may possibly be the factor responsible for the antithyroid activity of these drugs. 4. Glutathione, cysteine hydrochloride, and ascorbic acid inhibit cytochrome oxidase activity (paraphenylendiamine oxidase) of thyroid tissue *in vitro*. 5. Paraminobenzoic acid and paraxanthin do not influence the cytochrome oxidase activity of the thyroid gland.

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Antibody Response of Swine to Repeated Vaccination with Formalin-Inactivated, Purified Swine Influenza Virus.*

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The results of studies on the relation of antibody response of swine to dose of purified swine influenza virus treated with formalin have been described in a recent report.[1] Within the range of dosage employed, 0.1 to 5.0 mg of formolized virus per 100 lb of animal weight, the relation of log antibody titer to log dose was essentially linear. In a single vaccination, a 10-fold difference in dose was associated with a 2.5-fold difference in titer. The maximum titer of serum taken at weekly intervals occurred in the serum obtained 1 week after vaccination; within 3 weeks after vaccination the average titers associated with all doses had declined to similar levels, which were about three 2-fold dilutions below the maximum average titer of the animals receiving the largest doses.

Because of the relatively small influence of the magnitude of the dose and the surprisingly rapid decline in titer, a second vaccination was made 6 weeks after the first. The relation of log antibody response to log dose was again apparently linear and a 10-fold difference in dose was associated with a 1.6-fold difference in titer. In contrast with the results of the first vaccination, however, the maximum titer, which occurred 7 to 9 days after the second vaccination, greatly exceeded that following the single vaccination. For comparable doses per unit weight, the antibody titer after the second vaccination rose to a level approximately three 2-fold dilutions above that seen after the first vaccination. Furthermore, the titers observed 6 weeks after the second vaccination, except those resulting from the smallest doses of vaccine, had not sunk to the level reached 3 weeks after the initial vaccination.

These and other results demonstrated that the degree of antibody response was far more dependent on the status of the host with respect to previous experience with the influenza virus or its derivatives than on the mass of formolized virus introduced at vaccination. In this respect it was observed that the maximum response to the dose of 0.125 mg per 100 lb at the second vaccination was approximately twice that to the dose of 2.0 mg given in the first vaccination. Inasmuch as the cost

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1 McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1945, **51**, 65.

and the difficulty of preparing vaccines of purified and concentrated influenza virus increase rapidly with the size of the dose, it is possible that repeated vaccination with small doses of vaccine would be more advantageous and desirable in obtaining a high antibody response than the use of a single large dose. Limitation of the dose is especially desirable in consideration of general reactions which have been observed both in men and swine[1] when the dose is increased beyond certain levels.

In the previous study repetition of vaccination was carried out at the period of 6 weeks after the initial administration of vaccine. The 6 weeks interval was employed because it was only after this period that the course of antibody response to the first vaccination was apparent. In order to learn more of the influence of the sequence of vaccination with respect to the interval between vaccinations, further study of the problem was undertaken. An investigation was made of the response of swine to 2 vaccinations spaced at intervals of 1 to 4 weeks, and the results obtained are described in the present report.

Materials and Methods. The virus used for the preparation of the vaccine was the same strain of egg-adapted swine influenza virus obtained from Dr. John F. Enders that was employed for the chemical, physical, biological, and immunological studies previously reported.[1-4]

Concentration of the virus and preparation of the vaccine. The vaccine employed in the present experiments was from a stock different from that used in the previous work.[1] Propagation and harvest of the virus was carried out as before. Concentration of the virus was accomplished with the modified[5] Sharples centrifuge in a manner similar to that previously described,[1] except that use was made of ultraviolet light for maintenance of aseptic conditions during centrifugation. The whole technic for getting the virus and concentrating it was essentially identical with that employed[5] in concentrating influenza viruses A and B for the preparation of vaccines. The virus from 16,100 ml of bacteriologically sterile chorio-allantoic fluid (yield from 1,700 eggs) was concentrated in 100 ml of sterile Ringer's solution, with a loss of only 9.5% of the starting hemagglutinative activity. Electron micrographs and sedimentation velocity studies of this preparation gave results indistinguishable from those previously reported[4] for this virus. The specific infectivity for chick embryos was $10^{-13.5}$ g of virus, and the specific hemagglutinative activity was $10^{-6.95}$ g of virus (based on a nitrogen factor[3] of 11.1). Cultures of the concentrate in beef extract broth and glucose agar showed no contaminating bacteria to be present. Anaerobic cultures were likewise negative.

To 86 ml of the concentrate containing 4.44 mg of virus per ml there was added sterile Ringer's solution containing 19.1 ml of 0.5% formalin and 1.91 ml of 1/500 solution of phenyl mercuric borate, to give a total volume of 191 ml of vaccine containing 0.05% formalin, 1/50,000 phenyl mercuric borate and 2.0 mg of virus per ml. After standing for 7 days at room temperature (28 to 30°C), the vaccine did not contain enough active virus to infect chick embryos. Cultures of the vaccine showed no viable bacteria to be present.

The vaccine was stored at the concentration of 2.0 mg per ml in a tightly stoppered bottle at 2-8°C for 4 months before final dilution for use. At the beginning of the present work 34 ml of the vaccine were diluted with 102 ml of sterile Ringer's solution containing 0.05% formalin and 1/50,000 phenyl mercuric borate to yield 136 ml of vaccine containing 0.5 mg of formolized virus per ml. This was partitioned in 17-ml volumes in vaccine bottles and stored at 2-8°C until used. An electron micrograph of the vaccine taken 11 months after preparation is shown in Fig. 1.

The experimental animals were swine obtained in Orange County, North Carolina. At the time of the first vaccination, the group

2 Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1944, **48**, 361.

3 Taylor, A. R., *J. Biol. Chem.*, 1944, **153**, 675.

4 Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1944, **156**, 585.

5 Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, **50**, 291.

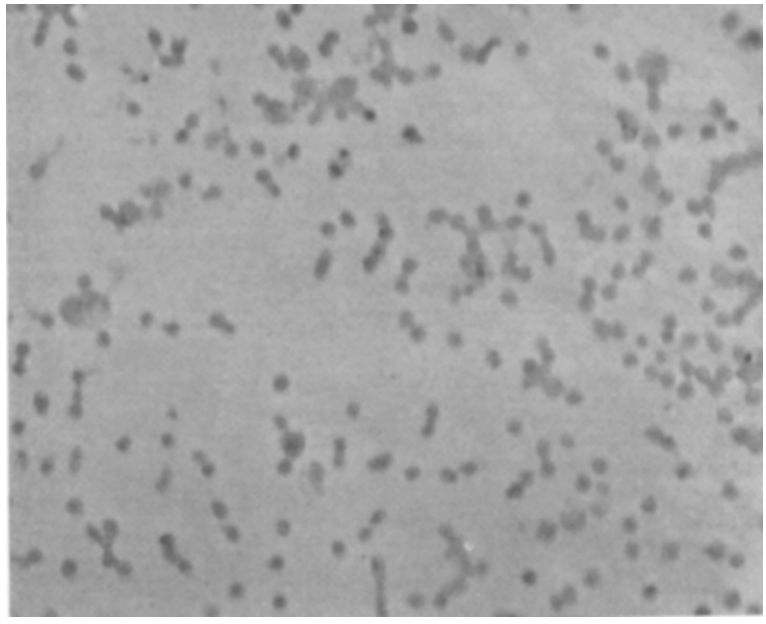

FIG. 1.

Swine influenza virus vaccine stored at 2-8°C for 11 months. The electron micrograph was taken at 55 kilovolts, and the total magnification is 19,700 ×. Comparison of this micrograph with that of 2, Fig. 2 reveals no essential difference between the appearance of the images of the formolized virus particles stored for this period and the images of the untreated, fully active particles immediately after purification.

of 64 varied in age from 16 to 20 weeks and weighed from 42 to 94 lb., with an average of 60.8 lb. There were 30 females and 34 males; 31 of the males had been castrated. The animals were predominently of Berkshire strain, with some admixture of Poland China, Hampshire, Duroc, and O.I.C.

The animals were divided into 4 groups of 16 each so that each subgroup was representative of the whole with respect to weight and sex. The average individual weights for the 4 groups, A, B, C, and D, were 59.1, 61.0, 61.7, and 61.4 lb, respectively. The pigs were kept on open range with sheds to provide protection from the rain and were fed from self feeders with a commercial balanced feed. Weight gain was satisfactory, being from 4.5 to 9 lb per week, and all of the animals appeared healthy throughout the experiment.

Vaccination was performed by introducing 1 ml of the vaccine into the subcutaneous tissue of the right posterior axillary region. No local or general reaction was observed in any instance. Weights were taken at the time of each vaccination and at the termination of the experiment, 6 weeks after the second injection. Blood was drawn from the femoral vein or artery immediately before each vaccination and 1, 2, 4, and 6 weeks after vaccination. The blood samples were left for about 18 hours at room temperature to allow for retraction of the clot, and the sera were then separated from the clots and centrifuged to remove suspended red blood cells. A sample of each serum was set aside for titration of antibody content before freezing and the remainder divided into 2 portions in individual vials, frozen, and stored at −10°C.

Serum antibody levels were estimated by a modification of the hemagglutinin-inhibition method of Hirst, Rickard, Whitman, and Horsfall[6] previously described.[1] It was found during the course of the work that while duplicate titrations set up at the same time with the same materials showed almost exact reproduction of endpoints, the results of titra-

[6] Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., *J. Exp. Med.*, 1942, **75**, 495.

TABLE I.
Dates of Vaccination and Bleeding.

Animal group	September						October										November			
	11	12	18	19	25	26	2	3	9	10	16	17	23	24	30	31	6	7	13	21
A	BV*		BV		B		B				B				B					
B	BV		B		BV		B		B				B				B			
C		BV		B		B		BV		B		B				B			B	
D		BV		B		B				BV		B		B				B		B

* B = Bled; V = Vaccinated.

tions of the same sera at different times using different preparations of cells and other materials exhibited a standard deviation of 0.6 of a 2-fold dilution interval, or 30% variation. In order that an overall comparison of results could be made, each serum was titrated in triplicate: (1) before it was frozen and within 4 days of the bleeding time (see Table I); (2) after storage in the frozen state until the completion of the experiment with a given group; and (3) after storage in the frozen state until the termination of the entire experiment. Freezing and storing made no significant difference in the level of antibody titer obtained by the hemagglutinin-inhibition method.

The titers referred to in this paper are the geometric means of the 3 titration endpoints observed. Common logarithms have been used for expression of the titers and for calculations throughout, rather than the arithmetic titers. The arithmetic means, being unduly influenced by the values of serums of very high titer, do not as closely approximate the medians and the modes of the groups as do the geometric means.

Results. The 4 groups of animals were given the first dose of vaccine at essentially the same time as shown in Table I. Subsequent vaccination was performed, Table I, at 1, 2, 3, and 4-week intervals for Groups A, B, C, and D, respectively. The sequence of bleeding in relation to the vaccination is likewise shown in Table I.

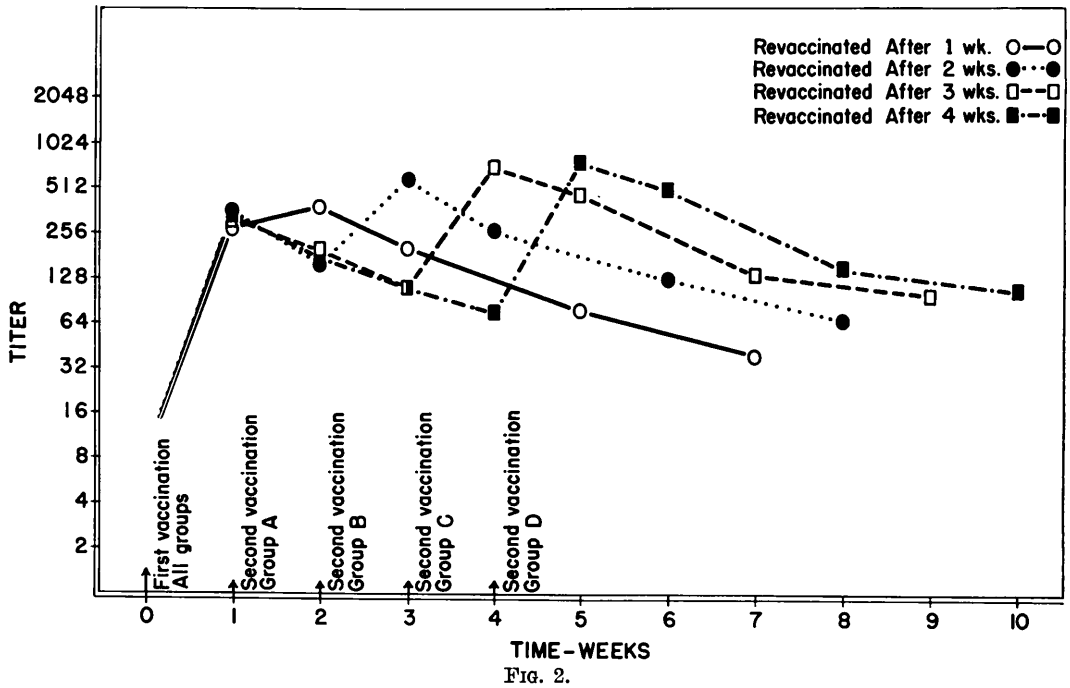

FIG. 2.

The antibody response to a single vaccination and to repeated vaccination at various intervals after the first.

The overall results of the experiment in terms of average group antibody response in relation to the sequence of vaccination are given in Fig. 2. The average antibody responses of the 4 groups of animals to the first vaccination were uniform, and the peak responses were observed in the serums taken 1 week after vaccination in the three groups, B, C, and D, which were not revaccinated at the 1-week interval. The similarity of the responses of the various groups to the single vaccination is seen in Fig. 2 and further demonstrated by the agreement—better than 0.3 of a 2-fold dilution interval—obtained between the mean log titers of the respective groups following one injection of vaccine.

In the present experiments, as in those previously described ([1], Fig. 4), the range of antibody titers of the serums of individual animals within the respective groups at a given bleeding was large, varying from about 2 to 4 2-fold dilution intervals, with an overall standard deviation from the means within groups of 1.48 2-fold dilutions. Analysis of the data of the triplicate titrations on each serum as described above showed a standard deviation of 0.56 of a 2-fold dilution interval which was attributable chiefly to variations in the technic of titration. From these two values, the standard deviation of the antibody titers of the serums within individual groups was estimated to be 0.8 of a 2-fold dilution interval. This variation is that related to differences in host response.

As noted above, the variation in the average antibody responses of the respective groups to the first vaccination was less than 0.3 of a 2-fold dilution. This value represents a variation which is less than would be expected from chance alone. The average of the mean responses of the entire group of 64 animals was taken as the best available estimate of the response to the single vaccination, and analyses of the effects of the second vaccination were made with this value as a basis.

Examination of the findings subsequent to the 1-week interval after the first vaccination reveals (1) a rapid, progressive decline in the average antibody titers of the respective groups of animals in the intervals between the first and second vaccinations; (2) the attainment of progressively higher maximum antibody *levels* in relation to the length of the interval between vaccinations; (3) the formation of progressively greater *amounts* of antibody in relation to the length of the interval between vaccinations; and (4) the tendency toward maintenance of higher antibody levels for longer periods in those animals held for the longer intervals between vaccinations.

The rapid decline in antibody titer following the initial vaccination in the present experiments was closely similar to analogous findings in the work already described.[1] The uniformity in the rate of decline in the average antibody levels of respective groups is evident.

The second vaccination resulted in all instances in an increase in the *average* antibody level of the respective groups above the maximum level reached after the first vaccination. The maximum level attained by each group after revaccination was observed, within the limits of the intervals in which bleedings were made, at the period of 1 week after the second vaccination. It is seen in Fig. 2, however, that the height of the maximum titer reached after the second vaccination was influenced by the interval elapsing between the two vaccinations, so that with increase in this interval a progressively higher average maximum titer was attained by the respective groups. Examination was made of the statistical significance of these progressively higher maximum antibody levels. Analysis of variance[7] of the pooled data of the entire experiment including the findings resulting from both first and second vaccinations indicated the least significant difference between group mean log titers ($t_{0.05}\ \bar{sd}$) to be 0.18 or 0.59 of a 2-fold dilution interval, a value which is much greater than the differences observed after the first vaccination. The average log antibody titer of the whole group of 64 animals 7 days after the first vaccination was 2.50 and the average log titers of the groups A, B, C, and D 7 days after the second injection were 2.57, 2.74, 2.83, and 2.86, respectively. The increases in the maximum mean log antibody titers of groups A, B, C, and D with revaccination above the mean log

[7] Snedecor, G. W., *Statistical Methods*, Ames, The Iowa State College Press, 3rd edition, 1940.

antibody titer of the whole group of 64 animals after a single vaccination thus were 0.07, 0.24, 0.33, and 0.36. From these values it is seen that differences between the increases in the titers of the adjacent groups, that is, between those of A and B or B and C, are too small to be of unequivocal significance. However, when the increase in log titer of Group D, 0.36, is compared with that of Group A, 0.07, the difference is seen to be a full 2-fold dilution, a difference which is highly significant. This finding, together with the trend shown in Fig. 2, provides strong evidence of the existence within the limits of the duration of the experiment of a direct relation between the maximum titer attained and the length of the interval separating the two vaccinations.

The dependence of the total average *amounts* of antibody formed by the different groups on the interval between vaccination is evident from inspection of Fig. 2. This finding is in accord with the observation in the previous work of the influence on response of prior experience of the host with the influenza virus or vaccine. The differences in the amounts of antibody formed by the respective groups in the present work were great. None of the animals used in the study showed any evidence of previous exposure to the swine influenza virus, since all of the titers before any vaccination were less than 16. Assuming that none of the serums contained any antibody before the first vaccination, the average *amount* of antibody formed in the whole group of 64 pigs as the result of the first vaccination was 315 units. The increases in antibody units of the various groups above the level present at the moment of second vaccination were 100, 400, 570, and 645 units for Groups A, B, C, and D, respectively. These amounts correspond to increases of 53, 223, 360, and 406 units above the average amount, 315 units, present one week after the first vaccination.

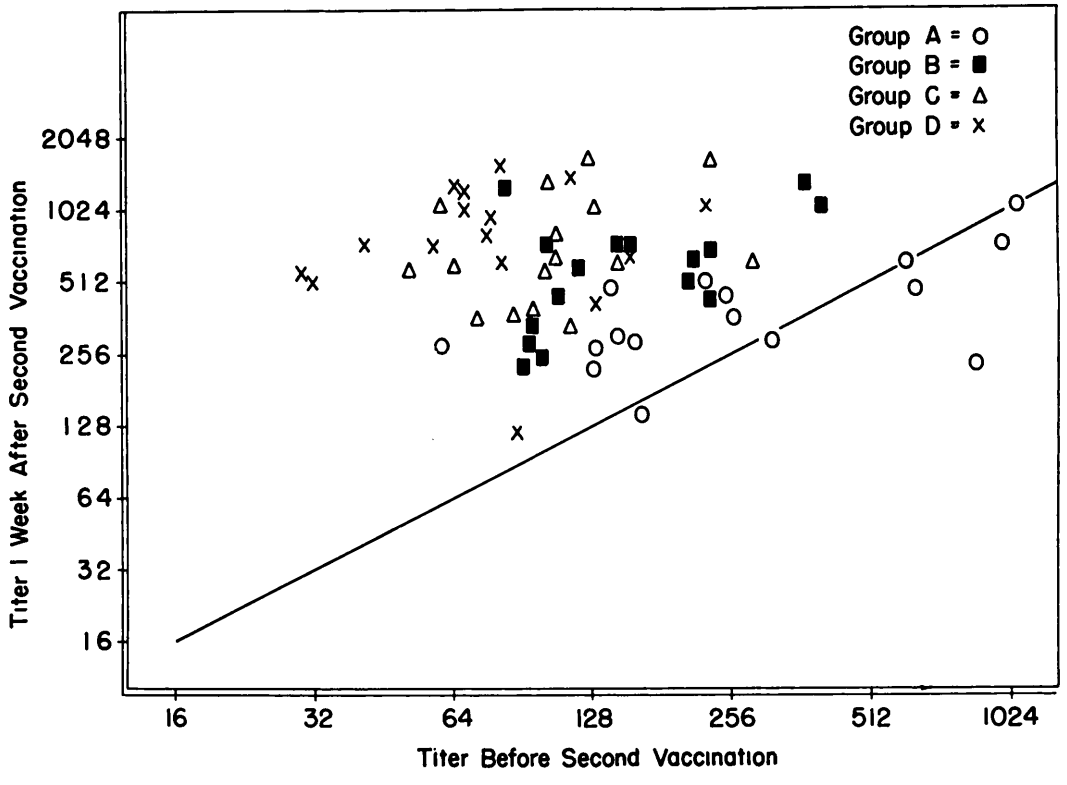

FIG. 3.

Individual antibody responses 7 days after second vaccination compared with the antibody titers immediately before second vaccination. The continuous line represents the base line of the antibody titers of the serums obtained immediately before the second vaccination.

The rate of decline in the average antibody level after the second vaccination was not greatly different in the various groups. As a consequence the residual titers of those groups having the higher titers following the second vaccination remained at higher levels during the later stages of the experiment. It appeared possible that the decline in Groups C and D was slower toward the end than that in Groups A and B, but the data were insufficient to provide an answer to this question.

The responses of the individual animals of the various groups to the second vaccination are shown in the spot chart of Fig. 3. Here the titers observed 7 days after the second vaccination are compared with the antibody titers of the individuals immediately before the second vaccination. The results given in Fig. 3, like those in Fig. 2, show that, though individual variation was great, quantitative response to second vaccination was greatly dependent on the time relations of previous experience of the animals with the vaccine. The animals with the lowest titers were those revaccinated after the longest period following first vaccination, and it was in these pigs that the largest amounts of antibodies were formed in response to the second vaccination. The poorest individual responses were seen in the animals of Group A which had the highest titers before second vaccination. As shown in Fig. 3, seven of the animals of this group either lost or experienced no gain in antibody titer after the second vaccination, while all of the animals of the other groups gained in antibody titer following revaccination.

Discussion. Previous studies on the vaccination of swine with formalin-inactivated swine influenza virus have shown that the magnitude of antibody response to a single vaccination is affected in relatively small degree by the size of the dose given. Similar relations were again apparent when vaccination was repeated in the same animals after a period of about 6 weeks from the time of the initial vaccination. In striking contrast to these results was the observation that the response to second vaccination with a given dose was much greater than that to the first. It was thus clear that the primary factors concerned with the height of antibody response resided in the host and that attempts to obtain maximum response should be directed more toward conditioning or preparation of the host, as by repeated vaccination, than toward manipulation of the dose of vaccine administered.

The results of the present work have shown that response is conditioned not only by the character of the stimulus but by the manner in which it is applied. As before, maximum response was observed after second vaccination. The magnitude of response, however, was found to be largely dependent on the interval elapsing between vaccinations. Within the limits of the period of study, the greater the interval before repetition of vaccination, the higher were the levels of antibody titer attained and the larger were the absolute amounts of antibody formed. It was observed, likewise, that a similar relation existed for the height of residual antibody titer at the 6-weeks period following revaccination, a relation due in large part to the similarities of the rates of antibody loss, which were relatively independent of the height of titer. The optimum interval between vaccination, from the point of view of the attainment and maintenance of the highest antibody levels in swine, would appear to be about 3 weeks. Choice of the proper interval is dependent on consideration both of the titer declining rapidly after the first vaccination and the magnitude of response to be achieved after the second vaccination.

Certain of these findings, in so far as a comparison can be made, are apparently contrary to those which Hirst, Rickard, Whitman, and Horsfall encountered in studies on the vaccination of man. From a study of the serums from 400 people, these authors concluded that "the amount of antibody produced by a group with a low prevaccination antibody level was very nearly the same as the amount produced by groups that had higher initial levels." In the present work this was by no means the case, for those animals having the lowest antibody titers before the second vaccination produced much greater amounts of antibody than those with the highest levels. The relation observed here, therefore, was concerned not with actual titer but with the immunological

status of the host. It should be emphasized, however, that the results in swine were related wholly to effects of formolized virus, whereas the findings in man were related to the effects of possible actual infection with active virus followed by vaccination. Further, the group of swine studied had been treated under controlled conditions in contrast with the people who were chosen at random. For example, in the present work the animals with the highest titer before second vaccination were those revaccinated after the shortest interval between vaccination when conditions for response were not optimum; in the group of people, those with the highest titers may have represented either the best reactors or those recently recovered from the disease.

The results of the experiments with swine were similar to those of Hirst, Rickard, Whitman and Horsfall[6] in indicating rapid loss in antibody titer subsequent to vaccination, regardless of the sequence of vaccination.

Summary. The antibody response of swine to second vaccination with formalin-inactivated swine influenza virus was greatly influenced by the period between vaccinations. Second vaccination at intervals of one to four weeks resulted in antibody titers and amounts progressively greater in direct relation to the length of the interval. In parallel higher antibody levels were maintained for longer periods in those animals receiving the second vaccination after the longer intervals. Considering the height of titer after the second vaccination, together with the rapid loss of antibody after the first injection, the interval between vaccinations optimum for maintenance of the highest antibody levels appeared to be about three weeks.

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Absorption of Penicillin from the Nose and Alimentary Canal.

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To obviate the necessity for parenteral injection of penicillin other routes have been sought. Administration by mouth, including enteric capsule and duodenal tube, and by rectum have been tried as summarized previously.[1] Although satisfactory absorption results from several such combinations or procedures, the efficiency falls short of that from injection of the drug.

In this report, the relative absorption by various routes in man and animals are compared.

Absorption from the Alimentary Canal of Rats. Under sodium pentobarbital anesthesia (35 mg per kg intraperitoneally), rats were given penicillin intramuscularly and in ligated sections of the alimentary tract as follows: esophagus, stomach, duodenum (3 cm), ileum (3 cm), colon (3 cm). Ten animals were used for each route, with doses of penicillin varying from 500 to 12,500 u. per kg, body weight. Blood was taken from the heart 30 minutes later for penicillin assay. Assays were made by the method of Wolohan and Cutting[2] on whole blood. The average results, in units per cc of blood, were as follows: esophagus 0.08, stomach 0.02, duodenum 0.02, ileum 0.03, colon 0.02, intramuscular 0.11.

It is evident that absorption in the rat occurs from the stomach, duodenum, ileum, and colon about equally, but only about one

1 Cutting, W. C., Halpern, R. M., Sultan, E. H., and Armstrong, C. D., *J. A. M. A.*, 1945, **129**, 425.

2 Wolohan, M. B., and Cutting, W. C., *J. Lab. and Clin. Med.*, 1945, **30**, 161.