Phylogeny of Immunoglobulin Structure and Function

IV. IMMUNOGLOBULINS OF THE GIANT GROUPER, *EPINEPHELUS ITAIRA*

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SUMMARY

Giant grouper serum was shown to contain 16 S (~3 mg per ml of serum) and 6.4 S (~6 mg per ml of serum) immunoglobulins. The 16 S immunoglobulin had a molecular weight of ~700,000, a relatively high hexose content, and was composed of approximately equimolar amounts of H and L polypeptide chains. It is suggested that this molecule resembles immunoglobulin M on the basis of polypeptide chain properties but that it is most likely a tetramer instead of the “typical” 19 S pentamer of immunoglobulin M. The 6.4 S immunoglobulin appeared structurally to represent a fragment of the 16 S molecule; the major difference being that the 6.4 S H chain was missing ~30,000 daltons.

In attempting to fit the class Osteichthyes into a scheme of vertebrate immunoglobulin evolution, it was suggested that the “primitive” form of polymeric immunoglobulin M may be a tetramer of subunits held together by both disulfide and noncovalent bonds.

The ability to synthesize humoral antibody in response to stimulation with complex antigens appears to be possessed by representatives of all true vertebrates (reviewed in References 2 and 3). Numerous studies have also been reported regarding the structural relationships between the immunoglobulins of “lower” vertebrates and those of “higher” animals. Certain cyclostomes (4) and elasmobranchs (5–8) apparently have only one class of immunoglobulin; this immunoglobulin resembles immunoglobulin M as defined for rabbits (9) and man (10). Certain amphibians (11) have two immunoglobulin classes; one resembles IgM and the other IgG of higher animals. Very little physicochemical data is available for the immunoglobulins of the numerically very large “intermediate” group, namely the class Osteichthyes, and thus the phylogenetic level at which multiple classes of immunoglobulin appeared is obscure. Therefore, in light of the paucity of data on bony fishes in general, and the finding that the giant grouper, *Epinephelus itaira*, yielded appreciable amounts of high and low molecular weight antibodies to the 2,4-dinitrophenyl determinant (12, 13), studies on the physicochemical properties of the immunoglobulins of this species were undertaken. This report presents the results of these studies.

MATERIALS AND METHODS

**Source of Grouper Serum**—The giant groupers used in this study were the same as those used in the study described in the following report (13). Normal serum was obtained from each animal prior to immunization. Supernatants from which antibodies to DNP had been removed by immune precipitation (13) were also employed and gave results similar to those obtained with normal serum.

**Fractionation Procedures**—DEAE-cellulose chromatography and Sephadex G-200 gel filtration were performed as described in detail previously (6). The exceptions were that the buffers for DEAE-cellulose chromatography contained 1 M urea and the NaCl gradients were linear.

**Analytical Procedures**—Antigenic analysis of grouper proteins, preparation of rabbit antisera, preparation of polypeptide chains by partial and extensive reduction, analytic ultracentrifugation, determination of extinction coefficients and hexose contents, and fingerprints of tryptic digests were all conducted as described in detail for similar studies on lemon shark immunoglobulins (6). Radioiodination of grouper proteins was performed by the chloramine-T method (14). Amino acid compositions were determined using an automatic amino acid analyzer (JLC-5AH Japan Optical Electronic Laboratories, Ltd., Tokyo) on acid hydrolysates, (6 N HCl, 105°, 20 hours), according to the method of Spackman, Stein, and Moore (15). Partial specific volumes (δ) were calculated from the amino acid composition (16). No corrections were made for losses during hydrolysis and a hexose to total carbohydrate (assumed carbohydrate δ = 0.61) ratio of 1:1.6 as for human IgM (10) was assumed for these calculations.

**RESULTS**

**Purification of Grouper Antibodies and Immunoglobulins**—Grouper 16 S and 6.4 S antibodies specific for the DNP determinant were purified by the dissolution of immune precipitates as described in the following report (13). In order to establish the
Physicochemical Properties—Grouper immunoglobulins prepared in the manner described above were assayed for purity by immunoelectrophoresis. Fig. 3 shows that each immunoglobulin was pure and exhibited a slow β mobility as seen with grouper antibodies (13). Also evident in this figure is the absence of cathodic moving proteins in grouper serum. The purified proteins were considered to be immunoglobulins by virtue of being antigenically identical to their specific antibody counterparts as shown in Fig. 4; also evident here is the antigenic deficiency of the 6.4 S immunoglobulin relative to the 16 S immunoglobulin as noted for specific antibodies (13).

Each of the grouper immunoglobulins was analyzed by sedimentation velocity and representative Schlieren patterns of these are shown in Fig. 5. Each immunoglobulin was judged to be homogeneous by this method. The $s_{20,w}$ of the 6.4 S immunoglobulin from a slight concentration dependence was taken to be 6.35 S (7.2 mg per ml = 5.7 S; 3.6 mg per ml = 6.05 S; 1.8 mg per ml = 6.3 S). The $s_{20,w}$ of the 16 S immunoglobulin exhibited considerable concentration dependence (10 mg per ml = 13.0 S; 5 mg per ml = 15.0 S; 2.5 mg per ml = 15.6 S). Therefore the $s_{20,w}$ of this latter protein was taken to be 16.1 S.

Grouper antibodies to DNP- and immunoglobulins were subjected to sedimentation equilibrium analysis. LnC versus $X^2$ plots of representative data (Fig. 6) indicate that the heterogeneity is minimal. Weight average molecular weights calculated from these data are presented in Table I. The partial specific volumes used in these calculations were determined from the amino acid compositions, i.e., 0.708 for the 16 S immunoglobulin and 0.716 for the 6.4 S immunoglobulin. There appeared to be no concentration dependence and thus the weights for each speed and protein concentration were averaged. The molecular weights of the grouper 16 S immunoglobulin and antibodies to DNP- each appear to be ~700,000 and of the 6.4 S immunoglobulin and anti-DNP- molecules to be ~120,000. The value of ~700,000 for the 16 S immunoglobulin was quite unexpected (see “Discussion”). Thus an experiment was performed wherein lemon shark 19 S immunoglobulin (mol wt ~900,000 (6)) and grouper 16 S immunoglobulin labeled with 125I were mixed and gel filtered on a column of Sepharose 4B. The elution profile of this column is presented in Fig. 7 and shows that the grouper 16 S immunoglobulin eluted after the lemon shark protein. This experiment supports the molecular weight data indicating the
Fig. 3. (left). Immunoelectrophoresis of grouper serum, immunoglobulins, and polypeptide chains. Rabbit antisera and grouper serum were used undiluted. The grouper protein concentrations were 2 to 3 mg per ml.

Fig. 4. (middle). Ouchterlony-type studies with purified grouper antibodies (Ab), immunoglobulins (Ig) and polypeptide chains (H). Rabbit antisera were used undiluted. Grouper proteins were used at 2 to 3 mg per ml.

Fig. 5. (right). Schlieren patterns of purified grouper immunoglobulins: A, partially reduced grouper 6.4 S immunoglobulin (3.5 mg per ml), $\theta_{20, w} = 6.1$ S; B, grouper 6.4 S immunoglobulin (5 mg per ml), $\theta_{20, w} = 5.8$ S; C, partially reduced grouper 16 S immunoglobulin (5 mg per ml), $\theta_{20, w} = 14.9$ S; D, grouper 16 S immunoglobulin (5 mg per ml), $\theta_{20, w} = 15.0$ S. The solvent employed was 0.14 M NaCl, 0.015 M Tris-HCl, pH 7.4.

Table I

Weight average molecular weights ($\times 10^{3}$) of grouper immunoglobulins and antibodies

<table>
<thead>
<tr>
<th>Protein</th>
<th>rpm</th>
<th>Concentration</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.25</td>
<td>0.50</td>
</tr>
<tr>
<td>16 S Ig</td>
<td>6,166</td>
<td>720</td>
<td>759</td>
</tr>
<tr>
<td></td>
<td>8,225</td>
<td>694</td>
<td>640</td>
</tr>
<tr>
<td>16 S Anti-DNP-</td>
<td>6,166</td>
<td>760</td>
<td>799</td>
</tr>
<tr>
<td></td>
<td>8,225</td>
<td>647</td>
<td>690</td>
</tr>
<tr>
<td>6.4 S Ig</td>
<td>13,410</td>
<td>123</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>16,200</td>
<td>120</td>
<td>116</td>
</tr>
<tr>
<td>6.4 S Anti-DNP-</td>
<td>13,410</td>
<td>122</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>16,200</td>
<td>121</td>
<td>108</td>
</tr>
</tbody>
</table>

grouper 16 S immunoglobulin to be smaller than the shark 19 S immunoglobulin.

Extinction coefficients and hexose contents of the two grouper immunoglobulins were measured and the results are given in Table II. The 16 S immunoglobulin has about three times more hexose than the 6.4 S molecule. It should be mentioned that these determinations were performed simultaneously with several control proteins (see Reference 17). The amino acid compositions of the grouper 16 S and 6.4 S immunoglobulins are given in Table III. The two proteins showed a remarkable degree of similarity with only slight differences in serine, glycine, and isoleucine content being evident.

Polypeptide Chain Structure—Each of the grouper immunoglobulins was extensively reduced and alkylated and then subjected to Sephadex G-200 gel filtration in the presence of 5.0 M guanidinium hydrochloride. The presence of $^{3}H$-labeled antibodies to DNP- in these immunoglobulin preparations permitted an evaluation of the elution volumes of antibody polypeptide chains. The elution profiles from two such columns are pre-
LEMON SHARK 19s lg +
E 04- 8
CJ f
I
50 60 70 60 90
FRACTION NUMBER
FIG. 7. Sepharose 4B gel filtration of a mixture of lemon shark 19 S immunoglobulin (mol wt ~900,000) and grouper 16 S immunoglobulin and 6.4 S immunoglobulin labeled with 151I.

**TABLE II**

Extinction coefficients in various solvents and hexose contents of purified grouper immunoglobulins

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Solvent</th>
<th>Extinction Coefficient $E_{280,1}$ on ± S.D.</th>
<th>Hexose %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grouper</td>
<td>0.3 m KCl</td>
<td>16.57 ± 0.19</td>
<td>1.1</td>
</tr>
<tr>
<td>6.4 S Ig</td>
<td>0.1 N NaOH</td>
<td>17.82 ± 0.18</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>6.0 m Guanidine hydrochloride</td>
<td>16.50 ± 0.31</td>
<td>1.1</td>
</tr>
<tr>
<td>Grouper</td>
<td>0.3 m KCl</td>
<td>13.78 ± 0.44</td>
<td>3.5</td>
</tr>
<tr>
<td>16 S Ig</td>
<td>0.1 N NaOH</td>
<td>15.03 ± 0.41</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>5.0 m Guanidine hydrochloride</td>
<td>13.53 ± 0.28</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**TABLE III**

Amino acid compositions of grouper 16 S and 6.4 S immunoglobulins

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>16 S Immunoglobulin</th>
<th>6.4 S Immunoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g per mg carbohydrate-free protein</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>5.1</td>
<td>4.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.6</td>
<td>4.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>14.2</td>
<td>13.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>11.0</td>
<td>11.1</td>
</tr>
<tr>
<td>Serine</td>
<td>7.3</td>
<td>8.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>12.1</td>
<td>12.0</td>
</tr>
<tr>
<td>Proline</td>
<td>5.8</td>
<td>5.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.4</td>
<td>6.4</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.7</td>
<td>4.1</td>
</tr>
<tr>
<td>Cysteine*</td>
<td>0.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Valine</td>
<td>7.2</td>
<td>6.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.0</td>
<td>7.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.6</td>
<td>5.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.6</td>
<td>4.4</td>
</tr>
</tbody>
</table>

* Based upon average of duplicate determinations.
\* Measured as cystine.

**Fig. 8.** Sephadex G-200 gel filtration in 5 m guanidine hydrochloride of extensively reduced and alkylated grouper immunoglobulins (—•—•) and antibodies to DNP. (○—○).

**Fig. 9.** Fingerprints of tryptic digests of grouper 16 S and 6.4 S H polypeptide chains. The left panels are photographs of the original chromatograms. The right panels are traced photographs showing spots visible to the eye on the originals. Designations employed are Lys, lysine marker for electrophoresis; red, phenol red marker for chromatography; y, yellow spots; B, brown spots; @ = spots unique to 6.4 S H chain; $\bigcirc$ = spots unique to 16 S H chain. The origin is in the lower right-hand portion of each pattern. Presented in Fig. 8; in each experiment ~95% of the applied material was recovered in the profiles presented here. The optical density (280 nm) elution profile of the 18 S immunoglobulin indicated that ~70% of the recovered material eluted in a volume corresponding to a molecular weight of ~70,000 (16 S H
chains). The remainder eluted in a volume indicative of molecular weight ~22,000 (L chains). The optical density profile of the 6.4 S Immunoglobulin showed 28% of the recovered material to elute in a volume corresponding to a molecular weight of ~40,000 (6.4 S H chains) with the remainder appearing as L chains. The radioactivity profiles of these columns indicated the antibody chains to have molecular weights indistinguishable from those of their immunoglobulin counterparts.

In view of the 2 H-2 L chain subunit structure that is probably ubiquitous among all immunoglobulins, efforts were made to study the subunit structure of the grouper immunoglobulins. Partial reduction (0.1 M dithioerythrom or cysteine) of the grouper 16 S and 6.4 S immunoglobulins followed by dialysis against neutral aqueous buffer did not alter their sedimentation characteristics as shown in Fig. 5. Subsequent gel filtration of such partially reduced proteins in 5 M guanidine hydrochloride (or 1 M propionic acid) resulted in the liberation of H and L chains. The failure of unreduced grouper immunoglobulins to dissociate in 5 M guanidine hydrochloride indicates that the partial reduction procedures employed had in fact broken some interchain disulfide bonds and therefore, the polypeptide chains were held together by both noncovalent and covalent interactions.

Antigenic Studies—As stated previously, partial reduction of grouper immunoglobulins followed by gel filtration in the presence of 5 M guanidine hydrochloride resulted in the liberation of H and L chains. Preliminary studies indicated these chains to be immunologically reactive although it was necessary to recycle the 6.4 S immunoglobulin chains to obtain pure preparations. Employing the technique of immunodiffusion in agar (Ouchterlony), rabbit antisera to the 16 S L chains and to whole grouper immunoglobulin showed 58% of the recovered material to elute in a volume corresponding to a molecular weight ~22,000 (L chains). Here again, assuming comparable extinction coefficients as for the 16 S immunoglobulin polypeptide chains, these appears to be equinolecular II and L chains, i.e. 2 H-2 L. The relationship of the grouper 6.4 S immunoglobulin to the grouper 16 S immunoglobulin appears to be that of a fragment wherein the H chain of the former is lacking a segment present on the latter. There are four experimental observations consistent with this hypothesis. (a) The antigenic data indicate that a portion of the 16 S H chain is not present on the 6.4 S H chain and (b) the molecular weight data indicate that the missing portion accounts for ~30,000 daltons. (c) The peptide maps indicate most of the 6.4 S H chain tryptic peptides to be present in the 16 S H chain; the one exception may represent the C-terminal peptide of the 6.4 S H chain. On the other hand, the 16 S H chain contains numerous unique peptides; these may account for the “missing” ~30,000 daltons. (d) The low hexose content of the 6.4 S immunoglobulin would be expected if this molecule represents an F(ab')2-like fragment of the 16 S molecule. Additional studies are required to establish the possible F(ab')2-like nature of the 6.4 S immunoglobulin and to establish any metabolic (anabolic versus catabolic) interrelationships between the grouper 16 S and 6.4 S immunoglobulins. In light of amino acid sequence studies which suggest that immunoglobulin polypeptide chains of other species are the products of several cistrons (for example see Reference 18), the finding of an animal synthesizing both intact and “partial” chains would certainly be exciting.

It would now seem appropriate to attempt to relate the grouper immunoglobulins to those of other species. First of all it should be stated that the most valid method to make such comparisons would entail the use of amino acid sequence data. This approach would be more apt to show that a phylogenetically conserved cistron (or cistrons) provided the genetic information for the synthesis of the polypeptide chains in question. In the absence of sequence data, any comparisons must be made on the basis of “grosser” molecular parameters, such as molecular weights of the whole molecule and its component polypeptide chains, carbohydrate content, and electrophoretic mobilities. Initial efforts in this direction with lower vertebrates focused on the 19 S and 7 S immunoglobulins of sharks (5-8). Such studies indicated that these two shark immunoglobulins belonged to the same immunoglobulin class and that this class closely resembled IgM as defined for higher vertebrates. On the other hand, amphibians, such as the bullfrog (11), had 19 S and 7 S immunoglobulins which were considered to be phylogenetic counterparts of IgM and IgG respectively. Somewhat “intermediate” between these two classes of vertebrates is the lungfish, a Dipnoid fish related to the Crossopterygii, i.e. an “uncle” of the tetrapods (19). The lungfish has recently been shown (20) to possess a 19 S immunoglobulin which, on the basis of its H chains, resembles IgM; molecular weights of the intact molecule were not obtained. The lungfish 5.9 S immunoglobulin appears to belong

**DISCUSSION**

The data presented here indicate that the grouper 16 S immunoglobulin has a molecular weight of ~700,000, has a relatively high hexose content, and is composed of disulfide linked II (mol wt ~70,000) and L (mol wt ~22,000) polypeptide chains. The polypeptide chain yields from extensively reduced material (based upon optical density readings) indicate there are probably equinomolar amounts of H and L chains in the intact 16 S molecule, i.e. 8 H and 8 L chains. The one disconcerting aspect of the studies on this molecule has been the unexpected failure to obtain reductive subunits; if, however, one assumes that the 2 H-2 L chain subunit is ubiquitous among all immunoglobulins, then the grouper 16 S molecule may be a tetramer of subunits held together by both covalent and noncovalent bonds.

The grouper 6.4 S immunoglobulin was found to have a molecular weight of ~120,000, a low carbohydrate content, and a composition of disulfide-linked H (mol wt ~40,000) and L (mol wt ~22,000) chains. Here again, assuming comparable extinction coefficients as for the 16 S immunoglobulin polypeptide chains, these appears to be equinmolecular II and L chains, i.e. 2 H-2 L. The relationship of the grouper 6.4 S immunoglobulin to the grouper 16 S immunoglobulin appears to be that of a fragment wherein the H chain of the former is lacking a segment present on the latter. There are four experimental observations consistent with this hypothesis. (a) The antigenic data indicate that a portion of the 16 S H chain is not present on the 6.4 S H chain and (b) the molecular weight data indicate that the missing portion accounts for ~30,000 daltons. (c) The peptide maps indicate most of the 6.4 S H chain tryptic peptides to be present in the 16 S H chain; the one exception may represent the C-terminal peptide of the 6.4 S H chain. On the other hand, the 16 S H chain contains numerous unique peptides; these may account for the “missing” ~30,000 daltons. (d) The low hexose content of the 6.4 S immunoglobulin would be expected if this molecule represents an F(ab')2-like fragment of the 16 S molecule. Additional studies are required to establish the possible F(ab')2-like nature of the 6.4 S immunoglobulin and to establish any metabolic (anabolic versus catabolic) interrelationships between the grouper 16 S and 6.4 S immunoglobulins. In light of amino acid sequence studies which suggest that immunoglobulin polypeptide chains of other species are the products of several cistrons (for example see Reference 18), the finding of an animal synthesizing both intact and “partial” chains would certainly be exciting.

It would now seem appropriate to attempt to relate the grouper immunoglobulins to those of other species. First of all it should be stated that the most valid method to make such comparisons would entail the use of amino acid sequence data. This approach would be more apt to show that a phylogenetically conserved cistron (or cistrons) provided the genetic information for the synthesis of the polypeptide chains in question. In the absence of sequence data, any comparisons must be made on the basis of “grosser” molecular parameters, such as molecular weights of the whole molecule and its component polypeptide chains, carbohydrate content, and electrophoretic mobilities. Initial efforts in this direction with lower vertebrates focused on the 19 S and 7 S immunoglobulins of sharks (5-8). Such studies indicated that these two shark immunoglobulins belonged to the same immunoglobulin class and that this class closely resembled IgM as defined for higher vertebrates. On the other hand, amphibians, such as the bullfrog (11), had 19 S and 7 S immunoglobulins which were considered to be phylogenetic counterparts of IgM and IgG respectively. Somewhat “intermediate” between these two classes of vertebrates is the lungfish, a Dipnoid fish related to the Crossopterygii, i.e. an “uncle” of the tetrapods (19). The lungfish has recently been shown (20) to possess a 19 S immunoglobulin which, on the basis of its H chains, resembles IgM; molecular weights of the intact molecule were not obtained. The lungfish 5.9 S immunoglobulin appears to belong
to a different immunoglobulin class than the 19 S molecule, but because it has an H chain with a molecular weight of only ~40,000, does not seem to have a readily identifiable mammalian counterpart (20); its relationship, if any, to a similar sized immunoglobulin in the duck (21) remains obscure.

When attempting to relate the immunoglobulins of a member of the class Osteichthyes to these other lower vertebrates, the one “certainty” that would have been based on the expected presence of a 19 S IgM (mol wt ~900,000); this was not found in the study reported here. The grouper 16 S immunoglobulin had a molecular weight of only ~700,000. Thus, with this protein, the only comparative statements must rely solely upon polypeptide chain properties. The 16 S H chain molecular weight (~70,000) and the relatively high hexose content are compatible with an IgM designation for the grouper 16 S immunoglobulin with the implication, as mentioned above, that based upon the molecular weight of the whole molecule it may be a tetramer of subunits instead of the “typical” pentameric 19 S form of IgM. In defense of this tenuous immunoglobulin class designation, it should be stated that molecular mass cannot be the only criterion for making such comparisons as evidenced by the presence of 7 S IgM in newborn (22, 23) and pathological (24) human sera and making such comparisons as evidenced by the presence of 7 S IgM in pathological human sera and making such comparisons as evidenced by the presence of 7 S IgM in pathological human sera. However, in light of statements in the literature that sharks do not resemble the p chain and thus the 14 S and 7 S molecules were considered to be IgM (4). Preliminary reports suggest that the molecular weight of the lamprey macroglobulin is considerably less than 900,000 (30), and thereby strongly indicates that the phylogenetically ancient version of what is probably IgM could not be a pentamer of 2 H-2 L chain subunits.

One additional interesting aspect of the lamprey immunoglobulin is the relative lack of interchain disulfide bond in favor of noncovalent interactions. The observed failure of partially reduced grouper (reported here) and paddlefish (29) immunoglobulins to dissociate in neutral aqueous buffers, suggests these intersubunit noncovalent interactions are still important at the level of the Osteichthyes. Recent data showing that intracellular mouse IgM exists only in the 7 S form and that polymerization occurs during release from the synthesizing cell (31), is consistent with the hypothesis that initial efforts (phylogenetically speaking) at polymerization may have resulted in the assembly of other than pentameric forms. The presence of strong noncovalent intersubunit bonds would be expected to help stabilize this hypothetical primitive molecule.

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REFERENCES


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