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Inhibition of Mitochondrial Respiration by Hydroxylamine and Its Relation to Energy Conservation*

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ABSTRACT: The inhibition of mitochondrial succinate oxidation by hydroxylamine is kinetically characteristic of an inhibitor of adenosine diphosphate phosphorylation, not of electron transport. When ascorbate and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine are used as electron donors, however, the hydroxylamine is an inhibitor of electron transport and is uncompetitive with respect to the dye. The inhibition of succinate oxidation is almost completely released and the inhibition of the *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidation is partially released by Ca^{2+} and uncouplers.

The release of hydroxylamine inhibition of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidation is effected by 1.3 moles of salicylanilide uncoupler per mole of cytochrome *a*, a titer which is independent of the rate of turnover of the cytochrome oxidase. This titer confirms the value obtained by the uncoupler dependent release of azide inhibition and is postulated to represent a titration by the uncoupler of a binding site on a specific protein associated with the respiratory chain.

In recent reports on the inhibition of mitochondrial respiration by hydroxylamine, Wikström and Saris (1969) have concluded that the inhibition of oxidative phosphorylation by hydroxylamine is specific for site 3 (cytochrome oxidase) while Utsumi and Oda (1969) have concluded more generally that the hydroxylamine interacts at an early, as yet unidentified, energy transfer step in mitochondria. These two conclusions are mutually inclusive and indicate that hydroxylamine may be a very useful tool in studying the mitochondrial energy conservation reactions. The properties described for the inhibition are similar but not identical with those of azide inhibition (Wilson and Chance, 1966, 1967; Wilson, 1967; Nicholls and Kimelberg, 1968). The comparisons of the behavior of the two inhibitors are not satisfactory since a quantitative kinetic analysis of the hydroxylamine inhibition has not been reported. Such a comparison is of great interest since azide has been shown to specifically inhibit electron transport between cytochromes *a* and a_3 and has been proposed to inhibit an intermediate common to the electron transfer and energy conservation reactions (Wilson and Chance, 1966, 1967; Wilson, 1967). The present communication presents a kinetic analysis of the hydroxylamine inhibition of respiration under conditions which permit quantitative comparison with azide inhibition. In addition the stoichiometric binding of the salicylanilide uncouplers to the respiratory chain (Wilson and Azzi, 1968; Wilson, 1969) is examined using the uncoupler dependent release of hydroxylamine inhibition.

Methods

Rat liver mitochondria were prepared in a 0.22 M mannitol, 0.07 M sucrose, and 0.2 mM EDTA medium (pH 7.2) essentially according to the method of Schneider (1948).

Mitochondrial respiration was measured polarographically at 23° in either a 0.2 M mannitol, 0.05 M sucrose, 0.02 M Tris, and 0.01 M KH_2PO_4 medium (MST-P_i) or a 0.12 M KCl, 0.02 M Tris, and 0.01 M KH_2PO_4 medium (KClT-P_i). The pH was 7.2 unless otherwise noted in the figure or table legends. The volume of the reaction vessel was 3.4 ml.

The indicated respiration rates are for initial conditions with a minimum time between the addition of the inhibitor and the rate measurement. For succinate inhibition, for example, the order of addition was medium, mitochondria, rotenone, ADP, hydroxylamine, then succinate.

The cytochrome *a* content of the mitochondrial preparation was measured as previously described (Wilson, 1969) assuming an extinction coefficient of 24 for reduced minus oxidized cytochrome *a* at 605 nm minus 630 nm (van Gelder, 1966).

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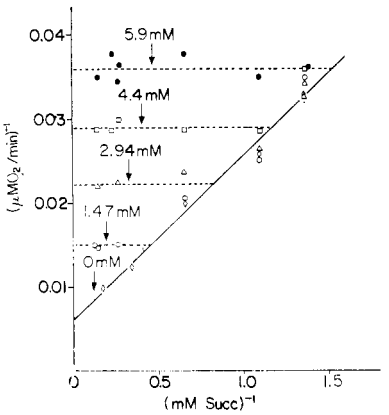

FIGURE 1: The double reciprocal plot of the kinetics of the hydroxylamine inhibition of succinate oxidation by rat liver mitochondria. The mitochondria were suspended at 0.23 μM cytochrome *a* in KCl-P_i, pH 7.2. Rotenone (3 μM), ADP (660 μM), and the designated hydroxylamine concentrations were added and then the respiration was started with sodium succinate. The reciprocal of the added succinate concentration is plotted on the abscissa and the reciprocal of the initial respiration rate is plotted on the ordinate.

The uncouplers 5-chloro-3-(*p*-chlorophenyl)-2′,4′,5′-trichlorosalicylanilide[1] and 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide were the generous gift of the agricultural division of Monsanto Chemical Co., St. Louis, Mo. The hydroxylamine hydrochloride was Baker Analyzed reagent grade and was neutralized immediately before use. All other reagents were the same as previously described (Wilson and Gilmour, 1967).

Experimental Results

The Inhibition of Succinate Oxidation by Hydroxylamine. When the inhibition of state 3 (ADP + P_i) succinate oxidation by hydroxylamine is plotted according to Lineweaver and Burk (Figure 1) we find that the inhibitor has almost no effect on either the K_m or V_m for succinate at low succinate concentrations.

The curve for each hydroxylamine concentration is biphasic. The portion of the curve for lower succinate concentrations is coincident for all hydroxylamine concentrations (*i.e.*, negligible inhibition by hydroxylamine) while the portion of the curve for higher succinate concentrations represents an electron flux which is independent of the succinate concentration. This failure to observe an effect on V_m or K_m could be explained by an inhibition of ADP phosphorylation but not of electron transport. The apparent inhibitor constant and molecular order (n) value may be obtained by plotting the intercept on the ordinate of the portion of the curves for higher succinate concentrations against the hydroxylamine concentration (Figure 2) and extrapolating to infinite succinate concentration. The hydroxylamine concentration required for 50% inhibition at infinite succinate concentration is 1.4 mM. The plot is a straight line and suggests that one hydroxylamine molecule reacts per inhibitory site (n = 1).

[1] Unusual abbreviations used are: S-10, 5-chloro-3-(*p*-chlorophenyl)-2′,4′,5′-trichlorosalicylanilide; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

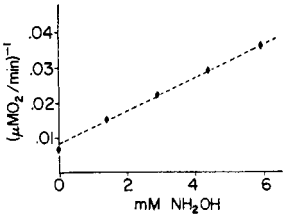

FIGURE 2: The hydroxylamine concentration dependence of the inhibition of succinate oxidation in rat liver mitochondria. The intercepts on the ordinate ($1/V_m$) from Figure 1 are plotted on the ordinate and the hydroxylamine concentration is plotted on the abscissa.

The pH Dependence of Hydroxylamine Inhibition. Wikström and Saris (1969) have reported that hydroxylamine inhibition is essentially independent of pH. We have determined the concentration of hydroxylamine required for 50% inhibition at infinite substrate concentration and obtained values of 1.1 mM at pH 6.8, and 0.8 mM at pH 7.8. This is in agreement with the report by Wikström and Saris (1969), and is consistent with the uncharged NH_2OH being the dominant reactive form of the inhibitor (pK = 5.9).

The Release of Hydroxylamine Inhibition by Uncouplers. When uncouplers are added to hydroxylamine inhibited state 3 mitochondria, the respiration increases (Utsumi and Oda, 1969; Wikström and Saris, 1969). In Figure 3 this is presented in a plot of the reciprocal of the measured respiration rate against hydroxylamine concentration. Two different succinate concentrations (1.5 and 5.9 mM) are presented and again it is apparent that hydroxylamine is inhibiting energy conservation but not electron transport. At hydroxylamine concentrations less than 3 mM the rate of oxidation of 5.9 mM succinate is inhibited but the rate of oxidation of 1.57 mM succinate is not. At hydroxylamine concentrations greater than 3 mM the respiration rate is the same for both 1.5 and 5.9 mM succinate. The addition of excess uncoupler causes an almost complete release of the hydroxylamine inhibition. In the presence of

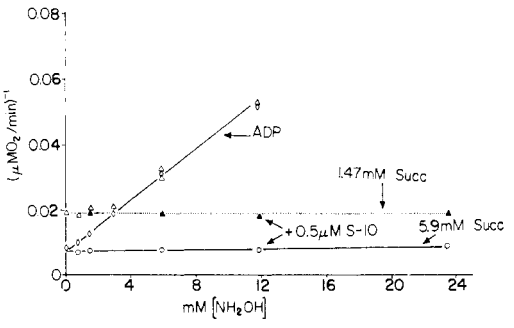

FIGURE 3: The release of hydroxylamine inhibition of succinate oxidation by uncouplers. Rat liver mitochondria were suspended at 0.22 μM cytochrome *a* in a KCl-P_i medium, pH 7.2. Rotenone (2 μM) and ADP (600 μM) were added and the reaction was started with sodium succinate. The uncoupler S-10 (when present) was added immediately before the succinate. The reciprocal of the initial respiration rate is plotted on the ordinate and the hydroxylamine concentration on the abscissa. The conditions were: (△) 1.47 mM Na-succinate, (▲) 1.47 mM Na-succinate plus 0.5 μM S-10, (◇) 5.9 mM Na-succinate, and (○) 5.9 mM Na-succinate plus 0.5 μM S-10.

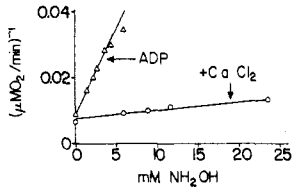

FIGURE 4: The Ca^{2+} dependent release of the inhibition of succinate oxidation by hydroxylamine. Rat liver mitochondria were suspended at 0.22 μM cytochrome *a* in MST–P_i medium. Rotenone (2 μM), ADP (600 μM), and 11.6 mM sodium succinate were added and the respiration rate was measured. The hydroxylamine was added at the indicated concentration immediately before the succinate and the respiration measured in this assay system (+ADP) or immediately after adding 1.1 mM $CaCl_2$ (+$CaCl_2$).

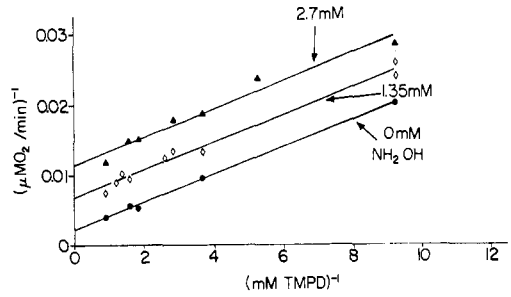

FIGURE 6: The inhibition of ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) oxidation by hydroxylamine. Rat liver mitochondria were suspended at 0.18 μM cytochrome *a* in a KCIT–P_i (with 200 μM EDTA) medium, pH 7.2. The assay medium contained 2 μM rotenone, 600 μM ADP, and 10.5 mM ascorbate. The respiration was started by adding TMPD; the reciprocal of the initial respiration rate is plotted on the ordinate and the reciprocal of the added TMPD concentration is plotted on the abscissa. The concentration of hydroxylamine is indicated in the figure.

5.9 mM succinate the hydroxylamine concentration required for 50% inhibition of respiration increases from 1.9 mM (ADP + P_i) to approximately 70 mM (+0.51 μM S-13).

The Release of Hydroxylamine Inhibition of Succinate Oxidation by Ca^{2+}. When $CaCl_2$ is added to mitochondria in state 4 (no ADP) in a medium containing inorganic phosphate, the respiration is stimulated during calcium transport (Saris, 1963; Chappell *et al.*, 1963). When it is added to mitochondria in the hydroxylamine inhibited state 3, the respiration is also stimulated as may be seen in Figure 4. In this case the hydroxylamine concentration required for 50% inhibition was 1.5 mM in state 3 and of the order of 40 mM when Ca^{2+} is present. The latter value is essentially that observed for uncoupled mitochondria. This is in agreement with the report of Utsumi and Oda (1969) that hydroxylamine inhibition is released by Ca^{2+}. It is in sharp contrast to azide inhibition for which the inhibition can be released by uncouplers (Wilson and Chance, 1967) but not by Ca^{2+}. When azide was used to inhibit succinate oxidation, 50% inhibition of state 3 (ADP + P_i) required 0.075 mM azide while 50% inhibition in the presence of Ca^{2+} required 0.17 mM azide (Figure 5). This is a ratio of 2:1 for Ca^{2+}:ADP + P_i as compared to a ratio of 13:1 found for the ratio of uncoupled:ADP + P_i. Calcium transport is therefore ineffective in releasing azide inhibition (Wilson and Chance, 1967).

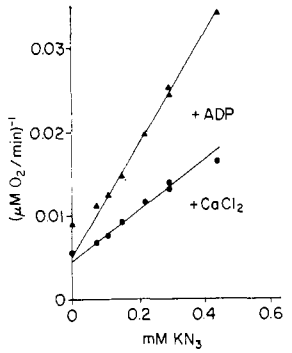

FIGURE 5: The effect of Ca^{2+} on the inhibition of succinate oxidation by azide. The rat liver mitochondria were suspended at 0.21 μM cytochrome *a* in MST–P_i, pH 7.2. Rotenone (2 μM), ADP (600 μM), and potassium azide were added, then the respiration was started with 11.8 mM succinate. The respiration was measured either in this assay system (+ADP) or immediately after adding 1.1 mM $CaCl_2$ (+$CaCl_2$). In the latter case, a linear rate was observed for approximately 1 min followed by a slow progressive increase in respiration. Only the initial linear rate was measured. The reciprocal of the respiration rate is plotted on the ordinate and the azide concentration is plotted on the abscissa.

The Inhibition of Ascorbate and N,N,N',N'-Tetramethyl-p-phenylenediamine Oxidation by Hydroxylamine. The inhibition of ascorbate–TMPD respiration by hydroxylamine is shown in Figure 6. The double reciprocal (Lineweaver–Burk) plots are parallel straight lines and show that the hydroxylamine changes both the K_m for TMPD and the maximal rate of electron transport (V_m). Unlike the inhibition of succinate oxidation this indicates that hydroxylamine inhibits primarily an electron carrier and not an energy conservation component. The apparent inhibitor constant calculated from the equations for uncompetitive inhibition is approximately 0.7 mM for state 3 (ADP + P_i). In contrast to the hydroxylamine inhibition of succinate oxidation the inhibition of ascorbate–TMPD oxidation is very similar to azide inhibition. The azide inhibition is also "uncompetitive" with respect to TMPD and an inhibitor constant of approximately 40 μM (pH 7.2) can be calculated. The hydroxylamine and azide differ, however, in that Ca^{2+} and uncouplers increase the calculated K_i for hydroxylamine by 2 or more while the K_i for azide is changed only by uncouplers.

The Uncoupler Concentration Dependence of the Release of Hydroxylamine Inhibition. When the uncoupler S-10 is added to a mitochondrial suspension in which succinate oxidation has been inhibited by hydroxylamine (Figure 7), a linear relationship is observed between the increase of respiration and the amount of added uncoupler up to a saturating amount of uncoupler. In the present work the uncoupler S-10 was found to be only slightly (10–20%) less active than S-13, as was reported by Williamson and Metcalf (1967). The slope of the portion of the curve representing uncoupler stimulation of respiration is independent of the succinate concentration but the maximal respiration rate attained increases with increasing succinate concentration. The maximal respiration is achieved at S-10 to cytochrome *a* ratios of 0.3, 0.6, 0.85, and 1.1 for succinate concentrations of 0.73, 1.46, 2.94, and 5.9 mM, respectively. This behavior is similar to the uncoupler

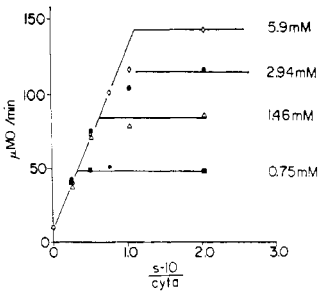

FIGURE 7: The titration of the release of hydroxylamine inhibition of succinate oxidation by S-10. The rat liver mitochondria were suspended at 0.24 μM cytochrome *a* in a KClT–P_i medium, pH 7.2. Rotenone (2 μM), ADP (660 μM), 17.6 mM hydroxylamine, and the indicated succinate concentration were added and then aliquots of S-10 were added. The measured respiration rates are plotted on the ordinate and the ratio of concentration of added S-10 to the concentration of cytochrome *a* are plotted on the abscissa. No more than two additions of S-10 were made per assay.

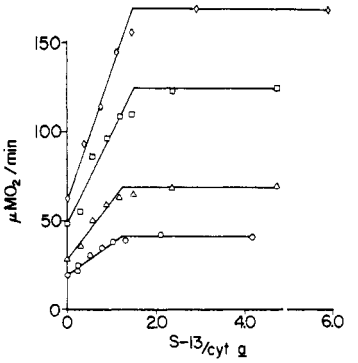

FIGURE 8: The electron flux dependence of the S-13 titer of the release of hydroxylamine-inhibited ascorbate–TMPD oxidation. Rat liver mitochondria were suspended in a MST–P_i medium, pH 7.2. Rotenone (2 μM), ADP (600 μM), and 5.9 mM sodium ascorbate were added in each case and the designated concentrations of hydroxylamine, TMPD, and S-13 added. The presented curves are normalized to 0.27 μM cytochrome *a* but the individual assay conditions were: (◊) 0.22 μM cytochrome *a*, 3.5 mM NH_2OH, 588 μM TMPD; (□) 0.27 μM cytochrome *a*, 5.88 mM NH_2OH, 294 μM TMPD; (△) 0.27 μM cytochrome *a*, 5.88 mM NH_2OH, 147 μM TMPD; (○) 0.33 μM cytochrome *a*, 7.06 mM NH_2OH, 73 μM TMPD.

dependent release of respiratory control (Wilson, 1969; Wilson and Azzi, 1968) except that slightly higher uncoupler to cytochrome *a* ratios are required to release hydroxylamine inhibition.

The observed S-13 titer for the release of azide inhibition of mitochondrial respiration and, in particular, the ascorbate–TMPD respiration has been used to demonstrate the stoichiometric binding of 1.35 moles of the uncoupler per mole of cytochrome *a*. When a similar titration was made of the release of hydroxylamine inhibited ascorbate–TMPD oxidation (Figure 8), it was found that the titer for the release of hydroxylamine inhibition was experimentally indistinguishable from the titer for the release of azide inhibition. For the release of both azide inhibition and hydroxylamine inhibition the uncoupler titer is independent of the rate of electron transport and of the rate of generation of high energy intermediates over a wide range of TMPD (0.07–1.1 mM) and hydroxylamine (3–10 mM) concentrations. The titrations in Figure 8 are typical for a single mitochondrial preparation. In this experiment the TMPD concentration was varied from 73 to 588 μM and the hydroxylamine from 3.5 to 7 mM. The resulting respiration rates in the presence of excess uncoupler varied from 41 μM O_2/min to 169 μM O_2/min, a factor of 4, and the change in respiration rate induced by S-13 varied from 21 μM O_2/min to 107 μM O_2/min, a factor of 5, while the S-13 to cytochrome *a* ratio was constant within experimental error.

Discussion

The hydroxylamine inhibition of mitochondrial respiration is valuable as a tool in the study of oxidative phosphorylation. The S-13 titer for the release of hydroxylamine inhibition of ascorbate–TMPD oxidation is independent of electron flux. The previous report of a stoichiometric binding of S-13 to a site which is present in a ratio of ~1.3 sites per cytochrome *a* (Wilson and Azzi, 1968; Wilson, 1969) is thus substantiated. In the release of hydroxylamine inhibition, as in the release of azide inhibition, the S-13 titer is independent of the rate of generation of high energy intermediates. This observation is not consistent with the chemiosmotic hypothesis in its present form (Mitchell, 1966). It is good evidence that the salicylanilides bind to a specific site in the respiratory chain (coupling protein?) and that this site has a profound effect on the electron transport through cytochrome oxidase.

Weinbach and Garbus (1969) have summarized the evidence that uncouplers are in general good protein binding agents. This is true also for the salicylanilides. Their coupling activity is completely reversed by subsequent addition of bovine serum albumin and after washing uncoupled mitochondria with bovine serum albumin the salicylanilide is quantitatively recovered in the albumin (D. F. Wilson, unpublished results).

The inhibition of mitochondrial respiration by hydroxylamine differs from that of any other described inhibitor. The ADP phosphorylation inhibitors oligomycin (Lardy *et al.*, 1958), *N,N'*-dicyclohexylcarbodiimide (Beechey *et al.*, 1967), and atractylate (Bruni *et al.*, 1962) are very similar to hydroxylamine with respect to the inhibition of state 3 succinate oxidation. In each case the kinetics of the inhibition are those of an inhibitor of energy conservation and not of electron transport. It is released almost completely by divalent ion transport (Ca^{2+}), gramicidin, and uncouplers, as shown by Utsumi and Oda (1969) and Wikström and Saris (1969).

In marked contrast to the inhibition of succinate oxidation the hydroxylamine is an uncompetitive electron transport inhibitor in the oxidation of ascorbate–TMPD. Neither oligomycin nor atractylate are inhibitors of ascorbate–TMPD oxidation (50% inhibition maximum) but the hydroxylamine inhibition is released by Ca^{2+}, gramicidin, and uncouplers. Azide, an electron transport inhibitor which is uncompetitive with respect to TMPD, is still very different from hydroxylamine since azide inhibition is uncompetitive with respect to succinate, is unaffected by Ca^{2+}, and is released by uncouplers.

Unfortunately, a survey of the known properties of hydroxylamine makes it unlikely that hydroxylamine itself is the primary reactive group. While hydroxylamine is known to

react with acyl phosphates, acylmercaptans, anhydrides, and carbonyl groups, it also forms complexes with transition metals including copper and iron. More important it is a powerful reducing agent which can undergo oxidation to products including N_2O, NO, and NO^-. Nitric oxide (NO) has a much higher affinity than hydroxylamine for heme or copper. Both NO and NO^- are powerful reductants and strong nucleophiles. In addition NO is an excellent ligand for cytochrome oxidase (Wainio, 1961), for catalase (Foulkes and Lemberg, 1949), and for hemoglobin (Gibson and Roughton, 1957). Utsumi and Oda (1969) have presented evidence that hydroxylamine is not reacting with acyl compounds but this still leaves many possibilities. Spectral and chemical studies are presented in the following paper (D. F. Wilson, G. R. Schonbaum, and E. Brooks, in preparation) which permit a partial resolution of the chemical entities which are responsible for the inhibition. A discussion of the possible mechanism for hydroxylamine inhibition is presented in the Discussion section of that paper.

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