

## THE RELATIONSHIP BETWEEN ASCORBIC ACID AND PHAGOCYTTIC ACTIVITY

W. J. NUNGESTER AND ADA MAY AMES

From Department of Bacteriology, University of Michigan, Ann Arbor, Michigan

Interest in the relationship between ascorbic acid and leucocyte activity was aroused during a series of studies on fundamental mechanisms governing phagocytosis. The repeated collection of blood tinged peritoneal exudate, containing few white cells which proved to be unusually fragile, directed attention to the fact that the donor animals, guinea pigs, were deficient in vitamin C.

A search of the literature contributed some positive information on the role of ascorbic acid in white cell physiology. Perla and Marmorsten<sup>1</sup> found the cellular response to intraperitoneal irritation to be poor in scorbutic guinea pigs. They did not mention, however, any lessening in the phagocytic activity of the few cells obtained. A definite impairment of the phagocytic activity of leucocytes from donors low in vitamin C has been reported by several workers including Lowrynowicz,<sup>2</sup> Busing,<sup>3</sup> and Cottingham and Mills.<sup>4</sup>

Vitamin C has long been known to be a component of leucocytes. Ralli and Sherry<sup>5</sup> emphasize in their paper on adult human scurvy that the concentration of blood vitamin C is higher in the white cells than in red cells or plasma.

The work of Crandon, Lund, and Dill<sup>6</sup> has shown that the white cell-platelet layer is the last fraction of the blood to be depleted of its vitamin content. The significance of these relationships to phagocytic activity is unexplained.

Mary Elizabeth Reid,<sup>7</sup> in her review article, describes the role of calcium and ascorbic acid in the proper functioning of the muscle cells. She then suggests the need for investigating the role of ascorbic acid in other tissues, such as the leucocytes, in whose proper functioning surface activities play an outstanding role.

This report deals with the relationship between the ascorbic acid level of peritoneal exudate obtained from guinea pigs and the fragility and phagocytic activity of the white cells in the exudate.

### METHODS

The guinea pigs used in these experiments received only a regular diet of a commercial ration\* for rabbits plus some dry alfalfa. The rabbit ration is claimed to contain all the elements needed for full growth and development of the animals except vitamin C. On this diet, the control animals soon developed symptoms of scurvy, namely, loss of weight, tendency to spontaneous hemorrhages, and paresis. If allowed to continue without treatment, the process was terminated by death, usually hastened by secondary infections. The remainder of the animals under obser-

Received for publication January 28, 1948.

This work was aided by a grant from U. S. Public Health Service.

1. Perla, D., and Marmorsten, J. 1937, *Arch. Path.*, **23**: 543-683.
2. Lowrynowicz, A. 1931, *J. de Physiol. et Path. Gen.* **29**: 270.
3. Busing, M. K. 1942, *Klin. Wchnschr.* **29**: 97.
4. Cottingham, E., and Mills, C. A. 1943 *J. Immunol.* **47**: 493-504.
5. Ralli, E. P., and Sherry, S. 1941, *Medicine* **20**: 251.

6. Crandon, J. H., Lund, C. C., and Dill, D. B. 1940, *New Eng. J. Med.* **223**: 353-369.
7. Reid, M. E. 1943, *Physiol. Rev.* **23**: 76-99.

\* Rockland Rabbit Ration.

vation received intraperitoneally, for varying periods of time, 50 mg of ascorbic acid per day. This amount was double that found to be the requirement of the guinea pig by Ecker and Pilemer<sup>8</sup> and was so increased in accordance with the work of Kyhos, Severinghaus, and Hagedorn<sup>9</sup> on the effect of large doses of ascorbic acid in restoring the vitamin C metabolism of depleted animals.

The white cells were obtained for study by inducing exudation into the peritoneal cavities of the donor animals. On the afternoon before the cells were to be used, 100 ml of Locke's solution containing 0.1% glucose was injected intraperitoneally into each animal. The following morning (15-16 hours later), the animals were exsanguinated by bleeding from the heart. The serum so recovered was used later in the phagocytic system. Following death, 5 ml of 10% sodium citrate solution was introduced into the peritoneal cavity to prevent the formation of fibrin clots in the exudate. Finally the abdomen was opened and the exudate collected. The exudate from several animals was pooled in a 250 ml centrifuge bottle. The bottles and contents were then chilled in a bath of ice water. From this point in their manipulation, until the cells were ready for incubation in the phagocytic systems, they were stored at about 5 C.

The cells were concentrated by gentle centrifugation (100 r.p.m., 134G) for five minutes. The supernatant fraction was removed, and the cells resuspended in Locke's solution containing 0.1% glucose. It was found necessary to repeat this procedure to free the cells of the sodium citrate, as well as any opsonic

substance which might have been present. The final suspension was standardized by direct cell counts to contain 45,000 to 50,000 leucocytes per cu mm. The data in the present work refer only to polymorphonuclear leucocytes which constituted 80 to 85% of the white cells in the final suspensions.

The phagocyte suspension was then subjected to various tests. About 5 ml of the cell suspension and of the serum were set aside in the deep freeze (-50 C) to await determination of ascorbic acid. This step was justified by the work of Kassan and Roe<sup>10</sup> on the preservation of ascorbic acid in drawn samples of blood and other fluids. They found that the loss of ascorbic acid from such specimens maintained in the frozen state was essentially nil. Further, the thawing process served to disintegrate the white cells, thus aiding in the vitamin C assay. When a number of serums and exudate samples had been accumulated, a day was set aside for the ascorbic acid determinations. The colorimetric method of Roe and Kuether<sup>11</sup> which depends upon the coupling of 2-4 dinitrophenylhydrazine with dehydroascorbic acid was found satisfactory for our purposes.

The bacterial suspension employed as antigen in the phagocytic tests contained a beta hemolytic streptococcus (New York 5) which had been grown in 0.1% glucose-peptone broth for 24 hours at 37 C. After the cells had been concentrated, and washed by repeated centrifugation and resuspension in Locke's solution, they were killed by heat (80 C for 30 minutes). The use of killed bacteria as test objects in the phagocytic systems has been common practice since the publication of Gab-

8. Ecker, C. E., and Pilemer, L. 1940, Proc. Soc. Biol. Med. **44**: 262.  
9. Kyhos, E. D., Severinghaus, E. L., and Hagedorn, D. R. 1945, Arch. Int. Med. **75**: 407-412.

10. Kassan and Roe, J. H. 1940, J. Biol. Chem. **133**: 579.  
11. Roe, J. H., and Kuether, C. A. 1943, J. Biol. Chem. **147**: 399.

ritchevsky.<sup>12</sup> The turbidity of the suspension was adjusted using a photoelectric colorimeter (Lumitron) to 37% transmission of light when compared to distilled water as 100%. Fresh suspensions were prepared every two or three weeks.

The system for study of the phagocytic activity of the white cells was set up in chemically clean tubes (73 × 8 mm). Each tube received:

- (a) 0.3 ml of the bacterial suspension.
- (b) 0.2 ml of the test substance. (This varied in each series from different dilutions of serum to plain Locke's solution.)
- (c) 0.5 ml of the phagocyte suspension.

The tubes were sealed with paraffin-dipped corks, and were gently rotated

cytes were examined in each smear for the condition of the cell, and for phagocytosis, by at least two, preferably three, individuals. The system used for evaluating activity was essentially that of Hamburger<sup>13</sup> in which the cells were counted plus or minus according to whether or not bacteria could be seen within them.

#### RESULTS

The exudates from deficient animals were strikingly lacking in white cells, and frequently the material was made even less satisfactory for phagocytosis studies by the presence of large numbers of red blood cells. This contamination was uniformly present in exudates found

TABLE 1.—*Effect of ascorbic acid on fragility and phagocytic activity of leucocytes.*

No. of tests	Animals per test	Ascorbic acid per day	No. days given	Vitamin C level of serum*	Vitamin C level of exudate*	Fragility of cells†	% activity of cells‡
6	3	0	—	0-.25	0-.30	80-90%	30%
4	3	50	1	0-.30	.25-.35	85%	30-35%
5	3	50	2	.10-.40	.30-.45	50%	50%
4	3	50	3	.10-.45	.55-.75	20%	75%
5	3	50	4	.20-.55	.90-1.20	10-12%	85-90%
6	3	50	5	.20-.60	1.0-1.25	10%	90%
5	3	50	6	.25-.60	1.0-1.25	10%	85-90%
5	3	50	7	.36-.90	1.0-1.25	5-10%	85-90%

Total number of animals used in tests = 120.

\* Quantity = mg/100 cc of the fluid.

† No serum present in the system.

‡ Homologous serum equals 20% of the phagocytic system.

end over end to insure optimum contact between the bacterial cells and the phagocytes at 37 C for 15 minutes. At the end of this time, the tubes were removed from the warm chamber and plunged at once into a bath of ice water to arrest further phagocytic activity. Duplicate films on slides were carefully made from the contents of each tube. After drying, the slides were stained with Wright's stain and examined.

In order to eliminate any element of personal bias and individual differences in counting, a serial number was assigned to each slide. At least 100 leuco-

to contain less than 0.30-0.35 mg of ascorbic acid per 100 ml of suspension. Such hemorrhage was never found accompanying exudates whose vitamin C content was above 0.45 mg per 100 ml.

The results of the fragility tests and phagocytic activity are given in table 1 and graphically presented in figure 1. The cells from vitamin C low donors proved to be singularly difficult to prepare for use. Further, when serum was not used in the phagocytic systems, the ease of rupture of the cells during incubation was found to bear an inverse

12. Gabritchevsky, G. 1890, Ann. Inst. Past. 4: 346.

13. Hamburger, H. J. 1912, Physikalisch-chemische Untersuchungen über Phagozyten. Wiesbaden.

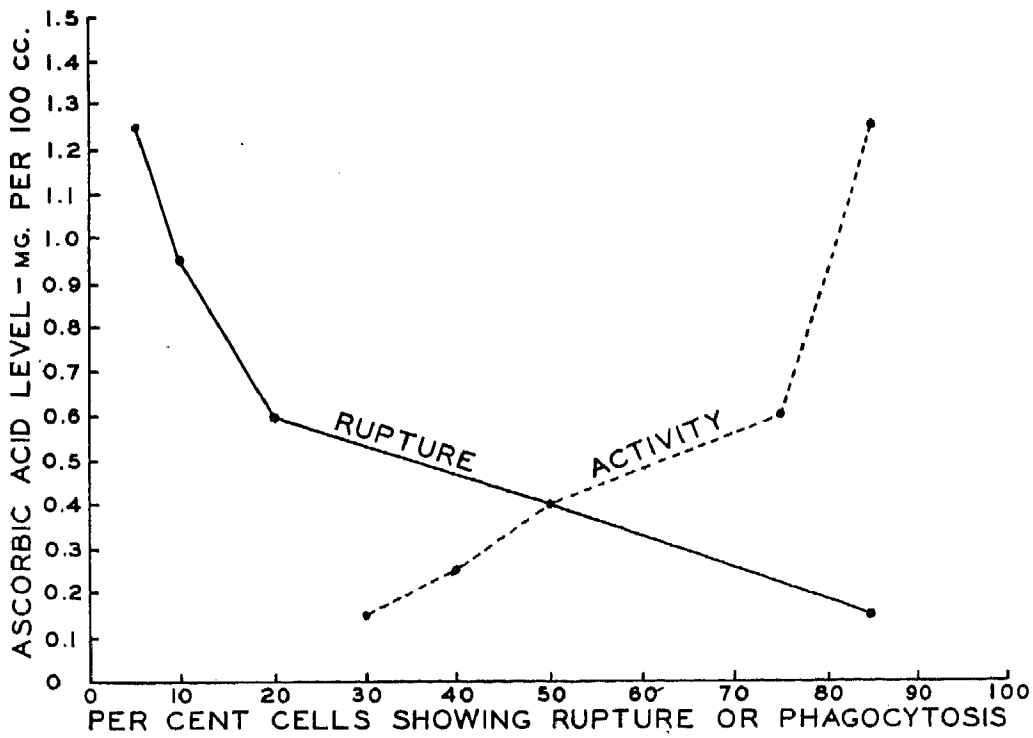


FIG. 1.—Phagocytosis and fragility of leucocytes as affected by ascorbic acid.

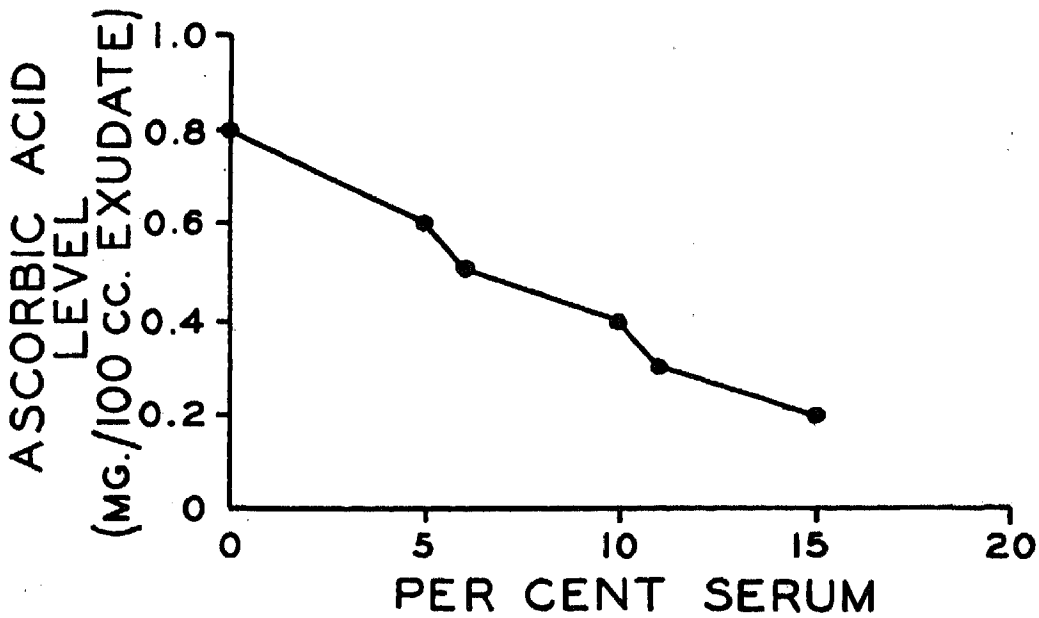


FIG. 2.—Amount of serum required in system to prevent rupture of cells.

ratio to the ascorbic acid level of the exudate. The rupturing was especially marked when the ascorbic acid level was between 0.15 mg and 0.60 mg per 100 ml of exudate. Above this level cells were much less fragile.

Within limits the amount of phagocytic activity was found to vary with the amount of ascorbic acid present. This effect, as in the fragility test, was most noticeable between concentrations of 0.10 mg and 0.60 mg of the vitamin per 100 ml of exudate. Above this latter ascorbic acid value, the amount of phagocytic activity tended to reach a constant level.

Serum in the phagocytic systems not only promoted activity, but was also found to have a protecting action for the cells. Figure 2 shows graphically the amount of serum necessary to prevent rupture of the cells as related to the ascorbic acid level of the exudate from which the phagocytes were obtained. It will be noted that this quantity varied inversely with the vitamin C content of the cells. At 0.25 mg or less per 100 ml

of cell suspension at least 15% serum was required in the system. Exudates found to contain 0.60 mg or more required as little as 5% serum to prevent rupture. At ascorbic acid levels of 0.80—1.10 mg per 100 ml of exudate, the tendency for the cells to rupture was minimum.

#### SUMMARY

The results of these studies demonstrate a relationship between the ascorbic acid content of exudative polymorphonuclear leucocytes from guinea pigs, and the fragility and the phagocytic activity of these cells. A parallel is also drawn between the level of vitamin C metabolism of the donor animals, and the quantity and quality of the exudate obtained from the injection of an irritating substance into their peritoneal cavities.

Serum in the system not only promotes phagocytic activity, but also tends to protect the cells from rupture. The amount necessary to afford such protection varies inversely with the vitamin C content of the cells.