BIOCHEMICAL AND GENETIC APPROACHES TO THE STUDY OF MITOCHONDRIAL BIOGENESIS IN MAMMALIAN CELLS

by

ROBERT BRUCE WALLACE, B.Sc.

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AUTHOR: Robert Bruce Wallace, B.Sc. Hons. (Simon Fraser University)

SUPERVISOR: Dr. K.B. Freeman, Professor and Chairman, Biochemistry

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ABSTRACT

I. A mammalian cell mutant of the Chinese hamster ovary cell line (designated tsH1) was shown by Thompson et al (1973) to have a temperature-sensitive leucyl-tRNA synthetase. At 40°C, a non-permissive temperature, protein synthesis continued at about 1% of the rate at 34°C, a permissive temperature. The possibility that this synthesis represented mitochondrial protein synthesis was confirmed by the following observations:

a) the protein synthesis at 40°C, but not at 34°C, was sensitive to tevenel, a specific inhibitor of mitochondrial protein synthesis;

b) protein synthesis by isolated mitochondria from tsH1 was not temperature-sensitive;

c) [^{14}C]leucine incorporation at 40°C was located specificially in the mitochondrial fraction;

d) [^{14}C]leucine incorporation into the mitochondrial fraction of tsH1 at 40°C was sensitive to tevenel but not cycloheximide;

e) the pattern of mitochondrial proteins synthesized at 40°C on SDS-polyacrylamide gels is very similar to those synthesized at 34°C in the presence of the inhibitor of cytosolic protein synthesis, cycloheximide.

These results, together with the demonstration of three species of leucyl-tRNA in hamster liver mitochondria different from the four cytosolic species (by RPC-5 chromatography), suggest that tsH1 mito-

chondria contain a unique, non-temperature-sensitive leucyl-tRNA synthetase. This cell line may prove to be very useful in the analysis of mammalian mitochondrial protein synthesis in the absence of inhibitors.

II. Interspecies somatic cell hybrids offer a unique system for the study of interactions between mitochondria and the nucleus. This is possible because one can distinguish mitochondrial components of the parental species, as well as the chromosomes of the parental species. In an attempt to characterize the differences between hamster mitochondrial DNA (mtDNA) and human mtDNA, a comparison of buoyant densities of the two mtDNAs was made. It was found that human mtDNA was more dense than hamster mtDNA by 0.008 g/cc. One human-hamster hybrid cell was examined and found to have mtDNA with a density similar to hamster mtDNA. This result, together with observations reported previously from this laboratory, should now allow the analysis of human-hamster hybrid cells for the maintenance and expression of mtDNA from the parental species.

Protein synthesis in mammalian cell mitochondria is initiated with fMet-$tRNA_f^{met}$, the formyl group being most likely donated by N^{10}-formyl H_4 folate. Methotrexate, which depletes the C_1-H_4 folate pools by inhibiting dihydrofolate reductase, might block the formylation reaction, inhibiting mitochondrial protein synthesis and indirectly cell growth. L cells grown in suspension culture were found to grow normally in the presence of 5×10^{-5} M methotrexate when supplied with metabolites whose synthesis requires the C_1-H_4 folate pools. Preincubation for 15 min with 10^{-4} M methotrexate does not affect the subsequent formylation of mitochondrial initiator tRNA. Also, there is no effect on the

synthesis of N-formylmethionylpuromycin in L cells grown for 160 hr in the presence of 5×10^{-6} M methotrexate, nor in KB cells grown for 72 hr in its presence. These results demonstrate that cell growth, the formylation of mitochondrial initiator tRNA and the initiation of mitochondrial protein synthesis are not inhibited by methotrexate when cells are supplied with metabolites whose *de novo* synthesis requires C_1-H_4 folate pools. The mechanism of continued formylation in the presence of methotrexate is not known.

III. The thymidine kinase deficient, LMTK$^-$ cell line incorporates BUdR specifically into mtDNA. The possibility that this specific incorporation of BUdR would induce mutations of the mitochondrial genome was investigated. Preliminary evidence suggests BUdR is effective in increasing the frequency of tevenel resistant cells, however, a large fraction of the LMTK$^-$ cell population studied ($4 - 9 \times 10^{-5}$) shows resistance to tevenel without BUdR treatment and these cells have been chosen for study.

After growth in suspension culture for 5 days in 100 µg tevenel/ml and subsequent plating in 100 µg tevenel/ml, LMTK$^-$ cells yielded resistant clones. As a control, L cells treated identically, yielded no clones. Three of the resistant clones were studied. Each resistant cell line had an identical growth rate in the presence and absence of 100 µg tevenel/ml; whereas, LMTK$^-$ ceased growing after 2 or 3 generations in 100 µg tevenel/ml. By plating efficiency analysis the resistant cells were found to be cross-resistant to D-chloramphenicol. The change responsible for resistance was found to be stable for at least 100 generations in the absence of the drug.

Protein synthesis by isolated mitochondria of resistant cells was found to be less inhibited by concentrations of both tevenel and D-chloramphenicol up to 200 μg/ml than the protein synthesis by LMTK$^-$ mitochondria. This resistance *in vitro* was not changed by incubation of the mitochondria in 0.01% Triton X-100.

These results suggest, but do not prove, that the resistant cells are mutants of the mitochondrial genome and that LMTK$^-$ cells may be important in the isolation of other mutants of mitochondrial function.

DEDICATION

To my wife Lynda, without whose love and willingness to sacrifice this work could never have been completed.

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LIST OF ABBREVIATIONS

A	adenosine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BUdR	5-bromo,2'deoxyuridine
C	cytidine
C_i	curie
C_1-H_4 folate	one carbon-tetrahydrofolate derivative
CPM	counts min^{-1}
CTP	cytosine triphosphate
cytmRNA	cytosolic messenger ribonucleate
cytrRNA	cytosolic ribosomal ribonucleate
DEAE	diethylaminoethyl
DNA	deoxyribonucleate
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetate
fMet	N-formylmethionine
fMet A	N-formylmethionyladenosine
fMet-puromycin	N-formylmethionylpuromycin
g	relative centrifugal force (1 g = 980 cm sec^{-2})
G	guanosine

g/cc	gram cm^{-3}
H-strand	heavy strand
HAT-medium	hypoxanthine, amethopterin (methotrexate), thymidine containing medium
HT-medium	hypoxanthine, thymidine containing medium
HGPGT	hypoxanthine guanine phosphoribosyl transferase
L-strand	light strand
MAK	methylated albumin kieselguhr
MEM	minimum essential medium
Met	methionine
Met A	methionyladenosine
Met-puromycin	methionylpuromycin
Met-$tRNA_f^{met}$	formylatable species of methionyl transfer ribonucleate
mRNA	messenger ribonucleate
mtDNA	mitochondrial deoxyribonucleate
mtmRNA	mitochondrial messenger ribonucleate
mtrRNA	mitochondrial ribosomal ribonucleate
mt-tRNA	mitochondrial transfer ribonucleate
µCi	micro curie
NAD	nicotinamide adenine dinucleotide
nucDNA	nuclear deoxyribonucleate
poly(A)	polymeric adenylate
PP_i	inorganic pyrophosphate
PrI_2	propidium diiodide
ψ	pseudo uridine
RNA	ribonucleate

RNase	ribonuclease
RPC	reverse phase chromatography
rRNA	ribosomal ribonucleate
rTp	ribothymidylate
S	sedimentation coefficient relative to 28S and 18S rRNA
S_E	electrophoretic mobility relative to that of 28S and 18S rRNA
SDS	sodium dodecylsulfate
TCA	trichloroacetic acid
TK	thymidine kinase
tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleate
U	uridine

PREFACE

Molecular biology is that part of the discipline of biochemistry which attempts to understand the basic mechanisms of gene expression and its control, as well as how these mechanisms explain, not only the relative simplicity of bacteriophages, but also the great organizational complexity of the eukaryotic cell. Molecular biologists attempt to find suitable systems to study gene expression; whatever the system, the questions are inevitably the same: What are the products (RNA and protein) of the expression of the genome? What controls the nature and relative proportions of the products? How do these products account for the phenotypic expression?

One of the most actively pursued areas in molecular biology is the study of the biogenesis of mitochondria. In part, this pursuit is due to an age-old curiosity about the origin of cell organelles. Ever since mitochondria were first observed within cells in the late 19th century (see Lehninger, 1965), it has been speculated that these organelles were derived from endo-symbionts of bacterial origin. The discovery of mitochondrial DNA and of mitochondrial protein synthesis adds support to this hypothesis. More recently, the mechanisms of the biogenesis of mitochondria has received considerable attention in an attempt to understand the relationships which exist between two physically separate genetic systems within one cell (Ashwell & Work, 1970; Borst, 1972; Mahler, 1973). An understanding of the expression and control of expression of the mitochondrial genome will certainly

add to some of the general concepts of molecular biology.

This thesis describes investigations into mitochondrial biogenesis in mammalian cells. While many approaches have been employed, the questions which these approaches have attempted to answer are the same. Broadly stated, these questions are: What is the nature of the products of the mitochondrial genome? How do the nuclear and mitochondrial genomes interact to control mitochondrial biogenesis? To what extent is the mitochondrion autonomous and how does it influence its own phenotype? The investigation will be considered in three chapters followed by a more general consideration of the significance of the results to the study of mitochondrial biogenesis.

CHAPTER I

Mitochondrial Protein Synthesis in a Conditional Mutant

INTRODUCTION

Mitochondria have long been known to be present in the cytoplasm of eukaryotic cells (see Lehninger, 1965, for a short historical review). While studies in the first half of this century were devoted mainly to establishing the biochemical role of these organelles (cellular oxidations), it was not until the early 1960's that the study of mitochondria turned to biogenetic aspects.

Today it is well established that mitochondria are semi-autonomous organelles (for reviews on mitochondrial biogenesis see Ashwell & Work, 1970a; Rabinowitz & Swift, 1970; Borst, 1972; Sager, 1972; Mahler, 1973). They are known to contain a small, but unique, genome which is replicated and transcribed by mitochondrial-specific enzymes. Mitochondria also contain all the appropriate machinery for the synthesis of proteins. It is the existence of this unique (and as will be discussed later, prokaryote-like) genetic system within the eukaryotic cell which greatly stimulated the interest in the study of mitochondrial biogenesis. I shall now briefly review what is known about the mitochondrial genome, and mitochondrial transcription and translation.

The Mitochondrial Genome

Unlike many scientific discoveries, the finding that mitochondria contain DNA was the result of an organized search (Nass, 1969; Ashwell & Work, 1970a; Rabinowitz & Swift, 1970; Mahler, 1973). This search was instigated due to a number of observations: 1) extrachromosomal, non-Mendelian inheritance in yeast "petite" mutants (Ephrussi & Hottinguer, 1951) and in *Neurospora crassa* "poky" mutants (Mitchell et al, 1953) were known to involve aspects of mitochondrial function such as the loss of cytochrome oxidase activity (Ashwell & Work, 1970; 2) isolated mitochondria had been shown to incorporate labelled amino acids into protein *in vitro* (McLean et al, 1958; and see below); 3) chloroplasts, cytoplasmic organelles of photosynthetic eukaryotes, were known to contain DNA (Chun et al, 1963); 4) the discovery of mitochondria in cells by Altman in 1890 (Lehninger, 1965) led him to speculate that mitochondria were endosymbiotic bacteria. This speculation was (and still is) a popular hypothesis of the evolutionary origin of mitochondria (Margulis, 1970) and suggests that mitochondria must have a degree of autonomy; and finally 5) mitochondria of *Neurospora* appear to arise from pre-existing mitochondria by a process which resembles growth and division (Luck, 1963a;b) and appear to arise from pro-mitochondria in yeast adapting to aerobic growth (Linnane et al, 1962; Schatz, 1963). These observations suggest a replicative capacity of these organelles.

The establishment of the existence of mitochondrial DNA (mtDNA) occurred by a number of independent routes (see Nass, 1969; Ashwell & Work, 1970a; Borst, 1972 for reviews). In the late 1940's and early 1950's, studies of mutants of yeast ("petites") characterized by their

slow growth (Ephrussi & Hottinguer, 1951) and of mutants of *Neurospora* ("poky") also showing slow growth (Mitchell et al, 1953) established a non-Mendelian (i.e. maternal) pattern of inheritance of these phenotypes. The slow growth of petites was found to be associated with a lack of a complete cytochrome complement and thus respiratory deficiency, and it was suggested that the mutants arose from "loss or inactivation of autonomous self-reproducing nonchromosomal elements" of the cytoplasm (Raut & Simpson, 1955). Studies by Raut and Simpson (1955) demonstrated that petite production was stimulated by ultraviolet light of 260 nm and it was suggested that the cytoplasmic elements were nucleic acid-containing mitochondria and that this nucleic acid determined the phenotype of the mutants.

DNA-like fibers were observed within the mitochondria of chick embryo cells by Nass and Nass (1962). The DNA nature of the fibers was confirmed by staining behaviour in electron microscopy and by the sensitivity of the fibers to DNase (Nass & Nass, 1963a;b). DNA was also shown to be a component of mitochondria from yeast (Schatz et al, 1964) and lamb liver (Kalf, 1964). This DNA was likely not a contaminent because a consistent content (μgDNA/mg mitochondrial protein) was found, it was insensitive to DNase treatment of whole mitochondria (Kalf, 1964) and highly purified mitochondria were used (Schatz et al, 1964).

DNA from mitochondria of *Neurospora* was also reported (Luck & Reich, 1964). This DNA was of a unique buoyant density and appeared to be associated with a DNA-dependent RNA polymerase (see also Kalf, 1964), suggesting that the mtDNA is transcribed in these cells.

Some of the [^{3}H]-thymidine incorporated into DNA of *Tetrahymena pyriformis* was found by electron microscopic-autoradiographic techniques

to be associated with mitochondria (Stone & Miller, 1965). The DNA was observed to be stable and present in all of the mitochondria in cells after division, suggesting that the DNA of the mitochondria was replicated. By 1965, Nass et al concluded that the mitochondria of all cells contain DNA.

While a wealth of literature has been devoted to the physical properties, replication and repair of mtDNA (cf. Borst, 1972; Borst & Flavell, 1972; Mahler, 1973), I shall consider only two aspects, the size and informational content of mtDNA, and the isolation of mtDNA. The latter will be discussed in Chapter II.

The most well characterized mtDNAs fall into two categories (Borst, 1972; Borst & Flavell, 1972), the mtDNA of vertebrates and other animals which is closed circular, with a contour length of approximately 5 μm (10^7 daltons) and the mtDNA of lower organisms such as yeast, *Neurospora* and *Tetrahymena* which is about 5-fold larger and may or may not be closed circular. Table I summarizes some aspects of the physical properties of mtDNA from various organisms.

More important than the physical size of mtDNA is its genetic complexity or informational content. This topic has been extensively reviewed by Borst and co-workers (Borst, 1971; Borst & Flavell, 1972; Borst, 1972). Renaturation kinetics of sheared mtDNA from many sources are second order, the time course of which, in each case, has approximated that expected for DNA molecules with a complexity equal to the observed length of the mtDNA. This has been summarized in Table II. It seems that physical size is a reflection of the genetic complexity of mtDNAs. The implication of the relatively small genome of mitochondria (the E.

TABLE I

PROPERTIES OF MtDNA

organism	contour length (μm)	circularity observed	density (g/cc) nucDNA	density (g/cc) mtDNA
Human	4.81	+	1.695 - 1.700	1.705
Rat	4.9	+	1.703	1.701
Mouse	5.1	+	1.703	1.698
Chick	5.35	+	1.701	1.708
Xenopus	5.40	+	1.702	1.704
House fly	5.2	+	-	-
Tetrahymena	15	-	1.683 - 1.692	1.683 - 1.686
Saccharomyces	25	+	1.698	1.684
Neurospora	20	+	1.713	1.698

Data summarized from: Borst & Kroon, 1970; Borst & Flavell, 1972; Borst, 1972.

TABLE II

COMPARISON OF KINETIC COMPLEXITY AND PHYSICAL SIZE OF MtDNA

Organism	Mol. Wt.* E.M.	Mol. Wt. renaturation
Rat	9.6	9.9
Guinea pig	11	11
Tetrahymena	29	30
Saccharomyces	49	50 - 63

Data after Borst & Flavell (1972). *Mol. Wt. E.M. (molecular weight determined by electron microscopy assuming 1 μm ≃ 2 x 10^6 daltons).

coli genome is approximately 800 μm (Cairns, 1963)) is that it can code for only a very few proteins. All studies of mitochondrial gene expression must bear this in mind.

Transcription of the Mitochondrial Genome

As indicated above, early studies indicated the presence of a DNA-dependent RNA polymerase (cf. Luck & Reich, 1964) in mitochondria. The possibility that mtDNA is transcribed was strengthened by the knowledge that isolated mitochondria incorporate labelled amino acids into protein (McLean et al, 1958; Roodyn et al, 1961). Today, while the identification of the nature of all mitochondrial transcripts is still not complete (Borst, 1972), it is clear that mitochondrial ribosomal RNA (mtrRNA) and certain mitochondrial transfer RNAs (mt-tRNA) are transcribed from mtDNA. Still a controversial topic concerns whether or not mtDNA codes for mitochondrial messenger RNA (mtmRNA) (contrast Hirsch & Penman, 1974a and Avadhani et al, 1974). We shall briefly review the observations concerning transcription of mtrRNA, mt-tRNA and mtmRNA from the mitochondrial genome.

a) MtrRNA and the mitochondrial ribosome

As already mentioned, mitochondria are known to contain DNA, known to synthesize protein *in vitro*, and known to synthesize RNA. Mitochondria would therefore be expected to contain ribosomes unless the synthesis of proteins proceeded by an as yet undiscovered mechanism. It was not surprising that mitochondrial ribosomes were discovered in a number of organisms not long after the demonstration of the existence of mtDNA. In 1967, Küntzel and Noll reported the isolation of ribosomes and polysomes

from *Neurospora* mitochondria (see also Rifkin et al, 1967). These structures were found to differ from the cytosolic structures. The mitochondrial ribosomes had a lower S value (73S) than the cytosolic equivalent (77S) as did the polysomes. The mitochondrial ribosome contained mtrRNAs of lower S value as well (23S and 16S versus 25S and 17S for the cytosolic rRNAs (cytrRNA)). The mtrRNA was found to have a unique base composition, characterized by a very low G+C content (38%). Finally, the mitochondrial polysomes were found to be associated with radioactivity when isolated mitochondria were pulse labelled with [^{14}C] leucine. This suggests a protein synthetic function for the polysome structures.

Also in 1967, O'Brien and Kalf (1967a;b) identified a particle from rat liver mitochondria with many properties expected for a ribosome, except that it sedimented at only 55S. Because of its apparent small size there was much doubt that it was the true mitochondrial ribosome. It was shown, however, to become labelled when isolated mitochondria were incubated with [^{14}C] leucine. This 55S (or 60S) particle was confirmed as the ribosome from animal mitochondria (Ashwell & Work, 1970b; Swanson & Dawid, 1970; Perlman & Penman, 1970; Attardi & Attardi, 1971; Brega & Vesco, 1971). It was found to contain rRNAs with low sedimentation values (16S and 12S) and of low G+C content. The ribosome dissociated into 40S and 30S subunits. The ribosome and presumptive polysomes became labelled when isolated mitochondria (Ashwell & Work, 1970b), HeLa cells (human) in the presence of inhibitors of cytosolic protein synthesis (Perlman & Penman, 1970; Brega & Vesco, 1971) or mitochondrial extracts (Swanson & Dawid, 1970) were pulse-labelled with radioactive amino acids, suggesting again that these ribosomes function in protein synthesis within the mitochondrion.

Ribosomes have since been found in the mitochondria of all organisms which have been examined. They have fallen into two categories: the ribosomes from fungi which are 70-75S, and the ribosones from animals which are 55-60S. These same phylogenetic differences appear in the size of the mtDNAs (Table I). As suggested by Swanson & Dawid (1970) perhaps the ribosomes from all organisms with 5 μm mtDNA will be 55-60S. The term "miniribosome" has been applied to it. Some of the properties of mitochondrial ribosomes have been summarized in Table III. It is of interest to note that the "miniribosome" from rat liver mitochondria has recently been shown (de Vries & Koogh-Schuuring, 1973) not to be small at all. By measurement of buoyant density (and therefore its RNA/protein ratio) and from a knowledge of the molecular weight of the mtrRNA, one can calculate that the mitochondrial ribosome should have a molecular weight close to that of *E. coli* ribosomes (70S). This was confirmed by noting the migration of the 55S ribosome in polyacrylamide-gradients, where a particle moves to a position in the gel corresponding to its volume. Mitochondrial ribosomes were found to be larger than *E. coli* and smaller than cytosolic ribosomes.

Recent studies from Kroon's laboratory (Datema et al, 1974; Agsteribbe et al, 1974) have demonstrated that presumptive ribosomes, which are 80S rather than 73S, can be isolated from *Neurospora* mitochondria, in the presence of the ribonuclease inhibitor heparin. By using various treatments, the 80S particles can be converted to 73S particles, but not vice versa. Both 80S and 73S particles are active in the chloramphenicol-sensitive peptidyl transferase reaction as expected for mitochondrial ribosomes (de Vries et al, 1971). All other properties of the 80S and 73S mitochondrial ribosomes are the same. It may be that mitochondrial

TABLE III

Summary of Properties of Mitochondrial Ribosomes and MtrRNAs

organism	mitochondrial ribosome S-value	mtrRNA S-values*	G+C content (%)	large subunit S-value	large subunit RNA S-value*	large subunit G+C† content (%)	small subunit S-value	small subunit RNA S-value*	small subunit G+C† content (%)	hybridization genes/mtDNA
HeLa	60	16,12	45	45	16	42-45	35	12	43-45	1.0
L cell	-	15,12.5	40-47	-	-	33	-	-	36	-
Rat	55	15,13	47	-	-	-	-	-	-	-
BHK-21	50	17,13	-	33	17	40	25	13	39	-
Xenopus	60	18,13	-	43	18	40	32	13	43	1.0
Tetrahymena	80 ?	21,14	-	55 ?	21 ?	28	55 ?	14 ?	31	1.4
Saccharomyces	74	22,15	~30	53-58	-	27-30	35-40	-	25-33	0.64
Neurospora	73	23,16	38	50	23	34	37	16	36	0.71
Aspergillus	67	23.5,15.5	-	50	-	31	32	-	32	-

Data summarized from: Borst & Grivell, 1971; Borst & Flavell, 1972; Freeman et al, 1973; Mahler, 1973. * Values represent sedimentation coefficients under various conditions and vary significantly under other conditions (e.g. of temperature, ionic strength, etc.). † G+C content of large or small component of mtrRNA, not necessarily the RNA extracted from the ribosomal subunit.

? The nature of the ribosomal subunits from *Tetrahymena* is still unclear although it has been suggested that they are both 55S particles, one containing 21S mtrRNA and the other 14S mtrRNA (Chi & Suyama, 1970).

ribosomes are prone to degradation upon isolation, and perhaps a re-evaluation of the properties of all mitochondrial ribosomes should be made.

In summary, it is clear that mitochondria contain ribosome-like particles and polysome-like structures. Moreover, it is apparent that these structures have the capacity to participate in mitochondrial-specific protein synthesis.

The mtrRNAs referred to above have been the subject of extensive research over the past eight years (Borst, 1972). The reason for the intense interest in these molecules is the demonstration that they are transcribed from mtDNA; mtrRNAs are one of the few "gene products" thus far recognized for the mitochondrial genome.

The study of mtrRNA from the unicellular eukaryotes followed a rather straightforward approach. Since the ascomycetes do not contain a highly developed endoplasmic reticular membrane system (Borst & Grivell, 1971), it was relatively easy to isolate mitochondria essentially free of cytosolic ribosomes. Mitochondrial ribosomes, with essentially the same properties as those described above for *Neurospora*, have been isolated from yeast (Stegeman et al, 1970; Schmitt, 1971; Morimoto & Halvorson, 1971) and *Aspergillus* (Edelman et al, 1970) (Table III). The mtrRNA could be isolated from the mitochondrial ribosomes in a relatively pure state. A similar approach was also successful for *Tetrahymena* (Chi & Suyama, 1970). Some of the properties of mtrRNA are also included in Table III. Of particular note is the fact that in all cases thus far examined, one cistron for each of the mtrRNAs exists on mtDNA. This is contrary to the situation in bacteria and the eukaryotic nucleus where many rRNA cistrons per genome are found (Attardi & Amaldi, 1970). This point will be important in later discussions.

The study of mtrRNAs in animal systems did not proceed by such a straightforward route (Attardi et al, 1970; Penman et al, 1970). Animal mitochondria are very difficult to isolate free of cytosolic contamination (Dubin, 1967; Bartoov et al, 1970; Malkin, 1972). Early studies with uridine (^{3}H or ^{14}C) labelled cells demonstrated considerable RNA associated with mitochondria (Dubin & Brown, 1967; Attardi & Attardi, 1968), RNA with S values ranging from 4 to 50S. Much of this RNA did not hybridize with mtDNA (Attardi & Attardi, 1969). Clarification of the situation came with the use of specific inhibitors of nuclear RNA synthesis such as mengovirus infection of HeLa cells (Vesco & Penman, 1969a) or low doses of actinomycin D (Dubin, 1967; Vesco & Penman, 1969b). Mitochondrial RNA (actinomycin-resistant) of animal cells in culture was demonstrated with S values of 17 - 16S, 13 - 12S and 4S (Dubin & Montenecourt, 1970; Attardi & Attardi, 1971) or with electrophoretic mobilities corresponding to $21S_E$, $12S_E$ and $4S_E$ (Vesco & Penman, 1969b). These RNAs were also isolated from highly purified mitochondria from the liver of rats labelled *in vivo* with [^{32}P] orthophosphate or [^{3}H] orotic acid (Bartoov et al, 1970). It has been concluded from studies on the mitochondrial ribosome that the 17 - 16S and 13 - 12S mitochondrial RNAs are the mtrRNAs (see above).

A second development which aided the analysis of mtrRNA was the demonstration by Zylber et al (1969) of the specific inhibition of mitochondrial-associated RNA synthesis ($21S_E$ and $12S_E$ particularly) by low doses of ethidium bromide. This specific effect is presumably due to the conformational changes induced in closed circular DNA by the intercalating phenanthridine dye (Radloff et al, 1967). Ethidium bromide has

since become an extremely important tool in the study of mitochondrial RNA synthesis, since inhibition of the synthesis of an RNA component by ethidium bromide suggests that it is transcribed from mtDNA.

Although high molecular weight mtrRNA has been observed in all eukaryotic organisms examined, the presence of a mitochondrial equivalent to 5S rRNA has not been demonstrated. Despite a rigorous search for 5S RNA in *Neurospora* mitochondria and mitochondrial ribosomes (Lizardi & Luck, 1971), none was found. Lizardi and Luck concluded that if a 5S rRNA equivalent exists in mitochondrial ribosomes, it must be concealed in RNA of other sedimentation values. Similarly, 5S RNA has not been observed in animal mitochondria (Zylber & Penman, 1969; Dubin & Montenecourt, 1970). It is very possible that, like mtrRNA, the 5S rRNA equivalent is smaller than the cytosol 5S rRNA and co-sediments with mitochondrial 4S RNA. In this connection, Dubin et al (1974) have recently reported the presence of a $3S_E$ RNA associated with mitochondria of BHK-21 cells (hamster). Although it was not demonstrated to be associated with mitochondrial ribosomes, its synthesis is ethidium bromide sensitive, it has the low G+C content characteristic of mtrRNA, it is present in an equimolar ratio with mtrRNA and was found to be unmethylated. These observations suggest that this molecule might be a 5S rRNA equivalent, but its association with ribosomes has yet to be demonstrated.

Thus, the mtrRNAs from mitochondria of unicellular organisms and from animals is unique to the organelle, transcribed from mtDNA and always smaller than the cytrRNA. The anomalous behaviour exhibited by these RNAs upon gel electrophoresis and sucrose gradient centrifugation (e.g. 16S and 12S versus $21S_E$ and $12S_E$ as noted above) under various conditions

(Edelman et al, 1971; Grivell et al, 1971; Mitra et al, 1972) is due, in part, to the low G+C content (Freeman et al, 1973). MtrRNA would be expected to contain secondary structures of low stability. The low G+C content does not, however, entirely explain the anomalous behaviour of this RNA. Dawid (1971) compared the thermal denaturation behaviour of *Xenopus* mtrRNA and *Drosophila* cytrRNA, RNAs with equal G+C content. The mtrRNAs were found to begin to melt at a lower temperature and continue over a broad temperature range while the cytrRNAs melted within a narrow temperature range. Similarly, the mobility of the *Drosophila* cytrRNA on polyacrylamide gels was not affected by temperature, while the mtrRNA mobility was affected. Whether or not the anomalous behaviours of mtrRNAs have functional significance remains to be seen (Freeman et al, 1973).

b) mt-tRNA

Research concerning mt-tRNA has proceeded along two separate lines: the study of mitochondrial 4S RNA, a fraction which presumably contains tRNA, and the study of aminoacyl-tRNA and aminoacyl-tRNA synthetases derived from mitochondria. These two areas of study yield results which can be interpreted quite separately, and offer a convenient division for discussion purposes.

i) *Mitochondrial 4S RNA*: This mitochondrial component was recognized very early in the study of mitochondrial-associated RNA (Suyama, 1967; Rifkin et al, 1967, Attardi & Attardi, 1967; Dubin & Brown, 1967; Zylber & Penman, 1969; Knight & Sugiyama, 1969). The functional significance of the mitochondrial 4S RNA was not known, although Knight & Sugiyama (1969) speculated that it was tRNA without offering any

evidence (see also Zylber & Penman, 1969; Dubin & Montenecourt, 1970).

The physical properties of the mitochondrial 4S RNA from animal cells have been actively pursued, particularly by Dubin and co-workers (cf. Dubin & Friend, 1972). The synthesis of the 4S RNA associated with mitochondria is partially sensitive to inhibition by ethidium bromide (Knight, 19691; Zylber & Penman, 1969; Attardi et al, 1970; Dubin & Friend, 1972). The residual, ethidium bromide-resistant 4S RNA is presumably of cytosolic origin (Zylber & Penman, 1969). The mitochondrial 4S RNA has a low degree of methylation (about 60% lower) when compared to cytosolic 4S RNA (Knight, 1969; Dubin & Montenecourt, 1970; Attardi & Attardi, 1971). Dubin and Friend (1974) have recently analyzed the methylated nucleotides in BHK-21 mt-tRNA labelled with $^{32}P_i$ and [^{3}H] or [^{14}C] methyl-methionine in the presence or absence of ethidium bromide. Establishment of an ethidium bromide-sensitive profile of nucleotides separated by electrophoresis gave results which represented mitochondrial specific methylated nucleotides. These differed significantly from cytoplasmic 4S RNA and from *E. coli* tRNA. One significant difference in terms of later discussions is the apparent lack of ribo-thymidine monophosphate (rTp) in mitochondrial 4S RNA.

The metabolic stability of mitochondrial 4S RNA has been examined by blocking further synthesis of mitochondrial RNA with ethidium bromide after a brief labelling period with [^{3}H] or [^{14}C] uridine (Zylber & Penman, 1969; Knight, 1969). The 4S RNA was found to be very stable. Similar experiments with mtrRNA of HeLa cells demonstrated that both 16S and 12S mtrRNAs are unstable, with a half-life of about three hours (Zylber et al, 1969; 1971).

Experiments to be discussed below suggest that mitochondria contain specific tRNAs. These molecules would be expected to co-sediment with the mitochondrial 4S RNA described above, or be identical with it. It was of interest, then, to determine the extent to which mitochondrial 4S RNA hybridized with mtDNA. Inhibition of mitochondrial 4S RNA synthesis by ethidium bromide as well as early hybridization experiments (Suyama, 1967; Attardi & Attardi, 1969) suggested that at least some of the 4S RNA was transcribed from mtDNA.

Hybridization-saturation experiments with 4S RNA of known specific activity have demonstrated 20 genes for 4S RNA on yeast mtDNA (Reijnders & Borst, 1972),15 genes on *Xenopus* mtDNA (Dawid, 1972), and 11 genes on HeLa cell mtDNA (Aloni & Attardi, 1971c). These values are very low and suggest that mtDNA does not code for a full complement of tRNA (recall that at least one of the 4S genes may be coding for a 5S rRNA equivalent).

The saturation values above may be underestimates for many reasons (Borst, 1972). One discrepancy is the molecular weight of mitochondrial 4S RNA assumed for calculation of gene numbers, 25,000 for yeast and HeLa cells and 28,000 for *Xenopus*. Dubin & Friend (1972) have suggested, on the basis of high resolution sucrose gradient and gel electrophoresis techniques under denaturing and non-denaturing conditions, that BHK-21 mitochondrial 4S RNA is smaller than cytosolic 4S RNA and was calculated to be 19,000 daltons. If this value is true for all animal mitochondrial 4S RNA, it brings the mitochondrial 4S RNA gene numbers to 15 for HeLa cells and 22 for *Xenopus*.

Hybridization experiments have been extended to studies of hybridization of mitochondrial 4S RNA to the separated strands of mtDNA

(due to differences in base composition of the two strands of animal mtDNA, they can be separated on alkaline CsCl density gradients (Borst & Flavell, 1972)). Although it has been demonstrated that HeLa cell mtDNA is transcribed symmetrically, virtually all the transcripts of the light strand (L-strand) are unstable (Aloni & Attardi, 1971b). It appears that most of the stable RNA species of animal mitochondria are transcripts of the heavy strand (H-strand) (Borst & Aaij, 1969; Aloni & Attardi, 1971a; Dawid, 1972). Both Aloni and Attardi (1971c) and Dawid (1972) have demonstrated that a small amount of the mitochondrial 4S RNA hybridizes to the L-strand (3 of the 11 genes reported by Aloni & Attardi, 1971c).

In an attempt to "visualize" the positions of the mitochondrial 4S RNA genes on the HeLa cell mitochondrial genome, Wu et al (1972) prepared mitochondrial 4S RNA covalently coupled to ferritin. This material was then hybridized to H- and L-strands of the HeLa cell mtDNA, the H-strand was also hybridized with mtrRNA. The DNA was spread on grids and examined in the electron microscope for presence of the duplex region (16S and 12S mtrRNA/DNA hybrids) and for ferritin. The H-strand could then be mapped for the positions of the ferritin coupled 4S RNA with respect to the mtrRNA duplex region (already shown to be on the H-strand (Robberson et al, 1972)). The L-strand was examined for ferritin coupled 4S RNA. Nine 4S genes were located on the H-strand, one of them between the 16S and 12S mtrRNA cistrons, possibly the site for a 5S rRNA equivalent. Three 4S genes were located on the L-strand. This result is represented as a circular map of the HeLa cell mitochondrial genome in Fig. 1. These results suggest that 12 genes for mitochondrial 4S RNA exist on the HeLa cell mtDNA, although it is

Figure 1

A schematic representation of the circular "map" of HeLa cell mtDNA as presented by Attardi et al (1974). H- and L-strands of the mtDNA were separated and hybridized with 16S and 12S mtrRNA or the portion of H-strand mtDNA coding for mtrRNA (mtrRNA genes) and ferritin coupled mitochondrial 4S RNA. When examined by electron microscopy, the DNA-RNA or DNA-DNA duplex regions define the mtrRNA genes (H-strand) or the anti-rRNA site (L-strand). These sites served to align the strands and the ferritin-4S RNA hybridization sites could be observed. Nine sites were found on the H-strand (H1 through H9) and three on the L-strand (L1 through L3).

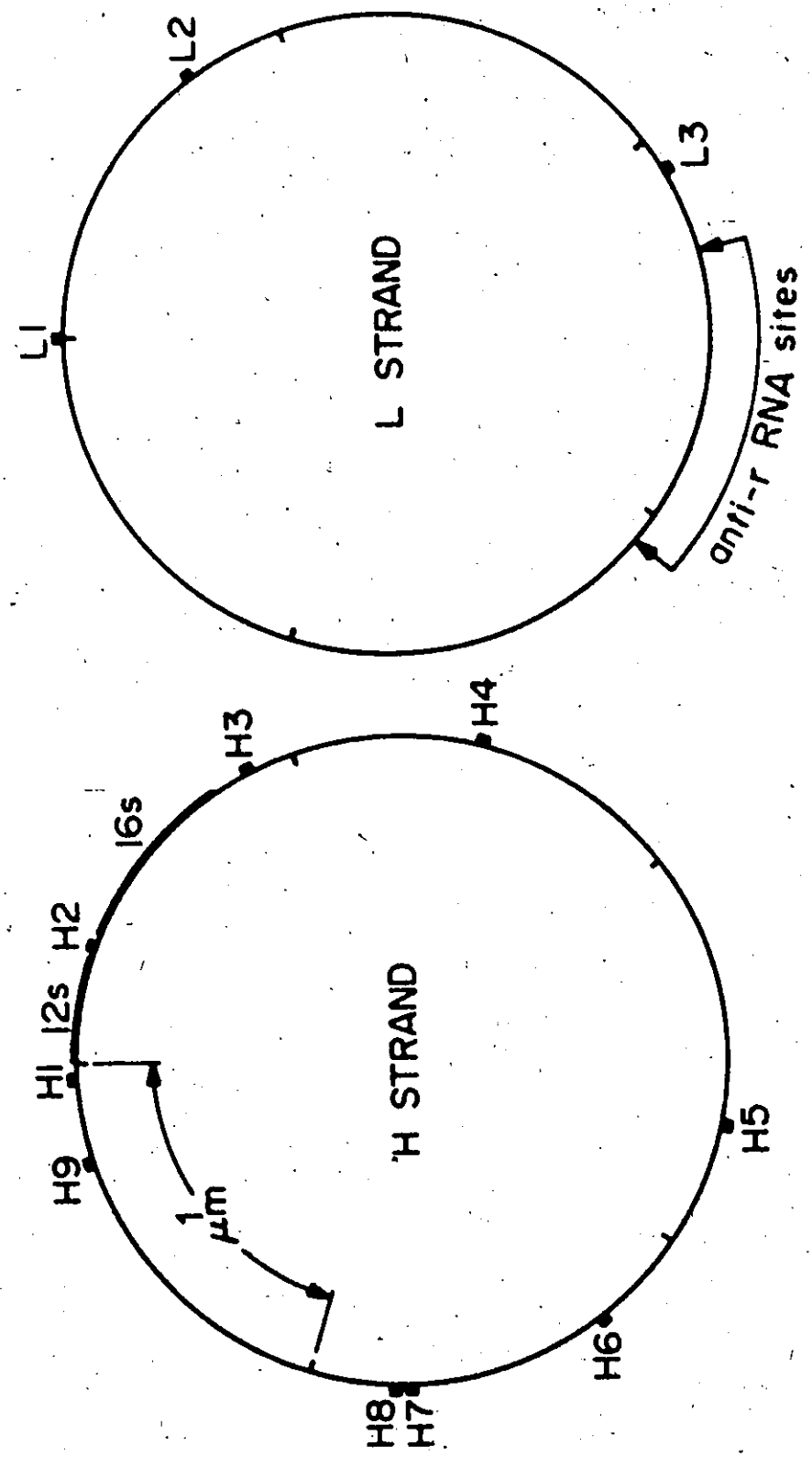
L1
L2
L3
L STRAND
ori-r RNA sites
H1
12s
H2
16s
H3
H4
H5
H6
H7
H8
H9
H STRAND
1 μm

Figure 1

possible that some RNA molecules exist in concentration too low to be detected by this technique.

In summary, it appears from hybridization experiments that there are between 12 and 22 genes for 4S RNA on the mtDNA of animals, and between 20 and 24 genes for 4S RNA on yeast mtDNA (Reijnders & Borst, 1972). Although these results may be underestimates, they suggest that mtDNA does not code for a full complement of tRNAs (at least 33 tRNAs are required for reading all 61 codons considering maximum wobble (Borst & Flavell, 1972)). The possibilities that mitochondria utilize fewer than 20 amino acids in protein synthesis (Costantino & Attardi, 1973) or import some tRNAs transcribed within the nucleus (Suyama et al, 1974) have been postulated, but to date there is little experimental evidence to support either hypothesis.

ii) *Mitochondrial amino acyl-tRNA and amino acyl-tRNA synthetases:* Very early studies on mitochondrial protein synthesis presented suggestive evidence for the presence of mitochondrial amino acylation of tRNA. Mager (1960) demonstrated that, while chloramphenicol inhibited protein synthesis in a mitochondrial fraction from *Tetrahymena*, it caused the accumulation of an unidentified [^{14}C] leucine labelled compound which, unlike protein, was precipitated by cold trichloroacetic acid (TCA) but destroyed if the TCA was heated. This compound was also destroyed (i.e. free leucine liberated) by treatment with 1N NaOH. The properties of this compound are consistent with those expected for leucyl-tRNA, although the results were not interpreted in that way.

Amino acyl-tRNA synthetases catalyze the following reactions:

$$aa_x + ATP \rightleftharpoons aa_x - AMP + PP_i$$

$$aa_x - AMP + tRNA^x \rightleftharpoons aa_x - tRNA^x + AMP$$

Early studies measured the amino acid stimulated ATP-[^{32}P]PP_i exchange reaction, rather than the formation of the amino acyl-tRNA. The amino acid stimulated exchange observed in mitochondrial preparations from rat liver (Reis et al, 1959), rat liver mitochondrial extracts such as pH 5 enzyme preparation (Roodyn et al, 1961; Craddock & Simpson, 1961) or carrot-root mitochondrial preparations (Davis & Novelli, 1958) was taken as evidence for the existence of amino acid activating enzymes (i.e. synthetases) within mitochondria. The exchange reactions observed, however, could simply be a result of contamination of the mitochondrial preparations with cytosolic synthetases.

A more convincing demonstration of mitochondrial amino acyl-tRNA synthetased was presented by Wintersberger (1965). He demonstrated that a synthetase preparation from yeast mitochondria acylated [^{14}C] leucine and [^{14}C] phenylalanine to homologous RNA more efficiently than to heterologous (cytosolic) RNA. Similarly, cytosolic synthetase acylated cytosolic RNA more efficiently.

Fournier and Simpson (1968) demonstrated the incorporation of [^{14}C] leucine into protein (hot TCA-stable incorporation) and into tRNA (cold TCA-stable, hot TCA-unstable incorporation). Both processes were resistant to ribonuclease (RNase) but only protein synthesis was inhibited by chloramphenicol. Attempts to characterize leucyl-tRNA by MAK chromatography were not particularly successful, probably due to degradation of the RNA. However, the MAK column profile of mitochondrial leucyl-tRNA did show differences with respect to the cytosolic leucyl-tRNA.

Extensive studies on tRNAs and amino acyl-tRNA synthetases from *Neurospora* mitochondria have been reported by Barnett and co-workers. Utilizing purified mitochondria, it was shown that chromatographically unique (e.g. DEAE-cellulose or hydroxylapatite chromatography) leucyl-, phenylanyl- and aspartyl-tRNA synthetases were associated with mitochondria and specifically acylated mitochondrial tRNA (Barnett et al, 1967). Also, amino acid acceptor activities for 18 amino acids were found associated with these *Neurospora* mitochondria (Barnett & Brown, 1967). Epler and Barnett (1967) have also demonstrated that *Neurospora* mitochondrial leucyl-tRNA has a unique counter-current distribution when compared with whole cell leucyl-tRNA. When examined for codon recognition, the mitochondrial leucyl-tRNA recognizes only UC-polymers but not UG-polymers. Further studies by Brown and Novelli (1968) and Epler (1969) utilizing reverse phase chromatography (RPC-2) showed the separation of 15 *Neurospora* mitochondrial amino acyl tRNAs from the corresponding cytosolic tRNAs. In the case of the two mitochondrial methionyl tRNAs (Brown & Novelli, 1968) resolved by RPC-2 chromatography, one was found to be formylatable by both *E. coli* transformylase and a mitochondrial specific transformylase (Epler et al, 1970). N-formylmethionyl $tRNA^{met}$ has been implicated in the initiation of protein synthesis in both chloroplasts and mitochondria as well as its well-known involvement in bacterial protein synthesis (see Chapter II for references and further discussion).

In an elegant series of experiments on rat liver mitochondria, Nass and Buck (Buck & Nass, 1968; 1969; Nass & Buck, 1969; 1970) demonstrated unique amino acyl-tRNAs and amino acyl-tRNA synthetases in

animal mitochondria. It was shown that species of mitochondrial leucyl-, tyrosyl-, aspartyl-, valyl-, and seryl-tRNAs could be separated from the corresponding cytosol tRNAs by MAK chromatography (Buck & Nass, 1968; 1969). Although multiple species of the mitochondrial tRNAs were seen, it was unclear whether some represented cytosolic contamination. Separation of mitochondrial and cytosolic phenylalanyl-tRNA was not observed by Buck and Nass; however, the two tRNAs were resolved by MAK chromatography as reported by Lietman (1968) also studying rat liver mitochondria.

In a later study it was found that the [^{3}H] leucyl- and [^{3}H] phenylalanyl-tRNAs hybridized to the H-strand of mtDNA, and [^{3}H] tyrosyl- and [^{3}H] seryl-tRNAs hybridized to the L-strand, while cytosolic tRNAs did not hybridize to mtDNA at all (Nass & Buck, 1969; 1970). It is of interest to note that two of the four amino acyl-tRNAs tested have been shown to hybridize to the L-strand, while only three genes for mitochondrial 4S RNA were determined for the HeLa cell L-strand (see above). It will be interesting to see if in the future, more amino acyl-tRNAs are found to hybridize to the L-strand of animal mtDNA. The results of Nass and Buck demonstrate the potential of mammalian mtDNA to code for a specific set of tRNAs.

Studies with *Tetrahymena* mitochondria have yielded similar results. Mitochondrial leucyl-tRNA when acylated with homologous synthetase was found to give a unique profile on MAK chromatography (Suyama & Eyer, 1967). As in the case of rat liver mitochondrial leucyl-tRNA synthetase (Buck & Nass, 1968; 1969), the *Tetrahymena* mitochondrial enzyme showed specificity to homologous tRNA. In a later study Chiu and Suyama (1973) demonstrated

that an antisera prepared against the mitochondrial leucyl-tRNA synthetase of *Tetrahymena* reacted only with the mitochondrial but not with the cytosolic synthetase. This was demonstrated by its ability to precipitate only the mitochondrial enzyme, inhibit only its activity and to retard only the mitochondrial enzyme on an affinity column prepared by covalently linking the antisera to agarose. This immunological evidence supports the hypothesis that the mitochondrial synthetases are unique proteins.

Utilizing the improved resolution of RPC-5 chromatography in resolving iso-accepting tRNA species, Chiu et al (1974a;b) demonstrated three leucyl-tRNA species unique to the *Tetrahymena* mitochondria. All three hybridize to the mtDNA; however, each compete with each other for hybridization, and it is not clear whether they share the same primary sequence. Codon recognition studies on the mitochondrial leucyl-tRNAs were ambiguous and further studies are required. It appeared that of the three species one recognized CUG, another CU(U,C,G) and the third CUU. A preliminary report from the same laboratory has demonstrated that other mitochondrial specific tRNAs are present in *Tetrahymena* which are not coded for by the mtDNA (Suyama et al, 1974). This result suggests that some mt-tRNAs may be transcribed in the nucleus and then transported into the mitochondria.

Studies on the mt-tRNAs of yeast by Rabinowitz and co-workers have utilized the same technique employed by Nass and Buck (1969; 1970) to study the hybridization of mitochondrial [^{3}H] amino acyl-tRNAs to mtDNA (Casey et al, 1972; Halbreich & Rabinowitz, 1971; Cohen et al, 1972; Cohen & Rabinowitz, 1972; Casey et al, 1974a;b). These studies have culminated in the demonstration that at least 14 amino acyl-tRNAs

hybridize with the yeast mitochondrial genome. These include leucyl-, valyl-, isoleucyl-, alanyl-, glycyl-, phenylalanyl-, N-formylmethionyl-, tyrosyl-, glutamyl-, aspartyl-, prolyl-, lysyl-, histidyl-, and seryl-tRNAs. It is interesting that nearly the same list of amino acyl-tRNAs were found to be uniquely mitochondrial by Epler (1969) studying *Neurospora* mitochondrial tRNAs by RPC-2 chromatography, except prolyl-tRNA was not reported by Epler while arginyl- and threonyl-tRNAs were. Also of interest is the demonstration by Suyama et al (1974) that valyl- and lysyl-tRNAs from *Tetrahymena* mitochondria do not hybridize to mtDNA.

One possibly conflicting result concerning the uniqueness of mitochondrial amino acyl-tRNA synthetases has been reported by Gross and co-workers. In the process of studying the biosynthesis of leucine and its control in *Neurospora* (Gross, 1965), a mutant was isolated and characterized as being at the leu-5 locus, presumably involved in the leucyl-tRNA synthetase production (Prinz & Gross, 1967). This mutant produces a cytosolic leucyl-tRNA synthetase with a reduced affinity for leucine but not tRNA, the mitochondrial enzyme activity is completely lost (Gross et al, 1968). This effect on both enzymes suggests, assuming the mutation to be a single point mutation, that one gene (leu-5) codes for at least one subunit of both cytosol and mitochondrial leucyl-tRNA synthetase. This idea was strengthened by a further study (Weeks & Gross, 1971) which indicated that a reversion at the leu-5 locus resulted in not only the recovery of mitochondrial leucyl-tRNA synthetase activity, but also an increased affinity of the cytosolic enzyme for leucine. These studies are difficult to interpret in terms of dual location of unique enzymes. Of course it is possible that the mitochondrial and

cytosol enzymes only share one subunit, but this subunit seems to be involved directly in activity of the enzyme. As already discussed, the *Tetrahymena* mitochondrial leucyl-tRNA synthetase is immunologically different from the cytosolic enzyme (Chiu & Suyama, 1973). Since Gross and co-workers have been able to isolate only one such mutant, it is possible that the results are due to a pleotropic effect of a point mutation. For example, perhaps the cytosolic leucyl-tRNA synthetase controls in some way the synthesis of the mitochondrial enzyme. Also, since *Neurospora* is obligatorily aerobic (Schwab, 1973), the activity of the mitochondrial synthetase must not be zero; otherwise, mitochondrial protein synthesis would be inhibited and growth would cease. Further genetic studies are necessary to clarify this situation.

In summary, it is clear that the mitochondria of all organisms examined contain at least some specific amino acyl-tRNAs and their synthetases. For yeast, the number of species of mt-tRNA transcribed from mtDNA approach a full complement, however the number of genes for each amino acyl-tRNA species has not been determined. As yet, the presence of mt-tRNAs and the number of species are not inconsistent with the number of mitochondrial 4S RNA genes determined by hybridization experiments. Much work remains to be done in this field, and as will be reported below and in Chapter II this thesis investigates some aspects of this problem.

c) mtmRNA

Evidence is accumulating that specific subunits of some mitochondrial enzymes (cytochrome c oxidase, cytochrome b and the ATPase

complex) are synthesized on mitochondrial ribosomes. It is not clear, however, what the origin of the mRNA for the mitochondrially-synthesized subunits is. Indeed, it might be that the role of the mitochondrial genome is to produce the mtrRNAs and some mt-tRNAs for a protein synthesizing apparatus which merely translates nuclearly-coded mRNAs. Experiments by Swanson (1971) and later by Gaitskhoki et al (1973) demonstrated that isolated mitochondria could take up exogenous RNA. Swanson showed *Xenopus* mitochondria could take up polyuridylic acid and translate it, and Gaitskhoki et al showed that rat liver mitochondrial ribosomes can become associated with added nuclear RNA. Dimitriadis and Georgatosos (1974) demonstrated that exogenous reticulocyte RNA can stimulate globin synthesis in isolated *Tetrahymena* mitochondria. The interpretation of these results is difficult since mitochondria synthesize specific proteins and certainly do not synthesize globin (Schatz & Mason, 1974).

Very recently, studies from the laboratories of Penman, Attardi and Avadhani have demonstrated at least some autonomy of the mitochondrial protein synthesizing machinery; however, their results differ enough to have made the situation unclear and very controversial. Perlman et al (1973) demonstrated an RNA fraction associated with mitochondrial protein synthesizing structures in HeLa cell mitochondria. This RNA fraction was found to contain a short polyadenylate (poly(A)) sequence previously thought to exist only in the cytosolic, nuclear or viral RNA in eukaryotic cells, but not in prokaryotic RNA. The poly(A) sequence was found to be shorter (approximately 60 nucleotides) than the corresponding poly(A) of the cytosolic mRNA (cytmRNA) (approximately 160 nucleotides). In later studies the poly(A) in HeLa cell mitochondria was found to be at the 3'

end of the RNA. The poly(A)-containing RNA could be resolved into eight species (also, similar species could be observed in hamster cell mitochondria as well as insect mitochondria) and it could be released from polysome-like structures with puromycin, suggesting an mRNA function (Hirsh & Penman, 1973; Hirsh et al, 1974; Hirsh & Penman, 1974a;b). Similar studies have been reported by Ojala and Attardi (1974a;b;c) also studying HeLa cell mitochondrial poly(A)-containing RNA. They have shown that the mitochondrial poly(A)-containing RNA resolves into eight species. One of these, the smallest, hybridizes to the L-strand of mtDNA while the others hybridize to the H-strand suggesting that they are coded by mtDNA. Ojala and Attardi (1974a) also find a "free" poly(A) fragment in HeLa cell mitochondria, but the significance of this is unknown.

Avadhani and co-workers studying Lettré Ehrlich ascites cells (mouse) also have reported poly(A)-containing RNA from mitochondria (Avadhani et al, 1973a;b; 1974). The synthesis of this RNA, however, is not inhibited by ethidium bromide but is by actinomycin D, suggesting a nuclear origin. The RNA is not synthesized in isolated organelles *in vitro* and does not hybridize to mtDNA but does to nuclear DNA. In the polysomes prepared from mitochondria from cells labelled with [^{3}H] ATP and [^{3}H] CTP an mRNA-like fraction can be isolated, at least some species of which contain poly(A). This RNA can be resolved into six species by polyacrylamide gel electrophoresis (similar to the results of Penman and of Attardi). The RNA labelled *in vitro*, on the other hand, resolves into three species none of which contain poly(A).

Avadhani and co-workers interpret these results to suggest

mitochondria translate proteins from mRNA, some of which is of nuclear origin and contains poly(A) and some of which is transcribed from mtDNA and which is without poly(A). This interpretation depends largely on the results obtained with isolated mitochondria. Poly(A) synthesis has recently been observed in mitochondria isolated from rat liver and Erlich ascites cells (Aujame & Freeman, 1975) suggesting that the results of Avadhani should be re-examined.

Poly(A)-containing RNA has also been reported from yeast mitochondria (Cooper & Avers, 1974). This poly(A) was also shown to be smaller than its cytosolic counterpart, and was thought to be associated with mitochondrial polysomes. Recently, however, the existence of poly(A)-containing RNA in yeast mitochondria has been challenged (Groot et al, 1974) and this situation remains unresolved.

It seems very likely, then, that mitochondrial protein synthesis utilizes mRNA coded by mtDNA. Ojala and Attardi (1974c) calculated from the apparent molecular weights of the eight presumptive mtmRNAs, the mtrRNAs and mt-tRNAs that 70% of the coding capacity of mtDNA has been used. Whether or not mitochondria also utilize mRNA of nuclear origin remains to be established. Mahler and Dawidowitz (1973) reported that in a mutant of yeast known to be temperature-sensitive for nuclear mRNA production, mitochondrial protein synthesis continued normally when cytmRNA synthesis was inhibited at the non-permissive temperature, suggesting mitochondrial protein synthesis does not utilize exogenous mRNA.

Mitochondrial Protein Synthesis

The existence of a mitochondrial specific protein synthetic apparatus has been continually referred to above. While other findings in the field of mitochondrial biogenesis have been just as significant, it was the original observations of incorporation of labelled amino acids by isolated organelles (McLean et al, 1958; Rendi, 1959; Reis et al, 1959; Mager, 1960; Roodyn et al, 1961) and the confirmation of these observations (Kroon et al, 1967; Beattie et al, 1967a) which stimulated most subsequent investigations in this field. Although it is now possible to study mitochondrial protein synthesis *in vivo*, analysis *in vitro* is still important. I shall discuss first some of the results obtained *in vitro* and then turn to a consideration of mitochondrial protein synthesis *in vivo*. I shall conclude this introduction by considering an alternative approach to conventional analysis of mitochondrial protein synthesis *in vivo*, through the use of a conditional mutant of mammalian cells. Mitochondrial protein synthesis has been the topic of an excellent recent review by Schatz and Mason (1974).

i) *Mitochondrial protein synthesis in vitro*: Interest in the subcellular sites of protein synthesis led to the observation that mitochondria isolated from rat liver (McLean et al, 1958; Reis et al, 1959; Roodyn et al, 1961; Truman & Korner, 1962; Kroon, 1963a) and from rat muscle (McLean et al, 1958) were capable of incorporation of labelled amino acids into protein *in vitro*. The main conclusions from these investigations were that the incorporation was dependent on ATP (or oxidizable substrates and ADP), it was resistant to RNase and it was highest into

the insoluble protein fraction. Other studies in the late 1950's demonstrated an aspect of mitochondrial protein synthesis which was to become very important, the observation that mitochondrial, but not microsomal protein synthesis was inhibited by chloramphenicol (Rendi, 1959; Mager, 1960).

The incorporation observed for the isolated mitochondrial preparations could easily have been due to microsomal or bacterial contamination. The studies by McLean et al (1958) and Roodyn et al (1961) seem to rule out the former, based on the resistance of the incorporation to RNase and the low level of microsomal contamination as measured by the absence of specific enzymes. The possibility of bacterial contamination could not be excluded, however. The inhibition of the mitochondrial protein synthesis by chloramphenicol, an antibiotic specific for bacterial protein synthesis (Vazquez, 1974), might suggest that the mitochondrial protein synthesis was actually due to the presence of bacteria in the mitochondrial preparation. Studies such as those by Beattie et al (1967a) and Kroon et al (1967) demonstrated that even mitochondria prepared under sterile conditions were active in protein synthesis. By the time the International Symposium on Biochemical Aspects of the Biogenesis of Mitochondria was held in 1967, little doubt existed that mitochondrial protein synthesis was not artifactual.

Extension of the observations of Rendi (1959) and Mager (1960) on the inhibition of mitochondrial protein synthesis by chloramphenicol soon led to the use of the antibiotic to distinguish mitochondrial and cytosolic protein synthesis. Kroon (1963a;b) and Kalf (1963) confirmed the inhibitory effects of chloramphenicol on mitochondrial protein synthesis. Kroon (1965) extended these results to include the inhibition

of protein synthesis in sub-mitochondrial particles by chloramphenicol and puromycin, suggesting the protein synthesis is ribosome mediated (see also Wintersberger, 1965). Another basic difference between mitochondrial and cytosolic protein synthesis is that, while cytosolic protein synthesis is inhibited by low concentrations of cycloheximide, the mitochondrial process is resistant to even high concentrations (Loeb & Hubby, 1968; Ashwell & Work, 1968; Beattie et al, 1967b). The differential effects of cycloheximide and chloramphenicol have become extremely important tools in the study of mitochondrial protein synthesis both *in vitro* and *in vivo*.

Mitochondrial protein synthesis in yeast is also sensitive to chloramphenicol (Wintersberger, 1965); this similarity to bacterial protein synthesis has been extended to include inhibition by other bacterial-specific antibiotics such as lincomycin, erythromycin, spiramycin, carbomycin and oleandomycin (Lamb et al, 1968). The sensitivity of mitochondrial but not cytosolic protein synthesis to these antibiotics strongly supports the endosymbiotic hypothesis for the evolutionary origin of mitochondria (Margulis, 1970); that is, mitochondrial protein synthesis seems to have "retained" a prokaryotic character. Mammalian mitochondria do not show sensitivity to antibiotics such as erythromycin or lincomycin (Firkin & Linnane, 1969). While Linnane and co-workers originally believed (and possibly still do (Towers et al, 1973)) that this represented another phylogenetic difference between fungi and animal mitochondria, the reason for the lack of inhibition of mammalian mitochondrial protein synthesis seems to be due to a permeability barrier of the mitochondrial membrane (Kroon

& DeVries, 1971; DeVries et al, 1973; Ibrahim et al, 1974).

The nature of the proteins synthesized by mitochondria *in vitro* has been the subject of many investigations. It appears that virtually all of the incorporation of labelled amino acids is into insoluble membrane proteins and not into soluble enzymes or cytochrome c (Simpson, 1962; Roodyn et al, 1962; Beattie, 1971). This incorporation has been shown to be into proteins of the inner mitochondrial membrane only and not of the outer membrane (Beattie et al, 1967b;Neupert et al, 1967; Neupert & Ludwig, 1971). In general, the membrane proteins which are synthesized are hydrophobic in nature. Some proteins are soluble in chloroform:methanol (15-40% of the total incorporation) and have been termed "proteolipid proteins" because of their solubility characteristics (Kadenbach, 1971; Murray & Linnane, 1972; Burke & Beattie, 1973; Kadenbach & Hadvary, 1973).

Analysis of proteins synthesized in mitochondria by SDS-polyacrylamide gel electrophoresis has demonstrated that the proteins are small with apparent molecular weights in the range of 50,000 to 10,000. In rat liver mitochondria the proteins labelled *in vitro* resolve into three molecular weight classes, 40,000, 27,000 and 20,000 respectively (Burke & Beattie, 1974). Proteins labelled in isolated *Neurospora* mitochondria are resolved into six molecular weight classes (Von Ruecker et al, 1974) and an almost identical pattern is observed from yeast mitochondria (Thomas & Williamson, 1971; Poyton & Groot, 1975).

Von Ruecker et al (1974) using isolated *Neurospora* mitochondria and Poyton & Groot (1975) using isolated yeast mitochondria have recently demonstrated the synthesis *in vitro* of the three subunits of

cytochrome c oxidase previously shown by studies *in vivo* to be mitochondrially-made (see below). Both of these investigations were feasible only because of the availability of specific antisera to the cytochrome c oxidase and because of a prior knowledge of which subunits are mitochondrially-made. In other words, the *in vitro* system has not by itself been a successful method for the analysis of specific mitochondrial proteins. This failure was partly due to the low synthetic activities of the isolated organelles and partly due to the uncoupling of any cytosolic controls which are normally imposed *in vivo* (Tzagoloff et al, 1973). These drawbacks do not seem to have been serious in the recent studies on synthesis of cytochrome c oxidase components *in vitro*, and this system may become useful in future analysis of mitochondrial translation products.

ii) *Mitochondrial protein synthesis in vivo*: Analysis of mitochondrial protein synthesis *in vivo* is based on the specificities of inhibitors of translation. As already discussed, cycloheximide inhibits cytosolic protein synthesis and chloramphenicol inhibits mitochondrial protein synthesis. For most studies these two inhibitors have been employed; however, anisomycin (Lizardi & Luck, 1972), emetine (Grollman, 1966; Perlman & Penman, 1970) and pederine (Brega & Baglioni, 1971) have been used as specific inhibitors of cytosolic protein synthesis, and erythromycin has been used as a specific inhibitor of mitochondrial translation in yeast (Mason & Schatz, 1973).

The analysis is relatively straightforward. An inhibitor of cytosolic protein synthesis is added to a cell suspension, allowed to

become effective and then a labelled amino acid is added. Proteins synthesized in the presence of inhibitors of cytosolic translation are presumed to be mitochondrial (Clark-Walker & Linnane, 1966; Ashwell & Work, 1968; Hawley & Greenawalt, 1970; Brega & Baglioni, 1971; Galper & Darnell, 1971; Coote & Work, 1971). This has been confirmed in each case by the inhibition of the synthesis of these proteins by chloramphenicol and by the localization of the labelled products in the mitochondrial fraction of cell-free homogenates.

The largest drawback to the analysis *in vivo* is that one must rely on the specificity of the inhibitors. Some inhibitors, however, are known to have side effects which may affect the interpretation of the results. For example, both chloramphenicol (Freeman & Haldar, 1967; 1968; Haldar & Freeman, 1968; Freeman, 1970a) and cycloheximide (Garber et al, 1973) have inhibitory effects on respiration and emetine inhibits mitochondrial protein synthesis at higher concentrations (Ibrahim et al, 1974). Another indirect effect of the inhibition of cytosolic protein synthesis is that the cytosolic translation products seem to be necessary for continued synthesis of mitochondrial proteins (Tzagoloff et al, 1973; Mason & Schatz, 1974). Tzagoloff and co-workers (for references see Tzagoloff et al, 1973) have found that one can stimulate the synthesis of yeast mitochondrial proteins *in vivo* by first inhibiting mitochondrial protein synthesis with chloramphenicol for a few hours, then removing the chloramphenicol, adding cycloheximide, and labelling the mitochondrial proteins. The interpretation of such results is that prior inhibition of mitochondrial translation allows the accumulation of cytosolic proteins; when the mitochondrial proteins are subsequently labelled, the pool of

cytosolic proteins is sufficiently large to allow a greater extent of mitochondrial synthesis than in untreated cells.

Despite the difficulties outlined above the analysis *in vivo* of mitochondrial translation products has been very successful. Studies in HeLa cells using pederine to inhibit cytosolic protein synthesis (Brega & Baglioni, 1971) have demonstrated that the mitochondrial proteins labelled *in vivo* are incorporated into the inner mitochondrial membrane. Galper & Darnell (1971) showed that in cycloheximide inhibited HeLa cells the mitochondrial products have apparent molecular weights of 50,000 to 10,000 (Galper, 1970) and that mitochondrial protein synthesis accounts for 20% of the total mitochondrial protein. Lederman and Attardi (1973) extended these results with emetine inhibited HeLa cells to show that the translation products *in vivo* have electrophoretic properties idential to mitochondrial proteins synthesized *in vitro*. Coote and Work (1971) have also demonstrated the similarity of mitochondrial proteins synthesized *in vivo* and *in vitro*. BHK-21 cells labelled *in vivo* in the presence of emetine and hamster liver mitochondria labelled *in vitro* gave essentially the same profile when mitochondrial proteins were analyzed on SDS-polyacrylamide gels, with molecular weights of 50,000 to 10,000. In an attempt to assign a function to some of the mitochondrial proteins labelled *in vivo*, Weiss et al (1972) purified cytochrome c oxidase from *Locusta migratoria* labelled in the presence and absence of cycloheximide. The results suggest that only one peptide (19,000 daltons) is synthesized within the mitochondria, while the other six peptides of the purified enzyme do not become labelled in the presence of cycloheximide. This result does not mean that the other six peptides are

synthesized in the cytosol (Schatz & Mason, 1974). One should show that their synthesis is resistant to chloramphenicol.

The situation in the fungi with respect to the nature of the mitochondrially-synthesized proteins is much more advanced (Schatz & Mason, 1974). It is beyond the scope of this introduction to go into an extensive review of this area. The results concerning the translational origin of subunits of cytochrome c oxidase, cytochrome b and the ATPase complex are, however, worthy of discussion.

Mitochondrial ATPase is a large enzyme complex which has been extensively studied biochemically for its role in oxidative phosphorylation (Racker, 1970). Tzagoloff and co-workers, studying the purified enzyme from yeast, have been interested in the site of synthesis of the subunit peptides. The ATPase complex consists of three components: ATPase (catalytic component, termed F_1), OSCP (oligomycin sensitivity conferring protein) and a membrane component consisting of four proteins (Tzagoloff & Meagher, 1972; Tzagoloff et al, 1974). This latter component has been shown to be synthesized in the mitochondria and consists of hydrophobic proteins with apparent molecular weights of approximately 30,000; 20,000; 20,000 and 7,000 (Schatz & Mason, 1974). The lowest molecular weight mitochondrially-synthesized subunit has been isolated in pure form and found to contain at least 14 amino acids, 76% of which are non-polar (Tzagoloff et al, 1974). Preliminary results from Jackl and Sebald (1974) suggest a similar situation exists in the ATPase complex from *Neurospora* mitochondria.

Cytochrome b of *Neurospora* is a component of the mitochondrial electron transport chain with an apparent molecular weight of 60,000 to

55,000. Although cytochrome b was originally believed to consist of three subunits with apparent molecular weights of 32,000; 11,000 and 10,000 (Weiss, 1972), it now appears that cytochrome b is a dimeric protein with two subunits each with a molecular weight of 30,000 (Weiss & Ziganke, 1974a;b). The site of translation of at least one of the 30,000 dalton subunits appears to be in the mitochondria since its synthesis is resistant to cycloheximide and sensitive to chloramphenicol (Weiss & Ziganke, 1974a;b). However, more recent experiments suggest that a cytosolic protein is one of the 30,000 dalton subunits as well. The synthesis of this cytosolic protein is difficult to observe in the presence of chloramphenicol, perhaps due to an effect of chloramphenicol on assembly of cytochrome b (Weiss, personal communication). Cytochrome b, therefore, may be a dimeric protein consisting of one mitochondrially-synthesized subunit and one cytosol-synthesized subunit.

The cytochrome c oxidase enzymes of both yeast and *Neurospora* are the best characterized protein complex with subunits of mitochondrial origin (Schatz & Mason, 1974). In both organisms the enzyme consists of seven polypeptides. In yeast the apparent molecular weights are: I, 40,000; II, 33,000; III, 22,000; IV, 14,000; V, 12,700; VI, 12,700; and VII, 4,600 (Poyton & Schatz, 1975a) and in *Neurospora* they are only slightly different (Sebald et al, 1973). In both organisms the three largest subunits are mitochondrially-synthesized and are present in equimolar amounts (Mason & Schatz, 1973; Weiss et al, 1971; Sebald et al, 1972; 1973). Amino acid composition of the two largest subunits (mitochondrially-synthesized) indicates that those subunits are relatively hydrophobic (Sebald et al, 1973; Poyton & Schatz, 1975a).

Although eight proteins have been identified as being synthesized in the mitochondria of yeast (and at least four in *Neurospora*), little is known about the function of these proteins. Poyton and Schatz (1975b) have demonstrated that an antisera prepared against subunit II of yeast cytochrome c oxidase can inhibit enzyme activity, suggesting that a mitochondrially-synthesized protein might be involved in enzyme activity. Antisera against subunit III of *Neurospora* cytochrome c oxidase cannot inhibit the enzyme (Werner, 1974).

No firm evidence is yet available to establish whether or not any of the eight mitochondrially-synthesized proteins are coded for by mtDNA. Interestingly enough, eight species of mtmRNA have been identified in HeLa cell mitochondria (see above, mtmRNA section). Assuming that all eight of the proteins thus far demonstrated as mitochondrial translation products are unique and are coded for by mtDNA, one can make a calculation as to how much DNA is necessary to code for these proteins. Table IV, taken from the review by Schatz and Mason (1974), summarizes the amount of mtDNA (in daltons) required to code for the eight proteins as well as mtrRNA and mt-tRNA (taking 20 as an average number of mt-tRNAs at a molecular weight of 28,000). Comparing the total amount of mtDNA required (6.84×10^6 daltons) with the molecular weight of mtDNA from animals (10^7 daltons) it is obvious that 30% of the mitochondrial genome remains, either to code for more tRNAs and/or proteins or to serve ancillary functions such as in control of replication or transcription.

TABLE IV

Can Animal mtDNA Code for all Mitochondrially Synthesized Proteins?

Mitochondrial product	Molecular weight	Daltons of double-stranded DNA required for coding
Large rRNA (17S-16S)	0.56×10^6	1.12×10^6
Small rRNA (13S-12S)	0.33×10^6	0.66×10^6
20 tRNAs	0.56×10^6	1.12×10^6
Cytochrome c oxidase subunits	4×10^4	0.80×10^6
	3×10^4	0.60×10^6
	2×10^4	0.40×10^6
ATPase complex subunits	3×10^4	0.60×10^6
	2×10^4	0.40×10^6
	2×10^4	0.40×10^6
	7×10^3	0.14×10^6
Cytochrome b subunit	3×10^4	0.60×10^6
		Total: 6.84×10^6

From Schatz and Mason (1974)

The Study of Mitochondrial Protein Synthesis in a Conditional Mutant

The study of biological systems has been greatly facilitated through the use of specific mutants. While it has been much easier to manipulate the genetics of microbes for such studies due, in part, to their haploid genomes and rapid growth rates, the study of biochemical genetics in higher organisms has recently grown considerably (Davidson, 1974). One type of mutant, the conditional mutant, has been particularly useful in biochemical studies since one can easily manipulate its phenotype by controlling some environmental factor such as temperature.

Recently, Thompson et al (1973) described a mutant of Chinese hamster ovary cells (CHO), designated tsH1, which was shown to be temperature-sensitive. This lesion resulted in the rapid cessation of protein synthesis when the growth temperature of the cell was shifted from the permissive temperature (34°C) to a non-permissive temperature ($\geq$38.5°C). An enzyme extract from tsH1 containing leucyl-tRNA synthetase was found to be sensitive to temperature when assayed *in vitro*, while that from the wild type (CHO) was not, implicating the leucyl-tRNA synthetase enzyme as the altered gene product of the mutation.

In terms of the study of mitochondrial biogenesis, it would be of interest to examine the tsH1 cell line to answer the following questions: (a) Does mitochondrial protein synthesis share the same temperature sensitivity as cytosolic protein synthesis? (b) If not, can temperature be used to specifically inhibit cytosolic protein synthesis? (c) As has been suggested for *Neurospora* (Weeks & Gross, 1971), are the mitochondrial and cytosolic leucyl-tRNA synthetases both altered by one mutation? and (d) How dependent is mitochondrial metabolism (protein,

RNA and DNA synthesis) on concomitant cytosolic protein synthesis? Studies aimed at answering, at least in part, questions (a), (b) and (c) are described below.

METHODS

Growth and Maintenance of Cells

The wild type Chinese hamster ovary (CHO) cell line and the tsH1 mutant were maintained in suspension culture in α-minimal essential medium (Stanners et al, 1971) supplemented with 10% fetal calf serum as described by Thompson et al (1973). The growth temperature for both was usually 34°C, although CHO was occasionally grown at 37°C. Both CHO and tsH1 were generous gifts of Dr. C.P. Stanners, The Ontario Cancer Institute.

Measurement of Protein Synthesis in Whole Cells

CHO or tsH1 cells were centrifuged at 1000xg for 5 min in an International PR-6 refrigerated centrifuge at 0°C, washed once in leucine-free minimum essential medium (Joklik's modification) and resuspended in the same medium at 10^7 or 2 x 10^7 cells/ml. The suspension was placed in a shaking-waterbath at 34°C or 40°C in the presence or absence of 100 µg tevenel/ml. Temperature for these and subsequent experiments was monitored with a Mettler TM 15 thermister and was always 33.8°C ± 0.2°C or 40.2 ± 0.2°C.

The cell suspension was preincubated for 10 min and then either L-[4,5-^{3}H]leucine (5 µCi/ml, 38Ci/mmole) or L-[U-^{14}C]leucine (1 µCi/ml, 348 mCi/mmole) added. Samples of 0.1 ml were removed at various times up to 30 min, placed onto Whatmann 3MM filter discs (2.3 cm) and then placed into ice cold 5% TCA containing 1 mg D,L-leucine/ml. At the end

of the incubation, the 5% TCA containing the filter discs was heated to 90°C for 20 min. The 5% TCA was then decanted and the discs washed two times with 5% TCA containing 1 mg D,L-leucine/ml at 0°C for 10 min, once with ethanol:ether (1:1) for 5 min, and once with ether for 5 min (Mans & Novelli, 1961). Finally, the discs were allowed to dry and then placed in a toluene-based scintillation fluid and the acid-insoluble radioactivity measured in a Nuclear Chicago Mark I Scintillation Spectrophotometer.

Measurement of Protein Synthesis by Isolated Mitochondria

Approximately 4 x 10^8 cells (tsH1 or CHO) were centrifuged at 1000xg for 5 min in an International PR-6 refrigerated centrifuge at 0°C. The cells were then washed twicewith 200 ml phosphate buffered saline (PBS), once with 40 ml Medium B and then suspended in 10 ml of Medium B and disrupted with an ultra-turrax as described by Freeman (1965). The mitochondria were then isolated as described by Lederman and Attardi (1970). Mitochondria were suspended in ST buffer and preincubated for 5 min by shaking in a water bath at either 34°C or 40°C in the presence or absence of tevenel (final concentration 100 µg/ml). An incubation medium containing all the components necessary for protein synthesis *in vitro* excluding the mitochondrial protein was also preincubated at 34°C or 40°C. Each incubation was initiated by combining mitochondrial suspension and incubation medium. Two incubation media were used. Medium 1 (Fig. 3A) contained in the final incubation: 1-2 mg mitochondrial protein/ml, 50 mM KCl, 20 mM potassium phosphate, 5mM $MgCl_2$, 100 mM sucrose, 10 mM sodium succinate, 2 mM ADP, 0.1 mM of all the amino acids

except leucine, 300 µg cycloheximide/ml to inhibit any residual cytosolic protein synthesis (Costantino & Attardi, 1973) and 17 µCi L-[4,5-^{3}H]-leucine (38 or 53 Ci/mmole)/ml,pH 7.2 (Haldar & Freeman, 1969). Medium 2 (Fig. 2B) was the same as Medium 1, except the ADP and succinate were replaced with 10 mM phosphoenolpyruvate, 2 mM ATP and 25 µg pyruvate kinase/ml (Spolsky & Eisenstadt, 1972). The kinetics of protein synthesis was measured by 50 µl aliquots at various times and placing them on filter discs. The filter discs were treated as described above. The incorporation was found to be inhibited about 75% by 100 µg tevenel/ml, indicative of mitochondrial specific protein synthesis (Freeman, 1970b).

Labelling of Mitochondrial Protein *in Vivo*

TsH1 cells, at a density of 2 x 10^{5} cells/ml were labelled overnight with L-[4,5-^{3}H]leucine (1 µCi/ml, 2.5 mCi/mmole). The cells were harvested, washed once with leucine-free minimum essential medium and suspended in the same medium at a density of 10^{7} cells/ml. Each 10 ml incubation was preincubated for 10 min at 34°C or 40°C in the presence or absence of cycloheximide (300 µg/ml) or tevenel (100 µg/ml) as described in the legends to the Tables and Figures. L-[U-^{14}C]leucine (1 µCi/ml, 348 mCi/mmole) was then added and incubation continued for 30 min. Cells were then centrifuged and mitochondria prepared by differential centrifugation as described by Freeman (1965). In most experiments, mitochondria were further purified by suspending in 1 ml Medium B, layering the suspension over a step-gradient of 2 ml 1.0 M sucrose in 2 x TE buffer over 2 ml 1.6 M sucrose in 2 x TE buffer, centrifuging in an SW 50.1 rotor in a Beckman L2-65B ultracentrifuge at 30,000 rpm

(73,500 x g_{av}) for 60 min and recovering the mitochondria from the lower interface by diluting with Medium B and centrifuging at 10,000 x g for 10 min. The mitochondria were then suspended in 0.1 ml of polyacrylamide gel electrophoresis buffer made 1% in SDS and mercaptoethanol, boiled 3-5 min to inactivate proteases and finally dialyzed against 1 liter of the same buffer overnight at 37°C. In the experiment described in Fig. 6, the cells were not labelled overnight but were labelled with L-[4,5-^{3}H]-leucine (20 µCi/ml, 38 Ci/mmole) rather than the [^{14}C]leucine to increase the specific activity of the protein.

SDS-polyacrylamide Gel Electrophoresis

Mitochondrial proteins were analyzed on 6 x 60 mm gels containing 6% acrylamide, 0.16% bis-acrylamide in polyacrylamide gel electrophoresis buffer essentially as described by Melnick et al (1973). Parallel gels containing 5 µg each of myosin, bovine serum albumin and either creatine kinase and cytochrome c or aspartate transcarbamylase were run for molecular weight standardization as described by Weber and Osborn (1969). The marker gels were stained at 37°C for 1 hr with 0.25% Coomassie blue in 10% acetic acid, 0.25% methanol and destained first for 1 hr at 65°C with two changes of 10% acetic acid and then overnight at room temperature in 10% acetic acid.

The gels containing the labelled mitochondrial protein were fractionated into 1 mm slices on a Gilson Aliquogel Fractionator and counted for ^{3}H and ^{14}C activities in 1 ml of water and 3 ml of PCS liquid scintillation fluid (Amersham-Searle) after overnight treatment of the gel aliquots with approximately 0.4 ml of 20% NH_4OH.

Isolation of Hamster Liver Mitochondria

Mitochondria were isolated from the livers of 6 week old Syrian hamsters by the differential centrifugation technique of Haldar and Freeman (1969), except mitochondria were recovered at 5,000 x g for 10 min in order to minimize microsomal contamination (Lederman & Attardi, 1970). Mitochondria were washed once with SE medium and once with ST buffer, each time centrifuging at 5,000 x g for 10 min.

Acylation of Mitochondrial $tRNA^{leu}$

Mitochondria were incubated under the conditions for protein synthesis as described by Haldar and Freeman (1969). The incubation medium contained 15 mg protein/ml, 0.05 M KCl, 0.005 M $MgCl_2$, 0.02 M potassium phosphate, 0.1 M sucrose, 0.01 M sodium succinate, 0.002 M ADP, 10^{-4} M of each of the 19 amino acids (less leucine), 100 µg tevenel/ml to inhibit mitochondrial protein synthesis (Freeman, 1970b), and L-[4,5-^{3}H]leucine (10 µCi/ml,51 Ci/mmole), pH 7.2. Incubation was continued at 30°C for 20 min and stopped by centrifuging the mitochondria at 10,000 x g for 10 min and resuspending them in 0.10 M sodium acetate, 0.005 M magnesium acetate, pH 5.0 containing 1% SDS. This suspension was extracted twice with one volume of buffer-saturated phenol at room temperature. The total nucleic acid (containing the [^{3}H]leucyl-tRNA) was recovered by precipitation with 2 volumes of ethanol at -20°C overnight.

Acylation of Cytosolic $tRNA^{leu}$

Approximately 2×10^8 tsH1 cells were centrifuged and suspended in 20 ml leucine-free minimal essential medium containing 200 µg cycloheximide/ml and L-[U-^{14}C]leucine (0.5 µCi/ml, 348 mCi/mmole). Cells were incubated at 30°C for 20 min, centrifuged and cytosolic leucyl-tRNA extracted and recovered as described above for the mitochondrial leucyl-tRNA.

RPC-5 Chromatography

RPC-5 chromatography was performed in a 9 x 300 mm Glenco column as described by Pearson et al (1971). The acylated tRNA was applied in 0.5 M sucorse, 0.45 M NaCl and eluted at 25°C with a 200 ml linear gradient from 0.45 M NaCl to 0.7 M NaCl in 0.01 M sodium acetate, 0.01 M $MgCl_2$, 0.002 M mercaptoethanol, pH 4.5 at a flow rate of 1 ml/min. Fractions of 1.3 ml were collected directly into scintillation vials and 0.02 ml aliquots taken for determination of the concentration of NaCl by refractive index. The fractions were counted directly after the addition of 3 ml PCS scintillation fluid.

SOLUTIONS

ST buffer:

0.25 M sucrose

0.01 M tris-HCl

pH 7.1

Medium B:

0.30 M sucrose

0.002 M tris-HCl

0.002 M EDTA

pH 7.4

TE buffer (1x):

0.01 M tris-HCl

0.001 M EDTA

pH 7.4

Polyacrylamide gel electrophoresis buffer:

0.04 M tris-HCl

0.02 M sodium acetate

0.002 M EDTA

0.1% SDS

pH 7.4

SE Medium:

0.3 M sucrose

0.002 EDTA

pH 7.2

MATERIALS

The source of most materials is tabulated below:

Materials	Source
acrylamide	Eastman Organic Chemical Co., Rochester, N.Y.
ADP	P-L Biochemicals, Milwaukee, Wis.
aspartate transcarbamylase	Dr. W.W. Chan, McMaster Univ.*
ATP	Boehringer Mannheim Corp., N.Y., N.Y.
bisacrylamide	Eastman Organic Chemical Co.
bovine serum albumin	Sigma Chemical Co., St. Louis, Mo.
Coomassie blue	Sigma Chemical Co.
creatin kinase	Worthington Biochemicals Corp., Freehold, N.J.
cycloheximide	Sigma Chemical Co.
cytochrome c	Sigma Chemical Co.
L-[4,5-^{3}H]leucine	Amersham Searle Corp., Arlington Heights, Ill.
L-[U-^{14}C]leucine	Amersham Searle Corp.
myosin	Dr. R. Keates, McMaster Univ.*
N,N,N',N'-tetramethyl-ethylenediamine	Eastman Organic Chemical Co.
PCS scintillation fluid	Amersham Searle Corp.
phosphoenol pyruvate	Calbiochem, La Jolla, Calif.
pyruvate kinase	Calbiochem
RPC-5	Astro Enterprises, Inc., Powell, Tenn.
tevenel	Dr. C.E. Hoffmann, E.I. DuPont de Nemours & Co. Incorp., Newark, Del.*
tissue culture supplies	Grand Island Biological Co., Grand Island, N.Y.

* Gift.

All other reagents and chemicals were highest quality available.

RESULTS

Whole Cell Protein Synthesis in tsH1

In the characterization of the tsH1 cell line, Thompson et al (1973) reported that, while protein synthesis was rapidly inhibited when the cells were shifted to a non-permissive temperature, even at the very high temperature of 40.5°C, protein synthesis continued at about 1% of the rate at 34°C. It seemed very possible, because of the known uniqueness of the mitochondrial leucyl-tRNA synthetases of many organisms, that this synthesis was due to non-temperature sensitive mitochondrial protein synthesis.

Fig. 2 shows the results of an experiment designed to test the effects of tevenel, a specific inhibitor of mitochondrial protein synthesis (Freeman, 1970b; Fettes et al, 1972; Wallace & Freeman, 1975), on whole cell protein synthesis. As can be seen, tevenel (100 µg/ml) has no effect on protein synthesis at 34°C (Fig. 2A, confirming the results of Haldar and Freeman (1968b)), while the residual protein synthesis remaining at 40°C after a 10 min preincubation is inhibited by about 50% by tevenel (Fig. 2B). For comparison, the incorporation of [^{3}H]leucine into protein by CHO at 40°C is presented in the insert of Fig. 2B. Cycloheximide (300 µg/ml), a specific inhibitor of cytosolic protein synthesis (Siegel & Sisler, 1965), was never found to inhibit the residual 40°C synthesis; however, in one series of experiments it was found to stimulate this synthesis with either [^{3}H]leucine or [^{3}H]valine as precursors. This result is difficult to explain, but may be due to a complex interaction

Figure 2

Kinetics of incorporation of [^{3}H] or [^{14}C]leucine into acid-insoluble material of tsH1 cells at 34°C or 40°C. A . TsH1 ~~cells~~ were centrifuged and then suspended in leucine-free medium at 10^{7} cells/ml. Suspension of cells were incubated for 10 min at 34°C in the presence or absence of tevenel or at 40°C. L-[4,5-^{3}H]leucine (5 µCi/ml, 38 Ci/mmole) was added to each incubation and aliquots (100 µl) were taken at various time intervals for the determination of acid insoluble radioactivity as described in the Methods. o——o, Incorporation at 34°C; x——x, incorporation at 34°C in the presence of 100 µg tevenel/ml; •——•, incorporation at 40°C. B. TsH1 cells and CHO cells were centrifuged and then suspended in leucine-free medium at 2 x 10^{7} cell/ml. Suspensions of tsH1 cells were incubated for 10 min at 40°C in the presence or absence of tevenel, while CHO was incubated for 10 min in the absence of tevenel. L-[U-^{14}C]leucine (1 µCi/ml, 348 mci/mmole) was added to each and aliquots (100 µl) were taken at various times for the determination of acid-insoluble radioactivity. o——o, Incorporation at 40°C in tsH1 cells; x——x, incorporation at 40°C in tsH1 cells in the presence of 100 µg tevenel/ml; insert, incorporation at 40°C in CHO cells.

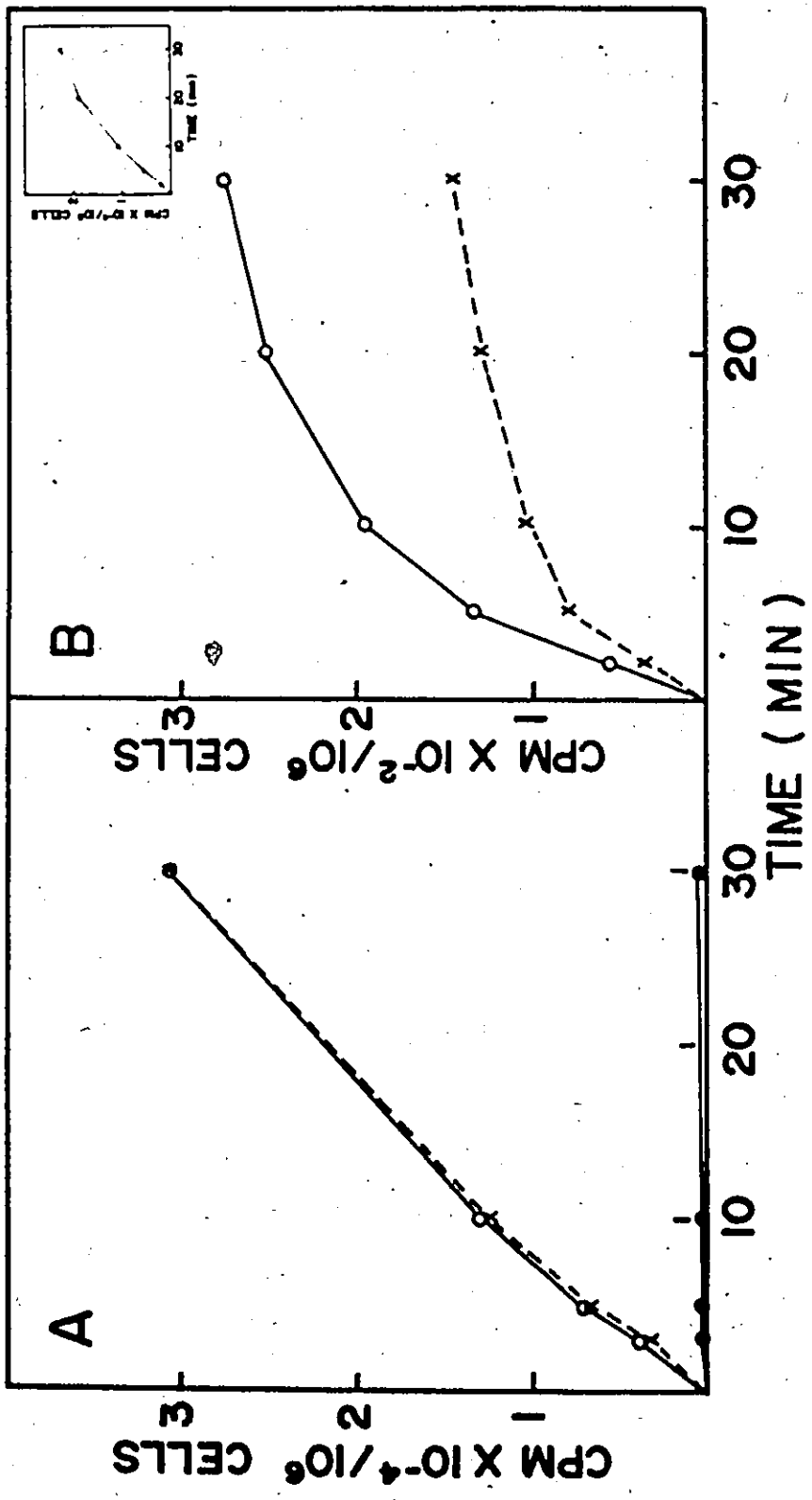

Figure 2

between the effects of cycloheximide and the effect of the mutation. The inhibition by tevenel of the residual protein synthesis at 40°C in tsH1 suggests that much if not all of this protein synthesis is mitochondrial. The fact that tevenel inhibited this synthesis by only 50%, while it inhibits mitochondrial protein synthesis *in vitro* by 75% at this concentration (Wallace & Freeman, 1975) suggests that a small amount of cytosolic protein synthesis may continue at 40°C.

In order to demonstrate mitochondrial location of the 40°C protein synthesis in tsH1, the following experiment was carried out. TsH1 cells were labelled overnight at 34°C with [^{3}H]leucine. The next morning the cells were harvested, suspended in leucine-free medium, preincubated at 40°C for 10 min and then labelled for 30 min at 40°C with [^{14}C]leucine. The cells were then disrupted and mitochondria prepared by differential centrifugation. Aliquots of the post-nuclear supernatant (1,900xg) and the post-mitochondrial supernatant (7,000xg) and the mitochondrial pellet were analyzed for their $^{14}C/^{3}H$ ratio, a reflection of specific activity. The results are presented in Table V. It can be seen that at 40°C the crude mitochondrial fraction has about a 3-fold greater $^{14}C/^{3}H$ ratio than the post-mitochondrial supernatant. In a parallel experiment it was found that further purification of the mitochondria by sucrose density gradient centrifugation increased the $^{14}C/^{3}H$ ratio of the mitochondrial fraction a further 6-fold (EXP. 2, Table V). These results strongly suggest that the mitochondria of tsH1 are the site of synthesis of protein at 40°C.

To determine whether mitochondrial protein synthesis of tsH1 cells was temperature-sensitive or not, protein synthesis by isolated

TABLE V

COMPARISON OF LABELLING OF MITOCHONDRIAL FRACTION AT 34°C AND 40°C IN tsH1

fraction	total [^{14}C]leucine incorporated		$^{14}C/^{3}H$ ratio x 10^3
	cpm	%	
EXP 1			
1900 x g supernatant	2610	100	3.2
7000 x g supernatant	1530	59	2.3
mitochondrial fraction	1100	42	7.1
EXP 2			
mitochondrial fraction[a] (step gradient)	-	-	43

TsH1 cells (1 x 10^8), labelled 16 hr with [^{3}H]leucine (1 mCi/ml, 2.5 µCi/mmole), were harvested, washed in leucine-free medium and suspended in 10 ml leucine-free medium. Cells were then preincubated at 40°C for 10 min before the addition of [^{14}C]leucine (1 µCi/ml, 348 mCi/mmole). Incubation was continued at 40°C for 30 min. Cells were then centrifuged, washed, disrupted and mitochondria isolated by differential centrifugation as described in the Methods. [a] EXP 2 was run in parallel to EXP 1, however the mitochondrial fraction was further purified by banding on a sucrose step gradient also as described in the Methods.

mitochondria from tsH1 and CHO was compared. The kinetics of [^{3}H]leucine incorporation into acid insoluble material by isolated mitochondria of tsH1 and CHO are presented in Fig. 3. It can be seen that, while the extent of protein synthesis at 40°C is less than at 34°C, the CHO and tsH1 mitochondrial protein synthesis behave identically. The reason that protein synthesis ceases after such a short time at 40°C is unclear. It is possible that oxidative phosphorylation is unstable at 40°C. This idea is supported by the results shown in Fig. 3B. Using an ATP-generating system rather than the ADP-succinate system (Fig. 3A), the extent of mitochondrial protein synthesis was enhanced at 40°C. Although protein synthesis by the isolated mitochondria continues for only about 5 min after addition of isotope at 40°C, the results of a similar experiment performed with whole cells to observe cytosolic protein synthesis at 34°C and 40°C is presented in Fig. 4. It can be seen that cytosolic protein synthesis in tsH1 is virtually completely inhibited by a 5 min preincubation at 40°C, while in CHO it continues at only a slightly slower rate at 40°C.

In order to compare the effects of non-permissive temperature and cycloheximide on the incorporation of leucine into mitochondrial protein of tsH1 cells, the following experiment was performed. Cells were labelled overnight with [^{3}H]leucine, harvested and suspended in leucine-free medium as described in Table V. Aliquots of 1 x 10^{8} cells were placed at 34°C or 40°C in the presence or absence of cycloheximide (300 µg/ml) or tevenel (100 µg/ml). Cells were preincubated for 10 min before labelling with [^{14}C]leucine for 30 min, and then mitochondria were isolated. The results of two such experiments are summarized in

Figure 3

Kinetics of incorporation at 34°C or 40°C of [^{3}H]leucine into acid-insoluble material by mitochondria isolated from tsH1 or CHO cells. Mitochondria were isolated from 4 x 10^8 tsH1 or CHO cells as described in the Methods, suspended in ST buffer and preincubated for 5 min at 34°C or 40°C before combining with Medium 1 (A) or Medium 2 (B). Aliquots (5 µl) were removed at various times for the determination of acid-insoluble material. o——o, Incorporation by mitochondria from CHO at 34°C; ●——●, incorporation by mitochondria from CHO at 40°C; Δ——Δ, incorporation by mitochondria from tsH1 at 34°C; ▲——▲, incorporation by mitochondria from tsH1 at 40°C. A. Medium 1 containing ADP and succinate. B. Medium 2 containing ATP, phosphoenol pyuruvate and pyuruvate kinase (see text).

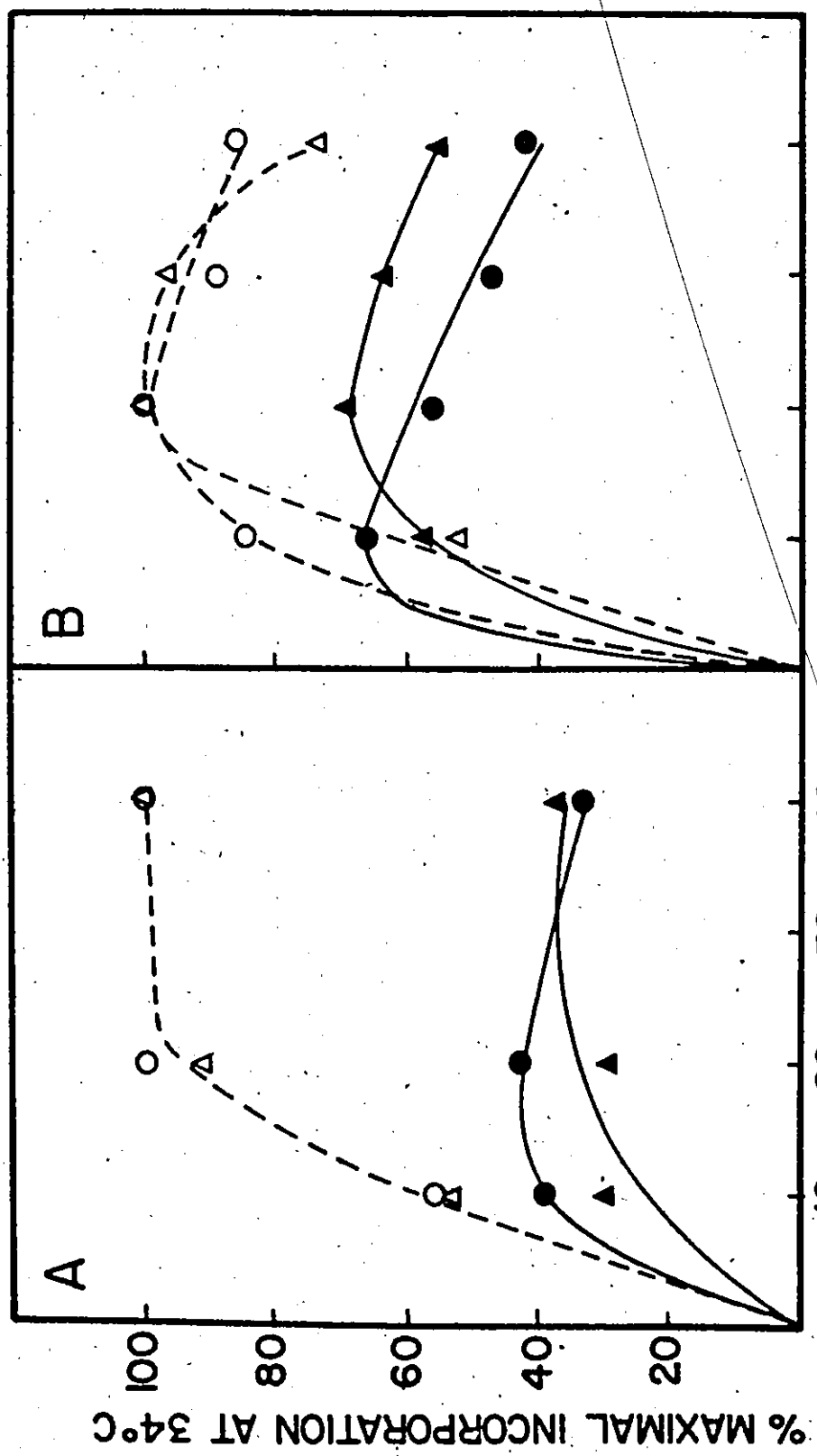
% MAXIMAL INCORPORATION AT 34°C
100
80
60
40
20
A
B
10
20
30
40
5
10
15
20
TIME (min)

Figure 3

Figure 4

Kinetics of incorporation of [^{3}H]leucine into acid-insoluble material of tsH1 or CHO cells after a preincubation at 34°C or 40°C for only 5 min. TsH1 and CHO cells were centrifuged and then suspended in leucine-free medium at 2 x 10^7 cells/ml. Suspensions of cells were incubated at 34°C or 40°C for 5 min. L-[4,5-^{3}H]leucine (17 µCi/ml, 38 Ci/mmole) was added and aliquots (100 µl) were removed at various times for the determination of acid-insoluble radioactivity. o——o, Incorporation by CHO cells at 34°C; •——•, incorporation by CHO cells at 40°C; Δ——Δ, incorporation by tsH1 cells at 34°C; ▲——▲, incorporation by tsH1 cells at 40°C.

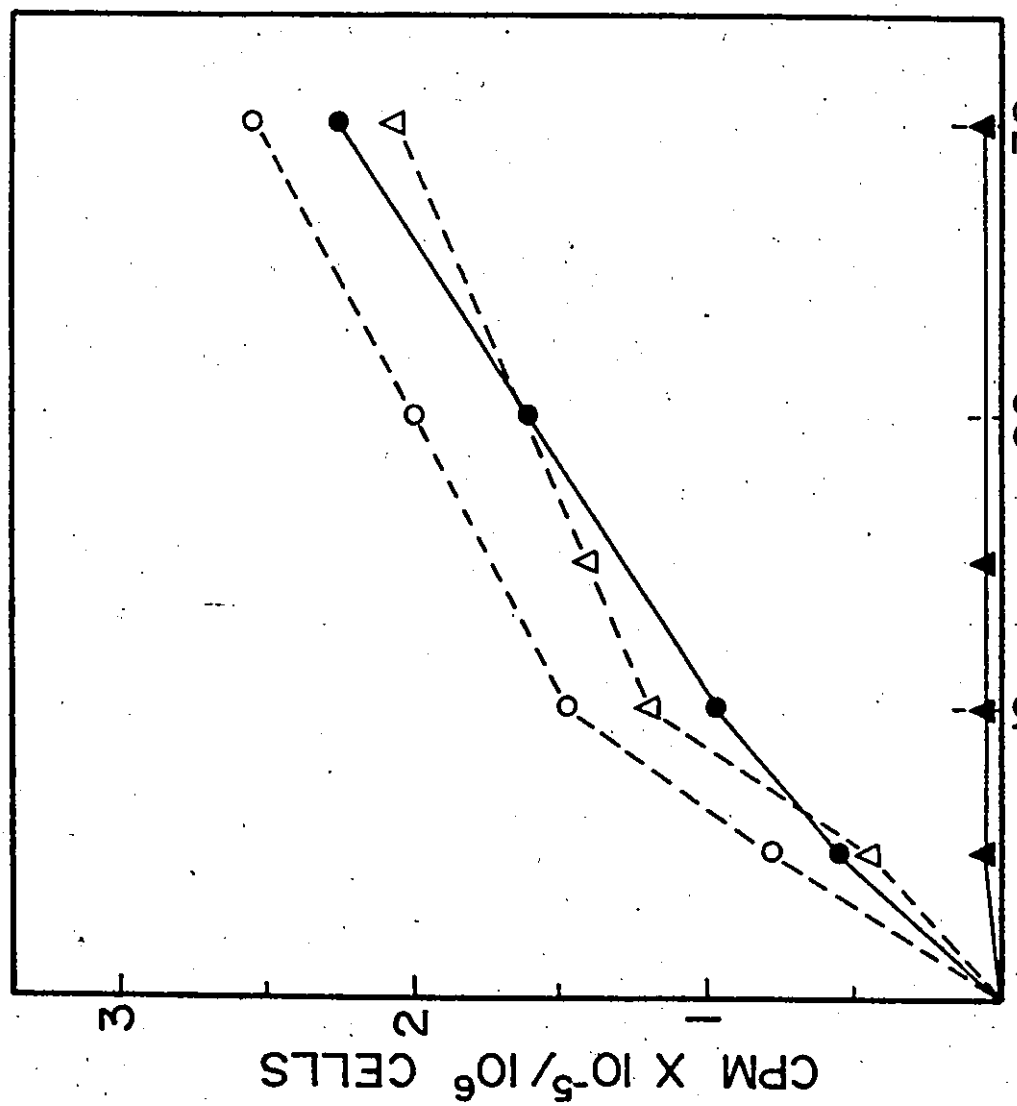

Figure 4

Table VI. It can be seen that cycloheximide treatment at 34°C inhibited the incorporation of [^{14}C]leucine by about 90%, as does labelling at 40°C, when compared to the incorporation at 34°C in the absence of cycloheximide (EXP. 1). The incorporation of [^{14}C]leucine in both these cases is presumably attributable to the mitochondrial protein synthetic apparatus. This suggestion is supported by the observation that the 40°C protein synthesis is inhibited by tevenel but not by cycloheximide (Table VI, EXP. 2).

To further characterize the nature of the proteins synthesized at 40°C in tsH1 mitochondria, the labelled mitochondrial fraction was dissolved in sodium dodecyl sulfate (SDS) and resolved by SDS-polyacrylamide gel electrophoresis as described in the Methods. Fig. 5 shows the [^{3}H] and [^{14}C] profiles of mitochondrial proteins labelled as described in EXP. 2 of Table VI. The [^{3}H] profile represents proteins labelled at 34°C overnight and the [^{14}C] profile represents the proteins labelled at 40°C. It can be seen that the profiles of cells labelled at 40°C in the presence and absence of cycloheximide are essentially identical, while in the presence of tevenel incorporation is drastically reduced. The proteins labelled at 40°C are poorly resolved. There is one group with an apparent molecular weight range of 40,000 to 20,000, and a second with a molecular weight range of 20,000 to 12,000 (Fig. 6). These results are very similar to those reported by Galper and Darnell (1971) and Lederman and Attardi (1973) for mitochondrial proteins labelled in the presence of cycloheximide and emetine respectively.

To compare the labelling of tsH1 mitochondrial proteins at the permissive temperature (34°C) in the presence of cycloheximide and at a

TABLE VI

THE EFFECT OF CYCLOHEXIMIDE TREATMENT AND TEMPERATURE ON THE INCORPORATION OF [^{14}C]LEUCINE INTO MITOCHONDRIAL PROTEIN OF tsH1

Inhibitor	Temperature (°C)	Incorporation[a] into mitochondrial protein (% control)
EXP 1		
none	34	100[b]
cycloheximide	34	11.7
none	40	9.4
tevenel	40	2.6
EXP 2		
none	40	100[c]
cycloheximide	40	96
tevenel	40	28

Each incubation represent 1 x 10^8 tsH1 cells. Labelling and preparation of mitochondria was as described in the legend to Table V, except the mitochondria were further purified on a step gradient. Cycloheximide (300 µg/ml) and tevenel (100 µg/ml) were added at the beginning of the 10 min preincubation. [a] Incorporation has been normalized to the recovery of [^{3}H]. [b] 100% represented approximately 40,000 cpm/10^8 cells. [c] 100% represents approximately 4,000 cpm/10^8 cells.

Figure 5

SDS-polyacrylamide gel profile of mitochondrial proteins of tsH1 cells labelled with [^{3}H] or [^{14}C]leucine at 34°C or 40°C. TsH1 cells were labelled overnight at 34°C with L-[4,5-^{3}H]leucine (1 µCi/ml, 2.5 mCi/mmole). 3 x 10^{8} cells were centrifuged and then resuspended in leucine-free medium at 1 x 10^{7} cells/ml. Ten ml of cell suspension was incubated for 10 min at 40°C: A, in the absence of inhibitors, B, in the presence of 300 µg cycloheximide/ml and C, in the presence of 100 µg tevenel/ml. At the end of the 10 min preincubation L-[U-^{14}C]leucine (1 µCi/ml, 348 mCi/mmole) was added and incubations continued for 30 min. Mitochondria were then isolated and the protein prepared for electrophoresis as described in the Methods. o——o, [^{3}H]leucine labelled proteins and ●——●, [^{14}C]leucine labelled proteins. Electrophoresis is from left to right and each fraction represents 1 mm of gel.

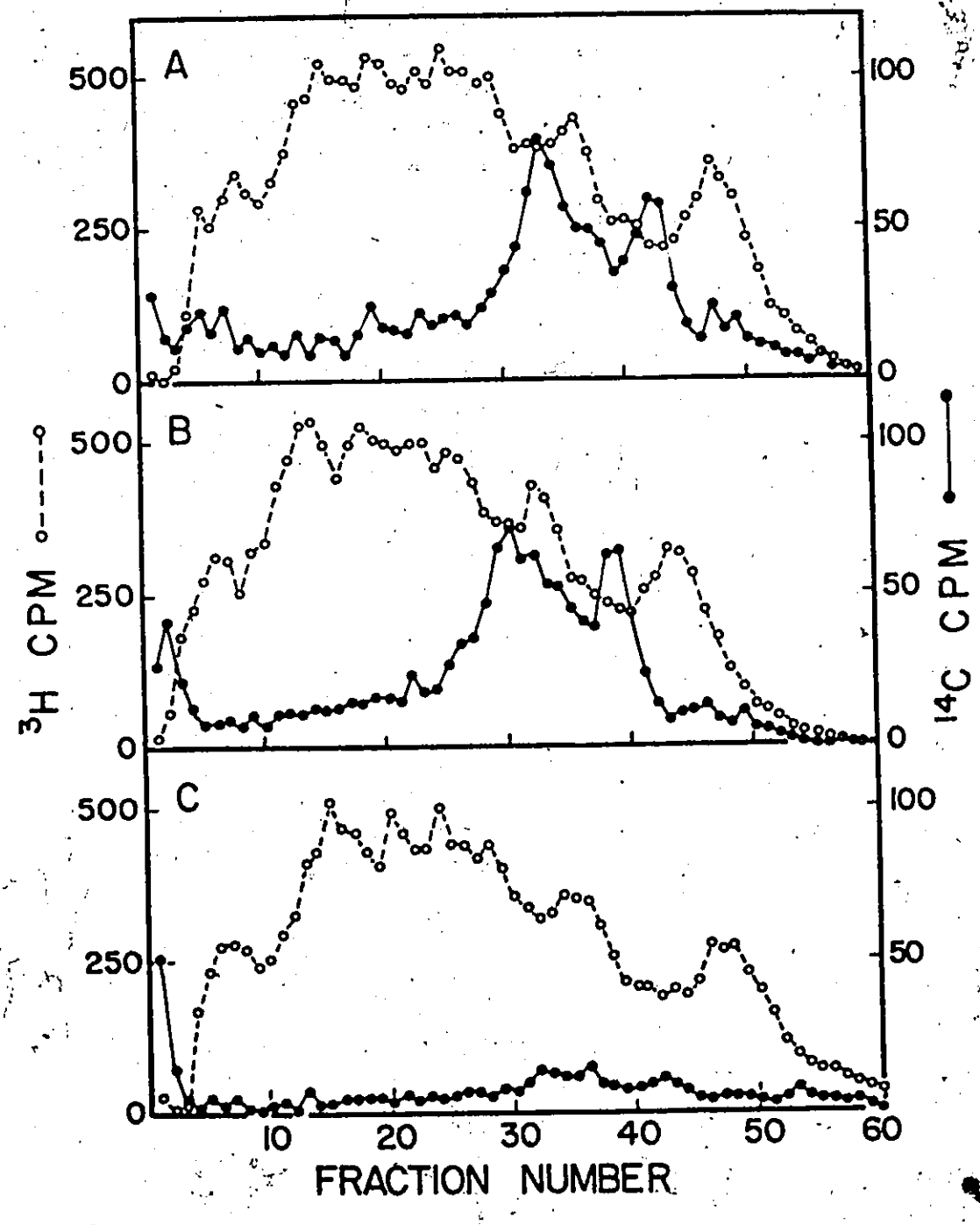
A
B
C
500
250
0
100
50
0
^{3}H CPM
^{14}C CPM
10
20
30
40
50
60
FRACTION NUMBER

Figure 5

Figure 6

SDS-polyacrylamide gel profile of mitochondrial proteins of tsH1 cells labelled with [^{3}H]leucine at 34°C or 40°C in the presence or absence of inhibitors of protein synthesis. 3 x 10^8 tsH1 cells were centrifuged and then suspended in leucine-free medium at 1 x 10^7 cells/ml. Ten ml of cell suspension was incubated for 10 min: A, at 34°C in the absence of inhibitors, B, at 34°C in the presence of 300 µg cycloheximide/ml and C, at 40°C in the presence (o——o) or absence (●——●) of 100 µg tevenel/ml. At the end of the 10 min preincubation L-[4,5-^{3}H]leucine (20 µCi/ml, 38 Ci/mmole) was added and incubation continued for 30 min. Mitochondria were isolated and the protein prepared for electrophoresis as described in the Methods. ●——● and o——o, Radioactivity; ▲——▲, plot of position of molecular weight marker proteins on a parallel gel. Electrophoresis was performed as described in Methods and the legend to Fig. 5.

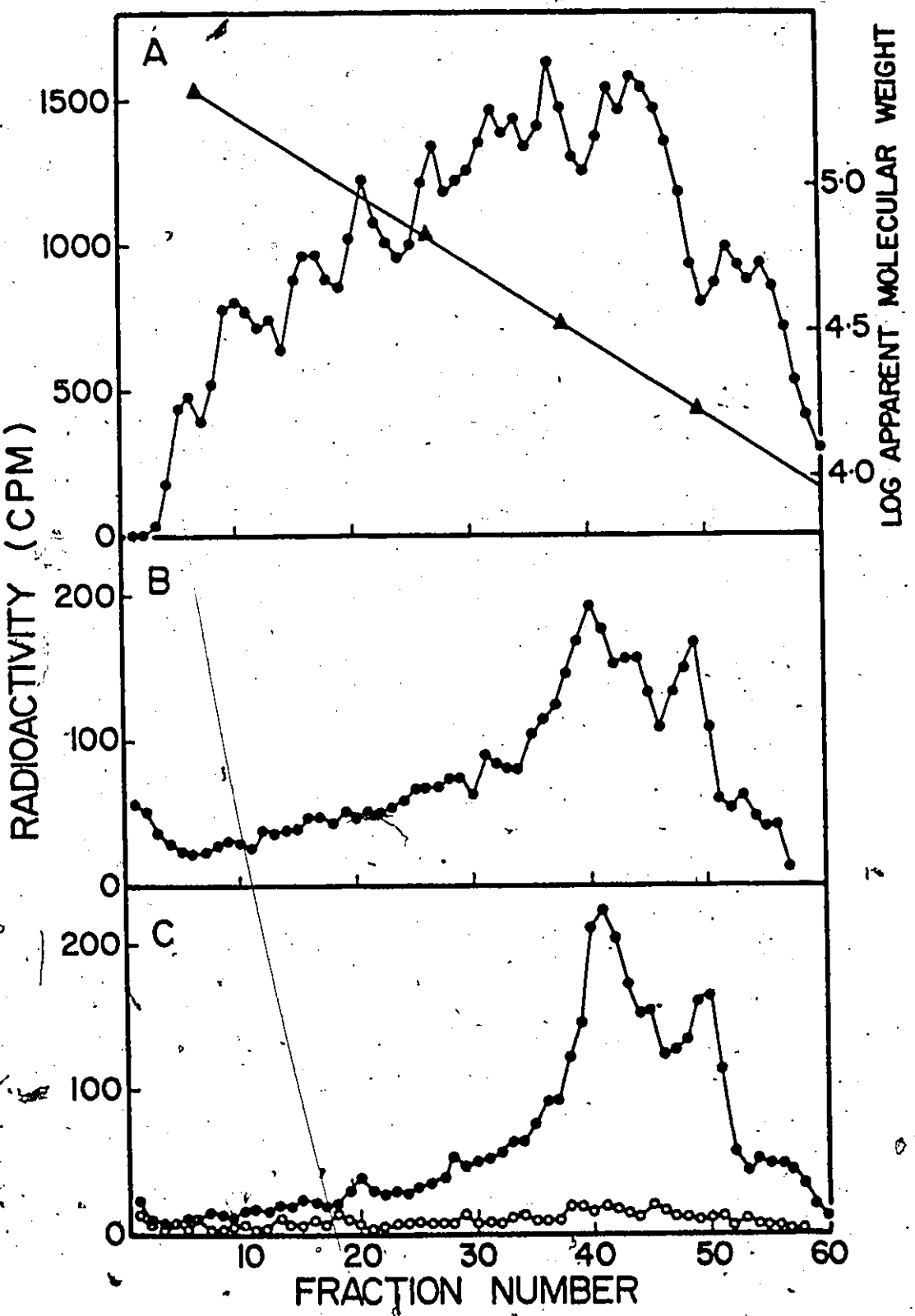
A
B
C
1500
1000
500
0
200
100
0
200
100
0
5·0
4·5
4·0
LOG APPARENT MOLECULAR WEIGHT
RADIOACTIVITY (CPM)
10
20
30
40
50
60
FRACTION NUMBER

Figure 6

non-permissive temperature (40°C) in its absence, tsH1 cells were labelled with [^{3}H]leucine at 34°C in the presence and absence of cycloheximide and at 40°C in the presence and absence of tevenel. The mitochondrial proteins were resolved in parallel SDS-polyacrylamide gels as described in Fig. 5. The results presented in Fig. 6 demonstrate that the inhibition of cytosolic protein synthesis of tsH1 by either cycloheximide or by the non-permissive temperature results in the incorporation of [^{3}H]leucine into mitochondrial proteins with the same molecular weight distributions (compare Fig. 6B and 6C). The proteins labelled at 40°C appear to be better resolved than those labelled at 34°C in the presence of cycloheximide. Also, there is less heterogeneous labelled protein in the 40°C products than in the 34°C plus cycloheximide products (compare fractions 1 to 35 in Fig. 6B and 6C). It is possible that the inhibition of cytosolic protein synthesis at 40°C is greater than with 300 µg cycloheximide/ml. The lack of total inhibition by cycloheximide has been observed previously in HeLa cells (Perlman & Penman, 1970). The total number of proteins synthesized at 40°C cannot be estimated from Fig. 6, but there are probably many protein species co-electrophoresing in the two molecular weight groups (Galper, 1970; Lederman & Attardi, 1973). Temperature seems to be an effective inhibitor of cytosolic protein synthesis in tsH1 and allows the differentiation of cytosolic and mitochondrial protein synthesis *in vivo*.

The results described above demonstrate that mitochondrial protein synthesis in tsH1 is not temperature-sensitive. This would suggest that the mitochondria contain a unique leucyl-tRNA synthetase, unaffected by the ts-mutation. This is different from the

Neurospora mitochondrial leucyl-tRNA synthetase described by Weeks and Gross (1971). A mutation affecting the affinity of the cytosolic enzyme for leucine resulted in the loss of activity of the mitochondrial enzyme, while in a "revertant", the mitochondrial enzyme activity returned. Some objections to the interpretation of Weeks and Gross have already been discussed in the introduction, but it may be that, if the mitochondrial and cytosolic enzymes in *Neurospora* do share a subunit, either they do not in mammals or if they do it is not that subunit which is affected by the mutation in tsH1.

In order to characterize the leucyl-tRNA in mammalian mitochondria, a procedure was developed to label, extract and resolve leucyl-tRNAs from whole cells and from isolated mitochondria. Although Buck and Nass (1969; 1970) have already reported that unique species of leucyl-tRNA and a unique leucyl-tRNA synthetase exist in rat liver mitochondria, it was not clear whether iso-accepting species of leucyl-tRNA existed or whether the mitochondria contained some cytosol-like leucyl-tRNAs. The following experiment suggests that iso-accepting species exist in mitochondria and that none are cytosol-like.

Mitochondria were isolated from hamster liver and incubated with [^{3}H]leucine under the conditions for mitochondrial protein synthesis, except protein synthesis was inhibited with tevenel to allow the accumulation of leucyl-tRNA. TsH1 cells were suspended in leucine-free medium and labelled at 34°C with [^{14}C]leucine in the presence of cycloheximide. Total RNA was extracted at pH 5.0 by the SDS-phenol method as described by Galper and Darnell (1969). Mitochondrial [^{3}H]leucyl-tRNA and cytosolic [^{14}C]leucyl-tRNA were then co-chromatographed on RPC-5

(reverse phase chromatography) as described by Pearson et al (1971). As can be seen in Fig. 7, four species of cytosolic (I, II, III and V) and three species of mitochondrial leucyl-tRNA (IV, VI and VII) are resolved. None of the mitochondrial leucyl-tRNAs co-elute with any cytosolic leucyl-tRNAs. These results suggest that mitochondria of hamster liver contain unique leucyl-tRNA species (at least three), while the investigations with tsH1 (also hamster) suggest that the mitochondria also contain a unique leucyl-tRNA synthetase. One important consideration is that the mitochondrial leucyl-tRNA was isolated from Syrian hamster mitochondria, while the cytosolic leucyl-tRNA was isolated from Chinese hamster cells (tsH1). It is not known whether or not this species difference will have affected the results obtained. Obviously, further work is necessary to extend these observations. It is of interest that RPC-5 chromatography also allowed the resolution of three mitochondrial leucyl-tRNAs from *Tetrahymena* (Chiu et al, 1973).

Figure 7

RPC-5 profile of [^{3}H]leucyl-tRNA from hamster mitochondria (●——●) co-chromatographed with cytosolic [^{14}C]leucyl-tRNA from tsH1 cells (o——o).

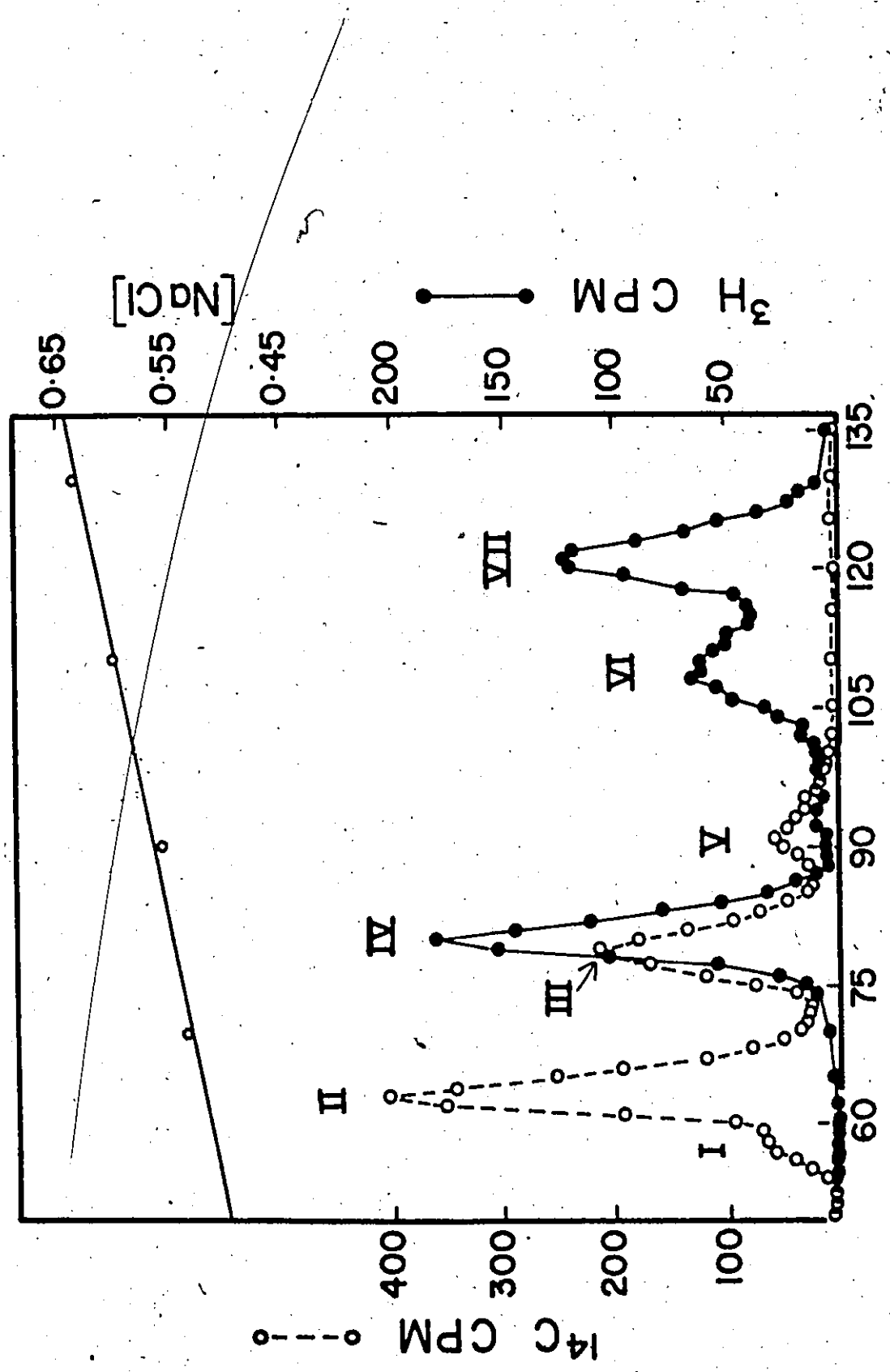
[NaCl]
0·65
0·55
0·45
^{3}H CPM
200
150
100
50
^{14}C CPM
400
300
200
100
FRACTION NUMBER
60
75
90
105
120
135
I
II
III
IV
V
VI
VII

Figure 7

DISCUSSION

As discussed in the Introduction, the objectives of this investigation were to answer questions concerning the relationship between mitochondrial and cytosolic leucyl-tRNA synthetase of tsH1 cells. While the results obtained are specific for tsH1 cells, certainly some of the conclusions to be drawn apply to all mammalian cells and their mitochondria. The approach used, that is the use of a conditional mutant to differentiate cytosolic and mitochondrial protein synthesis, could be applicable to all eukaryotic organisms, but relies on the availability of appropriate mutant cell lines and their characterization.

Returning to the questions outlined in the Introduction, the following can be stated:

a) Mitochondrial protein synthesis both *in vitro* (Fig. 4) and *in vivo* (Table VI) does not appear to be temperature-sensitive.

b) The specific effect of temperature on cytosolic protein synthesis allows the differentiation of mitochondrial and cytosolic protein synthesis previously only possible with specific inhibitors (Fig. 6)

c) No direct evidence has been obtained on the effects of inhibition of cytosolic protein synthesis on mitochondrial metabolism (cf. Storrie & Attardi, 1972). The results presented in Fig. 2, however, suggest that the rate of mitochondrial protein synthesis decays rapidly when cytosolic protein synthesis is inhibited by temperature. This is the expected result if, as suggested by Schatz and Mason (1974), mito-

chondrial protein synthesis only continues as long as a supply of cytosolic proteins, necessary for the assembly of mitochondrial components, is available. Recent results by Neupert (1974), however, suggest that mitochondrial protein synthesis is not inhibited *per se*; rather, the products do not accumulate; presumably they are degraded.

Results similar to those reported here have been obtained with two temperature-sensitive mutants of yeast (Schweyen & Kaudewitz, 1971). In these mutants, temperature-sensitive for protein synthesis, it was shown that the incorporation of [^{3}H]leucine into mitochondrial protein at 36°C (non-permissive temperature) was insensitive to cycloheximide. In comparing the incorporation into mitochondrial protein at 23°C (the permissive temperature), the 36°C incorporation accounts for 30% of the mitochondrial protein synthesis. This value seems very high, since in all systems studied, mitochondrial protein synthesis produces only about 10 - 15% of the total mitochondrial protein, as was also found for tsH1 above (Ashwell & Work, 1970a). This large discrepancy may, however, be simply a kinetic phenomenon due to the 13°C difference between the permissive and non-permissive temperatures. It may also be due to the fact that the temperature sensitivity is not complete. This is a strong possibility since incorporation into soluble protein is inhibited 95% by the temperature shift and a further 3.5% by cycloheximide. This was never observed for tsH1, although a very small residual cytosolic protein synthesis may be present at 40°C (Fig. 2). Since the report by Schweyen and Kaudewitz (1971), no further work with the mutants has been reported.

Other studies on mitochondrial biogenesis have employed conditional mutants. The studies by Mahler and Dawidowitz (1973), using a

yeast mutant temperature-sensitive for nuclear RNA synthesis, have already been described (mtmRNA section of the Introduction). Another very interesting report, by Thomas and Scragg (1973), describes a mutant of yeast with temperature-sensitive mitochondrial ribosomes. This mutant may be of considerable importance in further elucidation of the mechanism of mitochondrial protein synthesis.

The studies presented here demonstrate the usefulness of this conditional mutant in the study of mitochondrial biogenesis. The use of temperature rather than drugs to accomplish specific inhibition of cytosolic protein synthesis has some advantages. First, the temperature-sensitive lesion is much more specific than any drug can ever be. Side effects of cycloheximide include the inhibition of RNA polymerases (Timberlake et al, 1972; Higashenakagawa & Muramatsu, 1972), the stimulation of uridine kinase activity (Cihak & Cerna, 1972) and the inhibition of respiration in liver mitochondria (Garber et al, 1973). Emetine, another "specific" inhibitor of cytosolic protein synthesis (Perlman & Penman, 1970) has been shown to inhibit mitochondrial protein synthesis to some extent (Kroon & Arendzen, 1972; Ibrahim et al, 1974). Another advantage to the conditional mutant over the use of drugs is the ease of reversibility of the effects of the conditional lesion (cf. Thompson et al, 1973), while removal of drugs usually requires centrifugation and resuspension of the cells, which may not be totally effective (cf. Lansam et al, 1974).

In conclusion, the biochemical studies on the tsH1 cell line reported above have demonstrated the applicability of conditional mutants to the study of mitochondrial biogenesis in mammalian cells. The genetic

implication of the results is that the mitochondria must have a specific leucyl-tRNA synthetase.

CHAPTER II

Study of Mitochondrial Biogenesis in Somatic Cell Hybrids: The Implication to the Initiation of Mitochondrial Protein Synthesis

INTRODUCTION

The assembly and function of active mitochondria require the close cooperation of both the mitochondrial and the nuclear genetic apparatuses. In mammalian cells, analysis of mitochondrial-nuclear interactions is extremely difficult. Mammalian cells grow only under aerobic conditions and thus cannot live without functioning mitochondria, in contrast to the situation in yeast where even mutants lacking mtDNA can be grown and studied (Schatz & Mason, 1974). Also, manipulation of the mtDNA of mammalian cells to produce and isolate cytoplasmic mutants (e.g. drug-resistant mutants) was not possible until very recently and very little information has been obtained by this approach (see Chapter III for discussion). It seemed, therefore, that a convenient system for the analysis of mitochondrial-nuclear interactions might be interspecies somatic cell hybrids.

Somatic cell hybrids are produced by fusing, usually with an inactivated virus, cells of differing phenotypes (Harris, 1970). When these cells are of different species (interspecies hybrid), the resultant hybrid cell often eliminates (segregates) some of the chromosomes of one

of the "parental" cell types during propagation of the hybrid. If techniques are available to distinguish the chromosomes of the two species and to distinguish particular gene products of the two species, one can correlate the presence of particular chromosomes with gene products (Harris, 1970; Davidson, 1973). This genetic technique has been successful in the assignment of genetic loci to individual chromosomes in human cells and in other mammalian cells (cf. Ruddle, 1973).

To extend this approach to the study of mitochondrial biogenesis, one can analyze interspecies hybrid cells for the persistence of components of the mitochondria of the two parental species and to correlate the analyses with the chromosome complement (Clayton et al, 1971; Attardi & Attardi, 1972; Coon et al, 1973; van Heyningen et al, 1973; 1974). This type of analysis requires methods for distinguishing mitochondrial components of the two species.

Using this approach, Clayton et al (1971) analyzed human-mouse somatic cell hybrids. They found that human mtDNA differed from mouse mtDNA in buoyant density (a difference of 0.008 g/cc). In six human-mouse hybrids examined, all contained mtDNA of mouse density (i.e. less than 2% human mtDNA). These human-mouse hybrids all segregate human chromosomes, and at the time of analysis the six hybrids contained only 3 to 17 human chromosomes. This result suggests that either human mtDNA is lost because it replicates more slowly than mouse mtDNA or genes that are necessary for the maintenance of human mtDNA (e.g. replicative enzymes) are lost through the segregation of the human chromosomes. It is interesting that the machinery present in the hybrid for the maintenance of mouse mtDNA does not maintain human mtDNA. This was further

examined by incubating L cells (mouse) with human mitochondria. L cells are known to take up exogenously added mitochondria and chloroplasts into their cytoplasm (Nass, 1969). Under these conditions, human mtDNA could still be detected 72 hr later, but not thereafter, confirming that human mtDNA cannot be maintained by mouse replicative-machinery.

Similar experiments with human-mouse hybrids were reported by Attardi and Attardi (1972). Out of five hybrids, none were found by buoyant density analysis to contain human mtDNA. All the hybrids analyzed by Attardi and Attardi also segregated human chromosomes and contained between 6 and 30 human chromosomes. They interpret their results to suggest that either human chromosomes necessary for maintenance of human mtDNA are repressed or the loss of human chromosomes results in the loss of nuclear genes necessary for the survival of human mtDNA. If the latter is true and if chromosome loss is random (this may not be strictly true (Norum & Migeon, 1974)) then a large number of human chromosomes must be necessary for the maintenance of human mtDNA.

Van Heyningen et al (1973; 1974) examined human-mouse hybrids for the persistence of species-specific mitochondrial components other than mtDNA. Techniques were worked out to separate the mitochondrially-located enzymes, citrate synthase and mitochondrial NAD-malate dehydrogenase, as well as the cytosolic isozyme of NAD-malate dehydrogenase. Mouse and human citrate synthase and cytosolic malate dehydrogenase were separated by electrophoretic techniques, while the presence of human mitochondrial malate dehydrogenase was determined by immunological techniques using an antisera directed against purified human mitochondrial malate dehydrogenase. Van Heyningen et al (1973) analyzed nine human-mouse hybrids for the

presence of human enzyme. Three of the hybrids had been analyzed previously by Clayton et al (1971) and shown to lack human mtDNA. They found that four hybrids contained both human citrate synthase and human mitochondrial malate dehydrogenase, while one contained only human mitochondrial malate dehydrogenase. This correlation was not found for any other pair of enzymes and suggests that possibly the genes for these enzymes are linked on the same chromosome. Of the three hybrids which were known not to contain human mtDNA, two contained both human citrate synthase and human mitochondrial malate dehydrogenase while the other contained neither human enzyme. Van Heyningen and co-workers concluded that the presence of human mtDNA is not required for the assembly of mitochondria containing human enzymes. The specificity which seems to exist for the maintenance of human mtDNA does not appear to apply to some human mitochondrial enzymes.

The inability to observe human mtDNA in human-mouse hybrids was very discouraging, since it was not possible to observe and study interactions which might occur between human[1] and mouse mitochondria, and between the nucleus and human mitochondria. The problem was that the unidirectional segregation of human chromosomes resulted in a concurrent unidirectional loss of human mtDNA. This has not been a problem in more recent investigations.

Eliceiri (1973a; b) studied the persistence of species-specific mitochondrial components in hamster-mouse hybrids. Although hamster and mouse mtDNAs do not differ in buoyant density (see Results) they can be

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[1] Human mitochondria in this context refers to mitochondria containing human mtDNA.

distinguished from each other by a hybridization technique (Eliceiri, 1973b; Coon et al, 1973). *E. coli* RNA polymerase is used to transcribe complementary RNA (cRNA) from mtDNA of each of [redacted] species using [^{3}H] or α-[^{32}P] labelled nucleoside triphosphate as precursors. The purified cRNA hybridizes very specifically to the mtDNA of the species from which it was transcribed. The specific hybridization can then be used to assay for the presence of mtDNA of either species (or both) in a hybrid cell.

Using this technique, Eliceiri (1973b) showed that in nine hamster-mouse hybrids, five contained mainly hamster chromosomes and mainly hamster mtDNA, three contained mainly mouse chromosomes and mouse mtDNA, and one (denoted 10A) contained mainly mouse chromosomes (60 mouse, 44 hamster (Eliceiri, 1973a)) but mainly hamster mtDNA (80%). This last result is rather surprising and is an exception to the normally observed coordinate loss of the mtDNA of one species along with the corresponding chromosomes. It suggests that, either there is a sufficient complement of hamster chromosomes to maintain hamster mtDNA, or mouse replicative-machinery has the capacity to recognize and replicate hamster mtDNA. If this latter possibility were true, there must not be any selective advantage to the replication of mouse mtDNA, otherwise it would have obviously re-populated the cytoplasm. One could speculate that the replicative-machinery for mtDNA (which is likely coded for in the nucleus (Schatz & Mason, 1974)) was made up of some hamster and some mouse enzymes such that both types of mtDNA were replicated.

The 12S mtrRNA of hamster can be separated from that of mouse by polyacrylamide gel electrophoresis (Montenecourt et al, 1970; Mitra et al, 1972). Eliceiri (1973a) used this distinguishing feature to analyze

hamster-mouse hybrids for the expression of mtDNA. Five hybrids were analyzed, two contained mainly mouse chromosomes, mouse mtDNA (above) and mouse mtrRNA, two contained mainly hamster chromosomes,hamster mtDNA (above) and hamster mtrRNA, while the one containing mainly mouse chromosomes but hamster mtDNA hybrid (10A) was found to synthesize mostly hamster mtrRNA. This indicates that the mtDNA present in the hybrids is expressed. In hybrid 10A with 80% hamster mtDNA, the ratio of hamster to mouse mtrRNA is approximately the same as the ratio of the mtDNAs. As suggested above, to explain the persistence of hamster mtDNA in this hybrid, either sufficient hamster chromosomes are present to allow for the transcription of hamster mtDNA, or the mouse transcription-machinery recognizes and replicates hamster mtDNA.

In an elegant series of experiments, Dawid, Coon and Horak were able to analyze human-rodent (mouse and rat) hybrids without the problem of unidirectional loss of human chromosomes (Coon et al, 1973; Dawid et al, 1974; Horak et al, 1974). The hybrids were formed by the fusion of permanent human cell lines with newly established, highly differentiated rodent cell lines (Minna & Coon, 1974). The resulting hybrids most often shed rodent chromosomes, although those shedding human chromosomes are also observed.

Using the hybrids just described, Coon et al (1973) observed that both human-mouse and human-rat hybrids, when analyzed by the cRNA-hybridization method, contain rodent mtDNA, human mtDNA or both. A positive correlation could be observed between the segregation of chromosomes and mtDNA of one of the species. In the hybrids containing both parental mtDNAs, the persistence of both was observed for up to as

many as 150 cell doublings and even after subcloning. In some of these hybrids, when the mtDNA was analyzed by CsCl density gradient centrifugation and fractions of the gradients were hybridized to [^{3}H] cRNA transcribed from human mtDNA and [^{32}P] cRNA transcribed from rodent mtDNA, an interesting result was obtained. While one would expect the peak of the [^{3}H] and [^{32}P] activities to be separated by the density difference of human and rodent mtDNAs, some of the hybrids containing both human and rodent mtDNA showed [^{3}H] activity at densities approaching that of rodent mtDNA, and vice versa (Horak et al, 1974). Horak et al interpret this result to suggest that recombination of human and rodent mtDNA had occurred, creating mtDNA of intermediate densities. This is an extremely important observation. Recombination of gene markers on the mtDNA of petite mutants of yeast has been shown to be accompanied by formation of mtDNA of intermediate buoyant density (Michaelis et al, 1973). While gene markers are not yet available for mammalian mtDNAs (see Chapter III), the results of Horak et al suggest that a genetic approach to the study of mitochondrial biogenesis in mammalian cells may be through the use of hybrid cells (Dawid et al, 1974). This emphasizes the potential of the hybrid cell system in the study of mitochondrial biogenesis.

While human-mouse, human-rat and hamster-mouse somatic cell hybrids have been shown to be useful in the study of mitochondrial biogenesis, future application of hybrid cells to this study is limited to the ease with which one can analyze the species-specific mitochondrial components. The objective of this investigation was to examine human-hamster hybrids to determine whether or not the mtDNAs of the parental species could be distinguished by a buoyant density difference as could

human and mouse (Clayton et al, 1971) and human and rat mtDNAs (Horak et al, 1974). Previous results from this laboratory showed that the 12S mtrRNA of human cells could be separated from that of hamster cells by polyacrylamide gel electrophoresis (Mitra et al, 1972). If human mtDNA could be separated from that of hamster, it is possible that human-hamster hybrids could be analyzed not only for the persistence of both parental mtDNAs, but also the expression of the mtDNA (transcription of mtrRNA).

Initiation of Mitochondrial Protein Synthesis

In the isolation of somatic cell hybrids, one often takes advantage of a procedure which selects on the basis of the ability of hybrid cells to grow in a medium in which neither of the parental cells can grow (Harris, 1970). The selection procedure described by Littlefield (1964) takes advantage of the ability of folate analogues such as aminopterin to block *de novo* purine and pyrimidine synthesis through the inhibition of dihydrofolate reductase (Stokstad & Koch, 1967). The inhibition of dihydrofolate reductase depletes the supply of reduced folate derivatives necessary for the *de novo* synthesis of purine and pyrimidine nucleotides as well as the synthesis of thymidylic acid from deoxyuridylic acid (Stokstad & Koch, 1967). Only cells which can utilize exogenous purine and pyrimidine nucleotide precursors via thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT) can grow in the selective medium containing aminopterin, hypoxanthine, thymidine and all 20 amino acids (HAT medium (Littlefield, 1964)). The fusion of TK-deficient cells (TK^-) and HGPRT-deficient cells ($HGPRT^-$) produces hybrid cells which contain both enzymes and which can grow in

HAT medium while the TK^- cells and the $HGPRT^-$ cells cannot (Littlefield, 1964; Davidson & Ephrussi, 1965).

A question arises from the known ability of aminopterin (and other folate analogues) to inhibit one carbon (C_1-H_4 folate) metabolism (Blakley, 1969) and the participation of a formylated species of met-$tRNA_f^{met}$ to initiate protein synthesis in both mammalian (Smith & Marcker, 1968; Galper & Darnell, 1969; 1971) and fungal mitochondria (Smith & Marcker, 1968; Epler et al, 1970; Holbreich & Rabinowitz, 1971; Küntzel & Sala, 1969). Fig. 8 outlines the steps involved in the initiation of protein synthesis in *E. coli* (Dickerman, 1971), presumably similar steps are involved in mitochondrial protein synthesis (Sala & Küntzel, 1970). The source of the formyl group is N^{10} formyl-tetrahydrofolate (N^{10} formyl H_4 folate) both in *E. coli* (Dickerman et al, 1967) and in yeast mitochondria (Halbreich & Rabinowitz, 1971) and therefore is probably the same in mammalian mitochondria. Aminopterin would be expected to inhibit the synthesis of N^{10} formyl H_4 folate (as well as other C_1-H_4 folate derivatives) by inhibiting the enzyme dihydrofolate reductase (Blakeley, 1969). Does this result in the inhibition of formylation of initiator met-$tRNA_f^{met}$ and subsequently of the initiation of protein synthesis?

In the growth of $TK^+/HGPRT^+$ cells (such as hybrid cells) in HAT medium, metabolites whose *de novo* synthesis requires C_1-H_4 folate precursors (i.e. hypoxanthine, thymidine and all the amino acids) are supplied in order to alleviate the block in folate metabolism produced by aminopterin. Under these conditions one would expect the formylation of mitochondrial met-$tRNA_f^{met}$ to be inhibited since no source of N^{10}

Figure 8

Specificities of methionyl-tRNAs in *E. coli* protein synthesis. Step 1 depicts the reaction of both $tRNA_f^{met}$ with methionyl-tRNA synthetase; Step 2 shows that only met $tRNA_f^{met}$ can be formylated by *E. coli* transformylase. The formyl donor is N^{10} formyl H_4 folate; Step 3 shows that fMet-$tRNA_f^{met}$ is involved in initiation of protein synthesis; Step 4 depicts the elongation process. From Dickerman (1971).

Specificities of Methionyl tRNA's in *E. coli* Protein Synthesis

1. $\text{methionine} + \text{ATP} + \text{tRNA}^{met}$ (30%)* $\rightleftarrows$ $\text{met tRNA}^{met} + \text{AMP} + \text{PP}$
   $\text{methionine} + \text{ATP} + \text{tRNA}^{fmet}$ (70%)* $\rightleftarrows$ $\text{met tRNA}^{fmet} + \text{AMP} + \text{PP}$
2. $\text{met tRNA}^{met} + N^{10}$ formyl H_4 folate $\rightarrow X$
   $\text{met tRNA}^{fmet} + N^{10}$ formyl H_4 folate $\rightleftarrows$ N-formyl met $\text{tRNA}^{fmet} + H_4$ folate
3. N-formyl met tRNA^{fmet} + 30 S ribosomal subunit
   $\downarrow$ AUG, $f_1 + f_2$, GTP
   "initiation complex"
4. $\text{met tRNA}^{fmet} + T_u + \text{GTP} \rightarrow X$
   $\text{met tRNA}^{met} + T_u + \text{GTP} \rightarrow$ elongation complex
   other aminoacyl tRNA

* Percentage of total *E. coli* tRNA accepting methionine.

Figure 8

formyl H_4 folate should be available. A question which has not been adequately answered is whether or not formylation is required for efficient initiation of mitochondrial protein synthesis (Dickerman, 1971) and thus for normal cell growth. In *E. coli*, the inhibition of folate metabolism with trimethoprim (another folate analogue) results in the cessation of growth and of protein synthesis (Eisenstadt & Lengyel, 1966). The addition of N^5 formyl H_4 folate to a soluble extract, which can be converted to the N^{10} derivative, stimulates protein synthesis, suggesting that a formyl donor is required for the initiation of protein synthesis (see also Nixon & Bertino, 1970). On the other hand, formylation can be dispensed with in the folate-requiring bacteria *Streptococcus faecalis* R (Samuel et al, 1970; 1972; Samuel & Rabinowitz, 1974) and *Streptococcus faecium* (Pine et al, 1969; Sarimo & Pine, 1971) under conditions of folate-deficiency (in the absence of folate but in the presence of metabolites whose *de novo* synthesis requires C_1-H_4 folate derivatives). The initiator tRNA used in the initiation of protein synthesis in the absence of formylation appears to lack rTp in the 'GTψC' loop, while the formylated initiator contains rTp (Samuel & Rabinowitz, 1974; Delk & Rabonowitz, 1974). Interestingly enough, eukaryotic initiator tRNA, which is not formylated also lacks rTp in the 'GTψC' loop (Simsek et al, 1973; RajBhandary et al 1973; Ghosh et al, 1974). It is possible, however, that mt-tRNA does not contain rTp (Dubin & Friend, 1974, see Chapter I).

In an attempt to answer the question of whether or not formylation can be dispensed with in the initiation of mitochondrial protein synthesis, I have examined mammalian cells grown in culture in HAT medium. One would expect, since cells are known to grow in HAT medium (Littlefield, 1964;

Goldstein & Lin, 1972) and since mitochondrial protein synthesis is required for growth (Fettes et al, 1972), that mitochondrial protein synthesis is not inhibited in HAT medium. However, Galper and Darnell (1969) and Galper (1970) demonstrated that in HeLa cells incubated in HAT medium, cell growth was inhibited, the formylation of mitochondrial met-$tRNA_f^{met}$ was inhibited and the rate of mitochondrial protein synthesis was depressed. This result is clearly in conflict with the normal growth of hybrid cells in HAT medium observed by others, and a reevaluation was obviously necessary. An investigation of the methionyl-tRNAs from mitochondria was also made by comparing their elution profile on RPC-5 to that of cytosolic methionyl-tRNAs.

In that mitochondrial protein synthesis is prokaryote-like (Chapter I) the question is whether it requires formylation of initiator tRNA for peptide chain initiation like *E. coli* or whether it can dispense with formylation like folate-requiring bacteria; mammalian cells are also folate-requiring. Methotrexate was used in this investigation since it is more readily available than some other folate analogues, it is routinely used as a chemotherapeutic agent in humans.

METHODS

Growth and Maintenance of Cell Cultures

L cells (mouse), KB cells (human), BHK-21 cells (hamster) and LMTK$^-$ cells (TK-deficient L cells selected for resistance to 5-bromo, 2'-deoxyuridine (BUdR) (Kit et al, 1963)) were maintained in suspension culture in minimum essential medium, Joklik's modification (MEM-Joklik) supplemented with 5% fetal calf serum as described by Bartoov et al (1970). TG2 cells (HGPRT-deficient BHK cells selected for resistance to 6-thioguanine (Goldstein & Lin, 1972)), and a normal human fibroblast line were maintained in monolayer culture in minimum essential medium, Eagle's modification (MEM-Eagle) supplemented with 10% fetal calf serum. Human-hamster hybrids were a generous gift of Dr. S. Goldstein, Department of Medicine, McMaster University. They were maintained in monolayer culture in MEM-Eagle supplemented with 15% fetal calf serum, 10^{-4}M hypoxanthine, 1.7×10^{-5}M thymidine, 10^{-6}M methotrexate and all 20 amino acids (HAT medium (Goldstein & Lin, 1972)).

Labelling, Isolation and Analysis of mtDNA

BHK, KB and LMTK$^-$ mtDNAs were labelled in cells grown in suspension culture in MEM-Joklik supplemented with 5% fetal calf serum. BHK and KB cells were labelled for 16 or 40 hr with [^{14}C-CH$_3$]thymidine (0.1 μCi/ml, 62 mCi/mmole). LMTK$^-$ cells were labelled 40 hr with [^{3}H-CH$_3$]thymidine (1 μCi/ml, 18.5 Ci/mmole). TG2 and hybrid mtDNAs were labelled in cells in monolayer culture in MEM-Eagle supplemented with 10% fetal calf serum.

Both cell types were labelled for 40 hr with [^{3}H-CH$_3$]thymidine (2 μCi/ml, 18.5 Ci/mmole).

Cells were harvested, washed, disrupted and mitochondria isolated by differential centrifugation as described by Freeman (1965). Mitochondria were further purified on a sucrose-step gradient as described by Smith et al (1971). Closed circular mtDNA was extracted and isolated by the dye-buoyant density method of Radloff et al (1967). The procedure followed was that outlined by Smith et al (1971) in which the dye propidium diiodide (PrI_2) is used rather than ethidium bromide. The profile of a typical PrI_2-CsCl density gradient is shown in Fig. 9 for KB cell mtDNA. The material at approximately 1.57 g/cc is the closed circular mtDNA. It was pooled and the PrI_2 removed on a Dowex-50 column (Smith et al, 1971). This material was confirmed to be closed circular mtDNA by examination in the electron microscope by M.M.L. Davidson of this laboratory. The buoyant densities of mtDNAs from various cell types and human-hamster hybrid cells were compared by CsCl-equilibrium density gradient centrifugation in a Beckman type 65 angle rotor as described by Flamm et al (1966). Samples of [^{3}H]mtDNA from one cell were mixed with [^{14}C]mtDNA from another cell and *Micrococcus lysodeikticus* DNA was added as a density standard. The initial density was adjusted to 1.71 g/cc as measured by refractive index (Handbook of Biochemistry, 1968). Centrifugation was at 42,000 rpm for at least 42 hr at 20°C in a Beckman L2-65B ultracentrifuge. The gradients were fractionated into approximately 60 fractions. Aliquots were taken for determination of density (by refractive index), absorbance at 260 nm (for the location of the *M. lysodeikticus* DNA) and radioactivity. [^{3}H] and [^{14}C] activities were determined by separating the two isotopes

Figure 9

PrI_2-CsCl buoyant density gradient profile for the purification of closed circular mtDNA from human (KB) cell mitochondria. KB cells were labelled 40 hr with [^{14}C-CH_3]thymidine (0.1 µCi/ml, 62 mCi/mmole), and the mitochondria isolated and purified as described in the Methods. The mitochondria were lysed as described by Smith et al (1971) and the suspension brought to 4.5 ml, 400 µg/ml in PrI_2 and 1.56 g/cc. Centrifugation was for 40 hr at 42,000 rpm using a type 65 Beckman rotor in an L2-65B ultracentrifuge at 20°C. The bottom of the gradient is to the left. The fractions marked mtDNA were pooled and the PrI_2 removed as described in the Methods. ●——●, Radioactivity; ▲——▲, density (g/cc).

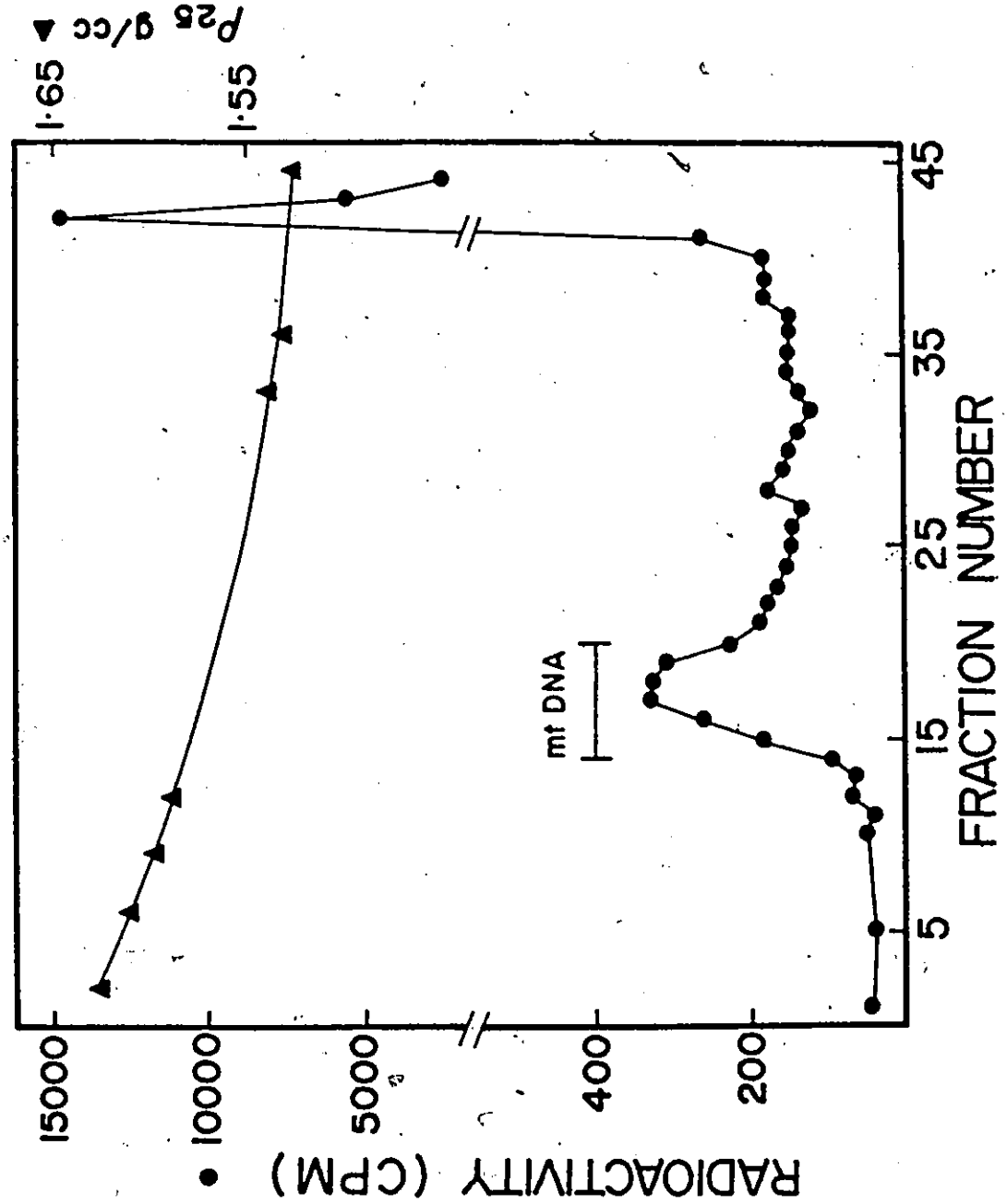
RADIOACTIVITY (CPM) ●
15000
10000
5000
400
200
mt DNA
5
15
25
35
45
FRACTION NUMBER
1·55
1·65
ρ_{25} g/cc ▲

Figure 9

in a Packard tri-carb oxidizer (Model 305) and counting in a Nuclear Chicago Mark I scintillation spectrophotometer.

Measurement of Cell Growth Rate

Growth measurements were made on L cells or KB cells grown in suspension culture in MEM-Joklik supplemented with 10% fetal calf serum, 10^{-4}M hypoxanthine, 1.7×10^{-5}M thymidine (HT-medium) and in the presence or absence of folate analogues. Cells were maintained in logarithmic phase of growth by regular dilution, and cell density was measured by hemocytometer or by Coulter counter.

Measurement of fMet-tRNA$_f^{met}$ levels

The effect of methotrexate on the production of fMet-tRNA$_f^{met}$ was measured in whole cells, since this tRNA is mitochondrial specific (Galper & Darnell, 1969), by measuring the amount of N-formylmethionine released from acylated tRNA (Samuel & Rabinowitz, 1974). L cells were harvested and then suspended in HT-medium lacking methionine but containing only 2% fetal calf serum. The cells were centrifuged and resuspended in the same medium at 1.5×10^7 cells/ml. The suspension was divided. To one-half of the culture, methotrexate was added to a final concentration of 10^{-4}M; to the other one-half nothing was added. After 15 min at 37°C [^{3}H-CH$_3$]methionine (5 Ci/mmole) was added to both to 5 µCi/ml and incubation continued for 15 min. The incubations were rapidly stopped by pouring each over frozen phosphate-buffered saline. The cells were collected by centrifugation, lysed by the addition of 0.1 M sodium acetate, 0.005 M magnesium acetate, 1% sodium dodecylsulfate, pH 5.0 and

extracted two times with buffer-saturated phenol at room temperature. The total nucleic acid was recovered by precipitation with two volumes of ethanol at -20°C. The nucleic acid was analyzed for fMet-tRNA as described by Samuel and Rabinowitz (1974), by hydrolysis in 0.5 M NH_4OH for 30 min at 37°C followed by addition of 1/4 volume of formic acid and extraction of the liberated N-formylmethionine into ethyl acetate. The N-formylmethionine was separated by high-voltage paper electrophoresis.

Measurement of N-formylmethionylpuromycin levels

N-formylmethionylpuromycin (fMet-puromycin) production was measured essentially as described by Galper and Darnell (1971). Cells were suspended in HT-medium lacking methionine and serum, centrifuged and then suspended in the same medium at 5 x 10^6 cells/ml. Methotrexate pretreatment was as described in the legends to the figures. [^{35}S]methionine (25 Ci/mmole) was added to 0.5 μCi/ml and the suspension incubated at 37°C for 2 min. The suspension was then divided. To one-half puromycin was added to a final concentration of 0.3 mg/ml, and to the other one-half nothing was added. The incubation was continued for 15 min and stopped by placing each at 0°C. The pH of each incubation was adjusted to 5.5 and then each was extracted twice with one volume of ethyl acetate. The ethyl acetate phase was concentrated under vacuum and analyzed for fMet-puromycin by high-voltage paper electrophoresis.

Synthesis of N-formylmethionine and fMet-puromycin

Labelled N-formylmethionine (fMet) was synthesized from [^{14}C-CH_3]-methionine as described by Sheehan and Yang (1958) except the reaction was scaled down by a factor of 100. The product was analyzed by NMR and

high-voltage paper electrophoresis, the results being consistent with fMet. fMet-puromycin (^{14}C-labelled) was synthesized from fMet (^{14}C-labelled) as described by Galper (1970).

Characterization of fMet-puromycin

The fMet-puromycin synthesized in cells was characterized by mild acid hydrolysis in 0.2 M HCl in methanol under nitrogen at 37°C for 20 hr (Galper, 1970). Analysis of the Met-puromycin produced was by high voltage paper electrophoresis.

High-voltage Paper Electrophoresis

fMet, methionine, fMet-puromycin, and Met-puromycin were separated by high-voltage paper electrophoresis (50 volts/cm) in pyridine acetate buffer, pH 3.5, for 90 - 150 min as described by Leder and Bursztyn (1966). The paper was then cut into 1 cm strips and the radioactivity measured either directly in a toluene based scintillation fluid (^{14}C or ^{35}S) or after oxidizing each strip in a Packard-Tricarb oxidizer (^{3}H).

Isolation of Mouse Liver Mitochondria

Livers were removed from 10 - 14 week old Swiss Webster mice and the mitochondria isolated as described by Haldar and Freeman (1969). The mitochondria were washed once with SE-medium and once with ST-medium. Each time the mitochondria were centrifuged at 5,000 x g_{av} for 10 min.

Isolation of Cytosolic Extract

Mouse livers were homogenized in 3 volumes of 0.01 M Tris-HCl,

0.01 M KCl, 0.001 M magnesium acetate, 0.006 M mercaptoethanol, pH 7.5. The extract was centrifuged at 30,000 x g_{av} for 10 min and the supernatant was passed through a Sephadex G-25 column equilibrated with the extraction buffer. The material eluting in the void volume was stored frozen and used within one week.

Acylation of tRNA

The cytosolic extract was incubated in a medium containing 15 mg protein/ml, 0.01 M Tris-HCl, 0.01 M KCl, 0.001 M magnesium acetate, 0.006 M mercaptoethanol, 0.008 M ATP, 0.004 M CTP and 20 µCi/ml [^{35}S]methionine (100 Ci/mmole), pH 7.5. The incubation was continued at 37°C for 10 min and stopped by diluting 10-fold with 0.10 M sodium acetate, 0.005 magnesium acetate, pH 5.0 containing 1% SDS, and extracting twice with one volume of buffer-saturated phenol at room temperature. The acylated endogenous tRNA was precipitated with 2 volumes of ethanol at -20°C overnight.

Mitochondria were incubated under the conditions for protein synthesis as described by Haldar and Freeman (1969). The incubation medium contained 15 mg protein/ml, 0.05 M KCl, 0.005 M $MgCl_2$, 0.02 M potassium phosphate, 0.1 M sucrose, 0.01 M sodium succinate, 0.002 M ADP, 10^{-4} M of each of the 19 amino acids (less methionine), 100 µg tevenel/ml to inhibit mitochondrial protein synthesis (Freeman, 1970b), and 10 µCi/ml [^{3}H-CH_3]methionine (5 Ci/mmole), pH 7.2. Incubation was continued at 30°C for 20 min and stopped by centrifuging the mitochondria at 10,000 x g for 10 min and resuspending them in 0.10 M sodium acetate, 0.005 M magnesium acetate, pH 5.0 containing 1% SDS. The acylated endogenous tRNA was extracted and precipitated as described above for the cytosolic tRNA.

Analysis for Formylated Met-tRNA$_f^{met}$

Acylated tRNA was analyzed for formylated species of met-tRNA$_f^{met}$ as described by Marcker and Sanger (1964). Pancreatic RNase digests of the acylated tRNA were subjected to high voltage paper electrophoresis at 60 volts/cm for 2 hr at pH 3.5. The paper was dried and then cut into 1 cm strips. The strips were either counted directly (^{35}S) in a toluene scintillation fluid or oxidized (^{3}H) in a Packard-tricarb sample oxidizer. The identity of N-formylmethionyladenosine (fMet A) and methionyladenosine (Met A) was inferred from the results of Smith and Marcker (1968) for rat liver mitochondria.

RPC-5 Chromatography

RPC-5 chromatography was performed as described by Pearson et al (1971). The acylated tRNA species were eluted at 25°C with a 200 ml linear gradient from 0.45 M NaCl to 0.7 M NaCl in 0.01 M sodium acetate, 0.01 M $MgCl_2$, 0.002 M mercaptoethanol, pH 4.5. For analytical purposes, 1.3 ml fractions were collected directly into scintillation vials and counted by adding 3 ml PCS scintillation fluid. For preparative purposes, 1.3 ml fractions were collected and 0.1 ml aliquots were counted. The remainder of each peak was pooled and precipitated with 2 volumes ethanol after adding 200 µg unlabelled *Escherichia coli* tRNA.

MATERIALS

The source of most materials is tabulated below:

Material	Source
5-bromo,2'-deoxyuridine	Sigma Chemical Co.
CsCl	Rare Earth Division, American Potash & Chemical Corp., West Chicago, Ill.
CTP	Boehringer Mannheim
E. Coli tRNA	General Biochemicals, Chagrin Falls, Ohio
hypoxanthine	Raylo Chemicals Ltd., Edmonton, Alta.
M. Lysodeikticus DNA (type X)	Sigma Chemical Co.
mercaptoethanol	Eastman Organic Chemical Co.
[^{14}C-CH$_3$]methionine	New England Nuclear, Boston, Mass.
[^{3}H-CH$_3$]methionine	Amersham Searle Corp.
[^{35}S]methionine	New England Nuclear
methotrexate	Lederle Products Dept., Cyanamide of Canada Ltd., Montreal, P.Q.
pancreatic RNase	Sigma Chemical Co.
propidium diiodide	Calbiochem
puromycin dihydrochloride	Nutritional Biochemicals Corp., Cleveland, Ohio
thymidine	Raylo Chemicals Ltd.
[^{14}C-CH$_3$]thymidine	Amersham Searle Corp.
[^{3}H-CH$_3$]thymidine	Amersham Searle Corp.

RESULTS

Comparison of Buoyant Density of mtDNA from Mouse, Human, Hamster and Human-hamster Hybrid Cells

MtDNA was isolated from human, hamster and human-hamster hybrid cells and the buoyant densities compared as described in Methods. Fig. 10A shows that human mtDNA can be separated from hamster mtDNA on CsCl-equilibrium density gradients. The buoyant density difference was approximately 0.008 g/cc. Fig.10B shows that mtDNA isolated from a human-hamster hybrid cell has a density resembling hamster mtDNA, with less than about 5% of the mtDNA banding at the density of human mtDNA. This hybrid cell line was found to contain a small number of human chromosomes before it was obtained from Dr. S. Goldstein. The shedding of human chromosomes in this hybrid, as in the case of human-mouse hybrids, results in the apparent loss of human mtDNA.

To further characterize the buoyant density of hamster mtDNA, mtDNA from human and mouse cells and from hamster and mouse cells were compared. As has been reported by others (Clayton et al,1971; Attardi & Attardi, 1972), human mtDNA can be separated from mouse mtDNA with a buoyant density difference of approximately 0.008 g/cc (Fig. 11B). As was to be expected from the results presented in Fig. 10A and Fig. 11B, hamster and mouse mtDNA have approximately the same buoyant densities and are not resolved on these CsCl-equilibrium density gradients (Fig. 11A).

These results demonstrate that like mtDNA of other rodents (mouse and rat) hamster mtDNA can be separated from human mtDNA on the basis of

Figure 10

CsCl equilibrium density gradient profile of human, hamster and hybrid cell mtDNA. A, [^{3}H-CH$_3$] thymidine-labelled human mtDNA and [^{14}C-CH$_3$]thymidine-labelled hamster mtDNA. B, [^{3}H-CH$_3$] thymidine-labelled human-hamster hybrid mtDNA and [^{14}C-CH$_3$]thymidine-labelled human mtDNA. The arrow indicates the position of *M. lysodeiktikus* DNA (1.731 g/cc). Centrifugation was for 42 hr at 42,000 rpm using a type 65 Beckman rotor in an L2-65B ultracentrifuge at 20°C. The bottom of the gradient is to the left, the volume of the gradient is 4.5 ml.

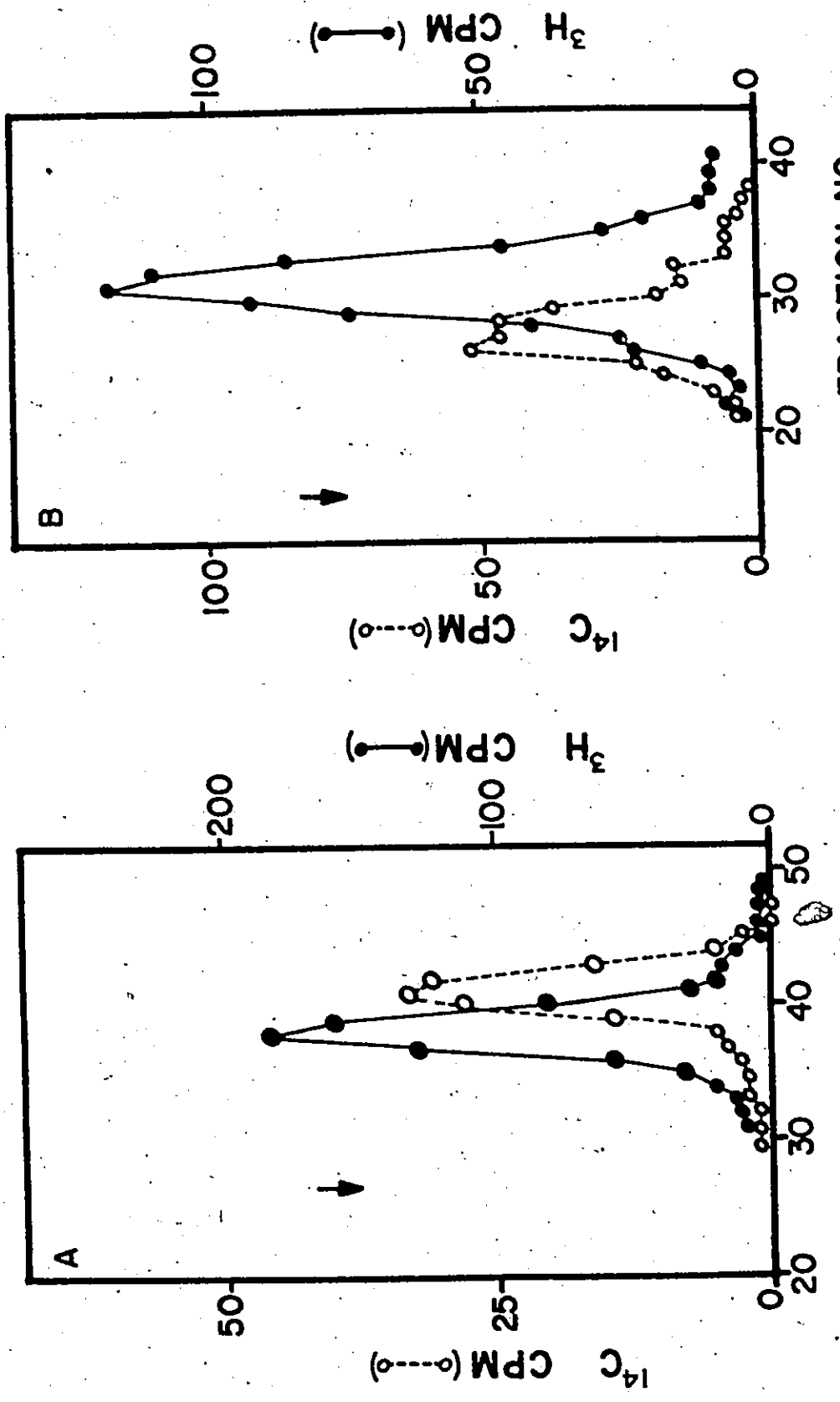

Figure 10.

Figure 11

CsCl equilibrium density gradient profile of human, mouse and hamster mtDNA. A, [^{3}H-CH$_3$] thymidine-labelled mouse mtDNA and [^{14}C-CH$_3$]thymidine-labelled hamster mtDNA. B, [^{3}H-CH$_3$] thymidine-labelled mouse mtDNA and [^{14}C-CH$_3$]thymidine-labelled human mtDNA. Conditions of centrifugation are as described in the legend to Fig. 10.

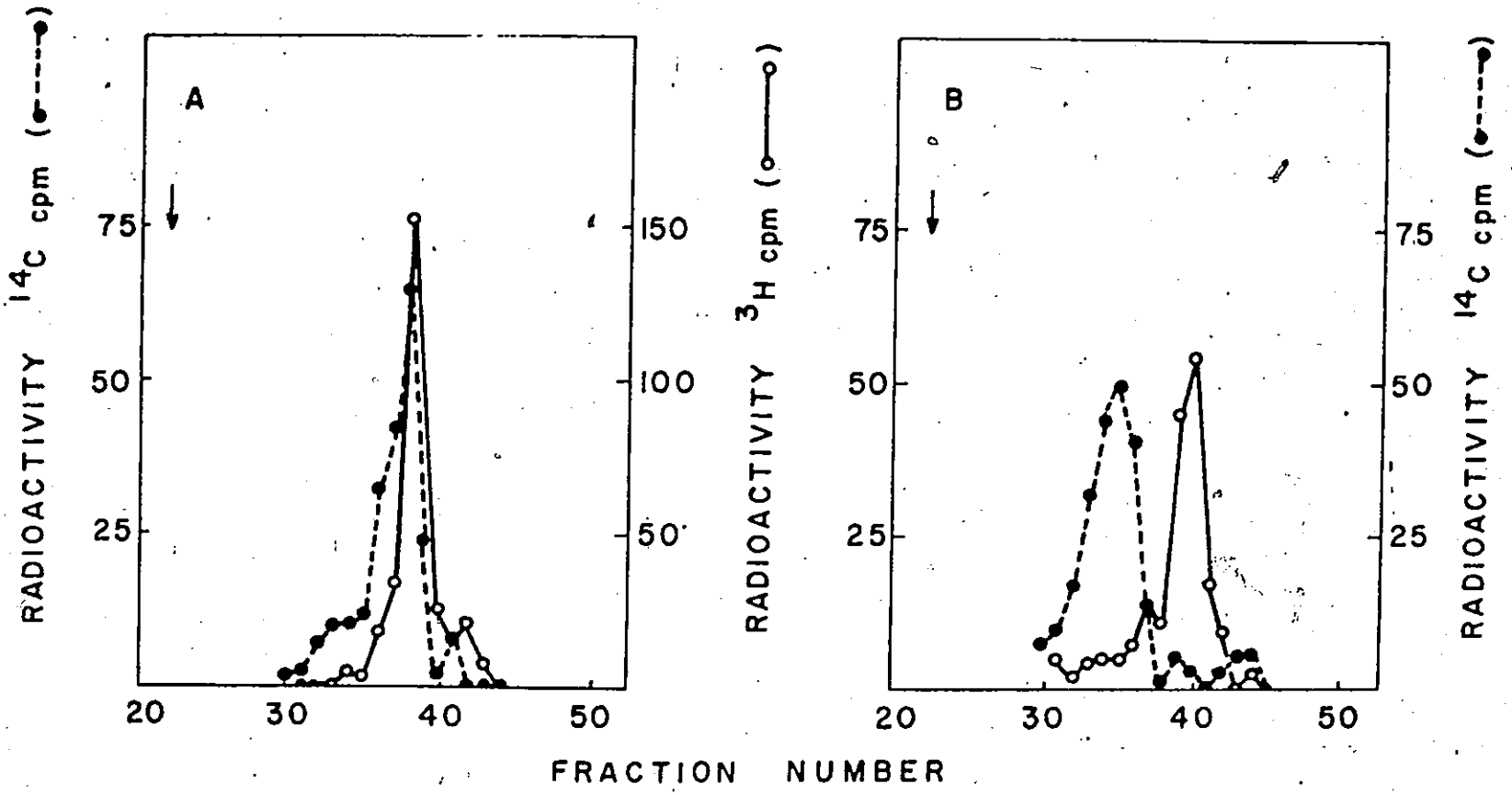
A
B
RADIOACTIVITY ^{14}C cpm (●----●)
RADIOACTIVITY ^{3}H cpm (○—○)
RADIOACTIVITY ^{14}C cpm (●----●)
75
50
25
150
100
50
20
30
40
50
FRACTION NUMBER

Figure 11

buoyant density. Perhaps all rodent mtDNAs will have a lower buoyant density when compared to human mtDNA.

Cell Growth in the Presence of Methotrexate

The hybrid cells described in the previous section were maintained in HAT medium (hypoxanthine, methotrexate (amethopterin) and thymidine) with no apparent effect on growth. To determine whether or not there is any effect of folate-analogues on cell growth, growth measurements were made on suspension cultures of L cells in HT-medium in the presence or absence of methotrexate. Concentrations of methotrexate as high as 5×10^{-5}M had no inhibitory effect on growth rate in HT-medium. Fig. 12 shows a growth experiment which demonstrates the ability of L cells to grow normally in HAT medium. The omission of hypoxanthine and thymidine from the growth medium results in a decrease in cell numbers in the presence of methotrexate. In one experiment L cells were found to grow normally for more than 13 generations in HAT medium, with no indication that the cells would not grow indefinitely. KB cells were also examined for their ability to grow in HAT medium. They were found to grow normally for approximately 4 to 5 generations and then growth was inhibited. This effect of methotrexate is not understood, but results to be presented later suggest that it is not due to an inhibition of mitochondrial protein synthesis through the inhibition of formylation of the mitochondrial initiator tRNA.

The lack of inhibition of the growth of L cells by methotrexate might have been due to the existence of a mitochondrial-specific enzyme which is relatively resistant to inhibition by methotrexate. Therefore,

Figure 12

Growth of L cells in the presence and absence of methotrexate. L cells were grown in suspension culture in HT-medium in the presence of 5×10^{-5} M methotrexate (o----o) or in its absence (o——o). Cells were grown in the presence of 5×10^{-6} M methotrexate but in the absence of hypoxanthine and thymidine (Δ——Δ). Duplicate cultures of each were maintained in logarithmic phase and cell counts made at appropriate intervals, the average of each count is plotted as number of generations.

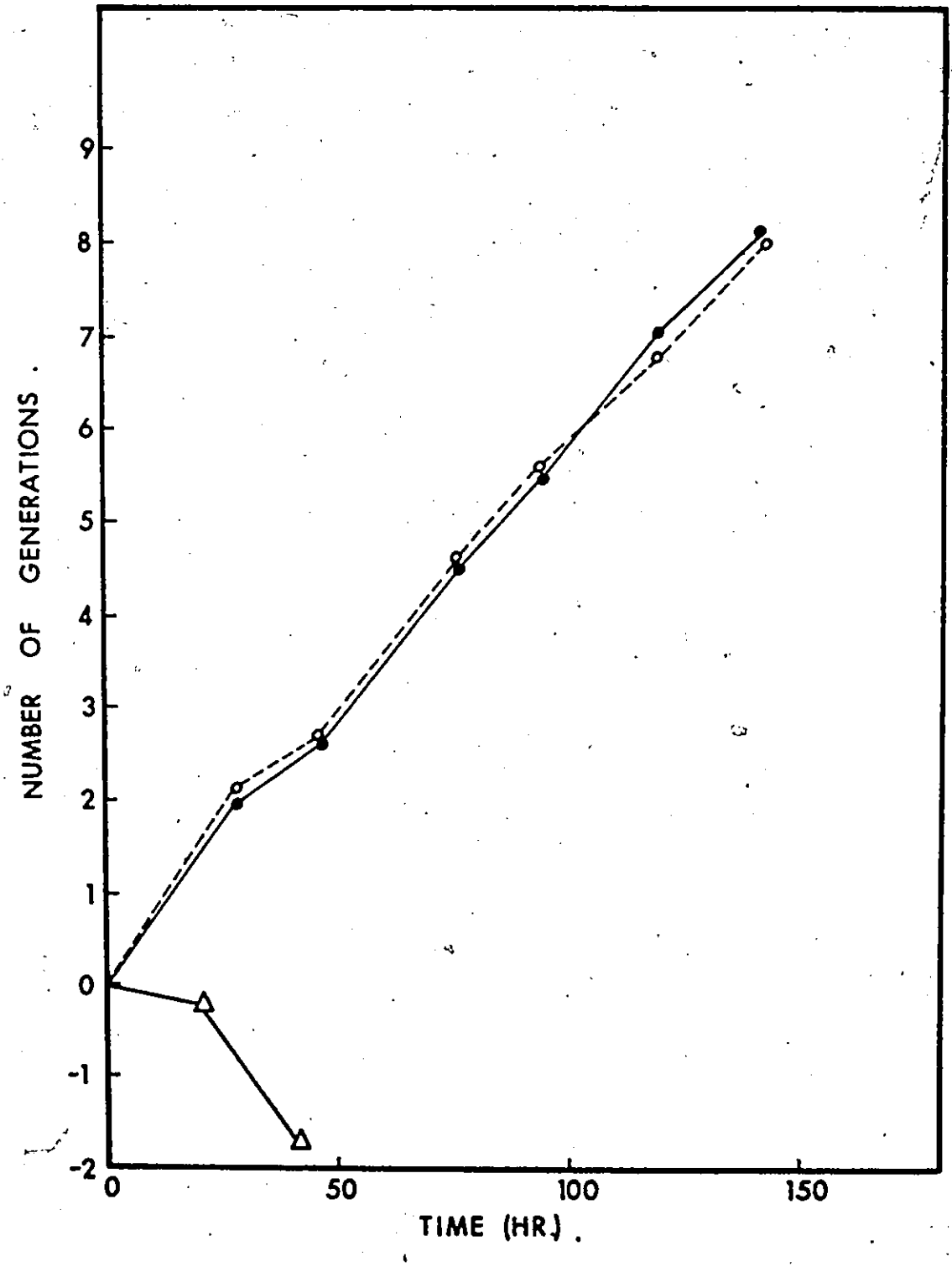

Figure 12

the effect of another folate analogue, trimethoprim, on the growth of L cells in HT medium was investigated. Trimethoprim is rather a poor inhibitor of mammalian dihydrofolate reductase, but is a potent inhibitor of the bacterial enzyme (Burchall & Hitchings, 1965). The growth experiment presented in Fig. 13 shows that L cells grow at an equal rate in the presence of 5×10^{-5}M methotrexate, 2.5×10^{-5}M trimethoprim or 2.5×10^{-5}M of each analogue.

The Effect of Methotrexate on Formylation of Met-tRNA$^{met}_{f}$

Since there appears to be no effect of methotrexate on the growth rate of L cells, then either an inhibition of formylation does not result in a subsequent inhibition of mitochondrial protein synthesis, or there is no inhibition of formylation. RNA extracted from cells incubated with or without methotrexate for 15 min and then labelled with [^{3}H-CH$_{3}$]-methionine contains the same amount of fMet as judged by electrophoresis of the alkali hydrosylates of total cell RNA (Fig. 14). Methotrexate, therefore, has essentially no effect on formylation within this time; whereas, an 80% inhibition has been reported for HeLa cells under these conditions (Galper, 1970).

The Effect of Methotrexate on the Initiation of Mitochondrial Protein Synthesis

The lack of effect of methotrexate on cell growth and on the formylation of Met-tRNA$^{met}_{f}$, led to an investigation of the formation of fMet-puromycin in cells exposed for long periods of time to methotrexate. It has been shown that the formation of the peptide, fMet-puromycin in

Figure 13

Growth of L cells in the presence of methotrexate, trimethoprim or both drugs. L cells were grown in suspension culture in HT-medium in the presence of 5×10^{-5} M methotrexate (●——●), 5×10^{-5} M trimethoprim (o——o) or 2.5×10^{-5} M methotrexate plus 2.5×10^{-5} M trimethoprim (Δ.-.-Δ). Measurement of growth was as described in the Methods and the legend to Fig. 12.

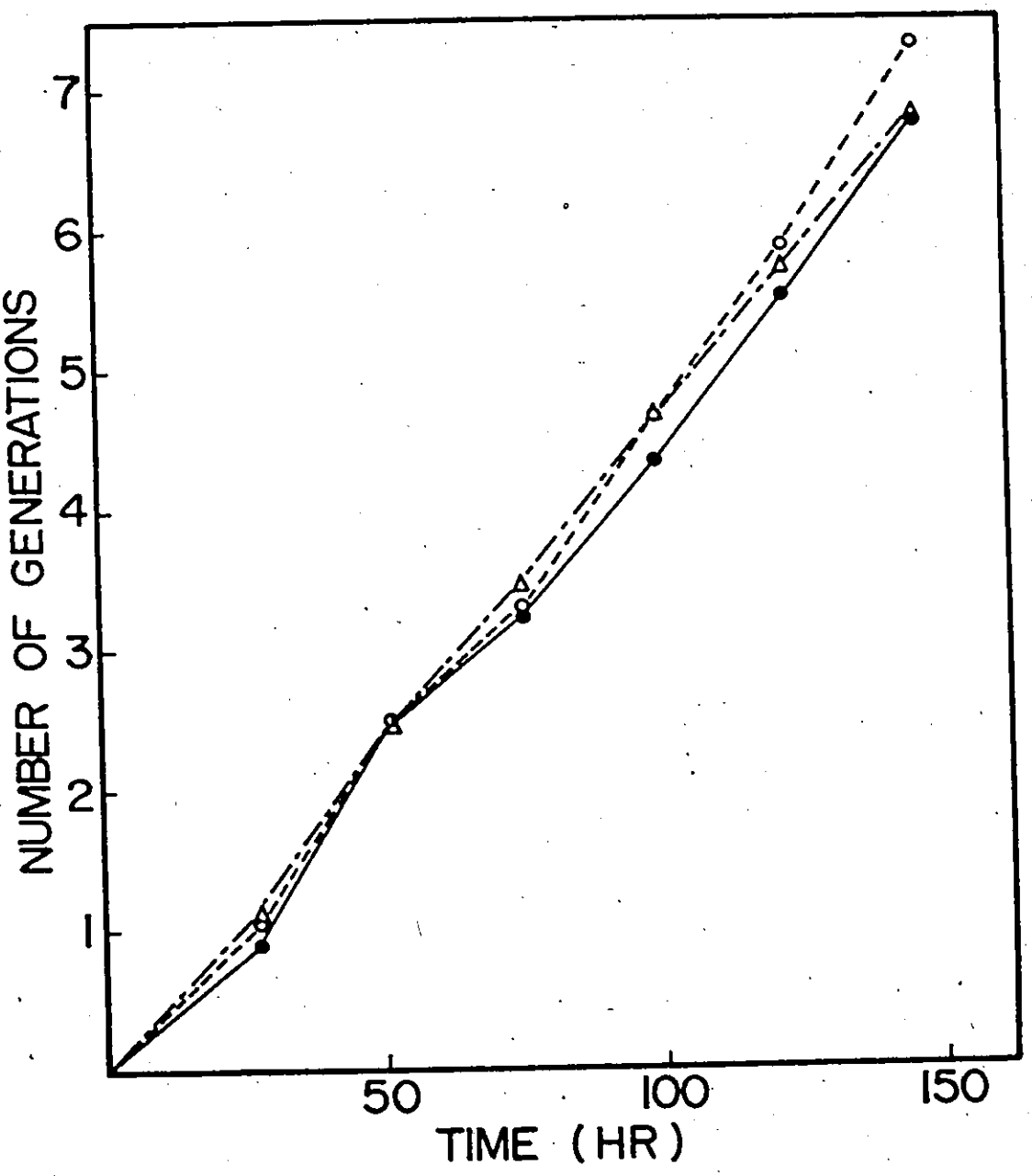

Figure 13

Figure 14

Effect of methotrexate on formylation of mitochondrial Met-tRNA$_f^{met}$. L cells were incubated in HT-medium, containing only 2% fetal calf serum, at a cell density of 1.5×10^7 cells/ml. To one-half of the culture methotrexate was added to 10^{-4} M. To the other one-half nothing was added. Both were incubated at 37°C for 15 min. [^{3}H-CH$_3$]methionine (5 Ci/mmole) was added to 5 µCi/ml and incubation continued for 15 min at 37°C. Acylated tRNA was isolated from each, hydrolysed in NH_4OH and the N-formylmethionine extracted into ethyl acetate as described in the Methods. The ethyl acetate phase was evaporated and the residue subjected to high voltage paper electrophoresis at pH 3.5 for 90 min at 50 volts/cm. The paper was cut into 1 cm strips and each strip oxidized in a Packard-tricarb oxidizer. o——o, Methotrexate treated cells; ●——●, control. M and FM refer to the mobilities of methionine and N-formylmethionine respectively.

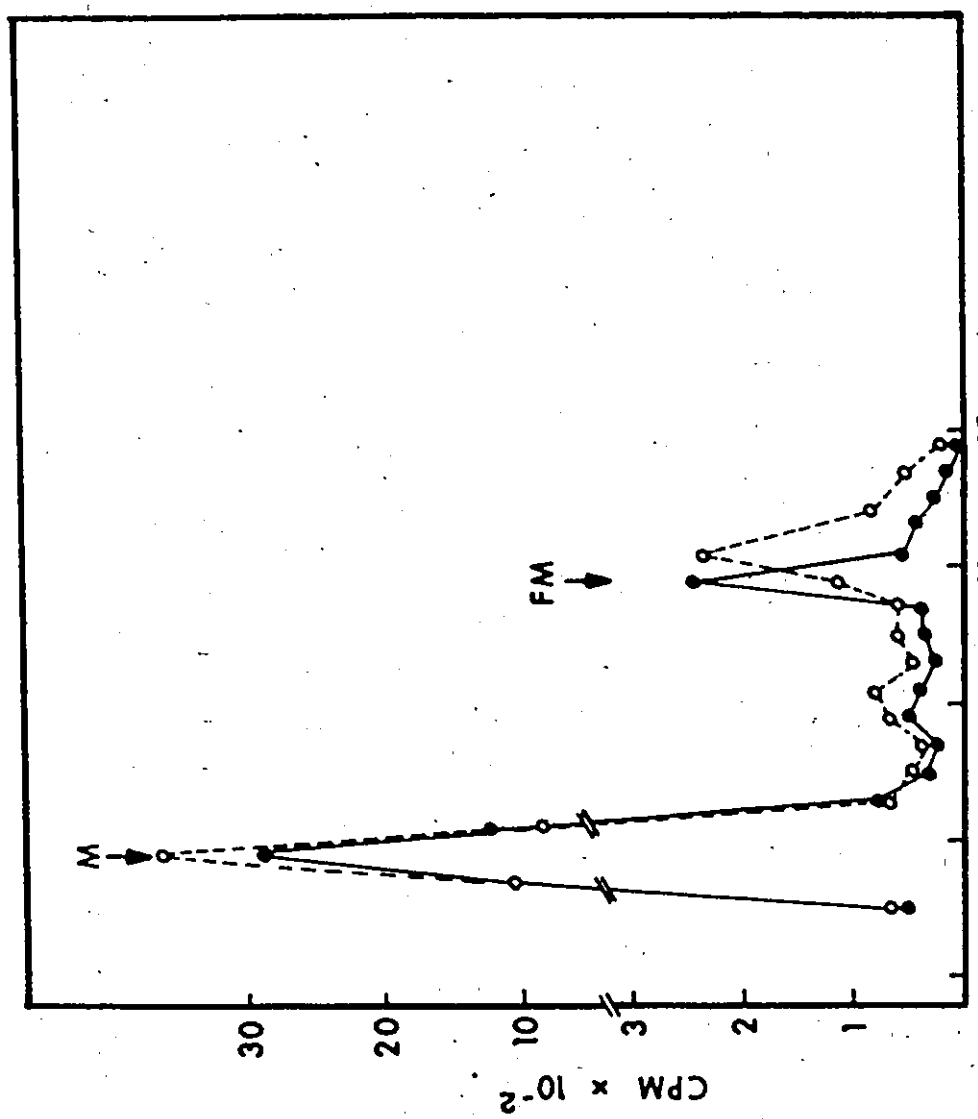

Figure 14

the presence of puromycin is a specific measure of the initiation of mitochondrial protein synthesis (Galper & Darnell, 1971). Any long-term effect of methotrexate on formylation should be reflected in decreased production of fMet-puromycin.

L cells incubated in the presence of puromycin produce fMet-puromycin. However, there is some unidentified compound, labelled with methionine and synthesized in the presence and absence of puromycin, which is extracted into ethyl acetate and which has an electrophoretic mobility close to that of fMet-puromycin. It is therefore difficult to quantitate the amounts of fMet-puromycin directly by electrophoresis. A modification of the procedure of Galper and Darnell (1971) was therefore developed. Cells incubated in the presence of puromycin and [^{35}S]methionine were extracted with ethyl acetate as usual; one-half of the extract was then hydrolysed in mild acid as described in the Methods, and both halves were analyzed for fMet-puromycin and Met-puromycin. The amount of Met-puromycin liberated by the acid treatment could be quantitated and is taken as the amount of fMet-puromycin produced by the cells. Fig. 15 shows an electrophoretic profile of the ethyl acetate extract (before and after acid hydrolysis) of L cells grown for eight generations in the presence of methotrexate and subsequently incubated in the presence of methotrexate, puromycin and [^{35}S]methionine. It is seen that there was still production of fMet-puromycin since Met-puromycin is released by the mild acid treatment. Further, as shown in Table VII, there was no apparent inhibition of fMet-puromycin production, implying that formylation was not inhibited.

It was possible that the inhibition of formylation observed by Galper (Galper & Darnell, 1969; Galper, 1970; 1974) in the presence of

Figure 15

Analysis of L cells, grown in the presence of methotrexate, for the production of fMet-puromycin. L cells were grown for 8 generations (160 hr) in the presence of 5×10^{-6} M methotrexate in HT medium. Cells were harvested and incubated in 5×10^{-6} M methotrexate, [^{35}S]methionine, and puromycin as described in the Methods for the production of fMet-puromycin. The cell extract was divided and the two portions evaporated to dryness. One portion was taken up in 0.2 M HCl in methanol and incubated in a sealed ampule at 37°C under N_2 for 20 hr, the other portion was stored at 4°C until needed. Each was subjected to electrophoresis at pH 3.5 for 150 min at 50 volts/cm. The paper was cut into 1 cm strips and each strip counted in a toluene-based scintillation fluid. The mild acid-treated sample (●——●) shows the presence of Met-puromycin (MP), the untreated sample (o----o) shows little Met-puromycin. fMet-puromycin has an electrophoretic mobility betweeen methionine (M) and an unidentified compound (?). The amount of fMet-puromycin produced by the cells is inferred from the amount of Met-puromycin released by mild acid hydrolysis.

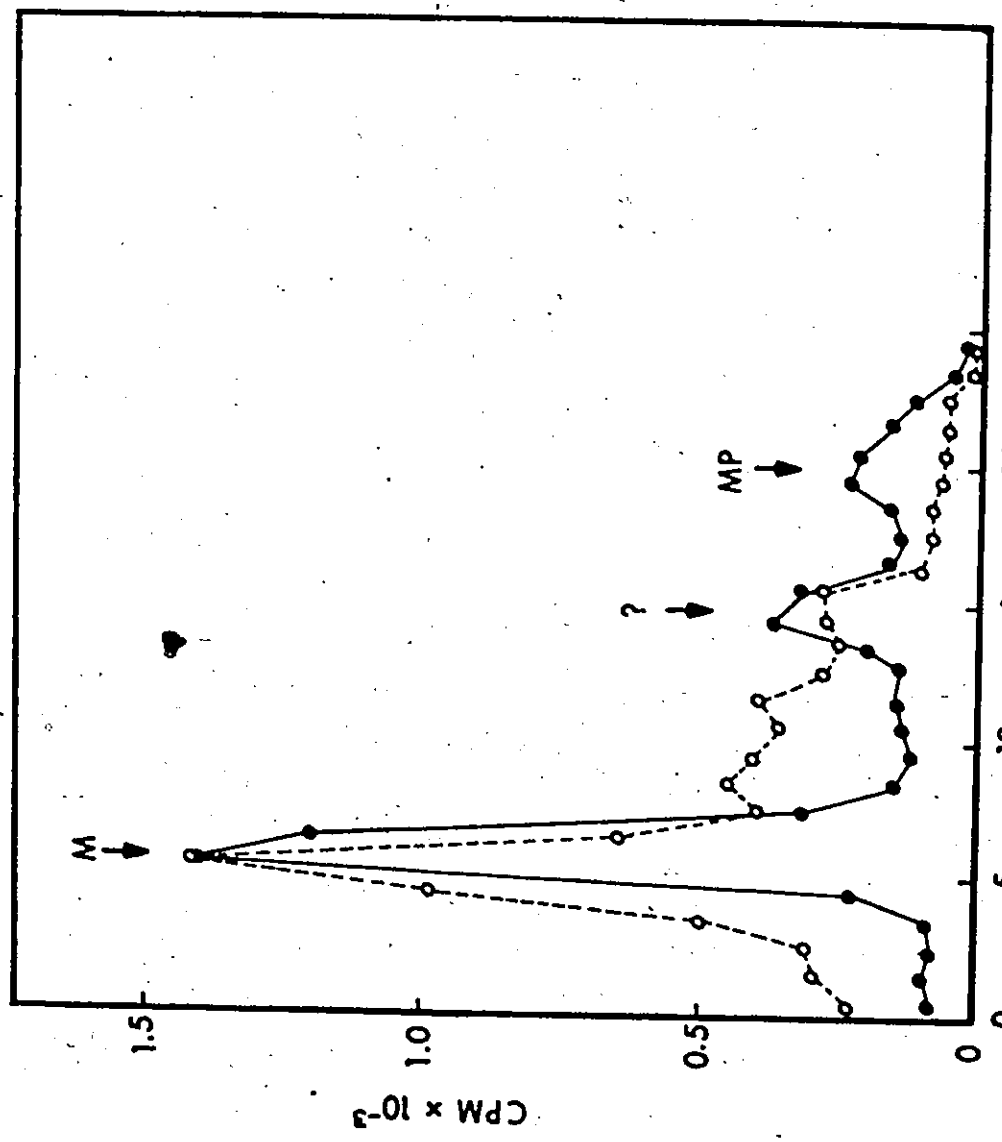

Figure 15

TABLE VII

A Summary of the Effects of Methotrexate on the Initiation of Protein Synthesis with a Formylated Species of Met-$tRNA_f^{met}$ in Mammalian Cell Mitochondria

Cell type	Duration of methotrexate treatment	fMet-puromycin (% control)
L cell	none	100
	160 hr	86
KB cell	none	100
	15 min	95
	60 min	98
	72 hr	116

The production of fMet-puromycin is estimated as described in the text and in Fig. 15 and Fig. 16. Methotrexate concentration is 5×10^{-6} M in each case and, in the case of the treated cultures, is present during the incubation with puromycin. Approximately 4000 cpm of fMet-puromycin (KB cells) or Met-puromycin (L cells) is recovered per 10^8 cells.

aminopterin and methotrexate is unique to human cell lines. Fig. 16 shows electrophoretic profiles of the ethyl acetate extracts of a human cell line (KB cells) incubated in the presence of puromycin and [^{35}S]-methionine after 15 (Fig. 16C) and 60 min (Fig. 16D) preincubation in the presence of methotrexate. It can be seen that there is no inhibition of fMet-puromycin synthesis (compare with control incubation (Fig. 16B)) even after one hr in the presence of methotrexate. A similar experiment to that reported in Fig. 16 was performed in KB cells grown for 72 hr in the presence of methotrexate. Again, no inhibition of fMet-puromycin was observed. These results are summarized in Table VII. KB cells, unlike L cells, do not produce the compound with an electrophoretic mobility in the region of fMet-puromycin. As can be seen in Fig. 17, essentially all of the fMet-puromycin produced by KB cells (Fig. 17A) can be converted to Met-puromycin after mild acid hydrolysis (Fig. 17B).

The lack of inhibition of fMet-puromycin production seen in KB cells grown in HAT medium for 72 hr (Table VII) suggests that the inhibition of growth seen after approximately 100 hr (4 generations) is not due to an inhibition of mitochondrial protein synthesis. The actual reason for the effect of methotrexate on growth of KB cells is not known; it is not seen in the growth of hybrid cells, nor other cells which have been examined.

Met-tRNA^{met} and fMet-tRNA$^{met}_f$ from Mouse Liver Mitochondria

To further characterize the initiation of mitochondrial protein synthesis, the methionyl-tRNAs from mitochondria were analyzed. Mitochondria from mouse liver were labelled *in vitro* with [^{3}H-CH$_3$]methionine

Figure 16

The effect of short preincubation in the presence of methotrexate on the production of fMet-puromycin in KB cells. KB cells were incubated in the presence of [^{35}S]methionine and puromycin in HT-medium lacking serum as described in the Methods for the measurement of fMet-puromycin (FMP) production: (a) puromycin omitted from the incubation shows the absence of the unidentified compound (?) found in L cells (cf. Fig. 15); (b) fMet-puromycin production in the absence of methotrexate; (c) 15 min preincubation in the presence of 5×10^{-6} M methotrexate; (d) 60 min preincubation in the presence of 5×10^{-6} M methotrexate. Methotrexate was also present during the incubation in the presence of puromycin in (c) and (d).

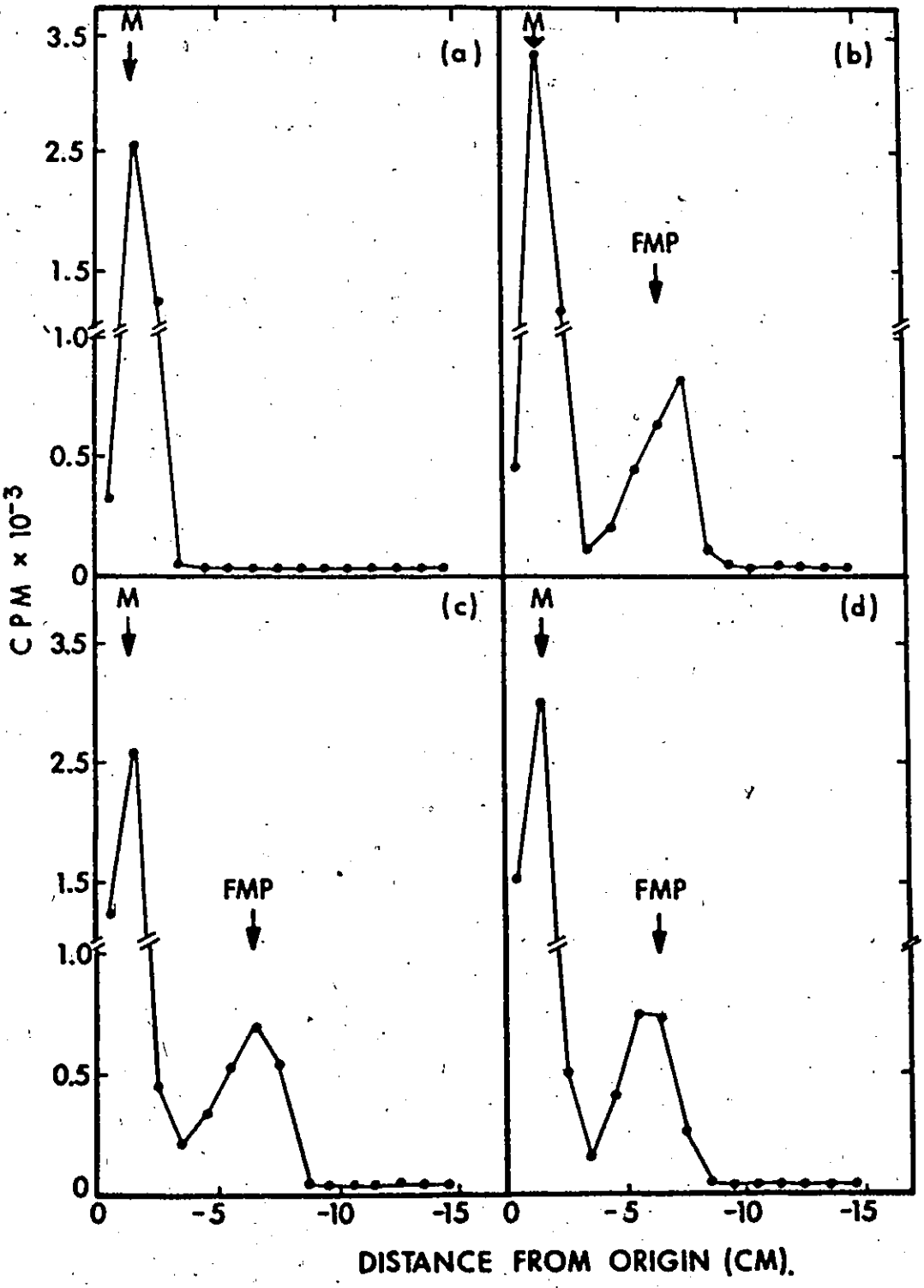
M
(a)
(b)
(c)
(d)
FMP
3.5
2.5
1.5
1.0
0.5
0
CPM x 10^{-3}
-5
-10
-15
DISTANCE FROM ORIGIN (CM).

Figure 16

Figure 17

Characterization of the fMet-puromycin produced by KB cells. KB cells were labelled with [^{35}S]methionine in the presence and absence of puromycin as described in the Methods and the legend to Fig. 16. A, High voltage electrophoresis of material labelled with [^{35}S]methionine in the presence (●——●) or absence (o——o) of puromycin; B, high voltage electrophoresis of material labelled with [^{35}S]methionine in the presence (●——●) or absence (o——o) of puromycin after mild acid hydrolysis in 0.2 N HCl in methanol at 37°C for 20 hr. M, FMP and MP refer to methionine, fMet-puromycin and Met-puromycin respectively.

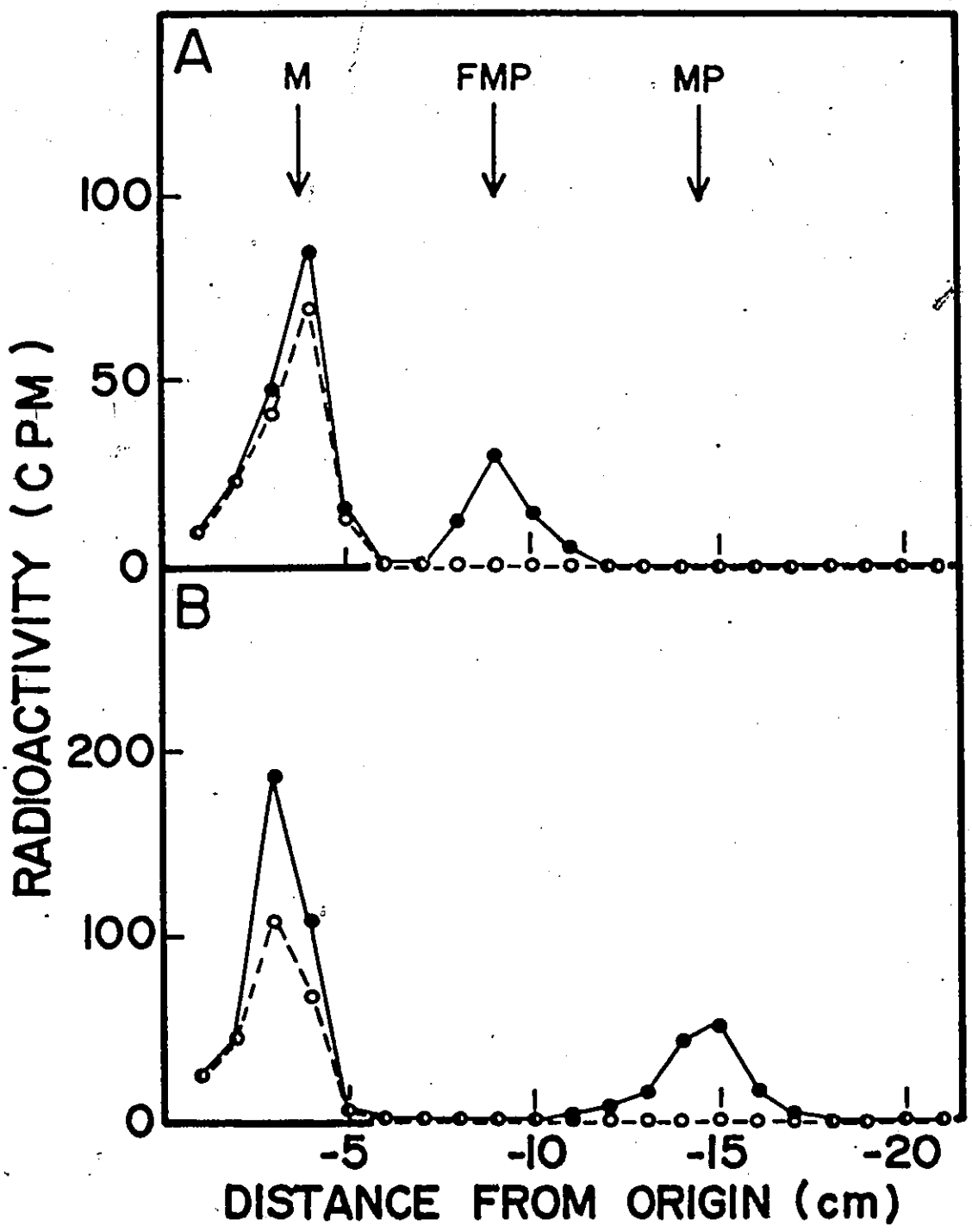
A
M
FMP
MP
100
50
0
B
200
100
0
RADIOACTIVITY (CPM)
-5
-10
-15
-20
DISTANCE FROM ORIGIN (cm)

Figure 17

and the methionyl-tRNA extracted as described in the Methods. The cytosolic methionyl-tRNAs were labelled with [^{35}S]methionine. When both the mitochondrial and cytosolic methionyl-tRNAs are digested with pancreatic RNase and subjected to high voltage electrophoresis, the cytosolic methionyl-tRNAs yield methionyladenosine and methionine (a deacylation product) while the mitochondrial methionyl-tRNAs yield methionyladenosine, N-formylmethionyladenosine and methionine (Fig. 18). As reported by Smith and Marcker (1968) for rat liver mitochondria and by Galper and Darnell (1969) for HeLa cell mitochondria, these results indicate that cytosolic methionyl-tRNAs are not formylated while mitochondrial methionyl-tRNAs are both formylated and unformylated.

When the mitochondrial and cytosolic methionyl-tRNAs were co-chromatographed on RPC-5 (Fig. 19), it was seen that the mitochondrial tRNA resolved into 2 major and 2 minor species (labelled II, IV, I and III respectively in Fig. 19) eluting prior to the 3 cytosolic species (labelled V, VI and VII in Fig. 19). Essentially the same RPC-5 profile is obtained from the mitochondria of Ehrlich ascites cells. When species I, II, III and IV were isolated and subjected to RNase digestion and high voltage paper electrophoresis, it was found that species I and II are not formylated while species III and IV are (Fig. 20). Fig. 18 already demonstrated that the cytosolic species (V, VI and VII) are not formylated.

Since mitochondrial initiator tRNA is a formylated methionyl-tRNA (Smith & Marcker, 1968; Galper & Darnell, 1969; 1971; Epler et al, 1970; Halbreich & Rabinowitz, 1971), species III, species IV or both must fulfill this role in mouse liver mitochondria.

Figure 18

An electrophoretic profile of the RNase digests of [^{35}S]methionine labelled cytosolic met-tRNA^{met} (o——o) and [^{3}H-CH$_3$]methionine labelled mitochondrial met-tRNA^{met} (●——●). Met, f-MetA and MetA refer to methionine, N-formylmethionyladenosine and methionyladenosine respectively.

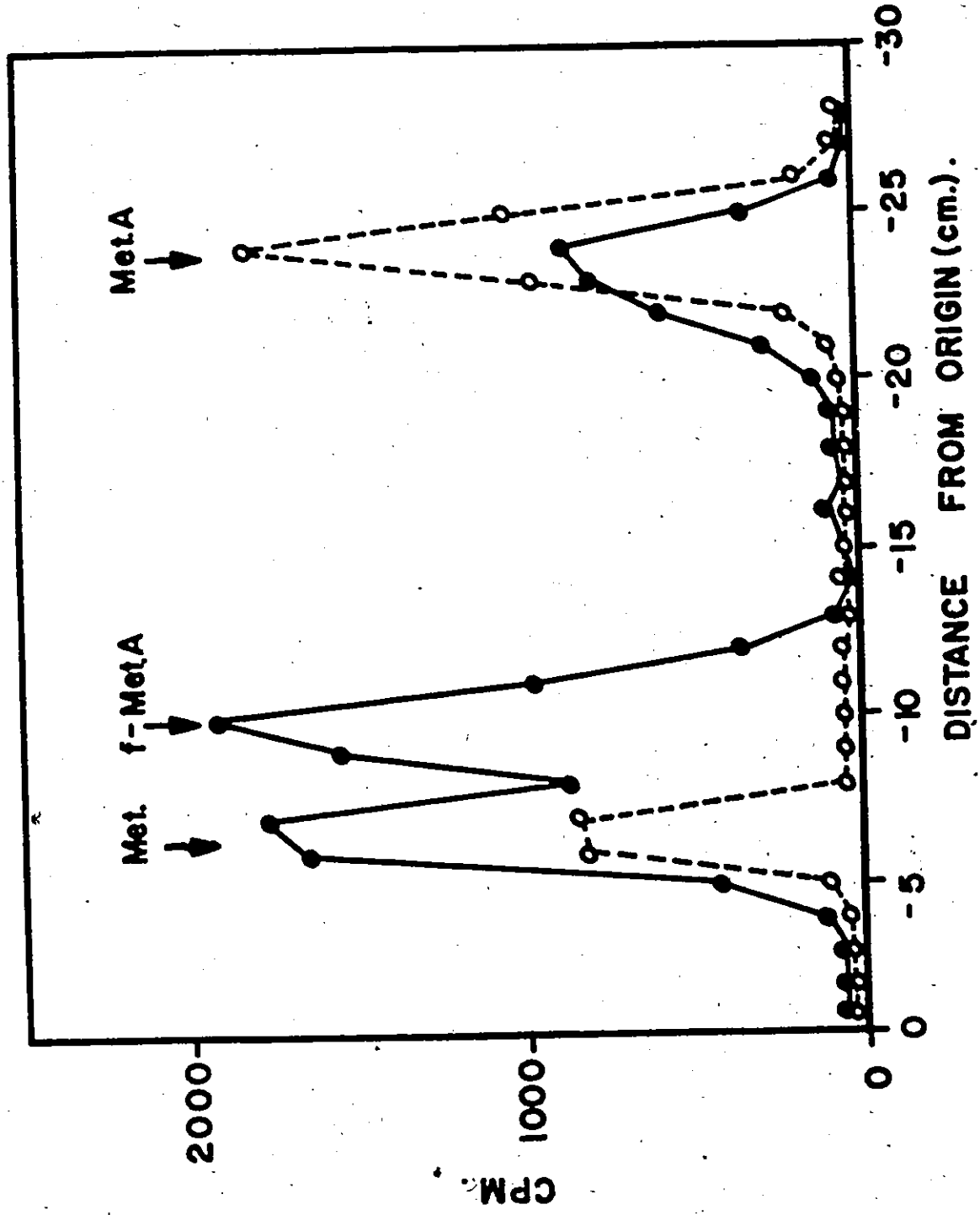
MetA
f-MetA
Met.
2000
1000
CPM
0
-5
-10
-15
-20
-25
-30
DISTANCE FROM ORIGIN (cm.).

Figure 18

Figure 19

RPC-5 profile of [^{3}H] methionyl-tRNA from mouse liver mitochondria (●——●) co-chromatographed with [^{35}S] methionyl-tRNA from mouse liver cytosol (o----o).

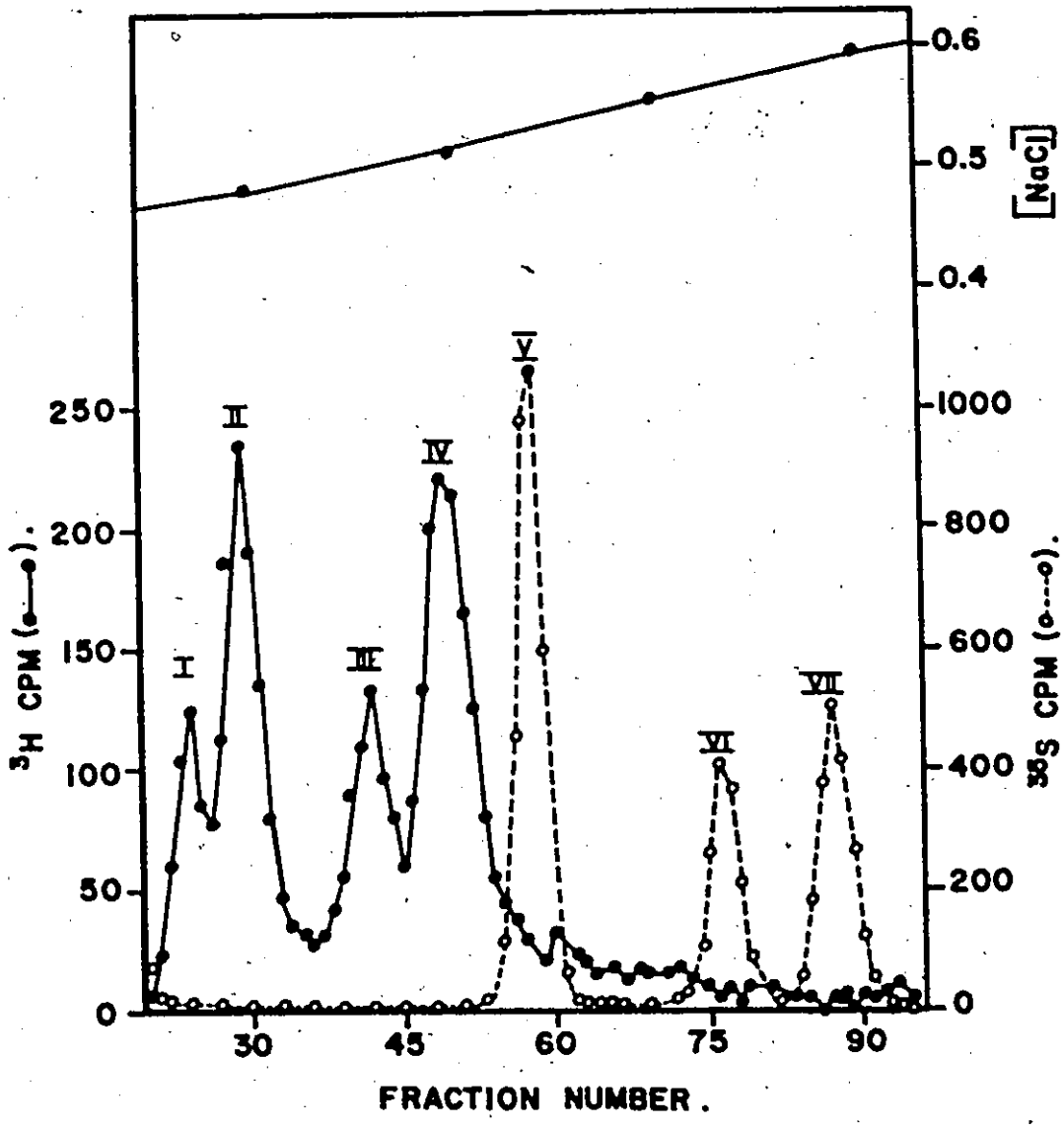
0.6
0.5
[NaCl]
0.4
250
200
150
100
50
0
1000
800
600
400
200
0
I
II
III
IV
V
VI
VII
30
45
60
75
90
FRACTION NUMBER.
^{3}H CPM (●—●).
^{35}S CPM (o---o).

Figure 19

Figure 20

The electrophoretic profiles of the RNase digests of the isolated [^{3}H] methionine labelled mitochondrial methionyl-tRNAs. I, II, III and IV refer to the four mitochondrial methionyl-tRNAs of Fig. 19. Met, f-Met A and Met A are defined in Fig. 18.

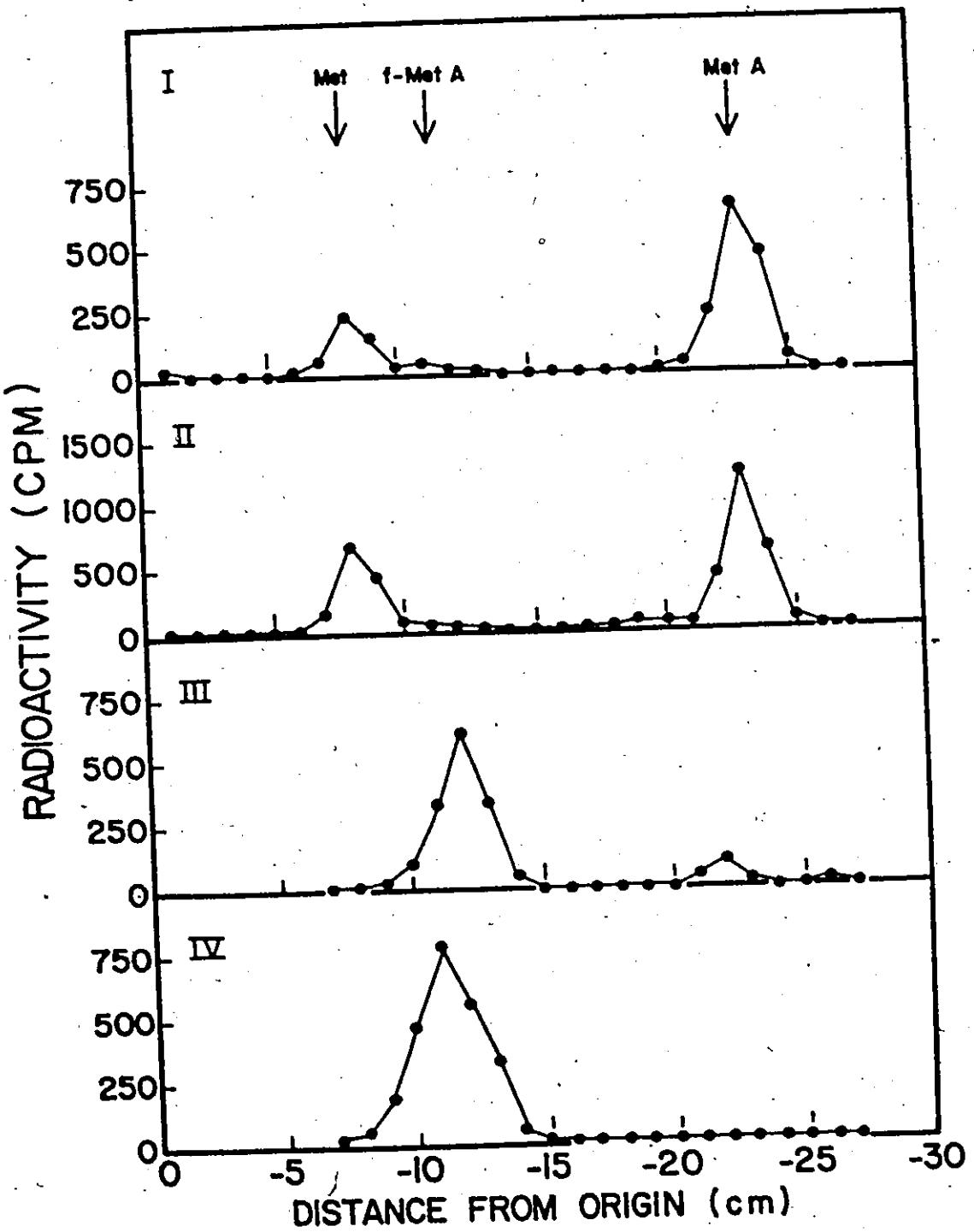
I
Met
f-Met A
Met A
750
500
250
0
II
1500
1000
500
0
III
750
500
250
0
IV
750
500
250
0
RADIOACTIVITY (CPM)
0
-5
-10
-15
-20
-25
-30
DISTANCE FROM ORIGIN (cm)

Figure 20

DISCUSSION

Separation of Hamster and Human mtDNA

The results reported above demonstrate the feasibility of separating mtDNA of hamster from that of human. Previous results from this laboratory have demonstrated the feasibility of separating 12S mtrRNA of hamster and human cells by gel electrophoresis (Mitra et al, 1972) as well as of separating human mitochondrial NAD-malate dehydrogenase, NADP-isocitrate dehydrogenase and glutamate-oxaloacetate transaminase from the hamster enzymes by starch gel electrophoresis (Strasberg et al, 1974). These three techniques should now allow the analysis of human-hamster hybrids for the persistence of parental mtDNAs, their expression and the incorporation of the three mitochondrial-specific enzymes. The success of the somatic cell hybrid system to add to the understanding of the study of mitochondrial biogenesis requires the availability of such techniques to analyze species specific mitochondrial components.

The one human-hamster hybrid cell examined appeared to segregate both human chromosomes as well as human mtDNA. To rule out that no sequence of human mtDNA were present in the mtDNA from the hybrid (recombination), one should analyze the mtDNA by the cRNA-hybridization technique described above.

The Initiation of Mitochondrial Protein Synthesis

The ability of hybrid cells and L cells (Fig. 12) to grow normally in HAT medium suggested that methotrexate does not interfere with mitochondrial protein synthesis (Fettes et al, 1972). This result is contrary

to the conclusions of Galper and Darnell (1969) and Galper (1970) studying the effects of methotrexate and aminopterin on the formylation of mitochondrial met-tRNA$_f^{met}$ and on mitochondrial protein synthesis in HeLa cells. They found that both aminopterin and methotrexate inhibited cell growth, mitochondrial protein synthesis and the formylation reaction and suggested that this was a direct effect on C_1-H_4 folate metabolism. The results reported here suggest that formylation and the initiation of mitochondrial protein synthesis continue in the presence of methotrexate and metabolites whose *de novo* synthesis requires C_1-H_4 folate derivatives.

The fact that formylation is not inhibited under conditions where C_1-H_4 folate pools should be depleted is paradoxical only if the formyl group is donated by a tetrahydrofolate derivative as in yeast mitochondria (Halbreich & Rabinowitz, 1971) and prokaryotes (Dickerman et al, 1967). Even so, there are other conceivable mechanisms by which a source of reduced folate could be maintained in the presence of methotrexate. For example, compartmentalization of dihydrofolate reductase, the target of methotrexate action, within mitochondria might allow sufficient reduced folate synthesis for the mitochondria to function normally. The actual mechanism of the formylation reaction under these conditions remains unknown.

The question whether mammalian mitochondria require a formylated initiator tRNA for efficient protein synthesis remains open. The observations reported here, however, demonstrate that L cells and KB cells maintain the formylation reaction under conditions of depleted C_1-H_4 folate pools, which may indicate that this reaction cannot be dispensed with. It appears from available data, that efficient initiation of

protein synthesis in the absence of formylation (as in eukaryotic cytoplasm (RajBhandary et al, 1973) and in folate-deficient *S. faecalis* R (Delk & Rabinowitz, 1974) requires an initiator tRNA of unique structure, different from that of prokaryotes which do require formylation (e.g. *E. coli* (Eisenstadt & Lengyel, 1966)) and different from initiator tRNA from folate-sufficient *S. faecalis* R (Samuel & Rabinowitz, 1974). These unformylated initiator tRNAs all lack rTp in their "TψCG" loop. Possibly, for mitochondria to dispense with formylation, a modified initiator tRNA would be required as in the folate-requiring bacterium *S. faecalis* R.

Studies of the effects of folate analogues on yeast have recently been reported. Wintersberger and Hirsch (1973a;b) studying the effects of methotrexate and Stone and Wilkie (1974) studying the effects of pyrimethamine on yeast, both find that these drugs inhibit growth and mitochondrial protein synthesis. Both authors postulate that the drugs inhibit mitochondrial protein synthesis by removing a source of C_1-H_4 folate for the formylation of mitochondrial Met-$tRNA^{met}_f$. It seems possible, however, that the inhibition of mitochondrial protein synthesis might be due to the inhibition of synthesis of nucleotides and thus of mtDNA and RNA synthesis. Yeast cannot utilize exogenous thymidine (Grivell & Jackson, 1968; Zeman & Lusena, 1974), so this possibility cannot be tested by adding purine and pyrimidine nucleotide precursors. The idea that nucleic acid synthesis is inhibited is supported by the observation in all three papers that methotrexate or pyrimethamine are potent inducers of petite mutants. Although the authors interpret this as a direct effect on mitochondrial protein synthesis, inhibition of mitochondrial translation *per se* is not very effective in producing

petite mutants (Carnevali et al, 1971; Williamson et al, 1971; Schatz & Mason, 1974). Therefore, the results in yeast as well as those in HeLa cells should be reevaluated in the light of the data presented here.

Multiple Species of Mitochondrial Methionyl-tRNAs

As in the case of mitochondrial leucyl-tRNAs reported in Chapter I, mitochondrial methionyl-tRNAs appear to exist as multiple species (Fig. 19). Although the four mitochondrial methionyl-tRNAs are resolved by RPC-5 chromatography, it is not known whether they share the same primary structure or what their transcriptional origin is. In total, seven species of mitochondrial tRNAs (three leucyl- and 4 methionyl-) have been identified in this investigation. Since mammalian mtDNA may code for as few as 12 - 15 tRNA molecules (see Introduction, Chapter I), multiple species of tRNA would clearly limit the coding capacity of mtDNA to only a few amino acyl-tRNAs. This is one of the many unsolved problems of mitochondrial biogenesis. Suyama et al (1974) have suggested that tRNAs are imported into the mitochondria from the cytosol. This would alleviate the problems of coding of tRNAs by mtDNA but raises the question of why mtDNA would code for some tRNAs and not others.

It is interesting that 2 species of mitochondrial Met-tRNA$^{met}_{f}$ are formylated. Presumably, both are utilized in the initiation of mitochondrial protein synthesis. It is possible that the 2 minor species (I and III in Fig. 19) represent differences in the extent of modification of the adjacent major species (II and IV respectively). *E. coli* fMet-tRNA$^{met}_{f}$ exists as a major and minor species, the only difference being in the replacement of 7-methyl guanosine with adenosine at position 47 of the

tRNAs (Dube et al, 1968; Egan et al, 1973). The possibility that these 4 species are artifacts due to diester bond cleavage seems to be ruled out by the fact that essentially the same RPC-5 pattern is obtained from Ehrlich ascites cell mitochondria.

CHAPTER III

An Approach to the Study of Mitochondrial Genetics in Mammalian Cells: Selection of Cells Resistant to Tevenel

INTRODUCTION

In Chapter I the usefulness of a conditional mutant to the study of mitochondrial biogenesis was demonstrated. In this Chapter, another application of mutants to the study of mitochondrial biogenesis will be considered, mutants whose genetic alterations reside on mtDNA. The study of mitochondrial genetics in yeast has become a very complex problem area. However, it has been very successful in producing information about mitochondrial biogenesis which could not have been obtained by any other approach (Dujon et al, 1974). The same success has not been achieved in the study of mitochondrial genetics in mammalian cells.

While a detailed discussion of the study of mitochondrial genetics in yeast is beyond the scope of this thesis (for reviews see Coen et al, 1970; Sager, 1972; Mahler, 1973; Linnane et al, 1973; Dujon et al, 1974), some of the conclusions reached from these studies are important and may be applicable to the study of mitochondrial genetics in mammalian cells. Other conclusions and speculations from studies in yeast are intimately related to yeast physiology and may not be applicable in mammalian cells. For example, crosses between two strains of yeast carrying allelic markers on their mtDNA often result in a bias of transmission of the

allele of one parent to the progeny (sometimes referred to as suppressiveness) (Coen et al, 1970). Also, when more than one genetic locus is involved in crosses, non-reciprocal recombination is often observed (Dujon et al, 1974). Although neither of these phenomena are well understood, they may be related to the same instability of the yeast mtDNA which results in petite formation (Perlman & Birky, 1974).

Mitochondrial Mutations in Yeast Conferring Resistance to Inhibitors of Mitochondrial Function

Studies in yeast have been successful in the isolation of cells resistant to inhibitors of mitochondrial function such as erythromycin, chloramphenicol, oligomycin and paromomycin (cf. Avner & Griffits, 1970; Stuart, 1970; Kutzleb et al, 1973; Molloy et al, 1973). Many of these phenotypes are not inherited in a Mendelian fashion when analyzed in crosses and the terms maternal, non-Mendelian or cytoplasmic inheritance have been applied (Coen et al, 1970). Available evidence has supported the hypothesis that the resistance-phenotypes are conferred by genetic loci on the mitochondrial genome (Dujon et al, 1974), although one cannot rule out the existence of non-mitochondrial genetic material in the cytoplasm (Guerinea et al, 1974).

As summarized by Dujon et al (1974), there are seven known genetic loci on yeast mtDNA, ω which is termed the sex locus by Slonimski and co-workers (although ω controls polarity of recombination and may not have anything to do with sexuality (Perlman & Birky, 1974)), R_I conferring resistance to chloramphenicol, R_{II} and R_{III} conferring resistance to erythromycin and spiramycin, O_I and O_{II} conferring resistance to

oligomycin and P_I conferring resistance to paromomycin. Each locus has many alleles which can be distinguished phenotypically but not by recombination. The ω, R_I, R_{II} and R_{III} have been shown to be linked by recombination analysis while the other three are unlinked.

The studies of these genetic loci and their recombination was based on the hypothesis that the information for the phenotype was coded by mtDNA. If this were true, one would expect that recombination of genetic loci was a process of the breaking of the phosphodiester backbones of two mtDNA molecules with the subsequent exchange of double stranded regions. Suggestive evidence for this process in somatic cell hybrids was already discussed in Chapter II (Horak et al, 1974). Michaelis et al (1973) studied the recombination of genetic markers in two petite mutants of yeast with mtDNAs of different buoyant densities[2]. The recombinant progeny were found to contain mtDNA of intermediate buoyant densities, suggesting recombination resulted from an exchange of regions of the mtDNA and supporting the hypothesis that the information for the observed phenotypes resides on mtDNA.

Mitochondrial genetics in *Paramecium* has also been studied recently, but the studies are not as advanced as those in yeast (see Beisson et al, 1974). Genetic crosses with mitochondria are achieved by a rather unique technique, in that mitochondria of one type can be introduced by microinjection into a cytoplasm of a cell containing a different

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2 Petite mutants of yeast result from the deletion of large aequences from mtDNA, which may change the buoyant density of the DNA (Perlman & Mahler, 1971; Lazowska et al, 1974). The mtDNA of these petites may, however, retain genetic information (Moumolon et al,1966; Borst,1974).

type of mitochondria. Studies have begun to yield results which suggest recombination of genes on mtDNA can be observed in *Paramecium* as well (Adoutte, 1974).

Studies of cytoplasmic genetics in *Neurospora* have not developed very far. The poky phenotype, which is a cytoplasmic-characteristic (Sager, 1972) is a rather ill-defined mutation resulting in slow growth. It appears to be associated with a deficiency in the small mitochondrial ribosomal subunit (Rifkin & Luck, 1971), which may account for the slow growth since the rate of mitochondrial protein synthesis is depressed (Sebald et al, 1968). Recently, Kuriyama and Luck (1974) have shown that in poky the processing of mtrRNA (see Kuriyama & Luck, 1973) is altered. Also, the methylation of both 25S and 19S mtrRNA is lower in poky cells than in the wild type cells and may be related to the alterations in processing and of the deficiency in the appearance of the small mitochondrial ribosomal subunits. However, Kuriyama and Luck (1974) conclude that "The true nature of the defect in poky and its relationship to cytoplasmic inheritance remain to be solved."

Biochemical Characterization of Mitochondrial Mutants of Yeast

Biochemical studies on yeast strains carrying mutations on mtDNA have not been successful in the identification of an altered gene product responsible for the phenotype (Schatz & Mason, 1974). However, there have been some preliminary results which suggest one of the altered gene products conferring resistance to chloramphenicol, erythromycin and spiramycin, might be mtrRNA.

Comparison of mitochondrial and cytosolic ribosomal proteins in

Neurospora by chromatographic methods (Küntzel, 1969) or by labelling in the presence of inhibitors of mitochondrial or cytosolic protein synthesis (Lizardi & Luck, 1972) have demonstrated that most if not all mitochondrial ribosomal proteins are different from the cytosolic ones and are products of cytosolic protein synthesis. Mutants of yeast which are resistant to inhibitors of mitochondrial protein synthesis and whose resistance is conferred by mtDNA are not likely to possess an altered mitochondrial ribosomal protein. This was confirmed by Grivell et al (1973) studying mutants of yeast resistant to chloramphenicol, erythromycin and spiramycin. Although all the mutants were a result of mutations of mtDNA and the resistance was found to be due to altered mitochondrial ribosomes (see also Grivell et al, 1971), no changes to mitochondrial ribosomal proteins could be detected by polyacrylamide gel electrophoresis. The authors postulate that the changes responsible for resistance to inhibitors of mitochondrial protein synthesis are within the mtrRNA cistrons already known to be on mtDNA. A second piece of evidence supporting this speculation comes from analysis of petite mutants of yeast (Nagley et al 1974). These authors observe that petites can be isolated in which large segments of mtDNA have been deleted but which have retained the loci for chloramphenicol and/or erythromycin resistance. In petites retaining chloramphenicol or erythromycin loci, the cistron for 23S mtrRNA is selectively retained; whereas, if the chloramphenicol and erythromycin loci are not retained, neither is the cistron for 23 mtrRNA. Nagley et al (1974) conclude that the mtrRNA cistron are close to or are part of the antibiotic resistance loci.

Alterations in rRNA in bacteria have been implicated in the

resistance to erythromycin in *Staphylococcus aureus* (Lai & Weisblum, 1971) or to kasugamycin in *E. coli* (Helser et al, 1971a; 1971b). Both of these changes in rRNA are due to alterations in methylation and probably the result of changes in an enzyme (methylase) rather than the rRNA cistron. Changes in the rRNA cistrons have not been observed to be responsible for antibiotic resistance in bacteria (Borst, 1972). Because of the large number of rRNA cistrons per genome (Amaldi & Attardi, 1971) a mutation in only one rRNA gene in bacteria would not be expressed as resistance (Borst, 1972). MtDNA, on the other hand, has only one cistron for each mtrRNA and a single mutation in a mtrRNA gene would, in the presence of antibiotic, be selected for and thus be easier to detect than in bacteria (Grivell et al, 1973).

Mammalian Cells Resistant to Inhibitors of Mitochondrial Function

No genetic studies of the mitochondrial genome in mammalian cells have yet been reported. This failure has been mainly due to the lack of mutants of mitochondrial function whose mutation resides on mtDNA and to the lack of available systems for "mating" mitochondria. Recently, Eisenstadt and co-workers have reported the selection of HeLa cells which are resistant to chloramphenicol (Spolsky & Eisenstadt, 1972). They employed ethidium bromide and suggested that it would act as a specific mutagen of mtDNA. Although ethidium bromide interacts with mtDNA (see Introduction, Chapter I) it is not known how this would be a mutagen, except by inducing large deletions in mtDNA as it does in yeast (Perlman & Mahler, 1971; Borst, 1972). Spolsky and Eisenstadt (1972) examined the effects of chloramphenicol on protein synthesis by isolated mitochondria

of sensitive and resistant cells. Protein synthesis by mitochondria of resistant cells was inhibited by chloramphenicol, but only by 50% even at the highest concentration, while protein synthesis by mitochondria of sensitive cells was inhibited 90% at the highest concentration. The resistance of isolated mitochondrial protein synthesis to chloramphenicol was not altered by treating the mitochondria with 0.01% Triton X-100, suggesting that a permeability barrier was not responsible for the observed resistance to the inhibitor. The authors suggest that the chloramphenicol-resistant HeLa cells are mutants of the mitochondrial genome.

In order to prove that the chloramphenicol resistance observed above was conferred by a mutation on mtDNA, Bunn et al (1974) attempted to show cytoplasmic inheritance of the resistance trait. Since systems were not available to mate cultured mammalian cells to observe non-Mendelian segregation, as in yeast, Bunn et al used an alternative approach. They isolated chloramphenicol-resistant mouse A9 cells ($HGPRT^-$) by the same procedure described for HeLa cells, enucleated these cells and then fused them with $LMTK^-$ cells (TK^-, chloramphenicol sensitive). The resulting "cybrids" (cytoplasmic hybrids) were plated in a selective medium containing both BUdR (to select against any remaining nucleated A9 cells) and chloramphenicol. The resulting colonies which arose were considered to occur from the transfer of cytoplasm (i.e. the mitochondria) of the chloramphenicol resistant A9 cells to the chloramphenicol sensitive $LMTK^-$ cells. This suggests that the resistance to chloramphenicol was conferred by a mutation on mtDNA. These authors found no colonies growing when $LMTK^-$ cells were plated in chloramphenicol;

I shall return to this point in the Discussion.

An Alternative Approach to the Selection of Mutants of Mitochondrial Function

The thymidine kinase-deficient mouse cell line LMTK$^-$ (Kit et al (1963) has been shown to contain a thymidine kinase activity associated with the mitochondria (Attardi & Attardi, 1972; Kit et al, 1973a;b). This enzyme is presumably responsible for the incorporation of BUdR (and [^{3}H]thymidine) specifically into mtDNA of these cells. The possibility exists that specific incorporation of BUdR into mtDNA may result in mutation of the mitochondrial genome. BUdR has been shown to be an effective mutagen in bacteriophage (Litman & Pardee, 1956) as well as in animal cell viruses (Sambrook et al, 1966; Basilico & Joklik, 1968).

LMTK$^-$ cells were therefore chosen in an attempt to induce mutations in the mitochondrial genome with BUdR specifically. Cells with a resistance to tevenel have been isolated both with and without BUdR treatment. Tevenel, the sulfamoyl analogue of chloramphenicol, is a potent inhibitor of mitochondrial protein synthesis (Freeman, 1970) but does not have the undesirable side effect of chloramphenicol, which inhibits respiration (Freeman & Haldar, 1967; 1968; Haldar & Freeman, 1968). Cells selected for resistance to tevenel without prior BUdR treatment will be described below. Preliminary characterization of the effects of BUdR in inducing mutations will also be described.

METHODS

Growth and Maintenance of Cell Cultures

The LMTK$^-$ cell line, originally derived by Kit et al (1963) was obtained from Dr. B.L. Hillcoat of this Department. The cells were maintained in suspension culture in MEM-Joklik supplemented with 5% fetal calf serum as described by Bartoov et al (1970). Cells resistant to tevenel were also maintained in this way, however the medium usually contained 100 µg tevenel/ml as well (see below).

Selection of Cells Resistant to Tevenel

Initial experiments were designed to use BUdR as a specific mutagen of mtDNA in the LMTK$^-$ cell line. In the course of these experiments it was found that a significant proportion of the LMTK$^-$ cell population could be selected for resistance to tevenel without prior BUdR treatment. In these early experiments BUdR was not found to increase the yield of resistant clones. In later experiments, however, a modification of the BUdR treatment procedure (selection procedure B, below) produced an increase in resistant clones. The resistant cells to be described here, however, were selected without prior BUdR treatment.

Selection Procedure A

The resistant cells to be described here were selected by the following procedure (selection procedure A of Fig. 21): LMTK$^-$ cells growing in suspension culture were treated for 5 days with 100 µg

Figure 21

A schematic representation of two procedures for the selection of tevenel resistant LMTK$^-$ cells. + BUdR means the growth medium contained 25 µg BUdR/ml, + TEV means the growth medium contained 100 µg tevenel/ml. See text for a fuller explanation of the procedures.

Selection Procedure A:

Spinner Culture	Spinner Culture	Monolayer Culture	Colony Growth	Growth of Clonal
± BUdR	+ TEV	+ TEV	+ TEV	Isolates + TEV
24 hours	5 days	2 weeks	2 weeks	2 weeks

Selection Procedure B:

Spinner Culture	Monolayer Culture	Colony Growth	Growth of Clonal
± BUdR	+ TEV	+ TEV	Isolates + TEV
96 hours	3 weeks	2 weeks	2 weeks

Figure 21

tevenel/ml. At the end of the 5 day treatment the cells had essentially stopped growing (cf. Fig. 23). The cells were then plated onto 100 mm Petri dishes and maintained in MEM-Eagle supplemented with 10% fetal calf serum and 100 µg tevenel/ml. The medium was changed frequently since it became very acidic. As a control, mouse L cells were treated in an identical manner. After 2 weeks of growth in tevenel, the $LMTK^-$ cell line yielded approximately 80 resistant clones from the 5×10^5 cells plated, while the L cell line yielded none. The Petri dishes containing the resistant $LMTK^-$ cells were allowed to grow to confluence, harvested and clones selected as described by Puck et al (1956). Three of these clones, selected without BUdR treatment (denoted T2, T8 and T22), were chosen for analysis. Stained Petri dishes showing the clones formed with $LMTK^-$ cells (Fig. 22B) and an L cell control plate (Fig. 22A) are shown in Fig. 22.

Selection Procedure B

In order to study the effects of BUdR on the production of resistant cells, an alternative selection procedure was developed. This procedure has been schematically represented in Fig. 21 (selection procedure B).

Cells were treated with 25 µg BUdR/ml for a longer period of time than in procedure A. The cells were then immediately plated in the presence of 100 µg tevenel/ml, rather than first growing them for 5 days in suspension culture in the presence of tevenel. The number of clones produced under these conditions should reflect the number of resistant cells in the population at the time of plating.

Figure 22

Stained Petri dishes showing resistant clones formed in selection procedure A. LMTK$^-$ cells (B) or L cells (A) were treated as described in the Methods and Fig. 21 by selection procedure B. No BUdR treatment was used. The clones which appeared two weeks after plating 5×10^5 cells, are stained with 0.2% methylene blue. No clones are observed for L cells (A) while approximately 80 are observed for LMTK$^-$ cells (B).

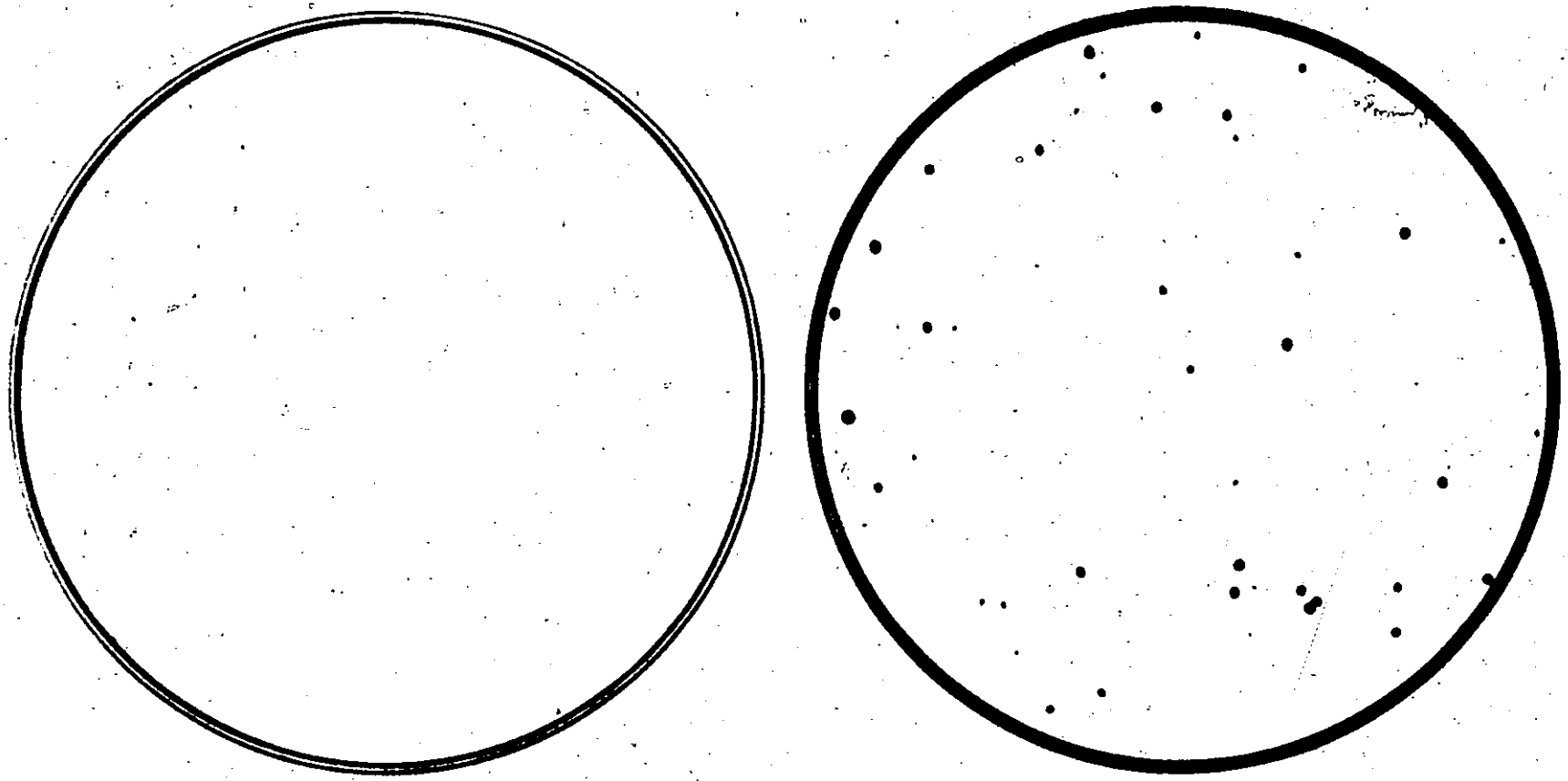

Figure 22

Measurement of Cell Growth

Cell growth in the presence and absence of 100 µg tevenel/ml was measured in suspension cultures of LMTK$^-$ or tevenel resistant cells as described in Chapter II.

To determine whether the cells resistant to tevenel retained their thymidine kinase deficiency, the ability of the cells to grow in (a) MEM-Eagle supplemented with 10% fetal calf serum and 25 µg BUdR/ml (Kit et al, 1963), or (b) HAT medium (10^{-4}M hypoxanthine, 5×10^{-5}M thymidine and 10^{-6}M methotrexate) (Littlefield, 1964) was examined. Cells deficient in thymidine kinase are able to grow in 25 µg BUdR/ml but not in HAT medium.

Measurement of Relative Plating Efficiency

Plating efficiency of LMTK$^-$ or resistant cells was determined by scoring the number of clones formed on plates innoculated with 200 cells. The absolute plating efficiency was variable but usually in the range of 70 - 90%. The relative plating efficiency is reported in order to make the results comparable from experiment to experiment and from cell type to cell type.

Measurement of Protein Synthesis by Isolated Mitochondria

Mitochondria were isolated from 2×10^8 cells essentially as described by Constantino and Attardi (1973) except the cells were washed and disrupted in Medium B using an Ultra-Turrax homogenizer as described by Freeman (1965). Mitochondrial protein synthesis was measured by incubating the mitochondrial fraction at 30°C in Medium 1 as described

in Chapter I. The effect of increasing concentrations of tevenel or D-chloramphenicol on mitochondrial protein synthesis was determined in a 30 min incubation. In experiments designed to determine the effect of Triton X-100 on mitochondrial protein synthesis, the incubation medium was modified. ADP and succinate were replaced with 10 mM phosphoenol-pyruvate, 2 mM ATP and 25 µg pyruvate kinase/ml and the incubations were performed in the presence of 0.01% Triton X-100 (Spolsky & Eisenstadt, 1972). Protein was determined by the method of Lowry et al (1951).

MATERIALS

The source of most materials is tabulated below:

Materials	Source
D- and L-chloramphenicol	Parke, Davis & Co., Research Division, Detroit 32, Mich.*
Triton X-100	Sigma Chemical Co.

* Gift.

RESULTS

As indicated in the Methods, initial experiments attempting to demonstrate a mutagenic effect of BUdR were not successful. Rather, a large number of cells resistant to tevenel were found to occur even in the absence of BUdR treatment. Three clones from the resistant population were selected for characterization.

Growth Characteristics of Resistant Cells

The growth of three tevenel resistant cell lines (T2, T8 and T22) were compared with that of LMTK$^-$ in the presence and absence of 100 µg tevenel/ml. A typical growth curve for LMTK$^-$ (A) and T8 (B) is shown in Fig. 23. While LMTK$^-$ ceased growing in 100 µg tevenel/ml, T8 continued to grow at the same rate in the presence or absence of tevenel. The same result was obtained for both T2 and T22. The resistant cell lines have been maintained for many months in the presence of tevenel without any apparent effect on cell growth.

To determine whether the resistant cells were cross-resistant to D-chloramphenicol, relative plating efficiencies were determined. In Table VIII it can be seen that T8 and T22 plate efficiently in 100 µg tevenel/ml and 50 µg D-chloramphenicol/ml, while no clones are observed for LMTK$^-$ under these conditions. The slightly lower plating efficiency of the resistant cells in D-chloramphenicol may not be due to a specific effect of the chloramphenicol on mitochondrial protein synthesis, since a lower efficiency was also seen in the presence of 50 µg L-

Figure 23

Growth curves of LMTK$^-$ cells (A) and T8 cells (B). Growth measurements were performed on cells growing in suspension culture as described in Methods of Chapter II and Fig. 12. Each cell count is the average from two cultures. Tevenel (100 µg/ml) was present (●——●) or absent (o——o) during growth.

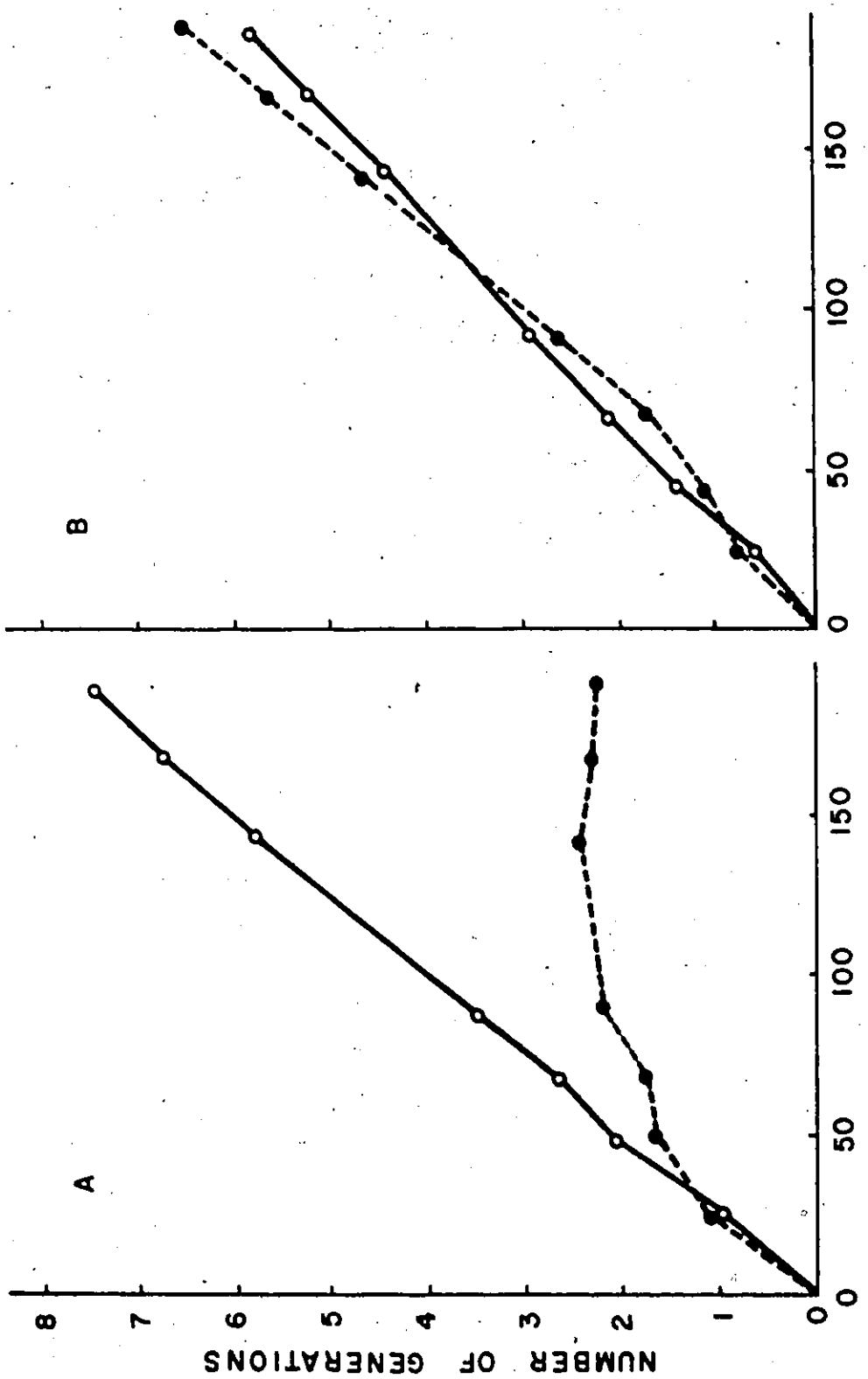

Figure 23

TABLE VIII

Effect of Chloramphenicol Analogues on the Relative Plating Efficiencies of LMTK$^-$, T8 and T22

	Relative Plating Efficiency		
Drug	LMTK$^-$	T8	T22
none	1.00±0.04	1.00±0.12	1.00±0.05
tevenel	0	0.96±0.08	0.87±0.07
D-chloramphenicol	0	0.74±0.08	0.68±0.08
L-chloramphenicol	0.71±0.07	0.86±0.13	0.78±0.11

Relative plating efficiency was determined as described in the Methods. The concentration of tevenel is 100 µg/ml, and the concentration of L- and D-chloramphenicol is 50 µg/ml. The data is presented as the mean of the relative plating efficiency plus or minus one standard deviation. The absolute plating efficiencies in the absence of drug were 76%, 78% and 68% for LMTK$^-$, T8 and T22 respectively.

chloramphenicol/ml for T8 and T22 as well as LMTK$^-$. L-chloramphenicol is not effective in inhibiting mitochondrial protein synthesis but it, as well as D-chloramphenicol, inhibits respiration (Freeman & Haldar, 1967; 1968; Haldar & Freeman, 1968).

The stability of the change responsible for the resistance of these cells was examined by comparing the relative plating efficiency in the presence and absence of 100 µg tevenel/ml. A culture of T22 grown for more than 100 days in the absence of tevenel (about 100 generations) plated with similar efficiencies in the presence and absence of tevenel, as did a culture of T22 maintained in tevenel for more than one month (Table IX). This result suggests that the change responsible for tevenel resistance is stable over the time interval examined and is inheritable. Similar results have been found for the other two resistant lines.

The resistant cell lines were examined for their ability to grow in the presence of BUdR and HAT. They were found to be resistant to 25 µg BUdR/ml and sensitive to HAT, consistent with retention of the thymidine kinase deficiency of the parent cell line (LMTK$^-$).

Mitochondrial Protein Synthesis

To determine that the growth characteristics of the resistant cells is not due to changes in cell membrane permeability to tevenel and D-chloramphenicol, the effect of these drugs on protein synthesis by isolated mitochondria was examined. As can be seen in Fig. 24, protein synthesis by mitochondria from both T8 and T22 shows a marked resistance to both tevenel and D-chloramphenicol with respect to the protein synthesis of LMTK$^-$ mitochondria. Although the resistance is not complete,

TABLE IX

Stability of the Resistance to Tevenel in T22

Drug	Relative Plating Efficiency	
	T22(-)*	T22(+)‡
none	1.00±0.06	1.00±0.05
tevenel	0.92±0.04	0.98±0.13

Relative plating efficiency was determined as described in Methods. The concentration of tevenel is 100 µg/ml. The data is presented as in Table VIII. The absolute plating efficiences in the absence of tevenel were 89% and 78% for T22(-) and T22(+) respectively.

* T22(-) maintained for 100 days in the absence of tevenel.

‡ T22(+) maintained for 35 days in the presence of 100 µg tevenel/ml.

Figure 24

Effect of increasing concentrations of tevenel (A) or D-chloramphenicol (B) on protein synthesis in isolated mitochondria from LMTK$^-$ (▽——▽), T8 (○——○), or T22 (●----●). Protein synthesis was measured as described in Methods; incubations were for 30 min at 30°C; 100% incorporation is approximately 100,000 cpm/mg protein/30 min.

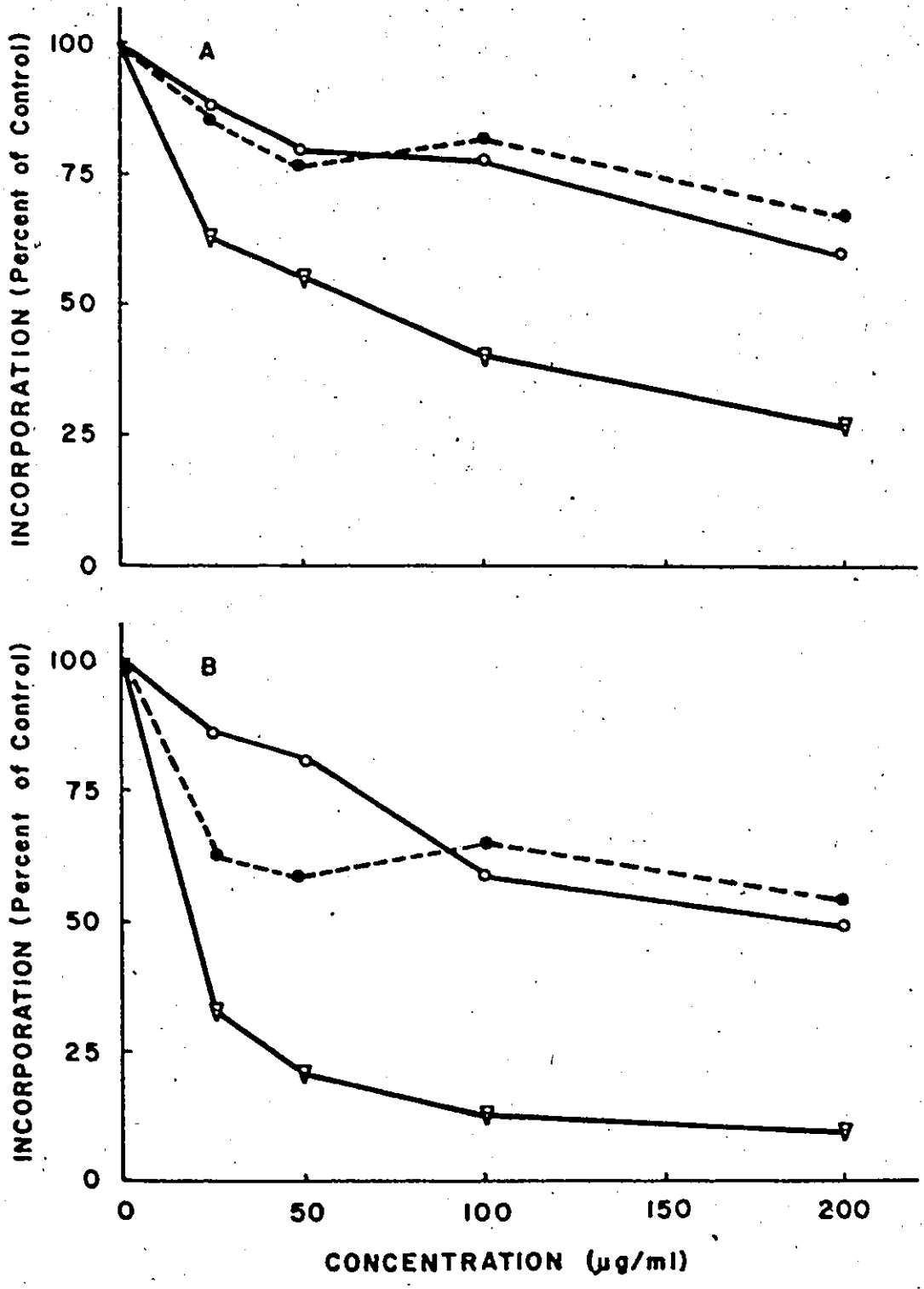
A
B
INCORPORATION (Percent of Control)
100
75
50
25
0
0
50
100
150
200
CONCENTRATION (µg/ml)

Figure 24

incomplete resistance has been observed in other antibiotic resistant cells (Spolsky & Eisenstadt, 1972; Molloy et al, 1973). The resistance to tevenel of the mitochondrial protein synthesis in T22 was not decreased by incubation in the presence of 0.01% Triton X-100 (Fig. 25), suggesting the resistance is not due to a change in the permeability of the mitochondrial membranes to the drug (Spolsky & Eisenstadt, 1972). Similar results were obtained with T8 mitochondria.

The Effect of BUdR on the Frequency of Colony Formation in the Presence of Tevenel

If the incorporation of BUdR specifically into mtDNA was responsible for the high frequency of tevenel resistant cells observed by selection procedure A (see Discussion for a possible mechanism), further treatment with BUdR should increase this frequency. This was not observed in initial experiments, but may have been due to the relatively short BUdR treatment (1 day) and due to the long period of growth in suspension culture prior to plating. Selection procedure B was designed in order to study the effects of BUdR on colony formation. Table X shows the results of one such experiment. When LMTK$^-$ cells were plated in 100 µg tevenel/ml, the population treated with BUdR was found to give about twice as many colonies, although this is not statistically significant. Further experiments are obviously required to determine if the incorporation of BUdR into mtDNA can act as a mutagen of the mitochondrial genome.

Figure 25

Effect of increasing concentrations of tevenel on Triton X-100 (0.01%)-treated mitochondria from LMTK$^-$ (●----●) or T22 (o——o). Protein synthesis was measured as described in Methods and Figure 24.

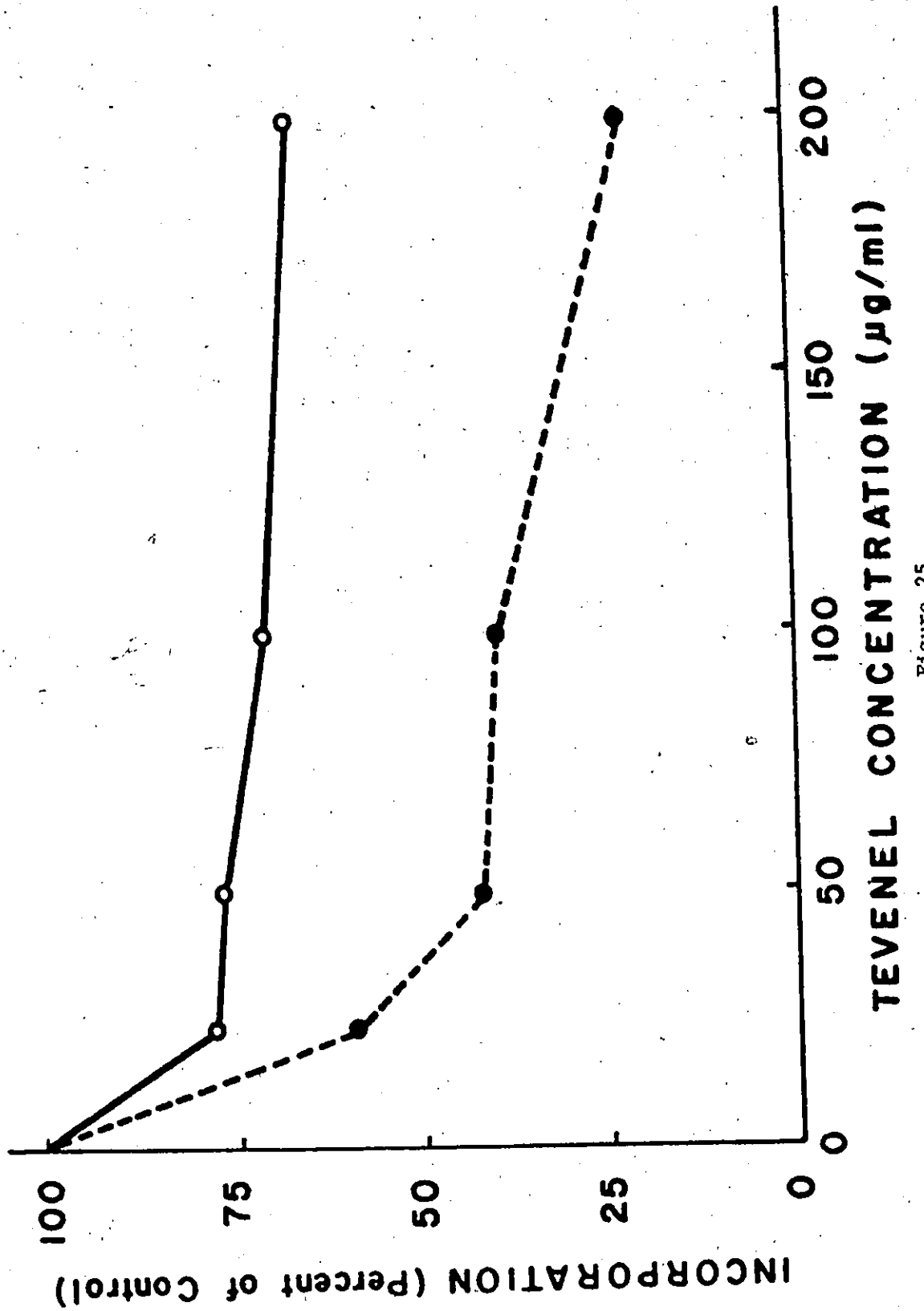
INCORPORATION (Percent of Control)
100
75
50
25
0
0
50
100
150
200
TEVENEL CONCENTRATION (µg/ml)

Figure 25

TABLE X

Effect of BUdR Treatment of LMTK$^-$ cells on the Number of Clones in the Presence of Tevenel

Number of Cells Plated	Number of Resistant Clones	
	BUdR treated Population	Untreated Population
10^5	15±9	9±4
5×10^5	42±13	20±15

LMTK$^-$ cells were treated with or without BUdR as described in selection procedure B in the Methods and Fig. 21. Five Petri dishes were plated for each population at the two cell densities. The data is presented as in Table VIII.

DISCUSSION

The tevenel resistant cells described above have many of the properties expected for cells carrying a cytoplasmic mutation. The resistance is stable; it is expressed at the level of isolated mitochondrial protein synthesis; it does not appear to be due to a lack of permeability of the mitochondrial membrane to tevenel and both cell growth and mitochondrial protein synthesis are cross-resistant to D-chloramphenicol. A proof that the resistance is a mutation within the mtDNA would be to demonstrate cytoplasmic inheritance of the characteristic, as has been shown for resistant mouse A9 cells by Bunn et al (1974). The ultimate proof would be to demonstrate an altered gene product of mtDNA.

The ease with which tevenel resistant cells can be isolated from an $LMTK^-$ population, may make this cell line useful for the isolation of cells resistant to other drugs which interfere with mitochondrial function. Isolation of such mutants would then allow studies on recombination of the mammalian mitochondrial genome. Evidence that recombination does occur in mammalian mtDNA has been discussed in Chapter II. Somatic cell hybrids may offer a convenient system for the study of recombination of antibiotic resistant markers in mammalian mtDNA.

A question which arises is, whether or not the resistant clones observed in the BUdR treated $LMTK^-$ population (selection procedure B) are similar to those observed and isolated in an untreated population (selection procedure A). An answer to this question awaits further analysis of tevenel resistant cells selected by procedure B. It is

possible that the LMTK$^-$ population contains a high frequency of resistant cells because of permanent, non-deleterious changes, which were produced in mtDNA in past growth of this cell line in BUdR (Kit et al, 1963). This speculation is supported by the fact that tevenel resistant L cells could not be isolated under the conditions of selection procedure A. To test this possibility, a clone of LMTK$^-$ cells should be isolated to determine whether it shows a low frequency of tevenel resistant cells and to see if BUdR can alter this frequency.

As discussed in the Introduction, Bunn et al (1974) did not observe resistant clones by plating LMTK$^-$ cells in chloramphenicol. While I have not attempted to select chloramphenicol resistant cells (the tevenel resistant cells are only cross resistant to chloramphenicol), it may be that it is much more difficult to select cells resistant to chloramphenicol than to tevenel due to the effects of chloramphenicol on respiration (see above). Also, Bunn et al (1974) plated between 2 to 9×10^6 cells per plate, which, in my experience, results in few resistant clones since the plates over-grow and cells detach from the surface.

The use of BUdR as a mutagen in other TK$^-$ cells may provide a convenient method for the production of mitochondrial mutants in a number of species, and may open the way for the study of mitochondrial genetics in mammalian cells.

CONCLUDING REMARKS

The study of mitochondrial biogenesis has long relied on the differences which exist between mitochondrial and extra-mitochondrial (cytosolic, nuclear, etc.) processes for biochemical analysis. Many such differences have been exploited or examined in this investigation: differences in the cytosolic and mitochondrial leucyl- and methionyl-tRNAs, differences in the mode of initiation of cytosolic and mitochondrial protein synthesis, differences in the physical properties of nucDNA and mtDNA, differences in mitochondrial and extra-mitochondrial thymidine kinase, differences in mitochondrial and cytosolic leucyl-tRNA synthetase and, finally, differences in the sensitivity of cytosolic and mitochondrial protein synthesis to various inhibitors. Despite a rather thorough understanding of these differences, it is still unclear why a mitochondrial genetic system exists at all. Certainly, the bulk of the mitochondrial proteins are coded for and translated outside of the mitochondria; why are all of them not? While many hypotheses exist to attempt to explain the existence of the mitochondrial genome on evolutionary grounds, little if any experimental evidence supports any of them (see Borst, 1971).

We are left, therefore, with the fact that mitochondria exist and contain a small but important genetic system. The importance of the mitochondrial genetic system to the existence of eukaryotic cells is exemplified by Fig. 23A. The inhibition of mitochondrial protein synthesis with tevenel results in the ultimate cessation of growth.

Similar results are obtained by the inhibition of mtDNA transcription and replication with ethidium bromide.

While the study of mitochondrial biogenesis in fungi, particularly yeast, has proceeded through both genetic and biochemical analysis since the early 1950's, genetic analysis in mammalian systems has been very slow to develop. This investigation has dealt with two genetic approaches to the study of mitochondrial biogenesis. The first, described in Chapter I, considers the study of mammalian cells with a nuclear mutation which will allow discrimination of mitochondrial and extra-mitochondrial processes. This is a powerful approach, particularly using conditional mutants, but has the great disadvantage that these mutants are difficult to isolate. With improvements in the isolation of mammalian cell mutants and their subsequent characterization, the more general application of this genetic approach to the study of mitochondrial biogenesis will certainly occur.

The second genetic approach, described in Chapter III, considers the study of mammalian cells with a mutation of the mitochondrial genome. The results of Chapter III do not prove that the tevenel-resistant LMTK$^-$ cells were mutants of mtDNA, but this is a strong possibility, as was discussed. If this is so, a more general significance of this approach might be in its application to the isolation of other mutants of the mitochondrial genome. Such mutants would be extremely useful in the establishment of the study of mitochondrial genetics in mammalian cells. Perhaps in the near future the genetic map of animal cell mtDNA (Fig. 1) will be expanded to include drug resistant loci as does yeast mtDNA.

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APPENDIX I

LIST OF PUBLICATIONS

Papers

1. P.M. Strasberg, M.M.L. Davidson, R.B. Wallace and K.B. Freeman. Comparison of mitochondrial components from human and hamster cell lines. Exptl. Cell Res. 89: 399-401 (1974).

2. R.B. Wallace and K.B. Freeman. Initiation of mammalian mitochondrial protein synthesis: The effect of methotrexate. Biochim. Biophys. Acta 366: 466-473 (1974).

3. R.B. Wallace and K.B. Freeman. Multiple species of methionyl-tRNA from mouse liver mitochondria. Biochem. Biophys. Res. Commun. 60: 1440-1445 (1974).

4. B.K. Waters, R.B. Wallace and K.B. Freeman. Synthesis of mammalian mitochondrial rRNA at low temperatures. Exptl. Cell Res. 90, 461-463 (1975).

5. B. Waters, R.B. Wallace and K.B. Freeman. Characterization of cytosol and mitochondrial rRNA of reptiles in relation to evolution. Comparative Biochem. and Physiol., 1975, in press.

6. R.B. Wallace and K.B. Freeman. Selection of mammalian cells resistant to a chloramphenicol analog. J. Cell Biol. 65, 492-498 (1975).

Abstracts

1. M.A. Shaw, M.R. Pudek, R.B. Wallace, M.S. Tsao and W.R. Richards. Evidence for a carrier protein in bacteriochlorophyll synthesis in *R. spheroides*. Proc. Amer. Soc. Photobiol. (1972).

2. R.B. Wallace, B. Waters and K.B. Freeman. The effect of chloramphenicol, cycloheximide and low temperature on the synthesis of mammalian mitochondrial ribosomal RNA. Proc. Can. Fed. Biol. Soc. 16, 22 (1973).

3. R.B. Wallace and K.B. Freeman. Initation of mitochondrial protein synthesis. Proc. Can. Fed. Biol. Soc. 17, 86 (1974).

4. P.M. Strasberg, M.M. Davidson, R.B. Wallace and K.B. Freeman. The fate of mitochondrial components in hamster-human cell hybrids. Proc. Can. Fed. Biol. Soc. 17, 69 (1974).

5. R.B. Wallace and K.B. Freeman. Mammalian cells resistant to a chloramphenicol analogue. Fed. Proc. 34, 510 (1975).

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