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Glucose-dependent Secretion and Destruction of Hydrogen Peroxide by *Mycoplasma pneumoniae*

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The secretion of H_2O_2 by *Mycoplasma pneumoniae* and *M. gallisepticum* was measured with the new catalase-aminotriazole method. Peroxide secretion by the mycoplasmas was stimulated by glucose. When catalase and aminotriazole were omitted and exogenous H_2O_2 was added to the mycoplasmas, a loss in H_2O_2 was noted with time; the addition of glucose speeded the disappearance of H_2O_2. The presence of this peroxidase-like activity in the mycoplasmas explains an observed failure of H_2O_2 to accumulate freely in the suspension medium.

As suggested by several investigators (5, 16), the virulence of *Mycoplasma pneumoniae* may be due, in part, to the toxic actions of H_2O_2 secreted onto the tissues of the respiratory tract. In this paper, we describe the results of a quantitative study of H_2O_2 secretion by *M. pneumoniae* and *M. gallisepticum*. The method used to measure H_2O_2 is a new one which employs catalase and 3-amino-1,2,4-triazole (AT); it has been described in detail (6). The method does not depend upon accumulation of H_2O_2 in the mycoplasma suspension medium and, therefore, it can be used to estimate H_2O_2 secretion in the presence of a peroxidase-like activity exhibited by the mycoplasmas.

MATERIALS AND METHODS

Reagents. All reagents were prepared as described elsewhere (6).

Organisms. The FH strain of *M. pneumoniae* and the S6 strain of *M. gallisepticum* (obtained from David Madden of the National Institutes of Health) were grown on the medium of Chanock et al. (2), but modified by the addition of 1% glucose and 0.002% phenol red. The colonies were grown in 500 ml of medium contained in 2-liter Povitsky bottles, where they adhered to the glass surface and formed a confluent layer (15) beneath the broth medium. The broth was removed by decantation, and the layer of organisms adhering to the surface was washed five times with 100-ml volumes of buffered saline. The colonies were removed by scraping and were suspended in 10 to 20 ml of buffered saline. We used cultures which were in the 15th to the 358th passage on artificial medium. In one series of experiments, *M. pneumoniae* suspensions were prepared 10 to 30 min before the studies were performed; in other experiments, the washed *M. pneumoniae* and *M. gallisepticum* suspensions were held at 0 to 5 C and sent from Bethesda to New York City, where experiments were performed after a 6- to 8-hr delay in transit.

Acid-forming units were determined by serial 10-fold dilutions of the mycoplasma suspension into broth containing 1% glucose and 0.002% phenol-red indicator. Growth and viability of the organisms were indicated by acid production which resulted in a decrease in *p*H and a consequent change in color of the indicator. The number of acid-forming units was given by the greatest dilution for which acid production was observed.

Measurement of H_2O_2-secretion rates. The rates of secretion of H_2O_2 by the mycoplasmas were measured by evaluating the H_2O_2-dependent rates of inhibition of catalase by AT, as previously described in detail (6). In general, 1.8 ml of buffered catalase-AT-glucose mixture was equilibrated with shaking at 37 C for 10 min in test tubes 11 mm in diameter, and then 0.2 ml of mycoplasma suspension was added. After 0.5 to 1 hr, samples of 0.1 ml were removed and analyzed for catalase activity. A calibration curve (6) was employed to convert the observed catalase inhibition rates into H_2O_2 secretion rates.

Measurement of H_2O_2 with peroxidase. The accumulation of secreted H_2O_2, or the loss of added H_2O_2, was measured with a standard peroxidase method with *o*-dianisidine as substrate (1). The washed mycoplasma suspensions were incubated at 37 C, and then they were centrifuged at 11,000 × *g* for 20 min at 5 C to sediment the organisms. Then, 1.5 ml of supernatant fluid was incubated at room temperature with 1.0 ml of a mixture of horseradish peroxidase (40 μg/ml) and *o*-dianisidine (0.4 μmole/ml) in buffered saline. After 2 min, 0.02 ml of 5 N HCl was added to stop the reaction. The optical densities of the samples

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were determined at 410 nm and compared to a standard curve prepared with reagent H_2O_2.

RESULTS

H_2O_2 secretion by M. pneumoniae. The rate of secretion of H_2O_2 was proportional to the number of organisms added to the system (Table 1, samples A–D). As expected, no loss of catalase activity occurred when AT was omitted (sample E). Since the H_2O_2-dependent mechanism of catalase inhibition proceeds through a catalase-H_2O_2 complex which can be decomposed by ethyl alcohol (4, 12), the intermediary presence of H_2O_2 in our experiments was confirmed by the ability of ethyl alcohol to prevent enzyme inhibition (sample F).

The inclusion of glucose in the medium stimulated H_2O_2 formation by the mycoplasmas. When glucose was omitted, no H_2O_2 was observed (sample G, as contrasted to sample D); as shown by sample A, the stimulation by glucose required the presence of the microorganisms. Heating the *M. pneumoniae* preparation at 90 to 100 C for 10 min resulted in complete loss of H_2O_2-generating activity (sample H).

The uninoculated culture medium used to grow *M. pneumoniae* contained a heat-stable substance which generated H_2O_2; that is, it inhibited catalase in the presence of AT. This substance was eliminated by the wash procedure. Absence of the interfering agent from the washed mycoplasma suspensions was verified by the inactivity of heated preparation (sample H).

TABLE 1. *H_2O_2 generation by M. pneumoniae*

Sample	Inhibition of catalase at 0.5 hr	Equivalent secretion rate for H_2O_2
	%	*μmoles/hr*
(A) Buffered catalase-aminotriazole-glucose mixture (control)	0	0.000
(B) + *M. pneumoniae* (0.05 ml)	24	0.015
(C) + *M. pneumoniae* (0.1 ml)	40	0.025
(D) + *M. pneumoniae* (0.2 ml)	76	0.048
(E) + *M. pneumoniae* (0.2 ml); AT omitted	0	—[a]
(F) + *M. pneumoniae* (0.2 ml) and ethyl alcohol (0.4 mmole)	3	—[a]
(G) + *M. pneumoniae* (0.2 ml); glucose omitted	0	0.000[b]
(H) + Heated *M. pneumoniae* (0.2 ml)[c]	0	0.000

[a] Catalase inhibition data for samples E and F cannot be converted into H_2O_2-generation rates because the deletion of AT or the addition of ethyl alcohol prevented a response in the H_2O_2 detection system despite the presence of H_2O_2.

[b] In other experiments, a small amount of H_2O_2 was detected in the absence of glucose (cf. Table 3).

[c] Heated at 90 to 100 C for 10 min.

The H_2O_2 detected in the *M. pneumoniae* suspension medium could have represented a metabolic end product of the organisms, or it could have been derived indirectly via the aerobic oxidation of some secreted, autoxidizable substance. To distinguish between these two possibilities, we performed the following experiment. Mycoplasma suspensions were incubated at 37 C for 1 hr. The cells were centrifuged at 11,000 × *g* for 20 min at 5 C, and 0.2 ml of cell-free supernatant fluid was tested in the catalase-AT system. If an autoxidizable agent had been present, the H_2O_2 generated slowly during subsequent aerobic oxidation would have been detected by the catalase-AT system. If, on the other hand, the supernatant fluid contained secreted H_2O_2, this would not be detected because the catalase-AT system is insensitive to the direct addition of H_2O_2 (6). In fact, no response was observed, and this result was consistent with the idea that H_2O_2 is secreted directly.

The data in Table 2 show measurements of H_2O_2 secretion by *M. pneumoniae* harvested after various days of growth. From the 3rd through the 5th day (samples 1–4), the amounts of H_2O_2 secreted in 1 hr by the total mycoplasma suspension ranged from 1.5 to 2.8 μmoles. On succeeding days, the viability, as measured in acid-forming units, declined markedly. On the 6th day (sample 5), despite the drop in viability, the ability to secrete H_2O_2 had not changed. However, by the 7th and 8th days (samples 6 and 7), H_2O_2 secretion was no longer detected. The latter result most probably reflected decreased metabolic activity, since the quantity of harvested microorganisms as measured by nitrogen (Kjeldahl) determination had not diminished. For example, samples 2 and 3 contained 0.06 and 0.20 mg of nitrogen per ml, respectively, whereas samples 5 and 6 contained 0.16 and 0.12 mg of nitrogen per ml, respectively.

In a separate series of experiments, washed cultures of *M. pneumoniae* harvested at various passage levels were prepared in Bethesda and then shipped in the cold to New York. We studied passage levels 15, 17, 19, 349, and 358, each harvested at 4 to 5 days of growth. H_2O_2 secretion was similar to that presented in Table 2.

Storage of washed mycoplasma suspensions. We studied the effect of storing mycoplasma suspesions at 0 to 5 C in the buffered medium employed

TABLE 2. *H_2O_2 secretion by M. pneumoniae, 339th passage level: effect of age of the culture*

Sample	Age of culture (days)	pH [a]	Acid-forming units[b]	H_2O_2[c]
1	3	7.25	10^9	1.5
2	4	6.65	10^{10}	2.8
3	5	5.30	10^9	1.8
4	5	5.25	10^{10}	2.8
5	6	5.30	10^7	2.0
6	7	5.30	10^7	<0.02[d]
7	8	5.30	10^4	<0.02[d]

[a] The *p*H listed is that of the culture fluid. The final *p*H of the washed suspension of organisms in buffer was 7.4.

[b] In a series of serial 10-fold dilutions of the mycoplasma suspensions, the number of acid-forming units (per milliliter of suspension) was given by the greatest dilution for which acid production was observed with a phenol red indicator. The measurement of acid-forming units is not better than ± 1 log unit. It is complicated by the tendency of mycoplasmas to cluster in a single acid-forming unit.

[c] Expressed as micromoles of H_2O_2 per hour per total suspension. Each sample consisted of a suspension of mycoplasma colonies scraped from a Povitsky bottle, washed, and then suspended in 10 ml (samples 1, 3, 4) or 20 ml (samples 2, 5, 6, 7) of buffered saline. Measurements of H_2O_2 secretion were started within 10 to 30 min after preparation of the suspension.

[d] No H_2O_2 was detected for samples 6 and 7. The values listed in the table are the limit of the method for 0.2 ml of cell suspension.

for preparation and shipment. Three preparations of *M. pneumoniae* showed no loss of activity after storage for 20 to 24 hr, whereas one preparation of *M. pneumoniae* and one of *M. gallisepticum* lost 40 to 60% of H_2O_2-secretion capacity during storage.

Accumulation of H_2O_2 in mycoplasma suspensions. *M. pneumoniae* and *M. gallisepticum* suspensions in buffered saline were incubated at 37 C with and without glucose. The changes in H_2O_2 concentrations from zero-time values were measured after 1 hr with a peroxidase method (Table 3). In the absence of glucose, some accumulation of H_2O_2 was seen in all specimens. The addition of glucose did not produce a consistent effect on the accumulation of H_2O_2. Two *M. pneumoniae* specimens (samples 13 and 14) and the *M. gallisepticum* preparation showed increased amounts of H_2O_2; the remaining *M. pneumoniae* preparations showed less H_2O_2 accumulation. One preparation (sample 10) actually showed a loss in H_2O_2 from that present at zero-time.

As shown in Table 3, we have compared H_2O_2 accumulation (peroxidase method) to H_2O_2 secretion (catalase-AT method). The addition of glucose markedly stimulated the secretion of H_2O_2 for all of the *M. pneumoniae* preparations; for the one preparation of *M. gallisepticum*, the effect of adding glucose was not ascertained because a maximal response (100% inhibition of catalase) had already occurred. From the measured rates of secretion, we expected very much greater accumulation of H_2O_2 than was observed with the peroxidase method. The amounts secreted in 1 hr exceeded the amounts that accumulated freely in the medium by roughly 10-fold or greater, except for

TABLE 3. *H_2O_2 accumulation (peroxidase method) compared to H_2O_2 secretion (catalase-aminotriazole method) for mycoplasma suspensions*[a]

Sample	Amt of H_2O_2 at zero-time	Peroxidase method for accumulation of H_2O_2[b]		Catalase-aminotriazole method for secretion of H_2O_2	
		Without glucose	With glucose	Without glucose	With glucose
	μmoles/ml				
M. pneumoniae					
8	0.038	0.022	0.010	0.044	0.112
9	0.031	0.013	0.007	0.065	>0.160[c]
10	0.034	0.012	(−) 0.023[d]	0.045	0.102
12	0.014	0.012	0.004	0.030	>0.160[c]
13	0.020	0.004	0.014	0.056	0.353
14	0.010	0.007	0.011	0.013	0.045
M. gallisepticum	0.007	0.014	0.018	>0.160[c]	>0.160[c]

[a] Expressed as micromoles per milliliter of mycoplasma suspension per hour.

[b] Concentration at 1 hr minus concentration at zero-time.

[c] Samples (0.2 ml) gave 100% inhibition of catalase at 1 hr.

[d] Decrease in H_2O_2 from zero-time value. This observation was confirmed in a repeat experiment.

sample 14, where only a fourfold differential was found. Sample 10 was particularly noteworthy in that no H_2O_2 accumulated despite a vigorous secretion of H_2O_2, as measured with the catalase-AT method. It should be emphasized that the H_2O_2 initially present did not interfere with secretion measurements because the catalase-AT system is insensitive to the direct addition of H_2O_2 (6).

Destruction of H_2O_2 by M. pneumoniae. In other experiments, exogenous H_2O_2 was added to the *M. pneumoniae* suspensions at zero-time. Changes in H_2O_2 concentrations were measured with the peroxidase method during subsequent incubation at 37 C for 1 hr (Table 4). For sample 10, without glucose, an H_2O_2 increment of 7% was noted; for sample 13, a decrement of 12% was noted. When glucose was added, the samples exhibited H_2O_2 losses of 46% and 25%, respectively; in other words, the addition of glucose stimulated a loss of H_2O_2.

DISCUSSION

We found that the secretion of H_2O_2 by the mycoplasmas was stimulated by glucose (Tables 1 and 3). Secretion of H_2O_2, stimulated by mannose or galactose, was reported for *M. gallisepticum* by Thomas and Bitensky (17). We have not attempted to identify the biochemical reactions in which H_2O_2 is formed. However, Smith et al. (14) have reported a reduced nicotinamide adenine dinucleotide (NADH) oxidase which generates H_2O_2 in *M. gallisepticum*; similar observations have been made for *Streptococcus faecalis* (8) and for human leukocytes during phagocytosis (9).

When catalase and AT were omitted so that the secreted H_2O_2 could accumulate in the mycoplasma suspensions, considerably less H_2O_2 collected than was predicted from the secretion rates (Table 3). These results could have been due, in part, to feedback control of H_2O_2 production; however, we observed a glucose-stimulated loss of H_2O_2 that had been added to the *M. pneumoniae* suspensions (Table 4). This glucose-stimulated, peroxidase-like activity may have been due to a NADH peroxidase, such as that described in *S. faecalis* (8, 13), or perhaps to an indirect dependency on reduced nicotinamide adenine dinucleotide phosphate, such as that exhibited by glutathione peroxidase of mammalian cells (3, 11).

TABLE 4. *Glucose-dependent destruction of exogenous H_2O_2 by M. pneumoniae*[a]

M. pneumoniae sample	Initial H_2O_2	H_2O_2 change[b]	
		Without glucose	With glucose
	μmoles/ml		
10	0.101	+0.007	−0.046
13	0.099	−0.012	−0.025

[a] Peroxidase method was used.

[b] Expressed as micromoles of H_2O_2 per milliliter per hour (measured 1 hr after zero-time).

The less-than-expected accumulation of H_2O_2 in the medium can be attributed to a dynamic interplay between the secretion and destruction of H_2O_2 by the mycoplasmas. In measurements made with the peroxidase method, continuous secretion and destruction of H_2O_2 took place simultaneously during the incubation period; undoubtedly, destruction interfered with accumulation of H_2O_2 in the medium (Table 3) and secretion interfered with measurements of the rate of destruction of added H_2O_2 (Table 4). On the other hand, secretion studies performed with the catalase-AT method were less liable to interference since the H_2O_2 did not accumulate, but was simultaneously measured and destroyed by the catalase in the medium during the entire incubation period; the method measures the steady-state concentration of the catalase-H_2O_2 complex (6), which is in dynamic equilibrium with residual free H_2O_2 in the medium. Destruction of H_2O_2 by catalase as it is secreted probably mimics the situation in vivo where secreted H_2O_2 impinges upon and reacts with adjacent tissues.

As a result of these observations, we recommend that H_2O_2-accumulation methods not be used to evaluate the potential for H_2O_2 secretion by microorganisms. The catalase-AT method can provide more reliable estimates of the capacity to secrete H_2O_2. These estimates would be of particular importance in attempting to evaluate the role of H_2O_2 in the virulence of microorganisms.

In pathogenesis, the accumulation of H_2O_2 may not be so critical as the maintenance of a low, but toxic, steady-state concentration of H_2O_2. It is known that reagent H_2O_2 delivered slowly and continuously to erythrocytes under steady-state conditions can cause oxidation of hemoglobin, lipid peroxidation, and lysis (3, 7). Methemoglobin formation and lysis of erythrocytes induced by mycoplasmas have been attributed to secretion of H_2O_2 (16, 17). In erythrocytes, the high concentrations of catalase would prevent accumulation of H_2O_2 and, therefore, the cellular damage is due, more likely, to the persistence of a low H_2O_2 concentration. As suggested elsewhere (5, 10, 16), secretion of H_2O_2 may be responsible for damage to the tissues of the respiratory tract during infection with *M. pneumoniae*.

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