Dialyzable Factor in Allantoic Fluid Affecting Virus Hemagglutination-Inhibition Reactions.* (17637)

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(Introduced by J. W. Beard.)
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The virus of swine influenza, purified by centrifugal procedures from the allantoic fluid of infected chick embryos(1), shares with human influenza viruses the capacity to agglutinate chicken red blood cells(2,3). The agglutination reaction is inhibited strongly by immune swine serum and weakly by normal serum, egg-white(4), and cow's milk(5). However, inhibition by the latter fluids, especially egg-white and milk, is considerably enhanced if purified virus heated at 53°C for 30 minutes is employed as the agglutinating agent(4,5). This increased susceptibility to inhibition, first described by Francis(6) for heated influenza virus B, has been attributed to an enzyme-like capacity of unheated virus to destroy inhibitor and to the loss of destructive activity on heating (7-9) (cf., however(10)).

Recently, it was found that swine influenza virus *in allantoic fluid* fails to develop on heating the high inhibition susceptibility characteristic of heated purified virus. Experiments carried out to elucidate this phenomenon, which would appear to have significance for numerous investigations employing unpurified virus, are described in the present report.

Materials and methods. Several sterile pools of normal allantoic fluid and of fluid infected with swine influenza virus (Shope's strain 15) or influenza virus A (PR8 strain) were employed. Embryonated 11-day eggs, obtained from a local hatchery, were inoculated with swine or PR8 virus and harvested as previously described(1,11) after 40 hours at 35°C (swine) or 37°C (PR8). Normal fluid was obtained from similar eggs after 40 hours at 35°C. The fluids were stored at 4°C without preservative. Before use, they were centrifuged at low speed, if necessary, to remove precipitates. Swine influenza virus was purified from a portion of one of the pools (Pool 1) by routine centrifugal procedures(12) with the modification that the pellets of the final cycle were taken up in 0.06 M phosphate buffer at pH 7.3 instead of Ringer solution. The product, labeled SF32-PB and stored at 0.217 mg N per ml at 4°C, possessed about 25,000 chick cell hemagglutinating doses per mg N and a 50% endpoint infectious unit of $10^{-13.7}$ g N on 10/17/49, 17 days after the purification was completed.

Hemagglutinative activity was determined by the method of Hirst(13), as modified in previous studies from this laboratory(14).

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1. 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, v48, 361.
2. Hirst, G. K., *Science*, 1941, v94, 22.
3. McClelland, L., and Hare, R., *Canad. Pub. Health J.*, 1941, v32, 530.
4. Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 312.
5. Lanni, F., Lanni, Y. T., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 227.
6. Francis, T., Jr., *J. Exp. Med.*, 1947, v85, 1.
7. Burnet, F. M., *Lancet*, 1948, v254, 7.
8. Hirst, G. K., *J. Exp. Med.*, 1948, v87, 301, 315.
9. Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 442.
10. Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F., and Stone, J. D., *Austr. J. Exp. Biol. and Med. Sci.*, 1948, v26, 403.
11. Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, v47, 261.
12. Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, v50, 291.
13. Hirst, G. K., *J. Exp. Med.*, 1942, v75, 49.
14. 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, v48, 129.

TABLE I.
Comparison of Purified (SF32-PB) and Unpurified Swine Influenza Virus with Respect to Development of Susceptibility to Egg-White Inhibition on Heating.

Virus	Conditions of heating				Inhibition titer§
	HD per ml	Diluent	pH‡	Min. at 53°C	
SF32-PB				0	20
"	24	Buff. saline*	7.3	30	49,000
"	24	Phos. buffer†	7.3	30	58,000
Pool 1				0	<20
"	96	None	8.5	30	50
"	32	Buff. saline	8.2	15	64
"	32	" "	8.2	30	80
"	32	" "	8.2	60	101
"	8	" "	7.6	30	115
Pool 2				0	<20
"	72	None	8.1	30	92
"	24	Phos. buffer	7.4	30	113
"	8	" "	7.3	30	450

* 0.81% NaCl and 0.005 M phosphate at pH 7.3.
† 0.06 M phosphate buffer at pH 7.3.
‡ By glass electrode.
§ Determined against 4 HD (hemagglutinating doses).

One *hemagglutinating dose* (HD) of virus is the amount required to give the standard two-plus (++) endpoint of agglutination with washed chicken red blood cells. *Heated virus* was prepared by immersing a rubber-stoppered test tube, containing a suitable dilution of virus, in a water thermostat at 53.0 ± 0.1°C. After a suitable period, usually 30 minutes, the tube was cooled in running tap water, and the virus was used immediately.

Inhibition titrations, employing a 20% solution of egg-white (EW) in buffered saline, preserved with 0.02% Merthiolate (Lilly), were carried out by the constant virus-varying inhibitor method(15). The *inhibition titer,* determined against 3 to 4 HD of virus, is expressed as the reciprocal of the final dilution of EW in the reaction mixture giving two-plus hemagglutination. Unless otherwise specified, the diluent was *buffered saline,* which consisted of 0.81% NaCl and 0.005 M phosphate at pH 7.3. A stronger buffer, consisting of 0.06 M phosphate at pH 7.3, was occasionally employed. Borate buffer at pH 7.4 was prepared according to Palitzsch, as given by Clark(16). All chemicals were of the reagent grade. Other details are given with the description of the experiments.

Experimental. Comparison of purified and unpurified virus. Samples of allantoic fluid infected with swine influenza virus (Pool 1, 96 HD per ml, and Pool 2, 72 HD per ml) and of virus (SF32-PB) purified from Pool 1 were heated at 53°C as shown in Table I and tested for susceptibility to EW inhibition by the routine method. The purified virus behaved typically(4) in developing a high susceptibility to inhibition on heating. The unpurified virus, on the other hand, developed only a low susceptibility. The inhibition titer against the latter was slightly improved by prolonging the period of heating to 60 minutes or by diluting the virus before heating (Table I). No greater increase in inhibition titer was obtained by heating unpurified virus at 56°C for 10 minutes; the rapid decrease in the hemagglutinative activity of the virus at the higher temperature precluded the use of longer periods of heating.

Adequate control of pH during heating was obtained by the use of 0.06 M phosphate buffer at pH 7.3 as diluent. The general similarity of the results obtained with this diluent and with the weaker buffered saline indicates

15. Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.,* 1949, v179, 1275.

16. Clark, W. M., *The Determination of Hydrogen Ions,* third ed., The Williams & Wilkins Company, Baltimore, 1928, 213.

TABLE II.
Effect of Heat on Dialyzed and Non-dialyzed Swine Influenza Virus in Allantoic Fluid (Pool 2). Reversibility of the Effect of Dialysis.

Mixture		Further treatment	Before titration, Mixture diluted 3-fold§ with	Inhibition titer
1 Vol.	2 Vol.			
Pool 2	Phos. buffer	None	Phos. buffer	<20
" "	" "	Heated‡	" "	101
" " dialyzed*	" "	None	" "	<20
" " "	" "	Heated	" "	64,000
" " "	Semi-dialysate†	None	" "	<20
" " "	" "	Heated	" "	127
" " "	Phos. buffer	"	1:3 Semi-dialysate	45,000
" " "	" "	None	1:3 Heated‡ semi-dialysate	<20
" " "	" "	Heated	1:3 " "	74,000

* Overnight in the cold against 100 volumes 0.06 M phosphate buffer at pH 7.3.
† Prepared by dialysis of Pool 2 against 1 volume of phosphate buffer.
‡ 30 min. at 53°C.
§ To give 4 HD in 0.5 ml. No significant decrease in hemagglutinative activity occurred during either dialysis or heating.

that pH is not a highly significant variable under the conditions of the experiments.

Effect of dialysis. The difference in behavior of purified and unpurified virus may be ascribed (a) to a difference in the properties of the virus particles or (b) to an extrinsic factor other than pH. Since the purified virus was obtained by relatively mild procedures, the latter alternative appeared the most likely explanation. Accordingly, virus-infected allantoic fluid was dialyzed in Visking casing overnight in the cold against 100 volumes of 0.06 M phosphate buffer at pH 7.3. A second sample was dialyzed against one volume of phosphate buffer under similar conditions in order to provide the dialyzable components of infected allantoic fluid in relatively high concentration; dialysate obtained in this way is referred to as *semi-dialysate.* In this experiment, phosphate buffer was used as diluent up to the point of the inhibition titrations.

Tests showed (Table II) that the thoroughly dialyzed virus developed on heating a high susceptibility to EW inhibition, comparable to that observed with centrifugally purified virus. Moreover, the effect of dialysis could be reversed by adding semi-dialysate to thoroughly dialyzed virus before heating; semi-dialysate, heated or not heated, added to heated dialyzed virus was without effect.

It appears, therefore, that the failure of unpurified virus to develop a high susceptibility to EW inhibition on heating may be ascribed to the influence of a dialyzable factor (or factors) in infected allantoic fluid. The results (Table II) show that this factor exerts its effect during the heating of the virus and not during the subsequent inhibition titration. The sensitivity of purified virus to heat (Table I) is readily explained on the reasonable assumption that the dialyzable factor, initially present in the infected allantoic fluid, is lost during the process of purification of the virus.

Occurrence of dialyzable factor in normal allantoic fluid. The dialyzable factor was found also in normal allantoic fluid. Furthermore, semi-dialysate from normal fluid was effective against virus purified by centrifugation (SF32-PB) as well as against dialyzed virus, as illustrated in Table III. These experiments followed the lines of the experiment recorded in Table II (above) and will not be described in detail.

The results indicate that the dialyzable factor is a normal constituent of allantoic fluid, independent of infection by influenza virus. The factor seems to be unrelated to the normal allantoic inhibitor of influenza virus hemagglutination (17) since (a) the normal inhibitor is not dialyzable and (b) thoroughly dialyzed normal allantoic fluid, although retaining inhibitory activity against

17. Svedmyr, A., *Ark. Kemi, Mineral. och Geol.*, 1947, v24B, No. 11.

TABLE III.
Effect of Dialysate from Normal Allantoic Fluid on Dialyzed Virus and Purified Virus (SF32-PB).

Virus	Treatment	Inhibition titer
Pool 1*	None	<20
Pool 1	Heated‡	200
Pool 1, dialyzed	None	<20
" "	Heated	76,000
" "	" with normal semi-dialysate	160
SF32-PB†	None	20
"	Heated	58,000
"	" with normal semi-dialysate	230

* Swine influenza virus in allantoic fluid.
† Diluted to approximately the same hemagglutinative activity as Pool 1.
‡ 30 min. at 53°C at a concentration of about 20 HD per ml.

TABLE IV.
Inhibitor Susceptibility of Thoroughly Dialyzed Swine Influenza Virus in Allantoic Fluid (Pool 3) Heated in the Presence of Ca^{++} or Mg^{++}.

2 vol. 1:3 dialyzed virus + 1 vol.	Further treatment	Inhibition titer
Buffered saline	None	<20
" "	53°C, 30 min.	68,000
Semi-dialysate*	" "	210
9×10^{-4} M $CaCl_2$†	" "	53
3×10^{-4} M "	" "	210
9×10^{-5} M "	" "	2,100
3×10^{-5} M "	" "	41,000
3×10^{-3} to $.3 \times 10^{-5}$ M $MgCl_2$	" "	68,000

* Obtained by dialysis of Pool 3 against 1 vol. of buffered saline.
† 3×10^{-3} M $CaCl_2$ in buffered saline formed a precipitate and was not tested.
No precipitation occurred in the other solutions; nor did the hemagglutinative activity decrease significantly during heating.

virus hemagglutination, does not exert the effect described above.

Identification of the dialyzable factor with Ca^{++}. Recent reports(18-23) have stressed the significance of Ca^{++} and calcium deionizing agents for certain *in vitro* properties of influenza viruses. Accordingly, Ca^{++}, which is reported (24) to be present in allantoic fluid in a concentration of about 0.019% (about 4.8×10^{-3} M), was tested for its capacity to substitute for the dialyzable factor. Mixtures of unpurified swine influenza virus (Pool 3; 80 HD per ml), thoroughly dialyzed against buffered saline, and of graded amounts of $CaCl_2$ in buffered saline were heated as usual for 30 minutes at 53°C. The products were then tested in the usual manner for susceptibility to inhibition by EW. Buffered saline, although containing phosphate and allowing the possibility of precipitation of the added calcium, was employed as diluent since phosphate was present in all the previous experiments.

The results, presented in Table IV, show that Ca^{++} is indeed capable of suppressing the development of inhibitor sensitivity by virus on heating. A highly significant effect was observed when virus was heated in the presence of 3×10^{-5} M $CaCl_2$, and a slight,

18. Briody, B. A., *J. Immunol.*, 1948, v59, 115.
19. Burnet, F. M., *Austr. J. Exp. Biol. and Med. Sci.*, 1948, v26, 389.
20. Woolley, D. W., *J. Exp. Med.*, 1949, v89, 11.
21. Stone, J. D., *Austr. J. Exp. Biol. and Med. Sci.*, 1949, v27, 229.
22. Edney, M., *Austr. J. Exp. Biol. and Med. Sci.*, 1949, v27, 253.
23. Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1949, v90, 475.
24. Needham, J., *Chemical Embryology*, Cambridge University Press, 1931, v3, 1538.

TABLE V.
Effect of Citrate, Oxalate, and Phosphate on the Development of Inhibitor Sensitivity by Non-dialyzed Swine Influenza Virus in Allantoic Fluid (Pool 4) on Heating (53°C, 30 Min.).

1:5 Pool 4* plus 0.2 vol.	pH before heating	pH after heating and 2-fold dil.† with 0.85% NaCl	Inhibition titer
.85% NaCl	7.6	7.7 (7.7)‡	140 (<20)‡
.128 M $Na_3C_6H_5O_7$	7.7	7.8	82,000
.032 "		7.7	34,000
.008 "		7.7	240
.002 "		7.7	200
.192 $Na_2C_2O_4$	7.6	7.7	82,000
.048 "		7.7	82,000
.012 "		7.7	200
.003 "		7.7	200
.192 Na_2HPO_4	7.7	7.8	900
.048 "		7.8	180
.012 "		7.7	180
.003 "		7.7	140
[Dialyzed control §]	7.6	7.7	82,000

* Diluted with borate buffer at pH 7.4.

† To give 4 HD in 0.5 ml. No significant decrease in hemagglutinative activity of any of the mixtures occurred during heating.

‡ Data in parentheses refer to unheated control with same composition.

§ Pool 4 dialyzed against 100 volumes buffered saline, diluted 5-fold with borate buffer, mixed with 0.2 volume 0.85% NaCl, heated, etc.

possibly insignificant effect was observed at 10^{-5} M. In the same experiment semi-dialysate was found equivalent to a 3×10^{-4} M solution of $CaCl_2$. In view of the presence of phosphate in the mixtures, these values are regarded to indicate only the maximal quantity of available Ca^{++}. In striking contrast with Ca^{++}, Mg^{++}, also present in allantoic fluid(24), was ineffective in substituting for semi-dialysate.

In other experiments undialyzed virus (Pool 4; 96 HD per ml) was heated in the presence of various amounts of citrate ($Na_3C_6H_5O_7 \cdot 5\frac{1}{2}\ H_2O$), oxalate ($Na_2C_2O_4$), and phosphate ($Na_2HPO_4$), as shown in Table V. The solutions at the highest concentrations shown were prepared in distilled water; the first 4-fold dilution of each was made with water in order to bring the ionic strength to a suitable level, and the subsequent dilutions were made with 0.85% NaCl. The mixtures were allowed to stand at room temperature about 10 minutes before heating. Within 5 minutes after preparation the mixtures containing oxalate in the two highest concentrations developed a faint turbidity, which did not increase appreciably during subsequent heating.

Both citrate and oxalate were effective in increasing the development of inhibitor sensitivity by virus on heating; phosphate was relatively ineffective. The maximal titer attained with virus treated with citrate or oxalate was identical with the titer attained with virus subjected to dialysis. The data (Table V) suggest that in certain equivalent concentrations oxalate might be more effective than citrate, a point which has been carefully checked for the conditions of the present experiments; however, it would be premature to conclude that this relation would hold under other conditions, in view of the high sensitivity of the system to the concentration of Ca^{++} (Table IV). These results with deionizing agents further support the conclusion that Ca^{++} is the agent in allantoic fluid responsible for the effects noted in Tables I to III.

Experiments with Human Influenza Virus A (PR8 Strain). The possibility that the foregoing results with swine influenza virus might be of general significance for influenza viruses was examined in experiments with allantoic fluid infected with the PR8 strain of human influenza virus A. It was found that this virus, in contrast with the swine virus, became highly susceptible to inhibition

TABLE VI.
Comparison of Swine Influenza Virus (Pool 3) and Human Influenza Virus A (PR8 Strain).

Mixture*		Further treatment	Inhibition titer
1 volume diluted dialyzed† virus	1 vol.		
Swine influenza	Buffered saline	None	<20
" "	" "	53°C, 30 min.	68,000
" "	Semi-dialysate (Swine)‡	" "	180
" "	" (PR8)‡	" "	130
PR8	Buffered saline	None	<20
"	" "	53°C, 30 min.	17,000§
"	Semi-dialysate (Swine)	" "	14,000
"	" (PR8)	" "	17,000

* All mixtures contained 16 HD per ml. The PR8 stock had about 170 HD per ml.
† Against 100 volumes of buffered saline.
‡ Prepared by dialysis against 1 volume of buffered saline. The pH was: Swine, 8.3; PR8, 8.1.
§ Identical titers were obtained with non-dialyzed virus heated 30 min. at 53°C and with dialyzed and non-dialyzed virus heated 30 min. at 56°C. In contrast with the swine virus, PR8 virus, whether dialyzed or not, lost 40 to 50% of its hemagglutinative activity on heating for 30 min. at 53°C or 56°C. Suitable adjustments were made prior to the inhibition tests.

by EW on heating and that dialysis prior to heating was without consequence.† The question therefore arose whether the dialyzable factor, present in normal and in swine-infected allantoic fluid, might be absent from PR8-infected fluid. Accordingly, semi-dialysate from each kind of virus-infected fluid was tested with both the homologous and the heterologous dialyzed viruses. As seen in Table VI, both semi-dialysates were effective against swine influenza virus and neither was effective against PR8 virus. In other experiments PR8 virus in allantoic fluid was heated in the presence of $CaCl_2$, added to give concentrations ranging from 10^{-5} to 3×10^{-3} M during heating. A slight effect was observed at the highest concentration only. It would appear, therefore, that PR8 and swine influenza viruses differ remarkably in their sensitivity to Ca^{++}.

Discussion. The primary object of the present investigation was to account for the difference in behavior of purified and unpurified swine influenza virus preparations heated for 30 minutes at 53°C. The titer of egg-white inhibitory activity against hemagglutination by heated purified virus is about 50,000. Against heated unpurified virus (virus in allantoic fluid) the titer is about 100. Against unheated virus of either sort the titer is 20 or less. The experimental results indicate that the difference between the two heated viruses is attributable to the calcium naturally occurring in the unpurified virus preparation. Ionized calcium, in low concentration depresses the development of inhibitor sensitivity by swine influenza virus on heating. The calcium may be removed by dialysis or neutralized by citrate or oxalate; phosphate is considerably less effective. Unpurified virus treated in these ways develops a high susceptibility to inhibition by egg-white on heating. In similar experiments human influenza virus A (PR8 strain) showed very little sensitivity to calcium. Burnet and his collaborators have shown that added calcium depresses the thermal development of inhibitor sensitivity by Lee virus(19) as well as the thermal inactivation of the receptor-destroying enzyme (RDE) of the cholera vibrio(25). The enzymatic activity of both of these agents against a mucoid inhibitor occurring in ovarian cyst fluid is enhanced by calcium and depressed by calcium de-ionizing agents(19,22,25; *cf.* (18)). The activity of the de-ionizing agents decreases along the series: hexametaphosphate, citrate, oxalate, phosphate, and fluoride(22). Stone(21), working with the cyst mucoid inhibitor, has

† Similar results were obtained in recent experiments with allantoic fluid infected with influenza virus B (Lee strain).

25. Burnet, F. M., and Stone, J. D., *Austr. J. Exp. Biol. and Med. Sci.*, 1947, v25, 227.

reported that unpurified swine influenza virus becomes highly susceptible to inhibition when heated at pH 8.5 for 2 hours at 37°C in borate buffer containing citrate. We have confirmed this observation only for dialyzed virus(26). Non-dialyzed virus does not develop high susceptibility to egg-white inhibition under these conditions; nor does dialyzed virus in the absence of citrate. No previous comparison of dialyzed and non-dialyzed virus with respect to development of inhibitor sensitivity on heating seems to have been made.‡ Several investigators have reported that influenza viruses in allantoic fluid differ in the extent to which they develop inhibitor sensitivity on heating(6,10,21,27,28). In particular, Bernkopf(28), employing egg-white as inhibitor, found that the PR8 virus was essentially unaffected by heat while the swine virus developed a high sensitivity to inhibitor, in contrast with the present results (Table VI).§ Hardy and Horsfall(29), however, reported a high inhibitory effect of egg-white against heated PR8 virus. These conflicting observations, while attributable to unrecognized factors, emphasize the necessity of explicit definition of the conditions under which a virus is heated. The importance of naturally occurring calcium for the properties of one virus and the lack of importance for another suggest a rational explanation for the variation among influenza viruses noted above and warn against the incautious use of unpurified virus in experiments of this sort. The mechanism by which calcium exerts its effect remains to be elucidated.

Summary. The difference between purified and unpurified swine influenza virus with respect to development of inhibitor sensitivity on heating is ascribed to a factor, identified as calcium, occurring in normal and virus-infected allantoic fluids. The factor, which is lost, or considerably diminished, during purification, may be removed also by dialysis or neutralized by citrate and oxalate. Calcium, in low concentration, depresses the development of inhibitor sensitivity by swine influenza virus on heating; magnesium is ineffective. In contrast with the swine virus, influenza virus A (PR8 strain) is relatively insensitive to calcium. This difference between two viruses suggests a possible explanation for the generally observed variation among influenza viruses with respect to development of inhibitor sensitivity on heating.

26. Lanni, Y. T., and Lanni, F., unpublished experiments.

‡ In a paper appearing after the present work was completed, Stone (*Austr. J. Exp. Biol and Med. Sci.*, 1949, v27, 337) reports that Lee virus, dialyzed against tap water, develops inhibitor sensitivity when incubated for 18 hours at 37°C in borate buffer (pH 8.5) containing citrate; this treatment was relatively ineffective against non-dialyzed virus.

27. Burnet, F. M., *Austr. J. Exp. Biol. and Med. Sci.*, 1948, v26, 371.

28. Bernkopf, H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 160.

§ The identity of the virus strains studied here was confirmed by means of hemagglutination-inhibition tests with specific immune sera. We are indebted to Dr. Fred Wrights for the PR8 antiserum.

29. Hardy, P. H., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, v88, 463.

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