Inhibition of Oxidative Phosphorylation in Brain Mitochondria by Electrical Currents and the Effect of Chelating Agents and other Substances

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In a previous study involving the inhibition of oxidative phosphorylation in brain mitochondria by electrical currents, it was observed that the effect varied with the kind of conductive substance used in the construction of the electrode (Abood, 1954). Electrodes constructed of either gold or an alloy of silver and gold were most effective, while silver and platinum electrodes were least effective. Although less polarization occurred with the former pair, it was not possible to account for the variation with the electrodes solely on the basis of electrolytic polarization. Another phenomenon known to depend on the nature of the conductive metal is electrolytic oxidation, whereby hydroxyl and other related oxidizing radicals are formed (Glasstone & Hickling, 1939). Since such oxidizing radicals, not to mention heavy-metal ions formed near the surface of the electrode, could oxidize thiol enzymes (see Barron, 1951 for review), it was necessary to determine to what extent electrolytic oxidations may have contributed to the inhibition of phosphorylation by electrical currents. In the course of such experiments it has been found that, although in the case of some metals, particularly the alloys of silver and copper, electrolytic oxidation does occur to some extent, the metabolic effects are by and large due to the electrical currents themselves. It has been found that, independently of any effect on heavy metals, glutathione and certain other chelating and reducing substances will protect against the inhibition of phosphorylation by electrical currents.

METHODS

The stimulating vessels used were essentially similar to those of McIlwain (1951). Details for the construction of the electrodes are shown in Fig. 1. Square wave pulses were delivered by means of a pulse generator attached to a Williamson type amplifier (HeathKit, Model WA-1). The externally applied potential was 10 v at a frequency of 300/sec. and pulse duration of 0.2 m-sec. An actual potential of from 4 to 6 v was measured between the inserted electrodes which were 2 mm. apart. The carbon electrodes consisted of two metal-free carbon plates, $1 \times 0.5 \times 0.1$ cm., separated by means of Lucite (polymethyl methacrylate) strips attached to the carbon by a cement made of Lucite and ethylene dichloride. Electrical contact with the Warburg vessel leads

was through platinum connexions which were coated with Lucite cement in order to insulate electrically that portion in contact with the flask contents. After frequent use an oxidized film occurred on the surface of the metallic electrodes, which could be removed by immersing the electrodes in conc. NH_3 for 1 min. Unless otherwise specified, the current was applied as soon as the vessels were placed in the Warburg bath.

Isolation of rat-brain mitochondria was done after a modification of a method described elsewhere (Abood, Gerard, Tschirgi & Banks, 1952). Adult Sprague-Dawley rats were killed by decapitation, their brains removed,

Fig. 1. Construction of electrodes. The dimensions of the Lucite plate were 1.3 cm. long, 0.5 cm. wide, and 0.15 cm. thick. Thirty-gauge wire was mounted on the plates by means of a Lucite cement (Lucite + ethylene dichloride).

washed for 1 min. in 20 vol. of ice-cold 0.25 M sucrose, and then homogenized (Potter-Elvehjem homogenizer) for 5 min. in 15 vol. of 0.25 M sucrose at 0°. Nuclei, blood cells, and unbroken cells were sedimented by two successive centrifugings at 1000 g for 5 min. The supernatant was centrifuged at 10000 g for 15 min., and the mitochondrial residue was resuspended in 6 vol. of 0.25 M sucrose and homogenized. All operations were performed at 0°.

Oxidative phosphorylation was determined as follows: each Warburg vessel contained (final concentration) 0.008 M potassium phosphate, pH 7.5, 5×10^{-4} M potassium adenosine triphosphate, 0.008 M magnesium chloride, 0.015 M potassium fluoride, 10^{-4} M diphosphopyridine nucleotide,

2×10^{-6} M cytochrome c, 0.02 M potassium pyruvate, 0.001 M potassium malate, 0.5 mg. yeast hexokinase (side arm), 0.004 M glucose (side arm), and brain mitochondria from 150 mg. fresh brain (prepared as above). The reaction mixture was kept at 33° for 5 min. before being introduced into the vessels. A thermo-barometer was run under identical conditions, and it was found that thermal equilibrium was reached in less than 6 min. After 3 min. incubation at 33°, the hexokinase and glucose were tipped in from the side arm, and the mixture incubated an additional 2 min. before the taps were closed. After oxidation was measured for 10 min., the reaction was stopped by tipping in 0.2 ml. of 50% (w/v) trichloroacetic acid (TCA). A 0.5 ml. portion of the vessel contents was pipetted into 3.0 ml. of ice-cold 10% (w/v) TCA for orthophosphate determination, according to the method of Fiske & Subbarow (1925).

a Büchner funnel with 500 ml. of 1.0 N-HCl and with 1 l. of distilled water before use. After incubation, a 0.5 ml. sample of the flask contents was pipetted into 2.5 ml. of 10% TCA and centrifuged. To 1.0 ml. of the supernatant were added 150 mg. of the ion-exchange resin and 1.0 ml. of distilled water. The contents were shaken for 0.5 min. and then immediately centrifuged in the cold. Orthophosphate was determined colorimetrically on 1.0 ml. of the supernatant which was free of ferrocyanide and ferricyanide ions. When the above conditions were duplicated with known concentrations of orthophosphate (2–50 μ g.) either in the presence or absence of ferricyanide, it was possible to show that no absorption of orthophosphate by the resin occurred at the acid concentration used, and that the standard curve exactly coincided with the one obtained without the resin. Yeast hexokinase was prepared according to the method of Berger, Stein, Colowick & Cori (1946) through step 3a.

Table 1. *Effect of electrical pulses on P/O ratio using various electrodes*

Electrode	No. of experiments	ΔO_2 (μ atoms)	ΔP (μ moles)	P/O	Inhibition of P/O ratio (%)
Control	28	6.2	18.1	2.9 (2.7–3.1)	—
Silver-copper*	22	6.8	10.2	1.5 (1.2–1.9)	48
Gold	8	6.7	14.0	2.1 (1.8–2.3)	27
Carbon	12	6.8	13.6	2.0 (1.7–2.2)	31
Gold-copper*	6	6.5	14.5	2.2 (1.9–2.4)	24
Silver	12	6.5	16.0	2.5 (2.2–2.7)	14
Platinum	10	6.4	16.5	2.6 (2.5–2.7)	10

* Contains about 8% copper.

Table 2. *Effect of electrical pulses on phosphorylation with ferricyanide as electron acceptor*

	No. of experiments	Δ [Fe(CN) ₆] ³⁻ (μ moles)	ΔP (μ moles)	P/2[Fe(CN) ₆] ³⁻	Inhibition of P/2[Fe(CN) ₆] ³⁻ (%)
Control	10	19.0	10.6	1.1 (0.9–1.3)	—
Silver-copper*	4	12.1	3.0	0.5 (0.4–0.6)	56
Carbon†	8	15.2	4.8	0.6 (0.5–0.7)	45
Gold-copper*	3	16.0	5.0	0.6 (0.5–0.7)	45
Silver†	3	9.6	2.6	0.5 (0.4–0.6)	56

* Contains about 8% copper.

† The addition of 10^{-3} M ethylenediaminetetraacetate or GSH did not alter the inhibition.

The complete system for the measurement of phosphorylation anaerobically was identical with that used aerobically except for the addition of 30 μ moles of potassium ferricyanide as electron acceptor and 50 μ moles of $KHCO_3$. For maximal inhibition of phosphorylation, the addition of 80 μ moles of KCl was essential. The gas phase was 95% N_2 and 5% CO_2 , and the incubation time was 15 min. Ferricyanide reduction was determined as follows: A 0.5 ml. sample was pipetted into 2.0 ml. of freshly prepared 10% TCA and centrifuged. Appropriate portions (0.2–1.0 ml.) of the supernatant were pipetted into test tubes containing 1.0 ml. of ferric iron reagent (Folin & Malmros, 1929) and 5.0 ml. of water. After 10 min. the optical density at 680 m μ . was measured on the Beckman model B spectrophotometer.

The analysis of orthophosphate in the ferricyanide system was accomplished by first removing the ferrocyanide and ferricyanide ions by means of an anion-exchange resin, Dowex-1 (200–400 mesh). The resin (100 g.) was washed on

RESULTS

The effect of electrical currents on the oxidative phosphorylation by brain mitochondria was determined using electrodes made of a variety of metals (Table 1). With electrodes made of an alloy of silver and copper (92.5% Ag, 7.5% Cu), 48% inhibition in the P/O ratio was obtained, and 31% with carbon electrodes. About a 25% decrease in the P/O ratio occurred with electrodes made of gold and those made of a gold and copper alloy (92% Au, 8% Cu), while with silver and platinum electrodes, the inhibition was 14 and 10% respectively. A slight increase in oxygen consumption, however, occurred with each type of electrode, the effect being greatest where the inhibition of phosphorylation was most marked. In so far as a phosphate-

deficient system was not used in the present study, a maximal stimulation of oxidation was not obtained (Abood, 1954). When ferricyanide was substituted for oxygen as electron acceptor in the system, the degree of inhibition in the $P/2 [Fe(CN)_6]^{3-}$ ratio induced by electrical pulses was over 45 %, regardless of the type of electrode (Table 2). Ferricyanide

Phosphorylation of liver and kidney mitochondrial suspensions was almost completely insensitive to electrical currents (carbon electrodes) when oxygen was used (Table 4). An inhibition of phosphorylation was observed in kidney mitochondria with silver alloy electrodes (Abood, Gerard & Ochs, 1952). When ferricyanide replaced

Table 3. *Effect of electrical current before measurement of phosphorylation on $P/2 [Fe(CN)_6]^{3-}$*

Some flasks received current only for period of 10 min. before and some only for a period of 10 min. following addition of side-arm contents. Total incubation time was 20 min. ΔP measured only for period of 10 min. Temp. = 34°. Values represent an average of three experiments agreeing within 5 %. Carbon electrodes were used.

Side-arm contents		$\Delta [Fe(CN)_6]^{3-}$ (μ moles)	ΔP (μ moles)	$P/2 [Fe(CN)_6]^{3-}$	Inhibition of $P/2 [Fe(CN)_6]^{3-}$ (%)
Hexokinase + ferricyanide	Control	18.2	9.0	0.99	—
	Current before ΔP measured	17.8	8.7	0.98	1
	Current during ΔP	14.2	4.0	0.56	44
Hexokinase + adenosine triphosphate	Control*	15.5	6.5	0.83	—
	Current* before ΔP measured	15.4	6.0	0.78	6
	Current* during ΔP	11.0	2.6	0.47	43

* Contained 20 % ferrocyanide and 80 % ferricyanide.

Table 4. *Comparison of inhibition of phosphorylation in rat liver and kidney mitochondria by electrical pulses, using oxygen and ferricyanide as electron acceptors*

Carbon electrodes were employed. Systems used as described in text: 2.0 mg. of liver mitochondrial N, and 1.3 mg. of kidney mitochondrial N used in each flask (average for three experiments).

		Oxygen				Inhibition of P/O (%)
		No. of experiments	ΔO_2 (μ atoms)	ΔP (μ moles)	P/O*	
Liver	Control	3	6.0	18.0	3.0	—
	Current	3	6.3	18.2	2.9	3
Kidney	Control	3	6.6	19.4	2.9	—
	Current	3	6.9	19.3	2.8	3

		Ferricyanide				Inhibition of $P/2 [Fe(CN)_6]^{3-}$ (%)
		No. of experiments	$\Delta [Fe(CN)_6]^{3-}$ (μ moles)	ΔP (μ moles)	$P/2 [Fe(CN)_6]^{3-}$	
Liver	Control	3	26.0	16.0	1.2	—
	Current	3	26.0	14.2	1.1	9
Kidney	Control	3	20.1	13.3	1.3	—
	Current	3	20.8	11.6	1.1	15

* P/O and $P/2 [Fe(CN)_6]^{3-}$ for three experiments agreed within 5 %.

reduction ($\Delta [Fe(CN)_6]^{3-}$) was also diminished, although to a considerably less extent than phosphorylation. With silver electrodes ferricyanide reduction was decreased to almost one-half the control value. Application of the current to suspensions of mitochondria before the period during which phosphorylation was measured did not alter phosphorylation, regardless of whether ferricyanide and ferrocyanide were present initially (Table 3).

oxygen, however, a 9 and 15 % decrease in the $P/2 [Fe(CN)_6]^{3-}$ ratio occurred in both liver and kidney. Ferricyanide reduction did not decrease in the case of either tissue.

A number of agents including sources of thiol groups were tested for their effect on the inhibition of phosphorylation by electrical stimulation (Table 5). Glutathione (GSH), sodium sulphide, diethyldithiocarbamate, and disodium ethylenediaminetetraacetate (Slater, 1953) in concentrations of

Table 5. *Reversal of inhibition of phosphorylation in brain mitochondria by various agents*

Oxygen was used as electron acceptor. Electrodes were of silver-copper alloy.

Agent added	Concentration of agent	ΔO (μ atoms)		ΔP (μ moles)		P/O		Inhibition (%)	Reversed (%)
		Control	Current	Control	Current	Control	Current		
None	—	6.3	6.5	18.0	9.6	2.9	1.5	49	—
GSH	$10^{-3}M$	6.4	6.6	18.1	18.0	2.9	2.8	4	91
Na_2S	$10^{-3}M$	6.1	6.4	18.0	17.9	3.0	2.8	6	87
Diethyldithiocarbamate	$5 \times 10^{-4}M$	6.0	6.2	18.0	17.5	3.0	2.9	3	94
Ethylenediaminetetraacetate	$10^{-3}M$	6.1	6.2	17.5	16.8	2.9	2.8	4	91
Cysteine	$10^{-3}M$	6.1	6.3	17.8	9.6	2.9	1.5	47	0
BAL	$5 \times 10^{-4}M$	5.2	5.3	15.0	8.1	2.9	1.5	47	0
Egg albumin	5 mg.	6.1	6.2	18.1	9.0	3.0	1.5	50	0
KCN	$5 \times 10^{-4}M$	4.2	4.4	12.2	6.4	3.0	1.5	50	0
Glycylglycine	$10^{-2}M$	6.2	6.4	17.9	9.2	2.9	1.5	47	0
Supernatant*	—	6.0	6.2	16.0	11.5	2.7	1.9	30	36
Ascorbate	$10^{-3}M$	6.1	6.3	17.5	9.4	2.9	1.5	47	0
Catalase	1 mg.	6.1	6.3	17.4	9.5	2.9	1.6	45	4

* Supernatant of sucrose homogenate after removal of mitochondria.

Table 6. *Effect of sulphhydryl inhibitors on P/O of rat-brain mitochondria*

Agent added	Concentration of agent (M)	No. of experiments	ΔO_2 (μ atoms)	ΔP (μ moles)	P/O	Inhibition of P/O (%)
None	—	16	6.1	18.0	3.0 (2.8-3.1)	—
$CuSO_4$	10^{-5}	4	4.5	13.9	3.1 (2.9-3.3)	0
$CuSO_4$	10^{-4}	4	2.8	7.8	2.8 (2.7-2.9)	7
$CuCl_2 + GSH^*$	10^{-4}	3	5.9	16.7	2.8 (2.7-3.0)	7
<i>p</i> -Chloromercuribenzoic acid	3×10^{-5}	3	4.8	14.0	2.9 (2.8-3.2)	3
<i>p</i> -Chloromercuribenzoic acid + GSH^*	—	4	6.0	17.5	2.9 (2.8-3.1)	3
Hydrogen peroxide	3×10^{-3}	4	3.2	9.0	2.8 (2.7-3.0)	7

* Concentration of GSH = $10^{-3}M$. Cysteine was as effective as GSH in the same concentration.

$10^{-3}M$ almost completely protected against an inhibition of oxidative phosphorylation. Other sources of thiol groups, such as cysteine, BAL, and egg albumin were without effect. Cyanide and glycylglycine were also ineffective. The supernatant fraction, which contained free thiol groups, was responsible for almost 40 % protection. Catalase and ascorbate were without effect.

The effect of GSH in preventing and reversing the electrically induced inhibition of phosphorylation in brain mitochondria is shown in Fig. 2. When present as soon as the current is applied, GSH will afford almost complete protection. In the absence of GSH, the rate of phosphorylation is depressed by 50 % by electrical pulses, but if it is added after 10 min. of current, the rate becomes almost normal. Copper and *p*-chloromercuribenzoic acid, both powerful inhibitors of sulphhydryl enzymes (Barron, 1951), depressed the oxidation of brain mitochondria about 25 % at $10^{-5}M$ and 50 % at $10^{-4}M$ (Table 6). Phosphorylation was inhibited to the same degree as oxidation, so that the P/O ratios did not differ from the control values. The addition of GSH or cysteine almost completely restored oxidation and phosphorylation to normal. Hydrogen peroxide at a concentration of $3 \times 10^{-3}M$ inhibited phosphorylation and oxidation about 50 %, the P/O ratio remaining unchanged.

Fig. 2. Reversal by glutathione of the inhibition of oxidative phosphorylation by brain mitochondria during electrical stimulation. O, Control; ●, stimulation + $10^{-3}M$ GSH present initially; ×, stimulation with GSH added at time indicated. Oxygen was the electron acceptor.

DISCUSSION

The present findings suggest that electrical currents act on enzyme systems of brain mitochondria related possibly to glutathione and other reducing substances of the cell, since the addition of substances protecting thiol groups will prevent as well as reverse the effects of currents on oxidative phosphorylation. Further support for this hypothesis results from the fact that when ferricyanide, an oxidant of thiol groups (Anson, 1941), is used as an electron acceptor, the inhibition of phosphorylation by electrical currents is maximal and more readily demonstrable. Whereas in previous investigations (Abood, 1954) the degree of inhibition of oxidative phosphorylation varied greatly with the metal used in the stimulating electrode, a constant effect could be obtained with all types of electrodes used, including metal-free carbon, when the ferricyanide system was employed in the measurement of phosphorylation. The addition of chelating agents to the ferricyanide system was without effect on the inhibition of phosphorylation.

Strong oxidizing radicals, such as OH and H_2O_2 , are able to react directly with mitochondrial sulphydryl groups, and thus interfere with normal oxidation-reduction mechanisms (Barron, 1951). The reversal of the inhibitory effect on brain mitochondrial phosphorylation by GSH, sodium sulphide, and diethyldithiocarbamate but not by cysteine, BAL, and other thiol-containing substances, makes it appear unlikely that merely thiol enzymes are involved, in so far as the latter substances will completely reverse the inhibitory effect of sulphydryl agents on metabolism. Furthermore, thiol reagents such as copper and *p*-chloromercuribenzoic acid apparently act primarily on oxidative enzymes, for the P/O ratio of brain mitochondria remains unchanged with increasing concentrations of the thiol agent. Since the addition of large concentrations of catalase did not alter the inhibitory effect of currents, hydrogen peroxide formation does not appear to be involved. Furthermore, with platinum electrodes the formation of oxidizing radicals is especially great, yet little or no inhibition of phosphorylation occurred. The addition of hydrogen peroxide to the vessels affected oxidation primarily, as in the case of the thiol inhibitors. As has been reported elsewhere (Abood, 1954), the uncoupling effect of electrical stimulation is reversible, disappearing as soon as the current is turned off, nor was phosphorylation impaired when mitochondria were exposed to currents prior to the period when phosphate esterification was measured. If sulphydryl enzymes had been oxidized, restoration of their activity would probably not take place without the addition of thiol-containing substances. Nor is it likely that reduction of oxidized GSH

could have occurred, since GSH reductase activity (Rall & Lehninger, 1953) is absent in brain mitochondria (unpublished).

The variation in the effect of different metallic electrodes upon phosphorylation with oxygen as electron acceptor as contrasted with the constant effect in the presence of ferricyanide suggests that mitochondrial thiol groups may be offering some protection against electrical currents, and that the thiol groups must be oxidized before currents can produce their metabolic effect. In the presence of ferricyanide many of these groups may be oxidized. In the system where oxygen was the electron acceptor it is conceivable that electrolytic artifacts could have occurred with specific metals, such as silver and gold, to sufficiently oxidize the protective thiol groups so that the phosphorylative system was made vulnerable to electrical stimulation. Undetectable traces of heavy metals or electrolytically formed oxidizing radicals could have catalysed thiol oxidation (Barron, 1951). The degree of electrolytic oxidation is known to vary greatly with the kind of metallic electrode used (Glasstone & Hickling, 1939).

The inability of Narayanaswami & McIlwain (1954) to obtain an inhibition of oxidative phosphorylation by electrically exciting brain mitochondria is apparently the result of the type of metal used in the electrodes. They were, however, able to obtain an inhibition of creatine phosphokinase by electrical excitation of brain suspensions, although the degree of inhibition varied considerably with the type of metallic electrode used. On the basis of these findings, it was concluded that the metabolic effects obtained from stimulating suspensions were essentially different from those derived from exposing cerebral slices to currents where the metabolic response was independent of the electrode. The present experiments employing the ferricyanide system for the measurement of phosphorylation suggest that the metabolic response of mitochondrial suspensions to electrical currents is similarly independent of the electrode.

If the metabolic responses to electrical pulses are dependent to an appreciable extent on the electrical pulses themselves, as they seem to be, then an explanation commensurate with this view should be forthcoming. It is conceivable that impulses, by virtue of their ability to move ions, can alter the concentration of some essential ion at the mitochondrial surface, thereby affecting the enzyme system dependent upon that ion. We have found that the K/Na ratio of rat-brain mitochondria can be decreased from a value of 8 to as low as 6.0 by electrical currents, while the uptake of ^{42}K is significantly decreased (Abood & Gerard, 1954). It remains to be seen whether or not this decrease is secondary to the inhibition of phosphorylation, in

so far as dinitrophenol will cause a drop in the K/Na ratio and ^{42}K uptake. Since the ability of brain mitochondria to phosphorylate is associated with a high potassium-low sodium concentration, a decrease in the intra-mitochondrial potassium could result in decreased phosphorylation (Abood & Gerard, 1954). It is noteworthy that during excitation of the nerve fibre the permeability to sodium is increased, while an equivalent amount of potassium must diffuse out to maintain electrical and osmotic balance (Hodgkin & Katz, 1949).

There is some evidence that GSH is essential for the preservation of the structural integrity of cells (Fegler, 1952), and we have observed that GSH in concentrations of 10^{-3}M will cause agglutination of brain mitochondria (unpublished). If the metabolic effects of electrical currents are in some way related to ion transport, it is possible that the physical alteration of cells or mitochondria induced by currents may be affected by GSH. Brain mitochondria do appear to have a 'membrane' potential and certain metabolic poisons such as dinitrophenol can affect it (Gerard, Abood, Taylor & Falk, 1953). It does not seem improbable, therefore, that mitochondria may be responsive to electrical phenomena. Studies along this general line are now in progress.

SUMMARY

1. The effect of electrical currents on oxidative phosphorylation of rat-brain mitochondria has been studied using a variety of metallic conductors.

2. Maximal inhibition of phosphorylation was obtained with silver-copper and gold electrodes, while with platinum and silver electrodes inhibition was almost absent.

3. When potassium ferricyanide replaced oxygen as electron acceptor in the system, an inhibition of about 45 % in the $\text{P}/2[\text{Fe}(\text{CN})_6]^{3-}$ ratio regardless of the kind of conducting substance used in the construction of the electrode.

4. Glutathione, sodium sulphide, and diethyldithiocarbamate, but not cysteine and many other reducing agents, were able to protect against inhibition of phosphorylation when oxygen was the electron acceptor.

5. A number of thiol-inhibiting agents were tested and were found, unlike electrical stimulation, primarily to depress oxidation, so that the P/O ratio was largely unchanged.

6. Evidence for believing that the effect on phosphorylation is due to the electrical currents and not electrolytic artifacts is discussed.

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Serratamic Acid, a Derivative of L-Serine Produced by Organisms of the *Serratia* Group

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During studies aimed at obtaining information on the biosynthetic route by which the pigment prodigiosin (Wrede & Rothaas, 1934) is produced by organisms of the *Serratia* group, attempts to isolate precursors of the pigment led to the discovery of another metabolic product of these organisms which is here given the trivial name of serratamic acid. The organism mainly studied

was isolated in this laboratory from coconut, and like other members of the *Serratia* group was difficult to classify exactly (Breed & Breed, 1926). In culture it resembled *S. indica* (Bergey, 1948). Other highly chromogenic strains, including *S. kiliense* (N.C.T.C. 4619) and *S. marcescens* (Woodbine, Kolasiewicz & Seaman, 1954) were also examined.