*IMMUNOGLOBULIN ASSEMBLY IN A MOUSE MYELOMA**

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Heavy and light chain subunits of immunoglobulin are synthesized on different size classes of polysomes in mouse myelomas[1-3] and immunized rabbit lymph nodes.[4-6] Nascent light chains are released from ribosomes and enter a pool of free light chain which eventually complements with heavy chain, forming the covalently associated immunoglobulin molecule, $(HL)_2$.[1-3, 7] Since the intracellular dimers HL,[2] H_2,[8] and L_2[9] have been proposed as synthetic intermediates in different myelomas, the pathway from nascent heavy and light chains to the finished tetramer has remained obscure, particularly with respect to the sequence of interchain disulfide bond formation. Part of the apparent contradiction could result from the experimental use of myeloma tumors with multiple stem lines.[10-12] By using an exponentially growing, cloned mouse myeloma tissue culture line, the most probable pathway of immunoglobulin biosynthesis and assembly was defined.

Materials and Methods.—The tissue culture line of MOPC21[13] was established from a clone by Kengo Horibata.[14] The line was maintained in Eagle's fortified medium[15] plus 10% horse serum at 37°C in 5% CO_2, 95% air. The cells had a doubling time of 15 hr and only secreted IgG_1 immunoglobulin. In some experiments, kappa-light chain secreted from MOPC41[13] was used as a marker. Secreted protein was prepared from the supernatant of a cell suspension after 4 hr in the presence of C^{14}-leucine as previously described.[10]

For amino acid labeling, exponentially growing cells were washed twice and resuspended in Eagle's medium minus leucine at 1×10^7 cells per ml. After 15 min at 37°C, the cells were labeled with H^3-leucine (45 mc/μmole, 20 μc/ml final concentration). For the preparation of polysomes, labeled cells were centrifuged and resuspended in pH 6.7 buffer[4] (0.025 *M* phosphate, 0.075 *M* NaCl, 0.01 *M* $MgCl_2$) containing 0.25 *M* sucrose. The cells were lysed with 1% Nonidet P.40,[2] the nuclei removed by centrifugation at $500 \times g$ for 5 min, and the supernatant fraction layered directly onto a 0.3–1 *M* sucrose gradient in the same buffer. The gradient was centrifuged at 5°C for 150 min (23,000 rpm in the 25.1 head of a Beckman model L-2 ultracentrifuge). OD_{260} was monitored through a Gilford continuous-flow spectrophotometer.

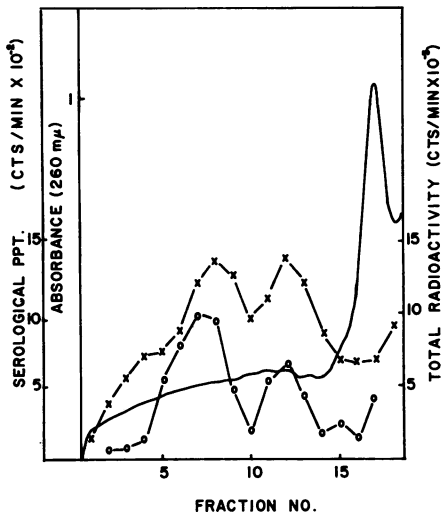
Serological precipitation was done by the indirect procedure. Fractions were diluted fivefold with saline, and 30 μl of rabbit anti-MOPC21 immunoglobulin were added and incubated for 15 min at 37°C. An excess of goat anti-rabbit immunoglobulin was then added, and incubation continued at 37°C for another hour. After refrigeration overnight, the precipitates were washed three times in cold saline. For nonspecific precipitation, a control of normal rabbit serum was used. All data are expressed as the difference between the two; the backgrounds are given in the figures. The rabbit antiserum was prepared against purified MOPC21 serum immunoglobulin, and reacted with kappa-type light chain, MOPC21 immunoglobulin, and the Fc fragment of normal mouse IgG. Precipitates were dissolved in 0.01 *N* NaOH, reprecipitated from 10% trichloroacetic acid, collected on Millipore filters, and counted in a scintillation counter.

Electrophoresis at pH 7.1 in 7.5% acrylamide, 0.5 *M* urea, and 0.1% sodium lauryl sulfate (SDS) was performed according to Maizel,[16] and fractions counted according to Choules and Zimm.[17] Serological precipitates were solubilized for electrophoresis by the procedure of Maizel;[16] 0.2 *M* 2-mercaptoethanol was used as a reducing agent when indicated. Electrophoresis was carried out with an internal control of C^{14}-labeled se-

creted MOPC21 immunoglobulin or C^{14}-labeled MOPC41 secreted light chain. Fractions are numbered from the negative to the positive electrodes. Purified secreted MOPC21 immunoglobulin had 65% of the leucine label in heavy chain.

Discontinuous sucrose gradients were prepared in 8 *M* urea and 0.05% SDS by successively layering 1.2-ml aliquots of 20, 15, 10, and 5%, or 30, 25, 20 and 15% sucrose in centrifuge tubes. Washed serological precipitates were dissolved in 0.2 ml 8 *M* urea and 0.05% SDS and layered on the gradient. Centrifugation was for 20 or 26 hr at 50,000 rpm in the SW65 head of a Beckman model L-2 at 23°C. Secreted C^{14}-labeled immunoglobulin and C^{14}-labeled MOPC41 light chain were serologically precipitated and used as internal controls. All data were corrected for counting overlap and efficiency.

Results.—The polysomal sites of heavy and light chain synthesis: Two sites of immunoglobulin-related synthesis, one for heavy chain and another for light chain, were defined on polysomes prepared from pulse-labeled cells by titration across the sucrose gradient with anti-MOPC21 (Fig. 1). Two polysome fractions involved in heavy and light chain synthesis were similarly implicated by other investigators.[1, 3] The peak fractions in the heavier and lighter polysome regions were separately pooled and precipitated with anti-MOPC21 immunoglobulin. The precipitates were then dissolved in SDS and urea, reduced, and electrophoresed in acrylamide with reduced C^{14}-leucine immunoglobulin as marker (Figs. 2*a* and *b*). Figure 2*c* shows the electrophoretic pattern of the



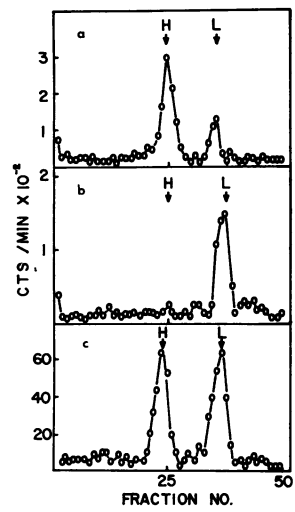


FIG. 1 (*left*).—Polysome titration profile of MOPC21. Cells were labeled for 10 min with H^3-leucine, polysomes prepared, and serological precipitations carried out as described in *Materials and Methods*. The nonspecific control was about 20% of the specific precipitate in tubes of maximum titer. (——), OD_{260}; (X-X-X), total TCA precipitate; (O-O-O), serological precipitate.

FIG. 2 (*right*).—Acrylamide electrophoresis of serological precipitates from the polysome gradient. Fractions were pooled from the heavy and light chain polysomes, and the supernatant fraction of the polysome gradient described in Fig. 1. The fractions were precipitated with anti-MOPC21 immunoglobulin, washed, reduced, and electrophoresed as described in *Materials and Methods*.

(*a*) Heavy chain polysomes; (*b*) light chain polysomes; (*c*) supernatant fraction. *H* marks the position of C^{14}-MOPC21 heavy chain and *L* that of C^{14}-MOPC21 light chain.

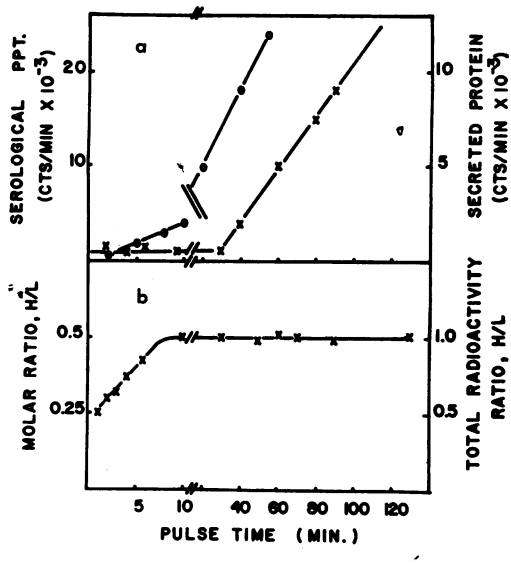


FIG. 3.—The heavy-to-light chain ratio found in the polysome supernatant at various times of labeling. MOPC21 cells were labeled with H^3-leucine for the indicated times, and the polysome supernatant precipitated and electrophoresed as described in Fig. 2. Secreted protein was determined by precipitating with TCA and counting a cell supernatant which contained only MOPC21 immunoglobulin as defined by G100 chromatography and acrylamide gel electrophoresis. (*a*) (O-O-O), Total amount of label serologically precipitated from the soluble gradient fraction; (X-X-X), secreted MOPC21 immunoglobulin.

(*b*) The calculated molar ratio and the total radioactivity ratio of heavy-to-light chain determined by the relative areas under the acrylamide peaks using as a reference purified, reduced C^{14}-leucine secreted protein. See text for detailed explanation.

serologically precipitable protein recovered from the top of the gradient (the polysome supernatant). The heavier and lighter polysome peaks defined by anti-MOPC21 are responsible for heavy and light chain synthesis, respectively, and light chains are also found associated with heavy chain polysomes. These findings extend those of other investigators.[1-3] A detailed account of the polysomal sites of immunoglobulin synthesis is presented elsewhere.[12]

Unbalanced synthesis of heavy and light chain: The free light chain pool tends to vary greatly with respect to intracellular immunoglobulin in different myelomas and immunized rabbit lymph nodes.[1-3, 8, 9, 18, 19] Since some of the myelomas secreted free light chain in addition to immunoglobulin, it was possible that they consisted of a mixture of two or more stem lines, some secreting immunoglobulin, and others, light chain only. In lymph nodes a heterogeneous population of cells is involved in antibody synthesis. None of these examples reflect immunoglobulin synthesis by a single plasma cell.

The pool of free light chains was determined in the cloned MOPC21 tissue culture line by labeling the cells for various lengths of time with H^3-leucine and examining the protein released into the polysome supernatant. Aliquots of the polysome supernatants were precipitated with anti-MOPC21, and the precipitates reduced and electrophoresed on acrylamide gels. The area under the heavy and light chain peaks were determined, and the molar heavy-to-light chain ratio calculated. The labeling kinetics of this growing cell line are shown in Figure 3. There was linear incorporation into serologically precipitable protein, and a lag of 25 minutes before the secretion of radioactive immunoglobulin (Fig. 3*a*). The calculated molar ratio of heavy-to-light chain increased linearly from 0.25 to 0.5 over a period of eight minutes, and remained constant for all longer labeling times (Fig. 3*b*). Since there is an equimolar ratio of heavy and light chains in immunoglobulin, the steady-state pool was two moles of free light

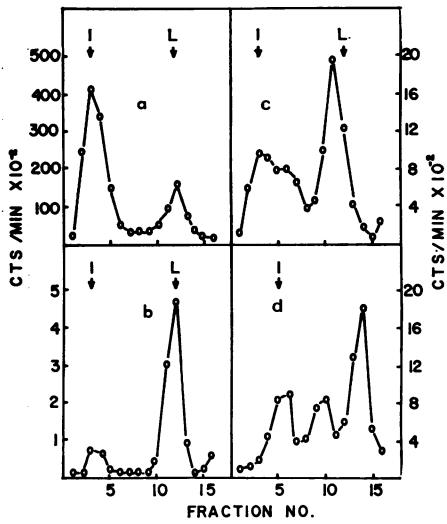


FIG. 4.—Sucrose gradients of protein serologically precipitated from polysome gradient fractions. Pulse-labeled polysomes were prepared, and the heavy chain, light chain, and soluble fractions precipitated as described in Fig. 2. The precipitates were dissolved in 8 *M* urea and 0.05% SDS, and layered onto either 5–20% (*a*, *b*, and *c*) or 15–30% (*d*) sucrose gradients in the same buffer. Centrifugation and monitoring are described in *Materials and Methods*.

(*a*) Polysome supernatant; (*b*) light chain polysome region; (*c*) heavy chain polysome region; (*d*) heavy chain polysome region, 26-hr centrifugation. *I* marks the position of C^{14}-MOPC21 immunoglobulin and *L* that of C^{14}-MOPC41 light chain.

chain for each mole of intracellular immunoglobulin. No detectable free light chain was secreted (less than 0.5% of the secreted immunoglobulin).

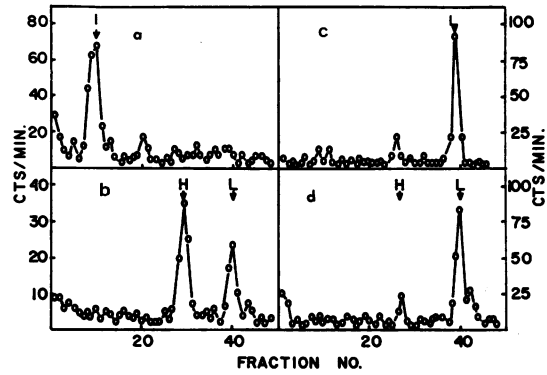
Identification of synthetic intermediates on polysomes and in the soluble cell fractions: The steady-state intermediates in immunoglobulin assembly were defined in MOPC21 labeled for ten minutes with H^3-leucine by the following procedure. Polysomes were prepared, and the heavy chain, light chain, and supernatant fractions were precipitated with anti-MOPC21. The precipitates were dissolved in urea and SDS and run on sucrose gradients in the same buffer (Fig. 4). Each component resolved in the gradients was then identified by acrylamide electrophoresis in the reduced and unreduced forms (Fig. 5). Secreted C^{14}-MOPC21 immunoglobulin and/or C^{14}-MOPC41 light chain were serologically precipitated and added as internal controls for both velocity sedimentation and electrophoresis. It will be shown that only immunoglobulin and free light chain are found in the polysome supernatant, while heavy chain polysomes contain monomeric heavy chain, HL, and $(HL)_2$.

The serologically precipitated protein found in the polysome supernatant consisted of immunoglobulin and free light chain monomers (Fig. 4*a*). The immunoglobulin had its interchain disulfide bonds intact, and upon reduction dissociated into a 1:1 molar ratio of heavy-to-light chain (Figs. 5*a* and *b*). (Neither free heavy chain nor HL was found in the polysome supernatant following a 1-min pulse, again showing that interchain disulfide bonds were formed prior to chain release.) Over 90 per cent of the material in the lighter peak on the gradient (Fig. 4*a*) consisted of free, monomeric light chains as defined by electrophoresis (Figs. 5*c* and *d*). The small amount of free heavy chain probably resulted from breakdown of some covalent interchain bonds, since 10 per cent of the C^{14}-labeled MOPC21 immunoglobulin marker broke down into heavy and light chain subunits when treated the same way; 0.1 per cent SDS increased this breakdown. This does not, however, rule out the possibility that less than 10 per cent of the immunoglobulin was released from the ribosomes without cova-

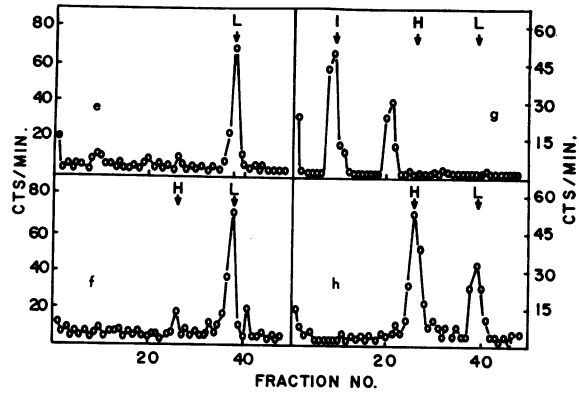
lent interchain bond formation. There was correspondingly little evidence for covalently associated L_2, HL, or H_2 dimers in the polysome supernatant.

The serologically precipitated protein from light chain polysomes (Fig. 4*b*) contained only light chain monomers and a trace of material the size of heavy

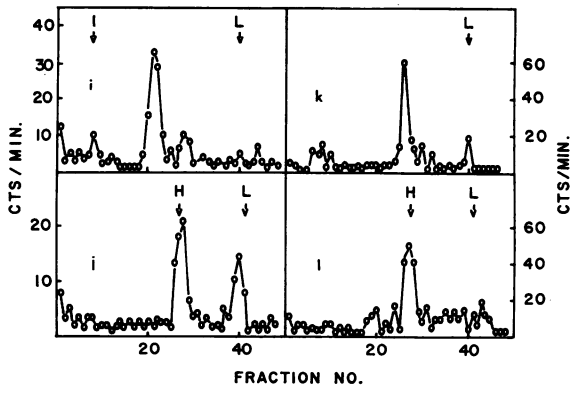
FIG. 5.—Identification of gradient components by acrylamide electrophoresis. Fractions from the gradients illustrated in Fig. 4 were dialyzed overnight against 0.01 *M* phosphate, pH 7.1, 0.5 *M* urea, and 0.1% SDS. One half of the sample was dialyzed against this buffer containing 1.0% 2-mercaptoethanol and the other half against buffer alone. The samples were electrophoresed as described in *Materials and Methods*.



(*a*) Immunoglobulin peak (*I*) of polysome supernatant, Fig. 4*a*, unreduced;
(*b*) Immunoglobulin peak (*I*) of polysome supernatant, Fig. 4*a*, reduced;
(*c*) Light chain peak (*L*) of polysome supernatant, Fig. 4*a*, unreduced;
(*d*) Light chain peak (*L*) of polysome supernatant, Fig. 4*a*, reduced;



(*e*) Light chain peak (*L*) of light chain polysomes, Fig. 4*b*, unreduced;
(*f*) Light chain peak (*L*) of light chain polysomes, Fig. 4*b*, reduced;
(*g*) Pooled fractions, immunoglobulin peak (*I*), Fig. 4*c*, unreduced;
(*h*) Pooled fractions, immunoglobulin peak (*I*), Fig. 4*c*, reduced;



(*i*) Intermediate peak between immunoglobulin (*I*) and heavy chain peak, Fig. 4*d*, unreduced;
(*j*) Intermediate peak between immunoglobulin (*I*) and heavy chain peak, Fig. 4*d*, reduced;
(*k*) Peak nearest light chain marker (*L*), Fig. 4*c*, unreduced;
(*l*) Peak nearest light chain marker (*L*), Fig. 4*c*, reduced.

chain and immunoglobulin (Figs. 5*e* and *f*). The latter probably resulted from polysome breakdown, bringing nascent protein found on heavy chain polysomes into the tetramer region of the gradient.

Three distinct molecular species were serologically precipitated from heavy chain polysomes (Figs. 4*c* and *d*). On the sucrose gradient, the largest ran identically with $(HL)_2$. The smallest protein ran slightly ahead of the light chain marker, was identified as heavy chain monomer by acrylamide electrophoresis, and constituted about 50 per cent of the label found on this polysome fraction (Figs. 5*k* and *l*). The two faster-sedimenting proteins were defined as HL and $(HL)_2$ by the following criteria. When the heavier, partially resolved, fractions from the 5 to 20 per cent sucrose gradient (Fig. 4*c*) were pooled, concentrated, and electrophoresed without reduction, two peaks were observed (Fig. 5*g*). The slower component electrophoresed identically with C^{14}-MOPC21 immunoglobulin and the faster component migrated more slowly than heavy chain. Since this acrylamide gel system separates primarily on the basis of size, it was possible to determine the approximate molecular weight of the faster component by using immunoglobulin, heavy chain, and light chain as markers.[20] Its calculated molecular weight was 70,000. Since the ratio of the areas under the two peaks in Figure 5*g* was 0.5, one would predict, upon reduction, a light-to-heavy chain ratio of 0.5 if these two proteins were $(HL)_2$ and HL, and a ratio of 0.28 if they were $(HL)_2$ and H_2. The observed ratio was 0.55 (Fig. 5*h*). By using a denser gradient it was possible to separate these two proteins (Fig. 4*d*). When electrophoresed in the unreduced form (Fig. 5*i*), the middle component of Figure 4*d* electrophoresed identically with the faster component in Figure 5*g*. Upon reduction, it dissociated into heavy and light chain subunits with a molar ratio of 1 (Fig. 5*j*), thus establishing that HL and not H_2 was found on heavy chain polysomes.

Discussion.—The following subunits of immunoglobulin were isolated from steady-state-labeled MOPC21 polysomes and soluble intracellular fractions: (1) light chain monomers were localized to light chain polysomes and to the soluble pool; (2) heavy chain monomers were found only on heavy chain polysomes; (3) HL was present only on heavy chain polysomes; (4) $(HL)_2$ was found on heavy chain polysomes and in the soluble pool. There was no evidence for H_2, L_2, and H_2L in any cell fraction, nor were significant amounts of heavy chains or HL dimers found released from ribosomes.

Assuming that light and heavy chain have but one growing point each, and that nascent light chain enters a pool from which it associates with the heavy chain,[1-3, 8, 18, 19] the following biosynthetic pathway may be inferred for MOPC21 immunoglobulin: (1) heavy and light chains are made on different size polysomes; (2) nascent light chain monomers are released, enter a pool, and complement with heavy chain monomers bound to the ribosomes of the heavy chain polysome, forming the covalently associated HL dimer; (3) the HL dimer complements with another dimer, either on the heavy chain polysome or directly after release to form the covalently associated immunoglobulin molecule; (4) the ribosome-bound immunoglobulin, $(HL)_2$, is released, and secreted after a 25-minute lag.

Because there is a synthetic excess of light chain in all of the myelomas studied,[1, 8, 9, 12] and also in immunized lymph nodes,[18, 19] it is most probable that immunoglobulin assembly in MOPC21 reflects the natural mechanism of antibody synthesis. It cannot be ruled out, however, that assembly proceeds through different intermediates, depending on the relative pool sizes of heavy and light chain. If, for example, heavy chain were made in excess, heavy chain dimerization might precede complementation with light chain.

The initial kinetics of amino acid labeling (Fig. 3) are best explained by assuming that eight minutes is the time required to saturate the light chain pool with labeled protein and to concomitantly remove incompletely labeled heavy chains from polysomes. There are twice as many leucines in the heavy as compared with the light chain. Therefore, under steady-state conditions, an equal amount of label in the heavy and light chain of protein serologically precipitated from polysome supernatants (Fig. 2*c*) would imply a molar heavy-to-light chain ratio of 0.5. At very short pulse times, however, an approximately *equal* amount of isotope would be incorporated into each previously initiated heavy and light chain prior to release. The ratio of total radioactivity in the light chain to total radioactivity in the heavy chain increased from 0.5 to 1.0 during the first eight minutes (Fig. 3*b*). Since the steady-state molar ratio of H/L is 0.5 and the molar rate of synthesis of H/L is also 0.5, the twofold excess of light over heavy chain cannot be due to differences in degradation rates nor to an only slightly higher rate of synthesis of light chain over heavy chain, coupled to accumulation of light chain in a pool which is diluted out by the increasing cell mass of the growing culture.

If the number of mRNA molecules coding for heavy chain were equal to those for light chain, and if the rate of translation were equal in both, the steady-state rate (moles of protein released from ribosomes per unit time) of heavy and light chain would be identical. Since this is not so, assuming the rate of translation to be a constant, there would be at least two mRNA molecules for light chain to one for heavy chain. There is, however, no *a priori* way to decide whether the inequality of synthetic rates is due to differences in translation, transcription, or the expression of two light chain genes and one heavy chain gene in these tetraploid cells. Whatever the regulatory control, light chain must be removed from the soluble pool at a rate of two moles for each mole of immunoglobulin secreted. The excess light chain could be degraded or converted into some other cell component; neither alternative is ruled out.

Additional support for the proposed immunoglobulin biosynthetic pathway is derived from a series of mouse myeloma mutants blocked at each of the various steps in assembly. Myelomas are known which are blocked in the complementation between HL dimers,[21] and also at the dimerization of heavy and light chain.[12] These mutants may have been generated by events leading to a change in tertiary structure which prohibits intracellular subunit complementation.

Summary.—It was shown that the biosynthesis of immunoglobulin by a cloned, exponentially growing mouse myeloma takes place through an HL intermediate associated with heavy chain polysomes. Light chain is synthesized

in a twofold molar excess over heavy chain, and is destroyed or incorporated into another cell component.

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