Correlation of Oxygen Consumption with Swelling and Lipid Peroxide Formation when Mitochondria Are Treated with the Swelling-inducing Agents Fe^{2+}, Glutathione, Ascorbate, or Phosphate*

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When isolated liver mitochondria are exposed to low concentrations of Fe^{2+} ion (10–20 μM) there is a rapid burst of O_2 consumption and lipid peroxidation. The process ends when Fe^{2+} is exhausted. These changes are correlated with rapid swelling or structural alterations in the mitochondria. High concentrations of Fe^{2+} inhibit initiation of lipid peroxidation. With 30–300 μM ascorbate extra O_2 consumption and lipid peroxidation occur more gradually. Quantitation of the extra O_2 consumption is difficult because the mitochondrial material simultaneously inhibits spontaneous oxidation of ascorbate. High concentrations of ascorbate prevent peroxidation and swelling-lysis by an antioxidant action and not by exhausting the O_2 supply. The presence of 5 mM oxidized glutathione accelerates the oxidation of 1 mM glutathione in medium alone. A trace metal may be involved, but added Cu^{2+} or Fe^{3+} alone in concentrations likely to be present does not substitute for oxidized glutathione, and ethylenediaminetetraacetate is a relatively poor inhibitor. The oxidized glutathione + glutathione mixture initiates O_2 consumption and lipid peroxidation in mitochondria. A small O_2 consumption is essential for PO_4-induced swelling of mitochondria. O_2 uptake usually increases owing to loss of respiratory control during the rapid phase of swelling, then declines due to loss of nicotinamide-adenine dinucleotide. There is no lipid peroxide formed.

Earlier work[1] demonstrated that small amounts of oxygen consumption or electron transport would support phosphate-induced swelling of isolated rat liver mitochondria. Initially the present study was undertaken to determine what was happening during phosphate-induced swelling. It was expanded to a more complete study of oxygen consumption when it was observed that lipid peroxides were formed during ascorbate or GSSG + GSH–induced swelling. It was of special interest to see what was occurring during the characteristic lag period before ascorbate- or GSH-induced swelling.

This paper reports experiments with all three swelling-inducing agents which lead to lipid peroxide formation: Fe^{2+}, GSSG + GSH, and ascorbate. The finding that extra oxygen consumption is correlated with the swelling-lysis process provides additional evidence that lipid peroxidation is intimately associated with the changes produced by these agents (Hunter *et al.*, 1963, 1964a,b). In some cases it is possible to estimate the oxygen consumption due to oxidation of the swelling-inducing agent and that due to oxidation of added mitochondrial material. Some interesting observations have been made concerning the mechanism by which GSSG hastens GSH-induced swelling, the concentrations of Fe^{2+} necessary to induce lipid peroxidation and swelling, and the rate of oxygen consumption with low and high concentrations of ascorbate, with and without mitochondria.

Experimental Methods

Mitochondrial Preparation.—Rat liver mitochondria were prepared as previously described (Hunter *et al.*, 1959a) with 0.33 M sucrose. EDTA, 0.1 mM, was used in the homogenizing fluid, but was absent from the washes. For some preparations the second washing and the final suspension were in 0.17 M KCl–0.025 M Tris, *p*H 7.4. Concentrated stock suspensions were maintained at 0°.

Chemicals.—All common chemicals were of analytical reagent grade. Ferrous ammonium sulfate was the source of ferrous ions. The stock solutions were freshly prepared in cold 0.17 M KCl as previously described (Hunter *et al.*, 1963). Distilled water was redistilled in a two-step all-quartz still. Special chemicals were of the highest grade obtainable from the Sigma Chemical Co., Nutritional Biochemicals Co., and Boehringer and Sons, Inc.

Measurement of Oxygen Consumption.—The Beckman micro oxygen electrode and physiological gas analyzer Model 160 were used for most measurements of oxygen tension. The changes were recorded with a Varian recorder. Some measurements were made with the Beckman macroelectrode. These electrodes permit measurement of oxygen consumption without stirring of the suspension.

Many oxygen-consumption studies during swelling induced by phosphate + β-hydroxybutyrate were carried out with a plastic-sheathed platinum wire electrode (collodion-coated tip) inserted through a specially designed plastic cap for the Beckman DU spectrophotometer cuvet. In most cases the platinum electrode was vibrated, but in some experiments a stationary electrode and rapid stirring of the solution were used.

Adaptation of Spectrophotometer Cuvets.—The cuvets used in the experiments with the microelectrode were Pyrex test tubes, 150 × 18 mm o.d., cut off to 57 mm length (i.d. = 16 mm). Stoppers were designed to reduce to a minimum the entry of air by diffusion or mixing. Holes were drilled in black rubber stoppers to accommodate a tight fit for three polyethylene tubes. The electrode was inserted through a tube with

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[1] A preliminary report of part of this work was given at the American Chemical Society Meeting, St. Louis, March, 1961. Abstracts p. 24C.

an inside diameter of 1.7 mm; a polyethylene stirring rod was inserted through one with an opening of 2.2 mm, allowing clearance for mixing via vertical movement of the stirrer; the third opening of 0.9 mm allowed for additions. The stopper was carefully inserted into the tube containing medium in a manner to eliminate residual air bubbles. The total volume was 8 ml. Additions to the cuvet, usually limited to 40–100 μl, were made by microsyringes through a No. 22 stainless steel needle inserted deep into the cuvet. The suspension was thoroughly mixed with the plastic stirrer after each addition, with care to avoid disturbing or touching the microelectrode.

Most experiments with the Beckman macro- or microelectrode were carried out with the cuvet tube immersed in a constant-temperature water bath at 24°. However, when OD_{520} and oxygen consumption were to be measured simultaneously, the cuvet was fitted into a modified Roto-Cell adapter for the Spectronic 20 spectrophotometer (Arthur H. Thomas Co. No. 9085–C.)[2] Water from the 24° batch was circulated through the jacket of the adapter.

In experiments using the Beckman DU spectrophotometer, a 3-ml Pyrex cuvet was fitted with a special plastic cap designed to admit a vibrating or a stationary platinum electrode, a silver electrode, and a plastic stirring rod. Additions were made through a small hole in the cap by means of a micro-syringe with stainless steel needle. The cuvets were used in a spectrophotometer equipped with circulating water-temperature-control plates for the cuvet chamber. The reaction volume was about 3.5 ml.

Media.—Most experiments were carried out in 0.175 M KCl–0.025 M Tris, *p*H 7.4. In certain cases, as indicated in the text, 0.33 M sucrose–0.025 M Tris was used with the microelectrode, while media containing 0.15 M sucrose, 0.075 M KCl, and 0.025 M Tris were standard with the collodion-coated platinum-wire electrode. Very small additions of cold solutions could be made without changes in the response of the electrode. However, when larger volumes had to be used, a small amount of solution was warmed in the water bath for 2 minutes before addition to the cuvet.

Lipid-Peroxide Determination.—A modification (Hunter *et al.*, 1963) of the thiobarbituric acid reaction used by Ottolenghi (1959) was employed to measure lipid peroxidation. This method actually measures malonaldehyde, formed primarily as a breakdown product when fatty acids with three or more double bonds undergo peroxidation (Dahle *et al.*, 1962).

Swelling. The density (OD_{520}) of the turbid suspension in the various cuvets was followed as a general measure of swelling (Hunter *et al.*, 1959a). When lipid peroxides were to be determined, readings were made on aliquots withdrawn from a larger tube (50 ml). The surface of the liquid in the tube was isolated from the air by a layer of mineral oil. After the OD_{520} reading was made, thiobarbituric acid reagent was added immediately to the aliquots.

Experimental Results

Ferrous Ion–induced Changes

Oxygen Consumption.—These studies were undertaken to determine (a) whether Fe^{2+} ion was oxidized during the lag period preceding rapid swelling-lysis, (b) whether there was oxidation of mitochondrial lipids during the lag period before swelling-lysis and the appearance of lipid peroxides as measured by the thiobarbituric acid color reaction, and (c) whether there was any change in the rate of oxidation during the period of rapid swelling-lysis.

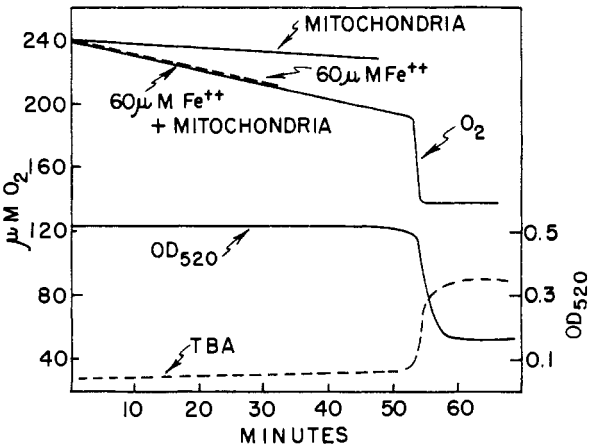

FIG. 1.—Correlation of oxygen consumption, lipid peroxidation (2-thiobarbituric acid [TBA] reaction), and swelling (ΔOD_{520}) owing to Fe^{2+} ion. KCl-Tris medium; mitochondrial protein ~150 μg/ml.

Early in this work it became apparent that the effect of Fe^{2+} ion was the same in aged as in fresh mitochondria (Hunter *et al.*, 1963). Therefore many of these experiments were done with preparations aged at 0° for 24–48 hours, since O_2 consumption due to endogenous substrates is largely eliminated in such preparations.

When 60 μM Fe^{2+} ion is added to the standard medium there is a slow O_2 consumption due to oxidation of Fe^{2+} to Fe^{3+} (Fig. 1). Most of the Fe^{2+} is converted to Fe^{3+} within 30–40 minutes. When mitochondria are added with the Fe^{+2}, the initial rate of O_2 uptake is similar to that with the same amount of Fe^{2+} alone. This rate remains fairly constant for 10–40 minutes, depending on the amount of Fe^{2+} added (30–120 μM). No O_2 consumption is seen when mitochondrial suspensions are exposed to Fe^{3+} ion alone (62–250 μM).

After the initial period of slow O_2 consumption there is a sudden increase to an extremely rapid rate when mitochondria are present (Fig. 1). This burst of O_2 consumption lasts only 2 or 3 minutes. Then O_2 uptake falls to a rate similar to the original rate or virtually ceases.

The magnitude of the burst of O_2 consumption is not related in a simple manner to the amount of Fe^{2+} added at the beginning of the experiment. Fe^{2+} from 8 to 62 μM may give essentially the same increment of O_2 consumption, but the lag periods preceding the burst are markedly different (Fig. 2). In fact, since higher Fe^{2+} concentrations (62 μM) give lags as long as 30–45 minutes, while low Fe^{2+} (8 μM) sometimes gives the same burst of oxidation after no lag period, it would appear that the burst may occur only after the Fe^{2+} concentration has fallen to about 10 μM as the result of oxidation of Fe^{2+} to Fe^{3+} during the lag period. However, the larger burst of O_2 consumption after a long lag with 125 μM Fe^{2+} (Fig. 2) does not fit with this hypothesis.

Since O_2 consumption bursts of similar magnitude with Fe^{2+} between 8 and 62 μM might result from exhaustion of the oxidizable material in the mitochondria by even the lowest Fe^{2+} (8 μM), experiments were carried out with larger amounts of Fe^{2+} (125 μM in Fig. 2) and with successive additions of more Fe^{2+} (Fig. 3). A second addition of Fe^{2+} resulted in a second burst of oxidation of similar magnitude. Sometimes two or three additional bursts of oxidation could be

[2] OD_{520} = absorbance of light by mitochondrial suspensions in tubes with either 10 or 16 mm light path.

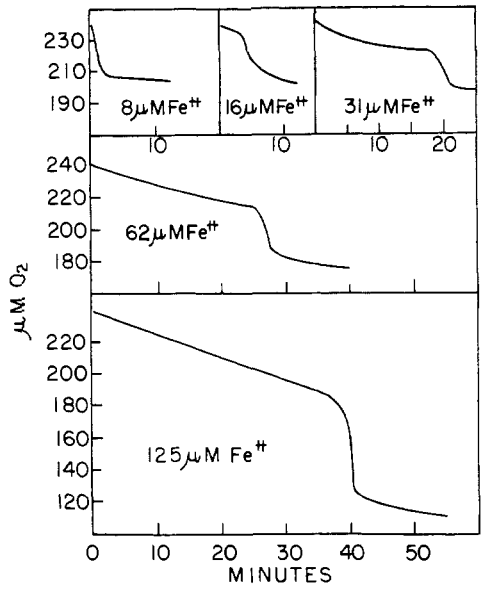

FIG. 2.—Oxygen consumption bursts with different amounts of Fe^{2+} ion. KCl-Tris medium; mitochondrial protein ~150 μg/ml.

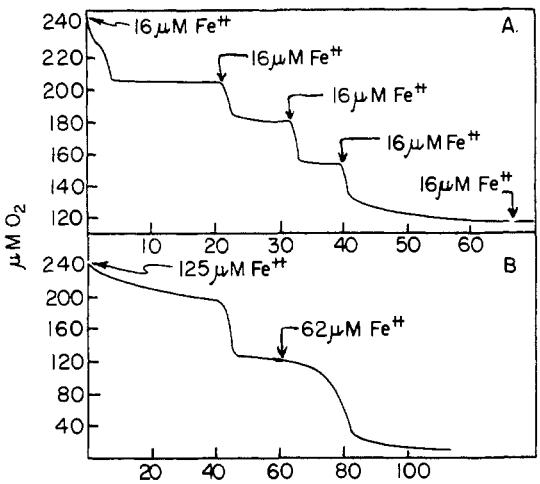

FIG. 3.—Oxygen consumption with multiple additions of Fe^{2+} ion. KCl-Tris medium; mitochondrial protein in (A), ~150 μg/ml; in (B), ~200 μg/ml.

obtained, but finally the suspension became unresponsive to addition of more Fe^{2+}, presumably because of exhaustion of the oxidizable material (lipid). These results indicate that the early bursts of oxidation are terminated because of the exhaustion of Fe^{2+}, not the exhaustion of mitochondrial material. Further support for this concept was obtained from experiments in which 125 μM Fe^{2+} (enough to exhaust mitochondrial lipid if added in smaller increments) was added initially. After a very long lag the typical burst of oxidation was seen. Another addition of 62 μM produced a second burst of oxidation (Fig. 3) in some experiments.

The total of the O_2 consumed with a series of small amounts of Fe^{2+} (8–16 μM) was usually two or three times that seen in initial single bursts, regardless of the amount of Fe^{2+} added to produce the single initial burst. Lag periods, as mentioned earlier, are correlated with the amount of Fe^{2+} added (8–125 μM) for the initial burst, but no lag period was seen for the subsequent additions of Fe^{2+} in the lower ranges (8–16 μM). With larger amounts of Fe^{2+} (31–125 μM), a distinct but usually shorter lag is seen with the second

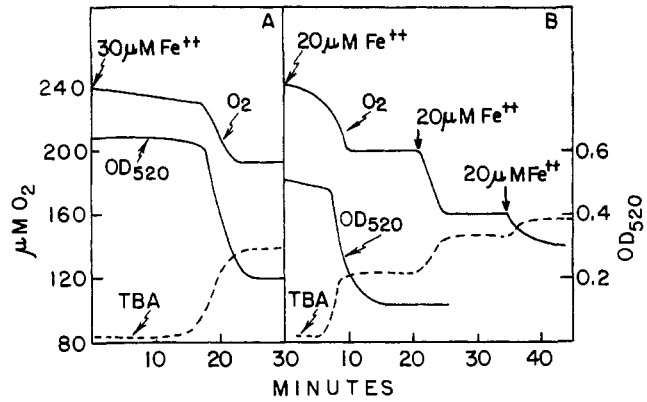

FIG. 4.—Correlation of oxygen consumption, lipid peroxidation, and swelling (ΔOD_{520}). KCl-Tris medium; mitochondrial protein ~150 μg/ml, in (A), with 30 μM Fe^{2+}; in (B), with multiple additions of 20 μM Fe^{2+}. In the latter case the peroxidizable lipid remaining was limiting with the last addition of Fe^{2+}. TBA = 2-thiobarbituric acid.

addition in most experiments (Fig. 3). The disappearance or shortening of the lag period with successive additions of Fe^{2+} appears to result in part from structural changes in the mitochondria.

Correlations with Swelling.—The first burst of O_2 consumption is closely correlated with the rapid phase of the swelling or OD_{520} change, as the two appear to coincide (Figs. 1 and 4). Small but significant oxidation of mitochondrial material may occur before the burst of oxidation and swelling, but it is too small to be clearly distinguished from the oxidation of Fe^{2+}. When very low (2–5 μM) Fe^{2+} is used, large OD_{520} changes (swelling?) without disintegration of the mitochondria occur with very limited amounts of O_2 consumption and lipid peroxidation (Gebicki and Hunter, 1964). A second or third burst of O_2 consumption does not cause a distinct additional fall in the OD_{520} reading (Fig. 4).

Correlations with Lipid Peroxidation.—With small amounts of Fe^{2+} the magnitude of the initial burst of O_2 consumption is much greater than could be explained by oxidation of the Fe^{2+} to Fe^{3+}, so there is little doubt that most of the burst is due to oxidation of mitochondrial material, presumably unsaturated fatty acids. Figures 1 and 4 illustrate the close correlation between appearance of lipid peroxides (thiobarbituric acid color material), the burst of oxidation, and the OD_{520} fall. Roughly quantitative estimates from initial bursts of oxidation indicate that 10–15 μmoles of oxygen are consumed per μmole of malonaldehyde formed from the 250–300 μmoles of lipid present per ml of mitochondrial suspension. Multiple bursts of oxidation result in similar bursts of lipid peroxidation until the lipid is exhausted, but the OD_{520} changes are associated almost entirely with the first burst.

GSSG + GSH-induced Changes

Studies on O_2 uptake during swelling-lysis of mitochondria induced by GSSG + GSH (Neubert and Lehninger, 1962; Hunter *et al.*, 1964a) brought out a number of points essential for full understanding of the reactions taking place.

GSH Alone.—In KCl-Tris medium without mitochondria GSH is oxidized at a slow rate. When mitochondria are present the rate of O_2 uptake with 1 mM GSH is increased but is still low (Hunter *et al.*, 1964a). This observation agrees with earlier work (Lehninger *et al.*, 1954; Maley and Lardy, 1954), indicating that added cytochrome *c* is virtually essential for oxidation of GSH by mitochondria.

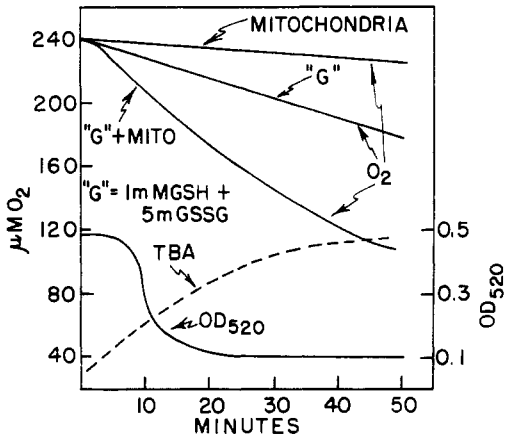

FIG. 5.—Correlation of oxygen consumption, lipid peroxidation, and swelling-lysis (ΔOD_{520}) with GSSG + GSH. KCl-Tris medium; mitochondrial protein ~150 μg/ml. TBA = 2-thiobarbituric acid.

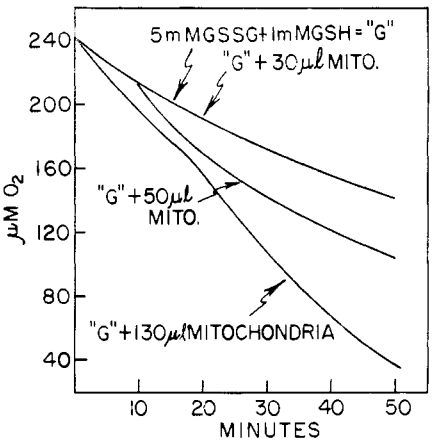

FIG. 6.—Oxygen consumption with GSSG + GSH without and with different amounts of mitochondria. KCl-Tris medium; 50 μl of mitochondria = 50 μg protein/ml.

GSSG Alone.—Five mM *GSSG* gave no O_2 consumption in medium alone and a very low rate with mitochondria (Fig. 4 in Hunter *et al.*, 1964a).

GSSG + GSH.—IN KCl-TRIS MEDIUM WITHOUT MITOCHONDRIA.—The rate of O_2 consumption when 5 mM GSSG is added to 1 mM GSH is usually increased 2- to 5- fold over that with 1 mM GSH alone (Fig. 4 in Hunter *et al.*, 1964a). This effect was seen with all samples of GSSG from Boehringer and Sons, Inc., and all except one from Sigma Chemical Co. One sample of lot 23B762 from Sigma produced the stimulation to a very limited degree. At first it was considered that trace-metal contamination of the GSSG solution was probably responsible for the stimulation of GSH oxidation whenever it was seen. However, direct investigation with added Cu^{2+}, Fe^{2+}, and Fe^{3+} has made it very doubtful that metal alone can be the correct explanation.

With 1 mM GSH in KCl-Tris medium, *p*H 7.4, the addition of 10^{-7}–10^{-5} M Cu^{2+} had very little effect on the rate of O_2 consumption. One $\times 10^{-4}$ M Cu^{2+} increased the rate, but the magnitude was variable. Fe^{2+} or Fe^{3+} at 10^{-6} and 10^{-5} M produced no effect. At 10^{-4} M, Fe^{2+} or Fe^{3+} stimulated GSH oxidation in some but not all experiments. The combination 10^{-5} M Cu^{2+} plus 10^{-5} M Fe^{3+} produced some stimulation.

When the mixture 5 mM GSSG + 1 mM GSH was studied in medium alone, 10^{-5} M Cu^{2+} had little effect on the O_2 consumption, but 10^{-4} M Cu^{2+} approximately doubled the rate. With Fe^{3+}, 10^{-5} M was without effect and 10^{-4} M showed a slight inhibition. Thus the effect of GSSG on the rate of GSH oxidation cannot be duplicated with Cu^{2+} or Fe^{3+} in trace amounts and is probably dependent on an effect of GSSG per se.

IN MEDIUM WITH MITOCHONDRIA.—When mitochondria are exposed to GSSG + GSH the O_2 consumption is considerably greater than that seen with the glutathione mixture alone (Fig. 5). This increased rate may or may not be preceded by a lag period. When there is a clear-cut lag period, it shows some correlation with the lag before the swelling reported previously (Neubert and Lehninger, 1962; Hunter *et al.*, 1964a). However, with GSSG + GSH the O_2 uptake and lipid peroxidation usually start significantly before the OD_{520} change (Fig. 5). With the one sample of GSSG mentioned (Sigma lot 23B762) there was less extra oxygen consumption, a late appearance of lipid peroxide, and a delayed and slowed OD_{520} change. Other samples from the same lot induced more rapid changes.

Because the 1 mM GSH present in the GSSG + GSH mixtures was more than sufficient to account for all the O_2 consumption, there was uncertainty as to how much was owing to GSH oxidation and how much to peroxidation of lipids in the mitochondria. For this reason experiments were carried out with different amounts of mitochondria to see whether bursts of O_2 consumption owing to lipid peroxidation could be observed, as in the case of Fe^{2+}. With very low amounts of mitochondria (30 μg protein/ml) it was impossible to measure a significant increment of O_2 consumption over that with GSSG + GSH in medium alone (Fig. 6). With intermediate concentrations of mitochondria (50–60 μg protein/ml) it was possible to observe a period of increased oxidation which was followed by return to a rate similar to that with GSSG + GSH without mitochondria. Since GSH is present in considerable excess relative to the O_2 available, this limited amount of extra oxidation must represent exhaustion of the peroxidizable lipid in the mitochondria. With larger amounts of mitochondria (130–160 μg protein/ml) the extra O_2 consumption appears to be proportionately greater. However, rate changes due to the low O_2 tension near the end of the experiment make determination of the exact value for extra O_2 consumption difficult.

Effect of Inhibitors on Oxygen Consumption with GSSG + GSH.—WITH MITOCHONDRIA.—In previous studies (Hunter *et al.*, 1964a,c) EDTA, phosphate, high concentrations of several electron-transport inhibitors, and antioxidants were shown to prevent the swelling-lysis and the malonaldehyde formation seen with GSSG + GSH. These inhibitors also eliminate the extra O_2 consumption which is caused by lipid peroxidation in the mitochondria. The actions of EDTA and phosphate have been illustrated previously (Fig. 2 in Hunter *et al.*, 1964c). Arsenate and citrate are shown in Figure 7. Butylated hydroxytoluene and butylated hydroxyanisole also eliminate the extra O_2 consumption. Whether these substances all act by the same mechanism is not known. Many apparently act by inhibiting lipid peroxidation in mitochondrial material. Others may complex a trace metal or interfere with an interaction between GSSG and GSH.

WITHOUT MITOCHONDRIA.—Since a number of metal-complexing agents and electron-transport inhibitors prevent swelling-lysis and lipid-peroxide formation when mitochondria are exposed to GSSG + GSH, it

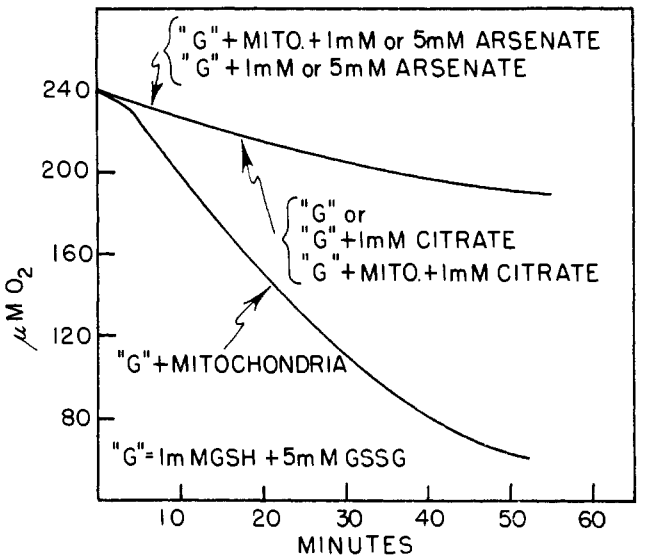

FIG. 7.—Effect of arsenate or citrate on the oxygen consumption with GSSG + GSH in the absence and in the presence of mitochondria. Sucrose-Tris medium; mitochondrial protein, 150 μg/ml.

was of interest to determine whether these agents prevented the spontaneous oxidation of GSH in KCl-Tris medium induced by the addition of GSSG. As may be seen in Figure 7 and Table I, concentrations of arsenate, citrate, antimycin A, 2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone, 2-nonyl-3-hydroxyquinoline-*N*-oxide, butylated hydroxytoluene, butylated hydroxyanisole, thyroxine, EDTA, and inorganic triphosphate, which are known to inhibit or prevent GSSG + GSH–initiated lipid peroxidation in mitochondria (Figs. 5, 6, 7, and 9 in Hunter *et al.*, 1964a; Figs. 1, 3, 4, 5, and 8 in Hunter *et al.*, 1964c), do not alter the spontaneous O_2 uptake with GSSG + GSH. Higher concentrations of some substances, such as 5 mM phosphate, 1–10 mM EDTA, and 1 mM inorganic triphosphate cause some reduction in O_2 consumption. Cyanide and diethyldithiocarbamate, on the other

TABLE I

EFFECT OF INHIBITORS ON OXYGEN CONSUMPTION WITH GSSG + GSH IN MEDIUM WITHOUT MITOCHONDRIA[a]

No inhibition:	
Arsenate, 5 mM	Amytal, 2.5 mM
Citrate, 1 mM	Rotenone, 1 μM
Antimycin A, 4–7 mM	Butylated hydroxyanisole, 10 μM
2-Hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone, 4–8 μM	Butylated hydroxytoluene, 10 μM
2-Nonyl-3-hydroxyquinoline-*N*-oxide, 4–7 μM	Thyroxine, 10 μM
Catalase, 100 μg/ml-variable	Mg^{2+}, 5 mM
Bovine serum albumin, 0.1% (partial inhibition after 15–20 min)	Mn^{2+}, 100 μM
Partial inhibition:	
Phosphate, 5 mM	25–30% inhibition
EDTA, 0.1 mM	0–10% inhibition
EDTA, 1–10 mM	0–50% inhibition
Inorganic triphosphate, 1 mM	0–30% inhibition
Inhibition:	
NaCN, 30 mM	75–80% inhibition
60 μM	90–100% inhibition
100 μM	100% inhibition
Diethyldithiocarbamate, 100 μM	100% inhibition

[a] 0.175 M KCl + 0.025 M Tris medium, *p*H 7.4: 5 mM GSSG + 1 mM GSH.

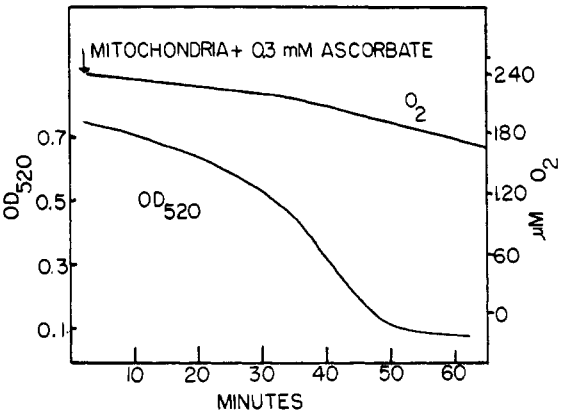

FIG. 8.—Oxygen consumption and OD_{520} change with 0.3 mM ascorbate. KCl-Tris medium; mitochondrial protein ~150 μg/ml.

hand, can cause complete inhibition in relatively low concentrations.

Oxygen Consumption with Ascorbate

Ascorbate (30–100 μM).—Low concentrations of ascorbate produce lipid peroxidation and swelling-lysis in a typical fashion after a characteristic lag period (Figs. 1, 8, and 9 in Hunter *et al.*, 1964b). However, the O_2 consumption with mitochondria + 30–100 μM ascorbate is not greatly different from that with ascorbate in the absence of mitochondria. Since work to be presented here indicates that the presence of mitochondrial material reduces the spontaneous oxidation of ascorbate, it is likely that the increment of O_2 uptake due to lipid peroxidation is offset by the decrease in spontaneous oxidation. There is support for this interpretation from the fact that in many experiments there is a slightly slower rate during the period which would correspond to the lag period before ascorbate-induced lysis. The rate then increases. There is definitely no sudden burst of O_2 consumption, as with Fe^{2+}, and the O_2 consumption is much smaller than with GSSG + GSH.

Ascorbate (0.3–0.6 mM).—When 0.3 mM ascorbate, the concentration used in many earlier swelling-lysis studies (Hunter *et al.*, 1964b), was added to the dilute mitochondrial suspension, there was an increased rate of O_2 consumption. The increase in rate sometimes occurred with no lag period, but in most cases there was a period of 5–30 minutes before the rate increased to the higher value (Fig. 8). This lag phase appears to be related to the lag period before ascorbate-induced swelling and lysis.

When 0.3–0.6 mM ascorbate was added first to the medium there was a relatively low rate of O_2 consumption owing to spontaneous oxidation of ascorbate. When mitochondria were then added, about half the experiments showed a 3-fold increase in rate of O_2 consumption with little or no lag. In the other half of the experiments, in which the spontaneous rate with ascorbate averaged slightly higher, addition of mitochondria resulted in a very distinct slowing of the O_2 consumption. After a lag period of 5–40 minutes at this lower rate the O_2 uptake increased 2- to 4-fold.

The results suggest that there is relatively little oxidation of mitochondrial material during the lag phase, but increased oxidation during and for some time after swelling-lysis. The rate falls off as the oxidizable material in the mitochondria is exhausted. Because of the problems introduced by the change in rate of spontaneous oxidation of ascorbate in the presence of mitochondria, the relatively low rate of

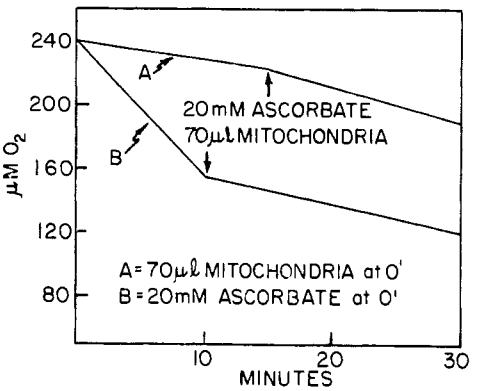

FIG. 9.—Oxygen consumption with 20 mM ascorbate. Sucrose-Tris medium; 70 μl of mitochondrial suspension represents ~125 μg protein/ml.

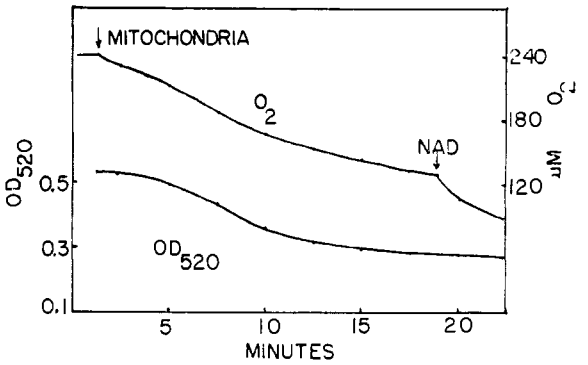

FIG. 10.—Oxygen consumption and swelling (ΔOD_{520}) with 5 mM PO_4 + 2 mM β-hydroxybutyrate. Medium: 150 mM sucrose + 75 mM KCl + 25 mM Tris; mitochondrial protein ~250 μg/ml; NAD, 2 mM.

O_2 uptake in the presence of mitochondria, and the somewhat capricious behavior of lag periods, it is not possible to make complete quantitative correlations with swelling-lysis.

Ascorbate (20 mM).—In sucrose-Tris medium without mitochondria, 20 mM ascorbate is oxidized at a rate which would consume the dissolved O_2 in 30–60 minutes (Fig. 9). When mitochondria are added after the 20 mM ascorbate, the rate of O_2 consumption immediately drops to 25–30% of that with ascorbate alone. If mitochondria are added first, the low rate of O_2 consumption typical for mitochondria alone is increased severalfold on addition of 20 mM ascorbate to a rate similar to that seen when the mitochondria are added last. These findings indicate that the spontaneous oxidation of 20 mM ascorbate is greatly decreased in the presence of mitochondrial material at 100–150 μg protein/ml. When mitochondria are present the rate of O_2 consumption does not exhaust the dissolved oxygen for several hours.

Oxygen Consumption during Swelling Induced by Phosphate

With the amount of mitochondria used to produce an initial OD_{520} of 0.500 for swelling experiments, the rate of O_2 consumption with the addition of PO_4 is very low. With addition of both PO_4 and β-hydroxybutyrate the O_2 consumption is greater but still low. More accurate estimates of the O_2 uptake can be obtained with somewhat more concentrated suspensions of mitochondria, which still show typical PO_4-induced swelling. Since there is no lipid peroxidation, the O_2 uptake represents substrate oxidation.

When mitochondria show some lag period before PO_4-induced swelling the O_2 consumption behaves as shown in Figure 10. There is a small but distinct increase in the rate of O_2 consumption as swelling begins. This may represent the loss of respiratory control, a change which appears to be essential for this type of swelling to occur (Hunter, 1963; Packer, 1963). Since mitochondria in such dilute suspensions lose their respiratory control very rapidly, PO_4-induced swelling frequently does not show a lag period. Such experiments show the most rapid O_2 consumption in the initial 5 minutes. In all cases the rate of O_2 consumption gradually falls off to a much slower rate. This change agrees with the observation that NAD is lost from mitochondria (Hunter *et al.*, 1959b) during PO_4-induced swelling, so that oxidation of NAD-dependent substrates would stop. The oxidation rate usually can be restored by adding NAD, but little further swelling occurs. With succinate the oxidation proceeds more rapidly and for a longer duration.

For the PO_4- or thyroxine-induced type of swelling O_2 is ordinarily necessary and some electron transport is absolutely essential, but the amount of electron transport required for the structural or permeability changes is not very large. With only endogenous substrate it can be estimated as about 3–5 mμmoles O_2/min per mg of mitochondrial protein. This may be compared to 40–50 mμmoles O_2/min per mg of protein in a complete system with ADP + Mg^{2+} + hexokinase + glucose.

DISCUSSION

The well-defined and limited bursts of O_2 consumption when mitochondria are treated with Fe^{2+} ion indicate that total lipid peroxidation, and not just that leading to malonaldehyde, is dependent on Fe^{2+} and ceases when this ion is exhausted. The mechanism by which Fe^{2+} initiates lipid peroxidation is not understood. An average burst of O_2 consumption represents 10–15 μmoles of O_2 per μmole malonaldehyde measured. The 3-to 5-fold excess of O_2 over the amount required to form malonaldehyde probably represents lipid peroxidation that does not yield malonaldehyde (Dahle *et al.*, 1962). In addition, values for malonaldehyde may be low because of losses in side reactions.

The O_2 consumption studies also indicate two important points concerning the action of Fe^{2+}. (a) The burst of O_2 consumption is not directly proportional to the added Fe^{2+}. It may be related to the Fe^{2+} concentration at the time of the burst. (b) Fe^{2+} concentrations over 10 μM tend to produce steadily increasing lag periods before the burst of oxidation, in exact parallel with earlier work in which malonaldehyde formation was studied (Fig. 3 in Hunter *et al.*, 1963; Fortney and Lynn, 1964). These observations support the suggestion that high Fe^{2+} concentrations are inhibitory until the Fe^{2+} falls as the results of oxidation during the lag period, and the final portion is consumed as it induces rapid lipid peroxidation. Oxygen consumption owing to Fe^{2+} oxidation during lag periods is clearly apparent with the higher Fe^{2+} concentrations. An alternative possibility is that the burst of oxidation occurs only at certain optimal Fe^{2+}/Fe^{3+} ratios. Too much Fe^{2+} or too high an Fe^{2+}/Fe^{3+} ratio obviously prevents the burst as well as the appearance of the thiobarbituric acid–reacting material (Hunter *et al.*, 1963). Thus high Fe^{2+} is influencing initiation of the whole process and not just the appearance of malonaldehyde as an end product. High Fe^{2+} might inhibit by a direct antioxidant effect. The inhibitory effect of Fe^{2+} is markedly decreased if the mitochondria have undergone some peroxidative change.

With low concentrations of ascorbate (30–300 μM) there is a distinct lag period followed by a rapid swelling-lysis phase in the OD_{520} curve (Fig. 1 in Hunter *et al.*, 1964b). The formation of lipid peroxide (malonaldehyde) is small, but it continues for some time after swelling is complete. The extra O_2 consumption associated with this lipid peroxidation is not large. It is difficult to measure because a decrease in the spontaneous rate of ascorbate oxidation upon the addition of mitochondria may compensate for most or all of the O_2 uptake owing to lipid peroxidation. This effect of mitochondria on the rate of spontaneous oxidation of ascorbate is dramatically evident when much higher concentrations of ascorbate (20 μM) are used. Since trace-metal catalysis plays a role in the spontaneous oxidation of ascorbate, binding of trace metals by the mitochondrial protein is the most likely explanation. Equal amounts of bovine serum albumin have relatively less effect. Decomposition of the product H_2O_2 by mitochondrial catalase could account for only part of the decrease in O_2 consumption when mitochondria are added with ascorbate. Although intact mitochondria may bind trace-metal ions, release of Fe from the mitochondria during the lag period may be responsible for initiation of ascorbate-induced swelling-lysis (Fortney and Lynn, 1964).

The experiments with 15–20 mM ascorbate and mitochondria demonstrate clearly that the failure of mitochondria to undergo lipid peroxidation and swelling-lysis with high concentrations of ascorbate is not owing to exhaustion of the dissolved oxygen in the suspension by spontaneous oxidation of the ascorbate. As previously suggested (Hunter *et al.*, 1964b), anti-oxidation action of the reducing power of ascorbate probably eliminates peroxidation and swelling-lysis.

When GSSG + GSH acts on mitochondria there is extra O_2 consumption which is undoubtedly associated with the rapid and continuing lipid peroxidation as judged by the malonaldehyde determinations (Fig. 5 and Hunter *et al.*, 1964a). However, since oxidation of the 1 mM GSH present could account for all of the O_2 consumption, quantitative assignment of a specific fraction to oxidation of mitochondrial lipids is difficult. While GSH alone is oxidized very slowly by normal mitochondria, lipid peroxides formed when GSSG is also present may react with part of the GSH.

Control experiments with GSSG + GSH without mitochondria revealed that GSSG markedly accelerates GSH oxidation in most cases. Although stimulation of GSH oxidation by metal impurities in the GSSG would be an explanation consistent with the effects of several inhibitors, the amount of added Cu^{2+} or Fe^{3+} required to produce similar increases in GSH oxidation under the conditions of these experiments was relatively high (100 μM). Such amounts are not likely to be present as impurities in GSSG. Boehringer[3] GSSG, prepared with Fe catalysis, is reported to show usually about 0.01% iron by analysis, with 0.03% being the maximum for an occasional sample. At the 5 mM GSSG concentration used in our experiments this would mean 6 μM and 18 μM iron. Ten μM Fe^{3+} or Cu^{2+} added to 1 mM GSH had little effect in our experiments. Moreover, other GSSG preparations (Sigma Chemical Co.) made without metal catalysis show stimulation of GSH oxidation. Finally, 0.1 mM EDTA, which stops lipid peroxidation in mitochondria, has little or no effect, and as high as 10 mM shows very variable inhibitory effects on the GSSG + GSH oxidation. An organic rather than metallic impurity might cause stimulation. However, Sigma GSSG shows only one ninhydrin-positive spot on paper chromatography. Other commercial preparations of GSSG show one to five additional spots.

The general behavior of several reactions involving GSSG + GSH suggests that there may be an interaction between GSSG and GSH. Such an intermediate might be responsible for the initiation of lipid peroxidation in mitochondria and in pure methyl arachidonate as well as for the rapid nonenzymatic reduction of cytochrome *c* (Froede *et al.*, 1964). Since the rapid reduction of cytochrome *c* by GSSG + GSH is not inhibited by cyanide or 10 mM EDTA, presumably there would be no trace-metal requirement for formation of the intermediate. However, there might be a trace-metal requirement for the intermediate to react with O_2, for 100 μM NaCN and 100 μM diethyldithiocarbamate can block the O_2 uptake completely. There is also the possibility that these powerful inhibitors react directly with the intermediate as reducing agents rather than with a metal, for EDTA at 10 mM only partially prevents oxidation of GSSG + GSH. Phosphate and pyrophosphate are also poor inhibitors of the reaction with oxygen.

There may also be a trace-metal requirement for GSSG + GSH to induce lipid peroxidation in mitochondria. NaCN can prevent this reaction, although 10-fold higher concentrations are required than to prevent oxidation of GSSG + GSH. Very low EDTA (10–100 μM) prevents the action of GSSG + GSH on mitochondria, but there are many indications that this represents a reaction with groups or metals in the mitochondrial membrane rather than chelation of a free trace metal. Both GSSG and a trace metal may be required for some of the reactions seen with GSSG + GSH.

The time course and quantitative aspects of both oxygen consumption and lipid peroxidation are different with Fe^{2+}, ascorbate, and GSSG + GSH. There is also some suggestion of a qualitative difference or selective effect on the mitochondrial lipids peroxidized with the different agents, so that structure or permeability may be affected in different ways. With equal amounts of malonaldehyde formation and possibly greater O_2 consumption, Fe^{2+} does not produce disintegration, while ascorbate and GSSG + GSH do. Continuing lipid peroxidation due either to a chain reaction or to persistance of ascorbate and GSH (in contrast to Fe^{2+}) may account for the subsequent disintegration. Swelling as measured by the OD_{520} change seems to lag behind initiation of O_2 uptake and lipid peroxidation with GSSG + GSH more than with Fe^{2+}. However, this may be more apparent than real because of the very rapid reactions with Fe^{2+}.

The work with Fe^{2+} indicates a controlled, possibly selective lipid peroxidation in isolated mitochondria that may be an interesting tool for certain studies in view of the key role that phospholipids play. *In vivo* Fe^{2+} is not likely to reach concentrations which would produce such effects except in certain pathological states (Reisman *et al.*, 1955). While ascorbate is widely distributed in tissues, most of its function is not understood. A role for ascorbate in a number of electron-transport and oxidative functions has been suggested. However, it is quite uncertain whether any of the peroxidative reaction observed in dilute mitochondrial suspensions *in vitro* ever occurs in the intact cell, for even *in vitro* it is almost completely suppressed in concentrated mitochondrial suspensions. The interaction of GSSG and GSH with membrane disulfide and thiol groups to produce significant changes in membrane structure *in vivo* is a very likely possibility (Lehninger, 1962). However, how much lipid peroxidation occurs

[3] Analyses supplied by Boehringer and Sons, Inc., Mannheim, Germany.

is uncertain, since it may be initiated only by direct interaction between GSSG and GSH. Failure to keep glutathione sufficiently reduced does seem to be the basis for pathological effects in certain cells (Beutler, 1960; Mills, 1959). Another interesting possibility is that interaction between disulfides and thiol groups in enzyme systems may provide more reactive intermediates for rapid transfer of electrons. Possibly such interactions account for the especially labile form of sulfur reported to be present in xanthine oxidase, liver aldehyde oxidase, dihydroorotic dehydrogenase, and some other enzymes (Handler *et al.*, 1964).

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Structure and Activity of Some Aryl *n*-Methyl Methylphosphoramidates as Cholinesterase Inhibitors

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A series of aryl *n*-methyl methylphosphoramidates were prepared and examined for inhibition of insect cholinesterase. This property was compared to the lability of the phosphorus-oxygen-aromatic bond. The correlation was excellent except for those compounds possessing a 4-*t*-butyl and the 3-*t*-butyl group on the phenyl ring. In these cases, enzyme inhibition was greater than could be accounted for by the lability of the P-O-phenyl linkage. A possible explanation is advanced for this discrepancy. An interpretation of the correlation between infrared stretching frequency and cholinesterase inhibition is also presented.

There has been a great deal of work connected with the mechanism of action of organophosphorus compounds as insecticides (see for example the work of Fukuto and Metcalf, 1956, and Metcalf and March, 1953, as well as the excellent books by O'Brien, 1960, and Heath, 1961). These workers have shown that toxicity of certain compounds to insects is sometimes associated with the cholinesterase enzyme system.

Following up these and earlier correlations, many investigators have studied the reaction between organophosphorus compounds and various esterases. Thus, Aldridge and Davison (1952) have shown that the inhibition of erythrocyte cholinesterase by paraxon and some of its analogs followed pseudo-first-order kinetics and was bimolecular. Furthermore, the bimolecular rate constants for this inhibition paralleled the rate of hydrolysis of these phosphates in water. These same authors proposed a mechanism for this reaction which may be depicted as follows:

$$\mathrm{EH} + (\mathrm{RO})_2\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}\!-\!\mathrm{X} \rightleftharpoons \mathrm{HE}(\mathrm{RO})_2\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}\!-\!\mathrm{X} \longrightarrow \mathrm{E}\!-\!\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}(\mathrm{OR})_2 + \mathrm{HX} \qquad (1)$$

where EH is the enzyme, R is any alkyl group, and X is any displaceable group such as halogen, alkoxy, or aryloxy. Such a mechanism implied that the inhibitory powers of the organophosphates were directly related to the lability of P–X bond. Indeed many such correlations have been made (Aldridge and Davison, 1952; Fukuto and Metcalf, 1956; Wilson, 1951, 1952).

In the present series of organophosphoramidates, 4-*t*-butyl-2-chlorophenyl *n*-methyl methylphosphoramidate (Ruelene, insecticide of the Dow Chemical Co.), appeared to have greater cholinesterase-inhibitory powers than could be explained on the basis of the lability of the P-O-phenyl linkage. It is the purpose of this investigation to explore the relationships be-

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