Evidence of a Phosphate-Transporter System in the Inner Membrane of Isolated Mitochondria

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1. The organic mercurial sodium mersalyl, formaldehyde, dicyclohexylcarbodiimide and tributyltin each blocked respiratory-chain-linked ATP synthesis in rat liver mitochondria. 2. Mersalyl and formaldehyde also blocked a number of other processes dependent on the entry of inorganic phosphate into mitochondria, including mitochondrial respiration and swelling stimulated by cations and phosphate, the substrate-level phosphorylation reaction of the citric acid cycle, and swelling in ammonium phosphate. 3. Dicyclohexylcarbodiimide and tributyltin did not inhibit the entry of phosphate into mitochondria. 4. Mersalyl and formaldehyde had a relatively slight effect on succinate oxidation and swelling stimulated by cations when phosphate was replaced by acetate, on succinate oxidation stimulated by uncoupling agents, and on swelling in solutions of ammonium salts other than phosphate or arsenate. 5. Formaldehyde blocked the oxidation of NAD-linked substrates in mitochondria treated with 2,4-dinitrophenol and the ATP-dependent reduction of NAD by succinate catalysed by ox heart submitochondrial particles. Both these effects appear to be due to an inhibition by formaldehyde of the NAD-flavin region of the respiratory chain. 6. Concentrations of dicyclohexylcarbodiimide or tributyltin sufficient to abolish ADP-stimulated respiration blocked the dinitrophenol-stimulated adenosine triphosphatase activity, whereas mersalyl and formaldehyde caused only partial inhibition of ATP hydrolysis. 7. When mitochondria were incubated with dinitrophenol and ATP, less than 10% of the total inorganic phosphate liberated was recovered in the mitochondria and no swelling occurred. In the presence of mersalyl or formaldehyde at least 80% of the total inorganic phosphate liberated was retained in the mitochondria and extensive swelling was observed. This swelling was inhibited by oligomycin but not by antimycin or rotenone. 8. The addition of mersalyl to mitochondria swollen by treatment with valinomycin, K^+ and phosphate blocked the contraction induced by dinitrophenol and caused an increase in the phosphate content of the mitochondria, but had no effect on the contraction of mitochondria when phosphate was replaced by acetate. 9. It is concluded that mitochondria contain a phosphate-transporter system, which catalyses the movement of phosphate in either direction across the mitochondrial membrane, and that this system is inactivated by organic mercurials and by formaldehyde. Evidence is presented that the phosphate-transporter system is situated in the inner membrane of rat liver mitochondria and is also present in other types of mammalian mitochondria.

According to one of four postulates of the chemiosmotic theory (Mitchell, 1961, 1966), mitochondria contain exchange-diffusion carriers that permit movements of the ionized substrates and products of respiratory-chain phosphorylation across the inner or coupling membrane without causing a discharge of the membrane potential. This postulate is supported by studies of mito-

chondrial swelling in iso-osmotic solutions of ammonium salts, which suggest that several substrate-transporting systems are present in liver mitochondria, including a system that permits the entry of phosphate or arsenate (Chappell & Crofts, 1965, 1966; Chappell & Haarhoff, 1967; Chappell, 1968). The existence of a phosphate transporter has also been inferred from observed differences

between the affinity for inorganic phosphate of the respiratory-chain phosphorylation system in mitochondria and that of the system in submitochondrial particles (Schatz & Racker, 1966; Bygrave & Lehninger, 1967).

Mitchell (1953, 1954) discovered a phosphate exchange-diffusion carrier in *Micrococcus pyogenes* that was strongly inhibited by organic mercurials. His theory and observations suggested that a study of the effect of organic mercurials on phosphate metabolism in isolated mitochondria might reveal the existence of a similar carrier in the mitochondrial membrane. Tyler (1968) found that the organic mercurial sodium mersalyl [sodium *o*-(3-hydroxymercuri-2-methoxypropyl)carbamoylphenoxyacetate] inhibited respiration and swelling dependent on the entry of inorganic phosphate into liver mitochondria, and that the effect of formaldehyde was similar to that of mersalyl. Comparable observations were made by Fonyo (1967, 1968), who used *p*-hydroxymercuribenzoate as inhibitor. It has been postulated that the influence of mercurials and formaldehyde is due to their ability to inactivate a phosphate exchange-diffusion carrier (Tyler, 1968).

The present paper describes studies of the action of mersalyl and formaldehyde on the respiration, swelling and ATPase* activities of isolated mitochondria before and after treatment to remove the outer mitochondrial membrane. The apparent distribution and movements of inorganic phosphate across the mitochondrial membrane, as indicated by measurements of mitochondrial swelling and contraction, have been confirmed by phosphate analyses after the separation of mitochondria from their suspending medium. The results indicate that the movement of inorganic phosphate in either direction across the inner mitochondrial membrane is catalysed by a phosphate-transporter system that can be inactivated by mercurials and by formaldehyde. The effect of these reagents demonstrates that the phosphate-transporter system is an essential component of the mechanism of respiratory-chain phosphorylation, as predicted by the chemiosmotic theory.

MATERIALS AND METHODS

Reagents. The following compounds were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.): antimycin, oligomycin, gramicidin, sodium mersalyl, ADP, ATP, cytochrome *c* (type III from horse heart) and bovine serum albumin. Rotenone, tris, ethylenedioxibis-(ethyleneamino)tetra-acetate and cysteine hydrochloride were from S. B. Penick and Co. (New York, N.Y., U.S.A.), Boehringer Corp. (London) Ltd. (London, W. 5), Koch-Light Laboratories Ltd. (Colnbrook, Bucks.) and the

California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.) respectively. Other reagents, including dicyclohexylcarbodi-imide, digitonin and formaldehyde, were from British Drug Houses Ltd. (Poole, Dorset). A.R.-grade reagents were used whenever possible. Valinomycin, carbonyl cyanide *m*-chlorophenylhydrazide and tributyltin acetate were generous gifts from Dr E. J. Harris, Dr B. C. Pressman and the Tin Research Institute (Greenford, Middx.) respectively.

Enzyme preparations. Rat liver and kidney-cortex mitochondria were isolated in 0.25 M-sucrose-5 mM-tris-HCl, pH 7.4 ('sucrose-tris solution'), by the method of Schneider (1953). The mitochondrial pellets were washed twice and again homogenized in this sucrose-tris solution to obtain a stock suspension of mitochondria containing 60-120 mg. of protein/ml., which was stored at 1° until used. Rat heart mitochondria were prepared by the method of Tyler & Gonze (1967). Inner-membrane-matrix preparation was obtained by fractionating rat liver mitochondria with digitonin by the method of Schnaitman, Erwin & Greenawalt (1967), as modified by Schnaitman & Pedersen (1968). A 2% (w/v) solution of digitonin was freshly prepared in warm sucrose-tris solution containing 0.5 mg. of bovine serum albumin/ml. and cooled to 1° just before use. The digitonin was fully soluble after recrystallization from ethanol. Ox heart submitochondrial particles were isolated by the method of Low & Vallin (1963). The protein content of the enzyme preparations was determined by the biuret method (Gornall, Bardawill & David, 1949) by using samples clarified with 0.2% (w/v) sodium cholate.

Enzyme assays. Enzymic activities and mitochondrial swelling and contraction were assayed at 22°. Respiration rates were recorded by the polarographic technique with a Clark oxygen electrode (Chappell, 1964) in a reaction vessel described by Estabrook (1967). The vessel was kindly provided by Professor R. W. Estabrook. The reaction mixture (3.2 ml.) contained sucrose (0.2 M) and tris-HCl buffer, pH 7.4 (20 mM); which is referred to as 'sucrose-tris buffer'. Other additions were made as indicated in the legends to the Figures and Tables. Stock solutions of oligomycin, dicyclohexylcarbodi-imide, valinomycin and gramicidin were prepared in ethanol, and tributyltin was dissolved in aq. 50% (v/v) ethanol. Sodium mersalyl was dissolved in sucrose-tris buffer. Formaldehyde solutions were prepared by diluting a stock A.R.-grade solution (37-40%, w/v), stated to contain about 11% (v/v) of methanol, with sucrose-tris buffer. The formaldehyde solution was standardized with the Nash (1953) reagent B, pH 6. Inhibitors were added in the form of small volumes (usually 10 µl.) of stock solutions. The amount of ethanol or methanol added with the inhibitor had no significant effect on the activities assayed.

ATPase activity was measured in a reaction mixture containing sucrose (0.2 M), tris-HCl buffer, pH 7.4 (20 mM), KCl (15 mM) and EDTA (1 mM), which is referred to as 'ATPase assay mixture', and mitochondria (1.3-1.8 mg. of protein/ml.). The mitochondria were incubated for 3 min. in the absence or presence of inhibitor, and the reaction was then started by the addition of 2,4-dinitrophenol (0.1 mM) and ATP (5 mM). When the total inorganic phosphate liberated during the reaction was measured, the ATPase activity was terminated after incubation for 4 min. by the addition of 0.1 ml. of 72% (w/v) HClO₄ to 2 ml. of reaction mixture. The samples were centrifuged and the phosphate

* Abbreviation: ATPase, adenosine triphosphatase.

content of the supernatants thus obtained was measured by the method of Fiske & Subbarow (1925). When the distribution of inorganic phosphate between the mitochondria and the reaction mixture was investigated, the ATPase activity was terminated by centrifugation. The samples (2 ml.) were placed in an MSE model HS17 refrigerated centrifuge fitted with an automatic starting device, and the mitochondria were sedimented at 1° by centrifugation for 10 min. at 8500g. The reaction mixture was decanted into clean centrifuge tubes and the mitochondria were homogenized in 2 ml. of water. The samples were deproteinized with 0.1 ml. of 72% (w/v) HClO_4 and centrifuged, and the phosphate content of the supernatants was measured as described above.

The phosphate content of mitochondria separated by centrifugation was corrected for the supernatant phosphate trapped in the mitochondrial pellet by assuming that the volume occupied by the mitochondrial pellet was the proportion 6.4 ml./g. of protein (Estabrook & Holowinsky, 1961), and that the extramitochondrial space of the pellet was equal to 30% of the total pellet volume (Share, 1960).

The ATP-dependent reduction of NAD^+ by succinate was assayed by the method of Low & Vallin (1963), and Mg^{2+} -activated ATPase activity was assayed by the method of Huijing & Slater (1961). NADH oxidase and succinate oxidase activities were assayed polarographically in 0.1 M-potassium phosphate buffer, pH 7.4, containing NADH (0.2 mM) or potassium succinate (15 mM). Monoamine oxidase activity was assayed by the method of Schnaitman *et al.* (1967), in which benzylamine is used as substrate and the product of the reaction, benzaldehyde, is measured in a spectrophotometer at 250 m μ . Before assay, samples containing 2 mg. of protein/ml. in 50 mM-potassium phosphate buffer, pH 7.6, were ultrasonically treated at 1° in an MSE Sonicator for two periods of 30 sec. with an interval for cooling. The specific monoamine oxidase activity of inner-membrane-matrix preparation was 6–15% (mean value 12%) of the activity of mitochondria, which catalysed the formation of 4.1–4.5 μ moles of benzaldehyde/min./g. of protein.

The extinction of mitochondrial suspensions was measured by the method of Tyler (1968).

RESULTS

Effect of mersalyl and formaldehyde on respiration.

Some features of the inhibitory action of mersalyl and formaldehyde on succinate oxidation by rat liver mitochondria suspended in a phosphate-buffered reaction mixture are shown in Fig. 1. The addition of mersalyl (10 μ moles/g. of protein; 12.5 μ M) (trace *b*) or formaldehyde (3 μ moles/mg. of protein; 4 mM) (trace *c*) had no significant effect on the controlled rate of respiration. Incubation with either reagent for 3 min. induced a marked inhibition of respiration stimulated by ADP or Ca^{2+} , but had a relatively small inhibitory effect on respiration stimulated by 2,4-dinitrophenol. Similar results were obtained when carbonyl cyanide *m*-chlorophenylhydrazone (0.5 μ M) was added instead of dinitrophenol and when *p*-chloromercuribenzoate (10 μ moles/g. of protein) was used instead of

mersalyl. The inhibition of respiration by mersalyl was reversed by the further addition of cysteine (trace *d*), and the P:O ratio, Ca^{2+} :O ratio and respiratory-control ratio thus obtained were similar to those observed in the control experiment (trace *a*). The ability of cysteine to reverse the action of mersalyl indicates that the inhibitory effect of the mercurial on mitochondrial respiration is not due to the promotion of irreversible changes in protein conformation or mitochondrial swelling. Organic mercurials induce swelling of rat liver mitochondria (Dickens & Salmony, 1956; Lehninger, 1959), but the concentration of mercurial used in these swelling experiments (0.33–1.0 mM) was much higher than that used in the present studies.

The first addition of Ca^{2+} or ADP to mitochondria treated with mersalyl or formaldehyde caused a very brief stimulation of oxygen uptake, whereas subsequent additions were without effect (Fig. 1). A similar response to Ca^{2+} or ADP was observed with mitochondria incubated in the absence of inhibitor and added inorganic phosphate. These

Fig. 1. Effect of mersalyl (*b* and *d*) and formaldehyde (*c*) on the respiration of liver mitochondria. (Trace *a* is a control, and trace *d* shows the effect of a subsequent addition of cysteine.) In each experiment the reaction mixture contained sucrose-tris buffer, potassium succinate (5 mM), KCl (15 mM), potassium phosphate buffer, pH 7.4 (5 mM), rotenone (5 μ g.) and cytochrome *c* (10 μ M). Additions were made where indicated as follows: *A*, liver mitochondria (4.4 mg. of protein); *B*, ADP (0.5 μ mole); *C*, CaCl_2 (0.5 μ mole); *D*, 2,4-dinitrophenol (0.1 mM); *E*, mersalyl (10 μ moles/g. of protein); *F*, formaldehyde (3 μ moles/mg. of protein); *G*, cysteine hydrochloride (30 μ moles/g. of protein).

Fig. 2. Effect of mersalyl concentration on succinate oxidation. The reaction mixture contained sucrose-tris buffer, tris succinate (5 mM), KCl (15 mM), rotenone (5 μ g.), cytochrome *c* (10 μ M) and liver mitochondria (4.2 mg. of protein). The mitochondria were incubated for 3 min. with the concentrations of mersalyl indicated before the addition of the compounds used to stimulate respiration. ○, ADP (0.5 μ mole) and tris phosphate (2.5 mM); Δ, CaCl₂ (0.5 μ mole) and tris phosphate (2.5 mM); ▲, CaCl₂ (0.5 μ mole) and tris acetate (25 mM); ●, 2,4-dinitrophenol (0.1 mM) and tris phosphate (2.5 mM).

Fig. 3. Effect of formaldehyde concentration on succinate oxidation. Liver mitochondria (4.2 mg. of protein) were incubated for 3 min. with the concentrations of formaldehyde indicated before the addition of the compounds used to stimulate respiration. The conditions and symbols used are specified in the legend to Fig. 2.

observations suggested that mersalyl and formaldehyde inhibit respiration by suppressing the movement of phosphate from the suspending medium into the mitochondria, either as a permeant anion during Ca²⁺ uptake or as a substrate for oxidative phosphorylation, without affecting the reaction between Ca²⁺ or ADP and endogenous phosphate within the mitochondria. This hypothesis is supported by the observation that concentrations of mersalyl and formaldehyde sufficient to block phosphate-dependent respiration had relatively slight effects on respiration stimulated by Ca²⁺ when acetate was used as the permeant anion instead of phosphate (Figs. 2 and 3). Similar results were obtained when valinomycin with K⁺ (Tyler, 1968) or gramicidin with Na⁺ and a permeant anion were used to stimulate respiration (Table 1). Further, mersalyl and formaldehyde caused only a small inhibition of succinate oxidation stimulated by gramicidin and ammonium ions (Table 1), a system that shows no requirement for a permeant anion (Chappell & Crofts, 1965). The inhibition of ADP-stimulated respiration (Figs. 2 and 3) was not modified when the external phosphate concentration was increased from 2.5 mM to 30 mM, indicating

that the inhibition of respiration by the reagents was non-competitive with respect to phosphate ions. The results in Figs. 2 and 3 indicate that mersalyl and formaldehyde partially inhibited uncoupled respiration in mitochondria suspended in phosphate-buffered reaction mixture, and in other experiments a similar effect occurred in the absence of added phosphate. This type of inhibition is unlikely to be due to the loss of cytochrome *c* from mitochondria treated with the inhibitors, because cytochrome *c* was added to the reaction mixture.

Mersalyl and formaldehyde had a similar inhibitory action on phosphate-dependent respiration catalysed by rat kidney and rat heart mitochondria (Table 2) and by rat skeletal-muscle mitochondria (J. Mockel & D. D. Tyler, unpublished work). Both reagents also inhibited the coupled oxidation of NAD-linked substrates (glutamate with malate) and of cytochrome-linked substrates (ascorbate with *NNN'*-tetramethyl-*p*-phenylenediamine and rotenone; Tyler, Estabrook & Sanadi, 1966), and the substrate-level phosphorylation reaction (glutamate with 2,4-dinitrophenol and oligomycin; Chappell & Greville, 1961). The rate of oxidation of NAD-linked substrates was stimulated when 2,4-dinitrophenol was added to mitochondria treated with mersalyl, but not when formaldehyde was present (Table 2). The partial inhibition by mersalyl of substrate oxidation by uncoupled

Table 1. *Effect of mersalyl and formaldehyde on respiration stimulated by gramicidin*

The reaction mixture contained tris succinate (5 mM), rotenone (5 μ g.), cytochrome *c* (10 μ M), liver mitochondria (4.5 mg. of protein), tris-HCl buffer, pH 7.4 (20 mM), and either sucrose (0.2 M) or NH_4Cl (0.1 M) as indicated. The mitochondria were incubated for 3 min. with or without mersalyl (10 μ moles/g. of protein) or formaldehyde (3 μ moles/mg. of protein) before the addition of gramicidin (1 μ g.), NaCl (15 mM), tris phosphate (2.5 mM) and tris acetate (25 mM) as indicated.

Reaction mixture	Additions	Rate of uptake of oxygen (μ g.atoms of O/min./g. of protein)		
		No inhibitor	With mersalyl	With formaldehyde
Sucrose	Gramicidin + NaCl	19	16	16
	Gramicidin + NaCl + phosphate	105	20	17
	Gramicidin + NaCl + acetate	85	75	72
Ammonium chloride	Gramicidin	110	100	85

mitochondria may be due either to an inhibition of the substrate-transporter systems postulated by Chappell (1968) or to the inhibition of the succinate- and NADH-oxidation systems by organic mercurials described previously (Barron & Singer, 1945; Minikami, Schindler & Estabrook, 1964; Tyler, Butow, Gonze & Estabrook, 1965).

Schnaitman *et al.* (1967) and Schnaitman & Pedersen (1968) described a method for the isolation of an inner-membrane-matrix preparation after the removal of the outer membrane by digitonin treatment. They concluded that monoamine oxidase activity is associated exclusively with the outer mitochondrial membrane. In agreement with Schnaitman & Pedersen (1968), I found that inner-membrane-matrix preparations (with about 12% of the monoamine oxidase activity of mitochondria) retained some degree of respiratory control during the oxidation of added substrates. Mersalyl and formaldehyde inhibited phosphate-dependent respiration by the inner-membrane-matrix preparation, indicating that the site of action of the reagents was located in the inner membrane of mitochondria and not in the outer membrane (Table 2).

Effect of dicyclohexylcarbodi-imide and tributyltin. Concentrations of these reagents sufficient to block the coupled oxidation of respiratory-chain-linked substrates (40 μ moles of dicyclohexylcarbodi-imide/g. of protein; 0.9 μ mole of tributyltin/g. of protein) caused about 10% inhibition of respiration stimulated by Ca^{2+} and phosphate and had no effect on the substrate-level phosphorylation reaction, indicating that these reagents have little or no effect on the entry of phosphate into mitochondria. The results obtained agree with observations (Beechey, Holloway, Knight & Robertson, 1966; Sone & Hagiwara, 1964) that these compounds block respiration stimulated by ADP and phosphate, and with the finding (Beechey, Robertson, Holloway &

Knight, 1967) that dicyclohexylcarbodi-imide has no effect on the substrate-level phosphorylation reaction in rat heart mitochondria.

Other experiments revealed that dicyclohexylcarbodi-imide and tributyltin are less specific inhibitors of phosphorylating respiration than is oligomycin. For example, both reagents caused about 10% inhibition of respiration stimulated by dinitrophenol, and tributyltin lowered the $\text{Ca}^{2+}:\text{O}$ ratio. Dicyclohexylcarbodi-imide at concentrations greater than 50 μ moles/g. of protein caused a stimulation of the controlled rate of respiration, apparently by an uncoupling action. None of these effects was observed when oligomycin was added at a concentration (1 μ g./mg. of protein) about ten times greater than that required to block respiration stimulated by ADP.

Mitochondrial swelling in solutions of ammonium salts. Chappell & Crofts (1966) and Chappell & Haarhoff (1967) concluded that the swelling of liver mitochondria in solutions of ammonium salts depends on the ability of the anion present to penetrate the mitochondrial membrane. The hypothesis that mersalyl and formaldehyde inhibit respiration by inhibiting the entry of phosphate into mitochondria was therefore tested by studying their effects on mitochondrial swelling. Concentrations of mersalyl or formaldehyde sufficient to block the phosphate-dependent respiration of mitochondria were found to inhibit specifically those types of swelling that require the entry of phosphate, including swelling in ammonium phosphate and the swelling induced by the addition of a low concentration of ammonium phosphate to mitochondria suspended in ammonium succinate (Table 3). Mersalyl and formaldehyde had no effect on swelling when acetate, β -hydroxybutyrate or azide was the permeant anion present, or on the slow rate of swelling in ammonium succinate alone (Table 3). The swelling behaviour of mitochondria suspended

Table 2. *Effect of mersalyl and formaldehyde on the respiration of mitochondria and inner-membrane-matrix preparation*

The reaction mixture contained either sucrose-tris buffer (Expts. 1-5) or mannitol (0.225 M), sucrose (0.075 M) and tris-HCl buffer, pH 7.4 (0.02 M) (Expt. 6); either potassium phosphate buffer, pH 7.4 (5 mM) (Expts. 1-3, 5 and 6), or potassium phosphate buffer, pH 7.4 (1.5 mM) (Expt. 4); and KCl (15 mM). Substrates added as indicated were potassium glutamate (5 mM) with potassium malate (5 mM) (Expts. 1 and 2); sodium ascorbate (8 mM) with *NNN'*-tetramethyl-*p*-phenylenediamine (0.15 mM), EDTA (1 mM) and rotenone (5 μ g.) (Expt. 3); potassium glutamate (5 mM) with EDTA (1 mM), 2,4-dinitrophenol (50 μ M) and oligomycin (5 μ g.) (Expt. 4); potassium succinate (10 mM) with rotenone (5 μ g.) (Expts. 5 and 6). The enzyme preparations used were liver mitochondria (3.7-7.2 mg. of protein); inner-membrane-matrix preparation (4.5 mg. of protein); kidney mitochondria (3.0 mg. of protein); and heart mitochondria (2.2 mg. of protein). The enzyme preparations were incubated for 3 min. with mersalyl (10 μ moles/g. of protein) or formaldehyde (3.5 μ moles/mg. of protein) before the addition of ADP (0.2-1.0 μ mole), Ca^{2+} (0.5-1.0 μ mole) and 2,4-dinitrophenol (50 μ M) as indicated.

Expt. no.	Enzyme preparation	Substrate	Addition	Rate of uptake of oxygen (μ g.atoms of O/min./g. of protein)		
				No inhibitor	With mersalyl	With formaldehyde
1	Liver mitochondria	Glutamate+malate	None	5.5	6.0	6.0
			ADP	35	6.0	7.0
			Ca^{2+}	48	7.5	7.0
			Dinitrophenol	44	33	8.0
2	Inner-membrane-matrix preparation	Glutamate+malate	None	15	11	9.0
			ADP	48	11	11
			Ca^{2+}	56	13	12
			Dinitrophenol	57	40	11
3	Liver mitochondria	Ascorbate+ tetra-methyl- <i>p</i> -phenylene-diamine	None	22	23	24
			ADP	35	24	24
			Ca^{2+}	40	23	24
			Dinitrophenol	40	38	39
4	Liver mitochondria	Glutamate	None	13	12	13
			ADP	23	14	14
5	Kidney mitochondria	Succinate	None	30	25	24
			ADP	102	26	24
			Ca^{2+}	113	25	32
			Dinitrophenol	155	115	105
6	Heart mitochondria	Succinate	None	40	36	35
			ADP	166	25	28
			Ca^{2+}	190	20	18
			Dinitrophenol	186	130	105

in ammonium malate or ammonium malonate and the effect of mersalyl and formaldehyde on these types of swelling were similar to those observed in ammonium succinate. The presence of oligomycin (5 μ g.) and 2,4-dinitrophenol (0.1 mM), as well as that of rotenone and antimycin, had no effect on the kinetics of swelling in ammonium phosphate, indicating that this swelling process is not dependent on respiration or energy-coupling reactions. The inhibition of swelling in ammonium phosphate by mersalyl and formaldehyde was also observed when inner-membrane-matrix preparation was used instead of mitochondria (Table 3), thus confirming the conclusion that the site of action is situated in the inner membrane. Both mersalyl and formaldehyde inhibited the swelling of mitochondria in

ammonium arsenate, indicating that the system sensitive to these reagents was able to mediate the entry of both phosphate and arsenate across the mitochondrial membrane (Table 3).

Measurements were made of the inorganic phosphate content of mitochondria after incubation in ammonium phosphate. In the absence of inhibitor, the phosphate content after incubation for 2 min. and for 20 min. differed by no more than 7%, in agreement with the kinetics of phosphate uptake indicated by swelling measurements, which show that near-maximal uptake is attained after a 2 min. incubation (Chappell & Haarhoff, 1967). Mersalyl and formaldehyde were found to inhibit the entry of phosphate when preincubated with mitochondria before addition to the reaction

Table 3. *Rates of mitochondrial swelling in solutions of ammonium salts*

Rates of swelling were measured by the method of Tyler (1968). Liver mitochondria (3.0 mg. of protein in 50 μ l.) (Expt. 1) or inner-membrane-matrix preparation (3.5 mg. of protein in 50 μ l.) (Expt. 2) were incubated for 2 min. with 10 μ l. of ice-cold sucrose-tris solution, with or without mersalyl (12 μ moles/g. of protein) or formaldehyde (3.3 μ moles/mg. of protein), before addition to a suspending medium (3 ml.) containing tris-HCl buffer, pH 7.4 (5 mM), ethylenedioxybis(ethylenamino)tetra-acetate (0.1 mM), rotenone (5 μ g.), antimycin (5 μ g.) and the concentrations of ammonium salts indicated.

Expt. no.	Suspending medium	Concn. of anion present (M)	Rate of swelling (extinction units/min.)		
			No inhibitor	With mersalyl	With formaldehyde
1	Ammonium phosphate	0.1	0.52	0.02	0.03
	Ammonium arsenate	0.1	0.58	0.02	0.03
	Ammonium succinate	0.1	0.01	0.01	0.01
	Ammonium succinate	0.1	0.16	0.01	0.01
	+ ammonium phosphate	0.005			
	Ammonium β -hydroxybutyrate	0.15	0.56	0.56	0.58
	Ammonium acetate	0.15	1.35	1.1	1.1
	Ammonium chloride	0.05	0.20	0.19	0.20
	+ sodium azide	0.08			
2	Ammonium phosphate	0.1	0.47	0.04	0.07

Table 4. *Phosphate content of mitochondria incubated in ammonium phosphate*

Liver mitochondria (3.2 mg. of protein in 50 μ l.) were incubated for 2 min. with 10 μ l. of ice-cold sucrose-tris solution, with or without mersalyl (12 μ moles/g. of protein) or formaldehyde (3.3 μ moles/mg. of protein), before addition to a centrifuge tube containing 3 ml. of a solution of ammonium phosphate (0.1 M), tris-HCl buffer (5 mM), ethylenedioxybis(ethylenamino)tetra-acetate (0.1 mM), rotenone (5 μ g.) and antimycin (5 μ g.), pH 7.4. The tubes were transferred to the centrifuge, which was started 2 min. after the addition of the mitochondria. The phosphate content of the sedimented mitochondria was measured as described in the Materials and Methods section.

Addition	Phosphate content (μ moles of P _i /g. of protein)
None	690
Mersalyl	390
Formaldehyde	410

mixture (Table 4), but had no effect when added to the reaction mixture 2 min. after the addition of mitochondria. The preincubation of mitochondria with 12 μ moles of mersalyl/g. of protein blocked the entry of about 300 μ moles of phosphate/g. of protein (Table 4).

Mitochondrial swelling induced by valinomycin. The increase in respiration that occurs when valinomycin is added to mitochondria incubated aerobically with substrate, potassium chloride and a permeant anion is accompanied by an extensive swelling of the mitochondria (Pressman, 1965). Chappell & Crofts (1965) found that a similar type

of swelling induced by gramicidin was reversed by the addition of uncoupling agents or antimycin, or by anaerobic conditions. They suggested that mitochondrial swelling induced by gramicidin or valinomycin was due to the osmotic effect of univalent cations and permeant anions accumulated in the mitochondria by an energy-dependent process and that the inhibition of energy-conservation reactions by uncoupling agents or antimycin caused the loss of accumulated ions and water from the swollen mitochondria and their consequent contraction.

In the present work, valinomycin in the presence of phosphate induced a phase of mitochondrial swelling that lasted for about 1 min., and mitochondrial contraction was then promoted by 2,4-dinitrophenol (Fig. 4, trace *a*). When mitochondria were pre-incubated with mersalyl (or formaldehyde) before the addition of valinomycin, swelling in a phosphate medium was strongly inhibited (Fig. 4, trace *c*). The addition of mersalyl to phosphate-buffered mitochondria in the swollen state blocked the contraction induced by dinitrophenol (Fig. 4, trace *b*), whereas formaldehyde caused a slow progressive contraction to occur in the absence of dinitrophenol. The inhibition of swelling and contraction by mersalyl was reversed by the addition of cysteine. When acetate was used as permeant anion instead of phosphate, mersalyl had no significant effect on either the swelling induced by valinomycin or the contraction induced by dinitrophenol (Fig. 4, trace *d*). Similar results were obtained when gramicidin was used instead of valinomycin and when antimycin (2 μ g.) was used instead of dinitrophenol.

Fig. 4. Effect of mersalyl on mitochondrial swelling and contraction. The Figure shows spectrophotometric traces of light-scattering, a downward deflexion representing swelling. The reaction mixture (3 ml.) contained sucrose-tris buffer, KCl (15 mM), rotenone (5 μ g.), liver mitochondria (3.0 mg. of protein) and either tris phosphate (2.5 mM) in Expts. (a), (b) and (c), or tris acetate (25 mM) in Expt. (d). In Expts. (c) and (d) the mitochondria were incubated for 3 min. with mersalyl (12 μ moles/g. of protein) before the addition of succinate. Additions were made where indicated as follows: A, tris succinate (5 mM); B, valinomycin (0.2 μ g.); C, 2,4-dinitrophenol (0.1 mM); D, mersalyl (12 μ moles/g. of protein); E, cysteine hydrochloride (35 μ moles/g. of protein).

The inhibition of mitochondrial contraction by mersalyl suggested that the mercurial abolishes the movement of phosphate ions from mitochondria treated with dinitrophenol. This hypothesis is supported by measurements of the phosphate content of mitochondria incubated under these conditions, which show that the addition of mersalyl to mitochondria swollen in a phosphate medium causes a retention of phosphate ions in the mitochondria (Table 5). No change in the phosphate content was detected after treatment with valinomycin alone, or when mersalyl was added before valinomycin, or when formaldehyde was added to phosphate-swollen mitochondria instead of mersalyl. The latter result indicates that the slow contraction induced by formaldehyde under these conditions is associated with a movement of phosphate ions from the mitochondria to the

Table 5. *Phosphate content of mitochondria treated with valinomycin*

Incubations were made in centrifuge tubes each containing 3 ml. of a solution containing sucrose-tris buffer, tris succinate (5 mM), KCl (15 mM), tris phosphate (2.5 mM) and rotenone (5 μ g.). Liver mitochondria (3.0 mg. of protein) were added at zero time. Further additions of valinomycin (0.2 μ g.), mersalyl (12 μ moles/g. of protein) and 2,4-dinitrophenol (0.1 mM) were made at the times indicated. The tubes were transferred to the centrifuge, which was started 6 min. after the addition of valinomycin. The phosphate content of the sedimented mitochondria was measured as described in the Materials and Methods section.

Expt. no.	Time (min.)	Addition	Phosphate content of mitochondria (μ moles of P_i /g. of protein)
1	1	Valinomycin	23.0
2	1	Valinomycin	21.2
	4	Dinitrophenol	
3	1	Mersalyl	19.1
	4	Valinomycin	
	8	Dinitrophenol	
4	1	Valinomycin	51.4
	2	Mersalyl	
	5	Dinitrophenol	

reaction mixture. In the absence of mersalyl, the uptake of phosphate ions aerobically by mitochondria treated with valinomycin and K^+ is presumably reversed when the mitochondria become anaerobic during centrifugation. These results confirm the conclusion (Chappell & Crofts, 1965) that phosphate ions are accumulated by an energy-dependent process in mitochondria treated with valinomycin and K^+ ions. The increment in the phosphate content of mitochondria induced by valinomycin, K^+ and mersalyl is about one-half the amount of added phosphate found by Harris, Höfer & Pressman (1967) to be required for a near-maximal stimulation of respiration by valinomycin and K^+ under similar experimental conditions.

ATPase activity. The dinitrophenol-stimulated ATPase activity of isolated mitochondria is generally believed to represent a reversal of the ATP-synthesis reaction of respiratory-chain phosphorylation, a conclusion supported by the observation that oligomycin blocks both the ATPase reaction and the ATP-synthesis reaction (Lardy, Johnson & McMurray, 1958). Concentrations of dicyclohexylcarbodi-imide and tributyltin sufficient to suppress the stimulation of respiration by ADP also abolished the dinitrophenol-stimulated ATPase activity (Table 6). This result confirms the view that, like that of oligomycin, the effect of these

reagents on phosphorylating respiration is due to an action on the reversible coupling ATPase (Beechey *et al.* 1966; Sone & Hagihara, 1964). In contrast,

Table 6. *Inhibition of dinitrophenol-stimulated ATPase*

The total inorganic phosphate liberated during ATPase activity was measured as described in the Materials and Methods section. Inhibitors were used at the following concentrations: oligomycin (5 μ g.), dicyclohexylcarbodi-imide (40 μ moles/g. of protein), mersalyl (10 μ moles/g. of protein) and formaldehyde (3.3 μ moles/mg. of protein).

Addition	ATPase activity (μ moles of P_i /hr./mg. of protein)	
	Without dinitrophenol	With dinitrophenol
None	0.28	5.9
Oligomycin	0.30	0.32
Dicyclohexylcarbodi-imide	0.30	0.35
Tributyltin	0.25	0.25
Mersalyl	0.30	3.4
Mersalyl + oligomycin	0.32	0.32
Formaldehyde	0.38	1.8
Formaldehyde + oligomycin	0.25	0.28

Fig. 5. Mitochondrial swelling induced by mersalyl and formaldehyde. The Figure shows spectrophotometric traces of mitochondrial light-scattering, a downward deflexion representing swelling. Liver mitochondria (3.0 mg. of protein) were added to 3 ml. of ATPase assay mixture containing 2,4-dinitrophenol (0.1 mM). The mitochondria were incubated for 3 min. with either mersalyl (12 μ moles/g. of protein) in Expts. (a) and (b), or formaldehyde (3 μ moles/mg. of protein) in Expts. (c) and (d). Further additions were made where indicated as follows: A, ATP (5 mM); B, cysteine hydrochloride (35 μ moles/g. of protein); C, oligomycin (10 μ g.).

Fig. 6. Phosphate distribution after dinitrophenol-stimulated ATPase activity. The Figure shows the effect of (a) mersalyl and (b) formaldehyde on the phosphate content of liver mitochondria (●) and of the reaction mixture (○), and on the sum of these two values (Δ), found after a period of ATPase activity. Liver mitochondria (3.5 mg. of protein) were incubated for 3 min. in ATPase assay mixture (2 ml.) containing 2,4-dinitrophenol (0.1 mM) and the concentrations of mersalyl and formaldehyde indicated, before the addition of ATP (5 mM). The centrifuge was started 3 min. after the addition of ATP and the phosphate content of the sedimented mitochondria and the suspending medium was measured as described in the Materials and Methods section.

mersalyl and formaldehyde only partially inhibited the ATPase activity stimulated by dinitrophenol (Table 6). A possible explanation of this result might be that these two reagents themselves induce an ATPase activity that is unrelated to the coupling ATPase. This explanation was excluded by the finding that mersalyl and formaldehyde had little or no effect on ATPase activity in the absence of 2,4-dinitrophenol. In addition, the dinitrophenol-stimulated ATPase activity resistant to the effect of mersalyl and formaldehyde was blocked by oligomycin (Table 6). Thus the powerful inhibition of phosphorylating respiration by mersalyl and formaldehyde cannot be attributed to an action on the coupling ATPase and is distinct from the action of oligomycin.

Inspection of the ATPase-assay samples revealed that mitochondria incubated with 2,4-dinitrophenol, ATP and either mersalyl or formaldehyde appeared to swell, and this observation was confirmed by spectrophotometric measurements (Fig. 5). No swelling was detected when mitochondria were incubated with dinitrophenol and ATP, dinitrophenol and mersalyl (or formaldehyde), or ATP and mersalyl (or formaldehyde). Swelling induced by mersalyl or formaldehyde was blocked by oligomycin and, in the case of mitochondria treated with mersalyl, the swelling was reversed by cysteine (Fig. 5). In other experiments, antimycin (5 μ g.) and rotenone (5 μ g.) had no effect on either the swelling induced by mersalyl or the contraction induced by cysteine, indicating that the volume changes observed were not dependent on respiration.

Table 7. *Effect of cysteine on the phosphate content of mitochondria treated with mersalyl*

Liver mitochondria (3.7 mg. of protein) were preincubated for 3 min. in centrifuge tubes containing samples (2 ml.) of ATPase assay mixture and mersalyl (12.5 μ moles/g. of protein). The reaction was started by the addition of 2,4-dinitrophenol (0.1 mM) and ATP (5 mM) at zero time. Further additions of oligomycin (10 μ g.) and cysteine hydrochloride (36 μ moles/g. of protein) were made as indicated. The tubes were transferred to the centrifuge, which was started 5 min. after the addition of oligomycin. The phosphate content of the sedimented mitochondria and the reaction mixture was measured as described in the Materials and Methods section.

Tube	Time (min.)	Addition	Phosphate content (μ moles of P_i)	
			Mitochondria	Reaction mixture
1	8	Oligomycin	0.87	0.22
2	8	Oligomycin		
	11	Cysteine	0.08	1.12

These measurements suggested that, when mitochondria are treated with mersalyl or formaldehyde, the inorganic phosphate liberated during ATPase activity is retained by the mitochondria, and that the swelling that occurs is due to the osmotic activity of the phosphate ions thus accumulated. The contraction induced by cysteine would then be due to the removal of mersalyl from the mitochondria and the subsequent movement of phosphate ions and water from the mitochondria to the reaction mixture. This explanation was confirmed by measurements of the phosphate content of the mitochondria and the reaction mixture, which showed that, in the absence of inhibitor, less than 10% of the phosphate liberated by ATPase activity was present in the mitochondria, and that the treatment of mitochondria with mersalyl or formaldehyde increased this proportion to more than 80% (Fig. 6). The normal distribution of phosphate was restored when cysteine was added to mitochondria treated with mersalyl after ATPase activity had been blocked by oligomycin (Table 7). The concentrations of mersalyl and formaldehyde required to prevent the movement of phosphate from the mitochondria to the reaction mixture were similar to those previously found to block phosphate entry.

Fig. 7. Effect of formaldehyde on the ATP-dependent reduction of NAD^+ by succinate. Ox heart submitochondrial particles (1.1 mg. of protein) were incubated for 3 min. with the concentrations of formaldehyde indicated before the assay of enzyme activities. $\circ$, ATP-dependent reduction of NAD^+ by succinate; $\bullet$, Mg^{2+} -activated ATPase activity; $\blacktriangle$, NADH oxidase activity; $\triangle$, succinate oxidase activity. The specific activities of samples incubated without formaldehyde were 40 μ moles of NAD^+ reduced/min./g. of protein, 6.2 μ moles of phosphate liberated/hr./mg. of protein, 1.5 μ g. atom of O/min./mg. of protein and 1.15 μ g. atom of O/min./mg. of protein respectively.

Energy-linked NAD^+ reduction. Van Buskirk & Frisell (1967) concluded that the inhibition of phosphorylating respiration of liver mitochondria by formaldehyde was due to an inhibition of an energy-transfer pathway situated between the respiratory chain and the locus of ATP synthesis. In support of their conclusion, they presented evidence that formaldehyde strongly inhibits the reduction of NAD^+ by succinate catalysed by ox heart submitochondrial particles incubated with ATP. The influence of formaldehyde concentration on the succinate- NAD^+ reductase activity and on several partial reactions of this system was therefore investigated. The patterns of inhibition by formaldehyde of the succinate- NAD^+ reductase and of the NADH oxidase activity of the preparation were very similar, whereas the ATPase activity (found in other experiments to be strongly inhibited by oligomycin) and succinate oxidase activity were only slightly modified (Fig. 7).

These results indicate that the site of action of formaldehyde is probably situated in the NAD-flavin region of the respiratory chain, and not in an energy-transfer pathway as Van Buskirk & Frisell (1967) assumed. The ability of formaldehyde to block NADH oxidation by the respiratory chain provides an explanation for the failure of 2,4-dinitrophenol to restore NAD-linked oxidation in mitochondria treated with formaldehyde (Table 2).

DISCUSSION

Mersalyl and formaldehyde inhibit the phosphate-dependent respiration of liver mitochondria catalysing respiratory-chain phosphorylation, the substrate-level phosphorylation reaction of the citric acid cycle, cation uptake from a phosphate-buffered medium, and the phosphate uptake and swelling that accompanies cation uptake. The results of phosphate analyses of the mitochondria and the suspending medium provide direct evidence that mersalyl and formaldehyde inhibit the movement of phosphate into mitochondria and substantiate the hypothesis (Tyler, 1968) that the influence of the reagents on mitochondrial respiration and swelling is due to their ability to block the entry of phosphate.

The observation that the entry of both phosphate and arsenate is sensitive to mersalyl and formaldehyde supports the suggestion (Chappell & Crofts, 1965) that the carrier mechanism for phosphate entry is also active with arsenate. It also confirms the conclusion (Tyler, 1968) that the inhibition of arsenate uncoupling by mercurials and formaldehyde (Fonyo & Bessman, 1966; Van Buskirk & Frisell, 1967) is due to the inhibition of the entry of arsenate into the mitochondria via the phosphate carrier. The reagents had a smaller effect on

activities dependent on the entry of anions other than phosphate or arsenate, including the respiration of mitochondria treated with dinitrophenol, cation-stimulated respiration and swelling with acetate as the permeant anion, and ATPase activity. These results indicate that the entry mechanism for phosphate and arsenate is distinct from that for other anions such as succinate, glutamate, acetate and adenine nucleotides, and that the inhibitory effects of the concentrations of mersalyl and formaldehyde used are largely confined to the phosphate-arsenate entry mechanism.

Concentrations of mersalyl or formaldehyde sufficient to inhibit the movement of phosphate from the suspending medium into the mitochondria also suppressed the movement of phosphate in the opposite direction. For example, they strongly inhibited the movement of phosphate, released by ATPase activity, from the mitochondria, and mersalyl prevented the exit of phosphate accumulated previously during univalent-cation uptake. It is evident from the results summarized above that liver mitochondria are not freely permeable to phosphate ions. The observations may be explained by postulating that the mitochondrial membrane contains a specific transporter mechanism that permits the movement of phosphate in either direction across the mitochondrial membrane, and that this mechanism is inactivated by mercurials and formaldehyde.

In one type of experiment, one molecule of mersalyl prevented the entry of about 25 molecules of inorganic phosphate, and it is therefore clear that mersalyl prevents phosphate entry by inactivating a catalytic mechanism and not by displacing phosphate from binding sites within the mitochondria. The phosphate-transporter system of rat liver mitochondria was blocked by $10\mu\text{moles}$ of mersalyl/g. of protein, which contains about $0.2\mu\text{mole}$ of cytochrome *a* (Estabrook & Holowinsky, 1961). These values indicate that rat liver mitochondria would contain about 50 phosphate-transporter systems/molecule of cytochrome *a* if each molecule of mersalyl was bound specifically to one transporter. However, the inhibitory effects of mersalyl on other enzyme activities suggests that mersalyl is bound to several components. For this reason it seems probable that the number of phosphate transporters/molecule of cytochrome *a* is less than 50, and that the number of phosphate molecules entering on each transporter in the experiment mentioned above is greater than 25.

Since a mercurial-sensitive entry of phosphate was found with all types of mitochondria examined, it appears probable that these conclusions are generally valid. The effect of mercurials on phosphate movements in bacteria (Mitchell, 1953, 1954) suggests that a basic similarity exists between

the mechanisms of phosphate transfer across the membranes of mitochondria and of bacteria. Spinach chloroplasts, which swell in ammonium acetate but do not swell in ammonium chloride (Crofts, Deamer & Packer, 1967), were found not to swell in ammonium phosphate (D. D. Tyler, unpublished work), a result suggesting that they may not contain a phosphate transporter of the same type as that found in mitochondria. Inhibitors of energy-coupling reactions were found to be without effect on either phosphate entry during swelling in ammonium phosphate or phosphate exit after the addition of cysteine to mitochondria incubated with mersalyl, ATP and di-nitrophenol. The observation that the movement of phosphate can occur in either direction independently of energy-coupling reactions is consistent with the hypothesis (Chappell & Crofts, 1965) that the transporter mechanism consists of an exchange of ions across the mitochondrial membrane, but details of the ionic species concerned in the process remain unknown. Gamble (1965) observed an approximately equivalent exchange of phosphate and citrate by rabbit liver mitochondria.

The pretreatment of mitochondria with mersalyl or formaldehyde before incubation in ammonium phosphate abolished swelling and decreased phosphate uptake by about 50%. These effects may be explained by postulating that mitochondria contain two spaces permeable to phosphate, similar to the two spaces permeable to adenine nucleotides described by Klingenberg & Pfaff (1966). According to this hypothesis, the entry of phosphate into the first space, between the outer and inner membranes of the mitochondria, has no osmotic effect, is insensitive to mersalyl and formaldehyde and therefore does not require the activity of the phosphate transporter. The entry of phosphate into the second osmotically active matrix space would be mediated by the phosphate transporter situated in the inner mitochondrial membrane. This hypothesis was confirmed by studies demonstrating that the ability of mersalyl and formaldehyde to block swelling in ammonium phosphate remained largely unaffected by the removal of the outer membrane. The observation that a partial entry of phosphate into mitochondria suspended in ammonium phosphate occurred even in the presence of mersalyl or formaldehyde is incompatible with the alternative view that the phosphate transporter is situated in the outer membrane, since if this were so the inhibitors would prevent phosphate entry completely.

The accumulation of phosphate ions in the second space also occurred during ATP hydrolysis when the phosphate transporter was inactivated by mersalyl or formaldehyde, because an extensive swelling was observed under these conditions.

Racker and his colleagues (Racker *et al.* 1965; Kagawa & Racker, 1966) demonstrated that the stalked spheres attached to the inner membrane of mitochondria and projecting into the matrix (Fernández-Morán, 1962) are most probably the coupling-ATPase molecules of mitochondria. This localization of the ATPase is consistent with the effects of mersalyl and formaldehyde, which show that during ATP hydrolysis phosphate is liberated in the matrix space and then passes into the reaction mixture via the phosphate transporter.

The inner membrane of mitochondria also contains the adenine nucleotide transporter (Winkler, Bygrave & Lehninger, 1968) and it is probable that all the substrate transporters of mitochondria are situated in this membrane (Chappell, 1968). The results of the present study and related studies therefore provide substantial evidence for the existence of transporters for the reactants of respiratory-chain phosphorylation in the inner or coupling membrane of mitochondria, as postulated by the chemiosmotic theory.

Inhibition of the adenine nucleotide transporter by atractyloside is competitive with ADP (Bruni, Contessa & Luciani, 1962; Winkler *et al.* 1968), whereas inhibition of the phosphate transporter by mercurials and formaldehyde appears to be of the non-competitive type, because it was not modified by increasing the external phosphate concentration and remained fully effective when the phosphate concentration was as high as 0.1M. Mersalyl is clearly a more specific and useful inhibitor than formaldehyde, since it was necessary to add about 300 times as much formaldehyde as mersalyl to inactivate the phosphate transporter, and this amount of formaldehyde also blocked NADH oxidation. The inhibition of the phosphate transporter is presumably due to the formation of complexes between mersalyl or formaldehyde and the thiol and amino groups of an inner-membrane protein or lipoprotein, resulting in the modification of groups catalysing ion-exchange processes during phosphate transport or in a less specific effect on the conformation of the protein concerned in the transporter mechanism. According to Schnaitman & Pedersen (1968), the inner-membrane-matrix preparation used in the present study is devoid of nucleoside diphosphokinase and adenylate kinase activity, and these enzymes are therefore probably not concerned in the phosphate-transporter mechanism. Certain properties of the protein isolated from mitochondria by Criddle, Bock, Green & Tisdale (1962) suggest that it may be concerned in phosphate transport. The protein is devoid of any known catalytic function, contains thiol groups (Woodward & Munkres, 1966), and is capable of binding inorganic phosphate (Hultin & Richardson, 1964).

The decrease in dinitrophenol-stimulated ATPase activity caused by mersalyl may be due to a binding of the mercurial that results in a modification of the ATPase molecule, to product inhibition of the enzyme by the high concentration of phosphate retained within the mitochondria, or to a combination of these effects. Winkler *et al.* (1968) found that organic mercurials (300 μ moles/g. of protein) were without effect on the adenine nucleotide transporter of rat liver mitochondria, and this transporter is thus unlikely to be affected by the concentration of mersalyl used.

The present studies of the action of dicyclohexylcarbodi-imide and tributyltin indicated that concentrations of these reagents sufficient to block respiratory-chain phosphorylation do not inhibit phosphate entry, and provide additional evidence that their effects are confined to an action on the reversible coupling ATPase. Guanidine derivatives (Pressman, 1963; Chappell, 1963) and azide (Bogucka & Wojtczak, 1966; Wilson & Chance, 1966) were once considered to inhibit an energy-transfer pathway between the respiratory chain and the coupling ATPase, but more recently it has been concluded that their effects are likely to be due to an inhibition of respiratory-chain activity after their energy-dependent uptake by mitochondria (Mitchell, 1966; Palmieri & Klingenberg, 1967). The influence of compounds that inhibit energy-linked reactions in mitochondria, apart from respiratory-chain inhibitors and uncoupling agents, therefore appears to be confined to the inactivation of the reversible coupling ATPase (oligomycin, dicyclohexylcarbodi-imide, organotin compounds) or the transporter systems for adenine nucleotides (atractyloside) and phosphate (organic mercurials, formaldehyde).

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REFERENCES

- Barron, E. S. G. & Singer, T. P. (1945). *J. biol. Chem.* **157**, 221.
- Beechey, R. B., Holloway, C. T., Knight, I. G. & Robertson, A. M. (1966). *Biochem. biophys. Res. Commun.* **23**, 75.
- Beechey, R. B., Robertson, A. M., Holloway, C. T. & Knight, I. G. (1967). *Biochemistry*, **6**, 3867.
- Bogucka, K. & Wojtczak, L. (1966). *Biochim. biophys. Acta*, **122**, 381.
- Bruni, A., Contessa, A. R. & Luciani, S. (1962). *Biochim. biophys. Acta*, **60**, 301.
- Bygrave, F. L. & Lehninger, A. L. (1967). *Proc. nat. Acad. Sci., Wash.*, **57**, 1409.
- Chappell, J. B. (1963). *J. biol. Chem.* **238**, 410.
- Chappell, J. B. (1964). *Biochem. J.* **90**, 225.
- Chappell, J. B. (1968). *Brit. med. Bull.* **24**, 150.
- Chappell, J. B. & Crofts, A. R. (1965). *Biochem. J.* **95**, 393.
- Chappell, J. B. & Crofts, A. R. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 293. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Chappell, J. B. & Greville, G. D. (1961). *Nature, Lond.*, **190**, 502.
- Chappell, J. B. & Haarhoff, K. N. (1967). In *Biochemistry of Mitochondria*, p. 75. Ed. by Slater, E. C., Kaniuga, Z. & Wojtczak, L. London: Academic Press (Inc.) Ltd.
- Criddle, R. S., Bock, R. M., Green, D. E. & Tisdale, H. D. (1962). *Biochemistry*, **1**, 827.
- Crofts, A. R., Deamer, D. W. & Packer, L. (1967). *Biochim. biophys. Acta*, **131**, 97.
- Dickens, F. & Salmony, D. (1956). *Biochem. J.* **64**, 645.
- Estabrook, R. W. (1967). In *Methods in Enzymology*, vol. 10, p. 41. Ed. by Estabrook, R. W. & Pullman, M. E. New York: Academic Press Inc.
- Estabrook, R. W. & Holowsinsky, A. (1961). *J. biophys. biochem. Cytol.* **9**, 19.
- Fernández-Morán, H. (1962). *Circulation*, **26**, 1039.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Fonyo, A. (1967). *Acta biochim. biophys. Acad. sci. hung.* **2** (Suppl.), 24.
- Fonyo, A. (1968). *Abstr. 5th Meet. Fed. Europ. biochem. Soc., Prague*, p. 5.
- Fonyo, A. & Bessman, S. P. (1966). *Biochem. biophys. Res. Commun.* **24**, 61.
- Gamble, J. L. (1965). *J. biol. Chem.* **240**, 2668.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Harris, E. J., Höfer, M. P. & Pressman, B. C. (1967). *Biochemistry*, **6**, 1348.
- Huijing, F. & Slater, E. C. (1961). *J. Biochem., Tokyo*, **49**, 493.
- Hultin, H. O. & Richardson, S. H. (1964). *Arch. Biochem. Biophys.* **105**, 288.
- Kagawa, Y. & Racker, E. (1966). *J. biol. Chem.* **241**, 2467.
- Klingenberg, M. & Pfaff, E. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 180. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Lardy, H. A., Johnson, D. & McMurray, W. C. (1958). *Arch. Biochem. Biophys.* **78**, 587.
- Lehninger, A. L. (1959). *J. biol. Chem.* **234**, 2465.
- Low, H. & Vallin, I. (1963). *Biochim. biophys. Acta*, **69**, 361.
- Minikami, S., Schindler, F. J. & Estabrook, R. W. (1964). *J. biol. Chem.* **239**, 2042.
- Mitchell, P. (1953). *J. gen. Microbiol.* **9**, 273.
- Mitchell, P. (1954). *J. gen. Microbiol.* **11**, 73.
- Mitchell, P. (1961). *Nature, Lond.*, **191**, 144.
- Mitchell, P. (1966). *Biol. Rev.* **41**, 445.
- Nash, T. (1953). *Biochem. J.* **55**, 416.
- Palmieri, F. & Klingenberg, M. (1967). *Europ. J. Biochem.* **1**, 439.
- Pressman, B. C. (1963). *J. biol. Chem.* **238**, 401.
- Pressman, B. C. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1076.

- Racker, E., Tyler, D. D., Conover, T. E., Estabrook, R. W., Parsons, D. F. & Chance, B. (1965). In *Oxidases and Related Oxidation-Reduction Systems*, vol. 2, p. 1077. Ed. by King, T. E., Mason, H. S. & Morrison, M. New York: John Wiley and Sons Inc.
- Schatz, G. & Racker, E. (1966). *J. biol. Chem.* **241**, 1429.
- Schnaitman, C. A., Erwin, V. G. & Greenawalt, J. W. (1967). *J. Cell Biol.* **32**, 719.
- Schnaitman, C. A. & Pedersen, P. L. (1968). *Biochem. biophys. Res. Commun.* **30**, 428.
- Schneider, W. C. (1953). *J. Histochem. Cytochem.* **1**, 212.
- Share, L. (1960). *Biochim. biophys. Acta*, **38**, 154.
- Sone, N. & Hagihara, B. (1964). *J. Biochem., Tokyo*, **56**, 151.
- Tyler, D. D. (1968). *Biochem. J.* **107**, 121.
- Tyler, D. D., Butow, R. A., Gonze, J. & Estabrook, R. W. (1965). *Biochem. biophys. Res. Commun.* **19**, 551.
- Tyler, D. D., Estabrook, R. W. & Sanadi, D. R. (1966). *Arch. Biochem. Biophys.* **114**, 239.
- Tyler, D. D. & Gonze, J. (1967). In *Methods in Enzymology*, vol. 10, p. 75. Ed. by Estabrook, R. W. & Pullman, M. E. New York: Academic Press Inc.
- Van Buskirk, J. J. & Frisell, W. R. (1967). *Biochim. biophys. Acta*, **143**, 292.
- Wilson, D. F. & Chance, B. (1966). *Biochem. biophys. Res. Commun.* **23**, 751.
- Winkler, H. H., Bygrave, F. L. & Lehninger, A. L. (1968). *J. biol. Chem.* **243**, 20.
- Woodward, D. O. & Munkres, K. D. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 872.