D-Amino Acids in Animals

Novel reactions and compounds unique to specific stages of development characterize this subject.

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Molecular asymmetry is fundamental to all known living systems. It plays a major role in enzymatic specificity and dictates the spatial architecture of many biological polymers. The concept of asymmetry grew out of observations on organic compounds of low molecular weight and was indispensable for the establishment of the tetrahedral geometry of the carbon atom. This review describes an extension of knowledge on asymmetry in animal metabolism, specifically, recent information on D-amino acids and structures containing D-amino acids (Fig. 1). The topic is relatively new and includes about 30 examples of amino acids found primarily among the invertebrates; this number has doubled in the past 5 years. In some cases, the D-amino acids are components of other metabolites and a functional role is implied. For most examples, the function is unknown and awaits future explication. Many of the observations point to yet undiscovered enzymatic reactions and may provide new ideas on regulation of cellular processes, particularly during the temporal enactment of genetic programs associated with insect differentiation.

Early Studies

Initially it was believed that only one optical isomer of each class of compounds occurred in nature. Accordingly, L-sugars and D-amino acids were regarded as laboratory artifacts and categorized as "unnatural isomers." This term is still widely used in texts of biochemistry but is contrary to fact. The discovery by Snell that D-alanine can replace vitamin B_6 in a defined growth medium for *Streptococcus faecalis* and *Lactobacillus casei* (*1*) led to a series of studies which ultimately resulted in the characterization of a peptidoglycan found in the cell walls of virtually all bacteria (*2*). The requirement for D-alanine is now understood since this amino acid is a component of the peptide portion and is synthesized by an enzyme, which racemizes alanine, isolated in 1951 by Wood and Gunsalus (*3*). Prior to and during this period, a number of other D-amino acids were found in microbial extracts and in many of the polypeptide antibiotics (*4*).

In an early report of a D-amino acid in animal tissue, Ackerman and Mohr claimed that D-ornithine could be extracted from the shark *Acanthias vulgaris* (*5*). At about the same time, Fuchs described the isolation of DL-glutamic acid from extracts of the flight muscle of the May beetle (genus *Melolontha*) and DL-alanine from anserine muscle (*6*). It is difficult to evaluate these reports published 30 years ago, before the introduction of chromatography and other modern fractionation techniques. They should be reinvestigated with contemporary analytical methods. The possibility of racemization occurring as an artifact of manipulation must be considered.

The first D-amino acid in animal tissues detected under less equivocal circumstances was free D-alanine found by Auclair and Patton in 1950 in the blood of the milkweed bug *Oncopeltus fasciatus* (*7*). The discovery was made on two-dimensional paper chromatograms of protein-free extracts of *Oncopeltus* blood. The papers were sprayed with a buffered solution of D-amino acid oxidase, and, after an incubation period, they were dried and treated with a solution of 2,4-dinitrophenylhydrazine. A large hydrazone spot which darkened when sprayed with alkali became visible at the location of alanine, and from this it was concluded that D-alanine was present. This conclusion was based on the premise that D-alanine in the blood had been oxidatively deaminated by the D-amino acid oxidase to pyruvic acid which yielded a phenylhydrazone derivative. No D-amino acid was detected in extracts of blood from two other insect species, the Mexican bean beetle *Epilachna varivestis* and the southern armyworm *Prodenia eridania*. An hydrolyzate of milkweed seeds did not contain D-alanine, an indication that the diet of *Oncopeltus* was not the source of the D-enantiomer.

During this period, Greenstein and his collaborators reexamined the question of the configuration of alanine in octopine (Fig. 1), a compound first isolated from octopus muscle by Morizawa (*8*). In 1957, they established that the alanine portion was of the D-configuration (*9*). Later work by Robin and Thoai showed that octopine biosynthesis involves the condensation of L-arginine and pyruvate to form a Schiff base, followed by an enzymatic reduction which yields the D-alanine moiety. It was postulated that, since the in vitro reaction is reversible, octopine may be a storage form for arginine in muscle and therefore is concerned in the supply of phosphoarginine (*10*). There is no evidence for free D-alanine in the tissues of the octopus or any other mollusk.

D-Serine in Annelids

The next development was due to the work of Ennor and his colleagues at the Australian National University in Canberra. In 1959, Beatty, Magrath, and Ennor reported the presence of a D-serine residue in lombricine found in the earthworm *Lumbricus terrestris* and they described procedures for the isolation and characterization of lombricine, serine ethanolamine phosphodiester, and free D-serine (*11*). Lombricine was first isolated together with *N*-phosphoryllombricine (Fig. 1) from earthworms by Thoai and Robin (*12*). In a series of papers, the Australian group described the biosynthesis of lombricine, with evidence that administration of ^{14}C-amidine-labeled arginine to earthworms resulted in substantial incorporation of label into the guanidinoethanol portion. Radioactive D- and L-serine were both incorporated into the serine component suggesting that a serine racemase may

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be present (*13*). Other studies were conducted on the purification and properties of several of the enzymes relevant to the metabolism of lombricine. The ATP-lombricine phosphotransferase was investigated (*14*). This enzyme catalyzes the synthesis of *N*-phosphoryllombricine according to the reaction:

ATP + lombricine ⇄ ADP + phosphoryllombricine

Both D- and L-lombricine serve as substrates while creatine, arginine, and guanidinoethanol are not phosphorylated (*15*). This reaction is of general interest since it is formally similar to the conversion of creatine to phosphocreatine in vertebrate muscle (*16*) and the conversion of arginine to phosphoarginine in crustacean muscle (*17*). All three of these reactions result in the formation of derivatives of phosphamic acid that have a high phosphate group transfer potential (*18*), and can react enzymatically with ADP to resynthesize ATP.

Investigations were also conducted on a serine ethanolamine phosphodiesterase that was purified from chicken kidney (*19*). Serine ethanolamine phosphodiester can be isolated from fish, amphibians, reptiles, and birds, and in these sources the serine configuration is L, whereas the D-enantiomer is characteristic of earthworms (*20*). The phosphodiesterase hydrolyzes both substrates, and the enzyme seems to be restricted to vertebrates containing L-serine ethanolamine phosphodiester. A recent report presents evidence for the synthesis of D-serine ethanolamine phosphodiester by homogenates prepared from the body wall of the earthworm *Megascolides cameroni*. The enzymatic reaction is specific for D-serine and requires CDP-ethanolamine:

CDP-ethanolamine + D-serine → D-serine ethanolamine phosphodiester + CMP

A similar enzyme prepared from the chicken can utilize either isomer, but L-serine is more efficiently incorporated (*21*).

Stereospecificity and Bioluminescence

One of the more fascinating aspects of comparative biochemistry is represented by the work of McElroy and his collaborators who succeeded in preparing crystalline luciferase and its substrate luciferin (Fig. 1) from fireflies (genus *Photinus*). The enzyme catalyzes the activation of luciferin with ATP to form an enzyme-bound luciferyl-adenylate and inorganic pyrophosphate. The luciferin is then oxidized, accompanied by the emission of light. In 1961, luciferin was synthesized in a series of steps, the last one of which required the reaction of 2-cyano-6-hydroxybenzthiazole with the amino acid cysteine. When D-cysteine was used in this reaction, the luciferin obtained was activated by the enzyme to form luciferyl-adenylate and pyrophosphate. The luciferyl-adenylate then reacted with molecular oxygen and emitted light. When L-cysteine was used for the last synthetic step, the luciferin was activated by the enzyme, and the luciferyl-adenylate reacted with molecular oxygen, but no light was emitted. In this unusual situation, both optical enantiomers of luciferin react with the enzyme with the same affinity, and both are oxidized to the product dehydroluciferin to the same extent (*22*). No studies have been reported on the biosynthesis of luciferin in the beetle, and it is not known if the insect contains free D-cysteine.

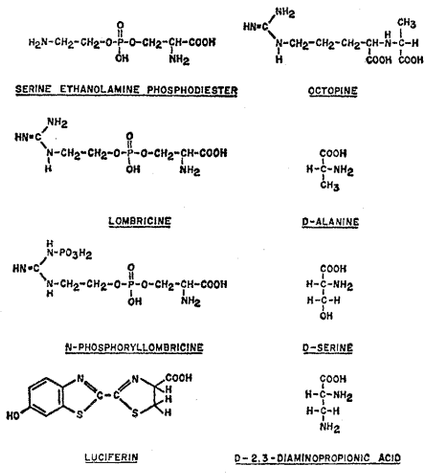

Fig. 1. Structures of D-amino acids and compounds containing D-amino acids isolated from animals.

Information on Mammals

Reports of D-amino acids in mammals are limited. D-Alanine was found in the blood of guinea pigs and mice by Hoeprich in 1965 (*23*). This observation was made during an investigation on the failure of the antibiotic D-cycloserine (D-4-amino-3-isoxazolidinone) to protect guinea pigs and mice from experimental infection with tuberculosis. When ultrafiltrates of serum from guinea pig and mouse blood were incubated with D-amino acid oxidase, subsequent addition of 2,4-dinitrophenylhydrazone gave color in excess of that observed in the control samples. It was concluded that this result was due to the presence of D-alanine. Independent determinations in my laboratory on samples sent by Hoeprich confirmed this conclusion. When serum from germfree mice and guinea pigs was analyzed, no D-alanine was found. Accordingly, it was proposed that the D-alanine in these animals arises from endogenous microbial flora. This work may provide a rationale for the existence of D-amino acid oxidase which is widely distributed in animals (*24*). We have detected this enzyme activity in various organs from a number of insect species (*25*).

Other information involving mammals concerns the cyclic compound pyrrolidone carboxylic acid (5-oxo-2-pyrrolidine carboxylic acid). It has long been known that when glutamic acid is boiled in aqueous solution it undergoes slow nonenzymatic cyclization to yield pyrrolidone carboxylic acid. In 1944, Ratner found that, when DL-glutamate was administered to rats, most of the D-isomer was excreted in the urine as D-pyrrolidone carboxylate (*26*). She suggested that the origin of the compound was enzymatic, and more recent studies by Wilson and Koeppe (*27*) provided data consistent with this view. In 1962, Meister and his co-workers discovered a new enzyme in kidney and liver tissues of the mouse, rat, and human which catalyzes the stereospecific cyclization of D-glutamate to D-pyrrolidone carboxylate (*28*). The distribution of this enzyme is similar to that of D-amino acid oxidase mentioned above, but the cyclizing enzyme requires manganese or magnesium ions for activity, while D-amino acid oxidase requires flavin-adenine dinucleotide and exhibits only slight activity toward D-glutamate (*4*). A surprising observation made during this work was the discovery of D-pyrrolidone carboxylic acid in samples of freshly voided human urine (*29*). The total daily excretion varied from 50 to 400 micromoles, and in one case the D-pyrrolidone carboxylate was determined before, during, and after a 2-day fast and found to be continually excreted. The L-enantiomer was virtually absent from the urine samples. It was suggested that the enzyme cyclizing D-glutamate may serve in conjunction with D-amino acid oxidase to act on D-amino acids originating

Table 1. D-Alanine in the milkweed bug.

Source	Alanine (μg)		D-Alanine (% of total)
	D-Isomer	Total	
Eggs, 50 mg	13	20	65
Nymphs, 50 mg	16	27	60
Adults, 1 g	260	480	54
Blood, 1 ml	560	1300	43
Blood, 1 ml	800	1600	50
Seed extract		31	

in the diet or resulting from the metabolism of microorganisms found in the gastrointestinal tract. The possibility was raised that a pathway requiring small quantities of D-amino acids may be operative in mammalian metabolism. It would be interesting to learn whether D-pyrrolidone carboxylate is present in mouse and rat tissues, since germ-free individuals of these species are available.

In this regard, it is pertinent to recall two past attempts to relate D-amino acids to broad biological problems. The first was a claim by Kögl and Erxleben that tumor proteins contain D-amino acids (*30*). Briefly, they stated that tumor proteins are dependent on the formation and incorporation of D-amino acids (primarily glutamate), and evidence in support of this statement continued to be published by Kögl and his co-workers, and by a few other laboratories through 1956 (*31*). Part of the evidence came from the isolation of

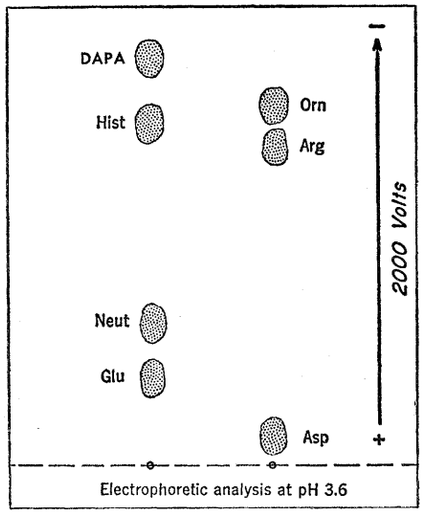

Fig. 2. Separation of D-2,3-diaminopropionic acid (*DAPA*) from other amino acids by high-voltage electrophoresis on paper (2 kilovolts, 2 hours, *p*H 3.6). Abbreviations: *Asp*, aspartic acid; *Glu*, glutamic acid; *Hist*, histidine; *Neut*, neutral amino acids; and *Orn*, ornithine.

D-glutamic acid after acid-catalyzed hydrolysis of tumor tissue, and part came from the isolation of D-pyrrolidone carboxylic acid from the urine of dogs which were fed tumor tissue. Many investigators were unable to verify these results, and the controversy has been reviewed by Miller (*32*). It remains an unconfirmed observation in the oncological literature.

The second attempt to relate D-amino acids to biological problems was concerned with senescence, and the principal ideas are available in an article by Kuhn (*33*). The argument was that as long as living organisms maintain a high degree of purity with regard to optical isomers in the various protein polymers, they remain healthy and vital. In the event of a slight deterioration of optical homogeneity, the organism declines and dies. In order to support this proposal, Kuhn hydrolyzed the keratinous protein from the hair of horses of different ages and determined the D-leucine content as a percentage of the total leucine. Small amounts of D-leucine were isolated, but there was no quantitative correlation with regard to the age of the horses. The data were consistent with the fact that small quantities of the D-isomers of several amino acids have repeatedly been found in hydrolyzates of protein. This is most frequently explained as racemization produced during hydrolysis (*34*). No unequivocal evidence has yet been presented for the occurrence of D-amino acid residues in proteins. Studies on this question will require enzymatic methods of hydrolysis combined with assays suitable for detecting small amounts of D-amino acids in the presence of large amounts of the L-enantiomers.

The Insects Revisited

In 1960, Meister, Srinivasan, and I (*35*) reinvestigated the report of the presence of D-alanine in the milkweed bug (*7*). Since we were limited to the collection of about 20 microliters of blood per insect, it was necessary to devise a more sensitive technique for the determination of D-alanine than that available. With D-amino acid oxidase highly purified from porcine kidney, we were able to directly couple the oxidative deamination of D-alanine to pyruvate with the reduction of pyruvate to lactate by lactic acid dehydrogenase. The reaction was followed by observing the oxidation of reduced nicotinamide-adenine dinucleotide (NADH) at 340 nanometers in a spectrophotometer. By this means, it was possible to determine 0.05 micromole of D-alanine (4.5 micrograms) in a 1-milliliter cuvette, as well as D-serine and D-α-aminobutyric acid, both of which reacted more slowly than D-alanine under our conditions (*35*). A series of analyses was carried out on amino acid fractions obtained from extracts of milkweed bugs. The results of these studies which confirmed Auclair and Patton's (*7*) discovery and extended the finding to every life stage of *Oncopeltus* are summarized in Table 1. The alanine samples were eluted from paper after the amino acids were separated by chromatography followed by high-voltage ionophoresis (*35*). Insect blood contains approximately 10 to 20 times as much free amino acid nitrogen as mammalian blood, based on volume; and 50 microliters of *Oncopeltus* blood were sufficient for each analysis. When insects were held for 5 days on a diet of milkweed seeds and water containing penicillin, DL-alanine was still present, suggesting that the D-enantiomer is synthesized by the insect rather than by indigenous microorganisms.

D-Serine in Insects

During the studies on *Oncopeltus*, we also had a colony of Japanese silkworms, *Bombyx mori*, in laboratory culture. A deproteinized sample of *Bombyx* blood was added to an assay cuvette and, to our surprise, all of the NADH was rapidly used up. After several control experiments had been carried out, it was apparent that the oxidation of NADH required both D-amino acid oxidase and lactate dehydrogenase. The reaction was quantitatively comparable when either deproteinized blood or an amino acid fraction separated from the blood with an ion-exchange resin was enzymatically assayed. In order to carry out systematic studies on the amino acids present, we obtained a second, larger, sample of blood. No activity was observed when extracts of this blood were assayed. Then we realized that the first sample had been collected from last instar larvae which were in the midst of silk production, while the second sample had been collected from younger larvae which were actively feeding.

A third sample of blood was pooled

from a large number of larvae engaged in silk production, and amino acid fractions from this blood were very reactive in the enzymatic assay. After further investigation, the source of the activity was identified as D-serine. Several criteria were applied to prove that the material eluted from chromatographic papers was serine. Besides cochromatography of the isolated material with authentic serine in five different solvent systems and formation of a characteristically colored derivative with ninhydrin, the substance from *Bombyx* showed the same retention time as serine when its volatile *N*-acetylamino-*n*-amyl ester was subjected to gas-liquid chromatography (*36*). Oxidation of the isolated compound with periodic acid resulted in the loss of ninhydrin reactivity and elimination of the response in the enzymatic assay. Optical rotatory dispersion data on the isolated serine were in agreement with values obtained from a mixture of authentic D- and L-serine in the same ratio and concentration as the material isolated from silkworm blood (*37*). Samples of L-serine were subjected to each of the analytical steps in order to rule out the possibility of racemization during the isolation procedure.

The endogenous synthesis of DL-serine in pupae was readily observed after subintegumental administration of ^{14}C-D-glucose (*38*). Injection of ^{14}C-L-serine into larvae in the silk-spinning stage was also followed by the appearance of ^{14}C-DL-serine. Further studies revealed that homogenates of whole pupae slowly catalyzed the conversion of L-serine to D-serine. This reaction was dependent on *p*H, with an optimum at about 9. No conversion of L- to D-serine was observed in homogenates prepared from feeding larvae. Most of the racemase activity was detected in homogenates of the fat body (analogous to liver) compared to those from gastrointestinal tissue, carcass, and blood. The reverse reaction, conversion of D-serine to L-serine was also detected in homogenates prepared from whole pupae, although the reaction was slower than that observed starting from L-serine. The data suggest that the D-serine arises by enzymatic racemization of L-serine. Other possibilities include the synthesis of D-serine from glycine by means of a D-specific serine transhydroxymethylase or the presence of a D-transaminase. These have not been rigorously excluded. However, injection of labeled glycine into pupae failed to yield labeled D-serine, and no D-serine was detected in transaminase experiments with β-hydroxypyruvic acid and either L-alanine, L-glutamate, D-alanine, or D-glutamate. If a racemase is present, it will be the first amino acid racemase in animal tissues and the first report of a serine racemase in any organism.

A phylogenetic survey disclosed the presence of D-serine in 11 out of 13 species of the order lepidoptera but none in species from the orders coleoptera, diptera, hemiptera, or hymenoptera. D-Alanine was also found in two of the lepidoptera, thereby extending its occurrence into another order (*35*). Intensive studies on the different stages of *Bombyx* revealed that, while individual larvae either lacked D-serine or contained not more than 1 percent D-serine of the total serine in their blood, pupae of the same individuals contained significant amounts of the D-isomer. This information corroborated the enzymatic observations described above. The presence of lombricine or serine ethanolamine phosphodiester in silkworms was considered, but neither compound was detected upon examination of larval and pupal blood and tissues with a chromatographic system used for these metabolites in earthworms (*39*). Control analyses consisted of cochromatographic separations of mixtures of *Bombyx* and *Lumbricus* extracts. The number of species found with D-serine or D-alanine to date is summarized in Table 2. The frequent occurrence of D-serine in lepidoptera suggests that interesting metabolic processes distinguish this order from the others.

D-2,3-Diaminopropionic Acid in Insects

While D-serine was being investigated, Wada and Toyota announced the discovery of a new D-amino acid, D-2,3-diaminopropionate, in the gastrointestinal fluid of *Bombyx mori* (*40*). Since this compound and serine are both derivatives of propionic acid, it became important to extend the scope of the work to possible relations between these metabolites. Wada and Toyota had isolated the diaminopropionic acid from relatively large numbers of larvae with ion-exchange chromatography. A method was required that would permit rapid analyses on small samples, and a technique was devised which is illustrated in Fig. 2. After electrophoretic separation of the diaminopropionic acid from other amino acids, it was eluted from the paper and incubated with D-amino acid oxidase. A repetition of the electrophoresis disclosed that all of the diaminopropionic acid had been destroyed by the enzyme. This confirmed enzymatically what Wada and Toyota had already established polarimetrically; that the new amino acid occurred in *Bombyx* only in the D-configuration (*41*). These observations led to further experiments in my laboratory. The use of ^{14}C-D-glucose provided evidence of synthesis in vivo, but the immediate precursor of diaminopropionate is unknown. After administration to larvae, glucose carbon atoms were incorporated into diaminopropionic acid at a slow rate. Radioactive D-diaminopropionate was found irrespective of whether the ^{14}C-glucose was administered orally or intracoelomically. The isolated, radio-

Table 2. Distribution of D-amino acids in animals.

Amino acid	Source	Chemical form	References
D-Alanine	Anserine muscle	Free	*6*
D-Alanine	Milkweed bug	Free	*7, 35*
D-Alanine	Two species of lepidopterous larvae	Free	*35*
D-Alanine	Guinea pig blood	Free	*23*
D-2,3-Diaminopropionic acid	Five species of lepidopterous larvae	Free	*35, 40, 44*
D-Glutamic acid	May beetle muscle	Free	*6*
D-Ornithine	Shark liver	Free	*5*
D-Pyrrolidone carboxylic acid	Human urine	Free	*29*
D-Serine	Eleven species of lepidopterous pupae	Free	*35*
D-Alanine	Octopus muscle	In octopine	*9*
D-Cysteine	Firefly lantern	In luciferin	*22*
D-Serine	Earthworm	In serine ethanolamine phosphodiester, lombricine, *N*-phosphoryllombricine, and free	*11, 13, 15, 19*

active material was destroyed by incubation with D-amino acid oxidase.

Analyses of larvae and pupae resulted in the interesting observation that all of the diaminopropionate was gone from the pupal stage. Studies show that the synthesis of this D-amino acid begins after the larva emerges from the egg and prior to the commencement of feeding. The exact time when this compound disappears is not precisely known, but the disappearance seems to be at about the time of cocoon production and coincident with the racemization of serine. Since diaminopropionate vanishes as D-serine appears, it is consistent to suggest that the former is a precursor of the latter. The racemase experiments summarized above argue against this, but the possibility is not completely excluded and is supported by the observation that the labeling of D-serine in vivo from ^{14}C-glucose lags behind the labeling of L-serine (*38*). Evidence against the possibility that diaminopropionate is a precursor of D-serine is the fact that D-serine is in a dynamic state in pupae and can be labeled with ^{14}C-glucose even though no diaminopropionate can be detected in this stage. Radioactive diaminopropionate is being prepared in order to obtain further information on its metabolic fate.

Although diaminopropionic acid is absent from hydrolyzates of silk protein, it was postulated that this amino acid is found in larvae because it is related in some way to the presence of silk. To test this hypothesis, it was necessary to prevent the release of silk without interrupting the subsequent pupation. This was accomplished by surgically excising the spinneret through which the viscous liquid in the silk gland is passed during the construction of a cocoon. After this operation, mature larvae showed all of the behavioral and physiological changes typical of cocoon-spinning but were unable to extrude any silk. Most of these larvae pupated and the excess silk protein in the glands was very slowly resorbed. During the ensuing days, a gradual increase in the amounts of serine, glycine, alanine, glutamine, and valine took place (Figs. 3 and 4). These photographs illustrate the free amino acid patterns of pupae 2 days into adult development out of a total period of 12 to 14 days. Figure 4 is from an insect forced to retain its silk protein. The compound labeled "X" above and to the right of glycine is seen only in these hyperproteinemic cases. Analysis of the configuration of serine revealed that, as the total serine increased, it was racemized so that the D- and L-enantiomers increased approximately together. This process continued until the total serine and the D-serine reached concentrations as much as four to six times higher than the amounts found in normal insects. Data from the results of serine determinations on amino acid extracts prepared from various pupae are contained in Table 3. When electrophoretic analysis (*p*H 3.6) was carried out on each of the extracts used for Table 3, no diaminopropionic acid was detected in any of the samples. In almost every case insects forced to retain their silk cease development and die about midway through the period of adult differentiation.

The disappearance of diaminopropionic acid was considered in relation to the expulsion (purge) of alkaline gastrointestinal fluid (*p*H 10) which comes at the end of the feeding period and just before the extrusion of silk. This expulsion results in the excretion of a volume of fluid equivalent to about 10 percent of the weight of the larva and is associated with a decrease of the gastrointestinal *p*H to 7. Analysis of the purge fluid shows that not over 10 percent of the total diaminopropionate is lost by this route. It seems conclusive that diaminopropionate is metabolized by enzymatic reactions rather than by being physically excreted.

The possibility that these D-amino acids are of microbial origin requires some comment. When larvae of *Bombyx* were raised on leaves impregnated with a solution of penicillin, the pupae contained D-serine. Furthermore, many pupae of other species with a variety of host plants and habitats also contain D-serine (Table 2). A recent paper by Rao *et al.* (*42*) confirms the presence of D-serine in *Bombyx* pupae raised on an aseptic diet in the laboratory of T.

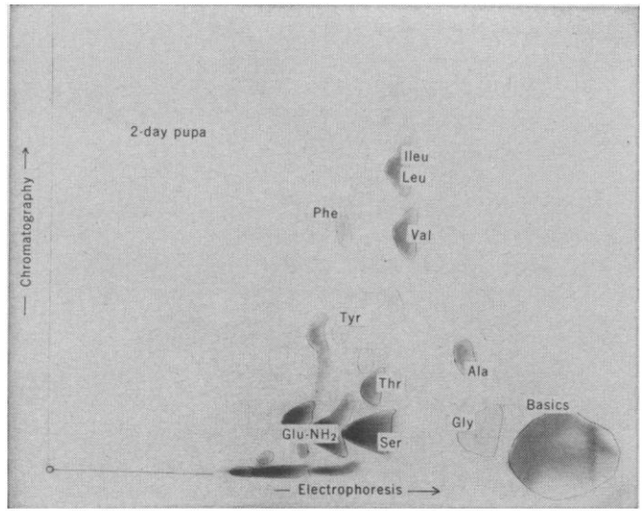

Fig. 3. The free amino acid pattern in an extract prepared from one normal pupa, 2 days into adult development. One-tenth of the extract was applied to the paper which was then subjected to chromatography in the vertical direction and electrophoresis in the horizontal direction with the negative electrode on the left. The amino acids were detected with ninhydrin (*35*). The D-serine content of this insect was 1.5 micromoles (157 micrograms).

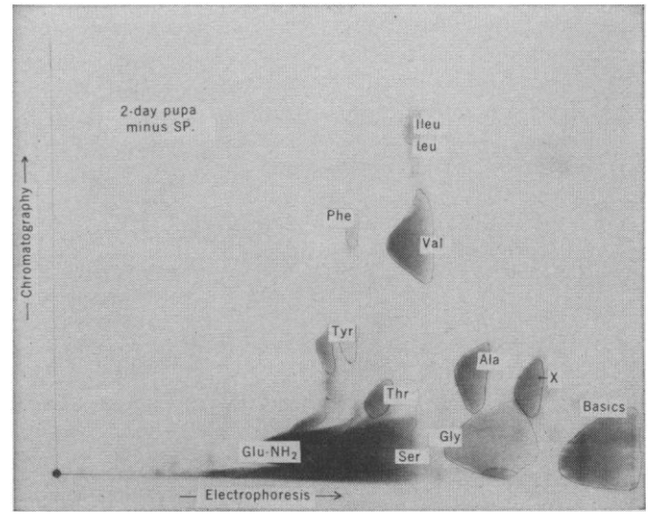

Fig. 4. The free amino acid pattern in an extract prepared from one pupa, 2 days into adult development. No silk extrusion occurred because the spinnerets were surgically excised. The analytical preparation was identical to that described for Fig. 3. The D-serine content of this insect was 6.2 micromoles (651 micrograms). Compound "X" to the right of and above glycine is seen only in insects forced to retain their silk protein.

Ito of the Sericultural Experiment Station, Tokyo, Japan. This report concludes with the statement that the large quantity of D-serine found in the silkworm has its origin in biosynthetic mechanisms which are an intrinsic part of *Bombyx*, in agreement with our conclusions (*38*). The endogenous origin of D-diaminopropionic acid is shown by its labeling from ^{14}C-D-glucose. Since it appears in newly hatched, unfed larvae and disappears prior to pupation, it seems unlikely that microbial sources would account for this close coupling to the larval stage. Several other species also contain D-diaminopropionate, including *Hyalophora cecropia*, *Antheraea pernyi*, *Antheraea polyphemus* [all giant saturniid silkworms (*43*)], and *Protoparce sexta* which does not spin a cocoon. Larvae of the genus *Antheraea* were found to contain substantially more D-diaminopropionate than representatives of the other three genera. In all of these cases, diaminopropionate cannot be detected in the pupal stage (*44*).

Discussion

From Fig. 1, it is apparent that lombricine and octopine resemble each other with respect to the D-amino acid residues as well as with regard to the guanidino groups common to each structure. These may prove to be examples of a class of compounds where the D-amino acid residues confer functional specificity on the molecules. Similarly, the remarkable ability of D-luciferin to emit light, compared to its L-enantiomer, may represent a case where the enzyme luciferase is capable of activating and oxidizing several substrates, but the D-cysteine is necessary for light emission which functions in the reproductive behavior of the beetles (*45*). In this sense, D-amino acids may be widespread and involved in special roles. New amino acids are continually being isolated from various animal sources, and the reports seldom specify the optical configuration.

The functional significance of D-amino acids in insects is unknown at this time, and no metabolite containing a D-amino residue has been isolated yet. A number of observations on biological effects of D-serine which may be relevant to its occurrence have been made. Several reports are devoted to its toxicity for a variety of organisms including bacteria (*46*), insects (*47*), and mammals (*48*). This is somewhat anomalous since most D-amino acids are less toxic than their corresponding L-isomers, as judged by experiments on intraperitoneal administration of synthetic mixtures to rats (*49*). With many bacteria, D-serine in the medium inhibits growth, and, in some cases, aberrant morphological forms have been detected (*50*). In this respect, D-serine may mimic some of the effects of penicillin. Recently, Strominger and his collaborators reported that certain D-amino acids including serine can reverse the transpeptidation reaction that links the peptide side chains during the synthesis of the microbial cell wall (*2*). The capacity of D-serine to inhibit larval growth in insects was discovered when this amino acid was added to synthetic diets during studies on nutrition (*47*). These facts make the occurrence of D-serine in lepidoptera particularly intriguing and suggest that it could play a role in protecting certain species from either microbial or insect parasites. In my laboratory, D-serine has been observed to inhibit the larval development of silkworms when administered with their host plant or injected in single large doses. Similar injections into pupae had no visible effect. However, as discussed above, mortality of pupae is high when silk extrusion is prevented, leading to the accumulation of DL-serine and certain other amino acids. In this case, the cause of death is unknown.

In 1942, Fishman and Artom reported that the daily administration of an aqueous solution of 100 mg of DL-serine to rats, resulted in anorexia, albuminuria, and hemorrhages under the nails and throughout the lungs (*51*). Subsequent investigation showed that the D-enantiomer was responsible for the symptoms and that single, daily doses produced renal necrosis with extensive tubular calcification (*52*). Since D-amino acid oxidase is concentrated in the renal tissue of mammals (*24*) and hydrogen peroxide is a product of oxidative deamination, it has been suggested that renal catalase is temporarily overloaded by acute doses of a D-amino acid. However, Fishman and Artom reported that DL-alanine failed to produce demonstrable lesions in renal tissue (*53*), and D-alanine is known to be deaminated more rapidly than D-serine by renal D-amino acid oxidase (*4*). These reports could be related to the interesting discovery that D-serine is an inhibitor of the uptake of ions by plant tissue (*54*). In 1963, Ellis, Joy, and Sutcliffe reported that a number of ions were affected including potassium, sodium, chloride, nitrate, phosphate, and sulfate. Salt uptake by washed slices of red beet root tissue was inhibited by 50 percent when the slices were exposed to D-serine at concentrations ranging from 1 to 5 millimolar. Ellis and his colleagues also observed that D-serine does not have to be present in the external medium in order to inhibit ion transport since pretreatment of plants with D-serine followed by an aqueous rinse produced similar inhibition. The influx of D-serine into beet slices was independent of the ions themselves, and was not affected by the simultaneous presence of potassium, sodium, chloride, nitrate, phosphate, and sulfate in the external medium. There may be clues here regarding a special function for D-serine in lepidoptera. The racemization of serine coincides with profound changes in the fluid balance and homeostatic state of the larvae after the abrupt expulsion of gastrointestinal contents which precedes silk extrusion.

In considering ion and fluid transport, it should be noted that serine and threonine are the only amino acids which contain an O-C-C-N system viewed along the α- and β-carbon atoms. When the nitrogen is positively charged, an electrostatic interaction takes place between it and the electronegative oxygen of the hydroxyl group. This sets up a staggered conformation about the axis of the carbon-carbon bond of the α- and β-carbon atoms. The projected angle between the nitrogen atom on the α-carbon and the hydroxyl-oxygen atom on the β-carbon then has a value of 60°, and the result is a *gauche* conformation. With other amino acids, no such interaction is found, and the projected angle

Table 3. Total serine and D-serine in normal *Bombyx* and in *Bombyx* forced to retain silk.

Pupal age	Sex	D-Serine (μmole)	Total serine (μmole)	D-Serine (% of total)
New (S)*	M	2.7	3.7	73
New (S)	F	2.0		
New	M	0.5	1.0	50
New	M	0.5	1.6	31
2 days (S)	M	6.2		
2 days	F	1.5	2.7	56
5 days (S)	M	6.6	11.5	57
5 days (S)	M	4.6	11.0	42
5 days	F	2.0	3.2	62
7 days (S)	M	11.8	21.0	56
7 days	M	2.7	3.5	77

* S, pupa forced to retain silk. The values are for the total insect in each case. The analytical methods are described in the text and in (*35*).

between the nitrogen atom on the α-carbon and the substituent on the β-carbon is 180° (the *anti* or *trans* conformation). The O-C-C-N system and the *gauche* conformation are structural features common to phospholipid components such as ethanolamine phosphate, serine phosphate, choline, and so forth, and neurologically active substances including acetylcholine and muscarine. This subject is thoroughly discussed by Sundaralingam (*55*). The existence of a stabilized *gauche* conformation relates serine to the membrane phospholipids and may prove significant in elucidating the role of D-serine.

The most striking observation regarding diaminopropionic acid is the fact of its rapid and total disappearance at the termination of the feeding period. In those species which contain it, this event is a metabolic seal, signifying the end of larval life and the preparation for metamorphosis (*44*). The D-isomer has been found only in insects. Consequently, other examples of this compound must be drawn from the limited reports on the L-isomer which is found in extracts of the seeds of *Mimosa palmeri* (*56*), *Vicia baicalensis* (*57*), and in hydrolyzates of the antibiotics viomycin (*58*) and edeine (*59*). It is noteworthy that L-diaminopropionate can be concentrated very rapidly by ascites carcinoma cells (*60*), and it would be interesting to learn if a similar transport rate is observed with the D-enantiomer. Diaminopropionate is a vicinal diamine, and compounds in this category are not common metabolites. The diamino groups permit chelate rings to form easily. The chelating properties can be seen in the fact that diaminopropionate shows high stability constants for a number of divalent cations including nickel, zinc, cobalt, iron, and magnesium (*49*, *61*). The higher this value, the more stable the complex formed. Authoritative coverage of this topic can be found in the excellent treatise by Greenstein and Winitz (*49*). It is quite possible that D-diaminopropionate plays a role in the transport of metal ions important for larval life. If the carboxyl group were esterified, thereby repressing its acidic nature, diaminopropionate would be similar to ethylenediamine which is used industrially as a chelating and solubilizing reagent.

Alternatively, biosynthetic substitution on the β-amino group of diaminopropionic acid could convert it into a molecule with behavior-modifying properties. Derivatives of this type occur in plants and show neurotoxic effects when administered to laboratory animals. In 1963, Adiga, Rao, and Sarma isolated β-*N*-oxalyl-L-α,β-diaminopropionic acid from various species of the genus *Lathyrus* (*62*) and demonstrated its ability to produce neurological symptoms after intraperitoneal administration to immature chickens. In 1967, Vega and Bell isolated β-*N*-methyl-α,β-diaminopropionic acid from seeds of the ancient plant *Cycas circinalis*. When this amino acid was injected into immature chickens, pronounced neurological effects were observed. It was suggested that these effects may be related to the high incidence of amyotrophic lateral sclerosis found among the inhabitants of Guam, where the seeds of cycads are a source of starch for human consumption (*63*). The *N*-methyldiaminopropionic acid could not be detected in extracts of *Bombyx* examined in my laboratory (*64*).

Conclusion

The natural occurrence of D-amino acids poses questions concerning their evolutionary significance, their role in animal metabolism, and their function in insect differentiation. From a teleological viewpoint, the advantage of these substances may be that they provide functional groups essential for special reactions without themselves being utilized for general metabolic purposes. Thus, in annelids, D-serine contains the chemical substituents required for the synthesis of lombricine, without being susceptible to incorporation into protein. Similar factors may explain the presence of D-alanine in octopine and D-cysteine in luciferin.

Insect development is one of several systems useful for exploring hormone action in relation to genetic expression. With this system, it has been possible to study morphological changes in nuclear chromatin (*65*), the activity of enzymes which synthesize precursors of DNA (*66*), changes in various species of RNA (*67*), and modifications in the metabolism of tyrosine (*68*) at the time adult development is initiated. To this information has now been added the discovery and synthesis of D-2,3-diaminopropionic acid and D-serine during metamorphosis. Because of their sequential appearance, these amino acids may serve as indices of activity for different regions of the gene.

The D-amino acids in insects account for a large fraction of the free amino acid nitrogen in body fluids. Recent analytical innovations include sensitive, stereospecific methods which promise to facilitate the quest for D-amino acids in animals (*69*). We shall undoubtedly see increasing reports of these compounds in the future.

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Photoperiod, Endocrinology and the Crustacean Molt Cycle

Seasonal changes in endocrine levels may alter the effect of photoperiod on the molt cycle of the crayfish.

D. E. Aiken

More than 2000 years ago Herodotus wrote: "Exposure to the sun is eminently necessary to those who are in need of building themselves up and putting on weight."

Whatever the merits of his counsel to mankind, he spoke the gospel for an assortment of lesser creatures. Directly or indirectly, most animal species respond to the influence of the sun. Dormant animals become active, lean animals become fat, small ones become larger, and complex activities associated with growth and reproduction wax and wane in harmony with the solar rhythm.

The timing of sunrise and sunset is a predictable event in an otherwise fickle environment, and this is extremely important to the many animal species which must time their vital functions to coincide with the appropriate seasons. Thus it is not surprising that so many have come to rely upon the relative lengths of the light and dark phases—photoperiod—for information on progression of the seasons. In the natural environment these light-dark cycles always approximate 24 hours, and this fact has a profound effect upon living systems. The physiologic functions of virtually all organisms, with the exception of bacteria and blue-green algae, show periodic oscillations (*1*). Such oscillations are termed "endogenous" if they persist in the absence of cues from the external environment, and "circadian" (*2*) if they have a period of about 1 day. Environmental stimuli "phase-set" these oscillations to keep them properly tuned to progression of the seasons. The concept of circadian periodicity has generated intense investigation into the nature of the timing mechanism (the biological clock) involved in the regulation of such cycles, and this subject appears to be at the root of a complete understanding of photoperiodism.

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