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Synthesis of Ascorbic Acid in Guinea Pigs by an Implanted Dialysis Bag Containing L-Gulonolactone Oxidase

PAUL H. SATO

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824

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SUMMARY

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Guinea pigs fed no ascorbic acid develop scurvy due to the inherited absence of the enzyme L-gulonolactone oxidase. However, by providing this enzyme together with its substrate L-gulonolactone, synthesis of the vitamin and prevention of scurvy are demonstrated in this species. Rat liver enzyme is placed in a dialysis bag which has been surgically implanted within the animals' peritoneal cavities. Substrate is then injected into the peritoneum. Plasma concentrations of ascorbic acid are markedly increased in enzyme-treated animals compared to similar bovine albumin-treated control animals. Adrenal gland, liver, and other tissue concentrations of the vitamin are also significantly elevated. Prolonged time of survival of enzyme-treated guinea pigs fed the ascorbic acid-deficient diet is shown. Enzyme placed within the dialysis bags does not appear to be antigenic, whereas unprotected enzyme is clearly antigenic.

INTRODUCTION

Unlike most species of mammals, guinea pigs, humans, and other primates require a dietary source of ascorbic acid. The reason for this is that they lack the ability to synthesize the vitamin. In mammals ascorbic acid is synthesized by the following pathway:

D-glucose → D-glucuronic acid →
L-gulonic acid → L-gulonolactone → L-ascorbic acid. (1)

It has been demonstrated that the defect in ascorbic acid-dependent species of animals is the absence of the enzyme L-gulonolactone oxidase (EC 1.1.3.8), which catalyzes the final step in this biosynthesis (2–4).

By supplying sufficient amounts of the vitamin to the diet of these enzyme-deficient species, scurvy can be prevented. If, alternatively, these scurvy-prone animals were supplied with the enzyme L-gulonolactone oxidase together with a source of L-gulonolactone, they should be able to synthesize their own ascorbic acid. Although scurvy is so easily treated by including ascorbic acid in the diet, this disease might provide a means for exploring methods of enzyme replacement therapy applicable to the other less treatable and more complex enzyme-deficiency diseases in humans. With this in mind, a simple method for administering an enzyme to an animal was devised which would provide at least partial protection of the enzyme from proteolytic attack and the animal from an immune reaction. A dialysis bag that allows passage of small molecules such as L-gulonolactone and ascorbic acid but is impermeable to macromolecules was implanted within a guinea pig and used to synthesize ascorbic acid.

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METHODS

Preparation of enzyme. L-Gulonolactone oxidase was partially purified through the Bio-Gel A 1.5 m column chromatography step as previously described (3, 5). Washed rat liver microsomes were digested with trypsin (0.3 mg/ml) overnight, and the 100,000*g* precipitate following digestion was solubilized with 1.5% Tween 20. After centrifugation the soluble supernatant was fractionated by ammonium sulfate precipitation, and the enzyme fraction was applied to the Bio-Gel A 1.5 m column. Gel fractions with the highest specific activity were concentrated to a protein concentration of about 35 mg/ml, dialyzed for 5 h against 1000 vol of Krebs bicarbonate buffer without glucose (6), and centrifuged at 20,000*g* for 30 min to remove insoluble material. The final enzyme preparation had a protein concentration of about 20 mg/ml and a specific activity of approximately 23 nmol of ascorbic acid formed/min/mg of protein.

Preparation of dialysis bags for implantation. Dialysis tubing (Spectrapor 2; dry cylindrical diameter, 15.9 mm; molecular weight cutoff, 14,000) was knotted on one end. Into the other end was inserted one end of a silastic tube (Dow Corning) (0.025-in. inner diameter, 0.047-in. outer diameter, 8-in. length) and the dialysis tubing was

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sealed around the silastic tubing by tying it with several loops of nylon monofilament. To prevent collapse of the silastic tubing at the point of this seal, a small stainless-steel tube was inserted into it at the point where the monofilament was tied. The sealed dialysis bag was 4 cm in length, had a flat width of 2.5 cm, and had a fully inflated capacity of approximately 10 ml. The prepared bags were placed in boiling water for about 10 min prior to implanting them within animals.

Guinea pigs. Male, English short-hair guinea pigs (inbred strain, Mdh: (SR[A])) weighing 140 g (age, 3 to 6 days) were purchased from the Michigan State Health Laboratories (Lansing, Mich.). Upon receipt, animals were given a normal ascorbic acid containing guinea pig diet until they appeared to adjust to the environment, and weight gain was noted (usually 2 days). Animals were then placed on an ascorbic acid-deficient diet (Nutritional Biochemical Corp., Division of ICN). After the animals were depleted of ascorbic acid for 7 days, surgical procedures were carried out. At this time animals weighed 190–210 g. Animals were weighed daily throughout the experiment.

Surgical procedures. Guinea pigs were anesthetized with pentobarbital (40 mg/kg) and a 5-cm-long midline incision into the peritoneal cavity was made. An empty dialysis bag was inserted into the peritoneal cavity and laid on the surface of the intestinal mass. The peritoneum and muscle were closed with sutures, so that the silastic access tube to the dialysis bag exited the peritoneal cavity between sutures. This tube was tunneled under the skin to the back of the animal's neck. The silastic tube was plugged when access to the bag was not desired. The skin was then closed with sutures. Animals intended for long-term treatment were given 60 mg of chloramphenicol palmitate (Parke–Davis, oral suspension), p.o. prior to surgery and for the 2 days following surgery. Animals used in plasma sampling experiments were catheterized by a carotid artery.

Enzyme and L-gulonolactone administrations. Enzyme (1.5 ml) or bovine albumin (Miles Laboratories, Inc.) (30 mg in 1.5 ml of Krebs bicarbonate buffer) was injected through the access tube into the dialysis bag. Air (1.0 cc) was then flushed through the tube. L-Gulonolactone (500 mg) was administered by intraperitoneal injections in 5 doses of 100 mg in 2.0 ml of water at hourly intervals.

Analytical methods. Ascorbic acid concentrations in plasma and other tissues were determined as previously described (7). All analyses were carried out in duplicate or triplicate. Plasma samples were obtained by drawing 1.0 ml of blood into heparin-treated centrifuge tubes through the carotid cannula. Blood was centrifuged for 3 min to separate the cells. Plasma (0.5 ml) was removed for analysis. In order to maintain adequate red cell volume in the animals, the cells were resuspended by gentle mixing with 0.5 ml of normal saline and returned to the animal through the cannula.

L-Gulonolactone oxidase activity, unless otherwise specified, was determined as previously described (8).

Protein was assayed as described by Lowry *et al.* (9) using bovine serum albumin as the standard.

Immunologic test. Immunologic testing was carried out using serum from the enzyme-treated guinea pigs. The animal that survived 37 days was bled immediately after death, and the second enzyme-treated animal was bled on day 46, 36 days after the first enzyme administration. A different guinea pig (420 gm) was injected i.p. with 400 μg of the same enzyme preparation used in the enzyme treatments. Three weeks later a second i.p. injection was made, and a week later the animal was sacrificed and antiserum was collected.

Ouchterlony double immunodiffusion tests were carried out using undiluted serum in agarose plates (IDF Cell II, Cordis Laboratories). Plates were developed for 24 h at room temperature. Some plates were stained for enzyme activity as previously described (10).

RESULTS

In order to determine the amount of activity enzyme contained in a dialysis bag would have, activity was first tested *in vitro*. Partially purified enzyme in a volume of 0.4 ml was placed in uniformly sized dialysis bags. These bags were placed in beakers that contained enough phosphate-buffered (0.05 M, pH 7.5) substrate solution to allow the bag to float freely (10 ml) and incubated with shaking at 37°C. Samples of the dialysate were removed at various time points for determination of the ascorbic acid concentration.

Activity after an initial lag period was linear for 30 min and proportional with the amount of enzyme in the bag. However, activity also varied with the size, surface area, and shape of the bag. During the linear phase activity was about half that of uncontained enzyme at a substrate concentration of 2.5 mM. After incubation for 200 min in this system the apparent rate of production of ascorbic acid decreased to about half that during the linear phase. On the other hand, enzyme incubated at 37°C without substrate has a half-life of the order of 6 days. The apparent K_m of enzyme contained within the dialysis bags was approximately 2.2 mM, whereas uncontained enzyme had an apparent K_m of 0.05 mM. Using these values the implanted dialysis bags should produce 54 μg of ascorbic acid/min.

In vivo experiments were then carried out to determine whether this device could also produce ascorbic acid within a guinea pig. Following implantation of the bag containing enzyme and administration of L-gulonolactone, tissue concentrations of the vitamin were measured. Plasma was sampled over a 390-min period (Fig. 1). During this period plasma concentrations of ascorbic acid increased about fourfold. In contrast, plasma levels in animals given L-gulonolactone and an inert protein, bovine serum albumin, in place of enzyme remained relatively constant over this period. Plasma levels of the vitamin at the end of the period of plasma sampling (0.37 mg%) approached the concentration reported for animals fed a diet high in ascorbic acid when analyzed by the method that was used (7). Therefore, these levels may approximate the maximum plasma concentrations that can be obtained (11).

The fact that plasma ascorbic acid concentrations increased suggested two possible explanations. Either the vitamin was being newly synthesized or it might be leaving tissues where it was more highly concentrated

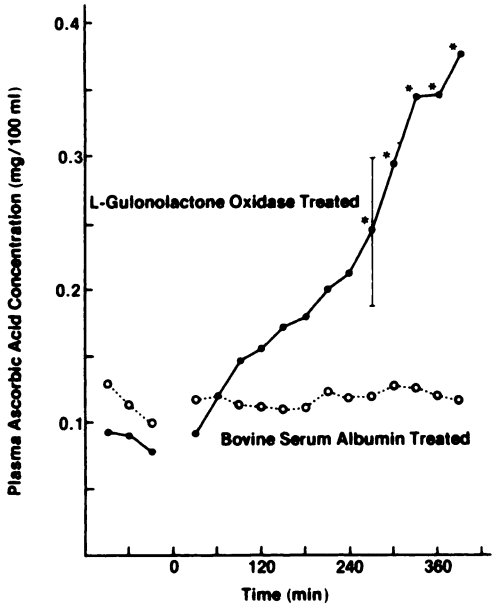

FIG. 1. *Plasma ascorbic acid concentrations in response to L-gulonolactone oxidase treatment of ascorbic acid-deficient guinea pigs*

Groups of four guinea pigs were treated with L-gulonolactone and either bovine serum albumin or L-gulonolactone oxidase. Plasma samples were taken and ascorbic acid concentrations determined at 30-min intervals. Three samples were drawn prior to enzyme or bovine albumin treatment. At 0 time, enzyme or bovine albumin and L-gulonolactone were administered, and L-gulonolactone was administered at hourly intervals thereafter for 4 h. Statistics were done by a factorial analysis of variance (17). Asterisks denote significant difference from control ($P < 0.05$) and the vertical bar denotes ±SEM.

and entering the plasma. This second possibility seemed unlikely since animals receiving bovine albumin did not show a corresponding increase. However, to further rule out this possibility, the animals were sacrificed at the end of the period of plasma sampling and ascorbic acid concentrations measured in adrenal gland, liver, lung, and spleen. These tissues were selected since their ascorbic acid contents appear to vary over the widest range and equilibrate most rapidly with plasma ascorbic acid (7, 12). As shown in Table 1 the concentration of ascorbic acid in each of these tissues from enzyme-treated animals was significantly higher than the corresponding tissue in bovine albumin treated animals.

TABLE 1

Tissue concentrations of ascorbic acid in response to L-gulonolactone oxidase treatment of ascorbic acid-deficient guinea pigs

Groups of four guinea pigs were treated with either bovine albumin or L-gulonolactone oxidase as described in Methods. Plasma samples were taken over a 390-min period, after which the animals were sacrificed, the tissues listed removed, and their ascorbic acid concentrations determined. Values are expressed as mg of ascorbic acid/100 g wet weight of tissue ± SEM. All values from enzyme-treated animals are significantly higher than comparable tissues from bovine albumin-treated controls ($P < 0.05$) (17).

Treatment	(*N*)	Adrenal	Liver	Lung	Spleen
Bovine albumin	4	5.7 ± 0.6	3.2 ± 0.4	3.0 ± 0.3	5.4 ± 0.6
L-Gulonolactone oxidase	4	21.3 ± 3.4	7.7 ± 1.1	4.8 ± 0.2	8.2 ± 0.4

Because scurvy is a lethal disease, it was possible to further test for *in vivo* synthesis of ascorbic acid by increasing the survival time of guinea pigs fed no ascorbic acid. In order to do this several modifications of the procedure were necessary. For example, multiple administrations of enzyme would be required, which was possible because the exteriorized tube allowed access to the dialysis bag. Furthermore, since the enzyme itself is isolated by the dialysis bag, antibody formation and a subsequent immune reaction should not occur. A bag was first implanted into 7-day ascorbic acid-deficient guinea pigs, and the animals were allowed to recover for 3 or 4 days before enzyme was placed in the bags. The animals were then given the enzyme preparation and L-gulonolactone as in the previous experiments. When subsequent enzyme treatments were carried out the contents of the dialysis bag were first removed, and the bag was rinsed with normal saline prior to placing a new enzyme preparation in the bag. On the days of enzyme administration and when judged necessary, 50 mg of chloramphenicol palmitate was given p.o. Also, on the day following treatments, 10 mg of the antibiotic was added to the contents of the bag in the hope of preventing growth of cellulytic microorganisms.

Figure 2 shows the growth curves of two enzyme-treated guinea pigs while on the ascorbic acid-deficient diet. One of these animals survived 47 days, nearly twice

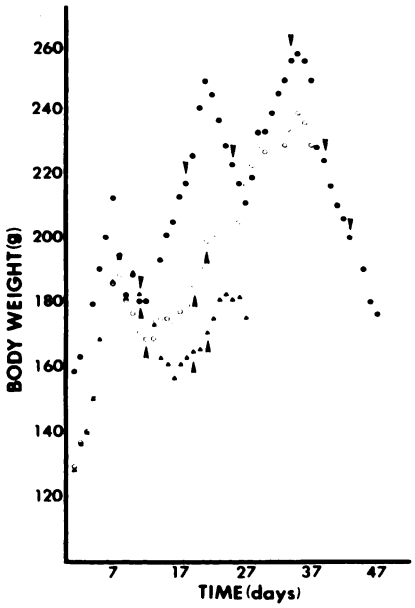

FIG. 2. *Growth curves of L-gulonolactone oxidase- and bovine serum albumin-treated guinea pigs*

Guinea pigs were started on the ascorbic acid-deficient diet and weighed daily. On day 8 a dialysis bag was surgically implanted within the peritoneal cavity of each animal. On day 11 or 12 partially pure preparations of L-gulonolactone oxidase were placed in the bags of two of the animals, curves 1 (○) and 2 (●), and BSA in the bag of the third guinea pig, curve 3 (▲). L-Gulonolactone was administered to each animal. On days marked with an arrow the contents of the bags were removed and replaced with fresh enzyme (animals 1 and 2), or bovine albumin preparations (animal 3), and L-gulonolactone was again administered. In other experiments five additional bovine albumin-treated animals have been tested, and curve 3, shown here, represents the animal that survived longest (27 days) under the regimen.

the normal time of survival of guinea pigs fed the ascorbic acid-deficient diet. A control animal given L-gulonolactone and bovine albumin in place of enzyme is shown for comparison. Because guinea pigs in this size range invariably die by the 29th day on an ascorbic acid-free diet (13–15), these animals further demonstrate *in vivo* synthesis of the vitamin by enzyme-treated animals.

Mapson (16) has reported that L-gulonolactone has no antiscorbutic activity. However, because large amounts of L-gulonolactone were administered, it was important to show that this precursor alone, when given according to the regimens used, was not responsible for the prolonged survival. Also, since the treated animals received chloramphenicol, any role of this antibiotic in preventing scurvy had to be ruled out. To do this four guinea pigs were placed on the deficient diet and given chloramphenicol palmitate and L-gulonolactone according to the same schedule as the long-term animal 2 in Fig. 2. These animals differed from the control animals in Fig. 2 in that no surgery was carried out and no proteins were administered. All four of these animals survived only the normal length of time (23 to 28 days) on the deficient diet.

It was assumed that foreign proteins contained within the dialysis bag would not be antigenic. The fact that the long-term animals survived several enzyme administrations suggests that this is, in fact, the case. To further test for an immune response to the administered enzyme, double immunodiffusion tests were carried out on serum isolated from these enzyme-treated animals. Figure 3 shows that the guinea pigs did not develop precipitating antibody against the administered proteins according to Ouchterlony immunoprecipitin tests. The unprotected enzyme preparation is, however, antigenic and yielded several precipitin bands. One of these precipitin bands could be stained for L-gulonolactone oxidase activity.

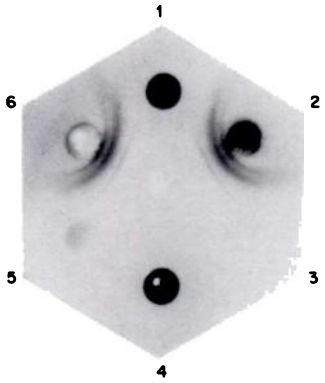

FIG. 3. *Ouchterlony double immunodiffusion analysis of serum from enzyme-treated guinea pigs*

The enzyme-treated animal represented by curve 1 in Fig. 2 was bled on day 37 immediately after death, and serum (15 μl) isolated from this animal was placed in well 1. Serum from the animal represented by curve 2 in Fig. 2 was prepared on day 46 and was similarly placed in well 4. Wells 2 and 6 contain 15 μl of the serum isolated from another guinea pig that received i.p. injections (28 and 7 days prior to bleeding) of the same partially pure L-gulonolactone oxidase preparation used in the replacement study. The center well contains 15 μl of this enzyme preparation (18 mg protein/ml). For improved visualization the plate was stained with Coomassie blue.

DISCUSSION

The fact that guinea pigs as well as primates are unable to synthesize ascorbic acid is due to an inherited enzyme deficiency. Since the trait is homozygous in this species, it provides a convenient model for studying an enzyme-deficiency disease in which experiments are not limited by the availability of diseased animals. In the present study this has allowed the development and testing of a procedure for administering the missing enzyme. The results of this study demonstrate synthesis of ascorbic acid in guinea pigs given L-gulonolactone and L-gulonolactone oxidase. The enzyme is placed in a dialysis bag that has been implanted within the peritoneal cavity of the animals. Synthesis is observed by both chemical analysis of vitamin concentrations in tissues and biologic assay.

The use of an implanted semipermeable enzyme container such as this has certain clinical potential. Notably, the time of survival of scorbutic guinea pigs is significantly prolonged using this procedure. Furthermore, guinea pigs in this study received multiple treatments with foreign proteins, and although conclusive immunologic data have not yet been obtained, containment of the proteins appears to at least decrease the immune response. Another possible advantage of a device of this nature is that it should allow for the containment together of more than one enzyme, possibly even entire metabolic pathways.

Unlike many inborn errors in metabolism, scurvy is not accompanied by a large accumulation of precursors. This is because the step prior to the oxidation of L-gulonolactone is reversible and the equilibrium favors the precursor (16), which can undergo further metabolism by a different pathway. In order to demonstrate synthesis by enzyme administration alone, the enzyme would probably have to be directed to specific sites in the body where L-gulonolactone concentrations are sufficiently high. For this reason it is probably necessary to supplement the animals with L-gulonolactone in addition to administering enzyme. In this study this was done by intraperitoneal injections. This would not be a concern, however, in replacing enzyme in certain other enzyme-deficiency diseases in which the metabolic block results in high precursor concentrations throughout the body.

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Send reprint requests to: Paul H. Sato, Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Mich. 48824.