Biochemical Pharmacology, Vol. 25, pp. 643–648. Pergamon Press, 1976. Printed in Great Britain.

EFFECTS OF CINNABARINIC ACID ON MITOCHONDRIAL RESPIRATION

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(*Received* 6 *May* 1975; *accepted* 10 *September* 1975)

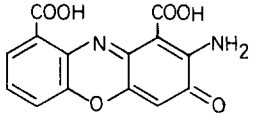
Abstract—The effects of cinnabarinic acid on the respiration of rat liver and beef heart mitochondria in the presence of various substrates were studied. Cinnabarinic acid inhibits respiration of rat liver mitochondria with α-ketoglutarate, malate, isocitrate, pyruvate and glutamate. Oxidation of succinate and β-hydroxybutyrate is not or only slightly inhibited. α-Ketoglutarate oxidation is most sensitive towards cinnabarinic acid followed by pyruvate, glutamate, malate and isocitrate oxidation. Respiration of beef heart mitochondria is inhibited in the presence of α-ketoglutarate, glutamate, β-hydroxybutyrate and malate. Cinnabarinic acid also activates the adenosine triphosphatase of intact rat liver mitochondria. Mitochondrial respiration inhibited either by rotenone, amytal or antimycin in the presence of β-hydroxybutrate or glutamate is restored by the addition of cinnabarinic acid. This cinnabarinic acid-mediated respiration is sensitive to dicumarol and cyanide. Oxidation of α-ketoglutarate blocked by these inhibitors and oxidation of succinate inhibited by antimycin is not restored. The cinnabarinic acid mediated respiration shows some degree of respiratory control. *P/O* ratios of 0·75 were observed. It is concluded that reducing equivalents are transferred from NADH upon the interaction of menadione reductase to cinnabarinic acid and enter the respiratory chain at the level of cytochrome cc_1.

Recently, Quagliariello and coworkers [1, 2] have shown that 3-hydroxyanilate, intermediate of tryptophan metabolism, inhibits the oxidation of NAD-dependent substrates in rat liver mitochondria. Nagasawa *et al.* [3] found that cytochrome c:O_2 oxidoreductase (EC 1.9.3.1) is an enzyme system capable of oxydizing *o*-aminophenols to iso-phenoxazines. We found [4, 5] that upon incubation of rat liver mitochondria with 3-hydroxyanthranilic acid a yellow–orange compound was formed which was identified with cinnabarinic acid. This raises the question of whether 3-hydroxyanthranilic acid is responsible for the effects reported by Quagliariello [1, 2]. In this paper the effects of cinnabarinic acid on the respiration of rat liver mitochondria and beef heart mitochondria in the presence of various substrates are reported. The results suggest that at least some of the effects of 3-hydroxyanthranilic acid are due to the formation of cinnabarinic acid from 3-hydroxyanthranilic acid.

MATERIALS AND METHODS

Cinnabarinic acid was synthesized according to Butenandt *et al* [6] and recrystallized from pyridine.

Abbreviations: RLM, rat liver mitochondria; BHM, beef heart mitochondria; BSA, bovine serum albumin; EGTA, ethylene glycol bis (β-aminoethyl)-*N*,*N*-tetra-acetic acid; EDTA, ethylene diamine tetraacetic acid; CA, cinnabarinic acid (2-amino,3-oxo-3H-phenoxazine-1,9-dicarbonic acid).



Cinnabarinic acid

Glutamate, succinate, α-ketoglutarate, ADP, ATP, NADH and antimycin were purchased from Boehringer und Söhne Mannheim. β-Hydroxybutyrate, rotenone, amytal, hexokinase type III (EC 2.7.1.1.) and sucrose were from Sigma Chemical Co., St Louis. All other reagents used were of analytical grade.

Rat liver mitochondria (RLM) were isolated according to Myers and Slater [7] in 0·25 M sucrose; beef heart mitochondria (BHM) were isolated by a modification of the nagarse (EC 3.4.4.16.) procedure of Hatefi *et al.* [8] as described by Settlemire *et al.* [9]. Oxygen uptake was measured either polarographically or manometrically. To measure the oxygen uptake polarographically mitochondria were incubated at 25° in medium composed of 6·6 mM PO_4^{3-} pH 7·4, 20 mM glycyl–glycine buffer pH 7·4, 1 mM EGTA, 80 mM KCl, 50 mM sucrose, 6·6 mM $MgCl_2$, 0·17 mM ADP, 6·6 mM β-hydroxybutyrate and succinate in a final volume of 3 ml.

To measure the oxygen uptake manometrically RLM were incubated at 30° in medium composed of 30 mM KCl, 60 mM sucrose, 1mM ATP, 20 mM Tris–HCl pH 7·4, 30 mM glucose, 20 mM PO_4^{3-} pH 7·4, 6·6 mM $MgCl_2$, 13·2 mM substrates and 36 I.U. of hexokinase. BHM were incubated at 30° in medium composed of 250 mM sucrose, 10 mM PO_4^{3-} pH 7·4, 5 mg BSA/ml, 36 I.U. of hexokinase, 13·2 mM substrates, 33 mM glucose, 1·67 mM ADP and 6·6 mM $MgCl_2$ in a final volume of 3 ml.

Protein was estimated by the Biuret method [10] using samples clarified with 0·2% sodium cholate as described by Tyler [11]. Inorganic phosphate was determined according to Lindeberg and Ernster [12].

RESULTS

Effect of cinnabarinic acid on mitochondrial respiration. Table 1 summarizes the effects of cinnabarinic

Table 1. Effect of cinnabarinic acid on the respiration of RLM and BHM with various substrates

	Substrate	Oxygen uptake (n-atoms × min^{-1} × mg protein^{-1}) control	50 μM CA	Inhibition (%)
RLM	Malate	74·7	22·8	69·5
	Isocitrate	61·2	33·8	44·5
	β-Hydroxybutyrate	55·3	46·8	15·4
	Pyruvate/malate	70·5	24·5	65·0
	Glutamate	74·0	28·8	61·0
	α-Ketoglutarate	60·0	10·1	83·2
	Succinate	89·3	83·4	6·6
BHM	β-Hydroxybutyrate	67·8	28·8	57·5
	Pyruvate/malate	107·0	52·3	51·0
	Glutamate	50·5	19·8	60·8
	α-Ketoglutarate/malonate	65·0	25·7	60·5
	Succinate	83·5	69·6	16·6

Oxygen uptake was determined manometrically. 13·2 mM substrates, 3·3 mM malate and malonate, with these substances used as initiators. RLM 5–6 mg protein, BHM 2–3 mg protein.

acid on state 3 respiration of rat liver and beef heart mitochondria in the presence of various substrates.

RLM. Glutamate, α-ketoglutarate, pyruvate, malate and isocitrate oxidation is inhibited by cinnabarinic acid. The oxidation of β-hydroxy-butyrate and succinate is not or only slightly affected. To evaluate the sensitivity of the oxidation of the various substrates the dependence of the inhibition on cinnabarinic acid concentration was studied (Fig. 1). Half-maximal inhibition of α-ketoglutarate and pyruvate oxidation was observed at 2 and 8 μM cinnabarinic acid, respectively (1·2 and 3·5 nmoles/mg protein, respectively).

BHM. Cinnabarinic acid inhibits to a similar degree, the respiration in the presence of glutamate, α-ketoglutarate, pyruvate and β-hydroxybutyrate whereas succinate oxidation is only slightly affected. Comparing the effects of cinnabarinic acid on RLM with those on BHM it becomes obvious that the respiration of BHM in the presence of β-hydroxybutyrate is also sensitive to cinnabarinic acid.

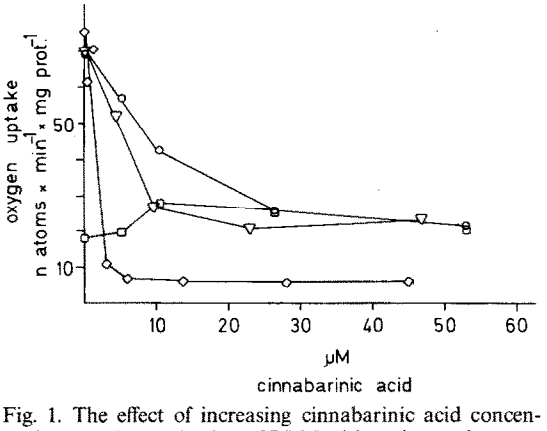


Fig. 1. The effect of increasing cinnabarinic acid concentrations on the respiration of RLM with various substrates. Experimental conditions as in Table 1. ○–○ glutamate oxidation, □–□ glutamate oxidation in the presence of 20 mM malonate, ▽–▽ pyruvate oxidation, ◇–◇ α-ketoglutarate oxidation.

It is well known that quinones react with SH-groups [13–16] and that Pi-transport [17–21], glutamate transport [22, 23] and electron transfer in the NADH–flavin region [19, 24] are sensitive to SH-reagents. Smith and Lester [15] reported that glutathione reverses the effects of 2,3-dimethoxy-5-methyl-*p*-benzoquinone on the oxidation of pyruvate and succinate and on oxidative phosphorylation in BHM. As cinnabarinec acid can be regarded as an iminoquinone the question arises whether the blocking of SH-groups by cinnabarinic acid is responsible for the observed effects.

A one hundred-fold excess of glutathione over cinnabarinic acid in the incubation medium does not protect RLM or BHM from the effects of cinnabarinic acid.

Effects of cinnabarinic acid on state 4 respiration. Cinnabarinic acid stimulates oxygen uptake of RLM respiring in state 4. The most pronounced effect is found with β-hydroxybutyrate as the oxidizable substrate. With this substrate a respiration rate up to 80 per cent of that of state 3 was found.

We know that stimulation of state 4 respiration may be due to uncoupling resulting in high ATPase activity. Therefore the effect of cinnabarinic acid on ATPase was studied.

From Fig. 2 it becomes evident that cinnabarinic acid stimulates ATPase about fifteen-fold, but it is doubtful that the enhanced ATPase activity is solely responsible for the observed stimulation of state 4 respiration.

Stimulation of succinate oxidation is only small in comparison with the stimulation of β-hydroxybutyrate or glutamate oxidation. Enhanced ATPase activity can therefore be responsible only for the effect on state 4 respiration with succinate and for a part of the stimulation of β-hydroxybutyrate oxidation. Another mechanism which leads to this stimulating effect must therefore exist.

Effects of cinnabarinic acid on the rotenone, amytal, antimycin or CN^- inhibited respiratory chain. Cinnabarinic acid restores the rotenone, amytal or antimycin inhibited respiration of rat liver mitochondria

Table 2. Cinnabarinic acid induced stimulation of state 4 oxidation of RLM

Substrate	Additions	Oxygen uptake (n-atoms × min^{-1} × mg $protein^{-1}$)
β-Hydroxybutyrate	—	1·8
β-Hydroxybutyrate	ADP	19·1
β-Hydroxybutyrate	CA	15·2
Succinate	—	14·6
Succinate	ADP	93·8
Succinate	CA	24·5
Glutamate	—	6·9
Glutamate	ADP	45·0
Glutamate	CA	19·6

Oxygen uptake was measured polarographically. 6·6 mM Hydroxybutyrate, succinate and glutamate, 0·17 mM ADP, 70 μM CA; 8·7 mg mitochondrial protein.

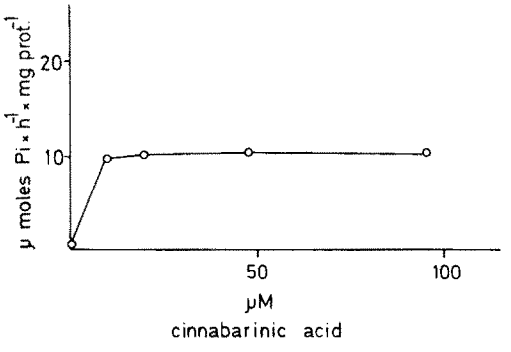


Fig. 2. Stimulation of ATPase by cinnabarinic acid. RLM (1·1 mg protein) were incubated at 25° in a medium of the following composition: 4 mM ATP, 76 mM KCl 1 mM EDTA, 108 mM sucrose, 3 mM $MgCl_2$, 20 mM Tris–HCl pH 7·4; final volume 1·5 ml. After 15 min the reaction was stopped by the addition of 0·5 ml 20% TCA.

supported by β-hydroxybutyrate. The cinnabarinic acid-mediated respiration is sensitive to cyanide and dicumarol. Similar results were found with glutamate but not with oxoglutarate or succinate.

The concentrations of dicumarol needed to inhibit the cinnabarinic acid shunt maximally are of the same order of magnitude as those which inhibit menadione reductase (EC 1.6.99.2.) [25] and menadione mediated respiration [26]. Such behaviour is taken as evidence for the participation of menadione reductase in cinnabarinic acid-mediated respiration.

The effect of increasing concentrations of cinnabarinic acid on the respiration rate in the presence of rotenone is shown in Fig. 3. Line A depicts this dependence for β-hydroxybutyrate oxidation. Below 32 nmoles/mg (50 μM) the concentration of cinnabarinic acid becomes rate-limiting; half-maximal recovery is found at 7 nmoles/mg (12 μM). With glutamate as oxidizable substrate (line B) about 40 per cent of

Table 3. Effect of cinnabarinic acid on the rotenone, amytal or antimycin inhibition of respiration with β-hydroxybutyrate or succinate

Substrate	Additions	Oxygen uptake (n-atoms × min^{-1} × mg $protein^{-1}$)
β-Hydroxybutyrate	none	23·3
β-Hydroxybutyrate	rotenone	2·6
β-Hydroxybutyrate	rotenone + cinnabarinic acid	21·9
β-Hydroxybutyrate	rotenone + cinnabarinic acid + dicumarol	3·9
β-Hydroxybutyrate	amytal	0·4
β-Hydroxybutyrate	amytal + cinnabarinic acid	14·0
β-Hydroxybutyrate	amytal + cinnabarinic acid + dicumarol	6·8
β-Hydroxybutyrate	antimycin	2·6
β-Hydroxybutyrate	antimycin + cinnabarinic acid	17·2
β-Hydroxybutyrate	antimycin + cinnabarinic acid + dicumarol	6·8
β-Hydroxybutyrate	KCN	4·9
β-Hydroxybutyrate	KCN + cinnabarinic acid	9·4
Succinate	none	103·4
Succinate	antimycin	4·4
Succinate	antimycin + cinnabarinic acid	21·5
Succinate	antimycin + cinnabarinic acid + dicumarol	13·4

Oxygen uptake was measured polarographically in a medium described in material and methods. The additions were as follows: β-hydroxybutyrate and succinate 20 μmoles, cinnabarinic acid 112 nmoles in the presence of β-hydroxybutyrate and 198 nmoles in the presence of succinate, 1 μg rotenone, 0·8 μg antimycin, 5.4 μmoles amytal, 3 μmoles CN^- and 30 nmoles dicumarol; 7·9 mg mitochondrial protein; final volume 3 ml.

Table 4. Effect of cinnabarinic acid on the respiration with glutamate and α-ketoglutarate

	Substrate	Additions	Oxygen uptake (n-atoms × min^{-1} × mg protein^{-1})
Expt. 1	Glutamate	none	73·5
	Glutamate	rotenone	2·8
	Glutamate	cinnabarinic acid	28·8
	Glutamate	rotenone + cinnabarinic acid	27·7
	Glutamate	rotenone + cinnabarinic acid + dicumarol	10·0
	Glutamate	antimycin	11·6
	Glutamate	antimycin + cinnababarinic acid	30·5
	Glutamate	amytal	8·0
	Glutamate	amytal + cinnabarinic acid	27·1
Expt. 2	α-Ketoglutarate	none	64·4
	α-Ketoglutarate	rotenone	2·8
	α-Ketoglutarate	cinnabarinic acid	1·4
	α-Ketoglutarate	rotenone + cinnabarinic acid	5·2

Oxygen uptake was measured manometrically, for 18 min. The amount of cinnabarinic acid was in Expt. 1 83·5 nmoles and Expt. 2 114 nmoles; mitochondrial protein, 5·5 mg in Expt. 1 and 5·7 mg in Expt. 2. For further detailes see materials and methods and Table 1.

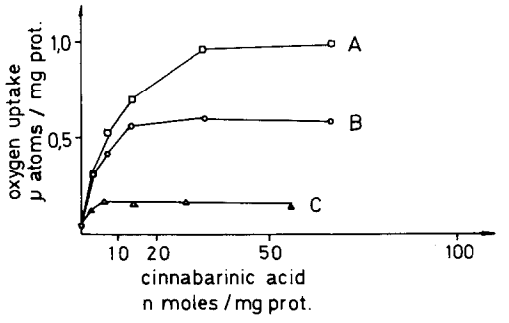


Fig. 3. Restoration of rotenone-inhibited respiration in the presence of increasing concentrations of cinnabarinic acid. Oxygen uptake was measured by the Warburg technique. 1μg rotenone, 13·2 mM β-hydroxybutyrate or glutamate; 5 mg mitochondrial protein. Line A, β-hydroxybutyrate oxidation; B, glutamate oxidation; C, endogenous respiration.

the normal uninhibited oxidation rate of glutamate is obtained on addition of cinnabarinic acid. The cinnabarinic acid-restored respiration proceeds linearly with time.

It is well established that glutamate is oxidized in isolated mitochondria predominantly via the transmination pathway. Oxidative deamination of glutamate occurs only if the transamination pathway is inhibited e.g. by blocking succinate dehydrogenase (EC 1.3.99.1.) with malonate [27]. The finding that cinnabarinic acid inhibits oxygen uptake in the presence of α-ketoglutarate indicates that cinnabarinic acid may activate the dehydrogenase pathway. This view is supported by the finding that during the cinnabarinic acid mediated glutamate oxidation ammonia generation is greatly enhanced whereas aspartate formation is nearly abolished (Table 5).

Like the oxidation of glutamate and β-hydroxybutyrate the endogenous respiration inhibited by rotenone, too, is restored on addition of cinnabarinic acid (line C of Fig. 3). 100 per cent recovery, however, is found at much lower concentrations of cinnabarinic acid (6 nmoles/mg (13 μM)). The malonate inhibition of succinate oxidation is not restored by cinnabarinic acid.

Energy conservation of the by-pass. It is well documented that antimycin inhibits the electron flux from cytochrome *b* to cytochrome *c* [30] and cyanide at the level of cytochrome a_3 [31]. Cyanide strongly inhibits cinnabarinic acid-mediated respiration whereas antimycin has no influence. This indicates that cytochrome *c* or cytochrome c_1 may be the electron

Table 5. Ammonia and aspartate formation during cinnabarinic acid mediated glutamate oxidation

Additions	Δ Aspartate (nmoles × min^{-1} × mg protein^{-1})	Δ Ammonia (nmoles × min^{-1} × mg protein^{-1})
Glutamate	20·9	0·5
Glutamate + rotenone + cinnabarinic acid	0·45	9·9

Mitochondria (4·9 mg protein) were incubated in the medium described for the manometric measurement of oxygen uptake at 30°; 13·2 mM glutamate, 60 μM cinnabarinic acid, 1 μg rotenone; final volume 3 ml. After 12 min the reaction was stopped by the addition of $HClO_4$ and aspartate was determined according to Bergmeyer *et al.* [28] and ammonia according to Kun and Kearney [29].

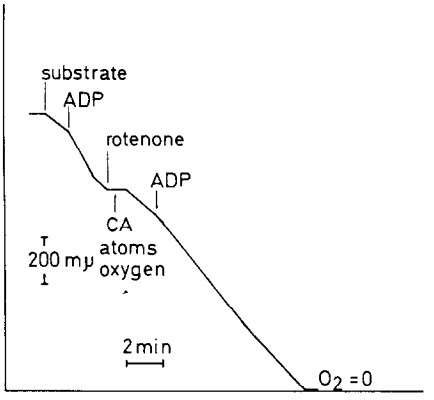


Fig. 4. Stimulation of the cinnabarinic acid-mediated by-pass by ADP. Oxygen uptake was measured polarographically. 138 nmoles cinnabarinic acid; 9·2 mg mitochondrial protein.

acceptor of the cinnabarinic acid-mediated pathway. In this case ATP synthesis or some degree of respiratory control should be observed. Stimulation of the by-pass with ADP could in fact be demonstrated (Fig. 4). Addition of ADP caused a 1·3- to 1·5-fold stimulation and respiration rate slowed down after the ADP was exhausted. On average an ADP/O ratio of 0·75 was found.

In the presence of an ATP trap (hexokinase plus glucose) a *P/O* ratio of 2·3 for the oxidation of β-hydroxybutyrate was found. Addition of 11 nmoles/mg (33·3 μM) of cinnabarinic acid lowered the *P/O* ratio non-significantly to 1·75. Therefore it is suspected that the cinnabarinic acid pathway competes with NADH-dehydrogenase (EC 1.6.99.3.) for NADH. In the absence of a respiratory chain inhibitor or in state 3 this competition is dominated by NADH-dehydrogenase.

Participation of menadione reductase in cinnabarinic acid mediated respiration. Menadione reductase was shown to be an integral part of the cinnabarinic acid shunt. Reducing equivalents are transferred, in a reconstituted system, from NADH to cytochrome *c* via cinnabarinic acid only in the presence of menadione reductase. In the absence of either cinnabarinic acid or menadione reductase no reducing equivalents are transferred. These findings also support the view that cytochrome *c* is the electron acceptor in the cinnabarinic acid shunt.

DISCUSSION

It was questioned whether the formation of cinnabarinic acid from 3-hydroxyanthranilic acid is responsible for the effects of 3-hydroxyanthranilic acid reported by Quagliariello *et al.* [1, 2].

The results presented in this paper indicate that at least some of the effects exerted by 3-hydroxyanthranilic acid are due to the formation of cinnabarinic acid. 1. Cinnabarinic acid is at least twenty times more effective than 3-hydroxyanthranilic acid in inhibiting respiration of rat liver mitochondria. 1 mM 3-hydroxyanthranilic acid decreases glutamate-, malate-, pyruvate- and isocitrate oxidation by about 40–70 per cent whereas 50 μM cinnabarinic acid gives the same effect. The differences are much more pronounced with α-ketoglutarate as the oxidizable substrate; to get half-maximal inhibition 250 μM 3-hydroxyanthranilic acid or 2 μM cinnabarinic acid, respectively, are needed. 2. The concentration of 3-hydroxyanthranilic acid needed to get half-maximal recovery of the inhibited respiration is about 10^{-4}M and maximal recovery is obtained at 10^{-3}M. It would be sufficient therefore if only a small percentage of the 3-hydroxyanthranilic acid is converted to cinnabarinic acid to exert the effects discussed above. 3. 3-Hydroxyanthranilic acid is rapidly oxidized by cytochrome *c* yielding cinnabarinic acid. Addition of cytochrome *c* reduces considerably the amount of 3-hydroxyanthranilic acid needed to get half-maximal or maximal recovery, to 10^{-5}M and 5×10^{-5}M respectively, values observed by use for cinnabarinic acid [2].

Two mechanisms are responsible for the formation of cinnabarinic acid; non-enzymic [32] and enzymic oxidation of 3-hydroxyanthranilic acid. The enzymatic formation of cinnabarinic acid is catalyzed by cytochrome c:O_2 oxidoreductase [3] and may be the main pathway for the formation of cinnabarinic acid upon incubation of isolated mitochondria. Besides the mitochondrial enzyme system, three other enzymes or enzyme systems are known to convert 3-hydroxyanthranilic acid to cinnabarinic acid. Subba Rao [33] isolated a cinnabarinic acid synthetase from rat liver nuclei, and Morgan *et al.* [34] reported that DOPA is

Table 6. Reconstitution of the cinnabarinic acid shunt

Additions	Cyt. *c* reduced (nmoles/min)
NADH	0
NADH + cinnabarinic acid	0·1
NADH + menadione reductase	3·8
NADH + menadione reductase + cinnabarinic acid	253·4
NADH + menadione reductase + cinnabarinic acid + dicumarol	11·9

To a solution of 50 μM cytochrome *c* in 0·1 M phosphate buffer pH 7·4 the following were added as indicated to give a final concentration of : 1 mM NADH, 60 μM cinnabarinic acid, 50 μM dicumarol and 0·2 U. menadione reductase; final volume 3 ml at 25°. Mitochondrial menadione reductase was isolated and determined according to the method of Conover *et al.* [26].

converted to DOPA-quinone by DOPA oxidase, and that DOPA-quinone oxidizes 3-hydroxyanthranilic acid as well as the presence of a cinnabarinic acid synthetase in the liver of poikilothermic animals [35].

It was assumed that a deranged tryptophan metabolism may be the cause of some tumors especially those of the bladder, [36] since it is known that abnormally high amounts of 3-hydroxyanthranilic acid and 3-hydroxykynurenine are excreted in the urine of patients with bladder tumors [37]. The present results indicate that 3-hydroxyanthranilic acid is converted to the more effective phenoxazone and an interaction of cinnabarinic acid with the oxidative processes in mitochondria may be involved in the induction of bladder tumours in man.

Acknowledgements. The author wishes to thank Prof. E. Schauenstein for his encouragement and helpful discussion and Mrs. G. Ranner for excellent technical assistance.

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