Chromatographic Evidence of Intraspecific Genetic Differences in the Eastern Oyster, *Crassostrea virginica* [1]

ROBERT E. HILLMAN

Introduction

The possibility of there being local genetic races of the Eastern Oyster, *Crassostrea virginica*, was first mentioned by Coe (1934) in relation to the alternation of sexuality in oysters. Following Coe's statement, the existence of such races was only speculated upon (Coe, 1938; Loosanoff and Engle, 1942; Nelson, 1947; Loosanoff and Tommers, 1948; Stauber, 1947, 1950) until Loosanoff and Nomejko (1951) demonstrated differences in spawning reactions in relation to temperature among certain stocks of oysters held in Long Island Sound. They regarded these results as conclusive evidence of the existence of physiological races among oysters. Although there is some objection to this conclusion (Ingle, 1951), it is now generally conceded (Korringa, 1952; Nelson, 1957; Loosanoff, 1960, 1962) that there are races of oysters which differ in their responses to environmental stimuli.

Although differences in response to the environment have been shown, actual genetic differences among stocks of American oysters have never been demonstrated. Imai and Sakai (1961), however, have produced hybrids among populations of the Japanese oyster, *C. gigas*. One technique that has been used recently to show genetic differences in closely related species of mollusks (Kirk et al., 1954; Wright, 1959; Collyer, 1961) is paper partition chromatography. The use of this technique for demonstrating genetic differences in oysters and other shellfish was suggested by Loosanoff (1960).

It is generally accepted that each species produces its own characteristic chromatographic pattern of free amino acids (Collyer, 1961), although it is known that individuals of the same species do not always produce exactly the same pattern. Schafer (1961) showed that pollution in the environment produced variations in the free amino acid patterns of two marine invertebrates. Different tissues from the same organism can produce different patterns, and Wright (1959) has described a seasonal variation in the chromatographic pattern of *Lymnaea palustris* bred in the laboratory. It is believed, however, that the pattern produced by any given set of conditions is genetically controlled.

For the purpose of this problem it was assumed that if each species could evolve a characteristic, free amino acid pattern, then isolated populations of the same species would also be able to evolve, eventually, a pattern characteristic of that population. This assumption was tested by using two stocks of oysters from widely separated geographic areas—Long Island Sound and James River, Virginia. "Seed" oysters from these populations were imported by the Chesapeake Biological Laboratory in the early spring of 1962 and held side by side in aquaria supplied with running seawater from the Patuxent River. This paper describes the results of the chromatographic determinations of patterns of free amino acids or small peptides in these two populations.

[1] Contribution No. 252, Natural Resources Institute, University of Maryland, Solomons, Maryland.

Materials and Methods

The two groups of oysters used in this study were indigenous to Long Island Sound and James River, Virginia, respectively. They have been held in running seawater in indoor aquaria since the spring of 1962. All oysters used were under 3 inches in greatest length. Random cytological examinations of the stocks failed to reveal any females. To insure against influence from variations within an aquarium, experimental oysters from each stock were held simultaneously in the same tray for several days prior to a chromatographic determination.

Several different papers were used in the early stages of the experiment, but Whatman No. 1 proved to be most satisfactory and was used more often than any of the others. Butanol : acetic acid : water (36 : 4 : 10) and 2,6-lutidine : ethanol : water (55 : 25 : 20) with 2 ml diethylamine added after mixing were the solvent systems used (see Block, Durrum and Zweig, 1958).

The test materials were applied to the paper in two ways: pressing the tissue directly into the paper (modified after Collyer, 1961), and by applying small drops of alcoholic extracts of the tissue.

To obtain material for pressing, oysters were shucked by inserting an oyster knife between the valves (left valve up) and severing the adductor muscle from the left valve as close to the shell as possible, leaving most of the muscle with the oyster on the right valve. The viscera were then dissected from the adductor muscle, leaving the right valve with the attached muscle. The muscles were washed with distilled water, and a small piece of tissue, about 1 mm^3, was cut from the opaque portion of the muscle. The tissue was gently blotted dry on clean filter paper.

The small piece of tissue was pressed into the prepared chromatographic paper with a clean glass rod that had one end melted into a small button-shaped tip. The flattened tissue was then removed from the paper, leaving a moist spot. At first, a separate glass tube was used for each spot, but there were no differences in the results using separate tubes from those obtained using a single tube that was rinsed between pressings. Material from the two populations was spotted alternately on the paper.

The paper was then air dried at room temperature or in an oven for 1 hour at 60°C, neither method having any apparent advantage.

To prepare alcoholic extracts, oysters were treated as above except that the entire opaque portion of the muscle was cut from the right valve and placed in a small test tube in 1 ml of 80% ethanol. The tissue was mashed in the bottom of the test tube with a pressing rod and allowed to stand overnight in a refrigerator.

The alcoholic extract was drawn up in a 5-μl micropipette and applied to the paper, keeping the spots as small as possible. In most determinations, two 5-μl aliquots were applied in each spot.

The material was applied 9 cm from the end of the papers, which were 57 cm in length. The spotted papers were replaced in a glass chamber saturated with solvent vapor and equilibrated for 2 hours. Solvent was then added to a trough for descending chromatography, and the runs were made in the machine direction of the paper for 24 hours.

The papers were removed from the chamber and air dried at room temperature or in an oven at 60°C, dipped in 0.3% ninhydrin in acetone, placed in an oven at 60°C for 30 minutes, and stored in the dark overnight prior to examination. No desalting or preliminary separation of amino acids on columns was done.

Results

Best results have been obtained on Whatman No. 1 paper run for 24 hours. From November through the early part of December, differences between the populations could be noted on any given chromatogram, although it was difficult to obtain any consistency in the patterns during this time.

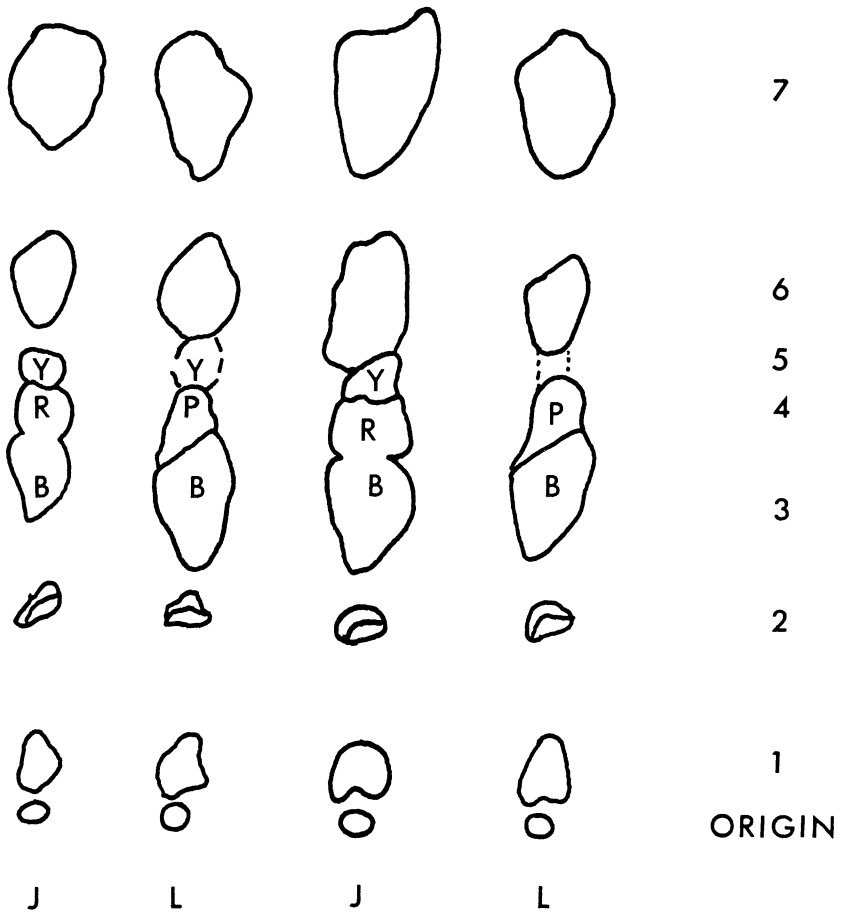

FIG. 1. Tracing of chromatogram of 4 individuals of *C. virginica* from two differnt populations (J—James River; L—Long Island Sound). Amino acids extracted with 80% ethanol. Solvent was butanol : acetic acid : water (36 : 4 : 10). Developed in ninhydrin. Key to abbreviations on spots: B—blue; R—reddish-blue; P—pale pink; Y—yellowish pink.

During this period the water temperature was dropping quite rapidly. In January, the water temperatures remained relatively constant, and consistent chromatograms were obtained from that time (Figs. 1 and 2). Lutidine : ethanol : water was the only solvent used from the beginning of February.

A large spot developed near the origin of the chromatograms run in lutidine : ethanol. This spot may be two spots that migrate at almost the same rate, but after 48 hours of solvent flow, it was not possible to separate them. Both stocks used in the study showed this spot. It was characterized by a reddish base and a blue flame-shaped top when developed in ninhydrin.

Spot No. 2 (Fig. 2) is the spot which is most consistently different between the two stocks. In the Long Island group, this spot was usually relatively small and blue, whereas in the James River stock, the spot was larger with a reddish color. It appeared as if in the James River stock there was either a small peptide (Fig. 2) or two spots migrating at nearly the same rate in lutidine : ethanol, but it was not possible to separate them under the experimental procedure used.

The other spots on the chromatogram

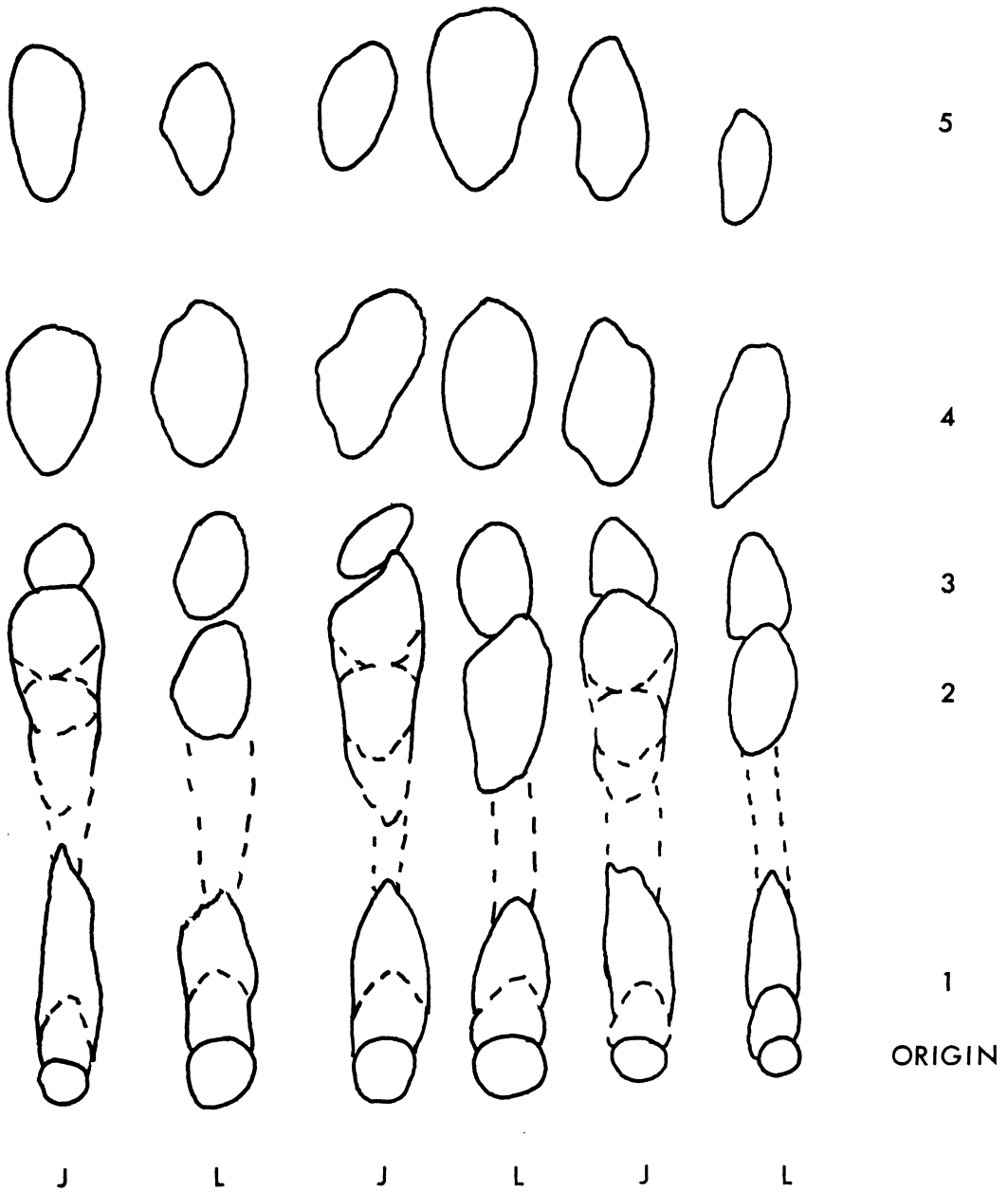

FIG. 2. Tracing of pattern of *C. virginica* from Long Island Sound (L) and James River (J). Dotted lines indicate diffuse color boundaries. Solvent was lutidine : ethanol : water (55 : 25 : 20). Developed in ninhydrin. Material pressed into paper. Band of spots farthest from origin not shown on this tracing.

were similar between the two populations of oysters. In general, 6 or 7 spots appeared for each oyster, by use of the methods described. In those chromatograms of the James River group run in butanol : acetic acid : water, a yellowish spot (developed with ninhydrin) appeared, but not always in the same place. The use of 2,6-lutidine : ethanol : water gave the best separation after 24 hours. At the end of this time, the solvent front had moved off the paper. This was allowed in order to achieve maximum separation.

Discussion

The results showed that under the conditions of the experiment, there is one reproducible difference in the free amino acid or small peptide pattern between the two stocks of oysters studied. It is recognized that many factors such as diet, temperature, and salinity will influence the particular chromatographic pattern of an organism like the oyster, but in view of the consistency and reproducibility of the patterns and the precautions taken to eliminate environmental differences between the two stocks, it is concluded that these results are evidence of intraspecific genetic difference between the two populations of oysters used in the experiment.

It is reasonable to assume that the presence and kind of free amino acids in tissues have some survival value. Kelly (1904) reported considerable amounts of taurine in the tissues of *Mytilus edulis*, and it has been found in other invertebrates since that time (Mendel, 1904; Henze, 1905; Kossel and Edlbacher, 1915; Okuda, 1920; Ackermann et al., 1924; Ackermann, 1935; Lewis, 1952; Kermach et al., 1955; Simpson et al., 1959). Camien et al. (1951) and Duchâteau et al., (1952) have demonstrated higher concentrations of free amino acids in marine forms than in freshwater forms. Duchâteau and Florkin (1955) and Shaw (1958a, 1958b, 1959) showed that the amino acid concentration in euryhaline crabs fluctuated in direct proportion with salinity.

Recent studies by Simpson et al. (1959) and Allen (1961b) and a review by Allen (1961a) indicate that free amino acids in aquatic invertebrates play a role in osmoregulation. Allen (1961b) reported that individual amino acids in *Rangia cuneata* increased in concentration as salinity increased and that this increase followed a definite pattern which was already established prior to a change to a higher salinity. This pattern was: alanine > glycine > glutamic acid > aspartic acid, regardless of the environment. This consistency is in itself indicative of genetic control.

Oysters, found primarily in estuaries, are subjected to wide ranges of salinity. An efficient osmoregulatory mechanism would then be of value to the oyster. Since all estuaries differ and the salinity ranges are not necessarily the same, it is possible that local populations of oysters could evolve mechanisms suitable to their particular location. Indeed, Korringa (1952) suggests that perhaps physiological races in regard to salinity exist in the same sense as those in relation to spawning temperatures.

One can go beyond the mechanism of osmoregulation in speculating upon the role of free amino acids in the tissues of oysters. It may be that some of the ninhydrin-positive compounds which show up chromatographically are by-products of metabolic reactions dealing with other aspects of the oyster's physiology. Since genes exert their effects through control of metabolism, the differences revealed are manifestations of differences in genetic control. Besides the differences in spawning reactions shown by Loosanoff and Nomejko (1951), these metabolic differences may also show up as variations in such factors as growth rate, shell shape, color, and thickness and possibly even resistance to parasites. Loosanoff (1960) discusses several other physiological manifestations of genetic differences in oysters and other shellfish.

Although it may not be possible to get unique chromatographic patterns from all populations of oysters because of chance

convergence and the mixing of stocks by commercial oystermen, it is possible to demonstrate differences in certain local stocks, particularly from natural oyster grounds. A great deal more work is required in this area, especially in determining the relationship of biochemical reactions to observations on growth and mortality made in the field.

Summary

Paper partition chromatography revealed a reproducible difference in the patterns of free amino acids or small peptides between two populations of the Eastern Oyster, *Crassostrea virginica*. Because of the consistency and reproducibility of the patterns, this difference is regarded as evidence of intraspecific genetic differences between the two populations.

Acknowledgments

The writer gratefully acknowledges the support and suggestions of Mr. G. F. Beaven and Dr. L. E. Cronin during this study and the help of Mr. Beaven, Mr. E. Dunnington, and Mr. C. D. Meyers in the preparation of the manuscript.

REFERENCES

ACKERMANN, D. 1935. Asterubin, a sulfur-containing guanidine occurring in nature. Chem. Abstr., 29:3694.

ACKERMANN, D., F. HOLTZ, AND F. KUTSCHER. 1924. Extractives of *Eledone moschata*. Chem. Abstr., 18:2387.

ALLEN, K. 1961a. Amino acids in the Mollusca. Am. Zool., 1:253–261.

1961b. The effect of salinity on the amino acid concentration in *Rangia cuneata* (Pelecypoda). Biol. Bull., 121:419–424.

BLOCK, R. J., E. L. DURRUM, AND G. ZWEIG. 1958. A manual of paper chromatography and paper electrophoresis. Academic Press, Inc., New York.

CAMIEN, M. N., H. SARLET, G. DUCHÂTEAU, AND M. FLORKIN. 1951. Non-protein amino acids in muscle and blood of marine and fresh water crustaceans. J. Biol. Chem., 193:881–885.

COE, W. R. 1934. Alternation of sexuality in oysters. Amer. Nat., 58:1–16.

1938. Primary sexual phases in the oviparous oyster (*Ostrea virginica*) Biol. Bull., 74:64–75.

COLLYER, DOROTHY M. 1961. Differences revealed by paper partition chromatography between the gastropod *Nassarius reticulatus* (L.) and specimens believed to be *N. nitida* (Jeffreys). J. Mar. Biol. Assoc. U. K., 41:683–693.

DUCHÂTEAU, G., AND M. FLORKIN. 1955. Concentration du milieu exterieur et etat stationnaire du pool des acides aminés non protéique des muscles d'*Eriocheir sinensis*. Arch. Intern. Physiol. et Biochim., 63:249–251.

DUCHÂTEAU, G., H. SARLET, M. N. CAMIEN, AND M. FLORKIN. 1952. Acides aminés non protéiques des tissues chez les Mollusques Lamellibranchs et chez les Vers. Comparison des formes marines et des formes dulcicoles. Arch. Intern. Physiol. et Biochim., 60:124–125.

HENZE, M. 1905. Beitrage zur Muskelchemie der Octopoden. Hoppe-Seyl. Zeitschr., 43:477–493.

IMAI, T., AND S. SAKAI. 1961. Study of breeding of Japanese oyster, *Crassostrea gigas*. Tohoku J. Agr. Res., 12:125–171.

INGLE, R. M. 1951. Spawning and setting of oysters in relation to seasonal environmental changes. Bull. Mar. Sci. Gulf. Carib., 1:111–135.

KELLY, A. 1904. Beobachtungen über das Vorkommen von Atherschwefel-säuren, von Taurin und Glycin bei niederen Tieren. Beitr. Chem. Physiol. Path., 5:377–383.

KERMACH, W. O., H. LEES, AND J. D. WOOD. 1955. Some non-protein constituents of the tissues of the lobster. Biochem. J., 50:424–428.

KIRK, R. L., A. R. MAIN, AND F. G. BEYER. 1954. The use of paper partition chromatography for taxonomic study of land snails. Biochem. J. 57:440–442.

KORRINGA, P. 1952. Recent advances in oyster biology. Quart. Rev. Biol., 27:266–308; 339–365.

KOSSEL, A., AND S. EDLBACHER. 1951. Beitrage zur chemischen Kenntnis der Echinodermen. Hoppe-Seyl. Zeitschr., 94:264–283.

LEWIS, P. R. 1952. The free amino acids of invertebrate nerve. Biochem. J., 52:330–338.

LOOSANOFF, V. L. 1960. Challenging problems in shellfish biology, p. 483–495. *In* A. A. Buzzati-Traverso [ed.], Perspectives in marine biology. Univ. of California Press, Berkeley and Los Angeles.

1962. Gametogenesis and spawning of the European oyster. *O. edulis*, in waters of Maine. Biol. Bull., 122:86–94.

LOOSANOFF, V. L., AND J. R. ENGLE. 1942. Accumulation and discharge of sperm by oysters living at different depths. Biol. Bull., 82:413–422.

LOOSANOFF, V. L., AND C. A. NOMEJKO. 1951. Existence of physiologically-different races of oysters, *Crassostrea virginica*. Biol. Bull., 101: 151–156.

LOOSANOFF, V. L., AND F. D. TOMMERS. 1948. Effect of suspended silt and other substances on the rate of feeding of oysters. Science, 107: 69–70.

MENDEL, L. B. 1904. Über das Vorkommen von Taurin in den Muskeln von Weichtieren. Beitr. Zeitschr. Chem. Physiol. Path., 5:582.

NELSON, T. C. 1947. Some contributions from the land in determining conditions of life in the sea. Ecol. Monogr., 17:337–346.

1957. Some scientific aids to the oyster industry. Am. Sci., 45:301–332.

OKUDA, Y. 1920. Extractive matters of *Palinurus japonicus* and *Loligo breekeri*. Chem. Abstr., 14:2827.

SCHAFER, RITA D. 1961. Effects of pollution on the free amino acid content of two marine invertebrates. Pacific Sci., 15:49–55.

SIMPSON, J. W., K. ALLEN, AND J. AWAPARA. 1959. Free amino acids in some aquatic invertebrates. Biol. Bull., 117:371–381.

SHAW, J. 1958a. Osmoregulation in the muscle fibers of *Carcinus maenas*. J. Exp. Biol., 35: 902–919.

1958b. Further studies on ionic regulation in the muscle fibers of *Carcinus maenas*. J. Exp. Biol., 35:920–929.

1959. Solute and water balance in the muscle fibers of the East African fresh water crab, *Potamon niloticus*. J. Exp. Biol., 36:145–156.

STAUBER, L. A. 1947. On the possible physiological species in the oyster *Ostrea virginica*. Anat. Rec., 99:614.

1950. The problem of physiological species with special references to oysters and oyster drills. Ecology, 31:109–118.

WRIGHT, C. A. 1959. The application of paper chromatography to a taxonomic study in the molluscan genus Lymnaea. J. Limn. Soc. (Zool.), 44:222–237.

ROBERT E. HILLMAN is on the staff of the Natural Resources Institute of the University of Maryland, Solomons, Maryland.