Table 2. Substrate support of 11β- and 19-hydroxylation of steroid by gerbil adrenal mitochondria in vitro

Precursor	Androstenedione (nmoles/15 min/mg mitochondrial protein)*		Testosterone	
Products	19-OHA	11β-OHA	19-OHT	11β-OHT
Additions**				
NADPH 1 mM	2.3	3.0	0.7	2.2
NADPH 1 mM + Ca^{2+} 10 mM	3.5	4.4	1.0	0.44
DL-Isocitrate	24.3	19.6	1.8	1.5
Malate	1.7	1.5	0.9	0.5
α-Ketogluterate	–	–	0.6	0.2
Succinate	–	–	0.3	0.2

* Average of duplicate incubations carried out in 0.15 KCl, 7 mM $MgCl_2$, 25 mM HEPES with 90 μM steroid; a separate experiment using testosterone as substrate gave results comparable to those listed.
** Substrate 10 mM final concentration.

Results and discussion. NADP-IDH activity was detected in all subcellular fractions tested (table 1). In fresh mitochondria the activity of NADP-IDH was 3-fold greater than that for NAD-IDH (table 1, exp. 2). Though mitochondrial disruption increased the activities of both IDH isozymes, the rise in activity of intramitochondrial NADP-IDH was the greater. NAD-IDH activity was modestly elevated by 1–10 mM ADP. NADP-IDH kinetics were studied in two separate experiments using different preparations of disrupted mitochondria; apparent K_m 0.58 mM, V_{max} 280 nmoles/min/mg mitochondrial protein. In frozen-thawed adrenal homogenate, the activity of malic enzyme was 2% that of NADP-IDH; virtually no malic enzyme was detectable in disrupted mitochondria. In contrast, in disrupted homogenate or mitochondria, high levels of malate dehydrogenase were evident (table 1). Thus mitochondrial dehydrogenations of isocitrate vs malate generate, respectively, NADPH vs NADH. Higher rates of mitochondrial steroid hydroxylation occur with the generation of intramitochondrial NADPH than with intramitochondrial NADH; reducing equivalents from NADH can be transferred to NADP with the aid of the energy dependent transhydrogenase[4,7]. These differences explain why rates of the 19- and 11β-hydroxylation of either A or T were greater in the presence of isocitrate than with malate (table 2). In this study A was a better precursor than T for steroid 19- and 11β-hydroxylation. Thus adrenal mitochondria of the gerbil, as in the rat[3] possess significant levels of NADP-IDH. In the intact adrenal cell it is possible that other substrates may gain entry to the mitochondria and be used to generate NADPH. However, the activity of NADP-IDH in the mitochondria is sufficient to support the levels of steroid 11β- and 19-hydroxylation.

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L-Gulonolactone oxidase is present in the invertebrate, *Limulus polyphemus*

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Summary. L-Gulonolactone oxidase (EC 1.1.3.8) which catalyzes oxidation of L-gulonolactone to L-ascorbic acid was detected in tissues of *Limulus polyphemus*.
Key words. Limulus polyphemus; horseshoe crab; invertebrate; L-gulonolactone oxidase; L-ascorbic acid.

Many vertebrates have the ability to synthesize L-ascorbic acid; those that do not do so have been shown to lack the enzyme L-gulonolactone oxidase (EC 1.1.3.8, GLO) which catalyzes the final step in the synthesis–oxidation of L-gulono-1,4-lactone to L-ascorbic acid[1–6]. Several invertebrate animals including certain insects[7–10] and penaeid shrimp[11], have been found to require dietary ascorbic acid and thus appear to be unable to synthesize it. Only one, the pink bollworm *(Pectinophora gossypiella)* has been reported to be an apparent synthesizer[10]; its content of L-ascorbic acid increased when it was maintained on a diet devoid of the vitamin. Possibly *Trogoderma granarium* and *Tribolium castaneum* also fall into this category[12]. GLO has been sought in homogenates of snails, prawns, crabs, spiders, annelids, centipedes and leeches but none of them was found to have it[3]. Claims of synthesis by insect tissue[13–15] have been discounted on methodological grounds[16]. The present study demonstrates that one invertebrate, the chelicerate arthropod, horseshoe crab *(Limulus polyphemus)* possesses an enzyme capable of synthesizing L-ascorbic acid from L-gulonolactone.

Materials and methods. Adult *Limulus* of both sexes were obtained from the Marine Biological Laboratory, Woods Hole, MA, in five separate shipments between 1976 and 1980. They were maintained at 12 °C without feeding in Instant Ocean, an artifical seawater preparation. Adult rats *(Rattus norvegicus,* Sprague-Dawley strain) were from stocks maintained in our facility and adult chickens *(Gallus gallus)* were obtained locally. All tissues were wrapped in parafilm immediately after removal and were frozen on dry ice or in a freezer and kept at −20 °C until assayed.

L-Gulono-1,4-lactone was synthesized by the method of Frush and Isbell[17] and the D-isomer was purchased from Sigma. Nuclear magnetic resonance spectra of these preparations were essentially identical and their optical rotations were of equal magnitude and opposite sign ($[\alpha]_D^{20} = +54°$ and $-54°$ for D- and L-respectively).

GLO activity was determined by a modification of the method of Ayaz et al.[18]. This method involves homogenization of the tissue in phosphate buffer, incubation of the homogenate with L-gulono-1,4-lactone at 37 °C, oxidation with charcoal, incubation with 2,4-dinitrophenylhydrazine (DNPH) at 47 °C and measurement of absorbance at 520 nm of the bis-dinitrophenylhydrazone of L-dehydroascorbic acid. Modifications in-

Table 1. L-Gulonolactone oxidase activity and ascorbate content in tissues of *Limulus polyphemus*

Tissue	Animals	GLO activity (μmoles/g/h) Range	Mean	SD	Ascorbate (μg/g) Range	Mean	SD
Muscle	5	0.12–0.65	0.33	0.20	4.7–11.6	7.8	3.2
Gut	3	0.32–0.47	0.42	0.08	12.3–20.8	15.4	4.7
Central nervous system	2	0.40–0.56			21.7–24.8		
Hepatopancreas	10*	0.13–0.25	0.19	0.04	5.4–23.8	12.4	6.4
heated	3**	nil					
Heart	12*	0.29–1.10	0.55	0.23	5.0–20.1	11.7	5.0
heated	3**	nil					

* Only eight of the hepatopancreas and 10 of the heart samples were analyzed for ascorbate content. ** Homogenates heated in boiling water bath for 5 or 10 min.

Table 2. Effect of substrate configuration on L-gulonolactone oxidase activity

Species (tissue)	Activity (μmoles/g/h) D-Gulonolactone	L-Gulonolactone
Rattus norvegicus (liver)	0.07	11.65
Gallus gallus (kidney)	0.00	34.00
Limulus polyphemus (heart)	nil	0.29

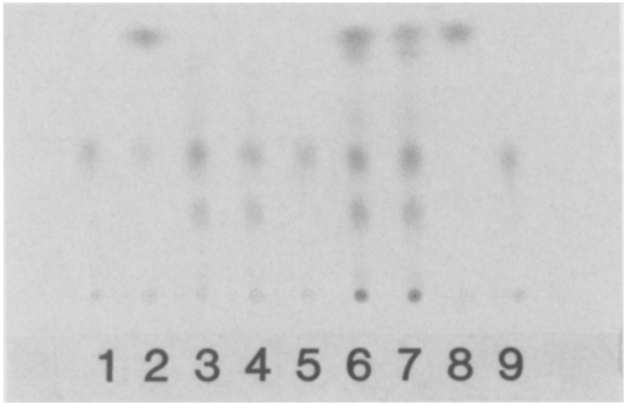
troduced in this study included reduction of the assay volume, elimination of sodium deoxycholate from the buffer and elimination of centrifuging of the homogenate. Sodium deoxycholate was eliminated because if was found to diminish the stability of GLO of *Limulus* at 37 °C.

DNPH derivatives for qualitative analyses were prepared from homogenates in 0.05 M sodium phosphate buffer, pH 7.4, of approximately 5 g of *Limulus* heart or 0.5 g of *Rattus* liver. Homogenates were centrifuged at 10,000 × g for 25 min to remove endogenous ascorbate; tests on supernatants from several successive resuspensions and recentrifuging of the pellet showed that virtually all of the endogenous ascorbate was removed in the first supernatant. The pellet was resuspended in 10 ml of fresh buffer, L-gulono-1,4-lactone was added and the mixture incubated for 7.5 h at 37 °C. Enzyme action was stopped and ascorbate oxidized by adding 18% HPO_3-16% TCA and charcoal. Charcoal and precipitated protein were removed by centrifuging. The supernatant was incubated with a solution of 2,4-dinitrophenylhydrazine (6 g/100 ml) and thiourea (0.4 g/100 ml) in 9 N sulfuric acid for 2 h at 47 °C. The derivatives were centrifuged at 15,000 × g for 12 min without refrigeration. The supernatant was poured off and the precipitate washed with distilled deionized water, freeze-dried, dissolved in acetone (approximately 5 mg/ml) and spotted (3–5 μl) on Kodak Silica Gel Sheets (No. 13181) which were then developed for 15–20 min with the solvent system of Navon and Levinson[19].

Results. Quantitative determinations of ascorbate content and GLO activity in several *Limulus* tissues are in table 1. All of the tissues analyzed contained ascorbic acid but the levels were much lower than those found in vertebrate tissues[20,21]. Heart had the highest GLO activity and thus was the tissue of choice for further experiments. GLO in heart and hepatopancreas was sensitive to heat; the activity was completely destroyed on exposure of the homogenate at 100 °C for 5 min.

The GLO activity in *Limulus* heart homogenates as well as in those of tissues from known synthesizers, *Rattus* and *Gallus*, is specific for the L-isomer of gulono-1,4-lactone (table 2).

Thin layer chromatography (fig.) showed spots from both the *Limulus* and *Rattus* preparations having $R_f = 0.54$, identical to that of the derivatives prepared from authentic L-ascorbic acid. The components of $R_f = 0.93$ were identified as unreacted dinitrophenylhydrazine and that of $R_f = 0.33$ is probably a derivative of glucose. The component with $R_f = 0.63$ is unidentified.



TLC of DNPH derivatives. 1, 5 and 9: Derivatives of authentic L-ascorbate. 2: Same as 1 plus unreacted dinitrophenylhydrazine. 3: 1 plus 4. 4: Derivative from rat liver. 6: Derivative from *Limulus* heart. 7: 1 plus 6. 8: Unreacted dinitrophenylhydrazine. All run on Eastman No. 13181 thin layer sheets with ethyl acetate, chloroform, glacial acetic acid, acetone in the volume ratio of 35:25:3.5:3.0.

Discussion. These experiments demonstrate the presence of GLO in the invertebrate *Limulus polyphemus*. Standard assays yielded low but consistent activity of this enzyme in a number of tissues. The activity in *Limulus* is abolished by heating and requires the L-isomer of substrate, thus indicating that it is enzyme catalyzed[22]. The activity is not confined to one or two tissues as in vertebrate liver and kidney. In this connection it is of interest that GLO has been reported widely distributed among tissues of chick embryos but is lost from all except kidney during development[23]. Unlike previous workers[13–15] who claimed biosynthesis of L-ascorbic acid by insects but failed to identify the product adequately, we have used TLC to confirm the formation of a derivative of ascorbic acid. To our knowledge, this is the first demonstration of the presence of L-gulonolactone oxidase activity in invertebrates. The evolutionary position of *Limulus* suggests that further studies on other protostomes could yield valuable new information on the evolution of the ability to synthesize ascorbic acid.

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