post partum. The mechanism of this hypoglycemia is unexplained.

(6) Administration of insulin was not required in order to obtain live, full term litters in 10 of the 11 pregnancies observed.

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Purification of Certain Viruses by Use of Protamine Sulfate. (17535)

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During attempts to improve the immunogenic potency and to increase the purity of Japanese encephalitis vaccine, it was noted that the addition of protamine sulfate to suspensions of infected chick embryo tissue produced a heavy precipitate, and, of particular interest, left almost all of the virus in the clear supernatant fluid. Subsequently, a number of viruses were studied with respect to their behavior in the presence of protamine. It was found that they fell into 2 groups, *i.e.*, those which were precipitated along with the tissue materials as has been observed by others(1,2) and those, like Japanese encephalitis virus, which remained in the supernatant fluid. For this latter group, protamine precipitation provides a rapid, simple and effective means for partial purification.

Methods. A considerable number of orienting experiments indicated that protamine precipitation could be carried out in the following manner: A chilled infected 10% suspension of mouse brain clarified by low-speed centrifugation or a 20% suspension of uncentrifuged chick embryo tissue, pH 7.0 to 7.2, is added to protamine sulfate (salmine) in the proportion of 1.0 ml of tissue suspension for each 5.0 mg of protamine. Either the readily soluble dry protamine powder or a solution of the material is used. A fine precipitate forms immediately on the addition of the tissue suspension to the protamine. The mixture is stored with occasional agitation in the refrigerator for 30 minutes after which the precipitate is separated by low-speed centrifugation at 3000 RPM for 15 minutes in an angle centrifuge. The resultant clear, pink supernatant fluid is decanted and saved; in the present report it is designated as "protamine supernate." Protamine supernates obtained by the method just described develop a granular precipitate during the first 24 hours of storage in the refrigerator. Furthermore, if formaldehyde is added in order to inactivate the virus, a heavy flocculation slowly develops. This flocculation apparently is due to the residual protamine in the preparations. It can be avoided if the excess protamine is removed by precipitating it with heparin. The optimum amount of heparin for this purpose is 3.8 mg per ml of supernate. Less than this amount fails to remove sufficient protamine whereas more produces a finely dispersed precipitate which sediments with difficulty. Thirteen viral agents were subjected to treatment with protamine. Their distribution in the various fractions thus obtained was determined by assaying the infectivity of the materials. In all but 2 instances this was done by intracerebral titration in mice in the usual manner. The exceptions were mumps virus, which was assayed in embryonated eggs by testing for the presence of viral hemagglutinins in the chorioallantoic fluids, and one strain of vaccinia virus, which was titrated on the chorioallantoic membrane.

Results. Action of protamine on infected suspensions. The viral agents which remain in the clarified supernatant fluid after treatment with protamine are listed in Table I, as are those which are precipitated along with tissue materials by this treatment. Others

1. Chambers, L. A., and Henle, W., PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 481.

2. Bawden, F. C., and Pirie, N. W., *Proc. Roy. Soc., Series B,* 1937, v123, 274.

TABLE I.
Infective Titer of Crude and Protamine-treated Suspensions of Virus Infected Tissues.

Virus	Source	Infective titer		
		Crude suspension	Protamine-treated Supernate	Sediment
	Agents not precipitated			
Colorado tick fever	Mouse brain	6.2	5.2	3.8
Equine encephalomyelitis (western type)	" "	8.4	8.2	N.D.
Encephalomyocarditis	" "	8.5	8.5	N.D.
Japanese encephalitis	" "	8.4	8.4	<6.0
	Chick embryo	7.3	7.2	<6.0
Poliomyelitis (Lansing)	Mouse brain	3.4	3.2	N.D.
	Cotton rat brain	3.0	3.0	N.D.
Russian Spring-Summer encephalitis	Mouse brain	8.7	8.5	N.D.
St. Louis encephalitis	" "	6.7	6.8	N.D.
West Nile virus	" "	7.6	7.6	N.D.
	Agents precipitated			
Herpes	Mouse brain	4.3	<1.5	3.8
Lymphocytic choriomeningitis	" "	5.2	<2.5	5.2
Murine encephalomyelitis (GD VII)	" "	7.4	<4.0	6.8
Rabies	" "	6.6	3.5	5.4
Vaccinia	" "	5.2	<3.0	N.D.
	Chick embryo	8.2	4.5	7.0

have shown that influenza(1) and tobacco mosaic(2) viruses are precipitated by protamine. Most of the observations on factors influencing the protamine reaction were made on suspensions of chick and mouse tissues infected with Japanese encephalitis virus. The amount of protein nitrogen, *i.e.*, acetic acid precipitable N, in the mouse brain protamine supernatant was about one-third that in the crude tissue suspension. When the protamine precipitation was performed at various temperatures ranging from 2°C to 30°C, there was little variation in the virus titer and total nitrogen in the protamine supernates. However, when the temperature was over 30°C, the supernates were never quite clear. We have chosen to perform the procedure in the cold to maintain maximum infectivity of the viral materials. Other observations indicated that the technic was equally effective when carried out over the pH range from 6.0 to 8.0.

Purity of protamine clarified materials. On the basis of infectivity and total nitrogen values the purity of preparations of Japanese encephalitis virus obtained by ultracentrifugation of protamine supernatants was appreciably greater than that of viral suspensions obtained by the usual differential centrifugation procedures. In 2 experiments aliquots of a suspension of mouse brain were treated by the 2 methods. Protamine supernate prepared from 1 aliquot was centrifuged at 100,000 G for 1 hour after which the sediment was resuspended and saved. The other aliquot was first clarified by centrifugation at 10,000 G for 1 hour and then subjected to the same treatment as the protamine supernate. The total nitrogen content of the ultrasediments, expressed as gamma/ml of suspension subjected to ultracentrifugation, was 7.0 and 3.0, respectively, for the 2 protamine-treated materials, and 73 and 40, respectively, for the materials concentrated by centrifugation alone. It should be emphasized that in one experiment the infective titers of the original suspension and of the resuspended sediments obtained by both procedures were of the same order of magnitude, *i.e.*, $10^{-7.0}$-$10^{-8.0}$.

Electron micrographs were made of preparations of Japanese encephalitis virus obtained from infected mouse brains by combined treatment with protamine and heparin followed by ultracentrifugation. These showed little other than numerous spherical particles with a diameter of approximately 20 mμ; the size of

TABLE II.
Comparative Potencies of Crude and Purified Japanese Encephalitis Vaccines (10% Mouse Brain).

Vaccine No.	Purification	Minimal immunogenic dose, ml
185A	Crude	.001
185A	Protamine*	.008
172	Protamine*	.007
176	Crude	.006
176	Protamine-heparin	.007
173	" "	.005
180B	" "	.002
181	Protamine-heparin + Ultracentrifugation	.004

* Vaccines inactivated by ultraviolet light. All other vaccines inactivated with formaldehyde.

Japanese encephalitis virus is about 15-22 mμ. Similar particles were also found in sediments from suspensions of normal mouse brain prepared in the same manner. Hence, it was not possible to differentiate between the Japanese encephalitis virus and particles of the sedimentable component which were present in normal mouse brain preparations.

Antigenicity of protamine-treated Japanese encephalitis vaccines. Japanese encephalitis vaccines were prepared from infected mouse brain and partially purified by treatment with (a) protamine, (b) protamine and heparin, or (c) by ultracentrifugation of protamine-heparin treated materials. In most of these the virus was killed by 0.2% formaldehyde. Two lots were inactivated by irradiation with ultraviolet light. The data summarized in Table II indicate that each of these preparations when assayed in mice would meet the potency requirements established for Japanese encephalitis vaccine.(3)

One of the lots of protamine-heparin treated vaccine (180B, Table II) was tested for its immunogenic activity in human beings. Seven volunteers were given 0.1 ml of the purified material intradermally while 8 received 1.0 ml subcutaneously. All of these persons had previously received one or more courses of crude chick embryo type Japanese encephalitis vaccine. The level of Japanese encephalitis neutralizing antibody before and after injection was determined by the intracerebral mouse test. Five of the volunteers had detectable amounts of specific antibody before receiving the present vaccine (neutralization indices between 100 and 650). All 5 of these showed a marked rise in titer following their booster dose with indices which now ranged between 2500 and 40,000. In addition, 6 of the 10 volunteers without detectable antibody prior to vaccination subsequently developed significant amounts of antibody with indices ranging from 100 to 15,000. The antibody response was similar in the groups who received vaccine intradermally and subcutaneously, and did not differ appreciably from that previously noted in persons receiving booster doses of the standard Japanese encephalitis vaccine by the same routes.(3)

It may be mentioned that vaccine 180B had a titer of 1:16 when used as antigen in a complement-fixation test performed according to the technic previously employed in this laboratory.(3) This is within the limits generally obtained for antigens prepared from mouse brain infected with this virus. Protamine-treated antigens of a different type have been used by Roberts(4) in a serological flocculation test for poliomyelitis.

Summary. Suspensions of a number of viral agents can be partially purified by treatment with protamine sulfate which precipitates much of the extraneous material leaving essentially all of the virus in the clarified fluid. Preparations obtained by this simple method have potential value as antigens, vaccines and starting material for studies on the physical and biochemical nature of selected viral agents.

3. Warren, J., Smadel, J. E., and Rasmussen, A. F., *J. Immunol.*, 1948, v58, 211.

4. Roberts, E. C., *Pub. Health Rep.*, 1949, v64, 212.

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