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PHOSPHORYLATION COUPLED TO THE OXIDATION OF TETRAMETHYL-*p*-PHENYLENEDIAMINE IN RAT-LIVER MITOCHONDRIA

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SUMMARY

1. The synthesis of ATP coupled to the oxidation of tetramethyl-*p*-phenylenediamine has been studied using rat-liver mitochondria. The system employed catalytic concentrations of tetramethyl-*p*-phenylenediamine kept reduced by an excess of ascorbate.

2. The efficiency of phosphorylation was found to be dependent on the concentration of tetramethyl-*p*-phenylenediamine with highest P:O ratios at low substrate concentrations.

3. P:O ratios, which at low substrate concentrations exceeded one, were diminished by one half by the addition of antimycin but not significantly by arsenite or 2-heptyl-4-hydroxyquinoline-*N*-oxide.

4. It is proposed that there are two phosphorylation sites spanned by the oxidation of low concentrations of tetramethyl-*p*-phenylenediamine and that one is eliminated by antimycin.

INTRODUCTION

Part of the energy derived from the oxidation of cytochrome *c* can be utilized in the esterification of phosphate[1-3]. However, the various technical problems associated with the measurement of ATP synthesis in the cytochrome oxidase region of the respiratory chain greatly retarded the development of understanding of the process. Studies of phosphorylation in the terminal region of the respiratory chain generally rely on the use of an external substrate to reduce mitochondrial cytochrome *c*. For example, *p*-phenylenediamine was shown by KEILIN AND HARTREE[4] to react with the cytochrome *c* of the respiratory chain. Its utility in phosphorylation studies, however, has been limited by the fact that it (or an oxidation product) was found by LEHNINGER[5] to uncouple phosphorylation associated with the oxidation of β-hydroxybutyrate. The use of hydroquinone or ascorbate was limited by the low reactivity of these substrates with the respiratory chain of intact mitochondria. The addition of soluble cytochrome *c* made it possible to use these substrates, but generated

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

the additional difficulty that little was known about the locus of reaction between the added cytochrome *c* and the intact chain.

However, in recent years several studies have shown substantial phosphorylation in the cytochrome oxidase region, using a variety of systems and substrates, and including the important demonstration by NIELSEN AND LEHNINGER[6] and by SLATER[7,8] of the synthesis of ATP coupled to the oxidation of added ferrocytochrome *c*. In all cases, however, P:O ratios reported have tended to be rather variable.

The recent development of a system including ascorbate and catalytic amounts of TMPD has, in the hands of JACOBS[9] and PACKER AND JACOBS[10], yielded high and consistent P:O ratios (approaching one) using rat-liver and rabbit-heart mitochondria, respectively. Their system circumvents the unreactivity of ascorbate with the intact chain by allowing TMPD to serve as a mobile electron carrier between external ascorbate and members of the respiratory chain. The possible uncoupling effect, which is believed to reside in the oxidized form of the dye, is circumvented by the combination of a low (catalytic) dye concentration and a large excess of the ultimate reducing agent, ascorbate. The ascorbate–TMPD system has proved useful, not only in studies of oxidative phosphorylation, but also in investigations of the energy-linked reversal of the respiratory chain[11–16] and of the mechanism of mitochondrial swelling[12]. Such studies depend, in part, on the ability to isolate the terminal portion of the respiratory chain, for example by the use of antimycin.

The present study deals with a further characterization of the ascorbate–TMPD system as used for the measurement of oxidative phosphorylation. Results are presented relating to the yield of ATP in the terminal portion of the respiratory chain and to the problem of isolating the region by the addition of antimycin.

METHODS

Rat-liver mitochondria were prepared in 0.25 M sucrose as described by MYERS AND SLATER[17]. Oxygen consumption was measured by the polarographic technique described by CHANCE AND WILLIAMS[18] using a Gilson Medical Electronics apparatus with a vibrating electrode. A value of 0.24 mM was taken as the oxygen concentration in air-saturated medium under the experimental conditions. Phosphorylation was estimated by the extent of the stimulated (State 3) respiration produced by the addition of a known amount of AMP, each mole of AMP reacting with endogenous ATP via myokinase to give 2 moles of ADP*. The AMP concentration was measured by the absorbancy at 259 mμ ($\varepsilon_M = 15.4 \cdot 10^3$). The standard reaction medium contained 15 mM KCl, 2 mM EDTA, 50 mM Tris–HCl buffer, 5 mM $MgCl_2$, 12.5 mM phosphate, 15 mM ascorbate, and 1.5–6 mg of protein in a final volume of 2 ml. The final pH was 7.4. When arsenite was used, it was first brought to pH 7.4. Antimycin was added as an ethanolic solution. In no case did the final ethanol concentration exceed 3% which was found to be without effect on oxidative phosphorylation.

P:O ratios were also determined by the method of SLATER AND HOLTON[19] where oxygen uptake was measured manometrically and phosphorylation estimated by allowing the ATP formed to be used in the synthesis of hexose monophosphate.

* Following the suggestion of Mr. A. KEMP, Jr., AMP was used instead of ADP because commercial ADP contains both AMP and ATP.

The reaction mixture was as described for polarographic experiments except that, in addition, it contained 0.1 mM ADP, 60 mM glucose, and 150 units of hexokinase in a final volume of 1 ml. The reaction time was 12 min at 25°.

Spectra were obtained using a Cary model-14 recording spectrophotometer. Digitonin fragments from beef-heart mitochondria were prepared according to the method of HAAS AND ELLIOTT[20] and were kindly supplied by Dr. D. HAAS.

One sample of antimycin A was kindly provided by the Kyowa Fermentation Industry Co. Ltd. (Tokyo) and the other was obtained from the Wisconsin Alumni Research Foundation. A sample of 2-heptyl-4-hydroxyquinoline-*N*-oxide was obtained through the generosity of Dr. J. LIGHTBOWN. Reagent grade *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride was obtained from The British Drug Houses Ltd.

Protein was determined using the biuret reagent[21] employing a standard of bovine serum albumin.

RESULTS

A reducing system composed of ascorbate and a catalytic concentration of TMPD is rapidly oxidized by respiratory-chain preparations[9]. Interpretation of much of the information gained from the use of the ascorbate–TMPD system depends on the

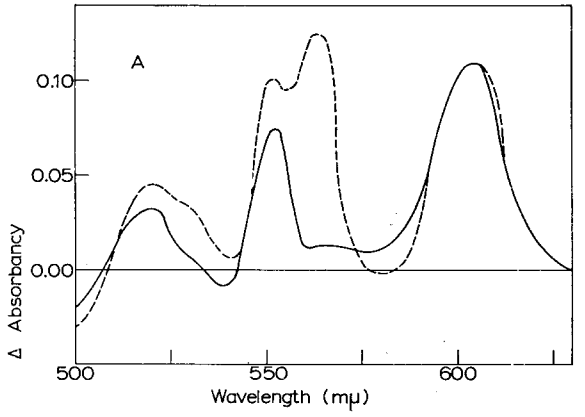


Fig. 1. A. Reduction minus oxidation difference spectrum of digitonin fragments in the presence of 5 μg antimycin/mg protein. ———, pigments reduced with 60 μM TMPD and 15 mM ascorbate; - - - -, pigments reduced with excess dithionite.

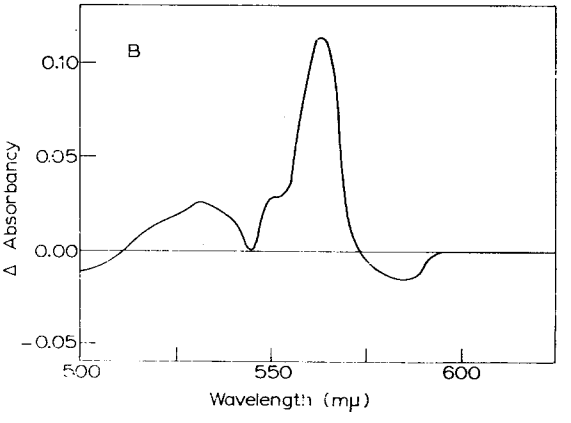


Fig. 1. B. Difference between the two spectra in A.

correct identification of the site of entry of electrons into the respiratory chain. Generally, the site is tacitly assumed to be cytochrome *c*, a view consistent both with the known reaction of *p*-phenylenediamine with endogenous cytochrome *c* (see ref. 4) and with the redox potential of TMPD which is 0.26 V (see ref. 9). However, the possibility remains that TMPD can react at other loci, either between cytochrome *c* and oxygen or below it, perhaps with cytochrome *b*. To eliminate the last eventuality, the spectra seen in Fig. 1 were obtained. There it is seen that ascorbate and TMPD effect the complete reduction of cytochrome *c* and *a* in the presence of antimycin. The cytochrome *b* peak appears only after the addition of dithionite and subtraction of the spectrum using ascorbate and TMPD from that using dithionite gives an almost pure cytochrome *b* spectrum with the suggestion of a peak in the *c* region.

Influence of TMPD concentration

Table I shows the pronounced dependence of the rate of oxidation on TMPD concentration in both State 3 (in the presence of AMP) and State 4 (absence of

TABLE I

INFLUENCE OF TMPD CONCENTRATION ON THE COUPLED OXIDATION OF TMPD

Reaction mixture as described in METHODS with addition of 1 mM arsenite and TMPD and antimycin as shown. Mitochondrial protein concentration was 2.9 mg/ml. Respiratory-control index is the ratio of State-3 (+ AMP) respiration to State-4 (after AMP is exhausted).

	O_2 consumption (μatoms/min)		Respiratory-control index	P:O
	−AMP	+AMP		
60 μM TMPD				
No antimycin	0.098	0.174	1.78	1.33
+ 0.33 μg antimycin/mg protein	0.133	0.145	1.09	0.66
300 μM TMPD				
No antimycin	0.284	0.498	1.78	1.05
+ 0.33 μg antimycin/mg protein	0.343	0.470	1.37	0.88

AMP)[18]. It is seen that respiratory control is maintained even at the higher TMPD concentration.

In addition, a pronounced effect of TMPD concentration on the P:O ratios was observed (Fig. 2). In the absence of antimycin, the efficiency of phosphorylation is much higher at low TMPD concentrations (and therefore low rates of oxidation). This is true both in the presence and absence of 1 mM arsenite which inhibits the oxidation of endogenous substrates[22]. It may be added that mitochondria which oxidize TMPD with the accompanying phosphorylation described above oxidize 60 mM succinate with P:O ratios of about 1.5 and exhibit respiratory-control ratios of about 4.

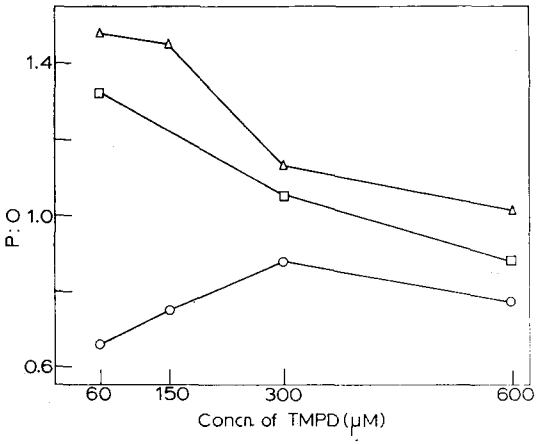


Fig. 2. Influence of TMPD concentration on P:O ratios. △—△, no additions; □—□, + 1 mM arsenite; ○—○, +0.33 μg antimycin/mg protein. Experimental conditions are as described in METHODS, with TMPD concentrations as shown, and 2.9 mg protein.

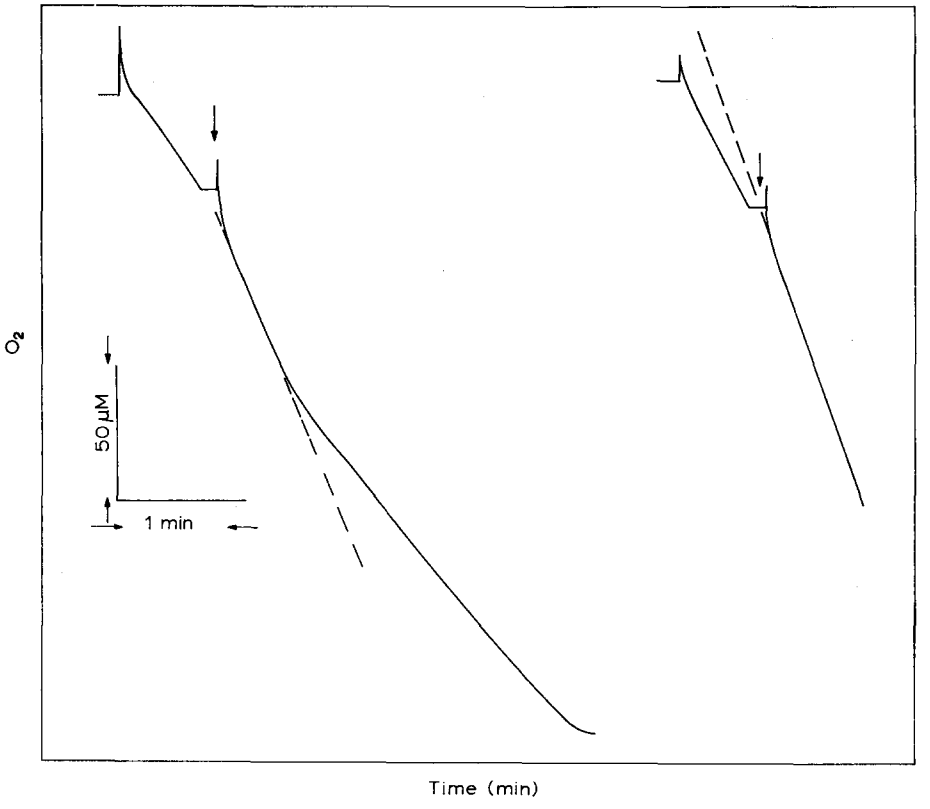


Fig. 3. Stimulation of TMPD oxidation by AMP and by antimycin. Tracings of Gilson Medical Electronics Oxygraph recordings. Conditions as described in METHODS with 3.6 mg protein. Left: arrow denotes the addition of 0.14 μmole AMP. Right: arrow indicates the addition of 4 μg antimycin.

The action of antimycin

The addition of antimycin has a marked effect. At low TMPD concentrations, 0.33 μg antimycin/mg protein lowers the P:O ratio to about one half of its control value with progressively less influence as the dye concentration is increased. An examination of Table I shows that the addition of antimycin also results in a sharp decline in the respiratory-control ratio, with slight inhibition of State-3 respiration and, most important, an increase in State-4 respiration (see also Fig. 3). Both the

TABLE II

INFLUENCE OF ANTIMYCIN IN THE PRESENCE OF 2-HEPTYL-4-HYDROXYQUINOLINE-*N*-OXIDE

Reaction mixture as described in METHODS with addition of 60 μM TMPD, 1 mM arsenite and 0.24 μg 2-heptyl-4-hydroxyquinoline-*N*-oxide/mg protein, a concentration found in the same experiment to be double that necessary to inhibit succinate oxidation completely. Protein concentration was 3.2 mg/ml.

	Respiratory-control index	*P:O*
No antimycin	1.64	1.26
+ 0.25 μg antimycin/mg protein	1.26	0.61

decline in P:O ratio and of respiratory control brought about by antimycin occur in the presence of 0.25 μg of 2-heptyl-4-hydroxyquinoline-*N*-oxide/mg protein, a concentration double that sufficient to inhibit succinate oxidation completely (Table II). Fig. 4 is a titration of the effect of 2-heptyl-4-hydroxyquinoline-*N*-oxide

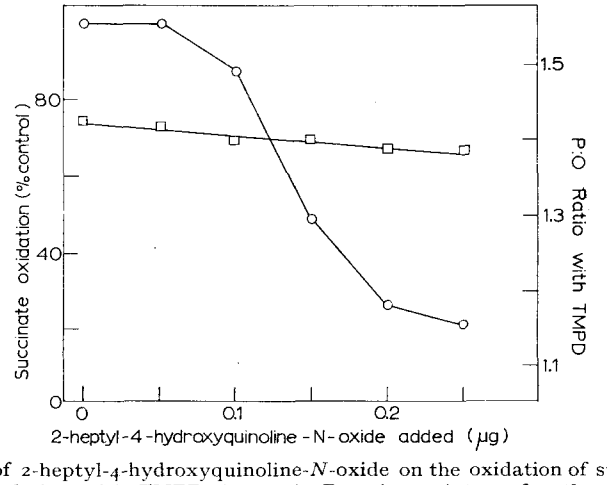


Fig. 4. Influence of 2-heptyl-4-hydroxyquinoline-*N*-oxide on the oxidation of succinate (○—○) and on phosphorylation with TMPD (□—□). Reaction mixture for the measurement of succinate oxidation included 15 mM KCl, 2 mM EDTA, 50 mM Tris–HCl buffer, 5 mM $MgCl_2$, 1 mM arsenite, 12.5 mM phosphate, 500 μM AMP, and 60 mM succinate in a final volume of 2 ml at pH 7.4. Conditions for TMPD oxidation as described in METHODS with 60 μM TMPD and 1 mM arsenite. In all cases, the reaction mixture contained 6.3 mg protein.

on both succinate oxidation and phosphorylation with TMPD. It is clear that the compound has only a slight effect on P:O ratios at concentrations that allow a drastic inhibition of succinate oxidation.

In addition, the effect of antimycin was examined in manometric experiments using an enzymic assay of hexose monophosphate. The experiments shown in Table III are in agreement with the polarographic, both with respect to the P:O ratios in

TABLE III

INFLUENCE OF ANTIMYCIN ON PHOSPHORYLATION MEASURED AS SYNTHESIS OF HEXOSE MONOPHOSPHATE

Reaction mixture as described in METHODS for manometric experiments with 60 μM TMPD and 1 mM ascorbate. Each flask contained 6.4 mg protein in the case of Expt. 1 and 5.6 mg in that of Expt. 2.

Expt.	*Additions*	*Δ O (μatoms)*	*Δ esterified P (μmoles)*	*P:O*
1.	No substrate	—	0.49	—
	No substrate + antimycin (0.8 μg)	—	0.18	—
	TMPD + ascorbate	2.56	3.02*	1.18
		2.67	3.14*	1.18
	TMPD + ascorbate + antimycin (0.8 μg)	2.70	2.06*	0.76
		2.83	2.34*	0.83
2.	No substrate	—	0.54	—
	No substrate + antimycin (0.6 μg)	—	0.27	—
	TMPD + ascorbate	3.18	3.51*	1.10
	TMPD + ascorbate + antimycin (0.2 μg)	2.77	2.89*	1.04
	TMPD + ascorbate + antimycin (0.4 μg)	3.26	2.17*	0.67
	TMPD + ascorbate + antimycin (0.6 μg)	3.12	1.96*	0.63
	TMPD + ascorbate + antimycin (0.8 μg)	3.12	2.12*	0.68

* Values for Δ esterified P are corrected for corresponding control without substrate.

excess of 1 at 60 μM TMPD in the absence of antimycin and to the significant effect of antimycin on the P:O ratio. The P:O ratios shown in Fig. 2 should be compared with the ratio of 1.01 reported by PACKER AND JACOBS[10] for rabbit-heart sarcosomes respiring with 200 μM TMPD and with that of 0.99 reported by JACOBS[9] for rat-liver mitochondria with 300 μM TMPD. The latter system was reported to be antimycin-insensitive. PACKER AND JACOBS report a respiratory-control index of 1.64 under their conditions.

Antimycin titration

Experiments thus far described employed concentrations of antimycin which are high when compared to the amount needed for the inhibition of succinate oxidation[23]. Titration of the decline in P:O ratios under the influence of antimycin (Fig. 5) showed a maximum effect at about 0.05 μg/mg protein. The effect amounted to approximately a halving of phosphorylation efficiency even when, in other experiments, the antimycin concentration exceeded the amount required for maximal

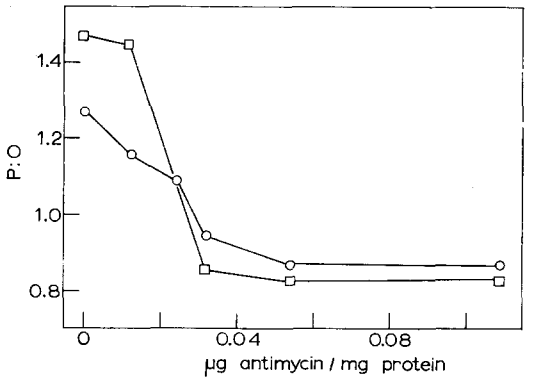


Fig. 5. Influence of antimycin on P:O ratio. □—□, no arsenite; ○—○, + 1 mM arsenite. Experimental conditions are as described in METHODS with 3.7 mg protein.

decline in ATP synthesis by about 10-fold (see Table I). The effects noted above have been obtained with samples of antimycin from both Kyowa Fermentation Industries and the Wisconsin Alumni Research Foundation and the halving of phosphorylation efficiency has also been confirmed in manometric experiments (Table III).

Oxidation of low concentrations of TMPD

Fig. 6 is a reciprocal velocity–concentration representation of oxidation rate from 60 to 600 μM TMPD. It is seen that States-3 and -4 respiration approximates a situation which is (not surprisingly) analogous to non-competitive inhibition. A

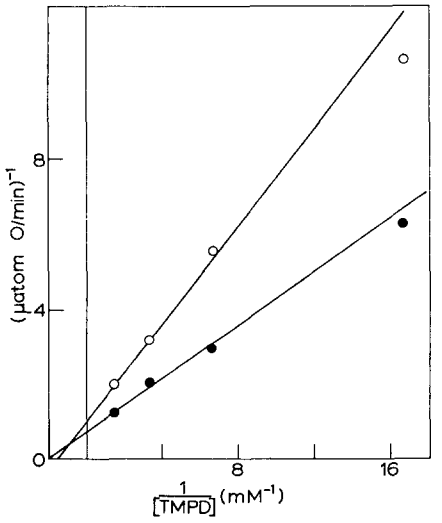


Fig. 6. Dependence of the rate of TMPD oxidation on dye concentration: reciprocal velocity–reciprocal substrate plot. ○—○, State 4; ●—●, State 3. Experimental conditions are as described in METHODS with 3.7 mg protein.

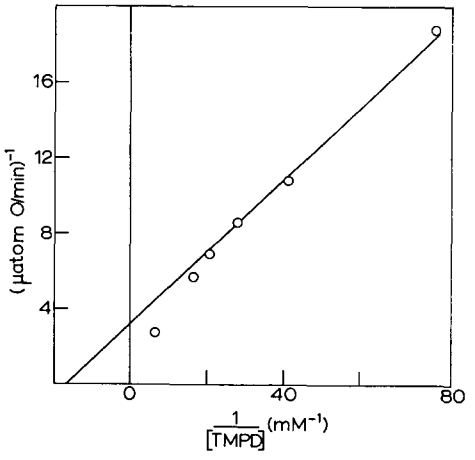


Fig. 7. Oxidation of low concentrations of TMPD: reciprocal velocity–reciprocal substrate concentration plot. Conditions as described in METHODS with 4 mg protein.

single apparent K_m is obtained from both respiratory States 3 and 4 of about 800 μM. It will be noted that deviations from linearity are noted at the lowest concentration (60 μM). Fig. 7 is a similar representation of State-3 respiration at TMPD concentrations of from 12 to 150 μM. In the range of 12–48 μM TMPD, a value of 60 μM for the apparent K_m is obtained.

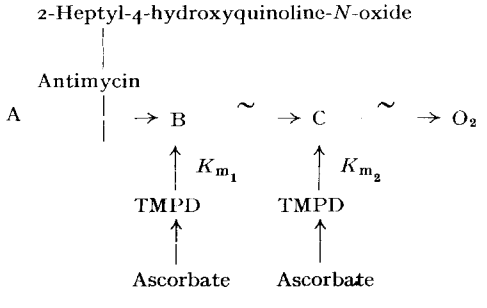
DISCUSSION

The present study has confirmed the P:O ratios of about 1 reported by JACOBS[9] and PACKER AND JACOBS[10] when their TMPD concentrations were employed. However at lower concentrations, the rate of oxidation diminished while the efficiency of phosphorylation increased giving a P:O ratio of about 1.3 at 60 μM TMPD. The addition of antimycin caused the P:O ratio to drop to about one half of this value and further addition of a large excess of antimycin caused little further decline. At the same time antimycin produced a sharp decline in the respiratory-control index, due, in part, to a stimulation of State-4 respiration. MINNAERT AND VAN KAMMEN-WERTHEIM[24] have warned that the oxidation of endogenous substrate can give rise to erroneously large P:O ratios when electron transport through the terminal part of the respiratory chain is slow. This is evidently not the explanation for the high P:O ratios described above. Neither 1 mM arsenite nor 2-heptyl-4-hydroxyquinoline-*N*-oxide have a significant effect although both should eliminate contribution by oxidation of endogenous substrate. Finally, this explanation obviously leaves out of account one of the most striking effects of antimycin, the stimulation of State-4 respiration.

PACKER AND JACOBS[10] reported P:O ratios of 1.0 for the oxidation of TMPD and regarded it as 100% of the theoretical yield. This corresponds to a P:electron ratio of 0.5 and it is probably unjustified at present to consider such a value the maximum yield of phosphorylation. Thus, it is not possible, from a consideration of

P:O ratios alone, to specify the number of sites involved in a phosphorylating system. The best evidence in the present study for two sites above the antimycin block comes, not from the absolute value of P:O ratios, but from the sensitivity of only one half of the phosphorylation to antimycin under conditions which strongly suggest uncoupling. SLATER[25] has calculated that one half of the free energy made available by the oxidation of NADH resides in the span from ferrocytochrome *c* to oxygen. Thus energy is at least available for phosphorylation at two sites above cytochrome *c* as there are, in fact, two below it. RAMIREZ[26,27] has presented evidence for the existence of two sites in the oxidase region, suggesting on the basis of cross-over experiments with intact cardiac muscle that they lie at the cytochrome *c*–*a* and at the cytochrome *a*–oxygen couples. The former corresponds to that proposed by CHANCE AND WILLIAMS[18] on kinetic grounds while the second corresponds to that regarded by SLATER[25] as being most likely on a thermodynamic basis.

The increasingly efficient synthesis of ATP at low TMPD concentrations might be understood either as uncoupling at higher TMPD levels or, alternatively, as the result of a shift in the locus of entry of TMPD into the respiratory chain. There is little basis for the first possibility as the respiratory-control ratio is maintained at the same value even at the highest TMPD concentrations used. Rather, it is suggested that the terminal portion of the respiratory chain can be drawn:



where A–C are unspecified members of the electron pathway, sites of phosphorylation are indicated by ~, and the K_m's are the apparent Michaelis constants for the interaction of TMPD with the respiratory chain. In addition, it is envisaged that $K_{m_1} < K_{m_2}$ so that, at low TMPD concentrations, the entry of electrons would be chiefly via carrier B. This view is consistent with the lower value for the K_m obtained at low TMPD concentrations. Phosphorylation between B and C would, in this view, be eliminated by antimycin, which is consistent with the greater uncoupling at lower TMPD concentrations. It may be added that, in systems where ascorbate reacts with the respiratory chain, it appears to react at more than one carrier giving rise, in the experiments of MINNAERT[28], to the complex kinetics for cytochrome *c* oxidase when activity is measured using ascorbate and added cytochrome *c*.

The concomitant increase in State-4 respiration and decline in phosphorylation on the addition of antimycin should probably be regarded as true uncoupling. Indeed, antimycin in higher concentrations is known to be a general uncoupler of oxidative phosphorylation and under certain conditions to stimulate mitochondrial

ATPase[29]. This apparent selectivity of antimycin as an uncoupler may serve as an argument against any theory of oxidative phosphorylation (such as that of MITCHELL[30]) which does not involve the existence of discrete sites.

It is clear that the action of antimycin on the phosphorylating respiratory chain is multiple. Caution should be exercised in situations where distinctions between inhibition and uncoupling are meaningful (*cf.* refs. 31, 32). Antimycin acting as an inhibitor at one point in the respiratory chain and as an uncoupler at another is strongly reminiscent of the suggestion made by HÜLSMANN[31] and HEMKER[29] that the difference between a respiratory inhibitor and an uncoupler resides only in the affinity between the compound and the target member of the respiratory apparatus. According to this view, antimycin binds strongly at a locus somewhere in the region of cytochrome *b* to produce its well-known inhibition and, loosely, at a point between that locus and oxygen producing the uncoupling described in the present study.

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