Inhibition of Mitochondrial Electron Transport by Piericidin A and Related Compounds*

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ABSTRACT: Piericidin A inhibits the electron transport system at two sites. It specifically reacts at very low concentrations at a site near the reduced nicotinamide–adenine dinucleotide (NADH) dehydrogenase which may be identical with the site of rotenone action. At high concentrations, it inhibits the succinic dehydrogenase system, and the inhibition can be partially reversed by coenzyme Q. Reduction of coenzyme Q by both dehydrogenase systems is inhibited by piericidin A. The pattern of cytochrome reduction in the presence of piericidin A is unusual in that cytochrome c_1 is reduced when NADH is the substrate and cytochrome *b* is reduced when succinate is the substrate. Several compounds related to piericidin A have been studied; side-chain-reduced piericidins A and B are inhibitory. Castor bean mitochondria show inhibition patterns similar to those of beef heart mitochondria.

Both the succinate and NADH oxidase system in *Azotobacter* electron transport particles are inhibited at high levels of piericidin A which are equivalent to levels which inhibit the succinoxidase of beef heart mitochondria.

Piericidin A is a substance which has been isolated from *Streptomyces mobaraensis* and shown to have insecticidal activity (Takahashi *et al.*, 1965). The organic structural resemblance between piericidin A and coenzyme Q had originally suggested the possibility that piericidin A might act as a competitive inhibitor of coenzyme Q. Indeed, it does act as an inhibitor of mitochondrial electron transport and it may be that its insecticidal and other lethal effects are based on inhibition of mitochondrial function (Hall *et al.*, 1966). It was found that the oxidation of both NADH[1] and succinate is inhibited, but the NADH oxidase system is much more sensitive to piericidin inhibition than the succinoxidase system.

We now present detailed evidence that piericidin A can inhibit at the coenzyme Q site in the succinoxidase system, and that the NADH oxidase system is sensitive to very low amounts of inhibitor so that an inhibition equivalent to that observed with the succinoxidase system cannot be observed. The site of inhibition in the NADH oxidase system appears to be similar to the site of inhibition of this system by rotenone (Ernster *et al.*, 1963).

A closely related natural product, piericidin B, as well as other structurally related compounds has also been studied for inhibitory effects. The structures of piericidin A and B and the other compounds are shown in Figure 1.

Materials and Methods

Preparation of Mitochondria. Beef heart mitochondria were prepared by a modified method of Löw and Vallin (1963). Cold, fresh beef hearts were trimmed of fat and connective tissue and ground in 0.25 M sucrose and maintained at pH 7.5 by addition of 0.1 M Tris. The Tris-sucrose mince was homogenized in a Waring Blendor at full speed for 30 sec. Tris (0.1 M) was occasionally added to keep the pH around 7.2–7.5. The homogenate was immediately centrifuged for 10 min at 1900 rpm (1600*g*) in a refrigerated International serum centrifuge (Model 13L). The red supernatant was decanted through a single layer of cheesecloth and the residue was discarded. The supernatant was then passed through a refrigerated Sharples supercentrifuge at 50,000 rpm (62,000*g*). The mitochondria which have been sedimented in the bowl of the centrifuge were scraped out in a cold room and homogenized with an equal volume of 0.25 M sucrose by means of a power-driven Potter-Elvehjem homogenizer. The mitochondrial suspension was frozen and stored at −15°. Castor bean mitochondria were prepared by the method of Breidenbach and Beevers (1967) and were kindly supplied by Dr. Breidenbach.

Sonication of Mitochondria. All manipulations were carried out within the range of 0–5°. The crude mitochondrial suspension in 0.25 M sucrose was diluted with a suitable amount of the same solution to a protein concentration ranging from 20 to 30 mg per ml and then subjected to sonic oscillation at 2–4 A for a total of 1.5–5 min in a Branson sonifier. The suspension was centrifuged for 20 min at 30,000 rpm (78,000*g*) in the Spinco preparative ultracentrifuge (no. 30). The resi-

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[1] Abbreviations not listed in *Biochemistry 5*, 1445 (1966), are: PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol; TMPD, *p*-tetramethylphenylenediamine.

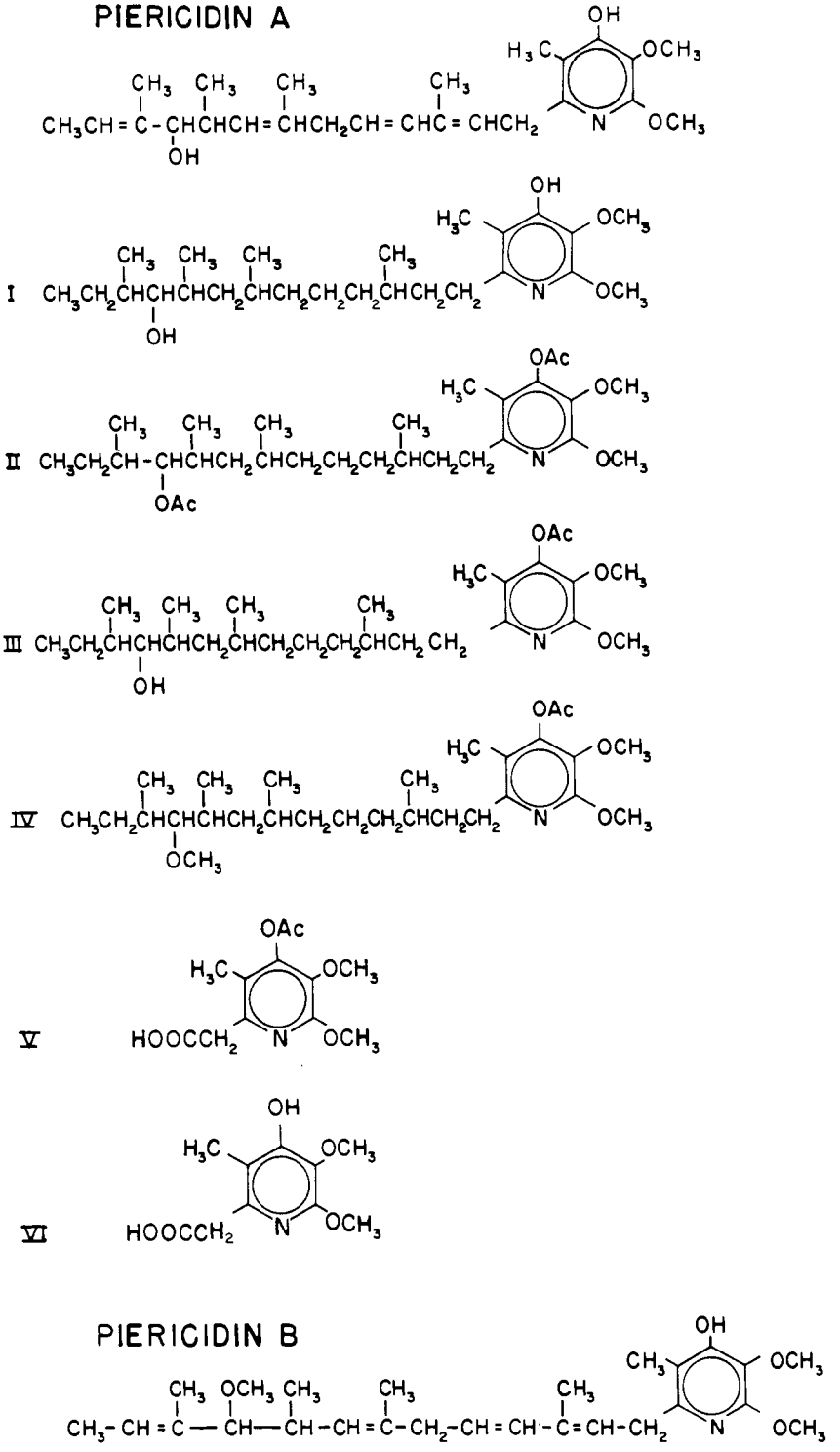

FIGURE: 1 The structure of piericidin A and B and the derivatives of piericidin A.

due was taken up in sucrose solution and homogenized with the power-driven Potter-Elvehjem homogenizer.

Preparation of the Electron Transport Particle. The crude mitochondrial suspension was adjusted to a protein concentration of 10–20 mg/ml and then subjected to sonic oscillation at 6–7 A for a total of 9 min by the use of a continuous-flow attachment in the Branson sonifier. The suspension was passed through the adapter at the rate of 10–20 ml/min. The first centrifugation was carried out in the no. 55–34 rotor of a Sorvall centrifuge at 15,000 rpm (27,000*g*) for 15 min. The supernatant was then centrifuged at 40,000 rpm (104,000*g*) in the no. 40 rotor of the Spinco centrifuge for 30 min. The residue was resuspended in 0.25 M sucrose by homogenizing with the Potter-Elvehjem power-driven homogenizer.

Preparation of Red Particles. A suspension of the electron transport particle in 0.25 M sucrose was mixed with an equal volume of 0.2 M Tris-Cl of pH 8.0. Deoxy-

cholate (10% solution) was then added in sufficient amount to achieve a concentration of 0.5 mg of deoxycholate/mg of protein. The suspension which was partially clarified by the addition of deoxycholate was then centrifuged for 45 min at 40,000 rpm (104,000*g*) in the Spinco preparative ultracentrifuge. After dialysis for 18 hr against 0.02 M phosphate buffer of pH 7.5, the red supernatant was centrifuged at 40,000 rpm (104,000*g*) for 30 min. The red precipitate was taken up in 0.25 M sucrose.

Preparation of NADH–CoQ Reductase. The preparation of NADH–CoQ reductase followed the method of Hatefi *et al.* (1962).

Preparation of the Electron Transport Particle of Azotobacter. The preparation of the electron transport particle of *Azotobacter* followed the method of Jurtshuk *et al.* (1967).

Assay Procedures

Protein Determination. Protein was determined by the biuret method (Gornall *et al.*, 1949).

NADH Oxidase. NADH oxidase activity was determined by the method of Slater (1950). The reagent concentrations were KPO_4^{2-} (1.8×10^{-3} or 7.5×10^{-4} M, pH 7.4) and NADH (2.3 or 3.53×10^{-3} M), respectively, per 3-ml volume for spectrophotometric assay and per 2-ml volume for polarographic assay.

NADH–Cytochrome c Reductase. This assay is based on the spectrophotometric measurement of a change in absorbancy at 550 mμ by means of the Unicam S.P. 800 recording spectrophotometer. The sample cuvet contained KPO_4^{2-} (1.8×10^{-3} M) (pH 7.4), cytochrome *c* (10–15 μg), and KCN (2.5×10^{-3} M) in a total volume of 2.8 ml against a blank containing all components except the substrate. The reaction started when 2.35×10^{-3} M NADH was added into the system. The molar extinction coefficients for reduced and oxidized cytochrome *c* at 550 mμ are taken to be, respectively, 2.87 and 0.9×10^7 cm$^2 \times$ mole^{-1}, the difference 1.97×10^7 cm$^2 \times$ mole^{-1} representing the change in absorbancy at 550 mμ which accompanies reduction of cytochrome *c*. The specific activity is defined as micromoles of cytochrome *c* reduced per milligram of protein per minute at 30°.

NADH–Coenzyme Q Reductase. The assay of NADH–coenzyme Q reductase activity followed the method of Hatefi *et al.* (1960). CoQ_2 was used as the intermediate instead of CoQ_1.

Reduction of PMS. The enzymatic oxidation of succinate by PMS was followed manometrically by measurement of oxygen uptake. The main compartment of the manometer flask contained 150 μmoles of Tris-Cl buffer (pH 7.0), 50–100 μmoles of succinate, 0.1–0.5 mg of enzyme, and 3 μmoles of KCN in a final volume of 3.0 ml. In the side arm, 0.1 ml of 1% PMS was added. The gas phase was air. After the manometer flasks had been incubated for 5 min at 38°, PMS was tipped in from the side arm and the rate of oxygen uptake was measured during three 2.5-min intervals, after an initial 2-min equilibration period with the taps open. The solution of PMS (in the side arm) must be protected from light at least until it is mixed with the contents of the flask.

Reduction of DCIP. The enzymatic oxidation of succinate by indophenol was followed spectrophotometrically at 600 mμ. A cuvet of 3-ml capacity and 1-cm light path was filled with 2.98 ml containing 50 μmoles of phosphate buffer (pH 7.4), 0.1–0.5 mg of enzyme, 2.5 μmoles of KCN, and 0.06 mg of 2,6-dichlorophenolindophenol. The reading at 600 mμ started when 50 μmoles of succinate in 0.02 ml was added. The rate of reaction is linear with respect to time for at least 2 min, and also linear with respect to enzyme concentration. Specific activity is defined as micromoles of indophenol reduced per milligram of protein per minute at 38°. The optical change at 600 mμ divided by 16.1 is the value for the amount of micromoles of indophenol reduced.

Reduction of Cytochrome c. The same procedure was followed as mentioned above (NADH–cytochrome *c* reductase), except with succinate instead of NADH as substrate.

Reduction of Ferricyanide. The enzymatic oxidation of succinate by ferricyanide was followed manometrically by measurement of carbon dioxide evolution. The main compartment of each manometer flask contained 100 μmoles of succinate, 0.1–0.5 mg of enzyme, and 50 μmoles of potassium bicarbonate in a final volume of 3.0 ml. The side arm contained 50 μmoles of ferricyanide in 0.2 ml. The gas phase was a mixture of 95% nitrogen and 5% carbon dioxide. After the manometers had been incubated for 5 min at 38°, ferricyanide was tipped in from the side arm and the rate of carbon dioxide evolution was measured after a 1-min equilibration period. The specific rate of carbon dioxide evolution is expressed as micromoles of carbon dioxide per milligram of protein per minute at 38°.

Oxidation of TMPD and Ascorbate. The rate of the oxidation of TMPD and ascorbate was measured polarographically at 38°. Oxygen in solution was determined by means of an oxygen electrode apparatus (Gibson Medical Electronics, Model K) employing a rapidly oscillating platinum electrode. The operating voltage was set at 0.6 V. Recording speed was 0.5 cm/sec. Freshly made TMPD (50 μmoles) was added to the electrode cell, which in a total volume of 2.5 ml contained 100 μmoles of phosphate buffer (pH 7.4), 0.33 mg of enzyme, and 100 μmoles of ascorbate. The activity of the system is estimated from the observed rate of decrease in the oxygen concentration of the medium at 38°.

Succinoxidase Activity. The rate of succinoxidase was measured polarographically as described above. The system contained 50 μmoles of phosphate buffer (pH 7.4), 0.1–0.5 mg of enzyme, and 0.1 ml of 1% cytochrome *c* in a total volume of 2.3 ml. The reaction was started by the addition of 0.2 ml of succinate (50–100 μmoles).

Difference Spectra. The difference spectra of the mitochondria were measured by the method of Chance (1952) in a Unicam S.P. 800 recording spectrophotometer at 20°. The cuvet contained 10–15 mg of mitochondrial protein and 50 μmoles of phosphate buffer (pH 7.4) in a total volume of 3.0 ml. Immediately after the addition of 50–100 μmoles of succinate or small amounts of NADH crystals to the sample cuvet, the

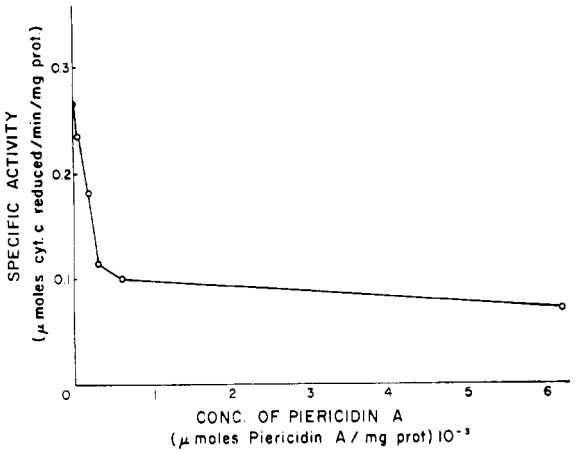

FIGURE 2: The inhibition of NADH–cytochrome *c* reductase activity by piericidin A.

suspension was scanned from 700 to 400 mμ. A complete scan from 700 to 400 mμ required 1.5 min.

The Oxidation–Reduction State of CoQ in Mitochondria. The determination of the oxidation–reduction state of CoQ at a steady state in the mitochondria followed the method described below. The beef heart mitochondria (1 ml) suspended in 0.25 M sucrose containing about 10–30 mg of protein incubated with 0.2 ml of 0.3 M succinate or 0.3 ml of 2×10^{-3} M NADH with or without the addition of 0.5 ml of KCN (0.5 M) was put in the Potter-Elvehjem homogenizer for 10 min. The preparation was denatured by rapid addition of absolute ethanol (ten times the volume). After stirring for 5 min the system was transferred to a 15-ml conical centrifuge tube and centrifuged in the International clinical centrifuge for 5 min. The extraction was repeated three times. The pooled supernatant was evaporated to dryness under vacuum. A small amount of isooctane was used to take up the CoQ from the precipitate. The isooctane solution was evaporated to dryness and resuspended in spectral grade absolute ethanol. The concentrations of CoQ was determined by a spectrophotometric assay which is based on the decrease in optical density at 275 mμ after reduction of the oxidized CoQ by addition of solid borohydride.

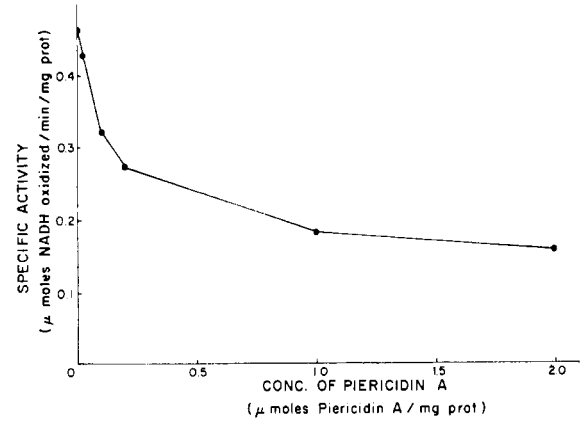

FIGURE 3: The inhibition of NADH–CoQ reductase activity by piericidin A.

Chemicals. Cytochrome *c* (types II, III, and VI) from horse heart, β-NADH, PMS, and CoQ_{10} were obtained from Sigma Chemical Co., St. Louis, Mo.

Coenzyme Q_2 was obtained from Dr. Folkers. Piericidins A and B and the derivatives were prepared by Drs. Takahashi and Tamura. Antimycin A was obtained from the Sigma Co., amobarbital from Eli Lilly, rotenone from British Drug House Ltd., and α-tocopherol was purchased from Distillation Products Industries, Rochester, N. Y.

Amobarbital and rotenone solutions were prepared according to Asano and Brodie (1964). Antimycin A, piericidin A, and its derivatives were dissolved in absolute ethanol and were added in small amounts in ethanolic solution.

TMPD and ascorbic acid were products of Eastman Kodak Co. and were used in aqueous solutions without further purification. Both TMPD and ascorbate solutions were freshly prepared just prior to use.

The phospholipids were prepared according to F. Sun and F. L. Crane (in preparation). The composition of phospholipid is as followed: 37% lecithin, 32% phosphotidylethanolamine, 18% of cardiolipin, and the remainder unknown phospholipid. The isolation is an acetone precipitation of phospholipids under nitrogen.

Results

Inhibition of NADH Oxidase by Piericidin A. Piericidin A is an especially potent inhibitor of NADH oxidase activity and shows complete inhibition at 0.036 mμmole/mg of mitochondrial protein (Table I). Coenzymes Q_2 and Q_{10}, vitamin $K_{2(10)}$, and phospholipid, singly or in combination, were unable to restore the inhibition.

Piericidin A inhibited NADH–cytochrome *c* reductase activity 90% as did antimycin A (Figure 2). Addition of antimycin A to a piericidin A inhibited system

TABLE I: Inhibition of NADH Oxidase Activity in Beef Heart Mitochondria by Piericidin A and Related Compounds.

Compound	Concn (μmoles/mg of protein)	% Inhibn of Initial Act.
Piericidin A	1.6×10^{-5}	0
Complete inhibition at about 0.036 mμmole/mg of protein	2.0×10^{-5}	30
	3.0×10^{-5}	69
	3.6×10^{-5}	100
	3.6×10^{-5}	100
Compound I	7.4×10^{-5}	0
	1.1×10^{-4}	100
	5.4×10^{-4}	100
Piericidin B	7×10^{-2}	36
	2×10^{-1}	88
	4×10^{-1}	90

TABLE II: Inhibition of Succinoxidase Activities in Beef Heart Mitochondria by Piericidin A and Its Derivatives and Piericidin B.

Compound	Concn (μmoles/mg of protein)	Succinoxidase Act. (μmoles of O_2/min mg of protein)
Piericidin A	None	0.65
	3.29×10^{-1}	0.28
	6.57×10^{-1}	0.28
	1.97	0.11
Compound I	None	0.58
	6.5×10^{-2}	0.52
	3.26×10^{-1}	0.21
	6.45×10^{-1}	0.00
Compound II	None	0.31
	2.69×10^{-1}	0.21
	8.07×10^{-1}	0.22
	1.34	0.13
Compound III	None	0.31
	2.93×10^{-1}	0.18
	8.79×10^{-1}	0.13
	1.46	0.05
Compound IV	None	0.31
	2.86×10^{-1}	0.22
	8.54×10^{-1}	0.17
	1.43	0.16
Compound V	None	0.31
	5.07×10^{-1}	0.26
	1.52	0.24
	2.53	0.17
Compound VI	None	0.31
	6.01×10^{-1}	0.21
	1.80	0.21
	3.01	0.21
Piericidin B	None	0.309
	2.43×10^{-2}	0.253
	7.30×10^{-2}	0.272
	1.22×10^{-1}	0.182
	2.43×10^{-1}	0.127
	7.30×10^{-1}	0.073
	1.22	0.073

did not increase the inhibition. The small amount of residual activity of NADH-cytochrome *c* reductase activity in the presence of piericidin A could be due to an antimycin A insensitive NADH-cytochrome *c* reductase present at one-tenth the level of the respiratory chain dehydrogenase.

Piericidin A inhibition of NADH oxidase activity shows evidence of stoichiometric titration of a component present in mitochondria in lower amounts than any other of the known redox agents (King *et al.*, 1964). In this respect its effect resembles rotenone inhibition and the amount required for complete inhibition is strikingly similar to saturating levels of rotenone (Ernster *et al.*, 1963), which has been shown to act at the level of the NADH dehydrogenase.

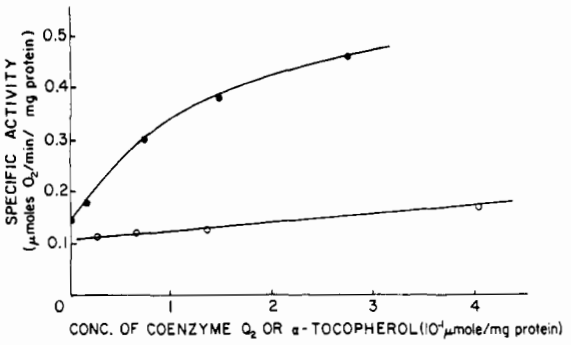

FIGURE 4: The restoration of piericidin A by different concentrations of CoQ_2 and α-tocopherol. No external cytochrome c was added for assay. (O–O–O–O) α-Tocopherol and (●–●–●) CoQ_2.

Compounds related to piericidin were used to ascertain what structural aspects were important in causing inhibition. Among the derivatives, compound I, which is most like piericidin A, showed the best inhibition, while the others showed little or no inhibition even at highly elevated levels (Table I). Piericidin B inhibits NADH oxidase at the level of 7×10^{-7} mole/mg of protein (Table I).

The NADH–coenzyme Q reductase particle described by Hatefi *et al.* (1962) represents the NADH dehydrogenase separated from the cytochromes. The activity of this enzyme preparation was also inhibited by piericidin A as shown in Figure 3. Maximum inhibition is only obtained at 2 μmoles/mg of protein in contrast to maximum inhibition at 3.6×10^{-5} μmole/mg of protein for inhibition of NADH oxidase activity in mitochondria. With this enzyme we also obtain only partial inhibition of activity. These effects indicate that the affinity of the enzyme for piericidin has been modified by purification. Incomplete inhibition may also indicate a partial bypass of the site of inhibition.

Inhibition of the Succinate Oxidase System by Piericidin. Succinoxidase activities of the sonicated beef heart mitochondria were greatly decreased when piericidin A was added to the assay system. The degree of inhibition varied within a limited range in different preparations of mitochondria. An increase in the amount of piericidin A added caused an increased inhibition of succinoxidase activity (*cf.* Table II).

Among the derivatives of piericidin A, compound I seemed to be a better inhibitor than piericidin A itself. Lower concentrations of compound I (0.645 μmole/mg of protein) were needed to completely abolish succinoxidase activity as compared to 80% inhibition at 2.0 μmoles/mg of protein of piericidin A. Compounds II to VI showed only slight inhibition of succinoxidase activity. Table II shows the inhibitory effects of piericidin A and its derivatives, and piericidin B on succinoxidase activity.

The inhibition of succinoxidase activity by piericidin A could be reversed by adding coenzyme Q when external cytochrome *c* is not added to the assay mixture. If cytochrome *c* was added then high levels of phos-

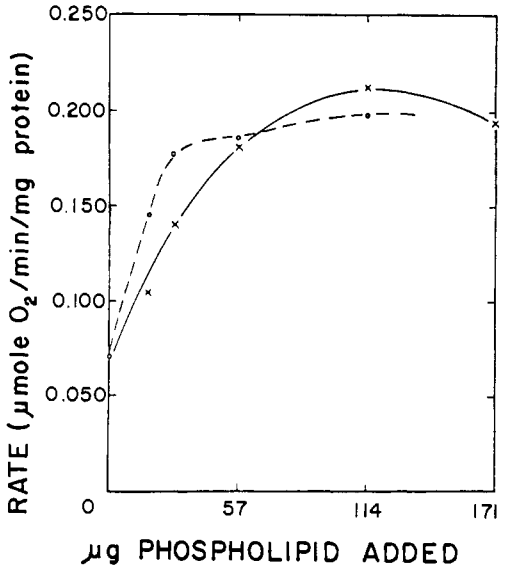

FIGURE 5: The restoration of the inhibition of piericidin A by different concentrations of phospholipid with or without CoQ_{10}. The solid line is for phospholipid and the dashed line for phospholipid with CoQ_{10}. External cytochrome *c* added for this assay.

pholipid or coenzyme Q acted to reverse the inhibition.

Addition of CoQ_2 to a piericidin-inhibited system restored succinoxidase activity, and the degree of the restoration was determined by the concentration of CoQ_2 added (Figure 4). Addition of increasing concentrations of CoQ_2 led to greater restoration of succinoxidase. Equimolar levels of α-tocopherol did not give the same restoration as CoQ_2 but did give a partial restoration at high concentration which might be related to reversal of nonspecific inhibition by the fatty part of the molecule as in the reversal of isooctane inhibition by α-tocopherol (Pollard and Bieri, 1958). CoQ_{10} was also effective in the reversal of the inhibition (Table III). Specific restoration of activity by CoQ occurs when no external cytochrome *c* is added to the assay system.

If external cytochrome c is added to the assay system addition of high levels of phospholipid will reverse the piericidin inhibition. Coenzyme Q will still show a restorative effect if the phospholipid level is kept low. Figure 5 shows the reversal of the inhibition of piericidin A by phospholipid and CoQ_{10}. Phospholipid would restore the succinoxidase activity when it was added alone. When CoQ_{10} is added along with phospholipid the rate approaches a maximum at a lower level of phospholipid than with phospholipid alone. When the assay system contained no exogenous cytochrome *c* phospholipid alone did not give any restora-

TABLE III: Reversal of Piericidin A Inhibition of Succinoxidase Activity by CoQ_2 in Beef Heart Mitochondria.[a]

Additions (μmoles/mg)	Succinoxidase Act. (μmoles of O_2/min mg of protein) −Piericidin A	+Piericidin A
Piericidin A at 1.28 μmoles/mg of Protein		
None	0.35	0.11
Coenzyme Q_2 (0.11)	0.34	0.16
Coenzyme Q_2 (0.22)	0.32	0.21
Coenzyme Q_2 (0.56)	0.30	0.24
Coenzyme Q_2 (0.22)	0.00	0.00
Piericidin A at 0.76 μmole/mg of Protein		
None	0.58	0.21
Coenzyme Q_{10} (1.2)	0.47	0.37
α-Tocopherol (1.3)	0.47	0.22
α-Tocopherol (4.1)	0.46	0.29

[a] No cytochrome *c* added.

TABLE IV: Inhibition of Succinate–Cytochrome *c* Reductases by Piericidin A and Its Derivatives in Beef Heart Mitochondria.

Addition	Concn (μmoles/mg of protein)	Sp Act. (μmoles of cytochrome *c*/min mg of protein)
Piericidin A	None	0.071
	0.548	0.032
	1.643	0.020
	2.739	0.020
Compound I	None	0.036
	0.645	0.024
	1.289	0.018
	1.934	0.006
	3.223	0.000
Compound II	None	0.037
	0.135	0.042
	0.269	0.035
	0.807	0.050
Compound III	None	0.037
	0.147	0.038
	0.293	0.032
	0.897	0.029
	1.466	0.024
Compound IV	None	0.037
	0.143	0.042
	0.285	0.041
	0.854	0.043
	1.423	0.033
Compound V	None	0.037
	0.254	0.042
	0.507	0.090
	1.520	0.055
Compound VI	None	0.037
	0.300	0.056
	0.601	0.085

TABLE V: The Inhibition of Succinate–Indophenol Reductase Activity by Piericidin A and Its Derivatives in Beef Heart Mitochondria and Reversal of Inhibition by Coenzyme Q and α-Tocopherol.

Addition	Concn of Inhibitor (μmoles/mg of protein)	Sp Act.[a]	Addition	Concn of Inhibitor (μmoles/mg of protein)	Sp Act.[a]
Piericidin A	None	0.043		1.803	0.028
	0.657	0.014	Piericidin A	None	0.043
	1.971	0.011		3.285	0.007
	3.285	0.007	Piericidin A + α-tocopherol (4.1 μmoles/mg)	3.285	0.012
Compound I	None	0.043			
	0.065	0.038			
	0.326	0.033			
	0.645	0.009	Piericidin A + CoQ_{10} (1.2 μmoles/mg)	3.285	0.014
Compound II	None	0.043			
	0.293	0.036			
	0.807	0.047	Piericidin A + CoQ_2 (0.11 μmole/mg)	3.285	0.020
	1.345	0.028			
Compound III	None	0.043			
	0.293	0.036	Compound I	None	0.043
	0.538	0.028		0.645	0.009
	0.807	0.034	Compound I + α-tocopherol (4.1 μmoles/mg)	0.645	0.020
Compound IV	None	0.043			
	0.285	0.029			
	0.570	0.036			
	0.854	0.023	Compound I + CoQ_{10} (1.2 μmoles/mg)	0.645	0.017
Compound V	None	0.043			
	0.507	0.034			
	1.014	0.054	Compound I + CoQ_2 (0.11 μmole/mg)	0.645	0.043
	1.520	0.037			
Compound VI	None	0.043			
	0.601	0.034	CoQ_2 (0.11 μmole/mg)	None	0.059
	1.202	0.047			

[a] Specific activity: micromoles of indophenol reduced per minute per milligram of protein.

tion, in contrast to the restorative effect of phospholipid when cytochrome c was present. The inhibition of succinoxidase by compound I could be restored by addition of CoQ_2 and CoQ_{10}.

Piericidin A inhibited succinate–cytochrome *c* reductase also, and the inhibition of this enzyme by compound I is better than that of piericidin A as in the case of succinoxidases. Compounds II, IV, V, and VI somewhat stimulate the succinate–cytochrome *c* reductase activity at certain concentrations. Particularly, compounds V and VI showed almost twofold stimulation of the activity compared with the activity of the untreated beef heart mitochondria (Table IV).

Piericidin A shows a strong inhibition of the reduction of indophenol by succinate. The same result was found with compound I. Other derivatives showed the same peculiar phenomena as in the succinate–cytochrome *c* reductase assay system (Table V). This activity was extremely sensitive to antimycin A when piericidin A and its derivatives were present in the assay system. The inhibition of reduction of indophenol by piericidin A was not reversed by CoQ_{10} or α-tocopherol but slightly by CoQ_2.

The reduction of ferricyanide and phenazine methosulfate by succinate were partially inhibited in the presence of piericidin A (Table VI). Reversal of the inhibition by CoQ_{10} was not as great as the reversal of inhibition of succinoxidase or succinate–cytochrome *c* reductase. Both reactions are sensitive to thenoyl trifluoroacetone.

The addition of piericidin A will decrease the oxidation of tetramethylphenylenediamine (TMPD) and ascorbate by cytochrome *c* oxidase. The activity (80%) of the enzyme was abolished at 1.10 μmoles of piericidin A/mg of protein. Addition of antimycin A will further inhibit the activity of the enzyme system (Table VII).

Cytochrome oxidase is not inhibited by piericidin A at levels which inhibit succinoxidase activity. Slight inhibition of cytochrome *c* oxidase is observed at levels of 50–100 times greater than the concentration which inhibits succinoxidase.

Difference Spectrum. Spectrophometric determina-

TABLE VI: Inhibition of Succinate-Based Reduction of Ferricyanide and PMS by Piericidin A in Beef Heart Mitochondria and Reversal of Inhibition by Coenzyme Q_2.[a]

Addition	Concn (μmoles/mg of protein)	Activity (Succinate)	
		Ferricyanide (μmoles of CO_2/min mg of protein)	PMS (μatoms/min mg of protein)
None	None	4.7	2.1
Piericidin A	0.255	4.5	1.4
	0.765	3.4	1.4
	1.275	2.5	0.5
	2.550	2.8	
None	None	6.7	2.9
None	None + CoQ_2 (0.33 μmole/mg)	8.5	3.3
Piericidin A	1.275	3.1	1.9
Piericidin A	1.275 + CoQ_2 (0.33 μmole/mg)	6.4	2.5

[a] Coenzyme Q_2 concentration given in micromoles per milligram of protein.

tion of cytochrome reduction in the sonicated beef heart mitochondrial preparation was used. The difference spectra were measured in a Unicam S.P. 800 recording spectrophotometer at 38° as described in Methods. Reduction of mitochondrial cytochrome was inhibited at the level of piericidin A which inhibits the activities of succinoxidases. Succinate was used as the substrate (Figures 6–9).

Figure 6 shows the normal reduction of mitochondrial cytochromes when succinate was added to the system without piericidin A. Cytochromes *b* and *c* and a small amount of cytochrome *a* are reduced immediately after addition of succinate. The full reduction of cytochromes was obtained 2.5 min later. Dithionite was added to the system in order to compare the total amount of cytochromes in the mitochondria to the amount of cytochromes which underwent oxidation–reduction during electron transfer. An extra amount of cytochrome a_3 appeared at the Soret region and small amounts of additional cytochrome *b* were observed after addition of dithionite.

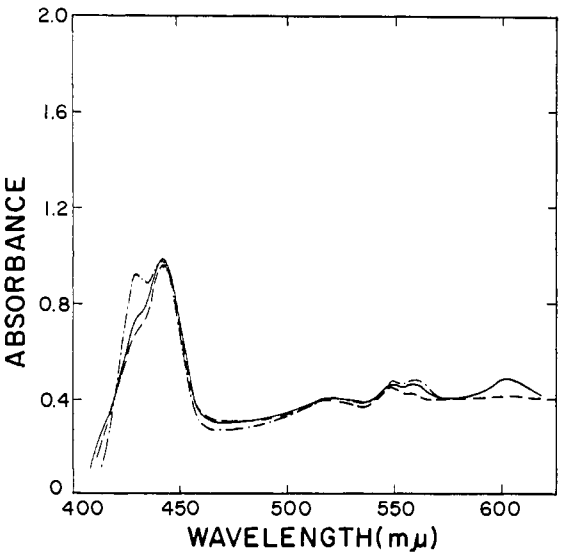

FIGURE 6: The difference spectra of beef heart mitochondria without piericidin A or antimycin A added. Succinate as substrate. (– – – –) The reduction of cytochromes immediately after addition of succinate; (———) 2.5 min later; (– · – ·) reduction by dithionite. The cell contains 50 μmoles of phosphate buffer (pH 7.4), 12 mg of mitochondrial protein, and 80 μmoles of succinate. The total volume is 3 ml. A few crystals of dithionite are added.

Addition of the amount of piericidin A which inhibited succinoxidase activity delayed the reduction of the cytochromes. Cytochrome *b* was first observed at 1.5 min after addition of succinate. A partial reduction of cytochrome *a* was seen 4.5 min after the addition of substrate. The full reduction of all cytochromes oc-

TABLE VII: Inhibition of the Oxidation of TMPD and Ascorbate by Beef Heart Mitochondria.

Addition	Concn (μmoles/mg of protein)	Act. (μmoles of O_2/min mg of protein)
Piericidin A	None	0.236
	0.22	0.118
	0.66	0.168
	1.10	0.042
Antimycin A	0.55	0.143
Piericidin A + antimycin A	0.66 + 0.55	0.084

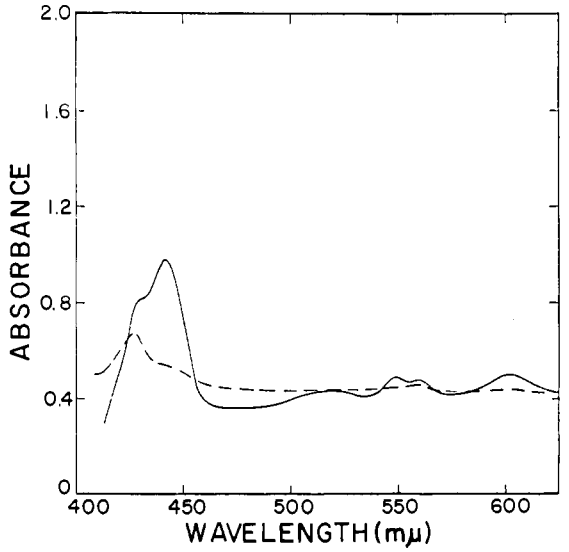

FIGURE 7: The difference spectra of beef heart mitochondria when piericidin A is added. Succinate as substrate. (- - - -) 1.5 min after addition of succinate; (———) 6 min after addition of succinate. The content of the cell is the same as that of the cell in Figure 4. Piericidin A (2×10^{-8} mole) is added.

curred around 1.5 min later, namely, 6 min after addition of succinate. The difference spectrum which was taken in the presence of piericidin A was slightly different from the normal difference spectrum at 550 (cytochrome *c*) and 430 mμ (Figure 7).

Figure 8 shows the difference spectrum after addition of antimycin A to mitochondria with succinate as the substrate. Cytochrome *b* was the only cytochrome to get reduced. Figure 9 is the difference spectrum when piericidin A and antimycin A were added together to the system. An unusual spectrum was obtained. Only cytochrome *c* was reduced to a small extent. Addition of dithionite which was expected to fully reduce the cytochromes surprisingly did not cause appearance of any bands of cytochrome *a*. Instead, the bands of cytochrome *a* shifted to 590 mμ which corresponds to the bands of a modified form of cytochrome *a* resulting from interactions between components suggested by Jacobs *et al.* (1964).

The difference spectra were also taken when NADH

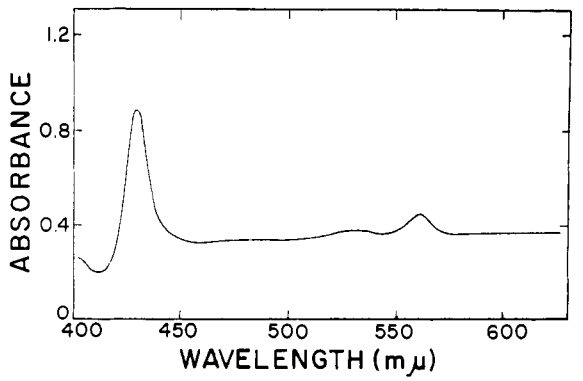

FIGURE 8: The difference spectrum of beef heart mitochondria when antimycin A is added. Succinate as substrate. Antimycin A (1 mg) is added in the reaction mixture.

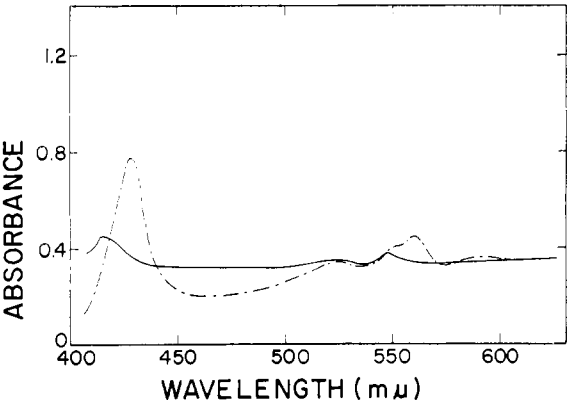

FIGURE 9: The difference spectra of beef heart mitochondria when piericidin A and antimycin A are present. (———) Right after addition of succinate. (- · - ·) Reduction by dithionite. The reaction mixture is the same as that in Figure 6.

was used as the substrate or electron donor. Figure 10 is the normal difference spectrum in which cytochromes *a*, *b*, and *c* showed up clearly when NADH was added to the system. Addition of piericidin A at the level which inhibited NADH oxidase activity slowed down reduction of mitochondrial cytochromes. The full-scale reduction was obtained at 6 min after addition of NADH. Cytochrome *b* completely disappeared from the spectrum (Figure 11).

Figure 12 is the difference spectrum of the system. Succinate was added to the system after the reaction of NADH oxidase was through. The appearance of cytochrome *b* in the spectrum suggested that cytochrome *b* was not destroyed by adding piericidin A, and cytochrome *b* was not in the reduced state.

Addition of a suitable amount of rotenone completely abolished NADH oxidase activity. No cytochrome peaks appeared in the spectrum.

Red Particles. Succinate–cytochrome *c* reductase activity in the red particles which were isolated from ETP by deoxycholate treatment of ETP as described in Methods was also inhibited by piericidin A. Addition of 2.006 μmoles of piericidin A/mg of protein would abolish 75% of the activity in the red particles which are responsible for the succinate dehydrogenases part of the electron

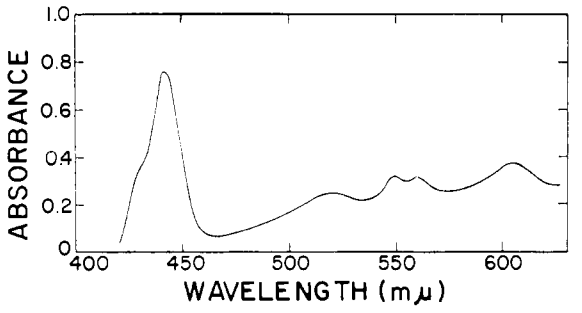

FIGURE 10: The difference spectrum of beef heart mitochondria when NADH is used as the substrate. The reaction mixture contains 50 μmoles of phosphate buffer (pH 7.4), 12 mg of mitochondrial protein, and 6 mg of NADH. The total volume is 3 ml.

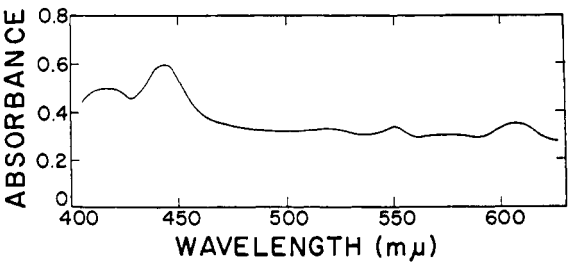

FIGURE 11: The difference spectrum of beef heart mitochondria in the presence of piericidin A with NADH as the substrate. The reaction mixture contains the same as in Figure 8, except 1.2×10^{-7} mole of piericidin A is added in addition.

transport system. Twofold increase of activity was obtained by adding CoQ_2 to the untreated system while more than fourfold increments of activity were shown when the same level of CoQ_2 was added to the system which was already inhibited by piericidin A (Table VIII).

Electron Transport Particle from Azotobacter. The inhibition of the electron transport particle from *Azotobacter* is quite different from the beef heart mitochondria. The levels of piericidin A which are needed to inhibit the succinoxidase activity are equivalent to the levels required to inhibit the NADH oxidase. In NADH system, 1.39 μmoles of piericidin A/mg of protein is needed to get complete inhibition, while in the succinate system 1.55 μmoles of piericidin A/mg of protein completely inhibit the succinoxidase activity (Figure 13).

Castor Bean Mitochrondria. Piericidin A has been used to check the effect on castor bean mitochondria. It was found that piericidin A completely inhibits the NADH oxidase of castor bean mitochondria at the low levels (1.35×10^{-2} μmole of piericidin A per mg of protein) while the higher concentrations of piericidin A are needed to inhibit the succinoxidase system (Figure 14).

Reduction of Coenzyme Q. The measurement of the oxidation–reduction state of CoQ during the electron transfer followed the procedure in Methods. Three different preparations were made: (a) a normal reduced preparation without adding KCN, (b) preparation reduced by substrate in the presence of KCN, and (c) the same as preparation a except piericidin A was added. In preparation a NADH reduced 32% and succinate 80% of the coenzyme Q. In preparation b 55 and 90%, respectively, were found reduced whereas in preparation c with piericidin no coenzyme Q was found reduced with either substrate.

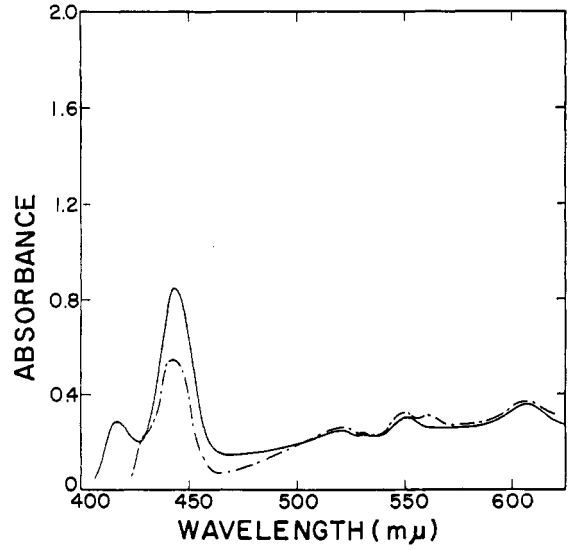

FIGURE 12: The difference spectra of beef heart mitochondria in presence of piericidin A with NADH as the substrate. Succinate is added to the system later. (———) After addition of NADH. (– · – ·) Reduction when succinate is added after NADH.

TABLE VIII: Inhibition of Succinic Cytochrome *c* Reductase Activity by Piericidin A and Restoration by CoQ_2 in the Succinic Dehydrogenase Complex.

Additions	Concn (μmoles/mg of protein)	Act. (μmoles of O_2/min mg of protein)
Piericidin A	None	0.45
	0.207	0.36
	0.413	0.32
	1.239	0.21
	2.066	0.12
CoQ_2	0.179	0.88
Piericidin A + CoQ_2	2.066	0.49

Discussion

Piericidin A inhibition of the NADH and succinoxidase systems occurs, in each case, at a site close to the primary dehydrogenase. In each system, it prevents reduction of coenzyme Q by the dehydrogenase. The great difference in concentration required for inhibition of the two systems indicates that different sites are affected. In the NADH oxidase system, similar concentrations of rotenone and piericidin A are required for inhibition, and apparently the two inhibitors react with this system at the same site. Rotenone inhibits reduction of both cytochromes *b* and c_1 while piericidin A only inhibits reduction of *b* when NADH is the substrate. Therefore, we recognize one difference in their sites of inhibition and consider that these sites of inhibition may be very close to each other. Since a rotenone-sensitive site is not found in the succinoxidase system, it is not surprising that low concentrations of piericidin A also do not inhibit succinoxidase. The fact that coenzyme Q tends to reverse the inhibition of succinoxidase induced by high concentrations of piericidin A indicates that the inhibition is caused by interference in the interaction of the dehydrogenase with coenzyme Q. This may reflect a simple displacement of coenzyme Q from a site adjacent to the dehydrogenase. The partial inhibition of ferricyanide and PMS reduction by piericidin A can either indicate that part of the electron flow from the dehydrogenase to these acceptors goes

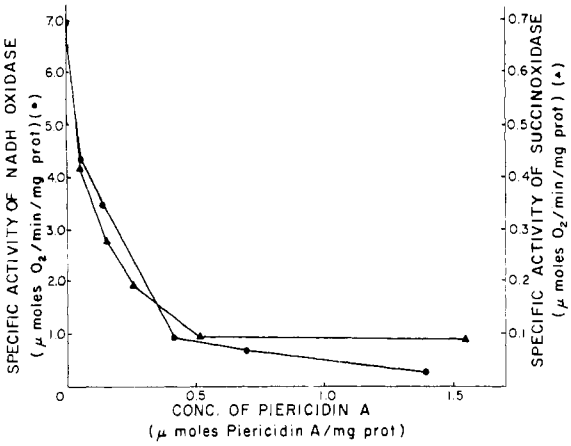

FIGURE 13: The inhibition of NADH oxidase and succinoxidase activities of electron transport particles of *Azotobacter* by piericidin A.

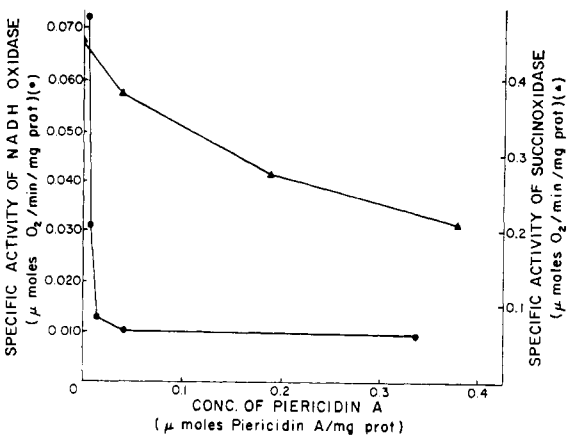

FIGURE 14: The inhibition of NADH oxidase and succinoxidase activities of castor bean mitochondria by piericidin A.

through coenzyme Q or that the site occupied by piericidin A blocks interaction of the acceptors with the dehydrogenase. The evidence that coenzyme Q is required for interaction of succinic dehydrogenase with ferricyanide in mitochondria after extraction of coenzyme Q with solvent is consistent with the idea that piericidin A inhibits ferricyanide reduction by interference at the coenzyme Q site. The effect of piericidin A on PMS reduction is unexpected since there has been no previous evidence for coenzyme Q function in this reduction (Crane and Ehrlich, 1961).

By direct measurement of NADH- or succinate-dependent coenzyme Q reduction inhibition between the flavoprotein and coenzyme Q is observed. With both substrates coenzyme Q is found to be completely oxidized in the presence of the inhibitor.

The reduction of the cytochromes in the presence of piericidin A shows a pattern which is not entirely consistent with a simple inhibition of a linear chain of carriers at a site before the cytochromes. With NADH as substrate, there appears to be considerable reduction of cytochromes c_1 and *a* although the reduction of these carriers must be slow since there is no flow to oxygen or to external cytochrome *c*. The failure of cytochrome *b* to be reduced under these conditions does suggest that some bypass electron flow may go directly to cytochrome c_1. With succinate as substrate, a different pattern emerges. Instead of reduction of cytochrome c_1 we observe the reduction of cytochrome *b* shortly after adding substrate. After a longer period, we observe reduction of all the cytochromes which is consistent with the fact that piericidin A inhibition of succinoxidase is not complete. The results suggest that piericidin A blocks a pathway from NADH dehydrogenase to cytochrome *b* and partially blocks a pathway from succinic dehydrogenase to cytochrome c_1. Since piericidin A does not inhibit cytochrome oxidase at the levels used it is doubtful that reduction of cytochromes in the chain can be attributed to a very slow leak of electrons past coenzyme Q and accumulation of electrons in the cytochromes because their reoxidation is blocked.

A more complex picture is presented when both antimycin A and piericidin A are combined to inhibit succinoxidase. With antimycin A added alone, cytochrome *b* is reduced when succinate is added. If both piericidin A and antimycin A are used, then cytochrome c_1 is reduced when succinate is added and cytochrome *b* is not reduced. It would appear that piericidin A modifies the response of the system to antimycin A. A further analysis of cytochrome reduction in the presence of piericidin A should await a study of the kinetics of these reactions. We point out the phenomena here to indicate that piericidin A may be a useful tool for future study of interaction between cytochromes.

The specificity of piericidin A inhibition of the NADH oxidase is quite clear. Acetylation of the hydroxyl group on the ring or replacement of the fatty side chain with a carboxyl group eliminates the inhibitory effect. The compound with a saturated side chain is equally inhibitory as the unsaturated. Replacement of the hydroxyl group on the side chain with methoxyl as in piericidin B reduces the inhibition slightly.

In the succinoxidase system, all of the compounds with long side chains show some inhibition at high concentrations. The hydrogenated piericidin A is a stronger inhibitor than piericidin A itself. In the succinate–cytochrome *c* reductase system, only piericidin A and hydrogenated piericidin A cause inhibition, the other compounds actually cause some stimulation of activity. These later compounds appear to inhibit succinoxidase similar to the opening effect of deoxycholate in that they cause a better interaction of cytochrome *c* with the particle but reduce electron transport through the whole chain to oxygen.

Piericidin A inhibits the NADH–CoQ reductase particle, but the level which inhibits the NADH–CoQ reductase is much higher than the concentration which inhibits the NADH oxidase of beef heart mitochondria. Perhaps the purification of the particle changes the affinity of the enzyme for the inhibitors.

Piericidin A inhibits castor bean mitochondria as it does beef heart mitochondria. Low levels inhibit NADH oxidase activity and high levels inhibit succinoxidase activity. With the electron transport particle from *Azotobacter*, the inhibition of succinoxidase is found at

levels of piericidin A equivalent to levels required to inhibit NADH oxidase. This low sensitivity of the NADH oxidase in *Azotobacter* particles may indicate that this system is different from the NADH system in mitochondria.

Acknowledgments

We wish to thank Mrs. Mildred Wetzel for supervision of mitochondrial preparation.

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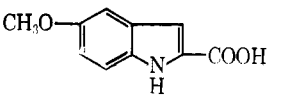
Inhibition of Gluconeogenesis and α-Keto Oxidation by 5-Methoxyindole-2-carboxylic Acid*

Norman Bauman and C. J. Hill

ABSTRACT: 5-Methoxyindole-2-carboxylic acid (MICA) is known to produce hypoglycemia *in vivo* by inhibition of hepatic gluconeogenesis. Its effects in liver slices and subcellular systems have been examined. MICA blocks gluconeogenesis at the level of mitochondrial CO_2 fixation, but does not affect solubilized pyruvate carboxylase.

MICA also specifically blocks mitochondrial oxidation of pyruvate and α-ketoglutarate, an effect reversible *in vitro* by lipoic acid.

5-Methoxyindole-2-carboxylic acid (MICA)[1] produces hypoglycemia in animals through inhibition of hepatic gluconeogenesis (Bauman *et al.*, 1967; Hanson *et al.*, 1967). In particular, it has been shown that it inhibits glucose production by the isolated perfused rat liver and at the same time decreases oxidation of lactate (N. Bauman and B. Pease, unpublished data). The present work extends these studies to liver slices and subcellular systems, demonstrating that MICA blocks mitochondrial oxidation of α-keto acids, an effect reversible *in vitro* by lipoic acid.



MICA

Experimental Section

Materials and Methods

MICA was purchased from the Aldrich Chemical Co. and recrystallized from water. It was dissolved in 0.15 M sodium bicarbonate or, in experiments where bicarbonate concentration might be critical, was suspended in water, to which 1 N KOH was added dropwise until the acid was dissolved and the pH was 7.5

* From the Biochemistry Research Department, Lederle Laboratories, American Cyanamid Company, Pearl River, New York. *Received December 11, 1967.* A preliminary report of part of these data was presented at the 51st Annual Meeting of the Federation of American Societies for Experimental Biology Chicago, Ill., 1967.

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: MICA, 5-methoxyindole-2-carboxylic acid; PEP, phosphoenolpyruvate.