The Inhibition of Oxidative Phosphorylation

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Loomis & Lipmann (1948) showed that low concentrations of 2:4-dinitrophenol (DNP) reversibly uncouple the phosphorylation associated with the oxidation of glutamate. This supported the hypothesis that agents such as DNP, which prevent the use of the energy provided by respiration and glycolysis, do so by inhibiting the formation of high-energy phosphate bonds (Lardy & Elvehjem, 1945; McElroy, 1947). The acceleration of respiration and glycolysis of intact cells by low concentrations of DNP (see Peiss & Field, 1948, for references) might thus be explained in terms of an increased availability of orthophosphate and of adeninenucleotide acceptors which normally limit the rate of carbohydrate breakdown (cf. Meyerhof & Geliazkowa, 1947). Johnson (1941) has suggested that the Pasteur effect is a consequence of the greater efficiency of aerobic phosphorylation compared with that associated with glycolysis, a view which is consistent with the inhibition of the Pasteur effect by DNP (Dodds & Greville, 1934). Lynen (1941) has indeed shown that respiration reduces the amount of orthophosphate available for yeast fermentation.

Some interest is therefore attached to the action of other compounds known to accelerate respiration and/or aerobic glycolysis on oxidative phosphorylation. For example, phenosafranine in suitable concentration will increase the aerobic glycolysis of rat-brain and tumour slices without affecting the respiration. Dyestuffs such as thionine accelerate the respiration of many tissues without influencing the aerobic glycolysis (Dickens, 1936*a*, *b*), while 4:6-dinitro-*o*-cresol increases both respiration and aerobic glycolysis (Dickens, 1939). We have, therefore, investigated the action of a number of known inhibitors of the Pasteur effect, as well as some metabolites of DNP, on the coupling between oxidation and phosphorylation. Two hormones, thyroxine and insulin, which we thought might possibly influence oxidative phosphorylation, have also been tested.

The enzyme systems that we have used in this investigation were those contained in cyclophorase preparations and in mitochondria of rat and rabbit liver and kidney. Hogeboom, Schneider & Pallade (1948) and Kennedy & Lehninger (1948, 1949) have shown that most of the enzymes of the tricarboxylic acid cycle, and also those catalysing fatty acid oxidation, are localized in the mitochondria. We have examined the possibility that the activity of crude preparations of tissue particles such as the 'cyclophorase system' (Green, Loomis & Auerbach, 1948) resides in the mitochondria.

METHODS

Enzyme preparations. Washed kidney and liver particles ('cyclophorase') were prepared by the method of Green *et al.* (1948). Purer preparations of the active particles were obtained by isolating the mitochondria according to the methods described by Schneider (1948). These methods were applied with success to rabbit and rat liver and kidney. The necessity for neutralization during homogenization (Green *et al.* 1948) is not apparent when the procedure is carried out in sucrose. All preparations were carried out in a cold room at 2°, a Servall SS1-*a* high-speed centrifuge being used for the mitochondrial isolations.

Hexokinase was prepared from baker's yeast by two methods. A crude preparation was obtained by a single ethanol precipitation (van Heyningen, 1942) and stored as a dry powder *in vacuo* at 2°. For use, 100 mg. of the powder was rubbed up with 10 ml. ice-cold water and centrifuged; 0·2 ml. of the supernatant was used per flask. A purer preparation of the enzyme was obtained by the method of Berger, Slein, Colowick & Cori (1946) as described by Cross, Covo, Taggart & Green (1949); the preparation was stored as a frozen solution at −10°, and retained its activity for many months. The conditions for autolysis of baker's yeast described by Berger *et al.* (1946) were found to be unsatisfactory and those described by Bailey & Webb (1948) were used instead.

Estimations. Inorganic orthophosphate was determined either by the method of Fiske & Subbarow (1925) or that of Lowry & Lopez (1946). Glucose was determined by the method of Nelson (1944). O_2 uptakes were determined manometrically, using Warburg flasks equipped with one side arm; the gas phase was air.

For the estimation of the P/O ratio (atoms of inorganic orthophosphate P disappearing/atoms O taken up from free O_2), a fortified medium was used containing the following: 0·001 M-adenosine-5-phosphate (AMP); 0·0067 M-$MgSO_4$; 0·02 M-potassium orthophosphate, pH 7·4; 0·01 M-substrate; 0·026 M-fructose and 0·2 ml. hexokinase solution.

The flasks were kept ice cold and the enzyme preparation was added immediately it was prepared. NaF was then added to give a final concentration of 0·013 M. The final volume in the centre compartments of the flasks was 3·0 ml. When a 'cyclophorase' preparation was used, this was

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added as 1·0 ml. of suspension in 0·9% (w/v) KCl. Where mitochondria were used, they were added to the flasks suspended in 0·25M-sucrose (0·5 ml., corresponding to 250–500 mg. fresh tissue), and in this case cytochrome *c* and KCl were added to the flasks to give final concentrations of 0·00001 and 0·025M respectively.

Trichloroacetic acid (0·5 ml. of 87·5%, w/v) was placed in the side arm of each flask. Immediately all components had been added to the flasks they were placed in the bath at 25 or 38° and allowed to equilibrate for 5 or 10 min. Four flasks were used for each determination. Two were deproteinized at the end of the equilibration period by tipping in the trichloroacetic acid, while the O_2 uptake was determined in the second pair for given periods (usually about 10 min.). These were then deproteinized. The contents of all flasks were made to 5·0 ml. with water, the precipitates centrifuged off and the supernatants were then analysed for inorganic ortho-P. In addition, samples were frequently analysed for the presence of acid-labile P (hydrolysis for 10 min. at 100° in N-HCl) and more specifically for inorganic pyro-P by the method of Cohn & Kolthoff (1942), which was modified so that pyro-P could be determined spectrophotometrically after hydrolysis. All results quoted are the means of duplicate determinations. Hexokinase activity was determined in small centrifuge tubes, in the presence of veronal-acetate buffer, pH 7·4, under the conditions stated by Bailey & Webb (1948), by estimation of glucose consumption or of the change in easily hydrolysable P.

Adenosinetriphosphatase (ATP-ase) and inorganic pyrophosphatase were determined in small tubes under the conditions summarized in Table 2.

Reagents. All solutions were made up in glass-distilled water, and neutralized to pH 7 if necessary by the use of KOH or HCl. Adenosinetriphosphate (ATP) was obtained as the dibarium salt from Boots Pure Drug Co. Ltd. Since some preparations contained inhibitory substances they were purified by reprecipitation as the Hg and Ba salts by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). The purified product yielded the theoretical amount of orthophosphate after hydrolysis in N-HCl at 100° for 10 min. After incubation with a four-times precipitated myosin preparation (Bailey, 1942), 48% of the theoretical pyrophosphate-P appeared as ortho-P. Adenosine-5-phosphate was obtained from Light & Co. Ltd. Cozymase was prepared by the method of Williamson & Green (1940). Purity determined spectrophotometrically after enzymic reduction was approx. 50%. Octanoic acid was purified by redistillation *in vacuo*. 'Hexose monophosphate' (an equilibrium mixture of glucose-6-phosphate and fructose-6-phosphate) was prepared by the method of Dubois & Potter (1943). Aneurin pyrophosphate was obtained from Roche Products Ltd. Cytochrome *c* was prepared by the method of Keilin & Hartree (1937). Most of the dyestuffs were obtained from G. Gurr Ltd. We are indebted to Dr H. McIlwain for a sample of ethyl red presented to him by Dr F. Hamer; to Dr H. King, F.R.S., for a specimen of 1:11-diamidinoundecane and to Dr J. M. Barnes for specimens of nitrophenols. Thyroglobulin was prepared from sheep thyroid glands by the method of Rossiter (1940). L-Thyroxine was a gift from Sir Charles Harington, F.R.S. 4:4′-Di(*p*-chlorophenyl)-1:1:1-trichloroethane (DDT) was suspended by the method described by Judah (1949). In all cases where effectors were dissolved in solvents other than water, the appropriate controls were run. Veronal-acetate buffer was prepared by the method of Michaelis (1931).

RESULTS

Initial experiments were carried out by the method of Potter (1945, 1947), in which water homogenates are used as a source of enzyme. Though oxygen uptakes were in good agreement with those reported by Potter, no phosphate uptake was ever observed. The experimental procedure described by Potter was rigidly adhered to.

Goranson & Erulkar (1949) have recently reported experiments with this type of preparation in which phosphorylation was obtained. Their value of 0·3 for the P/O ratio with succinate as substrate is unacceptably low. In view of the fact that homogenization in water destroys the mitochondria (Hogeboom *et al.* 1948) these results are not surprising.

Preliminary experiments with 'cyclophorase' and mitochondria on the conditions necessary for obtaining optimum values for the P/O ratio are in substantial agreement with those of Cross *et al.* (1949), and need not be discussed further. Table 1 shows the values for the P/O ratio obtained with different substrates and preparations at 25 and 38°. One finding justifies comment. It will be seen that with octanoate as substrate a clear-cut phosphorylation was observed, the phosphate uptake being many times the value expected from the oxidation of succinate added to prime the reaction. The latter experiments were conducted in the absence of fluoride which completely abolishes fatty acid oxidation at the concentrations used and which decreases glutamate oxidation by some 50% at 25°. It can be seen that in the absence of fluoride the P/O ratio with glutamate as substrate is markedly reduced. In many of these experiments, analyses for inorganic pyro-P were carried out. In no case was an accumulation of this compound observed. On a few occasions, inorganic pyro-P (10 μmol.) was added to flasks, and, upon estimation at the end of the experiment, no change in concentration was found. These observations clearly do not rule out the possibility that the compound was undergoing exchange with inorganic ortho-P in the suspension medium.

Since the results obtained with 'cyclophorase' could be duplicated with mitochondria, it was reasonable to suppose that the latter were the active agents in the 'cyclophorase' system. This was confirmed by resuspending the centrifuged particles in 0·88M-sucrose and subjecting them to differential centrifugation. The ability to carry out oxidative phosphorylation was recovered quantitatively in the mitochondrial fraction, the cytological characteristics of which were confirmed by phase-contrast microscopy and differential staining.

Some authors have corrected the observed P/O ratio for loss of esterified phosphorus by ATP-ase

Table 1. P/O *ratios with different substrates and preparations*

(P/O ratio = atoms of inorganic orthophosphate P disappearing/atoms O taken up from free O_2. The systems used are described in the text. 30 μmol. of substrate added in each case, except for succinate (100 μmol.) and octanoate (6·0 μmol.)

Substrate	Enzyme preparation	Temp. (°)	O_2 uptake (μg. atoms)	Inorganic ortho-phosphate (μg. atoms)	P/O ratio	Duration of experiment (min.)
Glutamate	Rabbit kidney 'cyclophorase'	25	8·85	15·9	1·8	10
Glutamate	Rabbit kidney 'cyclophorase'	25	4·53	11·3	2·5	5
Glutamate	Rat liver 'cyclophorase'	25	6·15	10·7	1·75	6
Glutamate	Rat liver 'cyclophorase'	25	5·35	13·5	2·52	7
Glutamate	Rat liver mitochondria	38	8·2	17·2	2·1	20
Glutamate	Rat liver mitochondria	38	21	22·2	1·05*	10
Glutamate	Rat liver mitochondria (Same preparation)	38	16·8	30·4	1·81	10
Succinate	Rabbit kidney 'cyclophorase'	38	33·2	29·4	0·88	8
β-Hydroxybutyrate	Rabbit kidney 'cyclophorase'	38	7·4	8·05	1·09	30
β-Hydroxybutyrate	Rabbit kidney mitochondria	38	15·1	15·1	1·0	43
β-Hydroxybutyrate	Rat liver mitochondria	38	17·8	24·0	1·35	8
Octanoate + 1 μmol. succinate	Rat liver mitochondria	38	44·5	25·0	0·56*	20
Pyruvate	Rat liver mitochondria	25	7·0	16·8	2·4	10

* No NaF added.

Table 2. *ATP-ase and inorganic pyrophosphatase of particles*

(The enzyme suspension (0·5 ml.) was added to a medium consisting of 0·017 M-veronal-HCl buffer, pH 7·4; 0·0067 M-$MgSO_4$; ATP (equivalent to 140 μg. easily hydrolysable P), or $Na_4P_2O_7$ (equivalent to 120 μg. pyro-P) and NaF as indicated. Final volume 1·2 ml. Duration of experiment 10 min.)

Enzyme preparation	Temp. (°)	Substrate	Hydrolysis (%) +0·02 M-NaF	Hydrolysis (%) No NaF
Rat liver 'cyclophorase'	25	ATP	44	99·5
Rat liver 'cyclophorase'	25	$Na_4P_2O_7$	8·0	86·0
Rabbit kidney 'cyclophorase'	25	ATP	33·0	65·0

action in the enzyme preparation. Ochoa (1943) has published evidence that the ATP-ase activity of heart homogenates is not completely inhibited by 0·02 M-fluoride. Ogston & Smithies (1948) have objected to the use of such corrected P/O ratios and have suggested that the sample of ATP used by Ochoa was heavily contaminated with inorganic pyrophosphate. It was further suggested by these authors that ATP-ase was completely inhibited, while the inorganic pyrophosphatase was unaffected by the concentrations of fluoride used.

We have investigated the influence of 0·02 M-sodium fluoride on the ATP-ase and inorganic pyrophosphatase activity of 'cyclophorase' isolated from liver and kidney. The ATP used was shown by enzymic dephosphorylation with four-times precipitated myosin to be practically free of inorganic pyrophosphate. Table 2 summarizes the results of these experiments, which demonstrate that the ATP-ase of liver and kidney is partially inhibited by 0·02 M-fluoride, while the inorganic pyrophosphatase is more powerfully inhibited.

Other experiments showed that the dephosphorylation of hexose mono- and di-phosphate was not marked in cyclophorase, and that 0·02 M-fluoride depressed this appreciably.

The P/O ratios recorded in this paper are not corrected for phosphatase action.

Inhibitors of oxidative phosphorylation

Unless otherwise stated, all experiments on the depression of oxidative phosphorylation were carried out at 25° with glutamate as substrate. Initial experiments confirmed the observation of Loomis & Lipmann (1948) that low concentrations of DNP powerfully inhibit the rate of phosphorus uptake while leaving respiration practically unaltered. Typical experiments are recorded in Table 3, which also shows that a similar effect was obtained with succinate and β-hydroxybutyrate as substrates, though in the case of these some inhibition of respiration was observed. A number of related nitrophenols were tested in this system. Table 4 shows that whereas 4-amino-2-nitrophenol and 2-amino-4-nitrophenol, known metabolites of 2:4-dinitrophenol (Guerbet & Meyer, 1932) did not affect the P/O ratio at the concentrations tested, 2:4-dinitro-1-naphthol-7-sulphonic acid and picramic acid were inhibitory, but not to such an extent as DNP.

Table 5 summarizes the results of experiments on the influence of various Pasteur inhibitors and related compounds on the P/O ratio. A number of

Table 3. *Depression of* P/O *ratio by DNP*

(Systems as described in text and Table 1.)

Substrate	Enzyme preparation	Concn. of DNP $\times 10^{-4}$ M	Temp. (°)	O_2 uptake (μg. atoms)	Inorganic ortho-phosphate uptake (μg. atoms)	P/O ratio
Glutamate	Rabbit kidney 'cyclophorase'	Nil	25	4·53	11·3	2·5
		1·0		4·55	0·0	—
Glutamate	Rabbit kidney 'cyclophorase'	Nil	25	3·54	6·15	1·74
		0·1		3·84	1·55	0·405
Succinate	Rabbit kidney 'cyclophorase'	Nil	38	33·2	29·4	0·88
		0·5		24·1	1·1	0·045
β-Hydroxybutyrate	Rabbit kidney 'cyclophorase'	Nil	38	7·4	8·05	1·09
		1·0		4·15	0·0	—
β-Hydroxybutyrate	Rat liver mitochondria	Nil	38	17·8	24·0	1·35
		1·0		13·0	0·0	—

Table 4. *Action of certain nitrophenols on* P/O *ratio*

(Rabbit kidney 'cyclophorase' used in every case, with glutamate as substrate.)

Compound	Concn. $\times 10^{-4}$ M	Percentage increase (+) or decrease (−) of O_2 uptake	Decrease of P uptake (%)	Depression of P/O ratio (%)
Picramic acid	1·0	−4	100	100
Picramic acid	2·0	−2	86	85·5
2:4-Dinitro-1-naphthol-7-sulphonic acid	1·0	+5·8	25	29·0
2:4-Dinitro-1-naphthol-7-sulphonic acid	2·0	−8·0	50	45·0
4-Amino-2-nitrophenol	2·0	−4	0·0	0·0
2-Amino-4-nitrophenol	2·0	+5·0	0·0	4·0
2:5-Dinitrophenol	1·0	−6·0	6·0	0·0

dyestuffs were found to be powerful inhibitors of phosphorus esterification at concentrations at which oxygen uptake was unaffected, namely, thionine, methylene blue, brilliant cresyl blue, pyocyanine, janus green B and phenosafranine. With the exception of the last, the results were reproducible. Using 'cyclophorase', concentrations of phenosafranine varying from 1 to 3×10^{-5} M inhibited the phosphate uptake of some preparations, while leaving that of others practically unaffected. Experiments with higher concentrations of the dyestuff were complicated by inhibitions of the oxygen uptake (50% inhibition at 10^{-4} M-phenosafranine with glutamate as substrate). McIlwain (1949) has shown that the breakdown of cozymase by rat-brain preparations is powerfully inhibited by low concentrations of phenosafranine. Although the enzyme system responsible for the breakdown of cozymase by rat brain is apparently quite different from that of rabbit kidney (McIlwain & Rodnight, 1949; Kornberg & Lindberg, 1948), it was considered that phenosafranine action might be related to the breakdown of cozymase by the 'cyclophorase'. The influence of the dye on the P/O ratio of a system, in which cozymase was added as a source of adenine nucleotide, was therefore investigated. It was found that 3×10^{-5} M-phenosafranine did not affect the replacement of adenosine-5-phosphate by cozymase and inhibited the P/O ratio to the same extent (40%) in the presence of either nucleotide. A possible reason for the inconsistent results with 'cyclophorase' may be that phenosafranine is strongly adsorbed by the preparations. More consistent inhibitions of the P/O ratio were obtained with purified mitochondrial preparations (Table 5), and this might be expected on the basis that 'cyclophorase' contains much debris which could effectively remove the dye from solution.

Of the other substances tested, ethyl red, guanidine, methyl guanidine, dimethyl guanidine, ethyl urethane and DDT affected neither respiration nor phosphate uptake in the concentrations used, 1:11-diamidinoundecane inhibited phosphate uptake at concentrations which did not depress the respiration. It is evident from the data included in Table 5 that the depression of the P/O ratio is not a simple function of the oxidation-reduction potential of the dyestuffs. Table 6 shows that neither thyroxine nor thyroglobulin when added *in vitro* affected the respiration or phosphate uptake. Similarly, insulin was devoid of influence on the P/O ratio, and did not affect the inhibition by DNP.

Hexokinase inhibition. It is evident that a lowering of the P/O ratio in the systems used in this in-

Table 5. *Depression of* P/O *ratio by Pasteur effect inhibitors and related compounds*

(Substrate glutamate throughout.)

Compound	Concn. $\times 10^{-4}$ M	Increase (+) or decrease (−) in O_2 uptake (%)	Decrease (−) or increase (+) in P uptake (%)	Depression of P/O ratio (%)	Oxidation/reduction potential E'_0 at pH 7	Enzyme preparation
Ethyl red	0·1	+20	0·0	− 12	?	Rabbit kidney 'cyclophorase'
	0·2	0·0	− 9·0	− 9·0	—	Rabbit kidney 'cyclophorase'
Phenosafranine	0·1	+10	− 90·0	− 89·0	−0·252	Rabbit kidney 'cyclophorase'
	0·1	− 2·0	− 45·0	− 43·0	—	Rabbit kidney 'cyclophorase'
	0·3	0·0	− 6·0	− 6·0	—	Rabbit kidney 'cyclophorase'
	0·3	− 3·0	− 42·0	− 40·0	—	Rabbit kidney 'cyclophorase'
	0·3	−14·0	− 42·5	− 36·0	—	Rat liver mitochondria
	0·3	0·0	− 40·0	− 40·0	—	Rat liver mitochondria
	0·3	0·0	− 43·0	− 43·0	—	Rat liver mitochondria
Janus green	0·2	0·0	−100·0	−100·0	−0·256	Rabbit kidney 'cyclophorase'
	0·2	0·0	− 60·0	− 60·0	—	Rabbit kidney 'cyclophorase'
	0·2	−33·0	− 79·0	− 68·5	—	Rat liver mitochondria
	0·2	−29·0	− 72·0	− 61·0	—	Rat liver mitochondria
Guanidine-HCl	10·0	+18	+ 57·5	+ 30	—	Rabbit kidney 'cyclophorase'
	20·0	+44	+ 5	− 29	—	Rabbit kidney 'cyclophorase'
Methyl guanidine	9·1	− 3	− 30	− 27	—	Rabbit kidney 'cyclophorase'
	45	− 6	+ 35	+ 11	—	Rabbit kidney 'cyclophorase'
Dimethyl guanidine	8·0	− 3	− 19	− 13·5	—	Rabbit kidney 'cyclophorase'
	16	+12	− 10	− 20	—	Rabbit kidney 'cyclophorase'
Pyocyanine	4	+15	− 77	− 80	−0·034	Rabbit kidney 'cyclophorase'
	4	+ 3·8	− 78	− 79	—	Rabbit kidney 'cyclophorase'
	4	+ 2·0	− 46	− 44	—	Rabbit kidney 'cyclophorase'
1:11-Diamidinoundecane	1·0	+12·0	− 25·0	− 34·0	—	Rabbit kidney 'cyclophorase'
	1·0	−23·0	− 44·0	− 30·0	—	Rabbit kidney 'cyclophorase'
Methylene blue	0·2	0·0	− 11·0	− 11·0	0·011	Rabbit kidney 'cyclophorase'
	0·4	+13·0	− 31·0	− 39·0	—	Rabbit kidney 'cyclophorase'
Thionine	0·2	+17·0	− 50·0	− 58·0	0·062	Rabbit kidney 'cyclophorase'
	0·2	+ 9·7	− 54·0	− 58·0	—	Rabbit kidney 'cyclophorase'
	0·2	+10·8	− 44·0	− 48·0	—	Rabbit kidney 'cyclophorase'
	0·2	+ 5·0	− 61·5	− 63·0	—	Rabbit kidney 'cyclophorase'
Brilliant cresyl blue	0·5	+18·5	− 40·0	− 50·0	0·045	Rabbit kidney 'cyclophorase'
	0·4	− 2·0	− 40·0	− 41·0	—	Rabbit kidney 'cyclophorase'
Neutral red	1·4	+14·6	− 16·0	− 19·0	−0·340	Rabbit kidney 'cyclophorase'
Toluylene blue	1·4	+14·4	− 16·0	− 17·0	0·115	Rabbit kidney 'cyclophorase'

vestigation could be the result of an inhibition of the yeast hexokinase which is added, rather than a true uncoupling of phosphorylation from oxidation, or it might be a combination of both. All substances found to be inhibitors of the P/O ratio were therefore tested for their effect on the hexokinase preparation used. In no case was any significant inhibition observed.

Inhibition of fatty acid oxidation. Cross *et al.* (1949) have shown that low concentrations of DNP were powerful inhibitors of fatty acid oxidation by 'cyclophorase', and suggested that the inhibition was due to the lack of high energy phosphate bonds required to prime and maintain the reaction rather than an inhibition of the fatty acid oxidases. Thus it seemed likely that inhibitors of oxidative phosphorylation would also inhibit the oxidation of fatty acids. The substances were tested in a system with acetate as substrate and glutamate as primer, and the results of the experiments are summarized in Table 7. The inhibitory activity towards acetate oxidation was generally of the same order as the depression of the P/O ratio. The oxidation of acetate by 'cyclophorase' was found to be sensitive to the presence of fluoride, but it was not completely abolished as was octanoate oxidation. It can be seen from Table 7 that in the presence of 0·0067 M-fluoride, the inhibition of acetate oxidation by DNP is reduced some 40%.

In several experiments the inhibitors were tipped in from the side arms of the flasks after measuring the oxygen uptake for 20 min. (after which time the oxidation of the primer was nearly complete). It was found that whereas DNP and other nitrophenols

Table 6. *Action of insulin, L-thyroxine and thyroglobulin on* P/O *ratio*

('Cyclophorase' used in both experiments.)

System	P/O ratio	Percentage depression of P/O ratio
Control	1·8	—
+Insulin*	1·66	7·5
$+10^{-4}$M-DNP	0·18	90·0
$+10^{-4}$M-DNP+insulin	0·116	96·0
Control	1·46	—
+L-Thyroxine $2·3 \times 10^{-4}$M	1·32	10·0
+Thyroglobulin 3·9 mg./ml.	1·30	11·0

* Insulin (Burroughs-Wellcome Ltd.) was added as follows: 8·5 mg. was dissolved in 0·75 ml. 0·003M-HCl and made up to 15·0 ml. with 0·1M-phosphate buffer (pH 7·4). 0·15 ml. of this solution was added where indicated and the appropriate controls were run.

Mr J. H. Ottaway of the Department of Biochemistry, University College, London, has kindly informed us that this specimen of insulin was effective in enhancing the glucose uptake and glycogen synthesis of isolated rat diaphragms. The control system was identical with that described in the text and Table 1.

found to be true only in the cases where fluoride had been added to the reaction mixture. It can be seen from the results in Table 8 that in the absence of fluoride, 10^{-4}M-DNP did not stimulate the respiration of phosphate-deficient systems to the same extent as inorganic ortho-P, the absence of fluoride permitting a much more rapid respiration in the control flasks. The oxidation of glutamate in the absence as in the presence of fluoride was unaffected by DNP if phosphate were present in the suspension medium.

Many of the compounds tested as depressors of the P/O ratio were also examined for their ability to stimulate the respiration of phosphate-deficient systems. The results of the experiments are summarized in Table 8. It will be seen that picramic acid, methylene blue, brilliant cresyl blue and arsenate produced substantial acceleration of respiration, while phenosafranine, janus green, pyocyanine and 2:4-dinitro-1-naphthol-7-sulphonic acid had much less effect. Ethyl red was found to have no action on glutamate oxidation in phosphate-deficient systems.

Table 7. *Inhibition of acetate oxidation by* P/O *inhibitors*

(Rabbit kidney 'cyclophorase' used throughout. 30 μmol. of acetate as substrate, primed with 1 μmol. of glutamate. O_2 uptakes corrected for oxidation of the latter. In addition, the flasks contained 0·001M-AMP, 0·0067M-$MgSO_4$, 0·02M-phosphate, pH 7·4 and inhibitors as indicated.)

Compound	Concentration	O_2 uptake (μl.)	Inhibition of O_2 uptake (%)	Depression of P/O ratio (%)
Nil	—	283	—	—
Ethyl red	2×10^{-5}	262	8·0	<20
1:11-Diamidinoundecane	10^{-4}	156	45·0	35
Picramic acid	2×10^{-4}	0·0	100·0	90·0
2:4-Dinitro-1-naphthol-7-sulphonic acid	2×10^{-4}	104	63·0	45·0
Nil	—	215	—	—
Phenosafranine	3×10^{-5}	124	43·0	40·0
Nil	—	300	—	—
DNP	10^{-4}	0·0	100·0	98·0
Nil	—	248	—	—
Thionine	2×10^{-5}	190	40·0	60·0
Nil+NaF, 0·0067M	—	250	—	—
DNP+NaF, 0·0067M	10^{-4}	139	56·0	—

cause an immediate inhibition of acetate oxidation, the inhibition by phenosafranine and janus green is small in the first 5 min., and increases rapidly with time.

Stimulation of the oxygen uptake of phosphate deficient systems

The oxidation of glutamate by either 'cyclophorase' or mitochondria was found to be much lower in systems to which inorganic ortho-P had not been added, the rate being some 35 % of the maximal. In confirmation of the observation of Loomis & Lipmann (1948), it was found that DNP stimulated the respiration of phosphate deficient systems to about the same extent as ortho-P itself. This was

The oxidation of succinate, however, is but slightly affected by the omission of phosphate from the medium, the rate being 70 % of the maximal. From Table 8 it can be seen that a small enhancement of the oxidation of succinate by phosphate deficient systems was observed in the presence of DNP. The interpretation of such experiments is complicated by the fact that DNP often caused inhibition of succinate oxidation. The oxidation of acetate was also found to be dependent on the presence of inorganic ortho-P. Table 8 shows that when this component was omitted from the suspension medium, acetate oxidation was practically abolished and neither DNP nor arsenate stimulated the oxidation.

Table 8. *Stimulation of O_2 uptake in P-deficient systems by P/O inhibitors*

(Rabbit kidney 'cyclophorase' used throughout; except where indicated, the systems were as described in Table 7. Where P was omitted, KCl was added to the same final molar concentration.)

Compound	NaF	Substrate	O_2 uptake (μl.)		Acceleration in absence of P by compound (%)
			P present	No P	
Nil	Nil	Acetate	162·0	1·0	—
DNP, 10^{-4}M	Nil	Acetate	0·0	0·0	—
Arsenate, 0·01M	Nil	Acetate	0·0	0·0	—
Nil	0·013M	Glutamate	255·0	80·5	—
DNP, 10^{-4}M	0·013M	Glutamate	258·0	228·0	184
Nil	0·013M	Glutamate	197·0	59·0	—
Nil	Nil	Glutamate	296·0	118·0	—
DNP, 10^{-4}M	0·013M	Glutamate	197·0	195·0	203
DNP, 10^{-4}M	Nil	Glutamate	295·0	210·0	79
Nil	0·013M	Glutamate	180·0	65·0	—
Picramic acid, 2×10^{-4}M	0·013M	Glutamate	—	167·0	157
2:4-Dinitro-1-naphthol-7-sulphonic acid, 2×10^{-4}M	0·013M	Glutamate	—	110·0	70
Phenosafranine, 3×10^{-5}M	0·013M	Glutamate	—	80·0	23
Janus green, 2×10^{-5}M	0·013M	Glutamate	—	78·0	20
Ethyl red, 2×10^{-5}M	0·013M	Glutamate	—	65·0	0·0
Pyocyanine, 4×10^{-4}M	0·013M	Glutamate	—	91·0	40·0
Arsenate, 10^{-2}M	0·013M	Glutamate	—	181·0	179·0
Nil	0·013M	Glutamate	90·0	30·0	—
Methylene blue, 8×10^{-5}M	0·013M	Glutamate	—	81·0	170·0
Brilliant cresyl blue, 8×10^{-5}M	0·013M	Glutamate	—	77·0	156·0
Nil	0·013M	Succinate	237·0	168·0	—
DNP, 2×10^{-5}M	0·013M	Succinate	218·0	205·0	22·0

Stimulation of systems deficient in adenine nucleotide

The oxidation of glutamate by 'cyclophorase' or mitochondria to which inorganic orthophosphate but not a source of adenine nucleotide had been added was depressed to about the same extent as that of phosphate-deficient systems. It was found that DNP will accelerate the oxidation of glutamate in such systems, although the 'replacement' of adenine nucleotide is never complete. Fluoride affected the oxidation of glutamate under such conditions in the same manner as in phosphate-deficient systems, and the same was true of the enhancement of their respiration by DNP. The experiments summarized in Table 9 show that brilliant cresyl blue and methylene blue also increased the respiration of adenine nucleotide-deficient systems. Phenosafranine, however, did not affect glutamate oxidation under these conditions.

If inorganic phosphate and adenine nucleotide are together omitted from the reaction mixture, the stimulation of glutamate oxidation by DNP is maximal.

The procedures normally used for the isolation of either 'cyclophorase' or mitochondria yield suspensions which, despite repeated washing, contain appreciable amounts of adenine nucleotide and substances which appear as inorganic ortho-P either by the method of Fiske & Subbarow (1925) or by that of Lowry & Lopez (1946). The following technique yielded a suspension apparently free of adenine nucleotide.

Table 9. *Stimulation of O_2 uptake in AMP-deficient systems*

(Rabbit kidney 'cyclophorase' used throughout The 'complete system' was identical with that described in Table 7, except that glutamate (30 μmol.) was substrate.)

Components	O_2 uptake (μl.)	Acceleration by compound in absence of AMP (%)
Complete system	255·0	—
No AMP	99·0	—
No AMP + 0·0005M-cozymase	183·0	85·0
No AMP + 10^{-4}M-DNP	179·0	81·0
No AMP, no P + 10^{-4}M-DNP	254·0	157·0
Complete system	197·0	—
No NaF	296·0	—
No AMP	79·0	—
No AMP, no NaF	103·0	—
No AMP + 10^{-4}M-DNP	131·0	66·0
No AMP, no NaF + 10^{-4}M-DNP	128·0	24·0
Complete system	213·0	—
No AMP	82·0	—
No AMP + 8×10^{-5}M-methylene blue	155·0	89·0
No AMP + 8×10^{-5}M-brilliant cresyl blue	138·0	69·0

Two rat livers (15 g.) were homogenized in a Waring blender in 0·25M-sucrose at 0°. Nuclei and other debris were removed by centrifugation at 600 *g*

and the mitochondria were sedimented at 9000 *g* (15 min.). The particles were suspended in 0·9% (w/v) potassium chloride, containing 0·02M-veronal-hydrochloric acid buffer (pH 7·7). They were subjected to four washings with this medium. The pellets resulting from the final centrifugation were then resuspended in 0·25M-sucrose and sedimented at 9000 *g*, and then finally suspended in 15 ml. 0·25M-sucrose. The suspension still contained material appearing as inorganic ortho-P (equivalent to 20 μg./ml.) by the method of Lowry & Lopez (1946). The suspension (0·5 ml.) was added to Warburg flasks and supplemented as usual in the case of the controls.

Table 10 shows that when adenosine-5-phosphate was omitted, the oxygen uptake was only 10% that of the complete system and the addition of 10^{-4}M-DNP failed to stimulate the respiration. In a phosphate-deficient system with added adenine nucleotide, however, the 'replacement' was maximal. Thus it appears that the 'replacement' of adenine nucleotide by DNP is dependent on the presence of a certain minimal amount of the former.

Table 10. *Failure of DNP to stimulate AMP-deficient systems*

(See text for description of method. Complete system as in Table 7.)

	O_2 uptake (μl.)/30 min.		
Compound added	Complete system	No P	No AMP
Nil	300	137	26
10^{-4}M-DNP	—	282	33

An attempt to remove all inorganic ortho-P was also made. The technique was similar to that used above, but 0·25M-sucrose containing 0·02M-veronal buffer (pH 7·7) was substituted for the potassium chloride solution. The particle preparation was indeed found to be free of ortho-P, but upon incubation in the Warburg flasks, the respiration fell off rapidly after 10 min. It was found on analysis that a considerable amount of ortho-P had been liberated from the mitochondria (approx. 100 μg. P), which had obviously been severely injured in some way.

DISCUSSION

It is impossible to over-emphasize the complexity of response of intact cells to the *in vitro* addition of the agents we have tested. The action of some of the latter varies from tissue to tissue and from species to species. The ionic composition of the suspension medium and the substrate employed also influence the results. Thus guanidine is an inhibitor of the Pasteur effect in brain slices, but does not have this effect in a number of other tissues (Dickens, 1939), while pyocyanine inhibits the aerobic glycolysis of a number of tumours (Friedheim, 1934; Dickens, 1936*a*), but accelerates that of brain slices (Young, 1937). Again, the aerobic glycolysis of rat kidney is unaffected by 6×10^{-5}M-thionine in the presence of glucose, but is considerably increased when lactate is present. The latter substance itself evokes a considerable aerobic glycolysis (Dickens, 1936*a*).

Seits & Engelhardt (1949), from a study of DNP, azide, arsenate, ethyl carbylamine and sodium nitrite on respiration, glycolysis and phosphorylation in yeasts, erythrocytes and tumours, have concluded that agents abolishing the Pasteur effect are, in general, inhibitors of respiratory phosphorylation. Many of the compounds listed in this paper as inhibitors of oxidative phosphorylation are indeed Pasteur-effect inhibitors in certain tissues, but it seems clear that, in view of the findings with intact cells, no comparison should be drawn between their activities in a cell-free system and on tissue slices. In addition, the question of tissue and species specificity remains an obstacle to generalization.

The action of the dyestuffs methylene blue, brilliant cresyl blue and phenosafranine merits further attention. The first two accelerate the respiration of slices, and it is often assumed that they do so by acting as supplementary electron carriers in respiratory systems (Harrop & Barron, 1928). The possibility must now be raised that their action is due to a depression of aerobic phosphorylation in the cells concerned.

With regard to phenosafranine, McIlwain (1949) has suggested that its action may be associated with its ability to inhibit the enzymic degradation of cozymase which was described by him. It should be pointed out that at least two mechanisms of cozymase breakdown exist (Kornberg & Lindberg, 1948; Kornberg & Pricer, 1950), and it is not yet known whether Kornberg's nucleotide pyrophosphatase is inhibited by phenosafranine. Furthermore, it is not certain that either of these degradative enzymes will act on coenzyme tightly bound to particles as they will when the substance is free in solution.

Our finding that the *in vitro* addition of thyroxine and thyroglobulin does not affect the P/O ratio strongly suggests that the thyroid hormone does not accelerate metabolic processes by interfering with oxidative phosphorylation in a manner similar to DNP.

We were unable to show any effect of insulin on oxidative phosphorylation. Recently, Polis, Polis, Kerrigan & Jedeikin (1949) have reported that insulin often increases the P uptake coupled with α-ketoglutarate oxidation by 'cyclophorase'. In order to demonstrate this effect they either employed systems with suboptimal amounts of adenosine-5-phosphate or added a magnesium-activated ATP-ase. In either case, the P/O ratios they obtained were far from maximal. We have not attempted to corro-

borate these experiments. It is of some interest that insulin did not reverse the inhibitory action of DNP.

In considering the apparent 'replacement' of inorganic ortho-P by DNP and other agents in systems which are deficient in ortho-P, the problem of the physical state of the particles must arise. Slater (1949) has demonstrated that the heart and kidney succinoxidase preparations used by him are sensitive to the phosphate concentration of the medium. He has found that substances such as denatured globin will accelerate the oxygen uptake of systems which are deficient in inorganic ortho-P, and suggests that phosphate has an effect on the structure of the particles composing the enzyme system. It seems impossible to rule out the operation of such factors in experiments with mitochondria or 'cyclophorase' suspended in similar media. Slater's results serve to emphasize the caution required in the explanation of results obtained from such complex systems as have been considered.

Our demonstration of the stimulation of respiration in the adenine nucleotide-deficient system by DNP, on the other hand, is not easily explained in the same way. The fact that DNP cannot enhance the respiration of adenine nucleotide-deficient systems except in the presence of a certain small amount of the latter is of interest. It might imply that adenosine-5-phosphate is required for the action of the respiratory enzymes as such, or that DNP does not completely circumvent transphosphorylation between adenine nucleotide and a hypothetical primary ester.

SUMMARY

1. The enzymes catalysing the esterification of inorganic phosphate coincident with the oxidation of glutamate have been found to be localized in the mitochondrial fraction of liver and kidney. Phosphate esterification coupled with fatty acid oxidation has been demonstrated in preparations of rat-liver mitochondria.

2. The depression of oxidative phosphorylation by dinitrophenol (DNP) has been confirmed using a number of substrates. Two metabolic products of DNP were found to be without influence on oxidative phosphorylation, which was markedly inhibited, however, by low concentrations of a number of dyestuffs and also by 1:11-diamidinoundecane.

3. The degree of stimulation by DNP of the oxidation of glutamate by kidney particles deficient in inorganic phosphate and/or adenine nucleotide has been found to be strongly dependent on the presence of fluoride in the suspension medium. In general, substances which depress oxidative phosphorylation also 'replace' inorganic phosphate and/or adenine nucleotide in this system.

4. Acetate oxidation catalysed by kidney particles is inhibited by substances which disrupt the linkage between oxidation and phosphorylation. The inhibition of acetate oxidation by DNP is considerably diminished in the presence of fluoride.

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The Use of Organic Solvents at Low Temperature for the Separation of Enzymes. Application to Aqueous Rabbit Muscle Extract

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Enzymes may be separated and purified by a number of methods, such as fractional adsorption and precipitation. The most widely used method has been that of salting out the proteins with neutral salts (cf. review of the literature by Cohn, Strong, Hughes, Mulford, Ashworth, Melin & Taylor, 1946). This method is safe, but the salting-out coefficient depends on rather unspecific characteristics, such as the shape and size of the protein molecule.

Organic solvents have not been applied so generally for the separation of enzymes, since they denature proteins at room temperature. During the war, Cohn, Leutscher, Oncley, Armstrong & Davis (1940) and Cohn *et al.* (1946) extensively developed the ethanol precipitation method of Mellanby (1908) and Hardy & Gardiner (1910). By working at low temperatures and low ionic strengths numerous protein fractions were isolated from blood plasma. Ethanol fractionation has various advantages over the salting-out method as pointed out by Edsall (1947). At low ionic strengths, the interaction between proteins and salts depends on specific properties of protein molecules, such as the distribution of electric charges. Small variations in dielectric constant and ionic strength will produce large and specific changes in the solubilities of proteins.

The present investigation was undertaken to study the separation of enzymes in tissue extracts from water-organic solvent mixtures. Enzyme tests provide a simpler and more specific measure of purity than the usual physical methods (such as solubility measurements and electrophoresis) which were applied by Cohn *et al.* (1946). Simple activity determinations will show where a protein is separating out and how much denaturation (as measured by loss of biological activity) results from the fractionation.

Aqueous extract of rabbit muscle was chosen as source of enzymes. No systematic protein fractionation of rabbit-muscle extract has been reported, although much is known about its enzyme systems and a number of studies have appeared recently, e.g. salting-out curves (Distèche, 1948), electrophoretic patterns (Jacob, 1947, 1948; Dubuisson, 1950), and ultracentrifugal studies (Amberson, Erdös, Chinn & Ludes, 1949). Most of the glycolysis enzymes are part of the myogen complex with albumin-like properties and a very high solubility in water-salt mixtures. For these enzymes separation by organic solvents, with a lowered dielectric constant, should be useful.

METHODS

Preparation of rabbit-muscle extract

Minced rabbit muscle was extracted at 0° with an equal volume of distilled water for 20 min. with gentle stirring. After centrifuging for 15 min., the supernatant was dialysed first against cold running water for 3 hr., and then against 20 times its volume of distilled water at 4° for 6 hr. The conductivity then equalled that of about 0·007 M-KCl. The pH was adjusted to the required value with 0·05 M-acetate buffer (pH 4), or $NaHCO_3$. This extract will be referred to as 'original extract' in the text.

Fractionation with organic solvents, at 0° and below

The insoluble precipitate formed on dialysis (ppt. *D*, containing some myosin, globulin *X* and denatured proteins) was removed by centrifugation. Fractionation of the supernatant was carried out in a metal vessel to permit rapid heat