On the Purity of Ascorbigen Preparations and Their Antiscorbutic Effect

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On account of the astounding claim of Procházka and Feldheim [1] that our synthetic preparation of ascorbigen must have contained ten times as much free ascorbic acid as was reported in our communication, and that the antiscorbutic effect of the preparation was due to this, we wish briefly to emphasize the following facts:

(1) An experiment with guinea-pigs according to which 26 mg of ascorbigen has a pronounced curative effect on scorbutic guinea-pigs was presented in our communication [2]. Experiments were not performed with other amounts of ascorbigen, and hence nothing could, of course, be said in the communication about the minimum curative doses of ascorbigen. The criticism levelled can thus only apply to the effect of this amount of ascorbigen.

(2) The preparation of ascorbigen used by us contained 5 μg of free ascorbic acid per mg of preparation, as measured by the method of Roe [3]. Paper chromatography, which is more specific, gave a value of ~ 3 μg. The value 5 μg was given in our communication, however. By shaking the ethyl acetate solution of ascorbigen several times with water, the content of free ascorbic acid in the preparation of ascorbigen can be reduced to the required level. Since both ascorbic acid and its compound with formaldehyde travel at the same speed on the paper chromatogram in the solvent system butanol-water-oxalic acid, the content of the ascorbigen preparation in regard to these substances can easily be determined by paper chromatography (Fig. 1). In our communications on the antiscorbutic effect of ascorbigen the purity of the preparations of ascorbigen means, of course, the content of free ascorbic acid.

(3) The growth curves of the control group presented in our communication reveal that the food given in our experiment with guinea-pigs could not have

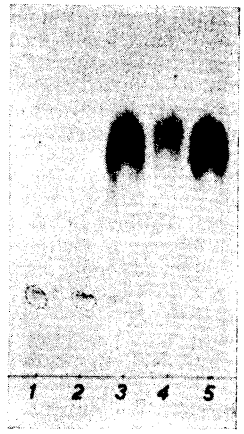

Fig. 1. Paper chromatogram of the preparations of ascorbigen. Solvent: butanol-oxalic acid-water (200:10:300). Spraying solution: 15 % $(NH_4)_2MoO_4$ in 1 % NH_4OH; H_2SO_4 added until the pH is 3.8.[4] *1.* 2 μg of ascorbic acid, *2.* 4 μg of ascorbic acid, *3.* 1 mg of ascorbigen prepared from indole, formaldehyde, and ascorbic acid, *4.* 0.5 mg of the same preparation, *5.* 1 mg of ascorbigen prepared from 3-hydroxymethylindole and ascorbic acid.

Fig. 2. Paper chromatogram of hay extracts and ascorbigen preparation 5 in Fig. 1. Solvent and spraying solution as in Fig. 1. *1.* Extract prepared from 200 mg of the hay used in the experiments with guinea-pigs. *2.* Hay extract + 2 μg of ascorbic acid, *3.* 2 μg of ascorbic acid, *4.* 1 mg of ascorbigen prepared from 3-hydroxymethylindole and ascorbic acid.

contained more than 0.1 mg of ascorbic acid, although the method of Roe gave a value of 10 μg of ascorbic acid per mg for the heat-treated hay used. According to the paper chromatogram, no ascorbic acid was present in the hay (Fig. 2).

We consider that our results, according to which 26 mg of ascorbigen has a curative effect on scorbutic guinea-pigs, is not due to the presence of free ascorbic acid in the ascorbigen preparation used or in the diet. This was confirmed by further tests in which the quantitative effect of ascorbigen as vitamin C was revealed. In the following communication the results of these tests are presented.

A detailed description of our methods to synthesize and purify the ascorbigen preparations used in the feeding experiments is given for the sake of safety.

The preparation of ascorbigen used in the new tests with guinea-pigs 1. From hydroxymethylindole. 4.5 g of ascorbic acid was dissolved in 400 ml of citrate buffer (*Solution A:* 21.015 g of citric acid/l. *Solution B:* 28.40 g of Na_2HPO_4/l. 245.8 ml of solution A + 154.2 ml of solution B were mixed and 2 N NaOH was added dropwise until the pH was exactly 4.0). 3.8 g of hydroxymethylindole was added to the solution, which was kept at 37°C for 1 h. The mixture was then cooled, filtered, and shaken three times with ethyl ether (200, 200, and 150 ml). The ascorbigen was transferred from the aqueous phase to the ethyl acetate by shaking it five times with freshly distilled ethyl acetate (300, 300, 200, 200, and 150 ml). Each fraction was dried with about 60 g of Na_2SO_4 and filtered into a 1 l evaporating flask. The ethyl acetate was evaporated, and the residue dissolved in 40 ml of ethyl acetate and transferred to a 100 ml dropping funnel. The solution was then washed in the funnel five times with 1 ml of distilled water. The ethyl acetate phase was poured into a 100 ml Erlenmeyer flask through a small cotton wool plug and evaporated to dryness in a vacuum.

The ascorbic acid was determined by paper chromatography in the product thus obtained. If it exceeded 1.5 μg/mg, the purification procedure was repeated.

2. From indole and formaldehyde. 30 g of ascorbic acid was dissolved in 400 ml of citrate buffer, and the pH of the solution adjusted to 4.0 as before. 10 g of indole and 14.5 ml of 35 % formaldehyde was added and the mixture was kept at 54°C for 5 or 6 h. The mixture was filtered after cooling and shaken three times with ethyl ether (200, 200, and 150 ml), and then four times with freshly distilled ethyl acetate (300, 200, 200, and 150 ml). Each fraction of ethyl acetate was dried with about 60 g of Na_2SO_4. The combined fractions were evaporated to dryness after filtering. If the residue contained more than 3 to 5 μg/mg of free ascorbic acid according to the method of Roe and to paper chromatography, it was redissolved in 30 ml of water and shaken three times with ethyl acetate (20, 20, and 20 ml). The fractions were dried with Na_2SO_4, and the solvent evaporated as before.

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Synthetic Ascorbigen as a Source of Vitamin C for Guinea-Pigs. II

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The effect of 26 mg of ascorbigen prepared from indole, formaldehyde, and ascorbic acid on guinea-pigs was reported in a preliminary communication[1]. Quantitative experiments with different doses of ascorbigen prepared from 3-hydroxymethylindole and ascorbic acid, as well as from indole, formaldehyde, and ascorbic acid, are reported in this paper. Paper chromatograms of both preparations were published in the preceding communication[2]. According to paper chromatography, the ascorbigen preparations contained < 1 μg of free ascorbic acid per mg.

In the animal experiments two different nutrient mixtures were used, neither of which contained any ascorbic acid according to paper chromatographic analysis.