INHIBITION OF SUCCINIC OXIDASE BY DIHYDROXYCOUMARINS*

P.ZBOŘIL and A.SVOBODA

Department of Biochemistry, J. E. Purkyně University, 611 37 Brno

Dedicated to Prof. V. Morávek on the occasion of his 80th birthday.

Received August 26th, 1976

Daphnetin (7,8-dihydroxycoumarin) or esculetin (6,7-dihydroxycoumarin) do not inhibit by themselves succinic oxidase activity in nonphosphorylating fragments of mitochondria. Only after treatment of these *ortho*-dihydroxycoumarins with oxidants (ferricytochrome c, diphenol oxidase, ferricyanide) very unstable products are formed which cause a strong and time-dependent inhibition of succinic oxidase. The site of action of these products is primary succinic dehydrogenase. This inhibition does not involve the reaction with the SH-groups of the enzyme. *Ortho*-Phenylene diamine protects succinic oxidase against inhibition yet does not possess a reactivating effect.

Studies on the inhibition of succinic oxidase by coumarin derivatives provided knowledge of the structural prerequisites necessary for this inhibiton[1] to take place. The inhibiting derivatives bore either a lipophilic (isoprenic) substituent in the neighborhood of oxygen (hydroxyl, methoxyl) or contained 2 hydroxy groups (*ortho*-derivatives were more efficient than *meta*-derivatives). Of the monohydroxy derivatives 4-hydroxycoumarin only showed weak inhibition. Additional studies have shown that the inhibition by isoprenic derivatives is not governed by the same mechanism yet that these derivatives fall into two groups at least, as evidenced by reactivation experiments[1]. Typical representatives of the individual coumarin groups were ostruthin (6-geranyl-7-hydroxycoumarin) and ammoresinol (3-pharnesyl-4,7-dihydroxycoumarin). The former is an efficient uncoupler of oxidative phosphorylation in mitochondria[2]; a maximum is observed with 20—30 nmol of ostruthin/mg of protein. Its interaction with mitochondrial membranes is strictly hydrophobic, dependent on the presence of a lipophilic chain; its binding capacity, 39—50 nmol/mg of protein well corresponds to its uncoupling effects[3]. Ostruthin inhibits succinic oxidase nonspecifically; such an inhibition often represents the effect of uncoupling agents at higher concentrations[4]. By contrast, ammoresinol was a strong inhibitor of electron transport in the cytochrome b domain; the mode of its interaction with mitochondrial membranes resembles its interaction with bovine serum albumin and is different from the strictly hydrophobic interaction of ostruthin[5].

The last group of coumarin inhibitors form dihydro derivatives lacking a lipophilic substituent. The low activity of *m*-dihydroxycoumarin compared to that of *o*-derivatives (represented by daphnetin and esculetin) led to the assumption of participation of oxidizing-reducing or chelating processes in the inhibition

* Part XIII in the series Antibiotic Efficiency of Natural Coumarins; Part XII: This Journal *40*, 1257 (1975).

mechanism. These inhibitors considerably differed from isoprenic inhibitors when assayed in reactivating experiments; special interest deserves the finding that the inhibition of succinic oxidase by daphnetin requires the presence of exogeneous cytochrome c (ref.[1]). The actual inhibitor can therefore be *a*) daphnetin modified by cytochrome c, *b*) cytochrome c modified by daphnetin, *c*) a complex of both compounds. This paper deals with these problems.

EXPERIMENTAL

Material

Mitochondrial fragments (preparation of Keilin and Hartree) were obtained from pig hearts according to Slater[6] by high-speed centrifugation of the neutral phosphate extract instead of acidification. The preparation was stored one week at the most at +3°C.

Soluble succinic dehydrogenase was prepared according to Der Vartanian and Veeger[7] and was stored 1 day at +3°C. Potato diphenol oxidase was prepared by a modification of the method of Balasingham and Ferdinand[8]. The final preparation contained 34 mg of proteins/ml and had a specific activity of 3·96 IU/mg of protein (20 mM pyrocatechol); it was kept at +3°C. Urease was prepared by the method of Sumner[9] and kept lyophilized at −10°C. Peroxidase was a commercial preparation (type II, Sigma, USA), RZ = 0·6.

Coumarin derivatives were obtained as described elsewhere[1]; esculetin (Lachema, Brno) was recrystallized twice. Horse heart cytochrome c, type II, was from Sigma, USA. Sodium *p*-chloromercuribenzoate, phenazine methosulfate, *o*-phenylene diamine, and 1,2-naphthoquinone-4-sulfonate were products of Lachema, Brno. 2-Mercaptoethanol was from Fluka, Switzerland, N-ethylmaleimide from Koch-Light, England, 2-thenoyl-3,3,3-trifluoroacetone from BHD, England, bovine serum albumin from Mann Research Laboratories, USA. The remaining chemicals used were commercial preparations of analytical purity.

Methods

The rate of oxygen uptake was measured polarographically (OH-102 Polarograph, Radelkis Hungary, with adapter) or manometrically by the direct Warburg method.

Inhibition of primary succinic dehydrogenase by esculetin oxidized by cytochrome c. A mixture of primary succinic dehydrogenase (0·2 mg of protein/ml) with esculetin and cytochrome c (ratio 1 : 1·5) in 0·06M phosphate buffer, pH 7·4, was incubated 15 min at room temperature. The samples were then placed in an ice bath and the activity determined polarographically at 30°C in the following medium (2 ml): 0·1M phosphate buffer pH 7·4, 0·1 mg of proteins of primary succinic dehydrogenase (*i.e.* 0·5 ml of incubation mixture), 25 mM succinate, 0·5 mg of phenazine methosulfate. Oxidation of esculetin by ferricyanide, procedure *a*. The incubation mixture contained 0·05M phosphate buffer, pH 7·4, succinic dehydrogenase (0·5 mg of proteins/ml), esculetin and ferricyanide at a ratio of 1 : 2. The incubation and activity determination were the same as with cytochrome c; 0·2 ml of the incubation mixture was taken (0·1 mg of the enzyme proteins). Procedure *b*: esculetin was incubated with ferricyanide 5 min in 0·1M phosphate buffer, pH 7·4 and an equal volume of the solution of primary succinic dehydrogenase (1 mg of proteins/ml) was added; the subsequent procedure was the same as described under *a*.

Urease activity was examined by titration of liberated ammonia in a weakly buffered medium (0·1M-HCl, TTT2 Autotitrator, Radiometer, Denmark). The reaction mixture (1·8 ml) contained:

1 mM phosphate buffer, pH 7·4, pH 7·4, 600 μmol of urea, 1 μmol of cytochrome c. The reaction was started by the addition of 0·4 ml of 1% urease and the time profile of the titration was recorded. Daphnetin was added during the reaction.

Reduced cytochrome c was prepared by the reduction by hydrosulfite and removal of accompanying products on Sephadex G-50. For the purpose of conventional measurements of the effect of cytochrome c on enzymatic activities and formation of products of inhibition, the original commercial preparation was regarded as oxidized (its concentration was not determined in special experiments, the values given are nominal).

Proteins were determined by the biuret method, the values were corrected for the turbidity of the preparation after decolorization with solid KCN (ref.[10]).

Free amino groups were determined by the ninhydrin method with the addition of KCN; after dilution with 50% propanol the absorbance at 570 nm was measured (Pulfrich photometer with ELPHO adapter) in the range of linear dependence on glycine concentration[11].

Ferrocyanide was determined after conversion into berlinate by colorimetry at 710 nm (Pulfrich) in the range of 0·1—1 μmol of ferrocyanide in sample. Free SH-groups were determined according to Boyer[12] by titration with *p*-chloromercuribenzoate, the formation of mercaptide was examined photometrically at 250 nm (Beckman DU Spectrophotometer).

Absorption spectra were recorded in Cary 118 C Spectrophotometer (Varian, U.S.A.).

RESULTS

Effect of Exogeneous Cytochrome c on Inhibition of Succinic Oxidase by Daphnetin

The dependence of the activity of succinic oxidase on the concentration of cytochrome c in the presence and absence of daphnetin is shown in Fig. 1. The mitochondrial fragments were incubated in the reaction vessel with cytochrome c and/or with daphnetin; the reaction was started approximately 5 min afterwards (equilibration of electrode) by the addition of succinate. Whereas cytochrome c speeds up the oxygen uptake in the absence of daphnetin, as recorded in literature[6], it causes an abrupt decrease of the activity of succinic oxidase to 15% of its original value in the presence of daphnetin. The same results were obtained with esculetin as inhibitor. The time profile of the inhibition of succinic dehydrogenase by daphnetin and cytochrome c (Fig. 2 and 3) shows that the activity gradually drops to a value corresponding to 10% of the original value; the rate of the decrease is proportional to both the concentration of cytochrome c and daphnetin. Cytochrome c was added in these experiments to the reaction medium only during the oxidation of succinate (approximately 2 min after the start), not before the succinate as in the experiments summarized in Fig. 1.

Daphnetin and esculetin react with cytochrome c, as manifested in the spectrum of the oxidized form of cytochrome. Because of the presence of *o*-dihydroxycoumarins a peak is observed at 550 nm which is characteristic of the reduced form; its height increases after the reduction with solid $Na_2S_2O_4$ to the same value as that observed

with cytochrome itself. *o*-Dihydroxycoumarins had no effect on the spectrum of reduced cytochrome c. It was likely that oxidized and reduced cytochrome c would differ also in their role they play in the process of influencing succinic oxidase activity by coumarin derivatives. A comparison of the effect of both forms of cytochrome c on the inhibition of succinic oxidase by daphnetin has shown that the latter inhibits in the presence of oxidized cytochrome c only; the reduced form is practically without any effect (Table I).

Formation of Products of Inhibition by Oxidation of o-*Dihydroxycoumarins*

It has been observed that *o*-diphenol oxidase from potatoes oxidizes both daphnetin and esculetin (the latter, however, at a slower rate). The products of this oxidation strongly inhibit succinic oxidase activity (Fig. 4). Unlike in the preceding experiments the reaction here was started by the addition of mitochondrial fragments to a mixture containing in addition to the buffer and succinate also daphnetin and diphenol oxidase (the reaction was started at different time intervals after the addition of diphenol oxidase). Similarly to cytochrome c here too the inhibition gradually increases

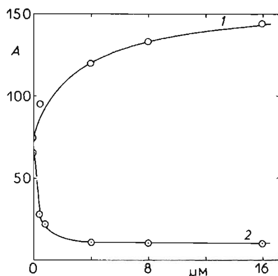

FIG. 1

Effect of Exogeneous Cytochrome c and Daphnetin on Activity of Succinic Oxidase

Polarography at 30°C. The reaction medium (2 ml) contained 0·1M phosphate buffer at pH 7·4, 0·8 mg of proteins of mitochondrial fragments, 25 mM succinate, cytochrome c, as shown, in μM. *A* activity in nmol O_2/min/mg of protein. *1* in the absence of daphnetin, *2* in the presence of 80 nmol of daphnetin.

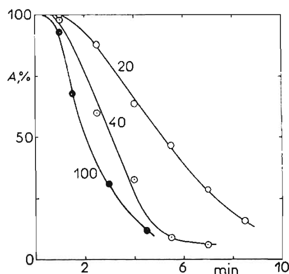

FIG. 2

Time Profile of Inhibition of Succinic Oxidase by Daphnetin as Function of Concentration of Exogeneous Cytochrome c

The conditions are those shown in Fig. 1. 0·4 mg of proteins of mitochondrial fragments, 80 nmol of daphnetin. Cytochrome c was added (*t* 0) 2 min after the start of the reaction; its final reaction, in μM, is shown with the corresponding curves.

TABLE I

Effect of Oxidized and Reduced Cytochrome c on Inhibition of Succinic Oxidase by Daphnetin

The oxygen uptake was measured manometrically at 30°C. The reaction medium (2 ml) contained: 0·1M phosphate buffer, pH 7·4, 2 mg of proteins of mitochondrial fragments, 18 μM cytochrome c, 2·5% ethanol, 20 μM daphnetin where shown, 25 mM succinate. The mixture was incubated in the absence of succinate for about 10 min (thermostating), subsequently the reaction was triggered by the addition of succinate from the side arm to the main compartment. Two enzyme preparations of different activity were used.

Cytochrome c	μl O_2/min/mg prot.		%
	without daphnetin	with daphnetin	
Oxidized	132	45	34
	435	131	30
Reduced	135	147	109
	402	384	96

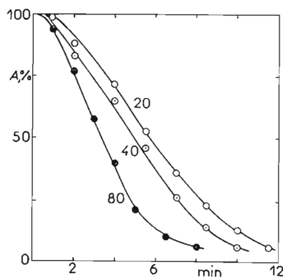

FIG. 3

Time Profile of Inhibition of Succinic Oxidase by Daphnetin as Function of its Concentration

The conditions are those shown in Fig. 2. Cytochrome c (20 μM) was added (t 0) 2 min after the start of the reaction. The concentration of daphnetin is shown with the corresponding curves.

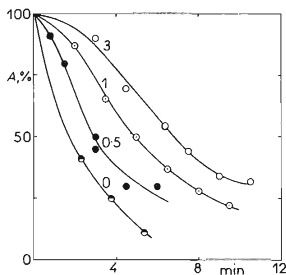

FIG. 4

Time Profile of Inhibition of Succinic Oxidase by Products of Oxidation of Daphnetin by Diphenol Oxidase

The reaction conditions are those shown in Fig. 1. The quantity of daphnetin was 160 nmol. The oxidation of daphnetin was started by the addition of 50 μl of diphenol oxidase, the reaction was triggered by the addition of mitochondrial fragments (t0). The intervals (in min) between the addition of diphenol oxidase and the mitochondrial preparation are shown with the curves.

with time; the inhibitor is most efficient if the mitochondrial fragments are added before phenol oxidase (curve 0 in Fig. 4). This shows that the inhibiting agent is formed during the oxidation of daphnetin by diphenol oxidase (the same holds for esculetin), its activity, however, drops unless the mitochondrial preparation is present during oxidation. The oxidation of daphnetin stopped before the complete uptake of the substrate, most likely because of the inhibitory effect of the product on diphenol oxidase. The presence of mitochondrial fragments enhanced the oxidation rate; it was therefore impossible to exactly determine the quantity of free product formed and to express it in terms of inhibition degree.

Since peroxidase oxidizes scopoletin[13] the oxidation of dihydroxycoumarins by this enzyme was most probable and it was expected that the oxidation degree could be determined from the quantity of the terminal electron acceptor (H_2O_2). Peroxidase in fact causes changes in the spectra of daphnetin, esculetin, and 4-methylesculetin (not shown); this indicates modification of these coumarins. After peroxide had been removed, the reaction products, however, were lacking any inhibitory effect on the activity of succinic oxidase when added to the mitochondrial fragments during the reaction.

When nonenzymatic oxidizing agents were selected the main criterion was the possibility of inhibition of succinic oxidase by these agents. Common artificial electron acceptors were therefore considered as the first ones. Phenazine methosulfate did not oxidize any of the *o*-dihydroxycoumarins at neutral pH. By contrast, ferricyanide in strongly reduced by *o*-dihydroxycoumarins and causes a decrease of the absorption maxima of these derivatives (Fig. 5). The reduction of ferricyanide took place even when its excess was higher than 2 mol per 1 mol of dihydroxycoumarin, *i.e.* where higher oxidation products than the corresponding quinones were formed. Efforts to decrease this additional oxidation, even in strongly acidic media (Table II) were unsuccessful. The products of oxidation of *o*-dihydroxycoumarins

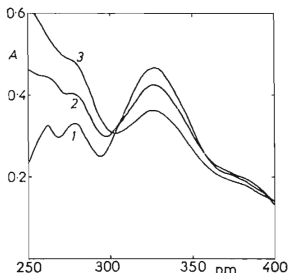

FIG. 5

Absorption Spectrum of Daphnetin Affected by Ferricyanide

The reaction medium contained: 25 mM Tris-HCl buffer, pH 7·4, 0·05 mM daphnetin, and ferricyanide (in mM): 1 0; 2 0·05; 3 0·1.

by ferricyanide showed inhibitory effects on succinic oxidase. The rate and degree of the activity decrease were proportional to the inhibitor concentration; the time profile of the inhibition, however, is somewhat different from that observed with the oxidation by cytochrome c or diphenol oxidase (the inhibitor concentrations, however, are lower) (Fig. 7). The effect of ferricyanide itself is eliminated by its presence in the control sample (taken for 100%). Daphnetin was as efficient as esculetin.

TABLE II

Effect of Medium on Reduction of Ferricyanide by Esculetin

The incubation mixture (5 ml) contained 2·5 μmol of esculetin in the media shown below. Aliquots (1 ml) were withdrawn after 10-min incubation at room temperature and ferrocyanide was determined. The results were practically the same after 35- and 50-min incubation. The results are given in μmol of ferrocyanide formed.

Ferricyanide Added μmol	0·1M-Tris-HCl, pH				HCl, mH		1% $FeCl_3$ 0·3M-HCl
	9·0	7·4	6·0	4·0	5	50	
2·5	2·38	2·18	2·00	2·18	2·25	2·13	0·37
5·0	3·83	3·75	3·67	3·83	4·10	3·92	0·31
10·0	8·34	8·19	8·01	8·45	8·79	8·52	0·32

TABLE III

Inhibition of Primary Succinic Dehydrogenase by Products of Oxidation of Esculetin

See Experimental for methods. The results are given in % of activity of controls (in the absence of esculetin).

Esculetin nmol/mg. prot.	Oxidizing agent		
	cytochrome c	ferricyanide	
		a	b
3	82		
4		90	107
15	49		
20		90	98
75	58		
100		89	94
250	36		
500		52	62

Site and Mode of Inhibitory Effect

It was observed in experiments with inhibition quenching that the addition of phenazine methosulfate (4 mg) does not restore succinate oxidation by mitochondrial fragments incubated with daphnetin (200 nmol/mg protein) and cytochrome c or diphenol oxidase. We were able to show that even a smaller quantity of phenazine methosulfate can completely restore succinate oxidation in case that succinic oxidase has been inhibited by KCN or ammoresinol, an inhibitor of the antimycin A type. The assumption that the inhibitor formed acts also on primary succinic dehydrogenase was confirmed in experiments with a preparation of soluble succinic dehydrogenase (Table III). The weak activity of ferricyanide can be accounted for by its

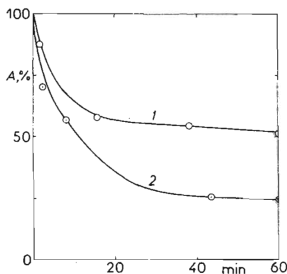

FIG. 6

Time Profile of Inhibition of Succinic Oxidase by Esculetin and Ferricyanide

Esculetin and ferricyanide were added (t 0) to 2 mg of protein of mitochondrial fragments per ml of 0·1M phosphate buffer, pH 7·4. The incubation was allowed to proceed at room temperature, the activity of succinic oxidase was determined polarographically at time t and 30°C in the following medium (2 ml): 0·1M phosphate buffer, pH 7·4, 14 μM cytochrome c, 1 mg of proteins of mitochondrial fragments (*i.e.* 0·5 ml of incubation medium), 25 mM succinate. The quantity of esculetin and ferricyanide was (in nmol/mg of protein): *1* 10 + 20; *2* 15 + 30. The results are given in % of activity of controls in the absence of esculetin.

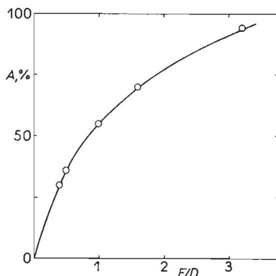

FIG. 7

Dependence of Activity of Succinic Oxidase on Molar Ratio of *o*-Phenylene Diamine and Daphnetin (F/D)

Conditions of experiment the same as those described in Fig. 4 curve *0*; 0·4 mg of proteins of mitochondrial fragments, 20 nmol of daphnetin, *o*-phenylene diamine, as shown, added before diphenol oxidase. The reaction was initiated after 10-min incubation of the medium by the addition of succinate.

reduction by succinate which is contained in soluble succinate dehydrogenase preparations as a protecting agent. Strongest inhibition was observed when oxidation was effected by diphenol oxidase (daphnetin concentration of 6 nmol/mg of protein caused an 83% inhibition of succinic dehydrogenase activity).

We have observed with incompletely inhibited succinic oxidase, however, that the addition of cytochrome c increases the rate of oxygen uptake (Table IV); this is typical of the so-called nonspecific inhibition of succinic oxidase[14]. The addition of phenazine methosulfate results in an additional increase of oxygen uptake. The relative activity is also slightly higher in the presence of cytochrome c and phenazine methosulfate.

Typical inhibitors of succinic dehydrogenase are compounds reacting with essential SH-groups of the enzyme. We checked on the possibility that *o*-dihydroxycoumarins yield upon oxidation products inhibiting *via* binding the SH-groups of the enzyme. Determining SH-groups photometrically according to Boyer[12], we did not observe any significant decrease of the number of SH-groups when mitochondrial fragments were incubated with *o*-dihydroxycoumarins oxidized by cytochrome c, diphenol oxidase, or ferricyanide. Likewise the addition of mercaptoethanol to completely inhibited mitochondrial fragments did not eliminate the inhibition of succinic oxidase. On the other hand, however, we found that daphnetin with cytochrome c inhibits the activity of urease, a typical sulfhydryl enzyme[25];

TABLE IV

Effect of Cytochrome c (cyt c) and Phenazine Methosulfate (PMS) on Esculetin-Inhibited Oxidation of Succinate by Mitochondrial Fragments

Conditions the same as those described in Fig. 6, 10 nmol of esculetim/mg of proteins. The activity was measured after 15-min incubation; cytochrome c was added during the measurement, phenazine methosulfate (0·5 mg) was added as the last. The results are expressed in rate of oxygen uptake.

Ferricyanide nmol/mg prot.	nmol O_2/min			%[a]		
	−cyt c	+cyt c	+PMS	−cyt c	+cyt c	+PMS
20[b]	19·7	30·2	30·5	100	100	100
20[b]	19·5	31·1	37·1			
10	15·7	22·7	27·9	80	75	92
20	9·3	16·9	22·0	47	56	67
30	6·5	12·8	17·0	33	42	56

[a] The values were corrected for changes of activity of controls with time on the assumption that these changes are linear, [b] control experiments in the absence of esculetin carried out in the begining and at the end of measurement.

TABLE V

Simultaneous Inhibition by Esculetin and SH Reagents

The incubation of mitochondrial fragments (2 mg of proteins/ml) was allowed to proceed in 0·1M phosphate, pH 7·4, at room temperature. After 15-min incubation with esculetin and ferricyanide (5 and 10 nmol/mg prot), *p*-chloromercuribenzoate (PCMB) and N-ethylmaleimide (NEM) were added at concentrations of 5 and 3 nmol/mg prot. respectively. The samples were placed in an ice bath 15 min later and their activity was determined as described in the legend to Fig. 6. Phenazine methosulfate (PMS, 0·5 mg) was added during the measurement. The inhibition is expressed in the following terms: a actual value calculated with respect to changes in the activity of controls with time; b value assumed for additive effect calculated as sum of inhibition by both inhibitors[16]; c minimal value for additive effect calculated from equation $100 - A_1 . A_2/100$, where A_1, A_2 are relative activities (in %) determined in the presence of the individual inhibitors.

Inhibitor	Succinic oxidase				Succinic oxidase + PMS			
	nmol O_2 . min^{-1}	inhibition, %			nmol O_2 . min^{-1}	inhibition, %		
		a	b	c		a	b	c
—	124	0	—	—	140	0	—	—
Esculetin	84	30	—	—	96	31	—	—
NEM	107	13	—	—	120	14	—	—
PCMB	75	39	—	—	85	39	—	—
Esculetin + NEM	98	20	43	40	106	24	45	41
Esculetin + PCMB	70	43	69	58	78	44	70	58
NEM + PCMB	59	53	52	48	67	52	53	48

TABLE VI

Simultaneous Inhibition by Esculetin and Thenoyltrifluoroacetone (TTFA)

The conditions and symbols are those described in the legend to Table V; the concentration of thenoyltrifluoroacetone was 2·5 nmol/mg protein.

Inhibitor	Succinic oxidase				Succinic Oxidase + PMS			
	nmol O_2 . min^{-1}	inhibition, %			nmol O_2 . min^{-1}	inhibition, %		
		a	b	c		a	b	c
—	291	0	—	—	359	0	—	—
—	230	0	—	—	294	0	—	—
Esculetin	229	17	—	—	285	17	—	—
TTFA	219	17	—	—	275	17	—	—
Esculetin + TTFA	196	23	34	31	242	24	34	31

this seems to indicate a reaction of the inhibitor with SH-groups. As the most sensitive test appeared simultaneous inhibition of succinic dehydrogenase with typical SH-inhibitors, *p*-chloromercuribenzoate and N-ethylmaleimide. As obvious from Table V the inhibition caused by these inhibitors is additive; we do not find, however, an additive action with esculetin and only a small increase of the inhibition degree is observed with *p*-chloromercuribenzoate. A fact deserving attention is that even though both *p*-chloromercuribenzoate and N-ethylmaleimide directly act on primary succinic dehydrogenase, the addition of phenazine methosulfate increases the rate of oxygen uptake by mitochondrial fragments in the presence of these inhibitors (the relative activities, however, remain unchanged).

Other characteristic inhibitors of succinic dehydrogenase are lipophilic chelating agents such as 2-thenoyl-3,3,3-trifluoroacetone. Simultaneous inhibition by thenoyltrifluoroacetone and esculetin oxidized by ferricyanide shows an increase not comparable, however, in magnitude to the additive action of two inhibitors[16] (Table VI). The measurement was complicated because the inhibition of succinic oxidase by thenoyltrifluoroacetone was also time-dependent and not always the activity was inhibited in the appropriate manner. Here too the addition of phenazine methosulfate causes an increase of the oxidation rate.

Another pathway of inhibitory action is a reaction with the amino groups of the enzyme. Enzymes are inhibited in such a manner, *e.g.* by 1,2-naphthoquinone 4-sulfonate[17]; the activity of succinic oxidase in mitochondrial fragments, however, was not affected by this compound. On the other hand, the incubation of mitochondrial fragments with esculetin and ferricyanide results in a decrease in the number of ninhydrin – positive groups of the preparation (Table VII). The presence of lysine

TABLE VII

Effect of Esculetin on Content of Ninhydrin-Positive Groups of Mitochondrial Fragments

The mitochondrial fragments (2 mg of proteins/ml) were incubated with esculetin and ferricyanide in 0·1M phosphate, pH 7·4, 15 min at room temperature. After two centrifugations (20 min at 25000 *g*) and washing with an equal volume of water the preparation was homogenized with the same volume of water. The content of proteins and ninhydrin positive groups of the samples was then determined.

Esculetin	Ferricyanide	A_{570}/mg prot.
nmol/mg prot.		
—	—	1·52
—	166·7	1·69
16·7	33·3	1·45
83·3	166·7	1·07

in the reaction mixture in which mitochondrial fragments were incubated with esculetin did not influence, however, the degree of inhibition of succinic oxidase. On the contrary, protective effects showed *o*-phenylene diamine against inhibition of succinic oxidase by daphnetin and diphenol oxidase. The protective activity here depends on the molar ratio of phenylene diamine to daphnetin and is 100% when this ratio lies between 3 and 4 (Fig. 3). Phenylene diamine did not show any reactivating activity; its addition to the inhibited enzyme preparation did not restore the activity. Phenylene diamine itself was not oxidized by diphenol oxidase and had no effect on succinic oxidase activity (unlike ethylene diamine which was considerably oxidized in the presence of mitochondrial fragments), it merely increased the oxygen uptake during daphnetin oxidation by diphenol oxidase.

DISCUSSION

The oxidized form only of cytochrome c converts *o*-dihydroxycoumarins into the inhibiting agent; simultaneously the oxidized form is reduced. This effect is not specific of cytochrome c which can be replaced by another agent oxidizing these coumarins, *e.g.* by diphenol oxidase or ferricyanide. This shows that the inhibitory mechanism does not involve the reaction with cytochrome c as functional link in the electron transfer chain. In spite of that we can state that the actual inhibitors of succinic oxidase are products of oxidation of *o*-dihydroxycoumarins. A similar formation of inhibitory products by enzymic oxidation of precursors has been reported with numerous substances and quinones have been identified with the active forms of polyphenolic inhibitors[18-21]. A survey of the effects of quinones on enzymes has been presented by Hoffmann-Ostenhof[22]. Generally the reaction with essential SH-groups of enzymes is regarded as the basis of the inhibition. With oxidizing-reducing enzymes and their systems there exist in addition to this mechanism other possibilities of interference with the electron transferator complex. Here inhibit analogs of the naturally occurring carriers of the quinone type[23,24], a typical group of inhibitors represent 2-hydroxy-3-alkyl-1,4-naphthoquinones inhibiting in the cytochrome b domain (ref.[25]) where also many 1,4-benzoquinone derivatives[26] are active.

The inhibitors derived from dihydroxycoumarins, investigated in this study, obviously differ from known quinone inhibitors of the electron transport. The site of interference of coumarin quinones is obviously primary succinic dehydrogenase whose activity they decrease especially during oxidation of dihydroxycoumarins by diphenol oxidase. Somewhat contradictory is the finding that the activity of the incompletely inhibited system increases after the addition of cytochrome c or phenazine methosulfate: this is typical of the so-called nonspecific inhibition of the succinic oxidase system[14]. In spite of that one can observe that the addition of phenazine methosulfate slightly increases the rate of oxygen uptake even in case of inhibition

of succinic oxidase by typical SH-inhibitors such as *p*-chloromercuribenzoate and N-ethylmaleimide whose action undoubtedly attacks primary succinic dehydrogenase. We can unambiguously conclude that primary succinic dehydrogenase is the main target for coumarin quinones since the completely inhibited enzyme (either soluble or bound to the structure) does not show activity after the addition of phenazine methosulfate. By contrast, a representative of the inhibiting 1,4-benzoquinone derivatives strongly stimulates the activity of succinic dehydrogenase[26].

Very unusual is the observation that the inhibition of succinic dehydrogenase by coumarin quinones does not involve the reaction with the SH-groups of the enzyme. The most clear-cut piece of evidence of this statement represent the results of simultaneous inhibition by esculetin with *p*-chloromercuribenzoate or N-ethylmaleimide. If there were a reaction with SH-groups (not determinable, *e.g.* because of the lability of the bond), the addition of these typical SH-inhibitors would necessarily result in additivity of the inhibitory effects; because more than 50% of activity was retained, *p*-chloromercuribenzoate and N-ethylmaleimide could have reacted with the remaining free SH-groups and the activity would then have decreased simultaneously. The resulting inhibition would then represent a sum of the effects of both inhibitors[16] or at least equal values calculated from equation c (Table V). Additive action has been shown for *p*-chloromercuribenzoate with N-ethylmaleimide; both react with SH-groups. The mechanism of action of esculetin is different. This finding cannot be negated either by the inhibition of urease by daphnetin and cytochrome c since the mechanism has not been examined in detail and need not necessarily be identical for both enzymes. We may therefore regard as proved that the inhibition of succinic dehydrogenase by products of oxidation of *o*-dihydroxycoumarins does not involve the reaction with the SH-groups of the enzyme.

In this respect a compound which resembles these inhibitors is rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), an inhibitor mainly of NADH oxidase which inhibits succinic oxidase only a little[27]. It does not act *via* SH-groups and its effect is assumed to involve rather mitochondrial components bound through lipids and non-heme iron. Such a mechanism is assumed to underlie the action of a characteristic inhibitor of the succinic oxidase system, thenoyltrifluoroacetone, which acts both on primary succcinic dehydrogenase and also at other sites of the system[28]. Under the experimental conditions used in this study the inhibition of succinic oxidase by thenoyltrifluoroacetone was the same even in the presence of phenazine methosulfate, in spite of the fact that various authors[28] report different sensitivity for succinic oxidase and succinic: phenazine methosulfate oxidoreductase (86 and 23% of inhibition respectively by 1 mM thenoyltrifluoroacetone); this can be caused by a low concentration of thenoyltrifluoroacetone in our experiments and by its preferential action on primary succinic dehydrogenase. The result of simultaneous action of esculetin (with ferricyanide) and thenoyltrifluoroacetone is not entirely unambiguous. An increase of inhibition permits us to consider a partial identity

in the mechanisms of action of both inhibitors. We can definitely say, however, that chelating, if any, of non-heme iron (if this is the case) is not the only mechanism governing the inhibition of succinic dehydrogenase by *o*-dihydroxycoumarins.

The results shown in Table VII indicate that a role in the inhibition mechanism may play a reaction with the amino groups of the preparation. Except for 1,2--naphthoquinone 4-sulfonate[17] we do not find with quinones an inhibition mechanism involving the reaction with amino groups of enzymes, even though the reaction between amino and carbonyl groups is common. We find that the reactions of quinones, formed by the oxidation of phenols by diphenol oxidase, with amino acids and peptides are one of the steps of humin synthesis; in model systems this reaction is most pronounced with proline and phenylalanine[29]. The decrease of the content of ninhydrin-positive groups as a result of incubation of mitochondrial fragments with esculetin and ferricyanide does not provide an unambiguous proof of this mechanism of action. The role of amino groups in the enzymatic activity of succinic dehydrogenase is not completely clear. It is sure that most of the free amino groups in the mitochondrial preparation are not in any relation to enzymatic activity; neither has been shown, however that the essential amino groups react with the inhibitor. These factors do not permit the content of amino groups to be correlated with enzymatic activity. Neither does the protective effect of *o*-phenylene diamine represent unambiguous evidence in favor of this mechanism since the reaction of phenylene diamine with quinones is of more general character[30]; this shows that the quinone formed reacts with *o*-phenylene diamine thus giving rise to a product (most likely a phenazine derivative) lacking inhibitory activity. Since lysine did not show a protective effect the reaction is most likely not a simple one; a role will play also other factors, such as, *e.g.* the character of substituents in the neighborhood, the reactivity of individual groups, *etc.* In spite of these objections the reaction with the amino groups of the enzyme preparation can be regarded as a very probable essence of the inhibition of succinic oxidase.

The authors thank Prof. Dr V. Dadák for his interest and valuable suggestions with which he followed this study.

REFERENCES

1. Zbořil P., Holasová J., Dadák V.: This Journal *35*, 2983 (1970).
2. Dadák V. in the book: *Wirkungsmechanismen von Fungiziden und Antibiotika* (H. Lyr, Ed.), p. 159. Akademie-Verlag, Berlin 1967.
3. Zbořil P., Dadák V.: Arch. Biochem. Biophys. *159*, 249 (1973).
4. Wilson D. F., Merz R. D.: Arch. Biochem. Biophys. *119*, 470 (1967).
5. Dadák V., Lochman I., Máchová L.: This Journal *40*, 1268 (1975).
6. Slater E. C.: Biochem. J. *45*, 1 (1949).
7. Der Vartanian D. V., Veeger C.: Biochim. Biophys. Acta *92*, 233 (1964).
8. Balasingham K., Ferdinand W.: Biochem. J. *118*, 15 (1970).

9. Sumner J. B.: J. Biol. Chem. *69*, 435 (1926).
10. Szarkowska L., Klingenberg M.: Biochem. Z. *338*, 674 (1963).
11. Homolka J.: *Klinické biochemické vyšetřovací metody*, p. 120. Published by Státní zdravotnické nakladatelství, Prague 1969.
12. Boyer P. D.: J. Amer. Chem. Soc. *76*, 4331 (1954).
13. Sirois J. C., Miller R. W.: Plant Physiol. *49*, 1012 (1972).
14. Slater E. C.: Biochem. J. *45*, 8 (1949).
15. Reithel F. J. in the book: *The Enzymes* (P. D. Boyer, Ed.), Vol. IV, p. 1, 3rd Ed. Academic Press, New York 1971.
16. Nijs P.: Biochem. Biophys. Acta *143*, 454 (1967).
17. Giri K. V., Nagabushanam A.: Naturwissenschaften *39*, 548 (1952).
18. Weaver R. F., Rajagopalan K. V., Handler P.: Arch. Biochem. Biophys. *149*, 541 (1972).
19. Metlitskii L. V., Savelyeva O. N., Ozerekhovskaya O. L., Baliauri V. D., Stom D. I., Timofeeva S. S. in the book: *Imunitet i Pokoi Rastenii* (L. V. Metlitskii, Ed.), p. 58. Nauka, Moscow 1972.
20. Gamburg K. Z., Osharova L. M., Stom D. I., Trubina T. P.: Biol. Nauki *16*, 88 (1973).
21. Meskhi A. B., Dzhokhadze D. I.: Fiziol. Rast. *20*, 1253 (1973).
22. Hoffmann-Ostenhof O. in the book: *Metabolic Inhibitors* (R. M. Hochster, J. H. Quastel, Eds), Vol. II, p. 145. Academic Press, New York 1963.
23. Folkers K., Catlin J. C., Daves G. D., jr: J. Med. Chem. *14*, 45 (1971).
24. Castelli A., Bertolli E.. Littaru G. P., Lenaz G., Folkers K.: Biochem. Biophys. Res. Commun. *42*, 806 (1971).
25. Howland J. L., Lichtman J. W., Settlemire C. T.: Biochim. Biophys. Acta *314*, 154 (1973).
26. Ozawa H., Momose K., Machida M.: Chem. Pharm. Bull. *15*, 1841 (1967).
27. Kean E. A.: Arch. Biochem. Biophys. *127*, 528 (1968).
28. Whittaker P. A., Redfearn E. R.: Biochem. J. *88*, 15P (1963).
29. Nefedova L. N.: Pochvovedenie *1970*, 61.
30. Stom D. I., Timofeeva S. S., Kolmakova E. F., Kalabina A. V.: Dokl. Akad. Nauk SSSR *205*, 989 (1972).

Translated by V. Kostka.