Amino Acid Composition of Some Calcified Proteins

Abstract. Amino-acid analysis shows that the proteins from two calcified tissues, enamel and snail shell, are related to collagen in that they also contain hydroxylysine. The presence of hydroxyproline is suggested but not confirmed. This evidence, together with other recent findings, suggests that hydroxylysine may have an important role in the calcification of each of these otherwise diverse proteins.

The calcified tissues of vertebrates are nearly all based on a collagen matrix. The nature of the collagen (*1*) and its possible role in the calcification process (*2*) are topics of considerable interest. A study of calcified tissues which do not contain a collagen matrix may give further information. Among vertebrates the most important instance of such tissues is the enamel, the outer layer of the teeth, produced by specialized epithelial cells. Among the invertebrate phyla there are many examples of calcified tissues which contain a matrix not generally classified among the collagens. As a typical example of invertebrate tissue I have studied the shell of the snail (*3*), *Australorbis globratus*. Pig embryos were selected as a convenient source of enamel (*4*).

The samples were placed in dialysis sacks and decalcified by suspension in 10-percent ethylenediaminetetraacetic acid, *p*H 7.5, for about 10 days at 5°C. The chelating agent was removed by dialysis, and insoluble protein was isolated by centrifugation. More than half of the protein of pig-embryo enamel was in solution. The soluble fraction was isolated by lyophilization after dialysis and analyzed separately. Acid hydrolysates of the proteins (6*N* HCl, 24 hours, 105°C) were analyzed for their amino-acid content by ion-exchange chromatography with an automatic instrument (*5*).

The data obtained from the snail-shell protein and the soluble portion of the pig embryo–enamel protein (*6*) are presented in Table 1. An analysis of human dentin, a typical calcified collagen, is included for comparison. Chromatograms of hydrolysates of both noncollagenous proteins showed a peak that was characteristic of hydroxylysine. Because this amino acid is partially epimerized during acid hydrolysis and the resulting diastereoisomers move at different rates on ion-exchange columns (*7*), a double peak is obtained which, together with its position relative to other amino acids, constitutes a unique reaction and permits certain identification. About two and six residues of hydroxylysine per 1000 total residues were found in the enamel and snail-shell proteins, respectively. The amount of hydroxylysine in the dentin collagen was typical of other calcified collagens, which contain 4 to 16 residues per 1000 (*1*).

Hydroxyproline was observed in the chromatograms of the snail-shell protein (Table 1) and in the insoluble portion of the pig embryo–enamel protein, but not in the soluble portion. Its absence in the soluble portion of the enamel matrix and the possibility of contamination of the insoluble fraction with collagen from dentin, makes this observation of doubtful significance (*6*). Burroughs (*8*), studying human enamel, and Mechanic and Glimcher (*9*), studying calf enamel, also have found small amounts of hydroxyproline. The possibility of contamination probably cannot be resolved until proteins of demonstrable homogeneity are prepared. Contamination of the snail-shell collagen seems less likely but is possible because snails contain a collagen as part of the soft tissue (*10*).

The hydroxylysine cannot reasonably be ascribed to the presence of collagen, since the high concentration of hydroxyproline in mammalian collagens would have resulted in the detection of much higher amounts of this imino acid than were found. In confirmation of my results, Mechanic and Glimcher (*9*) found a similar level of hydroxylysine in calf enamel matrix. Eastoe (*11*) analyzed protein from human fetal enamel. His results are similar to mine, but he did not report the presence of either hydroxyproline or hydroxylysine. However, they may easily have been missed, because the sensitivity of his methods may not be sufficient to detect the small amounts which are present.

There are other points of interest in the data in Table 1. The enamel protein has an exceptionally high imino-acid (proline) content, more than one-quarter of the total residues. The snail-shell protein has a high glycine content, also more than one-quarter of the total residues. Also unusual are the large amounts of aspartic acid in snail-shell protein and the large amounts of glutamic acid, methionine, and histidine in enamel protein. The dentin collagen is typical of other vertebrate collagens (*1*).

The significance of hydroxylysine in these tissues is not presently clear, but this observation suggests a common role for this amino acid in the calcification process. Consistent with this possibility is the finding by Glimcher (*2*) that native collagen fibrils appear to function in vitro, and perhaps also in vivo, as a nucleating agent for the ordered deposition of the inorganic phase. Specific sites appear to be involved. Of further interest is the observation that an unknown functional group of collagen can be phosphorylated by adenosine triphosphate and the suggestion that this reaction could provide a means of introducing an oriented phosphate group as the initial step in the nucleation (*12*).

Although the evidence described here is only circumstantial, it is sufficiently strong to suggest that a search for direct evidence for a role for hydroxylysine in calcification and perhaps other metabolic processes may be profitable.

Table 1. Amino-acid composition of several calcified proteins.

Amino acid	Number of residues per 1000 total residues		
	Snail-shell protein	Enamel protein*	Dentin collagen
Cysteic acid†	10	0	0
Hydroxyproline	3.2	0	99
Aspartic acid	183	29	46
Theonine	22	37	17
Serine	91	46	33
Glutamic acid	81	185	74
Proline	47	271	116
Glycine	265	49	329
Alanine	83	24	112
Valine	32	37	25
Methionine	1.1	47	5.3
Isoleucine	15	32	9.3
Leucine	34	94	24
Tyrosine	35	22	6.4
Phenylalanine	21	26	16
Hydroxylysine	6.2	1.6	9.6
Lysine	52	11	22
Histidine	3.8	72	4.7
Arginine	15	6.1	52
Tryptophan		12	0
Amide N			(38)

* The analysis is of the soluble fraction of pig-embryo enamel after decalcification. † A tentative identification. The cysteic acid presumably arose from cystine or cysteine by oxidation.

KARL A. PIEZ
National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland

References and Notes

1. K. A. Piez and R. C. Likins, in *Calcification in Biological Systems*, R. Sognnaes, Ed. (American Association for the Advancement of Science, Washington, D.C., 1960), p. 411.
2. M. J. Glimcher, *ibid.*, p. 421.
3. I am indebted to Elmer Berry of the National Institute of Allergy and Infectious Diseases for this sample. The growing edge was cut from the shells of snails, allowed to grow again, and then recut until sufficient material was obtained.
4. I am indebted to William Lefkowitz and Bernard Forscher of the University of Kansas City Dental School for this sample. Enamel was obtained free of cellular material, membranes, and dentin by microdissection of pig embryos.
5. K. A. Piez and L. Morris, *Anal. Biochem.* **1**, 187 (1960).
6. These enamel analyses together with similar

data for human teeth will be presented in detail elsewhere [K. A. Piez, in *Symposium on Keratinization*, E. O. Butcher, Ed. (American Association for the Advancement of Science, Washington, D.C., in preparation)].
7. K. A. Piez, *J. Biol. Chem.* **207**, 77 (1954); P. B. Hamilton and R. A. Anderson, *ibid.*, **213**, 249 (1955).
8. L. R. Burroughs, Intern. Assoc. for Dental Research, 39th general meeting, Boston (1961); *J. Dental Research*, in press.
9. G. L. Mechanic and M. J. Glimcher, personal communication.
10. A. P. Williams, *Biochem. J.* **74**, 304 (1960).
11. J. E. Eastoe, *Nature* **187**, 411 (1960).
12. S. Crane and M. J. Glimcher, presented before the Council for International Organizations of Medical Science "Symposium on the Use of Radioisotopes in the Study of Bone, Princeton, N.J., 1960," in press.

13 June 1961

A Function for Tissue Mast Cells

Abstract. Experimental elimination of mast cells from the peritoneal tissues of the rat by distilled water treatment inhibited the increase of vascular permeability which normally follows a passively induced antigen-antibody reaction in peritoneal tissue. Thus mast cells may contribute to the initiation of inflammation which follows antigen-antibody reactions.

The tissue mast cells have been shown to contain histamine, 5-hydroxytryptamine, heparin, and hyaluronic acid—substances having significant physiological action (*1*). However, functions for the tissue mast cells have not been demonstrated clearly. Information concerning their function should be obtainable by determining whether phenomena conceivably controlled or influenced by the mast cells in normal tissues are altered in tissues made substantially free of mast cells. This report is concerned with such an approach which uses the alterations in the permeability of blood vessels that have been observed to follow antigen-antibody reactions in tissue (*2*).

The serous tissues of the peritoneal cavity of Sprague-Dawley rats weighing about 150 g were studied. The mast cells of these tissues were destroyed by the intraperitoneal injection of 20 ml of distilled water. This treatment causes the immediate disruption of mast cells, the debris of which is phagocytized during the next 3 to 5 days. Thereupon the serous tissues of the peritoneal cavity are quite normal in appearance except that they lack mast cells (*3*). The effects of antigen-antibody reactions on vascular permeability as determined by the method outlined by Ovary (*4*) in normal rats (having an intact mast cell population in the serous tissues of the peritoneal cavity) were compared with rats treated with distilled water 3 to 8 days previously. In these tests, a dilution of antiserum (rabbit anti-egg albumin) was injected intraperitoneally in volumes of 1 or 5 ml. (No estimates were made of the activity of the antiserum in terms of antibody N per milliliter.) This was followed 3 hours later by the intravenous injection of 10 mg of crystallized egg albumin in 1.0 or 1.5 ml of a 1-percent solution of Evans Blue dye in 0.9-percent sodium chloride. The animals were killed 15 to 20 minutes later by ether anesthesia, and the peritoneal tissues were washed free of exudate with water and examined for leakage of the dye into the perivascular spaces. Corresponding dilutions of normal rabbit serum were used as the control for the antiserum. An arbitrary grading system was employed for visual evaluation of the degree of coloration of the tissues against a background of white cardboard. The notations 0 and 1+ to 4+ were used to cover the observed range of dye leakage.

The mesentery and parietal peritoneum had a 2+ or greater coloration in normal rats treated with rabbit anti-egg albumin serum and egg albumin. This coloration was markedly attenuated (1+) or absent (0) in rats receiving the antibody, antigen, and dye but previously treated with distilled water. No extravascular blue coloration was seen in these tissues in either normal or water-pretreated animals into which normal rabbit serum was injected instead of the antiserum. These observations are summarized in Table 1.

Table 1. Effect of distilled water pretreatment on the leakage of dye following an egg albumin–rabbit anti-egg albumin reaction in the peritoneal tissues of the rat.

Treatment	Rats (No.)	Degree of coloration (No. of rats)				
		0	1+	2+	3+	4+
Distilled water, antiserum	18	10	8			
Distilled water, normal serum	11	11				
Antiserum only	20			3	3	14
Normal serum	13	13				

* All animals received a subsequent intravenous injection of egg albumin and dye.

Substantially the same results were obtained when the skin was used as the site of the antigen-antibody reaction, the mast cells having been previously destroyed by the local intradermal injection of distilled water. Four to six animals were included in each experimental group in individual experiments. The above findings have appeared consistently in six repetitions of the experiment. Preliminary experiments suggest that results similar to, though less striking than those described above, may be found in rats in which mast cell destruction has been effected by the injection of antimast cell serum prepared in rabbits (*5*) or by repeated administration of the histamine liberator, compound 48/80.

We interpret the findings of the experiments reported here to indicate that the increased permeabiliy accompanying the antigen-antibody reaction depends, in part at least, upon the presence of the tissue mast cell, the release of its histamine or 5-hydroxytryptamine, or of both, being directly responsible for the alteration in permeability. The mechanism by which the release of these substances is brought about is not clear. It is known from previous experiments, however, that mast cells disrupt and release their granules into the surrounding tissue in passive anaphylaxis (*6*), and recently, Archer (*7*) has reported that a heat-labile substance is formed during an antigen-antibody reaction which causes the disruption of rat mast cells in vitro. That mast cells may initiate inflammatory processes following other tissue injury in rats is suggested by Sheldon and Bauer (*8*). The present results indicate that the mast cells may be of primary importance in initiating the inflammatory response accompanying antigen-antibody reactions (*9*).

LAURENCE R. DRAPER*
DOUGLAS E. SMITH
Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois

References and Notes

1. G. B. West, *J. Pharm. and Pharmacol.* **11**, 513 (1959).
2. J. P. Fisher and R. A. Cooke, *J. Allergy* **28**, 150 (1957).
3. D. W. Fawcett, *Anat. Record* **121**, 29 (1955); D. E. Smith and Y. S. Lewis, *Anat. Record* **132**, 93 (1958).
4. Z. Ovary, *Intern. Arch. Allergy Appl. Immunol.* **3**, 293 (1952).
5. D. E. Smith and Y. S. Lewis, *J. Exptl. Med.* **113**, 683 (1961).
6. J. H. Humphrey and I. Mota, *Immunology* **2**, 31 (1959).
7. G. T. Archer, *Australian J. Exptl. Biol. Med. Sci.* **38**, 147 (1960).
8. W. H. Sheldon and H. Bauer, *J. Exptl. Med.* **112**, 1069 (1960).
9. This work was performed under the auspices of the U.S. Atomic Energy Commission. Full details of these investigations will be published elsewhere.
* Present address: National Cancer Institute, National Institutes of Health, Bethesda, Md.

7 July 1961