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Immunoglobulin Synthesis in a Cell-Free System*

Peter Ralph† and Alexander Rich‡

ABSTRACT: A cell-free system prepared from rabbit lymph nodes synthesizes predominately immunoglobulin heavy and light chains. The enzymatic and ionic requirements for optimal protein synthesis are shown. The synthesis of complete heavy and light chains is directed by two different size classes of polysomes. The number of ribosomes in the polysomes is that expected for mRNA molecules coding for individual complete polypeptide chains. Synthesis of the heavy and light chains also occurs if lymph node polysomes are incubated with supernatant enzymes from rabbit reticulocytes, rabbit liver, or rat liver. Analysis of the cyanogen bromide cleavage peptides of the heavy chain suggests that the bulk of the amino acid incorpration occurs by a COOH-terminal completion of polypeptides already initiated on polysomes *in vivo*.

Antibodies are synthesized in the plasma cells of lymph nodes. These proteins are characteristically composed of two heavy chains, each containing about 440 amino acids, and two light chains with about 215 amino acids each (Edelman and Gall, 1969). In the completed IgG molecule, these are bound together by disulfide linkages. The NH_2-terminal regions of these two types of chains vary greatly in their amino acid sequence, whereas the rest of the molecule is relatively invariant within a species. It has been suggested that these two regions may be coded for by different genes (Dreyer and Bennett, 1965; Wang *et al.*, 1970). Kinetic labeling experiments with whole cells suggest that the heavy and light chains are made as single intact units (Fleischman, 1967; Lennox *et al.*, 1967). However, it is of interest to study this question by *in vitro* experiments with the protein synthetic apparatus of the cell.

In a hyperimmunized rabbit, more than 70% of the protein synthesized by the lymph nodes *in vivo* is immunoglobulin (Becker *et al.*, 1970). The maximum synthesis of immunoglobulin occurs 3–5 days after a booster injection of antigen when cell proliferation has begun to subside (Mach and Vassali, 1965). This can be correlated with the appearance of two classes of polysomes in the lymph nodes (Becker and Rich, 1966), the development of two rapidly labeled RNA species (Kuechler and Rich, 1969a,b), and a concomitant rapid increase in serum antibody titer. The small polysomes contain 6–8 ribosomes and the larger ones contain slightly over twice this number of ribosomes (Becker *et al.*, 1970). By comparing the size of the polysome with the size of the proteins being synthesized in other cell types (Warner *et al.*, 1963; Heywood and Rich, 1968), we can make an estimate of the molecular weight of protein made by these two classes of polysomes. The biphasic lymph node polysome pattern is consistent for a tissue synthesizing predominantly polypeptide components containing 210–220 residues (light chains) and 440–480 residues (heavy chains), respectively. Experiments have also been carried out on closely related myeloma tumors which support this interpretation (Shapiro *et al.*, 1966; Williamson and Askonas, 1967; Schubert, 1968).

In the present paper the cell-free system prepared from rabbit lymph nodes is described; a brief report has been made earlier (Ralph *et al.*, 1967). Here we demonstrate that this system synthesizes the intact immunoglobulin molecule. Incubation of separate heavy and light polysome fractions yields labeled heavy and light chains, respectively. Furthermore, substitution of soluble enzymes from other tissues yields the same products, showing that the specificity for synthesis resides in the polysomes, presumably through its mRNA,

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† Present address: Salk Institute for Biological Studies, La Jolla, Calif.

‡ To whom to address correspondence.

rather than elsewhere. Cleavage of the completed heavy chain at methionine residues by the use of cyanogen bromide is used to identify the peptide product. This analysis shows that the C-terminal peptides are more heavily labeled than those at the NH_2 terminus. The bulk of the cell-free synthesis is therefore probably due to peptide-chain completion even though a small amount of peptide-chain initiation may also occur.

Methods and Materials

Immunization and Lymph Node Extract. Rabbits were immunized by hind footpad injection of ovalbumin or bovine serum albumin in incomplete Freund's adjuvant as described previously (Becker and Rich, 1966). Although immunization with antigen in complete Freund's adjuvant gives a higher serum titer and larger lymph nodes, the resulting cell-free system is inferior in protein synthesizing activity. This may be due to the necrosis which develops in the footpads after injection of large amounts of the adjuvant. Animals were sacrificed 3–5 days after a booster injection of antigen. The two popliteal lymph nodes, each weighing about 1 g, were excised and teased through a 30-mesh screen in 3–5 ml of 0.25 M sucrose in TKM buffer (0.01 M Tris (pH 7.4)–0.1 M KCl–0.01 M $MgCl_2$. About 30–40% of the cells lyse during this procedure to yield an extract (Becker *et al.*, 1970). Where indicated, detergents were added to samples to final concentrations of 1% v/v Tween 40 and 0.5% w/v sodium deoxycholate, or 0.2% v/v Nonidet P40 (Shell Chemical Co.) to effect complete cell lysis.

Cell-Free Systems. Total ribosomes were prepared by layering 2.5 ml of a 10-min 10,000*g* supernatant of the lymph node extract over a discontinuous gradient containing 2 ml of 0.3 M sucrose in TKM buffer layered over 1 ml of 1.8 M sucrose in TKM buffer. This was centrifuged for 90 min at 50,000 rpm in a Spinco SW50 rotor. The centrifuge tube was then sliced using a Beckman tube slicer just above the cloudy layer at the 0.3–1.8 M sucrose interface. The lower solution containing the ribosomes and polysomes was saved. The activity of these ribosomes in the cell-free system was similar to that of ribosomes pelleted by centrifugation for 2 hr at 50,000 rpm in a Spinco SW50 rotor and then resuspended by gentle agitation.

Polysomes were prepared from a 10-min 10,000*g* supernatant by layering on a 27-ml 0.3–1.0 M linear sucrose gradient in TKM buffer. This was centrifuged for 2 hr at 25,000 rpm in a Spinco SW25.1 rotor at 4°. Polysome fractions from this sucrose density gradient were pelleted by centrifugation for 2.5 hr at 50,000 rpm in a Spinco 50 rotor and were then resuspended in TKM buffer. Ribosome concentrations were estimated by absorbance, $A_{260}^{mg/ml} = 12$ (Peterman, 1964).

Lymph node enzymes were prepared from postribosomal supernatants of lymph node extracts by two methods. (1) Enzymes were prepared by pH 5 precipitation and resuspension in TKM buffer containing 6 mM mercaptoethanol. This solution was brought to pH 7.6 by the addition of one-tenth volume of 0.25 M Tris·HCl (pH 8.1). (2) The entire supernatant was dialyzed for 16 hr against two changes of TKM buffer (pH 7.6) plus 6 mM mercaptoethanol. Rabbit reticulocyte enzymes were prepared from reticulocytes of phenylhydrazine-treated rabbits (Borsook *et al.*, 1952). The postribosomal supernatant was dialyzed for 2 days against TKM buffer (pH 7.6) containing 6 mM mercaptoethanol. It was mixed with an equal volume of 95% glycerol and stored at −20° to protect aminoacyl-tRNA synthetases (Muench and Berg, 1967). The preparation retained its amino acid incorporating activity upon the addition of exogenous polysomes for over 2 months. Supernatant enzymes from rabbit liver and rat liver were prepared and stored in a similar manner. Protein concentration was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

The standard cell-free incorporating system contained the following per milliliter of solution: 0.2–0.4 mg of ribosomes, 0.4–0.8 mg of supernatant enzyme protein, 2 μmoles of ATP, 10 μmoles of phosphoenolpyruvate (PEP), 0.5 μmole of GTP, 50 μg of pyruvate kinase, 1 mμmole each of 20 amino acids which contained 1 μCi of a [U-^{14}C]amino acid mixture (New England Nuclear Corp.), and either 6 μmoles of 2-mercaptoethanol or 2 μmoles of dithiothreitol. The standard final ion concentrations, including contributions from the reagents, were 0.02 M Tris (pH 7.6), 0.04 M KCl, 0.05 M NH_4Cl, 0.03 M NaCl, and 7 mM $MgCl_2$. The incubation volumes varied from 0.1 to 2.0 ml in different experiments. Incubations were carried out at 37°, generally for 2 hr. After incubation, acid-precipitable incorporation was assayed. Samples were added to an equal volume of 10% trichloroacetic acid, heated for 10 min at 90°, then cooled to 4°, and collected on Millipore filters for counting (Nuclear-Chicago low-background counter, 24% efficiency).

Cell-free systems were also prepared from unfractionated, undialyzed postmitochondrial supernatants (3,000–20,000*g* for 10 min) of lymph node extracts by adding the energy-generating system, amino acids, and a reducing agent as described above.

Ion-Exchange Chromatography and Gel Electrophoresis. Purification of IgG by DEAE-cellulose chromatography was performed as described previously (Becker *et al.*, 1970). Ribosome-free supernatants of cell-free incubations together with added carrier rabbit IgG (Pentex) were dialyzed and chromatographed on DEAE-cellulose columns in 0.018 M phosphate (pH 6.3).

Acrylamide gel electrophoresis was carried out in sodium dodecyl sulfate. Samples of cell-free-synthesized material were reduced with dithiothreitol and alkylated with iodoacetamide in 6 M guanidine hydrochloride (Small and Lamm, 1966). The preparations were dialyzed against 0.5 M urea to remove the guanidine hydrochloride, and then dialyzed and electrophoresed according to Shapiro *et al.* (1966) for 3–4 hr at 5 mA in 5% acrylamide gels containing 0.1% sodium dodecyl sulfate–0.5 M urea–0.1 M phosphate buffer (pH 7.2). Proteins enter the gel system with an electrophoretic mobility that varies inversely with molecular weight (Shapiro *et al.*, 1967). The position of carrier heavy (H) and light (L) chains was determined by staining with Amido Schwarz dye. The cylindrical gels were frozen on Dry Ice and sliced into 0.47-mm sections. These were dried and counted in a low-background counter with an 80–90% recovery of radioactivity. Fractions are numbered from the origin.

Cyanogen Bromide Cleavage. Heavy-chain cyanogen bromide peptides were prepared according to the method of Fleischman (1967). The IgG fraction from DEAE-cellulose chromatography was further purified by chromatography on Sephadex G-150 in 0.15 M NaCl. The fraction containing IgG after gel filtration was reduced by treatment with 0.2 M mercaptoethanol for 2 hr at 37° in 0.5 M Tris (pH 8.5) and alkylated by the addition of solid iodoacetamide to 0.26 M. Heavy chains were purified on Sephadex G-100 columns in 1 N propionic acid, lyophilized, and cleaved with two to three times their weight of cyanogen bromide in 70% formic acid for 24 hr at room temperature. The mixture was degassed

in a flash evaporator to remove volatile compounds and applied to a Sephadex G-100 column in 1 N acetic acid.

Immunoglobulin Synthesis by Lymph Node Cells in Culture. Whole cells teased from lymph nodes were collected and washed by centrifugation for 2 min at 3000*g*, and resuspended in 3 ml of minimal essential medium (Grand Island Biological Co., N. Y.) containing essential amino acids at about 0.4 mM. The cell concentration was 10^7/ml. [^{14}C]Amino acids (5 μCi) were added and the suspension was incubated 4 hr at 37°. Cells were removed by a 10-min centrifugation at 3000*g*.

Results

Properties of the Cell-Free System. The cell-free system prepared from the lymph nodes of hyperimmune rabbits is very active in protein synthesis. It has already been shown that this system is dependent on undegraded polysomes, supernatant enzymes, and an energy-generating system (Ralph *et al.*, 1967). The synthetic reaction is inhibited by puromycin and ribonuclease. These properties exclude any significant synthesis by contaminating cells or by a soluble, nonribosomal-incorporating system (Kaji *et al.*, 1963; Gill, 1967).

Data are presented in Figure 1 for the optimization of Mg^{2+}, K^+, and NH_4^+ ions. The optimum concentration of magnesium ion is 6–7 mM in this system, which is similar to that found in other cell-free systems in higher organisms. In the presence of minimal amounts of ammonium ion (0.02 M NH_4^+ from the pyruvate kinase solution), the cell-free system incorporated maximally at a potassium ion concentration of 0.1 M (Figure 1b). However, a more active system is obtained at a lower potassium concentration by optimizing for ammonium ion as seen in Figure 1c. Maximum incorporation is obtained at 0.05 M NH_4^+ for potassium levels of 0.03 M. Identical aliquots were used in the experiments shown in Figure 1b,c using lymph node enzymes. It can be seen that the maximum incorporation in Figure 1c is almost twice that found in Figure 1b, showing the stimulating effect of the NH_4^+ ions.

At one time it was felt that the protein synthetic system responsible for antibody production differed in many ways from other protein synthetic systems. We have explored one possible facet of this by using heterogeneous systems in which the lymph node polysomes are incubated with supernatant enzymes from other tissues. An ammonium ion optimization study using supernatant enzymes from rabbit liver is shown in Figure 1c. It can be seen that the maximum incorporation is similar to that seen with the lymph node enzymes. The nature of the protein synthesized will be discussed below.

It is generally believed that the polysomes for secretory proteins are bound to the lipid membranes of the endoplasmic reticulum. It is therefore not surprising that treatment of extracts of lymph node cells with detergents such as a mixture of Tween 40 and deoxycholate preferentially increase the yield of polysomes (Becker *et al.*, 1970). We have analyzed the effect of detergent treatment on amino acid incorporation in the cell-free system (Table I). Detergent-treated polysomes, after separation from the detergent mixture, show up to twice the incorporating activity of untreated polysomes on an equal weight basis (Table I, expt 1). However the detergent treatment abolished the enzymatic activity of the supernatant in protein synthesis even when dialyzed 16 hr against two changes of buffer. The activity of detergent-released polysomes is substantially greater than that found with untreated polysomes when enzymes are prepared either by pH 5 precipitation or by dialysis of the supernatant. The two sets of reconstituted cell-free systems in Table I (expt 1) showed stimulations of 20 and 80%; experiments typically ranged between 50 and 100% stimulation. Dialyzed supernatants were generally more active than the pH 5 enzymes as shown in the identical aliquots used in Table I (expt 1).

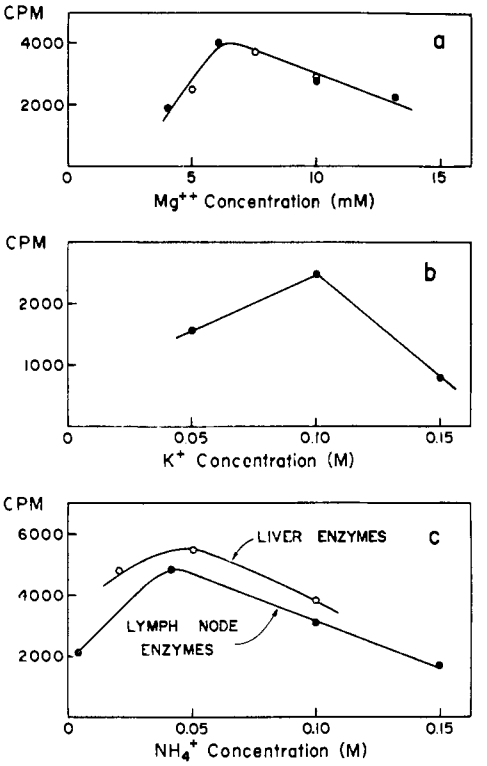

FIGURE 1: Dependence of cell-free amino acid incorporation on essential cations and enzyme preparations. (a) Variation of Mg^{2+}. (○) Reconstituted cell-free system: each point of the graph represents a 0.5-ml incubation containing 0.14 mg of lymph node ribosomes prepared from a 1.8 M sucrose cushion as described in Methods. Added to this were 0.22 mg of dialyzed supernatant enzyme protein, amino acids, and an energy-generating system. (●) Unfractionated cell-free system: a lymph node extract was centrifuged 10 min at 3000*g* as described in Methods. Amino acids and an energy-generating system were added to the supernatant. Each point represents a 0.1-ml incubation containing 0.05 mg of ribosomes and 0.1 mg of protein. Standard ionic conditions were used in both types of incubation. (b) Variation of K^+. Each point represents a 1-ml incubation containing 0.30 mg of ribosomes (from a 1.8 M sucrose cushion) and 0.38 mg of pH 5 protein at 0.02 M NH_4^+ with other ions at the standard concentration listed in Methods. (c) Variation of NH_4^+ and source of enzymes. (●) Lymph node enzymes: the system is the same as in part b. The incubation for the initial point, at 0.004 M NH_4^+, contained 0.1 M K^+. The remaining incubations were performed at 0.03 M K^+. (○) Liver enzymes: another preparation of lymph node polysomes was made by centrifuging into 1.8 M sucrose. Each point represents a 1-ml incubation containing 0.28 mg of ribosomes and 0.56 mg of dialyzed rabbit liver protein. The incubations all contained 0.01 M K^+ and other ions at their standard concentrations.

Cell-free systems were also prepared from the unfractionated postmitochondrial supernatants of lymph node extracts. These systems have the same Mg^{2+} optimum as the dialyzed supernatant systems (Figure 1a). When the nonionic detergent Nonidet P40 was added to unfractionated extracts the resulting cell-free system was stimulated 100% (Table 1, expt 2). This stimulation is presumably due to a greater activity of those ribosomes freed from enveloping membranes by the detergent, since Nonidet P40 partially inhibited the incorpora-

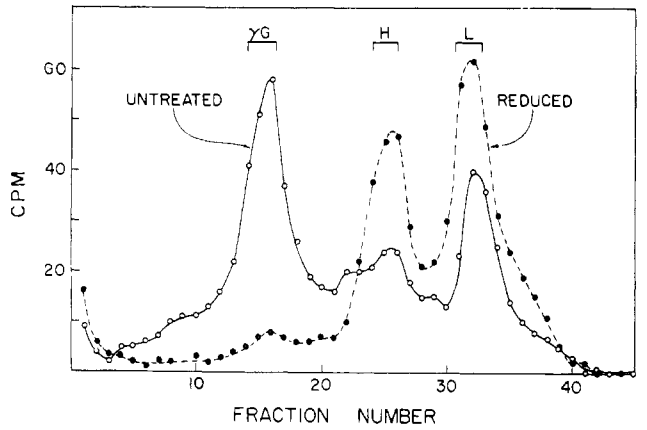

FIGURE 2: Acrylamide gel electrophoresis of DEAE-cellulose-purified proteins synthesized in the cell-free system. Radioactive proteins were synthesized in the system described in the legend to Table I (expt 2) using the detergent Nonidet P40. After incubation the solution was centrifuged 3 hr at 50,000 rpm in a Spinco 50 rotor. The ribosome-free supernatant, together with carrier rabbit γ-globulin (Pentex), was chromatographed on a DEAE-cellulose column (3 × 1.1 cm) as described in Methods; 7.5% of the incorporated radioactivity in the cell-free system supernatant was recovered in the IgG fraction. Half of the pooled IgG peak material was not treated (untreated); the other half was reduced and alkylated in guanidine hydrochloride (reduced). These were subsequently analyzed by electrophoresis in 5% acrylamide gels containing sodium dodecyl sulfate as described in Methods.

TABLE I: Effect of Detergents and Enzyme Preparation on Amino Acid Incorporation.

Polysomes	Enzymes	Incorporation (cpm) Total	No Ribosomes	Net
Expt 1. Reconstituted Cell-Free System[a]				
pH 5 Preparation				
a −Detergent	−detergent	1,420	230	1190
b +Deteregnt	−detergent	2,280	230	2050
c +Detergent	+detergent	104	0	
Dialyzed Supernatant				
d −Deteregnt	−detergent	2,820	430	2390
e +Detergent	−detergent	3,270	430	2840
f +Detergent	+detergent	930	0	
Expt 2. Unfractionated Cell-Free System[b]				
3,000*g* extract		7,462		
3,000*g* minus ATP, PEP,[c] kinase		492		
3,000*g* plus Nonidet P40		15,261		
20,000g extract		8,947		

[a] Incubations were carried out under standard conditions described in Methods, and contained 0.15 mg of ribosome and 0.36 mg of either pH 5 or dialyzed lymph node protein in 0.5-ml volumes. Ribosomes were omitted in parallel incubations. The detergent mixture, 1% Tween 40 and 0.5% deoxycholate, was added to the lymph node extract before preparing the polysomes or supernatant enzymes as indicated. [b] The unfractionated cell-free systems contained the supernatant of a lymph node extract after centrifuging for 10 min at 3000*g* or 20,000*g*. They were incubated under standard conditions as described in Methods. Each 1-ml incubation contained 0.4 mg of ribosomes and 1.2 mg of protein. Where indicated, Nonidet P40 was added to the lymph node extract to a concentration of 0.2% to release polysomes from membranes before low-speed centrifugation. Its final concentration in the cell-free system was 0.1%. [c] PEP = phosphoenolpyruvate.

tion by a similar cell-free system from reticulocytes, which do not have active, membrane-bound polysomes.

The most active cell-free systems were those obtained from unfractionated, undialyzed postmitochondrial supernatants, most of which incorporated 20,000–60,000 cpm of the mixture of labeled amino acids per mg of ribosomes (Table I, expt 2). Isotope dilution experiments with these systems indicate that the concentration of endogenous, unlabeled amino acids is about six times the concentration of the added labeled amino acids (Ralph, 1968). The total incorporation of amino acids in these experiments therefore represents 5–10 nmoles/mg of ribosomes, or 15–30 amino acids/ribosome. Short dialysis times (1–4 hr) sometimes increased the amount of radioactivity incorporated, but not the total synthesis since fewer unlabeled endogenous amino acids were incorporated.

In reconstituted systems with ribosomes and enzymes prepared separately, incorporation amounted to 10,000–30,000 cpm/mg of ribosomes or 0.5–1.0 nmole/mg. However, because there are few unlabeled amino acids in the system, this represents a tenfold decrease in incorporation compared with the unfractionated system.

Identification of Immunoglobulin Components as Soluble Products of the Cell-Free System. Experiments were carried out to identify the protein products synthesized in the cell-free system by lymph node polysomes. During incorporation, ^{14}C-labeled amino acids were taken up at a linear rate for 30–90 min. The amount of radioactivity in trichloroacetic acid precipitable material remained constant afterward. After the incorporation was complete, ribosomes were removed by centrifugation for 3 hr at 50,000 rpm. It was found that 50–75% of the incorporated radioactivity was released from the ribosomes in the soluble fraction. Complete reduction and alkylation of this fraction and subsequent analysis by Sephadex chromatography in guanidine and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed about half of the incorporated radioactivity to be in polypeptides of the size of immunoglobulin heavy and light chains (Ralph *et al.*, 1967).

To estimate the amount of complete immunoglobulin synthesized, the soluble products of the cell-free system were purified on a DEAE-cellulose column. Unlabeled carrier rabbit γ-globulin was added prior to the fractionation. About 8% of the trichloracetic acid precipitable radioactivity appeared in the IgG fraction. Analysis of this fraction by sodium dodecyl sulfate–polyacrylamide gel electrophoresis without further treatment shows that a major peak comprising 40% of the radioactive protein migrates with the carrier IgG molecules (Figure 2). The untreated sample also shows a peak containing 20% of the radioactivity which migrates with carrier light chains. This peak may represent light chains synthesized in the incubation which have not associated into completed molecules, since free light chains also elute from DEAE-cellulose in the IgG fraction. There is also some radioactivity in the region expected for IgG heavy chains and for heavy–light or heavy–heavy dimers. Some of these molecular forms may have dissociated in the sodium dodecyl sulfate

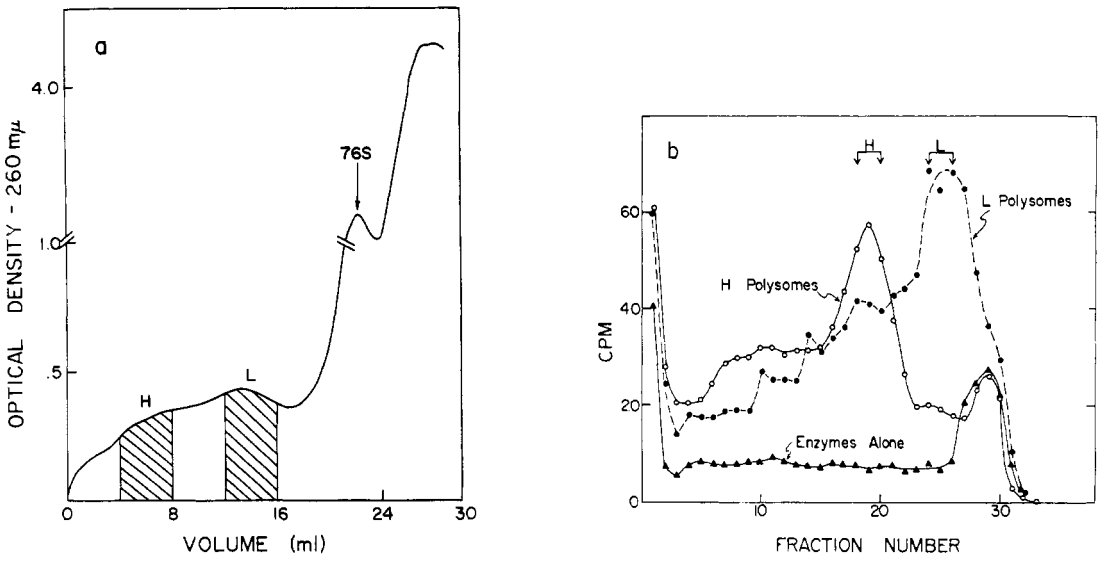

FIGURE 3: Identification of the polysomes synthesizing immunoglobulin heavy and light chains. (a) Polysomes used to demonstrate the separate syntheses of heavy and light chains. A lymph node extract was treated with 1% Tween 40 and 0.5% deoxycholate and centrifuged 10 min at 10,000*g*. The volume of the supernatant was 5 ml. Samples (2 ml) were layered on each of two 27-ml sucrose gradients for polysome preparation as described in Methods. One milliliter was diluted with 1 ml of 0.25 M sucrose in the extraction buffer and it was layered on a third gradient tube for analysis of the polysomes. The optical density tracing from this gradient is shown in part a. Polysomes from the regions marked H and L were used in separate cell-free systems. (b) Polyacrylamide gel electrophoresis of the proteins from cell-free systems containing heavy and light polysomes. The polysome fractions from the heavy (H) and light (L) regions (a) were collected and pooled from the other two gradients and pelleted as described in Methods. Three 0.7-ml cell-free systems were prepared as described in Methods. These contained 0.4 mg of reticulocyte enzymes and 0.17 mg of lymph node heavy polysomes, 0.20 mg of light polysomes, or no polysomes. After 30 min at 37°, 20-μl samples showed 129 cpm of trichloroacetic acid precipitable material incorporated by the heavy polysomes and 150 cpm by the light polysomes. Without the addition of polysomes, 26 cpm was incorporated by the enzyme preparation alone. The remainder of the incubations were analyzed by gel electrophoresis as described in Methods.

gel from IgG molecules which had not formed covalent disulfide bonds between the subunits.

An equal portion of the DEAE–IgG fraction was reduced and alkylated before electrophoresis. Figure 2 shows that 31 and 46% of the counts now appear in heavy- and light-chain peaks, respectively, with a concomitant loss of IgG radioactivity. This suggests that IgG molecules were assembled in the *in vitro* system from heavy and light chains both of which contained incorporated radioactivity. The increase in radioactivity in the heavy and light peaks following reduction and alkylation is approximately equal. Since the heavy chain is twice the length of the light chain, these results suggest that the light chains are more radioactive than the heavy chains.

Identification of the Polysomes Synthesizing Heavy and Light Chains. Cells synthesizing either antibody molecules or the closely related immunoglobulins of myeloma tumors characteristically have biphasic polysomes, with two different sizes (Becker and Rich, 1966; Schubert, 1968). In extracts of lymph nodes of hyperimmunized rabbits, these two polysome distributions are seen by measurements both of absorbance at 260 mμ and of radioactivity found in labeled nascent polypeptide chains following sucrose density centrifugation (Ralph *et al.*, 1967). The "light" polysomes each contain 6–8 ribosomes and the "heavy" polysomes contain slightly over twice as many as shown by their sedimentation properties and visualization in the electron microscope (Becker *et al.*, 1970).

To study the role of these polysomes in protein synthesis, lymph node polysomes were prepared on a sucrose gradient. Different fractions of the polysome region were pooled and tested in cell-free systems for their ability to synthesize immunoglobulin polypeptides, as measured by sodium dodecyl sulfate–polyacrylamide gel analysis. A typical experiment is shown in Figure 3. The two polysome groups marked H and L (Figure 3a) were incubated separately with rabbit reticulocyte enzymes. After incubation of the fractions, carrier IgG was added and the total mixtures were reduced and alkylated. The electrophoretic gel patterns of the incubation mixtures using these polysome are shown in Figure 3b. The control incubation containing only reticulocyte enzymes, but with no lymph node polysomes, showed only a small radioactive peak with the approximate mobility of the globin subunit of hemoglobin. When heavy or light polysomes were included in the incubation mixture together with reticulocyte enzymes, the amino acid incorporation was stimulated fivefold. Electrophoresis of these products showed prominent peaks of radioactivity which migrated with heavy and light chains, respectively. A limited synthesis of globin-like peptides is also evident in the presence of the lymph node polysomes. Thus the protein synthetic product of the large polysomes is a polypeptide chain which migrates with carrier heavy chains; the protein synthetic product of the small polysomes migrates with carrier light chains. Both the incubation containing H polysomes and that containing L polysomes show some labeled polypeptides migrating slower than heavy chains during electrophoresis. It is likely that these arise from incompletely reduced and alkylated molecules in which the chains have not separated completely. Single ribosomes, marked 76 S in Figure 3a, show very little amino acid incorporation in cell-free systems. Both heavy and light chains are produced by polysomes intermediate in size between the H and L polysomes.

Similar experiments were carried out using H and L polysomes with enzymes from the lymph node and from rat liver. The results were similar to those presented in Figure 3b, except that the contribution due to the enzymes alone showed a low background upon gel electrophoresis and did not yield

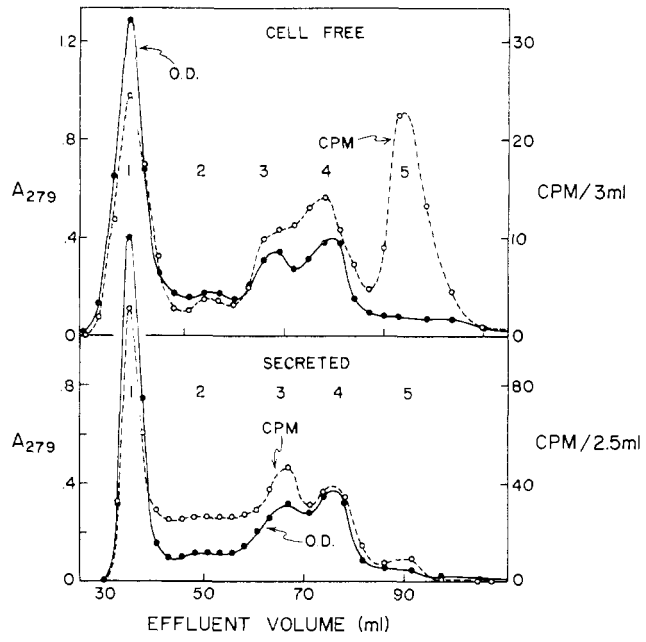

FIGURE 4: Cyanogen bromide peptides of completed heavy chains. Cell-free synthesis: an aliquot of the cell-free system supernatant described in the legend to Figure 2 was chromatographed with 40 mg of rabbit γ-globulin (Pentex) on a DEAE-cellulose column as described in Methods. The IgG fraction contained 8% of the initial radioactive protein. This fraction was then chromatographed on a Sephadex G-150 column (2 × 35 cm) in 0.15 M NaCl. The IgG peak contained 49% of the radioactivity and 86% of the OD_{279}, the latter due mostly to carrier portein. The IgG peak was mildly reduced and alkylated as described in Methods, and then chromatographed on a Sephadex G-100 column (2 × 35 cm) in 1 N propionic acid. The heavy-chain peak contained 47% of the applied radioactivity and 76% of the OD_{279}. Recoveries of radioactivity and uv absorbance from the Sephadex columns were greater than 90%. The heavy-chain fraction was lyophilized, cleaved with cyanogen bromide, and chromatographed on a Sephadex G-100 column (1.4 × 60 cm) in 1 N acetic acid as described in Methods. There was a 67% recovery of radioactivity following cyanogen bromide cleavage and an 87% recovery of applied uv absorbance. Cyanogen bromide peptides are labeled 1–5. Intact cell synthesis: a control study was carried out using labeled immunoglobulin secreted by intact lymph node cells cultured for 4 hr as described in Methods. Purification with carrier immunoglobulin by chromatography on DEAE-cellulose and Sephadex G-150 as above yielded 70–80% of the initial radioactivity and uv absorbance in the IgG fraction. As above, it was reduced and alkylated, and the purified heavy chains contained 75% of the radioactivity and 77% of the uv absorbance. The cyanogen bromide peptides were chromatographed on the same G-100 column as above with a recovery of 84% of the initial radioactivity and 89% of initial uv absorbance.

a peak in the position expected for globin molecules as seen in the reticulocyte enzyme preparation.

Distribution of Radioactivity in the Cyanogen Bromide Peptides of Heavy Chains. The demonstration of protein synthesized in a cell-free system with the proper mobility in polyacrylamide gels and ion-exchange chromatographic properties is suggestive, but not conclusive, evidence for immunoglobulin. Accordingly, experiments were carried out in which the properties of the protein depend on its amino acid sequence. This was accomplished by using the method of cyanogen bromide cleavage, which selectively attacks the methionine residues of polypeptide chains. This reaction is reported to be almost 100% complete and very specific (Givol and Porter, 1965). Heavy chains of rabbit IgG have 3 methionine residues in the COOH-terminal, constant half of the molecule. A minor fraction of these chains also contain additional methionine residues in their NH_2-terminal region (Cebra, 1967). Following cyanogen bromide cleavage of the heavy chain of IgG molecules, some of the resultant peptides are cross-linked by disulfide bonds and only five peaks of uv absorbance are seen by Sephadex chromatography.

Cell-free synthesis of heavy chains accounts for as much as 30% of the incorporated radioactivity in the soluble fraction released from ribosomes, but many of the molecules may be incompletely or improperly disulfide bonded. Thus a purification was first carried out to recover only those heavy-chain molecules found in the complete immunoglobulin. Passage of the soluble products of the cell-free system through DEAE-cellulose yielded 8% of the radioactivity with the ionic properties of IgG, as described above. Subsequent chromatography on Sephadex G-150 in 0.15 M saline showed one-half of this radioactive material to have the proper size for the 7S molecule (150,000 molecular weight). Analogous results are seen in Figure 2 in which the IgG peak comprises 40% of the untreated material. The IgG peak obtained from the Sephadex filtration was mildly reduced and alkylated. The heavy chains were then purified by filtration through Sephadex G-100 in 1 M propionic acid. A peak containing 47% of the radioactivity cochromatographed with the carrier heavy chains. The heavy chains were next cleaved with cyanogen bromide, and analyzed by gel filtration in Sephadex G-100 using 1 M acetic acid. This yields five peptide fractions which are shown in Figure 4 (cell free). Peaks 1 and 2 come from the NH_2-terminal half of the heavy chain; peak 2 is represented in only a fraction of all the molecules since they do not all have identical methionines (Cebra, 1967). The peptides in peaks 3, 4, and 5 comprise the COOH-terminal, homogeneous half of the heavy chains, in their natural NH_2- to COOH-terminal order. Peak 5 contains the COOH-terminal octadecapeptide.

The cell-free results shown in Figure 4 demonstrate two things. First, the peaks of radioactivity correspond to the absorbance profile of the carrier heavy-chain peptides. This indicates that amino acids were incorporated into all regions of the heavy chain by the cell-free system. It also indicates that a radioactive polypeptide chain has been synthesized with methionine residues in the same position as the unlabeled carrier IgG molecule. Second, there is a striking abundance of radioactivity in the COOH-terminal peptides of the molecule, peaks 4 and 5.

A control experiment was performed with uniformly labeled heavy chains synthesized by intact lymph node cells incubated for 4 hr with [^{14}C]amino acids. Heavy chains were purified from the secreted IgG and the cyanogen bromide peptides were prepared and fractionated as described for the cell-free heavy chains. The results are shown in Figure 4 (secreted). Here the radioactivity more closely follows the absorption profile. The radioactivity is due to the mixture of labeled amino acids; the absorbance at 279 nm is mainly due to tryptophan and tyrosine. The COOH-terminal peptide in peak 5 represents 4% of the amino acid residues of the heavy chain, and contains approximately the same fraction (4.6%) of the radioactivity in the molecules uniformly labeled by intact cells in culture. In contrast, 28% of the cell-free incorporated radioactivity elutes in this peak.

The data in Figure 4 can be presented in another fashion to show the relative distribution of protein synthetic activity in the cell-free system compared to the synthesis carried out by incubated intact cells secreting γ-globulins. The radioactivity in peaks 1 + 2, 3, 4, and 5 were integrated, and are plotted as specific activity by dividing the counts per minute

by the number of amino acids in each peptide fragment of the heavy chain. Peak 2 is obtained from only a fraction of all heavy chains, due to the variable presence of methionine residues in the NH_2 terminus (Cebra, 1967). Therefore the data for peaks 1 and 2 were pooled, since together they represent the total cyanogen bromide fragments from the NH_2-terminal end of the molecule. In Figure 5, the specific activity is plotted against the actual length of the various cyanogen bromide fragments for both secreted and cell-free synthesis.

Discussion

Cell-free systems should contribute information to help answer some of the basic problems in immunoglobulin synthesis. Among the important questions are the following: (a) Are heavy or light chains synthesized on templates coding for complete polypeptides or are they synthesized separately in the constant and variable regions? (b) Is there translational control of amino acid assembly? (c) What regulates heavy- and light-chain synthesis? (d) What is the mechanism of immunoglobulin assembly from heavy and light chains? In this paper we describe several features of the cell-free system which are relevant to these basic questions.

The cell-free system from the lymph nodes of hyperimmunized rabbits seems to reflect the activity of the intact cell, although only to a limited extent. In the hyperimmunized rabbit lymph node, over 70% of the protein products are IgG immunoglobulins (Becker *et al.*, 1970). This high degree of specialization is responsible for the characteristic biphasic polysome distribution which is maintained in the intact cell. In contrast to this about half of the soluble products of the cell-free system coelectrophorese with complete heavy and light chains (Ralph *et al.*, 1967). However, only 8% of the labeled protein has the charge properties of the characteristic IgG immunoglobulin as seen by DEAE-cellulose fractionation (Figure 2). This difference may be due to an imperfect folding of the heavy and light chains or an incomplete assembly so that it does not form completed IgG molecules. In its present form, this system appears not to be suitable for studying problems of immunoglobulin assembly. In the cell-free system of Lisowska-Bernstein *et al.* (1970) there is a similar proportion of IgG synthesis when mouse myeloma microsomes are used. The cell-free system thus characteristically produces a fraction of recognizable immunoglobulin about tenfold less than is seen in the intact cell. Furthermore, it does this at the expense of the polysomes. When cell-free synthesis terminates, the polysomes are almost entirely degraded (unpublished results). These results could be due to both nuclease contamination in the preparation as well as failure to initiate the synthesis of new proteins as discussed below.

In the intact node, equimolar amounts of heavy and light chains are synthesized (Becker *et al.*, 1970). In contrast to this, an excess of radioactivity is incorporated into light chains by the cell-free system (Figure 2 reduced). Microsomes from rat lymph nodes (Vassalli *et al.*, 1967) and from mouse myeloma cells (Lisowska-Bernstein *et al.*, 1970) also incorporate more label into light chains than heavy chains. This difference might be due to an alternation (or loss) of regulating mechanisms associated with heavy and light-chain synthesis, but most likely it reflects the fact that few of the heavy chains initiated *in vivo* are completed in the cell-free system (Figure 5), and a larger number of light-chain ribosomes may find their way to the end of the mRNA chain. Low levels of nuclease would bias the output in this direction by cleaving

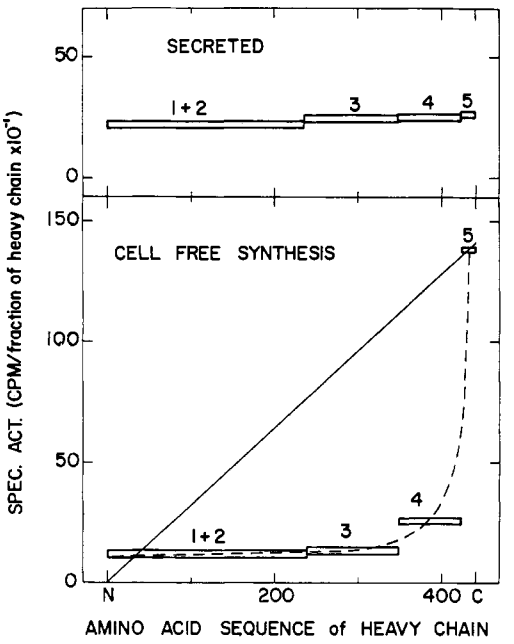

FIGURE 5: Specific activity of heavy-chain cyanogen bromide peptides secreted from intact cells as well as synthesized in the cell-free system. The data from the cyanogen bromide peptides of Figure 4 are plotted in terms of radioactivity per amino acid residue (or fraction of the heavy chain). The data from fractions 1 and 2 were pooled as described in the text. Data at the top show the distribution for heavy chains synthesized by intact cells in culture. At the bottom, the cell-free data are presented. The solid line passing through the origin represents the theoretical yield if there were no initiation in the system and all ribosomes, randomly placed on the mRNA, completed heavy-chain synthesis. Arbitrary amounts of radioactivity from secreted or cell-free heavy-chain material were applied to the columns. Thus only the shape of the two distributions is important, not their relative specific activities.

the longer (and therefore more vulnerable) heavy-chain polysomes to a greater extent. If more light chains are synthesized, they would tend to be more radioactive since the later chains would have more radioactive amino acids at the NH_2 terminus of the chain. This effect may contribute to the fact that the completed IgG molecules showed equal amounts of radioactivity in the separated heavy and light chains, even though one would expect a ratio of 2:1 if they both had the same specific activity.

The fidelity of translation in the cell-free system is shown by a study of the heavy-chain peptides. As shown in Figure 4, both intact cells and the *in vitro* system synthesize heavy chains which cleave in the same manner as added carrier rabbit γ-globulin. However, the pattern of synthesis differs substantially as shown graphically in Figure 5. The secreted heavy-chain peptides were all approximately equally labeled. There is a slight rise toward the C-terminal end of the molecule. This would be expected for proteins synthesized *in vivo* since the first proteins to be excreted would be C-terminal labeled, and only later proteins would be uniformly labeled. However, the pattern of cell-free synthesis is quite different, with over a tenfold increase in specific activity of fragment 5 compared to fragment 1 + 2. A very large portion of the cell-free synthesis is thus due to the completion of polypeptide chains already attached to polysomes at the start of the incubation. If there were no initiation in the cell-free system and the randomly distributed ribosomes on the mRNA all completed polypeptide-chain synthesis, the specific activity

curve would be the solid diagonal line shown in Figure 5 in the cell-free synthesis portion. The observed dashed line shows considerable departure from this, indicating that the probability of polypeptide-chain completion is much greater for a ribosome near the COOH-terminal end of the mRNA than for a ribosome near the end corresponding to the NH_2 terminus. This effect could be due to many factors, such as nuclease cleavage of the mRNA, or to an artificial release of the ribosome prior to chain completion. Extrapolation of the observed dashed curve to the left in Figure 5 does not go through the origin. If there were no initiation in the system, we would expect this curve to pass through the origin; therefore it is possible that there is a small amount of initiation in the cell-free system. Unfortunately due to the fact that the NH_2-terminal half of the molecule (fragments 1 + 2) had to be treated as one piece, we do not have enough information to state with assurance that a small amount of initiation occurred.

It should be noted that the radioactive peptides analyzed in Figure 4 are derived from heavy chains purified from whole IgG molecules and therefore represent only a fraction of all heavy chains synthesized in the cell-free system. Polypeptides completed early in the incubation may be preferentially assembled into H_2L_2 structures, and would thus show the predominance of labeled COOH-terminal peptides. Analysis of the total population of heavy chains may show considerably more NH_2-terminal labeling. However, it is important to note that the entire H chain is labeled, and there is no suggestion that the mRNA in the polysomes individually specifies smaller segments of the entire polypeptide chain. Even though there is a gradient of labeling in going from the NH_2 to the COOH terminus of the heavy chain, there are no discontinuities.

The most active cell-free systems described here incorporate 5–10 nmoles of amino acids/mg of ribosomes, or an average of 20 amino acids/ribosome. Assuming that only the polysomes are active, which represent about 50% of all the ribosomes (Becker *et al.*, 1970), the cell-free system probably incorporates an average of 40 amino acids/active ribosome. Heavy and light chains contain about 440 and 210 amino acids, respectively; thus cell-free incorporation only accounts for the synthesis of a part of each chain on the average. This does not exclude the possibility that a small fraction of polysomes is synthesizing complete chains as is suggested in Figure 5.

Overall improvements may be necessary in the efficiency of the system before enough incorporation is obtained in the NH_2-terminal regions of the molecule to be able to test any translational regulation of antibody specificity. The ability of enzyme fractions from reticulocytes to complete the synthesis of heavy and light chains from lymph node polysomes (Figure 3) shows there is no gross difference in protein synthesis between these two cell types. It should be mentioned that any regulation at the level of initiation of chain synthesis probably would not be seen in these experiments, and possible differences in carbohydrate addition, disulfide-bond formation, or special membrane functions were not investigated.

In a further study of the specificity of the cytoplasmic translation process, cell-free systems were reconstituted with identical aliquots of rabbit lymph node polysomes and different sources of enzymes—rabbit lymph node, rabbit liver, rabbit reticulocyte, and rat liver (Ralph, 1968). The soluble products of these incubations were reduced, alkylated, and analyzed in sodium dodecyl sulfate–polyacrylamide gels. It was found that radioactivity in heavy- and light-chain polypeptides predominated in all systems, with no systematic difference in the ratio of radioactivity in the heavy and light chains. Control experiments were carried out with cell-free-incorporating systems in which the polysomes were prepared from chick embryo muscle, rat liver, and rabbit reticulocytes. In contrast, these products showed no peaks of radioactivity which migrated with carrier heavy and light immunoglobulin chains. Thus the synthesis of these two classes of proteins requires the participation of lymph node polysomes, and is apparently not dependent on soluble factors specific to lymph node tissue.

Incubation of separated polysome fractions from the two major heavy and light peaks yielded predominantly heavy- and light-chain material respectively (Figure 3). If variable and constant regions were synthesized on different polysomes, appreciable quantities of these fragments should be seen, especially since the reticulocyte source of enzymes would not be expected to provide the joining mechanism to form complete light or heavy chains. These results are in agreement with the binding of antibodies against heavy or light chains (Williamson and Askonas, 1967), as well as the analysis of nascent polypeptide chains on large and small polysomes (Shapiro *et al.*, 1966; Schubert, 1968) in mouse myelomas. All of these results suggest that the heavy chain is synthesized as one long molecule on the large polysome, and the light chain on the smaller polysome. The number of ribosomes on the heavy and light polysomes are consistent with the relationship between polysome size and polypeptide size found in hemoglobin synthesis (Warner *et al.*, 1963) and in the synthesis of myosin, actin, and tropomyosin (Heywood and Rich, 1968). This does not rule out the possibility of separate genes specifying constant and variable regions with a joining mechanism either at the DNA or mRNA level before translation (Lennox and Cohn, 1967; Dreyer and Bennett, 1965), but clearly makes a joining mechanism unlikely with the completed polypeptide chains. Experiments have been carried out to analyze the rapidly labeled RNA in polysomes of intact rabbit lymph nodes. Two fractions are found which have a size and temporal distribution such that they are good candidates for the heavy- and light-chain mRNAs (Kuechler and Rich, 1969a,b). These observations also reinforce the interpretation that only complete heavy and light chains are made during translation.

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Mercury Derivatives of the Fab and Fc Fragments of a Human Myeloma Protein*

Lisa A. Steiner† and Peter M. Blumberg

ABSTRACT: To explore the role of the covalent interchain bonding in the structural organization of immunoglobulins, and to develop a general method for attaching heavy metals to these proteins, the reactions of the interchain disulfide bridges with mercuric ion have been investigated. In the Fab and Fc fragments of a human myeloma protein of subclass IgG1, a single atom of mercury forms a stable complex with the pair of cysteine residues derived from each interchain bridge. The mercury derivative of Fab resembles native Fab except that the disulfide bond joining light chain to Fd is replaced by a bridge of the type S–Hg–S. In contrast, the introduction of mercury into Fc induces a rearrangement of the disulfide bonding. When the two closely spaced interchain bridges in this fragment are reacted with Hg^{2+}, they re-form as intrachain bridges with mercury held in a compact ring between two cysteine and two proline residues. The Fc fragment is accordingly converted into a pair of monomeric subunits. Provided that they are not exposed to dissociating solvents, the mercury derivatives are indistinguishable from the native proteins by a number of structural and immunochemical criteria. The crystal structure of the derivative of Fc was closely related to, but not isomorphous with, that of native Fc.

Immunoglobulins are composed of one or more units of a basic tetrameric molecule consisting of two heavy and two light polypeptide chains (reviewed by Edelman and Gall, 1969).[1] Although the heavy and light chains are generally cross-linked by disulfide bonds, noncovalent interactions among the chains are usually sufficient to preserve the tetrameric structure and the activity of these proteins. Indeed, cleavage of the interchain disulfide bonds of antibodies by reduction and alkylation may have little or no effect on antigen binding (Weir and Porter, 1966; Jaton *et al.*, 1968). Moreover, in certain classes of immunoglobulin, some of the light and heavy chains are not linked to each other by disulfide bridges, but are held together by noncovalent interactions alone (Abel and Grey, 1968; Grey *et al.*, 1968). However, some functions of antibodies, such as complement fixation, may be weakened by disruption of the interchain bonding (Schur and Christian, 1964).

To obtain further information on the role of the interchain bridges in the structural organization of these molecules, we have initiated studies in which these bonds are modified by the introduction of a bifunctional reagent. A simple reagent of this type with a high degree of specificity for the sulfhydryl group is the divalent mercuric ion. Several proteins have been modified by reduction and reaction with Hg^{2+} to form derivatives with one or more disulfide bonds replaced by bridges

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† To whom correspondence should be addressed.

[1] The nomenclature used is that recommended by the World Health Organization (1964, 1965, 1966).