Effect of High-Pressure Oxygen on the Steady State of Cytochromes in Rat-Liver Mitochondria

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1. The split-beam spectrophotometer was used to monitor changes in the steady state of cytochrome *c* and cytochromes *a* + *a*₃ during pressurization in pure oxygen. 2. High-pressure oxygen was found to cause oxidation of cytochrome *c* in rat-liver mitochondria, and of cytochromes *a* + *a*₃ at low pH. 3. No difference in these effects was found when various substrates were metabolized. 4. Lowering of pH markedly potentiated the high-pressure effect on the cytochromes. 5. Increased temperature and pressure hastened the reaction to high-pressure oxygen. 6. The oxidation of the cytochromes occurs on the substrate side of cytochrome *c*, probably at the dehydrogenase level, and the time-course of the reaction is compared with effects of oxygen toxicity *in vivo*.

High-pressure oxygen inactivates many enzyme systems, as indicated by a decrease in the oxygen consumption of tissue slices or homogenates, or by a decrease of specific enzyme activity (Bohr & Bean, 1940; Barron, 1936, 1955). Dickens (1946) and Mann & Quastel (1946) demonstrated a toxic effect of oxygen pressure on brain tissue *in vitro* and such studies were extended by Stadie, Riggs & Haugaard (1945*a,b,c,d*), who exposed slices, homogenates or isolated enzymes of several tissues to high pressures of oxygen and followed the subsequent inactivation of respiratory activity. All living systems were 'poisoned' gradually, and enzymes with essential thiol groups were found to be the most oxygen-sensitive, non-thiol enzymes being generally resistant. However, Stadie *et al.* (1945*a,b,c*) were concerned that inactivation developed only slowly, seemingly too slowly to explain the sequelae of symptoms typical of oxygen poisoning *in vivo*. Also, brain tissue of rats that had been exposed to high pressures of oxygen for 30 min. at 7 atm. showed no change in respiration although convulsions had occurred at earlier times. Thus the explanation for the deleterious effect of oxygen remained in doubt. Thomas, Neptune & Sudduth (1963) showed that oxygen at 5 atm. decreased metabolism of brain homogenates quite rapidly, with α -oxoglutarate oxidation being particularly sensitive: the respiratory activity with α -oxoglutarate as substrate declined to 50% of the control level in 30 min.

That enzyme inactivation by oxygen can occur *in vivo* was shown in studies by Jamieson & van den Brenk (1962*a*), who exposed rats to 5 atm. of oxygen

and measured dehydrogenase activity in lung homogenates at suitable intervals. Even at 15 min. there was a definite decrease of activity, well before signs of macroscopic lung damage were apparent.

These methods involved either the assay of the samples after decompression or the use of pressurized manometers in a large compression chamber. In the present investigation, we have followed spectrophotometrically the changes in the steady state of cytochrome reduction in mitochondria under pressure. With this method it was possible to pressurize simply and rapidly under a variety of conditions, and the kinetics of the changes could be observed immediately after compression.

METHODS

Preparation of mitochondria. Rat-liver mitochondria were prepared from Wistar albino rats weighing 200–250 g. Three rats were decapitated, and the livers were removed, minced with scissors and then homogenized rapidly at 0° in 50 ml. of a solution consisting of mannitol (225 mM), sucrose (75 mM) and EDTA (50 μ M), pH 7.0. The fractionation procedure was as follows: (1) 7 min. centrifugation at 480g, to remove cell debris; (2) centrifugation of the supernatant solution at 7700g for 14 min. to sediment mitochondria; (3) washing of the mitochondrial pellet by resuspension in the mannitol-sucrose-EDTA medium and centrifugation for 7 min. at 7700g; (4) two further washings of the mitochondria as in (3), but in EDTA-free medium. All centrifugation was carried out at 0°.

The mitochondria were then suspended in 3 ml. of the mannitol-sucrose medium with tris (10 mM) as a buffer at the appropriate pH (6.7 or 7.4).

Measurements of the steady state of cytochrome reduction. Mitochondria were further diluted before use to give approx. 2 mg. of protein/ml. The dilution medium consisted of mannitol (225 mM), sucrose (75 mM), tris-chloride buffer (10 or 30 mM), serum albumin (0.1%) and $MgCl_2$ (1 mM). The pH was adjusted as required. In preliminary experiments the mitochondrial preparations were titrated with azide after the addition of substrate (Chance & Williams, 1956). A final concentration of 0.5 mM-azide was found to give satisfactory reduction of cytochromes a ($+a_3$) and c , usually 30–60% of the anaerobic level, and this concentration of azide was used as a routine. This high concentration of azide is necessary for producing cytochrome reductions in the 'state 4' condition (Chance & Williams, 1956) and its effect is somewhat greater at lower pH. By poisoning the cytochromes in a semi-reduced state in this manner their absorption peaks can be clearly recorded and subsequent oxidations readily measured.

Substrates were adjusted to the required pH and added in amounts to give a final concentration of 20 mM, so that substrate depletion did not occur during the course of the experiment. Before the addition of substrate or inhibitor, 0.5 ml. of the diluted mitochondrial suspension was added to each cuvette. The split-beam spectrophotometer as described by Yang & Legallais (1953) was used, and an initial base line obtained with both test and reference cuvette containing mitochondria with cytochromes in the oxidized state ('state 1'; Chance & Williams, 1955b). The cuvettes used for these pressurization studies are shown in Fig. 1. Substrate and azide were then added to the test

cuvette and an absorption spectrum from 620 $m\mu$ to 340 $m\mu$ was recorded. This absorption spectrum was repeated, to ensure a steady level of cytochrome absorption peaks, and used as the initial 100% level for measuring the extent of reduction of cytochromes $a+a_3$ and cytochrome c . The test cuvette was flushed with oxygen, then compressed to the required pressure and shaken thoroughly to equilibrate the solution with oxygen. This procedure was completed in approx. 1 min. Another spectral scan was taken just after pressurization; subsequently spectra were recorded at suitable intervals to determine changes in the steady state of the cytochromes. Both the reference and the pressurized cuvette were shaken at frequent intervals to ensure that anaerobiosis did not occur in the mitochondrial suspension in the reference cuvette, and that the pO_2 was maintained constant in the test (pressurized) cuvette. Preliminary experiments showed that without stirring 10–15 min. was required for this concentration of mitochondria to use 1 atm. of oxygen. At the end of each experiment, the cuvette was decompressed and further substrate was added and another absorption spectrum was taken, to ensure that the observed oxidations were not caused by substrate depletion. In the earlier experiments controls in air, with substrate and azide, were also run as described above. No significant changes in controls were found, and therefore in later experiments a control cuvette was set up in air simultaneously with the cuvette for pressurization and the steady state of cytochrome reduction was measured in the control immediately before and after the experimental run was completed.

Correlation of changes in the steady state of cytochrome c reduction and substrate utilization. Simultaneous measurement of absorption due to cytochrome c and respiratory activity were made with the double-beam spectrophotometer fitted with a vibrating platinum electrode as described by Chance & Williams (1955a). The rat-liver mitochondria were diluted to approx. 3 mg. of protein/ml. in mannitol-sucrose-tris buffer, pH 7.2. Rotenone (final concn. 10 μM) was added to inhibit endogenous respiration. Azide (final concn. 0.5 mM) was added to the mitochondria and serial additions of succinate were made to give concentrations of 10 μM to 60 μM ; these concentrations fell within the rapidly changing portion of the plot of cytochrome reduction against substrate. Each subsequent addition of substrate was made when the previous amount of succinate had been fully depleted.

The wavelengths used for measurements of reduced cytochrome c were 550 $m\mu$ with reference to 540 $m\mu$. The rate of succinate oxidation was estimated by two techniques, first with the vibrating platinum electrode (Chance & Williams, 1955a) and secondly by the ' $t_{\frac{1}{2}}$ off' method, from the cytochrome c reduction kinetics, as described by Chance (1943). In the latter method, a proportionality would be expected with the steady-state concentration of reduced cytochrome c and the ratio of the initial succinate concentration to ' $t_{\frac{1}{2}}$ off' (the time from half-maximal reduction of cytochrome c to half-maximal reoxidation of cytochrome c as the substrate is depleted).

Measurement of oxygen uptake. Respiration rate was measured with the vibrating platinum electrode by the technique described by Chance & Williams (1955a). Mitochondria were diluted to approx. 3 mg. of protein/ml. in mannitol-sucrose-tris buffer and succinate (20 mM), pH 7.4. A sample of this suspension was compressed to 12 atm. for 30 min. at 24° and another sample was compressed to

Fig. 1. Pressure cuvette shown in the cuvette holder. The reference cuvette (A) on the left remains open to air. The test cuvette on the right (B) is kept under pressure directly (C) from an oxygen cylinder. The cuvette holder (D) is sealed with an 'O' ring seal (E).

12 atm. for 1 hr. at 24°. Control samples of mitochondria were maintained in air at 1 atm. for the same periods. Respiratory rates were measured in the presence of inorganic phosphate immediately after decompression.

Fig. 2. Correlation of the steady state of cytochrome *c* reduction in mitochondria with the substrate oxidation rate. Details are given in the text. ●, Substrate oxidation measured by '*t*_{1/2} off' method; ▲, respiration rate measured with platinum electrode.

RESULTS

*Substrate oxidation and the steady state of cytochrome *c* reduction.* The correlation between the rate of utilization of substrate (succinate) and the steady state of cytochrome *c* reduction is shown in Fig. 2. The extent of reduction of cytochrome *c* is plotted against the rate of oxidation of succinate at a given concentration of this substrate. There is a linear relationship between these parameters, whether rate of succinate oxidation is measured directly by the oxygen electrode or calculated by the '*t*_{1/2} off' method. Thus the changes in the steady state of cytochrome *c* may be taken as a measure of the rate of substrate oxidation.

*Changes in the steady state of cytochrome *c* and *a* + *a*₃ reduction in mitochondria exposed to high-pressure oxygen.* In this series of experiments we examined the effect of high oxygen pressure on the steady state of cytochrome reduction in mitochondria metabolizing various substrates at room temperature (24°) or 38°, and at pH 7.4 or pH 6.7 (Table 1).

After treatment with azide the cytochromes are most readily measured by their absorption in the Soret region, where prominent peaks at approx. 445 mμ and 415–420 mμ appear, due to reduced cytochromes *a* + *a*₃ and cytochrome *c* respectively (Chance & Williams, 1956). After oxygen pressurization the absorption peak of reduced cytochrome *c* in the Soret region gradually diminished and under certain conditions the cytochrome *a* + *a*₃ band also decreased, indicating a more oxidized state of these components in the respiratory chain. The broader α-band of cytochrome *c* was apparent after azide treatment and decreased during exposure to high-pressure oxygen, as did the Soret band, but the

Table 1. Changes in the steady state of cytochrome *c* and *a* + *a*₃ reduction induced by hyperbaric oxygen

The final concn. of substrate was 20 mM and that of azide 0.5 mM. Other details are given in the text. The results are given as means ± S.E.M.

Temp.	Pressure (atm.)	Substrate (no. of trials)	pH	Oxidation of cytochrome <i>c</i>		Oxidation of cytochrome <i>a</i>	
				Rate: <i>t</i> _{1/2} max. (min.)	Extent (percentage effect)	Rate: <i>t</i> _{1/2} max. (min.)	Extent (percentage effect)
24°	12	Succinate (10)	7.4	23 ± 2	75 ± 4	—	< 10
24	12	α-Oxoglutarate (3)	7.4	22 ± 5	76 ± 10	—	< 10
24	12	Malate + glutamate + malonate (3)	7.4	19 ± 2	65 ± 4	—	< 10
24	12	Succinate (3)	6.7	8 ± 3	70 ± 8	8 ± 3	25 ± 7
24	12	α-Oxoglutarate (5)	6.7	9 ± 2	89 ± 6	7 ± 1	60 ± 4
38	12	Succinate (3)	7.4	7 ± 1	85 ± 9	—	< 10
38	12	α-Oxoglutarate (2)	7.4	6 ± 1	90 ± 0	—	< 10
38	12	Succinate (3)	6.7	2 ± 0.5	95 ± 4	5 ± 1	35 ± 5
38	12	α-Oxoglutarate (2)	6.7	1.5 ± 0	93 ± 3	3.5 ± 0.5	65 ± 2
38	5	Succinate (3)	7.4	27 ± 3	57 ± 13	—	< 10
38	5	Succinate (3)	6.7	10 ± 2	83 ± 8	13 ± 4	20 ± 2

Fig. 3. (a) Difference spectra obtained with the split-beam apparatus, showing the gradual decline in size of the peaks for reduced cytochrome *c* and *a+a₃* after compression to 12 atm. The reference cuvette contained mitochondria without added substrate with cytochromes in the oxidized state, and succinate (final concn. 20 mM) and azide (final concn. 0.5 mM) were added to the test cuvette, pH 6.7, at room temperature. The uppermost trace (trace A) shows the difference spectrum before pressurization, with the cytochrome peaks indicated. Trace B was taken 3 min., trace C 8 min. and trace D 15 min. after compression. (b) Difference spectra with the preparation in the test cuvette allowed to become anaerobic before pressurization (i.e. an oxidized—reduced difference spectrum.)

smaller size of the absorption band in the α -band region made measurement of this latter peak more difficult. The changes in absorption spectra under conditions of high-pressure oxygen are shown in Fig. 3.

The rate of oxidation of the cytochromes in high-pressure oxygen was dependent on the incubation conditions of the mitochondria during the hyperbaric treatment and showed strong positive correlation with decreasing pH, increasing temperature and higher pressures.

*Effect of various factors on hyperbaric-oxygen-induced oxidation of cytochromes c and *a+a₃* in mitochondria.* (a) Effect of substrate. Three separate substrate systems were examined, namely the succinate-oxidation pathway, the NAD-linked malate-glutamate path and the more complex α -oxoglutarate-oxidase reaction, to attempt to establish whether the changes in the steady state of the cytochromes reflected differential sensitivity to oxygen at some critical substrate site, as suggested by the results of Thomas *et al.* (1963).

Fig. 4 compares the changes seen under pressure with succinate or malate-glutamate as the substrate, the latter being used in the presence of malonate. Both experiments were carried out with the same mitochondrial preparation and no difference was found when the various substrates were metabolized. A similar effect is seen in Fig. 5, where succinate and α -oxoglutarate were compared in an experiment with the same preparation, again at room temperature and pH 7.4. Table 1 shows that the changes in the steady state of cytochrome *c* (and *a+a₃*) reduction were independent of the substrate being metabolized during exposure to high pressures of oxygen.

(b) Effect of pH. Lowering the pH of the solution caused a dramatic increase in the reaction rate at high oxygen pressure. At pH 6.7 and room temperature, the oxidation of cytochrome *c* proceeded about two to three times as quickly as at pH 7.4 (Table 1). At 38° a similar and possibly even more marked effect of pH was observed. Again there appeared to be no substrate dependence in the

Fig. 4. Effect of substrate on the steady state of cytochrome *c* and *a*+*a*₃ reduction in mitochondria after compression to 12 atm. The substrates were: (a) succinate (final concn. 20 mM); (b) malate (final concn. 20 mM), glutamate (final concn. 20 mM) and malonate (final concn. 20 mM). The mitochondria were diluted in mannitol-sucrose plus tris buffer, pH 7.4 (10 mM), and the experiment was carried out at room temperature (24°). Azide (final concn. 0.5 mM) was added to the test cuvette. ●, Cytochrome *c* (pressure); ○, cytochromes *a*+*a*₃ (pressure); ■, cytochrome *c* (control in air); □, cytochromes *a*+*a*₃ (control in air). The same mitochondrial preparation was used for all scans.

Fig. 5. Effect of substrate on the steady state of cytochrome *c* and *a*+*a*₃ reduction in mitochondria after pressurization to 12 atm. The substrates were: (a) succinate (final concn. 20 mM); (b) α -oxoglutarate (final concn. 20 mM). The mitochondria were diluted in mannitol-sucrose plus tris buffer, pH 7.4 (10 mM), and the experiment was carried out at room temperature (24°). Azide (final concn. 0.5 mM) was added to the test cuvette. ●, Cytochrome *c* (pressure); ○, cytochromes *a*+*a*₃ (pressure); ■, cytochrome *c* (control in air); □, cytochromes *a*+*a*₃ (control in air). The same mitochondrial preparation was used for all scans.

oxygen-induced reaction. Another noteworthy finding was that the changes in the steady state of cytochromes *a*+*a*₃ were more apparent at the lower pH. It was difficult to see any definite change in the steady state of cytochromes *a*+*a*₃ at pH 7.4 even when the absorption of reduced cytochrome *c* was greatly diminished, but at pH 6.7 the steady state of cytochromes *a*+*a*₃ changed, with a *t*₁ maximum (time for half-maximal effect) somewhat slower than

that for cytochrome *c* at 38°, but with about the same *t*₁ maximum as that of cytochrome *c* for oxidation at room temperature. However, the changes in the steady state of cytochromes *a*+*a*₃ were never quite as pronounced as the change in the steady state of cytochrome *c* (Fig. 6).

(c) Effect of temperature. The oxidation of cytochrome *c* (and *a*+*a*₃) by high-pressure oxygen occurred much more rapidly at 38° than at room

Fig. 6. Effect of pH on the changes in the steady state of cytochrome *c* and *a* + *a*₃ reduction in mitochondria in 12atm. of oxygen: (a) pH 7.4; (b) pH 6.7. The highly increased rate of oxidation at pH 6.7 is clearly seen, and also the pH difference in the effect on cytochromes *a* + *a*₃. The substrate was α -oxoglutarate (final concn. 20mM). The mitochondria were diluted in mannitol-sucrose in tris buffer (10mM), and the experiment was carried out at 38°. Azide (final concn. 0.5mM) was added to the test cuvette. ●, Cytochrome *c* (pressure); ○, cytochromes *a* + *a*₃ (pressure); ■, cytochrome *c* (control in air); □, cytochromes *a* + *a*₃ (control in air). The same mitochondrial preparation was used for all scans.

temperature (Table 1). This effect is clearly seen by comparing the traces for α -oxoglutarate in Fig. 5 (room temperature) and Fig. 6 (38°).

(d) Effect of pressure. Most of these experiments were carried out at 12atm. of oxygen. At this pressure the reaction occurred rapidly, such that comparisons of substrates and pH values could be made conveniently at room temperature. Subsequently, it was considered necessary to study the kinetics of the changes in the steady state of the cytochromes at physiological temperatures, and owing to the rapidity of the changes at this temperature it was possible to use a lower pressure. Jamieson & van den Brenk (1962*a,b*, 1963) had previously carried out many investigations at 5atm. in rats, including studies of inactivation of dehydrogenases and oxidation of thiol groups, and Thomas *et al.* (1963) also tested brain tissues at 5atm. Therefore this pressure was chosen for comparison with effects at 12atm. Fig. 7 demonstrates that the changes in the steady state of cytochrome *c* occurred much more slowly at this lower pressure. In addition, the extent of the change in reduction of cytochrome *c* appeared to be less at this lower pressure, although this was possibly due to failure to reach a true plateau because of the slowness of the reaction (Table 1). At pH 7.4 and 38° the control (air) samples did not show deterioration during 1 hr. However,

at pH 6.7 and 38°, the control mitochondria also showed slight changes after 30–35min. incubation, as shown in Fig. 8, and more rapid changes and precipitation after this time-interval.

Control experiments. To ensure that the observed changes in the steady state of cytochrome reduction were not due to the effect of hyperbaric oxygen in oxidizing azide itself, solutions of azide of pH 7.4 or 6.7 were compressed to 12atm. for 60 and 30min. respectively and then compared with control azide solutions by titration with mitochondria.

Azide solutions (5mM) in mannitol-sucrose-tris buffer at the required pH were pressurized at room temperature, and were found to be no different from similar solutions of non-pressurized azide. The experiment was performed twice at each pH.

The corollary of this experiment gave a similar result, i.e. a sample of mitochondria was pressurized at pH 6.7 for 20min. at room temperature, without prior addition of azide. After decompression these mitochondria were titrated with azide and compared with mitochondria stored in air at 1atm., under similar conditions of pH and temperature. The pressurized mitochondria showed almost no cytochrome *c* absorption, and cytochrome *a* + *a*₃ bands were decreased to approx. 50% of the control values. These experiments showed that the changes in the steady state of the cytochromes were due to changes

Fig. 7. Effect of oxygen pressure on the rate of change in the steady state of cytochrome *c* and *a*+*a*₃ reduction in mitochondria. A much lower rate of oxidation of cytochrome *c* occurs at 5 atm. (b) than at 12 atm. (a). The substrate was succinate (final concn. 20 mM). The mitochondria were diluted in mannitol-sucrose in tris buffer, pH 7.4 (10 mM), and the experiment was carried out at 38°. Azide (final concn. 0.5 mM) was added to the test cuvette. ●, Cytochrome *c* (pressure); ○, cytochromes *a*+*a*₃ (pressure); ■, cytochrome *c* (control in air); □, cytochromes *a*+*a*₃ (control in air). The same mitochondrial preparation was used for all scans.

Fig. 8. Effect of lowered pH on the steady state of cytochrome *c* and *a*+*a*₃ reduction in mitochondria at 5 atm. A more rapid rate of oxidation occurred at pH 6.7 than at pH 7.4 (cf. Fig. 7b). The substrate was succinate (final concn. 20 mM). The mitochondria were diluted in mannitol-sucrose in tris buffer, pH 6.7 (10 mM), and the experiment was carried out at 38°. Azide (final concn. 0.5 mM) was added to the test cuvette. ●, Cytochrome *c* (pressure); ○, cytochromes *a*+*a*₃ (pressure); ■, cytochrome *c* (control in air); □, cytochromes *a*+*a*₃ (control in air). The same mitochondrial preparation was used for all scans.

in the mitochondria and were not due to destruction of azide by hyperbaric oxygen.

Respiratory rate of mitochondria after compression. Hyperbaric oxygen caused a time-dependent

Table 2. *Respiratory rate of mitochondria after compression to 12 atm. of oxygen at pH 7.4 and 24°*

The substrate was succinate (final concn. 10 mM). Other details are given in the text.

	Respiratory rate (μmoles of O ₂ /l./min.)		
	Initial	After 30 min.	After 60 min.
Sample 1			
Air at 1 atm.	24	24	22
O ₂ at 12 atm.		14	12
Sample 2			
Air at 1 atm.	26	26	22
O ₂ at 12 atm.		17	12

decrease in the respiration rate of mitochondria, and the results are shown in Table 2.

DISCUSSION

The changes in the steady state of cytochrome reduction provide a sensitive kinetic monitor of disturbances in the electron-transport chain. It is well known that inhibition at any site along the electron chain causes reduction of components on the substrate side and oxidation on the electron acceptor side of the inhibition. The site of action itself is termed the cross-over point (Chance, Holmes, Higgins & Connelly, 1958). Thus inhibition of electron transport at the terminal site produces reduction of all cytochromes, flavoproteins and nicotinamide nucleotides in the electron-transport

chain whereas inactivation of the dehydrogenase or substrate depletion leads to oxidation of all these components.

In the present study we have used these reactions to study the effects of high pressures of oxygen on isolated mitochondria. Azide, a terminal inhibitor of the electron-transport chain, was used in a concentration producing partial reduction of components of the electron-transport chain, so that the absorption peaks of the reduced cytochromes became apparent (Chance & Williams, 1956) and changes in their magnitude could readily be observed. For these experiments the steady state of cytochrome *c* was best poised in the direction of reduction, as occurs with azide, since inhibition of electron transfer would cause cytochrome oxidation. The results obtained in the present study (Fig. 2) show that under the experimental conditions employed there was a linear relationship between the extent of cytochrome *c* reduction and the rate of substrate oxidation, as calculated from analogue-computer studies (Chance, 1957).

In the present work the cytochrome components that have been studied in detail are cytochrome *c* and cytochromes *a* + *a*₃, and on exposure of mitochondria to hyperbaric oxygen the steady states of both these cytochromes changed in the direction of oxidation (decrease in size of the absorption peaks), indicating that inhibition of electron flow occurred on the substrate side of cytochrome *c*. In view of other data available on respiratory inhibition at high oxygen pressures, it seems appropriate to identify the site of inactivation at the dehydrogenase level. The observed oxidations are not due to reversal of azide inhibition by high-pressure oxygen as azide was unaffected by such treatment. In addition, independent tests with the platinum micro-electrode have shown that the respiratory rate of the mitochondria diminishes at high oxygen pressures. Thus the increased oxidation of cytochrome *c* is a measure of the inhibition of electron flow in the chain and spectrophotometric observation affords a convenient indication of this activity.

Several comparisons may be made between the experiments with isolated rat-liver mitochondria described above and oxygen poisoning in the whole animal. Increased acidity potentiates oxygen poisoning *in vivo* (Behnke, Shaw, Shilling, Thompson & Messer, 1934; Bean, 1945; Laborit, Brousselle & Perrimon-Touchet, 1957; Marshall & Lambertsen, 1961; van den Brenk & Jamieson, 1964), and in the present experiments lowering of the pH of the medium markedly decreased the time taken for cytochrome oxidations under hyperbaric conditions. Extreme dependence on temperature and pressure are shown in the present studies with isolated mitochondria, as is of course true *in vivo*.

In Fig. 9 we have compared the rate of oxidation

of cytochrome *c* under the present experimental conditions with previous data obtained *in vivo* and *in vitro* by various investigators. The present system studied by us shows changes before the homogenates or tissue slices examined by Stadie *et al.* (1945*a,b,c*). This is more prominent as our rates of inactivation for 5 atm. exceed the respiratory decline at 7 atm. in the work of Stadie and his co-workers. Probably coincidentally, our present results for 5 atm. of oxygen show a time-course of inactivation almost the same as that found for the depression of dehydrogenase activity in the lungs of rats that had been exposed to oxygen at 5 atm.

Thomas *et al.* (1963) found significant depressions of respiratory activity in brain slices after 30 min. at 5 atm. of oxygen when α -oxoglutarate was metabolized. Although their results with α -oxoglutarate agree reasonably well with the results presented above, a large difference in substrate effect is apparent between our results and those of Thomas *et al.* (1963), who found little effect at high oxygen pressure if succinate was being metabolized and that an inactivation of respiration occurred fairly rapidly when brain homogenates were exposed to high oxygen pressure with α -oxoglutarate or pyruvate as the substrate, thus substantiating previous work, also on brain tissue, by Dickens (1946) and Mann & Quastel (1946). This led Thomas *et al.* (1963) to postulate that a cofactor in these latter oxidase systems formed the critical point for oxygen toxicity, with lipoic acid as a strong candidate for this site. Our present results do not support this conclusion, for under a wide variety of conditions no differential substrate effect was observed,

Fig. 9. Comparison of the inactivation of respiratory activity in various systems at pH 7.4 and 37–38°. ▲, Respiration of cerebral cortical slices at 2.9 atm. (Dickens, 1946); ■, oxygen consumption of kidney slices at 7 atm. (Stadie *et al.* 1945*b*); ○, succinate-dehydrogenase activity of lungs of rats compressed *in vivo* at 5 atm. (Jamieson & van den Brenk, 1962*a*); ◆, changes in the steady state of cytochrome *c* reduction in rat-liver mitochondria at 5 atm.; ●, changes in the steady state of cytochrome *c* reduction in rat-liver mitochondria at 12 atm.

indicating that, if indeed the oxidation of cytochrome observed is due to action at a substrate level, all the dehydrogenases are fairly equally sensitive to oxygen inactivation. From previous investigations it appears that there may be a real difference between tissues with regard to substrate effects on oxygen toxicity, with brain being more sensitive when pyruvate or α -oxoglutarate is metabolized. The results of Wood & Watson (1963a,b, 1964) and Wood, Watson & Clydesdale (1963), who used γ -aminobutyric acid as a protective agent against oxygen-induced brain damage, appear to support this view.

Although the inhibition of electron-transport reactions appears to correlate reasonably with time for pulmonary damage to occur *in vivo*, the problem of poisoning of the central nervous system by hyperbaric oxygen remains unresolved. In animals dying from exposure to high oxygen pressures no decrease in brain oxygen consumption (Stadie *et al.* 1945a) or dehydrogenase activity (Jamieson & van den Brenk, 1962a) could be demonstrated. Thus it appears that an earlier biochemical lesion occurs, preceding the changes reported above or those found in previous studies. The rapid and reversible oxidation of nicotinamide nucleotides found *in vitro* and *in vivo* during exposure to high-pressure oxygen (Chance, Jamieson & Coles, 1965; Chance, Jamieson & Williamson, 1966) occurs earlier than dehydrogenase inactivations and it is possible that the inactivations at a substrate level are the consequence of this altered reduction-oxidation state.

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