

lating species referred to as *Aerobacter levanicum* (III) has been studied. All three produced levan from sucrose and raffinose. In I, levansucrase is formed adaptively; in III it is constitutive. In I, growing on sucrose agar, levansucrase is exocellular and diffuses through the medium; in II and III on the same medium, it is endocellular.

3. Levansucrase has been prepared cell-free from III as an aqueous solution, as well as in soluble dry form. The cell-free enzyme systems act on sucrose

with rapid formation of levan and of reducing sugar. Certain physical properties of cell-free levansucrase from I and III have been studied.

4. Levan has been prepared in substantial amount as a nitrogen-free powder by the action of sterile levansucrase from III on sucrose. Some properties of the enzymically formed polysaccharide are described.

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### Complement Activity and Vitamin C

By E. KODICEK, *Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*,  
AND B. TRAUB, *Department of Pathology, University of Cambridge*

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In recent years evidence has accumulated that deficiency of ascorbic acid impairs the defensive reaction of the organism towards infection. There have been many attempts to correlate this anti-infective effect more specifically with factors participating in immunological reactions [cf. Perla & Marmorstan, 1941]. The greatest attention perhaps has been paid to the possible correlation between the complement activity of the serum and the vitamin C intake.

Although Zilva [1919, 1936] found no significant change of complement titre in scorbutic guinea-pigs, Simola & Brunius [1933] and Marsh [1936] claimed a lowering of the complement activity with a low intake of vitamin C. Chakraborty [1937] could not confirm the finding that vitamin C deficiency affected the complement titre in guinea-pigs.

More recently, Ecker, Pillemer, Martiensen, Wer-

theimer & Grandis [1938a], Ecker, Pillemer & Wertheimer [1938b], Ecker, Pillemer, Griffiths & Schwartz [1939] and Ecker & Pillemer [1940] claim to have established a direct correlation between the complement activity of the serum and its concentration of vitamin C. They based their conclusions on average results without any statistical evaluation of the significance of the means. On the other hand, Maccolini [1939] and Agnew, Spink & Mickelsen [1942] found no correlation between the complement titres of guinea-pigs and their intake of ascorbic acid. In view of these conflicting findings, we have reinvestigated the question.

The method of titrating the complement was based on the determination of 50% haemolysis, and the results were statistically evaluated. It may be said in advance that no significant changes were found in the complement titres of guinea-pigs at different levels of vitamin C intake.

## EXPERIMENTAL

*Titration of complement.* The end-point of the titration ('complement titre') was taken as the volume of serum, expressed in units of which one unit is equal to 0.0001 ml. of undiluted serum, needed to produce 50% haemolysis in a standardized system described below. This arbitrary end-point, being on the steepest slope of the S-shaped titration curve, increases the accuracy of the titration [Brooks, 1920; Wadsworth, Maltaner & Maltaner, 1931]. The value so obtained expresses in reciprocal form the complementary activity of serum. Sheep cells derived from one source were used throughout the experiment and were standardized according to the procedure of Herbert [1941].

Blood samples measuring 2-3 ml. were obtained by cardiac puncture without anaesthesia. The serum obtained by clotting was diluted with 0.9% buffered saline of pH 7.4 in a ratio 1:30, and eight tubes were set up containing different amounts of undiluted serum, ranging from 0.025 to 0.002 ml. The volume of all the tubes was adjusted to 0.75 ml. by the addition of buffered saline and 0.5 ml. of a suspension of sensitized sheep red cells was added. It was prepared 15-20 min. before use by mixing equal volumes of rabbit anti-sheep haemolysin and of washed sheep red cells containing 50 mg. haemoglobin/ml. The tubes were incubated for 30 min. in a water-bath at 37°, cooled to 2-4°, and 2 ml. of buffered saline were added to facilitate

the subsequent reading. After the mixture had been centrifuged, the percentage of haemoglobin present in the supernatant fluid was estimated colorimetrically as described by Herbert [1941]. The results were plotted on squared paper, the haemolysis-serum volume curve fitted and the value for 50% haemolysis read by interpolation. The titrations were usually done in duplicate.

*Animals.* Two series of experiments were performed at different seasons of the year (June-July; Oct.-Dec.). Twenty-two guinea-pigs weighing 300-368 g. were used in the summer experiment. The second series, in winter, consisted of eighteen animals averaging 509 g.

*Basal diet.* The basal diet contained oatmeal, bran, dried yeast, egg yolk, salt mixture and radiostoleum, with water *ad lib.* The solution of pure crystalline vitamin C was prepared daily and pipetted into the mouths of the animals.

*Supplements of vitamin C.* As will be seen from Table 1, the eleven animals in group 1 (summer) were maintained without ascorbic acid; animals in group 2 (controls) were given a daily dose of 5 mg. of ascorbic acid plus 15 g. of cabbage.

In the winter experiment (Table 2) the diets of the guinea-pigs in group 3 (five animals), group 4 (seven animals), and group 5 (six animals) were supplemented with 0.5, 1.0 and 10.0 mg. of ascorbic acid, respectively. After 4 weeks the dose of ascorbic acid was interchanged in groups 3 and 5. The animals of group 3 then received 10 mg. and those of group 5 0.5 mg. daily.

Table 1. Complement titres and weights of guinea-pigs on diets deficient in vitamin C and supplemented with 5 mg. ascorbic acid plus cabbage

Group	No.	Ascorbic acid added mg./day	Complement titres (one unit = 0.0001 ml. of undiluted serum)			Wt. (g.)			
			Week			Week			
			2nd	3rd	4th	0	2nd	3rd	4th
Group 1	1	0	64	60	—	306	348	261	219*
	2	0	81	62	—	327	380	272	237*
	3	0	50	53	—	335	370	373	205*
	4	0	66	46	—	308	334	257	Died
	5	0	66	64	—	312	344	244	188*
	6	0	62	64	—	309	342	257	Died
	7	0	50	72	—	306	338	216	Died
	8	0	53	50	—	368	386	325	224*
	9	0	60	72	—	340	364	258	202*
	10	0	50	80	—	304	324	200	Died
	11	0	66	71	—	330	335	291	192*
	Mean		61	63					
	General mean		62						
Group 2	12	5 mg. + cabbage	60	37	64	335	346	367	372
	13	"	64	—	—	310	389	Died	—
	14	"	66	54	60	301	384	426	250
	15	"	66	61	70	320	382	410	441
	16	"	72	58	56	308	394	444	490
	17	"	54	43	50	306	360	366	380
	18	"	51	59	65	346	386	425	485
	19	"	55	64	54	343	409	439	495
	20	"	64	58	66	308	347	354	378
	21	"	58	63	72	339	386	404	442
	22	"	71	60	62	318	370	401	432
	Mean		62	56	62				
	General mean		60						

\* Animals died on 22nd-25th day of experiment. Weight is that on day of death.

Table 2. Complement titres and weights of guinea-pigs on scorbutogenic diets supplemented with 0.5, 1.0 and 10.0 mg. ascorbic acid

Group	Week	Dose of vit. C mg./day	Complement titres units (one unit=0.0001 ml. of undiluted serum) in animals					Mean	Wt. (g.) of animals									
			No. 23	24	25	26	27		No. 23	24	25	26	27					
3	0	0.5	—	—	—	—	—	69	470	450	375	550	570					
	2	0.5	—	—	—	55	78		525	485	457	470	386					
	4	0.5	92	63	70	57	—		515	483	380	432	Died					
	7	10.0	38	49	—	—	—		470	480	Died	Died	—					
	9	10.0	70	50	—	—	—		450	510	—	—	—					
4			No. 28	29	30	31	32	33	34	Mean	No. 28	29	30	31	32	33	34	
	0	1.0	—	—	—	—	—	—	—		60	640	570	570	520	530	485	450
	4	1.0	65	92	66	66	82	58	61		70	660	550	580	490	500	595	500
	7	1.0	85	68	51	55	65	36	50		59	665	575	610	580	367	630	505
	9	1.0	63	54	50	49	—	42	45		50	665	610	610	635	Died	625	550
5			No. 35	36	37	38	39	40	Mean	No. 35	36	37	38	39	40			
	0	—	—	—	—	—	—	—		—	500	460	450	490	525	555		
	2	10.0	—	50	—	—	—	—		—	450	480	500	540	550	600		
	4	10.0	57	55	72	67	70	72		61	440	545	510	580	570	575		
	7	0.5	—	80	197	49	—	—		—	470	445	435	585	620*	545*		
	8	0.5	46	61	63	—	64*	46*		80	470	460	420	595	650*	520*		
9	0.5	48	50	147	57	60*	—	—	485	465	380	600	600*	Died				

\* Guinea-pigs 39 and 40 supplemented with 10.0 mg. ascorbic acid daily throughout.

## RESULTS

*Exp. 1 (summer).* The complement titres (Table 1) of deficient guinea-pigs ranged from 50 to 81 units, with a mean of 61 in the second and 63 in the third week of deficiency. The average of all samples had a value of 62 units. The controls maintained on a supplement of 15 g. of cabbage plus 5 mg. of ascorbic acid showed an average for all samples of 60 units (Table 1).

While the weights of the controls increased steadily, the deficient animals started losing weight in the third week of the experiment and died in the following week.

*Exp. 2 (winter).* The guinea-pigs in group 3 (Table 2) receiving 0.5 mg. of ascorbic acid daily for the first 4 weeks, showed an average complement titre of 69 units. When the supplement of vitamin C was increased to 10 mg., their titre had a value of 53 units.

Group 4, having a supplement of 1 mg. of ascorbic acid, showed average titres of 70, 59, and 50 units in the fourth, seventh and ninth weeks, respectively. The general mean from all the samples of serum was found to be 60 units. The weight of the animals remained at a steady level or showed a slight increase.

The animals in group 5, supplemented with 10 mg. of ascorbic acid for 4 weeks, had an average titre of 61 units. After changing to a partially deficient dose (0.5 mg.), the average value of the titre was found to be 80 units. The weight of all but one animal increased steadily when adequately

supplemented with vitamin C. The gain, however, was less than that of the controls in *Exp. 1*, receiving 15 g. of cabbage in addition to the 5 mg. of ascorbic acid. When the guinea-pigs of group 5 were put on a partially deficient diet in the fourth week, they either lost weight or remained at an almost steady level.

### Statistical calculations

It has been claimed [Ecker *et al.* 1938b] that a comparison of complement titres between groups of guinea-pigs on different levels of vitamin C intake is unsatisfactory, and that data from the same animal with various levels of vitamin C intake are preferable. *Exp. 2* was therefore performed in such a way as to allow a statistical evaluation on this basis. In addition, the significance of the differences between groups on different levels of vitamin C intake was also calculated. The method of analysis of variance was employed throughout [Fisher, 1938; Goulden, 1939].

*Significance of differences between groups 1 and 2.* The average values of the complement titres of the deficient guinea-pigs (group 1) did not differ appreciably from the average values in group 2, which had received supplements of cabbage and vitamin C. The general mean (60 units) of all samples collected during a period of deficiency agreed almost exactly with the general mean (62 units) of the control group. This insignificant difference between the two means makes a statistical evaluation almost unnecessary. The mean difference between groups 1 and 2 amounts to -1.76. When the *t* test was applied, the difference was found to be insignificant ( $t=0.797$ , while *t* tabulated for  $P 0.05$  is 2.23). The standard error was calculated from the mean of titres of guinea-pigs bled on the same day.

*Significance of differences between guinea-pigs maintained on doses of 0.5, 1.0 and 10.0 mg. of ascorbic acid.* When the means of the complement titres for the different levels of

vitamin C intake were calculated, irrespectively of groups, the following values were found:

Mean of complement titres for

0.5 mg. ascorbic acid daily = 75.8,  $s \pm 10.16$  units

1.0 mg. ascorbic acid daily = 60.3,  $s \pm 3.19$  units

10.0 mg. ascorbic acid daily = 58.6,  $s \pm 3.03$  units

The above values may indicate a slight increase in complement activity with an increase of ascorbic acid in the diet. When, however, the value of  $F$ , i.e. the ratio of group mean square to error mean square, was calculated, it was found that  $F=2.25$ ; tabulated  $F$  for 5% point = 3.19. It may therefore be concluded that no significant differences had been established.

*Significance of differences within group 3.* Complement titres of partially deficient guinea-pigs were compared with those of the same animals in the later part of the experiment, when they were given an adequate amount of vitamin C. The variance ratio was  $F=2.62$ ; tabulated  $F$  for 5% pt. = 19.0.

There is therefore no significant difference between the titres of the same animals on different levels of intake of vitamin C.

*Significance of differences within group 4.* In this group, which had a supplement of 1.0 mg. of ascorbic acid throughout, the titres obtained at different periods of the experiment (4th, 7th and 9th weeks) were analysed. The variance ratio was  $F=3.91$ ; tabulated  $F$  for 5% pt. = 3.98.

As  $F$  found is only slightly below the value of  $F$  tabulated, the differences are almost significant. This conclusion is interesting as the complement activity increased slightly during the course of the experiment, which is contrary to the findings reported by Ecker & Pillemer [1940].

*Significance of differences within group 5.* Although the means of complement titres showed an apparent increase from 61 to 80 units when the dose of ascorbic acid was decreased from 10.0 to 0.5 mg. daily, the variance ratio for these findings was  $F=1.08$ ; tabulated  $F$  for 5% pt. = 4.35.

The change of complement titre is therefore not significant.

*Analysis of differences between individual guinea-pigs and error of method.* Complement titres of individual guinea-pigs within groups did not vary significantly when their variance was compared with the respective error variance. From duplicate samples the standard deviation of a single titration has been calculated ( $s=2.33$ ;  $\bar{x}=60.1$ ). There is therefore a probability of only 1 in 20 that the titres will be outside the limits of  $\pm 8\%$ .

## DISCUSSION

Experimental precautions were taken to eliminate variables other than vitamin C intake which might influence the result. Also by a proper statistical evaluation we eliminated to a certain extent variations due to causes other than vitamin C intake. Thus we took care to bleed the animals without using anaesthetics, which are believed to increase the concentration of ascorbic acid in the blood [Ecker *et al.* 1938*b*]. We also used the same animals as controls for their subsequent complement titres, by putting each animal as far as possible on two levels of vitamin C. The results, particularly those in groups 1 and 2, show without doubt that when a

reliable method of complement titration is used there is little, if any, difference between complement titres of individual guinea-pigs within a group, no matter whether they are maintained on a deficient or an optimal diet. We can confirm the conclusions of Brooks [1920] and Wadsworth *et al.* [1931] that the 50% haemolysis gives consistent results with a low degree of experimental error [Traub, 1943].

It is evident that we could find no influence of the intake of vitamin C on the complement titre. Only in group 4 were the differences between complement titres almost significant. In this group, the figures refer to samples of blood withdrawn from the same animals at different times of the experiment. It may, however, be noted that the complement activity of the sera was either unaltered or improved slightly, quite contrary to expectation based on the findings of Ecker *et al.* [1938*a*] and Ecker & Pillemer [1940]. The guinea-pigs had a supplement of only 1 mg. of ascorbic acid, which certainly was not sufficient to maintain optimal growth. If vitamin C influences complement titres in the way claimed there should therefore have been a decrease of complement activity in this group.

The agreement between the average complement titres of group 2 (Exp. 1) and those in Exp. 2 seems of interest. The animals in group 2 were maintained on a diet supplemented with cabbage as well as an adequate dose of ascorbic acid, and thus received other essential dietary factors [cf. Kohler, Elvehjem & Hart, 1938]. The influence of these could be observed from their improved gains in weight. Nevertheless, their complement titres did not differ from those of the guinea-pigs which received an adequate supplement of vitamin C alone (Exp. 2). Apart from this, although the two experiments were performed in the summer and winter months respectively, no seasonal influence, such as has been claimed, could be observed.

The weights of the guinea-pigs given in the tables show conclusively the influence of the different levels of vitamin C intake. Also, post-mortem findings confirmed the presence of scorbutic changes in the deficient animals.

Our conclusion that the state of nutrition with regard to vitamin C has no influence upon the complement activity of serum is in agreement with results of Crandon, Lund & Dill [1940] in human scurvy. Spink, Agnew & Mickelsen [1942] and Feller, Roberts, Ralli & Francis [1942] arrived at similar conclusions. Further indirect evidence of the lack of positive correlation between vitamin C and complement activity is the finding that although newborn infants have a higher vitamin C concentration in plasma than their mothers [Mindlin, 1940] they have a significantly lower complement activity [Traub, 1943].

Although we could not find any relation between complement and vitamin C, we do not wish to draw any conclusions as to the possible influence of the vitamin on other immunological processes. The exact significance of complement in immunology is in any case still uncertain.

#### SUMMARY

1. Complement titrations, using 50 % haemolysis as the end-point, were performed on forty guinea-pigs maintained on different levels of intake of vitamin C, and the results were evaluated statistically.

2. No significant change in complement was

found to occur in guinea-pigs partially or completely deficient in vitamin C.

3. Addition of cabbage to the diet did not result in any change in the complement titre as compared with that of guinea-pigs whose diet was supplemented with adequate amounts of synthetic vitamin C.

4. The significance of these results is discussed.

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## Antigenic Properties of Hyaluronic Acid

By J. H. HUMPHREY (Jenner Memorial Student), *The Lister Institute of Preventive Medicine, Elstree, Herts*

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Hyaluronic acid has been shown by Meyer and colleagues [Meyer & Palmer, 1936; Meyer, Smyth & Dawson, 1939; Meyer & Chaffee, 1941] to occur in the vitreous humour, synovial fluid, skin and umbilical cords of various mammals and by the author (unpublished) to occur in the lung tissue. Although the detailed structure of this substance has not been worked out, the work of Meyer and his collaborators [reviewed by Meyer, 1938] has shown hyaluronic acid to be a polysaccharide of high molecular weight, composed of units containing one residue of N-acetyl glucosamine linked with one of glucuronic acid. Existing evidence suggests that specimens of hyaluronic acid prepared from different

sources are chemically identical. A polysaccharide which is probably identical has been obtained from capsulated streptococci of groups A and C by Kendall, Heidelberger & Dawson [1937] and by Seastone [1939]. The capsules of these organisms are destroyed by an enzyme—hyaluronidase—obtained from other strains of streptococci or from various other sources [McClellan, 1941]. Their presence is associated with the virulent stage of the organisms, but when either whole organisms or purified capsular material were injected into rabbits they failed to give rise to detectable circulating antibodies against the capsules [Seastone, 1939].