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THE EFFECT OF METHEMOGLOGIN ON THE INHIBITION OF CYTOCHROME *c* OXIDASE BY CYANIDE, SULFIDE OR AZIDE

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Abstract—Under our experimental conditions sulfide was a more potent inhibitor of a particulate preparation of cytochrome oxidase than was cyanide; azide proved to be a relatively weak inhibitor, all of which is in agreement with the observations of others. The undissociated species (H_2S) appeared to be more inhibitory than the anionic species (HS^-) in accord with the conclusions of others about HCN and HN_3. Addition of methemoglobin to the oxidase inhibited by cyanide or sulfide restored the activity of the enzyme system, but the addition of methemoglobin to the azide-inhibited oxidase under the same conditions had little or no effect. Our results suggest that sulfide produces death in animals by inhibition of cytochrome oxidase, but such a mechanism seems unlikely in the case of azide.

In vitro experiments have shown that isolated preparations of cytochrome *c* oxidase (cytochrome a-a_3) are susceptible to inhibition by cyanide and azide; the inhibitory species in each case appeared to be the undissociated acid [1]. The exact mechanism of inhibition by these agents is not known. Studies of the EPR spectra of the complexes of these inhibitors with a purified preparation of the enzyme showed that azide, cyanide and sulfide can compete for binding site on the oxidase. The affinities of cyanide and sulfide for the binding site were similar and greater than that of azide [2]. Nicholls [3] calculated the K_i values for the undissociated acids at pH 7.4 and found the value for HN_3 to be much higher than that for either HCN or H_2S. A slow and complex pattern of reaction with the oxidase was exhibited by HCN; H_2S also bound slowly and with a high affinity [4–6]. Cyanide and sulfide produced similar shifts in absorption spectra when they were added to a purified preparation of the oxidase, but the addition of azide at least in concentrations of less than 1 mM resulted in shifts in the absorption spectrum in the opposite direction [7,8].

Inhibition of cytochrome *c* oxidase is widely accepted as the mechanism for the lethal effects of cyanide in man and other species [9], but it has not been established that the same is true for sulfide or azide. Indeed, it may not be technically feasible to obtain direct evidence for such a mechanism. Indirect evidence for the inhibition of cytochrome *c* oxidase as a lethal mechanism common to cyanide, sulfide and azide is ambiguous. Similarities in toxic signs are recognized, e.g., all produce hyperpnea secondary to stimulation of the carotid body chemoreceptors [9]. The acute LD_{50}'s in mice differ over only a 5-fold range. Azide, however, is unique in that it is a potent, directly acting vasodilator. Animals that survive the acute (asphyxial?) effects may die later in a state of collapse. Oxygen appeared to increase the proportion of animals dying in the early convulsive stage although it had no influence on the overall mortality [10]. Oxygen alone does not affect the course of acute sulfide or cyanide poisoning [11]. The clinical experience with azide poisoning suggests that circulatory collapse rather than acute respiratory failure may be the proximal cause of death whereas the opposite is true for cyanide and sulfide [12].

Cyanide, sulfide and azide ions all form complexes with the ferric heme groups of methemoglobin, and their binding to the iron is mutually competitive [13]. Chemically-induced methemoglobinemia in animals protects them against otherwise lethal doses of all three poisons [13], but a common protective mechanism does not prove the existence of a common lethal mechanism.

The magnitude of the protection afforded to mice by methemoglobinemia correlates with estimates of the respective dissociation constants of the individual complexes. Cyanmethemoglobin is the most stable complex, and methemoglobinemia is most effective in protecting against cyanide poisoning. The azide-methemoglobin complex is the least stable, and methemoglobinemia affords only marginal protection against azide poisoning [13]. The limited clinical experience is consistent with these findings. Methemoglobinemia is well established in the treatment of cyanide poisoning [9]; it has been used successfully in one case of sulfide poisoning [14]; and, it had no effect when it was induced late in the course of one case of azide poisoning [12].

To obtain more insight into the biological effects of these inhibitors we have examined their influence in a purified preparation of cytochrome *c* oxidase, and at the same time we have observed the effects of added methemoglobin.

MATERIALS AND METHODS

Glass redistilled water was used throughout. Stock solutions of M/1 KCN were prepared with AR grade material in N/1 NaOH and refrigerated. The cyanide content was checked periodically by the method of Bruce *et al.* [15]. Stock solutions approximately M/10 in Na_2S were filtered, refrigerated and standardized weekly by potentiometric titration; an Orion sulfide electrode was used as the indicator [16]. Stock solu-

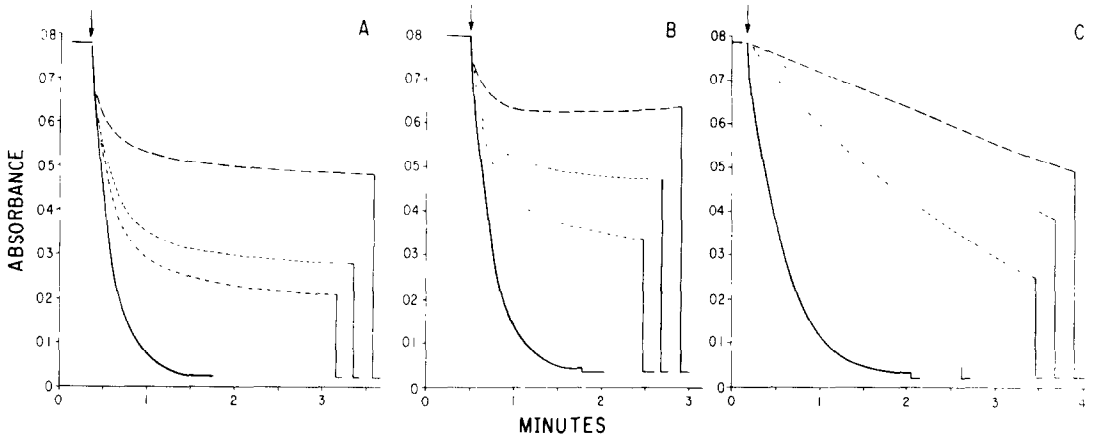

Fig. 1. The change in absorbance of cytochrome *c* at 550 nm with time in the presence of various concentrations of inhibitors. Cytochrome *c* was present in a concentration of 4.3 μM and cytochrome *c* oxidase was present in a concentration of about 10 nM. The arrows indicate the addition of cytochrome *c* oxidase. Panel A. The inhibitor is cyanide in the following concentrations: —— control (no cyanide), —·— 5 μM cyanide, - - - 10 μM cyanide, —— 50 μM cyanide. Panel B. The inhibitor is sulfide in the following concentrations: —— control (no sulfide), —·— 3.4 μM sulfide, - - - 6.8 μM sulfide, —— 13.6 μM sulfide. Panel C. The inhibitor is azide in the following concentrations: —— control (no azide), ------- 30 μM azide, —·— 500 μM azide, —— 1000 μM azide, —— 2000 μM azide.

tions of AR grade NaN_3 were prepared just prior to use.

Stock preparations of methemoglobin were prepared by incubating washed human red cells with a 2-fold molar excess of $NaNO_2$ relative to total heme for 1 hr at 37°. The cells were then washed three times in isotonic saline and resuspended to a concentration of about 5 mM heme in Krebs–Ringer phosphate buffer, pH 7.4. A minimal amount of saponin was then added to effect hemolysis. Spectrophotometric methods were used to estimate total hemoglobin [17] and per cent methemoglobin [18].

Submitochondrial particles were prepared from beef heart [19] and treated with deoxycholate (DOC) by adding 1 mg DOC/mg protein [20] to maximally expose the oxidase for reaction with cytochrome *c*. Cytochrome *c* was also prepared from beef heart [21] and purified by isoelectric focusing*.

Cytochrome oxidase activity in the presence and in the absence of inhibitors was assayed with a Cary 14 spectrophotometer by following the decrease in absorbance at 550 nm due to the oxidation of pure ferrocytochrome *c* by the enzyme [22]. Reactions were carried out in M/15 phosphate buffer at a pH of either 6 or 7 and at a temperature of 25°. Because the activity of cytochrome *c* oxidase is less at pH 7 than at pH 6, enzyme concentrations were diluted until their activities were equivalent at the two pH's.

In experiments in which methemoglobin was present at the start of the reaction (*e.g.*, Fig. 2) its ferric heme groups were necessarily in a state of equilibrium with respect to the inhibitor before the enzyme was added to initiate the reaction. Thus, the change in absorbance is a true estimate of the extent of the oxidation of reduced cytochrome *c*. Experiments in which methemoglobin was added after the effect of the inhibitor was manifested (Fig. 4) must be regarded as only semi-quantitative. Methemoglobin was always added to the reference cuvette, but an unknown fraction of methemoglobin in the experimental cuvette must have been complexed with inhibitor. Complex formation with any of the three inhibitors results in an increase in absorbance at the critical wavelength. Since the assay system involves a decrease in absorbance at 550 nm, a systematic error is introduced which leads to an underestimation of the effect of methemoglobin in relieving the inhibition.

* Unpublished method of G. S. McLain and L. Smith.

RESULTS

In the absence of inhibitors the reaction of cytochrome *c* oxidase with ferrocytochrome *c* obeys first

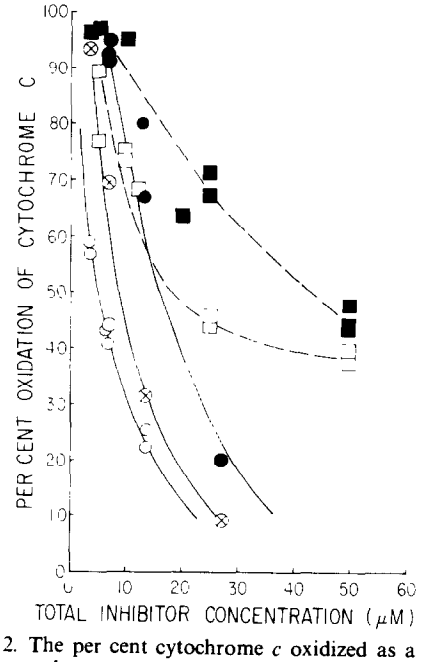

Fig. 2. The per cent cytochrome *c* oxidized as a function of the total concentration of cyanide or sulfide added. Zero per cent oxidation of cytochrome *c* represents a completely inhibited reaction. The effects of cyanide are shown without □ and with ■ added methemoglobin to 12 μM heme. The effects of sulfide are shown without ○ and with added methemoglobin to 6 μM heme ⊗ and 12 μM heme ●. Curved lines connect points with equal methemoglobin concentrations.

order kinetics [22]. In the presence of cyanide and sulfide, however, the reaction did not conform to first order kinetics. As previously observed [4,23], the inhibitory effects took time to appear. Characteristically, in the presence of inhibitors the reactions started rapidly, but eventually cyanide and sulfide in concentrations over the range from 3–50 μM brought reactions to a complete halt at some point short of completion (Fig. 1). The extent of the oxidation of ferrocytochrome *c* was therefore dependent upon the concentration of cyanide or sulfide. This pattern of inhibition was the same whether the reaction was initiated by the addition of cytochrome oxidase or by the addition of ferrocytochrome *c*. Our results are expressed as the per cent of cytochrome *c* oxidized when the reaction comes to a stop in the presence of cyanide or sulfide. A complete reaction is represented by the change in absorbance from its initial value to the value when all of the cytochrome *c* has been oxidized whether the reaction proceeds spontaneously or is brought to immediate completion by the addition of ferricyanide. Estimation of the amount of cytochrome *c* oxidized in the presence of sulfide was complicated by a slow reduction of oxidized cytochrome *c* which proceeded non-enzymatically. We corrected for this phenomenon by noting the increase in absorbance on addition of sulfide to control reaction mixtures in which the cytochrome *c* had been completely oxidized by cytochrome oxidase. This value was subtracted from the absorbance in the reaction mixtures where cytochrome *c* oxidase was the oxidant.

The pattern of inhibition obtained with similar concentrations of azide was different from that observed with cyanide or sulfide (Fig. 1). There was no initial lag before the inhibition was manifested, and the inhibited reaction approximated first order kinetics. Even concentrations as high as 2 mM in azide did not halt the reaction completely. Thus, in the case of azide it was possible to calculate first order rate constants in the presence of inhibitor and to express these as a per cent of the first order rate constant of the uninhibited reaction.

In Fig. 2 the per cent oxidation of ferrocytochrome *c* at the time of complete inhibition of cytochrome *c* oxidase by cyanide or sulfide has been plotted as a function of the total concentration of inhibitor initially added irrespective of whether or not methemoglobin was also present. A totally inhibited reaction is represented by zero per cent oxidation of cytochrome *c*. In each case the presence of methemoglobin resulted in an increased oxidation of cytochrome *c*, *i.e.*, reversal of the inhibition, except in cases where the inhibitor concentration was very much in excess of the methemoglobin heme concentration. The curved lines in Fig. 2 connect experimental points obtained with equal concentrations of methemoglobin. Increasing amounts of methemoglobin result in parallel shifts of these curves to the right by an increment approximately equal to the heme concentration. We were unable to produce significant reversal of an azide-inhibited system by similarly adding methemoglobin.

Figure 3 shows the inhibitory effects of cyanide, sulfide and azide on cytochrome *c* oxidation by cytochrome *c* oxidase at pH 6 and at pH 7. Both sulfide and azide are more effective inhibitors of cytochrome oxidase at pH 6 than at pH 7. No difference related

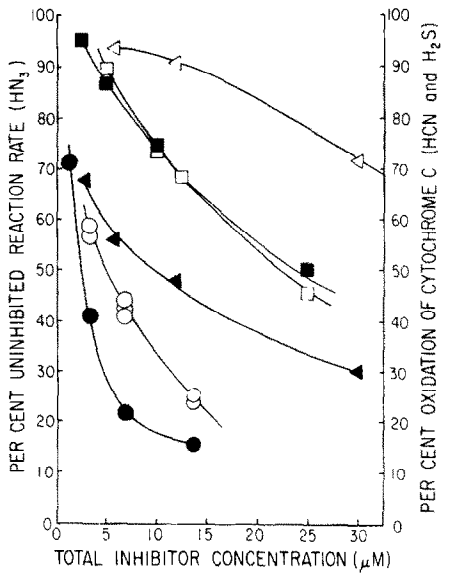

Fig. 3. The per cent cytochrome *c* oxidized as a function of the total concentration of inhibitor added: ○ sulfide, pH 7; ● sulfide, pH 6; □ cyanide, pH 7; ■ cyanide, pH 6; △ azide, pH 7; ▲ and azide, pH 6. See text for explanation of the abscissas.

to pH was apparent in the case of cyanide presumably because of the much higher pK_a of HCN. These results suggest that the undissociated acids, H_2S and HN_3, are more effective inhibitors of cytochrome *c* oxidase than are their respective anionic moieties. Others have already reached this conclusion in the cases of HCN and HN_3 [1,6]. At the same time it is clear from the work of others that methemoglobin complexes with the anionic forms: CN^-, N_3^- and HS^- [1,13]. The following pK_a values are relevant to the data of Fig. 3: H_2S = 7.1 [24], HCN = 9.2 [25] and HN_3 = 4.7 [26].

The effect of the addition of 11 μM methemoglobin heme to a reaction mixture inhibited by 14 μM sulfide is shown in Fig. 4. It can be seen that methemoglobin

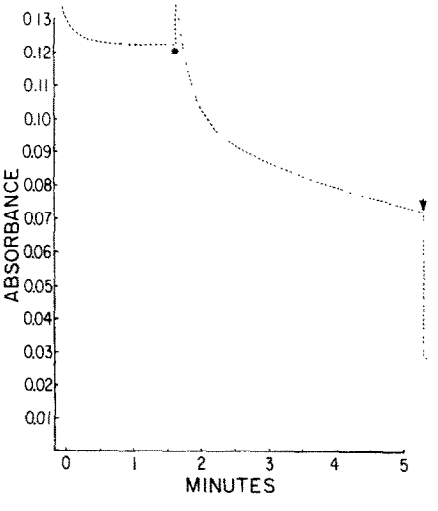

Fig. 4. The changes in absorbance at 550 nm with time for a reaction between cytochrome *c* (4.3 μM) and cytochrome oxidase (about 10 nM) in the presence of 14 μM sulfide. The asterisk indicates the addition of methemoglobin to 11 μM in heme to both blank and experimental cuvettes. The arrow indicates the addition of excess ferricyanide to the experimental reaction mixture.

re-initiated the oxidation of ferrocytochrome *c*. The addition of ferricyanide then oxidized the remaining ferrocytochrome *c*. Although these results can be regarded as only semi-quantitative for reasons explained above, a comparison of the results of Fig. 4 with those of Fig. 1 are consistent with the hypothesis that methemoglobin lowered the effective sulfide concentration of the system from 14–3 μM. The reaction end point reached after the addition of methemoglobin is approximately that which would have been predicted if the lower concentration of sulfide had been initially added to the reaction mixture in the absence of methemoglobin. Similar results were obtained with methemoglobin for cyanide-inhibited reactions, but the addition of methemoglobin to the azide-inhibited enzyme resulted in only a slight increase in the rate of the reaction (data not shown).

DISCUSSION

In confirmation of the results of others [3] we have found that sulfide is a more potent inhibitor of cytochrome oxidase than is cyanide. We have also confirmed [23] the relatively weak inhibitory effects of azide on purified preparations of the enzyme. Whereas concentrations of cyanide and sulfide in the μM range eventually bring to a complete halt the oxidation of cytochrome *c* by cytochrome oxidase, mM concentrations of azide merely slow the rate of the reaction.

Our results suggest that the undissociated acid, H_2S, is a more potent inhibitor of cytochrome oxidase than is its anionic moiety, HS^-. This conclusion is in accord with that of others for HCN and HN_3 [1,6]. No pH related difference was apparent in Fig. 3 in the case of cyanide; however, the pH's employed were 2–3 orders of magnitude below the pK_a for HCN whereas they were 1–2 orders of magnitude above the pK_a for HN_3. At physiological pH cyanide exists almost exclusively as the undissociated acid, sulfide is about half ionized and azide exists almost entirely in the ionized form.

Perhaps by coincidence the three inhibitors have similar acute toxicities in mice. The intraperitoneal LD_{50}'s in m-moles/kg of the respective sodium salts are 0.1 for cyanide, 0.3 for sulfide and 0.6 for azide [13]. Even if one assumes that all three indissociated acids are equi-effective as inhibitors of cytochrome oxidase, a difference in LD_{50}'s between cyanide and azide of about 2 orders of magnitude would be predicted simply on the basis of the relative concentrations of the respective unionized forms at pH 7.4. Although distribution and metabolic detoxication may play unknown roles, these facts argue against the hypothesis that inhibition of cytochrome oxidase accounts for the high lethality of azide *in vivo*. In the case of sulfide poisoning, however, our findings are consistent with an inhibition of cytochrome oxidase as the acute lethal mechanism.

The effects of methemoglobin in relieving cytochrome oxidase inhibition by the three agents as observed here correlates well with the protective effects of methemoglobinemia against the three inhibitors as observed in mice [13]. Methemoglobinemia was highly efficacious in the case of sulfide and cyanide, but it was only marginally protective against azide. Perhaps the inferior results against azide *in vivo* may be ascribed to the relative instability of the azide-methemoglobin complex as compared to the cyanide- or sulfide-complexes. As evaluated *in vitro* a constant concentration of methemoglobin was less effective in the case of azide-inhibition than in the case of sulfide or cyanide simply because of the higher total concentration of azide required to effect a comparable degree of inhibition.

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REFERENCES

1. J. N. Stannard and B. L. Horecker, *J. biol. Chem.* **172**, 599 (1948).
2. R. Wever, B. F. Van Gelder and D. V. Dervartanian, *Biochim. biophys. Acta* **387**, 189 (1975).
3. P. Nicholls, *Biochim. biophys. Acta* **396**, 24 (1975).
4. P. Nicholls, K. J. H. Van Buuren and B. F. Van Gelder, *Biochim. biophys. Acta* **275**, 279 (1972).
5. K. J. H. Van Buuren, P. F. Zurrendonk, B. F. Van Gelder and A. O. Muijsers, *Biochim. biophys. Acta* **256**, 243 (1972).
6. T. Yonetani and G. S. Ray, *J. biol. Chem.* **240**, 3392 (1965).
7. P. Nicholls, L. C. Petersen, M. Miller and F. B. Hansen, *Biochim. biophys. Acta* **449**, 188 (1976).
8. M. V. Gilmour, D. F. Wilson and R. Lemberg, *Biochim. biophys. Acta* **143**, 487 (1967).
9. R. E. Gosselin, H. C. Hodge, R. P. Smith and M. N. Gleason, in *Clinical Toxicology of Commercial Products*, 4th Ed., pp. III–105–112 and pp. III–169–173. Williams and Wilkins Co., Baltimore, (1976).
10. R. P. Smith, R. E. Gosselin and R. Kruszyna, *Ann. Intern. Med.* **83**, 739 (1975).
11. R. P. Smith, R. Kruszyna and H. Kruszyna, *Arch. Environ. Hlth.* **31**, 166 (1976).
12. E. A. Emmett and J. A. Ricking, *Ann. Intern. Med.* **83**, 224 (1975).
13. R. P. Smith and R. E. Gosselin, *Toxic. appl. Pharmac.* **8**, 159 (1966).
14. R. J. Stine, B. Slosberg and B. E. Beacham, *Ann. Intern. Med.* **85**, 756 (1976).
15. R. B. Bruce, J. W. Howard and R. F. Hanzel, *Analyt. Chem.* **27**, 1346 (1955).
16. H. Kruszyna, R. Kruszyna and R. P. Smith, *Analyt. Biochem.* **69**, 643 (1975).
17. P. J. Beal, L. P. Cook and V. A. Lovric, *Pathology* **6**, 251 (1974).
18. K. A. Evelyn and H. T. Malloy, *J. biol. Chem.* **126**, 655 (1938).
19. C. P. Lee and L. Ernster, in *Methods in Enzymology* (Eds R. W. Estabrook and M. E. Pullman) Vol. 10, p. 543, Academic Press, New York, (1967).
20. L. Smith and P. W. Camerino, *Biochemistry* **2**, 1428 (1963).
21. E. Margoliash and O. F. Walasek, in *Methods in Enzymology* (Eds R. W. Estabrook and M. E. Pullman) Vol. 10, p. 339. Academic Press, New York, (1967).
22. L. Smith and H. C. Conrad, *Archs. Biochem. Biophys.* **63**, 403 (1956).
23. S. Yoshikawa and Y. Orii, *J. Biochem.* **71**, 859 (1972).
24. H. L. Loy and D. M. Himmelblau, *J. phys. Chem.* **65**, 264 (1961).
25. G. Andregg, *Helv. Chem. Acta* **40**, 1022 (1957).
26. R. C. Weast, *Handbook of Chemistry and Physics*, 46th edn., Chemical Rubber Company, Cleveland, Ohio, (1965).