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Thermal Denaturation of the Heart Muscle Preparation with Respect to Its Capacity for DPNH Oxidation*

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The effects of mild thermal treatment of the Keilin-Hartree heart muscle preparation on its catalytic activity toward DPNH oxidation by oxygen, cytochrome *c*, 2,6-dichlorophenolindophenol, and ferricyanide have been studied in the presence and absence of Amytal and antimycin A. DPNH → oxygen and DPNH → cytochrome *c* activities were most sensitive to the thermal inactivation, whereas cytochrome oxidase activity was almost unaffected. Both antimycin and Amytal failed to inhibit completely any of the activities studied. The inhibitor-insensitive activities for various acceptors exhibited markedly different thermal susceptibilities when compared with the corresponding inhibitor-sensitive activities. The results suggested that, on the respiratory chain of the non-phosphorylating system, essentially only one site is available for ferricyanide and this site is insensitive to Amytal and antimycin. There are two sites for cytochrome *c*: one is inhibited by Amytal and antimycin, whereas the other is not. Dichlorophenolindophenol may interact at three sites.

Information about the effect of aging on the oxidative capabilities of particulate preparations from heart muscle is, in general, very meager; this is especially true for the oxidation of DPNH. Slater (1949b) has reported that the succinate oxidase activity of the Keilin-Hartree preparation increases upon storage at 4° for a short period but declines after several days. Recently, Redfearn and Dixon (1961) have shown that the succinate oxidase and succinate phenazine reductase activities of the heart muscle particles prepared with a Waring blendor are unstable even when the preparation is stored at −20°.

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TABLE I
SUMMARY OF ASSAY PROCEDURES[a]

Donor	Conc. (mM)	Acceptor	Conc. (mM)	Buffer	*p*H	Reaction Measured at (mμ)	References
DPNH	0.177	Ferricyanide	0.63	Tris	8.1	420	King and Howard, 1960
DPNH	0.088	DCIP	0.04	Phosphate	7.4	600	Savage, 1957
DPNH	0.088	Cytochrome *c*	0.042	Phosphate	7.4	550	King and Howard, 1960
DPNH	0.088	Oxygen (air)		Phosphate	7.4	340	Slater, 1950
Reduced *c*	0.020	Oxygen (air)		Phosphate	7.0	550	Smith, 1955

[a] All assays were performed in 1-cm cuvets with a final volume of 3 ml at 25°. The system for the DPNH oxidase assay also contained 8 μM cytochrome *c*, and in all systems with acceptors other than oxygen, cyanide was added to 1 mM. The phosphate buffer was the Sorensen type containing a mixture of Na_2HPO_4 and KH_2PO_4. Tris buffer was prepared from Tris and acetic acid. All buffers were present at a final concentration of 0.067 M.

However, no kinetic studies similar to those on the thermal denaturation of simple enzymes have as yet been documented. Such studies not only would yield practical information about the stability of the preparation but also might shed some light on the sites of reaction of electron acceptors with the respiratory chain.

This paper will be concerned with the effect of mild thermal treatment on the catalytic activity of the Keilin-Hartree heart muscle preparation toward DPNH oxidation by ferricyanide, cytochrome *c*, 2,6-dichlorophenolindophenol (DCIP),[1] and oxygen.

METHODS AND MATERIALS

The Keilin-Hartree preparation was prepared from beef heart according to the method of Keilin and Hartree (1947) as adapted in this laboratory (King, 1961). The washed heart mince was extracted once with phosphate buffer, *p*H 7.4. The pellet from centrifugation was suspended in a phosphate-borate mixture, 0.1 M with respect to both Na_2HPO_4 and H_3BO_3 (King, 1961), to give a final protein concentration of about 20 mg per ml. The borate-phosphate buffer was used in preference to phosphate alone because of its weak antibacterial action (Bonner, 1954).

The thermal treatment was carried out by rapidly heating the heart muscle preparation at a concentration of 20 mg per ml to the desired temperature and then maintaining it in a thermostat within ±0.05°. Samples were then removed after appropriate time intervals and immediately cooled at 0°. Since the thermal inactivation showed a high temperature coefficient, this method sufficed to prevent any further inactivation.

The systems used for activity determinations are summarized in Table I. The activity of the preparation was calculated from the initial, zero-order rate of reaction in assay.

[1] Abbreviations used: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; DCIP, 2,6-dichlorophenolindophenol; Tris, tris(hydroxymethyl)-aminomethane.

Protein was determined either with trichloroacetic acid (Slater, 1949a) or with the biuret reagent (Gornall *et al.*, 1949), in the presence of 2% deoxycholate. Ninhydrin-reactive substances were determined according to Moore and Stein (1954). The coenzyme Q (ubiquinone) content of the preparation was determined after the method of Redfearn and Pumphrey (1960).

RESULTS

The Effect of Antimycin A and Amytal upon DPNH Oxidation by the Unaged Preparation.—Figure 1 shows the effect of antimycin A and Amytal upon the DPNH → cytochrome *c* activity of the Keilin-Hartree preparation. This activity was not completely blocked by either inhibitor. The addition of antimycin alone up to 0.2 μg/mg of enzyme protein caused a sharp fall in the observed DPNH → cytochrome *c* activity to about 15% of the uninhibited value. However, beyond this point, further increase of antimycin, up to 0.5 μg/mg protein, resulted in no further inhibition. Similar results were obtained with Amytal. At concentrations of Amytal up to 2 mM, the DPNH → cytochrome *c* activity fell rapidly to 15% of the original level. Further increase of the Amytal concentration to 5 mM caused no additional inhibition. It was also noted that the effects of these two inhibitors were not additive. Thus when successively larger quantities of antimycin were added to a preparation containing 3 mM Amytal, the activity remained constant at 15% of its original value.

Analogous effects were observed for the DPNH → DCIP activity. As shown in Figure 2, this activity was reduced by antimycin at 0.2 μg/mg of protein to 85% of its original value but no further inhibition was produced even when the antibiotic was increased to 0.5 μg/mg. Amytal at 2 mM reduced the activity to 67% of its original level with no further decrease when the concentration was raised to 5 mM. Again, the inhibitory effects were not additive; the addition of antimycin to the preparation which had been inhibited by 3 mM Amytal caused no further inhibition.

Table II summarizes the inhibitory actions of antimycin and Amytal on the catalytic activity of the heart muscle preparation. The results for DPNH → O_2 were similar to those noted for DPNH → cytochrome *c*. However, DPNH → ferricyanide was much less sensitive to the inhibitors. Antimycin had practically no effect at the concentrations used here, and Amytal inhibited at the most only 8% of the activity.

TABLE II

THE EFFECT OF AMYTAL AND ANTIMYCIN A UPON THE DPNH → CYTOCHROME *c*, DPNH → DCIP, DPNH → FERRICYANIDE, AND DPNH → O_2 ACTIVITIES OF THE HEART MUSCLE PREPARATION[a]

	% Control Activity			
Acceptor	Control	+ Antimycin	+ Amytal	+ Amytal + Antimycin
Cytochrome *c*	100	15	15	15
DCIP	100	85	65	65
Ferricyanide	100	100	>95	>95
Oxygen	100	5	5	5

[a] Details of the assay systems are given in Table I. Where indicated, Amytal was added to the assay medium to 3 mM. The enzyme was preincubated with antimycin at 0.5 μg/mg protein for 30 minutes at 0°.

Redfearn and Pumphrey (1960), using pig heart sarcosomal preparations, have succeeded in quantitively extracting the endogenous coenzyme Q by means of a mixture of methanol and petroleum ether. By their method, fully aerated samples of the heart muscle preparation were reduced by DPNH in the presence of cyanide. The cyanide was used to inhibit the reoxidation of reduced endogenous coenzyme Q by cytochrome oxidase. After a suitable time interval, the reaction was stopped by the addition of cold methanol and then petroleum ether and the content of reduced coenzyme Q was determined spectrophotometrically. Figure 3 shows the results thus obtained. At 0° in the absence of Amytal, DPNH rapidly reduced a portion of the total coenzyme Q, the reduction being essentially complete in 30 seconds. However, in the presence of Amytal, no reduction of coenzyme Q was observed even after a prolonged reaction.

Thermal Inactivation of the Keilin-Hartree Preparation.—The preparation is remarkably stable. Very little change of oxidative activity could be detected when the preparation was aged below 30° for 12 hours. However, above this temperature a significant loss of catalytic activity was observed which became relatively rapid at 40°. This effect is illustrated in Figure 4. The rates of loss of DPNH → O_2 and DPNH → cytochrome *c* were rapid, whereas DPNH → ferricyanide and cytochrome *c* → O_2 activities were lost much less readily. The rate of loss of DPNH → DCIP activity was intermediate between the two extremes. In general, apart from a slight initial rise at the beginning of the thermal treatment, the fall in activity obeyed first-order kinetics. The first-order rate constant, k_1, was fairly constant throughout the aging process.

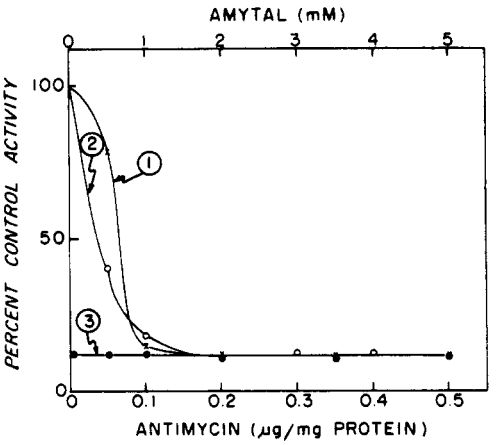

FIG. 1.—The effect of Amytal and antimycin A on the DPNH → cytochrome *c* activity of the heart muscle preparation. Curves (1) antimycin, (2) Amytal, and (3) antimycin in the presence of 3 mM Amytal. Assays were performed as in Table I. Amytal was added directly to the assay mixture. Antimycin was preincubated with the enzyme for 30 minutes at 0° before assay.

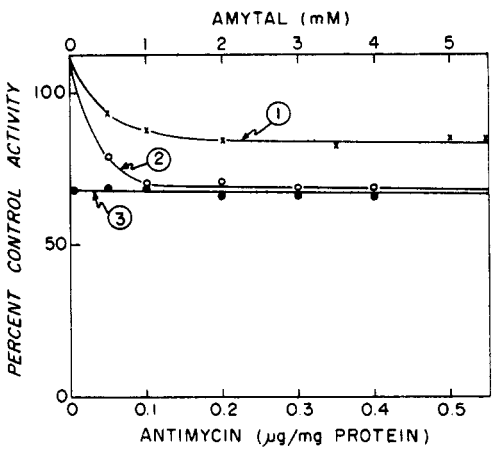

FIG. 2.—The effect of Amytal and antimycin A on the DPNH → DCIP activity of the heart muscle preparation. Curves (1) antimycin, (2) Amytal, and (3) antimycin in the presence of 3 mM Amytal. Other experimental details are as described in the legend of Figure 1.

In view of the apparent complexity of the overall thermal decay curves, a study was also made of the decay of the DPNH → cytochrome *c*, DPNH → DCIP, and DPNH → O_2 activities in the presence of Amytal and antimycin. Table III and Figure 5 summarize the experimental results. When cytochrome *c* was the acceptor (Fig. 5), a very large difference was noted in the rates of inactivation of the Amytal-sensitive and

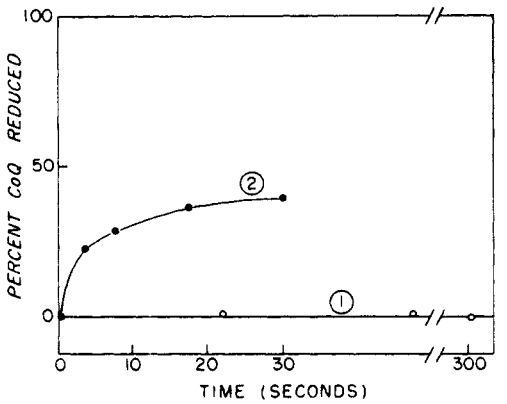

FIG. 3.—The effect of Amytal on the reduction of endogenous coenzyme Q by DPNH. The reaction mixture contained the heart muscle preparation, 20 mg/ml in borate-phosphate buffer, *p*H 8.0; DPNH, 4.4 mM; and KCN, 7.6 mM; in the presence (curve 1) and absence (curve 2) of 6 mM Amytal. The reactions were carried out at 0°. The coenzyme Q content in the heart muscle preparation was found to be 6.5 μmoles/g protein.

Amytal-insensitive activities. Similar differences were observed for the DPNH → DCIP activities; the antimycin-insensitive but Amytal-sensitive activity, for example, decayed much faster than the antimycin- and Amytal-insensitive activity (column 4 *vs.* column 3 of Table III). DPNH → ferricyanide, Amytal-insensitive DPNH → cytochrome *c*, and Amytal-insensitive DPNH → DCIP activities (Fig. 6) were all lost at comparable rates.

The possibility that the denaturation of the heart muscle preparation was due to the action of contaminating proteolytic enzymes was considered. Measurements of the trichloracetic acid–soluble and ninhydrin-reactive components present in the preparation showed, however, no increase in either component with age. Thus, it was considered unlikely that the thermal inactivation was due to proteolysis.

It was observed that the denaturation of the Keilin-Hartree preparation was very temperature dependent, and subsequent calculation showed that the activation energies of the denaturation process for the various acceptors were high. By assuming that the denaturation processes followed first-order kinetics, plots of the logarithm of the rate constants (log k_i) against the reciprocal of the temperature ($1/T$) were obtained. The plots were linear for denaturation carried out at three different temperatures between 38° and 45°. Table IV summarizes the activation energies thus calculated according to the usual Arrhenius equation. Owing to experimental scatter, the absolute magnitudes of these parameters are accurate to within ±20%.

Estabrook and Minakami (personal communication, 1962) have shown recently that the DPNH → cytochrome *c* activity of the heart muscle preparation exhibits a pronounced lag period upon assay. Thus, after the addition of DPNH to the assay mixture, the rate of oxidation of DPNH gradually increased with time to a limiting, linear rate. Preincubation of the enzyme with DPNH and cytochrome *c* abolished the effect, which was not observed with any other acceptors. We confirmed these findings from almost 100 batches of the Keilin-Hartree preparation in the oxidation of DPNH by oxygen and by cytochrome *c*, but not by ferricyanide or DCIP, and noted also that the extent of the lag period increased upon thermal denaturation.

TABLE III

THE EFFECT OF AGING OF HEART MUSCLE PREPARATION ON ITS DPNH → DCIP ACTIVITY IN THE PRESENCE OF ANTIMYCIN A AND AMYTAL[a]

	1	2	3	4	5
	Control	+ Antimycin	+ Antimycin + Amytal	(2) Minus (3)	(1) Minus (2)
Age (Min.)		Antimycin-Insensitive	Antimycin- and Amytal-Insensitive	Antimycin-Insensitive but Amytal-Sensitive	Antimycin-Sensitive
	mμmoles DPNH oxidized/minute/mg of heart muscle preparation protein				
0	109.5	93.9	63.9	30.0	15.6
10	103.2	89.4	62.7	26.7	13.8
30	97.8	81.3	60.3	21.0	16.5
50	92.1	76.2	57.0	19.2	15.9
80	80.7	69.6	52.8	16.8	11.1
110	74.1	62.4	49.5	12.9	11.7
170	60.9	51.0	45.9	5.1	9.9

[a] See Table I for assay details. Amytal was added directly to the assay mixture at 3 mM. The enzyme was preincubated with antimycin at 0.5 μg/mg protein for 30 minutes at 0°. The aging temperature was 40.0°, with 10 minutes' preincubation prior to removal of the first sample.

TABLE IV

ARRHENIUS ACTIVATION ENERGIES FOR THE THERMAL DENATURATION OF THE HEART MUSCLE PREPARATION WITH RESPECT TO ITS DPNH → CYTOCHROME *c*, DPNH → DCIP, DPNH → FERRICYANIDE, AND DPNH → OXYGEN ACTIVITIES[a]

Acceptor	E_A (Kcal/mole)
Cytochrome *c*	92
DCIP	112
Ferricyanide	114
Oxygen	92

[a] E_A was calculated for each acceptor from the slope of a plot of log k_i against $1/T°K$ in the range of 38–45°. Outside of this range, the plots were no longer linear. Results were the average of two experiments.

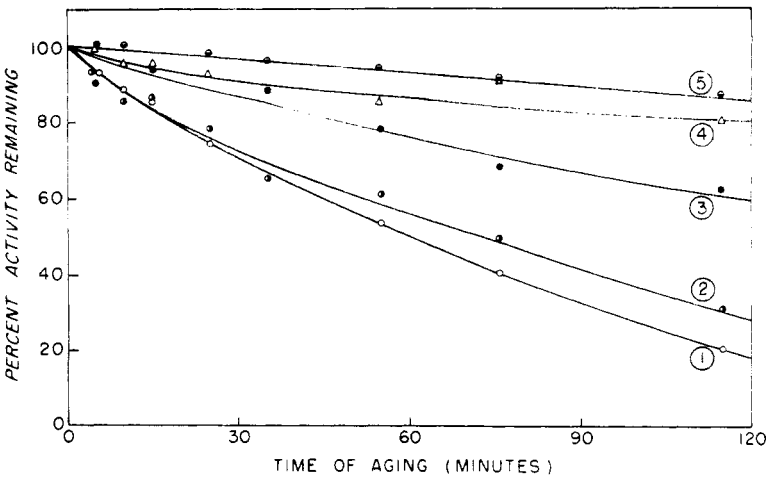

FIG. 4.—Over-all rates of loss of activities on thermal denaturation of the heart muscle preparation. Curves (1) DPNH → O_2, (2) DPNH → cytochrome *c*, (3) DPNH → DCIP, (4) cytochrome → O_2, and (5) DPNH → ferricyanide. See Table I for the assay system. Thermal denaturation was conducted at 42.2°. The heart muscle preparation was preincubated for 5 minutes at this temperature prior to removal of the first sample.

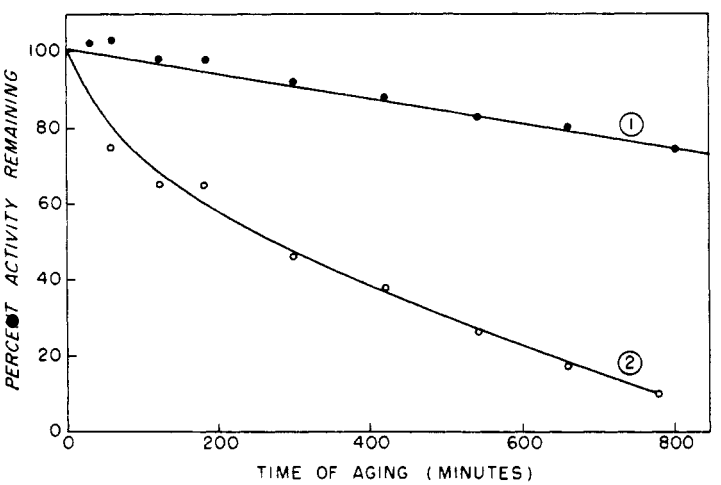

FIG. 5.—Relative rates of loss of DPNH → cytochrome *c* activities upon thermal denaturation of the heart muscle preparation. Curve (1) the Amytal-insensitive activity and (2) the Amytal-sensitive activity. The aging was performed at 35.3°, and the first sample was removed after incubation at 35.3° for 3 minutes. Amytal was added directly to the assay mixture to the final concentration of 3 mM. See Table I for the assay details.

DISCUSSION

The Keilin-Hartree preparation was selected for this study because it contains a powerful respiratory DPNH oxidizing system (Slater, 1950) and it has been used as a source in the isolation of a number of DPNH dehydrogenating enzymes (*cf.* King *et al.*, 1961).

Thermal denaturation of simple proteins and enzymes is characterized by high values of the Arrhenius activation parameters; values of the activation energy (E_A) in the range of 50–100 kcal/mole are common (Laidler, 1958). The activation energies for the thermal inactivation of the Keilin-Hartree preparation (Table IV) with respect to its catalytic activity in DPNH oxidation fall within the expected range, indicating the similarity of the denaturation process with that operating in the simpler systems. The fact that the DPNH → cytochrome *c* activity paralleled the loss of DPNH → O_2 activity and that both were greater than the loss of cytochrome oxidase and DPNH → ferricyanide activities (Fig. 4) strongly suggests that the main point of thermal inactivation lies between the DPNH flavoprotein and the main site (*vide infra*) of interaction of cytochrome *c* with the chain. It is not possible, however, with the present information to localize it further.

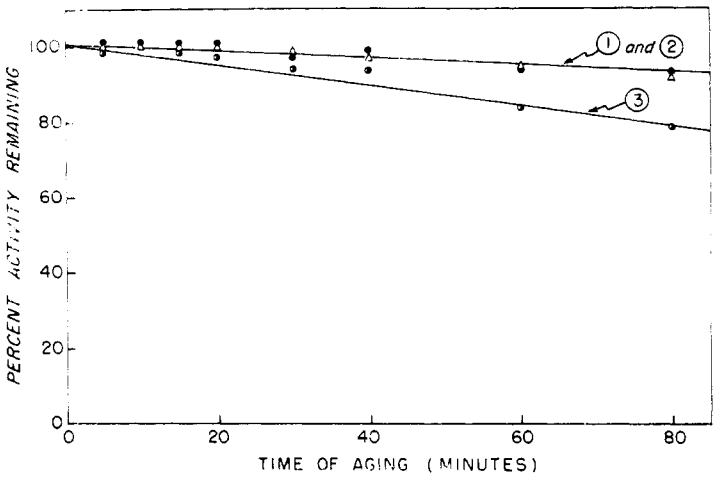

FIG. 6.—Thermal denaturation of the DPNH → ferricyanide, Amytal-insensitive DPNH → DCIP, and Amytal-sensitive DPNH → cytochrome *c* activities of the heart muscle preparation. Curve (1) DPNH → ferricyanide, curve (2) Amytal-insensitive DPNH → DCIP, and curve (3) Amytal-sensitive DPNH → cytochrome *c*. Conditions and assay systems as described in Tables I and III. The temperature during aging was 42.2°.

Use of specific inhibitors in electron transport studies has yielded valuable information. Keilin (1927) recognized that narcotics inhibit intracellular respiration and used this fact as one of the means in assigning the sequence of cytochromes on the respiratory chain. Later, Chance (1956) found that in phosphorylating systems such as ascites cells and intact mitochondria, Amytal acts on the chain somewhere near the flavoenzyme or even directly on it. But Estabrook (1957) has shown that in nonphosphorylating systems the rate of reduction of both cytochromes *b* and c_1 by DPNH decreases to almost zero in the presence of Amytal, whereas the rate of reduction of flavoprotein appears to be as fast as in the uninhibited state. Estabrook has thus proposed that the site of Amytal inhibition is located at a point on the oxygen side of the dehydrogenase flavoprotein. Ernster *et al.* (1955) have, on the other hand, demonstrated in liver an Amytal-insensitive pathway for DPNH oxidation in addition to the usual sensitive route.

Since the discovery (Ahmad *et al.*, 1950; Potter and Reif, 1952) of antimycin A as a potent respiratory inhibitor, numerous studies have appeared in an attempt to determine its reaction site on the electron transport chain. Although no definite conclusions can yet be drawn as to the details of the inhibition mechanism, it is rather well established that the point sensitive to the antibiotic must be close to cytochrome *b* and on the substrate side of cytochrome c_1 (Chance and Williams, 1955, 1956; Estabrook, 1957; Slater, 1958). Since antimycin inhibits the oxidation of reduced coenzyme Q (Redfearn and Pumphrey, 1960), it must act on component(s) on the oxygen side of the quinone on the chain. Partial insensitivity of concentrated (2–4 mg/ml) sarcosomal preparations to antimycin was attributed (Estabrook, 1957; Thorn, 1956; Hatefi *et al.*, 1961) to the distribution of the inhibitor in the *protein* of the preparation leading to the reduction in its effective concentration at the sensitive site. This explanation does not seem sufficient to account for the inhibitor-insensitive activities described in this paper, since the protein concentrations employed (0.03 mg/ml in the assay mixture) were much lower than those used by the above workers. Two alternative explanations may be advanced to explain our results.

It may be suggested that the blocks imposed by antimycin or by Amytal at their respective points of action may not be complete. In other words, the inhibited site may allow reducing equivalents to "leak" through, giving rise to the inhibitor-insensitive activities noted. Clearly this hypothesis does not readily account for the observations on, for example, the different sensitivities of DPNH → DCIP and DPNH → cytochrome *c* activities toward the same inhibitor at the same concentration. Alternatively, it could be postulated that at the point of action of antimycin or Amytal the block is complete but that the inhibitor-insensitive activities arise from the chain from sites on the substrate side of the blocked point. Thus, in the case of DPNH → cytochrome *c* it would be necessary to envisage two sites at which cytochrome *c* could interact with the chain. The first, on the DPNH side of the Amytal inhibition point, would give rise to 15% of the activity insensitive to both Amytal and antimycin, while the second, comprising the bulk (85%) of the activity, would arise from a point on the oxygen side of the antimycin-sensitive site (*cf.* Table II and Fig. 8). Similar reasoning could be applied to the results observed for the DPNH → DCIP activity, although here the situation is more complex. Examination of Table II leads to the con-

clusion that the over-all DPNH → DCIP activity may be composed of three portions—firstly, that portion insensitive to both Amytal and antimycin; secondly, that sensitive to Amytal but insensitive to antimycin; and, finally, that sensitive to antimycin alone. Quantitatively, these activities were estimated approximately to be (1) 65% of the total from the DPNH side of the Amytal inhibition point, (2) 20% from the chain between the two inhibition points, and (3) 15% from the oxygen side of the antimycin inhibition point.

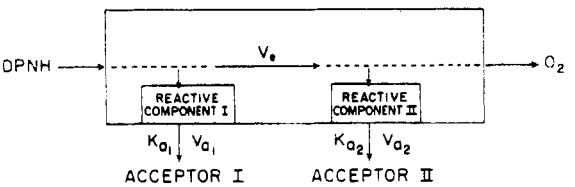

FIG. 7.—Hypothetical scheme of the interaction of artificial acceptors with the respiratory chain. Full arrows represent the flow of electrons. See text for the significance of the symbols.

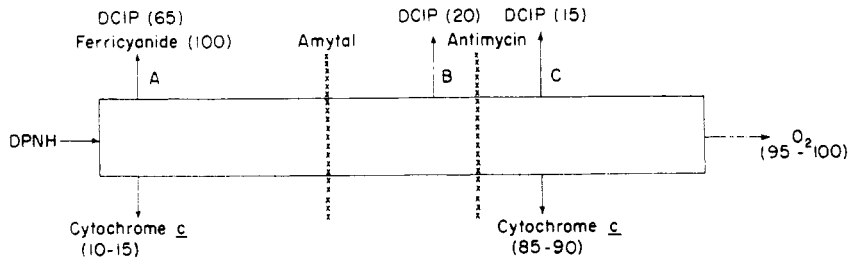

FIG. 8.—A diagrammatic representation of the acceptor loci on the non-phosphorylating respiratory chain. Lines with crosses (×) indicate inhibition. Full arrows denote the flow of electrons. Numbers in parentheses are the approximate percentages arising from each site of the total activity towards a given acceptor; *e.g.*, of the total DPNH → DCIP activity, 65% arises from site A, 20% from site B, and 15% from site C.

Although it is not yet clear whether coenzyme Q is on the main pathway of electron flow in nonphosphorylating particles, it is generally accepted that the quinone is in oxidation-reduction equilibrium with certain components of the main respiratory chain (Redfearn, 1960; Green, 1959, 1960; Chance, 1959, 1960; Slater *et al.*, 1960). The rate of reduction of endogenous coenzyme Q by DPNH was therefore measured. The results in Figure 3 clearly show that the block imposed on the chain by Amytal was complete and therefore that the aforementioned second hypothesis is probably correct, namely, that cytochrome *c* and DCIP interact with the chain at more than one site. The fact that there is a great difference in thermal susceptibilities between the Amytal-sensitive DPNH → cytochrome *c* and Amytal-insensitive DPNH → cytochrome *c* reactions, as depicted in Figure 5 and Table III, clearly rules out the possibility that cytochrome *c* is interacting at only one site. These observations can indeed serve as additional, independent evidence for the hypothesis. Comparison of the several DPNH → DCIP activities (Table III) leads to conclusions of a similar nature. There was a great difference between the rate of loss of the Amytal-insensitive activity and the rate of loss of the Amytal-sensitive, antimycin-insensitive activity.

On the other hand, all the results suggest that the electrons from DPNH may flow through essentially only one egress to ferricyanide, probably through the DPNH dehydrogenase flavoprotein. If any appreciable portion of the ferricyanide activity arose from the chain at points other than this one, the decay with age should have been much greater than that which was experimentally observed. However, these findings are in contrast to the results recently reported by Estabrook (1961) from experiments on intact mitochondria. Using ferricyanide concentrations in the range of 0.3–0.9 mM, Estabrook has found that the rate of the ferricyanide reduction by DPN-linked substrates is more than 90% inhibited by both Amytal and antimycin. Consequently, he has proposed that ferricyanide is reduced at a point on the oxygen side of the antimycin-sensitive site. Since exogenous cytochrome *c* stimulated the rate of reduction, Estabrook further argued that ferricyanide reacts directly with reduced cytochrome *c*. This discrepancy between results of the two schools may be due to the fact that different tissues (liver *vs.* heart) were used, or that in the conversion of mitochondria into non-phosphorylating particles a new site is exposed for ferricyanide interaction. The disparity in oxidative behavior between liver and heart preparations has been abundantly documented (see Slater, 1958; Ernster *et al.*, 1955). Likewise, variations between intact mitochondria and non-phosphorylating preparations with respect to their specificity toward electron acceptors and their reactions toward inhibitors are well known (*e.g.* Chance and Williams, 1956; Estabrook, 1957, 1961; Slater, 1958).

The validity of the above arguments is not affected by the fact that activities were measured at a finite concentration of the acceptor. However, under conditions described, direct comparison of the rates of thermal denaturation of different acceptor activities is not capable of yielding unequivocal information. For example, the rates of loss of DPNH → ferricyanide and Amytal-

insensitive DPNH → DCIP activities were practically identical (Fig. 6), and it is tempting to suggest that these two acceptor activities arise from the same site. Although this conclusion is probably true, it cannot necessarily be drawn from these experiments alone. The following reasoning illustrates this point. Adopting a classical enzyme-substrate model for the interaction of an acceptor with the chain, and postulating the existence of a reactive component on the chain responsible for interacting with the acceptor, the scheme shown in Figure 7 may be drawn. Here, V_e, V_{a_1}, and V_{a_2} are the maximum velocities of transfer of reducing equivalents through the chain, and from the chain components to each acceptor respectively. The symbols K_{a_1} and K_{a_2} represent the affinities of the acceptors for the reactive components of the chain. Any one of these quantities may be rate limiting, and all are, in principle, susceptible to thermal destruction. A fortuitous combination of thermal changes in K_a and V_a values for two different acceptors could clearly lead to apparently identical thermal susceptibilities when measured at only one acceptor concentration. Simultaneous elimination of the effects of K_{a_1} and K_{a_2} is possible by means of assays extrapolated to infinite acceptor concentration. However, this still leaves three quantities, V_e, V_{a_1}, and V_{a_2}, of which only two are accessible to experimental investigation.

Summarizing all arguments, a diagram may be constructed (Fig. 8) to depict the acceptor loci on the respiratory chain. The electrons from DPNH may flow through at least three outlets to DCIP, two to cytochrome *c*, and essentially only one to ferricyanide, probably *via* the DPNH dehydrogenase flavoprotein. The numbers in parenthesis in Figure 8 are the percentages of the DPNH oxidation by a particular electron acceptor at a given site. They were the averages obtained from a number of experiments. It is evident that the interaction of artificial acceptors with the respiratory chain *is far less simple* than has previously been assumed.

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