AMINO ACIDS IN THE HAEMOLYMPH OF SMALLER EUROPEAN ELM BARK BEETLE LARVAE, *SCOLYTUS MULTISTRIATUS* (MARSHAM) (COLEOPTERA: SCOLYTIDAE)[1, 2]

GAIL F. KLEINER AND JOHN W. PEACOCK

Northeastern Forest Experiment Station, Forest Service, U. S. Dept. Agriculture, Delaware, Ohio 43015

ABSTRACT

Nineteen amino acids and two amides were detected in the haemolymph of last-instar *Scolytus multistriatus* larvae by thin-layer chromatography. Glycine, arginine, glutamine, lysine, ornithine, histidine, asparagine, glutamic acid, serine, proline, alanine, valine, isoleucine, and leucine were readily detected. Taurine, phenylalanine, tyrosine, α- and β-amino butyric acid, and aspartic acid were less readily detected on some chromatograms, and at times were apparently absent in the haemolymph extract. Cysteine, cystine, and cysteic acid were not adequately separated by any of the methods tried, but their presence was confirmed through co-chromatography.

INTRODUCTION

The free amino acids of insects have been studied as an indication of physiological condition (Pickett and Friend, 1966; Corrigan and Kearns, 1963), as a

[1]Mention of a specific product in this paper does not imply its endorsement by the U. S. Department of Agriculture.

[2]Manuscript received December 19, 1969.

taxonomic tool (Micks and Gibson, 1957; Schaefer and Wallace, 1967), and for the determination of nutritional requirements (Kastings and McGinnis, 1962). Our investigation of the amino acids present in the larval haemolymph of the smaller European elm bark beetle *Scolytus multistriatus* (Marsham) may be useful in nutritional studies of this insect.

Paper-partition chromatography has been used extensively in the determination of amino acids in insect haemolymph. However, since the development of thin-layer chromatography, several authors (Pickett and Friend, 1966; Kastings and McGinnis, 1962; Schaefer, 1964; and Schaefer and Wallace, 1967) have used this technique for amino-acid analysis of insect haemolymph. Thin-layer chromatography is more sensitive, has shorter running times, and develops more compact spots than does conventional paper chromatography. For these reasons, we used thin-layer chromatography to determine the free amino acids in the haemolymph of *S. multistriatus* larvae.

MATERIALS AND METHODS

Rearing and Collection of Larvae

All *Scolytus multistriatus* larvae used in this study were obtained from a laboratory culture and were reared on elm bolts in plastic-coated paper cartons according to the method described by Peacock, Kennedy, and Fisk (1967). Bolts were removed from the rearing cartons approximately three weeks after infestation, and the bark was removed to expose the larvae. Larvae were removed from elm bolts daily during the period of collection, and were placed in petri dishes on filter paper moistened with distilled water.

Sample Preparation

The larvae were killed by refrigerating at 5° C for 10 to 30 minutes. They were then rinsed in 50% ethanol to remove any debris from the integument. Each specimen was blotted dry and placed on its side under a microscope, and the integument of the dorsal thorax was punctured with a dissecting needle. The larva then was turned on its ventral side, and its body was squeezed gently until a drop of clear haemolymph appeared at the puncture. The exuded haemolymph was collected in a disposable 2-μliter capillary pipette. The haemolymph was emptied into a 12-ml centrifuge tube, and the pipette was rinsed three times with 80% ethanol. The washings were added to the haemolymph in the centrifuge tube. When the desired amount of haemolymph was collected, the volume in the centrifuge tube was brought up to 3.0 ml with 80% ethanol.

The haemolymph was deproteinized by the method described by Schaefer (1964). The centrifuge tube containing the haemolymph in 80% ethanol was heated in a water-bath to 87°C. The sample was then centrifuged for 10 min at 2700 x *g*, and the supernatant containing the deproteinized extract was transferred to a 15-ml screw-capped glass vial. The residue was heated and centrifuged twice more, once in 80% ethanol and once in deionized distilled water, and the supernatants were pooled and frozen.

The deproteinized extract was then fractionated on a column of Amberlite IR–120 cation-exchange resin in the H^+ form. The amino acids retained on the column were removed by successive elution with 10% ammonium hydroxide, 30% ammonium hydroxide, and deionized distilled water. The pooled eluant of the three solvents was then reduced in volume by flash-evaporation at 40°C in a vacuum, dried under a stream of nitrogen, and redissolved in 2 ml of 10% isopropanol. This amino-acid fraction of the deproteinized haemolymph extract was then analyzed by thin-layer chromatography.

Chromatography

Glass plates were coated with cellulose powder MN 300 (Brinkman Instruments, Inc., Cantiague Road, Westbury, New York) as described by Jones and

Heathcote (1966). The adsorbent layer was prepared by mixing 15 g of the powder with a solution of 75 ml deionized distilled water and 10 ml absolute ethanol in a Model PB–5A Waring Blender. The blender was operated at high speed for 45 sec and then at medium speed for 45 sec. The resulting slurry was poured into a Desaga spreader and left standing for 4 min to allow the air bubbles to break.

This mixture was enough to coat five glass plates, 20 x 20 cm in size, with a layer 300 μ thick. The coated plates were placed in a horizontal position for 30 min to allow the coating to set, and then were transferred to a chromorack and air-dried in a vertical position at room temperature overnight. The plates were stored in a desiccating cabinet over silica gel. Before use, the plates were activated by heating them in a vertical position in an oven at 110°C for 30 min.

Standard amino acids were dissolved at the rate of 0.3% in deionized distilled

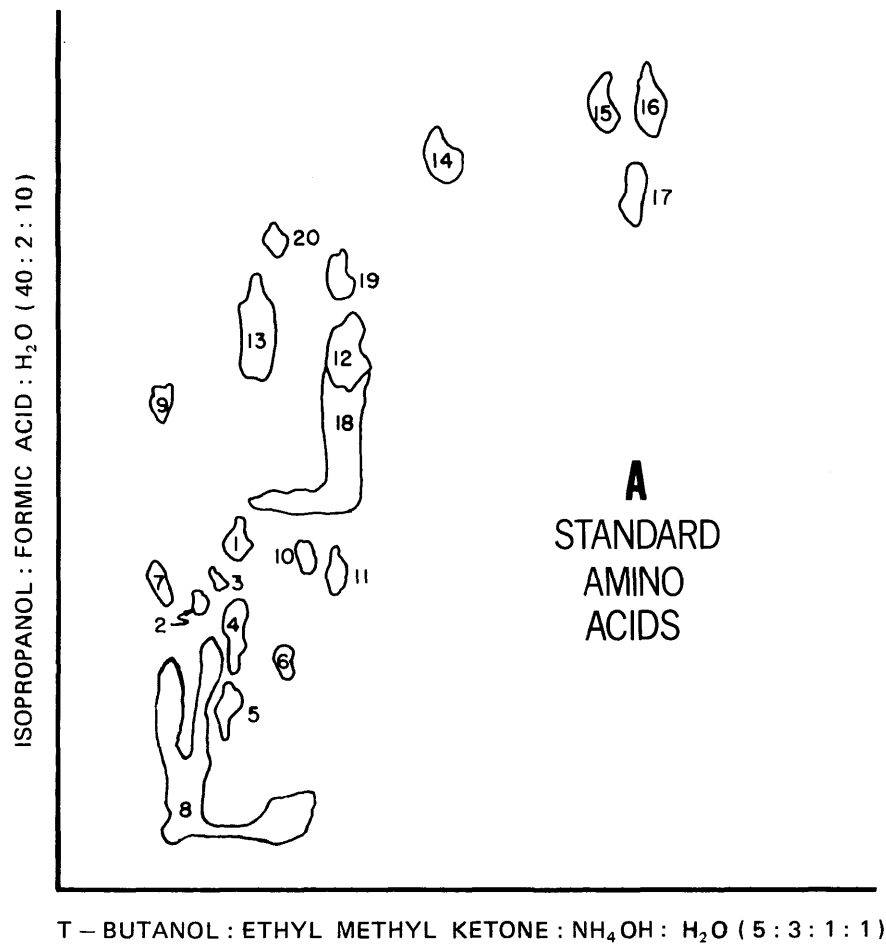
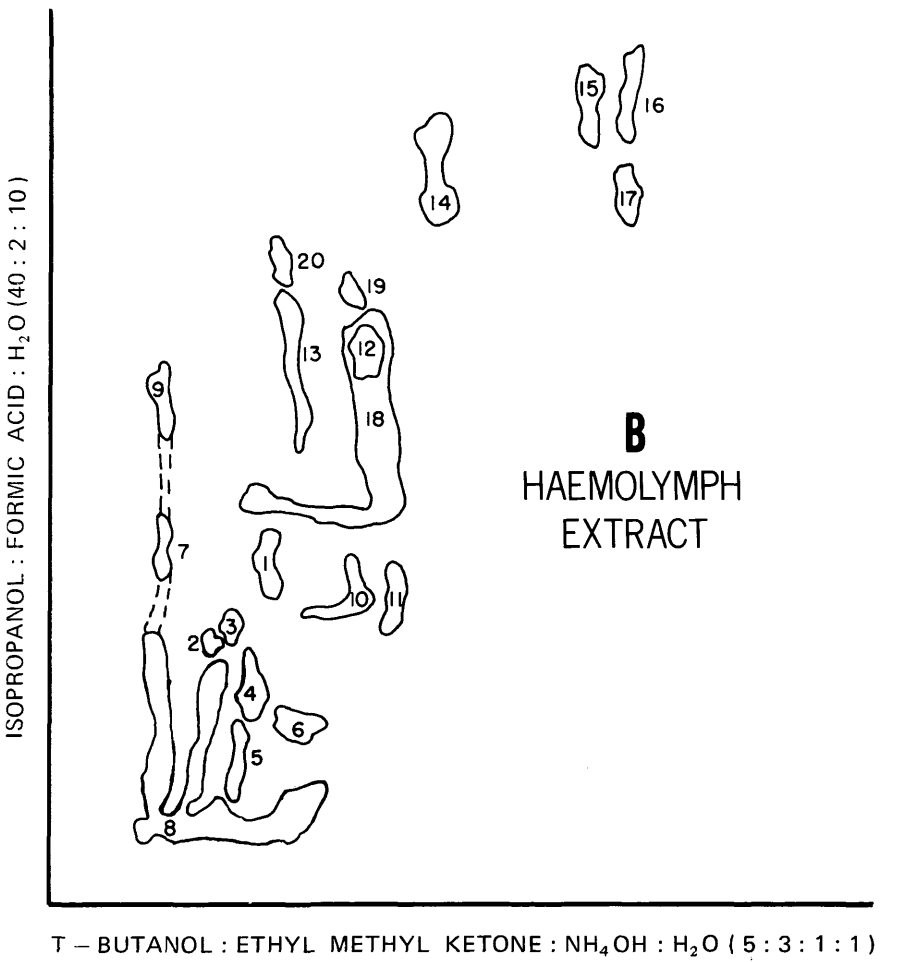


FIGURE 1. Chromatograms showing separation of (A) standard amino acids and amides and (B) haemolymph extract in isopropanol/tert-butanol system. Numbers represent: 1=glycine; 2=arginine; 3=glutamine; 4=lysine; 5=ornithine; 6=histidine; 7=asparagine and aspartic acid; 8=cysteic acid, cysteine, and cystine; 9=glutamic acid; 10=serine; 11=taurine; 12=proline; 13=alanine; 14=valine; 15=isoleucine; 16=leucine; 17=phenylalanine; 18=tyrosine; 19=α-amino-n-butyric acid; 20=β-aminobutyric acid.

water or 10% isopropanol, depending on their solubility (Smith, 1960), and were spotted in amounts varying from 1 to 5 μliters. Initially, the haemolymph extract was spotted at rates of 10, 25, 35, and 50 μliters. A volume of 35 μliters proved to be the most satisfactory.

The solvent system used was isopropanol: formic acid: water (40:2:10) in the first direction, and tert-butanol: methyl ethyl ketone: 0.88 ammonium hydroxide: water (5:3:1:1) in the second direction (Jones and Heathcote, 1966). Before development, the plates were pre-washed in the first direction with the isopropanol system.



Although adequate separation of the amino-acid standards was achieved by conventional two-dimensional methods using the above solvent system, considerable difficulty was encountered in the separation of the the haemolymph extract. Separation of the extract was greatly improved by running the plates twice in each solvent system. The isopropanol system was used in the first and third directions, and the tert-butanol system was used in the second and fourth directions.

The isopropanol solvent was always run in the direction opposite to that of the adsorbent layer application. The tert-butanol solvent was run at right angles

to the run in isopropanol. The plates were air-dried and stored overnight in a desiccator between runs.

Using this method, most of the free amino acids in the haemolymph were separated and identified by comparison with the standards. However, certain of the amino acids overlapped and could not be readily identified. Several two-dimensional systems reported in the literature were tried, but in all cases, considerable tailing and poor separation were encountered. However, development of the plates in n-butanol: glacial acetic acid: water (4:1:1) (BuA) in both directions adequately separated those amino acids that were not separated by using the isopropanol-tert-butanol solvent system described previously. Separation was improved further by running the plates twice in each direction, as described above.

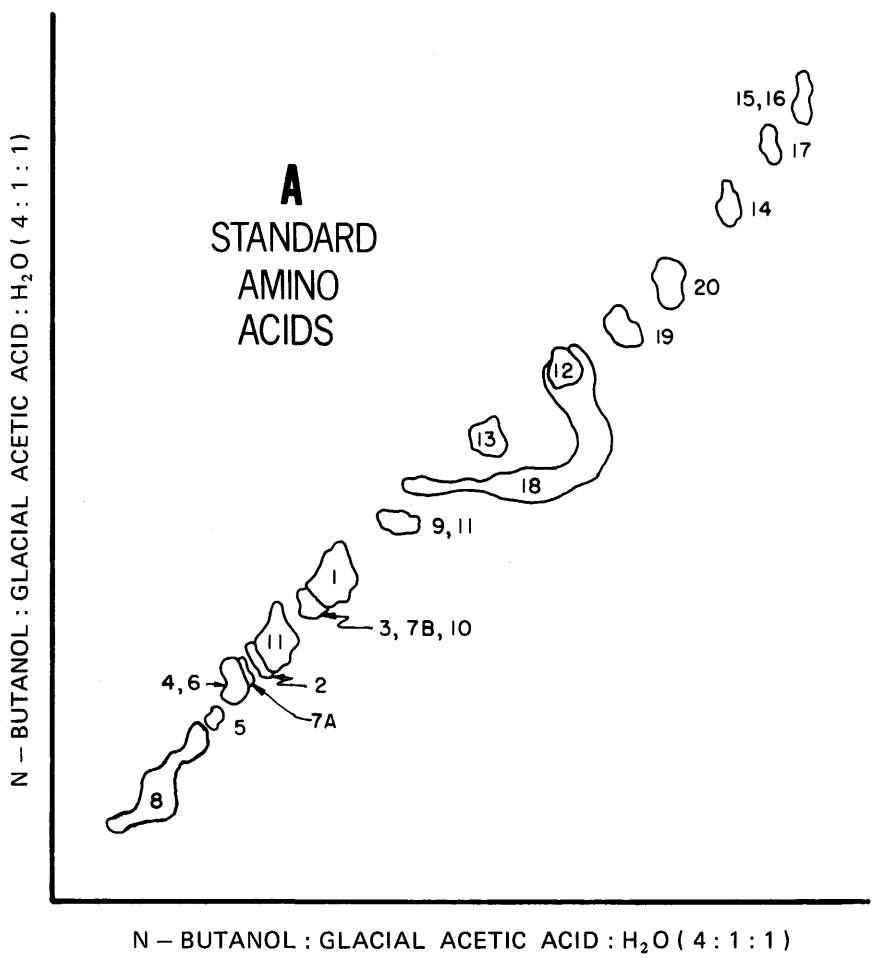
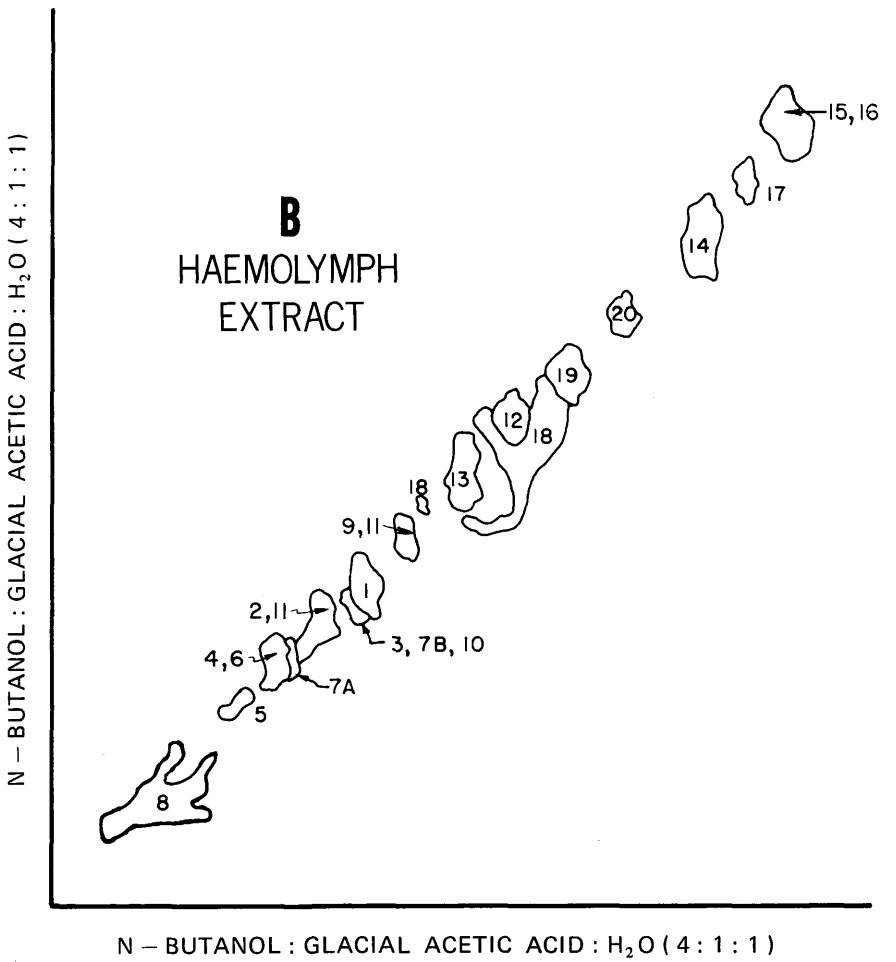


FIGURE 2. Chromatograms showing separation of (A) standard amino acids and amides and (B) haemolymph extract in BuA system. Numbers represent: 1=glycine; 2=arginine; 3=glutamine; 4=lysine; 5=ornithine; 6=histidine; 7=(A) asparagine, (B) aspartic acid; 8=cysteic acid, cysteine, and cystine; 9=glutamic acid; 10=serine; 11=taurine; 12=proline; 13=alanine; 14=valine; 15=isoleucine; 16=leucine; 17=phenylalanine; 18=tyrosine; 19=α-amino-n-butyric acid; 20=β-aminobutyric acid.

Amino acids were detected on the developed plates by using the ninhydrin-cupric nitrate chromogenic reagent described by Moffat and Lytle (1959). The reagent was prepared fresh for each use by mixing 100 ml of solution 1 (a freshly prepared mixture of 100 ml of 0.2% ninhydrin in absolute ethanol, 20 ml glacial acetic acid, and 4.0 ml 2,4,6-trimethylpyridine) with 6.0 ml of stock solution 2 (1% cupric nitrate trihydrate in absolute ethanol). After chromatography, the plates were air-dried, sprayed with the reagent, air-dried again, and developed by heating 5 to 10 min in an oven at 110°C.



The haemolymph amino acids were identified by direct comparison with the positions and colors of the known pure standards and by co-chromatography. No attempt was made at quantitative analysis, but concentration was estimated from spot size and color intensity.

A permanent record of each plate was made with a Xerox copy machine after each spot was circled with a needle. Each plate was also photographed on color film.

RESULTS

Nineteen amino acids and two amides were positively identified in the haemolymph of *Scolytus multistriatus* larvae. Two solvent systems were required for aequate separation and identification of all of these compounds. Figure 1 shows the separation of (A) the amino acid and amide standards and (B) the larval haemolymph extract in the isopropanol/tert-butanol solvent system. Separation of the standards and the extract in the BuA system is shown in Figure 2. The isopropanol/tert-butanol system was satisfactory for separation of most of the amino acids and amides present in the haemolymph. However, the amino acid pairs, citrulline and arginine, α-amino-n-butyric acid and β-aminobutyric acid, and the amide asparagine and the amino acid, aspartic acid, were not readily separated using this solvent system, and were separated by the BuA system.

The concentrations of the amino acids, glycine, arginine, lysine, ornithine, histidine, glutamic acid, serine, proline, alanine, valine, isoleucine, and leucine, and the amides, glutamine and asparagine, were great enough to be readily detected in all tests. Certain other amino acids were less readily detected on some chromatograms. These, in order of descending concentration, were: taurine, phenylalanine, tyrosine, α-amino-n-butyric acid, β-amino butryic acid, and aspartic acid.

Differences in the concentrations of the amino acids in some chromatograms could be attributed to variations in host-plant material on which the larvae were reared, since haemolymph samples were collected at three different times over a period of two years. In addition, these amino acids may have been overlooked on certain plates due to their low concentrations and corresponding faint color reactions.

Cysteine, cystine, and cysteic acid in the haemolymph were not adequately separated by any of the solvent systems. However, co-chromatography of the haemolymph with standard solutions of cysteine, cystine, and cysteic acid led to the conclusion that these amino acids were present in the larval haemolymph. It is possible that cysteine, cystine, and cysteic acid represent a portion of the spots labeled "8" in the figures and that one or more unidentified compounds were also present. Cysteine and cystine are interconvertible in all animals by oxidation or reduction, according to Gilmour (1961). Because cysteine is easily oxidized aerobically to cystine and cysteic acid, isolation of these last two is no proof that they exist in the living organism (Robinson, 1963).

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