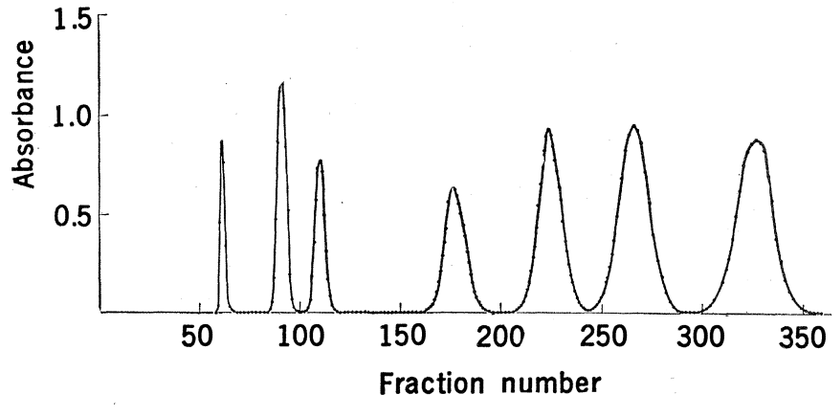

Fig. 2. Separation of DNP amino acids. Peaks (partition coefficients are shown in parentheses) in order of elution are: dinitrophenyl-L-ornithine (> 100), dinitrophenyl-L-serine (3.8), dinitrophenyl-L-threonine (2.4), *N,N'*-di(dinitrophenyl)cystine (0.94), dinitrophenyl-β-alanine (0.71), dinitrophenyl-L-alanine (0.56), and dinitrophenyl-L-proline (0.45).

can be used to deliver a constant flow of droplets. The rate is set at the highest flow compatible with a steady generation of discrete droplets. The highest permissible flow gives droplets which are barely separated.

Figure 1 shows an assembly used to separate dinitrophenyl (DNP) amino acids. It consists of 300 silylated glass columns of standard Pyrex tubing (0.6 mm in wall thickness and 3 mm in outside diameter) 60 cm long connected by AWG 24 standard-wall Teflon tubing (*3*). The system has a capacity of about 460 ml (excluding the volume of moving phase in the Teflon tubing, which is 80 ml). Solvents [in this case, chloroform, acetic acid, and O.1*N* HCl, 2 : 2 : 1 (by volume)] were mixed and allowed to equilibrate in a separatory funnel. The chloroform layer (stationary phase) was pumped into the assembly. The DNP amino acids, 2 to 10 mg each, were dissolved in 3 ml of a 1:1 mixture of both phases, and this solution was placed in a 5-ml sample tube (for example, a modified 5-ml Mohr pipette with suitable high-pressure fittings). The aqueous phase was then pumped through the top of this tube at a flow rate of 16 ml/hour (10.2 atm). Fractions, each 3 ml, were collected, and their absorbances were determined at 350 nm.

Symmetrical, completely resolved peaks were obtained for each component in the mixture (Fig. 2). The efficiency of the separation calculated from the peak of dinitrophenyl alanine was 900 theoretical plates (*4*). The analysis time of 70 hours could not be shortened with the present design without decreasing the number of columns, as moving phase was pumped at a near maximum rate. Although 30 mg of mixture was used in the present study, 300 mg have been separated with little loss in resolution. The capacity of the technique may be increased by the use of columns of larger bore, but it is not yet known to what extent this modification affects resolution. Resolution is, of course, proportional to the number of columns used; the necessary number of columns depends on the separation desired.

Droplet countercurrent chromatography, which may be compared to the classic separation technique of countercurrent distribution (*1*), has high resolving power, compactness, simplicity of design, and ease of operation. It would appear to be well suited to the separation of milligram quantities of peptides, ribonucleic acids, lipids, and similar substances.

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3. The Teflon tubing connecting the columns passed through a Delrin fitting which with suitable tubing adapters secured it to the glass tubing. The base of the fitting was fitted over the glass tubing which was then flanged. A sleeve of polyethylene tubing (2.92 mm, inside diameter; 3.73 mm, outside diameter) approximately 1 cm long was placed over the glass to adapt the Delrin to the flange. Inserted into the glass tubing was a 1-cm length of AWG No. 18 standard-wall Teflon tubing containing a polyethylene tip (0.76, inside diameter; 1.22 mm, outside diameter) for the production of droplets of suitable size. The Teflon transfer tubing passed through the male threaded head of the Delrin fitting and into the Teflon adapter in the glass tubing. The transfer tubing had a tendency to slip out under high pressure. It was held in place with a short sleeve of shrinkable Teflon 1203 Penntube WTF. When shrunk, the sleeve bound to the transfer tubing and provided an anchor to the male Delrin fitting.
4. We calculated the number of theoretical plates using the formula
$$N = (4R/W)^2$$
where N is the number of theoretical plates, R is the elution volume, and W is the peak width volume. The elution volume does not include the volume of the moving phase in the Teflon tubing, as this volume is merely in transit and not involved in the partitioning process.

10 April 1970

Macroglobulin Structure: Variable Sequence of Light and Heavy Chains

Abstract. *The variable regions of the light and heavy chains on the same macroglobulin (immunoglobulin M) molecule are no more related in amino acid sequence than are the variable regions of the light and heavy chains of different immunoglobulin molecules. Subgroups of μ chains are similar in their variable sequence to subgroups of γ chains.*

In the 5 years since the primary structure of Bence Jones proteins was first reported (*1, 2*) it has been established that the polypeptide chains of all immunoglobulins are divided into a variable NH_2-terminal region and a constant COOH-terminal region. In the κ and λ antigenic classes of Bence Jones proteins, which are equivalent to the two classes of immunoglobulin light chains, the variable region comprises some 110 amino acid residues or approximately the first half of the molecule, and the constant region constitutes the second half (*3*). Many changes in sequence occur in the variable region of different light chains of the same antigenic class, but none or only one or two occur in the constant region. Studies of the sequence of μ and γ heavy chains have shown a similar division into a variable region and a constant region (*4–10*). Although the variable region is about the same length as in light chains,

the constant region of μ and γ chains is almost three times as long. Whereas the κ and λ variable regions are characteristic of the antigenic class of light chain, the variable region of μ and γ chains appears to be independent of the class of heavy chain (*4*, *7–9*).

Knowledge of the sequence of the complete variable part of immunoglobulin molecules is important because the antigen-combining site is thought to reside in the variable regions of the light and heavy chains of the antibody molecule. This raises the question whether the variable regions of the light and heavy chains of the same immunoglobulin molecule are more related in primary structure than are the corresponding regions of chains on different molecules. Up to now, the complete sequence of the variable regions of the light and heavy chains on the same molecule has been reported for only one case—a γG1 myeloma globulin (*5*). We now report the tentative sequence of the variable region of the κ light chain of a human γM globulin for which the sequence of the NH_2-terminal 105 residues of the variable region of the μ heavy chain has been published (*7*). We also present the sequence around the disulfide bridge between the light and heavy chains; this differs markedly from the sequence at the light-heavy bridge in γG1 globulin but closely resembles that in γG4 globulin (*11*).

The purified γM globulin Ou (*7*, *12*) was completely reduced with mercaptoethanol and was aminoethylated with [^{14}C]ethylenimine in 7*M* guanidine hydrochloride, so that all disulfide bridges were broken and all half-cystine residues were converted to [^{14}C]-aminoethylcysteine (*13*). The heavy and light chains were separated on Sephadex G-100 in 4*M* guanidine hydrochloride at *p*H 3.0. The two radioactive chains were separately digested with thermolysin for 1 hour (*14*, *15*). The peptides in each digest were fractionated on Sephadex G-25 into a series of major pools with continuous monitoring of the optical absorbance at 280 nm and of the radioactivity with use of a Nuclear-Chicago flow-through scintillation counter. The peptides in each pool were purified by ion-exchange chromatography with a Technicon peptide analyzer to which a flow-through counter was also connected. In many cases the peptides were further purified by a second chromatographic procedure, either with different gradients, or with resins of different cross-linkage, or by chromatography or electrophoresis on paper. By these methods more than 120 different thermolysin peptides were obtained from the heavy chain and about 40 from the light chain. The sequences

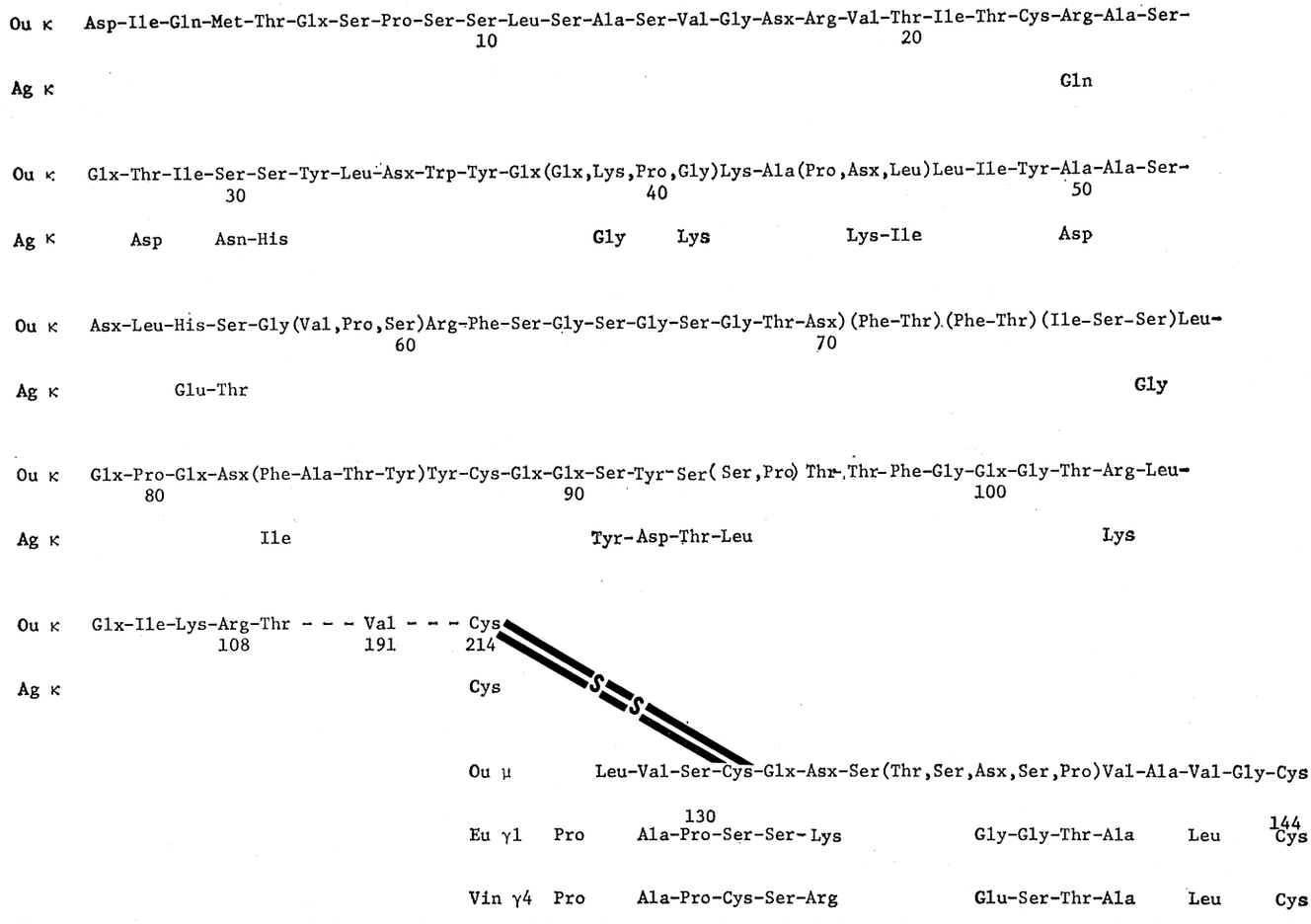

Fig. 1. Amino acid sequence of the variable region of the κ light chain Ou and of the region in the μ heavy chain to which it is linked by a disulfide bridge in the human γM globulin Ou. For comparison to other human κ light chains, only the residues that differ are shown for the κ Bence Jones protein Ag (*17*). Residues 109 through 214 in the constant region are identical in the two κ chains. For comparison to other human heavy chains, only the residues that differ in the homologous region are shown for the γ1 chain Eu (*5*) and the γ4 chain Vin (*10*) with the exception of Cys-144, which is used as a position marker. The disulfide bridge is drawn schematically. The sequence given around the disulfide bridge accords with a partial sequence given for a different γM globulin (*11*).

of many of these were determined by the dansylation procedure (*16*).

The radioactivity was followed at each step of purification of peptides containing [^{14}C]aminoethylcysteine. Thus, we isolated peptides representing five different positions of this tracer in the κ light chain and eleven different positions in the heavy chain. These included the two peptides that form the disulfide bridge between the light and heavy chains. In another experiment, trypsin was used to cleave light chain in which the five half-cystine residues had been converted to aminoethylcysteine but were nonradioactive. In this case we isolated about three-fourths of the theoretical tryptic peptides.

By means of the overlapping of tryptic and thermolysin peptides and with the help of homology in placing these peptides, we obtained the tentative sequence of the κ light chain of the γM globulin Ou (Fig. 1). Only the variable portion of the sequence is shown, for the constant portion was identical to that of protein Ag. The light chain was of the Inv (*3*) type (*12*) and, as expected, valine is present at position 191.

The sequence of the light chain of the γM globulin Ou is of the κI subgroup for which protein Ag is the reference (*17*); only 18 positions differ in the variable regions of these two κ chains. The light chain of the γG1 globulin Eu (*5*) is likewise of the κI subgroup. Yet, although the variable regions of the light chains of Ou and Eu are closely homologous in sequence, the variable regions of the two heavy chains have only a 30 percent identity (*18*). In these two immunoglobulins of different classes there is no closer similarity in sequence between the light and heavy chains on the same molecule than there is between the light and heavy chains from different molecules.

The sequence of the variable region of the μ and γ chains is not characteristic of the class of the heavy chain, whereas the sequence of the constant region is. The variable regions of the Ou μ chain and the Daw γ1 chain (*4*, *7*) have a 73 percent identity, but the constant regions of these two chains of different classes differ greatly in sequence. Conversely, the heavy chains of Daw and Eu which are of the same subclass (*1*) have only a 33 percent identity in their variable regions though they have only one known amino acid difference in their constant regions (*4*).

We have evidence that there are subgroups of μ chains, that these are similar in their variable sequence to subgroups of γ chains, and, furthermore, that there is no restriction on the combination within the same molecule of light chains of any antigenic type or sequence subgroup with heavy chains of any class or subgroup. The heavy chain subgroups are analogous to the subgroups of variable sequence in light chains for which the classification κI, κII, and κIII has been proposed (*3*, *19*). The existence of heavy chain subgroups based on sequence homology of the variable regions of heavy chains was first proposed because of the strong homology in the amino terminal 105 residues of the human γG1 proteins Daw and Cor with the human γM protein Ou (*4*, *7*, *18*). Subgroups within heavy chains of the same class have been reported for γ chains by Cunningham *et al.* (*8*), who found a third γG1 chain with a variable sequence similar to that of Daw, Cor,

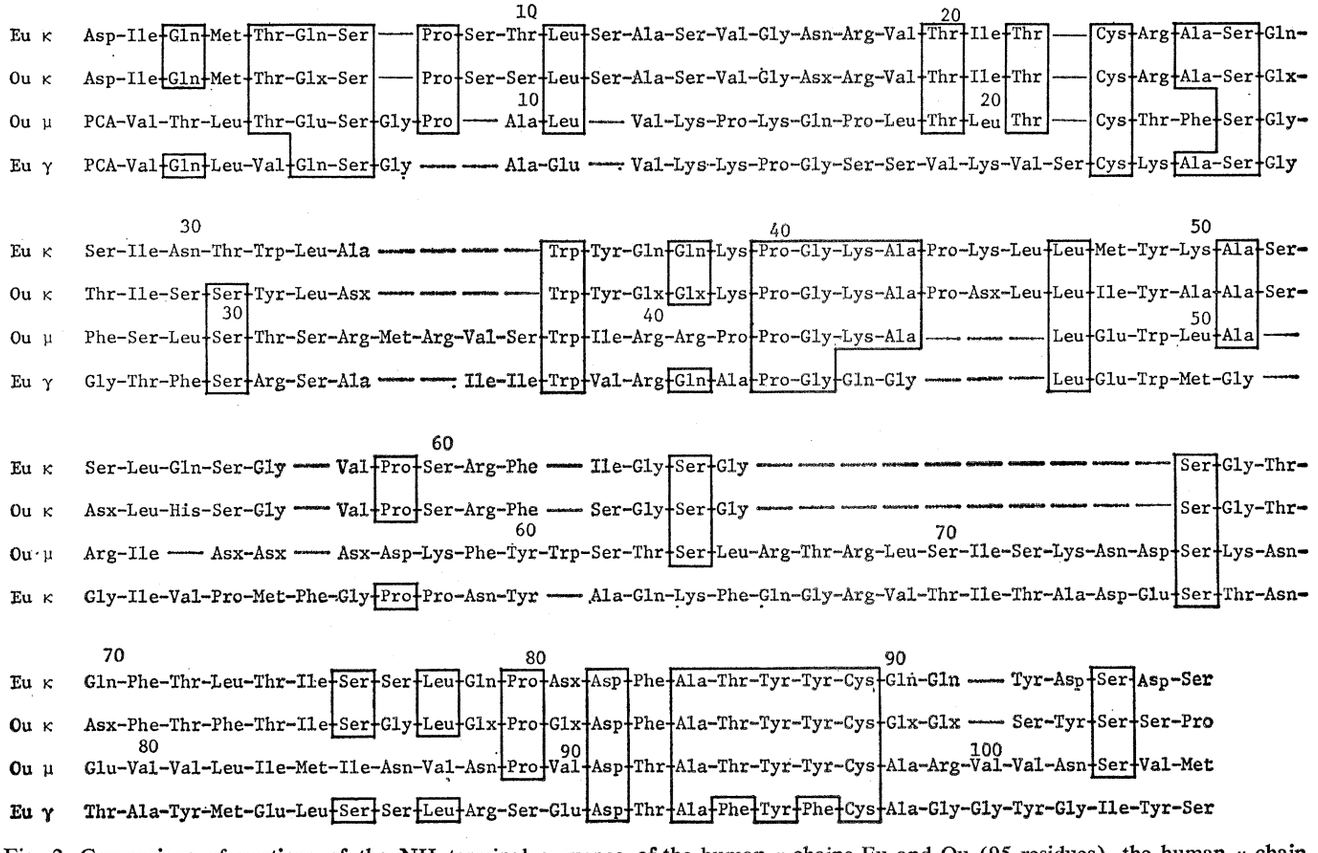

Fig. 2. Comparison of portions of the NH_2-terminal sequence of the human κ chains Eu and Ou (95 residues), the human μ chain Ou (105 residues) (*7*), and the human γ1 chain Eu (104 residues) (*5*). Identical residues in all four or in any three chains are included in boxes. Gaps have been introduced in the sequences to secure the maximum number of identities. The numbering system used for κ chains is given on the top line and for the μ chain on the third line.

and Ou. Another subgroup is based on similarity to the variable sequence of the γ1 heavy chain Eu. This second kind Cunningham *et al.* (*8*) refer to as subgroup I and the kind first discovered as subgroup II. We have extensive sequence data on μ chains that show that both of these subgroups are found in γM immunoglobulins, and we have discovered a third subgroup that differs from the first two in having a free NH_2-terminal residue of glutamic acid and its own characteristic variable region sequence (*20, 21*). This new subgroup, which we have designated subgroup III (*22*), includes two μ chains and an α_1 chain sequenced in our laboratory and a γ4 chain sequenced by Pink *et al.* (*10*). The light chains in each of these four immunoglobulins are all of different subgroups.

Our data for the constant region of the μ chain (*21*) indicate that the latter has less than 40 percent identity in amino acid sequence with the constant region of γ chains. Figure 1 gives evidence that the μ chain constant region may be somewhat more related in structure to that of subclass γ4 than to γ1. The disulfide bridge to the light chain involves a half-cystine that is homologous in μ and γ4 chains but which is replaced by serine in γ1 chains (*4–7*). As a result, the bridge between the light and heavy chains in γ1 is located at Cys-220 instead of at Cys-131 (*23*). In human γG1 globulin two disulfide bridges link the pair of μ chains.

This demonstration of a unique sequence for the variable regions of the light and heavy chains of the same γM molecule without any evidence for sequence ambiguity excludes a duality in the primary structure of macroglobulins. Such a duality has been suggested by the finding of two categories of binding sites, five weak and five strong, in the γM pentamer (*24*). This heterogeneity of binding sites is thus probably attributable to stereochemical masking of sites in such multivalent molecules.

The results illustrated in Fig. 2 show that the same subgroup of light chain (the κI type) can combine with heavy chains of different class and subgroup. Although antibody activity has not been demonstrated in these two molecules, this finding raises three questions: (i) Is antigen-combining specificity dependent on the subgroup of either the light or heavy chain? If so, does the light chain have an equal role in specificity for antigen or only a stabilizing function for heavy chain? (ii) Is the subgroup and even the variable sequence identical on the μ and γ chains, respectively, of the γM and γG antibodies formed successively in the primary and secondary immune responses to the same antigen? (iii) In the latter case is the light chain identical on the γM and γG antibodies against the same antigen?

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22. The term subgroup has been approved by a nomenclature discussion group of the World Health Organization to signify a set of similar variable-region sequences in heavy chains analogous to those in light chains. However, in heavy chains the subgroups are independent of the class of the chain, which is determined by the sequence of the constant region, whereas in light chains the kappa subgroups are restricted to kappa chains and lambda subgroups to lambda chains. The nomenclature for heavy chain subgroups is still undecided [*Bull. World Health Org.* **41**, 975 (1969)].
23. Abbreviations for amino acid residues: Ae-Cys, aminoethylcysteine; Lys, lysine; His, histidine; Arg, arginine; Asp, aspartic acid; Asn, asparagine; Asx, aspartic acid or asparagine, identity not established; Thr, threonine; Ser, serine; Glu, glutamic acid; Gln, glutamine; Glx, glutamic acid or glutamine, identity not established; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; Cys, half-cystine; and PCA, pyrrolidonecarboxylic acid. The first amino acid of the chain is designated Asp-1 and so forth.
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Sulfate-Binding Protein from Salmonella typhimurium: Physical Properties

Abstract. *This protein binds sulfate strongly and is implicated in sulfate transport in* Salmonella typhimurium. *It has a molecular weight of 32,000 and an axial ratio of 4 : 1. Crystals are elongate prisms up to 0.5 millimeter. X-ray diffraction photographs give discrete crystalline reflections to a spacing of at least 2 angstroms. The unit cell is orthorhombic* $P2_12_12_1$, *with four molecules per unit cell of 40.8 by 47.5 by 136 angstroms. This is consistent with a highly asymmetric molecule such as the prolate ellipsoid suggested by the other physical measurements. Addition of sulfate had minimum effects on the physical properties as measured by light absorption, optical rotary dispersion, circular dichroism, fluorescence and its depolarization, nuclear magnetic resonance, and sedimentation velocity.*

A protein that binds sulfate strongly and specifically has been isolated and crystallized (*1*) from *Salmonella typhimurium*. It appears to be characteristic of a class of similar proteins that may function in transport of the bound substrate (*2*). Preliminary physical and chemical characterization of the sulfate-binding protein has been reported (*3*). We report here detailed studies, par-