STUDIES ON CHOLINE DEHYDROGENASE*

III. ORGANIZATION OF THE RESPIRATORY CHAIN IN CHOLINE OXIDASE

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SUMMARY

1. Titration of rat-liver mitochondria with azide, antimycin A, 2-*n*-heptyl-4-hydroxyquinoline N-oxide, and cyanide showed quantitatively very different effects on succinic and on choline oxidase activities. The former enzyme system was very much more sensitive to each of these inhibitors and reached complete inhibition at relatively low concentrations of these inhibitors; the latter showed a significant activity even at high concentrations of all the inhibitors except cyanide.

2. While choline oxidase was partially resistant to antimycin A and quinoline oxide, the reduction of cytochrome *c* by choline was completely inhibited.

3. Added cytochrome *c* stimulated succinate oxidation several fold without affecting choline oxidase.

4. Anaerobically, no oxidation of choline by fumarate could be demonstrated.

5. In untreated rat-liver mitochondria the succinic and choline oxidase and the corresponding cytochrome *c* reductase activities are additive, not competitive. When the amount of active cytochrome a_3 was limited by titration with azide, the competition of succinic and choline dehydrogenases for the respiratory chain could be demonstrated. Limitation of the rate of the cytochrome *b* to c_1 step by antimycin A or quinoline oxide, however, failed to produce competition between the two oxidases.

6. The differential effects of respiratory chain inhibitors and of added cytochrome *c* were shown to be due to the greater activity of succinic oxidase, as compared with choline oxidase. Following equalization of the activities of the two enzyme systems by titration with malonate these differences disappeared largely, but not entirely.

7. The conditions necessary for the development of the inhibitor-resistant oxidation of choline have been studied. With excess azide present, choline and succinic oxidases compete for the azide-resistant, autooxidizable component. With excess antimycin A or quinoline oxide present, however, succinate oxidation is completely abolished, but significant choline oxidase activity remains.

Abbreviation: DPNH, reduced diphosphopyridine nucleotide.

* A part of this work was presented at the 50th annual meeting of the American Society of Biological Chemists, Atlantic City, April 17, 1959. A preliminary note has been published[1].

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8. On the basis of these observations, a scheme has been presented for the interrelations of the choline and succinic oxidase chains in liver mitochondria. It is proposed that the two oxidases operate through respiratory chains which are interlinked between cytochrome c_1 and O_2, but not at the oxidation level of cytochrome *b*. It is suggested that the *b* component of the choline chain may become autooxidizable under suitable conditions and thus account for the incomplete inhibition of choline oxidase by respiratory chain inhibitors.

INTRODUCTION

In a recent publication from this laboratory[2], by means of a kinetic approach, the question was explored as to whether a common or separate and independent chains serve succinic and α-glycerophosphoric dehydrogenases in brain mitochondria. It was concluded that a common respiratory chain serves the two dehydrogenases from cytochrome *b* to O_2, or, if the two enzymes are linked to separate cytochrome chains, that a crossover must occur between the chains in the course of normal electron transport. Similar conclusions have been reached independently by WU AND TSOU for the interrelation of succinic and DPNH oxidases in heart muscle preparations[3].

As briefly reported in an abstract[4], when the same kinetic approach was applied to the study of the relations of choline and succinic oxidases in rat-liver mitochondria, the results did not seem to be readily reconcilable with the operation of a common cytochrome chain in the two enzyme systems. Thus, titrations with respiratory chain inhibitors had quantitatively very different effects on succinic and choline oxidase activities; external cytochrome increased the activity of the former several fold without affecting the latter; no competition of the two enzymes for the respiratory chain could be demonstrated; and, anaerobically, no oxidation of choline with fumarate as electron acceptor was observed.

The present paper is a detailed account of this work and of further experiments which lead to an explanation of these observations. It is concluded that functionally interlinked respiratory chains serve choline and succinic dehydrogenases, but the cytochrome *b* moiety serving the two enzymes may be unique to each system.

MATERIALS AND METHODS

The materials and experimental procedures used were as described in previous papers[2,5]. Except as noted, mitochondria were used immediately after preparation.

RESULTS

Titration with respiratory chain inhibitors

The observations which initiated the present investigation are summarized in Figs. 1 to 3 and in Table I. Titration of rat-liver mitochondria with azide or quinoline oxide* (Fig. 1) shows a much greater sensitivity of succinic than of choline oxidase toward these inhibitors. At concentrations of these inhibitors which abolish succinate oxidation almost completely, the respiration on choline is little or not at all affected.

* The abbreviation quinoline oxide denotes 2-*n*-heptyl-4-hydroxyquinoline N-oxide.

TABLE I

COMPARISON OF THE EFFECTS OF CYANIDE ON CHOLINE AND SUCCINIC OXIDASES DURING INITIAL REACTION PERIOD

Conditions: Manometric assays during initial 5 min after addition of the substrate. Each vessel contained 16 mg sucrose mitochondria (biuret basis) in 0.01 *M* KCl–0.015 *M* phosphate, pH 7.6, 0.5 mg cytochrome *c*, and 0.02 *M* succinate or 0.017 *M* choline. Temperature, 38°.

Cyanide concentration (M)	*O_2 uptake*		*Inhibition*	
	Succinate μl	*Choline* μl	*Succinate* %	*Choline* %
None	118.7	48.8		
$3.3 \cdot 10^{-5}$	71.6	44.0	39.7	9.9
$6.7 \cdot 10^{-5}$	66.9	43.3	43.6	11.3
$1.3 \cdot 10^{-4}$	35.7	30.5	70.0	37.5
$3.3 \cdot 10^{-4}$	15.4	9.1	87.0	83.4
$6.7 \cdot 10^{-4}$	6.4	4.3	94.6	91.2

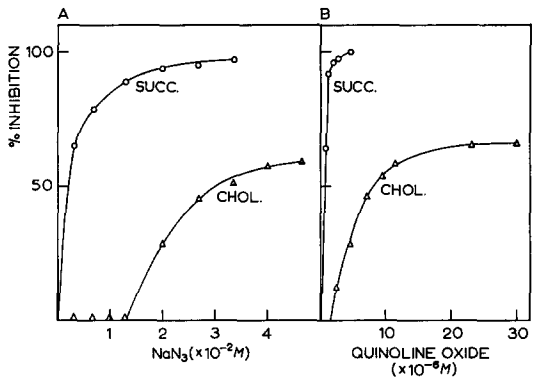


Fig. 1. Titration of succinic and choline oxidase activities of rat-liver mitochondria with azide and quinoline oxide. Manometric determination of O_2 uptake at 30°. Each Warburg vessel contained 150 μmoles phosphate, pH 7.6, sucrose mitochondria in the amounts indicated below, 1.25 mg cytochrome *c*, and inhibitor as indicated in the main compartment, 45 to 50 μmoles choline or 60 μmoles succinate in the side arm, in a total volume of 3 ml. The contents of the side arm were tipped after 7 min temperature equilibration. Activities were calculated from the linear rate obtained between 5 and 20 min after the start of the reaction. In comparing the effects of inhibitors on the two enzymes it was necessary to keep the ratio of inhibitor to mitochondrial protein constant. The much higher activity of succinic than of choline oxidase caused considerable difficulty in meeting this requirement. The problem was circumvented in this and subsequent experiments by using a fixed concentration of mitochondria in all vessels where inhibitor was present, but the activity of the uninhibited succinic oxidase control was calculated from measurements at lower concentrations of mitochondria. Thus, in Fig. 1A all vessels contained 18.4 mg mitochondrial protein (biuret basis) and in Fig. 1B 16.1 mg protein, except the succinic oxidase controls, which contained 5.5 and 3.2 mg protein in experiments A and B, respectively.

Further, while the former enzyme system is completely inhibited at low concentrations of quinoline oxide and over 96% inhibited by 0.03 to 0.04 *M* azide, the inhibition of the latter system does not reach completion, and a significant respiration remains which is insensitive to these agents. As shown in Fig. 2, similar results were obtained with antimycin A as inhibitor. In titrations with cyanide, the inhibition of both enzyme systems was virtually complete, but at relatively low concentrations of cyanide the much greater sensitivity of succinic oxidase than of choline oxidase was again apparent.

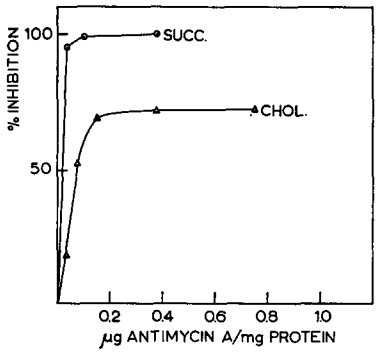


Fig. 2. Titration of succinic and choline oxidase activities with antimycin A. Experimental conditions, as in Fig. 1, except that the temperature was 38° and the amount of mitochondrial protein 10.7 mg in each vessel. The activities were corrected for the slight inhibition produced by the ethanol used as solvent for the antimycin A.

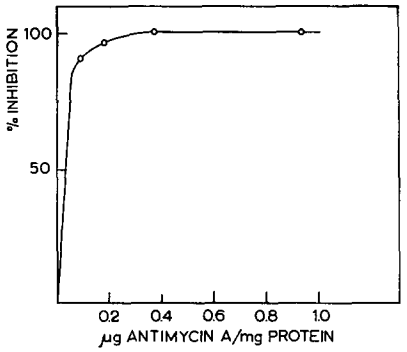


Fig. 3. Titration of choline–cytochrome *c* reductase activity with antimycin A. The experiment was conducted with a Process and Instruments Company Model RS-3 recording spectrophotometer, thermostated at 30°. Each cuvette contained 150 μmoles phosphate, pH 7.6, 30 μmoles HCN, 100 μmoles choline, 2 mg cytochrome *c*, 8.6 mg mitochondrial protein, and inhibitor as indicated in a total volume of 3 ml. All components except cytochrome *c* were preincubated until temperature equilibrium was reached and the reaction was started by the addition of the latter. Activity was calculated from the increase in O.D. at 550 mμ and was corrected for the inhibition caused by the ethanol in which the antimycin was dissolved.

In contrast to the incomplete inhibitions observed with antimycin A and quinoline oxide when O_2 was the electron acceptor (Figs. 1 and 2), when added cytochrome *c* served as the terminal oxidant the inhibition of choline oxidation was complete, although at low concentrations of these inhibitors the greater sensitivity of succinic oxidase was readily demonstrable. Comparison of Figs. 2 and 3 shows that at a concentration of antimycin A which inhibits the choline-cytochrome *c* reaction completely (0.4 μg/mg protein), the choline–O_2 reaction is maintained at about 30 % of the uninhibited rate.

In Figs. 1 and 2, inhibition was calculated from the linear part of the reaction rate (5 to 20-min interval after addition of the substrate), since during the initial 5 min at 30° or 38° the rates of both succinate and of choline oxidation increased markedly. The reason for the increase in the succinic oxidase activity with time is probably the previously described activation process[6], while the rate of choline oxidation increases during the initial period because of the limited permeability of mitochondria to choline, as discussed below. If one compares the response of succinic and choline oxidases to azide (Fig. 4), quinoline oxide (Fig. 5), or antimycin during this initial 5-min reaction period, it is found that both enzymes are completely or nearly completely inhibited, but succinic oxidase is still significantly more sensitive to these inhibitors, particularly at low inhibitor concentrations. Thus, the resistance of choline oxidase to respiratory chain inhibitors observed during the linear reaction period develops with time, and its extent depends on the ionic composition and the physiological state of the mitochondria (*cf.* below). In experiments employing devices which measure only the rate during a brief initial period, therefore, some of the differences mentioned would escape notice.

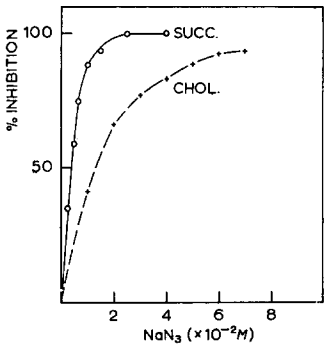


Fig. 4. Extent of inhibition of succinic and choline oxidase activities by azide during the initial phase of oxidation. The experiment utilized a rotating platinum microelectrode connected to a Kin-Tel Model 202B microvoltmeter-amplifier and a Varian Model 11B strip chart recorder. The reaction mixture contained in 2 ml volume 200 μmoles KCl, 30 μmoles phosphate, pH 7.6, 5.1 mg mitochondrial protein, 20 μmoles succinate or 25 μmoles choline, 1.5 μmoles $CaCl_2$, and NaN_3 as indicated, temperature 22°. The reaction was started by the addition of substrate. Rates were calculated from the initial reaction period, which varied from 1 to 5 min depending on the activity of the sample.

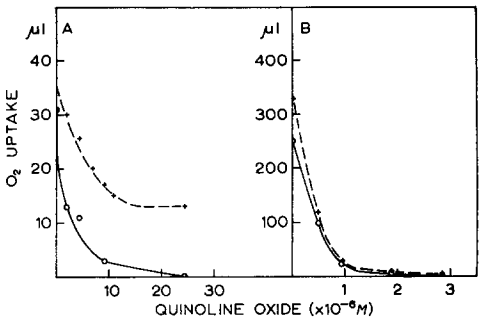


Fig. 5. Inhibition of choline oxidase (A) and of succinic oxidase (B) by quinoline oxide during initial 5 min of reaction and during later period. Conditions as in Fig. 1, B. Circles, rate of O_2 uptake during first 5 min after addition of substrate; crosses, rate during 5 to 20 min interval after addition of substrate (linear phase). Rates are expressed as μl O_2 uptake/5 min/ml mitochondrial suspension (= 32.2 mg protein).

Attempts at demonstrating a common respiratory chain

The close interrelations of the succinic and α-glycerophosphoric oxidase chains in brain mitochondria are readily demonstrated by the anaerobic oxidation of α-glycerophosphate by fumarate and by the fact that the oxidations of succinate and α-glycerophosphate are mutually inhibitory whether O_2 or external cytochrome *c* serves as electron acceptor[2]. Attempts to demonstrate a similar relationship between the succinic and choline oxidase chains were not successful. In experiments designed to show the anaerobic oxidation of choline with fumarate as terminal electron acceptor, the formation of the oxidation product, betaine aldehyde, was followed by the 2,4-dinitrophenylhydrazine reaction[7]. Although in control experiments added betaine aldehyde was satisfactorily recovered and a sufficient quantity of mitochondria were employed to detect 0.1 % of the aerobic rate of choline oxidation, no formation of betaine aldehyde was found. Further, in experiments involving the simultaneous oxidation of choline and succinate with either O_2 or cytochrome *c* as electron acceptor, and with a limiting amount of mitochondria present, the rates of oxidation of the two substrates were additive and no evidence for competition was observed.

Other disparities between the choline and succinic oxidase systems were the sensitivity of choline oxidase to amytal[8], and, as discussed in the next section, the stimulation of succinic but not of choline oxidase activity by added cytochrome *c*.

Stimulation by cytochrome c

That rat-liver mitochondria prepared in 0.25 *M* sucrose are partially deficient in cytochrome *c* is evident from the considerable stimulation of succinate oxidation

by added cytochrome *c* (Fig. 6). Choline oxidation, however, is unaffected by the addition of cytochrome *c*.

One possible explanation of this and of the other discrepancies noted in the behavior of the choline and succinic oxidase systems is that the two chains operate independently of each other. One could readily visualize the co-existence in the same

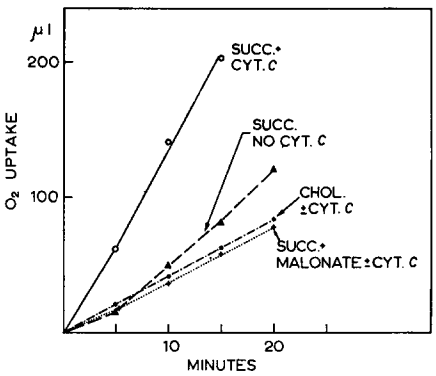


Fig. 6. Effect of added cytochrome *c* on succinic and choline oxidase activities of rat-liver mitochondria. Conditions as in Fig. 1, except as follows: 20 mg mitochondrial protein per vessel; 1.25 mg cytochrome *c* added only where indicated; temperature, 24°. In the lowest curve (+), succinic oxidase activity was depressed with $8.3 \cdot 10^{-4}$ *M* malonate approximately to the level of choline oxidase activity.

mitochondrion of a cytochrome chain with a loosely held cytochrome *c* moiety, serving succinic dehydrogenase, and of another chain, serving choline dehydrogenase, in which the cytochrome *c* is more tightly bound. Another explanation of the differential effects of added cytochrome *c* on the two oxidase systems might lie in the much greater activity of succinic dehydrogenase compared with that of choline dehydrogenase and of the consequently greater demand the former enzyme might put on the activity of a common respiratory chain. Thus, the significant concentration of cytochrome *c* retained in liver mitochondria isolated in 0.25 *M* sucrose[8] might suffice to support the full potential rate of choline oxidation, but not of succinate oxidation.

In order to decide between these alternatives, succinic oxidase activity was specifically depressed by titration with malonate until it was equal to that of choline oxidase (Fig. 6). Following equalization of the succinic and choline oxidase activities in this manner, the two systems behaved identically, *i.e.*, neither was affected by the addition of cytochrome *c*.

Titration with inhibitors in malonate-treated preparations

Since the experiment just described demonstrated that at least one of the discrepancies in the behavior of the two oxidase systems is due to the relatively higher activity of succinic oxidase, the titrations with respiratory chain inhibitors were repeated with preparations in which the succinic and choline dehydrogenase activities were made approximately equal by means of malonate. As seen in Fig. 7, this treatment brings the titration curves of the two enzyme systems much closer together. The differences in the titration curves of the two oxidases at high inhibitor concen-

trations, particularly with quinoline oxide, are again related to the inhibitor-resistant oxidation of choline, although this was intentionally minimized in the present experiment by employing a low concentration of phosphate (*cf.* below). Complete coincidence of the titration curves of succinic and choline oxidases with these inhibitors may be obtained, following equalization of the control activities with malonate

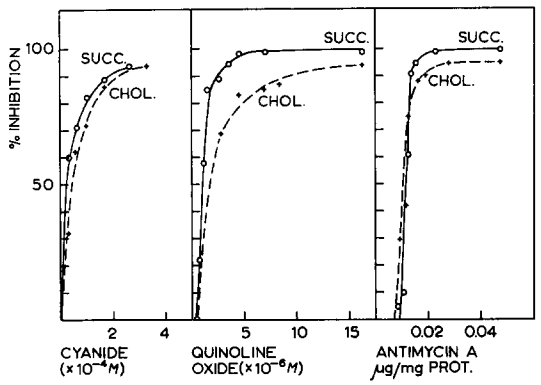


Fig. 7. Comparison of titrations of choline and succinic oxidases with respiratory chain inhibitors following equalization of their activities. Conditions as in Fig. 1 except that the temperature was 28°, and 12.5 µmoles phosphate and 50 µmoles KCl were present per vessel. The succinic oxidase activity was depressed by the addition of malonate to the level of choline oxidase activity. Cyanide experiment, 14.4 mg mitochondrial protein, $1.5 \cdot 10^{-3}$ *M* malonate; quinoline oxide experiment, 10.9 mg protein and $1.5 \cdot 10^{-3}$ *M* malonate; antimycin A experiment, 23.3 mg protein, $1.7 \cdot 10^{-3}$ *M* malonate.

and with Ca^{++} present to aid penetration of choline, during the initial 5-min interval after addition of the substrate, since the resistance to inhibitors is not manifest during this period.

Competition in inhibitor-treated preparations

At this point, it appeared possible that previous failure to demonstrate a competition between the two enzyme systems might have been the consequence of the fact that their combined activities did not exceed the turnover rate of the cytochrome chain in liver mitochondria. If this explanation were correct, and provided that some part of the respiratory chains serving the two dehydrogenases were in common or in intercommunication, the selective suppression of the activities of given members of the cytochrome system should make it possible to demonstrate the competition of the two enzymes. Expt. 1 in Table II shows that when the effective concentration of cytochrome a_3 was reduced by the addition of a low concentration of azide, a competition arose between succinic and choline oxidases, and, as in the competition between succinic and α-glycerophosphoric oxidases[2], the combined rate with both substrates present was less than with the faster one alone. (An explanation of this behavior has been proposed in a previous paper[2].) That the inhibition of succinate oxidation was caused by the operation of choline oxidase and not by choline itself was shown by the fact that the addition of amytal, an inhibitor of choline oxidase, re-established the original rate of succinate oxidation and of fumarate formation. It may be concluded from this experiment that at the oxidation level of cytochrome a_3

TABLE II

COMPETITION OF SUCCINIC AND CHOLINE DEHYDROGENASES FOR THE RESPIRATORY CHAIN

Conditions: Manometric assays at 30°, pH 7.6, in 0.05 *M* phosphate, with 0.02 *M* succinate and/or 0.017 *M* choline present. In Expts. 1, 3, and 4, 0.5 mg cytochrome *c* was added; and in Expt. 2, succinic dehydrogenase activity was depressed with $5 \cdot 10^{-4}$ *M* malonate to the level of choline dehydrogenase. The amount of mitochondrial protein (biuret basis) per vessel was 6.3 mg in Expt. 1, 3.8 mg in Expt. 2, and 7.8 mg in Expts. 3 and 4. The results are expressed in each case for a 15-min reaction period and 1 ml mitochondrial suspension (25.5 ± 0.3 mg protein).

Experiment	*Electron acceptor*	*Substrate*	*O_2 uptake µatoms*	*Fumarate accumulation µmoles*
1	Respiratory chain	Succinate	40.0	36.0
	cytochrome a_3 limiting	Choline	20.0	
	(3.3 m*M* azide)	Succinate + choline	35.6	28.0
2	Phenazine methosulfate	Succinate	51.2	
		Choline	55.4	
		Succinate + choline	112.0	
3	Respiratory chain	Succinate	22.2	22.8
	cytochrome $b \rightarrow c_1$ limiting	Choline	12.5	
	($9.3 \cdot 10^{-7}$ *M* quinoline oxide)	Succinate + choline	36.1	24.6
4	Respiratory chain	Succinate	20.2	19.7
	cytochrome $b \rightarrow c_1$ limiting	Choline	11.3	
	(0.11 µg antimycin A)	Succinate + choline	33.4	24.6

the two oxidase chains do not act independently of each other, but are functionally interrelated.

When phenazine methosulfate served as the electron acceptor (Expt. 2, Table II), the rates of oxidation of succinate and choline were additive, as expected from the fact that this dye interacts directly with the two dehydrogenases. The lack of competition was evident whether or not the activities of the two dehydrogenases were equalized with malonate.

Contrary to expectation, however, when the rate of the cytochrome *b* to c_1 step in the respiratory chain was lowered by treatment with low concentrations of quinoline oxide or antimycin A (Expts. 3 and 4), no competition but, instead, strict additiveness of the two oxidase activities was observed. Fumarate analysis confirmed that the oxidation of choline under these conditions failed to inhibit the oxidation of succinate. It appears from these results that at the cytochrome *b* to c_1 step in the respiratory chain the flow of electrons from the two dehydrogenases does not follow a common path.

Nature of the inhibitor-resistant respiration

The relevant observations on the nature of the inhibitor-resistant respiration are as follows. At 30 to 38° the full rate of the azide-, antimycin-, and quinoline oxide-resistant oxidation of choline does not develop until about 5 min after the addition of the substrate (Figs. 5 and 8) and at 20 to 25° may take up to 15 min to develop. The inhibitor-resistant oxidation is best observed in the presence of high phosphate concentration and with fresh mitochondria (Table III); in aged or frozen-thawed preparations it is small or insignificant.

While in the presence of an excess of antimycin A or of quinoline oxide an inhibitor-resistant respiration develops, under identical conditions the choline-cytochrome *c* reaction is fully inhibited. In view of this fact and the lack of auto-oxidizability of choline dehydrogenase[5], the resistant respiration appears to be due to the

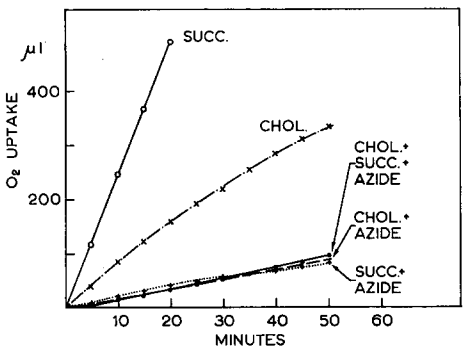


Fig. 8. Competition of succinic and choline dehydrogenases in the azide-resistant respiration of rat-liver mitochondria. Conditions as in Fig. 1, except as follows. Each vessel contained 14 mg mitochondrial protein and 0.5 mg cytochrome *c*, and 150 μmoles azide where indicated. The activities recorded are the actual amounts of O_2 uptake measured.

TABLE III

EFFECT OF EXPERIMENTAL CONDITIONS ON THE AZIDE-RESISTANT RESPIRATION OF LIVER MITOCHONDRIA

Conditions: Manometric determination of O_2 uptake with 0.05 *M* choline as substrate during linear phase of reaction (5 to 20 min after tipping of substrate). Each vessel contained 18.6 mg mitochondria (biuret basis); azide, where present, $3.3 \cdot 10^{-2}$ *M*; pH, 7.6; temperature, 30°.

Mitochondrial preparation	*Rate of respiration (μl O_2/15 min)*			
	In 0.05 M phosphate		*In 0.015 M phosphate–0.1 M KCl*	
	No azide	*+ azide*	*No azide*	*+ azide*
Fresh	34.5	14.0	31.0	7.5
After 24 h storage 0°	33.0	6.5	29.0	5.7
After overnight storage in frozen state	31.5	7.5	28.0	4.2

autooxidation of a respiratory chain component acting between the dehydrogenase and cytochrome *c*.

The inhibitor-resistant oxidation of choline has been regularly observed in the presence of excess azide, antimycin A, quinoline oxide, and, to a more limited extent, cyanide. In identical experiments the oxidation of succinate was completely inhibited by antimycin A and quinoline oxide, but not by azide. As illustrated in Fig. 8, the azide-resistant respiration proceeds at the same absolute rate with either succinate or choline or both present as substrate, which would suggest that the same auto-oxidizable component is limiting the rate in the azide-resistant oxidation of both substrates. Since the activity of succinic oxidase is much greater than that of choline oxidase in the absence of inhibitors, when the same amount of azide-resistant respiration is expressed as a per cent of the activity of the control samples, as in Fig. 1A,

the inhibitor-resistant oxidation of succinate is far less significant than that of choline.

The implications of the observations described in this section on the possible identity of the autooxidizable component of the respiratory chain are dealt with in the DISCUSSION.

The effect of Ca^{++}

It was reported by WILLIAMS[9] that in rat-liver mitochondria isolated in 0.25 *M* sucrose the oxidation of choline is extremely slow or absent, but that following freezing and thawing or the addition of Ca^{++} the apparent permeability barrier to choline is abolished and a rapid oxidation ensues. The observations of WILLIAMS were limited to initial reaction rates since he used a platinum micro-electrode for the measurement of O_2 uptake.

The extremely slow initial rate of choline oxidation in fresh mitochondria and the rapid initiation of O_2 uptake following the addition of $7.5 \cdot 10^{-4}$ *M* Ca^{++} were readily confirmed (Fig. 9). As is apparent from this figure, however, following the initial lag the oxidation of choline begins also in the sample not containing added Ca^{++}, and within 10 min following the addition of choline to fresh mitochondria the rates are equal with and without added Ca^{++}. The rate eventually declines in both samples owing to the accumulation of the inhibitor betaine aldehyde[5].

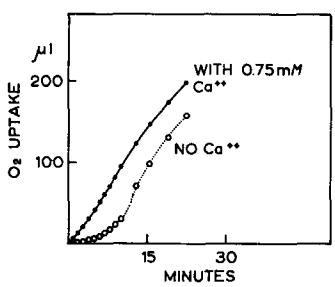


Fig. 9. Rate of appearance of choline oxidase activity in rat liver mitochondria with and without Ca^{++}. Manometric assays at 38°. Each vessel contained 0.8 ml mitochondrial suspension in 0.25 *M* sucrose (27.7 mg protein) in the side arm, 50 µmoles each of choline, and KCl, and 7.5 µmoles phosphate in the main compartment. Total volume, 3 ml. A 10-min temperature equilibration without shaking preceded the tipping (zero time).

Numerous experiments designed to explore the initial lag in the oxidation of choline in intact mitochondria led to the conclusion that while its underlying cause may be a restricted permeability of the mitochondrial membrane to choline, the phenomenon is a transient one and disappears spontaneously without resorting to conditions which are known to destroy the mitochondrial membrane. Thus, if a relatively concentrated mitochondrial suspension (25 to 30 mg/ml) in 0.25 *M* sucrose is added to choline-buffer mixture, no O_2 uptake is evident during the first 5 min (Fig. 9), whereas if the mitochondria are preincubated for about 10 min in a more dilute suspension (2.5 to 5 mg/ml) oxidation begins immediately upon the addition of choline. The circumstance that the former technique approximates the conditions apt to be employed in the measurement of respiration with an oxygen electrode, whereas the latter technique parallels the conditions of the manometric assay of

choline oxidase employed in this laboratory, may explain why the lag described by WILLIAMS[9] was not encountered in our earlier work[5].

DISCUSSION

The question whether the respiratory chains linking the various dehydrogenases in liver mitochondria to O_2 are the same, completely separate, or separate but interlinked may be at least partially answered on the basis of the experiments presented in this and in the preceding paper[8]. It has been shown that the cytochrome components reduced in the steady state during the oxidation of choline are spectrophotometrically indistinguishable from those operative in the oxidation of succinate and DPNH[8]. The spectrophotometric data presented in the previous paper did not rule out the possibility, however, that the choline, succinic, and DPNH oxidase systems each possess their own cytochrome moieties which are not functionally interlinked. The observations reported in the first part of this paper, in fact, did not appear compatible at first with the operation of a common respiratory chain in choline and succinic oxidases. The quantitative differences noted in the relative response of these two enzyme systems to respiratory chain inhibitors and the qualitative difference in their response to added cytochrome *c* have been satisfactorily explained by the higher activity of succinic oxidase and of the consequently greater demand it puts on the operation of the cytochrome chain. The fact that choline oxidase is completely inhibited by amytal, but succinic oxidase is unaffected by this reagent[8,10], has been explained, without the need for postulating separate respiratory chains for the two enzymes, by the finding that amytal interrupts the flow of electrons from choline dehydrogenase (flavoprotein) to the cytochrome chain, and, therefore, acts prior to the possible junction of the two chains[8].

Direct evidence for a functional interconnection of choline and succinic oxidases at the cytochrome a_3 level is provided by the competitive behavior of the two systems under conditions where the functional cytochrome a_3 content of the mitochondria is limited by titration with azide (Table II). The competition of the two systems for the azide-resistant, autooxidizable component of the respiratory chain (Fig. 8) provides further evidence for their close interrelation.

There remain three observations which cannot be readily interpreted if one assumes that all the members of the respiratory chain are in common—or in functional interconnection—in the choline and succinic oxidase systems. The first of these is the failure to demonstrate the thermodynamically feasible anaerobic oxidation of choline by fumarate. Second, when the rate of the cytochrome *b* to c_1 step is lowered with antimycin A or quinoline oxide to a point where the oxidation of succinate is significantly inhibited, the activities of choline and succinic oxidases are still additive, rather than competitive. Third, with a great excess of either of these inhibitors present, the oxidation of succinate is completely abolished, but appreciable choline oxidase activity remains.

A satisfactory explanation of these discrepancies may be offered on the basis of two assumptions: first, that the cytochrome *b* components serving choline and succinic dehydrogenases, respectively, are not the same and are not directly interlinked, and, second, that the component which becomes autooxidizable when the inhibitor-resistant oxidation of choline appears is the cytochrome *b* component of

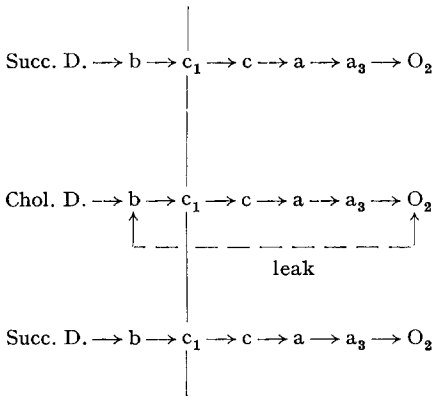
the choline oxidase chain (Scheme 1). In terms of this scheme it may be readily visualized why the two enzyme systems do not compete in the presence of low concentrations of antimycin A or quinoline oxide. These inhibitors, acting between cytochromes *b* and c_1, interrupt the flow of electrons from any dehydrogenase molecule which is attached to one of the respiratory chains which had been inhibited, and there remains a great excess of cytochromes c_1, *c*, *a*, and a_3 to serve those dehydrogenase molecules (choline or succinic), whose cytochrome *b* to c_1 step has not been affected. As to the oxidation of choline but not of succinate in the presence of either of these inhibitors, complete interruption of the *b* to c_1 step would leave no pathway for the electrons originating from succinate to reach the autooxidizable component of the choline chain, whereas the oxidation of choline would still occur to the extent that the *b* component of the choline oxidase chain is autooxidizable, since the electrons originating from choline would not have to pass the *b* to c_1 barrier. In the presence of an excess of azide, however, electrons originating from either dehydrogenase could reach the autooxidizable *b* moiety, those from choline dehydrogenase directly, those from succinic dehydrogenase by the interconnection at the c_1 level. Finally, the lack of a significant rate of choline oxidation by fumarate might be caused by the absence of a direct interconnection between the two chains at the cytochrome *b* level, in contrast to the succinic and α-glycerophosphoric oxidases of brain or succinic and DPNH oxidases of heart, which appear to be interlinked at the cytochrome *b* level[2,3], inasmuch as their anaerobic cross reactions are not antimycin-sensitive[2]. Even in these systems the rate of the anaerobic cross reaction is only 2% of the aerobic activity of the slower of the two interacting oxidases, and the further restriction imposed by the indirect path postulated in Scheme 1 might reduce the rate of interaction of choline and succinic dehydrogenases to an insignificant level.

The reason why choline oxidation in the presence of excess azide, antimycin A, or quinoline oxide is thought to be mediated by a component at the oxidation level of cytochrome *b* is that inhibition by amytal (acting between choline dehydrogenase and cytochrome *b* (see ref. 8)) is complete, whereas a significant level of choline oxidation is observed in the presence of inhibitors which act between cytochromes *b* and c_1. The component which becomes autooxidizable when the inhibitor-resistant respiration develops does not have to be cytochrome *b* itself, of course, but may be an as yet unrecognized electron transport component functioning between the amytal-sensitive site and cytochrome c_1.

Since the early observations of KEILIN AND HARTREE[11], the autooxidizability of cytochromes of the *b* type has been often invoked as an explanation of the inhibitor-resistant respiration of certain tissues, particularly higher plants[12–14]. Not infrequently this explanation has been countered with the "excess oxidase" hypothesis. (For a detailed discussion *cf.* CHANCE AND HACKETT[15].) Applied to choline oxidase, this hypothesis might be paraphrased as follows: since the turnover rate of choline oxidase in the presence of respiratory chain inhibitors is very low compared with the turnover rate of the respiratory chain component being titrated with an inhibitor, if an insufficient excess of inhibitor is used, the small fraction of residual respiratory chain activity might account for the limited rate of choline oxidation. This explanation is clearly not tenable in the present instance, for a variety of reasons, only one of which will be mentioned. Choline oxidase is completely inhibited during the

first few minutes following addition of the inhibitor and part of the activity returns gradually, a behavior which strongly suggests the transformation of a respiratory chain component to an autooxidizable form.

Scheme I and the discussion presented fail to account for one property of the inhibitor-resistant respiration, namely, that the inhibition of choline oxidase by cyanide is much more complete than by the other inhibitors tested. Although cytochrome *b* is not thought to form a stable complex with cyanide, conceivably in some manner not yet understood cyanide interferes with the transformation of the *b* component of choline oxidase to an autooxidizable form.



Scheme I. Postulated interrelations of the succinic and choline oxidase chains in rat liver mitochondria. The vertical lines interconnecting the chains are meant to indicate free electron transfer between the chains at one or more points between cytochromes c_1 and a_3.

In the conventional scheme of the mammalian respiratory chain two or more dehydrogenases are pictured as feeding into a single cytochrome chain at some point. In the case of the succinic and choline oxidase chains the bifurcation would occur at the c_1 level. In this type of representation no mechanism is clearly implied for free electron transfer between adjacent chains. Therefore, it would be difficult to see why partial inactivation of the cytochrome a_3 content of a mitochondrion by azide establishes a competition between choline and succinic oxidases. If the sum of the activities of succinic and choline dehydrogenases is less than the turnover rate of a_3 in an intact mitochondrion, as is clearly the case, the addition of azide should merely lower both activities (by eliminating some of the respiratory chains), but the activities of the remaining choline and succinic oxidase chains should remain additive. The postulated scheme, on the other hand, visualizes the co-existence of parallel respiratory chains in liver mitochondria, interlinked, in the case of the choline–succinate pair, at one or more points between cytochrome c_1 and O_2. The competition established between the two enzyme systems by lowering the functional a_3 content of the mitochondria is clearly more compatible with this visualization of the organization in liver mitochondria.

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