TABLE II. Results of Pre-Inoculation Treatment of Lymphosarcoma Cell Suspensions with Rat and Rabbit Small Intestinal Fractions. Subcutaneous injection of tumor cells and tissue fractions in CBA mice.

Tissue fraction		No. cases	Survivals	Deaths
Rat intestine:	Fraction 90	20	19	1
	Supernatant	20	0	20
	Controls	11	0	11
Rabbit "	Fraction 90	26	0	26
	" 4	6	0	6
	Supernatant	16	0	16
	Controls	48	0	48

days and death ensued in 17 to 27 days after inoculation. Death in all instances was due to uncontrolled lymphosarcoma. The treated mice have survived as long as 180 days after inoculation in good condition and with no evidence of tumors.

From the data it is evident that the maximum inhibition occurs in animals inoculated with tumor cell suspensions incubated with Fraction 90. Instances of survival in animals given tumor cell suspensions exposed to Fraction 4 or to the supernatant from Fraction 90 occurred in early experiments. Data from later experiments suggest that these survivals were due to overlapping during the fractionation procedure. Improved centrifugation technics in subsequent experiments have eliminated such results.

Corresponding fractions prepared in identical fashion to the above from other normal tissues (spleen, muscle, liver) showed no inhibitory properties. Fractions prepared from rabbit small intestine were ineffective, though this may be due to technical insufficiencies rather than to the absence of inhibitor.

In order to establish further that the inhibitory activity is due to specific substances in Fraction 90 rather than to bacterial contamination, proteolytic enzymes, mechanical or irritating effects or pH differences, appropriate experiments have been conducted to rule out these factors. Fraction 90 appears to be non-toxic since large intraperitoneal doses yielded no deleterious effects.

A limited number of experiments using Sarcoma 180 as the stock tumor indicated a response similar to that found with Gardner lymphosarcoma.

The data reported suggest that a non-toxic substance inhibitory to the development of lymphosarcoma is present in mouse and rat small intestine and that this material is recoverable by ultracentrifugal fractionation.

Further experiments are in progress to determine the nature of the inhibitory substance and to prepare it in a manner that will permit treatment of animals bearing established tumors.

1. Ladd, R. L., Goldstine, M. T., and Burns, O. R., *Growth*, 1950, v14, 265.

2. Tyler, D. B., *Science*, 1950, v112, 456.

3. Schneider, W. C. and Hogeboom, G. H., *Cancer Research*, 1951, v11, 1.

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Stabilizing Action of Glycerine on Hemagglutination of Egg-Adapted Mumps, Newcastle Disease and Influenza Viruses. (19222)

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It has long been recognized that glycerine either destroys or inhibits certain bacterial contaminants and it has been extensively used as a bacteriostatic and preservative agent in virus infected tissues since its introduction by Copeman(1) for the purification of calf lymph vaccine. Levaditi and Harvier(2) reported that herpes, vaccinia, rabies, and poliomyelitis viruses remained virulent for weeks and even months *in vitro* when placed in pure glycerine or glycerine diluted with an equal volume of normal saline and stored at 4°C. Their findings were confirmed by Doerr and others(3,4). According to Rivers(5) the preservative ac-

tion of glycerine probably depends upon the inhibition it exerts on autolysis of virus infected tissues. Various authors have also reported the preservation of viability of some bacterial agents in pure glycerine for several years. McKinley and Verder(6) cite the preservation of green streptococci in glycerine for as long as four years. The same authors report a personal communication by Francis stating that he had maintained viability and virulence of *B. tularense* in 100% glycerine for more than 6 years and *B. pestis* for over 7 years. when the glycerinated preparations were kept in the cold. Glycerine has also been employed for the preservation of anti-sheep-cell hemolysin for serological work. It has been used as a preservative in the preparation of certain bacterial antigens, as for example the *B. abortus* stained antigen used in the ring test for Brucellosis(7). Takaki and others(8) used a glycerine extracted saline suspension of rabies infected brain as antigen in the complement-fixation test. So far as we are aware, however, there are no other references in the literature to the use of glycerine for the preservation and stabilization of viral sero-diagnostic antigens. Formalin and phenol, on the other hand, have been widely used against bacterial contaminants in the preservation of sero-diagnostic antigens; while these compounds are effective in checking bacterial growth they may adversely affect both sero-diagnostic and immunizing antigenic properties and titers eventually decrease despite such precautions as cold storage. The use of the hemagglutination-inhibition test as a diagnostic tool in testing for certain red cell agglutinating viruses is somewhat limited by the fact that the hemagglutinating property of the viral antigen may decrease quite rapidly upon storage at 4 C. In such cases a relatively fresh supply of infected material must always be kept on hand.

The purpose of this paper is to report some experimental observations on the stabilizing action of glycerine on the hemagglutinating activity of chick embryo propagated mumps, Newcastle disease and influenza viruses.

Materials and methods. Virus strains. All strains of virus included in this study were adapted to grow in the allantoic cavity of embryonated hen's eggs. Two strains of mumps virus were used: Strain H*(9) in its 25th egg passage and strain E*(10) in its 42nd passage in eggs. The Newcastle disease virus employed in this series of experiments was from a supply pool representing the second egg passage of the Isele strain isolated in these laboratories from a field case of the disease. The 13th egg passage of the PR^8 strain of influenza virus was also included in this study.

Methods of concentration. When concentrated virus suspensions were used the concentration was carried out by either one of two methods: Sharples centrifugation, as already described by others(11-13), or alcohol precipitation as described by Cox *et al.*(14) for the concentration and purification of influenza virus. *Titration method.* All virus samples were titrated by the Salk(15) hemagglutination procedure, using a 0.25% chicken red cell suspension. *Glycerine and saline.* The glycerine used throughout this study was a chemically pure, neutral, reagent glycerine having a specific gravity of 1.24. All glycerine mixtures were made gravimetrically rather than volumetrically in order to avoid errors from loss of glycerine on the pipette wall. Control mixtures were made in buffered saline(16) at pH 7.0. Formalin in 0.1% concentration was added to all saline and glycerine mixtures.

Experimental. Exp. I was set up in order to determine the effect of a 50% glycerine concentration on the hemagglutinating factor of egg-propagated mumps virus, when virus-glycerine mixtures were held at 4°C, 37°C and room temperature (22° to 24°C). Three preparations of each of the 2 mumps strains (E and H) were used: unconcentrated infected allantoic fluids, Sharples centrifuged concentrates and alcohol precipitates. Each of these virus suspensions was treated in the following manner: 60 ml of infected material were divided into 2 equal portions. To the first par, 30 ml of buffered saline were added; this mixture served as a control. The second part was mixed with 30 ml of undiluted glycer-

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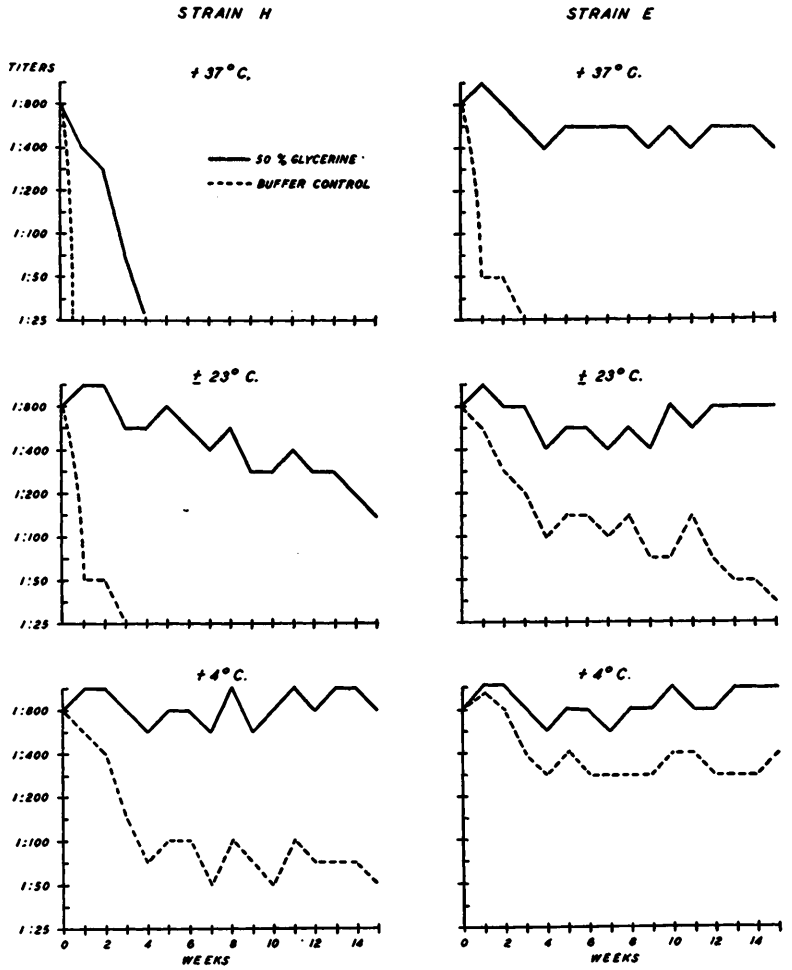

FIG. 1. Action of 50% glycerine on the hemagglutinin of mumps virus at three different temperatures.

ine, thus bringing it to a 50% concentration. Enough formalin was added to both buffered saline and glycerine mixtures to give a final concentration of 0.1%. Formalin was added mainly to protect the buffered saline suspension against bacterial action and was also used in the glycerine mixture in order to equalize the 2 preparations. Each of these 2 preparations was in turn divided into three portions of 20 ml each and these were placed at 4°C, 37°C and room temperature respectively. Titration of hemagglutinin was done at weekly intervals for 16 weeks, all samples being replaced at their respective temperatures as soon as enough material had been taken out for the day's test.

No differences in the titer behavior were observed among the three types of material used. To avoid repetition only the results obtained with the Sharples centrifuged concentrates of both mumps strains have been summarized in Fig. 1.

It can be seen that the hemagglutinating principle of mumps strain H is very labile in buffered saline and is destroyed overnight at 37°C. At room temperature its titer decreases quite rapidly during the first week and becomes completely negative by the third week. It is somewhat more stable at 4°C, but reaches an appreciably lower titer by the fourth week. With the addition of glycerine, however, the sample maintained at 37°C retained its he-

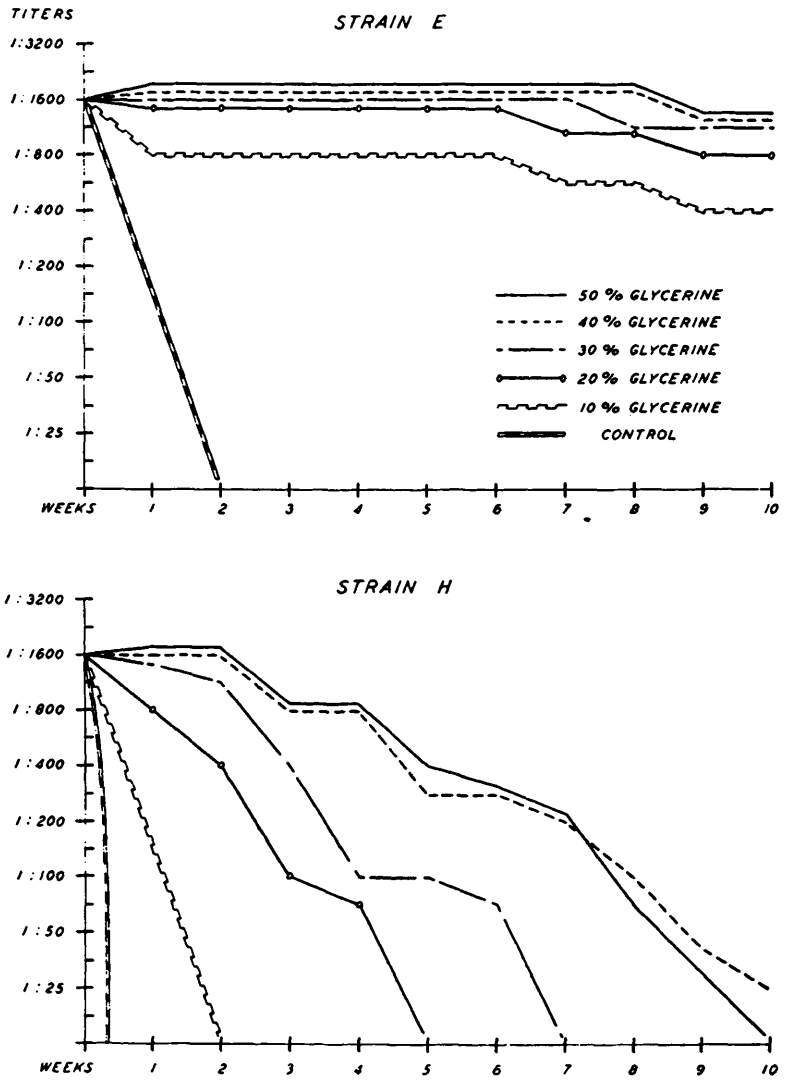

FIG. 2. Action of increasing concentrations of glycerine on the hemagglutinin of mumps virus at 37°C.

magglutinating activity for 4 weeks. The room temperature sample decreased in titer at a much slower rate and was still appreciably active after 16 weeks, while the 4°C specimen maintained its titer for the total observation period of 16 weeks.

The hemagglutinating capacity of mumps strain E is much more stable than that of strain H, even in buffered saline and lost its activity completely only after 3 weeks at 37°C. Its titer decreased at a slower rate at room temperature but reached a very low level after 16 weeks. At 4°C, it suffered only a 2-fold decrease after four weeks and then maintained the same level to the end of the experiment. The addition of glycerine seemed to preserve the titer of strain E just as well at all 3 temperatures.

Exp. II was devised to determine the lowest concentration of glycerine capable of stabilizing hemagglutinin titers. Sharples centrifuged concentrates were prepared from both E and H strains of mumps virus. Each batch was divided into 6 parts and glycerine mixtures of increasing concentrations from 10 to 50% were made; the sixth part was made up in

buffered saline to serve as a control. Formalin was added at a concentration of 0.1% to all 6 preparations of each strain. All mixtures were placed at 37°C and titrations were made at weekly intervals for 10 weeks. Results for both strains E and H are summarized in Fig. 2.

The lability of strain H again can be seen, since glycerine concentrations of 10, 20 and 30% delayed deterioration only partially. Even with 40 and 50% glycerine concentrations the titers became completely negative after nine to ten weeks. In contrast, 10% glycerine was enough to stabilize strain E to an appreciable extent and as little as 20 per cent was sufficient to maintain it practically at the same level for 10 weeks at 37°C.

Similar but less detailed observations carried out on suspensions of Newcastle disease and influenza (PR8) viruses, prepared in the same manner as the mumps virus suspensions, indicate that the hemagglutinating capacity of these viruses is also stabilized to a considerable degree by the addition of 50% glycerine. Buffered saline suspensions of Newcastle disease virus suffered a 128-fold loss of titer in less than 2 weeks at 37°C, whereas under the same temperature conditions those containing 50% glycerine showed not more than a 2-fold decrease in titer during a 5-week interval. Deterioration without glycerine was less rapid at room temperature than at 37°C, but a 32-fold drop in titer was observed in 4 weeks while at the same temperature the glycerinated sample had fallen by only a single 2-fold dilution at the end of 8 weeks. It was also observed that a suspension of PR8 influenza virus containing 5% glycerine suffered a 32-fold loss in titer at 37°C during an interval of approximately 8 weeks, while a comparable preparation containing 50% glycerine showed only a 4-fold drop in titer under the same temperature conditions.

It may be of interest to note here that a one liter batch of mumps antigen, prepared by the Sharples centrifugation method in October, 1949, and containing 50% glycerine, has maintained its original titer up to the present date, October 1951. During these 2 years, it has been constantly stored at 4°C. Similarly, a batch of Newcastle disease diagnostic antigen prepared with 50% glycerine had a titer of 1:3200 in November, 1949, and its titer in September, 1951, was 1:2240. This lot has been held at 4°C but has been in continuous use for routine tests during most of the 2-year interval.

Conclusions. (1) Experimental observations based on the behavior of two strains of mumps virus having hemagglutinins of markedly different stability show that the addition of glycerin has a marked stabilizing action on the hemagglutinating antigen. Without glycerine, at 37°C the H strain of mumps virus in buffered saline containing 0.1% formalin, loses its hemagglutinating activity overnight and the E strain in 3 to 4 weeks. In contrast, the H strain in 50 per cent glycerine suspension, also containing 0.1 formalin, maintained useable titers for 2 to 3 weeks at 37°C, and under the same conditions the E strain showed little or no loss of activity at the end of 16 weeks. (2) Glycerine concentrations as low as 10% markedly enhanced the stability of the E strain while the stability of hemagglutinating activity of the H strain was dependent to a much greater degree upon an increased concentration of glycerine. (3) The hemagglutinating activity of antigens prepared from suspensions of Newcastle disease virus and influenza virus (PR8) was similarly stabilized by the addition of 50% glycerine.

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1. Copeman, S. M., *Vaccination,* London, MacMillan and Co., Ltd., 1899, 153-188.

2. Levaditi, C., and Harvier, P., *Compt. rend. Soc. de biol.,* 1920, v83, 354.

3. Doerr, R., and Schnabel, A., *Z. f. Hyg. u. Infektionskr.,* 1921, v94, 29.

4. Doerr, R., and Berger, W., *Schweiz. med. Wchnschr.,* 1922, v52, 862.

5. Rivers, T. M., *Filterable Viruses,* Baltimore, Williams and Wilkins Co., 1928.

6. McKinley, E. B., and Verder, E., PROC. SOC. EXP. BIOL. AND MED., 1933, v31, 297.

7. Roepke, M. H., Clausen, L. B., and Walsh, A. L., *Proc. U. S. Livestock San. Assn.,* 52nd Ann. Meet., Oct. 1948, 147.

8. Takaki, J., Bonis, A., and Koref, O., *Z. immun. Forsch.*, 1926, v47, 431.
9. Habel, K., *Pub. Health Rep.*, 1945, v60, 201.
10. Levens, J. H., and Enders, J. F., *Science*, 1945, v102, 117.
11. Stanley, W. M., *J. Exp. Med.*, 1944, v79, 255.
12. Stanley, W. M., *J. Exp. Med.*, 1945, v81, 193.
13. Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, v50, 291.
14. Cox, H. R., Van der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, v56, 149.
15. Salk, J. E., *J. Immunol.*, 1944, v49, 87.
16. Eagle, H., *The Laboratory Diagnosis of Syphilis*, St. Louis, 1937, The C. V. Mosby Co., 149.

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Separation, Concentration, and Transfusion of Platelets.* (19223)

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Evaluation of the therapeutic usefulness of blood platelets has been hindered by the lack of a method which permits the efficient separation and concentration of these elements physiologically intact in quantities suitable for transfusion. The method described below has met these requirements reasonably well, and may serve as a useful point of departure for others interested in this field. It has been kept sufficiently simple that no unusual technical skill or equipment are required.

Materials and methods. The method is based on the use of di-sodium ethylene diamine tetra-acetate (Sequesterene Na_2†) as anticoagulant, and the use of differential centrifugation for the separation and concentration of the platelets. One g of Sequesterene and 0.7 g of sodium chloride were dissolved in 100 cc of distilled water. Nine parts of blood were added to one part of the Sequesterene solution. Venous blood was collected from humans, and arterial or venous blood from dogs, by gravity flow through a coated 15 gauge nylon-hub electro-polished needle‡ and plastic tubing‡ into 200 ml siliconed centrifuge bottles, each containing 20 cc of the Sequesterene solution. The guinea pig blood was aspirated from the heart into a syringe containing the Sequesterene solution and transferred to the centrifuge bottles. The bottles were carefully balanced and centrifuged at 30 x g for 50 minutes at 5°C. The supernatant platelet rich plasma was then siphoned into another 200 ml siliconed glass centrifuge bottle, using siliconed glass and plastic tubing as the siphon. This plasma was centrifuged at 300 x g for 50 minutes at 5°C, and the supernatant siphoned off leaving the platelets as a loosely packed mass in the bottom of the tube. The platelets were then resuspended in an aliquot of the supernatant plasma. Resuspension was readily accomplished by aspirating and expelling the platelet-plasma suspension 3 or 4 times in a siliconed sterilized cotton plugged pipette with a tip bore of 1 mm or more. All platelet counts were done by the method of Brecher and Cronkite(1). To test the viability of the platelets, animals were usually given the equivalent of their normal total platelet content by transfusion, the volume of suspension varying from 1-2 ml in guinea pigs and 10-25 ml in dogs. Sixty-three transfusions of pooled platelets were given to guinea pigs, and 19 were given to dogs; 4 of the guinea pigs were normal, 13 were irradiated, 3 of the dogs were normal, and 3 were irradiated. The transfusions were given within one hour of the preparation of the suspension and within 4-5 hours of drawing the blood. No platelet transfusions were at-

* The opinions and assertions are those of the authors and are not to be construed as reflecting the policy or opinions of the Naval Service.

† Supplied by courtesy of the Alrose Chemical Co., Providence, R. I.

‡ Product of the Fenwal Co., Ashland, Mass.