QUANTITATIVE STUDIES OF COMPLEMENT FIXATION[1]

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I. INTRODUCTION

Since the initial description of the complement (C′)-fixation reaction in 1901 (8), progress in this area has advanced along two pathways. Investigations directed towards elucidating the mechanism of C′ action have been based primarily on the hemolytic reaction and the current status of this problem may be found in a recent review by Mayer (69). The other major effort has been channeled towards the design of improved C′ fixation procedures for the detection and estimation of antigens and antibodies as well as for studies of their mode of interaction in highly dilute systems. The accomplishments noted during the past two decades with respect to the latter problem will be the major concern of this article. More specifically, an effort will be made to summarize the contributions of quantitative immunochemistry to the development of methods for studying the loss of hemolytic activity of guinea pig C′ following its interaction with antigen and antibody. Some applications of these methods will be noted as they relate to areas of current immunological interest.

As a result of this limitation, several important areas that might otherwise fall within the confines of a discussion on the fixation of C′ will be omitted. Among these may be mentioned the studies on the weight uptake of complement nitrogen (C′N) by Heidelberger and Mayer (37), Maurer and Talmage (63, 64), and Weigle and Maurer (119–121), the participation of C′ in phenomena associated with natural resistance, recently reviewed by Skarnes and Watson (109), and the accumulated work on properdin, which was summarized in 1956 by Pillemer (88).

II. THE METHOD OF WADSWORTH, MALTANER, AND MALTANER[2]

The first efforts to develop a quantitative approach to the study of C′ fixation can be attributed to the extensive studies of Wadsworth and the Maltaners (59, 114, 115), Maltaner and Gnesh (60), Maltaner and de Almeida (61), Thompson *et al.* (111), Rice (97, 99–102), and Rice and Sickles (98). More recent discussions of these techniques, which embody some minor modifications, have been presented briefly in (4) and at greater length in (94). The major contribution of the innovators of this procedure (114) derives from the design of a technique based on the 50 per cent hemolytic unit of C′ ($C'H_{50}$). A similar approach had been suggested

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[2] It is a pleasure to thank Dr. Otto G. Bier, Escola Paulista de Medicina, São Paulo, Brazil, for his contributions to the evaluation of the method of Wadsworth, Maltaner, and Maltaner.

TABLE 1

Comparison of procedures for determination of serum antibody titers

Serum Dilution Technique	Maltaner Technique
(1) A series of serum dilutions is tested with a single amount of antigen previously shown to yield the highest serum titers for the immune system under study.	(1) Several serum dilutions are used, each with its corresponding optimally reactive level of antigen.
(2) The amount of C′ initially available for the reaction is maintained at a constant level, *e.g.*, 5 $C'H_{50}$.	(2) The amount of C′ is not kept constant but is varied. Each level of the immune complex is reacted with a different amount of C′ so as to obtain partial lysis in at least one tube of the series.
(3) The serum titer is expressed as the highest serum dilution which fixes 4 out of 5 $C'H_{50}$.	(3) The serum titer may be expressed as a ratio of the $C'H_{50}$ for a tube containing serum plus antigen, and that for the serum control.

by Calmette and Massol (11) but later French investigators (6, 48) who used this method failed to apply the von Krogh equation (113) for estimating the hemolytic activity of C′ in the range of partial lysis (51) and were therefore unable to realize the degree of precision inherent in the method.

The Maltaner procedure for the determination of serum antibody titers is based upon the assumption that the amount of C′ required for 50 per cent hemolysis following interaction with an immune complex is directly proportional to the amount of serum under test when the antigen is maintained at optimally reactive levels for each different amount of serum. The method involves several C′ titrations in the presence of serum plus antigen, and controls with serum alone. The ratio of the two values has been considered to be an expression of the serum titer. The essential differences between the Maltaner technique and a conventional serum dilution method such as previously outlined (80) are summarized in table 1.

Several modifications of the original description of this procedure, including the application of spectrophotometric methods, have been offered (3, 4, 115).

In addition, the term "isofixation curves" has been introduced to describe the lines drawn through the several tubes of optimal fixation in the traditional type of two-dimensional bloc titrations such as those used so productively by Dean (16, 17).

Although the application by the Maltaners of the equation derived by von Krogh culminated in a technique superior to those available at the time, this method also embodied some severe limitations. For one, the range of quantitation was still restricted to about 1 $C'H_{50}$ since the reagents were so manipulated as to leave only enough lytic activity after fixation to provide results in the partial range of hemolysis. This restriction in turn created the need to set up a rather cumbersome system of conversion factors based upon different values for the constant $1/n$ in the von Krogh equation, for each immune system and fixation condition under study.

The difficulties resulting from this circumstance may be summarized as follows. The value for the constant $1/n$ describes the slope of the dose-response curve for immune hemolysis and permits estimations of the $C'H_{50}$ unit from experimental points in the 20 to 80 per cent range of lysis. If then, the value for $1/n$ determined under one set of experimental conditions, *e.g.*, the titration of C′, deviates markedly from that obtained for the residual C′ after fixation, the curves for the two titrations will have different slopes, thus rendering uncertain the comparison of $C'H_{50}$ values. The value for $1/n$ has been found on an empirical basis to vary but slightly from 0.2 in many laboratories (66, 69, 73, 116). As one consequence of this almost fortuitous finding, the development of an improved C′ fixation technique must rest in large measure on the precision and reproducible behavior of the hemolytic system, *i.e.*, a constant

value for $1/n$ is required for estimations of the hemolytic unit of C′ under all conditions of the test.

It has been demonstrated repeatedly that exposure of C′ to the action of an immune complex may produce a marked disarrangement in the relative proportions of the four known C′ components (7, 19, 34, 39, 55, 62, 78, 87, 107, 108). When this type of derangement is sufficiently intense, variations from 0.2, the value of $1/n$, may be expected. The variations in $1/n$ values encountered by Wadsworth, the Maltaners, and their associates are due in large measure to the fact that the fixation of 2, 5, 8, or 11 $C'H_{50}$ out of the 3, 6, 9, or 12 units initially added creates a serious alteration in component interrelationships. Plescia *et al.* (89) and Cavallo *et al.* (12) have recently discussed this problem and have suggested that inhibitors also contribute to this complication. The isolation of these inhibitors and the further clarification of their role certainly merit further studies in this regard. However, with the knowledge presently available, it may be noted that fluctuations in $1/n$ values may be induced by the conditions discussed above as well as by variations in other components of the hemolytic system, such as the hemolysin concentration, the number of erythrocytes in the assay procedure, and the concentration of divalent cations, particularly Ca^{++}. With respect to the latter, the proponents of the procedure under discussion seem to have wavering misgivings. Thus, despite the repeated verification of the divalent cation requirements for the fixation of C'_1, C'_4, and C'_2 (13, 44, 46, 47, 49, 50), sodium chloride or borate buffers continue to be used as diluents for all reagents other than the test serum (4, 61). On the one hand, these workers maintain that the failure to incorporate these cations does not influence the numerical value of the serum titer expressed as a ratio. This view has been opposed recently by Rapport and Graf (94), who nevertheless refrain from adding Ca^{++} and Mg^{++} to the buffered diluent. In another instance, the use of mouse-liver powder has been recommended as a supplement to overcome the cation requirements for C′ action (24). The latter suggestion has not received general acceptance (9, 49, 80).

The use of saline or borate diluent in C′ fixation studies renders the fixation and hemolytic systems subject to chance variations in Ca^{++} and Mg^{++} concentrations, which may be expressed as uncontrolled fluctuations in $1/n$ values (61). Further, experience indicates clearly that uncontrolled diminution or enhancement in C′ activity such as observed by Friedewald (22) can be effectively minimized through the use of optimal Ca^{++} and Mg^{++} concentrations (66). Finally, the use of normal human serum as a diluent for the test serum (115) might also be reconsidered in view of the findings with regard to its inhibitory effect in some reactions (84), and its possible influence on the fixation of C′ (12, 89).

As indicated above, the calculation of serum titers by the method of Wadsworth, Maltaner, and Maltaner rests on a supposed linear function between the amount of antibody in the test and the C′ required for 50 per cent lysis. Careful quantitative studies have shown that this relationship is in fact characterized by a sigmoid curve and that the slope of the central linear portion will vary markedly with different sera of similar specificity (105, 117). Figure 1 (taken from reference 117) illustrates these findings, which will be enlarged upon in a subsequent section. Almeida *et al.* (3) have confirmed the sigmoid shape of the plot, C′ fixed *vs.* serum, under experimental conditions similar to those employed by Wallace *et al.* (117) but reaffirm the linear relationship obtained by their method within a limited fixation range of 2 to 14 units. It is pertinent to note that C′-fixation studies by Shulman (105) also yield a sigmoid relationship in the plot of C′ fixed against antibody, when different levels of the latter are tested with optimally reactive amounts of antigen. The sigmoid nature of these dose-response curves may also be deduced from the following speculative considerations.

With small amounts of antibody and an excess of C′, the uptake of C'_1 and C'_4 will be relatively meager so that their loss may be barely demonstable. Therefore, this segment of the reaction curve would tend to show little if any fixation of C′. However, with further increments of immune complex, significant portions of the available C'_1, C'_4, and some C'_2 will be fixed. Threshold limits are exceeded and a relationship is attained where the disappearance of lytic activity is proportional to the amount of immune reactants. The relation may be described by a straight line whose slope will depend upon the combining properties of the antibody, its

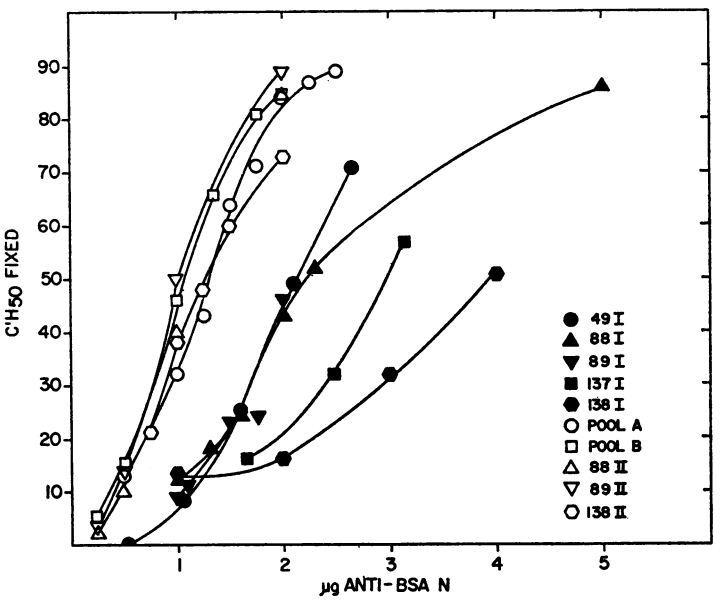

Figure 1. Maximal number of $C'H_{50}$ fixed with different amounts of anti-BSA N and the corresponding quantity of BSA yielding maximal uptake of C′.

relative content of nonprecipitating or coprecipitating antibody, the molecular weight of the antigen, the structure and rate of formation of the aggregate, etc. Indeed, the total amount of antibody may be but one of many factors influencing the slope of this line. As the amount of immune serum and antigen are increased still further, it is conceivable that the utilization of C'_1 and C'_4 becomes less efficient per unit of weight of immune complex, thus contributing to the terminal asymptotic portion of the sigmoid curve. Fortunately, methods such as the kinetic flow analysis devised by Hoffmann *et al.* (40 and *unpublished data*) are now available so that the speculations set forth above can be subjected to experimental scrutiny.

In summary then, it would appear that the method of Wadsworth, Maltaner, and Maltaner provided an important impetus towards the development of a quantitative approach to the study of C′ fixation. As presently constituted and despite the relatively minor changes that have been introduced (3, 4, 60), this procedure has not advantageously applied some of the more recent theoretical findings relative to the mechanism of C′ action. For the practical requirements of a clinical laboratory, the results obtained by this method do not yield more information than can be obtained with the conventional serum dilution titrations performed with the simpler techniques (80). The inherent theoretical limitations discussed above may have unknowingly contributed to the jocular defeatism expressed by Rapport and Graf (94), as typified by the warning, "the soundest advice that one can give to those who would employ C′ fixation (in cancer studies)[3] is to avoid it." Nor can the writer agree with the following thought, "It is thus not possible on the basis of loss of C′ activity *per se* to distinguish a true immunochemical reaction from one that comes about through other, ill-defined, mechanisms that can inactivate one of the C′ components" (93). It will be the aim of the remaining portion of this paper to indicate that the C′ fixation reaction may be uniquely applicable to the study of some immunochemical problems, provided there is an awareness of its utility and limitations.

III. THE QUANTITATIVE C′-FIXATION PROCEDURE

A somewhat different approach to the study of C′ fixation was initiated in Heidelberger's

[3] The parentheses are not in the original statement.

laboratory about 12 years ago (66–68). This procedure evolved from the then recent demonstration of the metal requirements for C′ activity (66) and the use of spectrophotometric techniques for the estimation of the 50 per cent hemolytic unit of C′ (45, 65). The application of these findings to the design of a precisely standardized hemolytic system of uniform reactivity eliminated many of the technical uncertainties inherent in earlier techniques and provided the basis for a C′-fixation assay characterized by a high degree of precision and reproducibility. This method departs from conventional techniques in several respects. In the latter instances, the primary immune reaction occurs in the presence of approximately two 100 per cent units of C′, roughly equivalent to 5 $C'H_{50}$. The extent of fixation is gauged by the addition of sensitized erythrocytes to the reaction mixture. Quantitation of the ensuing lytic process is thus possible only when more than 3.5 of the 5 $C'H_{50}$ have been fixed by the immune system under study. Thus, assay of the C′-fixing properties of an antigen-antibody reaction is confined to a fairly narrow range (*cf.* 94). The conventional methods are further limited in that they fail to detect an immune reaction when less than 3.5 $C'H_{50}$ are fixed, since the remaining, free C′ suffices for complete lysis of the sensitized erythrocytes. In contrast, the quantitative method utilizes a relative excess of C′ (*e.g.*, 50 to 100 $C'H_{50}$) of which only part of the hemolytic activity is fixed. If then, two C′ titrations are performed, before and after the fixation process, the numerical difference between these two activity measurements has been expressed as the number of $C'H_{50}$ fixed by the immune reactants. Table 2 outlines a typical protocol. Further technical details may be found (68, 82, 82a, 117). In any single experiment, the amount of C′ reacted with antigen and antibody is maintained at a constant level sufficient to provide an adequate excess over a wide range of concentrations. The fixation of C′ may therefore be studied as a system of three independent variables. The experimental manipulation of each of these parameters has yielded data which served to clarify several aspects of the C′-fixation reaction and has provided further information pertaining to antigen-antibody interaction in highly dilute systems approximating 0.2 μg of antibody N per ml of reaction mixture. Some applications of this method to the detection, estimation, and characterization of antigens and antibodies will now be briefly reviewed.

During the initial period in the design and application of the quantitative C′-fixation procedure, it was of considerable interest to note that the value of 0.20 for the constant $1/n$ in the von Krogh equation, was maintained uniformly despite wide variations in the level of C′, and concentrations or specificity of the immune reactants. The elucidation of the reaction steps in immune hemolysis by Levine *et al.* (52, 52a, 53, 55), Levine and Mayer (54), and Rapp (91) subsequently provided a clearer understanding of some of the factors which might influence the value of $1/n$. Briefly, these findings, in addition to those discussed above, indicate that the rate and extent of hemolysis by C′ cannot be explained in terms of a single limiting component of C′, as proposed by Hegedüs and Greiner (29). Rather, the final outcome of the hemolytic reaction reflects among other things, the availability and concentration of all four components, the sequence of their interaction, and the stability of the intermediate compounds. The shape of the sigmoid dose-response curve is highly sensitive to the concentration of C'_3. Consequently, when an excess of C′ is used for the primary fixation reaction as in the quantitative method, the residual C′ available for the second titration may still contain a relative, if not greater excess of C'_3 with respect to the other components. In this fashion, marked deviations in the shape of the sigmoid response curve, which might otherwise be due to this factor, are controlled (*cf.* 39, 69). This interpretation places primary emphasis on the loss of C'_1, C'_4, and C'_2 in reaction mixtures containing only soluble immune aggregates and has been extrapolated from findings in more concentrated immune systems and in immune hemolysis. Studies of component fixation in the more dilute instances await further confirmation in rigorous experimentation of the type that has been reported (12, 89). Moreover, since Rapp (91, 92) and Amiraian *et al.* (5) have presented evidence concerning the possible multiple nature of the reaction step hitherto considered to involve only a single component, C'_3, it would appear that further study will be needed to elucidate the component relationships to $1/n$ values in the complex series of events leading to fixation of C′ components on

TABLE 2

Fixation of guinea pig C′ by a constant amount, 2.36 μg, of human Wassermann antibody N (serum P. J.) and varying amounts of human and beef heart cardiolipins

Serum: 1.0 ml of 1 → 20.0 dilution.
C′ added: 50 $C'H_{50}$.
Fixation reaction: 90 min at 37 C in a volume of 10.0 ml.
Lytic reaction: 60 min at 37 C in a volume of 7.5 ml.

	Vol of Diluted Fixation Mixture Used for Hemolytic Estimation with 1.0 ml of Sensitized Erythrocytes*	Hemolysis†	von Krogh Factor for $1/n = 0.20$‡	$C'H_{50}$ Left in Entire Reaction Mixture§	$C'H_{50}$ Fixed¶
	ml	%			
Controls:					
C′ and buffer	2.0	34.6	0.881	48.4	
	2.5	64.9	1.131	49.8	
C′ and serum	2.5	38.6	0.912	40.1	
	3.0	59.7	1.082	39.7	
C′ and human heart cardiolipin‖ (23.4 μg)	2.0	34.4	0.879	48.3	
	2.5	64.2	1.124	49.4	
C′ and beef heart cardiolipin (25.6 μg)	2.0	34.4	0.879	48.3	
	2.5	65.6	1.138	50.0	
Fixation mixtures with 2.36 μg antibody N and:					
Human heart cardiolipin					
(a) 1.46 μg	4.0	33.1	0.869	23.9	21
	5.0	54.0	1.033	22.7	
(b) 8.8 μg	5.0	27.5	0.824	9.9	38
	6.5	57.9	1.066	9.8	
Beef heart cardiolipin					
(c) 1.6 μg	3.0	16.8	0.726	26.6	17
	4.0	42.0	0.937	25.8	
(d) 9.6 μg	5.0	45.7	0.966	11.6	35
	6.5	82.6	1.366	12.6	

* All reaction mixtures except (b) and (d) were diluted 2.0 + 20.0 (1 → 11) for estimation of residual hemolytic activity. Tubes (b) and (d) were diluted 4.0 + 20.0 (1 → 6).

† The erythrocyte suspension was standardized to yield a lysate with an O.D. of 0.680 in a Beckman spectrophotometer at a wave length of 5410 A in a cuvette with a light path of 10 mm (117), and sensitized with rabbit antibody to boiled sheep stromata (90).

‡ See Mayer *et al.* (66) for table of conversion factors.

§ Calculated as follows: factor × (dilution of fixation mixture)/(vol of fixation mixture) × 10.

¶ All values are corrected to 50 $C'H_{50}$ (*i.e.*, × 50 ÷ 39.9); 39.9 = the mean value for the serum control.

‖ Cardiolipin antigens prepared with cholesterol and L-α-(dimyristoyl) lecithin.

the surface of a sensitized erythrocyte or in the three-dimensional immune aggregate.

IV. PARALLELISMS IN C′ FIXATION AND SPECIFIC PRECIPITATION

When the fixation of C′ is studied as a function of increasing antigen concentrations reacting with a constant level of antibody in the presence of a uniform amount of C′, the reaction curve bears a striking resemblance to that for specific precipitation (68, 77–79). In both instances, peak values may be attained with a slight excess of antigen and inhibition of fixation and of precipitation occur as the antibody:antigen ratio diminishes (68, 77, 79, 97–101). These parallelisms have been noted with a variety of protein and polysaccharide antigens reacting with homologous rabbit antibody. They apply equally well in cross-reacting systems that have been studied by these two quantitative procedures, despite

the fact that the fixation reactions are conducted with less than 1 per cent of the specific reactants generally used in precipitin analyses.

An additional similarity in the two reactions emerged from the experiments previously reported (82, 82a). Heidelberger has proposed a mechanism of C′ fixation which depicts the fixation process as one in which molecules of C′ components are enmeshed in the three-dimensional lattice formed by the interaction of multivalent antibody with antigen (32, 36, 38). On the basis of this theory it was considered that release of hemolytically active C′, after fixation by an immune complex, might be produced through the dissociation of the latter. This prediction was borne out in experiments with a polysaccharide immune system. It was found that about 40 per cent of the initial C′ activity was freed from a hemolytically inert antigen-antibody-C′ complex by increasing the electrolyte concentration of the medium to favor dissociation of the immune aggregate. In an analogous fashion, when specific aggregates were dissociated with excess antigen, a release of C'_1 has also been noted (121).

The data obtained in the time course studies of C′ fixation (82, 82a) were of interest in demonstrating an additional similarity between C′ fixation and specific precipitation. Maximal fixation rates for different sera tested under a variety of experimental conditions were compared in terms of "t_{50}," the time in minutes required for the fixation of 50 per cent of the maximum number of $C'H_{50}$ bound in a given experiment. For example, the "t_{50}" values for one serum at ratios of equivalence and antigen excess were 4.9 and 22.5 min, respectively, in a reaction medium containing 40 $C'H_{50}$ and 10 μg of anti-SIII N per 30 ml (figure 2). It was concluded that the inhibition of fixation by excess antigen was associated with a diminished initial rate, thus providing another analogy with specific precipitation. It need only be recalled that in the latter reaction the method of optimal proportions for antibody titration is based upon speed of flocculation. Moreover, in estimations of precipitable antibody N in regions of marked antigen excess, maximal values are attained after longer periods of incubation than are required at equivalence zone ratios.

Another correspondence in the properties of these two immunologic reactions emerges from observations of specific precipitation to the effect that the antibody molecules in an immune serum

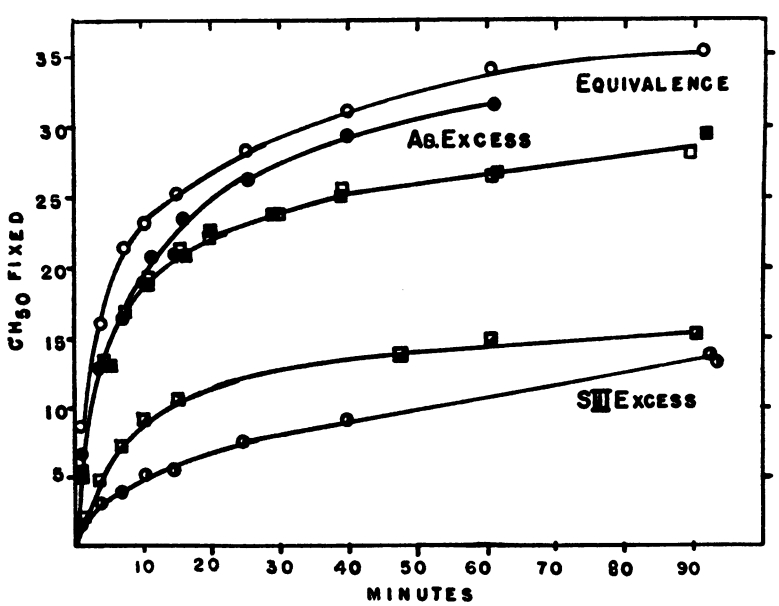

Figure 2. The fixation of C′ by a constant amount of antibody and varying quantities of SIII. *Open symbols*, equivalence zone ratios; *closed symbols*, antibody excess ratios; *semiclosed symbols*, antigen excess ratios; *circles*, 10 μg of anti-SIII N serum B56; *squares*, 20 μg of anti-SIII N serum (pool A). Temperature, 37 C; C′, 40 $C'H_{50}$.

may vary in their combining ratios with the homologous antigen (42, 43, 76). The experiments previously detailed (82, 82a) demonstrate a similar heterogeneity with respect to the fixation of C′.

In the light of these similarities, it was considered that the C′-fixation analyses, like those for specific precipitation, are based upon aggregate formation by antigen and antibody. In the former instance, the extent of the aggregation reaction is estimated by the loss in hemolytic activity of C′ exposed to this process. A logical consequence of this deduction would be that the C′-fixing potency of an immune serum might reflect the aggregating capacity and avidity of the antibody as well as its concentration. The experimental evidence that provided a valid basis for this hypothesis is described briefly in the following section.

V. RELATIONSHIP BETWEEN THE C′-FIXING POTENCY AND ANTIBODY NITROGEN CONTENT

By far the most frequent application of the C′-fixation reaction involves titrations of sera for a relative comparison of their antibody contents. For example, there are numerous studies that pertain to assays of the immunogenicity of vaccines based upon estimates of the C′-fixing potency of the antibody induced by the administration of these antigenic materials. Further, the diagnosis of some infectious diseases often rests solely upon an increase in the C′-fixing capacity of a convalescent stage serum as compared to that of a specimen taken during the acute phase of the illness. While these procedures frequently supply valuable clinical and epidemiological information, the interpretations drawn from the results are nevertheless based upon the underlying assumption that an increased C′-fixation titer reflects a corresponding increment in the amount of serum antibody. This hypothesis had never been subjected to critical experimental analysis. The availability of a highly reproducible quantitative C′-fixation technique afforded an opportunity to test this assumption through comparisons of antibody weight determinations with C′-fixing capacities of various sera.

This question had been studied earlier by Rice (97, 99–101) and Rice and Sickles (98), who found an apparent correlation between C′-fixation titers and antibody N values although marked deviations were recorded for individual sera. However, since precipitin studies had demonstrated that the combining proportions of antigen with antibody vary significantly in different antisera of the same specificity (30, 31, 42, 76) the relationship between antibody weight and C′-fixing potency was reinvestigated (117). For these experiments, bovine serum albumin was reacted with homologous rabbit antibody obtained after varying periods of immunization. The antisera were analyzed by the quantitative precipitin method prior to their assay by C′ fixation. The pertinent findings are summarized in figure 1, where it may be observed that sera taken after two courses of immunization totaling 30 injections (pool A, pool B, 88-II, 89-II, 138-II), manifest a fairly uniform C′-fixing activity on a unit weight basis. Thus for the five antisera, the $C'F_{50}$, *i.e.*, the weight of the antibody required to fix 50 out of 100 $C'H_{50}$ in the cold, varies from 1.00 to 1.32 μg of anti-BSA N. The corresponding 50 per cent intercept values for sera obtained after a single immunization course of 14 injections comprising approximately 44 mg of BSA are more widely dispersed, ranging from 2.11 to 3.96 μg of antibody N. These findings embody several noteworthy features with respect to the application of C′ fixation to antibody estimations. Of outstanding significance is the conclusion recently confirmed by Libretti *et al.* (57) that the C′-fixing titer of an antiserum does not necessarily reflect its antibody content. Thus, for the 10 rabbit anti-BSA sera plotted in figure 1, the $C'F_{50}$ unit varies from 1.00 to 3.96 μg of antibody N. A far greater range of variation may well be anticipated in the analysis of animal or human sera obtained after infection or following customary prophylactic immunization practice.

It was also observed during the course of this investigation that the optimally reactive quantity of antigen cannot be uniquely defined for a given immune system but may vary with individual antisera. In the conventional two-dimensional bloc titration with 5 $C'H_{50}$, it was found that for the second course anti-BSA rabbit sera, peak fixation occurred at an antibody:antigen ratio of 5, necessitating a critical selection of the optimal antigen level in comparative serum titrations. In contrast, maximal fixation with sera from the earlier bleedings was observed

with a wide range of BSA concentrations yielding ratios of antibody N:BSA N varying from 5 to less than 1. This observation indicates the inaccuracies that may result from calculations of antisera potencies based upon optimally reactive quantities of antigen (59).

Observations of this nature have been confirmed in part (94), and impose a further complication in routine analyses for diagnostic purposes carried out with a single "optimal" antigen level. Nor can this difficulty be neglected in investigations concerned with purification of antibodies as characterized by C′-fixation analyses. Finally, it is pertinent to note that this problem is completely averted in the quantitative procedure under discussion, which is generally conducted with a single level of antibody and varying antigen concentrations.

Comparison of the C′-fixing behavior of early and late antibodies yielded a further finding of interest with respect to the mechanism of C′ fixation in these highly dilute systems. It was noted that the reaction curves obtained with constant amounts of second course antisera and increasing quantities of BSA differed substantially from those of the first course antisera. In the former instance, as in the earlier studies, the curves rose sharply as the antigen reached equivalence zone proportions and then were characterized by marked inhibition with moderate amounts of excess antigen. With the earlier antisera, peak values were attained more gradually and in some cases did not diminish even with large increments of antigen. In their failure to show inhibition with excess antigen, the C′-fixation reaction curves obtained with the early bleedings were reminiscent of those obtained in studies of cross-reacting immune systems. Since the BSA studies were performed with an antigen of a high degree of purity, as judged by electrophoretic and ultracentrifugal analyses, an explanation for the C′-fixing behavior of the first course sera was sought in the properties of the antibody.

Several lines of evidence, in agreement with Goldsworthy's early observations (25), led to the belief that the C′-fixing potency of an antiserum might be associated with the aggregating properties of the antibody. This hypothesis was subjected to experimental verification by means of kinetic C′-fixation studies (82, 82a). As often noted, the extent of C′ fixation is governed by the ratio of specific reactants as well as by their absolute quantities (11, 16, 17, 74). In specific precipitation, inhibition by excess antigen has been attributed to the formation of soluble complexes. Since all of the antibody, in a given analysis, is combined with the antigen both at equivalence and at antigen excess ratios, the inference may be drawn that the inhibition of C′ fixation in the latter instance may also result from the smaller unit mass of the soluble complex. The validity of this deduction was demonstrated in the following manner. Many years ago, Goldsworthy showed that a greater amount of hemolytic activity could be removed from fresh guinea pig serum if this reagent was present at the initiation of the antigen-antibody interaction than when it was added to the preformed complex (25). This was confirmed by Goodner and Horsfall (26) and by Heidelberger *et al.* (33) and Heidelberger and Mayer (35) in studies on the weight uptake of C′ N. In the light of these considerations, it was anticipated that the C′-fixing potency of an immune complex formed with excess antigen and presumably deficient in extensive cross linkages would be relatively independent of the time of addition of C′. In contrast, the steric hindrance effect imposed by a preincubation of antigen and antibody at equivalence zone ratios, prior to the addition of C′, would be manifest by a marked decrease in the number of $C'H_{50}$ fixed. This finding was obtained in studies of the type shown in figure 3, where it may be seen that the fixation of C′ by 0.67 μg of anti-SIII N per ml and equivalent amounts of SIII is slower and less efficient when C′ is added 5 hr after initiation of the antigen-antibody reaction. At antigen-excess ratios with the same weight of antibody, the steric hindrance effect could not be elicited.

Further support for this contention emerged from experiments with intact pneumococci. Maximal fixation of C′ by a given quantity of homologous rabbit antibody occurred when C′ was present at the time of antigen and antibody admixture. The diminution in C′ uptake was roughly proportional to the size of the specific agglutinates when these were formed prior to the introduction of the C′. Also, with preincubated soluble immune complexes, the reduction in the number of $C'H_{50}$ fixed, varied directly with the interval of SIII-anti-SIII preincubation and with the amount of antibody incorporated in the

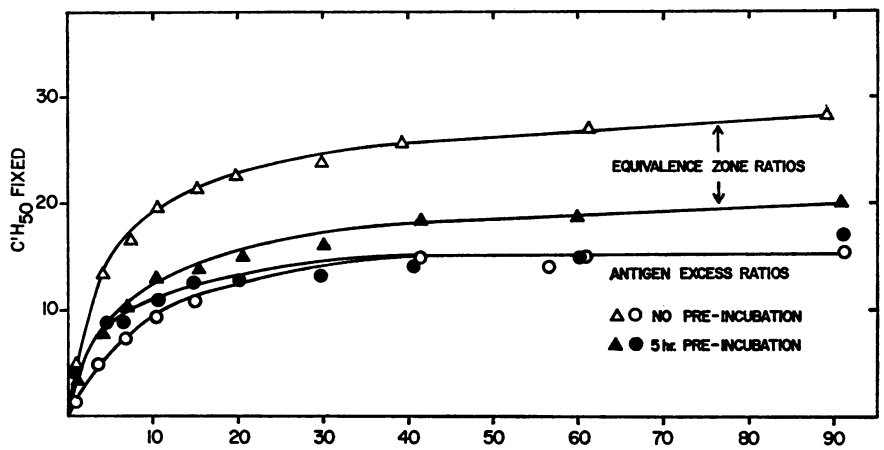

Figure 3. The effect of preincubation of 20 μg of anti-SIII N (pool A) at equivalence and SIII excess ratios. Temperature, 37 C; C′, 40 $C'H_{50}$.

reaction mixtures. Finally, in studies with individual sera, a positive correlation was found between the rate of flocculation of an antiserum, the C′-fixing potency per unit of antibody weight, and the degree of inhibition imposed by a steric hindrance effect created by incubation of the antigen and antibody prior to the addition of C′.

When experiments of the type described above were performed with partially absorbed antisera, it was observed that a marked degree of heterogeneity could be demonstrated in terms of specific C′-fixing potencies of different portions of the antibody in a single serum.

These observations provided a rational explanation for the earlier findings obtained by Wallace *et al.* (117), which pointed to the unreliability of C′-fixation tests as a means of antibody estimation. It would seem then that the difficulty in applying C′-fixation reactions for this purpose stems in part from variations in the aggregating properties of the antibody. Therefore, while precipitin analyses estimate the total antibody weight comprising both the precipitating and coprecipitating varieties, the degree of C′ fixation obtained with a given amount of antiserum is predominantly attributable to the relative concentration of that portion of the antibody characterized by greater affinity for the antigen, as judged by its aggregating capacity. These considerations also help explain the uniform results obtained in the earlier studies, where it was found that different sera fixed essentially similar numbers of $C'H_{50}$ on a unit weight basis (77–79). For these experiments, the sera were obtained after intensive immunization and presumably were relatively devoid of antibody components of inefficient C′-fixing properties. It would be of interest in this connection to evaluate the specific C′-fixing potencies of well-characterized preparations containing only nonprecipitating antibody, or rabbit sera of the type described by Feinberg (21), which fail to precipitate all of the added antigen at antibody excess ratios.

On the basis of these considerations, it seems difficult to provide an unequivocal interpretation to serum antibody titers such as obtained in clinical C′-fixation tests. There is little doubt that the increase in C′-fixation titers observed with sera of convalescent patients may well represent an actual increase in antibody levels. However, the possibility must be entertained that in some cases a rise in titer may simply reflect a change in the C′-fixing quality of the antibody, thus diminishing the diagnostic value of comparisons between acute and convalescent sera which are frequently interpreted only as an index of increased antibody levels and therefore of recent infection.

Shulman (105) has recently published some interesting experiments in which the quantitative C′-fixation procedure was applied to studies on the mechanism of antigen-antibody interaction in thrombocytopenic purpura. This highly complex system involves quinidine as the putative antigen or hapten, platelets as the adsorbent, antibody-containing human serum, and guinea

pig C′. In one sense this reaction resembles the lysis of erythrocytes coated with unrelated antigens in that the attachment of quinidine to the platelets and the reaction of these formed elements with the antibody leads to destruction of the thrombocytes. The fixation of C′ in this reaction has been used as a means of antibody estimations and is unfortunately subject to the same limitations discussed above. The antibody units, as previously defined (105), can have only a relative meaning with respect to other sera and should not be considered in absolute terms.

There are at least two other aspects of this provocative study which may be worthy of comment with respect to the C′-fixation problem. The function of quinidine in the system may resemble that of a monovalent hapten capable of combining with antibody to form soluble complexes in the absence of platelets. These complexes may be deficient or possibly even devoid of C′-fixing properties. It would seem then that the characterization of any serum with respect to the fixation of C′ would require careful studies with different concentrations of this alkaloid to avoid the likelihood of inhibition. The data given by Shulman (see figure 7 of reference 105) on the rate and extent of C′ fixation as a function of quinidine concentration seem to support the notion that a single level of quinidine is not optimal for different dilutions of a single serum. With sera from different patients, this effect may be even more pronounced.

Finally, the data (82, 82a) may be recalled with respect to the heterogeneity of C′-fixing potencies of the antibody molecules in a single serum. In this light the attempt to compare the amount of antibody in a serum, before and after partial absorption, by C′ fixation (see figure 6 in reference 105) yields data which at best may represent but a first approximation to the actual antibody values.

VI. ANTIGEN ESTIMATION AND CHARACTERIZATION BY MEANS OF THE QUANTITATIVE C′-FIXATION PROCEDURE

The curves which describe the course of C′ fixation by a constant level of antibody reacting with increasing amounts of antigen at uniform C′ concentration, are characterized by a steep initial segment which tapers off as equivalence zone ratios are approached. At ratios corresponding to marked antibody excess, C′ uptake is highly responsive to minute changes in antigen, thus providing a sensitive and reproducible means of antigen estimation and characterization. Applications of this nature were demonstrated in reciprocal cross-reaction studies between the capsular polysaccharides of pneumococcus types III and VIII as well as with chicken and duck ovalbumins (77–79). As predicted from knowledge of the chemical composition of the polysaccharide antigens, the results obtained in C′-fixation experiments as in specific precipitation, showed that SIII was a more efficient cross-reacting antigen with anti-SVIII than was SVIII in the reciprocal situation. The diminished cross-reactivity of SVIII as compared to SIII was attributed to the presence of two molecules of hexose for each cellobiuronic acid unit in the polymer, since a polymer of the latter alone accounts fully for the structure of SIII.

With the protein antigens, however, a close similarity was found in the antigenic composition of the two egg albumins, both by the precipitin and C′-fixation studies. The marked immunological relationship between the chicken and duck ovalbumins was also reflected in the observation that the course of specific precipitation between rabbit anti-duck ovalbumin and the homologous or heterologous protein could be described by the same form of the Heidelberger-Kendall equation.

The results of these C′-fixation studies also brought to light considerations which seem pertinent in the interpretation of the numerous studies concerned with the serological relationships between various bacterial, viral, and tissue antigens. Cross-reactivity depends not only upon the specificity and antigenic homogeneity of the antigen on a molecular level but also upon the characteristics of the antibody formed in different species, in different animals of the same species, and at different stages of immunization in the same individual (42, 43). Unless these variables are adequately controlled, misleading conclusions may be drawn with respect to antigenic relationships, particularly from C′-fixation experiments based upon the conventional serum dilution end point titers. Thus with rabbit anti-pneumococcus sera, the extent of cross-fixation with heterologous polysaccharides per unit weight of antibody varies sharply among individual sera and is independent of the homologous antibody content. Further, minor antigenic differences that are

readily demonstrable in the quantitative technique might not be discernible in the customary serum or antigen titration procedures. It is also of interest that with some heterologous immune systems, a much greater excess of the cross-reacting than of the homologous antigen is required to inhibit the fixation of C′. This finding implies that in comparative titrations of a single or pooled immune serum with different but related antigens, highest serum titers are not necessarily obtained with the homologous system, since inhibition in the latter case may be attained with but a slight excess of antigen.

Indeed, antigens of more distant immunological relationship may occasionally yield the higher C′-fixation titers. The frequency of this occurrence may be increased when only a single antigen level is used in the comparative studies. In the light of these considerations, the greater expenditure of time and reagents required for the quantitative studies may be justified in providing information that does not emerge from experiments with the conventional type of C′-fixation test.

An illustrative example in this connection may be drawn from studies with the Wassermann antigen. This investigation was concerned with the immunochemical characterization of phospholipid antigens reactive with the Wassermann antibody in humans. Preliminary studies were conducted with alcoholic extracts of human and beef cardiac tissue in C′-fixation tests with 5 $C'H_{50}$ (81). The accumulated data suggested that the Wassermann antibody in the sera of humans with syphilis or in sera of the "biologic false positive" type (71, 72) might represent a response to a human tissue component. This tentative hypothesis was advanced with the realization that relatively significant antigenic dissimilarities between the human and bovine tissue extracts might not be detected with a titration technique inherently incapable of great precision. The question was therefore submitted to more rigorous experimental analysis with two precise procedures, *i.e.*, quantitative precipitin and C′ fixation, using purified, non-nitrogenous phospholipid antigens isolated from beef hearts, from human hearts and livers, and from wheat germ (84, 85). Data on specific precipitation were obtained from estimations of the Wassermann antibody in absolute weight units by means of the highly sensitive, colorimetric ninhydrin procedure. These analyses showed that virtually identical amounts of antibody N (± 1 μg) were absorbed from sera of human syphilitics with either the bovine- or human-heart cardiolipins. Moreover, the curves describing the course of specific precipitation through the zones of antibody excess and equivalence were almost identical for each antigen. In contrast, the plant phospholipid exhibited the characteristics of a cross-reacting antigen in its inability to precipitate as much antibody as did the mammalian antigens. Nevertheless, the wheat-germ phospholipid had been reported as equivalent in sensitivity to the beef-heart cardiolipin in titration procedures used for the laboratory diagnosis of syphilis ((96, 112) and Osler, Strauss, and Lowenstein, *unpublished data*). These serum-dilution titrations lacked the definition required to distinguish between two antigens that cross reacted to the extent of about 70 per cent.

Quantitative C′-fixation studies with the several phospholipid antigens provided further definitive evidence to the effect that in most cases, the Wassermann antibody in human sera of the biologic false-positive type, or from patients with treponemal infections fails to differentiate between the phospholipid antigens of human or bovine origin. A typical experiment is depicted in figure 4.

Furthermore, the sera of rabbits immunized with the bovine or human cardiolipins reacted equally well with either antigen throughout a wide range of concentrations. The C′-fixation studies also showed that the Wassermann antibody in sera of patients without a clinical history of treponemal infection showed the same specificity relationships as did the antibody formed during syphilis. Since antibody levels are generally higher in the latter group, it was suggested that the Wassermann antibody may have a dual origin in syphilitic infections as a response to an as yet undetected phospholipid antigen in *Treponema pallidum* and to a tissue cardiolipin. In the absence of syphilis, as in patients of the biologic false-positive type, only the latter antigen or a related phospholipid from still another source may stimulate Wassermann antibody production and to lower levels than in the treponematoses.

These studies may also be discussed in relation to those summarized in (94), which deal with antigens from normal and malignant rat tissues, and to an investigation of autoimmune reactions with human tissue extracts (23, 58, 81). All of

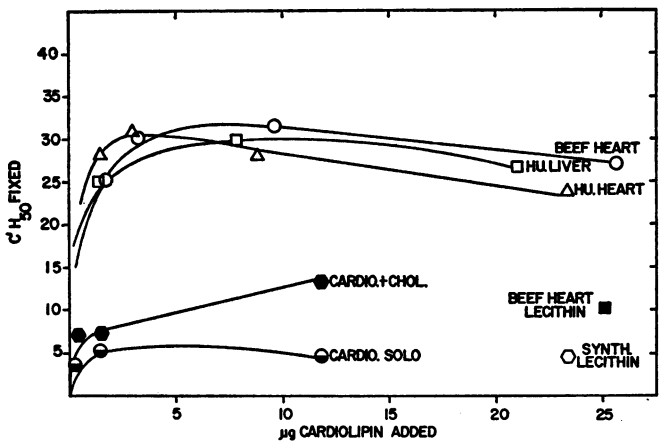

Figure 4. Fixation of complement by human syphilis serum (J. H.) with human liver, human heart and beef heart cardiolipins, plus synthetic lecithin; with human heart cardiolipin alone, and with cholesterol; with beef heart and synthetic lecithin.

these investigations seem to be limited by the use of methods capable of uncovering only rather gross differences between the relatively crude antigen preparations used. In the experiments with tissue antigens based upon mitochondrial preparations, the inability to arrive at unequivocal results may be attributed in part to the method and to the multiplicity of immune systems at play in the C′-fixation experiments. In studies with an antigen specific for malignant tissues (95), the C′-fixing properties of this substance might well be masked under such experimental conditions that were employed (94). Studies by Burrows (10) do in fact point to the possibility that some malignant tissues may be differentiated from their normal sites of tissue origin by immunological procedures. The continued efforts of Yagi and Pressman (122) may be cited as but one of innumerable examples which demonstrate the complex antigenic composition of animal tissues, findings which cannot be lightly dismissed in immunological studies of cancer, no matter what type of C′-fixation procedure is employed (*cf.* page 249 above).

The applicability of the quantitative C′-fixation procedure to the problem of immunochemical homogeneity may also be viewed in the light of the recent findings by Levine *et al.* (56). In an investigation of low density plasma lipoproteins, the C′-fixation studies contributed significantly to the demonstration of the extensive cross reactivity among six different classes of ultracentrifugally separable lipoproteins. As was the case with the studies of the Wassermann antigen, the quantitative C′-fixation experiments also yielded data which were entirely analogous to those obtained by specific precipitation. It was found that the low density human plasma lipoproteins represent a family of macromolecules more readily differentiated by ultracentrifugal than by immunochemical methods (*cf.* 1, 2). Lipoproteins of high density have also been studied by DeLalla *et al.* (18) with particular reference to the action of surface active agents on the C′-fixing properties of those macromolecules.

It may be inferred from these C′-fixation studies that the role of lipids in immunochemical reactions requires further study both with respect to the contribution of this class of compounds as antigenic determinants, and, as was the case with lecithin and cholesterol (85), as enhancers of antigen-antibody reactions (*cf.* 38). In another study, the quantity of heat-labile-α-2 glycoprotein in human sera was estimated by means of C′-fixation analyses with a reference rabbit antiserum to the purified glycoprotein (86). Evidence for the validity of this technique as a means of obtaining reproducible protein assays was presented by these authors with another immune system, human serum albumin and its homologous rabbit antibody. The hemolytic C′-uptake estimations for human serum albumin agreed within 10 per cent of the determinations obtained electrophoretically. As pointed out by Levine, the use of C′-fixation analyses is economical of materials since assays

can be performed with microgram quantities of antibody N and correspondingly small amounts of proteins or other antigens. This factor might well be considered in the choice of an analytical procedure for an antigenic substance, especially when the availability of purified material for the preparation of antisera is limited. Estimates of gamma globulin and other proteins in cerebrospinal fluids by means of quantitative C′ fixation might thus be substituted for the more laborious N analyses of specific precipitates. Thus, a concurrent study of the interaction of C-reactive proteins with rabbit antisera (57) by means of quantitative precipitin and C′-fixation analyses is pertinent to some of these considerations. These authors also observed the lack of correspondence between precipitable antibody N levels and C′-fixing potency. Further, the C′-fixation experiments provided striking confirmation of the results obtained with the much more concentrated precipitating system in demonstrating the identical specificities of C-reactive proteins from pleural fluids in cases of Hodgkins disease and bronchiogenic carcinoma, and from an abdominal exudate drawn from a patient with cirrhosis of the liver. Thus with protein, polysaccharide, or lipid-soluble immune systems, the C′-fixation studies yielded valid results, with an economy of time, labor, and materials when compared to the reference procedure of specific precipitation.

VII. SOME COMMENTS PERTAINING TO THE SENSITIVITY OF C′-FIXATION TESTS

The development of C′-fixation tests for clinical and diagnostic purposes has been punctuated by numerous modifications, most of which were designed to increase the sensitivity of this immunological reaction for the detection of either antigen or antibody. One of the earlier and important improvements emerged from the demonstration by Jacobstahl (41) that more C′ was fixed by a given quantity of immune complex in reactions carried out for 18 to 20 hr at refrigerator temperatures as compared to shorter periods at 37 C. This observation has been confirmed in many laboratories and has found widespread application in clinical laboratory tests.

Another well-known variable of marked influence upon the outcome of a C′-fixation reaction concerns the amount of C′ initially available for the reaction. Alterations in the level of this reagent form the basis for many of the present modifications so that the laboratory worker today is faced with the choice of techniques which utilize as few as 2 (110) or as many as 100 $C'H_{50}$ (68). The selection of an individual procedure must, of course, be guided by the nature of the problem under study. For routine clinical studies, a method utilizing 5 $C'H_{50}$, such as that described in (80), has proved highly satisfactory. However, it should be pointed out that greater sensitivity of the test procedure does not necessarily follow the use of smaller amounts of C′ for the primary fixation reaction. It was previously noted that a given weight of immune complex will bind more C′ when the amount of C′ initially available for the reaction is increased. Under these conditions, a smaller proportion, but a greater number, of the total available C′ units are fixed. Thus, when 1.9 μg of anti-SIII N were reacted with 0.20 μg of SIII at 37 C for 90 min, the number of $C'H_{50}$ fixed increased from 29 to 36 to 53 as the initial level of C′ was varied from 50 to 100 to 200 $C'H_{50}$ (68). The implications of this finding with respect to considerations of sensitivity may be viewed as follows: In the conventional type of test (80, 110, 115) sensitized erythrocytes are added to the entire reaction mixture at the termination of the fixation process. Consequently, as the initial level of C′ is diminished, *e.g.*, from 6 to 5 to 2 units, a greater proportion of the C′ will have been bound by a given amount of immune complex so that the amount left for the hemolytic process may be less than 0.6 $C'H_{50}$, the quantity required for barely visible lysis (*ca.* 5 per cent). Thus, if in a given case, an antigen-antibody reaction fixes only 1.5 $C'H_{50}$, the outcome of tests utilizing 5 or 6 $C'H_{50}$ will be reported as negative while only the technique of Stein and van Ngu, in which only 2 $C'H_{50}$ are added, will probably succeed in detecting the immune reaction.

In this sense, therefore, the Stein and van Ngu procedure would warrant much more extensive study. One serious limitation of this technique might involve sera whose anticomplementary properties are sufficiently high to prevent effectively any estimations of antibody levels. Fixation tests with threshold levels of C′ also provide rather narrow limits for manipulation and quantitation of the immune reactants. However, this method would appear well suited for studies concerned with estimation of antigens when a single large pool of serum is used as reference, as in the

assay of virus preparations during the course of purification or in the characterization of different viral antigens in tissue culture (70, 104). An elaborate micro technique has been described which permits the estimation of polysaccharide antigens at levels approximating 10^{-3} μg in a volume of 12 mm^3 (19a).

In the quantitative procedures, the amount of residual C′ is estimated with only an aliquot of the fixation mixture rather than by the addition of sensitized erythrocytes to the entire contents. Since the reproducibility of this method approximates 5 per cent, it is readily apparent that the fixation of as few as 5 $C'H_{50}$ may be detected in a reaction mixture containing 50 $C'H_{50}$. Moreover, the presence of C′ in excess may enhance the immune reaction by suppressing the dissociation, which may be significant at these low levels of antigen and antibody. This technique can therefore demonstrate antigen-antibody interaction when only a small proportion of the available C′ is bound and may consequently exceed the limits of sensitivity of the former methods. A valuable illustration in this regard concerns the misconception that horse anti-pneumococcal sera do not fix C′ in their reaction with the specific polysaccharides (6a, 26, 106, 123). This question was recently reinvestigated in our laboratory (83). C′-fixation tests were conducted with horse antibody to the pneumococcus capsular antigen at 37 C with 5 and 50 $C'H_{50}$. Although as much as 42 μg of anti-SIII N showed no detectable fixation in the conventional test with 5 $C'H_{50}$, the quantitative method indicated that 25 μg of the same antibody fixed 22 out of 50 $C'H_{50}$. Similar results were obtained with a type VIII horse antiserum and the reaction was shown to be specific for the homologous polysaccharide in every instance. Although this degree of fixation is greatly inferior to that obtained with rabbit antiserum, these results indicate that for these horse antisera the quantitative method is more sensitive than the classical type of test. A further example of the applicability of the quantitative procedure in detecting low levels of fixation may be found in the recent studies of Hare and Warren (27) with poliovirus. Comparative complement-fixation analyses with human sera containing antibodies to antigens isolated from the anthrax bacillus also demonstrated that tests with 50 $C'H_{50}$ were more sensitive than those with 5 $C'H_{50}$ (Norman, Rafter, and Osler, *unpublished data*).

VIII. SOME SPECULATIONS CONCERNING FUTURE DEVELOPMENTS

There is little doubt that the major hindrance to further refinements and understanding of the C′-fixation reaction centers about precise measurement of the elusive components of complement. It is currently acknowledged by all workers in this field that the individual C′ components react in a disproportionate and sequential fashion when they are simultaneously exposed to an antigen-antibody reaction that occurs either on a cell surface or in dilute solutions. Therefore, present C′-fixation techniques are necessarily limited to those conditions where enough of C'_1, C'_4, and C'_2 have been taken up to produce a significant over-all loss in lytic activity of the C′. Further, the mode of interaction of the components, which is characterized by a high degree of mutual interdependence, also indicates that the present methods of studying C′ fixation may not have attained the ultimate degree of sensitivity. Thus, it may be anticipated that tests designed to detect the interaction of C'_1, C'_2, or C'_4 singly or sequentially, with an immune complex may eventually replace present day C′-fixation analyses.

Experiments of this type might be of great utility in studies attempting to correlate C′ levels with the clinical events in certain disease processes. Many of these have been carried out but with essentially equivocal results. This is perhaps not unexpected, since *in vivo* fixation of C′ is likely to take place in the presence of a huge excess of C′, so that changes of only a relatively minor magnitude with respect to the total available C′ cannot be demonstrated. Further, if only slight amounts of C'_1 and C'_4 were fixed, the net effect might not be apparent in titrations of total hemolytic activity.

When these circumstances are considered in the light of the rapid reappearance of C′ activity after depletion (83), the failure of many observers to note substantial diminution of lytic C′ activity in a variety of syndromes with a presumed immunological etiology is more readily understood. As discussed above, the ability to detect the activity losses of a single component might provide a potentially more fruitful approach (*cf.* 28). A recent and pertinent illustration of variations in serum C′ levels in renal disease is provided by the studies of Walton and Ellis (20, 118).

The availability of highly sensitive and valid

methods for component assays might also be utilized in studies dealing with the cellular site and mechanism of antibody formation in tissue culture. Methods using purified component reagents would also permit an extension of experiments designed to establish the enzymatic activities of some C′ components. The possibility could then be envisaged that the availability of component reagents of this type would also provide greater flexibility in the interchange of C′ components from different species. This may result in the application of C′-fixation procedures to the characterization of immune systems involving fowl or equine antibody, or other immune systems to which C′-fixation studies have not been readily applicable. (For a discussion of the specificity and differences of C′ from different species see references 14, 15, 40, 75, and 103.)

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