PATHWAYS OF OXIDATION IN CELL-FREE POTATO FRACTIONS. II. PROPERTIES OF THE SOLUBLE PYRIDINE NUCLEOTIDE OXIDASE SYSTEM[1,2]

DAVID P. HACKETT

DEPARTMENT OF BIOLOGY, UNIVERSITY OF BUFFALO, BUFFALO, NEW YORK

In the previous paper (11), the oxidation of reduced diphosphopyridine nucleotide (DPNH) by isolated potato tuber mitochondria was examined. It was also shown that the supernatant fraction rapidly attacks DPNH by a reaction that does not involve molecular oxygen. More recently, it has been shown that the inclusion of cysteine in the isolating medium greatly enhances the ability of potato mitochondria to oxidize organic acids (10, 23). Accordingly, it was of interest to know whether the presence of cysteine has any effect on the reaction between the supernatant fraction and DPNH. The present study indicates that the cysteine-containing fraction does not catalyze the anaerobic DPNH attack, but it can, on the other hand, oxidize DPNH very rapidly by a reaction that requires oxygen. Thus, the soluble fraction can catalyze either an aerobic or an anaerobic reaction with DPNH, depending on whether or not cysteine is included in the grinding medium. This paper describes the properties of the oxidase system, and evidence that ascorbic acid is involved in the reaction is presented.

MATERIALS AND METHODS

Potato tubers (*Solanum tuberosum*, var. Katahdin) were shipped from Maine and stored at 10° C. Fifty grams of peeled tissue were sliced, passed through a meat-grinder, and then ground in a glass mortar with sand. The 30 ml of grinding medium was 0.5 M in sucrose, 0.05 M in phosphate buffer (pH 7.0), and 0.001 M in $MgSO_4$; when cysteine was included, its final concentration was 0.01 M. After filtering through cheesecloth, the homogenate was centrifuged at 2,000 × G for 5 minutes (Servall SS-1) to sediment the large particles. In most experiments the supernatant fraction was then spun at 100,000 × G for 1 hour in a refrigerated Spinco ultracentrifuge (Model L) and the soluble fraction decanted for use as the enzyme solution. When the intracellular localization was examined, the homogenate was centrifuged successively at 20,000 × G for 30 minutes and 100,000 × G for 1 hour; the residue at each stage was resuspended in grinding medium and recentrifuged to give washed mitochondrial and microsomal fractions respectively. Throughout all the above operations, the temperatures of the homogenate and fractions were maintained close to 0° C. The final soluble fraction was frequently frozen and stored at − 20° C for later use.

The oxidation of reduced pyridine nucleotides (PNH) was followed by measuring the decrease in optical density (OD) at 340 mμ with a Beckman DU spectrophotometer. Reactions were performed at room temperature in 1-cm cuvettes, to which the enzyme solution was added with a hypodermic syringe at zero time. When reactions were carried out in the presence of a gas other than air, a vaccine-stoppered cuvette was flushed with the appropriate gas mixture. To obtain strictly anaerobic conditions, the special cell of Lazarow and Cooperstein (14) was employed; the enzyme solution was kept in the side-arm during a 10-minute flushing with nitrogen. All reagents, coenzymes and gases were obtained from commercial sources. Reaction rates were calculated from the earliest linear portions of the time course curves; rates are expressed as OD units per minute, or micromoles PNH per minute per gram of initial tissue.

RESULTS

GENERAL PROPERTIES OF REACTION WHEN HOMOGENATE CONTAINS CYSTEINE: The addition of a small volume of cysteine-containing homogenate to a solution of DPNH is followed by a rapid decrease in the optical density at 340 mμ; reaction rates were of the order of 5×10^{-2} micromoles DPNH/minute × g fresh weight. This activity is roughly 10 times greater, on a fresh weight basis, than that associated with the heavy particle or mitochondrial fraction (11). More than 90 % of the activity was recovered in the final supernatant fraction, after centrifuging for 1 hour at 100,000 × G; this soluble fraction was used in all subsequent experiments. The washed microsomal pellet showed essentially no reaction with DPNH. The rapid reaction between DPNH and the soluble fraction is probably enzymatic in nature, since boiling destroys all the activity. The reaction rate remains essentially constant throughout and falls off in velocity only when the substrate is virtually completely oxidized (fig 1, curve A). The soluble system is relatively stable at low temperatures, the reaction rate declining only 35 % after storage for one week at − 20° C; many of the following experiments were carried out with previously frozen preparations. Reduced triphosphopyridine nucleotide (TPNH) can also be attacked by the soluble fraction and the ratio of rates of oxidation of DPNH/TPNH is roughly 1.5.

As shown in figure 1, curve A, the reaction with DPNH does not proceed under anaerobic conditions, but it goes rapidly to completion after the addition of oxygen, indicating that a true DPNH oxidase system is involved. The reaction thus differs sharply from the attack on DPNH which is catalyzed by a soluble fraction prepared in the absence of cysteine; in this case, the attack proceeds rapidly in the absence of oxygen (fig 1, curve B). Some indication of the relative oxygen affinity of the terminal enzyme in the DPNH oxidase system was obtained by carrying out the reaction in solutions flushed with various gas mix-

[1] Received May 16, 1957.

[2] This work was supported in part by a grant from the National Science Foundation.

tures. The rates of reaction in 5, 20, and 100 % oxygen were 22, 29, and 50 OD units/minute respectively. The fact that the rate in 20 % O_2 is only 60 % of the rate in 100 % O_2 suggests a relatively low oxygen affinity, comparable to that of flavin or copper-containing oxidases.

In order to demonstrate that DPN is the reaction product, an attempt was made to regenerate the DPNH. After the initial DPNH had been completely oxidized at pH 7, 1.5 ml of the reaction mixture was transferred to a cuvette containing TRIS buffer, pH 9; the oxidase is 90 % inhibited at the higher pH. Malate was then added as a hydrogen donor, since the endogenous malic dehydrogenase can catalyze the reduction of DPN at pH 9. The addition of malate resulted in an increase in OD at 340 mμ, indicating that DPNH was regenerated (fig 2). The theoretical amount that could be regenerated was calculated from the equilibrium constant, K^1 = (oxalacetate) (DPNH)/(malate) (DPN), and the concentration of the reactants. The constant, K^1, is markedly dependent on pH, and its value was calculated from the expression of Burton and Wilson (5), $K^1 = K_c \times$ antilog pH, using their mean value of 7.5×10^{-13} for K_c. The concentration of malate added is known (0.02 M), the DPN concentration is assumed to be equal to the original DPNH (from initial OD), and the amount of oxalacetate is assumed to be negligible. In order to prevent the breakdown of DPN by DPNase, 0.1 M nicotinamide was included in the original reaction mixture. Under these conditions, DPNH regeneration was 90 % of the theoretical in one experiment (fig 2). Clearly, DPN is the major product of the DPNH oxidase reaction.

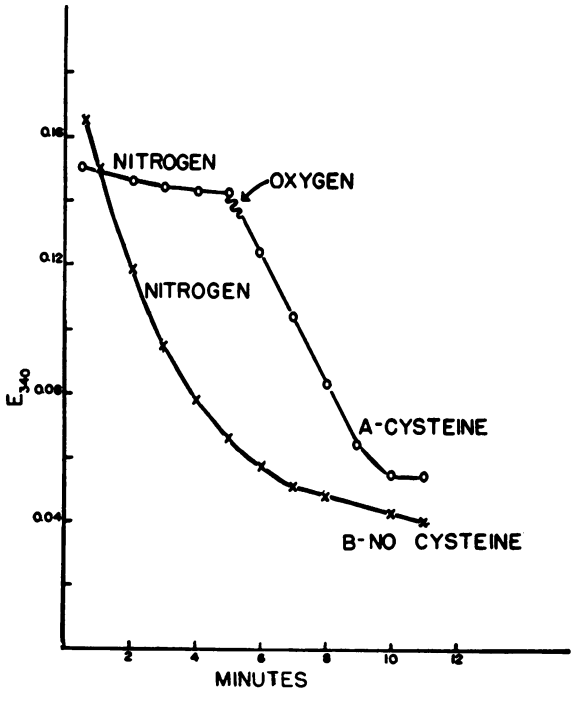

FIG. 1. The effect of anaerobic conditions on the reaction between DPNH and potato soluble fractions (A) with and (B) without cysteine. Cells contained DPNH, 0.25 M sucrose, 0.025 M phosphate buffer (pH 7). 0.3 ml enzyme added to side-arm of special cell (14) and tipped in after flushing with N_2 for 10 min. Final concentration of cysteine in (A) was 0.001 M.

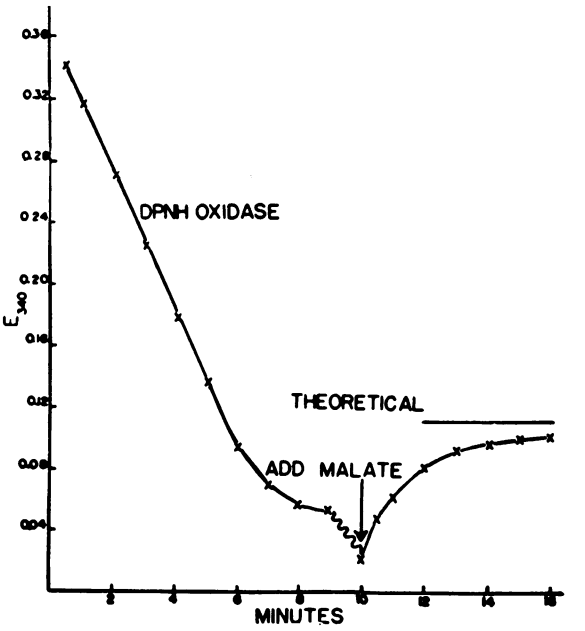

FIG. 2. Demonstration that DPN is a reaction product. Initially, cuvette contained 5.7×10^{-5} M DPNH, 0.25 M sucrose, 0.025 M phosphate buffer (pH 7), 0.1 M nicotinamide, and 0.3 ml enzyme. After 9 min, 1.5 ml reaction solution transferred to cuvette containing 1 ml of 0.15 M TRIS buffer (pH 9.3); malate added to give 0.02 M in 3 ml.

EFFECTS OF INHIBITORS: Table I shows the effects of some compounds which inhibit the activities of metal-containing enzymes. The fact that cyanide, at a concentration of 10^{-3} M, can essentially completely block the soluble DPNH oxidase system suggests that a metal-containing oxidase is involved. The oxidation of TPNH can also be prevented by cyanide. Nevertheless, the cyanide-sensitivity of this oxidase is considerably less than that of the mitochondrial cytochrome c oxidase, which is inhibited 95 % by 10^{-4} M cyanide (11). The fact that the copper-binding agent diethyl dithiocarbamate (DIECA) is an even more powerful inhibitor than cyanide suggests that the soluble oxidase contains copper. Phenylthiourea (PTU), which inhibits isolated polyphenol oxidase 50 % at 2.5×10^{-7} M (9), only inhibited the DPNH oxidase 29 % when used at 10^{-3} M. A further piece of evidence against the participation of polyphenol oxidase is the lack of carbon monoxide inhibition. The results suggest that the terminal oxidase may be a copper enzyme such as ascorbic acid oxidase.

A variety of inhibitors were tested in an effort to

TABLE I

EFFECTS OF SOME INHIBITORS OF (1) METAL-CONTAINING ENZYMES AND (2) SULFHYDRYL GROUPS ON THE DPNH OXIDASE SYSTEM

	INHIBITOR	PERCENT INHIBITION *	
(1)	0.0001 M NaCN	57	(2)
	0.001 M NaCN	90	(4)
	0.0001 M Diethyldithiocarbamate	82	(2)
	0.001 M Diethyldithiocarbamate	91	(2)
	0.001 M Ethylenediaminetetra acetate	12	(1)
	0.01 M Ethylenediaminetetra acetate	77	(2)
	0.001 M Phenylthiourea	29	(1)
	95/5 CO/O_2	0	(1)
(2)	0.001 M Iodoacetate	0	(1)
	0.0001 M Phenylmercuric acetate	39	(1)
	0.001 M Phenylmercuric acetate	100	(2)
	0.0001 M Parachloromercuri benzoate	0	(1)
	0.001 M Parachloromercuri benzoate	100	(1)

* Relative to initial rate of oxidation in control, which contained DPNH (3×10^{-5} M), sucrose-phosphate buffer (pH 7.0), and potato soluble fraction with cysteine. Values represent averages of number of experiments indicated in brackets.

block the hydrogen transport at some point between DPNH and the terminal oxidase. The following compounds had essentially no inhibitory effect: 5×10^{-4} M atabrine, 1.7 μg/ml Antimycin A, 1×10^{-3} M amytal, 1×10^{-4} M 2,4-dinitrophenol (DNP), 1×10^{-4} M $ZnSO_4$. It is not surprising that inhibitors which act on various mitochondrial oxidations are without effect on the soluble fraction. Neither the DNP-sensitive quinone reductase (27) nor glutathione reductase, which is inhibited by Zn ions (26), seems to be involved. Evidence was sought for a requirement of an essential sulfhydryl (-SH) group (table I). The complete inhibition by the two mercurials strongly suggests such a requirement. The apparent insensitivity of certain -SH inhibitors, e.g., iodoacetate, may be correlated with the presence of 10^{-3} M cysteine, which is introduced into the reaction mixture by the soluble fraction itself.

EXPERIMENTS WITH DIALYZED FRACTIONS: Dialysis of the soluble fraction against TRIS (hydroxymethyl-aminomethane, 0.05 M) buffer, pH 7, for 24 hours at 4° C completely eliminates its ability to oxidize DPNH and TPNH (fig 3). The activity can be partially restored by adding an equal aliquot of a boiled fraction, which alone shows no activity (fig 3). This reconstructed system oxidized DPNH at roughly half the rate of the undialyzed control (kept 24 hours at 4° C); the enzyme is diluted 30 % during the dialysis and this would account for some of the difference. It is clear that the original DPNH oxidase system requires the participation of both a dialyzable, heat-stable factor and non-dialyzable, heat-labile components. Accordingly, a variety of compounds were added to the dialyzed preparation in an attempt to reactivate the DPNH oxidase. The following inorganic compounds had essentially no effect: 3×10^{-3} M $NaNO_3$, 10^{-3} M $MgSO_4$, 10^{-3} M $MnSO_4$, 3×10^{-4} M H_2O_2; the absence of any effect with the last two suggests that the original reaction does not involve a DPNH peroxidase. The addition of cysteine, cystine, oxidized and reduced glutathione, at concentrations of 10^{-2} and 10^{-3} M, activated little or no DPNH oxidation. Apparently the soluble fraction lacks enzymes which can catalyze the transfer of hydrogen from DPNH to either cystine or oxidized glutathione.

In the presence of a suitable hydrogen (or electron) acceptor, the dialyzed fraction can readily oxidize DPNH. For example, the inclusion of 10^{-3} M pyruvate in the reaction mixture permits the oxidation of DPNH at a rate comparable to that measured with the undialyzed fraction. This finding confirms the report that potato extracts contain an active lactic dehydrogenase (7). Since this reaction is not affected by 10^{-3} M cyanide, it cannot be invoked to explain the original activity. DPNH is also rapidly oxidized by the dialyzed fraction in the presence of ferricyanide or 2,6-dichlorophenol-indophenol (fig 3); there was no reaction with methylene blue. The rate of the diaphorase reaction was similar to that of the DPNH oxidase in the undialyzed supernatant fraction. Added mammalian cytochrome c, 2 to 6×10^{-5} M, had no effect (fig 3), indicating that this soluble prepara-

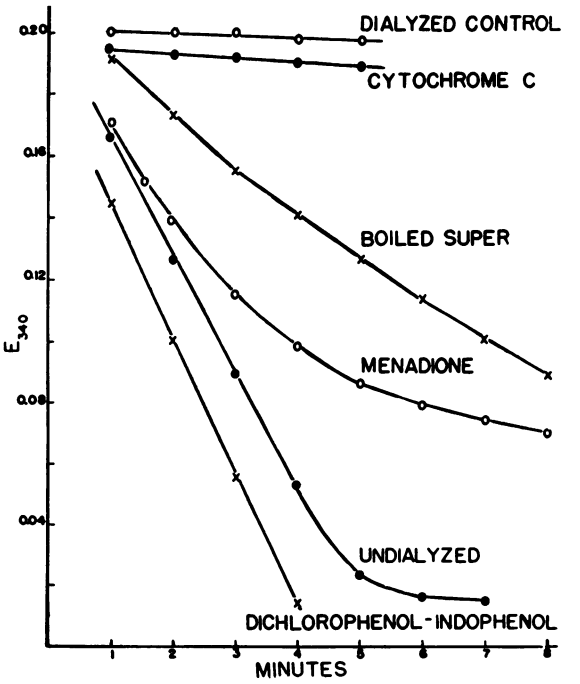

FIG. 3. The effect of dialysis on the DPNH oxidase reaction, and effects of additions to the dialyzed fraction. All cuvettes contained DPNH, 0.25 M sucrose, 0.025 M phosphate buffer (pH 7) and 0.3 ml dialyzed fraction (undialyzed where indicated). Final concentrations of additions: 2×10^{-5} M cytochrome c, 10^{-4} M menadione, 10^{-4} M 2,6-dichlorophenol-indophenol, and 0.3 ml boiled, undialyzed supernatant in 3.0 ml.

tion does not possess a DPNH-cytochrome c reductase system.

Menadione (2-methyl, 1,4-naphthoquinone) can serve as an effective hydrogen acceptor (fig 3). There is no reaction between DPNH and menadione either in the absence of the dialyzed fraction or in the presence of boiled enzyme, indicating that a DPNH-menadione reductase system is present. However, this reaction is inhibited only 20 % by 10^{-4} M dinitrophenol, in contrast to the DNP-sensitive quinone reductase described earlier (27). The DPNH-menadione reaction is completely prevented by 10^{-2} M cysteine, probably due to the formation of a product between cysteine and menadione (28), and this fact argues strongly against the participation of quinones as intermediate carriers in the DPNH oxidase system of the cysteine-containing supernatant.

Ascorbate-Activated Reaction: The effects of inhibitors on the DPNH oxidase system suggested that ascorbic acid oxidase might be involved in the reaction. This possibility is supported by the fact that addition of a suitable concentration of ascorbic acid to the dialyzed supernatant results in a rapid oxidation of DPNH (fig 4). The optimum ascorbate concentration is from 10^{-2} to 10^{-3} M, and the maximum oxidation rates were roughly one-half those measured with an equivalent amount of the original undialyzed fraction, i.e., around 2×10^{-2} micromoles DPNH/min × g. In one experiment, the rate of ascorbate-activated DPNH oxidation, corrected for dilution of enzyme during dialysis, was 73 % of the original rate. This suggests strongly that much, if not all, of the DPNH oxidase requires the participation of ascorbic acid. Ascorbate was also able to activate the oxidation of TPNH by the dialyzed fraction. It is of considerable interest that dehydroascorbic acid is unable to activate DPNH oxidation.

The properties of the ascorbate-activated reaction were studied in order to permit a direct comparison with the original DPNH oxidase. There is no reaction under anaerobic conditions, and the participation of an oxidase is clearly indicated by the fact that it proceeds rapidly when oxygen is added. As with the undialyzed preparation, the rate was markedly increased when 100 % O_2 was substituted for air in the reaction mixture. Using malate as a hydrogen donor, it was possible to show that DPN is a product of the reaction. The system is not inactivated by freezing for several days. The following inhibitions of the ascorbate-activated reaction were measured: 10^{-3} M cyanide, 44 %; 10^{-3} M DIECA, 75 %; 10^{-4} M PTU, 9 %; 10^{-4} M phenylmercuricacetate, 100 %; 10^{-4} M parachloromercuribenzoate (PCMBA), 100 %. These results suggest that both a copper-containing enzyme (oxidase) and an essential -SH group are involved in the ascorbate-activated reaction.

Reaction in Absence of Cysteine: It was reported earlier that the 10,000 × G supernatant fraction from a sucrose-phosphate homogenate also attacks DPNH rapidly, as indicated by the decrease in OD at 340 mμ (11). Subsequent experiments have

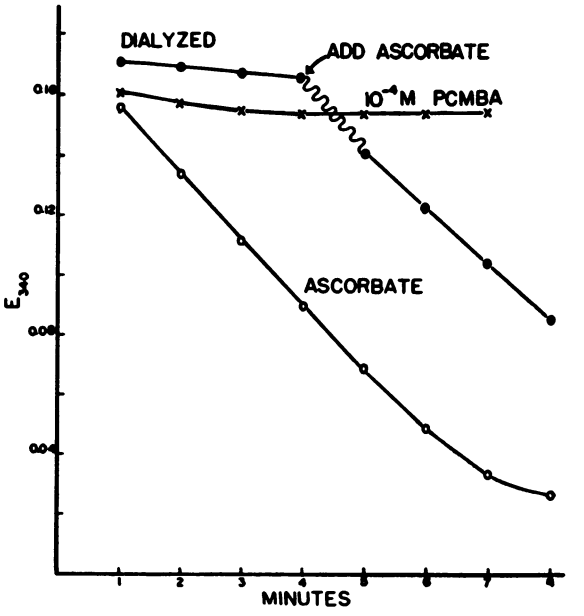

Fig. 4. Activation of the DPNH oxidase reaction in the dialyzed fraction by ascorbate, and its inhibition by parachloromercuribenzoate (PCMBA). All cuvettes contained DPNH, 0.25 M sucrose, 0.025 M phosphate buffer (pH 7), and 0.3 ml enzyme. 0.001 M ascorbate present initially or added after 4 min (where indicated); PCMBA, 10^{-4} M; final volume, 3.0 ml.

shown that this reaction is catalyzed by soluble components which are not sedimented at 100,000 × G (1 hour). The rate of DPNH attack by this system is in the range 3 to 4×10^{-2} micromoles DPNH/min × g fresh weight, or roughly half that of the DPNH oxidase described above. Unlike the DPNH oxidase reaction, the time course curve does not remain linear (fig 1). The system is completely inactivated by boiling, but freezing and storage for several days at − 20° C has no effect. Dialysis removes all the activity, but the control kept for 24 hours at 4° C also shows a greatly diminished activity.

The reaction between DPNH and this fraction proceeds under anaerobic conditions (fig 1, curve B), indicating that it does not involve an oxidase. This conclusion is supported by the fact that the reaction is relatively insensitive to typical oxidase inhibitors. For example, the following inhibitions were measured: 5×10^{-4} M cyanide, 21 %; 10^{-3} M cyanide, 32 %; 10^{-4} M DIECA, 6 %; 10^{-3} M DIECA, 13 %. A comparison of these results with earlier data (table I) emphasizes the difference between this reaction and the DPNH oxidase of the cysteine-containing supernatant fraction. The reaction is also relatively insensitive to -SH inhibitors: 10^{-4} M PCMBA had essentially no effect, and 10^{-3} M phenylmercuricacetate inhibited roughly 60 %. Cysteine (0.01 M) in the reaction mixture does not block the anaerobic DPNH attack. The quinone and glutathione reductase inhibitors, 10^{-4} M DNP and 10^{-4} M $ZnSO_4$, did not in-

hibit. The reaction also proceeded normally when DPN was included to give a DPN/DPNH ratio of 2/1; this argues against a modification reaction of the type described by Rafter et al (22).

Discussion

All of the evidence suggests that the terminal reaction with oxygen in the soluble DPNH oxidase system is mediated by ascorbic acid oxidase. The linear reaction rate observed here is characteristic of ascorbic acid oxidation (12). The oxygen affinity of the DPNH oxidase is similar to that of purified ascorbic acid oxidase (25). There is a close correspondence in inhibitor sensitivities, for it is well known that ascorbic oxidation both by plant juices (24) and by the purified oxidase (8) is effectively blocked by cyanide and DIECA. Further evidence comes from the finding that ascorbic acid can activate the dialyzed supernatant fraction to catalyze a DPNH oxidation that is both qualitatively and quantitatively similar to the reaction in the undialyzed, cysteine-containing fraction. Assuming that the amount of ascorbic acid in potato tubers is 15 mg/100 g (3, 4), the concentration in the homogenates would be between 10^{-3} and 10^{-4} M; a similar concentration range is effective in activating the dialyzed supernatant. The oxidation rate of 5×10^{-2} micromoles DPNH/min × g fresh weight would correspond to an oxygen consumption of 34 μl/hr × g.

In some plant extracts, the oxidation of reduced pyridine nucleotides (PNH) can be coupled to ascorbic acid oxidase by intermediate sulfhydryl carriers, such as cysteine and glutathione, which reduce dehydroascorbic acid. Such a mechanism does not seem to be involved in the potato extract; neither cystine nor oxidized glutathione served as an effective hydrogen acceptor for DPNH. In general, glutathione reductase is specific for TPNH (17, 26). The finding that ascorbic acid alone, and not dehydroascorbic acid, activates the DPNH oxidase also argues against this type of coupling mechanism.

Matthews (19) first noted that PNH oxidation by plant extracts may be linked to ascorbic acid by a reaction which does not utilize dehydroascorbic acid. Several workers, (2, 13, 20) have now shown that the oxidation of ascorbate by its oxidase generates a labile product (monodehydroascorbate?) which can accept hydrogen from PNH. Although the details of this reaction have not been worked out, there is evidence that it involves a flavoprotein (13) and a component that is very sensitive to -SH reagents (2); the latter factor may be concerned with the reduction of flavin by PNH (21). All of the evidence obtained with the potato extracts—the participation of ascorbic acid oxidase, the activation by ascorbate alone, the presence of an active diaphorase (flavoprotein), the sensitivity to -SH inhibitors—suggests that this type of reaction is involved in the DPNH oxidase system. The reaction with TPNH was not investigated in detail, but it is probably oxidized by a similar pathway. Although TPNH could not be substituted for DPNH in the cucumber oxidase system (2), other workers (13, 19, 20) have indicated that TPNH oxidation can be directly coupled to the ascorbic acid oxidase system. The TPNH oxidase of Conn et al (6), which involves a peroxidase and is inhibited by ascorbate, is probably not responsible for the reaction studied here, although its participation has not been ruled out.

The demonstration that ascorbic acid can act as a component of an extracted DPNH oxidase does not establish its role as a respiratory carrier in vivo. Indeed, at least 70 % of the respiration of freshly cut potato slices is mediated by cytochrome oxidase (15, 25), and the remaining cyanide-insensitive fraction probably does not involve ascorbic acid oxidase. The indirect evidence for a respiratory role based on alterations in the levels of glutathione and ascorbate in potato tubers (1) is not conclusive on this point. Thus, it is most likely that the active DPNH oxidase examined here is an "isolation artifact" which is not physiologically important for respiration. This conclusion may also obtain for the PNH-ascorbic acid oxidase systems which have been demonstrated in various soluble (16, 18) and particulate (29) plant fractions.

The rapid DPNH attack by the homogenate which does not contain cysteine involves an entirely different type of reaction; it proceeds anaerobically and is much less sensitive to -SH inhibitors. The evidence suggests that the anaerobic and aerobic reactions with DPNH are mutually exclusive, since the conditions which favor one prevent the other. This could be explained as follows: in the absence of cysteine, endogenous ascorbate is all oxidized to dehydroascorbic acid and no DPNH oxidase can be demonstrated; on the other hand, a variety of oxidized compounds, e.g., quinones, are formed, and these can serve as hydrogen acceptors under anaerobic conditions. In the presence of cysteine, ascorbate is kept reduced and it can participate in the DPNH oxidase system; no suitable hydrogen acceptors for the anaerobic reaction are formed. It may be that such an effect of cysteine, i.e., prevention of the formation of potentially injurious oxidation products, accounts for its protective action during the isolation of potato mitochondria.

Summary

The soluble fraction prepared from a sucrose-phosphate-cysteine homogenate of potato tuber tissue catalyzes the rapid oxidation of DPNH and TPNH. The reaction requires molecular oxygen and can be inhibited by cyanide and diethyldithiocarbamate; it is also blocked by certain -SH group inhibitors. Dialysis of the fraction removes all activity. The dialyzed fraction can oxidize DPNH in the presence of added ascorbate or a suitable hydrogen acceptor, such as pyruvate, 2,6-dichlorophenol-indophenol, ferricyanide, or menadione. The ascorbate-activated reaction is qualitatively similar to that catalyzed by the undialyzed fraction. It is concluded that the original DPNH (TPNH) oxidase reaction involves both ascorbic acid and a step that is sensitive to -SH inhibitors.

The soluble fraction prepared in the absence of cysteine also attacks DPNH rapidly, but this reaction does not require oxygen and is less sensitive to the inhibitors tested. It is suggested that the presence of cysteine in the grinding medium not only permits the soluble DPNH oxidase to operate, but also prevents the formation of components required for the anaerobic DPNH attack.

The author gratefully acknowledges the able assistance of Mr. Darrell W. Haas and Mr. Donald J. Niederpruem.

Literature Cited

1. Barker, J. and Mapson, L. W. The ascorbic acid content of potato tubers. III. The influence of storage in nitrogen, air and pure oxygen. New Phytol. 51: 90–115. 1952.
2. Beevers, H. The oxidation of reduced diphosphopyridine nucleotide by an ascorbate system from cucumber. Plant Physiol. 29: 265–269. 1954.
3. Bessey, O. A. A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances. Jour. Biol. Chem. 126: 771–784. 1938.
4. Birch, T. W., Harris, L. J. and Ray, S. N. A microchemical method for determining the hexuronic acid (Vitamin C) content of foodstuffs, etc. Biochem. Jour. 27: 590–594. 1933.
5. Burton, K. and Wilson, T. H. The free-energy changes for the reduction of diphosphopyridine nucleotide and the dehydrogenation of L-malate and L-glycerol 1-phosphate. Biochem. Jour. 54: 86–94. 1953.
6. Conn, E. E., Kraemer, L. M., Liu, Pei-Nan and Vennesland, B. The aerobic oxidation of reduced triphosphopyridine nucleotide by a wheat germ enzyme system. Jour. Biol. Chem. 194: 143–151. 1952.
7. Conn, E. E. and Seki, S. L. Reactions of keto acids catalyzed by plant enzyme preparations. Federation Proc. 15: 235. 1956.
8. Dawson, C. R. and Tarpley, W. B. Copper oxidases. In: The Enzymes, J. B. Sumner and K. Myrback, eds. Pp. 454–498. Academic Press, New York 1951.
9. DuBois, K. P. and Erway, W. F. Studies on the mechanisms of action of thiourea and related compounds. II. Inhibition of oxidative enzymes and oxidations catalyzed by copper. Jour. Biol. Chem. 165: 711–721. 1946.
10. Haas, D. W. and Hackett, D. P. Oxidative activities of potato tuber mitochondria. Plant Physiol. 31 suppl.: *xxv*. 1956.
11. Hackett, D. P. Pathways of oxidation in cell-free potato fractions. Plant Physiol. 31: 111–118. 1956.
12. Hopkins, F. G. and Morgan, E. J. Some relations between ascorbic acid and glutathione. Biochem. Jour. 30: 1446–1461. 1936.
13. Kern, M. and Racker, E. Activation of a DPNH oxidase by an oxidation product of ascorbic acid. Arch. Biochem. Biophys. 48: 235–236. 1954.
14. Lazarow, A. and Cooperstein, S. J. Versatile anaerobic spectrophotometer cell. Science 120: 674–675. 1954.
15. Levy, H., Schade, A. L., Bergmann, L. and Harris, S. Studies in the respiration of the white potato. II. Terminal oxidase system of potato tuber respiration. Arch. Biochem. 19: 273–286. 1948.
16. Mapson, L. W. Function of ascorbic acid in plants. Vitamins and Hormones 11: 1–28. 1953.
17. Mapson, L. W. and Goddard, D. R. The reduction of glutathione by plant tissues. Biochem. Jour. 29: 592–601. 1951.
18. Mapson, L. W. and Moustafa, E. M. Ascorbic acid and glutathione as respiratory carriers in the respiration of pea seedlings. Biochem. Jour. 62: 248–259. 1956.
19. Mathews, M. B. The oxidation of reduced diphosphopyridine nucleotide in green peas. Jour. Biol. Chem. 189: 695–704. 1951.
20. Nason, A., Wosilait, W. D. and Terrell, A. J. The enzymatic oxidation of reduced pyridine nucleotides by an oxidation product of ascorbic acid. Arch. Biochem. Biophys. 48: 233–235. 1954.
21. Nicholas, D. J. D. and Nason, A. Molybdenum as an electron carrier in nitrate reductase action. Arch. Biochem. Biophys. 51: 310–312. 1954.
22. Rafter, G. W., Chaykin, S. and Krebs, E. G. The action of glyceraldehyde-3-phosphate dehydrogenase on reduced diphosphopyridine nucleotide. Jour. Biol. Chem. 208: 799–811. 1954.
23. Sharpensteen, H. H. and Conn, E. E. Preparation and properties of potato mitochondria. Reported at Amer. Soc. Plant Physiol. Meeting. Sept. 5–9. Gainesville, Florida 1954.
24. Stotz, E., Harrer, C. J. and King, G. G. A study of "ascorbic acid oxidase" in relation to copper. Jour. Biol. Chem. 119: 511–522. 1937.
25. Thimann, K. V., Yocum, C. S. and Hackett, D. P. Terminal oxidases and growth in plant tissues. III. Terminal oxidation in potato tuber tissue. Arch. Biochem. Biophys. 53: 239–257. 1954.
26. Vennesland, B. and Conn, E. E. The enzymatic oxidation and reduction of glutathione. In: Glutathione. S. P. Colowick et al. eds. Pp. 105–126. Academic Press, New York 1954.
27. Wosilait, W. D. and Nason, A. Pyridine nucleotide-quinone reductase. I. Purification and properties of the enzyme from pea seeds. Jour. Biol. Chem. 206: 255–270. 1954.
28. Wosilait, W. D. and Nason, A. Pyridine nucleotide-menadione reductase from *Escherichia coli*. Jour. Biol. Chem. 208: 785–798. 1954.
29. Young, L. C. T. and Conn, E. E. The reduction and oxidation of glutathione by plant mitochondria. Plant Physiol. 31: 205–211. 1956.