# Effect of Ethanol on the Clearance of Airborne Pneumococci and the Rate of Pneumococcal Transformations in the Lung

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The rate of clearance of pneumococci from the lungs of mice infected by aerosols was found to vary inversely with the virulence of the pneumococci. Treatment of of the mice with ethanol delayed the clearance and increased the rate of pneumococcal transformations in vivo.

The transfer of genetic information from one *Pneumococcus* to another by transformation with freshly released deoxyribonucleic acid is known to occur both in vitro (1, 10, 16) and in vivo (4, 8, 18, 19). Conant and Sawyer (4) have recently reported that it will occasionally occur in mice infected by intrabronchial inoculation. The objective of the present study has been to design an experimental model in which pneumococcal transformation will occur consistently in the lungs. The design is based on the evidence that (i) pneumococci must multiply rapidly and accumulate in relatively large numbers for transformation to be efficient (4) and (ii) that ethanol intoxication will slow the pulmonary clearance of airborne bacteria (6, 12, 20, 23).

## MATERIALS AND METHODS

**Mice.** Strain CD-1, male, pathogen-free albino mice weighing 21 to 25 g were obtained from Charles River Laboratories, Wilmington, Mass. The lungs of these mice are essentially devoid of bacteria.

**Antibiotics.** Streptomycin sulfate (Sm; Eli Lilly & Co., Indianapolis, Ind.) was added to blood-agar medium to a final concentration of 500 µg/ml. Erythromycin gluceptate (ery; Eli Lilly & Co.) was added to a final concentration of 0.1 µg/ml.

**Deoxyribonuclease.** Pancreatic deoxyribonuclease (1X recrystallized, Worthington Biochemical Corp., Freehold, N.J.) was used in a final concentration of 10 µg/ml together with a final concentration of 0.007% $MgSO_4 \cdot 7H_2O$.

**Ethanol and its determination in murine blood.** Absolute ethanol, diluted to 10% in saline, was in-

jected intraperitoneally in doses of 0.25, 0.50, or 0.75% of body weight. Saline was used for the controls. Blood alcohol levels were measured by a colorimetric micro procedure (Determatube C-ALC; Worthington Biochemical Corp.) and expressed as per cent blood alcohol (grams of alcohol/100 ml of blood) based on the average value in ten mice.

**Pneumococcal strains.** The strains used have been fully described (19). The pertinent characteristics of these strains are presented in Table 1.

**Growth media and conditions.** Fluid cultures were grown in freshly prepared beef heart infusion broth (1 lb. of fresh beef heart per liter of water) containing 1% Neopeptone (Difco Laboratories, Inc., Detroit, Mich.) and adjusted to pH 7.6 to 7.8. (BHI). Stock cultures were grown in BHI containing 5% fresh defibrinated rabbit blood. Cultures for animal inoculation were grown at 37 C for 8 to 16 hr in BHI containing 10% human serum. Trypticase Soy Agar (BBL, adjusted to pH 7.8) with 5% sterile citrated horse blood and with streptomycin or erythromycin or both was used for selection of parental and transformed bacteria. Dilutions were made with Trypticase Soy Broth (BBL).

Cultures were concentrated by centrifugation at 1,500 × g for 1 hr at 4 C and resuspension in BHI.

**Estimation of bacteria in murine tissues.** Lungs were removed aseptically and homogenized in 2 ml of Trypticase Soy Broth with a sterile mortar and pestle. Dilutions of the homogenate were distributed over the surfaces of blood agar plates with a spreader. Sectors of selective blood agar plates were also streaked with 0.01 ml of cardiac blood. All plates were incubated for 24 to 48 hr at 37 C.

**Intranasal inoculation of mice.** Approximately 0.04 ml of bacterial suspension was dropped from a 27-gauge needle onto the nose of each mouse (anesthetized by ether) to permit inhalation.

**Aerosol inoculation of mice.** A modification of the aerosol apparatus of Schulman and Kilbourne (22) was constructed to achieve an airborne infection of the murine lung (Fig. 1). Mercuric chloride in 70% ethanol (1:100, wt/v) was placed in the gas washing

FIG. 1. Aerosol apparatus.

TABLE 1. Characteristics of pneumococcal strains

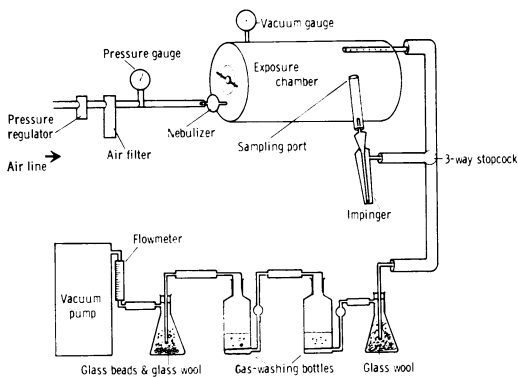| Strain designation | Streptomycin resistance (1 mg/ml) | Erythromycin resistance (0.1 µg/ml) | Appearance of colony on agar |
|---|---|---|---|
| R36NCSm$^r$ | +$^a$ | −$^b$ | Rough |
| R36NCery$^r$ | − | + | Rough |
| IIIS-1R6 | − | − | Smooth (small) |
| IIIS-R36NCSm$^r$ | + | − | Smooth (large) |
| CD-VII-Sm$^r$ | + | − | Smooth (small) |
| CD-VII-Sm$^r$ery$^r$ | + | + | Smooth (small) |

$^a$ Denotes resistance.
$^b$ Denotes sensitivity.

bottles as a disinfectant. Mice were exposed to aerosols of varying concentrations of pneumococci for 20 min. To determine the dose administered, air in the exposure chamber was sampled with an impinger type of sampling device during the last 2 min of aerosolization.

**Pulmonary clearance of pneumococci.** The term clearance is used to express loss of viable organisms from the lung. The bacteria may have been either killed or removed from the respiratory tract. Exposure of mice to an aerosol of pneumococci resulted in the deposition of approximately $10^{5.3}$ colony forming units (CFU) per lung when concentrated suspensions of strains R36NC, IIIS-1R6, and CD-VII-Sm$^r$ were nebulized. Because the large amount of capsular material enveloping cells of strain IIIS-A/66 prevented efficient concentration, only $10^{4.5}$ CFU per lung of this strain could be deposited.

After exposure to the aerosol, mice were injected intraperitoneally either with saline or with 10% ethanol. At intervals, eight or more mice were sacrificed, and the number of viable bacteria per lung was determined.

**Transformation system.** Two combinations of pneumococcal strains were used: R36NCery$^r$ with CD-VII-Sm$^r$ and R36NCSm$^r$ with IIIS-1R6. Transformants in the former system were CD-III-Sm$^r$ery$^r$, and transformants in the latter were IIIS-R36NCSm$^r$ (Table 1). The same protocol was followed for both. The rough pneumococci were inoculated intranasally into mice which were then exposed to an aerosol of the encapsulated strain. Some animals were injected intraperitoneally with saline and some with ethanol (0.75% body weight). Mice were sacrificed at 6, 12, 24, 48, and 72 hr after the infections, at which times heart blood and lungs were assayed for parental and transformed bacteria.

**Controls.** Individual cultures (with substitution of sterile BHI for the second strain) were used as controls. Deoxyribonuclease was shown to destroy transforming activity in the lung in the following way. At 8 hr after infection, 0.5 ml of serum broth (with or without deoxyribonuclease) was injected into the lungs through a needle inserted into the trachea of the anesthetized (sodium pentobarbital) mouse. The

intact lungs were removed, covered with serum broth (with or without deoxyribonuclease), and incubated at 37 C for 12 hr. The lungs were ground with mortar and pestle and assayed for transformants as described.

## RESULTS

**Aerosol apparatus.** The aerosol apparatus used in these experiments was extremely efficient. Figure 2 illustrates the reproducibility of the airborne inoculum. Each point represents an individual experiment in which 10 mice were exposed to an aerosol, sacrificed, and assayed for pneumococci deposited in their lungs. Samples of nebulizer fluid and sampler fluid were also assayed. There is a linear increase in the number of bacteria in the air (reflected by sampler assay) and in the lungs in response to increasing concentrations of bacteria in the nebulizer. In each experiment, all the mice received approximately the same number of bacteria as manifested by the small standard deviation.

**Effect of ethanol on pneumococcal pulmonary infections.** As long as 4 hr after the intraperitoneal injection of increasing amounts of ethanol, the level of alcohol in the blood reflected the initial dose of drug. No alcohol was detected in the blood of control animals (Fig. 3). A direct correlation was found between the dosage of ethanol and the percentage of rough pneumococci retained in the lungs 4 hr after administration of alcohol (Fig. 4). Although injection of 0.75% ethanol significantly impaired the pulmonary clearance of strains CD-VII-Sm$^r$ and IIIS-1R6, the results with lower doses were equivocal. Hence, 0.75% alcohol was used in all subsequent experiments. Since the blood content of alcohol after the dose decreased linearly with time (Fig. 5), studies of the clearance of the
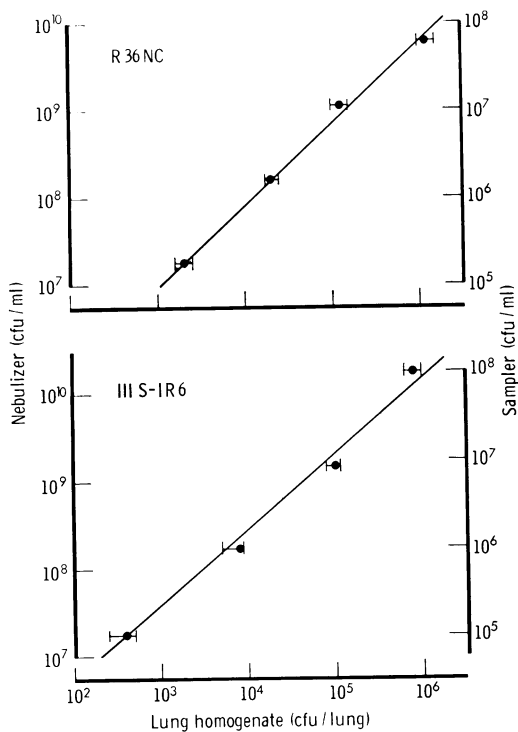
FIG. 2. *Number of pneumococci in nebulizer, sampler, and mouse lung after exposure to aerosol.*



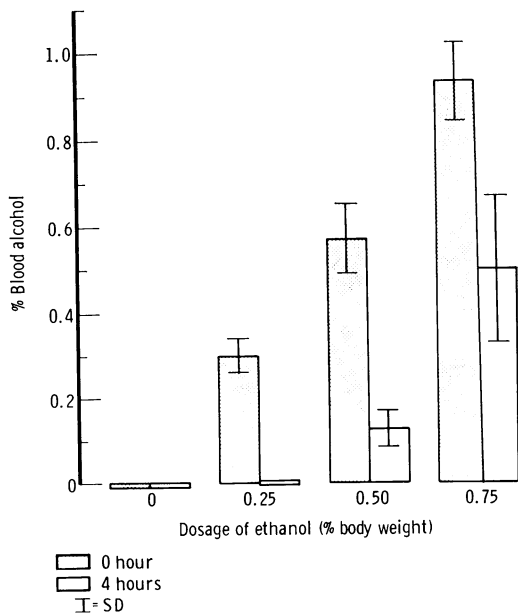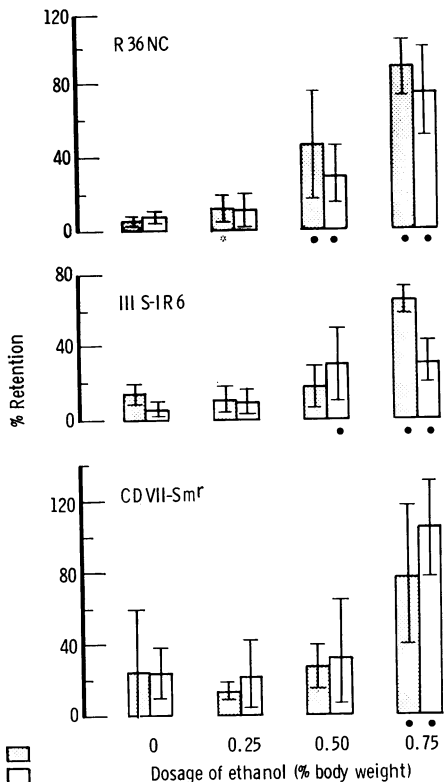FIG. 4. *Per cent pneumococci retained at 4 hr after varying doses of ethanol given intraperitoneally.*



FIG. 3. *Levels of alcohol in murine blood after varying doses of ethanol given intraperitoneally.*

different bacterial strains were done within 48 hr of the alcohol injection (Fig. 6 and 7).

The clearance of bacteria from the lungs of control animals (injected with saline) varied with the virulence of the infecting *Pneumococcus* (Fig. 7). The avirulent unencapsulated R36NC strain was cleared rapidly, whereas the encapsulated IIIS-1R6 was cleared more slowly. Pulmonary clearance of CD-VII-Sm$^r$, a strain of somewhat greater virulence than IIIS-1R6, was still slower. Forty-eight hours after infection CD-VII-Sm$^r$ pneumococci could still be cultured from the lungs of approximately half the mice. Bacteria of the fully virulent strain IIIS-A/66 were partially cleared during the first 4 hr after aerosol infection, but thereafter replication resumed.

During the first 4 hr after administration, the ethanol inhibited the pulmonary clearance of all four of the pneumococcal strains studied; thereafter, it had no demonstrable effect (Fig 6 and 7).
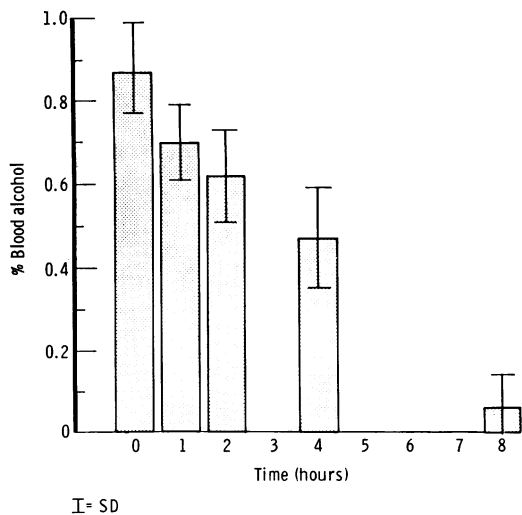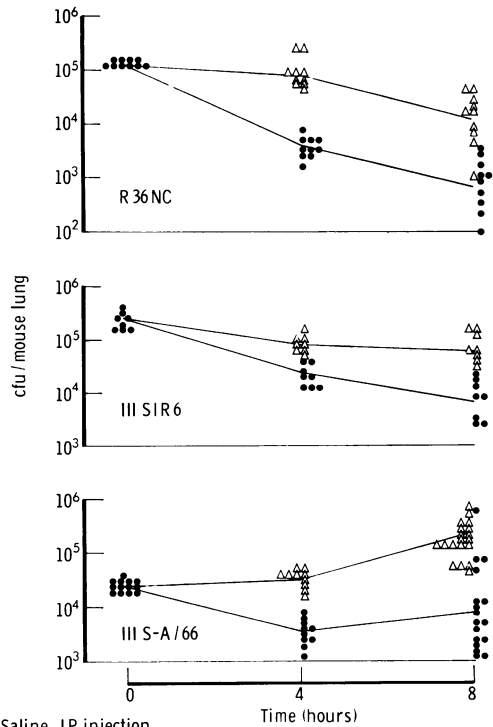
FIG. 5. *Levels of alcohol in murine blood after intraperitoneal administration of 0.75% ethanol (body weight).*



- Saline, I.P. injection
△ Ethanol, I.P. (0.75% body weight)

FIG. 6. *Pulmonary clearance of pneumococci (at 4 hr, difference between ethanol and saline-treated mice is significant: t test, P < 0.05).*

The ethanol also affected both the initiation and the progression of the double pneumococcal infections produced in the transformation studies. The resulting bacteremias are compared in Table 2.

**Occurrence of intrapulmonary transformations in the presence and absence of ethanol.** Mice were inoculated intranasally with $10^{7.8}$ CFU of R36NCery$^r$ followed by exposure to an aerosol which deposited $10^{5.1}$ CFU of CD-VII-Sm$^r$ in the lungs. The animals were then injected intraperitoneally with either saline or ethanol. Heart blood and lungs were cultured at various intervals thereafter for both parental and transformed pneumococci. Ethanol increased the incidence of detectable transformation from 9 to 30% (Table 3). Transformants were isolated most frequently at 24 hr.

Although intraperitoneal transformations between strains R36NCSm$^r$ and IIIS-1R6 have been reported in mice (4, 18, 19), we detected such a transformation in the doubly infected lungs of only 1 mouse among 117 injected with ethanol and 91 injected with saline (*see* Discussion). In contrast to the observations of Conant and Sawyer (4), no transformants were isolated from the blood.

**Control experiments.** In order to determine whether or not the recombinant genotype (CD-VII-Sm$^r$ery$^r$) resulted from deoxyribonucleic acid (DNA)-mediated transformation, control studies were done with single infections (CD-VII-Sm$^r$ alone and R36NCery$^r$ alone) and with

double infection (both strains) and deoxyribonuclease. In the single infections, no recombinants were cultured from the blood or lungs (Table 3).

In the double infections, deoxyribonuclease was administered by the combined in vivo-in vitro method described under Materials and Methods. It significantly lowered the incidence of intrapulmonary transformations (Table 4). The lung tissue which was incubated in vitro appeared to be a rich medium for growth of pneumococci; this closed system may have allowed increased mixing of bacteria and, hence, an exceptionally high incidence of transformation in the preparations without deoxyribonuclease.

**DISCUSSION**

The inhibitory effect of alcohol on the pulmonary clearance of pneumococci, observed in these studies, has been reported with *Staphylococcus aureus, S. albus,* and *Proteus mirabilis* (6, 7, 13).

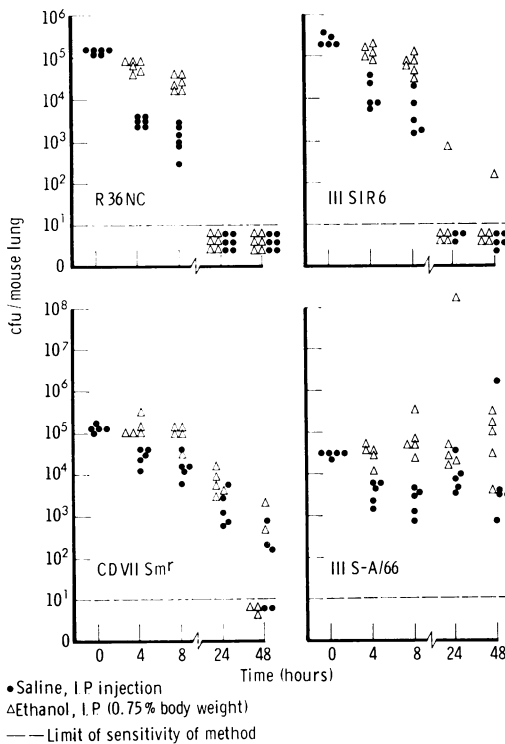The mechanisms by which alcohol depresses resistance to bacterial infection are only partially

• Saline, I.P injection
△ Ethanol, I.P. (0.75% body weight)
— — Limit of sensitivity of method

FIG. 7. *Effect of ethanol on pneumococcal infection of murine lung.*

TABLE 2. *Pneumococcal bacteremia during mixed infection (CD-VII-Sm$^r$ and R36NCery$^r$)$^a$*

| Type of bacteremia$^b$ | No. of mice with bacteremia | |
|---|---|---|
| | Saline group | Ethanol group (0.75% body weight) |
| CD-VII-Sm$^r$ | 10 (5)$^c$ | 35 (16) |
| R36NCery$^r$ | 0 | 2 |
| CD-VII-Sm$^r$ and R36NCery$^r$ | 1 | 23 (8) |
| None | 53 (1) | 31 (3) |
| Total number of mice | 64 (6) | 91 (27) |

$^a$ The incidence of bacteremia in intoxicated mice was significantly greater than in saline controls 24 hr after infection (X$^2$ test, $P < 0.001$).

$^b$ At least 1 colony-forming unit/0.01 ml of heart blood at 24 hr after infection.

$^c$ Numbers in parentheses equal number of mice from the lungs of which transformant bacteria were isolated.

understood. Inhibition of the vascular inflammatory response, resulting in decreased mobilization of phagocytic cells, has been repeatedly reported (2, 9, 14, 20). The results of studies on

TABLE 3. *Summary of in vivo transformation experiments*

| Infecting strains | No. of mice with transformant (CD-VII-Sm$^r$ery$^r$) / total no. of mice infected | |
|---|---|---|
| | Saline group | Ethanol group (0.75% body weight) |
| CD-VII-Sm$^r$ and R36NCery$^r$ | 6/64 | 27/91$^a$ |
| CD-VII-Sm$^{rb}$ alone (control) | 0/30 | 0/30 |
| R36NCery$^{rb}$ alone (control) | 0/27 | 0/27 |

$^a$ Significantly greater than fraction of saline treated mice with transformants (X$^2$ test, $0.01 > P > 0.001$).

$^b$ The difference between experimental and control data is significant (X$^2$ test, $P < 0.001$).

TABLE 4. *Effect of deoxyribonuclease on transformation in vivo in the murine lungs$^a$*

| Expt no. | No. of mice with transformant (CD-VII-Sm$^r$ery$^r$) / total no. of mice infected$^b$ | |
|---|---|---|
| | Without deoxyribonuclease$^c$ | With deoxyribonuclease$^d$ |
| 1 | 5/5 | 2/5 |
| 2 | 4/4 | 1/7 |
| 3 | 6/9 | 0/7 |
| 4 | 8/11 | 1/10 |
| Total | 23/29 | 4/29 |

$^a$ There were significantly fewer transformants in mice receiving deoxyribonuclease than in those without deoxyribonuclease (X$^2$ test, $P < 0.001$).

$^b$ Numerator indicates number of mice yielding transformants and denominator the number of mice infected.

$^c$ Received equivalent volume of broth (*see* Materials and Methods).

$^d$ Ten micrograms of deoxyribonuclease/ml.

phagocytosis, on the other hand, have been contradictory. Some have claimed that both phagocytosis and intracellular killing are depressed by alcoholic intoxication, whereas others have concluded that neither is affected (2, 13, 14).

In the present experiments, the rate of bacterial clearance from the lungs was shown, first, to be inversely related to the virulence of the pneumococci and, second, to be significantly depressed by ethanol. The treatment with ethanol facilitated both the initiation and progression of the pulmonary infection, thereby increasing the interbacterial contacts and transformation. The

frequency of transformation could not be calculated because the time of transformation, and, hence, the number of times a given transformant had divided were unknown.

Since treatment with deoxyribonuclease virtually eliminated recombinants and no recombinants were isolated in control experiments with single strains, it was concluded that the observed transformations were DNA-mediated and not the result of spontaneous mutations.

The frequency of transformation in the murine lung varied with different pneumococcal strains, as previously observed in the peritoneal cavity (19). Transformation of CD-VII-Sm$^r$, for example, was more common than transformation of IIIS-1R6. Frequency of transformation seemed to depend on the nature of the pulmonary infection produced; i.e., strain CD-VII-Sm$^r$ caused a more prolonged infection than strain IIIS-1R6 (Fig. 7). The relative competence of the two strains was not studied, but it, too, may affect the frequency of transformation in vivo (24).

Studies on the effect of viral infections (5, 11) on intrapulmonary transformation would be of interest because some viral infections, like alcoholic intoxication (3, 15, 21, 25), lower resistance to bacterial pneumonia in man as well as in rodents and, therefore, may enhance in vivo transformations (4, 10, 17, 19).

## LITERATURE CITED

1. Avery, O. T., C. M. MacLeod, and M. McCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. J. Exp. Med. 79:137–157.
2. Brayton, R. G., P. E. Stokes, M. Schwartz, and D. B. Louria. 1970. Effect of alcohol and various diseases on leukocyte mobilization, phagocytosis, and intracellular killing. N. Engl. J. Med. 282:123–128.
3. Capps, J. A., and G. H. Coleman. 1923. Influence of alcohol on the prognosis of pneumonia in Cook County Hospital. J. Amer. Med. Ass. 80:750–752.
4. Conant, J. E., and W. D. Sawyer. 1967. Transformation during mixed pneumococcal infection of mice. J. Bacteriol. 93:1869–1875.
5. Green, G. M. 1965. Patterns of bacterial clearance in murine influenza. Antimicrob. Ag. Chemother. 5:26–29.
6. Green, G. M., and E. H. Kass. 1964. Factors influencing the clearance of bacteria by the lung. J. Clin. Invest. 43:769–776.
7. Green, G. M., and E. H. Kass. 1965. The influence of bacterial species on pulmonary resistance to infection in mice subjected to hypoxia, cold stress and ethanolic intoxication. Brit. J. Exp. Pathol. 46:360–366.
8. Griffith, F. 1928. The significance of pneumococcal types. J. Hyg. 27:113–159.
9. Guarneri, J. J., and G. A. Laurenzi. 1968. Effect of alcohol on the mobilization of alveolar macrophages. J. Lab. Clin. Med. 72:40–51.
10. Hotchkiss, R. D. 1951. Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures. Cold Spring Harbor Symp. Quant. Biol. 16:457–460.
11. Klein, J. O., G. M. Green, J. G. Tilles, E. H. Kass, and M. Finland. 1969. Effect of intranasal reovirus infection on antibacterial activity of mouse lung. J. Infec. Dis. 119:43–50.
12. Laurenzi, G. A., and J. J. Guarneri. 1966. A study of the mechanisms of pulmonary resistance to infection: the relationship of bacterial clearance to ciliary and alveolar macrophage function. Amer. Rev. Resp. Dis. 43:134–141.
13. Laurenzi, G. A., J. J. Guarneri, and R. B. Endriga. 1965. Important determinants in pulmonary resistance to bacterial infection. Med. Thoracalis 22:48–59.
14. Louria, D. B. 1963. Susceptibility to infection during experimental alcohol intoxication. Trans. Ass. Amer. Physicians Philadelphia 76:102–110.
15. Osler, W. 1930. Principles and practice of medicine, 11th ed. Appleton and Co., New York, p. 82.
16. Ottolenghi, E., and R. D. Hotchkiss. 1960. Appearance of genetic transforming activity in pneumococcal cultures. Science 132:1257–1258.
17. Ottolenghi, E., and R. D. Hotchkiss. 1962. Release of genetic transforming agent from pneumococcal cultures during growth and disintegration. J. Exp. Med. 116:491–520.
18. Ottolenghi, E., and C. M. MacLeod. 1963. Genetic transformation among living pneumococci in the mouse. Proc. Nat. Acad. Sci. U.S.A. 50:417–419.
19. Ottolenghi-Nightingale, E. 1969. Spontaneously occurring bacterial transformations in mice. J. Bacteriol. 100:445–452.
20. Pickrell, K. L. 1938. The effect of alcohol intoxication and ether anesthesia on resistance to pneumococcal infection. Bull. Johns Hopkins Hosp. 63:238–260.
21. Rush, G. 1785. An inquiry into the effect of ardent spirits upon the human body and mind with an account of the means of preventing and the remedies for curing them. Reprinted in Quart. J. Stud. Alc. 1943, 4:321–341.
22. Schluman, J. L., and E. D. Kilbourne. 1963. Experimental transmission of influenza virus infected mice. J. Exp. Med. 118:257–266.
23. Stillman, E. G. 1924. Persistence of inspired bacteria in the lungs of alcoholized mice. J. Exp. Med. 40:353–361.
24. Tomasz, A., and R. D. Hotchkiss. 1964. Regulation of the transformability of pneumococcal cultures by macromolecular cell products. Proc. Nat. Acad. Sci. U.S.A. 51:480–487.
25. Van Metre, T. E., Jr. 1954. Pneumococcal pneumonia treated with antibiotics. New Engl. J. Med. 251:1048–1052.