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Raymond L. Libby

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A NEW AND RAPID QUANTITATIVE TECHNIC FOR THE DETERMINATION OF THE POTENCY OF TYPES I AND II ANTIPNEUMOCOCCAL SERUM

RAYMOND L. LIBBY

From the Lederle Laboratories, Inc., Pearl River, New York

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It is the purpose of this report to describe in detail a new and simple quantitative method for the determination of the potency of types I and II antipneumococcal serum; a rapid method for estimating the amount of the homologous capsular polysaccharide (SSS), and a method for determining the purity of a preparation of SSS and its suitability for titrating antipneumococcal serum.

All of the quantitative methods at present employed in the study of specific precipitation, or in the determination of the antibody-potency of a serum are laborious, require considerable technical skill for accurate determinations, and are thus subject to mechanical errors.

In any field of research the advancement of knowledge closely parallels the development of new and simplified technics adapted to the study of the phenomena involved. A new instrument, the "Photronreflectometer" (I), has been developed to permit accurate measurement of degrees of turbidity or opalescence produced by the precipitation of antigen-antibody mixtures. Determination of the equivalent combining proportions of the antigen-antibody systems under discussion in the region of slight antibody-excess, combined with a rapid measurement of the degree of turbidity produced in this zone by the reactants by means of the photronreflectometer permits a simple, rapid and accurate determination of the antibody-potency of a sample of serum.

Throughout this report all serum-potencies have been expressed in terms of mouse-protective units. They can, of course,

be as readily expressed in terms of actual milligrams of protective antibody, as has been suggested by Heidelberger and others.

MATERIALS

All measurements were made by means of the photronreflectometer, which in this particular instance, was utilized to determine differences in degree of turbidity produced by the precipitation of serum and SSS. Pneumococcal polysaccharides, types I and II, prepared by ultrafiltration and repeated alcoholic precipitation were employed as antigens and were found to be type-specific. Types I and II univalent and multivalent antipneumococcal horse sera, both unrefined and refined (over 300 samples) were used for potency-tests. All reactions were carried out in 3 cc. refraction-cells, having inside dimensions of 40 x 16 x 5.5 mm.; 0.85 per cent salt solution containing 0.5 per cent ether-phenol, was used as the diluent.

METHODS

1. *Determination of the equivalent combining proportions of serum and SSS in the zone of antibody-excess.* One cubic centimeter of a constant dilution of serum, on which mouse-protective units had been well established, was mixed with 1.0 cc. aliquots of increasing dilutions of the homologous SSS in 3.0 cc. refraction-cells. The resulting turbidities were read, after a five-minute period of incubation at room temperature, by means of the photronreflectometer. If the galvanometer-readings are plotted against the milligram of SSS in the reacting mixture a typical antigen-antibody precipitation-curve is obtained as shown in figure 1. The equivalent combining proportion of serum and SSS was determined at a point just below the point of maximal turbidity as indicated by the maximal galvanometer-reading. This point corresponds approximately to Heidelberger's "equivalence zone" (2), and is indicated by an arrow in figure 1. This point was picked to obviate the possibility of obtaining false readings due to inhibition by excessive antigen.

2. *Establishment of the relationship between varying dilutions of constant proportions of serum and SSS, and galvanometric reading.*

Having determined the combining proportions of antibody in terms of serum-dilutions, and milligram of SSS and galvanometer-reading, it is then necessary to establish the relationship between various dilutions with constant proportions of serum and SSS and galvanometer-readings as shown in table 1. If the dilution of both the serum and the homologous SSS is halved and 1.0 cc. portions of each are mixed and read after five minutes' incubation the galvanometer-reading will be one-half. This relationship

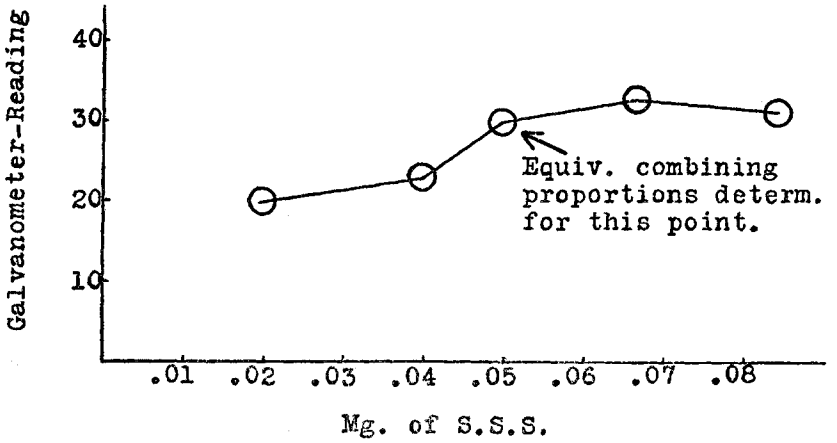

FIG. 1. TYPICAL DATA OBTAINED BY PHOTRONREFLECTOMETRIC TITRATIONS OF CONSTANT SERUM WITH VARYING DILUTIONS OF HOMOLOGOUS S.S.S.

Equivalent combining proportions determined for point indicated (type II); 10 per cent serum-dilution; 150 mouse-units.

holds for a comparatively broad zone of combining proportions of serum and SSS just below the point of maximal turbidity.

That the direct relationship between galvanometric-reading and constant proportions of serum and SSS also holds for small differences in dilution as well as halved dilutions, is demonstrated in table 2 and figure 2. This table and this figure illustrate a typical series of readings obtained by mixing 1.0 cc. quantities of serum and of solutions of SSS at constant proportions, incubating five minutes at room temperature, and reading the degree of turbidity. The actual readings on the galvanometer are compared in table 2 with the calculated figures which were obtained

from the first determination; made with 1.0 cc. of 1 per cent serum plus 1.0 cc. (0.04 mgm.) of SSS which read 41.5, by direct proportion (*i.e.*, per cent serum x 41.5 equals calculated galvanometric-reading). For the data shown the standard error of the calculated readings was ±1.006. All serum and SSS-dilutions

TABLE 1

Relation between galvanometer-reading and halved dilutions of serum and S.S.S. at constant proportions

SERUM NUMBER	SERUM-DILUTION	MILLIGRAM S.S.S. PER CUBIC CENTIMETER	GALVANOMETER-READING
	per cent		
7654	10.0	0.05	30.0
	5.0	0.025	14.9
H55	10.0	0.04	41.5
	5.0	0.02	20.0
1498	2.0	0.04	42.0
	1.0	0.02	21.1
1437	10.0	0.04	29.0
	5.0	0.02	15.0
6236	40.0	0.04	23.8
	20.0	0.02	11.5
7665	40.0	0.04	39.0
	20.0	0.02	20.5
7659	20.0	0.04	22.5
	10.0	0.02	10.5

Mean whole dilutions	32.54
Mean halved dilutions	16.2 = 49.8%

were prepared with non-standardized blow-out pipettes. It will be noted from figure 2, that although the relationship found is not exactly linear, for all practical purposes the approximation to a linear relationship is well within the limits of error of the test.

If we examine the actual readings we find that differences of as little as 5 per cent of serum, or 0.001 mgm. of SSS can be ac-

TABLE 2

Calculation of galvanometer-reading with constant proportions of antigen and antibody (serum H 55)

1.0 cc. of serum*	1.0 cc. of S.S.S.	Reading of galvanometer at 5 minutes	
		Calculated	Actual
per cent	*mgm.*		
1.000	0.04		41.5
0.909	0.0364	37.7	37.0
0.834	0.0333	34.6	34.0
0.770	0.0308	32.0	33.0
0.712	0.0285	29.5	30.5
0.667	0.0267	27.7	29.0
0.625	0.0250	25.9	27.0
0.588	0.0235	24.4	26.0
0.556	0.0222	23.1	24.1
0.526	0.0210	21.8	21.2
0.500	0.0200	20.7	20.0

Standard error ±1.006.

*1.0 per cent serum indicates 1:100 dilution, etc.

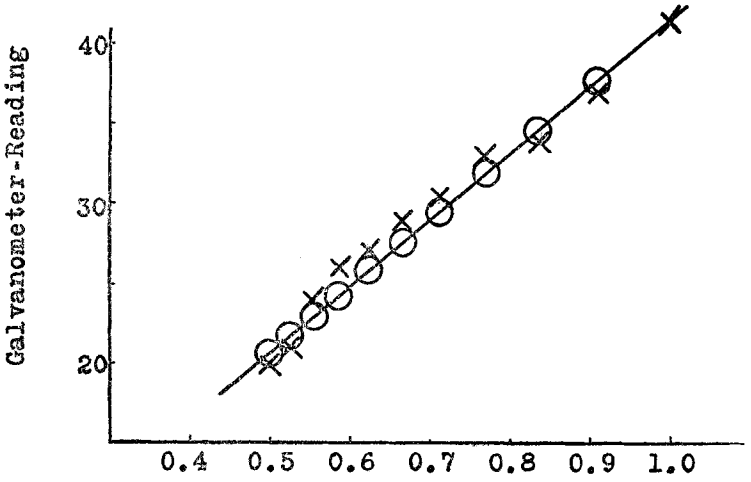

FIG. 2. COMPARISON OF CALCULATED AND ACTUAL GALVANOMETRIC READINGS WITH CONSTANT PROPORTIONS OF SERUM AND S.S.S.
O, calculated; × actual

curately detected by this method. This sensitivity exceeds any of the methods previously in use in studying antigen-antibody reactions.

It should be noted that over a wider range of readings the output of the photoelectric cell, as a measure of the degree of turbidity, is not exactly linear, and for accurate determinations it is necessary to correct for this; however, for a comparatively short range this correction is insignificant and well within the probable error of preparing dilutions.

3. *Calculation of the unit, SSS and galvanometer-reading equivalents.* If we assume that the mouse-protective units of a serum will be directly proportional to the concentration of the serum, *i.e.*, if a serum containing 100 mouse-protective units per cubic centimeter is diluted with an equal volume of diluent, the units per volume will decrease one-half then this fact, in addition to the relationships established in (1) and (2) makes it possible to calculate either mouse-protective units or mg. of SSS or galvanometric-reading by means of the following equation:

$$U = \frac{G}{K_{1/N}} = K_2(S)$$

where U = Mouse-protective units;
G = Galvanometer-reading;
S = Milligram of SSS per cubic centimeter;
$1/N$ = New serum-dilution;
K_1 = Ratio of units to galvanometric-reading x a particular serum-dilution;
K_2 = Ratio of units to milligram of SSS per cubic centimeter.

Thus, by taking samples of serum on which the mouse-protective units have been determined, and establishing the relationships detailed in (1) and (2) it is possible to calculate the various equivalents as shown in table 3. In actual practice the unit-equivalents have been worked out for 1:1.25, 1:2.5, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 dilutions of serum for both types I and II.

4. *Determination of the potency of a sample of serum.* The method employed in determining the potency of a serum consists essentially in titrating known quantities of the homologous SSS with varying dilutions of serum to a galvanometric reading cor-

responding to an equivalent dilution of SSS. An arbitrary dilution of serum is prepared, 1.0 cc. portions of this dilution are mixed with 1.0 cc. portions of dilutions containing 0.02, 0.04, 0.05, 0.0667, and 0.0834 mgm. of the homologous polysaccharide, incubated at room temperature for five minutes, and the degree

TABLE 3

Equivalent units, milligram of S.S.S. and galvanometer-reading

EQUIVALENT DILUTION OF S.S.S.	GALVANOMETER-READING	UNITS* FOR SERUM-DILUTION OF			
		Type I		Type II	
		1:5	1:20	1:5	1:20
0.0167	10.2	48	184	25	101
0.0200	12.1	56	224	30	121
0.0250	15.1	70	280	38	152
0.0333	20.3	92	368	51	202
0.0357	21.7	100	400	54	216
0.0370	22.5	104	416	56	225
0.0385	23.3	108	432	58	233
0.0400	24.2	112	448	60	242
0.0417	25.3	116	464	63	252
0.0435	26.4	120	480	66	263
0.0455	27.7	128	512	69	275
0.0477	29.0	136	544	72	288
0.0500	30.5	141	560	76	304
0.0526	32.0	148	592	79	318
0.0556	33.7	156	624	84	336
0.0588	35.7	165	660	89	355
0.0625	38.0	176	704	94	378
0.0667	40.4	187	736	101	404
0.0715	43.4	200	800	108	432
0.0770	46.7	216	864	117	466
0.0834	50.6	234	928	126	504

* Given in whole numbers only.

of turbidity read in the photronreflectometer. These readings are then compared with the calculated figures for corresponding dilutions of SSS on the table (see table 3). If all of the galvanometer-readings, corresponding to the above SSS dilutions, are less than the calculated figures it is necessary to increase the concentration of the serum and retest as above; if all the galvanom-

eter-readings are greater than the corresponding calculated figures it is necessary to repeat, decreasing the concentration of the serum. However, if the readings, as compared with the calculated readings, cross so that they are higher than the calculated readings on one side and lower on the other, the units for the particular unknown serum will lie between the two dilutions of

TABLE 4

Random sample of photronreflectometric titrations of antipneumococcal sera

(Refer to table 3 for estimation of units)

Type I

S.S.S.	1:5 No. 6452		1:5 No. 7491		1:20 No. 6441		1:5 No. 7959		1:5 No. 7570	
	Galvanometer reading	Estimated units	Galvanometer reading	Estimated units	Galvanometer reading	Estimated units	Galvanometer reading	Estimated units	Galvanometer reading	Estimated units
mgm.										
0.0200	15.0	70	18.0		14.0	380	24.9		15.5	
0.0400	19.0		26.0		21.9		33.0		26.0	120
0.0500	20.8		31.0	150	25.9		35.2		27.0	
0.0667	28.2		36.2		28.0		41.9	200	34.2	
0.0834	30.5		42.0		29.8		46.8		40.0	
0.0333					20.5	380				
0.0357					21.2					

Type II

	1:20 No. 7661		1:5 No. 7722		1:5 No. 7917		1:20 No. 7757		1:5 No. 7668	
0.0200	18.2		22.5		14.5	55	20.0		23.2	
0.0400	24.0	240	28.9	70	22.5		28.0	290	33.9	
0.0500	23.0		28.0		21.5		28.9		37.2	
0.0667	20.0		27.5		16.0		26.0		42.0	110
0.0834	14.0				12.0		24.0		47.2	

SSS where the readings cross. If a more accurate determination of units is desirable, further tests may be run by using dilutions of SSS intercalated between the dilutions determined as above. The actual units for the unknown serum are read opposite the corresponding galvanometric reading in the proper column of the serum-dilutions. (See table 3.)

Table 4, shows a random sample of the type of results obtained by photronreflectometric titrations of over 300 samples of types I and II serum. For example, a 1:20 dilution of serum 6441, type I, when tested with the dilutions of SSS shown, gave an actual reading of 14.0 when tested with 0.02 mgm. of SSS, which is higher than the calculated reading of 12.1 for the corresponding SSS dilution on table 3; and when tested with 0.04 mgm. of SSS gave a reading of 21.9, which is less than the calculated reading of 24.2 for the same SSS dilution (table 3). Therefore, the mouse-protective units of the unknown serum must be somewhere

TABLE 5

Photronreflectometric-titration units vs. mouse-protective units per cubic centimeter

TYPE I			TYPE II		
Sample number	Photronreflectometric units per cubic centimeter	Mouse-protective units per cubic centimeter	Sample number	Photronreflectometric units per cubic centimeter	Mouse-protective units per cubic centimeter
1466	184	200	1466	138	125
1437	440	425	1437	290	275
1468a	360	350	746s	275	300
1468b	240	250	7642	175	175
1418	2,700	2,750	7648	175	135
1475	2,600	2,700	6640	300	275
1422	2,300	2,250	6494	225	225
6450	240	325	1418	1,728	1,750
Mean.....	1,133	1,156	Mean.....	413	408
Standard error ±53			Standard error ±21.6		

between these two readings. Referring to table 3, type I, 1:20 dilution of serum, we find that the units are more than 224 and less than 448. It was estimated that they were about 380. To check this estimate further, the 1:20 dilution was reacted with 0.0333 and 0.0357 mgm. of SSS, as can be seen by comparing the actual readings obtained, as above, the unknown units actually lie between 368 and 400, indicating that the original estimate of 380 units was approximately correct.

5. *Accuracy of photronreflectometric titrations.* Table 5 shows a random sample of a series of tests on serum of known mouse-

protective units and gives a comparison with the units determined by photronreflectometric titration. Very close agreement is obtained in most instances. The standard error of the units determined by means of photronreflectometric titrations for type I was ±53, and for type II ±21.6. These figures represent a deviation of approximately 5 per cent in both instances. As may be seen, some of the individual comparisons may vary as much as 35.5 per cent (sample 6450, type I); however, it is recognized that unit-determinations with the method of mouse-protection show great variations at times. It is probable that in these cases most of this difference may be accounted for by the many variable factors in the mouse-test.

If all of the dilutions of SSS shown in table 3 are utilized in titrating, the maximal possible error is 8 per cent. This represents the maximal difference between consecutive unit-values shown in table 3. However, since one may estimate between the dilutions of the SSS and galvanometric equivalents, actually the error in estimating units is in most instances much less than this figure.

6. *Measurement of SSS.* The actual quantity of homologous SSS in a solution may be estimated with the same degree of accuracy as the potency of a serum. In this case it is necessary to utilize a serum whose potency has been established. The actual photronreflectometric titration of the SSS consists of testing varying dilutions of the unknown SSS with the standardized serum until equivalents of unit and galvanometer-reading are obtained (table 3). Knowing the dilution made from the original sample of SSS one may calculate the milligrams of SSS per cubic centimeter in the original.

All new dilutions of SSS are standardized by the above method before being used to titrate serum-potency.

7. *Determination of purity of the SSS preparation and its suitability for use in photronreflectometric titrations.* If equivalent combining proportions of serum and SSS are determined as in (1) and the consistency of these combining proportions are tested by halving the dilutions as in (2), it will be found that if the sample of SSS contains traces of the "C" substance the galvanometer will not give a reading of one-half as would be expected, but

will be considerable more or less than half. This is due to the fact that each of the constituents of the SSS preparation will have its own particular combining equivalent thus precluding any correlation with the turbidity as measured by the galvanometer. Thus, this method offers a simple means of testing the purity of an SSS preparation and its suitability for use in the photronreflectometric method of titrating serum.

SUMMARY

A simple quantitative technic has been described for the mechanical determination of the potency of antipneumococcal serum, types I and II, in terms of mouse-protective units. It consists essentially in titrating, by means of the photronreflectometer (1), known dilutions of the homologous polysaccharide with varying dilutions of serum to a galvanometric-reading corresponding to a dilution of SSS equivalent to a certain amount of a standard serum (see table 3). Similarly, milligrams per cubic centimeter of polysaccharide in an unknown solution may be determined by testing varying dilutions of the unknown with a serum on which the unit-potency has been established and titrating to equivalent unit and galvanometer-reading (table 3).

A rapid method for testing the purity of a preparation of polysaccharide and its suitability for use in the above photronreflectometric titrations is described. Essentially this method consists of: first, determining the equivalent combining proportions of serum and homologous polysaccharide (see (1)) in the zone of slight excess of antibody and then determining whether or not these combining proportions hold for halved dilutions of the serum and SSS (see (2)). If they do not hold, then the preparation contains other constituents than the homologous SSS and cannot be used in the photronreflectometric titrations described.

The chemical aspects of the data presented in this report will be discussed in a subsequent publication.

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