ASPECTS OF REGULATION OF MITOCHONDRIAL DNA

REPLICATION AND TRANSCRIPTION

IN MAMMALIAN CELLS

Thesis by

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Dedicated to my parents,

Eddie and Esther

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ABSTRACT

The first part of the thesis deals with a study of
the synthesis of RNA in isolated HeLa cell mitochondria.
Isolated mitochondria were capable of supporting close to
linear incorporation of the radioactive precursor
$[5-^3H]$UTP or $[5-^3H]$ATP for at least 1 hour. Virtually
all of the RNA labeled in vitro was shown to consist of
mitochondrial DNA transcripts complementary to one or the
other of the two strands. At least 81% of the RNA
labeled in the presence of $[5-^3H]$UTP and 72% labeled in
the presence of $[5-^3H]$ATP hybridized to mitochondrial DNA;
70% of the RNA homologous to mitochondrial DNA hybridized
to the "H" strand and 30% to the "L" strand. Sucrose
gradient analysis of the products labeled with either
$[5-^3H]$UTP or $[5-^3H]$ATP showed the presence of mitochondria-
specific ribosomal 16S and 12S RNAs.

The effect of cycloheximide pretreatment of the cells
on the RNA synthetic capactiy of isolated organelles was
investigated. Incorporation of $[5-^3H]$UTP into RNA of
mitochondria isolated from HeLa cells treated with
200 µg cycloheximide/ml up to 4 hours was found to decrease
very modestly. Addition of normal cytoplasm to these
mitochondria resulted in a stimulatory effect on RNA
synthesis independent of the length of cycloheximide
treatment.

The second part of the thesis concerns an investigation of the timing of mitochondrial DNA synthesis during the cell cycle in mouse cells. In LM(TK⁻) Cl1D cells synchronized by selective detachment, a fairly constant rate of incorporation of [methyl-^{3}H]thymidine or [5-^{3}H]deoxycytidine into mitochondrial DNA was observed. Due to low levels of uptake of the radioactive precursors, however, the mitochondrial triphosphate precursor pool specific activities could not be measured and, thus, the rate of mitochondrial DNA replication could not be determined. On the other hand, in A9 cells, the rate of incorporation of [methyl-^{3}H]thymidine into mitochondrial DNA was found to increase by at least a factor of 5 during the late-S and G2 phases relative to the G1 phase. In addition, the mitochondrial pool specific activity decreased by a factor of 3 during the same period, indicating a substantial increase in the rate of mitochondrial DNA synthesis in the late-S and G2 phase cells in agreement with previous evidence obtained in HeLa cells. A mathematical discussion is presented which indicates that a recent study of unsynchronized A9 cells (Bogenhagen and Clayton, Cell 11, 719, 1977), erroneously interpreted as indicating a constant rate of mitochondrial DNA replication during the cell cycle, is not inconsistent with the results presented here.

In the third part of the thesis, the results are presented of preliminary experiments with the goal of developing an approach for studying the nature of the cell cycle dependence of mitochondrial DNA synthesis. This approach is based on an analysis of mitochondrial DNA synthesis in heterokaryons formed by fusion of mouse L cells at different stages of the cell cycle. The mitochondrial DNAs of the two parental cells are distinguished by using, as one of the parental cell types in the fusion, synchronized cells which had been grown in the presence of 30 μg BrdU/ml; the BrdU substituted mitochondrial DNA of these cells is separable from unsubstituted DNA by CsCl density gradient centrifugation. Cell fusion with a fast sedimenting fraction of Sendai virus was shown to result in a much higher proportion of parental cells in heterokaryons than in fusions produced by standard Sendai virus. The results of a pilot experiment carried out with C11D cells using the above described approach are presented.

TABLE OF CONTENTS

CHAPTER I

General Introduction on the Organization,

Replication and Transcription of

Animal Mitochondrial DNA

1. Structure and Replication of Mitochondrial DNA

In animal cells, mitochondria contain unique mitochondrial DNA (mit-DNA) molecules which are double stranded and circular, with a contour length of about 5 μm (Borst, 1972). Measurements of the contour length of mit-DNA from a variety of species, assuming φX174 has a length of 5386 base pairs (Sanger et al., 1978). would indicate a range of mit-DNA lengths from 15,400 base pairs to 19,000 base pairs, including sea urchin (Pikó et al, 1968; Brown, 1976), rat (Sinclair et al., 1967; Brown, 1976), hamster (Oda, 1968; Brown, 1976), pekin duck, bush baby, rabbit, chimpanzee (Brown, 1976), monkey (Suyama and Miura, 1968; Brown, 1976), human (Nass, 1969b; Brown, 1976), chicken (Nass 1969b; Brown, 1976), various amphibia (Wolstenholme and Dawid, 1968), Xenopus (Wolstenholme and Dawid, 1968; Dawid, 1972a,b; Brown, 1976), Urechis (Dawid and Brown, 1970) and Drosophila (Bultman and Laird, 1973; Peacock et al., 1973).

The %(G+C) composition of most animal mit-DNAs that have been analyzed is between 30% and 50% (Borst and Kroon, 1969; Brown, 1976). The mit-DNAs of mouse LA9 cells (37%(G+C)) and of HeLa cells (44%(G+C)) have buoyant densities in CsCl of 1.692 g/cm^3 and 1.70 g/cm^3, respectively (Brown, 1976). Drosophila has a %(G+C) content of

22% and a buoyant density of 1.68 g/cm^3 (Peacock et al., 1973). Because of the difference in base composition between complementary mit-DNA strands, heavy, "H", and light, "L", strands can be separated on alkaline CsCl equilibrium density gradients (Corneo et al., 1968; Aloni and Attardi, 1971a).

Renaturation kinetics of mit-DNA from rat (Borst, 1970) and guinea-pig (Borst, 1971) were shown to follow ideal second-order kinetics and were interpreted as being consistent with a unique sequence the length of a mit-DNA molecule. Clayton et al. (1970) demonstrated that renatured HeLa mit-DNA does not contain single-stranded regions longer than 100 nucleotides. In Drosophila, one fourth of the length of mit-DNA has been shown to be an AT rich region (Peacock et al., 1973).

Replication of mit-DNA is understood in some detail. By ordering the mit-DNA forms visualized by electron microscopy, mit-DNA replication in mouse cells (Robberson et al., 1972), rat and chicken cells (Wolstenholme et al., 1973; Koike et al., 1976) and sea urchin (Matsumoto et al., 1974) has been shown to occur in a modified Cairns mode (Kasamatsu and Vinograd, 1974; Cairns, 1963). Replication begins at a unique site and is unidirectional (Robberson and Clayton, 1972; Robberson et al., 1974). The nucleotide sequence in the vicinity of the origin of replication of

HeLa cell mit-DNA has been determined (Crews et al., 1978; Ojala and Attardi, 1978); sequence data indicate a possible stable hairpin structure including the site at which replication begins. The origin of replication is on the "L" strand near the 3'-end of the 12S rRNA "H" strand coding sequence (see below) and displacement synthesis of the new "H" strand proceeds away from the 12S rRNA coding site.

In most of the animal cells investigated, there is present a stable intermediate replicative form, the D-loop, containing the first 450-700 nucleotide sequence (7S DNA) of the new "H" strand hydrogen bonded to the original "L" strand (Kasamatsu et al., 1971). The proportion of the mit-DNA in the D-loop configuration is higher in growing mouse cells, with about 60% D-loop (Kasamatsu et al., 1971), rat liver (Arnberg et al., 1973) and growing sea urchin oocytes (Matsumoto et al., 1974) than it is in stationary phase mouse cells, with about 2% D-loop (Kasamatsu et al., 1973) and mature oocytes (Matsumoto et al., 1974). In Drosophila the D-loop form has not been observed and thus may not be a "holding point" in mit-DNA replication (Klukas and Dawid, 1976; Rubenstein et al., 1977).

From the D-loop stage, a continuation of the new "H" strand occurs, forming an expanded D-loop form in which the new "H" strand displaces the parental "H" strand; new "L" strand synthesis does not begin until 2/3 of the "H" strand

has been completed (Robberson et al., 1972; Robberson et al., 1974). The parental strands form closed circles during replication, indicating that a nicking and closing process must be occurring (Robberson and Clayton, 1972). Berk and Clayton have demonstrated that the daughter molecules segregate as open circular molecules (Berk and Clayton, 1974), are then converted to a form denoted E-mit-DNA (Kasamatsu et al., 1971; Berk and Clayton, 1974), which has a low superhelix density ($\sigma \sim 0$), and by nicking and closing they are converted to some topological variant of the form C-mit-DNA (Berk and Clayton, 1976), which was estimated to have about 50 superhelical turns per molecule ($\sigma \sim -0.03$); they are finally converted to D-mit-DNA, which contains the 7S strand, without additional nicking (Berk and Clayton, 1976). The magnitude of the superhelical density is a point of contention (Pulleyblank and Morgan, 1975; Wang, 1974).

Gently lysed mitochondria have been shown to release mit-DNA molecules with a protein-containing structure and a membrane-like patch at a location in the vicinity of the origin of replication (Albring et al., 1977). These structures are likely to be involved in attachment sites of mit-DNA to the mitochondrial membrane observed by electron microscopy by Nass (1969a).

Concatenated forms of mit-DNA are found at different

frequencies in a variety of organisms (Kasamatsu and Vinograd, 1974). The function of these forms is not known, but it has been argued that concatenates and monomers are rapidly interconverted (Berk and Clayton, 1976).

Evidence of recombination in mit-DNA has been reported in mammalian mitochondria (Dawid et al., 1974). Mouse-human cell hybrids were induced and after about 50 generations, their mit-DNAs were found to have the hybridization and buoyant density properties of recombinant molecules. Inheritance of mit-DNA appears to be maternal, even in Xenopus in which mitochondria are contained in the fertilzing sperm (Dawid and Blackler, 1972).

Several laboratories have reported partial purification of two DNA polymerases from animal cell mitochondria, one polymerase reported to be a unique mit-DNA polymerase of molecular weight around 105,000 (Kalf and Ch'ih, 1968; Fry and Weissbach, 1973; Tibbetts and Vinograd, 1973; Wang et al., 1975). Recently, however, it has been reported that some of these cell lines were mycoplasma contaminated (Radsak and Seidel, 1976) and that the only enzyme associated with the mitochondrion might be the γ-polymerase, molecular weight 110,000 (Bolden et al., 1977), one of the three major DNA polymerases found in vertebrate cells (Weissbach, 1975).

2. Products of Mitochondrial DNA Transcription

It has been demonstrated in HeLa cells that there is complete transcription of both the "L" and "H" mit-DNA strands (Aloni and Attardi, 1971a; Aloni and Attardi, 1971b; Murphy et al., 1975). The more stable mitochondrial RNA (mit-RNA) species detected are ribosomal RNA (rRNA) and transfer RNA (tRNA) molecules.

The 16S and 12S rRNA species (by sedimentation in sucrose gradients; 5.4×10^5 and 3.5×10^5 daltons; Attardi and Attardi, 1971; Vesco and Penman, 1969; Robberson et al., 1971) are found in the major 45S and the minor 35S subunits, respectively, of the 60S mitochondrial ribosomes (Attardi and Ojala, 1971; Brega and Vesco, 1971). rRNA species of similar size have also been observed in Xenopus (Dawid and Chase, 1972).

In HeLa cells, nineteen 4S RNA (3×10^4 daltons) coding sites have been located by electron microscopy, 12 on the "H" strand and 7 on the "L" strand (Wu et al., 1972; Angerer et al., 1976). 17 of the tRNA species which hybridize to HeLa mit-DNA are specific for 16 amino acids (Lynch and Attardi, 1976) and it is not yet clear if there are mitochondrial tRNAs for all 20 common amino acids (Costantino and Attardi, 1973; Ching et al., 1977). In Xenopus it has been estimated that there are at least fifteen mitochondrial 4S RNA genes (Dawid, 1972a).

Stable RNA species and the origin of replication have
been located on a map of restriction enzyme fragments of
HeLa mit-DNA (Ojala and Attardi, 1977; Ojala and Attardi,
1978; Wu et al., 1972; Angerer et al., 1976). The origin
of replication is on the "L" strand and is located
approximately 400 nucleotides from the 3'-end of the
12S rRNA coding site on the "H" strand. Farther from
the origin of replication is the 16S rRNA coding site,
which is on the "H" strand and separated from the 12S site
by a segment of 160 nucleotides that includes a 4S RNA
coding site. The 3'-end of the 12S RNA coding site and
the 5'-end of the 16S RNA coding site are flanked by 4S RNA
genes and the remaining 4S RNA genes are scattered uniformly
on the "H" and "L" strands in the numbers given above.
There may be additional 4S RNA genes as yet undetected.
The order of transcription of rRNA genes, based on the in-
formation given above, should be the 12S RNA gene followed
by the 16S RNA gene. In Drosophila and in Xenopus, the
rRNA genes are, likewise, adjacent and in Xenopus the order
of transcription is the same as in HeLa cells (Klukas and
Dawid, 1976; Dawid et al., 1976).

In addition to rRNA and tRNA, other discrete mit-DNA
transcripts have been detected in HeLa cells. RNA contain-
ing a sequence of poly(A) as the 3'-terminus has been
detected (Perlman et al., 1973; Ojala and Attardi, 1974)

and the length of the poly(A) stretch, which is not transcribed from mit-DNA, has been determined to be about 56 residues long (Hirsch and Penman, 1973). At least 18 discrete poly(A)-containing RNA species and 14 discrete species lacking poly(A) have been identified in the molecular weight range of 9×10^4 to 3.4×10^6 daltons (Amalric et al., 1978); there is evidence that some of these species will turn out to be precursors of other species. In addition, it is conjectured that the poly(A)-containing molecules may function as mRNA, as is true of cytoplasmic poly(A)-containing RNAs (Darnell et al., 1971). Poly(A)-containing RNAs have also been reported in Drosophila (Spradling et al., 1977).

Detection and purification of a mitochondrial poly(A) polymerase has been reported in rat liver (Jacob et al., 1972; Jacob and Schindler, 1972) and the occurrence in mitochondria of "free" poly(A) sequences, of about the same size as poly(A) in poly(A)-containing RNA, has been reported in HeLa cells (Ojala and Attardi, 1974); post-transcriptional addition of poly(A) to mit-DNA-coded RNA has been suggested.

It has been reported that mitochondrial DNA-dependent RNA polymerase has been isolated in Xenopus (molecular weight 46,000; Wu and Dawid, 1972) and in rat liver (molecular weight 65,000; Gallerani et al., 1972; Mukerjee and Goldfeder, 1973); these enzymes are insensitive to

α-amanitin and sensitive to rifampicin or one of its
derivatives.

Mit-DNA-RNA complexes in HeLa cell mitochondria have
been observed by electron microscopy and further charac-
terized as transcription complexes (Aloni and Attardi, 1972;
Carré and Attardi, 1978).

3. Nuclear Control of Mitochondrial DNA Replication
   and Transcription

It has been observed that HeLa cell mitochondrial nucleic acid synthesis is under nuclear genome control. Selective inhibition of cytoplasmic protein synthesis with 200 µg cycloheximide/ml causes a decrease in the rates of mit-DNA and mit-RNA synthesis by over 50% and by over 75%, respectively (Storrie and Attardi, 1972). Also consistent with this dependence of mitochondrial function on cytoplasmic protein synthesis is the observation that mit-RNA synthesis in green monkey cells, enucleated with cytochalasin B, decreases progressively until after 20 hours, synthesis is undetectable (Attardi et al., 1977).

Both mit-DNA and mit-RNA synthesis are under cell cycle dependent control. In physiologically synchronized cells, the rates of mit-DNA and mit-RNA synthesis have been shown to increase by at least a factor of 5 as the cells progress from the G1 phase to the late-S and G2 phases of the cell cycle (Pica-Mattoccia and Attardi, 1971, 1972) and a similar increase in mouse mit-DNA synthesis has been demonstrated (Chapter III, this thesis). A recent study on unsynchronized mouse cells (Bogenhagen and Clayton, 1977) inaccurately concluded that there must be a constant rate of mit-DNA synthesis during the cell cycle (Chapter III, this thesis).

Although in normally growing cells nuclear DNA synthesis precedes mit-DNA synthesis, the persistence of mit-DNA replication in the absence of nuclear DNA replication after cells enter the stationary phase of growth (Bogenhagen and Clayton, 1976) suggests that nuclear DNA synthesis, per se, is not the prerequisite for mit-DNA synthesis.

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CHAPTER II

Transcription of HeLa Cell Mitochondrial DNA

in Isolated Mitochondria

1. Introduction

In animal systems, due to the lack of appropriate mutants, the only approaches available for the study of nuclear control of mitochondrial DNA (mit-DNA) transcription have been the use of synchronized cells (Pica-Mattoccia and Attardi, 1971), drug treatment (Storrie and Attardi, 1972) or enucleation (Attardi et al., 1977) of unsynchronized cells, and analysis of RNA synthesis in isolated mitochondria. The only mammalian system in which RNA synthesized in isolated mitochondria (in vitro) has been partially characterized is rat liver.

In rat liver, using a variety of conditions for the in vitro incubation, several laboratories have shown that there is labeling of RNA in the presence of a radioactive precursor (Neubert et al., 1968; Saccone et al., 1968; Fukamaki et al., 1970; Aaij et al., 1970). There is evidence that this RNA is a mitochondrial DNA transcription product. Aaij et al.(1970) have shown that at least 85% of RNA labeled in swollen isolated mitochondria in the presence of ^{3}H-UMP hybridized to purified, denatured mit-DNA. Hybridization to the separated complementary strands of mit-DNA demonstrated that some (> 8%) of the labeled RNA was transcribed from the light ("L") DNA strand, while probably most (> 33%) was transcribed from the heavy ("H") strand. Characterization of RNA labeled

in isolated mitochondria in the presence of ^{3}H-UTP revealed
RNA species migrating on a gel as 21S and 12S (Fukamaki
et al., 1970), which correspond to stable mitochondrial RNA
species observed in vivo.

There are differing reports on how incubation condi-
tions affect incorporation into RNA. This may be due to the
variety of incubation systems used. Some investigators have
used (besides nucleoside triphosphates) pyruvate kinase and
its substrate, phosphoenolpyruvate (Saccone et al., 1968),
others have added pyruvate, succinate and malate (Fukamaki
et al., 1970), while still others have added no energy
generating system (Neubert et al., 1968).

Saccone et al. (1968), using ^{14}C-ATP and pyruvate
kinase at 30°C, have demonstrated linear incorporation for
at least 1 hour. Neubert et al. (1966), using ^{14}C-UTP
and no energy generating system at 37°C, have observed a
linear incorporation for only 5-15 minutes.

The rate of ^{14}C-ATP incorporation, using the pyruvate
kinase system, has been shown by Saccone et al. (1968) to
increase linearly with increasing amounts of mitochondrial
protein up to 0.5 mg protein/ml, followed by a less than
linear increase with increasing protein.

The incubation pH optimum appeared to be in the range
of pH 7.8-9 for ^{14}C-ATP (Saccone et al., 1968) or ^{14}C-UTP
(Neubert et al., 1968) incorporation. At pH 7, the in-
corporation was a factor of two lower.

Neubert et al. (1968) observed that, with increasing amounts of ^{3}H-UTP, incorporation reached a plateau around 0.1 mM; Saccone et al. (1968) observed a similar phenomenon with ^{14}C-ATP. The rate of incorporation was about twice as high at 37°C as at 25-30°C (Saccone et al., 1968).

When the radioactive precursor was ^{3}H-ATP, partial inhibition was observed in the presence of atractyloside, a specific inhibitor of mitochondrial ATP-ADP translocase (Saccone et al., 1968). Ethidium bromide, which, at 1 µg/ml, preferentially inhibits mit-RNA synthesis in vivo relative to nuclear RNA synthesis (Zylber et al., 1969), inhibited in vitro incorporation of the labeled precursor by 70% at 0.33 µg/ml (Fukamaki et al., 1970). Rifampicin, a specific inhibitor of some RNA polymerases, was found to inhibit incorporation into swollen mitochondria but not into intact mitochondria (Saccone and Quagliariello, 1975).

In HeLa cells, studies on mit-DNA transcription in synchronized cells and in drug treated unsynchronized cells have indicated that there is nuclear control. In HeLa cells grown in the presence of 200 µg cycloheximide/ml, a selective inhibitor of cytoplasmic protein synthesis, the rate of mitochondrial RNA synthesis decreased by more than 75% after 4 hours (Storrie and Attardi, 1972). In contrast, in cells grown in 40 µg chloramphenicol/ml, a potent inhibitor of mitochondrial protein synthesis, the rate of mitochondrial RNA synthesis decreased by only 30% after

3 days (Storrie and Attardi, 1972). These results indicate
that mitochondrial transcription is dependent on cytoplasmic
proteins. In support of this conclusion, mitochondrial RNA
synthesis in green monkey cells, enucleated with cytochalasin
B, decreased progressively until, after 20 hours, synthesis
was undetectable (Attardi et al., 1977). Regulation of
transcription has been observed only at the level of cell
cycle dependent control. In HeLa cells synchronized by
selective detachment (Pica-Mattoccia and Attardi, 1971),
the rate of mitochondrial RNA synthesis has been shown to in-
crease by a factor of 5 from early labeling periods (mostly
G1 cells) to late labeling periods (mostly late-S and G2
cells). The mechanism of cytoplasmic cell cycle dependent
control of mitochondrial RNA polymerase activity is unknown.

The purpose of the following study is to characterize
RNA synthesis in isolated HeLa cell mitochondria.

2. Methods and Materials

(a) Solutions[1]

The solution designations are as follows: (1) DET: 2% SDS, 1% Na desoxycholate, 1% Na cholate, 0.25% bentonite in TKV. (2) HP: 0.1% hydroxyquinoline in hydrated, distilled phenol. (3) NKM: 0.13 M NaCl, 5 mM KCl, 7.5 mM $MgCl_2$. (4) Low ionic strength TKV: 10 mM KCl, 10 mM Tris buffer (pH 6.7 at 25°C), 0.1 mM EDTA. (5) SDS buffer: 0.5% SDS, 10 mM Tris buffer (pH 7.0 at 25°C), 0.1 M NaCl, 1 mM EDTA. (6) Low ionic strength TKM: 10 mM KCl, 10 mM Tris buffer (pH 6.7 at 25°C), 0.15 mM $MgCl_2$. (7) SSC: 0.15 M NaCl, 0.015 M Na citrate. (8) STE: 0.25 M sucrose, 10 mM Tris buffer (pH 6.7 at 25°C), 0.1 mM EDTA. (9) STM: 0.25 M sucrose, 10 mM Tris buffer (pH 6.7 at 25°C), 0.15 mM $MgCl_2$. (10) TD: 0.137 M NaCl, 5 mM KCl, 25 mM Tris buffer (pH 7.4 at 25°C). (11) TKM: 0.05 M Tris buffer (pH 6.7 at 25°C), 0.025 M KCl, 2.5 mM $MgCl_2$.

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[1]Abbreviations used in this chapter are: (1) ATP: adenosine 5'-triphosphate. (2) CTP: cytidine 5'-triphosphate. (3) DNase: deoxyribonuclease. (4) EDTA: ethylenediaminetetraacetate. (5) GTP: guanosine 5'-triphosphate. (6) mit-DNA: mitochondrial DNA. (7) mit-RNA: mitochondrial RNA. (8) mRNA: messenger RNA. (9) PCA: perchloric acid. (10) POPOP: 1,4-bis-2-(5-phenyloxazolyl)-benzene. (11) PPO: 2,5-diphenyloxazole. (12) RNase A: pancreatic ribonuclease A. (13) rRNA: ribosomal RNA. (14) SDS: sodium dodecyl sulphate. (15) TCA: trichloroacetic acid. (16) tRNA: transfer RNA. (17) UTP: uridine 5'-triphosphate.

(12) TKV: 0.05 M Tris buffer (pH 6.8 at 25°C), 0.025
M KCl, 10 mM EDTA.

(b) Conditions of cell growth
and labeling

Strain S3 of HeLa cells (Amaldi and Attardi, 1968)
was grown in suspension at 37°C in a modified Eagle's
phosphate medium (Levintow and Darnell, 1960; MED-69191,
Grand Island Biological) supplemented with 5% calf serum;
if a radioactive precursor was added to the growth medium,
5% calf serum dialyzed against TD was utilized. Only
exponentially growing cells (no more than 4×10^5 cells/ml)
were utilized, except for the preparation of unlabeled
mit-DNA. The cultures used here were free of any detect-
able contamination by pleuropneumonia-like organisms
(PPLO, mycoplasma).

Cycloheximide treatment of cultures was carried out
at 200 µg/ml for up to 4 hours. The control culture
for this experiment was cooled on ice, supplemented with
cycloheximide and immediately harvested.

To label mit-RNA in vivo (Attardi et al., 1970),
HeLa cell cultures were treated with 40 ng Actinomycin
D/ml for 30 minutes and then exposed to 1.25 µCi/ml
[5-^{3}H]uridine (30 Ci/mmole, Amersham) for 2 hours, always

in the presence of the drug. In the experiments in
which the effect of ethidium bromide had to be tested,
this drug was added to the medium at 1 µg/ml 15 minutes
before addition of the [5-^{3}H]uridine. For the labeling
of cytoplasmic rRNA, cultures were grown in [2-^{14}C]uridine
(58 mCi/mmole, Amersham) for 2 days. Labeled mit-DNA
was extracted from cultures grown for 3 days in the
presence of 0.025 µCi/ml [2-^{14}C]thymidine (40-60 mCi/
mmole, Amersham).

(c)   Subcellular fractionation

Preparation of the mitochondrial fraction (Attardi,
Cravioto and Attardi, 1969) for the study of RNA synthesis
in isolated organelles was accomplished under semisterile
conditions. All buffers were either autoclaved or
filtered and all glassware was autoclaved. All operations
described below were carried out between 0°C and 4°C
unless otherwise noted. Cells were washed 3 times in
NKM and pelleted at 900 rpm (180 g) in plastic conical
tubes for 5 minutes in an International centrifuge.
The cells were swollen in 6 volumes of low ionic strength
TKM for 2 minutes before breakage with a motor driven
glass-teflon homogenizer. After homogenization (approx-
imately 60% cell breakage), the sucrose concentration

was promptly brought to 0.25 M. Intact cells, nuclei and other big cell debris were removed by centrifugating twice at 2,500 rpm (1,400 g) for 3 minutes; mitochondria were pelleted in the Sorvall at 8,600 rpm (9,100 g) for 10 minutes. After resuspension in 4 ml STM/ml original packed cells, another low speed centrifugation was carried out to remove residual nuclei and the mitochondria were again pelleted and resuspended in an appropriate buffer.

The mitochondrial fraction preparation for mit-DNA extraction was similar to the procedure given above except that the buffers contained EDTA: the cells were swollen in low ionic strength TKV, the homogenization was more extensive (approximately 90% cell breakage) and the first mitochondrial resuspension was in STE.

The first 9,100 g supernatant from cells homogenized in low ionic strength TKM was centrifuged for 20 minutes at 12,000 rpm (17,800 g) in a Sorvall rotor to separate the postmitochondrial supernatant ("cytoplasmic fraction").

(d)   Incubation of isolated

mitochondria

Pelleted mitochondria from unlabeled cells were resuspended in STM. In some instances, the mitochondrial

fraction (at 2 mg protein/ml) was pretreated with ethidium bromide in STM for 30 minutes on ice. [5-^{3}H]UTP and [5-^{3}H]ATP were desiccated before use to remove alcohol.

There were two types of incubation mixtures. One type utilized "KCl medium" which contained 75 to 100 mM KCl, 7.5 to 10 mM MgCl$_2$, 35 to 50 mM Tris buffer (pH 7.4 at 25°C), 0 to 0.1 mM CTP, GTP and UTP, 0 to 2.5 mM ATP, 0.1 to 2 mg mitochondrial fraction protein/ml and either [5-^{3}H]ATP or [5-^{3}H]UTP, as described below. In some cases, one half of the incubation mixture was cytoplasmic fraction. The other type was the sucrose incubation mixture which contained 0.15 to 0.25 M sucrose, 50 mM Tris buffer (pH 7.4 at 25°C), 1 to 25 mM MgCl$_2$, 0 to 2 mM each ATP, CTP and GTP, 0.1 to 1 mg mitochondrial fraction protein/ml and [5-^{3}H]UTP.

The incubations were begun by the addition of the mitochondrial fraction to the complete incubation mixture on ice and placing the mixture in a 30°C shaking water bath. Incubations were terminated by placing the mixture on ice and diluting the radioactive precursor with cold UTP or ATP; the mitochondrial fraction was washed in STM. Agar plating of portions of the incubation mixture at the end of selected incubations showed no or very few

bacterial colonies; no 23S RNA was detected in extracted
RNA.

The TCA insoluble radioactivity incorporated into
a mitochondrial suspension was determined by adding SDS
to 0.5%, precipitating with cold 15% TCA, collecting the
precipitate on a Millipore filter and counting. Protein
content of the mitochondrial fraction was determined by
a modified Lowry method (Oyama and Eagle, 1956).

The PCA soluble fraction of a mitochondrial resuspen-
sion was obtained by bringing the suspension to 0.5 N PCA,
keeping it on ice for one hour and pelleting the precip-
itate by centrifugation at 12,000 rpm (17,800 g) for
15 minutes in the Sorvall. The supernatant was neutralized
with KOH, the $KClO_4$ precipitate was removed by centrifuga-
tion and the resulting supernatant counted.

(e) Extraction and analysis
of RNA

The mitochondrial pellet was resuspended in SDS
buffer, incubated in the presence of 40 µg pronase/ml
at room temperature for 60 minutes, and mixed with an
equal volume of cold DET. (Pronase had been predigested
at 2 mg/ml in 20 mM Tris buffer (pH 8 at 25°C) at 37°C
for 2 hours and particulate matter removed by centrifuga-
tion.) The sample was then extracted for 15 minutes with

HP (Attardi, Parnas, Hwang and Attardi, 1966) in the cold and the aqueous phase separated by centrifugation; the procedure was repeated until no protein interface was visible (typically 3 extractions). After the NaCl concentration was brought to 0.1 M, the sample was precipitated with 2 volumes of ethanol at -20°C for at least 2 hours, spun down at 13,000 rpm (20,900 g) for 15 minutes and resuspended in TKM. This procedure is referred to as SDS-pronase-phenol extraction. Three ethanol precipitations were performed before reading the optical density at 260 nm.

RNA to be used in RNA-DNA hybridizations was incubated with 40 µg/ml of electrophoretically purified DNase (Worthington) in TKM buffer at room temperature for 1 hour (Aloni and Attardi, 1971a). The sample was brought to 0.5% SDS, phenol extracted, ethanol precipitated and run on a 0.9x55 cm G100 Sephadex column equilibrated with 0.3 M NaCl, 10 mM Tris buffer (pH 7.4 at 25°C). Fractions of the void volume were pooled, brought to 0.1 M NaCl, precipitated with 2 volumes of ethanol, pelleted by centrifugation and resuspended in 10 mM EDTA, 10 mM Tris buffer (pH 7.4 at 25°C).

For analysis of the sedimentation properties of RNA, samples were brought to 0.5% SDS and layered on

14 ml of a 15%-30% sucrose gradient in SDS buffer with

a cushion of 2 ml of 64% sucrose in SDS buffer in a

1.59x10.16 cm tube and centrifuged in a Beckman SW27 rotor

either at 25,000 rpm for 12.5 hours or at 27,000 rpm

for 11 hours.

(f) Isolation of mit-DNA

For the extraction of mit-DNA (Hudson and Vinograd,

1967), the mitochondrial pellet was resuspended in 1.2%

SDS, 10 mM EDTA, 10 mM Tris buffer (pH 7.4 at 25°C),

brought to 1 M CsCl and after 30 minutes on ice, the

precipitate was removed by centrifugation in the Sorvall

at 12,000 rpm (17,800 g) for 15 minutes. The solution

was brought to 4.5 M with CsCl to remove remaining protein

and the sample was again left one-half hour on ice and

centrifuged as described above. Ethidium bromide was

added to 200 µg/ml, the CsCl concentration was adjusted

for an index of refraction of 1.389 (25°C), and the

sample was centrifuged to equilibrium in the Beckman 65

rotor at 40,000 rpm for 2 days at 20°C. The closed

circular mit-DNA band was collected, and recentrifuged

to equilibrium. The resulting closed circular mit-DNA

band was pooled, the ethidium bromide was extracted by

shaking (3 times) with an equal volume of isoamyl alcohol

and the sample was then dialyzed against SSC/10.

The upper DNA band of the first ethidium bromide density gradient was likewise extracted with isoamyl alcohol and dialyzed against 10 mM EDTA, 10 mM Tris buffer (pH 7.4 at 25°C): this material was predominantly nuclear DNA.

Separation of the complementary strands of mit-DNA was accomplished by bringing a solution of closed circular mit-DNA to 0.055 M K_3PO_4, 0.01% SDS and adjusting the pH to 12.4 (at 25°C) and the index of refraction to 1.405 (25°C) with CsCl (Aloni and Attardi, 1971a). Centrifugation in a polyallomer tube in the Beckman 65 rotor at 42,000 rpm for 42 hours at 20°C resulted in strand separation. The fractions were assayed for TCA precipitable radioactivity, promptly pooled and brought to pH 8 (at 25°C) with 1 M Tris buffer. Samples were dialyzed for 1 day against 3 changes of SSC/10 and the optical density at 260 determined (the equivalence 48 µg DNA/ml = 1.0 OD_{260} unit was used).

(g) RNA-DNA hybridization

Immediately prior to the hybridizations, RNA samples and nuclear DNA samples were separately denatured at 90°C for 5 minutes in 0.01 M EDTA, 0.01 M Tris buffer

(pH 7.4 at 25°C), then cooled quickly on ice. The
hybridization mixtures contained in 0.5 ml of 0.4 M NaCl,
10 mM Tris buffer (pH 8 at 25°C), 10 mM EDTA, 100-200
[3]H-cpm of RNA, and different amounts of heavy or light
strand mit-DNA or nuclear DNA as specified below (Aloni
and Attardi, 1971a). Incubation was carried out in
sealed vials at 66°C for 4 hours and stopped by quick
cooling. Unhybridized RNA was degraded by treatment
with 5 μg RNase A/ml at room temperature for 20 minutes.
(RNase A, pancreatic ribonuclease, had been heated
previously at 80°C for 10 minutes at 2 mg/ml in 0.1 M
NaCl, 10 mM acetate buffer (pH 5 at 25°C) to destroy any
DNase activity.) The hybrids were collected on nitro-
cellulose membranes (Bac-T-Flex type B6, Schleicher and
Schuell) and subsequently washed with 100 ml of 2xSSC,
and counted. Samples of input ^{3}H-RNA and input ^{14}C-DNA
were plated on similarly washed filters and counted.

(h) TCA precipitation and scintillation counting

For the determination of acid precipitable radio-
activity in solution, except where noted, samples were
plated on Whatman 3 MM filter circles, dried, washed
successively in 5% TCA (with SDS, 15% TCA), 95% and

absolute ethanol to remove TCA, and finally in ether before drying. Filters were counted in toluene-based scintillation fluid (15.16 g PPO/l and 0.10 g POPOP/l of toluene).

The radioactivity data contained in the figures represent the total radioactivity in the appropriate incubation mixture or gradient fraction.

3. Results

(a) <u>In vitro incubation conditions for isolated mitochondria and characterization of the labeled RNA</u>

<u>In</u> <u>vitro</u> incubation of isolated mitochondria in the presence of radioactive precursors has been used in order to specifically label the products of mitochondrial DNA transcription. Several precautions were taken in preparing the mitochondrial fraction for use in <u>in</u> <u>vitro</u> experiments. The cells used were growing exponentially. All of the solutions and glassware were sterilized to minimize bacterial contamination. Homogenization was moderate (approximately 60% cell breakage) in order to minimize breakage of nuclei. The homogenate was subjected to low speed centrifugation three times to minimize contamination by intact cells and nuclei.

(i) <u>The effect of incubation conditions on in vitro incorporation of [5-^{3}H]UTP or [5-^{3}H]ATP into RNA of isolated mitochondria</u>

Two types of media were used for <u>in</u> <u>vitro</u> incubation of isolated mitochondria in the presence of radioactive ribonucleoside triphosphate precursors: "KCl medium,"

containing, in addition to other components, 75 mM KCl, 7.5 mM $MgCl_2$ and no sucrose, and "sucrose medium," containing 0.15 M sucrose, 1 mM $MgCl_2$ and no KCl. Both types of media were found to be satisfactory and, as shown below, gave fairly comparable rates of incorporation. In all experiments described below, unless otherwise specified, "KCl medium" was utilized.

Conditions of incubation in "KCl medium". The standard "KCl medium" for in vitro incubation consisted of 75 mM KCl, 7.5 mM $MgCl_2$, 35 mM Tris buffer (pH 7.4 at 25°C), 0.1 mM CTP, 0.1 mM GTP, 2.5 mM ATP and 3.3 μM $[5-^3H]UTP$. The incubations were carried out at 30°C. The effects of varying either $MgCl_2$ or KCl concentration or pH on the level of incorporation of $[5-^3H]UTP$ into RNA are shown in Figure 1. For all of the conditions tested, the rate of incorporation of the precursors in a 10 minute incubation did not vary by more than a factor of 2. The optimum $MgCl_2$ concentration was found to be 10 mM (Figure 1a). The optimum KCl concentration was 50 mM (Figure 1b, ●——●); in addition, when sucrose was added to compensate for the lower osmolarity[1] in the mixtures containing less than 75 mM KCl (Figure 1b,

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[1]The added sucrose concentration was 0 mM at 75 mM KCl, 50 mM at 50 mM KCl, 100 mM at 25 mM KCl and 150 mM at 0 mM KCl.
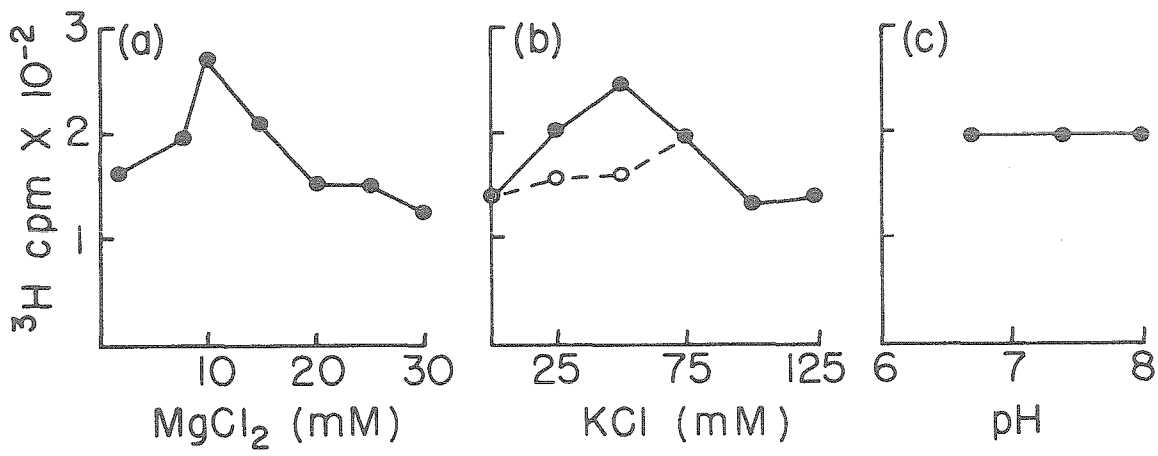
Figure 1

LEGEND TO FIGURE 1

Effect of the concentration of $MgCl_2$ (a) or KCl (b) and of pH (c) on [5-^{3}H]UTP incorporation into RNA during incubation of isolated mitochondria in "KCl medium."

Portions of an isolated HeLa cell mitochondrial fraction (0.1 mg protein each) were incubated in 1 ml mixtures containing varying concentrations KCl and $MgCl_2$, 35 mM Tris buffer (at varying pH as specified below), 0.1 mM CTP, 0.1 mM GTP, 2.5 mM ATP and 3.3 μM [5-^{3}H]UTP (50 μCi/ml, 15 Ci/mmole, Schwarz) at 30°C for 10 minutes. After incubation, the mitochondrial fractions were pelleted, and aliquots were spotted on 3 MM filters, washed in 5% TCA and ethanol and counted.

(a) 75 mM KCl, Tris buffer (pH 7.4 at 25°C) with varying $MgCl_2$ concentration. (b) 7.5 mM $MgCl_2$, Tris buffer (pH 7.4 at 25°C) with varying concentrations of KCl (●——●). In the samples indicated with open circles, as the KCl concentration was lowered to 50 mM, 25 mM and 0 mM, sucrose was added to concentrations of 50 mM, 100 mM and 150 mM respectively (o---o). (c) 75 mM KCl, 7.5 mM $MgCl_2$, Tris buffer with a pH (at 25°C) of 6.7, 7.4 or 8.0.

o----o), the rate of incorporation appeared to be fairly

constant for different KCl concentrations, with a hint

of a peak at 75 mM. Varying the pH between 6.7 and 8.0

had no detectable effect on the rate of [5-^{3}H]UTP incor-

poration (Figure 1c). The effect of adding increasing

amounts of cold ATP to incubation mixtures containing a

constant amount of [5-^{3}H]ATP (32 µCi/ml, 22.7 Ci/mmole)

is shown in Figure 2, where the abscissa indicates the

total ATP concentration. One can see that with all the

ATP concentrations tested in the range between 0.1 and

10 mM, the level of incorporation is much higher than

expected from the [5-^{3}H]ATP dilution. This suggests that

with increasing ATP concentration, exogeneous ATP is

entering the organelles more readily due to the more

favorable concentration differential; in addition there

may be other effects of high ATP concentration on the

utilization of the precursor.

Conditions of incubation in "sucrose medium". Several

experiments were performed to study the effect of various

parameters on the level of [5-^{3}H]-UTP incorporation into RNA

in "sucrose medium." The detailed incubation conditions

in each experiment are mentioned in the legend of Figure 3.

As shown in Figure 3a, varying the sucrose concentration

in the range 50-350 mM had moderate effects on the level
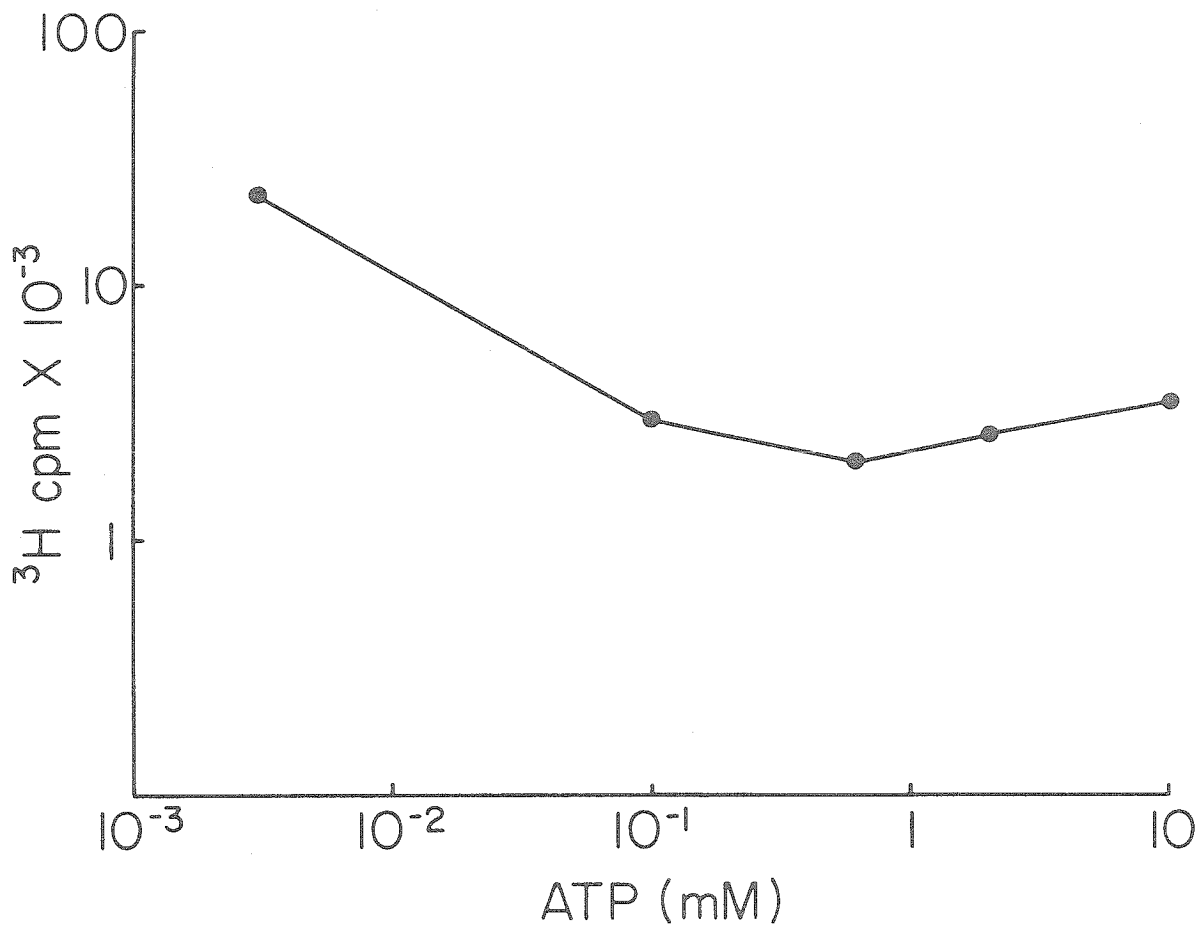
Figure 2

LEGEND TO FIGURE 2

Effect of ATP concentration on the incorporation of [5-^{3}H]ATP into RNA during incubation of isolated mitochondria in "KCl medium" conditions.

Portions of a HeLa cell mitochondrial fraction (0.2 mg protein each) were incubated in a 0.5 ml mixture containing 75 mM KCl, 7.5 mM MgCl$_2$, 35 mM Tris buffer (pH 7.4 at 25°C), 0.27 mM each CTP, GTP, selected concentrations of unlabeled ATP, and [5-^{3}H]ATP (32 μCi/ml, 22.7 Ci/mmole, Schwarz) at 30°C for 60 minutes. The total ATP concentrations of the incubation mixtures were 2.8 μM, 100 μM, 670 μM, 2 mM and 10 mM. After incubation, the RNA was SDS-pronase-phenol extracted, spotted on 3 MM filters, washed in 5% TCA and ethanol and counted.

Figure 3

LEGEND TO FIGURE 3

Effect of the concentrations of $MgCl_2$, KCl, sucrose, UTP, CTP, ATP and mitochondrial fraction protein, and effect of pH and temperature on the incorporation of isolated mitochondria in "sucrose medium."
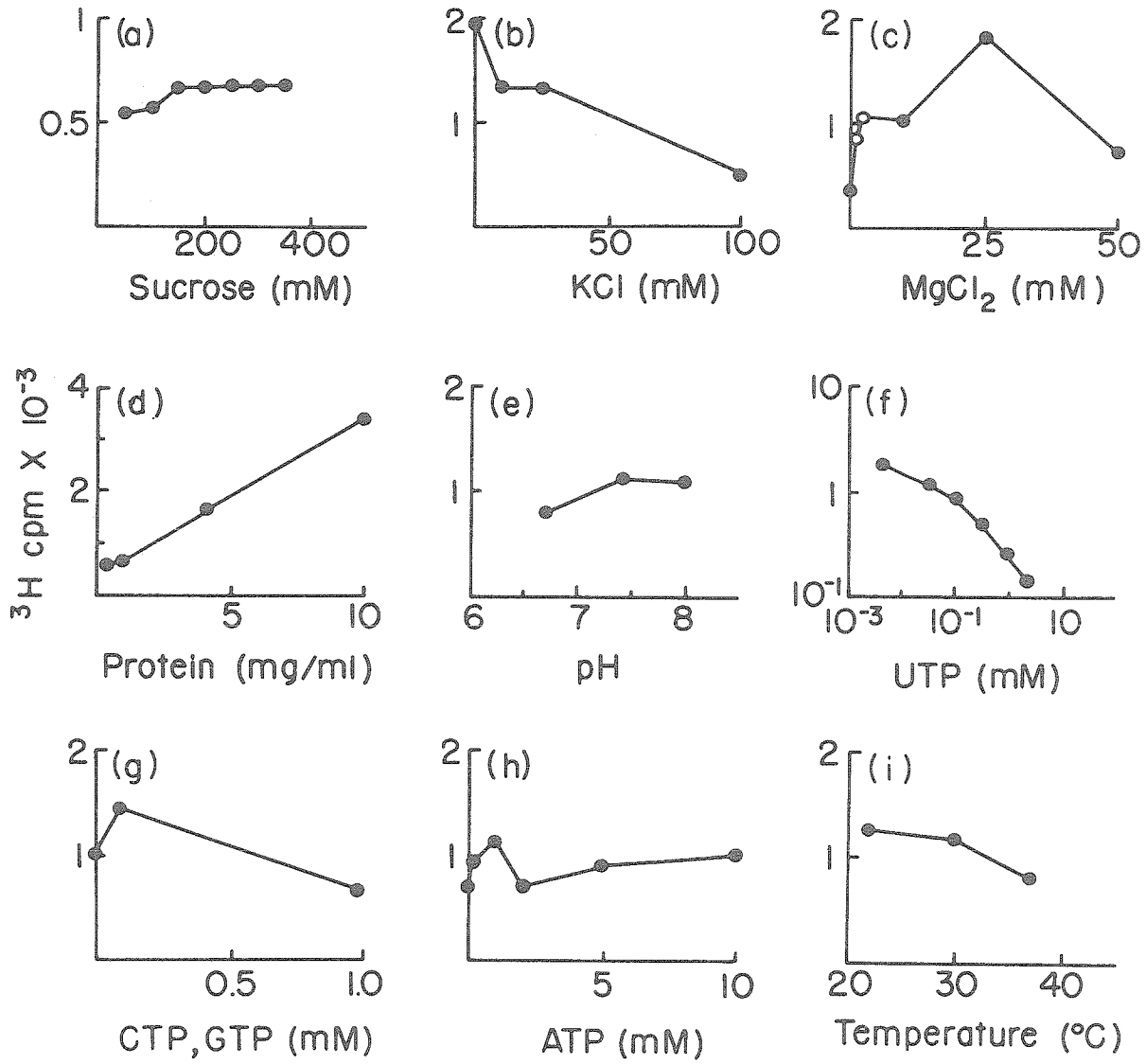
(a), (c, ●——●), (d), (g):   Portions of a HeLa cell mitochondrial fraction (0.5 mg protein each) were incubated in 1 ml of 0.25 M sucrose, 50 mM Tris buffer (pH 7.4 at 25°C), 2.5 mM $MgCl_2$, 1 mM each of CTP, GTP and ATP, and 33 μM [5-^{3}H]UTP (0.13 mCi/ml, 15 Ci/mmole, Schwarz), incubation at 30°C for 60 minutes, varying sucrose concentration (a), varying $MgCl_2$ concentration (c), varying the amount of mitochondrial fraction protein (d), varying CTP and GTP concentration (g).

(b):   Portions of another mitochondrial fraction (0.8 mg protein each) was incubated, in 0.5 ml of 0.15 M sucrose, 50 mM Tris buffer (pH 7.4 at 25°C), 1 mM $MgCl_2$, varying KCl concentration, 2 mM ATP and 33 μM [5-^{3}H]UTP (0.5 mCi/ml, 15 Ci/mmole, Schwarz), incubation at 30°C for 60 minutes.

(c, o——o, (e), (f), (h), (i):   Portions of mitochondrial fraction (0.15 mg protein each) were incubated, in 0.5 ml, under conditions varying by one factor from the following: 0.15 M sucrose, 50 mM Tris buffer

(LEGEND TO FIGURE 3, CONTINUED)

(pH 7.4 at 25°C), 25 mM $MgCl_2$, 1 mM ATP and 33 µM
$[5-^3H]UTP$ (67 µCi/ml, 2 Ci/mmole, Schwarz) at 30°C for
60 minutes. Varied are the concentrations of $MgCl_2$,
(c, o————o), the pH (e) determined by the Tris buffer
(pH at 25°C) used, the final concentration of UTP (f)
reached by the addition of unlabeled UTP, the concentra-
tion of ATP, (h), and the temperature, (i).

The RNA was SDS-pronase-phenol extracted, ethanol
precipitated and aliquots were spotted on 3MM filters,
washed in 5% TCA and ethanol and counted.

of incorporation of [5-^{3}H]UTP, with a broad plateau

between 150 and 350 mM. At 150 mM sucrose and 1 mM

MgCl$_2$, addition of KCl appeared to have an inhibitory

effect that became pronounced (an approximately 75%

decrease) at 100 mM (Figure 3b). At 150 mM sucrose

and no KCl, the optimum MgCl$_2$ concentration was at

25 mM (Figure 3c); the optimum around this concentration

was observed also in a separate experiment (not shown).

At 250 mM sucrose, the level of total [5-^{3}H]UTP incor-

poration increased linearly with increasing mitochondrial

fraction protein concentration (Figure 3d). With regard

to the effect of pH, a moderate increase in the level

of incorporation was detected at pH 7.4-8.0 (25°C) as

compared to pH 6.7 (Figure 3e). Slight effects, of

uncertain significance, were observed by varying in

parallel the CTP and GTP concentration in the range

from 0 to 1.0 mM each (Figure 3g), and the ATP concentra-

tion in the range between 0 and 10 mM (Figure 3h). In

order to study the effect of the total concentration of

UTP in the incubation mixture, increasing amounts of cold

UTP were added to individual samples, containing a constant

amount of [5-^{3}H]UTP (67 µCi/ml, 2 Ci/mmole), up to the

total final concentration shown in Figure 3f. As seen

above in the case of [5-^{3}H]ATP incorporation in the

presence of increasing amounts of cold ATP, the extent
of incorporation with increasing UTP concentration was
found to be greater than would be expected by straight
dilution of [5-^{3}H]UTP. For example, as the external
UTP dilution increased by 3 orders of magnitude, the
radioactivity incorporated decreased by only 1 order
of magnitude, again suggesting an increased uptake of
the precursor. As for the temperature effects, the
extent of incorporation of [5-^{3}H]UTP at 37°C was found
to be somewhat lower than at 22°C or 30°C (Figure 3i).

Comparison of incorporation in vitro using the
"KCl medium" and the "sucrose medium". The "sucrose
medium" and the "KCl medium," in experiments utilizing
the same precursor of identical specific activity and the
same mitochondrial preparation, gave comparable levels
of incorporation. For example, in an experiment using
a "sucrose medium" like that of the control in Figure 3f
and a "KCl medium" as in Figure 6b (except for the omission
of CTP and GTP), both sets of conditions being near-to-
optimal, the levels of incorporation were within 10% of
each other.

The dependence of incorporation on $MgCl_2$ and KCl
concentrations is quite different in "KCl medium"
(Figures 1a, 1b) and in "sucrose medium" (Figures 3b, 3c).

In "KCl medium," the KCl concentration optimum (50 mM)
may well represent that which allows an optimal degree of
swelling; no KCl, on the contrary, appears to be necessary
when the required osmolarity is insured by sucrose. The
optimal $MgCl_2$ concentration was also different in the
two types of media, 10 mM in "KCl medium" and 25 mM in
"sucrose medium."

The in vitro RNA synthesis experiments, in which
the RNA was analyzed in its sedimentation and hybridiza-
tion properties (Sections a-iii, a-iv), were carried out
by using 75 mM KCl, 7.5 mM $MgCl_2$ and pH 7.4, which are
at or near the optimum. The in vitro incubations in the
cycloheximide inhibition experiments, discussed below,
were performed at 75 mM KCl and 10 mM $MgCl_2$.

(ii) Kinetics of in vitro $[5-^3H]$UTP and $[5-^3H]$ATP
     incorporation into RNA of isolated organelles

Isolated mitochondria can support incorporation
of a radioactive precursor for 1-2 hours. Figure 4 shows
that $[5-^3H]$UTP incorporation into the mitochondrial
fraction (1 mg protein/ml) incubated in "sucrose medium"
is almost linear for the first hour; thereafter the
reaction slows down fairly rapidly. In one experiment,
$[5-^3H]$ATP incorporation, in "KCl medium" and using 1.7 mg

Figure 4

LEGEND TO FIGURE 4

Kinetics of $[5-^3H]$UTP incorporation into RNA synthesized in isolated mitochondria during incubation in "sucrose medium."

Portion of an isolated HeLa cell mitochondrial fraction (0.5 mg protein) were incubated in identical mixtures containing, in 0.5 ml, 0.15 M sucrose, 1 mM $MgCl_2$, 50 mM Tris buffer (pH 7.4 at 25°C), 2 mM ATP, 33 μM $[5-^3H]$UTP (0.5 mCi/ml, 15 Ci/mmole, Schwarz) at 30°C. At selected times, the incubation mixtures were cooled in ice and the mitochondrial fractions were washed. The labeled RNA was SDS-pronase-phenol extracted, ethanol precipitated and analyzed for acid-insoluble radioactivity.

protein of the mitochondrial fraction per ml, was found

to be linear for 2 hours (Figure 5). These results

indicate that, at least under the conditions of the

above described experiments, incubation gives an almost

linear incorporation of the precursor for up to at least

60 minutes.

(iii) Sedimentation behavior of the RNA labeled in vitro

and ethidium bromide sensitivity of its synthesis

The RNA extracted from the in vitro incubation

mixtures was purified by SDS-pronase-phenol extraction,

ethanol precipitation and sedimentation through a sucrose

gradient. In order to compare the sedimentation patterns

thus observed with those obtained for in vitro labeled

RNA, one culture was labeled for 2 hours with $[5-^3H]$uridine

in the presence of actinomycin D (0.04 µg/ml). These

conditions have shown to label preferentially mit-DNA

species as a result of the actinomycin D inhibition of

the synthesis of the nuclear rRNA (Dubin, 1967; Perry,

1964; Penman et al., 1968) and partial suppression of

the HnRNA and mRNA synthesis. As a control, an identical

culture was similarly labeled in the presence of

actinomycin D and 1 µg of ethidium bromide/ml. The
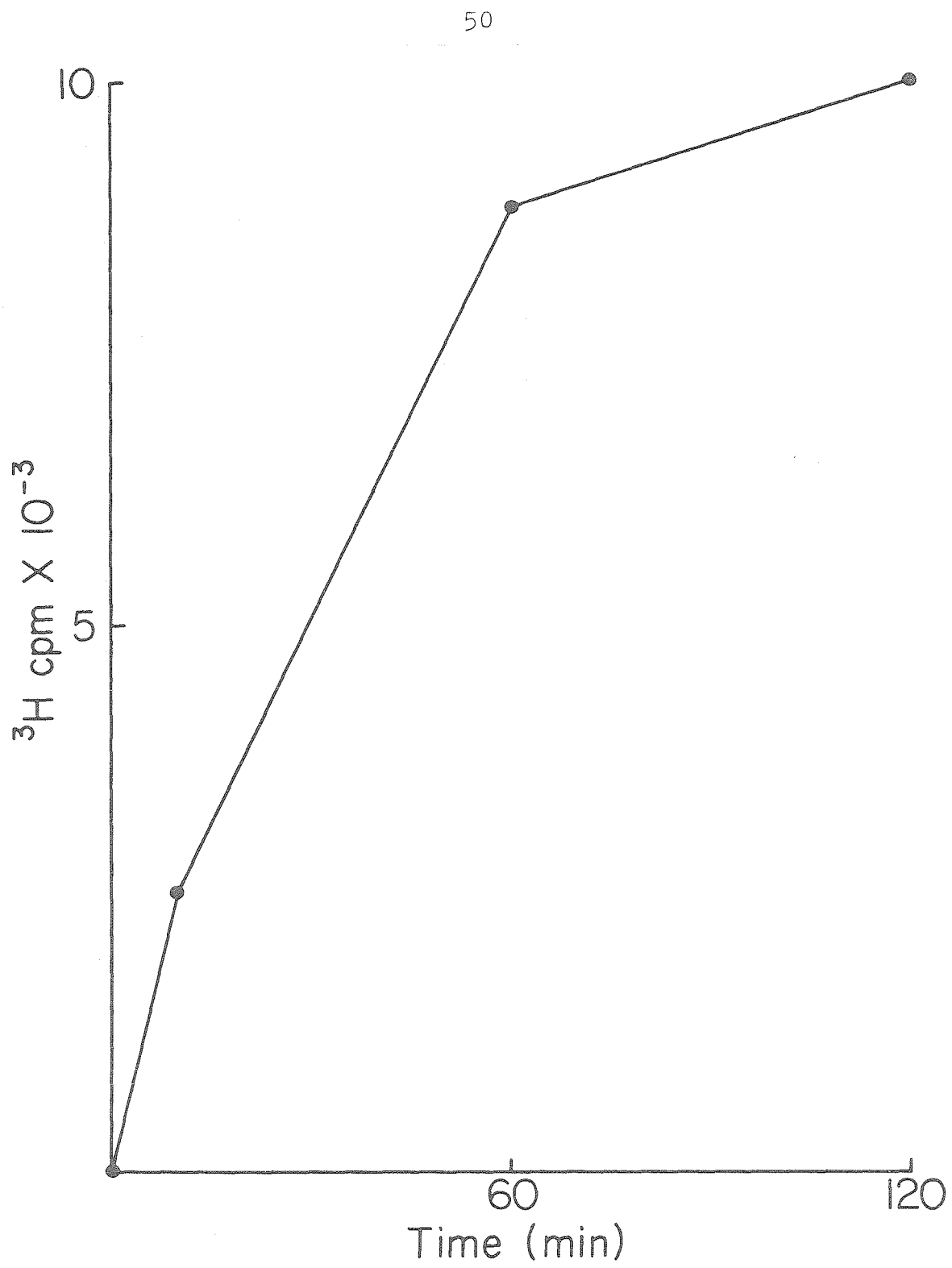
latter drug has been shown to inhibit selectively the
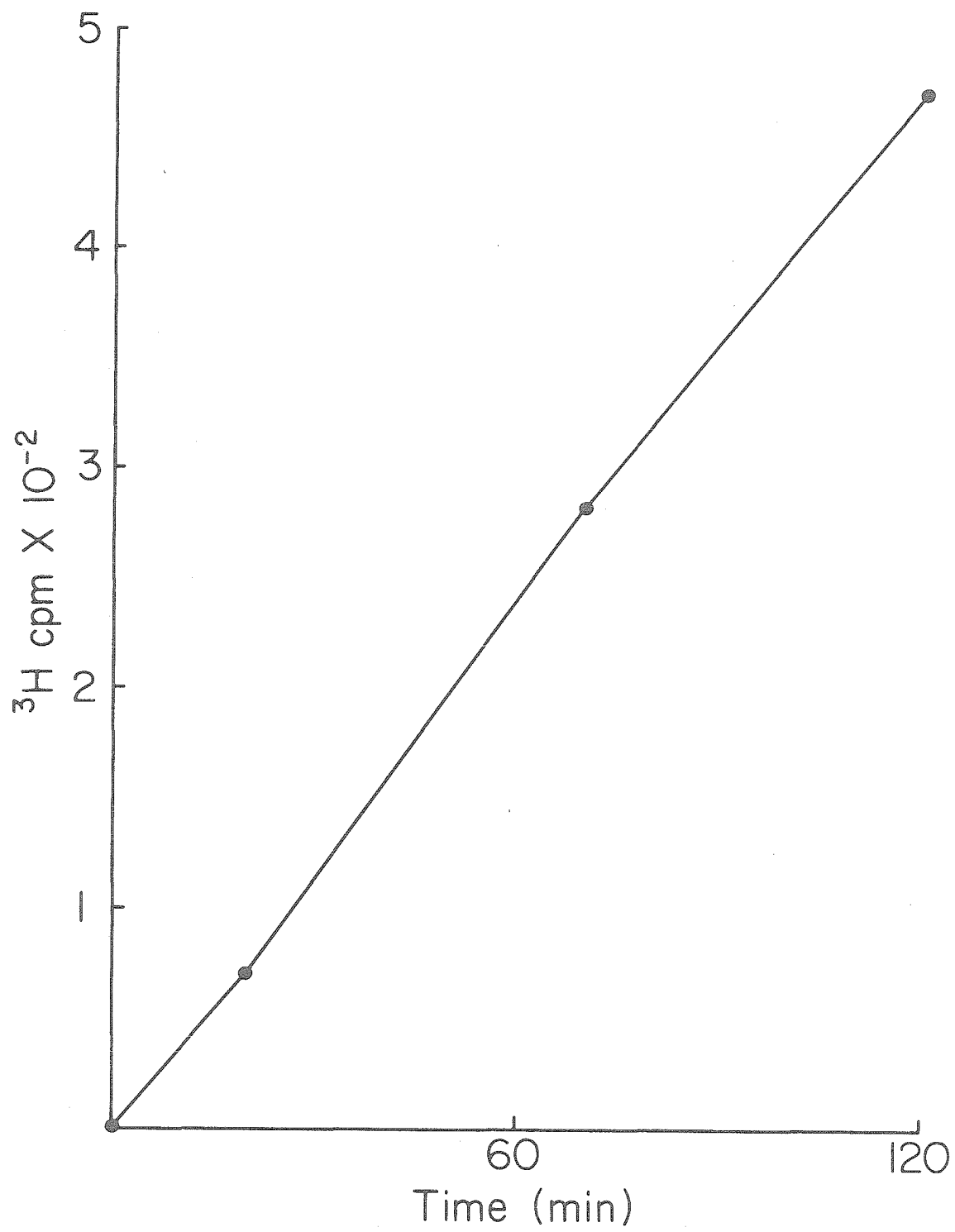
Figure 5

LEGEND TO FIGURE 5

Kinetics of [5-^{3}H]ATP incorporation into RNA
synthesized in isolated mitochondria during incubation
in "KCl medium."

Portions of an isolated HeLa cell mitochondria
fraction (0.44 mg protein) were incubated in identical
mixtures containing, in 0.25 ml, 100 mM KCl, 10 mM MgCl$_2$,
50 mM Tris buffer (pH 7.4 at 25°C), 0.8 mM each CTP,
GTP, UTP, 2 mM ATP, 0.5 mM EDTA, 1 mM phosphate buffer
(pH 7.4 at 25°C), 0.48 µM [5-^{3}H]ATP (10 µCi/ml, 20.7
Ci/mmole, Schwarz) at 30°C. At selected times, the
incubations were stopped by precipitation with 5% TCA
and 10 mM pyrophosphate and counted.

synthesis of mit-DNA-coded RNA (Zylber et al., 1969).

As shown in Figure 6, the sedimentation patterns of the 2 hour in vivo labeled RNA and of the RNA labeled in vitro for 1 hour with [5-^{3}H]UTP or [5-^{3}H]ATP are very similar. In all patterns, one can see a broad peak of radioactivity centered around 16S (corresponding to the major mitochondrial rRNA species) with a more or less defined shoulder at about 12S (corresponding to the minor rRNA species). Moreover, in the pattern of in vivo labeled RNA and in that of the RNA labeled in vitro with [5-^{3}H]ATP (Figure 6c), there is a well-defined 4S RNA peak; the in vitro [5-^{3}H]UTP labeled RNA shows at the same position only the hint of a shoulder (Figure 6b). After a longer centrifugation time, the sedimentation pattern of the RNA labeled in vitro for 1 hour with [5-^{3}H]UTP (Figure 7a) shows clear peaks at 16S and 12S, while a 4S RNA peak is absent and there is only a slight indication of a shoulder at that position. In all patterns, there is heavier heterogeneous material which accumulates against the sucrose cushion. This material presumably represents transcription complexes (Aloni and Attardi, 1972a,b; Carré and Attardi, 1978) and large unprocessed RNA transcripts.

As illustrated in Figure 6a, ethidium bromide at 1 μg/ml, inhibits by 71% the labeling of mit-DNA-coded
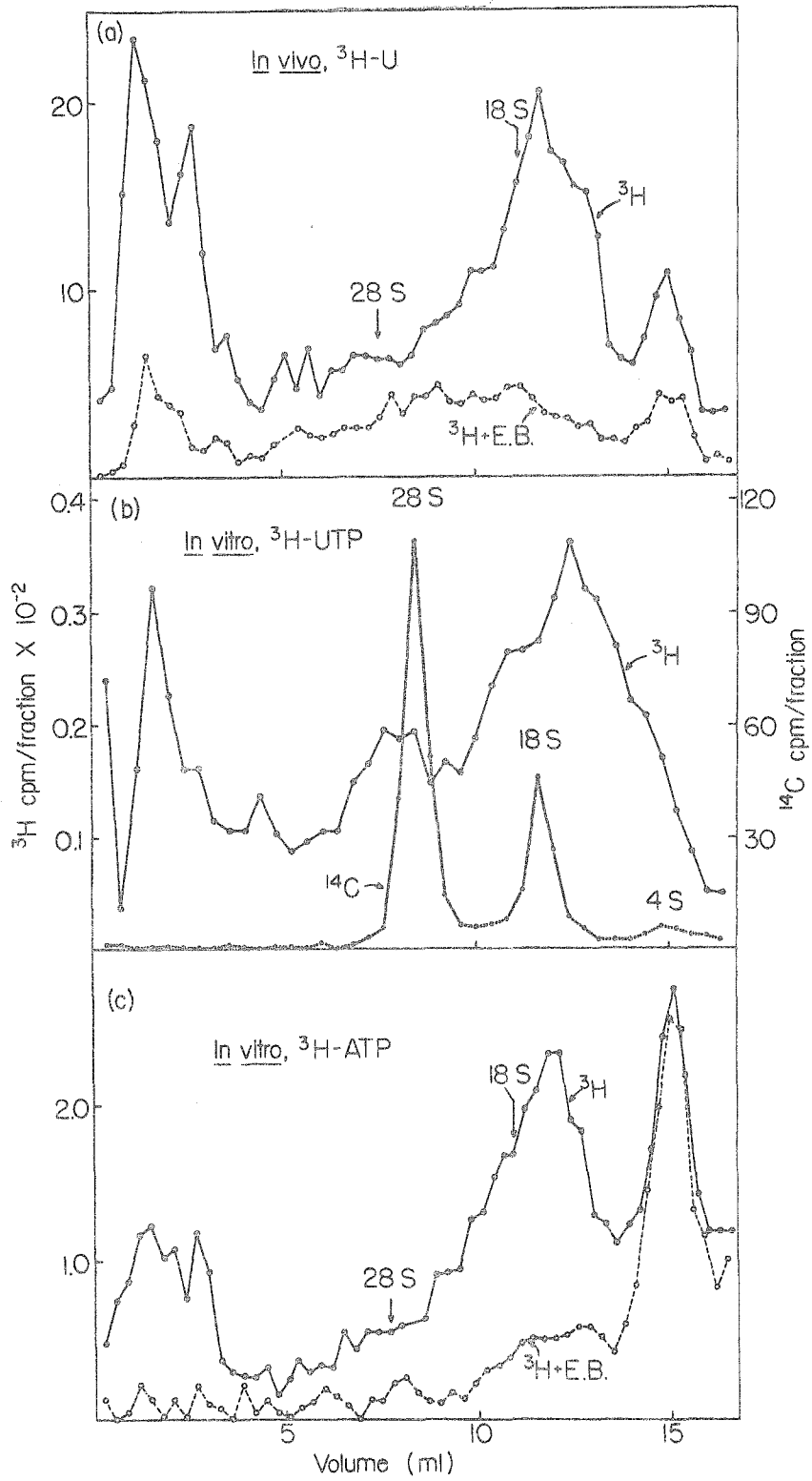
Figure 6

LEGEND TO FIGURE 6

Sedimentation pattern of RNA extracted from the
mitochondrial fraction of HeLa cells labeled in vivo
with [5-^{3}H]uridine (a) or from the mitochondrial fraction
incubated in vitro in the presence of [5-^{3}H]UTP (b), or
[5-^{3}H]ATP (c).

(a) Two cell suspensions (each 400 ml at 2x10^5
cells/ml) in growth medium were treated with 0.04 µg
actinomycin D/ml for 30 minutes and then labeled with
[5-^{3}H]uridine, always in the presence of the drug. To
one suspension 1 µg ethidium bromide/ml was added
15 minutes before labeling. RNA was phenol extracted
from the mitochondrial fractions, ethanol precipitated
and dissolved in SDS buffer. The samples were layered
on sucrose gradients in SDS buffer with a cushion of
64% sucrose in the same buffer. Centrifugation was in
the Beckman SW27 rotor at 25,000 rpm for 12.5 hours.
Fractions were spotted on filters, batch washed in 5%
TCA and ethanol and counted. No ethidium bromide
treatment, ●——●; ethidium bromide treatment, o———o.

(b) The HeLa cell mitochondrial fraction was incubated
at 1.3 mg protein/ml in 1 ml of 75 mM KCl, 7.5 mM MgCl$_2$,
35 mM Tris buffer (pH 7.4 at 25°C), 0.1 mM each

(LEGEND TO FIGURE 6 CONTINUED)

ATP, CTP, GTP and 0.028 mM [5-^{3}H]UTP (0.43 mCi/ml,
15 Ci/mmole, Schwarz) at 30°C for 60 minutes. The RNA
was SDS-pronase-phenol extracted, ethanol precipitated
and redissolved in SDS buffer. The sample (●——●), after
addition of ^{14}C-labeled HeLa cell rRNA (●···●) as a
sedimentation marker, was centrifuged through a sucrose
gradient at 27,000 rpm for 11 hours. The fractions were
treated with 5% TCA and the precipitates collected on
Millipore filters and counted.

(c) The mitochondrial fraction was incubated in
1.5 ml of 75 mM KCl, 7.5 mM MgCl$_2$, 35 mM Tris buffer
(pH 7.4 at 25°C), 0.027 mM each CTP, GTP, UTP, and 0.067
mM [5-^{3}H]ATP (0.5 mCi/ml, 18.3 Ci/mmole, Schwarz), at
30°C for 60 minutes. The RNA was extracted and run on a
sucrose gradient as in (a). The radioactivity data
plotted in the three panels refer to the total in the
in vivo or in vitro labeled samples. No ethidium bromide
treatment, ●——●; ethidium bromide treatment, o---o.
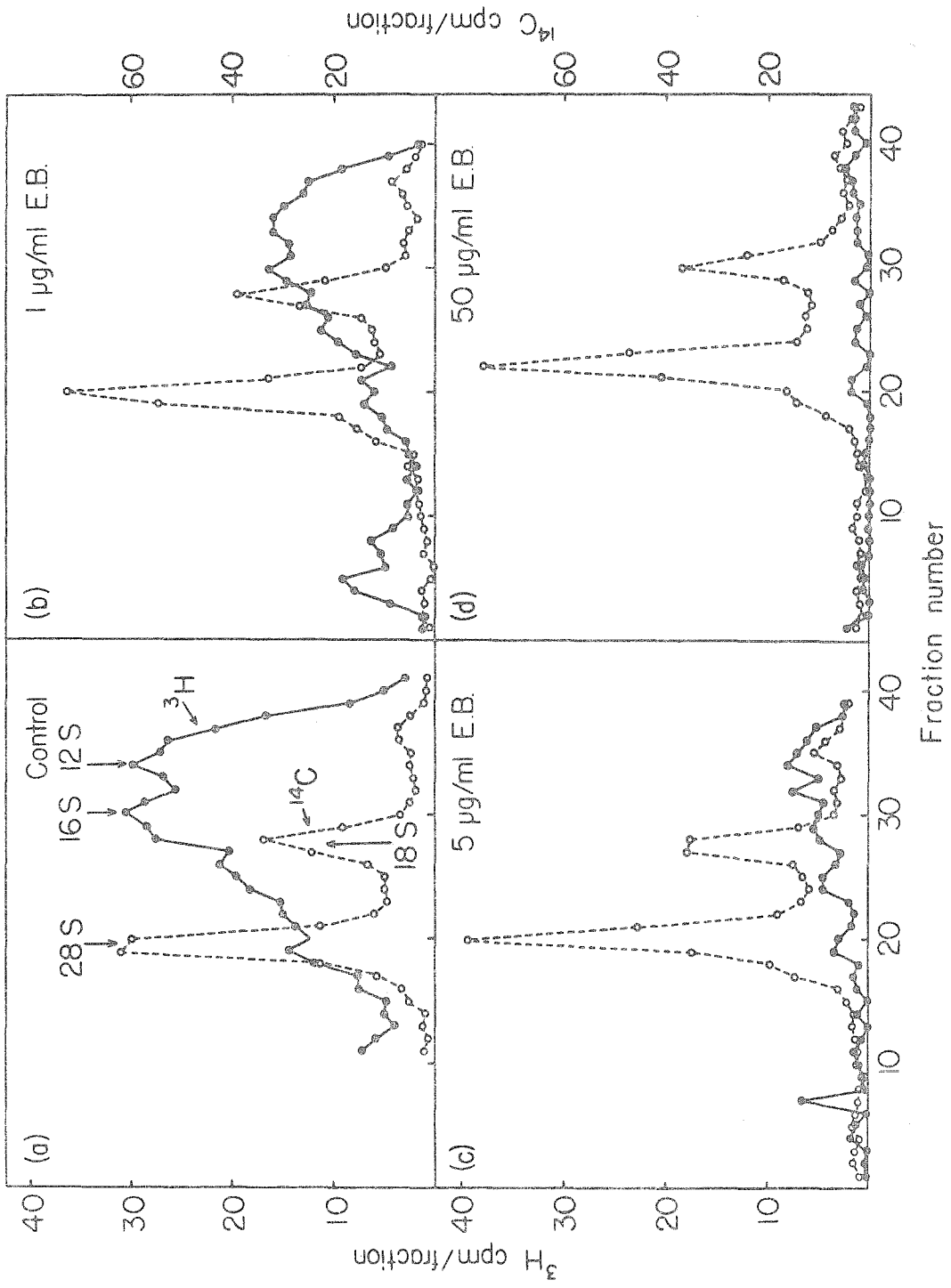
Figure 7

LEGEND TO FIGURE 7

Sedimentation pattern of RNA labeled with [5-^{3}H]UTP
in isolated mitochondria in the presence of ethidium
bromide at various concentrations.

The mitochondrial fraction was isolated from HeLa
cells (800 ml, 2x10^5 cells/ml), divided into 4 portions
which were then incubated for 30 minutes on ice with
various concentrations of ethidium bromide: 0 (a),
1 (b), 5 (c), and 50 (d) µg/ml. Each sample was then
incubated, for 60 minutes at 30°C in 0.5 ml of 75 mM
KCl, 7.5 mM MgCl$_2$, 35 mM Tris buffer (pH 7.4 at 25°C),
0.1 mM each ATP, CTP, GTP, 0.033 mM [5-^{3}H]UTP (0.5 mCi/ml,
15 Ci/mmole, Schwarz), and the same ethidium bromide
concentration as in pretreatment. RNA was SDS-pronase-
phenol extracted, ethanol precipitated and dissolved
in SDS buffer. The extracted RNA (●——●) was mixed
with ^{14}C-labeled HeLa cell rRNA (o----o) as a sedimentation
marker sample and layered on a sucrose gradient in SDS
buffer with a cushion of 64% sucrose in SDS buffer.
Centrifugation was in the SW27 Beckman rotor at 27,000
rpm for 11 hours. Fractions were treated with 5% TCA
and the precipitate collected on Millipore filters and
counted. The radioactivity data plotted in the figure
refer to the total samples and were not corrected for

61

(LEGEND TO FIGURE 7 CONTINUED)

small variations in the amount of protein measured in
each incubation (0.241 mg (a), 0.221 mg (b), 0.238 mg (c),
and 0.237 mg (d)).

RNA in vivo with [5-^{3}H]uridine, in agreement with previous
observations (Zylber et al., 1969; Attardi et al., 1970).
This indicates that under the experimental conditions
used, the bulk of the newly sythesized RNA associated
with the mitochondrial fraction is mit-DNA-coded. The
resistant portion presumably represents cytoplasmic or
nuclear RNA contaminants whose labeling was expected to
be only partially inhibited by 0.04 µg of actinomycin D/ml.
The effect of ethidium bromide on the in vitro RNA
synthesis was also investigated. In these experiments,
ethidium bromide treatment of isolated mitochondria
included a 30 minute pretreament to permit entry of the
dye into the mitochondria. As shown in Figure 7b and
Figure 8, ethidium bromide at 1 µg/ml inhibited only
about 50% of the labeling with [5-^{3}H]UTP of the in vitro
synthesized RNA. With the drug at 5 µg/ml, the inhibition
of labeling with [5-^{3}H]UTP of the in vitro synthesized
RNA reached about 88% (Figure 7c, Figure 8) and increased
only very slowly with higher doses of the drug (Figure 7d,
Figure 8). At all concentrations, the drug appeared to
affect uniformly the RNA species of different sedimentation
constants. The in vitro labeling with [5-^{3}H]ATP of the
in vitro synthesized RNA appeared to be substantially
inhibited by ethidium bromide at 1 µg/ml (approximately
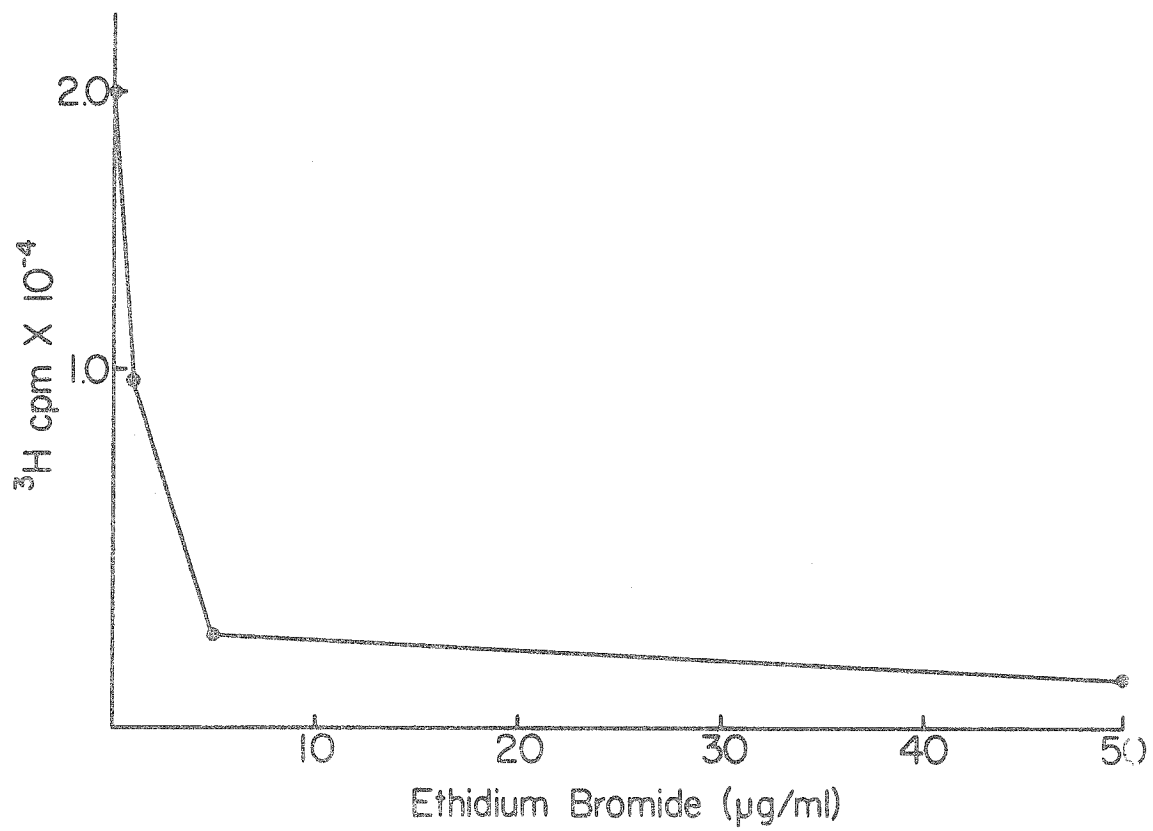
Figure 8

LEGEND TO FIGURE 8

Effect of ethidium bromide on total RNA labeling in isolated mitochondria incubated in the presence of $[5-^3H]$UTP.

The samples were from the experiment described in Figure 7. The total ^{3}H-cpm incorporated in each incubation mixture (ordinate) were determined by spotting a portion of the SDS-pronase-phenol extracted, ethanol precipitated RNA on a filter, batch washing in 5% TCA and ethanol and counting.

90% excluding the 4S RNA peak; Figure 6c); it is noteworthy that the labeling of the 4S RNA peak appeared to be insensitive to the drug at this concentration and, in some experiments, the labeling of this peak in the presence of the drug appeared to be even higher than in the controls. The significance of the labeling with [5-^{3}H]ATP of 4S RNA and its insensitivity to ethidium bromide will be discussed below.

The experiments described above indicated that ethidium bromide sensitivity of the in vitro labeled RNA was not an unambiguous indicator of the proportion of the RNA synthesized in vitro which is transcribed from mit-DNA. For the in vivo labeled RNA, the incomplete sensitivity to ethidium bromide at 1 µg/ml could be accounted for by the known occurrence of cytoplasmic and nuclear labeled RNA contaminants (Attardi et al., 1969). In contrast, there was no previous information available to judge whether the incomplete sensitivity to the drug of in vitro synthesis was due to alteration in the membrane permeability to the drug which reduced its effective intramitochondrial concentration, or to changes in the transcription apparatus after isolation of the organelles, or to a certain level of nuclear RNA synthesis occurring in residual nuclear fragments

contaminating the mitochondrial fraction.

(iv) Hybridization with separated mit-DNA strands of
     in vitro labeled RNA

In order to determine more directly the origin of the
in vitro labeled RNA, RNA-DNA hybridization experiments
were carried out in solution between in vitro $[5-^3H]UTP$
or $[5-^3H]ATP$ labeled RNA and increasing amounts of
complementary strands of $[2-^{14}C]$thymidine labeled HeLa
mit-DNA. The latter were separated by an alkaline CsCl
gradient as illustrated in Figure 9. The results of the
hybridization experiments are summarized in Figure 10.
One sees that 57% of the RNA labeled with $[5-^3H]UTP$
hybridized with the "H" strand and 24% with the "L"
strand (Figure 10a). In the case of $[5-^3H]ATP$ labeled
RNA, 52% hybridized with the "H" strand and more than
20% with the "L" strand (Figure 10b). In both series
of experiments, the lack of hybridization with total
HeLa DNA ruled out nonspecific binding to DNA. These
experiments gave, as minimum estimates of the percentage
of mit-DNA-coded RNA in the in vitro $[5-^3H]UTP$ and
$[5-^3H]ATP$ labeled RNA, 81% and 72% respectively. These
values are comparable to those obtained by RNA-DNA
hybridization experiments for in vivo nascent RNA chains
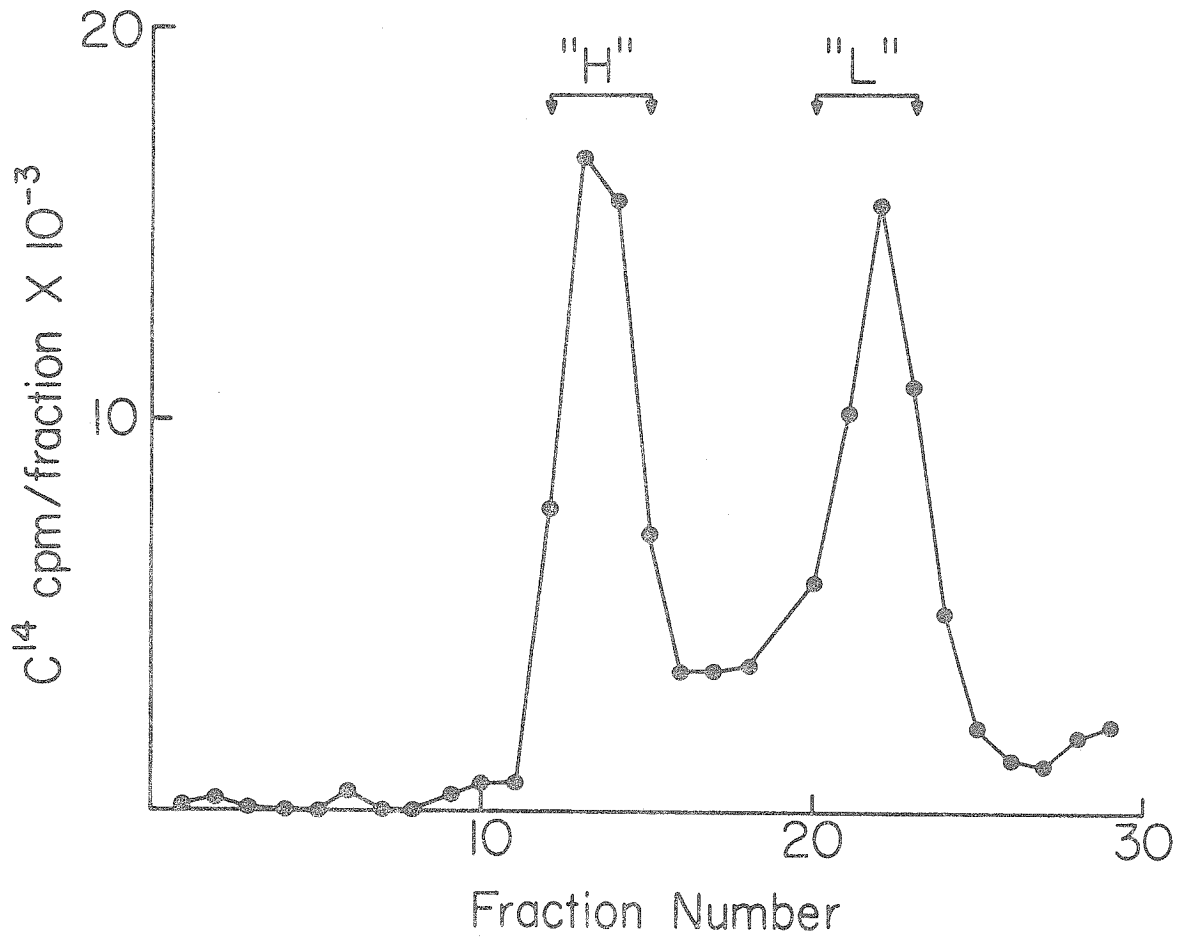
Figure 9

LEGEND TO FIGURE 9

Strand separation of mit-DNA.

HeLa cells were labeled with $[2-^{14}C]$thymidine (0.025 µCi/ml, 57 mCi/mmole, Amersham) for 3 days. Closed circular mit-DNA from these cells was purified by two successive equilibrium runs in a CsCl density gradient with 200 µg ethidium bromide/ml. The final sample, freed of ethidium bromide, was mixed with 40 µg of unlabeled closed circular mit-DNA, brought up to pH 12.4 with K_3PO_4 and run in the Beckman 65 rotor at 42,000 rpm for 42 hours. Aliquots of the fractions were analyzed for acid-precipitable radioactivity. The radioactivity data plotted refer to the total fractions. Pooled fractions of heavy ("H") strand or light ("L") strand mit-DNA are indicated by brackets.
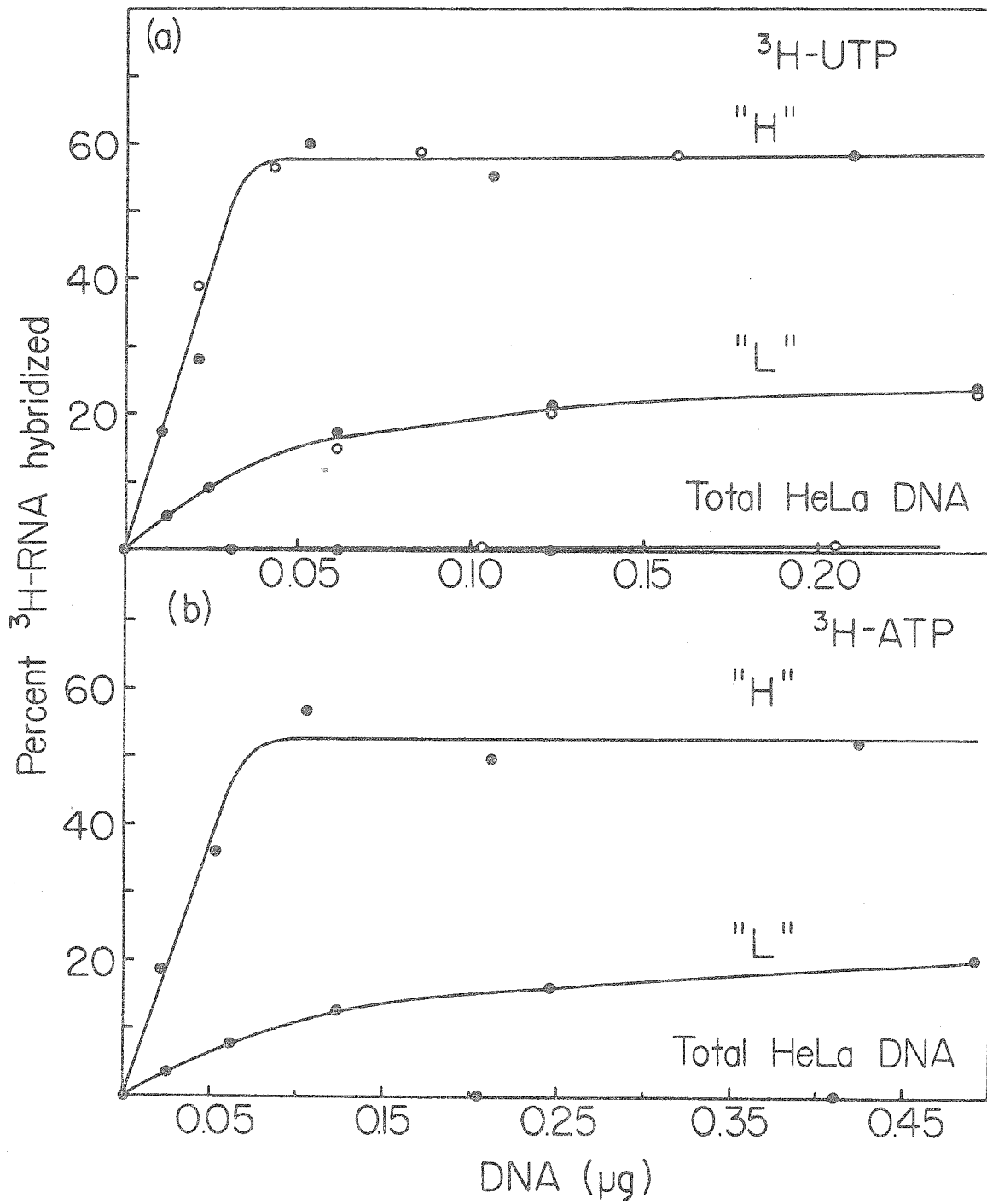
Figure 10

LEGEND TO FIGURE 10

Homology to separate mit-DNA strands of RNA labeled in isolated mitochondria incubated in the presence of $[5-^3H]$UTP (a) or $[5-^3H]$ATP (b).

SDS-pronase-phenol extracted and ethanol precipitated RNA, from the experiment described in Figure 6b, (a), and from an experiment similar to that described in Figure 6c, (b), was treated with RNase-free DNase and reextracted. The RNA was denatured by heating, and aliquots (100-200 cpm) were incubated with selected amounts of heavy strand mit-DNA ("H"), light strand mit-DNA ("L"), or heat denatured "nuclear" HeLa DNA (upper band in the first CsCl/ethidium bromide equilibrium density gradient described in the legend in Methods and Materials) in 0.4 M NaCl, 10 mM Tris buffer (pH 8.0 at 25°C), 10 mM EDTA, final volume 0.5 ml, at 66°C for 4 hours. After treatment with DNase-free RNase, the hybrids were collected on Schleicher and Schuell B6 filters, washed and counted as described in Materials and Methods. The percentage of the total input ^3H-cpm retained on the filter is plotted as a function of the amount of DNA placed in the hybridization mixture after correction for the background obtained without DNA (< 1% of input).

(LEGEND TO FIGURE 10 CONTINUED)

In (a), the results of two hybridization experiments utilizing the same RNA as shown. In (b) the incubation conditions for labeling the RNA were as those in the legend for Figure 6b, the labeled precursor was $[5-^3H]$ATP (0.023 mM, 0.43 mci/ml, 18.3 ci/mmole, Schwarz) and the unlabeled UTP concentration was 0.1 mM.

associated with transcription complexes (Aloni and
Attardi, 1971c).

(b) Effect on in vivo cycloheximide treatment
on in vitro RNA incorporation

In order to study the effect of the treatment of
HeLa cells with cycloheximide on the in vitro labeling
of mit-RNA, mitochondria were isolated from HeLa cells,
grown for up to 4 hours in the presence of the drug at
200 μg/ml and incubated with [5-^{3}H]UTP in "KCl
medium" under the conditions described in Figure 12.
Figure 11 shows that the recovery of protein of the
mitochondrial fraction was not affected by the drug
treatment. As shown in Figure 12a (●——●), the rate of
incorporation of [5-^{3}H]UTP into RNA of organelles isolated
from cells exposed to the drug for various times showed a
slight, progressive decrease as compared to that of untreated
cells, becoming 85% of the control after 4 hours. In
another experiment (not shown), the decrease appeared
to be somewhat more pronounced (∿ 25% after 4 hours).

The labeling of the mitochondrial PCA soluble fraction
in the experiment illustrated in Figure 11 and in another
experiment not shown was also determined (Figure 12b,
●——●). The in vitro labeling of the soluble pool in
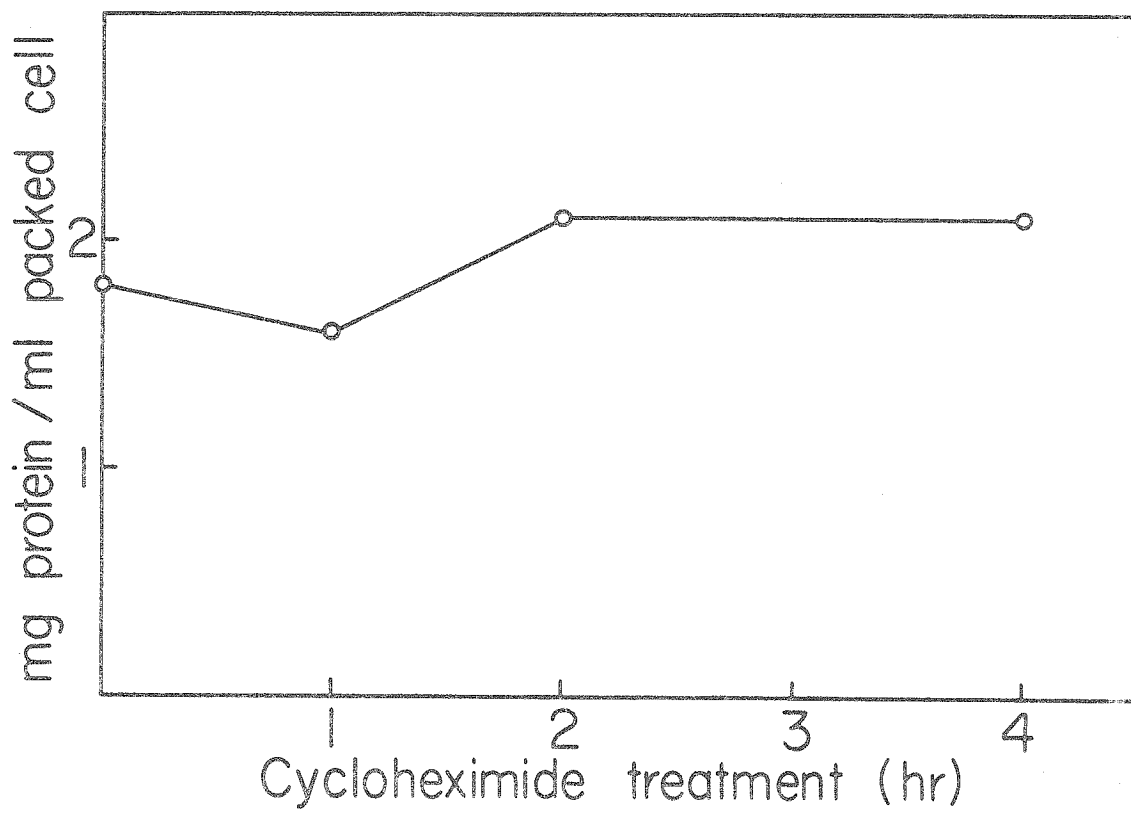
Figure 11

LEGEND TO FIGURE 11

Recovery of mitochondria from cycloheximide treated HeLa cells.

The protein concentration of the mitochondrial fractions was determined by a modified Lowry technique. The data are expressed as the total mg of protein in each mitochondrial preparation per ml of packed cell volume from which that mitochondrial fraction was isolated.
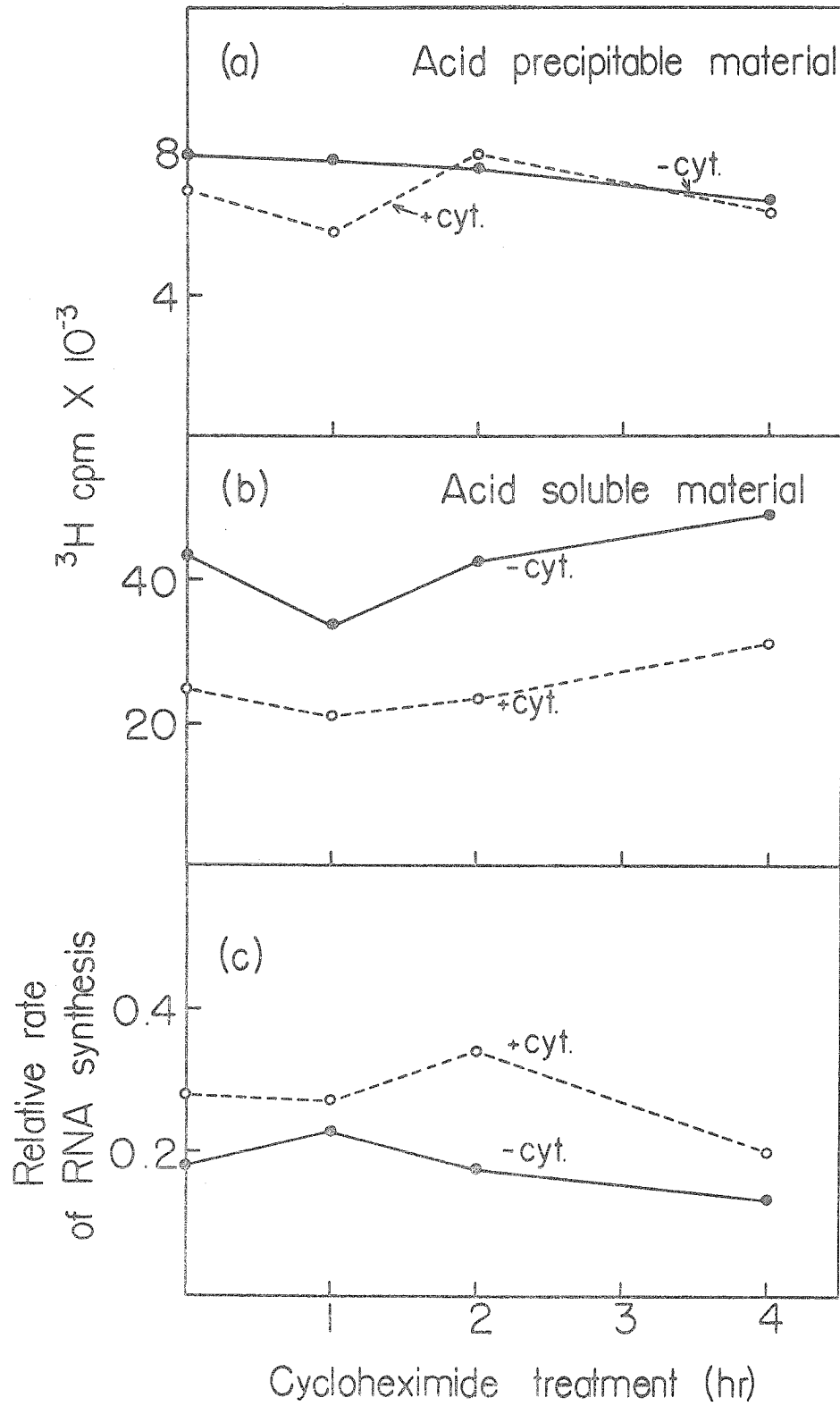
Figure 12

LEGEND TO FIGURE 12

Effect of a normal cytoplasmic fraction on the
labeling of the acid precipitable and acid soluble
fractions of isolated HeLa cell mitochondria incubated
with [5-^{3}H]UTP in "KCl medium."

Cells (400 ml, 2x10^5 cells/ml) were maintained either
in the absence, or for varying times (up to 4 hours) in
the presence, of 200 µg cycloheximide/ml, harvested and
a cytoplasmic fraction (17,000 g supernatant of the cell
homogenate) prepared as described in Materials and
Methods. The cycloheximide treated cultures were
harvested and the mitochondrial fractions were isolated.
Two portions of each preparation (0.5 mg protein) were
incubated in a final volume of 1 ml in 75 mM KCl, 10 mM
MgCl$_2$, 50 mM Tris buffer (pH 7.4 at 25°C), 2.5 mM ATP,
1.7 µM [5-^{3}H]UTP (0.025 mCi/ml, 15 Ci/mmole, Schwarz),
at 30°C for 15 minutes. The mitochondrial fractions
were then washed twice in STM and resuspended in STM.
The PCA soluble fraction of one half of the sample was
neutralized and counted. The TCA precipitable fraction
of the other half of the sample was collected on Millipore
filters and counted. TCA precipitable radioactivity (a);
PCA soluble radioactivity (b). TCA precipitable radio-
activity/PCA soluble radioactivity (c). o---o: with

(LEGEND TO FIGURE 12 CONTINUED)

added cytoplasmic fraction; ●——●: with no added cytoplas-
mic fraction.

mitochondria from cells exposed to cycloheximide for various times up to 4 hours showed some fluctuations with a certain tendency to increase relative to control samples at late times (2 and 4 hours). If the radioactivity in this soluble pool is proportional to the mitochondrial UTP specific activity, then they can be used to calculate the relative rate of RNA synthesis. One can see from Figure 12c (●——●) that the estimated rate of in vitro mit-RNA synthesis is not significantly affected by cycloheximide treatment of the cells up to 2 hours, showing a 25% decrease after 4 hours. In another experiment, the rate of synthesis after 2 or 4 hours was decreased about 65%.

In the experiment shown in Figure 12, the effect of the addition of the incubation mixtures of 17,800 g cytoplasmic fraction from normal cells was also investigated in order to test the capacity of this fraction to supply some factors which may have been depleted in the mitochondria of the cycloheximide treated cells. As shown in Figure 12a (o----o), the effect of the addition of normal cytoplasm on in vitro mitochondrial RNA labeling was negligible both in the mitochondrial sample isolated from untreated cells and in the samples isolated from drug-treated cells. There was, on the contrary, a consistent decrease in the labeling of the acid-soluble pool; this

decrease, if truly reflecting the labeling of the mito-
chondrial UTP pool, would indicate a stimulating effect
of the normal cytoplasm on mit-RNA synthesis independently
of in vivo cycloheximide treatment for up to 4 hours
(Figure 12c, o----o).

4. Discussion

(a) Characteristics of the System

(i) Uptake of precursors

It has been demonstrated here that isolated HeLa cell
mitochondria are capable of supporting mit-DNA transcrip-
tion and, possibly, some RNA processing. $[5-^3H]UTP$ and
$[5-^3H]ATP$ were utilized as precursors in in vitro assays
of mit-RNA synthesis in isolated mitochondria. Using a
rat liver mitochondrial fraction incubated under a variety
of conditions, several laboratories have previously shown
labeling of RNA in the presence of radioactive UTP (Neubert
et al., 1968; Fukamaki et al., 1970; Saccone et al., 1968),
UMP (Aaij et al., 1970), GTP (Neubert et al., 1968;
Saccone et al., 1968), AMP (Saccone et al., 1968), or
ADP (Saccone et al., 1968). Bosmann (1971) observed
^3H-uridine incorporation in isolated mitochondria from
mouse tissue culture cells. The present study, however,
is the first one in which an in vitro system from
mammalian cells in culture has been investigated in detail
and in which the in vitro products have been character-
ized as mit-RNA. This system offers obvious advantages
over the rat liver mitochondrial system because of the

large amount of information already available on the mechanism, products and regulation of transcription of mit-DNA in HeLa cells. In a more general sense, a system derived for cells in culture is more amenable to quantitative investigations and to comparative analysis of the effects of various experimental conditions in vivo and in vitro; also the effect of various treatments of the cells, prior to organelle isolation, on their subsequent RNA synthetic capacity can be more easily studied with cell culture systems.

It is not known in what form the labeled precursor utilized in the above mentioned and the present investigations entered the mitochondria. An ATP-ADP translocase has been identified in the inner mitochondrial membrane which can exchange labeled ADP for unlabeled ATP or labeled ATP for unlabeled ADP across the membrane; this translocase is strongly and specifically inhibited by atractyloside (Pfaff et al., 1969). Membrane transport for UTP has not been demonstrated so far. It has been observed in this laboratory that, after labeling isolated HeLa cell mitochondria in the presence of commercially available $[\gamma-P^{32}]$ATP, all or almost all the radioactivity found in RNA is in the α position, suggesting the occurrence of hydrolytic processes either inside or outside the

organelles (P. Young, personal communication). UTP
dephosphorylation activity has been detected in mitochon-
drial extracts (Racker, 1965). Whatever the precursor
utilized, in in vitro experiments any dephosphorylation
occurring outside or inside the mitochondria which would
generate nucleoside di- or monophosphates, must be followed
inside the organelles by phosphorylation to ribonucleoside
triphosphates, which are the substrates for RNA polymerase.

The above mentioned work by Aaij et al. (1970)
indicates that ribonucleoside monophosphates can indeed
be utilized as precursor for mitochondrial RNA synthesis.
Adenylate kinase has been reported to be located in the
space between the inner and outer membranes (Schnaitman
and Greenawalt, 1968). A mitochondrial thymidine kinase
is also known to exist (Attardi and Attardi, 1972; Clayton
and Teplitz, 1972); it would not be unexpected that uridine
kinase activity is also present.

(ii) Effect of in vitro incubation conditions on RNA incorporation

The effect of various parameters on the level of
$[5-^3H]$UTP incorporation in vitro has been investigated
here using two main sets of conditions. In the "KCl

medium," the main role of KCl was probably that of
insuring the appropriate osmolarity of the medium for
optimum functioning of the isolated organelles for RNA
synthesis; no requirement for KCl could be detected in
the "sucrose medium" when the necessary osmolarity was
obtained with sucrose. That the ionic environment was
important in these in vitro experiments, however, is
indicated by the different optimum of Mg^{++} concentration
in the two types of media.

There are contrasting reports in the literature on
how incubation conditions affect in vitro incorporation
of precursors into RNA of isolated rat liver mitochondria.
This may be due to the variety of incubation systems used.
Some include pyruvate kinase and its substrate, phosphoenol-
pyruvate, for the exogenous production of ATP. Other
investigators have used endogenous energy sources
(Fukamaki et al., 1970), including pyruvate, succinate
and malate, whereas still others have used ATP but no
ATP generating system (Neubert et al., 1966; Neubert
et al., 1968).

The effect of some incubation parameters have been
investigated in rat liver. Saccone et al. (1968) found
that ^{14}C-ATP incorporation increased linearly with
increasing mitochondrial protein up to 0.5 mg/ml and

then leveled off. In HeLa cells, the data presented here show that [5-^{3}H]UTP incorporation increased linearly with protein concentration between 1 mg/ml and 10 mg/ml. No obvious explanation can be suggested for these differences.

Both Saccone et al. (1968), using ^{14}C-ATP, and Neubert et al. (1968), using ^{3}H-UTP, reported that the rate of incorporation increased with increasing amounts of radioactive precursor and then leveled off at around 0.1 mM. Their studies covered a range of labeled ATP or UTP concentrations of about one order of magnitude. In the present experiments, the [5-^{3}H]UTP or [5-^{3}H]ATP concentration was kept constant, while the cold UTP, or respectively ATP, was added in increasing concentrations, up to 2.5 mM and 10 mM respectively; in both types of experiments, the concentration of the precursor covered 3-4 orders of magnitude. In the case of [5-^{3}H]UTP, its incorporation went down only by 1-2 orders of magnitude (16-fold) as the total concentration of UTP increased by nearly 3 orders of magnitude (7 μM to 2.5 mM). In the case of ATP, with increasing total concentration from 2.8 μM to 0.7 mM, incorporation of [5-^{3}H]ATP decreased 12 fold, whereas above 0.7 mM ATP, an increase in labeling more than compensated for the 14-fold dilution of the precursor. These results

suggest that, with an increasing concentration of exogenous UTP or ATP, the precursor entered the mitochondria in increasing amounts, although this increase in uptake was not proportional to the increase in the exogenous concentration; furthermore, the results imply that the amount of nucleoside triphosphate taken in was, for all concentrations tested, small in comparison with the mitochondrial pool.

In the incubations utilizing $[5-^3H]UTP$, the ATP concentration did not appear to have a pronounced effect on the level of incorporation. Therefore, it is suggested that, in the in vitro system described here, endogenous ATP concentration is probably not rate limiting as an RNA polymerase substrate or as any energy source; thus, the increase in $[5-^3H]ATP$ incorporation with increasing the ATP concentration may be due primarily to an increased specific activity of the mitochondrial pool.

(iii) Rate and kinetics of in vitro RNA synthesis

In the present experiments, the in vitro incorporation of labeled precursors into RNA was found to be low and comparable to that observed in rat liver (Saccone et al., 1968). Nothing, however, can be concluded from these data about the rate of RNA synthesis in vitro as compared to

the in vivo situation, since it is not known whether
the rate limiting step in the utilization of exogenous
precursors is the rate of entry or of modification of
these precursors or the actual rate of RNA synthesis in
the isolated organelles.

In the present work, the isolated mitochondria
were able to support linear or close to linear incorpor-
ation of the radioactive precursor for at least 1 hour
under the conditions used. These findings are therefore
comparable to those reported by Saccone et al., (1969)
who observed a linear incorporation for 1 hour at 30°C
using ^{14}C-ATP as a precursor, phosphoenolpyruvate and
pyruvate kinase as an ATP generating system and a
concentration of mitochondrial fraction protein of 3 mg/ml.

(iv) Nature of the products of in vitro RNA synthesis

Virtually all of the RNA labeled in vitro in the
present experiments has been shown to consist of mit-DNA
transcripts complementary to both strands; furthermore,
the labeled mitochondrial RNA which accumulated in vitro
had a gross sedimentation pattern similar to that of
in vivo synthesized RNA.

16S and 12S RNAs, known to be the relative stable
mitochondrial ribosomal RNAs (Attardi and Attardi, 1971),

appeared as products in the in vitro experiments utilizing both $[5-^3H]$UTP and $[5-^3H]$ATP (Figures 6b and 6c). Labeled 4S RNA was not present in detectable amounts when $[5-^3H]$UTP was used as a precursor, and the labeling of 4S species RNA with $[5-^3H]$ATP was ethidium bromide insensitive. These results, therefore, suggest that mit-DNA coded tRNA may not be synthesized or properly processed under the in vitro conditions used here.

Fukamaki et al. (1970) have previously reported the in vitro labeling with ^3H-UTP of discrete RNA species in isolated rat liver mitochondria. In particular, they detected labeled RNA species with sedimentation constants, estimated from their electrophoretic mobility, of $21S_E$ and $12S_E$: these species corresponded to the in vivo synthesized mitochondrial ribosomal RNAs. (It has been shown that the apparent molecular weights of mitochondrial ribosomal RNAs estimated by gel electrophoresis under certain conditions can be different from those estimated by sedimentation analysis through sucrose gradients, probably due to the effect of secondary structure (Robberson et al., 1971).) Fukamaki et al. also failed to observe any in vitro labeled 4S RNA. In the above mentioned experiments, the in vitro labeling of rat liver mitochondrial RNA was inhibited up to 70% by ethidium bromide at 0.33 µg/ml.

In the present work, RNA-DNA hybridization experiments showed that almost all (> 80%) of the RNA labeled in isolated HeLa cell mitochondria with [5-^{3}H]UTP during a 1 hour incubation consisted of mit-DNA transcripts, with a higher proportion of the transcript being homologous to the "H" strand (70%) than to the "L" strand (30%). When [5-^{3}H]ATP was used as a precursor, the RNA labeled during 1 hour showed about 72% homology to mit-DNA; of the labeled transcripts showing homology to mit-DNA, 72% were complementary to the "H" strand and over 28% were complementary to the "L" strand. The lower proportion of the [5-^{3}H]ATP labeled RNA which is homologous to mit-DNA is possibly due to the fact that a substantial fraction of the label was present in 4S RNA (27%), which may be at least in part no; mit-DNA-coded (see below). The proportion observed here of labeled "L" strand and "H" strand transcripts after a 60 minute in vitro incubation at 30°C is very similar to that observed in intact HeLa cells after a 45 minute ^{3}H-uridine pulse at 37°C (Aloni and Attardi, 1971c).

Aaij et al. (1970) showed that at least 85% of the RNA, labeled in swollen rat liver isolated mitochondria in the presence of ^{3}H-UMP, hybridized to rat liver mit-DNA. The hybridization of the RNA to separated mit-DNA strands was not carried out to exhaustion; their

observation, however, of 33% of the labeled product
being "H" strand transcripts and 8.5% being "L" strand
transcripts is consistent with the results reported here.
Their interpretation of the results was, however, that
only the "H" strand is usually transcribed and that the
transcription of the "L" strand was an in vitro artifact.
The same laboratory had similarly interpreted analogous
saturation hybridization experiments with in vivo labeled
rat liver mitochondrial RNA (Borst and Aaij, 1969). In
both cases they failed to realize that "L" strand
transcription occurs, as it does in HeLa cells in vivo
(Aloni and Attardi, 1971c) and in vitro as demonstrated
here.

In contrast to the results of RNA-DNA hybridization
experiments, ethidium bromide at 1 μg/ml was found in
the present work to inhibit [5-^{3}H]UTP incorporation
in vitro to the extent of 50%. This concentration of
the drug is known to be sufficient to inhibit essentially
completely mit-DNA-coded RNA synthesis in intact HeLa
cells (Zylber et al., 1969; Attardi et al., 1970).
In the present work, > 5 μg/ml were needed to inhibit
mit-RNA synthesis by 90%. Whether this is due to a lower
intramitochondrial concentration of ethidium bromide
(due to binding of the dye by the mitochondrial or other

membranes or to altered permeability of the organelles)
or to greater uptake of triphosphates by isolated
mitochondria in the presence of the dye remains to be
determined.

With regard to the labeled, ethidium bromide-resistant
4S RNA observed in the experiments using [5-^{3}H]ATP as a
precursor, it is not known whether they represent mitochon-
drial DNA transcripts. It seems reasonable to exclude the
possibility that they are newly synthesized species, since as
mentioned above, no labeling of 4S RNA with ^{3}H-UTP was
observed. If all or a portion of this material is mit-DNA-
coded, then the labeling of this portion is probably the
result of -CCA end labeling (Daniel and Littauer, 1963)
of mit-tRNA present in the mitochondria before labeling.
On the contrary, if all or a portion of the radioactive
4S material is not mit-DNA coded, then this portion may
either represent end-labeled nuclear-coded tRNA utilized
in the mitochondria (Lynch and Attardi, 1976; Ching et al.,
1977) or "free" poly A synthesized by mitochondrial
poly A polymerase (Jacob et al., 1974; Ojala and Attardi,
1974a). RNA/DNA hybridization experiments utilizing
4S RNA and analysis of the binding properties to oligo(dT)-
cellulose should be able to resolve these ambiguities.

(b) Effect of in vivo cycloheximide treatment

on in vitro RNA synthesis

In the present work, treatment of HeLa cells with

200 µg cycloheximide/ml for up to 4 hours had the effect

of decreasing slightly and progressively the level of

in vitro incorporation of [5-^{3}H]UTP into RNA of isolated

organelles, although there was some variation from

experiment to experiment. After correcting for the

changes observed in the specific activity of the mito-

chondrial and soluble pool, the rate of RNA synthesis

in mitochondria isolated from cells treated 4 hours

with cycloheximide was decreased by 25 to 68% relative

to the controls. These in vitro estimates depend on

the assumption that the cycloheximide treatment does

not change the relative proportion of uridine derivatives

including UTP in the mitochondrial soluble pool. The

effect on in vitro mitochondrial RNA synthesis of

in vivo treatment of the cells with cycloheximide

appears to be somewhat less than previously observed

for in vivo RNA synthesis after the same treatment

($\sim$ 70% after 4 hours of exposure of the cells to the drug,

Storrie and Attardi, 1972).   However, the lack of

information on the absolute rate of in vitro RNA synthesis

makes a direct comparison of the in vitro and in vivo

results difficult. In any case, the present results sug-
gest that whatever changes have been caused by the cyclo-
heximide in vivo treatment, the effect of these changes on
the rate of mitochondrial RNA synthesis continue to be
observed in isolated organelles.

When normal cytoplasm was added to the in vitro
incubation mixtures, there was a stimulatory effect on
RNA synthesis; this effect, however, was independent of
the length of cycloheximide treatment and therefore did
not appear to represent a compensation phenomenon for the
effects of being treated. The stimulation of RNA synthesis
by addition of cytoplasm cannot be explained by changes in
the concentration of the salts of the in vitro incubation
mixtures; in fact, any increase in the concentration of the
salts would have been expected to result in a lower level
of incorporation on the basis of the evidence discussed in
a previous section. Therefore, the levels of stimulation
observed are underestimates of the real effect. The
mechanism of this stimulatory effect has not been further
investigated.

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94

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CHAPTER III

Cell Cycle Dependence of Mitochondrial DNA

Replication in Mouse L Cells

1. Introduction

Although the occurrence of mitochondrial DNA synthe-
sis in mammalian mitochondria is well established (Kasamatsu
and Vinograd, 1974), studies on the timing of mammalian
mitochondrial DNA (mit-DNA) synthesis during the cell cycle
have yielded conflicting results. Several organisms have
been studied and a variety of synchronization techniques
have been used.

In the case of lower eukaryotic cells, mit-DNA synthe-
sis has been reported to occur throughout the cell cycle at
a constant rate, within a factor of two, in Tetrahymena
(Parsons and Rustad, 1968) and in Physarum polycephalum
(Guttes et al., 1967; Braun and Evans, 1969). Conflicting
results have been reported in yeast and have not yet been
resolved (Carter, 1975); the results of Smith et al. (1968)
and Cottrell and Avers (1970) indicate that mit-DNA syn-
thesis occurs predominantly just after nuclear DNA syn-
thesis whereas Williamson and Moustacchi (1971) report a
continuous mit-DNA synthesis throughout the cell cycle.

Chinese hamster CHO cells (Ley and Murphy, 1973),
Chang liver cells (Koch and Stokstad, 1967; Koch, 1969),
and HeLa cells (Volpe and Eremenko, 1973) have been studied
but the mitochondrial DNA nature of the labeled material
was not demonstrated. L5178Y mouse cells, synchronized by
a thymidine block followed by a colcemide block, were

reported to have peak periods of ^{3}H-thymidine incorporation into the DNA found in the mitochondrial fraction during the G2 phase and the S phase with in vivo labeling (five-fold increase) and to have a peak period in the G2 phase with in vitro labeling (Bosmann, 1971); however, the DNA was not well characterized as mitochondrial.

In HeLa cells, the labeled DNA found in the mitochondrial fraction has been more carefully characterized. Pica-Mattocia and Attardi (1972) showed that during the late-S and G2 phases of cells synchronized by selective detachment there was a five-fold increase in the rate of mit-DNA synthesis, based on incorporation of [^{3}H]thymidine into closed circular mit-DNA and on the mitochondrial thymidine pool specific activity. In the latter study, mit-DNA synthesis was found to be constant during the cell cycle when the cells were synchronized by the nonphysiological technique of a double thymidine block.

In a recent study on unsynchronized mouse A9 cells, the conclusion was reached that the rate of mit-DNA synthesis is constant during the cell cycle (Bogenhagen and Clayton, 1977); however, as will be discussed in detail below, their data are equally consistent with the opposite conclusion.

The original purpose of the present study was to determine the rate of mit-DNA synthesis during the cell cycle in the thymidine kinase deficient mouse L cell derivative

Cl1D in preparation for a study of mitochondrial regulation using cell fusion (see Chapter IV). In the course of this work, Cl1D cells were found to be unsuitable for the determination of the rates of mit-DNA synthesis in cells synchronized by selective detachment, as is explained below. Therefore, another L cell derivative, A9, was utilized. The present study demonstrates that the rate of mit-DNA synthesis in A9 mouse cells increases dramatically during late-S and G2 phases of the cell cycle.

2. Methods and Materials

(a) Solutions[1]

The solution designations are as follows. (1) NKM:
0.13 M NaCl, 5 mM KCl, 7.5 mM $MgCl_2$. (2) Low ionic

strength TKV: 10 mM KCl, 10 mM Tris buffer (pH 6.7 at

25°C), 0.1 mM EDTA. (3) SDS buffer: 0.5% SDS, 10 mM

Tris buffer (pH 7.0 at 25°C), 0.1 mM NaCl, 1 mM EDTA.

(4) STE: 0.25 M sucrose, 10 mM Tris buffer (pH 6.7

at 25°C), 0.1 mM EDTA. (5) STNM: 0.25 M sucrose, Tris

buffer (pH 7.2 at 25°C), 0.05 M NaCl, 1.5 mM $MgCl_2$.

(6) TD: 0.137 M NaCl, 5 mM KCl, 25 mM Tris buffer (pH 7.4

at 25°C).

(b) Growth and labeling conditions of

unsynchronized cell cultures

Two mouse L fibroblast cell line derivatives were

used (Attardi et al., 1975). A9 cells are resistant to

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[1]Abbreviations used in this chapter are: (1) ATP:
adenosine 5'-triphosphate. (2) BrdU: bromodeoxyuridine.
(3) CTP: cytidine 5'-triphosphate. (4) dCTP: deoxycy-
tidine triphosphate. (5) DNase: deoxyribonuclease.
(6) EDTA: ethylenediaminetetraacetate. (7) GTP:
guanosine 5'-triphosphate. (8) MeOH: anhydrous methanol.
(9) mit-DNA: mitochondrial DNA. (10) PCA: perchloric
acid. (11) PPO: 2,5-diphenyloxazole. (12) RNase A:
pancreatic ribonuclease A. (13) SDS: sodium dodecyl
sulphate. (14) TCA: trichloroacetic acid. (15) TDP:
thymidine 5'-diphosphate. (16) TMP: thymidine 5'-mono-
phosphate. (17) TTP: thymidine 5'-triphosphate.
(18) UTP: uridine 5'-triphosphate.

8-azaguanine and deficient in hypoxanthine-guanine
phosphoribosyltransferase (Littlefield, 1964; Kit et al.,
1963; Attardi and Attardi, 1972). LM(TK⁻) C11D cells
are deficient in extramitochondrial thymidine kinase
activity but retain mitochondria-associated thymidine
kinase activity; they can grow well in the presence of
30 µg BrdU/ml in the dark. The cell lines used were
found to be free of any detectable PPLO contamination
as determined by the method of Brown, Teplitz and Revel
(1974). For synchronization by selective detachment,
cells were grown at 37°C in monolayer on polysytrene
roller bottles (490 cm^2 surface area, Corning, rotating
at 2 rpm) with 100 ml of Eagle's medium (MED-72214, Grand
Island Biological) supplemented with 10% calf serum
(Irving). For labeling the acid-soluble pool uniformly
with ^{32}P-orthophosphate, the cells were grown for 1 day
in modified Eagle's medium containing 5×10^{-4} M phosphate
plus 10% calf serum in the presence of ^{32}P-orthophosphate
(3 µCi/ml, carrier free, ICN) (Pica-Mattoccia and
Attardi, 1972).

A9 and C11D cells were also grown in suspension at
37°C in modified Eagle's medium (Levintow and Darnell,
1960) (MED-69191, Grand Island Biological) supplemented
with 5% dialyzed calf serum (dialyzed against TD). The

initial cell concentration was adjusted so that it was

~ 10^6 cells/ml at the time the individual synchronized

cultures were harvested for subcellular fractionation.

A9 suspension cultures were exposed for 2 days to

0.014 µCi/ml [$2-^{14}$C]thymidine (53 mCi/mmole, Amersham).

C11D suspension cultures were exposed for 2 days to 0.013

µCi/ml of [$2-^{14}$C]thymidine (60 mCi/mmole, Amersham) or

for 2 days to 0.019 µCi/ml of [$2-^{14}$C]thymidine (61 mCi/

mmole, Amersham).

(c)   Selective detachment and labeling

of synchronized cells

During all steps of an experiment, the growth medium

of the monolayer cells remained unchanged (including the

presence or absence of ^{32}P), unless otherwise specified.

(i)   Synchronization experiments for A9 cell mit-DNA

labeling with ^{3}H-thymidine

In experiments aimed at measuring the rate of A9

mit-DNA labeling, all steps were carried out at 37°C.

Thirty roller bottles of monolayer cells were shaken

to selectively detach mitotic cells (Terasima and

Tolmach, 1963; Robbins and Marcus, 1964; England and

Attardi, 1974) at 1 hour intervals, the medium was

removed and fresh medium was added after each removal.
(The first two shakings were vigorous and the second two
were moderate.) The medium of the last shaking containing
detached cells, mostly in mitosis, was filtered through
Nitex 100 nylon mesh, the cells were pelleted at 1,500 rpm
(500 g) for 5 minutes in the International centrifuge,
and the cells resuspended in fresh medium at 4×10^5 cells/ml
for mit-DNA labeling or at 5×10^4 cells/ml, for mitotic
index and nuclear labeling determinations.

For the determination of the rate of mit-DNA labeling,
10 ml portions of the synchronized cell suspension at
4×10^5 cells/ml were placed in the 37°C CO_2 incubator in
10 cm petri dishes. At selected times after cultures
were placed in the incubator, individual cultures were
exposed for 1 hour to [methyl-^{3}H]thymidine (200 μCi/ml,
48.1 Ci/mmole, Amersham) in Eagle's medium with 10%
dialyzed calf serum.

For the determination of the percentage of cells
in S phase, 0.2 ml portions of the 5×10^4 cells/ml
suspension were dispensed into 3.5 cm petri dishes onto
coverslips in an area delimited by a glass ring which
was attached to the coverslip by vacuum grease. At
selected times after placement in the 37°C CO_2 incubator,
the medium was replaced with Eagle's medium plus 10%

dialyzed calf serum containing 200 μCi/ml [methyl-^{3}H]thymidine (48.1 Ci/mmole, Amersham) and the culture was incubated for 1 hour.

For the determination of the mitotic index, 0.2 ml portions of the suspension at 5×10^4 cells/ml were dispensed into 3.5 cm petri dishes onto coverslips. After 2 hours in the 37°C CO_2 incubator, 2 ml of growth medium was added to each culture. At various times, individual coverslips were taken for analysis.

(ii)   Synchronization experiments for A9 cell TTP pool
         labeling with [methyl-^{3}H]thymidine

In experiments aimed at measuring the rate of labeling of the A9 mitochondrial TTP pool, the selective detachment procedure differed from that described above as follows. Selective detachments of ^{32}P-labeled cells were carried out at 2 hour intervals; preliminary shakings, 2 vigorous and 1 moderate, were followed by 3 successive moderate detachments. The cells were pelleted at room temperature and then maintained at 15°C until the cells of the three last detachments were pooled; the cells were then adjusted to 4.5×10^5 and 5×10^4 cells/ml, and rapidly dispensed.

For the determination of the mitochondrial TTP pool labeling, 18 petri dishes were seeded with 10 ml of 4.5×10^5

cells/ml. At selected times after the cultures were placed in the 37°C CO_2 incubator, sets of 6 plates were exposed for 1 hour to 200 µCi/ml of [methyl-^{3}H]thymidine (48.1 Ci/mmole, Amersham) in Eagle's medium with 5×10^{-4} M phosphate, 10% dialyzed calf serum and 3 µCi/ml of ^{32}P-orthophosphate.

(iii) Synchronization experiments for C11D mit-DNA labeling with ^{3}H-thymidine

The procedure was similar to that described above in (i), except that the C11D detached cell population was centrifuged at room temperature and maintained at this temperature during resuspension at 3×10^5 cells/ml and dispensing in 10 ml aliquots in 10 cm petri dishes.

For mit-DNA labeling, at selected times after shifting cultures to the 37°C CO_2 incubator, single dishes were exposed for 2 hours to 200 µCi/ml [methyl-^{3}H]thymidine (48.1 Ci/mmole, Amersham) in Eagle's medium supplemented with 10% dialyzed calf serum.

(iv) Synchronization experiment for C11D mit-DNA labeling with [5-^{3}H]deoxycytidine

The procedure was similar to that described above in (iii), except that selective detachment and all

subsequent culture manipulations were carried out at 37°C. For mit-DNA labeling, individual cultures (4×10^6 cells) were exposed for 1 hour to 200 µCi/ml of [5-^{3}H]deoxycytidine (24 Ci/mmole, Amersham) in Eagle's medium with 10% dialyzed calf serum.

(d) Subcellular fractionation

Monolayer cells were removed from 10 cm petri dishes by replacing the growth medium with 10 ml of TD with 1 mM EDTA and 0.05% trypsin and incubating for 1 minute at 37°C. Cells were gently pipetted off of the dish, the trypsin action was stopped with ice cold medium containing 10% calf serum, and the cells were pelleted. All further steps were carried out between 0°C and 4°C, unless otherwise specified. ^{14}C-labeled suspension culture cells were pelleted and mixed with the ^{3}H-labeled monolayer cells. The cells were washed 3 times in NKM (Attardi, Cravioto and Attardi, 1969) and pelleted at 900 rpm (180 g) for 5 minutes in plastic conical tubes in the International centrifuge. The cells were swollen in 6 volumes of low ionic strength TKV for 5 minutes before breakage with a motor driven glass-teflon homogenizer. After homogenization which gave 70% cell breakage, the sucrose concentration was promptly brought to 0.25 M.

After removal of cells and nuclei by centrifuging at
2,400 rpm (1,300 g) for 3 minutes in plastic conical
tubes in the International centrifuge, mitochondria
were pelleted in the Sorvall at 9,000 rpm (10,000 g)
for 10 minutes and resuspended in 4 ml of STE/ml of the
original packed cell volume. This suspension was subjected
to 2,200 rpm (1,100 rpm) centrifugation for 2 minutes
in the International, and the mitochondrial fraction was
then pelleted at 9,000 rpm (10,000 g) for 10 minutes in
the Sorvall.

(e)   Mit-DNA isolation and analysis

The mitochondrial pellet was resuspended in 4 ml
of STNM/ml of the original packed cell volume.   The
solution was brought to 100 µg DNase I/ml and 100 µg
RNase A/ml and incubated at 0°C for 1 hour. (RNase A,
pancreatic nuclease, had been previously heated at 80°C
for 10 minutes at 2 mg/ml in 0.1 M NaCl, 10 mM acetate
buffer (pH 5 at 25°C) to denature any possible DNase
contaminant.) The mitochondria were washed in 0.25 M
sucrose, 10 mM Tris buffer (pH 6.7 at 25°C) and
pelleted in the Sorvall at 9,600 rpm (11,300 g) for
10 minutes.

The mitochondrial pellet was resuspended in 10 mM
EDTA, 10 mM Tris buffer (pH 7.4 at 25°C) and immediately

brought to 1% SDS. Pronase was added to 75 µg/ml, the lysate was incubated at 37°C for 30 minutes, CsCl was brought to 1 M and the sample left on ice for 30 minutes. (Pronase had been predigested at 2 mg/ml in 20 mM Tris buffer (pH 8 at 25°C) at 37°C for 2 hours and particulate matter was removed by centrifugation.) The precipitate was removed by centrifugation in the Sorvall at 13,000 rpm (20,900 g) for 15 minutes.

The resulting supernatant was layered on a two-step CsCl/ethidium bromide gradient (Storrie and Attardi, 1972) consisting of 1 ml of CsCl (ρ=1.76 g/cm^3) and 3 ml of CsCl (ρ=1.4 g/cc), both in 10 mM EDTA, 10 mM Tris buffer (pH 7.4 at 25°C), 200 µg ethidium bromide/ml in 1.3x5.1 cm polyallomer tubes presoaked in 5 mM EDTA. The tubes were centrifuged in the Beckman SW65 or SW50.1 rotor at 38,000 rpm for 5 hours at 20°C. After puncturing the bottom of the tube, fractions were collected directly onto Whatman 3MM filters which were batch washed in 5% TCA and ethanol. Dried filters were counted in toluene-based scintillation fluid.

(f) TTP pool extraction

The samples analyzed were the mitochondrial pellet resuspended in STM, the first nuclear pellet resuspended

in STM, and the supernatant of the first 8,600 rpm
(9,100 g) centrifugation after removal of residual
organelles by centrifugation at 12,000 rpm (17,800 g)
for 15 minutes ("cytoplasmic fraction").

The samples were brought to 0.5 N PCA. After
30 minutes on ice, the precipitate was removed by centri-
fugation in the Sorvall at 13,000 rpm (20,900 g) for
15 minutes. Desalting the sample prior to its application
was found to be an important step. Charcoal absorption
of the PCA soluble portion of the mitochondrial fraction
was found to be the most effective desalting procedure
when it was compared with a Dowex column or a G50 column.
TTP was absorbed on HCl-washed charcoal during 5 minutes
on ice. After the charcoal was washed several times in
cold H_2O and pelleted, TTP was effectively eluted with
50% ethanol, 0.1% NH_4OH for 30 minutes at 37°C, conditions
under which TTP was found to be stable. The charcoal was
removed by centrifugation in the Sorvall at 13,000 rpm
(20,900 g) for 15 minutes. The sample was dried under
an air flow and then resuspended in no more than 100 µl
of 50% ethanol, 0.1% NH_4OH.

(g) Two-dimensional thin-layer chromatography

TTP was purified from the PCA soluble fraction by two-dimensional thin-layer chromatography (Neuhard et al., 1965). 20x20 cm x 0.1 mm PEI-cellulose plates with plastic backing (Brinkmann) were washed by ascending runs in 10% NaCl and in H_2O and dried. The radioactive sample was combined with 10 μl of 5 mM of each of TTP, TDP, TMP, dCTP, ATP, CTP, GTP and UTP as ultraviolet markers and was spotted without intermediate drying onto the PEI-cellulose plate. Plates were washed for 8 minutes in MeOH and dried.

In the first dimension, the first ascending chromatography was carried out at room temperature in 1 M LiCl saturated with H_3BO_3 (pH 7 at 25°C), up to a front migration of 12 cm, followed by drying, two 15 minutes rinses in MeOH, drying and a second ascending run at 4°C with fresh 1 N HCOOH, 0.8 M LiCl up to a front migration of 12 cm. Plates were washed for 5 minutes in 1.2 g Trisma base/l of MeOH and were rinsed twice for 10 minutes in MeOH and dried. The second dimension at 4°C was ascending chromatography in 0.5 M $(NH_4)_2SO_4$ to a front migration of 4 cm above the spot origin, then 0.7 M $(NH_4)_2SO_4$ to 12 cm.

The TTP spot was located under short wavelength ultraviolet light, cut out (sometimes subdivided as inner circle and outer border) and the radioactive TTP quantitatively eluted with 1 ml of 0.7 M $MgCl_2$, 0.02 M Tris buffer (pH 7.4 at 25°C) at room temperature for 30 minutes. The elution buffer (1 ml) was then counted in 10 ml of "Savusol" scintillation fluid (12 g PPO, 3 l xylene, 1 l Triton X-100).

(h) Autoradiography

For the determination of the percentage of cells in S phase, monolayer cells were [methyl-^{3}H]thymidine labeled on a portion of a coverslip delimited by an 8 mm glass ring as described above. Labeling was terminated by removing the glass rings, the coverslips were washed 3 times in NKM, and the cells were fixed in fresh ethanol-glacial acetic acid (3:1,v/v) for 30 minutes and stored in 70% ethanol at 4°C.

Vacuum grease was wiped off of the coverslips, which were slowly hydrated in H_2O. Coverslips were taped to slides and covered, emulsion side down, with Kodak AR10 autoradiographic stripping film, which had been briefly floated on 20 g sucrose/l, 10 mg KBr/l. The film was air dried and exposed for 4-9 days in the dark. The

film was developed in Kodak D-19, developed for 4 minutes, fixed in Kodak Hypo (diluted 1:1), rinsed 3 times in H_2O and stained for 5 minutes in 0.04% toluidine blue.

After drying, the coverslips were mounted on slides with Gurr's DePex and checked for nuclear DNA labeling by phase contrast microscopy. Approximately 1,000 cells were examined per time point to determine the percentage of labeled nuclei.

(i) Determination of mitotic index

For the determination of the mitotic index, cells grown on coverslips were carefully inverted on a drop of 1% orcein, 75% acetic acid and sealed with fingernail polish. After 30 minutes, the percentage of cells in metaphase, anaphase or telophase was determined by examining by phase contrast microscopy approximately 1,000 cells for each time point.

3. Results

(a) Cl1D cells

Cl1D mouse cells, an L cell derivative which lacks extramitochondrial thymidine kinase activity (Kit et al., 1963), was chosen for the initial experiments because mit-DNA can be labeled in these cells selectively with exogenous ^{3}H-thymidine (Clayton and Teplitz, 1972). These cells can be well synchronized by selective detachment from monolayers grown in roller bottles. Figure 1 shows the percentage of cells in S phase and the mitotic index of a Cl1D cell population synchronized by this method. The increase in the percentage of cells in S from 5% (at t=2.5 hours; t_o corresponds to the beginning of incubation of the detached cells in the 37°C CO_2 incubator) to 92% (at t=10.5 hours) and the increase in the mitotic index from 0.5% (at t=2 hours) to 9.6% (at t=16 hours) indicate a high degree of synchrony.

From Figure 1 it was calculated that the G1 phase, under the experimental conditions investigated here, lasted between 6.0 and 7.0 hours, the S phase about 7.5 hours, the G2 phase 2.0 hours, and M 30 minutes. In particular, the average length of time a cell spent in S was estimated by determining the area under the curve "percentage of cells in S" over one generation (i.e.,
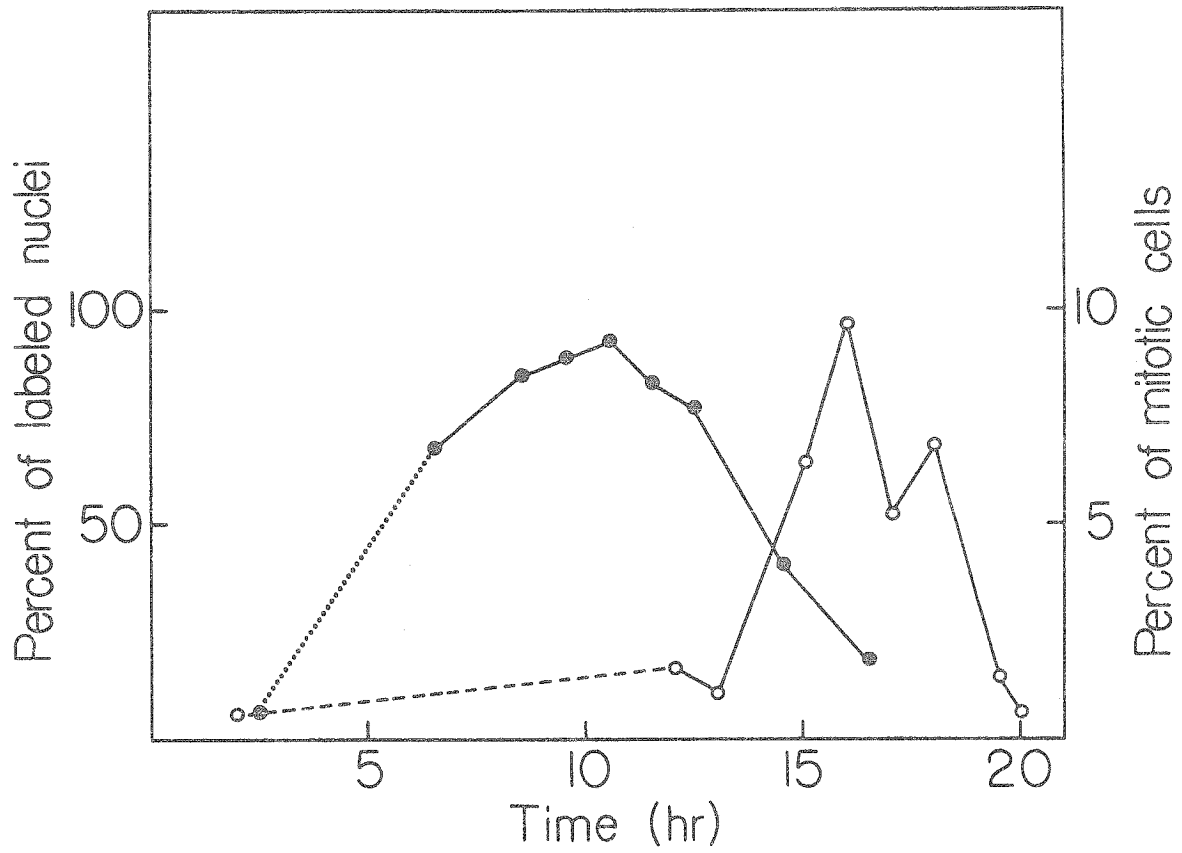
Figure 1

LEGEND TO FIGURE 1

Percentage of labeled nuclei and of mitotic cells
in a synchronized mouse Cl1D cell population.

Cl1D mouse cells were grown in monolayer on a plastic
roller bottle in Eagles' medium supplemented with 10%
calf serum. A population rich in mitotic cells was
detached by one shaking. The synchronized population
was divided into parallel cultures which were grown at
37°C.

For the determination of the percentage of the cells
in S phase ($\bullet\!-\!-\!\bullet$), 10^4 cells were grown on coverslips
in an area delimited by a glass ring. After different
times of incubation in the 37°C incubator, the cells
were labeled with 200 µCi/ml of [methyl-^{3}H]thymidine
(48.1 Ci/mmole, Amersham) for one hour. The cells were
then washed, fixed, covered with Kodak AR-10 stripping
film and exposed for 9 days. The film was developed
and the cells were then stained with 0.04% toluidine
blue. Approximately 1,000 cells were examined per time
point to determine the percentage of labeled nuclei.
The time points plotted here are the midpoints of the
labeling periods.

For the determination of the mitotic index (o$-\!-\!-$o),
10^4 cells were grown on coverslips. At various times,

(LEGEND TO FIGURE 1 CONTINUED)

individual coverslips were stained with 1% orcein in 75% acetic acid and the percentage of cells in metaphase, anaphase or telophase was determined by examining approximately 1,000 cells for each time point.

Time zero is the time at which the synchronized populations were placed into the $37°C$ CO_2 incubator, which was, on the average, 1 hour after the selective detachment step.

the time interval between 0 and 19 hours) and dividing
the result by 100%. The midpoint of S was represented
by the time, t, which bisected the area under the curve.
Likewise, the length of the M phase was estimated for the
generation time delimited by t=5 hours and t=24 hours
by determining the area under the mitotic index curve
(multiplied by the factor 1.44 to correct for the
overrepresentation of post-mitotic G1 cells at later
times; Stanners and Till, 1960) and dividing the result
by 100%. The M phase midpoint was 19.2 hours after t_o.
Except for the estimate of the length of the M phase,
the correction for the overrepresentation of G1 cells
has only a minor effect and has not been applied. For
G1 a minimum length of 6 hours was estimated to correspond
to the period between t_o and the beginning of S; a maximum
length of 7 hours was estimated by including 1 hour spent
by the cells at room temperature between detachment and
t_o. G2 was estimated as the period between the end of
S and the beginning of M.

In order to measure the rate of mit-DNA synthesis
in different phases of the cell cycle, it was considered
important to use a method for mit-DNA isolation which
would allow the analysis of different forms of mit-DNA;
the method chosen involves extensive treatment of the

mitochondrial fraction with DNase I to destroy any
contaminating nuclear DNA, followed by centrifugation
of the mit-DNA through a two-step CsCl/ethidium bromide
gradient.[2] During this centrifugation, the different
forms of mit-DNA are partially resolved on the basis of
both their molecular weight and hydrodynamic parameters
and their density in the presence of ethidium bromide
(Storrie and Attardi, 1972; Croizat and Attardi, 1975;
Carré and Attardi, 1978). Thus, as shown in Figure 2,
one recognizes a peak of closed circular monomers (Ia),
concatenated forms or double length circles forming a
shoulder on the heavy side of the closed circular DNA
(Ib), a peak of open circular monomers (II), and some
material between peaks Ia and II which consists presumably
of concatenated forms of mixed open and closed circular
configurations and possibly replicative forms. Residues
of partially degraded nuclear DNA form a large peak near
the meniscus, well separated from the mit-DNA forms.

A population of C11D cells was synchronized by
selective detachment, and parallel samples of the detached
cell population were incubated with [methyl-^{3}H]thymidine
starting 2, 5.5, 9.5, 12.5, 14.5, and 19 hours after t_o,

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[2]"Two-step" refers to the composition of the gradient
mixture prior to centrifugation.
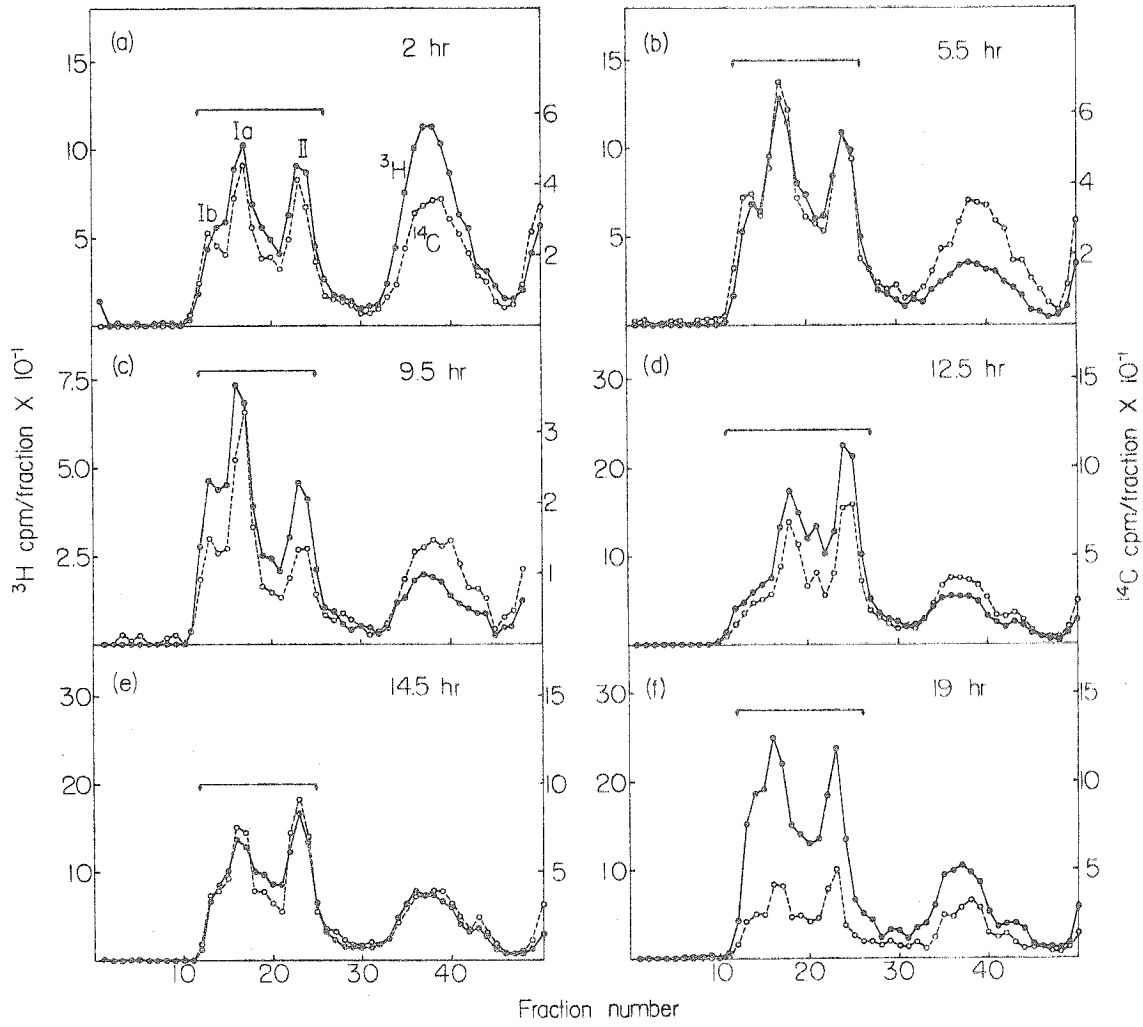
Figure 2

LEGEND TO FIGURE 2

Sedimentation pattern in a CsCl/ethidium bromide gradient of the [methyl-^{3}H]thymidine pulse-labeled DNA from the DNase treated mitochondrial fraction of synchronized mouse C11D cells at different stages of the cell cycle.

C11D cells were grown in monolayer on plastic roller bottles in Eagle's medium with 10% calf serum. A population rich in mitotic cells was detached from the bottles by one shaking per bottle at 37°C and resuspended at 3×10^5 cells/ml. 10 ml aliquots of the suspension were dispensed into 10 cm petri dishes and incubated at 37°C.

At selected times, individual cultures were exposed for 2 hours to 200 µCi/ml of [methyl-^{3}H]thymidine (46 Ci/mmole, Amersham) in Eagle's medium with 10% dialyzed calf serum. In order to correct the [methyl-^{3}H]thymidine incorporation data for variations in mit-DNA recovery, a C11D cell suspension (5×10^8 cells), grown for 2 days in Eagle's phosphate medium supplemented with 5% dialyzed calf serum in the presence of [2-^{14}C]thymidine (0.013 µCi/ml, 60 mCi/mmole, Amersham), was mixed with the [methyl-^{3}H]thymidine labeled cell sample.

(LEGEND TO FIGURE 2 CONTINUED)

The mitochondrial fraction was isolated, treated with DNase and RNase, dissolved in SDS, treated with pronase and brought to 1 M CsCl. After removal of the protein precipitate, the supernatant was layered on a step gradient of 1 ml of CsCl, ρ = 1.76, and 3 ml of CsCl, ρ = 1.4, both in 10 mM EDTA, 10 mM Tris buffer (pH 7.4 at 25°C), 200 µg ethidium bromide/ml, and centrifugation was carried out in the Beckman SW65 rotor at 38,000 rpm for 5 hours at 20°C. The fractions were collected on Whatman 3 MM filters, which were batch washed in 5% TCA and ethanol. (●——●), ^{3}H cpm; (o---o), ^{14}C cpm.

Time zero is the time at which the synchronized populations were placed into the 37°C CO_2 incubator, an average of 2.25 hours after the selective detachment step. The 2 hour [methyl-^{3}H]thymidine labeling began 2 hours (a), 5.5 hours (b), 9.5 hours (c), 12.5 hours (d), 14.5 hours (e), and 19 hours (f) after time zero.

the beginning of incubation of the detached cell population in the 37°C CO_2 incubator. Prior to homogenization, each [methyl-^{3}H]thymidine labeled sample was mixed with a constant amount of [2-^{14}C]thymidine labeled C11D cells to provide an internal sedimentation marker in the analysis of mit-DNA and a reference for normalization purposes. The mit-DNA was extracted from DNase treated mitochondria and sedimented in two-step CsCl/ethidium bromide gradients, as shown in Figure 2. The bracketed region in each profile was used to determine the ^{3}H/^{14}C ratio for each time point. These ratios are plotted in Figure 3 (●——●). One can see that the relative rate of incorporation of [methyl-^{3}H]thymidine remains fairly constant during the cell cycle, apparently doubling over a period of a few hours.

Since the pattern of incorporation shown in Figure 4 appeared to be very different from that reported for HeLa cell mit-DNA (Pica-Mattoccia and Attardi, 1972), and since complications due to pool equilibration problems (Berk and Clayton, 1973) could conceivably be responsible for the difference, an experiment similar to that described above was carried out utilizing [5-^{3}H]deoxycytidine as a precursor. Samples of a synchronized C11D cell population were labeled for 1 hour with [5-^{3}H]deoxycytidine starting at 2, 4, 13, and 15 hours after t_o. The mit-DNA patterns obtained in CsCl/ethidium bromide gradients are shown
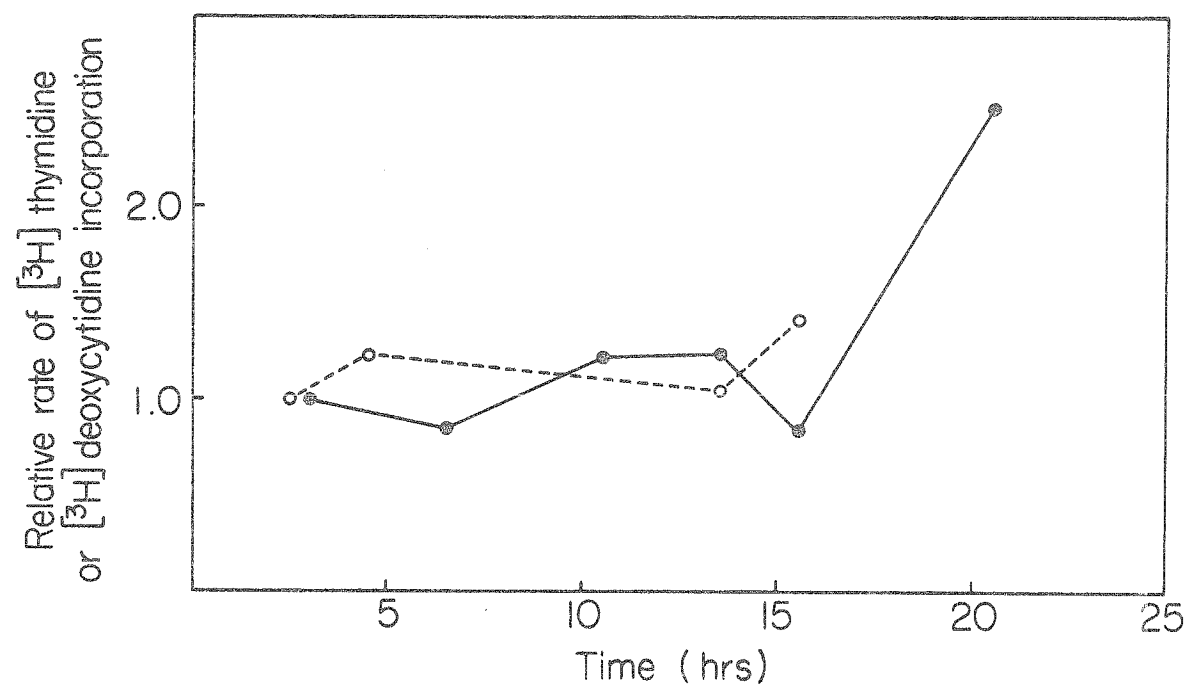
Figure 3

LEGEND TO FIGURE 3

Rates of [methyl-^{3}H]thymidine and of [5-^{3}H]deoxy-cytidine incorporation into mit-DNA in synchronized C11D cells.

The relative rates of [methyl-^{3}H]thymidine or of [5-^{3}H]deoxycytidine incorporation into mit-DNA is the ^{3}H/^{14}C ratio of the CsCl gradient fractions indicated by arrows in Figure 2 (for [methyl-^{3}H]thymidine incorporation (●——●)) and in Figure 4 (for [5-^{3}H]deoxycytidine incorporation (o----o)). The time points plotted here are the midpoints of the pulse-labeled periods.
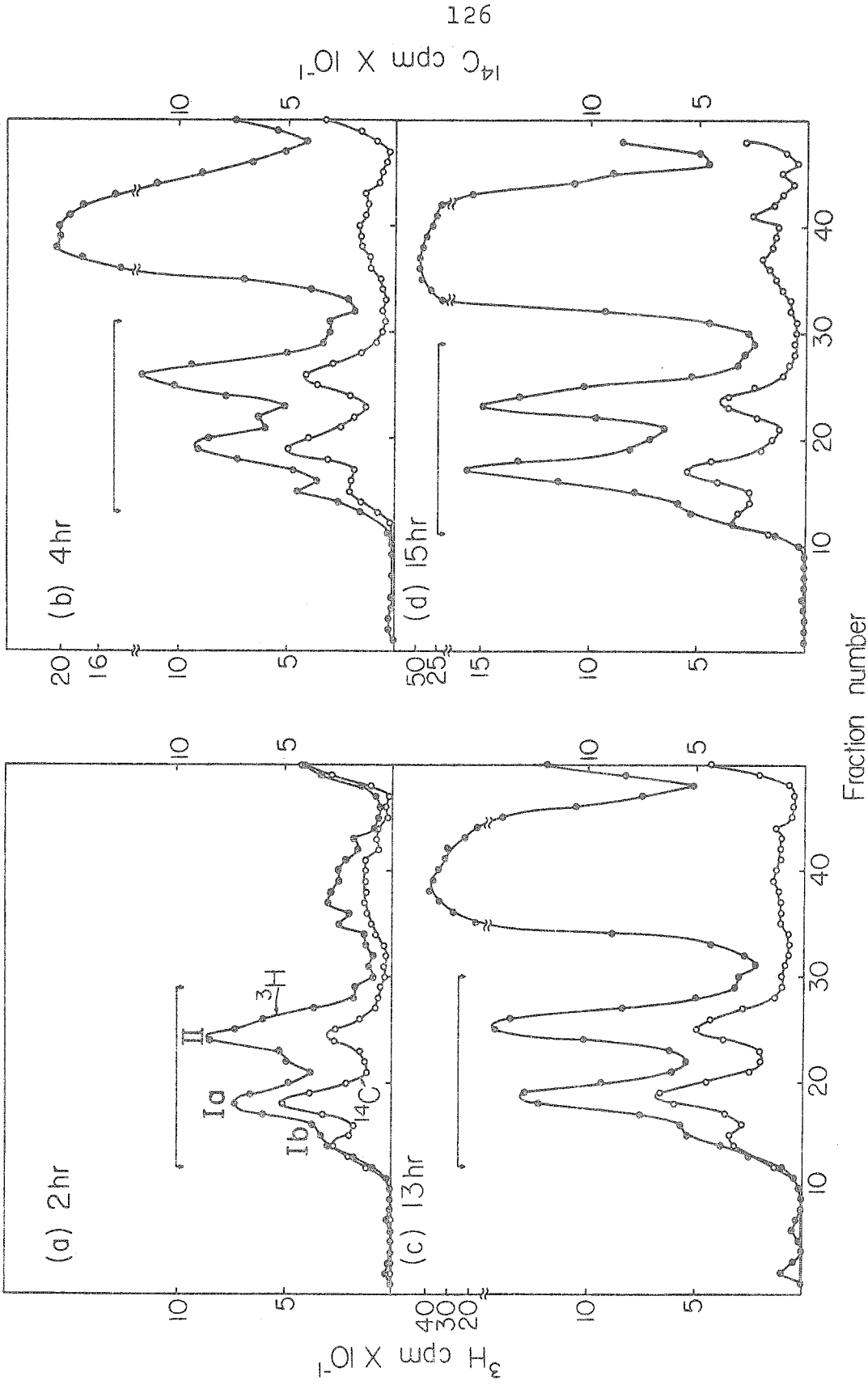
Figure 4

LEGEND TO FIGURE 4

Sedimentation pattern in a CsCl/ethidium bromide gradient of the [5-^{3}H]deoxycytidine pulse-labeled DNA from the DNase treated mitochondrial fraction of synchronized mouse C11D cells at different stages of the cell cycle.

C11D cells were grown in monolayer on plastic roller bottles in Eagle's medium with 10% calf serum. A population rich in mitotic cells was detached from the bottles by one shaking per bottle at 37°C, centrifuged and resuspended at 37°C in fresh medium at $4x10^5$ cells/ml. 10 ml aliquots of the suspension were dispensed into 10 cm petri dishes and incubated at 37°C.

At selected times after plating of the synchronized cell population, individual cultures ($4x10^6$ cells) were exposed for 1 hour to 200 µCi/ml of [5-^{3}H]deoxycytidine (24 Ci/mmole, Amersham) in Eagle's medium with 10% dialyzed calf serum. After trypsinization and harvesting, the cultures were mixed with a constant amount ($5x10^8$) of cells labeled for 2 days with [2-^{14}C]thymidine (0.019 µCi/ml, 61 mCi/mmole, Amersham).

The mitochondrial fraction was isolated, treated with DNase and RNase, lysed in SDS, pronase digested, brought to 1 M CsCl on ice and the precipitate was

(LEGEND TO FIGURE 4 CONTINUED)

pelleted out.   The supernatant was layered on a two-step
CsCl/ethidium bromide gradient and centrifuged in the
Beckman SW65 rotor at 38,000 rpm for 5 hours at 20°C.
(●——●), ^3H cpm; (o——o), ^{14}C cpm.

Time zero is the time at which the synchronized
populations were placed into the 37°C CO_2 incubator,
an average of 1.25 hours after the selective detachment
step.   In different cultures, the 1 hour [5-^3H]deoxy-
cytidine pulse-labeling was started 2 hours (a), 4 hours
(b), 13 hours (c) and 15 hours (d) after time zero.

in Figure 4. The bracketed regions indicate the fractions used to calculate the $^3H/^{14}C$ ratio for each time point. As shown in Figure 4 (o——o), the rate of incorporation of [5-^{3}H]deoxycytidine into mit-DNA, like that of [methyl-^{3}H]thymidine, is fairly constant throughout a major portion of the cell cycle.

Although the above described labeling experiments with the two precursors seem to agree in indicating a constant rate of incorporation of precursor into mit-DNA during the cell cycle, in order to interpret the incorporation data in terms of rates of synthesis, it was essential to correct the labeling data for changes during the cell cycle in specific activities of the mitochondrial pool of TTP or dCTP. For example, it is conceivable that the endogenous synthesis of thymidilic acid and/or the utilization of exogenous thymidine could vary during the cell cycle. In order to test the feasibility of this pool analysis, preliminary experiments were carried out utilizing unsynchronized cells in suspension to determine the labeling of the TTP and dCTP mitochondrial pools after 1 hour exposure of the cells to [methyl-^{3}H]thymidine and [5-^{3}H]deoxycytidine, respectively. In these experiments, the acid soluble mitochondrial pool, isolated as described in Materials and Methods, was fractionated by two-dimensional thin layer chromotography under conditions which

give a separation of the deoxyribonucleotide triphosphates (Neuhard et al., 1965).

Unfortunately, the labeling of the mitochondrial triphosphate precursor pool with either [methyl-^{3}H]thymidine or [5-^{3}H]deoxycytidine was found to be too low to allow meaningful measurements, even when the amount of cells utilized was much greater than practical for cells synchronized by selective detachment (3×10^7 cells per sample). It has been previously reported (Berk and Clayton, 1973) that ^{3}H-thymidine uptake in C11D cells is very much reduced as compared to that of A9 cells. Because of this difficulty, it has not been possible to interpret the incorporation data shown above in any conclusive way concerning the cell cycle dependence of the rate of mit-DNA synthesis in C11D cells.

(b) A9 cells

Because of the difficulties encountered with C11D cells, it was decided to approach the same problem with another L-cell derivative, A9. A9 cells were also found to be well synchronized by selective detachment from monolayers grown in roller bottles. Figure 5 shows the percentage of cells in S phase and the mitotic index of an A9 cell population synchronized by this method. The increase in the percentage of cells in S from 0.3% ± 0.2%
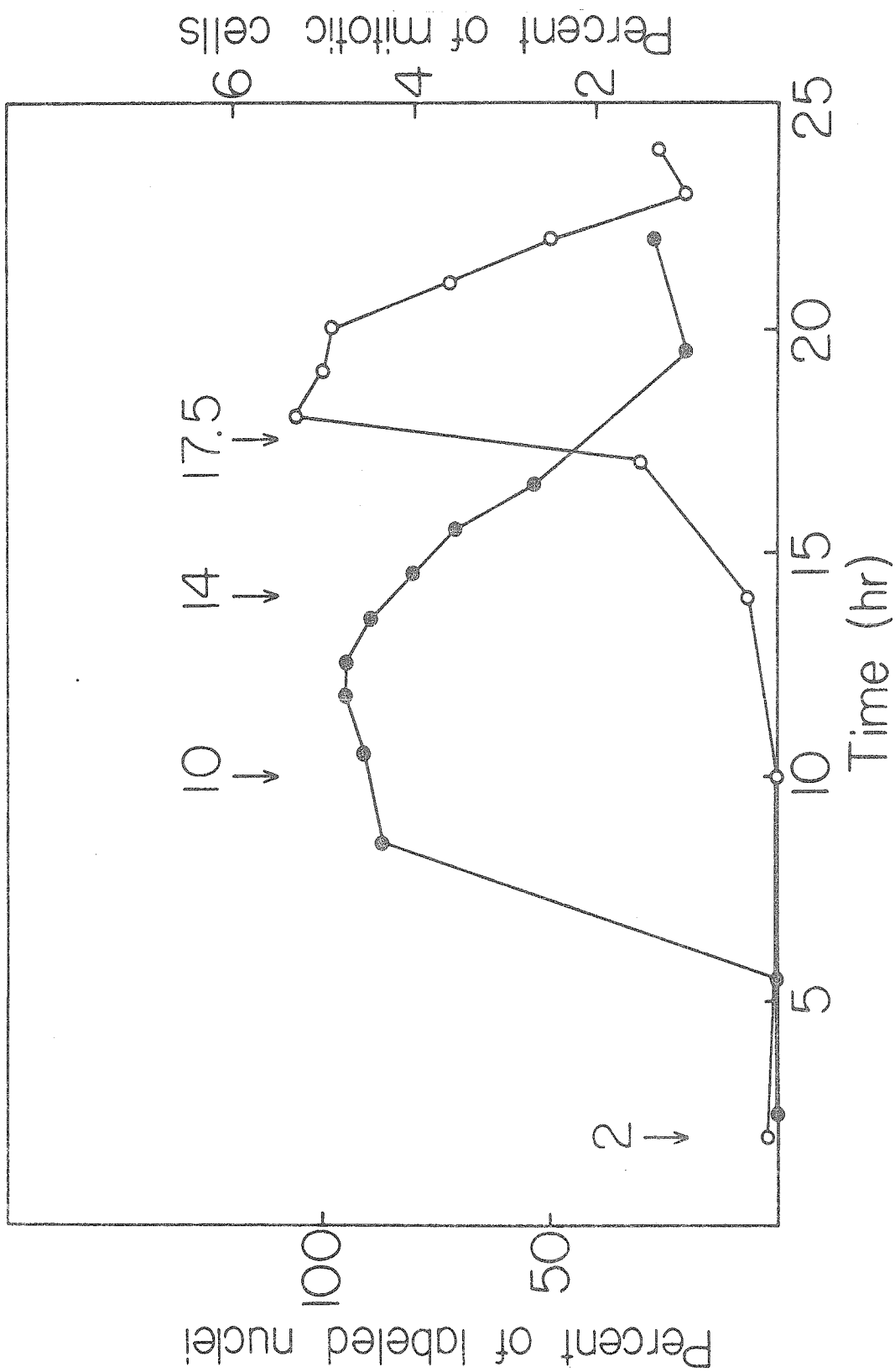
Figure 5

LEGEND TO FIGURE 5

Percentage of labeled nuclei and of mitotic cells in the synchronized A9 cell population utilized for the determination of the rate of incorporation of [methyl-^{3}H]thymidine into mit-DNA.

A9 cells were grown in monolayer on plastic roller bottles in Eagle's medium with 10% calf serum. A population rich in mitotic cells was selected by one single shaking per bottle at 37°C, centrifuged and resuspended at 37°C in fresh medium; two cell suspensions were prepared, one at 4×10^5 cells/ml and the other at 5×10^4 cells/ml.

For the determination of the percentage of cells in S phase (●———●), 10^4 cells were grown on coverslips in an area delimited by a glass ring. After different times of incubation in the 37°C incubator, the cells were labeled with 200 μCi/ml of [methyl-^{3}H]thymidine (48.1 Ci/mmole, Amersham) for one hour. The cells were then washed, fixed, covered with Kodak AR-10 stripping film and exposed for 4 days. The film was developed and the cells were then stained with 0.04% toluidine blue. Approximately 1,000 cells were examined per time point to determine the percentage of labeled nuclei. The time points plotted here are the midpoints of the labeling

(LEGEND TO FIGURE 5 CONTINUED)

periods.

For the determination of the mitotic index (o——o), 10^4 cells were grown on coverslips. At various times, individual coverslips were stained with 1% orcein in 75% acetic acid and the percentage of cells in metaphase, anaphase or telophase was determined by examining approximately 1,000 cells for each time point.

Time zero is the time at which the synchronized populations were placed into the 37°C CO_2 incubator, which was, on the average, 1.75 hours after the selective detachment step. Arrows indicate times at which labeling of mit-DNA with [methyl-^{3}H]thymidine began (see Figure 7).

at t=2.5 hours) to 95% ± 3% (at t=12.5 hours) and the increase in the mitotic index from 0.1% ± 0.1% (at t=2 hours) to 5.3% ± 0.7% (at t=18 hours) indicate a very high degree of synchrony. From Figure 5 it was calculated (as described above) that, in the population analyzed here, G1 lasted 7.5-9.3 hours, S about 9.4 hours, G2 2.1 hours and M 23 minutes. Results similar to these were obtained in another A9 synchronization (see below).

The cell cycle phase composition of the cell population for any chosen labeling period could be determined from Figure 5 (Table 1, Experiment 1) as explained in the legend for the table. The t=2-3 hours population consisted of about 99% G1 cells; by t=10-11 hours, 91% of the cells had entered S phase while 9% remained in G1 phase; by t=14-15 hours, almost all cells had left G1 phase, 80% were still in S phase, 16% were in G2 phase, about 1% were in M phase and 3% were cells in the next G1 phase; and by t=17.5-18.5 hours, 45% of the cells were in S phase, 25% in G2 phase, 5% in M phase and 25% were in the next G1 phase.

Similar calculations were made for the experiment shown in Figure 6. The increase in the percentage of cells in S from 0.1% ± 0.1% (at t=2.5 hours) to 95% ± 3%

TABLE 1

ESTIMATES OF PERCENTAGES[a] OF CELLS IN DIFFERENT
PHASES OF THE CELL CYCLE DURING SELECTED LABELING
PERIODS FOR THE CELL POPULATIONS USED IN THE
MIT-DNA INCORPORATION EXPERIMENT[b] AND IN
THE MITOCHONDRIAL TTP POOL EXPERIMENT[c]

Experiment 1: Mit-DNA Incorporation Experiment[d]

Cell Cycle Phase	Labeling Period			
	2-3 hr	10-11 hr	14-15 hr	17.5-18.5 hr
G1 (8 hr)	99	9	0	0
S (8 hr)	0	91	80	45
G2 (3 hr)	0	0	16	25
M (1/2 hr)	0	0	1	5
G1 (8 hr)	0	0	3	25

Experiment 2: Mitochondrial TTP Pool Experiment[e]

Cell Cycle Phase	Labeling Period		
	2-3 hr	10-11 hr	14-15 hr
G1	99	16	0
S	1	84	87
G2	0	0	2
M	0	0	1
G1	0	0	10

136

(TABLE 1 CONTINUED)

The estimates for the mit-DNA incorporation experiment
were made as follows. The 2-3 hour population was judged
to be about 99% G1 phase cells since the 0.3% ± 0.2%
S phase cells and 0.1% ± 0.1% M phase cells (both less
than sampling error) indicated that there were probably
less than 1% asynchronous cells in the population. At
t=10-11 hours, 91% ± 3% of the cells were observed to be
in the S phase; most of the remaining 9% must still have
been in G1 phase, because the mitotic index was 0.0% ± 0.1%,
the average of 7.5 hours necessary for them to have entered
and left the S phase had hardly passed since t=2-3 hours,
and they had not had the almost 17 hours required on the
average after mitosis to pass through both G1 and S. At
t=14-15 hours, 80% ± 3% of the cells were found to be in
the S phase and 0.5% ± 0.2% in the M phase. 3.4% of the
cells were calculated to have been in the next G1 phase
and 15.7% were assigned to the G2 phase; the rationale is
identical to that described in Appendix II.

During the labeling time t=17.5-18.5 hours, 45% ± 2%
of the cells are observed to be in the S phase, 5.3% ± 0.7%
to be in the M phase and the area under the mitotic index
curve from 5 hours to 17.6 hours in 5.0%-hr (the area
under the mitotic index curve for one generation is

(TABLE 1 CONTINUED)

26.1%-hr). The percentage of cells in the t=17.5-18.5
hour population which are in the next G1 phase is
approximated using the area under the mitotic index
curve as described in Appendix II. About 25% of the
cells were estimated to have been in the next G1 and
the remaining 25% were assigned to G2.

The estimates for the mitochondrial TTP pool exper-
iment were made in the same manner.

    (a)   The estimates are rounded off to the nearest
         percent.

    (b)   See Figures 5, 7, 8a.

    (c)   See Figures 6, 8b.

    (d)   Estimates are based on data in Figure 5.

    (e)   Estimates are based on data in Figure 6.

Figure 6

138
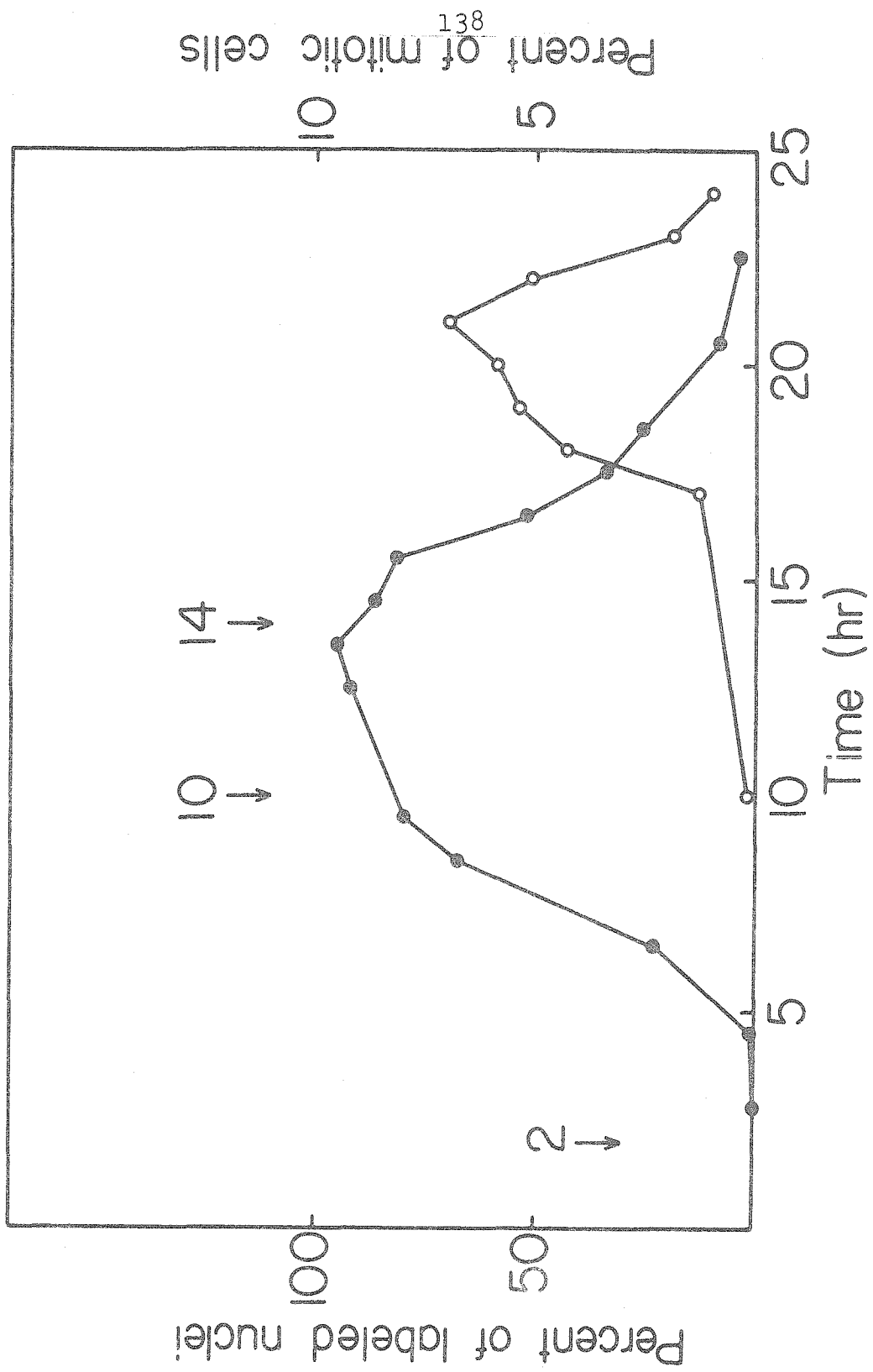
LEGEND TO FIGURE 6

Percentage of labeled nuclei and of mitotic cells in the synchronized A9 cell population utilized for the determination of the specific activity of the mitochondrial TTP pool.

A9 cells were grown for one day in monolayer on plastic roller bottles in modified Eagle's medium with 5×10^{-4} M phosphate supplemented with 10% calf serum in the presence of ^{32}P-orthophosphate (3 μCi/ml, carrier free, ICN). A population rich in mitotic cells was detached, the cells being kept at 15°C before utilization. The synchronized populations were pooled and grown at 37°C in fresh ^{32}P-medium as described above.

For the determination of the percentage of cells in S phase (●——●), 10^4 cells were grown on coverslips in an area delimited by a glass ring. After different times of incubation in the 37°C incubator, the cells were labeled in 200 μCi/ml of [methyl-^{3}H]thymidine (48.1 Ci/mmole, Amersham) for one hour. The cells were then washed, fixed, covered with Kodak AR-10 stripping film and exposed for 4 days. The film was developed and the cells were then stained with 0.04% toluidine blue. Approximately 1,000 cells were examined per time point to determine the percentage of labeled

(LEGEND TO FIGURE 6 CONTINUED)

nuclei. The time points plotted here are the midpoints of the labeling periods.

For the determination of the mitotic index (o——o), 10^4 cells were grown on coverslips. At various times, individual coverslips were stained with 1% orcein in 75% acetic acid and the percentage of cells in metaphase, anaphase or telophase was determined by examining approximately 1,000 cells for each time point.

Time zero is the time at which the synchronized populations were placed into the 37°C CO_2 incubator, which was, on the average, 1.75 hours after the selective detachment step. Arrows indicate times at which labeling of the mitochondrial TTP pool with [methyl-^{3}H]thymidine began (see Methods and Materials, Section 2(c)ii).

(at t=13.5 hours) and the increase in the mitotic index from 0.2% ± 0.1% (at t=10 hours) to 7.0% ± 0.8% (at t=21 hours) indicate a very high degree of synchrony. It was calculated, as described above, that in the population analyzed here, G1 lasted 8.0-9.0 hours, S about 8.8 hours, G2 2.8 hours and M 33 minutes. As shown in Table 1, Experiment 2, at t=2-3 hours about 99% of the cells were in G1 and 1% in S; at t=10-11 hours 16% were in G1 and 84% ± 3% of the cells were in S; and at t=14-15 hours 87% ± 3% of the cells were in S, 2% in G2 phase, 1% in M phase and 10% had entered the next G1 phase.

In the mit-DNA incorporation experiment, the mitotic cells were collected by single shakings of the bottles and all steps were performed at 37°C, and the average interval between the shakings and the beginning of incubation in the 37°C CO_2 incubator (t_o) was about 1.75 hour.

Parallel cultures of the synchronized A9 population described in Figure 5 were labeled with [methyl-^{3}H]thymidine for 1 hour, beginning at 2, 10, 14, and 17.5 hours after t_o. Prior to cell homogenization, each [methyl-^{3}H]thymidine labeled sample was mixed with a constant amount of [2-^{14}C]thymidine labeled A9 cells to provide ^{14}C-mit-DNA as a sedimentation marker and for normalization purposes,

as described above. The mit-DNA was extracted from DNase
treated mitochondria and run through two-step CsCl/ethidium
bromide gradients. The patterns obtained are shown in
Figure 7. The relative rates of incorporation of
[methyl-^{3}H]thymidine were determined by calculating the
^{3}H/^{14}C ratios for both the closed circular mit-DNA and
for total mit-DNA, as indicated by the bracketed regions
in the profiles shown in Figure 7; the results are
presented in Figure 8a. The relative rate of incorporation
into the closed circular mit-DNA peak shows a substantial
increase during the cell cycle, from 1 at the t=2-3 hour
point (G1 cells) to about 5 at the later time points
(mostly S and G2 cells). A marked increase in the rate
of incorporation during the cell cycle, somewhat greater
than that mentioned above, was also estimated for total
mit-DNA. It is not clear whether the differences in the
two estimates are significant; the estimate for closed
circular mit-DNA is considered more reliable, however,
because of the overlapping of the degraded nuclear DNA
peak and the open circular mit-DNA peak in some of the
patterns.

In order to convert the rates of [methyl-^{3}H]thymidine
incorporation into rates of mit-DNA synthesis, the
labeling of the mitochondrial TTP pool with [methyl-^{3}H]thy-
midine was analyzed to measure possible changes in the
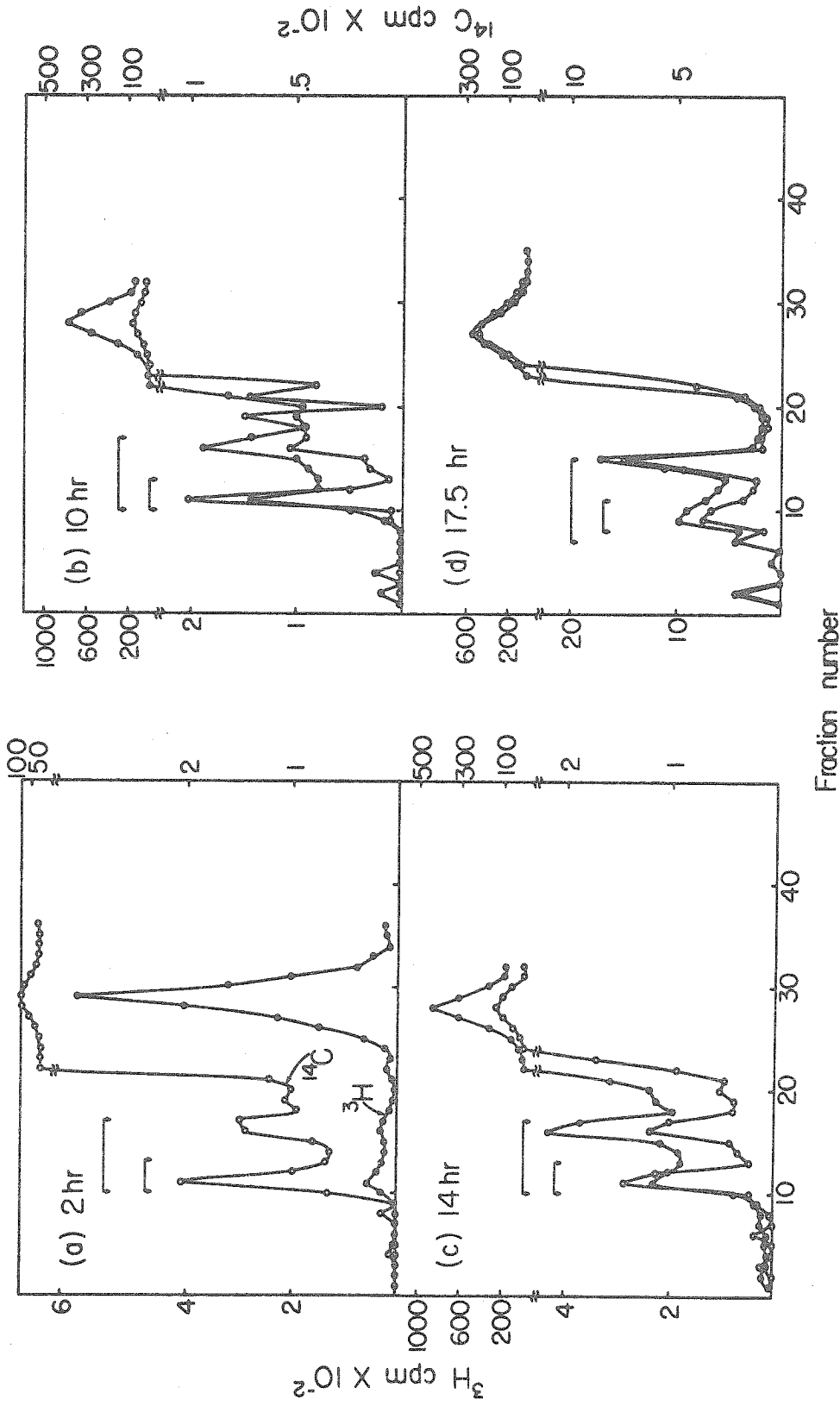
Figure 7

LEGEND TO FIGURE 7

Sedimentation pattern in a CsCl/ethidium bromide
solution of the [methyl-^{3}H]thymidine pulse-labeled DNA
from the DNase treated mitochondrial fraction of
synchronized A9 cells at different stages of the cell
cycle.

10 ml portions of the synchronized cell suspension
(4×10^5 cells/ml) described in Figure 5 were incubated
at 37°C in 10 cm petri dishes. Beginning at selected
times (indicated by arrows in Figure 5), individual
cultures were exposed for 1 hour to [methyl-^{3}H]thymidine
(200 µCi/ml, 48.1 Ci/mmole, Amersham) in Eagle's medium
with 10% dialyzed calf serum. An A9 cell suspension
(10^9 cells) grown for 2 days in Eagle's phosphate
medium with 5% dialyzed calf serum in the presence of
[2-^{14}C]thymidine (0.014 µCi/ml, 53 mCi/mmole, Amersham)
was mixed with the [methyl-^{3}H]thymidine labeled cell
sample for each time point to correct for mit-DNA
recovery.

The mitochondrial fraction was isolated, treated
with DNase and RNase, dissolved in SDS, treated with
pronase and brought to 1 M CsCl. After removal of
the protein precipitate, the supernatant was layered
on a step gradient of 1 ml of CsCl, $\rho = 1.76$, and 3 ml

(LEGEND TO FIGURE 7 CONTINUED)

of CsCl, ρ = 1.4, both in 10 mM EDTA, 10 mM Tris buffer
(pH 7.4 at 25°C), 200 µg ethidium bromide/ml, and
centrifugation was carried out in the Beckman SW50.1
rotor at 38.000 rpm for 5 hours at 20°C. Fractions
were collected on Whatman 3 MM filters, which were
batch washed in 5% TCA and ethanol.

Times specified are the periods the synchronized
cultures were in the 37°C CO_2 incubator before the
beginning of the 1 hour pulse labeling with [methyl-^{3}H]thy-
midine: 2 hours (a), 10 hours (b), 14 hours (c) and
17.5 hours (d). (●——●), ^{3}H cpm; (o——o), ^{14}C cpm.
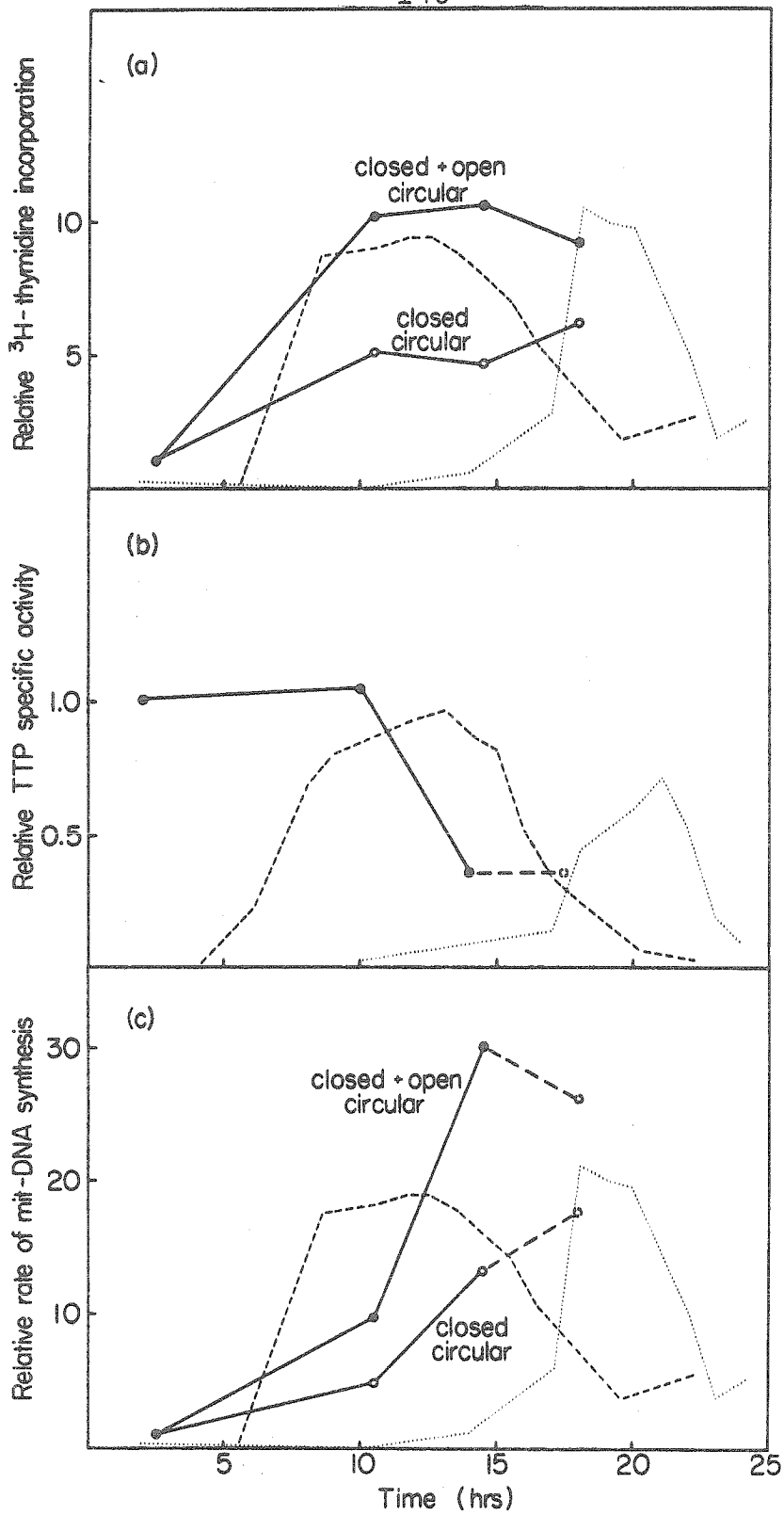
Figure 8

LEGEND TO FIGURE 8

Rate of [methyl-^{3}H]thymidine incorporation into mit-DNA (a), mitochondrial TTP specific activity (b), and an estimated rate of mit-DNA synthesis (c) in synchronized A9 cells.

(a) The relative rate of [methyl-^{3}H]thymidine incorporation into mit-DNA was determined from the ^{3}H/^{14}C ratio of CsCl gradient fractions bracketed in Figure 7, including the total mit-DNA (o——o) and the closed circular form alone (o——o). The percent of cells in S (---) and the mitotic index (···) are from Figure 5.

(b) The relative mitochondrial TTP pool specific activity (o——o) is the ^{3}H/^{32}P ratio in the TTP spot after two-dimensional thin layer chromatography of the PCA soluble, charcoal absorbed, constituents of the mitochondrial fraction. The pool specific activity at t=17.5 was tentatively estimated to be equal to that at t=14.5. The percent of cells in S (---) and the mitotic index (···) are from Figure 6.

(c) The relative rate of mitochondrial DNA synthesis was determined by dividing the relative rate of [methyl-^{3}H]thymidine incorporation by the relative

(LEGEND TO FIGURE 8 CONTINUED)

TTP pool specific activity. The percent of cells in

S (---) and the mitotic index ($\cdots$) are from Figure 5.

The time points plotted are the midpoints of the

pulse-labeling periods.

specific activity during the cell cycle. In contrast to
the situation observed for C11D cells, preliminary
experiments with unsynchronized population revealed that
with A9 cells an adequate labeling of the mitochondrial
pool could be obtained. For the determination of the
mitochondrial TTP pool specific activity in different
phases of the cell cycle, the synchronized A9 cell popula-
tion shown in Figure 6 was used.

For normalization purposes in the analysis of the
labeling of the mitochondrial TTP pool, the cells had
been grown for 24 hours prior to detachment in medium
containing ^{32}P-orthophosphate and 5×10^{-4} M unlabeled
phosphate. Due to the need for relatively large samples
of synchronized cells to perform pool labeling deter-
minations, the mitotic cells were collected by multiple
shakings of the same bottles. The shakings and the cell
centrifugations were performed at room temperature (the
total time spent by each cell sample at room temperature
varied between 1 and 2 hours), the detached populations
were stored after each shaking before utilization at 15°C
(England and Attardi, 1974) and the average time spent
at 15°C by each sample was 2 hours (range 0 to 4 hours).
The similarity of the time relationship to t_o of the
S curve and of the mitotic index curve observed in the pool
labeling experiment (Figure 6) as compared to the mit-DNA

labeling experiment (Figure 5) suggests that staying at
room temperature and 15°C for the times indicated above
allowed the cells in mitosis to progress to the same
extent as at 37°C after staying for 1.5 to 2 hours.

At t=2 hours, 10 and 14 hours, parallel cultures
were pulse labeled for 1 hour with [methyl-^{3}H]thymidine,
the acid soluble pool extracted and analyzed by two-
dimensional thin layer chromatography, and the ^{3}H/^{32}P
ratio of the TTP spot determined. As shown in Figure 8b,
the mitochondrial TTP pool specific activity is very
similar at t=2-3 and t=10-11 hour points (mostly G1 and
early S cells), and decreases by a factor of 3 at t=14-15
hours (mostly late-S and G2 cells). In the absence of
pool specific activity data for a t=17.5-18.5 hour point,
the data for the t=14-15 hour point was used for the
purpose of estimating the rate of mit-DNA synthesis during
that labeling period.

The relative rates of mit-DNA synthesis during the
cell cycle, estimated as ratios of [methyl-^{3}H]thymidine
incorporation into mit-DNA to TTP pool specific activity
for each labeling period, are shown in Figure 8c for
both closed circular mit-DNA and total mit-DNA. The
estimated rate of synthesis, thus calculated, into closed
circular mit-DNA appears to increase from t=2-3 hours

(G1 cells) to t=10-11 hours (G1 and early S cells) by
a factor of 5, and from t=2-3 hours to the later time
points (mostly late-S and G2 cells) by a factor of 13-18.
For total mit-DNA, the estimated rate increased from
t=2-3 hours to t=10-11 hours by 10-fold and from t=2-3
to the later time points by about 26-30-fold.

4. Discussion

Selective detachment of rounded cells near mitosis
from a solid substrate is a technique unique in yielding
a synchronized cell population with a minimum of physio-
logical perturbation. Synchronization of growing cells
by drug treatment has the disadvantage that, while one
metabolic process may be inhibited, other cell cycle
dependent functions may continue; furthermore, the cells
may enter an abnormal state (enzyme activities, pool
sizes, etc.) and it may take the cells a substantial
amount of time to recover from the effects of the drug
after its removal. In HeLa cells, after selective
detachment both mit-DNA synthesis and nuclear DNA synthesis
were found to be synchronized; in contrast, after a double
thymidine block, only nuclear DNA synthesis appeared to
be synchronized (Pica-Mattoccia and Attardi, 1972). This
result was interpreted as indicating a failure of the
double thymidine block to inhibit mit-DNA synthesis; an
alternative explanation was that the mit-DNA synthesis
might be blocked but that the development of the
mitochondria might be arrested at different stages of
their life cycle and thus recover asynchronously in
mit-DNA synthetic capacity. Subsequent experiments

indicated that the double thymidine block does indeed inhibit
mit-DNA synthesis, pointing therefore to the validity of the
second explanation (Pica-Mattoccia and Roberti, 1974).
Because of the difficulties mentioned above, only selective
detachment was used in the experiments presented here.

The LM(TK⁻) C11D cell line, which is deficient in
cytoplasmic thymidine kinase but retains mitochondrial
thymidine kinase activity, was considered to be a
particularly suitable cell line for studying the rate
of mit-DNA synthesis during the cell cycle because, in
experiments using ^{3}H-thymidine as a precursor, contamina-
tion of the mitochondrial fraction by labeled nuclear
DNA would be minimized. In addition, determining the
rate of mit-DNA synthesis in C11D was important in view
of the simultaneous study of mitochondrial regulation
using cell fusion (see Chapter IV, this thesis). Mouse
C11D cells, therefore, were the initial choice for the
present studies.

Mit-DNA was purified by DNase treatment of the
mitochondrial fraction, followed by lysis of the organelles
and centrifugation through a two-step CsCl/ethidium
bromide gradient. This technique separates mit-DNA from
degradation products of nuclear DNA contaminating the
mitochondrial fraction and also fractionates the mit-DNA

species both on the basis of sedimentation properties and density in a CsCl/ethidium bromide gradient.

The rate of incorporation of either [methyl-^{3}H]thymidine or [5-^{3}H]deoxycytidine into mit-DNA in C11D cells was found to be fairly constant throughout the cell cycle. In order to determine whether or not this reflected the true rate of mit-DNA synthesis, it was considered necessary to determine the mit-TTP pool specific activity. Unfortunately, incorporation of both [methyl-^{3}H]thymidine and [5-^{3}H]deoxycytidine into the mit-TTP pool was found to be too low to be detected in the small cell samples obtainable by selective detachment. A lower rate ($\sim$ 40%) of ^{3}H-thymidine uptake in C11D cells than in A9 cells has been previously reported (Berk and Clayton, 1973). The low level of triphosphate precursor pool labeling with [methyl-^{3}H]thymidine which is indicated by the present observations may thus be due in part to a decreased rate of thymidine transport across the cell membrane; however there is evidence in the data reported by the cited authors that other factors besides transport of exogenous thymidine into the cell are responsible for the low level of labeling with ^{3}H-thymidine of mit-DNA in C11D cells. For example, a lack in the cytoplasm of thymidine kinase activity required for phosphorylation of thymidine might

retard its entry into mitochondria if such phosphorylation
were required for transport. It is interesting that the
same low level of labeling of the mitochondrial triphos-
phate pool was also observed with [5-^{3}H]deoxycytidine.
Because of the problems discussed above, it has been
impossible in the present work to measure the labeling
of the TTP and dCTP pools in synchronized C11D cells and
then to determine the rate of mit-DNA synthesis in these
cells at different stages of the cell cycle. Because of
these difficulties, A9 cells were investigated.

Estimates for the lengths of the phases of the cell
cycle of A9 cells in the present work were 7.5-8.0 hours
for the G1 phase, 8.8-9.4 hours for the S phase, 2.1-3.0
hours for the G2 phase, and 0.4-0.5 hours for the M phase.
Because the labeling of nuclei for autoradiographic
determination of the percentage of cells in the S phase
lasted for 1 hour, cells leaving the S phase and spending
part of the hour in the G2 phase, for example, might be
counted as S phase cells; this probably results in an
overestimate of the length of the S phase by 0.5-1 hour
at each end of the S phase. G1 and G2 phases would be
correspondingly underestimated. The length of the G1
phase is also probably underestimated, by at most 1-2
hours, by growth (presumably slowed down, at least in

the experiment involving exposure of the cells to temperatures < 37°C) in the period between cell detachment and t_o. These observations lead to best estimates of the lengths of cell cycle phases, from the experimental technique used here, of roughly an 8.5-10.5 hour G1 phase, 7-8 hour S phase, 3-4 hour G2 phase and 0.5 hour M phase. These results are consistent with the observation of Stanners and Till (1960) on L929 mouse cells (generation time 20 hours) that the S phase was 6-8 hours long, G2 phase was 3-4 hours long and M phase was 0.3-0.7 hour long.

In A9 cells, the rate of incorporation of [methyl-^{3}H]thymidine into closed circular mit-DNA was found to increase, relative to the value at the t=2-3 hour point (G1 cells), by a factor of about 5 at t=10-11 hours and t=14-15 hours (S and G2 cells) and by a factor of 6 at t=17.5-18.5 hours. This increase in the rate of A9 mit-DNA labeling during the cell cycle is similar to the 5-fold increase reported for HeLa cells synchronized by selective detachment (Pica-Mattoccia and Attardi, 1972). The specific activity of A9 mitochondrial TTP at t=2-3 hours (G1 cells) and t=10-11 hours (mostly early-S and G1 cells) was higher, by a factor of 3, than the specific activity at t=14-15 hours (mostly late-S and G2 cells), a result which is similar to that reported for HeLa cells:

in these cells, the specific activity of thymidine and
its derivatives was shown to decrease by a factor of 2
from the early labeling period to the late labeling peri-
od (Pica-Mattoccia and Attardi, 1972). Using the estab-
lished specific activity of the TTP pool, it was calculated
that the rate of synthesis into closed circular A9 mit-DNA
increases from the t=2-3 hour point to the t=10-11 hour
point by a factor of 5 and to the t=14-15 hour and
t=17.5-18.5 hour points by a factor of 13-18.

The above mentioned data pertaining to closed
circular mit-DNA have been further analyzed in terms of
the relative contribution of cells in different phases
of the cell cycle to the total labeling of mit-DNA
observed at the various time points. Because 99% of
the cells during the t=2-3 hour labeling periods are G1
cells, it seems likely that there is a true, though low,
level of mit-DNA synthesis during the G1 phase. The rate
of synthesis increased by a factor of 5 in the t=10-11
hour population which consisted of some G1 and mostly
early-S phase cells (defined as nuclear DNA synthesizing
cells up to the midpoint of S, t=12.2 hours), suggesting
that there is mit-DNA synthesis in S phase at a higher
level than in G1 phase (barring the unlikely possibility
of an enormous activity in late G1 cells). The t=14-15

hour population, which consisted of 80% late-S cells
(nuclear DNA synthesizing cells after the midpoint of S)
and 16% G2 cells, gave a 13-fold higher rate of synthesis
than the population at t=2-3 hours; although the mit-DNA
labeling and TTP pools specific activity data referred
to the total population at t=14-15 hours and not to the
components of the population at different stages of the
cell cycle, the data suggest that the rate of synthesis
is higher in late-S and/or G2 cells than in early-S cells.
With the same qualification made above, the rate of mit-DNA
synthesis would be still higher in the t=17.5-18.5
population, in which the G2 cells represent a still larger
fraction of the population, if one assumes that the TTP
pool specific activity at t=17.5-18.5 is not higher than
at t=14-15. Although no conclusive statement can be made
about the relative rate of synthesis in late-S and G2
cells, it seems possible, therefore, that the rate of
mit-DNA synthesis increases throughout the cell cycle.

It has been recently reported that the rate of mouse
mit-DNA synthesis is constant throughout the cell cycle
(Bogenhagen and Clayton, 1977). The experimental design
used by these authors was to label unsynchronized cells
for 2 hours with ^{3}H-thymidine, chase them with cold
thymidine for various times and then expose them for

4 hours to BrdU; they collected data on the percentage

of the labeled mit-DNA shifted in density by BrdU

incorporation for chosen chase times. The pattern of

these data was interpreted as a straight, horizontal

line, and it was concluded that mit-DNA molecules which

had replicated during the labeling period with ^{3}H-thymidine

were recruited again for replication at random throughout

the cell cycle, and that replication occurs at a constant

rate during the cell cycle.[3] Both conclusions are

dubious because the experimental data presented on the

percentage of mit-DNA molecules density shifted as a

function of chase time had indeed a pseudosinusoidal

appearance, which would be predicted from models of

nonconstant mit-DNA synthesis during the cell cycle;

furthermore, the equations derived to predict the results

according to various possible models were incorrect.

Finally their interpretation of the data did not include

adequate consideration of pool labeling phenomena. It is

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[3]Under the interpretation of their data given by
the authors, the rate of mit-DNA replication per cell
would be expected to increase exponentially during the
cell cycle, rather than to remain constant as they
imply in their description of theoretical models (see
Appendix II).

clear in fact that any reduction in the specific activity

of the intramitochondrial TTP pool in late-S and G2, as

detected in the present work and in the previous work

in HeLa cells, would decrease the contribution to the

total labeling during the ^{3}H-thymidine labeling period

of the cells in S and G2 phases: this would have the

effect of reducing the sinusoidal behavior in the data

expected from a rate of synthesis of mit-DNA increasing

throughout the cell cycle more than expected from the

increase in the number of templates. Furthermore if

there were any reduction in the BrdUTP to TTP ratio in

mitochondria in late-S and G2 cells, this would have

the effect of reducing the density difference of the

mit-DNA molecules replicating in late-S and G2, excluding

them at least in part from consideration. For further

discussion, see Appendix II. On the basis of the

arguments presented here and in Appendix II, the data

reported in the above mentioned paper are not sufficient

to justify the conclusion that the probability of mit-DNA

replication per mit-DNA molecule is constant during the

cell cycle. Furthermore, in view of the scatter in the

data, the approach followed by the cited authors is

not sensitive enough to distinguish between the constant

rate of synthesis hypothesis and the cell cycle dependence

of the rate of mit-DNA synthesis indicated by the present work.

The conclusion by Bogenhagen and Clayton (1977) that mit-DNA molecules are selected at random for replication throughout the cell cycle is also in disagreement with a previous report. A study by Flory and Vinograd (1973) revealed that in HeLa cells grown in BrdU for 24 hours (about one generation), 11% of mit-DNA was light-light (LL), 7% heavy-heavy (HH), and 82% of intermediate densities. If the mit-DNA molecules were chosen for replication independently of the timing of their replication (so that some molecules replicate more than once during the period of one generation time, while others not at all), then after one generation one would expect the ratio of HH:HL:LL to be ¼:½:¼ (see Appendix III). The Flory and Vinograd data, in part explainable in terms of the variability of generation times within the population as suggested by the nuclear DNA labeling data, do in part suggest a certain degree of random replication of mit-DNA molecules. In any case it seems clear that the random replication compatible with the data of Flory and Vinograd is far below that expected if molecules were selected at random for replication throughout the cell cycle. This is in contrast with the interpretation

proposed by Bogenhagen and Clayton.

If mit-DNA synthesis is tied to the cell cycle with regard to the rate of synthesis, the question arises as to how it is regulated. One possible mechanism which would account for increasing mit-DNA synthesis during the cell cycle would be the production in the cytoplasm of a labile factor during the late stages of the cell cycle, which would in part persist through mitosis and into the next G1 phase. Evidence for a tight dependence of mit-DNA synthesis on cytoplasmic protein synthesis in HeLa cells has been previously obtained (Storrie and Attardi, 1972).

5. References

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APPENDIX I

CALCULATION OF THE PROPORTION OF CELLS IN A
POPULATION, SYNCHRONIZED BY SELECTIVE
DETACHMENT, WHICH AT VARIOUS TIMES
HAVE PROCEEDED THROUGH ONE CELL
CYCLE AND ARE IN THE SECOND
G1 PHASE

(a)   The rationale

Let us consider a hypothetical cell population
synchronized by selective detachment which at t=2-3 hours
after the beginning of incubation ($=t_o$) consists exclu-
sively of G1 cells (similar to the A9 synchronized
populations discussed in Chapter III, this thesis). Let
us further assume that the cells will not double at the next
M phase but will each give rise to only one new G1 cell.
At time t after t_o, the proportions of this hypothetical
population which will be in the G1, S, G2 and M phases
and the next G1 phase of the cell cycle are designated
$a(t)$, $b(t)$, $c(t)$, $d(t)$ and $e(t)$. For times, t, greater
than 2 hours and such that no cells have entered the next
S phase, it will be true that $a(t)+b(t)+c(t)+d(t)+e(t)=1$.

On the other hand, the number of cells in the actual
synchronized cell population increases since the cells
double upon leaving the M phase; therefore at time, t,
defined as described above, the number of cells increases

over the number of cells in the original G1 population
by the factor of 1+e(t). The measurements made on the
synchronized population are the proportion of cells
with labeled nuclei at a particular time, S(t), and
the proportion of cells in mitosis at a particular time,
M(t). These two quantities can be expressed as

$$S(t) = \frac{b(t)}{1+e(t)} \tag{1}$$

$$M(t) = \frac{d(t)}{1+e(t)} . \tag{2}$$

The actual proportion of the cells in the synchronized
population which is in the next G1 at a particular time,
E(t), taking into consideration the doubling of the cells
leaving M, is given by

$$E(t) = \frac{2e(t)}{1+e(t)} . \tag{3}$$

For any time, t, in the cell cycle, it is estimated
that the proportion of the original G1 population which
has passed through M and entered the next G1, e(t), is
approximated by the ratio of the area under the curve
e(t) up to time t, $\theta(t)$, to the total area under the
curve e(t) over one generation, ω, thus

$$e(t) = \frac{\theta(t)}{\omega} . \tag{4}$$

It has been shown that the relative length of the
M phase, ℓ_m, can be given in terms of M(t), (Stanners
and Till, 1960), that is:

$$\ell_m = \int e(t)dt = \beta \int M(t)dt \tag{5}$$

where β is determined by the limits of integration and,
in particular, is equal to 1/(ln2) for integration over
one generation.

The correction factor β for any time, t, is
approximated as follows: (a) β is 1/(ln2) $\overset{\sim}{=}$ 1.44 for
the integral over one generation (for example, t=5 to t=24
hours), and would be approximately 1.0 if the integral were
calculated over a short period of time in the left tail
of the M(t) curve (t=11 to t=12, for example); (b) the
approximation is made that the correction factor increases
linearly with increasing proportion of the area under
the curve M(t).

(b)   Calculations for the t=17.5-18.5

hour point

Let us consider the generation time between t=5
and t=24 hours. Therefore ω is given by

$$\omega = \int_5^{24} e(t)dt \ . \tag{6}$$

For t=18,

$$\theta(18) \stackrel{\sim}{=} \int_5^{18-\ell_m} e(t)dt \qquad (7)$$

where ℓ_m is subtracted from 18 to make an approximate correction for the fact that it takes a finite period of time, ℓ_m, for a cell to pass through mitosis. From Figure 6 and equation (5) and taking ℓ_m as 0.4 hour (Chapter III, Results), it can be calculated that

$$\theta(18) \stackrel{\sim}{=} \beta \int_5^{17.6} M(t)dt \stackrel{\sim}{=} 0.050\beta \qquad (8)$$

and

$$\omega = \frac{1}{\ln2} \int_5^{24} M(t)dt = \frac{0.261}{\ln2} . \qquad (9)$$

Consequently,

$$\frac{\theta(18)}{\omega} \stackrel{\sim}{=} 0.19 \ \beta\ln2 \qquad (10)$$

For about 0.19 of the area under M(t), the correction factor should be approximately

$$\beta \stackrel{\sim}{=} 1 + 0.44 \ x \ 0.19 \stackrel{\sim}{=} 1.08 \qquad (11)$$

and it follows that

$$e(18) = \frac{\theta(18)}{\omega} \cong \frac{1.08 \times 0.05}{1.44 \times 0.261} \cong 0.144 \qquad (12)$$

Therefore, the proportion of the t=17.5-18.5 population which are G1 cells (Table 1, Experiment 1, column 4, line 5) can be approximated as

$$E(18) = \frac{2e(18)}{1+e(18)} \cong 0.25 \ . \qquad (13)$$

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APPENDIX II

REANALYSIS OF THE CONCLUSIONS OF BOGENHAGEN
AND CLAYTON (1977) REGARDING THE TIMING OF
MITOCHONDRIAL DNA SYNTHESIS DURING THE CELL CYCLE

1. General approach

The papers of Bogenhagen and Clayton (1976; 1977) claim
to have shown that the rate of mit-DNA synthesis in A9 cells
is constant during the cell cycle and that molecules are
chosen at random for replication.

The conclusions they present are in disagreement with
the data previously published for HeLa cells (Pica-Mattoccia
and Attardi, 1972) and the data presented here on A9 cells.
It is the purpose of this review to evaluate whether the
data of Bogenhagen and Clayton are sufficient to justify
their conclusions.

Their experimental design was to pulse-label unsyn-
chronized cells with ^{3}H-thymidine for 2 hours, chase them
with cold thymidine for various lengths of time and then
grow them in the presence of BrdU for 4 hours. In analyzing
their data, they gave mathematical equations which were to
provide theoretical values for the percentage of labeled
mit-DNA that is density shifted for chosen chase times.

In the mathematical models considered, some dubious
assumptions were made. One was that the mit-TTP pool
specific activity during the cell cycle is constant, con-
trary to the published data (Pica-Mattoccia and Attardi,
1972) and to the data presented here. Another assumption
is that molecules recently replicated may be randomly
selected for another round of replication. There is no

evidence available to support this assumption, and the only
published report on this subject (Flory and Vinograd, 1973)
suggests that this assumption may not be valid. The
mathematical formulas were intended to determine the
percentage of labeled molecules expected to be shifted
in density, as a function of chase time, for different
theoretical patterns of mit-DNA synthesis during the cell
cycle. Such an approach is very indirect, and although the
experimental data may be shown to be consistent with one
model, care must be taken in excluding other models, es-
pecially those making different basic assumptions.

In addition, in the present case, some of the equations
used to determine the percentage of ^{3}H-mit-DNA shifted in
density are incorrectly formulated (see below). The authors
did not present the evidence justifying their conclusion
of a significantly better fit of their data to the expecta-
tions for a constant rate of mit-DNA synthesis during the
cell cycle, as compared to other models. Their data do,
in fact, seem to reveal a pseudo-sinusoidal appear-
ance, which would be predicted from many models of the
timing of mit-DNA synthesis. Several models, exhibiting
striking changes in the rate of mit-DNA synthesis during
the cell cycle, fit the authors' data as well as or better
than the constant rate model they proposed. Furthermore,
one model, predicting mit-DNA synthesis only in S and G2,
that does not fit the data as well is an extreme

interpretation of published rate of incorporation data,
which suggest a low level of incorporation during G1 and
probably a continually increasing rate of incorporation
during the rest of the cell cycle.

2. Corrected Equations

The following is a discussion and corrected derivation of the equations from Appendix I of Bogenhagen and Clayton (1977) used to predict their experimental results, assuming the mitochondrial TTP and BrdUTP pool specific activities remain constant during the cell cycle.

The experimental protocol considered here is as follows. The cell population was radioactively labeled with ^{3}H-thymidine from t=-2 to t=0 hours. Nonradioactive thymidine was added as a chase at t=0, BrdU was added to the cultures at $t=t_c$ resulting in denser mit-DNA molecules and the cells were harvested at $t=t_c+4$ hours.

Consider an asynchronous population of cells growing exponentially. At time t, the number of cells, N(t), is given by

$$N(t) = N_o e^{\alpha t} \tag{1}$$

where $\alpha = (\ln 2)/T_g$, T_g is the generation time of the cells and N_o is the number of cells at t=0.

The cell age is defined as the time since the last mitosis involving that cell. The probability density distribution of a cell in the population having cell age τ is given by the function, $n(\tau)$, with

$$n(\tau) = 2\alpha e^{-\alpha\tau} \qquad \text{for } 0 \leqq \tau < T_g \tag{2}$$

as given by Stanners and Till (1960). $n(\tau)$ satisfies the

equation

$$\int_0^{T_g} n(\tau)d\tau = 1. \tag{3}$$

For any cell, let z be the cell age at t=0. At time t, the cell age of a cell that was, or whose ancestor was, of cell age z at t=0 is given by

$$\phi(z + t) = z + t - kT_g \tag{4}$$

where k is the integer which satisfies the inequality

$$kT_g \leqq z + t < (k + 1)T_g. \tag{5}$$

The number of cells of cell age z at t=0 and their descendants is

$$N(t)n(\phi(z + t)) = 2^{k+1}\alpha N_0 e^{-\alpha z} \tag{6}$$

where k is the integer which satisfies equation (5). $N(t)n(\phi(z + t))$ is a step function increasing by a factor of two at each mitosis.

Let $q(\tau)$ be the average rate of mit-DNA replication (mit-DNA molecules/cell/hour) in cells of cell age τ and let the number of mit-DNA molecules synthesized per cell over one generation ($\tau=0$ to $\tau=T_g$) be a constant, c. It follows that

$$\int_0^{T_g} q(\tau)d\tau = c. \tag{7}$$

Bogenhagen and Clayton (1977) have considered the special
case with $q(\tau) = Hr(\tau)$ where H is a constant equal to
c ÷ (the length of the period during the cell cycle during
which replication occurs) and $r(\tau)$ has the value 0 for
cell ages, τ, at which cells do not engage in mit-DNA
replication and has the value 1 for cell ages at which
cells do engage in mit-DNA replication. The equations
presented here are equally valid for either the special
case, with $Hr(\tau)$, or the more general $q(\tau)$. $m(\tau)$, the
average number of mit-DNA molecules per cell in cells of
cell age τ, is

$$m(\tau) = c + \int_0^\tau q(y)dy. \qquad (8)$$

In order to simplify the derivation presented here,
the assumption is made that the replication of a mit-DNA
molecule occurs instantaneously (the only exception to
this assumption is pointed out below).

The number of radioactive mit-DNA strands synthesized
during the ^{3}H-thymidine labeling period from t=-2 to t=0
in cells which will have, or whose descendants will have,
cell age z at t=0, $P^*(z)$, is given by

$$P^*(z) = 2\int_{-2}^0 N(t)n(\phi(z + t)) \, q(\phi(z + t))dt. \qquad (9)$$

Alternatively, $P^*(z)$ can be expressed as

$$P^*(z) = \begin{cases} 4\alpha N_o \int_{-2}^{0} e^{-\alpha z} q(z + t) \, dt & \text{if } 2 \leqq z < T_g, \\[2em] 4\alpha N_o \left[e^{-\alpha T_g} \int_{-2}^{-z} e^{-\alpha z} q(z + t) dt \right. \\[1em] \left. \quad + \int_{-z}^{0} e^{-\alpha z} q(z + t) dt \right] & \text{if } 0 \leqq z < 2. \end{cases} \qquad (10)$$

The total number of ^{3}H-labeled mit-DNA strands is, therefore,

$$\int_{0}^{T_g} P^*(z) dz = 2 \int_{0}^{T_g} \int_{-2}^{0} N(t) n(\phi(z+t)) \, q(\phi(z+t)) dt dz. \quad (11)$$

After a thymidine chase time of t_c, BrdU is added at $t = t_c$ and the cells are harvested at $t = t_c + 4$. For the population of cells which had cell age z at $t = 0$ and their descendants, the average number of radioactive mit-DNA strands per mit-DNA molecule at time t is

$$F^*(z,t) = \frac{P^*(z)}{N(t) n(\phi(z+t)) m(\phi(z+t))} \qquad (12)$$

Specifically for determining the limits of integration for the following equation, it is assumed that the process of mit-DNA replication lasts for 1 hour. Due to the mit-DNA isolation procedures used, a mit-DNA molecule shifted in density due to BrdU substitution will be observed only if replication ends by $t = t_c + 4$, and thus only if replication begins by $t = t_c + 3$. If the entire period of replication of a molecule must occur in the presence of BrdU for that

molecule to be observed to be shifted in density, then the replication must begin after $t=t_c$[5]. Therefore, for cells of cell age z at t=0 and their descendants, the number of radioactive molecules which are radioactive in one strand and contain maximum BrdU substitution in the other strand, for $t_c>1$ hour, is estimated[6] by

$$G^*(z,t_c) = \int_{t_c}^{t_c+3} F^*(z,t)N(t)n(\phi(z+t))q(\phi(z+t))dt. \quad (13)$$

From equations (12) and (13), it can be shown that

$$G^*(z,t_c) = P^*(z)\int_{t_c}^{t_c+3} \frac{q(\phi(z+t))}{m(\phi(z+t))} dt. \quad (14)$$

It should be noted that neither $F^*(z,t)$ nor $G^*(z,t)$ is a function only of z+t.

The total number of molecules labeled with ^{3}H in one strand and substituted with BrdU in the other strand is

$$\int_0^{T_g} G^*(z,t_c)dz. \quad (15)$$

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[5] If only a portion (e.g. one half) of the replication of a mit-DNA molecule must occur in the presence of BrdU for the molecule to be observed to be shifted in density, then a smaller value for the lower limit of integration (e.g. $t_c-1/2$) might be a judicious improvement.

[6] A ^{3}H-labeled mit-DNA strand may be involved in more than one replication, but, as a ^{3}H-labeled, BrdU-substituted mit-DNA molecule, would be counted as only one replication in the final analysis. This aspect would tend to make $G^*(z,t_c)$ an overestimate of the number of heavy, labeled molecules expected to be observed.

Therefore the fraction of the observed mit-DNA molecules with a radioactively labeled strand which also have a BrdU substituted strand, $S(t_c)$ is given by

$$\frac{\int_0^{T_g} G^*(z,t_c)dz}{\int_0^{T_g} P^*(z)dz} \, , \qquad (16)$$

and thus

$$S(t_c) = \frac{\int_0^{T_g}\left[\int_{-2}^0 N(t)n(\phi(z+t))q(\phi(z+t))dt \int_{t_c}^{t_c+3} \frac{q(\phi(z+t))}{m(\phi(z+t))}dt\right]dz}{\int_0^{T_g}\int_{-2}^0 N(t)n(\phi(z+t))q(\phi(z+t))dtdz} . \qquad (17)$$

Consequently, if $q(\tau)$ is equal to a constant, for $0 \le \tau < T_g$, then $S(t_c)$ is not equal to a constant as t_c changes.

3. Comparisons of theoretical predictions
with the published data

The predicted percentage of mit-DNA expected to be shifted in density by BrdU labeling after chosen chase times, $S(t_c)$, has been calculated for six different models represented by six chosen temporal patterns of rate of mit-DNA replication, $q(\tau)$, for cells of age τ since the last mitosis. Since the data analyzed here (estimated from Figure 6, Bogenhagen and Clayton, 1977) are consistently lower than the values predicted by all models, the assumption is made, following the authors' suggestion, that a constant proportion of the theoretically expected hybrid mit-DNA is actually observed as light DNA, due to quarter-heavy concatenates. Therefore, the theoretical values must be multiplied by a factor less than 1 to fit the observed data. The factor was chosen, for each model, to be that which gave the minimum sum of squares, $SS = \Sigma(\% \text{ observed} - \% \text{ predicted} \times \text{factor})^2$ with the summation including all experimental data points.

Model a, $q(\tau) = 2\alpha e^{\alpha(\tau - Tg)}$, in which the rate of mit-DNA synthesis is proportional to the number of mit-DNA molecules, is the model which yields a constant value of $S(t_c)$ for any t_c. (A different model considered by Bogenhagen and Clayton with a constant rate of mit-DNA

replication per cell, from cell ages $\tau=0$ to $\tau=T_g$, yields
an oscillating, though very flat, $S(t_c)$ curve.) As can
be seen in Figure 1, model 1 appears to fit the data
(SS=31) better than model b (SS=128) for which there is
absolutely no synthesis in the G1 phase and then a
constant rate per cell during S and G2 phases, considered
as a possible alternative in Bogenhagen and Clayton (1977).
This latter model, however, contrary to the interpretation
of the above authors does not accurately reflect the
rate of mit-DNA synthesis during the cell cycle in A9
cells (Chapter III, this thesis) or in HeLa cells
(Pica-Mattoccia and Attardi, 1972). In model c, there
is no mit-DNA synthesis during the early G1 phase, a
constantly increasing rate during the late G1 and S
phases, and a constant and maximal rate during the G2
phase; in model d, there is a residual rate of synthesis
during the G1 phase and a rate 5 times higher during the
S and G2 phases; in model e, there is a residual rate
of synthesis during the early G1 phases, a steadily
increasing rate during the late G1 and S phases and a
constant and maximal rate of synthesis during the G2
phase; in model f, the rate of mit-DNA synthesis begins at
zero and increases at a constant rate during the cell cycle.
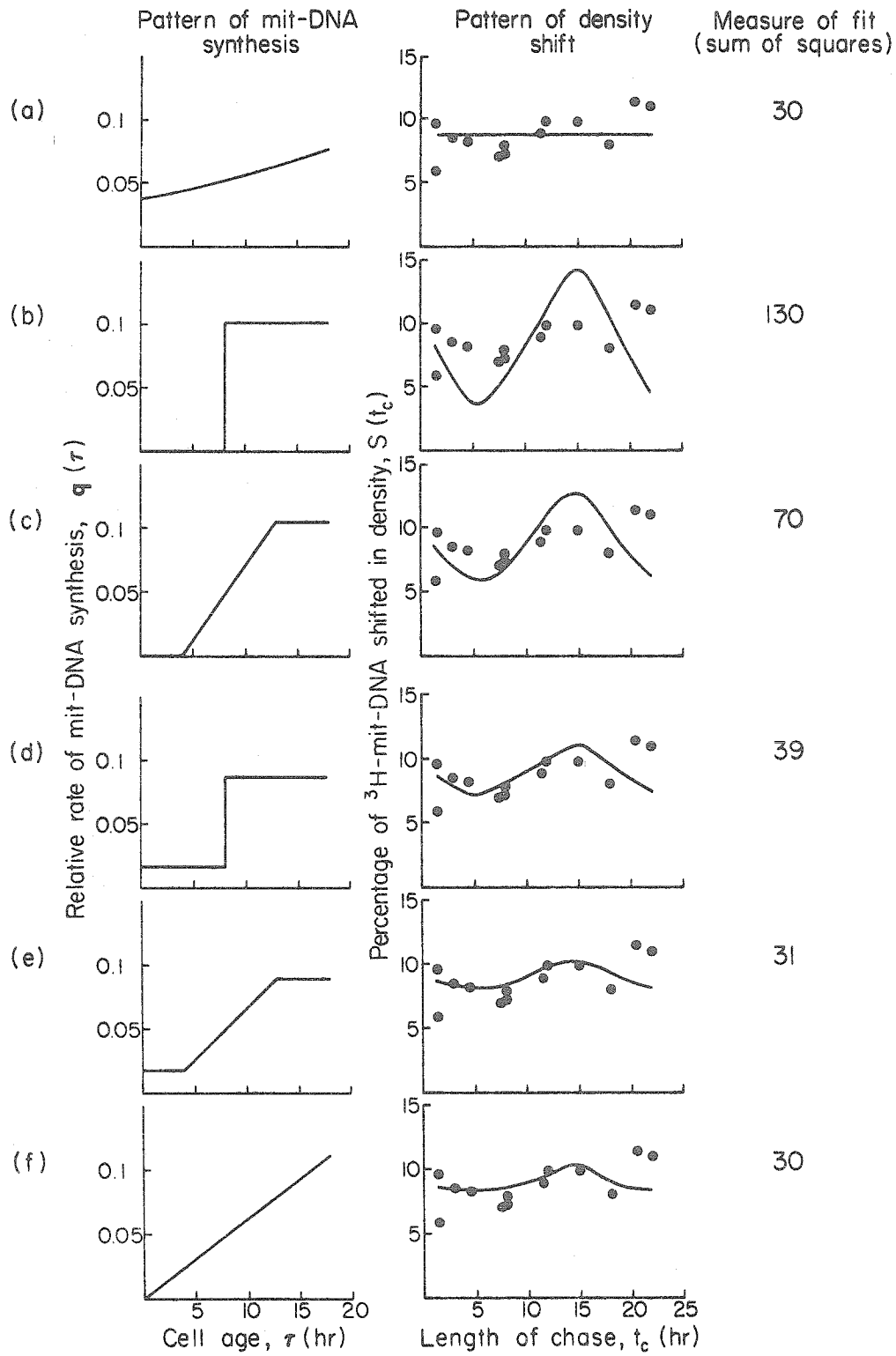Models more representative of the latter observations

Figure 1

LEGEND TO FIGURE 1

Computer derivation of theoretical curves describing the density shift of mit-DNA for six models, each characterized by a different temporal pattern of the rate of mit-DNA synthesis during the cell cycle.

The diagrams on the left represent the chosen temporal patterns of the rate of mit-DNA replication during the cell cycle, $q(\tau)$, where τ is the cell age since the last mitosis. The diagrams on the right show, for each model, the percentage of ^{3}H-mit-DNA expected to be shifted in density by BrdU labeling, $S(t_c)$, as a function of the length of the thymidine chase, t_c, and, for comparison, the experimental data of Bogenhagen and Clayton (Figure 6, 1977). Since the data are consistently lower than the values predicted by all models, the assumption is made, following Bogenhagen and Clayton's suggestion, that a constant proportion of the theoretically expected hybrid mit-DNA is actually observed as light DNA, due to quarter-heavy concatenates. Therefore, the theoretical values must be multiplied by a factor less than 1 to fit the observed data. The factor was chosen to be that which gave the minimum sum of squares, SS = Σ(% observed - % predicted x factor)^2 with the summation to include

(LEGEND TO FIGURE 1 CONTINUED)

all experimental data points. SS is given to the right
of each diagram. The values of the factors were 0.75-0.78.
● ●, observed percentage of mit-DNA shifted at various
chase times; ——, theoretically predicted percentage of
mit-DNA shifted at various chase times.

are models d, e and f (SS=30-39); those models, although
quite different from model 1, fit the data almost as well
or better than model a.[5] Despite the fact that there is
obvious scatter in the experimental data of Bogenhagen
and Clayton and that no model explains the high points
for t_c > 20 hours, it is conceivable that the tendency of
a sinusoidal shape in the data up to 15 hours may reflect
a rate of replication similar to the last three models.
Mention was made in the Discussion of Chapter III (this
thesis) of the possible effect of the pool changes in
alternating the sinusoidal pattern of the experimental
observations.

It is concluded from the above observations that
the experimental technique of Bogenhagen and Clayton
(1977) is quite insensitive to the precise temporal
pattern of the rate of mit-DNA replication during the
cell cycle and that their experimental data are not
inconsistent with direct measurements on the rate of
mit-DNA synthesis during the cell cycle presented in
Chapter III (this thesis) and published previously.

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[5]The reason why model 2 gives a poorer fit to the
data (SS=130) is probably its period of absolutely no
replication, as shown by the striking difference in
$S(t_c)$ and the sum of square between models b and d, and,
between models c and e. Both in the present work and
in the previous work, a residual mit-DNA synthesis was
observed early in the cell cycle.

188

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189

APPENDIX III

THE PRODUCTS OF SEMI-CONSERVATIVE

MITOCHONDRIAL DNA REPLICATION

AFTER ONE GENERATION

IN THE PRESENCE OF BrdU

ASSUMING RANDOM REPLICATION

Mitochondrial DNA (mit-DNA) replication is considered
in a normally growing cell which is transferred at time
t=0 to medium containing BrdU. For the purpose of these
calculations, it is assumed that at any particular time,
any of the mit-DNA molecules of the cell, or its progeny,
are equally likely to begin replication and it is further
assumed that replication is completed instantaneously.

Initially, all molecules consist of two light
(L; non-BrdU substituted) strands. Replication of one
of these molecules in the presence of BrdU results in two
molecules, each with one light strand and one heavy (H; BrdU
substituted strand). Replication of an HL molecule results
in one HL and one HH molecule. At any instant in time, t,
the rate of replication of molecules in a particular class
(LL, HL, HH) is assumed to be proportional to the number of
molecules in that class. The relative number of LL molecules
is denoted X, of HL molecules is Y, of HH molecules is Z.
It follows that

$$\frac{dX}{dt} = -kX,$$

$$\frac{dY}{dt} = 2kX, \text{ and}$$

$$\frac{dZ}{dt} = k(Y+Z)$$

where k is a constant. The boundary conditions are X=1, Y=0, Z=0 at t=0. The solution to these equations is

$$X = e^{-kt},$$

$$Y = 2(1-e^{-kt}), \text{ and}$$
$$Z = e^{kt} + e^{-kt} - 2.$$

It is assumed that the number of mit-DNA molecules doubles over one generation, during which time the cell will have undergone one mitosis. The composition of the mit-DNA molecule population in these two cells is determined by $X+Y+Z = e^{kt} = 2$. It follows that X = 1/2, Y = 1 and Z = 1/2 and therefore the ratio of LL:HL:HH molecules is 1/4:1/2:1/4 after one generation in the presence of BrdU.

CHAPTER IV

An Approach for Testing the Nature of the Cell

Cycle Dependent Regulation of Mitochondrial

DNA in Mammalian Cells

1. Introduction

Study of the regulation of mammalian mitochondrial bio-
genesis is hindered by the lack of mutants affecting mito-
chondrial replication, transcription and translation. One
available approach is the use of drugs that modify cellular
functions but they have the disadvantage that there are
documented, as well as unknown, side effects of such treat-
ments.

An attractive approach to the study of mammalian tissue
culture cell-cycle dependent regulation has been used on
HeLa cells by Rao and Johnson (1970). By fusing cells in
different stages of the cell cycle (the nuclei of one
population lightly prelabeled) and monitoring microscopically
DNA synthesis and mitosis, they were able to show that in
G1/S heterokaryons, the G1 phase nucleus was induced to
enter the S phase prematurely. They concluded that certain
S phase cell components migrate into the G1 phase nucleus
and induce DNA synthesis (positive control). Mitosis in
S/G2 heterokaryons, on the other hand, was delayed in G2
phase nucleus until the S phase nuclei had aged sufficiently
to enter M. Mitosis, they suggested, may require a minimum
concentration of an inducer which is diluted out by the
presence of S cell components.

Mitochondrial DNA replication (Chapter III, this thesis;
Pica-Mattoccia and Attardi, 1972) and transcription

(Pica-Mattoccia and Attardi, 1971) are subject to cell cycle
dependent regulation. It is known that HeLa cell mitochon-
drial replication and transcription are inhibited by cyclo-
heximide (Storrie and Attardi, 1972), a potent inhibitor of
cytoplasmic protein synthesis and it is quite likely that
nuclear coded genes are involved in the cell cycle dependent
regulation of mitochondrial nucleic acid biosynthesis.

It is the purpose of this study to propose an approach
for revealing the nature of cell cycle dependent control
of mit-DNA synthesis. In this approach, populations of
synchronized mouse cells at different stages of the cell
cycle are fused with Sendai virus. The mit-DNA of one popu-
lation has had thymidine substituted for by BrdU for sub-
sequent identification of its origin. The resulting hetero-
karyons are pulse labeled and each of the two mit-DNA species
is analyzed for changes in its relative labeling due to its
presence in a heterokaryon containing the nucleus and
cytoplasm from a cell of a different phase of the cycle.
In this manner information can be obtained concerning a
possible positive or negative control of mit-DNA synthesis
by exogenous factors.

2. Methods and Materials

(a) Solutions[1]

The solution designations are as follows. (1) PBS:
0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4,
10^{-4} M phenol red, 200 units penicillin/ml, 50 µg strepto-
mycin (S2)/ml. (2) TD: 0.137 M NaCl, 5 mM KCl, 25 mM Tris
buffer (pH 7.4 at 25°C).

(b) Cell growth and labeling conditions

LM(TK⁻)C11D cells are a mouse L fibroblast cell line
derivative which are deficient in extramitochondrial
thymidine kinase activity and can grow normally in the
presence of 30 µg/ml BrdU despite the presence of mitochon-
dria-associated enzyme activity (Kit et al., 1963; Attardi
and Attardi, 1972). Monolayer cultures were grown at 37°C
in Eagle's medium (MED-72214, Grand Island Biological)
supplemented with 10% calf serum in 10 cm plastic petri
dishes (57 cm^2 surface area, Nunclon) or in Corning poly-
styrene roller bottles (490 cm^2 surface area) rotating at
2 rpm. Suspension cultures were grown at 37°C in a modified
Eagle's phosphate medium (Levintow and Darnell, 1960;
MED-6919, Grand Island Biological) supplemented with 5%

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[1](1) BrdU: bromodeoxyuridine, (2) EDTA: ethylenedi-
aminetetracetate, (3) HAU: hemagglutinating units, (4)
mit-DNA: mitochondrial DNA.

calf serum. If a radioactive precursor was to be added to the medium, dialyzed calf serum was used. To maintain exponential growth, the cell concentration was kept below 10^5 cells/cm^2 in monolayer cultures and below 10^6 cells/ml in suspension cultures. Some of the cultures, as specified below, were maintained, in the dark in medium containing 30 µg BrdU/ml.

For the labeling of monolayer cells, C11D cells were grown in the presence of 30 µg/ml BrdU for at least 10 generations up to a concentration of 2×10^7 cells/roller bottle. The medium was replaced with 200 uCi/ml of [methyl-^{3}H]thymidine (45 Ci/mmole, Amersham) in Eagle's medium plus 10% dialyzed calf serum and the cells incubated for 2 hours at 37°C. For the labeling of suspension grown cells, a spinner culture was grown in modified Eagle's phosphate medium with 5% dialyzed calf serum in the presence of 30 µg/ml BrdU for more than 10 generations up to a concentration of 5×10^5 cells/ml; 2 µCi/ml [methyl-^{3}H]thymidine (54 Ci/mmole, Amersham) was then added and the cells were grown for an additional 15 hours. For both labeling experiments described above, in order to provide an internal marker of non-BrdU substituted mit-DNA, [methyl-^{3}H]thymidine labeled cells were mixed at the time of harvesting, with a suspension of C11D cells (4 or 8×10^8 cells) maintained in the absence of BrdU and labeled for 16 hours in 4 or 16 nCi/ml of [2-^{14}C] thymidine (57 mCi/mmole, Amersham) in

medium not containing BrdU.

(c) Growth and harvesting of Sendai virus

Sendai virus was grown and harvested under sterile
conditions. The allantoic sacs of 5 dozen 10 day old
fertile chicken eggs were each inoculated with 0.1
hemagglutinating units (in 0.2 ml of PBS) of Sendai virus.
The allantoic fluid was harvested following a 3 day incuba-
tion at 37°C (Henle, 1953). After the red blood cells were
removed by centrifugation at 2,000 rpm (500 g) for 10
minutes, the virus was pelleted by centrifugation at 15,800
rpm (30,500 g) for 30 minutes in the Sorvall SS-34 rotor.
The pellet was resuspended in 5 ml of PBS and the remaining
cells removed by centrifugation at 500 g for 10 minutes;
the resulting supernatant will be referred to as "standard
virus preparation."

2 ml portions of the virus suspension were each layered
on a 32 ml 15%-30% sucrose gradient (in 2.54 x 8.89 cm
nitrocellulose tubes with a 2 ml cushion of 50% sucrose)
in 1 mM EDTA, 10 mM Tris buffer (pH 6.7 at 25°C), 90% D_2O.
Centrifugation was in the Beckman SW27 rotor at 17,500 rpm
for 40 minutes at 20°C (Kingsbury et al., 1970). The
gradients were collected by eye in 4 unequal fractions, as
specified below; each fraction was diluted with an equal
volume of PBS and each centrifuged at 30,500 g for 15
minutes and the corresponding pellet resuspended in

1 to 5 ml PBS.

To determine the amount of hemagglutinating units/ml of a virus sample, serial dilutions in PBS were mixed with an equal volume of 1% chicken red blood cells in PBS. The maximum dilution factor which caused agglutination of the red blood cells was designated as the number of hemagglutinating units/ml of the original suspension. Sendai virus was inactivated by treatment with ultraviolet light (Sylvania G15T8; at 3.5 cm) in petri dishes with 1 ml of virus/20 cm^2.

(d) Cell fusion

Monolayer C11D cultures were detached with TD plus 1 mM EDTA and 0.05% trypsin for 1 minute, and the cells were pelleted after the addition of Eagle's medium supplemented with 10% calf serum. The cells were resuspended in the same medium, repelleted and finally resuspended in a modified Eagle's medium (brought to 10 mM HEPES, 10 mM HEPPS, 10 mM TES and pH 8) without calf serum. Cell fusion was carried out in 12 x 75 mm polystyrene tubes (Falcon) in 0.2 ml of the Eagle's pH 8.0 medium containing inactivated Sendai virus at selected concentrations and C11D cells at a concentration of 10^7 cells/ml. The mixture was placed on ice for 20 minutes, then shaken for 15 minutes at 37°C. One portion was incubated with an equal volume of 0.2% Trypan Blue at 37°C for 10 minutes and examined in the phase contrast microscope to estimate the percentage of live (dye-excluding)

cells. Another portion of the mixture was placed on cover-
slips in petri dishes with 2 ml of Eagle's medium plus 10%
calf serum and kept in a 37°C CO_2 incubator for 2-24 hours;
already after 2 hours almost all cells appeared to be
attached and spread on the coverslips.

(e)   Analysis of heterokaryons

Coverslips to which fused cell samples had attached
were washed in TD and fixed in anhydrous methanol for one
half hour. The samples were stained for 2 minutes in
freshly filtered May-Grünwald and for 5 minutes in freshly
filtered Giemsa (diluted 1:5), conditions which give optimal
visualization of CllD nuclear membranes. After dehydration
in acetone-toluene, samples were mounted with Gurr's DePex.
Approximately 500 cells were analyzed by phase contrast
microscopy and the number of nuclei per cell was determined.

(f)   Mit-DNA analysis

The mitochondrial fraction was isolated and the mit-DNA
run on two-step gradients in the Beckman SW65 rotor as
described earlier (Chapter III, this thesis). The two-step
gradients were collected, samples counted and the closed
and open circular mit-DNA region was pooled. The ethidium
bromide was extracted from the solution of pooled fractions
3 times with isoamyl alcohol; the residual alcohol in the
final aqueous phase was removed by ether which was separated

by centrifugation; the residues of ether in the final sample were removed by bubbling N_2 through the solution. The solution was suitably diluted with 10 mM EDTA, 10 mM Tris buffer (pH 7.4 at 25°C) and then brought to 1.72-1.73 g/cc with solid CsCl. Centrifugation was in the Beckman SW65 rotor at 30,000 rpm for 2-3 days. The refractive index was determined on samples of selected fractions and converted to density by the formula $\rho = 10.875 \, n_D^{25} - 13.5178$. The entire fractions were then spotted on Whatman 3MM filters, washed in 5% TCA and ethanol, and counted as described earlier (Chapter III, this thesis).

(g) G1/G2 cell fusion

C11D cells to be synchronized were grown in monolayer on the inner surface of borosilicate roller bottles (Bellco) in Eagle's medium supplemented with 10% dialyzed calf serum up to a concentration of less than 5×10^6 cells/bottle. One set of bottles contained CL10 cells which had been growing for more than 10 generations in the dark in media containing 30 µg BrdU/ml and were maintained in BrdU throughout the selective detachment procedure described below. A second set of bottles contained CL10 cells which had been maintained for many generations in the absence of BrdU.

C11D cells to be used as carrier ($\sim 3.2 \times 10^8$ cells)

which had been maintained for more than 10 generations in
the absence of BrdU, were grown in suspension in Eagle's
phosphate medium without BrdU supplemented with dialyzed
calf serum. The cultures were exposed to $[2-^{14}C]$thymidine
(0.016 μCi/ml, 61 mCi/mmole, Amersham) for 2 days.

For each CL1D population (grown in the presence or
absence of BrdU) eight roller bottles of monolayer cells
were subjected to a vigorous shaking to remove easily
detachable dead cells; after fresh medium addition and
one hour incubation, three moderately vigorous shakings
were made at 1 hour intervals to selectively detach mitotic
cells (Terasima and Tolmach, 1963; Robbins and Marcus, 1964;
England and Attardi, 1974). The medium of the latter three
shakings, containing detached cells, mostly in mitosis, was
filtered through Nitex 100 nylon mesh; the cells from each
shaking were pelleted at 1,500 rpm (500 g) for 5 minutes
in the International centrifuge at room temperature, resus-
pended in a small volume of Eagle's medium without BrdU and
maintained at 15°C until the cells released by the three
shakings were pooled. The cell concentration was then
adjusted to 5×10^5 cells/ml with Eagle's medium without
BrdU and the cell suspension (∿20 ml) was rapidly dispensed
into two 10 cm petri dishes and placed in the 37°C CO_2
incubator. Cells obtained by selective detachment from
the cell population grown in the presence of BrdU were placed
in the 37°C incubator 15.8 hours before the cell fusion

procedure began (described below), and cells obtained by
selective detachment from the cell population grown in the
absence of BrdU were placed in the 37°C incubator 2.8 hours
before the cell fusion procedure began.

At the appropriate time, the cell monolayers were
removed from petri dishes with trypsin in the presence of
EDTA and washed with Eagle's medium without BrdU as described
above (Chapter IV, Section 2d). Cell fusions were carried
out in 0.8 ml medium as described earlier with 1000 HAU/ml
of fast sedimenting fraction Sendai virus and (1) 4×10^6 G1
population cells plus 4×10^6 G2 population cells ("G1/G2"
fusion), or (2) 8×10^6 G1 population cells ("G1/G1" fusion),
or (3) 8×10^6 G2 population cells ("G2/G2" fusion). After
cell fusion, the cells were diluted with fresh Eagle's
medium without BrdU with 10% calf serum. A small portion
of each fusion was used for analysis of heterokaryon forma-
tion as described above (Section 2e), while the bulk of
the mixture was placed in the 37°C CO_2 incubator for 2 hours,
during which time the cells attached to the petri dish. The
medium was replaced with Eagle's medium without BrdU supple-
mented with 10% dialyzed calf serum and containing 200
μCi/ml of [5-^{3}H]deoxycytidine (24 Ci/mmole, Amersham), and
the cultures were further incubated for 75 minutes. The
[5-^{3}H]deoxycytidine labeled samples were removed by tryp-
sinization from the petri dishes and mixed with equal amounts
($\sim 2 \times 10^7$) C11D cells from the suspension culture long-term

labeled with [2-^{14}C]thymidine. The mitochondrial fractions were isolated, the mit-DNA isolated and run on two-step CsCl/ethidium bromide gradients and the high molecular weight mit-DNA run in equilibrium density gradients as described above (Section 2f). The amount of radioactivity in mit-DNA, both BrdU substituted (originally from G1 population cells) and unsubstituted (originally from G2 population cells), was determined for the samples from each cell fusion by counting aliquots spotted on 3MM filters and washed with TCA as described above.

3. Results

(a) The approach

As mentioned above, the purpose of the present experiments was to develop an approach for studying the effect of the phase in the cell cycle on mit-DNA synthesis by analysis of the process in heterokaryons formed by fusion of cells at different cellular stages. The technical demands of such an approach are exceedingly high. First, the mit-DNA of the two parent cells must be distinguishable by some physical technique. Second, the cells must be synchronized by selective detachment which is the only physiological synchronization technique available; this technique can be applied with varying efficiency on different cell lines, and unfortunately can only yield small samples of synchronized cells. Finally, the cells must be fused by a technique which causes a high proportion of the parental cells to form heterokaryons of mixed origin. The experiments described below aimed at finding conditions which satisfy the requirements mentioned above. A preliminary experiment of heterokaryon formation is described at the end of this section.

(b) Choice of the cells

The mouse L cell derivative, ClID, which lacks the cytoplasmic (Kit et al., 1963) but not the mitochondrial

thymidine kinase (Clayton and Teplitz, 1972) and therefore
can be labeled selectively in its mit-DNA with BrdU, appeared
to be a suitable choice for the proposed experiments. In
particular, by inducing heterokaryon formation between
synchronized cells grown in the presence and synchronized
cells grown in the absence of BrdU it was hoped that it
would be possible to distinguish newly synthesized mit-DNA
derived from BrdU-loaded and non-BrdU-loaded mit-DNA in the
heterokaryons. To achieve this aim, BrdU loading must give
a sufficient density shift to permit separation of sub-
stituted and unsubstituted mit-DNA on CsCl density gradients.

Since selective detachment is a technique which can
be used only with monolayer cells, preliminary experiments
were performed to determine the extent of the substitution
of BrdU for thymidine in mit-DNA after monolayer cells. The
C11D cells were grown for more than ten generations in the
presence of 30 µg BrdU/ml and then labeled for 2 hours with
[methyl-^{3}H]thymidine in the presence of BrdU. The cells
were harvested and mixed with cells grown in [2-^{14}C]thy-
midine in the absence of BrdU, the mitochondrial fraction
was isolated and treated with DNase to degrade contaminating
nuclear DNA, and the mit-DNA was extracted with 1% SDS,
pronase treatment and precipitation of SDS and residual
protein with CsCl on ice. The mit-DNA was separated from
nuclear fragments by sedimentation through a two-step CsCl/
ethidium bromide gradient. After analyzing the fractions

for acid precipitable radioactivity the fractions containing
mit-DNA were pooled and freed of the dye as described in
Methods and Materials. The mit-DNA was then centrifuged
in CsCl to equilibrium, the gradients were fractionated and
radioactivity of each fraction determined. The results,
shown in Figure 1a, indicate that the ^{14}C-labeled non-BrdU-
loaded mit-DNA formed a peak at a density of about 1.68 g/cm^3,
while the ^{3}H-mit-DNA from monolayer cells grown in BrdU had
a density of ∿1.70 g/cm^3, which is about 0.02 g/cm^3 greater
than the density of the non-BrdU-substituted DNA. This
density difference is marginally adequate for the quantita-
tive determination of the amount of BrdU loaded and unloaded
mit-DNA in a sample, a requirement discussed above. In
addition, this density difference is substantially less than
that previously reported with mit-DNA from C11D suspension
culture cells (Attardi and Attardi, 1972), for reasons which
are not clear. To confirm this difference in the extent of
BrdU substitution for thymidine in mit-DNA
from cells grown in suspension or in monolayers, ^{3}H-mit-DNA
was prepared from a C11D cell suspension as described above.
The results of the CsCl gradient equilibrium run, presented
in Figure 1b, indicate that BrdU-substituted mit-DNA formed
a broad peak covering the density from 1.72 to 1.76 g/cm^3
with a median value of about 1.74. The latter value corre-
sponds to a difference in density from the median value of
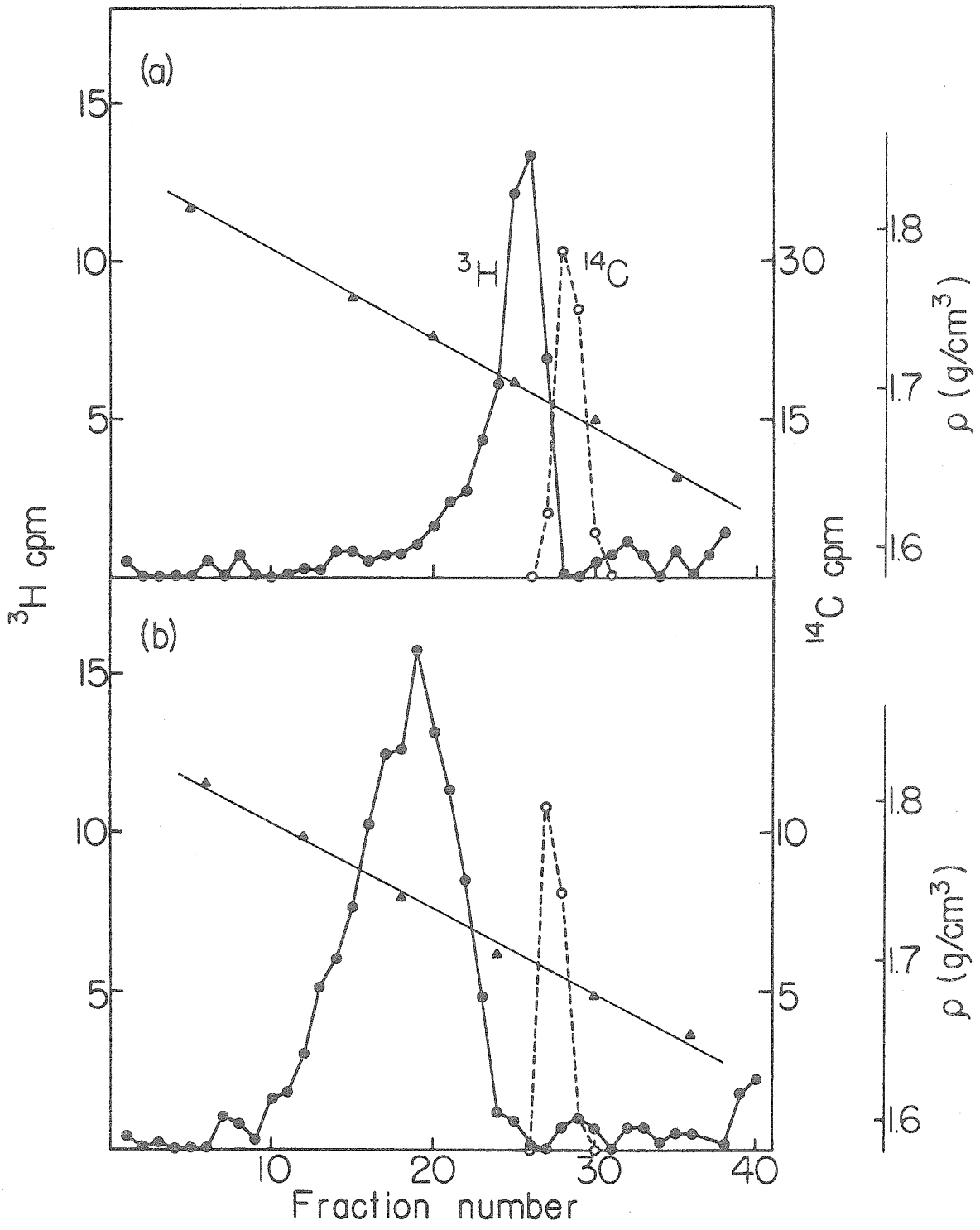unsubstituted DNA of about 0.05 g/cm^3, while the range of

Figure 1

LEGEND TO FIGURE 1

Density of mit-DNA in CsCl gradients from C11D cells

grown in the presence of BrdU either in monolayer (a) or

in suspension (b).

(a) Monolayer C11D cells were grown in the dark in

Eagle's medium supplemented with 10% calf serum and 30 µg

BrdU/ml in petri dishes to 4×10^6 cells/dish. The medium

was then replaced with Eagle's medium plus 10% dialyzed

calf serum, 30 µg BrdU/ml and containing 200 µCi/ml of

[methyl-^{3}H] thymidine (4.4 µM, 45 Ci/mmole, Amersham).

After incubation for 2 hours at 37°C, the cells ($\sim 2 \times 10^7$

cells) were trypsinized and mixed with C11D cells

(4×10^8) grown in Eagle's phosphate medium supplemented

with 5% dialyzed calf serum in the presence of [2-^{14}C]thy-

midine (0.07 µM, 4 nCi/ml, 57 mCi/mmole, Amersham). The

mitochondrial fraction was isolated, treated with DNase

and RNase, lysed in 1% SDS, pronase digested, brought

to 1M CsCl and the precipitate pelleted. The supernatant

was layered on a two-step CsCl/ethidium bromide gradient,

centrifuged at 38,000 rpm for 5 hours in a Beckman SW65

rotor. Closed and open circular mit-DNA were pooled,

and after removal of the dye, centrifuged in a CsCl

solution (density 1.72 g/cc) at 30,000 rpm for 2 days

(LEGEND TO FIGURE 1 CONTINUED)

in the Beckman SW65 rotor. The refractive index was
read and converted to CsCl density ($\blacktriangle$——$\blacktriangle$). The samples
were spotted on Whatman 3MM filters, batch washed in 5%
TCA and ethanol, and counted.

(b) Cl1D cells were grown up to 5 x 10^5 cells/ml in
suspension in Eagle's phosphate medium supplemented with
5% dialyzed calf serum plus 30 μg BrdU/ml, and subsequently
for 15 hours in the presence of 2 μCi/ml [methyl-^{3}H]thy-
midine (0.037 μM, 54 Ci/mmole, Amersham). The cells
($\sim$ 2 x 10^8 cells) were then mixed with Cl1D (8x10^8 cells)
labeled for 16 hours in 16 nCi/ml [2-^{14}C]thymidine (0.28
μM, 57 mCi/mmole, Amersham). The mitochondrial DNA was
extracted as described above, layered on two-step
CsCl/ethidium bromide gradient, and centrifuged in an
SW65 rotor at 38,000 rpm for 5 hours at 20°C. The closed
and open circular DNA was pooled, ethidium bromide was
removed and the CsCl was brought up to a density of
1.73 g/cc. The sample was then centrifuged to equilbrium
at 30,000 rpm for 3 days in the SW65 rotor. The density
and the acid precipitable radioactivity in the individual
fractions were determined as described above.

($\bullet$——$\bullet$), ^{3}H cpm, labeling in the presence of BrdU;
(o----o), ^{14}C cpm, labeling in the absence of BrdU.

density differences is 0.02 to 0.07 g/cm^3.

Based on the observation of a 0.2 g/cm^3 separation of poly[d(A-T)] and poly[d(A-BrdU)] (Wake and Baldwin, 1962) and on an estimated thymidine content of mit-DNA of 33% (W. Brown, 1976), the density shift in monolayer cells was due to an average of 15% substitution of BrdU for thymidine and the density shift in suspension culture cells was due to a mean of 35% substitution with a range of 20-55% substitution.

Since suspension cultures cannot be used directly for selective detachment experiments it was decided to examine the extent of BrdU substituted for thymidine in suspension cultures which had been recently adapted to grow on mono-layers. It was observed that the median density shift of mit-DNA was still about 0.03 g/cm^3 two days after cells had been transferred from suspension culture to monolayer (data not shown). Furthermore, already one day following the transfer the transferred cells could be well synchronized by selective detachment. Additionally, it was determined that the labeling of C11D cells was 5- to 10-fold lower than A9 cells under comparable labeling conditions (also observed by Berk and Clayton, 1973), which could be a substantial limitation when working with small numbers of cells from a selective detachment.

(c) Preparation of Sendai virus for
cell fusion

Inactivated Sendai virus has been successfully used
to induce cell fusion between a variety of tissue culture
cell lines. A glycoprotein, F, of the viral envelope is
apparently responsible for both hemolytic and cell fusion
properties of the virus (Scheid and Choppin, 1974). Sendai
virus propagated in chicken eggs and harvested early con-
tains glycoprotein F_O, a precursor of F which lacks
hemolytic and cell fusion capacity; this capacity can
be restored by cleavage of F_O to F through trypsinization
(Homma and Tamagawa, 1973) or freeze-thawing of the virus.

In the present work, preliminary tests were performed
with Sendai virus prepared from chicken eggs to induce cell
fusion in an asynchronous population of C11D cells. At
between 500 and 2000 hemagglution units/ml (HAU/ml),
there was only modest cell fusion[2] (10-25%) with moderate
cell killing (0 to 20% with different virus preparations
and different virus concentration); at higher concentrations
of virus, cell killing was extensive, with no significant
improvement in cell fusion. For the purpose of the experi-
ment planned in this work (see the Introduction of this
Chapter), the extent of fusion achieved with these virus

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[2]The "percentage fusion" is defined as the proportion
of the cells observed to be multinucleate.

preparations was not adequate. Therefore, an effort was made to obtain a Sendai virus preparation endowed with a substantially higher fusion capacity and a tolerable toxicity. In the hope that the standard virus preparation could be fractionated to yield a portion with the desired properties, the virus was sedimented through a 15 to 20% sucrose gradient in 90% D_2O. This type of fractionation had been previously applied to Sendai virus for the purpose of analyzing of RNA content of complete and incomplete virus particles (Kingsbury et al., 1970). As shown in Figure 2, there is material banding in the middle of the gradient, which includes complete (1000S) and incomplete (400S) virus particles (Kingsbury et al., 1970); sedimenting against the D_2O-sucrose cushion there is another virus fraction which was previously shown to consist of virus aggregates (Kingsbury et al., 1970). An analysis of the fusion capacity and toxicity of this fast sedimenting material revealed unexpectedly that this fraction had satisfactory properties for the purpose of the present work. In particular, the banding fraction, the fast sedimenting material, the allantoic fluid and the standard virus preparation were assayed for their ability to promote cell (C11D-C11D) fusion in a population of C11D cells, as described in Methods and Materials. Samples were evaluated for the extent of fusion after the mixtures were plated and left in a 37°C CO_2
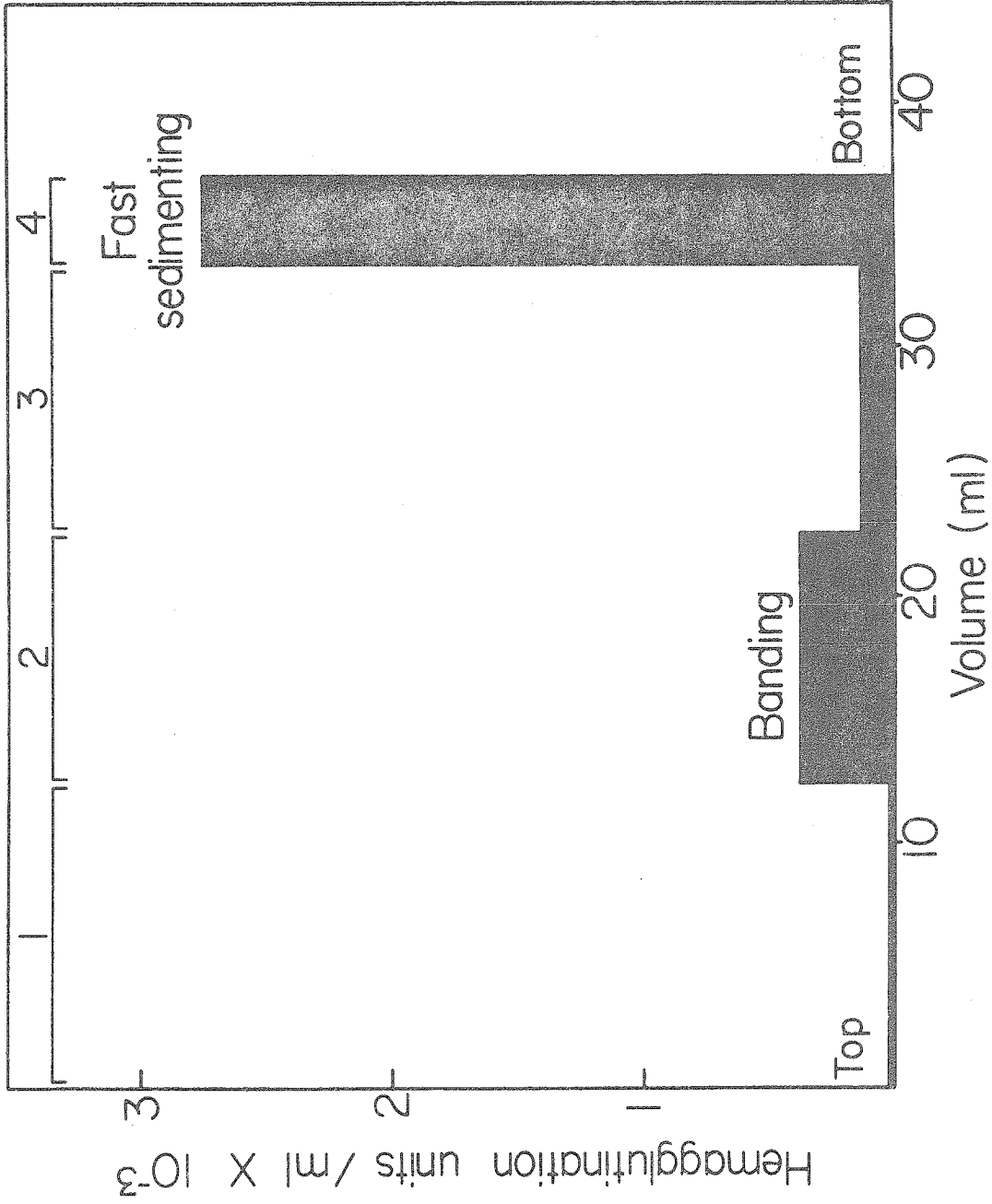
Figure 2

LEGEND TO FIGURE 2

Sedimentation pattern of hemagglutination units from the allantoic fluid of Sendai virus infected chicken embryos.

The allantoic sacs of 5 dozen 10-day old chicken embryos were innoculated with 0.1 hemagglutinating units (0.5 HAU/ml of PBS) of live Sendai virus. The allantoic fluid was harvested following a 3-day incubation at 37°C. After the red blood cells were removed by low speed centrifugation, the virus was pelleted by centrifugation at 15,750 rpm in the Sorvall SS-34 rotor (30,000 g). The pellet was resuspended in 5 ml of PBS and 2 ml portions were each layered on a 32 ml 15%-30% sucrose gradient (with a 2 ml cushion of 50% sucrose) in 1 mM EDTA, 10 mM Tris buffer (pH 6.7 at 25°C), 90% D_2O. Centrifugation was in the Beckman SW27 rotor at 17,500 rpm for 40 minutes at 20°C. The gradients were collected by eye in 4 unequal fractions, which were assayed for their capacity to agglutinate chicken red blood cells. Fractions 1 to 4 are indicated by brackets; the top of the gradient is on the left.

incubator for 2.5 hours (Figure 3), or for 24 hours
(Figure 4). As shown in Figure 3, fast sedimenting virus
was able to induce up to 75 percent fusion, with up to 89%
of the original nuclei ending up in multinucleate cells.
This is a substantial improvement over the standard virus
preparation which gave up to 23 percent fusion and up to
38% of the nuclei in heterokaryons. The banding virus and
the allantoic preparation gave values of percent fusion
similar to the standard preparation.

When the fusion mixtures were left at 37°C for 24 hours
(1.3 cell doubling times), the percentage fusion by the
fast sedimenting virus decreased from 75% to 32% (Figure 4);
this is probably due to the fact that the majority of the
multinucleated cells did not divide during the 24 hour
period, in contrast to the mononucleated cells.

The fast sedimenting virus not only induced more
heterokaryons but also larger ones, some containing more
than 10 nuclei per cell (Figure 5). The average number of
nuclei/cell was found to be 3.9 for the fast sedimenting
virus, and 1.4 for the standard virus. An analysis of the
average number of nuclei/multinucleates versus the percen-
tage fusion in different virus fractions obtained by
sucrose-D_2O gradient centrifugation and for different
virus concentrations revealed a rough correlation between
the average number of nuclei per multinucleate cell and
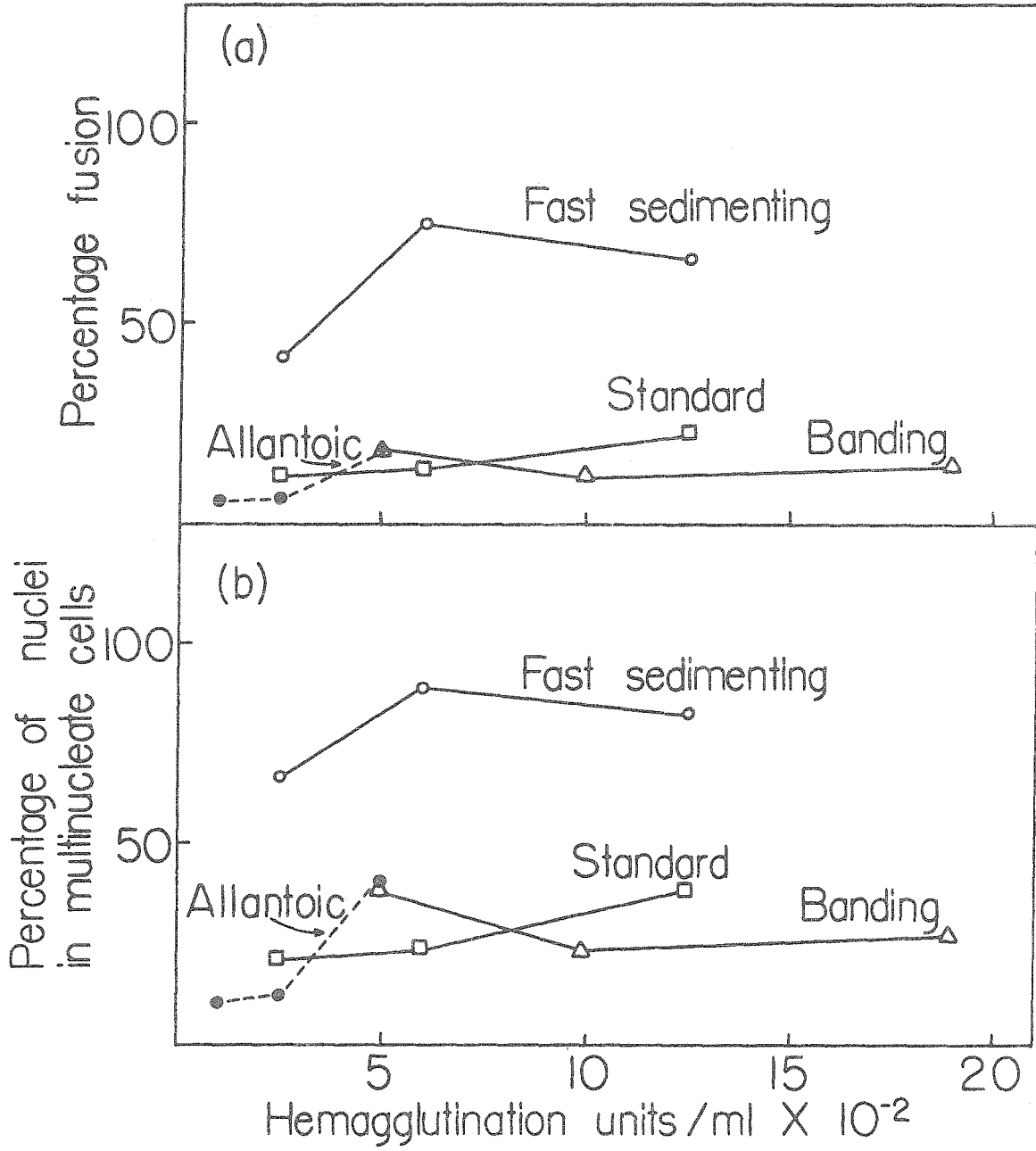the percentage fusion (Figure 6).

Figure 3

LEGEND TO FIGURE 3

The extent of heterokaryon formation in mouse C11D cells 2.5 hours after fusion with Sendai virus.

C11D cells, grown in monolayer, were trypsinized, washed with Eagle's medium plus 10% calf serum and then mixed, at 10^6 cells/ml, with different amounts of the UV-inactivated Sendai virus in 0.2 ml of Eagle's medium (adjusted to pH 8 as detailed in Materials and Methods), incubated without shaking at 0°C for 20 minutes, and then shaken at 37°C for 15 minutes. The cells were subsequently incubated on coverslips in the 37°C CO_2 incubator for 2.5 hours in Eagle's medium with 10% calf serum, fixed in methanol, stained with May-Grünwald-Giemsa and examined by phase contrast microscopy.

The percentage of cells which contained more than one nucleus, called the percentage fusion, is shown in Figure 3a at several concentrations of UV-inactivated Sendai virus from four different sources: (i) allantoic fluid with red blood cells removed, (ii) a standard preparation obtained by differential centrifugation, (iii) material banding in the sucrose gradients, and (iv) material pelleting against the sucrose cushion of the gradient (fast sedimenting).

The percentage of the nuclei found in heterokaryons was also determined for each fusion mixture (Figure 3b).

(LEGEND TO FIGURE 3 CONTINUED)

o——o, fast sedimenting virus (fraction 4);

Δ——Δ, banding virus (fraction 2);

□——□, standard virus preparation;
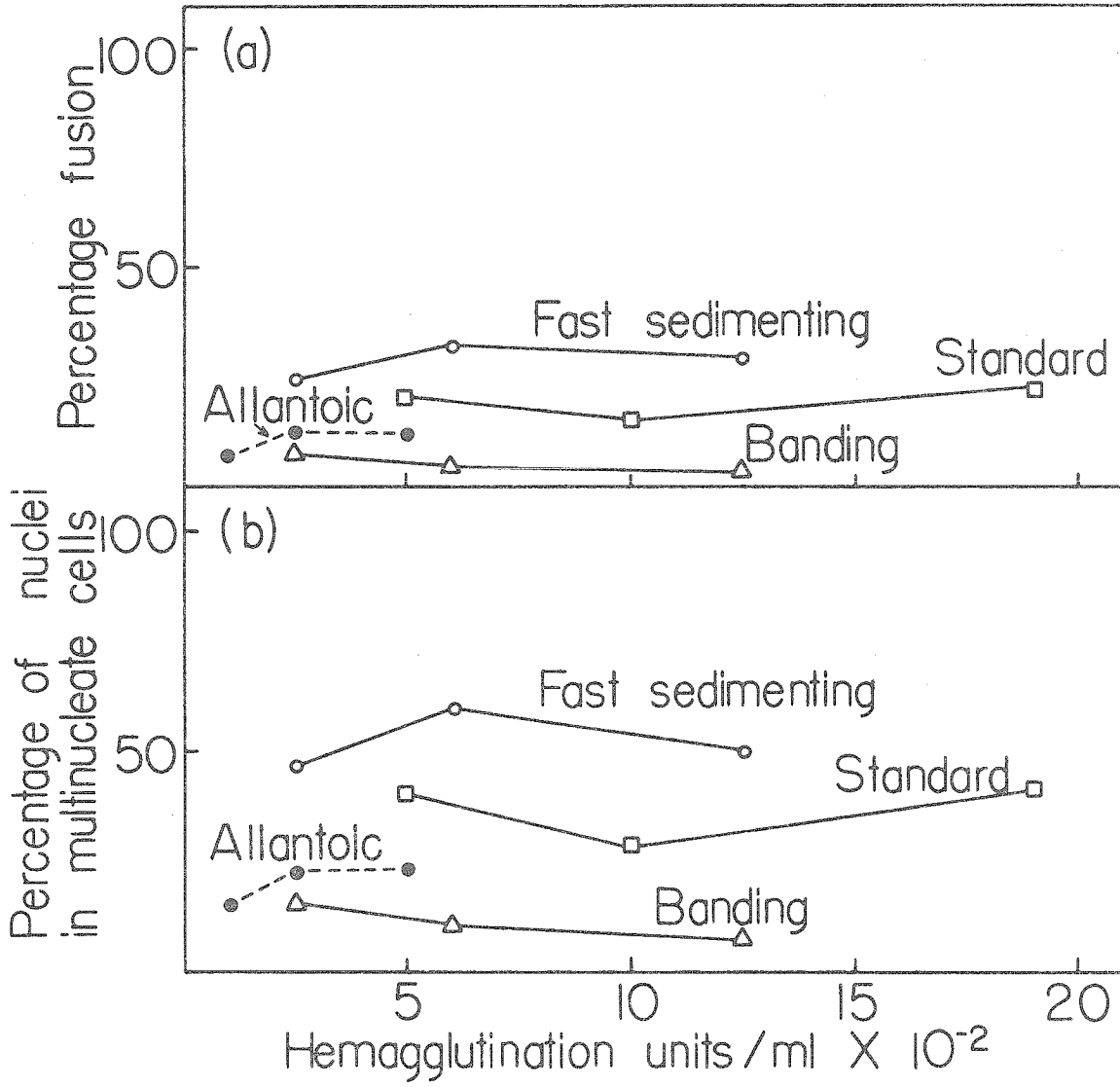
●——●, allantoic fluid.

Figure 4

LEGEND TO FIGURE 4

The extent of heterokaryon formation in C11D mouse cells 24 hours after fusion with Sendai virus.

The samples are identical to those of Figure 3 except that after fusion the cells were incubated at 37°C for 24 hours before fixation.

o———o, fast sedimenting virus (fraction 4); Δ———Δ, banding virus (fraction 2); □———□, standard virus preparation; ●----●, allantoic fluid.
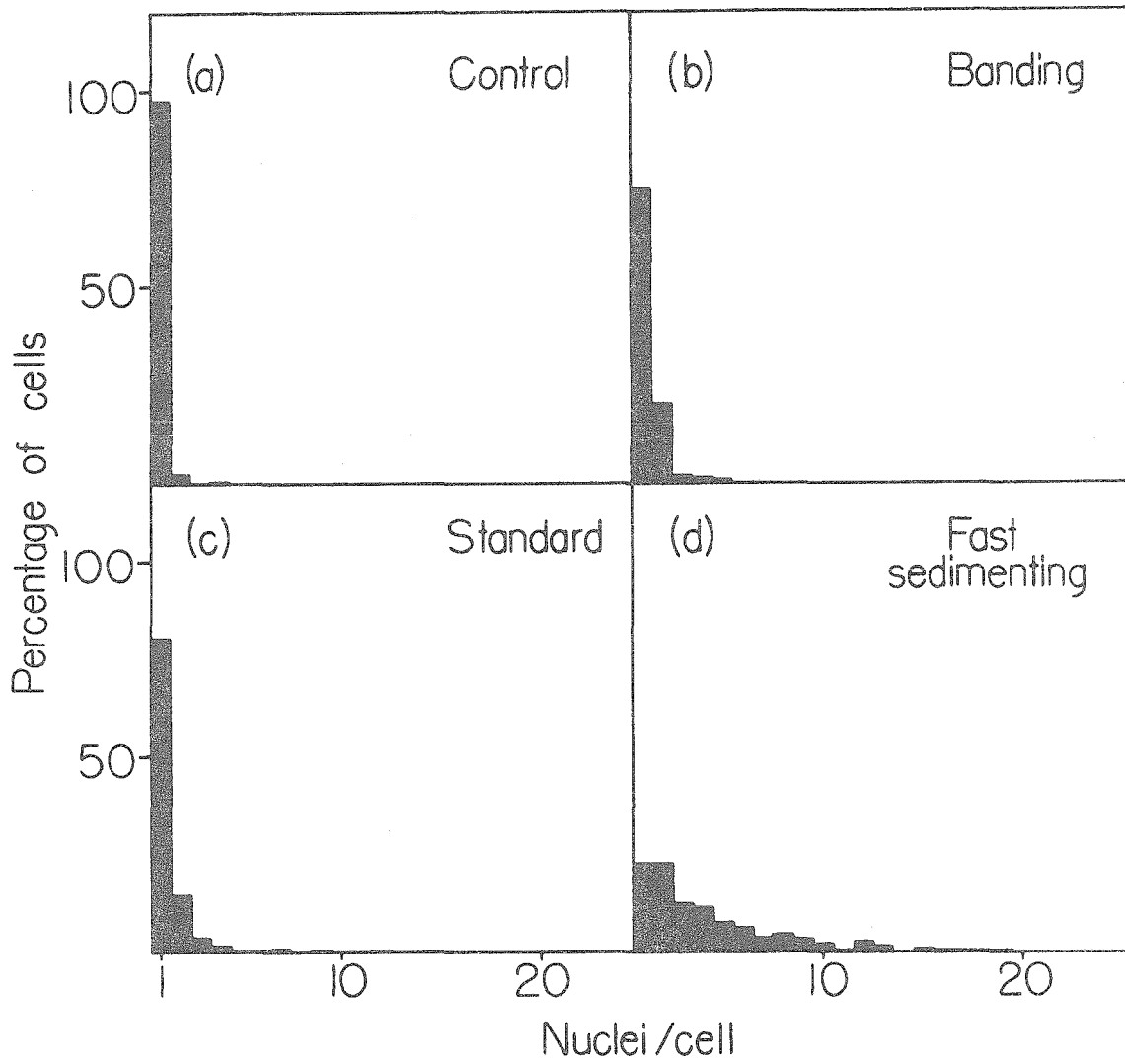
Figure 5

LEGEND TO FIGURE 5

Distribution of the number of nuclei per cell in

C11D cells after Sendai virus-induced heterokaryon

formation.

The percentage of the cell population is plotted

against the number of nuclei per cell in the fusion

mixtures of the experiment described in Figure 3, using

standard (c), banding (b) and fast sedimenting (d) virus.

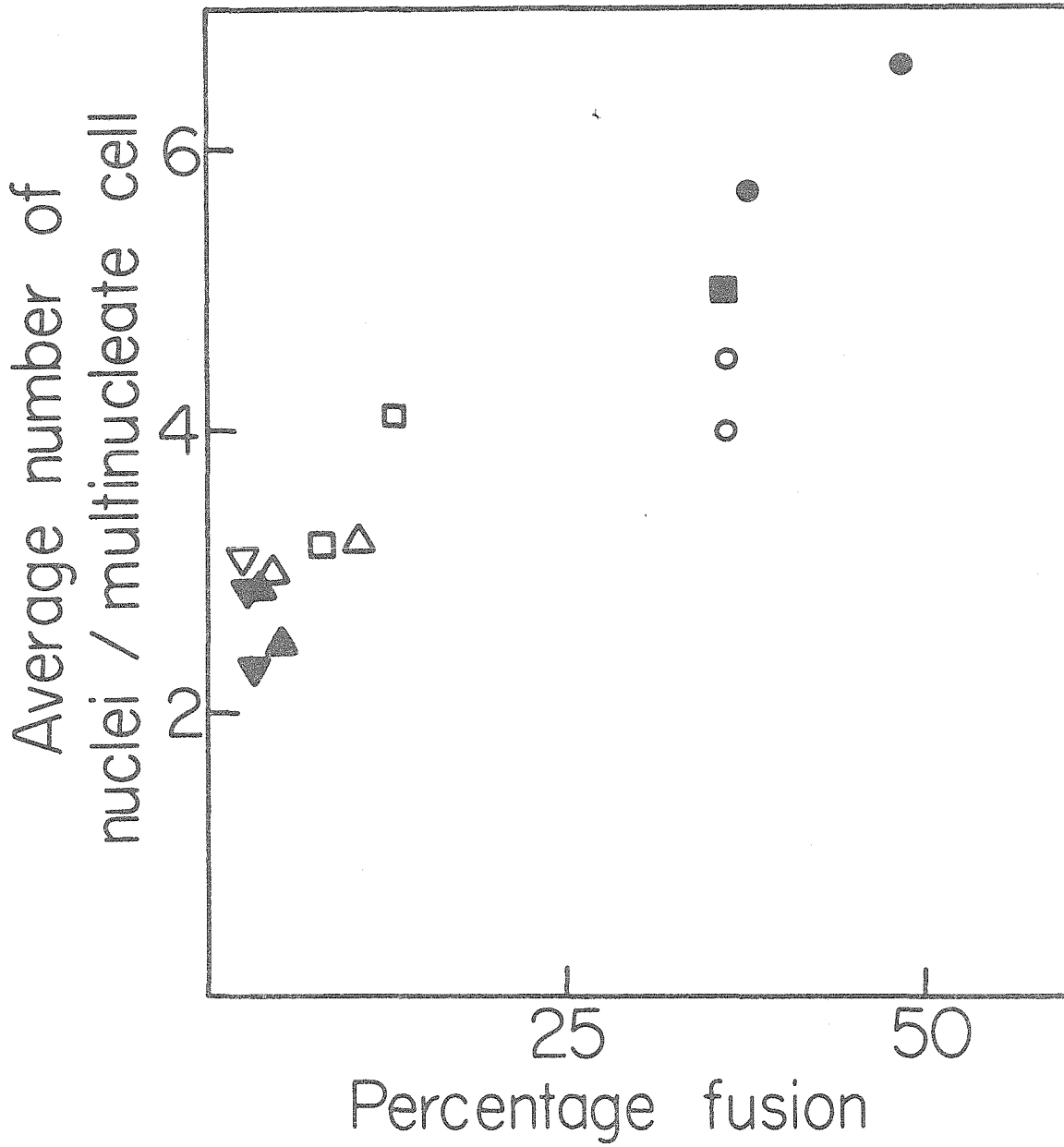The control mixture (a) contained no virus.

Figure 6

LEGEND TO FIGURE 6

Relationship between average number of nuclei per cell in multinucleated cells and percentage fusion.

The average number of nuclei per multinucleate cell is plotted as a function of the percentage of cells which contained more than one nucleus (percentage fusion).

Table 1 summarizes the data on the percentage fusion
and cell survival, as determined by Trypan Blue exclusion,
of ClID cell preparations exposed to different fractions
of virus sedimented through sucrose-D_2O gradient. The
data refer to tests carried out after storing the virus
fractions at 4°C for 4 or 30 days or storing them for 30
days at -50°C. The increase in cell fusion capacity in
fraction 3 (collected in the region of the gradient between
banding virus and fast sedimenting virus) and in fraction 4
(fast sedimenting virus) as compared to the banding virus
(fraction 2) and the virus collected in the upper portion
of the gradient (fraction 1), is clearly illustrated by
the data in the table. No obvious effect of the storage
conditions on cell fusion capacity of the various fractions
was observed. Most batches of inactivated virus increased
in their hemolytic capacity with extended storage at -50°C,
losing their usefulness for cell fusion studies in 3-12
months.

(d)   Pilot experiment: Rate of labeling of the two

parental mit-DNAs in G1/G2 heterokaryons

At an early stage during the development of the tech-
niques described above, the pilot experiment described
here was performed to test the feasibility of the experi-
mental design discussed in the Introduction of this Chapter.

TABLE 1

EFFECT OF AGING AND FREEZING OF SENDAI VIRUS

ON CELL FUSION AND CELL KILLING

	Day	Control (No Virus)	Standard Virus	Sucrose Gradient Fractions			
				Fraction 1	Banding	Fraction 3	Fast Sedimenting
Percentage Cell Survival	4	100	97	110	84	99	61
	30	100	74			86	80
	30*	100	56			100	94
Percentage Fusion	4	2.8	8.1	5.4	2.6	29.8	19.8
	30	3.6	4.0			36.0	48.1
	30*	3.6	10.6			8.0	36.4
Percentage of nuclei in multi-nucleate cells	4	10.3	18.6	14.1	6.2	60.1	46.2
	30	8.0	10.6			73.9	85.9
	30*	8.0	27.4			21.9	69.7
Average number of nuclei per cell in multi-nucleate cells	4	2.3	2.6	2.8	2.5	3.5	3.5
	30	2.3	2.9			5.0	6.6
	30*		3.2			3.2	4.0

*Frozen

(TABLE 1 CONTINUED)

The standard virus was obtained by differential centrifu-
gation of allantoic fluid from innoculated chicken eggs. A
portion of this preparation was sedimented through a sucrose
gradient, which was fractionated by eye into fraction 1 ($\sim$2 cm
layer near the top of the centrifuge tube), fraction 2 (visible
banding virus), fraction 3 (next $\sim$2 cm layer), and fraction 4,
the bottom ($\sim$2 cm) portion of the tube (fast sedimenting
virus). Virus preparations were stored until used either for
4 or 30 days at 4°C, or for 30 days at -50°C. Monolayer C11D
cells were harvested by trypsinization, washed in Eagle's
medium without calf serum and resuspended in pH 8 Eagle's
medium (see Section 2d). Cell fusion was carried out in
12 x 75 mm polystyrene tubes in 0.2 ml of pH 8 Eagle's medium
containing 4 x 10^6 C11D cells and UV inactivated Sendai virus
at various concentrations, prepared as described in Section
2c. The fusion mixtures were placed on ice for 20 minutes,
then shaken for 15 minutes at 37°C. One portion of each mix-
ture was incubated with an equal volume of 0.2% Trypan Blue
at 37°C for 10 minutes, and the percentage of cells not ab-
sorbing the dye (viable) was determined (approximately 100
cells were counted). The remainder of each mixture was dis-
pensed onto a coverslip in a petri dish with Eagle's medium
supplemented with 10% calf serum, which was placed in a 37°C
CO_2 incubator for 2.5 hours. The cells attached to the
coverslips were fixed in methanol, stained with May-Grünwald-

(TABLE 1 CONTINUED)

Giemsa, and mounted in Depex. For each slide, the per-
centage fusion, the percentage of nuclei observed in multi-
nucleate cells, and the average number of nuclei per cell
in multinucleate cells were determined (approximately 1000
cells were analyzed).

To determine the relative rates of [methyl-^{3}H]thymidine incorporation into the two parental mit-DNAs in G1 phase/G2 phase heterokaryons of C11D cells, the procedure diagrammed in Figure 7 was followed. Two cell populations, which had been grown in monolayer cultures, were synchronized by selective detachment, so that the detached cells would have been in the incubator for 2.8 hours ("G1" population) or 15.8 hours ("G2" population), respectively, by the beginning of the cell fusion step. The cell population from which the G2 cells were derived had been grown for more than 10 generations in the presence of 30 µg BrdU/ml and the G1 cells were maintained up to the time of fusion in the presence of the analogue.

The synchronized G1 and G2 populations were divided into three mixtures for cell fusion, one with equal numbers of cells from the G1 and G2 populations and two control mixtures with either G1 or G2 population cells. A sample of each fusion mixture was examined, after 2 hours at 37°C, by May-Grünwald-Geimsa staining. 52-55% of the nuclei were found to be in multinucleate cells and from the distribution of the number of nuclei per multinucleate cell it could be estimated that if G1 and G2 cells fuse to each other as readily as among themselves, then 36-38% of the nuclei were in mixed heterokaryons. The fusion mixtures were suspended in fresh medium, incubated for 2 hours in the 37°C CO_2 incubator and then labeled with [5-^{3}H]deoxycytidine for

PROTOCOL OF A G1/G2 CELL FUSION EXPERIMENT
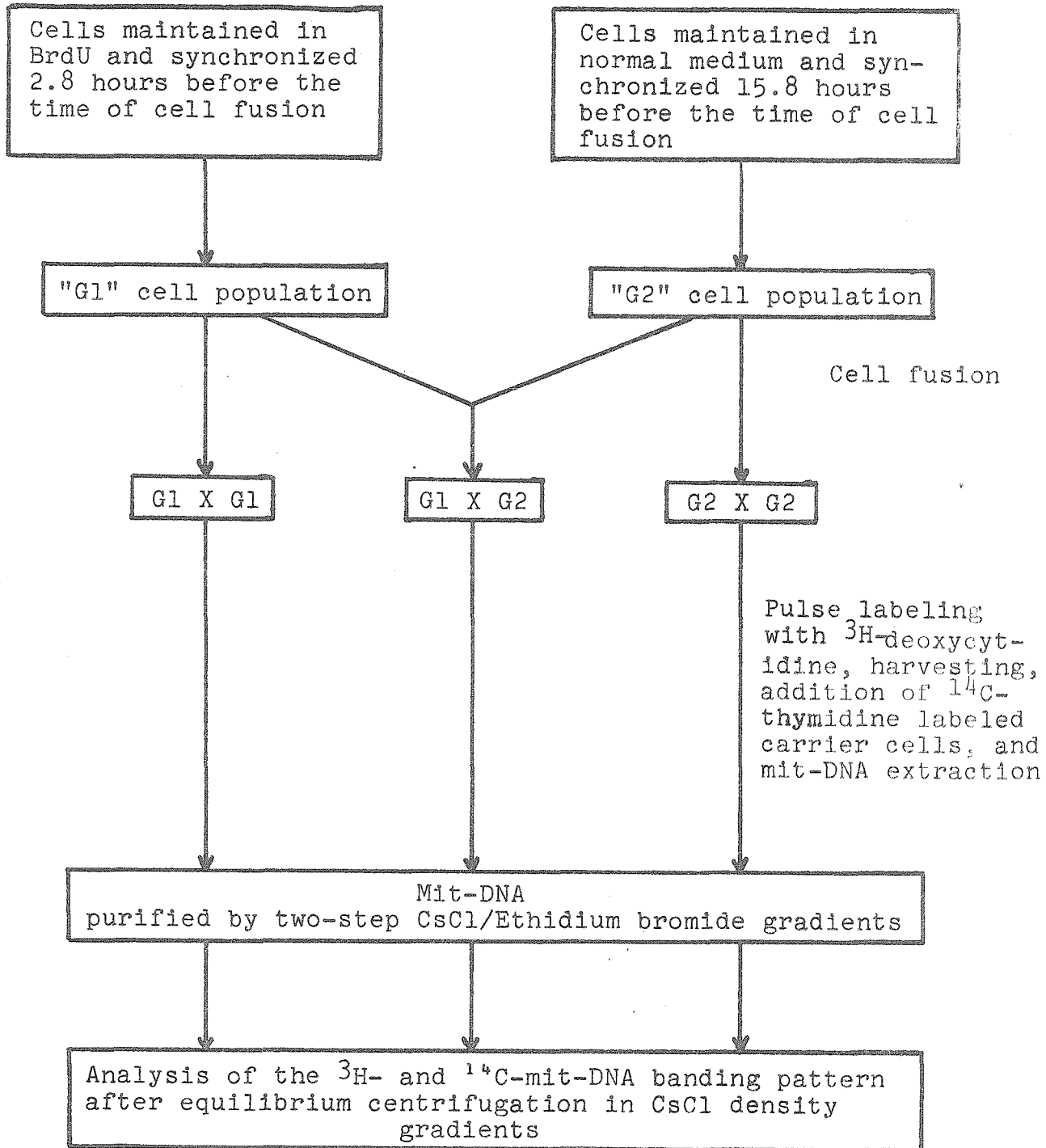
WITH C11D CELLS

Figure 7

75 minutes. The cells of each fusion mixture were harvested,
mixed with a constant amount of cells labeled with
$[2-^{14}C]$thymidine in the absence of BrdU for the purpose
of correcting for variations in the yield of mit-DNA during
the extraction procedure and to provide a marker mit-DNA in
CsCl density gradients. Mit-DNA was extracted from DNase-
treated mitochondrial fractions and sedimented through
two-step CsCl/ethidium bromide gradients to purify mit-DNA
from nuclear DNA fragments. Fractions containing mit-DNA
were pooled, the dye was removed, and the mit-DNA was
centrifuged to equilibrium in CsCl density gradients. The
amount of ^{3}H- and ^{14}C-DNA in each fraction was determined.

The distribution of ^{3}H- and ^{14}C-mit-DNA in the CsCl
equilibrium gradient containing mit-DNA from the G1/G1 fusion
mixture is shown in the left portion of Figure 8a. The
unsubstituted ^{14}C-mit-DNA density marker has a ρ_{av} of
1.69 g/cm^3. As is shown in the right portion of Figure 8a,
where the ^{3}H-labeled material is decomposed into "light"
(unsubstituted) and "heavy" DNA on the basis of the pattern
of ^{14}C-mit-DNA (as described in the Legend). The ^{3}H-mit-DNA
is all, or almost all, partially BrdU substituted DNA
(ρ_{av} = 1.70 g/cm^3). In particular, 91% of the ^{3}H-mit-DNA
is clearly BrdU substituted, as expected for G1 population
cells. The 9% of ^{3}H-mit-DNA which appears as "light" DNA
by the decomposition applied here may in fact be substituted
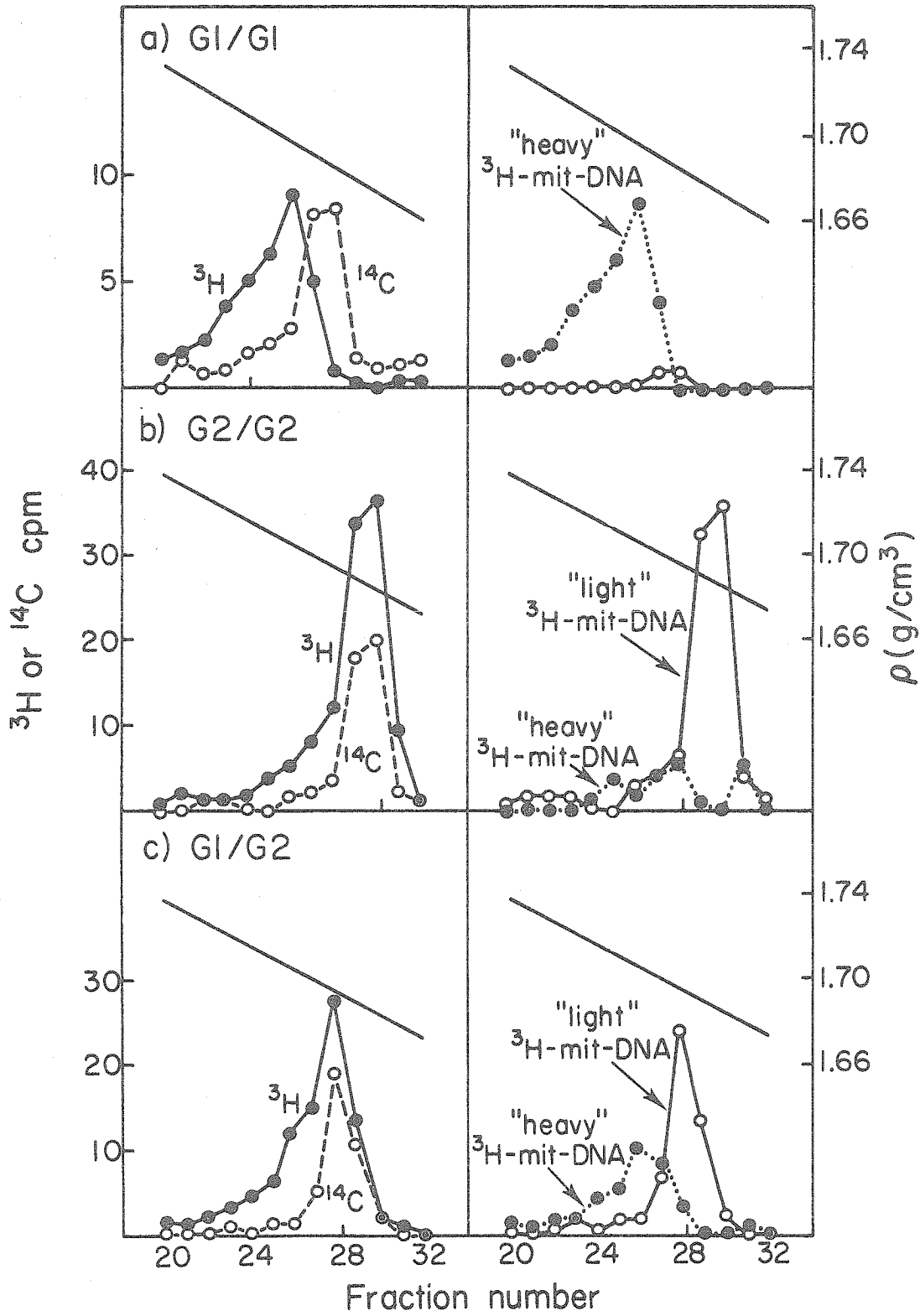DNA, since the modest density separation between substituted

Figure 8

LEGEND TO FIGURE 8

Density banding pattern in CsCl equilibrium gradients of ^{3}H-mit-DNA labeled in G1/G1 (a), G2/G2 (b) and G1/G2 (c) cell population mixtures after cell fusion.

To obtain the G1 population, one culture of C11D cells was grown in monolayer in the presence of 30 µg BrdU/ml for more than 10 generations and a synchronized population was produced by selective detachment 2.8 hours prior to the cell fusion step. To obtain the G2 population, a second culture of C11D cells was grown in monolayer in the absence of BrdU for many generations and a synchronized population was produced by selective detachment 15.8 hours prior to the cell fusion step. Portions of these two cell populations were divided into 3 tubes containing G1 population cells only, G2 population cells only, and a mixture of equal numbers of G1 and G2 population cells. Cell fusion was induced with fast sedimenting fraction Sendai virus and the fusion mixtures were incubated at 37°C for 2 hours. Each mixture was then exposed to [5-^{3}H]deoxycytidine (200 µCi/ml, 24 Ci/mmole, Amersham) for 75 minutes; the cells were harvested and each mixture was combined with cells ($\sim$2 x 10^7) labeled for 2 days with [2-^{14}C]thymidine (15 nCi/ml, 61 mCi/mmole, Amersham). The mitochondrial fractions were isolated, treated with DNase and RNase, lysed in 1% SDS, pronase digested, brought to 1 M CsCl and

LEGEND TO FIGURE 8 (CONTINUED)

centrifuged to pellet precipitate thus formed. The resul-
ting supernatants were layered on two-step CsCl/ethidium
bromide gradients, centrifuged at 38,000 rpm for 5 hours
in a Beckman SW65 rotor. The fractions corresponding to
closed and open circular mit-DNA were pooled, and after
removal of the dye, centrifuged in a CsCl solution (density
1.73 g/cc) at 30,000 rpm for 3 days in the Beckman SW65
rotor. The amount of ^{3}H ($\bullet$——$\bullet$) and ^{14}C (o---o) radio-
activity in each fraction was determined. The radioactivity
values for the fractions in the region of the peaks are
shown in the left portion of each panel in Figure 8: (a)
mit-DNA from the G1/G1 fusion mixture, (b) mit-DNA from
the G2/G2 fusion mixture and (c) mit-DNA from the G1/G2
fusion mixture. The refractive index was read, converted
to CsCl density and the line fit to all fractions measured
is shown (——).

In the right portion of each panel the profile of
^{3}H-mit-DNA was decomposed into a component with the density
pattern of unsubstituted ("light") mit-DNA ($\bullet$——$\bullet$) and a
heavier DNA component (o$\cdot\cdot$o). This decomposition was per-
formed as follows: (1) of the two fractions in the ^{14}C-
mit-DNA pattern with the highest cpm, the fraction with the
lower CsCl density was chosen, (2) it was assumed that all
^{3}H cpm in that fraction were due to unsubstituted ("light")

LEGEND TO FIGURE 8 (CONTINUED)

mit-DNA and that the pattern of this unsubstituted mit-DNA in various fractions was identical to that in the ^{14}C pattern, (3) the remaining ^{3}H cpm was designated as heavier ("heavy") mit-DNA.

and unsubstituted mit-DNA of C11D monolayer cells (discussed above) results in significant overlapping of the density profiles. In the case of mit-DNA from the G2/G2 fusion mixture (Figure 8b, left), most of the ^{3}H-mit-DNA and ^{14}C-mit-DNA marker has a ρ_{av} of 1.69 g/cm^3, as expected for G2 population cells. After decomposition (Figure 8b, right), a portion (17%) of the ^{3}H-mit-DNA appears as "heavy" DNA. This material is probably in good proportion transcription complexes of mit-DNA (Carré and Attardi, 1978) and replicative intermediates but the contribution of nuclear DNA contaminant (which has a higher density in CsCl than mit-DNA) cannot be excluded. For the purpose of the calculation made below, this material has been considered to be mit-DNA. The ^{3}H-mit-DNA from the G1/G2 fusion (Figure 8c, left) shows a distribution which to a great extent corresponds to that of the light mit-DNA. This distribution can be decomposed, as described above (Figure 8c), into "light" mit-DNA (ρ_{av} = 1.69 g/cm^3 of "G2" cell population origin) and "heavy" mit-DNA (ρ_{av} = 1.70 g/cm^3 of "G1" cell population origin).

Table 2A shows, for the mit-DNA from each fusion mixture, the amount of substituted ^{3}H-mit-DNA and of unsubstituted ^{3}H-mit-DNA, as determined by the decomposition applied here, each normalized for 100 cpm of total ^{14}C-mit-DNA in the gradient. From the data in Table 2A, it can be determined that the relative rates of

TABLE 2

LABELING OF MIT-DNA IN CELL CYCLE PHASE HETEROKARYONS

A. Decomposition of mit-DNA into "light" (non-BrdU-
    substituted) and "heavy" DNA based on CsCl gradient
    banding patterns

Cell mixture labeled after fusion	Density banding pattern[3] (^{3}H cpm/100 ^{14}C cpm)	
	"Heavy" mit-DNA	"Light" mit-DNA
G1/G1	104	11
G2/G2	37	178
G1/G2	84	128

B. Estimate of the origin of ^{3}H-mit-DNA in labeled fusion
    mixtures

Cell mixture labeled after fusion	Parental origin of ^{3}H-mit-DNA (^{3}H cpm/100 ^{14}C cpm)	
	"G1" population	"G2" population
G1/G1	115	0
G2/G2	0	215
G1/G2	65	147
G1/G2 prediction for a mixture of G1 and G2 cells without fusion	58	108

[3]Observed ^{3}H cpm were normalized to ^{3}H cpm per 100 ^{14}C
  cpm on the gradient. Actual ^{14}C cpm were 30-50 cpm
  on each gradient.

(TABLE 2 CONTINUED)

The values in Table 2A are determined from the ^{3}H-mit-DNA patterns in CsCl equilibrium density gradients of mit-DNA labeled in G1/G1, G2/G2 and G1/G2 fusion mixtures shown in Figure 8. From the decomposition of the ^{3}H-mit-DNA patterns, shown on the right side of each panel, the amount of radioactivity in the "unsubstituted" mit-DNA pattern and the heavier "substituted" mit-DNA patterns were determined and expressed as ^{3}H cpm in the appropriate fractions per 100 ^{14}C cpm on the entire gradient (actual ^{14}C cpm were 30-50 cpm on each gradient).

As mentioned in the Results section, the "light" ^{3}H material from the G1/G1 mixture (11 ^{3}H cpm/100 ^{14}C cpm; 9.2% of the ^{3}H-DNA) is an artifact due to the decomposition of two overlapping patterns. Similarly, the "heavy" ^{3}H material from the G2/G2 mixture (37 ^{3}H cpm/100 ^{14}C cpm; 17% of the ^{3}H-DNA) is probably for the most part RNA-DNA complex or replicative intermediates of unsubstituted DNA. All of the ^{3}H-mit-DNA in the G1/G1 fusion mixture is due to G1 mit-DNA (115 ^{3}H cpm/100 ^{14}C cpm) and all ^{3}H-mit-DNA in the G2/G2 fusion mixture is due to the G2 mit-DNA (215 ^{3}H cpm/100 ^{14}C cpm), as shown in Table 2B.

The relative contributions of "G1" population substituted mit-DNA and "G2" population unsubstituted mit-DNA to the ^{3}H-mit-DNA pattern of the G1/G2 fusion mixture were

determined by solving two simultaneous equations:

$$ax + by = c \qquad \text{and}$$

$$dx + ey = f$$

where $x = {}^{3}H$ cpm due to the G1 population mit-DNA per 100 ${}^{14}C$ cpm on the G1/G2 equilibrium gradient, $y = {}^{3}H$ cpm due to the G2 population mit-DNA per 100 ${}^{14}C$ cpm on the G1/G2 equilibrium gradient, $a=0.908$, $b=0.17$, $c=83.8$ ${}^{3}H$ cpm/100 ${}^{14}C$ cpm, $d=0.092$, $e=0.83$ and $f=128.1$ ${}^{3}H$ cpm/100 ${}^{14}C$ cpm. The solution is $x=65$ ${}^{3}H$ cpm/100 ${}^{14}C$ cpm and $y=147$ ${}^{3}H$ cpm/ 100 ${}^{14}C$ cpm (Table 2B). The prediction for the ${}^{3}H$-mit-DNA of a mixture of G1 and G2 population cells without fusion is calculated as one-half of the ${}^{3}H$ cpm seen in the G1/G1 fusion mixture plus one-half of the ${}^{3}H$ cpm seen in the G2/G2 fusion mixture.

$[5-^3H]$deoxycytidine incorporation into mit-DNA of G1/G1 and G2/G2 fusion mixtures were 115 and 215, in units of ^3H cpm/100 ^{14}C cpm (Table 2B). This apparent increase, by a factor of two, in the rate of incorporation of precursor into mit-DNA of G2/G2 fusion mixtures relative to the G1/G1 fusion mixture is in good agreement with the doubling over one generation seen in the rate of $[5-^3H]$deoxycytidine incorporation into mit-DNA of C11D cells in the absence of cell fusion (Chapter III, this thesis, Figure 3).

As mentioned above, a small fraction of the ^3H-mit-DNA from the G1/G1 cell fusion appears to be possibly unsubstituted DNA due to the overlap in the density patterns of substituted and unsubstituted mit-DNA, and a fraction of the ^3H-mit-DNA from the G2/G2 cell fusion bands at the density of substituted DNA. Therefore, the amounts of ^3H-mit-DNA from the G1/G2 cell fusion which are of G1 or G2 origin was determined by solving two simultaneous equations, as described in Table 2. 65 and 147 ^3H cpm/100 ^{14}C cpm were estimated to be due to G1 and G2 populations, respectively. If G1 and G2 population cells had been mixed without cell fusion, it would be predicted that 58 and 108 ^3H cpm/100 ^{14}C cpm would be due to G1 and G2 population cells, respectively. Further work is needed to establish if these differences are significant.

4. Discussion

An approach for the study of the regulation of cell cycle dependent mit-DNA synthesis in mammalian cell hetero-karyons has been presented here. It has been shown that the technical requirements of the approach, as mentioned above, can be satisfied. In particular (1) the cells could be fused so as to generate a high proportion of hetero-karyons using fast sedimenting Sendai virus, (2) the mit-DNAs of the two parental cells were distinguishable in density when one cell line was grown in BrdU under the appropriate conditions, and (3) the cells could be well synchronized by selective detachment.

The planned approach requires extensive heterokaryon formation between two populations in different stages of the cell cycle. The fraction of a Sendai virus preparation with a higher sedimentation coefficient than single virus particles was found to be capable of inducing fusion resulting in 50-90% of the nuclei to be contained in multi-nucleated cells. It has been shown by Rao and Johnson (1970) that G1/G2 heterokaryons are induced by Sendai virus; Stadler and Adelberg (1972), however, have presented evidence suggesting that the extent of heterokaryon formation may vary depending upon the cell cycle stage of the cells used. It remains to be determined, therefore, for the cell lines utilized here, what the relative amounts of G1/G1,

G2/G2 and G1/G2 heterokaryons are in a mixture of G1 and G2 cells treated with Sendai virus. It has recently been demonstrated that polyethylene glycol is an effective and versatile cell fusion agent (Davidson and Gerald, 1976); if it proves to be satisfactory for the experiments discussed here, the problems of increasing cytotoxicity of fast sedimenting Sendai virus with age could be avoided.

C11D cells were initially chosen for the present experiments because of the possibility of labeling preferentially their mit-DNA with BrdU resulting from the absence of the cytoplasmic thymidine kinase. C11D monolayer cells can be well synchronized by selective detachment (see Chapter III, this thesis); however, as observed in the G1/G2 pilot fusion experiment with monolayer cells, there is significant overlapping in density between unsubstituted and BrdU substituted mit-DNA, making the determination of the parental origin of mit-DNA difficult. The limited extent of BrdU substitution in monolayer C11D cells was unexpected, because extensive substitution had been demonstrated previously (Attardi and Attardi, 1972); further experiments demonstrated that there was a difference in extent of BrdU substitution in mit-DNA between cells grown in monolayers and suspension cultures. The nature of this effect of the growth conditions on deoxyribonucleotide metabolism is not presently understood.

It was shown here that C11D cells could be grown for

many generations in suspension culture in the presence of BrdU, transferred to roller bottles (monolayers) for 1-2 days and still retain a 0.3-0.4 g/cm^3 difference in density of mit-DNA relative to unsubstituted mit-DNA; at this time after the transfer the cultures could be synchronized by selective detachment.

Cl1D cells have the disadvantage, however, that there is a low level of labeling of mit-DNA in the presence of ^{3}H-thymidine and ^{3}H-deoxycytidine; this restricts the accuracy of further analysis, due to the limited number of cells obtainable by selective detachment. Likewise, it was subsequently determined that the level of mitochondrial TTP and dCTP pool labeling is not high enough to make determination of Cl1D mitochondrial TTP and dCTP specific activities possible in selectively detached Cl1D cells (see Chapter III, this thesis); this makes the mit-DNA labeling data more difficult to interpret. Because of the ease of labeling of mit-DNA and precursor pools in A9 mouse cells and the clearly measurable cell cycle changes in the rate of A9 mit-DNA replication (Chapter III, this thesis), A9 cells appear to be a good choice to replace Cl1D cells in this approach. A9 cells have been shown to grow at a normal rate for 2 days in the presence of BrdU (Attardi et al., 1975).

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