CONTRASTING EFFECTS OF CYCLOHEXIMIDE ON MITOCHONDRIAL PROTEIN SYNTHESIS 'IN VIVO' AND 'IN VITRO'

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Although mitochondria are no longer regarded as completely self-reproducing organelles, the fact that they can incorporate radioactive amino acids into non-terminal peptide linkages when incubated 'in vitro' cannot be disputed. However, a certain number of mitochondrial proteins are thought to be made on the cytoplasmic ribosomes as shown by pulse-chase labelling experiments with whole cells (see Work, Coote and Ashwell, 1968).

If mitochondria which have been labelled 'in vitro' are fractionated using Triton X-100 into a Triton-soluble and Triton-insoluble fraction, very little label is associated with the soluble fraction (Roodyn, 1962). Labelling experiments 'in vivo' by Beattie et al. (1966) using rat liver and rat kidney mitochondria have also indicated that, in the intact cell, the soluble and insoluble proteins have very different kinetics of labelling and this suggests that the labelling pattern of isolated mitochondria represents the 'in vivo' situation.

The protein synthesizing machinery of mitochondria has been compared to that of bacteria, partly on account of the similarity between mitochondrial DNA and bacterial DNA and partly because protein synthesis in bacteria and mitochondria showsimilarity in response to certain drugs (Roodyn and Wilkie, 1968). In the case of chloramphenicol, this has been attributed to the similarity in size between bacterial and mitochondrial ribosomes.

In the same way, the apparent insensitivity of protein synthesizing system of isolated mitochondria to cycloheximide (Borst et al., 1967; Grivell, 1968; Beattie, Basford and Koritz, 1967; Clarke-Walker and Linnane, 1967) can be compared with the resistance of bacteria to this drug (Whiffen, 1948; Ennis and Lubin, 1964). However, cycloheximide is toxic to a wide variety of organisms including fungi, yeasts, higher plants and mammalian cells (see Sisler and Siegel, 1967, for a full review), and it is thought to inhibit microsomal protein synthesis by inhibiting the association of peptide synthetase with cytoplasmic (80S) ribosomes (Heintz, Sales and Schweet, 1968).

It was thought that the apparent selective effects exhibited by cycloheximide 'in vitro' might be used to distinguish between those proteins made by mitochondrial ribosomes and those made on cytoplasmic ribosomes in the intact cell. We have shown that although cycloheximide does appear to have no inhibitory effect when mitochondria are incubated 'in vitro' (even though cycloheximide uptake can be demonstrated), the 'in vivo' results using Krebs II mouse ascites tumour cells show that cycloheximide is an effective inhibitor of both microsomal and mitochondrial protein synthesis. Possible explanations for this discrepancy are discussed.

MATERIALS AND METHODS

Isolated rat liver experiments: 60 g. Sprague-Dawley rats were used for the isolation of rat liver mitochondria, using the method of Roodyn, Reis and Work (1961). They were freed from contaminating microsomes by the method of Freeman (1965).

Krebs cell experiments: Krebs II mouse ascites tumour cells were propagated, collected, washed and counted as described by Martin, Malec, Sved and Work (1961). The cells were disrupted using the Ultra-Turrax disintegrator as described by Freeman (1965) and the mitochondrial fraction purified as above. When cells were used for labelling 'in vivo' the mitochondrial, microsomal and cell sap fractions were separated (Freeman, 1965) and the specific activity of the protein of each fraction determined (Simkin and Work, 1957). Protein determinations were made using the method of Lowry et al., (1951).

Isolated mitochondrial incubation media: Medium 1 was identical to Medium B used by Roodyn et al., (1961) with the following additions: 1 mg./ml. bovine plasma albumin (Armour Pharmaceutical Co.) and 0.2 mg./ml. cytochrome c (C.F. Boehringer and Soehne). In this medium the energy required for mitochondrial protein synthesis is supplied by succinate.

Medium 2 was the incubation medium used by Beattie et al. (1967) where an external ATP-generating system supplies the energy.

Isolated rat liver mitochondria and isolated Krebs cell mitochondria were both incubated at a concentration of 2 mg./ml. at 30°.

Cycloheximide assay: (see Whiffen, 1948) was performed on mitochondria subjected to ultrasonics after incubation with the drug for 40 min.

Density gradient centrifugation of the mitochondria from Krebs cells was performed by a slightly modified Freeman procedure (1965).

Fractionation of mitochondria with 0.1 per cent Triton X-100 (B.D.H.) was performed as described by Roodyn (1962).

L[^{14}C]-leucine (specific activity 305 mC/mM) was used for all experiments, and obtained from the Radiochemical Centre, Amersham, Bucks., England.

Cycloheximide (CH) was obtained from Koch-Light Laboratories (Colnbrook, Bucks, England).

Chloramphenicol (CAP) was obtained from Parke Davis & Co. (Hounslow, Middx., England).

RESULTS

'In vitro' experiments. Table 1 shows the results obtained when isolated mitochondria were incubated in the presence of either cycloheximide or chloramphenicol. Results for both isolated rat liver mitochondria and isolated Krebs II mitochondria are given. It is clear that cycloheximide even at a concentration of 500 μg./ml. has no inhibitory effect whilst chloramphenicol at 50 μg./ml. causes considerable inhibition of ^{14}C-leucine incorporation into mitochondrial protein. The energy source appears to bear no relation to the inhibitory effects observed.

Table 1

Source of mitochondria	Incubation medium	CH concn. (μg./ml.)	CAP cocn. (μg./ml.)	Time (min.)	cpm/mg. protein	% of control value
Rat liver	1	-	-	20	300	-
		-	-	40	460	-
		25	-	20	310	103
		250	-	20	261	87
		-	50	20	130	43.2
Rat liver	2	-	-	20	293	-
		-	-	40	573	-
		25	-	20	298	102
		250	-	20	258	88
		500	-	40	561	98
		-	50	20	129	44
		-	50	40	160	18.5
Krebs cells	2	-	-	40	372	-
		500	-	40	352	94.6
		-	50	40	71.7	19.3

^{14}C-leucine (0.248 μc./ml.) was present during all incubations.

Cycloheximide uptake by isolated mitochondria: There was an assayable quantity of CH present in the mitochondrial lysate when the applied CH concentration was 10 or 20 μg./ml. Using the data of Werkheiser and Bartley (1957) for the value of the inter-mitochondrial space in a tightly packed pellet, it was calculated that cycloheximide either penetrated the mitochondrial membrane or was irreversibly bound by it.

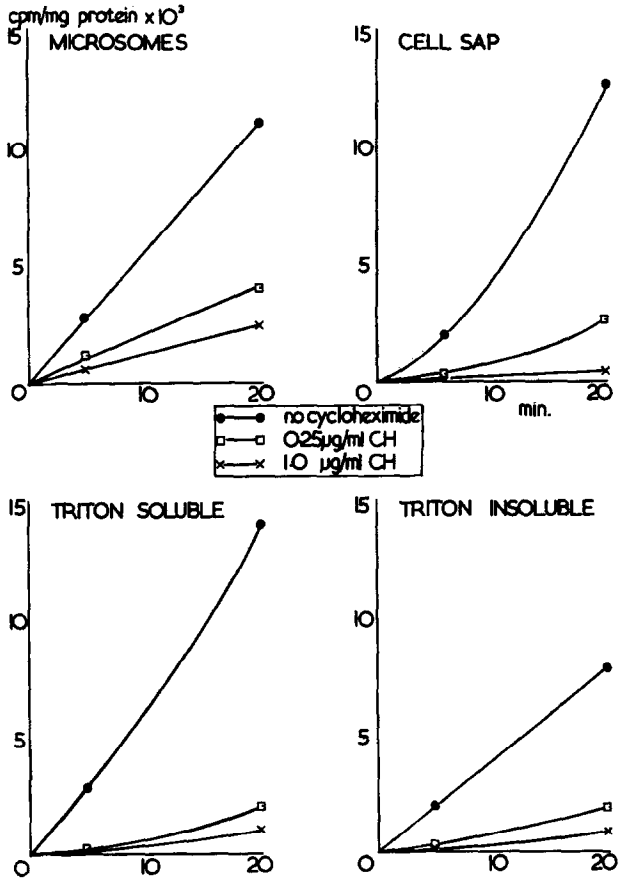

Fig. 1: Krebs II mouse ascites tumour cells were collected and washed in the usual way (Martin, et al., 1961). They were resuspended in Earles medium (Martin, et al., 1961) at a concentration of 2 x 10^7 cells/ml. ^{14}C-leucine was added to a final concentration of 0.248 µc./ml. Samples of cells were incubated at 22° for 5 or 20 min. in the presence of cycloheximide at the concentrations indicated. The specific activities of proteins from different subcellular and submitochondrial fractions were determined as described in the text.

'In vivo' experiments: Fig. 1 shows the results obtained when intact Krebs cells are incubated at 22° in the presence of ^{14}C-leucine and cycloheximide. Even at a concentration as low as 0.25 µg./ml., considerable inhibition of incorporation into the Triton X-100 insoluble fraction occurs, i.e. that fraction which is labelled almost exclusively when mitochondria are incubated 'in vitro'. In the presence of cycloheximide, incorporation into all fractions proceeds at a linear, although much reduced, rate during the first 20 min. incubation. Similar results were obtained using cycloheximide at a concentration up to 500 µg./ml. and incubation periods of up to 80 min.

To check that the inhibition of the mitochondrial fraction was not due to microsomal contamination, the mitochondrial fractions were purified by density gradient centrifugation. The mitochondrial peaks obtained compared well with those obtained by Freeman (1965) and inhibition of ^{14}C-leucine incorporation was definitely associated with the mitochondrial peak.

DISCUSSION

In considering the above results, the first possibility that must be considered is the limited permeability of the cell plasma membrane and the mitochondrial membrane to cycloheximide. If the cycloheximide molecule could not penetrate the membrane system of mitochondria '*in vitro*', then its lack of inhibitory influence could perhaps be explained. Our experiments measuring cycloheximide uptake by isolated mitochondria show that this explanation is improbable.

Does cycloheximide, like chloramphenicol, act as an inhibitor of energy metabolism in general? Freeman and Haldar (1967) have found that chloramphenicol at concentrations greater than 3×10^{-4} M inhibits protein synthesis in Ehrlich Lettre ascites tumour cells but the effect on protein synthesis appears to be a secondary effect since concentrations of chloramphenicol greater than 10^{-4} M inhibit NADH oxidation in isolated rat liver mitochondria and inhibition of protein synthesis parallels inhibition of respiration. This does not appear to be the case with cycloheximide, as Freeman (1968) has shown that a concentration of at least 100 µg./ml. cycloheximide is needed to show any inhibition of beef heart mitochondrial NADH oxidase. Since the cycloheximide effect on protein synthesis is easily demonstrated at 0.25 µg./ml. then it seems that this must be a primary action.

If one can assume that cycloheximide enters mitochondria '*in vivo*' and '*in vitro*' and that its primary action is on protein synthesis, there are two other explanations that might account for our results. The first possibility is the existence of a control mechanism within the cell which couples the two protein synthesizing systems. If this is the case, then primary inhibition of the microsomal system by cycloheximide would cause secondary inhibition of the mitochondrial system. A similar hypothesis has been proposed by Mahler, Perlman, Henson and Weber (1968) to account for the results of their experiments on formation of yeast mitochondrial proteins during derepression. Both cycloheximide and chloramphenicol appear to inhibit certain enzyme activities regardless of the location of the enzyme in the cell, or its supposed mitochondrial or cytoplasmic origin. Yu, Lukins and Linnane (1967), also working with yeast, have studied the formation of cytochromes *a* and a_3 (presumed to be mitochondrial in origin) during de-

repression of cells previously grown under anaerobic conditions. Their results also indicate complex interactions between the two protein synthesizing systems in the cell.

The other explanation for our results with cycloheximide would be that the proteins produced by mitochondrial DNA in the intact cell are a very small percentage of the total mitochondrial proteins and that 'in vivo' the majority of the mitochondrial proteins are made on cytoplasmic ribosomes and therefore susceptible to cycloheximide inhibition. Hensen, Weber and Mahler (1968) have studied ^{14}C-phenylalanine incorporation into subcellular and submitochondrial fractions of yeast in the presence of chloramphenicol and cycloheximide and their extensive findings indicate a minor role for mitochondrial DNA in the formation of mitochondrial proteins.

It is clear that the inter-relations between the mitochondrial protein synthesis and microsomal protein synthesis, and the possible significance of mitochondrial protein synthesis in the intact cell require further investigation.

SUMMARY

1) Our results show that cycloheximide at concentrations up to 500 µg./ml. does not inhibit ^{14}C-leucine incorporation into the protein of isolated mitochondria from either rat liver of Krebs II mouse ascites tumour cells.

2) However, when Krebs cells are labelled 'in vivo' in the presence of cycloheximide at concentrations as low as 0.25 µg./ml., considerable inhibition of ^{14}C-leucine incorporation into the mitochondrial fraction is observed. Even when the mitochondria are subfractionated with Triton X-100, both soluble and insoluble fractions still show considerable inhibition of ^{14}C-leucine incorporation.

3) Possible explanations for these observations are given and discussed with special reference to the results of other workers who have attempted to use cycloheximide as a selective inhibitor of microsomal protein synthesis.

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