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Studies on the Reduction of a Human 19s Immunoglobulin M

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1. Reduction of a 19s immunoglobulin M with 3mM-mercaptoethanol or 0·05–0·5mM-dithiothreitol followed by alkylation gave sedimentation patterns indicating products compatible with structures consisting of one, two, three, four and five 7s sub-units. This supports the concept of a five-sub-unit structure for immunoglobulin M. 2. Reduction with 0·125mM-dithiothreitol or 20mM-cysteine produced 7s sub-units that could not be dissociated into chains in M-propionic acid. 3. By labelling (with iodo[2-^{14}C]acetic acid) the thiol groups liberated during reduction with 0·125mM-dithiothreitol, it was possible to identify the tryptic peptides involved in the disulphide bridges that link the 7s sub-units together (inter-sub-unit bridges). 4. By further reducing and labelling (with iodo[2-^{14}C]acetic acid) the 7s sub-units produced by 0·125mM-dithiothreitol, it was possible to identify tryptic peptides derived from intra-sub-unit bridges. 5. Sub-units produced by reduction with 20mM-cysteine proved to be unsuitable for distinguishing between inter-sub-unit bridges and intra-sub-unit bridges. 6. The possible arrangement of the interchain disulphide bridges was deduced.

Deutsch & Morton (1957) first described the dissociation of 19s IgM* by thiol reducing agents and put forward the idea of a sub-unit structure linked together by cystine disulphide bridges. The investigations by Lamm & Small (1966) and Miller & Metzger (1965*a*,*b*) showed that IgM probably consists of five 7s sub-units each containing two heavy (μ) polypeptide chains and two light (κ or λ) polypeptide chains. However, Suzuki & Deutsch (1967) suggested that IgM might have five 8s sub-units each consisting of two heavy chains and three light chains, two of which could be readily removed to give 7s sub-units. In either structure, two types of interchain disulphide bridges can be postulated: those that join chains within a sub-unit (intra-sub-unit bridges) and those that join chains from different sub-units (inter-sub-unit bridges).

Miller & Metzger (1965*b*) investigated the possibility of selectively reducing some of the interchain bridges, and found that 20mM-cysteine produced 7s sub-units that could not be dissociated into chains unless further reduction was carried out. However, they obtained no selective reduction with dithiothreitol even with concentrations as low as 1mM.

The term 'sub-unit' or '7s sub-unit' has been used in the literature indiscriminately, regardless of the exact nature of the sub-unit and its method of preparation from 19s IgM. With other immunoglobulins, such as 7s immunoglobulin G, the term 'sub-unit' has been used to refer to 5s half-molecules and to individual polypeptide chains. In this paper the term 'sub-unit' or '7s sub-unit' is used to refer to any reduction product of 19s IgM sedimenting at approx. 7s and composed of light and heavy polypeptide chains (e.g. that shown in Fig. 8). However, the reduction conditions under which the sub-unit was produced are always stated. In particular, we characterized a sub-unit produced by selectively reducing human 19s IgM with 0·125mM-dithiothreitol. This made it possible to distinguish between inter- and intra-sub-unit bridges.

* Abbreviation: IgM, immunoglobulin M (macroglobulin).

MATERIALS AND METHODS

Enzymes. Trypsin was a twice-recrystallized preparation and chymotrypsin a three-times-recrystallized preparation from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Reagents. Pyridine, butan-1-ol and guanidinium carbonate were A.R. products from Hopkin and Williams Ltd., Chadwell Heath, Essex. Dithiothreitol (Cleland's reagent) was obtained from Calbiochem, Los Angeles, Calif., U.S.A., and iodo[2-^{14}C]acetic acid from The Radiochemical Centre, Amersham, Bucks. Other reagents were general-purpose products from Hopkin and Williams Ltd.

Preparation of IgM. Serum from a patient with Waldenström's (1944) macroglobulinaemia was diluted tenfold with water to give a precipitate of euglobulin. After centrifugation the precipitate was dissolved in a minimal volume of 0·2M-NaCl and reprecipitated four times with water. The purified IgM was stored at −20° as a 5% solution in 0·25M-NaCl–0·025M-tris–HCl buffer, pH 8. The

preparation was examined on the analytical ultracentrifuge and by immunoelectrophoresis.

Sedimentation measurements. These were carried out on a Beckman model E analytical ultracentrifuge with schlieren optics. In correcting observed sedimentation coefficients to water at 20° a partial specific volume of 0·725 was assumed (Koenig, 1950; Dayhoff, Perlmann & MacInnes, 1952).

Immunochemical techniques. Immunoelectrophoresis was carried out by the method of Grabar & Burtin (1960) as modified by Pierce & Feinstein (1965). Sheep antiserum against human light chains and sheep antiserum against human serum were prepared by intramuscular injection of the appropriate antigen in complete Freund's adjuvant. Specific antisera against κ- and λ-chains were made by absorbing the sheep anti-(human light chains) with λ and κ Bence-Jones proteins respectively.

Reduction of IgM with dithiothreitol. Seven samples (2ml.) of a 1% solution of IgM in 0·3M-NaCl–0·2M-tris–HCl buffer, pH8, were flushed with N_2 for 30min., and dithiothreitol solution (50mM in the same buffer flushed with N_2) was added to give final concentrations of 1·0, 0·5, 0·25, 0·125, 0·075, 0·05 and 0·025mM. The samples were again flushed with N_2 for a few minutes and kept in tightly stoppered tubes for 1hr. at room temperature. Freshly prepared iodoacetic acid solution (0·1M in 0·2M-tris–HCl buffer, pH8) was added to give a fivefold molar excess over the dithiothreitol in each of the samples, which were then left at room temperature for a further 1hr. Dialysis against 0·15M-NaCl was then carried out overnight at 4°, and the reduction products were examined on the analytical ultracentrifuge.

Preparation of sub-units by selective reduction of IgM. A 50mg. sample of IgM as a 1% solution in 0·3M-NaCl–0·2M-tris–HCl buffer, pH8, was made 0·125mM with respect to dithiothreitol after thorough flushing with N_2. The reaction mixture was left at room temperature for 1hr. and then divided into two equal samples (*A* and *B*). Sample *A* was then made 0·275mM with respect to iodo[2-^{14}C]acetic acid (0·5mc/m-mole) and sample *B* was made 0·275mM with respect to unlabelled iodoacetic acid. After the samples had stood for 1hr. at room temperature sufficient unlabelled iodoacetic acid was added to both samples *A* and *B* to make the total iodoacetic acid concentration a fivefold molar excess over the dithiothreitol. The samples were dialysed against 0·15M-NaCl and then against 0·5M-NaCl–0·5M-tris–HCl buffer, pH8. Each sample was then subjected to gel filtration on a column (2cm.×140cm.) of Sephadex G-200 and eluted with the NaCl–tris buffer. Fractions (3ml.) were collected and protein was detected by measuring E_{280}. Selected fractions were pooled and the pools were concentrated by pressure dialysis through a Diaflo membrane (Amicon Corp., Cambridge, Mass., U.S.A.). The sedimentation and dissociation properties of the isolated sub-units were then examined.

The sub-units obtained from sample *A* were then further reduced by using a 1% solution in 0·3M-NaCl–0·2M-tris–HCl buffer, pH8, and making it 5mM with respect to dithiothreitol after flushing with N_2. The reaction mixture was left for 1hr. and then sufficient unlabelled iodoacetic acid was added to make the final concentration 11mM. The sub-units from sample *B* were further reduced in a similar manner except that iodo[2-^{14}C]acetic acid was used. After the reaction mixtures had stood for a further 1hr., sufficient unlabelled iodoacetic acid was added to make the total concentration of iodoacetic acid a fivefold molar excess over the dithiothreitol. Samples were dialysed overnight against 0·15M-NaCl.

Separation of polypeptide chains. Samples that had been reduced with 5mM-dithiothreitol and alkylated with iodoacetic acid were dialysed against M-propionic acid. Chain separation was then carried out in a manner similar to that described by Fleischman, Pain & Porter (1962). A column (4cm.×60cm.) of Sephadex G-75 was used and eluted with M-propionic acid. Fractions (2ml.) were collected and protein was detected by measuring E_{280}. Selected fractions were pooled, dialysed against 0·15M-NaCl and then three times against water, and freeze-dried.

[^{14}C]Carboxymethylated chains were obtained directly from IgM by reducing a 1% solution in 0·3M-NaCl–0·2M-tris–HCl buffer, pH8, with 5mM-dithiothreitol in an atmosphere of N_2. After 1hr. sufficient iodo[2-^{14}C]acetic acid was added to make the final concentration 11mM in the reaction mixture, which was then left for a further 1hr. The concentration of iodoacetic acid was then increased by the addition of unlabelled iodoacetic acid until a fivefold molar excess over dithiothreitol was present. The sample was dialysed against 0·15M-NaCl and then M-propionic acid, and the peptide chains were separated as described above.

Reduction of IgM with mercaptoethanol and cysteine. By following the method of Deutsch & Morton (1957) IgM was reduced with 0·1M-2-mercaptoethanol and alkylated with iodoacetic acid. The effect of lowering the concentration of reducing reagent to 3mM was also studied.

IgM was also reduced with 20mM-cysteine in a similar manner to that described by Miller & Metzger (1965*b*). A 1% solution of IgM in 0·2M-NaCl–0·2M-tris–HCl buffer, pH8, was made 20mM with respect to cysteine and left at room temperature for 1hr. The reaction mixture was then made 0·05M with respect to iodoacetic acid, and after standing for a further 1hr. it was dialysed against 0·15M-NaCl. Sub-units were separated by gel filtration on Sephadex G-200 as described above. The purified sub-units were further reduced with 5mM-dithiothreitol and alkylated with iodo[2-^{14}C]acetic acid as described above under 'Separation of polypeptide chains'.

Complete reduction of chains and sub-units. Freeze-dried samples were made up as 1% solutions in 6M-guanidinium chloride prepared by the method of Anson (1941). Sufficient dithiothreitol (200mM in 6M-guanidinium chloride flushed with N_2) was added to give a final concentration of 20mM in the reaction mixtures, which were left at room temperature for 1hr. Iodoacetic acid was then added to give a concentration of 60mM, and after standing for 1hr. the alkylated samples were dialysed overnight against 0·15M-NaCl.

Digestion with trypsin. Completely reduced samples were dialysed against 0·1M-NH_4HCO_3, pH8·4, and then trypsin solution (2mg./ml. in water) was added to give a protein/enzyme ratio 50:1. Digestion was carried out at 37° for 4hr., after which the digest was immediately freeze-dried. The material was dissolved in 0·2–0·3ml. of pH3·5 buffer (pyridine–water–acetic acid, 1:89:10, by vol.) and insoluble peptides were centrifuged off. The supernatant containing the soluble tryptic peptides was evaporated in a vacuum desiccator over NaOH pellets and conc. H_2SO_4.

Digestion of insoluble peptides with chymotrypsin. The

insoluble peptides from the tryptic digest were washed three times with pH 3·5 buffer (see above) and three times with water. The material was then dissolved or suspended in 0·1 M-NH_4HCO_3, pH 8·4, to give a concentration of approx. 1%. Chymotrypsin solution (2 mg./ml. in water) was then added to give a protein/enzyme ratio 50:1, and digestion was carried out for 6 hr. at 37°. The chymotryptic digest was then evaporated to dryness in a vacuum desiccator.

Separation of peptides. This was carried out as described by Beale (1967), by using high-voltage paper electrophoresis at pH 3·5 at 60 v/cm. for 1–1½ hr. Peptides were also examined by ascending and descending chromatography in butan-1-ol–pyridine–water–acetic acid (15:10:12:3, by vol.), but this did not achieve any further resolution of labelled peptides.

^{14}C-labelled peptides were detected by radioautography by using Kodak Industrial G X-ray films for 2–4 days. Unlabelled peptides were detected by dipping the electrophoretograms into 0·2% ninhydrin in acetone and heating them in a cabinet at 80° for 5 min.

RESULTS

Pathological IgM. Examination of the purified pathological IgM on the analytical ultracentrifuge showed that 85% of the material sedimented at approx. 18s (Fig. 1). The remainder of the material consisted of a 25s component, a 30s component and a small amount of a 15–16s component. The main peak had $S_{20,w}$ 17·9s at a protein concentration of 0·64%.

Immunoelectrophoresis of the IgM with antiserum against human serum showed that the preparation was free from other serum proteins. The IgM was shown to be of the κ type by the fact that it gave a precipitin line with antiserum to human light chains made specific for κ-chains but did not react with antiserum made specific for λ-chains.

Reduction of IgM. Treatment of IgM with 0·1 M-mercaptoethanol followed by alkylation with iodoacetic acid converted all the material into a component sedimenting at approx. 7s. Reduction with 3 mM-mercaptoethanol followed by alkylation produced only a small amount of 7s material together with traces of components sedimenting between 7s and 18s (Fig. 2). Component (*a*) had $S_{20,w}$ 7·3s, component (*b*) had $S_{20,w}$ 11s, component (*c*) had $S_{20,w}$ 13·2s, component (*d*) had $S_{20,w}$ 15·3s and component (*e*) had $S_{20,w}$ 17s at a total protein concentration of 1%. After dilution of the sample to a protein concentration of 0·4%, component (*a*) had $S_{20,w}$ 7·7s and component (*e*) had $S_{20,w}$ 18·6s. These results could be explained if component (*e*) consisted of undissociated IgM having a five-sub-unit structure and component (*a*) contained single sub-units. Component (*b*) and component (*c*) would then represent traces of material consisting of two- and three-sub-unit structures respectively. Component (*d*) most probably represented a structure of four sub-units or a sterically modified five-sub-unit structure.

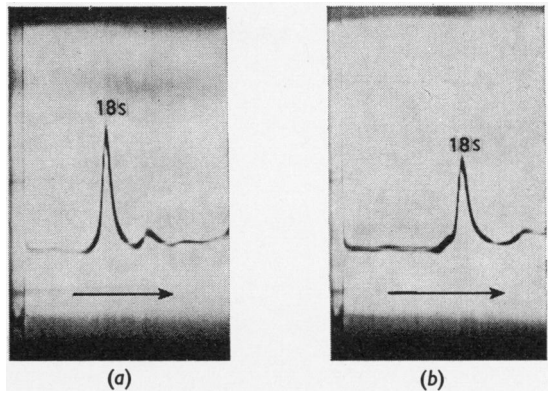

Fig. 1. Sedimentation of a human IgM in NaCl–phosphate buffer, pH 7·8 and I 0·1. Sedimentation was at 59 780 rev./min. The bar angle was 50°. Photographs were taken (*a*) 15 min. after maximum speed was reached and (*b*) 24 min. after maximum speed was reached. The protein concentration was 0·64%.

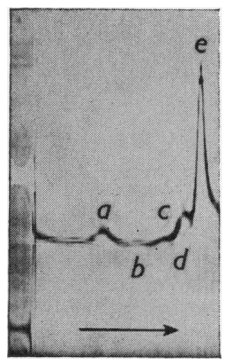

Fig. 2. Sedimentation of reaction products formed by the reduction of IgM with 3 mM-2-mercaptoethanol followed by alkylation, in NaCl–phosphate buffer, pH 7·8 and I 0·1. Sedimentation was at 59 780 rev./min. and the bar angle was 45°. Photographs were taken 40 min. after maximum speed was reached. The protein concentration was 1%. Components had $S_{20,w}$ values: (*a*) 7·3s; (*b*) 11s; (*c*) 13·2s; (*d*) 15·3s; (*e*) 17s.

Similar results were obtained when IgM was reduced with 0·05 mM-dithiothreitol (Fig. 3*a*, top). Most of the material consisted of a component (*e*), which had $S_{20,w}$ 16·8s at a total protein concentration of 0·57%. The other components, (*a*), (*b*), (*c*) and (*d*), had $S_{20,w}$ 7·5s, 11·1s, 13·3s and 14·9s respectively. Fig. 3(*a*, bottom) shows the effect of 0·025 mM-dithiothreitol.

Figs. 3(*b*), 3(*c*) and 3(*d*) show the sedimentation

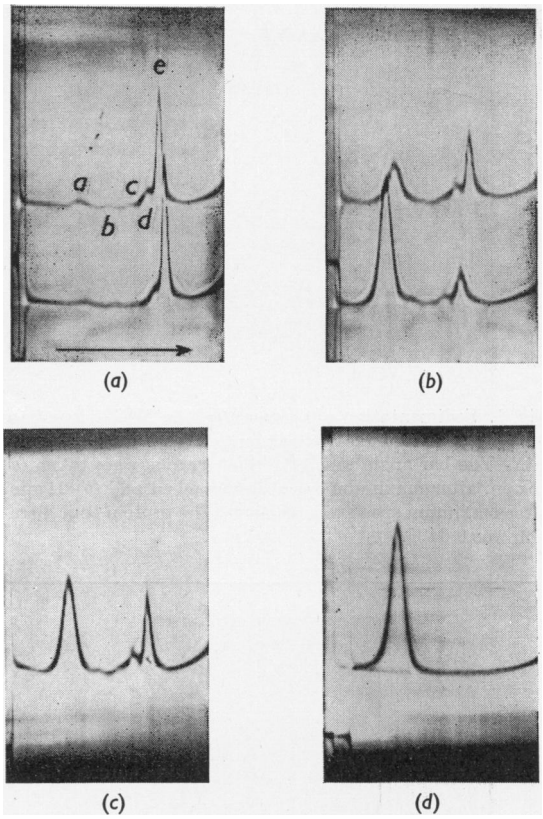

Fig. 3. Sedimentation of the reaction products after reduction of IgM with 0·025–1·0 mM-dithiothreitol followed by alkylation. Sedimentation was at 59780 rev./min. (*a*) Sedimentation was in 0·3 M-NaCl–0·02 M-tris–HCl buffer, pH 8·0. The bar angle was 55° and photographs were taken 32 min. after maximum speed was reached. Top: reduction was with 0·05 mM-dithiothreitol; the protein concentration was 0·57%. Components had $S_{20,w}$ values: (*a*) 7·5 s; (*b*) 11·1 s; (*c*) 13·3 s; (*d*) 14·9 s; (*e*) 16·8 s. Bottom: reduction was with 0·025 mM-dithiothreitol; the protein concentration was 0·62%. (*b*) Sedimentation was in 0·3 M-NaCl–0·02 M-tris–HCl buffer, pH 8·0. The bar angle was 55° and photographs were taken 24 min. after maximum speed was reached. Top: reduction was with 0·125 mM-dithiothreitol; the protein concentration was 0·54%. Bottom: reduction was with 0·5 mM-dithiothreitol; the protein concentration was 0·76%. (*c*) Sedimentation was in 0·5 M-NaCl–0·05 M-tris–HCl buffer, pH 8·0. The bar angle was 50° and photographs were taken 37 min. after maximum speed was reached. Reduction was with 0·25 mM-dithiothreitol; the protein concentration was 0·69%. (*d*) Sedimentation was in NaCl–phosphate buffer, pH 7·8 and *I* 0·1. The bar angle was 55° and photographs were taken 28 min. after maximum speed was reached. Reduction was with 1 mM-dithiothreitol; the protein concentration was 0·76%.

runs of IgM after reduction with 0·125 mM-, 0·25 mM-, 0·5 mM- and 1 mM-dithiothreitol followed by alkylation, and Fig. 4 (bottom) shows the effect

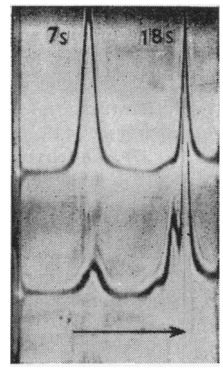

Fig. 4. Sedimentation of reduction products from IgM. Sedimentation was at 59780 rev./min. in 0·15 M-NaCl with a bar angle of 50°. Photographs were taken 40 min. after maximum speed was reached. Top: reduction was with 20 mM-cysteine followed by alkylation. Bottom: reduction was with 0·075 mM-dithiothreitol followed by alkylation.

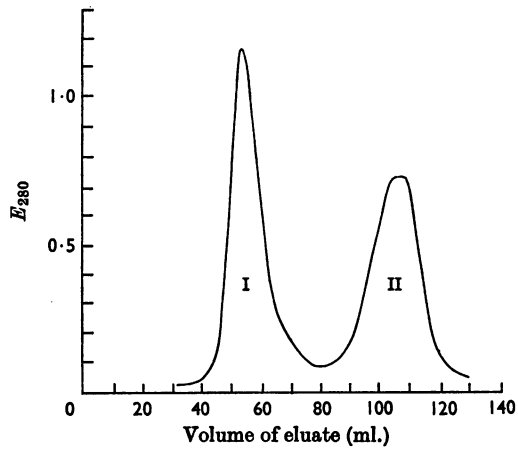

Fig. 5. Sephadex G-200 gel filtration of the reduction products obtained by treating IgM with 0·125 mM-dithiothreitol and alkylating the products with iodoacetic acid. The column was eluted with 0·5 M-NaCl–0·05 M-tris–HCl buffer, pH 8.

of 0·075 mM-dithiothreitol. As the concentration of reducing reagent increased the amount of material sedimenting at approx. 7 s increased and the amount of 18 s material decreased. The material sedimenting at approx. 15 s was prominent when 0·075 mM-dithiothreitol was used, and it remained until all the 18 s material had disappeared. At no stage were more than traces of components sedimenting at approx. 11 s and 13 s ever observed.

Fig. 4 (top) shows the sedimentation run of IgM treated with 20 mM-cysteine followed by alkylation.

The amounts of 7s and 18s components were in the ratio approx. 2:1 and there were only traces of material with an intermediate sedimentation coefficient.

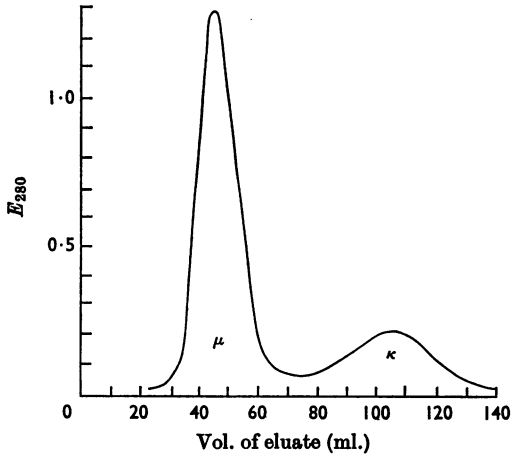

Fig. 6. Separation of IgM chains on Sephadex G-75 eluted with M-propionic acid. IgM was reduced with 5mM-dithiothreitol and alkylated with iodoacetic acid before being run on the column.

IgM sub-units and chains. Fig. 5 shows the elution pattern from Sephadex G-200 after IgM was reduced with 0·125mM-dithiothreitol and alkylated. Peak I consisted of undissociated 18s IgM and peak II contained 7s sub-units. Samples *A* and *B* (see above) gave identical elution patterns and the sub-units could not be dissociated into chains in the presence of M-propionic acid.

IgM after reduction with 20mM-cysteine followed by alkylation gave a similar elution pattern except that there was approximately twice as much 7s material as 18s material. The 7s sub-units could not be dissociated into chains in the presence of M-propionic acid.

When 7s sub-units produced by 0·125mM-dithiothreitol or 20mM-cysteine were further reduced with 5mM-dithiothreitol, dissociation into chains was achieved. These results showed that the sub-units must have contained some intact interchain disulphide bridges. Such bridges can be assumed to be intra-sub-unit bridges because all the inter-sub-unit bridges must have been broken to form 7s sub-units.

IgM or 7s sub-units after reduction with 5mM-dithiothreitol followed by alkylation were separated into light and heavy chains by Sephadex G-75 gel filtration in M-propionic acid as shown in Fig. 6.

Peptide analysis. The results of peptide analysis

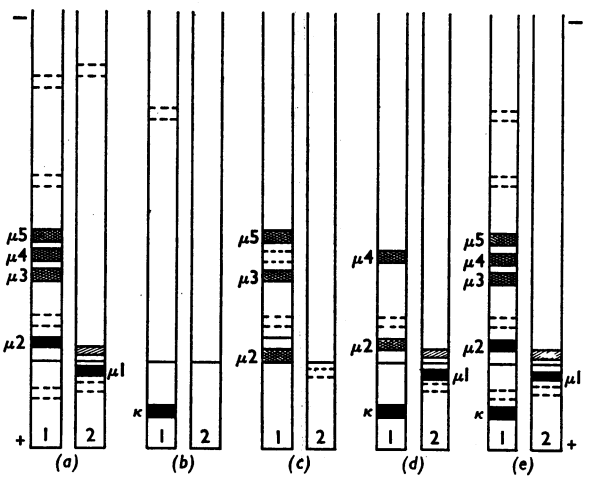

Fig. 7. High-voltage paper electrophoresis of [^{14}C]carboxymethyl-peptides obtained from IgM chains and sub-units. Electrophoresis was carried out at pH 3·5 at 60 v/cm. for 1½ hr. Only the labelled peptides are shown. 1, Soluble tryptic peptides; 2, insoluble tryptic peptides digested further with chymotrypsin. (*a*) Peptides from heavy (μ) chain; (*b*) peptides from light (κ) chain; (*c*) peptides from 7s sub-units produced by reduction with 0·125mM-dithiothreitol and alkylation with iodo[2-^{14}C]acetic acid, and then further reduced with 5mM-dithiothreitol and alkylated with unlabelled iodoacetic acid (sample *A*); (*d*) peptides from 7s sub-units produced by reduction with 0·125mM-dithiothreitol and alkylation with unlabelled iodoacetic acid, and then further reduced with 5mM-dithiothreitol and alkylated with iodo[2-^{14}C]acetic acid (sample *B*); (*e*) peptides from 7s sub-units produced by reduction with 20mM-cysteine and alkylation with unlabelled iodoacetic acid, and then further reduced with 5mM-dithiothreitol and alkylated with iodo[2-^{14}C]acetic acid.

Table 1. *Characteristics of* 7s *sub-units produced by the reduction of* 19s *IgM with dithiothreitol*

Reduction and alkylation conditions	Products of dissociation in M-propionic acid	Peptides labelled	Conclusions
5mM-Dithiothreitol and iodo[2-^{14}C]acetic acid	κ-Chains and μ-chains	κ, μ1, μ2, μ3, μ4 and μ5	These peptides form interchain disulphide bridges
(*a*) 0·125mM-Dithiothreitol and iodo[2-^{14}C]acetic acid;	No dissociation		
(*b*) 5mM-Dithiothreitol and iodoacetic acid	κ-Chains and μ-chains	μ2, μ3 and μ5	Peptides κ, μ1 and μ4 form intra-sub-unit disulphide bridges
(*a*) 0·125mM-Dithiothreitol and iodoacetic acid;	No dissociation		
(*b*) 5mM-Dithiothreitol and iodo[2-^{14}C]acetic acid	κ-Chains and μ-chains	κ, μ1, μ2 and μ4	Peptides κ, μ1, μ2 and μ4 form intra-sub-unit bridges. Peptides μ3 and μ5 form inter-sub-unit bridges

of IgM chains and sub-units are shown in Table 1 and Fig. 7. The heavy chain from IgM reduced with 5mM-dithiothreitol and alkylated with iodo-[2-^{14}C]acetic acid gave four major soluble labelled peptides, μ2, μ3, μ4 and μ5 (Fig. 7*a*1), and one major insoluble labelled peptide, μ1 (Fig. 7*a*2). The light chain gave only one soluble labelled peptide κ (Fig. 7*b*1), but nothing of significance was found among the insoluble peptides (Fig. 7*b*2).

Sub-units produced by 0·125mM-dithiothreitol and alkylated with iodo[2-^{14}C]acetic acid (sample *A*), and then further reduced with 5mM-dithiothreitol and alkylated with unlabelled iodoacetic acid, gave labelled peptides μ2, μ3 and μ5 (Figs. 7*c*1 and 7*c*2). Peptides κ, μ1 and μ4, which were unlabelled, must have remained as intact disulphide bridges during the formation of the sub-units and these bridges must therefore be intra-sub-unit bridges. Peptide κ and one of the other peptides must form the light–heavy-chain disulphide bridge, so that the third peptide (μ1 or μ4) must form a symmetrical inter-heavy-chain bridge.

Sub-units produced by 0·125mM-dithiothreitol and alkylated with unlabelled iodoacetic acid (sample *B*), and then further reduced with 5mM-dithiothreitol and alkylated with iodo[2-^{14}C]acetic acid gave labelled peptides κ, μ1, μ2 and μ4 (Figs. 7*d*1 and 7*d*2). Such peptides must have arisen from intact intra-sub-unit disulphide bridges in the 7s sub-units.

The appearance of labelled peptide μ2 in both the above experiments can be explained if some of the 7s sub-units have the disulphide bridge formed by this peptide still intact whereas others have it broken.

Sub-units produced by 20mM-cysteine and alkylated with unlabelled iodoacetic acid, then further reduced with 5mM-dithiothreitol and alkylated with iodo[2-^{14}C]acetic acid, gave all the labelled peptides, κ, μ1, μ2, μ3, μ4 and μ5 (Figs. 7*e*1 and 7*e*2), which must have arisen from intact disulphide bridges remaining after the production of sub-units by cysteine.

DISCUSSION

The most important result to emerge from these studies seems to be that a very low concentration of dithiothreitol (0·125mM) can selectively reduce some of the inter-heavy-chain disulphide bridges of IgM. Examination of the 7s sub-units produced by selective reduction and alkylation shows that there are a number of intra-sub-unit bridges, which give rise to the peptides κ, μ1, μ2 and μ4. The peptide κ is the only labelled one from the light chain and presumably partners the heavy-chain peptide μ1 or μ4 in forming the light–heavy-chain bridge. Peptide μ2 cannot be involved in this bridge since it can be labelled without peptide κ being labelled. Beale & Feinstein (1968), in a brief report on the tryptic fragments of IgM, provided evidence that in fact it is peptide μ1 that participates. Peptides μ2 and μ4 therefore most probably form inter-heavy-chain bridges, and, since peptide μ2 can be labelled without peptide μ4 being labelled, the bridges are likely to be symmetrical. This has also been confirmed by tryptic fragmentation of IgM (Beale & Feinstein, 1968).

To form 7s sub-units by selective reduction with 0·125mM-dithiothreitol, it seems to be necessary to break the disulphide bridges involving peptides μ3 and μ5. Although this first suggests that there are at least two inter-sub-unit bridges, analysis of these peptides (D. Beale, unpublished work) shows that they most probably arise from the same part of the heavy chain and differ from each other only in the nature of the attached carbohydrate. Selective reduction therefore distinguishes between three groups of inter-chain disulphide bridges: those that need to be broken to form 7s sub-units and that give rise to peptides μ3 and μ5; those that remain intact in all sub-units and give rise to peptides κ, μ1 and

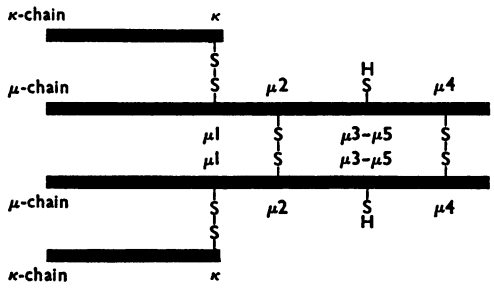

Fig. 8. Possible arrangement of interchain disulphide bridges in a 7s sub-unit produced by the reduction of IgM with 0·125mM-dithiothreitol. The arrangement is based on the results described in the present paper, results from Beale & Feinstein (1968) on the tryptic fragments of IgM, and unpublished work by D. Beale on the amino acid analysis of IgM chains and tryptic peptides. The arrangement is similar to one of the models suggested by Miller & Metzger (1965*b*). The lettering (κ, μ1, μ2, μ3, μ4 and μ5) indicates the approximate positions of peptides referred to in the text. Peptides μ3 and μ5 are believed to arise from the same region of the heavy chain. The relative positions of peptides μ4 and μ3–μ5 are uncertain.

μ4; and those that are broken in some sub-units but not in others and give rise to peptide μ2 (see Fig. 8).

In most previous work on IgM, sub-units were produced under conditions resulting in the reduction of all interchain disulphide bridges. Such sub-units might arise from rearrangement of the polypeptide chains. In sub-units produced by selective reduction with 0·125mM-dithiothreitol, most of the interchain disulphide bridges remain intact, so that the sub-units are more likely to correspond to those occurring in the original IgM. Some support for this can be obtained from the observation that the sub-units produced with 0·125mM-dithiothreitol, when further reduced so that all the inter-heavy-chain bridges are broken, give mainly material that still sediments at 7s, although some material sedimenting at 5s is also formed. This indicates that there is a non-covalent interaction between the polypeptide chains that must be satisfied within the sub-units, otherwise sub-unit aggregation would occur. Such sub-unit aggregation was never observed. This non-covalent interaction also presumably plays some role in determining the assembly of polypeptide chains within the IgM molecule.

Other workers have described a 7s sub-unit prepared by reducing IgM with 20mM-cysteine and alkylating the fragments (Miller & Metzger, 1965*b*; Cooper, 1967); these sub-units did not dissociate in M-propionic acid. The results given in this paper show that, when such sub-units were further reduced with 5mM-dithiothreitol and alkylated with iodo-[2-^{14}C]acetic acid, no indication of selective reduction of interchain disulphide bridges was obtained. The peptides κ, μ1, μ2, μ3, μ4 and μ5 were all labelled, showing that they had been involved in the formation of disulphide bridges in the 7s sub-units. Therefore, if selective reduction occurs, some of the cysteine presumably remains as half-cystine, bridged to half-cystine residues in the peptide chains. However, reduction could have been non-selective, possibly owing to disulphide interchange causing random formation of bridges or sub-units.

Examination of the sedimentation properties of the products formed after the treatment of IgM with low concentrations of mercaptoethanol (3mM) and dithiothreitol (0·05–0·5mM) revealed the presence of five components that had sedimentation coefficients compatible with those expected for structures consisting of one sub-unit, two sub-units, three sub-units, four sub-units and five sub-units. The formation of five components during the process of limited reduction therefore adds support to the concept of a five-sub-unit structure for IgM.

Although the alkylated sub-units described in this paper are referred to as approximately 7s, low concentrations of the sub-units produced by 3mM-mercaptoethanol and 0·05mM-dithiothreitol had $S_{20,w}$ values of 7·5s and 7·7s. Suzuki & Deutsch (1967), who used 0·1M-mercaptoethanol, gave $S^0_{20,w}$ values of 7·85s and 7·76s for their unalkylated sub-units and $S^0_{20,w}$ values of 6·82s and 6·71s for alkylated sub-units. Miller & Metzger (1965*a*), who also used 0·1M-mercaptoethanol, quoted $S^0_{20,w}$ 7·07s for alkylated sub-units. Suzuki & Deutsch (1967) proposed that their alkylated sub-units contained one light chain and two heavy chains and that their unalkylated sub-units consisted of three light chains and two heavy chains. Miller & Metzger (1965*a*) suggested that their alkylated sub-units contained two light chains and two heavy chains. Studies on the sub-units described in this paper have so far been compatible with a structure consisting of two light chains and two heavy chains (Fig. 8), although the amount of each chain has not yet been assessed by direct quantitative measurement.

Of the five components formed during limited reduction of IgM only those sedimenting at approx. 7s and 18s were observed in significant amounts. As the concentration of reducing agent was increased the amount of 18s material decreased and the amount of 7s material increased. No appreciable formation of components sedimenting between 18s and 7s was observed, although a small amount of 15s material was consistently present until all the 18s material had disappeared. The 11s and 13s components were only observed as traces. If IgM consists of five equal sub-units linked by disulphide bridges, it seems reasonable to assume

in the first instance that all the inter-sub-unit bridges would be equally accessible to reducing agent, so that during the process of limited reduction the formation of appreciable amounts of components consisting of two, three and four sub-units would be observed at some stage. The fact that this was not observed suggests that the sub-unit structure of IgM is arranged in some specific way, perhaps coiled or cyclic, and that the reduction of any inter-sub-unit bridge causes an alteration in this arrangement, perhaps to a linear structure. This might then make the remainder of the inter-sub-unit bridges more accessible to reducing agent, thereby producing a rapid conversion into sub-units. Some electron-microscopic studies of IgM (Munn & Feinstein, 1968; Chesebro, Bloth & Svehag, 1968) have revealed the possibility of a coiled or cyclic structure.

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