16849

Influence of Detergents on Egg-White Inhibition of Hemagglutination by Formolized Swine Influenza Virus.*

FRANK LANNI, EDWARD A. ECKERT, AND J. W. BEARD.

From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

During the past year investigations have been made of the capacity of hen's egg-white (EW) to inhibit hemagglutination by purified swine influenza virus and derivatives of this virus obtained by heating or treatment with formaldehyde.[1,2] Many of the studies involved the use of purified, formolized swine influenza virus[3] which had been prepared for vaccine and stored for approximately 4 years at 4°C. The use of this preparation as a reagent for titrating inhibitor was encouraged since vaccine hemagglutination was found highly susceptible to EW inhibition,[1,2] and since the preparation could be regarded to have become stabilized during the prolonged storage.

Recently, certain irregularities in the results of these inhibition titrations have made it necessary to search for the factors concerned. This study has revealed a strong influence of soaps and synthetic ionic detergents on the inhibition reaction.

Materials and methods. The formolized virus (vaccine) employed in the present work, as well as the method of preparation of egg-white and the methods for estimation of hemagglutinative or inhibiting capacities, have been described in a previous report.[2] The details of significant deviations from the routine procedures will be included in the description of the experiments given here.

Experimental. The irregularities stimulating the present work may be illustrated by the results of a set of duplicate anti-vaccine inhibition titrations carried out on three successive days by the methods previously described.[2] Table I shows a titration, I*a*, characterized by excellent duplication, *steep* endpoint gradient, and *high* conventional (++) inhibition titer. Titration I*b* is characterized by excellent duplication, *shallow* endpoint gradient, and *low* conventional titer; however, inhibition, as shown by the occurrence of agglutination numbers smaller than the maximal number (++++), is evident in high EW dilutions. In contrast with these, titration I*c* shows irregularities which preclude any useful statement of titer. The analytically smooth character of titrations I*a* and I*b* suggested that the irregularities evident in titration I*c* were to be attributed to a factor other than the technic of the titrations.

During the period in which the initial investigations[1,2] were carried out, the titration tubes were cleaned in a solution of "green" soap (Medicinal Soft Soap, U.S.P.) and a commercial synthetic detergent (Orvus, Procter and Gamble). Later, hot chromic acid ("cleaning" solution) was substituted for the detergent mixture. Since the onset of the analytical irregularities was approximately coincident with this change in cleaning method, it appeared possible that extraordinary disagreement of duplicates might have resulted from the inadvertent use of tubes cleaned in the two different ways for the two members of a duplicate.

The experiment recorded in Table II was performed as a test. Titration II*a* was carried out in tubes cleaned with hot chromic acid, washed thoroughly in tap water followed by distilled water, and dried in the oven. Titration II*b* was carried out in tubes cleaned first with acid and dried, as above, and then cleaned again in green soap-Orvus mixture,

* This work was aided by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y., and by the Dorothy Beard Research Fund.

1 Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 312.

2 Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 442.

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

TABLE I.
Three EW Anti-Vaccine Inhibition Titrations,* in Duplicate, Illustrating the Analytical Irregularities Which Stimulated the Present Investigation.

Titration	Reciprocal of final EW dilution†						
	1,600	3,200	6,400	12,800	25,600	51,200	102,400
I*a*	0	0	0	+	++	+++	++++
(9/15/48)	0	0	0	+	++	+++	++++
I*b*	++±	++±	+++	+++	+++	+++±	+++±
(9/16/48	++±	++±	+++	+++	+++	+++±	++++
I*c*	+++	+++	+++±	+±	+++±	+++	+++±
(9/17/48)	++	+++±	+	+++	+++±	+++±	+++±

* Carried out at room temperature (28°C) with 4 HD (hemagglutinating doses) of vaccine. The EW dilutions and vaccine were incubated together for 40 minutes before RBC were added.
† Final EW dilution refers to the dilution of EW in the final reaction mixture including RBC.

TABLE II.
Influence of the Method of Cleaning Titration Tubes and of "Green" Soap on the EW Anti-Vaccine Inhibition Titration.

Titration	Cleaning method	Reciprocal of final EW dilution									
		200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	†
II*a*	Acid	0	0	++	++±	++±	+++	+++	+++±	+++±	++++
		0	±	++	++±	++±	+++	+++	+++±	+++±	++++
II*b*	Detergent	0	±	+±	++	++±	++±	+++	++	+++±	++++
		0	±	++	±	++	++±	+±	+++±	+++±	++++
II*c**	Acid	0	0	0	0	0	0	0	+±	+++	++++
		0	0	0	0	0	0	0	+±	+++	++++

* Green soap was present in 1:80,000 (V/V) final dilution in all tubes.
† Indicates mixtures devoid of EW.

with thorough rinsing in tap and distilled water. Titration II*c* was carried out in acid-cleaned tubes in the presence of a constant, known amount of green soap; the soap was used in a final dilution, 1:80,000 (V/V), well below the hemolytic limit (see below), which was 1:8,000. Other experimental factors were held constant. A volume of 0.25 ml of buffered saline (II*a* and II*b*) or 0.25 ml of a 1:10,000 dilution of green soap in buffered saline (II*c*) was added first to the tubes, followed by 0.25 ml of the EW dilutions, which had been prepared in bulk in acid-cleaned tubes, and then by 0.5 ml vaccine dilution containing the customary 8 HD (hemagglutinating doses) per ml. These mixtures were incubated for 35 minutes at room temperature (25°C), and 1.0 ml of 2% chicken RBC was then added. After an additional hour at room temperature, the readings were made in the usual manner.[4]

Result II*a* (Table II) shows excellent duplication, shallow gradient, and low conventional titer. II*b*, while similar on the whole to II*a*, shows several poor duplications. II*c* shows excellent duplication, steep end-point gradient, and high conventional titer. From these results it is evident that the irregularities present in II*b* may be explained as deviations from the pattern of II*a* in the direction of II*c*, and, accordingly, it is reasonable to attribute the irregularity of II*b* to an irregular residuum of soap in the tubes used in this titration.

An explanation may now be given for results of the sort shown in Table I. It may be supposed that titration I*a* was carried out in tubes which had been cleaned with detergent and in which the residuum of detergent was

[4] Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

TABLE III.
Influence of "Green" Soap on the EW Anti-Vaccine Inhibition Titration; Dependence on Detergent Concentration.*

Reciprocal of final green soap dilution × 10-3	Reciprocal of final EW dilution									
	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	204,800	†
20	0	0	0	0	0	+	++±	+++±	++++	++++
40	0	0	0	0	0	+	+++	+++±	++++	++++
80	0	0	0	0	0	++	+++	+++±	++++	++++
160	0	0	0	0	0	++	+++	+++±	++++	++++
320	0	0	0	0	±	++	+++	+++±	++++	++++
640	±	±	+±	+±	++	++±	+++	+++±	++++	++++
1,280	+	+±	++	++	++±	++±	+++±	++++	++++	++++
†	+	+±	++	++±	++±	+++	+++±	+++±	++++	++++

* For this experiment, EW dilutions and soap dilutions were mixed first, then 4 HD vaccine was added to each tube; after 30 minutes at room temperature (27°C), RBC were added. Mixtures devoid of EW or soap are indicated (†), representing an infinite dilution of the respective reagents.

great enough and uniform enough to cause considerable and uniform depression of the agglutination; that titration I*b* was carried out in tubes which had been cleaned with acid; and that titration I*c* was carried out in a mixture of the two kinds of tubes.

It was important to investigate next the dependence of the inhibition on the quantity of detergent present. Anti-vaccine inhibition titrations were carried out in acid-cleaned tubes in the presence of graded amounts of green soap or Duponol PC (du Pont), a synthetic detergent consisting chiefly of sodium dodecyl sulfate, with about 8-10% sodium sulfate and sodium chloride, a small amount of sodium tetradecyl sulfate, and perhaps traces of other alcohol sulfates.[5] Since the two detergents gave essentially the same results, only those obtained with green soap are presented (Table III). Reference to Table III shows that a significant effect was detectable with green soap in a final dilution of 1:640,000; with Duponol PC, a significant effect was observed with the highest dilution tested, 1:1,920,000. Furthermore, as the amount of detergent was increased, the effect on the inhibition increased rapidly at first and then more slowly until a concentration was reached beyond which the change was very gradual; at the same time, the titration gradient progressed from a shallow gradient at low detergent concentration to a steep gradient at high detergent concentration. Approximately the same plateau inhibition titer was attained with the two detergents; expressed as the reciprocal of the final EW dilution, this titer was approximately 40,000.

A calculation shows that the amount of detergent needed for a great effect on the inhibition is of the order of the amount which might reasonably remain in detergent-cleaned tubes after rinsing. Duponol PC, in a concentration of approximately 1 γ per ml of final reaction mixture, corresponding to a final dilution of 1:1,000,000, exerts a great, almost maximal, effect. Since the reaction mixture has a volume of 2 ml, the total quantity of detergent needed for this effect is 2 γ. The internal surface area of a titration tube of the sort used in the present experiments is approximately 30 cm^2. On the assumption that the detergent molecules have an average molecular weight of 300 and that each molecule can occupy 20 sq. Å of glass surface when close-packed with other molecules, the quantity of detergent needed to form one complete unimolecular layer on the glass may be calculated as $\frac{(30 \times 10^{16})\ (300 \times 10^{6})}{20\ (6 \times 10^{23})} = 7.5\ \gamma$. The amount of detergent, 2 γ, needed for a great detergent effect is thus approximately ¼ of the amount which will cover the internal surface of a titration tube with a single, close-packed unimolecular layer. A similar result may be calculated for green soap if reasonable assumptions are made about the composition

[5] Personal communication from Dr. J. H. Shipp of the Fine Chemicals Division, E. I. du Pont de Nemours and Company, Inc., Wilmington, Del.

of this complex material.[6] Since it is known[7] that in concentrated solution detergents exist as aggregates (micelles) in equilibrium with unassociated ions, it is possible that aggregates, as well as free ions, are adsorbed to the glass during treatment with concentrated detergent solution and that portions of these aggregates survive the after-rinsing. In this way an amount of detergent greater than the amount needed to form a unimolecular layer could be made available. Furthermore, the recorded effective concentrations of detergent are probably in excess of the actual concentrations, since an undetermined amount may be supposed to have adhered to the walls of pipettes and vessels other than the titration tubes during the preparation and distribution of the detergent dilutions.

It was of interest next to determine whether the detergent effect on EW anti-vaccine titrations could be demonstrated with detergents of different kinds. For this experiment, 0.5 ml vaccine (4 HD) was first placed in each tube, and then 0.25 ml of a dilution of detergent well beyond the hemolytic limit (see below) was added, followed after 15 minutes by 0.25 ml of two-fold EW dilutions. After a further period of 20 minutes at room temperature, 1.0 ml 2% RBC was added, and the agglutination readings were made as usual after one hour. The *hemolytic limit* of each detergent was determined by mixing 1.0 ml 2% RBC with 1.0 ml of each of several two-fold dilutions of detergent; the endpoint was taken as the highest dilution of detergent at which hemolysis could be detected visually after one hour at room temperature. The detergents not previously tested included the anionic detergents sodium oleate (Fisher, U.S.P.), Orvus (Procter and Gamble), Aerosol OT (American Cyanamid), and Sodium "Lorol" Sulfate PT† (du Pont), a purified, essentially salt-free detergent, consisting chiefly of sodium dodecyl sulfate; the cationic detergent Zephiran chloride (Winthrop-Stearns); and the non-ionic detergent Tween 80‡ (Atlas Powder). It was found that all of the ionic detergents exerted a great and strikingly similar effect on the EW anti-vaccine titrations; in other experiments, Zephiran chloride, which was noted to agglutinate RBC in high dilution of the detergent, gave irregular results which have not yet been further investigated. Tween 80, the only non-ionic detergent tested thus far, gave, in contrast with the other materials, a slight depression of inhibition, which has been confirmed but not further studied.

Discussion. A review of the data of Tables II and III shows that, in the range of concentrations in which they modify the EW inhibition of vaccine hemagglutination, the detergents, typified by "green" soap, do not inhibit hemagglutination by vaccine in the absence of EW; nor do they, in these concentrations, agglutinate RBC or cause hemolysis. On the other hand, EW, in the absence of detergent, gives evidence of *bona fide* inhibitor activity in high dilution, although the conventional (++) endpoint is reached in relatively low dilution. Indeed, examination of the results of many titrations indicates that, in general, EW and detergent together do not cause inhibition in EW dilutions appreciably beyond that dilution at which EW can be effective alone. Accordingly, we may provisionally interpret the detergent effect, which may be described as a sharpening of the inhibition endpoint, as an effect on the interaction of EW inhibitor and vaccine.

Summary. Investigation of the origin of irregular titration results has led to the observation that soaps and synthetic ionic detergents exert a great effect on the character of inhibition titrations involving purified, formolized swine influenza virus (vaccine) and the egg-white (EW) inhibitor of vaccine hemagglutination. The amount of detergent needed for such an effect is of the order of the amount which is sufficient to cover the internal glass surface of a titration tube with

[6] Remington's Practice of Pharmacy, ninth ed., by Cook, E. F., and Martin, E. W., The Mack Publishing Company, Easton, Pa., 1948.

[7] Putnam, F. W., *Advances in Protein Chem.*, 1948, 4, 79.

† Obtained through the courtesy of Dr. J. H. Shipp of the E. I. du Pont de Nemours and Co., Wilmington, Del.

‡ Kindly furnished by Dr. Hilda Pope of the Duke University School of Medicine.

a single, close-packed unimolecular layer. While the detergent effect shows a dependence on detergent concentration, there exists a broad range of detergent concentrations over which the effect on the inhibition is essentially constant. For reasons indicated, the effect of detergent has been provisionally interpreted as an effect on the interaction of EW inhibitor and vaccine.

The mechanism of the detergent effect is being investigated and will be the subject of a subsequent report.

16850 P

Assay for Antistiffness Activity with Guinea Pigs Depleted on Solid Rations.

WALDEMAR DASLER* AND CLIFFORD D. BAUER. (Introduced by Richard G. Roberts.)
From Nutrition Research Laboratories, Chicago.

Oleson *et al.*[1] have described a solid basal ration with which they depleted guinea pigs for the purpose of conducting assays for antistiffness activity. Their results, however, seem to indicate shortcomings in the assay method. Thus, 13% of their negative controls showed marked improvement and an additional 17% showed slight improvement in stiffness. Furthermore, their homologous series of ergostanyl esters showed wide, inexplicable differences in activity.

Petering *et al.*[2] have recently described an apparently satisfactory assay method in which they used a solid, semipurified diet and with which they claim to have confirmed the antistiffness activity of ergostanyl acetate.

We have confirmed the production of wrist stiffness in guinea pigs with solid rations. Both the Lederle basal ration described by Oleson *et al.*[1] and the Rockland stock diet for guinea pigs† were successfully used to produce wrist stiffness. On the basis of our results, however, it is questionable whether the condition produced in guinea pigs on these rations is identical to the Wulzen stiffness syndrome which develops in animals fed the skim milk diets.[3-5] In our hands, in fact, these commercial, pelleted rations proved unsatisfactory for the assay of anti-stiffness activity.

That the condition which develops in guinea pigs on the Rockland and Lederle rations is not identical to that which has been produced by the Oregon group on skim milk diets is indicated by the fact that in our animals the easily hydrolyzable phosphorus of the liver did not decrease as drastically as in the experiments reported by van Wagtendonk[5] and van Wagtendonk and Wulzen.[6] In fact, we found no correlation between wrist stiffness and the easily hydrolyzable phosphorus of the liver in animals fed these rations.

We have nevertheless attempted to determine the antistiffness activity of a number of materials in assays involving several hundred animals fed these diets. Among the substances assayed were the previously reported active compounds listed in Table I.

Animals having a 3+ stiffness[1] were used for the therapeutic assays which, except for the diet, were conducted by the method of van Wagtendonk and Wulzen.[4,6] Supplements were dissolved in cottonseed oil and adminis-

* Present address: Department of Biochemistry, Chicago Medical School, Chicago, Ill.

1 Oleson, J. J., Van Donk, E. E., Bernstein, S., Dorfman, L., and SubbaRow, Y., *J. Biol. Chem.*, 1947, **171**, 1.

2 Petering, H. G., Stubberfield, L., and Delor, R. A., *Arch. Biochem.*, 1948, **18**, 487.

† Arcady Farms Milling Company, Chicago, Ill.

3 Wulzen, R., and Bahrs, A. M., *Am. J. Physiol.*, 1941, **133**, P500.

4 van Wagtendonk, W. J., and Wulzen, R., *Arch. Biochem.*, 1943, **1**, 373.

5 van Wagtendonk, W. J., *J. Biol. Chem.*, 1944, **155**, 337.

6 van Wagtendonk, W. J., and Wulzen, R., *J. Biol. Chem.*, 1946, **164**, 597.