phosphate, *p*H 7.3, it does not reform any heavier units as acid-dissociated 22*S* protein does (*15*). Moreover, the immunochemical experiment indicates that cross-reactivity with antiserum against 22*S* protein resides in the fast-sedimenting fraction rather than in the slow-sedimenting fraction. Finally, a major part of the slow-sedimenting fraction is precipitable by calcium at neutrality, whereas the 22*S* protein, as well as the fast-sedimenting peak in the acid extract, are soluble under these conditions.

Because precipitability by calcium is a characteristic both of microtubule protein of cilia and flagella (*13* and above) and of the protein obtained by Sakai from mitotic apparatus (*4*) and proposed by Kiefer *et al.* to be the microtubule protein (*6*), we have determined the solubility of the acid extract after neutralization and addition of 0.01*M* calcium chloride in various buffers. Under these conditions 50 to 75 percent of the extract (4 to 6 percent of the original mitotic apparatus) precipitates rapidly. Sucrose gradient analysis of the calcium-soluble fraction and of the insoluble fraction after redissolution in acid shows that the calcium-insoluble fraction is derived solely from the slow-sedimenting material, whereas the rapidly sedimenting material is soluble in calcium.

Preliminary sedimentation analysis of the calcium precipitate from mitotic apparatus (dissolved in acid) suggests that this material comes from both the 4*S* material of the acid extract and the material that remains near the meniscus. Similarly, the acid extract of outer doublet microtubules of sperm flagella shows two components on analytical ultracentrifugation while still in acid. One has a sedimentation coefficient of 4.6*S*, and one sediments much more slowly. Both components are insoluble in calcium, and our experiments with sequential acid and mercuhydrin extraction indicate that both components are forms of the microtubule protein. Therefore, the sedimentation profiles in acid of the calcium-precipitable protein from mitotic apparatus and the microtubule protein from sperm tail are comparable.

Our preliminary data indicate that in both cases the relative amounts of the two components depend on the amount of calcium ion present in the acid. Thus, the addition of ethylenediaminetetraacetic acid to the acid in which the calcium precipitate is redissolved causes the material to appear almost exclusively as the lighter component, whereas addition of calcium in concentrations as low as $10^{-4}M$ to the acid used to extract the sperm tail microtubules causes the lighter component to be absent.

The data suggest that the calcium-precipitable protein from mitotic apparatus is the microtubule protein and has properties similar to those of the protein of outer doublet microtubules from sperm flagella. Extraction with HCl at *p*H 3 dissolves the microtubule protein from sperm tail; it causes the disappearance of the microtubules from isolated mitotic apparatus. The major component of the mitotic apparatus extract resembles the sperm tail microtubule protein in its precipitation with calcium and, approximately, in its sedimentation pattern in acid.

The calcium-precipitable protein also has points of resemblance to proteins previously extracted from mitotic apparatus. The protein obtained by Sakai (*4*) is also insoluble in calcium. It has been recovered in two different forms, either with a sedimentation coefficient of 3.5*S*, or as a material that remains near the meniscus (*3*, *4*). We have not yet studied the sedimentation of this protein under the acid conditions used in our work. Sakai's protein constitutes a much higher percentage of mitotic apparatus than ours does (*6*), but it was obtained from mitotic apparatus isolated by a different method, so this does not exclude identity of the proteins.

The extracts of mitotic apparatus isolated in hexylene glycol obtained (*5*, *15*) in 0.6*M* potassium chloride show as a minor component a heterogeneous material with sedimentation coefficient of approximately 4*S*. This material constitutes about the same percentage of mitotic apparatus as our calcium-precipitate does (4 to 6 percent). Stephens (*12*) has recently reported that this protein has an amino acid composition very similar to that of actin, as does microtubule protein from sperm flagella. Finally, Borisy and Taylor (*7*) have calculated that 5 to 15 percent of mitotic apparatus isolated by the hexylene glycol method is a protein that binds colchicine and is proposed to be the microtubule protein, a percentage which agrees fairly well with the percentage of calcium-precipitated protein which we recover.

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Antigenic Specificities Related to the Cold Agglutinin Activity of Gamma M Globulins

Abstract. *Certain antiserums prepared against isolated cold agglutinins demonstrate specificity for gamma M globulins with this activity and not for similar gamma M globulins lacking such activity. The cold agglutinin specific antigens fall into several major and minor groups. Their exact relation to the presumed antibody-combining sites remains to be determined.*

The delineation of a wide variety of antigenic determinants among the immunoglobulins has aided greatly in the classification of these proteins with respect to major subdivisions of both the heavy and light chains (*1–3*). Chemical studies, particularly those involving sequence analyses, have brought forward the structural basis for many such differences (*4*). However, no antigenic determinant which relates to the antibody specificity of the immunoglobulins has been observed. Part of the difficulty stems from the very apparent heterogeneity of most antibodies, but even with certain very homogeneous antibodies no such relationship has been apparent. Instead, each antibody shows "individual antigenic specificity" (*5*). Studies with antibodies to A substance in humans (*5*, *6*) and with antibodies to proteus in rabbits (*7*) have revealed only "individual antigenic specificity" with no cross specificity among these

antibodies which would relate to their common antibody-combining properties.

A number of observations have indicated that cold agglutinins might be especially suitable for studying the relation between antibody specificity and antigenic properties, including chemical structure. (i) In a variety of properties they show a homogeneity close to that of myeloma proteins or Waldenstrom macroglobulins and with red-cell specificities involving the I antigens that appear typical of antibodies of the gamma M class. (ii) The vast majority have kappa-type light chains (*8*), suggesting a possible relationship between light chain type and their agglutinin activity.

In our study antiserums were produced against isolated cold agglutinins, which after absorption showed the expected strong specificity for the immunizing antigen. However, in addition, most of these antiserums also reacted with heterologous cold agglutinins but not with other macroglobulins that lacked the cold agglutinin property.

The cold agglutinins were primarily obtained from patients with idiopathic cold agglutinin disease. Each serum showed marked elevation of γM with a sharp macroglobulin band visible on electrophoresis. Antiserums against isolated cold agglutinins were produced by immunizing rabbits either with eluates from red-cell stroma or isolated macroglobulin fractions. All preparations were mixed with equal volumes of complete Freund's adjuvant before injection. Antiserums were usually absorbed with 0.3 ml of normal human serum per milliliter and 3 mg of a pool of three Waldenstrom macroglobulins. Such absorbed antiserums were tested in Ouchterlony analysis with a panel of cold agglutinins isolated as eluates from human red-cell stroma and, in a separate set of experiments, separated as serologically active peaks of cold agglutinin after zone electrophoresis (*3*). A number of other cold agglutinins were further purified by separation on Sephadex G-200 columns or by density-gradient ultracentrifugation. For comparison, 50 isolated Waldenstrom's γM proteins, as well as a variety of human serums showing polyclonal γM components, were also studied. Concentrated preparations of normal human γM were obtained from a pool of 50 normal serums by the procedure of Chaplin *et al.* (*9*).

Ten antiserums made against different isolated cold agglutinins were used. In the unabsorbed state or after absorption with pooled γ-globulin, all exhibited strong specificity for macroglobulin determinants. After absorption with normal serum and a mixture of Waldenstrom macroglobulins without cold agglutinin activity, all reactivity for Waldenstrom macroglobulins and concentrated normal macroglobulins in agar plate analysis was lost. However, reactivity for a number of isolated cold agglutinins was retained by some of these antiserums. Figure 1 illustrates the strong precipitin lines obtained for six different isolated cold agglutinins with absorbed antiserum to cold agglutinin B.M. and the absence of lines with six Waldenstrom macroglobulins at the same concentration.

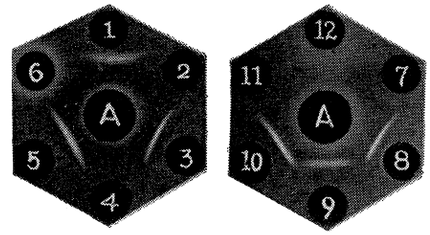

Fig. 1. Precipitin lines obtained with six different isolated cold agglutinin macroglobulins and their absence with six macroglobulins lacking this property. Wells 1, 3, 5, 8, 9, and 10 contained cold agglutinins Low., Hak., Sch., Nys., Ber., and Gre., respectively. The central wells (*A*) contained absorbed antiserum to cold agglutinin B.M.

Table 1 summarizes the results with 20 cold agglutinins. Absorbed antiserum B.M. showed reactivity with the majority of cold agglutinins but not with any macroglobulin that lacked the cold agglutinin property. Isolation of cold agglutinins by three different procedures gave identical results. Antiserum Hal. (Table 1) as well as three others showed a very similar pattern of reactivity. With each antiserum, spurs were often clearly visible between different cold agglutinins. The gradations in Table 1 are based on these spurs and the results of experiments involving absorption with certain cold agglutinins. Antiserum Lon. (Table 1) showed a different pattern of reactivity. Only three cold agglutinins failed to show a line with at least one antiserum. Several major groups of antigens were involved along with a number of subsidiary types. Four antiserums failed to bring out any of these specificities.

Table 1. Comparison of the reactivity of 20 cold agglutinins with three absorbed antiserums to cold agglutinins.

Cold aggl.	Antiserums		
	B.M.	Hal.	Lon.
Hak.	+++	++++	0
Nys.	++++	++	0
Low.	++++	++	+
Sch.	+++	+	0
Gre.	++++	+++	0
Too.	+++	++	0
Ber.	+++	++	0
Baa.	+++	++	0
B.M.	+++++*	+++	0
Sau.	+	+	+
Grb.	+	+	0
Myr.	++	++++	0
Joh.	0	0	+++
Kof.	0	0	++
Nor.	0	0	++
Her.	0	0	++
Lon.	0	0	+++++*
Kro.	0	0	0
Gra.	0	0	0
Mar.	0	0	0

* Individual specificity.

No reactivity with these absorbed antiserums was obtained with any of 50 monoclonal macroglobulins devoid of cold agglutinin activity. Several of these had other serologic activities. Concentrated preparations of macroglobulins isolated from normal serums failed to show direct precipitation, but evidence for slight inhibition of the cold agglutinin precipitin reaction was obtained. The amount of normal macroglobulin necessary to produce an effect was more than 100 times the amount of isolated cold agglutinin.

Evidence was obtained that not all of the macroglobulin molecules from the macroglobulin bands in the cold agglutinin serums reacted with the specific antiserums described above. Cold agglutinin eluates contained a considerably higher proportion of reactive molecules. A parallelism was noted between a fall in cold agglutinin titer in stored serums and a decrease in the amount of γM which showed the specific reactivity.

Efforts at localizing the cold agglutinin antigens to specific areas of the macroglobulin molecule were only partially successful. Isolated cold agglutinin light chains had no activity even in inhibition systems. In addition, cold agglutinin Sch., which contained only lambda light chains gave a similar reaction to the other cold agglutinins which contained only kappa chains. Precipitating ability was lost when the cold agglutinin was reduced with mercaptoethanol or cysteine. Inhibiting activity remained, and this was found in low molecular weight fractions. Isolated

heavy chains were only slightly active; isolated FAB fragments produced by papain were more active.

Seven of the cold agglutinins which reacted with antiserum B.M. (Table 1) showed both I and i red-cell specificity. Four showed only I specificity and one (Myr.) showed primarily i specificity. Four showed an additional reactivity with rabbit red cells at 37°C. The cold agglutinins isolated from serums of patients with atypical pneumonia did not react with the antiserum B.M.

Efforts were made to study the effect of combination with red cells on the specific antigenic determinants. Two isolated cold agglutinins when attached to red cells were considerably less effective in absorbing out the specific reactivity of antiserum to B.M. than an equivalent amount of free cold agglutinin.

These studies indicate that there are a group of antigens characteristic of macroglobulins with cold agglutinin activity. The term "cross specificity" has been applied to this antigenic similarity, distinguishing it from the "individual antigenic specificity," which is readily observed with cold agglutinins as well as with some antibodies produced by direct immunization (*5*). Previous studies on various other antibodies (*5*, *7*) failed to reveal cross specificity of the type observed for the cold agglutinins. However, these antibodies lacked the homogeneity of the cold agglutinins.

The major question raised by the above observations is whether the specific antigens characterizing cold agglutinins relate directly to the presumed antibody-combining site of these proteins. A complete answer is not yet available, and the main evidence depends simply on direct correlation. The fact that these antigens are at least partially blocked by combination of the cold agglutinin with its antigen favors such a concept. However, no clear relation was apparent between these antigens of the cold agglutinins and the exact specificity of the cold agglutinins for red cells of different types.

Alternatively the γM cold agglutinins might belong to a minor subgroup of the macroglobulins which includes antibodies of other specificities. There is precedence for such a concept in the case of antibodies to dextran where the vast majority belong to the minor γG2 subgroup (*10*). Against this view is the finding of an assortment of different specific antigens in cold agglutinins, whereas subgroup antigens are usually extremely similar from one protein to another. A third possibility is that some unusual antigenic material is linked to these macroglobulins that is involved in their cold agglutinin property. Studies on another group of homogeneous antibodies such as the antibodies to γ-globulins should help decide between these alternatives. Regardless of the outcome of further studies, the determination of the chemical basis for the various antigens should be feasible and should prove of considerable interest.

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Hemoglobin Variant Common to Chinese and North American Indians: $\alpha_2\beta_2^{22\ Glu\rightarrow Ala}$

Abstract. *An electrophoretically slow hemoglobin variant, in which the structural change involves the replacement of a glutamyl residue by alanyl at position beta-22, was reported in two groups of North American Indians: hemoglobin-G Coushatta, in Alabama-Coushatta Indians in Texas; and hemoglobin-G Saskatoon, in descendants of Santee Indians living in Canada. Hemoglobin-G Hsin-Chu, found in Taiwan in a Chinese from the northern Chinese province of Liaoning, is now shown to have the same structural anomaly.*

An electrophoretically slow variant typical of G-type hemoglobins was found in Taiwan in 1964 during a survey of presumably normal Chinese (*1*); one Chinese male had the G variant and normal hemoglobin-A in approximately equal amounts. Study of the structure of the hemoglobin, which was named G Hsin-Chu, now shows that the structural anomaly occurs at the beta-22 position where the glutamyl residue is replaced by an alanyl residue: $\alpha_2\beta_2^{22\ Glu\rightarrow Ala}$. Thus this variant appears to be identical with two others whose structures were reported recently in North American Indians: hemoglobin-G Coushatta (*2*) and hemoglobin-G Saskatoon (*3*).

The studies of structure used reported procedures (*4*). The purified mixture of A+G hemoglobin was subjected to tryptic digestion, and the resultant peptides were examined by peptide-mapping procedures (*5*) on Whatman 3MM paper (*6*). A new arginine-positive (*7*) peptide was present near the normal βT3 peptide from hemoglobin-A; its electrophoretic mobility

Table 1. Amino acid composition of abnormal βT3 peptide from hemoglobin-G Hsin-Chu.

Amino acid residue	Normal βT3 molar ratio	Observed for the abnormal βT3 peptide		
		Micro-mole	Molar ratio	Nearest integer
Arg	1	0.033	1.0	1
Asp	2	.064	1.9	2
Glu	2	.042	1.3	1
Gly	3	.094	2.9	3
Ala	1	.066	2.0	2
Val	3	.095	2.9	3
Leu	1	.035	1.1	1