6. Shetlar, M. R., Shetlar, C. L., Payne, R. W., Neher, G. M., Swenson, C. B., *ibid.*, 1958, v98, 254.

7. Kushner, D. A., Honig, K., Dubin, A., Dyniewiez, H. A., Bronsky, D., de la Huerga, J., Popper, H., *J. Lab. Clin. Med.*, 1956, v47, 409.

8. Shetlar, M. R., Ryan, R. S., Foster, J. V., Shetlar, C. L., Everett, M. R., Proc. Soc. Exp. Biol. and Med., 1949, v72, 294.

9. Winzler, R. J., in *Methods of Biochemical Analysis,* Vol. II, D. Glick, Ed., Interscience Publishers, N. Y., 1955.

10. Weiner, H. E., Voge, M., Quinn, F. A., Redlich-Moshin, J., Proc. Soc. Exp. Biol. and Med., 1955, v90, 494.

11. Rooney, J. R., *Am. J. Vet. Res.,* 1957, v18, 67.

12. Köiw, E., Grönwall, A., *Scand. J. Clin. Lab. Invest.,* 1952, v4, 244.

13. Shetlar, M. R., Foster, J. V., Everett, M. R., Proc. Soc. Exp. Biol. and Med., 1948, v67, 125.

14. Shetlar, M. R., Foster, J. V., Kelly, K. H., Everett, M. R., *ibid.,* 1948, v69, 507.

15. Larson, B. L., Touchberry, R. W., *J. Anim. Sci.,* 1959, v18, 983.

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Effect of Sodium Chloride Concentration on Staphylococcal Agglutination.* (25370)

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Since it has been shown that concentration of salts affects direct bacterial agglutination (1,2), it was decided to evaluate the degree of this effect in macroscopic staphylococcal agglutination. Spontaneous agglutination, the quantity of agglutination as measured by highest agglutination titer and the quality of agglutination as determined both by reproducibility and sharpness of endpoint and gross particulation of aggregates were observed.

Method and materials. Four different strains of hemolytic coagulase positive staphylococci representing the following phage types were used†

Strain	Phage type
a	29
b	81
c	4, 6, 29, 29A, 31, 79, 44, 52, 52A, 71, 3A, 42E, 44A, 73, 75, 51, 81
d	51, 42B, 81

Antisera were obtained from rabbits immunized intravenously with these strains grown in broth and killed with 0.25% phenol. Human sera were obtained from adult male volunteers. The term agglutination reaction is restricted to visible (second) stage of agglutination and precipitation as determined macroscopically, and does not imply consideration of combination or dissociation of antibody and antigen. Antigen concentration was that giving a density equal to that of the Standard Typhoid Vaccine (1000 million organisms/ml) supplied by Division of Laboratories and Research, N. Y. State Dept. of Health. Agglutination reactions were carried out as follows: Serial 2-fold dilutions of serum were made in standard agglutination tubes (½ x 3 inch) with the desired concentration of salt solution. One half ml was left in each tube. The organisms were kept in frozen skim milk until used. The original or first subculture from skim milk was used as antigen. After overnight incubation on rabbit agar, the growth was removed with a sterile cotton swab from surface of plate and suspended in desired concentration of sodium chloride solution. After the suspension was filtered through a small cotton plug, it was adjusted to a density twice that of optimal antigen concentration. The antigen suspension was then added to the serial 2-fold dilutions of serum in a ratio of 1:1 thus giving final volume of one milliliter. The mixture was incubated in water bath at

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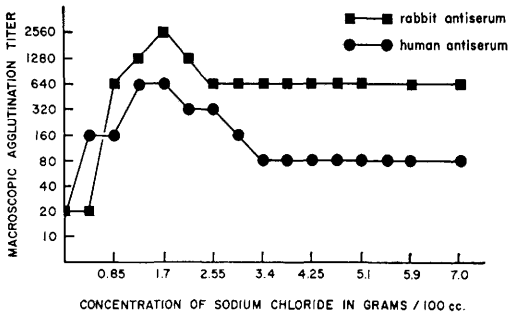

FIG. 1. Optimal concentration of NaCl for agglutination of hemolytic *Staphylococcus aureus* by antisera.

37° for one hour, placed in refrigerator overnight and observed the following morning for agglutination with the aid of a hand lens.

Results. Optimal concentration of sodium chloride in staphylococcal agglutination. Fig. 1 illustrates the macroscopic agglutination titer of a representative suspension of hemolytic *Staphylococcus aureus* by homologous immune rabbit serum and by human serum in various dilutions and at various concentrations of sodium chloride, the amount of antigen being constant. The highest serum titer in rabbit antiserum occurred at salt concentration of 1.7% sodium chloride. In human sera, again with constant amounts of antigen, highest serum titer was reached at salt concentration of from 1.3% to 1.7%. Agglutination titers in general were maximal at NaCl concentration between 1.3 and 2.1 and were significantly less below and above this range.

With human sera, there was improvement in quality of agglutination at 1.3% and 1.7% sodium chloride. Endpoints were sharper and were more easily reproduced. A tendency of aggregates to be ropy and difficult to read was observed at and below 0.85% sodium chloride. This did not occur at 1.3% or at greater concentrations of the salt. In rabbit antisera, marked improvement in quality of agglutination consistently occurred in concentrations of 3.2% sodium chloride and above. Spontaneous agglutination of control antigens was irregularly observed in saline concentrations of 0.85% and less.

These data show that variations in staphylococcal agglutination occur with changes in salt concentration as Duncan(1) found with *Bacterium dysenteriae*, but in different ranges of concentration.

Solubility of antigen. Solubility of agglutinating antigen of the staphylococcus is shown in Tables I and II. Organisms suspended in salt free solution, in 0.85%, in 1.3% and in 3.2% sodium chloride concentrations were immediately separated from the respective solutions by centrifugation, washed and resuspended in 1.3% sodium chloride solution and used for agglutination reactions with antiserum of known titer. Table I represents variation in agglutination titer of a homologous antiserum (rabbit) using these organisms as antigens. The titers were markedly reduced in the case of organisms previously suspended in concentrations of 0.85% sodium chloride and less. This is in agreement with Oeding (3) who used both autoclaved and live staphylococci in 0.85% sodium chloride. On the other hand, little reduction of titer occurred when the organisms were previously suspended in solutions of 1.3% sodium chloride and greater. Thus the agglutinating antigen appears to be soluble in salt concentrations of 0.85% and less.

To confirm this, the respective supernatant solutions in which the organisms were originally suspended were cleared by centrifugation and passage through a Seitz filter and were used in the standard antibody absorption test. For final titering, unwashed organisms suspended in 1.3% sodium chloride constituted the antigen. Table II shows the typical results from such a test. Agglutinin is absorbed by the supernatant solutions of suspensions of organisms in concentrations of sodium chloride at 0.85% and below, and remains practically unaffected in concentrations at and above 1.3%.

TABLE I. Effect of Previous Exposure of Staphylococci to Various Concentrations of NaCl upon Their Agglutination by Immune Rabbit Serum.

Conc. of NaCl to which organisms were exposed (%)	Max agglutination titer of organisms after exposure
0 (distilled water)	80
.85	0
1.3	320
3.4	320
Unexposed	640

TABLE II. Absorption of Agglutinins from Immune Rabbit Serum by Washings of Staphylococci with Various Salt Concentrations.

Absorbing antigen (washing) according to its conc. of NaCl (%)	Max agglutination titer of antiserum after absorption
0 (distilled water)	40
.85	0
1.3	640
3.4	640
Unabsorbed	1280

It appears, therefore, that the change in agglutination demonstrated is related to the release of antigen into the solution since it is demonstrable there.

Summary. Optimal concentration of sodium chloride for quantitative evaluation of staphylococcal agglutinins was 1.3% to 1.7%. This applies to rabbit and human antisera. At this concentration, macroscopic technics are reliable and spontaneous agglutination is inhibited. The quality of the reaction is best at these concentrations for human antisera, while in rabbit antisera, it is best at concentrations of 3.2% sodium chloride. Concentrations below the optimal dissolves the antigen to such extent that macroscopic agglutination is definitely decreased.

1. Duncan, J. T., *Brit. J. Exp. Path.*, 1932, v13, 489; 1937, v18, 108.

2. Northop, J. H., DeKruif, P. H., *J. Gen. Physiol.*, 1922, v4, 639, 19.

3. Oeding, P., *Acta pathologica et microbiol. Scandinav.*, 1957, v41, 310.

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Effects of Storage on Serum Non-Esterified Fatty Acid Concentrations.* (25371)

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Although Dole(1) pointed out that extracts for non-esterified fatty acids (NEFA) should be prepared "as soon as possible after separation of plasma," no studies on possible changes in NEFA concentrations under various storage conditions have been reported. The present study illustrates that such storage, even in the frozen state, is accompanied by steadily increasing NEFA concentrations.

Methods. Fasting blood samples were obtained from 4 randomly selected patients, and from 2 patients with idiopathic hyperlipemia. Hyperlipemic blood samples also were obtained from 2 patients following intravenous infusion of a fat emulsion.† All blood samples were allowed to clot for approximately one hour, and the sera separated. Aliquots were promptly analyzed for NEFA by the method of Dole(1), and for triglyceride by the method of Forbes and Forbes (in preparation for publication). The latter was carried out in order to know the concentration of triglyceride substrate available for possible lipoprotein lipase action. Additional samples of the same sera were promptly separated for storage at −15°C, + 10°C, and room temperature (approximately + 25°C), and were subsequently analyzed for NEFA by the method of Dole (1) on days 1, 3, 8, 15, and 30 (days 1 and 3 only for room temperature stored sera).

Results. The initial (zero day) values for serum triglyceride fatty acids and NEFA for each patient are shown in Table I. Fig. 1 presents the effects of storage at the 3 temperatures on NEFA concentrations. Even in the frozen state, NEFA concentrations consistently rose in the first 24 hours, in one case to +63%. Increasing the storage temperature to +10°C and +25°C augmented these rises. In all of the frozen sera and in 5 of 8 sera stored at +10°C, transient decreases in NEFA following the initial rises were observed between days 3 and 15. In sera containing "artificial" chylomicrons from infused fat emulsion, enormous rises during storage oc-

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