The Effect of Rotenone on Beef Liver Glutamic Dehydrogenase*

Ronald A. Butow

ABSTRACT: Crystalline beef liver glutamic dehydrogenase was demonstrated to be inhibited by the mitochondrial electron-transport inhibitor, rotenone. This inhibition is evident as a loss in glutamic dehydrogenase activity with the attendant emergence of alanine dehydrogenase activity. Inhibition of the initial velocity occurred at all pH values tested when the reaction was measured from α-ketoglutarate to glutamate; the reaction from glutamate to α-ketoglutarate was inhibited at all pH values tested except in the vicinity of pH 8.0. In the latter case, the activity was observed to decrease during turnover, reaching less than 25% of the control rate after 7–8 min. This inhibition was not due to a time-dependent inactivation *per se*. The enzyme can be "trapped" in an inhibited form by preincubation with rotenone when both substrate and coenzyme are present.

The activity of this inhibited form is slowly and spontaneously restored following dilution of the preincubation mixture. These results indicate that a reversibly inhibited rotenone-sensitive form of the enzyme accumulates during turnover.

Rotenone has been observed to inhibit mitochondrial electron transport in the region between the DPNH[1] dehydrogenase flavoprotein and cytochrome *b* (Burgos and Redfearn, 1965). The known physiological effects of rotenone had been confined to this region of the electron-transport chain until recently, when Balcavage and Mattoon (1967) observed an inhibition of yeast mitochondrial alcohol dehydrogenase by rotenone at concentrations of the inhibitor in excess of that required to inhibit DPNH oxidation in mammalian mitochondria.

The mechanism of inhibition and the identification of the redox components involved in the inhibitory effects of rotenone on electron transport are unknown. Inasmuch as both points are mutually inclusive, it was felt that some insight into the action of rotenone on mitochondrial electron transport could be gained by an examination of its possible effects on other, more well-defined systems.

Earlier studies (Yielding *et al.*, 1960a; Yielding and Tomkins, 1962) showed that a variety of small molecular weight compounds, including some of the steroid hormones, were effective inhibitors of beef liver glutamic dehydrogenase (L-glutamate:NAD(P) oxidoreductase (deaminating), EC 1.4.13). Of considerable interest is the fact that many of these are also inhibitors of mitochondrial DPNH oxidation (Yielding and Tomkins, 1959; Yielding *et al.*, 1960b). The sensitivity of these two systems to inhibition by these compounds prompted the investigation of the effects of rotenone on the reactions catalyzed by crystalline glutamic dehydrogenase. The present report describes the rotenone inhibition of the enzyme and some characteristics of this inhibition which have not yet been described for other inhibitors.

Experimental Procedure

Crystalline beef liver glutamic dehydrogenase was purchased from the Boehringer Mannheim Corp. as a suspension in 2.0 M ammonium sulfate or as a solution in 50% glycerol. Both preparations were used interchangeably and gave similar results. Unless noted otherwise, the enzyme was diluted in 50 mM potassium phosphate buffer, pH 7.6, containing 1×10^{-4} M EDTA prior to assay.

Rotenone was purchased from the Aldrich Chemical Co. Stock solutions were prepared at 2×10^{-3} M in absolute ethanol and stored at −20°.

DPN^+, DPNH, TPN^+, TPNH, pyruvate, α-ketoglutaric acid, and L-alanine were purchased from Sigma. L-Glutamic acid was obtained from Merck and Co.

Enzyme assays were carried out at room temperature by following the change in optical density at 340 mμ in 1-ml quartz cuvets with a 1-cm light path. Measurements were made with a Gilford Model 2000 recording spectrophotometer attached to a Beckman monochrometer. Initial velocities were determined from the linear portion of the curve during the first 30 sec of the reaction. Reactions were initiated either by the addition of enzyme or pyridine nucleotide (see text).

For the pH–activity measurements, potassium phosphate buffer was used from pH 6.7 to 8.0, Tris·HCl

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[1] Abbreviations used in this work: DPN^+ and TPN^+, oxidized di- and triphosphopyridine nucleotides, respectively; DPNH and TPNH, reduced di- and triphosphopyridine nucleotides, respectively; CoQ_1, coenzyme Q_1.

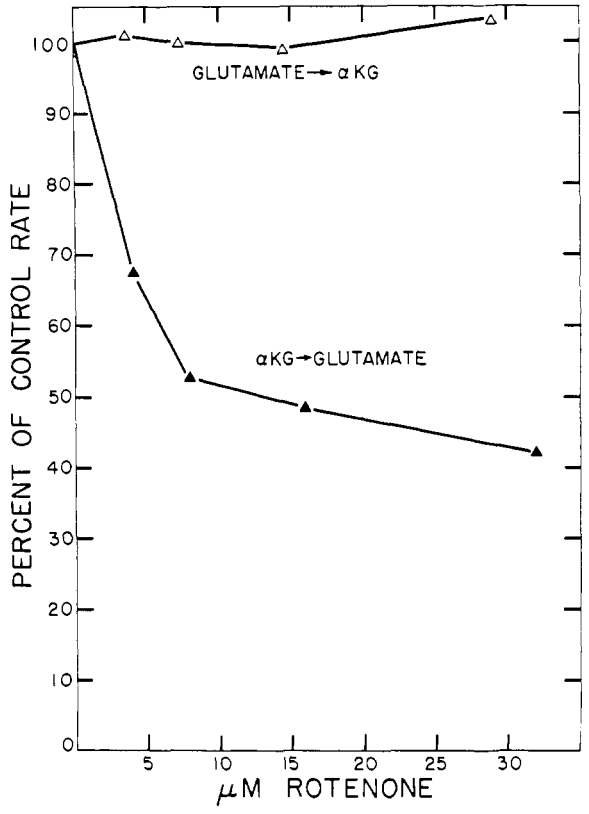

FIGURE 1: Effect of rotenone on glutamic dehydrogenase activity. The reaction mixture (glutamate to α-ketoglutarate) in a final volume of 1.0 ml contained: 0.1 M Tris·HCl (pH 8.0), 1×10^{-4} M EDTA, 2.5×10^{-2} M glutamate, 1×10^{-3} M DPN^+, 1 μg of enzyme, and rotenone as shown. The reaction mixture for the assay from α-ketoglutarate to glutamate in a final volume of 1.0 ml contained: 0.1 M Tris·HCl (pH 8.0), 0.15 M NH_4Cl, 2.5×10^{-2} M α-ketoglutarate, 1×10^{-4} M EDTA, 1.3×10^{-4} M DPNH, 1 μg of enzyme, and rotenone as shown. In both assay systems the reaction was initiated by pyridine nucleotide after a 2-min preincubation of the enzyme in the assay mixture.

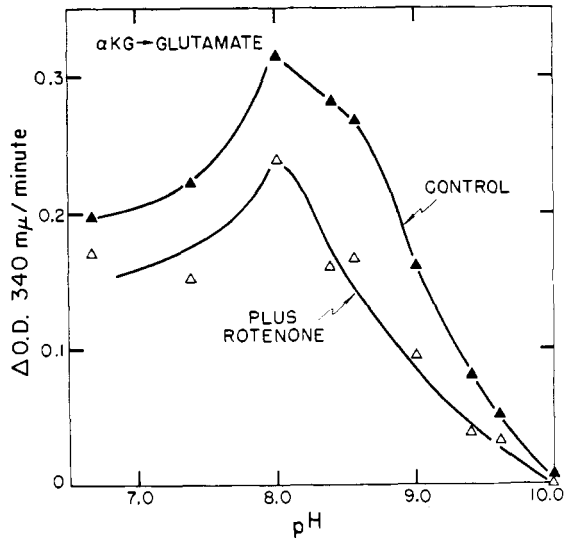

FIGURE 2: Effect of rotenone on the pH–activity curve of glutamic dehydrogenase. The reactions were carried out with 0.1 M buffer at the pH values shown. Otherwise, the assay mixture and conditions of preincubation of 1 μg of enzyme with 2×10^{-5} M rotenone are the same as that described in Figure 1 for the reaction from α-ketoglutarate to glutamate.

from pH 8.0 to 8.9, and 2-amino-2-methyl-1,3-propanediol from pH 9.0 to 10.0. In assaying the reductive amination of α-ketoglutarate or of pyruvate, NH_4Cl was added directly to the particular buffers employed prior to the final pH adjustment.

Results

The effects of rotenone on glutamic dehydrogenase activity were first examined at pH 8.0, close to the optimal pH for both the oxidative deamination of L-glutamate and the reverse reaction from α-ketoglutarate to glutamate. As illustrated in Figure 1, a 50–60% inhibition of the reductive amination of α-ketoglutarate occurs at concentrations of rotenone greater than 10^{-5} M. These results, obtained from initial velocity measurements, are similar to those reported by Balcavage and Mattoon (1967) for rotenone inhibition of crystalline yeast alcohol dehydrogenase, *i.e.*, maximal inhibition of about 60%, but are in contrast to the nearly complete inhibition of electron transport observed in intact mitochondria by Ernster *et al.* (1963a). Moreover, the rotenone titer in mitochondria was found to be less than stoichiometric with the respiratory carriers. More extensive inhibition of glutamic dehydrogenase by rotenone can be observed under conditions which are described below.

In the case of the glutamate to α-ketoglutarate reaction, no inhibition of the initial velocity was observed in the range from 3.6×10^{-6} to 2.8×10^{-5} M rotenone (Figure 1). Furthermore, 2×10^{-5} M rotenone did not significantly alter the K_m or V_{max} values for glutamate (varied from 2.5×10^{-5} to 1×10^{-2} M) or DPN^+[2] (varied from 1.25×10^{-5} to 1×10^{-3} M). No significant potentiation of rotenone inhibition was observed by inclusion of up to 0.13 mM DPNH in the assay mixture at either pH 8.0 or 9.0. Higher concentrations of DPNH were too inhibitory to assess any potentiating effect. Very similar results were obtained if both reactions were measured with the triphosphopyridine nucleotide coenzymes, except that somewhat less rotenone inhibition of the reductive amination of α-ketoglutarate was observed with TPNH rather than DPNH as the coenzyme.

[2] K_m and V_{max} values for DPN^+ were obtained from double-reciprocal plots by extrapolation of the values at high DPN^+ concentrations where activation of the reaction occurs.

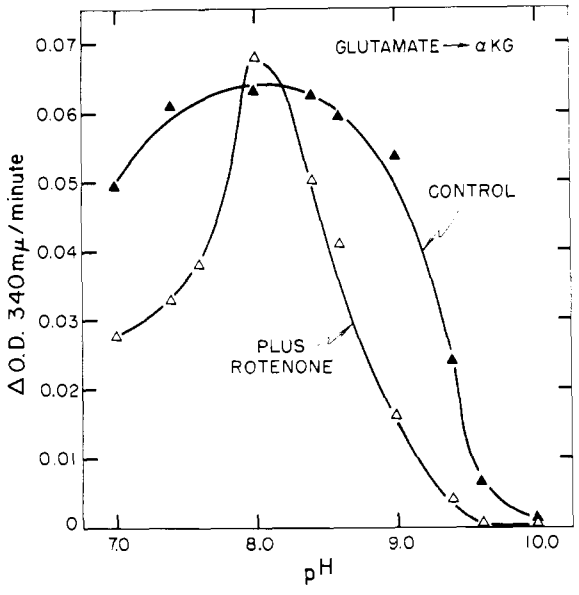

FIGURE 3: Effect of rotenone on the pH–activity curve of glutamic dehydrogenase. The reactions were carried out with 0.1 M buffer at the pH values shown. Otherwise, the assay mixture and conditions of preincubation of 1 μg of enzyme with 2×10^{-5} M rotenone is the same as that described in Figure 1 for the reaction from glutamate to α-ketoglutarate.

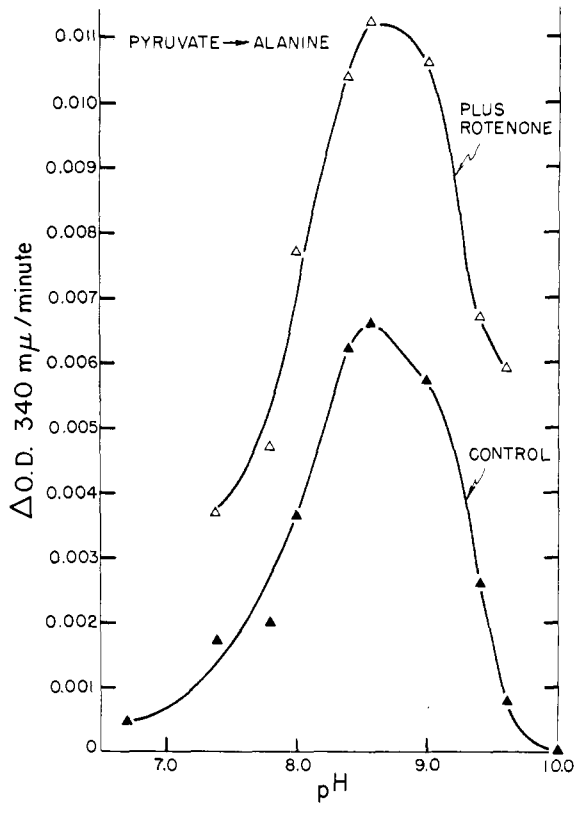

FIGURE 4: Effect of rotenone on the pH–activity curve of alanine dehydrogenase activity. The reaction mixture in a final volume of 1.0 ml contained: 0.1 M buffer, 0.15 M NH_4Cl, 5×10^{-2} M EDTA, 5×10^{-2} M pyruvate, and 1.3×10^{-4} M DPNH. The enzyme (5 μg) was preincubated for 2 min in the reaction mixture containing 2×10^{-5} M rotenone as shown in the figure, prior to addition of DPNH.

Effect of pH. Figures 2 and 3 illustrate the effects of varying the pH on the extent of rotenone inhibition of glutamic dehydrogenase when assayed with DPN^+ or DPNH as coenzyme. The data presented in Figure 2 show that rotenone inhibits the reaction from α-ketoglutarate to glutamate at all pH values tested; however, in the glutamate to α-ketoglutarate assay, rotenone inhibition occurred at all pH values except in the vicinity of pH 8.0 (Figure 3). These results are similar to those reported by Di Prisco *et al.* (1965) for inhibitors such as progesterone and thyroxine in that the extent of inhibition was shown to vary with pH. Diethylstilbesterol, however, appears to inhibit at all pH values tested (Bitensky *et al.*, 1965). In both cases, the assays were carried out with glutamate as the substrate.

It has been suggested (Frieden, 1963a; Bitensky *et al.*, 1965) that many of the inhibitory modifiers of glutamic dehydrogenase affect the equilibrium between monomeric forms of the enzyme, designated as x and y, where the x form represents the species active as glutamic dehydrogenase and the y form active as alanine dehydrogenase. These modifiers appear to shift the equilibrium toward the y form resulting in a reciprocal shift of the glutamate and alanine dehydrogenase activities. In this respect, rotenone acts similarly to these modifiers. Figure 4 shows that 20 μM rotenone will stimulate the alanine dehydrogenase activity of the enzyme at all pH values measured.

Kinetics of Rotenone Inhibition. All of the data reported thus far were obtained from initial velocity measurements. Figure 5 shows the results of an experiment where the time course of the reaction from glutamate to α-ketoglutarate was followed at pH 8.0. Under these conditions, where no inhibition of the initial velocity was observed in the presence of 10 and 20 μM rotenone, a progressive inactivation of the reaction is evident after the first minute. In order to determine if this was a time-dependent inactivation *per se*, or inhibition as a result of the formation of a rotenone-sensitive form of glutamic dehydrogenase, the enzyme was preincubated in the assay mixture with 10 μM rotenone for various time intervals prior to initiation of the reaction with DPN^+. Figure 5 shows that the observed initial velocity values, multiplied by the time of preincubation, coincide with a line constructed from an extrapolation of the initial rate of the control reaction. These results, in addition to the substrate and coenzyme requirements for inhibition described below, are consistent with the view that the progressive inactivation of the glutamate to α-ketoglutarate reaction is the result of the formation of a particular rote-

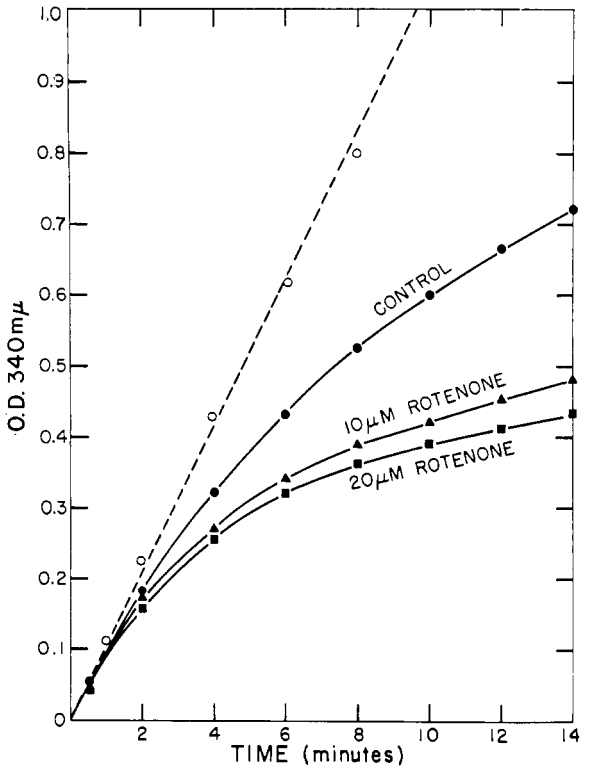

FIGURE 5: Effect of rotenone on the time course for the reaction from glutamate to α-ketoglutarate at pH 8.0. The same assay mixture described in Figure 1 was used. Rotenone was added as shown. The open circles represent initial velocity values multiplied by the time of preincubation of 1 μg of enzyme with 2×10^{-5} M rotenone in the assay mixture. In this case, the reaction was initiated at the times indicated with 1 μmole of DPN^+.

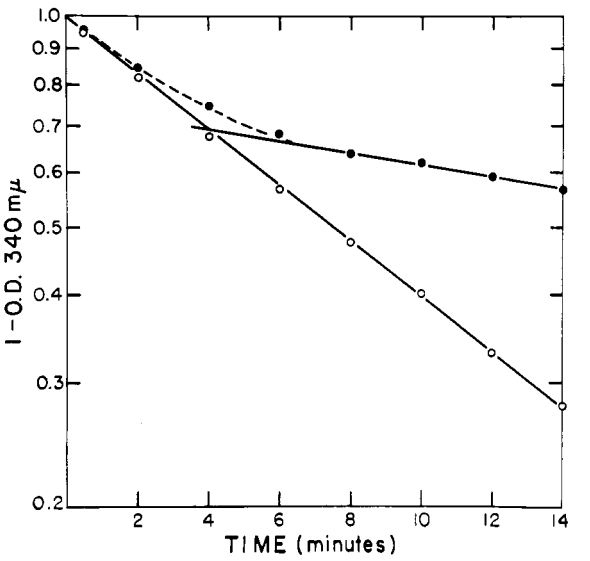

FIGURE 6: Semilog plot of the data obtained from Figure 5. The open circles represent the control and the closed circles, plus 2×10^{-5} M rotenone.

none-sensitive form of the enzyme during turnover.

The data obtained from the previous experiment for the control reaction and that containing 20 μM rotenone are plotted in Figure 6 to show the decrease in rate as an apparent first-order process. Maximal extent of inhibition, from 0.091 (for the control) to 0.020 min^{-1}, occurs after approximately 7 min. Thus, under these conditions, rotenone inhibits glutamic dehydrogenase by more than 75%, whereas no inhibition of the initial velocity is observed at pH 8.0.

Since these data suggest that a particular form of glutamic dehydrogenase was more susceptible to inhibition by rotenone, the possibility of "trapping" this reactive form was investigated. In this connection, glutamic dehydrogenase was preincubated at pH 9.0 with glutamate and DPN^+ in the presence and absence of 20 μM rotenone. Aliquots were then removed at various time intervals and assayed for the glutamate to α-ketoglutarate reaction at pH 8.0. Figure 7 (curve A) shows that under these preincubation conditions where turnover occurs, the enzyme is stable in the absence of rotenone. On the other hand, curve B shows that a progressive inactivation does indeed occur when the enzyme is preincubated with glutamate, DPN^+, and rotenone. Here, the inhibition after 5 min of preincubation reaches a value less than 20% of the original activity. It is significant to note that inhibition is observed although rotenone was diluted 50-fold in the assay mixture to a final concentration of 4×10^{-7} M, a concentration which is not inhibitory under the assay conditions employed. These results show that the enzyme can in fact be trapped in an inhibited form; however, the inhibition is clearly reversible as shown in Figure 7 (curve C). Within 5 min following dilution into the assay mixture, enzyme activity is spontaneously restored to nearly its original level. In this experiment the conditions of preincubation and assay were identical with that for the inactivation kinetics (curve B) except that the reaction was initiated at various time intervals by the addition of DPN^+.

The requirements for rotenone inhibition of enzyme activity under these conditions are shown in Table I. These data clearly demonstrate that both substrate and coenzyme are required in order to trap the enzyme in an inhibited form. Similar results can be obtained by preincubation at pH 8.0 rather than at pH 9.0 except that longer preincubation times are required before the onset of inhibition is observed.

Discussion

The results presented here demonstrate that the mitochondrial electron-transport inhibitor rotenone is an effective modifier of beef liver glutamic dehydrogenase. This is evident as a loss of glutamic dehydrogenase activity with the attendant emergence of alanine dehydrogenase activity. Bitensky *et al.* (1965) described this phenomenon for a number of modifiers of the

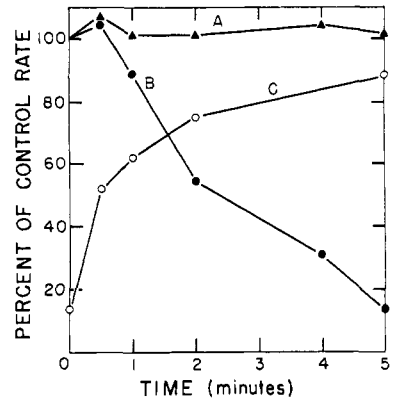

FIGURE 7: Reversibility of rotenone inhibition of glutamic dehydrogenase. The enzyme (200 μg) was preincubated in a reaction mixture which contained in a final volume of 1.0 ml ammediol buffer (pH 9.0), 1×10^{-4} M EDTA, 2.5×10^{-2} M glutamate, 1×10^{-3} M DPN^+ (curve A), and 2×10^{-5} M rotenone (curves B and C). At the times indicated, 20 μl was removed and added to cuvets containing the complete glutamate to α-ketoglutarate assay mixture (pH 8.0), as described in Figure 1. For curve C, DPN^+ was added to initiate the reaction following incubation of 20 μl of the preincubation mixture in the assay mixture for the times indicated.

enzyme and formulated their results as follows

$$\text{polymer} \underset{}{\overset{\text{I}}{\rightleftharpoons}} \text{monomer x} \overset{\text{II}}{\rightleftharpoons} \text{monomer y}$$

where x represents the glutamate and y the alanine dehydrogenase activity. The equilibrium represented by reaction II is most probably the result of modifier-induced conformational changes (Frieden, 1963a; Bitensky *et al.*, 1965; Talal *et al.*, 1964). The factors which affect this equilibrium and, in turn, the activation or inhibition of the activity of glutamic dehydrogenase monomer by a variety of modifier compounds, are complex. Among these include pH, ionic strength, and specific ions such as ammonium and phosphate (Di Prisco and Strecker, 1966).

The present experiments clearly show that rotenone modification of glutamic dehydrogenase varies with the substrate, coenzyme, and pH. It was shown that rotenone has no effect on the initial velocity of the glutamate to α-ketoglutarate reaction at pH 8.0 (Figures 1 and 3), whereas immediate inhibition was observed at other pH values (Figure 3) or at all pH values in the α-ketoglutarate to glutamate reaction (Figure 2).

The chemical nature of the interaction of small molecules with glutamic dehydrogenase, resulting in altered kinetic properties of the enzyme, is unknown. It is unlikely, however, that the *mechanism* of interaction and, in this case, of rotenone inhibition, differs with pH or the direction of the enzyme-catalyzed reaction. The fact that extensive inhibition of the glutamate to

TABLE I: Substrate–Coenzyme Requirements for Rotenone Inhibition.[a]

Composition of Preincubn Mixture	ΔOD at 340 mμ/min
Complete System	0.016
−Rotenone	0.037
−Glutamate	0.040
$-DPN^+$	0.049

[a] In the complete system, 100 μg of glutamic dehydrogenase was preincubated with 2×10^{-5} M rotenone in the reaction mixture described in Figure 7. After 1-min incubation, a 10-μl aliquot was removed and assayed for activity (glutamate to α-ketoglutarate) at pH 8.0 as described in Figure 1, except that the reaction was initiated by addition of the enzyme.

α-ketoglutarate reaction at pH 8.0 was only observed during turnover (Figure 5), rather than as a time-dependent inactivation *per se*, suggests the formation of a particular rotenone-sensitive form of the enzyme. Consistent with this view is the observation that both substrate and coenzyme were required in order to "trap" the enzyme in an inhibited form (Table I). The apparent directional effects reported here might reflect differences in the steady-state concentration of a rotenone-sensitive form of the enzyme which would be expected to vary with pH and the particular substrates and coenzyme employed. It should be added that although DPNH will facilitate the extent of inhibition of enzyme activity by purine nucleotides (Frieden, 1962), rotenone had no effect on the initial velocity of the glutamate to α-ketoglutarate reaction at pH 8.0 when measured in the presence of up to 1.3×10^{-4} M DPNH (R. A. Butow, unpublished observations); however, maximal inhibition by 20 μM rotenone occurs between 7 and 8 min when the concentration of the accumulated DPNH is only approximately 5×10^{-5} M. The type of reversible inhibition illustrated in Figure 7 has not previously been reported for other inhibitors of glutamic dehydrogenase. Further experiments are required to determine if the reactivation phenomenon represents a dissociation of rotenone from the enzyme or a slow conformation change from inactive to active form. The relationships between substrate, coenzyme, and modifier binding sites for glutamic dehydrogenase have been extensively documented (Frieden, 1959, 1963b; Coleman and Frieden, 1966a,b).

The significance of the observations presented in this paper lies, in part, in the relationship between the mechanism of rotenone inhibition of electron transport and its effect on glutamic dehydrogenase. Rotenone inhibition of mitochondrial electron transport is specific for DPNH oxidation (Lindahl and Oberg, 1961; Ernster *et al.*, 1963a,b). Moreover, the site of

inhibition has been localized between the DPNH dehydrogenase flavoprotein and cytochrome *b* (Burgos and Redfearn, 1965). Of considerable interest is the correlation which can be made between compounds which inhibit electron transport in this region of the respiratory chain and those which modify glutamic dehydrogenase. For example, Yielding and Tomkins (1959) showed that a variety of steroids were inhibitors of mitochondrial DPNH oxidation; as in the case of rotenone, the site of inhibition was shown to be between the DPNH dehydrogenase flavoprotein and cytochrome *b* (Yielding *et al.*, 1960a). Furthermore, these steroids were shown to be effective modifiers of glutamic dehydrogenase (Yielding *et al.*, 1960b). In addition to steroids, compounds such as *o*-phenanthroline and its analog phenanthridine are inhibitors of DPNH oxidation (Butow and Racker, 1965; Redfearn *et al.*, 1965; R. A. Butow, unpublished observations) as well as glutamic dehydrogenase (Yielding and Tomkins, 1962). Although many of these compounds will cause the dissociation of the polymer form of the enzyme to monomeric units (Yielding and Tomkins, 1960; Frieden, 1963a), this in itself does not appear to be the mechanism of inhibition since it has already been demonstrated that the low molecular weight form is catalytically active (Frieden, 1963a,b). By "stabilizing" a particular monomer form, however, dissociation is promoted by mass law effects.

Recent studies carried out with mitochondria isolated from the *Saccharomyces* strain of yeast have underlined an important relationship between rotenone inhibition of electron transport and first site oxidative phosphorylation. It appears that these mitochondria are not only lacking a first phosphorylation site, but also a rotenone-sensitive DPNH oxidase, as well as the $g = 1.94$ electron paramagnetic resonance (epr) signal of the nonheme iron located between the DPNH dehydrogenase flavoprotein and cytochrome *b* (Ohnishi *et al.*, 1966; Schatz and Racker, 1966a; Schatz *et al.*, 1966). Furthermore, it has been shown (Schatz and Racker, 1966b) that there is an excellent correlation between the loss of rotenone-sensitive DPNH-CoQ_1 reductase activity in beef heart submitochondrial particles with the loss of oxidative phosphorylation coupled to CoQ_1 reduction; *i.e.*, first site phosphorylation appears to be at or on the oxygen side of the rotenone-sensitive site. Studies on the mechanism of rotenone action, with glutamic dehydrogenase as a possible model system, should help to elucidate the functional relationship between the rotenone-sensitive electron-transport component and oxidative phosphorylation.

Acknowledgment

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