nitrogen metabolism of the slime mold Dictyostelium discoideum during growth and morphogenesis. Biol. Bull. 107, 226.

Gregg, J.H., and Brőnsweig, R.D. (1956). Biochemical events accompanying stalk formation in the slime mold Dictyostelium discoidum. J. Cell. Comp. Physiol. 48, 293.

Jones, T.H.D., and Wright, B.E. (1970). Partial purification and characterization of glycogen phosphorylase from Dictyostelium discoideum. J. Bacteriol. 104, 754.

Lineweaver, H., and Burk,D.(1943).The determination of enzyme dissociation constants.J.Amer.Chem.Soc 56, 658.

Loomis, W.F., Jr. (1968). The realtion between cytodifferentiation and inactivation of a developmentally controlled enzyme in Dictyostelium discoideum. Exp. Cell Res. 53, 283.

Loomis, W.F., Jr. (1969a). Acetylglucosaminidase, an early enzyme in the development of Dictyostelium discoideum. J. Bacteriol. 97, 1149.

Loomis, W.F., Jr. (1969b). Developmental regulation of alkaline phosphatase in Dictyostelium discoideum. J. Bacteriol. 100, 417.

Loomis, W.F., Jr. (1970a). Temporal control of differentiation in the slime mold Dictyostelium discoideum. Exp. Cell Res. 60, 285.

Loomis, W.F., Jr. (1970b). Developmental regulation of α -mannosidase in Dictyostelium discoideum. J. Bacteriol. 103, 375.

Loomis, W.F., Jr., and Sussman, M. (1966). Commitment to the synthesis of a specific enzyme during cellular slime mold development. J. Mol. Biol. 22, 401.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265.

McMahon, D., and Langstroth, P. Control of Macromolecular synthesis in Chlamydomonas reinhardi. I. The effects of starvation for arginine and of canavanine. Submitted for publication.

Marshall, R., Sargent, D., and Wright, B.E. (1970). Glycogen turnover in Dictyostelium discoideum. Biochemistry 9, 3087.

Mizukami, Y., and Iwabuchi, M. (1970). Effects of actinomycin D and cycloheximide on morphogenesis and synthesis of RNA and protein in the cellular slime mold, Dictyostelium discoideum. Exp. Cell Res. 63, 317.

Newell, P.C., and Sussman, M. (1969). Uridine diphosphate glucose pyrophosphorylase in Dictyostelium discoideum. J. Biol. Chem. 244, 2990.

Newell, P.C. and Sussman, M. (1970). Regulation of enzyme synthesis by slime mold cell assemblies embarked upon alternate developmental programs. J. Mol.Biol. 49,627.

Newell, P.C., Telser, A., and Sussman, M. (1969). Alternative developmental pathways determined by environmental conditions in the cellular slime mold Dictyostelium discoideum. J. Bacteriol. 100, 763.

Pannbacker, R.G. (1967). Uridine diphosphoglucose biosynthesis during differentiation in the cellular slime mold. I. In vivo measurements. Biochemistry 6, 1283.

Pischel, K., and Firtel, R.A. Characterization of glycogen synthetase during the developmental cycle of Dictyostelium discoideum. Submitted for publication.

Raper, K. (1935). Dictyostelium discoideum, a new species of slime mold from decaying forest leaves. J. Agar. Res. 50, 135.

Raper, K. (1940). Pseudoplasmodium formation and organization in Dictyostelium discoideum. J. Elisha Mitchell Sci. Soc. 56, 24.

Rosness, P.(1968). Cellulytic enzymes during morphogenesis in Dictyostelium discoideum. J.Bacteriol. 96, 639.

Roth, R.M., and Sussman, M. (1968). Trehalose-6-phosphate synthetase and its regulation during slime mold development. J. Biol. Chem. 243, 5087.

Roth, R., Ashworth,J.M., and Sussman,J.M.(1968). Periods of genetic transctiption required for the synthesis of three enzymes during cellular slime mold development. Proc. Nat. Acad. Sci., Wash. 59, 1235.

Sonneborn, D., White, G.,and Sussman,M.(1963). A mutation affecting both rate and pattern of morphogenesis in Dictyostelium discoideum. Develop. Biol. 7, 79.

Sussman, M. (1965). Inhibition of actidiene of protein synthesis and UDPGal: polysaccharide transferase accumulation in Dictyostelium discoideum. Biochem. Biophys. Res. Commun. 18, 763.

Sussman, M. (1966). Biochemical and genetic methods in the study of cellular slime mold development.In Methods in Cell Physiology,II,P.397. Academic Press, New York .

Sussman, M., Loomis, W.F., Jr., Ashworth, J.M., and Sussman, R.R. (1967). The effect of actinomycin D on cellular slime mold morphogenesis. Biochem. Biophys. Res. Commun. 26, 353.

Sussman, M. and Longren, N. (1965).Preferential release of the enzyme UDP Galactose polysaccharide transferase during cellular differentiation in the slime mold Dictyostelium discoideum. Exp. Cell Res. 38, 97.

Sussman, M., and Osborn, M.J. (1964). UDP Galactose: polysaccharide transferase in the cellular slime mold, D. discoideum. Proc. Nat. Acad. Sci. Wash. 52, 81.

Sussman, M., and Sussman, R.R. (1965). The regulatory program for UDPgalactose polysaccharide transferase activity during slime mold cytodifferentiation: requirement of a specific synthesis of ribonucleic acid. Biochim. Biophys. Acta. 108, 463.

Sussman, M., and Sussman, R. (1969). Patterns of RNA synthesis and of enzyme accumulation and disappearance during cellular slime mold cytodifferentiation Symp. Soc. Gen. Microbiol. XIX, 403.

Telser, A., and Sussman, M. (1971). Uridine diphosphate galactose-4-epimerase, a developmentally regulated enzyme in the cellular slime mold Dictyostelium discoideum. J. Biol. Chem. 246, 2252.

Watts, D.J., and Ashworth, J.M. (1970). Growth of myxamoebae of the cellular slime mold Dictyostelium discoideum in axenic culture. Biochem. J. 119, 171.

White, G.J., and Sussman, M. (1961). Metabolism of major cell components during slime mold morphogenesis. Biochim. Biophys. Acta 53, 285.

White, G.J., and Sussman, M. (1963a). Polysaccharides involved in slime mold development. I.Water-soluble glucose polymer(s). Biochim. Biophys. Acta 74, 173.

White, G.J., and Sussman, M. (1963b). Polysaccharides involved in slime mold development. II. Water-soluble acid mucopolysaccharide(s). Biochim. Biophys. Acta 74, 179.

Wiener, E. and Ashworth, J.M. (1970). The isolation and characterization of lysosomal particles from myxamoebae of the cellular slime mold Dictyostelium discoideum. Biochem. J. 118, 505.

Wright, B.E. (1964). Biochemistry of the Aerasiates. In Biochemistry and Physiology of Protozoa. III, p. 341, ed. S.H. Hunter, Academic Press, New York.

Wright, B.E., and Anderson, M.L. (1960). Protein and amino acid turnover during differentiation. I. Biochim. Biophys. Acta 43, 62.

Wright, B.E., and Dahlberg, D. (1967). Cell wall synthesis in Dictyostelium discoideum. II. Synthesis of soluble glycogen by a cytoplasmic enzyme. Biochemistry 6, 2074.

Wright, B.E., Dahlberg, D., and Ward, C. (1968). Cell wall synthesis in Dictyostelium discoideum. A model system for the synthesis of alkali-soluble cell wall glycogen during differentiation. Archives Biochem. Biophys. 124, 380.

Yanagasawa, K., Loomis, W.J., Jr., and Sussman, M. (1967). Developmental regulation of the enzyme UDPGal: polysaccharide transferase. Exptl. Cell Res. 46, 328.

CHAPTER IV

PARTIAL CHARACTERIZATION OF SEVERAL PROTEIN AND AMINO ACID METABOLIZING ENZYMES IN THE CELLULAR SLIME MOLD *DICTYOSTELIUM discoideum*

PARTIAL CHARACTERIZATION OF SEVERAL PROTEIN AND AMINO ACID METABOLIZING ENZYMES IN THE CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

Richard A. Firtel[1] and Robert W. Brackenbury[2]

Division of Biology, California Institute of Technology

Pasadena, California, 91109

[1] Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

[2] Present address: Department of Biology, Brandeis University, Waltham, Massachusetts.

Running Title: Amino acid metabolizing enzymes

Summary

The developmental kinetics of several amino acid and protein catabolizing enzymes have been studied. Aminopeptidase and alanine transaminase have been found to increase approximately 3 fold starting at the time of starvation and reaching maximum activity at 18 and 5 hours respectively. The increase of both enzymes is sensitive to actinomycin D and cycloheximide, suggesting that prior RNA and concomitant protein synthesis are necessary for the increase. Neither enzyme increases in a mutant which does not aggregate. Aminopeptidase but not alanine transaminase shows temporal regulation in the temporally deranged mutants FR-17 and GN-3. This paper also confirms that glutamate dehydrogenase and lactate dehydrogenase, and aspartate transaminase are not developmentally regulated. Aminopeptidase and alanine transaminase appear to be a single molecular species. GDH and LDH appear to have multiple isozymes.

INTRODUCTION

The cellular slime mold, Dictyostelium discoideum, grows vegetatively as single cell myxamoebae in presence of a food source. When the food supply is depleted or removed, the amoebae stop dividing and differentiate into a fruiting body containing two cell types: stalk cells and spore cells. During this process it appears that cell protein rather than carbohydrate is used as an energy source (Gregg et al., 1954; Gregg and Bransweig, 1956; Wright and Anderson, 1960; White and Sussman, 1961; Wright, 1964; Cleland and Coe, 1968; Sussman and Sussman, 1969; Mizukami and Iwabuchi, 1970). Carbohydrates which are stored during vegetative growth are used during culmination as precursors of stalk and spore cell-wall polysaccharides and for the synthesis of the storage disaccharide, trehalose (Clegg and Filosa, 1961; White and Sussman, 1963a,b; Wright, 1964; Sussman and Sussman, 1969). During the differentiation period, the amount of protein and RNA per cell decreases by one half while total carbohydrate per cell (anthrone positive material) decreases by only 24% (Gregg et al., 1954; Gregg and Bronsweig, 1956; Wright and Anderson, 1960; White and Sussman, 1961; White and Sussman, 1963a; Sussman and Sussman, 1969; Mizukami and Iwabuchi, 1970). Cleland and Coe (1968) suggest that the activities of the glycolytic enzymes involved in

glyconeogenesis are not great enough during differentiation to permit amino acids to be used to produce polysaccharide precursors. They show directly (Cleland and Coe, 1969) that little aspartic acid is converted into glucose during differentiation, again supporting the view that the amino acids produced by protein degradation are not used to synthesize polysaccharide precursors (see also Wright, 1964). It has also been shown, that even though the level of glutamic acid dehydrogenase does not increase during slime mold development, the rate of glutamic acid oxidation, apparently via the Krebs cycle, does increase during differentiation (Wright and Bard, 1963; Brühmüller and Wright, 1963, Wright 1964) also suggesting that during differentiation in this organism amino acids are actively being catabolized for use as an energy source.

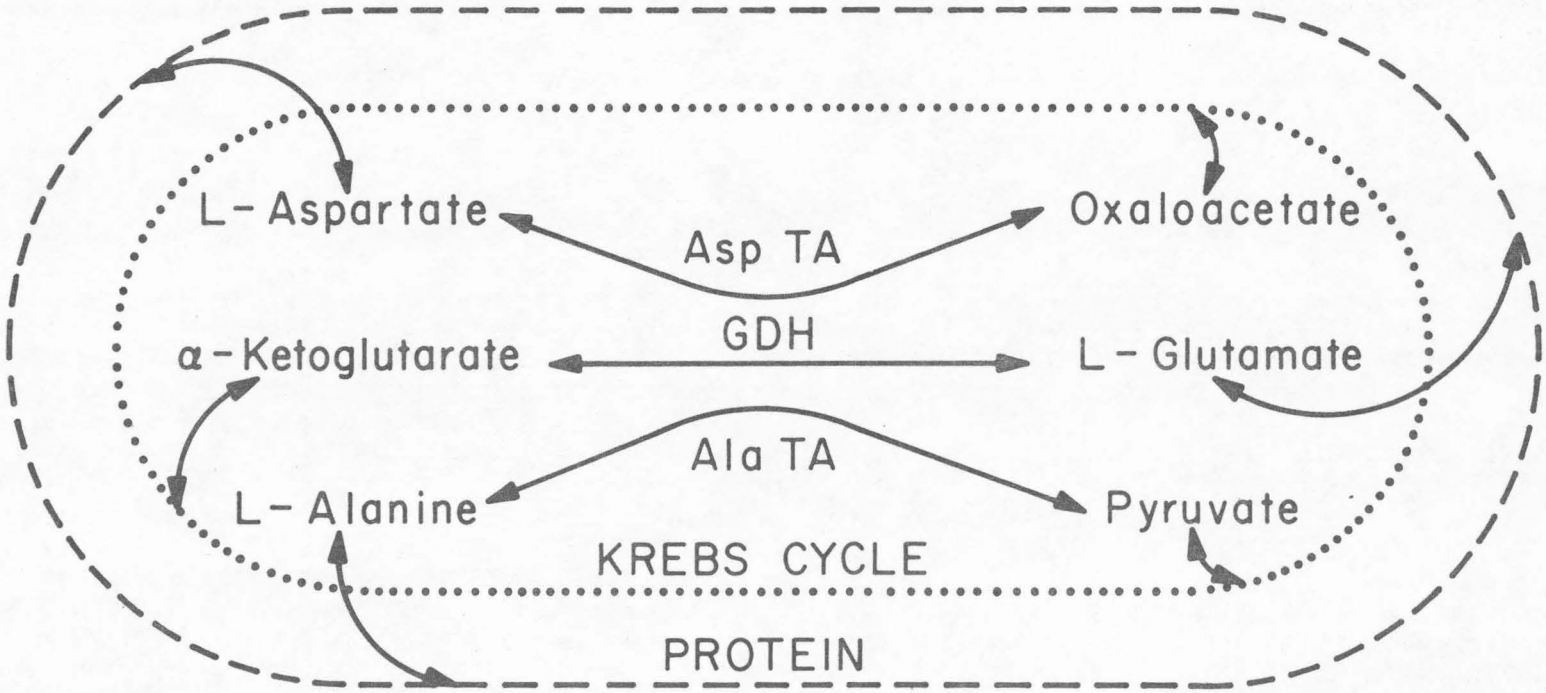
One method that has been used in attempts to understand more of the physiology and regulation of Dictyostelium differentiation has been the study of developmentally regulated enzymes, i.e., enzymes whose specific activities increase during development (Sussman, 1966a; Sussman and Sussman, 1969; Loomis, 1970a; Telser and Sussman, 1971; Firtel and Bonner, 1971a). Approximately 15 enzymes have been shown to be developmentally regulated. These include enzymes involved in polysaccharide degradation and synthesis and enzymes involved in protein and amino acid catabolism. Certain of these enzymes are undetectable

during logarithmic growth and appear in later development while others can be detected in the myxamoebae and then increase anywhere from two to more than fifty fold (Loomis, 1970a). In most of the cases studied, the increase in enzyme specific activity appears to require prior RNA and concomitant protein synthesis.

Since previous work suggests that amino acids represent an important source of metabolic energy during differentiation, it is of interest to examine enzymes involved in protein and amino acid metabolism including aminopeptidase and amino acid transaminases and to determine if these are developmentally regulated. The present paper considers aminopeptidase, EC 3.4.1.2; L-aspartate: α -ketoglutarate amino-transferase (aspartate transaminase), EC 2.6.1.1; L-alanine :α -ketoglutarate aminotransferase (alanine transaminase), EC 2.6.1.2; and glutamate: NAD oxidoreductase (deaminating) (glutamic acid dehydrogenase, GDH), EC 1.4.1.2; and L-lactate: oxidoreductase (lactate dehydrogenase, LDH) EC 1.1.27. These enzymes, as shown below are involved in the matabolism of the amino acids aspartate, alanine and glutamate (Figure 1).

This paper shows that the enzymes aminopeptidase and alanine transaminase are developmentally regulated and that their increase in activity appears to require RNA and protein synthesis.

Figure 1. Interrelationships between certain amino acids and Krebs Cycle intermediates.


L-Aspartate
Asp TA
Oxaloacetate
α-Ketoglutarate
GDH
L-Glutamate
Ala TA
L-Alanine
Pyruvate
KREBS CYCLE
PROTEIN

MATERIALS AND METHODS

Organisms and culturing conditions. A haploid strain of Dictyostelium discoideum NC-4 isolated by Raper (1935; 1940) was used. All mutants used were derived from this strain and were kindly supplied by Dr. William F. Loomis, Jr., Univ. of California, San Diego.

The organism was grown in association with Aerobacter aerogenes on SM agar plates (Sussman, 1966b; Firtel and Bonner, 1971a). To initiate synchronous development cells were washed free of bacteria and deposited on Whatman #50 filters supported by Millipore pads saturated in PDF buffer (0.04 M potassium phosphate, 1.5 g/l KCl, 0.61 g/l Mg SO_4, 0.5 g/l streptomycin sulfate)(Sussman, 1966b; Newell and Sussman, 1969). For further discussion of the developmental conditions see Firtel and Bonner (1971a).

For purification of some of the enzymes axenically grown strain AX-3 was used. See Firtel and Bonner (1971b) for growth conditions.

Assay conditions. (A) aminopeptidase. The cells from one filter containing approximately 1 x 10^8 cells were collected in water and used immediately or stored at -70°C until used. The crude extracts were prepared by slowly thawing the cells and sonicating for 2X 20 sec (with a 40 sec rest period) at 2 milliamps in a Branson sonifier at 0°C. The assays were performed within 30 min. The

reaction mixture contained, per 0.6 ml; 0.5 ml of 0.05 M potassium phosphate buffer, pH 7.2, containing 0.016 ml of a 0.24 M L-leucine-p-nitranilide (Sigma) solution in methanol, and 100 µl of crude cell extract containing 50-125 ug protein. The reaction mixture was incubated at 30°C for 30 min and stopped by addition of 0.5 ml of 1 M Na_2CO_3. The p-nitrophenol produced upon cleavage of the L-leucine-p-nitranilide was determined by reading the absorption at 420 nm in a spectrophotometer. An extinction coefficient of 18,300 was used for p-nitrophenol produced. The activity of the aminopeptidase was found to be the same in the presence of 0.01 M $MgCl_2$ or $MnCl_2$ or 0.01 M EDTA, pH 7.2. In this paper one enzyme unit is defined as the amount of enzyme required to produce one mµmole product/min.

The linearity of the assay with respect to protein concentration and time was determined in an automatically recording Beckman Kintrac VII spectrophotometer. The change in absorbance at 405 nm with time was shown to be constant for over 1 hr at 30°C. The rate of reaction was also found to be a linear function of the amount of crude extract added per assay up to a concentration of approximately 750 µg/ml.

The specific activities of all enzymes is expressed as units of enzyme activity/mg protein of crude extract. The protein was estimated using the method of Lowry et al., (1951). Bovine serum albumin was used as a standard.

Assay conditions. (B) Transaminases and dehydrogenases. The cells from one filter containing approximately 1 x 10^8 cells were collected in 15% glycerol, 0.2 mM dithiothreitol (DTT) and used immediately or stored at -70°C until use. The crude extracts were prepared as described above.

Alanine transaminase (Ala TA): The reaction mixture contained per 1 ml: 0.71 ml 0.1 M Tricine buffer, pH 7.6, 0.2 ml 1.0 M L-alanine, pH 7.5, 0.075 ml 0.021 M α -ketoglutarate, pH 7, 14 µg lactic acid dehydrogenase (LDH), (Sigma), 0.33 mg NADH (Calbiochem). 15 µl of extract containing 10-25 µg protein was used.

$$\alpha\text{-ketoglutarate } (\alpha KG) + \text{L-alanine} \xrightarrow{\text{Ala TA}} \text{L-glutamate } (\text{L-Glu}) + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

Aspartate transaminase (Asp-TA): The reastion mixture contained per 1 ml: 0.09 ml solution 0.1 M potassium phosphate, pH 7.4 containing 0.04 M aspartate; 0.33 mg NADH; 0.01 ml MDH (Boehringer-Mannheim) solution, 2 mg/ml; 0.07 ml 0.21 M -ketoglutarate, pH 7; and 15 µl of extract.

$$\alpha\text{-KG} + \text{L-aspartate} \xrightarrow{\text{Asp TA}} \text{L-Glu} + \text{oxaloacetate}$$

$$\text{oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

In assaying the dehydrogenases and transaminases the oxidation of NADH was monitored by measuring the decrease in absorbance at 340 nm on a Beckman Kintrac VII recording spectrophotometer at 28°C. An extinction coefficient of 6220 was used for NADH at 340 nm.

RESULTS

(A) Aminopeptidase

Developmental kinetics of aminopeptidase in wild type and mutant strains. Fig. 2 shows the developmental kinetics of aminopeptidase in wild type NC4 cells. The activity begins to increase within 30 min after initiation of starvation and rises 3.5-fold during the first 16-18 hrs of development. The specific activity of the enzyme remains constant thereafter.

Many developmental mutants of Dictyostelium discoideum have been isolated independently from wild type NC-4 cells. These mutants can be classified into two main groups; morphologically aberrant mutants (aggregation-minus, mutants which do not culminate, stalkless mutants, etc.), and temporally deranged mutants (mutants which take a longer or shorter time than wild type to complete morphogenesis). The developmental kinetics of aminopeptidase was studied in mutants of both classes (Figure 3). Although aminopeptidase starts to increase before any morphological signs of differentiation are apparent, there was no

Figure 2. Developmental kinetics of aminopeptidase in NC-4. The developmental stages are given on the abscissa.

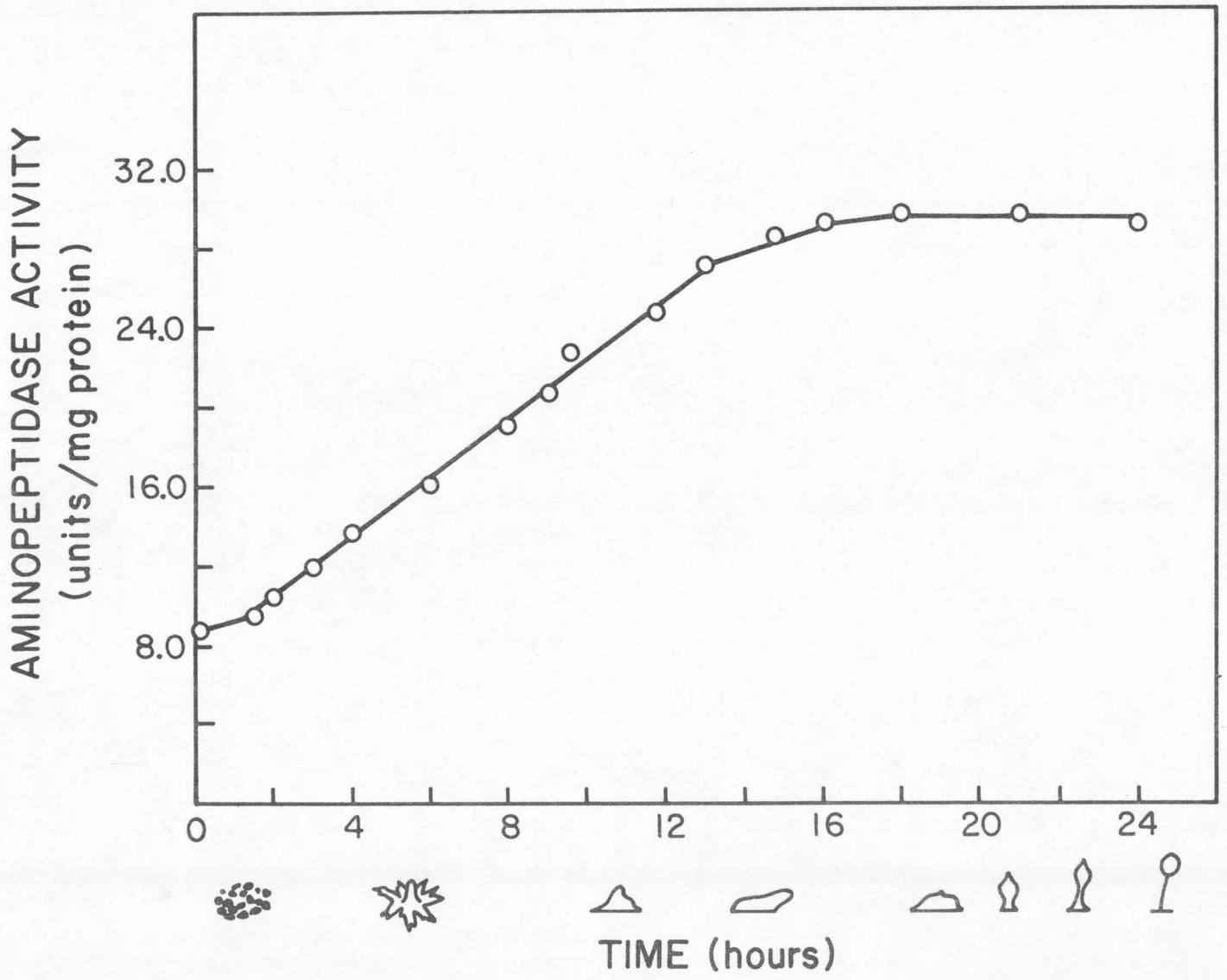
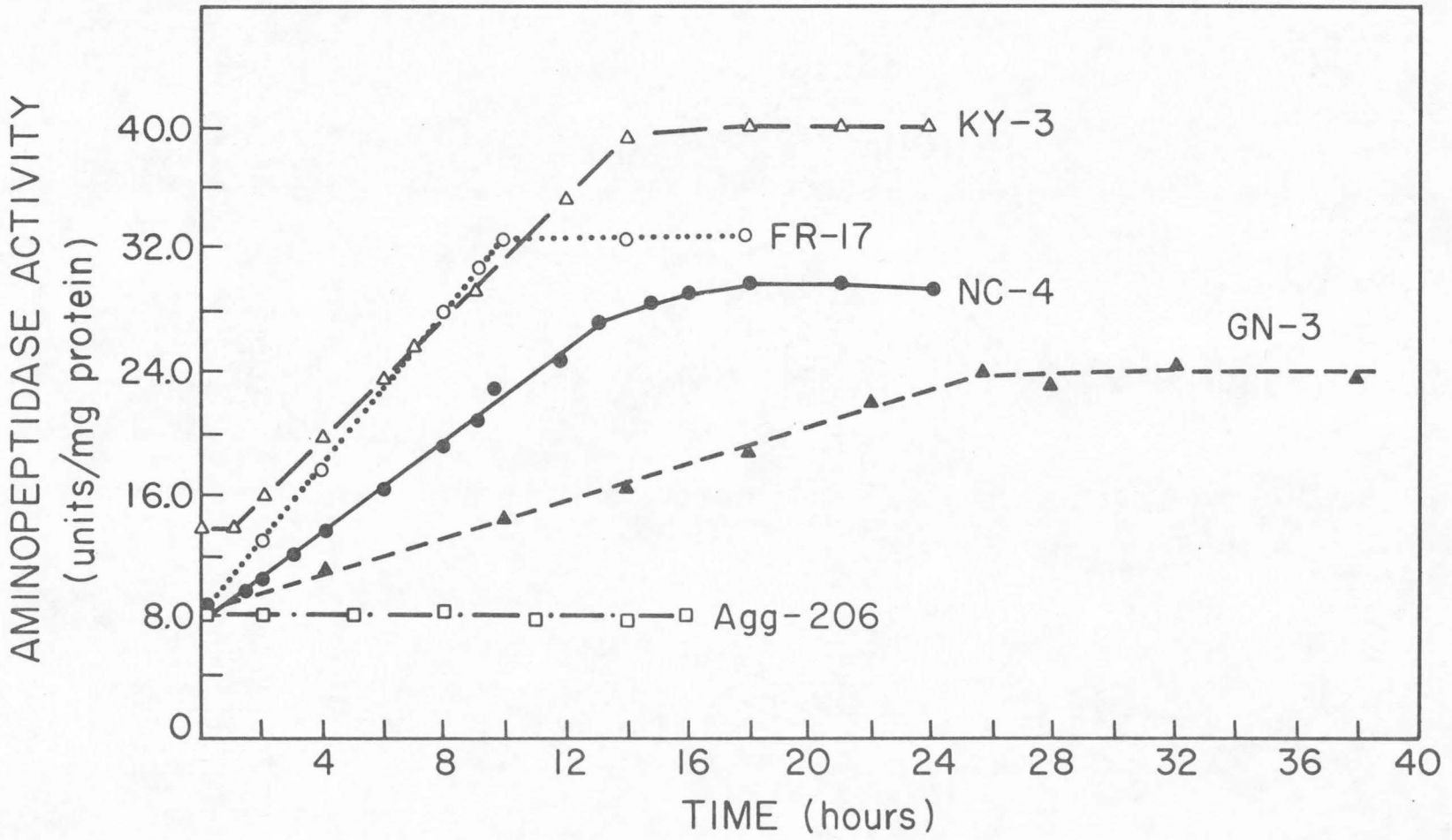

AMINOPEPTIDASE ACTIVITY
(units/mg protein)
32.0
24.0
16.0
8.0
0
4
8
12
16
20
24
TIME (hours)

Figure 3. Developmental kinetics of aminopeptidase in morphologically aberrant and temporally deranged mutants.


AMINOPEPTIDASE ACTIVITY
(units/mg protein)
40.0
32.0
24.0
16.0
8.0
0
4
8
12
16
20
24
28
32
36
40
TIME (hours)
KY-3
FR-17
NC-4
GN-3
Agg-206

increase or decrease in its activity in the non-aggregating mutant Agg-206. This was in contrast to the accumulation in Agg-206 of α -mannosidase which also starts to increase immediately after starvation in NC-4 (Loomis, 1970a). In mutant Agg-206 α -mannosidase does accumulate but reaches a level only approximately ½ that of NC-4. Mutant KY-3, which forms a pseudoplasmodium under normal culturing conditions but does not form a fruiting body (Yanagasawa et al., 1967), shows the same developmental kinetics as NC-4 except that the initial specific activity is approximately 1.6X higher. Mutant FR-17 and GN-3 are temporally deranged. While wild type cells complete morphogenesis in 24 hrs, FR-17 develops mature spore cells in 16 hours (Sonneborn et al., 1963), and GN-3 requires 60-70 hours to form a fruiting body (Loomis, 1970b). In FR-17, as expected, the maximum specific activity of aminopeptidase is reached by 10 hr. In GN-3 maximum activity is reached by approximately 24 to 26 hr. These results suggest that at least the early steps in morphogenesis are necessary for aminopeptidase accumulation and that the appearance of the enzyme is temporally linked with development.

Effect of actinomycin D, cycloheximide, and EDTA on accumulation of aminopeptidase. To determine whether RNA and protein synthesis are required for the increase in aminopeptidase specific activity, cells were treated with actinomycin D or cycloheximide at various times during

development. As discussed elsewhere (Firtel and Bonner, 1971a), conclusions from inhibitor studies must be interpreted cautiously because the drugs exert secondary effects on the cells. Actinomycin D has been shown to inhibit RNA synthesis by over 90% within 30 to 45 min in developing cells at all stages up to culmination (Sussman et al., 1967; Sussman and Sussman, 1965; Firtel unpublished observation). Cycloheximide inhibits protein synthesis (incorporation of amino acid into hot TCA precipitable material) by over 90% within 15 to 30 min (Firtel, unpublished observation). The effects of actinomycin D and cycloheximide on the increase in specific activity of aminopeptidase are shown in Fig. 4 and 5, respectively. In the presence of actinomycin D aminopeptidase continues to accumulate at a slightly slower rate for approximately 2 to 3 hours after which its activity remains constant. Cycloheximide inhibits the increase in enzyme activity within 30 min. The results are taken to suggest that prior RNA and concomitant protein synthesis are necessary for the increase in specific activity.

If developing Dictyostelium discoideum is placed on filters in PDF lacking divalent cations and in the presence of 1.5×10^{-2} M EDTA, pH 6.5, the cells do not aggregate and do not undergo development, although their viability and rate of incorporation of ^{14}C-amino acids into protein is not affected (Loomis and Sussman, 1966).

Figure 4. Effect of actinomycin D on the increase in specific activity of aminopeptidase in NC-4. Whatman #50 filters with developing Dictyostelium were transferred to pads saturated with PDF containing 125 µg/ml of actinomycin D. Cells were then harvested at various times after addition of the drug and aminopeptidase activity measured. All culturing in the presence of actinomycin D was done in darkness.

-o- Control NC-4 (no drug).

-Δ- Actinomycin D given at various times.

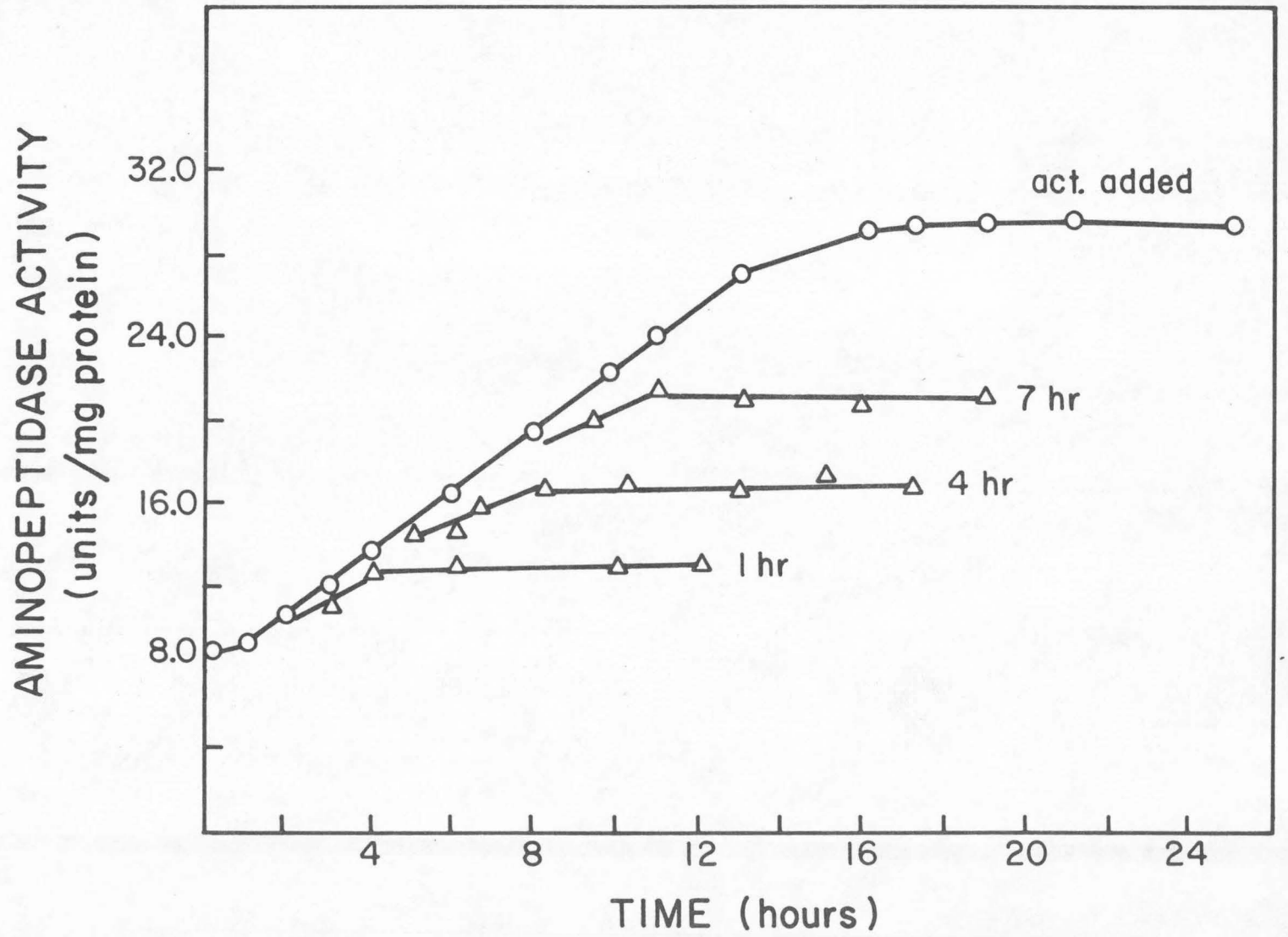

AMINOPEPTIDASE ACTIVITY
(units/mg protein)
32.0
24.0
16.0
8.0
act. added
7 hr
4 hr
1 hr
4
8
12
16
20
24
TIME (hours)

Figure 5. Effect of cycloheximide on the increase in specific activity of aminopeptidase in NC-4. Whatman #50 filters with developing cells were transfered to Millipore pads saturated with PDF containing 500 µg/ml cycloheximide. Cells were then harvested at various times after addition of the drug and aminopeptidase activity measured.

-O- Control NC-4 (no drug).

-●- Cycloheximide given at various times.

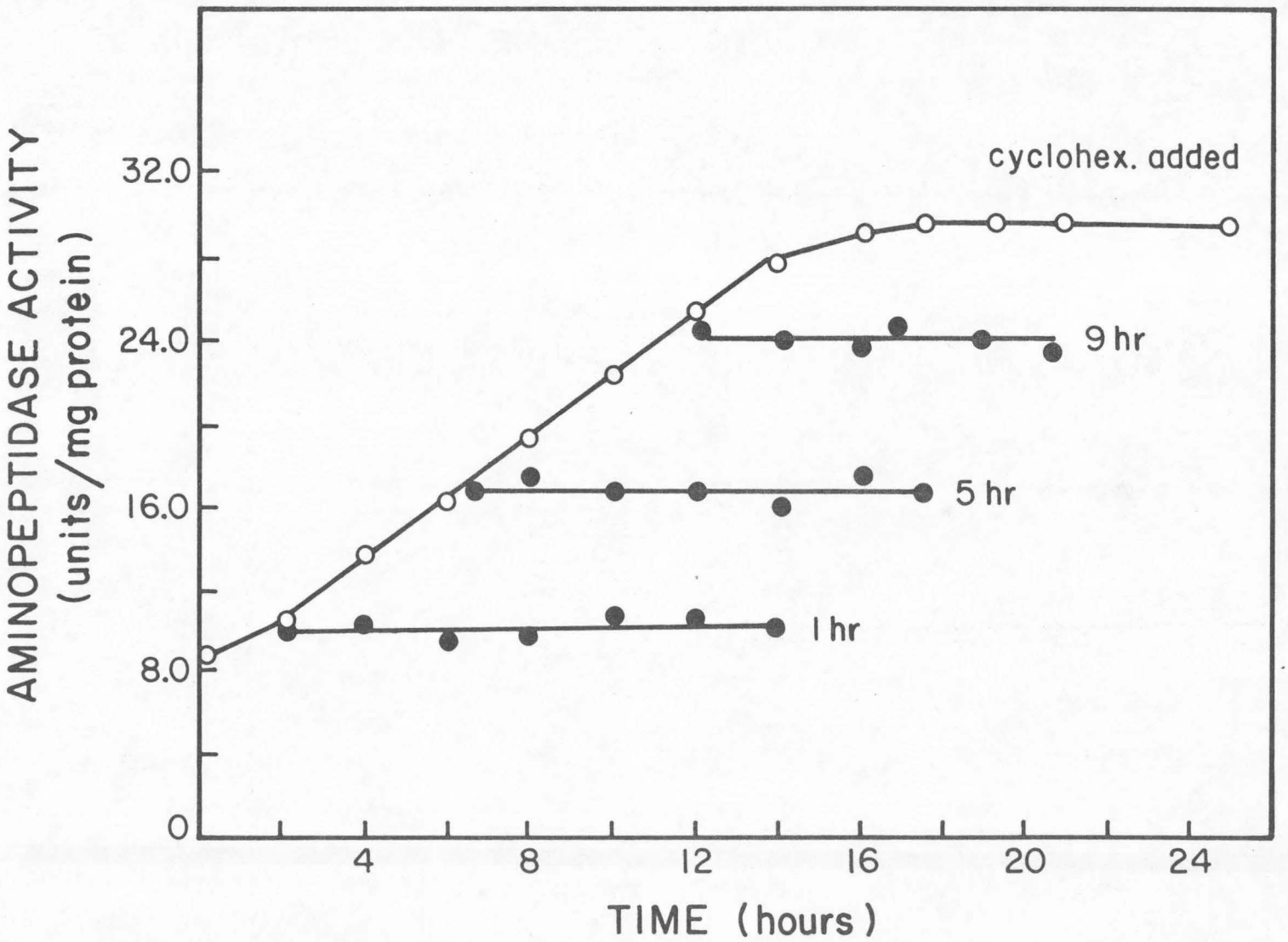

AMINOPEPTIDASE ACTIVITY
(units/mg protein)
32.0
24.0
16.0
8.0
0
4
8
12
16
20
24
TIME (hours)
cyclohex. added
9 hr
5 hr
1 hr

Figure 6. Lineweaver and Burk plot (1934) for determination of the Km of L-leucine-p-nitroanihide for partially purified aminopeptidase

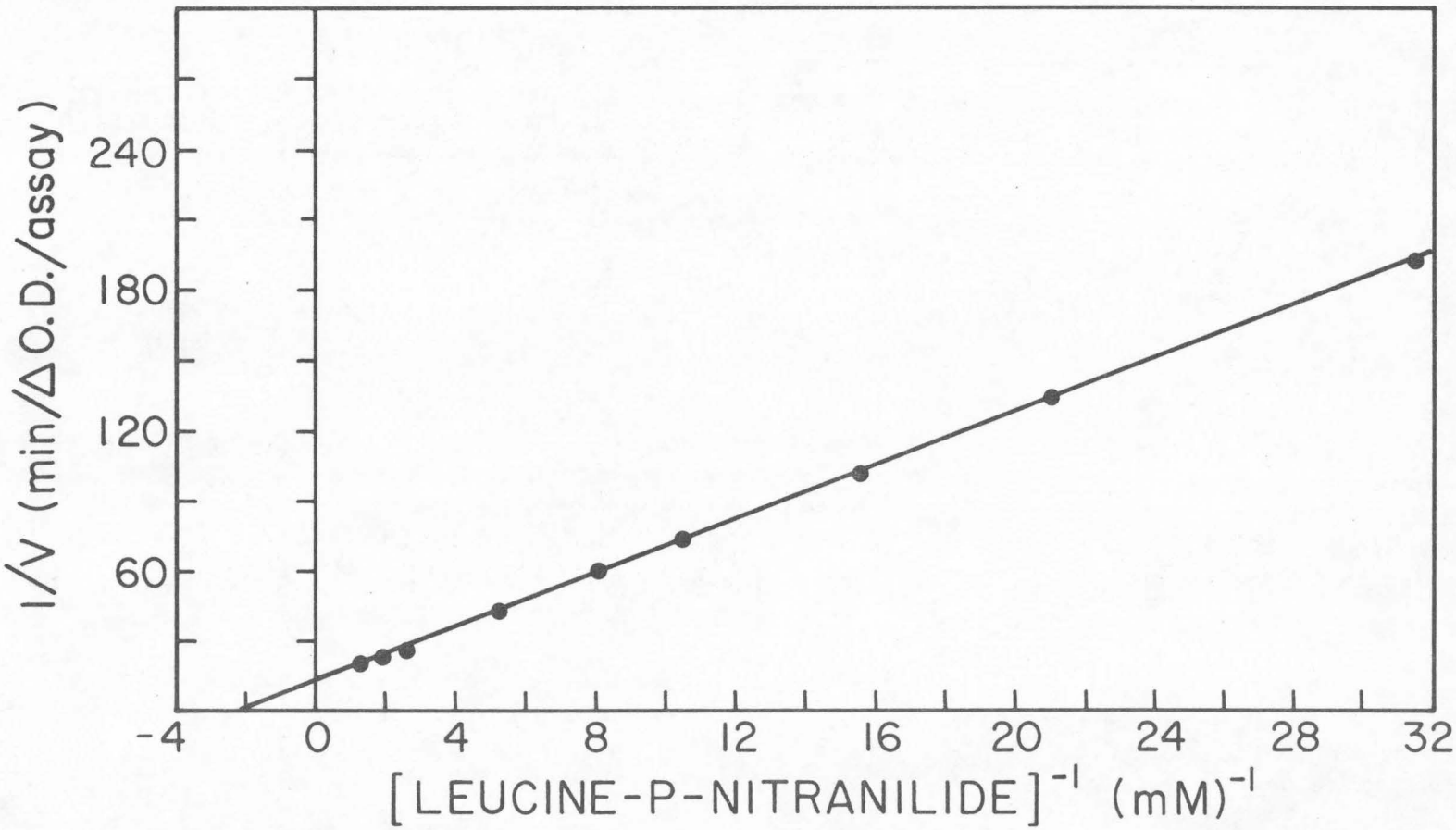
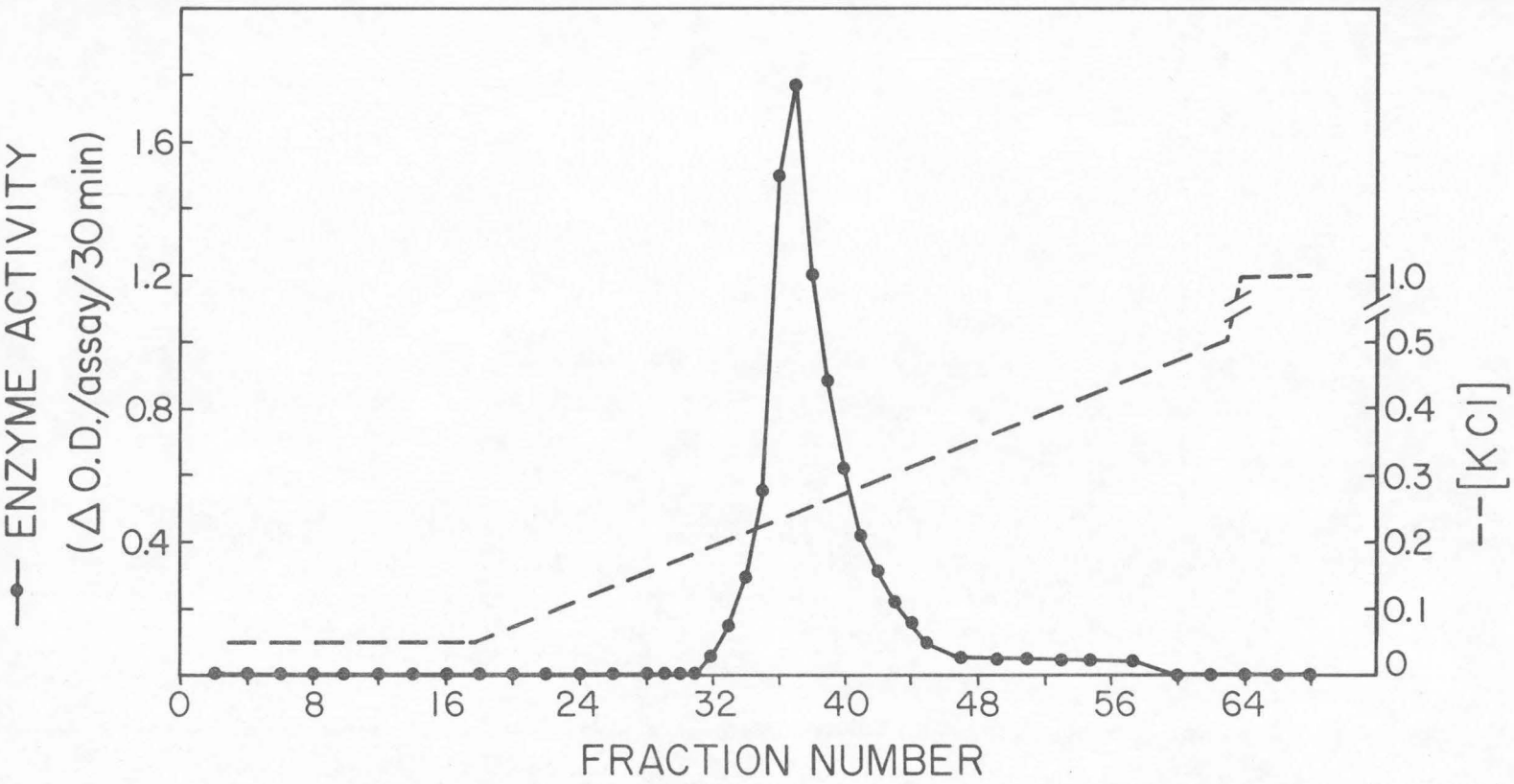

1/V (min/ΔO.D./assay)
240
180
120
60
-4
0
4
8
12
16
20
24
28
32
[LEUCINE-P-NITRANILIDE]$^{-1}$ (mM)$^{-1}$

Figure 7. DEAE cellulose chromatography of partially purified aminopeptidase. Sample was applied in 0.01 M KPO_4, pH 7.2, 0.05 M KCl. A linear KCl gradient was run.


ENZYME ACTIVITY
(Δ O.D./assay/30 min)
1.6
1.2
0.8
0.4
0
8
16
24
32
40
48
56
64
FRACTION NUMBER
1.0
0.5
0.4
0.3
0.2
0.1
0
[KCl]

Under these conditions the specific activity of aminopeptidase decreases from a level of 8 units/mg protein at 0 hr. to approximately 6 units/mg protein at 20 hr (graph not shown). It appears that treatment with EDTA inhibits the increase in specific activity, and this together with the results with mutant AGG-206, suggests that cellular morphogenesis is necessary for the accumulation of aminopeptidase activity.

Partial purification and physical properties of aminopeptidase on Biogel P-150. Sonicated extracts of logarithmically growing cells (Firtel and Bonner, 1971b) were made 1% with respect to streptomycin sulfate. This was allowed to stand for 30 min at 0°C and the precepitate removed by centrifugation at 10,000 rpm in a Serval SS-34 rotor. The supernatant was then centrifuged at 45,000 rpm (160,000 g) for 2 hr in a Spinco SW-50 rotor. All enzyme activity was found in the supernatant fraction. The enzyme was then chromatographed on BioGel P-150 in 0.1 M potassium phosphate buffer, pH 7.2. The enzyme eluted as a single peak near the excluded volume. The BioGel P-150 enzyme was used to determine the Michaelis constant (Km) for L-leucine-p-nitranilide. The Km was determined to be approximately 0.5 mM (see Fig. 6).

The enzyme was also analyzed by DEAE cellulose ion exchange chromatography and sucrose gradient centrifugation. Figure 7 shows the elution profile on DEAE cellulose of

aminopeptidase from the 160,000 g supernatant. The enzyme eluted as a single peak at 0.25 M KCl. Figure 8 shows the sucrose gradient of aminopeptidase 160,000 g supernatant of crude extract harvested at 0 hr and 18 hr of development. As can be seen, the enzymes of both stages showed the same sedimentation profile. The enzyme has a s value of approximately 5.6 S (bovine serum albumin, 4.3 S as marker). The 160,000 g supernatant of 0 hr and 18 hr developing cells were also subjected to polyacrylamide disc electrophoresis. The supernatant was dialyzed against 0.01 M phosphate buffer, 10% glycerol and layered over 7.5% polyacrylamide gels and electrophoresed according to the procedure of Loomis (1970a). The gel was sliced and assayed for aminopeptidase. A single peak of activity was found in both extracts and in both cases migrated 40% of the distance of the gel toward the anode, relative to the bromphenol blue marker. These results indicate that aminopeptidase is most likely a single molecular species which increases in specific activity during development.

(B) Transaminases and Dehydrogenases

Developmental kinetics of alanine transaminase and aspartate transaminase.

Figure 9 shows the developmental time course of alanine transaminase (Ala TA) and aspartate transaminase (Asp TA) in wild type NC-4 cells. As can be seen, the specific activity of aspartate transaminase remains

Figure 8. Sucrose gradient centrifugation of 160,000 g supernatant from 0 and 18 hour developing cells assayed for aminopeptidase activity. 5-20% sucrose gradient, 0.1 M KCl, 0.01 M KPO_4, pH 7.2, centrifuged 7 hr, 63,000 rpm, Spinco SW 65 rotor, 4°C.

-●- Extract from cells allowed to develop 18 hours.

-O- Extract from 0 hour, washed, bacterial grown NC-4.

-Δ- $O.D._{230}$ profile of second tube with BSA marker, 4.3 S.

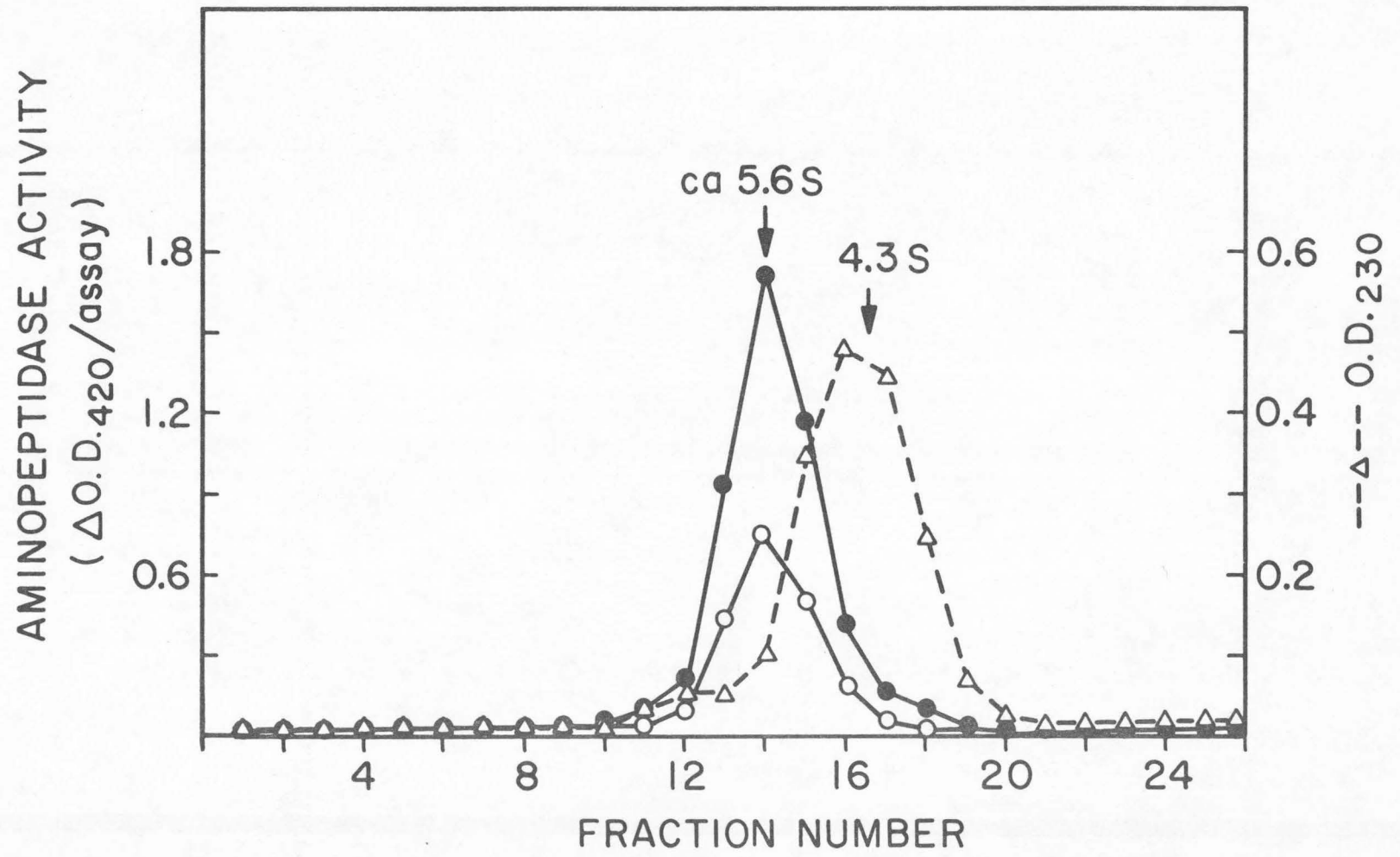
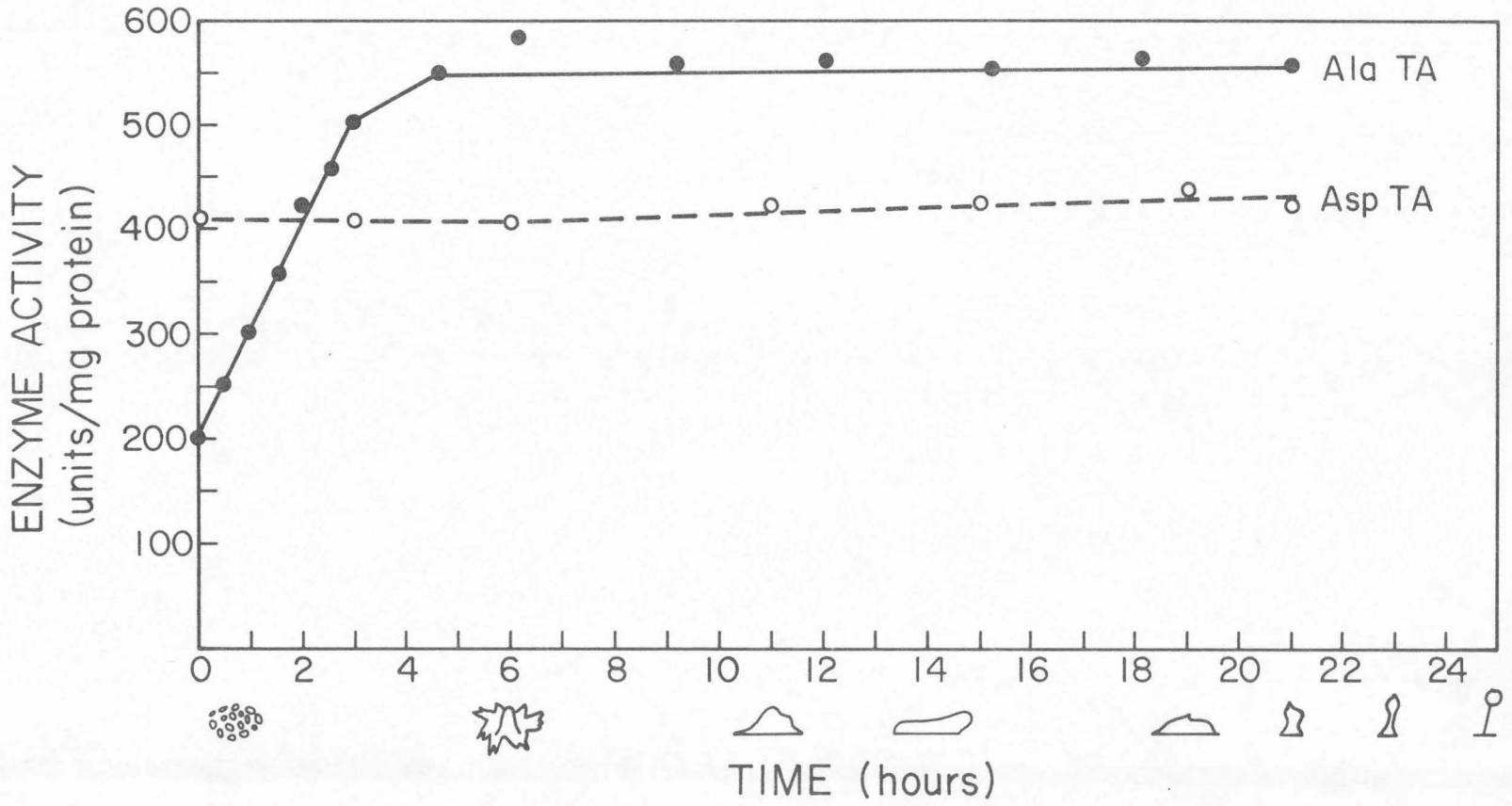

AMINOPEPTIDASE ACTIVITY
(ΔO.D.420/assay)
1.8
1.2
0.6
ca 5.6 S
4.3 S
0.6
0.4
0.2
--△-- O.D.230
4
8
12
16
20
24
FRACTION NUMBER

Figure 9. Developmental kinetics of alanine transaminase (Ala TA) and aspartate transaminase (Asp TA) in NC-4. The developmental stages are given on the abscissa.


ENZYME ACTIVITY
(units/mg protein)
600
500
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (hours)
Ala TA
Asp TA

constant through development while alanine transaminase increases by a factor of 2.5-3.3 during the first 4-5 hours of development. This increase is reproducible.

Since the assay for Ala-TA also contains the reactants for glutamic acid dehydrogenase (GDH), it is necessary to measure the GDH activity present under the conditions of the Ala-TA assay. Table I shows the amount of NADH oxidation as a result of GDH activity using the complete Ala-TA reaction mixture and the mixture free of alanine or alanine and LDH. As can be seen, the GDH activity measured in this assay system increases only very slightly over the time period of alanine transaminase increases. GDH activity represents approximately 20% of the alanine transaminase activity in 0 hr cells and approximately 12% of the activity in 6 hr cells.

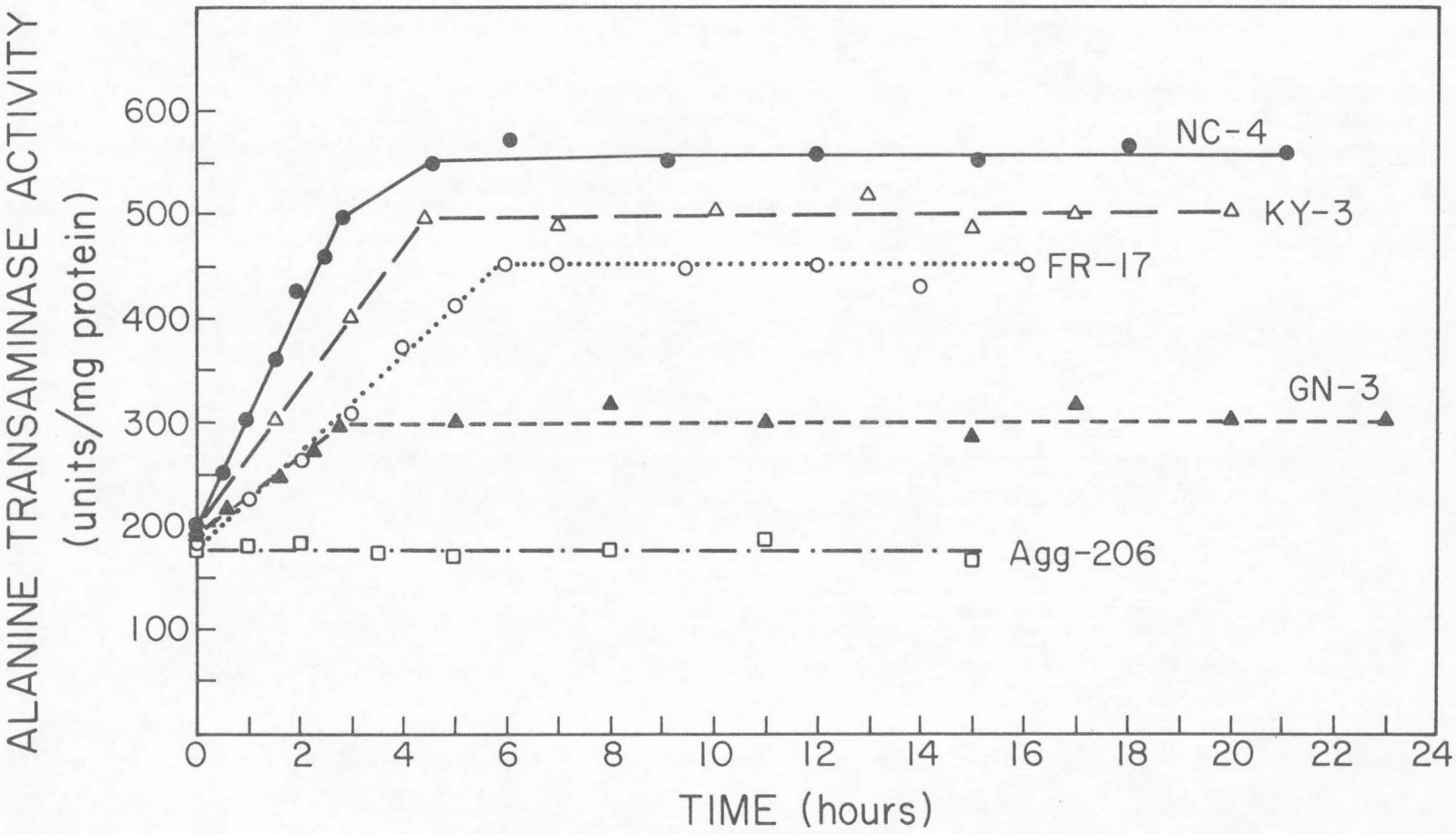
The developmental kinetics of alanine transaminase was measured in morphologically aberrant and temporally deranged mutants. As is shown in Fig. 10, the developmental kinetics of alanine transaminase in KY-3, FR-17 and GN-3 are essentially the same as in NC-4 except that in FR-17 cells the enzyme reproducibly reaches its maximum activity at 5-6 hours instead of 4.5 hours and in GN-3 it reaches maximum activity at 2-3 hours and increases only 1.2-1.3-fold. The enzymatic activity of Agg-206 does not show any change with time. The results suggest that alanine transaminase is regulated and that mutants which

TABLE I

Assay	Activity (mμmoles NADH oxidized/min/mg protein)	Enzyme being Measured
0 hr cells		
Complete	180	Ala TA and GDH
- alanine	31	GDH
- alanine, LDH*	30	GDH
Difference	150	Ala TA
6.3 hr cells		
Complete	422	Ala TA and GDH
- alanine	46	GDH
- alanine, LDH*	44	GDH
Difference	378	Ala TA
15 hr cells		
Complete	410	Ala TA and GDH
- alanine	40	GDH
- alanine	39	GDH
Difference	371	Ala TA

*Since LDH is in a 2.4 M $(NH_4)_2SO_4$ solution, an equivalent amount of $(NH_4)_2SO_4$ was added.

Figure 10. Developmental kinetics of alanine transaminase in morphologically aberrant and temporally deranged mutants.


ALANINE TRANSAMINASE ACTIVITY
(units/mg protein)
600
500
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (hours)
NC-4
KY-3
FR-17
GN-3
Agg-206

do not undergo any developmental change do not accumulate the enzyme. It is interesting to note that alanine transaminase is apparently not temporally regulated in mutants FR-17 and GN-3. All other developmentally regulated enzymes that have been tested so far have been shown to be temporally regulated in these mutants (Loomis, 1970b; Firtel and Bonner, 1971a).

Effects of actinomycin D and cycloheximide on increase in enzyme activity. Since alanine transaminase appears to be developmentally regulated, the effects of actinomycin D and cycloheximide on its accumulation were examined. As is shown in Fig 11 and 12, actinomycin D and cycloheximide do inhibit the developmental increase in enzyme activity in FR-17 and NC-4. The results with GN-3 are more tentative due to the small increase in specific activity in this mutant. The results, however, are reproducible and do suggest that the drugs inhibit the rise of activity in this mutant also. Prior RNA and protein synthesis are therefore necessary for the increase in the specific activity of the alanine transaminase in both wild type and mutant strains.

Levels of lactate dehydrogenase and glutamate dehydrogenase in NC-4. Table II and III show the specific activities of GDH and LDH in crude extracts of axenically grown cells under various assay conditions. Pyruvate appears to inhibit LDH at high substrate concentrations as

Figure 11. Effect of actinomycin D and cycloheximide on the use of alanine transaminase activity in wild type NC-4. For details of methods see legends to Figs. 4 and 5.

● Control NC-4 (No drug).
O Actinomycin D given at time indicated.
Δ Cycloheximide given at times indicated.

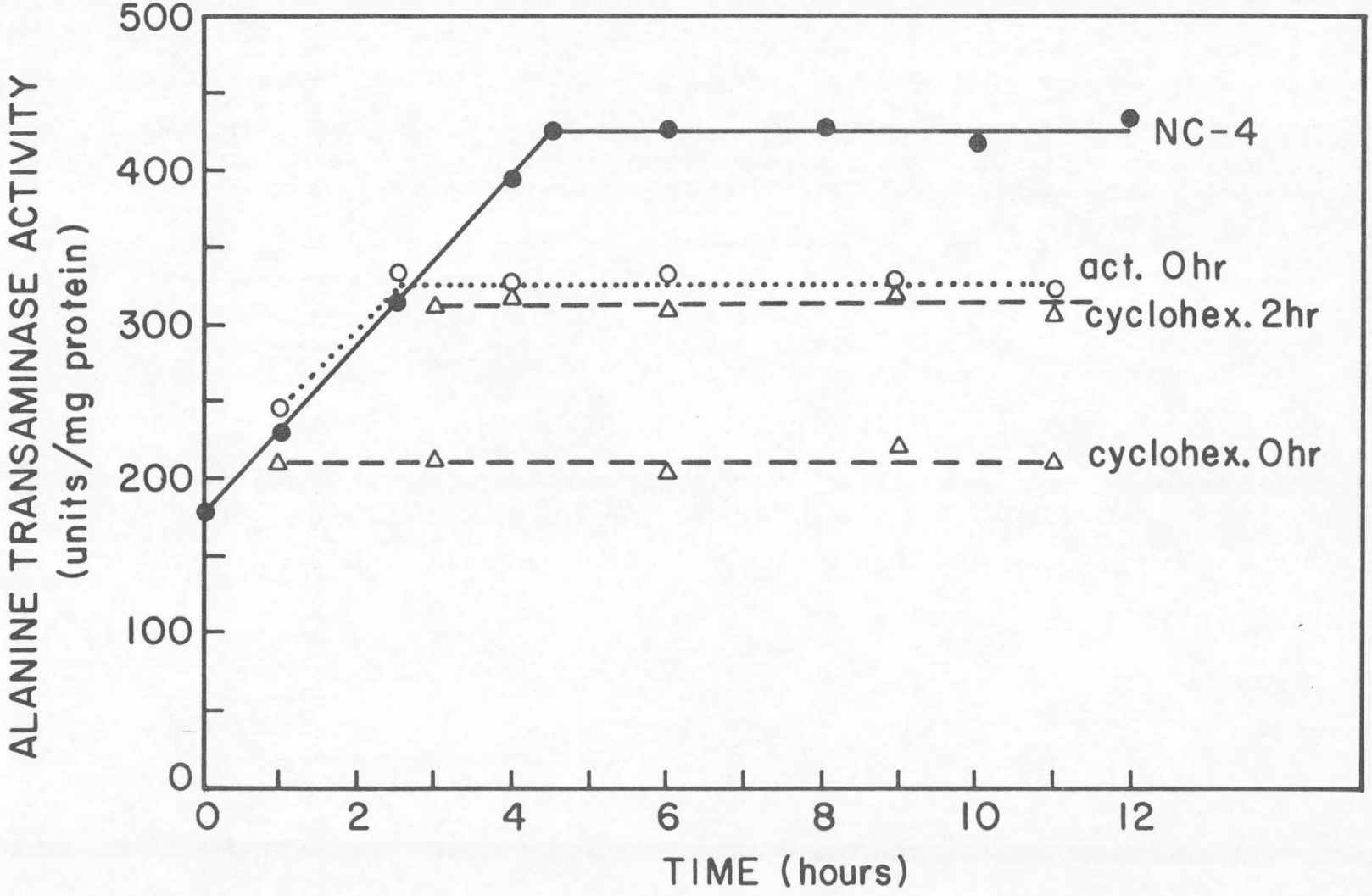

ALANINE TRANSAMINASE ACTIVITY
(units/mg protein)
500
400
300
200
100
0
0
2
4
6
8
10
12
TIME (hours)
NC-4
act. 0hr
cyclohex. 2hr
cyclohex. 0hr

Figure 12. Effect of actinomycin D and cycloheximide on the rise of alanine transaminase activity in temporally deranged mutants FR-17 and GN-3. For details of methods see legends to Figs. 3 and 4.

● Control (No drug).

O Actinomycin treated cells.

Δ Cycloheximide treated cells.

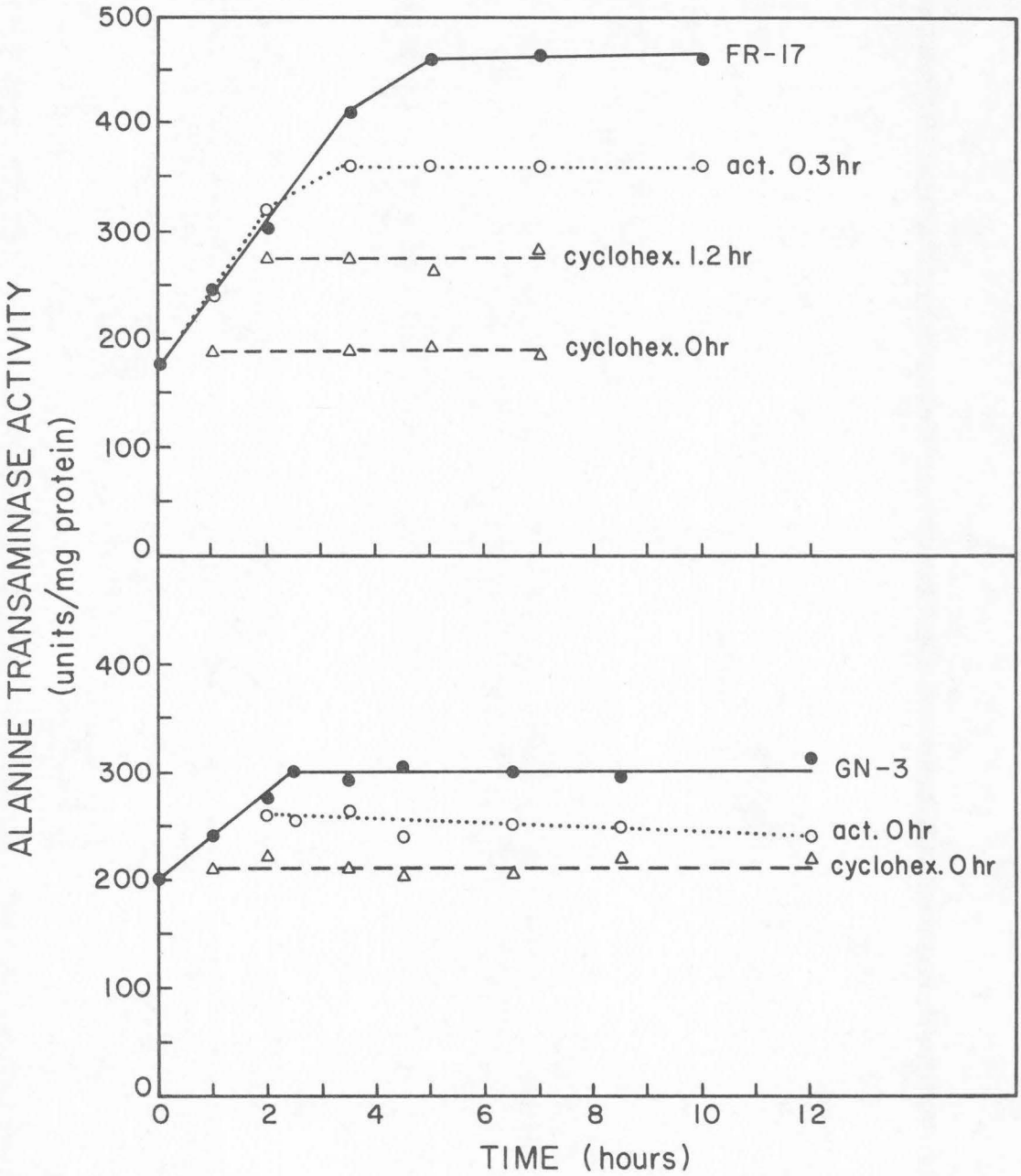

ALANINE TRANSAMINASE ACTIVITY
(units/mg protein)
500
400
300
200
100
0
FR-17
act. 0.3 hr
cyclohex. 1.2 hr
cyclohex. 0 hr
400
300
200
100
0
GN-3
act. 0 hr
cyclohex. 0 hr
0
2
4
6
8
10
12
TIME (hours)

TABLE II

Glutamic acid dehydrogenase

Assay system*	Activity (mμ moles NADH oxidized/min/ mg protein)
0.050 M NH_4^+	23.0
0.125 M NH_4^+	24.0
0.25 M NH_4^+	25.9
0.50 M NH_4^+	20.0
0.25 M NH_4^+, 0.04 M α-ketoglutarate	25.4
0.25 M NH_4^+, 0.062 M L-Alanine	26.0
0.04 M Tricine, pH 8.2	24.5

*Glutamic acid dehydrogenase assay:

0.04 M Tricine, pH 7.4; 0.02 M α-ketoglutarate, pH 7.0; 0.2 mg/ml NADH; .003 M EDTA, pH 7.4; and NH_4Ac (see above for concentrations), and approximately 25 ul of a crude extract from logarithmically growing axenic cells. The crude extract was made by sonicating the cells at a concentration of 2×10^8 cells/ml in 20% glycerol. Assays were performed at 28°C.

TABLE III

Lactic Acid Dehydrogenase

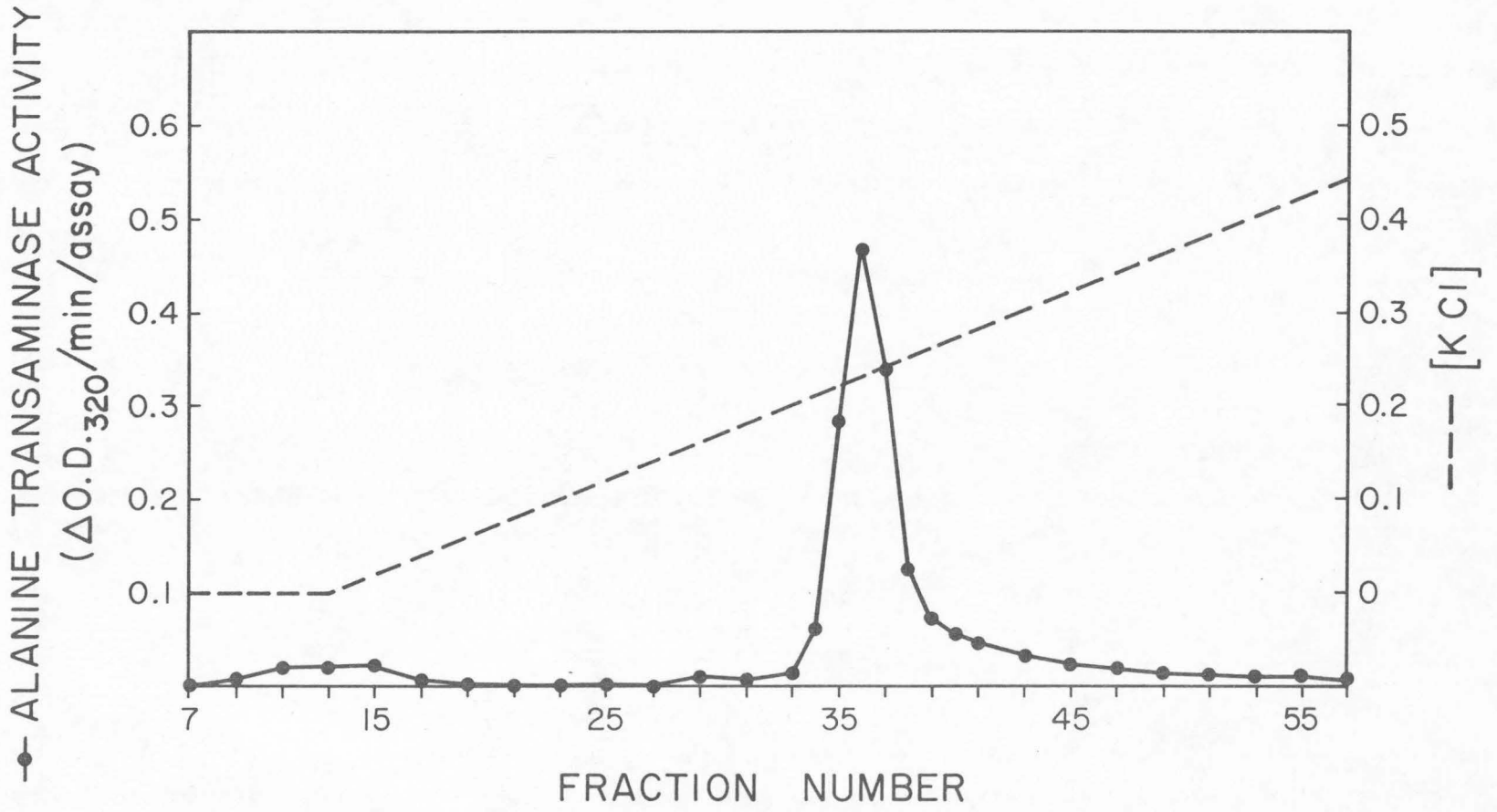
Assay system*	Activity (mμm NADH oxidized /min/mg protein)
1.27 mM pyruvate	10.6
2.54 mM pyruvate	9.2
6.62 mM pyruvate	6.3
13.2 mM pyruvate	5.7
2.54 mM pyruvate, 0.05 M NH_4^+	11.1
13.2 mM pyruvate, 0.07 M NH_4^+	9.3

*Assay conditions: 0.09 M potassium phosphate buffer, pH 7.0; 0.25 mg/ml NADH, and Na pyruvate (see above for concentrations) and approximately 50 ul of a crude extract prepared as described in Table II). Assays were performed at 28°C.

has been shown for mammalian LDH (see Schwert and Winer, 1963). NH_4Ac stimulates NADH oxidation, especially at higher substrate concentrations. The observed increase may be due to an actual stimulation of LDH or to the presence of a second enzyme, alanine dehydrogenase (ADH). Although it was not possible to distinguish between these two possibilities in crude extracts using the procedures described in this paper, further results to be discussed strongly suggest that at least some of the increase in activity is due to NH_4Ac stimulation of LDH. Other studies (not shown) indicate that the levels of GDH and LDH change only slightly during the developmental cycle of NC-4. GDH activity increases approximately 1.2-1.5 fold during the first 6-7 hours of development while LDH activity decreases by approximately 30% over the developmental cycle. The ratio of LDH to LDH assay with NH_4Ac for each point assayed remained constant for each sample.

Characterization of the various enzymes by DEAE chromatography, sucrose gradient centrifugation, and gel electrophoresis. All experiments were performed on 160,000 g supernatant of sonicated crude extracts of logarithmically growing axenic cells. Fig. 13 shows a DEAE cellulose chromatography profile of the crude extract for alanine transaminase. A single peak of activity elutes at 0.23 M KCl; the small peak near the beginning of the gradient is probably due to GDH activity (see below). Sucrose density

Figure 13. DEAE cellulose chromatography of 160,000 g supernatant of logarithmically growing axenic cells assayed for alanine transaminase. Sample was applied in 0.01 M Tricine, pH 7.6, 5% glycerol, 0.2 mM DTT. A linear KCl gradient was run.


ALANINE TRANSAMINASE ACTIVITY
(ΔO.D.320/min/assay)
0.6
0.5
0.4
0.3
0.2
0.1
0.5
0.4
0.3
0.2
0.1
0
[K Cl]
7
15
25
35
45
55
FRACTION NUMBER

gradient analysis (Fig. 14) also shows only one species with a sedimentation velocity of approximately 6 S relative to a BSA standard. Fig. 15 and 16 show the Lineweaver and Burk plots (1934) for the substrates α-ketoglutarate and alanine. The Km for α-ketoglutarate is approximately 0.1 mM and that for alanine approximately 0.4 mM.

Figure 17 shows a sucrose gradient of crude extracts assayed for GDH activity. For this extract, over 80% of the GDH activity sedimented at 12 S (Case I). In other crude extracts (not shown), the gradient showed two peaks of equal activity (Case II). When extracts yielding two equal peaks on sucrose gradients (Case II) are chromatographed on DEAE cellulose (Figure 18) three GDH activity peaks, a small peak eluting at very low salt and two peaks eluting between 0.18 and 0.23 M KCl, are formed. When the individual DEAE fractions are then centrifuged in a sucrose gradient (Figure 19), fraction I yields a peak which reproducibly sediments slower than the 6-7 S GDH peak. Fraction II yields a peak at 6-7 S, while fraction III yields peaks at 12 and 6-7 S. The DEAE cellulose chromatography of Case I crude extracts (Fig. 20) showed only two peaks, a very small peak eluting at the beginning of the KCl gradient, and one at 0.22 M KCl, approximately the concentration at which fraction III elutes. If these fractions are run on sucrose gradients, the small,

Figure 14. Sucrose gradient centrifugation of 160,000 g supernatants assayed for alanine transaminase activity. 5-20% sucrose gradient containing 0.02 M Tricine buffer, pH 7.6, 0.05 M KCl, 0.2 mM DTT centrifuged 7 hours, 63,000 rpm, Spinco SW 65 rotor, 4°C. The BSA marker was run in a second tube and located by measuring $O.D._{230}$.

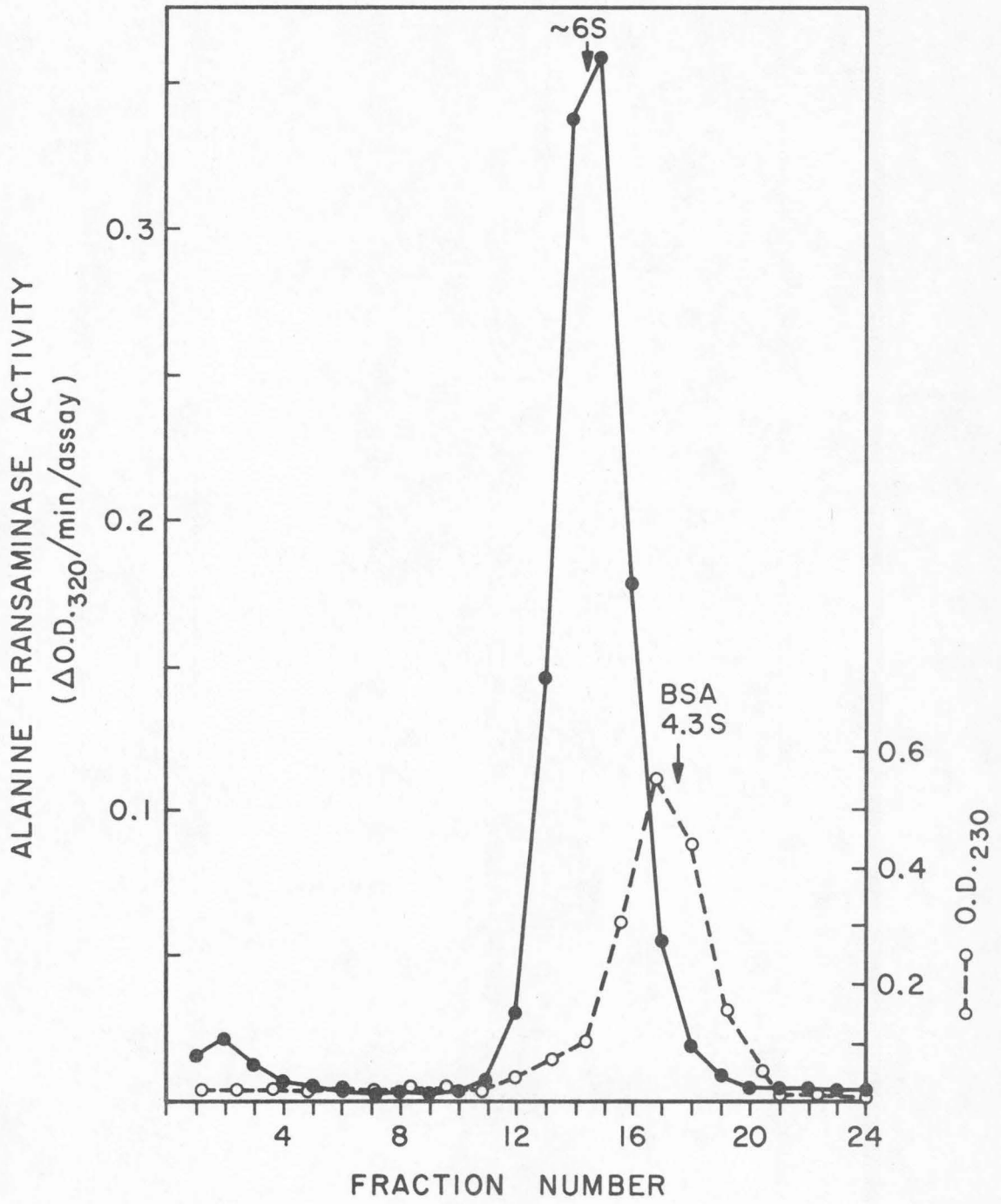

~6S
BSA
4.3S
0.3
0.2
0.1
0.6
0.4
0.2
4
8
12
16
20
24
FRACTION NUMBER
ALANINE TRANSAMINASE ACTIVITY
(ΔO.D.320/min/assay)
O.D.230

Figure 15. Lineweaver and Burk plot (1934) for determination of Km of α-ketoglutarate for alanine transaminase. Concentrations of all other reactants are given in Materials and Methods.

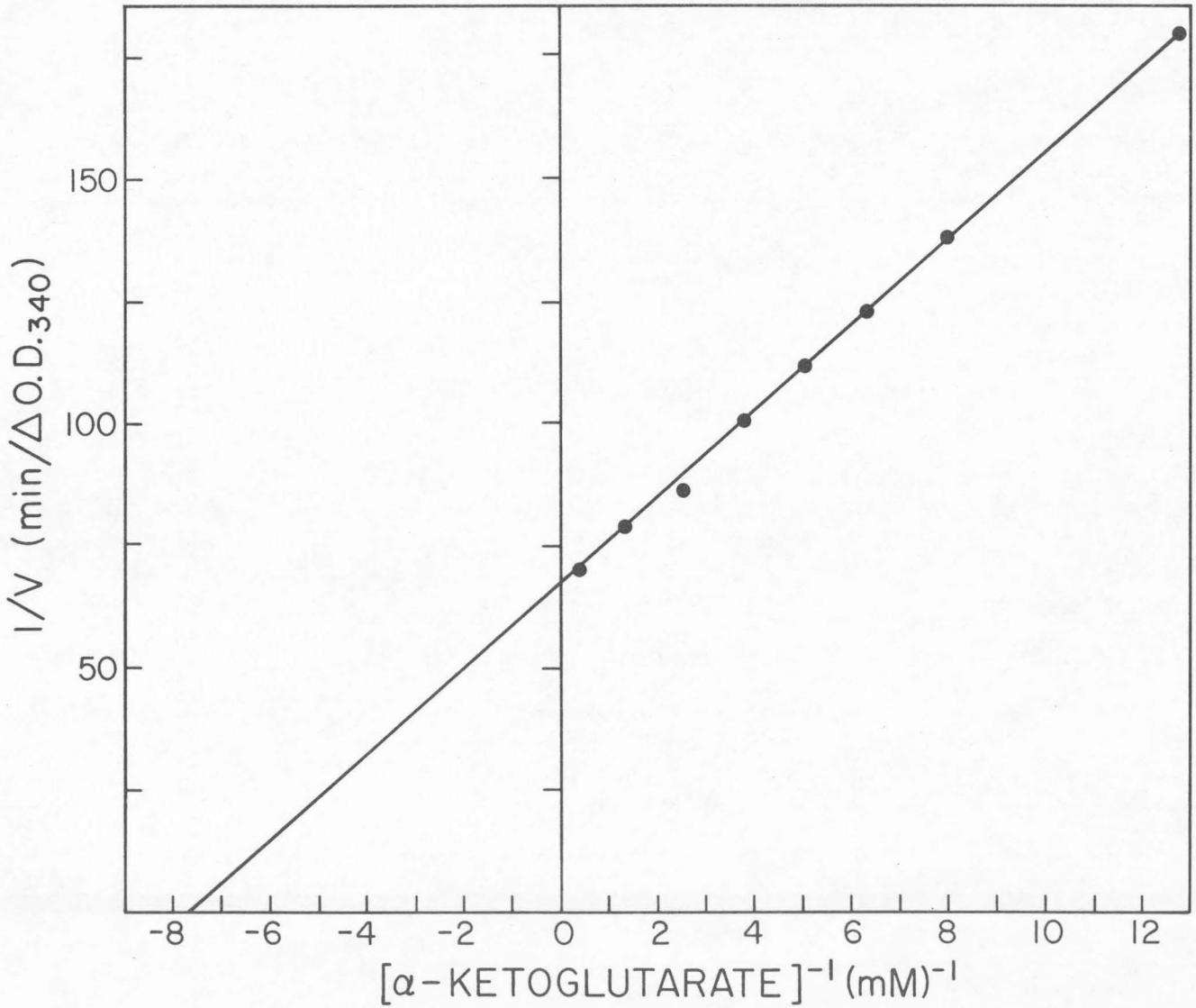

150
100
50
1/V (min/Δ O.D.340)
-8
-6
-4
-2
0
2
4
6
8
10
12
[α-KETOGLUTARATE]$^{-1}$ (mM)$^{-1}$

Figure 16. Lineweaver and Burk plot (1934) for the determination of the Km of L-alanine for alanine transaminase. Concentrations of other reactants given in Materials and Methods.

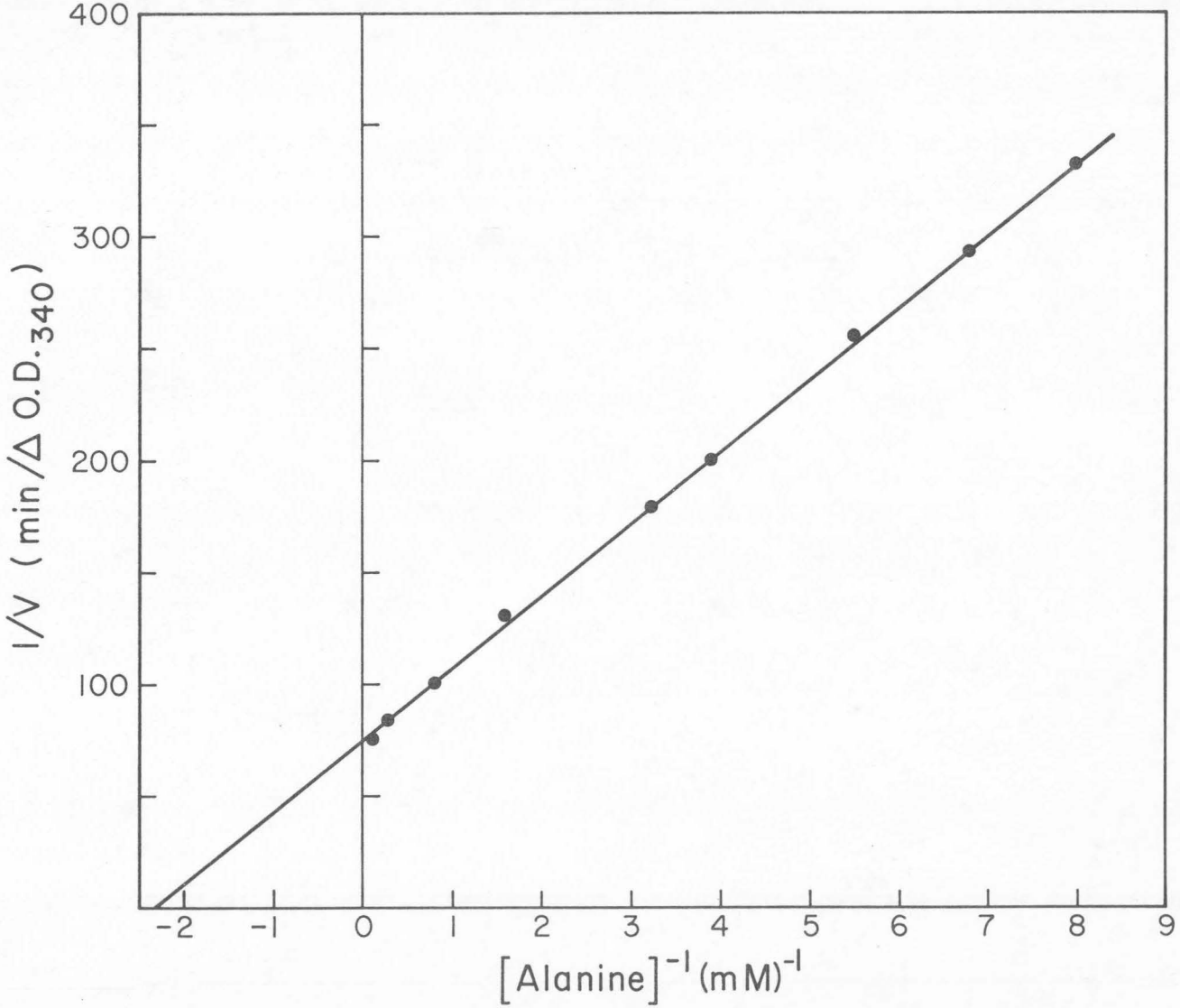

400
300
200
100
1/V (min/Δ O.D.340)
-2
-1
0
1
2
3
4
5
6
7
8
9
[Alanine]-1 (mM)-1

Figure 17. Sucrose gradient centrifugation of 160,000 g supernatant of sonicates of lagarithmically growing axenic cells assayed for GDH and LDH activity. 5-20% sucrose gradient containing 0.02 M Tricine, pH 7.4, 0.2 mM DTT, 0.05 M KCl centrifuged 5 hours, 60,000 rpm in Spinco SW 65 rotor at 4°C.

The enzymes were assayed as described in Tables II and III. 0.013 M pyruvate was used for the LDH assay. Markers: BSA, 4.3 S; catalase 11.2 S.

—●— GDH activity

—▲·— LDH activity

—△— LDH activity assayed in the presence of 0.07 M Na_4Ac.

···o··· $O.D._{230}$ of second tube containing BSA and catalase markers.

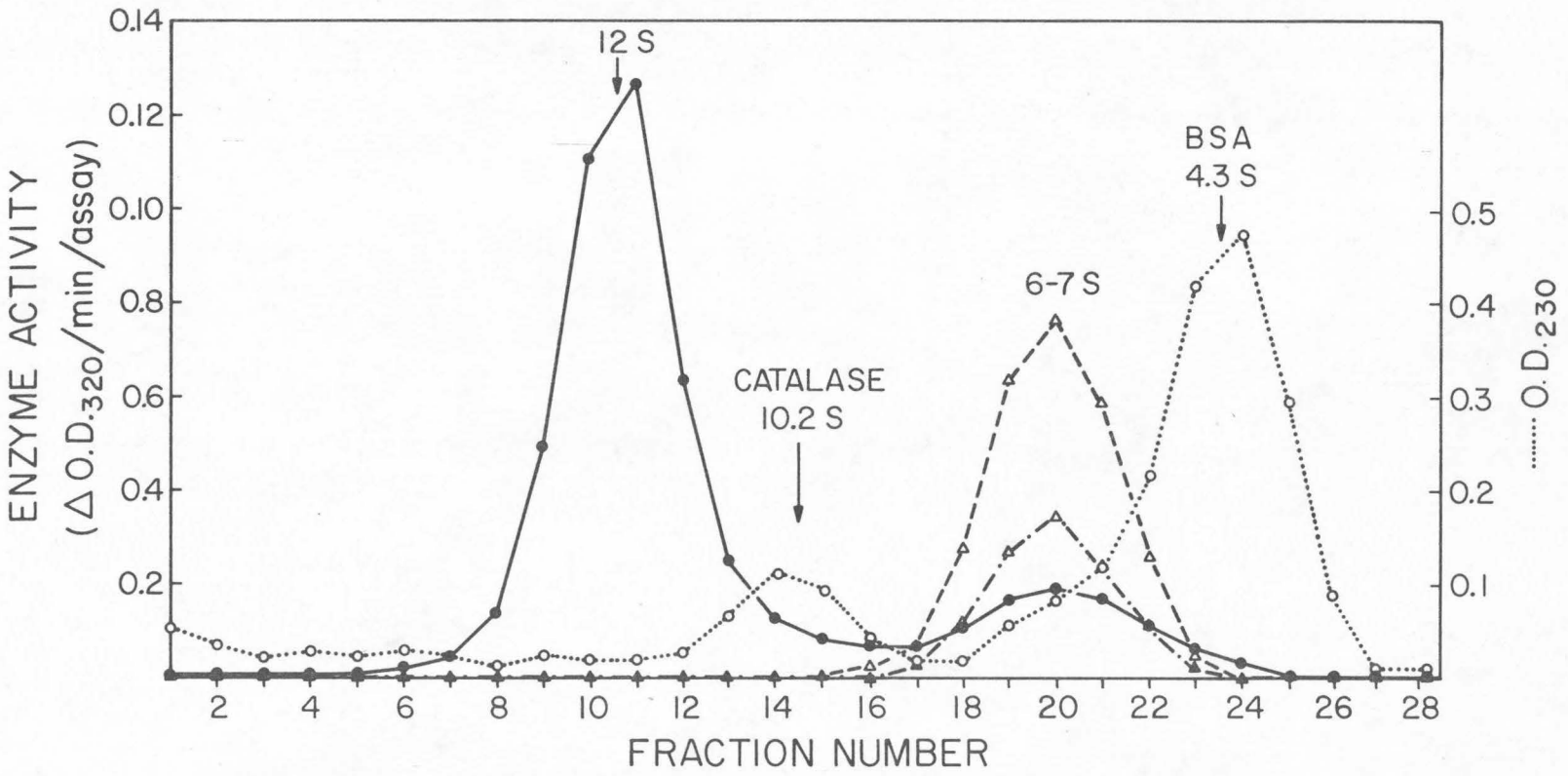

ENZYME ACTIVITY
(Δ O.D.320/min/assay)
0.14
0.12
0.10
0.8
0.6
0.4
0.2
12 S
CATALASE
10.2 S
6-7 S
BSA
4.3 S
0.5
0.4
0.3
0.2
0.1
O.D.230
2
4
6
8
10
12
14
16
18
20
22
24
26
28
FRACTION NUMBER

Figure 18. DEAE cellulose chemotography of 160,000 g supernatant of logarithmically growing axenic cells assayed for GDH activity. See legend to Figure 13 for details. See Table II for assay conditions.

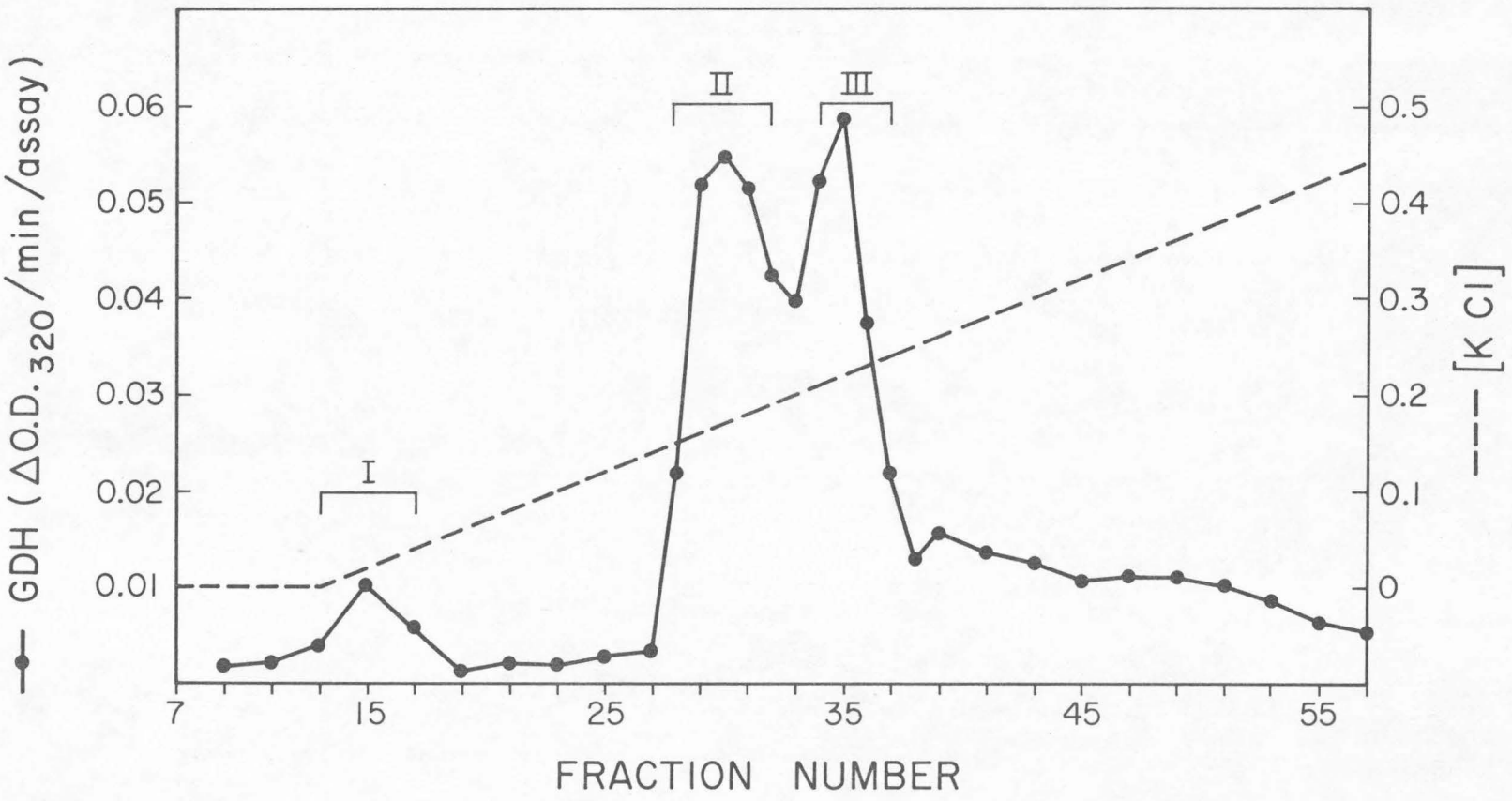

GDH (ΔO.D.320/min/assay)
0.06
0.05
0.04
0.03
0.02
0.01
I
II
III
0.5
0.4
0.3
0.2
0.1
0
[K Cl]
7
15
25
35
45
55
FRACTION NUMBER

Figure 19. Sucrose gradient centrifugation of DEAE column fractions shown in Figure 18 assayed for GDH activity. 5-20% sucrose gradient containing 0.02 M Tricine, pH 7.4, 0.2 mM DTT, 0.05 M KCl centrifuged 5 hours, 60,000 rpm in Spinco SW 65 rotor at 4°C.

··△·· Fraction I

-●- Fraction II

--o-- Fraction III

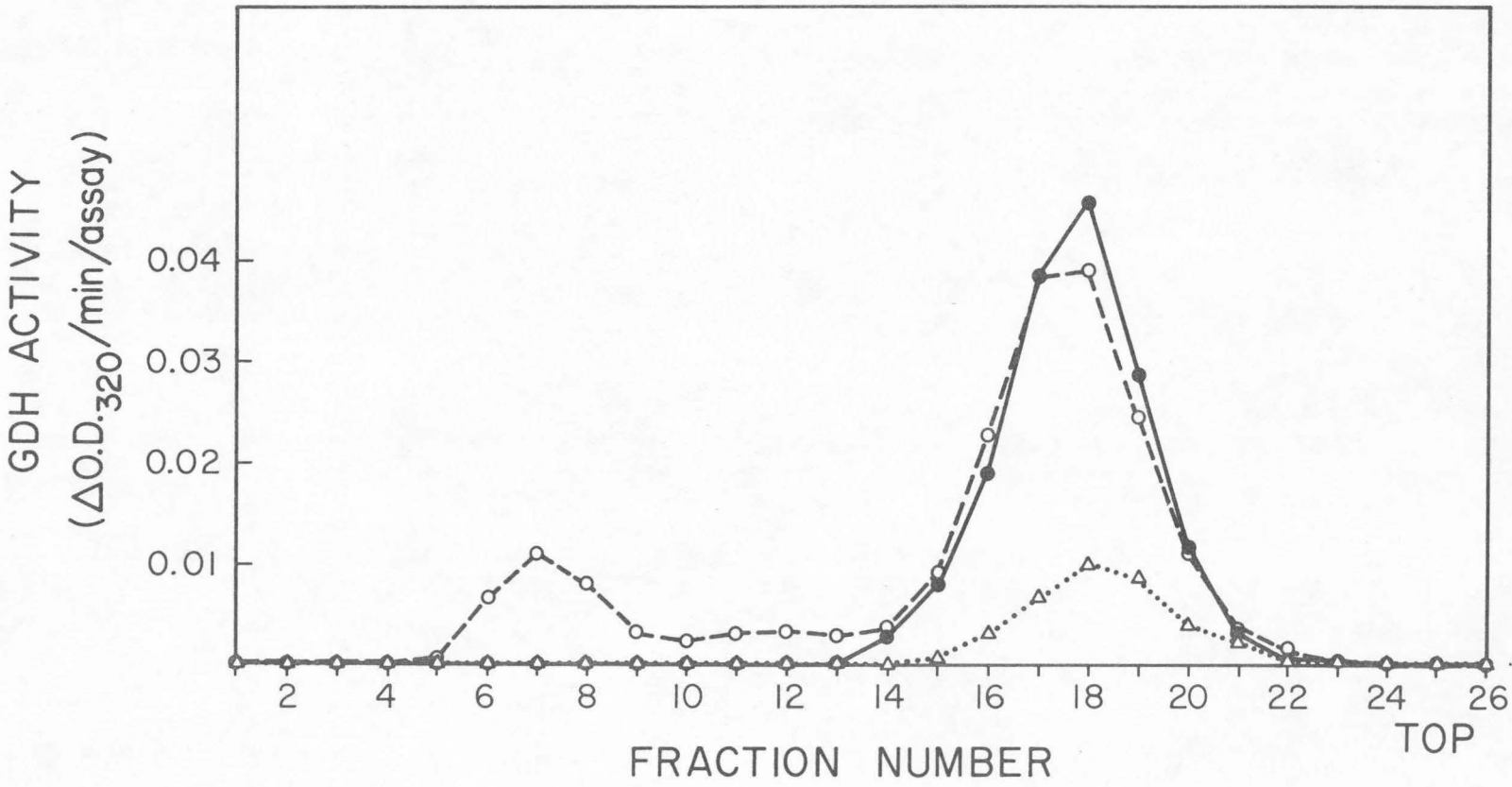
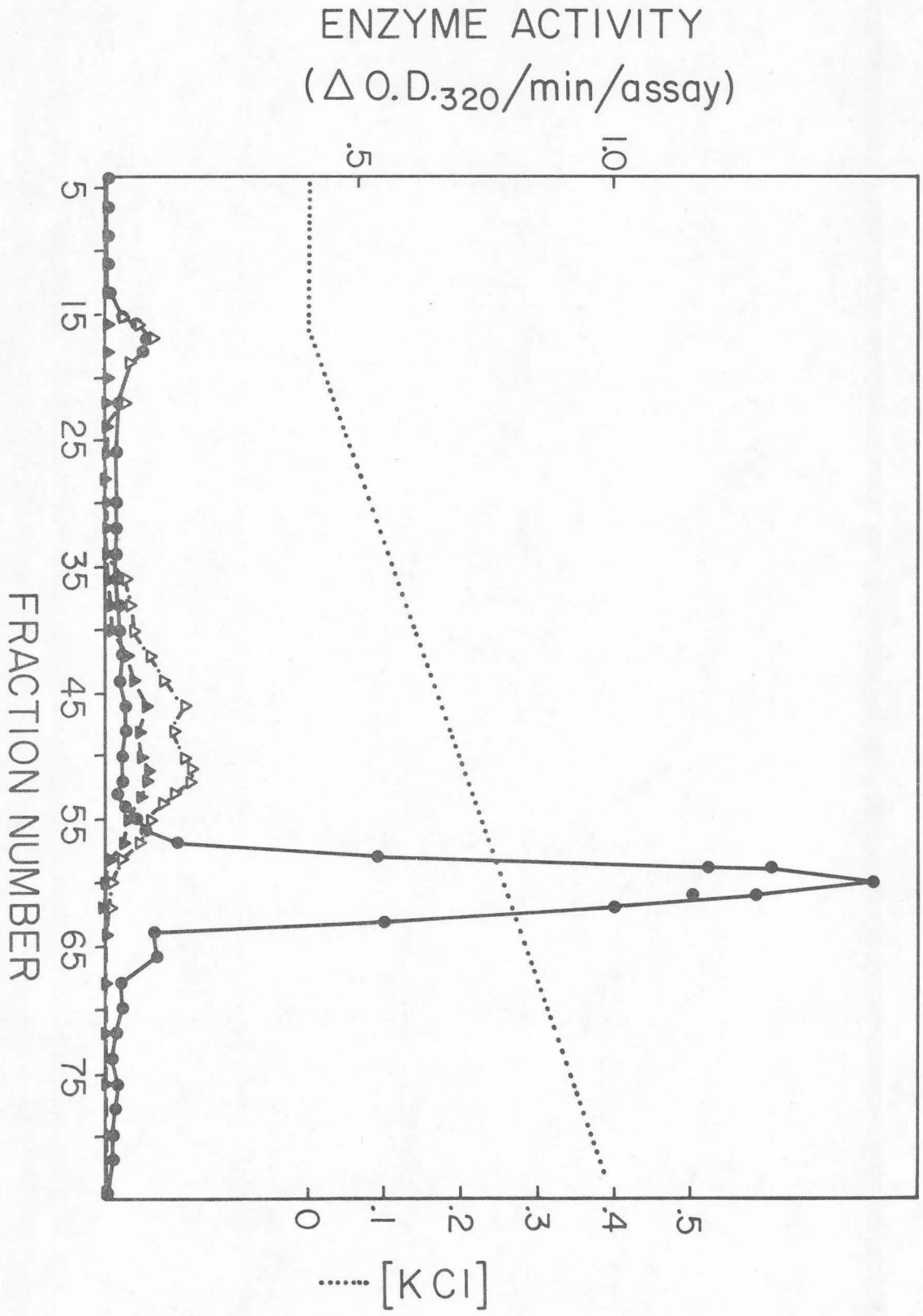

GDH ACTIVITY
(ΔO.D.320/min/assay)
0.04
0.03
0.02
0.01
2
4
6
8
10
12
14
16
18
20
22
24
26
TOP
FRACTION NUMBER

Figure 20. DEAE cellulose chromatography of 160,000 g supernatant of logarithmically growing axenic cells assayed for GDH and LDH. The sample was applied in 0.005 M KPO_4 buffer, 0.001 M EDTA, 10% glycerol, 0.2 mM DTT, pH 7.2. A linear gradient of KCl was run. The fractions were assayed for GDH and LDH activity as described in Table II and III. In assaying LDH activity, the pyruvate concentration was 0.013 M.

—o— GDH activity

—▲— LDH activity

—·△—·— LDH activity with 0.07 M NA_4Ac.


ENZYME ACTIVITY
(Δ O.D.320/min/assay)
.5
1.0
5
15
25
35
45
55
65
75
FRACTION NUMBER
0
.1
.2
.3
.4
.5
[KCl]

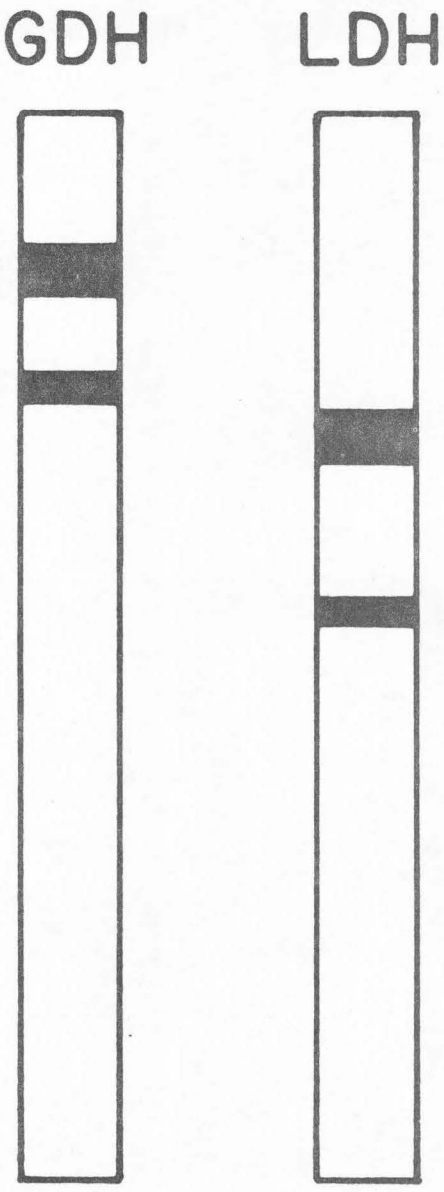
low salt peak again sediments more slowly than the 6-7 S material, while the 0.22 M peak shows the same distribution between 12 and 6-7 S material as does the original extract. Case I and case II samples were obtained from cells cultured and treated in exactly the same way. Of 7 different experiments, five came out as case I and two as case II.

Tompkins and Yielding (1961) found that partially purified Dictyostelium GDH is not very sensitive to diethylstibestriol (DES) unlike mammalian GDH. They found, however, that Dictyostelium GDH could be made sensitive to DES by incubation at 37°C. The native and sensitized enzymes were not physically characterized, but it is possible that the 12 S component represents the native enzyme and that the 6-7 S component may be the sensitized component produced during sonication of the crude extracts in the cases presented here.

The crude extract was run on polyacrylamide gel electrophoresis (Davis, 1964; E-C Apparatus Corp., Tech. Bull. 135) using Tris-citrate, pH 7.0 or Tris-glycine, pH 8.3 buffers. As can be seen in Figure 21, two GDH bands were found. When the 12 S and 6-7 S sucrose gradient peaks are run on gels, they migrate with the upper and lower bands respectfully (not shown).

Also shown in Figures 17 and 20 are the DEAE cellulose chromatography and sucrose gradient profiles of 160,000 g

Figure 21. Polyacrylamide gel electrophoresis of 160,000 g supernatant on crude and sonicates assayed for LDH and GDH activities. The gels shown were run in Tris-glycine buffer pH 8.1 (Davis, 1964). Other gels run in Tris-citrate buffer, pH 7.0, shows the same bands. Gels were stained specifically for the enzymes indicated according to E-C Apparatus Corp., Tech. Bull. 135 using L-glutamate and lactic acid as substrates.


GDH
LDH

crude extract supernatant assayed for LDH with and without NA_4Ac. There appear to be two activity peaks eluting between 0.18 and 0.20 M KCl on the DEAE columns. A third peak which shows little activity in the absence of NH_4Ac and may actually be ADH elutes near the runoff material and is superimposed on a GDH peak.

Acrylamide gels of crude extracts assayed for LDH activity show two distinct bands indicating that there are two LDH isozymes. Samples run toward the cathode showed no enzyme activity.

DISCUSSION

During the developmental cycle of Dictyostelium discoideum there is a net loss of over 50% in cellular protein which apparently is metabolized as an energy source (see Introduction). It has been previously shown that two amino acid metabolizing enzymes which may be involved in the process of protein degradation are developmentally regulated; tyrosine transaminase reaches a maximum activity during early culmination, and threonine dehydrolase reaches a maximum specific activity during the early pseudoplasmodium stage (Pong and Loomis, in preparation). In the present paper it is shown that aminopeptidase activity increases slowly during development and reaches a maximum at approximately the same time as tyrosine transaminase. It is conceivable that this enzyme is responsible for the

degradation of small peptides to single amino acids *in vivo*. It is interesting that no developmentally regulated general protease has been found in *Dictyostelium* (Sussman and Sussman, 1969). A pH 2 acid protease has been discovered but its activity remains constant during the developmental cycle (Sussman and Sussman, 1969; Wiener and Ashworth, 1970). Experimental results with cycloheximide and actinomycin D suggest that RNA and protein synthesis are required for the rise in specific activity of aminopeptidase. Further evidence that the enzyme is developmentally regulated (although it only increases by a few-fold), is found in the fact that the enzyme is temporally regulated in mutants FR-17 and GN-3 and does not increase in activity in the aggregation-minus mutant, Agg-206. Plating the cells on filters in the presence of EDTA to inhibit morphogenesis also prevents the increase in enzyme activity.

Because *Dictyostelium* is an obligatory aerobe (Gregg, 1950; Gerish, 1962) Krebs cycle-linked amino acid metabolizing enzymes have also been examined. The results presented here are in agreement with those of Wright (Wright and Anderson, 1959; Wright, 1960). The levels of glutamic acid dehydrogenase, lactate dehydrogenase, and aspartate transaminase do not change during the developmental cycle of *Dictyostelium discoideum*. Alanine transaminase, however, increases by 2.5-3.3 fold soon after

the initiation of starvation. The increase has been shown to require RNA and protein synthesis in wild type and in all mutants examined. The increase thus appears to be developmentally regulated and at least one mutant (Agg-206) which shows no signs of undergoing even the first steps in the developmental cycle does not accumulate increased amounts of alanine transaminase.

Although the level of glutamic acid dehydrogenase does not increase during the developmental cycle, Wright and Bard (1963) and Brühmüller and Wright (1963) have shown that the rate of oxidation of glutamate (production of ^{14}C-CO_2 from ^{14}C-glutamate) increases approximately 7-fold as intracellular levels of glutamic acid increase by a factor of approximately 10-fold. During this period the ratio of the glutamic acid concentration to the Km always remains less than 1, indicating that the rate of oxidation of glutamate through the Krebs cycle is a function of the concentration of this substrate, which appears to be rate-limiting. GDH (NAD-requiring) appears to be present in at least two molecular forms, one sedimenting at approximately 12 S and one at 6-7 S which may be an artifact due to experimental procedures. When NADP is used as substrate there is no detectable GDH activity. Previous studies by Tompkins and Yielding (1961) on Dictyostelium GDH found only one molecular species with an estimated molecular weight of approximately 200,000. These investigators

showed that, as with the mammalian GDH, the steroid hormone DES drastically inhibits GDH activity if the enzyme is sensitized at 37°C and that this inhibition can be overcome by AMP. AMP was also found to stimulate the partially purified GDH. Other work (Firtel, unpublished observation) indicates that some other amino acid degrading enzymes are not developmentally regulated, including histidase and homoserine dehydrolase. It is not yet clear why certain amino acid catabolizing enzymes are developmentally regulated and others are not. It is interesting that Dictyostelium is an obligatory aerobe and that it is capable of growing in axenic culture on proteose peptone and yeast extract without an added carbohydrate source. It is possible that Dictyostelium may maintain a generally high level of many of the amino acid catabolizing enzymes at all developmental stages. Dictyostelium discoideum alanine transaminase has one interesting property which differentiates it from the mammalian alanine transaminases previously examined. The Km of the Dictyostelium enzyme is approximately 4×10^{-4} M while that for mammalian systems is between $1\text{-}4 \times 10^{-2}$ M (Hopper and Segal, 1962; Bulos and Handler, 1965). The lower Km permits Dictyostelium to metabolize alanine at a faster rate at low substrate concentrations which may be advantageous if amino acids represent the principal energy source during differentiation.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. James Bonner and Miss Jessica Tuchman, California Institute of Technology, for helpful suggestions and for critically reading this manuscript. We would also like to thank Ken Pischel, California Institute of Technology, for technical assistance on some of the experiments.

During the period this work was done one of us (RAF) was a NSF predoctoral fellow. The work was supported in part by USPHS grant (to Dr. James Bonner) GM13762.

REFERENCES

Brühmüller, M., and Wright, B.E. (1963). Glutamate oxidation in the differentiating slime-mold. II. studies in vitro. Biochim. Biophys. Acta 71, 50.

Bulos, B. and Handler, P.(1965).Kinetics of beef heart glutamic-alanine transaminase. J.Biol.Chem. 240, 3283.

Clegg, J.S. and Filosa,M.F.(1961). Trehalose in the cellular slime mold Dictyostelium mucoroides. Nature 192, 1077.

Cleland, S.V.,and Coe,E.L.(1968).Activities of glycolytic enzymes during early stages of differentiation of Dictyostelium discoideum. Biochim Biophys.Acta 156,44.

Cleland,S.V.,and Coe,E.L.(1969).Conversion of aspartic acid to glucose during culmination in Dictyostelium discoideum. Biochim. Biophys. Acta 192,446.

Davis, B. (1964).Disc electrophoresis II. Method and application to human serum proteins. Ann.N.Y.Acad.Sci. 121, 404.

E-C Apparatus Corp.,Tech.Bull. No.135. Buffer and enzyme incubation recipes.

Firtel, R.A.,and Bonner,J.(1971a).Developmental control of α1-4 glucan phosporylase in the cellular slime mold Dictyostelium discoideum. Submitted for publication.

Firtel, R.A.and Bonner,J. (1971b). Characterization of the genome of the cellular slime mold Dictyostelium discoideum. Submitted for publication.

Gerish, G. (1962). Zellfunktionene und Zellfunktioswechsel

in der Entwicklung von Dictyostelium Discoideum VI. Inhibitoren der Aggregation ihr Einfluss auf Zellkontaktbildung und morphogenetische Bewegung. Exptl. Cell Res. 26, 426.

Gregg, J.H. (1950). Oxygen utilization in relation to growth and morphogenesis of the slime mold Dictyostelium discoideum. J. Exptl. Zool. 114, 173.

Gregg, J.H., Hackney, A.L., and Krivanek, J.O. (1954). Nitrogen metabolism of the slime mold Dictyostelium discoideum during growth and morphogenesis. Biol. Bull. 107, 226.

Gregg, J.H., and Bronsweig, R.D. (1956). Biochemical events accompanying stalk formation in the slime mold Dictyostelium discoideum. J. Cellular Comp. Physiol. 48, 293.

Hopper, S., and Segal, H.L. (1962). Kinetic studies of rat liver glutamic-alanine transaminase. J. Biol. Chem. 237. 3189.

Lineweaver, H., and Burk, D. (1934). The determination of enzyme dissociation constants. J. Amer.Chem. Soc. 56, 658.

Loomis, W.F., Jr. (1970a). Developmental regulation of α-mannosidase in Dictyostelium discoideum. J. Bacteriol. 103, 375.

Loomis, W.F., Jr. (1970b). Temporal control of differentiation in the slime mold Dictyostelium discoideum. Exptl. Cell Res. 60, 285.

Loomis, W.F., Jr., and Sussman, M. (1966). Commitment to the synthesis of a specific enzyme during cellular slime mold development. J. Mol. Biol. 22, 401.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265.

Mizukami, Y., and Iwabuchi, M. (1970). Effects of actinomycin D and cycloheximide on morphogenesis and synthesis of RNA and protein in the cellular slime mold Dictyostelium discoideum. Exptl. Cell Res. 63, 317.

Raper, K. (1935). Dictyostelium discoideum, a new species of slime mold from decaying forest leaves. J. Agr. Res. 50, 135.

Raper, K. (1940). Pseudoplasmodium formation and organization in Dictyostelium discoideum. J. Elisha Mitchell Sci. Soc. 56, 24.

Schwert, G.W., and Winer, A.D. (1963). Lactate dehydrogenase. In "The Enzymes" (Boger, P.D., Laidy, H., Myrback, eds.) vol. 7, chap. 6, pp.127.

Sonneborn, D., White, G., and Sussman, M. (1963). A mutation affecting both rate and pattern of morphogenesis in Dictyostelium discoideum. Develop. Biol. 7, 79.

Sussman, M. (1966a). Some genetic and biochemical aspects of the regulatory program for slime mold development. "Current Topics in Developmental Biology" (A.A.

Moscona and A. Monroy, eds.) vol. 1, p. 61. Academic Press, N.Y.

Sussman, M. (1966b) Biochemical and genetic methods in the study of cellular slime mold development. In "Methods in Cell Physiology," vol II, p.397. Academic Press, N.Y.

Sussman, M., Loomis, W.F., Jr., Ashworth, J.M., and Sussman, R.R. (1967). The effect of actinomycin D on cellular slime mold morphogenesis. Biochem. Biophys. Res. Commun. 26, 353.

Sussman, M., and Sussman, R.R. (1965). The regulatory program for UDP-galactose polysaccharide transferase activity during slime mold cytodifferentiation: requirement of a specific synthesis of ribonucleic acid. Biochem. Biophys. Acta 108, 463.

Sussman, M., and Sussman, R. (1969). Patterns of RNA synthesis and of enzyme accumulation and disappearance during cellular slime mold cytodifferentiation. Symp. Soc. Gen. Microbiol. XIX, 403.

Telser, A., and Sussman, M. (1971). Uridine diphosphate galactose-4-epimerase, a developmentally regulated enzyme in the cellular slime mold Dictyostelium discoideum. J. Biol. Chem. 246, 2252.

Tompkins, G.M., and Yielding, K.L. (1961). Regulation of the enzymatic activity of glutamic dehydrogenase mediated by changes in its structure. Cold Spring Harbor Symp. Quant. Biol. XXVI, 331.

White, G.J., and Sussman, M. (1961). Metabolism of major cell components during slime mold morphogenesis. Biochim. Biophys. Acta 53, 285.

White, G.J., and Sussman, M. (1963a). Polysaccharides involved in slime-mold development I. Water-soluble glucose polymer(s). Biochim. Biophys. Acta 74, 173.

White, G.J., and Sussman, M. (1963b). Polysaccharides involved in slime-mold development II. Water-soluble acid mucopolysaccharid(s). Biochim. Biophys. Acta 74, 179.

Wiener, E. and Ashworth, J.M. (1970). The isolation and characterization of lysosomal particles from myxamoebae of the cellular slime mold Dictyostelium discoideum. Biochem. J. 118, 505.

Wright, B.E. (1964). Biochemistry of the Acrasiales. In "Biochemistry and Physiology of Protozoa III" (S.H. Hunter, ed.) pp. 341. Academic Press, N.Y.

Wright, B.E., and Anderson, M.L. (1959). Biochemical differentiation in the slime mold. Biochim. Biophys. Acta 31, 310.

Wright, B.E., and Anderson, M.L. (1960). Protein and amino acid turnover during differentiation I. Biochim. Biophys. Acta 43, 62.

Wright, B.E., and Bard, S. (1963). Glutamate oxidation in the differentiating slime mold. I. Studies in vivo.

Yanigasawa, K., Loomis, W.J., Jr., and Sussman, M. (1967). Developmental regulation of the enzyme UDPGal: polysaccharide transferase. Exptl. Cell Res. 46, 328.

PART II

POLYSOMES AND RNA SYNTHESIS DURING EARLY DEVELOPMENT OF THE SURF CLAM *SPISULA solidissima*

Reprinted from DEVELOPMENTAL BIOLOGY, Volume 21, Nos. 1-2, January-February 1970

DEVELOPMENTAL BIOLOGY **21**, 87-104 (1970)

Polysomes and RNA Synthesis during Early Development of the Surf Clam *Spisula solidissima*[1]

RICHARD A. FIRTEL[2] AND ALBERTO MONROY[3]
Marine Biological Laboratory, Woods Hole, Massachusetts

Accepted July 14, 1969

INTRODUCTION

A comparative analysis of the molecular events of the early development in eggs belonging to different phyla may be of great importance to the understanding of the basic processes underlying embryogenesis. In particular, as far as the early postfertilization events are concerned, it is important to compare eggs which at the time of fertilization have reached a different stage of maturation. Most of the studies pertaining to the process of fertilization have been carried out on the sea urchin egg—an egg which at the time of fertilization has already completed its maturation. The egg of the mollusc *Spisula solidissima*, in which the onset of maturation is initiated by fertilization, is therefore a very convenient object for a comparative analysis. It has been shown that in *Spisula*, similar to what is known to occur in the sea urchin egg, the overall rate of protein synthesis increases immediately after fertilization (Bell and Reeder, 1967). For the sea urchin, this increase is known to accompany an increase in number of polysomes (Monroy and Tyler, 1963; Piatigorsky, 1967; Rinaldi and Monroy, 1969). The increase in protein synthesis is insensitive to actinomycin D (Gross and Cousineau, 1963) which indicates that the mRNA necessary for early polysome formation is already present in the unfertilized egg. Several hypotheses have been proposed as to the location of such masked mRNA before fertilization (see Tyler and Tyler, 1966; Piatigorsky, 1968).

In the sea urchin, however, RNA synthesis does begin soon after fertilization, and this newly synthesized RNA can be detected in

[1] This paper is dedicated to the memory of Albert Tyler. The work has been supported by NIH Grant T1-HD-26-07 (Fertilization and Gamete Physiology Training Program) Marine Biological Laboratory, Woods Hole, Massachusetts.

[2] Recipient of a National Science Foundation Predoctoral Fellowship, Present address: Division of Biology, California Institute of Technology, Pasadena, California.

[3] Present address: C.N.R. Laboratory of Molecular Embryology, Arco Felice (Napoli), Italy.

polysomes as early as the nuclear streak stage. By the 2–4-cell stage an appreciable amount of newly formed RNA is present in the light polysomes. The main site of protein synthesis (amino acid incorporation) is, however, the heavy polysomes (Rinaldi and Monroy, 1969). The newly formed RNA found associated with the polysomes during early cleavage has been shown to be sensitive to EDTA and upon extraction to be polydisperse and neither transfer nor ribosomal (Rinaldi and Monroy, 1969).

This paper is concerned with RNA and protein synthesis in the surf clam *Spisula solidissima* and compares these results with those found for sea urchins. The formation and activity of polysomes before and after fertilization and through early development have been studied with regard to protein and RNA synthesis. It will be shown that unfertilized *Spisula solidissima* eggs have a small number of polysomes which are active in protein synthesis. After fertilization and through development a progressively increasing number of ribosomes become engaged in polysomes. By the 2-cell stage, newly synthesized RNA is associated with polysomes. This RNA is polydisperse, nonribosomal, and non-4 S. Amino acid incorporation occurs mainly on the heavy polysomes while during a short pulse the newly synthesized RNA is mainly associated with light polysomes. Actinomycin D inhibits the formation of approximately 30% of the polysomes that normally appear during early development.

The egg of the surf clam *Spisula solidissima* is shed as an oocyte with the germinal vesicle intact. Fertilization activates the maturation process. By approximately 10–15 minutes after fertilization the germinal vesicle breaks down and maturation is completed by 40 minutes. The first cleavage occurs approximately 30 minutes later. The second cleavage takes about 40 minutes, and the next few cleavages about 30 minutes each. The embryos hatch out of the fertilization membrane and begin swimming by 5 hours. By 7 hours the trochophore stage is reached.

MATERIALS AND METHODS

Excised ovaries from the surf clam *Spisula solidissima* were cut open and suspended in Millipore-filtered seawater. The eggs were separated from the surrounding tissue by filtration through several layers of fine mesh gauze. The eggs were next washed six times by contrifugation in a hand centrifuge. All procedures as well as culturing were done in Millipore-filtered seawater containing 50 μg/ml of sulfadiazine, 150 units/ml of penicillin, and 50 μg/ml of streptomycin

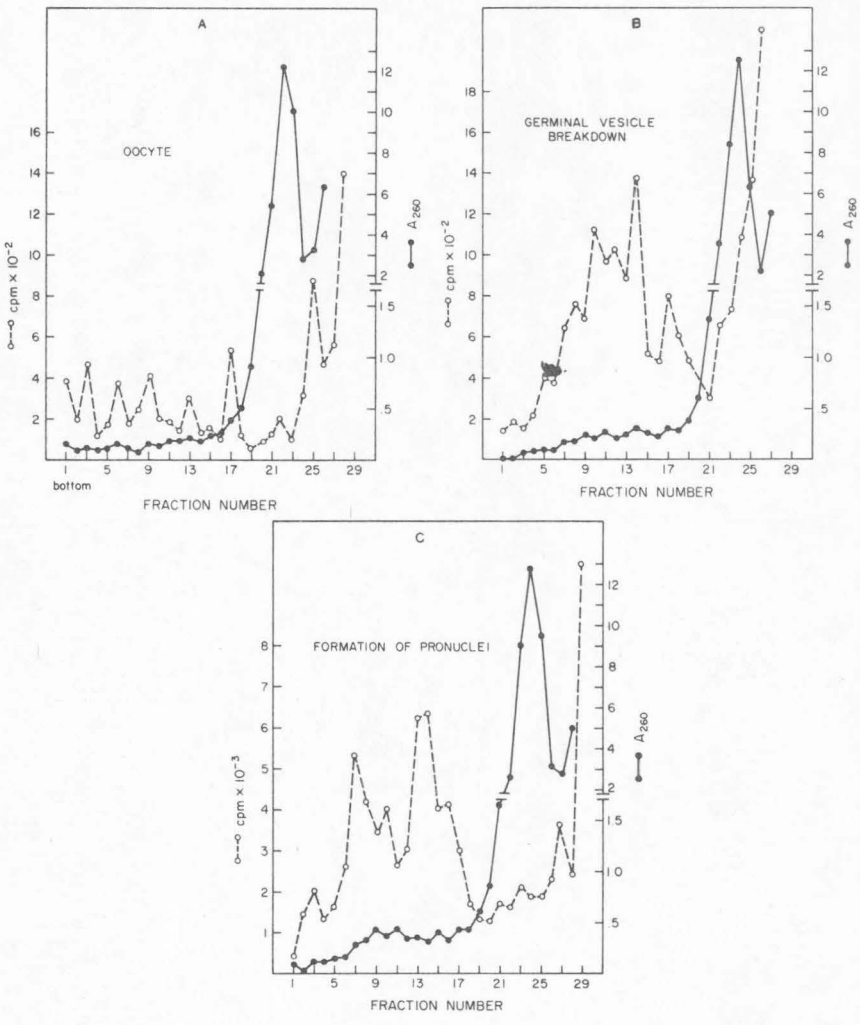


FIG. 1. a–c

FIG. 1. Sucrose gradient sedimentation profiles and leucine-^{3}H incorporation into polysomes at the various stages indicated in the figure. Embryos were incubated in a 7% culture (1 ml of packed embryos/14 ml of culturing medium) with 5 μC/ml of leucine-^{3}H. The oocytes and fertilized eggs immediately after germinal vesicle breakdown were pulsed for 20 minutes because of the low rate of amino acid uptake. Eggs labeled after pronuclei formation were pulsed for 5 minutes. All other stages were pulsed for 2 minutes. The 18-hour trochophores were incubated with 2 μC/ml of leucine-^{3}H. RNase incubation: Part of the low speed supernatant of the experiment shown in Fig. 1D (8-cell stage) was incubated for 10 minutes with 1.5 μg/ml of boiled RNase before layering on the sucrose gradient.

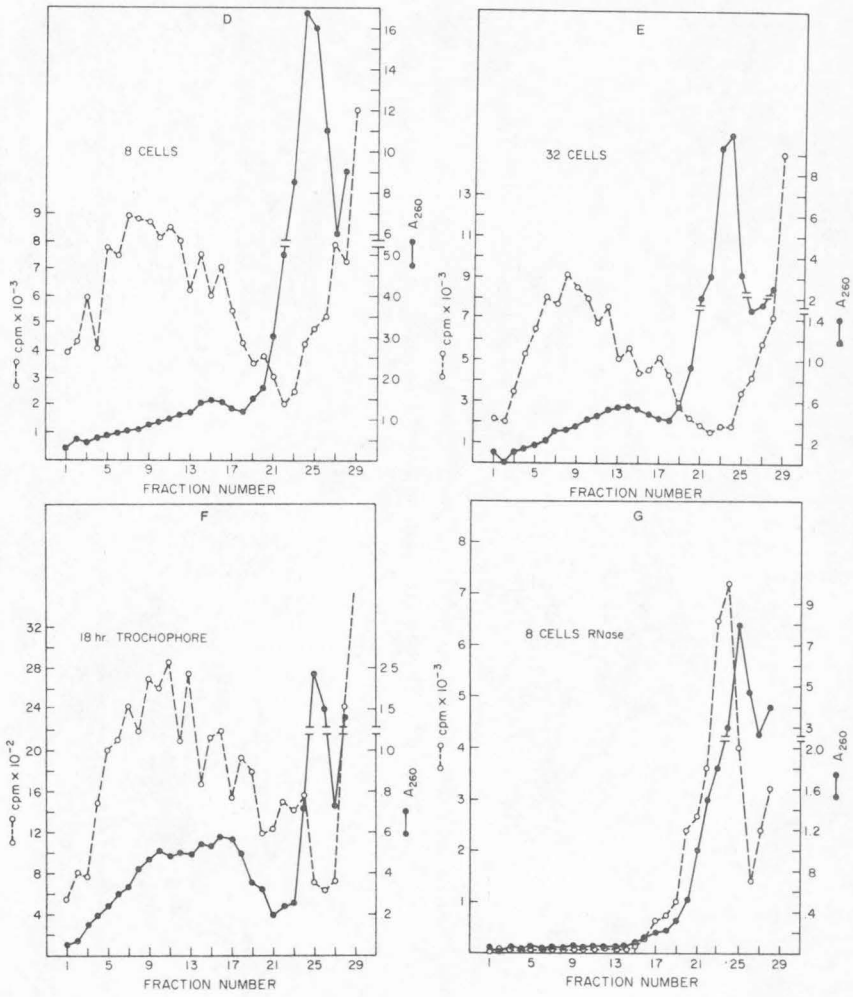


FIG. 1. d–g

(henceforth called antibiotic seawater). The eggs were suspended in 200 times the volume of antibiotic seawater and fertilized with 1 drop per 100 ml of egg suspension of a 5% sperm suspension. The sperm was collected dry by cutting excised testis and allowing the sperm to ooze out. Fertilizing eggs in a solution more concentrated than 1 ml of eggs per 100 ml of seawater resulted in a low efficiency of fertilization.

Labeling of eggs. The eggs were cultured until the proper stage of development, collected by settling or centrifugation, and then washed four times with antibiotic seawater. The eggs were then labeled for the specified time with either leucine-^{3}H (2 C/mmole, Schwartz BioResearch) or uridine-5-^{3}H (20 C/mmole, Schwartz BioResearch), and washed three times by centrifugation with cold antibiotic seawater and once with calcium- and magnesium-free artificial seawater (from the Marine Biological Laboratory). The conditions of incorporation of radioactive precursors are detailed in the legends of the figures. Actinomycin D was purchased from Mann Research Laboratories.

Polysome gradients. Homogenates and sucrose gradients were prepared similarly to the modification of the Cohen and Iverson (1967) technique described by Rinaldi and Monroy (1969). The eggs were suspended in 3–4 times their volume of ice-cold TKM buffer (0.06 *M* Tris, pH 7.7, 0.45 *M* KCl, and 0.019 *M* Mg acetate) containing 1.5 mg/ml of Macaloid and 0.5% Triton X-100. The detergent was found necessary for good homogenization in the isotonic buffer. Similar results were obtained with a combination of 0.5% DOC and 0.5% Tween 40, as well as 0.5% Nonidet P-40 (Shell Chemical Company).

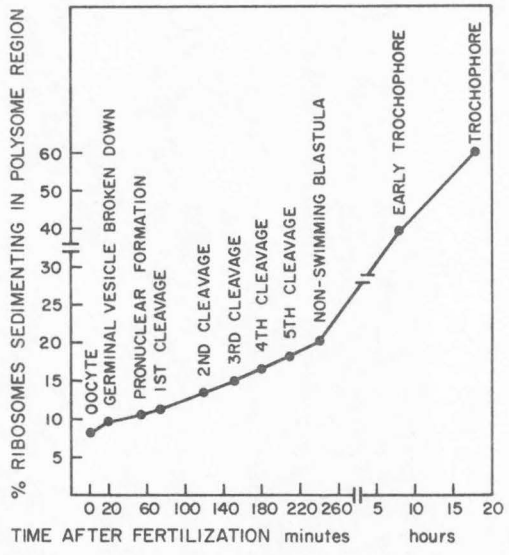


FIG. 2. Calculation of the percent of total ribosomes, as measured by A_{260}, sedimenting in the polysome region at different stages in development.

The eggs were homogenized using two up and down strokes of a loose-fitting Dounce homogenizer (Kontes Glass Co.). The homogenate was centrifuged at 12,000 *g* for 10 minutes to remove particulate material. Two milliliters of the supernatant was layered directly on 29 ml of a 17–50% linear sucrose gradient (TKM buffer) containing 1 ml of a 60% sucrose cushion. Ribonuclease-free sucrose was used (Schwartz BioResearch). The gradients were centrifuged at 24,000 rpm for 3.5 hours in a Spinco SW 25.1 rotor. One-milliliter fractions were collected, and absorption at 260 mμ (A_{260}) was determined with a Zeiss spectrophotometer. In some experiments the homogenates were first incubated for 15 minutes with 1.5 μg/ml of boiled RNase (5 times crystallized, Sigma Chemical Co.). Control experiments were done using mechanical breakage of the cells with 2 gentle strokes of a tight-fitting Dounce homogenizer and then treating the 12,000 *g* supernatant with 0.5% DOC. No change in the A_{260} of ^{3}H cpm profile of the polysomes results.

Measurement of radioactivity. The samples were precipitated with two volumes of ice-cold 12% TCA after the addition of 100 μg/ml of bovine serum albumin as a carrier. For amino acid incorporation studies the samples were allowed to stand 30 minutes in the cold and were then heated for 15 minutes at 92°C. The precipitate was collected on Millipore filters and washed with 20 ml of 5% TCA. For RNA incorporation studies, samples were precipitated as before but omitting the hot TCA step. They were then collected directly on Millipore filters and washed. RNA extraction from the polysome region was by the cold phenol method Gross *et al.* (1965). Bulk RNA of oocytes was used as carrier and as an optical density marker for sucrose gradients. The extracted RNA was centrifuged in a 5–20% sucrose gradient (0.1 *M* NaCl, 0.01 *M* Na acetate, pH 5.0) in a Spinco SW 25.1 centrifuge rotor at 24,000 rpm for 16 hours at 5°C.

RESULTS AND DISCUSSION

Preliminary experiments were done on the uptake and incorporation of leucine-^{3}H into unfertilized and fertilized eggs. The results confirm those reported by Bell and Reeder (1967) and will therefore be summarized only. Rate of amino acid uptake is the same in fertilized and unfertilized eggs until maturation is complete. As soon as the polar bodies are extruded, the rate of amino acid uptake by the fertilized egg increases 10- to 12-fold (Monroy and Tolis, 1964). Incorporation of amino acids into proteins by the unfertilized egg oc-

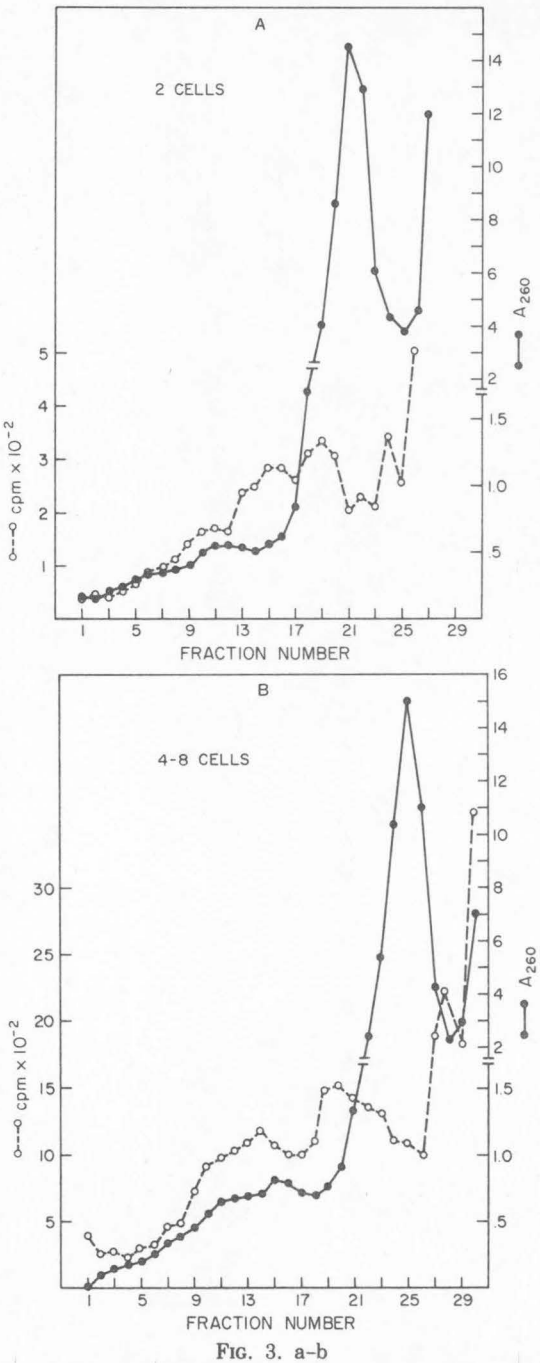


FIG. 3. a–b

FIG. 3. Sucrose gradient sedimentation analysis of uridine-^{3}H incorporation in polysomes at the various stages indicated. Embryos were incubated in a 7% culture for 30 minutes with 10 μC/ml of uridine-5-^{3}H (20 C/mmole).

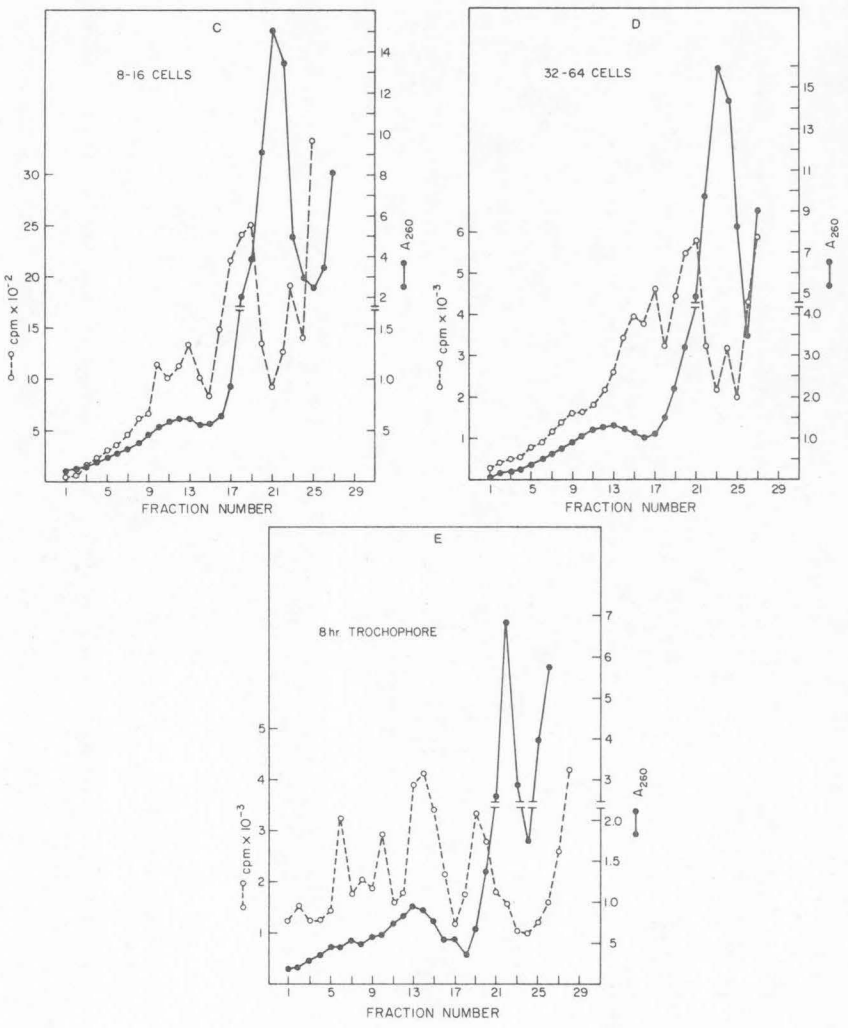


FIG. 3. c–e

curs at a low but measurable rate. Upon fertilization, a severalfold increase in incorporation becomes apparent when incorporation of amino acids into hot TCA-precipitable material is computed as percentage of label taken up by the egg. Rate of amino acid incorporation increases 5-fold upon completion of maturation, but this increase in rate is associated with the large increase in amino acid uptake. It

appears then that in this egg a major increase in rate of protein synthesis occurs at fertilization.

Further indication of a change in protein synthesizing activity during development is provided by the study of polysome profiles. Figure 1 shows the A_{260} and amino acid incorporation profiles for polysomes at several stages during early *Spisula* development. The oocyte (Fig. 1A) contains a small number of polysomes and exhibits a low but definite incorporation of amino acids evenly distributed through all polysome size classes. The considerable amount of label near the top of the gradient indicates that an appreciable amount of protein is synthesized and released during the labeling period. A small increase in the polysome:monosome ratio (calculated as percentage of total ribosomes, A_{260}, in the polysome area) is detected by 20 minutes after germinal vesicle breakdown (see Fig. 1B and Fig. 2). The percentage of ribosomes sedimenting as polysomes increases rapidly after fertilization (Fig. 2), and a concurrent increase of the radioactivity over the polysomes is observed. Thus, 30 minutes after fertilization the specific activity of the polysomes is 2.5 times greater than that of the unfertilized egg polysomes; and after formation of pronuclei (Fig. 1C) it is 4–5 times greater than that of polysomes before completion of maturation. Since, however, completion of maturation is accompanied by a marked increase in permeability (Monroy and Tolis, 1964) the interpretation of this latter observation is difficult.

Amino acid incorporation occurs principally on heavy polysomes

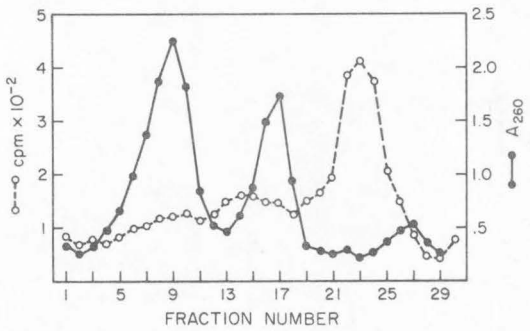


FIG. 4. Sucrose gradient sedimentation analysis of labeled RNA extracted from the polysomes of embryos at the 4-cell stage incubated for 35 minutes with 20 μC/ml of uridine-5-^{3}H. Total RNA extracted from oocytes was used as carrier and as a sedimentation marker.

throughout the 18 hours of development we have examined (Fig. 1D–F). In the early cleavage stages, polysomes of sedimentation coefficient 200–250 S increase in number more than do other classes. By the 32-cell stage, however, a slight shift in center of gravity toward heavier polysomes occurs. By the time the 18-hour (trochophore) stage is reached the mode of the polysomes has shifted still further toward the heavier region which predominates over that of the smaller polysomes. Figure 1G shows that a short treatment with RNase (1.5 μg/ml for 15 minutes) shifts practically all the optical densities from the polysome region toward the light polysomes and monosome region. The radioactivity is now present in a sharp peak slightly shifted ahead of the leading edge of the monosome peak. In the control (no RNase) experiments, it may be noted that little label appears in the monosomes. This indicates that little breakdown of polysomes has occurred during the preparation.

Figure 3 shows the incorporation of uridine-5-^{3}H into polysomes during a 30-minute pulse given at different stages. In eggs labeled at the time of the first cleavage, there is substantial incorporation of uridine into polysomes. As development proceeds, the amount of labeled RNA present in polysomes increases. One notes that in all the stages considered the radioactivity after a pulse of 30 minutes is present mainly in the lighter polysomes; some label is also present in the heavy polysomes. In the young (8-hour) trochophore (Fig. 3E), the uridine radioactivity is still mainly found in the lighter polysomes, although the amount of radioactivity on the heavier polysomes has increased. The radioactivity profile also indicates several peaks of radioactivity which on the whole coincide with the optical density profile of polysomes. A large peak is also present just ahead of the leading edge of the monosome peak. Even though ribosomal RNA synthesis has begun by this time in development (Firtel and Mirkes, unpublished) it is unlikely that during the short pulses used any appreciable labeling of ribosomal RNA in the cytoplasm would have occurred. Treatment with RNase shifts the uridine-^{3}H radioactivity to the heavy side of the monosomes and to the soluble region. To determine the classes of the newly synthesized RNA in or on the polysomes, RNA was extracted from polysomes of embryos which had been incubated at the 4-cell stage for 45 minutes with 15 μC/ml of uridine-5-^{3}H, RNA extracted from oocytes was used as carrier. Figure 4 presents a sucrose density gradient sedimentation profile of the extracted RNA. The labeled RNA is polydisperse and is

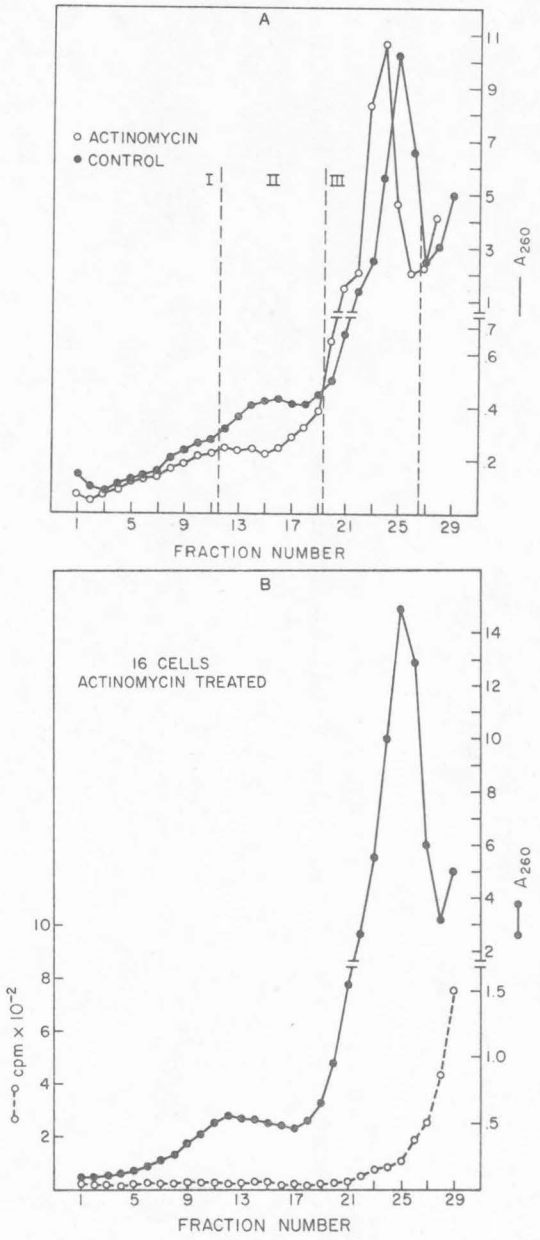


FIG. 5. The effects of actinomycin D treatment and RNA synthesis and polysome formation. (A) Experimental conditions are described in text. The results shown have been normalized so that both actinomycin D-treated and control embryos contain the same total A_{260} in the polysome and monosome regions combined. (B) Embryos were treated for 1 hour with 25 μg/ml of actinomycin D and labeled at the 16-cell stage with 10 μC/ml of uridine-5-^{3}H for 30 minutes.

neither 4 S nor ribosomal. The sedimentation coefficient of the main peak is approximately 11 S. An appreciable amount of heavy radioactive RNA is also present. The absence of radioactive peaks at 23 S and 16 S rules out bacterial contamination. These results are generally similar to those obtained by Rinaldi and Monroy (1969) for *Paracentrotus* at the 4–8-cell stage although *Spisula* appears to contain a larger amount of heavier species.

In another series of experiments the effects of actinomycin D on development and on RNA synthesis and polysome formation have been studied. Under the culture conditions used, embryos normally begin swimming at approximately 5 hours after fertilization. In concentrations of actinomycin D from 6–25 μg/ml embryos develop up to the swimming stage. They appear generally normal although they do not form cilia and do not swim. At actinomycin D concentrations of 2–3 μg/ml, approximately 5% of the embryos become swimmers; however, they do so at 6, rather than at 5, hours after fertilization.

Treatment of *Spisula* embryos with 12 μg/ml of actinomycin D for 45 minutes results in a reduction of 75% of uridine-^{3}H incorporated during a 20-minute pulse. Uridine uptake was not affected.

We have found that in the *Spisula* embryo actinomycin D exerts a differential effect on the various classes of polysomes. *Spisula* oocytes were fertilized and divided into two batches at the time of germinal vesicle breakdown. One batch was cultured in actinomycin D (25 μg/ml) while the other served as control. The embryos were cultured for 3.5 hours to the 16–32-cell stage, washed and labeled for 2 minutes with leucine-^{3}H. Polysome profiles were divided into 3 regions: I, heavy polysomes; II, light polysomes; III, monosomes. Figure 5A shows that the A_{260} profiles differ little in the region of the heavy polysomes whereas a marked difference is observed in the region of the light polysomes. This is clearly shown by the ratios of the A_{260} values of the actinomycin D to control polysomes in each one of the regions of the gradient. The ratios are: in region I, 0.91; in region II, 0.68; and in region III, 1.17. Thus actinomycin D causes a 9–10% reduction in the region of heavy polysomes but a 32% reduction in the light polysomes. There is an overall decrease of 26% in the total amount (A_{260}) of polysomes. There is also a small increase in the proportion of monosomes in the actinomycin-D treated preparation. The profile of amino acid incorporation (not shown) was similar for both the control and the experimental. Treatment with 25 μg/ml of actinomycin D caused an inhibition of over 85% in total uridine-^{3}H incorporation, and examination of sucrose gradients indicates no uri-

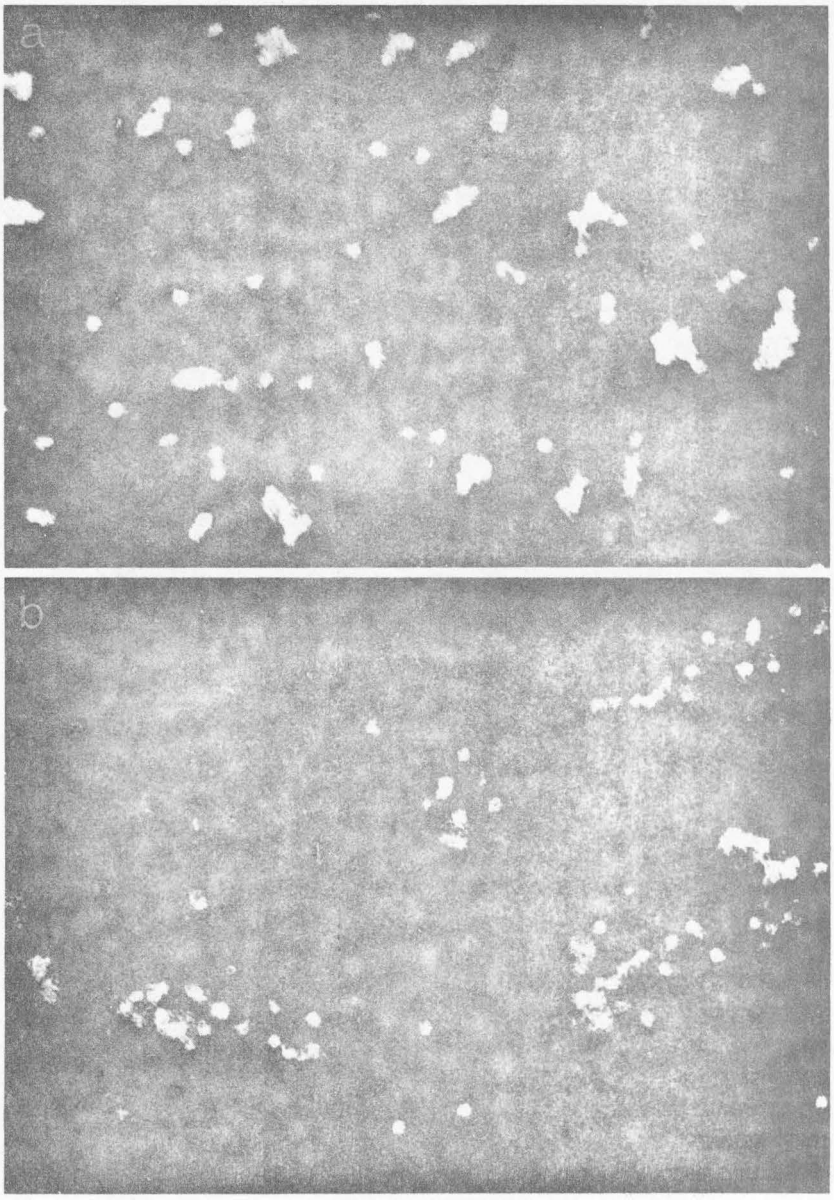


FIG. 6. a–b

FIG. 6. Electron micrographs of uranyl acetate-stained polysomes obtained from different fractions of the sucrose gradients: (a) fraction 13–15 (× 90,000); (b) fraction 8 (× 90,000); (c) fraction 4 (× 90,000); (d) fraction 2 (× 130,000).

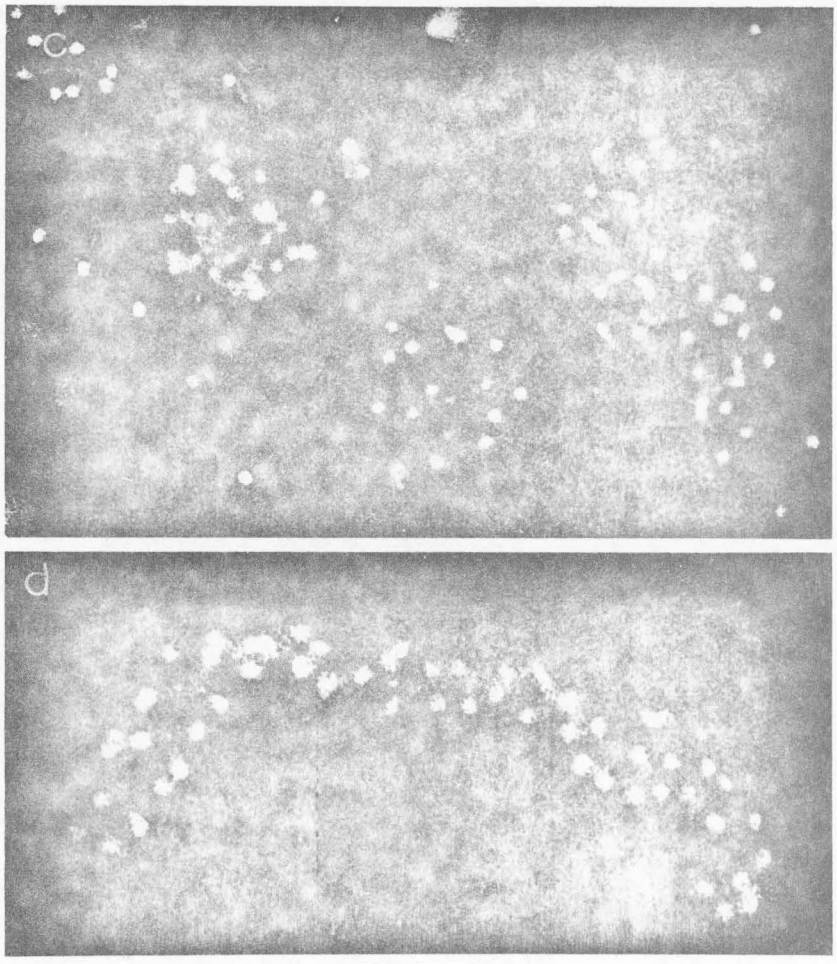


FIG. 6. c–d

dine-^{3}H incorporation into polysomes in actinomycin D-treated embryos (see Fig. 5B). In other experiments (not shown) it was found that when actinomycin D treatment was given between 2.5 and 4.5 hours after fertilization a more equal effect on light and heavy polysomes was observed (19% reduction of heavy polysomes and 21% reduction of light polysomes).

Electron micrographs of uranyl acetate stained polysomes reveal that polysomes of the main peak, fractions 13–15 (see Fig. 6A), contain 8–14 ribosomes. Polysomes of the heavy region (fraction 8, Fig.

6B) contain approximately 20 ribosomes. Polysomes of the very heavy region (fraction 4) contain over 30 ribosomes (Fig. 6C). Figure 6D shows a polysome taken from the heaviest region just above the cushion and which contains over 50 ribosomes. All polysomes in this region appear to have a minimum of 40 ribosomes. The polysomes appear somewhat helical and bent back on themselves.

CONCLUSION

The unfertilized egg of *Spisula* exhibits a low rate of protein synthesis and a small number of polysomes. After fertilization and as development proceeds, more and more ribosomes become engaged in polysome formation. Through the early swimming stages the quantity (A_{260}) of polysomes increases, and that of monosomes decreases. It is to be noted that no new ribosomes are formed until the trochophore stage (Firtel and Mirkes, unpublished). The increase in number of polysomes appears to be due both to the activation of stored maternal mRNA *and* to the formation of polysomes from newly synthesized mRNA. This is suggested by the observation that the suppression by actinomycin D of the polysome-associated RNA affects significantly the polysome profile. Our data show that especially in the early stages actinomycin affects mostly the class of the small polysomes. This together with the predominant accumulation of the uridine-^{3}H label on the light polysomes suggests that it is this class of polysomes that is predominantly formed on the newly synthesized RNA. In the early trochophore stage, a considerable amount of newly synthesized RNA is found on the heavier polysomes but the bulk is still to be found in the lighter polysome classes. Two explanations may be proposed. Spirin and Nemer (1965) and Infante and Nemer (1967) have suggested that in early sea urchin development the newly synthesized mRNA is stored in a class of inactive light polysomes. A second possibility, which is supported by recent findings on HeLa cells (Robbins and Borun, 1967) and sea urchin embryos (Nemer and Lindsay, 1969) is that the small polysomes are engaged in the synthesis of histones which must be particularly active during cleavage. The lower specific activity of amino acid incorporation by light polysomes may be due to the short chain length of the polypeptides on the ribosomes. Some caution has to be used in the interpretation of experiments showing uridine-^{3}H labeling on the polysomes; indeed the mere association of labeled RNA with polysomes is no proof that this is a message on polysomes (see also Plagemann, 1969). A possible source of misinterpretation of experiments of this

kind is leakage of nuclear RNA which would spuriously attach to the polysomes. Although our evidence on this problem is indirect, the following points may be cited. The first is that the same results were obtained either by the use of Triton X-100 or by homogenization with only a couple of strokes of the Dounce homogenizer (see page 92). Triton X-100 has also been shown to minimize nuclear leakage in L cells (Perry and Kelley, 1968). Further, even if nuclear RNA leakage should have occurred, the RNA-protein particles would not have sedimented mainly in particles greater than 100 S.

These observations support the view that some of the newly transcribed RNA is translated soon after it is synthesized. Whether this new RNA is transcribed by the same cistrons which are active during oogenesis [as has been indicated thus far in the sea urchin (Glišin *et al.*, 1966; Whiteley *et al.*, 1966)] or by different ones [as in *Xenopus* (Crippa and Gross, 1969)] is not known at this time. The data for *Spisula* do not indicate a class of inactive polysomes. The importance of early RNA synthesis and whether such synthesis is necessary for normal development through cleavage is unknown. Embryos appear to develop in a morphologically normal manner in actinomycin D. It is clear that they may not be biochemically normal since their development is arrested before the swimming and trochophore formation stages. The formation of trochophores in *Spisula* appears to be analogous to gastrulation in sea urchin development, and new RNA synthesis appears to be necessary for both of these major changes.

During the final preparation of this paper, the authors were able to examine a manuscript by Kedes and Gross (1969) on mRNA synthesis in early sea urchin development. They find that during a 30-minute pulse newly synthesized RNA is present mainly in light polysomes whereas after a 2-hour pulse the heavy and light polysomes were more uniformly labeled. According to the authors this may indicate a step that occurs at the level of the light polysomes and is necessary for heavy polysome formation. This newly synthesized RNA is polydiperse, non-4 S, and nonribosomal and the polysomes are sensitive to puromycin and EDTA treatment. Actinomycin D prevents the formation of approximately 40% of the polysomes present at the morula stage, and all classes of polysomes are affected. An analysis of the protein-synthesizing activity of the polysomes using the "tape theory" of Kuff and Roberts (1967) does not show a class of inactive polysomes.

SUMMARY

RNA and protein synthesis and polysome formation were studied during early development of *Spisula* embryos. The oocyte has a small number of polysomes and a low but measurable rate of protein synthesis (leucine-^{3}H incorporation). After fertilization, there is a continual increase in the percentage of ribosomes sedimenting in the polysome region. Newly synthesized RNA (uridine-5-^{3}H incorporation) was found in polysomes as early as the 2-cell stage. During cleavage, the newly formed RNA is associated mainly with the light polysomes.

RNA extracted from polysomes labeled at the 4-cell stage is polydisperse, nonribosomal, and non-4 S. Actinomycin D causes a reduction of about 30% of the polysomes formed between fertilization and the 16-cell stage.

In the early cleavage stages the light polysomes are mostly affected by actinomycin.

The authors are grateful to Dr. James Bonner and Mr. John Smart, California Institute of Technology, for critical reading of the manuscript. We would like to thank Mr. Philip Mirkes, University of Michigan, for assistance in some of the experiments.

REFERENCES

BELL, E., and REEDER, R. (1967). The effect of fertilization on protein synthesis in the egg of the surf clam, *Spisula solidissima*. *Biochim. Biophys. Acta* **142,** 500–511.

COHEN, G. H., and IVERSON, R. M. (1967). High-resolution density gradient analysis of sea urchin polysomes. *Biochem. Biophys. Res. Commun.* **29,** 349–355.

CRIPPA, M., and GROSS, P. R. (1969). Maternal and embryonic contributions to the functional messenger RNA of early development. *Proc. Natl. Acad. Sci. U.S.* **62,** 120–127.

GLISIN, V. R., GLIŠIN, M. V., and DOTY, P. (1966). The nature of messenger RNA in the early stages of sea urchin development. *Proc. Natl. Acad. Sci. U.S.* **56,** 285–289.

GROSS, P. R., and COUSINEAU, G. H. (1963). Effects of actinomycin D on macromolecular synthesis and early development in sea urchin eggs. *Biochem. Biophys. Res. Commun.* **10,** 321–326.

GROSS, P. R., MALKIN, L. I., and HUBBARD, M. (1965). Synthesis of RNA during oogenesis in the sea urchin. *J. Mol. Biol.* **13,** 463–481.

INFANTE, A. A., and NEMER, M. (1967). Accumulation of newly synthesized RNA templates in a unique class of polyribosomes during embryogenesis. *Proc. Natl. Acad. Sci. U.S.* **58,** 681–688.

KEDES, L. H., and GROSS, P. R. (1969). Synthesis and function of messenger RNA during early embryonic development. *J. Mol. Biol.* **42,** 559–576.

KUFF, E. L., and ROBERTS, N. E. (1967). *In vivo* labeling patterns of free polyribosomes: relationship to tape theory of messenger ribonucleic acid function. *J. Mol. Biol.* **26,** 211–225.

MONROY, A., and TOLIS, H. (1964). Uptake of radioactive glucose and amino acids and their utilization for incorporation into proteins during maturation and fertilization of the eggs of *Asterias forbesii* and *Spisula solidissima*. *Biol. Bull.* **126,** 456–466.

MONROY, A., and TYLER, A. (1963). Formation of active ribosomal aggregates (polysomes) upon fertilization and development of sea urchin eggs. *Arch. Biochem. Biophys.* **103,** 431–435.

NEMER, M., and LINDSAY, D. T. (1969). Evidence that the S-polysomes of early sea urchin embryos may be responsible for the synthesis of chromosomal histones. *Biochem. Biophys. Res. Commun.* **35,** 156–160.

PERRY, R. P., and KELLEY, D. E. (1968). Messenger RNA-protein complexes and newly snythesized ribosomal subunits: analysis of free particles and components of polyribosomes. *J. Mol. Biol.* **35,** 37–59.

PIATIGORSKY, J. (1967). Ph.D. Thesis. California Institute of Technology.

PIATIGORSKY, J. (1968). Ribonuclease and trypsin treatment of ribosomes and polyribosomes from sea urchin eggs. *Biochim. Biophys. Acta* **166,** 142–155.

PLAGEMANN, P. G. W. (1969). RNA synthesis in exponentially growing rat hepatoma cells. 1. A caution in equating pulse-labeled polyribosomal RNA with messenger RNA. *Biochim. Biophys. Acta* **183,** 46–56.

RINALDI, A. M., and MONROY, A. (1969). Polyribosome formation and RNA synthesis in the early post-fertilization stages of the sea urchin egg. *Develop. Biol.* **19,** 73–86.

ROBBINS, E., and BORUN, T. W. (1967). The cytoplasmic synthesis of histones in HeLa cells and its temporal relationship to DNA replication. *Proc. Natl. Acad. Sci. U.S.* **57,** 409–416.

SPIRIN, A. S., and NEMER, M. (1965). Messenger RNA in early sea urchin embryos; ctyoplasmic particles. *Science* **150,** 214–217.

TYLER, A., and TYLER, B. S. (1966). Physiology of fertilization and early development. *In* "Physiology of Echinodermata" (R. A. Boolotian, ed.), pp. 639–741. Wiley (Interscience), New York.

WHITELEY, A. H., MCCARTHY, B. J., and WHITELEY, H. R. (1966). Changing populations of messenger RNA during sea urchin development. *Proc. Natl. Acad. Sci. U.S.* **55,** 519–525.