THE IN VITRO INHIBITION OF CYTOCHROME OXIDASE BY AZIDE AND CYANIDE

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Differences in the action of the commonly employed respiratory inhibitors, cyanide and azide, have been described in studies on a number of tissues and microorganisms; *e.g.*, resting and active frog muscle (1, 2), mammalian salivary glands (3), embryonic fish hearts (4), and yeast (5). More recently differences in the action of these substances on the oxidation of various substrates by cell-free tissue preparations have been noted (6). Many of these findings and their interpretation were reviewed by Stotz (7) and Goddard (8) and specific examples were outlined in the preceding paper (9).

As a possible mechanism to account for these differences in the action of cyanide and azide on respiration, it has been postulated that a different pathway of metabolism, independent of the cytochrome-cytochrome oxidase system, may account for the azide-insensitive, cyanide-sensitive respiration. As discussed in the preceding paper, the possible nature of this pathway is still unclear, although it is not difficult to find examples of systems with these characteristics (6, 10).

As an alternative to the separate pathway hypothesis, Ball (11) has proposed that the oxidation-reduction potentials of the azide and cyanide complexes formed with cytochrome oxidase may be sufficiently different to account for the results. Specifically it was postulated that azide might combine with both the ferro and ferri forms of the enzyme, thus stabilizing the potential at a level sufficiently above that of cytochrome *c* to permit oxidation of the cytochromes at a diminished rate. In the presence of cyanide, on the other hand, the potential of the cytochrome oxidase system would be too low to allow any oxidation. Since cytochrome oxidase has not yet been isolated, direct measurement of the oxidation-reduction potentials is impossible and no experimental verification of Ball's suggestion is possible. However, it is clear that more fundamental study of the mode of action of these inhibitors, especially on cell-free preparations, is required. The present study was initiated with this general objective in view, and specifically to observe the effects of azide and cyanide on the oxidation of ferrocytochrome *c* measured spectrophotometrically.

EXPERIMENTAL

Enzyme Preparations—The majority of the experiments were carried out with a cytochrome oxidase preparation of rat heart, similar to that of Stotz and Hastings (12), except that the washings preceding the extraction were omitted. This preparation was found to be free of intact cells and could be pipetted readily. It was usable with some loss of activity for about 2 weeks if kept refrigerated. This was diluted 1:100 to 1:5000 in appropriate buffer, the dilution needed being determined by the pH and, to a lesser extent, the age of the preparation.

Rat brain and frog skeletal muscle were prepared as 2 per cent homogenates in distilled water with a Waring blendor, according to the technique described by Albaum, Tepperman, and Bodansky (13).

All enzyme preparations were kept iced at all times until pipetted into the absorption cells.

Reagents—The buffers used were 0.05 M phosphate (Sørensen), McIlvaine type, or 0.1 M borate (pH 9 to 9.1). The nature of the buffer altered somewhat the absolute rates of reaction at constant pH (the rates in phosphate buffer were higher than in McIlvaine's buffer at neutrality but lower at pH values below 6.5). However, the effects of azide and cyanide were independent of the nature of the buffer used.

Cyanide solutions were prepared fresh for each experiment from Baker's reagent grade KCN by use of volumetric flasks arranged to have only a negligible gas space when brought to volume. Stock solutions were made up in 0.1 N NaOH and dilutions from these prepared and adjusted to the desired pH just before the test. This procedure was found to minimize loss of HCN from the very dilute solutions employed in the final stage.

Sodium azide solutions were freshly prepared each day in the appropriate buffer.

Cytochrome *c* was prepared as described in the previous paper and estimation of its concentration and purity carried out in the same manner. Before use in the spectrophotometric test system a dilute solution was deoxygenated with previously purified nitrogen, reduced with a minimal quantity of $Na_2S_2O_4$, and any excess hydrosulfite removed by aeration. To check the possible formation of H_2O_2 from the small excess of hydrosulfite added in this procedure, preliminary tests were made in which the cytochrome was only partially reduced by $Na_2S_2O_4$. No change in results could be detected.

Spectrophotometry—The Beckman spectrophotometer was employed and density readings made at 5500 A as a function of time. All determinations with inhibitor present were paralleled by a control set prepared from the diluted enzyme at the same time. The oxidation of ferrocytochrome *c* was first order with respect to this substance, and the results are expressed as

the first order reaction velocity constants. On the basis of fundamental equations already described by Altschul, Abrams, and Hogness (14), the concentration of ferrocytochrome c in moles per liter at any given time was calculated from the relation

$$C \text{ (ferrocytochrome } c) = \frac{d_t - \frac{\epsilon_{ferri}}{\epsilon_{ferro}} \cdot d_{Na_2S_2O_4}}{(\epsilon_{ferro} - \epsilon_{ferri})l}$$

where C (ferrocytochrome c) is expressed in moles per liter, ϵ_{ferro} and ϵ_{ferri} represent the extinction coefficients of ferro- and ferricytochrome c respectively, $d_{Na_2S_2O_4}$ is the optical density after addition of a slight excess of hydrosulfite, and d_t the optical density at any time, t. This becomes for a length, l, of 1.0 cm. and ϵ_{ferro} and ϵ_{ferri} equal to 2.80×10^4 and 0.84×10^4, respectively, at 5500 A

$$C \text{ (ferrocytochrome } c) = \frac{d_t - 0.3d_{Na_2S_2O_4}}{1.96 \times 10^4}$$

Correction for autoxidation of ferrocytochrome c was not necessary in most experiments, since this rate was negligible in comparison with the enzymatic rate. However, such determinations were included under each set of experimental conditions. It was noted that cyanide completely inhibited autoxidation, while azide at higher concentrations had a tendency to accelerate autoxidation (9).

Manometric Experiments—These were carried out in a conventional Barcroft-Warburg apparatus with reaction vessels of about 7 ml. capacity. The thin hind limb muscles of the frog were carefully dissected the evening before the experiment and treated as described by Stannard (1).

Results

Fig. 1 illustrates the proportionality of reaction rate to concentration of enzyme with the rat heart preparation. The rates are strictly proportional except for the point at 15×10^{-5} ml. of enzyme, which presented a much more rapid rate of oxidation than was employed in the inhibitor experiments, and which was too rapid for reliable measurement. It is concluded that the rates are sufficiently proportional to enzyme concentration to permit quantitative analysis of the inhibitor experiments.

Effect of Azide—Fig. 2 presents a composite plot representing the per cent cytochrome oxidase activity remaining as a function of the concentration of N_3^- and of undissociated acid, HN_3, at a series of pH values. Fig. 2 contains points obtained for the rat heart preparation only, but identical results were obtained at pH 7.4 with a homogenate of frog skeletal muscle.

A notable feature of the results plotted in Fig. 2 is the progression of apparent effect with pH if N_3^- ion concentration is used as the basis for

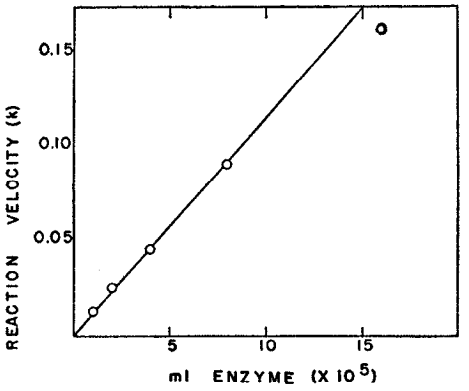

FIG. 1. Oxidation of ferrocytochrome *c* as a function of enzyme concentration (referred to the original enzyme preparation); pH 7.4, temperature 25°.

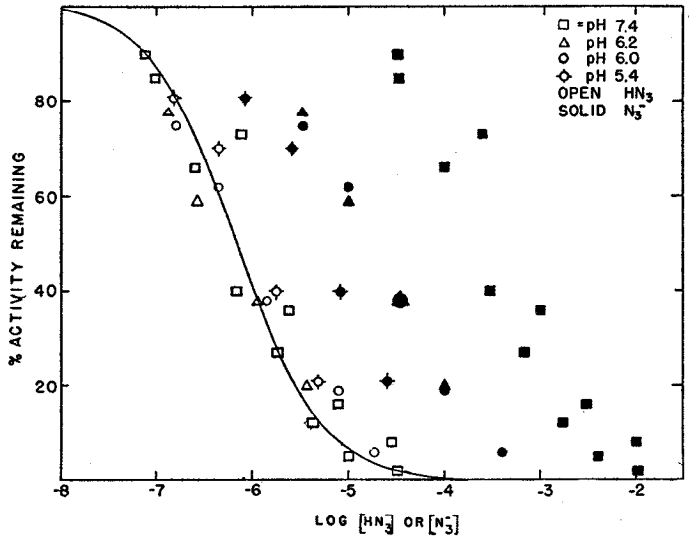

FIG. 2. The effect of azide on cytochrome oxidase activity as measured by oxidation of ferrocytochrome *c* in a rat heart preparation. The solid line is calculated from the mass law as described in the text, assuming a dissociation constant of 7×10^{-7}; temperature 25–26°.

comparison. Thus the solid symbols fit no single curve but would form a family of curves progressing to the left with decreasing pH. As a result there is a nearly hundred-fold difference in the N_3^- ion (and NaN_3)[1] con-

[1] Since the free acid is always a relatively small fraction of the total azide in the pH range employed (0.17 at pH 5.4, 0.002 at pH 7.4), the relationship of effect to total NaN_3 and to N_3^- is very similar.

centrations required to produce a given effect as the pH progresses from 7.4 to 5.4. Actually this only reflects quantitatively the change in free acid concentration and suggests strongly that inhibition is proportional to the HN_3 concentration. This is completely borne out by the good fit to theory (Fig. 2) obtained when computation is based upon the HN_3 concentration. For these reasons, the dissociation constant and other characteristics are reported on the basis of a reaction between the free acid (HN_3) and the

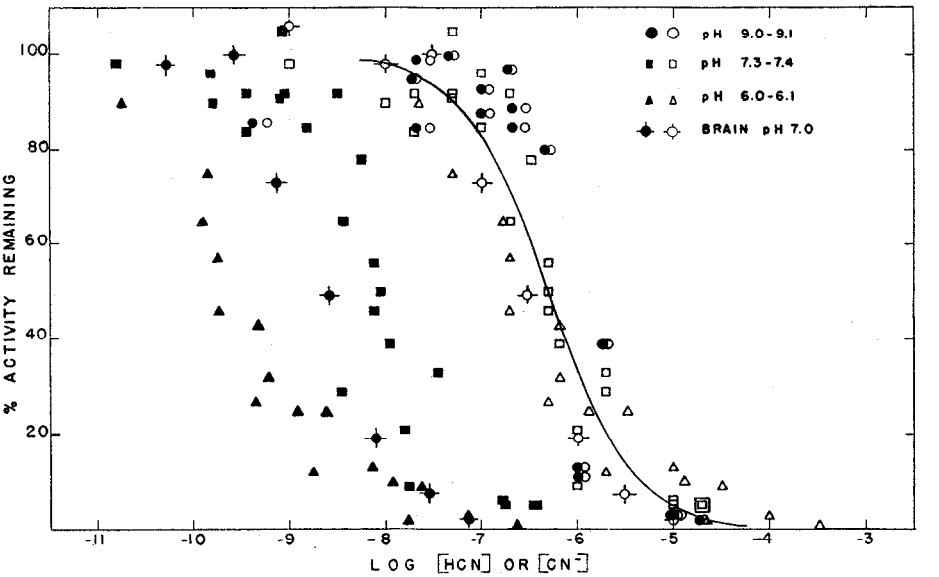

FIG. 3. The effect of cyanide on cytochrome oxidase activity of rat heart and brain preparations. The open symbols represent HCN concentrations, the solid symbols CN^- concentrations. The solid line is derived from the mass law as described in the text, assuming K for the enzyme-HCN complex = 5×10^{-7}; temperature 25–26°.

enzyme. As is indicated in the legend, the curve fitted to the open symbols is a theoretical one derived from the mass law and represents the expression

$$\frac{\text{(Free enzyme)}}{\text{(Combined enzyme)}} \cdot (HN_3)^n = K$$

where (free enzyme) is the fraction of activity remaining (combined enzyme), the fraction of activity inhibited, n the number of molecules of azide combining with each enzyme molecule (or active group), and K the dissociation constant of the enzyme-azide complex. In these calculations the dissociation constant of HN_3 was taken as 1.9×10^{-5} (15), $n = 1$, $K = 7 \times 10^{-7}$.

Effect of Cyanide—Fig. 3 presents a composite graph for experiments in

which the influence of cyanide on the enzymatic oxidation of ferrocytochrome *c* was determined at several H^+ ion concentrations. Rat heart and brain homogenates were employed as sources of the enzyme. The activity remaining is plotted as a function of log (CN^-) or log (HCN).

In spite of the precautions outlined in the experimental section for handling KCN solutions, the variability observed in these experiments was somewhat greater than in those with azide. Hence a larger number of tests was made. It is clear, however, that the effect of cyanide on both heart and brain preparations closely parallels that described above for azide. The points based on cyanide ion form a family of curves as a function of pH, while those relating effect to free HCN concentration fit reasonably well a theoretical curve derived from the mass law in the manner described above for azide. The dissociation constant for the enzyme-HCN complex is 5×10^{-7} and $n = 1$.[2] Thus the similarity in the action of azide and cyanide is marked and extends even to almost identical dissociation constants for the respective enzyme-inhibitor complexes.

Since the dissociation constant of HCN is very small (7.2×10^{-10} at 25° (15)), most of the cyanide is present as HCN except at the most alkaline pH. As a result there is very little difference in the inhibitory action of a given total KCN concentration at various pH values in the physiological range, a superficial contrast with the marked pH dependency seen with NaN_3. This same contrast has been observed in intact tissue studies (4). The basis for the phenomenon *in vitro* at least is now obvious.

Incidental to the above, it is also clear that, at pH 7.4 for example, cyanide will be a more effective inhibitor than azide on the basis of total salt at submaximal levels of inhibition. For example, at 10^{-3} M total salt and pH 7.4 the oxidase is completely bound to cyanide, but only 75 per cent is bound to azide. However, this is of importance only in determining the concentration range for exploration in experiments with intact tissues and does not represent a fundamental difference in action.

Manometric Experiments—A dependency of effect on pH was noted in Keilin's original observations on the effect of azide on yeast respiration (16), and, as noted above, both the presence of pH dependency in the case of azide and its absence in the case of cyanide were noted by Armstrong and Fisher (4). On the other hand, experiments with frog muscle (1) showed that the resting respiration remained insensitive to azide even when the pH of the medium was lowered to 4.6. In these earlier experiments, the respiration of stimulated muscle was studied at a single pH. In view of the hypothesis that cytochrome oxidase participates only in the respiration of

[2] The scatter of points in the cyanide tests necessitated trials of lines calculated on the basis of $n = 2$ and $n = \frac{1}{2}$, but the fit of these latter was obviously poorer than with $n = 1$.

stimulated muscle, a check of the effect of azide on the activity respiration at various pH values was considered of interest for comparison with the results *in vitro* already outlined.

Accordingly, the effect of selected NaN_3 concentrations on the oxygen uptake of frog muscle stimulated by immersion in 0.035 per cent caffeine (*cf.* (1)) was determined as a function of pH of the medium over the range 7.4 to 5.4. The results are summarized in Table I.

It is clear that there is a marked pH dependency in the effect of azide on the respiration of caffeinized frog muscle. Also it will be noted that the inhibition is not complete even at the lowest pH, since total respiration, not

TABLE I

Inhibition of O_2 Uptake of Caffeinized Frog Muscle by Azide at Various pH Values

Initial pH of medium	NaN_3	HN_3	Oxygen uptake (Q_{O_2})*			Per cent change	
			Control†	1st 30 min.	2nd 30 min.	1st 30 min.	2nd 30 min.
	$M \times 10^4$	$M \times 10^4$					
7.4	8.4	0.02	1.90	2.20	2.54	+16	+34
	8.2	0.02	1.56	1.80	2.16	+15	+39
7.1	8.9	0.04	4.57	4.16	3.72	−9	−18
	9.0	0.04	2.12	2.00	3.10	−6	+46
	9.0	0.04	2.56	2.62	3.08	+8	+20
	28.0	0.12	2.60	2.58	1.38	−1	−47
6.0	0.3	0.02	4.10	3.48	3.46	−15	−16
	9.1	0.45	5.76	1.78	1.18	−69	−80
5.6	8.2	0.94	2.74	1.18	0.76	−57	−72
	8.5	0.98	2.96	1.10	0.74	−63	−75
5.4	9.5	1.61	4.84	1.46	0.72	−70	−85
	9.5	1.61	6.98	2.26	1.44	−69	−79

* In c.mm. per mg. of wet weight per hour.

† Before addition of azide.

the increment due to caffeine, is reported in Table I. Since the intracellular H^+ concentration could not be determined, no attempt was made to place these data on the same basis as the spectrophotometric determinations. Also, penetration phenomena may modify the results obtained in experiments with intact tissues. However, the basic phenomenon is clearly present, and bears a close similarity to that observed in the oxidation of ferrocytochrome *c* measured spectrophotometrically.

DISCUSSION

The experiments presented above demonstrate that the enzymatic oxidation of ferrocytochrome *c* can be completely inhibited by both azide and cyanide when a spectrophotometric test system and cell-free homogenates

are employed. In addition, the properties of the reaction between enzyme and inhibitor are very similar with these two substances. There is no "azide-stable" fraction or other difference between azide and cyanide comparable to those seen in intact tissues (1–6) or to be expected from Ball's postulated behavior of the oxidation-reduction potentials of the enzyme-inhibitor complex (11). Unless the azide complex of cytochrome oxidase behaves differently *in vivo*, it is unable to oxidize cytochrome *c* and, therefore, could not account for the residual respiration obtained in the presence of azide. Thus other mechanisms, possibly still involving cytochrome *c*, but not the oxidase, must be sought for an explanation of the azide-insensitive, cyanide-sensitive respiration of intact tissues. The possibility that the inhibitor itself brings about qualitative changes in metabolism cannot be ignored (17), however, and further studies of this aspect with purified systems are in progress.

The effect of pH noted with azide in yeast and other intact cells and described above in muscle has frequently been attributed to permeability phenomena because of the more ready cellular penetration, in general, of uncharged particles. The appearance of this same phenomenon in cell-free preparations indicates that the explanation in intact tissues may be, in part at least, characteristic of the combination between enzyme and inhibitor. A study of the relative rôles of these two phenomena in intact cells is indicated, especially since it has been shown that certain local anesthetic bases enter *Arbacia* eggs as undissociated molecules but act in cationic form (18), while many barbituric acid derivatives both penetrate and act as undissociated molecules (19).

The experiments with cyanide were technically closely similar to those reported by Albaum, Tepperman, and Bodansky (13). Yet our results indicate a close agreement with equations derived from the mass law with *1* molecule of cyanide combining with 1 of enzyme, while Albaum *et al.* obtained a curve relating activity to log cyanide concentration, from which it would appear that 1 cyanide inactivates *4* enzyme molecules (or active groups). Consequently, their experiment with rat brain was duplicated exactly as described except for use of double the quantity of enzyme and slightly lower pH, and the results shown in Fig. 3 were obtained. Therefore, we are at a loss to account for the difference between these two series of experiments.

Except for the examples cited at the outset and above, it has not been possible to locate clear cases in the literature in which the reaction of an enzyme with cyanide or azide takes place with the free acid. Usually the form of combination is ignored, although many of the earlier studies on cyanide utilize terminology indicating that HCN was considered the active agent (*e.g.*, inhibition of *Atmungsferment* (20), catalase inhibition

(21), and peroxidase inhibiton (22)), but without formal proof or data which could be used for the necessary computations.

In the preceding paper (9), and in earlier work on the cyanide-cytochrome *c* complex (23), the reaction of azide and cyanide with ferricytochrome *c* was shown to involve the ionic forms, N_3^- and CN^-. Since the reaction with cytochrome oxidase involves the undissociated acid as shown here, the form of combination with ferrihemoglobin and ferrimyoglobin was considered of interest. The magnetic moment studies of Coryell, Stitt, and Pauling (24) show that the reaction between ferrihemoglobin and cyanide involves the CN^- ion. We have confirmed this finding, using a spectrophotometric method, and have extended the observations to include azide and ferrimyoglobin.[3] It was clear that the N_3^- and CN^- are the forms which combine with ferrihemoglobin and ferrimyoglobin. Thus the enzyme cytochrome oxidase appears in a somewhat anomalous position with respect to hemin type compounds of established structure. Interesting questions are raised, thereby, as to the nature of the combining groups in the enzyme and the structure of the oxidase as compared with well established hemin compounds.

SUMMARY

1. The oxidation of ferrocytochrome *c* by cytochrome oxidase preparations from rat heart and brain and from frog skeletal muscle was followed spectrophotometrically, and the effects of azide and cyanide investigated.

2. Both azide and cyanide can bring about complete inhibition of enzymatic cytochrome *c* oxidation.

3. Application of the mass law to the action of these substances at various pH values demonstrates that in both cases a complex is formed with the undissociated acids, HN_3 and HCN, while combination with ferricytochrome *c*, ferrihemoglobin, and ferrimyoglobin occurs with the respective ions. The dissociation constant for the enzyme-inhibitor complex was found to be 7×10^{-7} for hydrazoic acid and 5×10^{-7} for hydrocyanic acid.

4. The effect of azide on the oxygen uptake of stimulated frog muscle is dependent on the pH of the medium in qualitatively the same manner as the oxidation of ferrocytochrome *c* is in a cell-free homogenate.

5. The *in vitro* findings are compared with the action of azide and cyanide on intact tissues and microorganisms.

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