AMMORESINOL, A NEW INHIBITOR IN THE CYTOCHROME b REGION*

V. DADÁK, I. LOCHMAN and L. MÁCHOVÁ

Department of Biochemistry,
Purkyně University, 611 37 Brno

Dedicated to Professor F. Šantavý on the occasion of his 60th birthday.

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Ammoresinol is a highly potent inhibitor of phosphorylating succinate oxidase in rat liver mitochondria. The inhibitory effect on the oxidation of β-hydroxybutyrate is not observable at low ammoresinol concentrations. The higher sensitivity of succinate oxidase to ammoresinol consists most likely in its preferential binding to a phospholipid complex involving succinate dehydrogenase since solubilized succinate dehydrogenase itself is not inhibited by ammoresinol.

Ammoresinol acts more weakly on nonphosphorylating oxidases. Dicoumarol and 2,4-dinitrophenol at concentrations causing uncoupling of oxidative phosphorylation weaken the inhibitory effect of ammoresinol; adenosine 5′-triphosphatase, however, is not inhibited by ammoresinol. Changes in ammoresinol fluorescence indicate that ammoresinol binds to mitochondrial fragments in a manner similar to its binding to albumin which is an effective reactivator of inhibited oxidases. The binding of ammoresinol to rat liver mitochondria manifests itself by a specific inhibition of the respiratory chain at the antimycin sensitive site.

It was observed in earlier studies on phenolic derivatives of coumarin that ammoresinol (3-farnesyl-4,7-dihydroxycoumarin) is a strong inhibitor of succinate oxidase[1] and of the respiratory chain of membrane fragments of bacteria[2]. The action of ammoresinol on the nonphosphorylating fragments, however, cannot provide a complete picture of the mechanism of its effect since the transfer of reducing equivalents from the substrate to oxygen proceeding in this system is not coupled to the synthesis of adenosine 5′-triphosphate (ATP). The effect of different factors on electron transfer combined with the conservation of the energy liberated can be conveniently studied with isolated mitochondria. Oxidative phosphorylation is tightly bound to the structure of the inner mitochondrial membrane and a number of processes are known which play a role in the generation of ATP. Their high sensitivity to external factors permits us to characterize better the changes which parallel the binding of the inhibitor to the mitochondrial membrane. The fluorescence of coumarin derivatives has been used succesfully in studies on binding to soluble proteins[3] as

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well as to mitochondrial fragments[4]. In the present study, this approach was taken to compare the mode of binding of ammoresinol to an insoluble enzyme preparation with the mode of binding to bovine serum albumin. The results obtained permitted us to demonstrate the specific effect of ammoresinol on respiration as well as the site of its interference with the respiratory chain. Some of the results have been published in a preliminary report[2].

EXPERIMENTAL

Chemicals. Coumarin derivatives ammoresinol, diacetylammoresinol, and hexahydrodiacetylammoresinol were from the collection of Prof. E. Späth and were kindly provided by the workers of the Institute of Organic Chemistry, University of Vienna. These derivatives were added to the reaction medium as ethanolic solutions. The micellar solution of phospholipids was prepared from Asolectin (Assoc. Concentrates) by sonication of its aqueous suspension according to Fleischer[5]. Bovine serum albumin (Mann Research Laboratories) was not purified to remove lipid contaminants. The remaining chemicals were commercial preparations of analytical purity grade.

Biological material. Rat liver mitochondria were prepared by the method of Schneider and Hogeboom and suspended in a sucrose medium[6]. Sarcosomal fragments from pig heart served as a succinate oxidase preparation; they were prepared by the modified method of Keilin and Hartree and stored in a phosphate buffer[7]. Beef heart mitochondria were isolated by the method described by Smith[8] (procedure 1) and used for the preparation of electron transport particles (ETP). For this purpose, the mitochondria were kept overnight in 0·25M solution of sucrose, 0·2 mM EDTA, and 0·01M Tris-HCl buffer at pH 7·5 at −20°C. The thawed suspension was sonicated with cooling at 10 s intervals for 1 min in an ultrasonic homogenizer (MSE 3100) at maximum output of 500 W. The homogenate was freed from intact mitochondria by centrifugation (2°C, 10 min, 20000 *g*) and the supernatant was centrifuged at 3°C (Omega II Christ ultracentrifuge, 40 min, 120000 *g*). The sediment of ETP was suspended in one quarter the volume of the original sucrose solution and used for the determination of nonphosphorylating β-hydroxybutyrate oxidase activity. Soluble succinate dehydrogenase was liberated from the sarcosomal fragments by butanol in an atmosphere of nitrogen according to Dervartanian and Veeger[9]. The enzyme was purified by adsorption to a calcium phosphate gel and elution by 0·075M phosphate buffer at pH 7·8. The protein content was determined by the biuret method; the modification described by Szarkowska and Klingenberg[10] was used for insoluble preparations.

Determination of enzymatic activities. The activity of oxidases was measured polarographically in terms of oxygen uptake using a Clark electrode (Instrument Development Workshops, Prague), separated by a polyethylene membrane from the reaction medium. The activity of succinate dehydrogenase was determined spectrophotometrically as the absorbance decrease of potassium ferricyanide serving as an artificial electron acceptor[7]. The activity of adenosine 5′-triphosphatase in the rat liver mitochondria suspension was established from the quantity of inorganic phosphate released into the reaction medium by the method of Fiske and Subbarow, described by Lindberg and Ernster[11]. The exchange reaction between ATP and orthophosphate was determined radiometrically by measurement the degree of esterification of ^{32}P-labeled orthophosphate. The procedure introduced by Nielsen and Lehninger[12] was followed. To measure the activity of translocase systems for phosphate and succinate, we examined the swelling of respiratorily inhibited mitochondria in an isoosmotic medium containing ammonium ions. The time dependence of the change of turbidity at 546 nm is directly proportional to the activity of the

transferring agent, *i.e.* to the entry of the corresponding anion into the mitochondria[13]. The measurement was made in Unicam SP 1 800 Spectrophotometer.

The binding of ammoresinol to sarcosomal fragments and albumin was determined from the change of the emission of the fluorescence spectrum during the incubation. The fluorescence intensity was measured in Perkin-Elmer 203 spectrofluorimeter. The site of interference of ammoresinol with the cytochrome system of the respiratory chain was determined by measurement of spectra in the 400—630 nm range. The mitochondria suspension was pipetted both in the measuring and the reference cuvet; the difference between the absorbance of the sample in state of dynamic equilibrium with the substrate and the inhibitor was measured against a sample in oxidized state. The differences in absorbance of these solutions were recorded in Cary 118 C recording spectrophotometer.

RESULTS

Action of Ammoresinol on Respiration

The respiratory activity of the mitochondria was established by measurement of oxygen uptake as a function of time using succinate and β-hydroxybutyrate as substrates. We observed (Table I) that ammoresinol is a highly effective inhibitor of the phosphorylating succinate oxidase system (state 3 according to Chance and Williams[14]). The inhibitory power of ammoresinol on succinate oxidase in coupled mitochondria is of the same range as that of the most effective inhibitors, such as, *e.g.* antimycin A. The inhibition depends on the ratio of ammoresinol to the mitochondrial protein.

TABLE I
Inhibitory Action of Ammoresinol

Enzyme preparation	Substrate	I_{50} ammoresinol nmol/mg protein
Mitochondria[a] (state 3)	succinate	0·8
Mitochondria[a] (state 3)	β-hydroxybutyrate	3·2
Keilin–Hartree[b]	succinate	25·0
ETP[c]	β-hydroxybutyrate	80·0
Succinate dehydrogenase[d] solubilized	succinate	—[e]

[a] Conditions of the measurement as in Fig. 1; the concentration of β-hydroxybutyrate was the same as that of succinate. [b] Value taken from a previous study[1]. [c] The same as under *a*, ADP was replaced by 0·16 mM nicotinamide adenine dinucleotide. [d] Three ml of reaction mixture contained 0·1M phosphate buffer at pH 7·4, 0·03M sodium succinate, and 1·3 mM potassium ferricyanide. [e] Ammoresinol was used up to a concentration of 40 nmol/mg protein; under these conditions it caused a 14% inhibition of the enzyme. Inhibition increased to 30% in the presence of the micellar phospholipid solution[5] (1·25 μmol of inorganic phosphate/g protein).

Unlike with antimycin A, a hyperbolic rather than sigmoidal inhibition curve is obtained (Fig. 1). The same shape is found in the inhibition curve of diacetylammoresinol which is an equally strong inhibitor of succinate oxidase. This indicates that the dissociable hydroxyl groups do not take part in the inhibitory effect. Some decrease of inhibition and a change of its dependence on the concentration in case of hexahydrodiacetylammoresinol suggest that the presence of double bonds plays a role in the interaction of ammoresinol with the mitochondrial membrane.

The inhibitory effect of ammoresinol on the oxidation of β-hydroxybutyrate by mitochondria does not manifest itself at low concentrations. A similar observation was made in experiments studying the effect of ammoresinol on NADH oxidase in the membrane fraction of a *Proteus rettgeri* strain[2]. Mitochondrial oxidases are more sensitive to ammoresinol than the particle fraction of *P. rettgeri*[2]. A four times lower concentration will still cause a 50% inhibition of succinate oxidase. As can be seen in Table I, ammoresinol is even less effective on nonphosphorylating sarcosomal fragments, *i.e.* on a succinate oxidase preparation according to Keilin

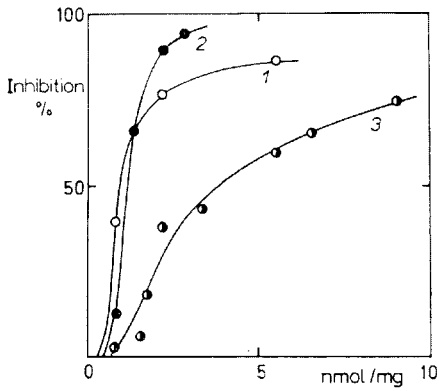

FIG. 1

Inhibitory Effect of Ammoresinol Derivatives on Succinate Oxidation in Rat Liver Mitochondria

The reaction medium (2 ml) contained 0·188M sucrose, 0·04M Tris-HCl buffer at pH 7·2, 1·25 mM KH_2PO_4, 0·15 mM EDTA, 2·5 mM $MgSO_4$, 5 mM sodium succinate, 0·2 mM ADP, 1 to 2 mg of protein of mitochondria and the inhibitor (nmol/mg protein). Oxygen uptake was measured polarographically at 25°C. *1* Ammoresinol; *2* diacetylammoresinol; *3* hexahydrodiacetylammoresinol.

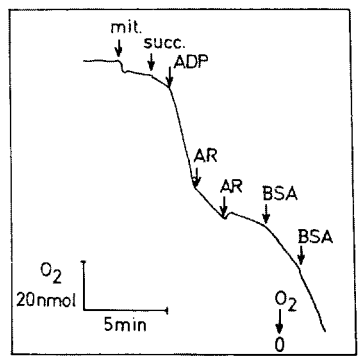

FIG. 2

Polarographic Record of Inhibitory Action of Ammoresinol on Succinate Oxidase and of Reactivation Effect of Albumin

Reaction medium and conditions of experiment the same as those given in legend to Fig. 1. The arrows indicate individual additions, mit. (0·9 mg of protein of rat liver mitochondria), AR (2·8 nmol of ammoresinol), BSA (3·6 nmol of bovine serum albumin).

and Hartree and electron transport particles from NAD-dependent dehydrogenases. Even here, however, succinate oxidase is inhibited to a higher degree.

We have demonstrated with a bacterial succinate oxidase[2] that the changes in the redox system of ubiquinone in the presence of various concentrations of ammoresinol suggest two different sites of interference with the succinate oxidase complex. The first of them, which manifests itself even at low ammoresinol concentrations, is localized on the substrate side of ubiquinone, the other on the oxygen side. The higher sensitivity of succinate oxidase may thus be ascribed to the action of ammoresinol on succinate dehydrogenase. A study of structurally bound succinate dehydrogenase with the aid of artificial electron acceptors is not likely to yield always a real picture of the actual inhibitory effect of phenolic coumarins. Hence we carried out experiments with soluble succinate dehydrogenase prepared by liberation from sarcosomal membrane fragments. As shown in Table I, soluble succinate dehydrogenase was not inhibited by ammoresinol. Inhibition was observed only after the micellar phospholipid solution had been added to the soluble enzyme. The enhanced inhibitory effect of ammoresinol on succinate oxidase, contrary to other oxidases, does not consist in a direct inhibition of succinate dehydrogenase but probably in binding to the phospholipid complex or to some other component of this region.

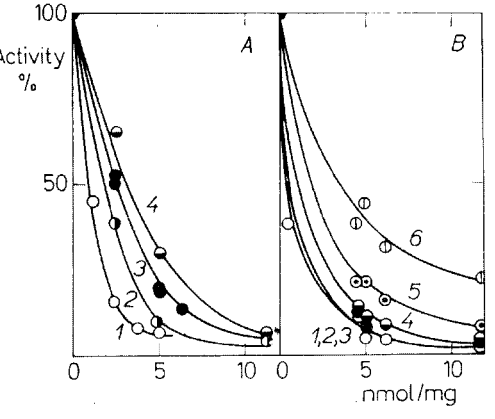

FIG. 3

Protective and Reactivation Effect of Albumin on Mitochondrial Succinate Oxidase in the Course of Inhibition by Ammoresinol

Albumin was added to the reaction medium (Fig. 1) before (*A*) and after (*B*) ammoresinol (nmol/mg). Concentrations of albumin (nmol/mg mitochondrial protein): *1* 0; *2* 0·015; *3* 0·15; *4* 0·30; *5* 1·0; *6* 3·0.

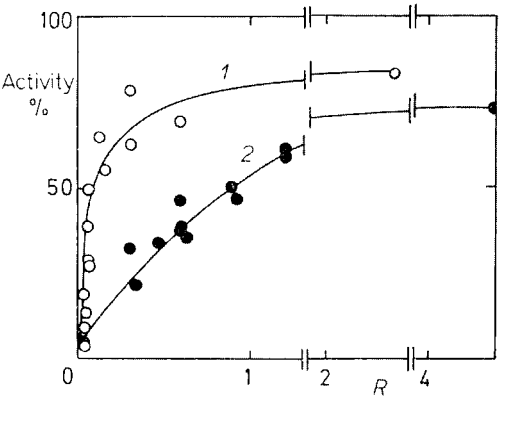

FIG. 4

Activity of Mitochondrial Succinate Oxidase as a Function of the Albumin to Ammoresinol Ratio

Conditions of measurement as in Fig. 1. *R* stands for molar ratio of bovine serum albumin to ammoresinol. Albumin added to reaction medium before (*1*) or after (*2*) ammoresinol.

Binding of Ammoresinol to Mitochondria and its Elimination by Albumin

We have observed earlier that bovine serum albumin efficiently restores succinate oxidase activity of sarcosomal fragments which has been inhibited by coumarin derivatives[1]. The rate of oxygen uptake (Fig. 2) shows that the inhibition of succinate oxidase by ammoresinol is decreased also in mitochondria after the addition of bovine serum albumin. Obviously, albumin can remove a part of ammoresinol bound to mitochondria and reactivates succinate oxidase which has been inhibited. The effect of albumin is greater if it is present in the incubation mixture before the addition of ammoresinol, in this case it exerts a protective action on succinate oxidase.

Fig. 3 shows the dependence of the activity of succinate oxidase on the concentration of ammoresinol and bovine serum albumin. If albumin has been added before ammoresinol, the inhibition is decreased even at very low albumin concentrations, in the opposite case inhibition is decreased only at albumin concentration many times higher. This difference becomes even more apparent if the activity of succinate oxidase is plotted *versus* the molar ratio of albumin to ammoresinol (Fig. 4). The activity of succinate oxidase increases more rapidly if albumin was present before the addition of ammoresinol. Hence, ammoresinol is firmly bound to mitochondria and its liberation requires a large excess of albumin. If the albumin is present beforehand in the incubation mixture, ammoresinol is distributed between the mitochondria and albumin; in this case, an approximately five times smaller quantity of albumin will produce the same protective effect.

The reactivation values given in Table II are calculated from values measured in experiments in which albumin was added after ammoresinol; they reflect therefore the actual reactivation. Since albumin itself does not affect the activity, we calculated reactivation according to a simplified formula given at the bottom of Table II.

TABLE II

Dependence of the Reactivation Effect of Albumin on its Ratio to Ammoresinol

Reaction medium and conditions of measurement as in Fig. 1 Albumin was added after ammoresinol. Reactivation (%) was calculated from $R = (A_{RI} - A_I/A - A_I) \cdot 100$. ($A$, original activity; A_I, activity in presence of inhibitor; A_{RI}, activity in presence of inhibitor and bovine serum albumin).

Molar ratio of albumin/ammoresinol	Reactivation %
0·297	27·5
0·595	44·0
0·892	47·0
1·211	57·4
4·237	71·8

Unlike the original equation, this one does not involve activity in the presence of reactivator, *i.e.* of albumin itself. Reactivation is proportional to the molar albumin to ammoresinol ratio; it did not exceed the value of 80% in any case, however. The degree of reactivation by albumin was higher with sarcosomal fragments[1] at equal molar ratios. This shows that ammoresinol binds more firmly to rat liver mitochondria than to the nonphosphorylating enzyme preparation.

In an effort to cast light on the character of binding to mitochondrial fragments and bovine serum albumin, we investigated changes in the fluorescence spectrum of ammoresinol during its incubation with these substances. The emission maximum of ammoresinol fluorescence lies at 410 nm in aqueous solutions, it disappears in ethanolic solutions, and a new maximum at 370 nm appears. We have observed a similar shift of emission maximum toward the shorter wavelengths earlier with ostruthin, not only in an alcoholic medium but also after the addition of mitochondrial fragments[4]. It was possible by this approach to prove and quantitatively evaluate the interaction of ostruthin with mitochondrial fragments which is of hydrophobic nature whereas the character of interaction of ostruthin with albumin was different[4].

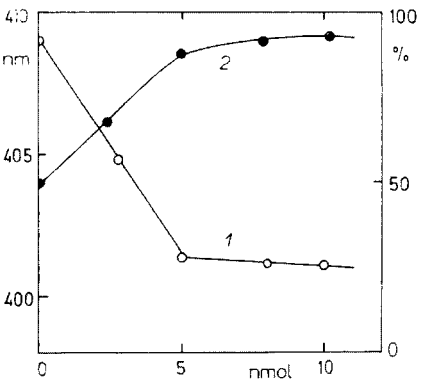

FIG. 5

Dependence of Intensity and Position of Emission of Ammoresinol Fluorescence on Added Albumin

Reaction medium (3 ml) contained: 0·1M phosphate buffer at pH 7·4, 10 nmol ammoresinol, and albumin as shown (nmol). *1* Position of emission maximum (nm); *2* intensity of fluorescence at maximum (%).

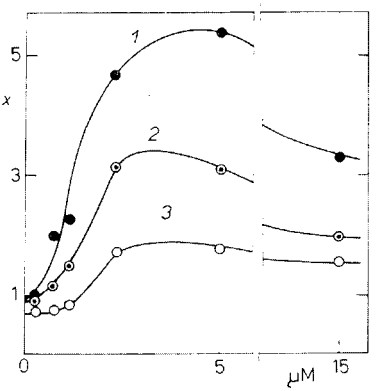

FIG. 6

Influence of Ammoresinol on Uncoupling Effect of Dicoumarol

Reaction medium and conditions of measurement the same as those given in legend to Fig. 1, ADP was omitted. Ammoresinol (nmol/mg protein of rat liver mitochondria): *1* 0; *2* 1·32; *3* 2·65. Concentration of dicoumarol in μM. *x*, Respiratory ratio.

We studied also the effect of serum albumin and sarcosomal fragments on the fluorescence spectrum of ammoresinol. In both cases an increase of fluorescence and a shift of the emission maximum toward shorter wavelengths take place; a new emission maximum, like that of ostruthin, was not observed. This provides evidence that the character of binding to albumin and sarcosomal fragments is similar. Unlike ostruthin, ammoresinol does not bind to the particulate enzyme preparation by a strictly hydrophobic bond which would give rise to a new emission maximum but by a bond similar to the ionic bond of aniline naphthalenesulfonate[15].

The dependence of the position of the emission maximum and intensity of ammoresinol fluorescence on the concentration of bovine serum albumin is demonstrated in Fig. 5. The parameters investigated vary up to a ratio of 5–6 nmol of albumin to 10 nmol of ammoresinol, any other increase of ammoresinol concentration is without effect. The results of fluorescence measurements carried out with bovine serum albumin show that one albumin molecule can bind two molecules of ammoresinol.

Effect of Uncouplers on Inhibitory Effect of Ammoresinol

The strong effect of ammoresinol on intact mitochondria compared to the low degree of inhibition of nonphosphorylating enzyme preparations suggests a possibility of its effect being weakened by compounds causing uncoupling of phosphorylating reaction from respiration (uncouplers). A number of inhibitors have been known to inhibit the respiration of intact mitochondria in the presence of inorganic phosphate and ADP yet they are practically without effect on enzymes of electron transfer not coupled to the synthesis of ATP. The best known representative of this group is oligomycin. Inhibitors of this type block reactions which convert the energy obtained in

TABLE III

Release of the Inhibitory Effect of Ammoresinol by Uncouplers in Rat Liver Mitochondria

Reaction medium and conditions of measurement as in Fig. 1. The concentration of ammoresinol causing 50% of inhibition in the absence of uncoupler is 0·8 nmol/mg protein for succinate oxidase and 3·2 nmol/mg protein for β-hydroxybutyrate oxidase.

Substrate	Uncoupler	Molarity	I_{50} Ammoresinol nmol/mg protein
Succinate	dicoumarol	$5 . 10^{-6}$	7·5
β-Hydroxybutyrate	dicoumarol	$5 . 10^{-6}$	27·5
Succinate	2,4-dinitrophenol	$1{\cdot}25 . 10^{-5}$	5·8
β-Hydroxybutyrate	2,4-dinitrophenol	$1{\cdot}25 . 10^{-5}$	27·0

the process of substrate oxidation to ATP and slow down also the reverse reaction, *i.e.* the hydrolysis of ATP by mitochondrial adenosine-5'-triphosphatase. Their action on the respiratory chain is only indirect. If the accumulated energy is removed in some manner, *e.g.* by the addition of an uncoupler, no inhibition of respiration appears.

As seen in Table III, dicoumarol and 2,4-dinitrophenol as typical uncouplers of oxidative phosphorylation considerably decrease the inhibitory effect of ammoresinol. However, ammoresinol cannot be classified as an inhibitor of the oligomycin type since it does not have an inhibitory effect on adenosine 5'-triphosphatase (not given) which is characteristic of this group. As shown in Fig. 6, the inhibition of mitochondrial succinate oxidase by ammoresinol results in a decrease of the uncoupling effect of dicoumarol. A similar influence on the effect of the uncoupler showed the inhibition of β-hydroxybutyrate oxidase. Ammoresinol affected the uncoupling effect of 2,4-dinitrophenol in the same manner. The maximum effect of both uncouplers remained in the same concentration range. In the presence of ammoresinol, maximum uncoupling of respiration could not be achieved; it is obviously partly inhibited by ammoresinol.

Ammoresinol at concentrations causing inhibition of the coupled electron transport slowed down only slightly the exchange reaction between ATP and inorganic phosphate. At these concentrations it was also without effect on translocase systems

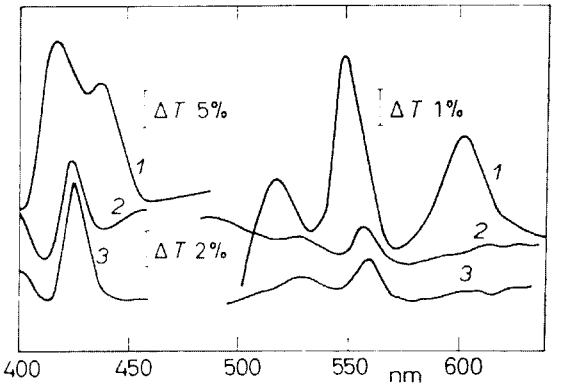

FIG. 7

Difference Spectra of Rat Liver Mitochondria with Succinate as Substrate

Reaction medium (1·5 ml) without succinate in the experimental and reference cell of 0·5 cm optical path contained 8·4 mg protein of rat liver mitochondria. Added to experimental cell: 1100 μmol succinate; *2* the same and 100 nmol of ammoresinol; *3* ammoresinol replaced with *1*t nmol of antimycin A. The spectrum (nm) was measured at room temperature as follows: after transition of reaction mixture to anaerobic state (5 min); *2* and *3* in the presence of oxygen in the experimental cell.

responsible for the transfer of inorganic phosphate and succinate anion through the mitochondrial membrane (not given). It may be thus stated that ammoresinol does not inhibit mitochondrial respiration by interfering with the translocase systems or by a reaction leading to ATP synthesis but that ammoresinol is a specific inhibitor of the electron transfer system.

Localization of the Site of Action of Ammoresinol in the Cytochrome System

The relations between structure and inhibitory effect of some inhibitors specifically interfering with the cytochrome system indicate that here too certain features of the molecule occur which are typical also of ammoresinol. First, this is the marked lipophilic character of one substituent bound to a relatively polar ring and second, the aromatic character of this ring with polar, mostly oxygen-containing substituents. It has been shown that it is especially the area of cytochrome b which is affected by the binding of these substances to such a degree that it no longer is capable of transferring electrons by which it is reduced.

We have been able to bypass the action of ammoresinol on mitochondrial oxidases by N,N,N′,N′-tetramethyl-*para*-phenylenediamine (TMPD) and ascorbate, *i.e.* by a donor system for the cytochrome c area. This means that the site of action of ammoresinol in the cytochrome chain precedes cytochrome c, *i.e.* is confined to the site of the complex of ubiquinone with cytochromes b. This view has been confirmed by measurement of the difference spectrum at the steady state of mitochondrial electron transfer. The addition of ammoresinol and substrate to one of the spectrophotometer cells leads to cytochrome b reduction, the other cytochromes remain oxidized (Fig. 7). Electron transfer from cytochrome b to cytochrome c is interrupted in the presence of ammoresinol, in analogy to the presence of antimycin A, a typical inhibitor of this region. The action of ammoresinol on the cytochrome system is entirely specific and is localized on the oxygen side of cytochrome b. Ammoresinol thus falls into the group of inhibitors of the antimycin type.

DISCUSSION

We have shown earlier that ammoresinol is a strong inhibitor of the bacterial respiratory chain, especially of succinate oxidase[2]. When the latter was inhibited by 70% no other oxidase of the membrane fraction of a *P. rettgeri* strain was inhibited to a detectable degree. If the concentration of ammoresinol is increased, first NADH oxidase and later formate oxidase become affected. The results obtained by the technique of rapid extraction and verified by direct photometric estimation of the redox state of the respiration components in metabolically active membranes showed that ubiquinone is completely oxidized even when succinate oxidase is inhibited

by 70%. This provides evidence that ammoresinol first interferes on the substrate side of ubiquinone, *i.e.* in the succinate dehydrogenase region. If the concentration is higher than 5 μmol/g protein, ammoresinol inhibits also NADH oxidase, the site of inhibition being on the oxygen side of ubiquinone which is reduced to a higher degree. We have shown that the other site of ammoresinol action lies in the cytochrome b region, in the so-called antimycin sensitive site. However, antimycin is less effective in bacterial respiratory chain. Several other inhibitors specifically bind to this area of the respiratory chain, the best known being the alkyl derivatives of 4-hydroxyquinoline-N-oxide and the alkyl derivatives of 3-hydroxy-1,4-naphthoquinone.

It is of interest that these chemically entirely different inhibitors retain a different polarity of the substituent and of the ring of the molecule. Hence, *e.g.* the derivatives of hydroxyquinoline-N-oxide show the strongest effect if the ring bears a nine or eleven-membered carbon atom chain which invests the molecule with lipophilic properties[16]. However, the removal of oxygen from the bond to nitrogen in a more polar ring also leads to a loss of activity. A similar dependence characterizes the alkyl derivatives of 3-hydroxy-1,4-naphthoquinone[17]. Similarly, the presence of a polar dilactone ring with a phenolic hydroxyl and other substituents, especially with an alkyl chain[18], is decisive for the activity of antimycins A_1 to A_3.

A characteristic feature of all the inhibitors described above is a firm bond to the site of their action. In spite of that, inhibition can be abolished for the most part by the addition of soluble proteins, *e.g.* of serum albumin[18]. The possibility of studying the interaction of ammoresinol with the insoluble enzyme preparation by fluorescence measurement has shown that ammoresinol does not bind to membrane fragments by a strictly hydrophobic bond as we have demonstrated for ostruthin[4]. Since the fluorescence of ammoresinol is far more lower, it was impossible to evaluate the binding in turbid suspensions of membrane fragments. Even so, however, it is possible to demonstrate a great similarity between the binding of ammoresinol to albumin and to the insoluble lipoprotein enzyme complex.

The explanation of a generally observed phenomenon namely, that the effect of inhibitors of the antimycin group can be weakened by substances causing a uncoupling of oxidative phosphorylation, is more difficult. This is also the case of ammoresinol. The decrease of inhibition as a result of uncoupling of oxidative phosphorylation seems to suggest that ammoresinol belongs to inhibitors of the oligomycin type. In contrast to oligomycin ammoresinol does not inhibit adenosine 5'-triphosphatase; this effect explains the release of oligomycin inhibition by uncouplers. Neither are certain other reactions involved in ATP synthesis inhibited by ammoresinol at the concentrations used. On the contrary, ammoresinol inhibits respiration even in the absence of ADP as acceptor of high-energy bonds (state 4 according to Chance and Williams) and strongly decreases the uncoupling ability of dicoumarol and 2,4-dinitrophenol; this indicates that it acts directly on the respiratory chain. At the same time, the maximum effect of both uncouplers is not

shifted toward higher or lower concentrations (Fig. 6); there is therefore no competition between ammoresinol and uncouplers for the binding capacity of the membrane.

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