AGGLUTINATING EFFICIENCY AND COMBINING CAPACITY OF SHIGELLA AND VIBRIO ANTISERA FROM RABBITS AT DIFFERENT STAGES OF IMMUNIZATION*

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Lack of quantitative relation between agglutinin titer and protective or bactericidal properties of antisera has been observed for some time by numerous workers studying immunity to bacterial infections in experimental animals (1–8 and unpublished data from this laboratory). Other workers (9–11) have found a good correlation between agglutinin titer and protective properties of antisera. In most of the studies quoted above, the antibody presumably involved in protective immunity was directed against a surface antigen (*e.g.*, O-antibody) and, despite the lack of quantitative correlation, protection was usually associated with the presence of agglutinins. In some instances however, sera collected a few days after immunization (6) or antisera prepared with heterologous antigen (2) were protective, even though no agglutinating antibody could be demonstrated. From these and other considerations to be discussed below, it appears that the agglutinin titer may misrepresent the concentration of antibody to surface antigens in a given serum qualitatively as well as quantitatively; *i.e.*, some antibodies, though directed against surface antigens, may not participate in agglutination at all, while others may affect the agglutinin titer to varying degrees. Conversely, some antibodies may be more protective than others. A necessary preliminary to the *in vitro* characterization and estimation of protective antibody is therefore the measurement and characterization of the antibody content of a given serum.

As pointed out recently by Talmage and Freter (12), most methods of antibody assay utilize 2-stage reactions, such as agglutination, precipitation, hemolysis, etc., as indicators of the primary antigen-antibody union. However, the intensity of a second stage reaction is not necessarily proportional to the number of antibody mole-

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cules absorbed by the antigen, and many reports may be cited of non-agglutinating (13, 14) and non-precipitating antibodies (15, 16), and of antibodies with different hemolytic efficiency (17). Another frequently used method for estimating total antibody, *i.e.*, the analysis of antibody nitrogen absorbed, involves a considerable error owing to the relatively large amount of antigen nitrogen, especially when the possibility of lysis of the bacteria cannot definitely be excluded. Moreover, it is known that antibodies of different specificities may have different molecular weights. In at least one case (17), antibodies of different efficiency in the second stage reaction (hemolysis) were shown to be of different molecular size, even though their specificities appeared to be identical or closely related. For these reasons, the amount of antibody nitrogen absorbed may not always provide a reliable measure of the relative number of antibody molecules that have combined with the antigen.

Recently, Talmage and Freter (12) developed a test for the study of sheep red blood cell antibodies which appears to be free from most of the shortcomings discussed above. The test measures the absorption of an I^{131} labelled antibody preparation by the antigen. If an unknown unlabelled antiserum is added to standard amounts of these reagents, the unknown antibody will compete with the labelled antibody for the antigenic sites available, resulting in a decreased absorption of radioactivity by the antigen. That amount of unknown serum which, under the conditions of this test, reduced the amount of radioactivity absorbed by 50 per cent was designated by these authors as one "combining unit." The "combining capacity" of the unknown serum was expressed in terms of combining units per milliliter. The reduction or blocking of the uptake of radioactivity appeared to be a function of the number of antigenic sites occupied by antibody from the unknown serum, essentially independent of second stage reactions and independent of the molecular size of the unknown antibody.

In the studies to be reported here, this test, referred to hereafter as "Talmage test," has been adapted for the study of antisera to *Vibrio cholerae* and *Shigella flexneri*, and the results obtained have been compared with the agglutinin titration. The investigations were begun prior to the publication of Talmage and Freter's studies (12), and the present writer is greatly indebted to these authors for their kindness in discussing with him work they had then in progress.

Materials and Methods

Antigens.—Antigens were prepared from a streptomycin-resistant strain of *Vibrio cholerae* and of *Shigella flexneri* type 2a, which have been described previously (18). The data given below refer to *Vibrio cholerae* antigens and antisera, and corresponding data for *Shigella flexneri*, if different, are shown in parenthesis. The organisms were grown for 18 hours at 37°C. on veal infusion agar (Difco) containing 1 mg./ml. streptomycin sulfate, suspended in 0.85 per cent saline, boiled in a round bottom flask over low heat for 2 hours (heated for 2 hours at 100°C. in the flowing steam), and washed 3 times. The suspensions were then adjusted with a Klett-Summerson colorimeter to a standard density, corresponding to about 20 mg./ml. dry

weight of bacterial substance. These preparations will be referred to as standard 100-antigens. Suspensions from similar cultures, heated for 15 minutes at 56°C., washed and standardized to the same density as above, will be designated standard 56-antigens. Antigens were stored at 4°C. without preservative; batches older than 3 weeks were discarded. On the day of use, a suitable dilution of standard antigen was prepared and washed again once with saline. All data given below in any one figure or table were derived from tests employing the same batch of antigen.

Antisera.—Female New Zealand Red rabbits weighing 5 to 7 pounds were immunized by the intravenous route at 4 to 5 day intervals, starting with a dose of 0.25 mg. (0.06 mg.) standard antigen diluted in saline, and doubling the amount of antigen with each successive injection. The 7th (8th) and all following injections consisted of 12 mg. (4 mg.) antigen. Eight to 9 ml. of blood was taken from the ear vein before the 1st, 3rd, 5th, etc., injection, the serum was separated, inactivated at 56°C. for 45 minutes and stored without preservative at −20°C. Normal rabbit serum used for diluting, etc., was inactivated and stored in the same way. Sera are designated by the number of the animal from which they were taken, followed by the number of antigen injections given. If necessary, the kind of antigen is indicated in parenthesis, *e.g.*, serum 9-5/4(56) was taken from rabbit 9-5 after 4 injections of 56-antigen. For reasons of convenience, sera taken early or late in the course of immunization will be referred to hereafter as "early" or "late" antisera.

Standard Agglutinin Titrations.—Agglutinin titers were determined in quadruplicate series of tests, using 2-fold serum dilutions and starting 2 series with a 1:100 dilution, the other 2 with a 1:140 dilution. Equal volumes of standard 100-antigen in 1:100 dilution were added. The titrations were carried out by 2 individuals, each preparing 2 dilution series of a given antiserum. The tests were read after overnight incubation at 37°C. *i.e.*, when the highest titer was obtained. The highest dilution at which agglutination could be detected by inspection with a hand lens was taken as the agglutinin titer. Readings were carried out by 2 individuals, one of whom was unaware of the identity of the sera. The reciprocals of agglutinin titers from all titrations of a given serum were averaged. Individual readings agreed closely and the error of the mean titers was well below 2-fold.

Labelled Antibody Eluate.—The globulin fraction of an antiserum was precipitated with 50 per cent saturated ammonium sulfate, washed, separated by centrifugation, and dialyzed against frequent changes of 0.85 per cent saline at 4°C. for 2 days. Ten mg. globulin in a volume of 0.5 ml. was then iodinated with 10 mc. I^{131} as described by Talmage, Baker, and Akeson (19). Ten to 60 mg. standard 100-antigen[1] was added to the 10 ml. of labelled globulin solution thus obtained, and the mixture allowed to stand at room temperature for 30 minutes. The antigen was then separated by centrifugation and washed 3 times at room temperature with a mixture containing 6 volumes of 0.85 per cent saline, 3 volumes normal rabbit serum, and 1 volume of 0.15 N KI. Depending on the globulin fraction used, the antigen retained 3 to 30 per cent of total radioactivity in the labelled globulin. The washed antigen was suspended in 2 ml. of the antiserum from which the original globulin fraction had been prepared and incubated with frequent agitation at 45°C. for 20 minutes. At this time 10 to 40 per cent of the labelled antibody absorbed by the antigen would elute, presumably owing to carrier exchange and to thermal dissociation. After centrifugation, the supernate was decanted and stored at 4°C. without preservative. This "antibody eluate" could be used for 2 to 6 weeks *i.e.*, until the radioactivity had decayed below the level of exact measurement. On the day of use the

[1] The exact amount of antigen to be added depends on the total antibody content of the labelled globulin fraction. Use of too much antigen resulted in insufficient elution of antibody in the subsequent step. More study is needed to determine the exact influence of the antigen: antibody ratio employed at this step of the procedure on avidity and other qualities of the antibody eluted.

eluate was diluted (usually 1:100) in 0.85 per cent saline containing 10 per cent normal rabbit serum and centrifuged to remove possible traces of bacterial contamination.

The radioactivity of samples was counted in 13 × 100 mm. test tubes in a well-type Texaco Geiger counter or in a well-type scintillation counter.

EXPERIMENTAL

Before using a freshly prepared antibody eluate, its quality was tested by studying the absorption of labelled antibody on standard antigen.

The eluate was diluted so that a 0.5 ml. sample gave several thousand counts per minute. One-half ml. amounts were then distributed into several 13 mm. test tubes, and different amounts of standard antigen in a 1.1 ml. volume added. The mixtures were allowed to stand at room temperature for 30 minutes. After centrifugation, sediments and supernates were separated by decanting, and the radioactivity in both fractions determined. A control tube containing only eluate in a 1.6 ml. volume was included to determine the fraction of supernate remaining in the tube after decanting (usually about 5 per cent). In order to correct for undecanted supernate in tubes containing the sediment, the following formula was used for calculating the percentage of radioactivity absorbed:

$$\text{Per cent} = \frac{p - (cs)}{p + s} \cdot 100 \qquad (1)$$

in which p = counts in sediment, s = counts in supernate, and c = fraction of undecanted supernate in the control tube. Fig. 1 illustrates absorption curves obtained with different eluates. Eluates E and L are typical examples, eluate Ve was derived from a very early antiserum and will be discussed later.

As can be seen, about 80 to 90 per cent of total radioactivity was absorbed from a typical eluate by an excess; *i.e.*, 1 ml. of undiluted standard antigen. Successive absorptions with this amount of antigen usually removed another 10 per cent of total radioactivity, indicating that only about 10 per cent of all I^{131} in the eluate was bound to non-antibody globulin. In the actual Talmage test as carried out here, 0.05 ml. (1 mg.) standard antigen was used throughout because the absorption curves of most eluates were steepest at this antigen concentration, indicating maximal sensitivity to a decrease in available antigen sites (Fig. 1). About 55 to 70 per cent of total radioactivity was absorbed from different eluates under these conditions. In order to obtain accurate assays of radioactivity, the concentration of eluate employed in the test had to be increased as the radioactivity decayed during storage. It was found that most eluates could be concentrated 2- to 8-fold without significantly decreasing the percentage of total radioactivity absorbed by 1 mg. standard antigen.

For testing the combining capacity of unknown sera, the following reagents were mixed in order: 0.5 ml. of a suitably diluted antibody eluate; varying amounts of unknown serum and 0.85 per cent saline to a combined volume of 1.1 ml.; and 1 mg. standard antigen in 0.5 ml. saline (total volume 1.6 ml.). Two control tubes were included with each series of tests, containing (*a*) 0.5 ml. eluate plus 1.1 ml. saline, to determine the fraction of supernate that would

remain after decanting; and (*b*) 0.5 ml. eluate, 0.6 ml. saline plus 1 mg. standard antigen in 0.5 ml. saline, to test the percentage of radioactivity absorbed by the antigen from eluate alone. The reactions reached equilibrium in 1 minute or less, but 30 minutes standing with occasional agitation at room temperature (24-27°C.) was allowed routinely. The tubes were then centrifuged for 10 minutes at about 1200 × gravity in a Servall type SP/X angle centrifuge, the supernates decanted from the sedimented antigen, and the radioactivity of both fractions counted. The percentage of radioactivity absorbed by the antigen was calculated

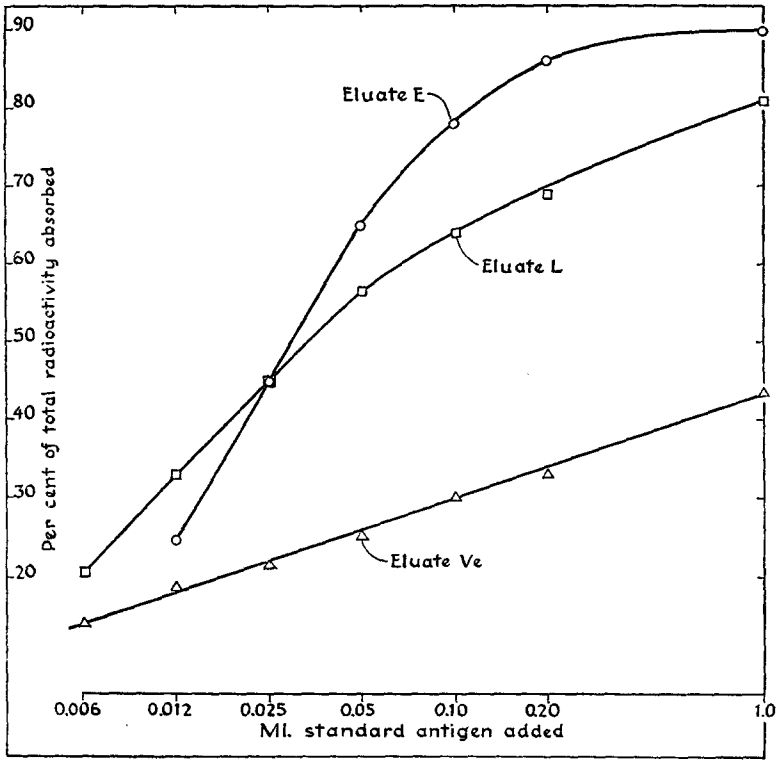

FIG. 1. Absorption by *Vibrio* 100-antigen of labelled antibody from different eluates

according to formula (1) and plotted against the logarithm of the amount of unknown serum (Fig. 2). The amount of unknown serum which reduced the uptake of radioactivity by the antigen to one-half of that absorbed from the eluate alone (control *b*) was taken from this "blocking curve" and designated as one combining unit.

The blocking curves of all antisera tested were s-shaped. However, only the straight part of the curves is shown in Fig. 2 because measurements with the present technique were most accurate in this region. The specificity of the Talmage test was indicated by the fact that labelled antibody from *Vibrio* antisera was not absorbed by *Shigella* antigen, and *vice versa*. Furthermore

normal or *Shigella* antiserum had little or no effect on the absorption of radioactivity from *Vibrio* antibody eluate by *Vibrio* antigen.

Table I gives some representative results obtained with antisera to *Vibrio cholerae*. The eluate used in these tests was obtained from an antiserum which had been prepared like serum 9-0/8(56) or 9-2/8(56), and 100-antigen was used in both Talmage test and agglutinin titration. Sera from rabbits 9-0, 9-2,

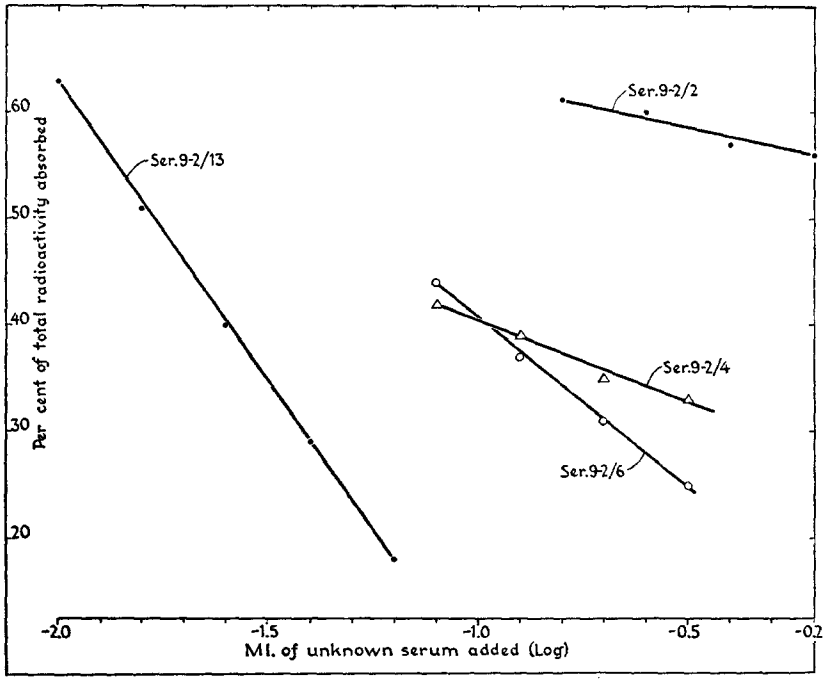

FIG. 2. Blocking curves of different *Vibrio* 56-antisera. Tested with a labelled antibody eluate prepared from 56-antiserum. (The designation 9-2/6 refers to an antiserum taken from rabbit 9-2 after 6 antigen injections).

9-5, and 9-6 were taken successively during the primary course of immunization as described above. Sera 6-6/A to 6-8/A were obtained from rabbits which had undergone a regular course of 8 injections of *Vibrio* 56-antigen, after which the animals were allowed to rest for 2 months. During this time the agglutinin titers of their sera dropped to about 1:800. The rabbits were then injected 4 times with 2, 6, and 12 mg. *Vibrio* 56-antigen at 4-day intervals and were bled from the heart 5 days after the last injection. As can be seen from Table I, high agglutinin titers were obtained in the primary course of immunization after 2 to 4 antigen injections. These early antisera had, however, a very low combining capacity (*i.e.* very few combining units per

ml.), and the ratio $\frac{a}{c} = \frac{\text{Mean reciprocal agglutinin titer}}{\text{Combining units per ml.}}$ was consequently very high. A steadily decreasing a/c ratio was regularly observed during the course of immunization indicating the appearance of antibodies which were able to combine with antigen under the conditions of the Talmage test, but which did not affect the agglutinin titer to any comparable degree. Antibody formed early during the anamnestic response to antigen (sera 6-6/A to 6-8/A)

TABLE I

Combining Capacities and Agglutinin Titers of Different Antisera to Vibrio cholerae

Serum No.		No. of antigen injections given						
		2	4	6	8	10	12	13
9-0(56)	*c**	<1	2.1	4.7	40	16	8.4	3.5
	a‡	3,840	4,400	4,640	9,920	3,280	1,800	1,440
	a/c	>3,840	2,100	987	248	205	214	412
9-2(56)	*c*	<1	3.4	5.6	34	22	22	30
	a	3,280	7,080	5,440	15,080	16,960	9,440	6,560
	a/c	>3,280	2,082	971	444	770	429	219
9-5(100)	*c*	<1	3.0	11	19	42	35	21
	a	5,120	5,440	8,640	9,780	17,280	18,560	6,080
	a/c	>5,120	1,813	785	515	411	530	290
9-6(100)	*c*	≪1	1	6.3	7.5	15	18	9.4
	a	200	2,720	6,560	3,280	3,840	2,320	2,080
	a/c	—	2,720	1,041	438	256	129	221
6-6/A	*c*	79	6-7/A	*c*	32	6-8/A	*c*	45
	a	14,540		*a*	7,200		*a*	18,555
	a/c	184		*a/c*	225		*a/c*	412

* *c* = combining capacity (number of combining units per milliliter).

‡ *a* = mean reciprocal agglutinin titer.

appeared to be of the same type (low a/c ratio) as that found in the late stages of the primary course of immunization. Results similar to those illustrated in Table I were obtained when *Shigella* 56- and 100-antisera were tested with a labelled antibody eluate prepared from 100-antiserum, using 100-antigen in Talmage tests and agglutinin titrations.

One possible criticism of the above results could be that the relatively low agglutinin titers of late antisera, for which the Talmage test showed large amounts of antibody, might have been due to insufficient concentration of some non-specific substance necessary for agglutination to occur. This possibility was ruled out by using a 1:1000 dilution of *Shigella* 100-antiserum

(agglutinin titer 1:12,800) as diluent in the agglutinin titration of *Vibrio* antisera 6-6/A and 9-6/12. The presence of this *Shigella* antiserum which should have supplied the suspected non-specific substance did not increase the agglutinin titer of the cholera antisera.

The following experiments were carried out to test the amount of antigen needed for absorption of antibody from early and late antisera.

Vibrio 100-antisera were diluted in normal rabbit serum to give approximately equal agglutinin titers. Varying amounts of homologous 100-antigen in 1 ml. saline were added to equal volumes of these sera and the mixtures allowed to stand with occasional agitation at room temperature for 30 minutes. The antigen was then separated by centrifugation and the agglutinin titers of the supernates determined by standard agglutinin tests.

TABLE II

Mean Reciprocal Agglutinin Titers of Vibrio 100-Antisera Absorbed with Different Amounts of Homologous Antigen

Serum No.	Standard antigen used for absorption, mg.				
	0	1.0	1.7	2.9	5.0
11-8/4 (early)	384	68	14.0	10.0	14.0
9-5/13 (late)	307	192	128	48	18.0

Table II illustrates representative results obtained with early and late antisera. As can be seen, late antiserum required more antigen for absorption than early antiserum of similar original agglutinin titer. This result might indicate (*a*) that late antiserum combines poorly with antigen, or (*b*) that late antiserum contains relatively more antibody than early antiserum. Since the Talmage test indicated a relatively higher combining capacity for late antisera, the second interpretation appears to be more likely.

In the present studies Talmage tests were conducted at antigen concentration and temperature different from those of standard agglutinin titrations. In order to study agglutination under conditions comparable to those of the Talmage test, the following experiments were carried out.

Antisera were diluted serially in 1 ml. amounts of saline and mixed with 1 mg. standard 100-antigen in 0.5 ml. saline. The tubes were then allowed to stand at room temperature, avoiding agitation as much as possible. At high serum concentrations the end-point was taken as the time at which agglutination could be detected by inspection with the unaided eye. At lower serum concentrations, this end-point was determined by using a hand lens.

Table III illustrates representative results obtained with early and late *Shigella* antisera. Serum 3/4(100), an early serum taken after 4 antigen injections, had an agglutinin titer of 1:12,800 in the standard agglutinin test and a combining capacity of 2.5 units per ml. in the Talmage test, both tests carried out with 100-antigen. The corresponding data for serum 3/13 are

a = 1600 and c = 25 units per ml. Under the experimental conditions used, serum 3/4 had a higher agglutinin titer than serum 3/13 (Table III), just as in the standard agglutinin titration. However, serum 3/13 at higher concentrations agglutinated the antigen much faster than serum 3/4. Similar differences in the speed of agglutination were regularly found with early and late antisera to *Shigella flexneri* and *Vibrio cholerae*. These results indicate that the differences in a/c ratios of early and late antisera (Table I) cannot be explained simply by assuming varying amounts of non-agglutinating "univalent" antibody. Boyd (20) in studies on blood typing found different speeds of agglutination in different antisera and stated that the agglutinin titer "does not tell the whole story." The present results appear to confirm this conclusion.

TABLE III

Speed of Agglutination of Shigella 100-Antigen by Early (3/4) and Late (3/13) 100-Antisera

Serum No.	Reciprocal of serum dilution											
	2	4	8	16	32	64	128	256	512	1024	2048	4096
3/4	(90″)*	(90″)	(4′)	60″‡	60″	2′	4′	4′	20′	47′	180′	§
3/13	(15″)	(30″)	(2′)	45″	60″	2′	4′	4′	30′	§		

* Figures in parenthesis indicate time for agglutination to become visible to the unaided eye.

‡ Figures without parenthesis indicate time for agglutination to become visible on inspection with a hand lens.

§ No agglutination within 24 hours.

DISCUSSION

Talmage, Freter, and Taliaferro (17) in their studies on hemolysins found a steadily decreasing ratio of hemolysin units to combining units during the course of immunization and explained this by the appearance of an antibody of low "hemolytic efficiency." Analogously, one might also suspect the occurrence of antibodies of greater or lesser "agglutinating efficiency." The present finding that late antisera appeared to contain relatively more antibody than early antisera of similar agglutinin titer (Tables I and II) may then be accounted for by assuming higher proportions of antibody with low agglutinating efficiency in late antisera.

The factor or factors responsible for agglutinating efficiency are, at present, still obscure. Agglutinating efficiency may be associated with the specificity of the antibody; *i.e.*, some surface antigens and their corresponding antibodies may contribute little or nothing towards agglutination. The present results would then indicate that the majority of antibodies in late antisera have such a specificity. However, the discrepancies in the speed of agglutination observed with early and late sera (Table III) appear to indicate that antibodies

of seemingly low agglutinating efficiency are extremely active in bringing about agglutination at high serum concentrations, a finding which can hardly be explained on the basis of specificity alone. Consequently, antibodies in early and late sera must differ in still other characteristics, and further studies are planned to investigate the nature of these differences.

It may also be noted that the present results may have some bearing on a widely held immunological concept; namely, that the anamnestic response to particulate antigen is much less pronounced in comparison to that obtained with soluble antigens (21). As can be seen from Table I, sera 6-6/A to 6-8/A had agglutinin titers which, in the average, were only 2.7 times higher than those of sera drawn after an equal number (4) of antigen injections given in the primary course of immunization. In contrast, the average combining capacities of the same groups of sera differed by a factor of 22. The secondary response, as judged by the rise in agglutinin titers, was thus indeed a poor one, not strikingly different from the primary response. However, the rise in combining capacities indicated a much better anamnestic response. The quality of the anamnestic response to particulate antigens may thus, at least in part, depend on the type of antibody, or the antibody characteristics, which one chooses to measure.

In general, the present experimental results indicate that large amounts of antibody may combine with the surface antigens of a bacterial cell without significantly affecting the agglutinin titer. Unlike most of the well known "blocking" or "univalent" antibodies, this type of antibody reached its highest concentration in late antisera. Furthermore, the proportion of antibodies with high and low agglutinating efficiency appeared to vary to such an extent that the agglutinin titer could not be used to estimate the relative concentrations of antibodies to surface antigens in different sera. It is thus not surprising to find discrepancies between agglutinin titer and other properties of antisera, such as the protective power referred to earlier, even in such cases as those in which the antigens involved in protective immunity are presumably located on the bacterial surface. Some degree of correlation may, however, be expected in comparisons of antibodies with similar agglutinating efficiency, as may be found in sera drawn at the same time during the course of immunization. This assumption is supported by the data in Table I which show that the a/c ratios of sera taken from different animals at the same stage of immunization agreed more closely than those of sera taken at different times from the same animal. It may also be noted in this connection that many investigators who found a good correlation between agglutinin titer and protective power were studying antisera drawn after a constant schedule of antigen injections (10, 11), while most workers who found discrepancies analyzed sera of less uniform history. It is thus possible that some of the discrepancies between

in vivo protective properties and *in vitro* behavior of antisera may be attributable to inadequacies of *in vitro* measurements, rather than to the complexities of host-parasite-antibody relationships. Further studies are planned to investigate the factor or factors which may account for the different characteristics of early and late sera, and to study the influence of these factors on the protective properties of early and late sera.

SUMMARY

Studies of the relative combining capacities of different antisera to *Vibrio cholerae* and *Shigella flexneri* were carried out using the Talmage test. In this test the absorption of an I^{131} labelled antibody preparation by antigen is blocked by the addition of unlabelled unknown serum.

Sera from rabbits in the early stages of immunization ("early antisera") had a high agglutinin titer, but low combining capacity. Sera from hyperimmune rabbits ("late antisera") had higher combining capacities than early antisera, but similar or lower agglutinin titers. More antigen was needed to absorb the agglutinins from late antisera than from early ones of the same agglutinin titer, indicating that late antisera contained relatively more antibody. At high concentrations, sera from hyperimmune rabbits agglutinated the homologous antigen more rapidly than did early antisera, even though early sera had similar or higher agglutinin titers. Sera drawn after the anamnestic response to bacterial antigen had the characteristics of late sera; *i.e.*, their combining capacities were high.

The possible significance of these findings for the *in vitro* estimation of protective antibody is discussed.

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