ON THE STATE OF MITOCHONDRIA IN PERFUSED LIVER: ACTION OF SODIUM AZIDE ON RESPIRATORY CARRIERS AND RESPIRATION

Helmut SIES, Bolko BRAUSER and Theodor BÜCHER
Institut für Physiologische Chemie und Physikalische Biochemie der Universität, München, Germany

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1. Introduction

Using azide, a respiratory inhibitor acting at the cytochrome oxidase site [1], Chance and Williams [2] have obtained shifts of the crossovers in redox changes of respiratory carriers in isolated mitochondria. The inhibitor has since been widely used, for example, in the study of redox patterns of mitochondrial components [3]. Azide is an interesting inhibitor because its actions are dependent upon the control state of mitochondria. While all investigators agree on this point, there are divergent views whether azide acts at the cytochrome oxidase site only [4] or at additional sites within the mitochondria ([5]; see [6] for recent refs.). Extramitochondrial action has also been considered to explain the complex effects of azide on respiration in perfused rat liver [7]. In order to gain an insight into the functional state of the redox carriers as present *in situ*, in the present study the inhibitor was employed in the hemoglobin-free perfused rat liver and redox levels of various cytochomes and of pyridine nucleotides were followed by dual-wavelength absorption and by fluorescence methods, respectively.

2. Experimental

Hemoglobin-free perfusion of livers from female Wistar rats, 120–180 g weight, fed on stock diet, was performed with a solution of Krebs-Henseleit buffer, pH 7.4, containing 7% dextran 40* [8]. No substrates were added. The perfusion fluid (33°C) was equilibrated with a gas mixture of O_2: CO_2: CO (91.5:5.0:3.5). Carbon monoxide was added to convert residual hemoglobin traces (<10 nmoles/g) to the redox-stable form of HbCO; overall respiration was not inhibited by this addition [8,9]. For anoxia, oxygen was replaced by argon.

Dual-wavelength transmission spectrophotometry [10] and surface fluorimetry was performed in a peripheral area of the lower left liver lobe (light path about 2 mm, see [11]). Oxygen concentration was measured in the perfusion fluid entering and leaving the liver with teflon-shielded Ag-Pt (20 μm)-microelectrodes.

3. Results and discussion

3.1. *Spectra in the region of the α-band of cytochrome a*

Azide causes a blue shift of the a^{2+}-α-band by 3–5 nm, first demonstrated at liquid nitrogen temperature [12] and later also in room temperature spectra [13]. This shift is shown in fig. 1 (33°C) for the Hb-free perfused liver system. Addition of azide in normoxia results in a reduction of *a* with a peak at 604 nm. The α-peak of *a* reduced in anoxia is observed at 607 nm in the presence or absence (not shown) of azide.

In view of the titration experiments shown below, it may be noted that the (605–630) nm OD-difference signal remains unchanged in the transition from normoxia plus azide to anoxia plus azide.

* Kindly supplied by Knoll Co., Ludwigshafen.

Table 1
Response of optical density at various wavelength settings to 30 sec azide pulse. Attempt to calculate a molar ratio of azide response for various redox carriers.

Wavelength pair (nm)	Predominant contributing redox carrier	Extinction coefficient[a] ($mM^{-1}cm^{-1}$)	OD change after 30 sec infusion of 0.5 mM sodium azide	'Molar ratio' of azide response [response of *a* taken as unity]
605–630	Cyt. *a*	16	+ 0.013	1
551–540	Cyt. *c*	18	+ 0.004	0.27
564–575	Cyt. *b*	22	+ 0.001	0.06
485–510	Fp	8.5	– 0.002	0.29

[a] Extinction coefficients used are those specific for the one redox carrier named in the second column.

3.2. *Azide titration of cytochromes, pyridine nucleotides and respiration*

The response of the cytochromes *a, c, b,* of *Fp*, of the PNH-dependent surface fluorescence, and of overall respiration is plotted in fig. 2 versus the concentration of sodium azide added to the perfusion fluid. The reduction of cytochrome *a* is observed to commence at azide concentrations <20 μM. Simultaneously, the overall respiration starts to *increase* to a maximum increment of 18% over the steady state rate, reached at about 0.7 mM azide. At this azide concentration, the cytochrome *a* reduction has progressed to about 80% of the final $OD_{(605-630\ nm)}$-increment.

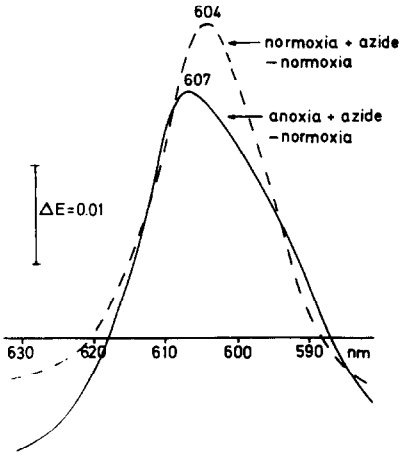

Fig. 1. Difference spectra of perfused liver in the α-band region of cytochrome oxidase. First, the absolute spectrum in normoxic steady state was recorded which serves as the baseline. Then the absolute spectrum was recorded after addition of 2 mM sodium azide to the perfusion fluid. The difference with respect to normoxia is shown as the dotted line. A subsequent anoxia yielded an absolute spectrum which is again plotted as a difference to normoxia (solid line). The peak shift from 604 to 607 nm is clearly visible.

The reduced pyridine nucleotide fluorescence signal remains unchanged up to this azide concentration, indicating that electron flow is not blocked. The PNH-pool becomes reduced at the deflection of the oxygen consumption curve. This is the first sign of an inhibition of respiration by azide in the perfused liver. The initial respiratory rate is reached again with 1.4 mM azide. The $OD_{(605-630\ nm)}$ has then reached its maximum, while PNH fluorescence has arrived at 65% of the anoxia response. A further increase of azide concentration leads to a depression of oxygen consumption by about 15% below the initial steady state rate.

While cytochrome *c* mainly follows the pattern of cytochrome *a*, cytochrome *b* constitutes an exception. Reduction of *b* occurs only when respiration becomes inhibited, just before the PHN increase. The extent of *b*-reduction is maximally 25% of the anoxia response, which is 50% of the antimycin response [11]. Moreover, a reoxidation of *b* is observed as the final level of inhibition of respiration is reached.

The special role of *b* is demontrated again in experiments in an open perfusion system where 30 sec 'azide pulses' (0.5 mM) were given and the different pigments were measured sequentially (table 1). The roughly calculated 'molar ratios' of the azide response show that *b* reacts sluggishly. The $OD_{(485-510)\ nm}$, which in part reflects the flavopro-

tein pool, follows the redox changes in *c* (table 1), while it follows *b* in the titration (fig. 2). This discrepancy requires further analysis.

The azide effects (spectral and respiratory) are readily reversible by perfusion with azide-free fluid. It should be mentioned that the pH in the perfusate declines from 7.4 to about 7.2 during the course of the titration, beginning also at 0.7 mM. The cause of this is under study. With infusion of bicarbonate to readjust the pH, the azide inhibition of respiration can be reversed, as is expected from earlier work by Stannard and Horecker [14]. The azide titration curve is not dependent on the time course of titration. The changes after addition of 0.5 mM azide are stable with time for at least 30 min.

3.3. *Extramitochondrial action of azide in perfused liver?*

In contrast to the experiments on isolated mitochondria, azide effects in perfused liver can, of course, be due partly to extramitochondrial events. One possibility is the inhibition of catalase which is known to occur at very low azide concentration. In analogy to Warburg's discussion of cyanide effects [15], an increase in respiration is expected. This effect (cf. fig. 2) was indeed interpreted in this way by Thurman and Chance [7] in a parallel study. However, when azide was added to livers of rats treated with 1 g 3-aminotriazole/kg body weight 2 hr prior to perfusion, the respiratory response was indentical to that of livers from untreated rats. The pretreatment was reported to inhibit liver catalase activity [16]. 3-Aminotriazole addition to the perfused liver caused slight respiratory increase (5% with 4 mM) but did not alter the azide titration curve.

Furthermore, we observed that in perfused livers inhibited by amobarbital or by rotenone and antimycin, where the tricarboxylic acid cycle and fatty acid oxidation are inhibited, the respiratory increase after addition of azide is abolished (3%). High azide concentrations (4 mM) cannot further inhibit respiration below the rotenone-antimycin level; however, cyanide can do so. These findings do not immediately support the catalase hypothesis under the present experimental conditions. Another explanation for the respiratory response to azide is given in section 3.4.

The extramitochondrial oxygen consuming system which carries out mixed function oxygenation within the microsomes does not appear to be involved. Induction of this system by prior treatment of the animals with phenobarbital (0.1 g/kg for 2 days) did not alter the azide response; subsequent stimulation of respiration by aminopyrine [9] was not affected by the presence of azide.

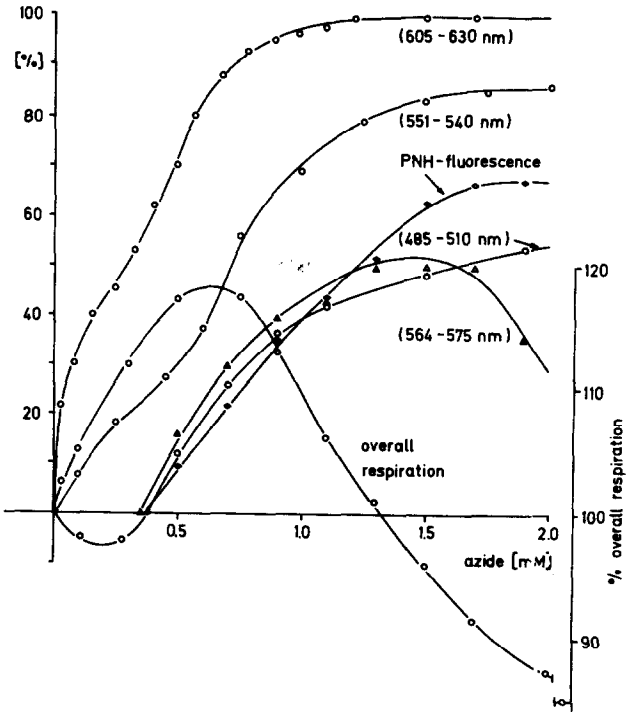

Fig. 2. Dose-response plot for cytochromes *a*, *c*, *b*, fo *Fp*, PNH-dependent surface fluorescence, and for overall respiration in perfused rat liver inhibited by sodium azide. Left ordinate: 100% is the OD-(anoxia after azide) minus OD(normoxia before azide) at the indicated wavelength pairs for cytochromes *a* and *c* and *Fp* (see table 1). For cytochrome *b*, this OD-value was corrected for the antimycin-insensitive portion (50%) of the anoxia response [11]. Note that the redox level in normoxia is taken as a basis (0%). The redox level in normoxia is <2%, 10% and 30% for cyt. *a*, *c* and *b*, respectively. That for *Fp* is unknown. For PNH-fluorescence, 100% represents the normoxia-anoxia response. The normoxic redox level of PNH is not known. Right ordinate: Overall respiration before azide (= 100%) is 220 μatom O/hr/g fresh weight, calculated from the A-V-difference in oxygen concentration and the volume of perfusate passing the liver per unit time. Abscissa: Concentration of azide added to the perfusion fluid.

3.4. *Stimulation of mitochondrial respiration by azide at the level of NAD-dependent oxidations*

Bogucka and Wojtczak [5] reported that in state 4 of isolated mitochondria (for definition see [17]) NAD-linked substrates are oxidized faster in the presence of of 1 mM azide than in its absence. These experiments were repeated here (fig. 3). The stimulation of state 4 respiration of rat liver mitochondria by azide is shown for endogenous substrate (trace A) and for glutamate oxidation (B,C). State 4 oxidation of succinate [12,5] and ascorbate + TMPD [5] is not stimulated by azide.

Moreover, the maximum respiratory increase due to azide was 4–6% in perfused livers from rats fasted for 24 hr compared with 18% in livers from fed rats. In the fasted state the oxidation of NAD-linked substrates is known to be lowered in favor of flavin-linked substrates (fatty acid oxidation).

Thus, a loosening of respiratory control at Site I of energy conservation under state 4 conditions [5] is one possible explanation of the stimulation of respiration by azide. This is supported by the rotenone-antimycin experiments mentioned in section 3.3. An additional factor may be the increased energy requirement due to azide uptake [4].

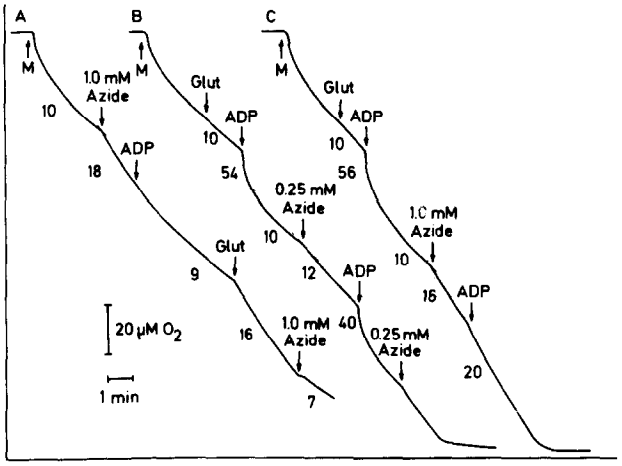

Fig. 3. Stimulation of state 4 oxidation of endogeneous substrate (A) and of glutamate (5 mM) (B,C) by sodium azide. Rat liver mitochondria (2 mg), prepared essentially according to [19], were suspended in 1 ml of assay buffer [20]. Other additions are shown in the figure (ADP was 0.12 mM). Temperature 25°C. Numbers indicate μmole O_2 consumed/min. Same results were obtained with 2 mM malonate present. State 4 oxidation of glutamate was stimulated by 20 and 60% with 0.25 and 1 mM azide, respectively. Note also the inhibition of state 3 respiration in trace C.

3.5. *Control state of perfused liver*

The perfused liver operates close to state 4. This was deduced earlier from the observation that cytochrome *a* could not be oxidized by the addition of amobarbital [18], and from the redox state of cytochrome *b* [11]. Since azide was shown to inhibit mainly state 3 respiration while state 4 respiration is either stimulated or just slightly inhibited [5,12], the maximum azide inhibition of respiration may be used to estimate the control state of perfused liver. When the respiration inhibited by amobarbital is taken as the mitochondrial contribution (70%), the maximum azide inhibition (15%) of total respiration is 22% of the mitochondrial contribution. Addition of 2,4-dinitrophenol (25-50 μM) to perfused liver caused an increase of total respiration to 165%. This increment reflects the uncoupling of mitochondrial respiration and serves as an approximation of state 3 activity (state 3_u). Thus, the mitochondrial respiration is almost doubled (195%) in the transition from normoxia → state 3_u). When the azide-sensitive portion of mitochondrial respiration (22%) is taken as the state 3 contribution to the normoxic steady state, it is seen that the perfused liver operates close to state 4 (130 vs. 260%; 100% being the 'state 4' after azide inhibition of state 3). This means that the respiratory rate is limited by the availability of energy acceptor.

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