Free Amino acid content and biosynthesis in eggs of the grasshopper Aulocara Elliotti Thomas during development
by Daryl Eugene Bunde

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Zoology
Montana State University

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VITA

Daryl Eugene Bunde was born to Mr. and Mrs. L. T. Bunde on October 29, 1937, in Sioux Falls, South Dakota. He attended high school in Sioux Falls, graduating in May, 1955. At Augustana College in Sioux Falls, he completed undergraduate study, receiving the degree of Bachelor of Arts in Biology in June, 1959. In September, 1959, he entered the Graduate School of The University of Texas and received the degree of Master of Arts in Zoology from there in January, 1962. His studies toward the doctoral degree began in May, 1962, when he entered the Graduate Division of Montana State College. He continued these studies with the assistance of a National Institutes of Health Predoctoral Fellowship.

In May, 1959, he was married to the former Marie Louise Erickson. They have three children, Steffen Maurice, age 5, Kermit Austin, age 3, and Jeannine Marie, age 1.

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ABSTRACT

Aulocara elliotti eggs from three wild and two greenhouse-reared populations were analyzed for free amino acid content and for *in vivo* synthesis of free amino acids from radioactive acetate. These analyses were performed on eggs in the prediapause, diapause, and postdiapause periods of development. Column chromatography, thin-layer chromatography, colorimetry, and liquid scintillation counting were used as analytical techniques. Fourteen free amino acids were present in each population egg sample throughout embryonic development. Five additional free amino acids and three unknown substances were found in some of the egg samples. The free amino acid content of each egg sample was found to increase considerably from the prediapause to the diapause period and then to decline during postdiapause development. Four amino acids were synthesized *in vivo* by all the egg samples during each period of development. Five additional ones were synthesized *in vivo* only in specific stages of development. All of the differences in free amino acid content and in the *in vivo* synthesis of free amino acids between the different egg samples at the same stage of development were not attributable to a direct effect of population density.

INTRODUCTION

The grasshopper species, Aulocara elliotti (Thomas), has been observed over an extended period of time to undergo considerable fluctuations in population density within a given habitat. Similar large periodic changes in population numbers have been recorded for other animals. Although various environmental conditions usually have been presumed to be the causative agents for these changes, more recent evidence indicates that intrinsic factors may be involved. In small mammal populations the stress of high densities has been suggested to be responsible for changes in the hormonal secretions within individuals, and also for changes in their behavior patterns. These in turn adversely effect the reproductive potential of the total population (Chitty, 1957, 1964; Christian, 1957, 1963 a, 1963b; Krebs, 1964).

Hastings and Pepper (1964) have indicated several factors including genetic segregation, physiological vigor, and environmental stress which may be associated with A. elliotti population fluctuations. They have also observed instances where mortality among newly hatched nymphs exceeded 99 percent with no apparent extrinsic cause for these high mortalities. This led them to conclude, (Pepper and Hastings, 1964), that the embryos from which the nymphs hatched were physiologically altered, and that this condition may have been due to stress imposed during the preceding adult stage which brought about a hormonal imbalance within the eggs. This imbalance in hormones, conceivably, could have effected the normal metabolsim of carbohydrate, lipid or protein materials

within the developing embryo. The result could be a physiologically deranged animal. Since the above was postulated, Svoboda (1964) investigated the lipid composition of A. elliotti eggs from different populations, but was unable to find any differences that could be correlated with population density; whereas, Van Horn (1963) followed the morphology of these eggs throughout embryonic development and discovered significant differences in the morphological variability and development rates in different populations.

Since the morphology of cells is associated with protein structure, it follows that abnormal morphological development may be the result of aberrant protein synthesis. In the cleiodic egg of a grasshopper the amino acids required for the synthesis of new embryonic proteins must either be synthesized by the embryo or come from the yolk material surrounding it. Thus the pattern of these unbound amino acid building blocks at any point in embryonic development represents an index of the protein metabolism of the egg at that stage of morphogenesis (Colombo et al., 1962).

Upon such a background, the present study was initiated to obtain some basic information on the free amino acid and protein metabolism of the egg. The pattern of distribution of the free amino acids throughout the development of the egg at prediapause, diapause, and postdiapause stages was investigated as were the patterns obtained from different wild and greenhouse-reared populations. Relationships were also sought between the free amino acid patterns and the density of the populations from which

the eggs were obtained.

The many investigations that have been done on the free amino acids extracted from mature insect tissues have been reviewed recently (Chen, 1962; Gilmour, 1961; Wyatt, 1961). These studies have shown that adult insect tissues contain a greater concentration of free amino acids than is normally found in other organisms. And also in insects, the level of concentration of each amino acids has been found to vary considerably both between different species and within one species examined at different times (Awapara, 1962; Duchateau and Florkin, 1955; Gilmour, 1961; Stephen and Steinhauer, 1959). Although special functions have been designated to this unique amino acid distribution, complete understanding of its significance is still lacking (Barton-Browne, 1964; Blackith, 1961; Bursell, 1963; Gilmour, 1961; Schoffeniels, 1960).

Additional evidence of the importance of free amino acids in the metabolism of insects has been found upon examination of immature stages. In the holometabolous insects, Hadorn and Mitchell (1955) studying the development of Drosophila melanogaster were the first to use paper chromatography to identify the free amino acids present in that species. Auclair and Dubreuil (1951) adapted this technique to the quantitative estimation of the free amino acids in the last larval instar of Galleria mellonella. Studies have also been reported on the free amino acids in immature forms of Aedes aegypti and Culex quinquefasciatus (Micks and Ellis, 1952), Bombyx mori (Drilhon and Busnel, 1952), C. pipiens (Chen, 1958), and D. melanogaster (Crone-Gloor, 1959).

Benz (1957), Chen and Hadorn (1955), Hadorn (1956, 1961), Hadorn and Stumm-Zollinger (1953), and Lewis (1954) established the usual pattern of occurrence of free amino acids in D. melanogaster throughout its embryonic development and detected deviations from this pattern in five lethal mutants, all of which died prior to reaching the adult stage. Similar evidence of the possible effect of free amino acids on embryonic development has been found in C. pipiens by Laven and Chen (1956) and in Ephestia kuhniella by Chen and Kuhn (1956). Various other studies have also illustrated other possible effects that alterations in free amino acid content can have upon specific physiological processes (Elvehjem, 1956; Lyman et al., 1964; March et al., 1964; Sidransky et al., 1964).

In the hemimetabolous insects, the presence of free amino acids in the grasshopper egg of Chortophaga viridifasciata has been shown by Shaw (1955). Fu (1957) noted the changes in concentration of sulfur containing amino acids in the developing grasshopper egg of Melanoplus differentialis. Twenty free amino acids were determined quantitatively by Colombo, et al., (1962) in eggs of the locust, Schistocerca gregaria, from ripe oocytes through to the time of hatching, and separately in yolk and embryos of later developmental stages. The results of these studies have shown that, in all of the holometabolous and hemimetabolous insects so far examined, the free amino acid content of the immature forms is high, and the pattern of distribution of these compounds varies with the stage of development.

To follow the biosynthesis of the amino acids in A. elliotti eggs and to determine which ones can be synthesized in vivo, various precursor compounds, containing radioactive isotopes, were introduced into intact organisms, and after varying lengths of time the free amino acids were examined for radioactivity. In recent reports, Kasting and McGinnis (1958, 1960, 1962) and Kasting et al., (1962 demonstrated the incorporation of C^{14} from uniformly labeled glucose into several amino acids in larvae of Agrotis orthogonia, Ctenicera destructor and Phormia regina. This technique was extended to other organisms using glucose-C^{14} and other radioactive precursors such as carbon dioxide-C^{14}, glycerol-C^{14}, and acetate-C^{14} (Awapara and Campbell, 1964; Mohri, 1964; Rothstein, 1963; Schaefer, 1964; Strong and Sakamoto, 1963). Generally, less than 11 of the common 20 amino acids were found to be synthesized by any organism.

To accomplish the purposes of this investigation into the amino acid metabolism of the A. elliotti eggs, the techniques of column chromatography, thin-layer chromatography, colorimetry, and radioactive tracing were selected.

PROCEDURE AND RESULTS

Biological samples

The eggs used in this study were obtained from three geographically separated wild populations, and from additional ones reared under greenhouse conditions at two different densities. The wild populations were located south of Chinook, in northcentral Montana, west of Simms, in central Montana, and northwest of Townsend, in central Montana. During the summer of 1964 the Simms population was the lowest in density while the Chinook one was the highest and the Townsend population was intermediate between the two.

The grasshoppers that were reared in cages in the greenhouse were collected as nymphs in late June from the population near Townsend. Twelve cages in all were used. Each had a bottom area of one square foot and was constructed with a circular wooden floor, cylindrical lucite wall, and a cheesecloth top. A removable nine inch aluminum pan, filled with soil in which the grasshoppers could oviposit, was inserted through a hole in the bottom of the cage. On alternate days fresh cuttings of Agropyron smithii, the preferred foodplant of A. elliotti (Anderson, 1964), were placed in three small vials positioned in the cage floor.

Eight cages were included in one series of tests with five pairs of adult grasshoppers in each cage. In a second series 15 pairs of adults were placed in each of four cages. On alternate days throughout July and August the soil in the pans was sifted and the egg pods removed. These pods were positioned vertically in vials partially filled with soil and

stored at 25°C.

Egg pods from the wild populations were collected in late August and stored at 25°C. in petri dishes containing soil taken from the collection site. In the first part of October these pods along with those from the greenhouse were transferred to a room held at 8°C. They were returned to the 25°C. chamber in early December. Each week throughout this period the pods were watered lightly to prevent dessication.

Samples of eggs from the three wild populations and the two greenhouse groups were analyzed for free amino acid content at prediapause, diapause, and postdiapause stages of embryonic development. One hundred eggs were used for each sample except those from the Simms population which were limited to 50 eggs. Only eggs which appeared viable and were in the desired stage of development were included in the sample. Diapause eggs were processed in the 8°C. room. They were immersed in distilled water and observed under a dissecting scope to determine the developmental stage of the embryo. This was accomplished by comparison with the morphological characteristics for each stage as outlined by Van Horn (1963).

Table I shows the numerical developmental age of the A. elliotti eggs from the different populations at the time of analysis. The use of two samples at each stage of development will be explained later. It will be noted from Table I that during the diapause and postdiapause periods, the numerically designated stage of development is quite

TABLE I

	Numerical Development Age				
	GH-5	GH-15	Chinook	Simms	Townsend
Prediapause	*11,12	12,13	15,16,17		16,17
	**12,13	12,13	13,14,15	13,14,15	14,15,16
Diapause	*18,19	18,19	18,19		18,19
	**18,19	18,19	18,19	18,19	18,19
			18,19***		
Postdiapause	*25,26	24,25	25		25
	**23,24,25	23,24,25	24,25	25	24,25

* Samples used in colorimetry analysis.

** Samples used in radioactive analysis.

*** Sample used in separation of yolk and embryo.

consistent for all populations. It was not possible, however, to obtain eggs from all the populations at the same stage during prediapause because there was no way of knowing when the wild eggs were deposited.

After the stage of development was determined, the eggs were air dried on filter paper for approximately 15 minutes and then weighed. The results are recorded in Table II.

Free amino acid extraction

After weighing, each sample was triturated in a microhomogenizer for five minutes with cold 80 percent ethanol. This mixture was heated for a few minutes to ensure denaturation of the protein and the total homogenate centrifuged at 5,000 rpm for 15 minutes. After decanting the supernatant into another tube, the residue was extracted with 50 percent ethanol and then with distilled water. This procedure was modified from that suggested by Schaefer (1963).

The supernatant and the two additional extractions were combined and passed through a short ion exchange column of Dowex 50Wx8 (H^+) following the steps outlined by Corrigan and Kearns (1963), Furuholmen et al., (1964), and Schaefer (1963). The samples containing the amino acids were then eluted with 50 milliliters of 4 N NH_4OH, dried in vacuo to near dryness, and made up to two milliliters with distilled water. All samples were stored in the deep freeze until assayed. A total of thirty samples were prepared.

TABLE II

Weight per 100 Egg Sample (gram)					
	GH-5	GH-15	Chinook	Simms	Townsend
Prediapause	*0.6406	0.6977	0.7593		0.6974
	**0.8935	0.8466	0.7005	0.9210	0.7737
Diapause	*1.0513	1.0800	0.9753		1.0277
	**1.0765	0.9664	0.9838	0.8280	1.0864
			1.0370***		
Postdiapause	*1.0728	1.1303	0.9644		1.0555
	**1.0588	1.0506	0.9887	0.8670	1.0134
Totals	5.7935	5.7766	6.4090	2.6160	5.6541
Averages	0.9656	0.9628	0.9156	0.8720	0.9424

* Samples used in colorimetry analysis.

** Samples used in radioactive analysis.

*** Sample used for separation of yolk and embryo.

Thin-layer chromatography

Paper chromatography was employed initially to separate and identify the free amino acids. This procedure, however, was discontinued in favor of thin-layer chromatography which is less time consuming and of greater sensitivity. The method of thin-layer chromatography as described by Randerath (1963a) was pursued. L-amino acid standards, all of reagent grade, were purchased from Mann Research Laboratories, New York, and from Sigma Chemical Company, St. Louis.

At first, two cellulose adsorbents (Cellulose MN 300 and MN 300 G, Brinkmann Instruments, Westbury, New York) were tried using the solvents proposed by Wollenweber (1962). Ten other solvents of the author's own derivation also were tested with these adsorbents. One-dimensional runs were satisfactory with a few of these solvents, but none produced usable results two-dimensionally.

The adsorbent Silica Gel G (Brinkmann Instruments) and the solvent systems developed by Brenner and Niederwieser (1960) and Fahmy et al., (1961) were tried also. Since the author was not able to reproduce the separation pattern using the two solvents that were especially recommended (Fahmy et al., p. 2022), several modifications were attempted. Finally, a more adequate two-dimensional separation of 24 amino acid standards was achieved by using chloroform-methanol-17 percent ammonia (CMA, 1:3:1 v/v) as the first solvent in combination with phenol-water (PW, 75:25 w/w) as the second solvent. Figure 1 illustrates this separation pattern. R_f

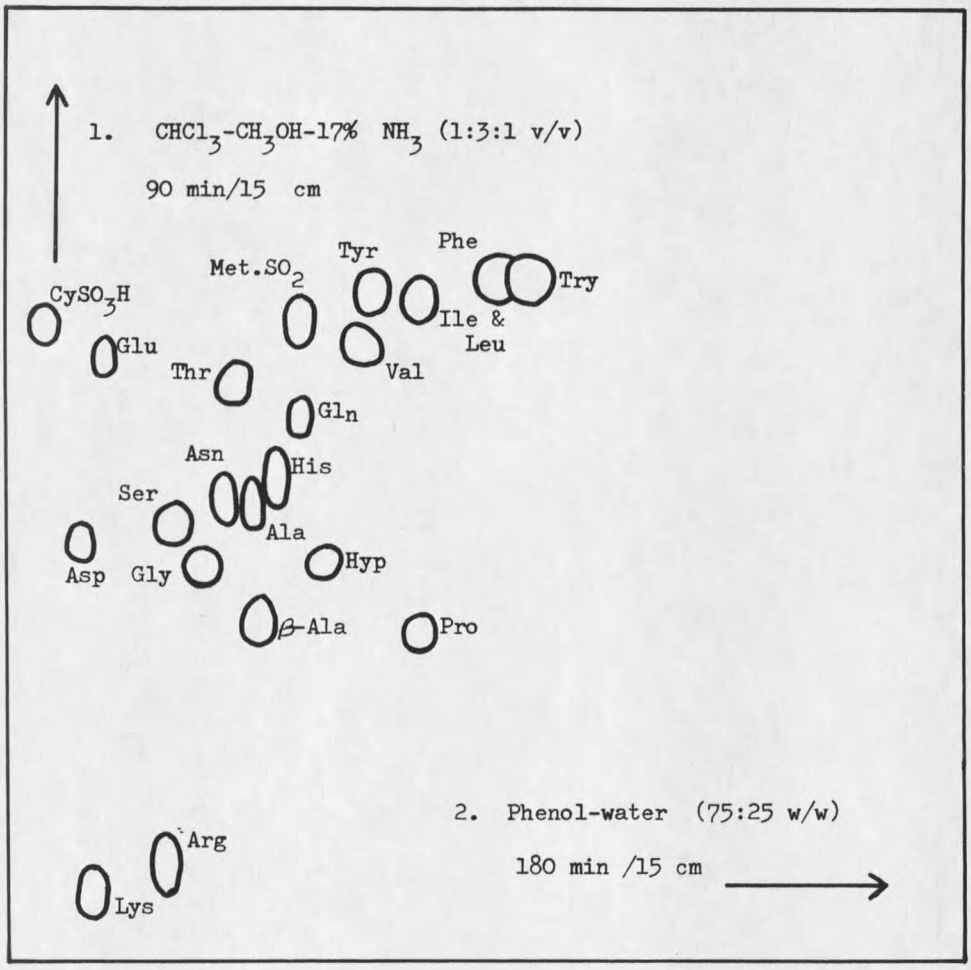

Figure 1. Two-dimensional separation of a mixture of 21 amino acids.

values for each amino acid in the CMA solvent is given in Table XI in the appendix.

Although the separation pattern produced by these two solvents (CMA and PW) was adequate, further attempts were made to improve the method. Another adsorbent, Anasil B, (Brinkmann Instruments) was tested with solvents reported by Cruickshank and Sheehan (1964). The results were unsatisfactory. A very useful separation pattern was finally obtained by substituting the solvent methyl ethyl ketone-propionic acid-water (4:3:2 v/v) used by Knight (1962) for the CMA solvent as the first dimension and phenol-water (75:25 w/w) as the second dimension with the silica gel adsorbent (Figure 2). The use of this combination was recently reported by Schaefer (1964). In all cases reagent grade solvents were employed but only one commercial phenol reagent (Mallinkrodt 88 percent phenol, liquefied, preservative free) was used. Richardson and Tolbert (1964) reported that with paper chromatography the commercial phenol reagent caused glycine to form stable condensation products.

Representative chromatographic distributions of free amino acids isolated from Chinook eggs at prediapause, diapause, and postdiapause development stages are shown in Figures 3, 4, and 5. In order to stay within the limits of the colorimetric quantitative test, the amount of sample used to obtain the chromatograph in Figure 3 was four times that in Figure 4 and two times that in Figure 5.

To detect and identify the individual amino acid spots on the

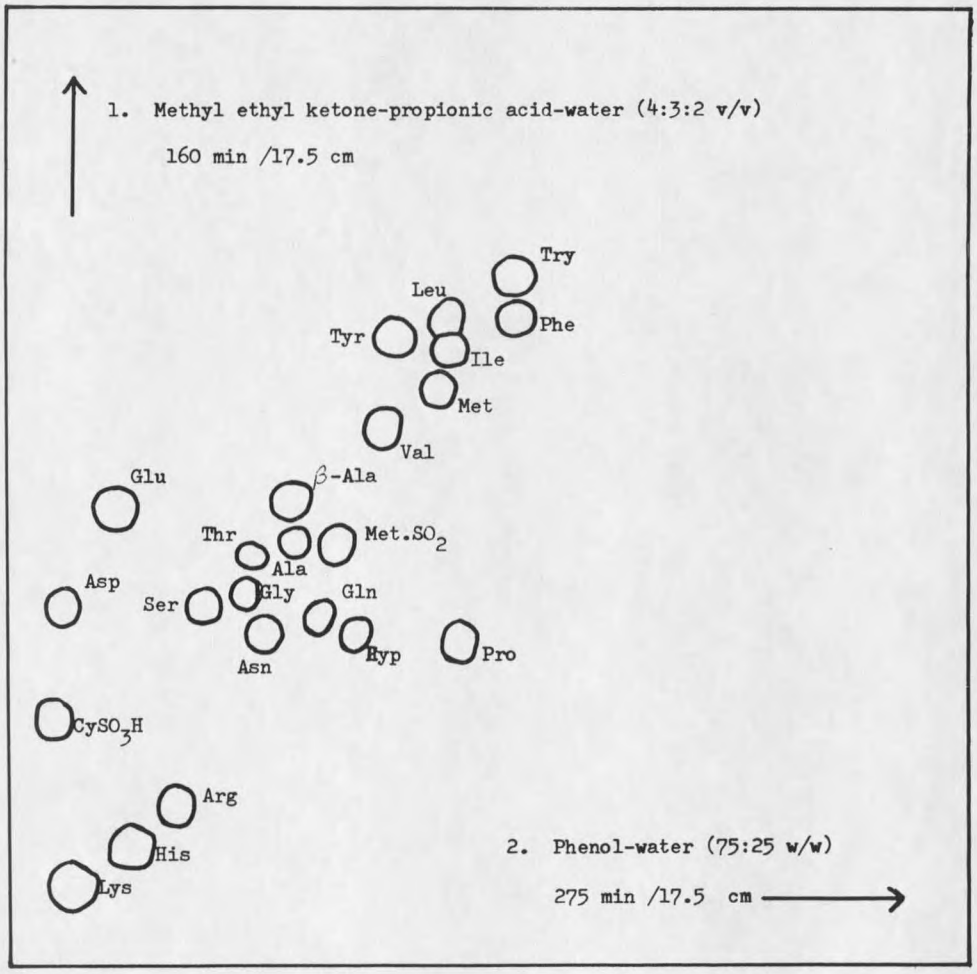

Figure 2. Two-dimensional separation of a mixture of 23 amino acids.

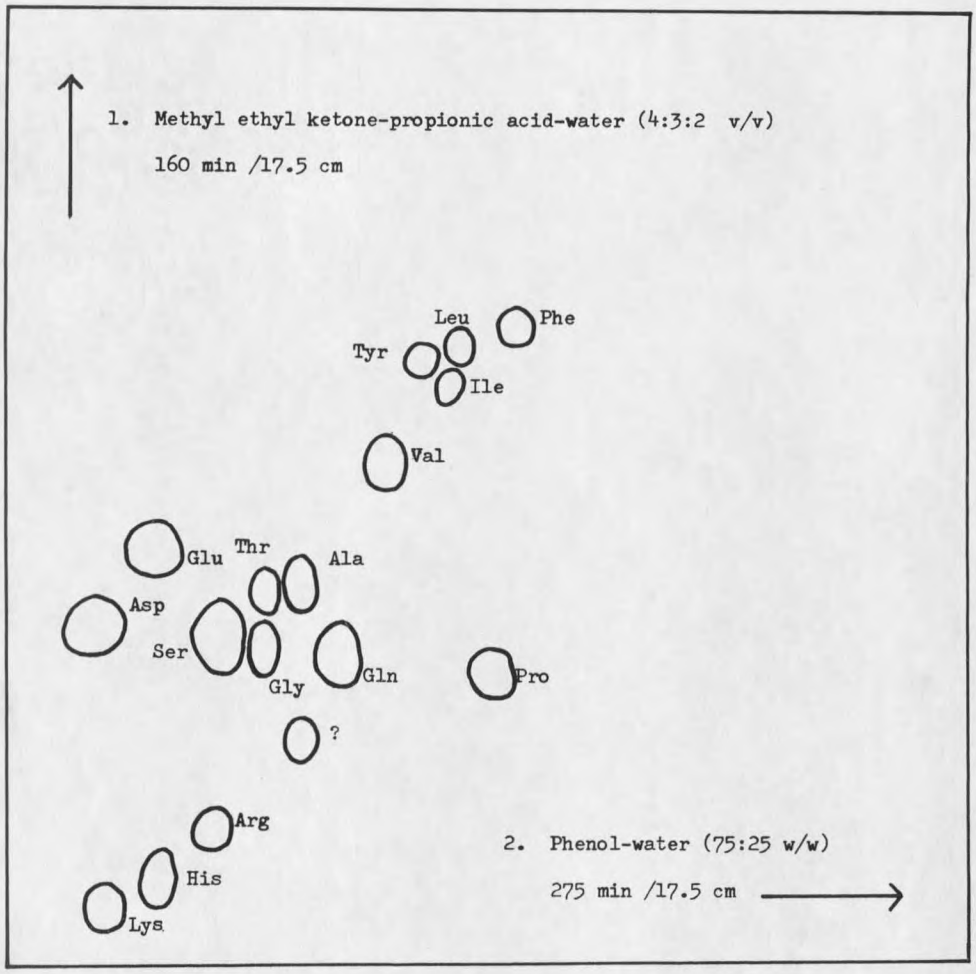

Figure 3. Two-dimensional separation of 16 free amino acids and one unknown substance from a sample of Chinook prediapause eggs.

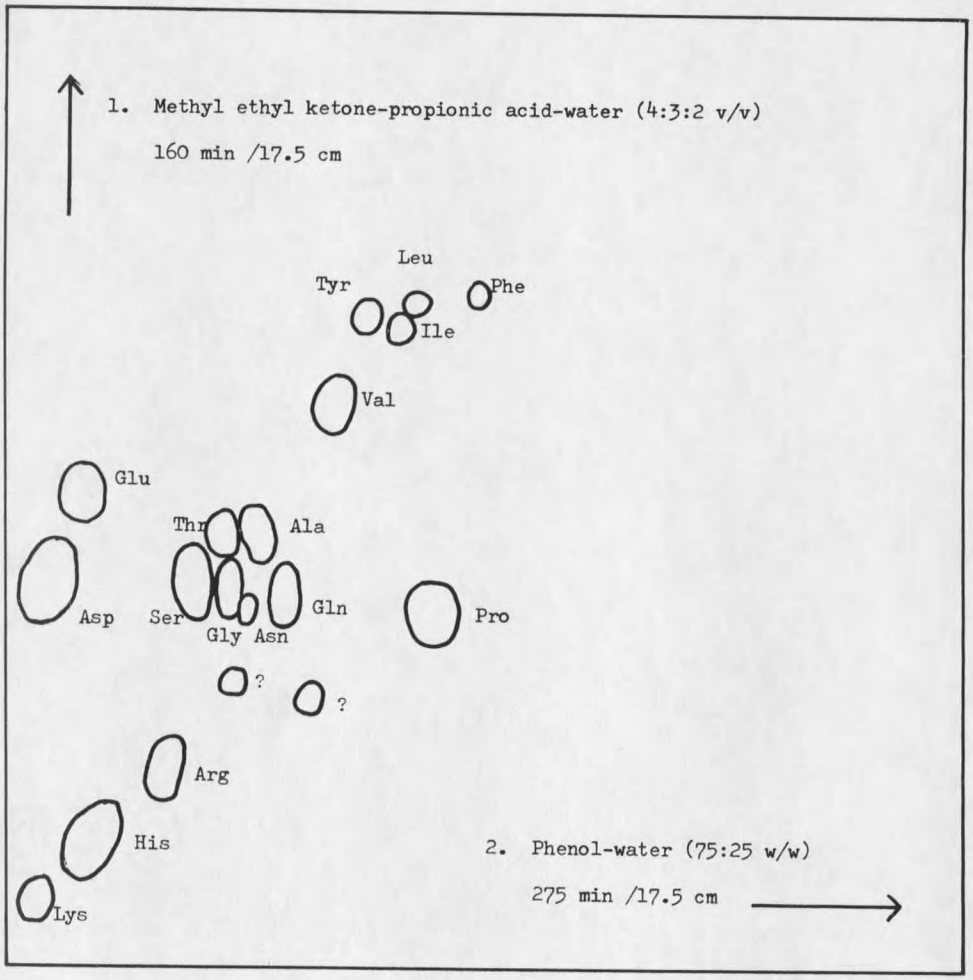

Figure 4. Two-dimensional separation of 17 free amino acids and two unknown substances from a sample of Chinook diapause eggs.

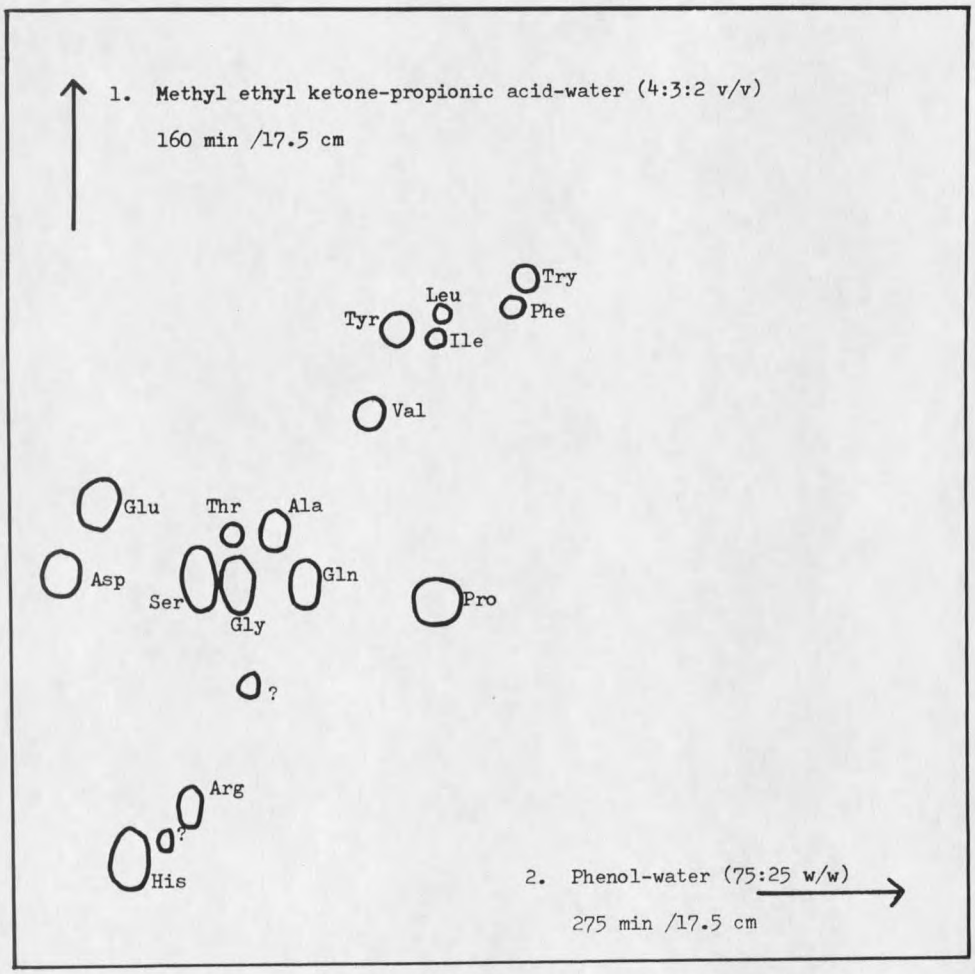

Figure 5. Two-dimensional separation of 16 free amino acids and two unknown substances from a sample of Chinook postdiapause eggs.