MECHANISM OF INHIBITION OF SUCCINATE OXIDASE BY PRODUCTS OF OXIDATION OF *o*-DIHYDROXYCOUMARINS*

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Received September 28th, 1976

Semiquinone is an intermediary product of the oxidation of daphnetin (7,8-dihydroxycoumarin) and esculetin (6,7-dihydroxycoumarin) by diphenol oxidase; its concentration rapidly decreases. When the oxidation is effected by ferricytochrome c, the concentration of the semiquinone remains practically constant for a long period. Similarly, the ability of the products of daphnetin oxidation by diphenol oxidase to inhibit succinate oxidase activity in mitochondrial fragments rapidly decreases with time; the decrease is considerably slower in the case of cytochrome c. The inhibitory activity of the product decreases with time also during esculetin oxidation by ferricyanide. This indicates that the inhibitory effects must be ascribed predominantly to the semiquinone, the quinone is less efficient. The inhibition of succinate oxidase or succinate dehydrogenase was strongly decreased when the enzyme preparation of Keilin and Hartree was incubated with esculetin and ferricyanide in the presence of KCN or under anaerobic conditions. This demonstrates that the reaction of the inhibitor with the enzyme either involves subsequent oxidations or that the inhibitor preferentially reacts with the oxidized form of the sensitive component of the respiratory chain. The second alternative is very little probable since there is no correlation between the degree of inhibition and the binding of the inhibitor to mitochondrial fragments.

We described in the preceding paper[1] the formation of strong inhibitors of succinate oxidase activity in KH by oxidation of two hydroxycoumarins, daphnetin and esculetin, by ferricytochrome c, diphenol oxidase, or ferricyanide. It was assumed that the actual inhibitors of succinate oxidase are the corresponding quinones since these compounds inhibit numerous enzymes[2]. The site of the inhibition was primary SDH; the mechanism of the inhibition, however, did not involve the reaction with SH-groups, typical of the quinones. Another fact deserving interest was that the inhibitory activity of these compounds decreased with time and that the inhibition itself was time-dependent. Quinones undergo changes in solution and form a number of intermediates. We decided to examine in more detail the mechanism of the inhibition of succinate oxidase by products of oxidation of *o*-dihydroxycoumarins in order to determine which products are the actual inhibitors.

* Part XIV in the series: Antibiotic Efficiency of Natural Coumarins; Part XIII: This Journal *42*, 3676 (1977).

EXPERIMENTAL

The data on the preparation of mitochondrial fragments (KH)* and diphenol oxidase, protein determination, and the chemicals used have been described in the preceding paper[1].

Cyanide-inhibited KH (KH-CN): KH (2 mg of protein/ml was incubated 15 min in 0·1M phosphate buffer, pH 7·4 with 0·01M-KCN, centrifuged 20 min at 20000g, washed with buffer, centrifuged, and homogenized with the same buffer.

The activity of succinate oxidase was determined polarographically at 30°C in the following medium (2 ml): 0·1M phosphate buffer, pH 7·4; 25 mM succinate; KH, cytochrome c and other additions as given. The activity of succinate dehydrogenase was determined under identical conditions as the oxidase activity, cytochrome c was replaced by 0·5 mg of phenazine methosulfate.

In several experiments KH was incubated with the inhibitors before the activity measurement (Figs 2, 4, and 6, Table II). The incubation was carried out at room temperature in the following medium: 0·1M phosphate buffer, pH 7·4; 2 mg of proteins of KH/ml; additions as given. After the given incubation time 0·5 ml of the mixture (*i.e.* 1 mg of proteins of KH) was transferred to the oxigraph vessel and the activity was immediately measured (if impossible the samples were stored in an ice bath).

The oxidation and determination of cytochrome c were carried out as described elsewhere[1]. The spectra were recorded in Cary 118C (Varian, USA) spectrophotometer.

The EPR spectra were measured in ER-9 (Zeiss, Jena) spectrophotometer. The reaction mixture contained (1 ml): 0·1M phosphate buffer, pH 7·4; 2·5 mM daphnetin; 7% ethanol. The reaction was triggered either by the addition of 0·1 ml of diphenol oxidase (0·17 mg of protein) or by 0·1 ml of 50 mM cytochrome c. The spectra were recorded at time intervals; the first record was made 90 s after the start of the reaction (time necessary for tuning up the instrument). The amplitude of the first spectrum is taken as the magnitude corresponding to relative semiquinone concentration (SO) equal 1·00.

The fluorescence measurements were made in the Perkin–Elmer 203 instrument.

RESULTS

Instability of Reaction Product

The inhibitory activity of daphnetin drops with time of its oxidation by phenol oxidase or ferricytochrome c before the addition of KH (Fig. 1). A rapid decrease of inhibitory activity, manifesting itself by a considerable prolongation of the period necessary to achieve a 50% inhibition, can be observed especially with diphenol oxidase. This decrease is far slower with cytochrome c. A decrease of the inhibitory activity of the product toward succinate oxidase can be observed also during the oxidation with ferricyanide (Fig. 2).

The most probable product of the oxidation of *o*-dihydroxycoumarins whose characteristic feature is its instability is the semiquinone. The presence of semiquinone during the oxidation of daphnetin both by ferricytochrome c or diphenol

* Abbreviations used: KH preparation of nonphosphorylating mitochrondrial fragments; KH-CN cyanide-inhibited KH; SDH succinate dehydrogenase.

oxidase was demonstrated by EPR spectroscopy. In the first case the amplitude of the spectrum remained constant for a long period, the spectrum lines were a little widened; this indicates the presence of another paramagnetic substance (oxygen) in the reaction mixture. This fact is not surprising since unlike with diphenol oxidase the oxidation by ferricytochrome c is accounted for by a change of the valence of iron. The concentration of the radical was higher (double height of the amplitude) during the oxidation by diphenol oxidase yet it rapidly decreased with time (Fig. 1). When *o*-phenylenediamine was present in the reaction mixture then the amplitude dropped approximately 20 times at a daphnetin to phenylenediamine ratio equal 2 : 1 and

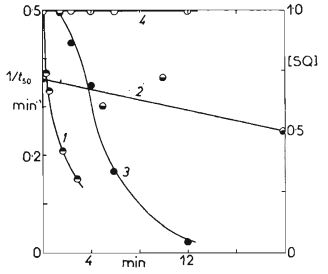

FIG. 1

Time Profile of Decrease of Inhibitory Activity *1*, 2 and Concentration of Daphnetin Semiquinone *3*, *4*

1 Daphnetin oxidized by diphenol oxidase. The reaction vessel contained a medium (*cf.* Experimental) without cytochrome c with 80 μM daphnetin, 50 μl of diphenol oxidase, and 0·8 mg of proteins of KH. 2 Daphnetin oxidized by cytochrome c. The reaction medium contained 50 μM cytochrome c, 40 μM daphnetin, and 0·4 mg of proteins of KH. Time $t = 0$ represents the time of mixing of daphnetin with the oxidant; KH was added at time *t* and the rate of succinate oxidation was recorded. The time during which this rate dropped to one half of the original rate is t_{50}. (SQ) — semiquinone concentration (see Experimental): *3* with diphenol oxidase, *4* with cytochrome c.

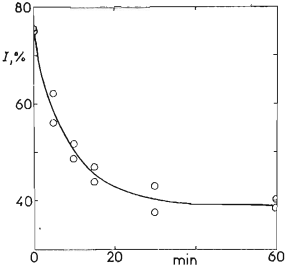

FIG. 2

Decrease of Inhibition of Succinate Oxidase as Function of Time for which Esculetin Was Oxidized by Ferricyanide in Absence of KH

Esculetin (80 nmol) was incubated with 160 nmol of ferricyanide at room temperature in 1·8 ml of 0·12M phosphate buffer, pH 7·4; 0·2 ml of KH (4 mg of proteins) was added at time *t*, and the incubation was continued for 15 min. Subsequently the activity was determined in an aliquot (1 mg of proteins), using 14 μM cytochrome c.

the EPR signal disappeared completely at a 1 : 1 ratio. *o*-Phenylenediamine hence reacts not only with quinones[3] yet even with the semiquinone. The cleavage constants were determined by simulation of the spectra in Minsk 22 computer; it was shown that the spectrum belongs to the anion radical of the semiquinone derived from daphnetin.

Effect of Ferricyanide on Inhibition of Succinate Oxidase by Esculetin

Evidence in favor of the view that semiquinone is the actual inhibitor of succinate oxidase could be sought in the conditions of oxidation of *o*-dihydroxycoumarins by various quantities of ferricyanide. The inhibitory effects of mixtures of esculetin with ferricyanide at various ratios are shown in Fig. 3 (the effect of the same concentrations of ferricyanide, *i.e.* in the absence of esculetin is shown in the Table below, ferrocyanide at the same concentrations did not affect the activity of succinate oxidase).

Ferricyanide, 10^{-4}M	1	2	4	6	8	10
Activity, %	96	79	74	72	72	72

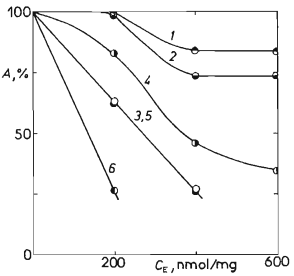

FIG. 3

Inhibition of Succinate Oxidase by Products of Esculetin Oxidation by Ferricyanide

Reaction mixture without cytochrome c, 1 mg proteins of KH. Mixtures of esculetin and ferricyanide (not later than 2 min after the mixing) were added during the oxidation of succinate. The molar ferricyanide to esculetin ratios were: *1* 0·5; *2* 1; *3* 2; *4* 3; *5* 4; *6* 10.

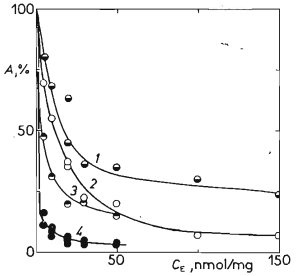

FIG. 4

Inhibition of Succinate Oxidase by Esculetin and Ferricyanide

KH was incubated with the inhibitors 15 min. The concentration of esculetin (C_E) as given; the molar ferricyanide to esculetin was: *1* 1; *2* 2; *3* 4. 4 the same samples yet after storage overnight at 4°C. The activity was determined with 14 μM cytochrome c.

Mixtures (not later than 2 min after the mixing) were added during the oxidation of succinate by KH. The inhibitory effect was far weaker than in the preceding cases when the dihydroxycoumarins were oxidized by cytochrome c or diphenol oxidase. The I_{50}-value (*i.e.* the inhibitor concentration required for a 50% inhibition) for daphnetin and 4-methyl-esculetin is 20 and 70 nmol/mg protein, respectively[4]. This value was obtained by measurement of the succinate oxidase activity by the method of Warburg; the inhibitors were incubated with KH and cytochrome c 10 min before the addition of the succinate. The I_{50}-value read from curve *3* (Fig. 3, esculetin to ferricyanide 1 : 2) is approximately 265 nmol/mg protein. If, however, we allow esculetin and ferricyanide to incubate 15 min with KH in the absence of succinate (Fig. 4), the inhibition is far stronger. The I_{50}-value for esculetin – ferricyanide at a ratio of 1 : 2 is in this case approximately 12 nmol/mg protein (curve 2). Ferricyanide itself incubated with KH in quantities of 100 and 200 nmol/mg protein gave an inhibition of 23 and 27%, respectively, ferrocyanide was practivally without effect (1 and 3% of inhibition, respectively). It is obvious from both experiments (Fig. 3 and 4) that the degree of inhibition depends on the ferricyanide–esculetin ratio. It is obvious in the latter case, however, that the inhibitory effect strongly increases after long-term incubation of esculetin and ferricyanide with KH (curve *4* in Fig. 4) and becomes practically independent of their ratio.

If we examine the effect of the molar ferricyanide to esculetin ratio on the degree of succinate oxidase inhibition it becomes apparent (Table I) that this degree increases up to a ratio of 2, then drops (most likely because of partial decomposition of esculetin) and subsequently starts again increasing. A marked increase of the inhibitory activity at a ferricyanide excess can be seen also in Fig. 4. The inhibition at a ratio of 4 is especially at low esculetin concentrations (curve *3*) considerably higher than at a ratio of 2 (curve 2).

TABLE I

Effect of Ratio of Ferricyanide to Esculetin on Inhibition of Succinate Oxidase

The results are given in % of inhibition of the enzyme.

Esculetin nmol/mg prot.	Molar ratio of ferricyanide to esculetin							
	0·3	0·5	1	1·5	2	3	4	10
200[a]	—	0	2	—	38	17	38	73
400[a]	—	16	27	—	74	54	72	—
10[b]	11	13	18	33	44	48	51	—

Conditions see Fig.: [a] 3, [b] 4.

Participation of Oxidation Processes on the Mechanism of Inhibition

Even though cytochrome c, diphenol oxidase, and ferricyanide are comparable as regards the inhibition which they cause, there are differences in their reactions with the coumarins. Hence, *e.g.* the oxidation by diphenol oxidase proceeded 10 to 15 min and the rate of oxygen uptake asymptotically approached zero. The reaction with ferricyanide was also completed in 15 min; its excess resulted in oxidative degradation of dihydroxycoumarins[1]. Likewise, the reaction with cytochrome c was finished in 10 min, proceeded, however, to a low degree only. Fig. 5 shows a part of the

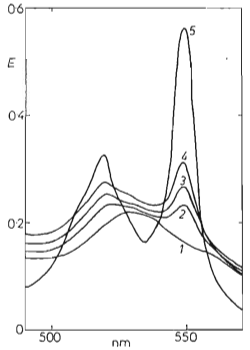

FIG. 5

Reduction of Cytochrome c by Esculetin

The absorption spectra were recorded after each esculetin addition, 4 times at 2 min intervals. *1* 20 μM ferricytochrome c in 3 ml of 0·1M phosphate buffer, pH 7·4; *2*, *3*, *4* addition of 10, 10, and 20 μl of 10 mM esculetin; *5* addition of solid $Na_2S_2O_4$.

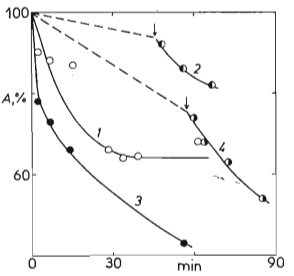

FIG. 6

Effect of Anaerobiosis on Time Profile of Inhibition of Succinate Oxidase by Esculetin and Ferricyanide

The incubation was allowed to proceed in the presence of 20 μM (curves *1*, 2) and 30 μM (curves *3*, *4*) esculetin and a double quantity of ferricyanide. *1*, *3* aerobic incubation from the start (addition of ferricyanide); 2, *4* ferricyanide added at time 0 from the side arm of a Thunberg tube after triple evacuation and filling with nitrogen (the anaerobic incubation is shown by a dashed line), the arrows indicate when the tubes were opened and the mixture flushed with a stream of air. The activity was determined with 14 μM cytochrome c.

spectrum of cytochrome c successively reduced by an excess of esculetin (the starting cytochrome was in oxidized form). If the molar esculetin over cytochrome c excess is six-fold, approximately 35% of the latter is reduced. Using the value of $E'_0 = 262$ mV for cytochrome c the value of $E'_0 = 350$ mV for esculetin was calculated. It may be assumed that the ferrocytochrome c formed is reoxidized in the presence of KH and is again capable of oxidizing the dihydrocoumarins; the efficiency of cytochrome c therefore equals that of diphenol oxidase and ferricyanide.

The fact that the inhibitory activity of esculetin increased at a ferricyanide excess (more than 2 mol per mol of esculetin) leads us to consider the possibility of reversible oxidation and reduction of dihydrocoumarins in the mechanism of the inhibition. To prevent the ferrocyanide formed from its possible reoxidation we examined the time profile of succinate dehydrogenase inhibition in KH in the presence of KCN (Table II). The activity of SDH dropped not only during the incubation with esculetin and ferricyanide but also with the control. The decrease of the activity of the control is caused by KCN in the absence of succinate[5] (which could not be added to the incubation mixture because it would have reduced the ferricyanide). It is quite obvious, though, that the inhibition will not exceed 20% in any case.

To avoid the effect of KCN on the activity of SDH, we incubated KH with esculetin and ferricyanide in an atmosphere of nitrogen. As can be seen in Fig. 6, the inhibition in the mixture incubated under anaerobic conditions is considerably lower than in the mixture incubated aerobically. After anaerobiosis had been terminated and a stream of air had been bubbled through the mixture, the activity started

TABLE II

Time Profile of Inhibition of Succinate Dehydrogenase Activity in KH in Presence of KCN

KH was incubated in the presence of 20 mM KCN, 40 μM esculetin, and 80 μM ferricyanide (inhibited sample). The control sample was incubated without esculetin.

Incubation min	Succinate dehydrogenase activity		
	nmol O_2/min		%
	control	inhibited	
3	240	225	94
14	219	202	92
32	190	157	83
47	177	149	84
66	159	143	90
90	150	134	89

decreasing; the decrease was similar to that observed in the mixture incubated under aerobic conditions from the very beginning of the experiment. Hence, atmospheric oxygen leads to a sharp increase of the inhibitory effect of ferricyanide-oxidized esculetin.

The possibility of oxidation of a component of KH by oxidized esculetin was checked by a test based on the fluorescence properties of esculetin. The excitation maximum of esculetin was found at 392 nm, the emission maximum at 463 nm. The plot of fluorescence intensity *versus* esculetin concentration is linear up to 20 μM concentration. The addition of ferrocyanide had practically no effect either on the shape of the spectra or on the fluorescence intensity up to five-fold molar excess of ferrocyanide over esculetin. Ferricyanide, however, decreased the fluorescence intensity by approximately 50% if its molar ratio to esculetin was 1 : 1; the fluorescence intensity at a ratio of 2 : 1 was about 6% (depending on the concentration of the solutions). It may be taken for granted that oxidized esculetin does not fluoresce.

TABLE III

Influence of KH and KH-CN on Oxidation of Esculetin by Ferricyanide

The components given were contained in 3 ml of 0·1M phosphate buffer, pH 7·4. KH and KH-CN were incubated 15 min at room temperature, then centrifuged (20 min at 20 000g), the supernatants subjected to absorbance measurement at 360 nm (the A_{360}-value was used for the calculation of total quantity of esculetin) and fluorescence measurement (the F_{463}-value was used for calculation of the quantity of reduced esculetin on condition that the reduced form only is fluorescent).

Composition of mixture nmol/mg prot.	A_{360}	F_{463}	Esculetin		
			total nmol	reduced	
				nmol	%
Esculetin, 40	0·125	100	40	40	100
Esculetin, 40 Ferricyanide, 80	0·105	2·4	40	1·0	2·5
dtto + KH, 2 mg	0·060	2·2	22·9	0·9	3·9
dtto + KH-CN, 2 mg	0·040	3·0	15·2	1·2	7·9
Esculetin, 100	0·366	218	100	100	100
Esculetin, 100 Ferricyanide, 200	0·251	1·5	100	0·7	0·7
dtto + KH, 2 mg	0·128	2·2	51·0	1·0	2·0
dtto + KH-CN, 2 mg	0·101	3·9	40·2	1·8	4·5

Table III shows the effect of KH and KH-CN on the relative distribution of reduced esculetin in the medium after incubation of the enzyme preparations with esculetin and ferricyanide. It can be seen that esculetin is either bound or adsorbed in a manner so far not characterized in detail both to native and cyanide-inhibited KH; it binds more to the latter even though succinate dehydrogenase is inhibited only very little in this case (Table II). The degree of the "binding" of esculetin to KH is therefore not related to its inhibitory effects. If we assume that the fluorescence intensity corresponds to the concentration of the reduced form of esculetin only then we can calculate both its absolute and relative distribution in the supernatant. It is shown in Table III that the relative distribution of the reduced form in the supernatant increased a little yet significantly as a result of the action of KH and especially KH-CN. An absolute increase of the concentration of the reduced form can moreover be observed with KH-CN.

DISCUSSION

We have shown in our inhibition studies that the inhibitors are most efficient if the oxidation of *o*-dihydroxycoumarins takes place in the presence of the enzyme with which they interact (KH, soluble succinate dehydrogenase). Likewise the oxidation rate is higher in the presence of KH because of removal of the product[1]. The inhibitory activity of the products is the lower the older the products are. This is especially marked with diphenol oxidase; if we allow to proceed the oxidation completely (about 10 min) in the absence of KH we do not observe any inhibition of succinate oxidase. This also explains the inactivity of the products of oxidation of *o*-dihydroxycoumarins by peroxidase[1]. This drop of activity is not so apparent with cytochrome c as shows a comparison of curves *1* and *2* in Fig. 1. Since we examine the inhibitory activity by measuring the time dependence of action of the arising inhibitory products we can explain the differences in activity in terms of differences between oxidation by cytochrome c and diphenol oxidase. The substrates are completely transformed in the case of diphenol oxidase (1 mol of O_2 roughly corresponds to 1 mol of *o*-dihydroxycoumarin) whereas cytochrome c oxidizes a small part of *o*-dihydroxycoumarin only (Fig. 5), its majority remaining unreacted in the equilibrium mixture for a long time. After the addition of KH the product is taken up with simultaneous oxidation of additional diphenol.

The instability of the inhibition product, observed mainly with diphenol oxidase, casts doubt on the opinion that the actual inhibitors of succinate oxidase are coumarin *o*-quinones. It is known that quinones (especially *ortho*) because of their high reactivity undergo in aqueous media transitions leading to polymers. The rate, however, at which the inhibitory activity decreases indicates that compounds even less stable than *o*-quinones could be involved. The autooxidation of esculetin (and of other hydroxycoumarins) in alkaline media leads to relatively stable anion radicals,

semiquinones[7]. We have demonstrated their formation by EPR spectroscopy also in the process of daphnetin and esculetin oxidation by diphenol oxidase or cytochrome c. When the oxidation was effected by diphenol oxidase the EPR signal disappeared relatively quickly unlike with cytochrome c where a longer viability of the semiquinone (even though at a lower concentration) was demonstrated. These results are in accordance with the longer viability of the inhibitory-active product during oxidation of *o*-dihydroxycoumarins by cytochrome c in comparison with diphenol oxidase where the inhibitor is rapidly inactivated at a rate corresponding to the viability of the semiquinone (about 10 min). As can be seen in Fig. 1 the viability of the semiquinone can be well correlated with the inhibitory activity of the oxidation products in both cases. This supports the view that the semiquinone is the actual inhibitor of succinate oxidase. Similarly, the inhibitory effect decreases if the *o*-dihydroxycoumarins are oxidized by ferricyanide (Fig. 2), but it does not disappear completely as in the case of diphenol oxidase yet drops approximately to one half of the original value. This weaker inhibition can be ascribed to the arising quinone; when diphenol oxidase is used as oxidant the quinone undergoes subsequent transformations or is bound by the enzyme itself.

The reactivation experiments with *o*-phenylenediamine seemingly indicated the participation of the quinones in the inhibition[1] yet the disappearance of the EPR signal in the presence of phenylenediamine means that this substance reacts even with the semiquinone. It has been observed moreover that phenylenediamine increases the quantity of oxygen taken up during the oxidation of daphnetin by diphenol oxidase high above the values necessary for the formation of quinone. We explain this phenomenon by the oxidation of phenylenediamine (mediated by semiquinone) to the diimine which can react with another molecule of phenylenediamine to 2,3-diaminodihydrophenazine which is subsequently oxidized to 2,3-diaminophenoazine. This fact complicates possible structural studies on the products of condensation of quinones with phenylenediamine.

It was intended to unequivocally determine in experiments with the inhibition and binding to KH of *o*-dihydroxycoumarins oxidized by various quantities of ferricyanide whether the inhibition of succinate oxidase is caused by the quinone or the semiquinone. Ferricyanide was used since it permitted the oxidation to be gradually controlled and since it reacted almost quantitatively up to a ratio of 2 mol per 1 mol of *o*-dihydroxycoumarin[1]. A certain drawback represents a deeper oxidation of dihydroxycoumarins at a ferricyanide excess. The inhibitory studies designed to determine whether the inhibition will increase only up to a ferricyanide to esculetin ratio equal 1 : 1 (corresponds to the action of semiquinone) or 2 : 1 (corresponds to the quinone) did not yield an unambiguous response. It may be concluded from the data given in Table I and Figs 3 and 4 that the inhibition should be ascribed rather to the quinone; the further increase of the inhibition at ratios above 2 indicates, however, that the process is more complicated. As shown by binding studies, the

binding of the inhibitor is not related to the inhibition. We assumed that the semiquinone can oxidize some component of KH without being bound, that the diphenol formed is again oxidized by excess ferricyanide, and that the inhibitory effect is thus increased. It can moreover be postulated that the ferrocyanide formed is oxidized by KH and thus to explain the strong influence of long-term incubation of KH with the inhibitors (Fig. 4). In the case of cytochrome c the reoxidation of its reduced form is also highly probable. Since the equilibrium is strongly shifted toward the ferricytochrome and the reduced dihydroxycoumarins, a mere withdrawal of the products most likely would not provide for an efficient inhibition of succinate oxidase; it is only the reoxidation of ferrocytochrome c which makes a sufficiently rapid course of the subsequent oxidation of the dihydroxycoumarins possible.

A proof of the role of the subsequent oxidations in the inhibitory mechanism provide Table II and especially Fig. 6. The inhibition of cytochrome oxidase by KCN makes the reoxidation of ferrocyanide by KH impossible; this manifests itself by a decrease of the inhibitory activity (20% inhibition of SDH *versus* 65% inhibition of succinate oxidase, both activities being almost identically sensitive to esculetin[1]). The inhibition of succinate oxidase under conditions of anaerobic incubation is weak (the oxidation of esculetin is limited by the quantity of ferrocyanide present), its substantial increase occurs only during aerobic incubation because of subsequent oxidations. The effect of subsequent oxidations is obviously one of the causes of the time profile of the inhibition. A similar mechanism of inhibition has been observed with propyl gallate[8] whose semiquinone oxidizes SH-groups; our inhibitors do not, however, react with these groups[1]. We cannot exclude the possibility, however, that the inhibitor preferentially reacts with the oxidized form of the sensitive component of KH; this would require, however, the binding of the inhibitor to this component. Since the inhibition is not in relation to the binding this mode of reaction seems improbable. The view that the oxidation of a component of KH is involved appears to be supported by the finding that incubation with KH and especially with KH-CN causes a partial reduction of oxidized esculetin. This model of action also explains why diphenol oxidase is the most efficient inductor of inhibition of soluble SDH (ref.[1]). Unlike KH primary SDH cannot reoxidize the arising ferrocytochrome c or ferrocyanide; the oxidation of dihydroxycoumarins by diphenol oxidase can follow a cyclic course in both cases.

In am indebted Prof. Dr V. Dadák for his valuable comments and interest in this study and to Dr P. Kubáček for the measurement and evaluation of the EPR spectra.

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Translated by V. Kostka.