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Succinate-linked Acetoacetate Reduction

II. ELECTRON TRANSFER PATHWAY AND ENERGY REQUIREMENT

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It has been demonstrated in previous papers (1–5) that isolated rat liver mitochondria catalyze an endergonic reduction of acetoacetate by succinate. The reaction was found to be sensitive to Amytal and rotenone, and to require a supply of high energy intermediates generated during the aerobic oxidation of succinate. It was concluded that the succinate-linked acetoacetate reduction proceeds by way of succinic dehydrogenase, the reduced diphosphopyridine nucleotide dehydrogenase flavoprotein, diphosphopyridine nucleotide, and β-hydroxybutyric dehydrogenase.

This paper is concerned with the electron transfer pathway and energy requirement involved in the succinate-linked acetoacetate reduction. Evidence already has been presented (1, 2) that the process is insensitive to antimycin A, and that it probably involves the expenditure of one high energy bond per molecule of acetoacetate reduced by succinate. Independent evidence concerning the insensitivity of the flavosubstrate-linked DPN reduction to antimycin A has been reported by Klingenberg and Schollmeyer (6), and by Löw, Krueger, and Ziegler (7). On the other hand, Chance and Hollunger (8, 9) have arrived at the conclusion that the reduction of DPN by succinate proceeds via the antimycin A-sensitive site of the respiratory chain. They have concluded (8, 10), furthermore, that the energy requirement of the process amounts to two or three high energy bond equivalents per molecule of DPN reduced.

Some new approaches to the above problems are reported here. From these studies our previous estimate (1, 2) of one high energy bond equivalent expended per molecule of acetoacetate reduced could be confirmed. Evidence will also be presented, based on the effect of vitamin K_3 on the succinate-linked acetoacetate reduction, that this process involves neither the antimycin A-sensitive site nor cytochrome *b*. An attempt will be made to explain the differences between the present conclusions and those of Chance and Hollunger (8–10) in their studies of the succinate-linked pyridine nucleotide reduction.

EXPERIMENTAL PROCEDURE

The experimental procedures employed were those described in Paper I (4).

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RESULTS

Energy Requirement for Succinate-linked Acetoacetate Reduction

Respiratory Stimulation Due to Succinate-linked Acetoacetate Reduction—It was concluded in previous papers (1–4) that the endergonic reduction of acetoacetate by succinate was dependent upon a supply of high energy intermediates generated during the aerobic oxidation of succinate. Hence, the acetoacetate reduction may be considered as an energy-trapping system whose action may be reflected, analogous to that of a P-acceptor system, in an increase in the rate of respiration. Data reported in Fig. 1 show that, indeed, addition of acetoacetate caused a slight but significant stimulation of the respiration with succinate in the absence of P-acceptor. No similar increase occurred when acetoacetate was added either in the absence of succinate or in the presence of succinate and P-acceptor (ATP, hexokinase, and glucose). It may also be noted that the acetoacetate-induced stimulation of the resting respiration in the presence of succinate was considerably below that induced by an excess of P-acceptor. This finding indicates that the energy-trapping capacity of the succinate-linked acetoacetate reduction is lower than the capacity of the energy-coupling system operating during the aerobic oxidation of succinate.

The assumption that the respiratory stimulation by acetoacetate was due to the succinate-linked acetoacetate reduction could be substantiated by showing that inhibition of the latter by Amytal (1–4) or rotenone (5) abolished the respiratory stimulation (Table I). Conversely, oligomycin, which was previously shown not to affect the succinate-linked acetoacetate reduction (1, 2, 4), also did not abolish the acetoacetate-induced respiratory stimulation (Table II). When succinate-linked acetoacetate reduction was abolished by P_i and P-acceptor and restored by oligomycin, the accompanying respiration again was higher than the corresponding respiration in the absence of acetoacetate.

Quantitative Evaluation of Respiratory Increase in Terms of Energy Requirement—Since it is well established that the aerobic oxidation of succinate can give rise to two high energy bonds per atom equivalent of oxygen consumed, it might be possible to calculate the number of high energy bond equivalents required per molecule of acetoacetate reduced (~:AcAc ratio), once the oxygen to acetoacetate (O:AcAc) ratio is known. A problem arises, however, as to the choice of the oxygen consumption value. Two alternatives are available for calculating the O:AcAc ratio: (*a*) on the basis of the *total* oxygen uptake of the

acetoacetate reducing system (Σ O); or (*b*) by considering only the *increase* in oxygen uptake due to the acetoacetate reduction (Δ O). Table III compares the ratios as computed according to the two alternatives from the data previously reported in Tables I and II. It can be seen that the Σ O:AcAc ratios are of the order of 1.5 to 2.0 (giving ~:AcAc ratios of 3.0 to 4.0), whereas the Δ O:AcAc ratios are in the vicinity of 0.5 (~:AcAc ratios of about 1).

In previous work from this laboratory (1, 2) alternative *b* was preferred. It was recognized that a similar procedure might not be applicable if the P:O ratio were to be calculated, in which case values close to 2 can be obtained on the basis of the total respiration even though the resting respiration may be relatively high (*cf.* reference (1)). In choosing alternative *b*, however, it was considered, that the low energy-trapping capacity of the

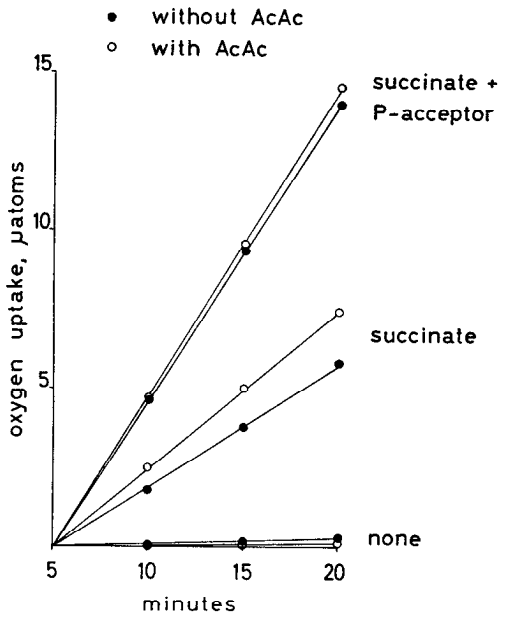

FIG. 1. Effect of acetoacetate (*AcAc*) on succinate oxidation in presence and absence of P-acceptor. Each vessel contained in a final volume of 2 ml: mitochondria from 400 mg of liver, 25 mM P_i (pH 7.5), 20 mM glycylglycine buffer (pH 7.5), 8 mM $MgCl_2$, 50 mM KCl, 62 mM sucrose and, when indicated, 5 mM acetoacetate, 10 mM succinate, 1 mM ATP, 30 mM glucose and yeast hexokinase in excess ("P-acceptor" stands for ATP + glucose + hexokinase).

TABLE I

Effect of Amytal and rotenone on respiratory stimulation due to acetoacetate reduction

Each vessel contained in a final volume of 2 ml: 10 mM succinate, 20 mM glycylglycine buffer (pH 7.5), 8 mM $MgCl_2$, 50 mM KCl, 50 mM sucrose, and, when indicated, 4.2 mM acetoacetate, 2 mM Amytal, and 4×10^{-7} M rotenone. Mitochondria from 300 mg of liver. Temperature, 30°. Time of incubation, 20 minutes.

Addition	Without acetoacetate: oxygen uptake	With acetoacetate	
		Oxygen uptake	Acetoacetate removed
	μatoms	*μatoms*	*μmoles*
None	6.4	8.7	4.4
Amytal	6.6	6.9	1.0
Rotenone	6.4	6.6	1.4

TABLE II

Lack of effect of oligomycin on respiratory stimulation due to acetoacetate reduction

Conditions as in Fig. 1; 10 mM succinate was added in all vessels, and, when indicated, 2 μg of oligomycin in 0.02 ml of absolute ethanol. Time of incubation, 20 minutes.

Additions	Without acetoacetate: oxygen uptake	With acetoacetate	
		Oxygen uptake	Acetoacetate removed
	μatoms	*μatoms*	*μmoles*
None	7.7	9.8	4.7
Oligomycin	6.7	9.1	5.7
ATP, hexokinase, glucose	18.5	19.3	0.0
ATP, hexokinase, glucose, oligomycin	7.2	10.1	5.4

acetoacetate reducing system might not be sufficient to overcome the waste of energy connected with the resting, "loosely coupled," respiration to the same extent as does the phosphorylating system in the presence of an excess of P_i and P-acceptor.

Data which support the justification of this way of reasoning, and simultaneously allow a more accurate determination of the ~:AcAc ratio, have now been obtained by investigating the influence of limiting amounts of P-acceptor on the P:O ratio obtained with succinate as substrate. Mitochondria were incubated with succinate, P_i, ATP, and glucose, and with varying amounts of added hexokinase. The respiration and P_i uptake were measured. The results of a typical experiment are shown in Fig. 2*a*. The resting respiration of the system was 2.6 μatoms of oxygen. Addition of increasing amounts of hexokinase gradually brought the oxygen uptake to a maximal level of 9.8 μatoms. The P:O ratio revealed a very marked increase with increasing capacity of the energy-trapping system, being only 0.44 when the amount of hexokinase was about $\frac{1}{16}$ of that needed for maximal respiration, and still slightly increasing (from 1.40 to 1.53) between "equivalent" and "2-fold excess" of hexokinase (under usual conditions 10- to 15-fold excess of hexokinase is used and the P:O ratio varies between 1.7 and 2.0).

When, in the same experiment, acetoacetate, rather than hexokinase, was added, the respiration raised from 2.6 to 3.8 μatoms, and the amount of acetoacetate removed was 2.7 μmoles. From this, the ~:AcAc ratio could be estimated by interpolating the extent of P_i uptake which occurred when hexokinase brought the respiration to the same level as prevailed in the acetoacetate system, *i.e.* 3.8 μatoms. This interpolation is illustrated in Fig. 2*b*, where the straight lines marked "oxygen" and "P_i" are magnified portions of the corresponding lines in Fig. 2*a*. It can be seen that the P_i uptake occurring at this level of respiration was 3.2 μmoles corresponding to a P:O ratio of 3.2/3.8 = 0.84, which gives a P_i:AcAc (= ~:AcAc) ratio of 1.19. This value thus is somewhat higher than that derived on the basis of the respiratory increase ($2 \times (3.8-2.6)/2.7 = 0.9$; *cf.* also Table III) but still it is in the vicinity of 1.

Electron Transfer Pathway

It has been reported (11) that addition of catalytic amounts of vitamin K_3 to liver mitochondria, whose respiration in the presence of DPN-linked substrates has been inhibited by Amytal

TABLE III

Calculation of oxygen to acetoacetate (AcAc) ratios

From data in	*a*. Conditions for measuring respiration as accompanied by succinate-linked acetoacetate reduction	*b*. Conditions for measuring respiration as *not* accompanied by succinate-linked acetoacetate reduction	Oxygen uptake under *a* (Σ O)	Difference in oxygen uptake between *a* and *b* (Δ O)	Acetoacetate removed	Oxygen to acetoacetate ratios	
						ΣO:AcAc	ΔO:AcAc
			μatoms	*μatoms*	*μmoles*		
Table I	Succinate, acetoacetate	Omission of acetoacetate	8.7	2.3	4.4	2.0	0.52
	Succinate, acetoacetate	Addition of Amytal	8.7	1.8	4.4	2.0	0.41
	Succinate, acetoacetate	Addition of rotenone	8.7	2.1	4.4	2.0	0.48
Table II	Succinate, acetoacetate	Omission of acetoacetate	9.8	2.1	4.7	2.1	0.45
	Succinate, acetoacetate, oligomycin	Omission of acetoacetate	9.1	2.4	5.7	1.6	0.42
	Succinate, acetoacetate, oligomycin, P-acceptor	Omission of acetoacetate	10.1	3.0	5.4	1.9	0.56

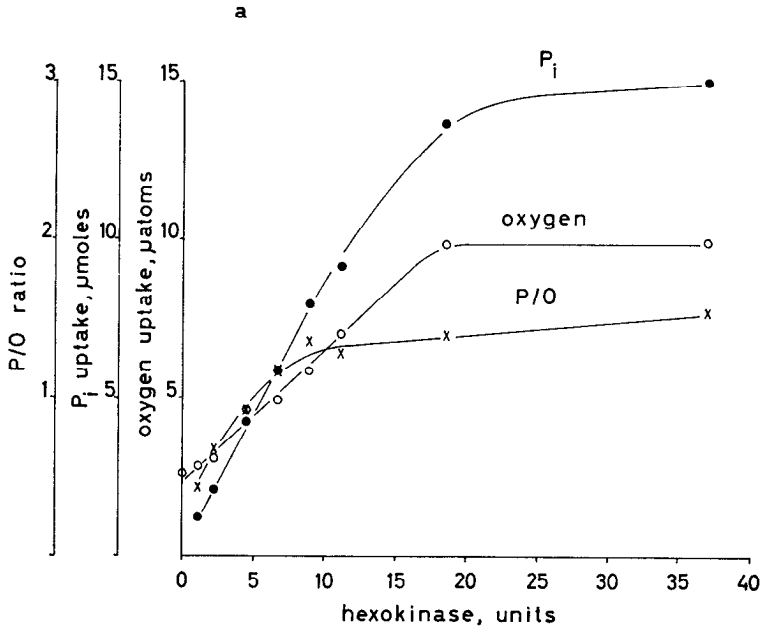

FIG. 2*a*. Influence of amount of hexokinase on respiration and P:O ratio with succinate as substrate. Each vessel contained (2 ml): mitochondria from 150 mg of liver, 10 mM succinate, 15 mM P_i^{32} (pH 7.5), 0.1 mM ATP, 20 mM glycylglycine buffer (pH 7.5), 8 mM $MgCl_2$, 50 mM KCl, 37 mM sucrose, 30 mM glucose, and yeast hexokinase as indicated. Sigma Type III hexokinase was used, containing 150 Kunitz-MacDonald units per mg. Temperature, 30°. Time of incubation, 25 minutes.

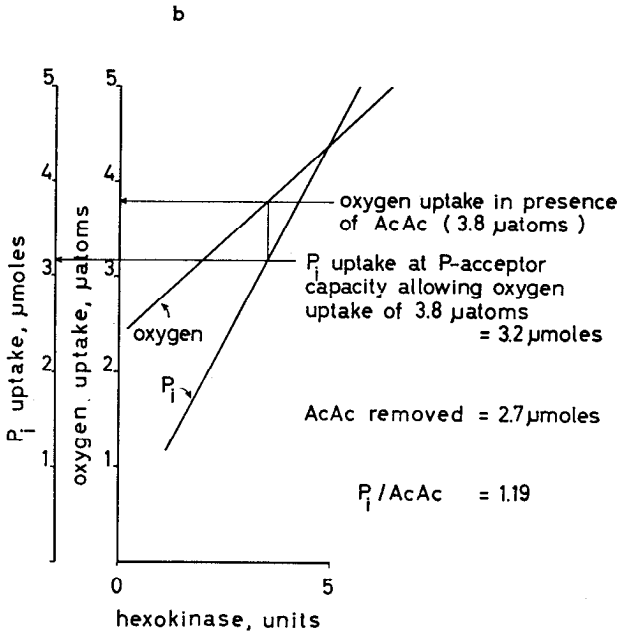

FIG. 2*b*. Computation of P_i:acetoacetate (*AcAc*) ratio. Acetoacetate removal was measured under conditions as in *a* except that ATP, glucose, and hexokinase were omitted, and 4.2 mM acetoacetate was added. For details of computation of P_i:acetoacetate ratio, see text.

(or rotenone (5)), induces an oxygen uptake by way of a flavoenzyme which is nonspecific with respect to pyridine nucleotide, the dicoumarol-sensitive "DT diaphorase." Phosphorylation coupled to the vitamin K_3-mediated respiration of the Amytal-blocked mitochondria was connected with a well marked respiratory control, but the P:O ratio was about 1 unit lower than that exhibited by the Amytal-sensitive respiratory pathway. The reoxidation of reduced vitamin K_3 was shown to involve cytochrome *b*, as revealed by spectrophotometric measurements, and to be sensitive to antimycin A.

From the above findings it was conceivable that investigation of the effects of catalytic amounts of vitamin K_3 on the succinate-linked acetoacetate reduction might provide information about the possible involvement of cytochrome *b* and of the antimycin A-sensitive site in the process. This approach was based on the assumption that vitamin K_3, when added to the succinate-linked acetoacetate reduction system, would trap electrons from DPNH (see schemes in Fig. 3). Two alternatives were envisaged:

Alternative a—The succinate-linked acetoacetate reduction involves cytochrome *b* or both cytochrome *b* and the antimycin A-sensitive site; in that case, any electrons trapped by vitamin K_3 from DPNH via DT diaphorase would enter the chain *at* or *before*, but not after, the site of entrance of electrons from succinic dehydrogenase. These electrons would then be refunneled into the electron flow directed toward DPN, and consequently, vitamin K_3 would cause no decrease in the rate of acetoacetate reduction.

Alternative b—The electrons from succinic dehydrogenase enter the chain before the level of cytochrome *b* (such as at the

DPNH dehydrogenase flavin, or at a quinone situated between the latter and cytochrome *b*), *i.e.* the succinate-linked acetoacetate reduction involves neither the antimycin A-sensitive site nor cytochrome *b*. In that case, any electrons trapped from DPNH by vitamin K_3 would be refunneled into the chain *after* the site of entrance of electrons from succinic dehydrogenase, and may therefore be drained by the terminal respiratory chain toward oxygen. As a consequence, there may occur a decrease in the rate of acetoacetate reduction by vitamin K_3, with a simultaneous increase in oxygen uptake.

The experimental findings reported below are in support of alternative *b*. As shown in Fig. 4, added vitamin K_3 markedly suppressed the succinate-linked acetoacetate reduction, and the concentration of vitamin K_3 required for maximal effect, 0.01 mM, was the same as that previously found (11) to induce maximal effect in overcoming Amytal inhibition of respiration. That the effect of vitamin K_3 involved the DT diaphorase pathway was demonstrated by the finding that it could be largely abolished by 10^{-6} M dicoumarol. This concentration of dicoumarol, in accordance with previous data (4), had no inhibitory effect on succinate-linked acetoacetate reduction by itself.

The suppressing effect of vitamin K_3 on the succinate-linked acetoacetate reduction was accompanied, as anticipated, by an increase in oxygen consumption (Fig. 5). Also this effect was abolished by 10^{-6} M dicoumarol (the general stimulation of oxygen uptake by this concentration of dicoumarol is probably due to a partial release of respiratory control).

The conclusion that the reduction of vitamin K_3 by succinate,

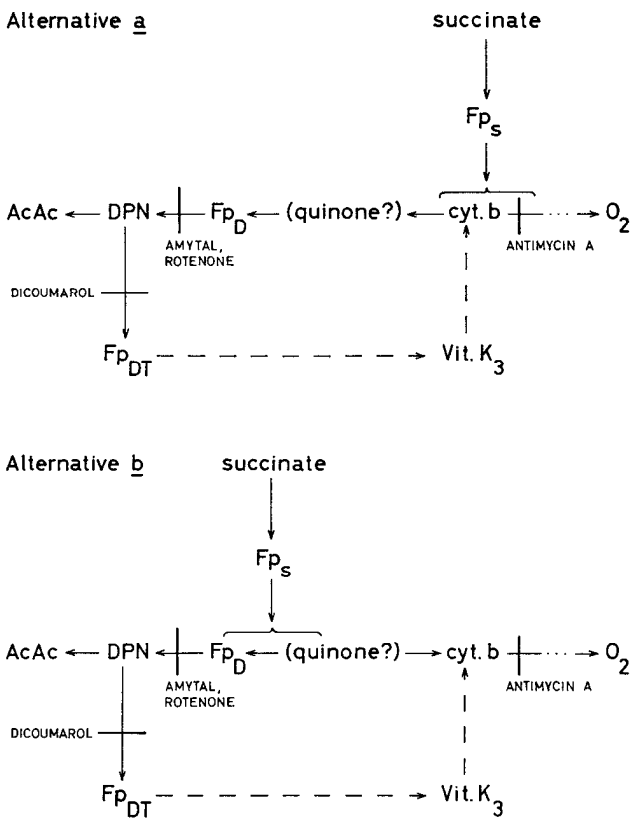

FIG. 3. Possible pathways of electron transfer in succinate-linked acetoacetate reduction. For explanation, see text. *AcAc* = acetoacetate; Fp_S = succinic dehydrogenase; Fp_D = DPNH dehydrogenase; Fp_{DT} = DT diaphorase.

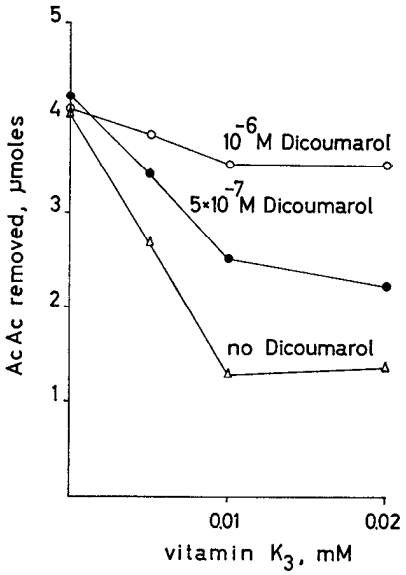

FIG. 4. Inhibition of acetoacetate removal by vitamin K_3 and abolition of vitamin K_3 effect by dicoumarol. Each vessel contained (2 ml): 4.2 mM acetoacetate, 10 mM succinate, 20 mM glycylglycine buffer (pH 7.5), 8 mM $MgCl_2$, 50 mM KCl, 50 mM sucrose and, when indicated, vitamin K_3 and dicoumarol. Mitochondria from 500 mg of liver. Temperature, 30°. Time of incubation, 10 minutes.

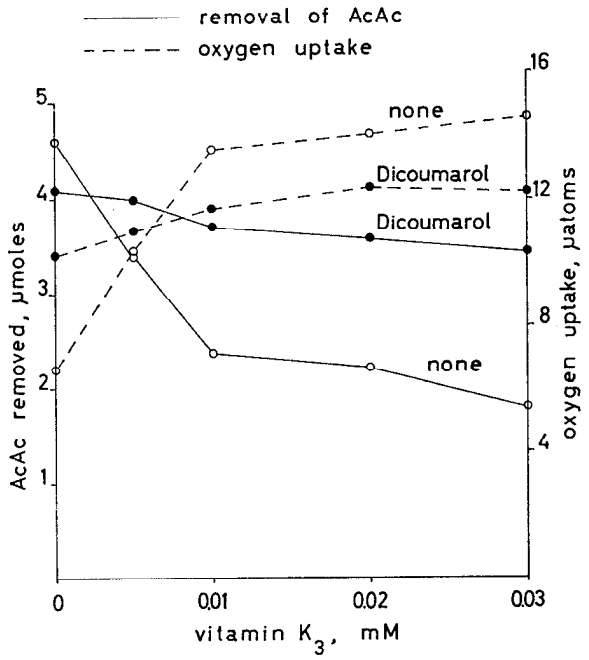

FIG. 5. Effect of vitamin K_3 on acetoacetate removal and succinate oxidation. Condition as in Fig. 4. Concentration of dicoumarol was 10^{-6} M. Mitochondria from 400 mg of liver. Temperature, 30°. Time of incubation, 15 minutes.

and the accompanying increase in respiration, were indeed proceeding by way of the reaction mechanism indicated in Fig. 3*b*, was further substantiated by the data reported in Table IV. Acetoacetate was omitted from the system and the effect of vitamin K_3 on the rate of succinate oxidation was tested in the absence and presence of P_i and P-acceptor. Vitamin K_3, 0.01 mM, caused a striking increase in the rate of the oxygen uptake

occurring in the absence of P_i and P-acceptor, but not in their presence. This effect of vitamin K_3 was abolished by 2 mM Amytal or 4×10^{-7} M rotenone, *i.e.* by agents which block the DPN-flavin electron transfer step (5, 13).

The above demonstration of an inhibition of the succinate-linked acetoacetate reduction by vitamin K_3 through the mechanism outlined in Fig. 3*b* had, in addition to proving the non-involvement of cytochrome *b* and antimycin A-sensitive site in the pathway of DPN reduction by succinate, also another important implication. The fact that there could occur a higher oxygen uptake in the presence of vitamin K_3 than in its absence implied that reduced vitamin K_3 could still be oxidized by way of the respiratory chain under conditions when the oxidation of succinate was limited by the efficiency of the energy-trapping system. In other words, this finding meant that the aerobic oxidations of reduced vitamin K_3 and of succinate, both of which give rise to two phosphorylations, may be subject to different extents of respiratory control. Data reported in Table V give an independent confirmation of this conclusion. Reduced vitamin K_3 was continuously generated by way of DT diaphorase in a rotenone-blocked system containing pyruvate plus malate as substrates.[1] The rate of the resting respiration in this system was 260 mμatoms of oxygen per minute. When in the same experiment succinate was used as a substrate the rate of the resting respiration was 170 mμatoms of oxygen per minute and it was virtually unaltered by the addition of rotenone or rotenone plus vitamin K_3. Thus, the respiratory control as compared on the basis of the extents of resting respiration was about 1.5 times more effective with succinate than with reduced vitamin K_3. The P:O ratios as anticipated were essentially equal with the two systems, 1.65 for the vitamin K_3-mediated respiration, and 1.73 for the oxidation of succinate in the presence of rotenone and vitamin K_3 (in the absence of rotenone a somewhat higher P:O ratio was obtained with succinate, due probably to the further oxidation of malate).

TABLE IV

Effect of Amytal and rotenone on stimulation of succinate oxidation by vitamin K_3

Each vessel contained in a final volume of 2 ml: 10 mM succinate, 20 mM glycylglycine buffer (pH 7.5), 8 mM $MgCl_2$, 50 mM KCl, 32 mM sucrose and, when indicated, 0.01 mM vitamin K_3, 2 mM Amytal, 4×10^{-7} M rotenone, 25 mM P_i (pH 7.5), 1 mM ATP, 30 mM glucose, and an excess of yeast hexokinase. Mitochondria from 250 mg of liver. Temperature, 30°. Respiration was measured for 20 minutes in the presence, and for 40 minutes in the absence, of P_i and P-acceptor (ATP, glucose, hexokinase). The respiratory rates were constant over the time measured.

Additions	Rate of respiration	
	Without P_i plus P-acceptor	With P_i plus P-acceptor
	mμatoms oxygen/minute	
Succinate	195	575
Succinate, vitamin K_3	420	580
Succinate, Amytal	245	625
Succinate, Amytal, vitamin K_3	240	655
Succinate, rotenone	195	685
Succinate, rotenone, vitamin K_3	200	675

TABLE V

Comparison between respiratory controls and P:O ratios for oxidation of pyruvate plus malate, succinate, and reduced vitamin K_3

Each vessel contained in a final volume of 2 ml: 20 mM glycylglycine buffer (pH 7.5), 8 mM $MgCl_2$, 50 mM KCl, 25 mM sucrose and, when indicated, 5 mM pyruvate, 5 mM L-malate, 10 mM succinate, 25 mM P_i^{32} (pH 7.5), 1 mM ATP, 30 mM glucose and an excess of yeast hexokinase, 0.01 mM vitamin K_3 and 4×10^{-7} M rotenone. Mitochondria from 200 mg of liver. Temperature, 30°. Respiration was measured 20 minutes in the presence, and for 40 minutes in the absence, of P_i and P-acceptor (ATP, glucose, hexokinase). The respiratory rates were constant over the time measured.

Additions	Rate of respiration		P:O ratio
	Without P_i plus P-acceptor	With P_i plus P-acceptor,	
	mμatoms oxygen/minute		
Pyruvate, malate	35	555	2.70
Pyruvate, malate, rotenone	0	0	
Pyruvate, malate, rotenone, vitamin K_3	260	385	1.65
Succinate	170	515	2.06
Succinate, rotenone	160	580	1.81
Succinate, rotenone, vitamin K_3	175	535	1.73

DISCUSSION

General agreement among investigators of the endergonic reduction of DPN by succinate seems to prevail concerning the involvement in this process of succinic dehydrogenase on one hand, and of the DPNH dehydrogenase flavoprotein on the other. Current discrepancies in views concern the electron path from succinic dehydrogenase to DPNH dehydrogenase.

According to one view, expressed both by our group (1 2, 14, 15) and by Klingenberg *et al.* (6, 14, 16–18) and Löw *et al.* (7), the reaction proceeds either by a direct interaction between the two flavoproteins or by way of a quinone, but in any case not through the cytochrome system. Another mechanism, postulated by Chance and Hollunger (8–10), involves both the antimycin A-sensitive site and cytochrome *b* as intermediate electron carriers between the two flavoproteins. The following lines of evidence seem to support the first pathway and to exclude the second:

1. Demonstration that Reduction of DPN by Flavosubstrates Is Not Inhibited by Concentrations of Antimycin A which are Sufficient to Block Respiratory Chain (1, 2, 6, 7)—Since block of the respiratory chain also means a suppression of the aerobic generation of high energy intermediates, it was of obvious importance in these experiments to secure an adequate supply of energy by other means. In the experiments of Klingenberg and Scholl-meyer (6) and of Löw *et al.* (7) energy could be supplied by added ATP. In our system, external ATP cannot provide sufficient energy for the succinate-linked DPN reduction, for reasons elaborated on elsewhere (1, 2, 19). Nevertheless the insensitivity of the reduction to antimycin A could be demonstrated (1, 2) by adding ferricyanide which allowed the generation of high energy intermediates.

In experiments with pigeon heart mitochondria, Chance and

[1] Rotenone rather than Amytal was used in these experiments because of the slight uncoupling effect of the latter (5).

Hollunger (9) have observed an inhibitory effect of antimycin A on both the rate and extent of reduction of DPN by succinate. Since in this system the succinate-linked DPN reduction was dependent upon added ATP, the suppression of the DPN reduction by antimycin A might not seem to be attributable to an inhibition of the energy supply. The following additional findings, however, raise some doubt as to the conclusiveness of the above observations in implicating the antimycin A-sensitive site as part of the succinate-DPN electron transfer pathway: (*a*) In an earlier paper Chance and Hagihara (20) had reported that pigeon heart mitochondria required ATP for the initiation of succinate-linked DPN reduction. Once started, however, the reaction proceeded more vigorously at the expense of high energy intermediates generated intramitochondrially than with externally added ATP as the only source of energy. Hence, the difference found by Chance and Hollunger (9) in the rates of DPN reduction under aerobic conditions and in the presence of antimycin A may reflect a difference in efficiency between the intra- and extramitochondrial energy sources. (*b*) Chance and Hollunger (9) offer no explanation for their finding (Fig. 6 of reference (9)) that addition of antimycin A to a system, in which maximal extent of DPN reduction has been attained by incubation with succinate and ATP, caused an instantaneous reoxidation of all DPN. Subsequent addition of ATP resulted in the formation of some DPNH, which, however, again was gradually reoxidized. These observations suggest, in our opinion, that some DPNH-oxidizing agent, *e.g.* oxaloacetate, must have been operating in the antimycin A-blocked system, and, thus, that the rate of DPN reduction in this system as estimated by Chance and Hollunger (9) is not a net rate but the resultant of a continuous reduction and reoxidation. (*c*) The antimycin A effect observed by Chance and Hollunger (9) does not appear to be consistently distinguishable from that of other respiratory chain inhibitors. For example, in a parallel paper, by Chance (21), a reduction-oxidation wave of DPN, very similar to that described by Chance and Hollunger with antimycin A, was shown (Fig. 10 of reference (21)) to occur when another inhibitor of electron transport, sodium sulfide, was added to succinate- and ATP-supplemented pigeon heart mitochondria.

2. Demonstration that One High Energy Bond Equivalent Is Expended per Molecule of DPN Reduced by Succinate ((1), (2), and this paper)—This ratio implies that the reversal of only the first of the three energy conservation reactions of the respiratory chain ought to be involved in the succinate-linked DPN reduction. The antimycin A-sensitive site is generally considered to be at or after, but not before, the site of the second energy conservation reaction of the respiratory chain. Thus, involvement of the antimycin A-sensitive site in the succinate-linked DPN reduction would not be compatible with the experimentally established energy requirement of the reaction.

Ratios of 2 to 3 high energy bond equivalents per molecule of DPN reduced have been postulated by Chance and Hollunger (8, 10) for the succinate-linked reduction of DPN. Since endergonic reduction of acetoacetate by succinate involves the reduction of DPN, and since it is very improbable that the oxidation of DPNH by acetoacetate would be connected with a generation of high energy bonds, our results imply that Chance and Hollunger's values must be overestimates. These values were derived from two types of experiments. In one approach, the calculation was based on estimations of the amount of ATP utilized in connection with the reduction of DPN by succinate in pigeon heart mitochondria under aerobic and anaerobic (Na_2S-inhibited) conditions. However, no satisfactory evidence was given to exclude the occurrence of side reactions, such as ATP formation by oxidative phosphorylation in the aerobic system, or splitting of ATP by way of *e.g.* the oxaloacetic carboxylase reaction in both the aerobic and anaerobic systems. In a second approach, the efficiency of the succinate-linked DPN reduction was calculated by Chance and Hollunger on the basis of the cytochrome *a* reduction-oxidation cycle after the addition of succinate to mitochondria in the controlled state. The energy expenditure was derived from titrations of the amount of ADP required for inducing an equal reduction-oxidation cycle of cytochrome *a*. However, the reduction of DPN in Chance and Hollunger's system was reported to proceed at a lower rate than that of the oxidation of succinate in the presence of P-acceptor. Hence, a direct comparison of the energy-trapping efficiencies of DPN reduction and of added ADP may not seem justified, just as it would not have been justified in our system to calculate the energy requirement of the acetoacetate reduction on the basis of the P:O ratio observed when a maximally efficient amount of hexokinase was present (*cf.* "Results" and Fig. 2).

3. Evidence that Electrons from DPNH, Generated by Succinate-linked DPN Reduction, Can Be Removed by Means of Added Vitamin K_3, and Funneled Into Respiratory Chain at Level of Cytochrome b—A competition with the succinate-linked acetoacetate reduction by way of this mechanism, as demonstrated in this paper, would not be feasible if the electrons from succinic dehydrogenase would enter the respiratory chain at or above the level of cytochrome *b*, since in that case the electrons trapped from DPNH by vitamin K_3 would be refed into the chain at the same site as they entered when coming from succinate. This finding, to our knowledge, is the first conclusive demonstration that cytochrome *b* is situated after, and not at or before, the merging point of the electron transfer routes from DPNH and succinate toward oxygen. The more efficient respiratory control observed with succinate in comparison to that found with reduced vitamin K_3 might be related to the previously described phenomenon of activation of succinate oxidation (1, 2, 14, 15, 22, 23). Further work in order to elucidate this relationship is in progress.

SUMMARY

Studies concerning the pathway of electron transfer and the energy requirement of the endergonic reduction of acetoacetate by succinate catalyzed by isolated rat liver mitochondria are reported.

It is shown that added vitamin K_3 can efficiently compete with acetoacetate in reoxidizing reduced diphosphopyridine nucleotide generated by the succinate-linked reduction, transferring the electrons by way of the terminal respiratory chain to oxygen, and that this effect of vitamin K_3 is abolished by dicoumarol. It is concluded that the entrance of electrons from succinic dehydrogenase into the respiratory chain occurs *before* (*i.e.* distal to oxygen) the site of entrance of electrons from reduced vitamin K_3, and consequently, that the succinate-linked acetoacetate reduction involves neither the antimycin A-sensitive site nor cytochrome *b*. Evidence is also presented that the aerobic oxidation of succinate and of reduced vitamin K_3, although both giving rise to two phosphorylations, obey different extents of respiratory control (the former more effective than

the latter) and the possible significance of this finding is considered.

Estimation of the energy requirement of the succinate-linked acetoacetate reduction is carried out on the basis of the respiratory increase caused by acetoacetate in a mitochondrial system oxidizing succinate in the absence of phosphate acceptor. The amount of acetoacetate reduced is compared with the phosphate uptake obtained in a parallel system where a corresponding respiratory increase was induced by the addition of adenosine triphosphate, glucose, and a limiting amount of hexokinase. From these measurements, the estimate of one high energy bond equivalent expended per molecule of acetoacetate reduced, previously arrived at in this laboratory, is confirmed. It is also shown that in mitochondria, which are in a "loosely coupled" state, the phosphate to oxygen ratio, or, in more general terms, the efficiency of the energy coupling mechanism, is greatly dependent upon the capacity of the energy-trapping system.

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