ASCORBIC ACID AND THE PRODUCTION OF ANTIBODY IN THE GUINEA-PIG.

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In discussing work on the relation of vitamins to immunity, Watson (1937) noted that "the voluminous literature, and specially such reviews as those of Clausen (1934) and Robertson (1934), reveal a mass of confusing and contradictory statements based on experiments carried out on an inadequate scale, and without regard to simple statistical requirements. The evidence, taken as a whole, suggests that Vitamins B and D have little, if any, influence on resistance, while in the case of Vitamin C the observations are particularly confusing and difficult to interpret." Perla and Marmorsten (1937) concluded that Vitamin C had no relation to the production of antibodies, either naturally or in response to active immunization.

At present ascorbic acid is generally considered to have but two uses, the prevention and treatment of scurvy (Pijoan and Lozner, 1944; Schneider, 1946). Wilson and Miles (1946) state: "If we attempt to assess the significance of the data at present available with regard to the influence of variations in vitamin intake on resistance to infection, we shall probably conclude that the evidence with regard to the effect of Vitamin C deficiency is so divergent that no sound conclusion is possible at the moment, though there are definite suggestions that animals such as the guinea-pigs, that are peculiarly susceptible to deprivation of the vitamin, are rendered more susceptible to infection when it is withheld."

Much of the confusion arises from failure to recognize the importance of the species of experimental animal. Vitamin C deficiency can be studied only in animals, which, like the guinea-pigs and the primates, are dependent on their diet for ascorbic acid, and which develop symptoms of deficiency if the vitamin is withheld. Withholding ascorbic acid from the diet of an animal which can synthesize Vitamin C does not make the animal deficient. Such animals can be used to study the effects of an excess, but not of a deficiency. Further confusion may arise unless a distinction is made between the response following a single injection of antigen (e.g. the primary response in the case of diphtheria toxoid) and the response to antigen of an animal that has already received one or more doses (the secondary response). Measurements of primary response, unless carefully made, are unlikely to yield significant results. The blood antibody response to a single dose of antigen is small, relatively slow in developing, rises and falls rapidly, and is less affected by the dose of antigen than the secondary response. The blood antibody response to more than one injection of antigen is large, develops quickly, rises rapidly, is maintained for a relatively long time,

and its magnitude depends largely on the dose of the antigen. It follows that real differences in primary responses tend to be small, and are in evidence for only a short period of time, and can be detected only by precise methods of estimating antibody. On the other hand, differences in the secondary response tend to be large, and persist for long periods, and can be detected by comparatively crude methods of estimating antibody.

With these considerations in mind, the effect of ascorbic acid deficiency on the primary and secondary antibody response of guinea-pigs to diphtheria toxoid was measured.

EXPERIMENTAL.

Three groups, each of 15 albino guinea-pigs of approximately 350 g. in weight, were given a dry, pelleted diet, containing very little ascorbic acid, but complete

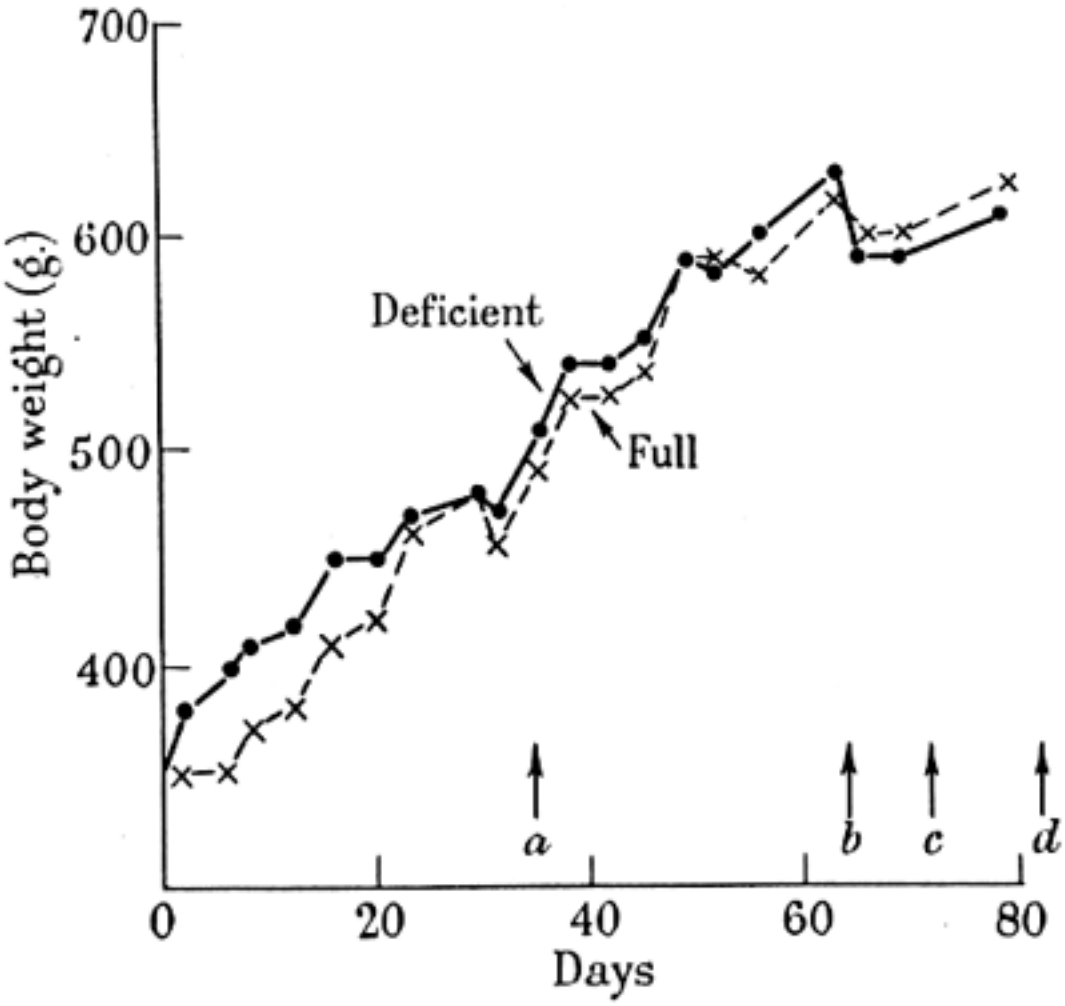

FIG. 1.—Changes in body weights of guinea-pigs given full diet and Vitamin C deficient diet. The arrows indicate the date of—
(*a*) primary toxoid stimulus ;
(*b*) intradermal tests of primary response ;
(*c*) secondary toxoid stimulus ;
(*d*) heart puncture for measurement of secondary response.

in all other respects (Bruce and Parkes, 1947). One group was given 5 mg. of ascorbic acid three times a week. To avoid oxidation of the vitamin, all solutions were made up freshly, and administered without delay by stomach-tube. The second group was given unlimited cabbage (Hartley, 1943) as a natural source of Vitamin C. The animals in all three groups gained weight at approximately the same rate. Fig. 1 records the weights of the vitamin excess and vitamin deficient groups. These results suggest that the vitamin deprivation had not produced a grossly deficient group of animals. Indeed, Bruce and Parkes (1947) believe that small, though slightly variable quantities of ascorbic acid are present in the diet. Post-mortem examination at the end of the experiment

confirmed this impression. There was no macroscopic evidence of deficiency, and none of delayed healing in the lesions made by the intradermal injection of diphtheria toxin used in the test of immunity.

On the thirty-fifth day all animals were immunized by the subcutaneous injection of 0·5 Lf of alum-precipitated diphtheria toxoid in 1 ml. of normal saline. On the sixty-third day a rough indication of the degree of ascorbic acid saturation of each group of animals was obtained by Rötter's (1938) test, which depends on the rate of decolorization of the redox dye 2:6-dichlorophenolindophenol injected intracutaneously. The solution of the dye, syringe, needle, site and method of injection were the same throughout, and there was

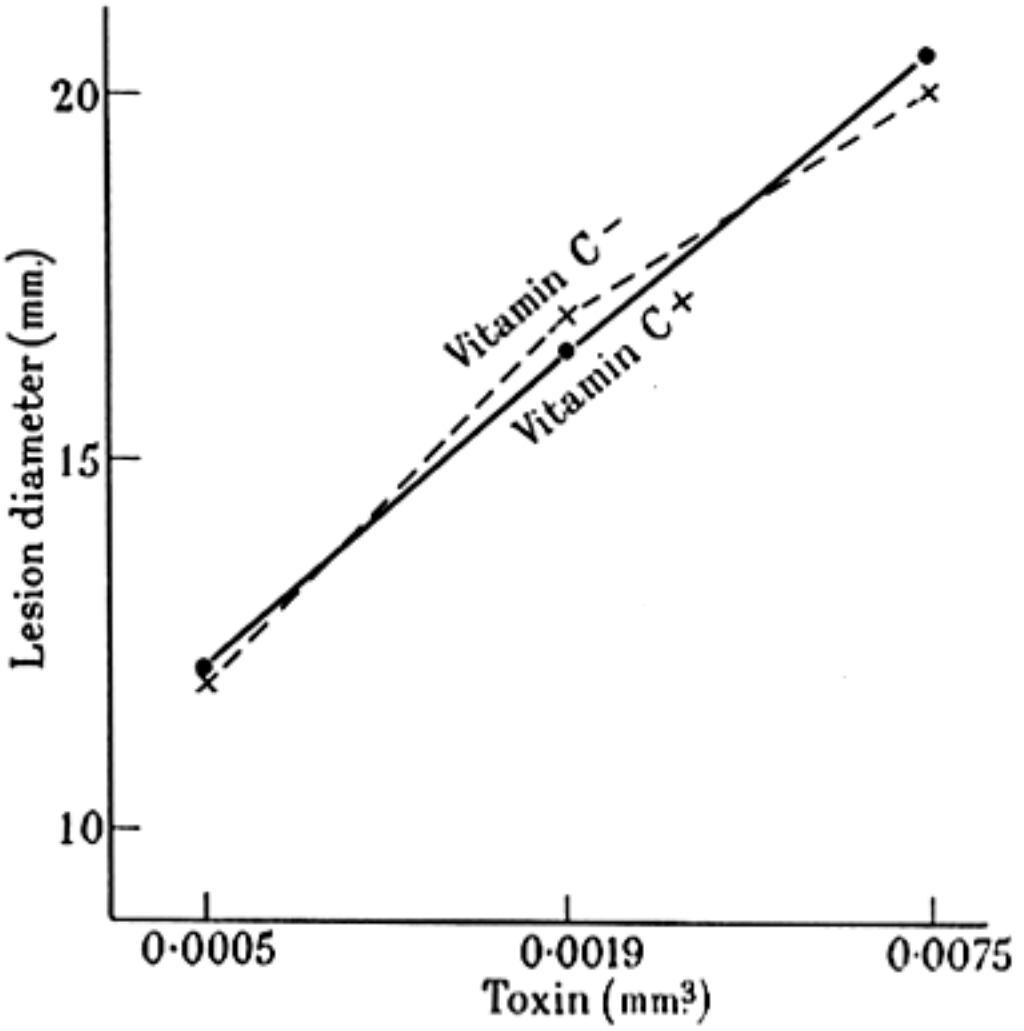

FIG. 2.—Susceptibility to intradermal diphtheria toxin of skin of guinea-pigs receiving full diet and Vitamin C deficient diet.

within groups close agreement of the times taken by individual animals to decolorize the dye. The difference between groups was striking. Animals given ascorbic acid took an average of two minutes, and those given cabbage five minutes, to decolorize the dye. In the deficient group the colour persisted for more than 30 minutes.

Primary response.—On the same day the primary response was measured by the intradermal injection of graded doses of diphtheria toxin. The response obtained when the inflammatory lesion-diameter at 24 hours is plotted against the log dose of toxin in passively-immunized guinea-pigs is linear; different dose-response curves are substantially parallel, and the shift in the dose-response curve is proportional to the antitoxin content of the blood (Miles, 1949). By fitting regression lines to the dose response curves so obtained, it is possible to detect relatively small, but significant differences, in the antitoxic immunity in different groups of animals. Subsequent tests made in this laboratory showed

the same relations held in actively-immunized animals (*cf.* Hartley, 1934). If skin reactivity to toxin were to be valid as a measure of antitoxic immunity, it was necessary to show that sensitivity to diphtheria toxin was not altered by ascorbic acid deficiency alone. Two groups of guinea-pigs were given the pellet diet, and in addition one group was given the 5 mg. of ascorbic acid three times a week, for 30 days. The skin reactivity tested by the intradermal injection of graded doses of diphtheria toxin was substantially the same in both groups (Fig. 2).

The three groups of immunized animals were accordingly tested by graded doses of intradermal toxin. Fig. 3 shows the results ; each point on the graphs represents the mean of readings from 15 animals. The results of this experiment were subjected to an analysis of variance (Table I). There is no significant

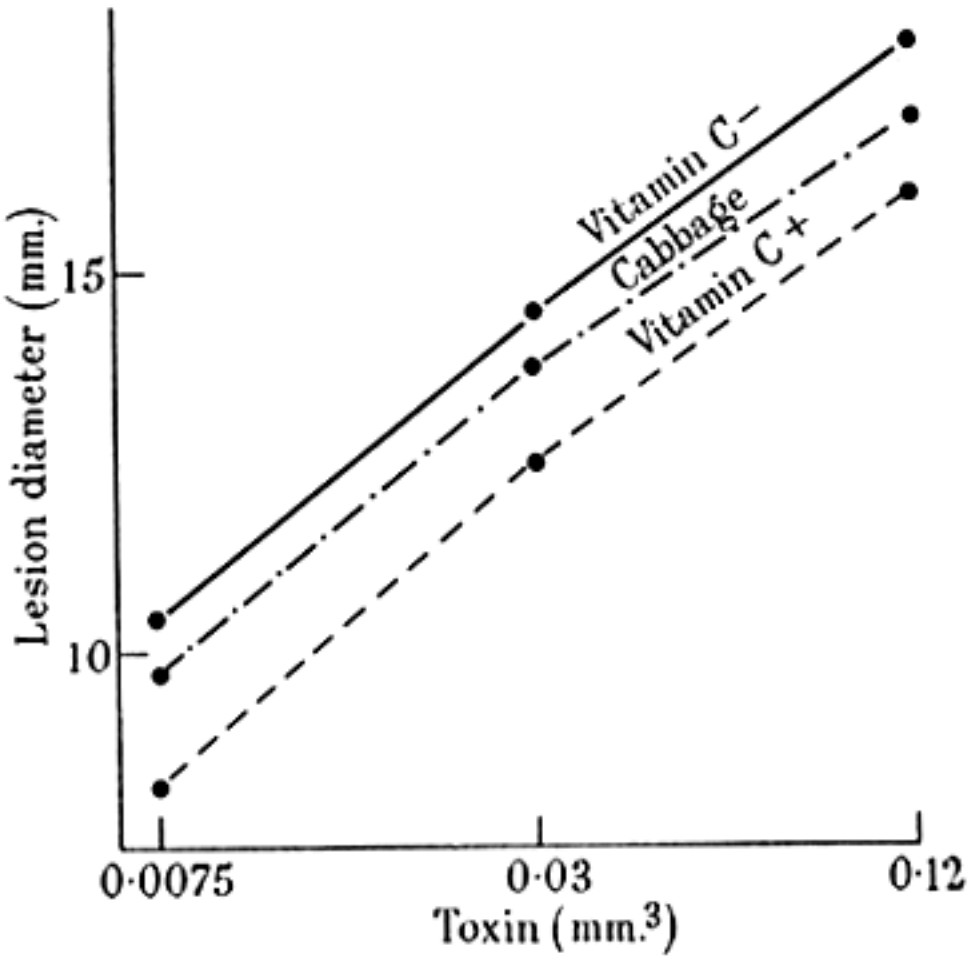

FIG. 3.—The response of guinea-pigs on various diets immunized by one injection of toxoid to graded doses of intradermal diphtheria toxin.

TABLE I.—*The Analysis of Variance of the Data of the Experiment Recorded in Fig.* 3.

	d.f.	Mean sq.	F.	P.
Between groups	2	47·5908	1·53	>0·05
,, doses	2	609·1685	19·55	<0·001
Linearity	1	1213·6694	38·95	<0·001
Departures	1	4·6676	0·15	>0·05
Dose/guinea-pig interaction	84	1·3646	0·04	>0·05
Group/dose interaction	4	0·5102	0·02	>0·05
Between animals within groups (error)	42	31·1582		
Total	134			

Slope = 6·09943.

difference between the three groups of animals. It should be noted, however, that the error term in the analysis is the variance between animals within groups, that there are large differences in the reactions of different animals. In addition, the variance ratios for the interactions are remarkably small, and this may also indicate a relatively large error term. It seems probable, indeed, that the differences between the three groups, which are comparatively regular (Fig. 3), are in fact real though small, and that an experiment with larger numbers of animals in each group would have produced statistically significant differences. It is noteworthy that if the method had not been amenable to statistical analysis

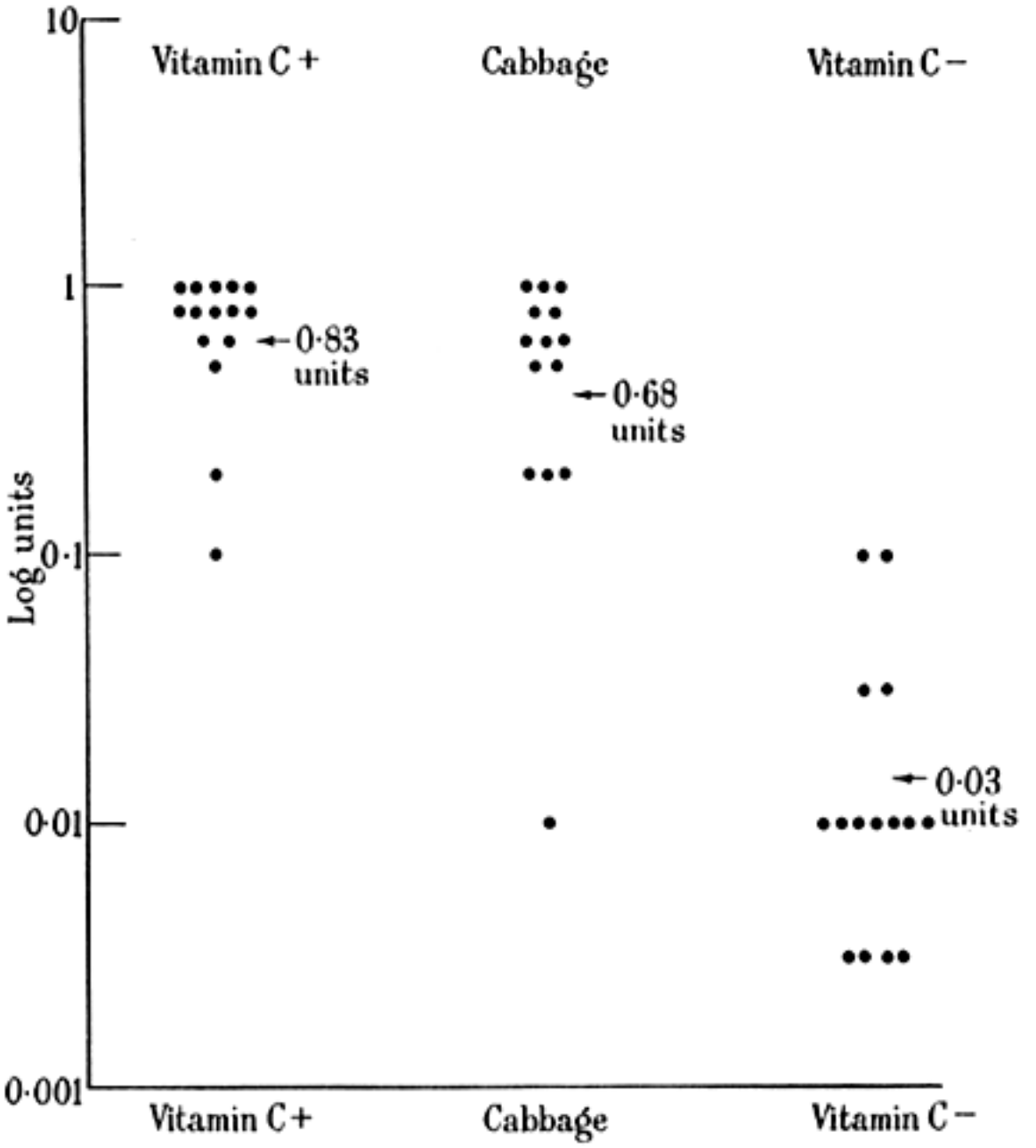

FIG. 4.—Serum antitoxin levels in guinea-pigs on various diets in response to a secondary stimulus of diphtheria toxoid.

and had been judged by an impression of the minimal reacting dose, as in the classical multiple Schick dose method (Glenny, Hopkins and Pope, 1924), a significant effect would have been deduced.

Secondary response.—The animals were given a second dose of antigen seven days after the intradermal injection of toxin. Ten days later the antitoxin content of the sera was estimated by the intradermal method (Römer and Sames, 1909).

The blood antitoxin clearly measures the secondary response, both to the

test toxin given intradermally and to the alum-precipitated diphtheria toxoid injected subcutaneously. The mean titre (Fig. 4) was very low in the ascorbic acid deficient animals, being about one-thirtieth of that in the controls; and animals given ascorbic acid had slightly more circulating antitoxin than animals receiving cabbage.

CONCLUSION.

In comparison with guinea-pigs receiving an excess of ascorbic acid—

(*a*) The primary antitoxin response of ascorbic acid deficient guinea-pigs to alum precipitated diphtheria toxoid was not significantly reduced, though it is probable that if larger groups of animals had been used in the experiment a slight reduction would have been shown.

(*b*) The secondary antitoxin response of the deficient animals was greatly reduced.

Since the indicating effect in the measurements of antibody is the neutralization of toxicity, the difference in antitoxin levels cannot be attributed to a modification of the serological (as distinct from the immunological) properties of circulating antibody as a result of vitamin deficiency; and almost certainly reflects a difference in the state of immunity.

It is clear that Vitamin C cannot be dismissed as having no direct influence on the immune state. The nature of the influence is obscure, though the comparatively small effect of deficiency on the primary response suggests that the vitamin is concerned more with the metabolic systems governing the production of antibodies in cells already in a state of "secondary responsiveness" than with the response of those cells to primary conditioning by the antigen.

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