The Toxic Effects of Oxygen on Brain Metabolism and on Tissue Enzymes

2. TISSUE ENZYMES

By F. DICKENS, *National Institute for Medical Research, London, N.W. 3, and Cancer Research Laboratory, Royal Victoria Infirmary, Newcastle-upon-Tyne*

(*Received* 23 *October* 1945)

The evidence in Part 1 (Dickens, 1946) enables the following working hypothesis of the mode of action of oxygen on brain metabolism to be advanced with some confidence.

(1) Exposure to unphysiologically high tensions of oxygen causes irreversible damage to brain respiration and glycolysis.

(2) This damage is sustained by the enzymes themselves rather than by their coenzymes, as shown by the lack of general protection in presence of excess of the more important coenzymes.

(3) The following conditions tend to protect against oxygen poisoning: Presence of (*a*) glucose or (as shown in this paper) manganese ions, which protect succinoxidase in finely divided brain tissue; (*b*) ions of manganese, cobalt, or magnesium (and probably calcium), which protect the whole oxidative system in brain slices, with decreasing activity in the order of the above series.

(4) The brain enzymes poisoned by oxygen are mainly those concerned in carbohydrate metabolism. Oxidation of glucose, fructose, lactate and pyruvate is inhibited, while the succinoxidase system, at least in intact tissue (slices) is much more resistant. There is support for the view that the inactivation of an —SH component of the pyruvate oxidase system may be a primary stage in oxygen poisoning, the resulting interference with resynthesis of adenosine triphosphate could then lead to general breakdown of aerobic and anaerobic carbohydrate metabolism.

(5) It is, however, not excluded that, in addition to the pyruvate oxidase, another and earlier stage of carbohydrate metabolism might be inhibited, thus poisoning directly the oxidation of glucose and fructose.

It is hoped later to study the effect of oxygen on components of the pyruvic oxidase system. In the present paper, some general evidence is collected on the mode of inactivation by oxygen of some other enzymes, particularly those which, like pyruvate oxidase, may play a part in brain metabolism. These experiments are of a preliminary and exploratory character, and serve mainly to define some conditions under which this type of inhibition may be expected to occur, and to investigate some examples of protection against oxygen poisoning in enzyme systems.

The general nature of the inactivation of enzymes by oxygen

A list has been compiled of enzymes known to be sensitive to oxygen, or capable of inactivation by comparatively mild oxidants, and therefore open to the suspicion of being oxygen-sensitive under the right conditions (Table 1). These observations were widely scattered, and probably others could be added: the only review found to bear on this subject, apart from the excellent one by Stadie, Riggs & Haugaard (1944) which appeared after this work was completed, is that of Hellerman (1937), which is limited to hydrolytic enzymes. As this author rightly remarks: 'In most enzyme studies, the effect of an ever present reagent, the oxygen of the air, has been generally neglected.'

There is another reason why such a list is inevitably incomplete, and that is the enormous effect on oxygen susceptibility of traces of contaminants, especially —SH compounds and metallic ions, but also other substances less well defined. The following

typical experiences are quoted as excellent examples of the extraordinary complexity of the oxygen poisoning of enzyme systems:

(*a*) *Urease.* Perlzweig (1932) showed that aeration of crystalline urease weakened its action, and believed this to be due to removal of a sulphydryl component by oxidation. The still more highly purified enzyme is, however, practically insensitive to the action of air, but is rendered sensitive again on the addition of minute amounts of copper salts (Hellerman, Perkins & Clark, 1933).

(*b*) *Arginase* behaves very similarly: cruder preparations are readily inactivated by oxygen, while purified ones are much less sensitive, or completely insensitive (Edlbacher, Kraus & Leuthardt, 1933; Klein & Zeise, 1933, 1935). Arginase preparations are activated by metallic ions (Mn, Fe, Co, Ni, Zn, Cd, and possibly others—cf. Richards & Hellerman, 1940). Hellerman (1937) believes that the action of these metals is to form co-ordination compounds linking the enzyme molecule with that of the substrate.

(*c*) *Phosphoglucomutase.* This enzyme, which is necessary for breakdown and synthesis of glycogen, is of peculiar interest, since the present work has shown that at different stages of purification it can exhibit a wide variation of sensitivity to air.

Gill & Lehmann (1939) showed that there was a very slight increase in the amount of Robison-ester formed from glycogen by muscle extract when the experiment was made in absence of air.

Like many other oxygen-sensitive enzymes, phosphoglucomutase is highly susceptible to the influence of traces of metals; cobalt, magnesium and manganese strongly accelerate, nickel feebly so, and zinc retards the activity of the enzyme. Concentrated solutions of reduced glutathione and oxidized glutathione respectively, accelerate, and retard its activity (Cori, Colowick & Cori, 1938; Gill & Lehmann, 1939; Lehmann, 1939; and present work). Although these properties suggest a direct connexion between oxido-reduction potential and activity, the relationship is, in fact, involved, e.g. substances such as insulin or caffeine are also inhibitory. The best way of circumventing

Table 1. *Enzymes sensitive to oxygen or to mild oxidants*

Enzyme nature*	Enzyme class	Authority
A. Inactivation by exposure to gaseous oxygen (or air)		
	(*a*) Proteolytic	
—SH	Cathepsin: tissue autolysis	Bailey, Belfer, Eder & Bradley (1942)
—SH	Papain	Hellerman (1937)
—SH	Proteases, Peptidase	Irving, Fruton & Bergmann (1942)
	(*b*) Hydrolytic ('deamidizing')	
M	Arginase	Edlbacher, Kraus & Leuthardt (1933), Hellerman (1937)
—SH	Urease	Perlzweig (1932), Sumner & Poland (1933)
	(*c*) Fe-porphyrins	
M	Hydrogenase	Stephenson & Stickland (1931), Hobermann & Rittenberg (1943)
M	'Pasteur enzyme'	Warburg (1926), Stern & Melnick (1941)
M	Catalase	Marks, G. W. (1936) (prolonged experiments)
M	Cytochrome oxidase	Dickens (this paper)
	(*d*) Oxidases, etc.	
—SH	Pyruvic oxidase	Barron (1936), Mann & Quastel (1946), Dickens (1946)
—SH	Succinoxidase	Lehmann, J. (1935), Liebricht & Massart (1937), Bohr & Bean (1940–1), Stadie (1943), Dickens (this paper)
—SH	Choline oxidase	Dickens (this paper)
F	Xanthine oxidase	Dixon (1925), Dixon & Kodama (1926)
F	*d*-Amino-acid oxidase	Stadie (1943)
F	Cytochrome-*c* reductase	Haas, Horecker & Hogness (1940): (possibly)
?	Fatty acid dehydrogenase	Shapiro & Wertheimer (1943)
—SH	Triosephosphate dehydrogenase	Dickens (this paper)
?	Formic acid dehydrogenase	Gale (1939)
	(*e*) Phosphorases	
—SH	Phosphoglucomutase	Gill & Lehmann (1939), Dickens (this paper)
—SH (?)	Neuberg ester phosphorase (phosphoglucokinase)	Engelhardt & Sakov (1943)
—SH (?)	Myokinase	Kalckar (1943)
—SH	Transaminase	Cohen, Hekhius & Sober (1942)
—SH	Triosephosphate dehydrogenase	Rapkine (1938), Rapkine & Trpinac (1939)
M	Zymohexase (of yeast)†	Warburg & Christian (1942)
—SH (?)	Cerebrosidase	Hellerman (1937, p. 466)
B. Inactivation by exposure to oxygen under special circumstances, or by mild oxidants: preservation by —SH compounds. (Less satisfactory evidence than the preceding.)		

* Enzyme nature. —SH indicates evidence that an intact sulphydryl group is considered necessary for activity. *M* indicates that the enzyme either contains a metal as part of its prosthetic group, or requires metallic ions for its activity. *F* indicates that the enzyme contains a flavoprotein. (The first and second of these classes are often combined, both an —SH group and an activating metal being necessary. The classification of some of these enzymes cannot be considered as final.)

† Zymohexase of animal tissues shows only inhibition by metallic ions, and is stable to oxidants (Herbert, Gordon, Subrahmanyan & Green, 1940).

the experimental difficulties has in practice proved to be: (*a*) preparation where possible of the enzyme in a state such that it is sensitive to oxygen; (*b*) study of conditions protecting enzymes of this kind against inhibition by oxygen. Experiments devised with this end in view are described below.

Table 1 shows that many different classes of enzyme are sensitive to oxygen. Although the presence of —SH groups, and/or metal activators is needed for the activity of many of these, some of the flavoprotein enzyme systems, which do not require metals, are also sensitive. There is also evidence that oxidative inactivation of enzymes may sometimes be due to the sensitivity of quite different groups; for example, pepsin inactivation by iodine may be due to iodination of tyrosine groups contained in the molecule of the enzyme, and oxidation of the phenolic group of tyrosine may account for the inactivation of alkaline phosphatase by moderately strong oxidants (Sizer, 1942). In general, however, the sensitivity of those enzymes which are weakened or inactivated by atmospheric oxygen seems to be related to either: (*a*) destruction of —SH groups by oxidation; (*b*) loss of catalytic function of essential metals, presumably by their conversion to higher valency forms; or (*c*) oxidative destruction of flavoproteins. Examples of each type of inactivation are listed in Table 1.

EXPERIMENTAL

(1) Oxidative enzymes

A. *Succinic oxidase system*

This system has been more fully studied hitherto in connexion with oxygen poisoning than any other (for references, see Table 1), and is known to be oxygen-sensitive. This has been confirmed, and the sensitivity of the components of the system oxidizing succinate has been determined. These are (*a*) cytochrome oxidase, (*b*) cytochrome *c*, (*c*) succinic dehydrogenase, and (*d*) a further component, which may be a flavoprotein (Axelrod, Potter & Elvehjem, 1942). The dehydrogenase itself is the most sensitive of the components *a*, *b* and *c* (component *d* cannot at present be studied separately); with longer exposure to H.O.P. the cytochrome oxidase may also be slightly weakened (Table 2).

The sensitivity to oxygen of succinoxidase contained in homogenized tissue has already been shown (Dickens, 1946) to be greater than that of the enzyme system when present in tissue slices. There are also wide variations in sensitivity from one enzyme extract to another (cf. Tables 2, 4). Bohr & Bean (1940–1), who made a similar observation, consider that this may be related to the susceptibility of the individual animals to oxygen, which is known to vary widely. In view of the dependence of sensitivity of these enzymes on their physical state, as well as on the presence or absence of sensitizers or protective substances as already described, it seems doubtful if this conclusion is justified on the available evidence. The data on oxygen inactivation of this enzyme are contained in Table 2.

Protection of succinoxidase against poisoning by oxygen

(*a*) *By malonate.* Malonic acid is a powerful competitive inhibitor for succinic dehydrogenase. If, during exposure of the enzyme to oxygen, malonate is present in concentration sufficient to 'block' the active groups of the dehydrogenase (*c.* 0·001M), and after exposure to oxygen the malonate is removed by washing, it is found that the enzymic activity has been completely protected (Table 3). The presence of malonate during the incubation also protects the enzyme against autolytic processes occurring in nitrogen, but these are less destructive of enzyme activity than the oxidative inactivation (Table 3); in some experiments there was little or no inactivation during incubation in nitrogen. Numerous other experiments, some under H.O.P.,* also showed similar protection of succinoxidase by malonate against oxygen poisoning. This result is interpreted (see Discussion) to mean that the sulphydryl groups of the dehydrogenase are involved in inactivation by oxygen. The succinic dehydrogenase system may require manganese for its activity (Massart, 1939), and if this is accepted as correct, the loss of manganese due to oxygen might be pictured as occurring similarly to that of manganese from arginase (Hellerman, 1937). The protection of succinoxidase activity against H.O.P. by manganese, described below, seems to be the first clear evidence of a possible function of this metal in the succinic acid system.

(*b*) *By manganese ions.* In view of the effects of metallic ions on the sensitivity of tissue respiration to oxygen (see Part 1), similar experiments were made with the succinoxidase system (Table 4). Whereas manganese ions (0·00025M concentration) gave almost complete protection of succinoxidase against oxygen, cobalt in the same concentration, or magnesium even in sixteen times higher concentration, did not. In fact, even at this very low concentration, cobalt proved to be quite toxic, both in oxygen and nitrogen. This illustrates further the great dependence of the effect of these metals on the exact circumstances of the test, for cobalt ions protect the whole oxidation system in brain slices, as well as the oxidation of pyruvate (Dickens, 1946), but these ions are unable to protect the succinic system in homogenized brain tissue. On the other hand, manganese ions protect both the total oxida-

* High oxygen pressure (see Dickens, 1946).

Table 2. *Inactivation of succinic oxidase system by oxygen*

(*a*) *Preparation from pig-heart muscle* by mechanical grinding of the washed tissue with sand, precipitation at pH 4·6 (cf. Straub, 1939), and resuspension in phosphate buffer at pH 7·4. 0·2 ml. enzyme/vessel; M/6 succinate; total vol. 3·0 ml. The enzyme was added from the side-bulb immediately after the first reading. Gas: air. Temp. 38°.

	Oxygen uptake (μl.) in 10 min. after the following exposure of enzyme					
		Nitrogen		Oxygen at 1 atm.	Oxygen at 4·4 atm.	
Contents of vessel during test:	None	2½ hr.	3 hr.	3 hr.	2 hr.	3 hr.
Enzyme alone (no succinate)	0·5	—	—	—	—	—
Enzyme + succinate (*A*)	102	100	78	51	41·5	30
Enzyme + succinate + meth. blue	106	93	85	66	—	49
Enzyme + succinate + meth. blue (0·03 %) + HCN 0·002 M (*B*)	53	51·5	—	41·5	—	29
Enzyme + succinate + HCN (no meth. blue)	2	—	—	—	—	—
Enzyme + *p*-phenylene diamine (*C*)	63·5	63	—	57	—	42·5

	As % of initial activities (mean results) after pre-treatment			
		1 atm.	4·4 atm. oxygen	
	Nitrogen (3 hr.)	oxygen (3 hr.)	(2 hr.)	(3 hr.)
Total system (*A*, above)	90	50	41	30
Succinic dehydrogenase (*B*)	97	78	—	55
Cytochrome oxidase (*C*)	99	90	—	67

(*b*) *From horse heart* (a more resistant preparation than the above). 0·1 ml. enzyme/vessel. Activity with succinate measured in air. Temp. 38°. pH 7·4.

	Oxygen uptake (μl.) in 10 min. after following exposure of the enzyme				
		Oxygen (1 atm.)		Oxygen (4·4 atm.)	
Control	Nitrogen (2 hr.)	(1 hr.)	(2 hr.)	(1 hr.)	(2 hr.)
129	124	124	117	112	116
% of initial activity remaining:					
(100)	96	96	91	87	90

(*c*) *Homogenized rat-brain tissue* (*cortex*). 40 mg. of moist tissue/vessel. Cytochrome *c* (0·1 ml.) added to give $2·2 \times 10^{-5}$ M solution.

	Oxygen uptake (μl.) in 10 min. after the following exposure of the homogenate		
None	Nitrogen (2 hr.)	Air (1 atm.) (2 hr.)	Oxygen (4·4 atm.) (2 hr.)
56	46	35	30
Without added cytochrome:			
21	—	—	8
Nitroprusside test (for —SH groups) in above after incubation:			
+ + + +	+ + +	±	-

tion system of brain slices and also the oxidation of succinate by homogenized brain. Much further work would be needed to arrive at an explanation; at present one can only draw attention to the fact that traces of metals have this profound influence on oxygen poisoning, and that under favourable conditions they may give complete protection of an otherwise susceptible enzyme.

The role of oxaloacetic acid in oxygen poisoning of succinoxidase

The succinoxidase preparations used in these experiments oxidized succinate only as far as fumarate and malate; the further stage of oxidation of malate to oxaloacetate does not occur with them to any considerable extent, the accumulation of

Table 3. *Protection of succinic dehydrogenase against the toxic effect of oxygen by malonic acid*

Method. Rat-brain cortex, homogenized in M/30 phosphate, pH 7·4.

Centrifuged, supernatant fluid discarded, precipitate re-suspended in M/30 phosphate buffer.

0·5 ml. of this suspension (equiv. to 100 mg. moist tissue) was incubated with the addition of either 0·05 ml. phosphate buffer, or 0·05 ml. 0·1 M-sodium malonate, for 2 hr. at 38° in either nitrogen or oxygen at 1 atm.

After this incubation, the contents of the vessels were rinsed into centrifuge tubes with 4·5 ml. of M/30 phosphate, centrifuged, supernatant discarded and the precipitate rinsed into Warburg vessels with 2 ml. M/30 phosphate, 0·2 ml. cytochrome *c*, and (in the side-bulbs) 0·1 ml. M-sodium succinate. Rates of succinate oxidation were measured at 1 atm. oxygen.

	Oxygen uptake of succinic oxidase system (μl. O_2/30 min.)			
	2 hr. oxygen		2 hr. nitrogen	
Period (min.)	No malonate	With malonate	No malonate	With malonate
0–30	15·5	102	49	97
30–60	15	98·5	49	93

minute traces of oxaloacetate being strongly inhibitory to succinic dehydrogenase. The accumulation of oxaloacetate requires an active malic dehydrogenase for which the presence of cozymase is necessary. Cozymase is rapidly broken down in homogenized brain tissue, and, in the case of the muscle preparations used in the above experiments, most of the cozymase had been removed by washing with acid. Swingle, Axelrod & Elvehjem (1942) have shown that calcium ions greatly accelerate the oxidation of succinate, their action being due to the fact that these ions promote the breakdown of cozymase, thus preventing the formation of oxaloacetate. They found that the inhibition of the enzyme by 0·2 μM-oxaloacetate was removed to a large extent by the presence of glutamate. This was due to the removal of the oxaloacetate by its interaction with glutamate, a reaction catalysed by the enzyme transaminase.

The possibility therefore arose that the oxygen poisoning of succinic dehydrogenase might be caused by excessive formation or accumulation of oxaloacetic acid. Decisive evidence on this point has not been obtained; although some results may be considered to support it, there is also evidence that other, irreversible, changes in the enzyme occur during exposure to oxygen, especially when the oxygen pressure is high or the exposure prolonged.

The influence of glutamate on the poisoning of succinoxidase by oxygen

If oxaloacetate inhibition of succinoxidase preparations were responsible for the poisoning of the dehydrogenase which occurs in oxygen, this poisoning would be expected to be reversed by glutamate. This, however, is only true as long as the transaminase present is not also poisoned by oxygen, for this enzyme is needed for the removal of oxaloacetate by glutamate. There is some reason to suspect that transaminase is, in fact, susceptible

Table 4. *Action of metallic ions on the oxygen sensitivity of brain succinoxidase*

Method. Rat-brain cortex, homogenized in M/30 phosphate buffer, pH 7·4. Each Warburg vessel contained 40 mg. of moist tissue in a total volume of 2 ml. M/30 phosphate, together with 0·1 ml. cytochrome *c* and 0·05 ml. of the solution of metallic salt to be tested.

The vessels were shaken at 38°, while filled with either nitrogen or oxygen at 1 atm.

After this incubation period, those vessels which had contained nitrogen were now filled with oxygen, and 0·1 ml. of M-sodium succinate was added from the side-bulbs. Readings of oxygen uptake were then taken as usual.

Exp. i. Oxygen uptake (μl./20 min.) after exposure to the following gases for 2 hr. at 38°.

	Oxygen				Nitrogen			
Metal present ...	Na only	Mn	Mg	Co	Na only	Mn	Mg	Co
Concentration of metal (molarity)	—	$2·5 \times 10^{-4}$	4×10^{-3}	$2·5 \times 10^{-4}$	—	$2·5 \times 10^{-4}$	4×10^{-3}	$2·5 \times 10^{-4}$
μl. O_2/20 min.	57	89	59	3·5	84·5	85	88	15·5
$\frac{\text{Activity after } O_2}{\text{Activity after } N_2}$:					67 %	*105* %	67 %	(23 %)

Exp. ii. Protection by $MnSO_4$ ($2·5 \times 10^{-4}$ M) of a more resistant brain homogenate. Exposure times, 2 and 4 hr. at 1 atm.

Gas	Oxygen				Nitrogen			
Metal	No Mn		+Mn		No Mn		+Mn	
Exposure (hr.)	2	4	2	4	2	4	2	4
Activity (μl. O_2/30 min.)	52	39	72	68·5	65	55·5	78	73
As % of corres. control exp.	80	70	92	94	—	—	—	—

to poisoning by oxygen (see Table 1). This probably accounts, in part, for the fact that, in certain experiments, some protective effect of glutamate was evident (Table 5, Exp. i), whereas in others it was not.

Table 5. *Influence of glutamate on the poisoning of succinoxidase by oxygen*

Exp. i. *Protection by glutamate*

Methods. Horse-heart succinoxidase preparation: 0·2 ml./vessel (in side-bulb). Main part of vessels contained 0·3 ml. 0·2 M-succinate, glutamate (0·1 ml. 0·2 M) if so stated below, and phosphate buffer, pH 7·4, to total of 2 ml. Oxaloacetic acid (as sodium salt) was added in some vessels to illustrate the poisoning of this particular enzyme by this substance, and the reversal by glutamate.

In some experiments the enzyme preparation, with or without the addition of glutamate (M/30) was exposed to H.O.P. (4·4 atm. oxygen, 3 hr.), and the activity then tested as above. (OAA = Oxaloacetate.)

	Oxygen uptake (μl./60 min.) of controls not exposed to H.O.P.
With succinate only	237
Succinate + oxaloacetate, 2×10^{-6} M	8
Succinate + glutamate (0·01 M) in addition to OAA	412
Succinate + oxaloacetate, 2×10^{-7} M	185

Exposed to O_2:	Oxygen uptake (μl./60 min.) after 3 hr. exposure to 4·4 atm. O_2	Inactivation
Without glutamate	93	61 %
With glutamate (during exposure and during measurement)	382	7 %

Exp. ii. *Lack of protection.* The same enzyme preparation after long keeping in the refrigerator was no longer protected by glutamate against oxygen poisoning. Several other negative results were obtained with other preparations.

Reversibility of oxaloacetate poisoning, and non-reversibility of oxygen poisoning, of succinoxidase

Whereas the poisoning of succinic dehydrogenase activity by oxaloacetate was readily reversed on removal of the oxaloacetate by washing (cf. poisoning by malonate, Table 3), even after the enzyme had been exposed to oxaloacetate for more than 1 hr., the poisoning of the same enzyme by oxygen was not so reversed (Table 6). Moreover, no oxaloacetate was detectable by the sensitive aniline-citrate test in a preparation of succinoxidase which had been exposed to 1 atm. oxygen for 2·5 hr.

Hence, if oxaloacetate formation is concerned in the poisoning of succinic dehydrogenase by oxygen, it can only partially explain the effect.

Table 6. *Restoration of succinoxidase activity after removal of added oxaloacetic acid, but not after poisoning by oxygen: failure to show OAA formation in oxygen*

Rat-brain cortex homogenate. Centrifuge and re-suspend in M/30 phosphate, pH 7·4. Activity test: 0·1 ml. cytochrome *c*, 0·1 ml. M-succinate added.

Exp. i. *Lack of reversal of oxygen poisoning on washing*

Exposure of homogenate	Oxygen (1 atm., 2 hr.)		Nitrogen (1 atm., 2 hr.)	
After exposure	Washed	Unwashed	Washed	Unwashed
Activity towards succinate; μl. oxygen uptake/30 min.	39·5	80	61	105

Ratio of activity: $\frac{\text{Washed} \times 100}{\text{Unwashed}}$ — After nitrogen: 58 %; After oxygen: 50 %

Exp. ii. *Reversal of oxaloacetate poisoning by washing*

Homogenized brain system. Succinate as substrate.

	Oxygen uptake (μl./10 min.)		Oxygen uptake (μl./10 min.)
(A) Without oxaloacetic acid	71	the same after washing	53·5
Oxaloacetic M/2500	13 (82 % inhibition)	,,	54
Oxaloacetic M/10,000	42·5 (40 % inhibition)	,,	56
(B) Without oxaloacetic acid	35	,,	21
Incubated at 37·5° with oxaloacetic acid M/2500 for 1½ hr.	18	,,	24

Exp. iii. *Lack of evidence of oxaloacetate accumulation in measurable amount*

Homogenized rat liver in M/30 phosphate buffer, pH 7·4 centrifuged and resuspended, shaken in oxygen for 2½ hr., in Warburg vessel. Temp. 38°.

After this period, the vessel contents were acidified by the addition of 0·5 ml. conc. citric acid solution, and 0·5 ml. of the aniline citrate reagent (Edson, 1935) was placed in the side-bulb. On tipping the reagent into the main part of the vessel, no measurable decarboxylation occurred. Hence no β-ketoacid had been accumulating under these conditions. This method would not detect with certainty less than 4×10^{-5} M-oxaloacetate, had this been present. The succinic oxidase activity of the same liver homogenate was inhibited by 51 % on the addition of 10^{-4} M-oxaloacetic acid.

Attempts made to reverse the oxygen poisoning of succinoxidase preparations by incubation under anaerobic conditions, with or without the addition of glutathione (cf. Hopkins & Morgan, 1938), were

Table 7. *Resistance of lactic dehydrogenase to* H.O.P.

(1) *In brain slices*

Method. Slices of rat-brain cortex were incubated for 2 hr. at 38°: (*a*) in air at 1 atm., (*b*) in oxygen at 4·4 atm. Medium: phosphate-Ringer's solution containing glucose. The slices were rinsed, and the oxygen-uptake rates measured in presence of lactate, cozymase, HCN, and methylene blue (Method i). Alternatively, the lactate dehydrogenase activity after exposure was measured anaerobically in presence of ferricyanide, HCN, and cozymase by the method of Quastel & Wheatley (1938) (Method ii).

Method i.	Air-incubated slices		4·4 atm. oxygen-incubated	
	Blank	With lactate added	Blank	With lactate added
Oxygen-uptake rate (Q_{O_2})	1·3	2·4	1·1	2·8

Method ii.	With lactate and cozymase	Without cozymase		With cozymase	
		−Lactate	+Lactate	−Lactate	+Lactate
CO_2 evolution rate (Q_{CO_2})	4·8	1·65	2·85	1·9	5·4

(2) *In purified lactic dehydrogenase solution*

Method. Dialyzed extract of acetone muscle powder (cf. Green, Needham & Dewan, 1937). Pig-heart flavoprotein of Straub (1939), cozymase prepared according to Williamson & Green (1940). Care was taken to use minimal amounts of dehydrogenase to secure maximum oxygen uptake; by avoiding any excess of this enzyme it becomes the factor limiting the rate of the reaction, hence any destruction of the enzyme would be clearly revealed.

Each vessel contained: 0·5–0·05 ml. enzyme, 0·2 ml. M-lactate, 0·8 mg. cozymase, 0·4 ml. M-HCN, 0·3 ml. flavoprotein solution, and sufficient 0·05M-phosphate, pH 7·4, to make the total volume 3·0 ml. 0·2 ml. methylene blue (0·5 %) was added from the side-bulb at the beginning of the readings of oxygen uptake.

Pre-treatment of enzyme	On ice	In nitrogen 38°, 3¼ hr.	In oxygen 4·4 atm. 38°, 3¼ hr.
Volume of enzyme solution, 0·5 ml./vessel:			
Rate of oxygen uptake (μl./30 min.)	103	103	99·5
Loss of activity in oxygen		3·5 %	
Volume of enzyme solution, 0·05 ml./vessel:			
Rate of oxygen uptake (μl./70 min.)	—	214	214
Loss of activity in oxygen		0 %	

(Volume of enzyme 0·01 ml.: oxygen uptake in control experiment falls to 81 μl./70 min., showing that excess enzyme was not used, particularly in latter of above experiments.)

unsuccessful. Unlike malonate, pyrophosphate, which also is an inhibitor of succinic dehydrogenase, did not, in the one experiment made, protect the enzyme against the poisonous effect of high-pressure oxygen.

B. *Lactic dehydrogenase*

The oxidation of lactate by tissue preparations is inhibited by oxygen (Dickens, 1946), but consideration of the mechanism of the oxidation shows how complicated the interpretation of this fact may be. In fact, lactic dehydrogenase itself is highly resistant to oxygen, as the following experiments show.

The activity of lactic dehydrogenase in slices of rat-brain cortex, after 2 hr. exposure to 4·4 atm. of oxygen, was compared with that of slices kept in air at 1 atm. There was no loss of activity of the dehydrogenase (Table 7). Other experiments confirmed this result (Table 7).

There is, therefore, no evidence to suggest that lactic dehydrogenase is at all sensitive to oxygen. This agrees with observations reported to us by Stadie (1943). The poisoning of lactate oxidation in tissues (Dickens, 1946) is probably accounted for by injury to other parts of the complex system by which lactate and pyruvate are metabolized in intact tissues.

C. *Malic dehydrogenase*

This enzyme was tested with the same preparation of lactic dehydrogenase from rabbit muscle, and under the same conditions, as the lactic dehydrogenase described above. The results of exposure to 4·4 atm. oxygen for 3·25 hr. showed no destruction, as compared with a portion of the enzyme incubated in nitrogen, and negligible loss of activity in either nitrogen or oxygen as compared with a control portion kept at 0° (Table 8). This dehydrogenase, like lactic dehydrogenase, is in our experience quite stable to oxygen, though according to Barron (1943) it is an —SH enzyme.

Table 8. *Malic acid dehydrogenase*

Method: same as for lactic dehydrogenase (Table 7) but with sodium *l*-malate as substrate instead of lactate. Each vessel contained 0·5 ml. enzyme, 0·8 mg. cozymase, 0·3 ml. heart flavoprotein, 0·2 ml. *l*-malate (M-solution), 0·4 ml. M-HCN, and M/20 phosphate buffer, pH 7·4. Total vol. 3 ml.

Oxygen uptakes were measured manometrically, immediately following the addition from the side-bulb of 0·2 ml. methylene blue (0·5 % solution).

Pre-treatment of enzyme	On ice	In nitrogen 38°, 3¼ hr.	In oxygen 4·4 atm. 38°, 3¼ hr.
Rate of oxygen uptake (μl./30 min.)	54·5	53	53

D. *Triosephosphate dehydrogenase*

The activity of this enzyme system was followed by the rate of the dismutation:

Triosephosphate + pyruvate → phosphoglyceric acid + lactate

(cf. Green, Needham & Dewan, 1937; Rapkine, 1938; Rapkine & Trpinac, 1939).

This reaction has the same rate in nitrogen as in oxygen (Green *et al.* 1937 and Corran, Green & Straub, 1939). After removal of cozymase by adsorption on charcoal from the enzyme, the latter is more sensitive to oxidants (Rapkine, 1938) and cozymase can probably protect this enzyme against oxidation. Rapkine & Trpinac (1939) showed that the inactivation of the enzyme by various oxidizing agents could be reversed by subsequent contact with reducing agents.

In studying the effect of oxygen on this enzyme, advantage has been taken of the above interesting properties. It was found that the triosephosphate dehydrogenase is susceptible to oxygen poisoning, but only becomes sensitive to this action after the cozymase has been removed. Table 9 shows that before removal of cozymase, exposure to 4·4 atm. of oxygen for 3 hr. caused only 4–9 % poisoning. After removal of cozymase by treatment with charcoal, a similar exposure to oxygen caused 24 % inactivation.

It is to be noted that the sulphydryl groups of this enzyme preparation are rather insensitive to oxygen; a weakly positive nitroprusside test, of about the same strength, being observed after exposure as above to either nitrogen or H.O.P. Probably only a small part of this reaction was due to the —SH groups of the enzyme itself, the remainder being due to contaminants. Specially made control tests gave no support to the idea that traces of metals introduced by the treatment with charcoal (which always contains metals) could have brought about the sensitization of the enzyme to oxygen. All charcoal was removed by centrifugation before the test was made, so that its presence was not responsible.

E. d-*Amino-acid oxidase, flavin-adenine-dinucleotide, and diaphorase*

Although *d*-amino-acid oxidase is not present in brain tissue, which oxidizes only glutamic acid among amino-acids, it is of interest (*a*) as a flavoprotein, a class which includes oxygen-sensitive (or at least H_2O_2-sensitive) enzymes (Table 1), and (*b*) because it was stated by Stadie (1943) to be highly susceptible to oxygen, when used in the form of homogenized tissue suspensions, but very resistant when used as tissue slices.

Our own experiments with homogenized kidney tissue were unsatisfactory as evidence of the stability or otherwise towards oxygen of this enzyme. This was because the rate of loss of activity of these preparations was very high, even when they were maintained in pure nitrogen. Hence comparison with the rate of destruction in oxygen was unreliable. After even 30 min. incubation at 38° in either nitrogen or oxygen the enzyme was inactivated, the rate of oxygen uptakes being then

Table 9. *Triosephosphate dehydrogenase*

Method: see text. 1 ml. muscle extract, 0·2 ml. M/2 sodium pyruvate, 0·3 ml. M/2 sodium bicarbonate, 0·7 ml. M/5 sodium fluoride, 1 mg. cozymase, and water to make the final volume 3 ml. The side-bulb contained 0·3 ml. *c.* M/10 hexose diphosphate solution, and the carbon dioxide evolution following the addition of this to the main part of the vessel was followed manometrically. Temp. 38°. Gas-space of manometer vessels contained 5 % (by vol.) of carbon dioxide.

(*a*) *Enzyme preparation not freed from cozymase*

Pre-treatment of enzyme	Untreated	Incubated 3 hr. in nitrogen	Incubated 3 hr. 4·4 atm. oxygen
Activity of dehydrogenase (μl. CO_2/15 min.)			
1. (No NaF added)	445	586	536
2. (In presence of NaF)	—	597	572

Percentage of control in N_2, after H.O.P.: 91 %, 96 % respectively.

(In the second of these experiments, the enzyme solution was neutralized to pH 7·4 but not in the first. This probably accounts for the apparently slightly higher activity in presence of NaF.)

(*b*) *Enzyme treated with charcoal to remove cozymase almost completely*

2·0 g. acetone powder from rabbit skeletal muscle, suspended in 24 ml. distilled water and dialyzed at 0° against distilled water for 24 hr. Volume now 30 ml. The clear centrifugate was shaken twice with 1 g. washed charcoal (Merck's Medicinal Ultra-carbon) each time, centrifugation being used to remove the charcoal. After neutralizing the solution to pH 7·4 with 0·16 ml. N-NaOH, and keeping at 0° for 2 days, a protein precipitate formed which carried down completely all residual traces of suspended carbon. The centrifuged solution (23·5 ml.) was then perfectly clear. Other conditions as for (*a*) above: NaF was present in the activity tests.

Activity of dehydrogenase (μl. CO_2/15 min.) measured in presence or absence of added cozymase.

Pre-treatment of enzyme:					
Kept on ice		Incubated at 38° for 3 hr. in nitrogen		Incubated at 38° for 3 hr. in 4·4 atm. oxygen	
Cozymase absent	Cozymase present	Cozymase absent	Cozymase present	Cozymase absent	Cozymase present
2·5	41	5	41	3	31
Inactivation:			0 %		24 %

almost equal whether alanine was added as substrate or not. In view of this result, the system in homogenized tissue was not further studied.

Instead, the behaviour of purified enzyme extracts was examined. The results (Table 10) showed that the enzyme separated from autolytic kidney enzymes is stable in both nitrogen and oxygen. The fairly crude extract was inactivated to the extent of 11 % after 3 hr. at 38° either in nitrogen, or in oxygen at 4·4 atm. Further purified, it was completely stable under the same conditions of exposure to H.O.P. or nitrogen. These experiments do not, therefore, provide any evidence that this enzyme is sensitive to oxygen. Barron (1943) states that this is an —SH enzyme.

Table 10. d-*Amino-acid oxidase*

Preparations:

(A) Crude extract. This was made from acetone-dried pig kidney by the method of Warburg & Christian (1938), and consisted of the centrifuged aqueous extract.

(B) Purified extract. Method of Negelein & Brömel (1939) carried as far as their 'Stage B'. The solution contained approximately 2·5 mg. protein/ml., and 0·2 ml. (*c.* 0·5 mg. protein) was used for each vessel. Both solutions were water-clear.

Method. The rates of oxygen uptake with *dl*-alanine as substrate were measured at 38° in air, using the Warburg apparatus. Each vessel contained the enzyme (0·5 ml. prep. 'A' or 0·2 ml. 'B'), 1·0 ml. M/10 pyrophosphate buffer of pH 8·3, water to make the total volume 2 ml. 0·2 ml. M/10 or M/20 alanine solution was added at the beginning of the readings, from the side-bulb of the vessels.

Results:

Preparation A. Without added alanine, oxygen uptake negligible (1 μl./10 min.).

Pre-treatment of enzyme solution:

	Control kept on ice	After 3 hr. at 38° in nitrogen	After 3 hr. at 38° in 4·4 atm. oxygen
Enzyme activity (μl. O_2/10 min.)	26	23	23
Percentage of ice control	—	89 %	89 %
Inactivation due to H.O.P.	—	—	0 %

Preparation B. Without added alanine, oxygen uptake nil.

Enzyme activity after pre-treatment as above:

	Control kept on ice	After 3 hr. at 38° in nitrogen	After 3 hr. at 38° in 4·4 atm. oxygen
(μl. O_2/10 min.)	55·5	55	55
			(half-quantity of enzyme = 27 μl. O_2)
Inactivation		Nil	Nil

Although brain tissue does not contain this enzyme, it does contain the same prosthetic group (flavin-adenine-dinucleotide; abbreviated F.A.D.). This is the prosthetic group of other important flavoproteins present in brain. Consequently, the F.A.D. content of brain tissue has been examined after exposure to H.O.P.

Slices of fresh brain cortex were cut as usual. These were weighed into phosphate-Ringer's solution containing glucose (2·5 ml. portions) so that each tube of the 'pressure-pot' apparatus contained one good slice of about 70 mg. moist weight. The slices were incubated for various periods in either air at 1 atm., or pure oxygen at 3·5 atm.

At the end of the incubation, the slices, which were quite intact, were carefully removed to centrifuge tubes, 1 ml. of water was added to each, and the whole heated to 80° for 15 min. to liberate the F.A.D.

The F.A.D. content of the liquid after centrifugation was estimated by measurement of the oxygen uptake produced by addition of 0·4 ml. of the solution to a preparation of amino-acid oxidase which had been freed from F.A.D. by acid precipitation (cf. Ochoa & Rossiter, 1939). Alanine was added as substrate.

The results are expressed for convenience as μg. of F.A.D./g. moist brain tissue. The *relative* values are correct, but the *absolute* amounts are only approximate, no standard preparation of F.A.D. being available. Instead of this, we have assumed that, as found by Ochoa & Rossiter (1939), an oxygen uptake of 180 μl./30 min. would correspond with the presence of 1 μg. F.A.D. This assumes that the enzyme used was of similar activity to theirs; an assumption at least approximately correct.

The results (Table 11) showed no destruction of the prosthetic group under these conditions.

Table 11. *Flavin-adenine-dinucleotide content of brain tissue*

Pre-treatment of brain slices:

None (fresh tissue)	Air, 1 atm., 38° for		Oxygen 3·5 atm., 38° for		
	2·25 hr.	4 hr.	2·25 hr.	3 hr.	4 hr.
Flavin-adenine-dinucleotide content (μg./g.)					
14·1	13·0	13·1	12·3	13·3	12·9

As a further example of a flavoprotein enzyme, that extracted from pig's heart (Straub, 1939) has been studied (diaphorase, coenzyme factor).

Diaphorase was made from pig's heart (Straub, 1939). The purification was carried as far as the elution from $C\gamma$ alumina. It was a clear yellow solution in phosphate buffer, pH 7·4, and was stored at 0°.

For testing its activity, a dialyzed aqueous extract of acetone powder made from rabbit skeletal muscle was used (cf. Green *et al.* 1937; Corran *et al.* 1939). The rates of oxygen uptake produced by the addition of various volumes of the diaphorase solution to this test system were found to be strictly proportional to the volumes added; hence any change of activity in the diaphorase would be clearly

revealed by the activity tests. The activity of the diaphorase was such that addition of 0·3 ml. caused an oxygen uptake of 59 μl./5 min. with the test system.

The diaphorase was incubated in nitrogen at 1 atm. or in oxygen at 4·4 atm. for 2·5 hr. at 38°. Activity tests upon these samples and upon the unincubated diaphorase were then made. For these tests, each Warburg vessel contained: 0·3 ml. diaphorase (in the side-bulb), 0·2 ml. M-sodium lactate, 0·8 mg. cozymase, 0·4 ml. M-HCN, 0·2 ml. methylene blue (0·5 % solution), and water to a total volume of 3 ml. The measurement of oxygen uptake was made in air at 38°.

This flavoprotein was not at all inactivated by 2·5 hr. exposure to 4·4 atm. of oxygen at 38° (Table 12).

Table 12. *Diaphorase (heart flavoprotein, 'coenzyme factor')*

Pre-treatment of enzyme solution:		
None (kept on ice)	Nitrogen, 2·5 hr. at 38° 1 atm.	Oxygen, 2·5 hr. at 38° 4·4 atm.
Oxygen uptake rates of test system (μl. O_2/5 min.):		
61	61	68

On the whole, therefore, the evidence presented here is against any great oxygen-susceptibility of these flavoprotein systems. It is, of course, probable that some flavoproteins are oxygen-sensitive (see Table 1), and it certainly cannot be maintained that a negative result with one particular preparation excludes the possibility that other preparations of the same enzyme may be inactivated by oxygen.

F. *Catalase*

In the absence of catalase, enzymes such as xanthine oxidase which form hydrogen peroxide are either themselves inactivated by H_2O_2 which accumulates, or they may inactivate other enzymes present, through the same mechanism. If catalase were to be destroyed in brain tissue by exposure to H.O.P., other brain enzymes might also succumb.

The estimation of catalase activity was as described by Fujita & Kodama (1931). This method gives rather variable results. It was especially noticed that after shaking brain tissue slices in Ringer's solution, part of the activity passed into the solution. As the activity of catalase in brain tissue is very low, while that of blood is high, this was probably due to slow washing out of traces of blood remaining in the tissue during slicing. The order of accuracy of the results is therefore only sufficient to detect a considerable inactivation of catalase.

Test of this point with brain cortex did not yield evidence of catalase inactivation. The results (Table 13) make it unlikely that destruction of catalase, if it occurs, plays any important part in the toxic effect of H.O.P. on brain metabolism. Exp. iii of Table 13 shows the catalase content of the tissue after it has been shaken for 3·5 hr. in Ringer's solution, removed from the solution, and the activity of the tissue alone (i.e. without the catalase washed into the solution) determined: these experiments are marked 'tissue only'. Comparison of the activity shown in these experiments, in air or oxygen at 1 atm., with those of Exp. ii under H.O.P. (where the tissue was similarly transferred to fresh Ringer's solution for the measurement of catalase activity) show that there is no greater fall with H.O.P. than in air or oxygen at 1 atm. More accurate results are unobtainable with brain tissue, for the reasons mentioned above. (The relevant figures are italicized in Table 13.)

Stadie (1943) also reports that purified preparations of catalase were highly stable to H.O.P.

Table 13. *Catalase content of brain tissue*

During the exposure to oxygen, the slices of rat-brain cortex were suspended in phosphate-Ringer's solution containing glucose.

Brain no.	Pre-treatment of brain slices		($Q_{catalase}$)
i	None (fresh tissue). Unwashed tissue		73
ii	3·5 atm. O_2: 75 min.		62
	Ditto	Tissue only (removed from suspension fluid)	*33*
	3·5 atm. O_2: 150 min.	,,	dupl. *39*, *25*
	3·5 atm. O_2: 210 min.	,,	dupl. *36*, *21·5*
iii	None. Unwashed		dupl. 56, 53
	The same. Shaken 210 min. in air (1 atm.)		
	Soln. + tissue		51
	Tissue only		*32*
	The same. Shaken 210 min. in O_2 (1 atm.)		
	Soln. + tissue		61
	Tissue only		*26*

G. *Choline oxidase*

This is an enzyme which is stated to depend for its activity on intact sulphydryl groups, and was tested because so many other —SH enzymes are sensitive to oxygen.

The preparation of the enzyme was based on the work of Mann & Quastel (1937) and Mann, Woodward & Quastel (1938). The liver of a rat was homogenized in 0·1 M-phosphate, pH 7·4, and the suspension strained through muslin. 1 ml. of extract was equivalent to 0·5 g. of fresh liver. Portions of the extract were shaken in air, or in 4·4 atm. pure oxygen, at 38° for 180 min. The activity of the choline oxidase was then estimated by the rate of oxygen uptake which occurred on addition of choline.

For the estimation each vessel contained: enzyme 0·5 ml., water to make the total volume 2 ml., and in the side-bulb 0·2 ml. of a solution containing 4 mg. choline chloride in 0·1 M-phosphate buffer, pH 7·4. Temperature 38°. Gas space, air.

This proved to be another example of an oxygen-sensitive —SH enzyme, and was inactivated to the extent of 60 % by 3 hr. exposure to 4·4 atm. of oxygen (Table 14).

Table 14. *Choline oxidase*

		Pre-treatment of enzyme: Air, 1 atm., 3 hr.	Pre-treatment of enzyme: Oxygen, 4·4 atm., 3 hr.
Oxygen uptake (μl./150 min.)	With choline	298	152
	No choline	54	56
Increase due to choline		244	96
Inhibition due to H.O.P.			61 %

(2) Non-oxidative enzymes

The above enzymes (A–G) are all oxidative in character. Two examples only have been studied of non-oxidative enzymes—hexokinase and phosphoglucomutase—both of which are believed to be of primary importance in carbohydrate metabolism. The former, although an —SH enzyme (Dixon, 1940), was not inhibited by exposure to oxygen; the latter was, but only under certain ill-defined conditions of purity.

H. *Hexokinase*

This enzyme, prepared according to Meyerhof (1927) and Colowick & Kalckar (1941), was obtained from baker's yeast as a white powder, completely soluble in water, of which 0·3 mg. was sufficiently active to give vigorous phosphorylation of glucose.

For tests of activity each vessel contained 0·3 mg. of hexokinase, 0·5 ml. of M-glucose, 0·1 ml. 1 % $MgCl_2$, 0·8 ml. 1·3 % $NaHCO_3$, water to make the total volume 3·0 ml. The solution of adenosine triphosphate (0·2 ml.) was added from the side-bulb at the beginning of the readings; it contained 0·85 mg. of pyrophosphate-P. The gas space of the vessels contained nitrogen with 5 % (by vol.) carbon dioxide, and the temperature was 38°. The activity curve of the hexokinase preparation tested under the same conditions, showed that 0·5 mg. hexokinase gave maximum activity (140 μl. CO_2 liberated in 20 min.). The basis of the manometric method of following the reaction rate is the liberation of one extra phosphoric acid group, which occurs in the reaction:

Glucose + adenosine triphosphate = glucose-monophosphate + adenosine diphosphate.

This acidic group liberates 1 mol. CO_2 from the $NaHCO_3$.

The aqueous hexokinase solution, plus various amounts of glucose, was incubated at 38° in nitrogen or oxygen, as stated, and afterwards the activity of the enzyme was determined as above. The activity remaining after exposure is expressed in terms of the weight of original enzyme which would be required to show the same activity as that observed; the latter being read from the weight-activity curve.

No evidence was obtained that it was inactivated by oxygen (Table 15). In the absence of glucose the enzyme is inactivated rapidly (Dixon, 1940), hence glucose had to be present as stabilizer. It is therefore not possible to say what its sensitivity to oxygen in absence of glucose might be.

Table 15. *Hexokinase*

Exp. no.	Glucose conc.	Period of exposure	0·3 mg. hexokinase originally taken. Wt. of hexokinase (mg.) remaining after: N_2 (1 atm.)	O_2 (1 atm.)	O_2 (4·4 atm.)
I	M/5	0·5 hr.	0·26	0·28	—
	M/100	,,	0·22	0·25	—
	M/500	,,	0·17	0·19	—
II	M/500	,,	0·17	0·13	—
	M/1000	,,	0·15	0·12	—
	M/5000	,,	0·06	0·03	—
III	M/1000	,,	0·11	0·05	—
	M/2000	,,	0·02	0·05	—
	M/4000	,,	0·00	0·00	—
IV	M/250	,,	0·18	0·16	—
	M/500	,,	0·14	0·16	—
	M/1000	,,	0·10	0·11	—
V	M/5	3 hr.	0·25	—	0·24
	M/25	,,	0·19	—	0·20
	M/100	,,	0·14	—	0·14
No glucose added:					
VI	0	5 min.	0·04	0·00	—
		10 min.	0·00	0·02	—

I. *Phosphoglucomutase*

Evidence suggesting that this enzyme might be sensitive to oxygen, even to atmospheric air, was presented by Gill & Lehmann (1939) and Lehmann (1939). The former authors report one experiment in which the Robison ester formation from glycogen was 8 % less in air than *in vacuo* after 1 hr., and

16 % less after 2 hr. of enzyme action. This enzyme system has since been separated from the crude muscle extract, as used by Gill & Lehmann, and its action has been cleared up by the work of Cori and co-workers.

It was shown by Cori *et al.* (1938) and Gill & Lehmann (1939) that phosphoglucomutase is probably an —SH enzyme and is highly susceptible to the influence of metals. Magnesium, manganese, cobalt and nickel greatly accelerate the action, while zinc (except in extremely low concentration, e.g. 10^{-7} M, when it may be accelerative), copper and iron salts are inhibitory. The electrodialyzed extract is practically inactive without added metals (Cori *et al.* 1938). Gill & Lehmann (1939) and Lehmann (1939) showed that caffeine, oxidized glutathione, various oxidizing agents or insulin inhibited the action of the enzyme, while reduced glutathione, and other reducing agents accelerated it. The action of insulin was ascribed by Cori *et al.* (1938) to its zinc content, but this is denied by Gill & Lehmann (1939), who observed a similar inhibition with insulin hydrochloride of very low zinc content.

These properties appear to have some relationship to the effect of some of the substances named above on oxygen convulsions, as observed by H. P. Marks (1944); hence they have been cited in some detail. For our experiments, the purification of the enzyme was by the method of Cori *et al.* (1938); followed by removal of other associated enzymes according to Colowick & Sutherland (1942). As already mentioned the oxygen sensitivity of this enzyme is greatly dependent on the method and stage of purification.

Undialyzed extract. One large rabbit was killed, the abdominal aorta exposed, and about 600 ml. warm 0·9 % NaCl was forced through to remove the blood, via the abdominal vein, from the hind limbs. The muscle of both hind limbs was excised, placed in ice, and passed twice through the ice-cold mincing machine: wt. 370 g. This mince was ground well with 370 ml. ice-cold distilled water, kept at 0° for 2 hr. and the extract expressed through cheese-cloth. A second similar extract was combined with the first; total volume, *Extract A*, 805 ml.

Dialyzed extract. 630 ml. of the above extract were dialyzed in cellophane sausage-skins at 12° for 5 hr., then for 16 hr. at 3° against 5 l. distilled water. After filtering from the precipitated globulins, the filtrate was used (*Extract B*). Inorganic P = 4·13 mg./100 ml.; easily hydrolyzable P (5 min. at 100° in N-sulphuric acid) = 0·73 mg./100 ml. (cf. Cori *et al.* 1938).

Purification of dialyzed extract (cf. Colowick & Sutherland, 1942). 530 ml. above extract were made 0·4 saturated with ammonium sulphate and warmed to 30°. The precipitate (phosphorylase) was removed; the clear solution contained the phosphoglucomutase together with isomerase. The latter was removed by precipitating the mixture with an addition of half the previous amount of ammonium sulphate, resuspending the precipitate in veronal buffer at pH 5·2, and heating to 52° for 30 min. The clear colourless filtrate was adjusted to pH 7·5, again filtered, and dialyzed at about 0° against distilled water for 5 days. The solution was concentrated by freezing, to 19 ml. (Extract C).

In addition, a further precipitate formed only slowly (overnight) on the addition of the ammonium sulphate in the last stage, just described. This was worked up separately as just described (Extract D), but was of lower activity than the main extract (Extract C).

Cori ester. The potassium salt of this ester (glucose-*l*-phosphoric acid) was made from starch with the aid of potato juice as described by Hanes (1940). Found: 'Apparent inorganic P' = 0·16 %. 5 min. hydrolyzable P = 7·96 %. Calc. 0 % and 8·33 % respectively.

Method of experiment. 5 ml. portions of the aqueous solution of the potassium salt of glucose-*l*-phosphate were measured into the vessels, and warmed to 38°. The solution contained approx. 1 mg./ml., the exact content being determined by phosphorus analyses.

The enzyme solution was added at the beginning of the experiment (0 min.). The experiments at 1 atm. were done in Warburg vessels; those at H.O.P. in 'pressure pots' (see Dickens, 1946). After the desired incubation times, the reaction was stopped by the addition of 2 ml. 10 % trichloroacetic acid. Blanks were run on the enzyme and Cori ester alone. Analyses were made of: (*a*) 'apparent inorganic P'; (*b*) easily hydrolyzable P (5 min. at 100° in N-sulphuric acid). The latter represents the unchanged Cori ester.

A control experiment showed that, without the addition of enzyme, the Cori ester was perfectly stable at 38° under the conditions of incubation adopted.

At first, when the crude undialyzed muscle extract made according to Cori *et al.* (1938) was used, oxygen had no effect on the activity (Table 16). After dialysis, the extract became oxygen sensitive. After further purification by the method of Colowick & Sutherland (1942), at various stages up to full purification by this method, the enzyme was still clearly sensitive to oxygen. The sensitivity to oxygen reached a maximum in the partially purified, freshly prepared enzyme. This was especially true of the immediate precipitate which occurred on adding ammonium sulphate to the solution freed from phosphorylase (Colowick & Sutherland, 1942). A portion of this precipitate which came down only after standing for some hours, was much less sensitive to oxygen, and was also of much lower activity. On keeping the highly oxygen-sensitive preparation in the cold room, it gradually deposited a white precipitate containing protein (cf. Cori *et al.* 1938). This was removed by centrifuging, when the supernatant clear solution, although still highly active as phosphoglucomutase, was no longer sensitive to oxygen. These results are collected in Table 16. Their general significance has been discussed in the Introduction.

In an attempt to re-sensitize the highly purified enzyme to oxygen, many experiments were made with metal-activated enzyme, comparing the ac-

Table 16. *Phosphoglucomutase*

Undialyzed enzyme (Extract A)

Amount of enzyme used: 0·1 ml./5 ml. Cori ester.

		P analyses: mg. P/vessel		
		'Apparent inorg. P'	Easily hydrol. P	% conversion of Cori ester
Initial. Enzyme only (0·1 ml.)		0·056	0	
Cori ester (5 ml.)		0·026	0·359	
	Total	0·082	0·359	
Incubated approx. 2 hr. at 38°:				
Exp. I	Oxygen 4·4 atm.	0·096	0·121	66
	Air 1 atm.	0·094	0·123	66
Exp. II	Oxygen 1 atm.	0·095	0·083	77
	Nitrogen 1 atm.*	0·095	0·082	77
Incubated 1 hr. at 38°:				
Exp. III	Oxygen 1 atm.	0·069	0·163	55
	Nitrogen 1 atm.*	0·066	0·151	58

Undialyzed enzyme is practically insensitive to oxygen.

Dialyzed enzyme (Extract B)

Initial. Enzyme only (0·1 ml.)		0·005	0	
Cori ester (5 ml.)		0·026	0·359	
	Total	0·031	0·359	
Incubated approx. 2 hr. at 38°:				
Exp. IV	Oxygen 4·4 atm. (Freshly made enzyme)	0·025	0·136	62
	Air 1 atm. ,,	0·026	0·140	61
Exp. V	Oxygen 1 atm. ,,	0·025	0·102	71·5
	Nitrogen 1 atm.* ,,	0·025	0·021	94
Exp. VI	Oxygen 1 atm. (Kept 2 days)	0·03	0·114	68·5
	Nitrogen 1 atm.* ,,	0·03	0·074	80
Exp. VII	Oxygen 1 atm. (Kept 5 days)	0·02	0·169	53
	Nitrogen 1 atm.* ,,	0·02	0·134	63

The freshly prepared dialyzed enzyme is highly susceptible to oxygen; though H.O.P. is no more inhibitory than is air at 1 atm. In nitrogen, however, the activity is much higher. The enzyme on keeping loses some of this susceptibility to oxygen, although the effect is still quite evident after 5 days.

Enzyme purified by ammonium sulphate, etc. (Extract C)

Amount of enzyme used: 0·1 ml. of 1 in 10 dilution (=0·01 ml. Extract C). This amount of enzyme contains negligible P.

		P analyses: mg. P/vessel		
		'Apparent inorg. P'	Easily hydrol. P	% conversion of Cori ester
Initial		0·02	0·359	
Incubated for 1 hr. at 38°. Freshly prepared enzyme:				
Exp. VIII	Oxygen 1 atm.	0·018	0·242	33
	Nitrogen 1 atm.*	0·017	0·126	65

Freshly prepared purified enzyme is highly susceptible to oxygen.

Incubated for 1 hr. at 38°. Enzyme had been kept 2 days; centrifuged from precipitate:				
Exp. IX	Oxygen 4·4 atm.	0·016	0·249	31
	Nitrogen 1 atm.†	0·018	0·229	36
Exp. X	Oxygen 1 atm.	0·02	0·239	33
	Nitrogen 1 atm. (triplicate)	0·02	0·220*	39
		0·02	0·226‡	37
		0·02	0·247†	31

Now almost completely insensitive to oxygen again.

* Nitrogen purified by hydrosulphite and 'silver-salt'.
† Unpurified cylinder nitrogen.
‡ Nitrogen purified by alkaline pyrogallol.

tivities in oxygen and nitrogen. Although the accelerating effects of traces of metals (manganese, cobalt, magnesium) and the inhibitory effects of other metals (copper, zinc) were fully confirmed, the presence of these metals did not alone bring back the oxygen-sensitivity to the enzyme preparation. The evidence makes it probable that the protein precipitate, mentioned above, which was formed on keeping the phosphoglutomutase in the cold room, was concerned in the sensitization to oxygen. It is of interest to note that Cori *et al.* (1938) observed that a similar kind of precipitate, added together with magnesium ions, greatly increased the catalytic of these ions on enzyme activity. Heating to 70° caused this precipitate to lose this co-activating action, and Cori *et al.* (1938) consider that the precipitate contained 'an unknown component' of the complete enzyme system. From our results it appears likely that this, or a similar, component may be needed to sensitize phosphoglucomutase to oxygen. Possibly such a component may play an important part in the behaviour towards oxygen of other enzymes: it may very well be itself an —SH protein.

DISCUSSION

The evidence collected shows that at least five of the enzymes believed to be concerned in the carbohydrate metabolism of brain tissue are capable of being inhibited by oxygen. These are: (1) phosphoglucomutase, (2) fructose-6-phosphate phosphorylase, (3) triosephosphate dehydrogenase, (4) succinic dehydrogenase, (5) pyruvic oxidase. In addition cytochrome oxidase may suffer slight damage after long exposure to oxygen. Zymohexase of yeast may be oxygen sensitive (Warburg & Christian, 1942), but the same enzyme from animal tissues is more stable (cf. Table 1).*

In the present paper, and the preceding one (Dickens, 1946), the oxygen sensitivity of enzymes, 1, 3, 4 and 5 has been demonstrated by their inhibition by molecular oxygen. That of enzyme 2 (phosphohexokinase), which was not investigated here, is presumed from the work of Engelhardt & Sakov (1943), available only in abstract form; it is not clear from the abstract whether this inactivation occurs only under artificial conditions, viz. by reason of the quinone formed from added polyphenol by succinoxidase preparations, in which case it would resemble the inactivation of urease and succinoxidase by quinones, already much studied (e.g. Quastel, 1933; Potter, 1942). The statement of Engelhardt & Sakov that phosphoglucomutase is unaffected by oxidizing agents is understandable in view of the great difference in oxygen sensitivity of different preparations of this enzyme described above.

Of these five enzymes, four at least are considered to rely upon intact sulphydryl groups within the enzyme for their activity (enzyme 1, Gill & Lehmann, 1939; Lehmann, 1939: enzyme 3, Rapkine, 1938; Rapkine & Trpinac, 1939: enzyme 4, Hopkins & Morgan, 1938; Hopkins, Morgan & Lutwak-Mann, 1938; Morgan & Friedmann, 1938: enzyme 5, Barron, 1936; Peters, 1940; Barron & Singer, 1943, 1945). The —SH nature of enzyme 2, phosphohexokinase, is made highly probable by the work of Engelhardt & Sakov (1943). Taken in conjunction with the evidence of poisoning by oxygen of these enzymes, these observations make clear a fairly general connexion between the oxygen poisoning and the —SH nature of the enzymes involved in carbohydrate metabolism. This agrees with the known oxygen-sensitivity of some other —SH enzymes not concerned in carbohydrate metabolism (cf. Table 1).

Of these five enzymes, the pyruvate oxidase and the succinic oxidase systems are believed to require in addition to the —SH component a flavoprotein. Evidence of the presence of a flavoprotein in alkali-inactivated succinoxidase has been provided by Straub (1941), but apparently this flavoprotein is not concerned in succinate metabolism directly, since Straub considered that it did not react with succinic dehydrogenase, but had a direct connexion with the oxidizing system, i.e. it was presumably a cytochrome *c* reductase capable of transporting hydrogen atoms from reduced cozymase. Potter & Albaum (1943) discuss the possibility that inactivation of such a flavoprotein might indirectly inhibit the succinic oxidase system. However this may be, it does not seem likely from Straub's experiments that the flavoprotein could be very labile, since it withstood an hour's exposure to pH 9, the conditions used to inactivate the dehydrogenase. So little is known, however, of the properties of the flavoprotein component of succinoxidase that there remains a possibility that it might be oxygen-sensitive, although experimental evidence of this is lacking. There is a similar ignorance about the stability to oxygen of the flavoprotein component of the pyruvate oxidase system.

The evidence obtained in the present work with other flavoproteins (diaphorase, *d*-amino-acid oxidase) and with flavine-adenine-dinucleotide of brain, has not demonstrated any sensitivity to oxygen of these substances. On the whole there is, as yet, very little positive evidence which would involve

* Preliminary reports (Colowick & Price, 1945*a*, 1945*b*) indicate that in muscle extracts dihydrocozymase and guanine may be essential coenzymes for the phosphorylation of glucose or fructose-6-phosphate. Although with *yeast* hexokinase no sensitivity to oxygen was observed in the present work, these new muscle systems involving dihydrocozymase would be expected to be oxygen-sensitive.

the flavoproteins in the oxygen poisoning of animal tissues.

The evidence involving the —SH enzymes, on the contrary, is quite strong. In addition to the known oxygen-sensitivity, further support is derived from consideration of the mode of protection against oxygen-poisoning by metals, inhibitors, and prosthetic groups.

The succinate system, though not that mainly affected by oxygen in animal tissues (Mann & Quastel, 1946; Dickens, 1946), is nevertheless the clearest example for discussion, because the fundamental work of Hopkins & Morgan (1938) and Hopkins *et al.* (1938) has made it the best understood. Besides revealing for the first time the essential —SH nature of the dehydrogenase, these authors showed that the oxidative inactivation of the enzyme by oxidized glutathione was prevented by malonate and, rather less effectively, by pyrophosphate. These reagents did not, however, prevent the reactivation of the inhibited (oxidized) form of the enzyme. It is usually considered (Leloir & Dixon, 1937) that these two inhibitors attach themselves to the active centres normally occupied by the substrate, which would account for the specificity of their action; Krebs (1943) however has stated that pyrophosphate, unlike malonate, does not cause any accumulation of succinate in metabolizing tissue, suggesting that these two poisons may have qualitatively different affinities. Adler, Euler, Günther & Plass (1939) consider, but reject, the possibility that pyrophosphate may poison an essential metal component (manganese or magnesium) of succinoxidase.

As regards the inactivation of succinic dehydrogenase by oxygen, the experiments described here, like those obtained independently by Stadie (1943), show clearly that the presence of malonate during the exposure to oxygen protects this enzyme, just as it is protected by malonate against inactivation by exposure to oxidized glutathione (Hopkins & Morgan, 1938) or benzoquinone (Potter & DuBois, 1943), and it is presumed that the same mechanism is involved. (In a single experiment, however, we observed no protection of heart muscle succinoxidase against high-pressure oxygen by pyrophosphate; this should be repeated as, if it were confirmed, it might indicate a different mechanism of combination of pyrophosphate from that of malonate.)

It is not yet possible to give more than a general picture of the manner of malonate protection. Malonate appears not to react with —SH groups, and it is necessary to assume that the latter are 'shielded' by the steric effects of the malonate molecules which are supposedly adsorbed at closely adjacent positions on the surface of the enzyme (cf. Potter & Du Bois, 1943), chemically perhaps not a very satisfying explanation. Nor is it known how the —SH group partakes in the activity of the enzyme; not by alternate oxidation and reduction, apparently, since the inactive form of the enzyme is not more readily reactivated in presence of succinate, rather the reverse (Hopkins *et al.* 1938; see, however, Potter & Du Bois, 1943).

It seems desirable to consider how the presence of an activating metal in the succinic dehydrogenase system would affect the interpretation of these results, though it is not suggested that the evidence available is sufficient to justify such an assumption. The fact, however, that the activity of the dehydrogenase is protected from inactivation by oxygen in presence of traces of manganese makes the participation of an activating metal at least a possibility. This metal need not be manganese; aluminium, for example, has also been considered to protect this enzyme against reagents which combine with —SH groups (Schneider & Potter, 1943). Chromium and rare earth metals also activate succinic oxidase, purified preparations of which contain aluminium and chromium (Horecker, Stotz & Hogness, 1939). The observations of Massart (1939) are quite inadequate to support his claim that manganese is a component. We have shown, however (Dickens, 1946; and present work) that cobalt, an activator of bacterial pyruvic oxidase, protects animal pyruvic oxidase against oxygen poisoning, while the whole glucose-oxidizing system of brain is similarly protected by magnesium, manganese or cobalt. Consequently much more extensive experiments than those recorded in this paper, which were designed with quite a different end in view, are highly desirable to see if further evidence of a metal component of the succinic system can be obtained. Such a metal could be a link between the —SH groups and the substrate (cf. Hellerman, 1937) or inhibitor, which would offer a possible explanation of the effect of inhibitors in holding the enzyme in the reduced form and thus protecting it against oxidants, while they do not prevent the reduction of the S-S form of the enzyme. There are difficulties in the way of this theory, such as the problem of what happens to the metal when the enzyme is in the oxidized form, but nevertheless it appears to merit some consideration.

It is necessary to point out that malonate does not exert its protective influence by virtue of the inhibition of enzyme activity which it produces. Activators can also protect this and other —SH enzymes, as we have seen. Similarly, it has been shown above that cozymase protects its enzyme, triosephosphate dehydrogenase, against inactivation by oxygen, and Rapkine (1938) and Rapkine & Trpinac (1939) showed a similar protection against the usual chemical oxidants. The metals which protect pyruvic oxidase have already been discussed. The protection of apoenzymes by their prosthetic

groups or substrates is a commonplace of enzyme chemistry. All these substances have one feature in common; they are specifically adsorbed at (or possibly adjacent to) the active centres of the dehydrogenases which they protect. Not every such substance, however, is necessarily protective. Zymohexase of yeast forms 'salts' with cobalt and iron which, in presence of cysteine, are reversibly inactivated by molecular oxygen, but the zinc compound of the same enzyme is not inactivated by oxygen (Warburg & Christian, 1942, 1943). Consequently the nature of the activating metal and its mode of combination, as well as the firmness with which it is bound, and the nature of the —SH 'contaminants', determine whether the enzyme is sensitive to oxygen or not. The great complexity introduced by these factors in studies of enzyme inactivation by oxygen is evident. They may explain the many confusing differences in the stability to oxygen of various preparations of the same enzyme, which have been repeatedly seen during the course of the present work and also by many other observers.

SUMMARY

1. A list has been compiled of enzymes known or considered to be oxygen-sensitive.

2. Succinic oxidase is inhibited by exposure to high oxygen tensions. The dehydrogenase, the most sensitive component, is apparently irreversibly poisoned. Longer exposure may also somewhat weaken the cytochrome oxidase. Evidence is presented that probably only a minor part of the poisoning could be accounted for by accumulation of oxaloacetate in oxygen. Malonate (10^{-3} M) or manganese ions ($2{\cdot}5 \times 10^{-4}$ M) protect the dehydrogenase against poisoning by oxygen.

3. Lactic and malic dehydrogenase preparations from animal tissues were very resistant to oxygen inactivation.

4. Triosephosphate dehydrogenase is poisoned by oxygen, but is sensitive only when the exposure occurs in absence of cozymase, the coenzyme of this dehydrogenase.

5. Typical flavoproteins (*d*-amino-acid oxidase, diaphorase) were not affected by exposure to 4·4 atm. of oxygen. The flavine-adenine-dinucleotide content of brain tissue was not affected by oxygen at 3·5 atm.

6. Catalase in brain tissue is insensitive to oxygen.

7. Choline oxidase, an —SH enzyme, is poisoned by oxygen.

8. The activity of yeast hexokinase was not affected by oxygen (glucose present).

9. Phosphoglucomutase is poisoned by oxygen, but only at certain stages of its purification.

10. The relationship between oxygen-sensitivity and the presence of essential —SH groups in many of the sensitive enzymes is pointed out. The possible mechanism of inactivation and of protection is discussed, and suggestions are advanced concerning the probably important part played by trace-metals in these processes.

This work, like that in the preceding paper, was done between March 1943 and March 1944 at the request of the Royal Naval Personnel Research Committee of the Medical Research Council. I am most grateful to the Council for giving me this opportunity of studying oxygen poisoning, and for the permission now granted to publish these results. Dr William C. Stadie has courteously given me permission to refer to some unpublished observations by himself and his colleagues.

REFERENCES

Adler, E., Euler, H. v., Günther, G. & Plass, M. (1939). *Biochem. J.* **33**, 1028.

Axelrod, A. E., Potter, V. R. & Elvehjem, C. A. (1942). *J. biol. Chem.* **142**, 85.

Bailey, B., Belfer, S., Eder, H. & Bradley, H. C. (1942). *J. biol. Chem.* **143**, 721.

Barron, E. S. G. (1936). *J. biol. Chem.* **113**, 695.

Barron, E. S. G. (1943). *Advances in Enzymology*, **3**, 149. New York: Interscience Publ. Inc.

Barron, E. S. G. & Singer, T. P. (1943). *Science*, **97**, 356.

Barron, E. S. G. & Singer, T. P. (1945). *J. biol. Chem.* **157**, 221.

Bohr, D. F. & Bean, J. W. (1940–1). *Amer. J. Physiol.* **131**, 388.

Cohen, P. P., Hekhius, G. L. & Sober, E. K. (1942). *Cancer Res.* **2**, 405.

Colowick, S. P. & Kalckar, H. M. (1941). *J. biol. Chem.* **137**, 789.

Colowick, S. P. & Price, W. H. (1945*a*). *J. biol. Chem.* **157**, 415.

Colowick, S. P. & Price, W. H. (1945*b*). *J. biol. Chem.* **159**, 563.

Colowick, S. P. & Sutherland, E. W. (1942). *J. biol. Chem.* **144**, 423.

Cori, G. T., Colowick, S. P. & Cori, C. F. (1938). *J. biol. Chem.* **124**, 543.

Corran, H. S., Green, D. E. & Straub, F. B. (1939). *Biochem. J.* **33**, 793.

Dickens, F. (1946). *Biochem. J.* **40**, 145.

Dixon, M. (1925). *Biochem. J.* **19**, 507.

Dixon, M. (1940). Personal communication.

Dixon, M. & Kodama, K. (1926). *Biochem. J.* **20**, 1104 (p. 1109).

Edlbacher, S., Kraus, J. & Leutdhardt, F. (1933). *Hoppe-Seyl. Z.* **217**, 85.

Edson, N. L. (1935). *Biochem. J.* **29**, 2082.

Engelhardt, V. A. & Sakov, N. E. (1943). *Biochimia*, **8**, 9 (*Brit. Chem. and Physiol. Abstr.* 1944, A III, 367).

Fujita, A. & Kodama, T. (1931). *Biochem. Z.* **232**, 20.

Gale, E. F. (1939). *Biochem. J.* **33**, 1012.

Gill, P. M. & Lehmann, H. (1939). *Biochem. J.* **33**, 1151.
Green, D. E., Needham, D. M. & Dewan, J. G. (1937). *Biochem. J.* **31**, 2327.
Haas, E., Horecker, B. L. & Hogness, T. R. (1940). *J. biol. Chem.* **136**, 747 (p. 773).
Hanes, C. S. (1940). *Proc. Roy. Soc.* B, **129**, 174.
Hellerman, L. (1937). *Physiol. Rev.* **17**, 454.
Hellerman, L., Perkins, M. E. & Clark, W. M. (1933). *Proc. Nat. Acad. Sci., Wash.*, **19**, 855.
Herbert, D., Gordan, H., Subrahmanyan, V. & Green, D. E. (1940). *Biochem. J.* **34**, 1108.
Hobermann, H. D. & Rittenberg, D. (1943). *J. biol. Chem.* **147**, 211.
Hopkins, F. G. & Morgan, E. J. (1938). *Biochem. J.* **32**, 611.
Hopkins, F. G., Morgan, E. J. & Lutwak-Mann, C. (1938). *Biochem. J.* **32**, 1829.
Horecker, B. L., Stotz, E. & Hogness, T. R. (1939). *J. biol. Chem.* **128**, 251.
Irving, G. W., Fruton, J. S. & Bergmann, M. (1942). *J. biol. Chem.* **144**, 161.
Kalckar, H. M. (1943). *J. biol. Chem.* **148**, 127.
Klein, G. & Zeise, W. (1933). *Hoppe-Seyl. Z.* **222**, 187.
Klein, G. & Zeise, W. (1935). *Klin. Wschr.* **14**, 205.
Krebs, H. A. (1943). *Advances in Enzymology*, **3**, 191. New York: Interscience Publ. Inc.
Lehmann, H. (1939). *Biochem. J.* **33**, 1240.
Lehmann, J. (1935). *Skand. Arch. Physiol.* **72**, 78.
Leloir, L. F. & Dixon, M. (1937). *Enzymologia*, **2**, 81.
Liebricht, W. & Massart, L. (1937). *C.R. Soc. Biol., Paris*, **124**, 299.
Mann, P. J. G. & Quastel, J. H. (1937). *Biochem. J.* **31**, 869.
Mann, P. J. G. & Quastel, J. H. (1946). *Biochem. J.* **40**, 139.
Mann, P. J. G., Woodward, H. E. & Quastel, J. H. (1938). *Biochem. J.* **32**, 1024.
Marks, G. W. (1936). *J. biol. Chem.* **115**, 299.
Marks, H. P. (1944). *Report to Royal Naval Personnel Research Committee, Med. Res. Coun.* no. 101.
Massart, L. (1939). *Hoppe-Seyl. Z.* **258**, 190.
Meyerhof, O. (1927). *Biochem. Z.* **183**, 178.
Morgan, E. J. & Friedmann, E. (1938). *Biochem. J.* **32**, 862.
Negelein, E. & Brömel, H. (1939). *Biochem. Z.* **300**, 225.
Ochoa, S. & Rossiter, R. J. (1939). *Biochem. J.* **33**, 2008.
Perlzweig, W. A. (1932). *Science*, **76**, 435.
Peters, R. A. (1940). Personal communication.
Potter, V. R. (1942). *Cancer Res.* **2**, 688.
Potter, V. R. & Albaum, H. G. (1943). *J. gen. Physiol.* **26**, 443.
Potter, V. R. & Du Bois, K. P. (1943). *J. gen. Physiol.* **26**, 391.
Quastel, J. H. (1933). *Biochem. J.* **27**, 1116.
Quastel, J. H. & Wheatley, A. H. M. (1938). *Biochem. J.* **32**, 936.
Rapkine, L. (1938). *Biochem. J.* **32**, 1729.
Rapkine, L. & Trpinac, P. (1939). *C.R. Soc. Biol., Paris*, **130**, 1516.
Richards, M. M. & Hellerman, L. (1940). *J. biol. Chem.* **134**, 237.
Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.
Shapiro, B. & Wertheimer, E. (1943). *Biochem. J.* **37**, 102.
Sizer, I. W. (1942). *J. biol. Chem.* **145**, 405.
Stadie, W. C. (1943). Personal communication.
Stadie, W. C., Riggs, B. C. & Haugaard, N. (1944). *Amer. J. Med. Sci.* **207**, 84.
Stephenson, M. & Stickland, H. (1931). *Biochem. J.* **25**, 205.
Stern, K. G. & Melnick, J. L. (1941). *J. biol. Chem.* **139**, 301.
Straub, F. B. (1939). *Biochem. J.* **33**, 787.
Straub, F. B. (1941). *Enzymologia*, **9**, 148 (cited from Potter & Albaum, 1943).
Sumner, J. B. & Poland, L. O. (1933). *Proc. Soc. Exp. Biol., N.Y.*, **30**, 553.
Swingle, K. F., Axelrod, A. E. & Elvehjem, C. A. (1942). *J. biol. Chem.* **145**, 581.
Warburg, O. (1926). *Biochem. Z.* **172**, 432.
Warburg, O. & Christian, W. (1938). *Biochem. Z.* **298**, 150.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **311**, 209.
Warburg, O. & Christian, W. (1943). *Biochem. Z.* **314**, 149.
Williamson, S. & Green, D. E. (1940). *J. biol. Chem.* **135**, 345.