The Occurrence of Certain D-Amino Acids in Insects*

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ABSTRACT: Paper chromatography and high-voltage electrophoresis were used to separate serine and alanine from the blood and tissues of four insect orders including 10 families (13 species) of Lepidoptera, 1 family (1 species) of Hymenoptera, 3 families (3 species) of Coleoptera, and 1 family (1 species) of Hemiptera. By the application of a sensitive assay dependent on D-amino acid oxidase, D-serine was demonstrated in 11 of the 13 species of Lepidoptera. Two of these species also contain D-alanine, an amino acid found in the milkweed bug, *Oncopeltus fasciatus*. It was shown that a significant increase in D-serine takes place when *Bombyx mori* ceases to feed and begins to spin a cocoon. Thus, when 11 individuals were examined none had more than 1% D-serine as larvae, while pupae of the same insects contained D-serine ranging from 5 to 50% of the total serine. D-Serine may be related to D-2,3-diaminopropionic acid recently discovered in *Bombyx*, since D-diaminopropionate is present in larvae and absent in pupae. The assay method is described, particularly its sensitivity and its use in differentiating between D-alanine, D-serine, and D-α-aminobutyric acid.

The discovery of D-alanine in the milkweed bug, *Oncopeltus fasciatus*, by Auclair and Patton (1950) was the first report of a free D-amino acid in animal tissues based on the use of a specific enzymatic method. Studies on the earthworm *Lumbricus terrestris* by Rosenberg and Ennor (1961) and by Beatty *et al.* (1961) revealed the presence of free D-serine together with lombricine and serine ethanolamine phosphodiester, both of which contain a D-serine residue. Recently, D-serine was discovered in significant quantities in the blood and tissue fluids of *Bombyx mori* and two other silkworms (Srinivasan *et al.*, 1962). Further experiments in which [^{14}C]-glucose, L-serine, and D-serine were injected into *Bombyx* provided evidence for the interconversion of D- and L-serine and for the biosynthesis of these amino acids from glucose (Srinivasan *et al.* 1965). In these studies, the evidence suggested that there is relatively little D-serine in the early larval stages of *Bombyx*, that the amount of D-serine increases at the time of cocoon spinning and pupation, and that the pupal tissues can catalyze the conversion of L-serine to the D-enantiomer while the larval tissues lack this ability. Subsequent experiments have included determinations of D-serine in hemolymph samples taken from individual larvae followed by determinations on similar samples taken from the pupal stage of the same insect. In this communication, we report the results of these determinations. In addition, we present evidence for the presence of D-serine and, in some cases, D-alanine in a total of eleven species of Lepidoptera including pupae of both moths and butterflies. Information is also given concerning the analytical methods, particularly the specificity of the enzymatic assay used for measuring certain D-amino acids.

Experimental Section

Materials. Amino acids were purchased from Sigma Chemical Co. and Schwarz BioResearch, Inc. DPNH[1] and FAD were purchased from Sigma chemical Co. Crystalline beef heart catalase was obtained from Worthington Biochemical Corp. and crystalline rabbit muscle lactic dehydrogenase was purchased from C. F. Boehringer and Sons. D-Amino acid oxidase was prepared from hog kidneys according to Massey *et al.* (1961). The high-voltage electrophoretic separations were carried out in a Lucite tank filled with Varsol, a mixture of aliphatic and aromatic hydrocarbons purchased from Esso Corp. (Katz *et al.*, 1959). DL-2,3-Diaminopropionic acid was kindly provided by Dr. Alton Meister.

The silkworms, *B. mori*, were from a colony maintained in this laboratory. Mulberry leaves were collected from trees in the vicinity of Boston and also, through the kindness of Dr. Richard A. Howard, from the Arnold Arboretum, Harvard University. During the winter months, leaves were obtained with the assistance of Mr. Jack Mead of the Sub-Tropical Experiment Station, University of Florida, Homestead, Fla. Occasional shipments of leaves were provided through the kindness of Dr. James MacBain Cameron of the Insect Pathology Research Institute, Sault Ste. Marie, Ont., Can. The oak silkworm, *Antheraea pernyi*, and

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[1] Abbreviations used: DPNH, reduced diphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; FAD, flavin-adenine dinucleotide.

cecropia silkworm, *Hyalophora cecropia*, were reared in this laboratory from eggs kindly donated by Dr. Carroll M. Williams of Harvard University. Black swallowtail butterfly pupae, *Papilio polyxenes*, were obtained through the courtesy of Dr. G. Staal, then associated with the Biological Laboratories, Harvard University. Cotton bollworm pupae, *Heliothis zea*, were provided through the kindness of Dr. Edward Lambremont of Louisiana State University. The Southern armyworm, *Prodenia eridania*, was reared in this laboratory from eggs kindly provided by Dr. Keith Ihde of the Hercules Powder Company. Gypsy moth pupae, *Porthetria dispar*, were reared from larvae kindly donated by Mr. Thomas McIntyre of the Agricultural Research Service, U. S. Department of Agriculture. Honeybee pupae, *Apis mellifera*, were generously donated by Dr. John Law of Harvard University. The Arctiid species (probably *Euchaetias egle*) and the Monarch butterfly, *Danaus plexippus*, were reared from larvae collected on milkweed plants. A number of *Danaus* pupae were obtained by rearing larvae from eggs oviposited by gravid female Monarch butterflies. Eastern tent caterpillar larvae, *Malacosoma americanum*, were collected from colonies in tents on cherry trees and reared to the pupal stage in the laboratory. The Noctuid was collected in a soil sample, the corn borer, *Ostrinia nubilalis*, was collected from mature field corn, and the tomato hornworm, *Protoparce quinquemaculata*, was collected from tomato plants. Two species of beetle larvae, the milkweed leaf beetle, *Labidomera clivicollis*, and the red milkweed beetle, *Tetraopes tetraophthalmus*, were collected from milkweed and the Japanese beetle, *Popillia japonica*, was taken from roses. The milkweed bug, *Oncopeltus fasciatus*, was from a colony maintained in this laboratory on milkweed seeds.

Preparation of Protein-Free Fractions. Amino acid fractions were prepared by homogenizing each species in an all-glass homogenizer with 1% picric acid and then removing the picric acid and isolating the amino acids with the aid of ion-exchange resins (Srinivasan *et al.*, 1965). The amino acid eluates were evaporated to dryness and the residues dissolved in water at a ratio of 1 ml of water/g of fresh weight of original tissue. Amino acid fractions from blood were prepared in a volume of water equivalent to the original blood volume.

Separation and Determination of Amino Acids. Aliquots (50–200 μl.) of the amino acid fraction were subjected to paper chromatography in one dimension followed by high-voltage electrophoresis in the other direction essentially as previously described (Srinivasan *et al.*, 1965). All the insect species studied were examined by separating the alanine and serine in this manner and extracting them from the paper with water. Total alanine was estimated by the quantitative ninhydrin method of Rosen (1957), and serine by the method of Frisell *et al.* (1954). D-Alanine and D-serine were determined by the spectrophotometric assay (Srinivasan *et al.*, 1965). In this procedure the reaction mixture contained sodium pyrophosphate buffer (40 μmoles; pH 8.3), crystalline beef heart catalase (10 units), crystalline rabbit muscle lactic dehydrogenase (4.2 units), FAD (2.5 μg), DPNH (0.1 μmole), porcine kidney D-amino oxidase (94 units, about 0.1 mg), and sample in a total volume of 1 ml. The reaction was followed to completion by reading the decrease in absorbance at 340 mμ against a reference solution lacking only DPNH. Under these conditions, as little as 0.05 μmole of D-alanine or D-serine could be detected. The 2,3-diaminopropionic acid was analyzed by one-dimensional high-voltage paper electrophoresis at pH 3.6.

For the determinations of total and D-serine in *Bombyx* larvae and pupae, blood was collected from 6-day-old last instar larvae by cutting off the tip of the dorsal horn which was later sealed with melted paraffin. The larvae were replaced on mulberry leaves and began to spin cocoons in about 4 days. After pupation had occurred, the pupae were removed and again exsanguinated.

Results

Specificity of the Spectrophotometric Assay. D-Amino acid oxidase and lactic dehydrogenase display broad specificity for D-amino acids (Krebs, 1935; Greenstein *et al.*, 1953) and α-keto acids (Meister, 1950, 1952), respectively. However, only a few D-amino acids yield α-keto acids that can be reduced at a significant rate under the conditions described above. Table I illustrates the relative specificity of the enzymatic assay for several D-amino acids. Only D-alanine, D-serine, D-α-aminobutyric acid, and D-threonine reacted at a rate sufficient for detection. Although α-keto-β-hydroxybutyric acid is reduced by lactic dehydrogenase almost as rapidly as pyruvic acid (Meister, 1965), the rate of oxidation of threonine by porcine kidney D-amino acid oxidase is about one-thirtieth that of D-alanine (Greenstein *et al.*,

TABLE I: Reactivity of Various D-Amino Acids in the Spectrophotometric Assay.[a]

Amino Acid Added	Time Required for Complete Reaction (min)
D-Alanine	12
D-Serine	60
DL-α-Aminobutyric acid	136
D-Threonine	300
D-Cystine	nr[b]
D-Homocystine	nr
DL-Methionine	nr
Glycine	nr

[a] Experimental details are given under Methods; 50 mμmoles of D-amino acid was added. [b] nr, no reaction.

1953), which probably accounts for the slow response of D-threonine in the assay. No reaction was observed with DL-methionine even when the concentration of lactic dehydrogenase was increased 10-fold in the assay cuvet. Although D-methionine is oxidized by D-amino acid oxidase about one and a half times faster than D-alanine (Greenstein *et al.*, 1953), α-keto-γ-methiolbutyric acid is reduced by lactic dehydrogenase at about one-twentieth the rate of pyruvic acid (Meister, 1952). The curves observed for the oxidation of DPNH in the assay were characteristic and different for D-alanine, D-serine, and D-α-aminobutyric acid (Figure 1). Hence, it was possible to distinguish pure samples of these three amino acids when each was assayed at the same molar concentration. No inhibition was observed when 0.05 μmole of D-serine or D-alanine was determined in the presence of 100 μmoles of L-serine or L-alanine or 10 μmoles of ethanolamine or *O*-phosphoethanolamine.

Serine Determinations in Blood from Larvae and Pupae of the Same Insect. These results (Table II) confirmed the observation that D-serine in *Bombyx* is either absent, or present in relatively small amounts during the early stages of larval life. Eight of the eleven insects contained no detectable D-serine in blood collected during the feeding phase of the last larval instar, while the remaining three had only 1%. Pupae of the same insects contained various amounts of D-serine ranging from 5 to 50% of the total serine. Similar studies on blood samples from individual larvae engaged in cocoon spinning gave values for D-serine ranging from 4 to 95% of the total serine.

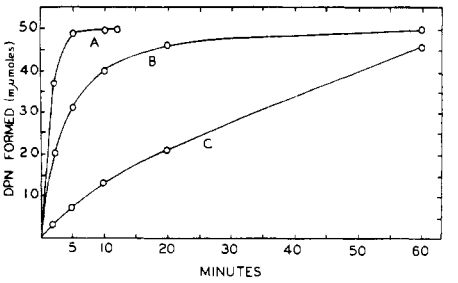


FIGURE 1: Conversion of DPNH to DPN$^+$ during the enzymatic assay of three D-amino acids. Each cuvet contained sodium pyrophosphate buffer (40 μmoles; pH 8.3), catalase (10 units), lactic dehydrogenase (4.2 units), FAD (2.5 μgm), DPNH (0.10 μmole), D-amino acid oxidase (94 units, about 0.1 mg), and sample in a total volume of 1 ml. Curve A, D-alanine (50 mμmoles), curve B, D-serine (50 mμmoles), curve C, DL-α-aminobutyric acid (100 mμmoles).

TABLE II: Total Serine and D-Serine in Larval and Pupal Blood from Individual Insects.[a]

Stage	Sex	Total Serine (mM)	% D of Total Serine
Larva		12	0
Pupa	M	7	19
Larva		15	0
Pupa	F	8	38
Larva		7	1
Pupa	M	6	15
Larva		18	0
Pupa	M	11	21
Larva		8	1
Pupa	M	26	50
Larva		15	0
Pupa	M	12	36
Larva		15	0
Pupa	M	6	24
Larva		15	0
Pupa	M	11	21
Larva		17	0
Pupa	F	14	46
Larva		6	0
Pupa	M	25	5
Larva		15	0
Pupa	M	10	17

[a] Each insect was bled as a larva and placed back on leaves until pupation had occurred. Pupal blood was then collected and serine was separated in each sample and analyzed as described under Methods. The larvae were 6-day-old last instars and the pupae were 4 days old.

Analysis of Other Species for D-Amino Acids. The result of a typical two-dimensional analysis of the amino acid fraction from *Bombyx mori* pupae is illustrated in Figure 2. Several ninhydrin-reactive compounds have not yet been identified. The unlabeled compound near the top center of the photograph is a reference spot of DL-serine. At this pH (2.2) the basic amino acids are not separated, but electrophoresis at pH 6.5 separates them (Richmond and Hartley, 1959). In each species examined, serine and alanine were separated by the method illustrated in Figure 2. A number of species were found to contain D-serine and in a few cases both D-serine and D-alanine were discovered. In most species, additional determinations were performed on the total amino acid fraction before and after treatment of the fraction with periodate. After treatment with periodate, only D-alanine was found. The results of this work are summarized in Table III. In the case of *Malacosoma*, both larvae and pupae were examined. Quantitative data for this and other species are presented in Table IV. A slight response was observed for *Malacosoma* in the spectrophotometric assay equivalent to approximately 1% D-serine based on the total serine present in the assay cuvet. This response was considered insufficient to report as positive since it is at the limit of sensitivity of the assay. In order to determine if other D-amino acids were present, aliquots of the amino acid fractions from pupae of *Bombyx* and *Danaus* were incubated with D-amino acid oxidase together with controls containing

TABLE III: D-Serine and D-Alanine in Various Insect Species.

Insect	Host Plant	Early Larvae		Late Larvae and Pupae	
		D-Serine	D-Alanine	D-Serine	D-Alanine
Lepidoptera					
Bombyx mori	Mulberry	−	−	+	−
Antheraea pernyi	Oak	+	−	+	−
Hyalophora cecropia	Cherry	−	−	+	−
Protoparce quinquemaculata	Tomato			+	−
Prodenia eridania	Potato			+	
Ostrinia nubilalis	Corn			+	−
Heliothis zea	Cotton			+	−
Papilio polyxenes	Parsley			+	−
Danaus plexippus	Milkweed	−	+	+	+
Arctiid	Milkweed	−	+	+	+
Noctuid	Unknown	−	+	+	+
Malacosoma americanum	Cherry			−	−
Porthetria dispar	Hemlock			−	−
Hymenoptera					
Apis mellifera	Multiple			−	−
Coleoptera					
Tetraopes tetrophthalmus	Milkweed			−	−
Labidomera clivicollis	Milkweed			−	−
Popillia japonica	Rose			−	−
Hemiptera		Nymphal instars		Adults	
Oncopeltus fasciatus	Milkweed seeds	−	+	−	+

[a] The amino acids were separated and analyzed as described under Methods. A positive was reported when the percent D of total amino acid was greater than 10%, or when the absorbance change at 340 mμ was greater than 0.15 in a 1-ml volume (corresponding to 24 mμmoles of D-amino acid).

heat-denatured enzyme. Two-dimensional paper analyses were prepared from these aliquots and treated with 0.25% ninhydrin in acetone. The only detectable decreases in color density were those observed for serine in *Bombyx* and alanine and serine in *Danaus*.

The recent discovery by Wada and Toyota (1965) of D-2,3-diaminopropionic acid in *Bombyx* indicates that an additional D-amino acid occurs in this species. This compound was found in high concentration (3.6 mM) in the gastrointestinal fluid of fasting larvae and reported to be low when larvae were feeding. Work in this laboratory (J. J. Corrigan, unpublished data) has confirmed the presence of 2,3-diaminopropionate in protein-free extracts prepared from gastrointestinal fluid, blood, and total homogenates of larvae. This compound was not found after the extracts were treated with D-amino acid oxidase. Administration of uniformly labeled [^{14}C]glucose to *Bombyx* larvae was followed by the appearance of ^{14}C in the isolated D-2,3-diaminopropionic acid. Pupae contain little or no diaminopropionate and the disappearance of this amino acid seems to take place during the period of cocoon spinning (J. J. Corrigan, unpublished data) coincident with the increase of D-serine in the blood.

Discussion

The determinations of D-serine in individual insects agree with other evidence that blood from feeding *Bombyx* larvae contains little if any D-serine and that an increase in this amino acid occurs at about the time of cocoon spinning. The wide variation in the percentage of D-serine may reflect an underlying biological process not subject to precise metabolic control. The occurrence of both D-alanine and D-serine in *Danaus* and in the Arctiid caterpillars is noteworthy. These species feed on milkweed plants in common with *Oncopeltus* which contains D-alanine but no D-serine. It is also of interest that D-alanine is present early in the last larval instar of *Danaus*, before D-serine can be detected, and persists into the pupal stage. The failure to detect significant amounts of D-serine in *Malacosoma americanum* and *Porthetria dispar* shows that although D-serine is widespread in Lepidoptera it is not universal in this order. Significant D-amino acid oxidase activity has not been detected in tissues from *Bombyx*, *Hyalophora*, and *Danaus* (J. J. Corrigan, unpublished data). This contrasts with the widespread occurrence of D-amino acid oxidase activity in many other insect species

TABLE IV: Concentration of D-Serine in Lepidoptera Blood.

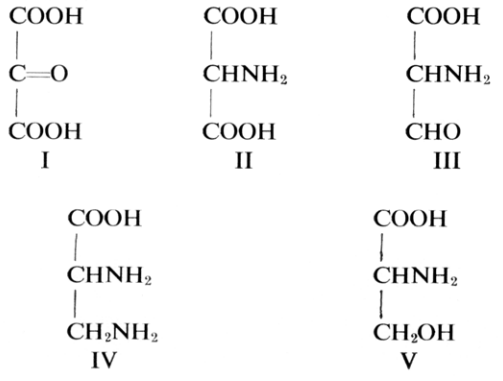
Insect	Stage	Total Serine Concn (mM)	D-Serine	
			Concn (mM)	% of Total Serine
Bombyx mori	P[a] (6)[b]	22	11	50
Antheraea pernyi	L[c] (1)	2.8	1.0	36
Hyalophora cecropia	P (2)	17	12	71
Protoparce quinquemaculata	P (1)	7.4	3.0	41
Prodenia eridania	P (5)[d]	1.0	0.55	55
Ostrinia nubilalis	L	7.2	1.9	26
Arctiid	P	29	7.2	25
Malacosoma americanum	P (17)	3.4	0.05	1
Malacosoma americanum	L (16)	18	0.24	1
Porthetria dispar	P (6)	2.7	0.0	0

[a] P, pupae. [b] The number of insects in the sample. [c] L, larvae. [d] Extract of total insect representing 1 g fresh weight/ml of extract.

(Corrigan *et al.*, 1963; Auclair, 1959; Desai and Kilby, 1958). It is possible that *Malacosoma* and *Porthetria* lack D-serine because they contain a D-amino acid metabolizing enzyme which destroys D-serine as fast as it is synthesized.

The discovery of D-2,3-diaminopropionic acid in *Bombyx* appears to be the first report of this isomer in nature. The L-enantiomer has been found in extracts of *Mimosa palmeri* (Gmelin *et al.*, 1959) and in hydrolysates of the antibiotic viomycin (Haskell *et al.*, 1952). Previously, evidence was obtained consistent with the presence of a serine racemase in *B. mori* (Srinivasan *et al.*, 1965). It was also reported that injection of [^{14}C]-glucose into *Bombyx* pupae led to L-serine possessing a specific activity that was initially greater than that of the isolated D-serine. After 8 hr, the specific activities of the isolated L- and D-serine were similar. These studies do not exclude the possibility that L- and D-serine are synthesized by separate path ways from glucose.

It is possible that α-aminomalonic semialdehyde (III) is an intermediate between D-serine (V) and D-2,3-diaminopropionic acid (IV). Thus, α-aminomalonic semialdehyde might be formed by oxidation of D-serine



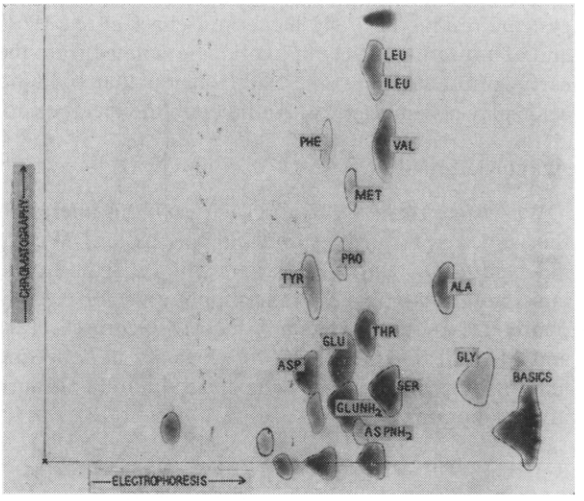


FIGURE 2: Two-dimensional separation of amino acids from *Bombyx* pupae. An amino acid fraction prepared from five *Bombyx* pupae was used and 50 μl was spotted on Whatman No. 1 paper. The amino acids were separated in the vertical direction by descending chromatography for 36 hr (2-butanol–acetic acid–water; 4:1:1) and in the horizontal direction by high voltage electrophoresis (pH 2.2, pyridine–formic acid–H_2O; 2500 v, 2.5 hr) (Richmond and Hartley, 1959). The amino acids were detected by treatment of the dried papers with 0.25% ninhydrin in acetone.

or by the oxidative deamination of D-2,3-diaminopropionic acid; these reactions could proceed in either direction.

On the other hand, it is conceivable that D-2,3-diaminopropionic acid (IV) is formed in larval tissues by a pathway independent of serine. Transamination of mesoxalic acid (I) with alanine to give α-aminomalonic

acid (II), *e.g.*, has been reported in homogenates of *Bombyx* posterior silkglands (Nagayama *et al.*, 1958). α-Aminomalonic acid (II) might be converted to α-aminomalonic semialdehyde (III) by a reaction analogous to the formation of aspartate-β-semialdehyde from aspartate. These and related possibilities are presently being investigated (J. J. Corrigan, unpublished data). It seems pertinent that 2,3-diaminopropionic acid has not been detected in extracts of gastrointestinal fluid or homogenates prepared from *Malacosoma* and *Porthetria* larvae. The fact that these species also lack D-serine is consistent with metabolic relationships such as those discussed above.

There is no obvious relationship between the occurrence of D-serine and the taxonomic positions of the species studied except that all those possessing D-serine are Lepidoptera. We have examined only 13 species from this order out of about 11,000 that are found in the United States and Canada. Since 11 of the 13 examined contain D-serine, it seems reasonable to suppose that a majority of the Lepidoptera contain one or more D-amino acids.

The present observations and the occurrence of a D-cysteine residue in firefly luciferin (Seliger *et al.*, 1961) and of a D-serine moiety in lombricine isolated from the earthworm (Beatty *et al.*, 1961) suggest that D-amino acids play a significant role in invertebrate metabolism.

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