QUANTITATIVE CHEMICAL STUDIES ON COMPLEMENT OR ALEXIN

I. A METHOD*†

BY MICHAEL HEIDELBERGER, PH.D.

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

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Whether complement or alexin be measured in hemolytic, opsonic, bactericidal, or combining units, these units refer only to the smallest volume of guinea pig or other serum producing the effect in question and give no indication of the actual amount of complement involved. The present study was undertaken in the hope of filling this gap and providing, in place of these relative and often variable units, an absolute measure of complement in weight units. The need for this seemed all the more indicated by the extension of knowledge and the results of theoretical and practical interest that followed substitution of the old relative dilution methods by quantitative, absolute methods of antigen and antibody estimation conforming to the criteria of analytical chemistry (1–3).

It has been known for many years that the titer of added complement or alexin is reduced by antigen-antibody precipitation in rabbit antisera; but the actual addition of a complement component to such precipitates has not been shown directly although hemolysis is generally interpreted as mediated by the addition of complement to the red cell. Indeed, Muir (4) has defined complement as "that labile substance of normal serum which is taken up by the combination of an antigen and its anti-substance (immune-body)." Owing to the accuracy with which specifically precipitable nitrogen may be measured, the quantitative precipitin method (5) seemed worthy of trial as a means of estimating any actual uptake of complement. It was thought that any difference between the amounts of specifically precipitable nitrogen found in the presence of active complement, on the one hand, and in the presence of inactivated complement on the other, might serve as a measure, in milligrams of nitrogen per milliliter,

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of complement as defined by Muir, or of the combining component or components, or "mid-piece," of a more complex complement. Only after considerable experimentation was it realized, however, that specific precipitates should be capable of combining with far more complement, in volume units, than had ordinarily been supposed, and that relatively large volumes of guinea pig serum would be needed if a weight unit for complement were to be established with any degree of accuracy. For this reason many earlier experiments are omitted.

Methods and Materials

Complement.—Sera from a number of large guinea pigs were pooled. The lots for Experiments 1 to 5 were neutralized in the cold to about pH 7 (phenol red) by dropwise addition of N HCl. In other experiments neutralization was either omitted or carried out by means of 0.15 N HCl in 0.9 per cent saline. Differences between the neutralized and the corresponding untreated samples were scarcely greater than the analytical error.

Inactivation by the standard procedure, 30 minutes at 56°C., did not always suffice to reduce to a minimum the amount of nitrogen taken up by specific precipitates from inactivated complement. For inactivation in the later experiments the guinea pig serum was rapidly heated in a water bath at 56° until a thermometer in the serum reached 56°, after which heating was continued for 45 to 50 minutes. The inactivated serum was chilled and, with the corresponding active serum, was allowed to stand in the cold overnight and centrifuged before use.

Sheep Red Cells and Hemolysin.—These were obtained from the Wassermann Laboratory of the Presbyterian Hospital[1] and were the usual 5 per cent sheep cell suspension and hemolysin dilution used for the Wassermann test. An equal volume of hemolysin dilution containing 2 "units" (estimated in the usual way with excess complement) per 0.1 ml. was stirred into the cell suspension. As so diluted the cell count varied from about 830,000 to 1,500,000 per c.mm.,[2] but this did not appear to affect the hemolytic titer found for the active guinea pig serum even though an equal volume of hemolysin dilution was used in each instance.

Hemolytic Titer of Complement.—This was estimated while the precipitin nitrogen analyses were being run by addition of varying quantities of 1:50 or 1:100 guinea pig serum to 0.2 ml. of the sensitized red cell suspension and incubation in a water bath at 37–39°C. for 20 minutes. The number of hemolytic units per milliliter of undiluted serum was calculated from the smallest volume giving complete hemolysis. Analyses are reported only on pools showing more than 150 per ml. of the "units" used.

Estimation of Specifically Precipitable Nitrogen.—Quantitative determinations were made (5) with proportions of antigen and rabbit antisera so chosen that not quite all of the antibody was precipitated. This avoided the formation of disc-like precipitates which are difficult to disintegrate and wash thoroughly. Since the measurement of a presumably small difference between two quantities of precipitate nitrogen was to be

[1] Through the kindness of Miss Edna Baker, in charge.

[2] Counts kindly made by Mrs. Katherine E. Smith of the Hematological Laboratory, Presbyterian Hospital.

attempted, analyses were run in triplicate, unless otherwise indicated, as an additional precaution to ensure the greatest possible accuracy. Moreover, all supernatants were again centrifuged, as in the quantitative agglutination procedure (6), and, on account of the relatively large amounts of guinea pig serum required, all tubes were washed three times with chilled saline instead of twice, as is usual for ordinary analyses (5, 6). Data given and conclusions drawn by Haurowitz (7) as a result of experiments along the same lines indicate that the technical difficulties involved were underestimated by this worker.

The rabbit antisera used were neutralized and inactivated for Experiments 1 to 5 and were of high antibody content, so that analyses could be made at dilutions which were not anticomplementary. In Experiments 1 to 5 1.0 ml. portions of rabbit antiserum dilution were added (each in triplicate) to 5 ml. samples of 0.9 per cent saline, 5.0 ml. portions of heat-inactivated guinea pig serum, and 5.0 ml. samples of an unheated portion of the same guinea pig serum pool. The contents of the tubes were mixed and 1.0 ml. of antigen dilution was added to each tube and mixed. Blank tubes were also set up with active or inactivated complement to which saline, antigen, or antiserum alone was added. After 1 hour at room temperature, or longer if aggregation did not occur within 1 hour in the active complement series, the tubes were centrifuged in the cold[3] and the analyses were completed as described above. In the later experiments neutralization and inactivation of the rabbit antisera were omitted owing to the high dilutions used.

Hemolytic units in the supernatants from the tubes which had contained active complement were computed from the largest non-anticomplementary volume failing to show appreciable hemolysis, and were therefore actually much less than the number indicated, except in Experiments 2 and 5, in which complete hemolysis was actually obtained at the level given. Washings from the precipitin tubes which had contained active complement were also tested and found free from complement, showing that dissociation did not take place during the washing procedure. This was also noted by earlier workers.

EXPERIMENTAL

Experiment 1.—Temperature, 23°C., 45 ml. of neutralized, centrifuged guinea pig serum (C')[4] used, of which one-half was inactivated (iC'). Anti-Pn[4] III rabbit serum 6.06_{2b}, 7.0 mg. antibody N per ml., diluted with 6 volumes saline, neutralized, and inactivated. S III in saline, 0.04 mg. per ml. C' "titer," 250 hemolytic units per ml.

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[3] Using a refrigerated centrifuge manufactured by the International Equipment Company, Boston.

[4] Owing to the frequent use of the symbol C to denote bacterial somatic specific polysaccharides it is proposed, in agreement with Dr. Ecker and Dr. Pillemer, to use the symbol C' for complement and iC' for inactivated complement. So called "mid-piece" would then be C'1 to denote first component, "end-piece" = C'2, and third and fourth components, C'3 and C'4. In the present papers the nitrogen added to specific precipitates is designated C'1 N, as it is generally stated that only mid-piece acts as combining component in such instances. However, this may be an oversimplification (communication from Dr. Ecker and Dr. Pillemer).

Pn is used for pneumococcus, S with the appropriate numeral for type-specific polysaccharide of Pn.

No. of tubes	1	1	2	1	1	3	3	3
C′, *ml.*	4.0	4.0						5.0
iC′, *ml.*				3.0	3.0		5.0	
Serum dilution, *ml.*	0.8		1.0	0.6		1.0	1.0	1.0
S III dilution, *ml.*		0.8			0.6	1.0	1.0	1.0
N precipitated, *mg.*	0.012	0.016	0	0.018	0.020	0.584 0.594 0.586	0.634 0.624 0.636	0.740 0.738 0.736
Mean	0.014			0.019		0.588	0.632	0.738
Subtraction of blank						0	0.032*	0.018†
Specific N pptd., *mg.*						0.588	0.600	0.720
Subtraction of iC′ series value								0.600
C′1 N pptd., *mg.*								0.12

Hemolytic units left in C′ series supernatants, <10 per 5 ml. C′ taken.
* 0.019 × 5/3.
† 0.014 × 5/4.

Experiment 2.—Temperature, 23–25°C. Neutralized, centrifuged guinea pig serum first filtered through gradocol membranes, 700 mμ average pore diameter.[5] 52 ml. filtrate, 250 hemolytic units per ml., 22 ml. C′ inactivated. Same rabbit serum diluted with 9 volumes saline, inactivated, filtered as above, neutralized. S III in saline, 0.028 mg. per ml.

No. of tubes	2	1	1	2	3	3	3	1
C′, *ml.*	5.0*	5.0					5.0	5.0
iC′, *ml.*			5.0			5.0		
Serum dilution, *ml.*	1.0*		1.0	1.0	1.0	1.0	1.0	0.5
S III dilution, *ml.*		0.5			1.0	1.0	1.0	0.5
Saline, *ml.*	1*		1	4	4			
N pptd., *mg.*	0.006	0.012†	0.016	0	0.392 0.388 0.386	0.422 0.416 0.426	0.570 0.574 0.578	0.274
Mean	0.009		0.016		0.389	0.421	0.574	0.274
Subtraction of blank					0	0.016	0.009	0
Specific N pptd., *mg.*					0.389	0.405	0.565	0.274
Subtraction of iC′ series value							0.405	0.203‡
C′ 1 N pptd., *mg.*							0.16	0.07

Hemolytic units left in C′ series supernatants, 40.
" " " " C′, 0.5 S III, 0.5 serum supernatants, 200.

* 0.9 of these quantities actually used in the second blank tube.

† The supernatant from this tube, after recentrifugation, was treated with 0.5 ml. of the rabbit anti-Pn III serum dilution, with the result given in the last column of the table.

‡ One-half of 0.405 (preceding column).

[5] The membranes and filtering apparatus were kindly supplied by Miss Katherine C. Mills of this department.

Experiment 3.—65 ml. of guinea pig serum were neutralized and filtered as in the preceding experiment. The filtrate contained 200 hemolytic units per ml. One-half was inactivated as before. Precipitin reactions were first carried out in both portions with anti-Pn horse serum, which is known not to fix complement (8, 9). Anti-Pn I horse serum 902,[6] containing 3.14 mg. of precipitin N per ml., was used after neutralization. The reactions were run as follows in 50 ml. centrifuge tubes, precipitates were centrifuged after 1 hour at 20°C. and washed 3 times with 10 ml. of chilled saline. All supernatants were recentrifuged. The washed precipitates were dissolved in alkali and rinsed into 20 ml. volumetric flasks. Two 6.0 ml. samples of each solution were analyzed.

C′, *ml.*	30				1.5
iC′, *ml.*		30			
Saline, *ml.*	1	1	30	30	
Anti-Pn I serum, *ml.*	1.0	1.0	1.0	1.0	
S I, 0.5 mg. per ml., *ml.*	1.0	1.0	1.0		0.05
N pptd. from aliquot, *mg.*	0.784 0.790	0.784 0.788	0.746 0.746	0.002	0*
N pptd. from entire sample†, *mg.*	2.62	2.61	2.48		
N pptd. per 5 ml. C′, iC′, saline, resp., *mg.*	0.437	0.435	0.413		

* Qualitative test.

† Less blank.

The C′ and iC′ supernatants recovered from the above reaction were then employed in a precipitin reaction with inactivated, neutralized rabbit anti-egg albumin (Ea) serum 3.87 II which had been diluted with 4 volumes of saline containing 1:10,000 merthiolate.[7]

No. of tubes	1	1	1	1	2	3	3	3
C′, *ml.*	5.5*	5.5*						5.5*
iC′, *ml.*			5.5*	5.5*			5.5*	
Anti-Ea serum dilution, *ml.*	1.0		1.0		1.0	1.0	1.0	1.0
Ea, 0.036 mg. N per ml., *ml.*		0.2		0.2		1.0	1.0	1.0
Saline, *ml.*					6.5	5.5		
N pptd., *mg.*	0.016	0.016	0.018	0.010	0	0.478 0.478 0.480	0.498 0.494 0.502	0.616 0.620 0.622
Mean	0.016		0.014			0.479	0.498	0.619
Subtraction of blank, *mg.*						0	0.014	0.016
Specific N pptd., *mg.*						0.479	0.484	0.603
Subtraction of iC′ series value								0.484
C′1 N pptd., *mg.*								0.12

Hemolytic units per 5 ml. of original C′	1000
" "† after addition of anti-Pn I horse serum	730
" "† " pptn. of S I-anti-S I	670
" "† " " " Ea-anti-Ea	≪75

* Corresponding to 5.0 ml. of original C′ or iC′.

† Calculated to original volume.

[6] Obtained through the courtesy of Dr. R. H. Muckenfuss, Miss A. Walter, and Dr. E. M. Schryver, of the New York City Department of Health.

[7] Manufactured by Eli Lilly & Company, Indianapolis.

Experiment 4.—Experiment 3 was repeated with omission of the filtration and use of only one-half the quantities of antipneumococcus horse serum and S I. The amounts of nitrogen precipitated in the presence of saline, 32 ml. of iC′, and 32 ml. of C′ were 1.136, 1.276, and 1.344 mg., respectively, or 0.210, 0.236, and 0.247 mg. per aliquot portion (6.5 ml. out of a total of 35 ml.) used in the second half of the experiment with Ea-anti-Ea. In this portion of the experiment the mean values for N precipitated in the presence of saline, iC′, and C′ were 0.482, 0.500, and 0.644 mg., respectively, giving 0.144 mg. as the amount of C′1 N found. In Table I there is added to this amount the 0.01 mg. C′1 N apparently brought down by the corresponding aliquot of the S I-anti-Pn I horse specific precipitate. 6.5 ml. of the supernatant from the S-anti S precipitate contained only 1100 hemolytic "units" although the number in the complement originally taken was 1480.[8] This last number is used in Table I in calculating the complement N precipitated per 1000 "units." After precipitation of the Ea-anti-Ea the supernatant contained ≪40 "units."

TABLE I

Complement Nitrogen Added to Specific Precipitates

Experiment No.	Total nitrogen precipitated		Total complement N precipitated (C′1 N)	Complement N precipitated (C′1 N)		
	in presence of active complement	in presence of inactivated complement		per 1000 hemolytic "units" taken up	per 5 ml. active guinea pig serum	per 1 ml. active guinea pig serum
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	0.720	0.600	0.120	0.096	0.120	0.024
2	0.565	0.405	0.160	0.132	0.160	0.032
3	0.603	0.484	0.119	0.119	0.119	0.024
4	0.644	0.500	0.154*	0.104	0.131	0.026
5	0.773	0.611	0.162	0.141	0.162	0.032

* 0.144 + 0.01 apparently added to the initial S I-anti-Pn I horse serum specific precipitate.

Experiment 5.—84 ml. of neutralized guinea pig serum were used, of which 28 ml. were left in the native state (C′), 28 ml. were inactivated in the usual way (iC′), and 28 ml. were warmed to 56°C. with the thermometer in the serum and left at 56° for 5 minutes, the serum being exposed to 50° and over for a total of 10 minutes (i′C′). Both iC′ and i′C′ were incapable of hemolyzing sensitized sheep cells. In order to test the validity of averaging the blanks, as had been done previously, a third blank tube was added in each series containing only the appropriate portion of guinea pig serum and saline. Owing to the increased number of blanks the precipitin determinations were run only in duplicate. After the original set of tubes and the supernatants had been centrifuged the supernatants from each set of antigen and antibody blanks were combined, as indicated in the protocol, giving another set of independent determinations with twice the quantity of C′, iC′, and i′C′. After 1 to 1½ hours these were also centrifuged and the analyses and hemolytic tests completed. The rabbit serum and dilution used were the same as in Experiment 1. The S III solution contained 0.042 mg. of S III per ml.

[8] When titrated as indicated in Table I of the following paper the original complement and the supernatant from the S I precipitate showed little difference.

No. of tubes.	2	1	1	1	1	1	1	1	1	1	2	2	2	2	1	1	1
C', *ml.*		5.0	5.0	5.0										5.0	10*		
iC', *ml.*					5.0	5.0	5.0						5.0			10*	
i'C', *ml.*								5.0	5.0	5.0		5.0					10*
Serum dilution, *ml.*	1.0	1.0			1.0			1.0			1.0	1.0	1.0	1.0	1*	1*	1*
S III, *ml.*			1.0			1.0			1.0		1.0	1.0	1.0	1.0	1*	1*	1*
Saline, *ml.*	1			1.0			1			1	5						
N pptd., *mg.*	0	0.014	0.014	0.014	0.018†	0.022	0.024	0.020	0.020	0.026	0.602 0.596	0.686 0.692	0.626 0.642	0.790 0.784	0.846	0.624	0.740
Mean, *mg.*		0.014			0.023			0.022			0.599	0.689	0.634	0.787			
Subtraction of blank, *mg.*											0	0.022	0.023	0.014			
Specific N pptd., *mg.*											0.599	0.667	0.611	0.773	0.863‡		
Subtraction of iC' N, *mg.*												0.611		0.611	0.611		0.611
C'1 N pptd., *mg.*												0.06		0.16	0.25		0.13

Hemolytic units taken in main series 1180
" " left in C' supernatants 30
" " taken in combined blank supernatants 2360
" " left " " " " 85

* The supernatants from each pair of S III and serum blanks were recentrifuged and then combined and mixed in a third set of tubes. Unavoidable losses would probably increase N pptd. by 3 to 5 per cent.

† Omitted, as Kjeldahl flask bumped during digestion.

‡ Corrected for 0.14 ml. removed for hemolytic test from each blank supernatant before mixing. Deducting the value 0.624 in the next column from this gives 0.24, in excellent agreement, and is perhaps more accurate since double quantities were used.

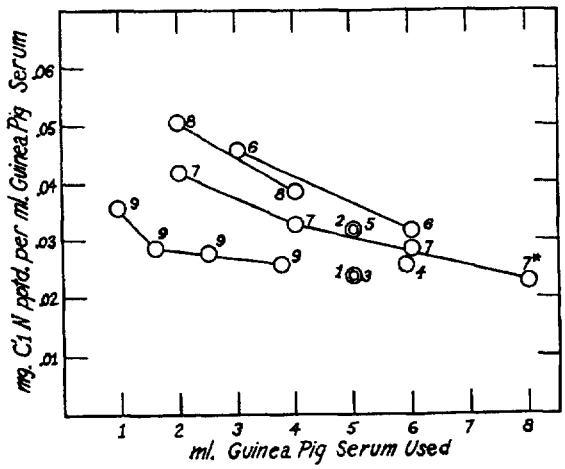
Experiment 6.—The guinea pig serum used was not neutralized. Titer: 400 "units" per ml. Possibly owing to the high activity, inactivation for 35 minutes at 56° destroyed only one-half of the combining power, although hemolytic activity had entirely disappeared. For this reason the iC' N value was calculated by addition of 0.01 mg. of N for each 3 ml. of guinea pig serum used to the amount of N precipitated by S III from anti-S III diluted with saline, this being approximately the increase between the salt and iC' runs calculated from Experiments 1 to 5. With this as a basis it was possible to estimate C'1 N from duplicate precipitations of S III-anti-S III in the presence of 3.0 and 6.0 ml. of the active guinea pig serum. It will be noted from Text-fig. 1 that more C'1 N per ml. was precipitated from 3 ml. of guinea pig serum than from 6 ml. (0.046 and 0.032 mg., respectively), although, judging by the hemolytic activity, less than 40 units of complement remained in the latter supernatant.

3.0 ml. portions of the same pool were also run at 0° for 24 hours, but only 0.037 mg. of C'1 N per ml. were added. Possibly maximal values would have been reached in 48 hours, as in the precipitin reaction (5).

Experiment 7.—Portions of another guinea pig serum pool were tested untreated (active pH 8.07; inactivated, pH 8.33), and neutralized with 0.15 N HCl in 0.9 per cent saline (active, pH 6.75; inactivated, pH 6.78 after addition of a little more acid to replace the CO_2 evolved). Titer during experiment: untreated, 290 "units;" neutralized, 250

"units." In this instance, also, inactivation for 30 minutes after the serum temperature reached 56° was insufficient to destroy all combining component, although inactivation in this respect was nearly complete in the alkaline, untreated portion. iC′ N was calculated as in the preceding experiment. With 4.0 ml. of untreated active guinea pig serum 0.035 mg. of C′1 N per ml. were precipitated, while from 6.0, 4.0, and 2.0 ml. of the neutralized pool, 0.029, 0.033, and 0.042 mg. of C′ N per ml. were precipitated by S III-anti-S III, corrected for the dilution attendant upon neutralization. Thus the same decrease per milliliter with increasing amounts of complement was noted in this instance (see text-figure) although in all cases practically all hemolytic activity was removed by the specific precipitate.

With this pool, also, a 24 hour run at 0° yielded slightly lower results than at room temperature.



TEXT-FIG. 1. Amount of complement combining component nitrogen (C′1 N) precipitated as a function of the volume of guinea pig serum used.

The numbers at each point refer to the experiment. 7* = combined serum and S III blank supernatants.

Experiment 8.—Complement not neutralized, merthiolate,[7] 1:10,000, added before allowing to stand in the cold overnight. Titer: 200 "units." Heated portion fully inactivated with respect to combining component in 50 min. at 56°. Data for 2.0 and 4.0 ml. of complement are plotted in the text-figure. Values in this series were compared undiluted and at a volume of 10 ml., 0.9 per cent saline being the diluent. The additional electrolyte slightly increased the amount of C′1 N found throughout the series, but the differences were scarcely outside the analytical error.

3.0 ml. of the supernatants from the precipitation of S III-anti-S III in 4.0 ml. of undiluted active[9] and inactivated complement were again set up, in duplicate, with

[9] The supernatant from the specific precipitation in active complement was, of course, inactive hemolytically.

appropriate blanks, with 1.0 ml. of anti-S III and S III dilutions. The amounts of nitrogen precipitated were: saline, anti-S III, S III, 0.538 mg.; iC′, anti-S III, S III, 0.552 mg.; C′, anti-S III, S III, 0.546 mg. Only traces of non-specific nitrogen were removed from the C′ and iC′ supernatants, showing the C′1 N to have been completely absorbed by the first precipitation.

Experiment 9.—Complement neutralized as in Experiment 7, merthiolate added. Titer: 170 "units" per ml. Inactivation as in Experiment 8. Data plotted in text-figure.

Fractionated Complement.—Complement was separated by dialysis or by dilution with water and precipitation with CO_2 into the precipitable combining component and so called "end-piece." The former, redissolved in saline, added nitrogen to antigen-antibody precipitates, much as did whole guinea pig serum, while the supernatant or "end-piece" behaved like inactivated complement. This phase of the work, which should be of value in the isolation of complement components, will be dealt with in a separate communication.

DISCUSSION

It was apparent even in the very first of the experiments described above that active complement affected specific precipitation differently from inactivated complement or a corresponding amount of saline. Particulation was greatly delayed in the tubes containing active complement and the precipitate remained more finely divided and settled less readily than in the other tubes. In later experiments, in which relatively larger amounts of saline were used as diluent, this effect was less marked.

The protocols of the runs, the summary in Table I, and the text-figure all show that appreciable quantities of nitrogen may be added to specific precipitates from rabbit antisera when these are formed in the presence of 2 ml. or more of active complement or alexin. Deposition of the same quantity of antigen-antibody nitrogen in the presence of complement fully inactivated by heat results in the separation of little or no more nitrogen than is precipitated when a similar volume of physiological salt solution is used as diluent. Proportions of reactants were so chosen that substantially all of the hemolytic activity of the complement disappeared during the precipitation. If, therefore, all of the nitrogen added by the active complement, as in Experiment 1, were actually derived from this substance, the increase would serve as a measure, in weight units, of complement as defined by Muir (4) or of one or more of its components, chiefly the so called "mid-piece," if complement is defined in terms of its hemolytic activity (10).

It might be objected, however, that in Experiment 1 the increased nitro-

gen or a part of it was due, not to the active complement that disappeared from solution during the precipitation, but to an invisible, non-centrifugable nitrogenous component of guinea pig serum which bore no relation to complement and which dissolved irreversibly when the serum was inactivated by heating at 56°C. Experiment 2 was carried out in order to test this possibility. It will be noted that a relatively large amount of guinea pig serum was filtered through gradocol membranes of 700 mμ average pore diameter with little loss of complement activity. The filtration should, of course, have removed any suspended solid such as the hypothetical substance in question. In spite of this, even more nitrogen was added to a smaller quantity of specific precipitate by the active complement pool used in this instance.

Since excess antibody was employed in the precipitin estimations, it might also be objected that active complement might permit antigen to combine more completely with antibody and so cause an increase in antibody nitrogen to be mistaken for C'1 N. Several types of experiment showing this objection, also, to be invalid, will be reported in another connection.

Experiment 3 was designed to test another possible objection, namely, that the added nitrogen might not be due to complement itself, but to an easily adsorbable soluble substance capable of adding to any precipitate formed in the solution. It was recalled that specific precipitates from antipneumococcus horse sera do not fix complement (8, 9). However, their general similarity to the rabbit precipitates made it appear possible that any easily adsorbable substance would be removed independently of the complement. Then, if the complement remained, and were specifically bound by an antigen-rabbit antibody precipitate formed in the supernatant any added nitrogen would seem more rigorously due to the complement itself. In order to repeat the test for the other interfering substance postulated in Experiment 2 the guinea pig serum pool was first filtered through gradocol membranes of 700 mμ A.P.D. before addition of Type I antipneumococcus horse serum containing antibody equivalent to the amount of rabbit antibody to be used later. It will be noted from the protocol (page 685) that practically identical quantities of nitrogen were precipitated by S I from the active and inactivated complement-anti-Pn I horse serum mixtures, a behavior in sharp contrast to that of the S III-anti-S III rabbit system. That suitably prepared S I absorbs complement with rabbit anti-S I had already been shown (9) and this was confirmed with the preparation used in the present experiments. It will be noted, also, that the nitrogen precipitated from the active and inactivated complement tubes exceeded that

from the saline tube by about 0.02 mg. per 5 ml. of active or inactivated complement, an amount comparable to the difference between the saline and inactive complement controls in the other experiments. This small difference may partly represent lipid nitrogen (*cf.* Horsfall and Goodner (11)) carried down by the specific precipitates from the guinea pig serum, the more so as these precipitates, dissolved in alkali, often formed initially slightly turbid solutions. The difference is also due in part to traces of non-specific nitrogen remaining in spite of the three washings (*cf.* last part of Experiment 8).

The egg albumin-rabbit anti-Ea precipitates formed in the second half of the experiment (page 685) not only dissolved in alkali to form clear solutions, indicating that lipid had been removed by the first precipitate, but also showed a difference between the active and inactivated complement tubes entirely comparable with that in Experiments 1 and 2. It would seem difficult to explain this outcome on any basis other than that the nitrogen difference was due to the active complement taken up. The experiment was repeated (No. 4) and gave a similar result.

It was noted in the experiments with antipneumococcus horse serum that the number of hemolytic "units" was reduced by about one-quarter by mere addition of the horse serum and by about one-third after the precipitation with S I even though the precipitate failed to add a measurable quantity of complement nitrogen. Since the amount of complement nitrogen expected from the original number of "units" was added to the rabbit specific precipitate subsequently formed in the active supernatant it would seem that the anticomplementary action of the horse serum was directed against a property concerned with hemolysis rather than against the combining property of the complement. If this were true the present method for the estimation of complement or its combining component would not only supply measurements in weight units but would also provide an independent means of measurement by which factors controlling the hemolytic properties of complement could be more accurately defined.

While it is hoped to carry out a detailed study along these lines in the near future, Experiment 5 was undertaken as a test of the feasibility of such a study. It was also desired to check the practice, followed up to this point, of averaging the blank values obtained on addition of antigen or antibody separately to the complement samples. It is evident from the protocol that this procedure is justifiable, since the blanks obtained in this way did not differ from those to which saline alone had been added.[10]

[10] This has also been confirmed in a subsequent run.

In this experiment the guinea pig serum pool was divided into three parts, one of which was used in the fully active state, another was inactivated in the usual way at 56° for 30 minutes, while the third portion was heated for 5 minutes at 56° with the thermometer in the serum, the temperature having risen above 50° for a total of 10 minutes. Although the hemolytic activity of this sample of inactivated complement was abolished as was that of the complement inactivated in the usual way, the combining properties had been only partially affected. As noted in the protocol 0.06 mg. more nitrogen was added to a specific precipitate formed in the presence of the non-hemolytic, partially inactivated complement than was added (0.01 mg.) to a precipitate thrown down in the presence of fully inactivated complement. It is therefore evident that nearly 40 per cent of the combining power (or component) of the complement remained available under these conditions although the hemolytic activity had been destroyed.

From the summary in Table I it would appear that the pools of normal guinea pig serum used (each from a different lot of animals) contained in each milliliter from 0.024 to 0.032 mg. of complement nitrogen, or nitrogen due to the combining component (C'1) or so called "mid-piece" of complement. At this stage it was considered that major technical difficulties had been surmounted and attempts were made to reduce the amount of guinea pig serum required for the estimation (Experiments 6 to 9).[11] Although absolute amounts of C'1 N found were less when smaller samples of a pool were used, the quantity of C'1 N per ml. was somewhat larger. This is shown graphically in Text-fig. 1. While the reason for this result remains obscure the effect is that of an enhanced solubility of the specific precipitate in active guinea pig serum. Inactivated complement showed no such effect, the small amounts of nitrogen added to specific precipitates being much more nearly proportional to the volume used (*cf.*, for example, protocol, Experiment 5).

Owing to the apparent solubility effect in active guinea pig serum the absolute amount of combining component nitrogen per milliliter remains somewhat uncertain. However, a rough extrapolation to zero volume of the data in Text-fig. 1 would give 0.04 to 0.06 mg. of C'1 N per ml. as the most probable values. There is much evidence that the combining component of complement is a globulin (4, 10, 12), so that these figures would correspond to 0.25 to 0.4 mg. of combining component protein per ml. If, on the basis of analyses made in this laboratory, guinea pig serum averages 60 to 70

[11] These experiments were carried out with the assistance of Mr. Manfred Mayer. The large volumes of guinea pig serum required throughout the work were furnished through the collaboration of Mr. Check M. Soo Hoo.

mg. of protein per ml., this would correspond to 0.4 to 0.7 per cent of the total protein content. The latter figure has just been given by Ecker and Pillemer (13) as a result of studies along totally different lines leading to the isolation of this component.

The actual mass of complement combining component or components present in this, the most strongly active serum of the common animal species, is accordingly quite small in contrast to the large number of hemolytic "units" ordinarily found because of the extreme delicacy of the hemolytic test. For purposes of rough comparison a column is included in Table I giving the quantity of nitrogen corresponding to 1000 of the hemolytic "units" used in the present study, although it is realized that "units" based, as these are, on total hemolysis, are quite different from "units" derived with a different relative proportion of hemolysin, or based on 50 per cent hemolysis, or on incipient hemolysis, as if often customary.

However variable the hemolytic "unit" may be it is believed that the new method affords a quantitative measure, in weight units, of the combining component(s) of active hemolytic complement. While the combining power has been shown to vary independently of the hemolytic power, the latter is the more labile property. A complete independence of hemolytic and combining properties could scarcely be expected, since the combining component of complement is essential for hemolysis. The method now proposed is sufficiently laborious to prevent its widespread use for routine purposes. It also consumes relatively large volumes of complement, especially if determinations are run with two or three different volumes (for instance, 2.0, 3.0, and 5.0 ml.) in order to make the extrapolation to zero volume giving the actual amount of combining component nitrogen in a guinea pig serum pool. The technique is, however, that of the quantitative precipitin (5) and agglutinin (6) methods which have come into general use for the acquisition of precise data, and it is hoped that the new extension of these methods will also serve as a useful tool for research and for the establishment of reference standards. Several immediate applications will be considered in the second paper.

SUMMARY

1. A quantitative micro method, conforming to the criteria of analytical chemistry, is proposed for the estimation of complement, or its combining component or components, in milligrams per milliliter instead of in the customary relative and variable volume units.

2. Data are given showing the range of accuracy and reproducibility of the new method.

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