The Significance of the Incorporation of [14C]Leucine into Different Protein Fractions by Isolated Ox Heart Mitochondria

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The problem of whether isolated mitochondria are able to synthesize specific proteins was investigated, particular consideration being paid to the possible contribution of micro-organisms to this activity. With ox heart mitochondria it was shown that: (1) The medium used for the incubations inhibits the exponential phase of bacterial growth for at least 8h either in the absence or the presence of fresh mitochondria, but the inhibition disappears after 4h when mitochondria damaged by freezing and thawing are used. (2) The incorporation of [14C]leucine into total proteins is linear up to at least 8h, although part of the radioactivity at the later periods might be due to some incorporation by resting-phase bacteria. (3) A contamination by as little as 800 cells/mg of mitochondrial protein is enough to contribute substantially to the total radioactivity incorporated by the mitochondrial preparations. (4) Purified cytochrome *b* and cytochrome oxidase are labelled even under conditions of minimal contamination by micro-organisms (less than 60 cells/mg of mitochondrial protein) and the contribution of bacterial proteins to the radioactivity found in cytochromes is negligible, as shown by double-labelling experiments. (5) At 4h the specific radioactivities of cytochrome *b* and cytochrome oxidase are seven- and 16-fold lower respectively than that of a structural protein-rich fraction, suggesting that the labelling of cytochromes is due to a residual contamination by these proteins.

Considerable attention has recently been focussed on the study of the mechanisms of mitochondrial biogenesis. One of the main problems has been to determine whether mitochondria can synthesize their own proteins. Since the first publication reporting the incorporation of radioactive amino acids into proteins by isolated liver mitochondria (McLean, Cohn, Brandt & Simpson, 1958) workers in a number of other laboratories have confirmed this finding and extended it to other tissues (for references see Roodyn, 1965; Roodyn & Wilkie, 1968).

Considering the very low activity of the mitochondrial preparations the possibility was raised that bacterial contamination could have a significant role in the results obtained. However, several authors (Roodyn, Reis & Work, 1961; Kroon, 1963; Roodyn, Freeman & Tata, 1965) concluded that the incorporation was mainly due to the mitochondria and therefore no special precautions were taken to avoid contamination. von der Decken, Löw & Sandell (1966) and Sandell, Löw & von der Decken (1967) have questioned this view, postulating that the activity was entirely the product of contaminating bacteria, since sterile rat liver mitochondria were totally unable to incorporate radioactive amino acids into proteins. A number of other authors working

with mitochondria from rat liver (Wheeldon, 1966; Kroon, Saccone & Botman, 1967; Beattie, Basford & Koritz, 1967; Work, Coote & Ashwell, 1968), rat brain (Yellin, Butler & Stein, 1967) and *Saccharomyces carlsbergensis* (Grivell, 1967) have demonstrated that, although contaminating bacteria do incorporate amino acids under the conditions of the incubation, mitochondria themselves can incorporate amino acids into proteins *in vitro*. Beattie *et al.* (1967) questioned the composition of the medium used by Sandell *et al.* (1967), claiming that it lacked some factors necessary for mitochondrial protein synthesis. Haldar & Freeman (1969) have even shown that this medium is unable to sustain the incorporation process owing to its high osmolarity.

Most of these earlier studies have been done with total proteins precipitated with trichloroacetic acid or other unspecified fractions. The incorporation of radioactivity in purified mitochondrial proteins satisfying some criteria of homogeneity has been demonstrated only in the case of the so-called 'structural proteins' (Roodyn, Suttie & Work, 1962; Haldar, Freeman & Work, 1966; Wheeldon & Lehninger, 1966; see also Green, Haard, Lenaz & Silman, 1968).

We have studied the contribution of bacteria to

the labelling of both the total mitochondrial proteins and two purified proteins, cytochrome b and cytochrome oxidase. Heart mitochondria were used for the present studies since most of the isolation and purification methods for these proteins described in the literature were worked out for this material from source. Therefore the assessment of the influence of bacterial contamination is particularly important in this case, since it is practically impossible to obtain the tissue under sterile conditions when slaughterhouse material is used.

Our results support the view that bacteria are not entirely responsible for the incorporation observed into total proteins and show that their contribution to the labelling detected in the purified cytochromes at very low levels of bacterial contamination is practically negligible. However, the rate of incorporation of [^{14}C]leucine into these cytochromes by isolated mitochondria is very slow compared with that of the fractions rich in structural proteins. Our results suggest that this low specific radioactivity in cytochrome b and cytochrome oxidase is due to a residual contamination by structural proteins.

MATERIALS AND METHODS

Chemicals. L-[U-^{14}C]Leucine (sp. radioactivity 311 mCi/mmol) and L-[G-^{3}H]leucine (sp. radioactivity 249 mCi/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). The [^{3}H]leucine was diluted with water and then evaporated to dryness to remove any labile ^{3}H.

The following biochemical compounds were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.): ADP (sodium salt), sodium succinate, deoxycholic acid, cholic acid, Triton X-100 and sodium dodecyl sulphate. The L-amino acids (chromatographic grade) were purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Culture media for bacteria were acquired from Difco Laboratories (Detroit, Mich., U.S.A.) as 'bacto-antibiotic medium 3' and 'bacto-nutrient broth'. All other chemicals were of analytical grade whenever commercially available.

Preparation of mitochondria. Ox heart was obtained from the slaughterhouse within the first hour after death under extreme precautions to keep bacterial contamination to a minimum and the heart was washed twice with sterile 0.25 M-sucrose. Mitochondria were prepared as described by Smith (1967) by using his procedure no. 3, starting usually from 1 kg of muscle; succinate was omitted from the medium of fractionation. Light mitochondria were only discarded in the experiment shown in Table 2. Aseptic operative tecnhiques and sterile solutions and materials were applied throughout the entire procedure. Sterilization was carried out by autoclaving at 120°C under steam, at 15 lb/in^2 for 20 min, except for the incubation medium, which was passed through a Millipore filter (HAWP 047 00).

Incubation of mitochondria. Mitochondria were suspended by gentle homogenization in the isolation medium (0.25 M - sucrose – 0.2 mM - EDTA – 0.01 M-tris – HCl buffer,

pH 7.8) at a concentration of 15–30 mg of protein/ml as measured by the method of Lowry, Rosebrough, Farr & Randall (1951), and used either immediately or after several weeks of storage at −20°C, according to the different experiments. The incubations were performed by adding to the medium used by Kroon (1965) one-tenth of its volume of mitochondrial suspension and shaking the mixture in a water bath at 37°C for various times either in test tubes (for 1.0 ml final volume) or in 250 ml conical flasks (for 25 ml final volume). Aseptic operative techniques and sterile solutions and materials were used and the tubes or flasks were loosely stoppered with cotton and gauze to allow access of bacteria-free air. The incubations were stopped by cooling down in an ice bath after addition of 0.1 vol. of a solution of unlabelled 0.1 M-leucine either in 0.15 M-KCl (Tables 1 and 2) or in 50% (w/v) trichloroacetic acid (Figs. 1–4).

Cultivation and incubation of bacteria. Samples (0.1 ml) were taken at different times during the incubation of mitochondria and transferred to plates of 'bacto nutrient broth' supplemented with 0.5% NaCl and 1.5% agar. Colonies were grown under aerobic conditions at 37°C for 48 h and then counted to assess the extent of bacterial contamination. Growth of bacteria for addition to incubation mixtures was carried out by transferring representative samples of these colonies (kept on the plates at 4°C for 2–4 weeks) to a rich liquid medium ('bacto-antibiotic medium 3') and incubating at 37°C until the exponential phase was reached.

Uptake of radioactive leucine by bacteria was determined either in the presence or in the absence of mitochondria under the same conditions as for the incubation of mitochondria. Before the addition of the trichloroacetic acid–leucine solution at the end of the incubations each tube containing bacteria alone received 2 mg of bovine serum albumin as protein carrier.

Extraction and purification of cytochromes. The incubated mitochondria were centrifuged at 450000g-min, the supernatants discarded and the pellets kept at −20°C until extraction of the cytochromes. They were homogenized in 0.9% KCl and extracted twice at 0°C for 15 min either before or after the addition of unlabelled mitochondria. The washings were separated by centrifugation at 450000g-min and pooled. Cytochrome b was isolated and purified by the procedure of Rieske & Tisdale (1967). In our hands this method was not entirely reproducible, probably as a result of the previous incubation of the mitochondria, and in the experiment described in Table 2 the cytochrome b-containing fraction did not behave as indicated in steps 5 and 6 of the original procedure. Therefore a suspension of crude cytochrome b was prepared with bacterial proteinase essentially as described by Ohnishi (1966). The precipitate obtained by 35% saturation with ammonium sulphate was dissolved in 1 mM-sodium dodecyl sulphate–10 mM-potassium phosphate buffer, pH 7.4 and the spectrum was recorded for both the oxidized and the reduced state. A further fraction containing cyrochrome b was obtained by precipitation at 50% saturation with ammonium sulphate from the 35%-saturation supernatant, and the spectrum was similarly recorded.

Cytochrome oxidase was obtained and purified by applying procedure no. 1 of Wharton & Tzagoloff (1967) to the sediment precipitated in step 2 of the method for

purification of cytochrome *b*. The spectrum was recorded as described by Griffiths & Wharton (1961).

Preparation of samples for radioactivity determinations. Several fractions obtained during mitochondrial washings from the purification of cytochrome *b* and cytochrome oxidase were precipitated with trichloroacetic acid (10%, w/v, final concn.) containing unlabelled leucine, either from the original suspensions or from suspensions of the pellets in distilled water. The KCl extract was previously passed through a Millipore filter (HAWP04700). These precipitates and the ones obtained directly from the incubation mixtures of mitochondria were washed as described by Campbell, Cooper & Hicks (1964), except that the ethanol contained potassium acetate (10%, w/v) to decrease protein losses (Munro & Downie, 1964; Steele, Okamura & Busch, 1964). The protein was dissolved by heating in conc. formic acid (A. R. grade) for 30 min at 90°C (water bath). The exact value of the protein concentration was obtained by plating 0.3 ml samples on pre-weighed 7 cm² aluminium discs, desiccating by heating with an i.r. lamp and weighing.

Samples were taken from the suspensions of the cytochromes for protein determination by the method of Lowry *et al.* (1951) and the remainder of each suspension was treated with the trichloroacetic acid–leucine solution. The precipitates were directly dissolved in formic acid as described above.

Radioactivity measurements. Samples (0.2–0.5 ml) of the formic acid solution were mixed with 10–12 ml of the liquid scintillator proposed by Patterson & Greene (1965), containing 2 vol. of toluene, with 0.4% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-(5-phenyloxazol-2-yl)-benzene, and 1 vol. of Triton X-100. The samples were counted in an automatic liquid scintillator–spectrometer (Nuclear–Chicago model 720) to a 1.5% statistical error at 68% probability. Efficiencies were determined for each sample by internal standardization.

RESULTS

Effect of bacteria on the incorporation of [¹⁴C]*leucine into total protein.* Our mitochondrial preparations when incubated in the medium of Kroon (1965) immediately after isolation showed a practically linear incorporation of [¹⁴C]leucine into protein for periods of up to at least 8h (Fig. 1). To test the effect of oxidizable substrates on the incorporation activity, succinate and glucose were added independently to final concentrations of 0.01 M and 0.28 M respectively. Both substrates decreased the incorporation between 2 and 4h but later in the glucose-supplemented medium the original rate was recovered. The replacement at 2h of the unsupplemented medium by a fresh one containing 0.01 M-succinate did not change the pattern of incorporation obtained when succinate was present from the start, with an increase after 4h (Fig. 2).

During 8h incubations of mitochondria in the unsupplemented medium, the number of contaminating bacteria increased very slightly with time (Fig. 3). When these micro-organisms were incubated alone (in the absence of mitochondria) under the same
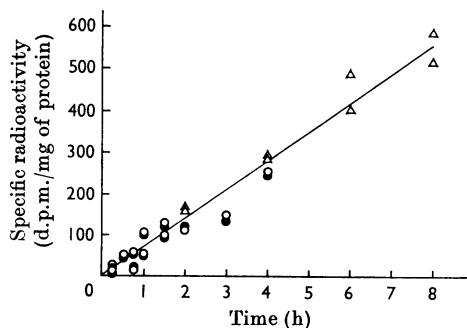

Fig. 1. Time-course of [¹⁴C]leucine incorporation into protein by isolated fresh mitochondria. The incubations were performed with 2.3–3.0 mg of mitochondrial protein in 1.0 ml of the medium described by Kroon (1965) containing (final concns.): 50 mM-tris–HCl buffer, pH 7.4, 50 mM-MgCl₂, 50 mM-KCl, 1 mM-EDTA, 30 mM-potassium phosphate buffer, 2 mM-ADP, 50 μg of a synthetic amino acid mixture lacking leucine (Roodyn *et al.* 1961) and 0.4 μCi of L-[U-¹⁴C]leucine. The points are the results of three separate experiments performed in duplicate. In the experiments represented by △ the number of bacteria/mg of mitochondrial protein increased from 470 at zero time to 830 at 8h. No controls for contamination were done in the experiments marked ○ and ●.
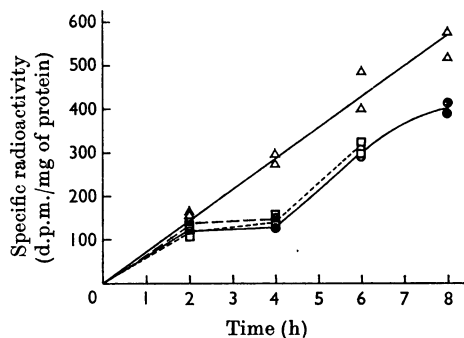

Fig. 2. Influence of substrate addition on the time-course of L-[U-¹⁴C]leucine incorporation into protein by isolated fresh mitochondria. The incubations were performed as described in Fig. 1. In the experiment marked △ normal medium was used. In the experiments marked ○ and □ it was supplemented with 0.01 M-succinate and 0.28 M-glucose respectively (final concns.). In the experiment marked ● normal medium was used for the first 2h and then it was replaced by the 0.01 M-succinate medium for the rest of the incubation.

conditions the results were similar. However, when the incubations were performed with mitochondria that had been kept frozen for 6 weeks at −20°C the number of cells remained approximately constant only during the first 4h and then it increased exponentially. The addition of bacteria obtained in the
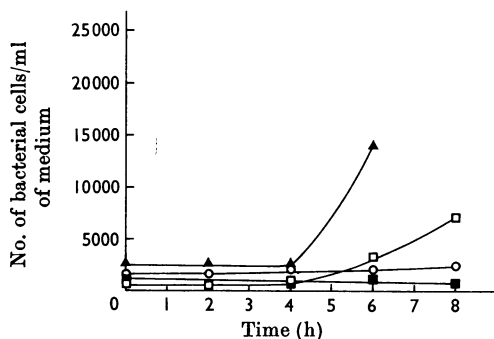
Fig. 3. Bacterial growth in the presence and absence of mitochondria. The incubations were performed in 1.0 ml of medium, with bacteria alone (■), added bacteria plus stored-frozen mitochondria (▲), stored-frozen mitochondria without addition of exogenous bacteria (□) or fresh mitochondria without addition of exogenous bacteria (○). In the experiment marked ▲ the value corresponding to 8 h could not be determined owing to the high number of colonies, but it certainly exceeds 30000 cells/ml.

Fig. 4. Time-course of incorporation of [^{14}C]leucine into protein by bacteria, contaminated mitochondria and contaminated mitochondria plus exogenous bacteria. The incubations were performed as described in Fig. 1. The incorporation by fresh mitochondria (----) is reproduced from Fig. 1. The incorporation by stored-frozen mitochondria is shown by ●. Bacteria alone (■) were incubated under the same conditions as the mitochondria, with 1200 cells/ml at zero time. Another experiment (□) was done with double this number. Bacteria plus stored-frozen mitochondria were treated in the same way, but the initial number was 2700 cells/ml (▲) or 5400 cells/ml (△).

phase of exponential growth to the latter kind of mitochondrial preparation showed a growth curve similar to that for the endogenous micro-organisms (Fig. 3).

A study of the kinetics of incorporation of [^{14}C]-leucine into protein by bacteria alone in the medium of Kroon (1965) at a concentration of 1200 cells/ml (similar to the level of contamination we usually found) showed that there was a considerable incorporation and that this was proportional to the amount of bacteria present (Fig. 4). Also, when bacteria were added to mitochondria stored at −20°C the radioactivity was also proportional to the total number of micro-organisms present. It can be calculated (from Fig. 4) that at 4 h of incubation the values of incorporation for 0.4 μCi of leucine in 1.0 ml of medium containing about 2500 bacteria, either in the absence or the presence of 3.0 mg of mitochondrial protein, were: bacteria alone, 600 d.p.m.; bacteria plus fresh mitochondria, 800 d.p.m.; bacteria plus frozen mitochondria, 900 d.p.m.

Effect of bacteria on the incorporation of [^{14}C]*leucine into purified cytochromes.* We wanted to study whether specific mitochondrial proteins, such as the cytochromes, were labelled during the incubation of mitochondria with little bacterial contamination. Cytochrome c is not synthesized by isolated rat liver mitochondria (Roodyn, *et al.* 1962) but rather by extramitochondrial ribosomes (González-Cadavid & Campbell, 1967; González-Cadavid, Bravo & Campbell, 1968), and, as for cytochrome c_1, the purification is not entirely satisfactory owing to the poor yield of the present methods (Rieske & Tisdale, 1967). For cytochrome b two procedures are described
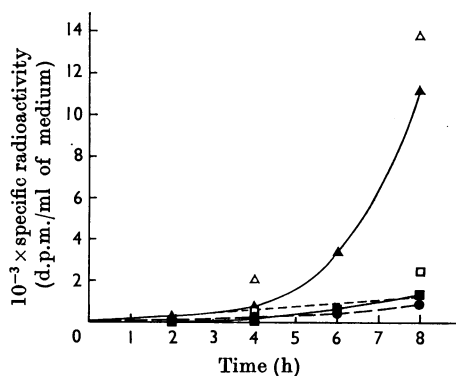
in the literature, both of which yield a product with a low molecular weight, free of other haemoproteins, flavins and, in general, substances absorbing in the visible regions of the spectrum (Ohnishi, 1966; Rieske & Tisdale, 1967). The product having a lower molecular weight and a higher haem/protein ratio is that obtained by the first method, but the cytochrome b prepared in this way probably contains also some mitochondrial structural proteins as contaminants. The E_{279} (ox.)/$E_{414.5}$ (ox.) ratio has a value of 0.77 and the molar extinction coefficient at 561.5 nm (red.) is $24.7 \times 10^3 M^{-1} cm^{-1}$. In the procedure of Rieske & Tisdale (1967) the ratio is not given and the molar extinction coefficient (in a slightly different solvent) for 562.5 nm (red.) − 600 nm (red.) is $20.7 \times 10^3 M^{-1} cm^{-1}$. When applying this technique to the purification of cytochrome b from non-incubated heart mitochondria or from incubated mitochondria mixed with four times its weight of non-incubated mitochondria added as a carrier (Table 1), we encountered some difficulties, but the final product was free from other haemoproteins and flavins as judged from the spectrum. Our preparation had an E_{279} (ox.)/$E_{414.5}$ (ox.) ratio 1.25, indicating that our product was more contaminated with proteins (probably structural) than that described by Ohnishi (1966). In the experiment shown in Table 2 the cytochrome b-containing fraction obtained in the procedure had to be purified further by the method of

Ohnishi (1966), as described in the previous section. The final product had the same characteristics as the one obtained in the other experiments.

With regard to cytochrome oxidase, the method described by Wharton & Tzagoloff (1967) yields a preparation meeting the same criteria of purity as the purified cytochrome b. In both experiments (Tables 1 and 2) the purified cytochrome oxidase had the same spectral properties as reported by Griffiths & Wharton (1961) and was therefore comparatively better purified than cytochrome b.

When freshly obtained ox heart mitochondria were incubated for 4h with [^{14}C]leucine, the residual insoluble proteins from the mitochondria attained a fairly high specific radioactivity (Table 1). If the specific radioactivity of the fractions obtained during the purification of the cytochromes is compared with the specific radioactivity of the residual insoluble proteins, it is seen that the values for the purified cytochromes were three- to four-fold lower. It is obvious that the fraction containing the highest specific radioactivity is therefore eliminated at the step when cytochrome b is separated from cytochrome oxidase and the latter crude fraction is precisely the one richest in structural proteins. This conclusion is supported by the fact that the residues from the purification of cytochrome b, containing variable amounts of structural proteins, are in general more labelled than the purified cytochrome itself.

To assess the real contribution of bacterial contamination to the labelling of total mitochondrial proteins and purified cytochromes an experiment was devised in which ^{3}H-labelled micro-organisms were added to mitochondria previously incubated with ^{14}C (Table 2). The experiment was conducted as follows. Extreme precautions were taken to

Table 1. Incorporation of [^{14}C]leucine into purified cytochrome b, cytochrome oxidase and other fractions by isolated ox heart mitochondria

Mitochondria were incubated in 300ml of medium at a concentration of 1.6mg of protein/ml in the presence of 0.4μCi of L-[U-^{14}C]leucine/ml. After 4h of incubation the supernatant was discarded. The residue was washed twice with iso-osmotic KCl(13% of total protein extracted) and then mixed with unlabelled mitochondria (1.72g of protein) previously washed with KCl. The different fractions were obtained as described in the text and specific radioactivities were corrected to account for the addition of unlabelled material. Bacterial contamination after 4h of incubation was 1950 bacteria/ml (average of four countings), equivalent to 1220 bacteria/mg of mitochondrial protein. The bacterial contaminants were identified as: Staphylococcus albus, Staphylococcus citrus and coliform bacteria.

Fraction	Specific radioactivity (d.p.m./mg of protein)
(1) Proteins extracted with KCl	236
(2) Residual insoluble proteins	630
(3) Main fractions obtained as residues from the purification of cytochrome b	137–294
(4) Purified cytochrome b	204
(5) Purified cytochrome oxidase	160

Table 2. Assessment of the contribution of contamination by structural proteins and micro-organisms to the ^{14}C labelling of purified cytochromes by isolated ox heart mitochondria

Mitochondria were incubated as described in Table 1 with the following differences: (a) 1.5μCi of L-[U-^{14}C]leucine/ml was used; (b) unlabelled mitochondria were incubated under the same conditions as described above and the addition of carrier (756mg of protein from unlabelled mitochondria added to 240mg of protein from labelled mitochondria) was done before the extraction with iso-osmotic KCl; (c) contamination expressed as number of cells/mg of mitochondrial protein was 62 at zero time and 93 at 4h (average of five countings); (d) contaminant micro-organisms were identified as Pseudomonas aeruginosa and yeast. Micro-organisms were obtained and grown as explained in the text and incubated for 4h at a concentration of 3×10^6 cells/ml in medium containing 12.5μCi of L-[G-^{3}H]leucine/ml and the radioactivity incorporated into protein was 316000 d.p.m./ml of medium. To the mitochondria 1.5$\times 10^7$ bacteria (5ml) were added, so that the ratio micro-organisms/mg of incubated mitochondrial protein was 6.2×10^4, roughly 700 times the endogenous contamination. The different mitochondrial fractions were obtained as described in the text and specific radioactivities were corrected to account for the addition of unlabelled material.

	Specific radioactivity		
Fraction	^{3}H (d.p.m./mg of protein)	^{14}C (d.p.m./mg of protein)	^{3}H/^{14}C specific radioactivity ratio
(1) Total proteins	5361	205	26.2
(2) Proteins extracted with KCl (second extraction)	11198	103	108.7
(3) Cytochrome b-containing fraction	2957	291	10.2
(4) Residual proteins obtained from the solubilization of cytochrome oxidase	16733	835	20.0
(5) Purified cytochrome b	1152	124	9.3
(6) Purified cytochrome oxidase	549	50	11.0

diminish the contamination of ox heart at the slaughterhouse, and in this way after 4h of incubation with [^{14}C]leucine the number of micro-organisms/mg of mitochondrial protein were 12-fold less than usual (93 cells/mg of mitochondrial proteins). The micro-organisms were isolated after incubation, grown in a rich liquid medium until exponential growth was attained and then incubated with [^{3}H]leucine. The ^{14}C-labelled mitochondria were mixed with the ^{3}H-labelled cells and after purification the radioactivity was determined in cytochrome b, cytochrome oxidase and other fractions. Table 2 shows that mitochondria themselves do synthesize proteins, since if the [^{14}C]leucine incorporation had only been due to bacterial activity the ^{3}H/^{14}C ratio would have been constant in all fractions. To evaluate the degree of contamination by bacterial protein in every fraction, we assumed, as the most unfavourable situation possible, that in the proteins extracted by potassium chloride (for which the ^{3}H/^{14}C ratio had the highest value) all the ^{14}C incorporated was due to bacteria. The low ^{3}H/^{14}C ratios of the remaining fractions, as compared with that of the potassium chloride-extracted proteins, show that the contamination by bacterial protein was minimal in the purified cytochromes and that most of the ^{14}C specific radioactivity in these fractions was actually due to a real synthesizing ability of the mitochondria themselves. However, comparison of the different ^{14}C specific radioactivities shows that fraction 4 (containing proteins obtained as residue from the solubilization of cytochrome oxidase), presumably rich in structural proteins as indicated by their known behaviour towards different detergents, had a specific radioactivity 16-fold and seven-fold higher than those observed in cytochrome oxidase and cytochrome b respectively. Similarly, in the cytochrome b-containing fraction (fraction 3), differing from purified cytochrome b (fraction 5) in having a higher content of structural proteins, the specific radioactivity was also over two-fold higher than the one corresponding to cytochrome b itself.

DISCUSSION

The studies reported in the literature on the influence of bacterial contamination on the incorporation of amino acids into protein by isolated mitochondria have been carried out with rat liver, yeast or plant mitochondria. The approach is relatively simple and consists of the preparation of sterile mitochondria followed by the application of aseptic techniques for the incubation. The evidence in support of the protein-synthesizing ability of sterile mitochondria is much more conclusive than the opposite view. However, when one is working with material such as ox heart or any other obtained

from sources where the usual laboratory precautions to avoid contamination completely cannot be applied, it is essential to determine the magnitude of the influence of this contamination on the rate of incorporation. This is precisely the point where more significant discrepancies can be found in the literature. Kroon et al. (1967) claimed that, in order to increase by 10% the amino acid incorporation by rat liver mitochondria in a standard 60min incubation, about 2.5×10^5 resting-phase bacteria/ml were necessary, and that 10^5 bacteria/ml did not modify the amount of radioactivity incorporated. On the other hand, Wheeldon (1966), working also with rat liver but under different conditions, showed that the same magnitude of contamination contributed 50% of the total incorporation observed. Essentially the same proportion (about 40%) can be calculated from the values given in Tables 2 and 3 of Beattie et al. (1967). However, it is remarkable that at 3.0mg of protein and 0.50μCi of [^{14}C]leucine/ml the specific radioactivity (in d.p.m./mg of protein) attained by mitochondria in 60min incubations were (recalculated from the original values) 145 (Wheeldon, 1966) and 2110 (Beattie et al. 1967). This is a nearly 15-fold difference in the specific radioactivities. Simpson, Fournier & Skinner (1967), working also with rat liver mitochondria, took an opposite view, claiming that contamination can be decreased from 60000–100000 cells/ml at zero incubation time to 40–1000 cells/ml without any loss in amino acid-incorporating activity.

It is therefore evident that, to determine the real significance of any incorporation of radioactivity into the purified cytochromes by our ox heart mitochondrial preparations, it is necessary to analyse the degree of contribution by the contaminating micro-organisms to the radioactivity of the total mitochondrial proteins. Our results in this respect can be summarized in the following conclusions.

(1) The medium of incubation used by Kroon (1965) and by ourselves in the present study inhibits the exponential phase of bacterial growth for at least 8h of incubation in the absence of mitochondria, even if the micro-organisms are transferred from an exponential-growth culture in a rich liquid medium. However, the incorporation of radioactivity by the bacteria in stationary phase increases, probably in preparation for a new exponential phase of growth.

(2) The inhibition of bacterial growth is not affected for the first 4h of incubation by the presence of fresh or stored-frozen mitochondria. From 4 to 8h there are two different situations: (a) when the mitochondria were stored at −20°C the micro-organisms can reach the exponential phase. The increase in the incorporation of radioactivity is also exponential and the rate is proportional to the growth rate of the micro-organisms. (b) When fresh mitochondria are present, the inhibition of bacterial growth by the

medium of Kroon (1965) is not counteracted and for 8h the bacteria remain practically in the stationary phase, with a slight and linear increase in the specific radioactivity of the proteins. This extends considerably the observations by Beattie *et al.* (1967) and Kroon *et al.* (1967) pointing out the constancy in the number of viable cells for 90 and 60min of incubation respectively. These results could be interpreted by admitting that some factors needed for bacterial growth are present in the medium when stored-frozen mitochondria are incubated. Two possible alternatives can be given to explain why fresh mitochondria do not allow an increase in the number of bacteria: (a) damage to the mitochondrial membrane caused by freezing and thawing is essential for the release of some of these growth factors; (b) a competitive monopoly by fresh mitochondria of some of the factors required by bacteria for entering the exponential phase. In fact, Wheeldon & Lehninger (1966) have shown that there is a very rapid uptake of leucine by isolated mitochondria and it is well known that mitochondria are able to concentrate substrates from the suspending medium (Van Dam & Tsou, 1968).

(3) Fresh ox heart mitochondria at a relatively low level of contamination (500–800 bacteria/mg of mitochondrial protein) show a linear incorporation of [^{14}C]leucine into protein for up to 8h of incubation. This is a period considerably longer than the usual one obtained with rat liver mitochondria (Beattie *et al.* 1967; Simpson *et al.* 1967) and even than the longest reported for ox heart mitochondria (Kroon 1965; Simpson, *et al.* 1967). However, the level of bacterial contamination, although acceptable by the standards of most other authors, has been shown to contribute to the amino acid incorporation observed, and therefore the possibility is open that at later times the incorporation of radioactivity by resting-phase bacteria could have masked a decrease in the mitochondrial activity.

(4) The addition of substrates such as 0.01M-succinate or 0.28M-glucose to the medium of Kroon (1965) inhibits after 2h the incorporation by fresh mitochondria. This confirms the observations of Kroon (1963) on the effect of added succinate and stresses the differences between ox heart and rat liver mitochondria, where an oxidizable substrate such as succinate is an essential constituent of the system (Roodyn, 1965). It is likely that the increase of incorporation observed after 4h reflects an increase in the number of micro-organisms.

(5) Our results after a 4h incubation give a much greater significance to bacterial incorporation than was concluded by other authors on the basis of assessments made during the first hour of incubation, when there is a considerable decrease in the number of micro-organisms. This would mean that conditions at this time are entirely unfavourable for bacterial

protein synthesis, although the situation is reversed later on, even during the stationary phase.

(6) When we purified cytochrome *b* and cytochrome oxidase from mitochondria incubated under conditions of relatively low contamination (Table 1), both cytochromes were labelled by ^{14}C. However, the application of the considerations discussed above lead to a great uncertainty about the role of bacteria in this labelling. The experiment performed with a very low bacterial contamination (Table 2) diminished these doubts since the contribution of micro-organisms to the total radioactivity becomes insignificant at this level of contamination. On the other hand, comparison of the ^{3}H/^{14}C ratios, apart from demonstrating the ability of mitochondria to synthesize proteins, ruled out any possible preferential contamination of the cytochromes by highly labelled bacterial proteins. This clearly demonstrates that the incorporation of [^{14}C]leucine into the cytochromes is due mainly to mitochondrial synthesizing activity. However, the rate of incorporation of [^{14}C]leucine into these proteins is very slow compared with that of the fractions rich in structural proteins. The linkage *in vivo* of the insoluble cytochromes with the structural proteins and the fact that the application of the available purification procedures does not assure a product of absolute purity makes probable the presence of traces of structural proteins in our preparations. This, together with the high labelling of the fractions rich in structural proteins, leads us to assume that the small amount of radioactivity incorporated in cytochrome *b* and cytochrome oxidase is due to contamination by the structural proteins. The relatively more significant incorporation of [^{14}C]leucine into cyrochrome *b* might be due to a higher content of structural proteins derived from the comparatively less satisfactory purification procedure.

The view that isolated mitochondria do not synthesize cytochrome *b* and cytochrome oxidase is consistent with recent results reported for yeast by Henson, Weber & Mahler (1968) and by Criddle & Schatz (1969), suggesting that the great majority of the proteins synthesized during the early phases of respiratory adaptation or release from glucose repression (mainly involving electron transport proteins) are assembled on cytoplasmic ribosomes.

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