Catalase-Dependent Peroxidative Metabolism in the Alveolar Macrophage during Phagocytosis

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ABSTRACT Evidence for the presence of peroxidative metabolism in rabbit alveolar macrophages (AM) has been obtained from the following observations: (*a*) catalase is present in high concentrations; (*b*) peroxidase activity could not be detected employing guaiacol as substrate; (*c*) the irreversible inhibition of AM catalase by aminotriazole served as a detection system for H_2O_2 and demonstrated increased intracellular H_2O_2 after phagocytosis; (*d*) formate oxidation, a marker of catalase-dependent peroxidations, occurs in resting AM and is increased by phagocytosis; (*e*) measurements of H_2O_2 accumulation in a dialysate of AM demonstrated twofold increase during phagocytosis; and (*f*) aminotriazole diminishes O_2 utilization and $^{14}CO_2$ production from labelled glucose and pyruvate. It is concluded that, while catalase-dependent H_2O_2 metabolism is not essential for particle entry, this pathway represents one of the metabolic pathways stimulated by particle entry in the AM.

INTRODUCTION

The alveolar macrophage (AM) plays an important role in pulmonary bacterial clearance by functioning as a phagocyte. While phagocytosis by the AM requires oxygen and stimulates both O_2 consumption (Q_{O_2}) and $^{14}CO_2$ production from labeled glucose (2), the relation of these metabolic pathways to H_2O_2 has not been defined in the AM. The classical work of Iyer, Islam, and Quastel (3) first indicated that hydrogen peroxide plays an important role in these pathways in another phagocytic cell, namely the polymorphonuclear leucocyte (PMN). Their evidence indicated that the oxidation of reduced pyridine nucleotides is accompanied by the generation of H_2O_2. The generation of H_2O_2 was detected indirectly employing formate oxidation as a marker. Further evidence for H_2O_2-dependent metabolism in the PMN has been provided by a number of workers notably Karnovsky (4), Roberts and Quastel (5), and Paul and Sbarra (6). This peroxidative metabolism has recently been inculpated in intracellular bactericidal mechanisms in the PMN (7, 8).

This study was designed to provide evidence concerning H_2O_2 metabolism in resting and phagocytosing AM. Several approaches have been used. First, inferences have been drawn from the presence of enzymes utilizing H_2O_2 as a substrate. Second, the catalase inhibitor aminotriazole (AT) may be used to detect intracellular H_2O_2 (9) since it causes an irreversible inhibition of catalase: H_2O_2 compound 1 as opposed to a reversible inhibition of free catalase (10). Third, formate oxidation may be used as a marker of catalase-dependent H_2O_2 metabolism (3) and changes in H_2O_2 concentration in intact cells may be detected by fluorimetric measurements of H_2O_2 concentration by the method of Paul and Sbarra (6).

METHODS

Alveolar macrophages. The AM were harvested by a modification of the technique of Myrvik, Leake, and Fariss (11) in which AM are obtained by pulmonary lavage from New Zealand white rabbits. The lavage fluid was Krebs Ringer phosphate solution (KRPS) (12) at pH 7.4 to which 5.5 mM glucose was added. Average cell yield was 1×10^8 cells/rabbit. Red cell contamination was less than 5%. Polymorphonuclear leucocytes were less than 2% of the nucleated cells.

Leucocytes and red blood cells. Human leucocytes were separated by sedimentation of five parts of heparinized blood with one part 6% dextran in normal saline. The plasma,

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which contained the leucocytes, was pipetted off and the cells separated by centrifugation at 3000 rpm at room temperature. The WBC were resuspended in KRPS before freeze-thawing for peroxidase determination. Red cell contamination of this WBC preparation varied between 5 and 15% of WBC. Rabbit red cells were prepared from heparinized blood by centrifugation. The buffy coat was removed and cells resuspended in KRPS.

Phagocytosis studies. Heat-killed (75°C for 30 min) coagulase-negative *Staphylococcus albus* were employed as test particles. Bacteria to cell ratios were approximately 20/1 unless otherwise specified. Phagocytosis was assessed by light microscopy of Gram's stained preparations obtained after 30 min incubation at 37°C in KRPS containing 5.5 mM glucose and 15% fresh rabbit serum. A semiquantitative expression of particle entry was employed, namely the number of cells containing 0-5, 6–10, and > 11 bacteria were counted. At least 50 cells were examined.

Catalase activity. Catalase activity was determined by the method of Feinstein (13), employing 0.1 M sodium perborate. Perborate utilization in 5 min was measured by titration with a 0.1 N solution of potassium permanganate after the reaction was stopped with 1 N H_2SO_4. The catalase activities of AM, red cells, and purified beef liver catalase (Worthington Biochemical Corp., Freehold, N. J.) were determined after incubation for 30 min in KRPS containing 15% homologous serum and 5.5 mM glucose at pH 7.4 Measurements were performed on two preparations of AM, namely hand homogenized preparations and after three cycles of freeze-thawing employing acetone–dry ice. Both total extract and the supernatant obtained by centrifuging both types of preparation at 8000 rpm in a Servall centrifuge RC2 (7710 *g*) for 10 min were assayed. Activity was expressed as milliequivalents of perborate utilized in 5 min; 1 U representing the utilization of 1 mEq of perborate. The method showed good reproducibility, a coefficient of variation ±2%, and linear concentration response when perborate utilization during 5 min was employed. Prolongation of incubation time of intact AM up to 3 hr did not increase the catalase activity. It should be noted that cell counts were measured in a limited number of experiments since all experiments to determine the effects of preparative procedures, AT, or particle exposure were internally controlled by comparison with control aliquots of the same AM preparations. In experiments employing aminotriazole, this was added at the beginning of the 30 min incubation period.

Dialysis experiments to assess the binding of aminotriazole to catalase were performed on 6-ml aliquots of a freeze-thaw extract of the AM in KRPS contained in a cellophane membrane. Dialysis was carried out for 3 hr against 100 ml of KRPS which changed hourly.

Additional experiments were performed to measure the effects of AT on cells previously exposed to bacteria. The procedure employed was the following; 4-ml aliquots of AM suspended in KRPS, containing 5.5 mM glucose and 15% fresh rabbit serum, were incubated for 30 min in the presence or absence of heat-killed *S. albus*. The cells were partially separated from bacteria by centrifugation at 1000 rpm for 15 min, washed in 10 ml of KRPS, recentrifuged, and resuspended in KRPS: serum: glucose with or without 20 mM AT. In some experiments aminotriazole-^{14}C was employed. After 30 min incubation at 37°C, the cells were separated, washed, and resuspended in 1 ml of KRPS for freeze-thawing. Aliquots were taken from freeze-thaw extracts for catalase measurements and for counting where aminotriazole-^{14}C was employed. Counting was performed in Bray's solution (14) containing 2% v/v of 10% hyamine.

Peroxidase activity. This was determined by a modification of the guaiacol method of Chance and Maehly (15). Whole extracts of freeze-thawed cells were employed. The assay medium contained 0.1 M phosphate buffer at pH 7.4, 0.5 ml of 100 mM guaiacol, 0.2 ml of extract sample, and 0.02 ml of ice-cold 10 mM H_2O_2. Absorbancy changes due to tetraguaiacol formation were measured at 750 mμ in a Zeiss spectrophotometer and the time required to produce an 0.05 U increase in absorbancy recorded. Results were expressed in reciprocal seconds per 10^9 cells. The method showed a coefficient of variation of ±7% and a linear response with time and number of cells. The effect of excess catalase on the estimation of peroxidase was examined in view of the presence of catalase in the AM and in view of the contamination of the white blood cell (WBC) preparation by catalase containing red cells. No effects on the peroxidase estimation performed on WBC preparations were observed under two conditions: (*a*) the addition of sufficient red cells to double the red cell contamination; and (*b*) the addition of purified beef liver catalase to increase threefold the initial catalase activity of the WBC preparation.

Hydrogen peroxide determination. Hydrogen peroxide was determined spectrophotometrically on dialysates of AM as described by Paul and Sbara (6). The nonfluorescent dye, diacetyl-2,7-dichlorofluorescin (LDADCF), was synthesized by the method of Brandt and Keston (16) and the fluorescence of the oxidized product of alkali-activated LDADCF was measured with an Aminco-Bowman spectrofluorimeter. The excitation wave length was 340 mμ and the emission wave length 525 mμ.

Aliquots of AM ($20–60 \times 10^6$ cells) in the presence and absence of heat-killed *S. albus* (bacteria to cell ratio, 150:1) were incubated at 37°C in KRPS containing 15% homologous serum, final volume 5 ml, in dialysis tubing immersed in 60 ml of KRPS. Samples were removed from the dialysate at appropriate times for determination of the H_2O_2 produced. Control experiments were also performed in which dialysate obtained from serum KRPS and, where appropriate, bacteria were assayed. The difference in fluorescence between experimental and the relevant control dialysate was taken as the measure of H_2O_2 produced by the AM. This was reduced by over 90% when commercial catalase (1 perborate U) was added to the cell suspension. Data were expressed as the quantity of H_2O_2 in the total dialysate per 1×10^6 cells in dialysis bag.

Metabolic studies. All studies were performed in KRPS containing 5.5 mM glucose and 15% homologous serum at pH 7.4. Approximately 25×10^6 cells were present in each flask in a final volume of 4 ml. Bacteria cell ratio was 150:1. O_2 consumption was measured for 1 hr in a modified Gilson respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.) after respirometer gassing with 100% O_2. Other studies were performed for 1 hr in a Dubnoff metabolic shaker with air as the gas phase.

Approximately 0.3 μCi of the following labeled substrates were employed without the addition of unlabeled substrate: sodium formate-^{14}C [1] (3 mCi/mmole), 1-sodium acetate-^{14}C [2] (40 mCi/mmole), and 1-sodium pyruvate-^{14}C [2] (4.0 mCi/mmole). The studies with 1-glucose-^{14}C [2] (4.0 mCi/mmole) and 6-glucose-^{14}C [2] (3.8 mCi/mmole) employed the addition of approximately 0.3 μCi of tracer glucose to each flask in which the total glucose concentration was 5.5 mM. The

[1] New England Nuclear Corp., Boston, Mass.

[2] Nuclear-Chicago Corporation, Des Plaines, Ill.

TABLE I

The Effect of Extraction Procedures on Catalase Activity of AM

Freeze-thawed extract		Homogenate	
Total	Supernatant	Total	Supernatant
0.55 ±0.05	0.52 ±0.05	0.22 ±0.04	0.02 ±0.01

Catalase activity expressed as milliequivalents of perborate utilized in 5 min. Mean ±1 SD of three duplicate experiments shown.

counts obtained with 6-glucose-^{14}C were adjusted to the same specific activity as 1-glucose-^{14}C by the ratio 1.05. $^{14}CO_2$ was released with 6 N H_2SO_4 in the closed flask and entrapped in 10% hyamine hydroxide in the center cup. The hyamine was transferred quantitatively into the counting vials containing 2,5-diphenyloxazole (PPO) and 1-4-bis-2-4-methyl-(5-phenyloxazolyl)benzene in toluene and counting was performed with a liquid scintillation counter (Tri-Carb, Packard Instruments Co., Downers Grove, Ill.). All experiments were performed in triplicate.

Aminotriazole. 3-amino-1,2,4-triazole was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio and recrystallized from ethanol. Labeled 5-aminotriazole-^{14}C (1.5 mCi/mmole) was obtained from Tracerlab Div. of Waltham, Mass.

RESULTS

Catalase and peroxidase activity. The catalase activity in two types of AM preparations is indicataed in Table I, which compares duplicate measurements of the activity present in total extract and supernatant obtained from aliquots of the same AM preparations after either homogenization or freeze-thawing. Two points emerge. First, freeze-thaw preparations demonstrate 2.5 times more catalase activity than homogenized preparations. Second, 95% of the activity was found in the supernatant after freeze-thawing while most of the activity remains in the particulate fraction after homogenization. The freeze-thaw supernatant was therefore employed in the ensuing measurements of the catalase activity. Further observations, not shown here, indicated that three cycles of freeze-thawing produced maximal catalase activity. No loss of catalase activity from the freeze-thaw supernatant obtained by centrifugation at 7710 *g* occurred after high speed centrifugation (up to 100,000 *g*) indicating that three cycles of freeze-thawing produced maximal solubilization of catalase.

TABLE II

The Effects of Aminotriazole on AM Catalase Activity

Experiment	Control	Aminotriazole	
		100 mM	*20 mM*
1	0.29	0.06	0.16
2	0.24	0.02	0.08

Means of two duplicate experiments are indicated; catalase activity expressed as milliequivalents of perborate utilized in 5 min.

The catalase activity, measured in 11 separate preparations of the AM freeze-thaw supernatant, was 37.3×10^{-2} U/10^6 cells with 1 SD of $\pm 3.1 \times 10^{-2}$ U. This is approximately 6 times more than rabbit red cells catalase whose activity was 6.3×10^{-2} U/10^6 cells with 1 SD of 0.7×10^{-2} U. Peroxidase measurements in the AM showed remarkably little activity, 6.8 ±0.9 reciprocal seconds/10^9 cells. This may be contrasted with the measured peroxidase activity of human polymorphonuclear leucocytes, namely 483 ±31 reciprocal seconds/10^9 cells. The minimal peroxidase activity of the AM preparation may reflect PMN contamination of these preparations.

The action of aminotriazole on catalase activity. The effects of AT on the catalase activity of the AM are indicated in Table II. Concentrations of 100 and 20 mM produce approximately 85 and 66% inhibition of the catalase activity respectively.

Evidence that the inhibition of catalase by AT is dependent on the presence of H_2O_2 (see Discussion) is provided by the data in Table III. The inhibition is shown to be of the irreversible type since it cannot be reversed by dialysis. Further, ethanol, which alone has no action on catalase, prevents the inhibitory action of subsequently added AT by decomposing catalase H_2O_2 compound 1 to free catalase (9). Ethanol did not diminish the inhibition of catalase pretreated with AT providing further evidence that the AT inhibition is of the irreversible type.

Particle entry in the presence of aminotriazole. The results of the semiquantitative intracellular bacterial counts in the presence or absence of 100 mM AT are presented in Table IV. No differences were observed.

TABLE III

Factors Influencing AT Inhibition of Catalase Activity

Experiment	Control	AT		Ethanol		
		No dialysis	Dialysis	Ethanol alone	AT after ethanol	AT before ethanol
1	0.75	0.04	0.04	0.75	0.73	0.04
2	0.75	0.05	0.06	0.74	0.72	0.05

Six aliquots of AM freeze-thaw extract were employed for each experiment. Duplicate measurements of catalase activity (milliequivalents of perborate used in 5 min) were obtained under the indicated conditions. AT and/or ethanol in final concentrations of 100 mM and 0.45 M respectively were added to the appropriate aliquots in the sequences indicated. The aliquots were allowed to stand at room temperatures for 30 min after each addition before assay or dialysis.

Particle entry and catalase activity. The possibility that particle entry increases H_2O_2 levels in the AM was next examined. Table V indicates a comparison of the catalase activity measured on aliquots of the same AM preparations at "rest" and during phagocytosis in the presence and absence of AT.

The data demonstrate the marked increase in the inhibition of catalase by AT in the AM which have phagocytosed the bacteria. The increased inhibition by AT after phagocytosis suggests an increase in H_2O_2 levels after phagocytosis. The possibility that this increased AT inhibition of catalase might be due to an increased intracellular AT concentration after phagocytosis is excluded by experiments 3 and 4 employing aminotriazole-^{14}C (Table V). The counts due to labelled aminotriazole recovered from aliquots of the AM extract employed for measurement of catalase activity were similar in the extracts from both resting and phagocytosing AM.

In all four duplicate experiments there was a small increase (12%) in the control catalase activity after phagocytosis. This change in catalase activity after phagocytosis was not due to loss of catalase during the washing procedures since, while the pooled washing fluid contained 8% of the final AM catalase activity, the amount of catalase detected was similar in the washings from "resting" and phagocytosing AM. Further, since no catalase activity could be detected in heat-killed *S. Albus,* it seems unlikely that this increased catalase is derived from the phagocytosed bacteria. The significance of this increase in intracellular catalase activity after phagocytosis is unclear.

Hydrogen peroxide. The quantities of H_2O_2 present in the dialysis fluid at varying incubation times in resting and phagocytosing AM are indicated in Fig. 1.

TABLE IV

The Effects of Aminotriazole on Bacterial Entry

Experiment	Bacteria per cell	Control			Aminotriazole		
		1–5	6–10	>11	1–5	6–10	>11
1	No. of AM	9	32	9	7	28	15
2		12	28	10	14	23	13
3		11	33	6	9	28	13
Mean		11	31	8	10	26	14

The number of AM containing the indicated number of particles are presented. 50 cells were examined in each of three paired experiments. 20 mM aminotriazole.

There was a progressive rise in the quantity of H_2O_2 recovered by dialysis as incubation is prolonged. Further the quantity recovered at any given time from phagocytosing AM was approximately double that observed in resting AM. In studies employing intact cells, up to 0.34 mμmoles of H_2O_2 were recovered from 10^6 AM during phagocytosis.

The effects of aminotriazole on O_2 utilization. The effect of AT on Q_{O_2} in "resting" and phagocytosing AM are presented in Table VI. Each experiment was performed on aliquots obtained from the same AM preparations and thus each experiment was internally controlled. AT diminishes the Q_{O_2} in the resting AM. Though these changes are small, they are consistent in all experiments and the mean values are significantly different by Student's t test for paired samples at $P < 0.01$ level. The maximum effect at concentrations of 0.1 M AT, was an approximately 14% reduction of Q_{O_2}. Particle entry consistently stimulated Q_{O_2} by 55%. ($P < 0.01$). The effects of AT on the Q_{O_2} increase

TABLE V

The Effects of Phagocytosis on Catalase Activity and on Aminotriazole Inhibition of Catalase

Experiment	Rest				Phagocytosis			
	Control	+AT	Inhibition	AT-^{14}C	Control	+AT	Inhibition	AT-^{14}C
			%	*cpm*			%	*cpm*
1	1.00	0.48	52	—	1.20	0.10	75	—
	0.98	0.48			1.22	0.08		
2	1.00	0.54	48	—	1.12	0.29	60	—
	1.00	0.54			1.14	0.35		
3	1.48	1.23	16	191	1.67	0.33	80	209
				190				191
4	1.80	1.11	37	187	1.94	0.65	65	184
	1.76	1.10		192	1.84	0.62		208
Average	1.31	0.82	39	190	1.48	0.45	75	198

Aminotriazole (AT) 20 mM, AT-^{14}C = 5-aminotriazole-^{14}C, 0.01 μCi of AT-^{14}C added. Duplicate measurements of catalase activity (milliequivalents of perborate utilized in 5 min) are indicated except for experiment 3. For procedures, see Methods.

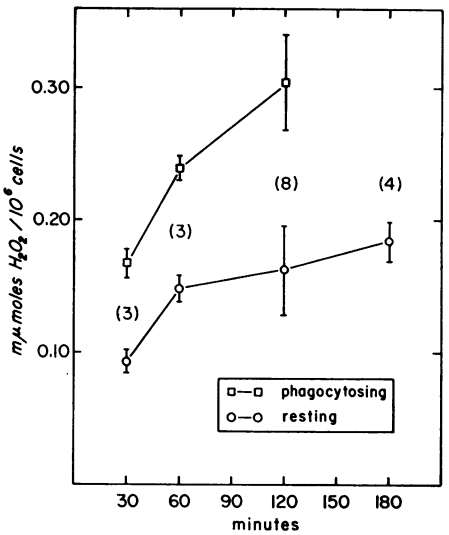

FIGURE 1 H_2O_2 recovery from resting and phagocytosing AM. H_2O_2 expressed as mμmoles/10^6 cells in dialysis bag. Mean ±1 SE are indicated; numbers in parentheses are number of observations.

specifically associated with phagocytosis are evident from a comparison of the right-hand two columns of Table V. The Q_{O_2} due to phagocytosis is greater in the absence of AT than in the presence of AT. The statistical significance of the differences in Q_{O_2} due to phagocytosis in the presence and absence of AT was examined by an analysis of variance (17). This analysis demonstrated that the "interactions" relating Q_{O_2} in resting and phagocyting AM in presence and absence of AT were statistically different at concentrations of 0.1 M AT (< 0.01) and at 0.04 M AT ($P < 0.05$) but not at 0.02 M AT ($P > 0.05$).

Formate oxidation. $^{14}CO_2$ production from sodium formate-^{14}C was employed to assess the activity of catalase/H_2O_2 compounds in the intact AM. The stimulation of $^{14}CO_2$ production from labeled formate by phagocytosis is demonstrated by the data in Table VII. The effects of AT on formate oxidation in the resting and phagocytosing AM are also shown in Table VII. AT causes an inhibition of formate oxidation both in resting and phagocytosing AM at both 100 mM and 20 mM concentration ($P < 0.01$ for both concentrations).

Oxidation of glucose, pyruvate, and acetate. The results of measurements of $^{14}CO_2$ production from 1-glucose-^{14}C, 6-glucose-^{14}C, 1-pyruvate-^{14}C, 1-acetate-^{14}C in resting and phagocytosing AM are presented in Table VIII. Data obtained in the absence of AT indicated the following: (*a*) resting AM generate 3–6 times more $^{14}CO_2$ from 1-glucose-^{14}C than from 6-glucose-^{14}C; (*b*) phagocytosis stimulates $^{14}CO_2$ production from both labeled forms of glucose, by a factor of varying between 2 and 4 times; (*c*) $^{14}CO_2$ production from pyruvate is approximataely doubled by phagocytosis; and (*d*) $^{14}CO_2$ production from acetate is not stimulated by phagocytosis.

The effects of AT on $^{14}CO_2$ production from these labeled substrates are also indicated in Table VIII. Concentrations of 100 mM AT diminished $^{14}CO_2$ production from pyruvate and both ^1C and ^6C-labeled glucose. Concentrations of 20 mM AT diminished $^{14}CO_2$ production from pyruvate alone. Neither concentration of AT affected acetate conversion to CO_2.

DISCUSSION

Evidence for the presence of H_2O_2 metabolism. The demonstration that catalase is present in high concentrations in the AM provides prima facia evidence for the existence of peroxidative metabolism. This was supported by using the catalase inhibitor, AT, and formate oxidation. The validity of these approaches is indicated by the following considerations. AT is known to produce two types of inhibition of catalase (10), a reversible inhibition of free catalase and an irreversible inhibition in which, according to Margoliash and Novogrodsky, the AT is bound to the protein moiety of catalase: H_2O_2 compound 1 (10). The distinction between these two types of inhibition is important, since the second type of inhibition has been used to detect the presence of H_2O_2

TABLE VI

The Effects of AT on Q_{O_2} in Resting and Phagocytosing AM

Concentration of AT	No. of Experiments	Resting		ΔQ_{O_2} due to AT in resting AM (A–B)	Phagocytosing		ΔQ_{O_2} due to phagocytosis AT absent (C–A)	ΔQ_{O_2} due to phagocytosis AT present (D–B)
		A Control	B AT		C Control	D AT		
M								
0.1	6	4.3 ±1.3	3.7 ±1.2	−0.6 ±0.08	6.7 ±1.4	5.1 ±1.3	+2.4 ±0.12	+1.4 ±0.12
0.04	5	3.9 ±0.61	3.6 ±0.71	−0.3 ±0.05	6.2 ±0.56	5.6 ±0.42	+2.3 ±0.10	+2.0 ±0.10
0.02	3	3.9 ±0.41	3.7 ±0.20	−0.2 ±0.07	6.1 ±0.42	5.7 ±0.36	+2.2 ±0.14	+2.0 ±0.10

Q_{O_2} expressed as μl/hr per 10^6 cells at 37°C and ambient pressure. Mean ±1 SE are indicated for A thru D; mean ±1 SE of differences between paired samples are indicated for ΔQ_{O_2} values.

in biologic systems, notably the red cell (9). The evidence that the inhibition is of the irreversible type in the AM is twofold. First, dialysis of the AT-treated extract failed to modify the catalase inhibition. Thus, the AT was bound to some component of the extract, presumably catalase protein (10). Second, pretreatment of the AM with ethanol completely prevented the AT inhibition and ethanol is known to decompose catalase: H_2O_2 compounds to free catalase and acetaldehyde (9, 18). These features of the AT inhibition of AM catalase, therefore, suggest the presence of catalase: H_2O_2 compound 1 which only exists in the presence of low concentrations of H_2O_2 (10). Evidence compatible with increased H_2O_2 levels after phagocytosis has also been obtained from the observation that AM catalase is more sensitive to AT inhibition in the presence of intracellular bacteria (Table IV). While the use of AT to detect H_2O_2 is indirect, it has the merit of demonstrating that H_2O_2 exists intracellularly. Thus in those experiments (Table V) in which washed AM were employed for the preparation of freeze-thaw extracts, it was demonstrated that the AT was irreversibly bound to intracellular catalase. Comparable observations of increased catalase sensitivity to AT after phagocytosis in the PMN have been obtained by Reed (19).

Formate oxidation has been used as a marker of catalase-dependent H_2O_2 metabolism in a number of tissues (20, 21) including the PMN (3, 5, 7) since Keilin and Hartree showed that catalase as opposed to a number of other hemoproteins was required for the coupled peroxidation of formate (18). The foregoing data indicate that formate oxidation by AM is almost completely inhibited by 100 mM AT, and is therefore presumably largely catalase-dependent. Phagocytosis has been shown to increase formate oxidation (Table VII) indicating increased H_2O_2 formation during phagocytosis.

TABLE VII

The Effects of Aminotriazole of $^{14}CO_2$ Production from Formate-^{14}C

Concentration of AT	Resting		Phagocytosing	
	Control	AT	Control	AT
M				
0.1	36.8 ±12.1	7.9 ±0.5	108.8 ±25.0	3.9 ±0.4
0.02	50.8 ± 5.1	23.0 ±2.7	96.9 ±21.0	32.5 ±3.6

Data expressed as cpm/10^6 cells per 200,000 cpm added to medium. Mean ±1 SE of two triplicate experiments are indicated.

This increase is quantitatively similar to that observed in the PMN (3).

The assay of H_2O_2 (Fig. 1) confirms that H_2O_2 is produced by resting AM. The increased quantity of H_2O_2 obtained from AM during phagocytosis suggests increased intracellular H_2O_2 concentrations. While changes in cell permeability to H_2O_2 during phagocytosis could explain the increased H_2O_2 observed during phagocytosis, the preceding lines of evidence suggest that increased intracellular H_2O_2 levels are the correct explanation. Our data for the AM are similar to those of Paul and Sbarra (6) who obtained values of 0.054 mmoles/10^6 cells from resting PMN and of 0.092 mmoles/10^6 cells from PMN phagocytosing *S. albus* after 30-min incubation periods.

It is noteworthy that the three indices of H_2O_2 formation, namely AT inhibition of catalase, formate oxidation, and H_2O_2 present in dialysates are all increased approximately twofold during phagocytosis.

Relation between peroxidative and glucose metabolism. The classical studies of Iyer and colleagues (3) on PMN first suggested the relationship between increased Q_{O_2}, increased H_2O_2 formation and increased glucose oxida-

TABLE VIII

The Effects of AT on $^{14}CO_2$ Production from Substrates-^{14}C

Substrate	No. of experiments	Concentration of AT	Resting			Phagocytosing		
			Control	AT	*P* value	Control	AT	*P* value
		M						
1-Glucose-^{14}C	4	0.1	22 ±7.1	8 ±3.1	<0.05	90 ±20.1	51 ±16.1	<0.01
	2	0.02	23 ±1.2	23 ±2.1	>0.1	76 ±3.1	70 ±4.2	>0.1
6-Glucose-^{14}C	3	0.1	2.3 ±0.3	0.5 ±0.1	<0.01	9.1 ±0.7	1.7 ±0.7	<0.01
	2	0.02	4.7 ±0.8	4.0 ±0.6	>0.1	10.5 ±1.3	10.1 ±1.3	>0.1
1-Pyruvate-^{14}C	3	0.1	162 ±14	101 ±6	<0.01	381 ±21	130 ±14	<0.01
	3	0.02	104 ±10	83 ±6	<0.05	179 ±19	116 ±12	<0.01
1-Acetate-^{14}C	3	0.1	35 ±6	32 ±5	>0.1	38 ±8	30 ±6	>0.1
	2	0.02	25 ±8	25 ±7	>0.1	23 ±5	27 ±5	>0.1

Data expressed as cpm/10^6 cells per hr of 200,000 initial counts; mean ±1 SE are indicated.

tion during phagocytosis. Our studies indicate a similar relationship in the AM by providing evidence of increased H_2O_2 metabolism during phagocytosis and by confirming the studies of Ouchi, Selvaraj, and Sbarra (2) on Q_{O_2} and glucose metabolism. The latter also demonstrated that phagocytosis by rabbit AM stimulates Q_{O_2} and $^{14}CO_2$ production from labeled glucose and pyruvate. Their observation that 1-4-succinate-^{14}C oxidation is unaffected by phagocytosis is in accord with our observations on acetate oxidation since both produce CO_2 specifically via the Krebs cycle. Both sets of observations are different from the results of Oren, Farnham, Saito, Milofsky, and Karnovsky (22), who observed smaller increases in Q_{O_2} and glucose conversion to CO_2 during phagocytosis by guinea pig AM. These differences may reflect either species differences or a difference in cell harvesting technique in that Oren employed minced guinea pig lung. Thus, at least in rabbit AM, there is an increase in Q_{O_2}, glucose oxidation, and H_2O_2 levels during phagocytosis and analogy with the PMN suggests these to be interrelated.

The definition of the role of catalase in these interrelations must await knowledge of the H_2O_2 generating pathway. Further communications [3] will provide evidence that, in the AM, H_2O_2 is in part generated by a D-amino acid oxidase, an enzyme recently described in PMN (23), and also that the H_2O_2 is coupled to triphosphopyridine nucleotide, reduced form of, (NADPH) in the AM by a glutathione system (24), similar to that described in the rat leucocyte (19). However, two comments may be made on the basis of the current studies. First, the virtual absence of myeloperoxidase activity precludes it serving as an H_2O_2-generating NADPH oxidase in the AM as has been proposed for the PMN (25). Second, while caution is required in interpreting evidence based on the actions of AT, it is possible that the actions of AT are relatively specific for catalase. At least AT has no general toxic actions on the AM since particle entry, cell viability (eosin uptake technique), acetate oxidation (Table VIII), and ATP concentrations (1) are uninfluenced by 100 mM AT. On the assumption of the catalase specificity two alternative explanations of the AT inhibition of Q_{O_2} and $^{14}CO_2$ production from glucose and pyruvate appear reasonable. First, catalase activity may determine the H_2O_2 production by an oxidase. The importance of the association of H_2O_2-generating oxidases and catalase has recently been stressed by deDuve and Baudhuin (21). Second, catalase may serve in coupled peroxidations linked to diphosphopyridine nucleotide, reduced form of, (NADH), as demonstrated by several workers (18, 20, 21) and NADH is a known regulator of glycolysis and pyruvate metabolism.

Significance of peroxidative metabolism. It is evident that inhibition of catalase activity does not seriously affect particle entry. Increased H_2O_2 metabolism, therefore, represents a secondary event after particle entry. Peroxidative metabolism represents a mechanism capable of increasing glucose metabolism in a manner similar to that observed in the liver, kidney, and red cells (20, 21, 26, 27). Finally, the role of H_2O_2 as an intracellular bactericidal agent in PMN has been recently emphasized (6, 7, 8). The tempting idea that H_2O_2 serves a similar role in the AM requires further study particularly since the AM lack white cell peroxidase activity. This enzyme represents an important component of the bactericidal peroxidative systems (8, 28, 29). Deficiency of this peroxidase in PMN is associated with impairment of Candidacidal activity and subnormal killing rates of certain bacteria (30–32).

[3] Vogt, M., C. Thomas, J. B. L. Gee, and R. E. Basford. Unpublished observations.

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REFERENCES

1. Gee, J. B. L., E. D. Robin, J. B. Field, J. D. Smith, A. R. Tanser, and J. Kaskin. 1968. Roles of ATP and of peroxidative metabolism during phagocytosis in alveolar macrophages *J. Clin. Invest.* **47**: 38a.
2. Ouchi, E., R. J. Selvaraj, and A. J. Sbarra. 1965. The biochemical activities of rabbit alveolar macrophages during phagocytosis. *Exp. Cell Res.* **40**: 456.
3. Iyer, G. Y. N., M. F. Islam, and J. H. Quastel. 1961. Biochemical aspects of phagocytosis. *Nature (London).* **192**: 535.
4. Karnovsky, M. L. 1962. Metabolic basis of phagocytic activity. *Physiol. Rev.* **42**: 143.
5. Roberts, J., and J. H. Quastel. 1964. Oxidation of reduced triphosphopyridine nucleotide by guinea pig polymorphonuclear leucocytes. *Nature (London).* **202**: 85.
6. Paul, B., and A. J. Sbarra. 1968. The role of the phagocyte in host-parasite interactions. XIII. The direct quantitative estimation of H_2O_2 in phagocytising cells. *Biochem. Biophys. Acta.* **156**: 168.
7. Holmes, B., A. R. Page, and R. A. Good. 1967. Studies of the metabolic activity of leucocytes from patients with a genetic abnormality of phagocytic function. *J. Clin. Invest.* **46**: 1422.
8. McRipley, R. J., and A. J. Sbarra. 1967. Role of the phagocyte in host-parasite interactions. XII. Hydrogen peroxide-myeloperoxidase bactericidal system in the phagocyte. *J. Bacteriol.* **94**: 1425.
9. Cohen, G., and P. Hochstein. 1964. Generation of hydrogen peroxide in erythrocytes by hemolytic agents. *Biochemistry.* **3**: 895.

10. Margoliash, E., and A. Novogrodsky. 1958. A study of the inhibition of catalase by 3-amino-1:2:4:-triazole. *Biochem. J.* **68:** 468.
11. Myrvik, Q. N., E. S. Leake, and B. Fariss. 1961. Studies on pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. *J. Immunol.* **86:** 128.
12. Umbreit, J. W., R. H. Burris, and J. F. Stauffer. 1964. Preparation of tissues. *In* Manometric Techniques. J. W. Umbreit, R. H. Burris, J. F. Stauffer, editors. Burgess Publishing Co., Minneapolis, Minn. 149.
13. Feinstein, R. N. 1949. Perborate as substrate in a new assay of catalase. *J. Biol. Chem.* **180:** 1197.
14. Bray, G. A. 1960. A simple efficient liquid scintillation for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1:** 279.
15. Chance, B., and A. C. Maehly. 1955. Assay of catalases and peroxidase. *In* Methods in Enzymology. S. Colowick, editor. Academic Press, Inc., New York. **2:** 764.
16. Brandt, R., and A. S. Keston. 1965. Synthesis of diacetyldichlorofluorescin: a stable reagent for fluorometric analysis. *Anal. Biochem.* **11:** 6.
17. Edwards, A. L. 1964. Experimental design in psychological research. Holt, Rinehart and Winston, New York. Chap. 9.
18. Keilin, D., and E. F. Hartree. 1955. Catalase, peroxidase and metmyoglobin as catalysts of coupled peroxidatic reactions. *Biochem. J.* **60:** 310.
19. Reed, P. W. 1969. Glutathione and the hexose monophosphate shunt in phagocytising and hydrogen peroxide treated rat leukocytes. *J. Biol. Chem.* **244:** 2459.
20. Portwich, F., and H. Aebi. 1960. Erfassung der Peroxybildung tierischer Gewebe mittels peroxydatischer Umsetzung. *Helv. Physiol. Pharmacol. Acta.* **18:** 312.
21. deDuve. C., and P. Baudhuin. 1966. Peroxisomes (microbodies and related particles). *Physiol. Rev.* **46:** 323.
22. Oren, R., A. E. Farnham, K. Saito, E. Milofsky, and M. L. Karnovsky. 1963. Metabolic patterns in three types of phagocytising cells. *J. Cell Biol.* **17:** 487.
23. Cline, M. J., and R. I. Lehrer. 1969. D-amino acid oxidase in leucocytes: a possible D-amino-acid-linked antimicrobial system. *Proc. Nat. Acad. Sci. U. S. A.* **62:** 756.
24. Gee, J. B. L., and R. E. Basford. 1969. Effects of sulfhydryl inhibitors on phagocytosis by alveolar macrophages (AM). *Fed. Proc.* **28:** 445.
25. Roberts, J., and Z. Camacho. 1967. Oxidation of NADPH by polymorphonuclear leucocytes during phagocytosis. *Nature (London).* **216:** 606.
26. Hochstein, P., and H. Utley. 1968. Hydrogen peroxide detoxication by glutathione peroxidase and catalase in rat liver homogenates. *Mol. Pharmacol.* **4:** 574.
27. Jacob, H. S., and J. H. Jandl. 1966. Effects of sulfhydryl inhibition on red blood cells. III. Glutathione in the regulation of the hexose monophosphate pathway. *J. Biol. Chem.* **241:** 4243.
28. Klebanoff, S. J. 1967. Iodination of bacteria: a bactericidal mechanism. *J. Exp. Med.* **126:** 1063.
29. Klebanoff, S. J., and L. R. White. 1969. Iodination defect in the leucocytes of a patient with chronic granulomatous disease of childhood. *N. Engl. J. Med.* **280:** 460.
30. Lehrer, R. I., and M. J. Cline. 1969. Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to *Candida* infection. *J. Clin. Invest.* **48:** 1478.
31. Lehrer, R. I., J. Hanifin, and M. J. Cline. 1969. Defective bactericidal activity in myeloperoxidase-deficient human neutrophils. *Nature (London).* **223:** 78.
32. Salmon, S. E., M. J. Cline, J. Schultz, and R. I. Lehrer. 1970. Myeloperoxidase deficiency: immunologic study of a genetic leukocyte defect. *N. Engl. J. Med.* **282:** 250.