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THE INTERACTION OF 2-PHENYLISATOGEN AND MENADIONE WITH RAT LIVER MITOCHONDRIAL NADH DEHYDROGENASE

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Abstract—The effects of 2-phenylisatogen and menadione on some mitochondrial reactions have been studied. The respiration with NAD^+-linked substrates was stimulated, but respiration with succinate was not affected. The stimulation of respiration was inhibited by *p*-hydroxymercuribenzoate, but not by other respiratory chain inhibitors. Potassium cyanide and sodium sulphide stimulated NADH oxidation in the presence of 2-phenylisatogen or menadione. 2-Phenylisatogen was found to be converted to 2-phenylindolone. It is concluded that both 2-phenylisatogen and manadione are acting as electron acceptors in the NADH dehydrogenase region of the respiratory chain. Possible mechanisms for the reaction and for the stimulation observed in the presence of cyanide are discussed.

THE HETEROCYCLIC molecule 2-phenylisatogen, a compound that possesses antimycoplasma and antibacterial activity,[1,2] has been shown to inhibit ADP-stimulated respiration and the 2,4-dinitrophenol-stimulated adenosinetriphosphatase activity of rat liver mitochondria at low concentrations.[3] In a preliminary communication we reported the finding that high concentrations of 2-phenylisatogen caused a stimulation of NADH oxidation in rat liver mitochondria.[4] We now wish to describe further experiments that we have carried out in order to investigate this phenomenon in greater detail.

MATERIALS AND METHODS

Enzyme preparations. Tightly-coupled rat liver mitochondria were prepared by the method of Chappell and Hansford[5] and suspended in 0·25 M sucrose and 3·4 mM Tris–HCl, pH 7·4, to give a protein concentration of 50 mg/ml. In the extraction and fluorescence experiments the mitochondria were suspended in 0·125 M potassium chloride. A post-mitochondrial fraction was prepared from rat liver homogenates by centrifuging the homogenate at 60,000 *g* for 10 min in the 8 × 25 ml rotor of an MSE Superspeed 40 centrifuge. The resulting supernatant was collected and will be referred to as the post-mitochondrial fraction.

Lyophilization and extraction of mitochondria. Mitochondria were lyophilized using an Edwards high vacuum pump fitted with a liquid air trap. After lyophilization, the mitochondria were extracted with 2 ml aliquots of chloroform and the combined chloroform extracts were concentrated over a boiling water bath, spotted onto glass plates coated with a layer of Kielselgel PF_{254} (0·25 mm thick) and eluted with a 50:50 mixture of ethylacetate and petroleum ether (b.p. fraction 60°–80°).

Enzyme activities. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge). NAD(P)H dehydrogenase was measured at 340 nm using a Pye-Unicam spectrophotometer. 2-Phenylindolone formation was measured with an Aminco-Bowman spectrofluorimeter; excitation wavelength 402 nm and a fluorescence maximum of 526 nm.

Protein. Protein was determined by the method of Gornall, Bardawill and David[6] after solubilization of the mitochondria with deoxycholate (0·16% w/v); bovine serum albumin was used as standard.

Chemicals. Rotenone was supplied by the Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Analytical grade laboratory chemicals and biochemicals were purchased from British Drug Houses Ltd., Poole, Dorset and Sigma Chemical Co., St. Louis, Mo., U.S.A. 2-Phenylisatogen was synthesized by the method of Bond and Hooper[7] and 2-phenylindolone by the method of Kalb and Bayer[8] as modified by Ch'ng.[9] 2-Phenylisatogen was added to the reaction media as a solution in absolute ethanol; control experiments carried out with equivalent amounts of ethanol showed that the solvent had no effect on the reactions under consideration.

RESULTS

Effect of 2-phenylisatogen on mitochondrial respiration. Figure 1a shows that NADH was not oxidized by the rat liver mitochondria, but when 2-phenylisatogen was added there was a marked stimulation of respiration which was not obtained in the absence of NADH (Fig. 1c). When the NADH was replaced by succinate, 2-phenylisatogen did not stimulate respiration (Fig. 1b). A possible explanation for these observations is that 2-phenylisatogen can interact with the mitochondrial NADH dehydrogenase to promote the interaction of NADH with the enzyme. To check that NADH was being oxidized under the conditions employed, NADH dehydrogenase activity was measured spectrophotometrically; a decrease in optical

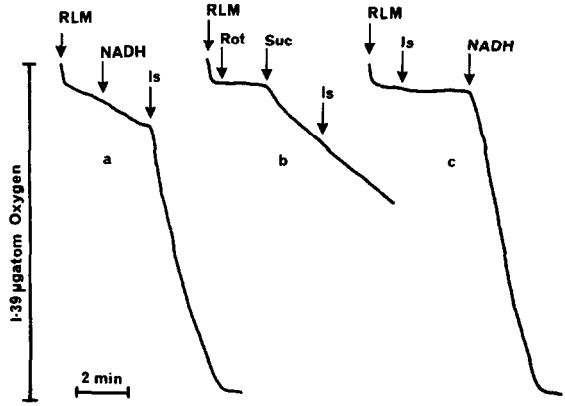

FIG. 1. Effect of 2-phenylisatogen on NADH and succinate oxidation in rat liver mitochondria. The reaction medium contained 675 μmoles of sucrose and 8·5 μmoles Tris–HCl, pH 7·4. Additions were: RLM, 5 mg liver mitochondrial protein; NADH, 2·6 μmoles NADH; Is, 1 μmole 2-phenylisatogen; Suc, 10 μmoles sodium succinate; Rot, 1 nmole rotenone. The final volume was 3 ml and the temperature 30°. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode. (a) 2-Phenylisatogen on NADH oxidation; (b) 2-phenylisatogen on succinate oxidation; (c) 2- phenylisatogen on NADH oxidation (2-phenylisatogen added before NADH).

density at 340 nm was used as an indication of the conversion of NADH to the oxidized coenzyme NAD^+. In agreement with the polarographic findings NADH was not oxidized by the mitochondria, but when 2-phenylisatogen was added to the mitochondrial suspension in the presence of NADH there was a rapid decrease in optical density at 340 nm. The results show that when mitochondria were exposed to NADH and 2-phenylisatogen there was an increase in oxygen consumption and a concomitant stimulation of NADH oxidation. NADPH was not oxidized by the mitochondria, either in the absence or in the presence of 2-phenylisatogen. This rules out the possibility of a direct chemical reaction between the isatogen and the adenine nucleotides. This conclusion is supported by the finding that in the absence of the mitochondrial preparation, 2-phenylisatogen did not promote the oxidation of either NADH or NADPH.

Effect of inhibitors. Two main groups of compounds are known to stimulate mitochondrial respiration. These are uncoupling agents, e.g. 2,4-dinitrophenol,[10] and electron acceptors, e.g. ferricyanide[11] and menadione.[12] Uncoupler-stimulated respiration typically shows no substrate specificity and is sensitive to respiratory chain inhibitors, whereas electron acceptors may exhibit substrate specificity and a variable pattern of sensitivity towards respiratory chain inhibitors. The effects of 2-phenylisatogen were specific for NADH and Table 1 shows that isatogen-stimulated NADH oxidation was not inhibited by rotenone, antimycin A, sodium sulphide or potassium cyanide at concentrations that completely inhibited the oxidation of NAD-linked substrates. The only compound found to inhibit the reaction was *p*-hydroxymercuribenzoate, a reagent that has been shown to interact with the mitochondrial NADH dehydrogenase on the substrate side of the rotenone-sensitive site.[13] The substrate specificity and pattern of inhibitor action were similar to those of the electron acceptor, menadione (see Table 1) and do not suggest an uncoupling action for the compound, a conclusion that is supported by the finding that there was no stimulation of the mitochondrial adenosinetriphosphatase reaction by the isatogen.[3]

In the presence of potassium cyanide or sodium sulphide, isatogen-stimulated NADH oxidation was further stimulated (Table 1). The degree of stimulation of respiration, either in the presence or in the absence of cyanide was dependant on the concentration of 2-phenylisatogen present (Fig. 2). The K_m (83 μM) was unchanged

TABLE 1. THE EFFECT OF RESPIRATORY CHAIN INHIBITORS ON 2-PHENYLISATOGEN-STIMULATED NADH OXIDATION IN RAT LIVER MITOCHONDRIA

	Oxygen consumption (ng atom oxygen/min/mg mitochondrial protein)	
Inhibitor	2-Phenylisatogen	Menadione
None	49	275
Rotenone (10 nmole)	50	270
Antimycin A (20 μg)	52	275
Potassium cyanide (10 μmole)	130	400
Sodium sulphide (10 μmole)	125	490
p-Hydroxymercuribenzoate (300nmole)	12	36

The experimental conditions were the same as those described in the legend to Fig. 1. The mitochondria (5 mg protein) were added to the reaction medium contained in the oxygen electrode chamber, followed immediately by the inhibitors (in the amounts shown), 2 min prior to the addition of 2-phenylisatogen (final concentration 166 μM) or menadione (final concentration 330 μM).

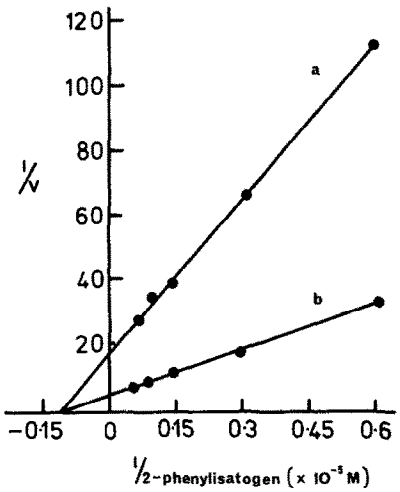

FIG. 2. Effect of potassium cyanide on 2-phenylisatogen-stimulated NADH oxidation in rat liver mitochondria. The reaction was measured as described in the legend to Fig. 1. (a) NADH plus 2-phenylisatogen; (b) NADH plus 2-phenylisatogen in the presence of potassium cyanide (final concentration 3·3 mM). Initial velocity, v, is expressed as μg atom oxygen/min/mg mitochondrial protein.

but the V_{max} was some three times greater in the presence of cyanide (50 ng atom/min/mg protein in the absence of cyanide and 167 ng atom/min/mg protein in the presence of cyanide). Similar results were obtained when the 2-phenylisatogen was replaced with menadione, and when the potassium cyanide was replaced with sodium sulphide. When succinate was used as substrate there was no stimulation of respiration by 2-phenylisatogen in the presence of potassium cyanide.

Post-mitochondrial fraction. It was thought to be a possibility that the observed enzyme activities were due to contamination of the mitochondria. To test this hypothesis the properties of the post-mitochondrial fraction were compared to those of the mitochondrial fraction (see Table 2). Three observations lead to the conclusion that the mitochondrial enzyme is not contaminated with enzymes from the post-mitochondrial fraction: (i) The post-mitochondrial fraction gave rates that were some three times greater than those of the mitochondrial enzyme; (ii) the mitochondrial

TABLE 2. COMPARISON OF THE EFFECTS OF 2-PHENYLISATOGEN ON NAD(P)H OXIDATIONS CATALYSED BY THE POST-MITOCHONDRIAL AND MITOCHONDRIAL FRACTIONS OF RAT LIVER HOMOGENATES

	Reaction rate (ng atom oxygen/min/mg protein)		
Enzyme preparation	Control	2-Phenylisatogen	2-Phenylisatogen + dicoumarol
Post-mitochondrial fraction:			
NADH	0	147	26
NADPH	0	242	12·5
Mitochondrial fraction:			
NADH	0	49	49
NADPH	0	0	0

Experimental conditions were as described in the legend to Fig. 1. Dicoumarol (4 nmole) was added 2 min prior to the addition of 2-phenylisatogen (final concentration 166 μM).

enzyme did not oxidize NADPH under any of the conditions tested, whereas the post-mitochondrial fraction oxidized NADPH at a rapid rate in the presence of 2-phenylisatogen; (iii) NADH oxidation catalysed by the mitochondrial fraction was not affected by dicoumarol, but this compound was a strong inhibitor of the reactions catalysed by the post-mitochondrial fraction. Similar results were obtained when 2-phenylisatogen was replaced with menadione.

TABLE 3. THE EFFECT OF RESPIRATORY CHAIN INHIBITORS ON 2-PHENYLINDOLONE FORMATION

Additions	μmoles of 2-Phenylindolone formed (after 10 min incubation)
None	0·43
Rotenone (50 nmole)	0·45
Antimycin A (100 μg)	0·39
Oxygen	0·16
Potassium cyanide (10 μmole)	0·88
Potassium cyanide (10 μmole) plus *p*-Hydroxymercuribenzoate (300 nmole)	0·54

The incubation medium contained 350 μmoles potassium chloride, 2·6 μmoles NADH, 7 mg mitochondrial protein, 1 μmole 2-phenylisatogen and inhibitors at the concentrations shown. The final volume was 3 ml and the temperature was 25°. The inhibitors were added 1 min before the isatogen and 100% oxygen was administered by gently bubbling the gas through the cuvette contents between fluorescence readings.

Identification of the products of the reaction. Samples of mitochondria were incubated with and without NADH, in the presence of 2-phenylisatogen and potassium cyanide. The mitochondria were then lyophilized and extracted as described in the Methods section; the resulting chloroform extracts were applied to thin layer chromatograms. The extract from the mitochondria incubated in the absence of NADH gave a single spot which corresponded to a 2-phenylisatogen reference spot, indicating that no change had taken place. It was not possible to detect any 2-phenylisatogen in the extract from the mitochondria incubated with NADH. The extract did, however, show a fluorescent spot which was found to correspond to a 2-phenylindolone reference. Determination of the ultraviolet and fluorescence spectra confirmed that the product of the reaction was 2-phenylindolone. The latter compound did not stimulate NADH, NADPH or succinate oxidation in the mitochondrial preparation.

2-Phenylindolone formation. The formation of the fluorescent molecule, 2-phenylindolone, from the non-fluorescent compound, 2-phenylisatogen, presented a direct method for studying indolone formation by measuring fluorescence changes. Table 3 shows that the respiratory chain inhibitors, rotenone and antimycin A, had no effect on 2-phenylindolone formation. When the reaction medium was prevented from becoming anaerobic, by bubbling oxygen through the solution, the extent of the reaction was markedly decreased. The presence of potassium cyanide stimulated the reaction, and this stimulation was inhibited when *p*-hydroxymercuribenzoate was included in the medium. 2-Phenylindolone formation therefore shows the same pattern of inhibitor sensitivities as does the stimulation of NADH oxidation by 2-phenylisatogen.

DISCUSSION

The simplest explanation for the findings outlined in this paper is that 2-phenylisatogen is capable of acting as an electron acceptor for the NADH dehydrogenase segment of the rat liver mitochondrial electron transport chain. The main pieces of evidence supporting this suggestion are that NADH oxidation, but not succinate oxidation, is stimulated by 2-phenylisatogen, and that the process is unaffected by either antimycin A or rotenone, but is inhibited by *p*-hydroxymercuribenzoate. This means that 2-phenylisatogen does not interact with the succinic dehydrogenase or cytochrome segments of the respiratory chain. The reaction of the compound with the respiratory chain must be on the NADH dehydrogenase system, either at the *p*-hydroxymercuribenzoate inhibition site, or between this site and the rotenone inhibition site as depicted in Fig. 3.

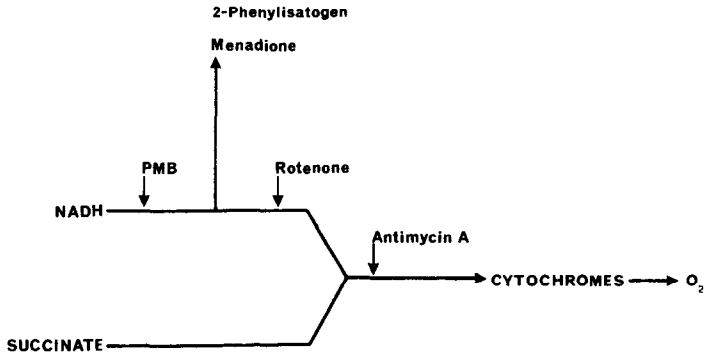

FIG. 3. Site of interaction of 2-phenylisatogen and menadione with the respiratory chain in rat liver mitochondria. PMB, *p*-hydroxymercuribenzoate.

Menadione, a compound that possesses a very similar redox potential to 2-phenylisatogen at pH 7·4,[14] has been shown to interact with NADH dehydrogenase;[12] a comparison was therefore made of the effects of the two compounds on mitochondrial reactions. When 2-phenylisatogen was replaced with menadione, a similar pattern of substrate and inhibitor specificity was observed. It is therefore concluded that 2-phenylisatogen and menadione react with the mitochondrial NADH dehydrogenase system at the same site.

The reduction of 2-phenylisatogen by NADH resulted in the formation of 2-phenylindolone and the oxidized coenzyme, NAD^+. 2-Phenylindolone, however, was not detected in the reaction medium until the reaction medium became anaerobic; bubbling oxygen through the solution also prevented 2-phenylindolone formation. In other words, although NADH was oxidized by 2-phenylisatogen the final reduction product, 2-phenylindolone, was not formed in the presence of oxygen. Under aerobic conditions more than one molecule of NADH was oxidized per molecule of 2-phenylisatogen added. It therefore seems likely that 2-phenylisatogen is reduced to an intermediate compound, which is readily oxidized back to 2-phenylisatogen in the presence of oxygen. When the medium becomes anaerobic the intermediate is converted to 2-phenylindolone in an irreversible reaction (once 2-phenylindolone had accumulated the addition of oxygen did not lower its concentration). A possible candidate for the intermediate compound, which could not be detected in chloroform

extracts of the mitochondrial suspensions, is the dihydroxy compound, 1,3-dihydroxy-2-phenylindole. This compound has been suggested as an intermediate in the formation of 2-phenylindolone from 2-phenylisatogen in the presence of a variety of reducing agents.[15] Figure 4 shows the reaction scheme proposed for the reduction of 2-phenylisatogen by the mitochondrial NADH dehydrogenase.

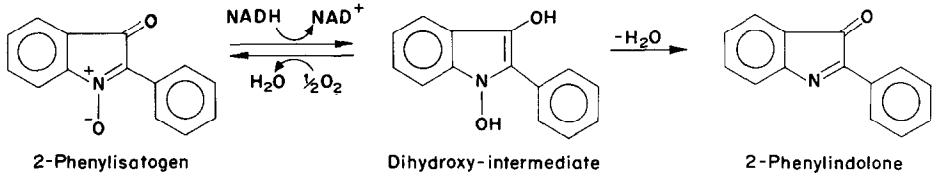

FIG. 4. Reaction scheme for the interaction of 2-phenylisatogen with rat liver mitochondrial NADH dehydrogenase.

An interesting feature of the reaction was the stimulation observed in the presence of potassium cyanide. It may be profitable in seeking an explanation for this phenomenon to consider the nature of the NADH dehydrogenase enzyme system, particularly of the region where interaction with 2-phenylisatogen occurs. Cremona and Kearney[16] and Tyler *et al.*[17] have demonstrated the presence of sulphydryl groups in the mitochondrial NADH dehydrogenase. The reduction of both 2-phenylisatogen and menadione was inhibited by the mercurial, *p*-hydroxymercuribenzoate, indicating that a sulphydryl group is involved in the reaction under consideration. Furthermore it has been suggested that menadione reacts with a sulphydryl group on the enzyme.[12] 2-Phenylisatogen could also be reacting with a sulphydryl group, since it has been shown to react under mild conditions with compounds such as glutathione.[1] Some of the sulphydryl groups on the NADH dehydrogenase could be in an inactive form, possibly as a mixed disulphide formed between a sulphydryl group on the enzyme and cysteine. Under these conditions, activation of the enzyme can be explained by nucleophilic attack by the cyanide, resulting in cleavage of the disulphide bond. A similar mechanism has been proposed for the activation by potassium cyanide of papain, a proteolytic enzyme containing a sulphydryl group at the active site.[18]

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