17167. The Precipitating Effect of Methanol on Viruses.*

MORRIS POLLARD, JOAN CONNOLLY, AND STANLEY FROMM.
From the University of Texas Medical School, Galveston, Texas.

Many technics have been applied to the problem of concentration of viruses. Those which utilize chemical and physical methods have been well reviewed by others.[1-3] Recently Cox *et al.*[4] reported on the application of the protein-precipitating property of methyl alcohol to the concentration of influenza B virus. By virtue of what seems to be a denaturating effect on somatic proteins and an innocuousness for virus protein, this technic appears to offer some promise of providing a method which might be useful with a wide range of viruses. It requires careful control over methanol concentration, temperature of reaction, and pH of the elution fluid.

The technic of virus precipitation with methanol is described in detail by Cox *et al.* and the procedure used in this study is essentially unchanged. The virus suspenion is maintained at 0°C to 5°C in an ice water bath. Chilled methanol (C.P.) is slowly mixed into the virus suspension and shortly thereafter a precipitate appears. The precipitation reaction is permitted to proceed for 3 hours. The precipitate is then sedimented in a chilled angle centrifuge at 4600 rpm for 30 minutes. The supernatant fluid is tested for virus activity and, since it is usually relatively free of virus, it is then discarded. The precipitate is resuspended to any volume desired in 0.2 M citrate or phosphate buffer, and the virus is permitted to elute at room temperature for one hour. The suspension is then centrifuged at 2500 rpm for 15 minutes at room temperature. The resulting supernatant fluid contains most of the virus.

Five viruses† were studied with this methanol precipitation technic, *viz:* 10% saline emulsion of Eastern equine encephalomyelitis virus (EEE) in mouse brain; 10% saline emulsion of human poliomyelitis virus (MEF) in mouse spinal cord; and influenza B, Newcastle disease, and mumps viruses in allantoic fluid. The EEE and MEF viruses were tested in decimal dilutions (0.03 ml per mouse) for infectivity by intracerebral inoculation into 10 g Swiss mice. Fifty percent lethal end points were determined according to the method described by Reed and Muench.[5] The 3 remaining viruses were tested for activity by the Hirst technic of chicken rbc agglutination.[6] In studying these five viruses some uniformity of pattern seems to have been manifested, particularly concerning the optimum concentration of methanol, and the optimum pH level at which virus elution occurs. All experiments re-

TABLE I.
Methanol Concentration and Elution pH for Optimal Yield of Virus.

Virus	Optimum	
	% methanol	Elution pH
EEE	25	7
MEF	25	8
Mumps	30	8
Influenza B	25	9
Newcastle disease	30	7

† Source of viruses:

MEF poliomyelitis—Dr. P. K. Olitsky, Rockefeller Institute, New York, N. Y.

Eastern equine encephalomyelitis—Local outbreak in equines during fall of 1947.

Mumps—Dr. J. F. Enders, Children's Hospital, Boston, Mass.

Influenza B—Dr. Joseph E. Smadel, Army Medical School, Washington, D.C.

Newcastle disease—Dr. L. T. Giltner, U.S.B.A.I., Pathological Division, Washington, D.C.

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1 Anderson, T. F., *Cold Spring Harbor Symposia on Quantitative Biology*, 1946, **11**, 1.

2 Pirie, N. W., *Annual Rev. of Biochem.*, 1946, **15**, 573.

3 Gard, Sven, *Acta Med. Scand. Supp.*, 1943, **143**, 1.

4 Cox, H. R., Van der Scheer, J., Aiston, S., and Bohuel, E., *J. Immunol.*, 1947, **56**, 148.

5 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

6 Hirst, G. K., *Science*, 1941, **94**, 22.

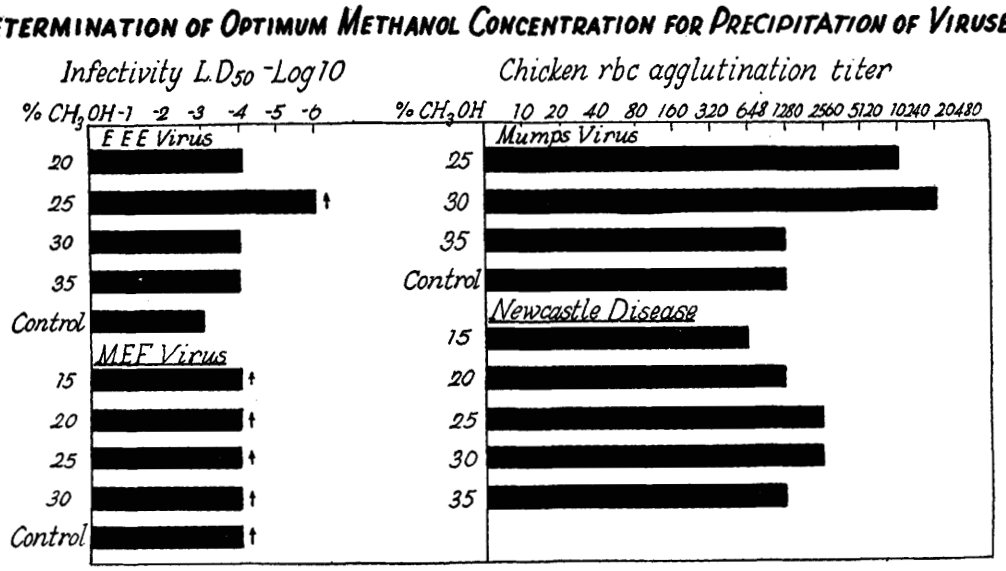

FIG. 1.

ported herein were repeated at least once, and the results were sufficiently in accord so as to warrant being published.

The optimum concentration of methanol was determined by adding 20, 25, 30, and 35% of methanol to aliquots of the virus suspension. By testing the resulting virus eluates in pH 7 buffer, the optimum methanol concentrations were arrived at and are listed in Table I: 25% methanol was optimal for EEE and MEF;‡ 30% for mumps virus and Newcastle disease virus. The concentration of methanol to be used for MEF virus was determined on the basis of complete extraction of the agent from the suspension by the precipitate and on this basis, 25% was found to be optimal. (Fig. 1).§ In accord with a previous report,[4] 25% methanol concentration was found to be optimal for influenza B virus.

Using the optimum concentration of methanol, as determined above for each virus, the pH at which each of the viruses would be eluted from the precipitate (or redissolved) most effectively was determined (Table I). It can be noted that the eluates were more potent as the pH of the eluting fluid was raised (Fig. 2). Each of the 5 viruses appeared to be more soluble in buffers at pH 7 or above than with buffers under pH 7. It was thought that some viruses might be inactivated at the lower pH levels, and so explain the lower titers of the eluates. However, when a precipitate containing EEE virus was eluted at pH 5 and then reeluted at pH 7, the virus was absent in the first elution but was recovered in the second step.

When virus suspensions were precipitated with methanol, the final eluate was found to contain less nitrogen than the original suspension. When the virus suspension was concentrated by reducing the relative volume of the final eluate, there was no proportional increment in content of nitrogen, while the virus content per ml was considerably increased. In fact, while there was considerable loss of nitrogen as a result of methanol precipitation, this loss did not appear to be at the expense of the virus. Table II illustrates some of the results of such microkjeldahl determinations.

Discussion. It can be noted that these methanol treated viruses have yielded eluates which are inordinately more potent than the

‡ In a recent report Gollan indicates that 33% methanol was suitable for precipitating rodent MM poliomyelitis virus, PROC. SOC. EXP. BIOL. AND MED., May 1948, **67**, 3.

§ The arrows in Fig. 1 and III indicate that an end point was not determined.

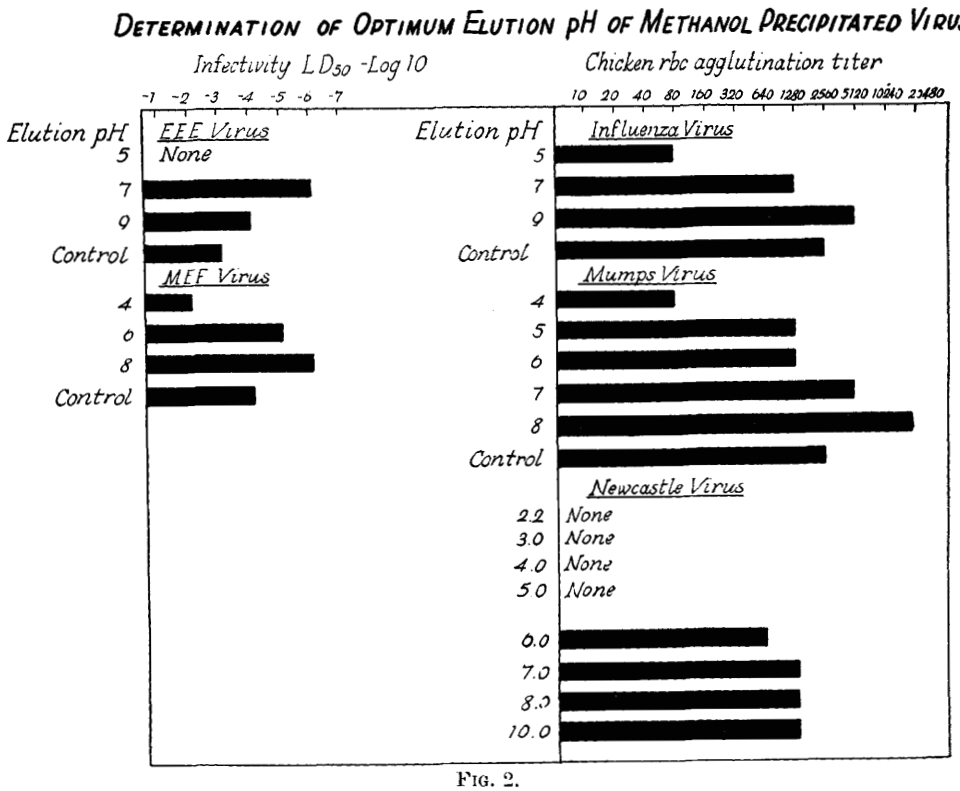

FIG. 2.

concentration factor would indicate. Our work has not yet offered any explanation for this phenomenon. The enhancement might be due to the disruption of infective aggregates or to the removal of some inhibiting substances as a result of this treatment with methanol. It may be that the viruses are manifesting an optimum pH activity point; however, Wenner[7] has reported that pH 4.6 is the optimum effective point for poliomyelitis. At this pH level, lower titratable endpoints were demonstrated in the eluates from methanol precipitated virus suspensions than were attained at the higher pH levels (Fig. 2). Horsfall[8] refers to autointerference agents which might provide an explanation for the enhancement of potency of the viruses following treatment with methanol. In all instances the nitrogen content of the virus suspension was reduced following methanol treatment, but not at the expense of the infective agent. This is in accord with the findings of Koprowski *et al.*[9] with methanol-precipitated viruses.

It may be that the viruses are less soluble at the lower pH levels because of the proximity to their isoelectric points; and in accordance with what has been demonstrated with enzyme proteins[10] and with animal serum proteins,[11] the solubility of the viruses increases as one moves away from this pH. As indicated above, while EEE virus was insoluble it was not destroyed after exposure

7 Wenner, H. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 104.

8 Horsfall, Frank L., Jr., Viral and Rickettsial Infections of Man, J. B. Lippincott Co., Philadelphia, 1948.

9 Koprowski, H., Black, J., and Cox, H. R., Proc. 4th Internat. Congress for Microbiol., July 1947.

10 Northrop, J. H., Crystalline enzymes, Columbia University Press, 1939.

11 Cohn, E. J., and Edsall, G., Proteins, Amino Acids and Peptides, Reinhold Publishing Corp., New York, 1943.

TABLE II.
Nitrogen Loss by Precipitation of Virus Suspension with Methanol.

Virus CAF*	Mg N crude	Fold. conc.	Mg N after precipitation	% N reduction
Newcastle	.64	0	0.20	68.8
"	.62	0	0.35	43.8
"	.62	10	0.42	99.2
Influenza B	.54	0	0.21	62.0
" A	.47	0	0.14	70.3
" A	.47	10	1.12	76.2
Mumps	.56	100	2.91	94.9
"	.56	100	1.47	97.3
"	.56	100	0.35	99.3

* CAF — Chorio-allantoic fluid.

to pH 5 for one hour, and it was thereafter recovered with appropriate pH solutions. It has been reported that poliomyelitis virus can tolerate exposure to pH 1.6,[12] and on this basis it seems probable that the MEF virus was not destroyed when it failed to elute at the lower pH levels. Additional studies of the pH stability of MEF poliomyelitis virus indicate that while the virus was most stable at pH 6-7 it was able to tolerate pH 3 for 10 weeks.[13]

Summary. Five viruses (Eastern equine encephalomyelitis, MEF human poliomyelitis mumps, influenza B, and Newcastle disease) were utilized in studying the precipitating effect on them of methanol. Optimal methanol concentrations and optimal pH for elution were determined for each virus and are tabulated (Table I). All of the viruses which were studied demonstrated a relationship between the pH of the elution fluid and solubility: as the pH was raised there was a corresponding increase in solubility of the virus. The methanol precipitation technic results in considerable loss of nitrogen from the virus suspension, but this loss does not appear to be at the expense of the virus.

[12] Loring H., and Schwerdt, C. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 173.

[13] Pollard, M., and Connolly, J., Tex. Reports on Biol. and Med., Spring 1949.

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17168. Comparison of Newcastle Virus in Hamsters Exposed by Intracerebral Injection and Intranasal Instillation.

REGINALD L. REAGAN, MARY G. LILLIE, DOROTHY E. SMITH, AND A. L. BRUECKNER.
From Maryland State Board of Agriculture, University of Maryland, College Park, Md.

Hamster-adapted Newcastle virus, California strain No. 11,914, has been continued through the 300th passage by serial intracerebral inoculation. Virus neutralization tests were conducted, using brain suspensions of the 245th and 300th intracerebral hamster passages with specific Newcastle virus immune and normal chicken sera, and with hamsters as test animals. Specific immune sera completely neutralized the hamster brain virus, whereas normal chicken serum had no effect. The virus of this 300th passage titred $10^{-4.77}$ intracerebrally in 4-week-old hamsters, in contrast to the virus of the 200th passage[1] which titred $10^{-4.25}$ in hamsters of the same age injected by the same route.

Newcastle virus infection in hamsters inoculated intranasally with 10% brain suspension of the 16th intracerebral hamster passage has been previously reported.[2] It was also reported that intranasal instillations

[1] Reagan, R. L., Lillie, M. G., Hauser, J. E., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 293.

[2] Reagan, R. L., Lillie, M. G., Poelma, L. J., and Brueckner, A. L., *Am. J. Vet. Res.*, **8**, 427.