Willson, R. L. (1970), *J. Chem. Soc., Chem. Commun.*, 1005.
Woollett, G. H., Davis, F. M., Jones, C. N., and Neil, M. (1937), *J. Am. Chem. Soc. 59*, 861.
Wynn, J., and Gibbs, R. (1963), *J. Biol. Chem. 238*, 3490.
Yamazaki, I., and Piette, L. H. (1963), *Biochim. Biophys. Acta 77*, 47.
Yonetani, T. (1966), *J. Biol. Chem. 241*, 2562.

A Spectroscopic and Kinetic Investigation of Anion Binding to Ascorbate Oxidase†

Ronald E. Strothkamp and Charles R. Dawson*

ABSTRACT: The binding of azide, fluoride, and cyanide to ascorbate oxidase has been investigated in detail. Both azide and fluoride inhibit the enzyme competitively with respect to ascorbic acid and noncompetitively with respect to oxygen. Cyanide inhibition is much more complex and also results in inactivation of the enzyme. The binding of azide and fluoride to the resting enzyme is partially competitive. Fluoride binds more strongly to the resting enzyme, while azide binds more strongly to the functioning enzyme. It is proposed that both azide and fluoride bind to type 2 copper and that this copper is also part of an ascorbate binding site. It seems likely that type 2 copper is a reductant binding site in all of the "blue" oxidases. This proposal is used to explain the effect of fluoride on these enzymes and also to suggest a mechanism for the internal electron transfer which is necessary for the reduction of oxygen to water.

Ascorbate oxidase (EC 1.10.3.3, L-ascorbate:O_2 oxidoreductase), along with laccase and ceruloplasmin, comprise a group of enzymes commonly known as "blue" copper oxidases. All members of this group have many similar properties and the state of copper in these enzymes has been reviewed (Malmström et al., 1975).

All of these enzymes contain three types of copper. Fungal laccase, containing a total of four copper atoms, has been studied in detail with respect to the enzyme properties associated with the copper (Malkin et al., 1968, 1969; Brändén and Reinhammar, 1975). It has been suggested, based on total copper content and electron paramagnetic resonance and visible spectral properties, that both ascorbate oxidase and ceruloplasmin contain twice as much of each type of copper as laccase and that these two enzymes possess two "laccase-type" active sites of four copper atoms each (Strothkamp and Dawson, 1974; Mondovi et al., 1975). In agreement with this type of arrangement is the presence of two, identical, half-molecular-weight subunits in both ascorbate oxidase (Strothkamp and Dawson, 1974) and ceruloplasmin (Mukasa et al., 1968; Freeman and Daniel, 1973). If this proposal is correct, the results concerning the copper in laccase can also be applied to the other two enzymes. It should be noted that other suggestions have also been made concerning the stoichiometry of copper (Deinum et al., 1974; Veldsema and VanGelder, 1973; Deinum and Vänngård, 1973) and quaternary structure (Rydén, 1972; Simons and Bearn, 1969) of ascorbate oxidase and ceruloplasmin.

The catalytic mechanism of the "blue" oxidases is very complex. Both ascorbate oxidase and laccase are reduced via several one-electron transfers from substrate, as shown by the detection of free radicals formed from the substrate during the reaction (Yamazaki and Piette, 1961; Broman et al., 1963). The reduced form of the enzyme then reduces molecular oxygen to water by the addition of four electrons, probably via two, two-electron transfers (Malmström, 1970; Andréasson et al., 1973a). That all three types of copper in these enzymes are involved in the catalytic mechanism has been shown for laccase (Malkin et al., 1968, 1969), and some kind of internal electron transfer must take place (Andréasson et al., 1973b).

A number of reports on laccase and ceruloplasmin have confirmed the binding of various anions (Malkin et al., 1968; Kasper, 1968; Brändén et al., 1973; Byers et al., 1973; Herve et al., 1975) with subsequent changes in spectral properties and loss of catalytic activity, but much of the data is conflicting. A recent report has indicated an interaction between ascorbate oxidase and azide (Mondovi et al., 1975). It was felt that a detailed study of the anion binding properties of ascorbate oxidase could reveal much about the catalytic mechanism of this enzyme, as well as confirm additional similarities between all of the "blue" oxidases.

Experimental Section

Materials. Ascorbate oxidase was obtained from green zucchini squash (*Cucurbita pepo medullosa*) as previously described (Lee and Dawson, 1973), and was homogeneous by polyacrylamide gel electrophoresis at pH 9.5, according to the procedure of Davis (1964). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, Mo.). All reagents were of the highest purity available and all aqueous solutions were prepared just prior to use using deionized water.

Methods. Protein concentration was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin as standard. Copper determinations were performed according to Stark and Dawson (1958). Enzymatic activity was measured at 25 °C in a 1.5-mL reaction volume using a Clark oxygen electrode on a Gilson Model KM Oxygraph, under concentration and buffer conditions previously described (Dawson and Magee, 1957).

Spectral titrations were carried out using a Cary 118C recording spectrophotometer equipped with a thermostated cell holder maintained at 25 ± 0.3 °C. Both sample and blank were identical solutions of oxidized enzyme in 0.20 M phosphate-citrate, pH 5.6. Each addition of ligand to the sample was matched by an identical amount of deionized water added to the blank to maintain equal protein concentrations. Absorbance values were corrected for dilution during the titrations.

† From the Department of Chemistry, Columbia University, New York, New York 10027. *Received October 28, 1976.*

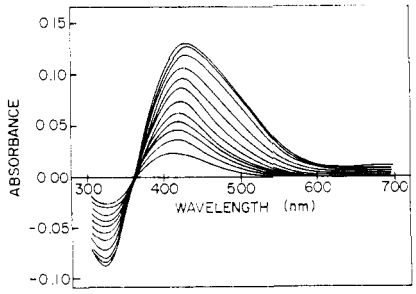

FIGURE 1: The visible spectrum of ascorbate oxidase treated with increasing amounts of sodium azide in 0.20 M phosphate-citrate (pH 5.6). The enzyme concentration is 28 μM and the blank is an identical solution of oxidized enzyme. Azide concentrations range from 0.30 to 49 mM. Increasing azide concentration parallels increasing absorbance at 430 nm. Further additions of azide in excess of 49 mM cause no additional increase in absorbance at 430 nm.

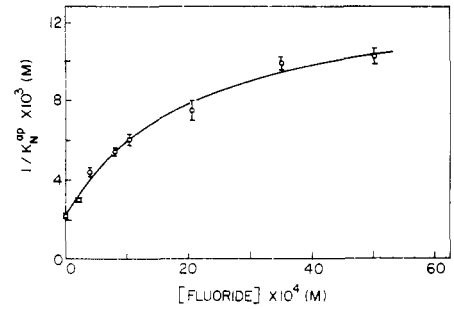

FIGURE 2: The relationship between the apparent azide equilibrium constant and fluoride concentration. The error bars indicate one standard deviation, as calculated by the method of least squares, and all values were determined at pH 5.6 in 0.20 M phosphate-citrate. The solid line is a theoretical fit, as explained in the text.

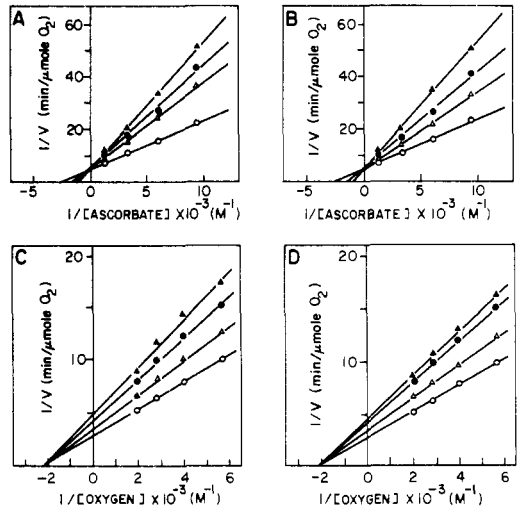

FIGURE 3: Patterns of inhibition for: (A) azide with respect to ascorbate at (○) 0, (△) 50, (●) 76, and (▲) 100 μM azide; (B) fluoride with respect to ascorbate at (○) 0, (△) 0.50, (●) 0.75, and (▲) 1.00 mM fluoride; (C) azide with respect to oxygen at (○) 0, (△) 50, (●) 100, and (▲) 150 μM azide; and (D) fluoride with respect to oxygen at (○) 0, (△) 0.51, (●) 1.00, and (▲) 1.49 mM fluoride. Ascorbate variation was done at 250 μM oxygen and oxygen variation at 750 μM ascorbate.

All kinetics experiments were performed using the Gilson Model KM Oxygraph and Clark oxygen electrode. The 1.5-mL reaction cell was thermostated at 25 ± 0.2 °C. The buffer used throughout was 0.20 M phosphate-citrate, pH 5.6, with 0.05% bovine serum albumin to keep the enzyme stable over the course of the experiment. Ascorbic acid solutions contained 0.1% metaphosphoric acid for greater stability (Dawson and Magee, 1957). Enzyme and buffer were equilibrated for several minutes before beginning the reaction, which was initiated with a small amount of ascorbic acid solution. When used, inhibitors were dissolved in deionized water and small volumes added just prior to reaction initiation. Oxygen variation was accomplished by passing oxygen or nitrogen gas over the reaction mixture in the oxygraph cell until the desired oxygen concentration was achieved, followed by immediate initiation with ascorbic acid solution.

Results

The result of titrating ascorbate oxidase with azide is shown in Figure 1. A new absorption band is formed at 430 nm and a negative band is seen at 330 nm. The new band at 430 nm has an extinction coefficient of 5000 M^{-1} cm^{-1}. The trough at 330 nm is due to a diminution in intensity of the band normally seen in the resting enzyme in this region (Strothkamp and Dawson, 1974). Double-reciprocal plots of absorbance vs. azide concentration at 430 nm are linear and the equilibrium constant is 464 M^{-1} at pH 5.6. The equilibrium constant is pH dependent, a value of 48 M^{-1} being obtained at pH 7.0.

Exhaustive dialysis removes azide from the enzyme and restores the original visible spectrum, with no loss of enzymatic copper or activity. Addition of azide to enzyme which has been reduced by an excess of ascorbate does not produce any new absorption bands in the visible region. Upon reoxidation with oxygen, the spectrum of the azide-complexed enzyme (Figure 1) appears.

Titration of the enzyme with fluoride does not produce any large changes in the visible spectrum. A small negative band at 380 nm and a small positive band at 310 nm can be seen with fluoride concentrations in excess of 10^{-3} M. Titration with azide when fluoride is present initially results in a decrease in the apparent azide binding constant. The effect of varying amounts of fluoride on the apparent azide binding constant, K_N^{app}, is shown in Figure 2. The plot of $1/K_N^{app}$ vs. fluoride concentration would be expected to be linear for the case of simple competitive binding. The actual curve obtained is explainable in terms of partially competitive binding. Both azide and fluoride can bind in the presence of the other, but with reduced binding constants. The equations for such a case are

$$\frac{1}{K_N^{app}} = \left(\frac{1}{K_N}\right)\left(\frac{1 + K_F[F^-]}{1 + K_F'[F^-]}\right) \quad (1)$$

and

$$K_N K_F' = K_N' K_F \quad (2)$$

where K_N and K_N' are azide binding constants to free and fluoride-complexed enzyme and K_F and K_F' are fluoride binding constants to free and azide-complexed enzyme.

The solid line in Figure 2 is the best simulated fit of eq 1 to the data. The values of K_F and K_F' are 3050 and 480 M^{-1}, respectively. Knowing that K_N is 464 M^{-1}, eq 2 yields a K_N' of 73 M^{-1}.

The patterns of inhibition by azide and fluoride with respect to both ascorbic acid and oxygen are shown in Figure 3. Data points for all plots are the average of at least three determinations. All replots of slopes and intercepts vs. inhibitor concentration are linear. Both inhibitors are competitive with respect to ascorbic acid and the binding constants are 16 400 and 1640 M^{-1} for azide and fluoride, respectively. With respect to oxygen, both inhibitors are noncompetitive with binding constants of 5000 and 435 M^{-1} for azide and fluoride, respectively. The two Michaelis constants for the uninhibited enzyme are 4.2×10^{-4} M for ascorbic acid at 250 μM oxygen and 4.5×10^{-4} M for oxygen at 750 μM ascorbate, under

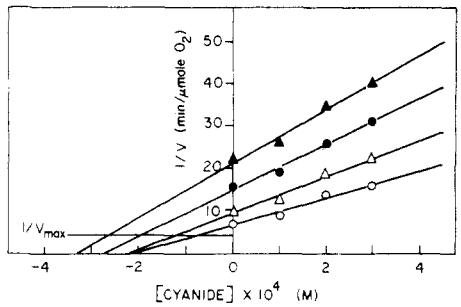

FIGURE 4: A Dixon plot (Dixon, 1953) of cyanide inhibition with respect to ascorbate at (○) 750, (△) 300, (●) 165, and (▲) 105 μM ascorbate. Oxygen concentration was 250 μM.

conditions cited under Materials and Methods. Both constants are a function of the fixed concentration of the second substrate.

Inhibition of ascorbate oxidase by cyanide is complicated by significant inactivation of the enzyme that occurs during the reaction. Initial velocities could not be determined as accurately as with the other inhibitors. The inhibition data for ascorbate variation is shown in Figure 4 in the form suggested by Dixon (1953). The lines are not parallel, which would indicate uncompetitive inhibition, nor are they convergent enough to show competitive or noncompetitive inhibition. The most reasonable interpretation is a mixture of uncompetitive and competitive inhibition. This would imply two binding sites and the binding constants can be found from replots of the standard double-reciprocal equation. The replots are linear with binding constants of 2140 and 7575 M^{-1} for the competitive and uncompetitive sites, respectively.

Cyanide data for oxygen variation gave lines that converged on or to the right of the y axis, indicating a situation approximating competitive inhibition. An estimation of the binding constant from this data gives 5260 M^{-1}.

Discussion

The spectral changes seen on binding of azide are qualitatively the same as those reported by Mondovi et al. (1975). They found a binding constant of 100 M^{-1} at pH 6.0 and electron paramagnetic resonance data suggested that azide was binding to type 2 copper. This idea is supported by our work in the presence of fluoride. Fluoride has been shown to bind to type 2 copper in both laccase (Malkin et al., 1968; Deinum and Vänngård, 1975) and ceruloplasmin (Andréasson and Vänngård, 1970). It is likely that it binds at the same site in ascorbate oxidase. The similarity of inhibition patterns and the binding competition between the two anions suggest that they do share a common binding site. Only partially competitive binding is seen because the type 2 copper can probably accommodate both anions simultaneously. Binding of two fluoride ions to the type 2 copper of laccase has been observed by electron paramagnetic resonance (Malkin et al., 1968), supporting the idea of two available coordination sites.

Similar azide binding properties have been reported for both laccase (Morpurgo et al., 1974; Holwerda and Gray, 1974) and ceruloplasmin (Andréasson and Vänngård, 1970; Manabe et al., 1971; Byers et al., 1973). Competition between azide and fluoride has been seen for ceruloplasmin (Andréasson and Vänngård, 1970), but not analyzed in detail.

Both azide and fluoride display the same types of inhibition with respect to both reducing and oxidizing substrates, but cyanide is much different. Cyanide also causes inactivation. Incubation of the enzyme with cyanide prior to starting the reaction has no effect on the rate or extent of inactivation, indicating that the mechanism of inactivation requires the enzyme to be functioning. It has previously been shown (Chang, 1970) that a large excess of cyanide both removes the copper and reduces the disulfide bonds of ascorbate oxidase. This effect may well be responsible for the inactivation seen here.

Since both azide and fluoride compete with ascorbic acid, it is likely that type 2 copper is an ascorbate binding site. Mondovi et al. (1975) have claimed that azide is uncompetitive with respect to ascorbic acid for ascorbate oxidase. However, they observed significant variations in the value of V_{max} from one experiment to the next, which casts doubt on their interpretation of the data. No such variation was seen in the present work. The lack of competition with oxygen suggests that type 2 copper is not an oxygen binding site.

The binding constants for azide and fluoride from both spectral studies and kinetics lead to certain observations. The difference in kinetically determined binding constants from ascorbate and oxygen variation data is predictable. The competitive ascorbate data gives the true binding constant. The constant resulting from noncompetitive oxygen data is a function of the fixed ascorbate concentration and will always be less than the true value. Azide binds much more strongly when the enzyme is functioning. Mondovi et al. (1975) also saw this. Several explanations are conceivable. It is possible that a conformational change may occur during turnover which increases the exposure of the azide binding site. Another possibility stems from the work of Holwerda and Gray (1974) on *Rhus* laccase. They have suggested the presence of a second azide binding site involving type 3 copper. A lack of significant absorbance change on binding of azide to this site would allow it to go unnoticed in spectroscopic experiments. However, type 3 copper, according to Holwerda and Gray, would be the stronger of the two binding sites and would be responsible for the inhibition seen with azide. This interpretation seems least satisfactory based on the work of Morpurgo et al. (1974), also using *Rhus* laccase. They found, as we have, a higher binding constant from kinetic studies than from spectral studies. However, both binding constants displayed very similar pH dependencies, making it most likely that azide binding to type 2 copper is responsible for both the spectral and kinetic effects.

Fluoride binds well to the resting enzyme, perhaps because the fluoride anion is smaller than the azide anion and does not require a conformational change to reach the binding site. Fluoride shows a decreased affinity during turnover. A recent report on laccase (Brändén and Reinhammar, 1975) indicates that type 2 copper may undergo reduction during the catalytic mechanism. This would explain the fluoride result as fluoride, in general, has a much lower affinity for copper(I).

Cyanide may very well bind at the oxygen site, although our results in this area are inconclusive. It has been shown for tyrosinase, containing only diamagnetic copper, that cyanide is competitive with oxygen (Gutteridge and Robb, 1975). Tyrosinase is similar to the "blue" oxidases in its ability to reduce oxygen all the way to water and it is reasonable to assume that cyanide might affect all of these enzymes in the same manner. If cyanide does actually compete with oxygen in ascorbate oxidase, it might be expected to display uncompetitive kinetics with respect to ascorbate, since it has been suggested, at least for laccase, that oxygen binds to a partially reduced form of the enzyme (Malmström et al., 1969). At pH 5.6, a small fraction of the cyanide is present as the anion. This may result in some binding to type 2 copper with resultant ascorbate competition. This would explain the less than parallel lines of Figure 4.

A number of suggestions have been made for the roles of the copper atoms in the catalytic mechanism of the "blue" oxidases. Type 1 copper is reduced first (Andréasson et al., 1973b) and must certainly be part of a reductant binding site. The diamagnetic copper, which is a two-electron acceptor, is reduced via an intramolecular step (Andréasson et al., 1973b). This site is then capable of transferring electrons in pairs to molecular oxygen (Malmström, 1970).

It has been unclear how a single type 1 copper can effect the reduction of the two-electron acceptor, and also how the type 2 copper becomes involved. Recent work has suggested that type 2 copper is involved in a reductant binding site (Holwerda and Gray, 1974; Brändén and Reinhammar, 1975). The present work provides additional support for this theory. The second electron reaches the two-electron acceptor via the type 2 copper. This theory can explain much of the past data in this area. It has been shown that fluoride, binding to type 2 copper, differentiates the electron accepting sites in laccase (Malkin et al., 1969). It could do this by inhibiting the binding of reductant at the type 2 copper site. Without this site as a source of electrons, the mechanism for reduction of the two-electron acceptor could not operate. There would no longer be an equilibration of added electrons among the various copper atoms. It was also observed in the same study that type 2 copper, normally the last site to be reduced, is reduced even more slowly in the presence of fluoride. This is because the reductant must compete with the fluoride in order to reduce this site.

It has been reported (Brändén et al., 1971) that dialysis of laccase against an ascorbate-containing buffer will remove fluoride from the inhibited enzyme. This would be the result of ascorbate competing with fluoride for the type 2 copper binding site. As fluoride is released, it is dialyzed away. This same type of activating effect has been observed for ascorbate oxidase as well (Gerwin et al., 1974).

The present work would suggest that the "blue" copper oxidases are highly complex, with the presence of two, nonequivalent reductant binding sites involving both type 1 and type 2 copper. The general features of anion binding to ascorbate oxidase are very similar to those observed for ceruloplasmin and laccase, providing further evidence for a close relationship between all of these enzymes (Dawson et al., 1975).

Acknowledgment

We are very much indebted to Prof. Stephen J. Lippard for making available to us the Cary 118C spectrophotometer, and also for helpful discussions concerning this work.

References

Andréasson, L.-E., Brändén, R., Malmström, B. G., and Vänngård, T. (1973a), *FEBS Lett. 32,* 187.

Andréasson, L.-E., Malmström, B. G., Strömberg, C., and Vänngård, T. (1973b), *Eur. J. Biochem. 34,* 434.

Andréasson, L.-E., and Vänngård, T. (1970), *Biochim. Biophys. Acta 200,* 247.

Brändén, R., Malmström, B. G., and Vänngård, T. (1971), *Eur. J. Biochem. 18,* 238.

Brändén, R., Malmström, B. G., and Vänngård, T. (1973), *Eur. J. Biochem. 36,* 195.

Brändén, R., and Reinhammar, B. (1975), *Biochim. Biophys. Acta 405,* 236.

Broman, L., Malmström, B. G., Aasa, R., and Vänngård, T. (1963), *Biochim. Biophys. Acta 75,* 365.

Byers, W., Curzon, G., Garbett, K., Speyer, B., Young, S., and Williams, R. (1973), *Biochim. Biophys. Acta 310,* 38.

Chang, H. T. (1970), Ph.D. Thesis, Columbia University.

Dawson, C. R., and Magee, R. J. (1957), *Methods Enzymol. 2,* 831.

Dawson, C. R., Strothkamp, K. G., and Krul, K. G. (1975), *Ann. N.Y. Acad. Sci. 258,* 209.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Deinum, J., Reinhammar, B., and Marchesini, A. (1974), *FEBS Lett. 42,* 241.

Deinum, J., and Vänngård, T. (1973), *Biochim. Biophys. Acta 310,* 321.

Deinum, J., and Vänngård, T. (1975), *FEBS Lett. 58,* 62.

Dixon, M. (1953), *Biochem. J. 55,* 170.

Freeman, S., and Daniel, E. (1973), *Biochemistry 12,* 4806.

Gerwin, B., Burstein, S. R., and Westley, J. (1974), *J. Biol. Chem. 249,* 2005.

Gutteridge, S., and Robb, D. (1975), *Eur. J. Biochem. 54,* 107.

Herve, M., Gernier, A., Tosi, L., and Steinbuch, M. (1975), *Biochim. Biophys. Acta 405,* 318.

Holwerda, R. A., and Gray, H. B. (1974), *J. Am. Chem. Soc. 96,* 6008.

Kasper, C/ B. (1968), *J. Biol. Chem. 243,* 3218.

Lee, M. H., and Dawson, C. R. (1973), *J. Biol. Chem. 248,* 6596.

Lowry, O. H., Rosebrough, N. J., Farr, A. A., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Malkin, R., Malmström, B. G., and Vänngård, T. (1968), *FEBS Lett. 1,* 50.

Malkin, R., Malmström, B. G., and Vänngård, T. (1969), *Eur. J. Biochem. 10,* 324.

Malmström, B. G. (1970), *Biochem. J. 117,* 15P.

Malmström, B. G., Agró, A., and Antonini, E. (1969), *Eur. J. Biochem. 9,* 383.

Malmström, B. G., Andréasson, L.-E., and Reinhammar, B. (1975), *Enzymes, 3rd Ed. 12,* 507.

Manabe, T., Manabe, N., Hiromi, K., and Hatano, H. (1971), *FEBS Lett. 16,* 201.

Mondovi, B., Avigliano, L., Rotilio, G., Agró, A., Gerosa, P., and Giovagnoli, C. (1975), *Mol. Cell. Biochem. 7,* 131.

Morpurgo, L., Rotilio, G., Agró, A., and Mondovi, B. (1974), *Biochim. Biophys. Acta 336,* 324.

Mukasa, H., Kajiyama, S., Sugiyama, K., Funakubo, K., Itoh, M., Nosoh, Y., and Sato, T. (1968), *Biochim. Biophys. Acta 168,* 132.

Rydén, L. (1972), *Eur. J. Biochem. 26,* 380.

Simons, K., and Bearn, A. G. (1969), *Biochim. Biophys. Acta 175,* 260.

Stark, G. R., and Dawson, C. R. (1958), *Anal. Chem. 30,* 191.

Strothkamp, K. G., and Dawson, C. R. (1974), *Biochemistry 13,* 434.

Veldsema, A., and VanGelder, B. F. (1973), *Biochim. Biophys. Acta 293,* 322.

Yamazaki, I., and Piette, L. H. (1961), *Biochim. Biophys. Acta 50,* 62.