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Barbiturates and Oxidative Phosphorylation

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It has been claimed that barbiturates uncouple oxidative phosphorylations (Brody & Bain, 1954; Brody, 1955). These authors have demonstrated that during the oxidation of pyruvate by liver and brain mitochondria, phosphate uptake was lowered proportionately more than oxygen uptake. Support for the uncoupling theory was derived from certain similarities between the barbiturates and 2:4-dinitrophenol (Brody & Bain, 1954). 'The slopes of the inhibition curves of these compounds are remarkably similar although dinitrophenol is the more potent agent. Both depress fatty acid oxidation and stimulate oxidative rate in a phosphate-deficient system. The addition of excess magnesium ion does not reverse the uncoupling action of either the barbiturate or dinitrophenol' (Brody, 1955). Further, barbiturate hypnosis is potentiated by 2:4-dinitrophenol (Killam, Brody & Bain, 1958). None of these arguments is conclusive evidence for uncoupling, and they ignore the major difference that, *in vitro*, barbiturates inhibit (Bain, 1952) whereas 2:4-dinitrophenol stimulates respiration (Loomis & Lipmann, 1948; Parker, 1958). This difference is readily demonstrated *in vivo*, for after administration of barbiturates oxygen consumption (Costa & Bonnycastle, 1955) and body temperature (Birnie & Grayson, 1952; Lessin & Parkes, 1957) fall, whereas 2:4-dinitrophenol causes a rise of both oxygen consumption (Cameron, 1958) and body temperature (Stoner, 1956). Other differences in behaviour in isolated systems have also been demonstrated (Johnson & Quastel, 1953; Jalling, Löw, Ernster & Lindberg, 1957; Messer, 1958). Although the supporting evidence for the uncoupling theory is not conclusive, Brody & Bain (1954) have nevertheless demonstrated a lowering of the phosphorylation quotient (P/O ratio).

Respiration, with pyruvate as substrate, of the liver mitochondria used in this paper can be inhibited 50% without any decrease of the P/O ratio (Aldridge, 1957). With such preparations we have re-examined the effect of oxy- and thio-barbiturates upon oxidative phosphorylation. Moreover, by dissociating the process of oxidation from that of oxidative phosphorylation by the intervention of 2:4-dinitrophenol (Loomis & Lipmann, 1948; Parker, 1958), we have studied the effects of barbiturates upon these processes separately. A preliminary report of these results has already appeared (Aldridge & Parker, 1958).

METHODS

Manometric experiments. The reasons for the composition and pH of the medium have been discussed by Aldridge (1957, 1958). For measurements of respiration each flask contained 3 ml. of a solution containing adenosine 5-phosphate (1·15 mM), adenosine triphosphate (ATP; 1·06 mM), KCl (10 mM), $MgCl_2$ (14 mM), ethylenediaminetetra-acetic acid (EDTA; 1·0 mM), potassium phosphate (50 mM), sucrose (30 mM) and substrates (10 mM, except fumarate mM). For measurements of oxidative phosphorylation, glucose (60 mM), glycylglycine (16·7 mM) and hexokinase (200–400 units) were added to the above-mentioned mixture. In each case the medium was adjusted to pH 6·7–6·8 with KOH. For either type of experiment 0·3 ml. of a suspension of mitochondria in 0·3 M-sucrose, equivalent to 150 mg. wet wt. of liver, was used. For experiments on oxidative phosphorylation, when a range of concentrations of barbiturate was examined (method 1), P uptake was measured between 10 and 22 min. after placing the flasks in the bath at 37°. Uptake of O_2 for the same period was calculated from the slope of the best straight line through manometer readings at 10 min., 13 min. 20 sec., 16 min. 40 sec. and 20 min. For more accurate determinations of P/O ratio six flasks were used for each determination (method 2). Manometer readings were taken at 10, 14, 18, 22 and 26 min. and the reaction was stopped by the addition of 6·5 ml. of ice-cold 5% (w/w) perchloric acid at 7, 11, 15, 19, 23 and 27 min. Thus P uptake was calculated from the slope of the regression line through six values. Owing to the successive removal of flasks for the determination of inorganic phosphate, the slope of the regression line for O_2 uptake was calculated from four readings at 10 min., four at 14 min., three at 18 min., two at 22 min. and one at 26 min. (a total of 14 readings). The standard errors of these slopes were calculated (Snedecor, 1946) and thus the phosphorylation quotient (P/O ratio) and its standard error could be determined (Langer, 1951). Since these P/O ratios have standard errors less than 10% of the ratio, the difference between the control and experimental values has been tested for significance (*t* test) on the basis of their being derived from a large sample (Snedecor, 1946).

Preparation of the mitochondria. Mitochondria were prepared as previously described (Aldridge, 1957, 1958), with a Potter–Elvehjem-type homogenizer with a smooth glass tube and Perspex pestle, with a total clearance of 0·02 in. for rat liver and 0·01 in. for rat brain.

Adenosine-triphosphatase activity. Each beaker contained 3 ml. of a solution containing ATP (3 mM), KCl (10 mM),

$MgCl_2$ (14 mM), EDTA (mM), and sucrose (30 mM). The beakers were shaken in air at 37°. After temperature equilibration mitochondria were added and the mixture was incubated for 10 min. The reaction was stopped by the addition of 0·5 ml. of ice-cold 65% (w/w) perchloric acid and inorganic phosphate was determined.

Special chemicals and reagents. The following chemicals were obtained from the sources indicated: adenosine 5-phosphate, glycylglycine, sodium pyruvate (Roche Products Ltd.); disodium salt of ATP (Sigma Chemical Co., St Louis, Mo., U.S.A.); glucose, sodium fumarate, 2:4-dinitrophenol (DNP; British Drug Houses Ltd.). Phenylarsenious acid was synthesized and used as described by Aldridge (1958). The following barbiturates have also been used: sodium 5-ethyl-5-phenyl barbiturate (Phenobarbital: British Drug Houses Ltd.); 5-(1-*cyclo*hexenyl)-1:5-dimethylbarbituric acid (Hexobarbital: May and Baker Ltd.); sodium 5-ethyl-5-*iso*amylbarbiturate (Amytal: Eli Lilly and Co. Ltd.); sodium 5-ethyl-5-(1-methylbutyl)-2-thiobarbiturate (Thiopental: Abbott Laboratories Ltd.); sodium 5-allyl-5-(2-*cyclo*hexenyl)-thiobarbiturate (Kemithal: Imperial Chemical Industries Ltd.); sodium 5-allyl-5-*iso*butylthiobarbiturate (Baytenal: Farbenfabriken Bayer, A.G., Wuppertal-Elberfeld, Germany).

Hexokinase was prepared from baker's yeast by a modification by V. H. Parker (unpublished work) of the method of Berger, Slein, Colowick & Cori (1946). The preparation was taken to the equivalent of step 3a and, when assayed by their procedure at 37° (instead of 30°), had an activity of 3500 units/ml. Potato apyrase was prepared by the method of Lee & Eiler (1951). This preparation liberated 1100 μg.atoms of inorganic phosphate/hr./ml.

Analytical methods. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925). Protein was measured by the biuret method of Robinson & Hogden (1940) as modified by Aldridge (1957), and has been expressed as mg. of albumin.

Purification of barbiturates. The sodium barbiturates as supplied were dissolved in water, converted into the free acids by the addition of HCl and the precipitated free acids were washed repeatedly with water. After drying they were recrystallized as follows: Phenobarbital from water–ethanol, m.p. 176° (uncorrected); Hexobarbital as supplied, m.p. 146·5–147·5°; Amytal from benzene, m.p. 156·5°; Thiopental from water–dimethylformamide, m.p. 161–162°; Baytenal from benzene, m.p. 147–148°. Kemithal was recrystallized from water–ethanol. It exists in two forms, one melting at 124° and the other at 140°; the former is converted into the latter on prolonged heating (*Brit. Vet. Codex*, p. 394, 1953). All barbiturates were prepared as stock solutions of the free acids in dimethylformamide and 0·03 ml. of various concentrations was added to the flasks, a constant final concentration of 1% (v/v) of dimethylformamide always being present.

Units. The oxidative activity of the mitochondria (q_{O_2}) is expressed as μl. of O_2/mg. of protein/hr. and ATPase activity as μg.atom of P liberated/mg. of protein/hr. The inhibitory power of a substance is given as its pI_{50} value, the negative logarithm of the molar concentration which will produce 50% inhibition. Where errors are given these are expressed as mean ± S.E. with the number of determinations upon separate preparations of mitochondria in parentheses.

RESULTS

Oxidative phosphorylation by liver mitochondria. A comparison of the inhibition of O_2 and P uptake produced by the oxybarbiturates Amytal, Hexobarbital and Phenobarbital is illustrated in Fig. 1.

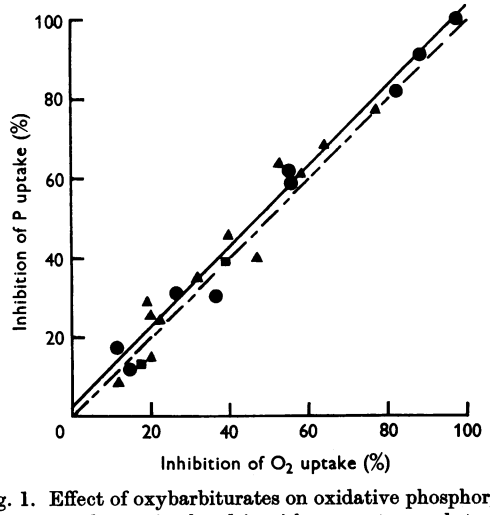

Fig. 1. Effect of oxybarbiturates on oxidative phosphorylation by rat-liver mitochondria with pyruvate as substrate. All values were obtained by method 1. The broken line shows where points should be if the P/O ratio is unchanged and the continuous line the calculated best straight line. ●, Amytal (0·06–0·5 mM); ▲, Hexobarbital (0·06–1·0 mM); ■, Phenobarbital (0·5–1·0 mM). The mean q_{O_2} was 113·2±4·6 (6) and the P/O ratio 2·67±0·08 (6).

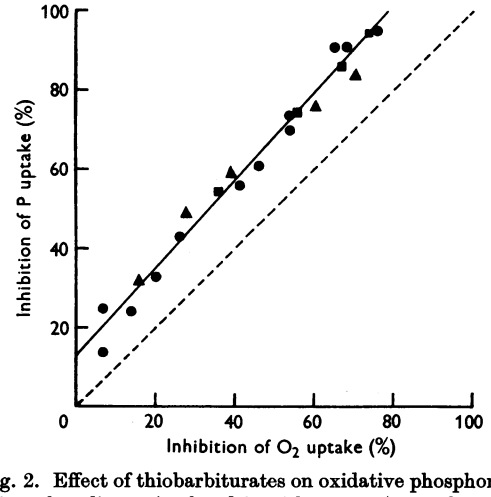

Fig. 2. Effect of thiobarbiturates on oxidative phosphorylation of rat-liver mitochondria with pyruvate as substrate. All values were obtained by method 1. The broken line shows where points should be if the P/O ratio is unchanged and the continuous line the calculated best straight line. ●, Thiopental (0·06–1·0 mM); ▲, Baytenal (0·2–1·0 mM); ■, Kemithal (0·1–0·4 mM). The mean q_{O_2} was 107·8±3·4 (5) and the P/O ratio 2·56±0·11 (5). When O_2 uptake is 50% inhibited, P uptake is inhibited by 68–69%.

Table 1. *Effect of barbiturates on pyruvate oxidation and coupled phosphorylation of liver mitochondria*

For details of technique used see method 2, described under Methods. The control and experimental values were obtained by parallel experiments upon the same preparation of mitochondria. Results are expressed as mean ± S.E. Mean q_{O_2} for controls is 90·5 ± 1·2 (12). pC = − log concn. (M).

Compound	Concn. (pC)	Inhibition of O_2 uptake (%)	Oxidative phosphorylation (P/O ratio) Control	Experimental	Difference	t
Amytal	3·70	44	2·71 ± 0·15	2·37 ± 0·11	0·34 ± 0·18	1·90 (P 0·06)
	3·70	47	2·58 ± 0·09	2·37 ± 0·10	0·21 ± 0·13	1·55 (P 0·13)
	3·91	25	2·59 ± 0·09	2·38 ± 0·05	0·21 ± 0·10	2·04 (P 0·05)
Hexobarbital	3·27	40	2·79 ± 0·11	2·66 ± 0·10	0·13 ± 0·15	0·87 (P 0·4)
Phenobarbital	3·00	58	2·53 ± 0·10	2·74 ± 0·24	0·21 ± 0·26	0·81 (P 0·41)
Thiopental	3·89	38	2·63 ± 0·11	1·57 ± 0·08	1·06 ± 0·13	8·18 ($P < 0·01$)
	3·89	27	2·98 ± 0·11	2·42 ± 0·18	0·56 ± 0·21	2·66 (P 0·013)
	3·70	32	2·63 ± 0·07	1·65 ± 0·15	0·98 ± 0·17	5·77 ($P < 0·01$)
Baytenal	3·00	65	2·48 ± 0·07	1·23 ± 0·10	1·25 ± 0·12	10·4 ($P < 0·01$)
	3·30	54	2·71 ± 0·05	1·53 ± 0·12	1·18 ± 0·13	9·1 ($P < 0·01$)
Kemithal	3·60	48	2·58 ± 0·09	1·66 ± 0·18	0·92 ± 0·20	4·6 ($P < 0·01$)
	4·00	18	2·70 ± 0·12	2·24 ± 0·15	0·46 ± 0·19	2·4 (P 0·02)

Table 2. *Influence of barbiturates on adenosine-triphosphatase activity, swelling and uptake of oxygen of unstimulated mitochondria*

Values for ATPase are all corrected for the activity of the mitochondria in the presence of solvent (1%, v/v, dimethylformamide), which was 0·73 ± 0·07 (6) μg.atom of P/mg. of protein/hr. In the presence of 0·03 mM-DNP their activity was 11·9 ± 0·13 (6). Swelling was measured by measurements of E at 530 mμ in a Unicam DG. spectrophotometer, the mitochondria being suspended and incubated at 37° for 10 min. under conditions identical with those used for the ATPase assay. Uptake of O_2 was measured in the presence of pyruvate and fumarate. Mean q_{O_2} was 22·4 ± 0·28 (6) and, in the presence of 0·03 mM-DNP, 82·9 ± 1·6 (6), giving a stimulation of 3·7-fold. pC = − log concn. (M).

Compound	Concn. (pC)	Activation of ATPase (μg.atoms of P/mg. of protein/hr.)	Swelling E	Uptake of O_2 increase (+) or decrease (−) (%)
Amytal	3·65	0·97	+0·01	−25
Phenobarbital	3·0	0·77	−0·01	−6
Hexobarbital	3·1	0·36	+0·02	−13
Thiopental	3·7	3·47	+0·01	+25
Kemithal	3·55	3·36	+0·01	+39
Baytenal	3·40	3·02	+0·01	+39

The broken line shows where the points will fall with an unchanged P/O ratio. There is clearly little indication that the inhibition of P uptake is any greater than that of O_2 uptake. In contrast, the thiobarbiturates Thiopental, Kemithal and Baytenal impaired P uptake more than O_2 uptake, so giving a lower P/O ratio (Fig. 2). As pointed out in the Methods section these results were obtained with a technique when the P/O ratio for each concentration of barbiturate is dependent upon measurements of O_2 uptake in one Warburg flask only. By using more flasks for the assessment of the rates of O_2 and P uptake, the standard error of each P/O ratio can be obtained. In Table 1 are shown the results of such experiments with six flasks for each concentration of barbiturate. For Hexobarbital and Phenobarbital the difference between the P/O ratios when O_2 uptake is 40–60% inhibited was not significant; the significance for Amytal was P approx. 0·1. For thiobarbiturates there was a significant difference between the P/O ratios. Even with quite a small lowering of O_2 uptake (cf. Kemithal 0·1 mM) there was a marked lowering of the P/O ratio.

Adenosine triphosphatase of liver mitochondria. DNP, as well as uncoupling oxidative phosphorylation, induces ATPase activity in liver mitochondria (Lardy & Wellman, 1953). It was therefore of interest to see whether the barbiturates, and particularly the thiobarbiturates, also share this property. At concentrations which inhibit O_2 uptake about 50%, Amytal, Phenobarbital and Hexobarbital gave an increase of ATPase activity of less than 1 μg.atom of P/mg. of protein/hr. (Table 2). Under similar conditions the thiobarbiturates induce an activity of more than

3 μg.atoms of P/mg. of protein/hr. This activation of ATPase was not associated with swelling of the mitochondria (Table 2). Indeed, concentrations four times as high as than those given in Table 2 did not produce measurable changes in the extinction of the mitochondrial suspension. The stimulation of ATPase activity by the thiobarbiturates was associated with stimulation of O_2 uptake (Table 2, Figs. 3 and 4) with pyruvate as substrate. No stimulation of O_2 uptake was found at any concentration of the oxybarbiturates (Table 2).

Since the ATPase activity elicited by DNP may be regarded as accounting for its uncoupling action (see Discussion), a comparison has been made between the action of the thiobarbiturates and of DNP in lowering the P/O ratio, by choosing concentrations of the different uncoupling agents that liberated equal activities of ATPase. When O_2 uptake is 50% inhibited by thiobarbiturates, P uptake is 69% inhibited: an increase of 19% over that required for the maintenance of a constant P/O ratio (Fig. 2). The concentration of thiobarbiturates inhibiting O_2 uptake during the measurements of oxidative phosphorylation was in the range pC 3·4–3·7 (Tables 1, 3). The mean ATPase liberated by these concentrations was 3·28 μg.atoms of P/mg. of protein/hr. (Table 2). The relationship between the concentration of DNP and the activity of ATPase which it induces was determined under identical experimental conditions. It was found that 4 μM-DNP induced the same ATPase activity as did the thiobarbiturates and inhibited P uptake by 25·2 ± 1·8% and O_2 uptake by 4·0 ± 1·6% (mean ± S.E. of three determinations upon the same preparation of mitochondria). The difference between the percentage inhibition of P and O_2 uptakes is 21·2, in agreement with 19·0 obtained with the thiobarbiturates. It was concluded that the relation between uncoupling action and ATPase production was the same for the thiobarbiturates as for DNP.

Adenosine-triphosphatase activity and uptake of oxygen induced by dinitrophenol. Uptake of O_2 in the presence of DNP reflects the activity of the non-phosphorylating respiratory chain and ATPase activity induced by DNP is a measure of the activity of the energy-transfer mechanism when it is dissociated from the respiratory chain; hence the use of DNP enables separate study of these two parts of the complete mechanism (see Discussion). A comparison has therefore been made of the action of the various barbiturates on O_2 uptake in the presence of DNP and the action of DNP in inducing ATPase activity. The results in Table 3 show that O_2 uptake in the presence of DNP was inhibited by concentrations of oxybarbiturates similar to those which inhibit O_2 uptake in the presence of hexokinase and glucose. The mitochondria were consistently rather more sensitive to barbiturates when in the presence of hexokinase and glucose than with DNP though in no case was the difference more than twofold. In contrast, ATPase activity induced by DNP is not inhibited by the similar concentrations of oxybarbiturates and even concentrations four times as high have no measurable effect (Table 3).

Experiments similar to the above have been carried out with the thiobarbiturates, but are more difficult to interpret. The results obtained with Kemithal illustrate the difficulties (Figs. 3, 4). Kemithal certainly inhibits O_2 uptake in the presence of DNP but the precise concentration at which a 50% inhibition occurs is difficult to assess. Taking the whole O_2 uptake in the presence of DNP, 50% inhibition is obtained at pC 3·30 (Fig. 3). If a correction is made for the stimulation of O_2 uptake by thiobarbiturates, 50% inhibition is at pC 3·55. Kemithal stimulates ATPase (Fig. 4) and this increases with concentration. Although concentrations of Kemithal which produce 50% inhibition of O_2 uptake (pI_{50} 3·60, cf. Table 3) give only a small lowering of DNP-activated ATPase, nevertheless, unlike the behaviour of the oxybarbiturates, a pronounced lowering occurs with higher concentrations. The interpretation of this effect depends on whether or not a correction should be made for ATPase activated by the barbiturates themselves (cf. Fig. 4).

Table 3. *Effect of barbiturates on uptake of oxygen and activity of adenosine triphosphatase of liver mitochondria in the presence of dinitrophenol*

Uptake of O_2 was measured with pyruvate as substrate. The mean q_{O_2} was 22·4 ± 0·28 (6) and, in the presence of 0·03 mM-DNP, 82·9 ± 1·6 (6). The values for pI_{50} in the presence of hexokinase and glucose are the ranges obtained from the experiments given in Table 1 and Figs. 1 and 2. ATPase activity in the absence of DNP and barbiturate but in the presence of solvent (1%, v/v, dimethylformamide) was 0·73 ± 0·07 (6) μg.atom of P/mg. of protein/hr., and, in the presence of 0·03 mM-DNP, 11·9 ± 0·13 (6). For the inhibition of ATPase in the presence of DNP concentrations of oxybarbiturates four times as high as those in column 2 were used.

Compound	Inhibition of O_2 uptake: In the presence of hexokinase and glucose (pI_{50})	Inhibition of O_2 uptake: In the presence of DNP (pI_{50})	Inhibition of ATPase in the presence of DNP (%)
Amytal	3·65–3·70	3·4	0
Phenobarbital	2·9–3·1	2·6	0
Hexobarbital	3·05–3·25	2·8	0
Thiopental	3·55–3·80	—	—
Kemithal	3·60–3·75	—	—
Baytenal	3·25–3·30	—	—

Succinate oxidation. It has previously been demonstrated that succinate oxidation is unaffected by barbiturates (Quastel & Wheatley, 1932–33) and more recently that phosphorylation associated with the oxidation of succinate is likewise not inhibited by Amytal (Eiler & McEwen, 1949; Löw, Ernster & Lindberg, 1955). In the presence of Amytal one atom of oxygen is consumed for every molecule of succinate oxidized (Fig. 5), indicating that other oxidations of the Krebs tricarboxylic acid cycle are suppressed. We have confirmed that phosphorylation with succinate as substrate is not affected by Amytal. Kemithal behaves in the same way with succinate oxidation, the oxygen consumed being one atom for every molecule of succinate. However, in agreement with the results obtained with pyruvate as substrate (Fig. 3), Kemithal also stimulates rate of O_2 uptake in the presence of succinate.

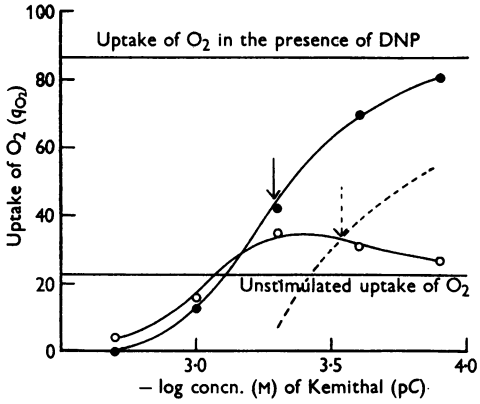

Fig. 3. Effect of Kemithal on unstimulated O_2 uptake and on O_2 uptake stimulated by 2:4-dinitrophenol. Liver mitochondria were used with pyruvate as substrate: ●, in the presence of 30 μM-DNP; ○, in the absence of DNP. The broken line is the difference between these curves in an attempt to correct for the stimulation of O_2 uptake by Kemithal. The arrows indicate the concentration where O_2 uptake is 50 % inhibited: a broken arrow for the uncorrected and continuous arrow for the corrected curves.

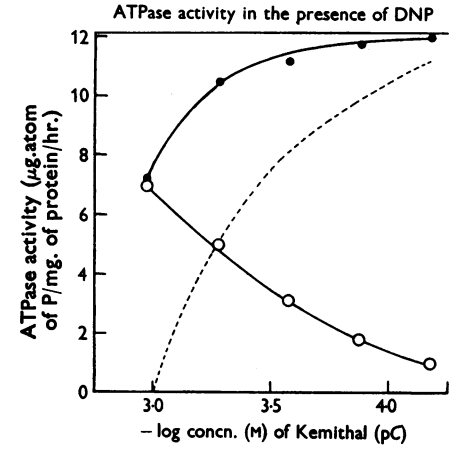

Fig. 4. Effect of Kemithal on ATPase with and without DNP. The ATPase activity in the absence of DNP and Kemithal but in the presence of solvent (1 %, v/v, dimethylformamide) was 0·63 μg.atom of P/mg. of protein/hr. This value has been subtracted from all results. ●, In the presence of 0·03 mM-DNP; ○, in the absence of DNP. The broken line is the difference between these curves.

Brain mitochondria. In view of the effects of barbiturates *in vivo* it would have been desirable to examine their influence upon the activities of brain mitochondria. However, unlike liver mitochondria, the respiration of our preparations of brain mitochondria declines during the course of the experiment (Aldridge, 1957). This limits the work one can do on oxidative phosphorylation. In addition, and presumably associated with their instability, phenylarsenious acid which is generally believed to inhibit α-oxo acid oxidases (Peters, 1955), inhibits P uptake of brain mitochondria more than O_2 uptake. A comparison of the effects of phenylarsenious acid with the barbiturates is shown in Fig. 6. These results indicate that for a given inhibition of O_2 uptake (less than 60 %) Amytal, Phenobarbital and Hexobarbital do not inhibit P uptake more than does phenylarsenious acid. In

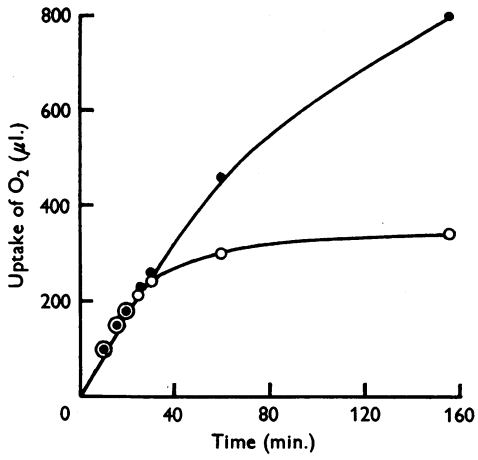

Fig. 5. Effect of Amytal on O_2 uptake with succinate as substrate. Potato apyrase (110 μg.atoms of P/hr.) was added to each flask to produce maximal O_2 uptake. Mitochondria (4·1 mg. of protein) equivalent to 150 mg. wet wt. of liver were added to each flask. The O_2 uptake during the 10 min. equilibration period was assumed to be at the same rate as during the following 5 min. period. Each contained 30 μmoles of succinate and, on the basis of 1 μg.atom of oxygen for each μmole of succinate, complete oxidation would require 336 μl. of O_2. ●, Control without Amytal; ○, with Amytal (1·0 mM).

contrast, the sulphur-containing barbiturates produce a much larger depression of P uptake. The interpretation of results upon unstable preparations is necessarily difficult but we consider that these results do not conflict qualitatively with the results obtained with liver mitochondria. The ATPase activity of our brain preparation is high without the addition of DNP; therefore studies of the effects of barbiturates upon DNP-activated ATPase were not carried out.

DISCUSSION

For the purposes of this paper the following definitions have been used. The respiratory chain is regarded as a physiological entity consisting of all the processes involved in the oxidation of substrates and the associated phosphorylation. The electron-transport chain consists of processes involving the transfer of electrons from coenzyme I through the flavin and cytochromes to oxygen. The energy-transferring chains are the processes whereby the energy of oxidation of electron-transport intermediates is used for the synthesis from inorganic phosphate of the terminal pyrophosphate bonds of ATP (cf. Fig. 7). Since phosphorylation is coupled to oxidation, certain processes are common to both the electron-transport and energy-transferring chains.

Recognition that the rate of oxidation by mitochondria is dependent upon the availability of phosphate acceptors (Potter & Recknagel, 1951; Lardy & Wellman, 1952; Siekevitz & Potter, 1953; Chance & Williams, 1955; Aldridge, 1957) has made it clear that, just as formation of ATP requires oxidation, oxidation requires formation of ATP. Hence, inhibition of an enzyme involved in the electron-transport chain or in the energy chain between electron transport and ATP formation will both lead to an inhibition of oxygen uptake (Aldridge, 1958). By measurements of oxygen and phosphorus uptakes, three possible results can be obtained: no inhibition of respiration but a lowering of the P/O ratio, inhibition of respiration with an unchanged P/O ratio and inhibition of respiration with a lowered P/O ratio. An example of the first of these is the uncoupling action of DNP. The second and third possibilities have been obtained with the oxy- and thio-barbiturates respectively. Therefore the oxybarbiturates inhibit respiration but do not uncouple, whereas the thiobarbiturates inhibit respiration but in addition uncouple oxidative phosphorylation. The demonstration of such a clear difference between oxy- and thiobarbiturates has only been made possible by the

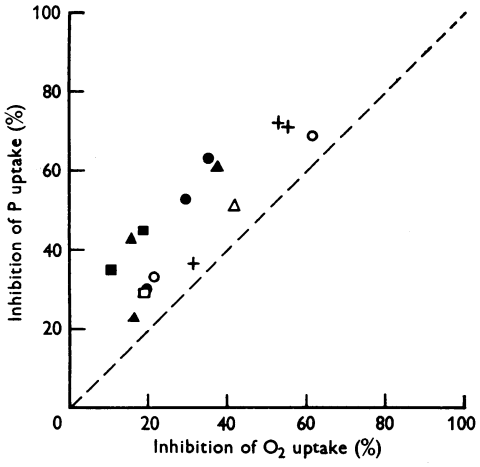

Fig. 6. Effect of barbiturates and phenylarsenious acid on oxidative phosphorylation by rat-brain mitochondria with pyruvate as substrate. The broken line shows where points would be if the P/O ratio were unchanged. All values were obtained by method 2. +, Phenylarsenious acid; ○, Amytal; □, Phenobarbital; △, Hexobarbital; ●, Thiopental; ■, Baytenal; ▲, Kemithal. The mean q_{O_2} was 106·3±6·8 (15) and the P/O ratio 2·26±0·05 (15).

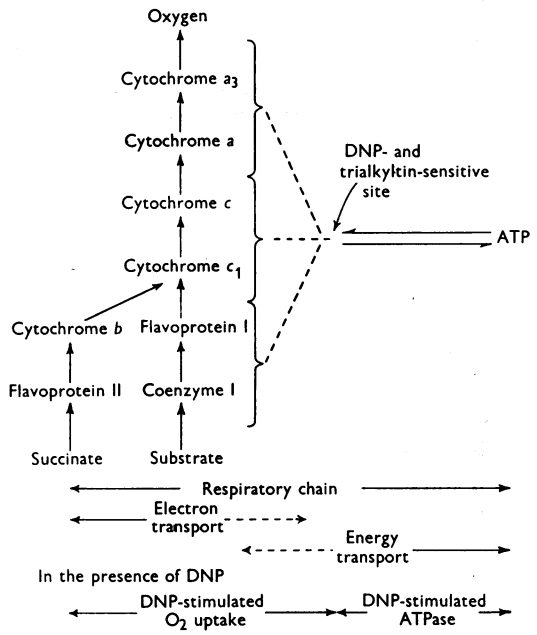

Fig. 7. Diagram illustrating the respiratory chain and the actions of drugs on it. Oxybarbiturates act in the area including flavoprotein I and coenzyme I and up to, but not including, the DNP-sensitive site. The electron-transport chain is taken from Slater (1958). The horizontal arrows in the lower half of the figure represent the boundaries of the processes defined and discussed in the text. The broken arrows indicate overlapping of electron and energy transport.

use of a stable preparation of mitochondria. The work with brain mitochondria illustrates the difficulties arising from the use of an unstable preparation. However, the results given by the oxybarbiturates with brain mitochondria are in general agreement with those with liver mitochondria and provide no positive evidence for uncoupling.

The concentrations of thiobarbiturates which inhibit respiration and lower the P/O ratio also activate some ATPase. An attempt has been made to assess the significance of this ATPase in relation to the uncoupling of oxidative phosphorylation by a comparison with the action of DNP. Before this assessment can be made it is important to know whether the ATPase activity induced by DNP is related to the lowering by DNP of phosphorus uptake during oxidative phosphorylation. The mean uptake of phosphorus for all the control experiments in this paper (Table 1, Figs. 1 and 2) during oxidative phosphorylation is 23·6 μg.atoms/mg. of protein/hr. This uptake is virtually eliminated by 0·03 mM-DNP and this same concentration in the absence of substrate induces an ATPase activity of (11·9–0·7) = 11·2 μg.atoms of phosphorus/mg. of protein/hr. (Table 3). This value is less than the uptake prevented by DNP during oxidative phosphorylation. However, higher values have been obtained (unpublished experiments) when the incubation time was reduced from 10 to 2 min., thus giving some indication of the initial rates of the reaction. These range between 17 and 19 μg.atoms of phosphorus/mg. of protein/hr. Thus there is reasonable agreement between the ATPase activity elicited by DNP and the uptake of phosphorus prevented during oxidative phosphorylation. Although there is agreement this does not indicate that the action of DNP upon oxidative phosphorylation involves the formation of ATP before its breakdown. The action of DNP could be due to the breakdown of a 'high-energy' intermediate in the energy-transferring chain and the ATPase results could be an experimental demonstration of the reaction sequence in reverse (Lardy, 1955). Experiments described in this paper have shown that concentrations of thiobarbiturates and DNP which induce the same ATPase activity cause the same depression of uptake of phosphorus in oxidative-phosphorylation experiments. Therefore on the basis of this comparison with DNP it is concluded that the ATPase activity induced by the thiobarbiturates could account for the observed uncoupling of oxidative phosphorylation. In contrast with DNP, the thiobarbiturates share with the oxybarbiturates the property of inhibiting respiration. However, it is not known if the activation of ATPase and the inhibition of respiration by the thiobarbiturates result from the same primary action. It is possible that the thiobarbiturates have two unrelated mechanisms of action, one inhibiting respiration by a similar mechanism to that of the oxybarbiturates and the other activating ATPase.

We have considered the possibility that the high fat-solubility of the thiobarbiturates (Mark *et al.* 1958) will favour their concentration in the mitochondria, followed by swelling and activation of ATPase. By the use of optical methods for the measurement of swelling (Cleland, 1952; Tedeschi & Harris, 1955), no change in extinction of the mitochondrial suspensions is found to occur after incubation in the medium used for ATPase assay. Although there are conditions when changes in extinction may not be quantitatively proportional to changes in mitochondrial volume (Tedeschi & Harris, 1958) it seems safe to conclude that no change in extinction is indicative of no change in mitochondrial volume. The dissociation constants (pK) of Phenobarbital, Amytal and Hexobarbital are 7·41, 7·94 and 8·4 respectively (Krahl, 1940; Brodie & Hogben, 1957). The increasing order of the pK values reflects a decreasing ionization at the pH of our experiments and therefore an increased tendency to partition into fat (cf. Brodie & Hogben, 1957; Brodie, 1952). However, little ATPase is activated by these oxybarbiturates; therefore fat solubility cannot be an important factor in this activation.

We have found that oxybarbiturates will also depress the oxidation stimulated by DNP but have no effect on ATPase stimulated by this drug. The interpretation of these results depends upon the current views of the respiratory chain, which are for the most part dependent upon a study of substances which influence its processes.

Before we can interpret the effects of barbiturates upon both oxidation and ATPase activity stimulated by DNP, it is necessary to consider the evidence that all the effects of DNP are symptoms of the same primary action. The suggestion (Hunter, 1951) that the ATPase activity in mitochondria induced by DNP is due to a modification of the normal energy-transferring chain which links electron transport and ATP formation has now been accepted by many workers (Potter & Recknagel, 1951; Lardy & Wellman, 1953; Potter, Siekevitz & Simonson, 1953; Myers & Slater, 1957*a*; Bronk & Kielley, 1958). With particles derived from rat-liver mitochondria by treatment with digitonin, Cooper & Lehninger (1957) have concluded that ATPase stimulated by DNP and the ATP–inorganic phosphate-exchange reaction are related to the enzymic mechanism responsible for oxidative phosphorylation. The evidence for this view is based primarily upon the specificity towards nucleotides of adenine of the three reaction systems, their equal sensitivity to DNP and their

similar pH-activity ranges. Studies of a number of nitro- and halo-phenols (Parker, 1958) and trialkyltins (Aldridge, 1958) indicate that both ATPase activity and uptake of oxygen induced in mitochondria by DNP are modifications of the respiratory chain. For each of several phenols ATPase activity and uptake of oxygen are stimulated to the maximum by the concentration which inhibits oxidative phosphorylation (Parker, 1958). These phenols therefore have a single common action. Studies with trialkyltin homologues have shown that, as well as inhibiting oxidative phosphorylation, these compounds depress both the ATPase activity and uptake of oxygen induced by DNP. For each of several trialkyltins, this ATPase activity and uptake of oxygen are both inhibited by that concentration which also inhibits oxidative phosphorylation (Aldridge, 1958). This indicates that the trialkyltins also have a single common action. The simplest hypothesis incorporating all these findings is that trialkyltins and DNP produce their effects by acting in different ways upon the same single site in the energy-transferring chain. The results with trialkyltins therefore reinforce the view that the observed effects of DNP are the result of a single primary action. Therefore studies of ATPase activity and uptake of oxygen stimulated by DNP are studies upon separate parts which between them involve the whole of the respiratory chain. If the effects of drugs upon these parts are to be logically compared and deductions made from them the experimental conditions must be as nearly identical as possible. The only difference in our procedures is the absence of inorganic phosphate in the assay of ATPase.

DNP uncouples oxidative phosphorylation associated with succinate oxidation (Hunter, 1951; Judah & Williams-Ashman, 1951). Phosphorylation associated both with the oxidation of reduced cytochrome *c* (Nielsen & Lehninger, 1955) and with the transfer of electrons from β-hydroxybutyrate to cytochrome *c* (Borgström, Sudduth & Lehninger, 1955) is completely inhibited by 0·1 mM-DNP. Trialkyltin prevents oxidation with succinate as a substrate (Aldridge, 1958). Further experiments (unpublished) have shown that tri-*n*-butyltin inhibits the one-step oxidation of succinate which takes place in the presence of Amytal (cf. Fig. 5). The effective concentrations of tri-*n*-butyltin are the same as those which inhibit oxidation in the presence of pyruvate. The simplest hypothesis to explain these observations is that, at the site where DNP and trialkyltins act, there is an intermediate or enzyme common to all three energy-transferring chains. If this is so then it is reasonable to conclude that, from this site at least, the three energy-transferring chains are identical (cf. Fig. 7).

This view is not in agreement with the opinion of other workers (Myers & Slater, 1957*a*, *b*; Hülsmann & Slater, 1957). Myers & Slater (1957*a*) have measured ATPase activities in liver mitochondria with pH optima of 6·3, 7·4, 8·5 and 9·4. The activities at the first three pH values are stimulated by DNP but only 'pH 6·3 ATPase' is clearly activated by 0·01 mM-DNP. The 'pH 7·4 ATPase and pH 8·5 ATPase' require concentrations of 0·1 and 1·0 mM respectively and the 'pH 8·9 ATPase' is not activated. On the basis of a demonstration of three peaks in the curve relating P/O ratio and pH it has been suggested that the ATPase activities with pH optima at 6·3, 7·4 and 8·5 correspond to the three steps of electron transport coupled to phosphorylation (Hülsmann & Slater, 1957; Myers & Slater, 1957*a*). The three ATPase activities with different pH optima have been confirmed in particles derived from rat-liver mitochondria by treatment with digitonin. However, the peaks in the curve relating oxidative phosphorylation to pH have not been found with these particles with β-hydroxybutyrate as substrate (Cooper & Lehninger, 1956; Purvis, 1959) or with rat-liver mitochondria (Chance, 1959; Chance & Conrad, 1959). It has been pointed out (Chance, 1959) that the magnitude of the optima (Hülsmann & Slater, 1957) is so small that an unusually high accuracy for the determination of the P/O ratio is required. In this work we have been surprised to find how difficult it is to obtain P/O ratios with a low standard error (cf. Table 1, controls; mean P/O 2·66, mean S.E. 0·10). It seems therefore that there is no certain evidence that the energy-transferring chains from the site of action of DNP and trialkyltins are not very similar or identical. The simpler hypothesis that the chains are identical is summarized in Fig. 7.

In the presence of Amytal (Fig. 5) or Kemithal, one atom of oxygen is absorbed for every molecule of succinate oxidized. The phosphorylation associated with this oxidation is not inhibited by Amytal (Löw *et al.* 1955; Eiler & McEwen, 1949). It has been claimed that barbiturates in general uncouple phosphorylation associated with succinate oxidation (Brody, 1955) but the only published experiments are with a thiobarbiturate, Thiopental (Brody & Bain, 1954). This result is to be expected, for Thiopental activates ATPase at the concentrations used (Table 2). In contrast with its effects with pyruvate as substrate Amytal does not inhibit uptakes of oxygen and phosphorus in the presence of succinate. This indicates that this drug acts in the respiratory chain below cytochrome c_1, i.e. between coenzyme I and cytochrome *c* and including flavoprotein (cf. Fig. 7). This conclusion is reminiscent of the earlier conclusions of Michaelis & Quastel (1941) and Grieg (1946) that barbiturates act at the flavoprotein level. Since ATPase stimulated by DNP is not influenced by oxybarbi-

turates, they must therefore act in the energy-transferring chain before the DNP-sensitive site (in the area on Fig. 7 bounded by cytochrome c_1, coenzyme I and DNP-sensitive site). This is the area where, as defined earlier, the electron-transport chain and energy-transferring chain overlap. Although it seems probable that the thiobarbiturates also act at the same site as the oxybarbiturates this conclusion is not at present certain. The activation of ATPase by the thiobarbiturates has made the interpretation of our experiments difficult; more information is needed on the nature of mitochondrial ATPase.

SUMMARY

1. The oxybarbiturates Phenobarbital, Amytal and Hexobarbital inhibit but do not uncouple oxidative phosphorylation of liver mitochondria with pyruvate as substrate.

2. The thiobarbiturates Thiopental, Baytenal and Kemithal inhibit and, to a certain extent, uncouple oxidative phosphorylation. The uncoupling appears to be correlated with their ability to activate mitochondrial adenosine triphosphatase.

3. Results with brain mitochondria are in qualitative agreement with those with liver mitochondria but owing to the instability of the preparation the interpretation of these results is uncertain.

4. The oxybarbiturates inhibited oxygen uptake stimulated by 2:4-dinitrophenol. In contrast adenosine-triphosphatase activity stimulated by 2:4-dinitrophenol was not inhibited. The thiobarbiturates inhibit the oxygen uptake and, to a lesser extent, the adenosine triphosphatase.

5. Neither Amytal nor Kemithal inhibits oxidation in the presence of succinate.

6. The interpretation of these results in the light of current views of the respiratory chain is discussed.

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The Activation of Plasminogen by Staphylokinase: Comparison with Streptokinase

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Plasminogen, the precursor of the proteolytic and fibrinolytic enzyme of blood, is activated by various substances, including the bacterial activators streptokinase (Milstone, 1941) and staphylokinase (Lack, 1948; Gerheim, Ferguson, Travis, Johnston & Boyles, 1948). Some animal plasminogens which are unaffected by streptokinase alone are activated by a mixture of streptokinase and human globulin (Müllertz & Lassen, 1953; Sherry, 1954). Müllertz has postulated the existence in human serum of a proactivator which is converted by streptokinase into an activator of plasminogen (Müllertz & Lassen, 1953; Müllertz, 1955). Staphylokinase activates a wider range of animal plasminogens (Gerheim & Ferguson, 1949) and may therefore not require proactivator. The activation of human and dog plasminogens by staphylokinase has been studied by Lewis & Ferguson (1951), Hayashi & Maekawa (1954) and Celander & Guest (1959).

This paper describes the activation by staphylokinase of human, rabbit and guinea-pig plasminogens. When sufficiently concentrated, staphylokinase acted as rapidly as streptokinase. It did not, however, activate ox plasminogen and it was unaffected by the addition of proactivator.

EXPERIMENTAL

Buffer. Palitzsch's borate buffer, pH 7·4, prepared as described by Norman (1957), was used throughout.

Casein. Light white soluble casein (British Drug Houses Ltd.) was used without further purification.

Human plasminogen. This was purified by the procedure of Kline (1953). This preparation was also used as a source of proactivator.

Euglobulin. Human and animal euglobulins were precipitated from serum as described by Norman (1957). They were resuspended in borate buffer in the original serum volume or, with ox euglobulin, in 0·4 vol.

Streptokinase. Dornokinase (Burroughs Wellcome and Co.) was used.

Activation and assay of plasminogen. This was carried out by the method described by Norman (1957). Perchloric acid was used instead of trichloroacetic acid to precipitate casein (Müllertz, 1955). This modification raised the extinction at 280 mμ due to the products of casein digestion by 50%. Measurements of E were made in the Unicam spectrophotometer model SP. 500. Activity was expressed in terms of a unit which gives, under the conditions of the test, an increase in E at 280 mμ of 1·00/min. of digestion.

Assay of staphylokinase. Several concentrations of the staphylokinase to be assayed were incubated for 30 min. at 37° with 50×10^{-3} unit of human plasminogen, in 1·1 ml. of borate buffer, pH 7·4. A portion (1 ml.) of 4% (w/v) casein was added and incubation continued for 30 min. Casein was then precipitated with 3 ml. of 10% perchloric acid and E at 280 mμ of the digestion products was measured. The staphylokinase activity was determined by interpolation. A unit of staphylokinase was defined as that activity which gives rise, under these conditions, to an increase in E of 0·300. This unit was approximately equal to one-third of a Christensen unit of streptokinase (Christensen, 1949) (Fig. 1). In the experimental results to be described, streptokinase activity also is expressed in these extinction units.

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