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SUCCINATE DEHYDROGENASE

I. ROLE OF PHOSPHOLIPIDS

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SUMMARY

Acidic phospholipids in the micellar state interact with succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) solubilized in the presence of succinate. An increase in enzymic activity ensues, which may be differentiated from "activation" by succinate. The phenomenon does not occur in succinate dehydrogenase in fragmented mitochondria which has the full lipid complement and if succinate is omitted during the solubilization procedure or is added only after solubilization of the enzyme. In preparations solubilized without succinate, however, phospholipids decrease the inhibition produced by oxaloacetate or by fumarate.

The reduction of fumarate by $FMNH_2$, catalyzed by succinate dehydrogenase solubilized in the absence of succinate, is inhibited by phospholipids.

The iron chelator, 2-thenoyltrifluoroacetone, abolishes the effects of phospholipids on the catalytic activity of the soluble flavoprotein. Moreover, in the presence of phospholipids, just as with the particle bound enzyme, the chelator inhibits both the forward and the reverse reaction catalyzed by the soluble dehydrogenase. This suggests that iron is involved in the interaction between enzyme and lipids. Phospholipids on the other hand may modify the hydrophobicity of the flavoprotein in the iron region and make iron available to the chelator.

INTRODUCTION

Much evidence has been produced showing that the activity of the respiratory chain of which succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) is part, and of several segments of it, depends on the presence of lipid components[1-6]. Close interactions exist between the catalysts, other proteins and

Abbreviations: PMS, phenazine methosulphate; DCIP, 2,6-dichlorophenolindophenol; TTA, 2-thenoyltrifluoroacetone.

lipid, resulting in a highly integrated, insoluble particulate system. The isolation of succinate dehydrogenase or other enzyme components from the particle is not easy. Differences in catalytic power and stability of the soluble enzyme as compared with the enzyme in the particulate form have been observed.

In previous studies we have shown that the decrease in catalytic activity upon solubilization of succinate dehydrogenase depends on the extraction of lipids from the original particulate preparations[7,8]. Subsequent work on the relationships between succinate dehydrogenase and lipids gave increasing evidence of the important role played by phospholipids in the catalytic efficiency of this enzyme, in its stability and in its binding to the particle. This appeared to be of interest since, except for their effect on cytochrome *c*, the influence of lipids had previously been demonstrated only on segments of the electron transport system containing several operational components, whereas here a single catalytic protein was involved.

The results mentioned above suggested that, when the succinic flavoprotein is separated from its highly hydrophobic physiological environment within the mitochondrion and is transferred to water as a soluble protein, its native structure and catalytic activity may be modified. Preliminary experiments indicated that the interaction with phospholipids may be of primary importance in maintaining the native properties of the flavoprotein[9,10]. The studies were therefore continued; the present paper deals with the effect of lipids on the catalytic activity of succinate dehydrogenase.

MATERIALS AND METHODS

Materials

The following materials were used. Fumaric acid, oxaloacetic acid, malonic acid, disodium succinate, trypsin crystallized, sodium dithionite (Merck & Co.); 2,6-dichlorophenolindophenol (DCIP) (Carlo Erba, RP grade); cholic acid (California Corporation for Biochemical Research); phenazine methosulphate (PMS), antimycin A, cytochrome *c*, α-chymotrypsin (3 times crystallized), ubiquinone 6, FMN (Sigma Chemical Co.); rotenone (British Drug Houses); 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TTA), synthetic β-γ-dipalmitoyl-L-α-glycerylphosphoryl ethanolamine, synthetic β-γ-dipalmitoyl-L-α-glycerylphosphorylcholine (Fluka AG); phosphatidic acid, lysophosphatidylcholine, sphingomyelin (Koch-Light Laboratories); natural glycerylphosphorylcholine and glycerylphosphoryl ethanolamine (kindly given by Dr. S. FLEISCHER, Vanderbilt University, Nashville, Tenn., U.S.A.); phosphatidylinositol, phosphatidyl-L-serine (Koch-Light, and gifts from Dr. G. PORCELLATI, Institute of Biological Chemistry, University of Pavia, Italy); natural cardiolipin (Sylvana Chemical Co., and gifts from Dr. S. FLEISCHER and Dr. J. H. DE BRUIJN, Rijksinstituut voor de Volksgezondheid, Utrecht, The Netherlands); synthetic cardiolipin (courtesy of Professor L. L. M. VAN DEENEN, Organisch Chemisch Laboratorium der Rijksuniversiteit, Utrecht).

Phospholipids were isolated from mitochondria or from whole heart tissue according to HANAHAN, DITTMER AND WARASHINA[11]. The phospholipid fraction was not fractionated further. Lipids were also extracted from mitochondria with aqueous acetone as described for the preparation of acetone powder (see below), the acetone phase being collected. This corresponds, with minor modifications, to the procedure

described by LESTER AND FLEISCHER[12]. The extract, predominantly phospholipids, was used without further purification.

All lipid preparations were dispersed to a micellar state according to FLEISCHER AND KLOUWEN[13] after being evaporated to dryness if necessary.

Chemical methods

Protein was determined either by the biuret[14] or by the micro-Kjeldahl method; crystalline bovine serum albumin served as a standard in both methods. Lipids were extracted from the trichloroacetic precipitate of enzyme preparations three times with chloroform–methanol (3:1, v/v); non-lipid phosphate contaminants were then removed from the extract using the procedure of FOLCH, LEES AND SLOANE-STANLEY[15]. Phospholipid phosphorus was estimated according to CHEN, TORIBARA AND WARNER[16]; in all calculations of phospholipid concentrations the phosphorus content of phospholipid was assumed to be 4%. Phospholipids were also assayed by thin-layer chromatography according to DAVISON AND GRAHAM-WOLFAARD[17], and to SKIPSKI, PETERSON AND BARCLAY[18].

Ubiquinone was assayed by the procedure of HATEFI *et al.*[19], or after extraction and purification according to CRANE *et al.*[20].

Peptide-bound flavins were assayed by differential fluorimetry as described previously[7,8], using a Farrand spectrofluorimeter.

Enzyme preparations

Mitochondria were prepared from beef heart[21]. Before use they were disrupted by freezing overnight in 10 mM KCl containing 1.2 mM phosphate buffer (pH 7.6) and then thawing. The pellet obtained by centrifuging at 59 000 × *g* was used in the subsequent steps.

Succinate dehydrogenase was solubilized either from the pellet by butanol treatment according to DERVARTANIAN AND VEEGER[22] or from acetone powders.

Acetone powders were prepared by slowly adding under continuous stirring in a refrigerated bath at −10°, 0.1 vol. of *tert.*-amyl alcohol and 10 vol. of acetone to the 59 000 × *g* precipitate suspended in 0.06 M phosphate buffer, pH 7.6. The mixture was filtered with suction, and the filter cake dried with acetone and ether. The acetone powder was then suspended in 0.05 M phosphate–0.05 M borate buffer (pH 10.3) for 10 min. The pH was adjusted to 8.3 with 1 M HCl and the preparation centrifuged at 59 000 × *g*. Soluble succinate dehydrogenase was present in the supernatant.

The solubilized enzymes were treated with calcium phosphate gel[22]. Further purification was carried out by ammonium sulphate fractionation[22].

When the enzymes were prepared in the presence of succinate, 40 mM succinate was added to all solutions after disruption of mitochondria. At the gel adsorption and elution stages the concentrations of buffer and of succinate were adjusted to maintain the ionic strengths indicated by DERVARTANIAN AND VEEGER[22].

Low temperatures (2–4°) and strict anaerobiosis by repeated evacuations and flushing with N_2 were maintained throughout the preparation of the enzyme, up to the very moment of assay. Unless otherwise stated, enzyme preparations were used as soon as ready or were stored in liquid nitrogen.

Enzymic assays

Succinate dehydrogenase activity was followed spectrophotometrically at 25° at 600 mμ in a Gilford Model 200 multiple-sample absorbance recorder equipped with a Beckman DU monochromator, or in a Beckman DB spectrophotometer equipped with Sargent DRL recorder. The recorder speed was usually 5 in. per min. The assay medium contained 20 mM sodium succinate, $8 \cdot 10^{-5}$ M 2,6-dichlorophenolindophenol, phenazine methosulphate from $1.5 \cdot 10^{-3}$ M to $1.9 \cdot 10^{-4}$ M and 0.8 mM KCN.

In the assay of particle-bound succinate dehydrogenase the enzyme was previously activated by preincubation for 20 min at 25° in 40 mM succinate.

Thermal equilibration at 25° of soluble enzyme preparations was obtained by adding the enzyme to the other reactants at 25° in the spectrophotometer cuvette to a final volume of 2.4 ml.

Fumarate reductase was assayed according to MASSEY AND SINGER[23] with minor modifications.

The activity of the enzyme in the forward or in the reverse reaction is expressed either as specific activity, *i.e.* mmoles succinate oxidized (or fumarate reduced) per min per g protein, or as catalytic-centre activity *i.e.* moles succinate oxidized (or fumarate reduced) per min per mole peptide-bound flavin. Since one mole of peptide-bound flavin is present per mole of succinate dehydrogenase, catalytic-centre activities allow one to compare preparations of different degrees of purity. When results refer to the same enzyme preparation, specific activity is enough for a fair comparison.

All activity measurements refer to initial rates, and are extrapolated to infinite concentration of the dye (PMS or $FMNH_2$).

Succinate oxidase activity was measured either manometrically at 38° according to BERNATH AND SINGER[21] or polarographically at 25° using a Clark oxygen electrode. For this assay the medium contained 10 mM sodium succinate, 60 mM potassium phosphate buffer (pH 7.6), and 1 mM cytochrome *c*.

RESULTS

Effect of extraction and addition of lipids

Table I shows the changes in some properties between bound and soluble succinate dehydrogenase.

The phospholipid concentration of the soluble preparations is considerably less than that of the original mitochondrial particle; a decrease in catalytic-centre activity accompanies solubilization; the greater decrease in activity occurs at the stage when the pellet is extracted with lipid solvents (butanol or acetone). These data suggest that phospholipids may influence the catalytic efficiency of the enzyme. This is confirmed by the observation that the addition of phospholipid strongly stimulates* lipid-extracted preparations.

The effect of the addition of phospholipid to different succinate dehydrogenase preparations is also shown in Table I. Lipid has no effect on the enzyme in particles where the lipid content is not markedly altered as compared with mitochondria[24]. A moderate stimulation is observed at finite phenazine concentration and decreases

* We use "stimulation" to indicate an increase in succinate dehydrogenase activity due to phospholipids, so as to avoid confusion with "activation" of the dehydrogenase by succinate or by competitive inhibitors.

TABLE I

CATALYTIC-CENTRE ACTIVITY AND PHOSPHOLIPID CONTENT OF SUCCINATE DEHYDROGENASE BEFORE AND AFTER SOLUBILIZATION

Phosphatidylserine, phosphatidylinositol, cardiolipin or phosphatidic acid (50 μg P per mg protein) or the mixed phospholipid fraction extracted from heart mitochondria (200 μg P per mg protein) were equilibrated with the enzyme preparations for 30 min at 2°. Other conditions are described in the text. Succinate dehydrogenase activity is given as moles succinate oxidized per min per mole peptide-bound flavin, at 25°. Native phospholipids were determined after extraction from the trichloroacetic precipitate of the enzyme preparations. Means ± standard deviation are shown.

Preparation	*Number of experiments*	*Catalytic-centre activity*		*Native phospholipid mg P per µmole peptide bound flavin*
		No addition	*Phospholipids added*	
Mitochondria (59 000 × *g* pellet) activated	7	12660 ± 1150	12500 ± 1100	218 ± 18
Succinate dehydrogenase solubilized by butanol treatment in succinate, gel eluate stage	10	9040 ± 980	15400 ± 1210	1.70 ± 0.28
Enzyme as above, after ammonium sulphate fractionation in succinate	9	8020 ± 870	13700 ± 1010	0.08 ± 0.02
Succinate dehydrogenase solubilized by butanol treatment without succinate, gel eluate stage, activated before the assay	7	6080 ± 1040	6000 ± 970	2.04 ± 0.32
Enzyme as above after ammonium sulphate fractionation without succinate, activated before the assay	7	5550 ± 1230	5500 ± 1330	
Acetone powder prepared in succinate	6	7900 ± 1370	14500 ± 1710	
Succinate dehydrogenase solubilized from the above	5	7700 ± 1120	14120 ± 1360	
Acetone powder prepared without succinate	5	4830 ± 1580	4650 ± 1530	
Succinate dehydrogenase solubilized from the above	5	4265 ± 1460	4300 ± 1480	

with increase in concentration of the dye. When rates are extrapolated to infinite phenazine concentration, no stimulation is evident.

On the other hand, succinate dehydrogenase preparations from which lipids have been extracted, such as those solubilized by butanol treatment or from acetone powder, and acetone powders, are stimulated by phospholipids, provided the preparations are made in the presence of succinate.

Conditions for lipid action

Stimulation of succinate dehydrogenase activity is obtained with the unpurified extract of lipids from heart mitochondria and with the phospholipid fraction isolated therefrom. Phospholipids extracted from other tissues, *e.g.* brain mitochondria, are also effective. Among the purified phospholipids that were tested, only acidic ones, such as cardiolipin, phosphatidylinositol, phosphatidic acid and phosphatidylserine are active. No stimulation is obtained with phosphatidylcholine, lysophosphatidylcholine or phosphatidyl ethanolamine (see Table IV).

A careful investigation of ubiquinone was carried out on all lipid preparations tested. In none was any evidence for the presence of ubiquinone obtained. It therefore seems unlikely that ubiquinone has any role in the phenomena reported here.

The micellar state is essential for the effect of lipid. Conditions which change the stability of the micelles, freezing and thawing for instance, either before or after equilibration with the enzyme, abolish the stimulation. Generally, after storage for one week at 2° the ability of the phospholipid preparations to stimulate the reaction decreases. This loss is probably related to changes in micellar organization. Inactive micellar preparations of phosphatidylserine which may even slightly inhibit the enzyme, stimulate it after the addition of Ca^{2+} (Table II, Expt. III). Ca^{2+} must be present during the period of equilibration of lipids with the enzyme. As shown in Fig. 1, optimal reactivation of the phospholipid preparation is achieved with a molar ratio of Ca^{2+} to phospholipid of 5:1. On the other hand, active phosphatidylserine preparations become inhibitory after the addition of Ca^{2+} (Table II, Expt. I). Similar

TABLE II

EFFECT OF Ca^{2+} ON THE STIMULATION OF SUCCINATE DEHYDROGENASE BY PHOSPHOLIPIDS

Succinate dehydrogenase in 40 mM succinate was incubated for 30 min at 2° with the lipid and/or with Ca^{2+} at the concentrations detailed in the legend. In the assay mixture there was from 9–16.4 μg protein per ml, and $CaCl_2$ from 62–124 μM. All other procedures and conditions are described in the text. Initial velocities are given as mmoles succinate oxidized per min per g protein at infinite PMS concentration (v_{max}).

Expt. No.	*Additions*			v_{max}	*% of control*	K_m *(PMS)* $(M \times 10^4)$
	Phospholipid	*μg P per mg protein*	$CaCl_2$ *(mM)*			
I. a	None	0	0	2.08	100	4.35
b	PS, lot A	99	0	2.80	135	4.95
c	PS, lot A	99	1.66	1.78	86	4.07
II. a	None	0	0	2.56	100	6.32
b	PI	76	0	4.35	170	10.12
c	PI	76	1.66	3.71	145	10.32
III. a	None	0	0	4.23	100	8.83
b	PS, lot B*	50	0	4.23	100	5.44
c	None	0	1.66	4.23	100	8.83
d	PS, lot B*	50	1.66	5.29	125	8.83
IV. a	None	0	0	2.23	100	4.18
b	PC	100	0	2.22	100	5.10
c	PC	100	1.66	2.25	101	4.96

* This preparation of phosphatidylserine, made from a different commercial lot from that used in Expt. I, did not stimulate dehydrogenase activity.

Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine.

events, though on a lesser scale, were observed with phosphatidylinositol. Lipids such as phosphatidylcholine, which by themselves do not interact with succinate dehydrogenase, are not affected in their behaviour by Ca^{2+} (Table II, Expt. IV).

Stimulation by phospholipids requires preincubation with the dehydrogenase. This is shown in Fig. 2. When lipids are added immediately before the assay the activity is the same as for succinate dehydrogenase assayed without any addition.

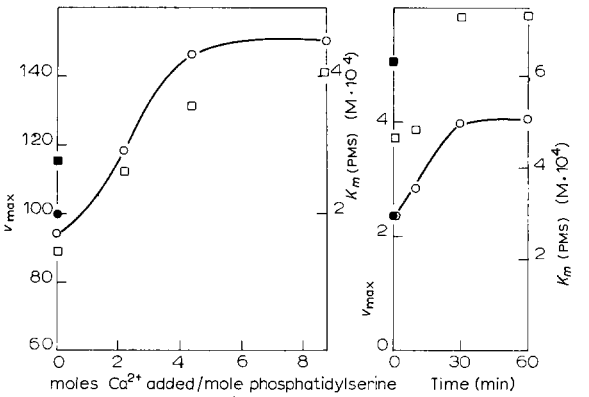

Fig. 1. Effect of Ca^{2+} on the stimulation by phospholipid of the activity of soluble succinate dehydrogenase. Succinate dehydrogenase solubilized in the presence of 40 mM succinate was preincubated for 30 min at 2° with $CaCl_2$, in the amount indicated by the abscissa, and with a preparation of phosphatidylserine (50 μg P per mg protein) which had lost the capacity of stimulating the activity of the dehydrogenase. Other conditions are detailed in the text. Left ordinate, initial velocity at infinite PMS concentration as percentage of the activity of the enzyme assayed without additions. Right ordinate, K_m (PMS).

Fig. 2. Influence of the preincubation time on the stimulation by phospholipid of the activity of soluble succinate dehydrogenase. Succinate dehydrogenase was solubilized as described for Fig. 2. Prior to assay it was incubated at 2° with phosphatidylinositol, 100 μg P per mg protein, for the period indicated by the abscissa. Other conditions are detailed in the text. Left ordinate, initial velocity at infinite PMS concentration in mmoles succinate oxidized per min per g protein. Solid symbols indicate, in Figs. 1 and 2, enzymes assayed without additions. Squares, K_m (phenazine).

Enzymes incubated with lipids for longer periods appear more active, and full effect is reached after 30–60 min preincubation. A standard period of 30 min was used in all other experiments, unless otherwise stated. We have routinely carried out the equilibration at 2–4°.

At a fixed protein concentration the effect increases with the lipid concentration. The curve of activity *vs.* total lipid concentration has a sigmoid shape (Fig. 3). The apparent K_m for phenazine increases with increasing stimulation. Purified phospholipids show maximal stimulation at about 50 μg phospholipid phosphorus per mg protein. With unfractionated mitochondrial phospholipid about four times as much is required; this may be because phosphatidylcholine and phosphatidyl ethanolamine, which do not stimulate succinate dehydrogenase, represent 68% of the total phospholipid of beef heart mitochondria[24] and may form mixed micelles with acidic phospholipids.

The degree of maximal stimulation varies with each preparation. It is also influenced by the state of the enzyme; in general it is higher with aged enzyme preparations. With high concentrations of phosphatidylinositol, stimulation of dehydrogenase activity disappears (Fig. 3).

Role of succinate and of competitive inhibitors in the stimulation by phospholipids

It is known that treatment of succinate dehydrogenase with succinate or with competitive inhibitors "activates" the enzyme.

Preparations made in the presence of succinate appear to be fully activated

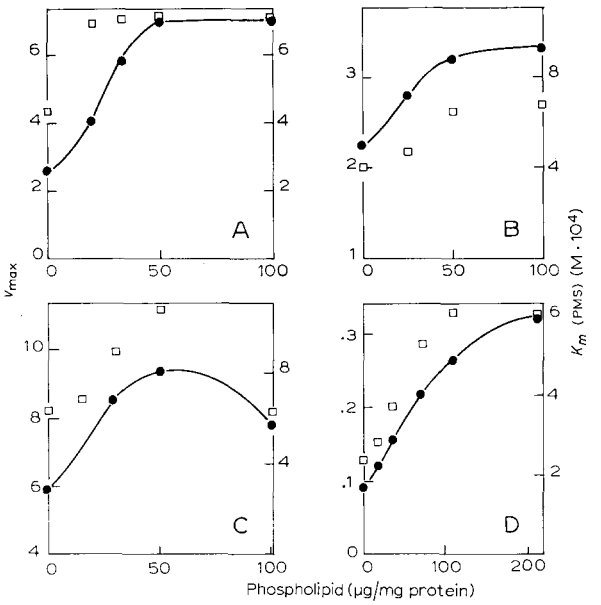

Fig. 3. Effect of increasing amounts of phospholipids on the activity of soluble succinate dehydrogenase. A, B and C were dehydrogenase preparations made by butanol treatment in the presence of 40 mM succinate, assayed at the gel eluate stage (A and B) or after ammonium sulphate fractionation (C). D was succinate dehydrogenase solubilized from an acetone powder in the presence of 40 mM succinate; this enzyme preparation was aged for 150 h at −20° under N before use. Cardiolipin (A), phosphatidylserine (B), phosphatidylinositol (C) and the total mitochondrial lipid extract (D) were preincubated, in the amounts indicated by the abscissa, with the enzyme preparation for 30 min (A, B, C) or 60 min (D) at 2°. Other conditions are detailed in the text. Abscissa, µg phospholipid P added per mg protein. Solid lines and circles, initial velocity (v_{max}) at infinite PMS concentration, in mmoles succinate oxidized per min per g protein. Squares, K_m (PMS).

and do not show an increase in activity when incubated at 25° with succinate at any concentration. They are stimulated by phospholipids. Enzymes solubilized in the absence of succinate are readily activated by succinate. They are not stimulated either by purified phospholipids or by total or fractionated extracts of mitochondrial lipids. As shown in Table III, activation by succinate does not restore to these enzyme preparations the ability to be stimulated by lipids.

Activation was performed by preincubating with 40 mM succinate for 20 min at 25°; in other experiments the time of preincubation with succinate was reduced, or the temperature was lowered to 2° so as to minimize thermal inactivation which is appreciable in soluble succinate dehydrogenase. Some typical experiments are reported in Table III. Activation effects are evident in various degrees but lipids do not stimulate enzymes prepared in the absence of succinate. Addition of lipids lowers the apparent K_m for phenazine; activity is not much affected in fully-activated preparations (*e.g.* Table III, Expt. I), but is significantly decreased if the preparation is only mildly activated (*e.g.* Table III, Expts. II and III).

An effect of lipids upon succinate dehydrogenase prepared without succinate becomes evident in the presence of oxaloacetate and of fumarate. Increasing phospholipid concentrations gradually remove the inhibition due to the presence of the

TABLE III

EFFECT OF PHOSPHOLIPID AND OF ACTIVATION ON SUCCINATE DEHYDROGENASE PREPARED WITHOUT SUCCINATE

Succinate dehydrogenase solubilized in the absence of succinate was activated with succinate either before or after incubation for 30 min at 2° with lipids. Dehydrogenase activity was then assayed as described in the text. In the assay succinate was 20 mM. Initial velocities are given as mmoles succinate oxidized per min per g protein at infinite PMS concentration (v_{max}).

Expt. No.	*Conditions at activation*			*Equilibration with lipids*			v_{max}	*% of control*	K_m *(PMS) (M × 10⁴)*
	Succinate (mM)	*Preincubation*		*Phospholipid*	*µg P per mg protein*	*Order of addition*			
		Time (min)	*Temp.*						
I	—	None	—	None		—	1.22	100	5.87
	40	10	25°	None		—	1.76	147	7.11
	40	10	25°	PS	17	After activation	1.76	147	5.73
II	—	None	—	None		—	1.23	100	6.39
	40	20	2°	None		—	1.59	129	6.71
	40	20	2°	PS	50	After activation	1.19	97	3.73
III	—	None	—	None		—	2.62	100	5.45
	5	0.5	25°	None		—	3.35	128	6.48
	5	0.5	25°	PI	98	Before activation	2.27	87	4.55

Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol.

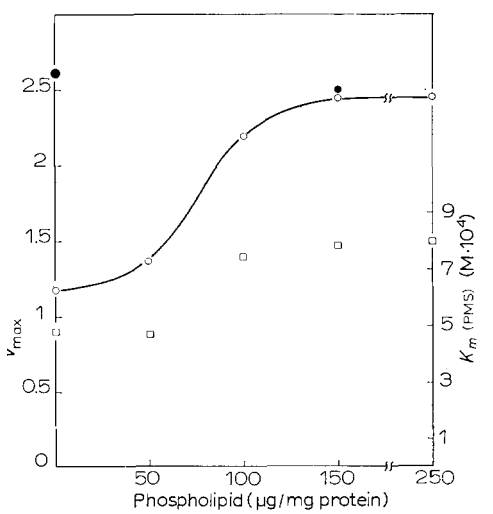

Fig. 4. Effect of phospholipid on the inhibition by oxaloacetate of soluble succinate dehydrogenase. Succinate dehydrogenase was solubilized in the absence of succinate. Where indicated it was preincubated for 30 min at 3° with the phospholipid fraction isolated from mitochondrial lipids. The preparation was then diluted into the spectrophotometer cuvette with 50 mM potassium phosphate buffer at 25° to 1.2 ml, then oxaloacetate was added, and after mixing the other reactants to a final volume of 2.4 ml. Final oxaloacetate concentration was $3.7 \cdot 10^{-7}$ M. Other conditions are detailed in the text. Abscissa, µg phospholipid P added per mg protein. Left ordinate, initial velocity (v_{max}) in mmoles succinate oxidized per min per g protein, at infinite phenazine concentration. Solid circles, v_{max} without addition of oxaloacetate. Open circles, v_{max}, and squares, K_m (phenazine), oxaloacetate added.

competitive inhibitor. The curve relating v_{max} to lipid added per mg protein (Fig. 4) has a shape similar to that obtained in the stimulation of non-inhibited succinate dehydrogenase prepared with succinate. An increase in K_m (phenazine) with increasing lipid action is also observed. The same phospholipids are active that are effective in stimulating the enzyme prepared in the presence of succinate (Table IV). Preincubation of phospholipids with the dehydrogenase is not mandatory; inhibition is also removed if they are added to the enzyme preparation just before the assay.

TABLE IV

EFFECT OF PURIFIED PHOSPHOLIPIDS ON SUCCINATE DEHYDROGENASE

Succinate dehydrogenase solubilized and purified either in the presence of or without succinate was preincubated with lipids (40–100 μg P per mg protein) for 30 min at 2°. For assays with competitive inhibitors 0.18 ml enzyme treated with lipids were diluted to 1.08 ml into the spectrophotometer cuvette with 60 mM potassium phosphate buffer (pH 7.6) at 25°. Subsequent additions were 0.12 ml $7.4 \cdot 10^{-6}$ M oxaloacetate or $6.6 \cdot 10^{-2}$ M fumarate, and after mixing, the other reactants in 1.2 ml at 25°. This started the reaction.

Lipid added	*Succinate dehydrogenase prepared in 40 mM succinate*	*Succinate dehydrogenase prepared without succinate*	
		+ Oxaloacetate	*+ Fumarate*
Cardiolipin	Stimulates	—	—
Phosphatidylserine	Stimulates	Inhibition removed	Inhibition removed
Phosphatidic acid	Stimulates	Inhibition removed	—
Phosphatidylinositol	Stimulates	Inhibition removed	—
Lysophosphatidylcholine	No stimulation	Inhibition remains	—
Phosphatidylethanolamine	No stimulation	Inhibition remains	—
Phosphatidylcholine	No stimulation	—	—
Sphingomyelin	No stimulation	—	—

We have attempted to determine at which step, in the isolation procedure, the presence of succinate is necessary in order to obtain an enzyme that can interact with lipids. If succinate is present at the addition of butanol and subsequent adsorption on calcium phosphate gel of the butanol-contaminated enzyme solution, but is omitted during the elution that follows, a preparation is obtained that does not interact with lipids and is slightly activable by succinate. However, if the elution from calcium phosphate gel is also done in the presence of succinate, succinate may be omitted during the subsequent fractionation with ammonium sulphate; the final precipitate between 0.30 and 0.45 saturation, dissolved in a buffer containing succinate, is readily stimulated by phospholipids.

We have investigated whether succinate might in part be transformed into fumarate or oxaloacetate during the isolation of the enzyme when preparations are made in succinate, so that stimulation by lipids in reality might consist in removal of an inhibition due to oxaloacetate or to fumarate. Rotenone (from 1–0.1 μmole/g protein) was added to all media during the preparation of mitochondria and the isolation of the enzyme in succinate so as at least to prevent formation of oxaloacetate. In these conditions phospholipids stimulate succinate dehydrogenase activity; the usual increase in apparent K_m for the dye was no longer observed (Table V).

TABLE V

STIMULATION BY LIPIDS OF SUCCINATE DEHYDROGENASE PREPARED IN THE PRESENCE OF ROTENONE AND OF SUCCINATE

Succinate dehydrogenase was solubilized by butanol treatment in the presence of succinate. Rotenone was present in all solutions from the preparation of the heart mince onwards; the amount was never less than 0.1 μmole/g protein. All other procedures and conditions are described in the text. Initial velocities are given as mmole succinate oxidized per min per g protein at infinite PMS concentration (v_{max}).

Addition		v_{max}	*% of control*	K_m *(PMS)* $(M \times 10^4)$
Phospholipid	*μg P per mg protein*			
None	—	7.55	100	15.2
Phosphatidic acid	83	9.94	132	15.2
Phosphatidylinositol	63	9.94	132	15.2

Effect of lipid addition on the reduction of fumarate

The ratio between the catalytic-centre activity in the forward and in the reverse directions, namely oxidation of succinate with phenazine as acceptor to reduction of fumarate with $FMNH_2$ as donor, drops from 70 in particle-bound succinate dehydrogenase to about 11 in soluble preparations (Table VI). In either direction values are calculated on mitochondria and soluble enzymes not activated. The results in Tables I and VI show that the ratio drops because upon extraction of lipids from the particulate preparation and solubilization of the flavoprotein the catalytic-centre activity in succinate dehydrogenation decreases and that in fumarate reduction increases.

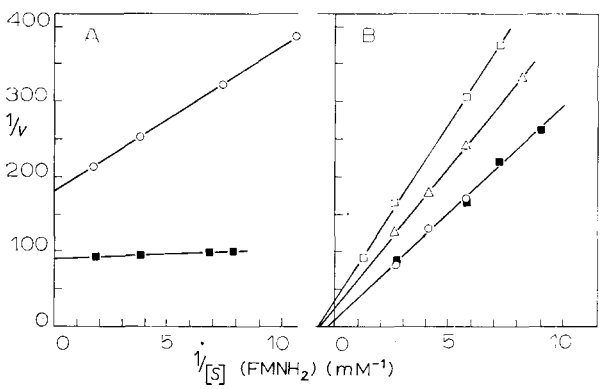

Fig. 5. Effect of thenoyltrifluoroacetone and of phosphatidylserine on the reduction of fumarate by particle-bound (A) and soluble succinate dehydrogenase (B). TTA was added to the enzyme in the main compartment of a Thunberg spectrophotometric cuvette and incubated for 2 min at 25° before the assay. Other conditions as in Table VII. Preincubation with phosphatidylserine was for 60 min at 2°. The enzyme used in A was particle-bound succinate dehydrogenase, 0.47 mg protein per ml in the assay. The enzyme used in B was succinate dehydrogenase solubilized in the absence of succinate, 0.38 mg protein per ml in the assay. A. ■—■, no additions; ○—○, TTA added, $1 \cdot 10^{-3}$ M, final concentration in the assay. B. ■—■, no additions; ○—○, TTA added, $1 \cdot 10^{-4}$ M, final concentration in the assay; □—□, phosphatidylserine added, 50 μg P per mg protein; △—△, phosphatidylserine added then $1 \cdot 10^{-4}$ M TTA. Ordinates: reciprocal initial velocity in (mmoles fumarate reduced per min per g protein)$^{-1}$. Abscissa: reciprocal $FMNH_2$ concentration in the assay in mM^{-1}.

TABLE VI

EFFECT OF PHOSPHOLIPIDS ON THE RATE OF FUMARATE REDUCTION AND ON THE RATIO OF CATALYTIC-CENTRE ACTIVITIES IN THE FORWARD AND REVERSE REACTION CATALYZED BY SUCCINATE DEHYDROGENASE

The assay of particulate succinate dehydrogenase was run on the 59 000 × *g* pellet of fragmented mitochondria. Soluble succinate dehydrogenase was prepared in the absence of succinate[22]. Fumarate reduction was measured in a Thunberg spectrophotometric cuvette. In the assay of particle-bound succinate dehydrogenase the main compartment contained the enzyme preparation and 60 mM phosphate buffer (pH 7.6) at 2°; fumarate and FMN were in separate side arms. $Na_2S_2O_4$ was added to FMN in a nitrogen atmosphere in slight excess over that needed for reduction of the dye to compensate for oxidized carriers in the particulate preparations. The cuvette was then equilibrated at 25°, and the reaction started by tipping in simultaneously $FMNH_2$ and fumarate. Soluble succinate dehydrogenase was assayed with a similar procedure, but fumarate was with the enzyme in the main compartment, and FMN was reduced by stoichiometric amounts of $Na_2S_2O_4$. Succinate dehydrogenase activity in the forward direction was measured after convenient storage of the enzyme at 2° under N_2, so as to account for the much longer time required in the assay of fumarate reduction. Preincubation of the soluble enzyme with phosphatidylserine (50 μg P per mg protein) was for 60 min at 2°. Initial velocities at infinite dye concentration are given as mmoles substrate transformed per min per g protein (v_{max}) or per mmole of peptide-bound flavin, (catalytic-centre activity). Ratios between catalytic-centre activities in succinate oxidation (SD) *vs.* fumarate reduction (FR) are given in the last column.

Enzyme preparation	*Addition*	*Fumarate reduction*		*Succinate dehydrogenation*		*(SD)*
		$v_{max} \times 10^2$	*Catalytic-centre activity*	$v_{max} \times 10^2$	*Catalytic-centre activity*	*(FR)*
Particulate succinate dehydrogenase	None	1.0	165	70	11550	70
Soluble succinate dehydrogenase	None	14.7	313	170	3600	11.5
Soluble succinate dehydrogenase	Phosphatidylserine	4.0	85	170*	3600	42.5

* The rate of succinate oxidation by soluble succinate dehydrogenase prepared in the absence of succinate is not enhanced by the addition of phospholipids.

Addition of phospholipids to soluble succinate dehydrogenase strongly inhibits the rate of fumarate reduction (Fig. 5). The ratio between catalytic-centre activities in the forward and reverse reactions is thus considerably increased in soluble preparations assayed after equilibration with lipids (Table VI).

Role of TTA and of lipids in succinate dehydrogenase

None of the phospholipids considered in this study, tested under standard conditions or with Ca^{2+} addition, makes soluble succinate dehydrogenase able to interact directly with DCIP. This reaction is shown only by the flavoprotein integrated in a particle, and requires some other component of the electron transport system[25].

Another difference between particle-bound and soluble succinate dehydrogenase is that TTA, at concentrations below millimolar, does not inhibit the soluble enzyme[10,26–28], while the dehydrogenase in particles is inhibited[10,26–29]. In the presence of lipids however 10^{-4} M TTA also becomes inhibitory upon the soluble enzyme. This is illustrated in Table VII.

TABLE VII

INHIBITION BY TTA OF SOLUBLE SUCCINATE DEHYDROGENASE IN THE PRESENCE OF PHOSPHOLIPIDS

Succinate dehydrogenase in 40 mM succinate was preincubated with lipids for 30 min at 2°. TTA was added either before or after incubation with lipids*. Dehydrogenase activity was then assayed as described in the text. The concentration of TTA at the assay was always $1 \cdot 10^{-4}$ M. All other procedures and conditions are those described in the text. Initial velocities are given as mmoles succinate oxidized per min per g protein at 25° at infinite PMS concentration (v_{max}).

Expt. No.	*Order of additions*				v_{max}	*% of control*	K_m *(PMS)* ($M \times 10^4$)
	TTA (M)	*Phospholipid*	*(µg P per mg protein)*	*TTA (M)*			
I. a	0	None	0	0	2.25	100	4.00
b	$2 \cdot 10^{-4}$	None	0	0	2.25	100	4.40
c	0	PS	99	0	3.31	147	6.80
d	$2 \cdot 10^{-4}$	PS	99	0	2.04	91	4.40
e	0	PS	99	$2 \cdot 10^{-4}$	2.67	119	5.43
II. a	0	None	0	0	2.57	100	8.28
b	0	PI**	100	0	2.40	93	7.96
c	$2 \cdot 10^{-4}$	PI**	100	0	1.62	63	5.73
d	0	PI**	100	$2 \cdot 10^{-4}$	2.27	89	8.58
III. a	0	None	0	0	4.88	100	7.76
b	$2 \cdot 10^{-4}$	None	0	0	4.88	100	7.76
c	0	PE	100	0	5.01	103	5.82
d	0	PE	100	$2 \cdot 10^{-4}$	3.86	79	3.43
e	0	PC	100	0	4.55	93	5.43
f	0	PC	100	$2 \cdot 10^{-4}$	4.55	93	4.66

* TTA was dissolved in absolute ethanol, and the volume added was always less than 1% as compared with the volume of enzyme preparation. After addition of TTA the preparation was heated for 30 sec to 1 min at 25°.

** This preparation of phosphatidylinositol did not stimulate dehydrogenase activity.

Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

Two effects can be identified with TTA. Low levels of TTA and addition of the inhibitor just before the assay, after equilibration of the enzyme with lipids, abolish the stimulation by lipids; no significant inhibition is however observed with respect to the basic activity of the enzyme not stimulated by lipids. However, when TTA is incubated with the flavoprotein together with lipids 30 min before assay, it removes the extra activity stimulated by phospholipids and also inhibits with respect to the activity of the enzyme not stimulated by lipids (*e.g.* Table VII, Expts. Id; IIc). Under conditions that allow a complete equilibration of the inhibitor with the enzyme, inhibition by TTA of soluble succinate dehydrogenase is also elicited by lipid preparations that have lost their power of stimulating the activity of the enzyme (Table VII, Expt. IIc), and by phospholipids such as phosphatidylethanolamine, which by themselves have no capacity for stimulating succinate dehydrogenase activity. Phospholipids with ionized basic groups, such as phosphatidylcholine, do not elicit any inhibition of succinate dehydrogenase by TTA (Table VII, Expt. III).

As shown in Fig. 5, TTA inhibits the reverse reaction catalyzed by succinate dehydrogenase, namely reduction of fumarate, when the enzyme is particle-bound, whereas it has no effect thereupon with the soluble enzyme. In the presence of

phospholipids TTA removes the inhibition by lipids of fumarate reduction, and apparently itself becomes inhibitory to the soluble enzyme also in this assay.

DISCUSSION

The results reported in the present paper suggest an effect of phospholipid on the catalytic efficiency of succinate dehydrogenase. Further aspects of the interaction are connected with the stability of the dehydrogenase and with its binding to the respiratory particle. These are examined in another paper[28]. The mechanism of phospholipid action suggested by ABDULLA AND DAVISON[30] has been discussed in a previous paper[31] and seems inadequate to explain the various effects brought out by phospholipids.

In the measurement of the enzyme activity the reactions that do not directly involve the enzyme are not rate limiting and the overall effect of lipids cannot be attributed to stimulation of any of them. All our results indicate that the effect of lipids depends upon the state of the enzyme, or at least that their action involves the molecule of the dehydrogenase. Indeed, under identical conditions of assay, lipids act upon enzymes prepared in succinate but no effect occurs if succinate is omitted during the preparation. In this preparation an effect of lipids appears in the presence of oxaloacetate or of fumarate. Stimulation by lipids disappears after addition of TTA, and none is observed on particulate preparations. A separate line of evidence confirming that lipids interact with the enzyme molecule is found in their ability to stabilize the dehydrogenase, preserving the activity to a level which is not duplicated if they are added after ageing the enzyme, before the assay[10,28]. Moreover, the observed increase in K_m (phenazine) indicates that lipids involve the protein in their effect; the apparent affinity for the dye is lowered. An effect of lipids also occurs when $FMNH_2$ interacts with the flavoprotein. Other artificial acceptors have not yet been studied; however, the effect of phospholipid on the rate of succinate oxidation should not depend on the choice of the acceptor since there is no evidence that the reaction between phenazine and flavoprotein is rate limiting in the assay of enzymic activity.

Under appropriate experimental conditions the various effects of lipids are qualitatively the same with enzymes isolated from different starting material (acetone powders or butanol-treated mitochondria) and of different purity (gel eluate and after ammonium sulphate fractionation), although a scatter in the values of v_{max} and K_m is observed, which probably depends on the purity and age of the preparations.

In the presence of high succinate concentrations, such as those used throughout the purification, succinate dehydrogenase exists mainly as the reduced enzyme–fumarate complex. This form interacts with lipids, and the effect is a stimulation of catalytic activity and an increased stability[10,28]. When the dehydrogenase is solubilized in the absence of succinate the reduced enzyme-fumarate complex can be produced, *e.g.* by incubating with succinate without an acceptor, but it does not appear to react with lipids judging from the absence of stimulation and of stabilization. Evidently the redox state of the flavin is not the unique condition for interaction of lipids with the dehydrogenase, and it is suggested that the conformational state of the flavoprotein is important. The effective state is maintained only when succinate is present throughout the purification. This might be correlated

with the observation that succinate improves the stability of the enzyme[28,32] and with the well-known effect of succinate on the ability of the dehydrogenase to interact with other components of the succinoxidase system[33]. The critical step at which the dehydrogenase molecule is more susceptible to external agents and succinate may prevent (or introduce) modifications, is probably at the separation of the succinic flavoprotein from the respiratory chain and at the first treatments it undergoes as a soluble protein. This interpretation agrees with the data of WANG AND WANG[34] on the inhibition of reconstitution by histidine and *o*-phenanthroline. Our results and those on reconstitution[33] show that the effect of succinate during purification does not coincide with activation (see also ref. 22).

The effect of lipids does not coincide with activation: as far as enzymic activity is concerned the form of succinate dehydrogenase solubilized in succinate appears to be already maximally "activated". Moreover phospholipids make soluble succinate dehydrogenase susceptible to TTA inhibition whereas activation does not[28].

With succinate dehydrogenase prepared in the absence of succinate an effect of lipids is apparent as a decrease in the inhibition by oxaloacetate and fumarate. A prolonged contact between lipids and flavoprotein is not necessary in this case: hence the mechanism of this effect may differ from that for stimulation of enzymes solubilized in succinate. The results so far do not allow us to state whether lipids simply compete with the inhibitor or whether they increase the dissociation of the enzyme-inhibitor complex. They may well do this since according to DERVARTANIAN AND VEEGER[22] the interaction between the inhibitors mentioned and succinate dehydrogenase probably gives rise to a charge transfer complex or at least to a change in polarity near the flavin. In either event lipids may have a destabilizing effect, by decreasing compression due *e.g.* to dipole forces that enhance charge transfer complex formation[35], or simply by originating a less-polar surrounding.

Only acidic phospholipids interact with succinate dehydrogenase. The interaction is probably at least in part electrostatic, with cationic groups on the protein. Our analytical results do not exclude some lipid being still associated with the soluble flavoprotein; added phospholipids may therefore also interact with this endogenous lipid and thereby give a suitable surface charge to the enzyme. The rate of reaction between protein and lipid as measured by the onset of stimulation is slow, and the rate-limiting step may be a rearrangement of lipids on the protein. Probably Ca^{2+} ions affect or reactivate the stimulatory power of phospholipid preparations by inducing a change in the micellar structure. The high efficiency of Ca^{2+} in this respect has been pointed out by several authors[36–38]. Some importance may also be attached to the influence of unsaturation in the fatty acids on the binding between phospholipid and Ca^{2+} (ref. 39). GREEN AND FLEISCHER[5] have pointed out that unsaturation is mandatory for lipids active on submitochondrial particles; we did not systematically investigate the relationships between degree of unsaturation and effect of phospholipid: it is our belief, however, that the decay in activity observed in aged lipid preparations may be due to the loss of double bonds.

TTA readily removes the effect of lipid upon the catalytic activity (stimulation of succinate oxidation or inhibition of fumarate reduction). This suggests that iron in the flavoprotein is involved in the interaction between the enzyme and lipid. TTA easily binds it without necessarily impeding the basic catalytic role of iron. Under proper conditions, however, TTA not only modifies the interaction between lipids

and enzyme, preventing stimulation of activity, but inhibits the enzyme itself. A convenient explanation is that, owing to its hydrophobic character the inhibitor cannot reach, into the soluble flavoprotein, the non-heme iron which is supposed to be the reaction site of TTA with succinate dehydrogenase[29]. Most likely, binding with lipids increases the hydrophobic character of the region of the flavoprotein where iron is located and makes it available to the inhibitor as it is in the particle-bound dehydrogenase. A role of lipids in the action of TTA as modifiers of the hydrophobic environment of the metal is suggested by studies on hydroxybutyrate dehydrogenase[40] and by the results of ZIEGLER[25] and of TAPPEL[41].

Experiments to be reported in detail elsewhere[42] favour the interpretation of TTA action suggested above. Thus, in fragmented mitochondria depleted of lipids by the action of lipolytic enzymes, the inhibitory power of TTA is significantly decreased. This cannot be attributed to the lack of some catalytic site for phenazine on which TTA is active, since the complete electron-transport system besides succinate dehydrogenase is present in the preparation.

Since the reduction of fumarate by particle-bound succinate dehydrogenase is inhibited by TTA, iron is probably also involved in the reverse reaction catalyzed by the flavoprotein. Previous reports[23], which excluded iron from the catalytic mechanism, are based on very indirect evidence.

It seems reasonable to assume that phospholipids inhibit the reduction of fumarate by soluble succinate dehydrogenase by the same mechanism by which they remove inhibition of the forward reaction by fumarate or by oxaloacetate, *i.e.* they increase the dissociation of the complex between enzyme and fumarate or they compete with fumarate. Since the rate of fumarate reduction is influenced by phospholipids, it follows that the increased activity towards $FMNH_2$ in soluble preparations of succinate dehydrogenase probably depends upon the removal of phospholipids.

The study of the reverse reaction ($FMNH_2$–fumarate) confirms that in the presence of TTA the effect of added lipid on the catalytic activity of the soluble flavoprotein vanishes. The residual inhibition is of the same degree as the one produced by TTA in the particulate enzyme. It is suggested that here too lipid makes the soluble dehydrogenase available to the inhibitor, and TTA itself inhibits the catalytic activity as it does in the particle.

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