Hemagglutinating 7S Subunits of 19S Cold Agglutinins

Abstract. *Two highly purified IgM cold agglutinins have been mildly reduced yielding 7S subunits, with interchain covalent bonds intact. These subunits retained most of the cold-agglutinin activity as well as the specificity of the parent antibodies. However, as might be anticipated from theories of the importance of antibody size and number of subunits for complement binding, the IgM subunits were only very weakly lytic compared with the intact cold agglutinins. The findings are consistent with the presence of ten antibody-combining sites on the IgM molecule.*

A presumptive test for IgM (immunoglobulin M) macroglobulins is their sensitivity to cleavage by sulfhydryl reducing reagents, such as mercaptoethanol and cysteine (*1*). These reagents split these macroglobulins into five 7*S* subunits, each consisting of two heavy and two light chains held together by noncovalent bonds (*2*). Such 7*S* subunits prepared from 19*S* antibodies by conventional methods lack the ability to agglutinate (*3–5*), to precipitate (*6*), and to bind complement (*7*). Miller and Metzger (*8*) have described a method for preparing 7*S* subunits from a Waldenström macroglobulin which retained intact covalent bonds between the heavy and light chains. I now describe the preparation of hemagglutinating 7*S* subunits from two highly purified IgM human cold agglutins.

Serum was obtained from two patients (H.D. and A.C.) with high-titer cold-hemagglutinin disease. The cold agglutinins were purified by adsorption and elution from erythrocyte stroma. The eluates were concentrated by vacuum dialysis and subjected to Sephadex G-166 filtration (*9*) at 4°C with *p*H 8.2 tris-buffered saline for elution. A single sharp peak in the macroglobulin region was obtained in each case. The protein fractions were concentrated and shown to contain only type kappa IgM macroglobulins by immunoelectrophoretic and Ouchterloney criteria when the usual variety of antihuman serums were used. The purified antibodies retained potent hemagglutinin and hemolysin activities. The purified antibodies were dialyzed against 0.2*M* tris buffer (*p*H 8.6), reduced with 0.02*M* cysteine at room temperature for 18 to 20 hours, and alkylated for 1 hour with a 10-percent excess of iodoacetamide. After dialysis against *p*H 8.2 tris-buffered saline, the reduced and alkylated antibodies were filtered through Sephadex G-166 at 4°C. For each preparation, two peaks appeared at similar positions. The first peaks appeared in the same position as the unreduced purified 19*S* antibodies and behaved in all respects as intact cold agglutinins. The second peaks appeared in the position of purified IgG (immunoglobulin G). In the case of one of the antibodies (A.C.), 50 percent of the eluted protein was present in each peak (Fig. 1), whereas with the other antibody (H.D.) 30 percent was in the 19*S* peak and 70 percent in the 7*S* peak. Immunoelectrophoresis with an antiserum to IgM showed in each case that the material in the second peak had a longer precipitin line which extended further toward the anode and diffused further toward the antiserum trough than the purified 19*S* antibody. Similarly, double-gradient immunodiffusion with an antiserum to IgM in a vertical trough and equal concentrations of the two antigens in separate horizontal troughs demonstrated that the material from the second peaks diffused much faster than the parent 19*S* antibodies, an indication of their much smaller size.

Analytic ultracentrifugation (Spinco Model E centrifuge) was performed with purified normal IgG (7 mg/ml) in one cell and material (7 mg/ml) from the second peak of antibody (H.D.) in the second cell. Photographs, taken at 8-minute intervals after the maximum speed of 59,780 rev/min was reached, revealed that most of the material in the second cell always traveled in a peak slightly in advance of the IgG peak (Fig. 2). This is consistent with the greater molecular weight of the IgM subunits (185,000) compared with that of IgG (160,000) (*2*). There was a very small shoulder on the slow side of the 7*S* peak, and there was also a very small amount of faster-sedimenting material which could represent unreduced cold agglutinin or reaggregated 7*S* subunits. Although serologic tests were performed within 2 days of Sephadex filtration, there was a delay of over a week before ultracentrifugation was performed, and a slight amount of reaggregation could have occurred during this time (*8*).

A sample of the 7*S* subunits (H.D.) was dialyzed against 1*N* acetic acid and filtered through Sephadex G-100 equilibrated with 1*N* acetic acid. A single sharp peak was obtained and indicated that the covalent bonds between heavy and light chains were intact.

Table 1 demonstrates the results of serologic tests on the intact 19*S* antibodies and identical concentrations of their 7*S* subunits. In each case, the 7*S* subunits with intact interchain bonds agglutinated normal adult erythrocytes in saline almost as well the intact 19*S* antibodies did. Furthermore, the 7*S* subunits retained their cold-agglutinin nature and also their anti-I specificity (*10*) as demonstrated by their higher titer against adult blood cells as compared with cord blood cells (not shown in Table 1). In order to preclude the possibility that the strong agglutination given by the 7*S* fraction was due to trace contamination by 19*S* antibody, the reduction and alkylation of one cold agglutinin (A.C.) was repeated and this again gave two peaks virtually identical to that shown in Fig. 1. However, instead of combining the tubes under each peak, every other tube was tested individually for cold agglutinin activity against normal adult erythrocytes. A biphasic pattern resulted with peaks

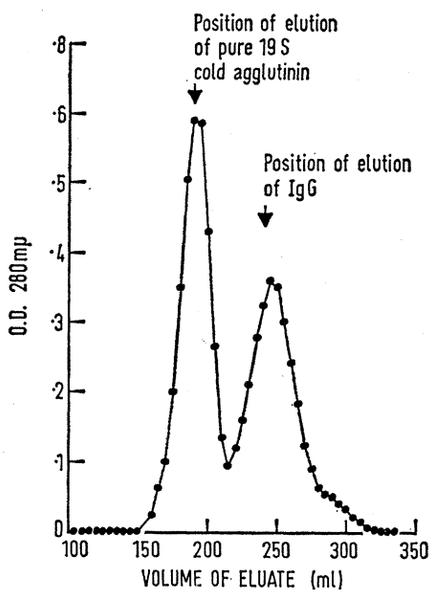

Fig. 1. Elution pattern of cold agglutinin (A.C.) from Sephadex G-166 after reduction with 0.02*M* cysteine for 20 hours followed by alkylation with iodoacetamide. First peak represents unreacted 19*S* cold agglutinin, and second peak represents 7*S* subunits with intact interchain bonds; 0.05*M* tris-HCl–buffered saline, *p*H 8.2. O.D., optical density.

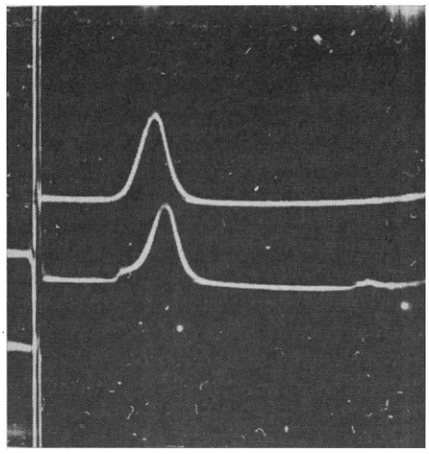

Fig. 2. Analytic ultracentrifugation comparing 7*S* subunits (H.D.) below with 6.6*S* IgG above. There is a small amount of unreduced or reaggregated fast material and also a small amount of slow material. No further peaks appeared. Direction of migration is to the right. Spinco Model E ultracentrifuge, speed 59,780 rev/min; 0.05*M* tris–HCl-buffered saline, *p*H 8.2; photograph taken at 32 minutes.

of agglutinin activity corresponding to the two peaked protein distribution. The tube corresponding to the trough between the two peaks had very low agglutinin activity. This proves that the agglutinin activity in the 7*S* peak was not due to trace amounts of 19*S* antibody carried over from the first peak.

In contrast to their agglutinating activity, the 7*S* subunits retained only a small fraction of the ability of the 19*S* antibodies to lyse enzyme-treated cells or to bind complement. Complement binding was measured by incubating the antibody, serially diluted in fresh normal human serum, *p*H 6.5, with a constant amount of normal human erythrocytes. After incubation for 1 hour at 20°C, the cells were washed with saline and then tested for the presence of bound complement with a rabbit antiserum capable of reacting with the third or fourth components of complement (C'_3 or C'_4). The titer reported is the reciprocal of the highest dilution of the antibody preparation which was capable of binding complement. The lysin- and the complement-binding titers of the 7*S* subunits were approximately 1/150 of those of the 19*S* antibodies on a weight basis and approximately 1/750 on a "per molecule" basis, if we assume a molecular weight of 900,000 for the 19*S* antibodies and 185,000 for the 7*S* subunits (*2*). The 7*S* subunits which had been further reduced and alkylated to cause cleavage of the interchain bonds retained some agglutinin activity, but no longer bound complement.

Evidence has been presented for the preparation of 7*S* subunits of two highly purified IgM cold agglutinins which appear to have retained their interchain covalent bonds. These are believed to be the first IgM subunits shown to have unequivocal hemagglutinating ability. Previously tested 7*S* subunits of IgM antibodies, including cold agglutinins (*3*), almost certainly were held together by noncovalent bonds. It would appear that the 7*S* subunits of IgM antibodies require intact interchain covalent bonds for maximum hemagglutinating activity. It is possible that IgG antibodies have stronger interchain noncovalent bonds than do the 7*S* subunits of IgM antibodies since the activity of the former is unchanged even after cleavage of the interchain bonds. In this respect, monomeric IgA antibodies are more akin to the 7*S* subunits of IgM since their agglutinating activity also appears to depend on the presence of intact interchain disulfide bonds (*11*). Some previously studied 7*S* subunits of IgM antibodies have been shown to bind to antigen (*5*, *6*, *12*) and have been thought to be univalent (*13*). However, the evidence presented here is most compatible with the theory that the IgM subunits are potentially bivalent as well as complete antibodies. This implies that the parent IgM molecule has ten potential combining sites. The finding that the 7*S* subunits are cold agglutinins like their parent antibodies indicates that the greater affinity for antigen in the cold is a property of the antibody-combining sites of the individual subunits rather than a special property of the macroglobulin molecule.

In general, IgM antibodies are much more potent binders of complement than IgG antibodies are. The current theory holds that, whereas one IgM molecule suffices for the initiation of complement binding, two adjacent IgG molecules are required (*14*, *15*). In a system where numerous antigenic sites are available, numerous IgG molecules would be required in order to occupy two adjacent sites. The superior complement-binding ability of the IgM antibodies is believed to depend on the presence of multiple subunits in the vicinity of each bound antigenic site (*16*). The preparation of serologically active subunits of complement binding IgM antibodies offers a unique opportunity to test this hypothesis, especially since the parent antibody and the subunits would be directed against the same antigenic site. The finding of a dissociation between agglutinating and complement-binding ability of the 7*S* subunits is supporting evidence in favor of the current hypothesis of the importance of antibody size and number of subunits present at each antigenic site. The weak lytic and complement binding ability shown by the 7*S* preparation is due to the presence of sufficient 7*S* subunits in the early tubes of the titrations to allow occasional molecules to be attached to adjacent antigenic sites. However, it is not possible to rule out a very small amount of contaminant or reaggregated 19*S* antibody sufficient to give the weak

Table 1. Serologic properties of purified 19*S* cold agglutinins and their 7*S* subunits. The numbers given are the reciprocals of the highest dilutions giving a positive result. The concentrations of 19*S* cold agglutinin and its subunits in the first tubes were 2.0 mg per milliliter for A.C. and 1.2 mg per milliliter for H.D. Agglutinin titers were performed in saline with normal adult erythrocytes. Lysin titers were performed in fresh acidified normal serum *p*H 6.5 with papain-treated normal adult erythrocytes. Indirect Coombs tests were performed as described in the text.

Preparation	Agglutinin titers at			Lysin titers (20°C)	Indirect Coombs titers (20°C)
	4°C	20°C	37°C		
Patient A.C.					
19*S* cold agglutinin	32,000	512	0	320	640
7*S* subunits (intact interchain bonds)	16,000	256	0	2	4
7*S* subunits (reduced and alkylated interchain bonds)	128	16	0	0	0
Patient H.D.					
19*S* cold agglutinin	4,000	1,000	0	320	250
7*S* subunits (intact interchain bonds)	2,000	512	0	2	16
7*S* subunits (reduced and alkylated interchain bonds)	256	32	0	0	0

lysis and complement binding. The complement-fixation and transfer test (*15*) should help to quantitate the complement ability of the 7*S* subunits.

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17. I thank Prof. J. V. Dacie for advice and encouragement, Mrs. J. Farnden and Miss R. Schein for their technical assistance, Dr. D. Robertson Smith for providing the serum from patient H.D., B. Brackenbury for the photography, and Dr. J. W. Prahl for suggestions and for performing the ultracentrifugation.

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Enhanced Protein Synthesis in a Cell-Free System from Hypertrophied Skeletal Muscle

Abstract. *Hypertrophy of the rat soleus muscle was induced by tenotomy of the synergistic muscles. Four days after the operation, the weight of the muscle had increased by 30 percent. The hypertrophied muscle had an increased concentration of DNA and RNA, when compared to the contralateral control soleus. Although the amount of myofibrillar protein increased during the 4-day period, the concentration of this component decreased. Microsomes prepared from hypertrophied muscle had an increased RNA concentration. The combined microsomes and pH-5 enzyme from hypertrophied muscle supported a faster rate of protein synthesis in vitro than the same system prepared from an equal weight of contralateral muscle which was used as a control.*

Hypertrophy of skeletal muscle in response to exercise indicates that protein metabolism in muscle is regulated by function. The hypertrophied heart in vivo (*1*) and the overloaded, perfused heart *in situ* (*2*) exhibit increased amino acid incorporation into protein. Kendrick-Jones and Perry (*3*) have reported an increased incorporation of amino acid into protein of an isolated frog muscle in response to repeated isometric contractions of the muscle. However, increased amino acid-incorporating activity has not been demonstrated in a cell-free system. Such a system should presumably be more amenable to biochemical studies.

We now describe initial studies of protein and nucleic acid metabolism in red skeletal muscle undergoing rapid compensatory hypertrophy induced by tenotomy of the synergistic muscles (see *4*).

We have compared some chemical characteristics of hypertrophied and normal muscle and shown that a cell-free system from hypertrophied muscle is much more active than the system from normal muscle in supporting incorporation of amino acid into protein.

The left gastrocnemius and plantaris tendons of male, Sprague-Dawley rats (250 to 300 g) were severed after intraperitoneal injection of chloral hydrate (35 mg per 100 g of body weight). A sham operation was performed on the contralateral limb. Four days after operation, the animals were killed by decapitation. The soleus muscle from each leg was dissected free. All procedures including killing of the animals were carried out at 4°C, unless otherwise stated. Muscles used for nitrogen and nucleic acid analysis were immediately frozen and pulverized in liquid nitrogen and then stored at −70°C until analyzed.

Paired groups of control and hypertrophied muscles were pooled to give sufficient quantities for analysis. Since histological examination of the muscles revealed a localized cellular infiltrate at the operative site in the hypertrophied muscles, RNA and DNA concentrations were also determined on the histologically normal, proximal segment of the hypertrophied muscles. Tissue nucleic acid was determined (*5*) after lipid extraction (*6*). The RNA determination was corrected for glucose (*7*). Dry weights were determined after lyophilization to constant weight.

Incorporation of labeled amino acid into protein by cell-free extracts was measured (*8*). After the incubation, protein was purified (*9*), and the radioactivity of the solubilized protein was determined (*10*) in a Packard Tri-Carb liquid-scintillation spectrometer. Protein (*11*) and RNA (*12*) content of the microsomes were determined (*11*). For the reason noted above, the concentrations of microsomal protein and RNA and the rates of the incorporation of amino acid in vitro were determined on fractions obtained from the whole muscle and from the proximal segment in the hypertrophied muscles. That the amino acids were incorporated into interior peptide chains was confirmed by dinitrophenylation (*8*). Free amino acids present after dinitrophenylation were determined by the method of Yemm and Cocking (*13*). The amounts of amino acid in whole muscle and in the subcellular fractions were also determined (*14*).

Four days after tenotomy the mean wet weight for solei from the tenotomized leg was 151 mg compared to 116 mg for solei from the control leg (Table 1).

The tissue nitrogen was fractionated into myofibrillar, sarcoplasmic, and stromal-protein nitrogen and nonprotein nitrogen on the basis of differential solubilities (*15, 16*). The nitrogen contents, determined by the micro-Kjeldahl method, of these fractions in five sets of paired hypertrophied and control solei (Table 1) show the concentration of nonprotein, sarcoplasmic, and stromal-protein nitrogen to be similar in both groups. The total tissue nitrogen and myofibrillar nitrogen concentration are both significantly lower in the hypertrophied muscle. From Table 1, it can be calculated that a hypertrophied muscle contains 2.32 mg of myofibrillar protein nitrogen per mus-