References and Notes

1. Marketed by National Instrument Laboratories, Bethesda, Md., as the N.I.L.–Saravis ultrafiltration apparatus. The unit used in these studies was 2.5 cm (inside diameter) by 30 cm; it held 140 ml of fluid. The total exchange area was 243 cm^2.
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5. The filtration unit was built to specifications supplied by Amicon.
6. Polypropylene tubing and fittings (Imperial Eastman) were obtained from A. E. Borden Co., Boston, Mass.
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28 June 1965

Hemolysin of Mycoplasma pneumoniae: Tentative Identification as a Peroxide

Abstract. Mycoplasma pneumoniae *produces a soluble hemolysin active against guinea pig erythrocytes. This hemolysin appears to be a peroxide, since catalase or peroxidase inhibits its activity. The action of catalase and peroxidase is specific, since heating the enzymes abolishes their effect on the hemolysin. In addition, 3-amino-1,2,4-triazole, a potent inhibitor of catalase, reverses the inhibitory effect of the enzyme. The hemolysin of* M. laidlawii *is also a peroxide. The hemolysins of* M. pneumoniae *and* M. laidlawii *seem unique for microbial organisms since the bacterial hemolysins which have been described have been protein or lipid in nature.*

Colonies of *Mycoplasma pneumoniae* and *M. laidlawii* produce a soluble hemolysin active against guinea pig and sheep erythrocytes (*1*). When an agar culture containing mycoplasma colonies is overlaid with agar containing red blood cells, each colony releases sufficient hemolysin to produce a circular area of complete or almost complete red cell lysis. We call these areas hemolytic plaques. Plaques also develop when horse, human, or rabbit erythrocytes are used, but with these cells hemolysis is often incomplete. We have speculated that the antibodies to red cells, which develop in some cases of primary atypical pneumonia (cold agglutinins) may be related to the action of the *M. pneumoniae* hemolysin (*2*).

The hemolysin of *M. pneumoniae* is extremely labile, because hemolysis does not occur when a mixture of red cells and agar is added to solid medium on which mycoplasma colonies had been grown and then removed. Omission of yeast extract from the mycoplasma growth medium, or incubation under reduced oxygen tension inhibits hemolysin production (*3*). That hemolysin passes through a viscose dialysis membrane indicates its low molecular weight and probable nonprotein nature.

We studied the nature of the hemolysin by incorporating specific enzymes in the erythrocyte-agar overlay and determined which enzymes inhibited lysis. Plastic dishes containing 5 to 6 ml of mycoplasma agar medium (*4*) were inoculated with a broth suspension of *M. pneumoniae*. The inoculum produced approximately 100 to 300 colonies after 7 to 10 days of aerobic incubation. In the first experiment, the addition of 12,000 units of catalase (*5*) into the erythrocyte-agar overlay completely suppressed the development of hemolytic plaques (Fig. 1). Plaque production could be prevented with 400, but not with 40, catalase units. The addition of boiled catalase did not affect plaque production. These observations indicated that peroxide was involved in the hemolytic reaction.

The incorporation of catalase directly into the overlay did not reduce the viability of the mycoplasma colonies. The contents of *M. pneumoniae* agar cultures overlaid with red cell-agar containing added catalase or boiled catalase were ground into a broth suspension (20 percent) which was then titrated for viable organisms. Decimal dilutions of the suspensions were inoculated onto mycoplasma agar medium. Cultures overlaid with either catalase or boiled catalase contained 2×10^9 colony-forming units.

Peroxide could produce hemolysis either by direct action on the red cells, or indirectly by reacting with constituents of the erythrocyte-agar medium to release hemolysin or induce its production. To determine whether the hemolysin itself was a peroxide, various enzyme preparations were enclosed in a double-dialysis membrane placed over the colonies. The hemolysin could diffuse through a dialysis membrane and still lyse guinea pig erythrocytes placed over the membrane (*3*). Viscose tubing (Visking brand, 44-mm flat width, 0.0254 mm thickness) was cut to fit the agar surface and moistened with Alsever's solution. Approximately 0.15 ml of various enzyme solutions, including catalase (30,000 unit/ml) and trypsin (25 mg/ml) were inserted between the layers of the dialysis membrane. To prevent bacterial contamination, 150 units of penicillin was added to each enzyme preparation. The solution was dispersed throughout the dialysis "bag" by exerting finger pressure. The dialysis bag was placed over the colony-containing agar surface, and the plates were then overlaid with 3 ml of a guinea pig erythrocyte and agar mixture. The catalase, acting as a barrier between the colonies on the agar surface and the erythrocyte in the agar overlay, completely suppressed hemolysis (Fig. 2). However, plaques of hemolyzed cells were observed in areas not covered by the membrane, particularly at the periphery of the plate. When trypsin or boiled catalase was placed in the dialysis bag, hemolytic plaques developed throughout the overlay. These findings indicate that the *M. pneumoniae* hemolysin is a peroxide.

The specificity of the catalase effect

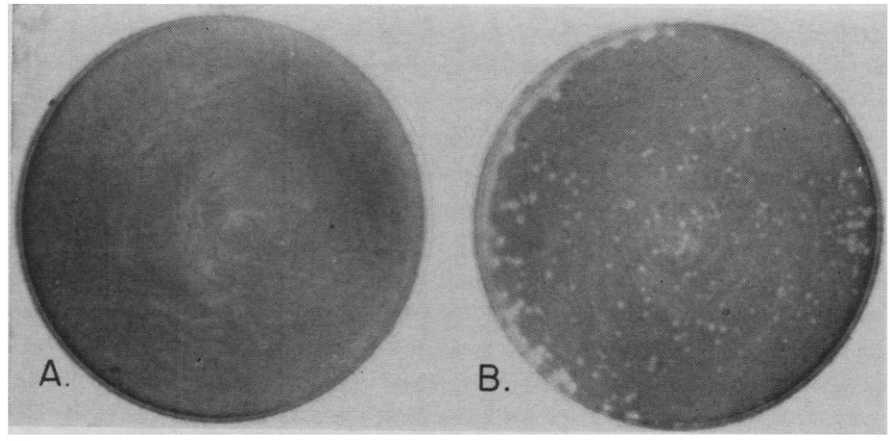

Fig. 1. Effect of catalase on development of *M. pneumoniae* hemolytic plaques. (*A*) 12,000 units of catalase added to guinea pig erythrocyte–agar overlay. (*B*) Catalase, boiled for 30 minutes, added to overlay.

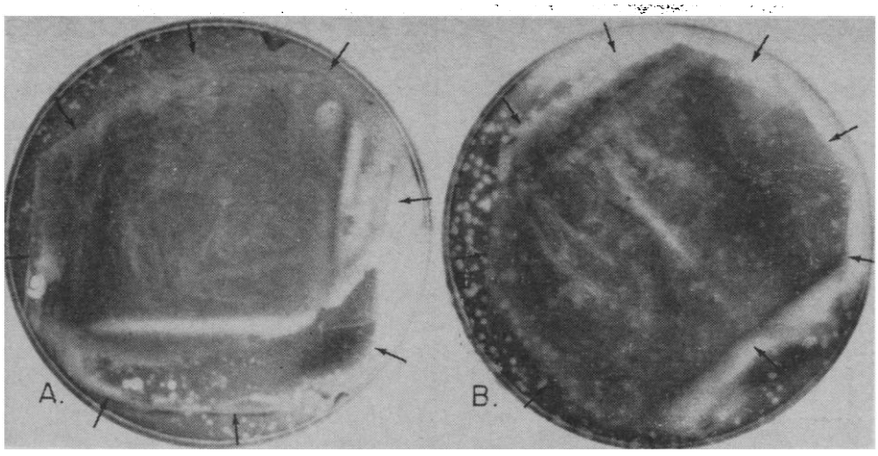

Fig. 2. Inactivation of *M. pneumoniae* hemolysin by catalase interposed between mycopiasma colonies and guinea pig erythrocyte–agar overlay. Catalase solution was placed in a dialysis bag which was then placed over the surface of an agar plate containing *M. pneumoniae* colonies. The plate was then overlaid with guinea pig erythrocytes in agar. (*A*) A portion of catalase solution (0.15 ml, 4500 units) was placed in the dialysis bag. (*B*) Same as *A* except catalase was boiled for 30 minutes. In *A*, hemolytic plaques are absent over the areas covered by the dialysis bag containing active catalase. However, plaques developed in the areas not covered by the dialysis bag or where erythrocyte–agar had seeped beneath the bag, that is, the lower 1/6th of the area covered by the dialysis bag in *A*. Plaques developed throughout the area covered by the dialysis bag containing heated catalase.

was further indicated when the potent catalase inhibitor, 3-amino-1,2,4-triazole (AT) (*6*) reversed catalase inhibition of hemolysis. This counter effect of AT was directly related to the ratio of enzyme to AT. In experiments in which catalase was incorporated into the erythrocyte-agar overlay, approximately 660 μg of AT completely reversed the inhibition produced by 200 units of catalase. With lower concentrations of AT a partial effect on catalase inhibition of hemolysis was observed, namely, smaller plaques, a delay in plaque formation, and incomplete lysis of red cells. In other experiments in which catalase and AT were placed within a "dialysis bag," 190 μg of AT reversed the effect of 560 units of catalase; the same phenomenon could be demonstrated with 375 μg of AT to inhibit the activity of 1125 μg of catalase. It has been suggested that AT irreversibly inhibits catalase only after formation of a catalase-peroxide complex (*6*). Some investigators have used AT to detect the presence of hydrogen peroxide (*7*). Thus, it appears unlikely that the action of the catalase in inhibiting hemolysis is due to its hematin nature or that the effect of the catalase is nonenzymatic.

The mycoplasma growth medium contains catalase activity, since the horse-serum component is not inactivated by heat before addition to the basal medium. However, this quantity of catalase is not sufficient to completely inhibit the action of *M. pneumoniae* hemolysin. Catalase in the agar medium appears to inhibit the action of hemolysin partially since the addition of AT to the erythrocyte-agar overlay resulted in an increase in the diameter of the hemolytic plaques; plaque size was proportional to the quantity of AT added (1.3 to 5.2 mg). The failure of red cell catalase to inhibit hemolysis is probably also explainable on the basis of quantitative considerations.

Horseradish peroxidase was used to confirm the identity of the *M. pneumoniae* hemolysin. Peroxidase inhibited the development of hemolytic plaques, whereas boiled peroxidase was without effect. In experiments in which peroxidase (4 mg) was added to the overlay, hydrogen donors such as ascorbic acid enhanced the inhibitory effect of peroxidase.

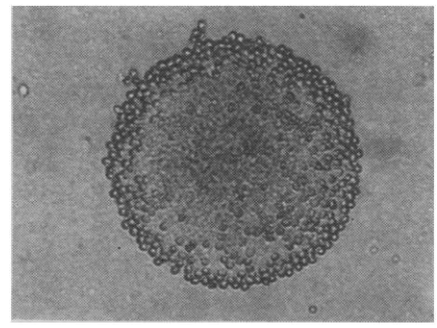

Fig. 3. Hemadsorption of guinea-pig erythrocytes to a colony of *M. pneumoniae* (× 165).

For comparison, the hemolysin produced by *M. laidlawii* was studied, and it also appears to be a peroxide. Addition of catalase to the erythrocyte-agar mixture inhibited plaque formation by *M. laidlawii*; boiled catalase was without effect.

Thus the hemolysins produced by *M. pneumoniae* and *M. laidlawii* seem to be peroxides. Both catalase and peroxidase are specific enzymes acting on the substrates hydrogen peroxide, ethyl peroxide, or methyl peroxide (*8*). The hemolysins of *M. pneumoniae* and *M. laidlawii* seem different from those of other microbial organisms; other bacterial hemolysins studied are protein or lipid in nature (*9*). Since *M. pneumoniae* has no catalase activity (*10*), hydrogen peroxide may be the terminal product of the aerobic glucose metabolism of this organism.

Del Guidice and Pavia (*11*) reported the hemadsorption of red blood cells to colonies of *M. pneumoniae*. We have confirmed these observations with both guinea-pig and human erythrocytes (Fig. 3). This direct contact between mycoplasma and red blood cells could allow in vivo diffusion of peroxide to the red cell membrane without exposure to the catalase activity found in most tissues. Peroxide toxicity for red blood cells has been amply documented. Peroxide can inhibit the glyoxylase activity of intact red cells (*12*). Peroxides decrease the resistance of red blood cells to hypotonicity (*13*). Thus, the peroxide produced by *M. pneumoniae* might affect red blood cells by altering their (i) biochemical activities, (ii) osmotic fragility, and (iii) antigenicity and type of hemoglobin (*14*). The erythrocytes, altered by the action of peroxide, could then stimulate the formation of cold agglutinins.

Among the human mycoplasma species *M. pneumoniae* produces the most rapid and complete lysis of erythrocytes. This organism is also the most virulent of the mycoplasmas that infect man (*2*). It is possible that virulence of *M. pneumoniae* is due, in part, to an action of peroxide on tissues of the respiratory tract.

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28 July 1965

Transfer of Learned Response by RNA Injection

In their report of an experiment on the performance of untrained rats injected with ribonucleic acid from the brains of trained rats [*Science* **149**, 656 (1965)], Babich, Jacobson, Bubash, and Jacobson properly caution that the data do not conclusively demonstrate transfer of training, and that we cannot be confident that RNA was the active factor in the injections. This being the case, the title of the report, "Transfer of a response to naive rats by injection of RNA extracted from trained rats," makes an excessive claim which could lead to misunderstanding.

Other problems of logic and design typical of much RNA-learning research are illustrated by the research in question. Let us look at some of the obvious requirements:

1) Precise definition of the response. In this study, rats were trained to *eat* from a food cup at the sound of the feeder. But a mere *approach* to the general area of the sound source was counted as a response in the injected rats. These are clearly not the same response patterns. Did the rats with experimental injections make consummatory responses, such as licking or chewing the food cup? Did they, indeed, pay any attention to the food cup at all?

2) Proper control groups. The authors say only that the experimental and control groups from which the RNA was obtained had been given equal amounts of food. Were the control rats also subjected to magazine clicks in equal number, and were they fed in the Skinner box without association with the clicks? Were they even adapted to the box? Were they handled equally often and in the same manner as the experimental rats? A deprived group of controls might well have fewer "activating" brain factors.

3) A behavioral criterion capable of discriminating between pseudoconditioning and learning. Generalized greater responsiveness is likely under the conditions employed, and the criterion must provide a test for true learning. Would the experimental injected rats also orient to a flashing light at the end of the cage opposite the food cup? A test of choice or discrimination behavior would have made possible a clear-cut distinction between simple activation and specific transfer.

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16 August 1965

Carney is in error in asserting that the principal response in question was eating from the food cup. As we stated in our report, the operant response to the discriminative stimulus (click) was running to the food cup. The rat was rewarded for this behavior with a food pellet, and consumption of the pellet was the last step in the chain of behavior. The link which concerned us was that between the click and the cup-approach response for which the click was discriminative.

We regret that Carney found our title misleading.

Carney is correct in pointing out the several differences between our experimental and control donor animals. This was the major reason we were cautious in our conclusions. In a subsequent experiment (*Science*, in press) this problem has been overcome: One group of donor rats was magazine-trained with click as discriminative stimulus, a second group with a blinking light (at the end of the chamber opposite the food cup) as a discriminative stimulus. Handling, box adaptation, and so forth were identical for the two groups. On testing, recipient rats responded (approached the food cup) predominantly to the stimulus with which their respective donor rats had been trained. In another experiment (*Proc. Nat. Acad. Sci.*, in press), control donors, instead of being untreated, were matched to experimental donors in terms of handling and adaptation to box and click. The behavioral differences between experimental and control recipients were similar to those described in our first *Science* report. The donor animals in this new experiment were hamsters and the recipient animals were rats. The RNA transfer effect has also held up in our laboratories (i) in a two-alternative maze apparatus, (ii) in classical conditioning of planarians (with pseudo-conditioning controls, and replicated four times), and (iii) with purified RNA in the case of planarians. An experiment with purified RNA in rats is now under way.

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14 September 1965