In 1943, Woolley and Krampitz postulated that 2,3-enediol-D-glucoheptono-1,4-lactone (D-glucoascorbic acid) was an antagonist of ascorbic acid, since rats and mice fed 10 per cent of the compound in a purified diet developed a scurvy-like syndrome. They noted diarrhea, extensive hemorrhage in the chest, leg, tail, and gingiva, and growth failure. They observed, however, that ascorbic acid did not reverse the effect of D-glucoascorbic acid, except in guinea pigs, and that a natural diet, containing certain factors present in liver, prevented the manifestations induced by D-glucoascorbic acid. Banerjee and Elvehjem confirmed the observation that diarrhea and growth failure developed and found that these disturbances were prevented by incorporating in the diet 6 per cent of 1:20 liver extract. Gould could find no antagonism between D-glucoascorbic acid and ascorbic acid as judged by histologic examination of the jaws of guinea pigs fed the analog or by estimation of the serum phosphatase levels. Gorlin added 5 per cent of D-glucoascorbic acid to the diet of mice. He observed diarrhea, growth cessation, perianal inflammation, and alopecia but no subcutaneous hemorrhages, hematomas, or inflamed gingiva and, in general, no histologic evidence of scurvy. This was confirmed by Schafer, who used rats, hamsters, and guinea pigs. On the other hand, Lan and Sealock showed that several other compounds, including hydroquinone, also increased tyrosine oxidation. According to Zilva, the analog is not retained in the body and has very little antiscorbutic activity. Sokoloff et al. reported that the addition of 1–2 per cent D-glucoascorbic acid to a Sherman-LaMer diet brought the ascorbic acid concentrations of blood plasma, adrenal, liver, and spleen close to zero in rats and mice without inducing diarrhea, loss of weight, or hemorrhagia. In a recent review, Woolley stated that “so far as present evidence goes it seems to indicate that D-glucoascorbic acid, when added to a highly purified ration, causes in guinea pigs a disease which is not exactly like scurvy but has some similarities to it. . . . This substance is undeniably analogue in structure to ascorbic acid. . . .”

**MATERIALS AND METHODS**

The British brown-white strain of rats, 106-AW, originally obtained from the Crocker Cancer Laboratory of Columbia University, black mice, strain C57N, originally supplied by the Jackson Memorial Laboratory, and guinea pigs of family 2 from the National Cancer Institute were used. Crocker (August) rat carcinoma, mouse adenocarcinoma E 0771, and liposarcoma of guinea pigs were used for testing the effect of D-glucoascorbic acid.

The Sherman-LaMer scorbutogenic diet, used in our experiments, was composed of: 18 gm. vitamin-free casein; 65 gm. corn starch; 5 gm. hydrogenated vegetable oil (Crisco); 2 gm. cod liver oil; 6 gm. dried brewers yeast; and 4 gm. salt mixture #1. Control animals were kept on Ralston Purina Chow.

For the blood plasma ascorbic acid determination, the technique of Farmer and Abt was employed. Blood from the tail of a rat or a mouse was mixed with potassium oxalate, centrifuged, deproteinized with fresh 5 per cent metaphosphoric acid solution, mixed, centrifuged again, and titrated with 2,6-dichlorophenolindophenol. For the tissue ascorbic acid determination, the various fresh organs were weighed, extracted with cold 4 per cent trichloroacetic acid, ground in a Ten Broeck tissue grinder, diluted, centrifuged, and filtered. Ascorbic acid was determined by the method of Roe and Kuetner (with dinitrophenylhydrazine and checked by the method of Ponting). The animals receiving D-gluco-ascorbic acid were fasted for 24–36 hours before ascorbic acid determinations.

For the citrovorum factor determination livers were removed, slices prepared with a Stadie-Riggs microtome and placed in 20-ml. beakers in regular sequence. Krebs-Ringer phosphate solution of pH 7.2 was added. The beakers were...
steamed for 2 minutes at 90°C, filtered and assayed by the method of Sauberlich and Baumann (8), with Leuconostoc citrovorum. Urine was collected under toluene during successive 48-hour periods and assayed for citrovorum factor according to Sauberlich and Baumann's method.

D-Glucoascorbic acid was used in a pure powdered form and added to the Sherman-LaMer scorbutogenic ration.¹

RESULTS

Toxicity bioassays.—Four groups of 80 male British white-brown rats, approximately 6 months of age, were used. Group I received Purina chow; Group II, the Sherman-LaMer scorbutogenic diet; Group III, the Sherman-LaMer diet to which 1 per cent of d-glucoascorbic acid was added; and Group IV, the Sherman-LaMer diet to which 2 per cent of d-glucoascorbic acid was added. The rats were fed ad libitum. They were weighed before and every 20 days during the 80-day experiment. Table 1 summarizes the results.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>AV. WEIGHT, GM.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial 40 days 80 days 90 days</td>
</tr>
<tr>
<td>I</td>
<td>105 177 218 113</td>
</tr>
<tr>
<td>II</td>
<td>107 179 216 104</td>
</tr>
<tr>
<td>III</td>
<td>111 178 211 100</td>
</tr>
<tr>
<td>IV</td>
<td>108 175 204 96</td>
</tr>
</tbody>
</table>

The average weight gain for Group II, fed the Sherman-LaMer diet for 80 days, was 3.8 per cent less than in the control Group I on Purina chow. The average weight gains in Group III on the Sherman-LaMer diet with 1 per cent of d-glucoascorbic acid and in Group IV with 2 per cent of the antimetabolite were, respectively, 11.3 per cent and 15.2 per cent less than in the control group. This difference between the control and treated groups was expected, but it was not large enough to indicate a markedly toxic effect of the ascorbic acid analog in these amounts. Moreover, there were no apparent manifestations of toxicity such as diarrhea or sluggishness, and no pathologic changes in organs or tissues were observed at autopsy when the experiment was terminated. The addition of 5 per cent or 10 per cent of d-glucoascorbic acid to the Sherman-LaMer diet produced a definite toxic effect with considerable loss of weight, diarrhea, and alopecia. The mortality rate in a group of 30 rats kept on the Sherman-LaMer diet with 10 per cent of d-glucoascorbic acid for 80 days was 40 per cent and 20 per cent in a comparable group given 5 per cent d-glucoascorbic acid. In view of the toxicity of these higher doses, we limited our experiments on cancerous animals to smaller doses (1–2 per cent) which we considered, for all practical purposes, nontoxic.

The ascorbic acid concentration of blood plasma.—One hundred male rats, averaging 8–9 months in age, were divided into four groups as in the previous experiment. The blood plasma ascorbic acid content was estimated on five rats of each group before they were placed on the various regimens, and blood was taken from ten different rats of each group during the 80-day diet period. Before taking blood, the animals were placed on a 24–36-hour fast, since the presence of d-glucoascorbic acid in blood interfered with the estimation of the ascorbic acid content.

The figures of Table 2 show that the Sherman-LaMer scorbutogenic diet slightly reduces the ascorbic acid concentration in blood plasma: from an average of 0.76 mg/100 cc for the rats on Purina chow to 0.61 mg/100 cc of blood plasma for the rats kept on Sherman-LaMer diet. D-Glucoascorbic acid added to the Sherman-LaMer diet at a level of 1 or 2 per cent brought the ascorbic acid concentration of blood plasma close to zero in 8–10 days. Similar results were obtained with female rats. The drop in the ascorbic acid values in female rats, kept on the Sherman-LaMer diet was much greater than the one which was observed in males: from an average of 0.44 mg/100 cc for the control group to 0.26 mg/100 cc for the group kept on the Sherman-LaMer diet. In general, the plasma ascorbic acid values for females were lower than for males. The effect of d-glucoascorbic acid on the ascorbic acid concentration of blood plasma in females was the same as in the case of males, since the addition of 1 or 2 per cent to the Sherman-LaMer diet brought the level close to zero.

A single parental injection of 20–30 mg. of glucoascorbate caused a slight decrease in the ascorbic acid concentration of blood plasma, bringing it down temporarily to 0.9 mg/100 cc. This was followed by a marked increase in ascorbic acid blood concentration above the normal level, in some instances as high as 0.95 mg/100 cc.

The ascorbic acid levels in spleen, liver, and adrenal.—Sixty male rats, averaging 10 months in age, were divided into four groups as in the previous experiments. Five rats of each group were killed after 10 days, while the remaining ten rats of each group were killed after 30 days. Their livers, spleens, and adrenals were dried in vacuo at low temperature and analyzed for their ascorbic acid content. Table 3 summarizes the results.

¹ We wish to thank Dr. Phillip P. Gray and Dr. Harold E. Smith of the Wallerstein Co., New York, for generous gifts of D-glucoascorbic acid.
There was only a slight decrease in the ascorbic acid concentration in liver, spleen, and adrenal from the rats on the Sherman-LaMer diet. The decrease was much more pronounced in the animals kept on the Sherman-LaMer diet with 1 or 2 per cent of d-glucoascorbic acid. Although after 30 days the ascorbic acid concentration in these organs remained higher than that of blood plasma, the ascorbic acid levels for the rats given 1 per cent of d-glucoascorbic acid for 30 days were reduced in the liver to one-thirteenth, in the spleen to about one-thirtieth, and in the adrenals to about one-fifth of those of the analogous tissues of the control animals. This drop was even more evident on a diet with 2 per cent of the antimetabolite.

The antitumor activity of d-glucoascorbic acid.—A standard test for activity against tumors was used for d-glucoascorbic acid. This test consists of intraperitoneal injections of the substance under investigation for 7 consecutive days, twice a day, starting 24 hours after transplantation. Mouse Sarcoma 180, Crocker rat carcinoma and mouse adenocarcinoma E 0771 were used. The animals, kept on a regular Purina chow diet, were given intraperitoneal injections of d-glucoascorbic acid, 125 mg/kg of body weight, twice a day for 7 days. The transplants of the tumor fragments were made subcutaneously in the lateral thoracic wall 24 hours before the tests were started. For each test, ten animals were used; five were given d-glucoascorbic acid, while five served as controls. After the test was terminated, the animals were killed, the weights of the tumors recorded, and the ascorbic acid concentrations in the tumorous tissues determined. Table 4 summarizes the results of this test in animals on a diet containing ascorbic acid.

These tests indicate no antitumor activity of d-glucoascorbic acid toward Sarcoma 180 and only
questionable activity toward Crocker rat carcinoma and mouse adenocarcinoma E 0771. There was only a moderate drop in the ascorbic acid values of blood plasma by the end of testing, and the levels of this substance in blood were quite high.

Since it was essential to find out whether a continuous maintenance of low levels of ascorbic acid in blood plasma and tissue had any effect on tumor growth, additional experiments were conducted with Crocker rat carcinoma, mouse adenocarcinoma E 0771, and guinea pig liposarcoma. Male British white-brown rats, about 10 months of age, average weight 255 gm., were divided into four groups of 30 rats each. The rats were housed separately on screens. Group I, on Purina chow, received 15.0 gm. of the diet/day/rat. Group II, on the Sherman-LaMer ration, Group III, on the Sherman-LaMer diet with 1 per cent of D-glucosaccharic acid, and Group IV, on the Sherman-LaMer diet with 2 per cent of D-glucosaccharic acid, each consumed 16.5 gm of diet/day/rat. Thus, the total caloric intake was approximately the same in each of the four groups. Tumor fragments from Crocker carcinoma were transplanted 3 days after the rats were housed. Four weeks after the transplantation the animals were killed, their tumors measured in three dimensions, and the ascorbic acid content of the tumors was determined. The same procedure was applied to C57BL mice, with transplanted adenocarcinoma E 0771. The mice were housed in groups of five: Group I, control, consumed 2.8 gm. of diet/day/mouse, and each of the three treated groups was given 3.1 gm. of the ration/day/mouse. Table 5 summarizes the results.

The figures of Table 5 indicate that while the Sherman-LaMer diet had no apparent effect on tumor growth, D-glucosaccharic acid, added to the diet at a 1 or 2 per cent level, exerted considerable inhibitory effect on Crocker rat carcinoma and on mouse adenocarcinoma E 0771. The tumor tissues of rats and mice kept on the Sherman-LaMer ration, while the rest of the animals were kept until the end of the 5th week of experimentation.

Chart 1 shows that there was an insignificant difference in the average weight of the tumors in Groups II and III. The tumors of guinea pigs receiving ascorbic acid (Group II) weighed an average of 26.5 gm., while the average weight of tumors of Group III receiving both ascorbic acid and D-glucosaccharic acid was 24.2 gm. The growth of liposarcoma in guinea pigs on the Sherman-LaMer diet (Group I) was very slow, and at the end of 30 days the average tumor weight was only 7.4 gm. The ascorbic acid values of the tumor tissue were as follows: Group I, 0.05 ± 0.02 mg/100 gm tissue; Group II, 23.23 ± 1.55 mg/100 gm tissue; and Group III, 19.12 ± 3.24 mg/100 gm tissue. Under the above described conditions it appears that D-glucosaccharic acid did not exert an appreciable antagonistic activity against injected ascorbic acid and produced no inhibitory effect on tumor growth. A vitamin C-free diet, however, retarded considerably the growth of liposarcoma and

We would like to express our gratitude to Dr. W. B. Heston of the National Cancer Institute for the generous supply of guinea pigs of family 2 and for the transplantable tumor, liposarcoma.

TABLE 5
THE EFFECT OF D-GLUCOSACCHARIC ACID ON CROCKER RAT CARCINOMA AND MOUSE E 0771
(120 male rats, and 120 C57 male mice. Four weeks after transplantation)

<table>
<thead>
<tr>
<th></th>
<th>CROCKER RAT CARCINOMA</th>
<th>MOUSE ADENOCARCINOMA E 0771</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Av. size (c. cm.)</td>
<td>Av. ascorbic acid conc. (mg/100 gm)</td>
</tr>
<tr>
<td>Group I, Purina chow</td>
<td>48.5</td>
<td>23.6 ± 1.55</td>
</tr>
<tr>
<td>Group II, Sherman-LaMer</td>
<td>40.3</td>
<td>43.4 ± 1.02</td>
</tr>
<tr>
<td>Group III, Sherman-LaMer</td>
<td>20.9</td>
<td>4.98 ± 0.82</td>
</tr>
<tr>
<td>Group IV, Sherman-LaMer plus 1 per cent glucosaccharic acid</td>
<td>15.4</td>
<td>9.7 ± 0.55</td>
</tr>
<tr>
<td>Group IV, Sherman-LaMer plus 2 per cent glucosaccharic acid</td>
<td>9.5</td>
<td>23.25 ± 1.55</td>
</tr>
</tbody>
</table>

We would like to express our gratitude to Dr. W. B. Heston of the National Cancer Institute for the generous supply of guinea pigs of family 2 and for the transplantable tumor, liposarcoma.
brought the ascorbic acid concentration of the tumor tissue close to zero. This observation confirms the findings of Robertson et al. (20) concerning the effect of a scorbutogenic diet on the growth of fibrosarcoma in guinea pigs.

The effect of D-glucoascorbic acid on the conversion of folic acid to citrovorum factor.—It was suggested that a reductive step may be involved in the enzymatic synthesis of citrovorum factor (27). Nichol and Welch (16, 17), Broquist et al. (2), Hill and Scott (12), and Welch et al. (28) found that ascorbic acid, a powerful reducing agent, enhanced the formation of this factor from folic acid. This section concerns the effect of D-glucoascorbic acid on the conversion of folic acid (PGA) to citrovorum factor (CF). Nichol and Welch (16, 17) reported the augmentation by glucoascorbate of the enzymic conversion of PGA to CF by liver tissue in vitro, and Welch et al. (28), administering glucoascorbate parenterally to rats, found the analog “to be as active as ascorbic acid” in this respect. Sauberlich and Baumann (22) demonstrated that the urinary excretion of CF by rats is proportional to the amount of PGA ingested.

A group of female British white-brown rats, 7–8 months of age, were raised on a purified diet consisting of: vitamin-free casein, 18; Cerelose 67.5; Celluloflo, 2; cystine, 0.2; succinylsulfathiazole, 2; vegetable oil, 4; fortified corn oil, 2; choline chloride, 0.1; salts, 4; thiamine, riboflavin, pyridoxine, nicotinic acid, and calcium pantothenate, each 0.3 mg/100 gm of ration; and containing 50 μg of PGA/100 gm. The animals were also fed this diet during the experiment. Six groups of rats, each containing five animals, were used. The rats of Groups IV, V, and VI were kept on this purified diet supplemented with 1 or 2 per cent of D-glucoascorbic acid for 10 days prior to the experiment. The ascorbic acid concentrations of blood plasma in these rats were brought down close to zero. Urine from each rat was collected under toluene during 48-hour periods and assayed with Leuconostoc citrovorum.

The results of these trials are summarized in Table 6.

Several facts emerge from these observations.

1. In confirmation of Nichol and Welch (14), the addition of ascorbic acid to a purified diet considerably enhanced the conversion of PGA to CF. In

TABLE 6

THE EFFECT OF D-GLUCOAASCORBIC ACID ON URINARY EXCRETION OF CITROVORUM FACTOR

(Female rats on a purified diet containing 50 mg/100 gm of folic acid. Av. 48-hour amount of CF for four collections, five rats.)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet Description</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Purified diet</td>
<td>35</td>
</tr>
<tr>
<td>II</td>
<td>Purified diet + 1.0 mg. of PGA daily</td>
<td>3,700</td>
</tr>
<tr>
<td>III</td>
<td>Purified diet + 1.0 mg. of PGA and 0.1 mg. of ascorbic acid daily</td>
<td>18,450</td>
</tr>
<tr>
<td>IV</td>
<td>Purified diet with 1 per cent of D-glucoascorbic acid plus 1.0 mg. of PGA daily</td>
<td>1,005</td>
</tr>
<tr>
<td>V</td>
<td>Purified diet with 2 per cent of D-glucoascorbic acid plus 1.0 mg. of PGA daily</td>
<td>697</td>
</tr>
<tr>
<td>VI</td>
<td>Purified diet with 1 per cent of D-glucoascorbic acid plus 1.0 mg. of PGA and 0.1 mg. ascorbic acid daily</td>
<td>17,000</td>
</tr>
</tbody>
</table>

3. When a purified diet containing 1 or 2 per cent of D-glucoascorbic acid was used and the ascorbic acid level was brought close to zero, not only was there no increase in the conversion of
ficiency in these animals. They remain in good health after many months of this diet. It appears this substance.

doubtful that a true scurvy could be induced in other investigators working with larger doses of this ascorbic acid did not produce the same symptoms. Miller et al. (15) observed that the plasma ascorbic acid concentration dropped to and remained at zero levels near zero are not indicative of scurvy. The observation of Miller et al. (15), who kept their patients on a vitamin C-free diet for a period from 4 to 6 months without any apparent signs of scurvy, confirm the previous findings of other investigators and suggest that this regime might be safely used for lowering the CF conversion for a period up to 6 months.

DISCUSSION

Woolley (30) tends to admit that the competitive action of D-glucoascorbic acid is not clearly manifested and that no inhibition index can be established in regard to the ascorbic acid-glucocorbidic acid antagonistic activity. He still adheres to his opinion, expressed in 1948, that D-glucoascorbic acid induces a scurvy-like condition in rats, mice, and guinea pigs, although this assumption has been contested by other investigators (Gould [11], Gorlin [10], Schafer [23]). Our observations have not disclosed any antagonistic activity of D-glucoascorbic acid to dietary vitamin C but have revealed that when this analog was administered in relatively small doses (1 or 2 per cent added to a purified diet) it decreased the ascorbic acid concentration in blood plasma, liver, spleen, and adrenals in rats and mice to values close to zero without inducing any of the toxic manifestations reported by Woolley and other investigators working with larger doses of this substance.

Thus, apparently a low concentration of ascorbic acid in blood and tissues in rats and mice does not necessarily induce any signs of vitamin C deficiency in these animals. They remain in good health after many months of this diet. It appears doubtful that a true scurvy could be induced in rats or mice by administration of the analog.

On the other hand, D-glucoascorbic acid appears to interfere with the biosynthesis of ascorbic acid, possibly by a suppressive action upon the ascorbic acid-forming tissue. The nature of this action has not yet been determined.

Our standard test with D-glucoascorbic acid on Sarcoma 180 disclosed no antitumor activity. An insignificantly small antitumor activity of D-glucoascorbic acid was observed in the standard tests with Crocker carcinoma and mouse adenocarcinoma E 0771. Under the condition of the standard test, when the analog is injected parenterally, the ascorbic acid concentration in blood plasma remains at a high level. It takes from 5 to 7 days on a special diet to bring the ascorbic acid level of blood plasma to values close to zero, and only a continuous maintenance of such low concentrations of ascorbic acid for a period of two weeks or more produces retardation in the growth of Crocker rat carcinoma and mouse adenocarcinoma E 0771. Since, in the trials with small nontoxic doses of D-glucoascorbic acid (1 or 2 per cent), no signs of vitamin C deficiency were evidenced and the animals were asymptomatic as far as the scurvy syndrome was concerned, one may conclude that the low concentrations of ascorbic acid in blood and tissue are unfavorable to the growth of certain malignant tumors. If the observation of Goldstein et al. (8), that ascorbic acid takes part in the formation of the deoxyribonucleic acid of the cell nucleus is confirmed, we might find a proper interpretation of the phenomenon described by us.

In man, a scorbutogenic diet or a diet low in vitamin C abates the conversion of folic acid to citrovorum factor, according to Broquist et al. (2), Gahwiler et al. (8), and Welch et al. (28). It may be added that, according to Crandon et al. (4), the first symptoms of scurvy syndrome appear in man only after more than 4 months on a vitamin C-free diet. They observed that the plasma ascorbic acid concentration dropped to and remained at zero after 41 days of the experimental vitamin C-free diet, even though the abnormal clinical signs of ascorbic acid deficiency, such as hyperkeratotic papules, did not appear before 132 days. Burrill (9) and Stamm et al. (27), on the basis of their extensive investigations of gingivitis, concluded that there is no justification for ascribing this condition to vitamin C deficiency alone, and that serum levels near zero are not indicative of scurvy. The observation of Miller et al. (15), who kept their patients on a vitamin C-free diet for a period from 4 to 6 months without any apparent signs of scurvy, confirm the previous findings of other investigators and suggest that this regime might be safely used for lowering the CF conversion for a period up to 6 months.
SUMMARY
When D-glucoascorbic acid was added to a Sherman-LaMer scorbutogenic diet at a level of 1 or 2 per cent, no apparent toxic effects such as diarrhea, alopecia, or hemorrhagia in rats and mice was produced, but on this diet the ascorbic acid concentrations of the blood plasma and certain tissues were lowered to nearly zero in 20 days.

Intraperitoneal injection of 250 mg/kg/day of D-glucoascorbic acid for 7 days did not retard the growth of mouse Sarcoma 180 and only slightly inhibited the Crocker rat carcinoma and mouse adenocarcinoma E 0771.

Continuous feeding of a Sherman-LaMer ration containing 1 or 2 per cent of D-glucoascorbic acid for 4 weeks considerably retarded the Crocker carcinoma and adenocarcinoma E 0771.

D-Glucoascorbic acid did not affect the growth of liposarcoma in guinea pigs receiving an adequate supply of vitamin C, although the tumor growth was retarded considerably in scorbutic guinea pigs.

In the absence of dietary ascorbic acid, the urinary excretion of the citrovorum factor in rats was decreased by a diet containing 1 or 2 per cent of the analog, but the antagonist did not significantly affect CF excretion in the presence of dietary ascorbic acid.

REFERENCES
Ascorbic Acid Analog in Experimental Cancer
