Further Studies on the Relationship Between Vitamin C and Thymic Humoral Factor (35256)

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(Introduced by P. D. Altland)

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The relationship between ascorbic acid and the differentiation, growth, and function of various cellular types have been investigated (1). We demonstrated during age involution of the lymphoid organs that while the total vitamin C concentration decreased, the percentage of dehydroascorbic acid remaining increased, and the percentage of ascorbic acid decreased (2). Treatment with adrenal cortical or androgenic steroid hormones to interrupt differentiation and growth of the lymphoid organs induced similar changes in the concentration and oxidation state of tissue vitamin C (1).

Recent reports have shown that the thymus, in addition to the adrenals and testes, elaborates humoral factors which influence the growth and immunologic function of other peripheral lymphoid organs (3). Subsequently it was demonstrated that cell-free, thymic extracts (thymic humoral factor or THF) were less active when prepared from guinea pigs maintained on lower, than on higher vitamin C supplements (4). The employed bioassay tested the capacity of THF to induce regeneration of weight and hexose monophosphate shunt enzyme activity in lymphoid organs in X-irradiated rats. The efficacy of this bioassay has since been confirmed by other investigators, who showed that the increase in spleen and lymph node weight of THF-treated, irradiated mice was accompanied by increased incorporation of tritiated thymidine or deoxycytidine into DNA (5).

While the initial study pointed out that dietary vitamin C influenced the bioassay for THF, one could not ascertain whether its function extended throughout thymic ontogeny for the production of THF, or whether vitamin C acted directly as a type of cofactor for the expression of THF activity. In an attempt to distinguish between these possibilities THF bioassays were performed using thymic extracts prepared from guinea pigs maintained 6 weeks on four known levels of daily vitamin C intake, or on dialyzed extracts of rat thymus from which the vitamin C had been partially removed. In addition the content of total, reduced, and oxidized vitamin C in the thymic preparations were measured and these parameters correlated with THF activity.

Materials and Methods. Male guinea pigs, 400–500 g, of the Hartley strain from NIH were maintained five to a cage and given a vitamin C deficient diet[1] and water, *ad libitum*. After 1 week they were divided into four groups and orally supplemented every other day with vitamin C equivalent to an intake of 0.25, 0.50, 1.00, or 5.00 mg/24 hr. The vitamin was freshly prepared as an aqueous solution of sodium ascorbate. Body weights were recorded throughout the study. After 6 weeks on the dietary regime, animals were sacrificed with chloroform and the thymus, spleen, liver, and adrenals were removed. Vitamin C from these organs was extracted with metaphosphoric acid and reduced, oxidized, and total vitamin C was determined by the indophenol method (6). Cell-free extracts of the thymus and spleen were prepared in 0.85% sterile saline as before (4), 0.1 ml of the supernate was analyzed for total protein (7), and the preparations were adjusted with sterile saline to a final concentration of 10 mg of protein/ml.

To determine whether ascorbic acid was

[1] Ascorbic acid deficient diet (guinea pig) from Nutritional Biochemicals Corporation, Cleveland, Ohio.

necessary for the expression of THF activity, cell-free saline extracts of rat thymuses were prepared in the same manner as guinea pig tissue extracts, except one portion was subjected to dialysis against 0.85% saline in the cold. Dialysis removed 50% of the ascorbic acid and the activity of this preparation was compared to that of nondialyzed THF.

THF bioassays were performed as outlined previously (4) by measuring regeneration of lymphoid organ weights and hexose monophosphate shunt enzyme activity in 7-week-old male Sprague-Dawley, pathogen-free rats

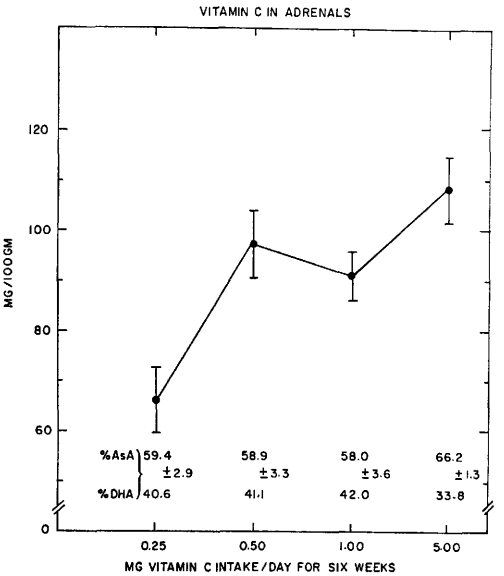

FIG. 1. Total, reduced (ASA or ascorbate), and oxidized (DHA or dehydroascorbate) vitamin C in the adrenals of guinea pigs maintained on four levels of vitamin C: means ± SEM, 10 animals/group.

following 300 R of whole body X-irradiation. Starting 6 hr postirradiation the rats were injected ip three times at 24-hr intervals with 0.5 ml of tissue extract, containing 5.0 mg of protein. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were determined by previously described methods (8) in the lymph nodes, thymuses, and spleens; the protein content of the supernates was analyzed, and activity was expressed in nanomoles of substrate transformed/milligram of protein/min (mUnits).

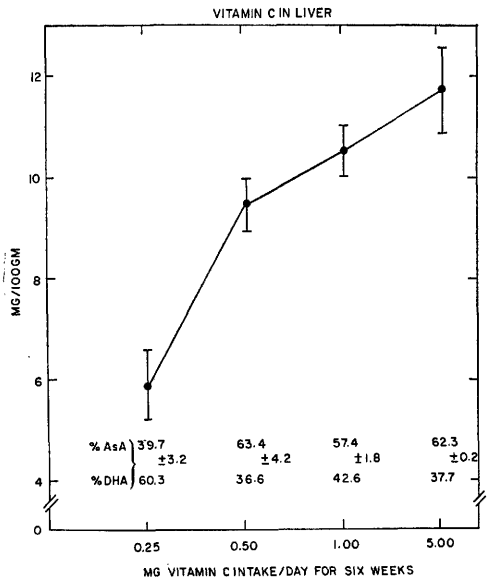

FIG. 2. Total, reduced (ASA or ascorbate), and oxidized (DHA or dehydroascorbate) vitamin C in the liver of guinea pigs maintained on four levels of vitamin C: means ± SEM, 10 animals/group.

Results. The guinea pigs given 0.5, 1.00, or 5.00 mg of vitamin C/day gained an equivalent amount of weight. Those maintained with 0.25 mg/day showed a 17% decrease in final body weight, the thymus and

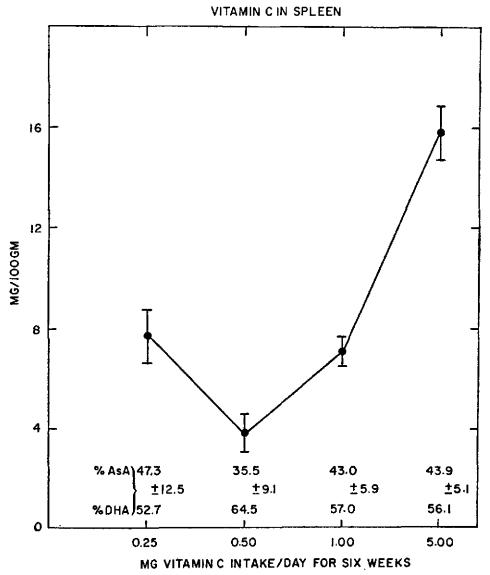

FIG. 3. Total, reduced (ASA or ascorbate), and oxidized (DHA or dehydroascorbate) vitamin C in the spleen of guinea pigs maintained on four levels of vitamin C: means ± SEM, 10 animals/group.

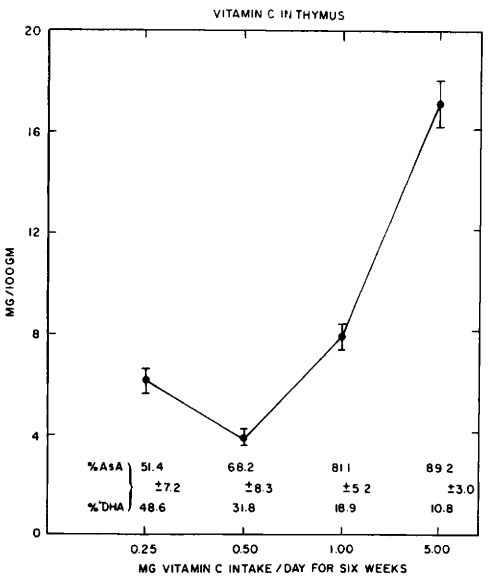

FIG. 4. Total, reduced (ASA or ascorbate), and oxidized (DHA or dehydroascorbate) vitamin C in the thymus of guinea pigs maintained on four levels of vitamin C: means ± SEM, 10 animals/group.

spleen weights did not differ, but the adrenal weights of those on 0.25 mg/day increased 25%.

Maintenance of guinea pigs on decreasing daily intakes of vitamin C resulted in ca. 50% decrease in the concentration of total vitamin C in the adrenals, liver, spleen, and thymus (Figs 1–4). However the only tissue showing a marked change in the oxidation state of vitamin C was the thymus (Fig. 4). There was a linear increase in the percentage of dehydroascorbate (DHA) and decrease in the percentage of ascorbate (ASA), corresponding to the decrease in the daily intake of vitamin C. Expression of total vitamin C, ascorbate, and dehydroascorbate on a total content basis (μg/thymus) did not result in values different from those cited above.

Thymic humoral factor bioassay. Figure 5 illustrates the increases in the lymphoid organ weights of X-irradiated rats following treatment with THF prepared from the thymuses of each of the 4 groups of guinea pigs. Following THF treatment there was a linear increase in the weight of the lymph nodes that was proportional to the level of vitamin C in the various thymic preparations. A somewhat linear relationship was also evidenced by the spleen weights, but the thymus weight was increased the same amount after all of the THF treatments. The increases in organ weight were significantly greater after THF treatment than after

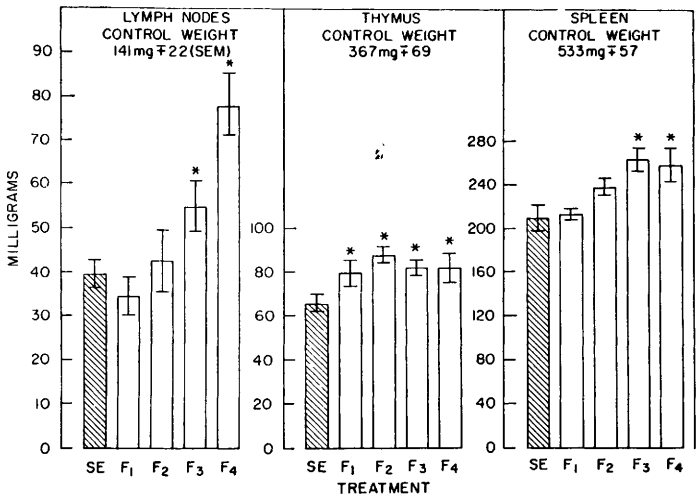

FIG. 5. Lymphoid organ weights of irradiated rats following injections of spleen extract or thymic humoral factor. The spleen extract (SE) was prepared from guinea pigs maintained on 5.0 mg of vitamin C, and the different peparations of thymic humoral factor (designated F_1 through F_4) from those maintained on 0.25, 0.50, 1.00, or 5.00 mg of vitamin C, respectively; mean ± SEM, sample numbers from 4 to 8. *Significantly different from values in irradiated rats injected with spleen extract, $p<0.05$, Student's t test.

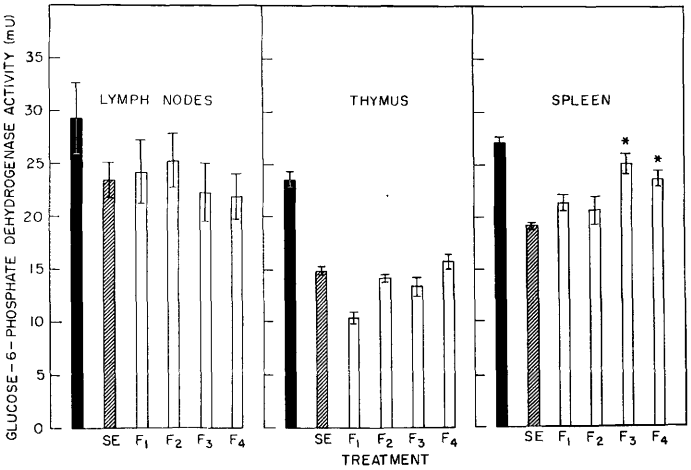

FIG. 6. Glucose-6-phosphate dehydrogenase activity in the lymphoid organs of irradiated rats following injections of spleen extract or thymic humoral factor. The closed bar represents values in nonirradiated rats. Abbreviations and other details explained in Fig. 5; mean ± SEM, sample numbers from 4 to 8. *Significantly different from values in irradiated rats injected with spleen extract, $p<0.05$, Student's t test.

spleen extract treatment in the lymph nodes (rats injected with preparations F_3 and F_4), thymus (all preparations), and in the spleen (preparations F_3 and F_4).

The increases in hexose monophosphate shunt enzyme activities after THF treatment of X-irradiated rats are illustrated in the lymphoid organs in Figs. 6 and 7. Glucose-6-phosphate dehydrogenase activity in the lymph nodes was not significantly greater in the irradiated rats treated with THF than in those treated with spleen extract. The increases in the activity of this enzyme in the thymus were proportional to the level of vitamin C in the various thymic preparations, but did not increase above the levels in rats

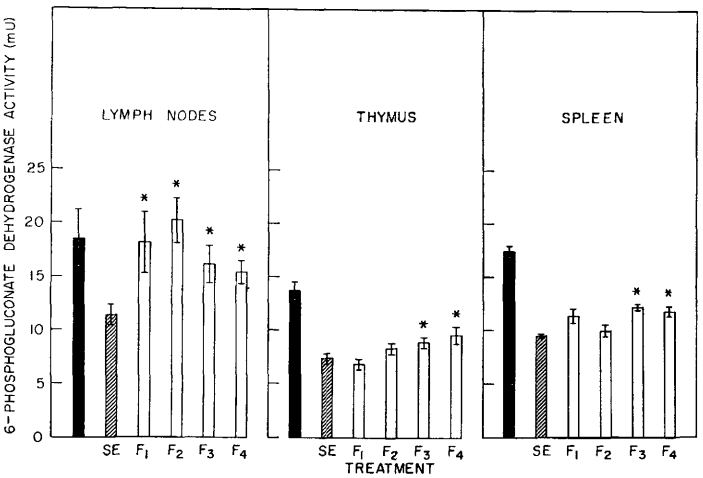

FIG. 7. 6-Phosphogluconate dehydrogenase activity in the lymphoid organs of irradiated rats following injections of spleen extract or thymic humoral factor. The closed bar represents values in nonirraidated rats. Abbreviations and other details explained in Fig. 5; mean ± SEM, sample numbers from 4 to 8. *Significantly different from values in irradiated rats injected with spleen extract, $p<0.05$, Student's t test.

treated with spleen extract. The activity of glucose-6-phosphate dehydrogenase in the spleens of rats treated with THF was somewhat proportional to the level of vitamin C in the thymic extracts, and that in the rats treated with preparations F_3 and F_4 was significantly greater than in rats treated with spleen extract.

The activity of 6-phosphogluconate dehydrogoenase in the rat lymphoid organs was more sensitive to THF treatment than that of glucose-6-phosphate dehydrogenase. In the lymph nodes treatment with any of the THF preparations induced significant increases in 6-phosphogluconate dehydrogenase activity above those in rats treated with spleen extract (Fig. 7). The increases of 6-phosphogluconate dehydrogenase activity in the thymus and spleen were proportional to the level of vitamin C in the thymic extracts; these changes were significant in both organs after injections of either THF preparations F_3 or F_4.

The results of the bioassay for THF activity using nondialyzed and dialyzed rat thymic preparations are presented in Table I. Although about 50% of the ascorbic acid was removed by dialysis, the activity of this preparation was not inhibited, and was in fact, somewhat greater than that of the nondialyzed preparation, even though an equivalent amount of protein was injected. In addition, the partial removal of ascorbic acid by dialysis did not inhibit the stimulation of hexose monophosphate shunt enzyme activity in the lymphoid organs of irradiated, THF-injected rats (not depicted in Table I).

Discussion. Prior to assay the partial removal of the vitamin C from rat thymic preparations did not inhibit THF activity, when bioassayed as before (4). It is possible that some of the ascorbic acid remaining in the dialyzed THF fraction (ca. 50%) is bound to protein, since in the determination of thymic ascorbic acid no distinction was made between the bound and unbound vitamin. In other tissues (muscle, liver) bound ascorbic acid accounts for only 10–15% of the total fraction (9, 10), but in the thymus this moiety could be necessary for the expression of THF activity. Nevertheless, the present data suggest that vitamin C is not needed for the expression of THF activity, *per se*, but that it is involved in some manner for the production of THF during thymic ontogeny.

During a period of rapid thymic development guinea pigs were maintained 6 weeks on daily intakes of 0.25–5.0 mg of vitamin C and their thymuses used to prepare THF. The bioassay showed there was a good correlation between the activity of the various

TABLE I. Content of Ascorbic Acid and Activity of Rat Thymic Humoral Factor (THF) in Dialyzed and Nondialyzed Preparations.

Means ± SEM, $n = 5$.

	Ascorbic acid (μg/ml) in THF preparations		
	Before	After 48 hr	After 72 hr
Dialyzed THF:	205	150	110
Nondialyzed THF:	195	200	180

	Activity of THF[a] [organ wt (mg)]		
Treatment	Lymph nodes	Thymus	Spleen
None	137 ± 12	510 ± 45	1188 ± 90
Irradiated			
+ spleen extract	46 ± 3	77 ± 5	451 ± 34
+ dialyzed THF	60 ± 3[b]	111 ± 12[b]	446 ± 22
+ nondialyzed THF	53 ± 4	90 ± 8	422 ± 30

[a] Index for THF activity: increase in organ weight above that in rats treated with spleen extract.

[b] Significantly different from mean immediately above, $p < .05$, Student's t test.

THF preparations and the level of vitamin C in the thymus. In a few instances the lymphoid organs of the irradiated rats injected with THF did not show increases in weight and hexose monophosphate shunt enzyme activity that were strictly proportional to the level of vitamin C in the THF preparations, *e.g.*, increases in hexose monophosphate shunt enzyme activity of the lymph nodes, and increases in the weight of the thymus. These inconsistencies are probably attributable to the heterogeneity of the THF preparations, the nature of the bioassay employed, etc., and are to be expected in biological assays of this type (19).

The guinea pigs serving as a source of THF in this study were maintained on much lower vitamin C levels than those of the preceding study (4), and the vitamin C content in the thymus is correspondingly less. It is pertinent to note that the bioassay responses in the present study were also less marked than in the former one. As before (4), the percentage of dehydroascorbate in the thymus was greater in animals on low than on high vitamin C intakes, and in fact, was inversely proportional to the level of vitamin C in the diet. Judging from the weights of the lymphoid organs and adrenals in the guinea pigs, there was little evidence to indicate that the hypovitaminotic diets elicited a stress response that could result in the release of adrenal cortical hormones, and directly or indirectly affect THF production. It follows then that the level and oxidation state of vitamin C in the thymus are by themselves critical for the optimal production of THF, or else that vitamin C is necessary for the maintenance of particular cells that are concerned with the production of THF.

Changes in the oxidation state of thymic ascorbic acid have been demonstrated during normal age involution (2), after lympholytic steroid hormone treatment (1), or after whole body X-irradiation (unpublished results). In each of these experimental situations, the decrease in lymphoid organ mass has been associated with increased tissue levels of dehydroascorbate and decreased tissue levels of ascorbate. In addition one can demonstrate the opposite shift in the oxidation state of tissue vitamin C (increased ratio of ascorbate to dehydroascorbate) as the thymus increases in mass during normal development (2). An extensive literature exists in both plants and animals that points out the correlation between enhanced cellular growth and the increased cellular concentration of ascorbic acid and decreased concentration of dehydroascorbic acid (11). Thus the association between normal cellular activity and the cellular concentration of ascorbate and dehydroascorbate appears to be universal. Perhaps in the thymus the deficiency of vitamin C augments protein breakdown (THF?) and diminishes protein resynthesis, as it has been shown to do in the liver, brain, and spleen (12). If the vitamin is in fact involved in some facet of protein turnover involving THF, it is apparent that the oxidation state of vitamin C in the thymus assumes particular importance in this regard.

As suggested earlier, vitamin C in the thymus may also function in the maintenance of certain cells that are concerned with the production of THF. In particular the thymic reticular cells, which require vitamin C for their differentiation (13), maintenance (14), and function (15) are likely candidates, and are suspected by some to elaborate THF directly (16), and by others to direct the maturation of lymphocytes (17), or to provide an architectural framework for lymphocyte reconstitution in the thymus (18).

Summary. Cell-free thymic extracts (thymic humoral factor or THF) were prepared from guinea pigs maintained 6 weeks on daily intakes of 0.25, 0.50, 1.00, or 5.00 mg of vitamin C. The concentration of dehydroascorbate in their thymuses increased, and that of ascorbate (and total vitamin C) decreased in direct proportion to the level of vitamin C in the diet. The activity of each preparation was then compared by measuring regeneration of lymphoid organ weight and hexose monophosphate shunt enzyme activity in irradiated, THF-injected rats, and was found to be directly correlated with the level of ascorbate, and inversely correlated with the level of dehydroascorbate in the THF preparations. Thus the data indicate that throughout the ontogeny of the thymus, the level, and particularly the oxidation state of thymic vitamin C is involved in some manner

for the production of THF. No evidence was found that vitamin C was required for the actual expression of THF activity, since prior to bioassay, the partial removal of ascorbic acid from similarly prepared rat THF preparations did not result in diminished reponses.

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