BBA 46205

PATHWAYS OF INTRACELLULAR HYDROGEN TRANSPORT IN THE WALKER CARCINOSARCOMA 256

I. THE INTRAMITOCHONDRIAL ELECTRON TRANSPORT AND THE TRANSLOCATION OF REDUCING EQUIVALENTS ACROSS THE MITOCHONDRIAL MEMBRANE

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(Received May 14th, 1971)

SUMMARY

1. Mitochondria isolated from the Walker carcinosarcoma 256 contain cytochromes *a*, a_3, *b* and *c plus* c_1 in an amount corresponding to 10–20% of that present in rat-liver mitochondria. However, the relative proportions of the cytochromes are similar to those of rat liver. Flavoproteins were not detected in the tumour mitochondria. Electron microscopic examination shows that the tumour mitochondria are fewer in number and are somewhat altered morphologically.

2. Rotenone, antimycin A and cyanide inhibited the respiration of tumour slices in the presence or absence of glucose by a maximum of 85–90%.

3. Oligomycin inhibited the endogenous respiration of tumour slices by a maximum of 48.5%, half-maximal inhibition being given at a concentration of about 0.1 μg/ml. The inhibition was only partially relieved by 2,4-dinitrophenol.

4. The activity of all enzymes (except mitochondrial malate dehydrogenase) concerned in the α-glycerophosphate and malate–aspartate redox "shuttles" was greatly reduced in the tumour. In reconstitution experiments on the malate–aspartate cycle the Walker tumour mitochondria were unable to carry out an active oxidation of added NADH. These "shuttles" cannot be very active in this tumour.

5. The present results show that, compared to normal liver, some of the factors involved in the regulation of the $NAD(P)^+$ redox potential are modified in the Walker carcinosarcoma 256. However, comparison with results obtained from other kinds of tumour cells suggests that these modifications may not be the factor responsible for changes in the oxidative metabolism of carbohydrates in this tumour.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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INTRODUCTION

The main efforts in the study of the control mechanisms of the energy metabolism (respiration and glycolysis) in tumour cells have, up to now, been concerned with attempts to demonstrate the important role of the intra- and extramitochondrial phosphate potential ($ATP/ADP \cdot P_i$) (see ref. 1 for review). However, regulation of the energy metabolism could also be achieved through the influence of the intracellular redox potential ($[NAD(P)^+]/[NAD(P)H]$). This, in turn, may be controlled by such factors as the phosphate potential, the translocation of reducing equivalents across the mitochondrial membrane, the mitochondrial and microsomal electron transport and the utilization of reducing power for biosynthetic processes.

In experiments designed to evaluate the possible role of the redox potential in the control of energy metabolism, we have now studied two aspects of the intracellular hydrogen transport in a solid tumour, the Walker carcinosarcoma 256; namely, the intramitochondrial electron transport and the translocation of reducing equivalents across the mitochondrial membrane. The hydrogen transport, carried out by the microsomal fraction, is described in the accompanying paper[2].

The results indicate that mitochondrial electron transport in these cells is sensitive to inhibitors acting at the level of the different coupling sites, and is rather efficiently controlled by the energy conservation reactions involved in the synthesis of ATP. However, "shuttle" mechanisms for the transfer of hydrogen across the mitochondrial membranes appear to be lacking.

MATERIALS AND METHODS

Walker carcinosarcoma 256 was grown in male or female albino rats of the Wistar strain. The tumour, finely minced, was inoculated subcutaneously into the dorsal part of the body of animals weighing 100–150 g. The tumours were used 4–6 weeks after the transplantation. Healthy albino rats, weighing 200–250 g, were used for rat-liver preparations. The tumours appeared white in colour, with small haemorragic areas and grossly visible necrosis restricted to the central part. Only the white regions (freed from the adhering capsule and foreign tissues) were used.

Rat-liver mitochondria were prepared by the method of CHANCE AND HAGIHARA[3]. Walker mitochondria were isolated in mannitol-sucrose medium, in the presence of 0.05 % bovine serum albumin and 2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.4*. Slices, 0.2–0.4 mm thick, were prepared freehand from the tumour tissue with a razor blade guided by a glass slide. Cell-free extracts were obtained by high-speed centrifugation (105000 × *g* for 10 min) of liver and tumour homogenates. Mitochondrial extracts were obtained by subjecting mitochondria to Ultra Turrax (Janke and Kunkel K. G.) disintegration for 8 periods of 15 sec each, at intervals of 30 sec. During the disintegration procedure mitochondria were held in tubes immersed in an ice–salt mixture (about −15°). Disrupted mitochondria were then centrifuged at 105000 × *g* for 30 min and the supernatant fraction collected[4].

Difference absorption spectra (anaerobic–aerobic) and kinetics of NADH (340–375 nm) in isolated mitochondria were performed with a Dual wavelength/split-beam

* According to an unpublished technique for isolation of mitochondria from the Morris hepatoma 3924A (L. PEDERSEN, personal communication).

Aminco–Chance spectrophotometer. Cytochrome concentrations were determined from the difference spectra using the millimolar extinction coefficients reported by ESTABROOK AND HOLOWINSKI[5], except that the cytochrome *a* concentration has been corrected as suggested by VAN GELDER[6].

Electron microscopic examinations were performed in a Philips-300 instrument. Oxygen consumption of tumour slices was measured in a conventional Warburg manometric apparatus during 60 min incubation at 38°. 15–30 mg dry weight of slices were placed in each flask containing 3 ml medium in the main compartment and 0.2 ml of 20 % KOH in the centre well. In the experiments with cyanide, the centre wells contained mixtures of KCN and KOH, as described by ROBBIE[7]. The rate of oxygen uptake was constant throughout the experiment. The incubation medium contained: 140 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 10 mM sodium phosphate, pH 7.4 (ref. 8). Rotenone, antimycin A and oligomycin were dissolved in absolute ethanol. The final ethanol concentration in the incubation medium did not exceed 0.7 %. This concentration of ethanol was also added to the control tumour slices.

The activities of the cytosolic enzymes were determined in the high-speed supernatant of cell-free extracts, and those of the mitochondrial enzymes in the soluble fraction of mechanically disrupted mitochondria (see above). α-Glycerophosphate dehydrogenase (L-glycerol 3-phosphate:NAD^+ oxidoreductase, EC 1.1.1.8) activity was measured in the presence of excess dihydroxyacetone phosphate. The activities of malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) and glutamate–oxalacetate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) were measured according to DELBRÜCK *et al.*[9]. The rate of NADH oxidation was followed at 366 nm in an Eppendorf photometer.

Reconstitution experiments of the malate–aspartate cycle were performed with intact mitochondria, as described by ROBINSON AND HALPERIN[10]. Proteins were determined by the biuret method[11].

Results given in the text and tables are expressed as mean ± standard error of mean with the number of observations given in parentheses.

Rotenone was purchased from K and K Lab. Plainview, N.Y., coenzymes and substrates for enzymic activity determinations were obtained from Boehringer und Soehne (Mannheim). All the other chemicals were products of Sigma (St. Louis) or E. Merck (Darmstadt).

RESULTS

Amount of respiratory pigments in isolated mitochondria

A difference absorption spectrum (380–630 nm) of intact mitochondria isolated from Walker carcinosarcoma 256 is shown in Fig. 1. The α, β and Soret bands of cytochromes $a + a_3$, b and $c + c_1$ are present, whereas the trough due to flavoproteins at 460 nm is lacking. The concentration of cytochromes and flavoproteins calculated from difference spectra of this type are shown in Table I, where the values for the tumour mitochondria are compared with those from rat liver. Besides the lack of flavoproteins, the concentration of the other pigments in the tumour mitochondria is 5–10 times less than that present in rat-liver mitochondria. This probably indicates that mitochondria from the former tissue contain a limited number of cristae. Accord-

ing to calculations of the number of respiratory chains per "average" mitochondrion, as made by ESTABROOK AND HOLOWINSKI[5] on the basis of the cytochrome *a* content of rat-liver mitochondria, one obtains about 1700 respiratory chains per Walker mitochondrion, compared to the 10500 of rat-liver mitochondrion. Table I also shows that the concentrations of cytochromes, relative to cytochrome *a*, are similar in the mitochondria from tumour and liver. The ratio between cytochrome a_3 and *a* is about 1.5 times lower in the Walker than in the liver mitochondria, but this may be due to

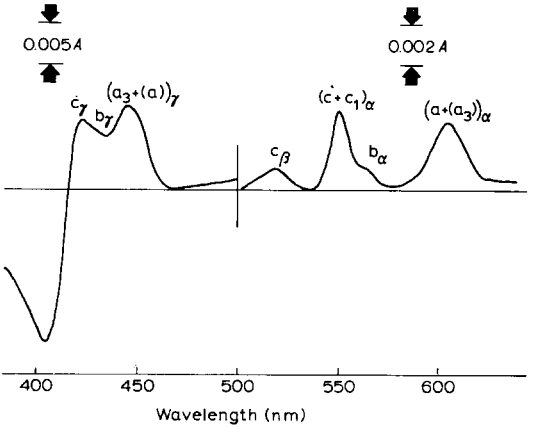

Fig. 1. Difference absorption spectrum of mitochondria isolated from Walker carcinosarcoma 256. 0.9 ml of a mitochondrial suspension containing 22.7 mg proteins per ml were diluted to 6.1 ml with a mannitol–sucrose medium (225 mM mannitol, 75 mM sucrose, 20 mM Tris–HCl, pH 7.4) and 1.7 mM potassium phosphate (pH 7.4) was then added to the suspension. Equal aliquots (3 ml) of the suspension were placed in two cuvettes and, after a base-line was recorded, they were treated differently. 6.7 mM succinate and 1 mM KCN were added to the measuring cuvette (reduced sample), 1 mM ADP was added to the reference cuvette (oxidized sample). The difference spectrum was recorded at room temperature.

TABLE I

CONCENTRATION OF RESPIRATORY PIGMENTS OF WALKER AND RAT-LIVER MITOCHONDRIA

The concentration of pigments of Walker carcinosarcoma 256 has been calculated from difference spectra of mitochondria similar to that presented in Fig. 1 using the molar extinction coefficients reported in MATERIALS AND METHODS. The values for rat-liver mitochondria are those of ESTABROOK AND HOLOWINSKY[5].

Pigment	*Concentration (nmoles/mg protein)*			*Relative concentration***	
	Walker	*Rat liver*	*Rat liver / Walker*	*Walker*	*Rat liver*
Cytochrome *a*	0.020	0.13	6.5	1.0	1.0
Cytochrome *b*	0.018	0.10	5.6	0.9	0.8
Cytochrome $c + c_1$	0.060	0.31	5.2	3.0	2.5
Cytochrome a_3	0.018	0.20	11.0	0.9	1.6
Flavoproteins	—*	0.69	—	—	5.5

* Not measurable.

** Concentration of cytochrome *a* is taken equal to 1 and the other values are relative to that of cytochrome *a*.

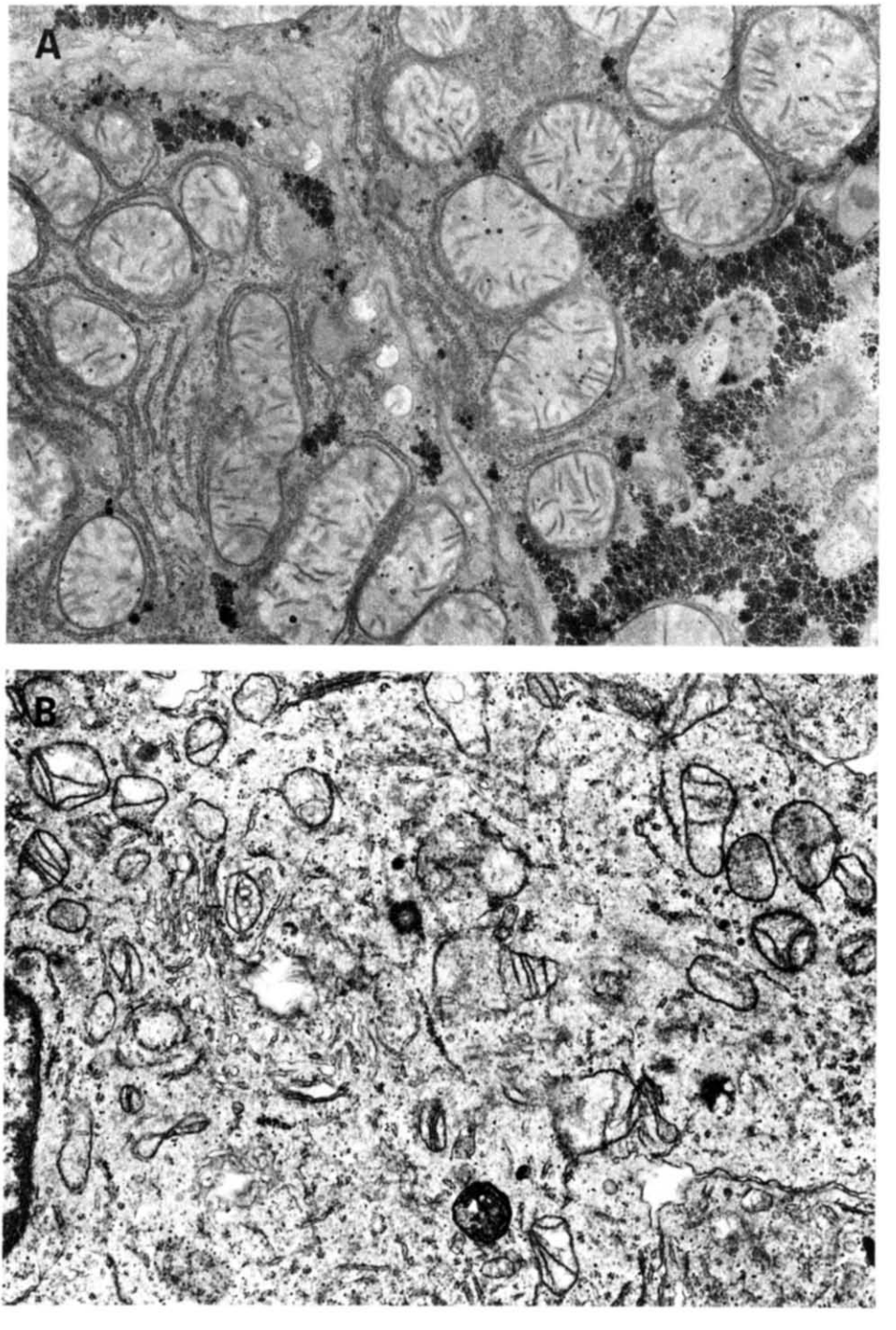

Fig. 2. Electron micrographs of rat-liver (A) and Walker carcinosarcoma 256 (B). The tissues were fixed in 2% glutaraldehyde, followed by 1.33% osmium and stained with 1% lead hydroxide. × 16000.

the value for cytochrome a_3 being made artificially high in the liver by virtue of the effect of the flavoprotein trough on the wavelength pair 444–455 nm.

Fig. 2 shows electron micrographs of rat-liver and Walker carcinosarcoma 256. Tumour mitochondria are decreased in number and are relatively smaller. They are also irregular in shape and have less cristae and matrix content. This is in agreement with our spectrophotometric measurements of cytochromes in isolated mitochondria and indicates moreover that the total mitochondrial protein content per unit tissue is greatly diminished in the tumour with respect to the rat liver.

The effect of inhibitors of the mitochondrial electron transfer on the respiration of slices in the presence and absence of glucose

The effect of inhibitors of the mitochondrial electron transfer (rotenone, antimycin A and cyanide) acting at the level of the three coupling sites has been tested on the respiration of slices prepared from the Walker carcinosarcoma 256. A titration curve of the rotenone inhibition is shown in Fig. 3. Half-maximal inhibition is given by 0.03 μM rotenone and maximal by 1 μM rotenone. However, the maximal inhibition is only about 85 %, and a significant O_2 uptake remains. A very similar rate of O_2 uptake also persists in the presence of antimycin A and cyanide (Table II). Glucose gives a slight Crabtree effect in this tumour which corresponds to an inhibition of

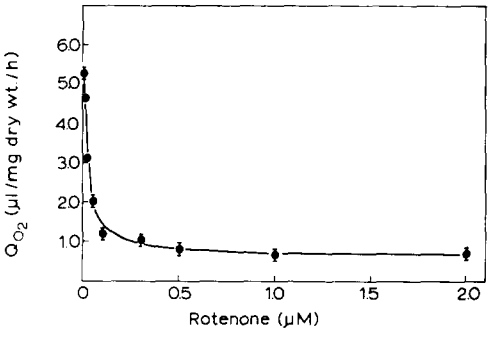

Fig. 3. Titration curve of rotenone inhibition of endogenous respiration in slices of Walker carcinosarcoma 256. Respiration was measured manometrically at 38°. Each point represents the mean of 4–7 experiments; the vertical lines correspond to twice the S.E.

TABLE II

THE EFFECT OF INHIBITORS OF MITOCHONDRIAL ELECTRON TRANSFER ON THE RESPIRATION OF SLICES OF WALKER CARCINOSARCOMA 256

Respiration of slices was measured manometrically during 60 min incubation at 38°. For other conditions see MATERIALS AND METHODS. The values represent the means ± S.E. (number of observations).

Inhibitors	*Respiration (µl O_2 per mg dry wt. per h)*	
	No glucose	*20 mM glucose*
None	5.26 ± 0.10 (13)	4.33 ± 0.28 (14)
Rotenone (2 µM)	0.69 ± 0.06 (7)	0.61 ± 0.04 (4)
Antimycin A (20 µg/ml)	0.54 ± 0.05 (5)	0.40 ± 0.02 (4)
CN^- (1 mM)	0.79 ± 0.09 (4)	0.69 ± 0.07 (7)

respiration by 18% (*cf.* QUASTEL AND BICKIS[12]); but it does not alter the inhibitor-resistant O_2 uptake. As discussed in the accompanying paper[2], the resistance to inhibitors of a portion of the O_2 consumption by the Walker carcinosarcoma cells is probably due to extramitochondrial oxidations similar to those described in rat-liver cells[13–16].

The effect of inhibitors of the energy conservation reactions on the respiration of slices

The use of inhibitors of the mitochondrial energy transduction such as uncouplers or oligomycin in the study of tumour slice respiration allows one to establish the nature of energy coupling in the intact cells. Titration curves of the oxygen consumption of slices of Walker carcinosarcoma in the presence of oligomycin and oligomycin *plus* 2,4-dinitrophenol are shown in the diagram of Fig. 4. A concentration of 1 μg/ml of oligomycin is already able to inhibit maximally (48.5 %) the endogenous respiration, the half-maximal inhibition being given at a concentration of 0.1 μg/ml. Similarly, *N,N'*-dicyclohexylcarbodiimide[17] inhibits the respiration by 43 %. 100 μM dinitrophenol stimulates the endogenous respiration by 8 %. Maximal stimulation of the respiration by the uncoupler is given at concentrations between 50 and 100 μM. The inhibition of the respiration by oligomycin is only partially released by dinitrophenol, so that about half of the respiration which is sensitive to oligomycin may be restored by the uncoupler. The behaviour of the respiration of these slices in the presence of oligomycin *plus* dinitrophenol is different from that of liver slices[18].

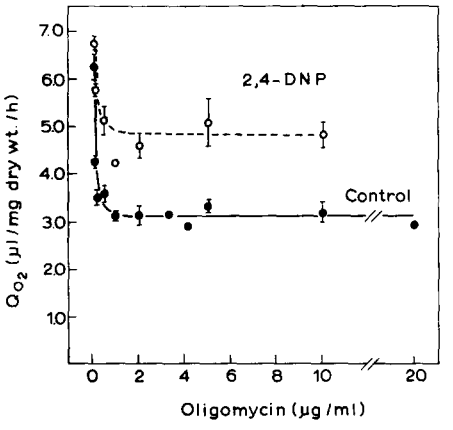

Fig. 4. Effect of different concentrations of oligomycin (●—●) and oligomycin *plus* 100 μM dinitrophenol (○---○) on the endogenous respiration of slices of Walker carcinosarcoma 256. The respiration was measured manometrically at 38°. The vertical lines represent twice the standard error of the mean of 2–11 experiments. 2,4-DNP, 2,4-dinitrophenol.

The transport of reducing equivalents across the mitochondrial membrane

The major mechanisms proposed for the transfer of reducing equivalents across the mitochondrial membrane in mammalian cells are the α-glycerophosphate[19] and the malate–aspartate[20] shuttles. In Table III the cytosolic and mitochondrial enzyme activities involved in these shuttles are compared for rat-liver and Walker carcinosarcoma 256. As far as the α-glycerophosphate shuttle is concerned, the activity of the soluble enzyme, the α-glycerophosphate dehydrogenase, is very low in the Walker

TABLE III

ENZYMIC ACTIVITIES IN THE CYTOSOL AND MITOCHONDRIA OF RAT-LIVER AND WALKER CARCINOSARCOMA 256

The enzymic activities, expressed in μmoles/h per mg protein, were determined as described in MATERIALS AND METHODS. The values represent the means ± S.E. (number of observations).

Tissue	*α-Glycerophosphate dehydrogenase*	*Malate dehydrogenase*		*Glutamate–oxalacetate transaminase*	
		Cytosol	*Mitochondria*	*Cytosol*	*Mitochond*
Rat liver	33.3 ± 3.1 (8)	147.2 ± 7.3 (7)	200.6 ± 30.6 (7)	26.1 ± 1.6 (7)	67.0 ± 5.8
Walker carcinosarcoma 256	0.48*	51.7 ± 4.6 (8)	177.9 ± 12.9 (7)	4.6 ± 0.7 (7)	16.6 ± 3.0

* From BOXER AND SHONK[22].

tumour[21,22]. This low value is not very different from that found in the Ehrlich Lettré ascites tumour cells, where the α-glycerophosphate shuttle does not operate *in vivo*[23,24]. The activities of both the cytosolic enzymes malate dehydrogenase and glutamate–oxalacetate transaminase related to the NAD-dependent shuttle are also much lower in the tumour than in rat liver. Differences in the mitochondrial activities between the two tissues are significant only for the glutamate–oxalacetate transaminase, the activity of this enzyme in the tumour being equal to only 25 % of that present in rat-liver mitochondria. These results indicate a poor functional role of these two shuttles in the Walker carcinosarcoma *in vivo*. Further support for this contention is given by the results of reconstitution experiments[10] of the malate–aspartate shuttle (BORST cycle)[20] performed in intact mitochondria. Fig. 5 shows an experiment carried out in mitochondria isolated from rat liver and Walker. 16.5 μM NADH is added to mitochondria supplemented with malate dehydrogenase and glu-

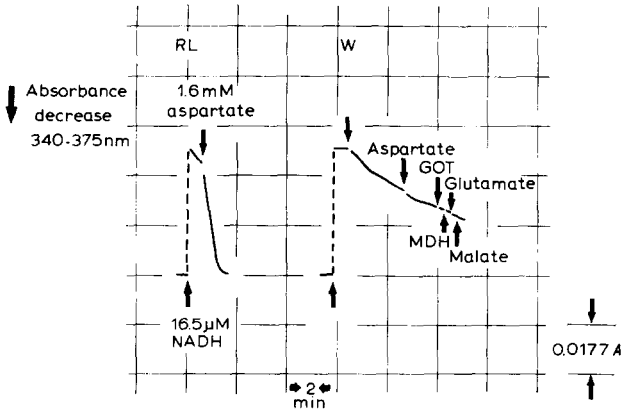

Fig. 5 Reconstitution experiments of the malate–aspartate shuttle with mitochondria isolated from rat liver (RL) and Walker carcinosarcoma 256 (W). The incubation mixture contained in a final volume of 3.0 ml: 125 mM KCl, 20 mM Tris–HCl, 1 mM potassium phosphate (pH 7.4), 1.3 mM ADP, 1.6 mM L-malate, 1.6 mM L-glutamate, dialysed malate dehydrogenase (3.6 units), dialysed glutamate–oxaloacetate transaminase (1.8 units) and 1.2 (RL) or 0.96 (W) mg mitochondrial proteins. NADH oxidation was followed spectrophotometrically at 340–375 nm. Temperature, 30°. GOT, glutamate–oxaloacetate transaminase; MDH, malate dehydrogenase.

tamate–oxalacetate transaminase, together with malate and glutamate. In these conditions the rate of NADH oxidation corresponds to 3.1 ± 0.4 (7) nmoles/min per mg protein for rat-liver mitochondria and is practically zero in Walker mitochondria. The value of NADH oxidation in rat-liver mitochondria thus obtained is 5–10 times higher than that found when NADH is added to mitochondria which have not been supplemented with enzymes and substrates of the shuttle. The subsequent addition of 1.6 mM aspartate strongly stimulates the NADH oxidation in rat-liver mitochondria (to 13.2 ± 1.4 (7) nmoles/min per mg protein), but has only a small, temporary effect in Walker mitochondria before the rate slows down to a value even lower than that observed in rat-liver mitochondria before the addition of the substrate. The extra addition of enzymes and substrates does not improve the system.

DISCUSSION

The control of energy metabolism by the cytosolic and mitochondrial redox potential

The intracellular $[NAD(P)^+]/[NAD(P)H]$ ratio may have a fundamental importance in the regulation of extra- and intramitochondrial metabolic processes. The redox potential of the cytosol represents a factor which tends to direct the reactions of carbohydrate metabolism of this compartment either towards glycolysis or gluconeogenesis. Similarly, in the mitochondria, the redox state of the various respiratory carriers plays a central role in the regulation of the electron flow from the substrates to oxygen or in the reverse direction.

Thus, a knowledge of the factors which contribute to the maintenance of the redox potential of $NAD(P)^+$ in the different intracellular compartments seems to be essential to the study of the control mechanisms of the energy metabolism in tumour cells. The relevant factors that have been studied in this paper will now be discussed.

Composition and organization of the mitochondrial respiratory chain

The spectroscopic determination of the type of respiratory pigments present and of their absolute and relative concentration has allowed us to establish the exact composition of the mitochondrial respiratory chain of the Walker carcinosarcoma 256. Flavoproteins could not be detected by absorption measurements in the mitochondria of this tumour, an observation which has already been reported for other kinds of tumours[25]. The results concerning the relative concentrations of the cytochromes suggest that the tumour has a respiratory chain of similar composition and organization to that of the liver mitochondria, and this hypothesis is supported by the results with site-specific inhibitors of electron and energy transfer. But the analysis of absolute cytochrome concentrations suggests that there are fewer chains per tumour mitochondria, a conclusion which is strengthened by the electron microscopic observation of fewer cristae in the tumour mitochondria than in the liver.

The nature of energy coupling in intact cells

Tumour slices utilizing endogenous substrates respire at a rate of 5–6 μl O_2 per mg dry wt. per h at 38°. This value is only about 2 units less than that found for rat-liver slices[26], despite the finding that the tumour mitochondria only contain 10–20 % of the cytochrome concentration found in liver. It is therefore clear that there is not

a direct relationship between the rate of endogenous respiration of the whole tissue and the concentration of mitochondrial pigments. This fact might have one of the following explanations: (i) a greater portion of the total tumour respiration is due to the activity of extramitochondrial oxidations; (ii) in the Walker tumour the coupling between respiration and phosphorylating reactions is seriously impaired and the oxygen consumption that we measure corresponds more closely to an uncoupled State 3 respiration (State 3u)[27], or (iii) only a partial amount of the respiratory enzymes of rat-liver mitochondria contributes to the maintenance of the resting respiration of the tissue slices. The first possibility may be ruled out on the basis of the experimental results with respiratory inhibitors, since the inhibitor-insensitive respiration may be taken (following results with liver preparations[13-16,28]) to represent the mixed-function oxidase activity of the microsomal fraction of the cell[16,28]. The experiments with oligomycin lead to the rejection of the second possible explanation, since the oligomycin-insensitive respiration (which may be taken as an indication of the degree of uncoupling from ATP synthesis[29,30]) is no more in the tumour cells than in normal tissues[18,31-36]. One unexpected finding, *i.e.*, the ineffectiveness of dinitrophenol in releasing completely the oligomycin inhibition in the tumour, has also been observed by TOBIN AND SLATER[36] in kidney slices.

In conclusion, the only possibility remaining for explaining the observed lack of a relationship between the rate of respiration of tissue slices and the concentration of respiratory pigments in isolated mitochondria is that the support of the basal oxygen consumption of the liver tissue does not require the activity of all the respiratory chains available.

Cytosolic-mitochondrial interactions

The kind of interaction which has been investigated in the Walker carcinosarcoma 256 is that relating the transport of reducing power across the mitochondrial membrane, and the results indicate the near absence of the "shuttle" mechanisms which are supposed to be operating in normal cells *via* flavin and NAD-dependent substrate couples.

As has been already emphasized in the INTRODUCTION, a complete analysis of the regulation system of the redox state of nicotinamide-adenine dinucleotides in Walker carcinosarcoma 256 requires the study of at least two more aspects of the cellular metabolism. They are the utilization of reducing power for biosynthetic reactions and the extramitochondrial electron transport. Studies of this last problem are reported in the accompanying paper[2]. At the moment, from the data presented in this paper, the following conclusions can be drawn: (a) certain features of the oxidative metabolism of the Walker carcinosarcoma 256, namely the intramitochondrial electron transport and the energy conservation reactions, appear to be similar in the conditions studied here to those of a normal tissue such as the rat liver; (b) the striking difference existing between rat liver and the Walker tumour in the activity of the shuttle mechanisms, even though it indicates the existence of a different regulation of the $NAD(P)^+$ redox state in the two kinds of tissue, may not be considered sufficient to account, by itself, for the high rate of aerobic production of lactate. This is in accordance with conclusions reached with other types of tumour cell (*e.g.* ascites tumour cells)[24,37].

ACKNOWLEDGEMENTS

We thank Mr. Guglielmo Palombini for skillful technical assistance and Drs. M. Cagossi and G. Cannella for their help during the first phase of this work. We are grateful to Drs. B. Chance and G. van Rossum for helpful criticism of the manuscript. The work was in part supported by a grant from Consiglio Nazionale delle Ricerche, Italy.

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