urine, TBA 81.5 mg%, BM-F 4 mg%, BM-T 81.5 mg%.

That the sulfonamides experience various metabolic changes including oxidations is well known,[3,4,5,6] though the precise nature of these has not been determined in all cases. The present work indicates that the pyrimidine ring in sulfadiazine experiences some change which blocks the TBA test and which can be reversed by reduction. However, when acetylsulfadiazine passes through the body, this change does not occur.

Summary. Thiobarbituric acid is a specific reagent for the determination of sulfadiazine (free or acetylated) among the sulfonamides because it reacts with the aminopyrimidine moiety. In most urines, however, the acetylated fraction which appears following the ingestion of sulfadiazine does not react unless the urine first be aged or be treated with a reducing agent. This suggests that the pyrimidine ring has been oxidized. Acetylsulfadiazine taken by mouth, when subsequently excreted in the urine, does not experience this change.

[3] Harris, J. S., *J. Clin. Invest.*, 1939, **18**, 521.

[4] Thorpe, W. V., Williams, R. T., and Shelswell, J., *Biochem. J.*, 1941, **35**, 52 and 61.

[5] Weber, C. J., Lalich, J. L., and Major, R. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 190.

[6] Scudi, J. V., and Jelnick, V. C., *J. Pharm. and Exp. Therap.*, 1944, **81**, 218.

14964

A Modification of the Technic for Determination of the Antistreptolysin Titer.*

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The determination of the antistreptolysin titer of human serum has proved to be a valuable tool for use in the study of hemolytic streptococcal infection and related disorders such as rheumatic fever. Its usefulness has been limited by certain technical difficulties inherent in the method most widely used, which was described by Coburn and Pauli,[1] as modified from Todd[2] and Hodge and Swift.[3]

Streptolysin or streptococcus hemolysin is unstable and is readily oxidized to an antigenically potent substance which is unable to hemolyze red blood cells. Previous technics have maintained a lysin for testing purposes in the active state by reduction with sodium hydrosulphite, followed by removal of oxygen in a vacuum, and storage under an oil seal in the cold room. This procedure is cumbersome and deterioration of the lysin occurs rapidly under any but the most favorable circumstances. Furthermore, it has been difficult to regularly produce a lysin of adequate potency because of variations in available culture medias and other factors.

Streptolysin, in the oxidized state, is stable and its hemolytic potency remains unchanged, even though it has been stored for many months in the refrigerator or for relatively long periods at room temperature before reduction. It may also be readily concentrated and partially purified.[4,5,6].

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The work described in this paper was done under contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development, and Stanford University.

[1] Coburn, A. F., and Pauli, R. H., *J. Exp. Med.*, 1935, **62**, 129.

[2] Todd, E. W., *J. Exp. Med.*, 1932, **55**, 267.

[3] Hodge, B. E., and Swift, H. F., *J. Exp. Med.*, 1933, **58**, 277.

[4] Rantz, L. A., unpublished observations.

TABLE I.

Ml diluted lysin	0.35	0.30	0.25	0.20	0.15	0.10
Ml buffered cysteine	0.15	0.20	0.25	0.30	0.35	0.40

These facts suggested a modification of the method for the determination of the antistreptolysin titer of serum which has proved to be extremely satisfactory in routine use and to give consistently reproducible values. It is based upon the preparation of a concentrated streptolysin which is stored in the oxidized state. Reduction to the active form is carried out at the time that tests are to be done.

Method. Preparation of lysin. A strain† of group A hemolytic streptococcus known to produce large amounts of hemolysin is grown for 18 hours in a meat infusion broth containing .2% of dextrose, 2% of proteose peptone (Difco), and buffered by the addition, after autoclaving, of .2% of sodium bicarbonate as a sterile solution. The original inoculum should be a large volume of rapidly growing organisms in a liquid medium.

After incubation the streptococci are removed in a centrifuge, the Sharples high speed type being most satisfactory if large volumes are to be processed. The lysin may now be sterilized by filtration through the Seitz filter and used, but the volume is large and the hemolytic activity may be insufficient. It is, therefore, very convenient to concentrate and partially purify the material.

The clarified culture is brought to .57% saturation by the addition of 429 g per liter of ammonium sulphate at 20°C. The resulting precipitate is removed in the regular or Sharples centrifuge, and dissolved in a phosphate buffer at pH 7.0 ($Na_2HPO_4 \cdot 12H_2O$, 14.2 g; KH_2PO_4, 3.63 g per liter of distilled water). The final volume should be about 4% of that of the original culture. This viscous brown solution is transferred to cellophane sacs and dialyzed for 18 hours against running tap water. This procedure is associated with a considerable increase in volume.

Nine g of sodium chloride is added to each liter of the dialyzed material which is then sterilized by passage through the Seitz filter. After aseptic transfer to sterile tubes, it is ready for use.

Determination of combining unit. The determination of the antigenic and hemolytic properties of the concentrated lysin requires its reduction at the time of use. This is accomplished by mixing it with an active reducing agent prepared in the following manner: 1.6 g of sodium hydroxide is added to 1 liter of an isotonic buffer of a pH of 6.5 (NaCl, 4.2 g; KH_2PO_4, 3.17 g; $Na_2HPO_4 \cdot 12H_2O$, 3.58 g per liter of distilled water). At the time of testing .15 g of cysteine hydrochloride is dissolved in 25 ml of this alkaline buffer to make a M/25 solution of the active agent.

To determine the combining unit the concentrated lysin is diluted serially from 1:2 to 1:12. Samples of each dilution are then set up in small tubes according to the schedule in Table I.

Reduction is permitted to proceed for 10 minutes when 1 ml of a dilution of standard antistreptolysin, containing 1 unit per cc, is added to each tube, thoroughly mixed and incubated for 15 minutes in the water bath. At the end of this time, 0.5 ml of a 5% suspension of thrice-washed defibrinated rabbit red cells are added to each tube. It is desirable to wash and suspend the cells in the isotonic buffer of pH 6.5 without added sodium hydroxide. After further incubation for 45 minutes the tubes are removed from the water and centrifuged. That amount of lysin which has just failed to produce hemolysis is the combining unit. In this laboratory it is usually .15 ml of the 1.8 dilution of the concentrated material.

Determination of antistreptolysin titer. The antistreptolysin titer of sera may be determined by the method described by Coburn and Pauli,[2] using the freshly reduced lysin. If this is to be done, the concentrated lysin is

[5] Herbert, D., and Todd, E. W., *Biochem. J.*, 1941, **352**, 1124.

[6] Smythe, C. V., and Harris, T. N., *J. Immun.*, 1940, **38**, 283.

† The Richards strain has been used in this laboratory. It and a supply of serum of known antistreptolysin titer were supplied by Dr. Rebecca C. Lancefield.

TABLE II.

	Serum dilution											
	1:10		1:100					1:500				
Ml of diluted serum	0.8	0.2	1.0	0.8	0.6	0.4	0.3	1.0	0.8	0.6	0.4	0.2
Ml of isotonic buffer	0.2	0.8	0.0	0.2	0.4	0.6	0.7	0.0	0.2	0.4	0.6	0.8
Ml of reduced lysin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Unit value of each tube	12	50	100	125	166	250	333	500	625	833	1250	2500

first diluted to the degree determined to be suitable by the previous test with isotonic buffer. A further dilution in the buffered cysteine solution is then performed at the time of use, so that the combining unit will be contained in a final volume of 0.5 ml. Thus a concentrated lysin might be diluted 1:8, 15 ml of this material being then added to 35 ml of the reducing solution. The lysin is ready for use after 10 minutes of exposure to the reducing agent and does not deteriorate within 60 minutes.

Lysin prepared in this way may be used in the usual technics, but it has been most convenient to eliminate the two-stage procedure previously described,[1,3] and to determine the antistreptolysin titer of each serum in a single operation, by means of a simplification of a procedure originally suggested by Hodge and Swift,[3] which does not appear to have been widely applied. This may be accomplished by preparing initial serum dilutions in isotonic buffer of 1:10, 1:100, and 1:500, samples of each of which are studied according to the scheme in Table II.

After 15 minutes of incubation at 37°C in the water bath, .15 ml of the previously described suspension of rabbit red cells are added and the whole maintained at the same temperature for an additional 45 minutes. The last tube in which lysis has not occurred indicates the antistreptolysin titer of the serum. A control serum of known antibody titer should always be included as a check on the potency of the lysin.

Discussion. The modified technic for the determination of the antistreptolysin titer described above, based upon the reduction of a concentrated standard lysin at the time of use, has certain advantages over methods previously described.

The lysin stored in the refrigerator and the reducing agent in the dry form remain stable for very long periods of time and are immediately ready for use. The complicated technic involving removal of dissolved oxygen in a vacuum and storage under oil is eliminated, as is the hazard of premature oxidation of the lysin before use. In addition, the presence of the reducing agent in adequate amounts during the actual testing, guarantees that the lytic agent will remain active during the interval required for the completion of the procedure.

Concentration of the lysin, while not essential, provides a product of suitable potency, makes sterilization by filtration more simple and decreases the volume for storage and shipment.

Other investigators[2] have indicated that lysin, after reduction, must be "aged" in order to obtain a stable product of maximum potency. Careful study of the technic described here demonstrates that this is not the case, and that the material is ready for use after exposure to the reducing agent for 10 minutes.

The suggested modification in which the antistreptolysin titer is determined as a single-stage operation greatly reduces the time involved in the performance of the tests. Larger than usual decrements of diluted serum are used but the values in units obtained are suitable for any investigative purpose except under circumstances involving the study of sera of very high antibody titer, when further determinations with a different dilution system may occasionally be desirable.

Many human and animal sera have been studied by the technic described and the results have been most satisfactory. Certain sera used as controls have been repeatedly titrated by different technicians, using several lots of lysin, with errors consistently less than might have been expected as the result of the dilutions involved. It has also been possible to ship lysin to other laboratories and to estab-

lish the method there with comparable results.

Summary. 1. A modified technic for the determination of the antistreptolysin titer of serum has been described which involves the use of a concentrated lysin reduced to the active form at the time of use.

14965

Effect of Heparin and Dicoumarol on Thrombosis Induced in the Presence of Venous Stasis.*

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The effect of heparin[1,2,3] and more recently dicoumarol[4,5,6] on the course of experimentally induced venous thrombosis has been extensively studied. However, the methods of producing intravascular thrombi used have not always been uniformly successful. For the evaluation of the effect of anticoagulants upon thrombosis a method of consistently producing thrombi is essential. In a preliminary report[7] the author states that he found stasis a prerequisite for the consistent production of occluding thrombosis by the insertion of intravascular foreign bodies. Rabinovitch and Pines[3] also found it necessary to add stasis (by partial ligature of the vein) to the intimal damage produced by stretching the vein to insure thrombosis. In view of the effect of stasis on experimental intravascular thrombosis[7,8] and the generally held clinical opinion as to stasis in the pathogenesis of thrombosis,[9] the antithrombotic action of heparin and dicoumarol [3,3'-methylenebis (4-hydroxycoumarin)] was studied in the presence of venous stasis.

Methods. Rabbits were used as the experimental animal. Prothrombin concentrations were estimated by the method of Quick[11] using rabbit brain thromboplastin in both whole and 16% plasma.[10,12] Coagulation times were determined by the capillary tube method using free-flowing venous blood. In preliminary experiments[7] various sclerosing agents and the intravascular insertion of darning cotton and wool yarn failed to consistently induce thrombosis in the absence of stasis and congestion. Soaking the wool yarn in defibrinated blood was found to augment thrombus formation to some degree. In the method of producing thrombosis finally adopted white wool knitting yarn was soaked in defibrinated dog's blood, thoroughly dried, and all gross masses of dried blood removed by gently pulling the yarn between the fingers. It was then unraveled into single strands; 2 strands loosely wound together were used as an intravascular foreign body. The neck of rabbits anesthetized

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1 Best, C. H., Cowan, C., and MacLean, D. L., *J. Physiol.*, 1938, **92**, 20.

2 Best, C. H., *Harvey Lectures*, 1940-41, **36**, 66.

3 Rabinovitch, J., and Pines, B., *Surgery*, 1943, **14**, 669.

4 Dale, D. U., and Jaques, L. B., *Can. Med. A. J.*, 1942, **46**, 546.

5 Richards, R. K., and Cortell, R., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 237.

6 Thrill, C. J., Stafford, W. T., Spooner, M., and Meyer, C. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 333.

7 Moses, C., *Fed. Proc.*, 1945, **4**, 52.

8 Guthrie, C. C., *Cleveland Med. J.*, 1912, **11**, 317.

9 Homans, J., *New Eng. J. Med.*, 1944, **231**, 51.

10 Overman, R. S., Stahlman, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1942, **142**, 941.

11 Quick, A. J., *The Hemorrhagic Diseases*, C. C. Thomas, Springfield, Ill., 1942.

12 Campbell, H. A., Smith, W. K., Roberts, W. I., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.