## Preparation of a Stable Non-Infective Soluble Influenza A Antigen. (18480)

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The soluble antigen of influenza A (PR8) prepared from the specifically infected chorioallantoic membranes(1) is an excellent antigen in the complement-fixation test for the laboratory diagnosis of influenza A. This soluble antigen can be rendered non-infective by the addition of formalin(2) or by ultraviolet irradiation(2,3). Ultraviolet irradiation requires special apparatus(4,5) while the use of formalin has been found to render the antigen anti-complementary on storage and unsuitable for lyophilization since the reconstituted material has little or no antigenicity.

In an attempt to develop a simple method of preparing a non-infective soluble influenza antigen that would remain stable on long storage, two procedures were developed. One is based on the use of hydrogen peroxide, the other on the use of formalin followed by its neutralization prior to storage or lyophilization.

*Materials and methods.* The soluble antigen was prepared from the chorioallantoic membranes of 13-day-old chick embryos which had been infected with the PR8 strain of influenza A virus by the allantoic route 2 days previously. To the pooled membranes was added 2.5 ml of saline per membrane and the mixture ground for 2 minutes in a Waring blendor. After a preliminary centrifugation for 10 minutes at 2000 r.p.m., the supernatant suspension was centrifuged for 1 hour at 15,000 r.p.m. The supernatant liquid was removed and used as the soluble antigen. It was tested for hemagglutinins by the procedure of Salk(6) and found to contain less than 20 units per ml. The $ID_{50}$ was $10^{7.2}$ when titrated in the allantoic cavity. Infectivity tests of control and treated samples were conducted by direct inoculation of undiluted and $10^{-2}$ diluted solutions into the amniotic sac of 11-12-day-old chick embryos and a subsequent incubation for 72 hours. Samples which after 2 successive amniotic passages yielded amniotic and allantoic fluids free of specific hemagglutinins were considered to be non-infective. Preliminary experiments had shown that this soluble antigen could be clarified by treating with Norit (Norit "A" from Fisher Scientific Co.) or certain concentrations of hydrogen peroxide (Eimer & Amend, reagent 30%) with little or no loss of the antigenicity. Although the Norit-treated fractions were still infective, some of the peroxide-treated ones were not. The following experiment was conducted to determine the optimal concentration of peroxide and experimental conditions to yield a soluble antigen which was non-infective, could be lyophilized, stored and then redissolved with a minimal loss of antigenicity.

Three temperatures, 0°, 22° and 37°C were selected for the initial peroxide-inactivation reaction. Aliquots of a common pool of the soluble antigen were placed in cotton stoppered Erlenmeyer flasks in water baths at the above temperatures. After 10 minutes, hydrogen peroxide was added to the antigens in the proportion of 1:10 to give the final peroxide concentrations shown in Table I. Normal membrane antigen was treated similarly as a control. After a given reaction period at the designated temperature, the flasks were placed in a cold room at 4°C overnight. To remove the excess hydrogen peroxide, the solutions were then shaken with 3% by weight of Norit at room temperature

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TABLE I. Treatment of PR8 Soluble Antigen with Hydrogen Peroxide.

| Antigen content of $H_2O_2$, % | Reaction temp., °C | Reaction time, hr | CF titer | Infectivity | After lyophilization: CF titer | After lyophilization: Infectivity |
|---|---|---|---|---|---|---|
| 0 (control) | 0 | 0 | 64 | + | 64 | + |
| 3 | 0 | 2 | 32 | + | 32 | + |
| 3 | 22 | ½ | 8 | — | <2 | — |
| 3 | 37 | ½ | 2 | — | <2 | — |
| 2 | 0 | 2 | 64 | + | 64 | + |
| 2 | 22 | 2 | 64 | + | 64 | — |
| 2 | 37 | ½ | 8 | — | 4 | — |
| 1 | 0 | 2 | 64 | + | 64 | + |
| 1 | 22 | 1 | 64 | + | 64 | + |
| 1 | 37 | 1 | 32 | + | 32 | + |
| 1 then increased to 2 | 37 | 1 | 32 | — | 32 | — |
| 1 then increased to 2 | 22 | 1 | | | | |

for one hour, alternately applying and removing a vacuum. The solutions were centrifuged for 5 minutes at 5000 r.p.m. and the clear supernatant liquids were removed. The antigenicity of these liquids was determined in the complement-fixation test(7), using 2 units of complement and 4 units of amboceptor. Aliquots were lyophilized, reconstituted to their original volume with distilled water and also tested. All samples were then tested for infectivity as described.

Although formaldehyde is a well-known virucidal agent, it was found that when the concentration of formaldehyde present in the soluble antigen was sufficient to destroy infectivity, lyophilization of the antigen for storage produced an almost insoluble material, suggestive of protein denaturation, which was no longer antigenic. It therefore seemed obvious that if, after the period of virus inactivation with formaldehyde, the excess formaldehyde could be removed or neutralized chemically, the lyophilization process might be more successful. Ammonia and ammonium salts are highly effective in converting formaldehyde to hexamethylenetetramine(8), especially at a neutral or slightly alkaline pH. Therefore, after the treatment of the antigen with formaldehyde, the amount of 5% ammonium hydroxide theoretically required from the equation:

$$6\ CH_2O + 4\ NH_3 \longrightarrow (CH_2)_6N_4 + 4\ H_2O$$

to convert all the formaldehyde to hexamine was added. Since some of the formaldehyde is consumed in reaction with the virus and protein, a slight excess of ammonia is present which is desirable for the conversion of formaldehyde to hexamine. Formaldehyde solution was added to the antigen in the proportion of 1:10 to give the final concentrations shown in Table II. The treated antigens were held at the designated temperatures for 2 hours and then placed in the cold room at 4°C overnight (16 hours). The excess formaldehyde in aliquots of each sample was neutralized by the addition of the theoretical amount of 5% ammonium hydroxide, the solutions maintained at 25°C for 1 hour and then the pH adjusted to 7.2-7.4 with 0.1 N sulphuric acid or isotonic dibasic sodium phosphate.

Aliquots were lyophilized and then redissolved to the original volume with distilled water. After testing for antigenicity and infectivity, samples were stored at 4°C in sealed

TABLE II. Treatment of PR8 Soluble Antigen with Formaldehyde.

| % HCHO | Temp., °C | CF titer | Infectivity | After lyophilization: CF titer | After lyophilization: Infectivity |
|---|---|---|---|---|---|
| 0 (control) | 25 | 16 | + | 16 | + |
| .5 | 25 | 32 | — | <2 | |
| .5 + $NH_3$ | 25 | 8 | — | 8 | |
| .25 | 25 | 16 | — | <2 | |
| .25 + $NH_3$ | 25 | 8 | — | 4 | |
| .1 | 25 | 16 | — | <2 | — |
| .1 + $NH_3$ | 25 | 16 | — | 16 | — |
| .1 | 37 | 16 | — | | |
| .1 + $NH_3$ | 37 | 16 | — | | |
| .03 | 25 | 16 | + | <2 | |
| .03 + $NH_3$ | 25 | 16 | — | 16 | |

7. Henle, W., Henle, G., Groupe, V., and Chambers, L. A., *J. Immunol.*, 1944, v48, 163.

8. Polley, J. R., Winkler, C. A., and Nicholls, R. V. V., *Can. J. Res.*, 1947, v25B, 525.

ampoules or lyophilized. The results by this procedure are shown in Table II.

As a final check on the virus inactivation by this procedure, a fresh lot of the soluble antigen was prepared in the usual manner. One aliquot of it was deliberately contaminated with PR8 virus by adding to it the pellet containing the virus which was obtained by centrifuging an equivalent volume of infected allantoic fluid. The soluble antigen had a hemagglutination titer of less than 20 units per ml and the $ID_{50}$ was $10^{-6.5}$ whereas the antigen plus virus had a hemagglutination titer of 2560 units per ml and the $ID_{50}$ was $10^{-8.7}$. The samples were then treated with 0.1% and 0.15% formaldehyde, both with and without subsequent neutralization. Infectivity tests showed all samples to be non-infective and the neutralized samples had lost no antigenicity before or after lyophilization.

At the time of writing, samples of antigens treated with hydrogen peroxide or formaldehyde plus neutralization have been stored for over 2 months in sealed ampoules and in the dried condition at 4°C and have lost none of their antigenicity nor have they become anti-complementary.

*Discussion.* It can be seen from Table I that (1) when the soluble influenza A (PR8) antigen contained 3% hydrogen peroxide, the infectivity was destroyed only under conditions which caused a large loss of the antigenicity (2) treatment with 2% peroxide at 22°C for 2 hours, followed by lyophilization, produced a non-infective antigen with little or no loss of antigenicity (3) treatment with 1% peroxide at 37°C for 1 hour followed by an increase to 2% peroxide at 22°C for 1 hour yielded an antigen which was non-infective after lyophilization and which had lost little or none of its antigenicity. In the 3 experiments done, this antigen was also non-infective prior to lyophilization.

From Table II it is evident that when the concentration of formaldehyde in the antigen is sufficient to destroy the infectivity, lyophilization destroys the antigenicity. However, when the excess formaldehyde is neutralized with ammonia prior to lyophilization, the antigenicity remains intact. The fact that the reaction temperature can be increased to 37°C for 2 hours and the ammonia concentration to pH 10 without loss of the antigenicity indicates that the antigen is quite stable to this method of treatment. Normal membrane antigen treated by this procedure gave no significant nonspecific antigen titer in the complement-fixation test.

Since the antigenicity is more stable to variations in temperature and reagent concentration with the formaldehyde treatment, it is considered to be the better method.

*Summary.* Two methods for the preparation of a stable non-infective soluble influenza A antigen have been developed. One is based on treatment with 2% hydrogen peroxide, the other on treatment with 0.1% formaldehyde followed by its neutralization prior to storage or lyophilization.

Received January 13, 1951. P.S.E.B.M., 1951, v76.

## *In Vitro* Cultivation of the Parasitic Phase of *Coccidioides immitis.* (18481)

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To date only partially successful results have been obtained in attempts to culture the parasitic phase of *Coccidioides immitis* which exists as a spherical endospore-producing organ (commonly known as 'spherule') *in vivo* and as a fluffy white mold *in vitro.*

* The author wishes to thank Prof. W. H. Weston for aid and counsel during the course of this work; Miss Betty Insley and Dr. G. Morel for tubes of coconut milk agar medium; Dr. E. E. Baker for suggestions concerning the research, and Dr. N. F. Conant for supplying 5 strains of *C. immitis.*