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Studies On The Replicative Organization Of Mammalian Dna

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STUDIES ON THE REPLICATIVE ORGANIZATION OF MAMMALIAN DNA

by

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ABSTRACT

The organization of the mammalian S phase was studied in synchronized mouse embryo cells in terms of the temporal order with which classes of replication units are synthesized and the spatial relationship between classes of replication units.

Results are summarized as follows: Nascent chain growth within replication units as measured by isopycnic centrifugation in alkaline Cs_2SO_4 - CsCl gradients constitutes the major factor determining the increase in the rate of DNA synthesis per cell during S phase.

Replication units comprising the main band of mouse nuclear DNA in neutral CsCl gradients are initiated in a definite temporal order, characterized by differences in base composition of DNA synthesized at different stages of the S phase. Initiation of replication units with a base composition characteristic of DNA synthesized in mid S depends upon completion of the synthesis of replication units with a base composition characteristic of DNA synthesized in early S phase. This indicates that duplication of the mammalian genome is, at least in part, a self-regulatory process.

The formation of high-molecular-weight nascent DNA strands several replication units in length was analyzed by velocity sedimentation in alkaline sucrose gradients and by isopycnic centrifugation in alkaline Cs_2SO_4 - CsCl gradients. Differential labeling with an isotopic and a density label shows that replication units synthesized at different stages of the S phase are not found within the same high-molecular-weight polynucleotide strand. It is thus concluded that

replication units duplicated at different stages of the S phase are spatially organized in clusters along the mammalian genome.

The rate of formation of high-molecular-weight nascent DNA strands is at least 4 - 8 times slower than that predicted from the spatial organization of replication units and the rate of chain growth within replication units. It is concluded that the process of merging of the nascent strands of adjacent replication units play a major role in the rate of completion of high-molecular-weight strands.

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Chapter 1

GENERAL INTRODUCTION

I.I THE PROKARYOTE MODEL

The basis for our understanding of the replication of eukaryotic DNA is the replication of the DNA of prokaryotes. Studies in this area provide the terms of reference and concepts used in describing eukaryotic DNA. Several findings are most significant in drawing a parallel between replicative organization in prokaryotes and eukaryotes. The chromosome of E. coli consists of a single, continuous, circular DNA molecule (Cairns, 1963) which is replicated semi-conservatively (Meselson & Stahl, 1958). The initiation of DNA synthesis occurs at a single, fixed, and genetically defined point (Bird & Lark, 1968) and proceeds bi-directionally (Masters & Broda, 1971; Prescott & Kuempel, 1972) by means of two synchronous replication forks. The rate of fork movement is 20-30μm/min. as measured by autoradiography (Cairns, 1963) and by centrifugation methods (Bonhoeffer & Gierer, 1963) Fork movement occurs by a discontinuous mechanism whereby short nascent strand fragments 1000-2000 nucleotides in length are synthesized with a 5' to 3' polarity on both parental strands (Okazaki et al., 1968; Sugino & Okazaki, 1972). Protein synthesis is required for the initiation of DNA synthesis but not for its completion (Lark, 1969; 1972). Jacob et al. (1963) proposed the concept of a unit of replication termed a replicon. A replicon is a unit of DNA that contains the elements of control over its own replication. This is carried out by means of two determinants on the DNA molecule, the initiator, and the replicator. The initiator is a structural gene that specifies an initiator protein which is capable of interacting with the nucleotide sequence at the replicator site to initiate DNA synthesis. In order to effectively regulate the frequency of initiation, the initiator protein has to be short-

lived, and its positive interaction with the replicator has to be coupled to cell growth, probably through involvement of the cell membrane. Such a model offers an explanation for the fundamental observation that only one copy of cellular DNA is produced per cell generation.

Thus, at least for certain prokaryotes, we understand to a reasonable degree both how initiation of duplication of the total genome is regulated, and the mechanics whereby this occurs.

Knowledge of the regulation of DNA synthesis in prokaryotes has been greatly facilitated by a sound basis in genetics. The ease of producing stable mutants and the availability of a well defined genetic map is the best means to identify gene functions involved in DNA synthesis. In higher eukaryotes this means is not yet available. Some lower eukaryotes such as yeasts have a sufficient genetic basis to make such an approach possible (Hereford & Hartwell, 1974). The only present alternative in the case of higher eukaryotes is the use of metabolic inhibitors which lack the specificity of mutants, and whose specificity must constantly be evaluated.

1.2 THE RELATIONSHIP OF DNA SYNTHESIS TO THE CELL CYCLE IN EUKARYOTES

In eukaryotes the synthesis of DNA is confined to a specific stage of the cell cycle termed the synthetic or S phase. This was first observed in randomly growing cell populations by autoradiography (Howard & Pelc, 1953; Stanners & Till, 1960) and subsequently confirmed in cell populations synchronized by various means. In mammalian cells the S phase is separated from mitosis by two gaps termed G1 and G2. Very little is known about the molecular events that occur during G1 and G2.

Several lines of evidence suggest that the events concerned with regulation of cell division occur in G1 (Prescott, 1968; 1970). Cells which are in a prolonged non-dividing state are arrested in G1. A G1 phase is generally found only in cell types that are highly differentiated. Thus it is hypothesized that cells which are required to perform a spacialized metabolic role are maintained in G1. On the other hand, cellular proliferation is also determined by events occuring in G1. The inhibition of protein or RNA synthesis during G1 prevents the entry of cells into S phase (Mueller & Kajiwara, 1965; Terasima et al., 1968; Fujiwara, 1967). Thus the primary step that determines whether or not a cell will divide occurs in G1. Nothing is definitely known as to the nature of this step nor how environmental or genetic determinants can influence it. Consequently, nothing is known of a mechanism analogous to the replicon concept of prokaryotes that would explain how the initiation of duplication of the total genome and subsequent cell division is regulated. Definite answers to this question will probably have to await the development of a genetics of somatic cells.

1.3 REPLICATIVE ORGANIZATION IN THE EUKARYOTIC S PHASE

How a eukaryotic cell regulates the duplication of its genome once it enters S phase is a question more approachable by present methods. The same limitations with respect to a genetic basis for regulation of DNA replication still apply. However, methods for the study of DNA synthesis are highly developed. Observation of the temporal and spatial order in DNA replication during the S phase allows the formation of hypotheses regarding mechanisms that regulate this process.

It is first of all evident that the concepts derived from the study of

prokaryotic DNA replication are not easily applied to eukaryotes for several reasons:

(i) Eukaryotes contain approximately 10^3 -fold more DNA than prokaryotes. For example, E. coli contains about 2.5×10^9 daltons of DNA corresponding to about 4×10^6 base pairs. A mammalian cell contains about $2\text{-}3 \times 10^{12}$ daltons of DNA, or about 3×10^9 base pairs. Mammalian DNA is distributed over several chromosomes, however even each of these still contains on the order of 10^2 -fold more DNA than the single chromosome of E. coli. This large amount of DNA naturally necessitates a more complex degree of replicative organization, as well as posing enormous technical problems in its analysis.

(ii) The chromosomes of eukaryotes contain many additional components in addition to DNA (histones and acidic proteins of uncertain function). Thus the replication of eukaryotic DNA must be co-ordinated with the production of these components (Weintraub, 1973; Seale & Simpson, 1975).

(iii) The greater amount of genetic material in eukaryotes is reflected in a greater phenotypic complexity. Higher eukaryotic cells have not only a greater phenotypic complexity than prokaryotes but have the capacity for enormous variation in phenotype through differentiation. The replicative organization of these cells of necessity must be adaptable to these variations.

It is the purpose of the remaining part of this section to review the known aspects of replicative organization in eukaryotes that are most pertinent to the work presented, and to point out the aims of this work. There is a large body of evidence that indicates that the progress of S phase in eukaryotic cells is a highly ordered process. This evidence will be reviewed from three aspects: (a) spatial order in replication, (b) temporal order of replication, and (c) regulation of the rate of DNA

synthesis during S phase.

(a) Spatial Order

The spatial order of DNA replication at the level of replication forks is similar to that of prokaryotes. DNA is replicated semi-conservatively (Taylor, 1963), by a fork-like growing point (Huberman & Riggs, 1968). The replication fork progresses by a discontinuous mechanism similar to that of prokaryotes (Schandl & Taylor, 1969, Huberman & Horwitz, 1973). Eukaryotic chromosomes appear to consist of continuous DNA helices (Kavenoff et al., 1973; Molitor et al., 1974). The major difference between prokaryotic and eukaryotic chromosomes is that replication of the eukaryotic chromosome is accomplished by the action of a large number of replication forks. This was first observed by Taylor (1959) by autoradiography of metaphase chromosomes within which DNA appeared to have been replicated simultaneously at many sites along the chromosome. Multiple repication sites were first observed at the level of individual DNA molecules by Cairns (1966). The fundamental model of the spatial organization of replication within individual DNA molecules comes from the work of Huberman & Riggs (1968). Using DNA fibre autoradiography in partially synchronized populations of Chinese hamster cells these authors confirmed the findings of Cairns (1966) with respect to multiple replication sites. In addition they found that replication forks in adjacent replication sections moved simultaneously in opposite directions away from a common origin. They proposed the term replication unit to describe such a pair of replication sections. (These units are sometimes referred to as replicons (Painter & Schaefer, 1969; 1971; Seale & Simpson, 1975). However, since there is no basis for assuming that these units have the characteristics defined by

Jacob et al. (1963), the term replication unit will be employed throughout this work.) The existence of similar diverging pairs of replication sections has been confirmed in a variety of mammalian cells by autoradiography (Callan, 1972; Hand & Tamm, 1973; McFarlane & Callan, 1973) and by physical methods (Weintraub, 1972a).

The size of replication units is determined by measurement of the inter-initiation distance between adjacent replication units in autoradiograms (Huberman & Riggs, 1968; Hand & Tamm, 1974). In randomly growing cells the size of replication units is heterogeneous. For example, Chinese hamster cells exhibit replication units ranging in size from 10μm* to 100μm, with an average value of 30μm (Huberman & Riggs, 1968). Replication unit size varies among different mammalian cell types (Hand & Tamm, 1974), however the average value does not exceed 60μm. The average size of replication units appears to remain constant throughout the S phase, and the same degree of size heterogeneity is maintained (Housman & Huberman, 1975). All evidence indicates that mammalian replication units are much smaller in size than the single replication unit of E. coli (1100-1300μm). Since a mammalian cell contains on the order of 1 m of DNA, there could be on the order of 10^4 such replication units in the genome.

DNA fibre autoradiography indicates evidence for the existence of clusters of replication units (Hori & Lark, 1973; Hand & Tamm, 1974; Hand, 1975). In other words groups of tandemly adjacent replication units initiate synthesis with a high degree of synchrony. The size of replication units and the rate of fork progression within the constituent replication units appears to be constant within any given cluster

* 1μm of extended double-stranded DNA is equivalent to about 2×10^6 daltons or 3000 base pairs.

(Hand, 1975). One of the aims of this work was to measure the degree of clustering of replication units in synchronized mammalian cells by physical methods. Differential labeling with an isotopic and a density label shows that replication units synthesized at different stages of the S phase are not found within the same high-molecular-weight polynucleotide strand. It is thus concluded that replication units duplicated at different stages of the S phase are spatially organized in clusters along the mammalian genome.

The rate of fork progression within replication units has been measured in a variety of unsynchronized and synchronized mammalian cell types by autoradiography (Cairns, 1966; Huberman & Riggs, 1968; Hand & Tam, 1973; Housman & Huberman, 1975) and by isopycnic centrifugation methods (Painter & Schaefer, 1969; 1971; Weintraub, 1972b). Estimates for different cells range from 5μm/min. to 0.5μm/min., with most estimates falling in the range of 0.5-1.0μm/min.

Very little is known of mechanisms involved in the completion of high-molecular-weight nascent strands during S phase. Autoradiography indicates that nascent strands of adjacent replication units grow to fusion, however autoradiography lacks the resolution to determine when actual physical continuity is established betwen nascent strands. One of the aims of this work was to determine the rate of formation of high-molecular-weight nascent DNA strands in synchronized cells. The rate of formation of high-molecular-weight nascent DNA strands, as determined by velocity sedimentation in alkaline sucrose gradients, is at least 4-8 times slower than that predicted from the spatial organization of replication units and the rate of chain growth within replication units. It is concluded that the process of merging of the nascent strands of adjacent replication units plays a major role in the rate of

completion of high-molecular-weight strands.

(b) Temporal Order

Several lines of evidence point to the fact that the timing of DNA synthesis during S phase within specific DNA molecules is regulated. Specific parts of the genome appear to always be replicated at the same time in S phase. This was first observed by autoradiography of metaphase chromosomes (Taylor, 1960), and in similar studies in synchronized cells (Hsu, 1964). Specific chromosomes and regions of chromosomes were engaged in DNA synthesis at specific times during S phase. Another observation supporting this point is the fact that there is a tendency in eukaryotic chromosomes for heterochromatic regions to replicate late in S phase (Brown, 1966; Hill & Yunis, 1967; Lima-de-Faria & Jaworska, 1968).

The temporal pattern of replication within specific DNA sequences has been studied by differential labeling with an isotopic and a density label in successive S phases (Braun et al., 1965; Mueller & Kajiwara, 1966). In such experiments there is a high degree of recovery of radioactive label in hybrid only when DNA is labeled during the same interval in both S phases.

There are a number of means of identifying specific DNA sequences that have been employed in studying the temporal order of DNA replication in the S phase. The most sensitive of these is hybridization. The time of replication of sequences complementary to ribosomal RNA has been studied in synchronized cells (Amaldi et al., 1969; Stambrook, 1974) and found to occur during a specific stage of S phase. However further studies of this sort are limited by unavailability of means to isolate specific unique sequence DNA or RNA molecules for hybridization. There is some

evidence that there is temporal order with respect to synthesis of DNA of some families of re-iterated sequences during S phase (May & Bello, 1974). Another method of distinguishing DNA sequences is by base composition. The base composition of native DNA is directly related to its buoyant density in CsCl (Schildkraut et al., 1962). This method has been employed to study the temporal order of DNA replication in several mammalian cell types synchronized by various means (Tobia et al., 1970; Bostock & Prescott, 1971 abc; Flamm et al., 1971). In all cases the base composition of newly synthesized main band DNA gradually shifts from GC -rich to AT - rich relative to bulk DNA as S phase progresses. In addition to this the highly re-iterated AT -rich sequences comprising the light satellite of mouse DNA are replicated predominantly in late S phase (Tobia et al., 1971).

All present evidence for temporal order in S phase in confined to a level of resolution above that of single replication units or clusters of replication units. In the one case that an attempt was made to reproduce temporal order at the level of replication unit origins by means of DNA fibre autoradiography in successive S phases, it was not successful (Amaldi et al., 1973). However, since the length of time necessary to complete the synthesis of an average replication unit is only a small proportion of the duration of S phase it is evident that temporal regulation is exercised at least at the level of broad classes of replication units.

Nothing is known of how the temporal order of DNA replication during S phase is determined. One of the aims of this work was to obtain some information on this point. It was found that replication units comprising the main band of mouse nuclear DNA in neutral CsCl gradients are initiated in a definite temporal order, characterized by differences in base composition of DNA synthesized at different stages of the S phase.

Initiation of replication units with a base composition characteristic of DNA synthesized in mid S depends upon completion of the synthesis of replication units with a base composition characteristic of DNA synthesized in early S phase. This indicates that duplication of the mammalian genome is, at least in part, a self-regulatory process.

(c) The Rate of DNA Synthesis During S Phase

The rate of DNA synthesis during S phase (and consequently the duration of S phase) is determined by three factors: the rate of chain growth within replication units, the frequency of initiation of synthesis throughout the genome, and the size of replication units. There is an increasing amount of evidence pointing to the fact that each of these factors can be regulated in eukaryotic cells to alter the overall rate of DNA synthesis under specific circumstances. Variations in the duration of S phase among different mammalian cell lines appears to be determined by the frequency of initiation of replication units during S phase (Painter & Schaefer, 1969). Variation in the duration of S phase at different developmental stages of a cell type appears to be determined by the size of replication units (Callan, 1972; Blumenthal et al., 1973). One of the aims of this work was to determine what factors regulate the rate of DNA synthesis during a single S phase. In at least one cultured mammalian cell type the average size of replication units at different stages of S phase remains constant (Housman & Huberman, 1975). Thus the rate of DNA synthesis during S could be determined by the rate of chain growth within replication units and/or the frequency of initiation of replication units. There is evidence from two methods of analysis to indicate that the rate of chain growth within replication units

increases during S phase (Painter & Schaefer, 1971; Housman & Huberman, 1975). At present there is no direct means of evaluating the frequency of initiation of replication units during S phase. However, the overall rate of DNA synthesis per S phase cell and the rate of chain growth within replication units can be measured in synchronized cells. This permits evaluation of the contribution of frequency of initiation of replication units to the overall rate of DNA synthesis during S phase. It was found that nascent chain growth within replication units as measured by isopycnic centrifugation in alkaline Cs_2SO_4 -CsCl gradients constitutes the major factor determining the increase in the rate of DNA synthesis per cell duging S phase.

Chapter 2

STUDIES ON THE REPLICATIVE ORGANIZATION OF MAMMALIAN DNA

2.1 MATERIALS AND METHODS

(a) Primary Cell Culture

All manipulations involving cell cultures (Sections a, b, c) up until the time of cell harvest after labelling were performed using sterile technique. Instruments, glassware, pipettes and some solutions were sterilized by autoclaving. Cell culture medium was sterilized by pressure filtration, and tested for sterility by overnight incubation at 37°C. All manipulations of cell cultures were performed in a sterile cabinet in a room equipped with overhead ultra-violet germical lamps. Cells were grown in incubators at 37°C in a moist atmosphere of 5% CO_2 in air.

The cell culture medium employed throughout is McCoy's 5A Medium (McCoy et al., 1959) (Modified) prepared from a commercial concentrate (Gibco in 20 litre lots.

Primary cultures of mouse embryo cells were prepared as follows:

1) A Swiss strain mouse 14-16 days pregnant was killed by breaking the neck and immersed in a beaker of Dettol.
2) The mouse was cut open, the uterus containing 4 to 12 embryos was placed in petri dish, and washed with PBS⁻ (Phosphate Buffered Saline, Dulbecco & Vogt (1954), containing no divalent cations).
3) The embryos were removed from the uterus and placed in another petri dish.
4) The heads were removed, the bodies placed in another petri dish and washed with PBS⁻.
5) The bodies were placed in a dry dish, chopped finely with scissors and poured into a stirring flask containing trypsin-citrate (0.125% trypsin (Gibco) in

[0.1% KCl - 0.44% sodium citrate]).

6) The tissue was incubated at 37°C for 10 minutes.

7) The trypsin-citrate was decanted, fresh trypsin-citrate added, and the tissue stirred for 1 hour at room temperature.

8) The mixture was filtered through sterile gauze into a centrifuge bottle, and a small amount of fetal calf serum was added to inactivate the trypsin.

9) Cells were centrifuged and resuspended in 20 ml. of medium and pipetted to break up remaining clumps.

10) Viable count - 0.1 ml. of cell suspension was added to 1.9 ml. citrate (0.1%KCl - 0.44% sodium citrate). 1ml. of this suspension was added to 1 ml. of crystal violet solution (0.1% crystal violet in 0.1 M sodium citrate) and incubated at 37°C for 5 min. Stained cells were counted on a hemacytometer.

11) Cells were bottled at 7.5×10^6 cells per bottle (75 cm^2 surface)(Falcon Plastics) in 30 ml. of McCoy's 5A Medium containing 10% (v/v) Fetal Calf Serum (Gibco), 1% (v/v) Penicillin - Streptomycin (Gibco), 1% (v/v) Fungizone (Gibco) and 1% (v/v) Anti PPLO agent (Gibco).

12) By 8 days of incubation the cells had formed monolayers of $1 - 1.2 \times 10^7$ cells/bottle.

(b) Secondary Cell Culture and Synchronization

Cell monolayers were dispersed with trypsin-citrate (2 ml./bottle), diluted to 10 ml. with medium and counted (hemocytometer or coulter counter). Secondary cultures were plated at 3×10^5 cells per 60 mm dish (Falcon Plastics) in 5 ml. of

medium containing 3% v/v Fetal calf serum (Gibco), of 1% (v/v) Penicillin - Streptomycin. The growth of cell cultures was monitored by counting duplicate cultures every day. Cultures grew to a concentration of $1.2 - 1.5 \times 10^6$ cells per dish over a period of 5 days. The cultures were allowed to remain at this density for 1-2 more days and then the serum concentration was increased to 10% (v/v) by changing the medium.

(c) Radioactive and Density Labelling of DNA.

DNA synthesized in synchronized cultures was radioactively labelled with [methyl - ^{3}H] thymidine ([^{3}H] dThd)(spec. act. 40-60 Ci./m-mole.) (New England Nuclear) or with [2 - ^{14}C] thymidine ([^{14}C] dThd)(spec. act. 57 mCi./m-mole) (Amersham Searle) added to the medium at the concentrations described in the figure legends. Unlabelled thymidine (dThd) was not added. Since McCoy's 5A medium contains no thymidine, approximate final specific activities can be calculated from these data. This calculation however is not completely accurate in most cases since labelling was usually carried out in the presence of serum which contains trace amounts of dThd.

Briefly, the rationale is as follows. Thymine enters DNA by means of two pathways; a salvage pathway and a de novo synthesis pathway. Both pathways involve the synthesis of thymidine monophosphate which is then converted by kinases into thymidine triphosphate which serves as the substrate for DNA polymerase. The salvage pathway utilizes exogenously supplied thymidine by converting thymidine to thymidine monophosphate via the enzyme thymidine kinase. The terminal step in the de novo pathway is the methylation of deoxyuridine

monophosphate by the enzyme thymidylate synthetase. However, de novo synthesis is inhibited allosterically by thymidine triphosphate. (Maley and Maley, 1962). Exogenously supplied [^{3}H] dThd is rapidly taken up by these cells and the intracellular pools of thymidine and thymidine phosphates are rapidly equilibrated. (Lindberg et al., 1969, Nordenskjöld et al., 1970). The predominant form of nucleotide in the pool is thymidine triphosphate. Our own measurements (Fig. 10) indicate that in these cells the intracellular pool of thymidine phosphates is equilibrated in 2-3 min. Nordenskjöld et al. (1970) employing a sensative assay, have measured the size of the intracellular thymidine triphosphate pool in these cells and estimate that it suffices for only 2 min. of DNA synthesis. In addition to this they find that the intracellular pool of thymidine triphosphate expands three-fold during S phase. However, the spacific activity of the pool remains constant. The significance of all of these observations for the present work is that 1) Exogenous [^{3}H] dThd incorporation measures the rate of DNA synthesis providing the exposure time is significantly longer than the equilibration time of the intracellular thymidine triphosphate pool, 2) Labelling with [^{3}H] dThd can be very efficiently "chased" with unlabelled dThd and 3) measurements of the rate of DNA synthesis based on the rate of [^{3}H] dThd incorporation into DNA are comparable to one another at all times during S phase.

Density labelling of DNA is carried out by effecting the substitution of 5 - Bromodeoxyuridine (BrdUrd) for dThd, thereby increasing the buoyant density of the DNA. BrdUrd is a synthetic, pyrimidine analog which enters DNA by the salvage pathway for thymidine since the enzymes in this pathway do not distinguish

the 5 - Bromo substitution from the 5 - methyl group of dThd. Thus exogenously supplied BrdUrd competes with dThd. Two conditions have to be met to effect efficient substitution: 1) exogenous dThd concentration must be reduced to nil and 2) de novo synthesis of thymidine monophosphate must be blocked. These conditions were met by 1) labelling in medium with no dThd and no serum and 2) Blocking the de novo synthesis of thymidine monophosphate with 5 - Fluorodeoxyuridine (FdUrd) (Simon, 1963). FdUrd is a synthetic pyrimidine analog which is taken up and converted to 5 - Fluorodeoxyuridylate via a salvage pathway. 5 - Fluorodeoxyuridylate forms a covalent compound with the enzyme thymidylate synthetase and immobilizes it. (Santi et al. 1974) thereby blocking the terminal step in the de novo synthesis of thymidine monophosphate. FdUrd is not incorporated into DNA since the vanderWaals radius of the 5 - Fluoro substitution, being more similar to that of hydrogen than of a methyl group, prevents its substitution for dThd and instead facilitates its competition with uridylate for thymidylate synthetase.

Density labelling of DNA was carried out by pretreating cell cultures for 30 min. with 6×10^{-5} M. FdUrd in medium with no serum, followed by the addition of BrdUrd to 1.6×10^{-5} M. The concentrations of pyrimidine analogs are those used to density label the DNA of mouse embryo cells in a previous report (Türler, 1974). Incorporation of BrdUrd was monitored by the addition of 5 - [6 - ^{3}H] Bromodeoxyuridine ([^{3}H]BrdUrd) (spec. act. 15 - 30 Ci/m-mole) (New England Nuclear) together with BrdUrd at the concentrations described in the figure legends. Since exposure of DNA to low wavelength light is known to cause breaks specifically in BrdUrd substituted regions (Regan et al., 1971; Weintraub, 1972a) exposure of substituted DNA to fluorescent lighting was avoided.

Chasing of both [^{3}H] dThd labelling and BrdUrd labelling was performed in the presence of 4×10^{-4} M dThd.

During the various medium changes involved in density labelling, radioactive labelling and chasing, cultures were repeatedly washed with pre-warmed medium containing the appropriate additions to effect complete medium change. Since the rate of DNA synthesis is drastically influenced by temperature, exposure of cultures to room temperature was kept to a minimum.

All pulses and chases were terminated by washing cultures with ice cold SSC (0.15 MNaCl - 0.015 M sodium citrate). Cells were dispersed by brief treatment with 0.125% trypsin (Gibco) in [0.1% KCl - 0.44% sodium citrate]. Cell suspensions were washed several times by centrifugation and resuspension with a vortex mixer and resuspended in ice cold SSC. Cell concentrations were determined with a Coulter counter (see sections a, b).

In cultures where total radioactivity was to be determined, pulse-labelling was terminated by washing the cultures with ice cold SSC followed by the addition of 3 ml. of [0.1M NaCl - 0.001 M EDTA - 0.01 M Tris (pH7.4) -0.5% (w/v) sodium dodecyl sulfate] directly to the culture dish. Total radioactivity was always measured in duplicate cultures, and averaged.

(d) Autoradiography

1. Sets of 3 cultures were labelled as described above with [^{3}H] dThd (2μCi/ml.) for 30 min. intervals.

2. Two cultures of each set were taken for determination of total radioactivity incorporated, as described in section c.

3. Cells from the remaining cultures were removed from the plate as described in section c and washed several times in PBS^-.

4. The cell pellet was resuspended in 0.5 ml. PBS^-, 0.5 ml distilled water was added, and the cell suspension incubated at 37° C. for 5 min.

5. This 1:1 dilution with distilled water followed by incubation at 37°C for 5 min. was repeated two more times. (i.e. final vol. = 4 ml., final dilution 1:8).

Lowering the salt concentration causes the cells to swell, thereby facilitating visualization of nuclei after straining.

6. Cells were resuspended in 4 ml. [75% ethanol - 25% acetic acid (v/v)] for 5 min.

7. The fixed cells were resuspended in 0.25 ml. of fixative (approx. 4×10^6 cells/ml.) and one drop of this suspension was placed near the end of a clean 75 x 25 mm. glass slide and allowed to dry thoroughly. Several slides were prepared from each culture.

8. The slide was washed in [75% ethanol - 25% acetic acid (v/v)] and dried.

9. Slides were stained for 5 min. with orcein (2% orcein (w/v) in [50% acetic acid - 50% water (v/v)], filtered through a 0.45 μm filter immediately before using).

10. The slide was flooded with ethanol to remove all excess stain, drained and dried.

11. Slides were washed in running tap water for 1-2 hrs. and dried thoroughly.

12. Steps 12 - 14 were done in the dark-room using a Wratten #2 safe-light filter. Nuclear Track Emulsion NTB-2 (Kodak) was melted at 45°C in a wide short

test tube. Each slide was briefly dipped in the emulsion and the back of the slide wiped clean.

13. When the emulsion was dry, slides were placed in a light-tight slide box together with some Drierite and taped. One complete set per box. Exposure was at 3-4°C for 4-7 days.

14. Slides were developed and fixed as follows: Kodak D19 developer (1:1) 18°C, 3.5 min.; distilled water, 18°C, 10 secs.; Kodak Fixer; 5 min.; running water 18°C, 1 hr.; dry.

15. Slides were scanned for nuclei with grains under low power magnification. Labelled and unlabelled nuclei were counted by screening the slide until a constant proportion of labelled nuclei was obtained. Minimum number of nuclei scored was 200. Nuclei of cells from unlabelled cultures showed no grains. Slides were also prepared from cultures labelled continuously throughout the course of the experiment. It was found that 25% of the cells in the cultures do not enter S phase by 24.5 hrs. post-serum. Data from pulse labelled cells was adjusted accordingly.

(e) Purification of DNA

For some experiments DNA was partially purified prior to centrifugation as described earlier (Cheevers et al.1972). Briefly, cell suspensions in SSC were lysed by gentle mixing with 0.1 volume of 10% (w/v) SDS. Lysates were poured onto 15 - 30% (w/w) sucrose gradients containing a 70% (w/v) sucrose cushion in [0.1M NaCl - 0.001 M EDTA - 0.01 M Tris pH 7.4 - 0.5% (w/v) SDS], centrifuged (SW27 rotor (Beckman) 23,000 rev./min, 12 hrs, 23°C), and the material on the cushion was collected. Cellular DNA is not fragmented extensively by this

procedure (Cheevers & Hiscock,1973), and over 95% of the radioactivity is found in the cushion. The sucrose cushion was diluted and the DNA sheared with a pipette. DNA precipitated by the addition of 2.5 volumes of ethanol at -20°C overnight was dissolved in 0.1 x SSC, returned to 0.15 M NaCl by addition of 0.1 volume of 10 x SSC, and stored at -80°C.

(f) Velocity Sedimentation in Alkaline Sucrose Gradients

Cell suspensions ($\leq 2.5 \times 10^5$ cells) in 0.1 ml. SSC were slowly pipetted into 0.4 ml. [0.3N NaOH- 0.001 M EDTA - 0.1% (w/v) sarcosyl] layered over a gradient of 15 - 30% (w/w) sucrose in [0.5M NaCl - 0.25N NaOH - 0.001 M EDTA - 0.1% (w/v) sarcosyl]. Cell lysis and DNA denaturation was allowed to proceed for a minimum of 8 hrs. at room temperature. Sucrose gradients were formed using an ISCO Density Gradient Former. It was found that this method resulted in a high degree of reproducibility both in uniformity of the gradient (from refractive index measurements) and in final volume. Gradients were centrifuged at 26,000 rev./min. for 6 hrs. at 23°C in an SW27.1 rotor (Beckman), and fractionated using an ISCO Density Gradient Fractionator.

Molecular weights were calculated by the formula $\log_{10} M = (\log_{10} S + 1.277)/0.400$ where M is the molecular weight in daltons and S the sedimentation coefficient (Studier,1965). Closed circular polyoma virus DNA (53S) (Weil & Vinograd, 1963) was used as a sedimentation marker. Polyoma DNA was centrifuged in one gradient of every rotor. A weight-average molecular weight was determined for each gradient according to the following formula:

$$\overline{M} = \sum_1^n \left[\left[\frac{cpm_i}{\sum_1^n cpm_i} \right] M_i \right]$$

where M is the molecular weight in daltons, n the total number of fractions in the

gradient, cpm the radioactivity in counts/min., and i any fraction.

(g) Isopycnic Centrifugation in Alkaline Cs_2SO_4 - CsCl Gradients

Cell suspensions ($\leq 2.5 \times 10^5$ cells) in 0.1 ml. SSC containing purified ^{14}C labelled marker DNA were slowly pipetted into 0.4 ml. [0.3N NaOH - 0.001 M EDTA - 0.1% (w/v) sarcosyl] layered in a polyallomer tube over a solution consisting of 1gm Cs_2SO_4, 4.8 gm CsCl and 4 ml. of alkaline SSC containing 0.1 volume [1N NaOH - 0.01 M EDTA]. After Cell lysis and denaturation of DNA for a minimum of 8 hrs. at room temperature, tubes were filled with paraffin oil and centrifuged in a Ti 50 rotor (Beckman)(40,000 rev./min., 40 hrs., 20°C). Tubes were then punctured and fractionated from the bottom.

(h) Isopycnic Centrifugation in Neutral CsCl Gradients

DNA in SSC was mixed with a saturated solution of CsCl in SSC and the density adjusted refractometrically to 1.710 gm./cm^3. 4.2 ml. of this solution was placed in a polyallomer tube, overlaid with paraffin oil and capped. Tubes were centrifuged using a Beckman 40.3 rotor at 32,000 rev./min. for 60 hrs. at 20° and fractionated from the bottom.

(i) Determination of Radioactivity

Radioactivity incorporated into DNA was measured by measuring radioactivity in the acid-insoluble fraction of cell lysates or gradient fractions. A solution of 5% trichloroacetic acid (TCA) precipitates protein and nucleic acids leaving small acid soluble molecules (e.g. nucleotides, amino acids, lipids) in solution.

Cells lysed directly in the plate were pipetted vigorously to break up viscosity of the lysate and made 5% in TCA. Fractions from alkaline sucrose gradients were neutralized by the addition of Tris-HCl. Bovine serum albumin was added as carrier and the DNA precipitated by the addition of an equal volume of 10% TCA. TCA-insoluble material was collected by filtration onto Whatman GF/C glass fiber papers and dried. Fractions from alkaline Cs_2SO_4 - CsCl and neutral CsCl gradients were collected directly onto GF/C filters, washed twice with 5% TCA, once with ethanol and dried.

Radioactivity on the filters was determined by submerging each filter in 4 ml. of non-aqueous scintillator solution [0.6% 2,5 diphenyloxazole - 0.01% 1,4 - bis (2-(4 - methyl - 5 - phenyloxazolyl)) benzene (w/v) in toluene] in a glass scintillation vial. Radioactivity was determined by counting each filter for a minimum of 1 min. per channel in a Nuclear Chicago "Isocap 300" Liquid Scintillation Counter. In all cases appropriate background values were determined and subtracted. In all experiments where ^{3}H and ^{14}C radioactivities were to be determined simultaneously, the ^{14}C radioactivity was adjusted to 10% of the ^{3}H radioactivity to minimize errors of quantitation resulting from channel spillover during liquid scintillation counting. The degree of channel spillover was for ^{3}H 0.05% and for ^{14}C 17%. Variation in quenching was monitored by measuring channel ratios in single label samples and by an External Standard Ratio program for double-label samples.

2.2 RESULTS

(a) Cell Synchrony

All experiments were performed in secondary cultures of mouse embryo cells synchronized by growth in a low concentration of serum (Fried & Pitts, 1968; Nordenskjöld et al., 1970). Fig. 1 shows the growth and synchrony properties of these cells. After 4-5 days of growth in medium containing 3% serum, the cultures reach a stationary state characterized by a constant sub-confluent cell number and a low level of DNA synthesis. Autoradiography measurements indicate that this residual incorporation is accounted for by approximately 10% of the cells, which may continue to cycle in conditioned medium. Treatment of cultures in this state with medium containing 10% serum results in a 7- fold increase in the rate of incorporation of [^{3}H] dThd, begining at 10 hrs. after addition of serum and reaching a maximum 5 hrs. later. This is closely paralleled by a 6-7 -fold increase in the number of S phase cells and followed by a near doubling in cell number. Estimation of the duration of S phase from autoradiography data yields a value of 8 hrs.

(b) Rate of DNA Synthesis per Cell as a Function of Rate of Chain Growth Within Replication Units and Frequency of Initiation of Replication Units.

The overall rate of DNA synthesis per cell was estimated from the experiment shown in Table 1. Measurement of changes in the rate of DNA synthesis from the rate of incorporation of [^{3}H] dThd is entirely dependent on the specific activity of the intracellular precursor pools and on their equilibration time. In mouse embryo cells synchronized by this method the specific activity of the intracellular dTTP pool

FIG. 1. Synchronization of secondary cultures of mouse embryo cells.

Secondary cultures of mouse embryo cells were plated in medium containing 3% serum. After 140 hrs., the serum concentration was increased to 10%. The rate of DNA synthesis was monitored by the incorporation of [^{3}H] dThd in 0.5 hr. pulses at a concentration of 3μCi/ml. The proportion of cells synthesizing DNA was monitored by autoradiography of cells labeled with [^{3}H] dThd for 0.5 hr. Cell number was monitored using a Model B Coulter counter. (●–●), cells per culture; (Δ–Δ), DNA synthesis in cells treated with 10% serum; (▲–▲), DNA synthesis in cells maintained in 3% serum; (x – x), proportion of S phase cells.

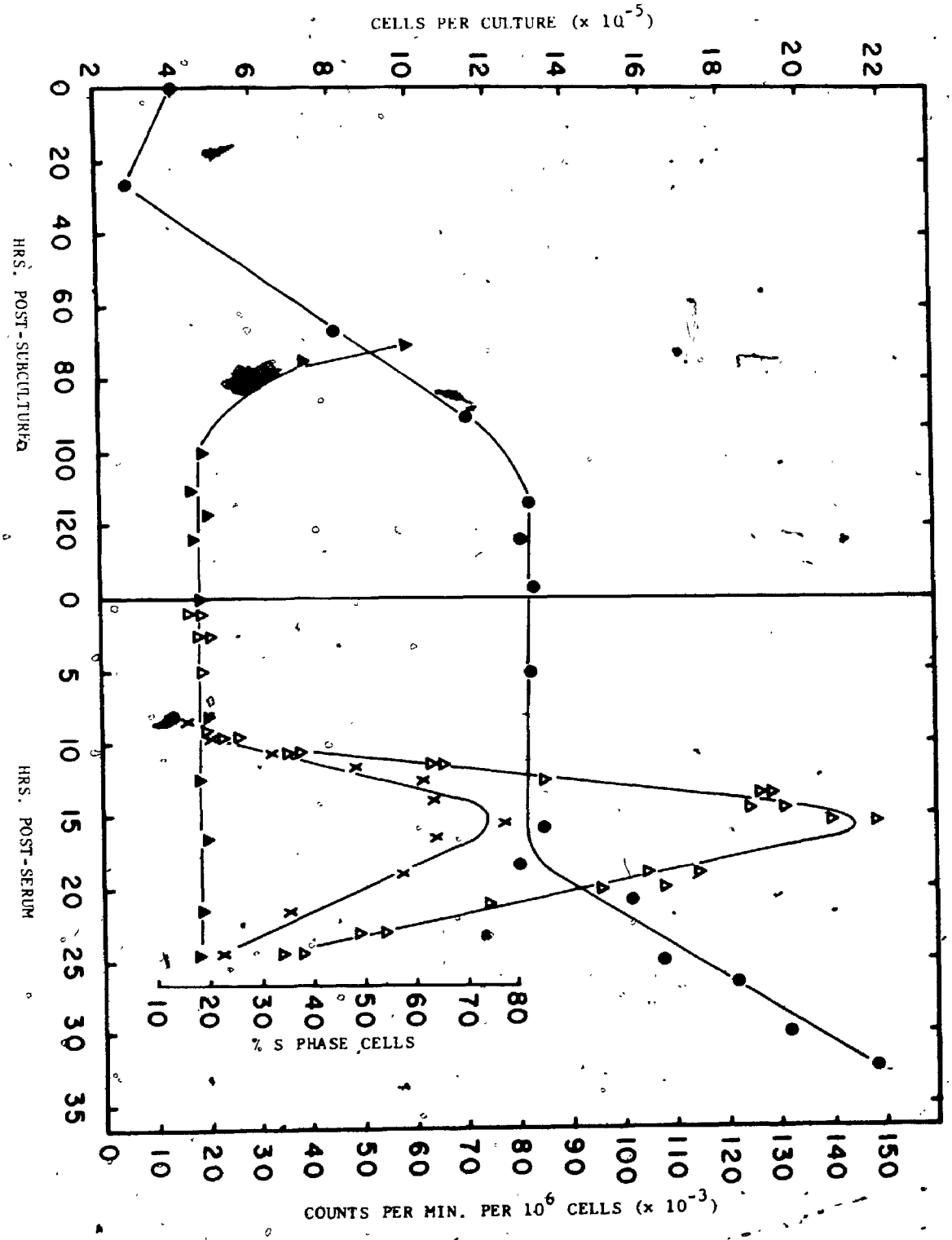

CELLS PER CULTURE (x 10^{-5})
2 4 6 8 10 12 14 16 18 20 22
0 20 40 60 80 100 120
HRS. POST-SUBCULTURE
0 5 10 15 20 25 30 35
HRS. POST-SERUM
10 20 30 40 50 60 70 80
% S PHASE CELLS
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
COUNTS PER MIN. PER 10^{6} CELLS (x 10^{-3})

FIGURE 1

TABLE I

Rate of DNA synthesis per cell

Time (Hrs. post-serum)	Cells in S/ culture ($\times 10^{-5}$)	C.p.m. / culture /0.5 hr. ($\times 10^{-4}$)	C.p.m./10^5 S phase cells /0.5 hr. ($\times 10^{-4}$)
9.5	2.3	2.15	0.93
10.5	3.0	3.82	1.27
11.5	4.4	5.75	1.31
14.5	5.7	9.15	1.61
16.5	6.2	9.85	1.59
21.5	3.9	4.60	1.18
24.5	3.2	3.05	0.95

DNA synthesized in cultures of mouse embryo cells synchronized as described in Fig. 1 was labeled during a 0.5 hr. pulse of [^{3}H] dThd (2 μCi/ml). Two cultures per time point were taken for determination of total radioactivity incorporated into DNA and for determination of cell number. Duplicate determinations were averaged. One culture per time point was prepared for autoradiography.

remains constant during S phase, despite a three - fold expansion in size. (Nordenskjöld et al., 1970). Our own measurements of the rate of equilibration of the intracellular pool of thymidine phosphates in these cells indicate an equilibration time of 2 - 3 min. (Fig. 10). On the basis of these findings we conclude that the data of Table I represents a valid measure of the rate of DNA synthesis per cell. This rate appears to increase less than 2 - fold during the course of S phase. The results obtained here are similar to the data of Nordenskjöld et al. (1970) for the same cell system.

The increase in overall rate of DNA synthesis can be accounted for by an expansion in the number of growing points per cell and/or an increase in the rate of polymerization of DNA chains within replication units. The rate of chain growth within replication units was measured by the method of Painter & Schaefer (1969, 1971) from the proportion of labeled DNA of greater than normal density synthesized in a 10 min. pulse of [^{3}H] dThd followed immediately by 3 hrs. of BrdUrd substitution (Fig. 2 and Table 2).

The value of F (the fraction of radioactivity of hybrid density), (Table 2) measures the rate of chain growth only if the ratio of initiation to termination of chains during the [^{3}H] dThd pulse is unity (Painter & Schaefer, 1971). Initiation during the pulse increases the proportion of labeled fragments of hybrid density, while termination during the pulse increases the proportion of light labeled fragments. Thus, the decrease in the value of F between 11 and 15 hrs. post-serum could be the result of: (i) an increase in the rate of chain growth per replication unit and/or (ii) a decrease in the ratio of initiations to terminations during the pulse. The data of Table I indicate that the rate of DNA synthesis per cell increases 1.2 - fold during

this time interval. Possibility (ii) implies that the number of operating replication units decreases during S phase; thus, it is inconsistent with the increase in the

FIG. 2. Alkaline Cs_2SO_4 - CsCl isopycnic centrifugation of DNA.

At (a) 11 hr. post-serum or (b) 15 hr. post-serum, cells were pulse-labeled for 10 min. with [^{3}H] dThd (66 μCi/ml) and then incubated for 3 hr. with medium containing BrdUrd and FdUrd. DNA was purified by velocity sedimentation in neutral sucrose gradients and centrifuged to equilibrium in alkaline Cs_2SO_4 -CsCl as described in Materials and Methods. [^{3}H] dThd-labeled DNA (O-O). [^{14}C] dThd-labeled DNA (●-●) and [^{3}H] BrdUrd-labeled DNA (Δ-Δ) were prepared from randomly growing cultures. ^{14}C radioactivity on the light side of the peak was adjusted to coincide with [^{3}H] dThd-labeled DNA.

FIGURE 2

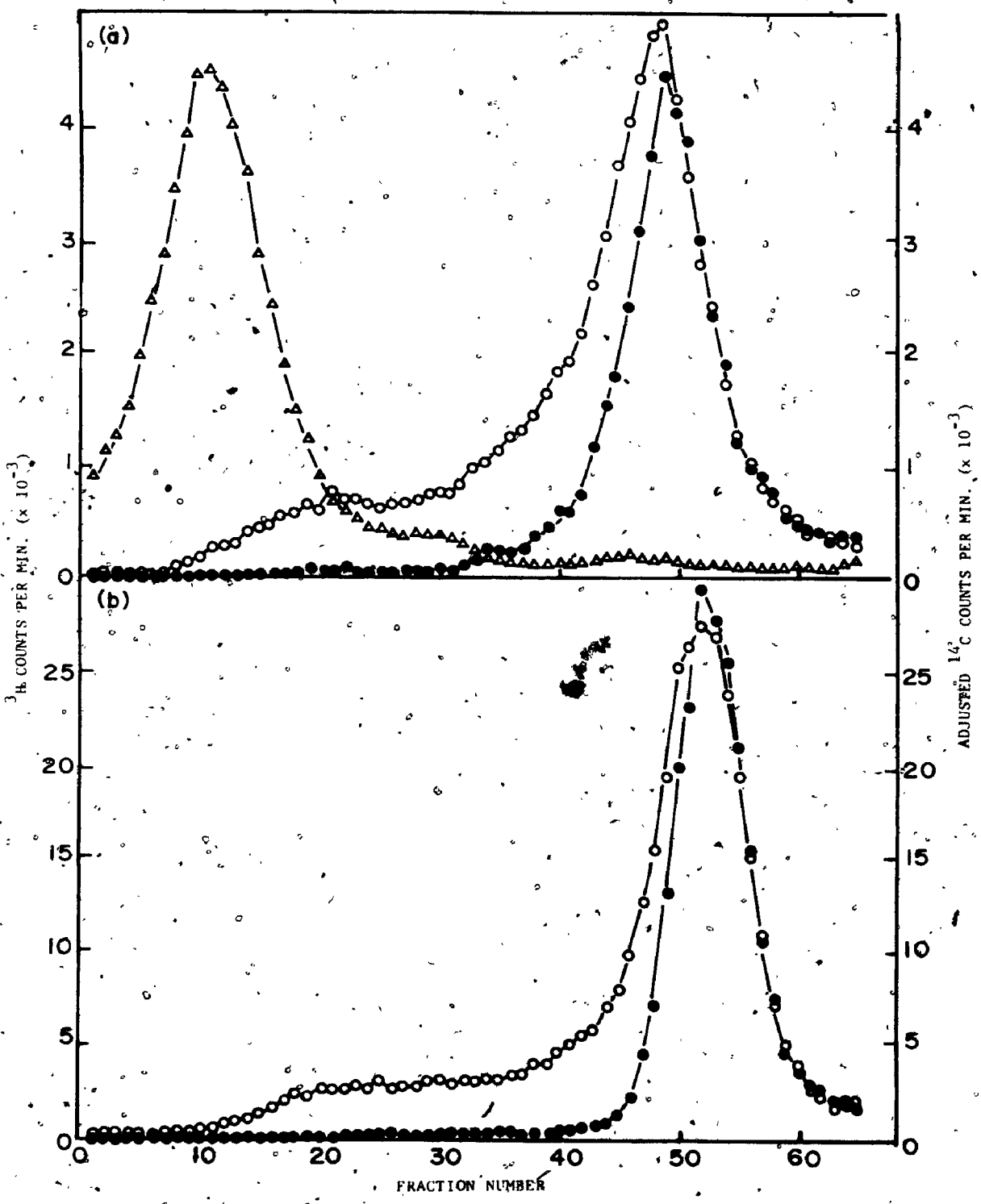

(a)
(b)
^{3}H COUNTS PER MIN. (x 10^{-3})
ADJUSTED ^{14}C COUNTS PER MIN. (x 10^{-3})
FRACTION NUMBER
0
10
20
30
40
50
60
0
1
2
3
4
0
5
10
15
20
25

TABLE 2

Rate of nascent DNA strand growth within replication units

Time (Hrs. post-serum)	t (min.)	F	F_{sh}	$F-F_{sh}$	B (Daltons $\times 10^{-7}$)	R (Daltons $\times 10^{-6}$/min./fork)
11	10	0.411	0.161	0.250	1.15	2.30
15	10	0.365	0.181	0.184	1.30	3.53

DNA was labeled and analyzed as described in Fig. 2. The [^{3}H] dThd pulse time is given by t. F. denotes the fraction of ^{3}H radioactivity of greater than normal density determined from Fig. 2, B, the average molecular weight of the labeled fragments determined by velocity centrifugation in alkaline sucrose gradients, and F_{sh}, the fraction of radioactivity of greater than normal density in DNA sheared at 50,000 p.s.i. to a single stranded molecular weight of 10^5 daltons. The rate of chain growth, R, was determined from the formula $R=B/2(F-F_{sh})t$ (Painter & Schaefer, 1969).

overall rate of DNA synthesis. The increase in the rate of DNA synthesis per cell is thus accounted for by an increase in the rate of strand growth within replication units.

(c) Temporal Organization of Initiation of Replication Units.

It was previously shown that the duplication of eukaryotic DNA is an orderly process characterized by differences in base composition of replication units initiated at different stages of S (Tobia et al., 1970; Bostock & Prescott, 1971 a b c; Flamm et al., 1971; Tobia et al., 1971). We next sought to confirm these observations in mouse cells synchronized by serum deprivation.

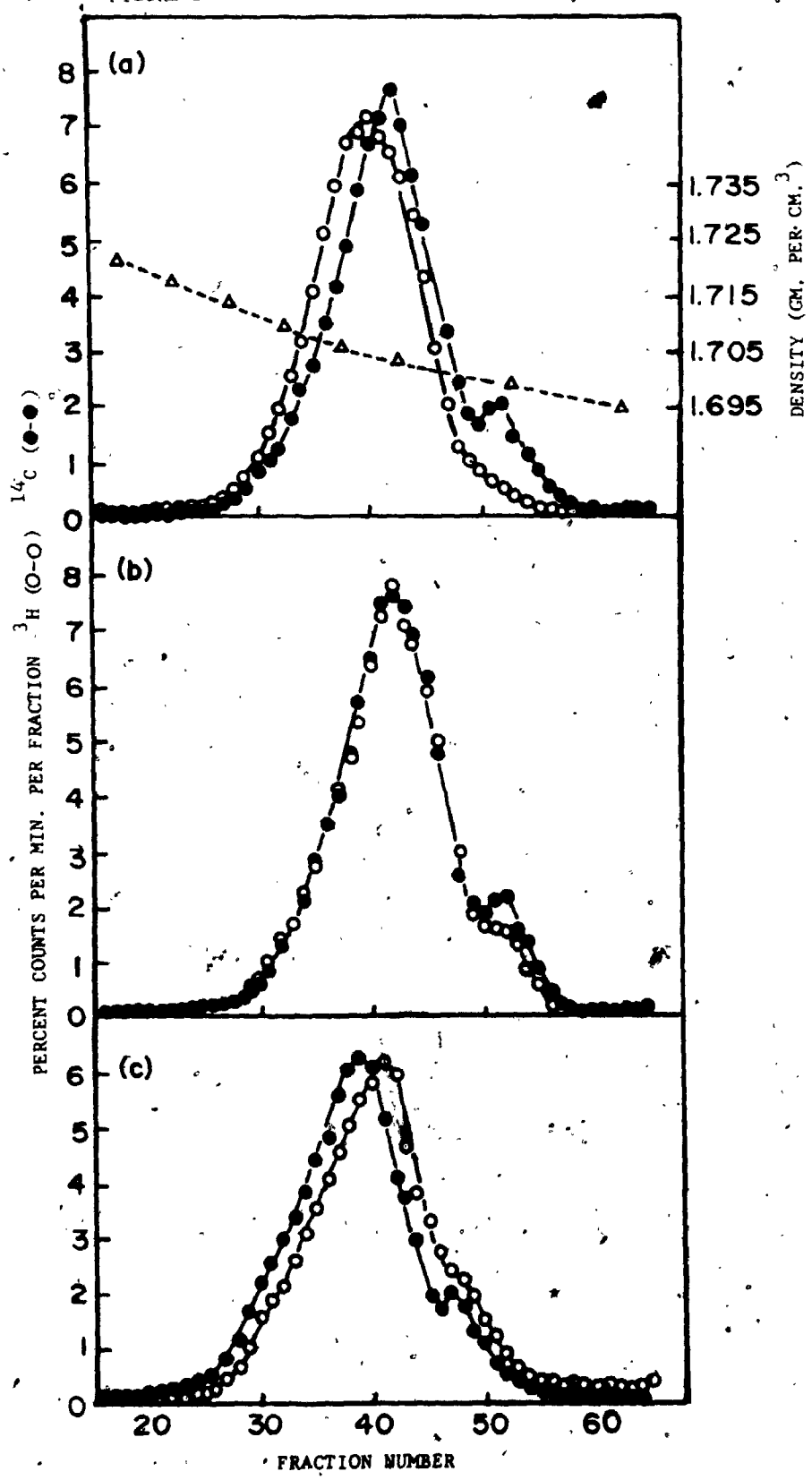
Fig. 3 shows that main band DNA(ρ = 1.702 gm./cm^3) is replicated in a definite temporal order, characterized by differences in buoyant density between replication units initiated at different stages of the serum-induced S phase. Replication units initiated during early S are GC -rich relative to the base composition of main band DNA labeled throughout the S phase (Fig. 3 (a)). Replication units initiated during a 30 min. pulse in mid S band coincidentally with main band DNA of buoyant density 1.702 gm./cm.^{3} (Fig.3(b)). Replication units initiated during late S are AT -rich relative to the average base composition of the total main band component of the genome (Fig. 3 (c)). Satellite DNA(ρ= 1.691 gm./cm.^{3}), constituting about 10% of the mouse genome, is replicated in mid and late S. These results are similar to those obtained using a variety of other kinds of mammalian cells synchronized by other means.

We next asked the significance of the order of initiation of replication units

FIG. 3 Isopycnic centrifugation in neutral CsCl gradients of DNA synthesized in synchronized cells.

DNA was pulse-labeled with [^{3}H] dThd (10 μCi/ml) for 0.5 hr. at 10.5 - 11 hrs. post-serum (a) (O–O), 15 - 15.5 hrs. post-serum (b) (O–O) and 20.5 - 21 hrs. post-serum (c) (O-O). [^{14}C] dThd labeled marker DNA (●-●) was purified from randomly growing cultures. The density of gradient fractions (Δ-Δ) was determined using a Jena refractometer.

FIGURE 3


(a)
(b)
(c)
PERCENT COUNTS PER MIN. PER FRACTION ^{3}H (O-O) ^{14}C (●-●)
DENSITY (GM. PER CM.^{3})
1.735
1.725
1.715
1.705
1.695
FRACTION NUMBER
20
30
40
50
60

outlined in Fig. 3 with regard to the control of the mammalian S phase. Specifically, the question was: Is it possible to induce cells to synthesize replication units characteristic of mid S before completion of the replication of early S replication units? To measure this, cells were allowed to enter S normally. Further entry of cells into S was then blocked using hydroxyurea, which immediately and reversibly blocks DNA synthesis (Skoog & Nordenskjöld, 1971), while allowing transcription and protein synthesis to proceed at normal rates (Young & Hodas, 1964; Mainprize & Cheevers, unpublished data). Hydroxyurea was removed when the cells would have normally been in mid S, and the base composition of DNA synthesized after reversal was determined by isopycnic centrifugation in neutral CsCl gradients.

Results are shown in Fig. 4. The addition of hydroxyurea at 9 hrs. post-serum to cultures synchronized as described in Fig. 1 immediately blocked the further entry of cells into S (Fig. 4 (a)). Upon reversal of hydroxyurea inhibition at 15 hrs. post-serum, the cells resumed DNA synthesis without a detectable lag and proceeded through an apparently normal S phase (Fig. 4 (a)). Neutral CsCl analysis of DNA pulse labeled with [^{3}H] dThd at 2-2.5 hrs. and 5.5-6 hrs. after hydroxyurea reversal (Fig. 4 (b) and (c)) shows that the base composition of DNA synthesized in the reversed cultures followed the same temporal pattern as that synthesized in untreated cultures allowed to enter S normally (Fig. 3): GC -rich main band DNA was synthesized first (Fig. 4 (b)) and was followed by a shift to the synthesis of sequences of average base composition and satellite DNA (Fig. 4 (c)).

These data show clearly that inhibition of the synthesis of replication units normally initiated between 9 and 15 hrs. post-serum does not alter the normal pattern of initiation of replication units when DNA synthesis is resumed. Thus, we conclude

FIG. 4. Effect of hydroxyurea on the order of initiation of replication units.

(a) Total DNA synthesis: Cultures were pulse-labeled with [^{3}H] dThd (2μCi/ml) for 1 hr. intervals at the indicated times in medium containing (●-●) or not containing (○-○) 0.01M hydroxyurea. Other cultures were treated with hydroxyurea between 9 and 15 hrs. post-serum, then washed and pulse-labeled at the indicated times (▲-▲).

(b) (c) Neutral CsCl isopycnic analysis of DNA synthesized after reversal of hydroxyurea inhibition: Cultures were treated with hydroxyurea between 9 and 15 hr. post-serum, washed and pulse-labeled with [^{3}H] dThd (20μCi/ml) at (b) 2 - 2.5 hr. or (c) 5.5 - 6 hr. post-reversal. [^{3}H]-DNA (○-○). ^{14}C marker DNA (●-●) was prepared by labeling cultures with [^{14}C] dThd (0.1μCi/ml plus 0.1μg/ml unlabeled dThd) between 9 and 25 hr. post-serum.

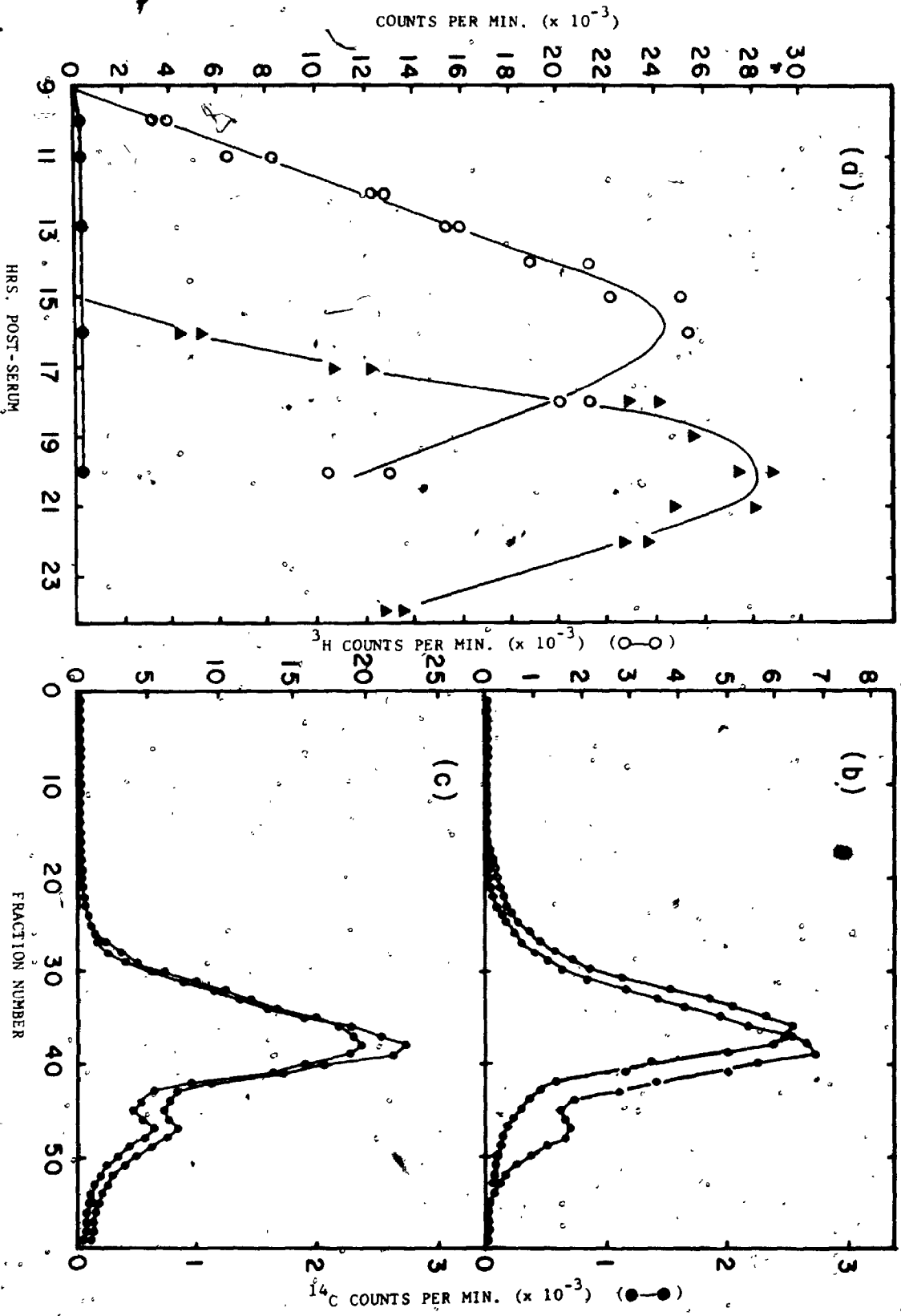

COUNTS PER MIN. (x 10^{-3})
HRS. POST-SERUM
(a)
^{3}H COUNTS PER MIN. (x 10^{-3}) (O—O)
(c)
(b)
FRACTION NUMBER
^{14}C COUNTS PER MIN. (x 10^{-3}) (●—●)

FIGURE 4

that the initiation of DNA sequences characteristic of mid S is dependent upon completion of replication units initiated during early S. This result argues against the possibility that the timing of initiation of various classes of replication units is programed entirely in the G1 phase of the cell cycle, suggesting instead that the S phase is, at least in part, a self-regulatory process. Further work on the effects of cycloheximide on replication unit initiation (Mainprize & Cheevers, unpublished data) suggest that the progress of the S phase depends upon the orderly synthesis of "initiator" proteins.

(d) Rate of Formation of High-Molecular-Weight Nascent Strands.

As an approach to the study of the spatial aspects of initiation of replication units during S phase, we sought to determine the rate of formation of high-molecular-weight nascent DNA strands by alkaline velocity sedimentation analysis. The preparation of fully denatured single strands of high-molecular-weight DNA and unambiguous measurement of their size is determined by the conditions of cell lysis and centrifugation (Cleaver, 1974; Hozier & Taylor, 1975). Under optimum conditions of cell lysis, single strands of 100 - 140 S can be obtained by alkaline sucrose gradient sedimentation free of artifacts resulting from incomplete denaturation (Cleaver, 1974). Changes in angular velocity acting on high-molecular-weight strands reduce the apparent molecular weight by generating asymmetrical distributions that give a low estimation of size (McBurney et al., 1971; Hozier & Taylor, 1975). However, even when centrifugation conditions are carefully controlled to provide the most accurate asessment of DNA of this size range, the maximum average molecular weight obtained is of the order of 1.7×10^8 daltons (Hozier & Taylor, 1975).

There is some question as to whether DNA strands of this discrete length occur in vivo and thus have functional significance (Hozier & Taylor, 1975), or whether they are the product of physical constraints on the extraction of high molecular weight single stranded DNA (Cleaver, 1974). They nevertheless represent the maximum size range of single stranded DNA amenable to analysis by centrifugation methods. DNA of this size is consistently obtained from cells labeled in exponential phase cultures prior to synchronization (Fig. 5 (a)), and thus represents the size of complete template strands obtained by our procedure (Cheevers et al., 1972; Cheevers & Hiscock, 1973).

DNA strands of molecular weight 1.7×10^8 daltons originate from a double stranded segment 170μm in length. The average size of replication units in various unsynchronized mammalian cell types varies between 20 and 60μm (Huberman & Riggs, 1968; Hand & Tamm, 1974; Housman & Huberman, 1975), and does not appear to vary during S phase (Housman & Huberman, 1975). Thus, we estimate that strands of 1.7×10^8 daltons result from joining of the nascent strands of a minimum of 3 average length replication units. Chain growth within nascent strands of this size, therefore, is a function of merging of nascent strands of adjacent replication units as well as initiation of chain growth within replication units.

Fig. 5 (a-e) shows the assimilation of nascent DNA pulse-labeled for 5 min. in early S into high-molecular-weight strands during a chase with unlabeled dThd. Fig. 5 (f-j) shows the assimilation of DNA pulse labeled for 5 min. in mid S. The average molecular weight of the DNA shown in these sedimentation distributions as well as other 5 min. pulse labels and chases not shown was determined as described in Materials and Methods. Results are shown in Fig. 6. The following points may be made from these data:

FIG. 5 Pulse-chase analysis by velocity sedimentation in alkaline sucrose gradients of DNA strands synthesized in synchronized cells.

Cells were labeled with [^{3}H] dThd (66 μCi/ml.) for 5 min at 11 hrs. post-serum (a) and chased with unlabeled dThd for 25 min. (b), 50 min. (c) 120 min. (d), and 480 min. (e). Cells were also labeled, as described above, at 15 hrs. post-serum (f) and chased with unlabeled dThd for 30 min. (g), 60 min. (h), 120 min. (i) and 240 min. (j).

Prior to administration of [^{3}H] dThd, cultures were incubated with [^{14}C] dThd during the period of exponential growth prior to synchronization (Fig. 1) to label template DNA strands ((a), (● - ●)). Labeled DNA strands were isolated and sedimented in alkaline sucrose gradients as described in Materials and Methods. The vertical dash line denotes the peak position of closed-circular polyoma virus DNA used as a sedimentation marker (see Fig. 7 (a)).

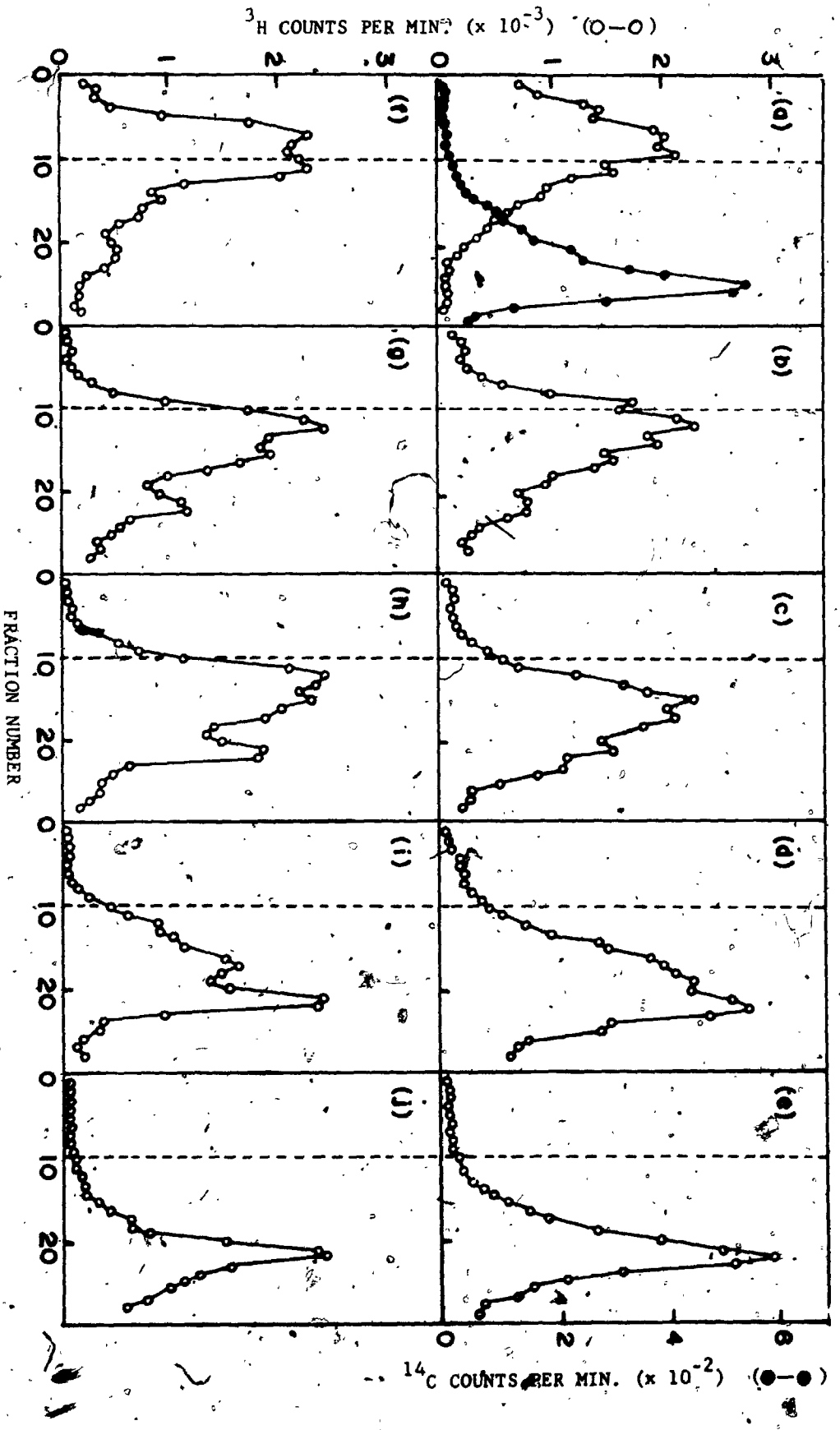
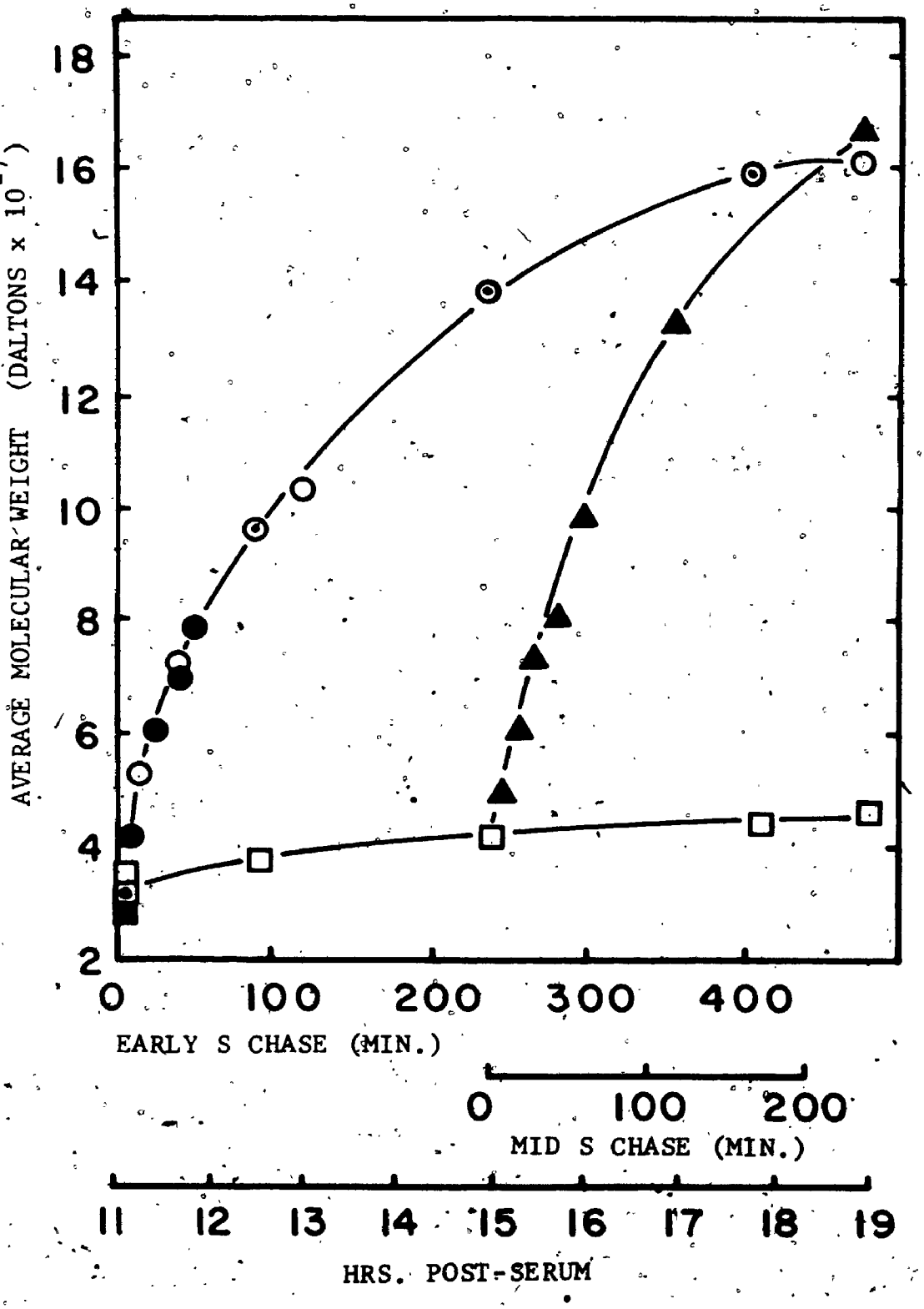

^{3}H COUNTS PER MIN. (x 10^{-3}) (O—O)
0
1
2
3
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
0
10
20
FRACTION NUMBER
0
2
4
6
^{14}C COUNTS PER MIN. (x 10^{-2}) (●—●)

FIGURE 5

FIG. 6 Rate of formation of high-molecular-weight nascent DNA strands in synchronized cells.

Cells were pulse-labeled with [^{3}H] dThd (66 μCi/ml) for 5 min. at the indicated times (□ ⊡ ■). Cultures labeled at 11 hr. and 15 hr. post-serum were chased with unlabeled dThd ((○ ⊙ ●) Chase of DNA labeled at 11 hr., (▲) Chase of DNA labeled at 15 hr.) Nascent DNA strands were analyzed by velocity sedimentation in alkaline sucrose gradients as described in Fig. 5, and average molecular weights were calculated as described in Materials and Methods. Different symbols denote separate experiments.

FIGURE 6


AVERAGE MOLECULAR WEIGHT (DALTONS x 10^{-7})
18
16
14
12
10
8
6
4
2
0
100
200
300
400
EARLY S CHASE (MIN.)
0
100
200
MID S CHASE (MIN.)
11
12
13
14
15
16
17
18
19
HRS. POST-SERUM

(i) A 5 min. pulse of [^{3}H] dThd at any point in the S phase labels strands with an average molecular weight of less than 5×10^7 daltons (Fig. 6). Considering the size of mamalian DNA replication units ($2-6 \times 10^7$ daltons of single stranded DNA), it is evident that the strand containing the labeled segments is of the order of one replication unit in length. The degree of heterogeneity in the size of these strands is similar to the heterogeneity in replication unit lengths observed by autoradiography. Thus we assume that nascent strands labeled during a 5 min. pulse represent single replication units in the process of replication. Nascent DNA strands of this size have been previously observed in alkaline sucrose gradients and their origin similarly interpreted (Huberman & Horwitz, 1973; Gautschi et. al, 1973)

(ii) The average molecular weight of these nascent strands increases during S phase. Such an increase in size could be attributable to an increase in replication unit size. Direct measurement of the size of replication units during S phase by autoradiography (Houseman & Huberman, 1975) indicates that there is no increase in the average inter-initiation distance. Thus the increase in size of the strands labeled by a 5 min. pulse most likely results from an increase in the rate of merging of nascent strands of adjacent replication units.

(iii) Pulse-chase analysis of the strands labeled in a 5 min. pulse of [^{3}H] dThd in early S phase (11 hrs. post-serum) (Fig. 5 (a-e)) shows their assimilation into high-molecular-weight strands. The fundamental observation is that the complete assimilation of replication unit size nascent strands into high-molecular-weight strands occurs over a period of 8 hrs. (Fig. 6). Assuming that the maximum size DNA is comprised of the nascent strands of at least 3 replication units, it would be formed by the movement of a minimum of 6 replication forks. Fork movement alone at the rate determined in Table 2 could result in the assimilation of replication unit-size nascent strands into high-molecular-weight DNA in a period of only 10-15 min. Fork movement at slower rates, such as those determined by autoradiography (Huberman & Riggs, 1968; Housman & Huberman, 1975) should lead to complete assimilation by 60 min. Huberman & Horwitz (1973) have observed the complete assimilation of replication unit-size strands into high-molecular-weight strands in 60 min. in asynchronously growing Chinese hamster cells. We have observed that in exponentially growing mouse embryo cells, the assimilation of similar strands is also complete by 60 min. (Fig. 11; Cheevers et al., 1972). The reasons for this difference between synchronized and unsynchronized cells are considered in the Discussion.

(iv) Pulse-chase analysis of nascent strands labeled in a 5 min. pulse of [^{3}H] dThd in mid S (15 hrs. post-serum) (Fig. 5 (f-j)) shows that their rate of assimilation into high-molecular-weight strands is much faster than that of strands labeled similarly in early S phase (Fig. 6). This is evident in Fig. 5 (f-j) where high-molecular-weight strands of maximum size obviously accumulate at a greater rate than in the early S chase (Fig. 5 (a-e)). Fig. 6 shows that maximum size strands

are formed in a period of 4 hrs. Thus, the rate of assimilation of nascent DNA strands the size of individual replication units into maximum size strands several replication units in length depends on the time in the S phase that the synthesis of these strands is initiated.

(e) Spatial Organization of Replication Units

The basic problem considered in this section is: What is the explanation for the fact that mid S replication units are assimilated into high-molecular-weight strands two times faster than early S replication units? This question may be answered, in part, in terms of the spatial arrangement of replication units within the mammalian genome. Thus, if mid S replication units are spatially interspersed among early S replication units, the rate of elongation of early S sequences into high-molecular-weight strands may be limited by the initiation of mid S sequences. Alternatively, if replication units are clustered along the genome, the slow rate of assimilation of early S sequences may involve a controlled process of joining of adjacent replication units.

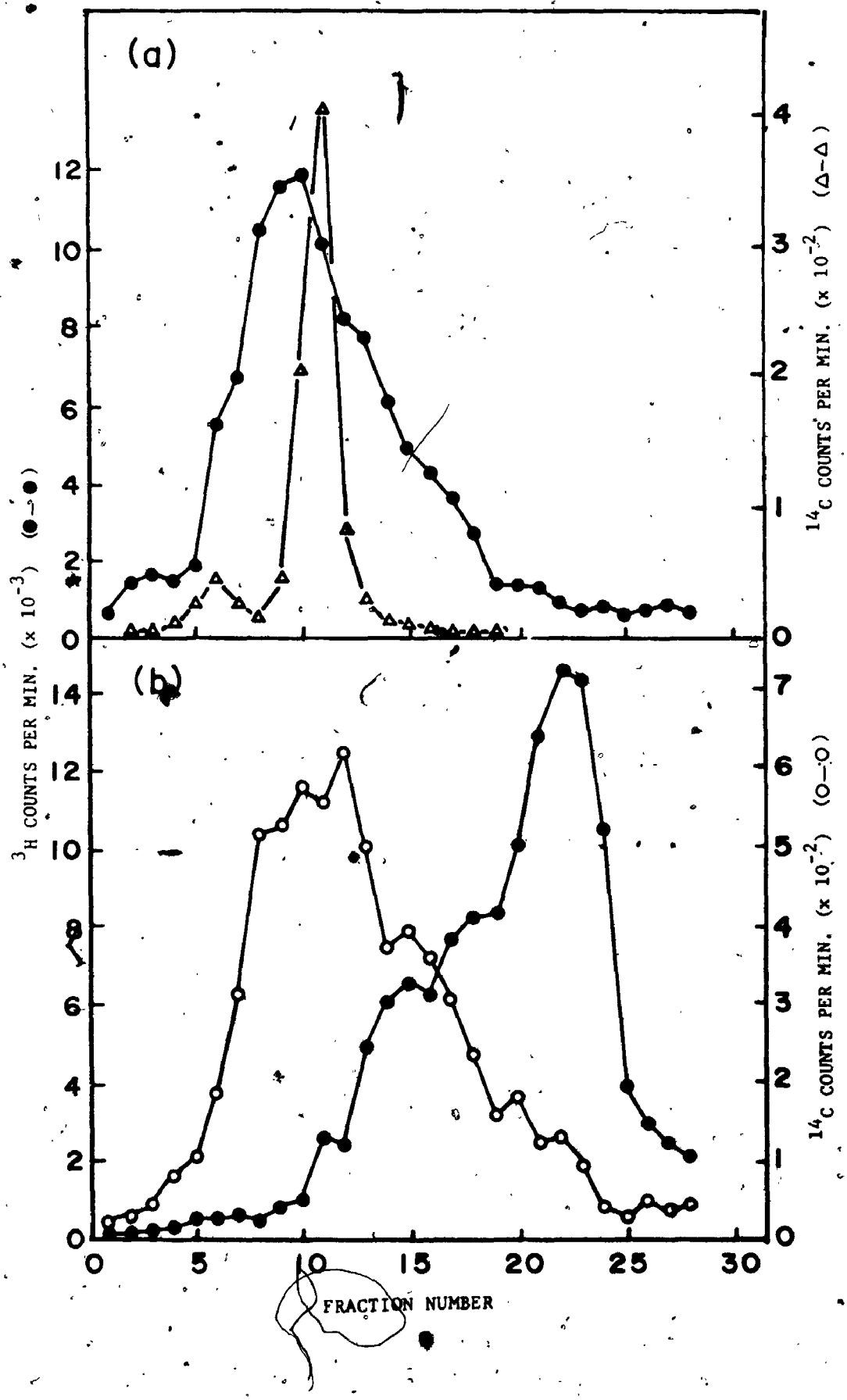
The following experiments attempt to measure the degree of clustering of initiation sites within the high-molecular-weight strands described in section (d). The question asked is: Does the assimilation into high-molecular-weight strands of replication units labeled in early S (Fig. 5 (a-e), Fig. 6) involve replication units initiated later in S?

Preliminary evidence for lack of interspersion of replication units was obtained in the experiment described in Fig. 7. In this experiment, DNA was pulse labeled for 30 min. with [^{3}H] dThd in early S and chased until mid S. In the last

FIG. 7. Alkaline sedimentation analysis of DNA synthesized in synchronized cells.

Cultures were labeled with [^{3}H] dThd (20 μCi/ml) for 30 min. at 11 hr. post-serum. The labeled medium was then replaced with medium containing unlabeled dThd, and incubation was continued until 16 hr. [^{14}C] dThd (2 μCi/ml) was added at 15.5 hrs. Labeled DNA was isolated and sedimented in alkaline sucrose gradients as described in Fig. 5. (a) DNA pulse-labeled at 11-11.5 hr. post-serum (●-●) plus [^{14}C]-labeled form I polyoma virus DNA (Δ-Δ) included as a sedimentation marker. (b) DNA isolated from chased cultures. (●-●) ^{3}H radioactivity. (o – o) ^{14}C rddioactivity.

FIGURE 7


(a)
(b)
^{3}H COUNTS PER MIN. (x 10^{-3}) (●–●)
^{14}C COUNTS PER MIN. (x 10^{-2}) (Δ–Δ)
^{3}H COUNTS PER MIN. (x 10^{-3}) (●–●)
^{14}C COUNTS PER MIN. (x 10^{-2}) (O–O)
FRACTION NUMBER

30 min. of the chase, [^{14}C] dThd was added to the same cultures to label replicating mid S sequences. Alkaline velocity sedimentation analysis showed, as expected, that the size distribution of nascent strands labeled by [^{3}H] dThd in early S was of the order of single replication units (Fig. 7 (a)) and that during a chase with unlabeled dThd, these strands were assimilated into DNA of higher molecular weight (Fig. 7 (b)). Note, however, that mid S sequences labeled with [^{14}C] dThd during the period of elongation of [^{3}H] - labeled strands sedimented predominantly as single replication units (Fig. 7 (b)). Some overlap of [^{14}C] - and [^{3}H] - labeled strands is apparent. Despite this, it is evident that a significant amount of [^{3}H] - labeled early S DNA was elongated without involvement of mid S sequences.

The results described in Fig. 7 support a clustered model of replication unit organization. An additional point is that DNA strands pulse labeled in early S for 30 min. are only slightly longer than those labeled for only 5 min. (compare Fig. 7 (a) and Fig. 5 (a)). In fact, extension of the labeling period to 1 hr. is still insufficient to show an increase in the size distribution of nascent strands comparable with that predicted by the rate of chain elongation (Fig. 12 (a)). These observations argue strongly for the view that the rate of assimilation of early S strands is limited by the joining of adjacent replication units within clusters.

Because of the ambiguity resulting from the overlap of [^{3}H] - and [^{14}C] - labeled DNA in Fig. 7, we sought a more sensitive approach to the question of whether replication units labeled in early and mid S are part of the same high-molecular-weight-polynucleotide strand. The method of distinguishing replication units synthesized at different stages of the S phase by base composition (section (c)) was tried, but was judged insufficient to distinguish the small changes in buoyant density

involved. Differential isotopic and density labeling provides a direct and probably the most sensitive means of clarifying this question. Accordingly, the following two protocols were established to differentially label early and mid S replication units: (a) Replicating DNA was labeled for 10 min. with [^{3}H] dThd at 11 hrs. post-serum, chased with unlabeled dThd and then substituted with BrdUrd between 13.5 and 18.5 hrs. post-serum. (b) In a reversal of this procedure, DNA was labeled with BrdUrd between 11 and 11.5 hrs. post-serum, chased with dThd, and labeled with [^{3}H] dThd between 13 and 18 hrs. post-serum.

In all experiments, incorporation of BrdUrd was monitored by parallel cultures labeled with [^{3}H] BrdUrd. Fig. 8 shows that the elongation of nascent strands labeled by both protocol (a) and (b) is similar to that described in Fig. 5 and Fig. 6. Thus, minipulations involved in density labeling did not prevent the assimilation into high-molecular-weight strands of DNA pulse labeled early in S phase.

The density of high-molecular-weight strands was measured by isopycnic centrifugation in alkaline Cs_2SO_4 - CsCl gradients as described in Fig. 9. Cells were layered on top of a Cs_2SO_4 - CsCl solution as described in Materials and Methods in a manner identical to that used to layer cells for velocity sedimentation. It is clear from Fig. 9 that high-molecular-weight nascent DNA strands labeled by both protocol (a) and (b) fail to show association of both isotopic and density label within the same polynucleotide strand. Consequently the assimilation of replication units pulse labeled early in S phase into high-molecular-weight strands does not involve replication units initiated between 2 and 7 hrs. later in S phase.

FIG. 8. . Alkaline sedimentation of high-molecular-weight DNA strands from synchronized cells incubated with [^{3}H] dThd and BrdUrd at different stages of S phase.

(a) Cultures were incubated with [^{3}H] dThd (33 μCi/ml.) for 10 min. at 11 hrs. post-serum, (●-●). High-molecular-weight marker DNA (○-○) was labeled with [^{14}C] dThd as described in Fig. 5 (a). (b) Cultures incubated with [^{3}H] dThd as described in (a) were chased with unlabeled dThd until 13.5 hrs. post-serum (●-●) and then incubated with BrdUrd until 18.5 hrs. post-serum, (○-○). (c) Cultures were incubated with [^{3}H] BrdUrd (25 μCi./ml.) between 11 and 11.5 hrs. post - serum (●-●). High-molecular-weight marker DNA (○-○) was prepared as in (a). (d) Cultures incubated with [^{3}H] BrdUrd as described in (c) were chased with dThd until 13 hrs. (●-●) and until 18 hrs. (○-○) post-serum.

FIGURE

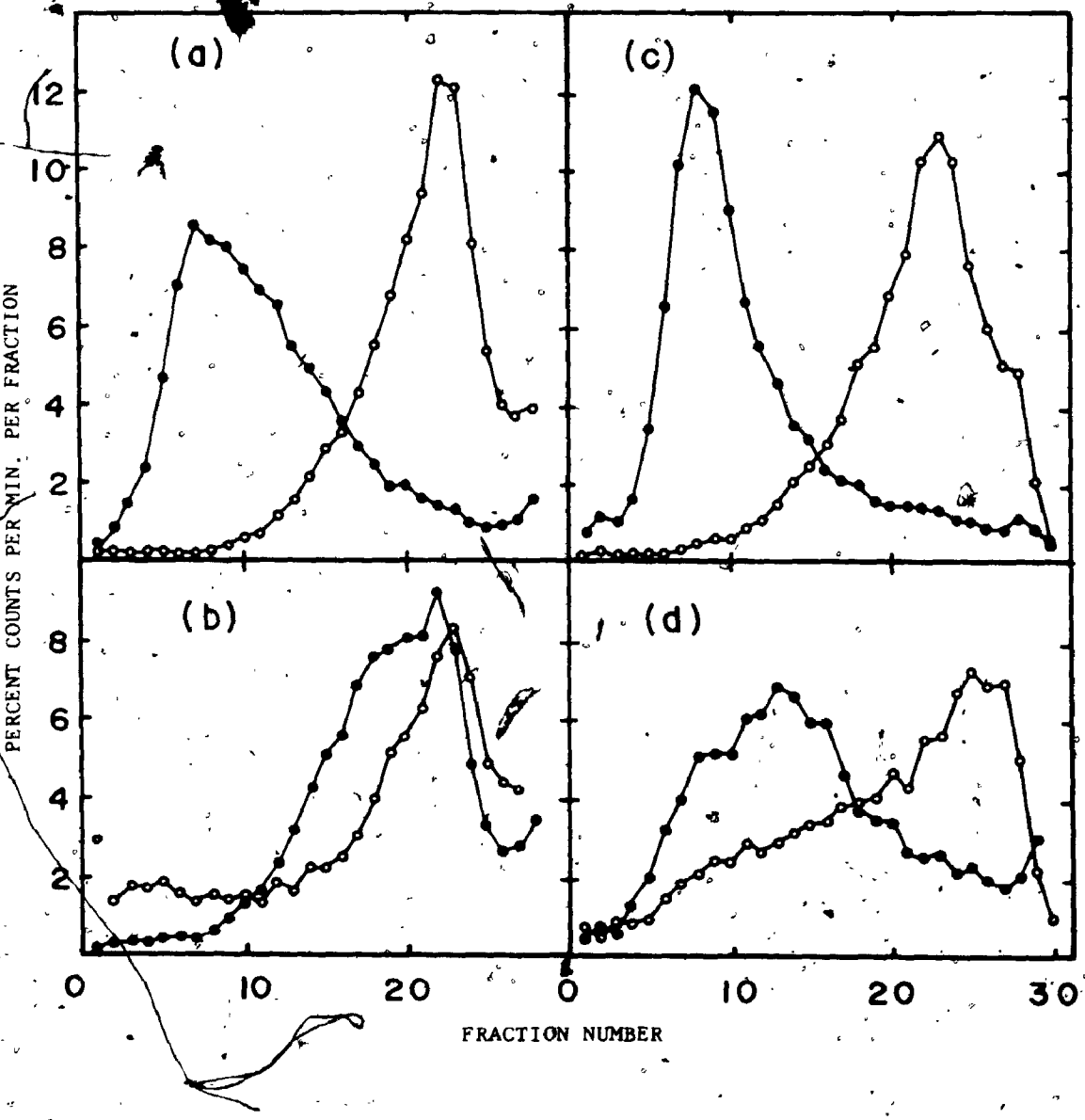
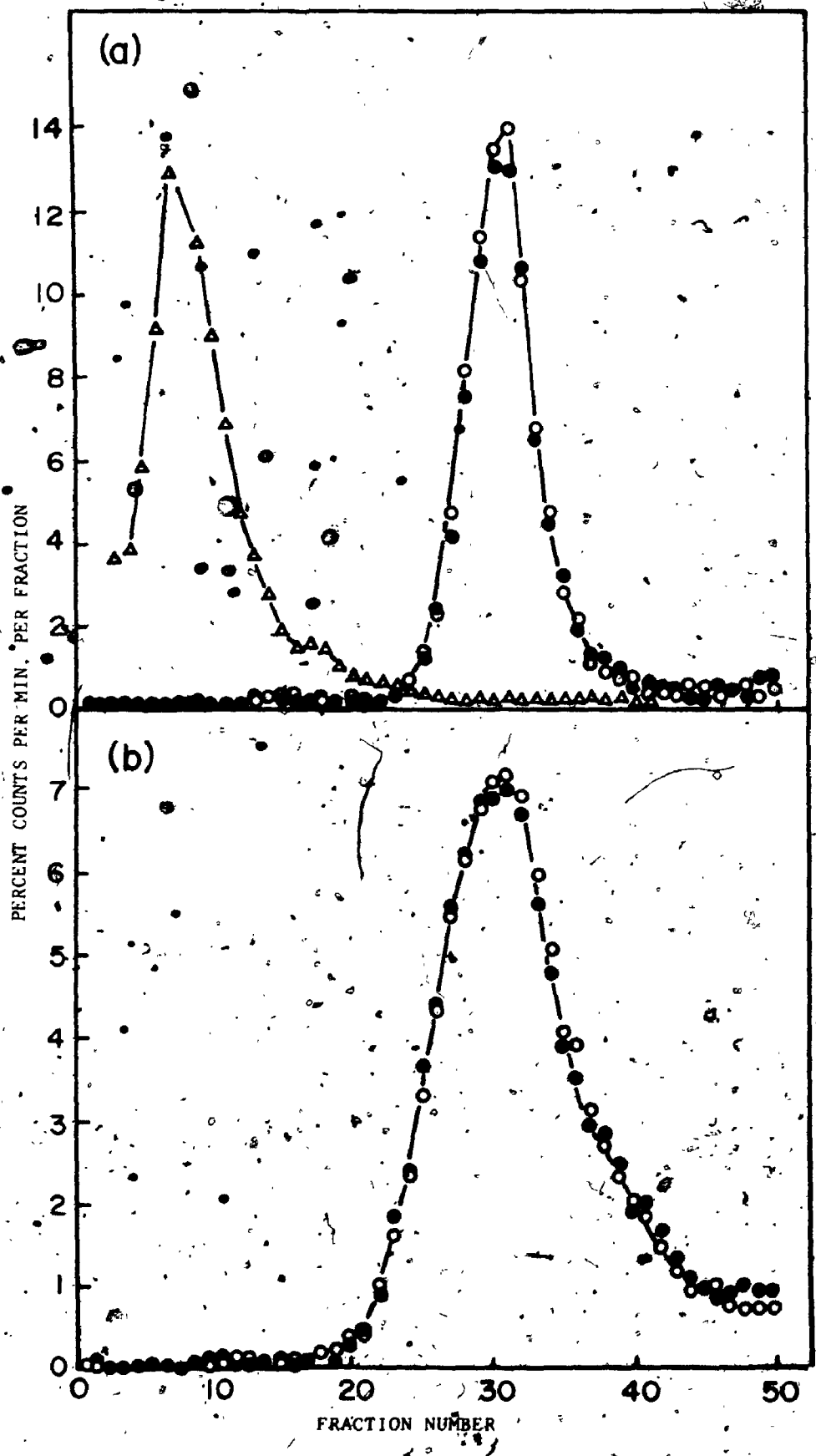

(a)
(c)
(b)
(d)
12
10
8
6
4
2
8
6
4
2
0
10
20
0
10
20
30
PERCENT COUNTS PER MIN. PER FRACTION
FRACTION NUMBER

FIG. 9. Alkaline Cs_2SO_4 - CsCl isopycnic centrifugation of high-molecular-weight DNA strands formed in synchronized cells incubated with [^{3}H] dThd and BrdUrd at different stages of S phase.

(a) Protocol (a). Synchronized cultures were incubated with [^{3}H] dThd (33 μCi./ml.) for 10 min. at 11 hrs. post-serum, chased with unlabeled dThd until 13.5 hrs. and incubated with BrdUrd until 18.5 hrs. post-serum (O-O). (b) Protocol (b). Synchronized cultures were incubated with BrdUrd between 11 and 11.5 hrs. post-serum, chased with dThd until 13 hrs., and incubated with [^{3}H] dThd (3 μCi./ml.) between 13 and 18 hrs. post-serum (O-O). [^{14}C] dThd - labeled DNA (●-●) and [^{3}H] BrdUrd - labeled DNA (▲-▲) used as density markers were purified from randomly growing cultures. Cell lysis and denaturation of DNA were performed by direct layering of cells onto an alkaline Cs_2SO_4 - CsCl solution as described in Materials and Methods.


(a)
(b)
PERCENT COUNTS PER MIN. PER FRACTION
FRACTION NUMBER
0
2
4
6
8
10
12
14
1
3
5
7
10
20
30
40
50

2.3 DISCUSSION

(a) Rate of DNA Synthesis per Cell as a Function of Chain Growth Within Replication Units and Frequency of Initiation of Replication Units.

In mouse embryo cells synchronized by growth in a low concentration of serum, the rate of DNA synthesis per cell increases by 20 - 50% during the S phase. We conclude that this increase is accounted for primarily by an increase in the rate of DNA chain growth within replication units, rather than by an expansion of the number of growing points per cell, on the basis of the following considerations: Accuracy in determination of the rate of chain growth by experiments of the type described in Fig. 2 and Table 2 depends upon a constant ratio of initiation to termination of replication units during the [^{3}H] dThd pulse. Since this ratio is unknown, one cannot precisely quantitate either the rate of chain growth or the number of replication units in operation during the S phase. However, the value of F, which represents the fraction of labeled DNA of hybrid density in Fig. 2 decreased in mid S relative to early S (Table 2). This decrease is due to either an increase in the rate of chain growth or to a decrease in the ratio of initiation to termination of replication units labeled during mid S as compared to early S. Assuming that chain growth is unchanged, a decrease of the initiation: termination ratio is equivalent to a reduction in the number of operating replication units, which is untenable with an increase in the rate of DNA synthesis per cell (Table 1). Therefore, the reduction in the value of F shown in Table 2 must have resulted from an increase rate of chain elongation within replication units. This increase fully accounts for the increase in the rate of DNA synthesis per cell during this interval

of S phase. This pattern of replication unit operation during the mammalian S phase supports previous conclusions regarding chain growth rates in HeLa cells synchronized by mitotic selection and double dThd block (Painter & Schaefer, 1971) as well as recent measurements of fork progression using a method independent of the ratio of initiation to termination of DNA chains (Housman & Huberman, 1975). The finding that the increase in the rate of fork progression fully accounts for an increase in the rate of DNA synthesis per cell is in conflict with the results obtained by Painter & Schaefer (1971) employing HeLa cells synchronized by the mitotic selection technique. These authors found that a 2-fold increase in the rate of fork progression was accompanied by a 5-fold increase in the rate of DNA synthesis per cell, indicating that a 2.5-fold increase in the rate of initiation of new growing points had occurred during S phase. In addition to this these authors found that synchronization of cells by a method involving blocking of DNA synthesis resulted in no increase in the rate of initiation of new growing points during the S phase that occurred after release of the block. The serum starvation technique of cell synchronization described in Fig. 1 probably does not represent a direct block in DNA synthesis since a lag period of 10 hrs. precedes the onset of DNA synthesis after serum stimulation. Thus it is not likely that the method of synchronization used in this study alters the normal pattern of initiation of replication units. The data of Fig. 3 support this conclusion. Thus there is no apparent reason for the discrepancy between our results and those of Painter & Schaefer (1971). Definitive elucidation of factors influencing the rate of DNA synthesis during S phase and of the mechanisms by which a cell alters its rate of DNA synthesis must await further investigation.

(b) Temporal Organization of Initiation of Replication Units

In agreement with previous work using other methods of cell synchronization that that described here (Tobia et al., 1970; Bostock & Prescott, 1971 abc; Flamm et al., 1971; Tobia et al., 1971) the present study shows that replication units comprising the mouse genome are initiated throughout the S phase in an orderly pattern, characterized by differences in base composition of sequiences initiated at different times in S. GC-rich sequences of main band nuclear DNA (ρ = 1.702 gm./cm.) are replicated first. These are followed by the initiation of sequences which gradually shifts the base composition of labeled DNA to AT-rich late in the S phase. Satellite DNA (ρ = 1.691 gm./cm.) is replicated in mid and late S.

The order with which sequences are initiated has allowed us to begin preliminary studies on the control of S phase. In this context, we have determined that the orderly progress of the S phase is at least in part, a self-regulatory process, i.e. the initiation of mid S replication units is dependent upon the replication of early S sequences.

Little is known concerning the mechanism by which replication of one class of replication units triggers the initiation of another class. It seems very likely however, that this mechanism is mediated by transcription and the orderly formation

of specific proteins, since continuous protein synthesis is required for both the initiation of replication units during S and maintenance of the polymerization of DNA strands within replication units (Mainprize & Cheevers, unpublished data).

(c) Spatial Organization of Replication Units

The data from these experiments provide a picture of the spatial aspects of the formation of high-molecular-weight nascent DNA strands during S phase. High-molecular-weight strands are defined as nascent strands of 1.7×10^8 daltons which represent a random sample of fragments, each consisting of a minimum of 3 average length replication units. The rate of formation of a nascent DNA strand several replication units in length is determined by three factors: (i) the frequency of initiation of replication over that segment of DNA, (ii) the rate of chain elongation within the constituent replication units, and (iii) the rate of ligation of completed nascent strands of adjacent replication units.

The data of Fig. 6 shows that the complete assimilation of nascent strands of replication units pulse labeled early in S phase covers a period of 8 hrs., a length of time similar to the duration of S phase. One explanation for this result is that the formation of these strands is continuously dependent on initiation of replication. We sought to answer this question by determining whether assimilation of nascent strands of replication units pulse labeled early in S phase involves replication units initiated later. It was found that despite the fact that the frequency of initiation in mid S is at least as high as that in early S, replication units labeled during a short pulse in early S are assimilated without the participation of these newly initiated sequences. These results indicate that there is no significant spatial interspersion of mid S

replication units among the replication units initiated in early S. Thus we conclude that the genome is characterized by a high degree of spatial clustering of replication sections which are independently replicated, at least through most of the S phase. A clustered model of genome organization is fundamentally in agreement with data derived by DNA fibre autoradiography (Huberman & Riggs, 1968; Hori & Lark, 1973; Hand & Tamm, 1974; Hand, 1975). Estimates of the size of replication unit clusters cannot be made because of physical constraints on the isolation of high-molecular-weight DNA. A minimum average value of 1.7×10^8 daltons of single-stranded DNA may be assigned, but they are probably longer.

The involvement in assimilation of chain growth within the constituent replication sections of early S clusters cannot be determined from Fig. 6, since alkaline sucrose gradient centrifugation as performed here does not directly measure DNA polymerization within replication units, but rather the overall process of assimilation of labeled segments into high-molecular-weight strands. The data of Table 2 provide a better measurement of rate of chain growth along replication units. These results indicate that this parameter does not play a rate limiting role in assimilation of early S replication units, since it is much higher than the overall rate of chain growth determined in Fig. 6. Even if a slower rate of chain growth within replication units is assumed, such as that measured by autoradiography (Huberman & Riggs, 1968; Housman & Huberman, 1975), complete assimilation would occur in a period of only about 1 hr.

Thus the following disparity is evident: The frequency of initiation of replication units within high-molecular-weight strands and the rate of chain growth within them are adequate to ensure the formation of high-molecular-weight DNA

strands within 1 hr. at most. Despite this, assimilation of the constituent replication units of early S clusters requires 8 times longer, i.e. essentially the entire S phase. To account for this, we propose that the process of merging nascent strands of adjacent replication sections plays a major role in the rate of completion of high-molecular-weight strands. This is strongly supported by the fact that a 12-fold increase in the time of exposure of S phase cells to [^{3}H] dThd labels DNA strands which fall markedly short of the size distribution predicted by the rate of strand growth alone (Fig. 12 (a)).

The assimilation of nascent strands of replication units labeled in mid S covers a period of 4 hr. The frequency of initiation of these replication units is at least as high as that occuring in early S and the rate of chain growth is faster than that of early S replication units. Thus, the same disparity noted in early S between the length of time necessary to assimilate the DNA and the prevailing rate of chain elongation is also evident in mid S.

Several factors may contribute to decrease the length of time required for complete assimilation of DNA strands at later stages of the S phase. First, of course, is the fact that the rate of strand growth along replication units increases. Secondly, the rate of merging of adjacent replication units may increase. This is supported by the fact that a 1-hr. pulse with [^{3}H] dThd administered at progressively later stages of the S phase labels DNA with a progressively increasing percentage of maximum-size strands (Fig. 12). Another factor which probably contributes to these results is the merging of replication unit clusters during later stages of the S phase. The present work shows no evidence for the merging of clusters, but this is not surprising if the length of clusters exceeds the maximum size of DNA strands

isolated. The greater the length of replication unit clusters relative to the molecular weight of the DNA analyzed, the more difficult it becomes to detect the strands containing the sequences of adjoining clusters.

We have previously observed that complete assimilation of replication unit size nascent strands into high-molecular-weight DNA occurs in exponentially growing mouse embryo cells at a much faster rate than that measured in Fig. 6 (Fig. 11; Cheevers et al., 1972). Huberman & Horwitz (1973) have also determined that assimilation is complete in randomly growing Chinese hamster cells within a period of 1 hr. Since randomly growing cultures contain cells in every stage of the S phase, they demonstrate a rate of DNA chain elongation which measures the average of that observed at various stages of S in synchronized cultures. The finding that assimilation in asynchronous cultures proceeds at an apparent rate of 8 times faster than early S-phase cells and 4 times faster than mid S-phase cells is consistent with the model of genome organization presented here. Such an increase would be predicted from the presence in a randomly growing culture of cells in the later stages of S, in which merging of adjacent replication units is maximal and in which the joining of previously replicated clusters is occurring.

APPENDIX

FIG. 10. Uptake and incorporation of [^{3}H] dThd by mouse embryo cells.

Medium containing [^{3}H] dThd (20 μCi./ml.; specific activity, 3.78×10^7 counts/min./nmole) was added to exponential phase mouse embryo cultures. At the indicated times, incorporation was stopped in duplicate cultures by addition of cold SSC. Cells were washed twice in cold SSC and lysed with 1% SDS. An equal volume of 10% trichloroacetic acid was added to the lysate, and the mixture filtered through Millipore filters. The acid-insoluble fraction retained on the filters was analyzed for radioactivity as described in Materials and Methods. Labeled dThd nucleotides were isolated from the acid-soluble filtrate fraction by DEAE-cellulose thin layer chromatography. 10 μl. of the 5% trichloroacetic acid soluble fraction were analyzed along with 1 μl. each of 0.05 M dThd and 0.03 M dTTP by ascending chromatography for 1 hr. in [0.005 M ammonium formate - 0.001 M formic acid] . . (●-●), Incorporation into acid-insoluble fraction. (O-O), uptake into acid-soluble nucleotide fraction.

FIGURE 10

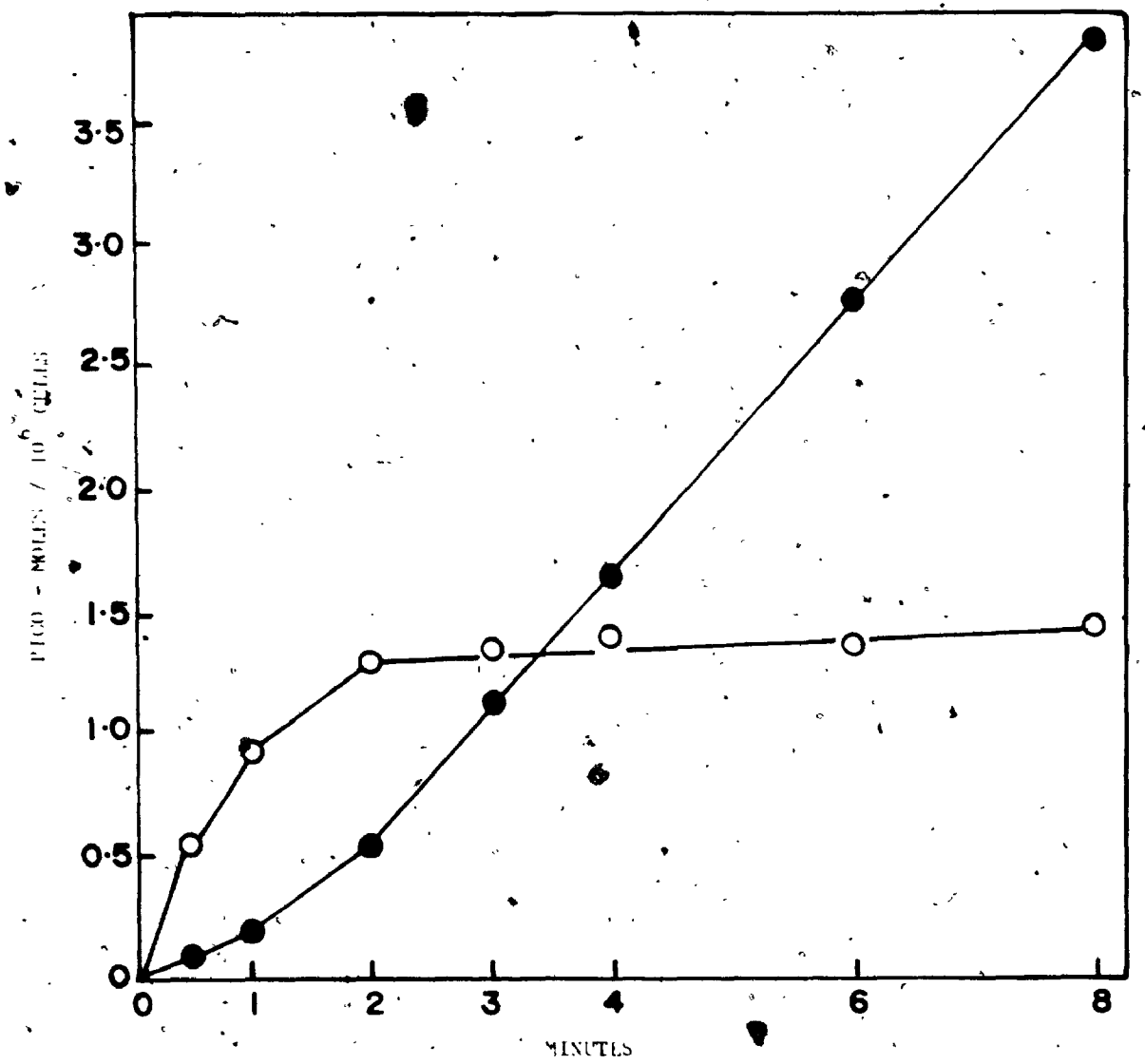
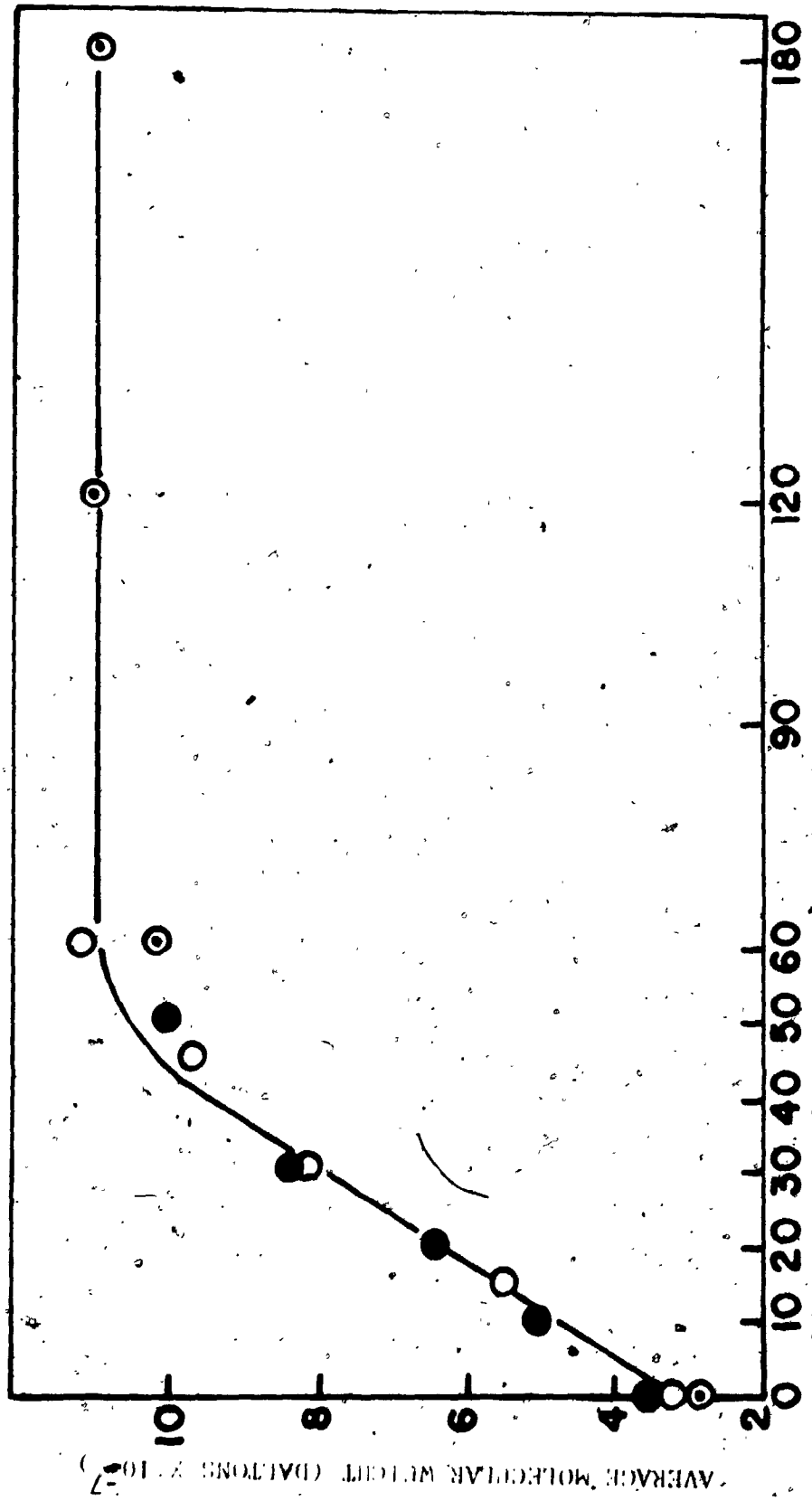


FIG. 11 Rate of formation of high-molecular-weight nascent DNA strands in unsynchronized cells.

Exponentially growing cultures of mouse embryo cells were pulse labeled with [^{3}H] dThd (33μCi./ml.) for 10 min. and chased with unlabeled dThd for the times indicated. Nascent DNA strands were analyzed by velocity sedimentation in alkaline sucrose gradients and average molecular weights were calculated as described in Materials and Methods. Different symbols denote separate experiments.

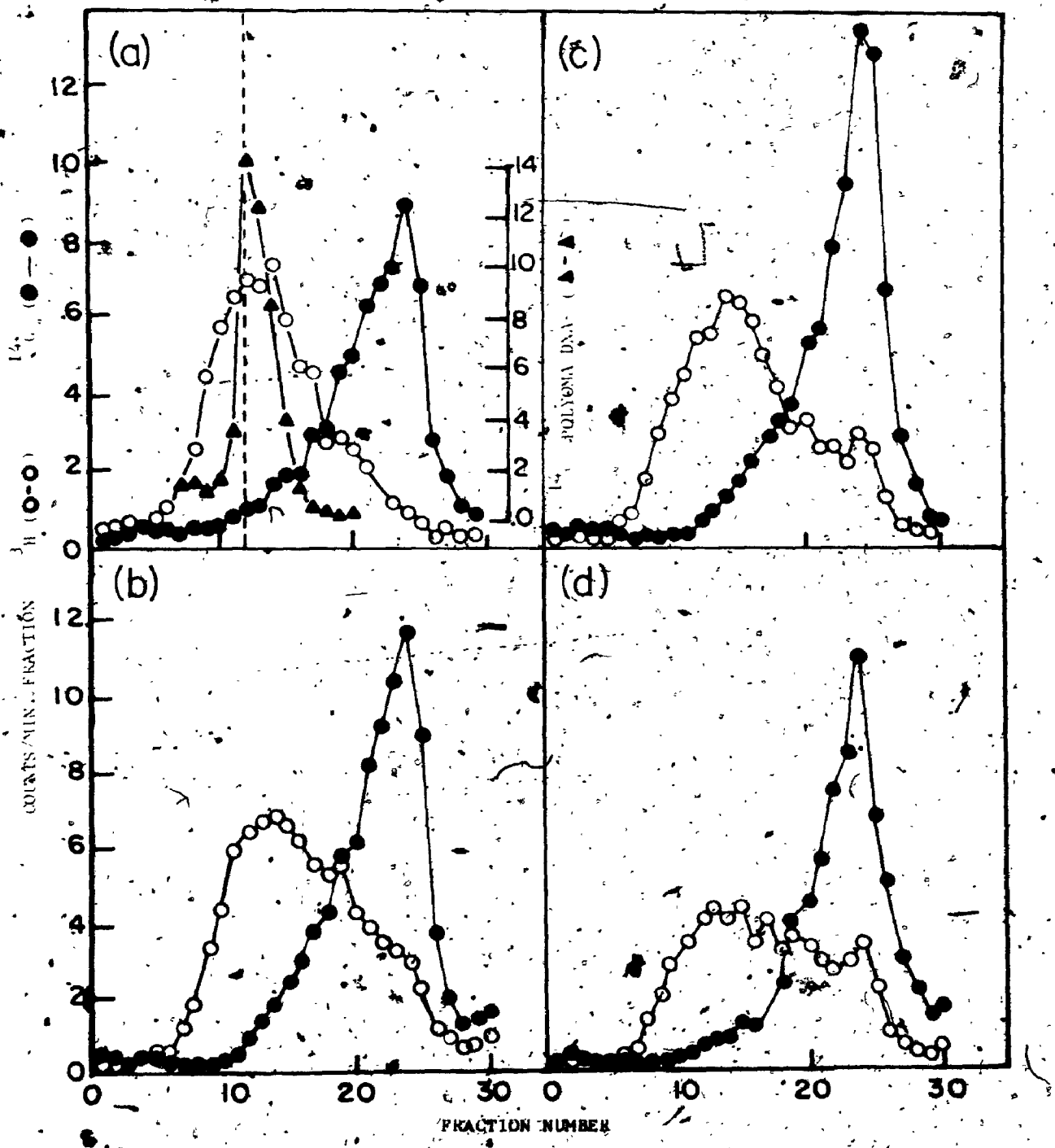


AVERAGE MOLECULAR WEIGHT (DALTONS X 10^{-7})

FIGURE 11.

FIG. 12 Analysis by velocity sedimentation in alkaline sucrose gradients of DNA synthesized in synchronized cells.

Cells were pulse labeled with [^{3}H] dThd (10 μCi./ml.) for 60 min. at: 10 - 11 hrs., (a); 14 - 15 hrs., (b); 18 - 19 hrs., (c); and 22 - 23 hrs., (d), post-serum. High-molecular-weight marker DNA was labeled with [^{14}C] dThd as described in Fig. 5 (a). DNA was analyzed by alkaline sucrose velocity sedimentation as described in Fig. 5. ^{14}C - labeled Form I polyoma virus DNA was included as a sedimentation marker. (○-○), ^{3}H - labeled DNA; (●-●), ^{14}C - labeled cell DNA; (▲-▲) ^{14}C - labeled polyoma virus DNA.


(a)
(b)
(c)
(d)
COUNTS/MIN./FRACTION
FRACTION NUMBER
0
10
20
30
2
4
6
8
12
14

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