Inhibition of Nonphosphorylating Electron Transfer by Zinc. The Problem of Delineating Interaction Sites*

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ABSTRACT: The inhibition of the nonphosphorylating respiratory chain by zinc was found to be similar to the inhibition in digitonin fragments and mitochondria. To observe this it was necessary to carry out the reactions under the same experimental conditions including similar zinc:protein ratios. In Keilin–Hartree heart muscle particles two types of zinc effect have been demonstrated, an initial one between cytochrome *b* and c_1 and a subsequent one at the flavoprotein level in the respiratory chain. The initial effect was reversible by EDTA.

The application of "crossover theorems" in defining the sites of action of inhibitors in the respiratory chain is discussed and the use of such criteria when analyzing the oxidation and reduction of the flavoprotein components is criticized.

Zinc has been reported to inhibit electron transfer in mitochondria (Skulachev *et al.*, 1967) and also affects digitonin particles obtained therefrom (A. N. Malviya and W. B. Elliott, manuscript in preparation). Skulachev and coworkers claimed the primary site of Zn^{2+} inhibition to be at the b–c_1 level, as with antimycin, with secondary sites at the flavoprotein and oxidase levels. In the digitonin particles the effect is variable, and depends on the rate of electron transfer and substrate employed. One of the sites of inhibition is at the flavoprotein level, resembling the effect of amytal.

In view of the reported differences between phosphorylating and nonphosphorylating systems in their sensitivity to inhibitors, including amytal (Chance and Hollunger, 1963) and also azide (Wilson and Chance, 1966), the effect of zinc on the nonphosphorylating Keilin–Hartree particle was studied. The results reported here throw light on some of the problems involved in determining sites of interaction in the respiratory chain. They are also pertinent to the question whether the respiratory chain can indeed be divided in a binary fashion by every inhibitor, with components above the interaction site reduced and components below oxidized (the "crossover" theorem). The existence of state 6 in calcium-treated mitochondria (Chance and Schoener, 1966) already shows the unlikelihood of this being true for all systems under all conditions.

Methods

Keilin–Hartree submitochondrial particles were prepared by the original procedure (Keilin and Hartree, 1940) modified by the substitution of blending (large stainless-steel Waring Blendor) for sand grinding and ultracentrifugation at pH 7.4 for low-speed centrifugation at pH 5.6. The preparation will be described elsewhere in more detail.

Double-beam measurements were made with the Aminco–Chance spectrophotometer. Spectra were obtained with the Cary 14 spectrophotometer with scattered transmittance accessory. Some activity measurements were carried out with a Zeiss PMQ II spectrophotometer with attached Sargent SRL recorder. Manometric observations of activity were made with the Gilson differential respirometer.

NADH was obtained from Sigma Chemical Co. (type III) as was Sigma type III horse heart cytochrome *c*. Phenazine methosulfate was obtained from California Biochemicals; tetramethyl-*p*-phenylenediamine and succinic acid from British Drug Houses. Zinc sulfate was Baker Analyzed Reagent grade.

Results

Spectrophotometric Observations. Figure 1 shows the change in steady states of cytochromes *b*, $c + c_1$, and "flavoprotein" (505–470 mμ) upon the addition of Zn^{2+} to Keilin–Hartree particles oxidizing succinate in the steady state. Under these conditions, the primary site of interaction appears to be at the same site as antimycin. Moreover, the decrease in turnover, as indicated by the increase in anaerobiosis time, was matched by corresponding decreases in the levels of oxidized cytochrome *b* and reduced cytochrome *c* (Table I).

For systems saturated with both substrate and oxygen, that is, in which active flavoprotein is

* From the Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York. *Received May 31, 1967.* The work described was supported by U. S. Public Health Service Grants 1-S01-FR-05400, GM 06241 (to Dr. W. B. Elliott), and GM 11691 (to P. N.). A. N. M. has a travel grant from C.S.I.R., New Delhi.

[1] Abbreviations used: SDH, succinic dehydrogenase; f_p, flavoprotein; *b*, cytochrome *b*; *c*, cytochrome *c*; NADH, diphosphopyridine nucleotide (reduced); a_3, cytochrome a_3; *a*, cytochrome *a*; PMS, phenazine methosulfate; TMPD, tetramethyl-*p*-phenylenediamine; DIB, *n*-butyl 3,5-diiodo-4-hydroxybenzoate.

TABLE I: Steady-State Changes and Inhibition of Electron Transfer by Zinc.

System	% Reduction in Steady State[a]					TN (sec^{-1})[e]	
	fp (NADH)	fp (suc)	b	$c + c_1$	a	Succinate	NADH
Normal[b]	45	34	57 (71)[c]	60 (57)	−(31)	7.2	7.9
+330 μM Zn^{2+}	13[d]	19[d]	74 (88)	45 (45)	−(23)	4.0	4.0
Per cent oxidized change	*d*	*d*	40 (58)			% inhibn	
Per cent reduced change	*d*	*d*		25 (21)	−(26)	45	50

[a] Reduction as per cent anaerobiosis figure. [b] Normal system contained ~2 μM heme *a* in 3.0 ml of 60 mM phosphate (pH 7.4) (~3 mg of particle protein), 25°, 3.3 mM succinate (all data except fp (NADH) reduction), and 0.83 mM NADH (reduction of fp (NADH). [c] Figures in parentheses indicate data from Cary 14 spectra. [d] See text under Discussion. [e] Electrons per second per heme *a*.

~100% reduced and a_3 is ~100% oxidized, an inhibition at site II will be characterized by such proportionate changes in *b* and *c*. The observations are therefore quite consistent with such an interaction as the primary one, with 50% inhibition between 0.3 and 0.35 mM Zn^{2+}.

Table I also indicates a "reversed crossover" site between fp and cytochrome *b*, suggesting further complications. It was concluded, however, that this effect was a spurious one, and did not reflect an interaction between zinc and flavoprotein (see below).

An approximate value for the apparent K_i for Zn^{2+} in this system may be obtained from eq 1

$$\frac{V_0[b^{2+}]_i[c^{3+}]_i}{V_i[b^{2+}]_0[c^{3+}]_0} = \frac{K_{i\,app} + [Zn^{2+}]}{K_{i\,app}} \quad (1)$$

where V_0 and V_i are the velocities of the uninhibited and inhibited reactions, respectively, and $[b^{2+}]$ and $[c^{3+}]$ the steady-state concentrations of reduced *b* and oxidized $c + c_1$ in the two reactions as indicated by the subscripts. For the values in Table I, this gives $K_{i\,app} \simeq 120$ μM. One of the difficulties involved in interpreting such data, however, is that the proportion of *b* reduced does not go on increasing indefinitely as $[Zn^{2+}]$ increases. Indeed, at high $[Zn^{2+}]$/[protein] ratios obtained either by increasing $[Zn^{2+}]$ or decreasing the quantity of particles present, the increased reduction of *b* disappears, to be replaced by an apparent oxidation, as

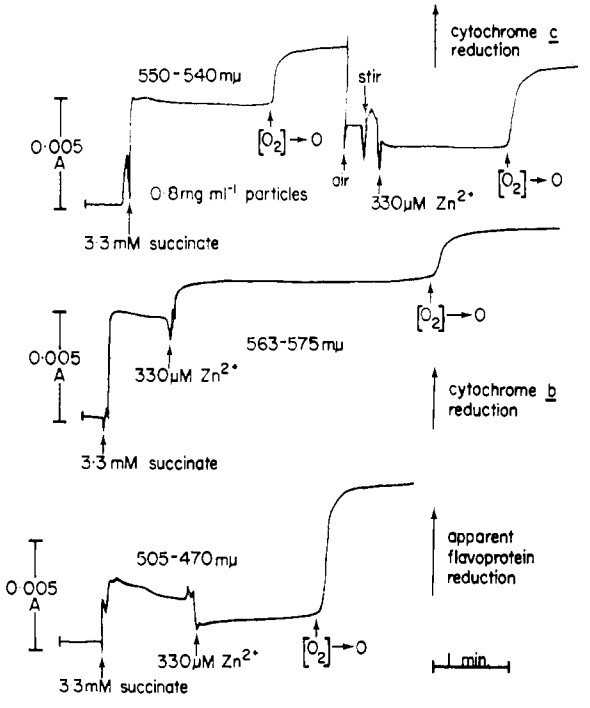

FIGURE 1: Effect of zinc on steady-state reductions of cytochromes *b*, $c + c_1$, and "flavoprotein" in Keilin–Hartree particles oxidizing succinate; pH 7.4, 60 mM phosphate buffer, 25°. Beef heart particles partially deficient in cytochrome *c* were employed; 3-ml final volume in 1-cm cuvet.

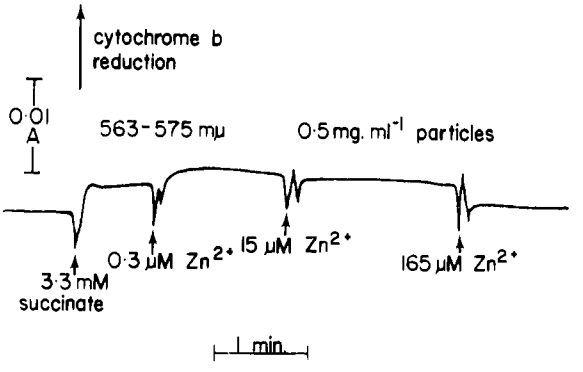

FIGURE 2: Effect of varying zinc concentration on the response of cytochrome *b* in Keilin–Hartree particles oxidizing succinate in the steady state. Conditions as in Figure 1.

shown in Figure 2. This failure of cytochrome *b* to become reduced may extend even to the anaerobic state, and to the other cytochromes. In the absence of zinc, succinate reduces the cytochromes of the Keilin–Hartree particle, on anaerobiosis, to the same extent as dithionite (except for failure to reduce a small fraction of the cytochrome *b*). In the presence of 3.3 mM Zn^{2+}, however, the cytochromes are only some 60–70% reduced on anaerobiosis with succinate, and dithionite addition is necessary to give full reduction. At such concentrations of zinc a physical action on the particles is indicated by a decrease in the light scattering of the suspension.

Evidently, there have developed here "secondary" effects of Zn^{2+} binding, effectively destroying the catalytic activity of the particles at the dehydrogenase level. These effects, as characterized by a general failure to reduce any cytochrome, occur more rapidly when NADH is used as substrate. That is, it is more difficult to find conditions for NADH giving steady-state changes analogous to those of Table I.

Manometric Observations. Parallel experiments carried out manometrically showed some kinetic differences from the spectrophotometric data. In the presence or absence of exogenous cytochrome *c*, with either succinate or NADH, Zn^{2+} tended to behave as a more powerful inhibitor in these systems. Table II compares the values observed for 50% inhibition of respiration with those obtained spectrophotometrically above, polarographically with digitonin particles (A. N. Malviya and W. B. Elliott, manuscript in preparation), and with mitochondria (Skulachev *et al.*, 1967). The sensitivity to zinc is evidently quite variable.

Although the concentration of protein was lower in the manometric experiments (0.25 mg ml^{-1}) than in the spectrophotometric ones (0.5–0.7 mg ml^{-1}), a manometric experiment using higher protein concentrations (~0.6 mg ml^{-1}), where succinoxidase activity was still linear with respect to protein, nevertheless gave a greater inhibition than under spectrophotometric conditions. If the manometric and polarographic inhibitions reflect secondary rather than primary processes, they may perhaps be expected to follow the relationship with K_i = 120 μM (eq 1) rather than the spectrophotometric phenomenological one. Great significance should not be given to the absolute values in Table II, however, because: (a) preincubation conditions were different, (b) the free Zn^{2+} concentration is unknown, depending on available protein and other binding sites, and (c) in some cases (A. N. Malviya and W. B. Elliott, manuscript in preparation), there was a residual respiration even at high zinc concentrations.

It was possible to show with the "bridging" compounds phenazine methosulfate (PMS) and tetramethyl-*p*-phenylenediamine (TMPD) that the inhibition observed manometrically was *not* simply a block at the level inhibited by antimycin (Table III). Thus, TMPD, an effective bypass for antimycin inhibition (Packer and Moustafa, 1966), is entirely without effect on the zinc inhibition of succinoxidase. TMPD does not react directly with the flavoprotein but probably at the cytochrome *b* level. PMS, a bypass for both antimycin and thenoyltrifluoracetone inhibitions, partially relieves the inhibition by Zn^{2+}. This acceptor can react directly with succinic dehydrogenase (Singer and Kearney, 1963).

TABLE II: Concentrations of Zinc Required for 50% Inhibition of Respiration.

Method	Particles	Concn Giving 50% Inhibn: Succinate as Substrate (μM Zn^{2+})	Concn Giving 50% Inhibn: NADH as Substrate (μM Zn^{2+})
Spectrophotometric[a]	Keilin–Hartree	~300	~300
Manometric[b]	Keilin–Hartree	10–15	20–40
Polarographic[c]	Digitonin	5–10	10–15
Polarographic[d]	Mitochondria (intact)	~5	~5

[a] Conditions as in Figure 1; this paper. [b] 0.06 M phosphate (pH 7.4), 30°, 6 μM cytochrome *c*, 27 mM succinate, or 3.3 mM NADH, using Zn^{2+} concentrations from 5 to 600 μM; 0.25 mg ml^{-1} of protein, using particles with maximal Q_{O_2} of about 600 (μl of O_2 hr^{-1} mg^{-1}); this paper. [c] A. N. Malviya and W. B. Elliott, manuscript in preparation. [d] Skulachev *et al.* (1967).

TABLE III: Inhibition of Succinate Oxidation by Zinc and the Effects of TMPD and PMS on This Inhibition Observed Manometrically.

Substrate (27 mM)	Inhibitor: None	3.3 μM Antimycin	380 μM TTFA	33 μM Zn^{2+}
Succinate	100[a]	0	3	10
+TMPD	102	53[b]	7	4
+PMS	124		50	20

[a] Uninhibited rate was from 9 to 11 μl min^{-1} mg^{-1} of protein, equivalent to a turnover number of ~14 sec^{-1} (electrons/heme *a*). [b] Increased to 100% as [TMPD] was increased to 400 μM. 30°, pH 7.4, 67 mM phosphate, 7.6 μM cytochrome *c*, ~0.4 mg of protein/flask (1.5 ml); 83 μM TMPD and 0.26 mg ml^{-1} PMS final concentrations.

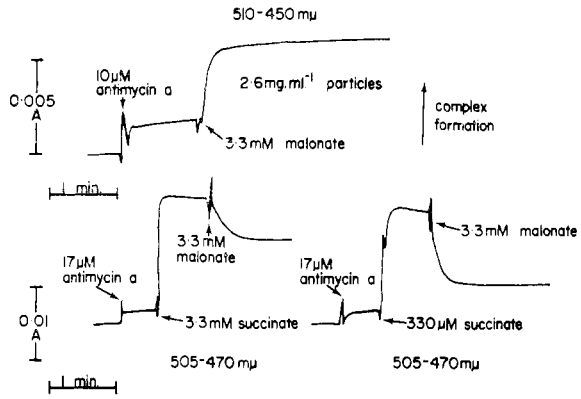

FIGURE 3: Spectral changes in Keilin–Hartree particles induced by malonate in the presence and absence of succinate. Conditions as in Figure 1. Malonate forms a spectroscopically detectable complex (510–450 mμ) but partially reverses the effect of succinate (505–470 mμ) depending on the succinate concentration.

TABLE IV: Authentic and Spurious Reductions Observed in the Flavoprotein Region.

	Δ Absorbancy (505–470 mμ)	
Substrate	Steady State	Anaerobiosis
Succinate	0.030	0.078
NADH	0.035	0.070
Succinate + NADH	0.057	0.113
Ascorbate + TMPD[a]	0.030	0.048 (→ 0.078)[b]
Succinate + antimycin	0.041	
Succinate + malonate	0.005	0.078
Succinate + antimycin + malonate	0.026	

[a] Plus 0.25 μM exogenous *c*. [b] Slow rise to this level. Approximately 3 mg of particles/3 ml, equivalent to 1.2 μM heme *a* or 0.23 μM SDH flavoprotein (see text); 3.3 mM succinate, 0.8 mM NADH, 8.3 mM ascorbate, 0.33 mM TMPD, 10 μM antimycin, and 3.3 mM malonate used where indicated. Final volume ~3 ml of 60 mM sodium potassium phosphate, pH 7.4, 25°.

The manometric results thus give, indirectly, a "crossover" site between flavoprotein and cytochrome *b*, consistent with the "secondary" effect obtained spectrophotometrically. What is the nature of this secondary effect? Does it result from changes in the particle following the binding of Zn^{2+} at the primary site, or does it require an attack by another Zn^{2+} ion? To study this question, a more detailed investigation of spectroscopic effects in the flavoprotein region was undertaken.

The Site between Flavoprotein and b. As indicated in Figure 1 and Table I, anomalous results were obtained from observations in the flavoprotein region (470–505 mμ), which indicated an apparent oxidation, instead of the expected reduction. Such an effect, of course, would suggest either a *promotion* by zinc of the flavoprotein–cytochrome *b* reaction, or a site of inhibition on the substrate side of the flavoprotein. The former is unlikely as zinc was never observed to stimulate respiration; the latter should lead to competition between zinc and substrate, also unexpected and unobserved. The flavoprotein reaction was therefore studied more closely.

As shown in Table IV both succinate and NADH reduce the "flavoprotein," the effects of the two being not quite additive. Table IV and Figure 1 also show that the apparent reduction of the flavoprotein in the steady state is only about 50% of that observed in anaerobiosis, despite the fact that a saturating amount of substrate has been used. This strongly suggests that the changes in the flavoprotein "region" reflect changes in the cytochromes as well as those in the flavoproteins proper. This was originally suggested by Klingenberg and Bücher (1959), and is also discussed in a recent review (Pullman and Schatz, 1967).

Indeed, it is possible to ask whether succinic dehydrogenase, for example, is reduced at all by succinate in these particles. Dervartanian and Veeger (1964, 1965) have shown that only a small spectroscopic change occurs on the binding of succinate to oxidized succinic dehydrogenase. Under what circumstances, then, will the spectrum of fully reduced dehydrogenase appear? Presumably, only on the addition of a non-substrate reductant, such as dithionite. On the other hand, electrons can flow from succinic dehydrogenase to other flavoproteins and reduce them. Conversely, electrons can flow from NADH dehydrogenase to succinic dehydrogenase (Slater, 1950) and presumably cause its complete reduction (Pullman and Schatz, 1967).

Table IV shows that an appreciable reduction occurs at the 470–505-mμ region with a substrate such as ascorbate–TMPD, reacting at the level of cytochrome *c* and c_1. Such an effect could be predicted by an inspection of the absorption data published by Margoliash and Frohwirt (1959). Similarly, the presence of antimycin, which increases the reduction of cytochrome *b* (Table IV), decreases the apparent reduction in the flavoprotein region (*cf.* antimycin "steady state" with normal anaerobiosis).

The absolute amount of succinic dehydrogenase may, nevertheless, be estimated approximately from the changes in the particles brought about by the addition of malonate (Dervartanian and Veeger, 1964). These spectral changes could be observed either from difference spectra in the Cary 14, using the expanded scale device, or from the 510–450-mμ difference in the double-beam machine. The ΔE_{mM}, oxidized minus malonate reacted, for this wavelength pair should be 5.2 approximately. Both methods gave 1.1–1.2 μM dehydrogenase

TABLE V: Calculated Contributions of Several Pigments to Observed Changes in the 505–470-mμ Region on Addition of Succinate.

Component	Reduced-Form Concn (μM)		505–470-mμ Contribution		
	Steady State	Anaerobiosis	ΔE_{mM}	Steady State	Anaerobiosis
$c + c_1$	0.2[a]	0.4[a]	5.3[b]	0.011	0.022
b	0.3[a]	0.6[a]	3.0[c]	0.009	0.018
fp (S)	0.23[d]	0.23[d]	7.6[e]	0.017	0.017
fp (D)	0[c]	0.20[c]	10[c]	0	0.020
	Sum of calculated changes (see Table III)			0.037	0.077
	observed changes with succinate			0.030[a]	0.078[a]

[a] Direct observation in double beam, using $\Delta E_{mM}^{550-540} = 19(c + c_1)$ and $\Delta E_{mM}^{563-575} = 16(b)$. [b] From Margoliash and Frohwirt (1959). [c] Assumed. [d] From malonate experiments (510–450 mμ) using Dervartanian and Veeger (1964). [e] From Dervartanian and Veeger (1964).

for a preparation containing 6–7 μM heme *a* and 5–6 μM cytochrome *b*. This value corresponds to about 0.2 μmole of SDH/g of protein. This may be compared with figures of 0.12 (King *et al.*, 1964) and 0.14 (Singer *et al.*, 1962) obtained by measuring the bound flavin present. These authors also emphasized the difficulty in determining flavin spectrophotometrically, even in digested preparations, when other pigments including heme compounds are present. Figure 3 shows the effect of malonate alone upon the particles, and its influence on the apparent reduction produced by succinate.

Table V breaks down the observed changes in absorption at 470–505 mμ and gives the contribution due to each component of the system. The large component in this region contributed by cytochrome *c* (*cf.* Klingenberg and Bücher, 1959) must be taken into account when considering claims such as that of Chance (1966), that the rates of flavoprotein and cytochrome reduction by succinate in cyanide-treated particles are equal. Evidently the changes in this region actually due to flavoprotein are so small, at least in the succinate reaction, that no useful conclusions can be drawn. Only the fluorometric method can therefore be depended upon to give useful data on the redox state of the flavoprotein. There is, in any case, no compelling reason to expect changes in the spectrum of flavoprotein when the flux between flavoprotein and *b* changes. The probable influence of zinc in this region (Figure 1, Table I) cannot therefore be determined by these methods.

Reversibility of Zinc Inhibition. The existence of "primary" and "secondary" interactions involving zinc might suggest that the heavy metal effect was irreversible, possibly catalytic in nature. Although this may eventually be so, Figure 4 shows that at least part of the initial effect may be reversible by the addition of a suitable chelating agent.

EDTA (1.6 mM) almost completely reverses the reduction of cytochrome *b* induced by 50 μM Zn^{2+} in the succinate steady state. A similar reversal can be observed in the NADH steady-state system. Manometric experiments also showed almost complete reversal by 1 mM EDTA of the inhibition produced by 133 μM Zn^{2+}.

Discussion

Is it now possible to define the site of inhibition by zinc in the respiratory chain? The idea of primary and secondary effects (Figures 1–3) may be represented by the schemes of Figure 5.

In the first stage of inhibition, recognized for mitochondria by Skulachev *et al.* (1967), an antimycin-like activity is observed. In the second stage, found in digitonin particles by A. N. Malviya and W. B. Elliott (manuscript in preparation), this is followed by an

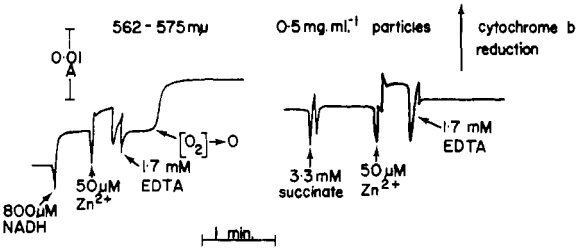

FIGURE 4: Reversal of Zn^{2+}-induced cytochrome *b* reduction by EDTA. Conditions as in Figure 1.

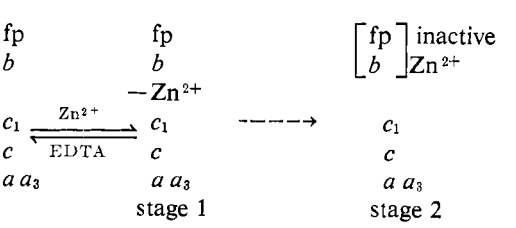

FIGURE 5: Patterns of inhibition by zinc in nonphosphorylating submitochondrial particles.

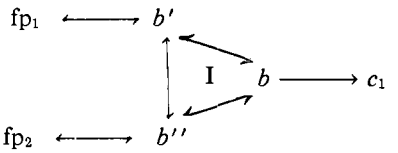

FIGURE 6: Possible pathways of electron transfer at the substrate end of the respiratory chain.

inactivation of electron transport at the flavoprotein end of the chain. Under these conditions, the interchain electron transfer is also cut down, and full reduction of all components does not occur, even upon anaerobiosis, unless dithionite is added.

Consider the electron-transfer pattern of Figure 6, where an inhibitor (I) blocks in varying degree the interactions between the several *b*-type components. Then if electrons are introduced at fp_1, "*b*" may appear more reduced (if *b'* predominates spectrally, and the *b'* → *b* transfer is inhibited) or more oxidized (if *b* or *b''* predominate spectrally). Similarly, flavoprotein may appear more reduced (if fp_1 is reduced during its activity) or more oxidized (if fp_2 is reduced during electron transfer) in the presence of I.

Furthermore, it is not necessary to assume, as do Skulachev *et al.* (1967), that "secondary" sites have lower affinities than "primary" sites. Such an assumption would make it difficult to interpret the manometric data (Tables II and III). It is possible that the "secondary" sites have a higher affinity, but lower reaction velocity, than the "primary" site. Alternatively, the secondary effect may be the result of an intraparticulate change following the primary binding, as suggested in Figure 5.

Finally we may ask: Is it meaningful to speak of of "above" and "below" at the level of flavoprotein? According to Pullman and Schatz (1967), "the application of the ingenious crossover theorem to the branched flavin region of the respiratory chain is not without ambiguity, especially since this region contains two spectrally indistinguishable flavins capable of mutual interaction." Nevertheless, Chance and Hollunger have claimed on the basis of spectrophotometric studies, that the site of action of amytal lies between NADH and flavoprotein in phosphorylating mitochondria, while in nonphosphorylating particles (Estabrook, 1957) the inhibition occurs between flavoprotein and cytochrome *b*. Changes in the 465–500-mμ region in phosphorylating mitochondria may, however, be due either to flavoproteins other than NADH dehydrogenase or to changes in the cytochrome (Table IV) (Pullman and Schatz, 1967). The experiments with nonphosphorylating particles (Estabrook, 1957) were carried out in the absence of cytochromes *c*, *a*, and a_3. The particles were initially completely oxidized, and the rates of reduction of flavoprotein, *b*, and c_1 were followed after the addition of substrate (NADH) in presence and absence of amytal. A reduction at the flavoprotein wavelength (465–500 mμ) preceded the very slow effects at the wavelengths characteristic of *b* and c_1.

It is possible to conclude that the site of inhibition by amytal may thus be between flavoprotein and cytochrome *b* in both phosphorylating and nonphosphorylating systems. The greater sensitivity of phosphorylating systems to amytal inhibition may then be amenable to a "kinetic" explanation. Thus, in the experiments considered (see Figures 2 and 4 of Chance and Hollunger, 1963), the initial rate of respiration in state 3 was almost twice that in the DIB uncoupled state.

Similarly, we are able to conclude that when acting as an inhibitor, zinc behaves in the same way toward Keilin–Hartree particles as it does toward digitonin fragments and intact mitochondria. To show the similarity, it is necessary to ensure the same experimental conditions, in particular to use approximately the same ratios of zinc to protein. Effects of zinc on ion uptake, either by being transported itself, or by affecting the transport of other cations, cannot of course be compared in Keilin–Hartree particles.

Acknowledgments

We express our appreciation to Dr. W. B. Elliott for providing facilities for the spectrophotometric experiments and for his interest and encouragement, and to Miss Mary Park for her technical assistance.

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