were transferred to larger wells (volume of culture, 1 ml) and, after further growth, to petri dishes (volume of culture, 4 ml), to yield sufficient material for the starch-gel enzyme tests. These were carried out 24 to 38 days after the original isolation of the 0.1-ml samples, depending on the growth rates of the different clones. Parasites were prepared for the enzyme tests by the method of Carter (*7*).

Table 1 shows that the original culture contained, at the time of dilution, a mixture of parasites of which approximately 70 percent were of type GPI-1 and 30 percent of type GPI-2 (all being ADA-1). The samples isolated into wells contained GPI-1, GPI-2, or GPI-1 plus GPI-2, the last presumably originating either from the simultaneous isolation of two different parasitized cells in the original 0.1-ml sample, in accordance with the Poisson distribution, or from the isolation of doubly infected cells. Analysis of the data in Table 1 shows that the results of experiment 2 agree well with the calculated expectations, whereas those of experiment 1 deviate considerably. It is assumed that growth of parasites in medium with a 2 percent hematocrit value is more regular than with a 10 or 7 percent value. As shown in Table 1, it is expected that with the dilutions chosen, 70 to 80 percent of the GPI-1 cultures and 85 to 90 percent of the GPI-2 cultures contained single clones.

Among the isolates obtained by this dilution procedure, five took 8 to 15 days longer than the remainder to yield enough material for enzyme typing. These isolates had a markedly slower growth rate, indicating that, in addition to enzyme variation, the original T9 isolate was heterogeneous with respect to growth-rate determining factors. Thus this isolate may have contained a larger number of clones than are shown by enzyme typing alone.

Electrophoretic forms of enzymes are particularly suitable as markers in this work because they are genetically stable and because mixed infections can be identified easily. *Plasmodium falciparum* is also heterogeneous with regard to antigens (*8*), and isolates that exhibit multiple drug resistance (*9*) may, in fact, be mixed infections of parasites exhibiting resistance to each drug separately. Cloned material will be important for analyzing the genetic composition of such isolates, as well as for other types of research, for example, for epidemiological and immunological studies aimed at the production of an antimalarial vaccine.

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Polyphenolic Substance of *Mytilus edulis:* Novel Adhesive Containing L-Dopa and Hydroxyproline

Abstract. *The fouling marine mussel* Mytilus edulis *attaches itself to various substrates by spinning byssal threads, the adhesive discs of which are rich in the amino acid 3,4-dihydroxyphenylalanine (dopa). An acid-soluble protein was extracted and purified from the phenol gland located in the byssus-secreting foot of the animal. This protein is highly basic and contains large amounts of lysine, dopa, and 3- and 4-hydroxyproline. The composition of this protein and its sticky tendencies in vitro strongly suggest that it contributes to byssal adhesion.*

The marine mussel *Mytilus edulis* successfully inhabits many of the most turbulent and inhospitable niches in the intertidal zone. This success is in part related to the animal's use of the byssus to glue itself securely to a wide variety of substrates (*1*). The byssus consists of a bunch of collagenous threads secreted by exocrine glands in the foot of the mussel (*2*). The adhesive ends of these threads are rich in a polyphenolic substance that is derived from the phenol gland located near the tip of the foot (*3–5*). The remarkably high tensile strength of the bond between the byssal adhesive and its substrate (*6*) has drawn the attention of an increasingly interdisciplinary research effort in recent years. Although the identification of the polyphenolic substance has been a prime target of research, the insoluble and sclerotized condition of the byssus has frustrated attempts to extract individual protein components from the structure. We have isolated the soluble polyphenolic substance from the phenol gland and report that it is a protein rich in 3,4-dihydroxyphenylalanine (dopa) and hydroxyproline.

In a study of structural proteins in *M. edulis*, a soluble polyphenolic substance resembling dopa was extracted from the phenol gland with dilute acid (*3*). The presence of dopa in hydrolysates of byssal discs as well as in acid-soluble protein from the phenol gland has now been confirmed (*7*). We dissected phenol glands from locally collected mussels, extracted them with neutral salt buffer, and homogenized the salt-insoluble material in 5 percent acetic acid. The acid-soluble proteins usually contained 25 to 40 μg of dopa per milligram of protein; dopa was associated with two polypeptide bands as judged by electrophoresis at acid *p*H (Fig. 1). The acid-soluble dopa proteins have a tendency to become adsorbed to chromatographic materials and to precipitate irreversibly at high ionic strength and neutral *p*H, during sample concentration, and in the presence of sodium dodecyl sulfate (SDS). For these reasons, we adopted the following procedures for purification: The dopa-containing material was applied to a column of SP-Sephadex. Using a linear gradient of guanidine hydrochloride we eluted a dopa-containing peak at about 8 percent guanidine hydrochloride (*8*). Peak fractions were pooled and fractionated on phenyl Sepharose 4B-CL and Sephadex G-200, respectively. Acid gel electrophoresis of the G-200 peak showed two bands (Fig. 1, bands a and b), both of which stained for protein and dopa. Since SDS gel electrophoresis proved useless for this protein, we turned to electrophoresis in the presence of cetylpyridinium chloride to estimate molecular weights. Bands a and b of Fig. 1 exhibit apparent molecular weights of 135,000 and 125,000, respectively, migrating between the α and β chains of type I collagen.

The presence of two polyphenolic proteins with similar mobilities on gel filtration, ion-exchange chromatography, and gel electrophoresis, at first suggested to us a two-subunit composition such as the α_1 and α_2 chains of type I collagen. However, upon repeated extraction of the polyphenolic protein from the phenol

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gland, we have found that the relative abundance of proteins a and b varies greatly, with protein b representing 50 to 90 percent of the dopa-containing protein. It is possible that protein b is derived from protein a by a limited proteolysis not sensitive to the protease inhibitors ordinarily used in these extractions.

Amino acid analysis of the hydrolyzed polyphenolic protein produced an unusual composition (Table 1). Dopa is present at about 105 residues per 1000, and 3- and 4-hydroxyproline together represent nearly 130 residues per 1000. Lysine is the most abundant amino acid at 210 residues per 1000, but large amounts of threonine, serine, and tyrosine are also present. No tryptophan or carbohydrate was detected in the hydrolyzate. Although dopa is also present in hydrolyzates of the byssal disc, the composition of the discs and the polyphenolic protein is not otherwise especially similar. This is probably due to two factors: (i) the byssal disc is an insoluble amalgam of polyphenolic protein and collagen (*4*), and (ii) the adhesive disc is quinone-tanned (*9*). The latter would presumably involve the oxidative modification of many dopa residues as well as those of other amino acids such as lysine (*10*). The polyphenolic protein resembles collagen in that it contains hydroxyproline, but the unusually low glycine content is not consistent with known collagen sequences in which glycine is present as every third amino acid residue (*11*). The hydroxyproline residues may conceivably be restricted to collagen-like domains in the polyphenolic protein. Such domains have been shown to exist in C1q complement protein (*12*) and in an acetylcholinesterase (*13*). In the polyphenolic protein, such domains may function to promote interactions with the collagenous moiety of the byssal thread.

Dopa is not known to occur widely in proteins, although it has been detected in periostracin (*14*), a structural protein in the periostracum of *Mytilus*. The most obvious role of dopa would seem to be that of a tanning agent by virtue of its facile oxidation to *o*-quinones (*15*). The presence of dopa in a protein rather than as a free amino acid has several adaptive advantages for the tanning of *Mytilus* byssus: intramolecular condensation of dopa with its own α-amino group is prevented (except for NH_2-terminal dopa), thereby allowing condensation with other residues such as lysines to occur. Moreover, solubilization and loss of the free amino acid in seawater is obviated by its inclusion in a protein that is insoluble in neutral salt solutions.

Although the polyphenolic protein is tightly adsorbed to many surfaces, and its composition resembles that of adhesive proteins in spider silk in its high content of lysine and hydroxyamino acids (*16*), it is premature to assume that it is solely responsible for adhesion of the byssus in *Mytilus*. There is growing histochemical and ultrastructural evidence for the presence of three other substances that may have a role in adhesion (*4, 17, 18*). A mucosubstance is secreted by the tip of the foot and applied, perhaps as a wetting agent, to the substrate surface. Wetting is a crucial element of

Table 1. Amino acid composition of the polyphenolic protein (Fig. 1, bands a and b) and the byssal adhesive disc are expressed as residues per 1000. Proteins were hydrolyzed in vacuo with 6 *M* HCl containing 1.0 percent (by volume) phenol for 24 and 48 hours at 110°C. Threonine, serine, tyrosine, and dopa were corrected for losses due to hydrolysis, by extrapolation to zero time. Values represent means and standard deviations for three different preparations. The absence of neutral sugars and tryptophan in the polyphenolic substance was ascertained in separate trials (*25, 26*).

Amino acid	Polyphenolic protein	Adhesive disc
3-Hydroxyproline	29.5 ± 2.8	Trace
4-Hydroxyproline	101.8 ± 2.6	22.5 ± 5.3
Aspartic acid	22.8 ± 0.3	91.5 ± 0.5
Threonine	116.8 ± 1.5	34.8 ± 1.4
Serine	102.2 ± 1.5	74.9 ± 4.1
Glutamic acid	8.7 ± 0.7	49.3 ± 0.2
Proline	81.2 ± 0.4	41.8 ± 3.0
Glycine	31.5 ± 0.5	194.7 ± 3.2
Alanine	81.1 ± 0.3	79.3 ± 1.5
Half-cysteine	0	20.1 ± 2.4
Valine	8.1 ± 2.3	40.0 ± 5.1
Methionine	0.8 ± 0.2	5.5 ± 0.5
Isoleucine	8.4 ± 0.8	24.5 ± 3.9
Leucine	11.1 ± 0.6	49.9 ± 1.8
Dopa	105.5 ± 1.2	13.2 ± 3.7
Tyrosine	65.8 ± 0.8	53.6 ± 4.1
Phenylalanine	0.5 ± 0.1	36.5 ± 1.6
Hydroxylysine	0	Trace
Histidine	7.5 ± 0.9	46.6 ± 3.8
Lysine	210.5 ± 2.4	53.6 ± 2.7
Arginine	6.5 ± 0.4	67.5 ± 0.9

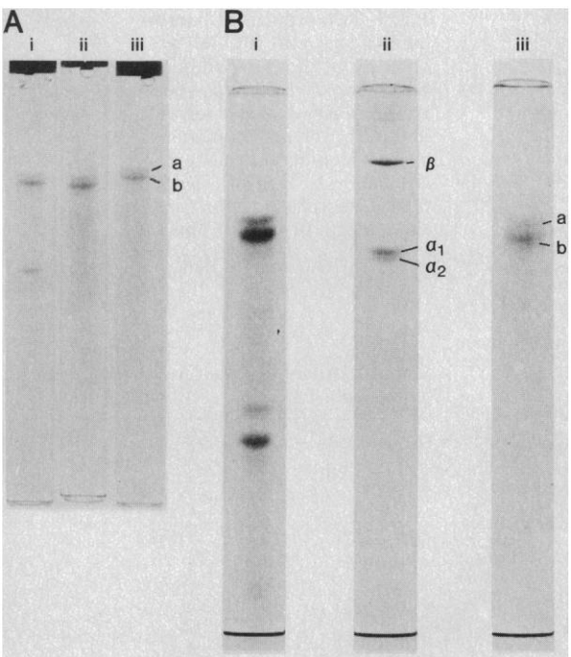

Fig. 1. Electrophoretic separation of the polyphenolic protein. (A) Polyacrylamide gel electrophoresis at acid *p*H [adapted from (*22*)]. Running buffer was 5 percent (by volume) acetic acid; acrylamide and methylenebisacrylamide concentrations were 6 and 0.15 percent (weight to volume), respectively. (Sample i) Acid-extracted phenol gland (10 μg of protein stained with Coomassie blue R-250); (sample ii) acid-extracted phenol gland (60 μg of protein stained for dopa); (sample iii) polyphenolic protein, G-200 peak (17 μg of protein stained with Coomassie blue R-250). The dopa stain was applied to acid gels as follows: gel was removed from the glass tube and immersed in one part nitrite solution (10 g $NaNO_3$ and $Na_2MoO_2 \cdot H_2O$ per 100 ml of distilled water) for 20 minutes and six parts 1*N* NaOH for 30 minutes. (B) Polyacrylamide gel electrophoresis in 0.3*M* glycinium acetate with 0.01 percent cetylpyridinium chloride (CPC) (*23*). (Sample i) Acid-extracted phenol gland (20 μg of protein); (sample ii) type I calfskin collagen (*24*) (15 μg of protein); (sample iii) polyphenolic protein, G-200 peak (6 μg of protein). All three were stained with Coomassie blue R-250. Before electrophoresis, the samples were denatured at 60°C for 5 minutes in buffer containing 0.3*M* glycinium acetate, *p*H 3.5, 0.1 percent CPC, and 1 percent (by volume) 2-mercaptoethanol.

adhesion and allows for the intimate association of the adhesive and the substrate (*19*). Onto this is applied a mixture of polyphenolic protein and collagen, with the former presumably acting as the adhesive, and the latter as a fibrous filler giving cohesive strength to the glue. Finally, the enzyme polyphenoloxidase is added to catalyze the quinone tanning of the adhesive, thus conferring permanence to the bond (*18*).

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Dictyota dichotoma (Phaeophyceae): Identification of the Sperm Attractant

Abstract. *Freshly released eggs of the marine brown alga* Dictyota dichotoma *secrete a substance that attracts spermatozoids. This compound has been identified as* n*-butyl-cyclohepta-2,5-diene. It is closely related to attractants in several other brown algae and confirms that a relation exists between phylogeny and attractant compounds.*

Various details of sexual reproduction have been studied in *Dictyota dichotoma*, a well-known species of marine brown algae found on temperate North Atlantic coasts. Spermatozoids of the algae are attracted by eggs, indicating sexual chemotaxis (*1*). Furthermore, in some localities liberation of eggs and spermatozoids has a pronounced semilunar periodicity, with mass discharge occurring on specific days of the lunar cycle (*2*, *3*).

A clonal culture of female gametophytes was derived from a tetrasporophyte of *Dictyota dichotoma* (Hudson) Lamour., which was collected in November 1959 at Helgoland (German Bight). The same strain was used for studies on lunar periodicity (*3*).

Female gametophytes (about 10 g, fresh weight) were placed in glass dishes and subjected to a regime of 14 hours of light and 10 hours of darkness. Every 28th cycle, the dark phase was replaced

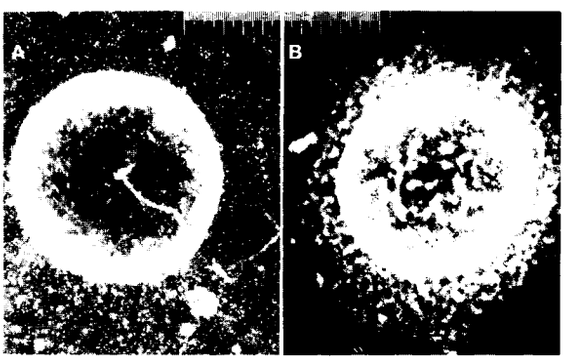

Fig. 1. Two droplets of fluorocarbon solvent FC-78 are shown 3 minutes after addition of *Dictyota dichotoma* spermatozoids in (A) blank solvent and (B) solvent containing synthetic *n*-butyl-cyclohepta-2,5-diene (1.5 mg/ml). Darkfield, microflash exposure. Total length of scale, 1 mm.

Table 1. Analytical data for *Dictyota* egg product, for ectocarpen, and for synthetic n-butyl-cyclohepta-2,5-diene.

Item	Retention index (elution temperature 80° ± 1°C)		Mass fragmentation
	OV 73	OV 61	
Dictyota egg product	1168.2 ± 0.5	1230.5 ± 0.4	150 (M^+; 12%); 135 (2%); 121 (6%); 107 (19%); 93 (87%); 91 (85%); 79 (100%)
Ectocarpen	1151.6 ± 0.4	1224.2 ± 0.2	148 (M^+; 12%); 133 (18%); 119 (15%); 105 (25%); 91 (89%); 79 (100%)
n-Butyl-cyclohepta-2,5-diene	1168.0 ± 0.6	1230.7 ± 0.3	150 (M^+; 10%); 135 (2%); 121 (4%); 107 (13%); 93 (57%); 91 (56%); 79 (100%)

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