

## ASCORBIC ACID AND IMMUNITY

### I. THE RELATION OF ASCORBIC ACID TO HUMAN COMPLEMENT<sup>1, 2, 3</sup>

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The correlation between vitamin C and the activity of complement is a controversial subject. Ecker and his associates (1938a) have reviewed the literature pertaining to this question and have also presented the results of their extensive investigations. They concluded that in the guinea pig the complementary activity of blood serum was related directly to the concentration of ascorbic acid. The relationship was apparent *in vivo* until concentrations of 1 mg per 100 ml of serum were reached. The deficiency in the activity of complement could be corrected *in vitro* by the addition of optimal quantities of ascorbic acid. Ecker and his group (1939) have extended their observations to include two cases of human scurvy. They stated that the serum from their scorbutic individuals contained "weakened complement" and that the complement could be reactivated by the addition of ascorbic acid. Ecker and his colleagues are of the opinion that the activity of guinea pig and human complement is regulated by a process of oxidation and reduction. When there is a deficiency of vitamin C, complement is present in the serum in an oxidized state. The *in vitro* or *in vivo* addition of ascorbic acid reactivates the latent activity of complement because of the reducing properties of the vitamin. Chu and Chow (1938) have reported that a qualitative relationship exists between the vitamin C intake and the complement titer in human plasma.

The conclusions of the foregoing workers are apparently in disagreement with those of others. Ardy (1939) and Maccolini (1939) were unable to establish a correlation between the activity of guinea pig complement and the status of their vitamin C nutrition. Cope and Kapnick (1940) observed that in the thyroidectomized and hypophysectomized rabbit the titer of complement in the blood serum decreased significantly, whereas in the unoperated rabbit injected with thyroxin the titer of complement increased. Kapnick and Cope (1940) then studied the relationship of cevitamic acid to complement in rabbits with altered thyroid activity. They found that in the thyroidectomized rabbit the concentration of serum complement fell although the level of cevitamic acid remained constant. In the rabbit made thyrotoxic, the titer of complement rose while there was almost a complete disappearance of cevitamic acid from the blood. They ascribed the differences between their findings and those of Ecker in the guinea pig to species variations. In one of the most unusual and well-controlled in-

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vestigations of experimental human scurvy, Crandon, Lund and Dill (1940) observed no fall in the titer of complement, even when signs of frank scurvy appeared.

While carrying out a group of studies pertaining to the effect of ascorbic acid on antibacterial immunity, we had occasion to repeat the studies of Ecker and his group in human beings and we were unable to confirm their results. In view of this, a larger number of observations were made and the results form the basis of this report.

#### METHOD OF STUDY

The individuals selected for study were all adult patients on the various services at the University Hospitals. None of the subjects had demonstrable infections. Venous blood was collected aseptically, allowed to clot at room-temperature, centrifuged and the serum withdrawn. To avoid the presence of traces of heavy metals, which may act as catalysts in the oxidation of ascorbic acid, all of the glassware used in the various procedures was thoroughly cleaned with distilled water, which was prepared in an all-glass still. Since hemoglobin products inhibit the action of complement and decrease the serum vitamin C, all sera containing visible amounts of hemoglobin were discarded.

The macroscopic, colorimetric method of Mindlin and Butler (1938) was used throughout in titrating the serum ascorbic acid, the readings being made with the aid of the Evelyn photoelectric colorimeter.

Two methods were used in determining the complement levels in the sera. In the standard method used in our laboratory, sheep cells always obtained from one source, were washed four times with sterile, physiological solution of sodium chloride.<sup>4</sup> The washed cells were freshly sensitized before doing each set of determinations by mixing 0.5 ml of a 2 per cent suspension with 0.5 ml of hemolysin (2 units) and allowing the mixture to stand at room-temperature for fifteen minutes. The same lot of rabbit antisheep hemolysin was used having a titer of 1 to 5,000 and containing 50 per cent glycerin as a preservative.<sup>4</sup> Ten tubes were used in the titration. To the first five were added 0.5 to 0.1 ml of a 1 to 10 dilution of serum, thus giving amounts of serum varying from 0.001 to 0.05 ml. One cubic milliliter of sensitized sheep cells was then added to each of the tubes. The final volume in each tube was made up to 3 ml by the addition of physiological saline solution. The contents of the tubes were thoroughly shaken and incubated in a water-bath at 37 C for one hour, and centrifuged. The lowest concentration of serum which showed a trace of hemolysis was used as the measure of complement activity. At least two observers, and more often three, recorded their readings independently.

Because the foregoing method of titrating complement was at variance with that described by Ecker and his colleagues (1938a), a group of observations were made in which simultaneous determinations of serum complement were done with the method just described and the following procedure of Ecker. Instead

<sup>4</sup> Prepared and supplied through the courtesy of the Minnesota Department of Health, Division of Preventable Diseases, Minneapolis.

of using physiological sodium chloride solution as a diluent, Ecker and his group employed Brooks' physiologically balanced solution (Brooks, 1920). Serum was diluted 1 to 10 and amounts from 0.15 to 0.01 ml were added to each of fifteen serological tubes. Five-tenths ml of a 5 per cent suspension of sheep cells was added to 0.5 ml of hemolysin. The sensitized cells were then added to the serum and Brooks' solution was added to make the final volume 1.15 ml in each tube. This final volume was almost one-half that used in our standard method.

In the first group of 24 subjects, the serum ascorbic acid and complement were titrated before and after the intravenous injection of crystalline ascorbic acid.<sup>5</sup> After a sample of venous blood was obtained, the needle was left in place in the vein, the syringe was removed, and a solution of crystalline ascorbic acid in 10 ml of physiological sodium chloride was injected with a second syringe. Within 5 to 10 minutes, the second sample of venous blood was collected.

A second group of 4 subjects were studied in the same manner as above, except that simultaneous titrations of complement were made by the two different methods as described.

Since Ecker and his associates (1938b) were able to activate complement in sera having a low titer of reduced ascorbic acid, an attempt was made to repeat this observation. One-tenth milliliter of a freshly prepared solution of crystalline ascorbic acid in physiological saline solution was added to 3.9 ml of serum so that the final concentration was between 1 and 2.5 mg of ascorbic acid per 100 ml of serum. The hydrogen-ion concentration of the serum was not altered by the addition of these small amounts of ascorbic acid as measured by the glass electrode method. Simultaneous ascorbic acid and complement titrations were made upon the sera with and without added ascorbic acid. The activity of complement was determined by the two described methods.

In the next series of observations, attempts were made in various ways to inactivate *in vitro* either ascorbic acid or complement without affecting the other. Since traces of copper will quickly oxidize ascorbic acid,  $\text{CuCl}_2$  in physiological sodium chloride solution was used. Several analyses revealed that 0.1 ml of the  $\text{CuCl}_2$  solution (0.22 mg) when added to 5 ml of fresh human serum would result in the oxidation of ascorbic acid within a short time. The sera of seven subjects were used and each was divided into three portions. The first served as a control; the second portion contained the added  $\text{CuCl}_2$ ; while the third had  $\text{CuCl}_2$  and an excess of added ascorbic acid. Simultaneous titrations of complement and ascorbic acid were then carried out on the three portions of each of the sera. The bactericidal activity of complement was also evaluated for normal serum and serum containing added  $\text{CuCl}_2$ . This was carried out by adding 0.1 ml of ten-fold dilutions of an *E. coli* broth culture to each of several tubes having 0.5 ml of serum. The tubes were incubated for 24 hours and then the contents plated out to determine the presence or absence of viable organisms.

The next step was to inactivate complement without oxidizing the ascorbic

<sup>5</sup> Crystalline ascorbic acid ("Cebione") supplied through the courtesy of Merck and Company.

acid, which was accomplished in two ways. The first method involved the heating of serum in a water-bath for 30 minutes at 56 C. Ascorbic acid and complement titrations were carried out upon unheated serum, and portions of the remaining serum heated for 5, 15 and 30 minutes. The bactericidal activity of unheated and heated serum was also determined using a culture of *E. coli*. The second method depended upon the phenomenon that the hemolytic activity of serum is completely lost when the pH is 4.3 or below (Osborn, 1937). This change in hydrogen-ion concentration was produced by adding 0.27 ml of 0.4 N HCl to each ml of serum, and allowing the mixture to stand for 30 minutes at

TABLE 1  
*Titrations of ascorbic acid and complement before and after intravenous ascorbic acid*

PATIENT	AGE AND SEX	SERUM ASCORBIC ACID BEFORE	COMPLEMENT BEFORE	ASCORBIC ACID INTRAVENOUSLY	SERUM ASCORBIC ACID AFTER	COMPLEMENT AFTER
		mg	ml	mg	mg	ml
1	57M	0.06	0.0073	1,000	3.47	0.00365
1A		0.0	0.01	1,000	3.112	0.01
2	19F	0.137	0.008	1,000	9.06	0.01
2A		1.099	0.02			
3	39F	1.18	0.01	1,000	7.09	0.01
4	38F	0.69	0.02	1,000	9.8	0.02
5	31F	0.035	0.04	1,000	6.94	0.04
6	38F	0.0176	0.03	1,000	6.72	0.03
7	60F	0.06	0.01	1,000	7.15	0.01
8	59F	0.00	0.02	1,000	5.2	0.02
9	66F	0.1	0.03	1,000	9.76	0.03
10	66F	0.0	0.01	500	2.1	0.01
11	21F	0.13	0.03	500	2.85	0.03
12	54F	0.08	0.006	500	5.6	0.006
13	37F	0.14	0.01	500	2.3	0.01
14	28F	0.03	0.006	500	4.36	0.006
15	37F	1.31	0.006	250	3.2	0.006
16	43F	0.19	0.006	250	2.74	0.006
17	39F	0.06	0.008	200	1.64	0.008
18	32F	0.20	0.004	200	1.76	0.004
19	65F	0.10	0.008	200	1.3	0.008
20	31F	0.03	0.02	200	2.0	0.02
21	70M	0.0	0.002	200	0.72	0.002
21A		0.10	0.002	200	1.6	0.002
22	60F	0.03	0.002	200	2.0	0.003
23	26F	0.1	0.006	200	1.9	0.010
24	18F	0.20	0.004	200	2.2	0.004

room-temperature. An equal quantity of 0.4 N NaOH solution was added to bring the hydrogen-ion concentration back to normal because it was observed that the acidified serum induced hemolysis of the erythrocytes without the addition of hemolysin.

#### RESULTS

The results of the simultaneous titrations of serum ascorbic acid and complement before and after the intravenous injection of ascorbic acid in 24 subjects are presented in table 1. The most outstanding feature is the failure to demon-

strate any significant change in the activity of complement even though there was a tremendous increase in the serum vitamin C level. In the first 9 subjects, 1000 mg of ascorbic acid were administered, which resulted in levels of serum ascorbic acid that exceed the values given for normal individuals. Recognizing the possibility that the activity of complement may be related to optimal levels of ascorbic acid, 6 subjects received 500 mg intravenously; 2 received 250 mg; and 8 had 200 mg injected. The final level of serum ascorbic acid did not appear to influence the titer of complement. Patient 1 received ascorbic acid on two occasions, each several days apart. Following the first injection of 1000 mg the titer of complement was elevated. But when the same dose was repeated no change in complement titer was observed. A similar observation is recorded for patient 21 who received a second injection of 200 mg of ascorbic acid in 24 hours. Patient 2 received an initial dose of 1000 mg of ascorbic acid, and in this instance, the level of complement was less than it had been before she received the ascorbic acid. Following daily intravenous injections of 750 mg of ascorbic

TABLE 2

Comparison of titers of serum complement as determined by method of authors (saline as diluent) and that of Ecker and associates (Brooks' solution as diluent)

PATIENT	AGE AND SEX	ASCORBIC ACID INTRAVENOUSLY mg	SERUM ASCORBIC ACID		COMPLEMENT			
			Before	After	Before		After	
			mg per 100 ml	mg per 100 ml	Saline ml	Brooks ml	Saline ml	Brooks ml
1	31F	200	0.03	2.0	0.02	0.010	0.02	0.010
2	70M	200	0.1	1.60	0.002	0.004	0.002	0.005
3	69F	200	0.03	2.0	0.002	0.002	0.002	0.002
5	26F	200	0.1	1.9	0.006	0.0036	0.01	0.0036

acid for 7 days, a normal value for serum ascorbic acid was obtained. There was no significant change in the titer of complement.

Since the method for titrating complement in the foregoing studies differed from that of Ecker and his associates, this variable was checked in a series of 4 observations using both methods. The results of these data are shown in table 2. Again, no significant differences in the amount of complement were detected. Attention is called to the higher values for complement with the method of Ecker, for which no satisfactory explanation is apparent.

The next step was to determine if complement could be reactivated by the *in vitro* addition of ascorbic acid to deficient serum, using the two methods for determining the titer of complement. The results of these observations using five different sera are summarized in table 3. The increase in the serum ascorbic acid to a normal range did not change the complement titer.

In the attempts to inactivate either ascorbic acid or complement *in vitro* without affecting the other, small amounts of copper were used which acted as a catalyst in the oxidation of ascorbic acid. In this connection, Ecker's group (1938b) observed that copper completely inactivated complement and the com-

plement could not be reactivated by the addition of ascorbic acid. In a series of observations with 7 different sera, we succeeded in completely oxidizing the ascorbic acid in 6 of the sera as determined by the colorimetric method without effecting any significant change in the hemolytic or bactericidal activity of complement. The addition of excessive amounts of ascorbic acid to samples of the same sera containing copper did not result in a change in the hemolytic activity of complement. The results of these studies are shown in table 4.

TABLE 3

*Ascorbic acid added in vitro to human serum and its effect upon complement (cells suspended in saline and Brooks' solution)*

OBSERVATION	NORMAL SERUM			NORMAL SERUM + ASCORBIC ACID		
	Ascorbic acid	Complement		Ascorbic acid	Complement	
		Saline	Brooks		Saline	Brooks
	mg	ml	ml	mg	ml	ml
1	0	0.002	0.004	1.80	0.002	0.008
2	0.1	0.002	0.004	2.30	0.002	0.004
3	0.03	0.002	0.002	1.40	0.002	0.002
4	0.01	0.006	0.012	1.3	0.006	0.012
5	0.10	0.006	0.0036	1.65	0.008	0.0042

TABLE 4

*The in vitro effect of copper upon ascorbic acid and complement in human serum*

OBSERVATION	NORMAL SERUM		NORMAL SERUM + CuCl <sub>2</sub>		SERUM + CuCl <sub>2</sub> + ASCORBIC ACID	
	Ascorbic acid	Complement	Ascorbic acid	Complement	Ascorbic acid	Complement
1	0.69	0.02	0.00	0.02	9.6	0.02
2	1.18	0.01	0.068	0.01	8.98	0.02
3	0.0176	0.03	0.00	0.03	7.86	0.03
4	0.035	0.04	0.00	0.03	7.32	0.03
5	0.06	0.01	0.00	0.008	8.96	0.008
6	0.13	0.03	0.00	0.03	10.24	0.03
7	0.08	0.006	0.00	0.006	18.4	0.006

The complement of two sera was inactivated by heating them in a water bath at 56 C for varying periods of time. The results are shown in table 5. Although there was some diminution in the concentration of ascorbic acid, a complete absence of the hemolytic and bactericidal activity of complement was demonstrated.

The complement of two sera was inactivated by lowering the hydrogen-ion concentration to pH 4.3 for 30 minutes and then the normal pH of the serum was restored. As summarized in table 6, there was some diminution of the ascorbic acid but the hemolytic activity of complement was absent.

TABLE 5

*Human serum heated at 56 C and its effect upon complement and ascorbic acid*

SERUM	ASCORBIC ACID	COMPLEMENT
	mg	ml
Normal.....	1.79	0.008
Heated 5 min.....	1.66	0.1
Heated 15 min.....	1.30	0
Heated 30 min.....	0.94	0

*Human serum heated at 56 C and its effect upon ascorbic acid, and the hemolytic and bactericidal activity of complement*

SERUM	ASCORBIC ACID	COMPLEMENT	BACTERICIDAL POWER (E. COLI)						
			10 <sup>1</sup>	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>
	mg	ml							
Normal.....	0.197	0.02	+	+	+	0	0	0	0
Heated 10 min.....	0.180	0	+	+	+	+	+	+	+
Heated 15 min.....	0.180	0	+	+	+	+	+	+	+

+ = growth.

TABLE 6

*Effect of changing pH of serum to 4.3 upon ascorbic acid and complement*

OBSERVATION	NORMAL SERUM		SERUM pH 4.3	
	Ascorbic acid	Complement	Ascorbic acid	Complement
	mg	ml	mg	ml
1	0.679	0.01	0.306	No hemolysis in 0.08 ml
2	0.247	0.008	0.026	No hemolysis in 0.2 ml

DISCUSSION

The foregoing data would indicate that it is highly doubtful if the activity of human complement is dependent upon the presence of reduced ascorbic acid. The complex nature of complement and the biological activity of ascorbic acid present many unknown factors which do not permit a final conclusion concerning the relationship of ascorbic acid to complement function. The observations that we have cited are in direct contrast to those of Ecker and his associates and are in agreement with the observations of Crandon, Lund and Dill (1940). We are unable to explain the discrepancy in the studies that have been reported and those that we are recording. We have also observed a similar discrepancy between our observations and those of Ecker and his group when guinea pig serum was investigated (Agnew, Spink and Mickelsen 1942). Ecker and his group have carried out an extensive series of observations with guinea pig serum. We have also extended our studies to include guinea pigs. These data will form the basis of another report. It can be stated that we failed to observe in this species of animal a direct relationship between the activity of complement and the presence or absence of ascorbic acid in the serum.

Ecker, Pillemer, Mortensen and Wertheimer (1938b) have presented the results of an interesting study on the effects of chemical agents on complement activity. Such agents as  $I_2$ ,  $H_2O_2$ , quinone,  $Cu_2O$ ,  $C_6H_6$ ,  $HgCl$  and  $O_2$  inactivated complement when sera were exposed to them. This complement could be reactivated in some instances by the addition of reductants such as  $H_2S$ ,  $KCN$  and ascorbic acid. We have attempted to repeat these observations but our results were inconsistent.

## SUMMARY

1. Contrary to the reports of Ecker and his workers, we have found that the intravenous injection of crystalline ascorbic acid into adults with a low serum vitamin C level produced no change in the titer of complement.

2. The *in vitro* addition of reduced ascorbic acid to deficient serum failed to result in an increase in complement activity.

3. Serum ascorbic acid was oxidized without affecting the titer of complement. The activity of complement was inhibited or abolished with but slight change in the concentration of ascorbic acid.

4. On the basis of the present study, it is doubtful that the *in vitro* activity of human complement is related to the presence of reduced ascorbic acid. The conclusions of Ecker and his associates that "the complementing activity of the serum of scorbutic guinea pigs and of men may be used as a biological index of the vitamin C deficiency" are open to considerable question.

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