large single crystals of calcite, with intercrystalline sheets of apparently nonmineralized organic material, found in *Murex*, strongly resemble the prismatic structure in bivalves (*8*). Atypical $CaCO_3$ polymorphs are found during shell repair (*2*), but the presence of the well-ordered framework, as well as calcite, in a normally aragonitic shell of gastropods has not been previously reported. Both the spherulites and the abnormal calcite structures are probably responses of the shell to pathological conditions. It is not yet known whether they persist as inclusions or are resorbed during restoration of the normal crossed-lamellar surface pattern.

E. O. MUZII*
H. CATHERINE W. SKINNER
Laboratory of Histology and Pathology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland

References and Notes

1. K. M. Wilbur and C. M. Yonge, *Physiology of Mollusca* (Academic Press, New York, 1964), vol. 1.
2. F. K. Mayer, *Jena. Z. Naturw.* **65**, 487 (1931); K. G. von Levetzow, *ibid.* **66**, 41 (1932); K. M. Wilbur and N. Watabe, *Ann. N.Y. Acad. Sci.* **109**, 82 (1963).
3. W. Biedermann, *Z. Allgem. Physiol.* **1**, 154 (1902); A. Abolins-Krogis, *Acta Zool. Stockholm* **39**, 19 (1958); N. Watabe and K. M. Wilbur, *Nature* **188**, 334 (1960).
4. A. A. Hirata, *Biol. Bull.* **104**, 394 (1953).
5. Dura Gloss; A. R. Winapick, Inc., New York.
6. Continuous microscopic observation of the decalcification of samples was made possible by mounting a sample on a glass slide and immersing it in water to which were added portions of a 10-percent solution of ethylenediaminetetraacetic acid (adjusted to *p*H 6.95 with 0.1*M* tris buffer) until the mineral birefringence disappeared.
7. N. Watabe, D. G. Sharp, K. M. Wilbur, *J. Biophys. Biochem. Cytol.* **4**, 281 (1958).
8. P. S. Galtsoff, "The American oyster," *U.S. Fish Wildlife Serv. Fishery Bull. Ser. 64* (Superintendent of Documents, Government Printing Office, Washington, D.C., 1964).
9. Work performed at the National Institute of Dental Research during tenure of a PHS international fellowship by E.O.M.
* Present address: Stazione Zoologica, Naples, Italy.

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Proteins and Disulfide Groups in the Aggregation of Dissociated Cells of Sea Sponges

Abstract. *A sponge extract that produced specific aggregation of dissociated cells was treated with various enzyme preparations to determine which enzymes would destroy its aggregating properties. The results indicate that proteins play a key role in the aggregating effect of the extract on dissociated, glutaraldehyde-fixed sponge cells. Further studies confirm the necessity of calcium for the aggregation and indicate the necessity of intact disulfide groups.*

Moscona (*1*) and Humphreys (*2*) have shown that extracts of certain sponges are capable of aggregating the dissociated cells of the same species of sponge. To determine the chemical nature of the responsible material in the extract, we studied a Chilian marine sponge (*Haliclona variabilis*), using a crude extract of this sponge and its chemically dissociated cells, according to Humphreys' techniques (*2*). Our object was to make advances in this field which could contribute to the understanding of several biological phenomena related to the cell surface in higher organisms, such as cell recognition and selective mutual attachment of cells (*3*); changes in cellular adhesiveness preceding cell movement in normal (*4*) and malignant tissues (*5*), which are particularly important in embryonic morphogenesis and tumor invasiveness; and possibly adhesion of circulating tumor cells to the vascular endothelium (*6*).

Preliminary studies by Moscona (*1*) indicate that the binding properties of the intercellular material in sea sponges are resistant to ribonuclease, deoxyribonuclease, and collagenase but susceptible to treatment with periodate and thus suggest that carbohydrates might play a role in aggregation of dissociated sea-sponge cells. However, because periodate is a very powerful oxidizing agent which may act upon other molecules, including proteins with hydroxyl groups (*7*), we decided to use milder agents to establish the chemical nature of the cell-binding material.

For this purpose, the crude extract, before being used in the aggregating experiments, was treated with a variety of enzymes. Chemically dissociated cells were fixed with 25-percent glutaraldehyde (7:1), washed, and suspended in normal, cold sea water. The crude extract was dialyzed at 2°C against 2 m*M* $CaCl_2$ for 72 hours and stored at 2°C. The cell aggregation test was performed at 2° to 4°C with a rotatory shaker (*8*). Under these conditions the fixed cells did not aggregate unless crude extract was added, as reported by Moscona (*1*). Calcium is necessary both for stabilization of the specific cell-binding substance present in the crude extract and for cell aggregation in the presence of this material (*1, 2*).

Chemical analysis of our crude extract revealed protein and sugar to be the two main components (Table 1). These results confirm those of Margoliash *et al.* (*9*), who performed chemical studies on similar but more highly purified material. To determine whether one or both components were involved in cell aggregation, the crude extract was treated with different hydrolases acting on peptide bonds (12 enzymes), or glycoside compounds (seven enzymes). In addition, ribonuclease and four hydrolases which split different kinds of ester bonds were also used. Most of the enzymes were dissolved in 2 m*M* $CaCl_2$, except for eight prepared with appropriate buffers. Usually they were used at the *p*H and concentration recommended (*10*). K-zyme, which is a filtrate from *Streptomyces fradiae* cultures (*11*), and a purified preparation of keratinase from this filtrate (*12*) were dissolved in 0.1*M* carbonate-bicarbonate buffer at *p*H 9.

Before the enzymatic incubation, 1 volume of the enzyme solution was mixed with 9 volumes of the calcium-stabilized crude extract. In controls, 1 volume of the enzyme solution was replaced by the same amount of 2 m*M* $CaCl_2$, or by 1 volume of a solution in which the enzyme was inactivated by different procedures. In each case the activity of the enzyme in 2 m*M* $CaCl_2$, with or without buffer, was tested with a suitable known biochemical or cytochemical system. Temperature and length of incubation were those usually recommended, and we began with the shortest incubation. If the crude extract proved to be resistant, incubation was prolonged at a lower temperature (30°C or 25°C), because of its thermosensitivity. After incubation, the mixture was chilled, and, if it contained buffer, it was dialyzed for 12 hours at 2°C against 2 m*M* $CaCl_2$.

For the cell aggregation test, 1.5 ml of the incubated mixture as just described was mixed with 1.5 ml of the sponge-cell suspension in a 25-ml erlenmeyer flask, and the flask was placed in a special rotatory shaker (New Brunswick Scientific Co., model S35) and rotated in a cold room (2° to

Table 1. Average chemical composition of six crude extracts of *Haliclona variabilis*.

Component	Concentration (μg/ml)	Method
Proteins	156	Folin-Ciocalteu (*20*)
Hexoses*	31	Cysteine-sulfate (*21*)
Fucose	6	Cysteine-sulfate
Hexosamines†	9	Elson-Morgan (*22*)
Uronic acids†	6	Dische (*23*)
Sialic acids		Ehrlich (direct) (*24*)
Nucleic acids	6	Warburg and Christian (*25*)

* Paper chromatography of the acid hydrolyzates revealed the presence of glucose and mannose. † Unidentified. It is probably neither glucuronate nor galacturonate.

4°C) at 80 rev/min for 72 hours; periodic observations were made with a Leitz inverted microscope. Since the enzyme used for treating the crude extract could not be removed from the incubating mixture, the enzyme, incubated similarly but in the absence of the crude extract, was added to the cell suspension to determine whether the enzyme alone would aggregate the cells.

Two types of results, summarized in Table 2, were obtained. One was normal aggregation of the cells. This occurred when the crude extract had been first incubated with one of the following enzymes: ribonuclease B, (*13*), deoxyribonuclease I, wheat germ lipase, wheat germ acid phosphatase, type 1 calf intestinal mucosa alkaline phosphatase, alpha amylase, egg white muramidase, *Vibrio cholerae* neuraminidase, beta glucuronidase, *Chalaropsis* *N*-acetyl-hexosaminidase (*14*), *Clostridium perfringens* glycosidases, leucine aminopeptidase, carboxypeptidase A, rennin, thrombin, or *Clostridium histolyticum* collagenase. In the other type of result, cell aggregation differed from the normal in varying degrees. It did so when crude extract had been incubated with one of the following enzymes: alpha chymotrypsin, K-zyme, and purified preparations of keratinase, papain, elastase, trypsin, and fibrinolysin. These results were reproducible, except for those with papain, which were erratic. The most efficient enzymes in order of their activity were chymotrypsin, K-zyme, keratinase, and papain (in positive experiments). Elastase and, particularly, trypsin were less effective, unless incubated for longer periods (5 hours instead of 40 minutes) or in high doses for 5 hours. Fibrinolysin had weak, variable activity. Crude extracts from sponges collected in early spring were much more susceptible to fibrinolysin than those obtained in summer or fall. Those obtained in summer and fall were unaffected by the enzyme, unless treated with high concentrations of fibrinolysin for 5 hours. When the crude extract was treated with heat-inactivated enzymes, changes in activity of the cell-binding substance did not occur. The untreated crude extract was always capable of aggregating cells and incapable of inhibiting the activity of the enzymes when the enzymes were tested against the proper substrate. The enzymes alone did not aggregate the sponge cells.

Active peptidases in our system may produce inactivation or destruction of the cell-binding substance by different mechanisms. They can act as esterases, splitting, among other linkages, ester glycosidic bonds. However, it is doubtful that they did so because trypsin, which is a powerful esterase (*15*), was less active than elastase which is considered to be a poor esterolytic agent (*16*). Because inactivated enzymes did not interfere with cell aggregation, it is also doubtful that peptidases can block active centers of the cell-binding material. Also unlikely is the possibility that soluble inhibitors resulting from digestion of protein contaminants may exert such a blocking effect. Dialysis against 2 m*M* $CaCl_2$ did not restore the activity of the crude extract previously treated with chymotrypsin.

Peptidases may disorganize the cell-binding protein by altering its primary structure through true proteolysis or by denaturation of other protein structures. Proteolysis may also liberate sugars attached to the resulting polypeptides; this possibility should be considered in interpreting results obtained with peptidases. Although there is a good correlation between the quantities of certain amino acids in the cell-binding substance found by other authors, chemical analyses (*9*) and the fact that our peptidases attack certain peptide bonds preferentially (*17*), it seems more probable that changes in cell aggregation are produced by denaturing effects than by enzymatic hydrolysis of peptide bonds in the cell-binding substance. Chymotrypsin and elastase may act by removing calcium from the cell-binding substance, in that a moderate increase of this cation (contained in 20 m*M* $CaCl_2$) renders the substance less susceptible to the enzymes but does not impair the peptidase activity of the enzymes. Other enzymes that have reductase activity (K-zyme and keratinase) (*11, 12*) or that had been incubated with a reducing activator (cysteine in papain preparations) seem to produce their effect by splitting disulfide bridges.

Table 2. Degree of cell aggregation by enzyme-treated crude extracts. +++, Most cells aggregate to form large, compact, smooth-surfaced masses; ++, fewer cells aggregate, producing loose, irregular, medium-sized clusters; +, smaller aggregates; +/−, loose clusters are very small and free cells abundant; −, only free cells are present. Results were obtained with 12 different crude extracts.

Enzyme	Degree of cell aggregation at		
	3 hr	24 hr	48 hr
50 μg/ml (40 minutes)			
Chymotrypsin*	−	−	−
K-zyme	−	−	−
Papain	+/−	+/−	+/−
Elastase	−	+	++
Trypsin	−	+++	+++
Fibrinolysin	+++	+++	+++
50 μg/ml (3 hours)			
Keratinase	−	−	
50 μg/ml (5 hours)			
Elastase	−	+/−	
Trypsin	−	+	
200 μg/ml (3 hours)			
Keratinase	−	−	
200 μg/ml (5 hours)			
Elastase	−	−	−
Trypsin	−	−	−
Fibrinolysin	++	++	++
None	+++	+++	+++

* Dialysis of the mixture did not change this result.

It is possible that the known disulfide-reducing activity of keratinase preparations reduced disulfide groups in the crude-extract protein. To determine whether or not such reduction would affect the aggregation, the crude extract was treated with the known reducing agents sodium thioglycolate (0.25*M*) and KCN (0.15*M*). Both were capable of destroying the aggregating activity in the extract, while salts without reducing properties were not. To exclude the possibility that these chemicals might act by removing calcium, the crude extract was dialyzed against distilled water at 2°C for 24 hours; then it was incubated for 48 hours at this low temperature in the presence of 0.25*M* sodium thioglycolate at *p*H 8.8, or with distilled water. After incubation, both mixtures were dialyzed at 2°C for 24 hours against 2 m*M* $CaCl_2$. The untreated crude extract was active, whereas no cell aggregation was observed when the thioglycolate-treated crude extract was added to the cell suspension.

These experiments indicate that cellular aggregation of the dissociated, glutaraldehyde-fixed, sponge cells depends primarily on the presence of intact disulfide groups and on protein integrity. The importance of the dithiol groups in the process suggests the need for an investigation of cysteine and disulfide linkages in the cell-binding protein. It is suggestive that our amino-acid analysis of acid hydrolyzates of six sponge extracts revealed the presence of cysteic acid and cysteine (*18*). Margoliash *et al.* (*9*) found that each molecule of glycoprotein in their purified aggregating principle contained 3 moles of sulfate. However, presence of cysteine was not determined.

Although none of the glycosidases destroyed the activity of the crude extract, the participation of sugars in the mechanism of the cell aggregation cannot be ruled out definitively because release of sugars has not been shown to occur without impairing the aggregating activity of the extract. However, because one of the enzyme mixtures (clostridial glycosidases) used is very active in removing sugars in other related cell-surface systems (*14, 19*), one could assume that such a removal, complete or incomplete, took place in our material.

GABRIEL J. GASIC*
NORBEL L. GALANTI
Department of Oncology, University of Chile School of Medicine, Santiago, and *Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia, 19104*

References and Notes

1. A. Moscona, *Proc. Nat. Acad. Sci. U.S.* **49**, 742 (1963).
2. T. Humphreys, *Develop. Biol.* **8**, 27 (1963).
3. A. A. Moscona, *J. Cell. Comp. Physiol., Suppl. 1* **60**, 65 (1962).
4. ———, *Sci. Am.* **205**, 143 (Sept. 1961).
5. D. R. Coman, *Cancer Res.* **13**, 397 (1953).
6. ———, *ibid.* **21**, 1436 (1961).
7. W. T. J. Morgan, in *Polysaccharides in Biology*, G. F. Springer, Ed. (Josiah Macy, Jr. Foundation, New York, 1955), p. 154.
8. A. Moscona, *Exp. Cell Res.* **22**, 455 (1961).
9. E. Margoliash, J. R. Schenck, M. P. Hargie, S. Burokas, W. R. Richter, G. H. Barlow, A. A. Moscona, *Biochem. Biophys. Res. Commun.* **20**, 383 (1965).
10. S. P. Colowick and N. O. Kaplan, Eds., *Methods in Enzymology* (Academic Press, New York, 1964).
11. J. J. Noval and W. J. Nickerson, *J. Bacteriol.* **77**, 251 (1959).
12. W. J. Nickerson, J. J. Noval, R. S. Robison, *Biochim. Biophys. Acta* **77**, 73 (1963).
13. The ribonuclease B, deoxyribonuclease I, leucine aminopeptidase, carboxypeptidase A, *Clostridium histolyticum* collagenase, papain, and elastase were obtained from the Worthington Biochemical Corp., Freehold, N.J.; the wheat germ lipase, egg white muramidase, rennin, thrombin, trypsin, and fibrinolysin from Mann Research Laboratories, New York, N.Y.; the wheat germ acid phosphatase and type 1 calf intestinal mucosa alkaline phosphatase from Sigma Chemical Co., St. Louis, Mo.; the alpha amylase and beta glucuronidase from Nutritional Biochemicals Corp., Cleveland, Ohio; the alpha chymotrypsin from General Biochemicals, Chagrin Falls, Ohio; and the *Vibrio cholerae* neuraminidase from Behringwerke Ag., Marburg-Lahn, Germany.
14. G. Gasic and T. Gasic, *Proc. Soc. Exp. Biol. Med.* **114**, 660 (1963).
15. C. H. Ryan and A. K. Balls, *Proc. Nat. Acad. Sci. U.S.* **48**, 1839 (1962).
16. R. L. Waldorf and B. Kickhofen, *Arch. Biochem. Biophys.* **98**, 191 (1962).
17. M. Dixon and E. C. Webb, *Enzymes* (Academic Press, New York, 1964), pp. 225–250.
18. G. J. Gasic and N. L. Galanti, unpublished.
19. W. T. J. Morgan, in *Mucous Secretion*, H. E. Whipple, Ed. (New York Academy of Sciences, New York, 1963), p. 177.
20. E. A. Kabat and M. N. Mayer, *Experimental Immunochemistry* (Thomas, Springfield, Ill., 1961), p. 556.
21. Z. Dische, in *Methods of Biochemical Analysis*, D. Glick, Ed. (Interscience, New York, 1955), p. 313; R. J. Winzler, *ibid.*, p. 279.
22. L. A. Elson and W. T. J. Morgan, *Biochem. J.* **27**, 1324 (1933).
23. Z. Dische, *J. Biol. Chem.* **167**, 189 (1947).
24. I. Werner and L. Odin, *Acta Soc. Med. Upsal.* **57**, 230 (1952).
25. O. Warburg and W. Christian, *Biochem. Z.* **310**, 384 (1941).
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* Present address: Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia 19104.

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Spontaneous Mutation Rates at Five Coat-Color Loci in Mice

Abstract. *Examination of 1.5 million mice yielded natural mutation rates estimated from 5.2 million gene reproductions at five specific coat-color loci. The average rates were 11.1 × 10^{-6} for forward mutations and 2.7 × 10^{-6} for reverse mutations. Differences between the frequencies of mutations at the individual loci were evident.*

Spontaneous or natural mutation rates are the rates of the occurrence of gene changes under normal conditions, excluding the deliberate use of mutagenic agents. Mutations may be either somatic or germinal. Germinal mutations occur in any cell of the germ line and result in a new allele not present in either parent. Somatic mutations involve body cells in any stage of development in an individual, and these cannot be transmitted to the next generation if they occur after the germ cells have been differentiated. The random changes induced by germinal mutations create an alteration in the genetic constitution of an individual and thus increase the genetic variability of a population. The change may be perpetuated for many generations and may, under the proper conditions of natural selection, appreciably alter the characteristics of a population. Information concerning the rate at which these gene changes occur under normal conditions can be obtained by studying specific loci at which a mutation is detectable.

The forward (wild-type to mutant) and reverse (recessive mutant to wild-type or to mutant of higher dominance) spontaneous mutation rates at five specific coat-color loci of mice are under investigation at the Jackson Laboratory (*1*). The mice in 18 inbred strains maintained by brother-sister matings and six F_1 hybrids produced by crossing various pairs of these strains are examined two to four times between birth and weaning for deviations from the expected coat color and other external characteristics. The variant mice are scored as mutations only if the trait is transmitted, as determined by genetic tests. Fifteen of the strains are homozygous for specific coat-color alleles, and, in particular, sufficient numbers of mice are reared and examined for mutations at the nonagouti (*a*), brown (*b*), albino (*c*), dilute (*d*), and leaden (*ln*) loci to warrant the calculation of reverse spontaneous mutation rates at these loci. In addition, these five loci are tested for forward mutations in the F_1 hybrids produced by crosses between wild-type and mutant-carrying strains.

This report summarizes the findings, based on an examination of 1.5 million mice for 5.2 million gene reproductions at the five specific coat-color loci, collected between August 1963 and January 1965. The strains, their coat-color genotypes, and the numbers of mice examined in each strain are shown in Table 1. A reverse mutation at any of these loci in the inbred strains will be visibly detectable in the offspring of the generation following its occurrence. The number of mutations m can thus be used to calculate directly the reverse mutation rate, v, by $v = m/2n$, where n is the number of animals examined. For instance, the DBA/2J strain yields information on the *a*, *b*, and *d* loci. The number of animals examined in this strain is included in the n used in calculating the