Biochem. J. (1965) **95**, 256

Preparation and Properties of the Peptide Chains of Normal Human 19s γ-Globulin (IgM)

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(*Received* 14 *September* 1964)

1. A method is described for preparing pure samples of 19s γ-globulin (IgM) from normal human serum by using successive steps of dialysis, density-gradient ultracentrifugation, chromatography on DEAE-cellulose, and gel filtration on Sephadex G-200. The yield of IgM (20–25mg./100ml. of serum) was equivalent to about one-quarter of that present in normal serum. 2. Analysis of the separated peptide chains of normal IgM and IgG (7s γ-globulin) showed considerable differences in the amino acid composition of A chains from the two proteins; their respective B chains, on the other hand, were similar in composition. The carbohydrate of both proteins is confined almost entirely to the A chains; the IgM A chain contains about four times as much carbohydrate as the IgG A chain. 3. These findings support the view that the different classes of human immunoglobulin have B chains that are identical and A chains that are chemically distinct.

The 19s γ-globulin (IgM) of human serum comprises about 1% of the total protein. Because of the difficulty of isolating this fraction from normal serum, chemical studies of IgM have been carried out mainly on the γ-macroglobulins that occur in high concentration in pathological sera. The isolation of normal IgM has, however, been greatly simplified by the recent introduction of gel-filtration methods (Flodin & Killander, 1962). When human serum is fractionated on Sephadex G-200, the leading peak contains a mixture of macroglobulins that includes 19s γglobulin, α_2-macroglobulin, lipoproteins and haptoglobin-2 (Flodin & Killander, 1962; Fireman, Vannier & Goodman, 1964; Killander, Bengtsson & Philipson, 1964). In the present study a method is described for separating 19s γ-globulin from other serum macroglobulins before purification by gel filtration on Sephadex G-200.

Recent studies (reviewed by Cohen & Porter, 1964*b*) have shown that IgG (7s γ-globulin) and IgM have a basically similar structure and after reduction can be separated into A (or H) and B (or L) peptide chains; for both normal and pathological proteins these chains are present in the same proportions in IgG and IgM. The results of electrophoretic analysis, as well as antigenic and allotypic analyses, indicate that the B chains of IgG and IgM are closely similar or identical, whereas their A chains are distinct. In the present study further evidence for the identity of B chains and the individuality of A chains has been obtained from amino acid and carbohydrate analyses performed on the separated chains of normal IgG and IgM immunoglobulin.

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MATERIALS AND METHODS

Preparation of IgM. Samples of IgM were prepared from (i) unhaemolysed plasma separated from normal donor blood collected in acid–citrate–dextrose solution. Bovine thrombin (Parke Davis and Co., Detroit, Mich., U.S.A.) was added (500 units in 10ml. of 1M-$CaCl_2$) to 500ml. of plasma, which was defibrinated by vigorous stirring in a water bath at 37°; (ii) pooled serum from healthy West African subjects living in the Gambia. Euglobulin precipitates were obtained from 400ml. samples of defibrinated plasma or serum by dialysis at 4° against three changes of 10vol. of 2mM-sodium phosphate buffer, pH6·0, for 24hr The precipitate was washed three times at 4° in the same buffer and was then resuspended in 1·0M-NaCl–0·5M-tris buffer, pH7·8–8·2, at 37°; the insoluble material was removed by centrifugation and the supernatant solution kept at room temperature. A solution containing NaCl (153g./l.) and KBr (354g./l.) (density 1·346) was then added to the euglobulin solution in a volume calculated to raise the density to 1·065; the mixture was centrifuged at 40000rev./min. for 22hr. at about 15°; the top lipid-containing and colourless intermediate layers were removed and the bottom blue–green layer, largely in the form of a gel, was resuspended in 0·15M-NaCl. Insoluble material was removed by centrifugation at 20000rev./min. for 30min. The clear supernatant solution was dialysed overnight against 0·1M-sodium phosphate buffer, pH6·3. A

sample containing up to 3g. of protein was applied to a DEAE-cellulose column (40cm.×2·3cm.) equilibrated against the same buffer. The column was eluted at room temperature with 0·1M-sodium phosphate buffer, pH6·3, until the eluate was almost protein-free, and a gradient elution was then commenced by using a 500ml. mixing vessel, with 0·45M-sodium phosphate buffer, pH4·2, in the reservoir. The selected fractions (Fig. 1) were concentrated by pressure dialysis against 1M-NaCl–0·05M-tris buffer, pH7·8, and each was separately applied to a Sephadex G-200 column (140cm.×3·3cm.) kept at room temperature and equilibrated with 1M-NaCl–0·05M-tris buffer, pH7·8. The leading fractions (Fig. 2) were concentrated by pressure dialysis at 4°.

Preparation of IgG. Normal IgG was isolated from human serum samples by chromatography on DEAE-cellulose (Sober & Peterson, 1958).

Reduction and isolation of A and B chains. Samples of IgM were reduced and fractionated on Sephadex G-75 according to the method of Fleischman, Pain & Porter (1962) by using 0·95M-mercaptoethanol as described by Cohen (1963*a*) for West African γ-globulin and 0·2M-mercaptoethanol for U.K. γ-globulin. Variation of the mercaptoethanol concentration within this range does not alter the yield of B chain.

Electrophoresis. Starch-gel electrophoresis was carried out in vertical trays using: (i) 8M-urea–0·05M-formic acid as described by Edelman & Poulik (1961); (ii) 8M-urea–0·035M-glycine buffer, pH8·8; the final pH of the gels was 7–8; (iii) 0·035M-glycine buffer, pH8·8. In the last two methods the electrode vessels contained 0·3M-boric acid–0·06N-NaOH buffer, pH8·2. Electrophoresis was continued for 15–24hr. at 5v/cm.

Protein content. The dry weight of protein samples used for amino acid and carbohydrate analyses was obtained by heating at 102° to constant weight (Chibnall, Rees & Williams, 1943). The concentrations of A and B chains from both IgG and IgM were determined by ultraviolet absorption, by using specific extinction coefficients ($E^{1\%}_{1cm.}$) 13·7 for A chains and 11·8 for B chains.

Immunoelectrophoresis. This was carried out according to the method of Scheidegger (1955) by using 0·02M-phosphate buffer, pH8·6, and a horse antiserum against human serum. Lipoprotein was identified by staining with Sudan Black B (Grabar & Burtin, 1960).

Ultracentrifugation. This was performed in a Spinco model E centrifuge at 59720rev./min. at approx. 25°. Protein solutions (10mg./ml.) were dialysed against 0·15M-NaCl–0·01M-tris buffer, pH7·6, before centrifugation.

Amino acid analysis. This was carried out in a Spinco amino acid analyser under the conditions described by Crumpton & Wilkinson (1963).

Carbohydrate analyses. Neutral sugars were identified by paper chromatography. A sample containing 80–150μg. of hexose was hydrolysed in 0·5N-HCl for 16hr. at 100°. The hydrolysate was passed through Dowex 1 (OH^- form) to remove the HCl and then through Dowex 50 (H^+ form) to remove peptides and amino acids. The resulting eluate containing neutral sugars was evaporated to dryness, taken up in 0·1ml. of water and chromatographed on Whatman no. 1 paper for 18hr. with butan-1-ol–pyridine–water (6:4:3, by vol.). The sugars were detected by spraying with the *p*-anisidine–phthalate reagent described by Rosevear & Smith (1961). Hexose was determined quantitatively by the orcinol technique (Winzler, 1955), with mannose as standard. Hexosamine was determined by Cessi's modification of the Elson–Morgan method, as described by Johansen, Marshall & Neuberger (1960), with glucosamine as standard. Sialic acid was estimated by the thiobarbituric acid method described by Warren (1959), with *N*-acetylneuraminic acid as standard.

RESULTS

Preparation of normal 19s *γ-globulin.* Samples of normal 19s γ-globulin were previously prepared from the euglobulin precipitates of normal West African serum by DEAE-cellulose chromatography followed by gel filtration on Sephadex G-200 (Cohen, 1963*a*). The pooled West African serum used for these preparations was almost free of haptoglobin and contained relatively low concentrations of β-lipoprotein. When the same preparative procedure was employed with defibrinated plasma from U.K. blood donors the final 19s γ-globulin fraction was found to be associated with significant amounts of β-lipoprotein, haptoglobin-2 and haptoglobin-2–haemoglobin complexes. Since the last three proteins were not separable from 19s γ-globulin by DEAE-cellulose chromatography or gel filtration, modification of the preparative procedure was necessary.

Dialysis of defibrinated plasma samples against running tap water for 48hr. and against distilled water for 48hr. leads to precipitation of 15–18% of the total protein; analysis by starch-gel electrophoresis, immunoelectrophoresis and ultracentrifugation showed that this euglobulin precipitate was free of α-macroglobulin but contained β-lipoprotein, haptoglobin-2 and haptoglobin-2–haemoglobin complexes and other serum proteins. Massayeff & Gombert (1964) have reported that IgM can be prepared from patients with trypanosomiasis by dialysis of the hyperglobulinaemic sera against 2mM-sodium phosphate buffer, pH6·0, followed by gel filtration. When the plasma of U.K. donors was dialysed for 17–24hr. against this buffer, 12% of the total protein was precipitated; this euglobulin mixture included both IgM and β-lipoprotein but was free of haptoglobin-2, haptoglobin-2–haemoglobin complexes and α-macroglobulin on starch-gel electrophoresis. The supernatant pseudoglobulin solution contained only small amounts of IgM as judged by immunoelectrophoresis. The β-lipoprotein present in the euglobulin fraction was largely removed by density-gradient centrifugation. When the rapidly sedimenting material obtained after centrifugation was fractionated on a DEAE-cellulose column, IgM was found in decreasing amounts throughout the protein peak obtained by gradient elution. Three successive cuts from the DEAE-cellulose effluent (Fig. 1) were fractionated on Sephadex

G-200 (Fig. 2), and in each case the leading peak was analysed by electrophoresis, ultracentrifugation and immunoelectrophoresis (Fig. 3). The three leading peaks obtained after gel filtration each gave a single band in the γ_1-globulin position on paper electrophoresis and each failed to migrate on starch-gel electrophoresis. Ultracentrifugation and immunoelectrophoresis showed a small amount of β-lipoprotein present in all three samples, but only the first was free of an antigenically distinct 11s component having the mobility of β-globulin; the second and third fractions contained 21 and 34% of this component respectively (Fig. 3).

The successive steps of euglobulin precipitation, density-gradient ultracentrifugation, DEAE-cellulose fractionation and gel filtration of the first DEAE-cellulose fraction (Fig. 1) on Sephadex G-200 gave a final protein component having γ_1-globulin mobility on paper electrophoresis and appearing as a single precipitin line corresponding to IgM on immunoelectrophoresis; the protein failed to enter the gel during starch-gel electrophoresis, and in the ultracentrifuge showed a peak with an S_{25} value of 19s (85%) together with smaller peaks of 25–35s (< 15%) (Fig. 3). The yields of IgM were 20–25mg./100ml. of defibrinated U.K. plasma and 30–40mg./100ml. of West African serum.

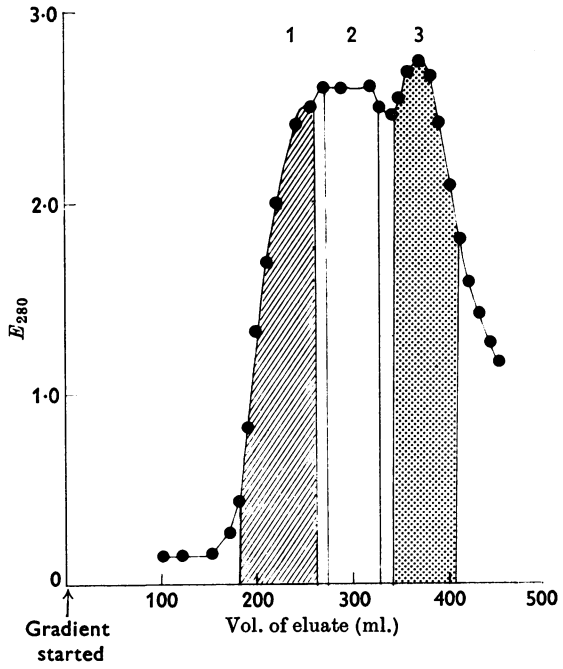

Fig. 1. Fractionation of a euglobulin solution on DEAE-cellulose (see the text for experimental details). The three fractions, 1, 2 and 3, were concentrated by pressure dialysis and fractionated on Sephadex G-200.

Reduction of IgM. Reduced IgM fractionated on Sephadex G-75 in normal acetic acid gives a pattern similar to that described for human IgG (Cohen, 1963*a*); the yield of B chain was 25% of the total protein as estimated by absorption at 280mμ. As previously shown, the B chains of normal IgG and IgM were identical on electrophoresis in urea–formic acid–starch and urea–glycine–starch gels (Cohen, 1963*a*; Cohen & Porter, 1964*a*). On electrophoresis in a urea–formic acid–starch gel the main component of the IgM A chain had a slower mobility than that of the IgG; an additional minor component with the same mobility as IgG A chain was present in all preparations of IgM A chain.

Amino acid analysis. The amino acid analysis of IgG and IgM shows significant differences, for example in lysine, arginine, serine and isoleucine (Table 1). Analysis of the separated chains shows that differences in amino acid composition are confined to the A chains, whereas the B chains of IgG and IgM appear to be chemically identical

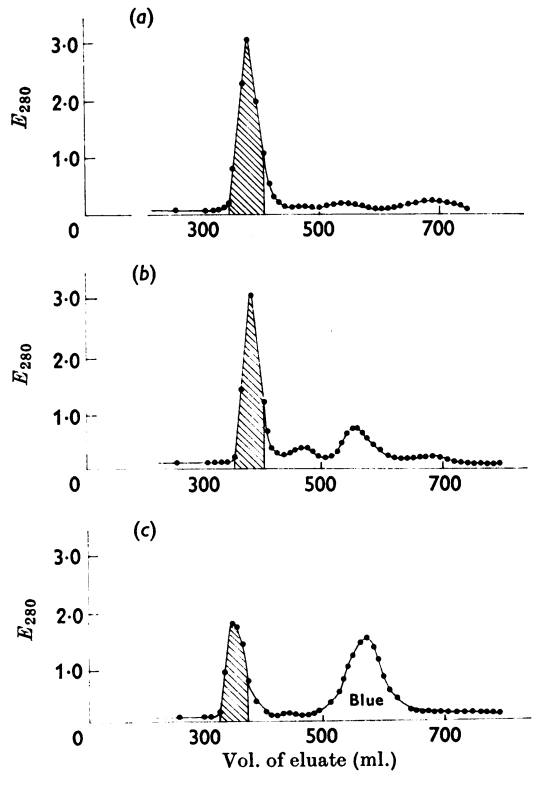

Fig. 2. Gel filtration of fractions 1, 2 and 3 shown in Fig. 1 on a column (140cm.×3·3cm.) of Sephadex G-200 in 1·0M-NaCl–0·05M-tris buffer, pH 7·8: (*a*) fraction 1; (*b*) fraction 2; (*c*) fraction 3. The leading hatched peak was, in each case, concentrated by pressure dialysis and analysed by ultracentrifugation and immunoelectrophoresis (Fig. 3).

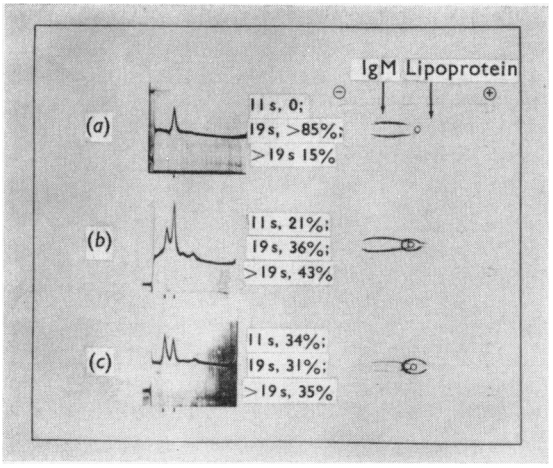

Fig. 3. Ultracentrifugal (left) and immunoelectrophoretic (right) analyses of the three IgM-containing fractions obtained on Sephadex G-200 and shown in Fig. 2: (*a*) leading peak obtained from fraction 1; (*b*) leading peak obtained from fraction 2; (*c*) leading peak obtained from fraction 3. Photographs of ultracentrifugal patterns were taken 8 min. after reaching full speed. Values in centre refer to the relative proportions of components separated in the ultracentrifuge. In fractions 2 and 3 the increasing concentration of an 11s component coincides with the appearance of a β-globulin precipitin line.

Table 1. *Amino acid analysis of normal human IgG and IgM*

IgM(1) was prepared from defibrinated plasma of U.K. blood donor; IgM(2) was prepared from West African serum. The amino acid analysis of IgG is taken from Crumpton & Wilkinson (1963). Values for carbohydrate content are taken from Müller-Eberhard, Kunkel & Franklin (1956) (for IgG) and Müller-Eberhard & Kunkel (1959) (for IgM).

	Amino acid content (g./100 g. of protein)		
	IgG	IgM(1)	IgM(2)
Lys	7·06	4·91	4·80
His	2·44	1·98	1·93
Arg	4·02	4·75	4·94
Asp	7·77	6·95	7·32
Thr	7·04	6·17	7·09
Ser	9·13	6·58	6·31
Glu	11·18	9·92	9·53
Pro	6·40	4·95	4·95
Gly	3·37	2·91	3·28
Ala	3·29	3·12	3·48
Val	7·92	5·77	6·55
Met	0·93	1·02	1·06
Ile	2·16	2·83	2·83
Leu	7·40	6·09	6·25
Tyr	5·76	4·01	4·23
Phe	4·07	3·85	3·86
Cys (half)	2·07	2·30	2·10
Trp	2·63	2·47	2·87
Carbohydrate	2·87	12·22	12·22
Total... ...	97·51	92·80	95·60

Table 2. *Amino acid analysis of the A and B chains of human IgG and IgM*

IgM(1) was prepared from defibrinated plasma of U.K. donor; IgM(2) was prepared from West African serum.

	Amino acid content (moles/mole of carbohydrate-free chain)					
Assumed mol. wt.....	A chain 50000			B chain 20000		
	IgG	IgM(1)	IgM(2)	IgG	IgM(1)	IgM(2)
Lys	29	20	21	9·7	9·6	9·5
His	9·6	7·6	8·3	2·5	2·4	2·7
Arg	12	17	18	5·8	5·3	4·6
Asp	33	33	35	13	13	14
Thr	34	38	38	15	15	15
Ser	50	42	39	24	24	24
Glu	39	40	41	20	19	19
Pro	33	29	28	11	11	12
Gly	28	28	29	11	12	13
Ala	19	26	28	12	12	12
Val	41	36	38	13	13	13
Met	3·9	5·3	5·0	0·6	0·7	0·6
Ile	8·2	13	13	4·9	4·7	4·6
Leu	31	30	32	13	13	12
Tyr	17	12	12	7·6	8·0	6·1
Phe	13	15	16	6·0	5·3	5·3
Cys (half)	6·9	7·4	5·7	2·9	3·3	
$CyS{\cdot}CH_2{\cdot}CO_2H$	3·6	3·2	4·5	0·9	1·1	1·3
Trp	7·5	7·6	7·8	2·3	2·7	2·8

Table 3. *Carbohydrate analysis of normal human IgG and IgM*

References: 1, present work (samples prepared from plasma of U.K. blood donor); 2, Müller-Eberhard *et al.* (1956); 3, Rosevear & Smith (1961); 4, Müller-Eberhard & Kunkel (1959); 5, Schultze *et al.* (1962); 6, Norberg (1964).

	Carbohydrate content (g./100g. of protein)						
	IgG			IgM			
Reference.....	1	2	3	1	4	5	6
Hexose	1·02	1·22	0·94	4·80	6·16	4·80	4·90
Fucose	Present	0·29	0·20	Present	0·74	0·80	—
Hexosamine	1·20	1·14	1·00	3·00	3·31	3·80	2·70
Sialic acid	0·03	0·22	0·20	1·06	2·01	1·80	1·70
Total	2·25	2·87	2·34	8·86	12·22	11·20	9·30

Table 4. *Carbohydrate analysis of the peptide chains of normal human IgG and IgM*

IgG and IgM immunoglobulins were prepared from plasma of a U.K. blood donor.

	Carbohydrate content (g./100g. of protein)			
	A chain		B chain	
	IgG	IgM	IgG	IgM
Hexose	1·42	7·00	0·26	—
Fucose	Present	Present	—	—
Hexosamine	1·55	4·40	0·21	0·30
Sialic acid	—	1·00	—	—

(Table 2). The alanine content of the separated chains was higher than expected from the analysis of whole IgM; this discrepancy is being investigated.

Carbohydrate analysis. Carbohydrate analyses of normal IgG and IgM immunoglobulins gave results similar to those previously reported (Table 3). Analysis of the separated A and B chains (Table 4) shows that almost all the carbohydrate is present on the A chain of both IgG and IgM; only trace amounts are found on the B chains from both preparations.

DISCUSSION

The method described for the preparation of normal IgM immunoglobulin was satisfactory with either West African human serum or human plasma obtained from banked donor blood. By dialysis of the serum against 2mM-sodium phosphate buffer, pH6 (Massayeff & Gombert, 1964), euglobulin precipitates were obtained free of haptoglobin and haptoglobin–haemoglobin complexes. This euglobulin preparation, when obtained from normal serum, contained β-lipoprotein, which was removed by ultracentrifugation in a density gradient before proceeding to the further purification of IgM by DEAE-cellulose chromatography and gel filtration. The yield of IgM (20–25mg./100ml.) was equivalent to only about one-quarter of that present in normal serum (Soothill, 1962; Fahey & Lawrence, 1963; Chodirker & Tomasi, 1963). The major loss of IgM resulted from selection of a narrow leading portion of the broad peak eluted from DEAE-cellulose. Immunoelectrophoresis showed that IgM was present in decreasing amounts throughout this peak. However, when samples from the latter part of the peak were fractionated on Sephadex G-200, the IgM preparations were always associated with appreciable amounts of an antigenically distinct 11s β-globulin (Fig. 3); the identity of this protein has not been established.

Normal IgG and IgM have a basically similar structure since after reduction both give A and B chains that are present in similar proportions in both proteins (Cohen, 1963*a*). Several experimental observations indicate that B chains from the two classes of immunoglobulins are closely similar, if not identical. The B chains of IgG and IgM have the same electrophoretic behaviour on urea–formic acid–starch gels, pH3·5 (Cohen, 1963*a*; Carbonara & Heremans, 1963), and on urea–glycine–starch gels at neutral pH (Cohen & Porter, 1964*a*). The B chains are also associated with the antigenic determinants of types I and II (Cohen, 1963*a*,*b*; Fahey, 1963) and the *Inv* allotypic specificity (Lawler & Cohen, 1965) common to all human immunoglobulins. In addition, as shown above, the amino acid analysis of B chains from normal IgG and IgM are closely similar and both

are almost free of carbohydrate. The A chains of IgG and IgM, on the other hand, are chemically and antigenically distinct. Thus the A chains carry class-specific antigenic determinants (Cohen, 1963*a*,*b*), and the IgG A chain is associated with *Gm* allotypic specificity that is characteristic of IgG immunoglobulin (Lawler & Cohen, 1965). In addition, the A chains of normal IgG and IgM have different mobilities in urea–formic acid–starch gels (Cohen, 1963*a*; Carbonara & Heremans, 1963) and they differ considerably in amino acid and carbohydrate composition.

Several carbohydrate analyses of human IgG and IgM have been published (Table 3). The observed differences may arise from contamination of the preparations used with other serum proteins, or be due to differences in analytical techniques. It is apparent, however, that the carbohydrate content of IgM is considerably greater than that of IgG. The available data suggest that the carbohydrate moiety of IgM contains relatively more sialic acid than the carbohydrate of IgG. As with rabbit IgG (Fleischman, Porter & Press, 1963), almost all the carbohydrate of human IgG and IgM is on the A chains (Table 4), which therefore differ considerably in carbohydrate content. Only trace amounts of carbohydrate were found on the B chains from IgG and IgM; this may result from contamination with A chains or be due to the fact that a small percentage of B-chain molecules in each preparation do contain carbohydrate.

Deutsch & Morton (1958) first demonstrated that 19s γ-globulin can be dissociated into 7s units by treatment with thiols; this indicated that the dissociation involved cleavage of disulphide bonds. Amino acid analyses (Table 2) show that after reduction and alkylation of IgM, the isolated B chain carries a single *S*-carboxymethylcysteine residue. Since it seems certain that A and B chains are joined by a disulphide bridge, it appears that the B chain is not involved in the covalent bonding of the 7s units of IgM. These units must therefore be united by disulphide linkages between their A chains; however, analyses of *S*-carboxymethylcysteine residues suggest that a similar number of disulphide linkages was broken during isolation of the IgG and IgM A chains. McDougall & Deutsch (1964*a*,*b*) reduced a human macroglobulin in 0·1M-mercaptoethanol and found 8 moles of *S*-carboxymethylcysteine/160000 mol. wt., which is slightly less than was found in the present study.

We thank Professor R. R. Porter, F.R.S., for his advice, Mr. J. M. Wilkinson for carrying out the amino acid analyses, Dr. R. H. Pain for ultracentrifugal analyses, and Dr I. A. McGregor for samples of West African serum. This work was made possible by grants from the U.S. National Science Foundation, the U.S. Public Health Service, the Wellcome Trust, the Medical Research Council, London, and the Nuffield Foundation. H. C. is a Commonwealth Fund Fellow, in receipt of U.S. Public Health Service Research Grant C-2918 (C7).

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