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# Purification of Equine Encephalomyelitis Virus by Ultracentrifugation and Maintenance of Its Activity with Cysteine.

F. B. BANG AND ROGER M. HERRIOTT. (Introduced by C. TenBroeck.)

*From the Department of Animal and Plant Pathology and the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N.J.*

Purification of enzymes[1] and viruses[2] is aided by quantitative methods of determining the activity and solids in the various fractions.

It has been reported[3] that the virus of equine encephalomyelitis has been concentrated and at least partially purified, but quantitative data showing the results of the various steps have not been published. We have carried out experiments designed to separate the virus from non-virus material by using the following procedures: (a) ultracentrifugation, (b) fractional precipitation by dialysis or dilution, (c) fractional precipitation by heparin. Since ultracentrifugation was the only procedure to yield consistent and desirable results as regards purification, discussion of the other experiments will be brief.

The results of a number of ultracentrifugation experiments are shown in Tables I and II. It may be seen from these tables that ultracentrifugation forces most of the virus into the pellet and that the titer per gram of nitrogen of the resuspended pellet is about 10 times higher than the starting material.

Since one of the major difficulties in purification of this virus is the maintenance of activity, we have also studied the effect of certain reducing agents on the stability of ultracentrifuge purified virus suspensions. The results of this study (Table III and Fig. 1) show that at pH 7.0-8.0 in the presence of M/10 cysteine the activity of ultracentrifuge-purified virus is relatively constant.

TABLE III.

Experimental procedure: To 2 ml of ultracentrifuge purified virus was added 2 ml of 0.2 molar reducing reagent in phosphate buffer so the final pH was 6.8-7.8. The thioglycollate was prepared by mixing solutions of calcium thioglycollate with $Na_2HPO_4$ and centrifuging off the precipitate.

| | Original | 8 days | 15 days | 100 days |
|---|---|---|---|---|
| Control | $10^{-7.8}$ | $<10^{5.5}$ | $10^{-2.3}$ | |
| Cysteine | | $10^{7.3}$ | $10^{7.8}$ | $10^{5.7}$ |
| Hydroquinone | | $<10^{5.5}$ | $<10^{2.5}$ | |
| Thioglycollate | | $<10^{5.5}$ | $<10^{1.5}$ | |

*Experimental.* Virus activity was followed by use of the 50% mortality endpoint of 10-day chick embryos, which allows frequent accurate titrations of activity.[4]

Our investigation was limited to the eastern variety of the virus, isolated in this laboratory from horses[5] in 1933. The solution of virus used as starting material was prepared as follows: 10-day chick embryos were infected by inoculating the chorioallantoic membrane with 1 drop of 1/100 dilution of infected guinea pig brain. The embryos were harvested at 23 hours, ground in a Waring Blendor in cold saline solution, buffered with phosphate to make a 20% suspension by weight. This suspension was cleared of large particles by centrifugation in a Swedish angle centrifuge at 4,000 R.P.M. for 20 minutes. Serial tenfold dilutions in cold buffered saline solution were made and each of 3 or 4 dilutions, which were likely to fall on either side of the 50% endpoint, was then inoculated on five 10-day embryos. The 50%mortality endpoint was calculated[6] after the eggs had been incubated 3 days.

1 Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739.

2 Stanley, W. M., *Science*, 1935, **81**, 644.

3 Sharp, D. G., Taylor, A. R., Beard, D., Finkelstein, H., and Beard, J. W., *Science*, 1940, **92**, 359; Taylor, A. R., Finkelstein, H., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 462; Taylor, A. R., Sharp, D. G., Finkelstein, H., Beard, D., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 648.

4 Bang, F. B., *J. Exp. Med.*, 1943, **77**, 337.

5 TenBroeck, C., and Merrill, M. H., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 217.

6 Reed, L. J., and Meunch, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.

| No. of centrifugings | Time of runs min | Titer* | | | |
|---|---|---|---|---|---|
| | | Original | Pellets resuspended in same vol. | First supernatant | Change in titer |
| 2 | 75 | 8.0 | 8.0 | | 0 |
| 2 | 45 | 8.0 | 6.4 | 4.3 | —1.6 |
| 3 | 30 | 7.5 | 7.8 | 6.0 | +0.3 |
| 2 | 45, 30 | 7.5 | 8.0 | 5.5 | +0.5 |
| 2 | 45, 30 | 8.5 | 7.8 | 4.2 | —0.7 |
| 1 | 45 | 8.4 | 8.0 | | —0.4 |
| 2 | 45, 30 | 8.2 | 7.6 | | —0.6 |
| 2 | 30 | 8.0 | 7.8 | | —0.2 |
| 2 | 45 | 7.5 | 7.6 | 4.4 | +0.1 |

*The figures shown below are exponents of 10. They represent the serial tenfold dilution of the original solution to produce 50% mortality.

TABLE II.

| No. of centrifugings | Original suspension | | Ultracentrifuged pellet resuspended | | Titer/g nitrogen | | |
|---|---|---|---|---|---|---|---|
| | Titer | Nitrogen, mg/ml | Titer | Nitrogen, mg ml | Original suspension | Resuspended pellet | Purification |
| 1 | $10^{8.1}$ | 0.8* | $10^{8.1}$ | 0.06 | $10^{11.2}$ | $10^{12.3}$ | 13× |
| 2 | $10^{8.3}$ | 0.8 | $10^{9.0}$ | 1.2 | $10^{11.4}$ | $10^{11.9}$ | 3× |
| 3 | $10^{7.5}$ | 0.8 | $10^{9.2}$ | 0.6 | $10^{10.6}$ | $10^{12.4}$ | 60× |
| 2 | $10^{8.0}$ | 0.8 | $10^{8.2}$ | 0.7 | $10^{11.1}$ | $10^{11.4}$ | 2× |
| 2 | $10^{7.6}$ | 0.7 | $10^{7.8}$ | 0.1 | $10^{10.8}$ | $10^{11.8}$ | 10× |

*This figure is based on a number of measurements made on suspensions prepared at other times which varied but slightly.

*Ultracentrifugation.* Table I presents the results of centrifugation of clarified 20% infected embryo suspensions in a Bauer and Pickels air-driven ultracentrifuge[7] spun at 30,000 R.P.M. Since the tubes are at an angle this speed produces 50,000-90,000 times gravity. The centrifuge head was precooled to 5°C. In these experiments the pellets so obtained were resuspended in cold normal buffered saline, cleared in the Swedish centrifuge at 4,000 R.P.M. and then immediately titered by serial tenfold dilutions or ultracentrifuged again.

The results in Table I show clearly that the virus is thrown down in the pellet and that it may be recovered from the pellet by resuspension in buffered saline. Previous experiments have shown that the titer of one solution when run in duplicate may vary[4] as much as $10^{0.6}$ and this error must be taken into account in appraising the above figures. The average loss in these experiments (with one low result excluded) is $10^{0.1}$. This indicates that most of the virus is recovered.

Since, as may be seen from Table I, most of the original virus activity is in the resuspended residue, while a fair proportion of the original protein is left in the supernatant liquid from the first ultracentrifugation, it follows that there has been at least a partial purification. This is seen more directly from the columns of Table II showing the virus activity per gram of nitrogen.

*Fractional Precipitation.* Precipitation was carried out by dialysis in a rocking dialyzer against distilled water for 36 hours. Ultracentrifuge purified virus was treated in this way but the results were inconstant. A variation of this method of precipitation was carried out by diluting the purified concentrates in distilled water, then centrifuging out the precipitate. This too yielded inconstant results, although individual experiments did show a significantly greater amount of activity of protein nitrogen in the precipitate.

Certain macromolecules, including plant

[7] Svedberg, T., and Pedersen, K. O., *The Ultracentrifuge,* Oxford Press, 1940.

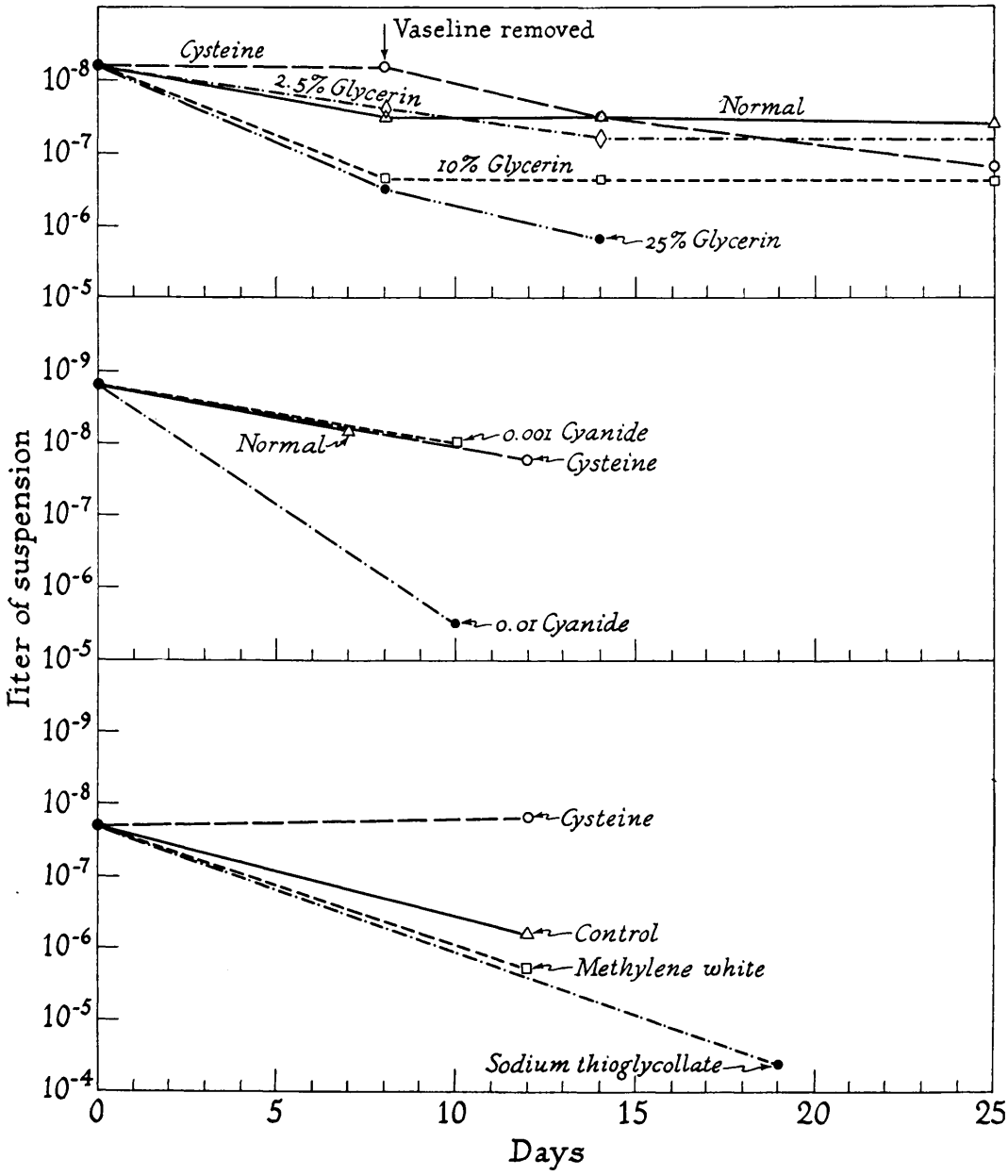


FIG. 1.

viruses, may be separated from solutions by adding heparin to make a 5% solution.[8] This was attempted on freshly prepared ultracentrifuged concentrates of equine encephalomyelitis and the resulting precipitate was then thrown down by low speed centrifugation. Titration of the precipitate and of the supernatant again showed no concentration of virus in the precipitate.

*Preservation of Virus Activity.* The previous work was made difficult by a frequent loss of activity of the virus during manipulation. This was particularly apparent when working with suspensions partially purified by centrifugation. For this reason we turned

[8] Cohen, S. S., *J. Biol. Chem.*, 1942, **144**, 353.

to a study of the factors affecting the stability of such suspensions. Other workers[9] have found that the stability is unaffected by a relatively wide pH range. Certain enzymes are active only within certain oxidation-reduction ranges.[10] Among viruses, that of tomato spotted wilt is highly susceptible to oxidation by air and may be protected by reducing agents such as cysteine and sodium thioglycollate.[11] Mueller found that the virus of Rous's sarcoma may be protected against oxidation by the addition of cysteine.[12]

Zinsser and Tang[13] have found that cysteine stabilizes herpes virus.

The results in Table III and Fig. 1 show the effect of certain reducing agents under vaseline and of glycerine in several concentrations on the stability of virus. Unless otherwise indicated the virus was ultracentrifuge purified; the pH was held at 7.0-7.6 with M/10 phosphate; the concentration of reducing agent was M/10; and the temperature was 5-10°C. Methylene white was prepared by reducing methylene blue with metallic zinc and hydrochloric acid followed by rapid neutralization and centrifugation of the precipitate. The supernatant was used. Cyanide was used in two instances to destroy some cellular enzymes which might be present and causing the inactivation. In several instances after the experiments were finished there was still some active reducing agent as detected with methylene blue.

In the experiments shown in Table III the temperature accidentally rose to something above 10°C. As a result of this accident, however, the stabilizing effect of cysteine was forcibly brought to our attention for there was only a perceptible drop of virus activity in the tube containing cysteine but a marked drop in all others including the control.

*Summary.* The virus of Eastern equine encephalomyelitis may be concentrated and partially purified by ultracentrifugation. M/10 cysteine retards spontaneous inactivation of the ultracentrifuge purified virus.

9 Finkelstein, H., Marx, W., Bridgers, W. H., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 103; Finkelstein, H., Marx, W., Beard, D., and Beard, J. W., *J. Inf. Dis.*, 1940, **66**, 117.

10 Hellerman, L., *Cold Spring Harbor Symp. Quant. Biol.*, 1939, **7**, 165.

11 Best, R., *Aust. J. Biol. and Med. Sci.*, 1939, **17**, 1.

12 Mueller, J. H., *J. Exp. Med.*, 1928, **48**, 343.

13 Zinsser, H., and Tang, F. F., *J. Immunol.*, 1929, **17**, 343.

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## Action of Promin and Diamino-Diphenyl Sulfone upon Tubercle Bacilli; Antipromin Action of p-Aminobenzoic Acid.

WM. STEENKEN, JR., AND FRED H. HEISE. (Introduced by L. U. Gardner.)
*From Research and Clinical Laboratory, Trudeau Sanatorium, Trudeau, N.Y.*

A series of test tube experiments was undertaken to determine the action of promin* and its parent substance, diamino-diphenyl sulfone, upon the tubercle bacillus, and to learn whether or not *p*-aminobenzoic acid would inhibit the effect of these compounds as it has been shown to inhibit the effect of sulfanilamide upon other organisms.

All these tests were made upon both virulent and avirulent mutants of the human strain $H_{37}$ used by Feldman, Hinshaw and Moses,[1-4]

* Promin used in this study was kindly supplied through the courtesy of Dr. E. A. Sharp, Parke, Davis and Company, Detroit, Mich.

1 Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Proc. Staff Meet., Mayo Clin.*, 1940, **15**, 695.

2 Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Ibid.*, 1941, **16**, 187.

3 Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Am. Rev. Tuberc.*, 1941, **45**, 303.

4 Feldman, W. H., Mann, F. C., and Hinshaw, H. C., *Ibid.*, 1942, **46**, 187.