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Genetics

Immunologic evidence that the gene for L-gulono-γ-lactone oxidase is not expressed in animals subject to scurvy

(ascorbic acid/vitamin C/genetics)

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ABSTRACT **L-Gulono-γ-lactone oxidase (L-gulono-γ-lactone:oxygen 2-oxidoreductase, EC 1.1.3.8) is the enzyme that catalyzes the terminal step of L-ascorbic acid biosynthesis in mammalian liver. The absence of the oxidase activity in primates and guinea pigs is the reason why these animals are subject to scurvy, which must be considered an inborn error of metabolism. Attempts were made to determine if a protein immunologically crossreactive with L-gulono-γ-lactone oxidase is present in these animals. Detergent-solubilized microsomal preparations from guinea pig and African green monkey liver did not precipitate the antisera directed to either rat or goat enzyme, nor did any of the other cell fractions obtained from guinea pig liver react with either antiserum. No crossreactive protein was detectable in guinea pig microsomes even with the sensitive procedure of micro-complement fixation. On the other hand, extracts of all 10 other mammalian (4 orders) liver microsomes tested were shown to contain L-gulono-γ-lactone oxidase activity that did crossreact with antibodies to the rat and goat enzymes. One explanation of these findings is that, in the guinea pig, and perhaps in primates too, the structural gene for L-gulono-γ-lactone oxidase is not expressed.**

The pathway for L-ascorbic acid biosynthesis in mammals is well documented (1) and is shown below:

D-glucose ⟶ D-glucuronic acid ⟶ L-gulonic acid ⟶ L-gulono-γ-lactone ⟶ L-ascorbic acid

Guinea pigs and primates are lacking in liver microsomal L-gulono-γ-lactone oxidase (L-gulono-γ-lactone:oxygen 2-oxidoreductase, EC 1.1.3.8) activity, which is required for the last step in the formation of the vitamin (1, 2).

The lack of an enzymatic activity could be due to a regulatory gene mutation or a structural gene defect. In the former instance, little activity will be observed, but if any enzyme is present it will behave normally. In a structural gene defect one might expect appreciable amounts of altered protein or incomplete polypeptides that might crossreact with antiserum against the native enzyme.

Recent studies on hereditary metabolic diseases have revealed that aberrant forms of an enzyme, which manifest altered kinetics, physical instability, or no activity at all, are present in many instances (3). Since the initial finding of the missing step in guinea pigs and primates, no information has appeared concerning the genetic basis of this deficiency.

It was of interest, therefore, to see whether those mammals that are unable to synthesize ascorbic acid have such defective enzymes. With the purification of L-gulono-γ-lactone oxidase from rat and goat liver (4), it was possible to prepare antisera to them and to use immunological methods to determine if crossreactive protein related to L-gulono-γ-lactone oxidase is present in guinea pigs and monkeys.

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MATERIALS AND METHODS

Preparation of Antisera to Rat and Goat L-Gulono-γ-lactone Oxidase. L-Gulono-γ-lactone oxidase was purified from liver microsomes by procedures which will be published elsewhere (4). Sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated that the preparations of both the rat and goat enzymes were essentially homogeneous. The rat enzyme was further purified by polyacrylamide gel electrophoresis (4), and the gel segment containing enzyme was used for antibody production according to the method of Hartman and Udenfriend (5). The goat enzyme could not be purified in this manner by gel electrophoresis. Fifty to 100 μg of enzyme in 0.5 ml of saline was mixed with 0.5 ml of Freund's complete adjuvant (Difco Laboratories) and injected into the footpad of a rabbit. An intradermal injection of the same mixture was administered into the back of the rabbit 3 weeks later. One week after the booster injection, antiserum was collected.

Preparation of Detergent-Solubilized Microsomes. Since sodium deoxycholate has been used to solubilize L-gulono-γ-lactone oxidase from microsomes with a high yield (6, 7), this detergent was employed for the solubilization of all the microsomes used in this study. Livers of rat, mouse, hamster, gerbil, guinea pig, rabbit, goat, sheep, cattle, dog, and African green monkey or kidneys of rat, bullfrog, and chick were homogenized in 4 volumes of 0.25 M sucrose using a Polytron homogenizer (Kinematica GmbH). The homogenate was centrifuged for 15 min at 10,000 × *g*, and the supernatant was centrifuged for 60 min at 100,000 × *g*. The sedimented microsomes were washed with 1.15% KCl and were solubilized at 5 mg of protein per ml in a solution containing 0.7% sodium deoxycholate, 50 mM potassium phosphate buffer (pH 7.5), and 1 mM EDTA. After centrifuging for 60 min at 100,000 × *g*, each solubilized microsomal preparation was dialyzed overnight against 200 volumes of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, and then centrifuged for 60 min at 100,000 × *g*. The supernatant was concentrated, using Aquacide II (Calbiochem), to a protein concentration of approximately 35 mg/ml.

The Ouchterlony test was carried out in agarose plates (IDF Cell II, Cordis Laboratories), which were developed for 20 hr at room temperature. L-Gulono-γ-lactone oxidase activity was stained for by incubating the plates in a reaction mixture containing 2.5 mM L-gulono-γ-lactone, 0.33 mM phenazine methosulfate, 0.12 mM nitroblue tetrazolium, 1 mM EDTA, and 50 mM potassium phosphate buffer (pH 7.5). The incubation was carried out in the dark for 10 min at room temperature.

The micro-complement fixation test was performed as described by Casey (8). For these studies, the deoxycholate-solubilized liver microsomes of rat, guinea pig, and monkey were prepared as described above, but EDTA was omitted and the samples were diluted to a protein concentration of 0.6 mg/ml.

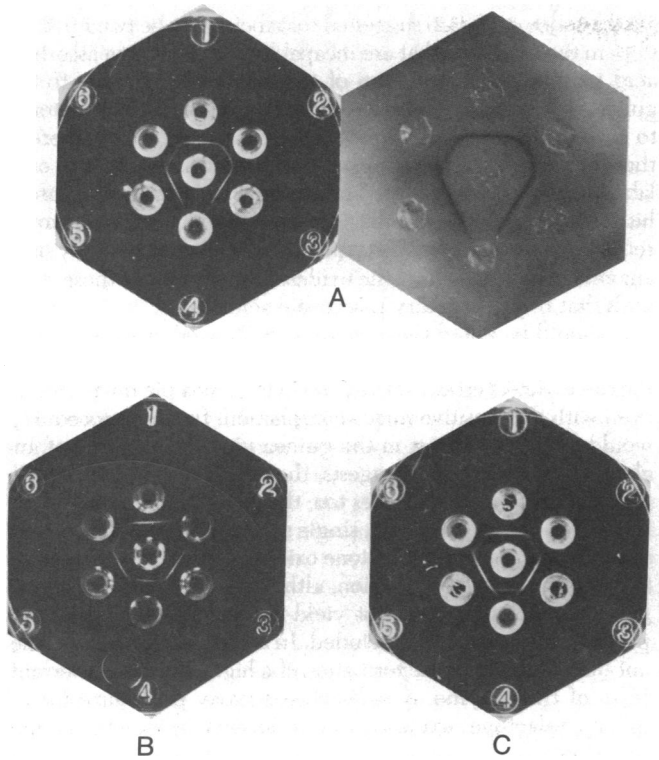

FIG 1. Ouchterlony test patterns of rabbit antiserum to rat L-gulono-γ-lactone oxidase with detergent-solubilized microsomes from various animals. Wells 1, 3, and 5 of all plates contained the purified rat L-gulono-γ-lactone oxidase (0.4 mg/ml), and the center wells of each plate contained undiluted rabbit antiserum to rat enzyme. (A) Well 2, rat; well 4, guinea pig; and well 6, mouse. The *right-hand* picture is the plate stained by enzymic activity. (B) Well 2, monkey; well 4, hamster; and well 6, gerbil. (C) Well 2, sheep; well 4, goat; and well 6, cattle.

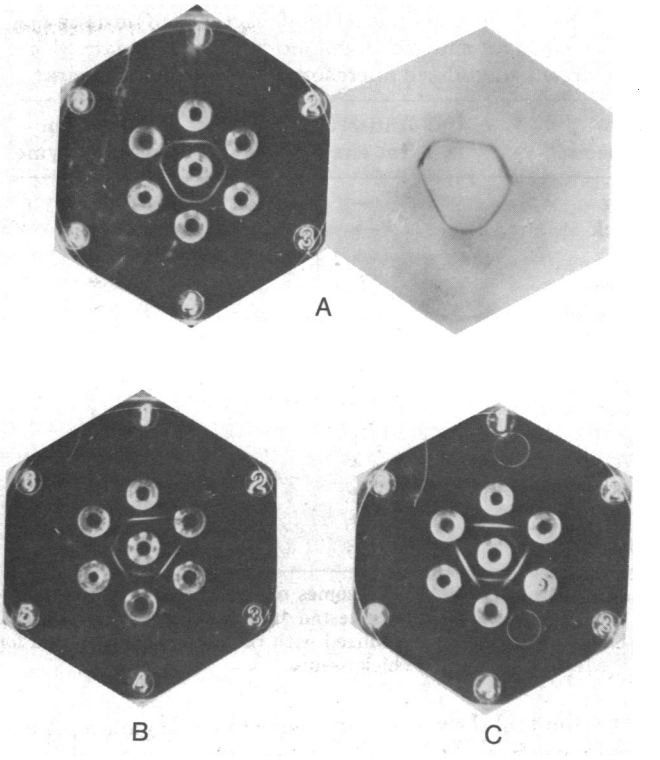

FIG 2. Ouchterlony test patterns of rabbit antiserum to goat L-gulono-γ-lactone oxidase with detergent-solubilized microsomes from various animals. Well 1, 3, and 5 of all the plates contained the purified goat enzyme (0.2 mg/ml) and the center wells of each plate contained rabbit antiserum to goat enzyme, diluted 2-fold. (A) Well 2, sheep; well 4, goat; and well 6, cattle. The *right-hand* picture is the plate stained by enzymic activity. (B) Well 2, monkey; well 4, hamster; and well 6, gerbil. (C) Well 2, rat; well 4, mouse; and well 6, guinea pig.

Protein was assayed by the Lowry method (9) except for the purified enzyme preparations, which were assayed by the fluorescamine method (10). With both reagents, bovine serum albumin served as the standard.

RESULTS

Fig. 1 shows examples of Ouchterlony test patterns with the antiserum directed to rat L-gulono-γ-lactone oxidase. As shown in Fig. 1A, the precipitin line formed with the detergent-solubilized rat liver microsomal preparation fused with the precipitin line of the purified rat enzyme, indicating the antigenic identity of the preparations. The precipitin line formed with detergent-solubilized mouse liver microsomes showed spur formation, indicating that the mouse enzyme is antigenically related but not identical to the rat enzyme. The precipitin lines were also stained by L-gulono-γ-lactone oxidase activity, indicating that the enzyme protein is, in fact, involved in the immunoprecipitation (Fig. 1A). Also, as shown in Fig. 1B and C, detergent-solubilized liver microsomal preparations from hamster, gerbil, goat, sheep, and cattle reacted with the antiserum to rat enzyme; these also formed spurs with the purified rat enzyme. These precipitin lines were also stained with enzyme activity (data not shown). In contrast, the antiserum did not precipitate detergent-solubilized liver microsomes from either the guinea pig or monkey or kidney microsomes from the rat; the rat kidney does not contain L-gulono-γ-lactone oxidase activity.

Crossreactivity of antiserum against goat L-gulono-γ-lactone oxidase was also examined by the Ouchterlony technique. Examples of the test are shown in Fig. 2A, B, and C. As with the antiserum directed to the rat enzyme, detergent-solubilized microsomes from the guinea pig and monkey showed no precipitin line, while samples from all other mammals tested gave immunoprecipitin lines that were enzymatically active (Fig. 2A). Results of the Ouchterlony test for extracts from all the animals investigated are summarized in Table 1. Frog kidney microsomes solubilized by the detergent, which were highly active with respect to L-gulono-γ-lactone oxidase activity, did not react with the antisera to either enzyme. On the other hand, chick kidney microsomes, which were also enzymatically active, reacted very well with antiserum against rat enzyme but did not form a precipitin line with the antiserum to the goat enzyme (data not shown). Microsomes from rabbit, dog, and cat liver were also found to crossreact with antisera to both the rat and goat enzymes. Other cell fractions of the guinea pig liver were also investigated, and it was found that extracts of nuclei, with mitochondria, and cytosol contain no protein crossreactive with antiserum to either the rat or goat enzyme.

To see whether guinea pig liver microsomes contain a crossreactive protein that does not form a visible precipitate with the antisera, a micro-complement fixation test was used. Antiserum to rat enzyme, which gave a positive test with rat microsomal extracts, even when the latter were diluted over 1 to 600, did not yield a positive complement fixation test with undiluted guinea pig liver microsomal extracts. Antiserum to goat enzyme also failed to give a positive complement fixation test with undiluted guinea pig liver microsomal extracts. With rat liver microsomal extracts, complement fixation was detected even at dilutions of over 1 to 10. The tests with both sera were

Table 1. Immunological crossreactivity of antisera directed to rat and goat L-gulono-γ-lactone oxidase with detergent-solubilized microsomes from various species*

Species	Antiserum to rat enzyme	Antiserum to goat enzyme
Frog	–	–
Chick	+	–
Rat	+	+
Mouse	+	+
Hamster	+	+
Gerbil	+	+
Guinea pig	–	–
Rabbit	+	+
Dog	+	+
Cat	+	+
Goat	+	+
Sheep	+	+
Cattle	+	+
Monkey	–	–

* Detergent-solubilized microsomes of livers from all animals except frog and chick were tested by the Ouchterlony method. Kidney microsomes solubilized with the detergent were used for the tests with frog and chick tissues.

at the limits of their sensitivity, indicating that guinea pig microsome extracts have no detectable crossreacting protein related to L-gulono-γ-lactone oxidase.

DISCUSSION

The immunoprecipitin studies with antisera to rat and goat L-gulono-γ-lactone oxidase indicate that extracts of guinea pig liver do not contain a crossreactive protein in microsomes or in any of the other cell fractions. Even the more sensitive micro-complement fixation test failed to detect any crossreactive protein in guinea pig liver microsomal extracts. Considering the very broad crossreactivity of the antisera among the mammalian species tested, these findings suggest that an aberrant form of L-gulono-γ-lactone oxidase is not formed or that there is a *major* alteration of the protein structure brought about by the genetic defect. In the case of the monkey, occurrence of a crossreactive protein that reacts but does not precipitate with antisera against the rat and goat enzymes has not yet been looked for by micro-complement fixation studies. Such micro-complement fixation experiments and radioimmunoassays can be used with tissues from monkeys and humans as well as with guinea pigs, to make absolutely certain that crossreactive protein is not present.

Antisera to goat L-gulono-γ-lactone oxidase did not form precipitates with frog or chick kidney microsomes, and the anti-rat enzyme serum did not react with frog kidney microsomes, although these microsomes were shown to have L-gulono-γ-lactone oxidase activity (11). These findings may be interpreted in terms of the vast difference in antigenic determinants to be expected between amphibia, birds, and mammals. It is noteworthy that antisera directed to the rat and goat enzymes were found to react with microsomal extracts from the rabbit, the animal used for antibody production. The phenomenon occurs, no doubt, because the rat and goat enzymes have antigenic determinants that are shared by the rabbit enzyme.

Chatterjee *et al.* (12) suggested that there may be two missing steps in those animals that are incapable of synthesizing ascorbic acid (namely, the reduction of D-glucurono-γ-lactone to L-gulono-γ-lactone as well as the oxidation of L-gulono-γ-lactone to L-ascorbic acid). Chatterjee and coworkers (6) considered that both steps must take place in microsomes. Studies in our laboratory‡ indicate that the findings on which Chatterjee based his conclusion concerning the absence of D-glucurono-γ-lactone reductase were wrongly interpreted. It appears that only one enzyme, L-gulono-γ-lactone oxidase, is deficient in those animals that require dietary L-ascorbic acid.

It should be noted that the antisera showed crossreactivity to all mammalian species listed, and even to the chick enzyme. Failure to detect crossreactive protein in guinea pig microsomes, even with the sensitive micro-complement fixation procedure, would rule out, at least in the guinea pig, the presence of an aberrant enzyme. This suggests, therefore, that in the guinea pig, and perhaps in primates too, the genetic defect in scurvy is the lack of expression of a single structural gene, the one responsible for L-gulono-γ-lactone oxidase. Very likely this is due to a regulatory gene mutation, although an altered structural gene product that does not yield precipitable crossreacting polypeptides cannot be excluded. In addition, one cannot rule out the possibility of the formation of a highly unstable aberrant form of the enzyme. A radioimmunoassay procedure for L-gulono-γ-lactone oxidase should answer these remaining questions.

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