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# INHIBITION OF OXIDATIVE PHOSPHORYLATION BY HYDROXYLAMINE IN SONICATED PARTICLES FROM BEEF-HEART MITOCHONDRIA

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SUMMARY

1. The effect of hydroxylamine on oxidative phosphorylation and respiration of sonicated submitochondrial particles from beef-heart mitochondria was studied and compared to the effects of azide, oligomycin and cyanide.

2. Hydroxylamine was found to inhibit oxidative phosphorylation without uncoupling effects. The inhibition was confined to the coupling site in the terminal region of the respiratory chain, as revealed from measurements of oxidative phosphorylation in the span from succinate to oxygen, or with the *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) shunt by-passing the second coupling site.

3. In contrast, cyanide improved the phosphorylation efficiency of the terminal coupling site by minimizing an "energy leak" associated with this site.

4. The inhibition of respiration by hydroxylamine was partially released by carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). "Reversed respiratory control" by ADP was demonstrated in the presence of hydroxylamine, this effect being also abolished by FCCP.

5. On the basis of the effects of hydroxylamine and cyanide, either cytochrome $a_3$ or copper of cytochrome *c* oxidase, or both, were suggested to participate in energy conservation and transfer at the terminal coupling site. The localization of the site affected by hydroxylamine between either cytochromes *a* and $a_3$, or $a_3$ and oxygen, was discussed in the light of the recent hypothesis for energy conservation proposed by STOREY[39].

## INTRODUCTION

Previous work from this laboratory has indicated that hydroxylamine is an inhibitor of oxidative phosphorylation apart from its weak inhibitory effect on cytochrome *c* oxidase[1-4]. Our primary results[1,2] have been essentially confirmed by UTSUMI AND ODA[5], who concluded that the inhibitor acts at an early energy-transfer step of oxidative phosphorylation. We have presented evidence in favour of the view that hydroxylamine leaves the primary energy-conservation step unaffected, but inhibits mainly energy transfer at a similar level as oligomycin, but differently from

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

the latter, specifically at the terminal coupling site of the respiratory chain[2–4,6]. The work of PIALOUX *et al.*[7] on the variation of free thiol groups depending on the energy state of mitochondria, included experiments with hydroxylamine that are in good agreement with our data. LINDBERG *et al.*[8] and GAUTHERON *et al.*[9] have also presented evidence that hydroxylamine inhibits oxidative phosphorylation. WILSON AND BROOKS[10] have more recently arrived at the same conclusion on the basis of kinetic data.

The site-specific action of hydroxylamine proposed by us is perhaps the most intriguing property of the inhibitor, because if this assumption is correct, some interesting conclusions may be drawn about the mechanism of oxidative phosphorylation in mitochondria.

The present work was undertaken in order to test whether the site specificity of inhibition by hydroxylamine can be found in sonicated submitochondrial particles. Possible transport effects of the inhibitor, which may have affected the results with intact mitochondria (but see ref. 4), can be neglected due to the opposite orientation of the membrane in sonicated particles in comparison with mitochondria[11,12].

## METHODS AND MATERIALS

### *Preparation of mitochondria and submitochondrial particles*

The mitochondria were isolated from beef heart, essentially as described by LÖW AND VALLIN[13]. $Mg^{2+}$-ATP particles from the heavy fraction of these mitochondria were prepared either as described by BEYER[14] (heavy electron transport particles, $Mg^{2+}$), or by the method of LÖW AND VALLIN[13].

### *Preparation of coupling factor $F_1 \cdot X$*

The coupling factor $F_1 \cdot X$ was prepared from the beef-heart mitochondria as described by VALLEJOS *et al.*[15]. The coupling factor preparation from "Extract 4" (ref. 15) was used.

### *Assay of oxidative phosphorylation*

Oxidative phosphorylation was followed by coupling the synthesis of ATP to formation of glucose-6-phosphate with glucose and hexokinase (EC 2.7.1.1), and by determination of formed glucose-6-phosphate either enzymatically with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and $NADP^+$, as described by SLATER[16], or by employing $^{32}P_i$ in the reaction mixture and extracting formed glucose-6-[$^{32}$P]phosphate with molybdate and isobutanol–benzene[17], followed by determination of the glucose-6-[$^{32}$P]phosphate by scintillation counting. Samples for determination of glucose-6-phosphate were withdrawn into cold $HClO_4$, usually when the oxygen was depleted from the reaction mixture. Control samples were similarily withdrawn after preincubation in the absence of substrate.

### *Spectrophotometrical, polarographical and analytical assays*

Polarographical and spectrophotometrical methods for the measurements of oxygen consumption and oxidoreduction of cytochromes and other respiratory carriers respectively, were used as described previously[4,6]. The ATPase (EC 3.6.1.3) activity of coupling factor $F_1 \cdot X$ was assayed by determination of liberated inorganic phos-

phate as described by LOWRY AND LOPEZ[18]. Protein concentrations were determined by the Folin procedure as described by LOWRY *et al.*[19].

*General experimental conditions*

The standard reaction mixture consisted of: 180 mM sucrose–50 mM glycylglycine–20 mM glucose–5 mM $KH_2PO_4$–3 mM $MgCl_2$. The pH was adjusted to 7.5 and the temperature was thermostated at 25°. Possible modifications of these general conditions are reported in the figure legends. The standard reaction mixture was taken to be 0.24 mM with respect to $O_2$.

*Reagents*

All reagents, except the carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), were commercial products of analytical grade. The FCCP was kindly supplied by Dr. P. G. Heytler of the E.I. du Pont de Nemours Company, Wilmington, Del. Hydroxylamine hydrochloride was purchased from E. Merck and Co., Darmstadt. A 4-M stock solution was stored at 4° for no longer than one week, and was neutralized with NaOH prior to use. The hexokinase (Calbiochem, A grade) was centrifuged at 10000 × *g* for 15 min, and the pellet resuspended in buffer consisting of 50 mM Tris–HCl (pH 7.4)–20 mM glucose–5 mM EDTA.

## RESULTS

*Experimental set-up*

In order to differentiate between the phosphorylation activities of coupling sites 2 and 3, two different systems of electron transport were employed experimentally. In both systems succinate was the respiratory substrate and rotenone was routinely present to prevent electron transfer across the first coupling site. Oxidative phosphorylation in the span from succinate to oxygen (coupling sites 2 *plus* 3) was assayed in the usual manner with no further basic additions. Oxidative phosphorylation associated with the third coupling site was assayed according to the procedure described by LEE *et al.*[20]. Antimycin was added to inhibit coupled electron transfer between cytochromes *b* and $c_1$; the amount of the inhibitor required for maximal inhibition was carefully titrated before each series of experiments, and a slight excess was added. The red/ox dye *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) was further added in its oxidized form (Wurster's blue) in order to shunt the reducing equivalents across the site of antimycin inhibition. In this manner the second coupling site is by-passed and phosphorylation associated only with the terminal coupling site is assayed[20]. This system is referred to as the TMPD shunt (or System 3), while the normal succinate respiration is referred to as System 2 + 3.

*Properties of the particles*

The $Mg^{2+}$-ATP particles used in the present study showed P/O ratios around 1.5 in the System 2 + 3 and around 0.5 with the TMPD shunt when high concentrations of ADP (2 mM) were employed. At low concentrations of ADP (0.1–0.2 mM), P/O ratios of approx. 0.6 and 0.3 were obtained with the Systems 2 + 3 and 3, respectively (*cf.* refs. 13 and 21). In addition, the particles prepared according to BEYER[14] had

a slightly lower phosphorylation efficiency than those prepared by the method of LÖW AND VALLIN[13].

*Rationale for inhibition of oxidative phosphorylation*

Since hydroxylamine is an inhibitor of cytochrome *c* oxidase, demonstration of a possible effect on energy conservation and oxidative phosphorylation in a system with low respiratory control, such as sonicated particles, is beset with considerable difficulties. TSOU AND VAN DAM[22] have demonstrated that pure inhibition of respiration in a partially uncoupled system results in lowered P/O ratios, because the straight line relating the phosphorylation rate to the rate of oxygen uptake does not go through the origin. With a true inhibitor of the respiratory chain, the slope of the line relating the phosphorylation and respiratory rates should not exceed the highest possible P/O ratio of the system[22]. On the other hand, titration of systems with poor respiratory control with a true inhibitor of oxidative phosphorylation such as oligomycin, results in a steep line. The slope of such a line will vary between the theoretical P/O value (in fully coupled systems) and infinity (in systems completely devoid of respiratory control). Thus it should be possible to differentiate between inhibition of respiration and oxidative phosphorylation by varying the rates with different concentrations of $NH_2OH$, and by plotting the data according to TSOU AND VAN DAM[22]. We have furthermore compared the effects of hydroxylamine with those of cyanide and oligomycin to see whether hydroxylamine behaves as a typical inhibitor of cytochrome *c* oxidase, or if it shows oligomycin-like effects as well.

*The inhibition of respiration by hydroxylamine*

A comparison between the effects of hydroxylamine on the rates of oxygen consumption in Systems 2 + 3 and 3, respectively, is shown in Fig. 1. The respiratory rates reported in this figure are average rates calculated from the time required to reach anaerobiosis. Average rates were used because oxygen consumption in the presence of hydroxylamine was non-linear (Fig. 2). The non-linearity was especially marked with the TMPD shunt, but was also present in System 2 + 3. It was still more marked with cyanide as the respiratory inhibitor, but with malonate, oxygen consumption always proceeded linearily. The non-linear oxygen consumption in the presence of hydroxylamine was characterized by a "double steady state" (Fig. 2), similar to that reported by NICHOLLS[23] for cyanide, but different from azide[23]. However, the respiratory rate cannot be inhibited by hydroxylamine to the same extent as with cyanide (see Figs. 1 and 4). We conclude that the inhibition of cytochrome *c* oxidase by hydroxylamine is, at least in some respects, similar to the inhibition by cyanide, but very different from the effect of azide. It therefore seems appropriate to compare the effects of these three inhibitors on oxidative phosphorylation.

*The inhibition of oxidative phosphorylation by hydroxylamine*

Fig. 3 shows the effect of increasing concentrations of $NH_2OH$ on the P/O ratios of Systems 2 + 3 and 3, respectively. It should be noted that the decrease of P/O in System 2 + 3 is strongly biphasic with approx. 60 % decrease already at low concentrations of the inhibitor, and weak inhibition of the remaining 40 %. In contrast, the P/O ratio of the TMPD shunt decreases in a monophasic fashion, approaching 100 % inhibition already at 3 mM of hydroxylamine. At this concentration the first

phase of inhibition in System 2 + 3 is completed. These data suggest that hydroxylamine inhibits oxidative phosphorylation specifically at the terminal coupling site at concentrations below 5 mM.

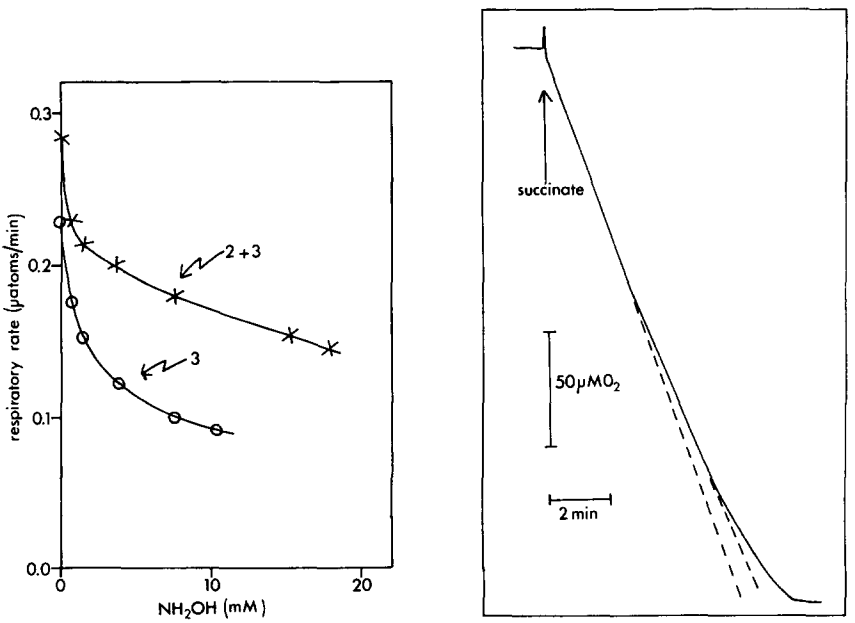


Fig. 1. Inhibition of respiration by hydroxylamine. The standard reaction mixture (see METHODS AND MATERIALS) was supplemented with 3 $\mu$M rotenone, 2 mM ADP (added as ATP), 50 enzyme units/ml hexokinase and 0.5 mg/ml particle protein (particle preparation according to LÖW AND VALLIN[13]). Carrier-free $^{32}P_i$ was added to the standard reaction mixture to a specific activity of approx. $3 \cdot 10^5$ disint. $\cdot$ min$^{-1} \cdot \mu$mole$^{-1}$. Hydroxylamine was present in the concentrations indicated. The reaction was started after 2 min preincubation by the addition of 10 mM Tris–succinate (Curve 2 + 3). In the TMPD shunt system (Curve 3), further initial additions were 100 $\mu$M TMPD and 0.35 $\mu$g/ml of antimycin. The average respiratory rates, calculated from the time required to reach anaerobiosis, were plotted in the figure.

Fig. 2. The kinetics of respiratory inhibition by hydroxylamine. The standard reaction mixture was supplemented with 3 $\mu$M rotenone, 2 mM ADP and 50 enzyme units/ml hexokinase. The particle protein concentration was 0.5 mg/ml. 12 mM of hydroxylamine was further added, and after 2 min preincubation the reaction was started by the addition of 10 mM Tris–succinate.

The plot of the rate of oxidative phosphorylation *versus* the respiratory rate is shown in Fig. 4. The rates were varied by different concentrations of hydroxylamine in the Systems 2 + 3 and 3, respectively. An experiment where the rates of System 3 were varied with cyanide is included for comparison. The right-hand portions of the curves labelled 2 + 3 and 3 approach straight lines with slopes of 1.8 and 0.9, respectively, probably indicating the maximal P/O ratio of the respective system. Thus only respiration was apparently inhibited at the lowest concentrations of $NH_2OH$. The extrapolation of these straight lines (shown in the figure) had approximately the same intercept with the ordinate, indicating that the maximal endogenous "energy leak" was the same in Systems 2 + 3 and 3 (*cf.* ref. 22). The steep portion of the Curve 2 + 3 indicates inhibition of oxidative phosphorylation without much effect on the respiratory rate. This part of the curve ($NH_2OH$ = 1.0–3.5 mM) corresponds to

the first phase of decrease in P/O shown in Fig. 3. The last portion of the 2 + 3 curve of Fig. 4 ($NH_2OH$ = 4–18 mM) has a slope of 1.8–2.0, which probably also indicates inhibition of oxidative phosphorylation although respiratory inhibition is relatively

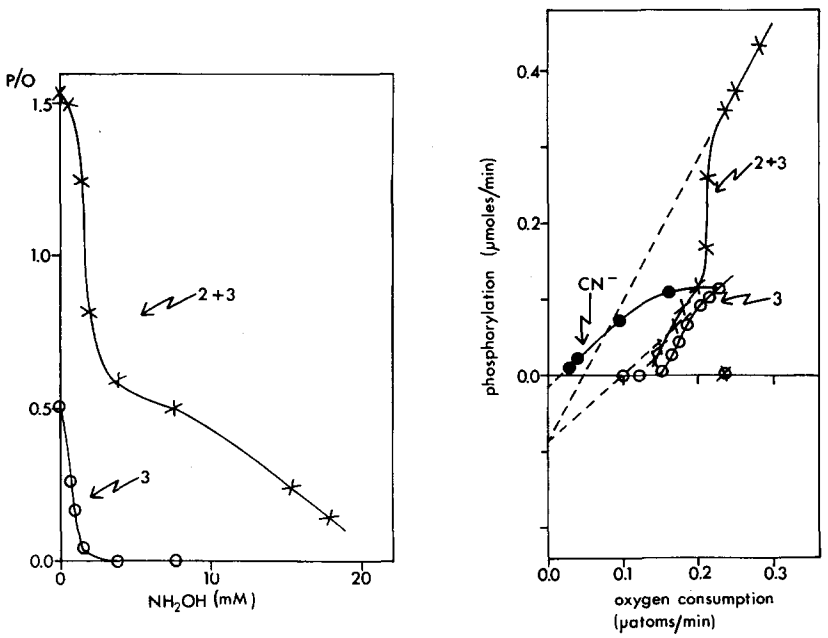


Fig. 3. Inhibition of oxidative phosphorylation by hydroxylamine. The experimental conditions were those described in the legend to Fig. 1. Formed glucose-6-[$^{32}$P]phosphate was determined as described in METHODS AND MATERIALS.

Fig. 4. Relationship between the rate of phosphorylation and the rate of oxygen consumption in particles inhibited by different concentrations of hydroxylamine or cyanide. The experimental conditions were those described in the legend to Fig. 1. The average rates of oxygen consumption were plotted against the average rates of phosphorylation at different concentrations of hydroxylamine or cyanide (added as the potassium salt, neutralized to pH 7.6 with HCl). The concentration of cyanide ranged between 0 and 125 $\mu$M, and the concentration of $NH_2OH$ between 0 and 18 mM (1.0–3.5 mM) and 0 and 7.7 mM (0.5–1.6 mM) in Systems 2 + 3 and 3, respectively. The values in parentheses correspond to the range of hydroxylamine concentrations where the steepest parts of the respective curves occurred. ×—×, System 2 + 3 inhibited by hydroxylamine; ○—○, System 3 inhibited by hydroxylamine; ●—●, System 3 inhibited by cyanide. The single point (⊗) was obtained with System 2 + 3 in the presence of 5 $\mu$g/ml of oligomycin.

high. This part corresponds to the latter phase of System 2 + 3 in Fig. 3. The steep part of Curve 3 in Fig. 4 ($NH_2OH$ = 0.5–1.5 mM) has a slope of about 2.0, clearly higher than the maximal P/O ratio possible with the TMPD shunt (compare with the slope of 0.9 with cyanide). Thus this result shows inhibition of oxidative phosphorylation in addition to the inhibition of respiration.

From the Curve 3 in Figs. 3 and 4 we know that 4 mM hydroxylamine inhibits oxidative phosphorylation completely at the terminal coupling site. The first phase of inhibition of oxidative phosphorylation in System 2 + 3 (Figs. 3 and 4) is thus best explained as due to inhibition at the terminal site, since it is also completed at 4 mM of $NH_2OH$, and since it does not seem justified to postulate a different affinity of hydroxylamine towards site 3 in the Systems 2 + 3 and 3. Thus it is site 2 phospho-

rylation that remains essentially unaffected at 4 mM of the inhibitor (Fig. 3) in agreement with the situation in rat-liver mitochondria[4].

The effect of cyanide (Fig. 4) is very interesting. The maximal slope is 0.9 as expected for a pure inhibitor of respiration in System 3. Thus the effect of cyanide definitely differs from hydroxylamine. In fact cyanide shows features opposite to those of hydroxylamine. Thus cyanide actually improves oxidative phosphorylation by displacing the straight line to the left. This effect is also seen in the System 2 + 3 (not shown). The intercept with the ordinate of the extrapolated cyanide line seems to indicate that cyanide effectively decreases the endogenous "energy leak" of the particles. Thus this "energy leak" may be associated mainly with the cytochrome *c* oxidase region of the respiratory chain.

*Comparison of the effects of hydroxylamine, azide, cyanide and oligomycin on oxidative phosphorylation*

Fig. 5 compares the effects of hydroxylamine, azide, cyanide and oligomycin on the P/O ratio of the TMPD shunt system. Hydroxylamine, azide and oligomycin all decreased the P/O ratio due to inhibition of oxidative phosphorylation as revealed by plots similar to that of Fig. 4. Such plots show that the inhibition of oxidative phosphorylation by azide is more clearly dissociated from inhibition of cytochrome *c* oxidase than when hydroxylamine is the inhibitor. Thus titration with azide gives

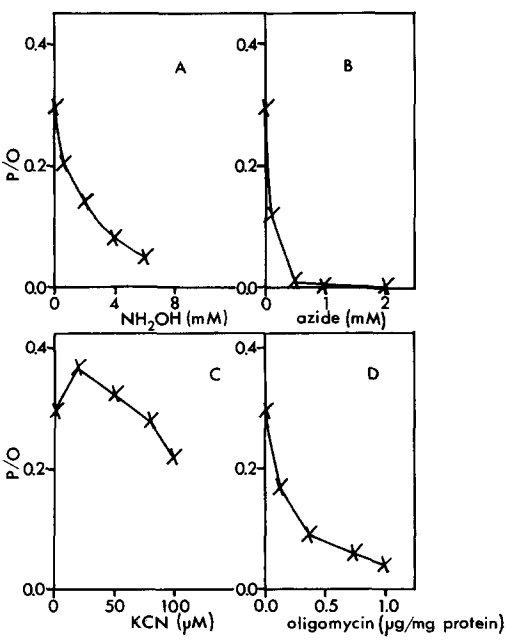


Fig. 5. Comparison of the effects of hydroxylamine, azide, cyanide and oligomycin on the P/O ratio of the TMPD shunt system. The standard reaction mixture was supplemented with 3 $\mu$M rotenone, 0.2 mM ADP, 50 enzyme units/ml hexokinase, 50 $\mu$M TMPD, 1.0 $\mu$g/ml antimycin and 0.7 mg/ml particle protein (preparation according to BEYER[14]). Different concentrations of hydroxylamine (A), azide (B), cyanide (C) and oligomycin (D) were further added. After 2 min preincubation the reaction was started by the addition of 10 mM Tris–succinate. The formed glucose-6-phosphate was assayed enzymatically (see METHODS AND MATERIALS).

steeper lines than hydroxylamine in the TSOU AND VAN DAM[22] plots with System 3. Oligomycin causes little inhibition of respiration, the corresponding line approaching infinity (see also the single point in Fig. 4). The effect of cyanide was discussed above. This pure inhibitor of cytochrome *c* oxidase causes an increase in the phosphorylation efficiency (Fig. 5C). The subsequent decrease in the P/O ratio at higher concentrations is due to inhibition of respiration (*cf.* Fig. 4). The effect of cyanide may be explained by a preferential inhibition of "non-phosphorylating respiratory chains". The data of Fig. 4 may further be taken to show that the activity inhibited by cyanide is not only "non-phosphorylating" but "energy-dissipating" as well (see DISCUSSION).

*The release of respiratory inhibition by FCCP and the effect of ADP*

Fig. 6A shows that the uncoupler FCCP partially released the respiratory inhibition by hydroxylamine in System 2 + 3. The factor of release ranged between 1.4 and 1.7. Addition of FCCP to the ADP-supplemented state in the absence of $NH_2OH$ had no effect on the respiratory rate. The partial release by FCCP of the inhibition induced by hydroxylamine indicates that the inhibitor causes a "high-energy state" in the particles that is dissipated by the uncoupler. It is interesting to note that the inhibitory factor of oligomycin in System 2 + 3 was only 1.2 under these conditions. The partial release of respiration by FCCP is associated with a transition of cytochrome *c* to a more oxidized state in the TMPD shunt system, as in intact mitochondria[6]. This suggests that the uncoupler-reversible inhibition by hydroxylamine is localized on the oxygen-side of cytochrome *c*. The addition of ADP to the hydroxylamine-supplemented particles (Fig. 6B) caused inhibition of the respiratory rate (and

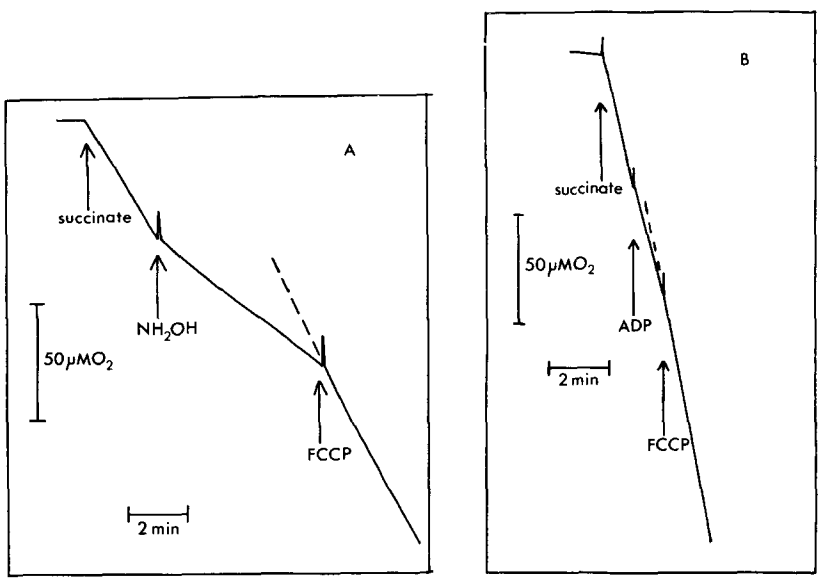


Fig. 6. The release of hydroxylamine-inhibited respiration by FCCP, and the respiratory inhibition by ADP. Standard reaction mixture. Further additions were (A) 3 μM rotenone, 0.5 mM ADP and 0.2 mg/ml particle protein (particles according to BEYER[14]). The additions indicated in the figure were 10 mM Tris–succinate, 12.5 mM hydroxylamine and 1 μM FCCP. (B) 3 μM rotenone, 1.6 mM hydroxylamine, 50 μM TMPD, 0.6 μg/ml antimycin and 0.45 mg/ml particle protein, with the further additions of 10 mM Tris–succinate, 0.5 mM ADP and 1 μM FCCP indicated in the figure.

reduction of cytochrome *c*). This effect, the so-called reversed respiratory control, was abolished by the further addition of FCCP. The total factor of respiratory release by FCCP is 1.5 in Fig. 6B. The release by FCCP in both Systems 2 + 3 and 3 in the presence of $NH_2OH$ is partially due to the abolishment of the reversed respiratory control as revealed by the finding that the release was less in the absence of ADP. The phenomenon of reversed respiratory control has been previously reported for azide by WILSON AND CHANCE[24], and has been interpreted by NICHOLLS AND KIMELBERG[25] as indication of a change in the mechanism of electron transfer.

*The mode of inhibition by hydroxylamine*

Inhibition of the synthesis of ATP by hydroxylamine as shown in the experiments above is not due to uncoupling of oxidative phosphorylation. Hydroxylamine is not an uncoupler in rat-liver mitochondria[4,6]. The results presented in Figs. 6A and 6B show that hydroxylamine on the contrary induces a more coupled state also in sonicated particles. Furthermore, hydroxylamine does not significantly affect the ATP-dependent reduction of cytochrome *b* by TMPD *plus* ascorbate, which proves that the inhibitor does not have uncoupling properties in submitochondrial particles. The effect of hydroxylamine on oxidative phosphorylation is thus clearly a true inhibition.

*The effect of hydroxylamine and azide on the ATP phosphohydrolase activity of coupling factor $F_1 \cdot X$*

Azide has been reported to inhibit the ATPase activity of coupling factor $F_1$ (ref. 26). We have found that it effectively inhibited also the activity of factor $F_1 \cdot X$. Hydroxylamine on the other hand had no effect on this activity. This finding would be expected for a site-specific inhibitor of oxidative phosphorylation.

## DISCUSSION

Our results strongly suggest that hydroxylamine inhibits oxidative phosphorylation specifically at the terminal coupling site of the respiratory chain, in good agreement with our previous results with intact rat-liver mitochondria[2–4,6]. The data presented in Figs. 3 and 4 indicate inhibition at site 2 as well, but at much higher concentrations of the inhibitor. When oxidative phosphorylation associated with site 2 was assayed directly in rat-liver mitochondria (ref. 4, Table II), negligible inhibition was found at 5 mM of $NH_2OH$. However, this concentration caused full inhibition of site 3 phosphorylation in sonicated particles (Figs. 3 and 4), and was sufficient to inhibit oxidative phosphorylation coupled to succinate oxidation by more than 85 % in rat-liver mitochondria[4].

There are numerous differences between the action of hydroxylamine and azide on respiration and oxidative phosphorylation (for a summary, see ref. 27). To these we may now add the different kinetics of inhibition of cytochrome *c* oxidase. Though WILSON AND BROOKS[10] and WILSON AND CHANCE[24] have shown that both hydroxylamine and azide are "uncompetitive" inhibitors of cytochrome *c* oxidase with respect to TMPD, the time-dependent kinetics of inhibition are quite different (Fig. 2 and ref. 23). In fact hydroxylamine resembles cyanide[23] in this respect, both inhibitors causing a "double steady state" on the way to anaerobiosis. A further difference

between hydroxylamine and azide is their effect on the ATPase of $F_1 \cdot X$. In this respect hydroxylamine resembles oligomycin[15]. However, hydroxylamine does not in contrast to oligomycin and azide inhibit the uncoupler-stimulated ATPase activities[4,5] or [$^{32}P_i$]ATP exchange[5].

Both hydroxylamine (M. K. F. WIKSTRÖM, unpublished results, and refs. 28, 29) and cyanide[29] combine with cytochrome $a_3$, and both show features of interference with the copper of cytochrome *c* oxidase as well[30,31]. The effects on oxidative phosphorylation coupled to the cytochrome *c* oxidase region are, however, quite different and indeed the opposite (Figs. 4 and 5). Hydroxylamine inhibits oxidative phosphorylation while cyanide appears to improve it. It may well be that the sonication procedure changes the properties of part of the cytochrome *c* oxidase molecules in such a way that they either (1) loose their energy-conservation capacity, or (2) do conserve energy that is subsequently dissipated. A distinction between these two possibilities may be obtained by analyzing the data of Fig. 4. If (1) would be the case, titration with a respiratory inhibitor, which does not discriminate between non-phosphorylating and phosphorylating respiratory assemblies, is expected to result in a straight line in the TSOU AND VAN DAM[22] plot, the slope of which is lower than the "theoretical" P/O value, the difference being proportional to the number of non-phosphorylating assemblies. A preferential inhibition of these "non-coupled" chains will result only in an increase of the slope without affecting the intercept with the ordinate of the extrapolated straight line. Inspection of Fig. 4 reveals immediately that this explanation is not applicable on the effect of cyanide. We conclude that the assemblies which are primarily affected by cyanide are not only non-phosphorylating, but do actually dissipate energy. Thus the endogenous "energy leak" observed under our conditions seems to be associated with the terminal coupling site and not for example with a generalized increase of membrane permeability towards monovalent cations[32], since the low concentrations of cyanide required would hardly affect the membrane permeabilities.

In view of the known properties of inhibition by cyanide[29–31] and hydroxylamine (M. K. F. WIKSTRÖM, unpublished results, and refs. 28–30), these results give strong support to the idea that either cytochrome $a_3$ or copper of cytochrome *c* oxidase, or both, are intimately involved in energy conservation at the terminal coupling site. The participation of cytochrome $a_3$ in the energy-coupling mechanism has been proposed recently[33,34]. Our results indicate further that the reactions of energy conservation, dissipation and transfer must differ in some respects at the different coupling sites (see *e.g.* Scheme 1 of ref. 4). Thus the presented evidence is at variance with the chemiosmotic theory of MITCHELL[35], which in its present form does not permit site specificity of the partial reactions of oxidative phosphorylation.

It has been pointed out by SLATER[36] that the terminal coupling site should reside between cytochrome $a_3$ and oxygen for thermodynamical reasons. Experimental evidence supporting this localization has been presented[33,37,38]. STOREY[39] has recently postulated that the free energy available from the oxidation of reduced cytochrome *c* oxidase by oxygen is conserved (coupling site 4), and fed into the energetically deficient sites 2 (between cytochromes *b* and $c_1$) and 3 (between cytochromes *a* and $a_3$), in order to account for both the thermodynamical and stoichiometrical requirements of oxidative phosphorylation. In view of this mechanism, inhibition at the site between cytochrome $a_3$ and oxygen (site 4) by hydroxylamine seems to be unlikely,

since this may be expected to cause inhibition at sites 2 and 3 as well, due to the coupling of site 4 to both sites 2 and 3 (ref. 39). Accordingly, hydroxylamine should inhibit at site 3, between cytochromes *a* and $a_3$. However, the thermodynamical and stoichiometrical requirements do not necessarily demand a mechanism such as that proposed by STOREY[39]. There is an alternative possibility that we feel suffers from less complex requirements on the molecular mechanism, and that fulfils the thermodynamics and stoichiometry of the process equally well. The fourth site between cytochrome $a_3$ and oxygen may be an independent coupling site similar to site 1, *i.e.* without coupling to sites 2 and 3. The latter two sites may well however be coupled together, each contributing one half of the free energy required for the synthesis of ATP. This proposal gains some experimental support from the findings[40,41] that inhibitors of the cytochrome $b$–$c_1$ region of the respiratory chain also inhibit oxidative phosphorylation coupled to the oxidation of TMPD *plus* ascorbate. If this possibility is true, hydroxylamine clearly inhibits oxidative phosphorylation at the site between cytochrome $a_3$ and oxygen. Our data do not allow us to differentiate between the two alternatives at present, but work is in progress to make the distinction possible.

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After preparation of this manuscript BLOKZIJL-HOMAN AND VAN GELDER[42] have reported that the EPR changes induced by hydroxylamine in cytochrome *c* oxidase are due to the formation of ferrocytochrome $a_3$–NO and not to a change in the EPR-detectable copper, as previously interpreted[30].

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