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Relation of Catalase to Substrate Utilization by *Mycoplasma pneumoniae*

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No catalase activity was detected in four strains of glucose-grown *Mycoplasma pneumoniae* at any time during the replication of the organism. Exogenous catalase dramatically increased the O_2 uptake with glycerol, presumably by releasing inhibition caused by hydrogen peroxide. The effect of added catalase on the O_2 uptake of washed organisms with glucose as substrate was moderate and variable in degree. The production of hydrogen peroxide was demonstrated by the quantitative enzymatic assay for inorganic peroxide and by the fact that added pyruvate, which is nonenzymatically oxidized by H_2O_2 to acetic acid and CO_2 could mimic the action of catalase.

Studies of metabolic patterns of *Mycoplasma pneumoniae* are of interest in that they may lead to a better understanding of the organism's relationship to other members of the genus *Mycoplasma* and to bacteria.

During our studies of carbohydrate metabolism of *M. pneumon ae*, two aspects became of interest: (i) the absence of catalase activity in *M. pneumoniae*, and (ii) the effect of added catalase on substrate utilization.

MATERIALS AND METHODS

The following strains of *M. pneumoniae* were used: (i) the Mac strain, isolated in the 1940's, cultured on artificial medium from the 79th chick embryo lung passage, (ii) the FH strain isolated by C. Liu in the mid-1950's, kindly provided by L. Hayflick, (iii) the Bru strain, ATCC 15377, isolated directly on artificial medium by W. A. Clyde, and (iv) the Bart strain, received as the third agar passage after isolation from a recent primary atypical pneumonia case by R. Kundsin. Most of the work was done with the Mac strain at the 14th passage on artificial medium, but both early and adapted agar passages were tested.

The organisms were grown in shaking cultures. The medium used was that of Chanock, Hayflick, and Barile (3), with the addition of 1% dextrose and phenol red and with decreased concentration of horse serum (from 20 to 15%) and yeast extract (from 10 to 7.5%).

Log-phase organisms were harvested when the *p*H had dropped from 7.8 to 7.2 but well before acid accumulation had reached toxic levels, as previously determined from growth curve studies (11). The decrease in *p*H occurred at 7 days for early passages and 3 to 4 days for well-adapted passages. The organisms were centrifuged in a Sorvall centrifuge at 37,000 × *g* for 1 hr, washed once in 0.01 M phosphate buffer, and then resuspended in the same buffer to give a 10% cell suspension by volume.

Since determinations of optical density, nitrogen, or dry weight did not reflect accurately the physiological state of the cultures, viable counts were made. Standard Warburg manometric determinations were done. Each flask contained 10^9 CFU (colony-forming units) of *M. pneumoniae* in 0.3 ml, with substrates or other reactants added in 0.1-ml volumes at the final concentrations indicated on each figure; appropriate amounts of phosphate-saline buffer were added to make a total volume of 1 ml. Since bacterial contamination could alter the Warburg results, sterility tests were done on each flask.

Catalase was determined by the method of Bonnichsen, Chance, and Theorell (2). The material to be assayed was added to a 0.01 M solution of H_2O_2 in phosphate buffer. The decomposition of H_2O_2 was measured, after the enzymatic reaction had been stopped with sulfuric acid, by titrating the remaining substrate with permanganate.

Inorganic peroxide was measured enzymatically with horseradish peroxidase and *O*-dianisidine at room temperature, as described in Bergmeyer's *Methods of Enzymatic Analysis* (1).

Purified commercial catalase preparations used were obtained from Calbiochem, Los Angeles, Calif., Mann Research Laboratories, New York, N.Y., and Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Presence of catalase. Unwashed and washed, as well as concentrated. suspensions of the four strains of *M. pneumoniae* showed no catalase activity at any time during the replication of this organism.

Substrate utilization. In the Warburg system, O_2 uptake with glycerol was negligible in the

absence of catalase. Only very rarely, mostly with the FH and Bru strain, could one observe a very small and brief O_2 uptake of 20 to 40 μliters of O_2 above endogenous levels. The addition of catalase allowed glycerol to be oxidized, presumably by removing inhibitory levels of H_2O_2 (Fig. 1). Catalase had no effect on the endogenous respiration. Also, controls containing substrates, with or without catalase, showed no oxygen uptake, whether run without or with inactivated cells.

It was also observed that added pyruvate mimmicked the action of catalase at varying substrate concentrations (Fig. 2 and 3). At the lowest glycerol concentrations of 1.25 and 2.5 mM, the addition of 5 mM pyruvate allowed even higher O_2 uptake than with catalase. With higher glycerol levels of 10 and 20 mM (Fig. 3), pyruvate at the 5 mM concentration was less effective than catalase, but doubling the pyruvate concentration allowed comparable O_2 uptake for the first 2 hr. The O_2 uptake due to pyruvate alone during the time of these experiments was usually negligible, as shown by the control line in Fig. 3. With one exception (shown in the second line of Fig. 2) where some O_2 uptake was noted, pyruvate, over a concentration range of 2 to 200 mM, rarely had an O_2 uptake higher than the "endogenous" respiration. These data once more suggested the production of H_2O_2, since it is known that pyruvate is nonenzymatically oxidized to acetic acid

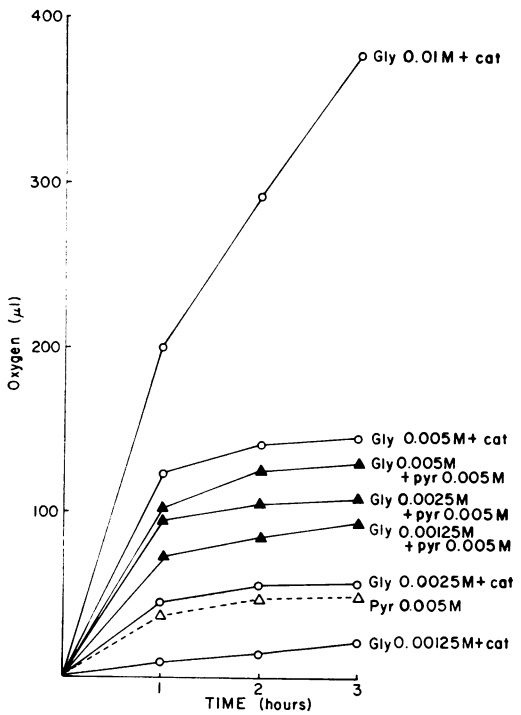

FIG. 2. *Warburg studies with Mycoplasma pneumoniae, Mac strain: effect on O_2 uptake of added catalase and pyruvate (pyr) with varying concentrations of glycerol as substrate.*

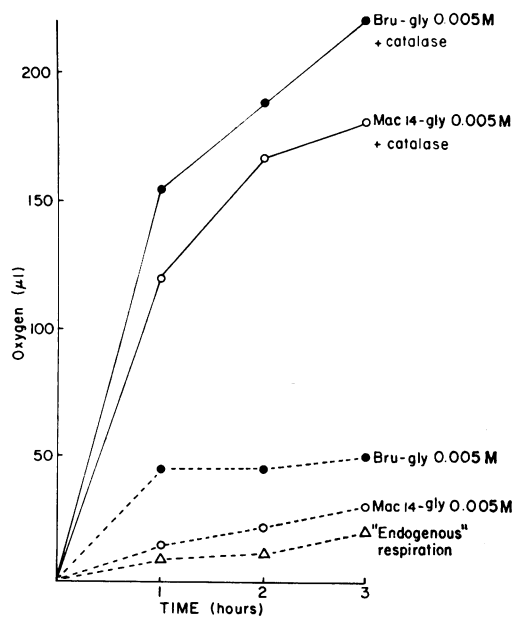

FIG. 1. *Warburg studies with Mycoplasma pneumoniae: effect of added catalase on O_2 uptake with glycerol (gly) as substrate.*

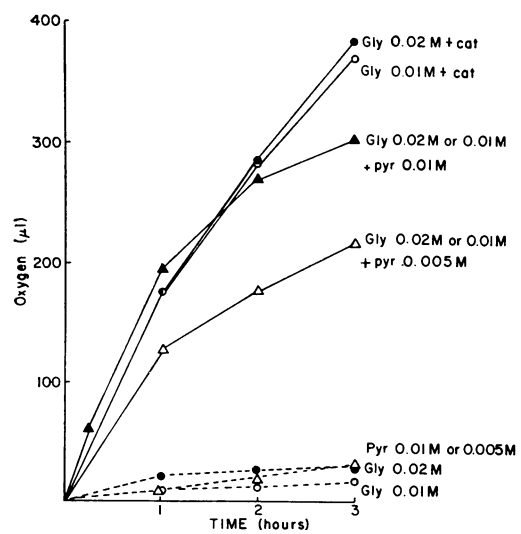

FIG. 3. *Warburg studies with Mycoplasma pneumoniae, Mac strain: effect on O_2 uptake with glycerol as substrate of added catalase and two concentrations of pyruvate.*

and CO_2. Some time after the completion of these studies, *M. pneumoniae* began to show O_2 uptake with pyruvate as substrate; we repeated all the experiments reported in this paper to assure that no other change had occurred in the metabolism of the organism.

Other evidence of H_2O_2 production was the direct titration with $KMnO_4$. In the flasks containing *M. pneumoniae*, glycerol, and catalase, no permanganate reacted beyond that expected from the blanks and controls. In the flasks with the organisms, glycerol, and heat-inactivated catalase, where O_2 uptake was inhibited, a small but significant permanganate titration was demonstrable; addition of fresh catalase prevented this permanganate reaction.

The enzymatic assay for inorganic peroxides provided more quantitative evidence for the production of a peroxide, presumably H_2O_2. Table 1 shows that 52 to 55 μg (approximately 1.5 μmole) of H_2O_2 formed per 10^9 glucose-grown, washed mycoplasmas, with glycerol as substrate over a 10-fold range of concentrations. A small amount of peroxide could be detected in the cells without substrate, but none was detected in those with glucose or catalase, in the heat-inactivated cell controls, or before incubation. The peroxide was formed within the 1st hr; further incubation had no effect on the total amount of H_2O_2 detected. Once the peroxide was formed, addition of catalase no longer stimulated O_2 uptake with glycerol as substrate; presumably this was caused by the toxic effect of the accumulated peroxide on the organism's respiration.

Enhancement of O_2 uptake by catalase in the presence of glucose was not as clear-cut, since glucose by itself markedly stimulated respiration with substrate utilization (Fig. 4). An average differential of 20 μliters of O_2 was statistically

TABLE 1. *Hydrogen peroxide determinations with Mycoplasma pneumoniae*[a]

Substrate	H_2O_2 (μg/ml)	
	Glucose-grown Mac or FH strain	Glycerol-grown FH strain
None	4	12
1% glucose (0.056 M)	0	20
1% glucose + catalase	0	0
Glycerol (0.01 M)	53–55	27–29
Glycerol (0.001 M)	52	26
Glycerol + catalase	0	0

[a] The organisms were present at a concentration of 10^9 colony-forming units per ml. No H_2O_2 was produced by heat-inactivated cells.

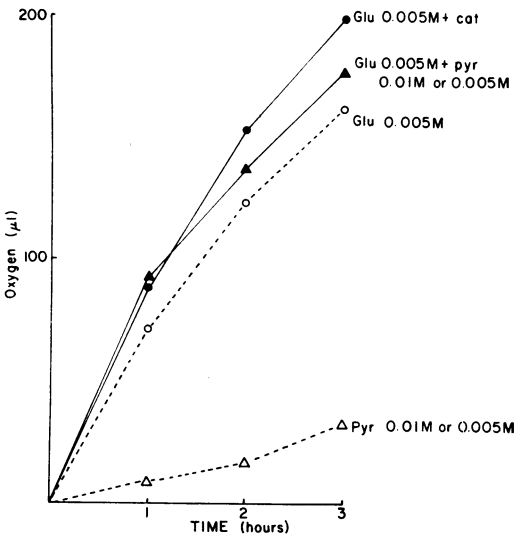

FIG. 4. *Warburg studies with Mycoplasma pneumoniae, Mac strain: effect of added catalase and pyruvate on O_2 uptake with glucose (glu) as substrate.*

significant. Addition of pyruvate also tended to increase glucose utilization, but the results were irregular. In short, the data are consistent with detectable H_2O_2 production that is quite variable and smaller in quantity than during glycerol oxidation.

That the amount of H_2O_2 freely available must be quite small is borne out by the data in Table 1, which show no detectable (i.e., less than 1 μM) H_2O_2 accumulation with glucose as substrate unless *M. pneumoniae* was pregrown in glycerol.

Experiments on the effect of exogenous H_2O_2 showed no inhibition of O_2 uptake with glucose until levels over 100 μM were reached. Concentrations of exogenous H_2O_2 up to approximately 300 μM became undetectable after incubation in flasks with viable organisms, as long as glucose was offered as substrate, but H_2O_2 was still present in those flasks with heat-inactivated *M. pneumoniae*.

Glycerol-grown cells. Recent studies with glycerol-grown (rather than the standard glucose-grown) cells showed marked inhibition of O_2 uptake with glucose unless catalase was added (Fig. 5). Table 1 shows that 20 μg of peroxide accumulated for the glycerol-grown organisms with glucose as substrate, approximately twice as much as without substrate.

The data would seem to indicate that the glycerol-grown cells, when tested as a resting-cell suspension with glycerol as substrate, produced only half as much H_2O_2 as glucose-grown organisms. However, it was extremely difficult to obtain

washed, concentrated "glycerol" cell suspensions with a comparable titer of 10^9 CFU/ml, and even those suspensions with a high titer were not as physiologically competent, as reflected by lower O_2 uptake. Both glycerol and H_2O_2 may have a toxic effect during the concentration and washing of the cells. Under the experimental conditions used, the relationship between cell concentration over a range of 10^8 to 5×10^9 CFU/ml and amount of H_2O_2 formed was not strictly proportional for either glucose- or glycerol-grown organisms.

In comparative studies with growing organisms, the complete Hayflick and Chanock medium was used. H_2O_2 formation could be measured with added glycerol but not when glucose was the main carbon source. With glycerol, 6 to 18 μg of H_2O_2 per ml could be detected when titers of 10^7 CFU/ml had been reached; the slower the growth rate, the more H_2O_2 was allowed to accumulate.

In addition to glucose, fructose and mannose were utilized by *M. pneumoniae*, as indicated by stimulation of O_2 uptake above endogenous levels and by acid production.

The standard glucose-grown cells showed no O_2 uptake with other monosaccharides, such as galactose, arabinose, ribose, and xylose, or with disaccharides, such as sucrose, maltose, and lactose, as substrates. Pregrowing the cells in the presence of galactose or the disaccharides had no effect. Since starch and dextrin had been reported as being fermented by *M. pneumoniae*, these two polysaccharides were tested, but only dextrin showed a small O_2 uptake. Other substrates involved in the Embden-Meyerhof pathway that showed O_2 uptake were lactate and, recently, pyruvate. In all these experiments, exogenous catalase had no demonstrable effect, except perhaps with fructose where a consistent but very small increment in O_2 uptake occurred.

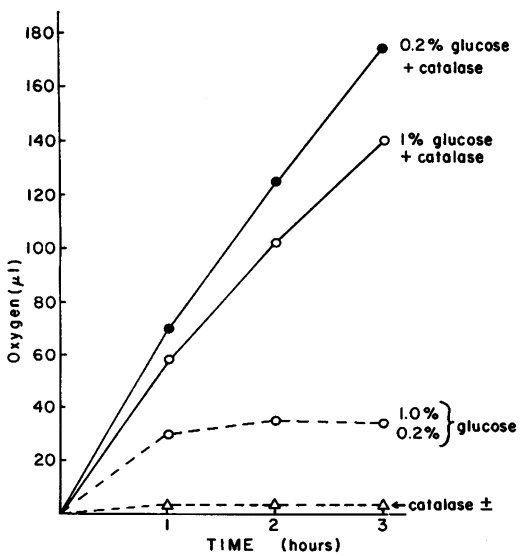

FIG. 5. *Effect of exogenous catalase on O_2 uptake with glucose as substrate in the Warburg system: glycerol-grown Mycoplasma pneumoniae FH strain.*

TABLE 2. *Inhibition of glycerol oxidation*

Inhibitor	Concn	Substrate	Inhibition
	M		%
Na azide	0.0001	Glycerol and pyruvate	0
	0.001		20
	0.01		30–40
	0.1		50–60
Iodoacetic acid	0.001	Glycerol and catalase	10
	0.001		50
	0.01		100
NaF	0.001	Glycerol and catalase	15
	0.003		15
	0.01		40[a]
	0.1		100

[a] Reversed by pyruvate, 0.05 M.

Inhibitor studies. Studies of the action of inhibitors on O_2 uptake with glycerol (Table 2) showed relative insensitivity to azide, a Fe^{+++} inhibitor. At 0.01 M concentration, only 30 to 40% inhibition had occurred, and even at 0.1 M azide nearly half the O_2 uptake was unimpaired. The O_2 uptake showed greatest sensitivity to iodoacetic acid, the SH group inhibitor used as a blocking agent of triose phosphate dehydrogenase activity. There was 50% inhibition at 0.001 M and a 100% inhibition at 0.01 M. The inhibitor studies showed moderate sensitivity to NaF, an inhibitor mainly of enolase activity; 100% inhibition was reached only at 0.1 M concentration. The 40% inhibition at 0.01 M could be completely reversed by 0.005 M pyruvate.

DISCUSSION

The lack of catalase activity in four strains of *M. pneumoniae* seemed unrelated to the stage of the growth cycle or the number of passages on artificial medium. This agrees with the findings for most other mycoplasmas (23). A notable exception was the demonstration of catalase activity in *M. mycoides* reported by Rodwell and Rodwell (15), who measured O_2 evolution from H_2O_2. However, Freundt (6) found no catalase activity with his strains of *M. mycoides*. The other positive report was Lecce and Morton's qualitative findings in the Campo strain (10) and in *M. hominis*. The presence of catalase in the latter

organism, a nonfermentative human mycoplasma, was confirmed recently by VanDemark and Smith (22). A word of caution has been introduced by VanDemark (21) in his report that the nature of the growth medium and method of cell washing were critical for catalase assays. He found 8 of 14 *Mycoplasma* strains catalase-positive. Also, with the usually catalase-negative group D streptococci, a few catalase-positive strains of *S. faecalis* isolated from silage were found to lose their catalase-like activity with serial transfers in broth (8). For these reasons, we examined both washed and unwashed cell suspensions, as well as recently isolated *M. pneumoniae* strains.

The data presented for the effect of exogenous catalase on glycerol utilization, as well as for the inhibitor studies, are in essential agreement with the pathway of glycerol oxidation worked out for *S. faecalis* by Gunsalus and Umbreit (7). A very similar pathway exists for *M. mycoides*, as was clearly shown by Rodwell and Rodwell (15). The glycerol was oxidized via glycerol phosphate, with H_2O_2 formation, and entered the Embden-Meyerhof pathway at the triose phosphate level.

There is a difference between results obtained with bacteria and those of the two *Mycoplasma* studies. In bacteria (7), exogenous catalase decomposes the H_2O_2, allowing higher levels of substrate oxidation. However, with half of the O_2 going into H_2O_2 formation, evolution of O_2 caused by catalase tends to balance uptake and net O_2 uptake is actually decreased. One exception was the glucose oxidation of another strain of *S. faecalis* (14), where the amount of H_2O_2 formed was quite variable, though some decrease in O_2 uptake was also noted with catalase. Keilin and Hartree (9) pointed out that the higher O_2 uptake may be due to secondary oxidation, but in their experiments glycerol did not undergo coupled oxidation in the presence of catalase nor did our own experiments show that any such peroxidation takes place.

For *M. pneumoniae*, calculations based on the actual peroxide levels found with glycerol would indicate a release of 34 to 36 μliters of O_2 upon the addition of catalase; this amount is probably completely "masked" by the ensuing O_2 uptake with glycerol once the inhibitory levels of H_2O_2 are removed.

The absence of peroxide accumulation with glucose as substrate could be explained by the very rapid reaction with pyruvic acid which is formed during respiration. However, with streptococci, earlier workers (5, 16, 17; E. C. Greisen and I. C. Gunsalus, J. Bacteriol. **45**:16, 1943), who were also puzzled that the flavin-terminated respiration did not always yield hydrogen peroxide, suggested that certain strains carry out non-hematin dependent peroxidase-like reactions in the presence of some organic substrates. Seeley and VanDemark (17) found the peroxidation mechanism with *Streptococcus faecalis* B33A to be acquired under highly aerobic conditions, so that no H_2O_2 accumulated and added H_2O_2 could be removed from the medium during the metabolism of certain substrates, including glucose.

Most of the literature on bacteria and hydrogen peroxide deals with the toxicity of exogenously added peroxide. The most extensive data are those of McLeod and Gordon (13), which give inhibitory levels of 27 to 85 μg/ml under aerobic conditions for the catalase-negative, peroxide-producing class of bacteria most comparable to *M. pneumoniae*.

Thus, the amounts of peroxide produced and the toxic levels of exogenous peroxide with *M. pneumoniae* are within the range of bacterial systems. However, a greater sensitivity may be implied since with the glycerol system of *S. faecalis* (7) a certain level of substrate utilization was allowed despite H_2O_2 formation, whereas with *M. pneumoniae* glycerol utilization cannot proceed without catalase addition. Further comparisons of inhibitory concentrations of peroxide between bacteria and mycoplasmas are difficult without proper quantitation of mass or viable numbers of the different organisms and evaluation of the effect of various medium components on the toxicity of peroxides.

Hydrogen peroxide production by *M. pneumoniae*, first reported in 1965 (I. E. Low, M. D. Eaton, and P. Proctor, Bacteriol. Proc., p. 77), has been made relevant by the interesting finding of Somerson et al. (19) that a peroxide is responsible for the rapid and complete lysis of certain red blood cells. Hemolysis is a characteristic distinguishing *M. pneumoniae* from other human mycoplasma, though it is also found in *M. laidlawii* and in such pathogenic animal strains as *M. gallisepticum* and *M. neurolyticum* (20).

These findings have been related to virulence by Chanock (4), who postulated that alteration of the red blood cell surface by the hemolysin resulted in a new antigen and production of antibody, specifically cold agglutinins, and by Somerson et al. (19), who suggested a direct action of the peroxide on cells of the respiratory tract. The similarities in metabolism of *M. pneumoniae* to *M. mycoides* and some of the streptococci continue to intrigue us, and we hope to continue to explore the physiology of *M. pneumoniae* and to discern other similarities and differences with reference to *M. pneumoniae's* theoretical bacterial ancestors and their L forms.

At present, the presence of an aerobic glycerol pathway with hydrogen peroxide formation, the lack of catalase, the resistance to methylene blue

inhibition, and the relative heat resistance in *M. pneumoniae* are taxonomic properties found in group D streptococci. The lack of a cell wall in *M. pneumoniae*, of course, predicts the absence of many other properties.

However, negative results so far from deoxyribonucleic acid homology (12, 18, 24) and serological studies, added to the fact that, at present, no confirmed reversions to bacterial forms have occurred with *M. pneumoniae*, point to the need for further study. Whether, then, the finding of pathways that are relatively unique for certain bacterial species is significant remains to be determined. The hope that several pathways of taxonomic significance can be demonstrated would make worthwhile a renewed search for genetic relationships between *M. pneumoniae* and bacteria.

As a practical corollary, these metabolic studies may lead to an understanding of the complex nutritional requirements and perhaps to a simplified and more defined medium for the growth of *M. pneumoniae*.

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