

lymphocytic neoplasms, the inverted chromosome 14 (when present in normal lymphocytes) is not invariably present in the subsequent malignant clone (14). It may be that the Ig V_H-TCR J_α rearrangement must fulfill additional criteria of functionality or specificity in order to contribute to malignant transformation. Both SUP-T1 and VP tumors contain other karyotypic abnormalities that could provide the primary event in malignant transformation (5, 15). The inv(14) in these two tumors might confer an additional selective advantage. It is striking that in both cases a hybrid Ig V_H-TCR J_α transcript with an open reading frame is generated. The message for this hybrid receptor could be translated into a protein capable of providing an abnormal mitogenic stimulus to the cell resulting in deregulated proliferation.

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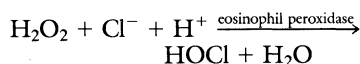
Brominating Oxidants Generated by Human Eosinophils

STEPHEN J. WEISS, SAMUEL T. TEST, CAREL M. ECKMANN, DIRK ROOS, SANDRA REGIANI

Eosinophils are white blood cells that in humans are found in association with helminthic infections and various inflammatory disease processes. These cells contain a unique lysosomal peroxidase that oxidizes halides to generate highly reactive and toxic hypohalous acids. Although chloride is found in vivo at concentrations at least 1000-fold greater than those of other halides, human eosinophils did not preferentially oxidize chloride under physiologic conditions. Instead, eosinophils used bromide, a halide with a hitherto unknown function in humans, to generate a halogenating oxidant with characteristics similar, if not identical, to those of hypobromous acid. These results indicate that physiological concentrations of bromide arm human eosinophils with the ability to generate and release an unusual oxidant capable of destroying a wide range of prokaryotic and eukaryotic targets.

THE HUMAN EOSINOPHIL CAN PLAY A unique and beneficial role in host defense by destroying parasitic worms and also a detrimental role in inflammatory disease states by damaging host tissues (1). In both cases the destructive effects exerted by the eosinophil are thought to be dependent on the cell's ability to release toxic lysosomal components and to generate reactive oxygen metabolites (1, 2). The specific processes used by the eosinophil to carry out its specialized functions are not known, but increasing attention has focused on one of the major lysosomal proteins of the cell, the heme-enzyme eosinophil peroxidase (1, 2). In cell-free systems, purified eosinophil peroxidase utilizes H₂O₂ to catalyze the peroxidation of halides to highly reactive halogenating intermediates capable of destroying a host of targets ranging from multicellular worms to mammalian cells (2-9).

Recent studies have directly demonstrated that purified eosinophil peroxidase can oxidize chloride to the powerful oxidant hypochlorous acid (10).



Because chloride is found in vivo at concentrations at least 1000-fold higher than those of any other halide (11), eosinophils might be expected to generate primarily HOCl under physiologic conditions. The ability of intact eosinophils to generate HOCl would allow the cell to mediate a variety of toxic effects (12), but chloride is oxidized less efficiently by purified eosinophil peroxidase than by myeloperoxidase, a lysosomal haloperoxidase that is found in the human neutrophil (2, 13-16). This information and the fact that these two peroxidases are distinct gene products with different heme and pro-

tein moieties (17, 18) have led to the suggestion that the true function of eosinophil peroxidase in the intact cell is still unknown (18). We have now shown that human eosinophils selectively utilize physiologic concentrations of bromide, a halide with a hitherto unknown function in humans, to generate a highly reactive and toxic oxidant with characteristics similar if not identical to those of hypobromous acid (HOBr).

In cell-free systems, purified eosinophil peroxidase can catalyze the H₂O₂-dependent oxidation of halides, but the ability of intact eosinophils to generate oxyhalides under physiologic conditions is unknown. Thus, purified eosinophils were isolated from the venous blood of normal volunteers (19), suspended in a physiologic, chloride-containing buffer (Dulbecco's phosphate-buffered saline, pH 7.4), and triggered to generate oxygen metabolites and release lysosomal components by the addition of either phorbol myristate acetate (PMA) (Consolidated Midland) or serum-opsonized zymosan particles (Sigma). As shown in Table 1, triggered eosinophils were able to generate significant quantities of HOCl after the addition of either stimulus. However, under identical conditions, triggered neutrophils produced even larger amounts of HOCl (Table 1). The attenuated ability of the eosinophil to generate HOCl relative to the neutrophil was not due to differences in the magnitude of the cells' respiratory burst.

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That is, PMA-triggered eosinophils were able to generate twice as much superoxide anion ($O_2^{\cdot-}$; the precursor of H_2O_2) than comparably treated neutrophils (Table 1). As expected, HOCl generation by eosinophils or neutrophils was almost completely inhibited if the granulocytes were triggered in the presence of catalase (reduces H_2O_2) (20) or azide (inhibits heme-enzyme peroxidases; see Table 1) (20).

Hydrogen peroxide and eosinophil peroxidase can mediate hypohalous acid-dependent effects in cell-free systems more efficiently when chloride is replaced by bromide (2, 3, 5, 7, 16). However, in vivo, extracellular fluids contain both halides in a mixture of approximately 100 mM chloride and 20 to 100 μM bromide (11). The possibility that these relatively small concentrations of bromide could alter eosinophil oxidative metabolism was examined by triggering cells in Dulbecco's phosphate-buffered saline (the chloride concentration in this buffer is 140 mM) supplemented with increasing concentrations of exogenous bromide (6.25 to 100 μM). Although the addition of bromide had no effect on HOCl generation by neutrophils, the halide exerted a dose-dependent inhibitory effect on the eosinophils (Fig. 1). At the highest concentration tested, 100 μM bromide had no effect on $O_2^{\cdot-}$ generation by the eosinophils. Because HOBr cannot be efficiently detected in our assay system for HOCl (21), these data indirectly support the premise that eosinophils, but not neutrophils, preferentially utilize bromide at physiologic halide concentrations.

In order to assess the brominating potential of intact eosinophils directly, we took advantage of the known ability of HOBr to

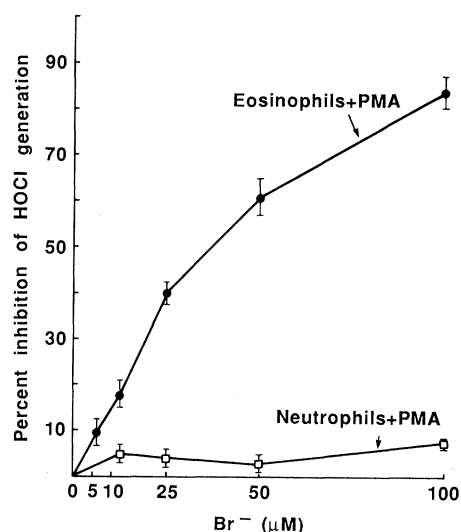


Fig. 1. The effect of exogenous bromide on HOCl generation by PMA-triggered eosinophils or neutrophils. Neutrophils ($\square$) or eosinophils ($\bullet$) (10^6 per milliliter in a final volume of 1.0 ml) were suspended in Dulbecco's buffer in the presence of PMA (50 ng/ml) and the indicated Br^- concentrations. HOCl generation was quantitated as described in Table 1. Neutron activation analysis revealed that the Dulbecco's buffer contained less than 5 μM Br^- as an endogenous contaminant. Results are expressed as the mean percent inhibition $\pm$ SEM of HOCl generated in the absence of Br^- in at least five paired experiments. In the absence of exogenous Br^- , the control values for PMA-triggered eosinophils (10^6 per milliliter) were 17.8 ± 1.5 nmol of HOCl (mean $\pm$ SEM, $n = 14$) and 62.0 ± 3.7 nmol of HOCl for PMA-triggered neutrophils ($n = 5$). If eosinophils isolated on Metrizamide gradients (34) were triggered with PMA in the absence or presence of 50 μM Br^- , HOCl generation similarly fell from 28.6 ± 5.2 nmol to 5.4 ± 0.5 nmol (mean $\pm$ SEM, $n = 3$).

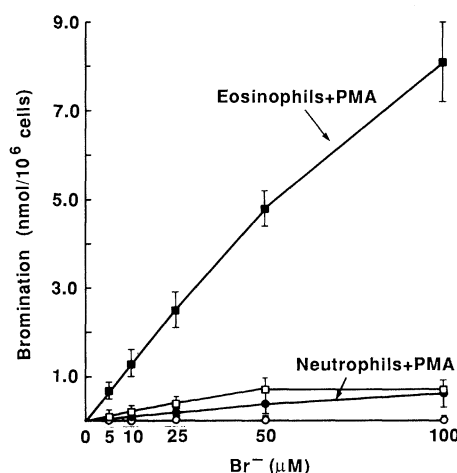
halogenate proteins (22). Thus, eosinophils or neutrophils were triggered in the presence of increasing concentrations of $^{82}Br^-$ in Dulbecco's buffer in the presence of an exogenous protein target (human albumin), and the incorporation of the radioactive halide into extracellular macromolecules was quantitated. Eosinophils stimulated with PMA produced at least ten times as much brominated product as comparably treated neutrophils at each bromide concentration tested (Fig. 2). Almost identical results were obtained with eosinophils prepared by an independent technique [eosinophils isolated by the method of Yazdanbakhsh *et al.* (23) and triggered with PMA in the presence of 50 μM $^{82}Br^-$ incorporated 8.8 ± 2.1 nmol of ^{82}Br ; $n = 3$]. Differences between eosinophil- and neutrophil-mediated bromination

reactions were not stimulus-dependent because similar findings were obtained with opsonized zymosan particles or serum-coated Sephadex beads, and they were not related to differences in the distribution (extracellular versus cell-associated) of the brominated products (Table 2) (24). In both cell populations, the pivotal role of the H_2O_2 -peroxidase-bromide system was demonstrated by the ability of catalase, azide, or the hypohalous acid scavenger methionine (12) to inhibit bromination almost completely (Table 2). Extracellular fluids also contain small quantities of free iodide (0.1 to 0.5 μM) (11) and iodinated thyroid hormones (the physiologic concentration of triiodothyronine and thyroxine is approximately 0.1 μM) (25), either of which might be utilized by eosinophil peroxidase. However,

Table 1. Generation of HOCl and $O_2^{\cdot-}$ by human eosinophils and neutrophils. Human eosinophils were isolated from peripheral venous blood by treating EDTA-anticoagulated whole blood with $10^{-7} M$ *N*-formyl-Met-Leu-Phe (Sigma) for 15 minutes at $37^\circ C$ and separated on a discontinuous Percoll gradient by the method of Roberts and Gallin (19). Cells ($\geq 92\%$ eosinophils, 3% to 4% lymphocytes, and 1% to 3% neutrophils, with a viability $\geq 95\%$) were suspended in Dulbecco's phosphate-buffered saline containing glucose (1 mg/ml) at pH 7.4. Neutrophils were isolated by a Ficoll-Hypaque separation and dextran sedimentation (20). HOCl generation by either cell population was quantitated by incubating granulocytes (10^5 per milliliter) with 15 mM taurine alone or in the presence of the triggering agents PMA (50 ng/ml) or opsonized zymosan particles (1.25 mg/ml) for 90 minutes at $37^\circ C$ (19). In selected experiments, cells were triggered in the presence of native catalase (25 $\mu g/ml$), heat-inactivated catalase (25 $\mu g/ml$), or azide (1 mM). HOCl was trapped as *N*-chlorotaurine, identified by its characteristic ultraviolet absorption maximum at 252 nm (32), and quantitated by its ability to oxidize 5-thionitrobenzoic acid to its disulfide derivative (20). Superoxide generation was quantitated by triggering cells (10^5 per milliliter) in the presence of 160 μM cytochrome *c* (Sigma) and catalase (40 $\mu g/ml$) in the absence or presence of superoxide dismutase (20 $\mu g/ml$) (33). Results are expressed as mean nanomoles generated $\pm$ SEM of the number of experiments indicated in parentheses. Similar results were obtained for superoxide or HOCl generation by human eosinophils prepared by techniques that did not necessitate treating whole blood with *N*-formyl-Met-Leu-Phe. Eosinophils isolated on discontinuous gradients of Metrizamide (34) and triggered with PMA generated 79.1 ± 1.8 nmol of superoxide ($n = 3$) and 31.8 ± 2.0 nmol of HOCl ($n = 3$). Slightly higher values for HOCl generation were obtained if eosinophils were prepared on the one-step Percoll gradient described by Yazdanbakhsh *et al.* (23). These eosinophils generated 43 ± 6 nmol of HOCl per 10^6 cells when triggered with either PMA ($n = 4$) or opsonized zymosan particles ($n = 5$). N.D., not determined.

Additive	Stimulus	HOCl (nmol per 10^6 cells)		$O_2^{\cdot-}$ (nmol per 10^5 cells)	
		Eosinophils	Neutrophils	Eosinophils	Neutrophils
Cells	None	3.8 ± 0.6 (9)	1.1 ± 0.4 (7)	4.7 ± 1.3 (6)	1.1 ± 0.6 (4)
Cells	PMA	16.2 ± 0.8 (26)	62.4 ± 3.2 (19)	83.2 ± 3.2 (19)	43.1 ± 1.5 (17)
Cells	Zymosan	25.8 ± 6.2 (4)	121.1 ± 5.4 (4)	18.4 ± 2.7 (7)	17.5 ± 1.4 (5)
Cells + catalase	PMA	2.7 ± 0.6 (12)	5.3 ± 1.0 (4)	N.D.	N.D.
Cells + heat-in-activated catalase	PMA	13.0 ± 1.3 (7)	61.8 ± 5.9 (3)	N.D.	N.D.
Cells + azide	PMA	0.0 ± 0.0 (11)	0.0 ± 0.0 (5)	N.D.	N.D.

Fig. 2. Bromination by purified eosinophils or neutrophils. Cell preparations and incubation conditions were as described in Table 1 except that human serum albumin (2 mg/ml) and $^{42}\text{K}^{82}\text{Br}$ (3.6 mCi/mg) (New England Nuclear) were added as indicated. [$^{42}\text{K}^{82}\text{Br}$ must be used in place of standard $\text{NH}_4^{82}\text{Br}$ to prevent the formation of potentially cytotoxic haloamines (35).] At the end of the 90-minute incubation period, cells were pelleted by centrifugation, and the supernatants were removed. Free $^{82}\text{Br}^-$ was separated from ^{82}Br incorporated into large molecular weight components by chromatography either over a column (1 $\times$ 20 cm) of Sephadex G-25 or a minispin column of Sephadex G-50 (Cooper Biomedical) or by trichloroacetic acid precipitation (15% w/v) (22). Identical results (values within 10%) were obtained with the three techniques. Cells (10^6 per milliliter) were incubated in Dulbecco's buffer supplemented with the indicated concentrations of $^{42}\text{K}^{82}\text{Br}$ (6.25 to 100 μM) and human serum albumin (2 mg/ml) in the absence or presence of PMA (50 ng/ml). (○) Resting neutrophils; (●) PMA-triggered neutrophils; (□) resting eosinophils; and (■) PMA-triggered eosinophils. Bromination is expressed as mean nanomoles of ^{82}Br incorporated $\pm$ SEM for ten experiments with eosinophils and six experiments with neutrophils (36).



none of these compounds significantly interfered with bromination (Table 2).

We showed that human eosinophils preferentially oxidized bromide to a halogenating intermediate in the presence of at least a 1000-fold excess of chloride. Because of recent evidence that haloperoxidases mediate halogenation reactions by generating free hypohalous acids (26), we conclude that HOBr is the brominating oxidant generated by the eosinophil in our system. Human eosinophils contain large amounts of eosinophil peroxidase (27), and it is known that the triggered cell coats adjacent targets with this highly cationic enzyme (1, 2). In concert with the eosinophil's demonstrated abil-

ity to release copious amounts of H_2O_2 and to utilize physiologic concentrations of bromide, the cell can generate an unusual, powerful, and toxic oxidant. Like HOCl, HOBr can react with thiols, thioethers, aromatics, amines, amino acids, and bioactive molecules (28), but HOBr also has unique characteristics that should provide new insights into eosinophil function. HOBr reacts faster than HOCl with many substrates and, in a functional sense, HOBr is the more powerful bleaching agent (28). The abilities of eosinophils to use HOBr to mediate toxicity (3, 5, 7), to generate highly reactive N-bromoamines (29) or singlet oxygen (30), and to form microbicidal brominated prod-

ucts (29) all deserve careful attention. Our findings indicate that the generation of brominated compounds, previously thought to be restricted to the realm of marine algae and prokaryotes (31), should now be considered a property of a distinct mammalian cell population. The identification of bromide as the halide preferentially used by eosinophil peroxidase in an intact cell system not only differentiates this peroxidase from myeloperoxidase on a functional level but also identifies a physiologic role for this halide in vivo. The results should provide important insights into the functions of the eosinophil and bromide in host defense and inflammation.

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- Although the product of HOCl and taurine, N-chlorotaurine, is stable in the presence of neutrophils or eosinophils, N-bromotaurine is rapidly reduced in the presence of intact cells (unpublished observation).
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- Eosinophils may utilize Cl^- more efficiently in the acidic environment (3) that exists within the confines of the phagocytic vacuole. Nonetheless, washed cell pellets recovered from 10^6 eosinophils triggered with opsonized zymosan particles in the presence of albumin (2 mg/ml) contained 3.9 ± 0.6

Table 2. Characteristics of bromination by eosinophils and neutrophils. Cell preparations and incubation conditions were as described in Table 1, except that human serum albumin (2 mg/ml) and $^{42}\text{K}^{82}\text{Br}$ (50 μM) were added as indicated. Cells were also triggered in the presence of catalase (25 $\mu\text{g/ml}$) or heat-inactivated catalase, 1 mM azide, 5 mM L-methionine, 0.5 μM I^- or a combination of $1.5 \times 10^{-7}\text{M}$ thyroxine (T4) and $1.5 \times 10^{-9}\text{M}$ 3,5,3'-triiodothyronine (T3). Radioactivity remaining associated with the cell pellets after three washes was determined by trichloroacetic acid precipitation and was completely inhibited if cells were triggered in the presence of either azide or methionine. Results are expressed as the mean nanomoles of ^{82}Br incorporated $\pm$ SEM of the number of experiments indicated in parentheses.

Preparation	Bromination (nmol of ^{82}Br incorporated per 10^6 cells)	
	Eosinophils	Neutrophils
Supernatants obtained from		
Resting cells	0.8 ± 0.2 (17)	0.0 ± 0.0 (5)
PMA-triggered cells	5.2 ± 0.5 (17)	0.4 ± 0.1 (5)
PMA-triggered cells without albumin	0.7 ± 0.1 (4)	N.D.
PMA-triggered cells		
+ catalase	0.8 ± 0.1 (5)	0.0 ± 0.0 (5)
+ heat-inactivated catalase	4.7 ± 0.6 (5)	0.4 ± 0.1 (5)
+ azide	0.0 ± 0.0 (8)	0.0 ± 0.0 (5)
+ methionine	0.3 ± 0.2 (6)	0.0 ± 0.0 (5)
+ I^-	4.2 ± 0.9 (4)	0.4 ± 0.1 (5)
+ T3 and T4	6.2 ± 1.0 (5)	N.D.
Cell pellet obtained from		
Resting cells	0.4 ± 0.1 (5)	0.0 ± 0.0 (3)
PMA-triggered cells	3.0 ± 0.2 (5)	0.2 ± 0.1 (3)

- nmol of trichloroacetic acid-precipitable, cell-associated ^{82}Br (mean $\pm$ SEM, $n = 3$).
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 36. The incorporation of ^{82}Br into albumin is an underestimate of the total amount of HOBr generated.

Bromination products of sulfhydryls, thioethers, or amines will not accumulate as stable adducts. For example, 25 nmol of HO^{82}Br [generated by incubating 25 nmol of HOCl with an excess of $^{82}\text{Br}^-$; (29)] incorporated 4.6 ± 0.6 nmol of ^{82}Br into albumin (2 mg/ml; $n = 5$) under conditions identical to those used for the eosinophil.

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Detoxification of Bacterial Lipopolysaccharides (Endotoxins) by a Human Neutrophil Enzyme

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Lipopolysaccharides in the cell walls of Gram-negative bacteria elicit toxic as well as potentially beneficial inflammatory responses in animals. It is now reported that tissue toxicity caused by lipopolysaccharides is preferentially reduced by an enzymatic activity in human neutrophils. Acyloxyacyl hydrolysis removes fatty acyl chains that are linked to the hydroxyl groups of 3-hydroxytetradecanoyl residues in the bioactive lipid A moiety of the lipopolysaccharides. Maximal acyloxyacyl hydrolysis reduced lipopolysaccharide tissue toxicity, as measured in the dermal Shwartzman reaction, by a factor of 100 or more. In contrast, the ability of the deacylated lipopolysaccharides to stimulate B lymphocytes to divide was decreased only by a factor of 12. It is suggested that during tissue invasion by Gram-negative bacteria acyloxyacyl hydrolysis may be a defense mechanism that reduces the toxicity of lipopolysaccharides while preserving some of their potentially beneficial inflammatory and immune stimuli.

ANIMALS MOUNT A COMPLEX ARRAY of inflammatory responses to tissue invasion by Gram-negative bacteria. Many of these responses are provoked by lipopolysaccharides (LPS) in the bacterial outer membrane. Much evidence suggests that the lipid A moiety of LPS stimulates various animal cells that, in turn, mediate the inflammatory changes (1). Some responses to LPS are toxic (hypotension, coagulation disturbances, death), while others may benefit the infected host (enhancement of antibody synthesis, mobilization of phagocytes, or acute-phase protein synthesis, for example). Although both humoral and cellular processes for detoxifying LPS have been proposed (2), no specific enzymatic mechanism has been described. We report here that partial deacylation of LPS by an enzymatic activity in human neutrophils greatly reduces the tissue toxicity of the molecules while preserving some of their immunostimulatory potency.

In LPS from enteric bacteria (such as *Salmonella* and *Escherichia*), lipid A is a glucosamine disaccharide that is phosphorylated at positions 1 and 4' and has six or seven covalently linked fatty acids (Fig. 1).

Four molecules of 3-hydroxytetradecanoate (3-OH-14:0) are attached to the glucosamine backbone at positions 2, 3, 2', and 3'; the hydroxyl groups of the 3-OH-14:0 residues at positions 2' and 3' (and some-

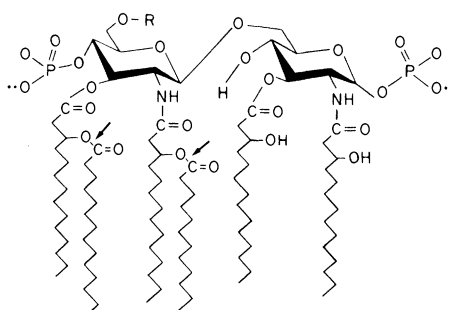


Fig. 1. Structure of *S. typhimurium* lipid A (3). R labels the site of attachment of the polysaccharide chain. We used three LPS that had different numbers of saccharides attached to lipid A. Rc (rough), SR (one O-repeat unit in the polysaccharide chain), and smooth (O-chains of different lengths) LPS had approximately 7, 15, and 11 to 151 or more saccharides, respectively. Aminoarabinose and phosphoethanolamine may be substituted to the phosphates at positions 1 and 4', respectively (18). The arrows show the sites of cleavage by acyloxyacyl hydrolases.

times 2) are substituted with nonhydroxylated fatty acids (NFA) (dodecanoate, tetradecanoate, and hexadecanoate) to form acyloxyacyl groups (3). It was previously reported that human peripheral blood neutrophils have an enzymatic activity that selectively removes the nonhydroxylated acyl chains from the lipid A moiety of *Salmonella typhimurium* LPS (acyloxyacyl hydrolysis) (4). Partial purification of large amounts of enzyme has now enabled us to determine the effects of acyloxyacyl hydrolysis on some of the bioactivities of LPS.

Acyloxyacyl hydrolase activity was partially purified (5) (approximately 1500-fold increase in specific activity) from HL-60 human promyelocytes (6). Biosynthetically radiolabeled LPS were prepared from *S. typhimurium* cells grown with [^3H]acetate and *N*-acetyl-1- ^{14}C]glucosamine (incorporated into fatty acids and the glucosamine backbone, respectively, of lipid A) as described (4).

The enzyme (or enzymes) released ^3H -labeled fatty acids, but not ^{14}C -labeled glucosamine, from the double-labeled LPS (Fig. 2). The reaction reached an apparent maximum when approximately 32% of the ^3H radioactivity was cleaved from the LPS; since 32% of the ^3H radioactivity in this preparation was in NFA, 32% deacylation was consistent with nearly complete removal of these residues. This conclusion was supported by analysis of the fatty acid composition of the substrate LPS and the reaction products: 68% of the radioactivity from the ^3H -labeled fatty acids in the LPS was in 3-hydroxytetradecanoate and 32% was in NFA, whereas the ^3H radioactivity that was enzymatically released from LPS was almost entirely (94%) in NFA, and 95% of the ^3H radioactivity in the partially deacylated LPS

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