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.

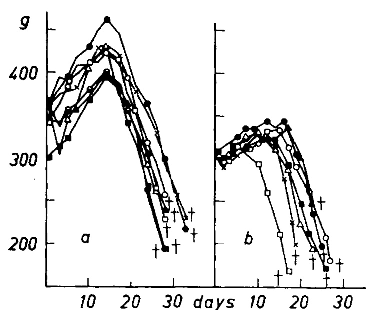

Fig. 1. Controls without ascorbic acid or ascorbigen. a. Diet I, b. diet II.

Nutrient mixture I. A mixture of rolled oats and wheat embryos (4:1) *ad libitum* (the amount eaten in the beginning was about 30 g/day/animal). In addition 20 g of timothy hay (day/animal) which had been kept at 45°C for 3 days. The hay was of the same batch as that used in the preliminary experiment. If the lack of ascorbic acid was made good, the animals grew much better on this diet than on nutrient mixture II. Guinea-pigs of a pure albino strain, weighing 280 to 380 g, were fed on nutrient mixture I for 12 days, when symptoms of ascorbic acid deficiency became apparent. The animals were now divided into groups, 7 or 8 animals in each. 1 ml of saccharose solution (given *per os* by pipette daily) was included in the diet of the control group. Ascorbigen doses of 3, 7, 10, and 20 mg/day/animal in 1 ml of saccharose solution were given to the other groups. The experiment also comprised a group which received ascorbic acid. The solutions of ascorbigen and ascorbic acid were prepared immediately before pipetting.

Nutrient mixture II. The ascorbic acid-free diet of Harris and Olliver [3]: Ground whole oats 72 %, wheat bran 16 %, dried egg yolk 9 %, NaCl 0.5 %, $CaCO_3$ 1.0 %, $MgSO_4$ 0.5 %, cod-liver oil 1 %. The procedure described by Harris and Olliver was followed. Albino guinea-pigs, weighing 200 to 250 g, were at first fed on diet II *ad libitum* (the amount eaten in the beginning was about 30 g/day/animal). 15 g of cabbage/day/animal was given in addition, until the weight of the animals had increased to 300 g. Eleven days after this, the animals were given the test substances in the same manner as when nutrient mixture I was used. The groups fed on this diet comprised 6 or 7 animals.

The weights of the animals during the test are shown in Figs. 1 to 6. Even3 to 5 mg of ascorbigen clearly retarded the development of scurvy. While the test animals on diet I died on the average 29 days (min. 26, max. 33 days) after the beginning of the experiment, and those on diet II after 23 days (min. 17, max. 27 days), the animals which were given 3 mg of ascorbigen (diet I) lived on the average 37 days (min. 28, max. 46 days) (Fig. 2a). 5 mg of ascorbigen lengthened the life of the animals on diet II from 23 to 39 days (min. 24, max. 56 days) (Fig. 5a). One animal in this group was still alive after 65 days, when it was killed. The odontoblast test revealed scurvy in this animal too, as could be concluded from the growth curve. Four animals died after 57 to 75 days in the group which had been given 7 mg of ascorbigen (diet I) (Fig. 2b). Three animals were still alive

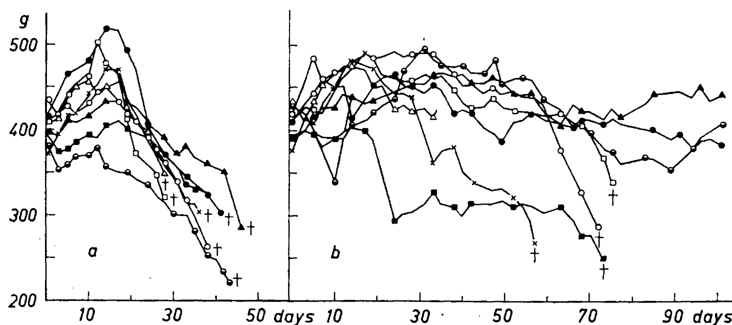

Fig. 2. Diet I. a. 3 mg of ascorbigen, b. 7 mg of ascorbigen. Both preparations synthesized from hydroxymethylindole.

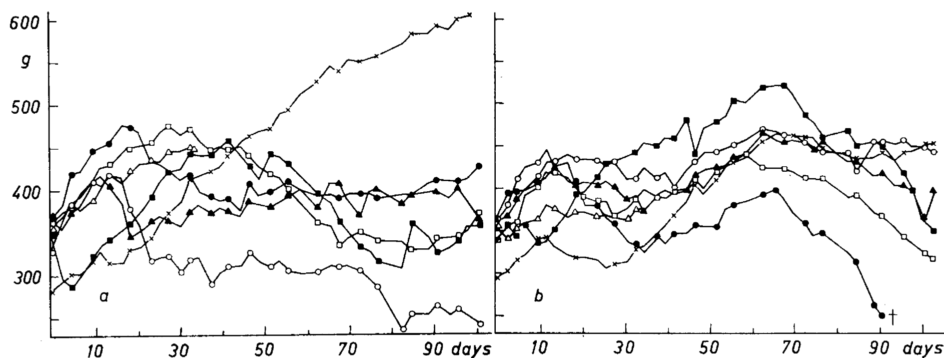

Fig. 3. Diet I. a. 10 mg of ascorbigen prepared from hydroxymethylindole, b. 10 mg of ascorbigen prepared from indole.

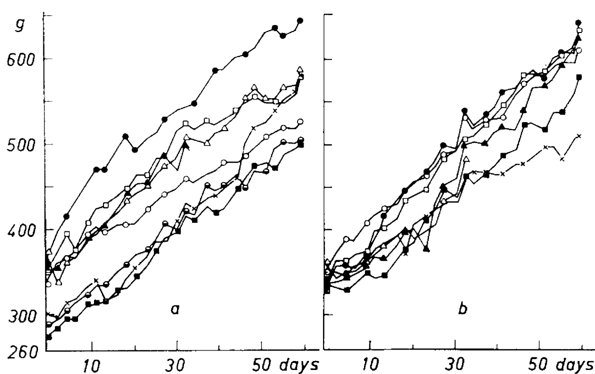

Fig. 4. Diet I. a. 20 mg of ascorbigen prepared from hydroxymethylindole, b. 2 mg of ascorbic acid.

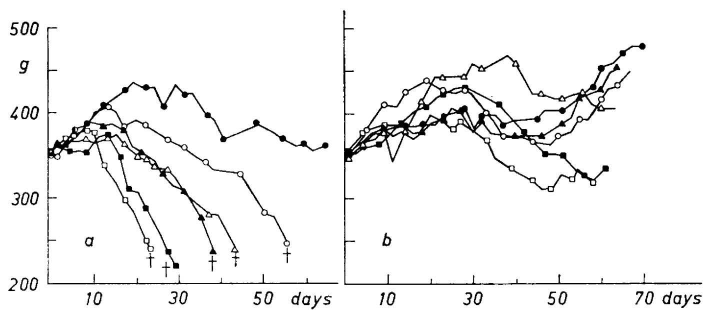

Fig. 5. Diet II. a. 5 mg of ascorbigen, b. 15 mg of ascorbigen. Both preparations synthesized from hydroxymethylindole.

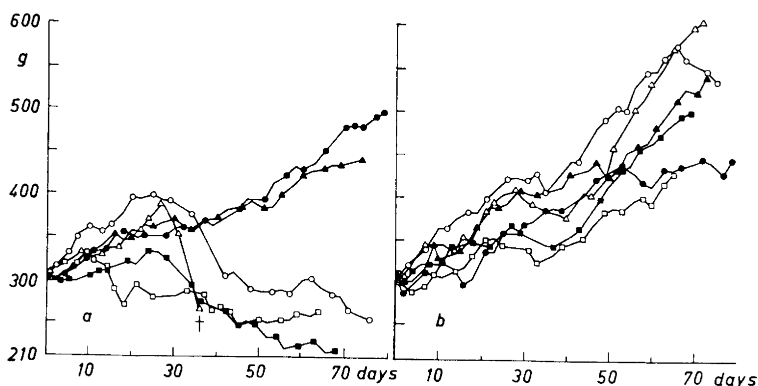

Fig. 6. Diet II. a. 0.6 mg of ascorbic acid, b. 3 mg of ascorbic acid.

after 100 days. The animals which had been given 10 mg of ascorbigen (diet I) (Fig. 3) survived the test time of 100 days; only one animal died after 91 days in the group in which ascorbigen prepared from indole, formaldehyde, and ascorbic acid (Fig. 3b) was given. In the parallel group in which 10 mg of ascorbigen prepared from 3-hydroxymethylindole and ascorbic acid (Fig. 3a) was given, all the animals survived and one attained a weight of 610 g, which corresponds to the growth of guinea-pigs which receive sufficient ascorbic acid. Whether a different acidity of the gastric juice should cause these variations was not investigated. The liberation of ascorbic acid from ascorbigen is a comparatively rapid reaction at the acidities of the gastric juice (Fig. 7).

The result thus indicates that some animals can differ greatly in their ability to utilize ascorbigen as a source of vitamin C. The animals which were given 10 mg of ascorbigen still suffered from lack of vitamin C, with the exception of the single, well-grown animal mentioned above. This was also revealed by the odontoblast test in the animals killed in each group after 33 days.

The guinea-pigs fed on diet II and given 15 mg of ascorbigen, grew on the average better than those which were given 0.6 mg of ascorbic acid (Figs. 5b and 6a). Two animals in the ascorbic acid group died (after 36 and 73 days), but none in the ascorbigen group.

The guinea-pigs grew as well on 20 mg of ascorbigen as on 2 mg of ascorbic acid

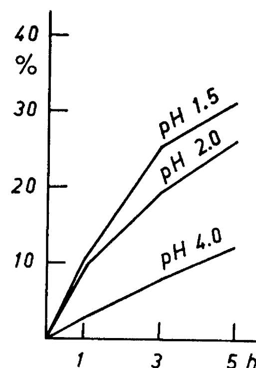

Fig. 7. The liberation of ascorbic acid from ascorbigen. The ascorbigen was hydrolyzed at 37°C in hydrochloric acid solutions, the pH values of which were 1.5, 2.0, and 4.0. The ascorbic acid was determined by titration both with dichlorophenolindophenol and dinitrophenylhydrazine according to the method of Roe. The results were confirmed by paper chromatography. The liberated ascorbic acid given as percentage of the bound ascorbic acid.

(Fig. 4). According to the odontoblast test, these animals had almost no further symptoms of deficiency after 33 days.

The diet (I) of the guinea-pigs which were given hay was apparently more suitable than diet II. The difference was not due to vitamin C, however, since the animals which were fed on diet II (Fig. 6b), and

which had also been given 3 mg of ascorbic acid, recovered slowly.

According to the results presented, guinea-pigs are on the average able to utilize about 10 to 15 % of the ascorbic acid bound in ascorbigen. The observation made in our first communication that 26 mg of ascorbigen has a curative effect on a scorbutic guinea-pig, was thus confirmed.

When using cabbage as a fresh vegetable, the formation of ascorbigen is of no practical importance. No decrease in ascorbic acid was found after normal chewing which lasted for 10—13 sec.

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The Separation of Pimaric and Isopimaric Acids by Partition Chromatography at Low Temperatures

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Partition chromatography employing silicic acid as the carrier, a mixture of 2-aminopyridine and furfuryl alcohol as the stationary phase, and isooctane as eluent has been successful since 1955 in the analysis of rosin acid mixtures such as oleorosins, gum rosin, wood rosin, tall oil rosin acids, and catalytically modified rosin acid mixtures (Ref.[1,2], and references cited in Ref.[2]). Although tetrahydroabietic, dihydroabietic, dehydroabietic, neoabietic, palustric, and abietic-levopimaric acids can be separated from one another satisfactorily by this method, pimaric and isopimaric acids are eluted together. In work on the analysis and synthesis of rosin acids and their mixtures, it would be of value to have a partition-chromatographic method which would permit the separation and analysis of the last two acids. We have found that by carrying out the partition chromatography at low temperatures, it is possible to alter the elution behaviour of the two acids sufficiently to effect their separation. The following results relate to the partition chromatography of an artificial mixture of the two pimaric acids at temperatures between + 1 and + 5°C.

The chromatographic column, the reservoir for the eluent and the tubes connecting them were kept at the temperature in question by means of a cooling medium consisting of a mixture of ethylene glycol and water which was circulated from a cryostat in which the reservoir for the eluent was immersed. The column with a inner diameter of 11 mm was filled with Mallincrodt's silicic acid (analytical reagent, 100 mesh) that had been treated with a mixture of 2-aminopyridine (m.p. 51—56°C; British Drug Houses Ltd. recrystallized from ligroin), and technical furfural alcohol (British Drug Houses Ltd.) that had been shaken with sodium hydroxide pellets and redistilled at 15 mm Hg. 9 ml of this stationary phase was mixed with 15 g of the silicic acid. The isooctane (n_D^{20} = 1.390—1.392) employed as eluent was a product of the British Drug Houses Ltd. It was purified by passing it through a column of aluminium oxide (for chromatography according to Brockmann, E. Merck AG) and then distilling it. This eluent was saturated with the stationary phase. A Parvalux micropump (The Distillers Company Ltd, England) was employed to move the eluent through the column.

The eluate was collected in 5-ml fractions by means of a siphon. The acids in the eluate fractions were determined by titration with 0.01 N tetrabutylammonium hydroxide (TBAH) in isooctane employing thymol blue as indicator.

The results obtained in three chromatographic runs are plotted in Figs. 1—3. It will be noted that isopimaric acid took a longer time to pass through the column than pimaric acid. The separation factor, the ratio of the peak volumes, varied in these cases from 1.10 to 1.12.

The idea that led to the preceding study was an observation made by one of us in 1954 [3] that some of the properties, *e.g.*