tein such as cytochrome c may functionally attract the opposite charges in the acidic protein, cytochrome c oxidase. The net result should be a trend toward charge neutrality.

Most compilations of the amino acid content of proteins list aspartic and glutamic acids in figures which actually represent Asp plus Asn and Glu plus Gln. This does not allow the necessary calculations to be made. We have summarized the composition of 68 completely sequenced proteins containing 12,170 amino acid residues. The compilation included 47 eukaryotic, 17 prokaryotic, and 4 virus proteins. Only one representative of each "family" of proteins, such as the globins, was included. These proteins contained, per 61 residues:

$$Ala_{5.3}\,Arg_{2.6}\,Asn_{3.0}\,Asp_{3.6}\,Cys_{1.3}$$
$$Gln_{2.4}\,Glu_{3.3}\,Gly_{4.8}\,His_{1.4}\,Ile_{3.1}$$
$$Leu_{4.7}\,Lys_{4.1}\,Met_{1.1}\,Phe_{2.3}\,Pro_{2.5}$$
$$Ser_{4.5}\,Thr_{3.7}\,Trp_{0.8}\,Tyr_{2.3}\,Val_{4.2}$$

Statistical analysis of these data forces the rejection of the null hypothesis that the distribution does not deviate from that expected from proportions of codons in the genetic code. Arginine plus lysine is 11.0 percent of the proteins, very close to 11.3 percent for aspartic plus glutamic acids.

If these amino acids were in proportion to their occurrence in the genetic code, there would be 9.8 percent arginine and 3.3 percent each of lysine, aspartic acid, and glutamic acid, an imbalance of 13.1 to 6.6 for basic to acidic amino acids. Clearly, natural selection counteracts the genetic code to neutralize the charge on proteins.

Furthermore, in the total of 11.0 percent for the basic amino acids (as opposed to the "code percentage" of 13.1), the amount of arginine, 4.3 percent, is maintained far below the level in the code, while the amount of lysine, 6.7 percent, is actually increased in proteins above the code level.

The imbalance between the ratio of arginine to lysine, in terms of the code, led to the suggestion that arginine was an evolutionary intruder that replaced ornithine in protein synthesis (*4*). Notwithstanding this imbalance, the sum of arginine plus lysine is almost identical to the sum of aspartic plus glutamic acids, which strengthens the proposal that evolutionary selection maintains charge neutrality. The net result is that the sum of basic plus acidic amino acids, 22.3 percent, is close to their representation in the genetic code, 19.7 percent.

As an explanation for the excess of "basic codons," there is a possibility that primitive forms of life existed at a higher *p*H than that encountered in present environments. Even today, the *p*H of seawater, 8.0 to 8.5, is higher than that of body fluids (~ 7.4), and the early oceans may have been even more alkaline. The quantitative relations between the charged amino acids in prokaryotes and eukaryotes and their relation to the structure of the genetic code have been described by one of us (*5*).

As regards the other amino acids, alanine is present at significantly "higher-than-code" levels, while histidine, cysteine, proline, serine, and leucine are significantly lower. Perhaps its small side chain makes alanine useful as a "filler" in protein molecules. Histidine, cysteine, and proline have special functions and properties, and this may diminish their use. The needs for serine and leucine may not be so great as to require six codons apiece. The other amino acids fall within expected ranges. To some extent, they may constitute a "pool" that can be augmented by mutations from arginine, cysteine, leucine, serine, proline, and histidine and diminished by mutations to lysine, aspartic acid, glutamic acid, and alanine. Selection would be responsible for maintaining the disparities noted between the frequencies in the code and in proteins, of certain amino acids. On the other hand, genetic drift would play a part in allowing neutral interchanges between, for example, isoleucine and valine, whose occurrences in the code and in proteins are at equal levels. Such a model combines selective and neutral mutations to give a picture of dynamic equilibrium in protein evolution, illustrated by the changes that take place incessantly in homologous proteins.

The distribution of amino acids in 207 proteins for which the analyses were reported by Reeck and Fisher (*6*) is as follows, per 61 residues: $Ala_{5.2}Arg_{2.7}(Asn + Asp)_{6.5}Cys_{1.4}(Gln + Glu)_{6.5}Gly_{4.9}His_{1.3}Ile_{3.0}$ $Leu_{3.9}\,Lys_{3.9}\,Met_{1.1}\,Phe_{2.3}\,Pro_{2.9}\,Ser_{3.8}\,Thr_{3.5}$ $Trp_{0.8}Tyr_{2.0}Val_{4.1}$. This distribution does not list separate values for asparagine, aspartic acid, glutamine, and glutamic acid. Apart from this, and for the values for proline and serine, the distribution is quite similar to our compilation, which, like that of Reeck and Fisher (*6*), includes only proteins containing 50 or more amino acid residues. Holmquist and Moise found (*7*) that the distribution of amino acids in proteins is not dependent on length or species of origin.

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Induced Adhesion in *Crassostrea virginica* Larvae

Abstract. *Normal motile veliger larvae of the oyster,* Crassostrea virginica, *were observed swimming in pairs or trios. Adhesion between animals is firm and has a specific orientation. This adhesion can be induced in low frequencies by culturing larvae at high densities, and in much higher frequencies by inclusion of an antibiotic mix in the seawater culture medium.*

The phenomenon of adhesion or aggregation of cells and tissues is an important area of investigation in plant and animal developmental biology. This report describes a chance observation (repeated many times since) of adhesion in larvae of the oyster, *Crassostrea virginica*, that has not been previously described. The information presented below leaves many unanswered questions, but we believe it should be reported now.

Adult oysters were spawned in the laboratory according to the methods described by Loosanoff and Davis (*1*). Fertilized eggs were screened on filters of graded pore-size (75, 54, 36 μm) nylon monofilament bolting cloth (Nitex) to remove large particles; the egg suspension was then washed and concentrated on a 20-μm screen with sterile (autoclaved) seawater. This fertilized egg suspension was counted in a Sedgwick-Rafter cell, aliquoted at 250 eggs per milliliter into covered Pyrex preparation dishes containing 200 ml of sterile seawater, and incubated at 24°C. About 24 hours later, the samples in each dish were washed with sterile seawater on a 36-μm Nitex screen, suspended in sterile seawater, and counted. At this time most of the eggs had developed to the straight-hinge veliger stage. The veligers were aliquoted into sterile Pyrex preparation dishes, containing 100 ml of sterile seawater, at whatever concentrations the experimental procedure

required. A small amount of algal food [0.5 ml of an enriched seawater culture of *Monochrysis lutheri* and *Isochrysis galbana* (*2*)] was added at this time. In some dishes the following GS Millipore-filtered solutions of antibiotics, individually or in combination, were included: chloramphenicol (Calbiochem), 50 μg/ml; polymyxin B sulfate (Nutritional Biochemicals, 6300 unit/mg), 120 μg/ml; and neomycin sulfate (Nutritional Biochemicals, 750 μg of base per milligram), 330 μg/ml. These antibiotics were found individually to be the most effective bacteriostatic and bactericidal agents against bacteria likely to appear in larval cultures at concentrations that did not affect normal larval development or behavior.

Observations at 48 hours showed that straight-hinge larvae were active with well-developed shells and swimming responses as expected (*3*). The surprising observation was that, in dishes to which a mixture of the three antibiotics was added, many larvae were swimming in pairs (Fig. 1A). The paired larvae had extended ciliated vela, and executed normal linear and rotational movements in unison with each other, looking very much like contented waltzing partners under the microscope. Larvae in pairs had similar orientations with the hinges aligned along the same axis in a straight line and the dorsoventral axis in the same orientation (only very rarely was the orientation in opposite directions resulting in the pairs spinning around in a circle). Adhesion of larvae in pairs at 48 hours was also observed if the antibiotics were added to the suspension of fertilized eggs rather than to 24-hour veligers. Trochophore larvae, however, never formed pairs. Adhesion between the larvae appeared to be fairly strong; for example, larvae did not drift apart from each other when crushed beneath a cover slip by pressure that was enough to crack the shell. Pairs of living larvae also remained adhered after 2 minutes in a Vortex test tube mixer. Adhesions remained firm in various fixatives [Lugol's fluid, formalin, Zenker's fluid, and even Carnoy's fluid (Fig. 1B) whose acid component deforms the shell] for long periods of time.

It became evident after numerous experiments that pairing of larvae could be in-

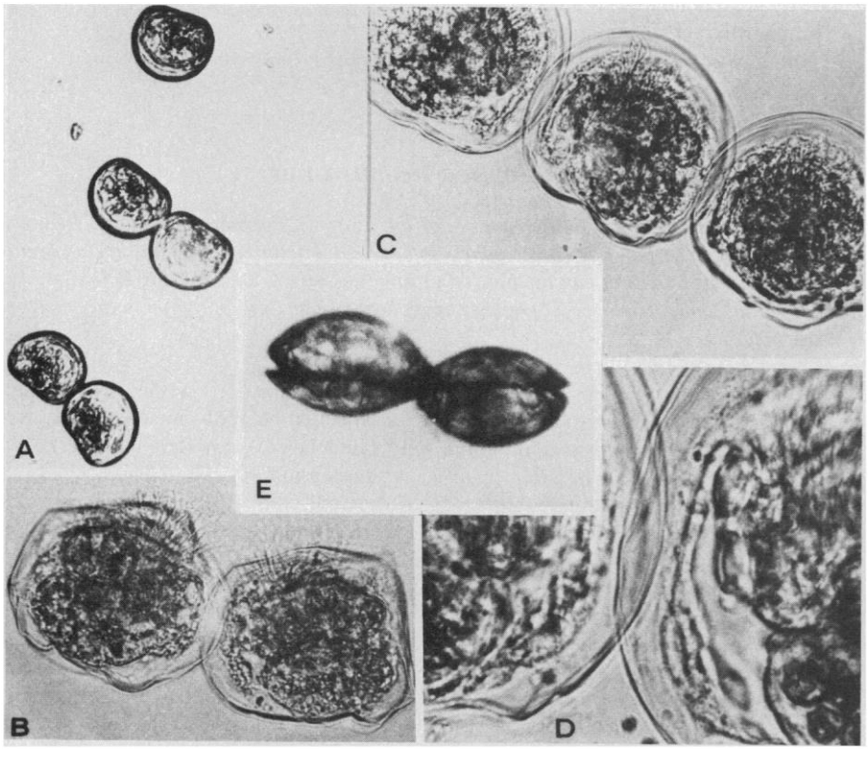
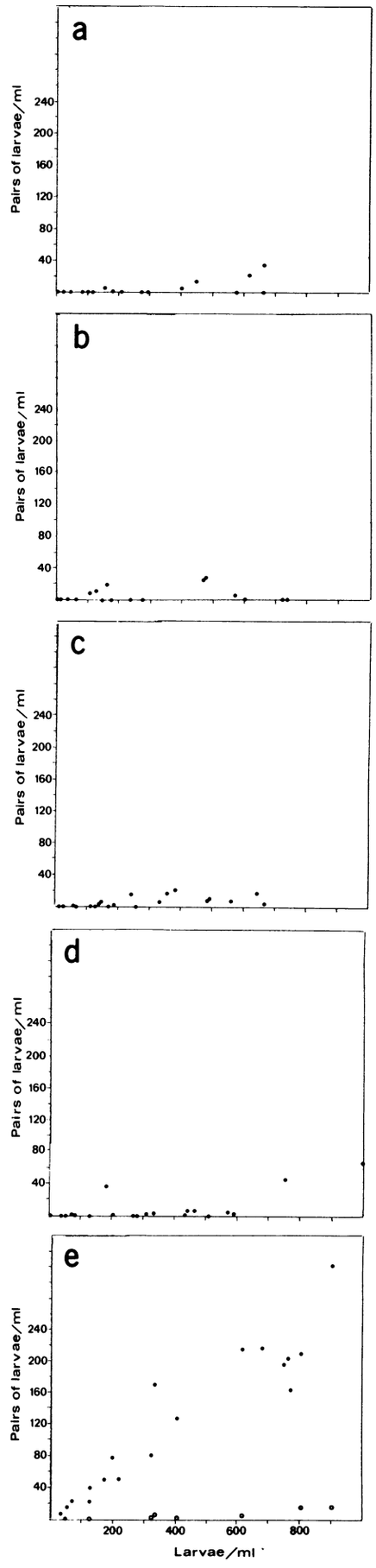


Fig. 1. (A) Veliger larvae, normal and paired, fixed in formalin (× 66); (B) veliger larvae, fixed in Carnoy's fluid (× 264); (C) trio of veliger larvae, fixed in Zenker's fluid (× 264), interference filter; (D) detail of adhesion site, fixed in iodine (× 660); (E) dorsal view of live, swimming, paired larvae (× 132).

Fig. 2. Numbers of paired larvae in various larval concentrations. (a) Control, (b) polymyxin B sulfate, (c) chloramphenicol, (d) neomycin sulfate, (e) combination of polymyxin B sulfate, chloramphenicol, and neomycin sulfate. In (e) the seven circles very close to the abscissa, at larval concentrations above 100 per milliliter, denote trios of larvae, rather than pairs.



duced by two factors—the concentration of veliger larvae and the simultaneous presence of all three antibiotics in a culture dish. Although some pairing was evident if antibiotics were added individually, and a few pairs were observed in very high concentrations of larvae with no antibiotics, by far the highest number of pairs was observed in dishes with high concentrations of larvae to which the triple antibiotic mix had been added (Fig. 2). In this latter experimental situation a group of three adhering larvae, all swimming in unison, was sometimes seen (Fig. 1C).

Shelled animals of the planktonic stage were observed in pairs beginning with the first smooth hyaline valves of the straight-hinge veliger larvae until the beginning of the umbo stage at about 7 to 10 days. Unfortunately, it was not possible to maintain cultures beyond this time because of the germination of mold spores in the dishes. (Antifungal agents, as nystatin, candicidin, $HgCl_2$, griseofulvin, amphotericin, and Merthiolate, in concentrations nontoxic to larvae, were not effective as control agents.)

The exact site or mechanism of adhesion is not clear from microscopic observations alone (Fig. 1D). The specificity of the adhesion site suggests that mechanism and site are interrelated. Since pairing is observed in the earliest stages of shell formation and not in the trochophore stage, adhesion must be associated with changes that occur after the transitional period between trochophore and veliger larvae. In summary, these changes are as follows: the prototroch develops into a velum, the larva develops a rudimentary foot which proliferates a shell gland and a shell, the shell differentiates into right and left valves, digestive diverticula appear, the intestine acquires loops, the stomach develops a crystalline style sac, three sets of retractor muscles develop, and the anterior adductor muscle which functions in closing the shell appears anteriorly (*4*). The observation that some adhesion of larvae occurs in very high larval density even without antibiotics suggests that random contact between swimming animals allows an adhesive site to make contact. Obviously, this is apt to occur with greater frequency in high-density cultures. One possibility is that the antibiotic mix potentiates this effect by acting on the adhesive site. An alternative possibility is that antibiotics create a malfunction in the adductor muscle which causes greater numbers of animals to have open valves than in untreated larvae, thereby increasing the possibility of natural adhesive sites making contact.

Adhesion occurs anteriorly at the widest place in the valve. Paired larvae appear to be attached in a position in which the vela are facing each other (Fig. 1B). Since dorsal and ventral alignments are always the same in members of a pair or trio, the lateral views of paired larvae are of right and left valves. In trios the third member of the group seems to have the velum facing in the same direction as the second member (Fig. 1C). This arrangement becomes especially evident in older veliger larvae in which the left valve is larger and more convex than the right (Fig. 1E). The adductor muscles suggest themselves as a site of adhesion since the anterior adductor muscles of paired animals are facing each other and in trios the anterior adductor muscle of the third member is adjacent to the posterior adductor of the middle member. As the anterior adductor muscle develops prior to the posterior muscle, it could explain the specificity and high frequency of the anterior site of adhesion. Contrary evidence, however, is provided by paired fixed animals in which the visceral mass has contracted away from the edge of the shell and reveals no shell-to-muscle or muscle-to-muscle contact.

Veliger larvae normally produce a "sticky" secretion from the byssus gland in the foot at the time of setting. As most of the foot is made up of gland cells packed with secretions (*5*), it is possible that some precursor of this material may also have "sticky" properties that could provide attachment in high-density culture even at this early stage. Alignment of the pairs, however, would argue against the area of the rudimentary foot being the site of larval attachment.

The most promising hypothesis is that the newly calcifying shells are adhering to each other; more specifically, the valve margin of one larva adheres to the mantle epithelium of the other, or both mantle epithelia to each other. In this regard it is of interest to note that Nelson (*6*) ascribed a role to the mantle in the process of attachment of *C. virginica* to the substrate. Calcification consists of deposition of calcium carbonate (aragonite) (*7*) in an organic matrix. The organic matrix, from which protein fractions and an acid mucopolysaccharide have been isolated, is secreted by the mantle into the extrapallial space [in *C. virginica* the extrapallial space has access to the external media (*8*)]. Differences in specificity of regions of the mantle epithelium in the synthesis of particular proteins, as well as morphological differences in cells of the mantle [see review by Wilbur (*8*)], suggest that an adhesive material, possibly a mucopolysaccharide, is being produced by specific regions in the periphery of the mantle and that this adhesive is acted upon synergistically by the triple antibiotic mix.

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21 February 1975

Control Factor of Nuclear Cycles in Ciliate Conjugation: Cell-to-Cell Transfer in Multicellular Complexes

Abstract. *Multicellular complexes of* Blepharisma intermedium *are regularly produced by treating doublet cells with the gamone of complementary mating type. Cells remain united without undergoing nuclear cycles of conjugation. However, if a cell of complementary mating type unites, nuclear cycles begin at the site of this union and propagate all through the multicellular complex.*

In the conjugation of ciliates, two cells temporarily unite and undergo a series of nuclear cycles including meiosis, fertilization, repeated mitosis, and development of new micro- and macronuclei. The whole process is a chain reaction initiated by the interaction between complementary mating types (*1–3*). If two such mating types of *Blepharisma intermedium*, 1 and 2, are mixed under appropriate conditions, cells unite in pairs after 2 hours of interaction (*4, 5*). Type 1 cells excrete gamone 1—blepharmone (*6*), which is a glycoprotein with a molecular weight of 20,000 and transforms type 2 cells so that they can unite. Type 2 cells excrete gamone 2—blepharismone (*7, 8*), which is calcium 3-(2′-formylamino-5′-hydroxybenzoyl) lactate and similarly transforms type 1 cells.

Transformed cells can unite in all three