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**Ascorbic Acid Deficiency in
Phytophagous Insects - Its Biochemical
Nature and Its Role in Insect Hostplant
Relationship**

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ABSTRACT

The Spodoptera littoralis larvae required for the study were reared on a new calcium-alginate diet. When ascorbic acid deficient, these larvae and that of Manduca sexta consume food less efficiently and the feces are abnormally wet. The cuticle of the ascorbic acid deficient M. sexta contains more ash. In such insects, lipids, neutral carbohydrates, acid soluble collagen, exuvial amino acids and tyrosine in the alkali-soluble protein decreased. The activities of the enzymes ribonuclease and phosphatase-activated phenolase differ markedly in the deficient animals.

In S. littoralis, deficient pupae have shorter wing pockets and adults crippled wings because of premature sclerotization. Feeding moths with D-isoascorbic acid caused males to produce malformed spermatophores albeit without reduced mating capacity. Females mated to the analog-fed males laid only few and infertile eggs. This effect could not be canceled when such females were subsequently fed L-ascorbic acid. In malformed spermatophores, both soluble proteins and phenols were high; the latter accumulated at the expense of aromatic compounds of the undigestible proteins. Structural proteins were fibers high in cysteine-cystine and proline, as well as collagen containing hydroxylysine. Amino acid deficits could be linked with failure to stabilize specific components of the spermatophore.

Effects of ascorbic acid deficiency on Manduca sexta and Spodoptera littoralis larval feeding and cuticle composition

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ABSTRACT - Dietary L-ascorbic acid deficiency caused in intermolt 5th instar Manduca sexta larvae was characterized within 3 days of feeding by higher levels of: food consumption, weight gain and fecal water content. In Spodoptera littoralis, this effect, as compared with the normal feeding behaviour of larvae, was more significant. Reduced level of L-ascorbic acid in the diet (0.25%) did not support pupation; the pupae failed to expand properly the cuticular wing pockets at postecdysis, and as a result, the adults wings were crippled and with reflexed edges. Chemical extractions of deficient and normal cuticles from the two insects revealed marked differences in protein solubility. These cuticle in the deficient M. sexta contained higher ash content, lower levels of cuticle tensile strength, and of lipids, neutral carbohydrates, acid-soluble collagen, 7 amino acids from larval exuviae and tyrosine in the alkali-soluble proteins. Titters of chitin, total phenols, neutral salt-soluble and non-soluble collagenous proteins did not differ between cuticles of deficient and repleted larvae. The marked effects of the chemical deficiencies on cuticular deformities, are discussed.

Key Word Index: Vitamin C, L-ascorbic acid, cuticle, nutritional deficiency, structural proteins.

¹On sabbatical leave from the Department of Entomology, ARQ, The Volcani Center, Bet Dagan, Israel

INTRODUCTION

Ascorbic acid deficiency symptoms in phytophagous insects were characterized by retarded growth and moulting failure (Dadd, 1960; Navon, 1978; Kramer et al., 1978), deformations in heavily sclerotized cuticle (Navon, 1978) and crippled wings in moths (Chippendale et al., 1965). Malformed serratophores produced by male moths was another deficiency caused by per os application of D-optically-active analogs of ascorbic acid (Navon and Levinson, 1976; Navon and Marcus, 1982). Physiological effects of ascorbic acid link blood level of vitamin C with the moult stage (Dadd, 1960, Navon, 1978). The high abundance in hemolymph of mature larvae (Navon, 1978; Kramer et al., 1980) was surpassed by the labial gland concentration (Kramer et al., 1980). Chemically, cuticular phenoloxidation (Navon, 1978) and blood carbohydrates (Bounias, 1980) were affected, but no clear picture of the role of vitamin C in the insect was reported. This paper describes morphological and chemical pathologies in the cuticle of Manduca sexta and Spodoptera littoralis induced by mild and severe ascorbic acid deficiencies.

MATERIALS AND METHODS

Insect colonies: M. sexta were reared from eggs supplied by Dr. J.P. Reinecke, Agricultural Research, Science and Education Administration, United States Department of Agriculture, Fargo, ND, U.S.A.

S. littoralis was reared on a calcium-alginate diet according to Navon and Keren (1980), at the Department of Entomology, ARO, The Volcani Center, Bet Dagan, Israel.

Nutritional procedures. M. sexta was reared on an agar-based diet containing ample levels of ascorbic acid by the method of Bell and Joachim (1976). Neonates were reared collectively on Petri dishes in an environmental chamber maintained at 27° C and 54% r.h, 15:9 LD program. Following 6 days of normal growth, larvae were transferred to individual plastic vials containing fresh diet prepared with or without ascorbic acid. Supplementation with ascorbic acid was at a level of 0.4%. When ecdysis was completed, the larvae were transferred to a semi-synthetic diet prepared with or without added vitamin C.

The diet contained wheat germ, 80 g; vitamin-free casein, 36 g; sucrose, 44 g; Wesson salts, 3.5 g; cholesterol, 4 g; sorbic acid, 2 g; Nipagin, mold inhibitor, 1.4 g; streptomycin sulfate, 0.2 g; agar, 50 g; formaldehyde (37%) 1 ml; raw linseed oil, 6 ml; vitamin solution, 20 ml and distilled water to 1 L. The vitamin solution contained: nicotinic acid, 150 mg; riboflavin, 75 mg; thiamin, 35 mg; pyridoxine, 35 mg; folic acid, 25 mg, biotin, 3 mg and distilled water to 150 ml. Supplementation with ascorbic acid was at a level of 0.5% w/w. Larvae were fed singly on this diet cubes of 2 cm³. Fresh food was offered daily. Three replicates of 15 larvae each were used per treatment.

Apolysed S. littoralis larvae were collected from the insect colony and were left to moult at 17°C. in an incubator. When ecdysis was completed the larvae were transferred to plastic vials each containing 2 cm³ piece of semi-synthetic diet without ascorbic acid (Navon, 1978). The control diet contained 0.5% vitamin C. The larvae were reared singly in an incubator at 25±1°C. and 15:9 LD program. Separately, neonates from the insect colony were reared on this diet containing 0.25% or 0.5% vitamin C. control diet did not contain ascorbic acid. Fifty larvae were used singly in two replicates for each of the dietary combinations. Pathologies caused in larvae, pupae and adults by the vitamin deficiency were recorded.

Chemical analysis. Cuticle structural protein and collagen were extracted by the methods of Navon et al., (1983) and Rhodes and Miller, 1978 respectively. Ash was prepared in a muffle furnace held at 600°C for 24 hr. Dry weight was established by desiccation at 25°C over phosphorus pentoxide. Amino acid analysis, lipid and natural sugar determinations were as described (Navon, et al., 1983).

RESULTS

The pathology of ascorbic acid deficiency in lepidopterous larvae was manifest by a diverse pathology, each symptom expressed at different stages of the insect's developmental program. The earliest visual consequence of vitamin inanition was a bloated appearance at the conclusion of the first day. The probable cause of this abnormality was retention of water since food intake was more or less identical in deficient and repleted subjects.

Fifth instar larvae of *Manduca* reared on adequate diet gained 1164±73 mg in 25 h (Fig. 1).

Fig. 1. Effect of 72 hr vitamin C deprivation on growth, food consumption and fecal excretion by fifth instar intermolt Manduca sexta. Values represent the mean $\pm$ S.E. per larva in two populations of twelve animals each. Solid lines and points - ascorbic acid sufficient; broken lines, ascorbic acid deficient. (Δ), (Δ)-larval fresh weight; ($\square$), ($\square$)-food consumption, fresh weight; ($\circ$), ($\circ$)-fecal fresh weight.

Insert: Daily efficiency of food utilization, fresh weight basis.

Solid boxes replete populations; open boxes, deficient animals.

Fifth instar larvae of *Manduca* reared on adequate diet gained 1164 ± 73 mg in 25 h (Fig. 1). If the larvae were reared on a deficient diet, on the other hand, weight increased to 1248 ± 61 mg in 25 h. On the succeeding day the time-course of weight increase was identical, and at the conclusion of day 3 of growth favored the replete population. Fig 1, also shows a striking loss of efficiency with respect to food utilization on the part of the deficient larvae. This effect was expressed as early as the second day of nutrient withdrawal. In spite of the noticeable decline in body weight compared to the control group on day 3 the inanates consumed 4659 ± 263 mg of food compared to 3697 ± 287 mg for the controls. For the entire four day feeding period, the average fifth instar larvae reared on a normal diet gained 5670 ± 217 mg while consuming 8967 ± 180 mg of diet, whereas the deprived larva gained 5100 ± 106 mg at the expense of 10131 ± 341 mg of diet.

The initial gain in weight of the deficient larvae cannot be ascribed to consistent and major differences in the rate of defecation (Fig. 1). On the other hand the inanimate subjects produced drier fecal pellets during the period of superior weight gain and retention of body water. The fecal dry weight (in percent of the fresh weight) for normal and deficient larvae, respectively, was 42.2 vs 36.0 on Day 1 and 30.0 vs 21.0 on Day 2. When the bloating symptom was in remission on Day 3, the dry weight values of the two samples was essentially identical.

S. littoralis larvae fed on ascorbic acid-omitted diet gained more weight and produced more feces than did the inanates, on each day of the feeding period (Fig. 1). Unlike M. sexta, larval weights on day 3 still differed between treatments; the deficient larvae gained 147 ± 12 mg and the weight increase in the healthy population

Fig. 2. Effect of 72 hr vitamin C deprivation on growth and fecal excretion by 6th instar intermoult Spodoptera littoralis. Values represent the mean $\pm$ S.E. in three replicates of 20 larvae each. Solid lines and points, ascorbic acid sufficient; broken lines, ascorbic acid deficient. (O), (O) - larval fresh weight; ($\square$), ($\square$) - fecal fresh weight. Numbers in parentheses indicate percent dry weight of feces.

was 102 ± 17 mg. Nevertheless, percent dry weight being higher in the inanates was similar in the two insects. Possibly, the deficient insects failed to reabsorb water from the feces.

Fig. 3. Malformations in pupal wing pockets and in adults wings caused by larval feeding on a diet containing reduced level of ascorbic acid (0.25%). In the deficient pupa (A) the wing pockets are incompletely expanded, leaving an exposed area of IVth abdominal segment (es_{IV}). (B) Normal pupa from a diet containing recommended level of vitamin C (0.5%). ($ti_1 + ta_2$) tibia and tarsus of 2nd pair of legs. (a) antenna. (w) wing. (p) proboscis. (as IV) abdominal segment IV.

At the moult stage when feeding and excretion are stopped, larval water balance is affected, as well; apolysed normal 4th instar (10 insects) weighing 73.7 ± 3.1 mg lost 11.1% of water during ecdysis and respectively 5th instans of 225 ± 5.0 mg lost 9.3% water after the new cuticle was expanded and hardened. Ascorbic acid-ommitted diet cubes of 1169 ± 27 mg each lost 3% of water within one day of feeding and respectively, 4.7% of water was evaporated from the diets containing vitamin C. These low levels of diet dehydration probably would not limit water uptake by the larvae.

Fig. 3 shows that feeding the larvae from neonates on a diet with reduced level of ascorbic acid (0.25%) caused pupal malformations in the wing pockets, at postecdysis of pupal moult stage. At first, ecdysis proceeded normally; the exuviae of head and trunk was shed off towards the distal part of the pharate pupa. While moving backwards, the exuviae "combed" the cuticular pockets of tibiae, tarsi, antennae, wings and proboscis (Fig. 3B). This caused the appendages to be placed in the right order of one organ being adjacent to the next. At this stage, the wing pockets covered the first and second abdominal segment only. Then, these pockets increased in size reaching the 4th segment. But, in the deficient pupa the cuticular wing cases were not stretched to the normal size, leaving an exposed area of the 4th segment (Fig. 3A). The white color of the fat body in the pupa was observed through the transparent exposed cuticle.

Table 1 - Malformations in pupae and adults caused by reduced level of ascorbic acid in larval diet (0.25%).

Level of ascorbic acid in the diet (%)	Range (mm) of exposed 4th abdominal segment	ADULT		
		Normal	Malformed wings	Failed to emerge
0.25	0.9-1.0	0	11	2
	0.6-0.8	2	8	1
	0.4-0.6	14	0	3
	0.0-0.4	6	1	2
	Total	<u>22</u>	<u>20</u>	<u>8</u>
0.5	0.4-0.6	0	1	0
	0.0-0.4	45	0	4
	Total	<u>45</u>	<u>1</u>	<u>4</u>
0	All larvae died with moulting difficulties			

Table 1 shows that when the exposed segment width ranged 0.6-1.0 mm 79% of the moths emerging from these pupae had malformed wings. A smaller width of an uncovered cuticle did not affect the adult wings.

The malformed wings were either crippled or with reflex edges, but coloration and picture of the wings were normal. Table 1 also shows that dietary ascorbic acid of 0.5% supported normal pupation, whereas omitting vitamin C from the diet caused total mortality of larvae.

Both M. sexta and S. littoralis show major differences in cuticle composition and physical properties as a consequence of ascorbic acid withdrawal. Following the exhaustion of lipid, protein and carbohydrate reserves, the integument constituted 14% of the wet weight of M. sexta larvae denied vitamin C (Table 2). Exoskeleton of inantes failed to one third the load as the controls in spite of the consumed intake of food by the scorbutic population. Apparently, the cuticle benefited physically from the superior efficiency of food utilization when ascorbic acid was included in the diet. In the case of M. sexta it is probable that the decline in tensile strength was caused by the replacement of chitin and structural protein by water and inorganic constituents (Hepburn and Joffe, 1976). In this respect, deficient cuticle contained 22% more water and five times more ash than the healthy larvae. Among the organic constituents, lipids declined six-fold in register with vitamin deprivation. In general, fluctuations accompanying ascorbic acid limitation produced identical effects in both species. In the case of other metabolites considerable specificity was encountered in the two lepidopterans. Deficient M. sexta cuticle contained only one third as much netural carbohydrates as the controls, whereas this discrepancy was not manifest in S. littoralis

The two species showed marked differences in solubility characteristics with respect to cuticle proteins. The response of M. sexta to vitamin deficiency

Table 2 - Gross composition of thoracic and abdominal cuticle from Manduca sexta and Spodoptera littoralis (fifth instar) larvae following ascorbic acid withdrawal.

Constituent	<u>Manduca sexta</u>		Interval vitamin withheld (days)	<u>Spodoptera littoralis</u>	
	<u>Normal</u>	<u>Deficient</u>		<u>Normal</u>	<u>Deficient</u>
Critical fracture point ¹	100	30	6	nd ²	nd
Integument/body wt (x100)	5.4	14.3	9		
Food utilization index ³	0.631±01	0.505±01	3	nd	nd
Lipid, % ⁴	6.6	1.1	9	3.2	0.5
Protein ⁵ , soluble in					
Guanidine, 6 M	367	197	9	69	9.
NaOH, 0.1 M	126	120	9	9	27
Collagen, acid-soluble	65	25	3	nd	nd
Pronase-susceptible	208	274	9	555	481
Insoluble	422	412	9	256	389
Neutral carbohydrate ⁶	292	113	3	50	59
Ash ⁷	75	370	9	nd	nd
Water (%)	24.8	19.1	3		
Pigmentation	Green	Blue green	6	brown-black	deep black
No significant differences	Chitin ⁸ , total phenols, neutral salt-soluble collagen, neutral salt-soluble non-collagen protein			nd	nd

¹ Foot notes in page 18.

Table 2 - cont.

¹Relative load, gm/cm²

²nd, not determined

³Weight gain/food consumed; gm/gm (wet weight) for 3 days vitamin withdrawal during fifth instar

⁴Head capsule with appendages.

⁵ugm/mgm cuticle.

⁶Conjugated hexoses and pentoses of soluble glycogen and glycoproteins.

⁷ugm/mgm residue remaining after protein solvent series.

⁸As acid-labil glucosamine.

consisted of a decline in acid soluble collagen, probably due to under-hydroxylation of the basement membrane. Aside from a decline in guanidine-soluble components, no major differences were observed in the NaOH, pronase-susceptible and residual protein depots.

S. littoralis on the other hand, showed reduced levels of quinoidine-soluble proteins and higher recoveries of alkali-soluble and residual components.

The extraction scheme, while indicating of major disorganization within the protein components of the cuticle, was insufficient sensitivity to detect obvious changes in chromo-protein distribution or composition. In both species aberrant coloration accompanied the exhaustion of ascorbic acid reserves.

The physical and chemical compositions described above imply faulty deposition of exoskeletal strata. The mechanism of protein synthesis does not permit major changes in amino acid composition of a given protein. Thus discrepancies in amino acid composition of homologous regions of normal and pathological specimen must indicate either under or overproduction of proteins assigned precise structural, enzymatic, reserve or regulatory function. The epi- and exocuticular polypeptides can be recovered by harvest of the exuviae providing ready access to compositional differences within these regions. Table 3 clearly shows that nine amino acids, namely aspartic acid, threonine, serine, glycine, proline, valine, isoleucine, lysine and arginine shows significant differences in recoveries. Since the epicuticle forms a minor portion of the casting, these differences probably signify improper fabrication of the sclerotized exocuticle.

Lepidopterous larval cuticle is non-uniform with respect to hardness and sensitivity to ascorbic acid withdrawal. This discrepancy is strongly expressed when the wing pockets of S. littoralis pupae and the head capsule of M. sexta are compared to homologous tissue from replete specimen. In the case of S. littoralis, a probable cause for the inefficient food utilization described in Table 1 is malformation of the mouth parts and head capsule (Navon, 1978). Table 4 presents the amino acids composition of the alkali-soluble proteins of the head capsule of M. sexta. It can be seen that the tissues differ in their content of tyrosine and arginine-rich components. Of particular interest is the near-identity of hydroxylysine titers. Synthesis of the latter from lysine is ascorbate dependent. It would appear that completion of the collagenous component of the basement membrane of the head dominates the demand for the vitamin even in the presence of marginal tissue levels.

Table 3 - Amino acid recoveries from exuviae of fifth instar Manduca sexta following nine days of ascorbic acid deficiency. Components of similar titers not listed. Values in nmoles/mg of thoracic and abdominal casting. Instrument accuracy $\pm 1.5\%$

<u>Amino acid</u>	<u>Normal</u>	<u>Deficient</u>
Aspartic acid ¹	194	89.1
Threonine	74	19
Serine	373	115
Glycine	26	88
Proline	28	103
Valine	102	56
Isoleucine	32	12
Lysine	56	25
Arginine	37	18

¹ Includes amides.

That the symptoms were related specifically to the scorbutic condition and was to general inanitation could be deduced from the maintenance of normal levels of chitin, phenols, neutral collagen, neutral salts (NaCl) - soluble proteins and fecal dry weight. Since the absence of ascorbic acid from the diet produced no measurable changes in the dry weight of the diet, the bloated appearance of the larva during the long deficiency syndrome could not be ascribed to differences in water intake.

Table 4 - Amino acid composition of 0.1 M NaOH-soluble proteins of the head and head appendages of Manduca sexta. Tissue extracted with 0.1 M KCl-phosphate, pH 7.4, and 6 M guanidine hydrochloride prior to extraction with 0.1 M NaOH at 2°C. Values in moles %. Instrument accuracy ±1.5%.

Amino acid	Ascorbic acid	
	<u>sufficient</u>	<u>deficient</u>
Aspartic acid ¹	9.5	11.7
Threonine	3.3	3.5
Serine	5.7	6.8
Glutamic acid ¹	14.2	16.2
Proline	6.1	6.1
Glycine	10.3	11.5
Alanine	15.9	19.2
Valine	2.1	3.2
Methionine	1.8	1.9
Isoleucine	1.2	1.6
Leucine	6.1	6.3
Tyrosine	11.4	3.0
Phenylalanine	3.1	1.9
Histidine	1.7	2.1
Lysine	4.4	5.1
Arginine	3.0	5.5
Hydroxylysine	0.24	0.32

¹ Includes amides.

DISCUSSION

In those warm-blooded animals sensitive to marginal levels of dietary ascorbic acid, the primary deficiency symptoms can in almost all instances, be ascribed to massive failure in the synthesis or maintenance of collagenous tissues (Barnes and Kodicek, 1976). This lesion dominates the pathology by virtue of collagen constituting the largest component of the protein complement of these organisms. In phytophagous lepidopterans, on the other hand, collagen is restricted to the thin basement membrane of the cuticle and the neurilemma of the brain and ganglia (Kramer and Seib, 1982). Even in the case of these two loci, the fibrils are diffuse and account for only a small portion of the protein titer. Although failure of these barriers might account for the rapid and massive depletion and structural elements (Bounias, 1980) the near identity of hydroxylysine titers in normal and replete populations suggest that the developmental inhibition has other causes (Table 4). That the tissues of ectodermal origin constitute the principal site of vitamin expression can be deduced from major differences in tensile strength, lipid levels and polypeptide distribution (Table 2). These findings extend previous studies on the spermatophore, a reproductive organ of identical embryonic origin (Navon et al., 1983). The decline in cuticle lipid, furthermore, is in accord with requirement for ascorbic acid by the fatty acids and hydrocarbon hydroxylases of the insect integument (Blomquist and Jackson, 1979). Other indications of exoskeletal involvement are: a. the high incidence of mortality due to moulting failure following larval depletion of ascorbic acid (Navon, 1978), b. pupal malformation of the cuticular wing pockets caused by moderate ascorbic acid deficiency and c. the striking alteration in the protein complement of the cast skin elaborated during ecdysis (Table 3).

Both S. littoralis and M. sexta show major loss of fat and cuticle integrity on prolonged culture in the absence of the vitamin (Navon, 1978; Kramer et al., 1978). The consequences of nutritional inadequacy in the two insects could be observed only after 72 hr after vitamin withdrawal as evidenced by discrepancies in the rate of the larval weight gain, fecal dry weight and food utilization

(Figs. 1, 2 and Table 2). Close examination of the inanate larvae as well as time-course of weight gain provide strong indications of early differences in water content at intermoult stage and this effect seems enhanced by natural loss of water at the moult stage. The sequestration of inorganic constituents by the hornworm integument is probably involved in disorganization of the fluid balances particularly with respect to carcass water (Table 2) and secretion of lubricant from the labial gland (Kramer et al., 1981). The changes expressed early in the deficiency syndrome eventually disturb the sequence of polypeptide deposition in the exoskeleton as expressed by moult-related morbidity. Both species failed to stabilize structural proteins normally insoluble in 6N guanidine (Table 2). In M. sexta, a significant fraction of the crosslinked (sclerotized) proteins of the head capsule are dissolved by dilute sodium hydroxide. It can be seen from Table 4 that a marked alteration in the protein complement followed vitamin inanition, particularly with respect to the retention of polypeptides rich in tyrosine. This observation confirms the maldistribution of aromatic components observed in the noctuid spermatophore (Navon et al., 1983). When considered in terms of the many systems that require ascorbic acid without involvement in collagenesis, it would appear that phytophagous arthropodes afford new and important entries to these vital metabolic systems..

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REFERENCES

- Barnes, M.J. and Kodicek, E. (1976) Biological hydroxylation and ascorbic acid with special reference to collagen metabolism. Vitams. Horm. 30: 1-41
- Bell, R.A. and Joachim, F.G. (1976) Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. Ann. Entomol. Soc. Am. 69: 365-373
- Blomquist, G.J. and Jackson, L.L. (1979) Chemistry and biochemistry of insect waxes. Prog. Lipid Res. 17: 319-345
- Bounias, M. (1980) Effects of ascorbic acid and dehydroascorbic acids per os on the larval glycemia and amino-acidemia of artificially fed Laspeyresia pomonella (Lepidoptera). Insect Biochem. 10: 521-527.
- Chippendale, G.M., Beck, S.D. and Strong, F.M. (1965) Nutrition of the cabbage looper Trichoplusia ni (Hubn.) - I. Some requirements for larval growth and wing development. J. Insect Physiol. 11: 211-223
- Dadd, R.H. (1960) Some effects of dietary ascorbic acid on locusts. Proc. R. Soc. B. 153: 128-143
- Hepburn, H.R. and Joffe, I. (1976) On the material properties of insect exoskeletons
In: The insect integument (Ed. Hepburn, H.R.) Chapter 12, Elsevier Scientific Pub. Co. Amsterdam.

- Kramer, K.J. and Seib, P.A. (1982) Ascorbic acid and the growth and development of insects. ACS Advances in Chemistry No. 200, pp. 276-291.
- Kramer, K.J. Mundall, E.C. and Law, J.H. (1980) Purification and properties of manducin, and amino acid storage protein of the hemolymph of larval and pupal Manduca sexta. Insect Biochem. 10: 279-288
- Kramer, K.J., Hendricks, L.H., Liang, Y.T. and Seib, P.A. (1978) Effect of ascorbic acid and related compounds on the tobacco hornworm, Manduca sexta. Johansson (Lepidoptera: Sphingidae). J. Agr. Food Chem. 26: 874-878
- Kramer, K.J., Spiers, R.D., Lookhart, G., Seib, P.A. and Liang, Y.T. (1981) Sequestration of ascorbic acid by larval labial gland and haemolymph of the tobacco hornworm, Manduca sexta L. (Lepidoptera: Sphingidae) Insect Biochem. 11: 93-96.
- Navon, A. (1978) Effects of dietary ascorbic acid on larvae of the Egyptian cotton leafworm Spodoptera littoralis. J. Insect Physiol. 24: 39-44
- Navon, A. and Levinsion, H.Z. (1976) Oral application of D-glucoascorbic acid to adult Spodoptera littoralis (Boisduval) inducing sterility by spermatophore malformation. Bull. ent. Res. 66: 437-442
- Navon, A. and Keren, S. (1980) Rearing the Egyptian cotton leafworm, Spodoptera littoralis, on a practical calcium-alginate diet. Phytoparasitica. 8: 205-207

Navon, A. and Marcus, R. (1982) D-isoascorbic acid fed to Spodoptera littoralis moths, induces sterility due to spermatophore malformation. J. Insect Physiol. 28: 823-828

Navon, A., Nesbitt, J., Henzel, W., Mulligan, K., Mullen, J.A., Sugarman, M. and Lipke, H. (1983) The effect of D-isoascorbic acid on spermatophore composition in Spodoptera littoralis. Insect Biochem. 13: 247-250

Rhodes, R.K. and Miller, E.J. (1978) Physicochemical characterization and molecular organization of collagen A and B chains. Biochemistry. 17: 3442-3448.

Scorbutic pathologies in Manduca sexta larvae

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Phytophagous insects require ascorbic acid for normal growth and maturation. In nature these animals derive the necessary ascorbic acid from the plants on which they feed. In the laboratory, larvae reared on diets deficient in ascorbic acid succumb to the deficiency. Typical scorbutic symptoms include loss of weight, morbid appearance, progressive loss of movement and finally death. The biochemical pathology of the scorbutic state in these animals is little understood. Two major symptoms resulting from ascorbic acid deficiency have been described by the grantees. Both involve dysfunctions in tissues derived from the ectoderm. The first is a reproductive failure due to malformation of the tanned spermatophore in the adult male (1). The second symptom is observed in the caterpillar where heavy mortality follows the larval moult due to faulty crosslinking of cuticle proteins. One cause of the fault is aberrant deposition of cuticle proteins; the finding that incorporation of a hydroxylsine-rich protein was abnormal in both integument and spermatophore (Navon et al., 1983a, b) suggest that turnover of the protein was effected. This amino acid occurs in collagenous elements of mammals and in vertebrates (2). Faulty turn over can be ascribed to errors in the rates of synthesis or disposal of the metabolite in question. Series of experiments on the lysosomal hydrolases of integumental scrapings (Table 1) revealed that the half life of RNA was seriously effected by the secretion of three times the normal level of the latent particulate acid ribonuclease. The activity of lysosomal polysaccharases was unchanged as was cathepsin activity. Apparently one of the causes of faulty cuticle development was a decline in

Table 1. Activity of lysosomal hydrolases during ascorbic acid withdrawal. Larvae (Stage V) fed synthetic diets with and without the vitamin for four days before sacrifice. Average of three experiments.

Enzyme	Specific activity EU/mg		
	Normal	Deficient	Starved
Acid ribonuclease	92	288	102
Cathepsin	77	67	96
β -glucuronidase	26	31	41

Table 2. Incorporation of ^{14}C -uridine following withdrawal from Vitamin C. Average of three experiments.

Diet	^{14}C , dpm/mg RNA
Adequate	11,470
Deficient	32,400
Starvation	14,600

translation rates leading to subnormal protein deposition. It was subsequently demonstrated that during the early stages of ascorbic acid deficiency the larvae temporarily compensated for the tissue lesion by production of exceptional levels of "nonfunctional" or "junk" RNA that was subsequently disposed of by the acid hydrolase. These findings were then extended to show that early in the deficiency syndrome, the levels of RNA are artificially maintained at normal levels by a compensatory mechanism that temporarily enhances RNA synthesis. Within a few days however the restorative mechanism fails and cuticle disruption ensues (Table 2).

Another cause of the failure of crosslinking of cuticle protein is unregulated oxidation of o-diphenols transferred from circulation to quinones (3). It has been known for a long time that in vitamin C deficiency tyrosine metabolites accumulate (4). The exact mechanism of this phenomenon has not been established. Vitamin C has also been implicated in several stages of catecholamine metabolism. It has been shown to protect dopamine in vitro (5), and has been implicated in keeping the catecholamines in the reduced state in vivo (6). Alterations in the activities of enzymes involved in tyrosine metabolism has been shown in scurvy (7). Insects use a variety of tyrosine metabolites for physiological functions; eg as neurotransmitters and as sclerotizing agents. An important enzyme involved in catecholamine metabolism in insects is tyrosinase. Tyrosinase is present in the hemolymph as well as the cuticle of insects. In lepidopterous larvae dopamine is connected to peptidylated quinones in the bridge complex by this means. In the studies that followed it was shown convincingly that ascorbic acid plays a major role in the regulation of this enzyme. The system revealed by Ramesh's studies, furthermore, operates by a route notably different from the popular speculation that the reducing potential of the ketopolyol

Table 3. Effect of neutral phosphatase activity on plasma phenolase¹

Treatment	Ascorbic acid nutrition	
	Normal	Deficient
None	378	22
+ dopamine	1114	460
+ alkaline phosphatase	352	318
+ dopamine + alkaline phosphatase	1009	1266
+ NaF	31	22
+ EDTA and oxalate	58	39
+ alkaline phosphatase + NaF	41	51

¹Values in $\Delta A_{415\text{nm}}/5 \text{ min } 10^3$

vitamin acts as a redox poise for the phenol-quinone couple. On the contrary, Table 3 shows that the phenolase is a phosphoprotein that must be dephosphorylated by a neutral or alkaline phosphoprotein phosphatase for activation. In the absence of dietary vitamin C the phosphatase is not secreted or synthesized at the proper time in the moult cycle resulting in the production of destabilized untanned cuticle proteins. Since the overall levels of o-diphenols and TOTAL phenolase (phosphophenolase plus dephosphophenolase) are unchanged it is not surprising that this effect has been overlooked by others. This observation was confirmed by gel electrophoresis of activated phenolase from normal and deficient larvae. No difference in the number of titer components oxidizing dopamine was observed.

The evidence in detail consists of the following: Plasma from replete individuals darkens gradually on exposure to air or darkens immediately on addition of dopamine. The reaction is inhibited by the addition of ATP which plays a dual role. In the presence of the hemocytes part of the ATP initiates a cascade via adenyl cyclase and a protein kinase. The product is an inactive phosphorylated prophenolase. A second role for the trinucleotide is the inhibition of the protein phosphatase either by an allosteric mechanism or by the presence of the pyrophosphate residue on the nucleoside triphosphate which serves an inhibiting or competing regulator. When the protein phosphatase is inhibited by F^- or by chelation of Mg^{2+} phenol oxidation is greatly reduced. This latter inhibition can be reversed by the addition of an exogenous phosphatase with pH optima overlapping with that of the phenolase (pH 6.8-8.8). The findings with plasma from ascorbic acid-deficient larvae are in accord with the facts outlined above. That the phosphorylated prophenolase is present in the light colored plasma at normal levels can be verified by the addition of exogenous phosphatases. Immediate darkening is observed. When the added phosphatase is inhibited

with F- or ATP, pigmentation is prevented. In contradistinction to normal plasma, the deficient plasma is virtually devoid of neutral or alkaline phosphatase activity. Phosphatase activity is readily uncoupled from phenolase by the addition of specific chelators of Cu^{2+} which prevent darkening but do not alter cleavage of phosphate esters. The lesion is clearly due to phosphatase absence rather than an ascorbate requirement for phosphatase activity since preincubation with the vitamin does not endow deficient plasma with phosphatase activity. In the scorbutic state the animal is progressively debilitated, at death enzymes are released, which accumulate in the circulation, often reflecting tissue damage. In fact Gould and Shwachman have developed a bioassay for Vitamin C in guinea pigs using elevation of phosphatase activity as the parameter (8). We measured acid RNase activities in the hemolymph. Acid RNase activity is very low in the hemolymph of normal animals, but increases sharply in deficient animals. However incorporation of ^3H -uridine was more in the RNA of deficient animals (Table 4).

Table 4. ^3H -uridine incorporation into RNA	
Sufficient	59,925 cpm/mg RNA
Deficient	108,370 cpm/mg RNA
Starved	14,950 cpm/mg RNA

Surprisingly the activities of acid phosphatase, aryl sulfatase and β -glucuronidase did not show great variation between the normal and deficient larvae.

It is known that osmotic fragility of the lysosomes is increased in several pathological and physiological conditions (9). We therefore looked at the osmotic fragility of the lysosome rich fractions. Larvae were dissected under cold saline and cleaned of body fat and gut. The remaining soft tissues composed principally of muscle, structural and connective tissue were scraped into 0.25M sucrose containing tris buffer and gently homogenized. After removing the cell debris, the lysosome rich fractions were obtained by centrifugation at 27,000g. This pellet was suspended in 0.25M sucrose containing

Fig. 1 (from Ramesh, 1982)

this buffer, an aliquot was drawn at different time intervals, lysosomes spun down and the supernate assayed for acid phosphatase and protein. The total enzyme activity and protein content was obtained by incubating the lysosome rich fraction in 0.5% Triton X-100 containing buffer. Triton X-100 disrupts the integrity of the lysosomal membranes thus liberating the entire contents of the lysosomes into the supernats.

Para-nitrophenyl phosphatase activity released at any time point from the lysosomes of deficient larvae was about twice that of sufficient or starved animals, (Fig. 1). This suggests that the fragility of the lysosomal fractions is increased in deficient animals. Lysosomes are membrane-bound organelles, hence their integrity may be affected by agents causing damage to the membrane. One effect may be peroxidative damage to the membrane. Vitamin C can act as a powerful antioxidant (10).

1. Navon, A., J. Nesbitt, W. Henzel, K. Mulligan, J.A. Mullen, M. Sugumaran and H. Lipke. Insect Biochem. 13, 247, 1983.
2. Piez, K. and J. Grase. Biochem. Biophys. Acta 34, 24, 1959.
3. Navon, A., J. Nesbitt, W. Henzel and H. Lipke, (see in this report)
4. Sealock, R.R. and H.E. Silberstein, J. Biol. Chem. 135, 251.
Sealock, R.R., J.D. Perkinson and D.H. Basinski, J. Biol. Chem. 140, 153, 1941.

Rogers W.F. and F.H. Gardner, J. Lab. Clin. Med. 34, 1491, 1949.

Morris, J.E., E.R. Harper and A. Goldbloom, J. Clin. Invest. 29, 325, 1950.
5. Heikkila, R.E. and F.S. Cabbat, Life Sciences 32, 847, 1983.
6. Njus, D., J. Knoth, C. Cook, P.M. Kelley, J. Biol. Chem. 258, 27, 1983.
7. Knox, W.E. and M.N.D. Goswami, J. Biol. Chem. 235, 2667, 1960.
8. Gould, B.S. and H. Shwachman, J. Biol. Chem. 151, 439, 1943.
9. Deter, R.L. and C. DeDuve, J. Cell. Biol. 33, 437, 1967.
10. Niki, E., T. Saito, Y. Kamiya, Chem. Lett 5, 631, 1, 1983.

EVALUATION OF THE RESEARCH ACHIEVEMENT WITH RESPECT TO THE ORIGINAL RESEARCH PROPOSAL

The title of the BARD award I-62 was: "Ascorbic acid deficiency in phytophagous insects its biochemical nature and its role in insect hostplant relationship". The grantee followed the BARD reviewer's recommendation to save the practical aspect of this work namely, the role of ascorbic acid in insect hostplant relationship, for another BARD program. Instead, the reviewers proposed a full occupation of the scientists with physiological and chemical experiments to elucidate functions of ascorbic acid in the phytophagous insect. As instructed in the research plan, insect colonies were established both in Bet Dagan and in Boston to supply eggs, larvae and moths for the experiments. Ascorbic acid deficiency diets were composed to breed ascorbic acid depleted insects. Deficiency symptoms in moths were produced by feeding adults with D- analogs of ascorbic acid causing the male to produce malformed spermatophores. Larval cuticles and male spermatophores, both organs being sclerotized tissues of ectodermal origin, were used for analyses of tissues and protein amino acids. Morphopathological symptoms in cuticle and spermatophore formation indicated premature sclerotization. The phosphatase-activated blood phenoloxidase was a vitamin C dependant enzyme system; ascorbic acid controls dephosphorilation of this tyrosinase.

As expected of this research a better understanding is gained of ascorbic acid dependant systems governing insect growth, sclerotization and cuticle formation. The sterility effect caused in males fed D- analogs of ascorbic acid in a novel phenomenon which in future time could prove useful in controlling moths' reproduction.

DESCRIPTION AND RESULTS OF COOPERATION

During the course of the project insect material was dispatched between the Bet Dagan and Boston laboratories to analyse the ascorbic acid-depleted tissues. The principal investigator, spent a sabbatical year during the second annual BARD study, in the laboratory of Prof. H. Lipke, at the University of Massachusetts - Boston. The grantees analysed the composition of ascorbic acid deficient cuticle and of malformed spermatophores produced by males feeding on D- analogs of ascorbic acid. The study was later published (Insect Biochem. 13:247-250, 1983). The collaboration of the investigators in Boston was concluded when Prof. H. Lipke visited the Bet Dagan laboratory for three months, in February-May, 1983. During this visit, the chemical analyses of the deficient cuticles was completed and a paper entitled "Effect of ascorbic acid deficiency on Manduca sexta and Spodoptera littoralis larval feeding and cuticle composition" was submitted for publication in J. Insect Physiol.

BENEFIT TO AGRICULTURE

The investigators have shown that: a) larvae feeding on ascorbic acid deficient diet consume food inefficiently and loose water in the feces. This effect would be useful in the study of insect hostplant relationship, as an indicator of primary ascorbic acid-deficiency symptoms.

b) D- analogs of ascorbic acid fed to male moths induce sterility in females paired with them. Mating capacity of males was not affected by the analogs. Since these insect-chemosterilants are not harmful to humans and serves as an approved additive to bakery products, its use in pheromone traps could afford a safe and rapid means of inducing sterility in the pest population. The use of the analog in the pheromone-bait station assures the specificity of the method and protects beneficial insects that are not attracted to the trap.

PUBLISHED REPRINTS AND MANUSCRIPTS

- Navon, A. and Keren, S. (1980) Rearing the Egyptian cotton leafworm, Spodoptera littoralis, on a practical calcium-alginate diet. Phytoparasitica 8:205-207.
- Navon, A. and Marcus, R. (1982) D-isoascorbic fed to Spodoptera littoralis moths, induces sterility due to spermatophore malformation. J. Insect Physiol. 28:823-828.
- Navon, A., Nesbitt, J., Henzel, W., Mulligan, K., Mullen, J.A., Sugarman, M. and Lipke, H. (1983) The effect of D-isoascorbic acid on spermatophore composition in Spodoptera littoralis. Insect Biochem. 13:247-250.
- Navon, A., Nesbitt, J., Henzel, W. and Lipke, H. (1984) Effects of ascorbic acid deficiency on Manduca sexta and Spodoptera littoralis larval feeding and cuticle composition. submitted for publication in J. Insect Physiol.
- Ramesh, N. and Lipke, H. (1982) Acid **Ribonuclease** activation in insect scurvy. Proc. XIII th Congress ESA and CSA, Toronto p. 64.
- Ramesh, N. and Lipke, H. (1983) Dephosphorylation of prophosphophenylase during zymogen activation. Science (in preparation).