P_i equivalent to the added PP_i does not support the growth of the three species of *Desulfotomaculum*. For optimal growth on PP_i, acetate, yeast extract, and sulfate are required; acetate and yeast extract probably meet the needs of *Desulfotomaculum* for cell carbon (*5*), and sulfate provides this microorganism with an electron sink with which to adjust the oxidation level of the supplied fixed carbon. The concentration of sulfate was only one-tenth of that in the usual lactate-sulfate medium, but the requirement for acetate was unexpectedly high, with little growth occurring below a concentration of 0.2 percent. The physiological basis for this high acetate requirement has not been investigated, but it may involve the bioenergetics of the permeation of acetate into the bacteria (*6*). The stimulatory effect of PP_i on growth does not appear to be due to the facilitation of anaerobic acetate oxidation (*7*) by PP_i, as the ratio of the disappearance of acetate to the production of sulfide was 3:14 rather than the expected ratio of 1:1. Similar growth responses to PP_i, acetate, yeast extract, and sulfate were found for the other two species of *Desulfotomaculum* (*D. ruminis* and *D. orientis*), and we conclude that PP_i serves as a source of energy for growth of these anaerobic sulfate-reducing bacteria.

The growth response of *D. ruminis* is proportional to PP_i concentrations up to 0.04 percent and growth is accompanied by the hydrolysis of PP_i to P_i (*3*). In the absence of growth, there is little hydrolysis of added PP_i. Above 0.05 percent PP_i, there appears to be inhibition of growth that may be due to alkalization of the medium (*p*H 8.5). This aspect of growth has not been investigated further; however, similar results were obtained with *D. nigrificans* and *D. orientis*.

It was of interest to determine whether the enzymatic complement of cells grown on the PP_i medium differed significantly from that of cells grown on the lactate-sulfate medium. The specific activities of various enzymes found in PP_i and in cells of *D. orientis* grown on lactate-sulfate medium are shown in Table 2. The reductases of respiratory sulfate reduction—APS reductase (*8*), thiosulfate reductase (*9*), bisulfite reductase (*10*), and ATP-sulfurylase (*11*)—have about the same levels of activity in each cell preparation. Fumarate reductase (*9*) is absent in both preparations, and nitrite reductase (*12*), formate dehydrogenase (*9*), PP_i:acetate kinase (*3*), pyrophosphatase (*3*), and pyruvate dehydrogenase (*9*) are present at similar specific activities. The reason for the significantly higher hydrogenase activity in cells grown on PP_i medium is not known, but it may be due to difficulties with the assay procedure. These observations (also confirmed for *D. ruminis* and *D. nigrificans*) indicate that the bacteria growing on PP_i and lactate-sulfate media exhibit no basic changes in their enzymatic patterns, as has been reported for *Desulfovibrio vulgaris* when grown on different media (*13*).

There is no a priori reason to believe that the utilization of PP_i as a source of energy for growth is limited to the genus *Desulfotomaculum*. We have, therefore, obtained mud samples from a salt water spartina marsh and used them to inoculate the basal medium supplemented with 2.5 percent NaCl, with and without PP_i, under aerobic and anaerobic conditions. Within 24 hours at 37°C only the anaerobic enrichments containing PP_i showed extensive microbial growth and contained an unexpected number of morphological types of microorganisms that persisted through as many as five serial transfers. Similar results have been obtained with PP_i enrichments from freshwater anaerobic environments (*14*). The observed morphotypes were both Gram-negative and Gram-positive and included motile and nonmotile rods of different sizes, with and without spores, and large and small vibrios and cocci. The diversity of cell types strongly suggests that PP_i respiration is not restricted to *Desulfotomaculum*, which are characterized as spore-forming rods. Five new genera of sulfate-reducing bacteria have recently been described by Widdel (*15*), and the diversity of bacterial types found in these enrichment cultures may be due to growth of these new microorganisms on PP_i; however, some initial isolates from these enrichments do not appear to be sulfate-reducing bacteria. We have been unable to find any reports on the existence of PP_i in marine environments, but the formation of polyphosphates is common in bacteria (*16*) and might supply PP_i for the growth of the sulfate-reducing and possibly other bacteria.

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N-β-Alanyldopamine: Major Role in Insect Cuticle Tanning

Abstract. N-β-*Alanyldopamine is the major tyrosine metabolite in the hemolymph and cuticle during pupal tanning in the tobacco hornworm,* Manduca sexta *L. Its concentration in hemolymph increases over 800-fold above larval levels by the start of tanning and decreases as the pupal cuticle darkens and hardens. It is a major catechol in species representing several insect orders and is the preferred substrate for pupal cuticular* o*-diphenol oxidase. In insects,* N-β-*alanyldopamine appears to be the main precursor for tanning chemicals at certain developmental stages.*

N-Acetyldopamine has been considered the principal catecholamine metabolite in sclerotization or tanning of insect cuticle (*1*). We now report that *N*-β-alanyldopamine (NBAD) is the major catecholamine metabolite in hemolymph and cuticle during tanning of pupal cuticle in the tobacco hornworm, *Manduca sexta*, and that *N*-acetyldopamine is a relatively minor component.

Hemolymph was extracted from *M. sexta*, and the catechols were analyzed by liquid chromatography with electrochemical detection (LCEC) (*2*). The major oxidizable substance in hemolymph extracts from pharate or newly ecdysed pupae was an unknown compound with a longer retention time than dopa, dopamine, or *N*-acetyldopamine (compound V in Fig. 1). Labeled compound V was

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Table 1. Tyrosine metabolite concentrations in hemolymph of *Manduca sexta* during pupal development. Data are the means of three to four insects ± standard error. The percentage conjugated is shown in parentheses.

Stage*	Tyrosine metabolite concentration (μ*M*)			
	Dopa	Dopamine	*N*-Acetyl-dopamine	*N*-β-Alanyl-dopamine
Wandering larva				
Heart exposed	2.8 ± 0.4 (43)	9.2 ± 3.9 (63)	20.5 ± 11.3 (96)	5.2 ± 1.6 (21)
Pharate pupa				
Ocellar retraction	9.5 ± 1.0 (33)	77.2 ± 21.9 (88)	14.5 ± 5.0 (100)	180.1 ± 47.4 (52)
Tanned patches	31.5 ± 12.4 (17)	721.6 ± 148.9 (99)	48.6 ± 19.0 (94)	4166.4 ± 581.5 (97)
Pupa				
Newly ecdysed	25.6 ± 8.1 (16)	630.8 ± 75.2 (97)	48.9 ± 15.2 (94)	3356.5 ± 643.1 (79)
1 hour	32.8 ± 4.4 (14)	659.0 ± 132.8 (96)	39.3 ± 7.3 (93)	4119.6 ± 502.4 (70)
6 hours	28.7 ± 5.6 (20)	478.0 ± 84.0 (91)	18.4 ± 7.1 (65)	2984.0 ± 235.7 (39)
24 hours	34.2 ± 9.1 (18)	161.0 ± 19.0 (41)	18.7 ± 3.8 (0)	561.8 ± 45.7 (2)
48 hours	33.5 ± 5.9 (20)	85.3 ± 33.6 (43)	7.5 ± 2.2 (79)	214.3 ± 82.1 (2)

*See (*12*) for description of developmental events.

isolated after wandering larvae or pharate pupae were injected with [U-^{14}C]tyrosine (50 μCi). The metabolite showed an ultraviolet absorption spectrum at *p*H 3 identical to that of dopamine (maximum wavelength, 278 nm). Products resulting from hydrolysis with HCl were dopamine and β-alanine, as indicated by LCEC and by an amino acid analyzer, respectively (*3*). When the hydrolyzate was silylated and analyzed by gas-liquid chromatography, two peaks with retention times identical to those of β-alanine and dopamine were observed (*4*). Synthetic NBAD, when subjected to LCEC and ultraviolet spectroscopy, exhibited a retention time and absorption spectrum identical to those of the unknown metabolite (*5*). This evidence demonstrated that the major catechol metabolite of tyrosine during pupal tanning of *M. sexta* was NBAD.

N-β-Alanyldopamine was present at low concentrations in hemolymph of last-stadium larvae of *M. sexta* during the feeding and wandering phases. Higher levels occurred in early pharate pupae and peaked with an increase of about 800-fold shortly before pupal ecdysis (Table 1). During the accumulation of NBAD in pharate pupae, much of the substance was present as a conjugate, probably as a β-glucoside. Mild acid hydrolysis of extracts (1.2*N* HCl for 10 minutes at 100°C) resulted in a large increase in NBAD, as did incubation with β-glucosidase (Sigma) (acetate buffer, *p*H 5.6, for 1 hour at 37°C). We have also found that tyrosine is conjugated with glucose in this species (*2*), and this modification may protect phenol and diphenol substituents from oxidative enzymes until tanning is initiated (*1*). Dopamine was the second most abundant catechol, while *N*-acetyldopamine and dopa were present at relatively low concentrations. Throughout the first hour after ecdysis NBAD remained at high levels in hemolymph; then, as the cuticle tanned over the next 24 to 48 hours, NBAD levels, and the percentage conjugated, declined (Table 1).

N-β-Alanyldopamine was also the major catechol in cuticle from tanning pupae. Scraped abdominal tergites from 3-hour pupae contained 145 nmole/g, whereas wing cuticle had 35 nmole/g. No *N*-acetyldopamine was detected in either extract. These data suggested that at least a portion of the large pool of NBAD in hemolymph is transported to cuticle, where it probably serves as the primary precursor for sclerotizing compounds.

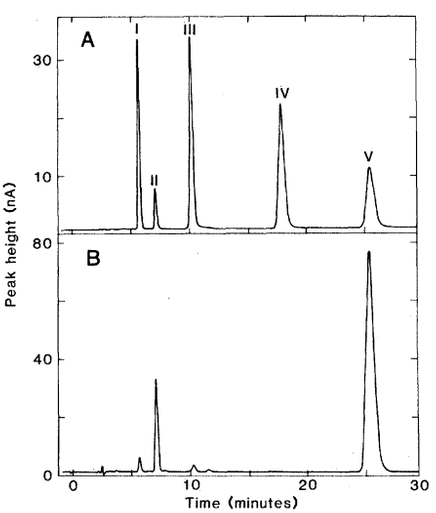

Fig. 1. (A) LCEC chromatogram of catechol standards. Internal standard, 30 ng; other catechols, 60 ng. *I*, dopa; *II*, 3,4-dihydroxybenzylamine (internal standard); *III*, dopamine; *IV*, *N*-acetyldopamine; *V*, *N*-β-alanyldopamine. (B) LCEC chromatogram of an unhydrolyzed extract of hemolymph from a newly ecdysed pupa of *Manduca sexta*. Liquid chromatography buffer was 5 percent methanol and 95 percent 0.1 m*M* potassium phosphate monobasic (*p*H 3), 0.2 m*M* sodium octyl sulfate, and 0.1 m*M* sodium EDTA. Flow rate of 1 ml/min at ambient temperature through a reverse phase octadecylsilane 5-μm spherical particle 25-cm column [Bioanalytical Systems LC 304 and electrochemical detector LC 4A (+0.72 V)].

In contrast, *N*-acetyldopamine was the primary catecholamine in hemolymph from newly ecdysed fifth-stadium larvae (708 μ*M*); dopamine and NBAD were less abundant (172 and 140 μ*M*, respectively). *N*-Acetyldopamine was also the predominant catechol in hemolymph of pharate adults close to ecdysis (2800 μ*M*), whereas NBAD was a minor constituent (20 μ*M*). Larval and adult cuticle of *M. sexta* may be sclerotized by compounds derived from *N*-acetyldopamine and pupal cuticle by metabolites of NBAD.

We used LCEC to survey seven other insect species from four different orders for the occurrence of NBAD (*6*). In all cases, NBAD was either the predominant catechol or a major component during tanning of pupal, puparial, or adult cuticles that formed brown sclerotins.

Phenoloxidases are enzymes that catalyze the formation of cross-linking agents for cuticle sclerotization (*1*). A phenol oxidase from blow fly cuticle has been reported to oxidize synthetic NBAD faster than it oxidizes *N*-acetyldopamine (*7*). We found a similar enzyme in pharate pupal integument of *M. sexta* (*8*). The relative turnover numbers (the ratio of the maximum velocity to the Michaelis constant) for NBAD, *N*-acetyldopamine, and dopamine were 0.87, 0.54, and 0.11 per minute, respectively. Thus NBAD was the preferred substrate for the hornworm enzyme also.

Our study demonstrates that NBAD is a naturally occurring metabolite in insects. β-Alanine, which has been isolated from the cuticles of many insect species, had previously been associated with the formation of brown cuticles (*1*). Its uptake and use during puparial tanning of Diptera apparently depends on

dopamine incorporation (*9*). Synthetic ^{14}C-labeled NBAD was incorporated into isolated larval cuticle of *Calliphora erythrocephala*, which became light brown (*10*). Another β-alanyl catechol, *N*-β-alanylnoradrenaline, has been identified from the yellow wing pigments of the swallowtail butterfly, *Papilio xuthus* (*11*).

Our evidence points toward a central role for NBAD in the pupal tanning of *M. sexta* and in other insects that form brown cuticle in pupae, puparia, or adults. Its occurrence as a major catecholamine metabolite in several insect orders suggests that conjugation of dopamine with β-alanine may be as important as acetylation for sclerotization processes in insects. The type of cuticle formed and its coloration, however, may depend on which form of dopamine is produced.

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3. Samples of compound V isolated by liquid chromatography and desalted on a Biogel P-2 column were hydrolyzed at reduced pressure in 6*N* HCl for 2 hours and 24 hours. Analysis of the constituent amino acids by liquid chromatography as their fluorescent *o*-phthaldialdehyde derivatives [D. W. Hill, *et al.*, *Anal. Biochem.* **51**, 1338 (1979)] gave a single peak eluting at the retention time of β-alanine (30.5 minutes). Analysis of the 6*N* HCl hydrolyzate by LCEC showed the disappearance of compound V and the appearance of dopamine.
4. Samples were hydrolyzed at reduced pressure in 2*N* HCl for 2 hours, silylated, and analyzed by gas chromatography [R. A. Wirtz and T. L. Hopkins, *J. Insect Physiol.* **20**, 1143 (1974)]. The retention times for silylated β-alanine and dopamine were 2 and 23 minutes, respectively, on a 5 percent OV-1 column with a column temperature of 200°C and a carrier gas flow rate of 84 ml/min.
5. Synthetic NBAD was prepared by coupling dopamine with *N*-α-*t*-butyloxycarbonyl-β-alanine-*N*-hydroxysuccinimide ester in potassium tetraborate buffer and deblocking the conjugate in 0.1*N* HCl. Identity was confirmed by LCEC and ultraviolet spectroscopy. Purity was > 99 percent.
6. Concentrations of hemolymph NBAD and *N*-acetyldopamine, respectively, for A, white adults; L, mature larvae; and P, white pupae or puparia were: *Periplaneta americana* (A), 91 and 63 μ*M*; *Sarcophaga bullata* (P), 254 and 53 μ*M*; *Diatraea grandiosella* (P), 310 and 10 μ*M*; and *Thyridopteryx ephemeraeformis* (L), 80 and 22 μ*M*. Whole body concentrations were *Plodia interpunctella* (P), 147 and 168 μmole/g; *Ephestia cautella* (P), 87 and 8 μmole/g; and *Tenebrio molitor* (P), 41 and 77 μmole/g.
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Size Analysis of Phospholipid Vesicle Preparations

Abstract. *Gel exclusion chromatography based on the use of Sephacryl S-1000 provides a quick and convenient method for determining the average diameter of phospholipid vesicles and an approximate measure of size heterogeneity.*

Recently, Kirkland *et al.* (*1*) pointed out the inconvenience associated with the use of electron microscopy for routine size analysis of phospholipid vesicle dispersions and demonstrated the applicability of the technique of sedimentation field flow fractionation to this problem. Since this is a separation method, it can be used not only for analytical purposes but also for the preparation of nearly homogeneous fractions from initially polydisperse mixtures. We report here that gel exclusion chromatography can also be used for both these purposes. This was one of the methods used by Huang (*2*) in his study of vesicle size. The vesicles prepared by Huang were very small (diameter < 25 nm), and the gel filtration media available at the time could not have handled particles much larger. Materials with larger pore size have been developed since then, and with Sephacryl S-1000 (Pharmacia, Inc., Piscataway, New Jersey) the size analysis has been extended to a diameter of about 300 nm. Much current research focuses on single-walled vesicles, which generally have diameters below this limit. For many practical projects, such as the use of phospholipid vesicles for drug delivery, it is necessary to have a reasonably accurate measure of average vesicle size but only a semiquantitative measure of polydispersity (*1*). Gel chromatography with Sephacryl S-1000 adequately meets these requirements.

The results reported here were obtained with single-walled vesicles prepared by detergent removal from detergent-solubilized egg yolk phosphatidylcholine. Mimms *et al.* (*3*) have described a preparative method in which octyl glucoside was used as solubilizing detergent, and either dialysis or gel chromatography (on a small-pore resin) was used for detergent removal. The data

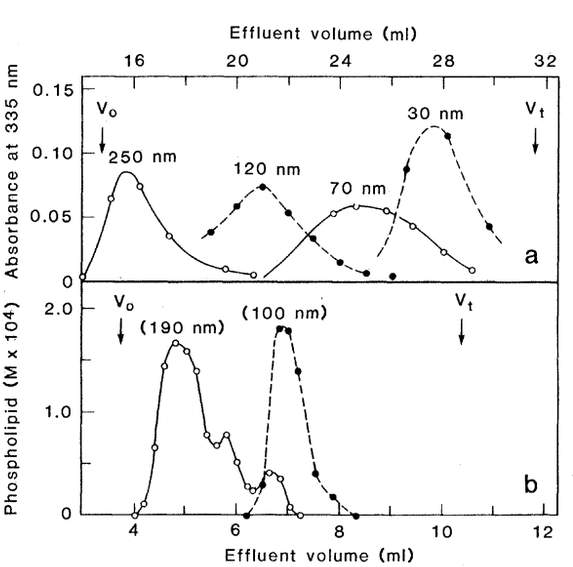

Fig. 1. (a) Elution patterns for four vesicle preparations, each determined separately on column A. A different detergent was used for each preparation: from left to right they were octyl glucoside (*2*), dodecyl octaethylene glycol monoether (*8*), polyoxyethylene 9 lauryl ether (Sigma Chemical Corporation, St. Louis), and sodium cholate (*8*). (b) Elution patterns on column B for two preparations, both made with octyl glucoside as the solubilizing detergent but differing in the manner and rate of detergent removal.

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