Characteristics of the Cofactor Requirements for the Superoxide-Generating NADPH Oxidase of Human Polymorphonuclear Leukocytes†

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ABSTRACT: Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and other cofactor requirements of the superoxide ($O_2^{-}\cdot$) generating activity evoked in human polymorphonuclear (PMN) leukocytes were characterized by utilizing subcellular particulate and solubilized preparations. Particulate preparations exhibited equal rates of $O_2^{-}\cdot$ generation irrespective of the stimulus, but the ratio of NADPH oxidation to $O_2^{-}\cdot$ generation was approximately 2-fold lower with opsonized zymosan than with phorbol myristate acetate as the stimulus. In contrast, the ratio of NADPH oxidation to $O_2^{-}\cdot$ generation was similar for preparations solubilized in ionic or nonionic detergents regardless of the stimulus. A greater isotope effect on V_{max} was observed when (4*R*)-[^{2}H]NADPH was substituted for (4*R*)-[^{1}H]NADPH in subcellular particles than in solubilized preparations, so that the extent to which the oxidation of NADPH is rate limiting is dependent on the physical state and purity of the oxidase. Both the rates of $O_2^{-}\cdot$ generation and of autoinactivation of the $O_2^{-}\cdot$-generating activity are accelerated by the presence of NADPH and by detergent solubilization of the subcellular particles, suggesting a change in the rate-limiting step rather than an alteration in the number of catalytic units. The dependence of the $O_2^{-}\cdot$-generating activity on a flavin cofactor capable of one-electron transfers was demonstrated by the stimulation of activity of Triton X-100 solubilized preparations by exogenous flavin adenine dinucleotide (FAD) and by the total inhibition of residual activity by 5-carba-5-deaza-FAD, which is only competent for two-electron transfers. Purified $O_2^{-}\cdot$-generating activity also contains a cytochrome with type *b* spectral characteristics which is the dominant chromophore, is readily oxidized by molecular oxygen ($k > 2.5 \times 10^6$ M^{-1} min^{-1}), and has an oxidation–reduction potential of -235 ± 9 mV. Although these data are consistent with the participation of the cytochrome *b* in the univalent reduction of oxygen, a functional role has not been established definitively.

The respiratory burst stimulated in human polymorphonuclear (PMN)[1] leukocytes engaged in the phagocytosis of opsonized particles or exposed to phorbol myristate acetate (PMA) or other soluble agents is characterized by increases in oxygen consumption and glucose oxidation through the hexose monophosphate (HMP) shunt and by augmentation of the production of superoxide ($O_2^{-}\cdot$), hydrogen peroxide, and hydroxyl radicals (Badwey & Karnovsky, 1980; Babior, 1978; Tauber & Babior, 1978). Subcellular particles obtained from homogenates of PMN leukocytes activated by either stimulus, solubilized preparations of the particles in detergents, and purified fractions of the solubilized preparations catalyze the reduction of oxygen to $O_2^{-}\cdot$ by utilizing electrons supplied by NADPH or NADH, and the respective K_m values indicate that NADPH is the preferred electron donor (Babior, 1978; Tauber & Goetzl, 1979). Further, the pH range for optimal activity, physicochemical characteristics, and susceptibility to inhibition by *p*-(chloromercuri)benzenesulfonic acid are similar for the $O_2^{-}\cdot$-generating activity in subcellular particles and in solubilized preparations of the particles from PMN leukocytes stimulated by exposure to opsonized particles or PMA (Tauber & Goetzl, 1979).

Constituent cofactors other than NADPH have been suggested in this $O_2^{-}\cdot$-generating system of the human PMN leukocyte. A role for flavin was proposed on the basis of solubilization procedures employing Triton X-100, where maximal reconstitution of $O_2^{-}\cdot$-generating activity was found to be dependent on the addition of flavin adenine dinucleotide (FAD) (Babior & Kipnes, 1977). A third proposed cofactor is a type *b* cytochrome isolated from partially purified preparations of plasma membranes and from phagocytic vacuoles of human PMN leukocytes (Segal & Jones, 1978). This cytochrome is not detectable in PMN leukocytes of some patients with chronic granulomatous disease (Segal & Jones, 1980; Borregaard et al., 1979) which fail to generate sufficient concentrations of $O_2^{-}\cdot$ and other oxidative metabolites to kill susceptible bacteria (Curnutte et al., 1975; Hohn & Lehrer, 1975). The data implicating NADPH, FAD, and possibly cytochrome *b* as cofactors for the $O_2^{-}\cdot$-generating activity suggested the importance of examining the kinetic characteristics and stereospecificity of NADPH oxidation and its stoichiometric relationship to $O_2^{-}\cdot$ generation, the functional requirement for flavin, and the role of a type *b* cytochrome that is found in association with the $O_2^{-}\cdot$-generating activity of the PMN leukocytes.

Materials and Methods

Materials. Ferricytochrome *c* (type VI), superoxide dismutase (SOD), zymosan, nicotinamide adenine dinucleotide phosphate in reduced form (NADPH), nicotinamide adenine dinucleotide in the reduced form (NADH), 2-(*N*-morpholino)ethanesulfonic acid (Mes), morpholinopropane-

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[1] Abbreviations used: $O_2^{-}\cdot$, superoxide radical; PMN, polymorphonuclear; HMP, hexose monophosphate; PMA, phorbol 12-myristate 13-acetate; OZ, opsonized zymosan; SOD, superoxide dismutase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide. The buffers used have their standard meaning.

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sulfonic acid (Mops), 4-(2-hydroxyethyl)-1-piperazine-2-ethanesulfonic acid (Hepes), flavin adenine dinucleotide (FAD), glucose 6-phosphate, glucose-6-phosphate dehydrogenase, malic enzyme, Triton X-100, phorbol 12-myristate 13-acetate (PMA), sodium deoxycholate, sodium azide, bilirubin, D-histidine hydrochloride, catalase, L-tryptophan, benzoic acid, and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO). 8-Chloro-8-demethylflavin adenine dinucleotide, 1-deazaflavin adenine dinucleotide, and 5-deazaflavin adenine dinucleotide were prepared from the riboflavin form of the analogues by the use of the flavokinase and FAD synthetase activity partially purified from *Brevibacterium ammoniagenes* as described previously (Light & Walsh, 1980; Spencer, 1978). The FAD analogues were purified by high-performance liquid chromatography (LC) and FAD was quantitated in solubilized fractions collected from Sephacryl S-200 gel filtration in like manner (Light et al., 1980). Indigotetrasulfonate, indigodisulfonate, and phenosafranin were generous gifts from Dr. William H. Orme-Johnson (Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA). [1-^{2}H]Glucose 6-phosphate was prepared by incubating [1-^{2}H]glucose (98% deuterio) with ATP and hexokinase and was purified as described (Light & Walsh, 1980). (4*S*)-[^{2}H]NADPH was generated in 0.13 M potassium phosphate (pH 7.0) by the action of 0.3 units of glucose-6-phosphate dehydrogenase on 1 mM [1-^{2}H]glucose 6-phosphate in the presence of 200 μM NADP. Racemic [2-^{2}H]malate and the control racemic [2-^{1}H]malate were prepared from oxaloacetate and either sodium borodeutride or sodium borohydride and were the generous gift of Dr. Carol Ryerson (Department of Nutrition and Food Science, Massachusetts Institute of Technology). (4*R*)-[^{2}H]NADPH was generated in 0.13 M potassium phosphate (pH 7.0) by the action of malic enzyme on 1 mM racemic [2-^{2}H]malate in the presence of 200 μM NADP. Zymosan particles were opsonized (OZ) just prior to use by incubation for 30 min at 37 °C in fresh autologous serum, washed twice, and resuspended at a concentration of 60 mg/mL in 0.02 M sodium phosphate buffered 0.13 M NaCl without Ca^{2+} or Mg (PBS) (Hohn & Lehrer, 1975). PMA was suspended at a concentration of 20 μg/mL in PBS containing 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$ (PBS^{2+}). Protein content of PMN leukocyte subcellular particles was assayed by a modification of the Folin–Lowry method (Lowry et al., 1951).

Preparation of PMN Leukocytes and Subcellular Particles from PMN Leukocytes and the Solubilization of Subcellular Particles. PMN leukocytes (Tauber & Babior, 1977) and eosinophils (Tauber et al., 1979a) were purified from citrate-anticoagulated venous blood obtained from normal adults and from hypereosinophilic patients, respectively. Portions of (2–8) × 10^8 purified leukocytes were suspended in 0.5–2 mL of PBS, prewarmed for 3 min at 37 °C, and incubated for an additional 6 min at 37 °C with 1–4 mL of PBS^{2+} and OZ at a final concentration of 2.0 mg/mL or of PMA at a final concentration of 0.7 μg/mL. For the preparation of subcellular particles, (2–8) × 10^8 leukocytes that had been incubated without or with OZ or PMA were pelleted by centrifugation, resuspended in 1–4 mL of 0.34 M sucrose, either homogenized in a glass tube with a Teflon pestle (Tri-R Instruments, Inc., Rockville Centre, NY) for 5 min at 4 °C or sonicated at 150 W for 30 s at 4 °C (Model 350, Branson Sonic Power Co., Danbury, CT), and centrifuged at 400*g* for 10 min at 4 °C. The supernatants were removed and centrifuged at 27000*g* for 20 min at 4 °C, and the 27000*g* pellets were washed and resuspended in 0.34 M sucrose at a protein concentration of 1.0–2.5 mg/mL (Tauber et al., 1979b). Portions of 1.5 mL from the 27000*g* pellet suspensions were mixed with either deoxycholate at a final concentration of 0.25 g/100 mL in 0.34 M sucrose, Triton X-100 at a final concentration of 0.4 g/100 mL in 0.34 M sucrose, or 0.34 M sucrose alone. FAD was added to some replicates of each preparation at a final concentration of 0.4 mM. One-milliliter portions of each preparation were then centrifuged at 100000*g* for 1 h, and the supernatants were utilized for studies of oxidative metabolism.

Determination of Oxygen Consumption, Superoxide ($O_2^{-}\cdot$) Generation, and Hexose Monophosphate (HMP) Shunt Activity in Intact PMN Leukocytes. For each measurement of oxygen consumption and $O_2^{-}\cdot$ generation, 2 × 10^7 PMN leukocytes in 50 μL of PBS and 2.8 mL of PBS^{2+} that had been prewarmed to 37 °C were preincubated with stirring for 2 min at 37 °C in a siliconized sample chamber of an oxygen electrode (Model 53, Yellow Springs Instrument Co., Yellow Springs, OH) that was calibrated according to the phenylhydrazine oxidation method (Misra & Fridovich, 1976). After the introduction of 0.1 mL of PBS^{2+} alone or containing 6 mg of OZ or 2.0 μg of PMA, the incubation was continued for 6 min. The maximal initial rate of oxygen consumption and the production of $O_2^{-}\cdot$ in the same chambers were assessed as described and expressed in nanomoles/minute (Curnutte et al., 1979). The rate of glucose oxidation by the HMP shunt was determined by the method of Stubbs et al. (1973). Duplicate portions of 2 × 10^7 PMN leukocytes in 0.9 mL of PBS^{2+} containing 20 μL of either 1.8 μCi of [6-^{14}C]-glucose/mL or 1.9 μCi of [1-^{14}C]glucose/mL were incubated for 10 min at 37 °C with 0.1 mL of PBS^{2+} alone or containing 2 mg of OZ or 0.7 μg of PMA. The $^{14}CO_2$ released was trapped in a 5 × 5 mm piece of filter paper saturated with 2 M NaOH that was counted in a Searle Mark III 6880 liquid scintillation counter (Tracor Analytical, Inc., Elk Grove Village, IL). The activity of the HMP shunt was expressed as nanomoles of $^{14}CO_2$ liberated per 20 min from [1-^{14}C]-glucose which exceeded the quantity liberated from [6-^{14}C]-glucose by 50-fold or more.

Quantitation of $O_2^{-}\cdot$-Generating Activity and NADPH-Oxidizing Activity of Subcellular Particles and Solubilized Preparations. The NADPH-dependent $O_2^{-}\cdot$-generating activity of 50–200 μg of protein from subcellular particles or solubilized preparations was assessed by measuring the superoxide dismutase (SOD) inhibitable reduction of cytochrome *c* as described (Curnutte et al., 1975; Tauber et al., 1979b). The difference between the increases in OD_{550nm} in paired cuvettes, one of which contained 30 μg of SOD, was recorded continuously from 5 to 15 min at 28 °C in a double beam spectrophotometer (Model 52, Perkin Elmer Co., Oak Brook, IL). The oxidation of NADPH to NADP was assessed at 28 °C for 5 min by measuring the difference between the decreases in OD_{340nm} in paired cuvettes, one of which contained 50–200 μg of protein from subcellular particles or solubilized preparations and the other of which contained only 0.8 mL of 0.13 M potassium phosphate buffer (pH 7.0) with 0.1 mM NADPH. The 2-min value was used to calculate NADPH oxidation after subtracting the difference in the decreases in OD_{340nm} of paired cuvettes that lacked NADPH. That NADPH oxidation accounted for the observed decreases in OD_{340nm} was confirmed by the reversal achieved when 0.5 units of isocitrate dehydrogenase and 5 μmol of sodium isocitrate were added after 5 min of incubation. $O_2^{-}\cdot$ generation and NADPH oxidation were calculated by utilizing millimolar extinction coefficients of 18.5 (Margoliash & Frohwirt, 1959)

Table I: Stimulation of the Oxidative Metabolism of Intact PMN Leukocytes by OZ and PMA

	oxygen consumption[a] (nmol of O_2/min)	$O_2^{-}\cdot$ generation[a] (nmol of $O_2^{-}\cdot$/min)	HMP shunt activity[a] (nmol of $^{14}CO_2$/20 min)
PBS^{2+} alone	1.2 ± 0.1[b]	0.5 ± 0.2	1.4 ± 0.3
OZ	119.2 ± 19.3	87.1 ± 17.5[c]	36.1 ± 10.0
PMA	114.8 ± 17.4	95.1 ± 17.8[c]	31.0 ± 3.4

[a] All values are expressed in terms of 10^7 PMN leukocytes. [b] Each value is the mean ± SEM of the results from three separate experiments. [c] At 28 °C, instead of at 37 °C, $O_2^{-}\cdot$ production was 58.7 ± 12.1 nmol/min with OZ and 65.0 ± 15.4 nmol/min with PMA.

and 6.22 (Sigma Chemical Co., 1974), respectively, and were expressed as nmol/(min·mg of protein). In experiments designed to evaluate the kinetic effect of deuteride substitution, 0.8 mL of freshly prepared (4*S*)-[^{1}H]NADPH and (4*S*)-[^{2}H]NADPH solutions, or of (4*R*)-[^{1}H]NADPH and (4*R*)-[^{2}H]NADPH solutions were used in place of buffer in the standard assay.

Anaerobic Titration and Enzymatic Reduction of the Type b Cytochrome in Subcellular Particles of PMN Leukocytes. All solutions for anaerobic work were placed in modified cuvettes or containers which could be sealed with a single serum stopper. Solutions were sequentially degassed under vacuum and reequilibrated with argon 6 times (Matheson Gas, Inc., Gloucester, MA); the argon had been freed of oxygen by passage through an oxygen adsorber (Oxisorb, Messer Griesheim, West Germany). All subsequent transfers were made with gas tight syringes (Hamilton, Inc.,Reno, NV). Reversible titrations were made by the serial injection of reductant (1 mM dithionite) and oxidant (0.25 mM oxygen). The midpoint potential of the cytochrome was determined by titrations in which the appropriate dye was added before the cuvette was degassed. In experiments designed to investigate the enzymatic reduction of the type *b* cytochrome, NADPH solutions were degassed separately and transferred anaerobically to the cuvette.

Results

Stoichiometric Relationships of Cytochrome c Reduction and NADPH Oxidation. The subcellular particulate preparation obtained from human PMN leukocytes stimulated with opsonized zymosan generates $O_2^{-}\cdot$ in the presence of NADPH. The stoichiometric relationship was found to follow

$$NADPH + 2O_2 \rightarrow 2O_2^{-}\cdot + NADP^+ + H^+$$

(Babior et al., 1976). We examined whether this relationship was also manifest in solubilized preparations and whether there was a stimulus dependence to the stoichiometry. The respiratory burst activity stimulated by the soluble activating agent, PMA, was compared with a phagocytosable particle, OZ, in both whole cell and broken cell preparations.

Comparable respiratory burst activity is seen with either PMA or OZ as a stimulating agent. Maximal rates were noted by 6 min of incubation with either agent, resulting in mean increases of approximately 100-fold in oxygen consumption and 150-fold in $O_2^{-}\cdot$ generation (Table I). In order to assess the putative source for NADPH reducing equivalents, HMP shunt activity was measured. As shown in Table I, comparable activities of the HMP shunt were seen upon stimulation with either OZ or PMA.

Subcellular particles were prepared from PMN leukocytes that had been stimulated by exposure to OZ or PMA in order to examine the influence of the stimulus on the ratio of NADPH oxidation to $O_2^{-}\cdot$ generation. Preliminary studies indicated that incubation of PMN leukocytes for 6 min with either stimulus yielded maximal NADPH-oxidizing activity in the particles, which was linearly related to the protein concentration from 50 to 200 μg/mL and was independent of pH from 5.5 to 8.0. In subsequent analyses, assays were performed at pH 7.0 with 100 μg of protein/mL.

Table II: $O_2^{-}\cdot$ Generation and NADPH Oxidation by Subcellular Particulate Preparations from PMN Leukocytes Activated by OZ or PMA

conditions	$O_2^{-}\cdot$ generation[a]	NADPH oxidation	NADPH oxidation/ $O_2^{-}\cdot$ generation[b]
OZ homogenate (n = 5)	24.8 ± 7.6	14.6 ± 6.2	0.64 ± 0.25
OZ sonicate (n = 7)	26.1 ± 9.2	21.7 ± 7.4	0.83 ± 0.13
PMA homogenate (n = 3)	27.1 ± 1.4	37.1 ± 3.1[c]	1.38 ± 0.27[d]
PMA sonicate (n = 12)	28.3 ± 4.1	39.6 ± 3.9[c]	1.54 ± 0.37[d]

[a] Nmol/min·mg of protein, mean ± SE. [b] Mean ± SD. [c] The levels of NADPH-oxidizing activity for the 27000*g* particulate preparations from homogenates and sonicates of PMA-activated PMN leukocytes were significantly higher than for the corresponding preparations from OZ-activated PMN leukocytes at $p < 0.015$ and < 0.025, respectively. [d] The ratios of NADPH-oxidizing activity/ $O_2^{-}\cdot$-generating activity for the 27000*g* particulate preparations from homogenates and sonicates of PMA activated PMN leukocytes were higher than for the corresponding preparations from OZ-activated PMN leukocytes at $p < 0.01$ and $p < 0.005$, respectively.

The ratio of NADPH oxidation to $O_2^{-}\cdot$ generation in subcellular particles prepared from PMN leukocytes was approximately 2-fold higher when PMA was the stimulus than when OZ was the stimulus (Table II). This highly significant difference was attributable solely to the greater rate of NADPH oxidation with PMA and was unrelated to the technique employed to disrupt the PMN leukocytes after stimulation. The $O_2^{-}\cdot$-generating activity from both sources exhibited the same preference for NADPH over NADH. The K_m for NADPH was 70 ± 27 μM (mean ± SD, n = 5) for PMA and 90 ± 10 μM for OZ at pH 7.0, and for NADH was 0.25 ± 0.1 mM (mean ± SD, n = 3) for PMA and 0.12 ± 0.02 mM for OZ at pH 7.0. At concentrations of NADPH that were 0.5, 1, and 3 times the value of the K_m for NADPH, the ratio of NADPH oxidation to $O_2^{-}\cdot$ generation (mean ± SEM, n = 4) were 1.67 ± 0.16, 1.70 ± 0.03, and 1.59 ± 0.23, respectively, for subcellular particles from PMA-stimulated PMN leukocytes, which were indistinguishable from the values obtained at an NADPH concentration equal to 1.6 times the K_m (Table II).

pH Dependence and Activation Kinetics of OZ- and PMA-Stimulated PMN Leukocytes. Subcellular particles were resuspended and assayed in a series of buffers ranging in pH from 5.3 to 8.6. Similar relationships were obtained with particles prepared from PMA-stimulated (Figure 1) and from OZ-stimulated PMN leukocytes. NADPH-oxidizing activity and $O_2^{-}\cdot$-generating activity reached peak values between pH 7.4 and 6.9 in particles from both PMA- and OZ-stimulated PMN leukocytes (Figure 1). The effects of pH were not predominantly a function of the anions of the buffer, as similar values were obtained for particles from PMA-stimulated cells at pH 7.0 with potassium phosphate, Mops, Mes, and Hepes buffer systems. The neutral pH optimum is similar to that previously reported (Babior et al., 1976) and

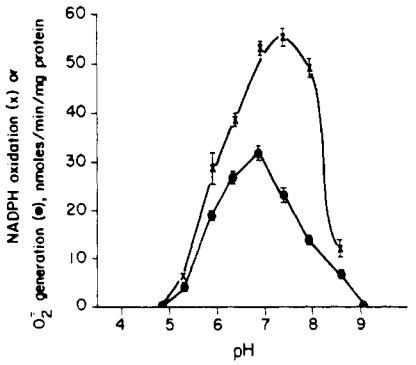

FIGURE 1: pH dependence of the O_2^-·-generating and NADPH-oxidizing activities of particulate preparations from PMA-activated PMN leukocytes. Each point and bracket represents the mean ± SE for the results of three experiments employing PMN leukocytes from different donors.

Table III: Effect of Detergent Treatment of 27000*g* Preparations of Subcellular Particles on O_2^-· Generation and NADPH Oxidation

	NADPH oxidation/O_2^-· generation (mean ± SD)
PMA activation	
0.34 M sucrose alone (n = 4)	1.40 ± 0.10[a]
0.25% deoxycholate (n = 3)	0.74 ± 0.17
0.4% Triton X-100 (n = 3)	0.57 ± 0.09
100000*g* supernatant, 0.25% deoxycholate (n = 4)	0.44 ± 0.16
OZ activation	
0.34 M sucrose alone (n = 3)	0.64 ± 0.08[a]
0.25% deoxycholate (n = 3)	0.60 ± 0.15
0.4% Triton X-100 (n = 3)	0.68 ± 0.28
100000*g* supernatant, 0.25% deoxycholate (n = 3)	0.59 ± 0.09

[a] The ratios of the NADPH-oxidizing activity to O_2^-·-generating activity differed significantly in relation to the stimulus ($p < 0.01$) only for the particulate preparations in 0.34 M sucrose without a detergent.

reflects the elimination of an artefact attributable to a manganese-catalyzed electron transfer, which exhibits a pH optimum of 5.5 (Curnutte et al., 1976).

That the ratios of NADPH-oxidizing activity to O_2^-·-generating activity in subcellular particles did not simply reflect differing rates of activation was shown by defining the time courses of activation by PMA and OZ. The mean ratios of NADPH oxidation to O_2^-· generation were 1.84, 1.40, and 1.52 at 1.5, 3, and 6 min of activation with PMA, and the corresponding ratios were not significantly different over 6 min with OZ.

Detergent Effect on NADPH Oxidation Stoichiometry. Triton X-100 (Gabig & Babior, 1979) and deoxycholate (Tauber & Goetzl, 1979) solubilize and enhance the O_2^-·-generating activity of PMN leukocyte derived subcellular particles; the effect of both detergents on the ratio of NADPH oxidation to O_2^-· generation was examined with particles from cells stimulated by PMA and OZ (Table III). The O_2^-·-generating activity of particles from PMA-activated cells was increased a mean of 2.3-fold with Triton X-100 and a mean of 2.7-fold with deoxycholate, while NADPH-oxidizing activity was unaffected. In contrast, the levels of NADPH oxidation and O_2^-· generation increased concomitantly with detergent treatment of particles from OZ-activated neutrophils. Thus, the ratios of NADPH oxidation to O_2^-· generation of PMA-stimulated preparations decreased with solubilization in detergents to the same level as the ratio characteristic of OZ-stimulated preparations.

Effect of Deuterium Analogues of NADPH on the V_{max} of O_2^-· Generation in Particulate and Solubilized Preparations. In order to determine whether the transfer of a hydride equivalent from NADPH to the next component of the respiratory chain in the O_2^-·-generating system might regulate the rate of reduction of molecular oxygen, (4*R*)-[^{2}H]NADPH and (4*S*)-[^{2}H]NADPH were utilized in place of the corresponding [^{1}H]NADPH cofactor. When the effect of the deuterium analogues is measured in particulate preparations of activated PMN leukocytes, V_{max} is significantly greater for (4*R*)-[^{1}H]NADPH than for (4*R*)-[^{2}H]NADPH (V_H/V_D = 3.9 ± 0.4). When the particulate preparation then was treated with 0.25% deoxycholate, a 3-fold increase in O_2^-·-generating activity was noted, but the isotope effect on V_{max} fell to 2.4 ± 0.4. Assays of the deoxycholate-solubilized O_2^-·-generating activity in the 100000*g* supernatant revealed a lesser, but significant, isotope effect with (4*R*)-[^{2}H]NADPH (1.8–2.0), but not with (4*S*)-[^{2}H]NADPH. The alteration in V_{max} by the 4*R* but not the 4*S* analogue in solubilized preparations establishes the stereospecificity of hydride removal from NADPH in this system, which constitutes a rate-limiting step in the generation of O_2^-·.

Lability and Autoinactivation of the O_2^-·-Generating Activity. The O_2^-·-generating activity is labile and rapidly loses activity at 25 °C ($t_{1/2}$ = 24 min) (Tauber & Goetzl, 1979). During the catalytic production of O_2^-· in the presence of oxygen and NADPH the rate of inactivation at 28 °C is even more rapid ($t_{1/2}$ = 1–3 min) and is increased during the enhanced catalytic turnover induced by deoxycholate (Figure 2, Table IV). Loss of activity is not due to the exhaustion of available substrates since the addition of neither cytochrome *c* nor NADPH restores O_2^-·-generating activity. Furthermore, the same autoinactivation kinetics are observed in the presence of an NADPH-generating system (10 mM glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase) which maintained the concentration of NADPH at 0.1 mM throughout the kinetic study. Since the number of catalytic units responsible for the generation of O_2^-· is unknown, the characteristics of inactivation have been analyzed on the basis of 10^7 PMN leukocyte equivalents. The half-life of inactivation (Table IV) is obtained from the slope of semilog plots of the observed rate vs. time (Figure 2). Upon detergent solubilization this half-life decreases in proportion to the increase in the initial rate of O_2^-· production such that the total amount of O_2^-· generated remains constant (Table IV). The number of catalytic turnover events per inactivation event is directly proportional to the total O_2^-· produced by 10^7 PMN leukocyte

Table IV: Autoinactivation Characteristics of the Particulate and Solubilized O_2^-·-Generating Activity

O_2^-·-generating activity	initial rate [nmol of O_2^-·/min (equivalent of 10^7 PMN leukocytes)]	percentage of rate in intact PMN leukocytes	half-time of inactivation (min)	O_2^-· generation/inactivation (nmol of O_2^-·/equivalent of 10^7 PMN leukocytes)
particulate preparation	15.4 ± 2.4[a]	23.7 ± 3.8	1.86 ± 0.5	39.3 ± 12.0
particulate preparation treated with 0.25% deoxycholate	43.4 ± 2.25	63.3 ± 6.0	0.75 ± 0.05	39.7 ± 3.2

[a] Each value is the mean ± SD of the results with preparations from three separate donors.

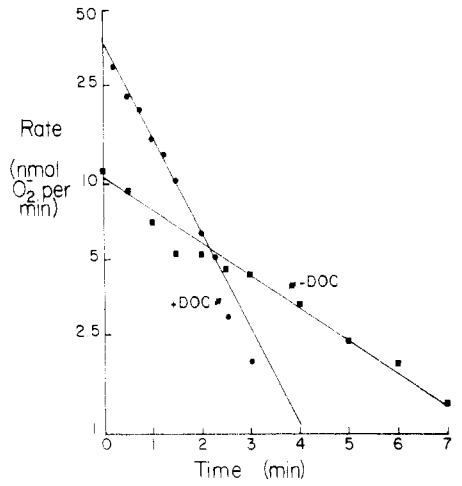

FIGURE 2: Autoinactivation of O_2^-·-generating activity in particulate preparations producing O_2^-·. Rates of inactivation were calculated from tangents to the tracings of O_2^-· generation. The reaction mixtures contained preparations from 0.8×10^7 PMN leukocytes, 0.16 mM cytochrome *c*, and 0.2 mM NADPH in 0.13 M potassium phosphate buffer (pH 7.0) with 30 μg of superoxide dismutase in the reference cuvette. The half-life for inactivation of the untreated particulate fraction was 2.3 min and for the 0.25% deoxycholate-treated particulate sample was 0.73 min.

Table V: Effect of FAD and FAD Analogues on O_2^-·-Generating Activity of Preparations Solubilized with Triton X-100

flavin	midpoint potential[a] (mV)	concn (μM)	% change (mean ± SD)
FAD	−208	0.4	+110
		1.6	+168
		8.0	+200 ± 16
FAD analogues			
8-chloro-FAD	−152	8.0	+360
1-deaza-FAD	−280	8.0	+90
5-deaza-FAD	−340	8.0	−50 ± 9
		16.0	−60 ± 10
		32.0	−100

[a] Midpoint potential of FAD and FAD analogues at the riboflavin level.

equivalents prior to complete inactivation and is thus independent of the rate of O_2^-· generation. Azide (1 mM) had no effect on the observed kinetics.

In order to ascertain whether autoinactivation was mediated by a product of the NADPH oxidase, various scavengers of oxygen metabolites were incubated with the preparation during turnover (Tauber et al., 1979b; Rosen & Klebanoff, 1977; Weiss et al., 1978). The effect of SOD was ascertained by measuring the oxidation of NADPH at OD_{340}, while other scavengers were assessed by the reduction of cytochrome *c* as assayed by changes in OD_{550}. Neither SOD (30 μg/mL), catalase (2000 units/mL), the OH· scavengers benzoic acid (5 mM), L-tryptophan (1 mM), or mannitol (0.15 M), nor the singlet oxygen scavengers bilirubin (124 mM) or L-histidine (200 μM) altered the kinetics of autoinactivation. Whether these results reflect the inability of the potentially effective scavengers to interact at a critical site in the oxidase, or rather demonstrate an inactivation process independent of oxygen-derived species, could not be discerned from these studies.

Effect of FAD and Analogues of FAD on the Solubilized O_2^-·-Generating System. FAD, a flavin with the capacity to transfer both one electron and two electrons, augmented the activity of the Triton X-100 solubilized preparation (Table V; Gabig & Babior, 1979) but not the deoxycholate solubilized preparation (Tauber & Goetzl, 1979). A mean maximum

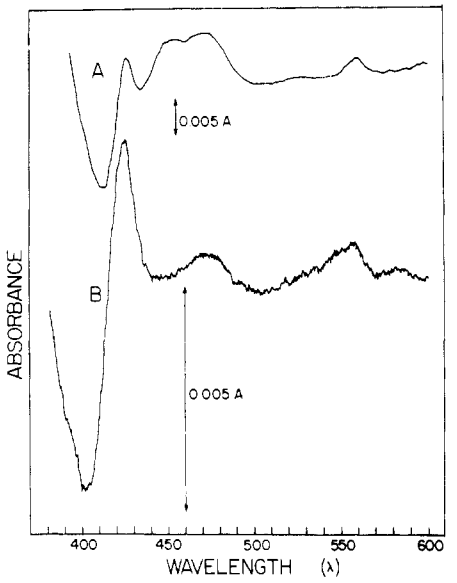

FIGURE 3: Difference spectra of subcellular preparations reduced with 1 mM dithionite. The respective characteristics of the dithionite-reduced minus oxidized spectra of cytochrome *b* and myeloperoxidase are λ_{min} = 410 nm, λ_{max} = 426 nm, 558 nm and λ_{max} = 473 nm, respectively. (A) Particulate preparation at a protein concentration of 0.47 mg/mL. (B) Peak of activity from Sephacryl S-200 column filtration of 0.25% deoxycholate-solubilized O_2^-·-generating activity at a protein concentration of 0.51 mg/mL.

enhancement by FAD of 200% can be achieved at concentrations of 8.0 μM (Table V). Comparison of the residual activity to the maximum activity observed during FAD titrations indicates that the preparation is 40–75% apoprotein after Triton X-100 treatment and that the dissociation constant for FAD is 0.2 μM or less. The stimulatory effects of FAD, 8-chloro-FAD, and 1-deaza-FAD, which are competent for one- and two-electron transfers, correlate with their oxidation–reduction potentials. This is the expected result if the reductive half-reaction of the FAD-sensitive step is partially rate-determining during turnover (Light & Walsh, 1980; Walsh et al., 1978; Spencer, 1978). Titration with 5-carba-5-deaza-FAD, which is only competent for two-electron transfers, inhibits the residual O_2^-·-generating activity with complete inhibition at 32 μM, suggesting that this analogue completely displaces residual FAD from the apoenzyme and that the physiological oxidant of the flavoenzyme is a one-electron acceptor.

Studies of a Type b Cytochrome Associated with the Purified Solubilized O_2^-·-Generating Activity. Up to 95% of the O_2^-·-generating activity in OZ- or PMA-activated PMN leukocytes was demonstrated to be associated with the particulate fraction which sediments between 400*g* and 27000*g* from 0.34 M sucrose–Tris buffer following sonication (Tauber & Goetzl, 1979). The preponderant chromophore in the resuspended 27000*g* pellet is myeloperoxidase (Figure 3, curve A) which has a broad absorbance around 473 nm (Wever et al., 1976). The spectral features characteristic of a cytochrome *b* (oxidized, λ_γ 410 nm; reduced, λ_α 558 nm; λ_γ 426 nm) also are clearly represented in the particulate preparation. The ratio of the moles of myeloperoxidase to the moles of the cytochrome *b* can be estimated by using the known spectral parameters of homogeneous myeloperoxidase [$\Delta\epsilon_{473-501}$ = 74000 M^{-1} cm^{-1} for the reduced minus oxidized spectrum (Wever et al., 1976)] and assuming a $\Delta\epsilon_{426-410}$ of 200000 M^{-1} cm^{-1} for the difference between the reduced and oxidized spectra of cytochrome *b*. The analogous extinction coefficient for rabbit liver microsomal cytochrome b_5 is 149000 M^{-1} (Strittmatter & Velick, 1956) and for rat liver cytosolic cy-

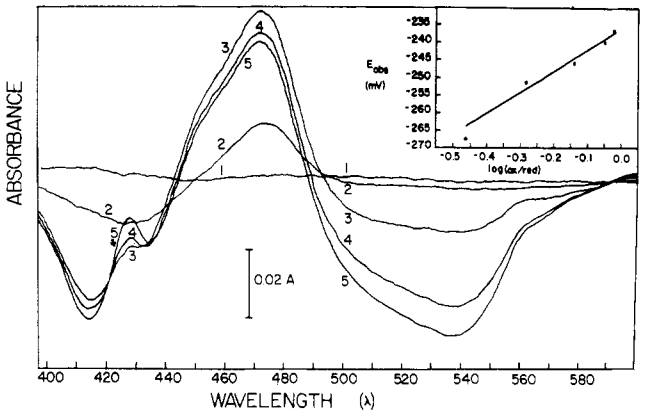

FIGURE 4: Oxidation–reduction titration of cytochrome *b* in particulate preparations of $O_2^{\cdot-}$-generating activity. Difference in dithionite-reduced minus oxidized spectra for the titration of approximately 0.15 μM cytochrome *b* in the presence of 5 μM phenosafranin. Scans represent (1) oxidized – oxidized (base line), (2) 2 nmol of dithionite – oxidized (myeloperoxidase is substantially reduced before the reduction of either cytochrome *b* or phenosafranin was detectable; phenosafranin λ_{max} 520 nm), (3) 3 nmol of dithionite – oxidized, (4) 5 nmol of dithionite – oxidized, and (5) 1000 nmol of dithionite – oxidized. Insert: Data points represent the observed log of the ratio of oxidized to reduced cytochrome *b* at various values for E_{obsd} derived by monitoring the spectrum of phenosafranin. The values for E_{obsd} are centered around the midpoint potential of phenosafranin ($E^{\circ\prime}$ = −252 mV). The line depicts a plot of the Nernst equation using the following values: $E^{\circ\prime}$ = −235 + 60 log (ox/red).

tochrome b_9 is 250000 M^{-1} (Kim, 1979), and until the cytochrome *b* from PMN leukocytes is purified and characterized we have found it convenient to assume that its absorbance properties are similar. The calculated ratio of myeloperoxidase to cytochrome *b* in the particulate fraction is 4.5.

After Sephacryl chromatography of the deoxycholate-solubilized preparation, the fractions with $O_2^{\cdot-}$-generating activity contain cytochrome *b* as evidenced by its oxidized peak at 410 nm. The ratio of the protein absorbance at 270 nm to the peak at 410 nm decreases during purification of the $O_2^{\cdot-}$-generating activity from 48.0 in the 100000*g* supernatant of the deoxycholate-treated particulate preparation to 8.9 in the Sephacryl fractions, indicating a 5.4-fold purification of the cytochrome *b*. Further, the ratio of myeloperoxidase to cytochrome *b* (0.28) drops 17-fold and the cytochrome *b* becomes the dominant chromophore (Figure 3). The ratio of the cytochrome *b* to protein is only 0.1 nmol/mg in these column fractions, and the fraction shown in Figure 3 which contains approximately 300 pmol of cytochrome *b* also contains 200 pmol of FAD.

Particulate preparation obtained from human eosinophils purified to 98% homogeniety contain an NADPH oxidase with similar characteristics to that found in PMN leukocytes (Tauber et al., 1979a). Solubilization of the particulate fraction in 0.25% deoxycholate demonstrated a cytochrome with the same spectral characteristics as the PMN leukocyte cytochrome *b*. The eosinophil cytochrome *b* is present in comparable quantity to that found in PMN leukocytes.

Assessment of the Oxidation–Reduction Potential of the Subcellular Particle Associated Cytochrome b. The midpoint oxidation–reduction potential of cytochrome *b* in particulate preparations was determined with phenosafranin dye ($E^{\circ\prime}$ = −252 mV) to be −235 ± 9 mV (Figure 4). The reduction of the cytochrome *b* is consistent with a one-electron process with a slope of 66 mV, which is close to the expected slope of 60 mV (Figure 4; insert). The midpoint potential of the cytochrome *b* in resting PMN leukocytes was identical with that obtained from PMN leukocytes stimulated by PMA. During anaerobic titrations with dithionite in the presence of indigotetrasulfonate ($E^{\circ\prime}$ = −46 mV) as well as indigodisulfonate ($E^{\circ\prime}$ = −125 mV), complete reduction of even the low potential dye occurred *before any reduction* of the cytochrome *b* occurred.

The extent of reduction of phenosafranin was monitored by comparison of the absorbance at two isosbestic points of the reduced minus oxidized spectrum of myeloperoxidase (Wever et al., 1976). Thus the decrease in absorbance at 501 nm due to phenosafranin reduction (oxidized, λ_{max} 519 nm) was subtracted from the absorbance at 591 nm (also an isosbestic point for phenosafranin). Two corrections were made to the $A_{426nm} - A_{410nm}$ due to the cytochrome *b* in the reduced minus oxidized spectrum. The first correction was made to account for the rather negligible absorbance contribution due to phenosafranin in this region (2–5%) and was calculated from the reduced minus oxidized spectrum of phenosafranin alone as (0.078) ($A_{591nm} - A_{510nm}$). The second and more substantial (30–50%) correction was made to account for the absorbance due to myeloperoxidase in this region, (0.20) ($A_{473nm} - A_{501nm}$). Simply using the uncorrected absorbance changes to monitor the cytochrome *b* ($A_{426nm} - A_{410nm}$) gave a less suitable fit to the Nernst equation, but the midpoint potential calculated in this manner was similar (−247 ± 7 mV). Myeloperoxidase in the particulate fraction was observed to be reduced during dithionite titrations in the presence of dyes of higher potentials, indicating that the potential of the myeloperoxidase is higher than that of the cytochrome *b*.

Failure To Reduce the Cytochrome b with NADPH. The measured oxidation–reduction potential of the cytochrome *b* is clearly high enough to allow complete reduction by NADPH ($E^{\circ\prime}$ = −320 mV). However, cytochrome *b* was not reduced by the addition of anaerobic NADPH to particulate preparations of $O_2^{\cdot-}$-generating activity in anaerobic cuvettes. Whether anaerobiosis was achieved by repetitive evacuation and flushing with argon alone or in the presence of 1 mM glucose and glucose oxidase, the addition of 350 μM NADPH (or NADH) resulted in slight reduction of myeloperoxidase (<10%), but no reduction of the cytochrome *b*, although the particles retained full $O_2^{\cdot-}$-generating activity when assayed in oxygenated buffers.

Since the possibility remained that enzymatic reduction of the cytochrome *b* was precluded by the presence of residual oxygen in the sample, a different approach to the enzymatic reduction of the cytochrome was attempted. Buffer containing NADPH (100 μM = final concentration) or NADH was saturated with carbon monoxide, injected into a cuvette containing 1.4×10^8 cell equivalents of the particulate preparation, and equilibrated with carbon monoxide to attempt to trap the reduced cytochrome *b* (Shingawa et al., 1966). At 8 and 30 min, respectively, a maximum of 20% and 38% of the cytochrome *b* could be reduced under these conditions when compared to the extent of reduction with dithionite (1.0 mM). Like studies were performed with deoxycholate-solubilized $O_2^{\cdot-}$-generating activity, and similar results were obtained (data not shown).

The Rate of Oxidation of the Reduced Cytochrome b by Molecular Oxygen. If the cytochrome *b* which is observed to copurify with the $O_2^{\cdot-}$-generating system is like cytochrome b_5, inert to reoxidation by molecular oxygen, it is an unlikely candidate for the terminus of the electron-transfer chain in this system. Therefore to test the oxygen reactivity of the PMN leukocyte cytochrome *b* a sample containing 0.4 μM cytochrome *b* was evacuated, flushed with argon, and titrated to full reduction by the addition of aliquots of dithionite. After

full reduction was observed, the instrument was placed in the double beam mode ($A_{426nm} - A_{410nm}$) to optimize the expected change in absorbance due to reoxidation. At this point, 100 μL of air-saturated buffer was injected through the serum stopper and the sample was mixed rapidly and inserted into the spectrophotometer (~10 s total elapsed time). Scanning the sample confirmed full reoxidation of the cytochrome *b* occurred within the first 10 s. Repetition of dithionite addition (1 μmol) fully reduced the cytochrome *b*, and brief shaking in air completely reoxidized the sample. The inability to observe the process of reoxidation implies that 10 s is greater than approximately 7 half-lives for the process. A rate constant of $k > 0.5$ s^{-1} at concentrations of 0.4 μM cytochrome *b* and 25 μM oxygen translates to a second-order rate constant of at least 2.5×10^6 M^{-1} min^{-1}.

Discussion

The respiratory burst of the human PMN leukocyte has been characterized as being due to an NADPH oxidase (Babior, 1978). We have determined that this particulate activity, in the presence of detergent, accounts for 57%–69% of the whole cell activity (Tables I and IV). Further studies to characterize the cofactors and source of reducing equivalents on this isolated activity are herein reported. While whole cell studies demonstrated an equivalent degree of HMP shunt activation, indicating an equal rate of NADPH reduction by this pathway for both OZ and PMA stimulation, the electron flux assessed by the direct measurement of NADPH oxidation was 2-fold greater in particles derived from PMA-activated PMN leukocytes than in those from OZ-activated cells. The excess NADPH oxidation may represent (a) an uncoupling from $O_2^-\cdot$ production (with electron flow to an unidentified acceptor or possibly oxygen-accepting multiple electrons) or (b) electron flow to other enzyme systems. The disproportionate increase in the $O_2^-\cdot$-generating activity of particles from PMA-activated PMN leukocytes that resulted from solubilization in Triton X-100 or deoxycholate decreased the ratios of NADPH oxidation to $O_2^-\cdot$ generation to values similar to those of preparations from OZ-activated PMN leukocytes before and after detergent treatment (Table III). Thus, the functional state of human PMN leukocyte $O_2^-\cdot$-generating activity in subcellular particles may be modulated not only by the specific stimulus but also by the physical state of the catalytic unit or units. The stoichiometric relationship of NADPH oxidation to $O_2^-\cdot$ generation in detergent-solubilized PMN leukocyte material indicates that the intrinsic activity of the putative respiratory burst enzyme complex of the PMN leukocytes couples NADPH oxidation exclusively to the one-electron reduction of oxygen to $O_2^-\cdot$. However, the expression of this activity depends on the physical state and the purity of the enzyme complex.

Two other findings are consistent with a functional alteration of the enzyme system upon detergent solubilization. Studies of the kinetic isotope effect on NADPH oxidation reveal a greater kinetic isotope effect on V_{max} when (4*R*)-[^{2}H]NADPH is used as the reductant in the particulate fraction (V_H/V_D = 3.9 ± 0.4) than either particulate material in the presence of detergent (V_H/V_D = 2.4 ± 0.4) or the solubilized activity (V_H/V_D = 1.9 ± 0.2). Upon solubilization or membrane disruption by detergent, hydride transfer from NADPH becomes less rate determining during overall turnover. Secondly, the nearly 3-fold enhancement of $O_2^-\cdot$-generating activity upon detergent treatment of the particulate fraction is offset by an analogous increase in the rate of turnover-dependent autoinactivation. Thus the total number of productive turnovers prior to complete inactivation remains constant. The simplest explanation is that the number of enzyme molecules involved and the ratio of turnovers per inactivation event remain constant and that the enhanced activity is due to a detergent-dependent change in the rate-determining step. Alternatively, the increase in the rate observed upon detergent solubilization may be due to an increase in the number of active catalytic units. In this model, the rate of autoinactivation would increase in parallel with the number of catalytic units to allow the same number of turnover events to occur before complete inactivation. That these two alterations would occur at equal rates appears less likely, unless autoinactivation is dependent on the generation of an oxygen-derived reactive species by the catalytic units. Attempts to demonstrate such a mechanism with various scavengers of oxygen metabolites were unsuccessful. Thus the change in kinetic isotope effect for hydride transfer from NADPH is entirely consistent with the constant ratio of successful turnovers per inactivation event observed during the marked increase in $O_2^-\cdot$-generating activity upon detergent treatment.

The rapid rate of autoinactivation of the NADPH oxidase reflects the rate of decline of the activities of the respiratory burst in intact PMN leukocytes, where linearity of the activity is observed for less than 1 min when either phagocytosable particles (Segal & Coade, 1978) or soluble stimuli (Simchowitz & Spilberg, 1979) are employed. Oxygen consumption and $O_2^-\cdot$ production rates are one-half maximal 5 min poststimulation (Curnutte, 1980), which is similar to that reported previously for particulate preparations (Babior et al., 1976). An appreciation of the kinetics of the $O_2^-\cdot$-generating activity in either whole cells or subcellular preparations is largely dependent on the ability to assess the initial and maximal rates of turnover. Similarities in the kinetics of inactivation of the respiratory burst of intact PMN leukocytes and of the partially isolated NADPH oxidase corroborate the central role of this catalytic activity in generating $O_2^-\cdot$ in the respiratory burst.

Superoxide-generating activity solubilized from the particulate fractions of stimulated PMN leukocytes by Triton X-100 can be enhanced by the addition of FAD (Gabig & Babior, 1979; this work). The dissociation constant for the added cofactor is <0.2 μM. The observation of a positive correlation between the enhancement of $O_2^-\cdot$-generating activity in the Triton X-100 solubilized fraction and the oxidation–reduction potential of the added FAD analogue (Table V) is the expected result if the reductive half-reaction of the FAD-sensitive step is at least partially rate determining during turnover (Light & Walsh, 1980). This observation is consistent with the kinetic isotope effect (1.8–2.0) measured with deuterated (4*R*)-[^{2}H]NADPH in the solubilized $O_2^-\cdot$-generating system. The FAD analogue 5-carba-5-deaza-FAD is observed to completely inhibit the $O_2^-\cdot$-generating activity (Table V). Thus all of the $O_2^-\cdot$-generating activity is dependent upon functional FAD. There are two properties of 5-deaza-FAD which could explain its ability to inhibit the $O_2^-\cdot$-generating activity, presumably after displacing residual FAD. Since 5-deaza-FAD is an obligate two-electron donor (Fisher et al., 1976), its inability to substitute for FAD could be due to its inability to reduce an obligate one-electron acceptor. Since reduced 5-deaza-FAD is inert with respect to reoxidation by molecular oxygen, the inhibition could also result from its inability to reduce oxygen. The present study does not distinguish between the two possible modes of inhibition and cannot answer the question of whether the FAD-sensitive step is also the site of the one-electron reduction of oxygen or whether the FAD-sensitive step is part of an electron-transfer

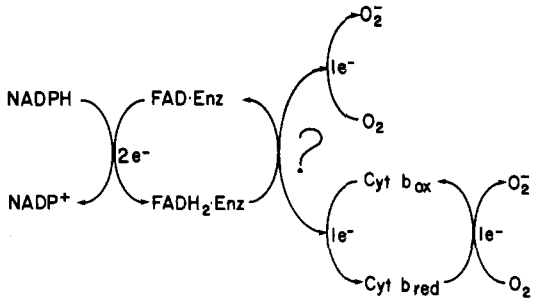

FIGURE 5: Schematic representation of possible routes of electron transfer during $O_2^{-}\cdot$ generation by activated PMN leukocytes.

chain to another site of oxygen reduction.

Biochemical sources of $O_2^{-}\cdot$ have been characterized, including flavins (Fridovich & Handler, 1961; Massey et al., 1969), hemoproteins (Wever et al., 1973; Berman et al., 1976), iron–sulfur proteins (Orme-Johnson & Beinert, 1969; Misra & Fridovich, 1971), and tetrahydropteridines (Nishikimi, 1975). That flavin is the site of $O_2^{-}\cdot$ formation in xanthine oxidase is well established (Komai et al., 1969), and of the simple flavoproteins, oxygen oxidation of reduced dehydrogenases results in $O_2^{-}\cdot$ formation while oxygen oxidation of oxidases does not (Massey et al., 1969; Ostrowski & Kistler, 1980). The rate of $O_2^{-}\cdot$ production catalyzed by free flavin (Ballou et al., 1969) and of tetrahydropteridine (Nishikimi, 1975) increases dramatically with increasing pH. Strittmatter first reported the ability of oxygen to slowly reoxidize reduced cytochrome b_5 purified from rabbit liver microsomes (Strittmatter & Velick, 1956), and subsequent quantitation of the rate of $O_2^{-}\cdot$ production by this process (Berman et al., 1976) revealed a nearly 300-fold increase in this rate as the pH was lowered from 7 to 4. The recently characterized soluble mammalian cytochrome *b* purified from rat liver cytosol is oxidized by oxygen at neutral pH (Kim, 1979). In this context, it is interesting to note that the pH optimum of the solubilized $O_2^{-}\cdot$-generating activity from activated leukocytes is 7.1 and falls off rapidly at higher and lower values (Figure 1) (Tauber & Goetzl, 1979; Gabig & Babior, 1979). It is not evident, however, that this represents the pH optimum of the active site of $O_2^{-}\cdot$ production but may rather reflect the pH optimum of a rate-determining step which precedes the interaction of oxygen with the enzyme system.

The oxidation–reduction potential determined for the cytochrome *b* in the particulate fraction from resting or activated cells is the same (-235 ± 9 mV) and is much lower than that observed for other mammalian *b* type cytochromes. While problems with the method of titration using dyes have been reported (Sullivan & Holloway, 1973), the potential of this cytochrome *b* is clearly lower than that of liver cytochrome b_5 (+30 mV) and the cytochrome b_5 in erythrocytes (−2 mV) since these potentials were determined by the use of the same dyes (Velick & Strittmatter, 1956; Abe & Sugita, 1979). This cytochrome *b* also differs from cytochrome b_5 in that its rate of oxidation by molecular oxygen (42000 M^{-1} s^{-1}) is 2000-fold greater than that seen for cytochrome b_5 (23 M^{-1} s^{-1}) (Berman et al., 1976). It should be noted that the observation of higher rates of reoxidation of cytochrome b_5 in microsomes vs. the soluble cytochrome *b* have been attributed to reverse electron flow back through a more readily oxidized intermediate (Ohnishi & Imai, 1975). Thus a direct comparison cannot be made until the cytochrome from leukocytes has been purified.

The inability to reduce the cytochrome *b* with pyridine nucleotide argues against its functional role in $O_2^{-}\cdot$ generation in this system. Even when the reduced cytochrome *b* is trapped in a complex with carbon monoxide (Shingawa et al., 1966), the rate of its reduction by NADPH or NADH in the particulate fraction is too slow to account for the rate of $O_2^{-}\cdot$ production. While the rate of reduction may differ in the presence of oxygen, the latter observation casts further doubt on the ability of this cytochrome *b* to function directly in $O_2^{-}\cdot$ production.

Since the evidence for the direct involvement of the cytochrome *b* in $O_2^{-}\cdot$ production is at best circumstantial, the scheme in Figure 5 is presented by way of summary. The present study does not determine whether either flavin or heme is the site of oxygen reduction nor does it assign a functional role for the *b* cytochrome. Studies demonstrating cytochrome *b* in the phagocytic vacuole (Segal & Jones, 1978), its reduction in whole cells stimulated with PMA (Segal & Jones, 1979), and its absence in some, but not all patients with chronic granulomatous disease (Borregaard et al., 1979) (who are unable to generate a respiratory burst) suggest that this component has a role in either the activation of the enzyme responsible for $O_2^{-}\cdot$ production or alternatively is directly involved in the electron reduction of molecular oxygen. However, in the characterization of the PMN leukocyte cytochrome in these studies, an attempt to functionally relate the cytochrome to $O_2^{-}\cdot$ generation was unsuccessful. Therefore, studies are currently in progress to (1) elucidate the role of this unique cytochrome *b* in the oxidative burst in PMN leukocytes, (2) purify and characterize the FAD sensitive component(s), and (3) determine the component(s) subject to turnover-dependent autoinactivation in the isolated system.

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Multiple Isotope Effect Probes of Glutamate Decarboxylase†

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ABSTRACT: The enzymatic decarboxylation of glutamic acid shows a carbon isotope effect $k^{12}/k^{13} = 1.018$ at 37 °C, pH 4.7. In D_2O under otherwise identical conditions, $k^{12}/k^{13} = 1.009$. Under the same conditions solvent isotope effects are $V_{max}^{H_2O}/V_{max}^{D_2O} = 5.0$ and $(V_{max}/K_m)^{H_2O}/(V_{max}/K_m)^{D_2O} = 2.6$. With the assumption that the carbon isotope effect on the decarboxylation step is in the usual range (1.05–1.07), it is possible to derive relative rates and solvent isotope effects for all steps in the enzyme mechanism. Substrate binding is ~2-fold weaker in H_2O than in D_2O, probably because of the desolvation which accompanies binding of the substrate to the enzyme. A proton inventory analysis of the reaction shows that the Schiff base interchange has a large solvent isotope effect composed of relatively small contributions from at least four separate sites. A conformation change probably accompanies this step. The decarboxylation step shows a solvent isotope effect of ~2. Schiff base interchange and decarboxylation are both partially rate determining. The pH dependence of the isotope effects indicates that the initial step in the reaction can occur by way of two different pathways.

Isotope effects are finding increasing use in studies of enzyme reaction mechanism (Cleland et al., 1977; Gandour & Schowen, 1978; Klinman, 1978). In most cases the observed isotope effect reflects some combination of the isotope effects on individual steps (called *intrinsic isotope effects*). The weighting factors which define this combination, called *partition factors*, are not isotopically sensitive.

Currently, the principal problem in applying isotope effects to problems in enzymology is the difficulty of extracting the intrinsic isotope effects and the partition factors from the observed effects. Several procedures for making this separation are currently in use. The important discovery by Northrop (1975, 1977) that deuterium and tritium isotope effects provide, by use of the Swain relationship (Swain et al., 1958), a separation of variables has given new impetus to studies of hydrogen isotope effects. However, the procedure is experimentally quite difficult and is not applicable in all cases (Albery & Knowles, 1977). In some cases it is possible to use pre-steady-state kinetics or partial reactions to determine isotope effects on individual reaction steps. This procedure has been used, among others, for aspartate aminotransferase (Jenkins & Harruff, 1979) and for serine *trans*-hydroxymethylase (Ulevitch & Kallen, 1977).

Manipulation of reaction conditions can sometimes provide the key to the separation. Use of abnormal substrates (Klinman, 1978; O'Leary & Piazza, 1978) or extreme pH

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