# QUANTITATIVE CHEMICAL STUDIES ON HEMOLYSINS

## I. The Estimation of Total Antibody in Antisera to Sheep Erythrocytes and Stromata*

By MICHAEL HEIDELBERGER and HENRY P. TREFFERS

*(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)*

(Received for publication, December 2, 1941)

Hemolysin in antisera is customarily estimated by "titer" and this measurement has served its purpose in the evaluation of antisera and in the determination of the relative degree of sensitization of erythrocytes. However, knowledge of the mode of action of hemolysins on red cells and of the quantitative relations of hemolysins to erythrocytes and complement has been difficult of access not only because of the complicated and sensitive nature of the reactions and their components, but also because methods of assay yielding end "titers" are, taken alone, without significance as to the actual weights of the reactants. Moreover, these methods do not even give relative values when different substances or functions are measured by end "titers," since the actual quantity of one substance required to produce its characteristic effect may greatly exceed that of another substance producing the same or a different effect. End "titers" are often, therefore, in opposite ratio to the actual quantities involved. Examples are the low "titers" of antipneumococcus sera of high antibody content as contrasted with the high "titers" of anti-*Salmonella* sera of low antibody content, and the lower "titer" but apparently higher content of first component in complement as compared with the higher "titer" but lower content of second and fourth components (1, 2). "Titers," then, depend upon the relative proportions in which the components of a given system interact, as well as upon the absolute quantities present, and are only interpretable, on passing from one reacting system to another, when reacting proportions as well as quantities are known. To return to the hemolytic system, since only "titers" of the components could be estimated in an effort to determine the very quantities which would necessarily have to be known before "titers" could be interpreted, lack of theoretical progress in this field is readily understandable.

The present communication deals with an attempt at the estimation of hemolysin in absolute, or weight units. Data of this kind would not only be useful in studying the quantitative relations between hemolysin, the red cell,

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

and complement, but would also supply a factor by which hemolysin "titers" could be converted into weight units. While this objective has not yet been attained, it has been possible to estimate total antibody in hemolytic antisera with a fair degree of accuracy and to use this value as a first approximation in the study of the relations sought (3).

The amount of nitrogen in the hemolytic unit has been estimated by Locke, Main, and Hirsch (4), by Brunius (5), and by Chow and Zia (6). The reasons for the large discrepancies in the estimates reached in the first two investigations are again (*cf.* 3) discussed below, together with an evaluation of the method used by Chow and Zia.

## EXPERIMENTAL

### *Materials and Methods*

*Sheep Cell Stromata.*—Lot 1: Three liters of sheep blood were defibrinated with glass beads immediately after bleeding, filtered through cloth, diluted with 4 volumes of saline, and centrifuged at room temperature in a Sharples centrifuge. The deposit of red cells was laked with 20 volumes of distilled water and centrifuged as before. The stromata were washed twice with water and twice with 0.9 per cent saline at room temperature, followed by two more washings with saline at 0°C.[1] The pale brown stromata were resuspended in saline containing 1:10,000 merthiolate.[2] The suspension was kept in the ice box and portions were washed at 0° before use.

Lots 2 and 3 were prepared similarly except that all operations, including the rather tedious process of centrifugation,[1] were carried out in the cold.

A portion of Lot 2 was heated at 56°C. for 40 minutes (7) (heated suspension).

*Antisera.*—Rabbits were injected intravenously with stromata suspensions (Lots 1 and 2) containing 0.5 mg. N per ml. The first course consisted of 13 injections averaging 0.75 ml. The second and third courses consisted of 10 and 12 injections respectively, of an average of 1.5 ml. of suspension. The fourth course comprised 10 injections and the fifth 11 injections of an average of 1.5 ml. of suspension (rabbits 7.62, 7.66). Rabbit 7.60 received only the first, third, and fifth courses. Rabbit 7.83 was injected subcutaneously instead of intravenously, receiving one course of 7 injections of 0.5 ml. followed by a month's rest, and a second course of 3 injections of 0.5 ml., 2 of 1 ml., 2 of 1.5 ml., and 4 of 2 ml. of suspension. A number of commercial antisera to sheep red cells were also used.[3]

*Analytical Methods.*—All antisera were inactivated at 56°C. for 50 minutes (thermometer in serum) (2), neutralized to pH 6.7–7.0 (glass electrode), and centrifuged in the cold shortly before use. Duplicate 1.0 ml. portions of undiluted antiserum, 2.0 ml. of saline, and 1.0 ml. of the stromata suspension were mixed at 0°C. Duplicate blanks were also run on 1.0 ml. of serum alone and with 1.0 ml. of stromata and 3.0 ml. saline alone. The tubes were allowed to stand in the ice box for 48 hours, with occasional mixing of the contents. The stromata usually showed pronounced agglutination in the presence of antiserum.

[1] In a refrigerated centrifuge manufactured by the International Equipment Company, Boston.

[2] Manufactured by Eli Lilly and Company, Indianapolis.

[3] Kindly supplied by E. R. Squibb and Sons, Inc., and by the Lederle Laboratories.

The determination of the antibody N precipitated was similar to that of the quantitative agglutinin procedure (8–10). The tubes were centrifuged in the cold and the precipitates washed three times with 0.9 per cent saline at 0°. The supernatants of duplicate determinations were combined and recentrifuged, and any deposits left were washed with the saline washings poured from the main tubes and finally recombined with the main precipitates. The initial supernatants, after the recentrifugation, were again set up with 1.0 ml. of stromata suspension and new blanks. The precipitates and the recentrifuged supernatants and saline washings from all stromata blanks were analyzed separately. The solutions always contained a small amount of N (about 0.04 mg.) due to soluble material remaining in the stromata in spite of the repeated preliminary washings.

Antibody N in the tubes containing serum and stromata was taken as the difference between the total N precipitated and the stromata N deposited in the stromata blank tubes after three washings and centrifugations. The antibody values obtained are probably slightly too high because of the antigen which dissolved. Correction would be difficult since it is not certain how much of the dissolved N represented inert material, or whether the same amount of N was washed out of the stromata which had combined with antibody.

*Hemolysin Titers.*—The hemolytic titers were determined from the dilution at which the sera would just sufficiently sensitize an equal volume of a 5 per cent suspension of washed sheep red cells to give complete hemolysis in 20 minutes at 37°C. in the presence of 2 units of complement.

Data obtained as above on a dilution of serum $7.66_5$ are given in Table I. Antibody N data for a number of sera are correlated with hemolytic titers in Table II.

*Effect of Experimental Conditions on Antibody Precipitation.*—(*a*) *Hydrogen Ion Concentration:* A dilution of serum 7.66 with a hemolysin titer of 1200 was absorbed with stromata after adjustment of one portion to pH 6.0 and another to pH 8.0 (glass electrode). The pH was redetermined after each absorption and slight readjustments to the original values were necessary for the first two. The total amounts of antibody N removed in three absorptions at low pH were 0.26, 0.01, and 0.02 mg. N, with a total of 0.29 mg. N, and in the more alkaline series, 0.25, 0.03, and 0.02 mg. N, with a total of 0.30 mg. N per ml. The fourth absorptions were negative in both cases. Hemolytic titers in both series were reduced to less than 6 units per ml. There were thus no differences in behavior in the absorptions at these two hydrogen ion concentrations and the precaution of neutralization, taken in most of these studies, would therefore seem unnecessary.

(*b*) *Effect of Temperature:* 1.0 ml. of undiluted rabbit serum 40H301 and 1.0 ml. of a 1:1 diluted donkey antiserum 186H102 to sheep red cells were each absorbed with stromata in parallel series at 0° and 37°C. The data are given in Table III. It will be noted that almost as much antibody N was removed at 37° as at 0°C.

(*c*) *Effect of Heat on Antigen Suspension:* Absorptions were made with heated and unheated suspensions of stromata. First bleedings from rabbits

TABLE I

*Estimation of Antibody in Anti-Sheep Cell Rabbit Hemolysin by Addition of Sheep Cell Stromata; 1.0 Ml. Serum, 0°C.*

| Absorption | Stromata N used* | Total N precipitated | Antibody N found (mean) | Calculated to 1.0 ml. of original serum |
|---|---|---|---|---|
| | *mg.* | *mg.* | *mg.* | *mg.* |
| 1st | 0.718<br>0.710 | 0.968<br>0.978 | 0.264 | 0.26 |
| 2nd‡ | 0.488<br>0.478<br>0.472 | 0.492‡ | 0.012 | 0.01 |
| 3rd§ | 0.486<br>0.484<br>0.492 | 0.530§ | 0.042 | 0.02 |
| 4th§ | 0.488<br>0.478<br>0.480 | 0.486§ | <0.01 | 0 |
| Total | | | | 0.29 |

* Values for washed stromata blanks, in duplicate or triplicate.

‡ On combined supernatants from first absorption.

§ On supernatant of preceding absorption.

TABLE II

*Absorption of Antibody in Anti-Sheep Cell Hemolysin by Sheep Stromata Suspensions, per 1.0 Ml. Undiluted Serum, 0°C.*

| Serum | Hemolytic titer* | 1st absorption | | 2nd absorption | | 3rd absorption | | Total antibody N | Hemolytic titer ÷ antibody N |
|---|---|---|---|---|---|---|---|---|---|
| | | Antibody N | Hemolytic titer | Antibody N | Hemolytic titer | Antibody N | Hemolytic titer | | |
| | | *mg.* | | *mg.* | | *mg.* | | *mg.* | |
| $L_1$ | 1,200 | 0.24 | <30 | 0.05 | <4 | 0 | | 0.29 | 4,100 |
| $L_2$ | 2,500 | 0.11 | ≪120 | 0 | <6 | | | 0.11 | 23,000 |
| $L_3$‡ | 3,000 | 0.71 | >240 | 0.11 | >240 | 0 | 100 | 0.82 | 3,500 |
| $L_4$ | 2,000 | 0.48 | <24 | 0.03 | <12 | | | 0.51 | 3,900 |
| $S_1$ | 1,200 | 0.36 | 130 | 0.10 | 36 | § | | 0.46 | 2,600 |
| $S_2$ | 750 | 0.20 | 60 | 0.02 | 40 | 0.02 | 24 | 0.24 | 3,000 |
| $7.60_4$ | 600 | 0.18 | <15 | 0.07 | <5 | 0.02 | | 0.27 | 2,200 |
| $7.62_4$ | 1,400 | 0.24 | 15 | 0.08 | <5 | 0.01 | | 0.33 | 4,200 |
| $7.66_4$ | 1,700 | 0.29 | 15 | 0.09 | <5 | 0.02 | | 0.40 | 4,300 |
| $7.83_1$‖ | 10 | 0.09 | 0 | 0.01 | 0 | 0 | | 0.10 | 100 |

* The titrations given are the reciprocals of the dilution at which the sera completely hemolyzed an equal volume of 5 per cent sheep red cell suspension in the presence of two units of guinea pig complement and are thus independent of the volumes chosen so long as this relation to the red cells is maintained. The antibody N estimations are all calculated to 1.0 ml. of the undiluted serum.

‡ Pony serum.

§ Determination lost.

‖ Rabbit injected subcutaneously with stromata.

injected with unheated stromata were used (Table IV). Although the total amounts of antibody N removed by heated suspensions approached those taken

TABLE III

*Estimation of Antibody in Anti-Sheep Cell Hemolysins by Stromata Suspensions at 37° and 0°C., per 1.0 Ml. Serum*

| Serum and temperature | Hemolytic titer | 1st absorption | | 2nd absorption | | 3rd absorption | | Total antibody N |
|---|---|---|---|---|---|---|---|---|
| | | Antibody N | Hemolytic titer | Antibody N | Hemolytic titer | Antibody N | Hemolytic titer | |
| | | *mg.* | | *mg.* | | *mg.* | | *mg.* |
| $L_5$, 37° | 1,600 | 0.26 | 200 | 0.06 | 90 | 0.03 | 50 | 0.40* |
| $L_5$, 0° | 1,600 | 0.29 | 400 | 0.10 | <225 | 0.04 | <25 | 0.43 |
| $L_6$,‡ 37° | 2,400 | 0.27 | 1,600 | 0.06 | 270 | 0.04 | 250 | 0.37 |
| $L_6$,‡ 0° | 2,400 | 0.28 | 1,000 | 0.09 | <225 | 0.05 | 50 | 0.42 |

Approximately equal, but accurately measured, quantities of stromata were used for each absorption.

* Includes 0.05 mg. N obtained on fourth and fifth absorptions. Final hemolytic titer 30

‡ Donkey serum. Determinations made and reported on basis of 1:1 diluted serum.

TABLE IV

*Absorption of Antibody in Anti-Sheep Stromata Rabbit Hemolysins by Heated and Unheated Sheep Stromata, per 1.0 Ml. Serum, 0°C.*

| Serum | Hemolytic titer | 1st absorption | | 2nd absorption | | Total antibody N |
|---|---|---|---|---|---|---|
| | | Antibody N | Hemolytic titer | Antibody N | Hemolytic titer | |
| | | *mg.* | | *mg.* | | *mg.* |
| Sera absorbed with heated stromata suspension | | | | | | |
| $7.60_1$ | <100 | 0.06 | >1 | 0.02 | <8 | 0.08 |
| $7.62_1$ | >1,000 | 0.09 | *ca.* 500 | 0.05 | >200 | 0.20* |
| $7.66_1$ | <1,000 | 0.08 | *ca.* 300 | 0.02 | *ca.* 10 | 0.13‡ |
| $7.57_1$ | <1,000 >500 | 0.09 | >500 | 0.04 | >64 | 0.17§ |
| Sera absorbed with same suspension, unheated | | | | | | |
| $7.60_1$ | <100 | 0.09 | 0 | | | 0.09 |
| $7.62_1$ | >1,000 | 0.22 | 75 | | | 0.22 |
| $7.66_1$ | <1,000 | 0.17 | <24 | | | 0.17 |
| $7.57_1$ | <1,000 >500 | 0.19 | 0 | | | 0.19 |

* Includes 0.06 mg. N from third and fourth absorptions.

‡ " 0.03 " " " a third absorption.

§ " 0.04 " " " " " "

out by unheated stromata, the unheated stromata suspension was the more active antigen per milligram of N used. 0.50 mg. N of the unheated suspension precipitated the same amount of antibody N in one absorption as did 2.99 mg. N of heated stromata in four absorptions. The data are summarized in Table IV.

*Combining Proportions of Stromata and Antibody.*—The existence of more than one combining ratio between stromata and antibody is indicated. For example, 1.0 ml. portions of a 1:1.5 dilution of serum $7.66_4$ were set up in duplicate with 1.0, 1.5, and 2.5 ml. of stromata suspension in a total volume of 8.0 ml.

| Stromata N added | 1st absorption | 2nd absorption* | Total antibody N precipitated |
|---|---|---|---|
| | Antibody N precipitated | Antibody N precipitated | |
| *mg.* | *mg.* | *mg.* | *mg.* |
| 0.476 | 0.23 | 0.04 | 0.27 |
| 0.714 | 0.24 | 0.03 | 0.27 |
| 1.188 | 0.29 | 0.01 | 0.30 |

* On pooled supernatants. Antibody N given is one-half of that found on addition of 0.476 mg. of stromata N in each case.

The smallest amount of antigen used removed practically as much antibody N as did a quantity 50 per cent larger, although neither completely exhausted the serum. Other particulate antigens, such as specific precipitates (10), show a similar behavior in their homologous antisera; the slope of the line representing antibody N/antigen, plotted against antigen N added, is usually much less than with soluble antigens.

*Removal of Complement by Stromata-Antibody Precipitation.*—In (3) it was mentioned that the combining component(s) of complement was taken up in the reaction of sheep cell stromata with hemolysin. The data upon which this statement was based are given in the protocol. The tubes were allowed to stand 2 hours at room temperature, after which they were centrifuged and washed three times with 2.5 ml. saline in the cold (*cf.* 2).

| No. of tubes | 2 | 2 | 3 | 3 | 3 |
|---|---|---|---|---|---|
| Guinea pig serum, C′, *ml.* | 3.0 | 3.0 | | | 3.0 |
| Inactivated* guinea pig serum, iC′, *ml.* | | | | 3.0 | |
| Rabbit hemolysin, *ml.* | 1.0 | | 1.0 | 1.0 | 1.0 |
| Sheep cell stromata, *ml.* | | 1.0 | 1.0 | 1.0 | 1.0 |
| Saline, *ml.* | 4 | 4 | 6 | 3 | 3 |
| Total N precipitated, *mg.* | 0 | 0.410 | 0.504 | 0.524 | 0.602 |
| | | | 0.508 | 0.520 | 0.606 |
| | 0 | 0.382 | 0.514 | 0.504 | 0.610 |
| Mean, *mg.* | 0 | 0.396 | 0.509 | 0.516 | 0.606 |
| Stromata N to be subtracted (column 3), *mg.* | | | 0.396 | | |
| Hemolysin N precipitated, *mg.* | | | 0.11 | | |
| iC′ to be subtracted, *mg.* | | | | | 0.516 |
| C′ N precipitated, *mg.* | | | | | 0.09 |

Hemolytic units of C′ taken 285.
" " " " remaining <1.

* At 56°C. for 50 minutes. The symbols used are explained in (2).

The quantity of C′ N removed is of the same order as that taken out by other specific precipitates (2). An estimation with S III-rabbit anti-S III at the same time gave 0.07 mg. of C′ N.

## DISCUSSION

Attempts have previously been made, by purification and isolation of the antibody, to define a weight unit for hemolysin. Locke, Main, and Hirsch (4) purified rabbit anti-sheep cell hemolysin and found 0.007 $\gamma$ of nitrogen per hemolytic unit in their best preparation. Brunius (5), on the other hand, using the Forssman antigen for selective extraction of the hemolysin, found only 0.0002 $\gamma$ of N in a smaller hemolytic unit. In both laboratories the preparations isolated usually contained ten times as much N per unit. As already pointed out (3) Brunius' value probably represents the actual hemolytic antibody unit more closely than Locke, Main, and Hirsch's figure, or that now reported, 0.03 $\gamma$ of N.[4] The latter, however, have the advantage of giving an insight into the actual weight of antibody deposited on the red cell from rabbit antisera.

These isolation techniques (4, 5) are naturally too laborious for analytical purposes. A method for the estimation of hemolysin in weight units has recently been described by Chow and Zia (6), who agglutinated sheep red cells with a measured volume of hemolysin, washed the deposit, and subsequently removed the hemoglobin by laking. The amounts of antibody found greatly exceeded those indicated in the present study, possibly because hemoglobin and other soluble products in the blanks without antiserum would be more easily washed out than from cells on which antibody had been deposited.

The method now proposed is a modification of the quantitative agglutination procedure (8–10) and depends upon the addition of antibody to sheep cell stromata from which as much as possible of the soluble components has been removed. Comparison of hemolytic titers of antisera before and after successive absorptions with stromata (Table II) shows that hemolysins are removed, as had been found by previous workers. Reproducible results are readily obtained, so that the method may serve, as a first approximation, for the estimation of hemolysin in weight units.

However, two factors combine to limit the utility of the method for its original object. One, of relatively minor consequence, tends to make the results somewhat too high. In spite of the pains taken to wash the stromata used small quantities of soluble material (about 0.04 mg. of N) were invariably found in the washings from the blank tubes. At least a portion of this material gave precipitin reactions in the antisera, but since it is uncertain whether or not similar amounts were washed out of the stromata-antiserum residues no correction has been made for this effect. It is therefore possible that the antibody N values found are too high by all or a portion of the N washed out of the con-

[4] Calculated from the figures in Table II for 0.1 ml. of hemolysin dilution.

trols. Stromata heated after the washing process according to Landsteiner and van der Scheer's method for red cells (7) are more easily centrifuged and appear to be slightly less soluble, but they cannot be used conveniently for the quantitative absorption of antibody as their antibody-binding capacity is too low (Table IV).

The second limiting factor is that the method, strictly speaking, is one for the estimation, not of hemolysin, but of total antibody in the hemolytic antisera. Sheep red cells and even washed sheep cell stromata are complex collections of antigens and only one or a limited number of these, presumably gives rise to true hemolysins. The remaining antibodies stimulated by injection of cells or stromata into rabbits add to the stromata suspension when this is mixed with antiserum, or to red cells, but probably take little part in hemolysis. This is probably the chief reason for the discrepancy in the values given (4, 5) for the quantity of N in the hemolytic unit. According to these values (p. 529) the antibody N figures may exceed true hemolysin N by a factor of 10 to 100, although these values are not to be taken too literally on account of the different units and end points compared. In spite of this, there does appear to be a fair proportionality between total antibody N content and hemolysin titer in rabbit anti-sheep hemolysin, as already noted by Chow and Zia (6) although on a different numerical basis. If one excludes the pony and donkey sera because of species differences, the serum of the subcutaneously injected rabbit and one other commercial rabbit serum, $L_2$, that was entirely out of line, ten anti-sheep cell and anti-stromata rabbit sera showed a titer: antibody N ratio of $3500 \pm 600$. In other words, an antiserum which, at a dilution of 1:3500, just completely hemolyzes an equal volume of 5 per cent sheep red cell suspension in the presence of 2 units of complement, should contain, within about 20 per cent, 1 mg. of antibody N per ml. None of the sera examined contained as much antibody N as this in a single milliliter. On this basis it might be more reasonable, both from the standpoint of purveyor and purchaser, to sell and buy hemolytic antisera by the milligram of total antibody nitrogen, rather than on the basis of units which might vary considerably from laboratory to laboratory and which provide no inkling of the actual antibody content.

If data obtained according to the new method are to be used in a consideration of the relation between hemolysin, red cells, and complement in the process of hemolysis, it must be remembered that the antibody N values are maximal and can only be used as hemolysin N as a first approximation, subject to correction by a factor still to be determined. This was emphasized in attempts to trace this relationship in (3). As there pointed out, the total antibody N value is of use in an understanding of the hemolytic process as actually carried out, for all of the antibody combines with the red cells present and also plays a major part in the fixation of complement, as shown in protocol 2 on p. 528.

Efforts are now being made to estimate accurately the actual amounts of hemolysin present in these antisera.

SUMMARY

1. Total antibody in hemolysins may be estimated from the nitrogen added to sheep stromata suspensions.

2. The method is applied to a number of hemolysins and a correlation, valid to within 20 per cent, established between hemolytic titer and total antibody.

3. When stromata combine with antibody in the presence of guinea pig complement they may take up at least 80 per cent of their weight of complement combining component(s).

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