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Generation of Hydrogen Peroxide on Oxidation of NADH by Hepatic Plasma Membranes

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Abstract

The oxidation of NADH by mouse liver plasma membranes was shown to be accompanied by the formation of H_2O_2. The rate of H_2O_2 formation was less than one-tenth the rate of oxygen uptake and much slower than the rate of reduction of artificial electron acceptors. The optimum pH for this reaction was 7.0 and the K_m value for NADH was found to be 3×10^{-6} M. The H_2O_2-generating system of plasma membranes was inhibited by quinacrine and azide, thus distinguishing it from similar activities in endoplasmic reticulum and mitochondria. Both NADH and NADPH served as substrates for plasma membrane H_2O_2 generation. Superoxide dismutase and adriamycin inhibited the reaction. Vanadate, known to stimulate the oxidation of NADH by plasma membranes, did not increase the formation of H_2O_2. In view of the growing evidence that H_2O_2 can be involved in metabolic control, the formation of H_2O_2 by a plasma membrane NAD(P)H oxidase system may be pertinent to control sites at the plasma membrane.

Key Words: Plasma membrane NADH oxidase; plasma membrane hydrogen peroxide formation; hydrogen peroxide formation by NADH oxidation; plasma membrane redox; quinacrine inhibition plasma membrane; NADPH oxidation-plasma membrane.

Introduction

The generation of H_2O_2 by a variety of cells and cellular organelles and the physiological role played by H_2O_2 have received increasing attention during the last decade. H_2O_2 or its possible precursor, superoxide, can function in several forms: defense against, or attack on, other cells; oxidative conversion of metabolites; proton shifts; peroxidation of membrane lipids or reduction of high-potential redox components (Crane *et al.*, 1979).

Intact mitochondria have been found to generate H_2O_2 (Loschen *et al.*, 1971; Boveris *et al.*, 1977), as determined by the scopoletin-horseradish

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peroxidase method (Andrea, 1955). The cytochrome *c* peroxidase method was used to demonstrate the utilization of succinate, malate + glutamate, palmitoyl-carnitine, and octanoate as substrates (Boveris *et al.*, 1972). Other substrates, α-glycerophosphate and choline, but not ascorbate, also support H_2O_2 generation by mitochondria (Swaroop and Ramasarma, 1981). These results indicate a common H_2O_2 generator at the ubiquinone site, since all the effective substrates donate electrons to ubiquinone (Turrens and Boveris, 1980; Boveris and Chance, 1973).

The capacity of endoplasmic reticulum to function as a source of H_2O_2 has been recognized for a long time (Gillette *et al.*, 1957) and is derived from enzymes involved in the mixed-function oxidation reactions of microsomes (Thurman *et al.*, 1972; Hildebrandt and Roots, 1975). In the absence of oxidizable substrates, microsomal oxidation of NADPH yielded H_2O_2 quantitatively, and therefore the well-known NADPH-cytochrome *c* reductase may indeed be the "microsomal H_2O_2 generator" (Boveris *et al.*, 1972), with a role for cytochrome *P*-450 in the oxidase function (Hildebrandt *et al.*, 1973; Kuthan *et al.*, 1978).

In the liver cell, the production of H_2O_2 occurs in peroxisomes, but it is destroyed to a large extent within the organelle (Boveris *et al.*, 1972) and, therefore, appears to be merely an end-product of catabolism.

There has been little evidence for H_2O_2 production in plasma membranes except in various leukocytes. It is now recognized that plasma membranes of a variety of cells possess redox functions involving NADH and NADPH (Löw and Crane, 1978). An active NADH dehydrogenase of liver plasma membranes has been shown to be distinct from that present in endoplasmic reticulum based on its response to inhibitors and hormones (Goldenberg *et al.*, 1977; Crane and Löw, 1976). The activity of this enzyme was high with ferricyanide but low with oxygen as the acceptor and it was not known whether H_2O_2 is the product of the latter reaction. There is a report indicating that NADPH oxidase of adipocyte plasma membranes could generate H_2O_2 (Mukherjee and Lynn, 1977). Recently Briggs *et al.* (1975) and Badwey and Karnovsky (1979) have reported the generation of superoxide and H_2O_2 by a membrane-bound NADH oxidase in human and guinea pig polymorphonuclear leukocytes. In this paper, we present evidence for the generation of H_2O_2 on oxidation of NADH by mouse liver plasma membranes and compare some of its properties with that of the dehydrogenase activity.

Materials and Methods

Methods

Plasma membranes were prepared from mouse liver by differential centrifugation in sucrose gradients according to the method of Yunghans and

Morré (1973). Smooth endoplasmic reticulum and Golgi apparatus were also prepared from mouse liver by the methods described by Morré (1973). The purity of the preparation was determined to be equivalent to membranes previously described (Goldenberg *et al.*, 1979).

The standard assay system for measuring the generation of H_2O_2 consisted of NADH (0.1 mM), potassium phosphate buffer (0.05 M, pH 7.4), scopoletin (1.5 nmoles), horseradish peroxidase (0.014 mg), and plasma membranes (0.15–0.30 mg protein) in a total volume of 1.5 ml. The rate of formation of H_2O_2 was measured at 30° by the disappearance of scopoletin fluorescence in an Aminco Spectroflouorimeter with excitation and emission wavelengths at 380 nm and 460 nm, respectively. The system is capable of oxidizing scopoletin by H_2O_2 at a rate of 4 nmol/min in the absence of NADH and plasma membranes (Boveris *et al.*, 1977). The fluorescence of scopoletin was proportional to concentration. The fluorescence yield was dependent on the pH of the solution, increasing in alkaline pH. For a 1 μM solution of scopoletin at the settings used, the relative fluorescence units were 112, 60, and 25 for pH values 8.0, 7.0, and 6.0, respectively.

A ratio of 1 is obtained for H_2O_2/scopoletin with the horseradish peroxidase system (Hildebrandt and Roots, 1975). This is confirmed in the present study, but in the presence of NADH or NADPH the ratio decreased. In the range of 0.04–0.10 mM NADH, a ratio of 0.25 is obtained. Formation of a scopoletin radical intermediate may reduce the fluorescence value. Under the conditions of assay, the decrease in fluorescence units of scopoletin on addition of known amounts of H_2O_2 (1–5 nmol) was standardized and used for the calculations.

The fluorescence of NADH is in the same region, but using an excitation wavelength of 380 nm, only 38% of maximum yield was obtained. Under the conditions employed in the present experiments, relative fluorescence on a molar basis for scopoletin is 500 times higher than for NADH. The contribution of added NADH to the total fluorescence was 13%. Since the oxidation rate of NADH is small, the loss due to NADH fluorescence had no effect on the large rates of fluorescence decrease due to scopoletin oxidation by H_2O_2. The rate of disappearance of NADH was also measured in some experiments by the decrease in absorbance at 340 nm in a Beckman spectrophotometer with a Gilford attachment.

Materials

Scopoletin, horseradish peroxidase, superoxide dismutase, catalase, NADH, NADPH, and other biochemicals were obtained from the Sigma Chemical Co., St. Louis, Missouri. Vanadium pentoxide was extracted with 0.2 N NaOH overnight and the resultant yellow solution known to contain decavanadate was used in these studies. Adriamycin was a gift from Dr. M. Ghione of Farmitalia, Milan, Italy.

Results

Generation of H_2O_2 by Plasma Membranes

The results in Fig. 1 demonstrate the generation of H_2O_2 by plasma membranes in the presence of NADH. The scopoletin fluorescence decreased on addition of plasma membranes, giving a rate of 0.05 nmol H_2O_2/min. The rate observed was dependent on both NADH and plasma membranes and was lost when heat-denatured plasma membranes were used.

The oxidation of NADH measured by decreased absorbance at 340 nm is shown to occur in the presence of scopoletin and horseradish peroxidase when plasma membrane is added (Fig. 2, top). These reagents do not inhibit NADH oxidation by plasma membrane. The loss of NADH when H_2O_2 is added to the scopoletin–peroxidase system is shown in Fig. 2 (bottom).

Comparison with Related Cellular Organelles

Smoth endoplasmic reticulum and Golgi apparatus were also prepared from mouse liver. The comparative rates of H_2O_2 generation by these membranes, using NADH and NADPH as the substrates, are shown in Table

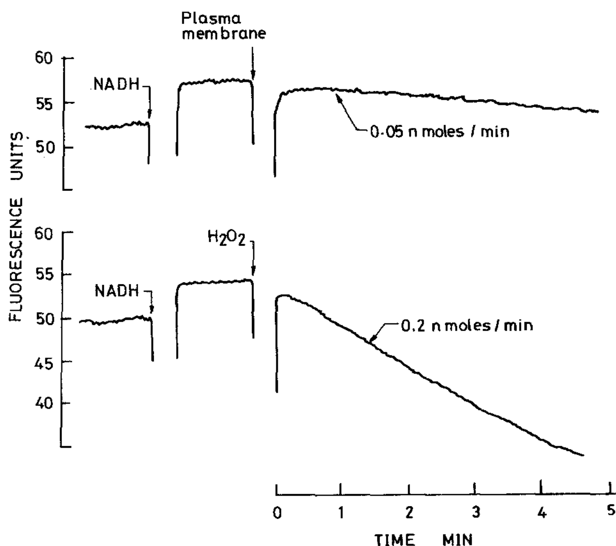

Fig. 1. H_2O_2 production by plasma membranes in presence of NADH as shown by decrease in scopoletin fluorescence. Standard assay conditions. (Top) rate with NADH alone followed by addition of 0.2 mg plasma membrane protein. (Bottom) maximum rate of fluorescence change with H_2O_2 added in the presence of NADH without plasma membrane.

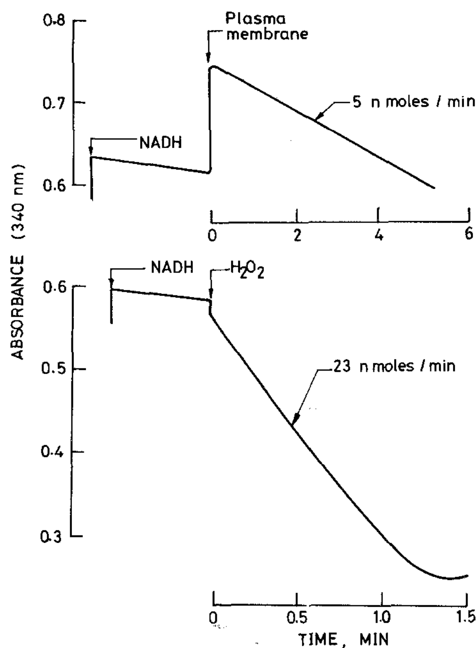

Fig. 2. The spectrophotometric demonstration of NADH oxidation by plasma membranes during H_2O_2 generation. The reaction mixture consisted of horseradish peroxidase (0.014 mg), scopoletin (20 nmol), and NADH (0.1 mM) in 1 ml of potassium phosphate buffer (pH 7.4, 0.05 M). The disappearance of NADH, measured by absorbance changes at 340 nm, is shown to be dependent on addition of plasma membranes (0.1 mg protein) (top) or on the presence of H_2O_2 (500 nmol) (bottom).

I. Plasma membrane shows a higher rate of NADH-dependent H_2O_2 generation than smooth endoplasmic reticulum. The NADPH system is obviously more active in endoplasmic reticulum. It is interesting to note that Golgi apparatus showed a low activity with NADH and none with NADPH.

Relative Rates with Different Acceptors

The relative rates of oxidation of NADH and NADPH by plasma membranes were measured in the presence of different acceptors. The decrease in absorbance at 340 nm was measured for the oxidation of the substrate and compared with the rates of H_2O_2 generation. The results in Table II show that the rates decrease in the order of ferricyanide, cytochrome *c*, and oxygen. Compared to NADH, the rates with NADPH are very low. The relatively high rate of H_2O_2 formation for NADPH oxidation suggests

Table I. The Generation of H_2O_2 by Cellular Organelles[a]

	nmol H_2O_2/min/mg protein	
Cellular organelle	NADPH	NADH
Plasma membranes	0.23	0.17
Smooth endoplasmic reticulum	0.41	0.07
Golgi apparatus	0	0.08

[a]The rates of the generation of H_2O_2 were measured under standard conditions with NADH or NADPH as substrates. The amounts of protein/ml were: plasma membranes (0.29 mg), smooth endoplasmic reticulum (0.13 mg), and Golgi bodies (0.28 mg).

that a separate H_2O_2-generating oxidase system can be present which is distinct from a part of the oxidases.

Effect of pH

The generation of H_2O_2 by plasma membranes has a pH optimum at 7.0 (Fig. 3), with decreasing rates at pH lower than 6.0 and higher than 8.0, similar to the broad pH optimum for NADH ferricyanide reductase activity (Ramasarma *et al.*, 1980). The same samples were tested for the disappearance of NADH by the decrease in absorbance at 340 nm with oxygen and with ferricyanide as the acceptor. The rates of NADH oxidase are between 20–40 times faster than the rates of H_2O_2 generation (Fig. 3), whereas the NADH–ferricyanide activity was a further 10-fold higher at each of the pH values tested.

Effect of NADH Concentration

Saturation of the H_2O_2-generating system in plasma membrane was obtained at a low NADH concentration. The K_m value is 3×10^{-6} M, calculated from the double reciprocal plot (Fig. 4).

Table II. Relative Rates of NADH Oxidation with Different Acceptors[a]

	nmol/min/mg protein	
Acceptor	NADPH	NADH
Potassium ferricyanide	30	346
Cytochrome *c*	3	51
Oxygen	<1	10
$O_2 \rightarrow H_2O_2$	0.41	0.31

[a]The rate of oxidation of NADH in plasma membranes by different acceptors was measured by an absorbance change at 340 nm. The generation of H_2O_2 was also measured with the same preparation under standard conditions where indicated as $O_2 \rightarrow H_2O_2$.

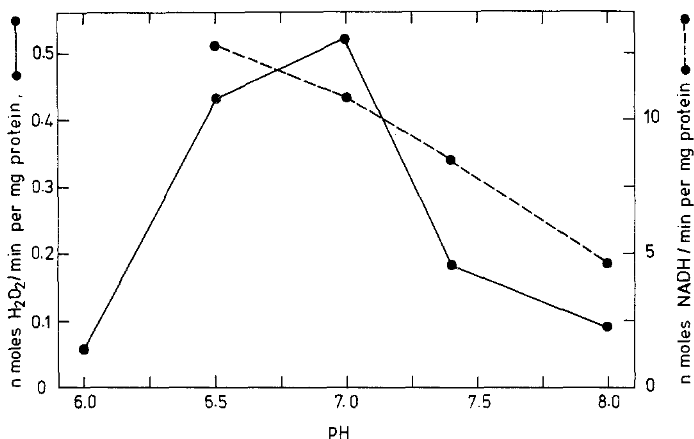

Fig. 3. The effect of pH on H_2O_2 generation by plasma membranes. The conditions are the same as in the standard assay except for the change in the pH of the phosphate buffer used. The small nonenzymic rates were first recorded and substracted from the total rate of the H_2O_2 generation obtained by adding plasma membranes. The same solutions were then tested for NADH oxidase activity by measuring the change in absorbance at 340 nm. The rates in all cases were linear for several minutes.

Effect of Some Inhibitors

The activity of NADH oxidase in plasma membranes was found to be different from that of endoplasmic reticulum or mitochondria in being more sensitive to inhibition by quinacrine and stimulation by azide (see Crane *et al.*, 1979, for a summary). These two compounds inhibit the H_2O_2 generation by plasma membrane at concentrations somewhat lower than required to inhibit NADH dehydrogenase, as shown in Table III.

Quinacrine at micromolar concentrations showed considerable inhibition of the generation of H_2O_2, whereas the reduction of other acceptors (dichloro-phenol-indophenol) required millimolar concentrations (Crane and Löw, 1976). In the absence of plasma membrane, quinacrine had little effect on the horseradish peroxidase system tested by adding H_2O_2. However, for each 100 nmol of quinacrine about 0.5 nmol of scopoletin becomes inert, as observed by the retention of the fluorescence. Quinacrine itself did not contribute to or affect the fluorescence of scopoletin under these conditions.

The effects with azide are more striking. At 100 mM, the generation of H_2O_2 was completely inhibited, whereas only about 40% inhibition was obtained with other acceptors (Crane and Löw, 1976; Ramasarma *et al.*, 1980). The addition of catalase inhibited the reaction, as expected for a reaction based on H_2O_2 formation. The low rates of H_2O_2 generation are not due to intrinsic catalase, since there was no increase when 1 mM azide was

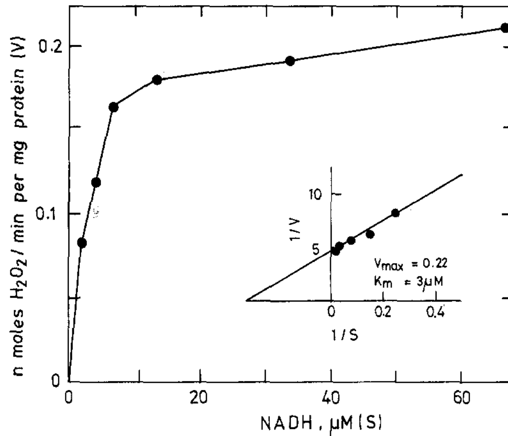

Fig. 4. The effect of NADH concentrations. The conditions are the same as described in the standard assay, except that the concentration of NADH is varied. The inset shows the double reciprocal plot from which V_{max} and K_m are calculated.

added, which is known to inhibit catalase activity. Azide, at 1 mM concentration, had no effect on the horseradish peroxidase reaction, and inhibited it about 30% at 100 mM, which still left sufficient capacity of this trapping system to detect the small quantities of H_2O_2 produced. It must also be pointed out that azide at 100 mM caused a 15% loss of fluorescence of scopoletin. Similar nonspecific loss of fluorescence was also reported for trichloroacetic acid (Hildebrandt and Roots, 1975). Like quinacrine, azide also makes a part of the fluorescence unresponsive to the reaction to the extent of 0.4 nmol scopoletin/100 μmol of azide.

The addition of KCN at 0.1 mM inhibited both the generation of H_2O_2 by plasma membrane and the horseradish peroxidase reaction by about 50%. Since the residual rate of the peroxidase was at least 100 times higher, inhibition of H_2O_2 generation may be by a hemoprotein. Potassium fericyanide, which acts as an artificial electron acceptor for the NADH oxidase of plasma membrane, totally abolished the H_2O_2 generation at 0.17 mM concentration without affecting the horseradish peroxidase reaction. Such a predominance of ferricyanide over oxygen as an electron acceptor for NADH dehydrogenase was also seen over other acceptors (Ramasarma *et al.*, 1980). Triiodothyronine and antimycin A had no effect. The lack of effect of triiodothyronine is in contrast to its reported inhibition of H_2O_2 generation by mitochondria (Swaroop and Ramasarma, 1981) and stimulation of NADH oxidase by rat liver plasma membrane (Gayda *et al.*, 1977).

Attempts to see the intermediate participation of superoxide in the H_2O_2

Table III. The Effect of Some Compounds on the Generation of H_2O_2[a]

Addition	Concentration	nmol H_2O_2/min/ mg protein	% of control
None		0.22	100
Quinacrine	30 μM	0.12	55
	60 μM	0.10	45
Sodium azide	1 mM	0.20	91
	10 mM	0.08	36
	100 mM	0	0
Potassium cyanide	0.1 mM	0.10	45
Potassium ferricyanide	0.17 mM	0	0
Triiodothyronine	10 μM	0.18	82
Antimycin A	1.5 μg/ml	0.22	100
None		0.14	100
Superoxide dismutase	13 μg/ml	0.04	29
Catalase	33 μg/ml	0	0
None		0.24	100
Adriamycin	60 μM	0.07	30
Decavanadate	0.5 mM	0.20	83

[a]Various compounds were added in the standard assay medium before the addition of plasma membranes, and the rate of H_2O_2 generated was recorded.

generation by plasma membrane did not meet with successs. Mn^{2+} could not be used as the quencher of O_2^-, as $MnCl_2$ inhibited the horseradish peroxidase reaction 50% at 4 μM and completely at 16 μM. H_2O_2 can be formed by the dismutation of superoxide, and the addition of superoxide dismutase can cause further increase in H_2O_2 generation. Indeed the addition of superoxide dismutase increased the rate of generation of H_2O_2 in mitochondria (Swaroop and Ramasarma, 1981), but it inhibited it in plasma membranes. An inhibition of a reaction by superoxide dismutase is widely considered to indicate the involvement of superoxide anions (McCord and Fridovich, 1969). In the present experiments the inhibition is perplexing because we are measuring the generation of H_2O_2, the very same reaction catalyzed by the dismutase. Recent incisive analysis by Fee (1980) on the role of superoxide dismutases questions whether the function of these metalloproteins is truly the dismutation of superoxide or whether they possess "alternative biological functions." In the present system both generation of H_2O_2 and H_2O_2-dependent oxidation of NADH by the plasma membrane, as described in Fig. 1, were inhibited by superoxide dismutase.

Effect of Adriamycin

The addition of adriamycin (40–60 μM), an antineoplastic anthracycline drug, was shown to stimulate NADH-dependent oxygen uptake by plasma

membranes and superoxide formation by the xanthine oxidase system (Crane *et al.*, 1980). But the generation of H_2O_2, which was only a fraction of the total oxygen uptake (see Fig. 1), was inhibited 70% at 60 μM concentration of adriamycin (Table III). The demonstration of this effect was beset with some difficulties, which revealed interesting reactions of adriamycin with the horseradish peroxidase–scopoletin system. First, the fluorescence decreased by an unspecific quenching to the extent of 1 nmol scopoletin/100 nmol of adriamycin. The addition of adriamycin to the standard assay system showed complete inhibition, but this was found to be due to its effect on horseradish peroxidase, whose action on scopoletin even in the presence of added H_2O_2 was prevented. However, on increasing its concentration, peroxidase seemed to titrate with the drug (50 mol adriamycin per mole of peroxidase). After this reaction, peroxidase became available for H_2O_2 measurement. In the above experiment, therefore, a concentration of 0.042 mg (1.05 nmol) horseradish peroxidase was used, thus ensuring sufficient activity of this enzyme to measure the H_2O_2 generated.

Effect of Vanadate

The addition of decavanadate was found to stimulate plasma membrane NADH oxidase (Menon *et al.*, 1980). It was of interest to see whether this large increase was accompanied by an increase in H_2O_2 generation. The addition of decavanadate (0.5 mM), which gave a 20-fold increase in the disappearance of NADH and uptake of oxygen, showed a small decrease in the generation of H_2O_2 (Table 3). Decavanadate also produced an unspecific decrease of scopoletin fluorescence (0.08 nmol scopoletin/μmol vanadate), but did not affect the horseradish peroxidase reaction. Thus, the vanadate-stimulated reaction leads to increased uptake of oxygen, which is reduced to either superoxide or directly to H_2O.

Discussion

The oxidation of NADH by plasma membranes has been well established (Crane *et al.*, 1979) in a variety of cells. The search continues for a meaningful function in cellular activities that justifies the utilization of energy in the process. The generation of H_2O_2 itself may be considered as a useful function in view of the growing evidence for metabolic importance of H_2O_2. This report gives evidence for H_2O_2 being at least one of the products of NADH oxidation by plasma membranes.

The generation of superoxide or H_2O_2 as a consequence of NAD(P)H oxidation in whole cells or by plasma membranes has been reported in

leucocytes (Goldstein *et al.*, 1977), neutrophils (Segal and Peters, 1976), eosinophils (De Chatelet *et al.*, 1977), and rodent malarial parasite (Etkin and Eaton, 1975). In most of these cases, H_2O_2 is considered to be produced via superoxide by dismutation, although direct reduction is not ruled out.

The present studies conclusively demonstrated the production of H_2O_2, albeit with small rates, on oxidation of NAD(P)H by plasma membranes. The natural acceptor seems to be oxygen and only a small part of the total oxygen reduced appears as H_2O_2. This is not due to the lack of superoxide dismutase, as the addition of this enzyme strangely inhibited the H_2O_2-generating reaction instead of promoting it. It is also not clear whether there is one NADH oxidase having different reactivities with acceptors or more than one enzyme exists to account for the variability expressed with different acceptors. The comparative study, shown in Table IV, indicates at least three levels of K_m and V_{max}. The rate of H_2O_2 generation is very low compared to the potential of the dehydrogenase, which is almost 2000 times higher, but the rates of H_2O_2 generation in cellular organelles are in the range of 0.1–0.8 nmol/min/mg protein (Boveris *et al.*, 1972; Boveris and Chance, 1973), not very much higher than that reported here for plasma membranes. Many properties of H_2O_2 generation in plasma membranes are different from that of mitochondria. H_2O_2 generation increases with an increase in pH up to pH

Table IV. A Comparative Study of the Properties of NADH Oxidase of Plasma Membranes with Different Acceptors[a]

	Acceptor				
Properties	Ferricyanide	Dichloro-phenol-indophenol	Cyto-chrome *c*	Oxygen (vanadate-stimulated)	$O_2 \rightarrow H_2O_2$
Optimum pH	5.5–7.0 (broad)	7.0	6.0–7.5 (broad)	5.0	7.0
V_{max} (nmol/min/mg protein)	500	27	35	455	0.3
K_m (NADH)	40	10	40	200	3
NADH/NADPH	12	—	17	—	0.8
		% Inhibition (concn.)			
Quinacrine	16 (100 μM)	50 (3 mM)	—	73 (60 μM)	45 (60 μM)
Azide	37 (0.1 M)	32 (0.1 M)	—	85 (0.1 M)	100 (0.1 M)
Triiodothyronine	20 (1 μM)	60 (1 μM)	—	20 (1 μM)	8 (10 μM)
Superoxide dismutase	none (20 μg/ml)	—	—	100 (1 μg/ml)	71 (13 μg/ml)
Adriamycin	58 (60 μM)	—	none (60 μM)	75 (45 μM)	70 (60 μM)

[a]The data are taken from Crane and Löw (1976), Ramasarma *et al.* (1980), and this work.

8.0 in mitochondria, while that in plasma membranes shows a pH optimum of 7.0. Inhibitions in plasma membranes were obtained with superoxide dismutase and in mitochondria with triiodothyronine, but not *vice versa* (Swaroop and Ramasarma, 1981). Antimycin A stimulates only in mitochondria (Boveris and Chance, 1973).

To our knowledge, there are only two reports on the generation of H_2O_2 by plasma membranes. One is that of Mukherjee and Lynn (1977) in rat adipocytes. They showed that NADPH oxidation yielded a 1:1 ratio of H_2O_2, measured by the glutathione peroxidase method, and that this activity increased on pretreatment of the cells with insulin. In view of the growing number of insulin-mimicking effects of H_2O_2 (Czech *et al.,* 1974; May and Haen, 1979a, b), a role of far greater significance for H_2O_2 is emerging with the status of another "second messenger" in hormone action. In another report Badwey and Karnovsky (1979) showed that a NADH oxidase of leukocytes, which is apparently loosely bound on the inner side of the plasma membrane, generated H_2O_2. This generation of H_2O_2 was modulated by nucleotides and divalent cations. These reports give the ubiquitous NADH oxidase of plasma membranes a possible metabolic importance similar to that of adenylate cyclase, especially in view of their coincidental relationship in showing hormone responses (Crane *et al.,* 1979). The oxidation of external NADPH by the malaria parasite is another type of plasma membrane oxidase which can generate H_2O_2 (Eckman and Eaton 1979, Friedman 1979).

Acknowledgments

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References

Andrea, W. A. (1955). *Nature* (London), **175,** 859–860.
Badwey, J. A., and Karnovsky, M. L. (1979). *J. Biol Chem.* **254,** 11,530–11,537.
Boveris, A., and Chance, B. (1973). *Biochem. J.* **134,** 707–716.
Boveris, A., Martino, E., and Stoppani, A. O. M. (1977). *Anal. Biochem.* **80,** 145–158.
Boveris, A., Oshino, N., and Chance, B. (1972). *Biochem. J.* **128,** 617–630.
Briggs, R. T., Drath, D. B., Karnovsky, M. L., and Karnovsky, M. J. (1975). *J. Cell Biol.* **67,** 566–586.
Crane, F. L., Goldberg, H., Morré, D. J., and Löw, H. (1979). *Sub-Cell. Biochem.* **6,** 345–379.

Crane, F. L., and Löw, H. (1976). *FEBS Lett.* **68,** 153–156.
Crane, F. L., MacKellar, W., Morré, D. J., Ramasarma, T., Goldenberg, H., Grebing, C., and Löw, H. (1980). *Biochem. Biophys. Res. Commun.* **93,** 746–754.
Czech, M. P., Lawrence, J. C., Jr., and Lynn, W. S. (1974). *J. Biol. Chem.* **249,** 5421–5427.
De Chatelet, L. R., Shirley, P. S., McPhail, L. C., Huntely, C. C., Muss, H. B., and Bass, D. (1977). *Blood* **50,** 525–535.
Eckman, J. R., and Eaton, J. W. (1979). *Nature (London)* **278,** 754–756.
Etkin, N., and Eaton J. (1975). In *Erythrocyte Structure and Function,* Vol. 21, G. J. Brewer, ed., A. R. Liss, New York, pp. 9–232.
Fee, J. A. (1980). In *Metal Ion Activation of Dioxygen,* T. G. Spiro, ed., Wiley, pp. 209–237.
Friedman, M. J. (1979). *Nature (London)* **286,** 245–247.
Gayda, D. P., Crane, F. L., Morré, D. J., and Löw, H. (1977). *Proc. Indiana Acad. Sci.* **86,** 385–390.
Gillette, J. R., Brodie, B. B. and La Du, B. N. (1957). *J. Pharmacol. Exp. Ther.* **119,** 532–540.
Goldenberg, H., Crane, F. L., and Morré, D. J. (1979). *J. Biol. Chem.* **254,** 2491–2498.
Goldenberg, H., Morré, D. J., and Crane, F. L. (1977). *J. Cell Biol.* **75,** 209a.
Goldstein, I. M., Cerqueira, M., Lind, S., and Kaplan, H. B. (1977). *J. Clin. Invest.* **59,** 249–254.
Hildebrandt, A. G., and Roots, I. (1975). *Arch. Biochem. Biophys.* **171,** 385–397.
Hildebrandt, A. G., Speck, M., and Roots, I. (1973). *Biochem. Biophys. Res. Commun.* **54,** 968–975.
Kuthan, H., TsuJi, H., Graf, H., Ullrich, V., Werringloer, J., and Estabrook, R. W. (1978). *FEBS Lett.* **91,** 343–345.
Loschen, G., Flohe, L., and Chance, B. (1971). *FEBS Lett.* **18,** 261–264.
Löw, H., and Crane, F. L. (1978). *Biochim. Biophys. Acta* **515,** 141–161.
May, J. M. and de Haen, C. (1979a). *J. Biol. Chem.* **254,** 9017–9021.
May, J. M., and de Haen, C. (1979b). *J. Biol. Chem.* **254,** 2214–2220.
McCord, J. M., and Fridovich, I. (1969). *J. Biol. Chem.* 6049–6055.
Menon, A. S., Meera Rau, Ramasarma, T., and Crane, F. L. (1980). *FEBS Lett.* **114,** 139–142.
Morré, D. J. (1973). In *Molecular Techniques and Approaches in Developmental Biology,* M. J. Chripeels, ed., Wiley, New York, pp. 1–27.
Mukherjee, S. P., and Lynn, W. S. (1977). *Arch. Biochem. Biophys.* **184,** 69–76.
Ramasarma, T., MacKellar, W., and Crane, F. L. (1980). *Indian J. Biochem. Biophys.* **17,** 163–167.
Segal, A. W., and Peters, T. J. (1976). *Lancet* **1,** 1363–1365.
Swaroop, A., and Ramasarma, T. (1981). *Biochem. J.* **194,** 657–665.
Thurman, R. G., Ley, H. G., and Scholz, R. (1972). *Eur. J. Biochem.* **25,** 420–430.
Turrens, J. F., and Boveris, A. (1980). *Biochem J.* **191,** 421–427.
Yunghans, W. N., and Morré, D. J. (1973). *Prep. Biochem.* **3,** 301–312.