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INHIBITORY ACTION OF RHEIN ON THE REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE-DEHYDROGENASE COMPLEX OF MITOCHONDRIAL PARTICLES AND ON OTHER DEHYDROGENASES

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Abstract—The compound, 4,5-dihydroxyanthraquinone-2-carboxylic acid (rhein) was found to be an inhibitor of NADH oxidation by the respiratory chain of beef-heart mitochondrial particles. Inhibition of succinate oxidation was minimal. Experiments on inhibition of NADH-ferricyanide reductase activity revealed that rhein specifically interferes with the redox function of the NADH-dehydrogenase complex, and indicated that the inhibition affects the binding of NADH to the enzyme. The related compounds, rhein anthrone and sennoside A, were less potent inhibitors than rhein. Lactic and alcohol dehydrogenases were also found to be susceptible to inhibition by rhein.

IT WAS reported elsewhere[1] that the compound, 4, 5-dihydroxyanthraquinone-2-carboxylic acid (rhein,* rheic acid, cassic acid, rhubarb yellow), acts as an inhibitor of mitochondrial oxidations. Prior to that report, cathartic properties[2,3] and weak antibacterial action[4] were the only notable biological activities associated with rhein.

Aspects of the inhibition described were: (1) a marked degree of specificity for NADH-linked oxidations; (2) lack of effect on coupled phosphorylations; (3) involvement of Complex I in the effect. Further, certain features (resistance to washing, reversal by NAD^+) differentiated inhibition by rhein from that by amytal and rotenone.

Further definition of the above findings was attempted in the investigation described here. Evidence is presented that, within the respiratory chain of submitochondrial particles, the action of rhein is on the NADH-dehydrogenase complex, resulting in decreased ferricyanide-reductase activity. This effect of rhein was compared with that of other inhibitors such as rotenone, Antimycin A and cyanide, and aspects of the inhibition kinetics were investigated.

Enzymes other than respiratory chain components were also found to be susceptible to inhibition by rhein by what appears to be a less specific mechanism. Two compounds related to rhein were also tested for inhibitory effects on electron transport.

MATERIALS AND METHODS

Compounds tested for inhibitory effects. Rhein, rhein anthrone and sennoside A were obtained from Sandoz Ltd., Basle. The structures of rhein and of sennoside A are

* The name rhein will be used.

given in the *Merck Index*. Rhein anthrone is 4,5-dihydroxy-9-oxo-10-dihydroanthracene-2-carboxylic acid (a partially reduced form of rhein); sennoside A is the related dianthrone diglucoside in which glucose is linked to the hydroxy groups on the 5- and 5'-positions of the anthrone nucleus. Some samples of rhein were prepared in our laboratories by a procedure adapted from Lemli.[5] To establish authenticity and purity, reliance was placed on published data[6] for R_f values from paper chromatography, and melting points. Rotenone (British Drug Houses, about 90 per cent pure) was recrystallized to a constant melting point of 163° from an acetone–water (10:1, v/v) mixture. Antimycin A was obtained from Nutritional Biochemicals.

Rhein was added to reaction mixtures as a solution in the buffer appropriate to the particular assay; rotenone and Antimycin A were prepared as solutions in ethanol, which were then diluted in the appropriate buffer. Ethanol thus added to reaction mixtures was insufficient to affect enzyme activities.

Preparation of mitochondrial particles from beef heart. The method of Crane *et al.*[7] as modified by Pharo *et al.*[8] was used for isolation of mixed "light" and "heavy" mitochondria; these were not further separated. Submitochondrial particles were prepared by sonication[8] and were stored as frozen suspensions in 0·25 M sucrose at −20°.

Assay procedures. Spectrophotometric assays of enzyme activity were performed with a direct-reading instrument (Spectrochem, Hilger and Watts Ltd.), designed to allow variable sensitivity over four ranges so that even high values of absorbancy could be read with a precision of approximately ±0·003. Each experiment was repeated two or more times to establish consistency; thus the data presented are the means of at least three sets of concordant results.

NADH- and succinate-cytochrome c reductase activity were determined according to Green and Ziegler.[9] The cytochrome c used (Sigma Chemical Co.) was first converted to the wholly oxidized state by treatment with a slight excess of $K_3Fe(CN)_6$, which was subsequently removed by exhaustive dialysis. In these assays, the addition of KCN to media was delayed until just prior to adding enzyme, in order to minimize formation of cytochrome c–cyanide complex.

NADH- and succinate-2,6-dichlorophenolindophenol (DCPIP) reductase were determined by methods based on those described by Mahler *et al.*[10] and Takemori and King[11] respectively.

The method of Minikami *et al.*[12] was used with minor modifications to determine NADH-ferricyanide reductase. The buffer was Tris-sulfate at 0·05 M final concentration instead of KH_2PO_4[12] or triethanolamine.[13] Also, a fixed concentration of oxidant (1·5 mM ferricyanide) was used in some assays rather than the varying concentrations required for the determination of V_{max} (ferricyanide).

Lactic dehydrogenase (crystallized, from rabbit muscle; Mann Biochemicals) was assayed as described by Kornberg.[14]

Alcohol dehydrogenase (lyophilized, from yeast; Sigma Chemical Co.) was assayed by a fluorimetric method adapted from the procedure described by Vallee and Hoch.[15] Initial reaction rates were derived from a continuous recording of fluorescence measured by a Turner fluorimeter (excitation wavelength, 360 mμ; emission at 460 mμ) as it increased with progressive reduction of NAD^+ in the medium. Fluorescence readings were converted to nanomoles of NADH by reference to calibration curves prepared by adding known amounts of NADH to the appropriate reaction media.

Absorption spectra. These were performed with a Zeiss PMQ 11 instrument. Rhein was dissolved at a concentration of 10 μM in 0·05 M Tris-sulfate buffer, pH 7·4. To observe whether reduction with sodium dithionite or sodium borohydride occurred, a few crystals of either reagent agent were added and spectra were determined again after about 5 min.

Protein determination was done by the biuret method[16] with bovine serum albumin as a standard.

RESULTS

NADH- and succinate-cytochrome c reductase. The effects of rhein on these two systems are shown in Fig. 1. Specific activities were found to be much lower than those usually reported for similar preparations; nevertheless, conclusions based on comparisons with relevant controls are very likely to be valid.

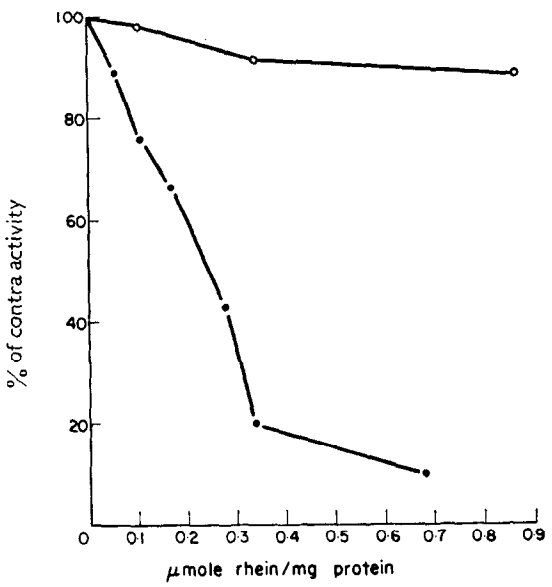

FIG. 1. Comparison between NADH- and succinate-cytochrome c reductase activity of mitochondrial particles as regards inhibition by rhein. Closed circles, NADH substrate; open circles, succinate substrate. Particles (0·22–0·44 mg protein) diluted in 1% bovine serum albumin were added at zero time and reduction of cytochrome c was monitored at 550 mμ for 15–45 sec. From the linear initial rate, calculations were based on the relationship: millimoles of NADH or succinate oxidized = $A_{550} \times 0{\cdot}0521$. Control values were 33·0 mμmoles NADH and 65·8 mμmoles succinate oxidized/min/mg protein.

In agreement with earlier findings, rhein was an effective inhibitor of the oxidation of NADH by the respiratory chain; in contrast, succinate oxidation was highly resistant. To illustrate, it can be seen from Fig. 1 that 0·34 μmole rhein/mg protein produced 80 and 8·7 per cent inhibition of NADH- and succinate-cytochrome c reductase activity respectively; nearly 2·5 times this amount of inhibitor (0·87 μmole/mg protein) produced only 11·5 per cent inhibition of the latter system. Maximum

inhibition of NADH-cytochrome c reductase was 90 per cent; this required 0·68 μmole rhein/mg of protein.

In Table 1 the effects of rhein, rhein anthrone and sennoside A on NADH-cytochrome c reductase are compared at equal concentrations. By this criterion, the anthrone proved to be less effective than rhein, while sennoside A was only slightly inhibitory.

DCPIP reductase. Rhein strongly inhibited the enzymic reduction of DCPIP by NADH, but hardly affected the reaction when succinate was the substrate (Table 2).

TABLE 1. EFFECT OF RHEIN AND RELATED COMPOUNDS ON NADH-CYTOCHROME C REDUCTASE ACTIVITY OF MITOCHONDRIAL PARTICLES*

Additions basic assay medium	Level of addition		Activity (% of control value)
	(μM)	(μmoles/mg protein)	
Rhein	50	0·28	42·7
Rhein anthrone	50	0·28	64·5
Sennoside A	50	0·28	89·0

* Reaction started with addition of particles (0·44 mg protein) to reaction mixtures. The control value was 33·0 mμmoles NADH oxidized/min/mg particle protein.

TABLE 2. EFFECT OF RHEIN ON NADH- AND SUCCINATE-DCPIP REDUCTASE ACTIVITY OF MITOCHONDRIAL PARTICLES*

Enzyme activity measured	Rhein added		Activity (% of control value)
	(μM)	(μmoles/mg protein)	
NADH-DCPIP reductase	10	0·11	85·6
	30	0·34	74·3
	60	0·68	55·6
Succinate-DCPIP reductase	10	0·14	104
	30	0·41	98·0
	60	0·82	88·3

* Reaction was started by addition of particles (0·22 mg protein) to reaction mixtures. Results were calculated from ΔA over the first 15–30 sec of reaction, assuming an absorbancy index of 21 mM^{-1} cm^{-1} for DCPIP at 600 mμ. Control values were 51·8 and 60·3 mμmoles NADH and succinate, respectively, oxidized/min/mg particle protein.

NADH-ferricyanide reductase. The data of Table 3 show the effects of rhein, Antimycin A, rotenone and cyanide on this system. With the very small amounts of enzyme used in these and subsequent experiments, inhibitor: protein ratios are considered to be of much less significance than in the case of NADH-cytochrome c and NADH-DCPIP reductase assays described above; instead the inhibitor concentration *per se*, as shown in the table, was considered a sufficiently meaningful parameter. Each compound was tested at or above levels known to be inhibitory to electron transport.

It is seen that marked inhibition was produced by rhein. Antimycin A inhibited only slightly. Cyanide and rotenone produced an apparent stimulation, although the

TABLE 3. EFFECTS OF RHEIN AND OTHER RESPIRATORY-CHAIN INHIBITORS ON NADH-FERRICYANIDE REDUCTASE ACTIVITY OF MITOCHONDRIAL PARTICLES*

Expt. no.	Inhibitor added	Inhibitor concn	Activity (μmoles NADH oxidized/min/mg protein)
1	None		4·73
	Rhein	10 μM	3·92
	Rhein	30 μM	2·88
	KCN	1·0 mM	5·36
2	None		4·30
	Rhein	30 μM	2·80
	Antimycin A	0·1 μg/ml	4·19
	Rotenone	0·33 μM	4·42

* Reaction media contained 150 μmoles Tris-sulfate (pH 7·8), 4·5 μmoles $K_3Fe(CN)_6$, and the additions indicated, in a total volume of 3·0 ml in a 1-cm cuvette. Temperature was 25°. After addition of 0·45 μmole NADH, nonenzymic reduction of ferricyanide was monitored at 420 mμ for 30 sec. Particles (26 μg protein) were added and the enzymic reaction rate was calculated for the subsequent 30–45 sec from the relationship: millimoles of NADH oxidized = $\frac{1}{3}A_{420\ m\mu}$.

TABLE 4. EFFECT OF RHEIN ON NADH-FERRICYANIDE REDUCTASE ACTIVITY OF MITOCHONDRIAL PARTICLES WITH AND WITHOUT PRE-TREATMENT WITH ROTENONE*

Inhibitor in contact with ETP prior assay	Inhibitor in assay medium	Concn of inhibitor in assay medium (μM)	Activity (μmoles NADH oxidized/min/mg protein)
None	None		2·54
None	Rhein	50	0·93
Rotenone	Rotenone	1·67	2·87
Rotenone	Rotenone + rhein	1·67 50	1·06

* Two separate samples of particles (0·28 mg protein each) were added to 10 ml of 0·075 M Tris-sulfate (pH 7·8) at 25°; rotenone, at 5·0 μM concentration, was included with one sample. After 1 min, aliquots were removed from each mixture for assay in the absence or presence of 50 μM rhein. The same conditions as for Table 3 applied, except that no estimates of nonenzymic rate were obtained.

effect of the latter was small. Similar results with Antimycin A and cyanide have been noted and discussed in detail by other workers.[12] The finding with rotenone was unexpected, but was confirmed by similar observations in the experiments next described.

In order to test whether rotenone and rhein shared a common reaction or binding site in the respiratory chain, particles were preincubated for 2–3 min with rotenone and then added to media designed for estimation of NADH-ferricyanide reductase, either with or without added rhein. It was considered that inhibition by rhein should be abolished or reduced if rotenone pre-empted target sites on the enzyme. It was found (Table 4) that inhibition by rhein was not measurably affected by preincubation of particles with rotenone.

Assays of NADH-ferricyanide reductase were performed at varying concentrations of ferricyanide, with NADH concentration fixed. From the results, plotted by the Lineweaver–Burk method in Fig. 2, the inhibition appeared to be noncompetitive with respect to the oxidant. V_{max} (ferricyanide) values were decreased by approximately 25 and 75 per cent, respectively, by 10 and 30 μM rhein.

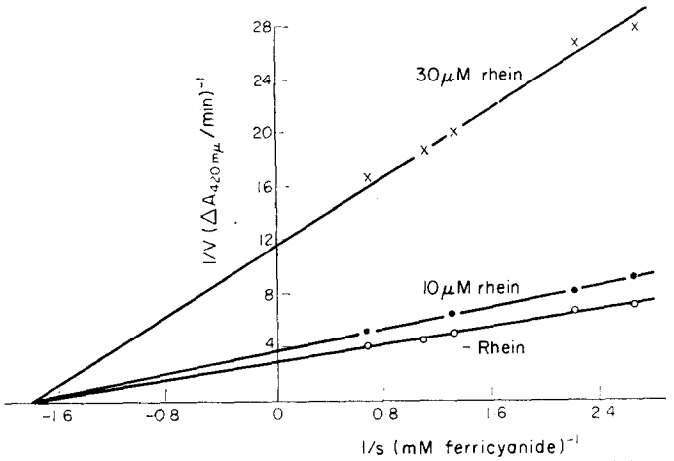

FIG. 2. Reciprocal plot showing inhibition of NADH-ferricyanide reductase activity of mitochondrial particles by rhein, with ferricyanide as the variable substrate. Activity was determined as for Table 3.

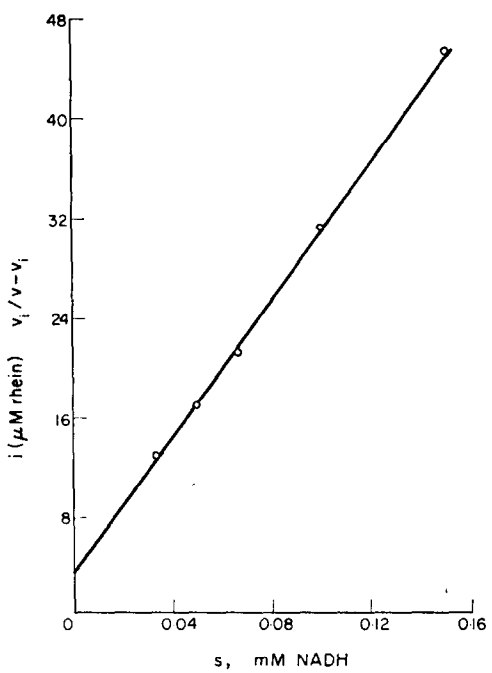

FIG. 3. Graphical indication of competitive inhibition by rhein of NADH-ferricyanide reductase activity in mitochondrial particles; NADH as variable substrate, method of Hunter and Downs[18] (see Results). The velocity in the absence of inhibitor is v, and v_i is the corresponding velocity in the presence of inhibitor. When present, inhibitor was either 10, 20 or 30μM concentration. Other conditions were as for Table 3.

In order to evaluate the interaction of NADH and rhein on the ferricyanide reductase system, data from several experiments were combined, in all of which ferricyanide concentration was fixed at 1·5 mM while various levels of NADH (ranging from 0·033 to 0·15 mM) and of inhibitor (ranging from 0 to 30 μM) were used. By applying a modification[17] of the method of Hunter and Downs,[8] values of $vi/v - vi$ were calculated and plotted against S for the various concentrations of inhibitor and substrate, where v_i and v are velocities (Δ A/min) in the presence and absence of inhibitor, respectively, at the same substrate concentration. It is seen (Fig. 3) that, as expected for

TABLE 5. EFFECT OF RHEIN ON YEAST ALCOHOL DEHYDROGENASE AND RABBIT MUSCLE LACTIC DEHYDROGENASE

Enzyme assayed	Concn of rhein added (μM)	Enzyme activity (μmoles NAD^+ reduced/min/mg enzyme protein)
Alcohol dehydrogenase*	0	121·3
(0·5 μg/ml)	15	122·0
	75	81·0
Lactic dehydrogenase†	0	57·8
(0·83 μg/ml)	15	51·0
	30	48·0
	60	34·2
	75	30·8

* Fluorimetric assay based on reference 15.
† Spectrophotometric assay based on reference 14.

competitive inhibition, a straight line of positive slope is obtained; noncompetitive inhibition would produce a line parallel to the abscissa. This result suggests that rhein interferes in some way with access of NADH to the enzyme. Both fully and partially competitive effects would yield qualitatively similar plots; hence, the interaction of rhein with the enzyme could be elsewhere than at substrate-binding sites.

It seemed of interest to determine whether enzymes functioning outside the respiratory chain, and especially NAD-linked dehydrogenases, could be affected by rhein. The data of Table 5 show that significant inhibition of rabbit muscle lactic dehydrogenase occurs even at relatively low concentrations of rhein, reaching nearly 47 per cent at 75 μM rhein. In the case of alcohol dehydrogenase, no inhibition occurred at 15 μM rhein, while 75 μM rhein produced an inhibition of 30 per cent. At this level of inhibitor, the effect was noncompetitive with respect to NAD^+ (Fig. 4).

Experiments were done to investigate the potential reactivity of rhein as an intermediate or terminal oxidant for NADH incubated with particles, since such an action might contribute to inhibitory effects on the normal functional sequence of the respiratory chain. In this connection, spectrophotometric evidence had been obtained (disappearance of absorption peaks at 230, 260 and 410 mμ) for the reduction of rhein by borohydride, but not by dithionite in aqueous media at pH 7·4. NADH (13·3 μM) was included in media containing rhein (60 μM); control cuvettes contained no rhein. Particles (0·05 mg protein) were added and oxidation of NADH was monitored at

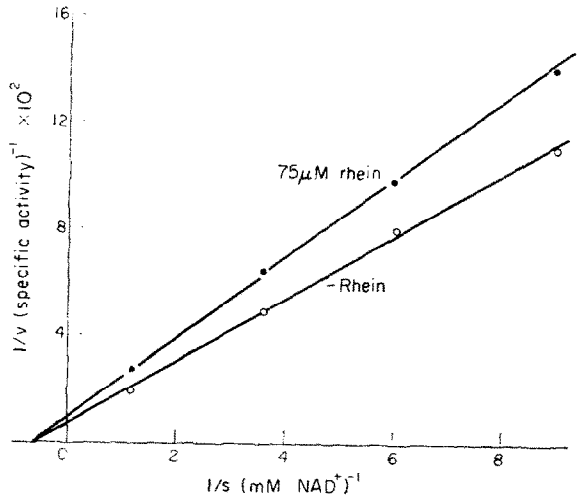

FIG. 4. Reciprocal plot of rhein inhibition of yeast alcohol dehydrogenase with NAD^+ as variable substrate. Fluorimetric measurement of velocity of NAD^+ reduction was performed on 4·0 ml of a mixture containing: sodium pyrophosphate (pH 8·8), 120 μmoles; ethanol, 1·33 m-moles; 2 μg enzyme; and the indicated amounts of NAD^+ and rhein. Temperature was 27°. Excitation wavelength, 360 mμ; emission measured at 460 mμ. Specific activity is μmoles NAD^+ reduced/min/mg of enzyme.

366 mμ. None was evident. A similar result was obtained when either cyanide, rotenone or Antimycin A was added to the medium along with rhein. It is therefore concluded that rhein did not serve as a terminal or mediator electron acceptor in these systems.

DISCUSSION

Cytochrome c reductase activity of beef heart mitochondrial particles was much more readily inhibited by rhein when NADH rather than succinate served as substrate. Thus, as previously shown for rat liver mitochondrial particles, Complex I of the respiratory chain, i.e. the span between NADH and coenzyme Q[19] contained the affected site(s).

Further, it was shown that NADH-DCPIP reductase activity was inhibited by rhein, while succinate-DCIP reductase activity was highly resistant. Since the former is a feature of the NADH-dehydrogenase flavoprotein complex in its various particulate or soluble forms,[20-23] it appeared that this particular flavoprotein possessed distinctive features which made it susceptible to inhibition by rhein.

Rhein inhibited NADH-ferricyanide reductase activity both under conditions of extrapolated infinite and fixed, finite concentrations of oxidant. These findings are significant in the light of arguments advanced by Minikami *et al.*,[12] who concluded that V_{max} (ferricyanide) specifically indicates the activity of a site in the flavoprotein NADH–dehydrogenase complex which is identical with or in close proximity to the grouping where NADH combines with the enzyme. By these criteria, rhein must be considered to inhibit NADH-dehydrogenase by some action which affects the substrate side of the enzyme complex, and would differ from amytal[12] and rotenone[24] in this respect.

Additional evidence that rhein and rotenone do not share a common target site was that preincubation of particles with rotenone, a firmly bound inhibitor,[25] failed to affect subsequent inhibition of this enzyme system by rhein.

The results of assays at fixed ferricyanide levels support these conclusions. Although it could be argued that the activity thus measured is partially due to respiratory chain components lying outside the flavoprotein complex[12] (as the Antimycin A inhibition and cyanide stimulation indicate), nevertheless, competitive interaction on the enzyme between NADH and rhein was evident.

On the basis that the sequence for electron transport through the NADH-dehydrogenase segment of the respiratory chain is NADH → flavoferroprotein → ferroprotein to coenzyme Q,[26–28] it seems reasonable to conclude that rhein inhibits the first step.

It was found that rhein inhibited rabbit muscle dehydrogenase and, at relatively high concentrations, alcohol dehydrogenase also. Since the inhibition of the latter enzyme was noncompetitive with respect to NAD^+, rhein did not apparently combine with coenzyme-binding groups of the enzyme. It is of interest that rotenone also inhibits alcohol dehydrogenase;[29] it inhibits glutamic dehydrogenase as well and, at relatively high concentrations, induces conformational changes in that enzyme.[30] Possibly, some similar mechanism could apply in the case of rhein inhibition.

The potency for inhibition of cytochrome c reductase was in decreasing order: rhein, rhein anthrone, sennoside A; hence, it appears that the loss of one oxo-group at position 10 of the anthracene nucleus weakened the inhibitory effect. Additional factors such as the nature of substituent groups on the anthracene nucleus may also be important.

It would appear that rhein is an interesting new agent for study of redox function in the NADH-dehydrogenase segment of the respiratory chain.

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