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S K Adeniyi-Jones, M L Karnovsky

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Research Article

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Oxidative Decarboxylation of Free and Peptide-linked Amino Acids in Phagocytizing Guinea Pig Granulocytes

S. K. ADENIYI-JONES and MANFRED L. KARNOVSKY, *Departments of Biological Chemistry and Pediatrics, Harvard Medical School; and Children's Hospital Medical Center, Boston, Massachusetts 02115*

ABSTRACT The oxidative decarboxylation of amino acids by a system consisting of myeloperoxidase-hydrogen peroxide-chloride has been demonstrated previously by others and the process has been considered to be part of the microbicidal armamentarium of some phagocytic leukocytes. We were able to translate these earlier observations, made on model systems, to intact guinea pig granulocytes. We could demonstrate differences in the cellular handling of peptide-linked amino acids as particles, compared with free amino acids. Specific inhibitors were used to explore two routes of oxidative decarboxylation: (*a*) the myeloperoxidase-catalyzed direct decarboxylation-deamination reaction, and (*b*) oxidation of α-keto acids after transamination of amino acids. These inhibitors were cyanide, azide, and tapazole for the former pathway, and amino-oxyacetate for the latter. Amino-oxyacetate profoundly inhibited the decarboxylation of free ^{14}C-amino acids (alanine and aspartate) in both resting and stimulated cells, but had only a minimal effect on $^{14}CO_2$ production from ingested insoluble ^{14}C-protein. On the other hand, the peroxidase inhibitors cyanide, azide, and tapazole dramatically inhibited the production of $^{14}CO_2$ from ingested particulate ^{14}C-protein, but had only small effects on the decarboxylation of free amino acid. Soluble, uniformly labeled ^{14}C-protein was not significantly converted to $^{14}CO_2$ even in the presence of phagocytizable polystyrene beads. These observations suggest that the amino acids taken up by phagocytosis (e.g., as denatured protein particles) are oxidatively decarboxylated and deaminated in the phagosomes by the myeloperoxidase-hydrogen peroxide-chloride system; soluble free amino acids that enter the cytoplasm by diffusion or transport are oxidatively decarboxylated after transamination by the normal cellular amino acid oxidative pathway.

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INTRODUCTION

It is now well accepted that the combination of myeloperoxidase (MPO),[1] H_2O_2 and a halide constitutes a potent antimicrobial and antitumor system, but the precise mechanism of action remains obscure (1–4). Starting in 1968, interest in one possible mechanism was aroused through the demonstration by Zgliczynski et al. (5) that MPO, prepared from leukocytes isolated from a patient with chronic granulocytic leukemia, could deaminate and decarboxylate amino acids in the presence of H_2O_2 and Cl^-. The products were aldehydes with one carbon less than the original amino acids. This was later confirmed by Strauss et al. (6) and Jacobs et al. (7) in a system in vitro that employed neutrophil granules as the source of MPO. These workers showed a relation between bactericidal activity and decarboxylation of L-alanine, and suggested that the aldehyde formed may itself be the bactericidal agent. Aldehydes are known to have such activity (8). It was demonstrated in these studies that the reaction was rather specific for MPO, since horseradish peroxidase failed to manifest appreciable activity under similar conditions. The same authors (9) demonstrated, with intact guinea pig leukocytes, a twofold increase in the decarboxylation of L-alanine during phagocytosis of latex beads. Subsequently, using leukocyte granules they demonstrated decarboxylation of [1,7^{14}C]diaminopimelic acid of bacteria that had incorporated this substance (10). Zgliczynski et al. (11) with a similar granule system showed the formation of an intermediate product, a chloramine, from amino acids. These unstable products decomposed spontaneously to yield NH_3, CO_2, and the corresponding aldehydes.

Although the data obtained are suggestive and make this system an attractive one in the context of granulo-

[1] *Abbreviations used in this paper*: AOA, amino-oxyacetate; BSA, bovine serum albumen; KRP, Krebs-Ringer phosphate; MPO, myeloperoxidase; PLP, pyridoxal phosphate; PMA, phorbol-12-myristate 13-acetate; PMN, polymorphonuclear neutrophilic leukocytes.

cyte function, there are some uncertainties regarding its relevance to intact granulocytes. All the studies cited above, with one exception, were done with soluble enzyme systems or isolated leukocyte granules. MPO-catalyzed decarboxylation-deamination of regular, protein (peptide-linked) amino acids has not been demonstrated with intact cells. The one study that did employ whole cells (9) did not take account of the possibility that the decarboxylation of the free alanine used as substrate occurred via the normal amino acid oxidative pathway after transamination. The pathways relevant to the observations above, and the findings of this paper are summarized in Fig. 1.

The uncertainties in the chain of evidence regarding the proposed MPO-catalyzed protein cleavage and deamination-decarboxylation reactions as potentially important in phagocytizing granulocytes may be summarized as follows:

(*a*) Lack of information as to whether soluble free amino acids and protein-bound amino acids (particularly of insoluble proteins accumulated by phagocytosis) are subject to the same reactions. This question concerns differences that might exist between reactions in the cytoplasm (free amino acids brought into the cell by diffusion or transport) or in the phagosomes (amino acids of insoluble protein accumulated by phagocytosis). The conditions (e.g., pH) and available enzymes are quite different in these two locations;

(*b*) Paucity of evidence concerning the extent of the relative contributions of the transaminase-α-keto acid dehydrogenase pathway and the MPO-H_2O_2-halide-mediated pathway.

(*c*) Lack of information as to whether the actual cleavage of ingested protein proceeds by the MPO-catalyzed reaction, or how much is due to released granule-bound proteases after fusion of the lysosomelike granules with the phagosome (12).

(*d*) Questions as to whether aldehydes derived from the relevant amino acids are importantly antibacterial (13) at the concentrations that might result from the MPO-catalyzed reaction, and what their fate might be.

In the present study we have explored the release of $^{14}CO_2$ from protein-bound amino acids, compared with that from free amino acids, using intact leukocytes. We have tried to determine the degree to which this occurs via transamination followed by decarboxylation, or via attack by the MPO-H_2O_2-halide system in each case, i.e., to cover points *a* and *b* above. Points *c* and *d* are mentioned only to indicate the scope of the problem. Inhibitors of transaminase (amino-oxyacetate [AOA]) or of peroxidase (cyanide, azide, methimazole [tapazole]) were employed to dissect the problem in whole cells.

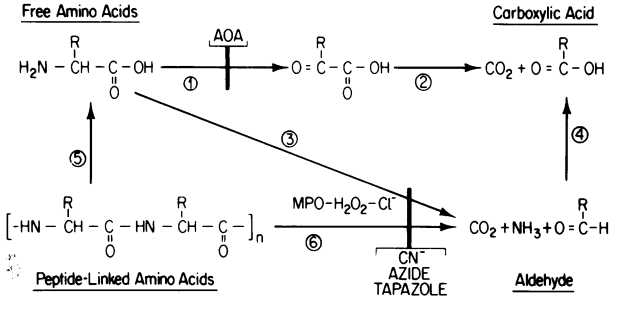

FIGURE 1 Two pathways for oxidative decarboxylation of amino acids. Free amino acids may be transaminated by reaction 1 involving PLP, and the resulting α-keto acid may be oxidatively decarboxylated by reaction 2, to yield a carboxylic acid one carbon shorter than the original amino acid. Alternatively, amino acids may be deaminated and decarboxylated by a peroxide- and peroxidase-dependent reaction to yield an aldehyde one carbon shorter than the original amino acid, ammonia, and CO_2 (reaction 3). The aldehyde may be enzymatically oxidized to a carboxylic acid (reaction 4; see Discussion). Peptide-linked amino acids may be cleaved by proteases to free amino acids (reaction 5) which are subject to decarboxylation reactions 1–3 above. Direct cleavage of peptides and proteins with simultaneous deamination and decarboxylation may also occur by the peroxidase pathway (reaction 6; see text). The steps blocked by the inhibitors used in this study are indicated by heavy bars.

METHODS

Materials. All reagents used were of analytical grade. Uniformly labeled ^{14}C-denatured algal (Chlorella) protein (58 mCi/m atom C) was obtained from the Radiochemical Centre Ltd., Amersham, England. ^{14}C-protein from *Escherichia coli* (0.053 mCi/mg) was obtained from New England Nuclear, Boston, Mass. The former organism was grown in an atmosphere of $^{14}CO_2$, the latter on a medium with [^{14}C]acetate. Both protein preparations were delipidated and freed of nucleic acids, carbohydrate, and small molecules. The purity of the latter protein was stipulated as >98.8%. [^3H]bovine serum albumin (BSA) (0.5 mCi/mg) was prepared by New England Nuclear by the Wilzbach technique. It was donated by Dr. John Udall, of the Shriners' Burns Institute, Boston, to whom we express our appreciation. The following were obtained from New England Nuclear: L-[U-^{14}C]alanine (0.05 mCi/0.025 mg); L-[U-^{14}C]aspartic acid (0.05 mCi/0.030 mg); L-[U-^{14}C]leucine (0.25 mCi/0.11 mg) and D-[1-^{14}C]glucose (0.05 mCi/1.3 mg). L-[1-^{14}C]alanine (0.63 mCi/mg) was obtained from ICN (ICN Chemical and Radioisotope Division, Irvine, Calif.). L-Alanine, L-aspartic acid, L-leucine, D-glucose, K-pyruvate, AOA, phorbol-12-myristate-13-acetate (PMA), 2,4-dinitro-phenylhydrazine, and BSA (essentially fatty acid free) were all obtained from Sigma Chemical Company, St. Louis, Mo. Bactolatex (0.81 μm) was from Difco Laboratories, Detroit, Mich.; Na azide was from Fisher Scientific Co., Pittsburgh, Pa.; Na cyanide from Mallinckrodt Inc., St. Louis, Mo.; and methimazole (tapazole) from Eli Lilly & Co., Indianapolis, Ind.

Guinea pig neutrophils were prepared from animals of either sex (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The animals weighed between 500–1,000 g. They were fed chow pellets and water ad lib. Each animal was injected intraperitoneally with 20–30 ml of a sterile solution of 12% (wt/vol) sodium caseinate in normal saline, and the cells were harvested 14–18 h later by the method reported previously from this laboratory (14). Contaminating erythrocytes were lysed by resuspending the pellets of cells, ob-

tained after centrifugation at 800 *g*, in cold water for 30 s, with mixing on a vortex agitator. Isotonicity was restored with an appropriate volume of 9% saline. The cells were then centrifuged once more and resuspended in Krebs-Ringer phosphate buffer (KRP). Cells were quantified by counting in a hemocytometer and resuspended in KRP with 5 mM glucose at pH 7.4.

Coagulated (denatured) protein particles for phagocytosis. Tritiated BSA was utilized for measurement of phagocytosis per se. For measurements of $^{14}CO_2$ production from ingested protein we used a mixture of ^{14}C-algal or bacterial protein and nonradioactive BSA since ^{14}C-labeled albumin was not available.

Crystallized [^{3}H]BSA was concentrated 10-fold, diluted, and reconcentrated 10-fold in a Minicon concentrator, B-15 (Amicon Corp., Lexington, Mass.). This process had previously been shown to yield a product that on a Biogel (Bio-rad Laboratories, Richmond, Calif.) P-60 column was found to be 90% pure intact BSA. It also removed residual exchangeable ^{3}H. Both the [^{3}H]BSA and a mixture of cold BSA and ^{14}C-algal or bacterial protein were heat denatured by a method previously described (15, 16). Briefly, the protein solution in physiological saline at pH 7.0 (1%; 30 ml) was heated to 75–80°C for 20 min. The mixture was cooled and precipitated with 0.1 N HCl. The precipitate was washed twice with isotonic saline adjusted to pH 5.4 with HCl. The denatured protein was resuspended in normal saline at pH 7.4. The resulting protein suspensions were designed to contain about 1 mg protein, 100,000 cpm (^{3}H) in 0.2 ml, or 1.5 mg protein, 300,000 cpm (for the algal-protein-BSA mixture), or 180,000 cpm (for the *E. coli* protein-BSA mixture) in 0.5 ml. Each mixture was made up fresh before each experiment. For control experiments with soluble ^{14}C-algal protein-BSA mixtures, the solution was dialyzed against three changes of saline over 48 h in the cold, in lieu of the precipitations performed in the preparation of the particulate protein (see above).

Phagocytosis of labeled, coagulated (denatured) protein. These experiments were done with guinea pig granulocytes in monolayers. The monolayers were prepared as previously described (17). Coagulated protein suspension (0.2 ml [^{3}H]-BSA) was added to the monolayers, to which 1 ml of KRP with 5 mM glucose had previously been added. After incubation and vigorous washing in five beakers of medium (previously shown to remove excess particles) (17), 1 ml of 0.5% NaOH was added, and the cells were allowed to digest overnight. Aliquots of the digest were counted in Aquasol Universal liquid-scintillation-counter solvent in an Ansitron II Liquid Scintillation Counter (Picker Corp., Nuclear Dept., Northford, Conn.). In experiments with inhibitors, the inhibitor was added to the medium 10 min before the coagulated protein was added. It has previously been demonstrated (17) that inhibitors of glycolysis block association of particles with the cells, an indication that phagocytosis per se was measured.

Production of $^{14}CO_2$ from ingested coagulated ^{14}C-protein. The experiments were carried out in special stoppered 24-ml Erlenmeyer flasks with center wells. A 2-ml suspension of cells containing 5×10^7 cells/ml in KRP with 5 mM glucose at pH 7.4 was used; 0.5 ml of the ^{14}C-labeled coagulated protein mixture was added to the outer compartment. The center well, where $^{14}CO_2$ was trapped, contained a small piece of filter paper soaked with 0.2 ml of 20% NaOH. Incubations were carried out over 2 h in a shaking water bath at 37°C. When inhibitors were used, they were placed in the outer compartment 10 min before the addition of the protein mixture. At the end of the incubation the flasks were chilled and 0.2 ml of 1 N H_2SO_4 was added to the outer compartment. The flasks were left to stand stoppered for 1 h, after which the filter paper was removed, the well washed, and counts determined on the paper and washings.

Cellular production of $^{14}CO_2$ from ^{14}C-amino acids or [^{14}C]glucose. In the experiments to measure $^{14}CO_2$ from ^{14}C-amino acids and [^{14}C]glucose, flasks similar to those described above were used. Cells were made up to 2×10^7/ml with KRP containing 5 mM glucose. Latex particles, when used as a stimulating agent, were prepared by dialyzing the commercial preparation twice against 0.9% NaCl, centrifuging, and resuspending the particles in one-half the original volume. A suspension of PMA was used in some cases to stimulate the cells. The original suspension containing 1 mg/ml was diluted 20-fold; 20 μl of this was used per milliliter (final) in each experiment. The final suspensions were made up to 3 ml by adding KRP with glucose. The labeled amino acids were added before the stimulating agent to give a concentration of 1.3 mM, unless otherwise specified, and to yield 1×10^6 cpm/flask.

Isolation of aldehydes and pyruvate. We wished to determine whether the oxidation of free amino acids proceeded via relevant aldehydes with one less carbon (MPO-mediated pathway; Fig. 1). In the case of alanine we also examined the possibility that pyruvate was involved (pyridoxal phosphate [PLP] mediated transamination, Fig. 1). For this purpose we used the fluid from the outer compartment of the flasks after centrifuging the cells and debris. Carrier aldehyde (~2 mg) was added and the dinitrophenylhydrazone derivative of the aldehyde was precipitated by the addition of 0.4% 2,4 dinitrophenylhydrazine in 2 N HCl. The recrystallized phenylhydrazone was counted. In experiments concerning the possible mediation of pyruvate in alanine oxidation, 5 mM pyruvate was added to the outer compartment before stimulation to trap any pyruvate formed. The dinitrophenylhydrazone was prepared as for the aldehydes and purified by thin-layer chromatography on silica gel plates in chloroform:acetic acid (100:7). The R_f was 0.3.

Extraction of [^{14}C]isovaleric acid and [^{14}C]acetic acid from the incubation mixture. We checked for the formation of oxidation products of deaminated-decarboxylated intermediates, e.g., isovaleric acid from free leucine and acetate from free alanine. For isovaleric acid, 2 mg of carrier isovaleric acid was added to the supernatant fluid from the outer compartment after centrifuging the cells. The acid was extracted on ice with two batches of diethyl ether. The ether was evaporated under N_2 at 30°C to a volume of 0.5 ml. To trap and isolate acetic acid, the original incubation mixture contained 2 mM potassium acetate; before extraction a further 1 mg of potassium acetate was added. The acidified solution was extracted with ether. The resulting ether extract was evaporated as above and the aqueous residue adjusted to pH 8. The ether extracts were applied onto a silica gel plate for thin-layer chromatography. Plates were developed with EtOH: NH_4OH:H_2O (8:1:1) solvent. After drying, the plates were placed in an iodine chamber to locate the product. The material in the appropriate spots was scraped off the plate into Aquasol Universal liquid-scintillation-counter cocktail and counted. Two controls each were simultaneously run with authentic acetate or isovalerate. In one control the acid was subjected to the extraction in ether as described above before being plated; in a second control the acid, dissolved in ether, was plated directly. The R_f value for acetic acid was 0.78, and for isovaleric acid it was 0.58.

O_2 consumption. O_2 consumption was measured polarographically on cell suspensions using a YS1 model 53 oxygen monitor with a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The bath unit was connected to a constant temperature circulator at 37°C.

Protein was determined by the method of Lowry et al. (18), with BSA as standard.

RESULTS

Uptake of denatured [^{3}H]BSA by polymorphonuclear neutrophilic leukocytes (PMN) in monolayers. To establish the pattern of uptake of coagulated albumin by granulocytes, these cells were exposed to tritiated protein particles. Fig. 2 shows that the uptake of tritiated heat-denatured BSA was linear for 30 min. This confirms observations by others (15) that this particle is suitable for studies of phagocytosis.

Effect of coagulated protein on O_2 consumption. Phagocytosis of these protein particles was accompanied by increased O_2 consumption. The addition of denatured BSA to a suspension of granulocytes caused a rise of 49±3% (n = 4) in the O_2 consumption, a reasonable figure for guinea pig granulocytes (4).

$^{14}CO_2$ from ^{14}C-labeled protein. Granulocytes in suspension were exposed to a mixture of BSA and ^{14}C-algal protein that had been coagulated together as indicated in Methods. Fig. 3 shows $^{14}CO_2$ production from this particulate mixture as a function of time. The result indicates that the maximum rate pertained in the first hour and diminished thereafter. 2 h was chosen as the standard period of reaction. The value obtained in seven experiments at 2 h (triplicate observations) was 63.4±4.0 cpm/10^7 cells per 10^6 cpm added.

A further stimulus in the form of latex particles applied to the suspension of cells and insoluble protein caused no further rise in $^{14}CO_2$ production (e.g., in a representative experiment done in triplicate 104±9 cpm/10^7 cells per 10^6 cpm [without latex], and 97±11 cpm/10^7 cells per 10^6 cpm [with latex]).

When we used uncoagulated (soluble) protein (^{14}C-algal protein-BSA mixture) with or without latex particles, $^{14}CO_2$ production by granulocytes was not increased above the zero-time or "no-cell" blanks (two experiments, each in triplicate).

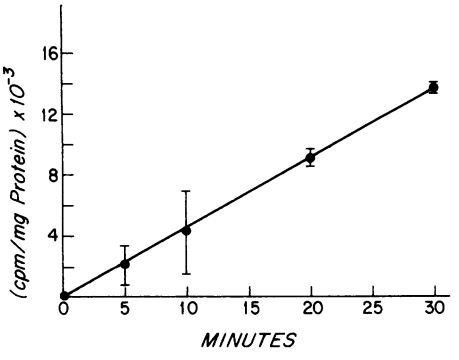

FIGURE 2 Time-course of uptake by granulocyte monolayers of insoluble, denatured [^{3}H]BSA. For conditions see Methods; 1 mg cellular protein is ~6.2 × 10^6 cells.

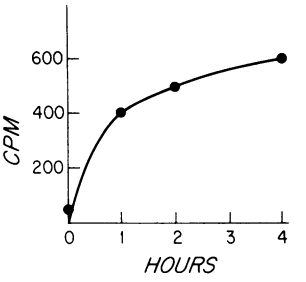

FIGURE 3 Time-course of appearance of $^{14}CO_2$ from a mixture of ^{14}C-algal-protein and BSA in particulate form. The cells were in suspension, and details are as given in Methods.

Effect of inhibitors of transaminase or of peroxidase on $^{14}CO_2$ production from ingested protein. To ascertain whether $^{14}CO_2$ production was due to attack by MPO on the protein (or amino acids released from protein particles) or was due to transamination and decarboxylation of released amino acids, we looked at the effect of a known, potent inhibitor of PLP-mediated amino acid transamination, AOA (19–21). As a preliminary step, the effect of AOA (1 mM) on actual uptake was checked. AOA had no effect on uptake of [^{3}H]BSA into guinea pig granulocytes in a monolayer. After 20 min the mean uptake without AOA was 4,066±561 cpm/mg protein, and with AOA it was 3,928 ±511 cpm/mg protein (six observations). The inhibitors of peroxidase have been shown previously by numerous investigators not to inhibit phagocytosis by these cells (e.g., 17, 22, 23).

We also examined the effect of AOA (1 mM) on both the basal and stimulated O_2 consumption and $^{14}CO_2$ production from D-[1-^{14}C]glucose as base-line observations. AOA had no effect on the O_2 consumption of resting cells, but did cause a 32±3% decrease in the O_2 consumption stimulated by latex or PMA, which suggests that only the respiratory burst (increase in O_2 uptake) was affected, but not the resting level. The production of $^{14}CO_2$ from D-[1-^{14}C]glucose by resting or stimulated cells was not depressed by AOA.

The effect of AOA (1 mM) on $^{14}CO_2$ production from ingested protein is shown in Fig. 4. AOA (1 mM) caused a 10±1% decrease in $^{14}CO_2$ production from the labeled protein mixture, which suggests that amino acid oxidation via transamination was not the major source of $^{14}CO_2$, since this concentration of AOA is known to be an effective blocker of transaminase (19), and was so under our conditions (see below).

The effect of inhibitors of peroxidase (azide, CN^-, and methimazole [tapazole]) on $^{14}CO_2$ production from ingested particulate protein was also examined, and is also shown in Fig. 4. Azide (2 mM) is considered the most potent of the three inhibitors (22, 23) and caused the most profound decrease in $^{14}CO_2$ production from labeled protein: 97±2% inhibition. 3 mM tapazole

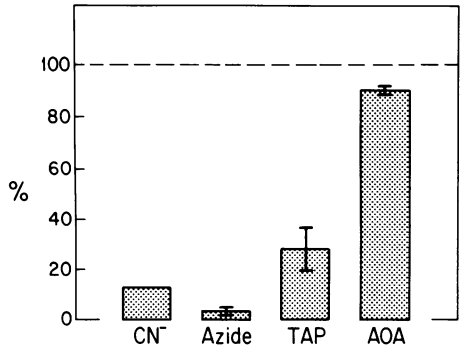

FIGURE 4 Effect of various metabolic inhibitors on $^{14}CO_2$ production from a ^{14}C-labeled particulate protein mixture. The concentrations of the inhibitors were: AOA, 1 mM; azide, 2 mM; CN^- and tapazole, 3 mM. The values for the controls were set at 100, and the experimental results expressed as a percentage. The bars indicate means±SEM. $n = 3$ experiments for AOA, 4 for azide, 1 for CN^-, and 3 for tapazole. All experiments were performed in triplicate.

caused a 72±10% inhibition and 3 mM CN^- an 86% inhibition. Three different concentrations of azide (0.5, 1, and 2 mM) were studied. At 0.5 mM there was a 93% inhibition, at 1 mM 100% and at 2 mM a 99% inhibition. The blockade is thus essentially complete at all three concentrations.

To establish that these effects were not associated with one particular labeled protein the experiments were repeated with *E. coli* protein-BSA coagulates, and two inhibitors—AOA (1 mM) and azide (2 mM). The data are given in Table I. It is clear that although the inhibitor of transaminase was without effect, the inhibitor of peroxidase depressed $^{14}CO_2$ production by 80%.

The profound inhibition caused by azide at low concentrations and by the two other known inhibitors of MPO suggests that $^{14}CO_2$ production from particulate ingested protein was mainly via the MPO-H_2O_2-halide system.

$^{14}CO_2$ production from ^{14}C-labeled free amino acids in resting and stimulated PMN, and the effect of inhibitors. There was no increase in $^{14}CO_2$ production from L-[U-^{14}C]leucine upon latex stimulation of the cells, in agreement with the study by Iyer et al. (24). There was, however, an increase with [^{14}C]alanine and [^{14}C]aspartate as substrates and with latex or PMA as stimulants. With latex there was a twofold increase in alanine oxidation, and a fivefold increase with PMA, as expected from earlier work (9, 24). Release of $^{14}CO_2$ from aspartic acid showed a twofold increase in response to PMA.

TABLE I
Production of $^{14}CO_2$ from Labeled Particulate E. coli Protein-Albumin

Conditions	$^{14}CO_2$	Inhibition
	cpm±SEM	%
−Cells ($n = 3$)	14±5	—
+Cells ($n = 6$)	190±7	—
+Cells + AOA ($n = 3$)	184±9	3
+Cells + Azide ($n = 3$)	51±3	80

Effect of AOA (1 mM) on $^{14}CO_2$ production. $^{14}CO_2$ production from both [^{14}C]alanine and [^{14}C]aspartic acid were examined. Fig. 5 illustrates the formation of $^{14}CO_2$ from L-[1-^{14}C]alanine, at two time intervals in the absence or presence of latex particles and AOA. The inhibitor effectively blocked the reaction. Because comparisons with U-^{14}C-protein were made, these experiments were repeated with [U-^{14}C]alanine and [U-^{14}C]aspartate.

Fig. 6 shows that, in experiments where PMA was the stimulant, AOA caused a 69% decrease in the basal $^{14}CO_2$ production from alanine. AOA caused a 91% reduction in $^{14}CO_2$ production when the cells were stimulated. With [U-^{14}C]aspartate as substrate and PMA as stimulant there was a 60±2% decrease in basal $^{14}CO_2$ and an 82±4% inhibition of the stimulated value when AOA was present at 1 mM. When latex was the stimulating agent, AOA caused an 84% decrease in the basal $^{14}CO_2$ production from [U-^{14}C]alanine and a 92% decrease in the stimulated value.

Effect of MPO inhibitors on $^{14}CO_2$ production from [U-^{14}C]alanine. Table II indicates that CN^- (3 mM) caused a decrease in the basal value (not significant statistically), but had no effect on the stimulated value. Azide (2 mM) exerted no effect on the basal $^{14}CO_2$ production from [^{14}C]alanine, but caused a 30% decrease in the stimulated value ($P < 0.01$). Tapazole (3 mM) had no real effect on either the resting or the stimulated values. Where effects were observed, these were clearly less dramatic than in the case of AOA (Figs. 5

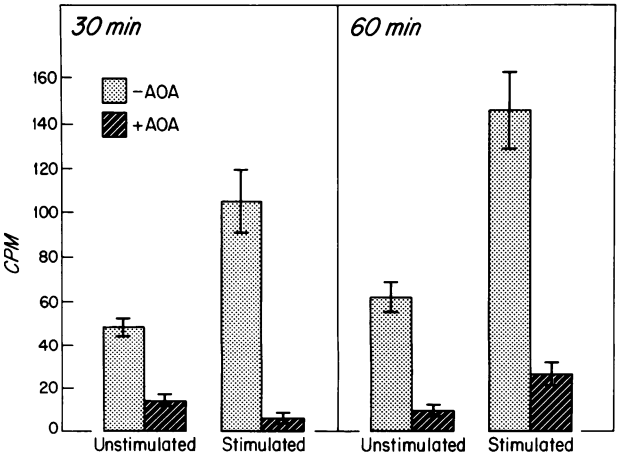

FIGURE 5 Effect of amino-oxyacetate on $^{14}CO_2$ production from L-[1-^{14}C]alanine (1 mM) by resting and phagocytizing PMN at two time intervals. For details see Methods.

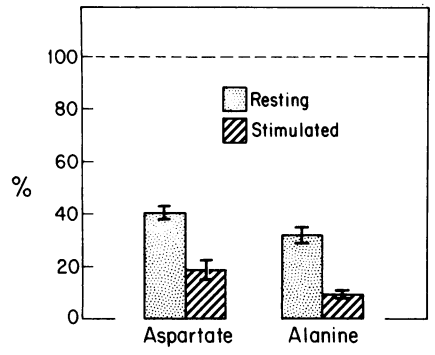

FIGURE 6 Effect of AOA on $^{14}CO_2$ production by PMN from labeled amino acids. Control cells are set at 100 (resting or stimulated, without inhibitor). Data for inhibitor-treated cells are expressed as percentages ± SEM. Concentrations of inhibitors are as in Fig. 4. $n = 6$ for aspartate, 3 for alanine unstimulated, and 2 for stimulated. The stimulating agent was PMA. Means and standard errors are given for aspartate, as elsewhere. For alanine, means ± average deviation are given.

and 6). The findings overall suggest that the bulk of the $^{14}CO_2$ production from free ^{14}C-amino acids by the stimulated cells occurs via the normal pyridoxal phosphate-dependent transamination pathway rather than, as in the case of the labeled proteins, via the MPO-H_2O_2-halide pathway.

The above experiments were performed with amino-acids at ~1 mM. When they were carried out with [^{14}C]alanine at 10 mM the picture was not significantly changed.

Additional observations on the oxidation of amino acids, and the effects of inhibitors. In the above studies $^{14}CO_2$ was collected and measured as a means of quantifying the oxidative metabolism of ^{14}C-labeled amino acids. We sought some information on the nature of the intermediates in the reaction. We looked for ^{14}C-label in stimulated cells either as isovaleraldehyde when [^{14}C]leucine was the substrate, or as acetaldehyde when [^{14}C]alanine was the substrate; i.e., the suspected products of MPO-catalyzed deamination-decarboxylation. No activity was found in these aldehydes. We were, however, able to detect radioactivity in the corresponding carboxylic acids, i.e., isovaleric acid (leucine) and acetic acid (alanine), when these were extracted. There was no increase in the appearance of [^{14}C]isovaleric acid with latex stimulation (as expected from the data of Iyer et al. [24]) but there was a twofold increase in [^{14}C]acetic acid with latex. These carboxylic acids would, however, be intermediates in either the MPO or the PLP-mediated pathways (Fig. 1). (Note the role of aldehyde oxidase [25] below.)

In experiments with labeled alanine we showed by the addition of cold pyruvate to the cell suspension to trap [^{14}C]pyruvate formed from [^{14}C]alanine and by subsequent isolation of the dinitrophenylhydrazone that an intermediate in the PLP-mediated pathway, labeled pyruvate, was indeed formed, and that there was a threefold increase in the radioactivity in this substance when the cells were stimulated with latex. AOA caused a diminution in the amount of [^{14}C]acetic acid recovered by extraction and thin-layer chromatography of both the resting and stimulated cells (55 and 77%, respectively).

These semiquantitative results are in agreement with the $^{14}CO_2$ data, and suggest that the oxidation of leucine was via isovaleric acid. But because no data were obtained with respect to the labeling of α-ketoisocaproic acid (the transamination product of leucine), the relevant pathway remains undecided. Alanine was clearly oxidized via pyruvate and acetic acid, i.e., via the known PLP-mediated pathway. This approach of examining possible intermediates was not applied in the experiments with labeled protein because of the inadequate specific activity of the protein, the multiplicity of products that could arise from all the labeled amino acids involved, and thus the low amount of radioactivity of each product.

TABLE II
Effect of Inhibitors of MPO on Production of $^{14}CO_2$ from L-[*U*-^{14}C]*alanine by Resting and Phagocytizing Granulocytes**

Cells	Ratio‡	
	Resting	Phagocytizing
Cyanide, 3 mM	0.60±0.19 (6)	1.06±0.26 (6)
Azide, 2 mM	1.09±0.18 (12)	0.70±0.08§ (14)
Tapazole, 3 mM	1.22±0.23 (8)	0.97±0.04 (8)

* The stimulus was polystyrene latex. (See text for details.)
‡ The ratio is that for $^{14}CO_2$ production in the presence of inhibitor/$^{14}CO_2$ in control (absence of inhibitor). Concentrations of inhibitors are as in Fig. 4.
§ P value for difference from unity < 0.01. No other values were significantly different from unity at P values of 0.1. n is given in parentheses and represents the number of paired observations made in each case.

DISCUSSION

We have demonstrated the ability of intact granulocytes to produce $^{14}CO_2$ from particulate ^{14}C-denatured protein, and have confirmed previous results that the cells can decarboxylate exogenously supplied free amino acids (9). Our data suggest, however, that free amino acids and those of particulate proteins are decarboxylated by different mechanisms in different locales in the intact granulocyte, as we indicated in the Introduction.

In the study with ^{14}C-denatured (insoluble) protein we showed that the protein is taken up and $^{14}CO_2$ released, and that this process was accompanied by an increase in O_2 consumption consistent with other observations on phagocytosis. The formation of $^{14}CO_2$ was almost totally abolished by azide, and inhibited profoundly by the other known inhibitors of MPO (CN^- and tapazole). The fact that all three known inhibitors of MPO show massive effects suggests that the process was MPO-mediated, and did not proceed significantly by any other mechanisms. Nevertheless, to test the possibility that amino acids liberated after hydrolytic cleavage of the ingested protein might be oxidized by more usual oxidative mechanisms, we looked at the effect of a potent transaminase inhibitor, AOA. AOA caused only a minimal (10%) decrease in the release of $^{14}CO_2$ from the amino acids of ingested particulate protein, which supports the idea that the decarboxylation of amino acids of particulate protein did not proceed significantly via transamination of liberated amino acids. Under our conditions, soluble ^{14}C-protein exposed to the cells was not significantly converted to $^{14}CO_2$ whether the cells were resting or stimulated. Others have noted that soluble protein, in contradistinction to particulate protein is not internalized to any extent by macrophages (26).

On the other hand, the oxidation of free soluble amino acids does appear to be via transamination, since AOA blocked most of the $^{14}CO_2$ production in resting cells or cells stimulated with particles or PMA when they were exposed to [^{14}C]alanine. With free labeled amino acid as substrate, only azide of the three inhibitors of MPO caused a real inhibition of the stimulated $^{14}CO_2$ production—and then only to the extent of 30%. The basis of this effect of azide, and indeed the nonsignificant but suggestive effect of cyanide on resting cells, remains to be explored further.

These observations with inhibitors of peroxidase suggest that the major pathway of decarboxylation of exogenous free amino acids is via transamination, in contrast to the situation with ingested particulate protein. Further evidence concerning the pathway followed by free amino acid was provided by the observation that [^{14}C]pyruvate and [^{14}C]acetic acid were produced during oxidation of [^{14}C]alanine, and that this was profoundly inhibited by AOA.

A rough calculation indicates that in a 1-h period, ~3% of the amino acids of the ingested particulate protein mixture were decarboxylated via the MPO system. We do not have a comparable figure for conversion of alanine to $^{14}CO_2$ by the PLP-mediated pathway during stimulation of the cells because uptake itself was not measured. On the basis of alanine added to the system, the value was also ~3%. Exact determinations of these conversions must follow but they are apparently of comparable orders of magnitude.

It is not evident whether the decarboxylation of the protein-bound amino acids occurs on the intact protein, i.e., without prior cleavage by proteases, or whether it occurs after such cleavage, i.e., on released (free) amino acids in the phagosome. The study of Selvaraj et al. (10) indicated direct cleavage of proteins and decarboxylation of 1,7-[^{14}C]diaminopimelate of labeled bacterial protein by the model MPO-H_2O_2-Cl-system in vitro. This would suggest that the system in intact cells is capable of directly decarboxylating protein-bound amino acids, though this was not proven because it is not known whether active proteases were also present in the system cited (10).

There are several ways by which decarboxylation of amino acids could play a role in the microbicidal process in granulocytes. If decarboxylation of amino acids was involved in antimicrobial action of the MPO-H_2O_2-halide system then it might be expected that the addition of amino acids to an in vitro system would affect the microbicidal effect. In studies done with glycine and alanine, microbicidal activity of the MPO-H_2O_2-Cl-system was inhibited (27). The effectiveness of aldehydes at the levels that would be produced has been questioned (13), and information on the relevance of chloramines in vivo is lacking. A suggestion has also been made concerning a possible role for "structural" aldehydes presumably formed by partial cleavage and oxidation of the protein of ingested microbes (10).

Recently, in our laboratory Badwey et al. (25) defined an oxidase capable of oxidizing aldehydes (particularly those expected from amino acids) to their corresponding carboxylic acids. The enzyme produces O_2^- in high yield as most of the electron transfer leads to O_2^- formation. The importance of this is that it might constitute a system for disposing of aldehydes that may be toxic to the phagocytizing cell. It would produce further quantities of O_2^- and H_2O_2, which could be utilized by the oxygen-dependent MPO-mediated microbicidal system, and could replenish the MPO-mediated attack on ingested protein-amino acids described above. Thus, aldehydes derived from amino acids might not themselves be potent bactericidal agents. The system outlined would provide a way of generating more active oxygen species that could be used for another round of activity, which one might term a "cidal cycle."

The $^{14}CO_2$ collected in our experiments is probably almost entirely representative of decarboxylation reactions, but some $^{14}CO_2$ from oxidation of the residual skeletons may be included since uniformly labeled substrates were sometimes used. The data from the experiments with metabolic inhibitors are, however, sufficiently clear-cut to validate the thesis that (*a*) the MPO-H_2O_2-halide mediated pathway for conversion of amino acids to $^{14}CO_2$ can occur in whole guinea pig PMN; (*b*) two different cellular locales and two different metabolic pathways are available in the oxida-

tion of amino acids, depending upon whether they are free and soluble or are associated with a particle. In the first case, diffusion or transport of L-alanine or L-aspartate, for example, into the cytoplasm results in transamination and oxidative decarboxylation of the resulting keto-acid. In the second case, deamination-decarboxylation to form an aldehyde would occur in the phagosome after phagocytosis of the particle and release of H_2O_2 and MPO, at acid pH (28–32). The pathway followed is presumably dictated by the cellular locale reached by the amino acid. Some free soluble amino acid could presumably reach the phagosome with a particle if the external concentration were high (>10 mM; see above), and could then undergo the MPO-mediated reaction. Entry of external medium into phagosomes is small, however (33). Furthermore, certain amino acids could be carried to the phagosome adsorbed to ingested particles. Finally, the MPO-mediated reaction could occur in the extracellular fluid because both MPO (30–31) and H_2O_2 (34) are released upon stimulation of the cells. These possible fates of free soluble amino acids and soluble protein involving MPO seem not to be of quantitative importance under our conditions using guinea pig granulocytes. However, an oxidative reaction mediated by MPO-H_2O_2-Cl^-—the conversion of methionine to sulfoxide—has been shown to occur both intracellularly and extracellularly in the case of stimulated human granulocytes (35). The comparative kinetics of the MPO-mediated deamination-decarboxylation reaction and of the conversion of methionine to sulfoxide is not known. In view of this and the very great quantitative differences in behavior between guinea pig and human granulocytes, e.g., with respect to O_2^- production (36), additional studies on these phenomena are clearly desirable. The objective of the present work, as stated in the Introduction—to examine the question of whether the decarboxylation of amino acids via the MPO-H_2O_2-Cl^- mediated pathway does indeed occur in intact cells—has resulted in a positive answer, and appears relevant to the fate of insoluble particulate protein.

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