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SITES OF INHIBITION OF MITOCHONDRIAL ELECTRON TRANSPORT BY RHEIN

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Abstract—The effect of rhein on the oxygen consumption, oxidative phosphorylation, ATPase activity and redox state of electron carriers of rat liver mitochondria has been studied. Rhein inhibits ADP- and uncoupler-stimulated respiration on various NAD-linked substrates and succinate, but stimulates state 4 respiration of mitochondria respiring on succinate. Experiments on specific segments of the respiratory chain showed that rhein does not inhibit electron flow through cytochrome oxidase. Electron flow through site 2, the ubiquinone–cytochrome *b*–cytochrome c_1 complex, was also unaffected by rhein, which failed to inhibit the oxidation of duroquinol. Rhein affects oxidative phosphorylation by inhibiting both electron transfer and ADP-driven H^+ uptake. The inhibition of succinate oxidation by rhein was found to take place at a point between succinate and ubiquinone, perhaps at the level of succinic dehydrogenase. Spectroscopic evidence demonstrated that rhein induces a NAD(P)H oxidation in mitochondria respiring either on endogenous substrates or on glutamate + malate, and an inhibition of the cytochrome *b* reduction by succinate. These observations, together with other evidence, suggest that rhein inhibits electron transport in rat liver mitochondria at the dehydrogenase-coenzyme level, particularly when the electron carriers are in a relatively oxidized state and/or when the inner membrane-matrix compartment is in the condensed state.

Rhein, 4,5-dihydroxyanthraquinone-2-carboxylic acid, is a naturally occurring substance with cathartic properties and an antibacterial action [1, 2]. Because of its structure (see Merck Index) the effect on the respiratory chain has been studied [3–5]. It has been reported that rhein interferes with electron transfer by inhibiting mitochondrial oxidation of NAD-linked substrates but not succinate oxidation and that its effect is unrelated to oxidative phosphorylation [1].

In the last few years, rhein and its diacetoxy-derivative have been successfully employed as anti-inflammatory drugs. Their mechanism of action differs from that of other non-steroidal anti-inflammatory agents. In fact, rhein does not affect the cyclo-oxygenase; it stimulates the biosynthesis of prostaglandins and their release, thus avoiding toxic side-effects; it inhibits the release of lysosomial enzymes and the activity of some proteases [6–8]. Furthermore, it has been shown that rhein affects the energy metabolism of neoplastic cells by decreasing the oxygen consumption and aerobic and anaerobic glycolysis (Floridi, manuscript in preparation).

On the basis of these new pharmacological activities of rhein, systematic investigations on its effect on the energy metabolism of normal and neoplastic cells have been undertaken.

This paper presents data on the effect of rhein on the substrates oxidation, the flow of electrons through specific segments of the respiratory chain, oxidative phosphorylation, ATPase activity and the redox state of endogenous $NAD(P)^+$ and cytochrome *b* in isolated rat liver mitochondria.

The experiments were essentially designed to obtain more information on the site(s) of action of rhein.

MATERIALS AND METHODS

Preparation of mitochondria. Rat liver mitochondria were isolated from adult male Sprague–Dawley rats, fasted overnight according to Pedersen *et al.* [9]. The mitochondria were resuspended in a minimal volume of H-medium (70 mM sucrose, 210 mM mannitol, 2.1 mM Li-HEPES,† pH 7.10) without BSA, because of its ability to bind rhein [6], at a concentration of 100 mg/ml. Protein content was determined according to Gornall *et al.* [10].

Assay of oxygen consumption, P/O *ratio, and ATPase activity.* The rates of oxygen consumption were determined with a Clark oxygen electrode (Yellow Spring Instruments Co.) equipped with an ultrathin Teflon membrane. The electrode was inserted horizontally in a thermostated, closed chamber of 2.0 ml and contained final concentrations of 180 mM sucrose, 40 mM KCl, 3 mM Li-HEPES (pH 7.10), 1 mM EGTA, 10 mM substrates and 3.0 mg of mitochondrial protein. Other additions are given in the figure legends. The temperature was 25°

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† Abbreviations used: AA, antimycin A; An, anaerobiosis; BSA, bovine serum albumin; DH, dehydrogenases; DHQ, duroquinol; EGTA, ethyleneglycol bis(β-aminoethyl ether)*N,N,N′,N′* tetracetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LND, lonidamine; RH, rhein; RLM, rat liver mitochondria; Rot, rotenone; Succ, succinate; TMPD, *N,N,N′N′* tetramethyl-*p*-phenylenediamine; TTFA, 2-thenoyltrifluoroacetone.

and the solubility of oxygen was taken 442 ngatoms ml^{-1} when the medium was air-equilibrated at 760 Torr [11].

The P/O ratio was determined by simultaneously evaluating the rates of oxygen consumption and phosphorylation [12, 13] in which the pH change resulting from the reaction $ADP + Pi + nH^+ \rightarrow ATP + H_2O$ is monitored. Assuming complete phosphorylation of the added ADP, provided that the pH change is small, the rates of H^+ consumption during oxidative phosphorylation are readily converted into rates of ATP synthesis. The main advantages of this method are (1) the rates of O_2 uptake and ATP formation can be continuously recorded; (2) relative rates of phosphorylation in the presence of inhibitors can be determined very accurately; (3) it is not necessary to assume the complete phosphorylation of ADP in the presence of inhibitors and uncouplers of oxidative phosphorylation; (4) it is not necessary to inhibit the adenylate kinase; (5) the initial rates of oxygen consumption and ATP synthesis can be compared during the steady-state period of phosphorylation.

The ATPase activity was evaluated by measuring the rate of H^+ ejection accompanying ATP hydrolysis [14]. The reaction medium contained final concentrations of 180 mM sucrose, 40 mM KCl, 3 mM Li-HEPES (pH 7.10), 4 μM rotenone, 0.2 nmol mg $protein^{-1}$ antimycin A, 0.01 μM diadenosinpentaphosphate to inhibit adenylate kinase, 1 mM EGTA and 3.0 mg of mitochondrial protein. After 30 sec at 25°, 1 μg of valinomycin was added with stirring and, 4 min later, the reaction was started by the addition of 200 nmol ATP; the rate of subsequent H^+ ejection was recorded. Known amounts of HCl were added as internal standard to calibrate the pH electrode response in all experiments.

The electron flow from succinate to respiratory chain was evaluated according to Alexandre and Lehninger [15].

Spectrophotometric determinations. The effect of rhein on the oxido-reduction state of mitochondrial NAD(P)$^+$ and cytochrome *b* was evaluated by dual-wavelength spectrophotometry (Aminco DW-2a). The cuvette, thermostated at 25°, was provided with magnetic stirring. The reaction medium (2.5 ml) contained 180 mM sucrose, 40 mM KCl, 3 mM Li-HEPES (pH 7.10), 1 mM EGTA and 3.0 mg of mitochondrial protein. The addition of substrates and other compounds, as indicated in the figure legends, was made by rapid injection from microsyringes in such a way as to achieve the shortest possible mixing time.

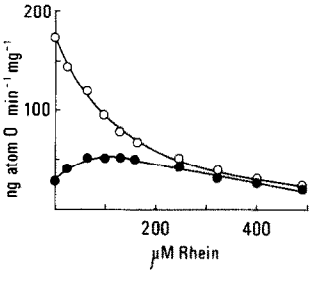

Fig. 1. Effect of rhein concentration on succinate oxidation by rat liver mitochondria in the presence (○) and in the absence (●) of FCCP. The final volume was 2.0 ml and temperature 25°. The mitochondria (3.0 mg protein) were preincubated for 1 min with the indicated concentrations of rhein in buffered medium (see Methods), 10 mM succinate was added and the rate of oxygen consumption was recorded. After 20% of the dissolved oxygen had been utilized, FCCP was added at a final concentration of 0.3 μM and the rate of oxygen consumption compared to that prior to the addition of FCCP. Each point was averaged from seven different mitochondrial preparations.

Chemicals. The following chemicals were purchased from the indicated sources: fatty acid-free BSA, TMPD, FCCP and ascorbic acid from Sigma Chemical Co. (St. Louis, MO); pyruvate, malate, glutamate, succinate, ADP, ATP, valinomycin, antimycin A from Boehringer-Mannheim, GmbH (F.R.G.); rotenone and duroquinol from K&K Laboratories (Plainview, NY). All other reagents were analytical grade and were purchased from BDH Italia (Milan, Italy). Rhein was obtained from Proter Laboratories, Opera (Milan, Italy).

RESULTS

Effect of rhein on the oxidation of various substrates by liver mitochondria

Figure 1 shows the effect of rhein concentration on the succinate oxidation by rat liver mitochondria in state 4 and after stimulation by FCCP. The mitochondria were preincubated for 1 min with different concentrations of rhein, and then 10 mM succinate was added and the rate of state 4 oxygen consumption was recorded. When about 20% of the available oxygen had been utilized, 0.3 μM FCCP was injected and the rate of the subsequent oxygen consumption was determined and compared to the stimulated rate in the absence of rhein. Figure 1 shows that the addition of rhein stimulates the state 4 rate of oxygen consumption which reaches a maximum and then declines. At 400 μM rhein the state 4 oxygen consumption is similar to that of the control. When the respiration is stimulated by FCCP, rhein strongly inhibits uncoupler-stimulated oxygen utilization, which decreases exponentially with drug concentration. Half-maximal inhibition was given by 80 μM rhein and almost complete inhibition of FCCP-stimulated respiration by 400 μM.

Table 1 shows the effect of rhein on the oxidation of some substrates which donate electrons to energy-conserving sites 1 and 3 of the respiratory chain. Rhein strongly inhibits FCCP-stimulated oxidation of pyruvate + malate and glutamate. With both substrates half-maximal inhibition was attained by 6 μM rhein. It must be pointed out that with substrates entering the energy-conserving site 1 the drug does not stimulate the state 4 respiration (data not shown).

The effect of rhein on electron flow through site 3, i.e. the cytochrome oxidase reaction, was investigated using ascorbate + TMPD as electron donor in the presence of antimycin A (0.2 nmol mg $protein^{-1}$) and 4 μM rotenone to block electron flow from endogenous site 1 and 2 substrates. The data of Table 1 show that the drug does not inhibit cytochrome oxidase; in the presence of FCCP, 650 μM

Table 1. Inhibition of state 3 respiration on various substrates by rhein

Substrate	FCCP	Control rate (ng atoms 0/min/mg)	Rhein giving half maximal inhibition (μM)
Pyruvate + malate	+	30 ± 3	6
Glutamate	+	27 ± 2	6
Succinate	+	175 ± 10	80
Ascorbate + TMPD	+	150 ± 8	No inhibition at 650 μM
Duroquinol	+	279 ± 15	No inhibition at 650 μM

The final concentration of FCCP was 0.3 μM. Each value ± SD was averaged from eight different experiments.

rhein does not influence at all the initial rate of the cytochrome oxidase reaction.

Rhein, therefore, does not inhibit electron flow in the cytochrome $c \rightarrow$ oxygen segment of the respiratory chain. A similar pattern was observed when respiration was ADP-stimulated (data not shown).

Effect of rhein on oxidative phosphorylation and on ATPase activity

Figure 2 shows representative O_2 and H^+ traces obtained with succinate as substrate in the absence of rhein (A) and in the presence of 163 μM rhein (B). The deflections observed on the chart recorder following phosphorylation of known quantities of ADP in the control (A) were used to determine the factor required to calculate the rate of phosphorylation.

The rates of oxygen consumption and phosphorylation of the control are 146 ngatoms 0 min^{-1} mg^{-1} and 260 nmol $H^+ min^{-1} mg^{-1}$ with a P/O ratio of 1.78. The addition of 163 μM rhein decreases both the rate of oxygen utilization (77 ngatoms 0 min^{-1} mg^{-1} and phosphorylation (54 nmol H^+ min^{-1} mg^{-1}), thus lowering the P/O ratio to 0.70. It should be noted that the drug inhibits the phosphorylation rate to a greater extent (−80%) than oxygen consumption (−47%). Figure 2C shows that P/O ratio decreases linearly with drug concentration from 1.95 to 0.4 at 200 μM rhein.

The effect of rhein on P/O ratio was confirmed by experiments with 3-hydroxybutyrate as substrate (Table 2), but it should be noted that, in this case, the

Table 2. Effect of rhein on P/O ratio of rat liver mitochondria respiring on β-hydroxybutyrate

Rhein (μM)	P/O ratio
0	2.52 ± 0.10
4	2.12 ± 0.08
8	1.81 ± 0.07
12	1.45 ± 0.09
16	1.21 ± 0.10
20	1.02 ± 0.05
28	0.93 ± 0.03

Each value ± SD was averaged from five different experiments.

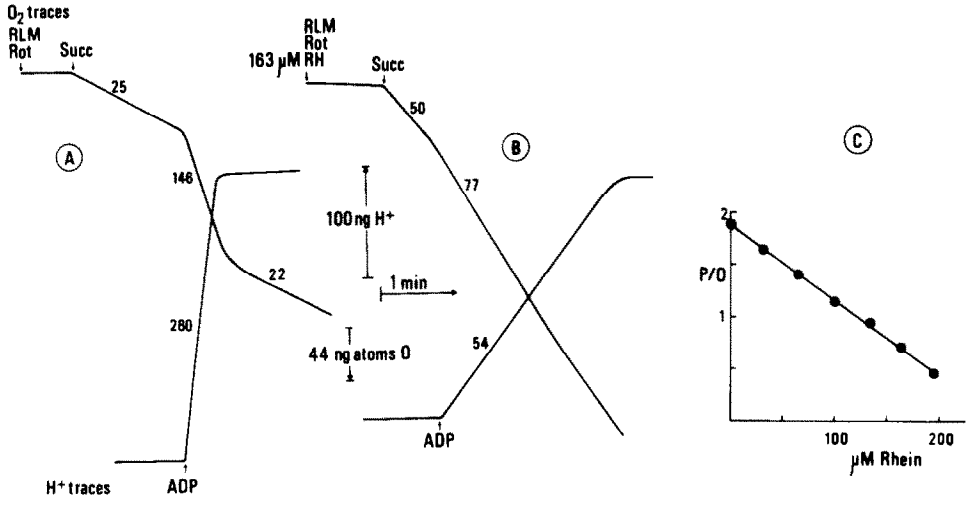

Fig. 2. Oxidative phosphorylation of rat liver mitochondria in the absence (A) and in the presence (B) of 163 μM rhein. Mitochondria were resuspended at 1.5 mg/ml in the buffered medium (see Materials and Methods). After approximately 20% of the dissolved oxygen had been consumed, 462 nmol ADP was added and the oxygen and H^+ uptake recorded. The rates of O_2 and phosphorylation (numbers along traces) were obtained from state 3 slopes. The O_2 uptake was expressed as ngatoms 0 min^{-1} mg^{-1}, and the rate of phosphorylation = 2.7 × ng-ions H^+ min^{-1} mg^{-1}. (C) Titration curve of the effect of rhein on P/O ratio. Each point was averaged from eight different mitochondrial preparations.

inhibition takes place at lower drug concentrations.

The effect on the vectorial ejection of H^+ coupled to activity of ATPase of rat liver mitochondria was also investigated. H^+ ejection coupled to the hydrolysis of added ATP was measured in the presence of antimycin A plus rotenone to inhibit endogenous electron flow, and K^+ + valinomycin to provide charge compensation for the ejected H^+. Figure 3 shows two representative traces of such an experiment. In the absence of the drug (Fig. 3A) the initial rate of H^+ is 211 ng-ions H^+ min^{-1} mg^{-1}. When the mitochondria were incubated with 163 μM rhein the rate of H^+ ejection was lowered to 87 ng-ions H^+ min^{-1} mg^{-1}. Figure 3C shows the effect of increasing drug concentrations on the ATPase activity. Rhein inhibits net H^+ ejection linearly with drug concentration up to 100 μM. Then the inhibitory effect becomes less marked and reaches a maximum at 250 μM rhein.

Therefore, the inhibition of H^+ ejection may be ascribed to an inhibition of F_0F_1 ATPase and/or adenine nucleotide translocase whose activity is required for ATP entry prior to ATPase action.

Effect of rhein on electron flow from succinate to respiratory chain

It has been reported that rhein affects the oxidation of those substrates entering energy-con-

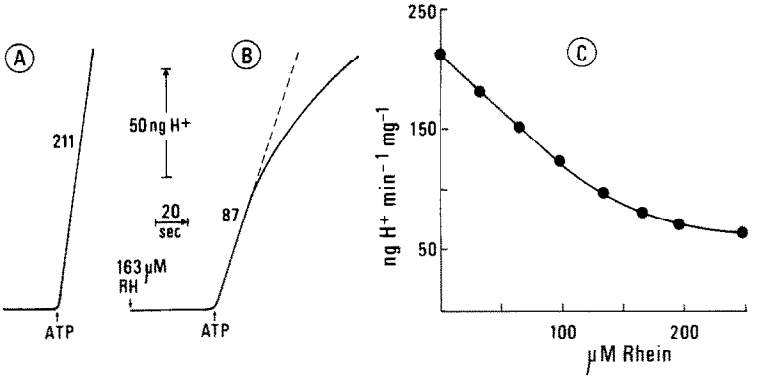

Fig. 3. H^+ ejection coupled to ATPase activity of rat liver mitochondria in the absence (A) and in the presence (B) of 100 μM rhein. Experimental details in Methods. (C): Titration curve of the effect of rhein on ATPase activity. Each point was averaged from five different mitochondrial preparations.

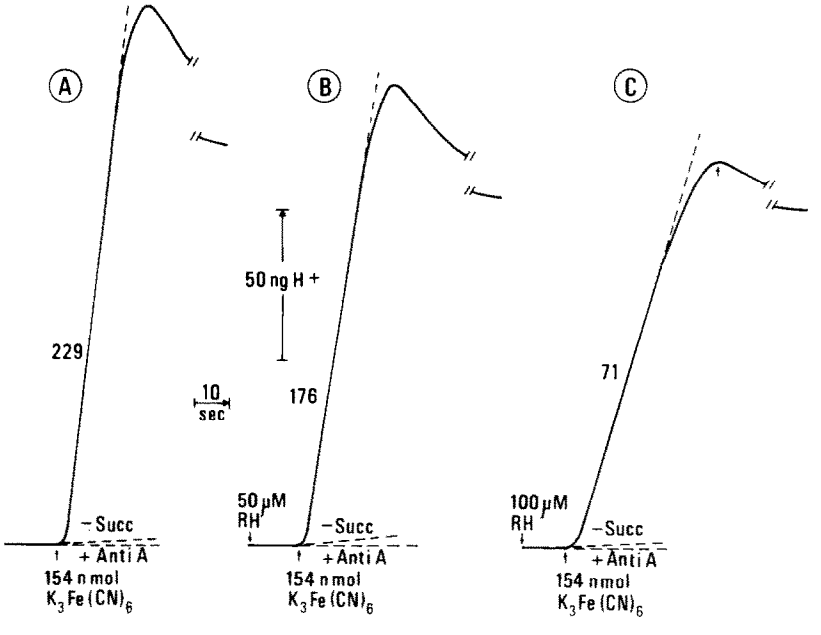

Fig. 4. Typical traces showing the effect of 50 μM (B) and 100 μM (C) rhein on H^+ translocation from succinate to ferricyanide. The mitochondria (3.0 mg protein) were incubated in 2.0 ml of buffered medium with 4 μM rotenone and 40 nmol/mg protein of *N*-ethylmaleimide. Valinomycin (1 nmol), KCN (0.6 mM) and succinate (0.5 mM) when at 2, 3 and 4 min respectively, after addition of mitochondria. The reaction was started at 5 min with a pulse of 154 nmol of ferricyanide. Numbers along traces are the rate of H^+ ejection, calculated from the linear part of the curve, expressed as ng-ions H^+ min^{-1} mg^{-1}. Experiments were repeated with five different mitochondria preparations and give reproducible results (±5%). (A) control.

serving site 1 of the respiratory chain but not succinate oxidation [1, 3, 4]. Nevertheless, the data reported in this communication (Figs 1 and 2, Table 1) clearly demonstrate that rhein inhibits equally well succinate oxidation even though in a manner not as sensitive as NADH-dependent substrates. Nevertheless, because of the inability of the drug to inhibit ascorbate + TMPD oxidation (Table 1) the inhibitory site of succinate oxidation cannot be localized in the cytochrome $c \rightarrow$ oxygen segment of the respiratory chain. The inhibition of oxygen consumption thus occurs at some point prior to cytochrome *c* or between succinate and respiratory carriers. To discriminate between these two possibilities three different experimental approaches were employed.

The first experimental approach was made by using duroquinol as the electron donor to site 2 of the respiratory chain. Table 1 shows the O_2-uptake data obtained when rhein was added to rat liver mitochondria with 0.5 mM duroquinol as electron donor in the presence of rotenone to inhibit electron flow from site 1 and FCCP to yield a maximal rate of duroquinol oxidation. It is seen that, up to 650 μM, rhein showed no inhibition of the very high rate of duroquinol oxidation. Thus, this direct test clearly demonstrates that rhein does not inhibit electron flow through site 2, i.e. the $Q \rightarrow c_1$ span.

The possibility that rhein might affect succinate oxidation between the substrate and Q was investigated by evaluating the effect of the drug on H^+ translocation coupled to electron flow from succinate to ferricyanide. In these experiments succinate was the electron donor and ferricyanide the electron acceptor. Rotenone was added to inhibit electron flow from the endogenous NAD-linked substrates and cyanide was present to block electron flow from cytochrome *c* to oxygen via cytochrome aa_3. K^+ + valinomycin was used as mobile permeant cation to compensate electrically for the H^+ translocated in the medium. *N*-Ethylmaleimide, an inhibitor of the $H^+/H_2PO_4^-$ symporter, was added to prevent interfering inward movement of H^+ caused by the presence of phosphate in the medium [16, 17]. Traces of such an experiment are shown in Fig. 4. Succinate was present in the medium during the preincubation period in the presence of K^+ + valinomycin. After the trace becomes stable a pulse of 154 nmol of ferricyanide was added and, following the ferricyanide reduction, the H^+ were ejected at an initial rate of 229 ng ions $H^+ \, min^{-1} \, mg^{-1}$ (Fig. 4A). Figure 4A also shows the results of some essential control experiments, indicated by dashed traces. Without succinate the rate of reduction of ferricyanide, and then the H^+ ejection, was insignificant as well as in the presence of antimycin A which inhibits the H^+ ejection by about 98%. When rat liver mitochondria were preincubated with 50 μM (Fig. 4B) or 100 μM rhein (Fig. 4C), the pulse of ferricyanide still induces H^+ ejection, but the initial rates are strongly inhibited. Yet, even in the presence of rhein, the omission of succinate from the incubation medium or the addition of antimycin A do not induce any significant ejection of H^+.

To localize better the site of rhein action in the succinate pathway the experiments of Fig. 4 were repeated, but valinomycin was replaced by the proton-conducting uncoupler, FCCP. The results are shown in Fig. 5. Since the presence of FCCP, protons equilibrate freely across the membrane and no net movement of vectorial H^+ from the matrix to the medium can be recorded. However, under these experimental conditions, the formation of H^+ arising from the dehydrogenation of succinate, delivered into matrix, can be detected by glass electrode in the medium because of the presence of FCCP. As shown in Fig. 5A addition of succinate to initiate electron flow is followed by the formation in the medium of H^+ from the methylene hydrogen atoms removed

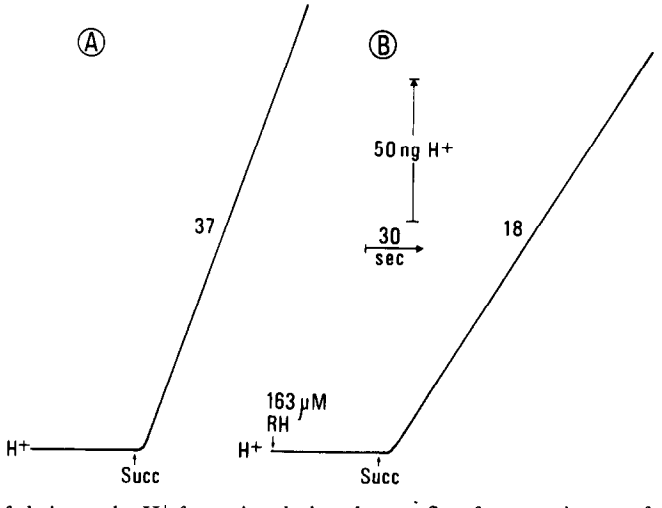

Fig. 5. Effect of rhein on the H^+ formation during electron flow from succinate to ferricyanide in the presence of FCCP. The experimental conditions were similar to those of Fig. 4 with the only exception that 400 nmol of ferricyanide were added after 4 min of incubation. The FCCP was present at a concentration of 1.2 μM. The reaction was started at 5 min with 0.5 mM succinate. Numbers along traces are the rate of H^+ formation expressed as ng-ions $H^+ \, min^{-1} \, mg^{-1}$.

from succinate by the dehydrogenase. When mitochondria are preincubated with rhein (Fig. 5B) the rate of H^+ formation is lowered to 18 ng ions $H^+ min^{-1} mg^{-1}$. It should be recalled that the degree of inhibition of H^+ is in agreement with the data of the inhibition of the oxygen consumption (Fig. 2).

Thus, these two types of experiments clearly show that the site of inhibition of succinate oxidation by rhein must be localized between the substrate and Q. This conclusion is further supported by other experiments.

Effect of rhein on the oxido-reduction state of mitochondrial electron carriers

To examine further the effect of rhein on the respiratory chain, its effect on the oxido-reduction level of $NAD(P)^+$ and cytochrome *b* in different metabolic states has been evaluated.

Figure 6A shows the effect of the drug on NAD(P)H oxidation in mitochondria in state 1 (no substrate, no ADP). Preincubation of the mitochondria results in an oxidation of NAD(P)H. When the trace becomes stable, the addition of 100 μM rhein ensued a rapid NAD(P)H oxidation which is blocked neither by rotenone nor by antimycin A. This experiment was repeated in the presence of increasing concentrations of rhein (Fig. 6B), thus showing that the rate of NAD(P)H oxidation increases with drug amount. Figure 6C shows that the addition of proton-conducting uncoupler FCCP to mitochondria in state 1 determines a fast oxidation of the total NAD(P)H, but the addition of rotenone induces a partial reduction of NAD^+, as demonstrated by the upward deflection of the spectrophotometric trace. Thus, this experiment shows that the drug inhibits electron transfer from the endogenous substrates to $NAD(P)^+$. Figure 7 shows the effect of rhein on mitochondria in state 4. The addition of glutamate + malate (Fig. 7A) to preincubated mitochondria gives a rapid reduction which, however, is not maintained. The injection of 130 μM rhein determines a prompt oxidation of NAD(P)H in a way similar to that observed with lonidamine (Fig. 7B), which inhibits the electron transfer from the substrates to respiratory carriers at the dehydrogenase-coenzyme level [19, 20]. Thus, these experiments clearly indicate that the site of action of rhein should be localized between substrates and $NAD(P)^+$.

Figure 8 shows the effect of rhein on the oxido-reduction level of cytochrome *b*. The preincubation of mitochondria (Fig. 8A) induces a slow oxidation of cytochrome *b* which becomes largely oxidized upon the addition of rotenone, which inhibits electron flow from the site 1 endogenous substrates. The addition of succinate, at the point shown, causes a fast reduction of cytochrome *b*. The addition of FCCP promptly reoxidizes the cytochrome *b* as respiration is stimulated by the uncoupler. Then, 2 min later, the dissolved oxygen is exhausted and cytochrome *b* becomes reduced as indicated by the upward deflection of the 430–410 nm trace. The addition of rhein to state 1 mitochondria with cytochrome *b* oxidized by rotenone does not induce any further modification in the spectrophotometric trace (Fig. 8B). The addition of succinate still reduces

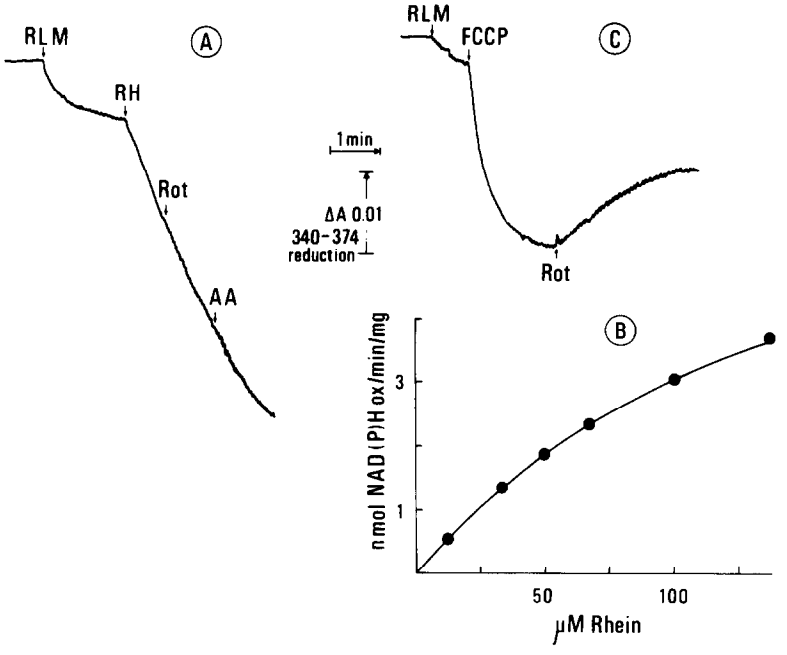

Fig. 6. Effect of rhein (A) and FCCP (C) on the oxido-reduction state of $NAD(P)^+$ of rat liver mitochondria in state 1. Mitochondria, 3.0 mg protein, were incubated in 2.5 ml of buffered medium at 25° to oxidize NAD(P)H. When the trace becomes stable 100 μM rhein was added at point shown. Rotenone, antimycin A and FCCP were added at final concentrations of 4 μM, 0.2 nmol mg protein^{-1} and 0.3 μM, respectively. (B) Titration curve of the effect of rhein concentration on NAD(P)H oxidation. Each point was averaged from four different mitochondrial preparations.

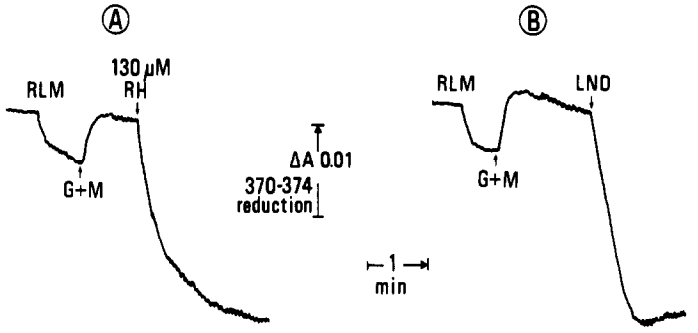

Fig. 7. Effect of rhein (A) and lonidamine (B) on the oxido-reduction state of NAD(P)$^+$ of rat liver mitochondria in state 4. Experimental conditions as in Fig. 6. Experiments were repeated with four different mitochondrial preparations and give reproducible results (±5%). G + M: glutamate plus malate.

cytochrome *b*, but both the extent and the rate of reduction are significantly lower. The reduced cytochrome *b* is then reoxidized by FCCP, but the time to reach the anaerobiosis is much longer (8 min) than in control.

The addition of rhein to mitochondria in state 1 (Fig. 8C) gives a pattern similar to that shown in Fig. 8B. Since rhein inhibits electron flow from site 1, the cytochrome *b* becomes oxidized to an extent overlapping that observed with rotenone. Nevertheless, the addition of succinate does not give a complete reduction of cytochrome *b* as when its oxidation is induced by rotenone, but both the extent and the time to anaerobiosis are similar to those

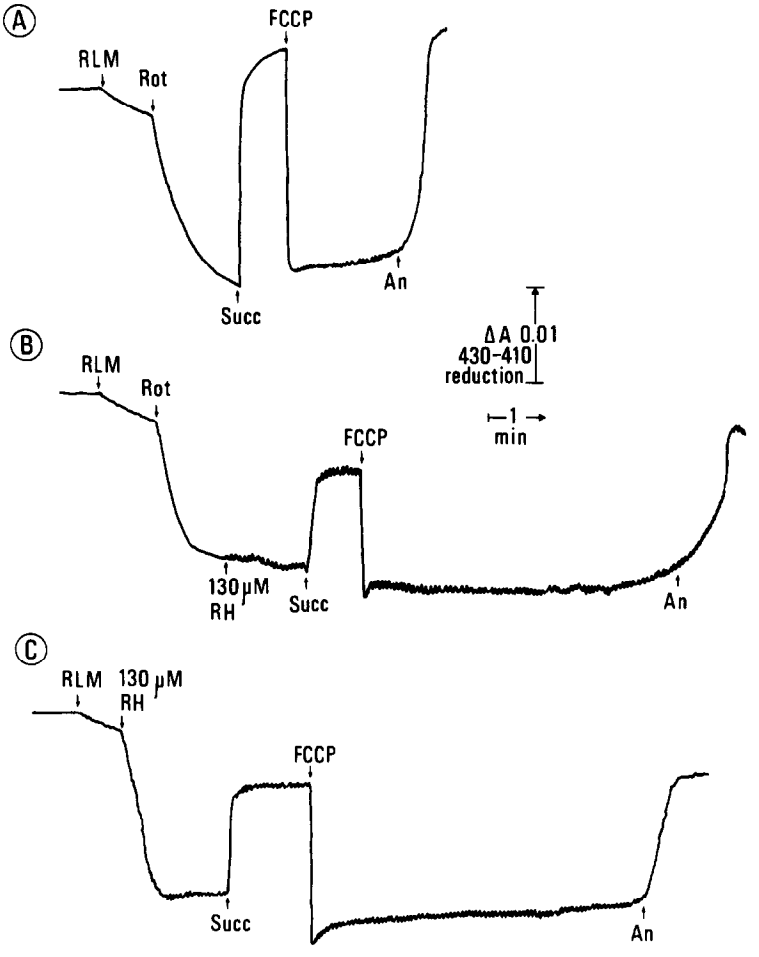

Fig. 8. Oxido-reduction state of cytochrome *b* in the absence (A) and in the presence of rotenone and rhein (B) or rhein alone (C). Traces B and C have been corrected for non-enzymic change in absorbance caused by rhein itself. Experimental conditions as in Fig. 6. Experiments were repeated with five different mitochondrial preparations and give reproducible results (±3%).

observed in Fig. 8B, thus confirming an inhibition of electron transfer from succinate to Q.

DISCUSSION

The observations recorded in this paper show that rhein, other than a potent inhibitor of the oxidation of various NAD-linked substrates, affects also succinate oxidation by rat liver mitochondria. The inhibition is exerted only on uncoupler- or ADP-stimulated respiration, but not on state 4 respiration, thus indicating an effect on electron transport.

Nevertheless, when respiration was ADP-stimulated the inhibition of the electron transfer (−47%) does not account for the higher decrease of ADP-stimulated H^+ uptake (−80%) (Fig. 2). These differences indicate that the inhibition of the uncoupler-stimulated respiration is solely due to inhibition of electron transport, while the decrease of P/O ratio is consequent to superimposition to inhibition of electron transport of a direct inhibitory action of rhein on ADP-driven H^+-inward transport. Tests of the effect of rhein on different segments of the respiratory chain showed that the energy-conserving site 3, the cytochrome oxidase reaction, is unaffected. Similarly, electron flow through the b–c_1 complex of site 2 is not inhibited by rhein, indicated by different types of experiments, and particularly by the failure of rhein to inhibit oxidation of duroquinol, which donates electrons directly to Q (Fig. 9).

More detailed studies of the site(s) of action of rhein were restricted to an examination of the point(s) at which the drug inhibits mitochondrial respiration. Spectrophotometric experiments demonstrate that the inhibition of the oxidation of NAD-linked substrates depends on a block of electron transfer from the substrates to respiratory chain (Fig. 9). In fact, rhein induces an NAD(P)H oxidation, which is not relieved by rotenone and antimycin A, in mitochondria oxidizing endogenous substrates (Fig. 6A). Furthermore, rhein causes the oxidation of NAD(P)H reduced by glutamate + malate (Fig. 7A), in a similar way to that observed with lonidamine, an antitumor drug [21–24] which inhibits electron transport in tumor and liver mitochondria at the dehydrogenase-coenzyme level [19, 20].

The measurements of the redox state of cytochrome b in mitochondria show that there must be also a second point at which rhein inhibits electron transfer (Fig. 9). In particular, the observation that rhein, when added after rotenone, greatly diminished the reduction of cytochrome b by succinate (Fig. 8B) indicates that it inhibits electron transfer at some points between succinate and Q. Such a conclusion is further supported by the results obtained when rhein was added to mitochondria in state 1 (Fig. 8C). In spite of the extensive oxidation of cytochrome b, due to a block by rhein of electron transfer from site 1, endogenous substrates, the cytochrome b reduction upon succinate addition overlaps that found in Fig. 8B, thus confirming that the drug inhibits electron transport from the substrate to respiratory chain. Since, in the presence of FCCP, the H^+ ejected in the medium arise only from succinate dehydrogenation, the ability of rhein to reduce the H^+ ejection rate suggests an inhibition of succinic dehydrogenase (Fig. 9).

While these data provide a clear evidence that rhein inhibits succinate oxidation and, consequently that the proposed specificity for site-1 phosphorylation is merely apparent, at the same time they evidence a higher sensitivity of NAD-linked substrates to rhein inhibition. The reduced sensitivity of succinate oxidation to rhein, as demonstrated by the higher $K_{i,\,app}$ (80 μM) may be essentially ascribed to (1) an intrinsic lower sensitivity of succinic dehydrogenase or (2) to a lower degree of accessibility to the site of action. Although the data reported here do not permit distinction between these two possibilities, there is some experimental evidence that makes the latter hypothesis more probable. In fact, if non-ionic detergent lubrol is added to mitochondria respiring on succinate, the inhibition of respiratory rate by rhein is increased, thus indicating that the permeabilization of membrane made the site of action more accessible to rhein [25].

Furthermore, the data reported in this paper support the concept that the inhibitory effect of rhein depends on some particular state of the respiratory chain of mitochondria. Oxidation of NAD-linked substrates as well as that of succinate is inhibited only when it is stimulated by FCCP or ADP. Since both these agents induce a more oxidized state of the electron carriers, rhein is an effective inhibitor only when the carriers are in a relatively oxidized state. Thus, it appears that mitochondria must be in some specific functional or morphological state to be susceptible to rhein inhibition either in the transition from the orthodox to the condensed state of the inner membrane-matrix compartment [26–28], in the transition to a more oxidized state of the dehydro-

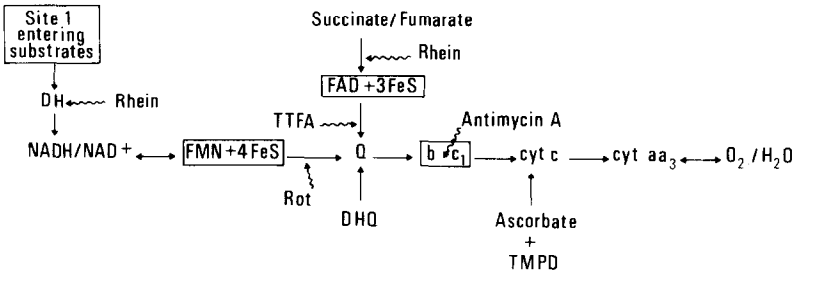

Fig. 9. Simplified scheme of the electron transport chain illustrating the suggested inhibition sites of rhein.

genase systems, or possibly when the intramitochondrial ATP/ADP ratio is lowered, as occurs in FCCP- or ADP-stimulated respiration. These possibilities may constitute the basis for further study of rhein action.

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