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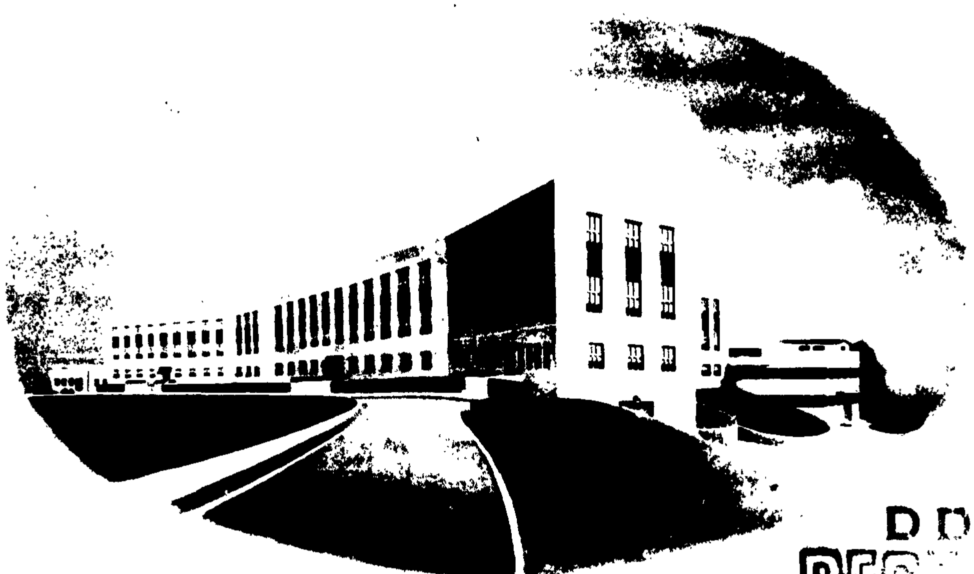

TOXIC EFFECTS OF OXYGEN AT HIGH PRESSURE ON THE METABOLISM OF D-GLUCOSE BY DISPERSIONS OF RAT BRAIN

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Toxic Effects of Oxygen at High Pressure on the Metabolism of D-Glucose by Dispersions of Rat Brain

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Previous extensive investigations of the neurochemical toxicity of oxygen at high pressure have resulted in a major problem: the neurotoxic phenomena *in vivo* occurred much more rapidly than did the neurochemical abnormalities *in vitro*. Exposures of men and animals to oxygen at pressures of from 3 to 8 atm. have resulted in the rapid occurrence of such severe neurotoxic phenomena as grand mal convulsions, commonly in less than 30 min. at the higher oxygen pressures (Donald, 1947*a*, *b*; Dickens, 1955). Experiments *in vitro* required much longer periods of exposure to oxygen at high pressure before toxic manifestations in brain metabolism could be detected. The respiration of brain slices or homogenates was not significantly inhibited for at least 1 hr. during incubations at even the highest of these oxygen pressures (Stadie, Riggs & Haugaard, 1944, 1945*a*, *b*; Haugaard, 1946, 1955; Dickens, 1946*a*, *b*, 1955). The review by Dickens (1955) reaffirms this problem and states that 'There is thus a discrepancy between the time course of the two processes [*in vivo* and *in vitro*] which is difficult to explain if poisoning of the brain respiration as a whole is the primary cause of oxygen poisoning'. With the advent of a better understanding of certain neurochemical processes as well as the use of techniques not available to previous workers, we have reinvestigated the problem of oxygen toxicity, beginning with the study of cell-free dispersions of rat brain.

Homogenates prepared from whole brain have demonstrated slightly more rapid alterations in the presence of oxygen at high pressure than have brain slices (Stadie *et al.* 1945*a*; Dickens, 1946*a*). Dissolved oxygen is more available to mitochondria, microsomes and other cellular components in dispersions, and the addition of critical enzymes and cofactors can promote and control the optimum utilization of glucose. The observation of a rapid increase in the production of lactate by cell-free dispersions of rat brain at 5 atm. p_{O_2} prompted the measurement of $^{14}CO_2$ production from ^{14}C-labelled substrates during incubation with oxygen at high pressure.

MATERIALS AND METHODS

Chemicals. Uniformly ^{14}C-labelled D-glucose (D-[U-^{14}C]-glucose), sodium [1-^{14}C]pyruvate, sodium [2-^{14}C]pyruvate, sodium [1,4-$^{14}C_2$]succinate, sodium α-oxo[5-^{14}C]glutarate and sodium DL-[1-^{14}C]glutamate were obtained from the California Corp. for Biochemical Research (Calbiochem), Los Angeles, Calif., U.S.A. Sodium [1-^{14}C]acetate and [1-^{14}C]glycine and additional D-[U-^{14}C]glucose were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Additional sodium [1-^{14}C]pyruvate, sodium [2-^{14}C]-pyruvate and sodium [1-^{14}C]acetate were obtained from Nichem Inc., Bethesda, Md., U.S.A. Sodium α-oxo[1,2-$^{14}C_2$]-glutarate was supplied by Merck, Sharp and Dohme of Canada Ltd., Montreal, Canada.

NAD$^+$, NADH, ATP, lactate dehydrogenase, L-glutamate dehydrogenase, *o*-dianisidine–HCl, hydrazine hydrate, glucose oxidase, peroxidase and phosphotransacetylase were obtained from Biochemica Boehringer, Mannheim, West Germany. Yeast hexokinase [type III, containing 150 000 Kunitz & McDonald (1946) units/g.], thiamine pyrophosphate and additional ATP and NAD^+ were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Catalase extracted from ox liver was supplied by Worthington Biochemical Corp., Freehold, N.J., U.S.A. Coenzyme A and additional NAD^+, NADH and ATP were purchased from Pabst Laboratories, Milwaukee, Wisc., U.S.A. Crystalline human serum albumin was obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A. Other organic and inorganic chemicals were procured from commercial sources and were of Analytic Grade (American Chemical Society).

Tissue preparation. Brains were obtained from decapitated unanaesthetized male hooded rats (160–260 g.) of a highly inbred strain maintained at this Institute. Rapid removal of three whole rat brains provided 5–6 g. of tissue, which was quickly rinsed in an ice-cold solution of 225 mM-sucrose containing potassium phosphate (20 mM) and EDTA (0·1 mM), at pH 7·4. Homogenates (10%, w/v) in similar solutions of sucrose buffered with phosphate were prepared at 1–3° in a Potter–Elvehjem-type homogenizer with a power-driven Teflon pestle. The homogenate was immediately centrifuged at 700*g* for 15 min. at 2° in an International refrigerated centrifuge. The decanted supernatant contained approx. 7·5 mg. of protein/ml. as determined by the biuret reaction, with a human serum albumin standard. One vol. of this cell-free dispersion was added to 2 vol. of standardized buffer solution. Where determinations of D-glucose were desired, 10% (w/v) homogenates were prepared in ice-cold solutions of 139 mM-KCl containing potassium phosphate (4 mM) and EDTA (0·1 mM), at pH 7·4, instead of in sucrose.

To obtain unwashed mitochondria for studies of α-oxoglutarate dehydrogenase and pyruvate oxidase, the cell-free dispersion was recentrifuged at 12 000*g* for 15 min. at 3° and the sediment equivalent to 1 g. of original tissue was resuspended in 1 ml. of 250 mM-sucrose solution.

Reaction mixtures. When only lactate was measured, 50 ml. of a standardized buffer solution was freshly prepared which contained all the necessary constituents in a variation (containing K^+ ions) of Krebs–Ringer phosphate solution. When 25 ml. of brain dispersion had been added, the final reaction mixture contained the following components (with final concentrations given in parentheses) at pH 7·4: K^+ ions (117 mM), Cl^- ions (86 mM), Mg^{2+} ions (0·8 mM), SO_4^{2-} ions (0·8 mM), Na^+ ions (3·3 mM), total inorganic phosphate (19·3 mM), D-glucose (10 mM), nicotinamide (27 mM), NAD^+ (0·1 mM), ATP (0·5 mM), EDTA (0·03 mM) and sucrose (62 mM). Yeast hexokinase was used in a concentration of 50 μg./ml., i.e. about 7·5 Kunitz & McDonald (1946) units/ml. This concentration of hexokinase was shown to stimulate maximum glycolysis, aerobically and anaerobically. When the homogenate was prepared in the KCl solution, the following altered concentrations were present: that of K^+ ions was 155 mM, that of Cl^- ions was 132 mM, that of inorganic phosphate was 14·7 mM, and sucrose was absent. When the reaction mixture was buffered at pH 7·4 with CO_2–bicarbonate as well as with phosphate, preparations in sucrose contained a final concentration of K^+ ions of 142 mM, and that of HCO_3^- ions was 25 mM. In all cases the osmolarity of the added constituents was less than 0·32. Twenty μmoles of ^{14}C-labelled substrates were added from the side arms of Warburg flasks to initiate the reactions in experiments where the production of $^{14}CO_2$ was determined, but, by using the same general techniques as above, the final concentrations of the constituents were not altered. D-Glucose was replaced by other ^{14}C-labelled substrates as noted below.

EXPERIMENTAL

Small-pressure-chamber techniques. For experiments on the production of lactate only, 2 ml. of the reaction mixture containing non-labelled D-glucose was incubated in each 25 ml. Erlenmyer flask. Gassing was usually done with nitrogen, oxygen or air, although the system was also tested with CO_2–bicarbonate buffer at pH 7·4 or 7·0 after gentle pregassing of the buffer solution with 5·6% CO_2 in nitrogen. For tests at atmospheric pressure the stoppered flasks were shaken at 100 cyc./min. in a constant-temperature water bath, usually at 37°. To study the production of lactate with oxygen at high pressure, gassed but unstoppered flasks were rapidly transferred to a small (100 l.) compression chamber and were incubated in a specially designed shaker under the same conditions as those with the control shaker operating at atmospheric pressure. The compression chamber was rapidly flushed with oxygen (or 5·6% CO_2 in oxygen) for 2 min. and then compression with pure oxygen was begun, usually to 5 atm. pressure (absolute) within 1 min.

Oxygen concentration at high pressure was determined by a Beckman oxygen analyser which recorded 97–99% oxygen in the chamber, except that when CO_2–bicarbonate buffer was added the oxygen concentration varied from 93 to 97%. In the latter experiments the partial pressure of CO_2 ranged between 35 and 45 mm. Hg as estimated by a Liston–Becker CO_2 analyser and checked by gasometric analysis with the micro-apparatus designed by Scholander (1947). A rapid return to 1 atm. pressure for inactivation of some of the flasks and recompression of the remaining active flasks could be accomplished in 3–4 min.

Techniques for studies of $^{14}CO_2$ production. For studies of the production of $^{14}CO_2$ from ^{14}C-labelled substrates, conventional (20 ml.) Warburg flasks with two side arms were incubated at 37° in two metabolic shakers, one at atmospheric pressure and the other under compression at 5 atm. absolute pressure inside a standard U.S. Navy recompression chamber. The main compartment of each flask initially contained 1·8 ml. of the reaction mixture without the ^{14}C-labelled substrate. One side arm contained 0·2 ml. of ^{14}C-labelled substrate, commonly a total of 20 μmoles, while the other side arm held 0·2 ml. of 50% (w/v) phosphoric acid. The centre well contained 0·2 ml. of CO_2-free 10% (w/v) NaOH and a filter-paper 'wick'. Divers trained in the techniques carried out at 5 atm. absolute pressure procedures identical with those done simultaneously at atmospheric pressure, namely, gassing the flasks with oxygen for over 20 sec., tightly stoppering the flasks, tipping in 0·2 ml. of substrate solution, inactivating the contents of the main compartment with 0·2 ml. of 50% phosphoric acid after completion of incubation and unstoppering the flasks after an additional 15 min. The contents of the centre well were analysed for total content of CO_2 and for $^{14}CO_2$, after removal and dilution to 2·5 ml. with washings of CO_2-free water. Total CO_2 in such samples was determined manometrically with Dixon–Keilin flasks. However, considerable quantities of atmospheric CO_2 were absorbed by the very alkaline samples during decompression and transfer to the Warburg apparatus. Despite the use of 'identical' control samples, results were erratic and not considered to be very accurate or precise.

Measurements of radioactivity. The metabolic $^{14}CO_2$ trapped in samples of the alkali from the centre well was precipitated as $Ba^{14}CO_3$, together with sufficient 'carrier' $BaCO_3$ from added Na_2CO_3 to produce 'infinitely thick' samples. The precipitated $BaCO_3$ was transferred to aluminium planchets. The weighed planchets were counted at an efficiency of approx. 15% in a proportional gas-flow counter that used an argon–methane carrier gas. The recorded counts were corrected by weight of $BaCO_3$ against a self-absorption curve. The initial radioactivity in ^{14}C-labelled substrates was determined by directly plating 0·1 ml. samples of aqueous solutions of the substrates on aluminium planchets and counting the samples as 'infinitely thin' specimens. For ease of interpretation initial radioactivity has been adjusted to 100 000 counts/min. in all experiments described in the present paper.

The data from the measurements of radioactivity have been treated in the following manner: (*a*) radioactivity of $^{14}CO_2$ as percentage of initial radioactivity equals 100 × [radioactivity (counts/min.) in $^{14}CO_2$/radioactivity (counts/min.) in ^{14}C-labelled substrate]; (*b*) minimum calculated amount (μmoles) of CO_2 from the particular carbon position labelled equals [(*a*) × initial μmoles of substrate]/100; (*c*) minimum calculated amount (μmoles) of CO_2 from D-[U-^{14}C]glucose equals [(*a*) × initial μg.atoms of carbon present in D-glucose].

Chemical determinations. For the majority of the experiments in which only lactate was measured, the method of Barker & Summerson (1941) was used after inactivation by 10% (w/v) trichloroacetic acid. Otherwise, unless noted, perchloric acid was used for inactivation at a final concentration of 3% (w/v). Lactate was analysed enzymically by measuring NAD^+ reduction at pH 9·0 during oxidation of lactate to pyruvate in the presence of lactate dehydrogenase and of hydrazine hydrate, which served as a carbonyl 'trapper' (Hess, 1956). Pyruvate was determined by measuring NADH oxidation in the presence of lactate dehydrogenase at pH 7·0 (Bauer, 1956; Ross, 1955). D-Glucose was determined by the use of D-glucose oxidase and peroxidase, with *o*-dianisidine–HCl serving as hydrogen donor and colour reagent (Huggett & Nixon, 1957*a*, *b*). A Beckman model DU spectrophotometer was used for these enzymic determinations. Acetyl phosphate was determined by the hydroxylamine reaction (Lipmann & Tuttle, 1945).

Assays of enzymic activity. The activity of pyruvate oxidase in unwashed rat-brain mitochondria was determined by the method suggested by Korkes, del Campillo & Ochoa (1952) and Korkes (1955). In this procedure the reactions shown in Scheme 1 were utilized. The method suggested by Korkes *et al.* (1952) and Korkes (1955) entails the use of Mn^{2+} ions, Mg^{2+} ions and cysteine. All these compounds have exerted various degrees of 'protection' against the toxic effects of oxygen at high pressure *in vitro* (Dickens, 1955). Preliminary experiments showed that the full activity of the coupled reaction system depended on the presence of lactate dehydrogenase, phosphotransacetylase, coenzyme A and thiamine pyrophosphate, but not on that of Mn^{2+} ions or 'thiol protectors' such as cysteine.

Thus, in the final assay system, Mg^{2+} ions were the sole bivalent cations added and 'thiol protectors' were often omitted. KCN in low concentration (0·2 mM) was added in many experiments, including all tests in which only the production of acetyl phosphate was measured. The cyanide was intended to prevent further oxidative metabolism of acetyl-coenzyme A as was done by the anaerobic atmosphere utilized by Korkes *et al.* (1952), which was of course not applicable to the present studies. This technique was at least partially successful in that production of acetyl phosphate in nitrogen at 1 atm. was almost identical with that in oxygen at 1 atm. when the system contained 0·2 mM-KCN. However, when production of $^{14}CO_2$ from [1-^{14}C]pyruvate was measured, cyanide was usually omitted, since the radioactivity in the CO_2 produced could only have been derived from the decarboxylation of pyruvate. The reaction measured by this technique ostensibly was confined to the pyruvate-oxidase system.

Brain mitochondria were also assayed for the activity of α-oxoglutarate dehydrogenase, the method suggested by Kaufman (1955) being adopted for use with crude preparations. This technique utilized the coupled reactions shown in Scheme 2. Measurement of the production of CO_2 from C-1 of α-oxoglutarate provides an assay of the enzymic activity. In our modification the oxidation of α-oxo[1,2-$^{14}C_2$]glutarate to $^{14}CO_2$ was measured. The proportion of the total $^{14}CO_2$ from C-2 was determined by equating it to the $^{14}CO_2$ produced in a parallel system in which [1,4-$^{14}C_2$]succinate was substituted for α-oxo[1,2-$^{14}C_2$]glutarate. Thus the CO_2 derived from only C-1 could be approximately estimated.

RESULTS

Studies of glycolysis. Initial experiments were concentrated on the effects of both anaerobiosis and oxygen at high pressure on the production of lactate by the dispersions of brain tissue. Measurement of the production of lactate was more con-

$$\text{Pyruvate} + NAD^+ + \text{HSCoA} \xrightarrow[\text{Mg}^{2+}\text{ ions}]{\text{Pyruvate oxidase}} \text{acetyl-SCoA} + CO_2 + NADH + H^+$$

$$\text{Pyruvate} + H^+ + NADH \xrightarrow[\text{dehydrogenase}]{\text{Lactate}} \text{lactate} + NAD^+$$

$$\text{Acetyl-SCoA} + \text{phosphate} \xrightarrow{\text{Phosphotransacetylase}} \text{acetyl phosphate} + \text{HSCoA}$$

$$\text{Sum: 2 Pyruvate} + \text{phosphate} \xrightarrow[\text{CoA}]{NAD^+} CO_2 + \text{lactate} + \text{acetyl phosphate}$$

Scheme 1

$$\alpha\text{-Oxoglutarate} + NAD^+ + HSCoA \xrightarrow[\text{dehydrogenase}]{\alpha\text{-Oxoglutarate}} \text{succinyl-SCoA} + CO_2 + NADH + H^+$$

$$H_2O + \text{succinyl-SCoA} \longrightarrow \text{succinate} + HSCoA$$

$$\alpha\text{-Oxoglutarate} + NADH + H^+ + NH_3 \xrightarrow[\text{dehydrogenase}]{\text{Glutamate}} \text{L-glutamate} + NAD^+ + H_2O$$

$$\text{Sum: } 2\ \alpha\text{-Oxoglutarate} + NH_3 \xrightarrow[HSCoA]{NAD^+} \text{succinate} + \text{L-glutamate} + CO_2$$

Scheme 2

Table 1. *Effect of oxygen at high pressure on the production of lactate by dispersions of rat brain buffered with phosphate at* pH 7·4

Experiments were conducted in triplicate in flasks which contained (final vol. 2·0 ml.) the following: the supernatant from a 10% (w/v) rat-brain homogenate (0·67 ml.), K^+ ions (117 mM), Cl^- ions (86 mM), Mg^{2+} ions (0·8 mM), SO_4^{2-} ions (0·8 mM), Na^+ ions (3·5 mM), total inorganic phosphate (19·3 mM), D-glucose (10 mM), nicotinamide (27 mM), NAD^+ (0·1 mM), ATP (0·5 mM) and yeast hexokinase (Sigma type III) (50 μg./ml.). In Expt. 4 no rate-accelerating hexokinase was added. Results represent the means of triplicates that agreed closely (differences between flasks less than 10%) and are expressed as μmoles of lactate formed from 5 to 35 min. of incubation.

	Lactate formed (μmoles)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Bath temp. ...	35°	30·5°	37°	37°
Gas phase				
Air (1 atm.)	3·7	1·8	5·7	1·8
N_2 (1 atm.)	7·6	6·8	10·3	3·9
O_2 (5 atm.)	6·2	5·0	12·2	3·6

venient in most experiments than was that of the utilization of D-glucose. The production of lactate was consistently and rapidly increased, not only during incubations in nitrogen or helium at atmospheric pressure, but also during incubations with oxygen at 5 atm. pressure. The similarity of the effects of both anaerobiosis and oxygen at high pressure is shown in Tables 1 and 2. The increases in the production of lactate were relatively greater in dispersions buffered by phosphate alone than in dispersions to which CO_2–bicarbonate buffer was added. Small variations in temperature below 37° or a lack of added hexokinase did not alter the definite and rapid increases in the production of lactate during exposure to oxygen at high pressure, nor did lowering the pH below 7·0 obviate such phenomena. Air and oxygen at atmospheric pressure did not differ significantly in effects on the production of lactate during the relatively brief, i.e. 30 min., periods of incubation. Incubations with oxygen at 3 atm. and at 7 atm. resulted in similar increases in the production of lactate. The rapidity of the increase in the production of lactate with oxygen at high pressure is shown in Fig. 1.

Increased production of lactate in oxygen at high pressure was not an effect of the mechanical pressure of gas (Table 3), since with a mixture of nitrogen and oxygen at 5 atm. total pressure, which has about the p_{O_2} of air at 1 atm., there was no significant effect on the production of lactate. Increased production of lactate with mixtures of nitrogen and oxygen at 5 atm. pressure only occurred when the p_{O_2} was less than 40 mm. Hg, which apparently resulted in a sufficient degree of anaerobiosis to increase the production of lactate.

The presence of inhibitors of electron transport, 1 mM-potassium cyanide or 1 mM-sodium azide, resulted in increases in the production of lactate in air at atmospheric pressure as would be expected. However, potassium cyanide or sodium azide in 1 mM concentration partially or totally prevented the increased production of lactate with oxygen at high pressure, whereas somewhat lower concentrations (0·1–0·01 mM) of potassium cyanide (but not of sodium azide) increased the effects of the hyperbaric oxygen on the production of lactate. In a typical experiment at 37° with bicarbonate–phosphate buffer at pH 7·4, the amounts (μmoles) of lactate produced in the first 15 min. were: at 1 atm. p_{O_2}, 7·3, 9·4 and 8·6 with cyanide concentrations of 0, 1 and 0·1 mM respectively; and at 5 atm. p_{O_2}, 9·3, 7·8 and 11·7 with cyanide concentrations of 0, 1 and 0·1 mM respectively.

Alterations in the total concentration of inorganic phosphate, from 7 to 33 mM, did not greatly alter the effect of oxygen at high pressure on the production of lactate. Variations in rate were observed, as might be expected from the rate-limiting effect of inorganic phosphate (Gatt & Racker, 1959; Racker, 1958). Pre-exposure of chilled dispersions of brain to 7 atm. p_{O_2} for 15 min. or to 5 atm. p_{O_2} for 30 min. had no consistent effect on subsequent production of lactate at 1 atm. p_{O_2}. Whether this was due to the absence of the effects of oxygen at high pressure on the 'inactive' preparation, or to rapid reversibility of the action of oxygen at high pressure, is not clear, although the latter was possibly indicated by two experiments that could not be consistently reproduced. The concentration of pyruvate was also significantly increased during incubations with

Table 2. *Effect of oxygen at high pressure on the production of lactate by the brain dispersions buffered with bicarbonate and phosphate*

Experiments were conducted in triplicate in flasks which contained (final vol. 2 ml.) the following: the supernatant from a 10% (w/v) rat-brain homogenate (0·67 ml.), K^+ ions (142 mM), Cl^- ions (86 mM), Mg^{2+} ions (0·8 mM), SO_4^{2-} ions (0·8 mM), Na^+ ions (3·5 mM), inorganic phosphate (18·3 mM), D-glucose (10 mM), nicotinamide (27 mM), NAD^+ (0·1 mM), ATP (0·5 mM), yeast hexokinase (Sigma type III) (50 μg./ml.) and HCO_3^- ions (25 mM for pH 7·4, or 12·5 mM for pH 7·0). Results represent the means of triplicates that agreed closely.

Expt. no.	Bath temp.	pH	Gas phase	Lactate formed (μmoles) From 5 to 20 min.	From 5 to 35 min.
1	36·5°	7·4	O_2 (1 atm.)	5·5	8·7
			N_2 (1 atm.)	7·1	11·3
			O_2 (5 atm.)	—	11·2
2	37·0	7·4	O_2 (1 atm.)	7·7	14·8
			O_2 (5 atm.)	11·2	18·6
3	34·5	7·4	O_2 (1 atm.)	4·2	6·8
			O_2 (5 atm.)	5·9	11·2
4	37·0	7·0	O_2 (1 atm.)	—	10·0
			O_2 (5 atm.)	—	15·3

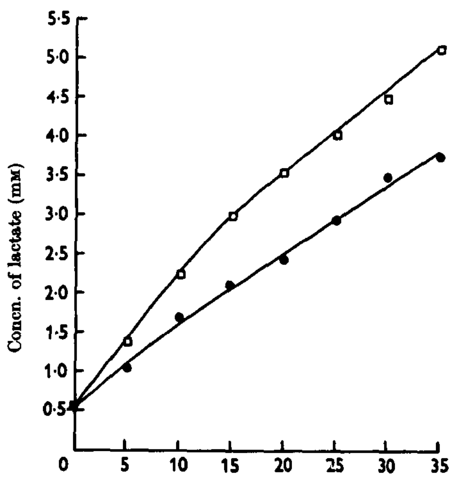

Fig. 1. Production of lactate during exposure to oxygen at 5 atm. pressure. Phosphate-buffered mixtures identical to those of Table 1 were incubated at pH 7·4. For the incubations with high oxygen pressure, gassing with O_2, stoppering of flasks and inactivating with 6% (w/v) perchloric acid were accomplished by a worker inside a recompression chamber at 5 atm. air pressure. Each point represents the mean of duplicates that agreed closely. ●, Concn. of lactate at 1 atm. p_{O_2}; □, concn. of lactate at 5 atm. p_{O_2}.

oxygen at high pressure, although concentrations of pyruvate greater than 0·5 mM were rarely observed.

Experiments with ¹⁴C-labelled substrates. The production of $^{14}CO_2$ from D-[U-^{14}C]glucose was greatly decreased in dispersions incubated for 30 min. in oxygen at 5 atm. (Table 4). The amount of $^{14}CO_2$ produced with oxygen at 5 atm. varied from one-fifth to one-third of that with oxygen at 1 atm. The addition of coenzyme A (0·05 mM) or of thiamine pyrophosphate (0·2 mM) failed to prevent this inhibition of the oxidation of D-[U-^{14}C]glucose. The presence of mercaptoethanol (50 mM) slightly increased the production of $^{14}CO_2$ from D-[U-^{14}C]-glucose with oxygen at 5 atm., but at the same time resulted in a 50% decrease in the production of $^{14}CO_2$ with oxygen at 1 atm. Thus any slight 'protection' by mercaptoethanol was more apparent than real. No improvement in the oxidation of D-[U-^{14}C]glucose during incubations in oxygen at 5 atm. was observed when ox-liver catalase was added in 0·1–1 μM concentrations. Concentrations of catalase greater than 1 μM markedly inhibited the ability of the system to oxidize D-glucose. The addition of 5 mM- and 25 mM-glutathione (reduced) did not result in any consistent improvement in the production of $^{14}CO_2$ from D-[U-^{14}C]glucose during incubations in oxygen at 5 atm. pressure. No decrease in the production of $^{14}CO_2$ from D-[U-^{14}C]-glucose was observed during incubations in air at 5 atm. pressure.

The production of $^{14}CO_2$ from both [1-^{14}C]-pyruvate and [2-^{14}C]pyruvate was markedly decreased during incubations performed with oxygen at 5 atm. (Table 5). The $^{14}CO_2$ produced was decreased at 5 atm. p_{O_2} to 50–70% of that produced at 1 atm. p_{O_2}. The inhibitions of the oxidation of pyruvate, though large, are not of the same order of magnitude as those of the oxidation of D-glucose. An explanation for this is suggested by the fact that concentrations of pyruvate rarely exceeded 0·5 mM during the experiments in which 10 mM-D-glucose was used to study glycolysis. It is

Table 3. *Effects of gas pressure on the production of lactate by the brain dispersions buffered with phosphate at* pH 7·4

Experiments were conducted in flasks whose contents were identical with those of Table 1. Increased production of lactate did not occur with nitrogen at 5 atm. unless the partial pressure of oxygen was less than 40 mm. Hg. Results are expressed in terms of μmoles of lactate formed between 5 and 35 min. of incubation. Variations shown are S.E.M., with the number of flasks tested given in parentheses.

Gas phase	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Air (1 atm.)	5·3 ± 0·3 (4)	4·8 ± 0·3 (4)	5·0 ± 0·3 (4)	7·6 ± 0·1 (3)
N_2 (1 atm.)	10·4 ± 0·8 (4)	11·4 ± 1·4 (4)	8·7 ± 0·2 (4)	12·1 ± 0·2 (3)
$N_2 + O_2$ (5 atm.)				
(a) p_{O_2} = 110 mm. Hg	5·6 ± 0·4 (4)	—	—	—
(b) p_{O_2} = 140 mm. Hg	—	4·6 ± 0·3 (4)	—	—
(c) p_{O_2} = 36 mm. Hg	—	—	7·1 ± 0·4 (4)	—
(d) p_{O_2} = 30 mm. Hg	—	—	—	12·2 ± 0·2 (3)

Table 4. *Effect of oxygen at* 5 *atm. pressure on the oxidation of* D-[U-^{14}C]*glucose*

Uniformly ^{14}C-labelled D-glucose (20 μmoles, containing 100 000 counts/min.) was incubated for 30 min. at 37° and pH 7·4 in reaction mixtures similar to those of Table 1. Variations are shown as S.E.M., with the number of flasks tested given in parentheses. *P* represents probability as determined by Student's *t* test.

Expt. no.	Additions (final concn.)	p_{O_2} (atm.)	Radioactivity of $^{14}CO_2$ produced (counts/min.)	Minimum calculated amount of CO_2 from D-glucose (μmoles)	*P*	Radioactivity of $^{14}CO_2$ (% of control)	Total CO_2 produced manometrically (μmoles)
1	—	1	2207 ± 100 (4)	2·65 ± 0·12	< 0·001	100	—
	—	5	577 ± 58 (4)	0·69 ± 0·07		26	—
2	—	1	1628 ± 118 (3)	1·95 ± 0·14	< 0·001	100	6·9 ± 0·1 (3)
	—	5	325 ± 18 (4)	0·39 ± 0·02		20	7·3 ± 0·1 (4)
3	—	1	1281 ± 50 (3)	1·54 ± 0·06	< 0·001	100	9·5 ± 0·6 (3)
	—	5	286 ± 19 (2)	0·34 ± 0·02		22	9·7 ± 0·8 (2)
	Coenzyme A (0·05 mM)	1	1071 ± 35 (3)	1·28 ± 0·04	< 0·001	84	—
	Coenzyme A (0·05 mM)	5	266 ± 8 (2)	0·32 ± 0·01		21	—

unlikely that the concentrations of pyruvate were much greater than 0·5 mM during the experiments with D-[U-^{14}C]glucose, in which the initial concentration of D-glucose was also 10 mM. In the experiments on the oxidation of ^{14}C-labelled pyruvate, the initial concentration of pyruvate was 10 mM, far larger than the 0·5 mM maximum concentration of pyruvate in the experiments on the production of $^{14}CO_2$ from D-[U-^{14}C]glucose. Such a significantly greater concentration of pyruvate conceivably could increase the oxidation of ^{14}C-labelled pyruvate during incubation with oxygen at high pressure through a mass-action effect, and thus partially overcome the inhibitory effects of oxygen at high pressure. Such an explanation is suggested by experiments such as that shown in Fig. 2, in which the magnitude of the inhibition of the oxidation of [2-^{14}C]pyruvate decreased as the initial concentration of pyruvate was increased. However, such phenomena were not observed when the initial concentration of pyruvate was varied in experiments in which [1-^{14}C]pyruvate was used. When fumarate or malate was present in relatively large concentrations (5 mM), the production of $^{14}CO_2$ from ^{14}C-labelled pyruvate was increased by 50–100 % in oxygen at atmospheric pressure, as might be expected from the results reported by Coxon, Liébecq & Peters (1949). With oxygen at 5 atm. pressure, however, much smaller increases in the production of $^{14}CO_2$ were observed when 5 mM-fumarate (or malate) was present. The apparent magnitude of inhibition by oxygen at high pressure was thus increased by the presence of 5 mM-fumarate; it was not altered by catalytic concentrations of malate (Table 5).

The presence of coenzyme A (0·05–0·25 mM) did not alter the inhibitory effects of oxygen at high pressure on the oxidation of ^{14}C-labelled pyruvate. The presence of thiamine pyrophosphate (0·2 mM) did not influence the inhibition of the oxidation of [1-^{14}C]pyruvate by hyperbaric oxygen, but apparently prevented the inhibition of production of $^{14}CO_2$ from [2-^{14}C]pyruvate (Table 5). However, experiments with oxygen at atmospheric pressure

Table 5. *Production of $^{14}CO_2$ from ^{14}C-labelled pyruvate in oxygen at 1 and 5 atm. pressure*

Experiments were conducted with reaction mixtures similar to those of Table 4, except that 20 μmoles of potassium pyruvate labelled with ^{14}C at either C-1 or C-2 and containing 100 000 counts/min. were employed instead of 20 μmoles of D-glucose. Flasks were incubated for 30 min. at 37°. All values of P are less than 0·01 except for that part of Expt. 2 concerned with [2-^{14}C]pyruvate. Variations shown are S.E.M., with the number of flasks tested given in parentheses.

Expt. no.	Additions (final concn.)	p_{O_2} (atm.)	Radioactivity of $^{14}CO_2$ from [1-^{14}C]pyruvate (counts/min.)	Minimum calculated amount of CO_2 from C-1 of pyruvate (μmoles)	Radioactivity of $^{14}CO_2$ (% of control)	Radioactivity of $^{14}CO_2$ from [2-^{14}C]pyruvate (counts/min.)	Minimum calculated amount of CO_2 from C-2 (μmole)	Radioactivity of $^{14}CO_2$ (% of control)	Total CO_2 produced manometrically (μmoles)
1	—	1	11 873 ± 881 (3)	2·37 ± 0·18	100	1676 ± 56 (3)	0·335 ± 0·011	100	10·2 ± 0·4 (6)
	—	5	7 147 ± 338 (3)	1·43 ± 0·08	60	897 ± 35 (3)	0·179 ± 0·007	54	12·3 ± 0·8 (6)
2	Thiamine pyrophosphate (0·2 mM)	1	8 835 ± 241 (3)	1·77 ± 0·03	100	451 ± 65 (2)	0·090 ± 0·013	100	10·6 ± 0·4 (3)
	Thiamine pyrophosphate (0·2 mM)	5	6 672 ± 342 (3)	1·34 ± 0·07	75	439 ± 33 (3)	0·088 ± 0·007	97	12·1 ± 0·2 (3)
3	—	1	38 194 ± 943 (2)	7·64 ± 0·18	100	524 ± 15 (3)	0·105 ± 0·003	100	11·0 ± 0·8 (4)
	—	5	23 195 ± 1313 (2)	4·64 ± 0·26	61	313 ± 22 (2)	0·063 ± 0·004	60	12·0 ± 1·8 (3)
4	Malate (0·25 mM)	1	19 206 ± 1052 (3)	3·84 ± 0·21	100	504 ± 15 (3)	0·101 ± 0·003	100	11·3 ± 0·9 (3)
	Malate (0·25 mM)	5	11 707 ± 209 (3)	2·34 ± 0·04	61	350 ± 37 (3)	0·070 ± 0·007	69	7·1 ± 0·8 (3)
5	—	1	7 384 ± 42 (3)	1·48 ± 0·01	100	—	—	—	—
	—	5	4 381 ± 80 (2)	0·88 ± 0·02	59	—	—	—	—
	Fumarate (5 mM)	1	14 635 ± 842 (4)	2·93 ± 0·16	100	—	—	—	—
	Fumarate (5 mM)	5	6 576 ± 766 (3)	1·03 ± 0·15	45	—	—	—	—

demonstrated that thiamine pyrophosphate in concentrations of 0·1–0·4 mM decreased the production of $^{14}CO_2$ from [2-^{14}C]pyruvate to 30–40% of control values (i.e. the production of $^{14}CO_2$ without addition of thiamine pyrophosphate). Thus the effects of oxygen at high pressure on the oxidation of [2-^{14}C]pyruvate are difficult to interpret when thiamine pyrophosphate has been added to this system.

The similarity of the α-oxoglutarate-dehydrogenase system to the pyruvate-oxidase system directed attention to the study of the oxidation of α-oxo[5-^{14}C]glutarate, of DL-[1-^{14}C]glutamate and of [1,4-$^{14}C_2$]succinate (Table 6). During incubations for 30 min. in oxygen at 5 atm. the production of $^{14}CO_2$ from α-oxo[5-^{14}C]glutarate was decreased to 45–70% of that produced with oxygen at 1 atm., a magnitude of inhibition comparable with that of the oxidation of ^{14}C-labelled pyruvate. The presence of DL-glutamate (10 mM), to block the pathway of oxidation of α-oxoglutarate through γ-aminobutyrate, did not affect the inhibition of the oxidation of α-oxo[5-^{14}C]glutarate during incubation with oxygen at 5 atm. Usually the production of $^{14}CO_2$ from [1,4-$^{14}C_2$]succinate

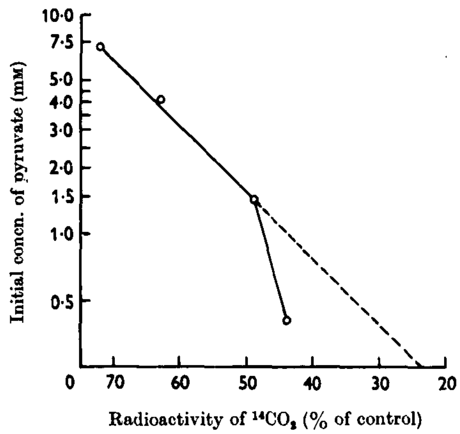

Fig. 2. Semilogarithmic plot of the effects of the initial pyruvate concentration on the apparent magnitude of inhibition of production of $^{14}CO_2$ from [2-^{14}C]pyruvate during incubation in oxygen at 5 atm. pressure. Other than the variation of the concentrations of pyruvate, this system was identical to those of Table 5. Each plotted point represents the ratio of the means of duplicates that agreed closely. A theoretical extrapolation of the initial linearity to the level of pyruvate concentrations observed during glycolysis in this system is portrayed by the broken line. The radioactivity of $^{14}CO_2$ (% of control) = 100 × [radioactivity (counts/min.) in $^{14}CO_2$ produced at 5 atm. p_{O_2}/radioactivity (counts/min.) in $^{14}CO_2$ produced at 1 atm. p_{O_2}].

was not significantly altered during the relatively brief, i.e. 30 min., periods of incubation in oxygen at high pressure. The $^{14}CO_2$ produced from DL-[1-^{14}C]glutamate was markedly and significantly inhibited during incubation in oxygen at 5 atm., though to a lesser extent than was that from α-oxo[5-^{14}C]glutarate. The study of the production of $^{14}CO_2$ from α-oxo[1,2-$^{14}C_2$]glutarate, a logical choice for the investigation of the effects of hyperbaric oxygen on the oxidative decarboxylation of α-oxoglutarate by this mixture, was reserved for the more specific studies presented below.

Oxygen at 5 atm. pressure caused small but consistently significant inhibitions of $^{14}CO_2$ production from [1-^{14}C]acetate (Table 7). The presence of coenzyme A (0·05 mM) exerted effects on the oxidation of [1-^{14}C]acetate in oxygen at 5 atm. that were suggestive of 'protection' but not conclusively ($0{\cdot}2 > P > 0{\cdot}1$). Although this system oxidized [1-^{14}C]glycine sluggishly, a mild decrease in $^{14}CO_2$ produced from this molecule was observed during incubations in oxygen at 5 atm., of a magnitude similar to the inhibitions of the oxidation of [1-^{14}C]acetate. (The oxidation of acetate by a dispersion of brain tissue is unusual.)

The utilization of D-[U-^{14}C]glucose during incubations for 30 min. in oxygen at 1 and 5 atm. pressure is summarized in Table 8, which presents the markedly decreased production of $^{14}CO_2$, the increased production of lactate and the slightly decreased utilization of D-glucose that occurred during incubations with hyperbaric oxygen. The

Table 6. *Production of* $^{14}CO_2$ *from α-oxo*[5-^{14}C]*glutarate*, [1,4-$^{14}C_2$]*succinate and* DL-[1-^{14}C]*glutamate in oxygen at* 1 *and* 5 *atm. pressure*

^{14}C-labelled substrate (16·25 μmoles, containing 100 000 counts/min.) was incubated for 30 min. at 37° and pH 7·4. The same reaction media as described in Table 4 were used except for the different ^{14}C-labelled substrates. Variations are shown as S.E.M., with the number of flasks tested given in parentheses. P represents probability as determined by Student's t test.

Expt. no.	Substrate	p_{O_2} (atm.)	Radioactivity of $^{14}CO_2$ collected (counts/min.)	Minimum calculated amount of CO_2 from labelled position (μmoles)	P	Radioactivity of $^{14}CO_2$ (% of control)	Total CO_2 produced manometrically (μmoles)
1	α-Oxo[5-^{14}C]glutarate	1	1 997 ± 2 (3)	0·325 ± 0·001	< 0·001	100	—
		5	1 013 ± 44 (3)	0·165 ± 0·007		51	—
	[1,4-$^{14}C_2$]Succinate	1	7 427 ± 192 (3)	1·21 ± 0·03	> 0·95	100	—
		5	7 076 ± 1046 (3)	1·15 ± 0·17		95	—
2	α-Oxo[5-^{14}C]glutarate	1	2 703 ± 124 (2)	0·44 ± 0·02	< 0·01	100	10·4 ± 0·9 (3)
		5	1 891 ± 52 (2)	0·31 ± 0·01		70	5·9 ± 1·2 (3)
	[1,4-$^{14}C_2$]Succinate	1	6 797 ± 107 (3)	1·10 ± 0·02	< 0·01	100	6·8 ± 0·8 (3)
		5	6 202 ± 28 (3)	1·01 ± 0·01		92	7·0 ± 0·4 (3)
3	α-Oxo[5-^{14}C]glutarate	1	1 390 ± 36 (3)	0·23 ± 0·01	< 0·001	100	5·8 ± 1·1 (3)
		5	634 ± 35 (3)	0·10 ± 0·01		46	3·8 ± 1·8 (3)
	DL-[1-^{14}C]Glutamate	1	10 394 ± 112 (3)	1·70 ± 0·02	< 0·001	100	12·6 ± 0·4 (3)
		5	6 529 ± 243 (3)	1·07 ± 0·04		63	8·3 ± 1·3 (3)

Table 7. *Production of* $^{14}CO_2$ *from* [1-^{14}C]*acetate in oxygen at* 1 *and* 5 *atm. pressure*

In these experiments [1-^{14}C]acetate (20 μmoles, containing 100 000 counts/min.) was used as the substrate during the incubation of 2 ml. of reaction mixture similar to that described in Table 4. Incubations were for 30 min. at 37° and pH 7·4. Variations are shown as S.E.M. with the number of flasks tested given in parentheses. P represents probability as determined by Student's t test.

Expt. no.	Additions (final concn.)	p_{O_2} (atm.)	Radioactivity of $^{14}CO_2$ collected (counts/min.)	Minimum calculated amount of CO_2 from C-1 of acetate (μmoles)	P	Radioactivity of $^{14}CO_2$ (% of control)	Total CO_2 produced manometrically (μmoles)
1	—	1	1076 ± 17 (3)	0·215 ± 0·003	< 0·001	100	—
	—	5	843 ± 7 (3)	0·169 ± 0·001		78	—
2	Glucose (10 mM)	1	1978 ± 40 (3)	0·400 ± 0·008	< 0·001	100	13·3 ± 2·2 (3)
	Glucose (10 mM)	5	1592 ± 58 (3)	0·318 ± 0·012		80	10·4 ± 3·3 (5)
3	—	1	2016 ± 8 (3)	0·403 ± 0·002	< 0·05	100	5·8 ± 2·1 (3)
	—	5	1658 ± 165 (3)	0·332 ± 0·033		82	5·3 ± 1·0 (3)
	Coenzyme A (0·05 mM)	1	1976 ± 50 (3)	0·395 ± 0·010	< 0·2	98	5·1 ± 1·4 (3)
	Coenzyme A (0·05 mM)	5	1866 ± 47 (3)	0·373 ± 0·009		93	4·6 ± 1·8 (3)

Table 8. *Utilization of* D-[U-^{14}C]*glucose in oxygen at* 1 *and* 5 *atm. pressure*

Each Warburg flask contained (final vol. 2 ml.) the following: the supernatant from a 10% (w/v) brain homogenate (0·67 ml.), EDTA (0·03 mM), K^+ ions (154 mM), Cl^- ions (131 mM), inorganic phosphate (14·7 mM), Na^+ ions (3·3 mM), Mg^{2+} ions (0·8 mM), SO_4^{2-} ions (0·8 mM), NAD^+ (0·1 mM), ATP (0·5 mM), nicotinamide (27 mM), yeast hexokinase (Sigma type III) (25 μg.) and D-glucose (10 mM) (20 μmoles of D-[U-^{14}C]glucose, containing 100 000 counts/min.). (In Expt. 3 63·8 μmoles of D-[U-^{14}C]glucose, containing 100 000 counts/min., was the initial substrate.) Incubations were for 30 min. at 37° and pH 7·4. Variations are shown as S.E.M., with the number of flasks tested given in parentheses.

Expt. no.	p_{O_2} (atm.)	Radioactivity of $^{14}CO_2$ collected (counts/min.)	Minimum calculated amount of CO_2 from D-glucose (μmoles)	Total CO_2 produced manometrically (μmoles)	D-Glucose utilized (μmoles)	Lactate produced (μmoles)
1	1	1619±50 (4)	1·94±0·06	12·5±2·5 (4)	17·1±0·2 (4)	6·9±0·1 (4)
	5	405±29 (4)	0·48±0·03	10·5±1·8 (4)	14·1±0·3 (4)	9·7±0·1 (4)
2	1	2286±127 (4)	2·74±0·15	5·5±1·0 (4)	15·8±0·3 (4)	9·7±0·4 (4)
	5	857±63 (4)	1·03±0·08	3·5±0·8 (4)	12·6±0·8 (4)	12·6±0·2 (4)
3	1	1114±38 (6)	4·26±0·16	13·0±1·3 (5)	23·5±1·4 (6)	8·3±0·9 (6)
	5	350±22 (6)	1·34±0·10	9·7±1·8 (5)	19·8±0·8 (6)	10·5±0·3 (6)

parameters measured obviously do not account for all of the D-glucose used. This may be explained, not only by the breakdown of D-glucose into the normal intermediate products of catabolism and conversion of such products into lipids and proteins, but also by the inadequacies of the measurements of total CO_2 produced and by the limitations of the assumptions used in the calculations derived from the $^{14}CO_2$ data.

Enzyme activity in rat-brain mitochondria. The mechanisms underlying the alterations in the oxidation of D-glucose by dispersions of rat brain during incubations in oxygen at high pressure were next sought in studies on the activity of pyruvate oxidase in rat-brain mitochondria. The production of acetyl phosphate by brain mitochondria in the system of coupled reactions described above (Scheme 1) was variably but very consistently inhibited in the presence of oxygen at 5 atm. pressure during the 30 and 60 min. incubations presented in Table 9. In separate experiments in air, however, acetyl phosphate was very unstable when incubated with phosphate buffer at 37°; therefore this technique was not as quantitatively valid as could be desired. This problem directed our attention to the measurement of $^{14}CO_2$ produced from [1-^{14}C]-pyruvate as a more reliable indicator of the activity of pyruvate oxidase. Table 10 provides several typical examples of the rapid decreases in the production of $^{14}CO_2$ that occurred when this mitochondrial preparation was incubated in oxygen at 5 atm. pressure. An explanation for the variations in the kinetics of the abnormalities produced by oxygen at high pressure is not readily apparent, although loss of oxygen from the stoppered Warburg flasks in the mildly-fluctuating high pressure of the recompression chamber was observed in certain cases. When cyanide (0·2 mM) was present in this mitochondrial preparation, the decrease in $^{14}CO_2$ produced in oxygen at 5 atm. was more moderate, although a significant depression in the production of $^{14}CO_2$ was still observed. This phenomenon remains to be investigated. The production of lactate was increased rather than decreased in the presence of oxygen at 5 atm. pressure. This apparent lack of correspondence with the decreased production of $^{14}CO_2$ implies abnormalities of the pyruvate-oxidase system during exposure to oxygen at high pressure. The discrepancies in the stoicheiometry of the results of these reactions (production of CO_2 was much greater than was the yield of lactate or of acetyl

Table 9. *Effect of oxygen at high pressure on the activity of pyruvate oxidase in rat-brain mitochondria*

Each 25 ml. flask contained (final vol. 2 ml.) the following: potassium phosphate (50 mM) at pH 7·4, mercaptoethanol (50 mM), NAD^+ (2·5 mM), potassium pyruvate (25 mM), coenzyme A (0·05 mM), thiamine pyrophosphate (0·1 mM), $MgCl_2$ (5 mM), KCN (0·2 mM), lactate dehydrogenase (2000 units), transacetylase (Worthington) diluted 1:10 (0·1 ml.) and unwashed mitochondria prepared from 0·5 g. original wet wt. of rat brain (0·5 ml.). Each result is the mean of two experiments.

		Acetyl phosphate produced (μmole)	
Expt. no.	p_{O_2} (atm.)	From 0 to 30 min.	From 0 to 60 min.
1	1	+0·13	+0·96
	5	−0·67	−0·54
2	1	+0·67	+0·93
	5	+0·56	+0·73
3	1	+0·23	+0·60
	5	+0·10	+0·36

Table 10. *Activity of pyruvate oxidase in rat-brain mitochondria as measured by the production of* $^{14}CO_2$ *from* [1-^{14}C]*pyruvate*

After the [1-^{14}C]pyruvate had been tipped into the main compartment to initiate the reactions, each Warburg flask contained (final vol. 2 ml.) the following: potassium [1-^{14}C]pyruvate (25 mM) (50 μmoles, containing 100 000 counts/min.), potassium phosphate (50 mM) at pH 7·4, NAD+ (0·5 mM), coenzyme A (0·05 mM), nicotinamide (25 mM), thiamine pyrophosphate (0·1 mM), $MgCl_2$ (2 mM), lactate dehydrogenase (2000 units), transacetylase (Worthington) diluted 1:10 (0·1 ml.) and unwashed mitochondria prepared from 0·5 g. original wet wt. of rat brain (0·5 ml.). Variations are shown as S.E.M., with the number of flasks tested given in parentheses. *P* represents probability as determined by Student's *t* test.

Expt. no.	Additions (final concn.)	p_{O_2} (atm.)	Incubation time (min.)	Radioactivity of $^{14}CO_2$ produced (counts/min.)	Minimum calculated amount of CO_2 from C-1 of pyruvate (μmoles)	*P*	Radioactivity of $^{14}CO_2$ (% of control)	Lactate produced (μmoles)
1	KCN (0·2 mM)	1	30	5895 ± 120 (3)	2·95 ± 0·6	< 0·01	100	0·31 ± 0·09 (2)
	KCN (0·2 mM)	5	30	5137 ± 29 (2)	2·57 ± 0·01		87	0·54 ± 0·04 (3)
	—	1	30	6216 ± 340 (3)	3·11 ± 0·17	< 0·01	100	0·32 ± 0·15 (3)
	—	5	30	4283 ± 301 (3)	2·15 ± 0·15		69	1·04 ± 0·12 (3)
2	—	1	15	3050 ± 80 (4)	1·53 ± 0·04	Not significant	100	—
	—	5	15	2964 ± 140 (3)	1·48 ± 0·07		97	—
	—	1	30	5536 ± 21 (4)	2·77 ± 0·01	< 0·001	100	—
	—	5	30	4975 ± 40 (4)	2·49 ± 0·02		89	—
3	—	1	15	4200 ± 348 (3)	2·10 ± 0·17	< 0·001	100	0·62 ± 0·02 (3)
	—	5	15	473 ± 112 (4)	0·24 ± 0·04		11	0·98 ± 0·08 (3)
	—	1	35	9065 ± 112 (3)	4·53 ± 0·06	< 0·001	100	1·24 ± 0·11 (4)
	—	5	35	7749 ± 47 (3)	3·87 ± 0·02		85	1·53 ± 0·15 (3)

Table 11. *Effect of oxygen at high pressure on the activity of α-oxoglutarate dehydrogenase in rat-brain mitochondria*

After the labelled substrate had been tipped into the main compartment to initiate the reactions, each Warburg flask contained (final vol. 2 ml.): potassium α-oxo[1,2-$^{14}C_2$]glutarate (8·5 mM) (16·9 μmoles, containing 100 000 counts/min.) or potassium [1,4-$^{14}C_2$]succinate (8·2 mM) (16·3 μmoles, containing 100 000 counts/min.), NH_4Cl (25 mM), coenzyme A (0·25 mM), NAD+ (0·5 mM), potassium phosphate (50 mM) at pH 7·4, nicotinamide (25 mM), glutamate dehydrogenase (0·2 mg.) and unwashed mitochondria prepared from 0·3 g. original wet wt. of rat brain (0·3 ml.). Variations are shown as S.E.M., with the number of flasks tested given in parentheses.

Substrate	Additions (final concn.)	p_{O_2} (atm.)	Radioactivity of $^{14}CO_2$ collected (counts/min.)	Minimum calculated amount of CO_2 from labelled positions (μmoles)	Minimum calculated amount of CO_2 from C-1 of α-oxoglutarate (μmoles)	Radioactivity of $^{14}CO_2$ (% of control)
α-Oxo[1,2-$^{14}C_2$]glutarate	—	1	14 188 ± 148 (3)	2·398 ± 0·025 (3)	2·29 ± 0·02	100
[1,4-$^{14}C_2$]Succinate	—	1	690 (1)	0·112 (1)	—	—
α-Oxo[1,2-$^{14}C_2$]glutarate	—	5	2 137 ± 1 089 (3)	0·361 ± 0·184 (3)	0·34 ± 0·18	15
[1,4-$^{14}C_2$]Succinate	—	5	145 (1)	0·024 (1)	—	—
α-Oxo[1,2-$^{14}C_2$]glutarate	KCN (0·2 mM)	1	15 280 ± 562 (3)	2·583 ± 0·095 (3)	2·54 ± 0·10	100
[1,4-$^{14}C_2$]Succinate	KCN (0·2 mM)	1	252 (1)	0·041 (1)	—	—
α-Oxo[1,2-$^{14}C_2$]glutarate	KCN (0·2 mM)	5	12 844 ± 124 (3)	2·171 ± 0·021 (3)	2·15 ± 0·02	85
[1,4-$^{14}C_2$]Succinate	KCN (0·2 mM)	5	142 (1)	0·023 (1)	—	—

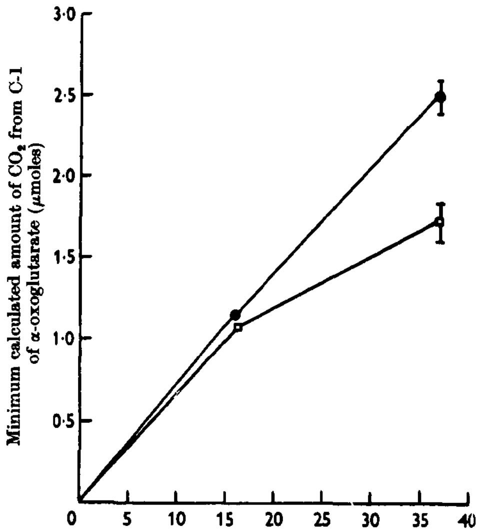

Fig. 3. Inhibition of α-oxoglutarate dehydrogenase by oxygen at 5 atm. pressure. This experiment was carried out in the presence of 0·2 mM-KCN and utilized a preparation identical with that presented in Table 11, except that the initial concentrations of α-oxoglutarate and of succinate were doubled, to 16·9 and 16·3 mM respectively. ●, Minimum calculated amount of CO_2 from C-1 of α-oxoglutarate at 1 atm. p_{O_2} (μmoles); □, minimum calculated amount of CO_2 from C-1 of α-oxoglutarate at 5 atm. p_{O_2} (μmoles). The vertical lines indicate S.E.M. ($n = 3$ for each point).

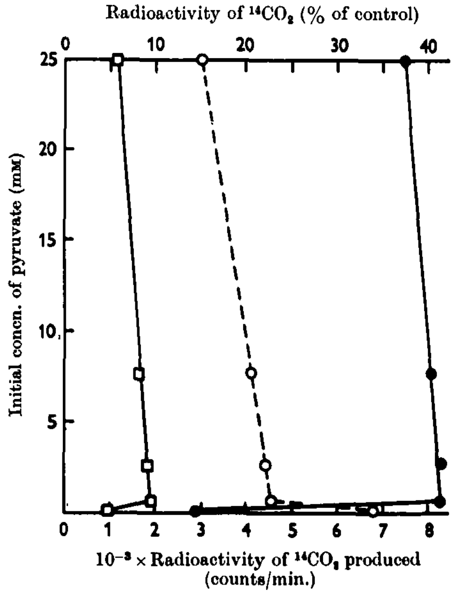

Fig. 4. Effect of the initial concentration of pyruvate on the activity of mitochondrial pyruvate oxidase during incubations in oxygen at 1 and at 5 atm., and on the apparent magnitude of inhibition observed at high oxygen pressure. Other than the different initial concentrations of pyruvate, this system was identical with those of Table 10. Each plotted point represents the mean of duplicates that agreed closely, or the ratios thereof. ●, Radioactivity in $^{14}CO_2$ produced in oxygen at 1 atm. (counts/min.); □, radioactivity in $^{14}CO_2$ produced in oxygen at 5 atm. (counts/min.); ○, radioactivity of $^{14}CO_2$ (% of control) = 100 × [radioactivity (counts/min.) in $^{14}CO_2$ produced in oxygen at 5 atm./radioactivity (counts/min.) in $^{14}CO_2$ produced in oxygen at 1 atm.].

phosphate) are similar to the inconsistencies encountered by Korkes *et al.* (1952). These workers attributed the relatively lower production of acetyl phosphate in their experiments to possible slow hydrolysis of acetyl-coenzyme A to free acetate. They also noted that brain slices and homogenates were the only tissue preparations able to form significant quantities of free acetate from pyruvate (Coxon *et al.* 1949).

The activity of α-oxoglutarate dehydrogenase was also decreased in preparations of rat-brain mitochondria incubated in oxygen at 5 atm. pressure, again to a less extent in the presence of 0·2 mM-potassium cyanide. Table 11 demonstrates such an experiment, together with the method used to estimate the number of μmoles of CO_2 derived from C-1 of α-oxoglutarate, where both C-1 and C-2 were originally labelled with ^{14}C. Such data are used in Fig. 3 to outline the kinetics of inhibition by oxygen at high pressure in a preparation containing cyanide (0·2 mM). The magnitude of the inhibitions produced by oxygen at high pressure in this enzyme system as well as in the pyruvate-oxidase system must be evaluated in the light of effects due to the concentration of substrate. In these assays of enzymic activity the substrates were added in supra-optimum concentrations, which may be significantly different from those existing during the metabolism of the dispersions of rat brain. The data of Fig. 4 represent an attempt to relate the magnitude of the inhibition of pyruvate oxidase to the initial concentration of the substrate. No relationship of any great importance was found in such experiments with the enzymic systems of pyruvate oxidase and α-oxoglutarate dehydrogenase in rat-brain mitochondria.

DISCUSSION

The incubation of cell-free dispersions of whole rat brain in oxygen at high pressure resulted in a rapid impairment of the oxidative degradation of

D-glucose. The rate of the appearance of biochemical alterations in these preparations during exposure to oxygen at high pressure showed good correlation with the rapid onset of marked neurological disturbances observed in mammals breathing oxygen at comparable pressure (Donald, 1947*a*, *b*; Stadie *et al.* 1944). Previous investigations *in vitro* have disclosed toxic alterations in the metabolism of brain tissue exposed to hyperbaric oxygen, but prolonged incubation (usually 1·5–2·5 hr.) in oxygen at high pressure was required before significant inhibitions of metabolism were detectable (Stadie *et al.* 1945*a*, *b*; Dickens, 1946*a*, *b*, 1955).

The results of the present experiments implicate two specific enzymic sites of inhibition by oxygen at high pressure: pyruvate oxidase and α-oxoglutarate dehydrogenase. Both of these enzyme systems require the presence of α-lipoic acid, as well as that of Mg^{2+} ions, NAD^{+}, coenzyme A and thiamine pyrophosphate. In these enzymic oxidative decarboxylations, there occur repetitive oxidations and reductions in the dithiol 'bridge' of α-lipoic acid. Greatly increased concentrations of oxygen should enhance the formation of the oxidized form of the dithiol moiety of α-lipoic acid and inhibit its reduction, thus interrupting the normal sequence of events in these two enzyme systems. The marked sensitivity of the thiol groups of α-lipoic acid to rapid oxidation by atmospheric air is only suggestive evidence. Many other important biological compounds contain thiol groups that are highly sensitive to oxidation by atmospheric air. However, a disproportionately great sensitivity of the thiol groups of α-lipoic acid to oxidation by oxygen in high concentrations would be most likely to result in inhibitions confined to these two particular enzyme systems. Disturbances at these two critical metabolic sites will be manifested by major and widespread alterations in cerebral energy metabolism. Thus a specific molecular mechanism may be postulated to explain the detrimental effects of hyperbaric oxygen on the oxidation of D-glucose by this particular system *in vitro*. Dickens (1946*a*, 1955) has suggested that an impairment of the activity of pyruvate oxidase might be the major mechanism for oxygen toxicity.

Although these observations *in vitro* cannot be directly extrapolated to provide the mechanism for the toxic action of hyperbaric oxygen *in vivo*, there are some common aspects of the two situations. Normal metabolism of D-glucose is essential for higher cerebral functions (Quastel, 1961; Maddock, Hawkins & Holmes, 1939; McIlwain, 1953*a*, *b*). Insulin-induced hypoglycaemia results in unconsciousness, with or without convulsions. Similarly, the administration of α-deoxyglucose, a competitive inhibitor of D-glucose, causes the experimental animal to lose consciousness (Sokoloff, 1959). Therefore it would not be surprising if similar inhibitions in the catabolism of D-glucose during exposure to oxygen at high pressure were to be found in future studies *in vivo*.

The manometric techniques employed in these experiments for the measurement of total production of CO_2 yielded results that were not accurate or consistent. Moreover, we were unable to demonstrate the striking and significant depressions of total production of CO_2 at high oxygen pressure that would be expected to occur in correlation with the results with $^{14}CO_2$. If all the data are nevertheless accepted as valid, it would appear that the homogenate is oxidizing an increased quantity of some substrate other than D-glucose. Endogenous D-glucose was negligible (less than 0·1 mM), which should make substrate-dilution errors insignificant in the estimation of minimum calculated μmoles of CO_2 from D-glucose. From measurements of the production of $^{14}CO_2$ from D-[U-^{14}C]glucose during perfusion of isolated cat brain, Geiger (1957) has calculated that only about 30% of the CO_2 produced by such a preparation '*in vivo*' could be traced directly to the oxidation of D-glucose, and, further, that most of the D-glucose utilized by the perfused cat brain during convulsions induced by pentylenetetrazol was accounted for by the lactate produced. Likewise studies of the oxidation of D-[U-^{14}C]glucose by human subjects suggest that a considerable portion of the CO_2 produced by brain is derived from substrates other than D-glucose and its catabolic intermediates (Sacks, 1957). Experiments *in vitro* that used slices and minces of brain have also resulted in disparities between the total CO_2 produced and the production of CO_2 as calculated from the $^{14}CO_2$ produced from known concentrations of ^{14}C-labelled D-glucose (Flexner, Flexner & Hellerman, 1956; Hotta, 1962; Sutherland, Burbridge & Elliot, 1955).

Numerous previous experiments have shown only delayed alterations in the metabolism of brain tissue *in vitro* with oxygen at high pressure (Dickens, 1946*a*, *b*; Stadie *et al.* 1945*a*, *b*; Van Goor & Jongbloed, 1949), and so the sensitivity of the present system is important but not entirely unexpected. The use of cell-free dispersions and of mitochondrial preparations obviates many of the problems of permeability that are inherent in the use of slices. The addition of optimum amounts of hexokinase, ATP and NAD^{+} (with nicotinamide protection) provides a system that is not only capable of rapid metabolism but also highly sensitive to environmental alterations. Moreover, the cell-free system provides better control of variables, and does not involve the controversial problems of the proper neuronal (and glial) environment (Gardner, 1961; Galambos, 1961; Van Harreveld, 1962; Tschirgi, 1962).

Certain assumptions were implied in the report by Haugaard, Hess & Itskovitz (1957), who studied the consumption of oxygen by homogenate of rat heart at 1 atm. p_{O_2} and observed a significant decrease in the uptake of oxygen that began after 1 hr. of incubation. However, the crude homogenate employed was derived from an organ rarely indicted as being susceptible to the direct effects of hyperbaric oxygen *in vivo*. Moreover, the premise that 1 atm. p_{O_2} in experiments utilizing homogenates could be equivalent to much higher oxygen pressures *in vivo* can be questioned. The concentrations of oxygen in experimental aqueous media are commonly lower than the p_{O_2} of the gas phase above the liquid would indicate, a difference that increases as the p_{O_2} of the overlying gas phase is increased (Van Goor & Jongbloed, 1949; Gilbert, Gerschman, Ruhm & Price, 1958). Partial pressures of oxygen of 1800–2200 mm. Hg have been observed in the arterial blood of human subjects respiring pure oxygen at 3·5 atm. pressure (Lambertsen, Ewing, Kough, Gould & Stroud, 1955; Lambertsen *et al.* 1953). Although the venous p_{O_2} in the jugular bulb of those subjects was commonly recorded as being less than 100 mm. Hg, the p_{O_2} in the tissues of many areas of the brain must have been increased many-fold. Sonnenschein, Stein & Perot (1953) employed micropolarimetry in the cerebral cortices of unanaesthetized cats to demonstrate 10- to 50-fold increases in the concentration of oxygen within 2–10 min. at 4–6 atm. p_{O_2}. Bean (1961) also used micropolarimetry in observing very marked and often fluctuating elevations of the concentration of oxygen in several areas of the brains of anaesthetized dogs breathing oxygen at 5·5 atm. pressure. However, none of these experiments have determined the absolute concentrations of oxygen in brain tissue during exposures to oxygen at high pressure.

Pyruvate oxidase and α-oxoglutarate dehydrogenase could also be inhibited by a mechanism involving a marked and detrimental oxidation of the reduced thiol group of coenzyme A. The lack of significant 'protection' against oxygen at high pressure by added coenzyme A in studies of the oxidation of acetate, pyruvate and D-glucose seems to indicate that direct effects of hyperbaric oxygen on coenzyme A play no major role in oxygen toxicity. However, it has been reported that parenterally administered coenzyme A provided some protection to mice exposed to oxygen at high pressure (Gerschman, Gilbert, Nye, Dwyer & Fenn, 1954).

The lack of close correlation of the magnitude of the inhibitions of the oxidation of D-glucose with the inhibitions of the oxidation of pyruvate and α-oxoglutarate does not present a matter of great concern in experiments of this type. Direct comparison of magnitudes of inhibition are not completely justified in this work, because the concentrations of reactants (including those of intermediate substrates) were not equivalent in experiments that used different substrates. It is also possible that inhibitions in the pentose phosphate pathway could contribute to the relatively large decreases in the oxidation of D-[U-^{14}C]glucose with oxygen at high pressure.

The increased production of lactate from D-glucose during incubations of dispersions of rat brain in oxygen at high pressure agrees with the significant elevations of lactate concentration in the venous blood of anaesthetized dogs exposed to oxygen at 5 atm. pressure (Bean & Haldi, 1932). Geiger (1958) has found increased production of lactate by perfused cat brain during intense activity, particularly during convulsive activity. Dawson & Richter (1950) consider increases of lactate to be a sensitive and significant index of altered neurochemical activity *in vivo*. The best explanation for the increased production of lactate with oxygen at high pressure is that there is a block in the oxidation of pyruvate while glycolytic mechanisms remain intact. The diminished production of $^{14}CO_2$ from ^{14}C-labelled pyruvate during incubations in oxygen at high pressure supports such an explanation, although a direct stimulation of glycolysis by hyperbaric oxygen has not been completely excluded. The production of lactate provided a better measure of overall glycolytic activity in this system than did the disappearance of D-glucose, because of the rapid conversion of D-glucose into glucose 6-phosphate in the presence of the added hexokinase. The effect of cyanide in low concentration in diminishing the increase in the production of lactate with oxygen at high pressure may be correlated with the decreases in the magnitude of inhibition of pyruvate oxidase and α-oxoglutarate dehydrogenase by hyperbaric oxygen that occurred when cyanide was present in low concentration. Such phenomena bring to mind the work of Riggs (1945), who observed that cyanide in low concentrations prevented the rapid relaxation of isolated pyloric sphincter muscle that usually occurred during exposure to oxygen at 8 atm. pressure. An explanation is not readily available for these effects of cyanide, which appear to conflict with our proposed mechanism for the inhibitions produced by oxygen in high concentrations.

The above explanation for oxygen toxicity does not exclude a physicochemical mechanism involving the extensive formation of free radicals in tissues exposed to high oxygen pressure (Gerschman, 1959). Both explanations are compatible with the 'protective' effects exhibited *in vivo* by agents that contain thiol groups. However, action through the formation of hydrogen peroxide from free radicals must be regarded with some scepticism. Dickens (1946*a*) reported the absence of the formation of hydrogen peroxide in brain slices incubated in oxygen at high pressure. The failure of added catalase to prevent the inhibition of the oxidation of D-glucose by oxygen at high pressure adds further evidence against a mechanism that involves the formation of peroxides. The possibility exists that glutathione reductase provides the principal mechanism in the central nervous system for the destruction of strong oxidants such as peroxides. Therefore the lack of 'protection' by added catalase is not conclusive evidence against a mechanism involving the formation of peroxides.

We have found by direct tests that oxygen at high pressure can rapidly disrupt certain of the oxidative mechanisms of the brain as a whole. However, different areas of the brain may possess different degrees of susceptibility to inhibition by hyperbaric oxygen. Moreover, the results reported by Bean (1961) and Lambertsen *et al.* (1953, 1955) indicate that exposure of mammals to oxygen at high pressure produces marked variations and gradients in the concentration of oxygen in different areas of the brain. Neurochemical alterations induced by oxygen at high pressure could vary in intensity within different areas of the brain, if only by virtue of marked differences in the concentrations of oxygen.

SUMMARY

1. Marked and rapid depressions in the catabolism of D-glucose have been observed in cell-free dispersions of rat brain during incubations in oxygen at high pressure, commonly 5 atm. These results have shown a better rate-correlation with phenomena *in vivo* than have previous neurochemical studies.

2. The production of lactate from D-glucose increased rapidly during incubations in oxygen at high pressure, with significant increases in the concentration of lactate occurring in less than 15 min.

3. During incubations in oxygen at 5 atm. pressure, oxidation of uniformly ^{14}C-labelled D-glucose to $^{14}CO_2$ decreased to 20–33 % of control values in oxygen at 1 atm. pressure within 30 min. This phenomenon was accompanied by a slightly decreased utilization of D-glucose, increased production of lactate and variable and usually insignificant alterations in the total production of CO_2.

4. The production of $^{14}CO_2$ from both [1-^{14}C]-pyruvate and [2-^{14}C]pyruvate decreased during incubations in oxygen at 5 atm. to 50–70 % of control $^{14}CO_2$ produced in oxygen at 1 atm., with again little significant alteration in total production of CO_2. Oxygen at 5 atm. pressure decreased the production of $^{14}CO_2$ from α-oxo[5-^{14}C]glutarate to 45–70 % of that produced in oxygen at 1 atm. and inhibited the production of $^{14}CO_2$ from DL-[1-^{14}C]-glutamate almost as much. The production of $^{14}CO_2$ from [1,4-$^{14}C_2$]succinate was not altered during incubations in oxygen at high pressure for 30 min.

5. The additon of coenzyme A, thiamine pyrophosphate, catalase, reduced glutathione or mercaptoethanol to the brain dispersions did not result in any significant 'protective effects' against the inhibitions in the oxidation of uniformly ^{14}C-labelled D-glucose and ^{14}C-labelled pyruvate caused by oxygen at high pressure.

6. Rapid depression of the activity of pyruvate oxidase in rat-brain mitochondria was observed during incubations in oxygen at 5 atm. pressure as measured by decreased production of $^{14}CO_2$ and acetyl phosphate from [1-^{14}C]pyruvate.

7. Rapid inhibition of the activity of α-oxoglutarate dehydrogenase in rat-brain mitochondria was observed during incubations in oxygen at 5 atm. pressure as determined by decreased production of $^{14}CO_2$ from α-oxo[1,2-$^{14}C_2$]glutarate.

8. A mechanism for the toxicity of high oxygen pressure is proposed in which direct oxidation of the dithiol moiety of α-lipoic acid by oxygen in high concentration interferes with the normal activity of pyruvate oxidase and α-oxoglutarate dehydrogenase.

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