MINOR COMPONENTS OF THE DNA OF *PHYSARUM POLYCEPHALUM*

Cellular Location and Metabolism

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ABSTRACT

DNA metabolism in the slime mold *Physarum polycephalum* was studied by centrifugation in CsCl of lysates of cultures labeled with radioactive thymidine at various times in the cell cycle. During the G_2 (premitotic) phase of the cell cycle, two components of the DNA are labeled. One component is lighter (buoyant density 1.686 g/cc) than the mean of the principal DNA (1.700 g/cc), and one is heavier (approximately 1.706 g/cc). The labeled light DNA was identified chemically by its denaturability, its susceptibility to DNase, and the recovery of its radioactivity in thymine. Cell fractionation studies showed that the heavy and the principal DNA components are located in the nucleus and that the light DNA is in the cytoplasm. The light DNA comprises approximately 10% of the DNA. About ⅓–½ of the light DNA is synthesized during the S period, and the remainder is synthesized throughout G_2 (there is no G_1 in *Physarum*). The light DNA is metabolically stable. A low, variable level of incorporation of radioactive thymidine into the principal, nuclear DNA component was observed during G_2.

The rates of DNA, RNA, and protein synthesis vary throughout the mitotic cycle in eucaryotic cells. Nuclear DNA synthesis is limited to a portion of the cycle, the "S phase" (Howard and Pelc, 1953), and both RNA and protein synthesis show minimal rates at the time of mitosis (Robbins and Scharff, 1966). A second minimum later in the cell cycle has been reported for *Physarum* (Mittermayer et al., 1964, 1966). Synthesis of certain enzymes has been shown to occur at particular times in the cycle (Gorman et al., 1964), and recent evidence confirms that the ratio of the rate of histone synthesis to the rate of DNA synthesis is remarkably constant throughout the cell cycle (Borun et al., 1967).

The discovery of cytoplasmic DNA has raised the question as to the extent to which the synthesis and function of this DNA are coupled to the events of the cell cycle. While information on this question can be provided by radioautographic studies, experiments with synchronously dividing systems have several advantages, one of them being the opportunity for a more complete characterization of macromolecules labeled by pulses of radioactivity.

We report here on the properties and metabolism of a cytoplasmic DNA from *Physarum polycephalum*. This acellular slime mold displays a natural precise synchrony in the nuclear division of each plasmodium (Howard, 1932). Mitosis is followed immediately by an S period of about 3 hr, and the succeeding G_2 period lasts 4–16 hr (Mittermayer et al., 1965; Nygaard et al., 1960). Methods have been developed by Rusch and collaborators

for growth of axenic cultures on a soluble medium. The mold can be maintained either in shaken flasks, in which case the culture as a whole is asynchronous, or on the surface of filter paper, in which case the culture is synchronous (Daniel and Rusch, 1961). Before the inception of the present experiments, Nygaard et al. (1960) showed that orotic acid is incorporated into acid-insoluble thymine residues during the G_2 portion of the cell cycle. In addition, Guttes and Guttes (1964) showed by radioautography that thymidine-^{3}H is incorporated into cytoplasmic bodies identified by light microscopy as mitochondria. The latter incorporation occurs at all times in the cell cycle, and the labeled material is RNase resistant and DNase sensitive. The continuously synthesized, cytoplasmic DNA described in the present work is likely to be identical with the material labeled during G_2 with orotic acid and to the mitochondrial DNA seen by radioautography.

In the course of the work, the existence of a heavy DNA component became apparent. This component is also synthesized during G_2 but is located in the nucleus.

MATERIALS AND METHODS

Culture Methods and Labeling

A culture line of *Physarum polycephalum* was initiated from axenic sclerotia (strain M3A) obtained from Dr. H. P. Rusch. Shaker cultures were maintained in 500-ml Erlenmeyer flasks containing 50 ml of a medium (Daniel et al., 1962) composed of 1% tryptone (Difco Laboratories, Detroit, Mich.), 1% glucose, 0.15% yeast extract (Difco Laboratories), 0.02 M citric acid, inorganic salts, 0.05% hematin (prepared from hemin chloride, Calbiochem, Los Angeles, Calif.), and KOH to pH 5.0. Surface cultures (Mittermayer et al., 1965) were prepared from shaker cultures which had a density of less than 0.5 ml of packed plasmodia/10 ml (this density is equivalent to about 2 mg dry weight/ml). Plasmodia were spun down, resuspended in twice their packed volume of the supernatant medium, and spread on filter paper (Schleicher and Schuell No. 595) supported by 3.5-mm glass beads in a 15 mm × 100 mm Petri dish. Approximately 14 ml of medium were added under the paper within a few minutes after spreading. Fusion was not inhibited by immediate addition of medium, as was evident both microscopically and from the excellent synchrony implied by the low level of nuclear label during G_2 (see Fig. 1). All cultures were grown in the dark at 22°C and had an interdivision time of approximately 12 hr. To determine the mitotic state of a surface culture, a small (about 1 mm^2) piece was smeared on a cover slip (Guttes et al., 1961), fixed in 95% ethanol (2 min), dehydrated in two changes of 100% ethanol (2 min each), and mounted in Euparal (Arthur H. Thomas Co., Philadelphia, Pa.). It is essential that the smear does not dry at any stage of preparation. The times given in relation to the time of the second metaphase (MII) after fusion are accurate to within 15 min.

Sterile solutions of thymidine-2-^{14}C (New England Nuclear Corp., Boston, Mass.) and thymidine-^{3}H (New England Nuclear Corp. and Schwarz Bio Research Inc., Orangeburg, N.J.) were diluted at least 20-fold with medium. Both general -^{3}H and methyl -^{3}H were used without noticeable difference in results. Surface cultures remained on the supporting filter paper during the labeling period. For label periods longer than 4 hr, the filter paper was supported by glass beads in the usual way, and 14 ml of labeled medium were added under the paper. For shorter label periods, excess unlabeled medium was first removed by placing the filter paper, mold up, on a piece of dry, sterile filter paper. The paper was then placed on a small volume of radioactive medium in a plastic Petri dish. To label 1 cm^2 of culture for 1 hr, 0.2 ml of labeled medium was used. "Fully labeled DNA" refers to DNA from a culture incubated for two or more doublings on labeled thymidine.

Preparation of Lysates without Cell Fractionation

Excess medium was removed as above, and the culture was placed on Parafilm M (American Can Company, New York). The mold was gently scraped off its paper with a spatula and transferred to an ice-cold Dounce homogenizer (Kontes Glass Co., Vineland, N.J.). 1 ml of cold lysis medium (0.05% sodium dodecyl sulfate, 0.025% sodium deoxycholate, 0.15 M NaCl, 0.01 M EDTA, 0.1 M Tris, adjusted to pH 7.4 with HCl) was added for each 4 cm^2 of mold. The tight fitting ("B") pestle was pressed down six times, and the resulting nearly clear lysate was stored frozen. Except where stated otherwise, such a lysate was used for CsCl fractionation without further treatment. In a few cases, lysates were deproteinized by shaking four times with chloroform-isoamyl alcohol (Sevag et al., 1938). The cold lysis medium contained a precipitate (presumably sodium dodecyl sulfate) that was suspended evenly and otherwise ignored. The precipitate was not present in the CsCl gradients which were run at 20°C. Lysates contained approximately 2 mg/ml of protein (Lowry et al., 1951) and 20 μg/ml of DNA (Burton, 1956).

Cell Fractionation

Nuclear and cytoplasmic fractions of a surface culture were prepared by a modification of procedures

described by Mohberg and Rusch (1964). Excess medium was removed, and the mold was scraped from the paper as described above. Homogenization also proceeded as above, except lysis medium was replaced with 1 ml of isolation medium (0.25 M sucrose, 0.01 M $CaCl_2$, 0.05 M Tris, 0.1% Nonidet P40 from Shell Chemical Limited of Australia, pH 7.2 with HCl) for each 3 cm^2 of surface culture. The homogenate was centrifuged at 1900 *g* for 10 min, yielding the nuclear (precipitate) and cytoplasmic (supernatant) fractions. This centrifugation was sometimes preceded by a very low speed centrifugation (30 *g*, 5 min) to remove clumps. The fractions were mixed with an equal volume of double-concentration lysis medium before storage or CsCl fractionation.

CsCl Fractionation

In a typical CsCl run, each tube contained the following components: lysate diluted to 0.55 ml with lysis medium, 1.923 g of CsCl (American Potash & Chemical Corp., Los Angeles, Calif.; Rb as Rb_2O = 0.115%; not optical grade), and 0.88 ml of 0.1 M Tris, pH 7.5, with 0.1 M EDTA. The final volume was approximately 2.0 ml. Centrifugation was done at 35,000 rpm for 25 hr at 20°C in an SW39 rotor in either the Spinco Model L (set at 48°F) or Model L2 (set at 17.4°C). DNA bands were no narrower when centrifugation time was extended to 41 hr total. In the experiments carried out to determine density by refractive index, the Model L2 centrifuge was used. Two drop (approximately 60 μl) fractions were collected into tubes or on filter paper discs (21 mm, Schleicher and Schuell #895E or Whatman 3MM). Whenever the last (top) fraction collected from a gradient is included in a figure, it is marked "T."

Acid Washing and Counting

Dried filter paper discs from one to three CsCl gradients were washed at 0°C in the same beaker (Bollum, 1959) with approximately 400 ml of 5% trichloroacetic acid containing 0.5% thymidine (15 min), twice with 5% trichloroacetic acid (300 ml, 10 min each), and twice with 100% ethanol (100 ml, 2 min each). The discs were dried again, and each was placed flat on the bottom of a counting vial containing 5 ml of a scintillation fluid containing 5 g of 2,5-diphenyl-oxazole, 0.25 g of 1,4-bis-2(5-phenyloxazolyl) benzene per liter of toluene. The ^3H counting efficiency in Fig. 1 was 13.9%, which was determined by counting a sample of purified DNA-^3H from *Escherichia coli* under the same conditions. When 50 μl of a crude unlabeled lysate (approximately 100 μg of protein) was included with the *E. coli* DNA, the counting efficiency was 9.5% before acid washing and 11.1% after acid washing.

Determination of Refractive Index from Gradients

Gradient fractions for density determination were collected directly into about 0.5 ml of mineral oil. A sample of about 35 μl of the fraction was later removed with a 50 μl capillary pipette, and its refractive index was immediately determined at 25°C in a Bausch and Lomb Abbé refractometer. A second measurement on the same sample yielded a value within 0.001 g/cc (the readability of the instrument) of the first measurement.

Specific Activity of Acid-Insoluble Material

To measure the acid-insoluble, alkali-stable radioactivity per microgram of protein, approximately 200 μl of a lysate were made 0.5 N in NaOH and 1 ml in volume. The alkaline solution was incubated for 2 hr at 37°C, 3 ml of cold 10% trichloroacetic acid were added, and after 15 min the precipitate was collected by centrifugation at 10,000 *g* for 10 min. The precipitate was washed twice by centrifugation with 5% trichloroacetic acid and then redissolved in 1.0 ml of 0.5 NaOH. 100 μl of the solution were used for protein determination (Lowry et al., 1951), and 100 μl were mixed with 1 ml of NCS (Nuclear Chicago Corp., Des Plaines, Ill.) reagent and counted with 20 ml of toluene scintillator fluid. The acid wash removed material which was present in the growth medium and which gave color on the protein assay. Lower centrifugation speeds gave erratic recoveries.

DNase Digestion

Fractions from CsCl were diluted 40-fold with 0.02 M $MgCl_2$, 0.01 M K-PO_4, pH 6.5. Electrophoretically purified DNase (Worthington Biochemical Corp., Freehold, N.J.) was added to a level of 25 μg/ml, and samples were removed for precipitation with 10 μg of bovine serum albumin and trichloroacetic acid (final concentration 5%). The precipitates were collected on Millipore filters (0.45 μ), dried, and counted in toluene scintillator fluid.

RESULTS

Synthesis of DNA during G_2

In order to characterize DNA synthesized during G_2, synchronous cultures were labeled, lysed, and fractionated on CsCl gradients. The ^3H on the gradient shown in Fig. 1 was derived from a surface culture labeled with thymidine-^3H during late G_2. A ^{14}C marker, derived from a culture labeled for approximately 2.5 doublings with thymidine-^{14}C, was banded in the same tube. The main feature of the ^3H profile is a sharp peak lo-

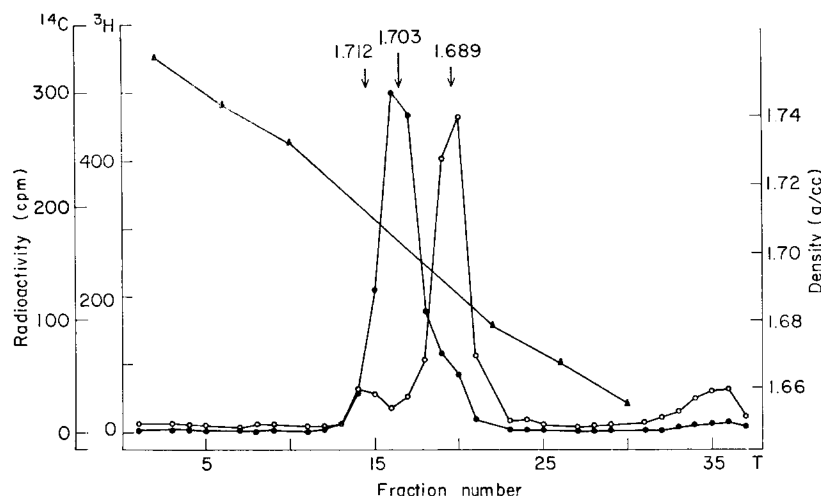

FIGURE 1 Labeling of DNA during G_2. The CsCl gradient contained 50 μl of a lysate of a culture labeled with 50 μc/ml of thymidine-^3H (12.5 c/mmole before addition to culture medium) for a 1.8 hr period ending 1 hr before M II, and 20 μl of a lysate of a culture labeled with 0.8 μc/ml of thymidine-^{14}C (30 mc/mmole before addition) for 33 hr. Two drop fractions were used either for counting on paper or for refractive index determination. Densities given in the figure were averaged with others to give the values in Table II. —▲—, density determined by refractive index; —●—, ^{14}C; —○—, ^3H.

cated at a lower density than the marker DNA. Additional smaller peaks are seen at a density slightly higher than the marker and at the top of the gradient. The peak at the top of the gradient is not DNA (see below). We conclude that there are two minor DNA components in *Physarum*, one lighter and one heavier than the mean of the principal DNA, and that the minor components are preferentially synthesized during the G_2 portion of the cell cycle. The remainder of this section is concerned with the elimination of alternative interpretations.

The very low activity in the principal DNA component (Fig. 1) could have arisen as an artifact of counting. Self-absorption on the filters by the precipitated principal DNA component could result in a lowered ^3H counting efficiency in the principal peak region (Carr and Rosenkranz, 1966). That this is not the case is shown by our finding that ^3H and ^{14}C profiles coincide when they arise from cultures labeled in the same way. We have also shown directly that the ^3H counting efficiency is essentially constant through the DNA region by first counting a set of papers in the usual way and then eluting the radioactivity from each paper and counting the eluant in a solution at known efficiency.

Labeling of DNA components during G_2 might also be explained by the presence of a contaminating organism in the cultures (Green et al., 1967), either in the culture media or in association with the plasmodia. Evidence against possible contamination of the medium is presented in Table I. The lysate of an unlabeled culture was mixed with (radioactive) medium of a labeled culture, and the level of acid-insoluble radioactivity was measured. The observed level was 2.6–2.9% of an equivalent amount of the culture incubated with thymidine-^3H. The level may be taken as an upper limit because the quantity of labeled medium added is more than the amount carried with a labeled culture. We conclude that the light DNA does not arise from a contaminant in the medium. Because the control lysate contained some acid-insoluble radioactivity, it was fractionated on CsCl. Even though the labeled culture *A* in Table I gave the heavy component on a CsCl gradient, the corresponding control gradient from culture *B* gave only a low level of radioactivity everywhere without any detectable peaks. Therefore, the heavy component does not arise from a contaminant in the medium.

The consistent finding of the labeled minor components could still be explained by the presence of a contaminating organism which grows only in contact with the mold and which grows

TABLE I

Level of Acid-Insoluble Radioactivity from Radioactive, Incubated Medium Plus Plasmodium

	Homogenized in lysis medium		Acid-insoluble ^3H in lysate	Control lysate
	Plasmodium	Radioactive culture medium		
			cpm	*% experimental*
A	15 cm² labeled during G2		30,432	
B	15 cm² not labeled	0.4 ml from *A*	884	2.9
C	12 cm² labeled during S		210,300	
D	12 cm² not labeled	0.5 ml from *C*	5,410	2.6

Cultures were labeled by incubating with 25 μc/ml of thymidine-^{3}H for a 3 hr and 10 min period terminating at prophase II (culture *A*) or for 3 hr and 24 min starting at M II (culture *C*). Lysates of the labeled cultures were made in the usual way. The unlabeled cultures (*B* and *D*) were also lysed in the usual way, except that culture medium as shown in the table was included with the lysis medium. Acid washing was carried out on paper. Blank papers which were acid washed with the samples did not pick up radioactivity.

with approximately the same doubling time as the plasmodia. However, the putative organism could not be capable of independent growth under a variety of conditions. Samples of the mold and culture medium showed no growth of any contaminating organism at 20°C and 37°C on thioglycollate sterility-testing broth, tryptone agar, heart-brain infusion agar, or tryptone-yeast extract agar. Absence of growth of such organisms was probably not due to inhibition by the mold or its products, because *E. coli* B, *Diplococcus pneumonii*, and various unidentified bacteria and yeasts grew vigorously when added to cultures.

Since the DNA was not purified, the peaks of the minor components could represent radioactivity in non-DNA materials such as polysaccharides. The principal and light DNA radioactivity were rendered 90–95% acid-soluble by DNase but not by RNase at the same concentration. (The RNase was prepared according to Marmur (1961); no $MgCl_2$ was added in the digestion.) The radioactivity is in thymine after acid hydrolysis (see Fig. 2 and below), and the labeled material increases in buoyant density on heat treatment followed by rapid cooling (see Results, Denaturation of the Light DNA). We conclude that the radioactivity is incorporated into DNA.

When large amounts (about 0.3 ml or more) of a crude lysate were centrifuged in CsCl, a band of opalescent material was seen close to the DNA position (see also Brunk and Hanawalt, 1966). The opalescent material is presumed to be polysaccharide, since analysis of the gradient fractions in the region of the DNA peak reveals a peak of sugar-containing material (Fig. 3 *a*). When a lysate was pretreated with α-amylase (Fig. 3 *b*), the polysaccharide peak was diminished, the sugar background rose, and the visible band was absent. The banding pattern of a preparation of DNA was the same whether amylase treatment was performed or not (Fig. 3 *a* and *b*). We conclude that the presence of the polysaccharide on the gradients described in this paper does not influence the radioactivity patterns observed.

The chemical nature of the ^{3}H-labeled peak at the top of the gradient was determined by acid hydrolysis and paper chromatography (Fig. 2). The radioactive product had an R_f of zero, which differs from that of the five common bases in the system used (Brown and Holt, 1967). The DNA-containing fractions, when hydrolyzed and chromatographed under the same conditions, gave a single radioactive peak with the R_f of thymine. The top peak radioactivity is present in both nuclear and cytoplasmic fractions. It varies in amount from about 2 to 10% of the total acid-insoluble radioactivity. In highly labeled cultures from long incubations, the radioactivity in the top peak represents a smaller fraction of the total radioactivity than in pulse-labeled cultures.

Density of Minor DNA Components

In this and following sections we report on studies which utilize preparations that are pref-

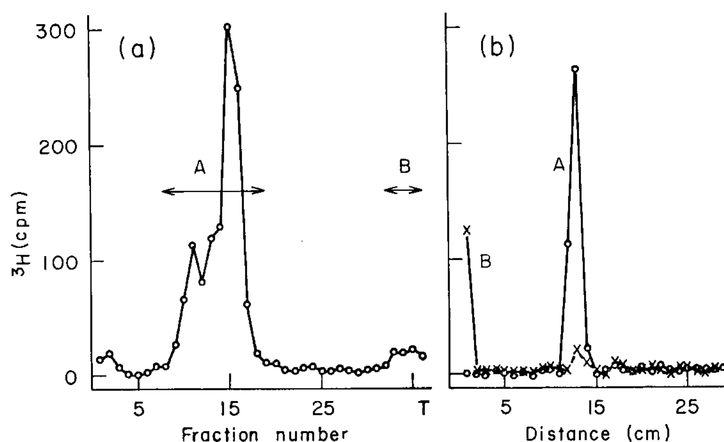

FIGURE 2 Chromatography of hydrolyzed peak fractions and top fractions. A crude lysate prepared from a culture labeled for a 64 min period beginning 6 hr after M II was centrifuged in CsCl. Each fraction was diluted with 150 μl of H_2O, 20-μl samples were taken for counting on papers (part *a* of graph). 5-μl aliquots from each of the diluted fractions shown by *A* were combined, and 25-μl aliquots from each of the diluted fractions shown by *B* were combined. The combined fractions were mixed with unlabeled homogenate as carrier, dissolved in alkali, precipitated with trichloroacetic acid, and acid washed by centrifugation. The acid precipitate was dissolved in 98–100% formic acid, hydrolyzed (170°C, 2 hr), and chromatographed on ion exchange paper (Brown and Holt, 1967). Strips 1 cm wide were cut out and counted in the toluene-based scintillator fluid (part *b* of graph). The R_f of thymine in this system is 0.44. (*a*) —O— ^{3}H; (*b*) —O—, ^{3}H from *A* region of gradient; —X—, ^{3}H from *B* region of gradient.

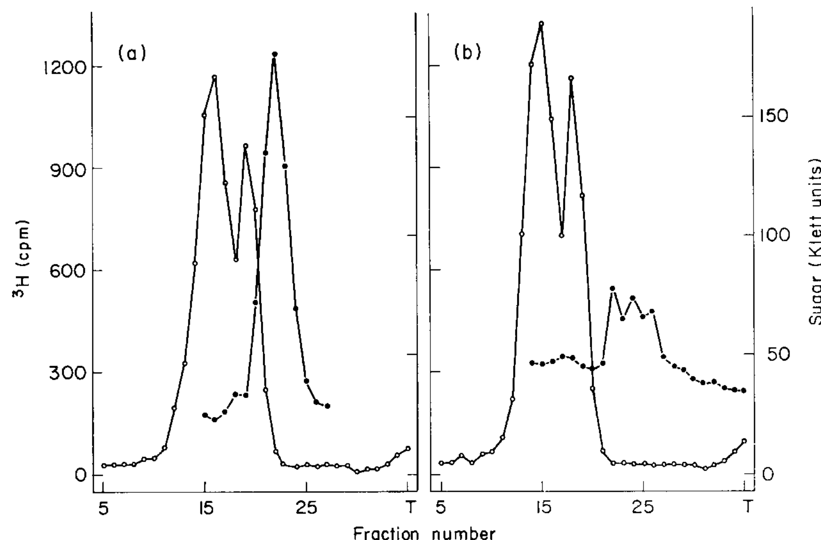

FIGURE 3 Effect of treating lysate with α-amylase. A lysate was prepared from a culture labeled with ^{3}H for 4.3 hr beginning 6 hr after M II. This culture is unusual because of the high proportion of ^{3}H in the principal DNA position. Two identical samples were prepared, each containing CsCl, Tris-EDTA and 750 μl of lysate. To one sample (*b*) 700 μg of α-amylase (from *Bacillus subtilis*, Sigma Chemical Co., St. Louis, Mo.) were added, and then both samples were incubated for 1 hr at 20°C. After centrifugation, fractions were collected in tubes, diluted with 200 μl of water each, and analyzed for radioactivity (10 μl of diluted fraction) or carbohydrate (270 μl). Sugar was estimated by the phenol-sulfuric acid method according to Smith and Montgomery (1956), except that the volumes were halved. Color was determined in a Klett colorimeter with No. 50 filter. —O—, DNA-^{3}H; —●—, polysaccharide; *a*, untreated lysate; *b*, α-amylase-treated lysate.

erentially labeled in either the light or the principal component. As described above, preparations labeled during G_2 usually have most of the radioactivity in the light DNA. Preparations labeled during S have nearly all of the radioactivity in the principal component (see below and Figs. 5 *d* and 6 *c*).

Buoyant densities were estimated by measuring the refractive indices of selected fractions from CsCl gradients (Fig. 1). When DNA-^{14}C from phage T7 was banded under the conditions (0.04 M NaCl, 0.007 M EDTA, 0.07 M Tris) used here, it gave a density of 1.703 g/cc when the usual formula (Weigle et al., 1959) for conversion of density at 20°C to refractive index at 25°C was used. Since T7 DNA has a density of 1.710 g/cc on the usual scale (Schildkraut et al., 1962), all densities reported here were calculated from the values given by the published formula plus 0.007 g/cc. For density measurements, the volume of lysate run on the gradient was less than 50 μl. Large amounts gave higher and less reproducible densities. The densities are reported in Table II along with values obtained by other workers. Only the values reported for the heavy component show substantial variation, presumably because of the presence of the principal DNA component in the preparations. The GC contents, on the basis of density, assuming no unusual bases, are 41% for the principal component and 27% for the light component (Schildkraut et al. 1962).

TABLE II

Buoyant Densities

DNA component	Densities			
	Present work	Braun et al. (1965)	Evans (1966)	Guttes et al. (1967)
	g/cc	*g/cc*	*g/cc*	*g/cc*
Heavy	1.706 ± 0.006	1.714		1.701
Principal	1.700 ± 0.002	1.703	1.700	1.697 ± 0.002
Light	1.686 ± 0.002		1.686	1.686 ± 0.002

The values obtained in the present work are averages of 4, 8, or 6 determinations, from heavy to light respectively, and the errors are 95% confidence limits.

Denaturation of the Light DNA

A crude preparation of G_2-labeled DNA was heated, rapidly cooled, and fractionated in a CsCl gradient with untreated, fully labeled DNA-^{14}C as a marker. The ^3H peak coincided with the marker DNA peak (Fig. 4 *b*), indicating that the light DNA component had increased in density by 0.014 g/cc and hence was double stranded before heating. When S-labeled DNA was denatured under the same conditions, only a small fraction of the radioactivity remained at the native principal position. Thus, the peak of ^3H in Fig. 4 *b* is due primarily or exclusively to the denatured light DNA component and not to the principal DNA which was partially labeled in the preparation (Fig. 4 *a*).

The profile of Fig. 4 *b* shows a shoulder on the heavy side of the peak, as expected from the presence oi labeled principal DNA in the preparation. The profile also shows a fraction of the radioactivity at densities much greater than that expected for denatured DNA. When the very dense material was treated with T1 ribonuclease and centrifuged again, it banded at or near the position expected for the denatured component. Thus, the very dense material appears to be an RNase-susceptible complex of RNA and DNA (Kubinski et al., 1966).

Cellular Location of the Minor DNA Components

Nuclei and cytoplasm were separated by the procedure described under Materials and Methods. When nuclei were suspended in the isolation medium, they maintained the same structure seen in ethanol-fixed smears of the mold. We note that in 0.25 M sucrose alone the nuclear structure as seen by phase-contrast microscopy breaks down over a period of minutes. The perinuclear chromatin and the nucleolus disappear, leaving finally an apparently empty spherical envelope of the same diameter as the original nucleus.

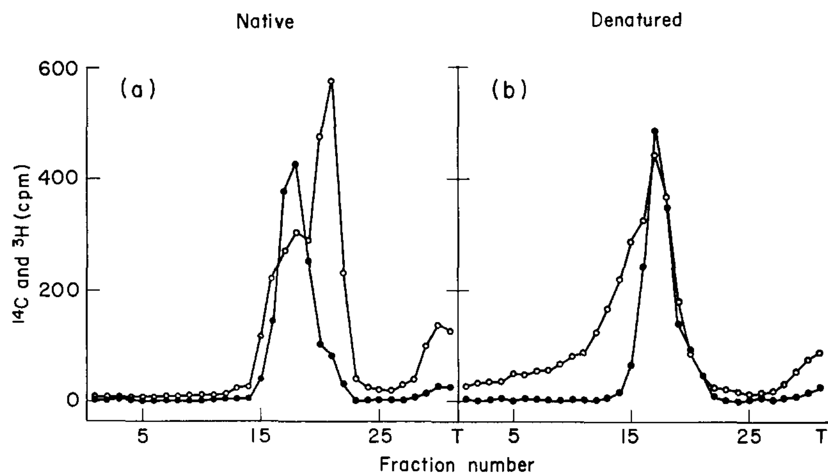

FIGURE 4 Denaturation of G_2-labeled DNA. *a*, The CsCl gradient contained 0.05 ml of a lysate from a culture fully labeled with ^{14}C and 0.3 ml of a lysate from a culture labeled with ^{3}H for a 3 hr period beginning 3.5 hr after M II (early G_2 period). *b*, The CsCl gradient was the same as above except that the ^{3}H lysate was heated to 100°C for 10 min and cooled in ice prior to mixing with ^{14}C lysate and CsCl. —●—, ^{14}C; —○—, ^{3}H.

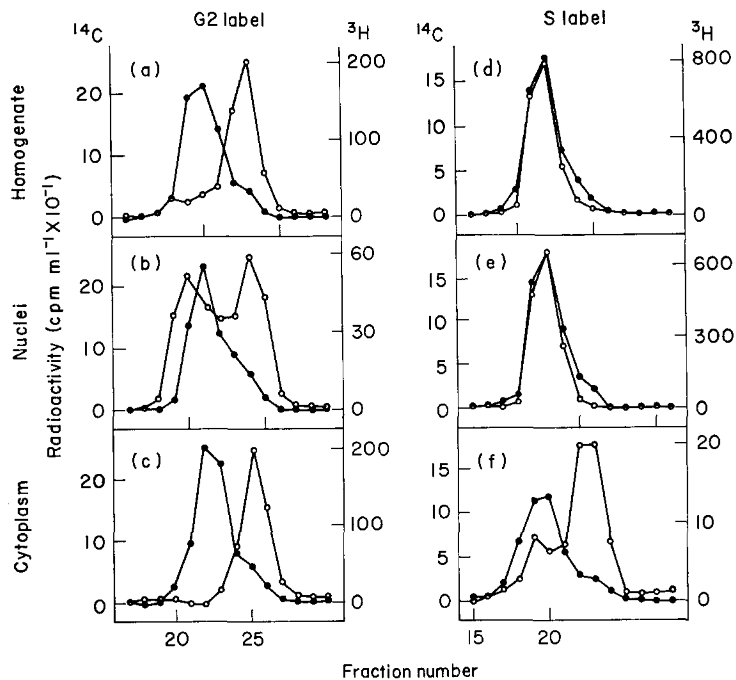

FIGURE 5 CsCl patterns of DNA from cell fractions. *a–c*, A culture was labeled with 75 μc/ml thymidine-^{3}H (2 c/mmole) for a 3 hr interval starting 4.3 hr after M II. The culture was fractionated into nuclei and cytoplasm, and lysates of the fractions were run on CsCl. *d–f*, The same as above, except that the culture was labeled with 25 μc/ ml of thymidine-^{3}H (2 c/mmole) for a 3 hr interval during the S period following M II. Gradient *a* contained 0.2 ml of the G_2-labeled homogenate, and *d* contained 0.1 ml of the S-labeled homogenate. —●—, ^{14}C derived from a lysate of a fully labeled culture and added separately to each gradient; —○—, ^{3}H.

Fig. 5 describes CsCl analysis of labeled DNA in the nuclear and cytoplasmic cell fractions produced by cell fractionation. The left hand column (*a–c*) shows the analysis of a G_2-labeled culture, and the right hand column (*d–f*) shows the analysis of an S-labeled culture.

The DNA of the cytoplasm (Fig. 5 *c* and *f*) is primarily the light DNA. There is no detectable heavy component; if 10% of the heavy DNA had been in the cytoplasmic fraction, it would have been detected (Fig. 5 *c*). Nuclear DNA can be detected in the cytoplasmic fraction after an S pulse (Fig. 5 *f*). However, this DNA represents less than 1% of the total nuclear DNA seen in the whole homogenate (Fig. 5 *d*). The simplest conclusion is that the cytoplasm contains only light DNA and that breakage of a small number of nuclei accounts for the low level of nuclear DNA.

The nuclear fraction contains essentially all of the heavy DNA (Fig. 5 *b*), all of the principal DNA components (Figs. 5 *d* and 5 *e*), as expected, and about 30% of the light DNA which may be trapped outside the nuclei. It may be possible to free the nuclear fraction of the light DNA component by washing it with isolation medium or by modifying the preliminary very low speed centrifugation.

The very low speed sediment (see Materials and Methods) contained between 5 and 30% of the total acid-insoluble radioactivity. The fraction was 6% (*a–c*) and 8% (*d–f*) in the two fractionations described in Fig. 5. The CsCl profile of the very low speed sediment was similar to that of the whole homogenate, indicating that the sediment arises from incompletely homogenized plasmodium.

Amount of the Light DNA

The fraction of total DNA represented by light DNA was estimated by analyzing the ^{14}C distribution of fully labeled DNA on CsCl gradients. We assume for this purpose that the thymidine-^{14}C specific activity is uniform among the DNA species.

The CsCl profile of fully labeled DNA shows a distinct shoulder on the light side (Fig. 6 *a*), presumably reflecting the presence of the light com-

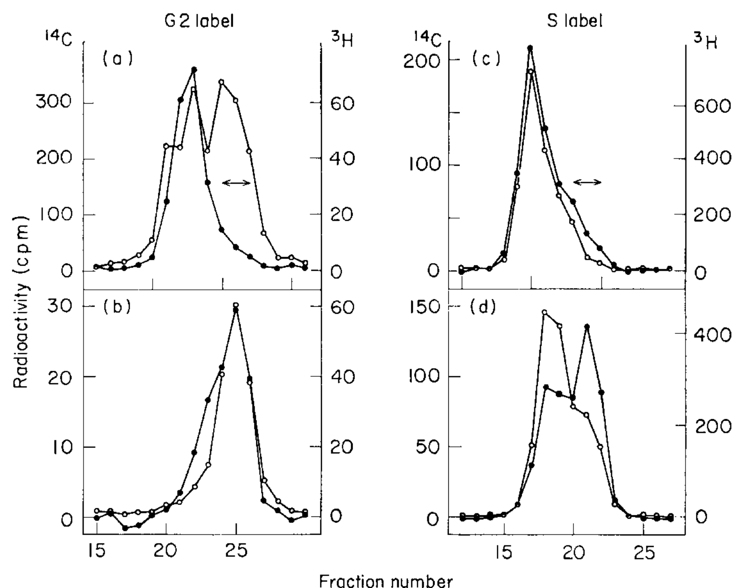

FIGURE 6 Recentrifugation of light region. Gradient *a* contained 0.4 ml of lysate from a culture labeled with thymidine-^3H for 3 hr beginning 3.5 hr after M II, and 0.22 ml lysate from a culture labeled for 33 hr with thymidine-^{14}C. Gradient fractions were diluted to 300 μl each. 40 μl of each diluted fraction were acid washed on paper and counted. Gradient *b* contained 200 μl of each of diluted fractions number 20, 21, and 22 (shown as ↔) from gradient *a*. Fractions from *b* were diluted to 120 μl, and 40 μl samples were acid washed and counted. Gradient *c* contained 0.3 ml of lysate of a culture labeled with thymidine-^3H for 3.5 hr during the S period, and 0.2 ml of the ^{14}C lysate described above. Gradient fractions were diluted and counted as in *a*. Gradient *d* contained 200 μl of each of the diluted fractions number 23, 24, and 25 (shown as ↔) in *c* and was collected directly onto filter papers for counting. —●—, ^{14}C; —○—, ^3H.

ponent. On rerunning the light region (Fig. 6 *b*), one finds that the bulk of the DNA-^{14}C in the region does reband with the ^{3}H-labeled light DNA which serves as a marker. Thus, the ^{14}C in the light region of the first gradient (Fig. 6 *a*) does approximate the amount of light DNA-^{14}C present on the gradient. The fractions of DNA-^{14}C in the light region were 12% (Fig. 6) and 11% for two preparations of fully labeled DNA. These values must be corrected for the difference in thymine content between the principal and light components. Assuming the per cent GC predicted from buoyant densities, the correction factor is 59/73. We estimate, therefore, that 9% of the DNA of *Physarum* is located in the cytoplasm.

An additional consideration is the possibility of unequal losses of principal and light DNA's. Losses were estimated by comparing the total acid-insoluble radioactivity applied to gradients with that recovered in the DNA peaks. The average recovery of fully labeled DNA-^{14}C was 90%, indicating that the principal component is recovered almost quantitatively. However, the ^{3}H recovery for G_2-labeled material was roughly half that for comparable S-labeled material. Thus, we have recovered at least half of the cytoplasmic DNA. Further experiments will be required to decide whether the lost radioactivity represents DNA trapped in the protein which forms a layer on top of the CsCl gradients, or whether it represents non-DNA material.

Time of Synthesis of DNA Components

The light DNA is synthesized continuously. Exposure of the mold to thymidine-^{3}H for periods of 1–4 hr during early, middle, and late G_2 consistently gave a demonstrable peak of radioactivity in the light region (Figs. 1, 2 *a*, 3, 4, 5 *a*, 6 *a*). Incorporation of radioactive thymidine into the light DNA during S period is apparent when cell fractionation is carried out prior to banding (Fig. 5 *f*). The rate of incorporation into the light DNA, determined as counts minute^{-1} (micrograms protein)$^{-1}$ after 1.8-hr pulses at various times in the cell cycle, was quite variable and showed no trend (Table III, third column). The rates given (Table III) for samples pulsed during the S period are uncertain, due to the predominance of labeled principal DNA on the relevant gradients. A somewhat different approach was used to determine more quantitatively the fraction of light DNA synthesized during the S period.

TABLE III

Rate of Thymidine Incorporation at Different Times in the Cell Cycle

Time 1.8-hr pulse started	^{3}H specific activity	
	Total	Light DNA
hr after M II	*counts min^{-1} µg protein^{-1}*	
−3	261	146
−1	2540	170
1	1264	77
3	340	93
5	266	137
7	163	76
9	193	56
11	185	93

Sections 1–2 cm^{2} in area were cut from a single culture at the times indicated and labeled with 50 µc/ml thymidine-^{3}H (12.5 c/mmole before addition) for 1 hr 50 min each. Total acid-insoluble, alkali-stable counts and protein were determined on lysates as described under Materials and Methods. A sample of each lysate was run on CsCl, and the fraction of the recovered counts in the light region was calculated. This fraction was multiplied by the total specific activity to give the light DNA specific activity.

Fig. 6 *c* describes a CsCl gradient which contained DNA-^{3}H labeled during the entire S period and fully labeled DNA-^{14}C. The data are plotted in such a way that the ^{3}H and ^{14}C curves essentially coincide in the principal peak region. If all of the light DNA synthesis occurred during S, the ^{14}C and ^{3}H curves would also coincide in the light region. In fact, the curve for S-labeled DNA is lower than the curve for fully labeled DNA in the light region. The normalized ratio of ^{3}H/^{14}C in the light region is 0.49, where the ^{3}H/^{14}C ratio was taken from recentrifugation data (Fig. 6 *d*) and normalized so as to make the principal peaks coincide exactly. Thus, no more than 49% of the light DNA that is synthesized during a cell cycle is synthesized during S. The actual fraction may be somewhat less and would be lowest if the ^{14}C culture were harvested at the end of the S period. However, one can show that this would not make a large difference in the result, and that the fraction of light DNA synthesized during the S period is in the range of 32–49%.

It is clear from the data presented here (Table III) and from that of others (Nygaard et al., 1960; Braun et al., 1965) that synthesis of the principal,

nuclear DNA occurs primarily in the first 3–4 hr of the cell cycle in *Physarum*. There is also some principal DNA synthesis during G_2. The level of labeling of the principal nuclear component during G_2 was variable. There was a tendency for cultures labeled late in G_2 to show little or none of this component (Fig. 1), but little or none was also observed in one case of a 3-hr label period during early G_2 (Fig. 5 *a*). One possible explanation for the principal component labeling is that small regions of the culture are not fused to the body of the culture and are out of synchrony. To test this possibility, a culture labeled during G_2 was divided into three equal parts, and each was run separately on CsCl. The ratio of the light component peak height to the principal peak height was 2.9, 3.0, and 3.2 in the three cases. These ratios are not significantly different so that regions out of synchrony, if they exist, must be spread evenly throughout the culture. Guttes et al. (1967), using radioautography, observed a few highly labeled nuclei in cultures incubated with thymidine-^{3}H during G_2. Our observations are consistent with their suggestion that the labeling of the principal component during G_2 arises from these highly labeled nuclei.

The heavy DNA component can be seen as a peak only when the principal component radioactivity is low, as in Fig. 1 and Fig. 5 *a*. However, the ^{3}H profile of a G_2 pulse always shows a greater fraction of radioactivity on the heavy side than would be expected from the shape of the fully labeled marker alone (see Fig. 4 *a*). Thus, like the light DNA, the heavy DNA is synthesized throughout G_2. We do not know whether the heavy DNA is synthesized during the S period.

Stability of the Light DNA

Evidence for conservation of the light DNA was obtained by chasing a G_2-pulsed culture, for one doubling time, into the next G_2 period. The removal of radioactivity from the pools was probably not complete, since the ^{3}H pattern (Fig. 7) shows a greater fraction of radioactivity in the principal component than is usual after a G_2 pulse without chase. This radioactivity presumably entered the DNA during the S period which occurred during the chase. Nevertheless, there is still a distinct peak of ^{3}H in the light component region, which is never seen with cultures labeled during S or for long periods of time. Thus, the experiment failed to detect significant breakdown of light DNA. However, the results are also consistent with breakdown and selective reuse, within the cytoplasm, of the labeled product of breakdown.

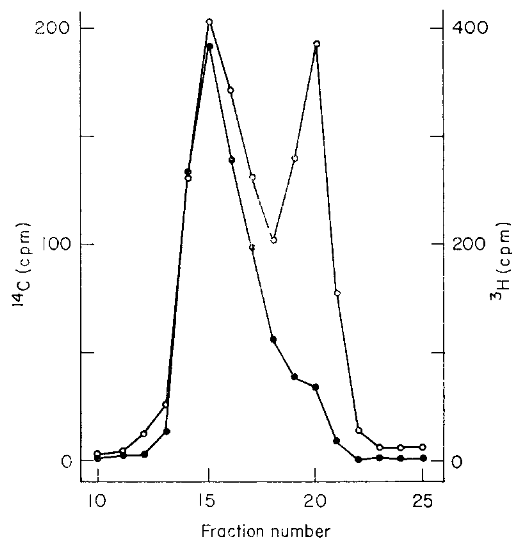

FIGURE 7 G_2 pulse followed by chase. A culture was exposed to 25 μc/ml thymidine-^{3}H for a 3 hr period beginning 5 hr after M II. Excess medium was then removed from the filter paper support, and the culture was incubated in fresh, nonradioactive medium for 14.7 hr. A lysate was made and run on CsCl. —●—, ^{14}C from a lysate of a fully labeled culture; —○—, ^{3}H.

DISCUSSION

We have observed preferential labeling of a light DNA component with labeling periods of 1–4 hr at all times during G_2. The phenomenon has also been observed in two other laboratories (Evans, 1966; Guttes et al., 1967). In addition, we have observed labeling of the light DNA during the S period both by cell fractionation (Fig. 5) and by recentrifugation of the light region (Fig. 6). The light DNA thus shares the properties of continuous synthesis and cytoplasmic location with the mitochondrial DNA seen by radioautography (Guttes and Guttes, 1964; Guttes et al., 1967). It seems probable, then, that the light DNA is indeed located only in mitochondria. However, the argument depends on the assumption that the predominant labeled species seen by radioautography is the predominant labeled species seen by biochemical techniques. The assumption is somewhat difficult to justify because of the difficulties of quantitative radioautography (Perry, 1964). When mitochondrial fractions were obtained by several

conventional techniques and banded isopycnically on sucrose or Renografin (Schatz et al., 1964) gradients, we found that the light DNA did not band with the bodies presumed to be mitochondria. Guttes et al. (1967) also found that the labeled DNA seen in mitochondria by radioautography was lost when attempts were made to purify the mitochondria. On the other hand, a mitochondrial fraction obtained by Brewer et al., (1967) gave a CsCl band identified as light DNA. However, in view of the difficulties described above and the possibilities of polysaccharide contamination (Brunk and Hanawalt, 1966; Guttes et al., 1967), further characterization of the cell fraction and the UV-absorbing peak would be desirable.

Studies on the amount of light DNA are of interest particularly because of the importance of establishing the number of molecules of DNA per mitochondria. Other authors (Guttes et al., 1967) have raised the possibility that there may be selective losses of minor DNA components during extraction. Because we isolate as DNA-^{3}H at least half of the acid-insoluble radioactivity from G_2-labeled preparations, it is possible to put a limit on the losses that could occur. The lost radioactivity is presumed to be located in the precipitate that forms between the CsCl solution and the overlying mineral oil. Although the labeled material in the interfacial precipitate has not been analyzed, it is likely that the lost radioactivity is not in DNA but is rather in some component which is labeled to an extent which does not depend on time of incubation. This possibility would explain (*a*) the existence of the non-DNA peak which is observed on top of the gradient, (*b*) the high recoveries from long-labeled or S-labeled gradients, and (*c*) our finding (Brown, R. D., and C. E. Holt. Unpublished data) that as much as 50% of the acid-insoluble ^{3}H in G_2-labeled cultures is not thymine, when analyzed by acid hydrolysis and paper chromatography. Thus, our figure of 9% as the fraction of DNA in the cytoplasm is a minimum estimate and is low, by at most a factor of about two, if one considers the possibility of selective losses. However, the figure must be considered tentative on other grounds. We have assumed that the pool specific activities are the same for all fractions of DNA, an assumption which has not been verified. Second, one would expect that the per cent cytoplasmic DNA would vary through the cell cycle, reaching a maximum just before the S period and a minimum at the end of S. It will therefore be necessary to make measurements on preparations in which the mitotic stage is known.

The presence of a heavy DNA component in *Physarum* was first reported by Braun et al. (1965), who observed a shoulder on the ultraviolet absorption profile of nuclear DNA centrifuged in CsCl in the analytical ultracentrifuge. Guttes et al. (1967) showed that when the heavy region of the principal component was run again on CsCl, the DNA banded heavier than the mean of the principal component. However, it is not clear what part of the rather complex pattern observed on recentrifugation should be termed "heavy component." The present work demonstrates the existence of a heavy DNA component which has labeling kinetics different from the principal component and which is located in the nucleus. In a few cases, the extent of specific labeling has been sufficient to assign the heavy DNA an approximate density.

If one assumes that the synthesis of the heavy DNA proceeds throughout the cell cycle with a pattern similar to that of the light DNA, then it can be concluded (Fig. 1) that the heavy DNA represents about 1% of the total DNA. However, we have no way of determining whether there is selective loss of this component. Therefore, 1% must be viewed as a minimum estimate.

In contrast to Guttes et al. (1967), we do observe the heavy DNA in preparations which show a preferential labeling of the light DNA. However, the ease with which a small amount of principal component labeling obscures the heavy component (see Fig. 4 *a*) may account for this apparent discrepancy. For the same reason, we do not know whether the heavy DNA is synthesized during the S period.

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