THE EFFECT OF DIPHTHERIA TOXIN UPON VITAMIN C IN VITRO

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Harde (1) reported that the adrenals of guinea pigs injected with diphtheria toxin gave a reaction with silver nitrate similar to that occurring in scurvy. Others have confirmed her observations and extended our information concerning the relationship of diphtheria intoxication to the presence of vitamin C in the tissues. Data accumulated indicate that the vitamin C reserves of the adrenals of guinea pigs dying from the effects of diphtheria toxin are greatly diminished (2, 3, 4), that test animals partially depleted of vitamin C survive the injection of this toxin for shorter periods of time than do those fed an adequate diet (5), and that guinea pigs given large doses of purified vitamin C may survive multiple lethal doses of diphtheria toxin (6, 7). Finally, it is claimed that under certain conditions diphtheria toxin is destroyed *in vitro* by solutions of ascorbic acid (6, 7). This, however, has been questioned (8, 9).

The data available up to this time have suggested a direct relation between these substances. Indeed, Agnoli (10) has assumed that they indicate neutralization of the diphtheria toxin in the body by the vitamin. This assumption, however, of necessity, postulates combination of the toxin and the ascorbic acid in order to account for the inactivation of the vitamin. The claim that ascorbic acid destroys the toxicity of filtrates of the diphtheria bacillus *in vitro* offered an approach to the study of their interaction as a chemical phenomenon outside of the body. These experiments were undertaken for this purpose.

Methods

The presence of vitamin C was determined by titration with fresh 0.05 per cent solutions of 2,6-dichlorophenol indophenol,

standardized against ascorbic acid crystals. When aqueous solutions of the synthetic vitamin were found to be too unstable for observations extending over considerable intervals of time, diluted lemon juice was substituted. Several different samples of diphtheria toxin produced in routine and experimental media in this laboratory and standardized by the usual procedures were

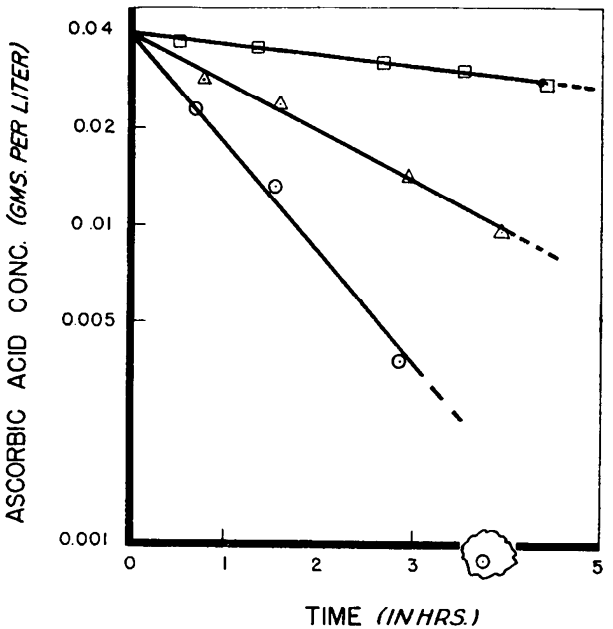

FIG. 1. The effect of a toxic filtrate of a culture of diphtheria bacillus and of the medium in which it was grown upon the ascorbic acid in 25 cc. samples of diluted lemon juice, pH 6.8, plus ⊡ 5 cc. of 0.85 per cent sodium chloride solution; △ 5 cc. of uninoculated infusion-free peptone medium; ⊙ 5 cc. of diphtheria Toxin 588, M.L.D. −0.002 cc., Lf 15.2.

used. The toxicity of some of these preparations was destroyed by boiling for 3 minutes or by heating at 80° for 30 minutes. To avoid the effect of marked changes in hydrogen ion concentrations, the lemon juice and toxin, before being mixed, were adjusted separately to pH 6.8 to 7.0. Both substances are relatively stable at this point. All receptacles containing toxin were pro-

tected from light except during actual manipulation of their contents.

Results

In the preliminary experiments it was found that the vitamin C content of mixtures of lemon juice and toxic filtrates of the diph-

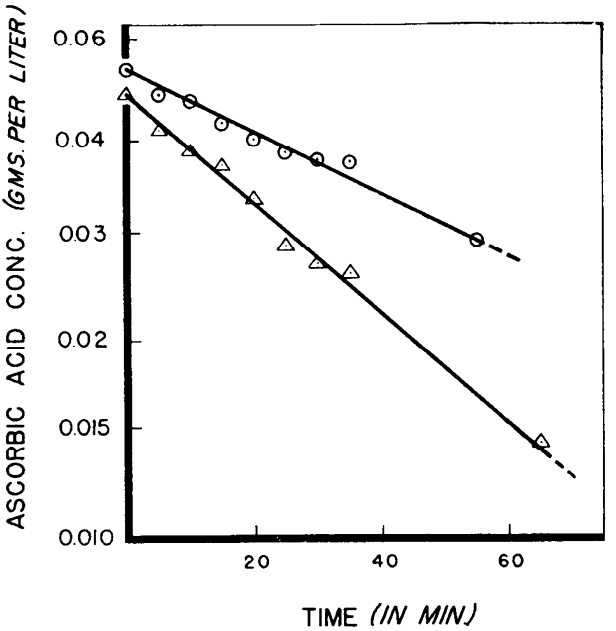

FIG. 2. The effect of diphtheria toxins of different potencies upon the ascorbic acid in 25 cc. samples of diluted lemon juice, pH 6.8, plus ⊙ 25 cc. of diphtheria Toxin 589, infusion-free peptone medium containing maltose and sodium acetate, M.L.D. +0.001 cc., Lf 28.8; △ 25 cc. of diphtheria Toxin 586, veal infusion medium, M.L.D. −0.002 cc., Lf 16.0.

theria bacillus progressively declined. Uninoculated toxin media had a similar action of less intensity (Fig. 1). While the rate of destruction of ascorbic acid caused by two toxins of different potencies might be roughly proportional to their toxicity, this was not always the case (Fig. 2). Diphtheria toxins heated sufficiently to destroy their toxicity for the guinea pig, however, were more active against the vitamin than were the same toxins

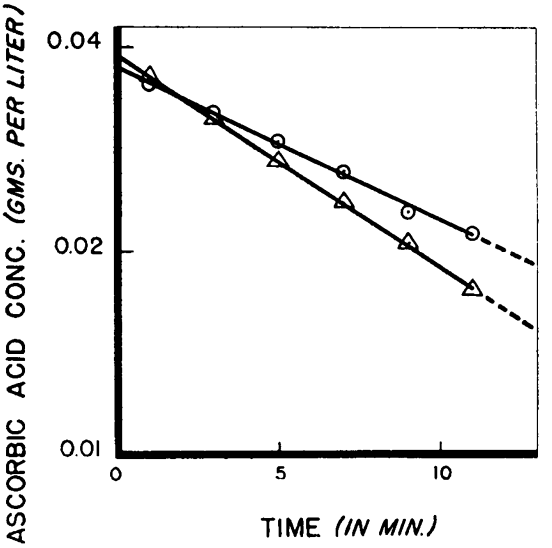

FIG. 3. The effect of diphtheria toxin, before and after heating, upon the ascorbic acid in 50 cc. samples of diluted lemon juice, pH 7.0, plus ⊙ 10 cc. of diphtheria Toxin 589, infusion-free peptone medium containing maltose and sodium acetate, M.L.D. +0.001 cc., Lf 28.8; △ 10 cc. of diphtheria Toxin 589, heated in a boiling water bath for 3 minutes.

TABLE I

Effect of Heated and Unheated Diphtheria Toxin upon Vitamin C Content of Guinea Pig Adrenals

No. of animals	Material injected	Amount of vitamin C			Deviation from normal
		Maximum	Minimum	Average	
		mg.	*mg.*	*mg.*	*per cent*
10*	Controls	0.136	0.042	0.083	0
10*	Diphtheria toxin	0.015	0.008	0.012	−85
3	Controls	0.110	0.049	0.071	0
2	Heated diphtheria toxin†	0.086	0.052	0.069	−3

* From an earlier study (4).

† Volume equivalent to ten lethal doses of unheated toxin.

before heating (Fig. 3). When guinea pigs were injected with such a heated toxin in 10 times the volume of the lethal dose of unheated toxin, the effect on the vitamin C content of their adrenals was negligible (Table I).

Diphtheria toxin allowed to stand in contact with lemon juice was found to have lost none of its original potency as measured by its flocculation titer. In repeated experiments at hydrogen ion concentrations near those of the body, no loss could be demonstrated in the toxicity of toxin-lemon juice mixtures by either the subcutaneous or intracutaneous injection of suitable dilutions.

To determine, if possible, the mode of destruction of the ascorbic acid by these toxic filtrates, toxin-lemon juice mixtures were allowed to stand until the ascorbic acid content had declined more than half and were then saturated with hydrogen sulfide. After the removal of this gas the titratable form of the vitamin was

TABLE II

Fate of Ascorbic Acid in Toxin-Lemon Juice Mixtures

Material	Ascorbic acid			
	On combining	Lost in 2 hrs.	Recovered with H_2S	
	mg.	*mg.*	*mg.*	*per cent*
Diphtheria Toxin 586, lemon juice, and sodium metaphosphate*	1.585	0.960	0.700	73
Diphtheria Toxin 589, lemon juice, and sodium metaphosphate*	1.660	1.097	0.562	51

* Added to inhibit the catalytic activity of the copper ion.

recovered in a fairly high concentration, which indicated that it had been oxidized to the dehydroxy form (Table II).

SUMMARY

If, as has been suggested, ascorbic acid acts directly upon the toxin in ameliorating the severity of diphtheria intoxication, it must follow that the vitamin combines with this substance because the vitamin is diminished in the bodies of animals dying from such intoxication. When a similar combination was sought in mixtures of toxin and lemon juice outside of the body, the ascorbic acid was found to have no effect upon the toxin in the pH range of mammalian tissue. While the ascorbic acid content of such mixtures decreased, the effect of different toxins did not vary in proportion to their toxicity for the guinea pig. Culture filtrates heated to destroy their toxicity had an action similar to unheated

toxin. Such heated filtrates did not, however, affect the vitamin C in the animal body. Finally, the destruction of the ascorbic acid by toxic filtrates *in vitro* was found to be reversible.

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