

SEQUESTRATION OF ASCORBIC ACID BY THE LARVAL LABIAL GLAND AND HAEMOLYMPH OF THE TOBACCO HORNWORM, *MANDUCA SEXTA* L. (LEPIDOPTERA: SPHINGIDAE)*

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Abstract—Tissues from *Manduca sexta* were examined for the presence of L-ascorbic acid and L-gulonolactone oxidase. L-Ascorbic acid was found in eggs, larval labial gland, haemolymph, gut, muscle, cuticle, adult nervous tissue and gonads at concentrations ranging from < 10 to > 150 mg per 100 g wet tissue. No ascorbate was detected in larval fat body and Malpighian tubule or adult salivary gland. Concentrations in labial gland and haemolymph increased 80- and 10-fold, respectively, during the fifth larval instar such that the labial gland surpassed all other tissues in ascorbate concentration. Since tissues from insects reared on an L-ascorbate-deficient diet contained no detectable vitamin C and L-gulonolactone oxidase was absent from tissue extracts, the hornworm apparently acquired L-ascorbate solely from the diet.

Key Word Index: Ascorbic acid, labial gland, *Manduca sexta*, tobacco hornworm, L-gulonolactone oxidase, vitamin C, haemolymph.

INTRODUCTION

FEW data are available on the requirement of insects for L-ascorbic acid. Dietary vitamin C is needed for normal growth, moulting, and fertility of several insects and this, or another compound with similar biological properties, may be an essential growth factor for all species. Although most insects subsisting on green plants need L-ascorbate to develop fully (DADD, 1973; HOUSE, 1974; CHATTERJEE *et al.*, 1975; CHIPPENDALE, 1978; KRAMER *et al.*, 1978; NAVON, 1978), it has been proposed that some species may not require the vitamin or may synthesize it *de novo* or rely on symbiotic organisms as a source (DAY, 1949; GAMO and SEKI, 1954; PIERRE, 1962; BRIGGS, 1962; RAYCHAUDHURI and BANERJEE, 1968). The ability of certain insects (or their symbionts) to synthesize the vitamin has not been demonstrated adequately. The present paper reports of experiments to determine diet and tissue levels of L-ascorbate for the tobacco hornworm, *Manduca sexta* L. and other species, and also to ascertain whether specific tissues are capable of converting a precursor, L- γ -gulonolactone, to vitamin C.

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MATERIALS AND METHODS

Animals

M. sexta eggs were obtained from Dr. J. P. REINECKE (Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Fargo, ND). Larvae were reared on agar-based diet (BELL and JOACHIM, 1976) at 28°C and 60% r.h. with a 16-hr photophase. *Plodia interpunctella* Hubner and *Periplaneta americana* L. were obtained from laboratory cultures. Dissection was performed under anesthesia by cooling to 5°C (SCHNEIDERMAN, 1967).

Paper chromatography

One-tenth gram of tissue was homogenized in 0.25 ml of 2% (w/v) metaphosphoric acid at 4°C. L-Ascorbic acid was separated by paper chromatography using ethyl acetate-acetic acid-water (6:3:2) as developing solvent. Chromatograms were sprayed with or dipped sequentially in (1) 0.10 ml saturated silver nitrate mixed with 20 ml acetone containing 0.1 ml of concentrated ammonium hydroxide, (2) 1 N NaOH in 95% ethanol, (3) 0.2 M aqueous sodium thiosulphate, (4) water (TREVELYAN *et al.*, 1950). The minimum amount detectable was 2 μ g after chromatography.

High performance liquid chromatography

Tissue extracts were analyzed with a Varian model 5020 liquid chromatograph equipped with a Rheodyne model 7120 loop injector valve, Tracor 970 variable wavelength detector set at 257 nm, a Hewlett-Packard 3385A printer-plotter automation system for determining the retention times and peak areas, and a Waters μ Bondapak column (3.9 mm i.d. \times 300 mm) for carbohydrate analysis. The buffer was eluted isocratically at 1 ml per min with a 1:4 (v/v) mixture of 0.01 M NaH₂PO₄, pH 4.46, and methanol. The minimum amount detectable was 10 ng.

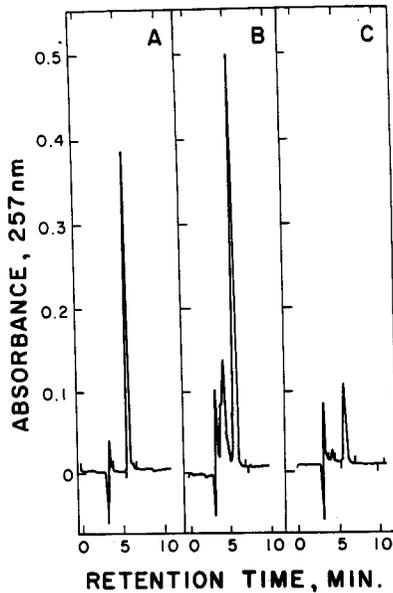


Fig. 1. High pressure liquid chromatography of L-ascorbic acid from insect tissues. A. L-ascorbic acid, 1.7 μ g; B. *M. sexta* haemolymph extract, 0.01 ml; C. *M. sexta* labial gland extract, 1.4 mg wet weight.

L-Gulonolactone oxidase assay

Tissues were assayed for L-gulonolactone oxidase by the method of AZAZ *et al.* (1976). Weighed portions of tissue (50–200 mg) were homogenized in 2 ml of 50 mM sodium phosphate pH 7.4 containing 0.2% sodium deoxycholate. Homogenates were centrifuged at 5000 g for 10 min at 4°C and 1 ml aliquots of the supernatant were incubated with 2 mM L-gulonolactone (Sigma) for 60 min at 35°C. Ascorbate was measured by the 2,4-dinitrophenyl hydrazine method of ROE and KUETHER (1943) as modified by GESCHWIND *et al.*

(1951). Chicken kidney was assayed as a control tissue rich in L-gulonolactone oxidase.

RESULTS AND DISCUSSION

Ascorbate levels in tissues

Previously it has been reported that *M. sexta* required 0.5 mM L-ascorbic acid in the diet for normal development (KRAMER *et al.*, 1978), suggesting that ascorbic acid was as important in insect nutrition as in vertebrate animal nutrition. The present paper surveys several tissues from the hornworm for the vitamin. Extracts were prepared in 2% metaphosphoric acid and analyzed by high pressure liquid chromatography (HPLC) and by paper chromatography. The HPLC retention time for ascorbic acid was 5.9 min (Fig. 1) while the R_f value on paper was 0.45. Neither of those methods, however, detects the second active form of vitamin C, dehydro-L-ascorbic acid, which may also be present in insect tissues. Preliminary evidence obtained using HPLC indicated that the dehydro form is present at about one-tenth the level of ascorbic acid. However, contaminating substances with similar retention times prevented an accurate determination. L-Ascorbate was abundant in the larval labial gland and haemolymph, ranging from < 1 to 10 mM in both tissues (Fig. 1 and Table 1). Vitamin C was also present in eggs, larval gut, muscle, cuticle, adult nervous tissue and gonads. For comparison, L-ascorbate in diet and faecal matter was assayed at 24 and 5 mg/100 g, respectively. This indicated that 80% of the vitamin was probably absorbed and/or metabolized by hornworm tissues. No ascorbate was observed in larval fat body and Malpighian tubules or adult salivary gland. The latter tissue is derived from the vitamin-rich larval labial gland during metamorphosis (HAKIM, 1976).

Table 1. L-Ascorbic acid content of tissues from *M. sexta*

Tissue	Stage	L-Ascorbate content*
Labial gland	L5	2–172 (24)
	L3	69 \pm 10 (4)
Haemolymph	L5	10–97 (24)
Brain and nerve cord	A	41 \pm 30 (4)
	A	63 \pm 8 (2)
Gonad	A	60 \pm 14 (2)
	—	43 \pm 3 (4)
Egg	—	39 \pm 8 (6)
Gut	L5	27 \pm 11 (4)
Muscle	L5	22 \pm 15 (4)
Cuticle	L5	15 \pm 4 (2)
Mouth exudate	L5	< 1 (4)
Fat body	L5	< 1 (3)
Malpighian tubule	L5	< 1 (2)
Salivary gland	A	< 1 (4)
L-Ascorbate deficient diet		
Labial gland	L5†	< 1 (6)
	L3‡	< 1 (6)
Haemolymph	L5†	< 1 (4)

* Units are mg L-ascorbic acid/100 g wet tissue or 100 ml haemolymph \pm S.D. Amounts of tissue or haemolymph analyzed were 20–300 mg or 0.3–0.5 ml, respectively. L = larva, A = adult, 3 = third instar, 5 = fifth instar. Number of determinations listed in parentheses.

† Hornworm reared on ascorbate deficient diet from middle of fourth larval instar.

‡ Hornworm reared on ascorbate deficient diet from neonate stage.

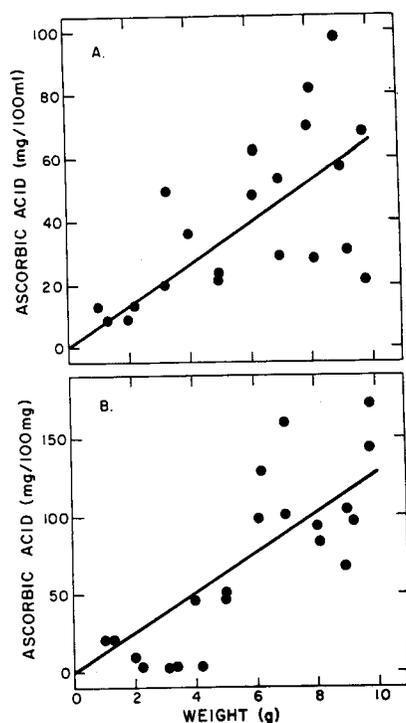


Fig. 2. Changes in the content of L-ascorbic acid in haemolymph and labial gland during larval development of *M. sexta*. A. Haemolymph: regression analysis yielded line defined as larval weight = 6.5 [ascorbic acid] at $\alpha = 0.01$ level and $R^2 = 0.83$. B. Labial gland: regression analysis yielded line defined as larval weight = 12.7 [ascorbic acid] at $\alpha = 0.01$ level and $R^2 = 0.87$.

The titre of L-ascorbic acid in several tissues during development was also examined. During the fifth larval instar, L-ascorbate concentration increased about 80-fold in the labial gland and 10-fold in the haemolymph (Fig. 2). Regression analysis revealed the labial gland accumulated vitamin C about twice as fast as did haemolymph. It is probable that an active transport mechanism is involved in the sequestration of L-ascorbic acid in the labial gland. A unidirectional influx of the vitamin has been demonstrated previously in vertebrate tissues such as mucosal border of guinea pig and human ileum (STEVENSON 1974; MELLORS *et al.*, 1977). Labial gland obtained from a third instar larva also contained a high titre of L-ascorbate (Table 1). It may be that vitamin C is depleted during the intermoult period, after which feeding recommences and tissue accumulation occurs. Three other phytophagous insects, *Spodoptera littoralis* (Boisduval) (NAVON, 1978), *Bombyx mori* (L.) (GAMO and SEKI, 1954) and *Schistocerca gregaria* (Forsk.) (DADD, 1960) showed high ascorbic acid levels during the instar and low levels at the moulting stage.

L-Ascorbic acid was also analyzed in tissues from hornworms fed a vitamin C-deficient diet. Without L-ascorbic acid neonate larvae grow to the third instar but they die before the next moult. These larvae retained little or no vitamin in the labial gland or haemolymph (Table 1). A similar result was characteristic of fifth instar larvae reared on a vitamin-free diet beginning at mid fourth instar. These larvae failed to complete

pupation. Those results illustrate further the importance of vitamin C in the development of *M. sexta*. Tissues become depleted of ascorbic acid in only a few days with major pathological consequences.

The observation that, among the tissues examined, larval labial gland sequestered the highest levels of L-ascorbic acid was unexpected in view of the minor role in development the organ plays. This tissue was analyzed because initially it was expected that the gland would be lacking in ascorbic acid. However, the vitamin concentration measured was among the highest reported even for tissues from other sources such as rat, guinea pig, and human (HORNIG, 1975).

Labial glands show great diversity of structure and function in insects (SNODGRASS, 1935). In almost all species the most common function is production of saliva. In some orders, the glands secrete silk, while in others they may function in excretion, in the reduction of haemolymph volume (TATCHELL, 1967) or in extraintestinal digestion (PETRALIA *et al.*, 1980). In *M. sexta*, the larval labial gland is divided into a large distal segment which produces a proteinaceous secretion and a smaller proximal segment in the form of a cuticle-lined duct (HAKIM and KAFATOS, 1974, 1976). After pupation the distal segment degenerates, whereas the duct differentiates into the adult salivary gland.

The physiological significance of the larval labial gland of *M. sexta* serving as a reservoir of L-ascorbic acid is unknown. Silk is not produced by this species except to a small degree during the first larval instar. Labial glands actively producing silk in the Indian meal moth, *Plodia interpunctella* Hubner, did not contain detectable levels of vitamin C (< 1 mg/100 g tissue). The contents of the hornworm gland may be secreted for other functions such as burrowing, which precedes pupation and probably requires an external lubricant (HAKIM and KAFATOS, 1976). In addition, fifth instar larvae undergo body wetting which occurs after the cessation of feeding (REINECKE *et al.*, 1980). In the latter case all accessible dorsal and lateral surfaces are coated in preparation for pupal ecdysis. Examination of the mouth parts during wetting revealed at least a portion of the fluid was derived from the labial gland duct opening. The fluid present on the mouth parts, or already deposited on the body contained L-ascorbic acid at a level of 15 mg/100 ml (mouth exudate, Table 1). Also, less than 20 mg L-ascorbate/100 g tissue was present in labial glands after the contents had been secreted.

L-Gulonolactone oxidase in insect tissues

The presence in insect tissues of L-gulonolactone oxidase, the enzyme catalyzing the final step in the biosynthesis of L-ascorbic acid from glucose, has been examined. Chicken kidney and liver served as controls; the former synthesized 10 μ g ascorbate/mg/hr, while the latter was inactive (< 0.1 μ g/mg/hr) as reported by CHAUDHURI and CHATTERJEE (1969). Within the limits of the assay, no evidence was found for L-gulonolactone oxidase in tissue homogenates from *M. sexta*, *Plodia interpunctella*, and *Periplaneta americana* L. (< 0.2 μ g/mg/hr). It is possible that some insects synthesize the vitamin or a similar factor at a rate too slow to measure, that they utilize a unique synthetic pathway, or that the assay procedure which

was developed for vertebrate tissue failed to detect the insect enzyme. Concerning proteolytic degradation, addition of the protease inhibitor trasylol to tissue homogenates was without effect (EISENTRAUT *et al.*, 1968).

The presence of L-ascorbic acid in specific insect tissues and the absence of the terminal enzyme in its biosynthetic pathway suggests that the insects examined here lack the ability to synthesize vitamin C and therefore derive ascorbate from the diet. Insects consume many highly specialized diets and some such as stored grain insects may be particularly adapted to utilize the vitamin efficiently since their food resource is deficient in ascorbate for certain vertebrate animals (WATT and MERRILL, 1975).

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