Glycerine as a Stabilizer of Some Complement-Fixing Antigens of Viral and Rickettsial Origin.* (20593)

ALEXANDER L. TERZIN. (Introduced by Herald R. Cox.)

From the Yugoslav Regional Center for Influenza, Institute of Hygiene, Belgrade.

In the course of our work we were faced with the problem of preserving the stability of complement-fixing antigens during shipping or long-term storage without refrigeration under unfavorable summer temperatures. Besides, the tendency of the soluble antigens to flocculate after storage in the frozen state and to deteriorate fairly rapidly at plus 4°C presented a serious difficulty. In their paper on the stabilizing action of glycerine on the hemagglutinating capacity of different viruses, Cabasso, Markham, and Cox(1), gave several references on the use of glycerine for the preservation of different biological materials. Hoyle and Fairbrother(2), Enders and coworkers(3), Shepard(4), and other authors have reported on the flocculation of influenza, mumps, and typhus antigens of the soluble types at different temperatures, and have discussed the influence of pH, agitation, dilution and other factors both on the formation of turbidity and on the serologic activity of the antigens.

We tested the stabilizing action of glycerine on the complement-fixing capacity of the following antigens: Q Fever (Henzerling), Q Fever (Nine Mile), Typhus Fever, epidemic (soluble type), Typhus Fever, murine (soluble type), all 4 made by Lederle Laboratories, U.S.A.; influenza A-prime (soluble type), Influenza A (soluble type), Influenza B (soluble type) antigens, all made from chorioallantoic membranes in our laboratory; and Mumps and LCM antigens. The influence of glycerine in preventing the visible flocculation of heated antigens was also investigated.

Materials and methods. Glycerine and saline antigens. The glycerine solutions were made up volumetrically, usually in 2-ml amounts as follows: to one ml of the antigen, either undiluted glycerine or a 40% glycerine solution in saline (Glycerin, Konc., rein D 1, 25-26 (Bayer)) was added up to the 2-ml mark of calibrated narrow test tubes. In that way 1:2 dilutions of the antigens were obtained which contained 50 or 20% glycerine. The control antigens were made in a similar fashion, substituting saline for glycerine. *Titration method.* If not stated otherwise, the antigens were titrated in serial 2-fold dilutions against about 8 units of the homologous human convalescent serum. The complement-fixation technic followed that used at the Virus Reference Laboratory, Colindale (Director, Dr. F. O. MacCallum)(5). The volume unit was 0.1 ml. The complement is titrated for each lot of sensitized cells (2% cells with 2 units of hemolysin) and the 2 units of complement to be used in the test are strictly controlled (control-tubes are set up to contain 2, 1, ½, and ¼ units of complement, respectively). All antigens (except LCM) were tested by 90-minutes fixation at 37°C, addition of sensitized red cells and a second incubation of 30 minutes, with one shaking after the first 5 minutes. For the LCM antigen, overnight fixation at 4°C was used before the addition of the sensitized cells. Controls of serum, antigen, sensitized cells and the exact amount of complement used in the test were included in each experiment. Dilutions of serum samples used in comparative studies were made in large quantities after the usual inactivation for 30 minutes at 56°C, and before distribution in the respective sets of test tubes. *Heating of antigens.* The antigen solutions were heated in sealed ampoules of thin, neutral glass, containing usually one-ml amounts. During heating, the ampoules were held under water level in either a water-bath with well controlled temperature, in boiling distilled water or in the autoclave. The heated ampoules were shaken thoroughly before opening. *Recording of results.* Results of the complement-fixation tests were recorded as 3, 2, 1, ± or negative. These were used to designate absence of

* The technical assistance of Mrs. Marthe Milivojevich is gratefully acknowledged.

TABLE I. Heat Resistance of Typhus Antigens (Epidemic, Soluble Type).

Antigens	Min.	Reciprocals of the titers of Saline-antigens	Glycerine-antigens
Unheated		32	32
Heated at 56°C	30	16	32
	120	8 (16++)	32
" 100°C	10	4	8
	30	2	8
	80	1-	8
	120	1-	4

hemolysis, or 25, 50. 75. and 100% hemolysis, respectively. End points of titrations are expressed by the reciprocal of the highest antigen-dilution showing 3 or 2+ fixation (25% hemolysis or less). Results recorded as 16, 16++, 16+, 16±, or 16— are to be respectively interpreted as 3, 2, or 1+, ± or negative at a 1:16 antigen dilution. In some instances, the formation of turbidity and the degree of flocculation of both heated saline and glycerine preparations were recorded.

Q Fever antigen (Henzerling). Upon testing the heat resistance of both the saline and glycerine preparations at different temperatures, surprising stability of Q Fever antigen was observed. After heating at 56°C for 270 minutes, at 100°C for 160 minutes, or even after autoclaving at 110°C for 30 minutes, no change in titers of the heated antigens could be detected when compared with unheated preparations. The only difference was that after heating both antigens at 120°C for 30 minutes, a flocculation developed in the saline antigen while the glycerine antigen remained unchanged.

Typhus fever antigen (epidemic, soluble type). Table I illustrates the sensitivity of the saline antigen which was rapidly lost during heating in boiling water: an 8-fold drop in the complement-fixing titer took place in 10 minutes at 100°C, whereas boiling for 80 minutes completely destroyed its complement-fixing ability. In contrast, glycerinated antigen maintained a fair titer after being exposed to the same temperature for the same length of time. Table II illustrates the results of 4 checkerboard titrations of both the saline and glycerine antigens before and after heating for 30 minutes at 100°C. Both preparations were titrated against the different dilutions of the same serum. Following exposure to 120°C for 30 minutes, the saline antigen showed a marked flocculation, whereas the glycerine antigen remained quite clear.

Influenza A-prime (strain FM 1 antigen). Table III illustrates well the stability of influenza antigen after exposure to 56°C for different lengths of time. Complete destruction of activity of the saline antigen was observed after 30 minutes, whereas the glycerine antigen remained unchanged even after 2 hours of heating. In addition, Influenza A-prime antigens containing 0, 20, and 50% glycerine, respectively, were prepared in 4-ml amounts. Each of the 3 antigens was divided into 2 equal portions. The first was kept in well-stoppered tubes in the incubator at 37°C, and the second was stored in icebox at +4°C. After different time intervals aliquots from

TABLE II. Checkerboard-Titrations of Typhus Antigens.

Dilutions of serum	Dilutions of unheated antigens: Saline-antigen 1/2	1/4	1/8	1/16	1/32	Glycerine-antigen 1/2	1/4	1/8	1/16	1/32
1/100	3	3	3	3	1	3	3	3	3	2
1/200	1	3	3	3	±	2	3	3	3	±
1/400	—	—	1	2	±	—	—	2	2	±
1/800	—	—	—	—	—	—	—	—	—	—
	Dilutions of antigens heated for 30 min. at 100°C: Saline-antigen 1/2	1/4	1/8	1/16	1/32	Glycerine-antigen 1/2	1/4	1/8	1/16	1/32
1/25	1	±	—	—	—	3	3	3	1	—
1/50	±	—	—	—	—	3	3	3	—	—
1/100	—	—	—	—	—	2	1	—	—	—
1/200	—	—	—	—	—	—	—	—	—	—

TABLE III. Heat Resistance of Influenza A-prime Antigens.

Antigens	Min.	Reciprocals of the titers of Saline-antigens	Glycerine-antigens
Untreated		32 (64+)	64
Heated at 56°C	10	16 (32+)	64
	30	2–	64
	60	2–	64
	120	1–	64++

the 6 samples of antigen were titrated against portions of the same serum, which was stored in the frozen state in small amounts at all times. The serum was always inactivated just before use.

Table IV shows the results obtained during a 115-day observation period. All 3 samples of antigens show very similar behavior when kept at +4°C, with a slightly slower rate of deterioration of the glycerine preparation when compared with the saline antigen. The saline antigen, when kept at 37°C for periods longer than one month, completely loses its activity, whereas with 50% glycerine it retains its titer fairly well under the same conditions. A greater rate of deterioration took place in the sample containing 20% glycerine, which was somewhat more stable, however, than the saline control.

Table V presents the data on the flocculation observed in both saline and glycerine antigens after exposure to different temperatures for different time intervals. The activity of the saline antigen exposed to a temperature of 80°C for one minute was completely destroyed, while the glycerine antigen showed just about a 2-fold decrease in titer following the same treatment.

Other antigens. Experiments similar to those described above were also performed with other antigens. A pronounced protective effect of glycerine on the heat stability and keeping quality of the following antigens was observed: Typhus-fever antigen (murine, soluble type), made by Lederle Labs., U.S.A.; influenza A antigen (strain PR8, soluble type), influenza B antigen (strain Yugoslavia B 1, soluble type), and LCM antigen (soluble type, prepared from infected guinea pig spleens), all 3 prepared in our laboratory. A very marked effect of glycerine in preventing flocculation after exposure to raised temperatures was also observed with these 4 antigens. The commercial Q Fever antigen (Nine Mile strain, made by Lederle Labs.) was tested in the same way as the Henzerling antigen, and was found to possess the same remarkable heat stability. However, glycerine was of value in preventing flocculation of the Nine Mile Q Fever antigen after autoclaving for 30 minutes at 110°C or 120°C. The stability of mumps antigen of the viral type was also investigated, using either a commercially available antigen (Lederle Labs.) or crude virus infected allantoic fluid prepared in our laboratory. No reproducible results were obtained in a few experiments with either of the 2 mumps antigens tested.

It might be of interest to report that 50% glycerine was also found capable of preventing both flocculation and deterioration in the tested samples of soluble antigens (*e.g.*, LCM), which tended to flocculate after having been stored at temperatures of about —30°C.

Discussion. In the experiments described above, no pH determinations were made on

TABLE V. Appearance of Influenza A-prime Antigens after Heating.

Heating Time, min.	Heating °C	Saline-antigen	Glycerine-antigen
20	56	turbidity	unchanged
120	56	flocculation	"
1	80	coagulation	slight turbidity
1	100	"	coagulation

TABLE IV. Resistance of Influenza A-prime Antigens.

% of glycerine in the antigens	Kept at, °C	Time in days 0	1	3	6	12	19	35	69	115
0	4	32	16	16++	8	8	8	8	8	8
0	37	32	8	8	8	4	4	2++	0	0
20	37	32	16+	16++	8	8	8	8++	8	4
50	37	32	16++	16	16++	16	16+	16++	16+	8

the antigens prior to heating. The amount and dilution of the antigens exposed to heating, as well as titer and protein content of the different samples of the same antigen, were not kept strictly constant when repeating the same experiments.

The results reported refer to single experiments, but very similar, if not identical, results were obtained repeatedly with the same kinds of antigen, under the same conditions. The only exception was experienced when testing mumps antigens, with which no reproducible results were obtained under the conditions used.

Although glycerine was found to have a protective effect against flocculation of Q Fever antigen at higher temperatures, glycerinated preparations do not seem to present any advantage because of the remarkable heat stability of this antigen even in the absence of glycerine.

Glycerine alone or in combination with any of the antigens tested, as well as glycerine mixed with positive or negative sera, consistently failed to give either pro- or anti-complementary effects.

After autoclaving for 30 minutes at 110°C or 120°C, both saline and glycerinated preparations of some antigens (*e.g.*, Nine Mile Q Fever antigen) became somewhat anti-complementary. However, the anti-complementary level never reached that of the specific antigen titer and since it appeared only after exposure to temperatures higher than 100°C, it seems evident that it does not add to the problem at hand.

The results obtained seem to justify therefore the addition of 50% glycerine as preservative to the complement-fixing antigens tested so far in our laboratory.

Summary. The stabilizing action of glycerine on some complement-fixing antigens of viral and rickettsial origin was investigated. Its ability to prevent rapid deterioration of antigen-titers and visible flocculation following long storage or exposure to unduly high temperatures has been reported.

1. Cabasso, V. J., Markham, F. S., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 791.

2. Hoyle, L., and Fairbrother, R. W., *J. Hygiene,* 1937, v37, 512.

3. Enders, J. F., Kane, L. W., Cohen, S., and Levens, J. H., *J. Exp. Med.,* 1945, v81, 93.

4. Shepard, C. C., *Nat. Inst. Health Bull.,* 1945, v183, 98.

5. Andrews, B. E., personal communication.

Received August 7, 1953. P.S.E.B.M., 1953, v84.

Binding of Histamine by Animal Proteins. (20594)

E. H. KAPLAN AND JANE DAVIS. (Introduced by R. W. Schayer.)

From the Rheumatic Fever Research Institute, Northwestern University Medical School, Chicago, Ill.

Recent reports(1,2) have given evidence for binding of histamine by components of normal human blood serum, especially γ-globulin. The availability of C^{14}-histamine* made it possible to check these results by the dialysis-equilibrium technic(3).

Guinea pig lung has been reported(4) to contain relatively high concentrations of histamine. However, it was demonstrated(5) in experiments with isotopically labeled histamine and *L*-histidine that injected histamine is rapidly metabolized and excreted in the urine by guinea pigs and does not appear to any appreciable extent in the internal organs, whereas injected *L*-histidine is converted to histamine which is present in the organs for long periods. It was therefore of interest to determine whether added C^{14}-histamine would be bound by guinea pig lung homogenates or equilibrate with histamine already present.

Experimental. Human plasma proteins†

* We are grateful to Dr. R. W. Schayer of this Institute for supplying the labeled histamine.

† These human plasma fractions were generously furnished by Dr. E. J. Cohn of Harvard University Medical School.