ABSORPTION SPECTROPHOTOMETRY OF PERFUSED RAT LIVER APPLIED TO FRUCTOSE-INDUCED INHIBITION OF RESPIRATION

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SUMMARY

In a hemoglobin-free perfusion of rat liver, a single addition of 5 mM fructose caused inhibition of respiration. Simultaneously, the intramitochondrial NAD, fluorescent flavoproteins and cytochrome c were reduced and cytochrome b oxidized. In a rotenone-blocked liver ethanol did not cause any reduction of cytochrome b, indicating that the hydrogen transfer mechanisms between cytosolic NADH and the mitochondrial respiratory chain do not operate above the level of the first phosphorylation site. The relative significance of the respiratory chain and regulation at substrate level in the control of respiration are discussed.

In studies of the oxidative metabolism at cellular or whole-organ level the role of the mitochondrial respiratory chain has been overlooked, probably because of the methodological difficulties encountered. The nicotinamide nucleotides are the only respiratory carriers, in which the metabolic transitions have been extensively studied. An overall picture of the metabolic state of mitochondria expressed as redox potential can be assessed by these methods, but they do not afford direct insight into the respiratory chain itself. The application of fluorometry to intact tissues (1) has been elegantly used by the Chance group (2) and by Scholz and Bücher (3, 4) in studies having metabolic aspects. Reflection spectrophotometry (3) has also been used but the method is hampered by the strong wavelength dependence of its resolution (5). Absorption spectrophotometry, on the other hand has found only sporadic use (6).

The present study was prompted by our observations of respiratory inhibition elicited by injection of fructose into a perfused liver (7). It was evident that the phenomenon was an expression of a mechanism of a mutual regulation between glycolysis and respiration, such as has previously been found in ascites tumor cells (8). In this communication we present evidence for a direct effect of some metabolites of fructose

on the mitochondrial respiratory chain, the latter showing a cross-over phenomenon (9) between the respiratory carriers. The results are relevant to the mechanism of regulation of oxidative metabolism and seem to settle some of the questions that have arisen out of the many hypotheses presented hitherto.

MATERIAL AND METHODS

Male white rats (150-200 g) of the Wistar strain were used as experimental animals. Food was available ad libitum. The perfusion technique was essentially that described by Scholz and Bücher (3). A thermostated rotating disc type oxygenator was used. The perfusion medium consisted of 137 mM NaCl, 3 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.7 mM NaH_2PO_4, and 24 mM $NaHCO_3$, equilibrated with 95 % O_2, 5 % CO_2 at 32°. The flow of the medium was adjusted to 30 ml/min. A perspex cuvette was constructed, which had an optic window and a chamber for an oxygen electrode. The dual wavelength spectrophotometer was constructed at this department by I. Hassinen. A spectral bandwidth of 3.3 nm was used at the measuring wavelength. The image of the slits was focused on a liver lobe in an area 2-3 mm thick. The same perspex cuvette could be used for surface fluorometry of the liver in a Zeiss PMQ II - ZFM 4 spectrofluorometer.

RESULTS AND DISCUSSION

The absorption spectrum of the perfused liver was recorded by applying repetitive 5-min pulses of anoxia, the gas phase being replaced with 95 % N_2, 5 % CO_2 and recording the change in absorbance compared to a reference wavelength (540 nm). The spectrum is closely reminiscent of that of isolated mitochondria and the contribution of the microsomal cytochromes seems to be insignificant (Fig. 1). However, the spectrum shows some distortion in the Soret region, possibly because of a light-scattering artifact due to a change of the effective light path with changing wavelength in the wedge-shaped liver lobe. This does not interfere with the interpretation of the data, since only percentage reductions of the respiratory carriers are meaningful. The redox levels of the cytochromes during endogenous respiration were estimated by using rotenone to approximate a complete oxidation of the cytochromes and NaCN to reduce them completely (Fig. 2). Cytochrome a measured at 605-630 nm was about 25 % reduced in the normoxic state. Cytochrome c measured at 550-540 nm in the same conditions was only 7 % reduced and cytochrome b measured at 434-400 nm closely 50% reduced. Consistent results were also obtained at the α-absorption maximum of cytochrome

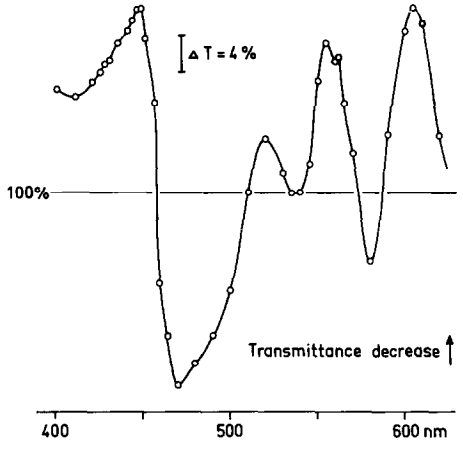

Fig. 1. Anoxic/normoxic difference spectrum of a hemoglobin-free perfused rat liver.
Measuring wavelength was changed between 5-min pulses of anoxia, separated by 10-min phases of normoxia. Reference wavelength 540 nm.

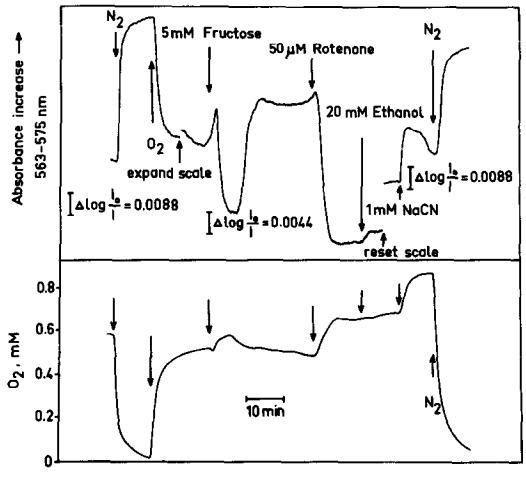

Fig. 2. Redox level of cytochrome b and oxygen consumption of hemoglobin-free perfused rat liver.
Simultaneous measurement of cytochrome b absorbance (563 minus 575 nm)(upper trace) and the oxygen concentration (lower trace) in the outlet cannula in the vena cava inferior. An upward deflection in the upper curve indicates reduction of cytochrome b. The arterial oxygen concentration was 1.01 mM, except in the two cycles of anoxia.

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b (Fig. 2). These results are easily reconciled with the location of the control sites in the respiratory chain and with the redox potentials of the

carriers. Our results are at variance with the results of Scholz and Bücher (3), who with reflectance spectrophotometry obtained lower reduction values.

Primarily the observation of a fructose-induced inhibition of respiration was made during a study of the interactions of the metabolism of fructose and ethanol in liver, and was characterized by a biphasic response (7). Oxygen consumption was initially stimulated and subsequently inhibited. In the initial phase there was concomitant oxidation of NADH and of the fluorescent flavoproteins. In the second phase the same components were reduced. Thus, it was plausible to assume that the phenomenon was due to an effect on the respiratory chain above the fluorescent flavoproteins. It was remarkable that the temporal pattern of the phenomenon was extremely reproducible in experiments with different livers, so that a multiple-parameter approach was possible by comparing parallel experiments. Data from such experiments are plotted on the same

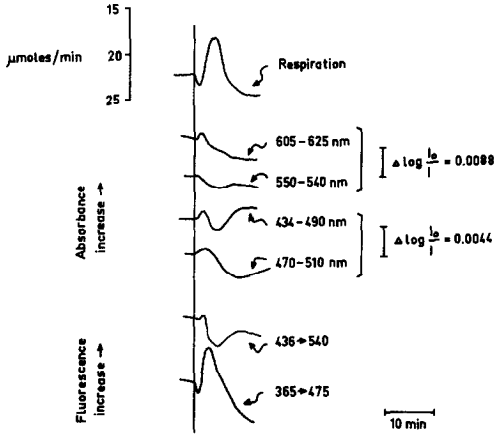

Fig. 3. Fructose-induced changes of respiration and of the redox states of the mitochondrial respiratory carriers in a perfused rat liver.
Fructose (5 mM) was injected to the perfusion medium at the time indicated by the vertical line. Comparison of individual experiments. Fluorescence emission of the nicotinamide nucleotides was measured at 475 nm, with excitation at 365 nm. Fluorescence of the flavins was measured at 540 nm, with excitation at 436 nm. An upward deflection indicates reduction of the cytochromes and NAD(P) and oxidation of flavins.

time scale in Fig. 3. There is a clear cross-over phenomenon between the fluorescent flavoproteins and cytochrome b, and a negative cross-over between cytochromes b and c, The absorbance changes at the wavelengths commonly used for flavoproteins in absorption spectrophotometry show different kinetics. One possible reason for this is that the reduced cytochromes also have an absorbance minimum in the region of the "flavoprotein trough". Another cause of an anomalous response is that the 510 nm region used for a reference point is not kinetically homogenous but shows rapid biphasic changes followed by isosbestic behavior when compared with the 535-540 nm region. This difference from isolated mitochondria from liver must be due to some interfering pigments showing redox-dependent absorption changes in this wavelength region. The fluorescent flavoproteins show the same kinetics as NADH. This, however, is only an indication of the subcellular localization of the NADH response observed, i.e. the mitochondria, in which the fluorescent flavoproteins are in equilibrium with the NADH/NAD couple (10).

It is plausible to assume that the cross-over observed between the fluorescent flavoproteins (10) and cytochrome b is actually effected on the first energy-coupling site of the respiratory chain. The difference between the responses of cytochromes c and a is more difficult to assess. There seems to be a reduction of cytochrome a during the phase of stimulation of respiration and a small oxidation when respiration is inhibited. Actually, this is what is seen in isolated mitochondria during the transition from state 3 to state 4 (11).

According to our knowledge the present data are the first observations in non-cancerous mammalian tissue of a truly respiratory chain-linked phenomenon reminiscent of the Crabtree effect in ascites tumor cells. Previously, retinal tissue seemed to be the only normal tissue in which a Crabtree effect, i.e. inhibition of respiration by glycolysis, has been observed (12). Only indirect evidence of the mechanism of the Crabtree effect has been obtained. The present results seem to provide a means to determine the relative importances of the alternatives offered as explanations of the mutual regulation between respiration and glycolysis. In ascites tumor cells a cross-over behavior of the components of the respiratory chain has been observed, and ADP identified as the cause of respiratory inhibition (13) on the basis of indirect evidence and some analogies with the behavior of isolated mitochondria. However, chemical analyses of inorganic phosphate, AMP, ADP and ATP (14) identify inorganic phosphate as the rate-limiting compound in the inhibition of respiration by fructose in the perfused liver. The steady-state redox

levels of the cytochromes establish the role of the mitochondrial coupling mechanism in the control of respiration in intact tissue. This mechanism can be contrasted with the possibility that processes at the substrate level regulate the terminal oxidations in mitochondria. The synthesis of citrate has been shown to be a rate-limiting step in the tricarboxylic acid cycle (15) and there are many ways in which an energy-linked regulation of this metabolite could be effected in the cell (16). So it seems plausible that the respiration occurring via the tricarboxylic acid cycle is also partly controlled by the direct interaction of the adenylate system with the respiratory chain. The fact that a Crabtree effect is produced in liver tissue with fructose but not with glucose can probably be explained by the higher rate of phosphorylation of fructose (17); another possible explanation is that fructose evades the regulatory step of phosphofructokinase (18). It is also evident that the response observed is not due to biochemical oscillation. The two phases of the response show differing mechanisms of alteration of the phosphate potential: a primary generation of ADP and a secondary depletion of phosphate.

Another important point was easily demonstrated in the experiment presented in Fig. 2. After the mitochondrial NADH dehydrogenase has been blocked with rotenone there seems to be no connection between cytosolic NADH and the mitochondrial cytochromes. This rules out the existence of any functioning hydrogen transport cycles (19) above the first phosphorylation site. As an indicator of the mitochondrial redox state, absorption spectrophotometry of the cytochromes seems to be a more reliable method than the previously used fluorometry of the mitochondrial flavoproteins. The latter method is hampered by the swamping of any redox changes of the flavoproteins by the reduction caused by endogenous substrates in the presence of rotenone, when it is applied to an experiment as in Fig. 2.

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