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Reduced Nicotinamide Adenine Dinucleotide and Reduced Nicotinamide Adenine Dinucleotide Phosphate Diaphorase Activity in Human Polymorphonuclear Leukocytes

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Total reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activities were examined in human neutrophils. Approximately two-thirds of each enzyme activity was located in the granule fraction with the remainder in the soluble. The activities in a 27,000 × *g* supernatant from a sonic extract of human polymorphonuclear leukocytes were characterized. Both NADH and NADPH diaphorase were insensitive to cyanide and azide and showed greater activity at acid pH. K_m values for nitroblue tetrazolium were not markedly different (33 μM with NADH and 12 μM with NADPH), but there was a 40-fold difference in K_m for the reduced pyridine nucleotides (10 μM with NADH and 400 μM for NADPH). Since the intracellular concentration of both nucleotides is estimated to be about 50 μM, it is much more likely, from a kinetic argument, that the respiratory burst of phagocytosis is intiated by the oxidation of NADH rather than of NADPH.

The term "diaphorase," as used here, is a descriptive one which is applied to any enzyme that can transfer electrons from reduced pyridine nucleotides to an artificial (i.e., nonphysiological) acceptor molecule. Thus, it is evident that a "reduced nicotinamide adenine dinucleotide (NADH) diaphorase activity" does not describe a single unique enzyme activity; in fact, there may be a number of different enzymes within a single cell (including pyridine nucleotide oxidases) which exhibit diaphorase activity. Furthermore, since the activity is defined in terms of an artificial acceptor molecule, the true function of the enzyme(s) within the cell is not known.

Baehner and Nathan (8) devised a test revolving around the ability of a cell to reduce nitroblue tetrazolium (NBT) dye as a measure of the metabolic competency of the cell. Normal cells reduce a certain amount of the dye under resting conditions, and the degree of reduction increases markedly during phagocytosis. Metabolically deficient cells, as in chronic granulomatous disease (CGD) or leukocyte glucose-6-phosphate dehydrogenase deficiency, fail to reduce the dye under either resting or phagocytizing conditions (7, 11), and this phenomenon has been used successfully as a screening test for CGD. The inability of the cells to reduce NBT dye does not appear to be due to a defect in either ingestion or degranulation (6). Furthermore, Baehner and Nathan have reported that the diaphorase activity of broken cell preparations from patients with CGD is essentially normal, when NADH or reduced nicotinamide adenine dinucleotide phosphate (NADPH) is employed as a cofactor (21). Similar results have been observed in severe glucose-6-phosphate dehydrogenase deficiency (11). Thus, a genetic defect resulting in a totally inactive diaphorase seems relatively unlikely. The demonstration that normal diaphorase activity was present in the defective cells was made under optimal conditions of enzyme activity. It seems most likely that a subtle change in the enzyme might have occurred, resulting in an inability of the enzyme to express its activity under conditions of substrate concentration, etc., which exist within the cell.

This study describes some of the properties of the normal diaphorase enzymes found in human polymorphonuclear leukocytes (PMNL). This is necessary so that future work may compare the properties of enzymes from normal and deficient neutrophils.

MATERIALS AND METHODS

Preparation of leukocyte extracts. Leukocytes were isolated from the blood of apparently healthy volunteer subjects by a method described previously

(12). Contaminating erythrocytes were removed by hypotonic lysis, and the leukocyte pellet was washed two times with Dulbecco phosphate-buffered saline, containing both magnesium and calcium ions, and adjusted to a final concentration of 5×10^7 phagocytes per ml by the addition of phosphate-buffered saline. Phagocytes are defined here as segmented neutrophils, band neutrophils, eosinophils, and monocytes. Lymphocytes accounted for less than 10% of the isolated cell suspension. Lymphocytes were separated from normal human blood in 95% purity by centrifugation in a Ficoll-Hypaque gradient (10). The cell suspensions were disrupted by sonic treatment for 45 s in three 15-s intervals with cooling in between. A Branson sonifier with a power output of 20 W was used for disruption. Cellular debris was removed by centrifugation at 27,000 $\times$ *g* for 15 min; the clear supernatant solution was assayed immediately because of loss of activity during storage. Attempts to release latent activity with 0.1% Triton X-100 were unsuccessful due to enzyme inhibition by the detergent.

Determination of diaphorase activities. Leukocyte diaphorase activity was determined by a modification of the assay of Nishikimi et al. (22) for superoxide dismutase activity. The following materials were incubated in a total volume of 3.0 ml in a glass cuvette: 50 μmol of sodium phosphate (pH 7.0), 0.15 μmol of NBT dye, and 0.23 μmol of NADH or 5.40 μmol of NADPH. Reaction was initiated by the addition of leukocyte sonic extract (usually 0.10 ml containing 0.2 to 0.5 mg of protein), and the increase in absorbancy at 560 nm was followed for at least 5 min on a recording spectrophotometer. Initial rates of NBT reduction were determined from the slopes of the lines.

Lysozyme was assayed with a recording spectrophotometer by following the decrease in absorbancy at 450 nm due to lysis of a suspension of *Micrococcus lysodeikticus* in 0.10 M phosphate buffer, pH 6.2 (19). Beta-glucuronidase activity was assayed by measuring the liberation of free phenolphthalein from phenolphthalein glucuronide at pH 5.0 (27). Protein concentrations of the leukocytic extracts were determined by the biuret procedure of Gornall et al. (13), using bovine serum albumin as a standard.

RESULTS

The specific activities of both NADH and NADPH diaphorase in sonic extracts of human leukocytes are shown in Table 1. The activity of sonic extracts from PMNL with NADH as substrate was generally slightly greater than that obtained with NADPH as substrate, although there was considerable variation from sample to sample. In two experiments, the diaphorase activities of sonic extracts from lymphocytes were of the same order of magnitude as those from the PMNL. Since the purity of the PMNL suspensions was always 90% or greater, the contribution of lymphocytic enzyme to the total activity measured was less than 10%.

Although the data in Table 1 were derived from whole sonic extracts, the specific activities of both diaphorase enzymes were comparable in the whole sonic extract or in a 27,000 $\times$ *g* supernatant of the whole sonic extract (data not shown). Attempts to release additional enzyme into the soluble fraction by the use of detergents (including Triton X-100, sodium dodecyl sulfate, and deoxycholate) were unsuccessful due to inhibition of the enzyme by the compounds in question. Similarly, sonic treatment in alkaline isotonic KCl (20) did not release any more activity than did sonic treatment in phosphate-buffered saline. Because of turbidity problems encountered when the whole sonic extract was employed, subsequent assays were performed on the 27,000 $\times$ *g* supernatant of the sonic extract.

To localize the diaphorase activities within the cell, a modified procedure was employed. Because a relatively large number of cells was required, a patient with polycythemia vera was used as the donor; however, we have not observed any reproducible differences (either qualitative or quantitative) between diaphorase activities of normal or polycythemia vera cells to date. Cells were isolated from 150 ml of blood without being subjected to hypotonic lysis. The packed cells (0.60 ml) were suspended in 0.34 M sucrose to a final concentration of 1.5×10^8 cells per ml. The cells were disrupted by homogenization in a Potter-Elvejhem-type homogenizer until approximately 90% disruption was achieved, as estimated by phase microscopy. Intact cells and cellular debris were removed by centrifugation at 400 $\times$ *g* for 10 min. The supernatant was centrifuged at 12,000 $\times$ *g* to sediment the lysosomes. The lysosomal pellet was suspended in 8.0 ml of 0.34 M sucrose and subjected to five cycles of freezing and thawing in liquid nitrogen. The 12,000 $\times$ *g* supernatant was centrifuged at 100,000 $\times$ *g* for 30 min, and enzyme assays were performed on the resuspended 12,000 $\times$ *g* pellet (lysosomal fraction) and 100,000 $\times$ *g* supernatant (soluble fraction).

TABLE 1. *Specific activity of human leukocyte diaphorases*

Sonic extract[a]	No. assayed	Δ OD per min per mg[b]	
		NADH	NADPH
PMNL	7	0.0183 ± 0.004	0.0142 ± 0.006
Lymphocyte	2	0.0154	0.0134

[a] Values for PMNL represent the mean ± standard deviation.

[b] Determined in triplicate at three different protein concentrations and averaged.

Both diaphorase activities were found primarily in the granule fraction, although there was significant activity in the soluble fraction as well (Table 2). Approximately two-thirds of each diaphorase activity was found to be associated with the lysosomal fraction under the conditions employed.

Because of the possibility that some of the soluble diaphorase activity might be due to erythrocyte contamination, the experiment was repeated with the following modifications. The isolated cells were subjected to hypotonic lysis to remove erythrocyte contamination prior to homogenization, and the lysosomal pellet was washed twice with phosphate-buffered saline prior to freeze-thawing to minimize the adsorption of soluble diaphorase activity to the lysosomes. The results exactly paralleled those illustrated in Table 2. The specific activity of lysozyme in the lysosomal fraction was 40 times that in the soluble fraction, whereas the specific activity of beta-glucuronidase was eight times higher in the lysosomal fraction. The diaphorase activities were observed in both the lysosomal and soluble fractions; the ratio of the specific activities of both NADH and NADPH diaphorase was approximately 3:1 (data not shown). These procedures do not differentiate between an enzyme that is localized in the granule membrane rather than in the contents of the granule. It is possible that the diaphorase activity, or a part of the activity, is actually membrane bound.

The effect of reduced pyridine nucleotide concentration on diaphorase activity is illustrated in Fig. 1. K_m values estimated from the graph (as the substrate concentration required for half-maximal velocity) are approximately 0.40 mM for NADPH and 0.01 mM for NADH. Although there was a 40-fold difference in K_m value, the maximal velocities were roughly equal (0.016 Δ optical density (OD) per min per mg for NADPH and 0.010 Δ OD per min per mg for NADH). Because of the very high affinity of the enzyme for NADH, it was difficult to determine initial velocities at nonsaturating concentrations of this nucleotide. At concentrations below about 20 μM, the absolute amount of nucleotide became limiting in the first minutes of reaction. The assay was complicated further by a relative lack of sensitivity due to the small extinction coefficient of reduced NBT. This resulted in a lack of precision when small amounts of reduced NBT were measured. For these reasons, the data could not be expressed as the usual double reciprocal plot. It should also be evident that the experimental K_m for NADH represented a maximal value; the actual K_m may be considerably lower than .01 mM, but this could not be demonstrated due to limitations in the assay procedure.

The effect of NBT concentration on the various diaphorase activities is illustrated in

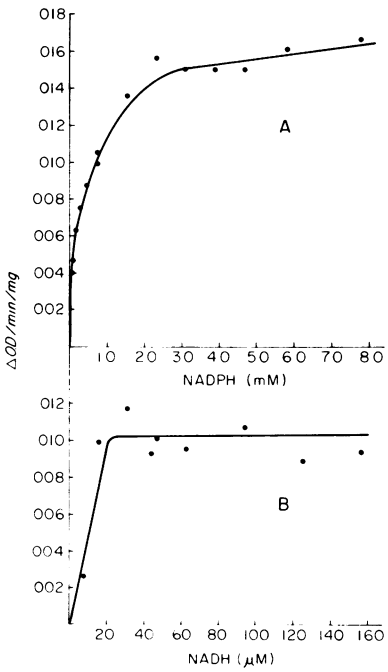

FIG. 1. *Effect of reduced pyridine nucleotide concentration on initial rate of diaphorase reaction. Concentration of NBT was 50 μM in all cases. A, effect of NADPH concentration; B, effect of NADH concentration.*

TABLE 2. *Localization of leukocyte diaphorase activity*[a]

Fraction	Δ OD per min per mg of protein			β-Glucuronidase (μmol of phenolphthalein per h per mg of protein)
	NADH diaphorase	NADPH diaphorase	Lysozyme	
Lysosomal (12,000 × *g* pellet)	0.0136	0.0181	0.2009	0.091
Soluble (100,000 × *g* supernatant)	0.0067	0.0060	0.0048	0.010

[a] Each value represents the mean of triplicate determinations run at different levels of protein concentration.

Fig. 2. Optimal concentrations of pyridine nucleotides were employed in this experiment (i.e, 78 μM NADH and 1.80 mM NADPH). In contrast to the preceding experiment, these data lend themselves quite nicely to the Lineweaver-Burke plot. The K_m for NBT with NADH as substrate was estimated to be approximately 33 μM, whereas the K_m with NADPH was somewhat less, approximately 12 μM. The maximal velocities obtained in this experiment are quite similar, 0.026 Δ OD per min per mg for the NADPH diaphorase and 0.021 Δ OD per min per mg for the NADH enzyme.

Because of these results, the standard conditions chosen for assay of diaphorase activity utilized 50 μM NBT, 78 μM NADH, and 1.80 mM NADPH, as outlined above.

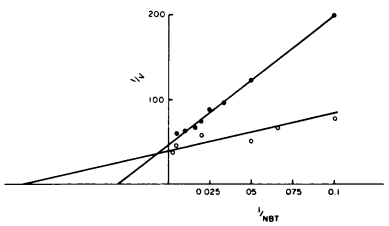

FIG. 2. *Double reciprocal plot of initial velocity versus NBT concentration. Velocity (V) is in Δ OD per min per mg; NBT concentration is in μM. Symbols: ●, 78 μM with NADH as substrate; O, 1.80 mM with NADPH as substrate.*

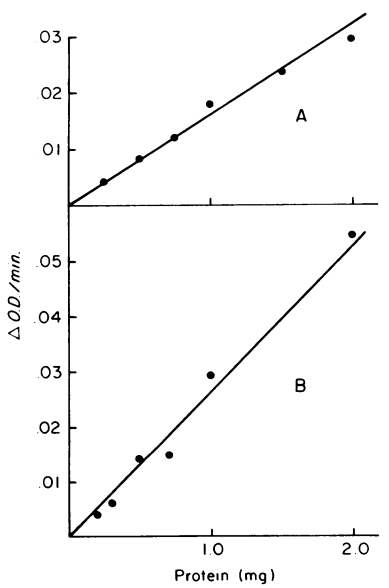

FIG. 3. *Effect of protein concentration on initial velocity of diaphorase reaction. A, NADPH concentration of 1.8 mM; B, NADH concentration of 78 μM. The concentration of NBT was 50 μM in all cases.*

The validity of this procedure for assay of the diaphorase activities is demonstrated in Fig. 3. Using these conditions, the change in absorption per minute was proportional to the protein concentration up to at least 2.0 mg of leukocyte protein. Similarly, at relatively low concentrations of protein (0.50 mg), the reaction was linear with respect to incubation time for at least 10 min (data not shown).

The pH profile of the two activities is illustrated in Fig. 4. Both the NADH- and the NADPH-dependent reactions showed increased activity at acidic pH, although the change was considerably greater and more abrupt in the case of the NADH-dependent diaphorase.

Both enzyme activities were completely insensitive to either cyanide or azide as illustrated in Table 3. The small degree of apparent stimulation observed in some cases was not considered significant.

DISCUSSION

The importance of diaphorase activities in the human PMNL is deduced from the fact that physiologically incompetent cells (e.g., from patients with CGD or leukocyte glucose-6-phosphate dehydrogenase deficiency) do not exhibit diaphorase activity during phagocytosis (7, 11, 14), and this is associated with an impaired ability to destroy certain types of bacterial pathogens (11, 17). Other metabolic changes, including increases in oxygen consumption, hexose monophosphate shunt activity, and hydrogen peroxide production, are likewise lacking during phagocytosis by these cells, although ingestion appears to be normal

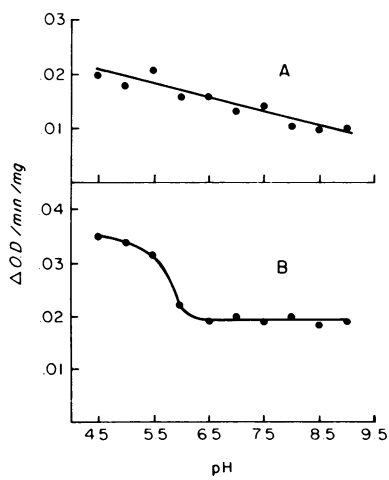

FIG. 4. *Effect of pH on initial velocity of diaphorase reaction. A, NADPH concentration of 1.8 mM; B, NADH concentration of 78 μM.*

TABLE 3. *Effect of cyanide and azide on leukocyte diaphorase activity*

Conditions	Δ OD per min per mg[a]	
	NADH	NADPH
Control	0.0231	0.0231
+ CN^- (0.33 mM)	0.0231	0.0254
+ CN^- (1.65 mM)	0.0233	0.0267
+ Azide (0.33 mM)	0.0280	0.0225
+ Azide (1.65 mM)	0.0278	0.0231

[a] Each value represents the mean of duplicate determinations.

(11, 14, 17). The precise basis for these metabolic changes is unknown, although most investigators feel that activation of an oxidase is the most likely explanation for the initiating event. Baehner and Karnovsky (5) have postulated that the initiating enzyme is NADH oxidase, based on their finding that cells from patients with CGD have low levels of this enzyme (about 50% of control) when compared with cells obtained from normal infected patients. Other investigators have disputed this finding (16), and some propose NADPH oxidase to be the critical enzyme (15). Indeed, a recent abstract (D. C. Hohn and R. I. Lehrer, Clin. Res. **22**:394A, 1974) has described a deficiency of this enzyme in CGD cells.

From a quantitative viewpoint, the activity of either diaphorase can readily explain the respiratory burst. It is possible to completely reduce NBT dye by the use of alkaline ascorbate. When this was done, we observed that 0.005 μmol of reduced NBT/ml gave an absorbancy change of 0.100 U. If the typical diaphorase activity is approximately 0.015 Δ OD per min per mg (Table 1), this corresponds to 2.3 nmol of reduced NBT per min per mg or 138 nmol per h per mg. Baehner et al. (3) have calculated that the change in oxygen consumption of human cells under similar conditions amounts to 63 nmol per h per mg, whereas the activity of NADH oxidase is 39 nmol per h per mg, and the change in glucose oxidation is still less at 20 nmol per h per mg. The diaphorase activities within the phagocytic vacuole might be expected to be significantly greater due to the lowered pH, in keeping with the data in Fig. 4. Thus, the activity of either NADH or NADPH diaphorase is more than sufficient to explain the respiratory burst.

The reduction of NBT is highly nonspecific and can be accomplished in a number of ways that would not require a specific diaphorase. In particular, superoxide anion (O_2^-), which can be generated by PMNL (2), will itself cause the reduction of NBT (9). This does not seem to be a problem in the present system, since reduction of the dye is absolutely dependent upon the addition of reduced pyridine nucleotides. Furthermore, the addition of purified superoxide dismutase (Truett Labs, Dallas, Tex.) gave minimal inhibition of the reduction of NBT (less than 20% inhibition) under the assay conditions employed (data not shown). Similarly, the granule diaphorase activity cannot be ascribed to myeloperoxidase, since purified myeloperoxidase (kindly supplied by Julius Schultz) failed to reduce NBT in either the presence or absence of CN^-.

It seems reasonable that at least a portion of the diaphorase activity of human PMNL is due to NADH and NADPH oxidase activity (13). We undertook to investigate the diaphorase activity for several reasons. First, the oxidase enzymes are low in activity and are difficult to measure. Furthermore, it is extremely difficult to adequately control the reaction since the only reactants are oxygen and reduced pyridine nucleotide; other cellular reactions could be responsible for the changes in oxygen consumption or oxidation of reduced nucleotide by which the oxidase activities are typically measured. Finally, since a defect in NBT reduction is well documented in phagocytizing CGD cells, this reaction might provide the best opportunity to probe the metabolic basis of this particular disease. Although the reduction of NBT by CGD cells is lacking during phagocytosis, the cells apparently have a normal complement of active enzyme when measured in a cell-free system (21). Thus, a more subtle defect than a simple enzyme deficiency is implicated. Such a defect might involve a deficiency in a particular isoenzyme which might not be observed when the total activity is determined, or it might be the result of a change in the properties of an enzyme so that it is unable to function at conditions which are present within the cell.

The present investigation reports some of the properties of the diaphorase activities of normal cells. Both NADH and NADPH diaphorase were found in both the granule and soluble fractions of the cell (Table 2). The soluble activity could not be ascribed to leakage due to lysosomal damage, since a far smaller percentage of lysosomal marker enzymes (beta-glucuronidase and lysozyme) was seen in the soluble fraction. The dual location suggests that there were at least two diaphorases, not distinguishable by the catalytic assay, which were located differently within the cell. It seems

likely that the granule diaphorase activity is one of importance during phagocytosis, since Nathan et al. have reported that the reduction of NBT dye occurs only within the phagocytic vacuole (21). The diaphorase activity would be delivered to the vacuole during the process of degranulation.

Some of the properties of the various diaphorases are of interest. Both NADH and NADPH diaphorase were totally insensitive to both cyanide and azide; this is consistent with a role for either in initiating the respiratory burst, which is itself cyanide insensitive.

Both enzyme activities were more active at acid pH, although the NADH diaphorase showed the more dramatic change. This might serve as an initiating mechanism, since the intravacuolar pH is known to decrease during phagocytosis (25).

Perhaps the most interesting data is obtained in the kinetic studies. Although both NADH and NADPH diaphorase had about the same affinity for NBT (Fig. 2), there was a marked difference in the affinity of the enzymes for their respective pyridine nucleotide substrates. The K_m value for NADH was about 10 μM, whereas the corresponding value for NADPH was about 400 μM. It is revealing to compare these values with the intracellular concentrations of these compounds. Baehner (4) has reported that the concentration of both NADH and NADPH is 2.4 nmol per 10^8 PMNL. Assuming that the cell is 70% water and that 1.0 ml of packed cells equals 1.5×10^9 PMNL (L. DeChatelet, unpublished data), then the intracellular concentrations of both nucleotides may be calculated to be about 51 μM. Although this concentration is sufficient to saturate the NADH diaphorase, it is far below the optimal concentration for the NADPH-requiring enzyme. Although not conclusive, these data argue in favor of an NADH oxidase as initiator of the respiratory burst which accompanies phagocytosis. Factors which might militate against this conclusion include activation of a specific diaphorase by phagocytosis, or the presence of different isozymes of a diaphorase with greatly different K_m values. Patriarca et al. (24) have reported that the K_m for NADPH oxidase in guinea pig PMNL decreases by a factor of 10 during phagocytosis. If confirmed, this would make the values for the two enzymes considerably closer, although the NADH-requiring enzyme would still have a lower K_m by a factor of four. A recent abstract (D. C. Hohn and R. I. Lehrer, Clin. Res. **22**:394A, 1974) suggests that a similar activation NADPH oxidase might occur in phagocytizing human PMNL. It will be important to determine whether the K_m values for the diaphorase enzymes change with phagocytosis.

Also, Holmes (16) has demonstrated that there are four isozymes of NADPH diaphorase and three of NADH diaphorase in the human PMNL. It is quite possible that only a specific isozyme is involved in the respiratory burst. Since the present investigation was concerned only with total diaphorase activity, we cannot exclude the possibility that a specific isozyme of NADPH has a much lower K_m than the average value for the total NADPH diaphorase activity.

The linearity observed with increasing protein concentration (Fig. 3) demonstrates that the conditions employed gave a valid estimate for enzyme activity. This assay system should prove useful in determining whether cells that demonstrate a high histochemical reduction of NBT (as in severe infection [23] or polycythemia vera [1]) do so because of an increased quantity of diaphorase, or because of some other change in the metabolism of the cell.

Future experiments include an examination of the various isozymes of the diaphorase activities in both normal and CGD cells. Alterations in enzyme activity upon phagocytosis will also be examined.

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