The mode of action of dinitrophenols on the different phosphorylating steps

Reprinted from
Biochimica et Biophysica Acta
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands

BBA 12283

THE MODE OF ACTION OF DINITROPHENOLS ON THE DIFFERENT PHOSPHORYLATING STEPS*

H. C. HEMKER

Laboratory of Physiological Chemistry, University of Amsterdam, Amsterdam (The Netherlands)

(Received May 17th, 1963)

SUMMARY

1. Determinations of the concentration $(c_{aq})_{opt}$ of a dinitrophenol that induced maximal respiration in a medium deficient in phosphate and phosphate acceptor was carried out with different alkylnitrophenols and substrates (succinate, pyruvate (+ malate), glutamate, β-hydroxybutyrate) at various pH's.

2. The pH optimum of maximally uncoupled succinate oxidation was 7.4, of maximally uncoupled pyruvate oxidation 6.8.

3. The $p(c_1)_{opt}$, where c_1 is the calculated concentration of uncoupling phenol in the lipid phase, is a linear function of pH, the slope of the line relating these quantities being 0 with succinate and -0.5 for NAD-linked substrates; an exceptional case is formed when the respiration of pyruvate (+ malate) is uncoupled by alkyldinitrophenols, in which case the slope is 0.

4. From a consideration of the reaction kinetics it appears that for systems in which $dp(c_1)_{opt}/dpH$ is zero, uncoupling and inhibition by phenols (Φ) is best explained by the reactions:

$$A \sim I + \Phi \rightarrow A + I - \Phi \qquad I - \Phi \rightleftharpoons I + \Phi$$

where A is an oxidized member of the respiratory chain, and $A \sim I$ an intermediate of oxidative phosphorylation. When the slope is -0.5 the sequence

$$A \sim I + \Phi \rightarrow A + I - \Phi \qquad I - \Phi + H^+ \rightleftharpoons I + \Phi$$

appears to be the most likely.

5. On basis of the difference in the relation between $p(c_{aq})_{opt}$ and pH for different NAD-linked substrates it is concluded that mitochondrial NAD^+ associated with the different substrates is present in different compartments, and that the whole phosphorylation system connected with a particular substrate must be localized in the same mitochondrial compartment. The compartments differ in their lipophilic character.

* This work was part of the M.D. thesis of the author which was published in Dutch in April 1962 (ref. 1).

INTRODUCTION

In the preceding paper[2] it was concluded (1) that dinitrophenols have a specific inhibitory action on the uncoupled respiration and the ATPase of intact rat-liver mitochondria; (2) that dinitrophenols uncouple by reaction with a high-energy intermediate normally involved in the transfer of the energy derived from electron transport to ATP synthesis. At present there are no reasons to assume that the high-energy intermediate involved does not contain a member of the respiratory chain (A) and it will be denoted as $A \sim I$, although it may well be that the coupling process is much more complicated than suggested by the minimum scheme shown in the preceding paper.

The experiments leading to the recognition of the role of lipid solubility of uncoupling agents[3,4] and those described in the preceding papers[2,5] revealed a variation of $(c_l)_{opt}$[4] with pH which remained to be investigated further. In the present paper, this variation is analysed in more detail in an attempt to clarify the mechanism of uncoupling. The results will be discussed in terms of the general theory of uncoupling proposed in the preceding paper[2].

METHODS

The methods were essentially the same as described in the preceding paper[2]. The incubation mixtures are indicated in the legends to the figures.

RESULTS

Determination of $(c_{aq})_{opt}$ at different pH's for the oxidation of succinate

In these experiments increasing concentrations of nitrophenol were added to the reaction mixture in the special vessel adapted to the oxygen polarograph described previously[6]. After each addition, the rate of O_2 uptake was measured for a short period, after which a further amount of nitrophenol was added and the measurement of the O_2 uptake repeated. Thus, a range of dinitrophenol concentrations was used in a single experiment. The concentration of nitrophenol inducing the highest rate of O_2 consumption in an experiment at a given pH is indicated by a point in Fig. 1. The neighbouring concentrations tested that induced a lower rate of O_2 uptake are indicated by the ends of the horizontal line drawn through the point. The true $(c_{aq})_{opt}$ must, then, lie between the extremes indicated by the horizontal line.

It has been shown previously[3,4] that Eqn. 1

$$pc_l = pc_{aq} + pH + pQ + \log(K + [H^+] + fQ[H^+]) \quad (1)$$

describes the relationship between the concentration (c_l) of an uncoupling phenol in a lipid phase within the mitochondrion and the concentration (c_{aq}) in the surrounding aqueous medium, where Q is the partition coefficient of the undissociated phenol molecule between lipid and water, K is its acid dissociation constant, and f is the ratio of the volumes of lipid and aqueous phases.

For dinitrophenols, the limiting case given by Eqn. 2 applies

$$pc_l = pc_{aq} + pQ + pH - pK \quad (2)$$

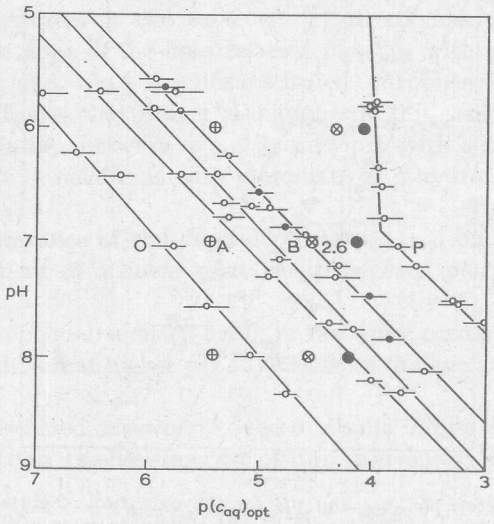

Fig. 1. The relation between $p(c_{aq})_{opt}$ and pH for the oxidation of succinate. Reaction medium: 15 mM KCl, 2 mM EDTA, 50 mM Tris–HCl, 5 mM $MgCl_2$, 50 mM sucrose, 60 mM succinate, 3 mM ATP (when present), 1.2 mM Amytal. —●—, indicate the experiments where ATP was added, —○—, where it was not added. The diagonals connect the values obtained with the same nitrophenol. O, 4-isooctyl-2,6-dinitrophenol; A, 4-isoamyl-2,6-dinitrophenol; B, 4-isobutyl-2,6-dinitrophenol; P, *p*-nitrophenol; 2.6, 2,6-dinitrophenol. When $(c_1)_{opt}$ is calculated from $(c_{aq})_{opt}$ with use of the partition coefficient for a xylene–water system the points ● are obtained for alkyldinitrophenols, ⊗ for *p*-nitrophenol and ⊕ for 2,6-dinitrophenol.

This equation succesfully described the variation with pH of the ATPase induced by various phenols. The facts illustrated in Fig. 1, *viz.* (1) that the effect of an uncoupling phenol on succinate oxidation depends upon the lipid solubility of the phenol, and (2) that the slope of the pH–concentration curve for *p*-nitrophenol changes abruptly at a pH equal to the pK of *p*-nitrophenol, support the application of the equation to the effects on succinate oxidation. When the values for pQ obtained in a xylene–water[4] system are applied to the data in Fig. 1, the values of $p(c_1)_{opt}$ are about the same for all the alkyldinitrophenols tested.

Although not too much significance is given to the actual value of $(c_1)_{opt}$ calculated from these data, since there is no reason to expect that the values of Q obtained for the xylene–water system apply to the mitochondria, the agreement between the values for $(c_1)_{opt}$ for different alkyldinitrophenols supports the application of Eqn. 2 and indicates further that there is no fundamental difference in the mechanism of action of the different alkyldinitrophenols.

Fig. 1 shows that the relationship between pH and $p(c_{aq})_{opt}$ for any one dinitrophenol may be described by Eqn. 3

$$p(c_{aq})_{opt} = -pH + b \qquad (3)$$

where b is a constant. Combining Eqns. 2 and 3 yields Eqn. 4

$$p(c_1)_{opt} = b + pQ - pK \qquad (4)$$

i.e. $p(c_1)_{opt}$ is independent of pH.

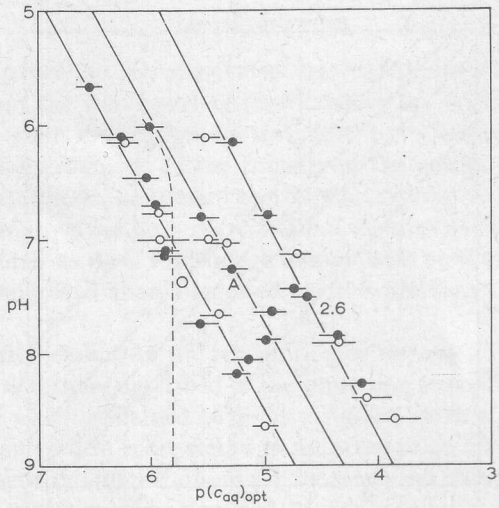

Fig. 2. The relation between $p(c_{aq})_{opt}$ and pH for the oxidation of glutamate and β-hydroxybutyrate. Reaction medium as in Fig. 1 with 20 mM glutamate or 20 mM β-hydroxybutyrate in place of succinate and no Amytal added. The letters denote the phenol used as in Fig. 1. —○—, β-hydroxybutyrate; —●—, glutamate; - - - -, the left-hand line from Fig. 3 shown for comparison.

Determination of $(c_{aq})_{opt}$ at different pH's for the oxidation of NAD-linked substrates

With glutamate or β-hydroxybutyrate as substrate (Fig. 2), the relationship between $p(c_{aq})_{opt}$ and pH is given by Eqn. 5

$$p(c_{aq})_{opt} = -0.5\ pH + b \qquad (5)$$

The results with pyruvate + malate as substrate (Fig. 3) differ from those obtained

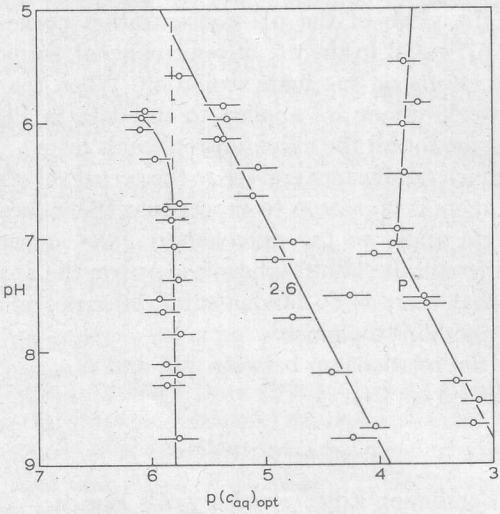

Fig. 3. The relation between $p(c_{aq})_{opt}$ and pH for the oxidation of pyruvate (+ malate). Reaction medium as in Fig. 2, with 20 mM pyruvate and 20 mM malate as substrate. P and 2.6 denote the phenol used as in Fig. 1. The left-hand (vertical) line connects the values obtained with all alkyl-substituted dinitrophenols.

with the other substrates in two respects: (1) all alkyldinitrophenols fall on the same curve; (2) the slope of the lines relating $p(c_{aq})_{opt}$ with pH is 0 in the case of alkyldinitrophenols and -0.5 for unsubstituted nitrophenols. However, the following evidence indicates that Eqn. 1 also applies in this case:

(1) The relationship between $p(c_{aq})_{opt}$ and pH with 2,6-dinitrophenol is the same with pyruvate + malate as with glutamate or β-hydroxybutyrate (*cf.* Figs. 2 and 3).

(2) The disappearance of the dependence of $p(c_{aq})_{opt}$ on Q goes hand in hand with the establishment of a linear relationship between $p(c_{aq})_{opt}$ and the amount of mitochondria[4].

(3) With *p*-nitrophenol, a sharp break in the curve occurs at a pH equal to the pK of *p*-nitrophenol, and at higher pH's the slope of the line again becomes equal to -0.5.

As already discussed elsewhere[4], these results imply that when pyruvate (+ malate) is substrate, the limiting case of Eqn. 1 given by Eqn. 6 aplies

$$p(c_1)_{opt} = p(c_{aq})_{opt} + \log f \qquad (6)$$

i.e. $fQ[H^+] \gg [H^+]$ and $\gg K$.

Thus, when pyruvate (+ malate) is substrate and an alkyldinitrophenol is the uncoupler, $fQ[H^+] \gg [H^+]$ and $\gg K$. Under all other conditions, $fQ[H^+] \ll [H^+]$ and $\ll K$.

The pH–activity curve of the maximally stimulated respiring system

Since the concentration of uncoupler that maximally stimulates succinate oxidation is strongly dependent upon the pH, three different concentrations of uncoupler were tested at each pH, all chosen to be close to the expected $(c_{aq})_{opt}$. The highest of the three values was taken to be the maximal activity at the pH tested. The optimal pH was about 7.4 (Fig. 4), the same as that found for the Amytal-

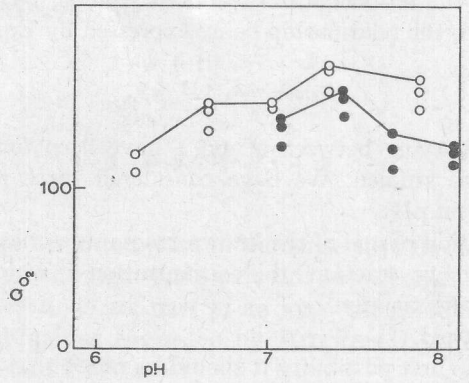

Fig. 4. The pH–activity curve of uncoupled succinate oxidation. The reaction velocities were measured manometrically. The reaction mixture was the same as in Fig. 1. The black and white circles indicate two separate sets of experiments carried out with different mitochondrial preparations. No ATP added. At each pH 3 concentrations of 4-isobutyl-2,6-dinitrophenol chosen to be close to $(c_{aq})_{opt}$ were used. The highest value was taken to be the maximum activity at the given pH, and the lines shown are drawn through these values. The other values are shown by the other points.

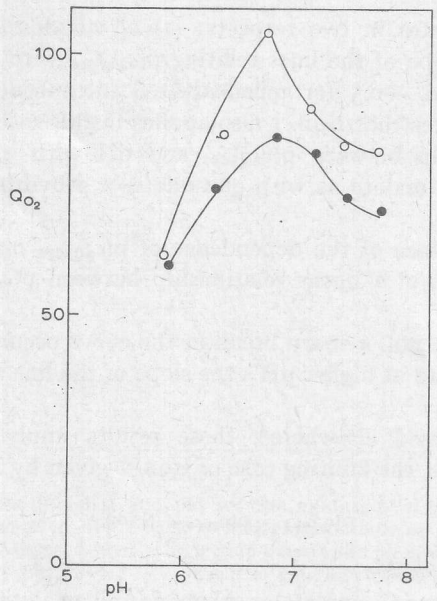

Fig. 5. The pH optimum of uncoupled pyruvate oxidation. Reaction medium as in Fig. 3. The reaction velocity was measured manometrically. ●—●, with 1.5 μM 4-isooctyl-2,6-dinitrophenol; ○—○, with 3 μM 4-isooctyl-2,6-dinitrophenol.

resistant ATPase[5]. Since $(c_{aq})_{opt}$ is independent of pH with pyruvate and alkyldinitrophenol, it was possible to determine the pH–activity curve at a single concentration of uncoupler. The optimal pH was about 6.8 (Fig. 5).

Theoretical considerations

In the case of all the mitochondrial reactions studied, $p(c_1)_{opt}$ was found to be a linear function of pH, the relationship being expressed by Eqn. 7

$$p(c_1)_{opt} = a_1 \cdot pH + b \qquad (7)$$

Various values of a_1 varying between 0 and 1 have been found for the different mitochondrial reactions studied. We have considered three possible explanations for a dependence of c_1 on pH:

(1) The concentration of one of the known reactants varies with pH.

(2) H^+ or an unknown reactant, the concentration of which is dependent upon the pH, takes part in the reaction.

(3) pQ varies with pH.

With regard to the first possibility it should be noted that the amounts of ATP or of substrate used were saturating (*cf.* refs. 7, 8). Although the amount of an active enzyme can vary with pH, such an effect would be revealed by a bend in the $p(c_{aq})_{opt}$–pH curve. This was not found. The third possibility was discarded as an explanation, since it seems very unlikely that the physical properties of the mitochondrion could be so altered—and reversibly—to account for a 100-fold variation

of Q. For these reasons, we have preferred to interpret our results in terms of the second possibility. The concentration of the unknown reactant could be either linearly or inversely related to the concentration of H^+. H^+ and OH^- will be taken as representatives of these two cases.

If we assume that H^+ or OH^- takes part in the reaction, we must also consider the possibility that the active species of the phenol is the phenolate ion, the concentration of which will be directly related to that of the undissociated phenol in the mitochondrial lipid (not to the concentration of phenolate ions in the medium).

The mechanism of uncoupling and inhibition by uncouplers proposed by HÜLSMANN[9] will be used as our working hypothesis.

$$AH_2 + B + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A \sim I + BH_2 \qquad (1a)$$

or

$$ATP + A + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A \sim I + ADP + P_i \qquad (1b)$$

$$A \sim I + \Phi \xrightarrow{k_3} A + I{-}\Phi \qquad (2)$$

$$I{-}\Phi \underset{k_6}{\overset{k_5}{\rightleftharpoons}} I + \Phi \qquad (3)$$

An alternative explanation of the inhibition, in which it is assumed that $k_6 = 0$, and the reverse of Reaction 3 is replaced by Reaction 4

$$I{-}\Phi + \Phi \underset{k_8}{\overset{k_7}{\rightleftharpoons}} I{-}\Phi{-}\Phi \qquad (4)$$

will also be considered.

The most likely point of action of H^+ or OH^- is on the splitting of I—Φ in Reaction 3, which may be written

$$I{-}\Phi + H^+ \underset{k_6}{\overset{k_5}{\rightleftharpoons}} I + \Phi \qquad (5)$$

or

$$I{-}\Phi + OH^- \underset{k_6}{\overset{k_5}{\rightleftharpoons}} I + \Phi \qquad (5')$$

Of course, these reactions are incomplete in the sense that the reaction partners on the left-hand side and on the right-hand side are not electrically equivalent. One possibility is that the reactions are part of an ionic-transport mechanism through the mitochondrial membrane as envisaged by MITCHELL[10]. Alternatively, I might be an acid or base (*cf.* SLATER[11]).

Two assumptions have to be made before a kinetic treatment of Reactions 1–5 can be given.

(1) The velocity of Reaction 2 from right to left is negligibly small. This is a reasonable assumption, since it is proposed that the high-energy compound A $\sim$ I is converted into the low-energy I—Φ.

(2) The concentrations of ATP and A are not rate-limiting. This is known to be the case with ATP[7]. That A is in excess is indicated by the finding of WADKINS AND LEHNINGER[12] and CHEFURKA[13] that only very rigorous reduction of the components of the respiratory chain leads to inhibition of the dinitrophenol-induced ATPase.

On the basis of these assumptions, we may write for the HÜLSMANN mechanism of the phenol-induced ATPase

$$\frac{d[A \sim I]}{dt} = k_1[I] - k_3[A \sim I]\,[\Phi]$$

$$\frac{d[I{-}\Phi]}{dt} = k_3[A \sim I]\,[\Phi] - k_5[I{-}\Phi]\,[w] + k_6[I]\,[\Phi]$$

where w represents H^+ or OH^-, according to whether Reaction 5 or 5′ is operating. In the steady state, $d[A \sim I]/dt = 0$, $d[I{-}\Phi]/dt = 0$, and $v = k_3[A \sim I]\,[\Phi]$. From this it follows that

$$v = \frac{k_1 k_3 k_5 i[\Phi]\,[w]}{k_3 k_6[\Phi]^2 + (k_1 k_3 + k_3 k_5[w])\,[\Phi] + k_1 k_5[w]}$$

where $i = [I] + [A \sim I] + [I{-}\Phi]$.
v will be a maximum at varying Φ when $dv/d\Phi = 0$, *i.e.* when

$$[\Phi] = \sqrt{\frac{k_1 k_5[w]}{k_3 k_6}}$$

An analogous treatment of the alternative explanation of the inhibition yields

$$v = \frac{k_1 k_3 k_5 k_8 i[\Phi][w]}{k_1 k_3 k_7[\Phi]^2 + k_3 k_8(k_1 + k_5 w)\,[\Phi] + k_1 k_5 k_8 w}$$

and

$$[\Phi]_{opt} = \sqrt{\frac{k_5 k_8[w]}{k_3 k_7}}$$

The respiration experiments can be treated similarly*, but a different value of k_1 applies. Since $(c_1)_{opt}$ differs for ATPase and respiration, k_1 would be expected to appear in the value of $[\Phi]_{opt}$. This is the case with the HÜLSMANN mechanism, but not with the alternative mechanism for the inhibition which has been considered, although in view of the various assumptions necessary in the kinetic treatment the latter mechanism cannot be excluded by these considerations.

Thus $[\Phi]_{opt} = k\sqrt{[w]}$, where w represents H^+ or OH^-. If Reaction 3 takes place without the participation of H^+ or OH^-, $[w]$ disappears from this relationship and $[\Phi]_{opt}$ becomes independent of pH.

* The additional assumption must be made that the velocity of Reaction 1a depends only on the concentration of I.

TABLE I

SUMMARY OF THE THEORETICALLY POSSIBLE VALUES OF $dp(c_1)_{opt}/dpH$

Combination	Phenol		Water ion			$(c_1)_{opt}$	$dp(c_1)_{opt}/dpH$
	undissociated molecule	ion	H^+	OH^-	neither		
a	+		+			$k\sqrt{[H^+]}$	+0.5
b	+			+		$k\sqrt{\frac{10^{-14}}{[H^+]}}$	−0.5
c	+				+	k	0
d		+	+			$\frac{k}{K}\cdot[H^+]^{1.5}$	+1.5
e		+		+		$\frac{k}{K}\sqrt{[H^+]10^{-14}}$	+0.5
f		+			+	$\frac{k}{K}\cdot[H^+]$	+1.0

TABLE II

SUMMARY OF THE DATA ON THE SLOPES (a) OF THE LINES RELATING $p(c_1)_{opt}$ AND pH

Column 1 states whether unsubstituted (U) or substituted (S) uncouplers were used. Column 2 gives the slopes (a_{aq}) of the lines found for the relation between $p(c_{aq})_{opt}$ and pH when $pH \gg pK$. Column 3 shows whether there is a bend when $pH = pK$. As there were no substituted compounds with a pK in the region 5–8 this is not known for the substituted uncouplers. Columns 4 and 5 show, respectively, whether there is a variation of $(c_{aq})_{opt}$ with the partition coefficient or with the amount of mitochondria present. Column 7 gives the values of a_1, which can be calculated from a_{aq} (Column 2) by substitution of the empirical relationship $p(c_{aq})_{opt} = a\text{pH} + b$ in Eqn. 1

$$pc_1 = pc_{aq} + \text{pH} + pQ + \log(K + [H^+] + fQ[H^+]) \qquad (1)$$

This equation has three limiting cases[3]

I $pc_1 = pc_{aq} + \text{pH} + pQ - pK$
II $pc_1 = pc_{aq} + pQ$
III $pc_1 = pc_{aq} + \log f$

Which of the three has to be used is derived from Columns 4 and 5. The result is given in Column 6.

Column 8 gives the combination (Table I) proposed for the uncoupling system studied.

System used		Column 1	2	3	4	5	6	7	8
Succinate	U	1	+	+	−		I	0	c
respiration	S	1	?	+	−		I	0	c
Pyruvate	U	−0.5	+	+	−		I	+0.5	a(e)
respiration	S	0	?	−	+		III	0	c
β-Hydroxybutyrate	U	−0.5	+	+	−		I	+0.5	a(e)
respiration	S	−0.5	?	+	−		I	+0.5	a(e)
Glutamate	U	−0.5	+	+	−		I	+0.5	a(e)
respiration	S	−0.5	?	+	−		I	+0.5	a(e)
ATPase	U	−0.5	+	+	−		I	+0.5	a(e)
	S	−0.25	?	+	?		I & III	0, 0.5	a(e), c

It is now necessary to relate $[\Phi]$ to c_1. If the reacting phenol is the undissociated molecule, $[\Phi] = c_1$. If, however, it is the phenolate ion,

$$[\Phi] = \frac{K}{[H^+]} \cdot c_1.$$

Thus the relationship between $p(c_1)_{opt}$ and pH will depend upon (i) whether H^+, OH^- or neither is involved in Reaction 3, and (ii) whether the undissociated phenol or the phenolate ion is involved in Reactions 2 and 3.

The 6 possible combinations and the relationship between $p(c_1)_{opt}$ and pH applicable to each combination are shown in Table I. Table II summarizes the experimental data obtained relevant to these considerations.

DISCUSSION

Phosphorylation step between flavoprotein and cytochrome c

Since inhibition in the cytochrome oxidase region is not observed with the concentrations of uncouplers used, and oxidation of NAD-linked substrates is inhibited by the Amytal present, the value of a_1 obtained with succinate as substrate is a property of the phosphorylation step between flavoprotein and cytochrome *c*. Since $a_1 = 0$, it appears, on the basis of our assumptions, that the undissociated phenol molecule reacts with $A \sim I$, and the $\Phi - I$ compound dissociates without mediation of H^+ or OH^-.

The optimum pH of maximally uncoupled succinate oxidation by rat-liver mitochondria (Fig. 4), of the Amytal-resistant dinitrophenol-induced ATPase of rat-liver mitochondria[5], of the P:O ratio with rat-heart sarcosomes oxidizing succinate[9], and of the succinate oxidase of KEILIN AND HARTREE heart-muscle preparation is in every case close to pH 7.4.

Phosphorylation step between NADH and flavoprotein

In a previous paper[5], it was concluded that the dinitrophenol-induced ATPase was made up of contributions from the first two phosphorylation steps of the res-

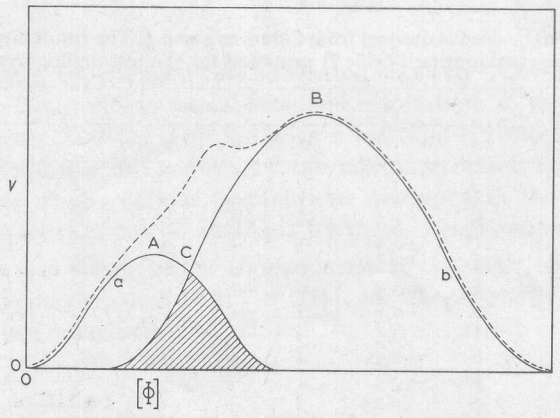

Fig. 6. The combined effect of uncoupling in two phosphorylating steps. For explanation see text.

piratory chain, the first (*i.e.* the one between NADH and flavoprotein) contributing about two-thirds. Higher concentrations of dinitrophenols were required to induce this ATPase. Schematically, these findings can be represented as in Fig. 6, where curves b and a represent the contribution of the first and second phosphorylation step, respectively, and the dotted line the total activity. Two peaks are shown on this curve, but the dominant peak is that due to the first phosphorylation step.

The situation is quite different in respiration experiments, since the least active step of the respiratory chain limits the overall rate of respiration. If Fig. 6 now represents the respiratory activity of the two steps, the borders of the area in which both curves overlap (the striated area) represent the respiration–[Φ]curve. Maximum activity will be found at C, which is not far from A, the peak of the less active step. Unfortunately, it is not easy to be sure which this is. Since, however, the behaviour of succinate respiration with respect to the variables summarized in Table II differs from that of the respiration with other substrates, it seems likely that the effects of variation of lipid solubility of the uncoupling phenol and of pH of the reaction medium on the rate of oxidation of NAD-linked substrates reflect the behaviour of the first phosphorylation step.

The difference between pyruvate (+ malate) and the other NAD-linked substrates has already been noted. It was concluded that $fQ[H^+] \gg [H^+]$ and K in the case of pyruvate (+ malate) and is $\ll [H^+]$ and K in the case of the other substrates. Since the experiments were carried out in the same pH range and with the same concentration of mitochondria (*i.e.* f), it must be concluded that Q differs in the two cases, being much greater when pyruvate (+ malate) is substrate. This leads to the conclusion that the enzymes involved in oxidative phosphorylation with pyruvate (+ malate) as substrate are located in a much more lipophilic region of the mitochondrion than is the case when glutamate or β-hydroxybutyrate is substrate.

This is an unexpected conclusion, but would provide a physical basis for the postulate of CHANCE[14,15] and KLINGENBERG[16] of compartmentation of mitochondrial NAD, based on the different degrees of reduction obtained with different substrates.

In all cases of NAD-linked substrates, except pyruvate (+ malate) in the presence of the lipophilic alkyldinitrophenols, the value of a_1 calculated from the observed values of a_{aq} was found to be 0.5 (see Table II). This can be explained by either combination a (undissociated phenol and H^+) or combination e (phenolate ion and OH^-),

i.e.

$$NAD \sim I + \Phi\text{–}OH \rightarrow NAD^+ + \Phi - I$$

$$\Phi - I + H^+ \rightleftharpoons \Phi\text{–}OH + I$$

or

$$NAD \sim I + \Phi\text{–}O^- \rightarrow NAD^+ + \Phi\text{–}I$$

$$\Phi\text{–}I + OH^- \rightleftharpoons \Phi\text{–}OH + I$$

With pyruvate (+ malate) and alkyldinitrophenols, $a_1 = 0$, so that the mechanism is presumably

$$NAD \sim I + \Phi\text{–}OH \rightleftharpoons NAD^+ + \Phi\text{–}I$$

$$\Phi\text{–}I \rightleftharpoons \Phi + I$$

similar in principle to that operating in the second phosphorylation step (see above).

The conclusion that neither H^+ nor OH^- plays a role in the dissociation of Φ—I is in agreement with the conclusion that this reaction takes place in a more lipophilic region of the mitochondrion.

Although no final decision can be made between combinations a and e, there is one consideration which favours the former. Since the undissociated phenol appears to be involved in Reaction 2 in the second phosphorylation step, and with the first phosphorylation step with pyruvate (+ malate) and alkyldinitrophenols, there is some reason to expect that this would also be so in the other cases.

The value for a_{aq} for the ATPase induced by unsubstituted nitrophenols is -0.5, giving $+0.5$ for a_1 (Table II). Since, as explained above, this corresponds to the first phosphorylation step, this value is in agreement with the conclusions drawn from the behaviour of respiration uncoupled with unsubstituted nitrophenols. With alkyldinitrophenols, however, values of a_{aq} between -0.2 and -0.3 were obtained, indicating rather strongly that two parallel systems are operating, one with a_{aq} of 0 and the other of -0.5. These two systems are probably the two mechanisms linked with the NADH–flavoprotein region located in the different mitochondrial compartments as discussed above.

The pH optimum of the maximally uncoupled pyruvate (+ malate) oxidation was found to be 6.8 (Fig. 5), identical with the pH optimum of the dinitrophenol-induced ATPase[5].

ACKNOWLEDGEMENTS

The author is very grateful to Professor E. C. SLATER and Dr. W. C. HÜLSMANN for many helpful discussions and criticism and to Professor E. C. SLATER and Dr. J. P. COLPA-BOONSTRA for reading and discussing the manuscript. The expert technical assistance of Miss W. NUWENHOF is also gratefully acknowledged.

This work was supported in part by the U.S. Public Health Service grant RG 6569..

REFERENCES

1 H. C. HEMKER, *Het mechanisme van de werking van ontkoppelende fenolen op de ademhalingsketenfosforylering*. M.D. Thesis, Klein Offset Drukkerij, Amsterdam, 1962.
2 H. C. HEMKER, *Biochim. Biophys. Acta*, 81 (1964) 1.
3 H. C. HEMKER AND W. C. HÜLSMANN, *Biochim. Biophys. Acta*, 48 (1961) 221.
4 H. C. HEMKER, *Biochim. Biophys. Acta*, 63 (1962) 46.
5 H. C. HEMKER, *Biochim. Biophys. Acta*, 73 (1963) 311.
6 H. C. HEMKER, *Biochim. Biophys. Acta*, 60 (1962) 646.
7 E. C. SLATER, *Proc. Intern. Symp. Enzyme Chem., Tokyo and Kyoto, 1957*, Maruzen, Tokyo, 1958, p. 288.
8 P. GREENGARD, K. MINNAERT, E. C. SLATER AND I. BETEL, *Biochem. J.*, 73 (1959) 637.
9 W. C. HÜLSMANN, *Over het mechanisme van de ademhalingsketenfosforylering*, M.D. Thesis, Klein Offset Drukkerij, Amsterdam, 1958.
10 P. MITCHELL, *Nature*, 191 (1961) 144.
11 E. C. SLATER, *Rev. Pure Appl. Chem.*, 8 (1958) 221.
12 C. L. WADKINS AND A. L. LEHNINGER, *J. Biol. Chem.*, 234 (1959) 681.
13 W. CHEFURKA, *Can. J. Biochem. Physiol.*, 38 (1960) 1195.
14 B. CHANCE AND G. HOLLUNGER, *Federation Proc.*, 20 (1961) 1.
15 B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 236 (1961) 1534, 1555, 1562, 1577.
16 M. KLINGENBERG AND P. SCHOLMEYER, *Biochem. Z.*, 333 (1960) 335.