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Mineralogic Changes during Growth in the Red Alga, Clathromorphum compactum

Abstract. *The amount of magnesium in the skeletal calcite of the encrusting marine red alga* Clathromorphum compactum *varies seasonally in response to changes in water temperature. X-ray diffraction analyses of serial samples of this alga collected in the Gulf of Maine indicate more than a 40-percent change in composition during a year and demonstrate a more rapid calcification during warmer periods.*

Marine organisms deposit skeletal parts composed of a wide variety of mineral forms. Among the carbonate-secreting groups, the minerals calcite, aragonite, and a variety of magnesium calcites (*1*) are common (*2, 3*). Green algae deposit aragonite exclusively. Red algae deposit both aragonite and magnesium calcites, the latter containing up to 30 mole percent magnesium, calculated as $MgCO_3$, in solid solution in the calcite (*1*). The coccolithophores deposit a calcite very low in magnesium.

Clarke and Wheeler (*4*) and Chave (*2*) have shown that in calcite-secreting groups of marine organisms, the magnesium content of the whole calcitic tests increases almost linearly with water temperature over the range 0° to 30°C. Thus tropical forms of a given taxon have a higher magnesium content than their boreal counterparts. During an attempt to determine the rate of growth and calcification in an individual encrustation of the red alga *Clathromorphum compactum,* we found that seasonal changes in the magnesium content of the skeletal calcite could be detected.

Because of the small radius of the Mg^{++} ion, relative to the Ca^{++} ion, an increase in the number of Mg^{++} ions in solid solution causes a decrease in the lattice spacings which can be quantitatively evaluated by means of x-ray diffraction techniques. The $d(112)$ spacing of the calcite was measured by using the $d(111)$ of fluorite (CaF_2), added as an internal reference standard. The composition of the calcite was determined from Fig. 1 of Goldsmith *et al.* (*5*). The accuracy of the analytical technique is about ± 0.2-percent $MgCO_3$. Contamination by dried salts from the sea or organically bound magnesium is no problem because only magnesium in the calcite lattice is measured by this technique.

Serial samples of a compact encrustation of *C. compactum* were taken from the surface inward, by cutting into the specimen with a fine file. The depth of the cut was measured with a micrometer ocular. The material removed, in 100- to 200-μ units, was collected, mixed with powdered fluorite, and mounted on glass slides for x-ray analysis. The results obtained are shown in Fig. 1. Layers deposited in the fall contain the gametangia, identified by *C* in the figure.

In the Gulf of Maine, where water temperatures range from near 0° to 13°C, *C. compactum* deposits skeletal calcite containing from 9.5 to 14 mole percent magnesium, calculated as $MgCO_3$. The alga deposits approximately 500 μ of carbonate annually, thicker layers being deposited in the summer and thinner layers in the winter. Since we have not yet determined whether growth and calcification occur when the water temperatures are at their lowest for this region, we do not know whether a full temperature record is preserved in the specimen.

In the case of *C. compactum,* seasonal growth increments are easily identified by the morphology of the colony, particularly by localization of gametangia. The technique described could be applied to other algae, in which the morphologic characteristics of growth are not so definitive, so that growth and calcification rates could be determined.

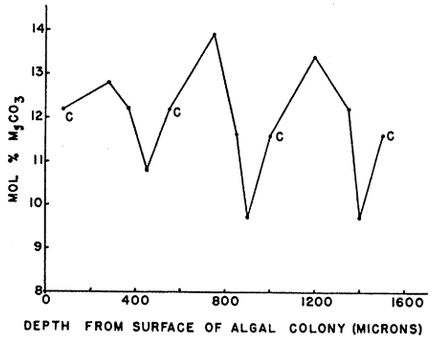

Fig. 1. Magnesium content of successive layers of skeletal calcite in *Clathromorphum compactum.* Fall layers containing gametangia are indicated by *C.*

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Hemoglobin Polymerization in Mice

Abstract. *Polymerization of certain mouse hemoglobins to eight-chain double molecules is completely inhibited by iodoacetamide. Each double molecule appears to consist of two* $\alpha_2\beta_2$*-units linked by way of their* β*-chains with two disulfide bridges.*

Hemoglobins from various strains of mice have been termed either "single" or "diffuse" according to the appearance of electrophoretic patterns of the hemolyzates (*1*). Diffuse mouse hemoglobins have a component sedimenting at approximately 7*S* which increases in quantity during storage (*2*). Discovery that formation of 7*S* components in frog and turtle hemoglobins can be prevented by sulfhydryl (—SH) reagents (*3*) suggested that formation of 7*S* components in mouse hemoglobins might be similarly inhibited. My experiments were designed to test this hypothesis and to determine the number of reactive —SH groups, the number of groups believed to be involved in disulfide (—S—S—) linkages, and distribution of these groups between the α- and β-chains. Initial experiments showed that hemoglobins from each of three strains of mice possess a total of eight cysteine or half-cystine residues per $\alpha_2\beta_2$ unit, of which four freely react with iodoacetamide in a fresh preparation. Hemoglobin from one strain was examined further; the 7*S* component was isolated as described below. It was hypothesized that some of the four reactive —SH groups took part in —S—S— linkage between the two tetramers composing the 7*S* component, and that other —SH groups

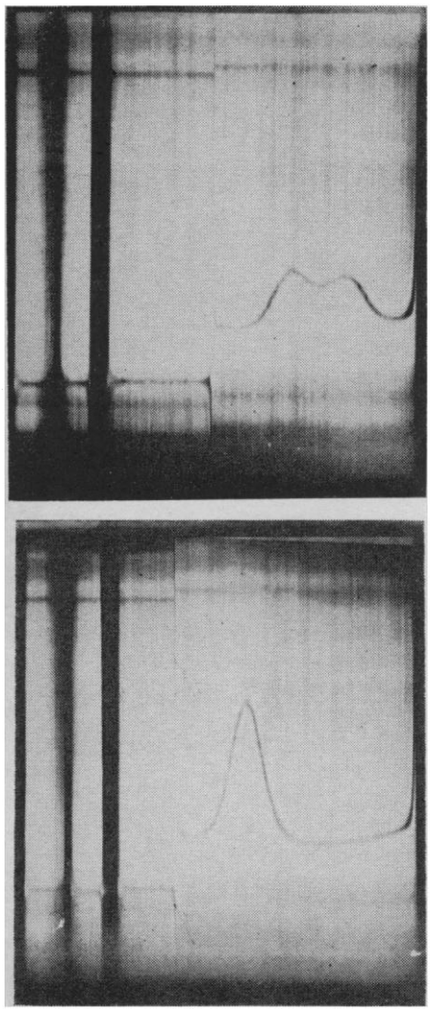

Fig. 1. (Top) Sedimentation pattern of BALB/cJ mouse hemoglobin 70 minutes after reaching 52,640 rev/min. (Bottom) Same as top, but treated with iodoacetamide at time of red-cell hemolysis.

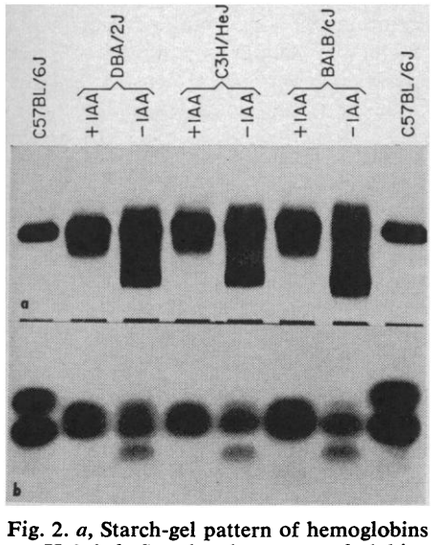

Fig. 2. *a*, Starch-gel pattern of hemoglobins at *p*H 8.6. *b*, Starch-gel patterns of globins at *p*H 1.8; exactly the same order from left to right as in *a*. Electrophoretic migration is upward.

were still freely reactive. Initial treatment of the 7*S* component with *N*-ethylmaleimide (NEM) should result in reaction of all available —SH groups not involved in —S—S— linkage. Treatment with mercaptoethanol after removal of excess NEM should reduce available —S—S— linkages, split the 7*S* component into two tetramers, and make available some —SH groups in the protein. The number of —SH groups so liberated could then be measured by reaction with iodoacetamide.

This sequence of operations shows that two cysteinyl residues in each tetramer in the 7*S* component are still freely reactive to NEM. Reaction with mercaptoethanol of molecules already treated with NEM results in transformation of all 7*S* component into 4*S* component and the appearance of two —SH groups per tetramer, which react with iodoacetamide. Subsequent separation of the α- and β-chains shows that both the —S—S— linkages and the remaining reactive —SH groups of the 7*S* component are associated with the β-chain. No reaction of either NEM or iodoacetamide occurred with cysteinyl residues of the α-chain. The experiments were not designed to locate the unreactive or "unavailable" four cysteinyl or half-cystine residues in the tetramer and gave no information concerning their distribution; presumably they are internal.

Hemolyzates were prepared from three inbred strains of mice, BALB/cJ, C3H/HeJ, and DBA/2J (*4*). The cells were washed three times with 0.9 percent NaCl, and were lysed by addition of an equal volume of glass-distilled water; cell debris was removed by centrifugation. The hemolyzates were saturated with carbon monoxide and diluted with distilled water to a hemoglobin concentration of 2.5 percent. The hemolyzate from each strain was then halved; an equal volume of 0.2*M* phosphate buffer, *p*H 7.0, was added to one half, and an equal volume of the same buffer containing iodoacetamide at 6.84 mg/ml to the other half. This corresponded to a ratio of ~10 moles of iodoacetamide per mole of hemoglobin, if a molecular weight of 64,000 was assumed for mouse hemoglobin (*5*). All six preparations were stored for 24 hours at 0°C under carbon monoxide, dialyzed for 24 hours against 0.1*M* phosphate buffer of *p*H 7.0 saturated with carbon monoxide, and then stored at 2°C until used. The sedimentation and starch-

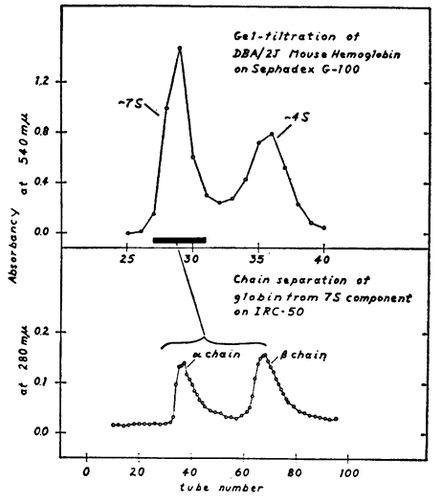

Fig. 3. Separation of the 7*S* from the 4*S* component on Sephadex G-100, and of the α- and β-chains of the 7*S* component by urea-gradient IRC-50 chromatography; treatment of the 7*S* component prior to chain separation is discussed in the text. Sephadex separation, 6 ml per tube; IRC-50 chromatography, 6.5 ml per tube, 20 ml/hr.

gel electrophoreses were carried out 3 to 7 days after this dialysis.

The sedimentation pattern of the hemoglobin, with and without iodoacetamide, from the BALB/cJ mice is shown in Fig. 1. Treatment with iodoacetamide effectively prevents polymerization: only a single symmetrical peak is present. The C3H/HeJ and DBA/2J mice returned virtually identical results. The sedimentation constants are given in Table 1. Addition of iodoacetamide to solutions containing a 7*S* component had no dissociating action on polymer already formed. Starch-gel electrophoresis (*6*) was carried out at *p*H 8.6 (Fig. 2*a*); the untreated hemoglobin from each strain shows a slow component which is blurred with the material migrating ahead of it. This slow component is absent in the hemoglobins treated with iodoacetamide; also absent is part of the diffuse appearance in the gel pattern.

Starch-gel electrophoresis at *p*H 1.8 (*7*) of the globins (*8*) from each of the three strains of mice is shown in Fig. 2*b*; globin from nonpolymerizing C57BL/6J hemoglobin (*5*) is included for comparison. Globins from the hemoglobins untreated with iodoacetamide possess three bands (Fig. 2*b*). The globin component with the lowest electrophoretic mobility on starch gel appears to form from the fastest component.

S-Carboxymethyl cysteine was deter-

mined by hydrolysis and amino acid analysis of the globins from each of the three strains of mice after reaction of the fresh hemolyzate with iodoacetamide. This analysis gave 1.9 *S*-carboxymethyl cysteine residues for the α β-unit from each strain. Analyses of portions not treated with IAA gave 3.8 to 4.1 cysteic acid residues (*5*) for the α β-unit from each strain. In each of these strains of mice the total number of cysteinyl or half-cystine residues is four in each α β-unit, of which two are reactive in the native molecule.

The 4*S* and 7*S* components of a DBA/2J hemolyzate were separated by passage through a column of Sephadex G-100 (2.5 × 43 cm) equilibrated with 0.2*M* NaCl (Fig. 3). The 7*S* component (26.7 mg in 30 ml of 0.2*M* NaCl) was reacted with 1.06 mg of *N*-ethylmaleimide in 1.0 ml of 0.1*M* phosphate of *p*H 6.5 for 4.5 hours under carbon monoxide at 25°C. The solution was treated with 0.5 mmole of β-mercaptoethanol in 3.5 ml of H_2O for 2 hours at 25°C after dialysis against 1 liter of 0.1*M* phosphate buffer, *p*H 6.5, saturated with carbon monoxide, for 65 hours; this treatment completely transformed the isolated 7*S* component into the 4*S* component. Iodoacetamide (370 mg) was then added and allowed to react for 2 hours at 25°C. The solution was dialyzed at 2°C for 24 hours against 2 liters of 0.1*M* phosphate of *p*H 6.5 and for 24 hours against 2 liters of glass-distilled water before final concentration to 5 ml by vacuum filtration. Globin was then prepared (*8*), and the chains were separated by a slight modification of the urea-gradient method (*9*). An IRC-50 column (0.9 × 50 cm) was equilibrated with 2*M* urea (*p*H 1.8) before application of the sample. The gradient was established with a 250-ml mixing chamber filled with 2*M* urea of *p*H 1.8 into which flowed 8*M* urea, *p*H 1.8. The two fractions (Fig. 3) were dialyzed in acetylated Visking dialysis sacs (*10*) against glass-distilled water until no urea was detectable by the α-isonitrosopropiophenone method (*11*). Portions were then lyophilized and hydrolyzed in 6*N* HCl for 24 hours at 110°C.

Table 1. Sedimentation constants of hemoglobins from three strains of mice. Measurements were made at 20°C, corrected to water as solvent; total protein concentration, 1.25 percent; IAA, iodoacetamide.

Treatment (time of hemolysis)	Sedimentation constant	
	Light	Heavy
	BALB/cJ	
+ IAA	3.93	
− IAA	4.10	7.45
	C3H/HeJ	
+ IAA	4.24	
− IAA	3.88	6.83
	DBA/2J	
+ IAA	3.93	
− IAA	3.98	6.32

Table 2. Amino acid analyses of separated chains of DBA/2J mouse hemoglobin after treatment described in text.

Amino acid	Amino acid residues per molecule*	
	α-Chain	β-Chain
Lysine	11.7	11.4
Histidine	11.2	8.6
Arginine	3.1	3.0
Aspartic acid	12.5	15.0
Threonine	6.7	5.7
Serine	13.0	8.1
Glutamic acid	6.6	8.4
Proline	6.5	3.0
Glycine	12.0	13.2
Alanine	20.9	18.7
Valine	9.8	12.5
Methionine	0.4	1.7
Isoleucine	2.0	3.4
Leucine	16.5	17.0
Tyrosine	2.7	2.5
Phenylalanine	6.6	6.6
Cysteic acid	0.4	0.2
S-Carboxymethyl cysteine	0	0.91
S-Succinyl cysteine	0	0.90†
Cystine/2	0.2	0

* On basis of 15,500 molecular weight. † Corrected as described in text.

The amino acid analysis of each chain, determined with the Spinco amino acid analyzer, is given in Table 2. The values are close to those previously given for the chains of C57BL/6 mouse hemoglobin (*5*), except for cysteine. The recovery of *S*-succinyl cysteine has been corrected by dividing by 0.58 because of the finding (*12*) that 22-hour hydrolysis is sufficient to convert only 58 percent of the reaction product of NEM and reduced ribonuclease to *S*-succinyl cysteine under the same conditions.

The very similar data on turtle and frog hemoglobins (*3*) together with the present data suggest that 7*S* components present in the hemolyzates of turtles, frogs, and mice result from the formation of double molecules, usually after hemolysis, apparently by formation of intermolecular disulfide bonds. The "available" —SH groups are on the so-called β-chains in those mammalian hemoglobins so far examined (*13*) and in at least one of the mouse hemoglobins examined here. Genetic and tryptic peptide studies (*14*) indicate that alleles at the "single-diffuse" (Hb) locus determine mouse β-chain characteristics. The present experiments strongly suggest that the formation of 7*S* molecules involves β—β linkages. Hutton *et al.* (*15*) isolated two distinct hemoglobins, having different β-chains, from each of two inbred strains, AKR and FL, which are homozygous for "diffuse" hemoglobin, but they did not determine the sedimentation characteristics of their fractions.

The hemolyzates in the present work may contain two hemoglobins with very similar electrophoretic properties. One hemoglobin may be able to polymerize but the other cannot. Several strains of "diffuse" and "single" mouse hemoglobins have recently been chromatographed on IRC-50 (*16*). Some of the reported components probably are double molecules. The nature of the "diffuse" pattern is not clear, but may indicate the existence of a mobile equilibrium between subunits. Dissociations of tetramers into subunits are known to occur in mammalian hemoglobins near neutrality and at low ionic strength (*17*).

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