THE BIOCHEMICAL GENETICS OF MITOCHONDRIA IN MAMMALIAN CELLS

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by

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This is for you who are to come, with time,
And gaze upon our ruins with strange eyes.

Stephen Vincent Benét

ABSTRACT

THE BIOCHEMICAL GENETICS OF MITOCHONDRIA IN MAMMALIAN CELLS

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A cell line resistant to antimycin A at a concentration of 15 μM has been isolated following the mutagenesis of the human cell line D98 with N-methyl-N'-nitro-N-nitrosoguanidine. The cell line, designated MA 6.5, has been cloned twice in 15 μM antimycin A, a concentration about ten times that required to kill all parental cells. MA 6.5 is fully sensitive to chloramphenicol and triethyl tin and has an active Krebs cycle. Oxygen consumption of whole cells is unaffected by antimycin at a concentration of 15 μM, although the *in vitro* activity of succinate-cytochrome c reductase appears to be as sensitive to antimycin as the parental cell enzyme. The resistance phenotype of the mutant is stable in the absence of selection.

The proteins synthesised by the mitochondria of MA 6.5 have been examined by sodium dodecyl sulphate electrophoresis and isoelectric focusing. No qualitatively variant protein has been detected, but there are consistent quantitative differences between the profiles of the mutant and parental cell lines on isoelectric focusing. The implications of these findings for the mechanism of resistance are discussed.

Preliminary genetic analysis suggests that it is possible to transfer resistance to a closely related cell line employing enucleated cell fragments of MA 6.5 as donors.

Some observations on mitochondrially synthesised proteins in a variety of cell lines and hybrids are discussed. These support the view that interspecific differences in the products of mitochondrial protein synthesis on electrophoresis are not solely a result of post-translational modification. Two "reverse segregant" hybrid cell lines, which retain large numbers of human chromosomes, were found to have a predominantly human profile of mitochondrially synthesised proteins on sodium dodecyl sulphate electrophoresis.

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ABBREVIATIONS USED IN THIS THESIS

BUdR	:	5 bromodeoxyuridine
CAP	:	chloramphenicol
CH1	:	cycloheximide
c.p.m.	:	counts per minute
d.p.m.	:	decompositions per minute
EM	:	emetine
HGPRT	:	hypoxanthine-guanine phosphoribosyl transferase
I_{50}	:	The concentration of an agent required to give half maximal effect
PBS	:	phosphate buffered saline
PEG	:	polyethylene glycol
PI_{50}	:	The concentration of an agent required to give half the maximal effect when values are expressed as percentages
r.p.m.	:	revolutions per minute
s.d.s.	:	sodium dodecyl sulphate
6TG	:	six thio-guanine
TK	:	thymidine kinase
Tris	:	Tris-(hydroxymethyl)-methylamine
v/v	:	volume per volume
w/v	:	weight per volume
Butyl PBD		2-(4'-tert-butylphenyl)-5-(4"biphenylyl) 1,3,4 oxadiazole
TCA	:	Trichloroacetic acid
NP40	:	Nonidet-P40

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CHAPTER 1

INTRODUCTION

Organelle biogenesis is an active area of research, as testified by the large number of reviews and texts which have appeared recently on its various aspects (Borst, 1972; Ashwell and Work, 1974; Gilham, 1974; Kroon and Saccone, 1974; Schatz and Mason, 1974; Birky et al., 1975; Bucher et al., 1976; Packer and Gomez-Payou, 1976; Buetow and Woods, 1978). Because of the availability of these reviews, this chapter is a brief review of current concepts, focusing on a limited number of areas of particular interest, rather than an exhaustive summary of the literature.

A) Mitochondrial DNA

Metazoan mitochondrial DNA from a variety of different animals is double stranded and circular, varying in contour length from 4.45 μm in a sea urchin to 5.85 μm in the worm Urechis capo (Borst, 1972). Mammalian mitochondrial DNA is a circle of contour length 5 μm, and molecular weight of about 1×10^7 daltons. A greater range of size and of gross structural organisation is found in the Protists. In the Fungi, yeast mitochondrial DNA has a contour length of 25 μm, and can be isolated as circular molecules by osmotic lysis of mitochondria, although most isolation procedures yield also linear molecules of heterogeneous size (Hollenberg et al., 1970). Neurospora crassa mitochondrial DNA appears to be a circle of 19 μm contour length (Agsteribbe et al., 1972). Mitochondrial DNA isolated from Acanthamoeba castellanii is a 12.8 μm circle (Bonhert, 1973) while that from Tetrahymena is a linear molecule 17.6 μm long (Suyama

and Miura, 1968). The fact that this is circularly permuted (Arnberg et al., 1972) suggests that at some point (e.g. replication) it may circularise.

Mitochondrial DNA exists in multiple copies in mitochondria; thus mouse L cells contain about 1,100 molecules of mitochondrial DNA, each cell containing about 250 mitochondria, whereas Hela cells contain about 8,800 mitochondrial DNA molecules (Bogenhagen and Clayton, 1974). In total, mitochondrial DNA accounts for about 0.3% of the cellular DNA in mammalian cultured cells. Although mitochondrial DNA released from cells by osmotic lysis appears to be free of large amounts of attached proteins such as histones, the association of mitochondrial DNA released by triton X 100 lysis of Hela cell mitochondria with a protein structure attached to the inner membrane has been reported (Albring et al., 1977). In addition, transient association with enzymes associated with DNA replication and transcription is presumed.

Mitochondrial DNA molecules replicate on average once per cell doubling in exponentially growing cultures (Berk and Clayton, 1974), replication occurring preferentially in S and G_2 phase in both mouse and human cells (Bosmann, 1971; Picca-Mattoccia and Attardi, 1972). The molecular mechanism of DNA replication is unusual. Replication is highly asynchronous with synthesis on one strand preceding that on the other. In these studies, and in those on transcription from mitochondrial DNA discussed later, the ability to separate the two strands of mitochondrial DNA by their buoyant density has proved invaluable. In this case, heavy strand synthesis has been found to precede light strand synthesis (Berk and Clayton, 1974). Replication commences with the synthesis of a short DNA strand using a specific point on the light (L) strand as template. The heavy (H) strand is displaced

at this site to form a single stranded "D loop". Continued synthesis of the nascent H strand occurs unidirectionally from the loop, the displaced H strand then being used as a template for L strand synthesis (Borst, 1972; Robberson and Clayton, 1972; Robberson et al., 1974; Berk and Clayton, 1974). The whole process takes about 120 minutes.

The sequence organisation of mitochondrial DNA has been studied in a variety of ways with a number of specific questions in mind. The existence of a large number of mitochondrial DNA molecules per cell raises the question of whether there is any nucleotide sequence heterogeneity between them. That this does not exist at a gross level is suggested by the almost perfect second order renaturation kinetics of Tetrahymena, yeast and pea mitochondrial DNA (Borst, 1970; 1972; Kolodner and Tewari, 1972; Locker et al. 1974) although such studies do not exclude small scale heterogeneity.

By partial alkaline denaturation, it has been shown that about 50% of the yeast mitochondrial genome consists of AT rich sequences, these being interspersed between GC rich regions 0.1-5 μm long. It has been suggested that these AT rich regions are non-coding spacers between the GC rich coding sequences (Piperno et al., 1972; Prunell and Bernardi, 1974; Prunell et al., 1977; Prunell and Bernardi, 1977). Similar regions have not been detected in mammalian mitochondrial DNA, but there is good evidence that the five times greater size of the yeast mitochondrial genome is not due to the synthesis of five times as many mitochondrially coded products (see later), so these "spacer" regions may be a peculiarity of the large yeast mitochondrial genome.

Restriction enzyme analysis of mitochondrial DNA will probably reveal much of interest about the arrangement and evolution of sequences within it. Both mammalian (human, monkey, dog,

horse, donkey and mouse) and fungal mitochondrial DNA has been examined using specific restriction endonucleases (Brown and Vinograd, 1974; Robberson et al., 1974; Potter et al., 1975; Sanders et al., 1975). Quantitative analysis of the results indicates that the mitochondrial DNA from a single individual mammal is predominantly a single molecular clone. The patterns of restriction fragments on gels are a fairly sensitive way of detecting differences in the mitochondrial DNA. Mitochondrial DNAs from distantly related mammals (e.g. donkey and dog) share few bands on a Hae III digest, which produces some 30 fragments from Hela mitochondrial DNA, and even closely related mammals (e.g. donkey and horse) shared only about 50% of the bands. Interestingly, intraspecies differences were picked up on human samples, with six distinct patterns being found. This result parallels findings by Upholt and Dawid (1977) that intraspecific differences in restriction fragment patterns are found in sheep and goat populations. These results indicate that mitochondrial DNA sequences have evolved very rapidly.

The limits to the information content of mitochondrial DNA can be assessed directly from the molecular weight. The 5 μm circles in mammalian mitochondria contain some 15,000 base pairs, which could code for a maximum of 555,000 daltons of protein. Lehninger (1964) has estimated that the basic mitochondrial respiratory assembly has a molecular weight in excess of 10^6 daltons, so it is clear that the mitochondrial genome can only be responsible for a minor fraction of mitochondrial proteins.

B) Mitochondrial transcription and its products

Mitochondria contain an RNA polymerase distinct from the nuclear polymerases. In both Neurospora and mammalian cells, the polymerase is sensitive to inhibition by rifampicin (Gadaleta et

al., 1970; Kuntzel and Schafer, 1971), as are the bacterial polymerases. The isolated Neurospora enzyme is one of the simplest polymerases isolated, consisting of a single polypeptide with a molecular weight of 64,000 daltons. This may reflect the fact that complex transcriptional controls requiring the recognition and differentiation between different sets of promoters is not a feature of mitochondrial transcription (see later).

The RNA products of mitochondrial transcription are two mitochondrial ribosomal RNAs, with sedimentation coefficients of 16S and 12S in mammalian cells (Aloni and Attardi, 1971a), 23S and 16S in yeast (Reijnders et al., 1972), a number (20 in yeast, about 19 in Hela) of 4S RNAs probably acting as transfer RNAs (Reijnders and Borst, 1972; Angerer et al., 1976) and a heterogeneous RNA fraction probably representing mitochondrial message.

The positions of the 4S and ribosomal genes have been studied by hybridising the RNAs covalently labelled with the electron opaque material ferritin back to separated H and L strands. Earlier reports suggested the existence of only 11-12 genes on Hela mitochondrial DNA coding for 4S RNAs (Aloni and Attardi, 1971a; Wu et al., 1972). Subsequent results using tRNAs aminoacylated with radioactive amino acids suggested the existence of at least 12 genes on the H strand and 5 on the L strand coding for tRNAs of 16 different amino acid specificities (Lynch and Attardi, 1976), and a further electron microscopic study using a newly developed labelling technique confirmed the presence of at least 19 4S RNA genes on the Hela mitochondrial genome (Angerer et al., 1976). Twelve of these are on the H strand, 7 on the L, these genes being distributed relatively uniformly around the genome.

Less controversy has surrounded the studies on the 12S and

16S ribosomal RNAs. A single gene for each is located on the H strand, separated by one gene coding for a 4S RNA species. The regular arrangement of 4S RNA genes around the genome in Hela is in contrast to the position in yeast, where 11 of the 12 genes mapped thus far appear to be clustered in about one-third of the genome (Monnerot et al., 1977). The stability (Zylber and Penman, 1969; Knight, 1969; Attardi and Attardi, 1971) of these 4S molecules, together with the fact that they can be charged with amino acids (Lynch and Attardi, 1976), leaves little doubt that at least most of them function as transfer RNAs. At least 20 tRNAs are required if a protein synthesising system is to be able to use every amino acid. There have been suggestions in the past that mitochondrially synthesised proteins contain a restricted set of amino acids (Wu et al., 1972; Costantino and Attardi, 1973), based on the belief that only 12 tRNA's were coded by mitochondrial DNA. There is evidence for the import of tRNA from the cytoplasm in Tetrahymena (Chiu et al., 1975). Even given the existence of 19 tRNAs synthesised within the mitochondria of Hela cells, the import of tRNA from the cytoplasm will still be required to make up a degenerate set, especially if the finding of 3 isoaccepting tRNAs for the same amino acid in Tetrahymena proves to be a general phenomenon (Chiu et al., 1974). Attardi et al. (1976) have presented preliminary evidence for the existence of two isoaccepting serine tRNAs in Hela mitochondria.

In Hela cells, the heterogeneous RNA fraction contains a 60 nucleotide poly A sequence which is added post transcriptionally (Hirsch and Penman, 1973; 1974a). This tail is shorter than those found in cytoplasmic mRNA which are about 160 nucleotides long, and the process by which it is added is distinct from that of the cytoplasmic message in that it is relatively much less sensitive to inhibition by cordycepin (Hirsch and Penman, 1974a). Attardi

et al. (1976) report that a fraction of the heterogeneous sequences cannot be bound to oligo d T columns, and so presumably either lack poly A tails or contain much shorter ones. The heterogeneous fraction of RNA's can be resolved into a large number of peaks on gel electrophoresis. The relation of these peaks to the messenger species, and the relation between the fractions containing and lacking detectable poly A tails are problems currently under investigation. Support for the view that at least some of these components represent mitochondrial messenger RNAs comes from the observation that after lysis of mitochondria, some components of this fraction are found sedimenting with polysome like structures (Hirsch and Penman, 1974b).

The mechanism of transcription of mitochondrial DNA presents some unusual features. Although after long periods of labelling, most labelled RNA associates with the H strand of Hela mitochondrial DNA, RNA labelled with short pulses hybridises to both the L and H strands at approximately equal levels (Aloni and Attardi, 1971b). Short pulse labelled RNA can anneal to itself to give RNA duplexes of lengths greater than 5 μm. These observations, together with evidence that the H strand is almost completely transcribed (Aloni and Attardi, 1971a) suggest that both H and L strands are transcribed, but that the transcript of the L strand is rapidly degraded, with the exception, presumably, of the 4S RNA molecules and any messenger species originating from the L strand. A similar transcription mechanism from two strands, followed by the degradation of the transcript from one strand, has been reported for SV40. (Aloni, 1972)[1].

The stability of the various products of mitochondrial transcription have been studied using preferential inhibitors of nuclear or mitochondrial RNA synthesis (Abelson and Penman, 1972;

1. Aloni, Y (1972). Proc. Nat. Acad. Sci. (U.S.A). 69 2404-2409.

Hirsch and Penman, 1974b). Mitochondrial 4S RNAs were found to be stable, and mitochondrial rRNAs to decay with a half life of about 3 hours (Borst, 1972). The heterogeneous RNA fraction, at least some of which is presumptive message, is less stable, with a half life of 1.5-2 hours. The half lives of all species are less in the presence of ethidium bromide, which suggests that it directly stimulates the degradation of mitochondrial RNAs (Hirsch and Penman, 1974b).

C) Protein synthesis

Mitochondria contain ribosomes which are distinct from those of the cytoplasmic protein synthesising system. Animal mitochondrial ribosomes sediment at 55S and are thus considerably smaller even than bacterial ribosomes. Thus far only the two rRNAs mentioned, 16S and 12S, have been found in mitochondrial ribosomes, so it is possible that they lack an equivalent of the small (5S) species found in bacterial and 80S eukaryotic ribosomes. Most or all of the proteins in both subunits are different from those in the cytoplasmic ribosomes in Neurospora (Kuntzel, 1969) and Xenopus (Leister and Dawid, 1974).

It has frequently been stressed by different authors that the pattern of antibiotic sensitivity of mitochondrial ribosomes is more similar to that of bacterial than that of eukaryotic 80S ribosomes (Roodyn and Wilkie, 1968; Beattie, 1971; Davidowicz and Mahler, 1973; Schatz and Mason, 1974). Mitochondrial ribosomes share the prokaryotic sensitivity to chloramphenicol, mikamycin and lincomycin, and are insensitive to inhibitors of 80S ribosome mediated protein synthesis, such as emetine and cycloheximide. Another feature shared by mitochondrial and bacterial protein synthesis is the initiation of polypeptides with N-formyl

methionine (Galper and Darnell, 1969; 1971).

The initiating formyl methionyl tRNA of Neurospora crassa has been sequenced recently (Heckman et al., 1978). This tRNA can be aminoacylated and formylated by E. coli enzymes, and is capable of initiating protein synthesis in E. coli cell free systems. Although in overall sequence this tRNA is more homologous to prokaryotic (56-60%) than to eukaryotic (45-51%) initiator tRNAs, it is still considerably different from either. In two distinguishing features it resembles eukaryotic tRNA more than prokaryotic tRNA, namely, the ability to form a base pair between the 5' terminal nucleotide and the fifth nucleotide from the 3' end, and the lack of the sequence T Ψ C G (or A) in loop 4. However, the corresponding sequence in the mitochondrial tRNA is not AU (or Ψ) CG, as in eukaryotic initiator tRNAs, but UGCA. One should beware of too facile an identification of the characteristics of mitochondrial protein synthesis with bacterial synthesis.

The products of mitochondrial protein synthesis in all cases examined seem to be a restricted number of polypeptides, 7-10 in mammalian species (Lederman and Attardi, 1973; Jeffries and Craig, 1976; Yatscoff and Freeman, 1977), and about 20 in yeast (Douglas and Butow, 1976). There is a certain amount of circumstantial evidence that these proteins are coded by the mitochondrial genome. Direct evidence for this conclusion has been provided by Moorman et al. (1978) who showed that antisera against known yeast mitochondrially synthesised proteins (cytochrome a/a3 and ATPase subunits) could precipitate the products synthesised by an in vitro translation system primed with various RNA fractions. These RNA fractions were physically mapped by annealing to restriction fragments of mitochondrial DNA.

Given these data, it seems unnecessary to postulate, as some have done, that some or all mitochondrially synthesised proteins are translation products of nuclear messages. The balance of evidence suggests that the translation products are encoded by mitochondrial DNA, transcribed by mitochondrion specific enzymes, and translated on mitochondrial ribosomes.

The nature and function of these products, as well as the reasons for synthesising them separately from other cellular proteins, are currently fields of active research. They are known to be generally small insoluble proteins, which are associated with the mitochondrial inner membrane (Beattie et al., 1967; Brega and Baglioni, 1971). Studies with cytoplasmic petite mutants in yeast, which lack a functional mitochondrial protein synthesis apparatus, suggested that these cells lacked cytochrome c oxidase, cytochromes b or c_1, and oligomycin sensitive ATPase, though they contained some succinate or NADH-cytochrome c reductase activity, and some matrix enzymes such as those of the Krebs cycle (Groot et al., 1971; Getz, 1972). It thus appeared that mitochondrially synthesised products might be associated with the final stages of the electron transport chain, from cytochrome b to cytochrome oxidase, and with the oligomycin sensitive ATPase. Other mitochondrial enzymes are presumed to be nuclearly coded and cytoplasmically synthesised.

Detailed biochemical studies on a number of enzyme complexes have supported this general hypothesis. Cytochrome oxidase from both fungal and mammalian sources has been shown to contain one or two (human) or three (yeast) mitochondrially synthesised subunits (Mason et al., 1973; Sebald et al., 1973; Tzagoloff et al., 1974; Jeffries and Craig, 1977; Yatscoff et al., 1977). The membrane factor required to bind the F_1 ATPase to the inner mem-

brane in reconstitution experiments using yeast mitochondria has been shown to consist of four mitochondrially synthesised polypeptides (Tzagoloff and Meagher, 1972; Ebner et al., 1973). Mitochondrially synthesised proteins have been associated with cytochrome b (Weiss and Ziganke, 1974) in Neurospora. With the exception of the studies of Jeffries and Craig, and of Yatscoff et al., comparable biochemical studies using mammalian material have not been made. Although one might anticipate that a similar association of mitochondrially synthesised products with the terminal stages of the mitochondrial energy transduction apparatus will be found, there is an 80% decrease in mitochondrial genome size in passing from fungi to mammals, and a great evolutionary gap separates these organisms. This should caution one against overgeneralisation.

It has been suggested that "core" membrane proteins of mitochondrial origin are necessary for the correct integration of cytoplasmically synthesised subunits of membranous enzyme complexes, (Mason et al., 1972) and that mitochondrial proteins are synthesised in the mitochondrion because they are too hydrophobic to be transported through the cytoplasm to the inner membrane (Bogorad, 1975). Such speculations must await more detailed characterisation of the nature and function of these proteins before they can be evaluated.

D) Genetics and transmission

The study of the transmission and recombination of mitochondrial genomes by formal genetic analysis is most advanced in the Fungi, especially in Saccharomyces. A large number of markers on the yeast mitochondrial genome have been isolated, including resistance to various drugs (Coen et al., 1970; Bolotin et al.,

1971; Avner et al., 1973; Wolf et al., 1973; Avner and Griffiths, 1973; 1973a; Lancashire and Griffiths, 1975), defects in electron transport and the oligomycin sensitive ATPase (Tzagoloff et al., 1975), and temperature sensitivity (Bolotin-Fukuhara et al., 1977). The availability of these markers has led to the construction of a genetic map of the mitochondrial genome (Dujon et al., 1974; Slonimski and Tzagoloff, 1976), and the identification of various genes determining in part the outcome of genetic recombination between mitochondrial genomes. These include a mitochondrial locus, ω, and at least one nuclear gene (Avner et al., 1973; Callen, 1974; Waxman and Eaton, 1974; Grimes et al., 1974; Forster and Kleese, 1975; Gunge, 1975).

A number of interesting studies have also been made of the transmission of mitochondria between Paramecium species (Beale et al., 1972; Beale and Knowles, 1976), where mitochondrially determined resistance to chloramphenicol and erythromycin has been used as a marker. Transfer of mitochondria in this organism takes place naturally as a result of conjugation, and has been accomplished artificially by microinjection. While transfer of mitochondria between some species results in a high frequency of transmission of resistance, between other species only a very low (less than 10%) proportion of recipient cells acquire resistance. However, retransfer of mitochondria from such rare resistant recipients to other sensitive cells of the same species now results in a high frequency of transfer of resistance. These sort of results have been interpreted in terms of an initial incompatibility of the mitochondria and cytoplasm from certain pairs of species, followed by a modification to compatibility of the received mitochondria at a low frequency (Beisson et al., 1974). This incompatibility appears to be determined primarily by the

nuclear genome, though a role for the mitochondrial genome was not excluded by Beale and Knowles (1976). Although recombination between mitochondrial genomes in Paramecium was thought not to occur (Beale et al., 1972; Perasso and Adoutte, 1974), Adoutte (1975) presented preliminary evidence for such an event.

Until recently, the study of mitochondrial genetics in mammalian cells had been neglected in favour of work with lower organisms. This partly reflects the greater intrinsic difficulty of isolating mitochondrial mutants, and partly the lack of a means of discriminating between nuclear and cytoplasmic inheritance in cultured cells. However, in recent years a number of mutant cell lines resistant to inhibitors of mitochondrial protein synthesis have been isolated (discussed at greater length in the Introduction to Chapter 3), and techniques of enucleating cells to leave cytoplasmic fragments which may then be fused to recipient cells have been developed. These techniques allow discrimination between nuclear and cytoplasmic inheritance of drug resistance markers (Bunn et al., 1974; Wallace et al., 1975; Munro et al., 1978).

A few studies have been made exploiting these techniques. In studies on mouse (Bunn et al., 1977) and human (Wallace et al., 1977) cell lines marked with chloramphenicol resistance, these authors studied the behaviour of the marked mitochondria in both whole cell and cytoplast-cell fusions. Initially, when mouse CAP^r cells were fused with CAP^s mouse cells, all the hybrids were resistant, indicating that the trait is dominant. This resistance was lost (segregated) on continued growth of the hybrids when the CAP^s cell was of different tissue origin from the CAP^r cell. In contrast, hybrids using CAP^s cells of the same tissue origin were stably resistant. Similar results were found using cytoplasts to

transfer the resistance. When the same kinds of experiments were performed using CAP^s and CAP^r Hela cells, a similar stability of intrastrain hybrids or cybrids was found, and a similar instability when the CAP^s parent was a non Hela human cell line. Some of the unstable hybrids were found to become stably resistant.

Munro et al. (1978) were similarly unable to transfer CAP^r mitochondria from a human cell line into a mouse cell line by cytoplast fusion, although they report the transfer at low frequency of CAP^r mitochondria from a Hela derived CAP resistant cell line to a transformed human fibroblast cell line.

In general, these results are reminiscent of those described for interspecies transfer of mitochondria in Paramecium (Beale et al., 1972; Beisson et al., 1974) and suggest several possible explanations. The mitochondria carrying the CAP^r determinants might be unable to utilise nuclearly coded mitochondrial proteins from strains of cells different from their own. They may lose their CAP^r determinants by a gene conversion system similar to that proposed for Saccharomyces (Dujon et al., 1974; Dujon et al., 1976), or finally there may be a restriction system which removes non-cognate mitochondria. The correct hypothesis must account for the observed change of some interstrain hybrids or cybrids from instability to stability. The latter two systems would appear to offer the best accommodation of this observation. Further results using other markers as they become available may be expected to elucidate the rules governing the maintenance of mitochondrial populations in cells.

The possibility of recombination between mitochondrial genomes of the same species in mammalian cells cannot be investigated until such mutants are isolated. However, the possibility of detecting interspecific recombination between mitochondrial DNA from mouse

and human sources by using physical differences in their DNA and proteins is discussed further in Chapter 7.

E) Interactions between mitochondria and cytoplasm

Mitochondria are the product of co-operation between their own genomes and the nuclear genome of the cell harbouring them, which must provide the majority of their structural and enzymic components. This implies that there must be a coupling between the rate of synthesis of mitochondrially and cytoplasmically synthesised components such that neither is present in excess. There are a number of indications that mitochondrial protein synthesis is intimately related to cytoplasmic protein synthesis and a variety of control phenomena serve to regulate them co-ordinately.

Growth of Neurospora in concentrations of chloramphenicol sufficient to inhibit mitochondrial protein synthesis has been shown to lead to a specific increase in a number of cytoplasmically synthesised proteins normally located in the mitochondrion. These include mitochondrial RNA polymerase, methionyl tRNA transformylase, mitoribosomal proteins and mitochondrial tRNA synthetases; (Barath and Kuntzel, 1972; Barath and Kuntzel, 1972a; Beauchamp and Gross, 1976). To explain these observations, Barath and Kuntzel (op.cit.) proposed a model in which mitochondria synthesise a repressor which specifically prevents transcription of nuclear genes coding for mitochondrially destined proteins. The increase in the amount of these proteins observed on growth in chloroamphenicol reflects the release of this inhibition as the repressor is no longer synthesised. This hypothesis also explains the observation that a mitochondrial mutant of N. crassa, Mi-1, overproduces mitochondrial components synthesised on cytoplasmic

ribosomes if the mutation is assumed to be in the repressor protein (Sheir-Neiss et al., 1975).

A reciprocal interaction, whereby cytoplasmically synthesised proteins affect the rate of mitochondrial protein synthesis, has been proposed by Tzagoloff et al. (1973), and Poyton and Kavanagh (1976) provided evidence in support of this hypothesis. They showed that amino acid incorporation by isolated mitochondria of Saccharomyces cerevisiae was dependent on the presence of a pool of cytoplasmically synthesised proteins present in the mitochondria at the time of isolation. When this pool was exhausted, protein synthesis in the mitochondria ceased, but could be restored by the addition of a proteinaceous post-mitochondrial supernatant to the system. Some specificity, and the presence of more than one stimulating protein, could be inferred from experiments in which fractions of the post-mitochondrial supernatant stimulated the synthesis of different mitochondrially synthesised proteins. This work provided evidence that the cytoplasmically synthesised subunits of cytochrome oxidase stimulate specifically the production of the mitochondrially synthesised subunits.

Whether these regulatory mechanisms are general is not known. The level at which the stimulation of mitochondrial protein synthesis by cytoplasmic proteins operates is uncertain, although both transcriptional and translational control modes can be envisaged.

Costantino and Attardi (1977) found evidence for a dependence of mitochondrial on cytoplasmic protein synthesis in Hela cells. The rate of mitochondrial protein synthesis was found to drop rapidly after the inhibition of cytoplasmic protein synthesis with cycloheximide. All the discrete products of mitochondrial protein synthesis were affected in parallel. This study also revealed

an interdependence of the two protein synthesis systems at a different level, in that the stability of the labelled mitochondrial products in a cold chase in the absence of cytoplasmic protein synthesis declined. If cytoplasmic protein synthesis is allowed to continue after labelling the mitochondrially synthesised proteins, the stability of the products varies from a half life of 3-4 hours to 3 days, but in the presence of a persistent block in cytoplasmic protein synthesis, all except one of the products become greatly destabilised. The authors suggest that this may reflect a lack of proper integration of these proteins into the membrane in the absence of cytoplasmically synthesised components. This is in accord with the known dual origin of several mitochondrial enzyme complexes. A functional interaction between the products of both protein synthesising systems is also indicated by the observation that a mutation in the mitochondrial genome can suppress the effect of a nuclear gene mutation in Paramecium (Sainsard, 1975).

Interactions between the mitochondrion and the rest of the cell may thus be expressed by an exchange of macromolecules controlling the rate of protein synthesis in the alternate compartment, or by the functional integration of the products of each genome to form an enzyme complex. That events in the mitochondrion can have a profound effect on the whole cell is also suggested by the work of Adoutte and Doussier (1978). These authors found that a series of mutations in the mitochondrial genome of Paramecium conferring resistance to erythromycin could be ranked according to the rate at which they were lost from mixed populations of resistant and sensitive mitochondria under non-selective growth conditions. This rate was very variable, some alleles being eliminated in 10 cell generations, others still being present after 90 cell generations. The basis of this segregation was

apparently the lower rate of replication of E^r mitochondria, and the associated mitochondrial defects (lower cytochrome oxidase levels, increased cyanide insensitive respiration and lowered P:O ratios) were due to a lower rate of mitochondrial protein synthesis. The observation of significance is that the growth rates of the mutants were absolutely determined by the growth rates of their mitochondria, a classification of the mutants by their growth rates paralleling exactly a classification of the rate at which the alleles they bore were lost from a mixed population. Thus the growth rate of the cells in this case is tightly coupled to the replication rate of the mitochondrion. This "control" may be mediated by mitochondrial energy production, in other words by the supply of ATP, or more elaborate regulatory modes may be envisaged.

A similar dramatic effect of events in the mitochondrion on the life of the whole cell is the production of lethal mitochondrial genotypes in Podospora anserina by crossing a pair of mutant cell lines (Belcour and Begel, 1978). Lethality in this case was interpreted as a result of an increased probability of conversion of the mitochondrial genome to a ρ^- suppressive petite, which would spread and eventually render all the mitochondrial DNA molecules defective. As Podospora cannot exist solely by fermentation, the death of the cell results. The general relevance of this observation is questionable, but it is an interesting example of the dependence of this organism on fully functional mitochondria, which may be lost by a specific type of mitochondrial mutation.

The existence in mitochondria of a large number of cytoplasmically synthesised proteins, some of which have analogues in the cytoplasm which are distinguishable from them, raises the

question of the specific localisation of these proteins in the mitochondrion. There appears to be no evidence of the mechanism by which the localisation is accomplished in mammalian cells, although Kellems and Butow (1972; 1974) have presented evidence that cytoplasmic 80S ribosomes are associated with the outer membrane of yeast mitochondria, and at least some of their products can be chased by puromycin into the mitochondrion. In this case, some variant of the signal mechanism suggested by Blobel and Sabatini (1971) may be in operation. Further characterisation of nascent cytoplasmically synthesised mitochondrially destined proteins might reveal whether these proteins contain instruction sequences for localisation which are subsequently removed.

OBJECTIVES OF THIS RESEARCH

The work in this thesis is principally concerned with the nature, function and transmission of mitochondrially synthesised proteins. This interest was pursued in two ways.

1) The isolation of mutants of mammalian cells which might have altered mitochondrially synthesised proteins, and which might be used in genetic analysis of mitochondrial transmission.

2) Procedures for the analysis of mitochondrially synthesised proteins were developed and used to examine the products of mitochondrial protein synthesis in such cell lines.

CHAPTER 2

GENERAL MATERIALS AND METHODS

1. General materials

Erythromycin, emetine hydrochloride and cycloheximide were obtained from Sigma. D-threo-chloramphenicol was a generous gift of Dr. H.E. Marchamer (Parke, Davis and Company, Detroit, Michigan). Antimycin A was obtained from Sigma. The detergents used in electrophoresis and isoelectric focusing were sodium dodecyl sulphate (specially pure grade, BDH Chemicals) and Nonidet P-40 (BDH Chemicals) respectively.

Scintillation fluid chemicals were butyl PBD (Ciba); napthalene, 2-methoxyethanol (May and Baker) and analytical grade toluene (Fisons).

Reagents used in forming polyacrylamide gels were: specially purified acrylamide (BDH Chemicals), N N'-methylene bis acrylamide (Sigma), N N N'N' tetramethylethylenediamine (TEMED, BDH Chemicals) analytical grade tris (Fisons) and glycine (Koch-Light).

^{35}S L-methionine (600-1,100 Ci/mmol), ^{14}C L-leucine[1] (59 mCi/mmol), ^{3}H thymidine[2] (48 Ci/mmol) and 2-^{14}C-pyruvate (sodium salt, 8.2 mCi/mmol) were obtained from the Radiochemical Centre, Amersham.

Non-radioactive amino acids were purchased from Koch-Light.

1. L-1^{14}C leucine
2 methyl ^{3}H thymidine

2. Cell culture

Cell lines were grown in RPMI 1640 medium (Biocult) supplemented with 10% (v/v) foetal calf serum (Biocult). Antibiotics to inhibit bacterial growth were not added routinely to

culture medium. Cells were cultured at 37° in either glass bottles or in plastic flasks (Falcon Plastics Limited. Oxnard, California). Culture vessels were gassed with a 95% air/5% CO_2 mixture. When large quantities of cells were required, they were grown in 2.5 litre roller bottles. When cells had been plated at low densities in plastic dishes (Nunc Limited)[1] either for cloning or for plating efficiency assays, the dishes were kept in a humidified incubator at 37° gassed with a 95% air/5% CO_2 mixture.

Cell lines resistant to antimetabolites were grown continuously in the presence of the appropriate antimetabolite to prevent the accumulation of non-resistant revertants. 6-thioguanine (6TG) was used at a final concentration of 2×10^{-5}M and 5-bromodeoxyuridine (BUdR) at 3.25×10^{-5}M. HMT medium for the growth of hybrids of parental cell lines bearing TK and HGPRT deficiencies contained hypoxanthine at 1×10^{-4}M, methotrexate at 1×10^{-5}M, and thymidine at 1.6×10^{-5}M.

Cells were harvested by treatment with trypsin solution (0.25% (w/v) in tris/saline pH 7.7) for a few minutes at 37°. The trypsin was removed, and medium with serum was added to wash the detached cells from the surface. Clumps of cells were broken up by slapping the bottle. Cell suspensions were pelleted in an MSE minor centrifuge at 1,000 r.p.m. (250g) for 5 minutes and washed by suspension in PBS (phosphate buffered saline; 145 mM NaCl, 2.6 mM KH_2PO_4, 10.4 mM Na_2HPO_4, pH 7.4) followed by recentrifugation. Cell densities were determined using a model FN Coulter counter calibrated for each cell line.

Cell stocks were preserved by suspension at about 2×10^6/ml in freezing mixture (foetal calf serum + 10% (v/v) dimethyl sul-

1. Nunc A/S
Kamstrupveg
Kamstrup
DK 4000
Denmark

Table 2.1

Cell lines used in these studies

Cell	Description	Reference
A) Human Lines		
D98/AH2	8 azaguanine^r Hela derivative	Szybalski et al. 1962
Hela B	Subclone of Hela S	"
MC63	CAP^r Hela derivative	Siegel et al. 1976
BU 25	Hela derivative resistant to 30 µg/ml BUdR	Kit et al. 1966
IMR 32	Neuroblastoma	Tumilowicz et al. 1976
VA2	Transformed fibroblast	
LNSV	Transformed fibroblast	
B) Mouse Lines		
1R	8 azaguanine^r L cell derivative	Nabholz et al. 1969
PG19	Derived from C57.BL.mel. 6 thioguanine^r	Jonasson et al. 1977
NA	C1300 neuroblastoma derivative. 8 azaguanine resistant	McMorris and Ruddle, 1974
3T3	Resistant to 30 µg/ml BUdR	Matsuya and Green, 1969
X63 (P3-NSI/1 Ag 4-1)	8 azaguanine resistant derivative of MOPC 21 myeloma	Kohler et al. 1976
Clone 1 D	Fibroblast resistant to 30 µg/ml BUdR	Berk and Clayton, 1974

phoxide, Fisons) and freezing overnight in small vials at -70°. The following day these vials were transferred to storage under liquid nitrogen.

Table 2.1 lists and briefly describes the cell lines used in the course of the work described in this thesis.

3. Cell fractionation

Crude mitochondrial fractions were prepared from cultured cells as follows. Radioactively labelled cells were pooled with a large excess of cold carrier cells of the same line, and were homogenised by hand with a teflon/glass homogeniser on ice. Cells were homogenised in SP buffer (0.25M sucrose, 10 mM KH_2PO_4 pH 7.2 with KOH) and samples were observed at intervals during the homogenisation with a phase contrast microscope to check the degree of cell breakage.

The homogenate was spun for 5 minutes at 1500g max, and the pellet was rehomogenised in SP buffer. After spinning the re-homogenised pellet again at 1500g max for 5 minutes, pooled low speed supernatants were spun at 2,000g max for 15 minutes. The supernatant of this spin was then centrifuged at 23,000g max for 25 minutes in an MSE High Speed 18 centrifuge at 4°C. The mitochondrial pellet was washed once by resuspension in SP buffer and repelleted. Mitochondrial pellets were stored at -70°C until used.

4. Protein assays

The protein content of samples was assayed by the method of Lowry *et al*. (1951). A calibration curve using 0-60 µg Fraction V bovine serum albumin (Sigma) was constructed for each assay. The blue colour formed was measured at 750nm using a Unicam SP 500 Series 2 spectrophotometer corrected against a protein free blank.

5. Determination of radioactivity in TCA insoluble material

Aliquots of samples (usually whole cells) labelled with ^{3}H-thymidine, ^{35}S-methionine or ^{14}C-leucine were incubated at 37° with 0.2 ml 1M sodium hydroxide for 10 minutes. This was then neutralised with 0.2 ml 1M hydrochloric acid, and usually 25 µl of 10 mg/ml bovine serum albumin were added as cold carrier. Samples were precipitated with 5 ml of 10% trichloroacetic acid (TCA) containing 10 mM L-methionine or 10 mM L-leucine, and the suspension was filtered by suction through a Whatman 2.5 cm diameter GF/C glass fibre filter. The filter was washed with 20 ml of TCA and placed in 7 ml of scintillation mixture in a plastic scintillation vial.

The scintillation mixture contained 8% napthalene, 0.8% butyl PBD dissolved in toluene/methoxyethanol (3:2 (v/v) ratio).

After dehydration of the damp filter by shaking in the scintillation mixture (about 0.5 ml of aqueous sample was miscible with 10 ml of scintillation fluid) the vials were counted in an LKB Wallac 81000 Liquid Scintillation Counter. ^{14}C and ^{35}S were detected at an efficiency of about 75%, and ^{3}H was detected at an efficiency of 37%. The background with the plastic scintillation vials was less than 5 c.p.m. All counts were efficiency corrected and are expressed as d.p.m.

6. S.d.s. polyacrylamide gel electrophoresis

Electrophoresis was performed in a Bio-Rad model 220 slab gel apparatus using the tris-glycine-s.d.s. discontinuous gel system of Laemmli (1970). The gel was cast between two glass plates of dimensions 15 x 12 cm, separated by two formers of 1.5 mm width.

The following stock solutions were made up, following the procedure of O'Farrell (1975):

Resolving gel buffer; 1.5M tris-HCl pH 8.8 containing 0.4% (w/v) s.d.s.

Stacking gel buffer; 0.5M tris-HCl pH 6.8 containing 0.4% (w/v) s.d.s.

30% acrylamide stock; 29.2% (w/v) acrylamide and 0.8% (w/v) bis acrylamide in distilled water.

Sample buffer; 10% (w/v) glycerol, 5% (v/v) β-mercapto-ethanol, 2.3% (w/v) s.d.s. and 0.0625M tris-HCl pH 6.8. 0.0001% (w/v) of bromophenol blue marker dye was added.

Gels of concentration 5-22% acrylamide containing 0.1% (w/v) s.d.s. and 0.375M tris-HCl pH 8.8 were cast by mixing 0.25 volumes of lower gel buffer with 0.75 volumes of acrylamide stock solution plus distilled water, the proportions of acrylamide stock and water being varied according to the gel concentration required. This mixture was degassed, and after the addition of 0.0033 volumes of 10% (w/v) ammonium persulphate and 0.0005 volumes of TEMED, was poured into the gel apparatus. The mixture was gently overlaid with distilled water. When the resolving gel had set, the stacking gel, containing 4.75% acrylamide, was made up by adding 0.25 volumes of stacking gel buffer to 0.15 volumes of acrylamide stock and 0.6 volumes of distilled water. 0.0033 volumes of 10% (w/v) ammonium persulphate and 0.001 volume of TEMED were added, and the mixture was poured on top of the resolving gel after removing the distilled water. Sample slots were formed by the insertion of a slot former containing 10 slot forming projections prior to the setting of the gel. This slot former was removed when the stacking gel had set. Generally, there were about 1.5 cm of stacking gel from the base of the slots to the top of the resolving

gel.

The resolving gels contained either 11% homogeneous acrylamide, or a linear gradient of 10-15% acrylamide. Linear gradients were poured using a home-made gradient maker consisting of two 25 ml syringe barrels connected by a tube at the bottom. The chamber distal to the apparatus contained the dense solution, which was formed using 75% (v/v) glycerol in distilled water as the diluent in place of distilled water. The chamber proximal to the apparatus was the mixing chamber, which contained initially the less dense acrylamide solution. The chambers were filled with equal volumes of acrylamide mixture of the appropriate concentration, while a clamp on the tube connecting them prevented premature mixing. This clamp was removed when both chambers were filled, and a few minutes were allowed for complete hydrostatic equilibrium to be reached. The mixing chamber was stirred by a vibratory mixer, and the acrylamide mixture was pumped out from the bottom of the chamber using a peristaltic pump at a rate of about 1 ml/minute. Acrylamide mixture was pumped into the bottom of the apparatus via a syringe inserted into the self-sealing rubber base. The lightest part of the mixture entered first and was progressively displaced upwards by the entering denser solution. When gradient gels were poured, the concentration of TEMED in the mixture was halved to allow time to pour the gradient before the gel set. Generally, gradient gels were poured in about 20 minutes and set in about 45 minutes.

Samples (usually whole cells) were solubilised in up to 100 µl of sample buffer. Cell pellets in 0.3 ml Beckman tubes were vigorously flicked with a finger to loosen them before the addition of sample buffer. This simple procedure greatly improved

the resulting solubilisation. After the addition of buffer, the Beckman tubes were heated in a boiling water bath for 5 minutes to solubilise protein. Samples were routinely spun in a Beckman microfuge after heating to remove insoluble material, although it was generally observed that all material could be solubilised and no visible pellets resulted from these centrifugations. Up to 10 samples were loaded onto the stacking gel, empty slots being filled by the addition of 50 µl sample buffer. The samples were overlain with running buffer (25mM tris, 0.192M glycine, 0.1% (w/v) s.d.s. pH 8.3) and electrophoresis begun. Electrophoresis continued at 20 mA constant current for 4-5 hours. It was terminated when the bromophenol blue marker dye reached the end of the gel.

The gel was removed from the apparatus and fixed overnight in 50% (w/v) TCA. It was stained for protein with 0.1% (w/v) Coomassie Brilliant Blue (Gurr) dissolved in 50% TCA for 30 minutes, and destained with several changes of 7% (v/v) acetic acid solution. Gels were dried on a Bio-Rad heated slab gel drier and then autoradiographed.

7. Isoelectric focusing gels

Isoelectric focusing in polyacrylamide gels was performed by adapting the first dimension system of O'Farrell (1975) to the Bio-Rad 220 slab gel apparatus. A 30% stock acrylamide mixture was made containing 28.4% (w/v) acrylamide and 1.6% (w/v) bis acrylamide. Isoelectric focusing was performed in 4% gels containing 8M urea, 2% (v/v) Nonidet P40 and 2% (w/v) ampholines (LKB Limited, Sweden). The ampholines usually consisted of 2% pH range 3.5-10, or 0.4% pH range 3.5-10 plus 1.6% pH range 5-7. The gel mixture was degassed, and prior to pouring it between the

slab forming plates, 0.00033 volumes of 10% ammonium persulphate and 0.00025 volumes of TEMED were added. The mixture was poured into the apparatus to the top (total volume 25 ml) with the slot forming comb in place.

After polymerisation, the slots were filled with lysis buffer (9.5M urea, 2% NP40 and 2% (w/v) ampholines, 1.6% pH range 5-7, 0.4% pH range 3.5-10) and overlain with 0.02M sodium hydroxide. The bottom of the gel was immersed in 0.01M phosphoric acid, and the gels were pre-run as follows:

200 volts for 15 minutes

300 volts for 30 minutes

400 volts for 30 minutes.

Samples were solubilised in up to 100 µl of lysis buffer by three cycles of freezing and thawing, and were routinely spun for 5 minutes in a Beckman microfuge (15,000g), although generally no turbidity could be observed. When the pre-running of the gel was complete, the sample wells and upper buffer compartment of the apparatus were emptied. The samples were applied, and gently overlain with sample overlay buffer (8M urea, 1% (w/v) ampholines, 0.8% pH range 5-7, 0.2% pH range 3.5-10). This was in turn overlain with fresh 0.02M sodium hydroxide (degassed).

Fresh degassed sodium hydroxide (0.02M) was added to the upper buffer compartment and isoelectric focusing commenced. Isoelectric focusing was carried out at room temperature, the apparatus being cooled by tap water. Gels were run at a constant voltage of 400 volts for 12 hours, after which the voltage was raised to 800 volts for one hour. During the course of isoelectric focusing the current generally dropped to very low values, 1-2 mA. When the procedure was complete, the power was switched off and the gels removed for fixing and staining as described for the treatment

of s.d.s. gels.

8. Autoradiography

Dried gels were sandwiched between two glass plates with a sheet of X ray film, wrapped in aluminium foil and kept at 4°C in the dark. Exposed film was developed for 5 minutes with Kodak D19 developer, and fixed for 5 minutes in Hypam (Ilford). In the earlier part of this work Kodak Blue Brand medical X ray film was used, but it was later found that a lower background could be obtained with Kodak Kodirex film and this was routinely used. When 30,000 dpm was applied per sample, good autoradiographs could be obtained in one week.

X ray films were scanned on a Joyce-Loebl microdensitometer which recorded a continuous trace of the optical density across the film.

9. Miscellaneous techniques

Solutions were sterilised by filtration through 0.22 µM Millipore GSWP filters. Glassware was sterilised by autoclaving. Inorganic solutions and media were kept at 4°C. Biological samples, such as labelled cells awaiting electrophoresis, were stored at -70°C.

The pH of solutions was adjusted on a Vibret model 3920 pH meter calibrated using standard buffer solutions of pH 4.0, 7.0 and 9.2, supplied by Electronic Instruments Limited.

The pH gradient across isoelectric focusing gels was measured on a strip of the gel removed prior to fixing. This strip was sliced into 12 x 0.75 mm sections, which were shaken with 2 ml of neutralised distilled water in a capped bottle. The pH of the water was measured after 10 minutes. No change in the pH register-

ed was found to take place if these samples were measured after greater intervals.

Centrifugations were performed in a controlled temperature MSE Superspeed 75 centrifuge.

Cell lines were screened for Mycoplasma by the method of Russell et al., 1975.

CHAPTER 3

THE ISOLATION OF ANTIMYCIN RESISTANT HUMAN CELLS

INTRODUCTION

The isolation of cell lines bearing mitochondrially transmitted mutations is of interest for two reasons. First, they may shed light on the contribution made to the genesis of the organelle by the mitochondrial genome. Specifically, this means the assignment of functions to individual mitochondrially coded products. Second, the existence of such cell lines would enable one to follow the fate of cytoplasm in genetic crosses, to elucidate the restrictions, if any, on the maintenance of organelles in a "foreign" cellular background, and to examine the question of whether mitochondrial DNAs from different sources can interact by recombination.

Much work, prompted by such considerations, has been carried out with fungi (see Introduction). Less has been done on mammalian cell lines, partly because the genetic techniques for discriminating between mitochondrial and nuclear transmission of markers have only been evolved relatively recently, and partly because of the intrinsically greater difficulty of working with mammalian cells than with fungi.

From the known functions of the mitochondrial genome, several categories of mutant phenotype specified by mitochondrial DNA might be envisaged;

1) Cells whose mitochondrial translation apparatus is affected.

2) Cells affected in the apparatus of ATP synthesis.

3) Cells affected at some point in electron transport.

Other aspects of mitochondrial biochemistry, such as Krebs cycle functioning, are less likely to form a discrete class of mitochondrially inherited mutations, as most components of these are soluble enzymes which are known to be produced cytoplasmically under the direction of nuclear genes (e.g. van Heyningen et al., 1973). It is conceivable that they could be affected secondarily by alterations in mitochondrial membrane permeability caused by a variant protein specified mitochondrially, but there appear to be no precedents in either fungal or mammalian systems.

Of these classes, all three are well represented in the fungi, as discussed in the Introduction. Mammalian cell lines resistant to a number of drugs affecting specifically mitochondrial functions have been isolated, and they all fall into either of the first two classes. Mammalian cell lines resistant to the following inhibitors of mitochondrial protein synthesis have been isolated: chloramphenicol (Spolsky and Eisenstadt, 1972; Mitchell et al. 1975; Siegel et al., 1976), tevenel (Wallace and Freeman, 1975), and carbomycin (Bunn and Eisenstadt, 1977). In none of these cases have any variant translational products of the mitochondrial genome been identified. Siegel et al. (1976) examined the profile of radioactive proteins on polyacrylamide gels synthesised by the Hela B CAP resistant mutant MC63 after inhibition of cytoplasmic protein synthesis with emetine and were unable to find any difference from the parental cell line. They also examined this cell line for the existence of detoxifying mechanisms with negative results. Since the characteristic is transmissible cytoplasmically (Munro et al., 1978) this raises the possibility that the affected mitochondrial product is a ribosomal RNA.

Another type of mutant affected in mitochondrial protein synthesis has been identified by Ditta et al. (1977). This, however, appears to be a pleiotropic effect, as cytochrome oxidase activity, ATPase activity and Krebs cycle activity are all affected. There appears to be a quantitative decrease in the amounts of cytochromes a/a_3 and b present. This is clearly a complex of phenomena, and it is not known whether the gene(s) determining it are mitochondrial or nuclear.

The second category of mitochondrial mutants is represented in mammalian cells by rutamycin resistant cells derived from the mouse cell line clone 1 D by Lichtor and Getz (1978). Rutamycin has been shown to inhibit the mitochondrial F_1 ATPase (ATP phosphohydrolase E.C.3.6.1.3) by binding to a protein subunit known as the oligomycin sensitivity conferring protein (OSCP) (Kagawa and Racker, 1966; Tzagoloff et al., 1968; MacLennan and Tzagoloff, 1968). One of the four subclones isolated by Lichtor and Getz was shown to retain resistance to rutamycin in an in vitro assay system, thus excluding the formal possibility that resistance was due to an alteration in membrane permeability. It has proved possible to transfer this trait at high frequency cytoplasmically. Thus far, this is the only mammalian cell line that has been reported with a presumptive alteration in the F_1 ATPase which has been shown to be mitochondrially transmitted. However, at least one subclone has been shown in this laboratory to have complete cross resistance to chloramphenicol (E. Munro, personal communication).

The third category, that of cells with an altered electron transport system, is not represented in mammalian system by drug resistant mutants at all. An interesting class of mutants, defective in NADH-Coenzyme Q reductase or in succinate dehydrogenase

has been isolated (De Francesco et al., 1976; Soderberg et al., 1977) but there is no evidence that these lesions are mitochondrially specified. The electron transport chain is, however, a potentially interesting system for investigation by the use of specific mutants. It is a membranous complex of enzymes, some of which (e.g. cytochrome c, Sherman, 1967) are known to be coded by nuclear genes, others of which (e.g. cytochrome a/a_3, Jeffries and Craig, 1977) contain both cytoplasmically and mitochondrially synthesised subunits. The elucidation of the precise contribution of the mitochondrial genome to such complexes was cited as one reason for the isolation of mitochondrial mutants defective or altered in the mitochondrial contribution. It is also conceivable that such mutants may shed light on the actual mechanism of electron transport and the structure of the electron transport chain. Certain studies with fungal mutants have, for example, shed light on the relative functions of different cytochromes of the b type (e.g. Bandlow, 1975).

One of the least understood regions of the electron transport chain is that including the region between coenzyme Q and cytochrome b. The precise number of b type cytochromes is in doubt (Chance, 1958; Yu et al., 1975; Weiss, 1976) and the polypeptide structure of the major species, b556, is uncertain (Weiss, 1972; Weiss and Ziganke, 1976). This region of the electron transport chain also includes the antimycin A binding site whose relation to the b type cytochromes is not known certainly (Das Gupta and Rieske, 1973; von Jagow and Bohrer, 1975; Bandlow, 1975). There has also been confusion over the question of the role played by b type cytochromes in electron transport, several hypotheses having been proposed relegating cytochrome b to a minor branch or pool of the electron transport chain (Chance, 1952; Kroger and

Klingenberg, 1967). It is also known that some components of this region of the chain are synthesised mitochondrially in fungi (Weiss and Ziganke, 1974; Lin and Beattie, 1976). There is thus considerable interest in this region of the electron transport chain, and the availability of the potent inhibitor, antimycin A, which acts at this region, led to the attempt to isolate mammalian mutants resistant to the drug described in this chapter.

There are two major problems associated with the selection of mutants with lesions specifically in mitochondrial DNA. The first of these is that a non-specific mutagenising procedure will produce a large background of nuclear mutations. The second is that there may be over a thousand copies of mitochondrial DNA per cell (Bogenhagen and Clayton, 1974) and it is implausible to imagine that the same specific lesion could be produced in all, or even in a sizeable proportion of them simultaneously. Thus any newly mutagenised mitochondrial DNA molecule will probably be in a minority against a background of either normal or differently mutagenised molecules. Presumably drug selection can work only at the level of the whole cell, not at the level of the mitochondrion. Thus, if selection is applied immediately to mutagenised cells, mutant mitochondria may be lost when the cell harbouring them dies because the mutant mitochondria which might have rescued it were present in too few copies to be effective. What is required is repopulation of the cell with mitochondria of the variant type to such an extent that their phenotype is expressed at the level of the whole cell.

There are a number of imponderables involved. First of all, it may not be true that selection is only effective at the level of the whole cell. Thus in the case of chloramphenicol resistant

mitochondria against a background of chloramphenicol sensitive mitochondria, the loss of the mitochondrially coded components in the sensitive mitochondria when selection is begun probably deprive these mitochondria of essential structural elements. It is conceivable that such mitochondria replicate inefficiently, if at all, and are lost, while the chloramphenicol resistant mitochondria are able to replicate and eventually come to repopulate the cell. This might explain, first, why the immediate imposition of chloramphenicol selection after mutagenesis has produced any resistant cells at all (Spolsky and Eisenstadt, 1972) and second, why it takes so long in such cases for resistant colonies to appear (Spolsky and Eisenstadt, 1972, in which 2.5 months elapsed before resistant colonies were seen, and Siegel, 1977, in which 6 months elapsed).

The second consideration is that it is not known whether there are any molecular mechanisms available to hasten the process of repopulation. Such mechanisms might involve the active removal of mitochondrial DNA molecules bearing certain types of gross structural damage, leaving a smaller population of mutagenised molecules with less severe lesions. A second possibility might be the spread of a given mutation through the population of mitochondrial DNA molecules by means of a biased directional recombination mechanism, similar to that believed to occur in heterosexual $\omega^+ \times \omega^-$ crosses in yeast (Dujon et al., 1974; Dujon et al., 1976). However, the occurrence of recombination between the mitochondrial genomes of mammalian cells is not well characterised. The extent of the process and its limitations, if it occurs, are factors which could affect the spread of a new mutation through the population.

The only experimental approach of any likely value in

attempting to bias the repopulation of cells with mutant mitochondria is to reduce the number of functional mitochondrial genomes in the cell prior to mutagenesis. After mutagenesis, repopulation of the cell from a limited number of mutagenised mitochondria will maximise the chance of a given mutant coming to represent a large proportion of the population. This approach was used by Lichtor and Getz (1978) who irradiated cells with ultra violet light after allowing the incorporation of BUdR into mitochondrial, though not nuclear, DNA. This treatment results in the breakage, and presumably the degradation and loss, of DNA which contains BUdR.

There are a number of techniques available which may bias mutagenesis in favour of mitochondrial DNA. Workers with yeast have exploited the sensitivity of the yeast mitochondrial DNA polymerase to manganese (Iwashima and Rabinowitz, 1969), low levels of which appear to lead to misincorporation of bases in DNA synthesis. This technique has yielded a variety of types of mutant in fungi, including ρ^- (Putrament et al., 1973), resistance to Janus green (Kruszewska and Szczesniak, 1978), resistance to chloramphenicol (Putrament et al., 1973) and defects in electron transport and in the rutamycin sensitive ATPase (Tzagoloff et al., 1975; Eccleshall et al., 1978). Ethidium bromide has also been used as a selective mutagen for mitochondria in fungi and in mammalian cells, although it is not clear that it interacts specifically with organelle DNA (Smith, 1977).

As has been mentioned, BUdR may be selectively introduced into the mitochondrial DNA of cells lacking a cytoplasmic thymidine kinase. As well as allowing the destruction of a proportion of the mitochondrial genomes by uv irradiation, BUdR is a potent mutagen, and has been used alone to obtain mitochondrial mutants.

The major aim of the work reported here was to produce cells resistant to the electron transport inhibitor antimycin A. From fungal precedents, it appeared likely that mutations conferring resistance to this drug would be mitochondrially coded. In an attempt to mutagenise selectively the mitochondrial genome, a mutagenic system using N-methyl-N'-nitro-N nitrosoguanidine (MNNG) in combination with cycloheximide was used. MNNG is believed to mutagenise selectively at replicating forks in DNA, a property that has been used to map bacterial genes by sequential mutagenesis (Cerda-olmedo et al., 1968). Cycloheximide is reported to have a rapid and reversible effect in inhibiting nuclear DNA synthesis though not mitochondrial DNA synthesis (Storrie and Attardi, 1972). Thus mutagenesis with MNNG in the presence of cycloheximide should preferentially affect mitochondrial DNA.

This chapter describes the isolation after mutagenesis of cells resistant to antimycin A from the parental Hela derived cell line D98. This line was chosen for this work as it grows rapidly, is easy to handle, and carries a selectable nuclear marker ($HGPRT^-$). Further discussion of antimycin and its effects is deferred to Chapter 4.

MATERIALS AND METHODS

1. MNNG. N-methyl-N'-nitro-nitrosoguanidine was purchased in crystalline form from Sigma. It was dissolved in ethanol in a sterile bottle at a stock concentration of 10 mg/ml immediately before use, and brought to working concentrations by sterile serial dilutions.

2. Cycloheximide. Cycloheximide was purchased from Sigma. It was made up as a stock solution of 10 mg/ml in PBS and sterilised

by filtration. The stock solution was made up immediately before use and was used at a working concentration of 200 µg/ml.

3. Antimycin A. Antimycin A was purchased from Sigma. It was dissolved in ethanol at a concentration of 1.8 mg/ml in a sterile bottle. This stock solution was stored at -20°C and added directly to medium in appropriate dilutions for use.

4. Cell growth media. These were as described in Chapter 2. For measurements of cell survival, cells were plated at low densities (~5 x 10^2 - 1 x 10^3) in 5 cm diameter plastic dishes (Nunclone Ltd.). Colonies were stained with Giemsa (Fisons Ltd.) and counted, usually at about the 64 cell stage, and results were expressed as a percentage of untreated controls.

5. Measurement of DNA synthesis. Tritiated thymidine was purchased from the Radiochemical Centre, Amersham. After incorporation, cells were solubilised in 1 M sodium hydroxide, neutralised in 1 M hydrochloric acid, and precipitated through GFC filters (Whatman Ltd.) using 10% trichloroacetic acid. Dried filters were counted in an LKB-Wallac liquid scintillation counter as described in Chapter 2.

6. Cloning. When colonies were to be selected for cloning, the medium in the flask containing them was first changed. The position of the colonies was marked, and a bent Pasteur pipette with a heat rounded tip was used to suck up cells and medium in a gentle "vacuuming" action. Picked colonies were placed in separate small Falcon flasks. Only colonies well isolated from others were selected for isolation.

RESULTS

A. Mutagenic System

Experiments were first performed to confirm the inhibitory effect of cycloheximide on total DNA synthesis. A time course experiment showing the effect of pretreatment of cells with 200 µg/ml cycloheximide for varying periods before labelling with a two minute pulse of ^{3}H labelled thymidine confirmed the rapid effect of cycloheximide on total DNA synthesis (Figure 3.1). After two minutes of pretreatment with cycloheximide, the total d.p.m. incorporated into TCA precipitable material were reduced to about 10% of control values, and even treatment with cycloheximide for eight hours prior to labelling failed to reduce this value significantly. The absolute levels of incorporation in the presence of cycloheximide are still greater than those reported earlier (Storrie and Attardi, 1972) but incorporation into whole cells followed by cell solubilisation and precipitation is a relatively crude measure of DNA synthesis. It is likely that the true level of incorporation is reduced below those values shown in Figure 3.1. However, in the context of this study, the most significant feature of these results is that virtually all the effect of cycloheximide is exerted in the first two minutes of incubation, thus rendering a long pre-exposure before mutagenesis unnecessary.

To test the discrimination between nuclear and mitochondrial DNA synthesis exerted by cycloheximide, bulk labelling of cells with ^{3}H thymidine was carried out in the presence of cycloheximide, followed by cell fractionation. Once again it should be emphasised that fractionation into mitochondrial and nuclear pellets is a relatively crude way of separating mitochondrial

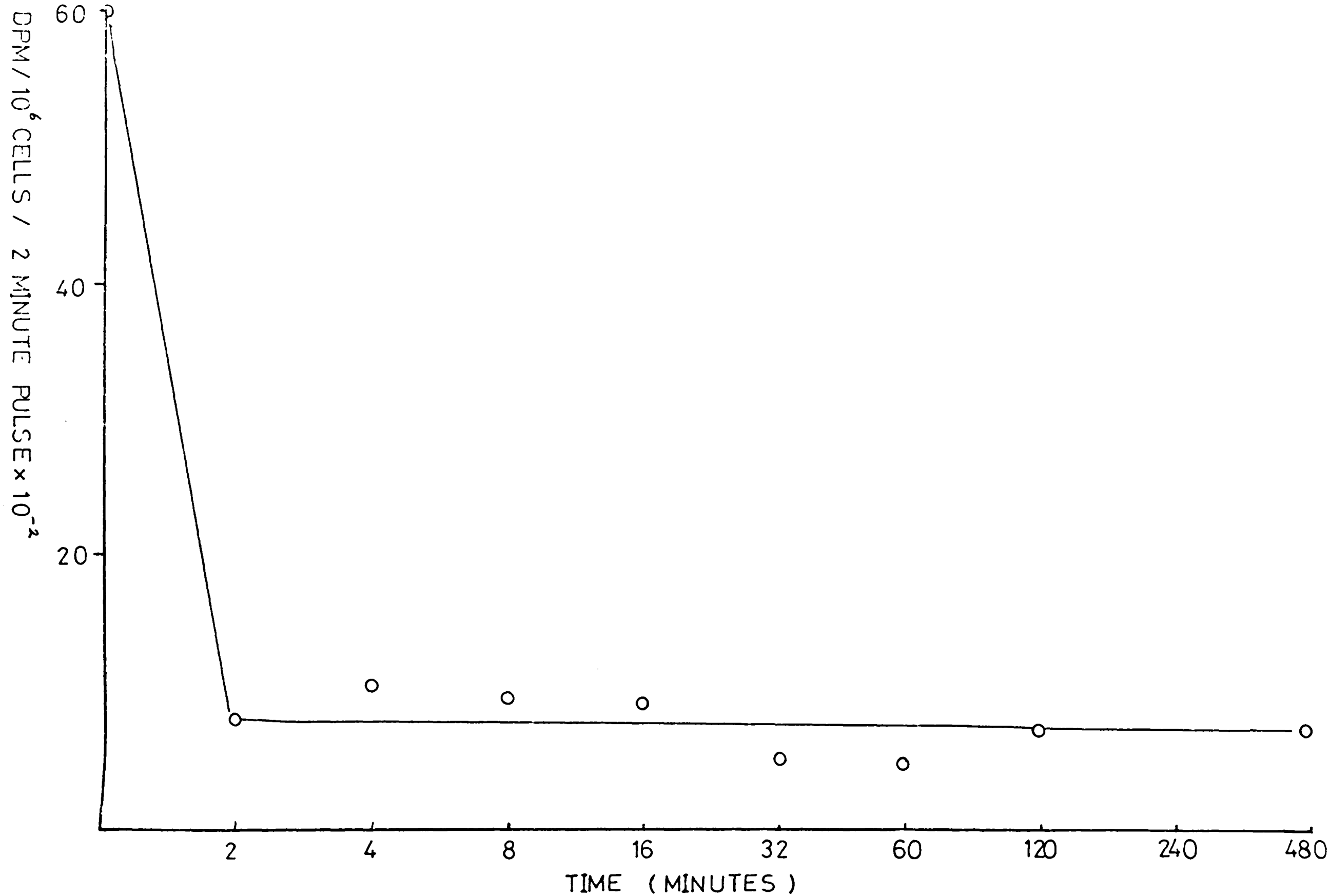

60
40
20
DPM / 10^6 CELLS / 2 MINUTE PULSE × 10^{-2}
2
4
8
16
32
60
120
240
480
TIME (MINUTES)

Figure 3.1 Time course of the effect of cycloheximide on the incorporation of labelled thymidine by cultured cells

2.5×10^5 D98 cells were plated out for varying periods in RPM1 1640 medium containing 200 µg/ml cycloheximide, before the addition of 1 µCi of ^3H labelled thymidine. Cells were allowed to incorporate for 2 minutes, after which the medium was removed and they were immediately solubilised in buffer containing 2% s.d.s. Labelled material was precipitated with 10% T.C.A. and collected on filters, which were counted as described in Chapter 2.

from nuclear DNA. Nevertheless, the results indicated that after thirty minutes of preincubation with cycloheximide, mitochondrial DNA synthesis continued at fairly high levels (35-40% of controls) during which period nuclear DNA synthesis was reduced to about 7% of control levels. In relative terms, mitochondrial DNA synthesis rose in these experiments to about 15% of total DNA synthesis compared with its normal level of about 1% (Storrie and Attardi, 1972). Thus inclusion of cycloheximide in the mutagenic system appears to bias DNA synthesis in favour of the mitochondrion compared with a system lacking cycloheximide.

In order to select a suitable concentration of MNNG for mutagenesis, plating experiments were carried out after treatment of cells with different concentrations of the mutagen to assess their survival. Experiments were carried out in the presence and absence of cycloheximide, the time of exposure to the mutagen being kept constant at three hours. It is clear from the results presented in Figure 3.2 that the inclusion of cycloheximide in the medium has a considerable effect in depressing survival below that of non cycloheximide treated cells. Treatment for three hours with cycloheximide alone reduced the plating efficiency to about 33% of control values. However, that a certain amount of protection against the effects of MNNG is afforded by the presence of cycloheximide is suggested by the way in which the survival of the cells does not markedly decline in the range of MNNG concentration from 0.01 to 0.1 µg/ml, over which range the survival of non cycloheximide treated cells falls by almost 60%.

Similar experiments over a more restricted range of MNNG concentration (Figure 3.3) showed that in the presence of 200 µg/ml cycloheximide, a concentration of 2.0 µg/ml MNNG gave a survival rate of about 1%. In order to maximise the chance of recovering cells which had been mutagenesised, this concentration of MNNG

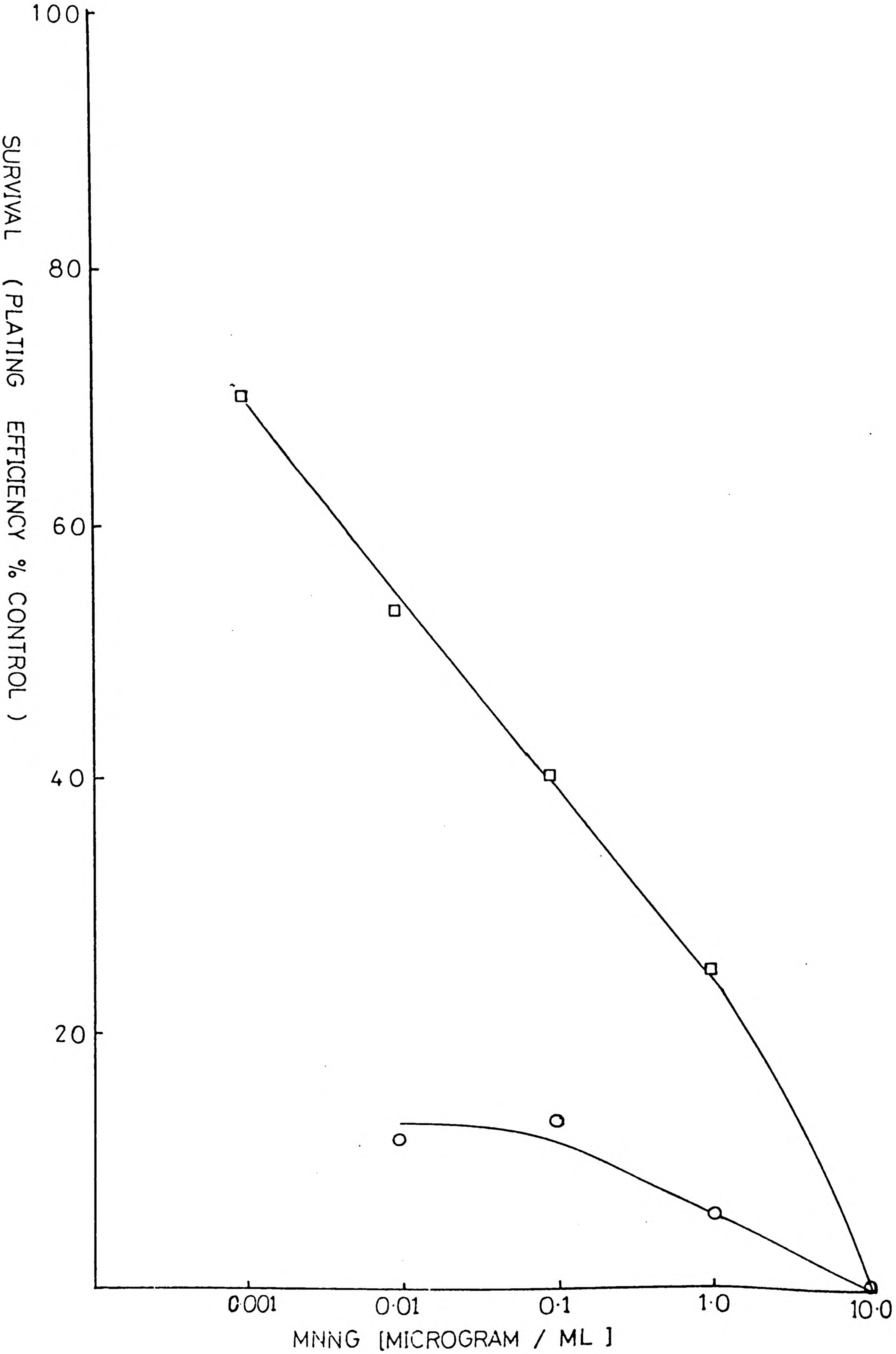
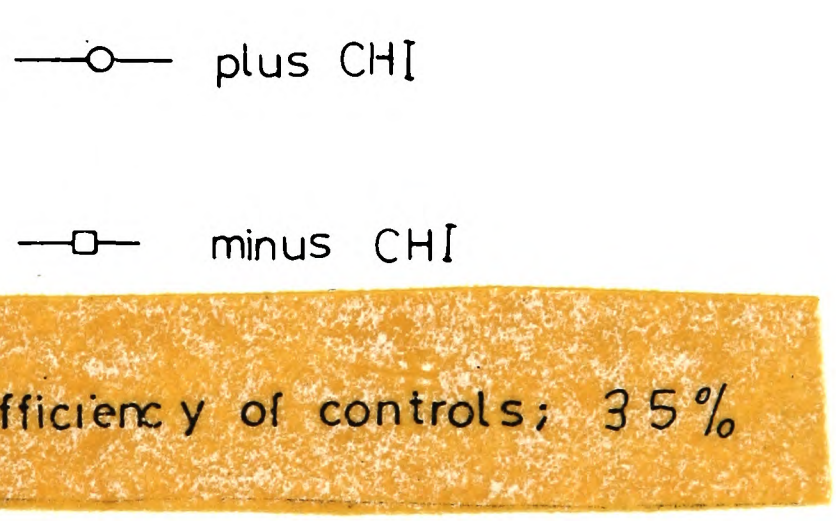

100
80
60
40
20
SURVIVAL (PLATING EFFICIENCY % CONTROL)
0·001
0·01
0·1
1·0
10·0
MNNG [MICROGRAM / ML]

Figure 3.2 Dose response of D98 to MNNG in the presence and absence of cycloheximide

2.5×10^5 D98 cells were plated out in small falcon flasks. The following day, the medium on all flasks was replaced by fresh medium either with or without 200 µg/ml cycloheximide. The flasks were incubated for 15 minutes, and then appropriate quantities of MNNG were added to each flask except controls. After 3 hours of treatment with mutagen, the medium was removed. The cells were washed once *in situ* with PBS, and then were released with trypsin and plated at low densities in plastic dishes to score survival.



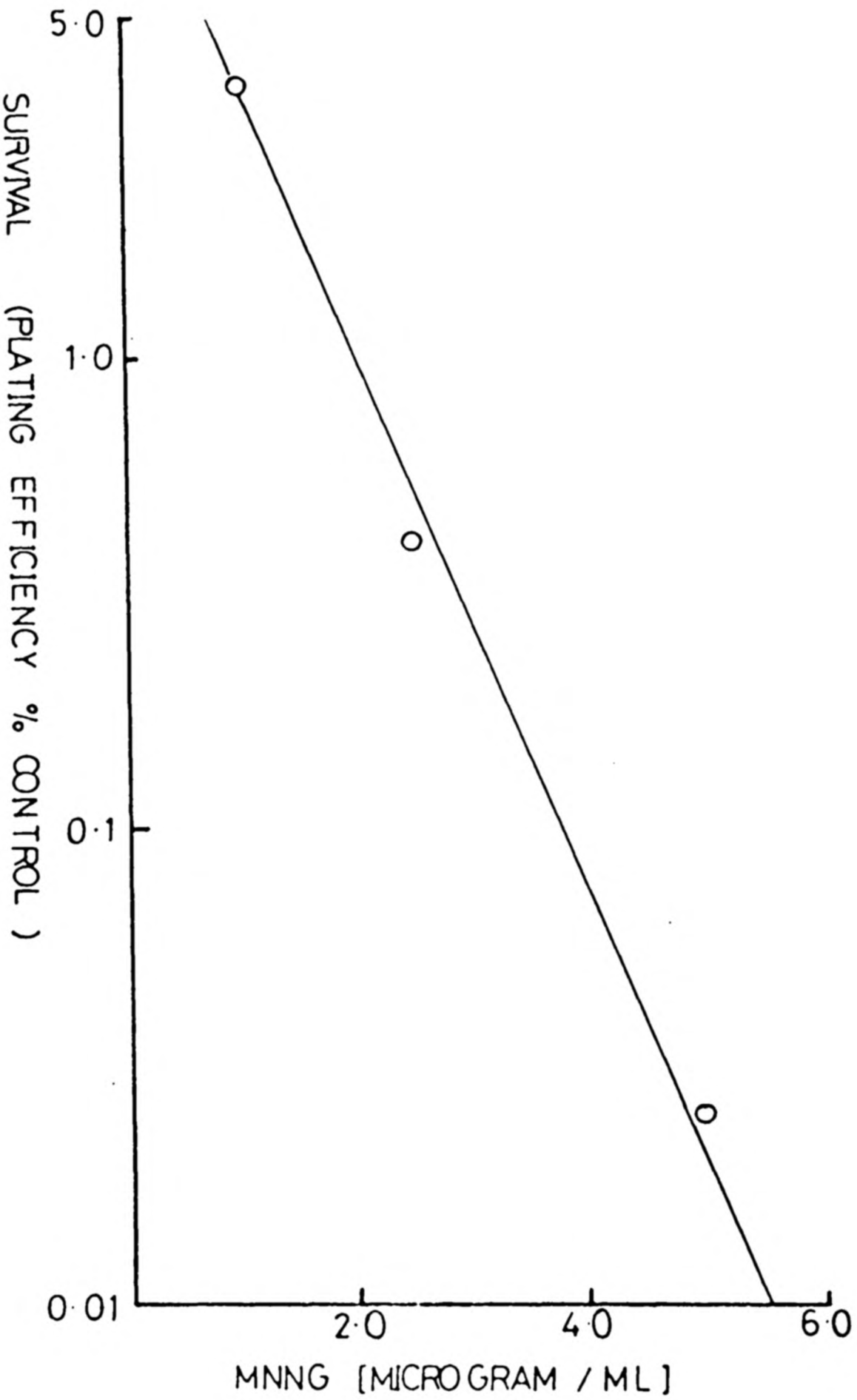

5·0
1·0
0·1
0·01
SURVIVAL (PLATING EFFICIENCY % CONTROL)
2·0
4·0
6·0
MNNG [MICROGRAM / ML]

Figure 3.3 The dose response of D98 to MNNG in the presence of cycloheximide

Cells were treated as described in the legend to Figure 3.2, except that all flasks contained cycloheximide at 200 µg/ml. The cells were exposed to mutagen for 3 hours before plating to assess survival.

Average plating efficiency of controls; 37%

was selected for use in the mutagenesis of parental D98 cells.

B. The effect of antimycin on the survival of D98 cells

Before embarking on the selection of antimycin resistant cells, a dose response curve was constructed by plating assay (Figure 3.4). There appears to be an almost linear response of survival against dosage, with a p I_{50} of about 0.016 µM antimycin A. A concentration of 1.2 µM antimycin suffices to reduce survival to between 1 and 3% of controls, and no cells at all were able to form colonies at 2.4 µM antimycin.

The final procedure adopted for mutagenesis and selection is described below.

C. Mutagenesis and selection

A total of 3.6×10^8 cells were mutagenesised with 2 µg/ml MNNG dissolved in RPMI 1640 medium supplemented with 10% foetal calf serum in the presence of 200 µg/ml cycloheximide. Mutagenesis was performed on attached cells growing in roller bottles and was continued for three hours. After removal of the mutagenic medium, the cells were washed once *in situ* with sterile PBS, released with trypsin, and placed in fresh medium in flat gassed flasks. To allow time for the loss of dead and dying cells, and to allow outgrowth and expression of any mitochondrial mutants that had been produced, unselected growth of the surviving cells was allowed for ten days.

Selection was begun by the addition of medium containing 5 µM antimycin A to all flasks. This concentration is between 2 and 3 times that required to kill all cells in experiments performed to construct the dose response curve (Figure 3.4). This initial selection was performed on bulk uncloned cells. Almost all of these died during the course of the next twenty days, leaving a

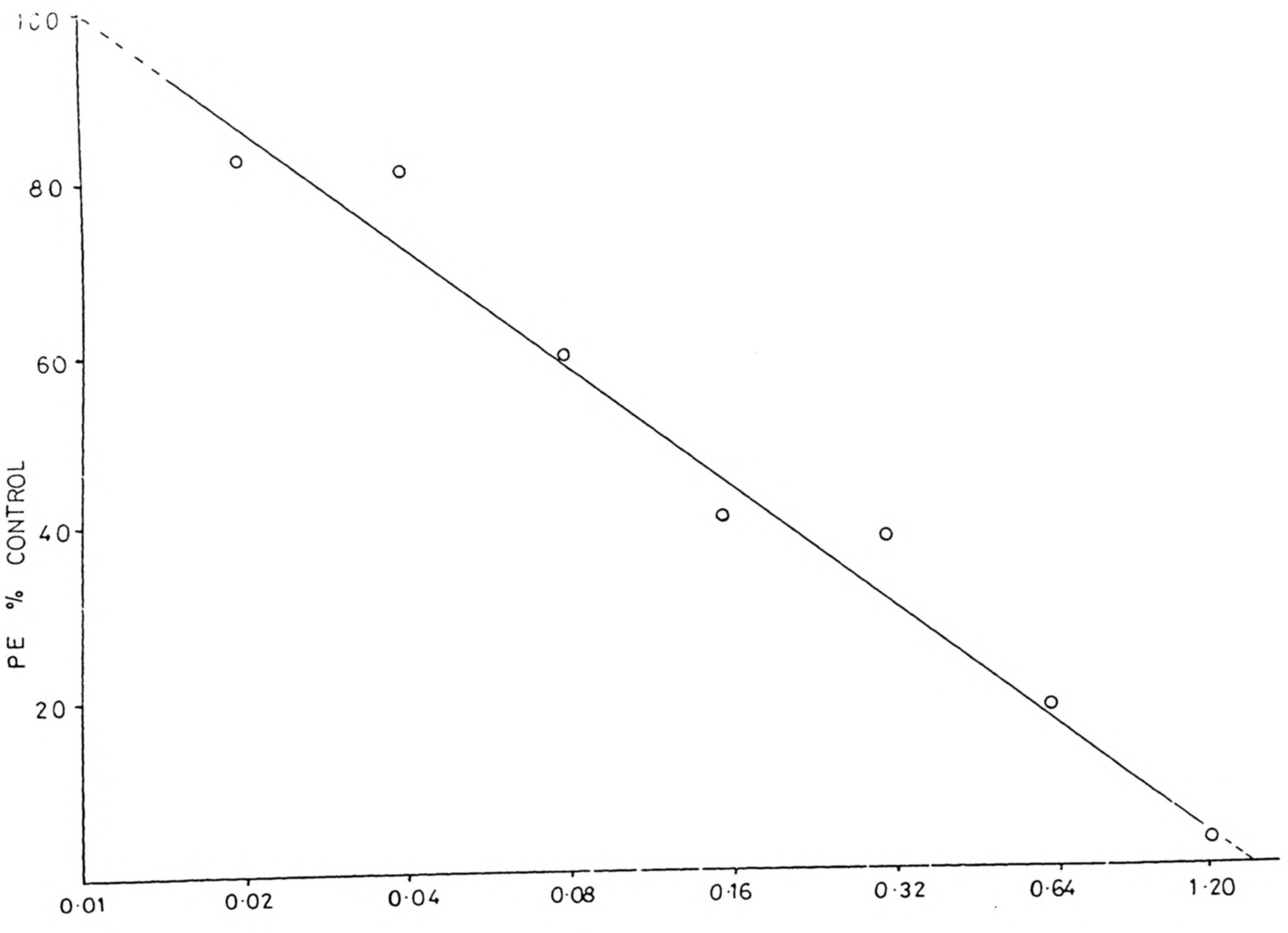

100
80
60
40
20
PE % CONTROL
0·01
0·02
0·04
0·08
0·16
0·32
0·64
1·20

Figure 3.4 The effect of antimycin on the plating efficiency of D98 cells

Equal aliquots containing approximately 10^3 D98 were plated out in plastic dishes of 5 cm diameter. 24 hours later the medium on all plates was changed to medium containing the appropriate concentration of antimycin, except for drugless controls. Plates were fixed, stained and counted 7-12 days later. Plating efficiency is expressed as a percentage of the number of colonies formed by controls lacking antimycin.

Average plating efficiency of controls; 35%

few colonies growing in otherwise empty plastic flasks. These cells were not cloned, but were grown for a further two weeks, at which time the concentration of antimycin A in the medium was raised from 5 to 15 μM, a concentration between seven and ten times that required to kill all parental cells. Once again, a large number of cells from the uncloned population died and were removed in routine changing of the medium. About one month after increasing the antimycin concentration to 15 μM, colonies were observed growing in the flasks.

D. First cloning

The colonies observed growing in 15 μM antimycin A were allowed to grow for between 10 and 15 days, at which point colonies were picked as described previously, and transferred to small Falcon flasks. A total of 23 clones were picked from 9 Falcon flasks. No flask contained more than 5 colonies.

After the cloning, cells were grown for one month in medium containing 10 μM antimycin A, and this concentration was then again reduced to 5 μM for routine growth of the established clones. One clone in particular grew well and was selected for further work (Clone 6).

E. Second cloning

Cells from clone 6 were again plated at low density in 15 μM antimycin A, and subclones were picked. The most rapidly growing of these was selected for detailed investigation. All the work described in this thesis was carried out on clone 6.5 (D98 antimycin resistant clone 6.5) hereafter known as MA 6.5. The remaining clones were frozen and stored in liquid nitrogen. All these cells were found to grow more rapidly at a concentration of 5 μM antimycin A than at 15 μM, and hence 5 μM supplemented medium

was used for routine growth.

CHAPTER 4

THE CHARACTERISATION OF ANTIMYCIN RESISTANT HUMAN CELLS

INTRODUCTION

The region of the mitochondrial electron transport chain catalysing the reduction of cytochrome c by ubiquinol can be isolated as a physically homogeneous, structurally intact entity, complex III (Hatefi et al., 1962; Rieske, 1976), containing cytochromes c_1 and b. This region of the electron transfer chain is of particular interest in the study of mitochondrial biogenesis as it has been shown that at least one of its components, cytochrome b, is translated on mitochondrial ribosomes in Neurospora (Weiss and Ziganke, 1974) and Saccharomyces (Lin and Beattie, 1976). It is also of interest in the study of bioenergetics as it corresponds to a site of energy coupling (Rieske, 1976). Several inhibitors are available which appear to inhibit specifically electron transport in this segment of the chain, the best characterised being antimycin A. The potential of a genetic approach to clarify the contribution of mitochondrial DNA to complex III and to elucidate the functional roles of the components of this region of the respiratory chain have led to the isolation by several workers of fungal mutants resistant to antimycin A. I will discuss briefly current ideas on the structure and function of this region of the chain before proceeding to review the studies with fungal mutants. Reviews of several of the topics discussed have appeared in recent years (Slater, 1973; Wikström, 1973; Rieske, 1976; Weiss, 1976).

Antimycin A is a family of closely related antibiotics differing only in the length and degree of branching of the acyl

or alkyl substituents of the molecule (Strong et al., 1960). Since the first observation of the effect of antimycin on the succinic oxidase activity of rat liver homogenates by Ahmad et al. (1950), antimycin has been widely used as a specific inhibitor of electron transport. Purified succinate dehydrogenase was shown to be insensitive to antimycin A (Singer et al., 1956), and Keilin and Hartree (1959) subsequently localised the site of action between cytochromes b and c_1, as reduction of a heart muscle preparation with succinate in the presence of antimycin led to the appearance in an absorption spectrum of an α peak for cytochrome b, but not for the combined peaks of cytochromes c and c_1.

The precise binding site of antimycin and its mode of action are not yet known. There are direct effects on cytochrome b, such as a red shift in the α peak maximum in the absorption spectrum, a lowering of the standard redox potential of cytochrome b_{562}, and alterations in the steady state aerobic reduction of the molecule (Berden and Opperdoes, 1972; Grimmelikhuijzen et al., 1975) and these effects are, under some conditions, separable from effects on electron transport. It has been proposed by Storey (1972) that antimycin forms a complex with cytochrome b analogous to the complex formed between cytochrome oxidase and carbon monoxide. However, there are several arguments against a direct interaction of antimycin with cytochrome b. On the assumption that the quenching of fluorescence of antimycin by cytochrome b is due to energy transfer to the haem from the benzene ring of antimycin, Berden and Slater (1972) estimated the distance between the haem and the benzene ring to be about 2 nm, a value approximately ten times greater than that predicted by the type of binding in Storey's model. In addition, estimates by several authors of the molar ratio of antimycin to cytochrome b required for full inhibi-

tion suggest the existence of one antimycin binding site for every two cytochrome b molecules (Pumphrey, 1962; von Jagow and Bohrer, 1975).

Evidence that cytochrome b is not the binding site for antimycin A was obtained by Gupta and Rieske (1973) who used a radioactively labelled deformamido derivative of antimycin to label covalently the antimycin binding protein in complex III preparations of beef heart. On s.d.s.polyacrylamide gels these preparations appeared to consist of seven major bands, which were identified with the components of complex III by comparisons with preparations enriched for various individual components. Most of the radioactivity was found in the fastest moving band with a molecular weight of 11,500 which did not correspond to any of the bands associated with identified components. These authors obtained an apparent molecular weight for cytochrome b of 15,000, which is about half the values reported by other authors for the purified molecule (Goldberger et al., 1961; Ohnishi, 1966; von Jagow et al., 1976). However, this assignment was criticised by Weiss (1976) who pointed out that it was based on assumptions about the quantitative staining of proteins with Coomassie brilliant blue which may not be valid for cytochrome b. However, the conclusion that cytochrome b does not bind antimycin is still valid, as it is unlikely that the true molecular weight of cytochrome b in these authors hands is any less than 15,000.

The characteristics of antimycin binding by submitochondrial particles have been studied by Berden and Slater (1972). Their results indicate that oxidised particles have a higher affinity for low concentrations of antimycin than reduced particles. They also reveal that antimycin is bound non-cooperatively to oxidised particles, but co-operatively to succinate reduced particles,

although binding to isolated complex III reduced by succinate is non-co-operative. Slater (1973) interprets these results as indicating interactions between individual molecules of QH_2-cytochrome c reductase in substrate reduced mitochondrial membranes. A further complication is provided by reports that there may be two antimycin binding sites with different affinities for antimycin (Potter and Reif, 1952; Burger et al., 1975).

There is uncertainty about the precise number of species of cytochrome b. Chance (1958) suggested the existence of three species of cytochrome b as a result of observed differences in the reducibility of components with α bands in the b spectral region at 556, 562 and 566 nm. The only cytochrome b readily reducible by succinate is cytochrome b 562, which is the classic b type cytochrome observed by MacMunn (1884), the other two requiring the presence of ATP or antimycin to render them succinate reducible. It is uncertain whether b-558 and b-566 represent a double α band of a single component, or two distinct species of cytochrome b. Although there have been reports of other minor cytochrome b-like components detected spectroscopically, most recent investigations have generally supported Chance's observations of two or three main spectral species.

Preparations of complex III in a number of workers' hands contain only two molecules of cytochrome b per mole of complex (Hatefi et al., 1962; Zaug and Rieske, 1962). Nelson and Gellefors (1974) observed two species of cytochrome b in their complex III preparation, absorbing at 562 and 565 nm, and each contributing about 50% to the total spectrally detectable cytochrome b. Most data support the concept that complex III contains two molecules of cytochrome b with two distinct sets of spectral, kinetic and thermodynamic properties. It is not known whether these differences

are generated through interactions of the same molecule with different local environments, or whether the two species are chemically different. It is noteworthy in this respect that even when starting from complex III containing two distinct cytochromes b, homogeneous preparations of purified cytochrome b of only one species have been obtained (Ohnishi, 1966; Weiss, 1972; Erecinska, 1973; Yu et al., 1975). Weiss (1976) proposes that cytochrome b is in fact a single molecular species. It is a dimeric haem protein with a molecular weight of 50,000-55,000 daltons, composed of two polypeptides of similar size, each binding one protohaem. Weiss suggests that this dimeric protein is the basis of two functionally distinct cytochrome b species. It is not yet certain whether the two subunits are identical. Weiss et al. (1975) observed on some s.d.s.gels a splitting of cytochrome b into two bands, which might indicate a difference in molecular weight of 1,000-2,000 daltons. Although cytochrome b could be eluted as two distinct peaks on hydroxyapatite chromatography, cyanogen bromide cleaved peptide fragments yielded identical elution profiles on gel filtration, indicating at least a similar primary structure. If the subunits are identical, interactions in situ with different polypeptides could lead to different conformations of the subunits, which in turn could give rise to different properties of the haem centres.

There are a number of unresolved questions concerning the role of cytochrome b in electron transport. On kinetic grounds, several authors have held that cytochrome b is not an obligatory component of the electron transport chain (e.g. Chance, 1952; Storey, 1968). However, a number of these studies have been conducted with sonicated submitochondrial particles, and it is possible that progressive disruption of the mitochondrial inner

membrane by extensive sonication is responsible for artifactual results. Conclusions from two studies with drug resistant mutants are relevant here. In the oligomycin resistant mutant isolated by Bandlow (1975), cytochrome b-566 is lost, though cytochrome b-558 is retained. As this mutant grows on glycerol, cytochrome b-566 must not be essential to electron transport and can be dispensed with, at least in the mutant. It should be noted, however, that all other mutants lacking cytochrome b-566 isolated by Bandlow have also lost the capacity to transport electrons. Grimmelikhuijzen et al. (1975) report that in an antimycin resistant mutant of Candida utilis, the amounts of antimycin required to affect cytochrome b are much lower than those required to inhibit electron transport. The demonstration that antimycin can cause a complete reduction of cytochrome b without affecting electron transport indicates that the mutant can transfer electrons from substrate to oxygen without passing them via cytochrome b. However, these authors admit that cytochrome b may be necessary for coupling to occur at complex III. They propose a model of antimycin binding to two sites with different dissociation constants similar to that of Burger et al. (1975).

It does not seem possible on the basis of the data currently available to arrive at a final conclusion on the role of cytochrome b in electron transport. It may be that a number of alternative pathways of electron transport exist, as proposed by Berden (1972), Norling et al. (1972) and Wikström (1973), but that cytochrome b is necessary for coupling electron transport through complex III to energy transduction.

Mutants resistant to antimycin have been isolated in Torulopsis utilis (Butow and Zeydel, 1968), Candida utilis (Grimmelikhuijzen and Slater, 1973), Schizzosaccharomyces pombe

(Lang et al., 1975) and Saccharomyces cerevisiae (Burger et al., 1976). These have been used both to study the inheritance of components of complex III, and directly to study the altered biochemistry and physiology of the mutant mitochondria.

Mitochondrial inheritance of antimycin resistance has been shown for a number of these (Burger et al., 1976; Pratje and Michaelis, 1977), although a complex pattern of inheritance was found by Lang et al. (1975) in mutants of Schizzosaccharomyces pombe. A mendelian inheritance of resistance was found, although some mitotic segregation of sensitive cells from nuclearly homozygous resistant cells occurred, suggesting that a cytoplasmic gene was involved in the expression of resistance. Dual nuclear and mitochondrial control of high levels of antimycin resistance has also been postulated by Pratje and Michaelis (1977a).

Pratje and Michaelis (1977a) conducted allelism studies on 15 mutants of Saccharomyces cerevisiae resistant to antimycin A, and 19 resistant to the related inhibitor funiculosin. They found that all these mutations mapped to one of two loci, designated AI and AII. All except one antimycin resistance determinant mapped at AI, and resistance specified by this locus appeared to be higher than that specified by AII. All the funiculosin resistance mutations mapped to AII. Recombination occurred between the two loci at high frequencies (8-21%) and the double recombinant ARI FUNR II showed the highest degree of resistance to antimycin, being the only class able to grow on plates containing 1 µg/ml antimycin. Neither locus was closely linked to the other known loci on the yeast mitochondrial genome.

In vitro resistance of electron transport to antimycin has been demonstrated in a number of these mutants (Bucow and Zeydel, 1968; Grimmelikhuijzen and Slater, 1973). The

mutant of Saccharomyces cerevisiae isolated by Burger et al. (1976) is interesting in that although the amount of antimycin required for 90% inhibition of NADH oxidase activity in sub-mitochondrial particles is the same in both the mutant and the wild type, the addition of bovine serum albumin to the mutant preparation restored activity, though this had no effect on the wild type preparation.

Direct demonstrations of lowered antimycin binding by sub-mitochondrial particles were provided by Butow and Zeydel (1968) and Grimmelikhuijzen and Slater (1973) by the quenching of fluorescence of a b.s.a./antimycin complex on the addition of mitochondrial protein. Particles prepared from the mutants caused less quenching than wild type particles, indicating that they bound less anti-mycin.

An accidental selection of mutants of Saccharomyces cerevisiae resistant to antimycin was discovered when Groot Obink et al. (1977) found that commercial preparations of mikamycin, a protein synthesis inhibitor, contained small quantities of an antimycin A-like substance as an impurity. Yeast cells were shown to be essentially impermeable to mikamycin, so the resistance of the mutants was in fact to the contaminant. The gene which had been thought to be responsible for mikamycin resistance maps close to the cyb locus, the presumptive structural gene for cytochrome b (Cobon et al., 1976; Nagley et al., 1976). Bunn et al. (1970) had previously suggested that in view of the map position of the gene, it might encode a membrane protein rather than a ribosome component.

It is clear that although these studies have provided much information of interest, several important questions remain. The number of mitochondrial genes for components of the bc_1 segment of

the respiratory chain, the number of cytochrome b species, and the gene product relationship of the various loci identified as conferring antimycin resistance with cytochrome b and the antimycin binding component remain to be established.

No work on the isolation and characterisation of mammalian cells resistant to antimycin has thus far been reported, though an interesting series of papers (Scheffler, 1974; Ditta et al., 1976; De Francesco et al., 1976; Soderberg et al., 1977) reports the isolation of a series of Chinese hamster mutants defective in respiration, and apparently lacking appreciable NADH-coenzyme Q reductase (De Francesco) or succinate dehydrogenase (Soderberg) activities. These may thus be the first mammalian cell mutants isolated with defective electron transport.

It seems important to establish whether mammalian cells resistant to antimycin can be produced, and to compare them with the fungal precedents. Such work might establish the general validity of conclusions already reached from work on fungi.

In this chapter, the detailed characterisation of the antimycin resistant cells whose isolation was described in Chapter 3 is reported.

MATERIALS AND METHODS

1. Antibiotics

D-threo-chloramphenicol was a generous gift from Dr. Marchamer, Parke, Davis. It was made up as a stock solution in distilled water at a concentration of 2.5 mg/ml and sterilised by filtration. The stock solution was made up immediately before use and added in appropriate quantities to medium.

Triethyl tin sulphate was a generous gift of the Tin Research

Institute, Greenford, Middlesex. It was dissolved in ethanol at a concentration of 1.2 mM. This was diluted with medium to a concentration of 1.2 µM by three successive serial dilutions of one tenth. Further dilutions were made as required.

2. Assay of mitochondrial protein synthesis

All operations were carried out sterilely. Cells were removed from culture bottles with trypsin and washed once by resuspension in RPMI 1640 medium supplemented with 10% foetal calf serum. They were then spun down at 1,000 g for five minutes and washed by resuspension in PBS. After this wash cells were again pelleted and resuspended in methionineless incorporation medium at $2 \times 10^6 - 4 \times 10^6$ cells/ml. 2 ml samples were placed in sterile centrifuge tubes and inhibitors were added as required from sterile stock solutions. Cells were incubated at 37^o for 20 minutes in the presence of inhibitors on a rotating table which gently agitated them. ^{35}S methionine was added, usually to about 2 µCi/ml. Cells were allowed to incorporate for one hour after which incorporation was stopped by the addition of 2 ml of ice cold PBS containing 1 mM L methionine. Cells were spun down, washed once by resuspension in PBS, and then pelleted. Cell pellets were dissolved by warming at 37^o for 10 minutes in the presence of 0.2 ml 1 M sodium hydroxide. After neutralisation with 0.2 ml 1 M hydrochloric acid, protein was precipitated with 10% trichloroacetic acid, collected on filter papers and counted in a liquid scintillation counter as described in Chapter 2.

3. Measurement of oxygen consumption

Oxygen consumption of whole cells was measured using a Clarke oxygen electrode with a polythene membrane (Snappies, Woolworths Ltd.). The output from the electrode was coupled to

a Servoscribe chart recorder. The recorder was calibrated by the addition of a few crystals of sodium dithionite to distilled water in the sample chamber to give a zero value, and by bubbling air through it to give a 100% level. The concentration of oxygen in the sample chamber is linearly related to the value displayed on the chart recorder, so, given the concentration of oxygen in air saturated water (0.255 mM), values may be directly converted to nanomoles of oxygen consumed. The electrode and sample chamber were kept at a constant temperature by means of a water jacket connected to a water bath, and the sample was stirred gently with a magnetic stirrer. It was not found to be necessary to conduct these measurements sterilely.

Cells were suspended in medium, usually at concentrations of 1×10^6 to 3×10^6 per ml, and 1 ml samples of such suspensions were assayed, each assay proceeding usually for about ten minutes. During the period of the assay, linear responses were continuously recorded. All values for oxygen consumption were corrected to nanomoles of oxygen consumed/minute/10^6 cells.

4. Assay of Krebs cycle activity

A modification of the method of Scheffler (1973) was used to assay the release of $^{14}CO_2$ from labelled pyruvic acid. The apparatus consisted of a 25 ml glass scintillation vial containing a short (1.5-2 cm) glass tube of diameter 0.5 cm which in turn contained the bottom half of a snapped Durham fermentation tube. The apparatus was sealed at the top using an airtight rubber seal (Suba-seal, Gallenkamp Ltd.). The scintillation vial containing the glass tube was sterilised by autoclaving and the Durham tube containing 100 µl of hydroxide of hyamine (Packard Ltd.) was subsequently placed inside the glass tube. This operation was carried out in a sterile hood using sterile forceps to avoid

contamination of the interior of the scintillation vial.

2 ml of cell suspension was placed sterilely in the bottom of the scintillation vial, outside the glass tube, and the apparatus was sealed by insertion of the suba-seal, the bottom of which was sterilised by washing with alcohol. Substrate was added by injection through the self-sealing rubber top using a Hamilton syringe. The apparatus was placed in an agitated water bath at 37^{o} for the duration of the assay, which was terminated and the cells killed by the injection of 0.5 ml of 10% trichloroacetic acid into the cell suspension. Vials were agitated gently for 30 minutes before removal of the seal, to allow time for the $^{14}CO_2$ released to be absorbed by the hydroxide of hyamine. The Durham tube was then removed using forceps and added directly to a scintillation vial containing 7 ml of scintillation liquid. This was counted in an LKB liquid scintillation counter as described in Chapter 2.

Assays were performed on cells suspended in the TD buffer of Scheffler (1973) (8.0 g NaCl/l, 0.38 g KCl/l, 0.25 g $Na_2HPO_4.12H_2O$/l, 3.0 g tris/l, pH 7.4 with HCl). According to Scheffler, similar results are obtained in this assay whether it is performed in MEM or TD buffer, and in the presence or absence of glucose. About 5×10^5 cells were assayed per vial.

TD buffer was made 0.6 mM with respect to unlabelled pyruvate. Usually 0.2 to 0.5 µCi of 2-^{14}C labelled pyruvate (Radiochemical Centre, Amersham) was added per ml. The specific activity of the pyruvate was 8.2 µCi/mMol. All results were converted to nmoles CO_2 released from pyruvate per 10^6 cells, taking into account the dilution of labelled with unlabelled substrate.

5. Assay of succinate-cytochrome c oxidoreductase

Electron transport activity, using succinate as substrate and cytochrome c as acceptor, was measured by the method of Rabinowitz and De Bernard (1957). The assay was performed in 40 mM potassium phosphate buffer, pH 7.4, made 0.5 mM with respect to potassium cyanide to inhibit cytochrome oxidase. 1 ml of buffer was incubated at 37^{o} with 0.01 ml of cell extract. 0.04 ml of cytochrome c stock solution (9.225 mg/ml phosphate buffer) was added, and the change in absorbance at 550 nm was monitored in a recording spectrophotometer[1]. The reaction was started by the addition of 0.02 ml of 1 M disodium succinate and the reaction was followed by the change in absorbance at 550 M as cytochrome c became reduced.

In preliminary assays, the extract used was whole cells sonicated (4 x 15 s cycles) in phosphate buffer, pH 7.4. However, these extracts were found to have a very high endogenous substrate activity. Subsequent assays were performed on membranes spun down at 105,000 g for 30 minutes after sonication of whole cells in phosphate buffer, pH 7.4. The pellet was resuspended in assay buffer at a protein concentration of 10-20 mg/ml, and this was used as extract. No endogeneous substrate activity was detected in these preparations. All activities were expressed as µmol cytochrome c reduced/minute/mg protein.

Antimycin was added as an ethanolic solution where required. The concentration of ethanol in the assay mixture at no time exceeded 1%.

1. Unicam SP 500 series 2

RESULTS

A. Response to antimycin A

Figure 4.1 shows the effect of antimycin A on the plating efficiencies of D98 (Parental) and MA 6.5 (antimycin resistant) cells. Two features require comment. First, it is clear that there is a considerable discriminatory effect of antimycin A between the parental and the variant cells. Approximately 80% survival of MA 6.5 is found at a concentration of antimycin A which kills all D98 cells. However, it is also apparent that antimycin A is not without effect on MA 6.5. There is a low but progressive decrease in plating efficiency with increasing antimycin concentration. At a concentration of 15 μM antimycin A, at which MA 6.5 has been cloned twice, the plating efficiency varies quite widely from between 45-70% of control values. It is not clear why these cells, which have been twice selected at 15 μM antimycin A, should retain any sensitivity to the drug at all at these concentrations. Antimycin was added to the medium used to construct the dose response curves as an ethanolic solution, and although the ethanol concentration did not exceed 1% at its maximum, it is possible that the progressive increase of ethanol with antimycin concentration may decrease the plating efficiency of MA 6.5. It is also possible that there are minor impurities in the antimycin which affect MA 6.5 at high concentrations.

Casual observations on MA 6.5 suggest that it does not respond well to being plated at low densities irrespective of the presence or absence of inhibitor, so it is conceivable that it is artifactually sensitive to antimycin under the conditions of this type of plating assay. This suggestion receives support from the observations of the effect of antimycin on oxygen consumption by the mutant reported later, where it is shown that antimycin at a

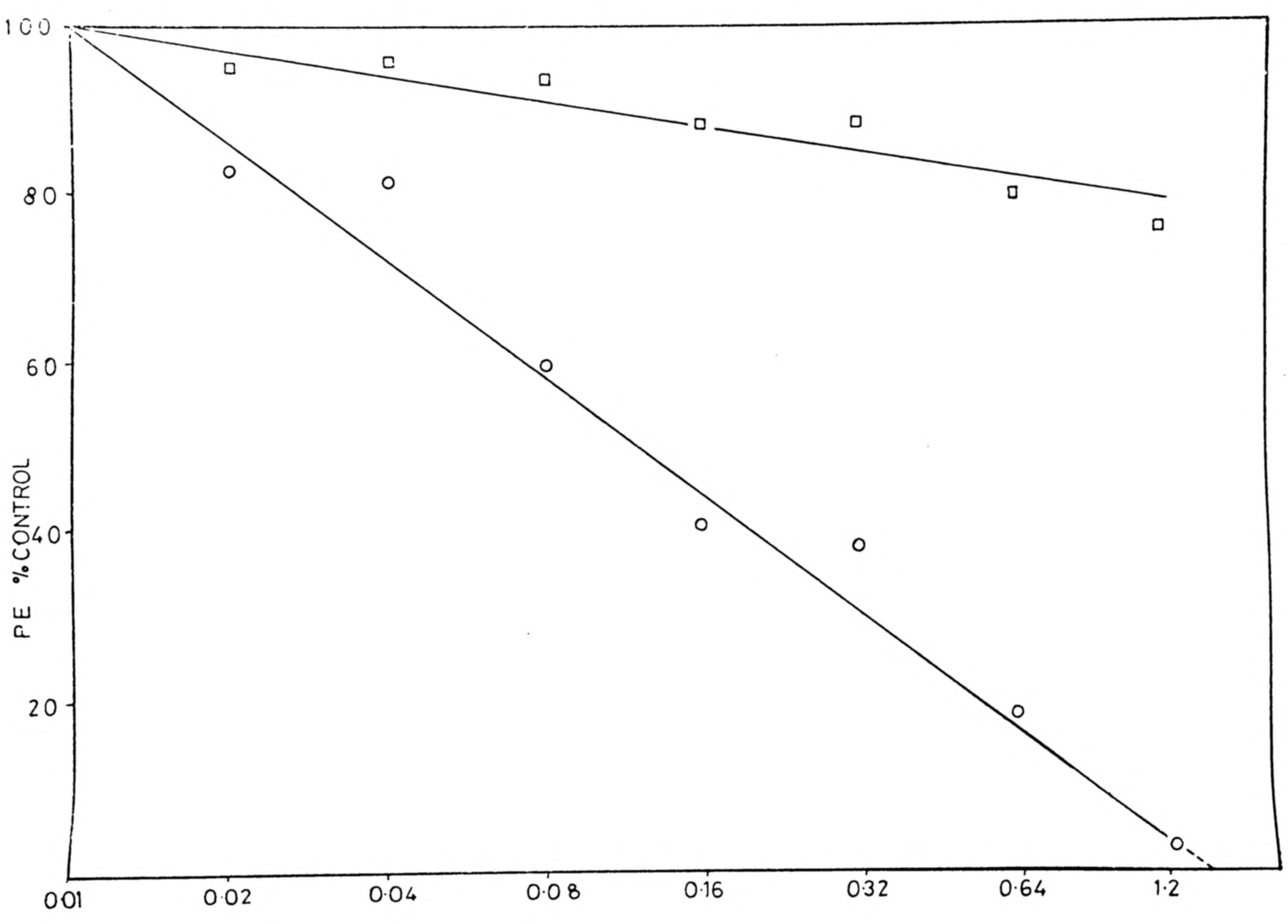
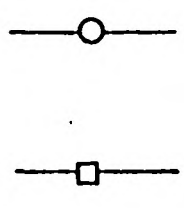

100
80
60
40
20
PE % CONTROL
0·01
0·02
0·04
0·08
0·16
0·32
0·64
1·2

Figure 4.1 The effect of antimycin on the plating efficiencies of MA 6.5 and D98

Equal aliquots containing approximately 2×10^3 MA 6.5 were plated out in plastic dishes of 5 cm diameter. 24 hours later the medium on all dishes was changed to medium containing the appropriate concentration of antimycin, except for drugless controls. Plates were fixed, stained and counted 7-9 days later. Results are expressed as a percentage of the number of colonies on drugless control plates. The data for D98 were replotted for this figure from Figure 3.4.



Average plating efficiency of controls;

D 98 35%
MA 6·5 25%

concentration of 15 μM has very little effect on respiration in these cells.

Whatever the reasons for these observations, the response of MA 6.5 to antimycin is clearly differentiated from the response of D98 over the concentration range in which the viability of D98 falls from 100% to 0. This observation supports the view that MA 6.5 is different from its parental cell line.

B. Growth rate of MA 6.5

A growth curve for MA 6.5 in the presence and absence of 5 μM antimycin A is shown in Figure 4.2. These cells grow fairly rapidly, with a doubling time of about 26 hours irrespective of the presence or absence of antimycin A. This doubling time is slightly longer than that shown by D98 under optimal conditions, which may be as low as 18 hours.

C. Response to other antimitochondrial antibiotics

There are two reasons for investigating the response of a drug resistant mutant to drugs other than that in which it was originally selected. First, if cross resistance to another drug is shown, genetic analysis using other cells resistant to this drug cannot be performed as the genome from only one parent will express resistance to both drugs and genuine recombinants cannot be selected. An example of this has been encountered in this laboratory (E. Munro, personal communication). One of the four subclones of mouse cells selected by Lichtor and Getz (1978) for resistance to oligomycin has been shown to be completely cross resistant to chloramphenicol. This means that these cells cannot be used for genetic analysis in combination with chloramphenicol resistant cell lines developed in this laboratory. In their paper, Lichtor and Getz reported that their oligomycin resistant cells

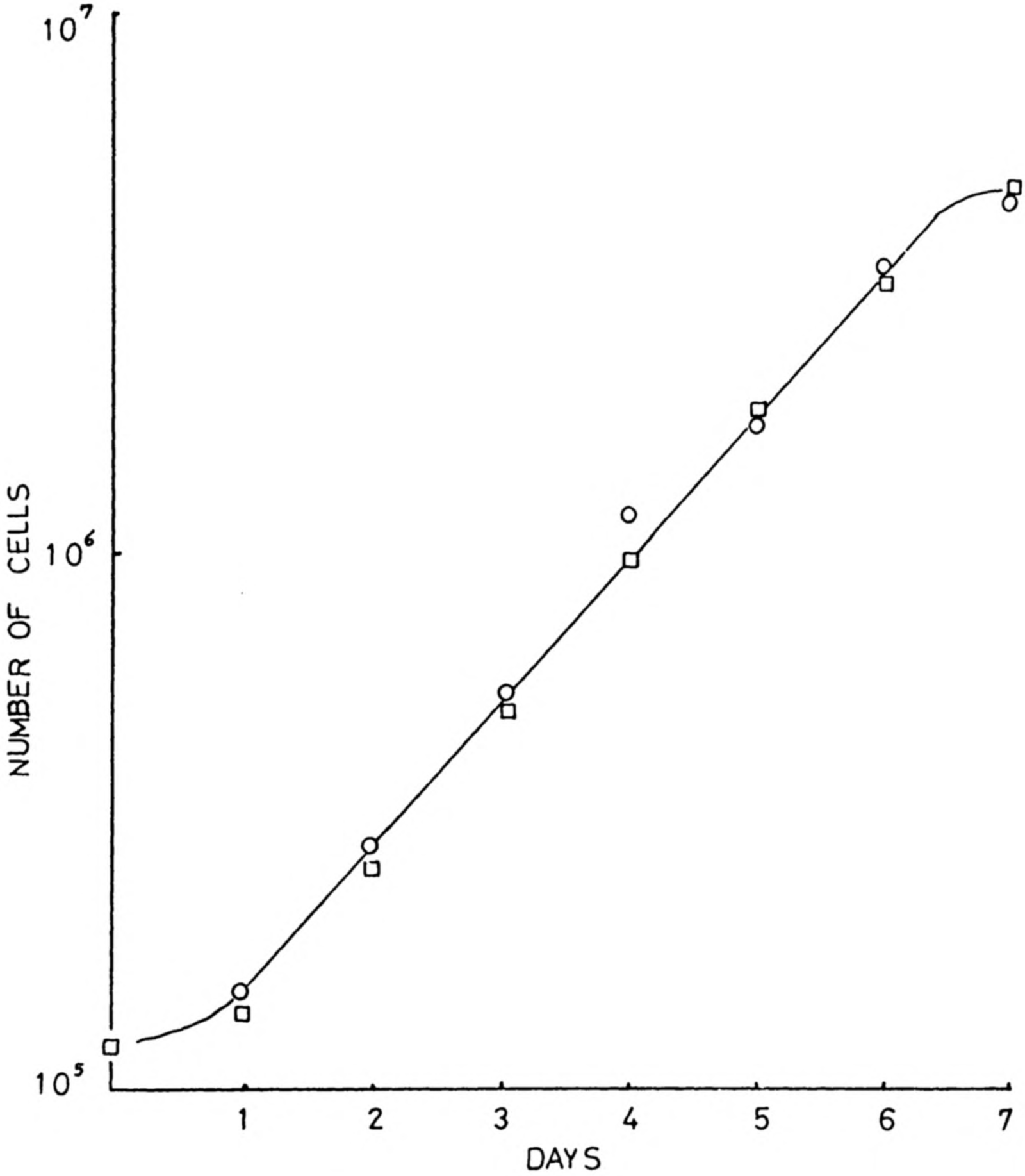


Figure 4.2: The growth rate of MA 6.5

The growth rate of MA 6.5 was determined in the presence (O) and absence (□) of antimycin (5 μM) as described in "Methods".

were sensitive to chloramphenicol at a concentration of 500 µg/ml, and this single observation was the basis of their comment that the cells do not show cross resistance. However, even the Hela derivative MC 63 selected for chloramphenicol resistance by Siegel et al. (1976) is affected by concentrations of chloramphenicol in excess of 100 µg/ml, and a concentration of 50 µg/ml has been found to be adequate for discriminating between resistant and sensitive cells. It is evident that the response of a drug resistant variant cell line to other drugs should be quantitated over a range which bears some relation to the effects of the drug on sensitive cells.

The second reason for investigating the response to other drugs is to elucidate the mechanism of resistance. There are a number of examples of pleiotropic mutations giving rise to a spectrum of resistance to several drugs (Bunn et al., 1970; Rank & Robbertson, 1975). In such cases, membrane permeability effects are usually invoked to explain the resistance patterns observed. These are usually of less interest to the investigator than specific alterations in either the target site or the metabolism of a single drug.

The response of MA 6.5 to two drugs, chloramphenicol and triethyl tin, was investigated. The response to chloramphenicol is of particular interest in view of the existence of mutants specifically resistant to it. Triethyl tin was selected for investigation as it appears to have a specific effect on mitochondrial ATPase (Lancashire and Griffiths, 1975), and is structurally unrelated either to antimycin or to chloramphenicol.

Figure 4.3 shows the results of an assay of mitochondrial protein synthesis and its response to chloramphenicol in MA 6.5.

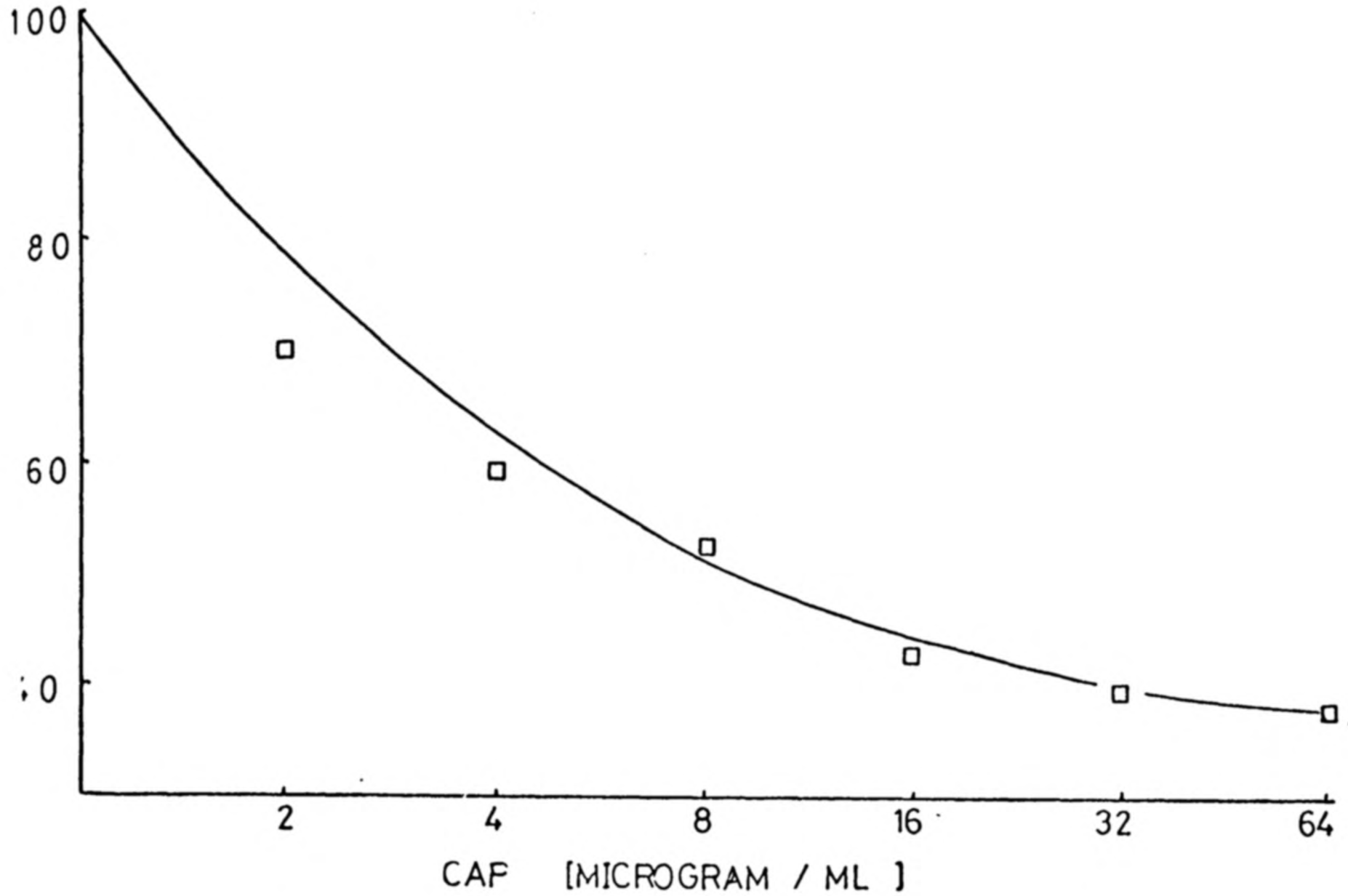


Figure 4.3: The sensitivity of MA 6.5 to chloramphenicol

The incorporation of ^{35}S methionine was measured as described in Methods. Cells were allowed to incorporate for one hour in the presence of emetine (100 µg/ml) and varying concentrations of chloramphenicol. Equal aliquots containing $4 \times 10^6 - 8 \times 10^6$ cells were used for each assay. All assays were performed in duplicate. An I_{50} value of about 8 µg/ml for MA 6.5 was found in these experiments, which was identical to that determined for D98 in similar experiments. The level of apparent incorporation insensitive to chloramphenicol was the same in both cell lines.

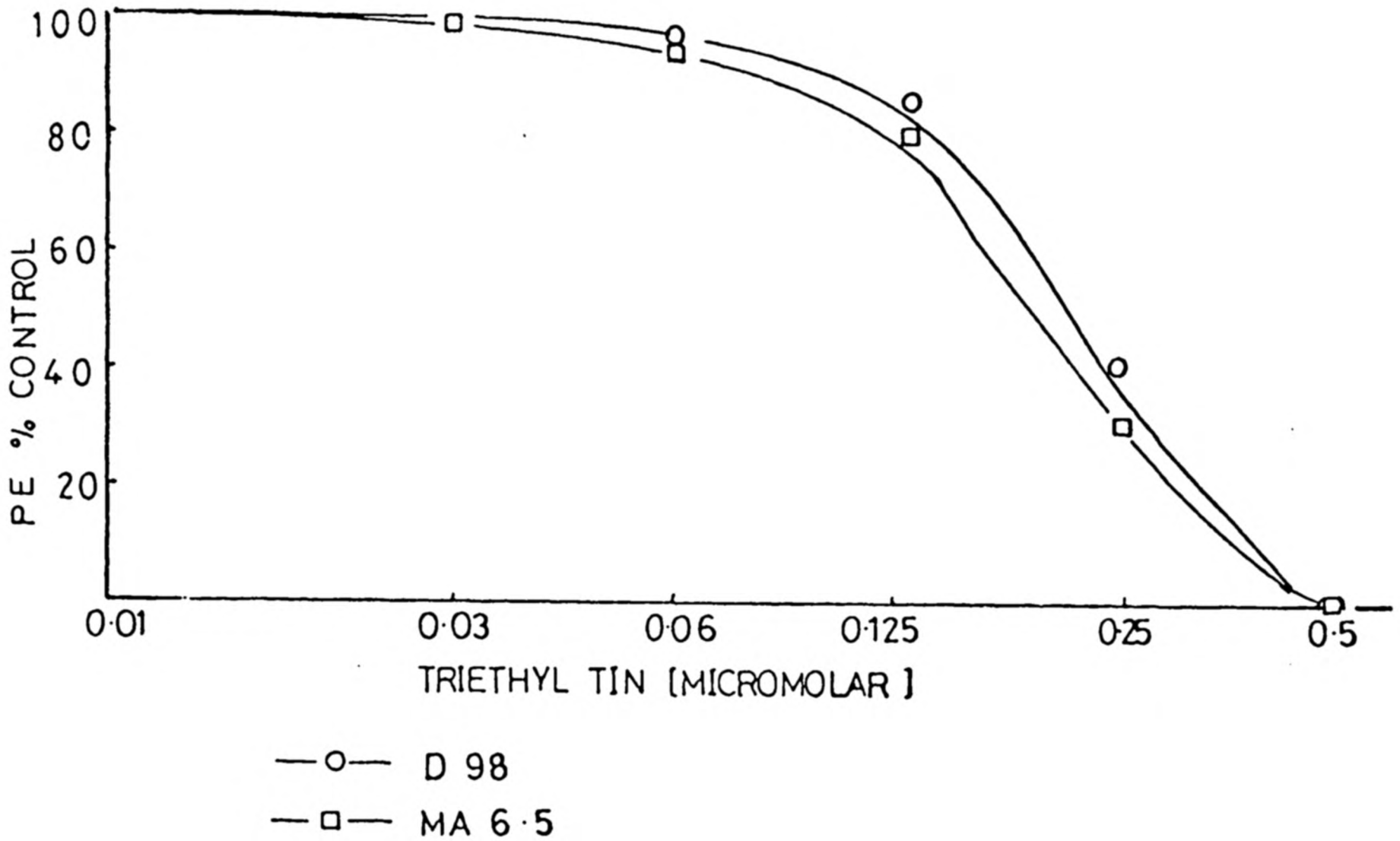


Figure 4.4: The effect of triethyl tin on the plating efficiencies of D98 and MA 6.5

24 hours after plating equal aliquots containing about 2×10^3 D98 or MA 6.5 in plastic dishes, medium was changed to medium containing appropriate concentrations of triethyl tin sulphate. Colonies were fixed, stained and counted after 9-13 days.

P.E. Plating efficiency.

Average plating efficiency of controls;

D 98	37%
MA 6·5	29%

Mitochondrial protein synthesis appeared to proceed at normal rates in MA 6.5 in that the emetine resistant chloramphenicol sensitive incorporation was approximately 1%-1.5% of uninhibited control values in both mutant and parental cell lines (Jeffries, 1975). The curve shown in Figure 4.3 is identical with that of D98, both curves having a similar I_{50} value of about 8 µg/ml chloramphenicol. It should be noted that it is not possible to reduce the amount of apparent incorporation to zero with any concentration of chloramphenicol. This residual incorporation is not sensitive to puromycin, and is found even in cells that have been killed (boiled, cyanide treated) prior to labelling (data not shown). It appears to have nothing to do with mitochondrial protein synthesis and is found in all cell lines examined. It is thus apparent that MA 6.5 is fully sensitive to chloramphenicol.

Figure 4.4 shows the results of a plating assay of the effect of triethyl tin on MA 6.5 and D98. The curves are identical, the concentration of triethyl tin required to reduce the plating efficiency to 50% of control values being about 0.2 µM in each cell line. From this it is concluded that MA 6.5 is as sensitive to triethyl tin as its parental cell line.

D. Oxidative metabolism of antimycin resistant cells

One possible explanation for the resistance of MA 6.5 to antimycin is that in these cells, electron transport, and consequently other aspects of oxidative metabolism, are largely or fully inhibited, but that the cells are able to survive by anaerobic metabolism, i.e. glycolysis (Ditta et al., 1976). Resistance to antimycin in this case would be effected by a switch from one type of metabolism to another. To test this possibility, the oxidative metabolism of antimycin resistant cells and of the

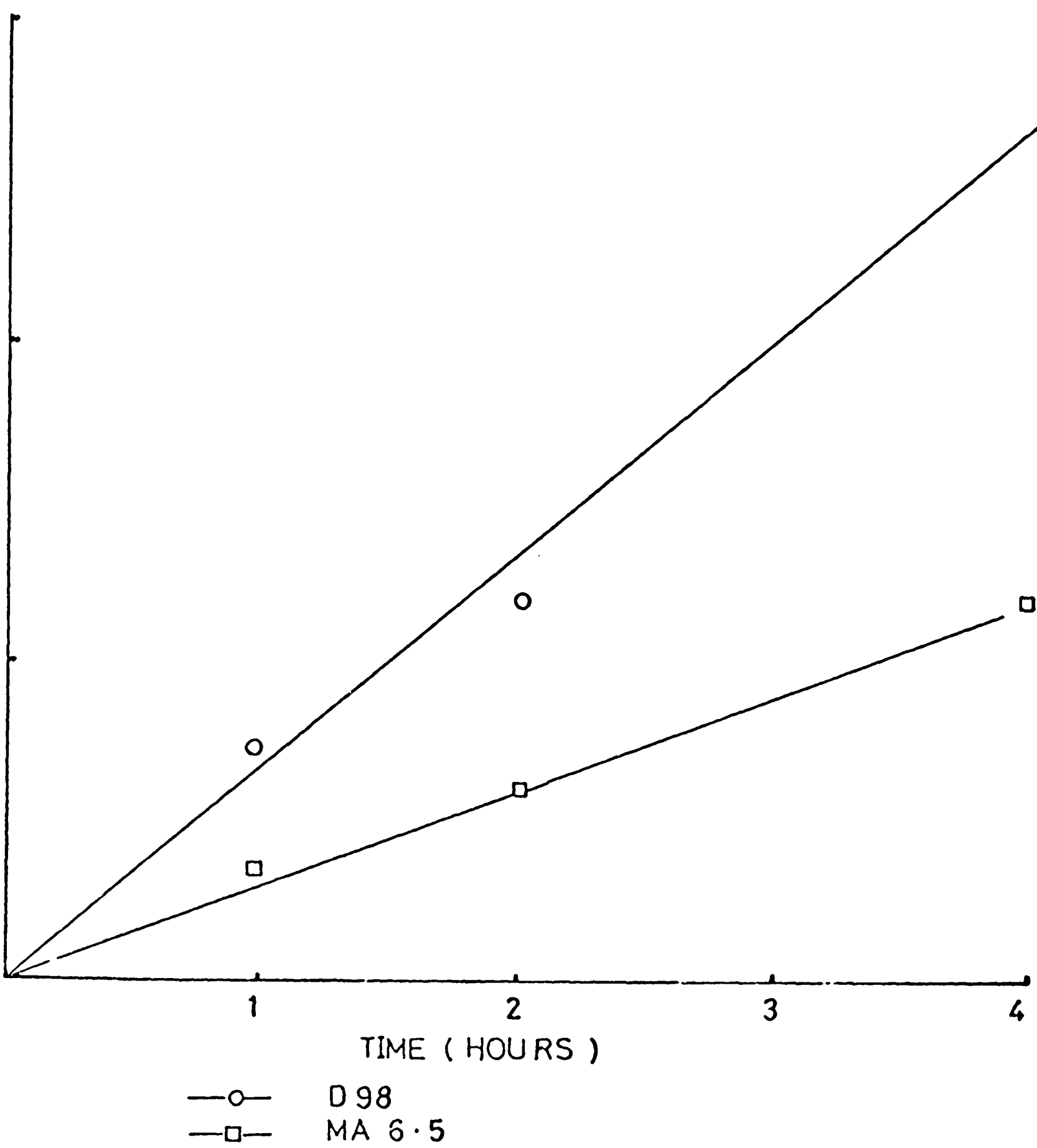


Figure 4.5 Release of carbon dioxide from 2-^{14}C labelled pyruvate by MA 6.5 and D98

Measurements were made as described in "Methods". Duplicate assays were performed on equal aliquots containing about 5×10^5 cells, and results were corrected to values per 10^6 cells.

parental line D98 was investigated.

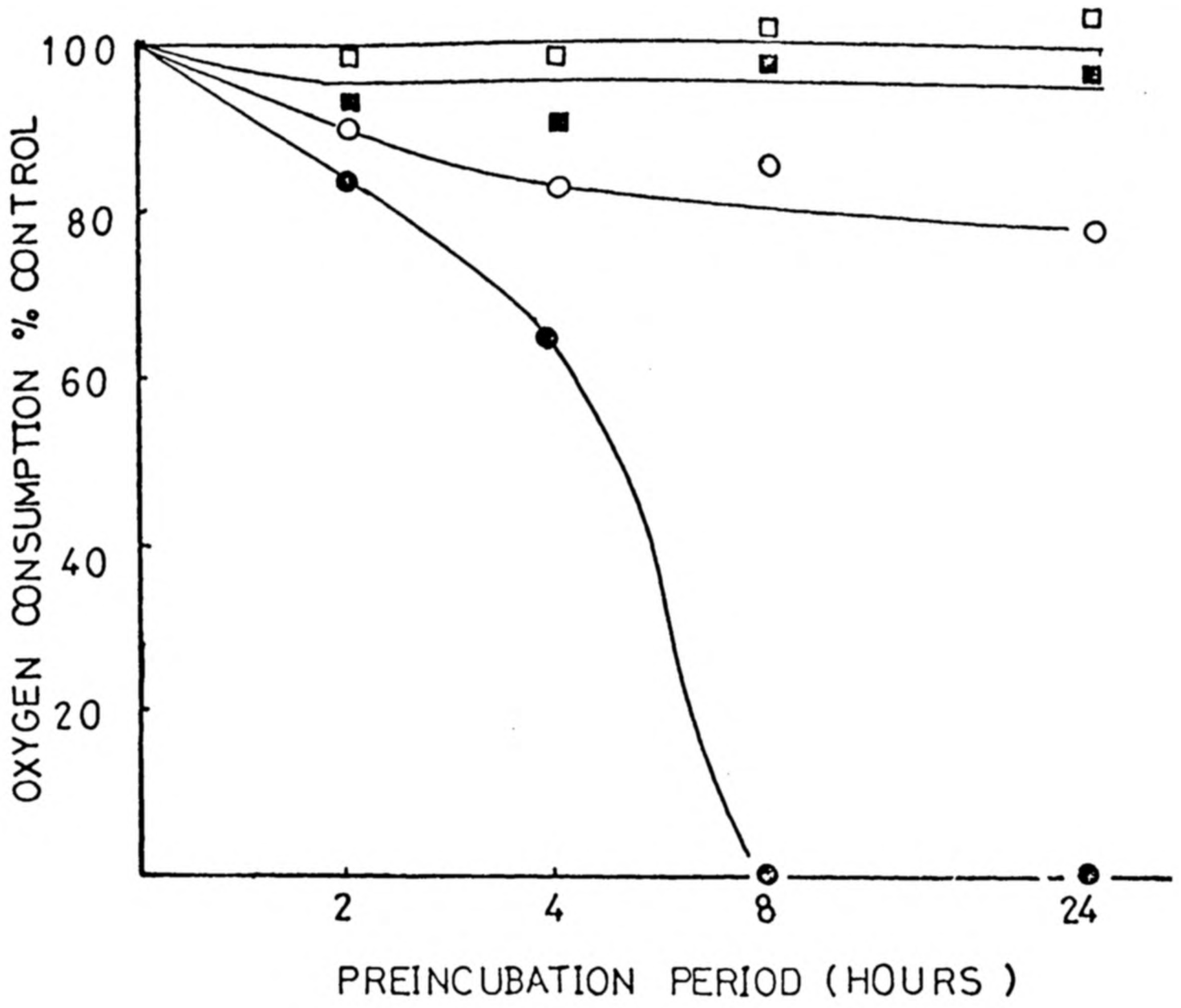
Krebs cycle activity was examined by the release of radioactive $^{14}CO_2$ from 2-^{14}C labelled pyruvate. The results of such an assay are presented in Figure 4.5, after correction for a low degree of non-biological $^{14}CO_2$ release found in blanks without cells. The linear response with time indicates that cells were viable during the period of the assay, and that substrate was not limiting. An absolute rate of 6.7 and 3.0 nmoles of CO_2 released per 10^6 cells per hour from pyruvate was calculated for D98 and MA 6.5 respectively. These values are low compared with the activity of some cell lines (e.g. Scheffler, 1973), but this may reflect variations in the method used to determine them.

It is clear that although MA 6.5 appears to release $^{14}CO_2$ from labelled pyruvate at only about half the rate of the parental cell line, there is still a degree of Krebs cycle activity at least roughly comparable in the two cases. For comparison, the respiration deficient Chinese hamster cells deficient in oxidative metabolism selected by Ditta et al. (1976) release $^{14}CO_2$ from labelled precursors at only 2% of the wild type rate. The explanation advanced by Ditta et al. for the low rate of Krebs cycle activity in their mutant is that the primary defect in NADH-Coenzyme Q reductase (complex 1 of electron transport) causes an accumulation of intramitochondrial NADH which feedback inhibits α ketoglutarate dehydrogenase activity. The relatively normal activity of the Krebs cycle in MA 6.5 may indicate that no such inhibition is occurring as a result of growth in antimycin, and hence that electron transport in vivo is proceeding at rates high enough to prevent the accumulation of intramitochondrial NADH. This point will be discussed later.

Oxygen consumption of both cell lines was measured as described in "Methods". Control values for both cell lines grown in the absence of antimycin ranged from 2.0-2.25 nmoles/minute/10^6 cells for D98, and from 2.7-3.2 nmoles/minute/10^6 cells for MA 6.5. Although rapidly growing cells which had not reached confluence were used for all these assays, the variations may reflect the varying health of the cells used.

In initial experiments to quantitate the effects of antimycin on oxygen consumption in the two cell lines, it was found that the addition of antimycin to respiring cells in the chamber of the oxygen electrode did not produce an immediate effect. This contrasts with the behaviour of cyanide, which inhibited oxygen consumption immediately on addition to respiring cells. Time course experiments were therefore performed with antimycin at two different concentrations, 5 μM and 15 μM. The results, shown in Figure 4.6, show that the effect of antimycin on sensitive cells is progressive with time. D98 cells were still consuming oxygen at about 75% of control rates after 24 hours in antimycin at a concentration of 5 μM, although the rate was still declining. A much more dramatic response to antimycin at a concentration of 15 μM was found. After 4 hours of preincubation, oxygen consumption in D98 had fallen to 65% of control values, and no oxygen consumption at all could be detected in cells preincubated for 8 hours. Oxygen consumption of MA 6.5, in contrast, showed no decline with time at a concentration of 5 μM antimycin A, and after an initial decline to 90% of control values after 2 hours in 15 μM antimycin, remained constant at this level for the remaining period of the experiment.

The effect of different concentrations of antimycin on cells with a constant preincubation period of 2 hours is illustrated in



—□— MA 6·5 5 MICROMOLAR ANTIMYCIN
—■— MA 6·5 15 " "
—○— D 98 5 " "
—●— D 98 15 " "

Figure 4.6 The effect of antimycin at two different concentrations on the oxygen consumption of MA 6.5 and D98 after incubation for varying periods

Measurements were made in duplicate as described in "Methods". 1×10^6-3×10^6 cells were assayed per measurement. Results were corrected to values per 10^6 cells and expressed as a percentage of untreated control values.

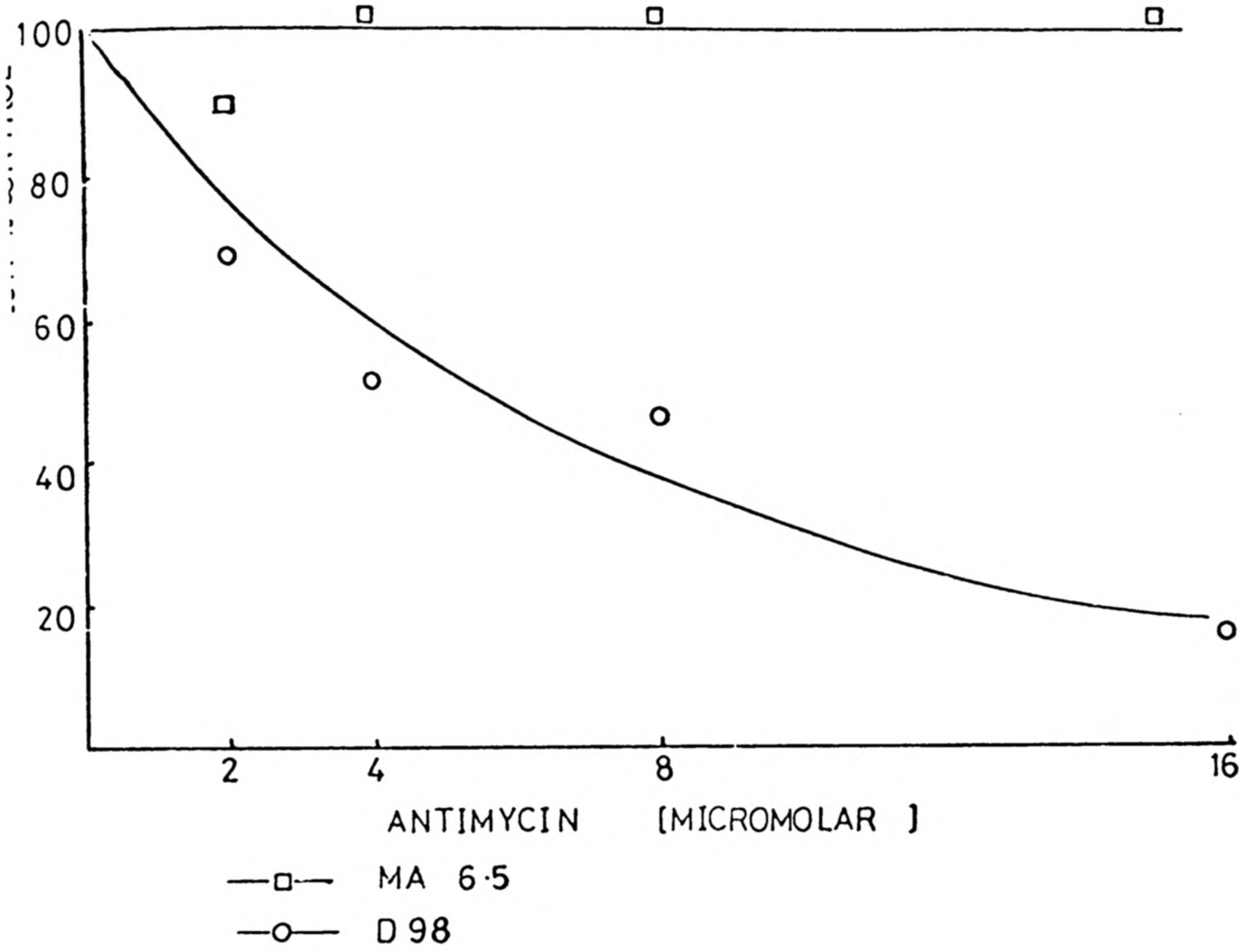


Figure 4.7 The response of oxygen consumption by MA 6.5 and D98 to different concentrations of antimycin

Measurements were made in duplicate as described in "Methods". Cells were preincubated in suspension for 2 hours with appropriate concentrations of antimycin prior to assay. 1×10^6-3×10^6 cells were used per assay. Results were corrected to values per 10^6 cells and are expressed as a percentage of untreated control values.

Figure 4.7. For these measurements, cells were preincubated in suspension with appropriate concentrations of antimycin, which may account for the more rapid effect of the drug than was observed in the time course experiments. Once again, it is clear that concentrations of antimycin of up to 16 μM have virtually no effect on MA 6.5, although over this concentration range the rate of oxygen consumption of D98 falls to under 20% of control values even with as short a preincubation period as 2 hours. Under these conditions, the concentration of antimycin required to inhibit 50% of the oxygen uptake of D98 is estimated to be 5.5 μM.

Taken together, these measurements show that the effect of antimycin on sensitive cells is progressive with time, at least up to 24 hours, and with concentration. Oxygen consumption can be completely inhibited by the drug at an appropriate concentration after a given period of preincubation. In contrast, the highest concentrations of antimycin used, and the longest preincubation periods, failed to affect markedly the oxygen consumption of MA 6.5.

From the results of these experiments, I conclude that oxidative metabolism in the antimycin resistant cells proceeds at a rate comparable with the parental cells, and that growth in antimycin fails to perturb either Krebs cycle activity or oxygen uptake.

E. Assay of succinate-cytochrome c oxidoreductase

The reduction of cytochrome c using a substrate such as succinate can be taken as an indication of a fully functional electron transport chain. Measurements of the activity of succinate-cytochrome c oxidoreductase were first performed on cells grown in the absence of antimycin. A certain degree of variability between assays was found, but an average value of 2.9 ± 0.3 μmol cytochrome c reduced/minute/mg protein was found for D98,

the equivalent value for MA 6.5 being 3.7 ± 0.2 µmol/minute/mg. These values cannot be directly compared with values obtained on purified submitochondrial particles, as these assays were all performed on crude 105,000 g pellets of whole sonicated cells, which presumably contain a large amount of non-mitochondrial protein.

The in vitro sensitivity of electron transport to antimycin A is shown in Figure 4.8. The response of these preparations to the addition of antimycin was immediate, and preincubation of the assay mixture with the inhibitor was not found to be necessary. It is clear from the figure that there is no significant difference between the two cell lines in response to antimycin. In both cases, the curves are strongly sigmoidal, which is in accordance with results on other systems (e.g. Torulopsis utilis, Butow and Zeydel 1968). The amount of antimycin required for 50% inhibition was about 0.4 ng/mg protein.

These results would appear to indicate that electron transport is fully sensitive to antimycin A in MA 6.5. However, it may be that the degree of inhibition of these membrane fragments does not accurately reflect the response of electron transport activity in vivo. Some support for this suggestion is given by the results shown in Table 4.1, where the activities of extracts of cells grown with and without antimycin are compared. D98 exposed to antimycin A at a concentration of 5 µM for 3 days had only 5% of the electron transport activity of controls not exposed to the drug, although MA 6.5 retained 50% of its control electron transport activity after an indefinite period in 5 µM antimycin A. The significance of these results is discussed later.

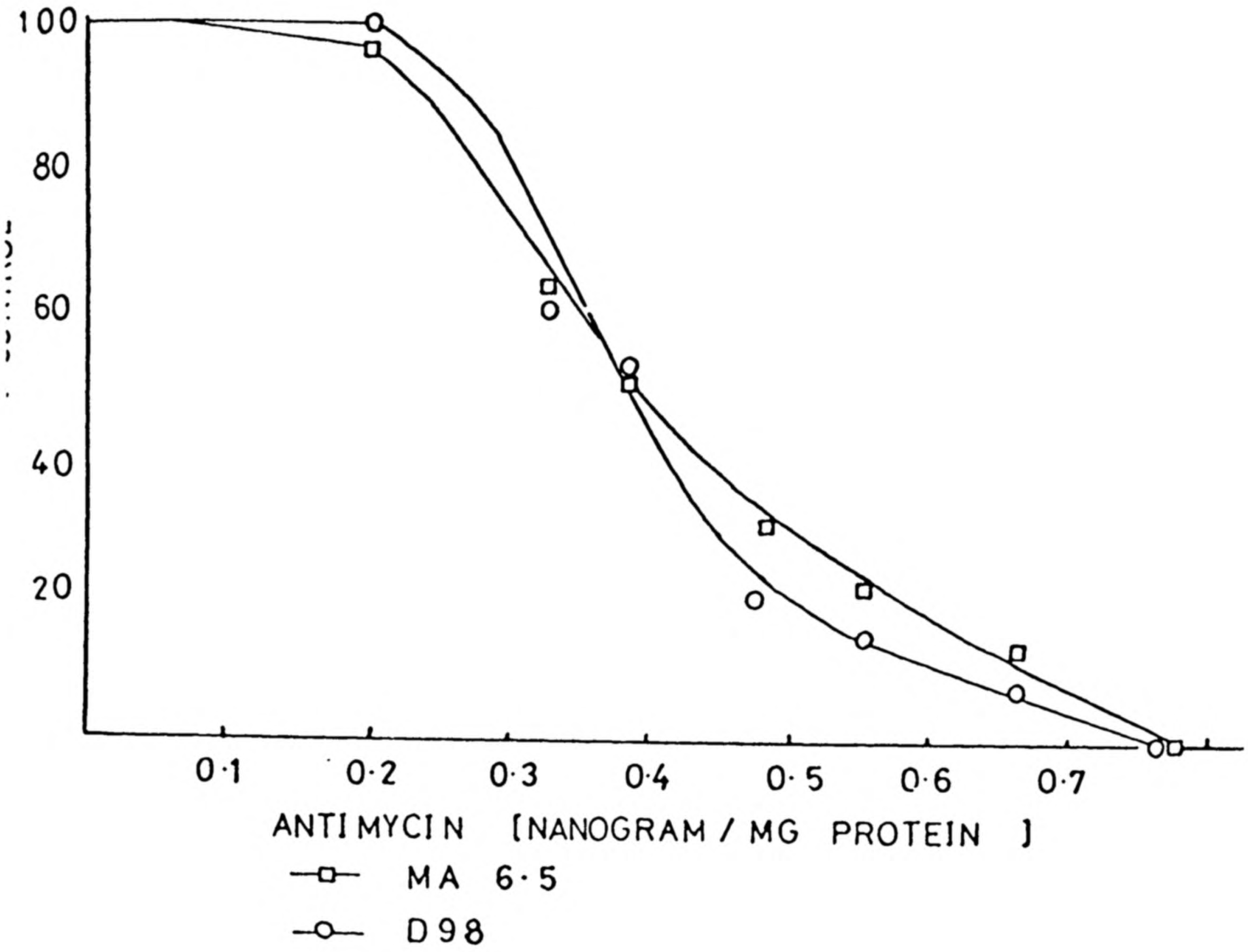


Figure 4.8 The sensitivity to antimycin of electron transport activity in membrane preparations of MA 6.5 and D98

Succinate-cytochrome C oxidoreductase activity of membrane preparations was measured as described in "Methods". Antimycin was added as an ethanolic solution. Points shown in the figure are averages of 4 (D98) and 5 (MA 6.5) determinations made on 2 independent membrane preparations from each cell line.

Table 4.1

The electron transport activity of membrane fractions prepared from MA 6.5 and D98 after growth in 5 μm antimycin

Cell line	Activity of membrenes of cells grown in the presence of antimycin(5 μM)	Activity of membranes of cells grown in the absence of antimycin	Percentage activity remaining after antimycin treatment
D 98	0·14	2·70	5
MA 6·5	1·70	3·40	50

Succinate-cytochrome c oxidcreductase activity of membrane fractions prepared from MA 6.5 and D98 was measured as described previously. D98 were exposed to 5 μM antimycin for 3 days prior to these assays. MA 6.5 cells used in these determinations had been grown in 5 μM antimycin for an indefinite, long period.

Assays were conducted in the absence of added antimycin in all cases.

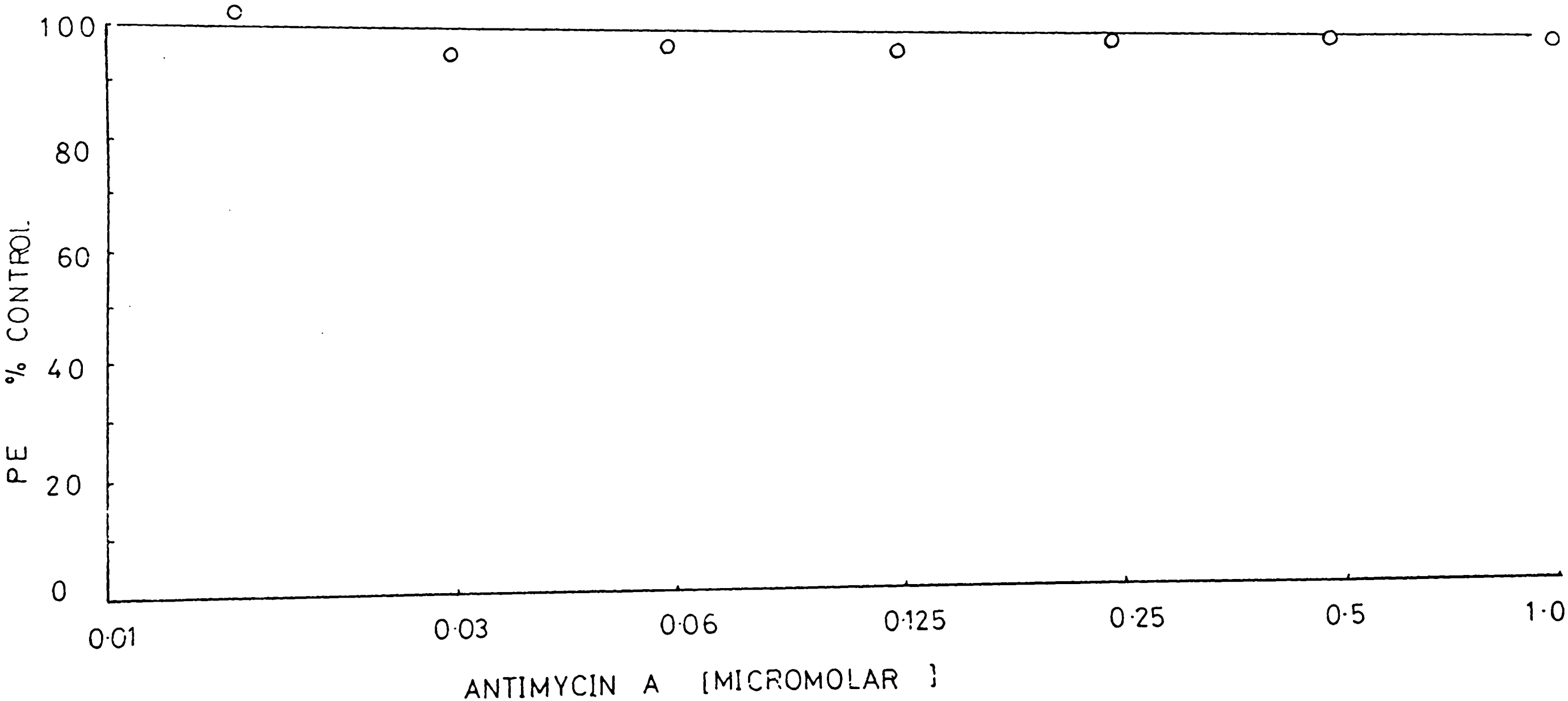

100
80
60
40
20
0
% CONTROL
PE
0·01
0·03
0·06
0·125
0·25
0·5
1·0
ANTIMYCIN A (MICROMOLAR)

Figure 4.9 The stability of antimycin resistance in MA 6.5

The effect of antimycin on the plating efficiency (P.E.) of MA 6.5 after growth in the absence of antimycin for 31 transfer generations was measured as described in the legend to Figure 4.1. All points were determined in triplicate. The plating efficiency was determined also at concentrations of 5 μM and 15 μM. These were 90% and 65% of control values respectively.

F. Stability of resistance in the absence of antimycin

A mutation is a stable, heritable variation in some character which persists in the absence of selection. The stability of the antimycin A resistance of MA 6.5 was tested by growing the cells in the absence of antimycin for 31 transfer generations (2½ months) and then repeating the plating assay of the response to antimycin A. No significant difference was found in the response when compared with the curve originally established (Figure 4.9). One is thus justified in regarding antimycin A resistance as a stable, heritable character, and hence in referring to MA 6.5 as an antimycin A resistant mutant.

DISCUSSION

The results presented in this chapter indicate that there is a clear, heritable and stable difference between the cell line MA 6.5 and the parental cell line from which it was derived, D98. The mutant has relatively normal rates of oxygen uptake and Krebs cycle activity, has the capacity for normal or even slightly elevated rates of electron transport, and shows no cross resistance to two other antibiotics acting on mitochondrial components. However, it survives at concentrations of antimycin well above those which kill the parental cell line, and both oxygen uptake and electron transport activity determined on cells exposed to antimycin are very much higher than those in parental controls.

It was disappointing that electron transport could not be shown to be resistant to antimycin when the antibiotic was added to the *in vitro* system, as this would have indicated that the site of the mutation was either in the antimycin binding site, cytochrome b, or some component of the electron transport system closely related to them. However, the antimycin resistant

Saccharomyces cerevisiae isolated by Burger et al. (1976) were apparently as sensitive in an in vitro assay of electron transport as the wild type cells, but differed in that inhibition could be reversed by the addition of bovine serum albumin to the assay mixture. This experiment was not attempted with MA 6.5, but this might be one line of future investigation.

This observation raises the question of the binding of antimycin by bovine serum albumin and similar proteins, which is relevant for two reasons. Routine growth medium used in these studies was supplemented by 10% foetal calf serum, which contains serum albumin at a concentration of approximately 10 mg/ml. Bovine serum albumin binds antimycin (Reporter 1966) and consequently the actual concentration of free antimycin in the medium is not as high as one would expect from the amount of the drug added, although no studies were made to determine the proportion of free to bound antimycin. This observation explains the much higher apparent concentrations required to kill sensitive cells than those used by workers with fungi. Thus, for example, only one of the doubly resistant classes of Saccharomyces cerevisiae with resistance alleles at two loci was able to grow at a concentration of 1 µg antimycin/ml in the studies of Pratje and Michaelis (1977). The routine apparent concentrations of antimycin for growth of MA 6.5, 5 µM, is about 2.5 µg/ml, and the cells will grow in concentrations three times this level. It is almost certain that the presence of bovine serum albumin in the growth medium of mammalian cells accounts for this discrepancy, as there is no reason to suppose that the mammalian cell is intrinsically more resistant to antimycin than the fungal cell.

The second reason for interest in the specific binding of

antimycin by proteins other than those of the antimycin binding site is that it suggests a possible mechanism for generating resistance to antimycin. Reporter and Ebert (1965) isolated a soluble protein factor, CAAF, from washed chicken mitochondria, which was capable of binding antimycin and reversing its effects on developing embryos. If such proteins exist in mammalian cells, resistance to antimycin A could be generated by overproduction, such that a great proportion of intracellular antimycin was bound and rendered unavailable to the antimycin binding site of the electron transport chain. (It has already been stated that the inhibition of electron transport depends on the ratio of antimycin to mitochondrial protein rather than on the absolute concentration of antimycin). If this were the case, one could explain why the enzyme is as sensitive in an <u>in vitro</u> assay, and yet still retains high levels of activity after growth of the cells in antimycin at concentrations sufficient to reduce the activity of the extract of sensitive cells to 5% of control values. This suggestion becomes of more interest by the demonstration in the next chapter that at least one mitochondrially synthesised protein is produced at higher levels by the mutant than by the wild type.

The data presented in this chapter are also consistent with the possibility that resistance in MA 6.5 is due to a permeability barrier. If this is the case, however, the barrier is relatively specific, in that the sensitivity to at least two other drugs known to act on mitochondrial components is identical in the mutant and the wild type cells. It is unfortunate that radioactively labelled antimycin is not yet commercially available, as it would be relatively easy to test the permeability barrier hypothesis if it were. Unfortunately, other methods of detecting antimycin, such as fluorescence, are not sensitive

enough for experiments designed to test the accessibility of mitochondria to antimycin.

Two final points require comment. First, although the absolute rates of oxygen consumption by D98 and MA 6.5 are comparable with published values for other cell lines (e.g. Soderberg et al., 1977; Ditta et al., 1976) the absolute rate of release of $^{14}CO_2$ from 2-^{14}C pyruvate is apparently much lower (Ditta, op. cit.). The reason for this discrepancy is unclear, but it may be due partly to placing the cells into the assay mixture directly from medium containing high glucose levels. If the cells already have high concentrations of intracellular substrate, the amount of label released will not accurately reflect the amount of CO_2 produced. Second, the effect of adding antimycin to whole cells in an oxygen electrode is not immediate, although the extracts used in the assay of electron transport responded immediately. There are two possible reasons for this; first, it may be another effect of the presence of antimycin binding proteins, either in the medium or intracellularly, and second, there may be a partial permeability barrier to the entrance of antimycin such that it only attains its final intracellular concentration after a period of equilibration.

CHAPTER 5

PROTEINS SYNTHESISED BY THE MITOCHONDRIA OF THE ANTIMYCIN RESISTANT CELL LINE MA 6.5

INTRODUCTION

The aim of the work discussed in this chapter was to examine the proteins synthesised by the mitochondria of the antimycin resistant cell line MA 6.5 and to compare these with the proteins synthesised by the antimycin sensitive line D98 from which MA 6.5 was derived. The proteins synthesised by the mitochondria of mammalian cells have been studied most frequently by their selective labelling with radioactive amino acids in the presence of inhibitors of cytoplasmic protein synthesis, followed by separation of the labelled material on s.d.s. gels (see Chapters 1 and 6 for a more extensive discussion). Typically, such procedures resolve a small number of polypeptides (7-10) of low molecular weight. (Lederman and Attardi, 1973; Jeffries and Craig, 1976; Yatscoff and Freeman, 1977). The profiles obtained on electrophoresis are consistent for any one cell line and species specific differences in the profiles have been reproducibly observed and used as a marker in interspecific crosses (Jeffries and Craig, 1974; Jeffries, 1975). These differences are discussed in greater detail in Chapter 6. Within any one species, the patterns of labelled proteins obtained on s.d.s. gels appear to be identical irrespective of the origin of the cell line studied.

The function of most of the proteins synthesised by mitochondria in mammalian cells is unknown. This is in marked contrast to the situation in fungi, where specific mitochondrially synthesised products are known to be associated with cytochrome c oxidase, cytochrome b, and the oligomycin sensitive ATPase (Poyton and Schatz, 1975; Schatz and Mason, 1974: Weiss and Ziganke, 1974; Griffiths and Houghton, 1974; Poyton and Groot, 1975; Tzagoloff et al., 1975). In general, most of the proteins synthesised by mammalian mitochondria are associated with the inner mitochondrial membrane (Work, 1968) and are apparently hydrophobic (Coote and Work, 1971). A biochemical approach was used by Jeffries and Craig (1977) to demonstrate that one subunit of purified human cytochrome oxidase comigrated on s.d.s. gels with a known mitochondrially synthesised polypeptide, and Yatscoff et al. (1977) provided immunological evidence that two of the subunits of mammalian cytochrome oxidase are mitochondrially synthesised.

There are several difficulties with this approach. First, it involves guessing at probable candidates for mitochondrial synthesis. Second, the biochemistry of a number of possible candidates is complicated by their hydrophobicity, by the fact that they are often part of enzyme complexes and do not exhibit enzyme activity when removed from these complexes, and by the fact that some of them may have structural rather than enzymic roles in the inner membrane. It is relatively very much more difficult to purify a protein present initially only in very small amounts when there is no reliable assay for it.

An alternative to this biochemical approach to the assignment of functions to mitochondrially synthesised proteins would be

possible if one could demonstrate a correlation between a given variant mitochondrial phenotype, such as a drug resistance, and a variant mitochondrial protein. A necessary prerequisite for this is the existence of cell lines with mutations in mitochondrial DNA affecting known mitochondrial functions. Drug resistant mutants may prove to be of particular utility in such studies, as the sites of action of many drugs are extremely well characterised. Human cell lines resistant to the inhibitor of mitochondrial protein synthesis, chloramphenicol, have been isolated (Spolsky and Eisenstadt, 1972; Mitchell et al., 1975; Siegel et al., 1977), as have mouse cell lines resistant to the F_1 ATPase inhibitor, oligomycin, (Lichtor and Getz, 1978). Unfortunately, examinations of the proteins synthesised by the mitochondria of these cells by conventional s.d.s. gel electrophoresis have not demonstrated any variant protein associated with these mutations.

The isolation of a human cell line resistant to antimycin A, reported in Chapter 3, was initially undertaken in an attempt to produce mammalian cells with a mutation affecting electron transport in the b-c_1 region of the chain. If in vitro resistance of this segment of the chain had been demonstrated, this would strongly implicate one of the protein components of this region of the chain in the mechanism of resistance, and had an examination of the mitochondrially synthesised proteins revealed an altered polypeptide, there would have been strong grounds for identifying that polypeptide as a component of the b-c_1 complex.

Disappointingly, in vitro resistance of electron transport to antimycin was not demonstrated. As reported in Chapter 4, the membrane fraction used in the assay was as sensitive to antimycin in the mutant as in the parental cell line, and one must thus

postulate a different mechanism of resistance. It is still important, however, to examine the proteins synthesised by the mitochondria of these cells, to see if any difference from the parental line might be detected.

S.d.s. electrophoresis separates proteins on the basis of their molecular weights, and it is thus unlikely that it would detect protein differences based on alterations to one or a few amino acids. Isoelectric focusing gels, in contrast, separate proteins on the basis of their isoelectric point, and can thus separate proteins of the same molecular weight. It is thus of potential use for the identification of charge alterations resulting from single amino acid alterations.

This chapter reports the results of examinations of the proteins synthesised by the mitochondria of the antimycin resistant cell line MA 6.5 and its sensitive parent D98. Selectively labelled proteins were examined both by s.d.s. electrophoresis and by the isoelectric focusing technique of O'Farrell (1975) adapted for a slab gel apparatus.

METHODS

1. Cell labelling media

Cells were labelled with ^{35}S L-methionine or with ^{14}C L-leucine (Radiochemical Centre, Amersham). Incorporation medium for ^{35}S-methionine was based on RPMI 1640 (Flow Laboratories) lacking L-methionine and supplemented with L-glutamine to 300 mg/l. Medium for incorporations using ^{14}C-leucine was based on minimum essential medium and was prepared from stock solutions of Earle's salts and vitamins, supplemented with essential amino acids minus leucine. Both media were further supplemented with

5% foetal calf serum plus 5% dialysed foetal calf serum, (Jeffries, 1975).

2. Preparative incorporation into cultured cells

All operations were performed sterilely. Cells were removed from culture flasks by trypsinisation, added to sterile centrifuge tubes and pelleted by centrifugation at 500 g for 5 minutes. They were washed once by suspension in PBS, pelleted again and resuspended in incorporation medium containing 100 µg/ml EM and 100 µg/ml ER (Erythromycin). Cells were typically suspended to between 1×10^6 and 3×10^6/ml in up to 8 ml of incorporation medium. Centrifuge tubes were placed in a slow rotating agitator at 37° for 40 minutes before the addition of labelled amino acid. ^{35}S L-methionine was added sterilely to 10 µCi/ml and incorporation was allowed to continue at 37° for between 3 and 5 hours. Labelled cells were cold chased by adding 1.0 ml of 10 mM L-methionine in PBS for 20 minutes after incorporation. The labelled cells were spun down, washed once with PBS, suspended in a small volume of PBS, aliquoted into 0.3 ml Beckman freezing tubes, and stored as small pellets at -70°.

Similar procedures were used for the incorporation of ^{14}C L-leucine, except that 1 µCi/ml of leucine was added, and the cold-chase PBS contained 10 mM L-leucine.

3. S.d.s. electrophoresis

Electrophoresis was performed using the tris-glycine-s.d.s. system of Laemlli (1970) as described in Chapter 2. Generally, either 11% homogeneous or 10-15% linear gradient gels were employed.

Labelled cell pellets were solubilised by heating at 100°C for 5 minutes in a boiling water bath in s.d.s. sample buffer.

Any residual turbidity (usually there was none) was precipitated by spinning in a Beckman microfuge. Up to 100 µl of the solubilised cells could be loaded per sample slot. Typically, between 20,000 and 30,000 d.p.m. were used in each sample, and never less than 10,000 d.p.m. The samples were gently overlain with electrode buffer, and electrophoresis was begun. The apparatus was run at room temperature but cooled by tap water. Electrophoresis was continued until the bromophenol blue marker dye had migrated to the end of the gel, which usually took about 5 hours. Subsequent treatment of the gel was as described in Chapter 2.

The mixture of marker proteins used for molecular weight determinations contained in 50 µl sample buffer 2 µg fraction 5 bovine serum albumin (Sigma) 5 µg beef liver glutamate dehydrogenase (Boehringer Mannheim), 5 µg rabbit lactate dehydrogenase (Sigma) and 10 µg horse heart cytochrome $_c$ type III (Sigma). Suburit molecular weights were taken as 68,000, 53,000, 35,000 and 12,400, respectively (Darnell and Klotz, 1972).

4. Isoelectric focusing gels

Isoelectric focusing in gels was effected employing the modification of O'Farrell's system (O'Farrell, 1975) described in Chapter 2. Equilibrium gels were used exclusively as the non-equilibrium pH gradient electrophoresis system described by O'Farrell *et al*. (1977) did not give a high resolution of mitochondrially synthesised proteins. Labelled cell pellets were suspended in lysis buffer. Three cycles of freezing and thawing appeared to dissolve cells very effectively, and usually no turbidity was observed. As with s.d.s. gels, up to 100 µl of sample could be loaded, and this usually contained 20,000-30,000 d.p.m. Samples were overlain with appropriate buffer as described

in Chapter 2, and focusing was carried out at room temperature, the apparatus being cooled by tap water. After 12 hours of focusing at 400 volts, the voltage was increased to 800 volts for 1 hour.

On termination of focusing, the gels were prepared for autoradiography as described in Chapter 2.

5. Determination of radioactivity in gel slices

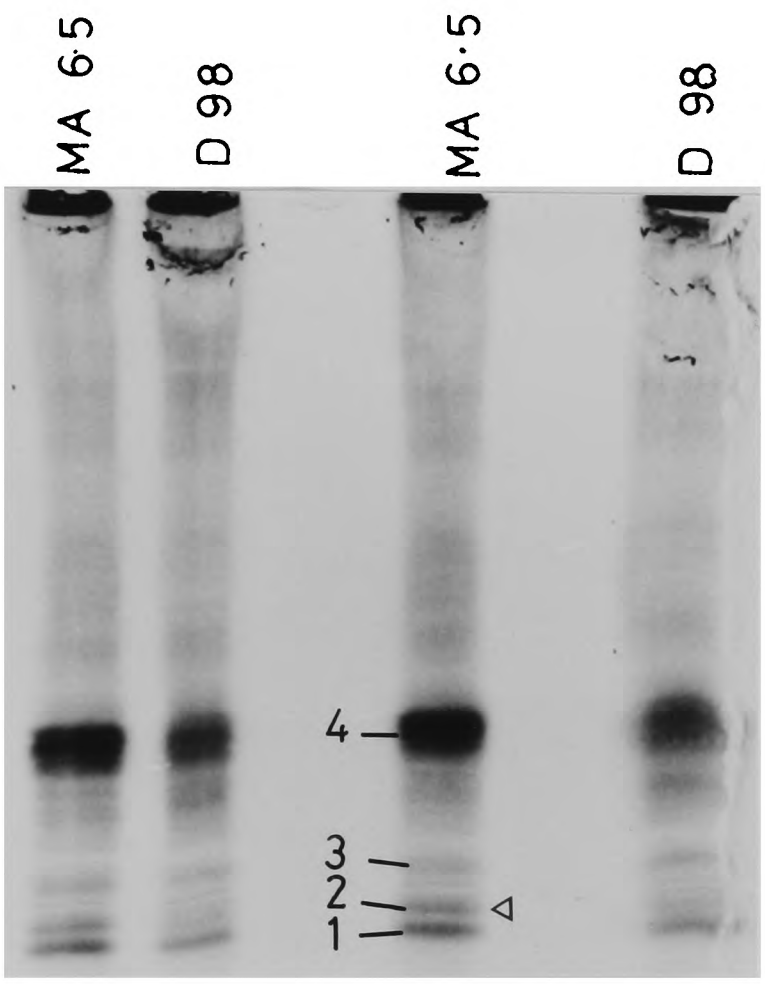
When necessary, the gel was cut into slices and samples of each slice were dissolved by treatment with full strength hydrogen peroxide at 55°C for 6 hours (Young and Fulhorst, 1965). Solubilised proteins were precipitated onto Whatman GF/C filters together with any undissolved gel residue. 100 µl of 10 mg/ml bovine serum albumin were added to act as carrier prior to the precipitation with 10% TCA. Counting was carried out as described in Chapter 2. Hydrogen peroxide was not found to be very effective in dissolving gel slices, and most filters carried small fragments of undissolved polyacrylamide.

RESULTS

A. Profiles of mitochondrially synthesised proteins on isoelectric focusing gels

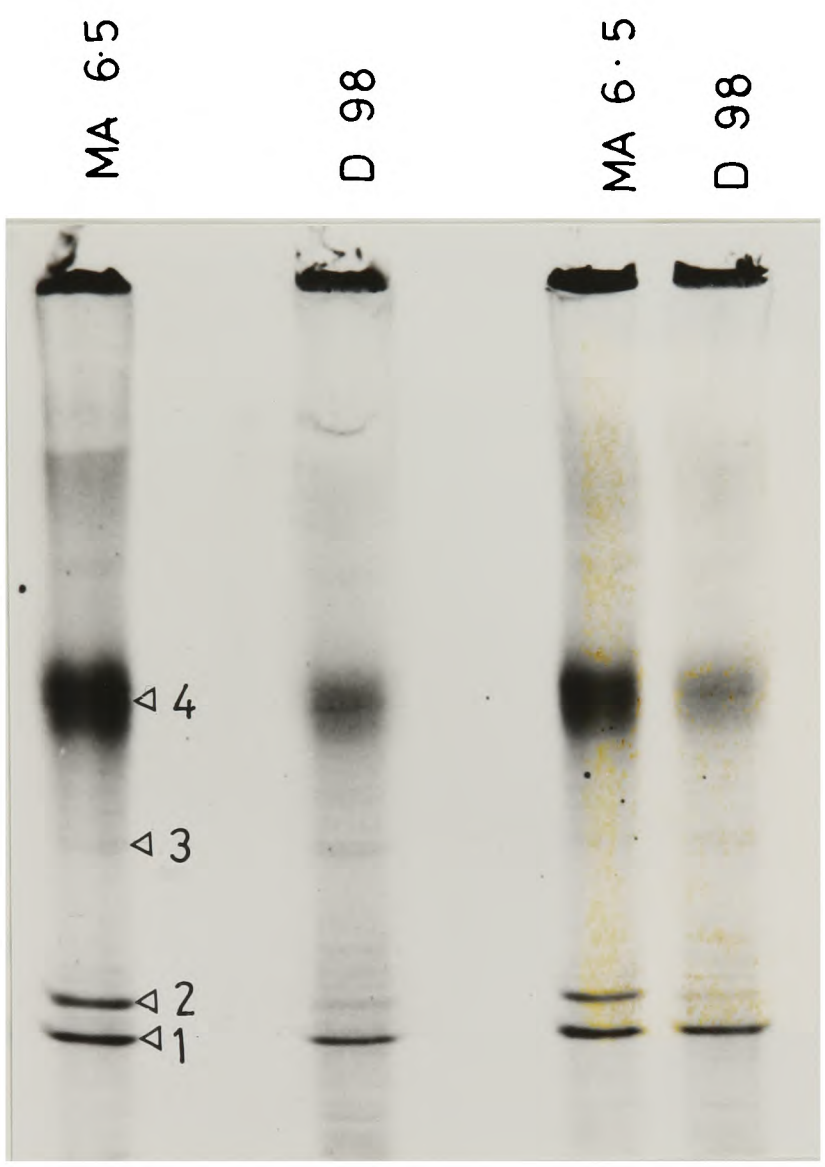
Figures 5.1 and 5.2 show the appearance of autoradiographs of isoelectric focusing gels of D98 (parental) and MA 6.5 (antimycin resistant) mitochondrially synthesised proteins on gels containing ampholines in the range 3.5-10 and 5-7 respectively. On the broad (3.5-10) pH range gels, most of the labelled material is in bands in the lower (acidic) end of the gel. It appears that there are four major labelled polypeptides, three of which are sharply focused, the fourth component, the uppermost of the four,

Figure 5.1



The profile of mitochondrially synthesised proteins on an isoelectric focusing gel containing ampholines in the range pH 3.5-10.

Figure 5.2



The profiles of mitochondrially synthesised proteins on an isoelectric focusing gel containing ampholines in the range pH 5-7.

being heavily labelled but relatively diffuse. There are a large number of faint bands which do not appear on all gels, and which probably represent the products of residual cytoplasmic protein synthesis. The major bands are labelled 1-4 in Figure 5.1 for ease of reference. The most striking difference between the parental and the resistant cell lines is the apparent absence in the former of band 2 (arrowed in Figure 5.1).

A disadvantage of isoelectric focusing to equilibrium in the presence of high concentrations of urea is the tendency of basic range ampholines to break down under these conditions (O'Farrell et al., 1977). It was to overcome this problem that O'Farrell et al. used the technique of non-equilibrium pH gradient electrophoresis. In this particular case, the apparent difference between the parental and the resistant cells were in the acidic region of the gel, below pH 6.0, and so equilibrium iselectric focusing using the restricted pH range 5-7 ampholines was used to give increased resolution in this region of the gel.

Figure 5.2 is an autoradiograph of an isoelectric focusing gel containing pH 5-7 ampholines as the main component. The same bands that can be seen on the broader range gel are present, but the separation is improved. Once again, band 4 is very diffuse, band 3 is a fairly faint band in the middle region of the gel, and bands 1 and 2 are tightly focused at the lower end. In this gel, it seems clear that the second band, which is heavily labelled in MA 6.5 is not absent in D98, but is very much fainter. There appears to be no difference in the position of any of the bands.

B. Chloramphenicol sensitivity of the bands appearing on isoelectric focusing gels

It is known that mitochondria synthesise only a limited number, usually estimated to be between 8 and 10, of their own

polypeptides (Jeffries and Craig, 1976; Yatscoff and Freeman, 1977). One potential source of artifact when dealing with the selective labelling of mammalian cells *in vitro* is contamination with *Mycoplasma*. Mycoplasmal protein synthesis is reported to be sensitive to inhibition by erythromycin (Tourtellotte *et al.*, 1967) which was routinely used in all incorporations. Another possible source of artifact is that resulting from residual cytoplasmic protein synthesis not fully inhibited by emetine. As the isoelectric focusing system described has not been used previously for the study of mitochondrially synthesised proteins, and thus the appearance of the latter has not been well characterised on such gels, it was possible that some of the bands appearing on these gels might be residual cytoplasmic products. If this were the case, such bands should be insensitive to inhibition by the protein synthesis inhibitor chloramphenicol. To test this, cells were harvested, spun down and washed as described in "Methods". Each cell line was then split into two aliquots containing equal numbers of cells, one of which was labelled in the presence of EM and ER, the other being labelled in the presence of EM, ER and CAP, all at a concentration of 100 µg/ml.

These samples were focused on an isoelectric focusing gel containing pH 5-7 ampholines as the main range (Figure 5.3). It can be seen that all the bands present in these gels are eliminated by CAP. It should be stressed that equal quantities of solubilised cells were loaded on to each track of this gel. This suggests that none of the major bands present on these autoradiograms are the results of residual cytoplasmic protein synthesis. The pattern of bands appears to be reproducible from gel to gel, and this gel also indicates the presence of a band in D98 corresponding to band 2 in MA 6.5, but much less intensely labelled.

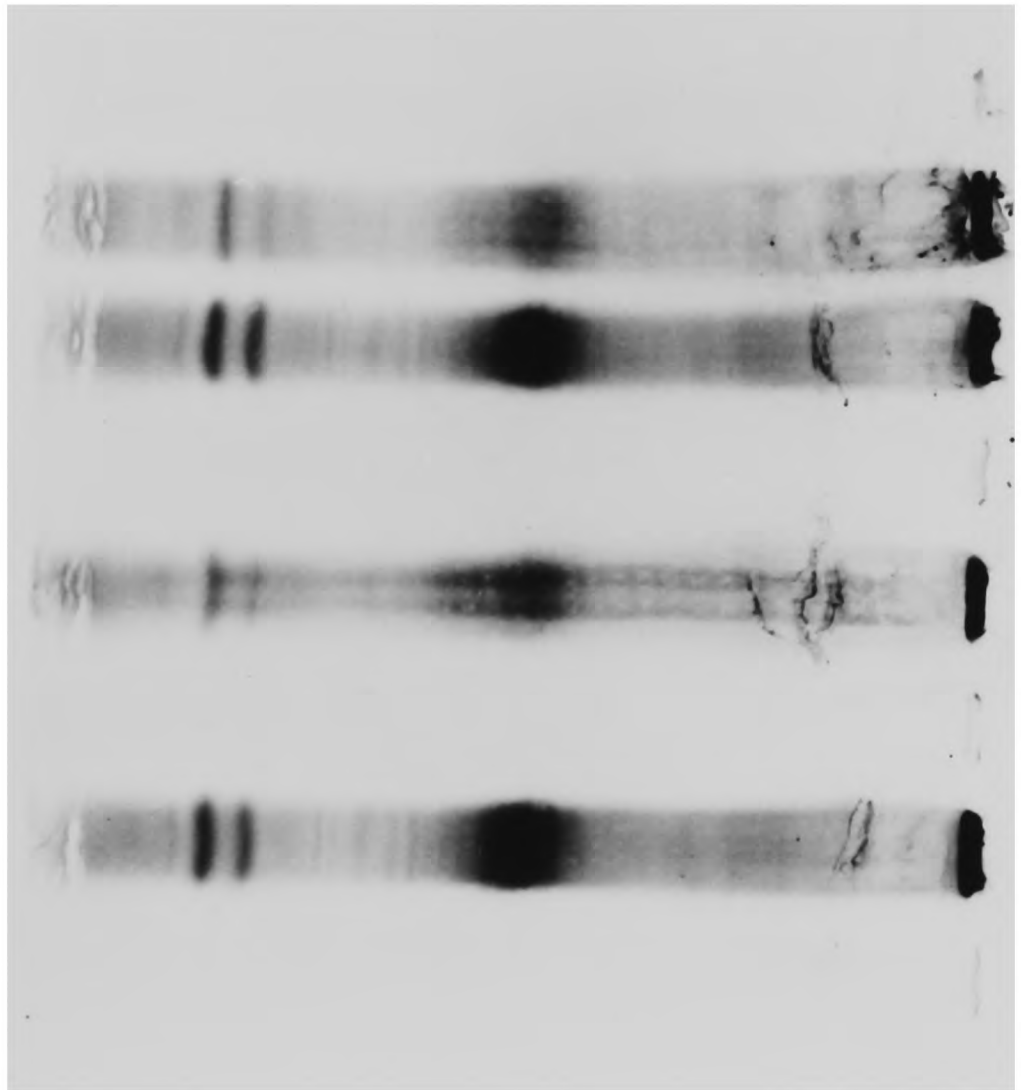

D98
MA 6·5
D98 CAP
D 98
MA 6·5 CAP
MA 6·5

Figure 5.3

The sensitivity to chloramphenicol of bands appearing on isoelectric focusing gels

Cells were labelled in the presence of emetine and in the presence or absence of chloramphenicol (CAP). Equal numbers of solubilised cells were used for each sample.

C. Isoelectric points of bands appearing on autoradiographs of labelled mitochondrial proteins

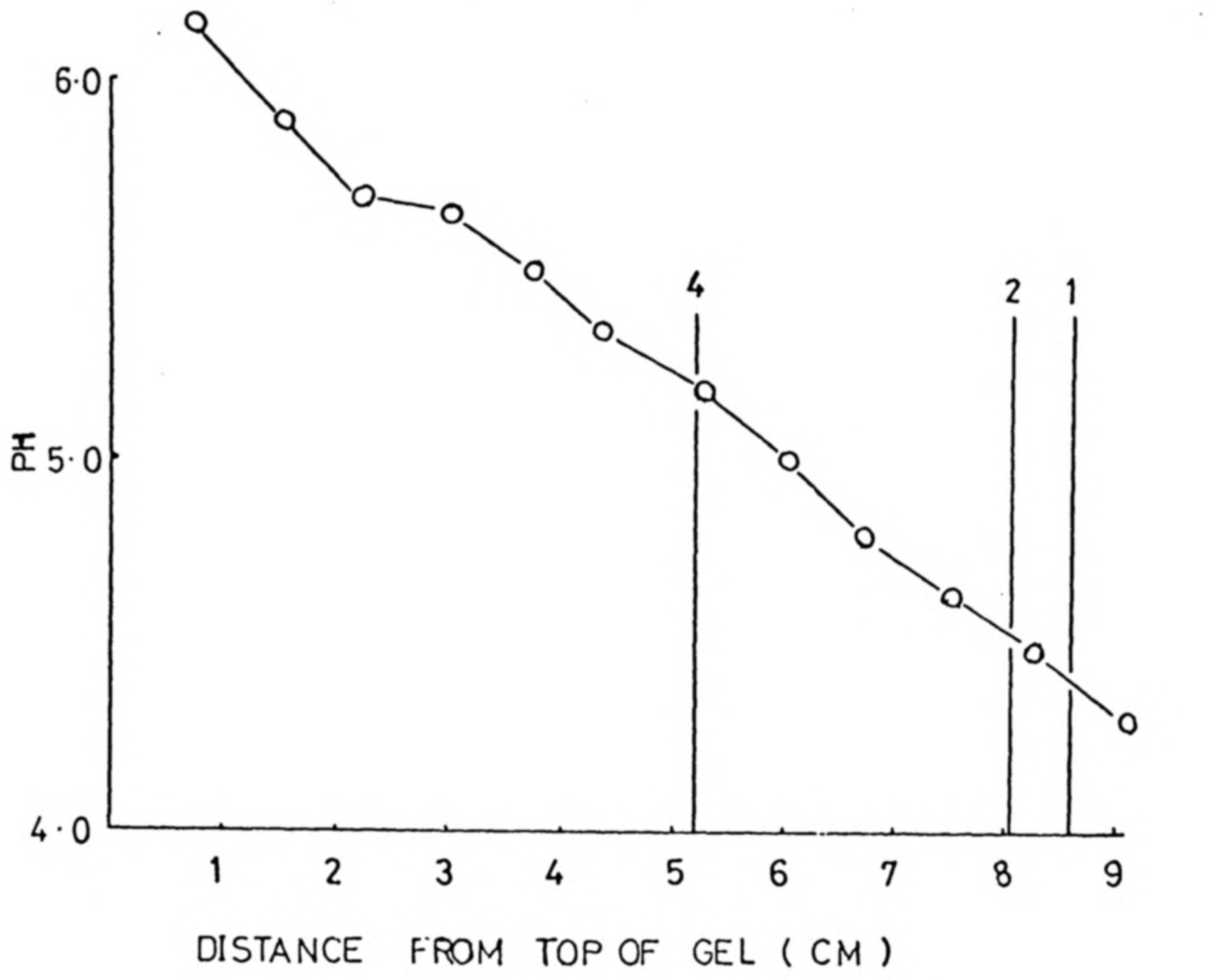
Further discussion of the isoelectric points of mitochondrially synthesised proteins will be made in Chapter 7. In the present context, the most significant band is that showing differential labelling between D98 and MA 6.5, band 2. Its isoelectric point was estimated by measuring the position of the band on autoradiographs and correlating this with a plot of the pH range across the gel, obtained as described in Chapter 2. Figure 5.4 shows such a plot, and indicates the position of the three most intensely labelled bands. The isoelectric point of protein appearing as band 2 is estimated from several gels to be 4.54 ± 0.05 and that of the protein below it at band 1 to be 4.44 ± 0.05. Irrespective of the absolute isoelectric points, the estimates of which vary as shown between different gels, the separation between bands 1 and 2 appears on all gels to be about 0.1 pH units.

D. The quantitative difference in band 2 between D98 and MA 6.5

No change can be detected in the positions of any of the bands shown on autoradiographs of isoelectric focusing gels of mitochondrially synthesised proteins when MA 6.5 and D98 are compared. This indicates that the isoelectric points of these proteins are identical in the mutant and the parental cell lines.

However, there is a major quantitative difference between the two cell lines in the intensity of labelling of band 2. In order to quantitate this difference, microdensitometer tracings were made from autoradiographs using a Joyce-Loebl gel scanner as described in Chapter 2. A typical trace of a pH 5-7 gel is shown in Figure 5.5. The relative intensities of labelling were quantitated from the relative weights of peaks cut from duplicated

Figure 5.4



The isoelectric points of three mitochondrially synthesised proteins in MA 6.5

The pH gradient across a gel containing ampholines in the range pH 5-7 was determined as described in "Methods". The positions of the bands were measured from the autoradiograph of this gel, and plotted on the pH gradient graph.

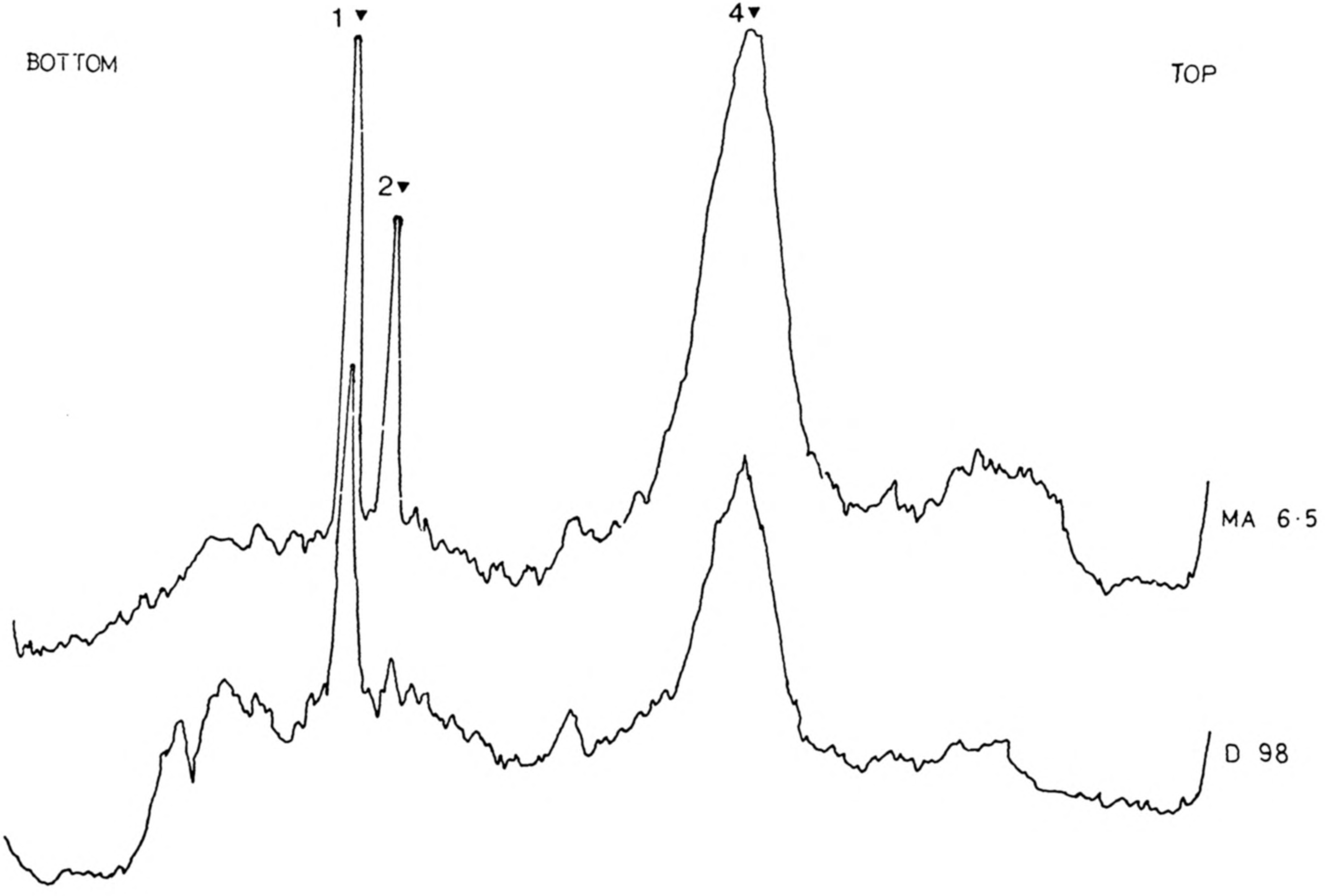

BOTTOM
TOP
1
2
4
MA 6·5
D 98

Figure 5.5 The quantitative difference between the isoelectric focusing profiles of mitochondrially synthesised proteins from MA 6.5 and D98

Microdensitometer tracings were made of the profiles observed on an isoelectric focusing gel containing ampholines in the range pH 5-7. This figure shows clearly the quantitative difference between the profiles of MA 6.5 and D98.

scans. It is not possible to compare directly the intensity of labelling in equivalent bands in two different tracks, because although approximately equal amounts of radioactivity were loaded on for each sample, slight variations in the effectiveness of solubilisation could result in spurious apparent differences. (It should be noted in this context that quite a lot of radioactivity does not enter the gel but remains at the top). However, it is possible to compare the relative intensities of bands on the same track, on the assumption that variations in the effectiveness of a given solubilisation will affect all the bands in an equivalent manner. On these gels, it would seem that the best comparison of the variant band 2 would be with the sharp band immediately below it, band 1. This band appears to be of similar intensity in both cell lines, and is tightly focused, in contrast to the diffuse band 4.

There is a degree of variation in the relative intensities of labelling between different gels. If the intensity of band 2 is expressed as a percentage of the intensity of band 1, then the value for D98 varies from 21% to 31%, and that of MA 6.5 is fairly constant at between 79% and 81%. At the time of writing, 8 gels of independently labelled samples have been examined.

E. Profiles of ^{14}C leucine labelled mitochondrial proteins on isoelectric focusing gels

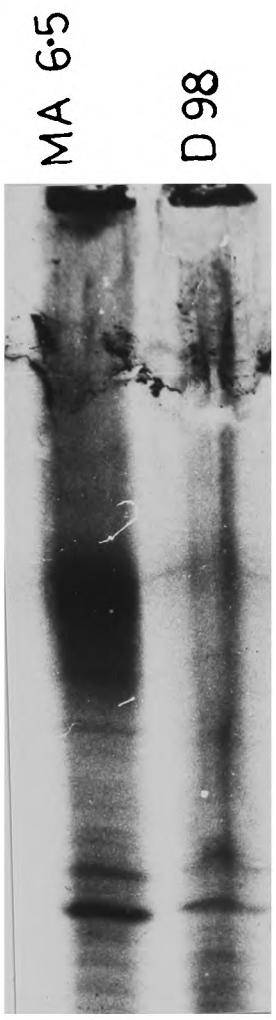
There are two possible explanations for the quantitative difference in the labelling intensity of band 2 between MA 6.5 and D98 described above. It is possible that the variant protein has suffered a mutational alteration which has resulted in an increased incorporation of methionine while leaving the isoelectric point unchanged. This could arise by the replacement of another uncharged amino acid by methionine. Alternatively, the mutant cell

line may be producing more of a protein which does not differ qualitatively from the parental protein. A way of discriminating between these two possibilities is by comparing the profiles obtained on gels of cells selectively labelled with a second amino acid. If the quantitative difference in the labelling of this protein is similar to that found employing ^{35}S methionine, then it is likely that the difference results from an increased synthesis by the resistant cell line of a protein identical with that of the parental cell line. It is extremely unlikely that the first possibility mentioned could embrace two different amino acids.

Cells were labelled with ^{14}C leucine as described in "Methods" and after solubilisation were isoelectrically focused on a pH 5-7 gel. Figure 5.6 shows the autoradiograph resulting from this gel. The same quantitative difference in band 2 observed with ^{35}S methionine labelling is present in the ^{14}C leucine labelled samples. Furthermore, it should be noted that there is no qualitative difference in the patterns obtained using different amino acids, indicating that all the proteins contain both amino acids. Jeffries and Craig (1975) observed that one minor ^{35}S labelled human mitochondrially synthesised protein was absent from a ^{14}C leucine autoradiograph, suggesting that the protein contained little or no leucine. All other polypeptides observed contained both amino acids, a result in agreement with that reported here.

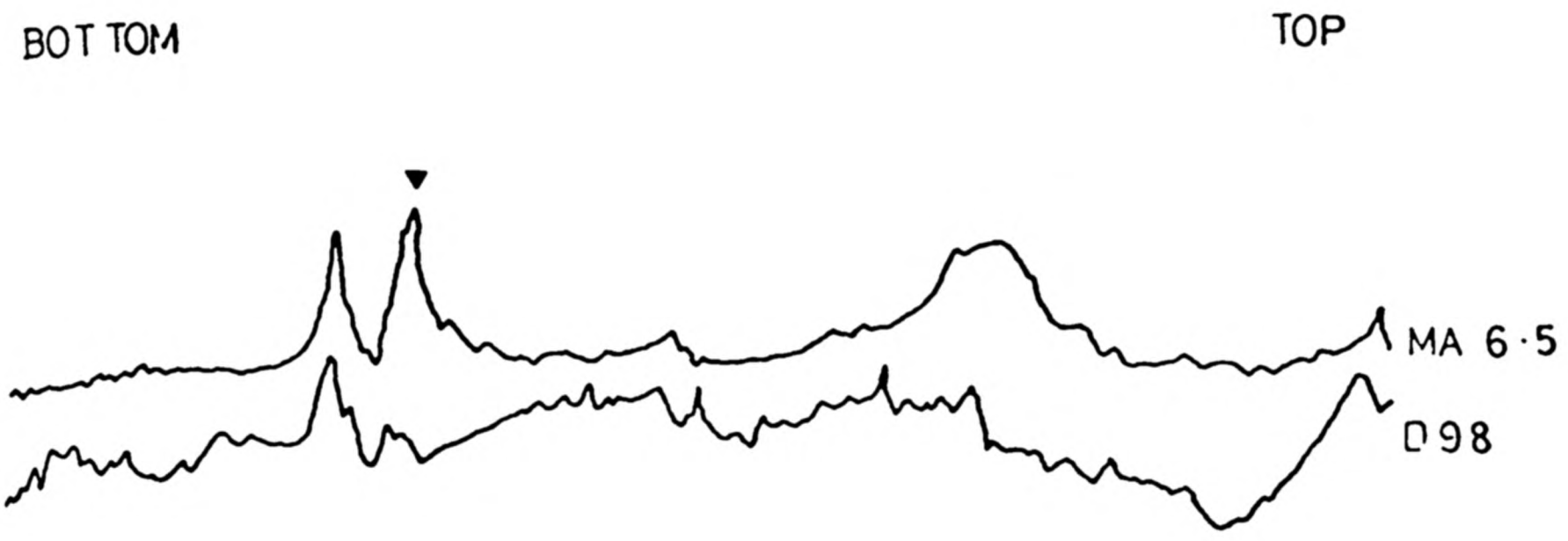
Microdensitometer tracings of the ^{14}C leucine labelled patterns for both cell lines are presented in Figure 4.7. The relative intensity of the variant band 2, estimated as described, was 25% for D98 and 90% for MA 6.5.

Figure 5.6



The profile of proteins selectively labelled with ^{14}C leucine in the presence of emetine on a narrow range isoelectric focusing gel.

Figure 5.7



Microdensitometer tracings of the profiles of mitochondrially synthesised proteins from MA 6.5 and D98 selectively labelled with ^{14}C leucine and separated on an isoelectric focusing gel containing ampholines in the range pH 5-7.

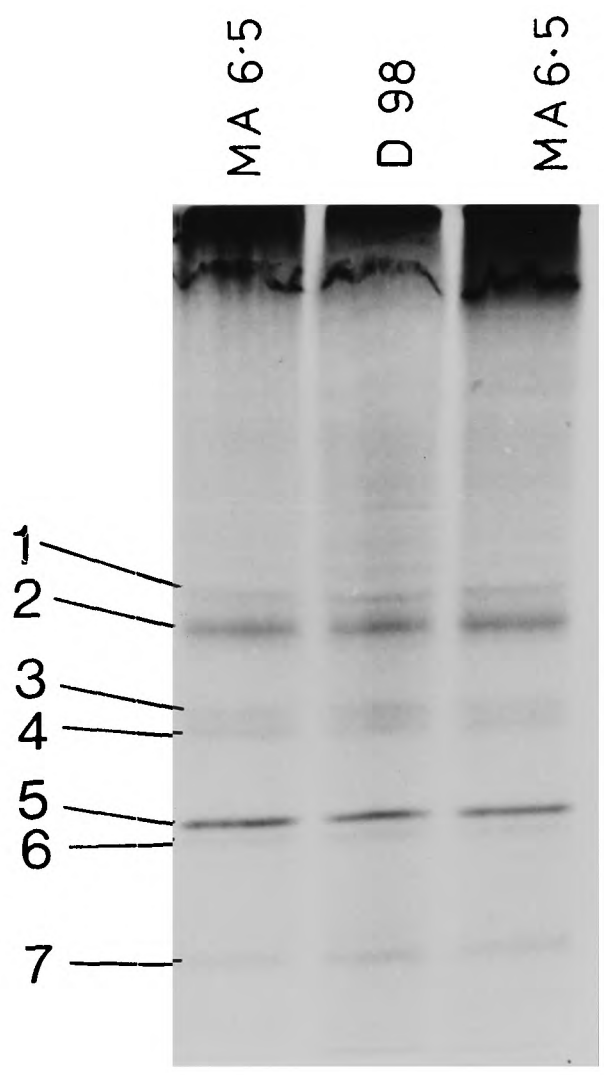
F. Profiles of mitochondrially synthesised proteins on s.d.s. gels

Figure 5.8 is an autoradiograph of a linear gradient s.d.s. gel of mitochondrial proteins from MA 6.5 and D98. Seven prominent bands are clearly resolved, which is a fairly characteristic observation for this type of gel. The molecular weights of these polypeptides were calculated on the basis of the mobility of reference proteins of known molecular weights. These molecular weights are shown in Table 5.1. Comparison of the data with similar determinations by other workers (Jeffries and Craig, 1975; Yatscoff and Freeman, 1977) reveal a broad agreement of the range of molecular weights. S.d.s. gels are known not to be a reliable tool for the determination of absolute molecular weights, and in the present context it does not appear to be important to argue which of these studies has produced the best estimates of the molecular weights for the mitochondrially synthesised proteins. The appearance and molecular weights of the proteins in this study were similar on a number of different gels, and the consistency of these results allows confidence that this system gives reproducibly good separations of mitochondrially synthesised polypeptides.

It is clear that there is no apparent change in the molecular weight of any band in MA 6.5 when compared with D98. It would have been surprising had a molecular weight difference been shown, as, with the exception of mutations causing premature chain termination, one would expect a mutagen such as MNNG to induce specific amino acid alterations.

It is more difficult to detect by eye differences in the relative intensities of these bands on s.d.s. gels than it is to detect differences on isoelectric focusing gels. This is partly

Figure 5.8



The profiles of mitochondrially synthesised proteins from MA 6.5 and D98 on a linear 10-15% gradient s.d.s. gel.

Table 5.1

The apparent molecular weights of mitochondrially synthesised proteins from MA 6.5 determined on s.d.s. gels

Band	Molecular weight (daltons)
1	43,000
2	39,500
3	31,000
4	29,000
5	21,000
6	19,500
7	15,000

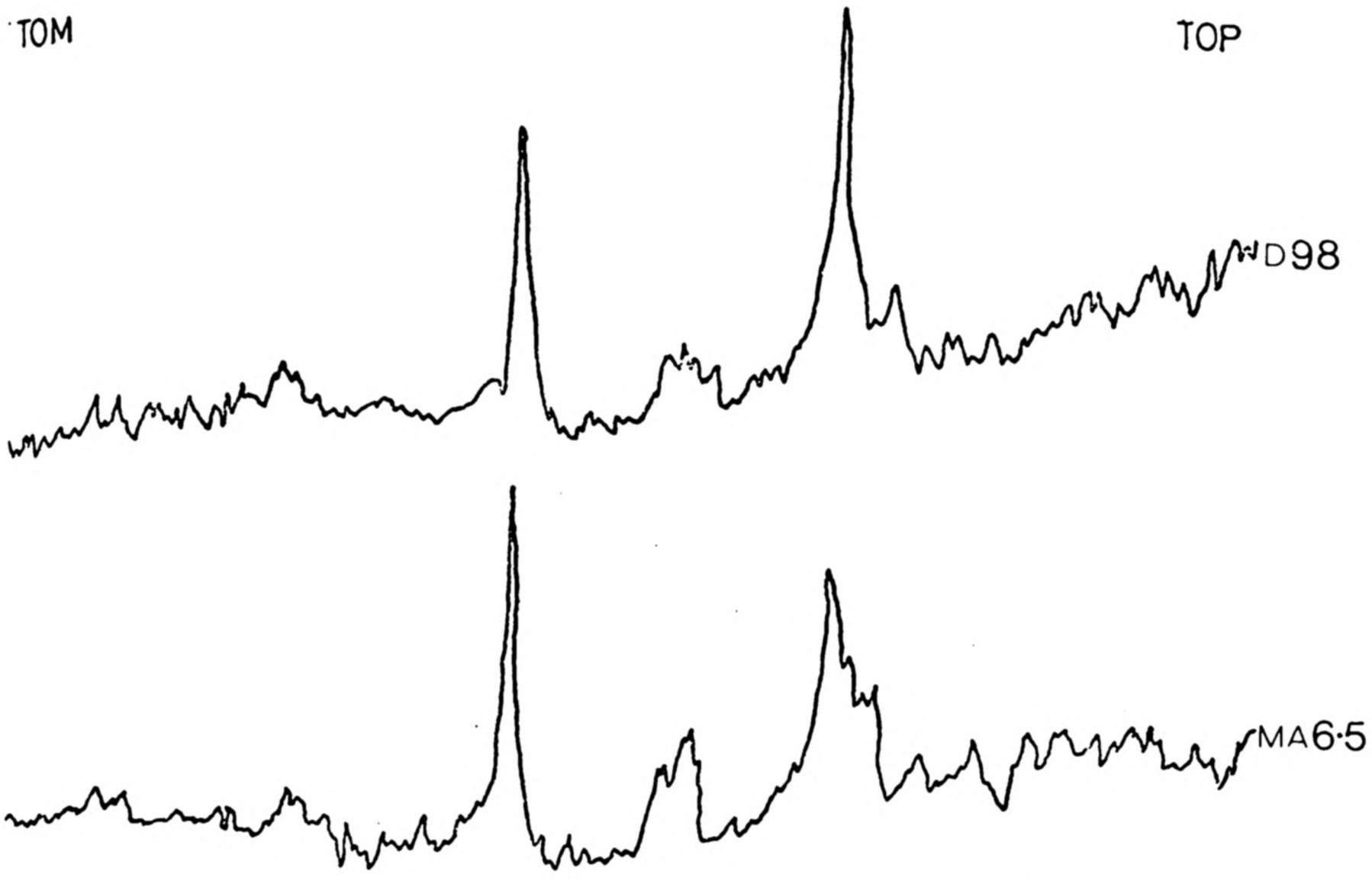


Figure 5.9

Microdensitometer tracings of the profiles of mitochondrially synthesised proteins from MA 6.5 and D98 on a linear 10-15% gradient s.d.s. gel.

because there are more bands, and partly because it is not clear which band should be chosen as a reference band. Gel scans obtained with a Joyce-Loebl gel scanner (Figure 5.9) indicate that there is an apparent difference in the intensity of a band whose molecular weight is 21,000. If this difference is normalised with respect to the band of molecular weight 39,500, then in D98 the intensity of the 21,000 dalton band is 40% of the reference band, the equivalent value for MA 6.5 being 65%. This determination is not as clearcut as that made from isoelectric focusing gels. One reason for this is that it is not clear which of the bands is varying, and it could be argued that the intensity of the band at 39,500 daltons should be expressed as a percentage of the band at 21,000 daltons. Another reason is that this difference is not as reproducibly observed on s.d.s. gels as the difference on isoelectric focusing gels.

Thus, in spite of an apparent difference between MA 6.5 and D98 in the relative intensities of a band on these gels, the data are not good enough to identify unequivocally the band which is observed to differ on isoelectric focusing gels.

G. The molecular weight of the variant band

In view of the difficulty of correlating unambiguously the profile of mitochondrially synthesised proteins from MA 6.5 on s.d.s. gels with that on isoelectric focusing gels, a direct approach to finding the molecular weight of the variant band was used. This consisted of running an isoelectric focusing gel with labelled mitochondrial proteins from MA 6.5 as the sole sample, determining the position of the bands by scintillation counting dissolved strips of the gel, cutting out the relevant bands, and applying them as sample to an s.d.s. gel.

For the first separation, an isoelectric focusing gel con-

taining ampholines in the range pH 5-7 was used. The gel was set up without sample slots, and a total of 400,000 d.p.m. of solubilised labelled MA 6.5 was applied. The gel was run to equilibrium overnight in the usual manner, and was then removed and cut up using a razor blade over the template shown in Figure 5.10. One strip along the side of the gel was immediately fixed for autoradiography, a similar strip on the opposite side was sliced and used to determine the pH gradient, and the remaining central portion of the gel was sliced from the bottom into 25 2mm thick sections. The average separation between the two bands 1 and 2 at the bottom of the gel had been determined from several gels to be about 4mm, and thus sections of 2mm width could not include material from both bands. One segment of 1 cm width was taken from each edge and from the middle of each strip. These three segments were pooled for each strip and used to determine the radioactivity in each region of the gel. The remaining pieces of gel, which comprised two pieces each of 3.5 cm length for each original strip of gel, were frozen at -70°C. All operations were carried out at room temperature as quickly as possible, to allow minimum time for the diffusion of proteins in the gel. The whole operation was completed within 15 minutes of switching off the current.

Radioactivity was determined for each slice as described in "Methods". The results of this determination, together with a pH gradient for the gel and an autoradiograph subsequently obtained from the fixed strip, are shown in Figure 5.11. From these data, the gel strips containing bands 1 and 2 were identified, and the strips for each band were pooled and used to determine the molecular weight.

The gel strips were forced many times through a syringe

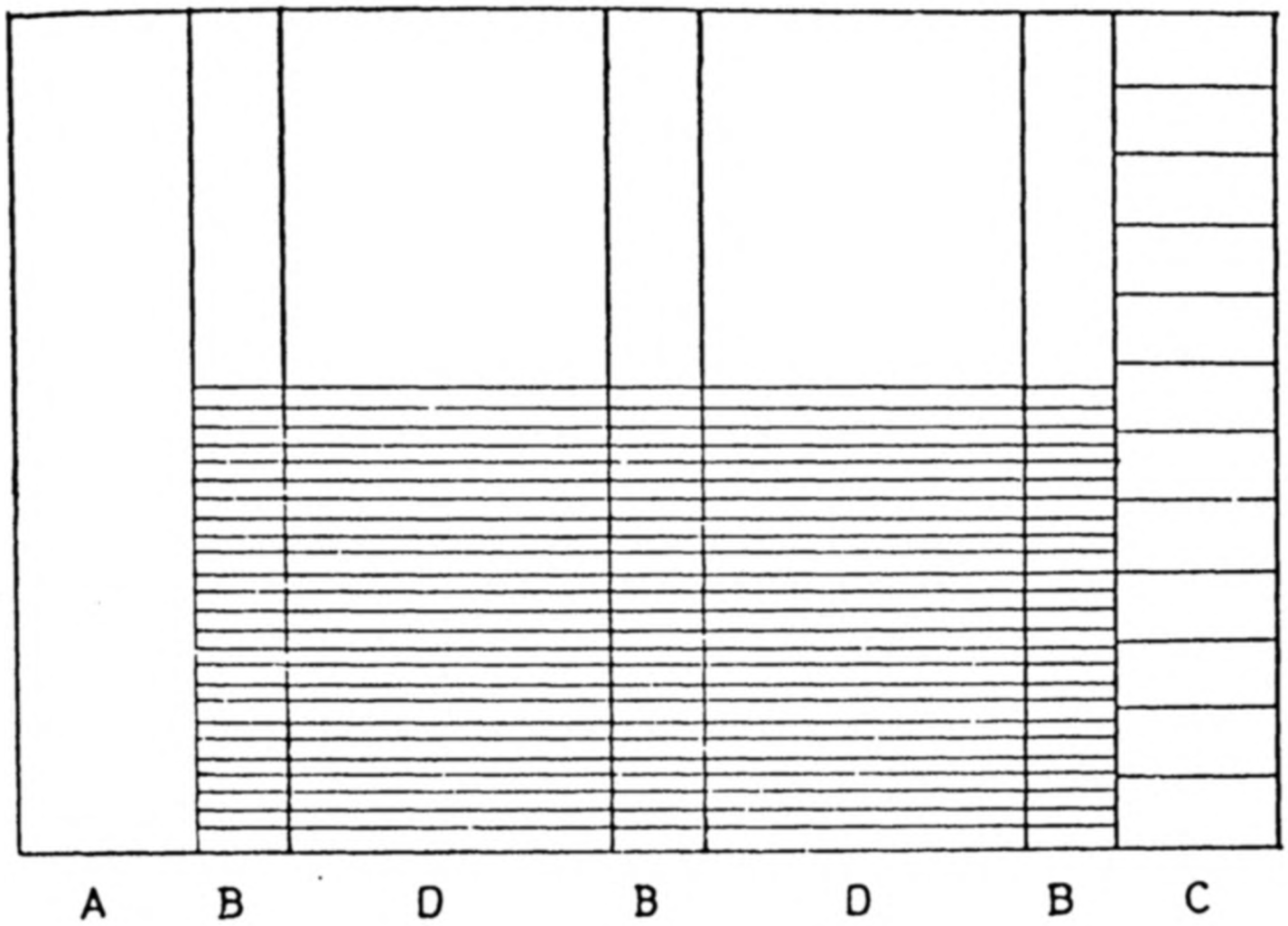


Figure 5.10

Treatment of a preparative isoelectric focusing gel

The figure shows the template used in the slicing of a preparative isoelectric focusing gel. Strip A was fixed and autoradiographed. Strip C was sliced as shown and used to determine the pH gradient across the gel. The pairs of longitudinal strips D were frozen and subsequently used as sample on an s.d.s. gel. The radioactivity in these strips was determined from the three segments B corresponding to each strip. Actual size.

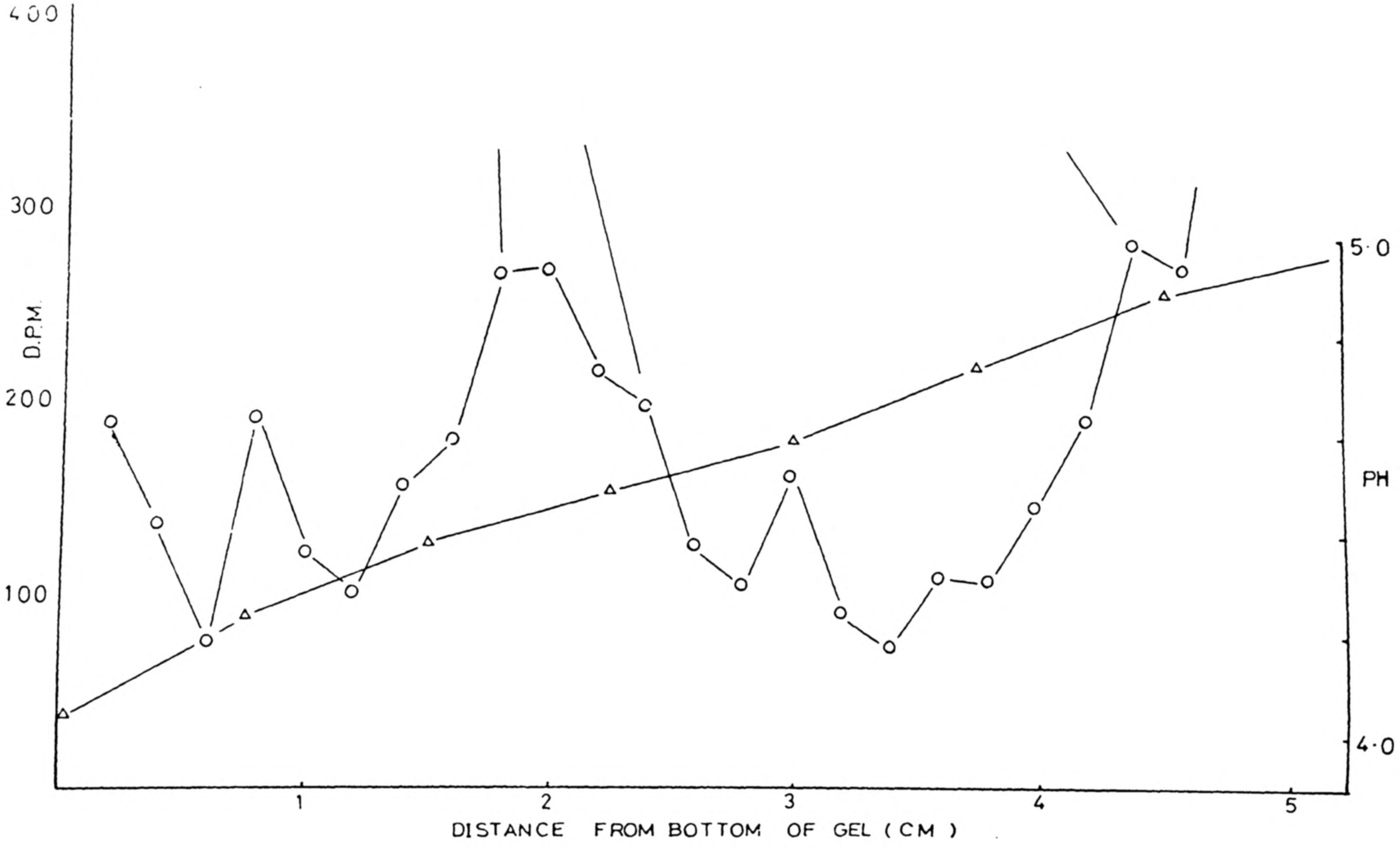
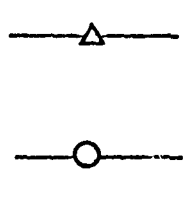

400
300
200
100
D.P.M.
5·0
PH
4·0
1
2
3
4
5
DISTANCE FROM BOTTOM OF GEL (CM)

Figure 5.11 Results of preparative isoelectric focusing gel

The pH gradient and radioactivity profile of the preparative isoelectric focusing gel described in the text are shown. The photograph shows an autoradiograph subsequently obtained from a fixed strip of the gel. The gel strips corresponding to the positions of bands were determined from this diagram and these were subsequently used as samples on an s.d.s. gel.

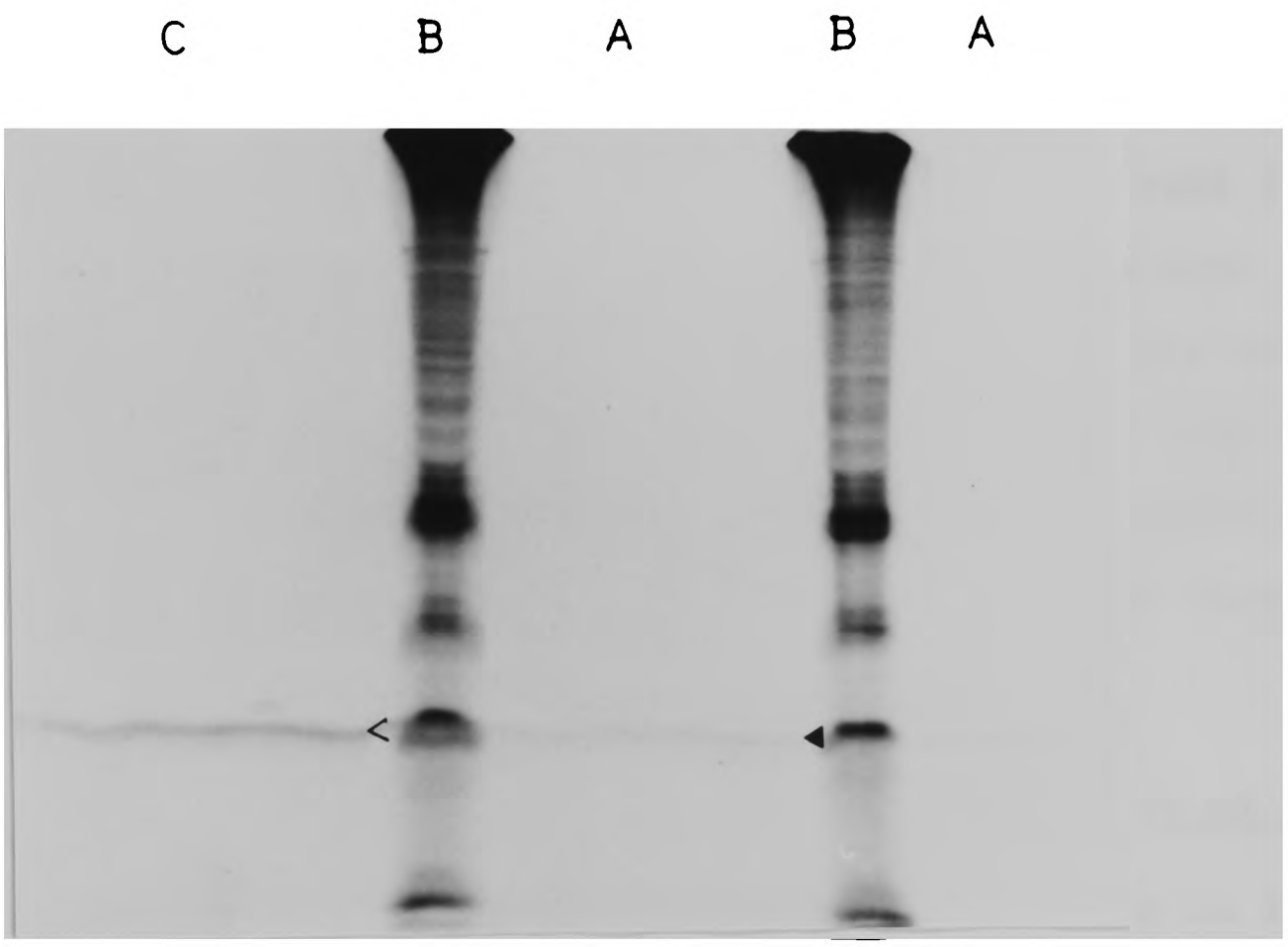


pH gradient

Radioactivity profile

Figure 5.12

The molecular weight of the variant band in MA 6.5



Strips cut from a preparative isoelectric focusing gel corresponding to the positions of the variant (A) and constant (C) bands were equilibrated with s.d.s. sample buffer and run on an 11% s.d.s. polyacrylamide gel. Solubilised selectively labelled MA 6.5 was also applied as sample on two slots (B). The time required to expose the constant band has resulted in a slight overexposure of B.

with approximately five times their own volume of s.d.s. sample buffer. This produced a semi-liquid mass which was heated at 100°C for two minutes in a boiling water bath, and then applied as sample to an s.d.s. gel.

An autoradiograph of this gel is shown in Figure 5.12. It is clear that the variant band co-electrophoreses with a known mitochondrial protein, which is additional evidence that the band is a genuine product of mitochondrial protein synthesis. From molecular weight calibrators run on the same gel, the molecular weight of the variant band was estimated as 22,000.

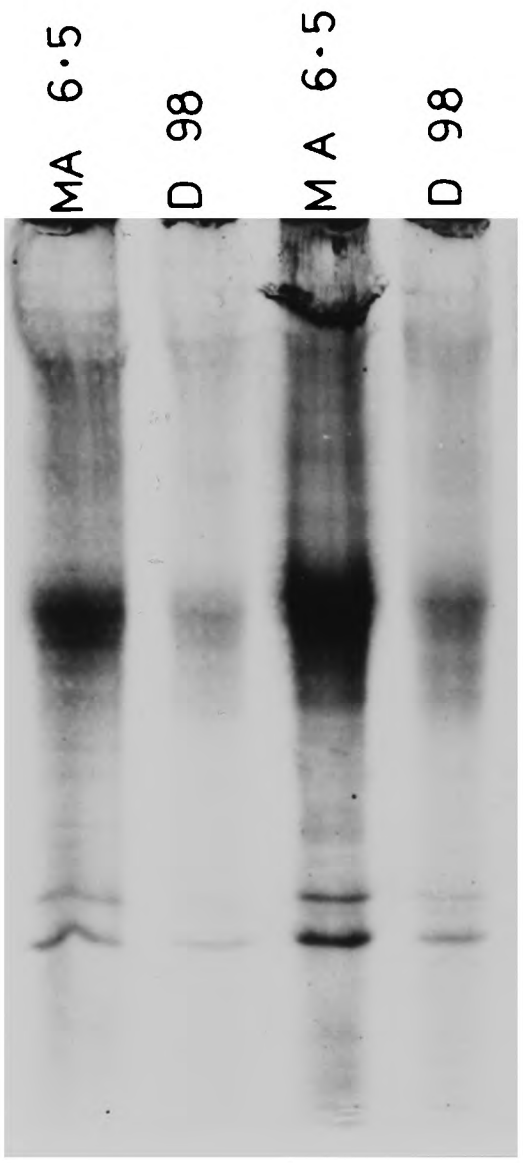
A surprising result of this experiment is that band 1 (isoelectric point 4.44) has a molecular weight identical to that of band 2, and thus conventional s.d.s. electrophoresis would not separate the two polypeptides. This observation may also explain why autoradiographs of s.d.s. gels do not reveal so clearly and consistently the difference in labelling intensity of band 2 shown on isoelectric focusing gels.

H. Stability of the variation in labelling intensity of band 2

A formal possibility to explain the variation in labelling intensity of band 2 between D98 and MA 6.5 is that more of this polypeptide is synthesised as a phenotypic response to the presence of antimycin A, and thus that these gels are revealing a control phenomenon rather than a genuine mutational alteration. To answer this objection, and also to examine the stability of the labelling pattern in MA 6.5 in the absence of selection, MA 6.5 were grown without antimycin for 29 transfer generations before labelling. D98 were treated at high population densities with 1 μM antimycin A for 3 days and, subsequently, with 5 μM antimycin A for four days. These cells were then labelled and applied as sample to an isoelectric focusing gel. Figure 5.13 illustrates that the high

Figure 5.13

The stability of the variant labelling intensity of a mitochondrially synthesised protein in MA 6.5



MA 6.5 were grown for 29 transfer generations in the absence of antimycin prior to labelling. D98 were pretreated with antimycin as described in the text prior to labelling.

labelling intensity of the variant band in MA 6.5 is stable in the absence of selection, and conversely that this cannot be produced in D98 by pretreatment of the cells for one week with antimycin A.

Taken together, these results indicate that the high labelling intensity of band 2 in MA 6.5 is due to a stable alteration, and not to a short term phenotypic adjustment to the presence of antimycin A.

DISCUSSION

An examination of the mitochondrially synthesised proteins of a mutant cell line would yield most information if it could be correlated with a well characterised alteration in some aspect of mitochondrial biochemistry. As shown in Chapter 4, the mechanism by which the cell line MA 6.5 has acquired antimycin resistance is still open to hypothesis. It does not appear to be due to an alteration in the antimycin binding protein leading to *in vitro* resistance of electron transport to antimycin, nor to an alteration in the electron transport chain such that it is functionally impaired.

The following conclusions may be drawn from the observations reported in this chapter:

1. MA 6.5 synthesises between 2.5 and 3.0 times as much of a specific mitochondrial polypeptide as its parental cell line. (See Discussion, Chapter 8).

2. The variant polypeptide is characterised by an iso-electric point of 4.54 ± 0.05 and an apparent molecular weight in s.d.s. gels of 22,000.

3. The higher labelling intensity of the variant band is stable in the absence of selection, and cannot be produced in the parental cell line by treatment with antimycin.

On the assumption that the increase in the level of synthesis of this particular polypeptide is not a coincidental observation of no relevance to the antimycin resistance of these cells, one must form a hypothesis to account for the relation of this polypeptide to the mechanism of resistance. It is unlikely that this protein represents either the antimycin binding site or cytochrome b, as it is probable that if increases in the level of these could produce cellular resistance, the resistance would be retained in vitro.

Two hypotheses seem plausible. The increased level of the variant protein in the inner mitochondrial membrane may decrease the permeability of the membrane to antimycin. The observed cellular resistance would then be a result of the exclusion of antimycin from the mitochondrion.

The second hypothesis is that MA 6.5 is more resistant to antimycin than the parental cell line because it synthesises more of a mitochondrial protein which, though not itself involved in electron transport, binds antimycin A. This binding may be functionally irrelevant, as is that of serum albumin, but acts to sequester antimycin from the antimycin binding site in the electron transport chain. The sensitivity in vitro of electron transport in MA 6.5 could be explained if the procedure used to prepare the membrane fragments on which the assays were performed altered the topographical relations of the electron transport chain and the hypothetical antimycin binding protein such that the latter no longer competed efficiently for the available antimycin.

This hypothesis suggests an easy experimental test, namely the purification of the protein and a direct binding assay using fluorescence quenching. As the protein has been characterised by molecular weight and isoelectric point, it should not be difficult to devise a purification procedure.

The first hypothesis would be easy to test if radioactive antimycin were available. Unfortunately, it was not commercially produced at the time this work was done, although it would probably be possible to label it by tritium exchange. This, together with the other suggestion, above, constitute potentially useful future lines of investigation.

CHAPTER 6

PRELIMINARY GENETIC ANALYSIS OF ANTIMYCIN RESISTANCE IN MA 6.5

INTRODUCTION

One of the reasons cited in Chapter 3 for the isolation of cell lines bearing mitochondrially transmitted mutations is that they would permit studies of the fate of the cytoplasm in genetic crosses. This would allow investigation of the restrictions, if any, imposed by different cellular backgrounds on the transfer and propagation of mitochondria, and also of the possibility of genetic recombination between mitochondrial genomes bearing different markers. Several studies of this type using a single mitochondrial marker in mammalian cells have been discussed in the Introduction to this thesis.

Before a mutant cell line can be used in this way, it must be established whether the variant character is coded by the nuclear or by a cytoplasmic genome.

Recently, techniques have been evolved which allow discrimination between cytoplasmic and nuclear transmission of markers in mammalian cells. These involve the physical separation of nuclei from enucleated cell fragments after treatment of cells with cytochalasin B (Carter, 1967; Croce and Koprowski, 1973; Shay et al. 1973), followed by the controlled, virus or polyethylene glycol induced, fusion of the enucleated fragments (cytoplasts) with recipient cells. If a given trait can be donated to the recipient cell line by cytoplast fusion alone, that trait may be presumed to be conferred by a cytoplasmic gene.

Although the mutagenesis used in the isolation of MA 6.5 was designed to induce preferentially mutations in mitochondrial DNA, and although at least one apparent change has been detected in a mitochondrially synthesised protein, no direct evidence has been presented so far that antimycin resistance is determined by a mitochondrial gene or genes. Accordingly, a genetic analysis was undertaken in an attempt to determine the mode of transmission of antimycin resistance from MA 6.5. This analysis was limited by the time available, and was confined to the question of whether antimycin resistance could be transferred to a closely related cell line by fusion of cytoplasts prepared from MA 6.5.

MATERIALS AND METHODS

1. Enucleation of MA 6.5

MA 6.5 cells were enucleated by a modification of the technique of Wigler and Weinstein (1975). All operations were performed sterilely. Cells were harvested by trypsinisation, washed once by resuspension in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum, and pelleted. The cells were resuspended in M.E.M. suspension medium (Gibco, Biocult) supplemented with 10% (v/v) foetal calf serum and containing 20 µg/ml cytochalasin B (Aldrich Chemical Company Inc., Milwaukee). They were incubated in suspension for 1 hour at 37^{o} at a density of about 4×10^{6}/ml.

After incubation, cells were pelleted by centrifugation at 250g for 5 minutes in an MSE Minor bench centrifuge. The pellet was gently resuspended in 4.5 ml serum free M.E.M. containing 12.5% (w/v) Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and 20 µg/ml cytochalasin B. 1.5 ml of this suspension was gently layered on top of each of three gradients of Ficoll in serum free

M.E.M. in 6.5 ml sterile centrifuge tubes. The composition of the gradients is shown in Figure 6.1. All the stages contained cytochalasin B at 20 µg/ml.

The gradients were spun at 130,000 g for 1.5 hours in a swinging bucket rotor. The centrifuge bowl and the rotor were pre-warmed to 37°, the temperature during the run being about 33°. The centrifuge was allowed to stop without braking. Different fractions could be seen as discrete bands in the gradients when the tubes were removed from the centrifuge, and these were collected sterilely by aspiration into a syringe. Usually equivalent fractions were pooled and diluted with fresh 10% RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum. The pellets obtained on centrifuging these fractions at 2000 g for 5 minutes were resuspended in 5 ml of medium. They were allowed to recover from the enucleation for 2-4 hours before fusion by incubation at 37° under 5% CO_2:95% air.

The percentage of cytoplasts in each fraction was estimated by plating 0.5 ml of the 5 ml suspension in 5 cm plastic dishes containing 5 ml of medium. The total contents were fixed and stained by allowing cells and some cytoplasts to attach overnight, pelleting those fragments remaining in the supernatant, resuspending them in 95% (v/v) methanol and returning them to the plastic dish. Cells and fragments were fixed with the 95% methanol, dried, and stained with Giemsa (Harlech, Liege, Belgium). The proportion of cytoplasts, nucleated fragments and whole cells was then determined.

2. Polyethylene glycol induced fusion

Cells and cytoplasts were fused in suspension using polyethylene glycol (PEG; BDH Ltd.) according to the method of Gefter *et al*. (1977). The cells and cytoplasts were mixed in round

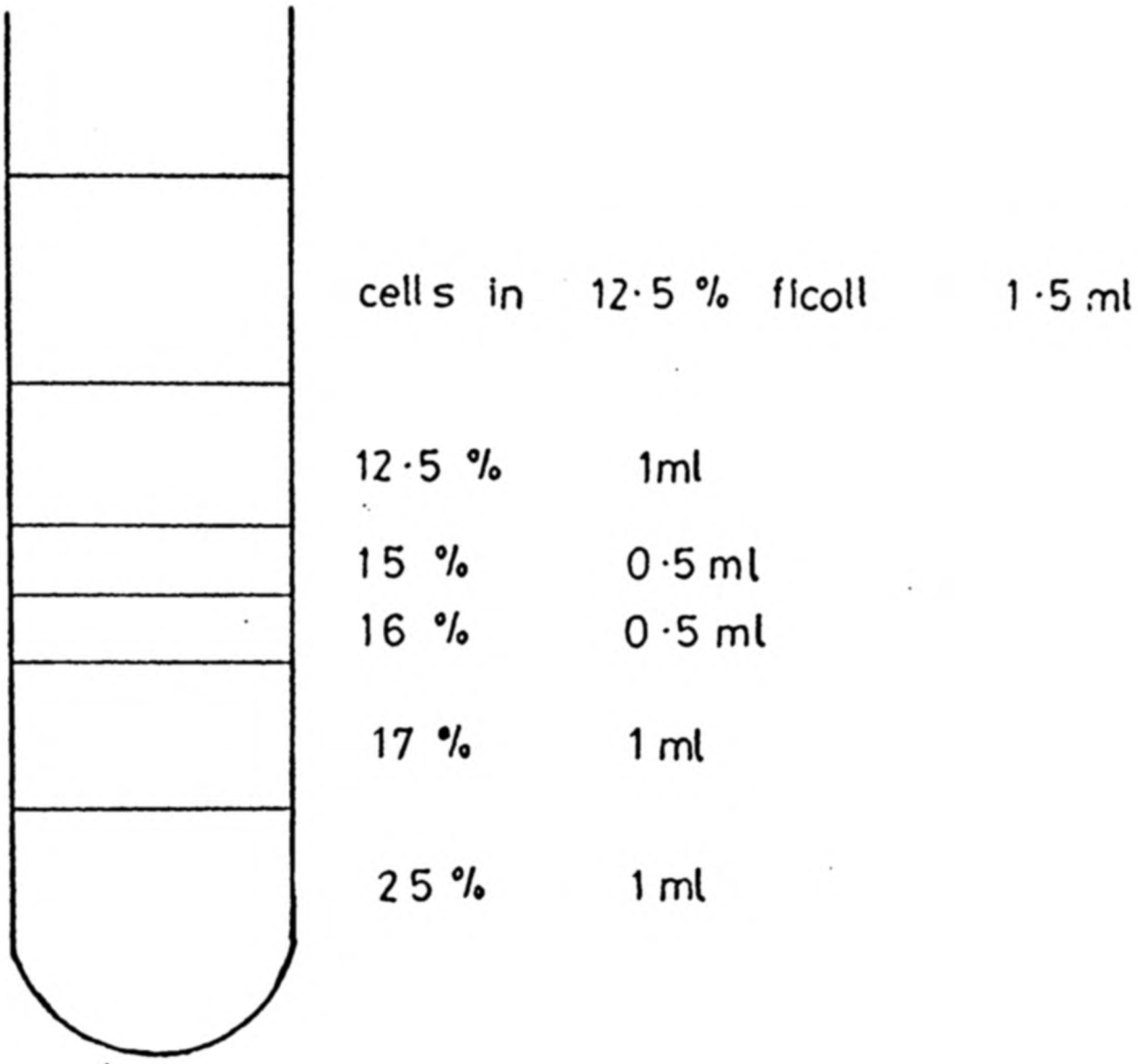


Figure 6.1: Composition of the Ficoll gradients used in cytoplast preparation

The volumes and Ficoll concentrations (% w/v) of the step gradients is shown. The total capacity of the centrifuge tube was 6.5 ml. The gradient was constructed sterilely.

bottomed tubes and pelleted by spinning at 1,000 g for 5 minutes. The supernatant was carefully removed, and 0.2 ml of 35% (w/v) PEG in serum free M.E.M. was added at time 0. The pellet was gently agitated to resuspend the cells in PEG, and then they were repelleted at 500 g for 3 minutes. After 8 minutes, 5 ml of serum free medium was added to the pelleted cells in PEG. They were gently pipetted to resuspend them, and then were spun down at 250 g for 5 minutes.

The supernatant was removed, and the cells were resuspended in fresh medium containing serum at a density of 5-10 x 10^5/ml. They were then aliquoted into medium sized Falcon flasks containing 15 ml of medium, at an average density of 4 x 10^4 per Falcon. They were incubated at 37°C under 5% CO_2:95% air for 48 hours, when the medium was changed to selective medium.

Colonies were fixed, stained and counted, after an appropriate period. Some individual colonies from each successful fusion were cloned as described in Chapter 3.

3. Cell lines

All fusions described in this Chapter involved only MA 6.5 and BU 25. BU 25 is described briefly in Table 2.1.

RESULTS AND DISCUSSION

A. Controls

BU 25 was chosen as a recipient in these experiments because it is closely related to MA 6.5, both cell lines being derived from Hela, and because of its selectable nuclear marker, resistance to BUdR (30 µg/ml). The sensitivity of BU 25 to antimycin was checked by plating out about 10^6 cells in each of 20 medium sized Falcon flasks, and changing the medium after 24 hours

to medium containing antimycin at either 10 μM or 15 μM. Both concentrations of the drug killed all BU 25 cells in such controls, although this took longer with 10 μM than with 15 μM antimycin.

It has been repeatedly observed in the course of this work that antimycin takes quite a long period to kill sensitive cells. At low concentrations (5 μM) these may undergo several divisions before being killed. At higher concentrations, the effect is more rapid, though not immediate. At a concentration of 10 μM antimycin A, up to 10 days elapsed before all control cells had died and been removed in routine changing of the medium. At a concentration of 15 μM, most cells died and became detached within 5 days of applying selection, but once again there was a low but variable background of cells which did not finally die and detach until 7-9 days after the initiation of selection.

No colonies of BU 25 survived in any of the control experiments at either concentration of antimycin. Routine changing of the medium was continued for about 15 days after microscopic examination of the control flasks suggested that all the cells were dead. This was done to allow growth of any spontaneously resistant cells. None were detected in 2×10^7 cells at either concentration. This establishes that antimycin is a good selective agent for discriminating between parental sensitive and resistant cells.

Reciprocal experiments to establish the sensitivity of MA 6.5 to BUdR at 30 μg/ml were carried out in the same manner. No resistant colonies were detected in a total of 10^7 cells screened.

It has been reported previously (Shin et al., 1973) that cells subjected to virus fusion techniques may give rise to drug resistant variants at increased frequency. Although PEG induced fusion was used exclusively in the experiments reported here, the

possibility that the fusion procedure itself might generate high frequencies of resistance to antimycin (BU 25) or BUdR (MA 6.5) was investigated. Mock fusions of both cell lines with themselves were carried out in parallel with the experimental fusions described in the next section. Typically, 2.5×10^6 cells of either line were fused and plated under selective conditions identical with those used in cytoplast/cell fusions. No colonies were found growing under such conditions. 5×10^6 cells of each parental line have been examined in this way.

To ensure that resistance could not be generated by growth of the two cell lines together without fusion, five flasks were set up containing 5×10^5 of both parental cell lines. The medium on these flasks was changed after 24 hours to medium containing antimycin at a concentration of 10 μM and BUdR at 30 μg/ml. No cells grew in such experiments.

These experiments establish that a selective regime employing antimycin at 10 μM and BUdR at 30 μg/ml kills unmodified MA 6.5 and BU 25 cells. It should thus only allow the growth of cells bearing the drug resistances of both parental cell lines.

B. Experimental fusions

Several fusions were initially performed using 2×10^6 BU 25 and about 4×10^6 cytoplasts. No colonies resulted from these fusions.

In a second set of three fusions, fewer BU 25 cells were fused with the cytoplasts. In these fusions, 5×10^5 BU 25 cells were fused with 5×10^6 - 1×10^7 cytoplast fragments.

This change was introduced when it was learned (Bunn and Eisenstadt, 1977) that increased frequencies of cybrid formation were given by raising the ratio of cytoplasts to cells.

The results of these fusions are shown in Table 6.1. Cybrid

Table 6.1

Fusion	Bu25	Cytoplasts	No. colonies	Colonies per 10^5 Bu25
1	5×10^5	9×10^6	638	127
2	5×10^5	5×10^6	724	156
3	5×10^5	1×10^7	2,936	587

Results of 3 fusions between BU 25 and cytoplasts prepared from MA 6.5.

colonies were obtained in these fusions at frequencies of 1-4 per 10^3 BU 25 parental cells, which is comparable with values for similar experiments using chloramphenicol resistance as the mitochondrial marker (Munro et al., 1978).

It is highly unlikely that these colonies result from the fusion of whole cells. The cytoplast fractions used in these fusions contained a low degree of contamination with nucleated fragments, estimated to be from 5-15% of the total fragments. However, the sensitivity of MA 6.5 to BUdR is based upon its possession of a nuclear coded enzyme, thymidine kinase. Whole cells have been shown to be sensitive to BUdR at the concentrations used in these experiments, and as intraspecific hybrids rarely segregate chromosomes extensively, it is likely that any hybrid resulting from the rusion of a nucleated fragment of MA 6.5 with a BU 25 cell would not survive the selective conditions imposed. Similarly, the observed frequency of colony formation is several orders of magnitude too high to be accounted for by spontaneous mutation of MA 6.5 to BUdR resistance, or of BU 25 to antimycin resistance. The control experiments already described indicate that such events must take place at a frequency less than 10^{-7}.

The possibility that artifactual results could be due to the instability and breakdown of antimycin A to some biologically inert substance was discounted. Medium containing antimycin at 5, 10 or 15 μM has been shown in the course of these experiments to kill sensitive cells after storage at 4^{o}C for 1 month, but to have no effect on MA 6.5.

The possibility of the transfer of antimycin resistance from MA 6.5 to BU 25 by a virus or other infectious agent has also been discounted by the results of the experiments in which BU 25 and MA 6.5 were plated together without fusion.

A number of colonies were cloned prior to staining and were

grown in medium containing BUdR and antimycin. This was to check that these presumptive cybrids were capable of continued growth in selective medium, and also to enable a karyotype analysis to be performed.

C. Discussion

The experiments reported in this chapter demonstrate that the antimycin resistance of MA 6.5 can be transferred at high frequencies (1 in 10^3) to a sensitive cell line by fusion of enucleated cell fragments. These experiments were performed under a selective regime which would prevent the survival of any hybrids resulting from whole cell fusions. By the accepted criteria, therefore, antimycin resistance may be said to be transmitted cytoplasmically in MA 6.5. It is reasonable to anticipate that cytoplasmically, in this case, means mitochondrially; the resistance is to a drug known to act at the mitochondrial inner membrane, it was generated after attempting to mutagenise specifically the mitochondrial genome, and at least one mitochondrially synthesised protein differs quantitatively from the parental cell line.

This work also suggests that antimycin resistance is a good selectable marker allowing clear discrimination between sensitive cells and those which have received resistance from MA 6.5. It is true that only Hela derived cell lines have been tested for sensitivity to antimycin (the chloramphenicol resistance mutant, MC 63, is sensitive to 5 μM antimycin) but there is every likelihood that other mammalian cell lines will exhibit similar levels of sensitivity. The establishment of this is clearly of importance for further genetic studies, but it appears from this preliminary analysis that MA 6.5 will prove to be of considerable value in future investigations of cytoplasmic genetics.

CHAPTER 7

THE GENERAL NATURE AND TRANSMISSION OF MITOCHONDRIALLY SYNTHESISED PROTEINS

INTRODUCTION

Since the earliest reports of the incorporation of labelled amino acids by mitochondria (reviewed by Ashwell and Work, 1970), it was apparent that the label incorporated was associated with a highly insoluble membrane fraction (Roodyn et al. 1962). It is now known that many of the products of mitochondrial protein synthesis are associated with the inner mitochondrial membrane (Beattie, 1971; Schatz and Mason, 1974). Since the introduction of the detergent s.d.s. and its inclusion in polyacrylamide gel electrophoresis (Shapiro et al., 1967; Weber and Osborn, 1969), the most frequently used approach to the investigation of mitochondrially synthesised proteins has been their labelling with radioactive amino acids, followed by their separation on s.d.s. polyacrylamide gels. As a result of such studies, it has been generally agreed that mitochondria in mammalian cells synthesise a small number (8-12) of hydrophobic proteins of fairly low molecular weight ($\lesssim$55,000 daltons) (Coote and Work, 1971; Lederman and Attardi, 1973; Jeffries and Craig, 1976; Yatscoff and Freeman, 1977).

The ability of s.d.s. to dissociate fully the products of mitochondrial protein synthesis has been questioned by Kuntzel and Blossey (1974), who found that the low molecular weight product of a submitochondrial protein synthesising system aggregated in the presence of 0.01% (w/v) s.d.s. However, the initial solubilisation of whole cells in the studies presented here and by others,

(e.g. Jeffries and Craig, 1976; Yatscoff and Freeman, 1977) is in s.d.s. concentrations equal to or greater than 2% (w/v), at which concentration no aggregation was observed by Kuntzel and Blossey, and the equilibrium concentration of s.d.s. in the gel system is 0.1% (w/v) which is ten times higher than the concentration at which they observed maximal aggregation. Although the possibility that aggregation artifacts may affect the interpretation of gel results should not be discounted, the reproducibility of species specific molecular weight differences in mitochondrially synthesised proteins, discussed below, and the fact that the mitochondrially synthesised subunit of purified cytochrome oxidase continues to migrate to the same position in s.d.s. gels as the labelled polypeptide solubilised from whole cells, argue that the patterns observed on such gels reflect at least in part the discrete products of mitochondrial protein synthesis.

Jeffries and Craig (1974) presented evidence that several mitochondrially synthesised proteins showed reproducible interspecific differences in apparent molecular weight on s.d.s. polyacrylamide gels. Jeffries (1975) presented more extensive data on a larger number of species, from Drosophila to man. Minor variations in the profiles of proteins on gels could be detected when the mitochondrial products of closely related species were compared, and these differences became larger as more distantly related species were considered. This result was taken to indicate an evolutionary divergence between the products of different species, although it is surprising that this divergence should affect the apparent molecular weight so extensively.

An alternative explanation for these results, which was considered by Jeffries (1975), is that the molecular weight differences are due to the existence of post translational modification of the primary translation products, these latter being

similar in all mammalian species. There is some evidence that some mitochondrially synthesised proteins may be glycosylated. Isolated liver or synoptosomal mitochondria can incorporate ^{14}C labelled hexoses into TCA insoluble material (Bosmann and Martin, 1969), and some of this material appears to co-electrophorese with some products of *in vitro* mitochondrial protein synthesis (Bosmann, 1971; Bosmann and Myers, 1974), although it was not shown that the ^{14}C label was still present as carbohydrate rather than being incorporated as ^{14}C amino acids derived from labelled sugars. If some mitochondrially synthesised proteins are glycosylated, interspecific molecular weight differences could be generated by the differential glycosylation of proteins of initially similar molecular weight.

The interspecific molecular weight differences of mitochondrially synthesised proteins observed by Jeffries and Craig (1974) have been used as markers for the mouse and human mitochondrial genome in hybrid cells. A well documented phenomenon, which is the basis of much of the genetic mapping of the human genome, is that most human-rodent hybrids characteristically possess a full complement of rodent chromosomes, but on prolonged culture lose most of their human chromosomes (Weiss and Green, 1967). This loss of human chromosomes appears to be random, and has been exploited in the assignment of genes to specific chromosomes (Ruddle, 1973; Craig, 1975). Fewer studies have been carried out on the fate of mitochondrial genomes in such hybrids, but Clayton *et al*. (1971) and Attardi and Attardi (1972) have shown that in several human-mouse hybrids which have lost a substantial proportion of their human chromosomes, only mouse mitochondrial DNA can be detected by CsCl buoyant density gradient centrifugation, a technique which allows separation of mouse and human mitochondrial DNA. This situation is in contrast to that observed in

hybrids obtained from the fusion of cultured human cells with primary rodent embryonic cells. These can lose either rodent or human chromosomes, and the loss of chromosomes is generally less than that observed in fusions where the rodent parental cells are from an established tissue culture line. In some of these hybrids, sequences of both human and rodent mitochondrial DNA can be detected (Coon et al., 1973).

Jeffries (1975) examined the profiles of mitochondrially synthesised proteins from a large number of hybrid human-mouse cells, which between them contained every single human chromosome. All of these cells showed a profile characteristic of the mouse, and no human type products were detected. From this it was concluded that no single chromosome is required for the retention of human mitochondria in hybrids.

The results presented in this chapter are relevant to a number of these issues, and fall generally into three categories. First, experiments were performed to test the hypothesis that the interspecific differences in molecular weight of mitochondrially synthesised proteins are due to the post translational addition of carbohydrate. Second, a number of human and mouse cell lines were compared on isoelectric focusing gels using the system developed for the examination of MA 6.5, in an attempt to characterise more completely the products of mitochondrial protein synthesis and to look for inter or intra specific differences in the profiles. Finally, the profiles of mitochondrially synthesised proteins on s.d.s. gels of two hybrid cell lines known to retain large numbers of human chromosomes were examined to see if human specific mitochondrial products could be observed.

MATERIALS AND METHODS

1. Inhibitors

2-deoxy-d-glucose and d-glucosamine hydrochloride were obtained from Sigma. They were dissolved in medium, filter sterilised, and added as stock solutions to medium at a final concentration of 5 mM (2-deoxyglucose) and 10 mM (glucosamine).

2. Selective labelling

Selective labelling was carried out in all cases as described in Chapter 5.

3. Gel analysis of mitochondrially synthesised proteins

Isoelectric focusing gels and s.d.s. gradient polyacrylamide gels were run as described in Chapter 2. 7-20% (w/v) horizontal exponential gradients of acrylamide were poured using the gradient maker described in Chapter 2 with a constant volume in the mixing chamber. The gel apparatus was rotated through 90^{o} during the pouring of the gel, which was poured from the top with the dense solution in the mixing chamber. A constant volume of 5.4 ml in the mixing chamber was maintained by pumping diluting acrylamide solution intc it at the same rate that the mixed acrylamide was removed to the gel apparatus (see Noll, 1969). The total volume of mixture in the gel was 18 ml. This shape and concentration range was empirically chosen to give a good separation of the mitochondrially synthesised proteins, and to ensure that the bands would be linear over a significant proportion of the gel.

RESULTS

A. Carbohydrate modification of mitochondrially synthesised proteins

There are a number of different ways of detecting carbohydrate groups attached to proteins. In cases where the proteins of interest are present in large amounts, it may be possible to stain gels for carbohydrate. However, mitochondrially synthesised proteins cannot usually be detected on gels of whole cells by staining for proteins with Coomassie Brilliant Blue, so the less efficient stains available for carbohydrate are unlikely to reveal anything about the existence of carbohydrate moieties attached to them. The binding of glycoproteins to lectin columns suggests another possible approach to this question, and has been attempted by A.J. Jeffries (personal communication) but with ambiguous results.

The failure or inapplicability of any of the usual methods of detecting carbohydrate attached to protein when these are applied to mitochondrially synthesised proteins suggested a more indirect approach, based on the anomalous migration of glycoproteins in s.d.s. polyacrylamide gels (Segrest and Jackson, 1972; Leach and Fish, 1974). Although the theory of protein separation by s.d.s. polyacrylamide gels does not appear to be fully understood, the separation is largely based on the binding by a wide variety of different proteins of a constant amount of the detergent per gram of protein (Pitt-Rivers and Impombate, 1968). The detergent is charged, and this charge is believed to mask any intrinsic charge on the protein. There is thus a constant charge:mass ratio for all proteins, but with increasing mass, molecular sieving through the matrix of the gel retards larger proteins more than small ones. The anomalous behaviour of

glycoproteins arises because the carbohydrate moiety binds little or no s.d.s., resulting in a decreased charge:mass ratio for the glycoprotein compared with standard proteins. This in turn leads to a decreased mobility on s.d.s. gels which is interpreted by the observer as a higher apparent molecular weight. However, at high concentrations of acrylamide, molecular sieving effects in the gel predominate over effects due to the charge on the protein, and the anomalously high molecular weight of the glycoprotein decreases, approaching in an asymptotic manner the true molecular weight (Biely et al., 1971; Segrest and Jackson, 1972). Thus in order to estimate the true molecular weight of a glycoprotein, it is usual to measure the apparent molecular weight obtained by electrophoresis in a variety of different gel concentrations, and to plot these values against the gel concentration to obtain the limiting value for the true molecular weight.

A different version of this method using only one gel is possible if a horizontal gradient of acrylamide concentration across the gel can be contrived. On such a gel, the bands observed due to normal unmodified proteins should behave in a similar manner, giving rise to a series of parallel or nearly parallel bands. Glycopolypeptides, in contrast, should give an anomalously high molecular weight at the low concentration end of the gel, but an increasingly true value as the higher concentrations are approached. For this reason, the bands observed due to glycopolypeptides should form bands which are not parallel with the bands due to ordinary polypeptides, and in practice, on a gel containing a large number of bands, those due to glycopolypeptides should cut across those due to ordinary polypeptides. This rationale has been used before to detect the presence of carbohydrate on proteins (Beckendorf and Kafatos, 1976).

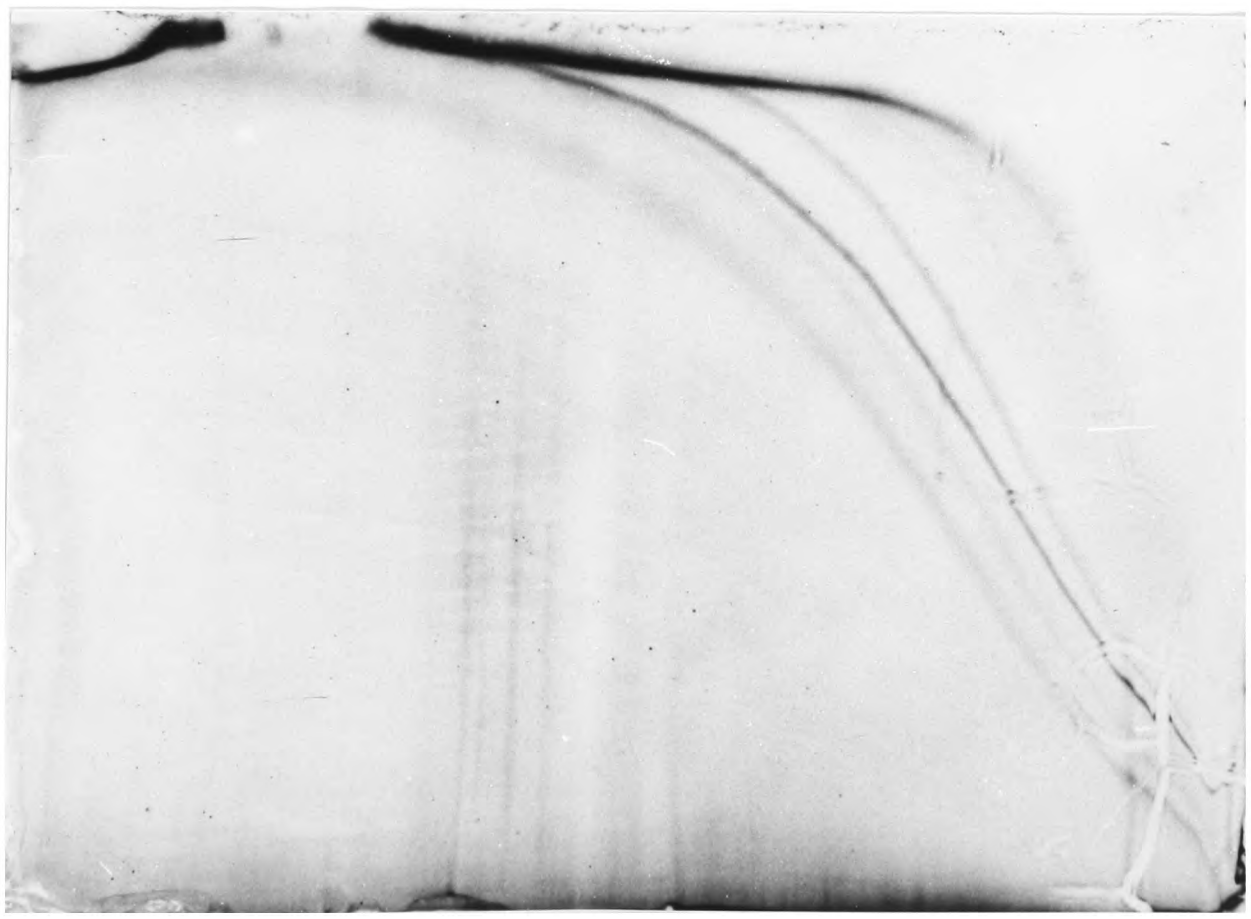


Figure 7.1 Profile of mitochondrially synthesised proteins on a horizontal gradient s.d.s. gel. Mouse (PG19) cells were labelled and applied as sample across the gel.

Figure 7.2 Profile of human (BU 25) mitochondrially synthesised proteins on a similar gel to that described in the legend to Figure 7.1.

Figures 7.1 and 7.2 show respectively the gel autoradiographs obtained following electrophoresis of labelled mouse (PG 19) and human (BU 25) cell lines on 7-20% (w/v) horizontal gradient gels. It can be seen that the mitochondrially synthesised proteins form a series of bands on such gels which show a regular and progressive divergence affecting all components in parallel. These bands followed in general outline the many bands which appeared on the protein stained gels (not shown). No obvious crossing between bands can be seen in the case of the 6 bands on the mouse gel or the 8-9 bands on the human gel which can be ascribed to mitochondrially synthesised proteins. A few faint high molecular weight bands also appear on the gel of BU 25. Similar bands have been observed by Jeffries (1975) and ascribed to residual cytoplasmic protein synthesis. It thus appears that by the criterion of anomalous migration in s.d.s. gels of different concentration, mitochondrially synthesised proteins in neither mouse nor human cells contain large amounts of carbohydrate. This conclusion is also supported by results of similar gels which were loaded with a mixture of selectively labelled mouse and human samples, and in which no obvious crossing could be seen (data not shown).

A second test of the hypothesis that the interspecies molecular weight differences in mitochondrially synthesised proteins are due to carbohydrate modifications is that it should be possible to alter the migration of these proteins in s.d.s. gels by interfering with the modification process. If post-translational modification is solely responsible for these differences, identical patterns on polyacrylamide gels for different species should be produced by inhibiting the modification.

A number of compounds are available which have been used to inhibit post-translational glycosylation of proteins, including

2-deoxy-d-glucose and glucosamine (Schmidt et al., 1976; Pouysségur et al., 1977). To test whether these glycosylation inhibitors could produce an alteration in the apparent molecular weight of mitochondrially synthesised proteins, two different mouse (PG 19 and clone 1 D) and human (D98 and LNSV) lines were grown for 24 hours in 10 mM glucosamine or 5 mM 2-deoxyglucose prior to labelling their mitochondrially synthesised proteins. When these were separated on gels, the results (Figures 7.3 and 7.4) gave no indication of any alteration in either the human or the mouse profiles. Two features should be noted about these gels. First, in some tracks there are faint high molecular weight bands, which may represent the products of residual cytoplasmic protein synthesis. Second, growth in these inhibitors resulted in much lower incorporation of labelled methionine into mitochondrially synthesised proteins, probably reflecting the known toxicity of these compounds (Bekesi and Winzler, 1969; Schmidt et al., 1976). This is evident in the faintness of the bands in some of the treated samples.

Although all mitochondrially synthesised bands cannot be seen in all tracks, those that can be seen migrate in a similar manner in all cases to the bands in untreated controls. Both of the human and both of the mouse cell lines give closely similar patterns when compared with the other cell line of the same species, and the profiles show the same interspecific differences irrespective of the treatment. Since the hypothesis under test is that the differences in apparent molecular weight on s.d.s. polyacrylamide gels between mouse and human mitochondrially synthesised polypeptides are due to carbohydrate additions to the primary translation products, the hypothesis is adequately refuted by finding that any bands which show interspecies differences are unaltered by inhibiting glycosylation. Even in the case

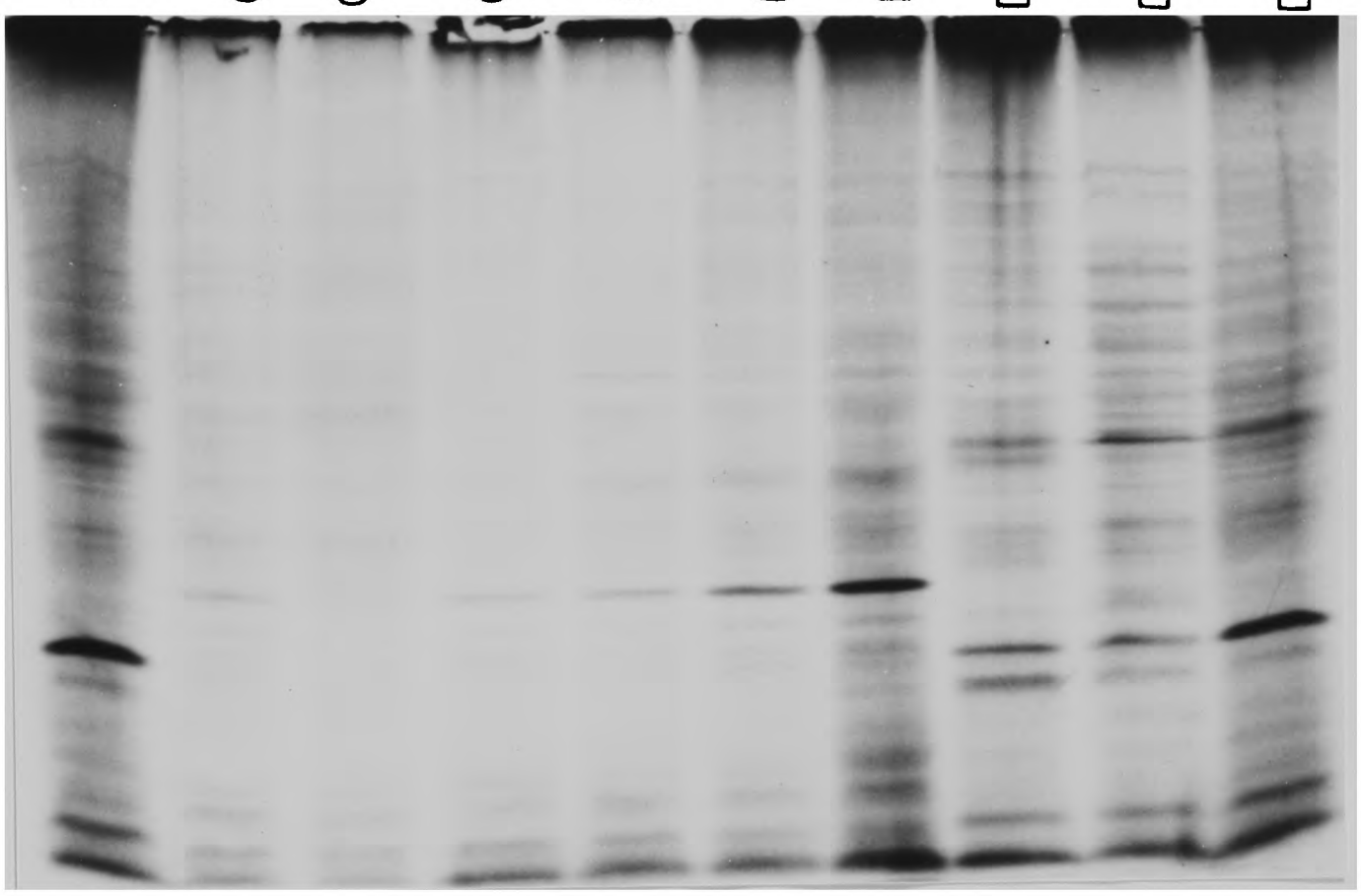


Figure 7.3

10-15% linear gradient s.d.s. gel of mitochondrial proteins from mouse and human cells treated with 2-deoxyglucose (5 mM) or glucosamine (10 mM) for 24 hours prior to selective labelling compared with untreated controls.

GA: glucosamine treated samples

2d: 2-deoxyglucose treated samples

Mouse cell lines: PG19 and Clone 1D

Human cell line: D98

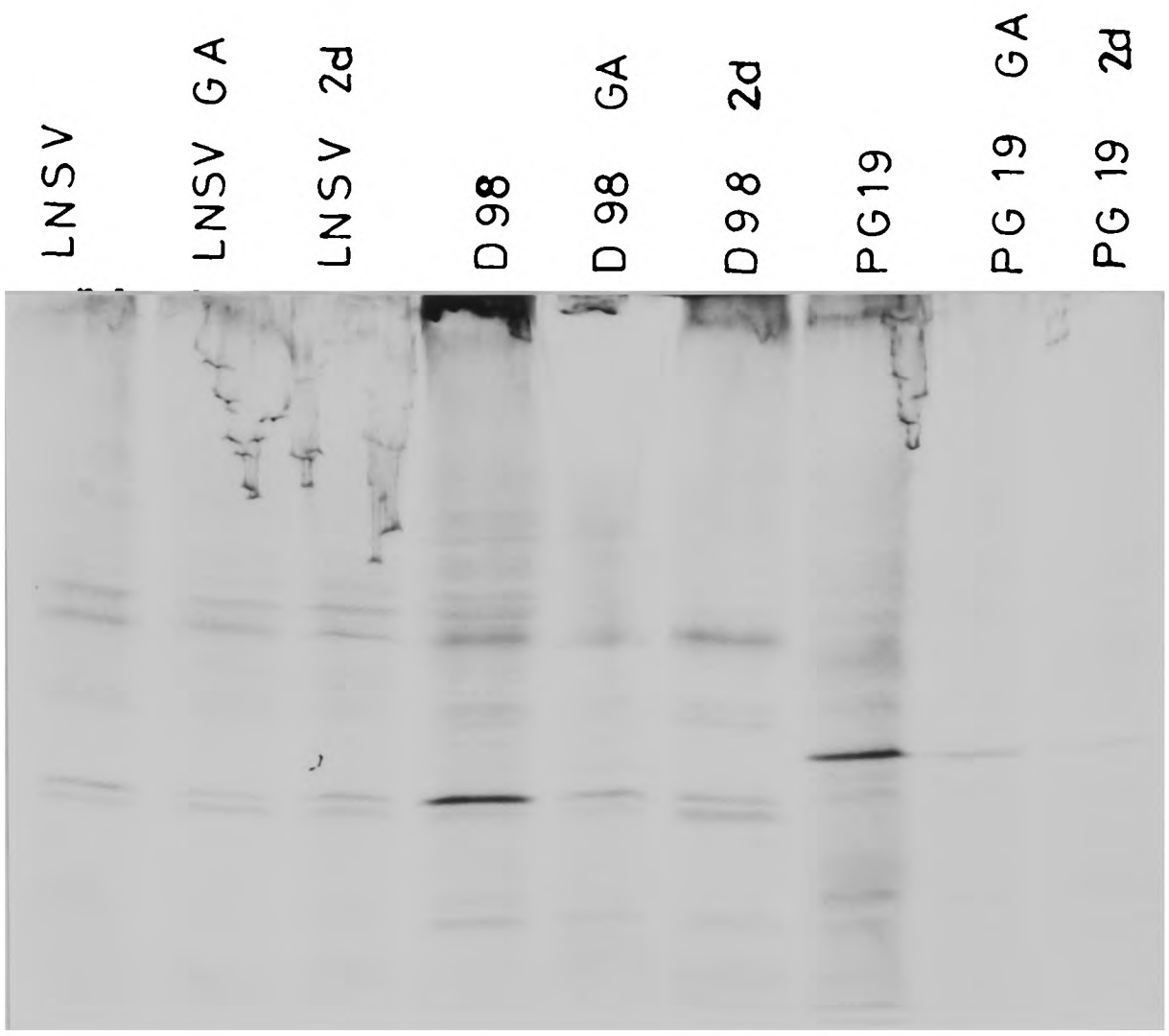


Figure 7.4

10-15% linear gradient s.d.s. gel of mitochondrially synthesised proteins from mouse and human cells treated as described in the legend to Figure 7.3.

Mouse cell line: PG19

Human cell lines: D98 and LNSV.

where fewest bands appear (clone 1 D), one such band is visible and unaltered by the treatments, and more bands are visible and similarly unaltered in the other cell lines.

These experiments are, by their nature, indicative rather than demonstrative. Only rigorous purification of the products of mitochondrial protein synthesis will finally answer questions of the existence and extent of carbohydrate modification. The results presented here nevertheless suggest that such modification, if it takes place, is not solely responsible for the interspecific molecular weight differences in these proteins. This allows some confidence that the different profiles on gels reflect different primary gene products from different species, and thus the use of these differences as markers for the mitochondrial genomes in interspecific crosses is justified.

B. Profiles of human and mouse mitochondrially synthesised proteins on isoelectric focusing gels

The isoelectric focusing system described in Chapter 5 was originally employed to examine the proteins synthesised by the mutant MA 6.5. As there appear to be no published studies on the isoelectric profiles of mitochondrially synthesised polypeptides in general, this system was used to examine the proteins synthesised by a number of mouse and human cell lines. This was done partly to characterise these proteins more fully, and partly to screen for intra and inter specific differences in the profiles. A variety of different cell types were examined, including fibroblasts, mouse and human neuroblastoma lines, and the mouse myeloma X63.

As was seen with the studies on MA 6.5, gels containing a full range of ampholines did not give adequate resolution in the low pH region of the gel, and in addition did not appear to contain

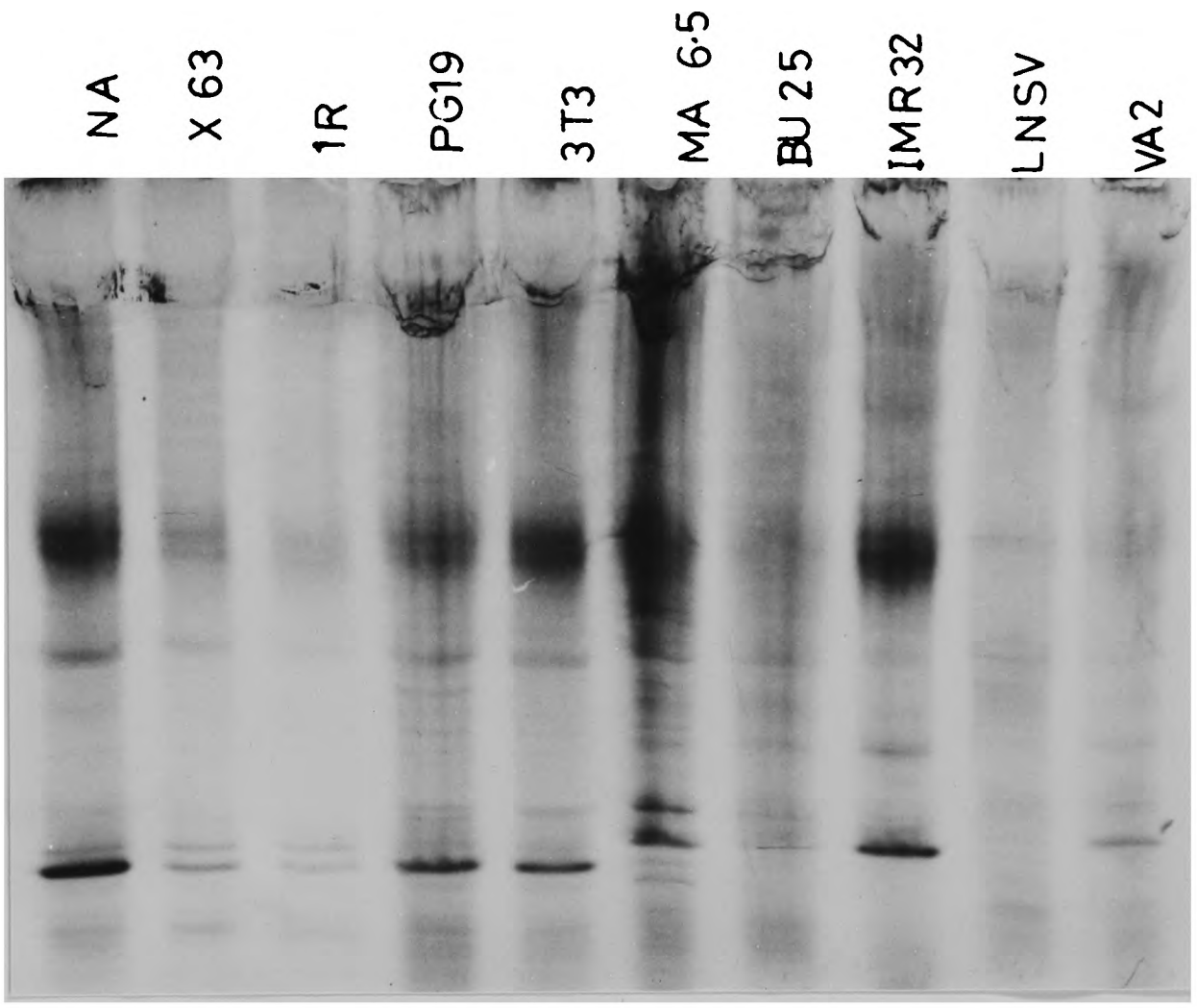


Figure 7.5

Isoelectric focusing profiles of mouse and human mitochondrially synthesised proteins on a gel containing ampholines in the pH range 5-7.

Mouse cell lines: NA, X63, 1R, PG19, 3T3

Human cell lines: MA 6.5, BU 25, 1MR32, LNSV, VA2.

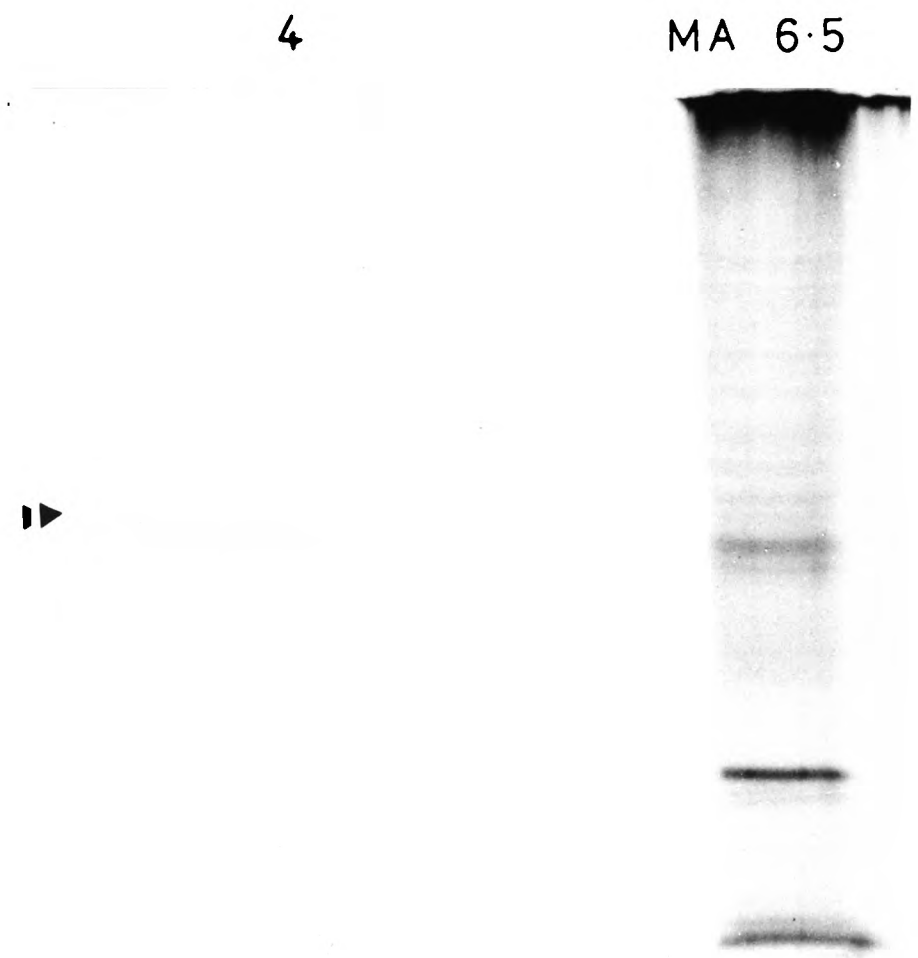


Figure 7.6

The molecular weight and polypeptide composition of band 4 of isoelectric focusing gels. Labelled solubilised whole cells (MA 6.5) were run as a control.

any focused material above the blurred band at pH 5.25 observed in all samples. The use of restricted range ampholines, from pH 5-7, offered improved resolution of the bands below pH 6.0, and did not entail the loss of any bands of interest above this pH. Figure 7.5 shows an autoradiograph of such a gel. Overall, there appear to be a similar number of bands in roughly the same position in the gel as were found in Chapter 5. These bands are numbered as in Chapter 5, from the band at the lowest pH upwards. The heavily labelled blurred band at pH 5.25 (4) is common to both human and mouse. It focuses equally badly in both cases, although in some samples it has the appearance of a poorly resolved double band. Pooled strips of the preparative isoelectric focusing gel described in Chapter 5 containing this band were run on an s.d.s. gel. This band(s) migrated as a single band in an s.d.s. gel with an apparent molecular weight of 41,000 (Figure 7.6). It is not known why this band is not well resolved in these gels. Its position is invariant in all the cell lines examined, as is that of the faint band beneath it (3). Once again, there is a problem in that there are some faint bands which do not consistently appear on all autoradiographs, and are not represented in all the cell lines of a given species. The mitochondrially synthesised status of these is doubtful, and this discussion will refer only to heavily labelled or consistently appearing bands.

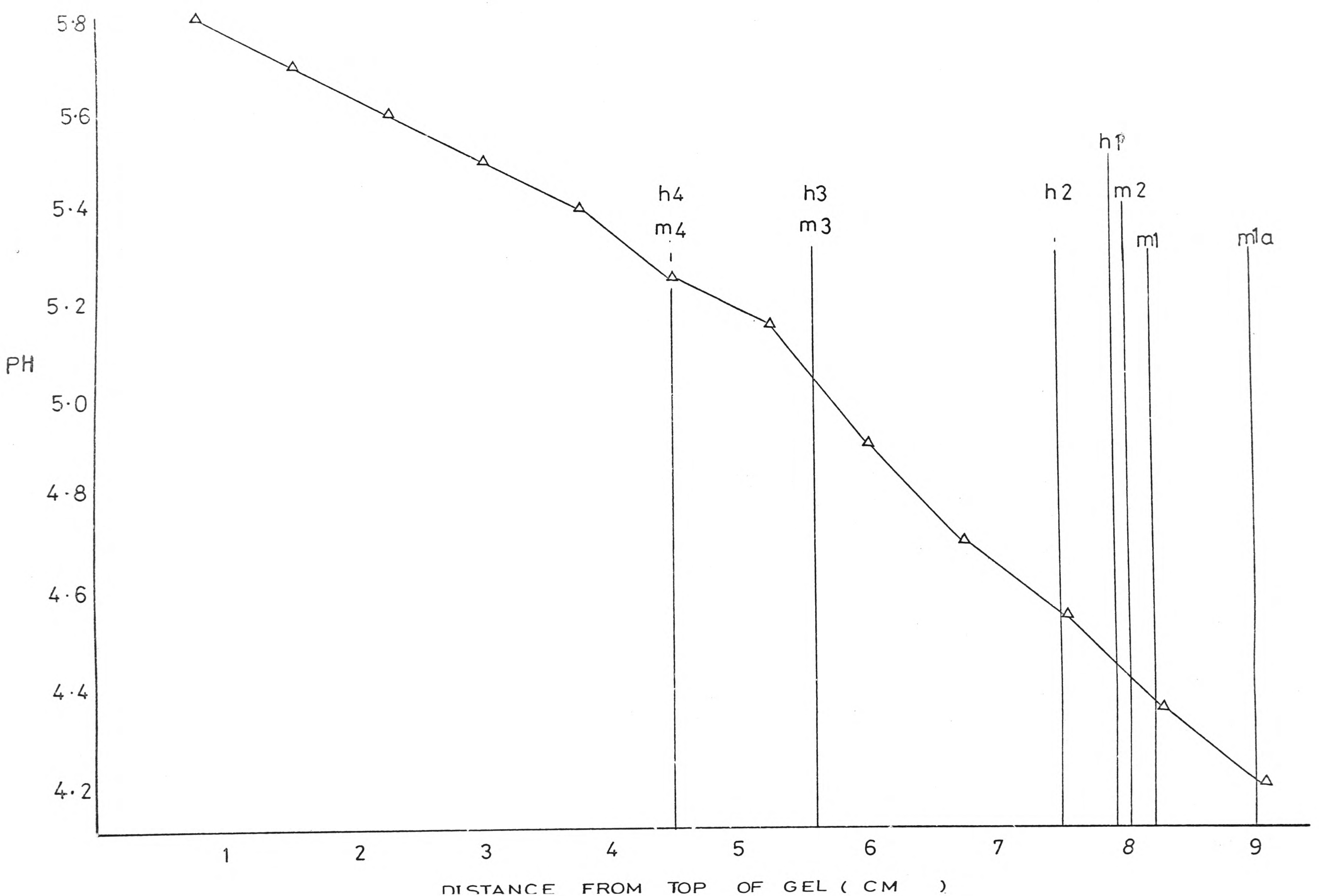
A consistent difference is seen between the mouse and human cell lines in the positions of bands 1 and 2. These are closer together in the mouse samples, and shifted below the equivalent human bands. In addition, there is in some mouse samples a faint extra band appearing below M1 (labelled M1a) which does not appear to be represented in any of the human samples. The iso-electric points of these bands were determined, as in Chapter 5,

by plotting their positions against a graph of the pH gradient across the gel. Such a plot is shown in Figure 7.7, and Table 7.1 shows the isoelectric points determined for these proteins. It has been noted that in all the isoelectric focusing gels run in the course of these studies, the pH gradients across the gels and the separation between bands are very consistent.

It is unfortunate that there should remain any bands of doubtful provenance on these gels. Probably further work is required to refine the labelling techniques such that no doubt remains of the origin of labelled bands. However, by concentrating on only the most heavily labelled and consistently appearing bands, I have confidence that the conclusions drawn are applicable to mitochondrially synthesised proteins. The co-migration of some of these bands with known mitochondrially synthesised proteins, shown in Chapter 5 and in Figure 7.6 supports this view, as does their co-purification with a mitochondrial pellet, shown later.

The positions of the bands corresponding to mitochondrially synthesised proteins are thus similar between different cell lines of the same species on these gels, although at least two of the bands show interspecific differences. Inspection of Figure 7.5 reveals that there are considerable intraspecific differences in the intensities with which individual bands are labelled. In part this may reflect variations in the efficiency of the solubilisation technique, but it has been shown in Chapter 5 that for any one cell line, the relative labelling intensities of the bands appearing on isoelectric focusing gels are fairly reproducible. It is thus likely that the variations between different cell lines shown in Figure 7.5 have some biological basis.

The general utility of this isoelectric focusing technique for the study of mitochondrially synthesised proteins is limited


5·8
5·6
5·4
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PH
5·0
4·8
4·6
4·4
4·2
h4
m4
h3
m3
h2
h1
m2
m1
m1a
1
2
3
4
5
6
7
8
9
DISTANCE FROM TOP OF GEL (CM)

Figure 7.7 Positions of mouse and human mitochondrially synthesised proteins on isoelectric focusing gels

The pH gradient across an isoelectric focusing gel was determined as previously described. The positions of bands corresponding to mouse and human proteins are indicated on the diagram.

h: human protein

m: mouse protein

TABLE 7.1

Isoelectric points of mitochondrially synthesised proteins from human and mouse cell lines

Mouse	IEP	Human	IEP
M 1a	4.2		
M 1	4.38	H 1	4.44
M 2	4.4	H 2	4.54
M 3	5.05	H 3	5.05
M 4	5.25	H 4	5.25

The isoelectric points (IEP) of bands appearing on autoradiographs was determined by plotting the positions of the bands on a graph of the pH gradient across the gel.

by the appearance on gels of only four (or five, in the case of some mouse cell lines) main bands. Two of these bands, at least in the human products, appear to be of identical molecular weight on s.d.s. gels. Thus only about one third of the products regularly appearing on s.d.s. gels are represented by bands on isoelectric focusing gels. Part of this problem may be due to the breakdown of basic ampholines in the presence of urea under the conditions of equilibrium isoelectric focusing. Even on gels containing ampholines in the pH range 3.5-10, the pH at the basic end of the gel has not been observed to exceed 7.75. Any protein with an isoelectric point above this value would presumably not be focused on these gels. Another problem may be that not all the mitochondrially synthesised proteins are solubilised by this system. On most gels, much labelled material can be seen stuck at the top of the gel, a problem long familiar to workers using exclusively s.d.s. polyacrylamide gels.

To overcome this problem, and to confirm that the bands represent proteins associated with the mitochondria, mitochondria were purified from labelled cells as described in Chapter 2, and the solubilised proteins examined by isoelectric focusing on a pH 5-7 gel. The result is shown in Figure 7.8, which shows both mouse and human patterns. The same components that are present in previous gels can be seen, but there are an additional two components in both the mouse and the human cell line. These additional components are arrowed in Figure 7.8, and are identical in relative position in both the mouse and the human cell line 5, one appearing between bands 1 and 2, the other above band 2. These components may occasionally be seen on gels where whole cells have been solubilised, although they are very faint. It thus appears that prior purification of mitochondria allows improved

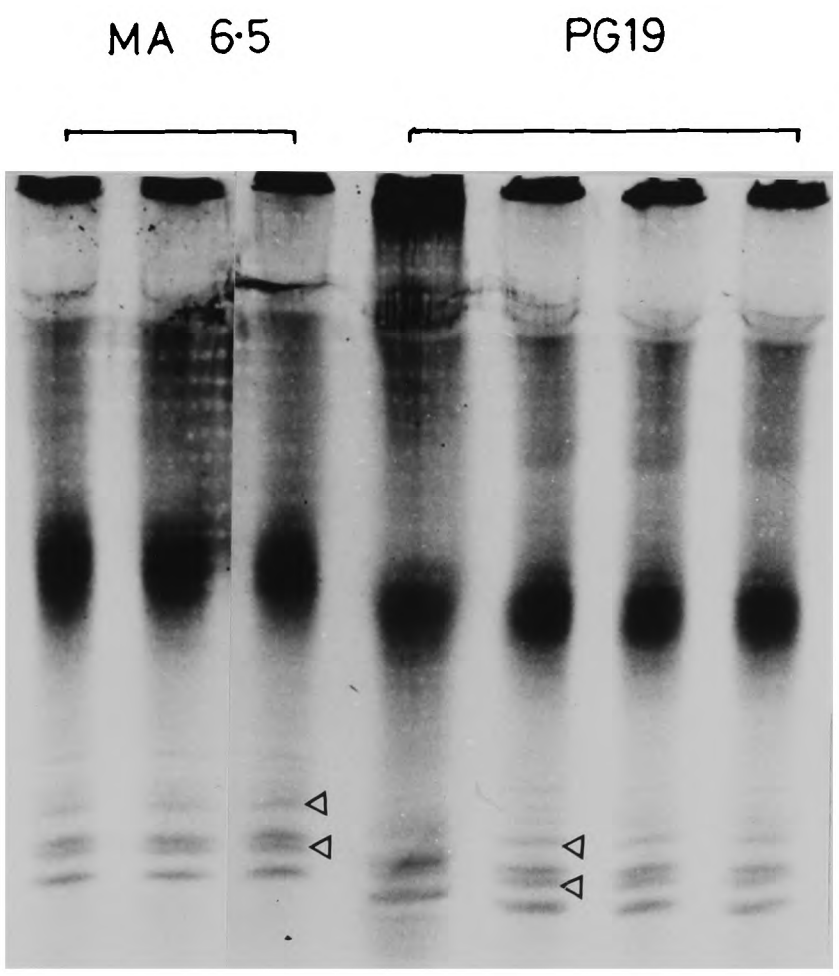

Figure 7.8

The appearance of mouse (PG 19) and human (MA 6.5) mitochondrially synthesised proteins on a pH 5-7 isoelectric focusing gel when purified mitochondria from labelled cells are used as sample.

solubilisation or resolution of mitochondrially synthesised proteins in this system. Perhaps the greatest significance of these results is that they provide evidence of the mitochondrial association of these bands, which supports the contention that they represent the products of mitochondrial protein synthesis.

C. The mitochondrially synthesised proteins of two "reverse segregant" hybrid cells

The loss of human mitochondrial DNA concomitant with the segregation and loss of human chromosomes in rodent-human hybrid cells has been shown (Clayton et al., 1971; Attardi and Attardi, 1972), and Jeffries (1975) presented evidence that such hybrid cells, containing only a few human chromosomes, show a typically mouse profile of mitochondrially synthesised proteins. Such co-segregation of mouse mitochondrially synthesised proteins with mouse mitochondrial DNA provides some genetic evidence that mitochondrially synthesised proteins are coded by mitochondrial DNA. The isolation of human-rodent hybrids retaining both large numbers of human chromosomes and human mitochondrial DNA (Coon et al., 1973) allows a further genetic test of this hypothesis. Subsequent reports that such cells contain hybrid mitochondrial DNA intermediate in density on CsCl gradients between the rodent and the human densities suggest the possibility of finding "recombinant" profiles of mitochondrially synthesised proteins on s.d.s. gels.

Two cell lines of this series, kindly provided by Dr. John Minna, were examined. Both arose from the fusion of embryonic mouse cells with the established human cell line VA2. The karyotypes of both cell lines have been examined in this laboratory, and are shown in Figures 7.9 and 7.10. Both cell lines contain mouse and human chromosomes, with a predominance of the latter.

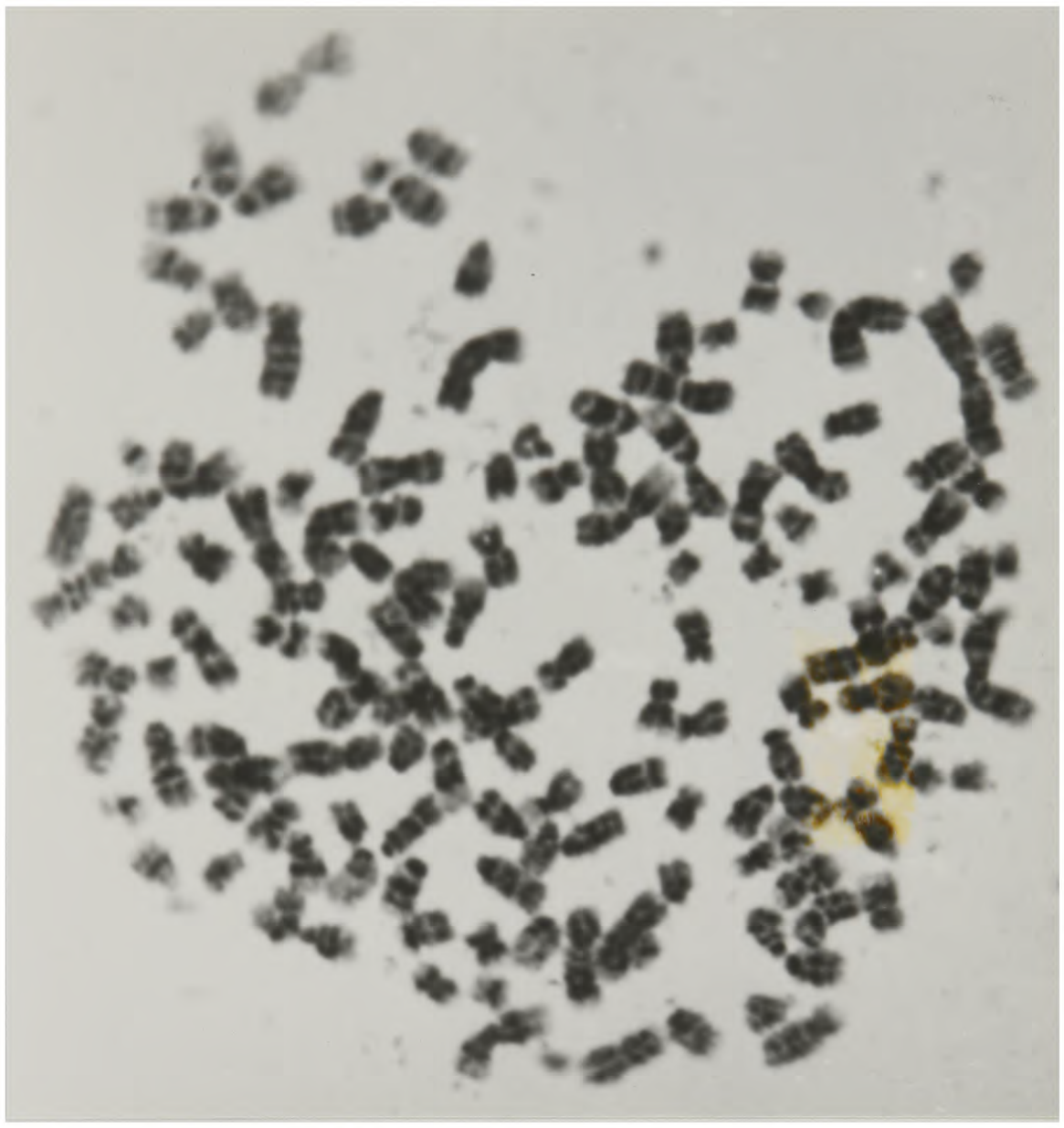

Figure 7.9: The Karyotype of the "reverse segregant" hybrid 18E11

The chromosomes have been trypsin banded. 38 standard human chromosomes are visible together with a number of rearranged human chromosomes. By courtesy of Jane Heritage.

Figure 7.10: The Karyotype of the "reverse segregant" hybrid line 15M9

The chromosomes have been G11 banded. The dark staining material is of mouse origin. Of a total of 82 chromosomes, 56 are of human origin. By courtesy of Ruth Brown.

Figure 7.11 presents the profiles of mitochondrially synthesised proteins in these cell lines, together with standard mouse and human lines as referents. It is clear that there are certain quantitative differences in the labelling of the bands between different cell lines. The general impression gained from this gel is that the patterns of the hybrids are similar to the human reference profile. This is particularly so in the lack of any bands corresponding to those labelled M1 and M2 in the hybrids, and also by the fact that the apparent doublet band M3/4 is represented both in the hybrids and in the human standards as a single heavily labelled band. There appear to be no characteristically mouse bands in the hybrid profile, and no bands appear in the hybrids which are not represented in the human referents. Thus two possibilities - the existence of a mixed profile containing both human and mouse bands, and the existence of a band(s) in the hybrid not corresponding to a band(s) of either parent, are not supported by this gel. It should be noted, however, that this gel is not as highly resolving as might be desired, so the possibility exists that a better gel would reveal minor differences between the hybrid cell profiles and those of the human parent.

It thus appears that the proteins synthesised by the mitochondria of these cells are more similar to the human than the mouse standards. This is the first observation of the predominant presence of human rather than mouse mitochondrial products in a hybrid cell line. Human mitochondrial DNA sequences have been detected in these hybrids (S. Kearsey, hybridisation data in preparation; see also Chapter 8), and thus these observations are additional evidence of the co-segregation of species-specific mitochondrial proteins with mitochondrial DNA. Further work to resolve the ambiguities in the results shown would primarily be of interest in establishing whether any genuinely "recombinant"

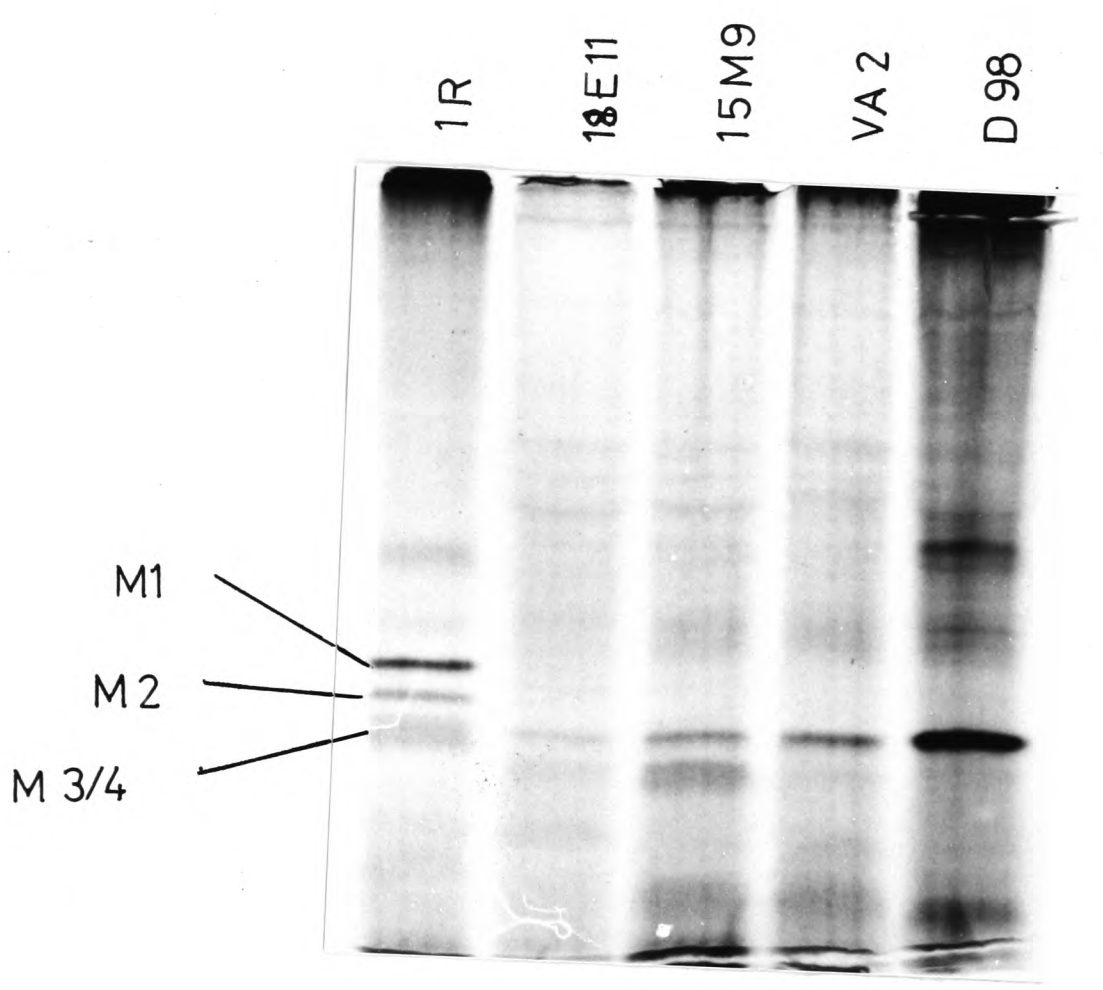

Figure 7.11

The mitochondrially synthesised proteins of two "reverse segregant" hybrid cells

18E11 and 15M9: "reverse segregant" hybrids.

D98 and VA2 : reference human profiles.

IR : reference mouse profile.

Samples were electrophoresed on an 11% homogeneous s.d.s. gel.

proteins exist in these cells.

DISCUSSION

The results described here arose out of the general interest in the nature and function of mitochondrially synthesised proteins in mammalian cells which has been a common theme throughout the work described in this thesis. They allow certain conclusions, though these are still tentative. It was not possible to find evidence that mitochondrially synthesised proteins contain substantial carbohydrate additions by techniques dependent on the anomalous migration of glycoproteins in s.d.s. gels. This negative result does not rule out the possibility that some or all mitochondrially synthesised proteins contain minor carbohydrate additions, as the horizontal gradient technique might not detect the presence of glycoproteins containing only small amounts of carbohydrate. If glycosylation had been prevented by the use of inhibitors in the second set of experiments, then the limiting factor on detection would be the resolving power of the gel. In general, although molecular weights determined on different gels agreed to within 10%, in any one gel, molecular weight differences of 1,000 daltons are easily detectable. Thus carbohydrate modifications which altered the apparent molecular weight of any of the mitochondrially synthesised proteins by at least this factor should have been detected, assuming the successful inhibition of glycosylation.

That these experiments are not conclusive is readily conceded. One major reservation is that the mechanism of glycosylation of mitochondrial proteins, if it exists, may be different from that of other cell glycoproteins, and may not be sensitive to the same inhibitors. In particular, the inhibitors, like other hexoses,

may be unable to enter the mitochondrion. The mechanism by which 2 deoxyglucose and glucosamine inhibit glycosylation is not fully understood; however it appears that 2 deoxyglucose may be incorporated at low levels into glycoprotein, but prevents chain elongation by the further addition of carbohydrate, whereas glucosamine is metabolised to a stable end product, UDP-N-acetyl glucosamine (Scholtissek, 1975), which accumulates and depletes the cellular pool of UTP available for sugar activation. The latter mechanism should affect glycosylation in the mitochondrion even if glucosamine does not enter.

These experiments provide evidence against the most extreme hypothesis that the molecular weight differences between mouse and human mitochondrially synthesised proteins are solely due to post-translational modification by the addition of carbohydrate. This strengthens the view that these differences reflect primary differences in the genes coding for the proteins. Genetic evidence has been discussed which supports the view that these genes are located on mitochondrial DNA. This is known to have evolved rapidly, as low levels of interspecific annealing (Coon et al., 1973) and differences in restriction patterns (Hutchison et al., 1974) of mitochondrial DNA from different mammalian species indicate considerable sequence divergence.

It appears that there is a consistent, though minor, difference in the profiles of mouse and human mitochondrially synthesised proteins on isoelectric focusing gels. It is interesting that the six bands on such gels have a restricted range of isoelectric points spanning only one pH unit, at the acidic end of the pH range. The significance of this observation is uncertain. Further

work on the isoelectric points of the mitochondrial products missing from these gels would be of interest.

The preliminary results on the proteins synthesised by the reverse segregant hybrids presented here should be extended by the use of more resolving gel systems, and also by the application of the isoelectric focusing techniques described. In this way, the existence of any mouse bands or bands intermediate between mouse and human might be established or disproved. The results of this preliminary study, however, suggest that these cells synthesise predominantly human type mitochondrial proteins.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

This thesis has described the isolation and characterisation of an antimycin resistant human cell line. It has been established that antimycin resistance is a stable property, expressed at the level of the cell, which is transmissible cytoplasmically to a related cell line. Membrane preparations from MA 6.5 appear to retain a normal capacity for electron transport through the antimycin sensitive region of the respiratory chain, this electron transport being fully sensitive to antimycin in an *in vitro* assay system. The mechanism by which antimycin resistance is conferred on whole cells has not been established.

There are a number of unresolved questions raised by this work. A mutagenic system was used which might be expected, on theoretical grounds, to yield an increased ratio of mitochondrial to nuclear mutations. It is difficult to assess the worth of this system from the data presented here. Unselected growth was allowed after mutagenesis, and the colonies which appeared after the initial selection at low (5 μM) concentrations of antimycin were neither counted nor cloned, but were allowed to grow to semi-confluence before the imposition of selection at high (15 μM) concentrations. For these reasons, no numerical estimate of the efficiency of the mutagenesis with respect to the mutation selected can be given. Antimycin resistance is an hitherto uncharacterised phenomenon in mammalian cells and it is not known how easy it is to generate, although the fusion controls described in Chapter 6 suggest a rate of spontaneous antimycin resistance lower than 10^{-7}. Chloramphenicol resistance, by contrast, is a better

characterised mitochondrial marker, and might form a useful model system for assessing the merits of different mutagenic procedures designed to affect the mitochondrial genome.

Another question concerns the relation between the increase in the relative labelling of a mitochondrially synthesised protein and the antimycin resistance of the mutant. The change in the labelling intensity of the variant protein in MA 6.5 may be caused by one of a number of factors:

1) The protein has suffered a mutational alteration resulting in the retention of the N-terminal formyl methionine.

2) The protein has suffered a point mutation resulting in the substitution by methionine of another uncharged amino acid.

3) The rate of turnover of the protein is altered in the mutant, allowing the accumulation of higher levels in the mitochondrion.

4) The protein is synthesised at an increased rate in the mutant.

The first two possibilities may be discounted in view of the results obtained using ^{14}C labelled leucine. Since the differential labelling intensity between the mutant and the parental cell line was observed using this amino acid, it seems probable that the explanation for the difference must involve an increased accumulation of the protein in the mutant. If the accumulation is due to an altered turnover in the mutant, it probably reflects a mutation altering the primary structure of the protein and making it less susceptible to degradation by proteases. In view of the apparent lack of any qualitative change in the protein, however, the final class of explanation is favoured. At a

molecular level, possible causes for the increased rate of synthesis of one specific protein fall into two classes. The number of structural genes for the protein may have been increased, or there may have been some alteration which conferred greater stability, altered RNA processing, or increased affinity for the ribosome on the message transcript specifying the protein. If the mutation was a result of MNNG treatment, then the second class of explanation, which would involve a change in sequence, but not in the number of copies of the gene, would be favoured.

If it is provisionally assumed that the increased level of this protein is related to the resistance observed, two mechanisms relating the observations may be envisaged.

1) The protein binds intracellular antimycin rendering it unavailable to the electron transport chain. The increased amount of the protein in the mutant allows more antimycin to be bound, and hence the cells will tolerate higher concentrations of antimycin in the medium.

2) Increased levels of this protein in the mitochondrial inner membrane may change its properties, making it more impermeable to antimycin.

Both these hypotheses have some merit. The first is in accord with the known existence of proteins able to bind antimycin in some cells (Reporter and Ebert, 1965) and with the unchanged response of the mutant to two other antimitochondrial antibiotics. The second invokes a well known mechanism of generating resistance to a number of drugs, which always exists as an *a priori* possibility in such studies.

An examination of the mitochondrial DNA of MA 6.5 by restriction digest analysis is in progress in this laboratory.

It is interesting to note in passing that a rare combination of symptoms in a patient with neuromuscular disease has been found to be associated with abnormally low levels of cytochrome b, which is a good candidate for being a mitochondrially synthesised protein (Morgan-Hughes et al., 1977). This suggests that quantitative variations in mitochondrially synthesised proteins may have profound effects on the individual, and encourages the view that this phenomenon in MA 6.5 may be of significance to its antimycin resistance.

Although I would have preferred to be able to demonstrate resistance to antimycin in an in vitro assay system, and perhaps to correlate this with a qualitatively variant mitochondrially synthesised protein, the observation that antimycin resistance can be transmitted cytoplasmically and constitutes a good selectable marker suggests that this cell line will be useful in a range of genetic experiments. Whatever the mechanism of resistance, it is the first mammalian cell line selected for resistance to an electron transport inhibitor, and it shows complete sensitivity to chloramphenicol. It should thus be of use in conjunction with chloramphenicol resistant cell lines for double marker genetic experiments. Finally, I hope that some of the data presented in this thesis will be of use in future attempts to isolate mutants resistant to antimycin.

In addition to the work discussed above on the isolation and characterisation of antimycin resistant human cells, some experiments have been reported which bear on the general nature of mitochondrially synthesised proteins. These generally support the concept that the mitochondrial products of mouse and human cells differ from each other not merely as a result of species specific modification mechanisms. This in turn indicates that these differences may be regarded as markers for the mitochondrial

genomes of the two species. A preliminary study using these markers indicates that two "reverse segregant" hybrid cells containing large numbers of human and some mouse chromosomes synthesise predominantly human mitochondrial proteins. It has been reported (Dawid et al., 1974) that the mitochondrial DNA from the rodent and human parents in these cell lines has undergone recombination. This report, based on the difference in buoyant density of the human and rodent parental mitochondrial DNA's is supported by the detection of both human and rodent mitochondrial DNA sequences in one such hybrid in this laboratory, using restriction enzyme analysis and radioactive probe DNA from either parent (S. Kearsey, personal communication).

Examination of the proteins synthesised by the mitochondria of these cells have not so far indicated the presence of mixed mouse/human profiles, or of proteins of intermediate molecular weight between those of the parental cells. This negative result is not in conflict with the data on the mitochondrial DNA of these cells, as recombination may not have affected parts of the genome coding for proteins showing species specific molecular weight differences. The observation that hybrid cells retaining a large proportion of human chromosomes also retain a human type profile of mitochondrial proteins is complementary to observations on the co-retention of mouse chromosomes and mitochondrial proteins made in more typical hybrid cells. It is quite striking that, irrespective of whether recombination has occurred between mitochondrial genomes, hybrid cells seem to have either mouse or human mitochondrially synthesised proteins. Stable mixtures do not seem to be found, even in the reverse segregant cells which retain a fairly large number (about 25) of mouse chromosomes in addition to their full human complements.

These observations suggest at least that a full or nearly full set of chromosomes from one species are required to retain the mitochondria of that species, and in this sense support the suggestions of Jeffries (1975).

It is also possible that more active mechanisms may exist by which alien mitochondria are removed from a cell. In this case, once a hybrid cell became predominantly mouse or predominantly human with respect to its chromosomal constitution, the mitochondria of the other species would be recognised in some way as being alien, and would be quickly removed, although recombination between genomes could rescue some sequences of the excluded mitochondrial DNA by associating them permanently with DNA of the cognate species. At the moment this is largely a speculative suggestion for mammalian cells, although the results of studies with Paramecium, and some genetic studies with mammalian cells, offer circumstantial support for such a view.

The proteins synthesised by mouse and human mitochondria have been examined using the isoelectric focusing technique first adapted for the study of MA 6.5. Although the limited number of proteins appearing on these gels limit their usefulness, at least some interspecific differences in the profiles were detected. The most useful part of this study is probably the determination of the isoelectric points for a number of these proteins. These, together with the molecular weights estimated from s.d.s. gels, should enable relatively rapid and easy purification techniques to be evolved. Such purification is probably the next step in determining more of the nature and function of the proteins synthesised by the mitochondria of mammalian cells.

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