The reliability of the method was checked by subjecting to this treatment organisms which had developed ability to multiply on agar containing different concentrations of penicillin. Multiplication always occurred in broth containing penicillin in the concentration just below that of the agar from which the inocula were taken.

Penicillin-fast strains of gonococcus showed distinct morphological changes, which were retained during subsequent cultivation on penicillin-free agar. They will be described in a subsequent report.

Penicillin resistance developed *in vitro* was not permanently retained during prolonged cultivation on penicillin-free agar. Its rate of loss has not yet been accurately determined.

Summary. Penicillin-fastness developed most rapidly in gonococci under conditions which permitted the greatest number of viable microörganisms to be transferred to a higher concentration of penicillin at each transfer. One strain of gonococcus acquired the ability to grow on media containing 21 units per ml. No appreciable increase in penicillin tolerance resulted from repeated exposure to bacteriostatic concentrations of penicillin.

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Studies on Action of Penicillin. V. Virulence of Penicillin Resistant Strains of Meningococcus.

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Several reports have appeared on the development of penicillin resistance *in vitro* and *in vivo* by staphylococci[1,2] and pneumococci,[3] but they do not agree on its effect on virulence.

Methods. The sensitivity of meningococci to the bacteriostatic action of penicillin* was determined by transferring them from routine media onto a series of blood agar plates[4] containing known concentrations of penicillin and incubating them in a candle jar. The highest concentration permitting the development of any colonies was considered the limit of that strain's resistance to penicillin. Increased resistance to penicillin was developed by the method described for gonococci.[5] Virulence was determined by inoculating mice intraperitoneally with mucin suspensions of meningococci.[6] Susceptibility to the therapeutic action of penicillin was estimated by infecting mice with 10,000-100,000 M.L.D. of meningococci and injecting them subcutaneously 3 and 6 hours thereafter with appropriate doses of penicillin.

Results. The natural resistance to penicillin was determined on 96 strains of meningococci and found to vary from 0.1 to 0.5 units per ml. Seven strains were selected at random for enhancement of penicillin resistance and were subcultured daily on media containing increasing concentrations of penicillin. The resistance of all 7 strains increased at about

1 McKee, Clara M., and Houck, Carol L., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 33.

2 Spink, Wesley W., Hall, Wendell H., and Ferris, Viola, *J. A. M. A.*, 1945, **128**, 555.

3 Schmidt, L. H., and Sesler, Clara L., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 353.

* The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

4 The medium is described by Miller, C. Phillip, Moeller, Velma, and Bohnhoff, Marjorie, PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 143.

5 Miller, C. Phillip, and Bohnhoff, Marjorie, PROC. SOC. EXP. BIOL. AND MED. (preceding communication).

6 Miller, C. Phillip, and Castles, Ruth, *J. Inf. Dis.*, 1936, **58**, 263.

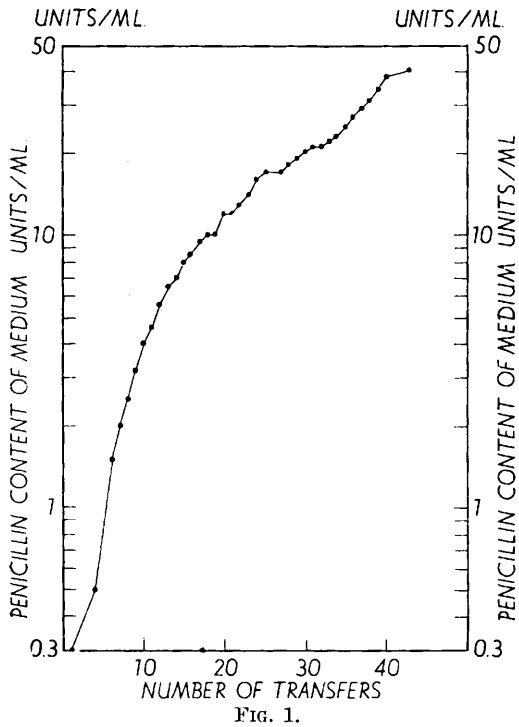

FIG. 1.

the same rate. None was carried to its limit. The one strain (No. 274) which was carried farthest became contaminated at a time when it was growing on media containing 41 units per ml. The results with this strain are shown in Fig. 1.

The virulence of all 7 strains was determined after they had acquired varying degrees of penicillin resistance up to 5 units per ml and all were found to have lost very little or none of their original virulence for mice.

Table I summarizes observations on the *in vitro* and *in vivo* action of penicillin on strain 274 up to the level at which it lost virulence. These include determinations of virulence and susceptibility to therapeutic action in the infected mouse. The slight diminution of virulence which occurred as penicillin resistance developed was no more than commonly occurs during repeated subcultivation on artificial media. But when the strain had acquired resistance to 18 units per ml virulence was lost completely and could not be restored by mouse passage. The strain had also acquired unusual physical properties although it still retained its ability to ferment glucose and maltose.

TABLE I.

Changes in Virulence and in Susceptibility to Treatment by Penicillin During the Development of Penicillin Resistance by Meningococcus.

Penicillin resistance units/ml	Virulence*	Smallest protective dose of penicillin† units
0.3	maximal	20-40
5	high	400
10	"	600
14	"	600
18	none	not done

* maximal—M.L.D. of 1-10 meningococci
high—M.L.D. of 10-100 meningococci.
none—M.L.D. of 10^9 meningococci.

† Smallest dose of penicillin which successfully protected mice infected with 100,000 of the meningococci described in the other columns.

The table also shows that as the strain increased its tolerance *in vitro*, the test infection (always produced by inoculating the same number of microörganisms) required larger doses of penicillin for successful treatment. Mice infected with the strain when it was resistant to 14 units per ml required 600 units of penicillin for its control, a dose 15-30 times the number which was effective before the strain had begun to acquire penicillin-resistance.

Summary. None of 96 strains of meningococci was found to be naturally resistant to penicillin *in vitro*. Seven strains all acquired penicillin resistance readily and at about the same rate during repeated subcultivation on agar containing increasing concentrations of penicillin. The strains remained virulent for mice as penicillin resistance increased, except 1 strain which lost virulence at 18 units per ml. Infections produced with penicillin-resistant strains required very large doses of penicillin for their control.

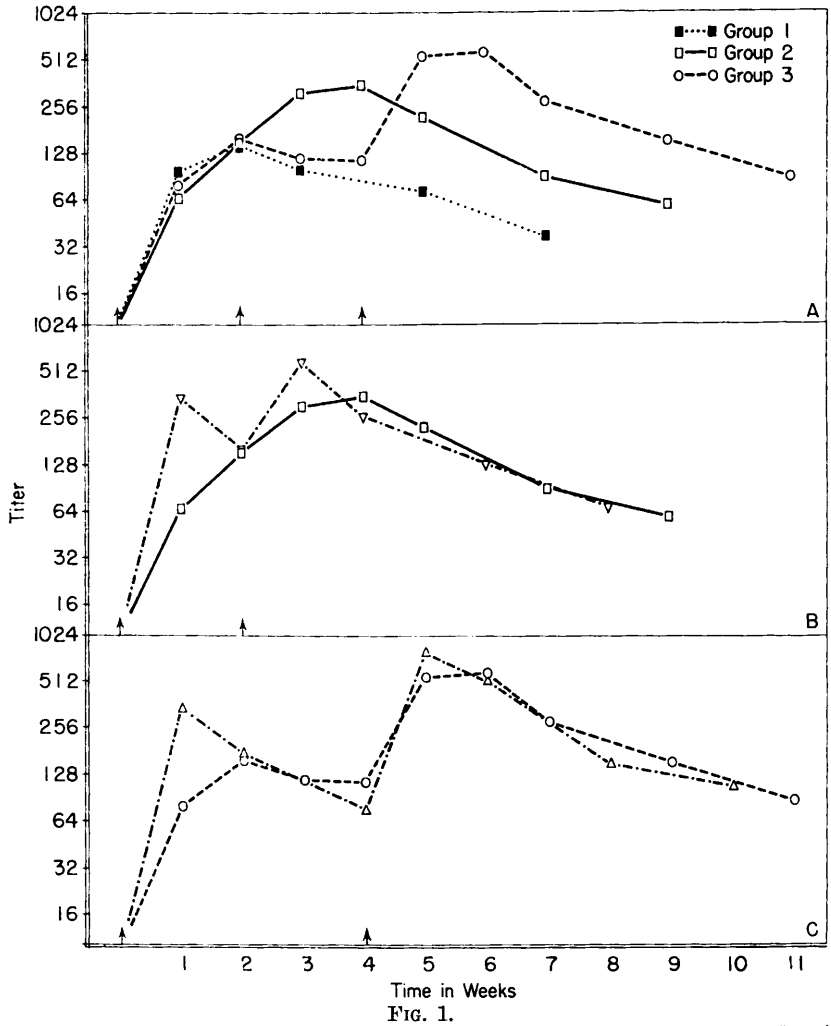

FIG. 1.

A. Antibody response of swine to vaccination with 0.5 mg of formolized swine influenza virus adsorbed on 1.1% alum. Group 1 received but one vaccination; in Groups 2 and 3 vaccination was repeated after 2 and 4 weeks, respectively.

B. Comparison of response to repeated vaccination after 2 weeks with alum-precipitated vaccine (Group 2, Fig. 1, A) with response to like vaccination with the same vaccine without alum (Group B, Fig. 2, previously reported[3]).

C. Comparison of response to repeated vaccination after 4 weeks with alum-precipitated vaccine (Group 3, Fig. 1, A) with response to like vaccination with the same vaccine without alum (Group D, Fig. 2, previously reported[3]).

right posterior axillary region where fat was avoided. Second injections were given similarly on the left side. No general reactions were observed, but all of the animals developed indurated nodules at the site of vaccination. The nodules were larger and more persistent with the larger amounts of alum. Those associated with the vaccine adsorbed on 1.1% alum were about 1.5 to 2 cm in diameter; about 70% of those initially present were still palpable after 7 weeks. The 0.6 and 0.3% alum vaccines produced nodules averaging 1 and 0.7 cm, respectively, which persisted for 7 weeks in 56 and 38% of the 2 groups of animals.

The pigs were weighed at the time of each vaccination and at the end of the experiment 7 weeks after the last vaccination; the average

gain in weight was 8 lb per animal per week.

Bleeding, preparation of sera, storage, and titration of the capacity of the sera to inhibit the hemagglutinative reaction were carried out as previously described.[2,3] Sera obtained immediately before, and 1, 2, 3, 5, and 7 weeks after, vaccination were titrated twice; (1) without freezing and within 4 days of the time of bleeding, and (2) after storage in the frozen state until the completion of both experiments. The titers of the 2 sets of titrations exhibited a standard deviation of 0.47 of a 2-fold dilution, and the values given here were the geometric means of the two endpoints.

Use was made of the geometric means of the titers of the individual pigs for the reasons previously given.[2]

Results. The experiments were set up in two categories. The purpose of the initial experiment was to measure the antibody response to the vaccine adsorbed on alum and to test the response to a second vaccination with the same material after intervals of 2 and 4 weeks. Three groups of 16 pigs each were given a single dose of Vaccine A (1.1% alum), and Groups 2 and 3 were revaccinated after intervals of 2 and 4 weeks, respectively. The sequence of vaccination and bleeding is shown in Table I.

In the second experiment the relation of the response to the quantity of alum employed for adsorption was investigated. Two groups of animals, 5 and 6, were used; Group 5 was given a single dose of Vaccine B (0.6% alum) and Group 6 a single dose of Vaccine C (0.3% alum). The time relations of vaccination and bleeding in this experiment are likewise shown in Table I.

The results of experiment 1, Groups 1, 2, and 3, are shown in Fig. 1, A. The antibody titer rose sharply during the first week after the initial injection and reached the maximum at the 2-weeks interval. The rate of antibody loss in subsequent weeks is illustrated in the findings with Group 1. Repetition of vaccination of Group 2 at the 2-weeks interval resulted in a continuation of the rise initiated by the first injection to a much higher level within 1 week and maintenance of this level for another 7 days. In the instance of Group 3, a considerable diminution in antibody occurred in the interval between first and second vaccinations; nevertheless, the second vaccination of this group resulted in a rapid increase in titer, which reached a level in 1 week still higher than that of the group revaccinated after the 2-weeks interval. The high level was maintained for a period of 7 days. The rate of antibody loss after final vaccination was nearly the same in all of the groups.

In Fig. 1, B and C, are shown comparisons of the responses to repeated vaccination at the intervals of 2 (Fig. 1, B) and 4 (Fig. 1, C) weeks with vaccine adsorbed on alum (Groups 2 and 3, Fig. 1, A), and with the same vaccine without alum (Fig. 2, Groups B and D).[3] The results shown in Fig. 1, B and C, show that the overall responses to the two sorts of vaccine were strikingly similar both in degree and duration. Differences were seen only in the rate of response and in the maximum immediate response. The highest titer after the initial vaccination with alum vaccine occurred at the 2-weeks interval; response was more rapid to the second vaccination, a high titer being reached in 7 days and maintained for another week before loss occurred. The rates of loss of antibody titer afterward were essentially identical in both instances.

The effects of one injection of vaccine adsorbed on various quantities of alum are shown in Fig. 2 (Experiment 2, Groups 4 and 5). The findings of Group 1 (Fig. 1, A) which received a single injection are repeated in Fig. 2 to compare more clearly the effects of 1.1% alum. It is seen again that the rate of response is relatively slow and, with all quantities of alum employed, the maximum titer was reached 2 weeks after vaccination. It is evident that the degree of response was considerably affected by the amount of alum employed. The highest mean titer observed with the vaccine in the presence of 0.3% alum was 277 and that with vaccine adsorbed on 1.1% alum was 147, a 2-fold difference in titer.

Discussion. Swine influenza virus vaccine adsorbed on alum elicited an antibody response in swine which differed in only two evident respects from the response to the identical kind and amount of the vaccine dispersed in saline solution. These differences were (1) a brief delay, approximately 1 week, in the

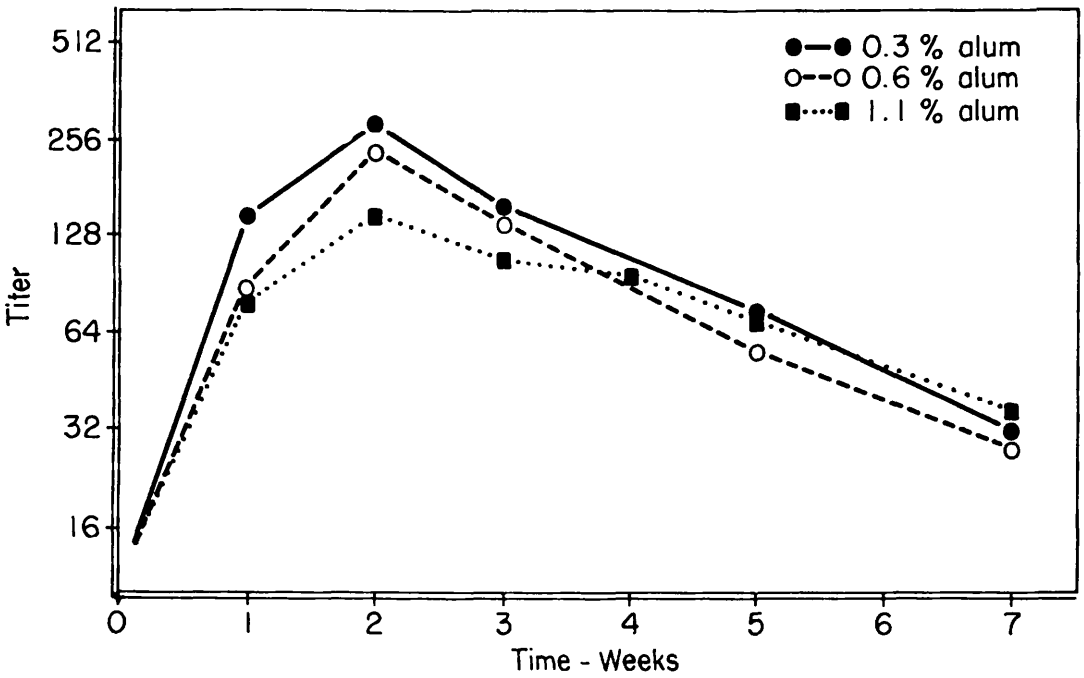

FIG. 2.
Effect of amount of alum present in vaccine on antibody response.

attainment of the maximum antibody level and (2) a lower antibody titer at the time of peak response. At no time was the response to the alum vaccine higher than that to the vaccine without alum. It is of interest to note that the maximum antibody titer induced by the vaccine with alum, which came at the interval of 2 weeks after vaccination, was essentially identical with the declining titer following the use of plain vaccine at the same interval. The results in swine were similar in this respect to those in man described by Bodily, Eaton and Corey,[10] for these authors measured antibody content at the interval of 2 weeks after vaccination with the two types of vaccine. Subsequent to the 2-weeks interval, the rates of antibody decline in swine were the same with the 2 vaccines and, consequently, the levels of antibody titer were alike with respect to time.

It was observed, further, that adsorption of the vaccine on alum may lessen the response to a given amount of the vaccine. In the present experiments, there appeared to be a definite relation between the height of response and the quantity of alum used. The response was least in the instance of 1.1% alum and greatest in that of 0.3% of the material. The present results as a whole indicate not only that adsorption of influenza virus vaccine on alum is of no benefit but that it may possibly be detrimental to the production and maintenance of anti-influenza antibody titer.

Summary. The antibody response of swine to formolized swine influenza virus adsorbed on alum is essentially the same as that to the vaccine dispersed in saline solution. The chief influence of the alum is a brief delay in the peak response. The higher concentrations of alum lessen the primary antibody response, but the subsequent antibody levels and the rates of diminution in titers are nearly identical with the two sorts of vaccine.