LOCALIZATION OF NADH OXIDASE ON THE SURFACE

OF HUMAN POLYMORPHONUCLEAR

LEUKOCYTES BY A NEW CYTOCHEMICAL METHOD

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ABSTRACT

The ultrastructural localization of NADH oxidase, a possible enzyme in the increased oxidative activity of polymorphonuclear leukocytes (PMN) during phagocytosis, was studied. A new cytochemical technique for the localization of H_2O_2, a product of NADH oxidase activity, was developed. Cerous ions, in the presence of peroxide, form an electron-dense precipitate. Resting and phagocytically stimulated PMN were exposed to cerous ions at pH 7.5 to demonstrate sites of NADH-dependent, cyanide-insensitive H_2O_2 production. Resting PMN exhibited slight activity on the plasma membrane; phagocytizing PMN had extensive deposits of reaction product localized within the phagosome and on the plasma membrane. Peroxide involvement was demonstrated by the inhibitory effect of catalase on cerium precipitation; the surface localization of the enzyme responsible was confirmed by using nonpenetrating inhibitors of enzymatic activity. A correlative study was performed with an NADH-dependent, tetrazolium-reducing system. As with cerium, formazan deposition on the surface of the cell was NADH dependent, cyanide insensitive, and stimulated by phagocytosis. Superoxide dismutase did not inhibit tetrazolium reduction, as observed cytochemically, indicating direct enzymatic dye reduction without superoxide interposition. These findings, combined with oxygen consumption studies on resting and stimulated PMN in the presence or absence of NADH, indicate that NADH oxidase is a surface enzyme in human PMN. It is internalized during phagocytosis and retains its peroxide-generating capacity within the phagocytic vacuole.

Phagocytosis by polymorphonuclear leukocytes (PMN)[1] is accompanied by specific alterations in the oxidative metabolism of the cell. Ingestion of particles or other stimulation of the surface triggers increases in oxygen consumption, hydrogen peroxide production, and hexose monophosphate

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[1] Abbreviations used in this paper: AT, 3-amino-1, 2, 4-triazole; Hanks' BSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; NADH, β-nicotinamide adenine dinucleotide phosphate, reduced form; NBT, nitroblue tetrazolium; O_2, superoxide; PCMBS, p-chloromercuribenzene sulfonic acid; PMN, polymorphonuclear leukocyte; PS, polystyrene; SOD, superoxide dismutase; TNBT, tetranitroblue tetrazolium.

shunt activity (for review, see reference 27). Al-
though some of the enzymatic mechanisms under-
lying these perturbations have been defined, uncer-
tainty remains as to the primary oxidase responsi-
ble for the increase in O_2 consumption and H_2O_2
production. An oxidase for NADPH has been
proposed by some workers (43, 50); others believe
that the respiratory burst is accounted for by the
oxidation of NADH by a flavoprotein oxidase
(3, 11). Both these enzymes are capable of a
cyanide-insensitive production of H_2O_2 (23), a
reaction product which is found both extracellu-
larly and intracellularly (42). In particular, H_2O_2
has been detected within the phagocytic vacuole
(10), where it presumably constitutes a part of the
bactericidal mechanism of the PMN (26, 28, 49).
The site of formation of H_2O_2 in the intact cell,
and therefore the site of oxidase activity, has not
been adequately resolved. NADPH-oxidizing ac-
tivity has been associated biochemically with the
azurophil granule fraction of PMN (39, 40), but no
specific localization has been determined for
NADH oxidase. It has been suggested that the
plasma membrane regulates peroxide production
(47, 48, 50), is the site of peroxide production (49,
56), or is the site of a reduced pyridine nucleotide
oxidase (35), but none of these has been conclu-
sively demonstrated. Plasma membrane involve-
ment in peroxide formation is of interest, for, as
pointed out by Root and Stossel (49), membrane
internalization during particle ingestion could then
result in H_2O_2 synthesis within the phagosome,
the important site of utilization by peroxidase re-
leased from the granules.

In order to explore the subcellular distribution
of NADH oxidase, we have devised a cytochemical
technique for the localization of the site of H_2O_2
production in unfixed, phagocytizing PMN. The
generation of reaction product is accomplished by
exposing the cells to cerium ions in the incubation
medium. H_2O_2 generated by the cell results in a
precipitate, presumably cerium perhydroxide
(Ce-[OH]$_2$OOH), with properties suitable for
ultrastructural cytochemistry. In conjunction with
this approach, a correlative electron microscope
study was done on NADH-dependent tetrazolium
reduction by PMN. In both instances, the primary
sites of cytochemical activity were the external
face of the plasma membrane and the internal
face of the phagocytic vacuole membrane. A brief
account of this work has been presented elsewhere
(9).

MATERIALS AND METHODS

The designs of major experiments are roughly
outlined in Fig. 1, stages I-IV.

Isolation and Preparation of PMN (Stage I)

The experiments were performed on human PMN
isolated by a modification of the technique of Harris (21).
Glass cover slips (22-mm diameter) were flooded with
blood from a pricked finger, placed in a moist chamber,
and incubated for 5 min at room temperature followed by
30 min at 37°C. The blood clot and erythrocytes were
rinsed from the cover slip with Hanks' balanced salt
solution (Hanks' BSS) (Microbiological Associates, Inc.,
Bethesda, Md.), pH 7.4, at 4°C. Numerous PMN and a
few monocytes and eosinophils remained attached to the
glass surface.

Metabolic Stimulation (Stage II)

(a) Phagocytic stimulation of PMN metabolism was
accomplished by exposing the cells to polystyrene spheres
(PS, 1.1 μm diameter) (Dow Chemical Co., Midland,
Mich.). These particles were prepared by extensive (72 h)
dialysis against twice distilled H_2O and were then diluted
to 2.5% of the original volume with sterile 0.9% NaCl.
For phagocytosis, a 1:20 dilution of the 2.5% stock was
prepared with Hanks' BSS plus additional glucose (1.0
mg/ml). Cover slip preparations were flooded with 0.5
ml of the suspension, resulting in an excess of particles to
cells, and incubated for 20 min at 37°C in a moist
chamber. At this time the response is known to be of
significant magnitude. (b) Metabolic stimulation without
using solid, phagocytizable particles was accomplished
with endotoxin (20). Lipopolysaccharide B of Escherichia
coli (LPS) (Difco Laboratories, Detroit, Mich.) was used
at a concentration of 50 μg/ml of Hanks' BSS with
additional glucose (1.0 mg/ml). The preparation was
sonicated before use to insure complete suspension. Each
cover slip received 0.5 ml of LPS and was incubated for
20 min at 37°C. After these incubations in PS or LPS,
the cell preparations were quickly but thoroughly rinsed
in several changes of cold (4°C) Hanks' BSS. Resting
(control) PMN were incubated in Hanks' BSS plus
glucose (1 mg/ml) at 37°C for 20 min, and then rinsed
like stimulated cells.

Hydrogen Peroxide Localization (Stage III)

Before incubation, resting or stimulated cells were
washed briefly at 4°C in 0.1 M Tris-maleate buffer with
7% sucrose at a pH identical to that of the cytochemical
medium. The unfixed cells were then preincubated for 10
min at 37°C in 0.1 M Tris-maleate with 7% sucrose, pH
7.5, containing 1 mM 3-amino-1,2,4-triazole (AT) (Al-
drich Chemical Co., Inc., Milwaukee, Wis.). The final
incubation medium consisted of 0.1 M Tris-maleate, pH
7.5, with 7% sucrose, 10 mM AT, 1 mM CeCl$_3$, and 0.71
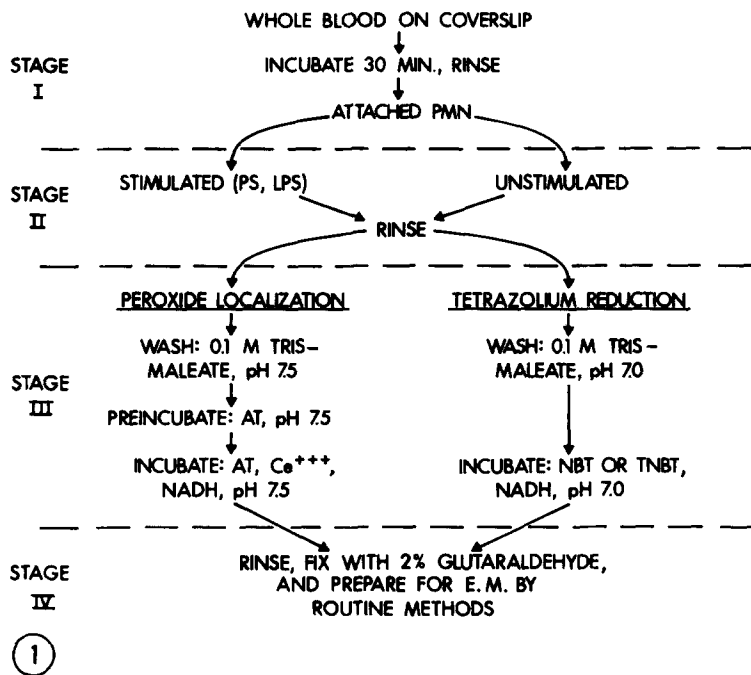mM NADH (Sigma Chemical Co., St. Louis, Mo.).

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                    WHOLE BLOOD ON COVERSLIP
                              ↓
STAGE               INCUBATE 30 MIN., RINSE
  I                           ↓
            _____ATTACHED PMN_____

STAGE     STIMULATED (PS, LPS)              UNSTIMULATED
  II
                          RINSE

            PEROXIDE LOCALIZATION        TETRAZOLIUM REDUCTION
                     ↓                           ↓
                WASH: 0.1 M TRIS-          WASH: 0.1 M TRIS-
STAGE           MALEATE, pH 7.5            MALEATE, pH 7.0
  III                ↓
            PREINCUBATE: AT, pH 7.5
                     ↓
            INCUBATE: AT, Ce⁺⁺⁺,          INCUBATE: NBT OR TNBT,
            NADH, pH 7.5                   NADH, pH 7.0

STAGE       RINSE, FIX WITH 2% GLUTARALDEHYDE,
  IV          AND PREPARE FOR E.M. BY
               ROUTINE METHODS

   (1)
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FIGURE 1   A schematic outline presenting the major experimental procedures used in the detection of sites of peroxide formation and tetrazolium reduction. Note that phagocytic stimulation was accomplished before cytochemical incubation. Additional details are given in the text.

PMN were incubated in this medium for 20 min at 37°C. Adjustments in the pH of the various solutions were made as necessary. All solutions containing cerium were filtered (0.45 μm, Millipore Corp., Bedford, Mass.) to remove the small amount of cerium hydroxide precipitate that formed at the alkaline pH. Any additional precipitate formed during the incubation period could be removed by a postfixation wash in slightly acidic (pH 6.0) buffer which did not alter the quantity or distribution of the reaction product. The stability of the incubation medium beyond several hours was not determined; all solutions were prepared just before use. A range of incubation times, from 10 to 60 min, was tested and 20 min was selected as being optimal for allowing accumulation of a sufficient amount of reaction product without unduly damaging the cellular ultrastructure. This was especially important when live, unfixed PMN were used.

NADH-Dependent Tetrazolium Reduction (Stage III)

In order to determine the sites of tetrazolium reduction, the cells were prepared and stimulated with PS as described above. The incubation medium was similar to the Ce⁺⁺⁺ medium, except that cerium and aminotriazole were omitted and 0.6 mM nitroblue tetrazolium (NBT) (Sigma Chemical Co.) or 0.88 mM tetranitroblue tetrazolium (TNBT) (Dajac Laboratories, Philadelphia,

Pa.) was included. In both cases, the medium was filtered before use (Millipore, 0.45 μm), since the tetrazoliums failed to dissolve completely; this resulted in a diminished final concentration of the tetrazolium. The pH of the medium was critical. In the presence of NADH, a spontaneous reduction of NBT would occur above pH 7.0 (see reference 55); this occurred with TNBT above pH 7.5. Therefore, in both instances, the pH of the medium was 7.0. An incubation time of 30 min at 37°C was found to be most effective in that it allowed for sufficient accumulation of reaction product without undue deterioration of cellular morphology, especially with regard to the integrity of the plasma membrane.

Controls

A variety of controls were performed in order to gain some insight into the specificity of the reactions. These will be elaborated in the appropriate sections of Results.

Preparations for Electron Microscopy (Stage IV)

After incubation, cover slips were washed briefly in 0.1 M Tris-maleate buffer with 7% sucrose at 4°C; the pH was the same as that of the incubation medium. The cells were then fixed in 2% glutaraldehyde (electron microscopy grade, Polysciences, Inc., Warrington, Pa.) in 0.1

M sodium cacodylate buffer, pH 7.3, with 5% sucrose at 4°C for 30–60 min. In the cerium precipitation series, cells were then washed 1 h at 4°C in 0.1 M cacodylate buffer with 5% sucrose at pH 6.0. All cells were washed overnight in 0.1 M cacodylate, pH 7.3, with 5% sucrose at 4°C, postfixed for 60 min in 2% osmium tetroxide in the same buffer, dehydrated in graded ethanols, and embedded in Epon 812 (30). The specimens were polymerized at 60°C for 24 h, then taken directly from the oven and immersed in liquid nitrogen for 10–15 s. This resulted in a clean separation of the Epon block from the cover slip.

Thin sections were prepared with an LKB ultramicrotome equipped with a diamond knife. Sections were routinely stained with 4% aqueous uranyl acetate and alkaline lead (60) (3 and 4 min, respectively) and examined in a Philips 200 electron microscope operated at 60 kV.

RESULTS

Morphology of Resting and Stimulated PMN

The morphology of the mature, human PMN has been described (7). These cells present a characteristic nuclear morphology, the cytoplasm contains a mixed population of primary and secondary granules, and there are a few mitochondria and scattered deposits of glycogen (Fig. 2). Typically PMN grown as monolayers exhibit numerous projections over most of the cell surface, and there are frequently membrane-limited, electron-lucent vesicles in the cytoplasm. Certain of these vesicles are surface-connected invaginations of the plasma membrane which appear vesicular due to the plane of the section.

Phagocytically stimulated PMN present an overall morphology similar to resting cells, except that there is a general depletion of the granule population due to fusion with phagocytic vacuoles. These vacuoles are membrane limited and typically appear partially empty due to the extraction of the polystyrene particle during preparation for electron microscopy (Fig. 3). PMN stimulated by LPS present an appearance similar to the resting PMN, as has been noted by others (20). In both cases, surface infoldings are not infrequent, giving rise to channels and vesicles in thin section.

Hydrogen Peroxide Detection by Cerium Precipitation

According to Feigl (19), cerium ions react with ammonia and hydrogen peroxide to precipitate cerium perhydroxide (Ce[OH]$_2$OOH and Ce[OH]$_3$OOH). This reaction, modified to eliminate the ammonia, was found to take place rapidly at near neutral pH in 0.1 M Tris-maleate buffer—a medium more suitable for enzyme and cellular preservation. The precipitate formed is quite electron dense and unaffected by the solvents used to prepare tissue for electron microscope examination.

The sequential scheme illustrated in Fig. 1 was employed; phagocytic stimulation and cerium incubation could not be performed simultaneously because phagocytosis was inhibited by the presence of cerium. This strategy eliminates the possibility that the treatments might interfere with the phagocytic process. Implicit in this method is the assumption that the stimulation will persist after the removal of the excess external particles.

H$_2$O$_2$ LOCALIZATION IN RESTING CELLS: Live unfixed PMN not exposed to PS or LPS exhibited only occasional deposits of reaction product on the external surface of the plasma membrane after incubation in complete medium (Fig. 4). Surface-connected channels and vesicles also showed very slight reactivity. No other cytoplasmic organelles were reactive for H$_2$O$_2$. Occasional cells were found with dense surface and vacuolar reaction product, and in some instances these were observed to have phagocytized cell fragments and other debris.

H$_2$O$_2$ LOCALIZATION IN PHAGOCYTICALLY STIMULATED PMN: The appearance of these cells was markedly different from the resting PMN after being tested for H$_2$O$_2$ with the complete medium. The phagocytic vacuoles contained deposits of reaction product which surrounded the particle and lay between the surface of the PS and the phagosomal membrane (Fig. 5). At sites of granule fusion, reaction product was found in conjunction with granule contents. The external surface of the plasma membrane was coated with a layer of electron-dense material (Fig. 6). In some instances this layer was continuous around the perimeter of the cell in section and in other cases it was discontinuous or restricted to only one region of the membrane. Channels connecting incompletely closed phagocytic vacuoles with the surface were typically lined with precipitate. There were occasional negative phagocytic vacuoles and vacuoles which were not completely lined with deposits. Other cytoplasmic organelles, including the primary and secondary granules, were not found to contain H$_2$O$_2$ by this method.
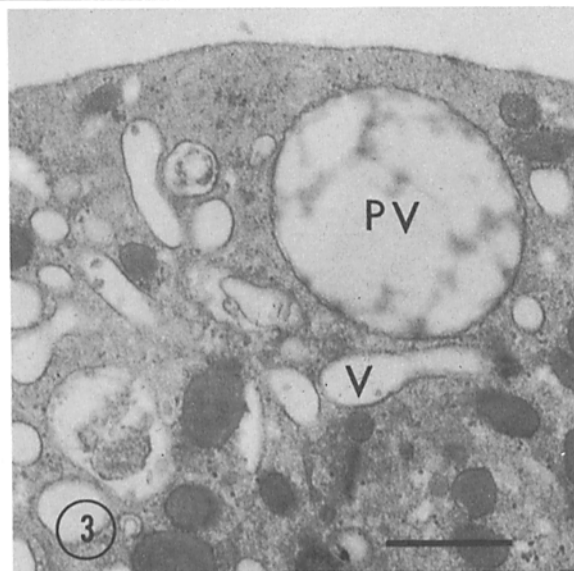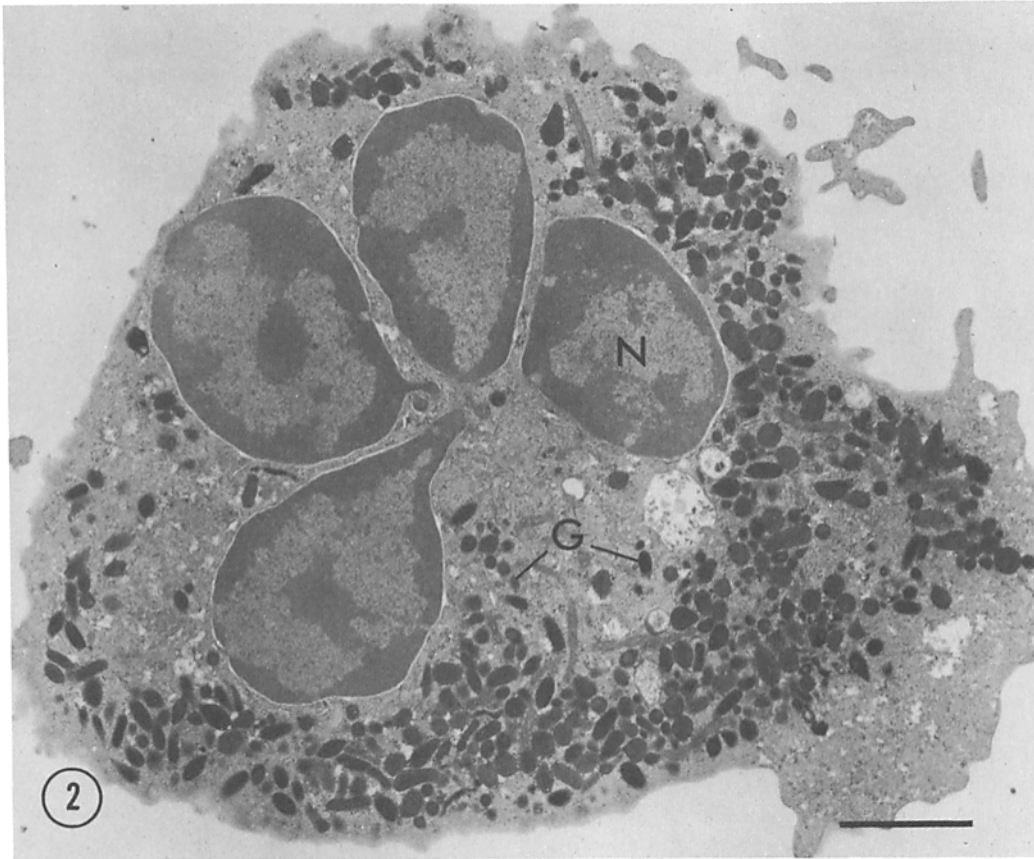
FIGURE 2   Resting human polymorphonuclear leukocyte (PMN) showing the characteristic morphology, which includes a multilobulated nucleus (N), a large population of morphologically diverse granules (G), few mitochondria, and scattered deposits of glycogen. × 8,700. Scale = 2.0 µm.

FIGURE 3   Portion of a phagocytically stimulated PMN, showing a polystyrene particle within a phagocytic vacuole (PV). These stimulated cells frequently exhibit an increased vesiculation (V) of the cytoplasm; at least some of these vesicles are profiles of surface-connected channels. The polystyrene is partially extracted during specimen preparation. × 33,100. Scale = 0.5 µm.

LPS-STIMULATED PMN: Morphologically similar to resting PMN, those which were prestimulated by LPS exposure can be characterized as being intermediate between resting and phagocytizing PMN with regard to peroxide reactivity (Fig. 7). Plasma membrane-bound reaction product varied from slight to moderate, and invaginations exhibited a moderate amount of precipitate.

SUBSTRATE DEPENDENCE: When NADH was eliminated from the incubation medium, the amount of cerium precipitation was dramatically reduced in PS-stimulated PMN (Fig. 8). Both resting and phagocytizing cells had light deposits of reaction product on the surface, but in addition the stimulated PMN exhibited phagocytic vacuoles that were slightly peroxide positive. It is clear that the generation of reaction product is strongly dependent on the presence of exogenous NADH.

CYANIDE INSENSITIVITY: Phagocytosis and the accompanying increase in O_2 consumption and H_2O_2 generation are insensitive to 1 mM KCN (23), as is NADH oxidase (3). To test the effect of cyanide on cerium precipitation, 1.0 mM KCN was included in all the incubation media, as well as AT. Resting and stimulated PMN, with and without NADH in the medium, showed similar amounts and distribution of reaction product to cell preparations lacking KCN (Fig. 9), which is consistent with the failure of KCN to interfere with the respiratory burst associated with phagocytosis. In the case of phagocytically stimulated PMN, the plasma membrane exhibited increased amounts of reaction product. This agrees with the stimulatory effect of KCN on NADH oxidase activity as noted biochemically (6).

EFFECT OF CATALASE ON REACTION PRODUCT FORMATION: To further assess the specificity of the reaction, catalase, which enzymatically destroys H_2O_2, was used to determine if cerium precipitation were dependent upon H_2O_2. In these experiments, 0.01% catalase (Sigma Chemical Co.) was included in the phagocytosis medium, washes, and all incubation media; aminotriazole was omitted because of its inhibitory effects on catalase. Catalase caused an almost complete elimination of reaction product from the cell surface, vesicles, and phagocytic vacuoles (Fig. 10). It was noted that the cerium formed a complex with the catalase, causing some precipitation. It was thought that the introduction of catalase might, in a nonenzymatic way, interfere with the precipitation of the cerium. To test this, the above experiment was performed with 10 mM amino-

triazole in the medium to inhibit the catalase. A normal distribution of reaction product was found, meaning that the complexing of cerium and catalase does not alter the effectiveness of either. It was concluded that cerium precipitation is indeed due to a reaction with H_2O_2, a reaction that can be interrupted by catalase.

EFFECTS OF ALDEHYDE FIXATION: The inhibitory effect of aldehyde fixation on some enzymes is well known. Prefixation of resting and stimulated PMN in 2% freshly depolymerized formaldehyde in 0.1 M cacodylate buffer, pH 7.3, plus 5% sucrose for 15 min at 4°C altered the normal distribution of reaction product. Deposit-containing phagocytic vacuoles appeared similar to those of unfixed cells, but there was a diminution in the amount of surface reactivity, as evidenced by reduced amounts of precipitate (Fig. 11). Exposure to 2% glutaraldehyde at 4°C for 15-30 min before the Stage III incubation resulted in a total elimination of reaction product from the cell. Both aldehydes apparently affect peroxide generation, presumably by inhibiting the activity of the enzyme. Additionally, formaldehyde prefixation (before Stage III) showed that intracellular reaction product, i.e. that present in the invaginations and phagocytic vacuoles, is formed *in situ*, for prefixation eliminates the possibility that the phagosomal deposits might be derived from the invagination of surface deposits and subsequent fusion with the phagosome. In this regard, preliminary investigations on phagocytic vacuoles isolated from guinea pig PMN, using an albumin-paraffin oil emulsion technique (57), show deposits of cerium within the vacuoles.

INHIBITION STUDIES: Enzyme inhibitors are useful to determine if the reaction product is generated enzymatically and to show the specificity of the reaction. Unfortunately, no specific inhibitor of NADH oxidase is available. As a result, the more nonspecific sulfhydryl reagents were tested. The effect of p-(hydroxymercuri)-benzoic acid (disodium salt, Aldrich Chemical Co., Inc., Milwaukee Wis.) at a concentration of 0.5 mM was examined on 2% paraformaldehyde-fixed cells by inclusion in all incubation media. This was found to reduce the number of reactive cells by approximately 60% beyond that induced by paraformaldehyde.

Two additional inhibitors were used to verify the localization of the peroxide-generating enzyme at the surface of the cell. It was reasoned that if nonpenetrating inhibitors could block reaction
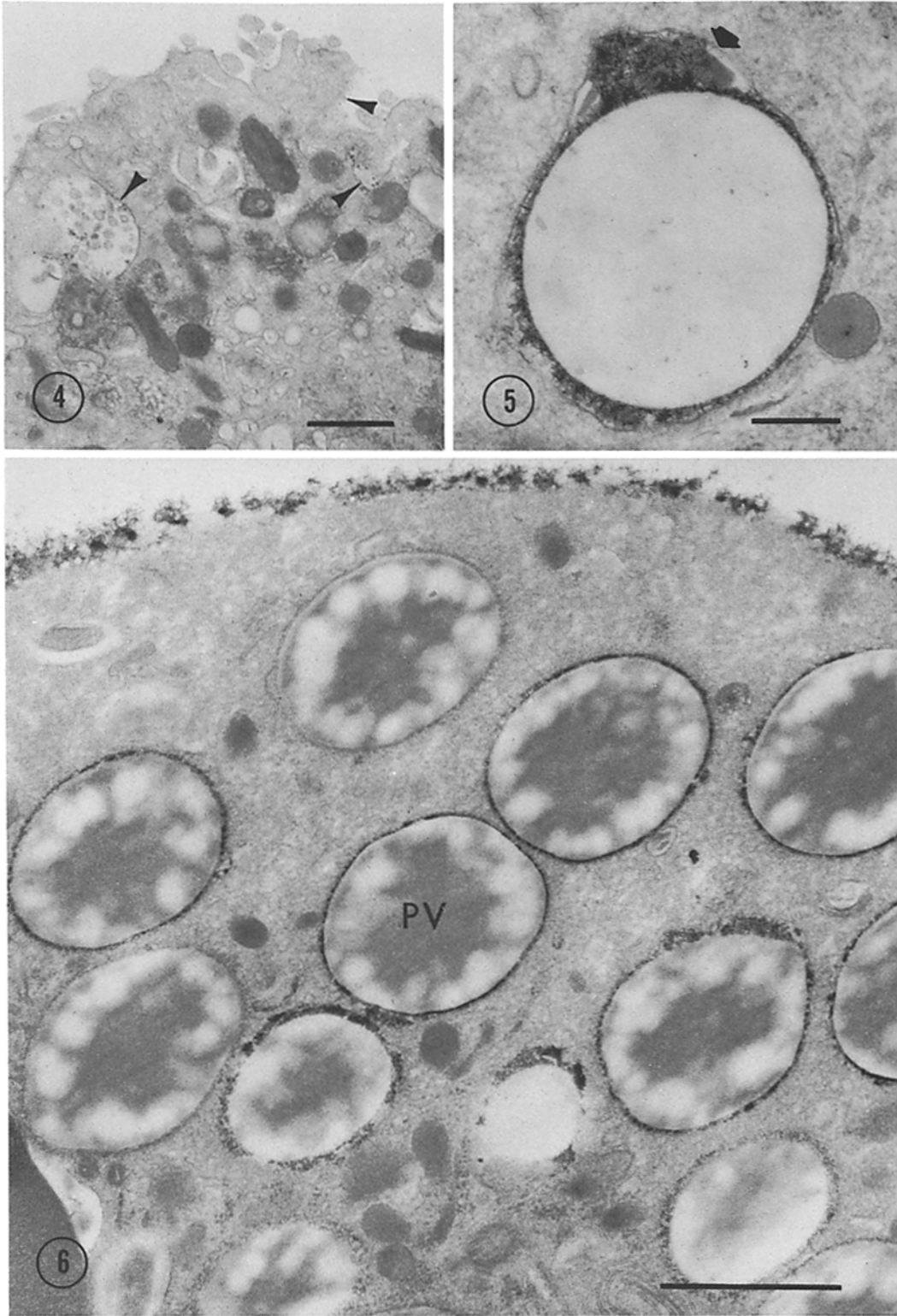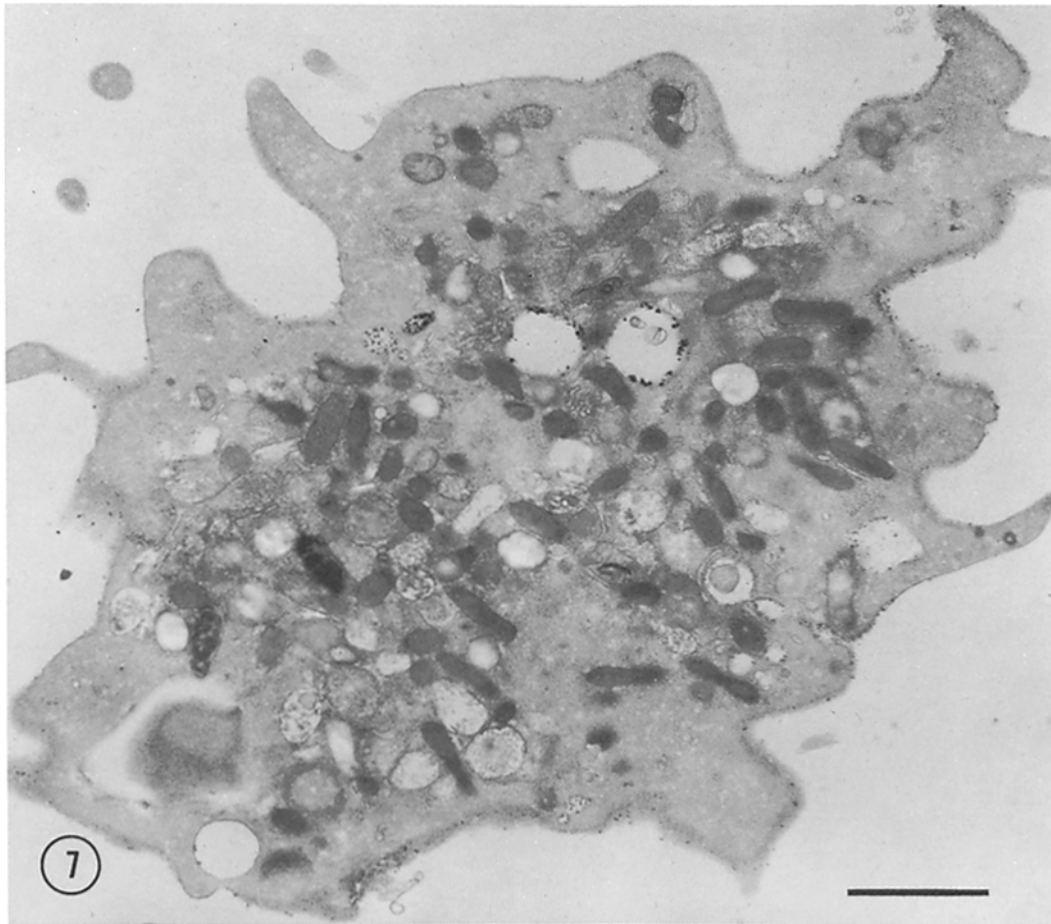
FIGURE 7   The metabolic response of PMN exposed to endotoxin is similar to that of the phagocytizing cell. Incubation of LPS-stimulated cells in the complete Ce^{+++} medium for H_2O_2 shows a distribution of reaction product on the plasma membrane and within some vesicles. This is similar to, although less extensive than the distribution found in phagocytizing PMN. × 18,500. Scale = 1.0 μm.

FIGURE 4   Incubation of resting PMN in a medium containing aminotriazole (AT), Ce^{+++}, and NADH to detect sites of peroxide generation results in only light, scattered deposits of reaction product principally on the surface of the cell and in vesicles (arrowheads). Peroxide was not detected in any other organelles. × 26,300. Scale = 0.5 μm.

FIGURE 5   One site of peroxide generation in phagocytically stimulated PMN is the internal surface of the phagosomal membrane. The cerium precipitate is deposited around the particle, lying between it and the membrane. Reaction product is also found in conjunction with granule contents (arrow). × 27,500. Scale = 0.5 μm.

FIGURE 6   An additional site of peroxide generation is the plasma membrane of the stimulated PMN. Reaction product is frequently found to cover large areas of the cell surface. A majority of the phagocytic vacuoles (PV) are peroxide positive, although occasional negative vacuoles are seen. × 27,800. Scale = 1.0 μm.
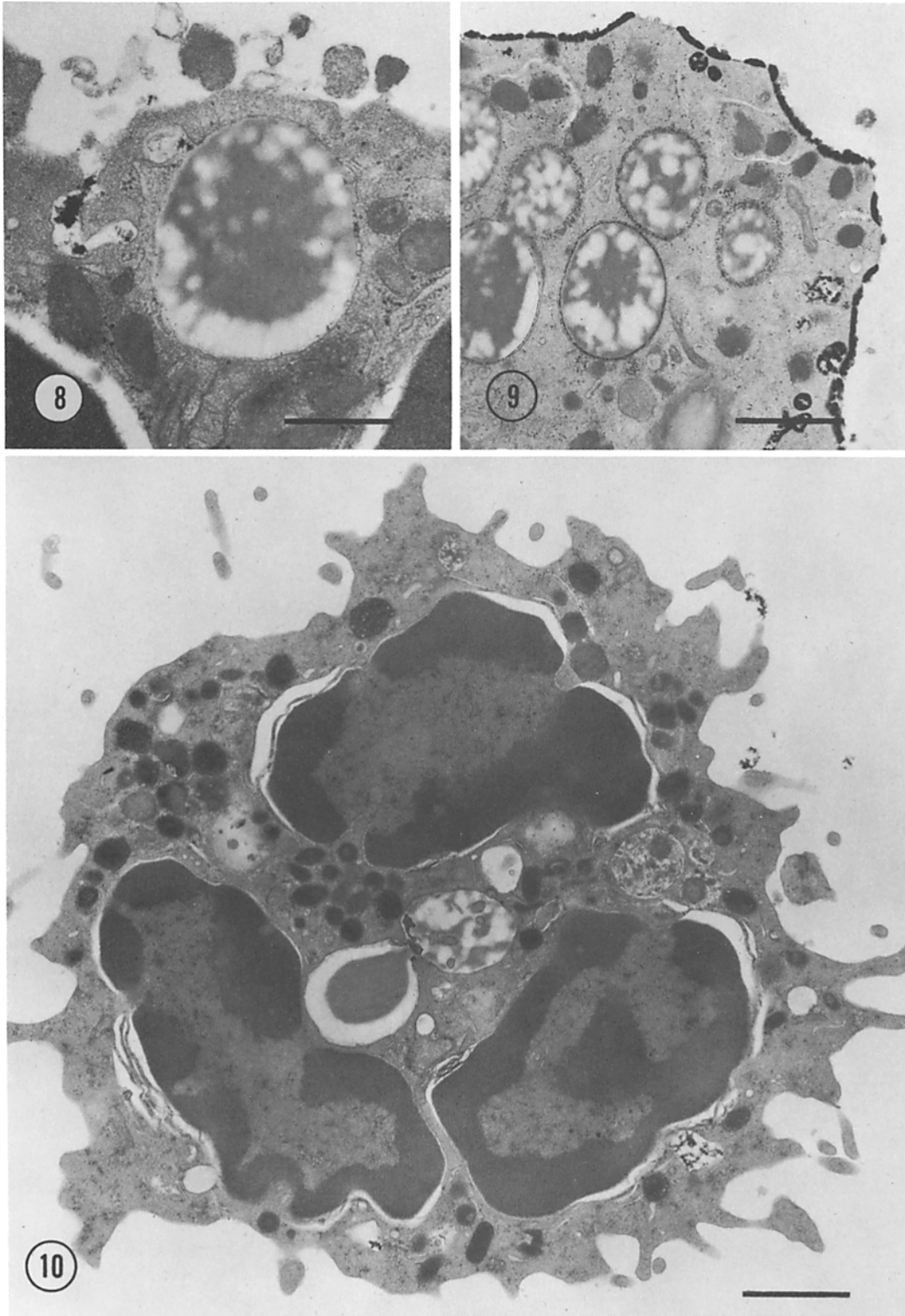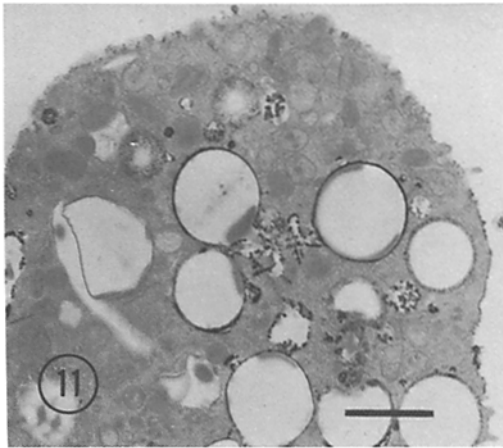
FIGURE 11 Brief fixation of PS-stimulated cells in 2% paraformaldehyde just before cytochemical incubation in the cerium medium produced a diminution of surface, but not phagosomal peroxide-generating activity. This implies a sensitivity of the enzyme to aldehyde fixation. Additionally, prefixation eliminates the possibility that intracellular deposits of cerium are due to an internalization of surface reaction product. × 11,700. Scale = 1.0 μm.

product formation on the surface of the cell, then the enzyme is probably located on the plasma membrane. Parachloromercuribenzenesulfonate (PCMBS) (Sigma Chemical Co.) was found to be a nonpenetrating sulfhydryl reagent capable of inhibiting glucose transport in erythrocytes (59). Stimulated, unfixed PMN preincubated 5 min at 37°C in 10 mM PCMBS in 0.1 M Tris-maleate buffer, pH 7.5, plus 7% sucrose and then incubated in the normal cytochemical medium containing 10

mM PCMBS were found to be essentially negative with regard to surface cerium deposits, although many phagosomes were reactive. The diazonium salt of sulfanilic acid, a second nonpenetrating enzyme inhibitor whose effects on the biochemistry of PMN have been well studied (16, 17), was also used. The reagent was prepared according to Berg (8), and cells were preincubated in 3.5 mM inhibitor in Krebs-Ringer phosphate for 10 min at 37°C, washed well in saline followed by Tris buffer, and then incubated normally. The conditions were chosen to permit virtual obliteration of surface enzyme activities while hardly affecting internal enzymes (17). Again, cell surface reaction product was eliminated with little diminution of phagosomal reactivity (Fig. 12). These results establish the enzymic dependence of reaction product formation. Additionally, since the surface reaction product is eliminated by reagents known not to penetrate the plasma membrane or to inhibit cytoplasmic enzymes, then peroxide generation must occur on the surface. The possibility of a cytoplasmically generated H_2O_2 diffusing to the surface of the cell and reacting with the extracellular cerium to give a surface-bound reaction product is thus greatly diminished.

BINDING OF EXOGENOUSLY GENERATED REACTION PRODUCT: It is important to determine if reaction product has a tendency to bind preferentially to one or more subcellular components. This was examined by two approaches. First, unfixed cells were preincubated for 10 min at 37°C in 1 mM $CeCl_3$ in standard buffer. The cover slip was drained and 0.45 ml fresh $CeCl_3$ was added, followed immediately by the addition of 0.05 ml of 1% H_2O_2 in buffer. At zero time and

FIGURE 8 Omission of NADH from the incubation medium resulted in a dramatic reduction in the amount of cerium precipitate found on the PS-stimulated PMN, showing a dependence on exogenous NADH. Most notably, reduced amounts of reaction product are found within the phagocytic vacuole, although some surface reactivity is occasionally seen. × 33,300. Scale = 0.5 μm.

FIGURE 9 Peroxide production by stimulated PMN is cyanide insensitive, and inclusion of 1 mM KCN in the cerium incubation medium did not inhibit reaction product formation on the surface of the cell or in the phagocytic vacuoles. In fact, heavier deposits than normal are seen on the surface, which is in agreement with the stimulatory effect of KCN on peroxide production by PMN. × 16,600. Scale = 1.0 μm.

FIGURE 10 The dependence of reaction product formation on H_2O_2 was verified by including 0.01% catalase, which destroys H_2O_2, in the phagocytosis medium and the cerium incubation medium. Aminotriazole was omitted from the latter due to its inhibitory effect on catalase. As seen, the presence of catalase resulted in an almost complete elimination of cerium precipitate on the cell surface and in the phagocytic vacuoles. × 16,500. Scale = 1.0 μm.
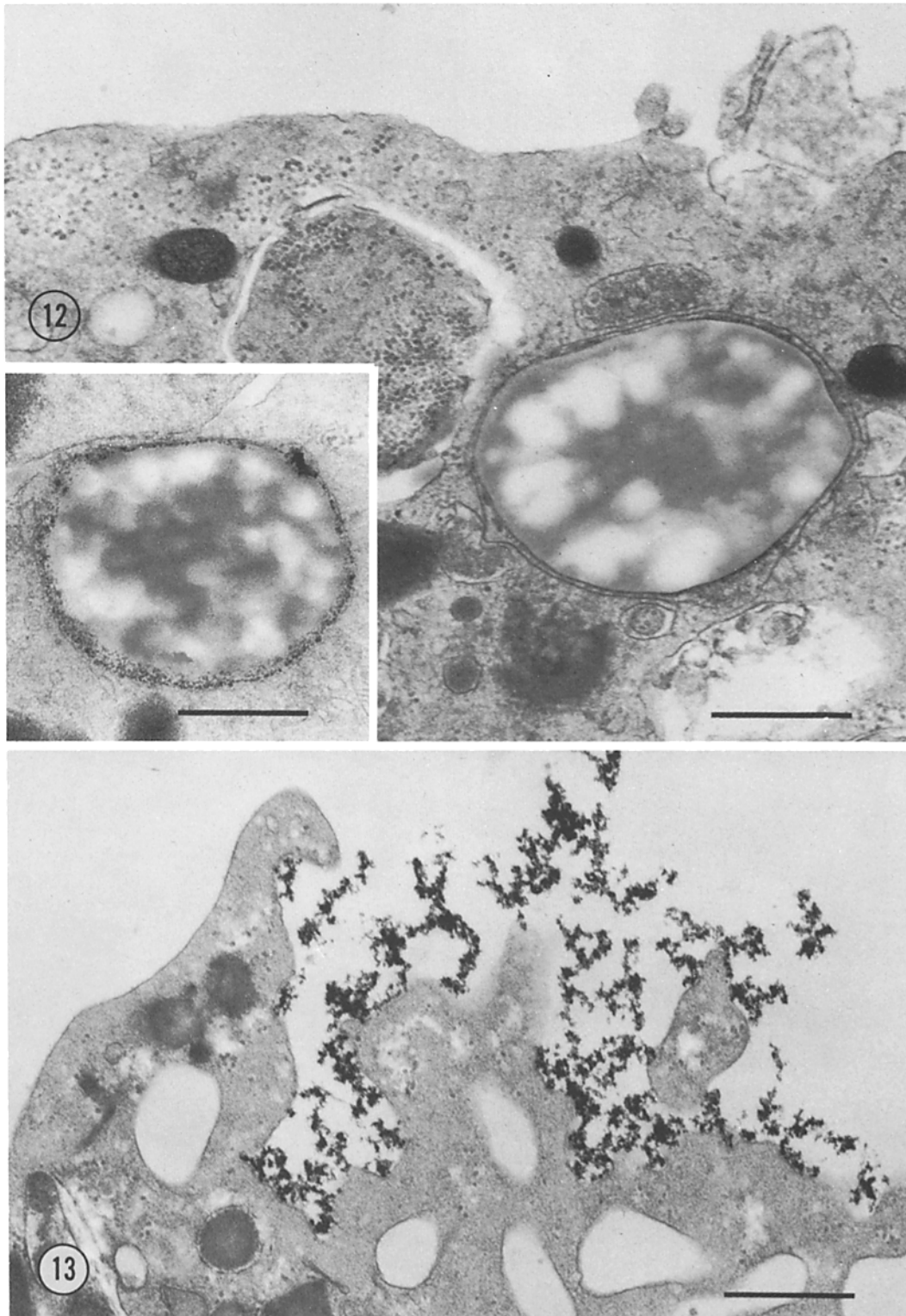
FIGURE 12   To verify the role of the plasma membrane in the NADH-dependent, enzymatic generation of H₂O₂, cells were exposed to 3.5 mM diazotized sulfanilic acid before cytochemical testing. This nonpenetrating inhibitor eliminated the formation of surface reaction product without disturbing phagosomal reactivity (*inset*). × 42,500. Scale = 0.5 μm. *Inset:* 39,400. Scale = 0.5 μm.

FIGURE 13   The possibility of a preferential binding of reaction product to the PMN was investigated by incubating cells in the cerium medium already precipitated by prior addition of H₂O₂. It is seen that the reaction product does not adhere closely and evenly to the cell membrane, an appearance quite distinct from that due to a proper cytochemical incubation. × 39,700. Scale = 0.5 μm.

after 10 min, the specimens were washed quickly and prepared for electron microscopy. The second approach involved a 5-min, 37°C incubation of resting and PS-stimulated PMN in the Ce^{+++} medium precipitated by prior addition of 0.1% H_2O_2. In both instances, the reaction product was physically similar to that generated by cellular activity, but its distribution was quite different. The precipitate did not adhere closely and evenly to the plasma membrane (Fig. 13). Additionally, the deposits were only infrequently found within the cell, although it does appear that they may eventually be phagocytized by the cell, as indicated by the membrane invaginations at the sites of precipitate adherence. These data imply that the deposits found after the cytochemical incubation are not adsorbed from the medium but are formed *in situ.*

ACCUMULATION OF CERIUM BY GUINEA PIG PMN: The above experiments raised the question of the permeability of living PMN to cerous ions. This was investigated in the following manner. Monolayers of guinea pig PMN were prepared according to Michell and co-workers (34) in Falcon plates. Guinea pig cells were used in order to have a sufficient volume of material. The incubation medium consisted of 0.1 M Tris-maleate buffer, pH 7.5, with 7% sucrose, 0.9 mM Ce^{+++} trace labeled with ^{141}Ce, and trace-labeled [^{14}C]inulin (1 mg/ml). The plates were incubated with and without 0.01% catalase for up to 1 h at 37°C. At the conclusion of each time period, plates were washed six times in saline, drained, and digested in 2 ml 0.5 N NaOH. Counting was performed on 0.1 ml of the digest in 15 ml of Buehler's solution with a Picker-Ansitron scintillation counter (Picker Corp., Cleveland, Ohio). Cellular protein was determined on the digests by the method of Lowry et al. (29), using bovine serum albumin as a standard. Inclusion of [^{14}C]inulin in the medium permitted us to determine that external medium was completely removed by the washing procedure, thus making corrections for Ce^{+++} in the entrapped medium unnecessary. The data were calculated on the premise that 1 mg of cell protein is equivalent to 4 mg of cell water. Plates taken at zero time, to determine the instantaneous attachment of cerium to the cells, showed a concentration of 0.7 mM cerium in the cell water. Over the next hour it was found that the concentration of cerium rose to 2.0 mM at 10 min, 2.5 mM at 20 min, and 3.5 mM at 60 min. When experiments were run at 0°C instead

of 37°C, it was found that although the zero time values declined by one-third, the accumulation of cerium after 20 min was identical to the values at 37°C. Similarly, in the presence of 1 mM iodoacetic acid, there was no real diminution of the cerium content of the monolayers after 20 min. A second series of experiments run at 0.2 mM cerium in the plates showed a similar time-dependent uptake of the cerium.

From these data it is concluded that there is no real uptake of cerium by the PMN in the sense that it is dependent neither on temperature nor on metabolism, but that there is a slow, progressive accumulation of cerium with time, either to the outside of the cell or within the cell. One reason these guinea pig PMN may slowly accumulate Ce^{+++} is because they may be "turned on" by the eliciting agent and may still be secreting H_2O_2, resulting in an accumulation of cerium precipitate. An additional indication that Ce^{+++} does penetrate PMN is provided cytochemically, i.e. reaction product can form within phagocytic vacuoles.

NADPH-DEPENDENT PEROXIDE GENERATION: It is known that NADH oxidase shows no activity with NADPH as a substrate (25). To determine if the enzyme under study could utilize NADPH, resting and stimulated cells were incubated in the standard medium containing 0.7 mM NADPH. Also included in this medium were 1 mM KCN and 1 mM $MnCl_2$, the latter was to supply the Mn^{++} ions necessary for optimal NADPH oxidation (45). Ultrastructural examination revealed no more reaction product than present in the substrate-free controls, meaning that the membrane-bound enzyme does not utilize NADPH, at least under these conditions. This does not necessarily imply that there is no NADPH oxidation or NADPH-dependent peroxide formation occurring in the cells.

NADH-Dependent Tetrazolium Reduction

A correlative study was performed to see if the sites of peroxide-generating activity could be localized with a second technique, i.e. tetrazolium reduction. It was reasoned that a tetrazolium could be substituted for oxygen as the hydrogen acceptor during NADH oxidation by NADH oxidase, resulting in formazan deposition at sites of enzyme activity. The conditions of the tetrazolium tests were made as similar as possible to those used in the cerium technique. Under such conditions, H_2O_2 would not, presumably, be formed.

SITES OF TETRAZOLIUM REDUCTION:

PMN stimulated by PS and incubated in the presence of NBT or TNBT and exogenous NADH exhibited formazan deposits on the external surface of the plasma membrane. The electron density of the reaction product was quite low.[2] Formazan deposits were most easily seen on cells sectioned perpendicularly to the plane of attachment to the cover slip (Fig. 14). With the low electron density of the reaction product, it was important to see the membrane clearly between the cytoplasm and the reaction product, and this method of sectioning reduced the percentage of the membrane being cut tangentially.

Reduced NBT appeared as a thin layer on the surface of the cell (Fig. 15), a layer which was by no means confluent around the perimeter of the cell; most frequently, the distribution was patchy and in some instances reaction product was limited to only a few areas on the membrane in a given thin section. Much more reaction product was deposited when TNBT was used, presumably because it is relatively more easily reduced than NBT (1). In this case, there were small "blobs" of reaction product at various points on the surface of the cell (Fig. 16). The presence or absence of reduced tetrazoliums in the vesicles and phagocytic vacuoles could not be determined with certainty due to the low electron density. Absence of intracellular formazan would not be surprising, due to

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[2] The formazans of NBT and TNBT are not osmiophilic, hence they are only slightly electron dense. BSPT, a nonosmiophilic tetrazolium that yields an osmiophilic formazan, was also tried, but, in the presence of NADH at pH 6.0–7.5, spontaneous reduction occurred.

the lack of penetration of tetrazoliums into PMN (36, 54) and the fact that the dye was not present during phagocytosis itself, i.e. could not leak in with the particles. The reasons for the topographical differences in localization between formazan and cerium deposits must be left open at this time because of the great physical and chemical differences between the precipitates.

CONTROLS OF TETRAZOLIUM REDUCTION: Omission of substrate (NADH) and/or phagocytic stimulation resulted in a complete loss of visible amounts of reaction product, implying that the reaction is dependent upon a phagocytically stimulated NADH-oxidizing system. When 1 mM KCN was included in the complete NBT incubation medium, stimulated PMN were capable of formazan generation to a degree similar to that when KCN was absent (Fig. 17). This control was not performed with TNBT, for cyanide caused a spontaneous reduction of this tetrazolium in the presence of NADH. An H_2O_2-dependent formazan production, presumably involving endogenous peroxidase, has been reported (37).

In the attempt to evaluate the specificity of the reaction, it was important to determine the role of superoxide (O_2^-), a known reducer of tetrazoliums (44), in the reaction sequence. Superoxide production by PMN and its stimulation by phagocytosis have been demonstrated (2, 12), although the mechanism of production is unknown. Since O_2^- is released by PMN into the extracellular medium, possibly from sites of synthesis on the surface and in the phagocytic vacuoles (52), formazan generation at the cell surface, where O_2^- and the

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FIGURES 14–18 The capacity of phagocytically stimulated PMN to reduce tetrazolium dyes, nitroblue tetrazolium (NBT), and tetranitroblue tetrazolium (TNBT) to insoluble formazans was examined in a correlative study. The results are shown in Figs. 14–18, which are of human PMN stimulated by PS and incubated in a medium containing a tetrazolium and NADH. Fig. 14: a normal PMN showing the appearance of the plasma membrane without any reaction product. × 40,700. Scale = 0.5 μm. Fig. 15: NBT reduction on the external surface of the plasma membrane occurs in stimulated PMN in the presence of NADH. The formazan is deposited as a thin layer (arrowheads) with low electron density. × 58,200. Scale = 0.5 μm. Fig. 16: TNBT, more easily reduced than NBT, produces more extensive deposits (arrowheads) on the surface of the PMN under conditions similar to Fig. 15. × 35,300. Scale = 0.5 μm. Fig. 17: the cyanide insensitivity of this reduction is shown by inclusion of 1 mM KCN in the NBT medium. The appearance and distribution of the formazan (arrowheads) is not altered. × 54,900. Scale = 0.5 μm. Fig. 18: tetrazoliums can be reduced by superoxide (O_2^-), a reactive anion generated by PMN. The possibility of formazan generation by this reaction is precluded by inclusion of superoxide dismutase in the cytochemical medium; this results in no alteration in the deposition of reduced TNBT (arrowheads). × 40,500. Scale = 0.5 μm.

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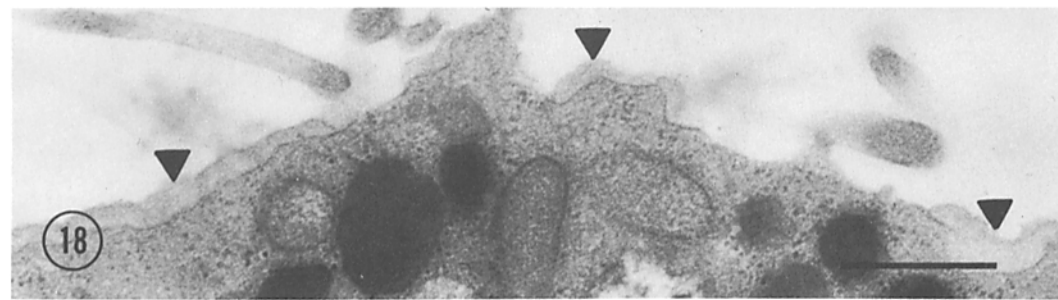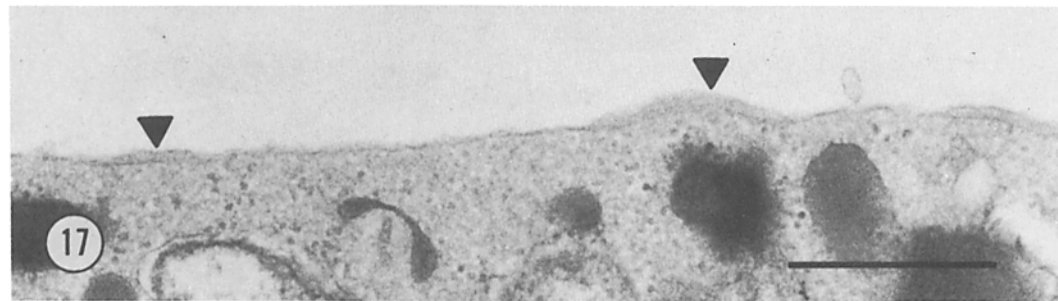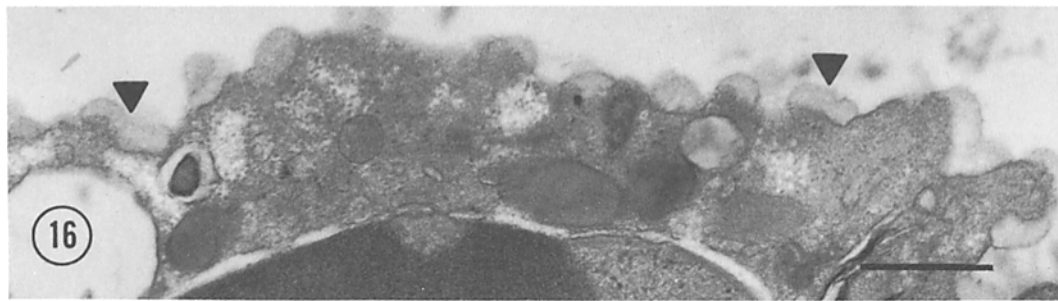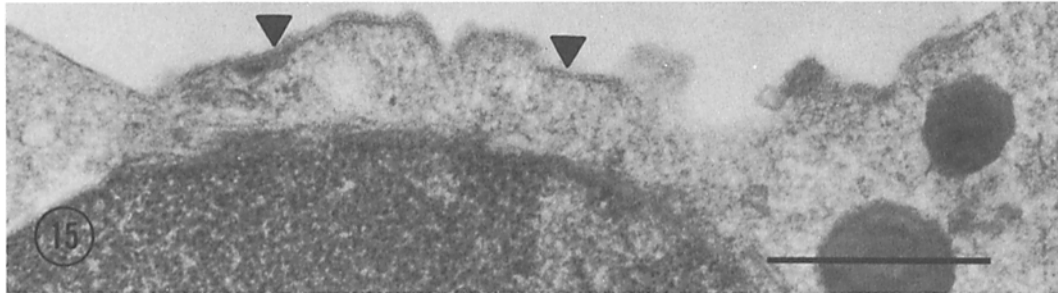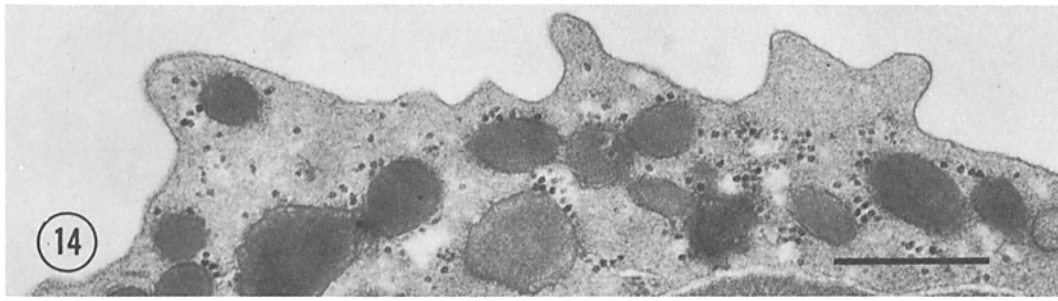
nonpenetrating tetrazolium meet, would be expected. This possibility was investigated by using superoxide dismutase to dismutate O_2^- to H_2O_2 and O_2. Superoxide dismutase (SOD, sp act = 3,000 + U/mg) (Truett Laboratories, Biochemical Division, Dallas, Tex.) was added to a complete TNBT medium at a final concentration of 125 U/ml. This control was applied to both resting and stimulated cells, and it was found that the dismutase did not block formazan generation in phagocytizing cells or alter its distribution (Fig. 18). Two important parameters of this experiment were examined. First, superoxide generation by PMN was examined by a modification (18) of the method of Babior et al. (2). Monolayers of resting and phagocytizing guinea pig PMN, elicited by peritoneal injection of sterile caseinate (38), were incubated in duplicate for 30 min with cytochrome c (Type VI) (Sigma Chemical Co.) in 0.1 M Tris-maleate buffer, pH 7.5, with 7% sucrose. Particles (Zymosan) and superoxide dismutase were added where appropriate. One preparation from each pair was maintained at 37°C while the other, which served as a blank, was kept at 0°C. After incubation, the medium was removed and centrifuged to remove nonadherent cells and particles. Superoxide-generating capacity or dismutase activity was measured by monitoring spectrophotometrically (550 nm) the levels of reduced cytochrome c, this reduction having been mediated by any superoxide formed. Additionally, the activity of the enzyme SOD was assured by using the same medium, without cells, but with xanthine and xanthine oxidase, a superoxide-generating system (33). In all instances, superoxide was released under the conditions imposed by these experiments (buffer, pH, etc.), although the levels were substantially lower than the release as measured in Krebs-Ringer phosphate or Hanks' BSS (18). Superoxide dismutase, however, remained active and in the amounts used (125 U/ml) totally inhibited the release of superoxide by cells or the generating system. Since SOD does not inhibit formazan deposition on the cell surface in the cytochemical test, direct enzymatic reduction of the tetrazolium is indicated, without interposition of O_2.

SIMULTANEOUS CE^{+++}-TNBT INCUBATION: Since the tetrazolium substitutes for oxygen as the hydrogen acceptor in the enzymatic oxidation of NADH, this should reduce or eliminate the generation of hydrogen peroxide. Stimulated PMN incubated in 1 mM Ce^{+++}, 0.88 mM TNBT, and

0.7 mM NADH in 0.1 M Tris-maleate buffer, pH 7.5, do not exhibit the peroxide-dependent cerium reaction product on the surface of the cell (Fig. 19). This is strong evidence that generation of reaction product in both techniques is dependent upon the same enzyme, NADH oxidase. Since the tetrazolium does not penetrate the plasma membrane and apparently cerium does, although slowly, the presence of cerium precipitate within intracellular phagosomes would be expected, and indeed it is seen.

Oxygen Consumption Studies

Since an oxidase of reduced pyridine nucleotide mediates the increased O_2 consumption and H_2O_2 generation in PMN, the effect of NADH and NADPH on oxygen consumption by resting and phagocytizing PMN was determined. We first employed guinea pig cells in order to have an adequate volume of material for numerous measurements that could be statistically evaluated. Later we compared human PMN with the guinea pig cells in three experiments. Guinea pig peritoneal PMN were isolated by the method of Oren et al. (38). Human PMN were isolated from peripheral blood by adding one part 0.08 M citric acid and 0.15 M Na citrate and one part 6% Dextran 70 in saline to five parts of blood. This was allowed to stand for 45 min at 37°C, then the supernate was removed and centrifuged at 750 g. Erythrocytes were lysed with distilled H_2O and the cells washed twice with KRP. The resulting preparation contained 90% PMN. The measurements were performed in 2.0 ml KRP containing 7.5 mM glucose, 3.0 mM NADH or NADPH, polystyrene particles (0.125% for guinea pig cells, 0.0125% for human cells), and 1–5 × 10^7 guinea pig PMN or 2.1 × 10^7 human PMN. Reduced pyridine nucleotide was added to resting cells or cells already being stimulated by PS. Similarly, PS was added to resting cells or to cells already exposed to NADH or NADPH. The incubation mixture was maintained at 37°C with constant stirring during all the measurements. Oxygen consumption was determined with an oxygen electrode (Instrumentation Laboratory, Lexington, Mass.); all results are expressed as microliters of oxygen consumed per 60 min per 10^7 cells.

As can be seen (Fig. 20), exogenous NADH added to either resting or phagocytizing guinea pig cells caused a significant increase in oxygen consumption, as did addition of particles to PMN already exposed to NADH. These data imply the
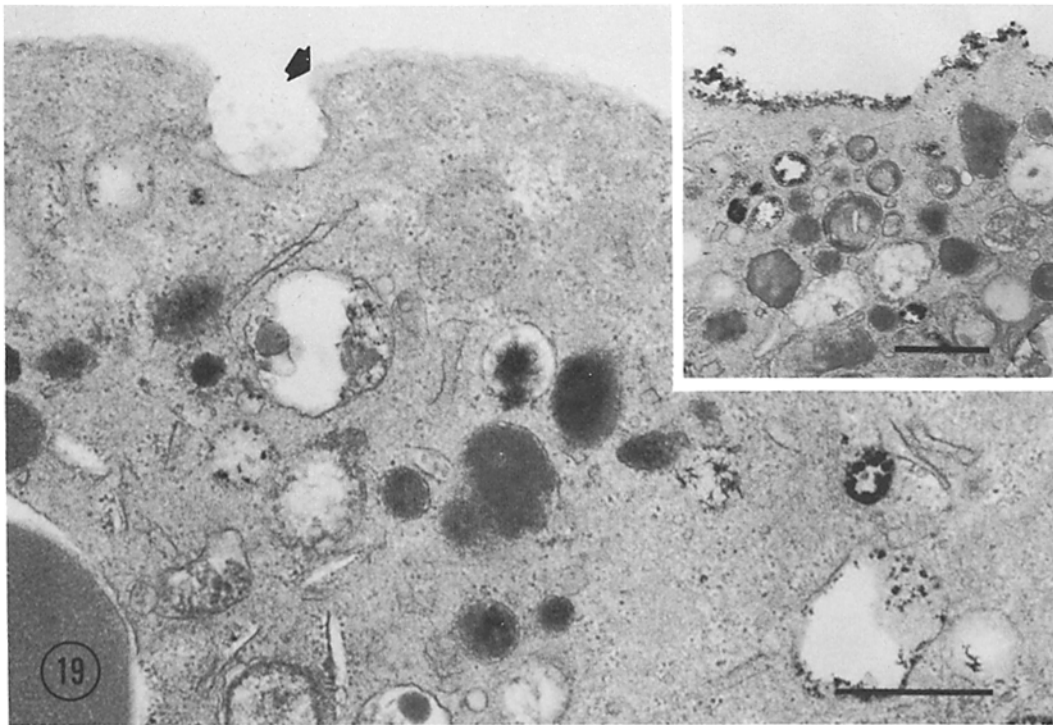
FIGURE 19 Stimulated PMN, incubated in a cytochemical medium containing both Ce^{+++} and TNBT, as well as NADH, lack a cerium precipitate on the surface (see inset for appearance of typical cerium reaction), although formazan deposits are present (arrow). This is strong evidence that the Ce^{+++} and tetrazolium reactions are dependent upon the same enzyme, for TNBT substitutes for O_2 as the hydrogen acceptor in the enzymatic oxidation of NADH, thereby eliminating H_2O_2 production. Since TNBT does not penetrate the plasma membrane, cerium precipitation can and does occur intracellularly. × 41,100. Scale = 0.5 μm. Inset: × 24,300. Scale = 0.5 μm.

activity of an enzyme to which the substrate has quick access, and one which is activated by phagocytosis. A surface-bound NADH oxidase would meet these requirements. NADPH, on the other hand, was without effect on the oxygen consumption of resting and phagocytizing cells, suggesting that access of substrate to an NADPH oxidase is restricted. When PS was added to cells already in the presence of NADPH, there was a large increase in oxygen consumption. Internalization of this substrate with the particles, resulting in access to an intracellular oxidase, might account for this observation. In the case of human PMN (see Table I), addition of NADH to resting cells caused a substantial increase in respiration; NADPH caused only a slight stimulation. When the reduced pyridine nucleotides were added to phagocytizing cells, NADH caused a real increase in respiration, while NADPH was somewhat depressive or had no effect. When cells already

exposed to reduced pyridine nucleotides were offered PS, the oxygen uptake increased in the case of both nucleotides. These data are consistent with those obtained with guinea pig cells. The only clear difference was the fact that with human cells the maximal respiratory response during phagocytosis was far higher than in guinea pig cells—reaching levels of 20 times resting values. The effects of reduced pyridine nucleotides were optimally detectable in these phagocytizing cells at respiratory levels 50% greater than resting values. Such conditions were achieved simply by using lower loads of polystyrene. The data on guinea pig and human PMN correlate well with those of Mandell and Sullivan (31), who measured NADH and NADPH oxidation under similar conditions.

DISCUSSION

The metabolic perturbations that occur in phagocytically stimulated PMN include increases in
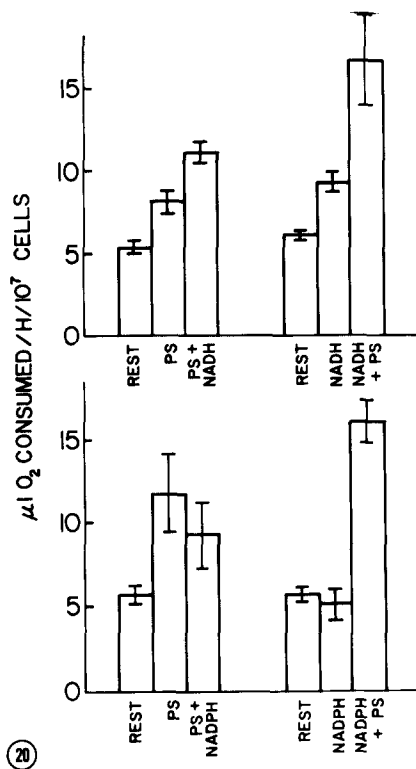
FIGURE 20 The effect of NADH and NADPH on the oxygen consumption of resting and phagocytizing guinea pig PMN was measured. NADH added to either resting or phagocytizing cells caused a significant increase in O_2 consumption ($P < 0.002$), as did the addition of particles of PMN already exposed to NADH ($P < 0.002$). NADPH was without significant effect on resting and phagocytizing cells, but phagocytic stimulation of cells in the presence of NADPH resulted in a substantial increase in oxygen uptake ($P < 0.01$). Standard errors of the mean are shown.

oxygen consumption and hydrogen peroxide production. Two enzymes, NADH oxidase (3, 11) and NADPH oxidase (43, 50), have been proposed as being responsible for this respiratory burst. We have examined NADH oxidase, a cyanide-insensitive enzyme which, when appropriately stimulated, catalyzes the reaction: $NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2$. The approach taken to define the ultrastructural location of this enzyme cytochemically was twofold. First, the site of enzyme activity was determined indirectly by utilizing cerium ions to form a precipitate with H_2O_2, a product of NADH oxidation. Secondly, a tetrazolium was substituted for oxygen as the hydrogen acceptor in the reaction, and the sites of reduced tetrazolium accumulation were assessed. The

tetrazolium-reducing capacity of phagocytically active PMN is well recognized and forms the basis of diagnostic tests of PMN function (6, 53). The specific reaction responsible for formazan production in these tests is uncertain, but it is correlated with the increased oxidative metabolism and peroxide production. As a result of the present study, the mechanism of tetrazolium reduction during phagocytosis becomes better defined. Application of these two dissimilar approaches and the correlation in the results constitute strong evidence that NADH oxidase is responsible for both reactions and that the enzyme is located on the surface of the PMN. NADH oxidase is internalized with the membrane during phagocytosis and, as part of the phagocytic vacuole membrane, maintains its peroxide-generating activity.

In evaluating the cytochemical technique, several factors combine to demonstrate that the test is enzyme dependent. (a) The amount of precipitate formed is directly proportional to the incubation time. (b) Reaction product formation is substrate dependent, and (c) it can be blocked by enzyme-inhibiting sulfhydryl reagents and aldehyde fixation. (d) Reaction product generated by incubation in the complete medium differs in distribution and adherence to the membrane from exogenously generated precipitate. From these observations, it is suggested that the observed localization depends on a plasma membrane-bound enzyme functioning as the site of formation of the cytochemical end product.

Analysis of the specificity of the cerium reaction demonstrates its dependence upon a hydrogen peroxide-producing enzyme, as shown by the catalase inhibition of reaction product formation. Evidence favors NADH oxidase as the source of this H_2O_2. Several biochemical characteristics of NADH oxidase from polymorphonuclear leukocytes have been described. It is a flavoprotein which reduces O_2 to H_2O_2 by the oxidation of NADH. The enzyme has a relatively high K_m for NADH; it has no activity with respect to NADPH (25). NADH oxidase is insensitive to 1 mM cyanide and may be stimulated by it (6). In addition, the capacity for oxidation of NADH by intact PMN (31) and extracts of PMN (3) is increased when the cells are phagocytically stimulated. The experimental data derived from the cytochemistry were found to agree with these biochemical characteristics of NADH oxidase. Reaction product deposition was dependent upon exogenous NADH; NADPH was not found to

TABLE I

Effect of Reduced Pyridine Nucleotides on Respiration of Resting and Phagocytizing Human PMN

	Resting	Reduced pyridine nucleotide	Phagocytizing
NADH	3.8 ± 0.3 (8)	6.0 ± 0.4 (8)	8.2 ± 0.4 (5)
NADPH	4.0 ± 0.2 (6)	4.6 ± 0.4 (6)	7.6 ± 1.2 (4)

	Resting	Phagocytizing	Reduced pyridine nucleotide
NADH	3.7 ± 0.3 (8)	6.4 ± 0.6 (7)	8.4 ± 0.6 (7)
NADPH	4.0 ± 0.4 (6)	7.4 ± 0.5 (6)	6.8 ± 0.6 (6)

Cells were obtained from three individuals and were examined separately. The number of observations made is given in parentheses. Data given as mean ± SE as microliters O_2 consumed per 60 min per 10^7 cells.

serve as a substrate for this enzyme under similar incubation conditions. The activity of the enzyme, as reflected by the amount of reaction product generated, was not inhibited by 1 mM cyanide and was increased by phagocytic stimulation of the PMN.

Correlative evidence for the localization of NADH oxidase by the cerium technique was obtained by examining the reduction of tetrazoliums under similar conditions. The capacity of phagocytically stimulated PMN to reduce tetrazoliums has been examined by many investigators, and formazan deposits have been found mainly in the phagocytic vacuoles. PMN exposed to a phagocytizable heparin-NBT complex showed suggestions of a surface-associated NBT-reducing capacity (32, 54), and the observations presented here definitely establish the activity of the plasma membrane in this regard. It was found that external NBT and TNBT were reduced on the outer surface of the plasma membrane, this reduction being cyanide insensitive, NADH dependent, and activated by phagocytosis. The biochemical basis for tetrazolium reduction by PMN has been uncertain. It can be reduced in the cell by dehydrogenase or diaphorase activity (51), part of the latter presumably being comprised by NADH oxidase, and by O_2^- (44). The tetrazolium-reducing capacity of PMN during phagocytosis has been found defective in patients with chronic granulomatous disease (6, 36). These cells have been shown biochemically to be deficient in NADH oxidase (4, 5), although conflicting data exist (22). The CGD cells have also been found to be deficient in O_2^- production (14). Data such as these have led to the proposal that pyridine nucleotide-dependent "NBT reductase" activity (36) or a cyanide-insensitive pyridine nucleotide oxidase (35) is responsible for tetrazolium reduction.

The weight of the combined evidence from

cerium precipitation and tetrazolium reduction suggests strongly that the cytochemical end products are due to NADH oxidase activity on the external surface of the plasma membrane. That the same enzyme is involved in both reactions has been shown by the inhibitory effect of TNBT on the peroxide-dependent deposition of the cerium precipitate on the PMN surface. The surface localization is strengthened by the fact that reaction product formation is blocked by diazotized sulfanilic acid, a nonpenetrating enzyme inhibitor. This militates against the possibility that leakage of cytoplasmically generated H_2O_2 into the medium permits the reaction with cerium. Additional rigorous tests are necessary to determine if NADH oxidase is a true ectoenzyme (15). It is important to note that both the cerium and tetrazolium techniques depend upon reaction product formation from substances which appear to penetrate the living PMN either slowly or not at all. This in no way jeopardizes the described cytochemical localizations, but it does leave open the possibility that intracellular sites of NADH oxidase activity are not recognized.

It has been shown that PMN undergo certain metabolic alterations when exposed to nonphagocytizable, surface-perturbing agents (20, 41, 46, 50). These alterations are similar to those of phagocytically stimulated PMN. The mechanism(s) that underlie the respiratory increment during these excitations, and those resulting in increased O_2^- and H_2O_2 production, are still not clearly understood. Recent data implicate myeloperoxidase (58) or O_2^- (13) as the agents for oxidation of reduced pyridine nucleotides, in addition to the older work that directly implicates oxidases for these substances (see 24). Observations such as the rapidity after stimulation of the onset of increased H_2O_2 production (47, 48), and the data presented in this paper combine to suggest

that the external surface of the plasma membrane, and ultimately the internal surface of the phagosomal membrane, are likely sites for an oxidation of reduced pyridine nucleotides that produces H_2O_2 (35, 47, 48, 49, 56, 57). The latter location would permit peroxide generation at the site of action of the peroxide-myeloperoxidase-halide bactericidal system in PMN (10, 26, 28, 49).

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