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Interchain Disulphide Bridges of Mouse Immunoglobulin M

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Mouse IgM (immunoglobulin M) was selectively and partially reduced and treated with iodo[2-^{14}C]acetate to label the interchain disulphide bridges. The carboxymethylation was studied in some detail. The labelled peptides were purified, sequenced and positioned by homology with human IgM. Only peptides originating from three interchain disulphide bridges were labelled, in contrast with the four labelled bridges obtained in human IgM under the same conditions. These peptides are homologous to human bridge peptides forming the heavy–light bridge and two inter-heavy bridges, one present in the $C_\mu 2$ region and the other in the *C*-terminal region. The inter-heavy bridge in the $C_\mu 2$ region was alone cleaved and radioactively labelled in selectively reduced IgM held together as a pentamer by non-covalent interactions. The same bridge was the only one to be totally cleaved in subunits released after more extensive, though still selective, reduction. In the light of these results a possible arrangement of the disulphide bridges of the mouse IgM pentamer is proposed.

The study of immunoglobulins has been frequently reviewed (see Porter, 1973). All immunoglobulins are formed of two types of chains, heavy and light, covalently linked by interchain disulphide bridges. The chains consist of repeating homologous sequences of about 110 residues, each of which is folded into a domain secured by an intrachain disulphide bridge. Each chain has an *N*-terminal domain that is variable and a number of constant domains that varies with the class of chain.

The mammalian IgM* molecule is constituted of ten heavy (μ) chains and ten light chains (reviewed by Metzger, 1970). In the human μ chain there are four constant homologous regions and an additional *C*-terminal sequence of 19 residues. There is at least one additional polypeptide chain (J) per molecule (Mestecky *et al.*, 1972), disulphide bridged to the additional *C*-terminal region (Mestecky & Schrohenloher, 1974). Only for human IgM, in studies of homogeneous preparations from patients with Waldenström's disease, has the μ chain been fully sequenced (Watanabe *et al.*, 1973; Putnam *et al.*, 1973) and the arrangement of interchain disulphide bridges described (Beale & Feinstein, 1969, 1970). A knowledge of the nature of the bridges of the IgM of other species should indicate how general is the arrangement found in human IgM. Homogeneous IgM can be isolated from mice carrying the transferable plasma-cell tumour MOPC 104E (McIntire *et al.*, 1965) and some structural studies have been reported (Parkhouse *et al.*, 1970; Robinson *et al.*, 1973). We have examined the IgM secreted by the same tumour line, and present here a study of the interchain disulphide bridges and the sequences of the bridge peptides.

* Abbreviations: IgM, immunoglobulin M (macroglobulin); IgA, immunoglobulin A; H, heavy chain; L, light chain; CmCys,Hse (in sequences, Figures and Tables), carboxymethylcysteine, homoserine.

Experimental

Preparation of mouse IgM

A euglobulin fraction was precipitated from the serum of mice bearing the plasmacytoma MOPC 104E, and lipoprotein was removed by preparative ultracentrifugation, by the method of Chaplin *et al.* (1965). The resulting crude IgM was purified by chromatography on a column (5cm×100cm) of Bio-Gel P-300 eluted with 0.5M-NaCl–0.02M-sodium phosphate buffer, pH7.0, containing 0.05% (w/v) NaN_3. The purity was demonstrated by serological tests, electrophoretic gels and analytical ultracentrifugation.

Selective and partial reduction and alkylation

A 1% (w/v) solution of IgM in 0.3M-NaCl–0.2M-Tris–HCl buffer, pH8.0, containing 10mM-EDTA was flushed with N_2 for 15min, and dithiothreitol solution in the same buffer flushed with N_2 was added to give a final concentration of 5mM (partial reduction) or 0.1mM (selective reduction). The samples were incubated at room temperature in stoppered vials for 1h. Sufficient iodo[2-^{14}C]acetate at 2mCi/mmol was then added to each to give a 1mM excess

over the added thiol, and the samples were alkylated under N_2 for 1h at room temperature. The concentration of iodoacetate was increased to a total 33mM by the addition of unlabelled reagent, and the samples were left for a further 1h at room temperature. Dialysis against 0.85% (w/v) NaCl–9mM-sodium phosphate buffer, pH7.2 (phosphate-buffered saline) was then carried out overnight at 4°C and the reaction products were examined in the analytical ultracentrifuge.

Alkylation studies

Time-course. IgM was partially reduced as described above and the alkylation with either 15 or 20mM-iodo[2-^{14}C]acetate of specific radioactivity 13mCi/mmol was followed for 5h 20min. Samples were removed at intervals of 10, 20, 40, 80, 160 and 320min, added to carrier and precipitated with 10% (w/v) trichloroacetic acid, on ice. The precipitate was filtered and washed with 3ml of 5% trichloroacetic acid three times, then with ether–ethanol (1:1, v/v), placed in a vial, dried and then the radioactivity was counted in a Packard scintillation counter by using Unisolve purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Short-term alkylation. IgM (5mg) was partially reduced as described above, but at a concentration of 20mg/ml. Iodo[2-^{14}C]acetate of high specific radioactivity (13mCi/mmol) was added to a final concentration of 15mM and alkylation was allowed to proceed for 45min at room temperature. The reaction was terminated by the addition of iodo[^{12}C]acetate to a final concentration of 28mM (excluding the initially added iodoacetate) and a drop of 2-mercaptoethanol. The excess of iodoacetate was eliminated by running the mixture through a column (1cm × 5cm) of Sephadex G-10 equilibrated with 5% (v/v) formic acid, and prepared for digestion with pepsin as described below.

Long-term alkylation. IgM partially reduced as described in the short-term alkylation experiment was carboxymethylated during the first 45min with 11mM-iodo[^{12}C]acetate and then iodo[2-^{14}C]acetate of specific radioactivity 13mCi/mmol was added to give a final concentration of 15mM (excluding the initially added iodoacetate). After 4h 30min the reaction was terminated and the rest of the procedure was followed as described under 'Short-term alkylation'.

Isolation of subunits after modified selective reduction

A 2% (w/v) solution of IgM in 0.3M-NaCl–0.2M-Tris–HCl buffer, pH8.0, containing 10mM-EDTA, was flushed with N_2 and made 0.4mM with dithiothreitol. Reduction under these conditions is less selective than that described above, but a higher yield (15%) of the IgM was released as 7S subunits when examined in the analytical ultracentrifuge in a non-dissociating medium, compared with 10% released as described above. After 1h at room temperature, one-half of the reduced mixture was carboxymethylated by adding iodoacetate to a final concentration of 33mM. After a further 1h the sample was applied to a column (1.4cm × 60cm) of Bio-Gel P-300 and eluted with 0.5M-NaCl–0.02M-sodium phosphate, pH7.0, containing 0.05% (w/v) NaN_3.

The other half of the reduced IgM was made 1.8mM in iodo[2-^{14}C]acetate and after a further 1h the concentration of iodoacetate was increased to 33mM by the addition of unlabelled reagent. After 1h the sample was applied to a similar column of Bio-Gel P-300 to that used for the first sample. In both cases fractions containing the separated subunits were pooled, and the concentrated pools were refractionated, concentrated by ultrafiltration to 0.5% (w/v) and dialysed against 0.3M-NaCl–0.2M-Tris–HCl buffer, pH8.0, containing 10mM-EDTA. Each preparation was then partially reduced and carboxymethylated as detailed above. The previously radioactively labelled sample was carboxymethylated with iodo[^{12}C]acetate, and the unlabelled preparation was carboxy[^{14}C]methylated with iodo[2-^{14}C]acetate.

Preparation of carboxymethylated chains

A 10ml portion of a 2% (w/v) solution of IgM was partially reduced and radioactively labelled as detailed above. After dialysis against phosphate-buffered saline for 16h at 4°C it was applied to a column (3.5cm × 100cm) of Sephadex G-100 and eluted with 1M-propionic acid. Fractions containing the separated heavy and light chains were pooled, dialysed against phosphate-buffered saline, then three times against water and finally freeze-dried. The carboxymethylated heavy chains were dissolved in sufficient 6M-guanidine hydrochloride, pH8.0, to give a 1% (w/v) solution. The sample was made 20mM with respect to dithiothreitol for 1h at room temperature (full reduction) and then carboxymethylated with a final concentration of 60mM-iodoacetate for 1h. The sample was then dialysed against phosphate-buffered saline. We will refer to this preparation as [^{14}C]μ.

Digestion with pepsin

Before digestion with pepsin all preparations were fully reduced and carboxymethylated as outlined above. The preparations were then dialysed against a minimum of 100vol. of phosphate-buffered saline, and three times against a minimum of 100vol. of formic acid–acetic acid–water (1:4:45, by vol.), pH2.1. Pepsin was added to give a protein/enzyme ratio of 50:1 (w/w) and the sample digested at 37°C for 16h. The peptides were then freeze-dried.

'Mapping' of peptic peptides

Soluble peptides from carboxymethylated preparations were applied as a band to a sheet of 3MM Whatman paper. The sample was run by high-voltage paper electrophoresis at pH3.5 in water–acetic acid–pyridine (189:10:1, by vol.) at 60V/cm for 1h 15min at room temperature. The strip containing the sample was cut out and sewn to a new sheet of paper and the sample was run at right angles by ascending or descending chromatography, as specified in the text, in butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.). Labelled peptides were located by radioautography and unlabelled peptides with 0.02% (w/v) ninhydrin in acetone.

Sequence studies

[^{14}C]μ was digested with pepsin as described above. Portions (100mg) of the digest were fractionated on a column (120cm×2cm) of Sephadex G-50 equilibrated with 5% (w/v) formic acid. The fractionation was followed at E_{280} by using an LKB recorder attached to an LKB 8300 Uvicord spectrophotometer, and the radioactivity of the eluate was measured in a Nuclear–Chicago gas-flow counter, model 4340. Samples from the radioactive fractions of the peptic digest of [^{14}C]μ were subjected to high-voltage paper electrophoresis at pH3.5 (see Plate 4*b*). Radioactively labelled peptides were located by radioautography and the patterns were used to select similar fractions to be pooled and freeze-dried for subsequent purification of the labelled peptides.

Specific radioactivity of peptides

This was determined by hydrolysing a sample of each peptide with 6M-HCl at 105°C for 19h. After drying down, one-tenth of the hydrolysed peptide was used for scintillation counting in a Packard Tri-Carb scintillation spectrometer model 2003 by using Unisolve, and the remaining nine-tenths was analysed in a Locarte automatic amino acid analyser for accurate determination of the quantity of peptide. The number of c.p.m./nmol of peptide was determined from the two sets of data.

Total acid hydrolysis

Peptides were hydrolysed with constant-boiling HCl, containing 1mg of phenol/ml, in evacuated sealed tubes at 105°C for 19h.

Amino acid composition

Quantitative amino acid analyses were preformed in an automatic Locarte amino acid analyser (Spackman *et al.*, 1958).

Edman degradations and N-terminal analyses

The procedure described by Gray (1967) was used. The Dns-amino acids were identified as described by Milstein & Feinstein (1968) but by using square layers one-quarter the area of those used by Woods & Wang (1967).

C-Terminal analyses

These were performed by using carboxypeptidase A at 37°C as described by Ambler (1967). As the enzyme/substrate ratio varied with different peptides, it will be given in the text in each case. The course of the reaction was followed by taking samples at given times and measuring the amount of amino acid released in the amino acid analyser.

Tryptic digest

Approx. 70nmol of peptide PIc was dissolved in 40μl of 1% (w/v) NH_4HCO_3, pH8.0, and digested with 20μg of trypsin at 37°C for 4h.

High-voltage paper electrophoresis

This was carried out as described by Milstein & Feinstein (1968).

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis with sodium dodecyl sulphate was performed by the technique of Summers *et al.* (1965). Protein dissolved in 0.01M-sodium phosphate buffer, pH7.8, containing 0.5M-urea and 0.1% (w/v) sodium dodecyl sulphate, was subjected to electrophoresis in 4.5% (w/v) gels (8cm×0.5cm) at a constant current of 10mA/gel for 3h at room temperature. The gels were then stained for 1h at room temperature in 0.1% (w/v) Amido Black in fixer (ethanol–acetic acid–water, 25:8:67, by vol.), and then destained in fixer at room temperature.

The results of sequence determination are shown by arrows under the peptides on which the experiments were performed. The sign $\rightharpoonup$ denotes that the nature of the residue has been established by the Dns–Edman procedure; $\leftharpoondown$ denotes a residue released by carboxypeptidase A digestion.

Results

Unreduced IgM barely penetrates the gel when examined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (Plate 1*a*). An identical pattern was observed after heating IgM at 100°C for 2min in 4M-urea–1% sodium dodecyl sulphate–0.1M-iodoacetamide. When a selectively reduced and carboxymethylated preparation was examined in the

analytical ultracentrifuge in a non-dissociating solvent, 90% sedimented as 19S molecules and 10% as 7S subunits. The selectivity of this reduction was confirmed when the same sample was examined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (Plate 1*b*). The pattern shows that after dissociation the covalently linked products are mainly oligomers and subunits (IgM_s) of a size corresponding to H_2L_2. Only very minor bands corresponding to HL, H or L units are present. A similar gel pattern was observed by Askonas & Parkhouse (1971) after reduction under similar conditions.

Plate 2(*a*) shows the radioactive peptides from a peptic digest of IgM that had been carboxy[^{14}C]-methylated after partial reduction to label the interchain disulphide bridges. The strongly labelled peptides that are designated were derived from the μ chain, and those not designated are from the λ light chain. This was shown by comparative electrophoresis at pH3.5 of the above-mentioned peptic digest with the digests of the separated μ and λ chains. Carboxy-[^{14}C]methylation after selective reduction of IgM with 0.1mM-dithiothreitol (Plate 2*b*) was restricted mainly to three of the μ-chain peptides (PIa, PIb, PIc). It will be shown below that these three peptides differ only in carbohydrate content. This result, together with those obtained by gel electrophoresis and ultracentrifugation, indicated that the three peptides labelled after selective reduction correspond to a single intersubunit bridge.

To determine more precisely to what extent this bridge is cleaved in subunits released by selective reduction, we performed a similar study to that described for human IgM (Beale & Feinstein, 1969). After a modified selective-reduction procedure, subunits were isolated and partially reduced as described in the Experimental section, and the peptic digests were examined. One preparation was carboxy-[^{14}C]methylated after selective reduction and carboxy[^{12}C]methylated after partial reduction (Plate 3*c*). As expected, electrophoresis revealed the three major labelled peptides, PIa, PIb and PIc. The other preparation was carboxy[^{12}C]methylated after selective reduction and carboxy[^{14}C]methylated after partial reduction (Plate 3*d*). Here PIa, PIb and PIc are the only peptides totally non-radioactive. This was clearly confirmed when the peptides were separated by chromatography in the second dimension. Thus these peptides are associated with a half-cystine residue in the released subunits corresponding to a bridge that is completely cleaved during selective reduction. The same result was obtained by using iodoacetamide as the alkylating agent. In similar experiments tryptic digestion also yielded three radioactive peptides and in all cases electrophoresis at pH6.5 gave a similar result.

In order to identify which 'intersubunit' bridge or bridges these peptides were associated with, the [^{14}C]μ chain was digested with pepsin and the labelled peptides were purified and sequenced.

Peptic digest

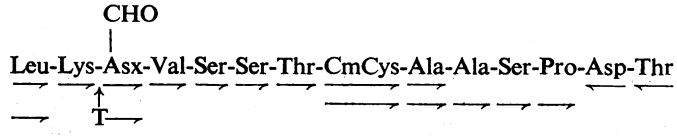
The peptic digest of [^{14}C]μ (see the Experimental section) was separated in a column of Sephadex G-50 into four radioactive fractions, which we called PI, PII, PIII and PIV (Plate 4*b*). The radioactive peptides obtained from these four fractions were purified as shown in Table 1, which also shows the amino acid composition, electrophoretic mobilities at different pH values, the *N*-terminus and the specific radioactivity of the isolated peptides.

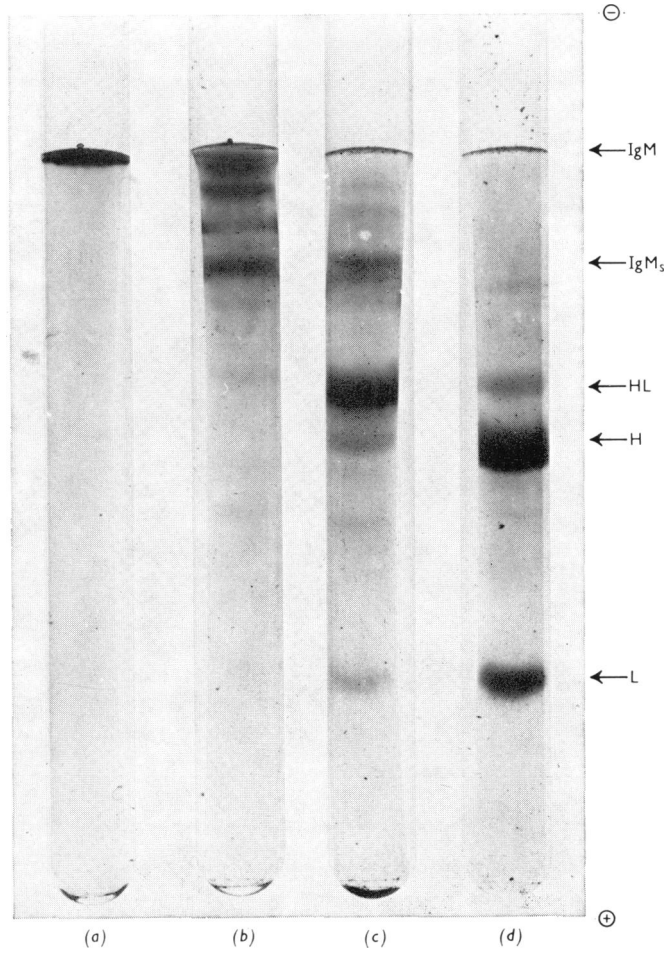
Fraction PI. This fraction gave three main radioactive bands when subjected to high-voltage paper electrophoresis at pH3.5 (Plate 4*b*). These three bands, PIa, PIb and PIc, were purified and analysed (Table 1). They all had the same *N*-terminus and amino acid composition, differing only in glucosamine content. The sequence of peptide PIa established by the Dns–Edman method was: Leu-Lys-Asx-Val-Ser-Ser-Thr-CmCys-Ala(Ala,Ser,Pro,Asx,Thr). Treatment with carboxypeptidase A showed the *C*-terminal sequence to be -Asp-Thr. The fact that carboxypeptidase A released the aspartic acid residue as such, rules out the possibility that carbohydrate was bound to that residue in peptide PIa.

Peptide PIb was used to establish the rest of the sequence. Seven Edman degradations were carried out; the nature of residues at positions 8 and 9 were confirmed as carboxymethylcysteine and alanine respectively, and the sequence that followed was Ala-Ser-Pro.

The position of lysine was confirmed by digesting peptide PIc with trypsin. This digestion was incomplete but we could identify a basic, non-radioactive peptide whose *N*-terminus was leucine and which had the composition Leu 0.9, Lys 1.1 (mol of residues/mol of peptide).

The combination of all these results led to the following sequence:

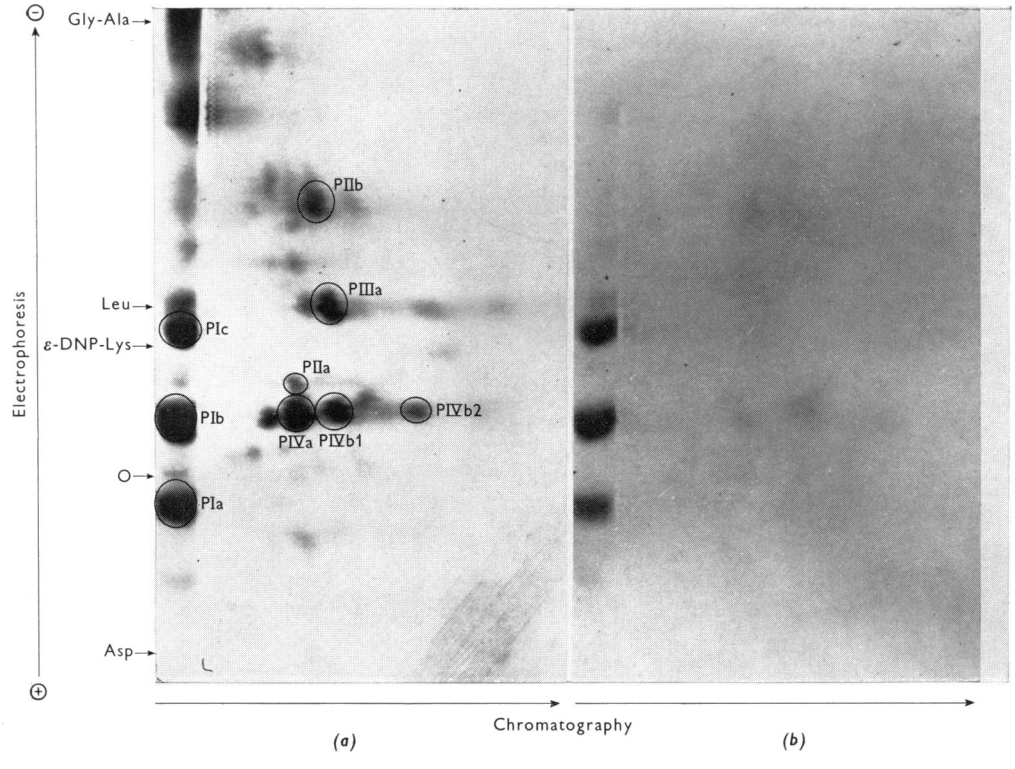




EXPLANATION OF PLATE 1

Analysis of reduced and alkylated IgM in sodium dodecyl sulphate–polyacrylamide gel

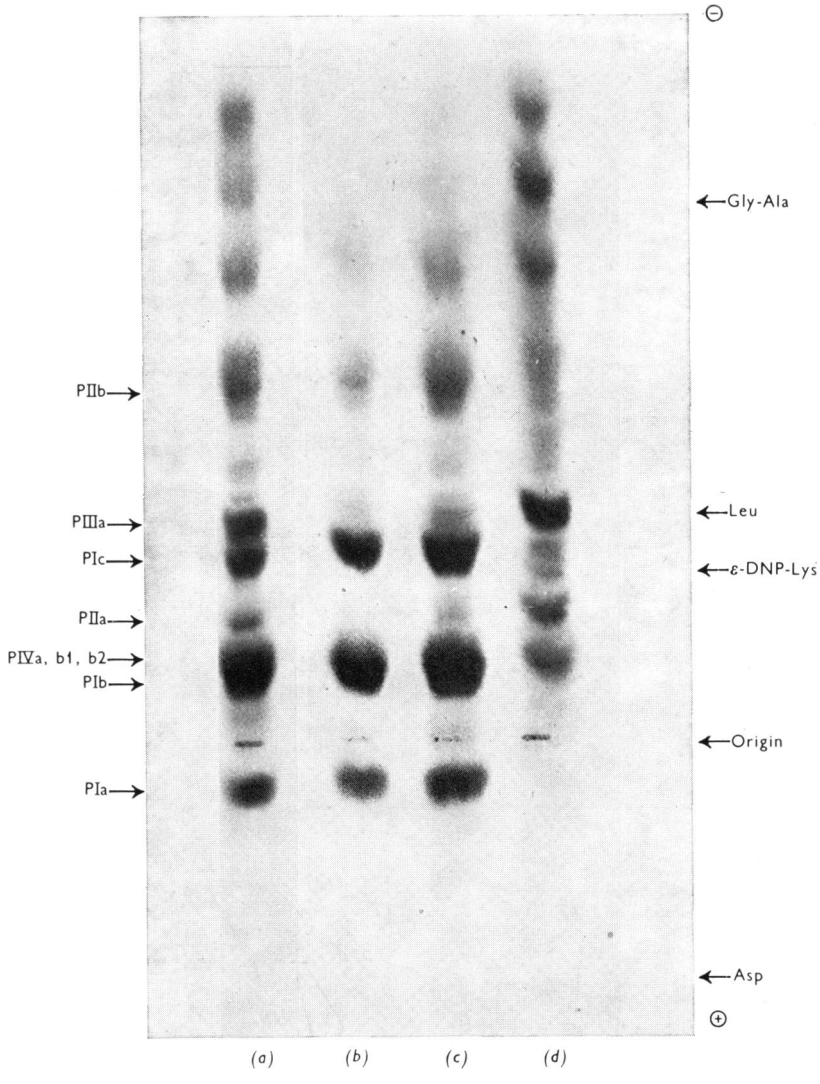
Electrophoresis was in 4.5% (w/v) polyacrylamide gels, 8cm long. (*a*) Unreduced IgM; (*b*) IgM reduced with 0.1mM-dithiothreitol and alkylated; (*c*) IgM reduced with 0.54mM-dithiothreitol and alkylated; (*d*) IgM reduced with 5mM-dithiothreitol and alkylated.



EXPLANATION OF PLATE 2

Radioautographs of peptide 'maps' of partially and selectively reduced carboxy[^{14}C]methylated IgM

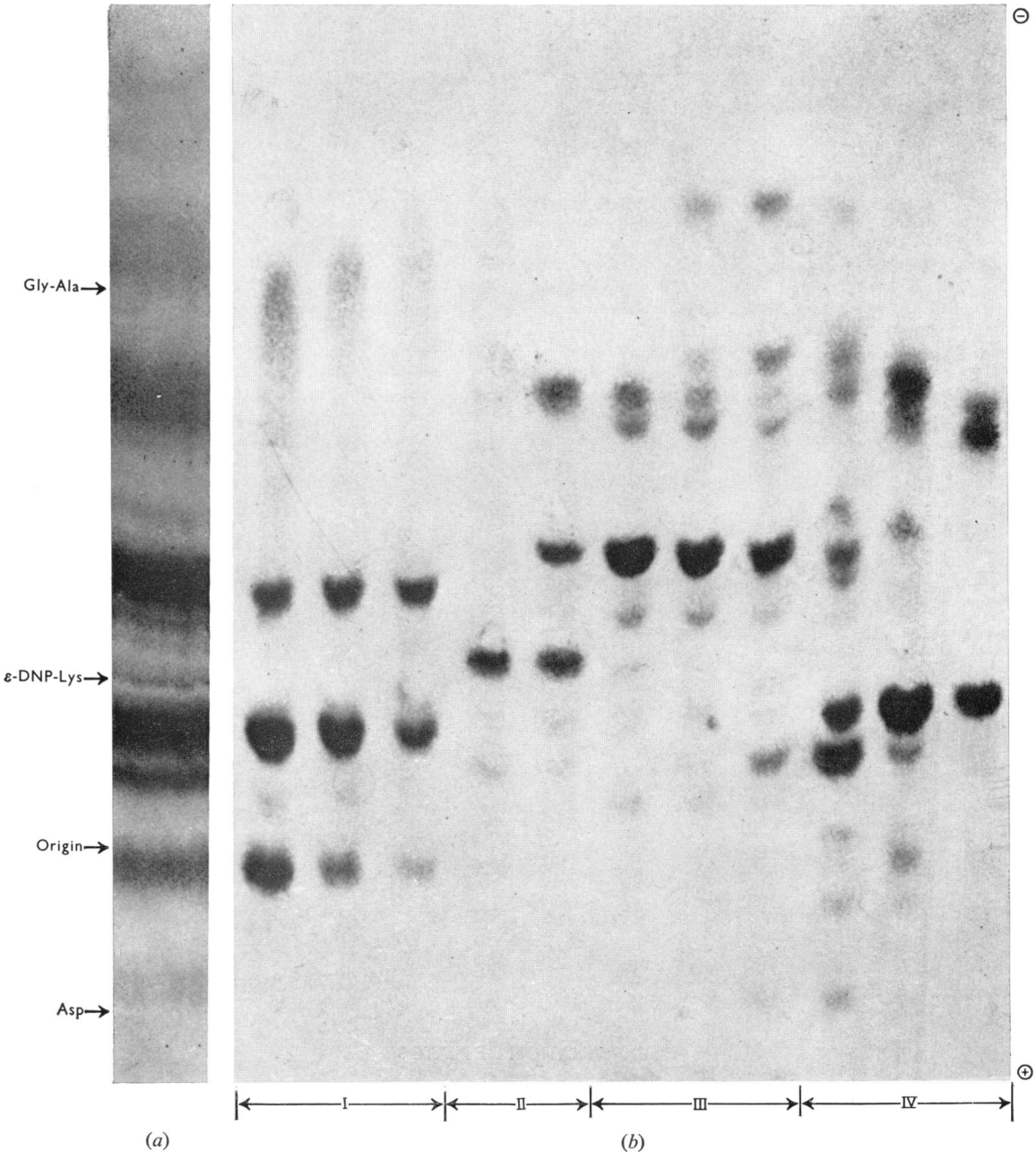
Electrophoresis of peptic digests at pH3.5 in one dimension was followed by ascending chromatography in butanol–acetic acid–water–pyridine in the second dimension. (*a*) Partially reduced IgM; (*b*) selectively reduced IgM.



EXPLANATION OF PLATE 3

Radioautographs obtained after electrophoresis at pH3.5 of peptic peptides of IgM

(*a*) Partially reduced IgM, carboxy[^{14}C]methylated; (*b*) selectively reduced IgM, carboxy[^{14}C]methylated; (*c*) after modified selective reduction and carboxy[^{14}C]methylation, subunits were separated, partially reduced and carboxy[^{12}C]methylated; (*d*) after modified selective reduction and carboxy[^{12}C]methylation, subunits were separated, partially reduced and carboxy-[^{14}C]methylated.



EXPLANATION OF PLATE 4

Radioautograph of carboxy[^{14}C]methylated peptides of mouse μ chain after electrophoresis at pH 3.5

A peptic digest of [^{14}C]μ chains (see the Experimental section) was fractionated in a Sephadex G-50 column. (*a*) Whole digest; (*b*) fractions. Pools were made as shown by the roman numerals. Major individual components within each pool were designated a to c in order of cathodic mobility.

Table 1. *Radioactive peptides obtained by peptic digestion of* [^{14}C]μ

In 'Purification procedure' the numbers refer to the pH at which high-voltage paper electrophoresis was done and BAWP (butanol–acetic acid–water–pyridine) refers to descending paper chromatography. Mobilities at pH3.5 were expressed relative to the mobility of alanylglycine in the same run, taking as mobility zero the midpoint of the taurine spot. Glucosamine values were not corrected for acid hydrolysis losses. N.D., Not determined.

Peptide	PIa	PIb	PIc	PIIa	PIIb	PIIIa	PIVa	PIVb1	PIVb2
Purification procedure	3.5, BAWP	3.5, BAWP	3.5, BAWP	BAWP, 2.1, 3.5	BAWP, 2.1, 3.5	BAWP, 3.5	3.5, BAWP	3.5, BAWP	3.5, BAWP
Mobility at pH3.5	–0.49	–0.15	0.22	0.03	–0.71	0.29	–0.23	–0.11	–0.11
Mobility at pH2.1	—	—	—	0.36	0.41	—	—	—	—
N-Terminus	Leu	Leu	Leu	CmCys	Lys	CmCys	N.D.	Ile	N.D.
	Composition (residues/molecule)								
Lys	1.2	1.0	0.9	1.1	1.0	1.1	—	—	—
CmCys	0.7	0.8	0.7	1.7	—	0.8	0.7	0.8	0.9
Asp	1.9	1.9	2.1	2.0	0.6	2.1	1.1	1.2	1.2
Thr	1.9	1.9	2.0	—	1.3	—	1.9	1.9	2.1
Ser	3.3	3.3	3.1	2.0	1.7	1.8	1.2	1.1	1.0
Hse	—	—	—	—	0.3	—	—	—	—
Glu	—	—	—	1.2	1.8	1.2	—	—	—
Pro	1.0	0.9	0.8	1.2	3.1	1.0	—	—	—
Gly	—	—	—	1.2	2.2	—	1.9	1.8	2.0
Ala	1.9	1.9	1.8	1.0	1.9	1.0	—	—	—
Val	1.1	0.9	1.1	0.9	1.2	1.0	—	—	—
Met	—	—	—	—	0.1	—	0.7	0.3	0.9
Ile	—	—	—	—	—	—	0.9	0.8	1.0
Leu	1.1	1.2	1.0	2.9	0.6	1.9	—	—	—
Tyr	—	—	—	—	0.9	—	—	0.7	1.0
Phe	—	—	—	—	0.9	—	—	—	—
GlcN	3.7	4.1	1.6	—	—	—	—	—	—
Specific radioactivity (c.p.m./nmol)	2464	2335	N.D.	1540	N.D.	1846	N.D.	2760	N.D.

Table 2. *Action of carboxypeptidase A on peptide PIIIa*

A 27nmol portion of the peptide was treated with 1 μg of carboxypeptidase A and samples were removed at the indicated intervals.

	Amino acid released (nmol)			
	2min	4min	6min	10min
Ala	5.6	5.9	6.4	6.5
Val	3.9	4.4	4.8	6.5
Leu	2.6	3.7	4.8	5.3

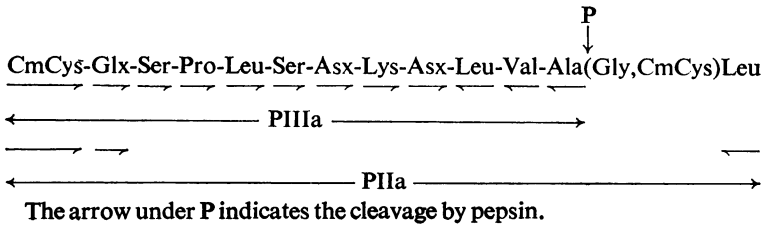
T indicates the site where trypsin split, and CHO indicates carbohydrate. There are only two Asp residues in this peptide, and the carboxypeptidase result with peptide PIa, mentioned above, shows that the penultimate residue cannot carry the carbohydrate in that variant of the peptide. The carbohydrate moiety for the PI peptide series has therefore been provisionally placed in the sequence Asx-Val-Ser. The sequence Asx-X-Ser is a well-known site for carbohydrate attachment. In human IgM the five oligosaccharide moieties are attached to aspartic acid or asparagine residues, four of which are followed by a sequence X-Ser as above.

Fractions PII and PIII. We will refer to these two fractions together because the major peptide obtained from each of them came from the same region of the molecule, peptide PIIIa being part of PIIa. Each fraction gave one major radioactive band on high-voltage paper electrophoresis (Plate 4*b*) and from the two bands peptides PIIa and PIIIa were purified (Table 1).

The sequence of peptide PIIIa was determined by manual Edman degradation as CmCys-Glx-Ser-Pro-Leu-Ser-Asx-Lys-Asx(Ala,Val,Leu). The *C*-terminal sequence was Leu-Val-Ala (see Table 2).

Peptide PIIa consisted of 15 amino acid residues, 12 of which were identical with the 12 residues composing peptide PIIIa (see Table 1). The *N*-terminus of these two peptides was identical and so was the amino acid residue which followed. A carboxypeptidase A digestion of peptide PIIa (12nmol+10 μg of enzyme) released only leucine, after 1h. This suggested that the next amino acid was difficult to release with this enzyme.

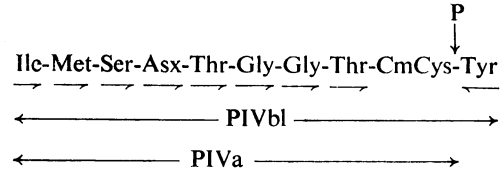
The assigned sequence of these two peptides was:



The arrow under P indicates the cleavage by pepsin.

The carboxymethylcysteine not sequenced was not radioactive, since the specific radioactivity of peptides PIIa and PIIIa was approximately the same (Table 1).

Fraction PIV. High-voltage paper electrophoresis at pH3.5 of this fraction showed two major radioactive bands, PIVa and PIVb (Plate 4*b*). Each band gave three radioactive bands on chromatography in butanol–acetic acid–water–pyridine. They all had the same *N*-terminus, and each set of three had the same amino acid composition except for the content of methionine, which decreased as the mobility increased (Harris, 1967). Peptide PIVb1 was the one obtained in best yield from band PIVb and was used to determine the sequence by Edman degradation and carboxypeptidase A digestion. Peptide PIVa was part of PIVb1, but lacked tyrosine. Frangione *et al.* (1971) reported a similar result for the tryptic peptide, T3b, from the homologous region in human IgM. The sequence was as follows:



The arrow under P shows the cleavage by pepsin.

We were expecting to find labelled peptides originating from four different interchain bridges from the μ chain, by analogy with human IgM (Beale & Feinstein, 1969). Moreover, although few experimental details are given, a value of 3.6 carboxymethylcysteine residues per MOPC 104E μ chain, after partial reduction and carboxymethylation, has been reported (Robinson *et al.*, 1973). However, we have found labelled peptides originating from only three different regions of the mouse μ chain. To make certain that carboxymethylation was complete in 1h, a time-course experiment (see the Experimental section) was performed. The main reaction occurred rapidly during the first 10–20min but continued at a slower velocity and was not complete after 5h 20min. The two different concentrations of alkylating agent gave similar curves. The above result suggested that a new peptide was being labelled during the slow-velocity reaction. To examine this idea, the short- and

long-term alkylating experiments described in the Experimental section were performed. The IgM thus treated was then fully reduced, digested with pepsin and subjected to electrophoresis and chromatography as described. As expected, no new radioactive spot was observed in the 'map' obtained after the short-term labelling experiment. However, the intensity of the radioactive spots originating from the *C*-terminal region changed, the intensity of peptides PIVa and PIVb1 having decreased, whereas the previously weak spot, PIVb2 (Plate 2*a*), was more intense. This spot had an amino acid composition identical with that of peptide PIVb1, except in the content of methionine (Table 1). Regarding the long-labelling experiments, the only radioactive spot that appeared was in the same position as, and had a similar amino acid composition to, a very weak one, peptide PIIb (Plate 2*a*), which we could not purify completely because of the low yield. A partial sequence was determined by the Dns–Edman method. This was:

Lys-Tyr-Val-Thr-Ser-Ala-Pro- -Pro-Glx-Pro-Gly-Ala

The residue at position 8 was not identified, but it is very likely that it is methionine, since this is the only amino acid residue that appeared in very low yield in the amino acid analysis (Table 1). Homoserine and *S*-carboxymethylhomocysteine were also present in small amounts. The low recoveries of methionine are explained by the presence of homoserine and *S*-carboxymethylhomocysteine, which are produced from methionine carboxymethylsulphonium salt during acid hydrolysis (Gundlach *et al.*, 1959). Further, by homology with human μ chain, peptide PIIb is very similar to peptide T4 reported by Frangione *et al.* (1971).

Discussion

It is well known that after partial reduction of immunoglobulins under the conditions used in the present study, most half-cystine residues carboxymethylated are those involved in interchain disulphide bridges (Fleishman *et al.*, 1963). Both to help place these bridges in the mouse IgM molecule and to see whether they are homologous to those in human IgM, we compared our sequences of the labelled peptides from mouse IgM with the regions of human μ chain containing the interchain disulphide bridges. We have found it convenient to use the nomenclature μ1–μ4 (Figs. 1 and 2) to designate either the inter-chain cystine residues that form the interchain bridges or the corresponding bridge peptides. Peptides PIa, PIb and PIc have the same sequence, so that the heterogeneity is presumably due to differences in the carbohydrate moiety. Similar heterogeneity in other immunoglobulin studies has also been attributed to carbohydrate differences (Rosevear & Smith, 1961; Clamp *et al.*, 1966; Frangione & Milstein, 1968;

Fig. 1. *Sequence of carboxymethylated peptides derived from the μ chain of partially reduced mouse IgM*

Homologous regions of the human μ chain (Watanabe *et al.*, 1973) are included for comparison. Unbroken lines indicate identical sequence. | indicates the interchain bridge.

		Alternative* terminology
	CHO | |	
Mouse peptides PIa, PIb and PIc	Leu-Lys-Asx-Val-Ser-Ser-Thr-Cys-Ala-Ala-Ser-Pro-Asp-Thr	Inter-heavy
	322 330	or
Human	Gln-Gln——Ala————Met——Val-Pro-Asp-Gln———	μ2
	|	
Mouse peptide PIIa	Cys-Glx-Ser-Pro-Leu-Ser-Asx-Lys-Asx-Leu-Val-Ala-Gly,Cys-Leu	Heavy–light
	130 140	or
Human	——Glu-Asx-Ser-Asx-Pro-Ser-Ser-Thr-Val-Ala-Val————	μ1
	|	*C*-Terminal
Mouse peptide PIVb1	Ile-Met-Ser-Asx-Thr-Gly-Gly-Thr-Cys-Tyr	inter-heavy
	560 565	or
Human	Val————Asp————Ala————	μ4
Mouse peptide PIIb	Lys-Tyr-Val-Thr-Ser-Ala-Pro-Met-Pro-Glx-Pro-Gly-Ala	Alkylated
	491 500	methionine
Human	————————————————————Glu——Gln——	491–503

* Beale & Feinstein (1969) designated as μ1, μ2 and μ4 the human tryptic peptides containing the corresponding bridges.

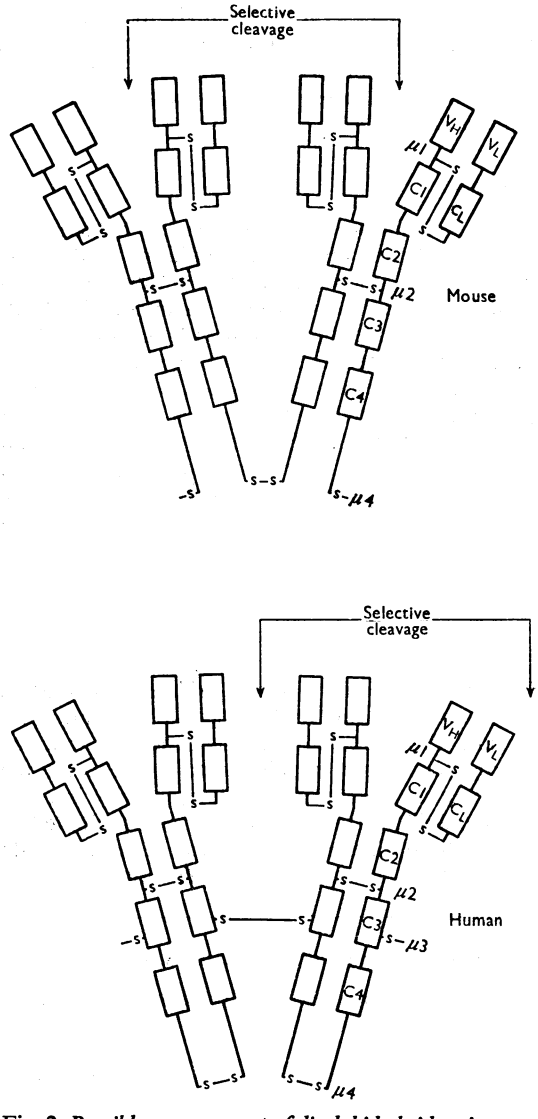


Fig. 2. *Possible arrangement of disulphide bridges in mouse IgM*

The generally accepted arrangement of disulphide bridges in human IgM is included for comparison. The bridges selectively cleaved and carboxymethylated are indicated. Variable (V) and constant (C) domains are shown.

Beale & Buttress, 1969; Milstein & Milstein, 1971; Shimizu *et al.*, 1971). The above-mentioned peptides were compared with those from human μ chains to decide from which part of the molecule they originated (Fig. 1). The most convincing homology is with the region that contains the inter-heavy-chain disulphide bridge of the $C_\mu 2$ domain and which stretches from residues 322 to 335 (Watanabe *et al.*, 1973). The homology in this region is 43% and the distance between the residue carrying the carbohydrate and the labelled cysteine is identical. On the basis of this homology the mouse peptide will be designated $\mu 2$ (Figs. 1 and 2). All the substituted amino acids except for the aspartic acid at position 332 could have arisen by a single base change in the coding triplet. The substitution of the aspartic acid residue for a serine residue at position 332 requires a two-step mutation.

The most convincing homology for peptide PIIa, when compared with human μ chain, is with the region containing the heavy–light disulphide bridge ($\mu 1$ in Figs. 1 and 2). The *N*-terminus of this peptide, carboxymethylcysteine, corresponds to the half-cystine residue joining the μ chain to the light chain. Thus in mouse, as in human, the light chain is bridged to the beginning of the $C_\mu 1$ domain. As in mouse $\mu 2$, all the substituted amino acids could have arisen by a single base change in the coding triplet, except for leucine at position 134, which is substituted for an aspartic acid or asparagine residue; this requires a two-step mutation. The isolated mouse peptide PIIa is 15 residues long. In spite of only 27% homology between human and mouse μ chains in this region, the distance between the half-cystine joining μ chain to light chain and the first intrachain half-cystine residue of the $C_\mu 1$ domain is the same.

Peptide PIVb1 ($\mu 4$, Figs. 1 and 2) is 70% homologous to the human *C*-terminal sequence which carries one of the inter-heavy-chain disulphide bridges. It forms part of the additional *C*-terminal 19 residues found in human μ and α chains (Watanabe *et al.*, 1973; Putnam *et al.*, 1973; Chuang *et al.*, 1973). It is of particular interest that in the polymeric forms of human IgA and IgM the polypeptide J chain is bridged to the half-cystine residue of the additional *C*-terminal sequence (Mendez *et al.*, 1973; Mestecky *et al.*, 1974; Mestecky & Schrohenloher, 1974), and presumably the mouse J chain is similarly attached to the half-cystine residue of peptide $\mu 4$. A homologous *C*-terminal tripeptide has been reported in mouse IgA (Abel & Grey, 1967).

Although we examined the weakly labelled peptides, we were unable to find a mouse peptide corresponding to $\mu 3$ in human, which carries the bridge linking the C3 domains of μ chains (Fig. 2). We considered various explanations for failing to detect an additional interchain bridge which might be present.

(1) The bridge might not be cleaved by partial reduction. This was ruled out, since the resulting μ chains move as monomers in a sodium dodecyl sulphate–polyacrylamide gel.

(2) Carboxymethylation might be very slow. We extended the time of carboxymethylation, but found that only a methionine peptide (PIIb, Fig. 1) was labelled.

(3) During partial reduction the bridge might rearrange to form a resistant intrachain bridge. The reason for the stability of intrachain bridges within homology regions is that they are buried in hydrophobic regions between pleated sheets. It is unlikely that a bridge formed by rearranging interchain bridges would be in a similar environment.

(4) The $\mu 3$ bridge in humans does not have a counterpart in the mouse.

The last explanation is the most likely, and suggests that only two types of inter-heavy chain bridge are present in the mouse IgM examined. If so, this has important implications, since the mouse IgM pentamer remains intact in a dissociating medium. This could only be explained if, as shown in Fig. 2, bridges $\mu 2$ and $\mu 4$ in mouse IgM are in series, i.e. they connect any μ chain to two different μ chains. In the generally accepted model of human IgM (Beale & Feinstein, 1969), however (Fig. 2), the corresponding two bridges are in parallel, i.e. they connect the same pair of μ chains.

The selective reduction experiments (Plates 1*b*, 2 and 3) indicated that peptides PIa, PIb and PIc were associated with the most labile bridge, which is cleaved in all the subunits released by selective reduction. As we have seen, these peptides correspond to a single bridge, and are homologous to human peptide $\mu 2$. The almost exclusive labelling of peptide $\mu 2$ indicates that a symmetrical bridge has been cleaved, and it follows that peptide $\mu 4$ must also form a symmetrical bridge.

After selective reduction, the IgM, which remained almost completely intact as shown by analytical ultracentrifugation or gel filtration in non-dissociating conditions, was dissociated into smaller units in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (Plate 1*b*). This confirms that non-covalent interactions hold together the selectively reduced IgM molecules (Tomasi, 1973; Parkhouse, 1975). This may be explained by the fact that the selectively cleaved bridge $\mu 2$ links pairs of μ chains which continue to interact non-covalently (Fig. 2).

In human IgM, in contrast with the mouse, it is bridge $\mu 3$ that is fully cleaved and carboxymethylated in the subunits released by selective reduction, and although bridge $\mu 2$ is fairly labile, it is cleaved and carboxymethylated in only some of the subunits released. The results obtained with mouse IgM suggest possible reinterpretations of the studies on human IgM disulphide bridges; as in the mouse, bridge $\mu 4$ may be in series with bridge $\mu 2$; one of these bridges would then be in parallel with bridge $\mu 3$. Since bridge $\mu 3$ alone appears to be fully cleaved and carboxymethylated after selective reduction, a rearrangement of other bridges would have to be involved. We have known for some time that in contrast with the restriction of radioactive labelling after selective reduction of both mouse and human IgM, electrophoresis of the products in sodium dodecyl sulphate gels demonstrates that a proportion of HL units are formed (Kownatzki, 1973; Parkhouse, 1975). This implies some rearrangement of bridges during selective reduction.

Alternatively there may be an unusual type of equilibrium between two forms of molecule, those in which bridges $\mu 3$ and $\mu 2$ are in series and others in which they are in parallel. This latter conformation is unlikely if we assume (Feinstein, 1974) that the different domains of human IgM have a quaternary structure similar to the constant domains of the Fab′ model (Poljak *et al.*, 1973). However, the model is only hypothetical, and until the tertiary structure of IgM is established this possibility remains open.

The experiments performed using different durations of labelling show that, in addition to the rapid carboxymethylation of cystine residues, the slower carboxymethylation of a methionine residue occurred. This is supported by the following observations.

(*a*) The short-term labelling experiments produced only peptides containing carboxymethylcysteine.

(*b*) Peptide PIIb is the only peptide labelled in the long-term labelling experiment. The radioactivity of this peptide is due to the presence of *S*-carboxymethylmethionine.

The carboxymethylation of the methionine residues of a protein and the instability of the sulphonium salt to acid hydrolysis were thoroughly studied by Gundlach *et al.* (1959). They found that at neutral pH the alkylation of the cysteine thiol groups is much faster than that of methionine.

The relative yields of the peptides originating from the *C*-terminal end (see the Results section) were changed in the short-term labelling experiment, peptide PIVb2 being in the highest yield although hardly appearing in the large-scale preparation. In the latter the reaction was stopped by the addition of iodoacetate, which was left for 1 h and then dialysed, whereas in the short-term labelling experiment the carboxymethylation was stopped by the addition of iodoacetate and the mixture immediately desalted by gel filtration. This last treatment not only avoided the carboxymethylation of methionine (see above, and compare the methionine values of peptides PIVb2 and PIVb1, Table 1) but also avoided or decreased considerably the oxidation of methionine and carboxymethylcysteine which occurs easily under mild oxidizing conditions (Harris, 1967).

Of the six methionine residues present in the mouse μ chain (Robinson *et al.*, 1973) only one was labelled under the conditions used. The peptide PIIb containing this methionine residue is 80% homologous to the sequence stretching from residues 491 to 503 of the human $C_\mu 4$ region, with the methionine residue at position 498. This methionine residue is also the only one labelled by Frangione *et al.* (1971) in human μ chain. The particular reactivity of this methionine

residue could be explained if it were the only one exposed to the solvent. Assuming that the four constant-homology regions in human IgM are folded similarly to the constant-homology regions in the Fab′ model of Poljak *et al.* (1973), it is noteworthy that whereas the labelled methionine residue lies between two proline residues within a β-pleated region, the unlabelled methionine residues all lie at the bends between strands of β-pleated sheets.

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