tococcus hemolyticus, viridans, or Pneumococcus inhibited coagulation; only the strains of *Streptococcus hemolyticus* induced fibrin dissolution.

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Bactericidal Action of Human Serum on Hemolytic Streptococci. Active Principle Obtained by Fractionation of Sera.

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The present report is part of an investigation concerning the capacity of serum from patients who are acutely ill to destroy hemolytic streptococci of the *beta* type. Previous reports[1] have demonstrated that the bactericidal property under consideration was demonstrable in the serum derived from patients during the period of active disease due to a variety of infections, but that following recovery from illness the streptococcidal activity was greatly diminished, as measured by the methods which were employed. Furthermore, normal sera have been found to be essentially devoid of streptococcidal activity and have served as controls throughout the observations.

The previous articles have described the technical procedures. In the present study a strain of *Streptococcus hemolyticus,* which has been found to be uniformly highly sensitive to the killing effect of patients' sera, has been used in all of the experiments. The samples of sera were derived from patients who were acutely, and usually severely ill from diseases such as pneumonia, or pyogenic infections due to different kinds of organisms.

Studies have been carried out in an attempt to isolate the active principle in serum which is responsible for the streptococcidal activity. Observations have been made with a protein-fraction and a non-protein constituent. The materials have been used separately and in combination.

The protein-fraction most regularly employed in the tests has been obtained by precipitation of serum at low temperatures with alcohol

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1 Tillett, W. S., *J. Exp. Med.*, 1937, **65**, 147, 163.

TABLE I.

Results of Streptococcidal Tests Performed with: A. Whole Serum; B. Protein-Fraction of Serum; C. Ultrafiltrate from Serum; D. Protein-Fraction Plus Ultrafiltrate.

Patient	No. 1		No. 2		No. 3		No. 4	
Test Material	6 hr.*	24 hr.*	6 hr.	24 hr.	6 hr.	24 hr.	6 hr.	24 hr.
P't serum (1 cc.)	†130	†—	250	7	2	—	11	—
P't Pr. (0.5 cc.) + Phys. S.S. (0.5 cc.)	† ∞	∞	∞	1000s	1000s	∞	320	∞
P't Ult. (1 cc.)	∞	600	∞	∞	∞	∞	∞	600
Norm. Ult. (1 cc.)	∞	1000s	∞	300	∞	∞		
P't Pr. (0.5 cc.) + P't Ult. (0.5 cc.)	46	1	400	1			26	—
P't Pr. (0.5 cc.) + Norm. Ult. (0.5 cc.)	160	6	500	4	23	—		
Normal	Normal		Normal		Normal		Normal	
Norm. Serum (1 cc.)	∞	∞	∞	∞	∞	∞	∞	∞
Norm. Pr. (0.5 cc.) + Phys. S.S.	∞	∞	∞	∞	1000s	∞	∞	∞
Norm. Pr. (0.5 cc.) + P't Ult. (0.5 cc.)	∞	1000s	∞	∞	∞	1000s	∞	∞
Norm. Pr. (0.5 cc.) + Norm. Ult. (0.5 cc.)	∞	∞	∞	280	∞	∞		

*Duration of incubation before subculture was made.

†Results of subculture. Numerals indicate number of colonies. — indicates no growth in subculture. 1000s and ∞ indicate innumerable colonies.

The number of colonies in subcultures taken at the beginning of the test was always 1000s to ∞.

according to the method described by Felton.[2] Sufficient 95% alcohol was added to serum to make a final concentration of 20%. The material was kept in the icebox overnight in order to obtain the maximal yield and was then spun down in a cold centrifuge. The supernatant fluid was drained as completely as possible from the packed sediment. The precipitate was redissolved in an amount of physiological salt solution equal to one-half the volume of the serum from which it was derived. The twofold concentration was, as the succeeding experiments will indicate, for the purpose of permitting the addition of non-protein constituents in equal quantity.

In determining the effect on hemolytic streptococci of the material obtained by alcoholic precipitation, 0.5 cc. of the protein solution was restored to the original volume of serum by the addition of 0.5 cc. of physiological salt solution or Ringer-Locke's solution and tested by the usual method for bactericidal action.

Data obtained from tests made with the protein fractions are contained in the table which also consolidates the results of experiments in which different materials were used separately and in combination. As the tabulated findings indicate, the alcoholic precipitate,

2 Felton, L. D., *J. Immunol.*, 1931, **21**, 357.

when restored to volume with physiological salt solution and used in the tests, has not been found to possess the active principle of serum. The results obtained with the globulin derived from 4 separate specimens of serum are given in the table and are representative of other similar experiments.

In 2 instances, the first precipitation with alcohol yielded a fraction which possessed some killing power. However, following reprecipitation, negative results were obtained. It seems likely that in the first procedure, there was an incomplete separation of the constituents. The fraction derived from reprecipitation, although inactive alone, was effectively streptococcidal when mixed with ultrafiltrate according to the procedures to be described later in this article.

Attempts were made to obtain the non-protein constituents of serum by ultrafiltration through cellophane number 300 and sausage-casings. The apparatus used was that described by Lavietes.[3] Nitrogen-pressure equal to that of a column of mercury 30-35 cm. high was used to force the material through the membrane. The apparatus was placed in an incubator at 37.5°C. for 18 to 24 hours. At the end of this time 4 to 6 cc. of water-clear material had filtered from 10 cc. of serum. The sera were obtained from both patients and normal persons. Sterile precautions were used throughout.

It is interesting to note that with some of the samples of ultrafiltrate, although chemical tests for protein were negative, precipitative tests with anti-human rabbit sera were positive. This suggestive evidence of the occasional presence of small amounts of protein was detectable in the material filtered through cellophane but not in that passed through sausage-casing. It seems probable that different lots of cellophane differed in porosity. No difference was noted in the streptococcidal properties of the products of ultrafiltration regardless of the presence or absence of small amounts of protein. This minor variation in the constitution of ultrafiltrate has not seemed to be of significance in the present study.

The ultrafiltrate, water-clear at the beginning of the test, became turbid in approximately 3 hours after streptococci were introduced. At this time both stained smears and estimations of growth by diluted subcultures revealed marked increase in the number of organisms, which continued for 6 to 8 hours. At the end of 24 hours, innumerable viable organisms were present with some samples of ultrafiltrates, whereas with other samples considerable bacterial destruction had taken place. The variations just mentioned occurred with specimens of ultrafiltrate from both normal and patients' sera.

[3] Lavietes, P. H., *J. Biol. Chem.*, 1937, **120**, 267.

In every test the initial increases in bacterial population were comparable. No explanation can be offered at the present time for the late bactericidal effect of some of the samples of ultrafiltrate. It should be noted, however, that the curve of rapid rise and fall of viable population which occurred in the occasional tests with ultrafiltrate was distinctly different from the progressive decrease in numbers of streptococci which have occurred in bactericidal tests with serum. It seems not unlikely, therefore, that the effect peculiar to some of the samples of ultrafiltrate may be dependent upon limitations in nutritional elements or some other undetermined factors rather than to the presence of an appreciable amount of the streptococcidal factor of whole serum. Further investigations are now in progress in an attempt to analyze the special effects of ultrafiltrate.

In addition to observations concerning the streptococcidal activity of the protein-fraction and the samples of ultrafiltrate used separately, tests have also been carried out with the 2 materials in combination. From the data presented in the table it may be seen that when the protein-fraction derived from patients' sera was combined with ultrafiltrate, the streptococcidal activity of the mixture was comparable to that of whole serum. It is interesting to note that in duplicating the bactericidal effect of serum, although the protein-element derived from patients' sera was a necessary constituent of the mixture, the ultrafiltrate from normal serum served equally as well as did the ultrafiltrate from patients' sera in restoring streptococcidal activity. The specific bactericidal principle seems, therefore, to be latently associated with the globulin of patients' sera obtained by precipitation with alcohol. In order to obtain bactericidal activity, however, the supplementing effect of a non-protein element, which could be derived from the serum of normal individuals as well as from that of patients, proved to be necessary.

That the bactericidal effect of the combined protein and ultrafiltrate operates through the same mechanism as that of serum, is further evidenced by the fact that the anaërobic conditions, which in previous studies were shown to inactivate the killing power of serum, also nullified the lethal action of protein-ultrafiltrate mixtures that were effective under aërobic conditions.

Additional comparative tests were made with serum and with the fractional mixtures with respect to the influence of hydrogen-ion concentration on the phenomenon. In a separate report,[4] results are presented which outline the ranges of pH in which the bactericidal

[4] Tillett, W. S., and Stock, C. C., *J. Exp. Med.* In press.

action of serum is effective. The importance of hydrogen-ion concentration is also indicated by the fact that the serological property may be inactivated by reducing the pH below the appropriate levels. In the present experiments, the pH of all the products, whether derived from patients or normal individuals, was comparable at the beginning of the experiments. Consequently, the difference in the effect of patients' protein plus ultrafiltrate as contrasted with that obtained with normal protein plus ultrafiltrate is not primarily dependent upon the initial pH.

A sample of ultrafiltrate was placed in boiling water and at intervals of 15, 30, 60 minutes, and at 2 hours, 0.5 cc. was removed, cooled, and added to 0.5 cc. of unheated protein-fraction derived from the serum of an acutely ill patient. The results were as follows:

	Subcultures	
	6 hr.	24 hr.
1. Patient's serum	210*	21
2. Pr. + Ult. unheated	250	10
3. Pr. + Ult. heated 15 min.	300	26
4. Pr. + Ult. " 30 "	400	120
5. Pr. + Ult. " 1 hr.	400	110
6. Pr. + Ult. " 2 "	∞	∞

*Number of colonies in subcultures.

The protocol indicates that the effect of heat was not significantly demonstrable until it had lasted for 2 hours. The observations just described have been repeated several times and in each instance the results have been essentially the same.

When the ultrafiltrate was heated, a faint but definite opalescence appeared after 15 to 20 minutes. The clouding did not however, appear to be associated with inactivation since the material was found after brief exposure to high temperature to retain the supplementing effect when added to protein.

In order to determine whether lipids were associated with the active principle present in ultrafiltrate, samples have been extracted with ether overnight and then tested in combination with a protein-fraction derived from patient's serum. The material treated with ether, plus protein, was found to be as potent as the original preparations.

When the preparations extracted with ether were heated, turbidity did not develop. It seems likely, therefore, that the clouding of untreated ultrafiltrate was due to the lipid constituent.

In all the experiments, in which fractions of serum have been tested separately and in combinations, material, composed of the

protein-fraction from patient's serum plus ultrafiltrate has been found regularly to possess a marked degree of killing power. The constituents individually, however, have not, except for the variations previously discussed, given comparable results. The results suggest, therefore, that the active principle responsible for the destruction of streptococci consists of elements which are separable into inactive individual constituents. However, when mixed together, the streptococcidal property becomes effective. Studies at the present time are too incomplete to permit final interpretation of the results just described. Questions concerning the solubility of the protein preparation, or the influence of the concentration of electrolytes, or other possible factors associated with the complex chemical composition of serum require further investigation before the nature of the active principle can be completely defined. From the results so far obtained the significant constituent of ultrafiltrate seems to be neither protein nor ether-soluble lipid.

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Menstruation Inhibiting Action of Testosterone.

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The following experiments were performed to test the Androgen Testosterone Ciba for its possible replacement value of progesterone as an inhibitor of the menstrual flow.

1. *Inhibition of menstruation in the normal cycle.*

Monkey No. 245 was selected for this experiment for the great regularity of her menstrual cycles. These ran as follows: 1935-36: 28, 25, 24, 24, 24, 25, 28 days; 1936-37: 26, 25, 25, 26, 25, 26 days. The female was likewise ovulating with regularity and was fertile, a 56-day embryo having been removed by hysterotomy January 9, 1934.

Last menstrual period, March 23, followed by ovulation before April 13 (exact day not known).

Beginning April 13, 10 mg. of testosterone* was administered

* The author wishes to thank the Ciba Company for the generous supply of Testosterone and Testosterone Proprionate placed at the disposal of the laboratory; likewise to E. R. Squibb and Sons for both material and the counsel of J. A. Morrell over a period of years.