

New Hemolytic Method for Determination of Antistreptolysin O in Whole Blood

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A new method is proposed for the determination of antistreptolysin O, based on the properties of streptolysin O. The reduced form of the toxin is hemolytic, whereas the oxidized form is not; this activity can be restored, however, by a reducing agent. Both forms maintain the capacity to bind specific antibodies. Unlike the classical method, the assay is performed on whole blood, using the patient's own erythrocytes as revealing agents. A procedure is proposed which limits the operation to a single dilution of a microvolume of blood (0.05 ml brought to 2 ml) and to the distribution of constant quantities in test tubes prefilled with different amounts of oxidized streptolysin O. After a few minutes, a reducing agent is added, and the reading is performed as in the classical technique after sedimentation of the erythrocytes. Correlation with the traditional method ($r = 0.987$) is evaluated on the basis of the results of samples from hospitalized patients.

Streptolysin O (SO) is an oxygen-labile protein toxin secreted into the culture medium during the growth of some strains of *Streptococcus* group A (1). SO belongs to the group of cytolytic toxins of bacterial origin which are able to cause in vitro destruction of cells such as mammalian erythrocytes or those from other animal species (3, 6, 8). SO is used in serology for the determination of antistreptolysin O (ASO). The first serological test for the determination of ASO titer was described by Todd (14), who demonstrated the presence of ASO in the sera of patients affected by various streptococcal diseases. This test was based on the neutralization of SO by serial amounts of the serum under study: the excess unneutralized SO was revealed by hemolysis of erythrocytes added to the system.

More recently, various modifications have been published simplifying the performance of the test (5, 9, 10, 16). As far as the hemolytic assay is concerned, we have attempted to improve the methodology by establishing a new method for the assay of ASO, using the patient's whole blood and making use of his own erythrocytes to reveal the reaction.

The method is based on the fact that the capacity of SO to hemolyze erythrocytes, but not that of binding specific antibodies, is lost when sulfhydryl groups are oxidized (Fig. 1). If the oxidation is carried out under mild conditions, a reducing agent is able to restore the original sulfhydryl groups. Consequently, the toxin not bound to specific antibodies recovers the ability to hemolyze erythrocytes (2, 4, 5).

MATERIALS AND METHODS

All the chemicals used were reagent grade.

Preparation of SO. For the preparation of SO, a hemolytic strain of *Streptococcus pyogenes* type 1 (group A) supplied by the Institute of Microbiology, University of Genoa, Italy, was used. The strain was stored freeze-dried at 4°C. Once reconstituted, it was streaked on agar containing 5% rabbit blood and incubated at 37°C for 18 h. A suspension was then made with four colonies per ml of saline; 2 ml was placed in an Erlenmeyer flask containing 100 ml of Todd-Hewitt medium and incubated for 8 h at 37°C. Eight liters of culture medium (beef heart infusion [Difco], 2.5%; peptone [Difco], 1%; glucose, 0.2%; NaHCO₃, 0.2%; NaCl, 0.2%; Na₂HPO₄·12H₂O, 0.1%) was inoculated with 100 ml of culture broth. After 18 h of growth at 37°C, the bacteria were killed by the addition of 0.01% Thimerosal and removed by centrifugation in a Sorvall centrifuge at 4,229 × *g* for 20 min at 4°C. Solid ammonium sulfate was added to the supernatant to 65% saturation. After 30 min at room temperature, the suspension was centrifuged at 4,229 × *g* for 20 min at 4°C, and the precipitate was dissolved and dialyzed against distilled water for 18 h at 4°C.

Determination of the hemolytic activity of SO. The method described by Kanbayashi and Makoto (7) was used, with some modifications. A 0.5-ml sample was subjected to serial 0.03-log dilutions with saline. A 1% (vol/vol) saline solution of 2-mercaptoethanol (0.5 ml) was added to each diluted sample. After activation of the SO for 15 min at 37°C, 1 ml of a 2% saline suspension of rabbit erythrocytes was added to each test tube. The mixtures were incubated at 37°C for 45 min, and after centrifugation the optical densities of the supernatants were read with a spectrophotometer at 541 nm. One hemolytic unit (HU) was arbitrarily defined as the amount of SO capable of

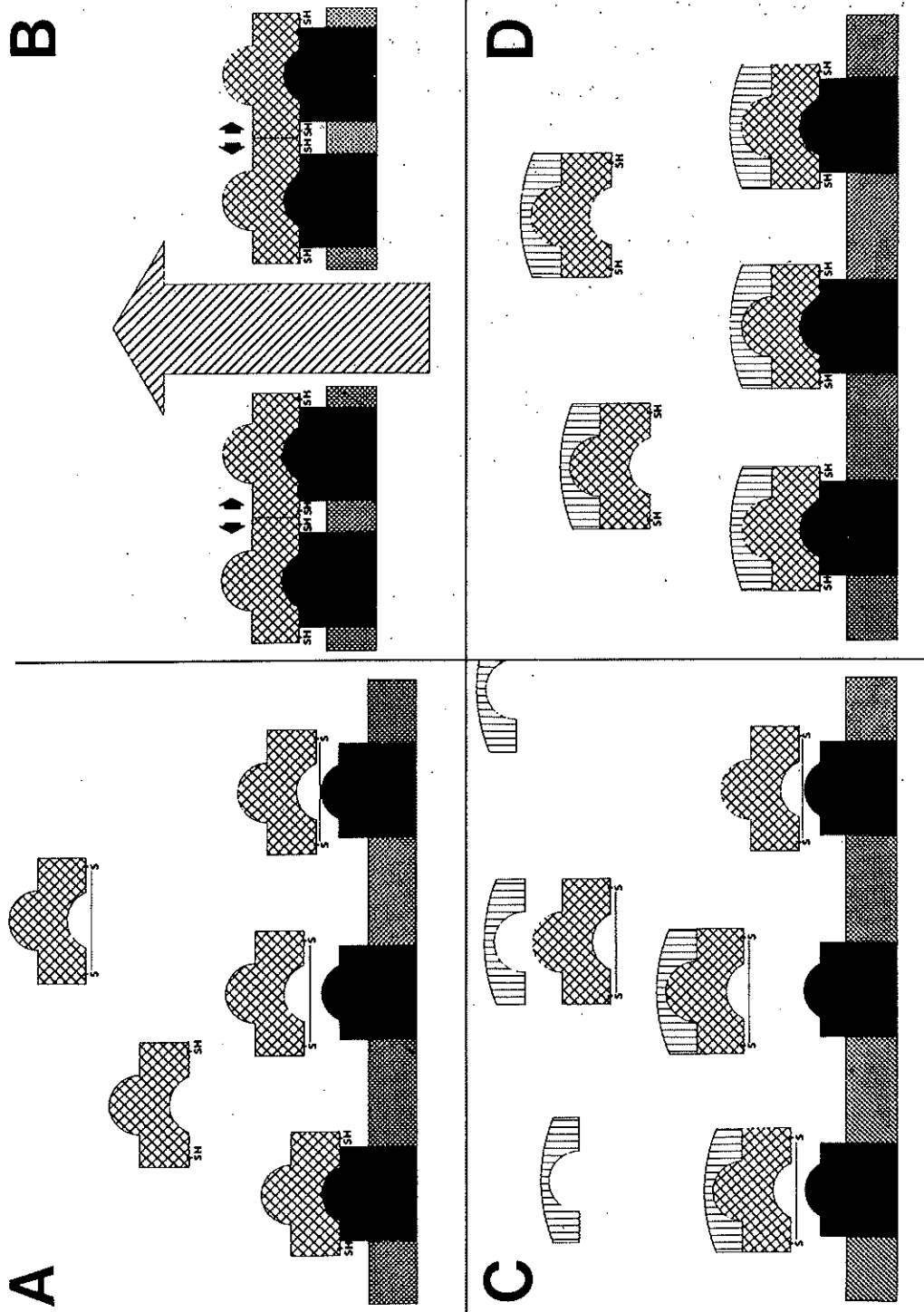


FIG. 1. Properties of reduced and oxidized SO. (A) The reduced SO can interact with the cell membrane through its sulfhydryl groups. Oxidized SO loses this capacity. (B) The interaction between the SO molecules bound to membrane receptors causes hemolysis. (C) Oxidized SO can bind the corresponding antibodies but cannot interact with the membrane. (D) Following reduction, the original sulfhydryl groups are restored and the SO-antibody complexes can bind to the membrane, but the interaction between SO molecules that causes hemolysis does not occur.

producing 50% lysis of 1 ml of 2% erythrocyte suspension after 45 min at 37°C. Oxidation of SO₂ was diluted in 0.001 M phosphate buffer (pH 7.6) to 640 HU/ml and cooled to 0°C. Cold H₂O₂ (36%, wt/vol) was added to reach a concentration of 12% (vol/vol). After 4 h at 0°C, the excess H₂O₂ was eliminated by dialysis for 18 h against distilled water at 4°C.

Inactivation of SO with CuCl₂. After oxidation, small amounts of unoxidized SO (6). The remaining unoxidized SO was inactivated by the addition of CuCl₂ at a final concentration of 5.10⁻³ M (15). The solution was maintained for 30 min at room temperature and then dialyzed against distilled water for 48 h at 4°C to eliminate the cupric ions that had not reacted. After dialysis, the solution was made isotonic by addition of NaCl, and the pH was brought to 7.0. Preparation of artificial blood to be used as reference. A hyperimmune horse serum rich in ASO was titrated by the technique of Schlangel (12), eliminating the serum dilutions below 150 IU and using commercial preparations (ISVT Sclavo, Italy) of SO and rabbit erythrocytes. After titration, the serum was diluted with saline to 150, 200, 300, 400, 600, 800, 1,200, and 1,600 IU, respectively. The sera were distributed in bottles (0.5 ml), freeze-dried, and stored at 4°C. At the time of use, they were reconstituted with 0.7 ml of a suspension of human erythrocytes, previously washed and suspended in saline at a concentration of 45%, as determined by the hematocrit value. The serum dilution in international units was determined in the supernatant after centrifugation of the reference artificial blood reconstituted with the erythrocyte suspension, using the method described by Schlangel (12). The reference artificial blood was used for the control of the new method.

Titration and distribution of SO. The oxidized and CuCl₂-treated SO was titrated, after reduction with 2-mercaptoethanol at a final concentration of 0.5%, by the International Standard Serum for ASO (10 IU/ml) supplied by the World Health Organization. The SO was diluted to 10.00, 7.50, 5.00, 3.75, 2.50, 1.87, 1.25, and 0.93 combining units (CU) per ml, where 1 CU is defined as the amount of SO neutralized by 1 IU of International Standard Serum. Samples of 0.4 ml of the above-mentioned diluted solutions of oxidized and CuCl₂-treated SO were distributed in test tubes (12 by 100 mm), frozen, and stored at -20°C. The titer was stable for at least 3 months.

Protein determination. The method of Lowry et al. (11) was used, using bovine serum albumin, fraction V, as standard.

The oxidized, frozen SO at various combining unit concentrations was tested by triple determinations with reference artificial blood. Figure 2 shows the results correlating the titration of the reference samples with the traditional method and the new technique. These titrations provided an evaluation of the relationship between the combining units of the oxidized SO and the values in international units of the reference artificial blood.

To test the new method in routine analyses, 468 blood samples were collected and tested with both the classical and new techniques. Of these, 109 samples had a titer higher than 150 IU in both titration methods; the remaining 359 samples were stable for at least 3 months.

TABLE 1. Characteristics of SO, oxidized SO, and oxidized and CuCl₂-treated SO solutions

Solution	Vol (ml)	HU/ml*	HU/ml	CU/ml*	Protein (mg/ml)	Potency (HU/mg of protein)
SO	350	2,560	320	108	17.5	146
Oxidized SO	980	640	8	28.5	5.7	112
CuCl ₂ -treated and oxidized SO	1,050	610	<1	26	5.6	108

* SO titrations performed after reduction with 2-mercaptoethanol. HU, Hemolytic units; CU, combining units.

RESULTS

Table 1 shows the results obtained from the titrations effected on SO, on oxidized SO, and on oxidized and CuCl₂-treated SO. The protein content remained practically constant during the oxidation and cupric chelation procedures, although there was a gradual loss of potency. Moreover, the residual hemolytic activity decreased to values less than 1 HU/ml. The total yield of the treatment was 73% in terms of potency.

The oxidized, frozen SO at various combining unit concentrations was tested by triple determinations with reference artificial blood. Figure 2 shows the results correlating the titration of the reference samples with the traditional method and the new technique. These titrations provided an evaluation of the relationship between the combining units of the oxidized SO and the values in international units of the reference artificial blood.

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Blood and sera collection. The samples to be analyzed were collected from adults and children of both sexes, hospitalized for various diseases. Part of the blood was heparinized, and part was allowed to clot for serum separation. Before testing, the sera were decomplemented at 56°C for 30 min.

Determination of the ASO with oxidized and CuCl₂-treated SO. A complete series of test tubes containing different concentrations of oxidized and CuCl₂-treated SO were thawed at room temperature. The heparinized blood under study was diluted 1/40 with saline solution, and 0.2 ml was dispensed in each test tube. The tubes were shaken and left for 15 to 20 min at room temperature; 0.1 ml of a 4% (vol/vol) saline solution of 2-mercaptoethanol was then added. The tubes were shaken and maintained at room temperature until complete sedimentation of the erythrocytes was obtained (about 1 h). The last tube without hemolysis represented the ASO titer in international units.

Statistical evaluation. The data were evaluated by linear regression and other elementary statistical methods (13).

ples had a titer lower than 150 IU, with either one or both techniques.

Figure 3 shows the correlation between the two methods in relation to the 109 samples at known titer. The correlation coefficient was 0.987 and was significantly different from 0 ($P < 0.001$). The regression coefficient was 0.984; this was significantly different from 0 ($P < 0.001$), and therefore the regression was significant. The regression coefficient did not differ significantly from 1 ($P > 0.05$). Moreover, the intercept was 0.0 with a standard error of 5.873. Of the other 359 samples analyzed, 94.5% had

a titer lower than 150 IU with both methods and 5.5% had a titer of 150 IU with the new technique and less than 150 IU with the traditional method.

DISCUSSION

The described method makes use of the oxidation of SO with H_2O_2 and reversible loss of its hemolytic activity. The oxidized SO containing variable amounts of combining units was allowed to react at room temperature with constant volumes of the diluted blood to be analyzed, to form the bond between ASO and SO; then the reducing agent (2-mercaptoethanol) was added. In the reaction, the patient's own erythrocytes were used as indicators. The reading was taken when the erythrocytes had sedimented. If the SO was not bound to ASO, after reduction it hemolyzed the patient's erythrocytes; there was no hemolysis if the SO had reacted with ASO.

During the SO oxidation procedure, a loss of specific activity was noted. This fact was probably due to irreversible oxidation by part of the SO; during the oxidation, in fact, some sulfhydryl groups might be transformed into groups with a higher degree of oxidation than disulfide groups.

It is known that reduced SO is inactivated by $FeCl_2$, $CuCl_2$, and $CaCl_2$ (12). When these salts react with oxidized SO, they have no effect on its potential activity (15). We adopted this mechanism to reduce the residue of hemolytic units after SO oxidation. The slight loss of specific activity after treatment with $CuCl_2$ may be attributed to the irreversible inactivation of unoxidized SO after treatment with H_2O_2 .

The results obtained with our method were in agreement with those obtained using the conventional one. The titers of those samples which were 150 IU with our method and below 150 IU with the method of Schlangel were always below the values considered suspect for clinical purposes.

During the study, 159 analyzed blood samples were tested for hematocrit. The values varied from 34 to 50% with an average of 40.3%. The titers in international units of these samples, analyzed both with the new method and the conventional one, were in agreement. In all probability, any errors in titration caused by the differences in erythrocyte volume of the various blood samples were lower than the sensitivity of the method.

One hundred fourteen of the samples were from patients affected by particular diseases, such as renal insufficiency, glomerulonephritis, gastroenteric infections, endocrine diseases, pneumonia, bronchitis, diseases of the cardiocir-

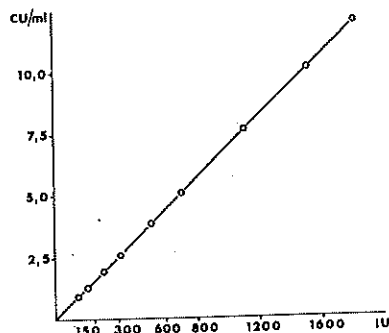


FIG. 2. Relationship between the combining units (CU) of the oxidized SO and the values in international units (IU) of the reference artificial blood. The abscissa reports the IU of the reference samples titrated using the technique of Schlangel. The ordinate shows the CU per milliliter of the SO solution, oxidized and treated with $CuCl_2$, in the last test tube that did not show hemolysis in the titration with the new technique.

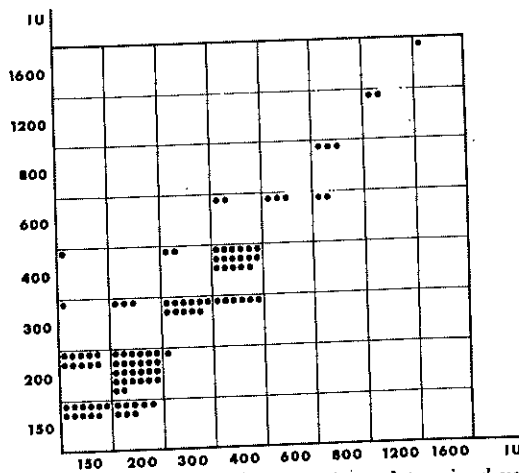


FIG. 3. Correlation between titers determined using the new (ordinate) and the classical (abscissa) methods, performed on 109 samples at known ASO titers.

- cularatory system, autoimmune diseases, and tumors. The titers found in these samples were also in agreement with those found with the classical method.
- The advantages of our method can be summarized as follows, (i) The reaction is performed on whole blood, and therefore it is not necessary to separate and decplement the serum. (ii) A microvolume (0.05 ml) of blood is sufficient and can therefore be taken by a finger or heel prick. It would thus appear to be particularly suitable for pediatric use. (iii) The blood is only diluted once, and a constant volume is distributed in each reaction vessel. The time-consuming dilutions and distributions of serum required by the conventional method are therefore unnecessary. Finally, (iv) fresh, washed rabbit or human erythrocytes are not needed, because the patient's own erythrocytes are used in the reaction.
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- LITERATURE CITED**
1. Alouf, J. E., and M. Raynaud. 1965. Un milieu simple pour la production de streptolydine O de titre elevé. *Ann. Inst. Pasteur 108:769-767.*
 2. Alouf, J. E., and M. Raynaud. 1968. Action de la streptolydine O sur les membranes cellulaires. *Ann. Inst. Pasteur 114:812-827.*
 3. Bernheimer, A. W. 1974. Interactions between membranes and cytolytic bacterial toxins. *Biochim. Biophys. Acta 344:27-50.*
 4. Bernheimer, A. W., and L. S. Avigad. 1970. Streptolydine O: activation by thiois. *Infect. Immun. 1:509-510.*
 5. Fasth, A. 1974. Determination of antistreptolydine O by reversed single radial immunodiffusion. *Acta Pathol. Microbiol. Scand. Sect. B 82:715-718.*
 6. Halberst, S. P. 1970. Streptolydine O, p. 69-94. In T. C. Montie, S. Kadis, and S. J. Aji (ed.), *Microbial toxins: a comprehensive treatise*; vol. III. Bacterial protein toxins. Academic Press Inc., New York.
 7. Kanbayashi, Y., and K. J. Makoto. 1972. Kinetic study on streptolydine O. *J. Biochem. (Tokyo) 71:227-237.*
 8. Klein, G. C., B. V. Addison, J. S. Boone, and M. D. Moody. 1968. Effect of type of red blood cells on antistreptolydine O titer. *Appl. Microbiol. 16:1761-1763.*
 9. Klein, G. C., C. N. Baker, and M. D. Moody. 1970. Comparison of antistreptolydine O latex screening test with the antistreptolydine O hemolytic test. *Appl. Microbiol. 19:60-61.*
 10. Klein, G. C., M. D. Moody, C. N. Baker, and B. V. Addison. 1968. Micro antistreptolydine O test. *Appl. Microbiol. 16:184.*
 11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193:265-275.*
 12. Schlangel, G. 1964. A rapid method for serum dilution in anti-streptolydine titers. *Am. J. Med. Technol. 30:195-198.*
 13. Snedecor, G. W., and W. G. Cochran. 1968. *Statistical methods*. The Iowa State University Press, Ames.
 14. Todd, E. W. 1932. Antigenic streptococcal hemolysin. *J. Exp. Med. 55:267-280.*
 15. Van Epps, D. E., and B. R. Andersen. 1971. Streptolydine O. II. Relationship of sulfhydryl groups to activity. *Infect. Immun. 3:648-652.*
 16. Vargues, R. 1966. Automatisation des dosages des antistreptolysines du serum humain. *Ann. Biol. Clin. 23:1235-1241.*