

Synthesis and Secretion of γ -Globulin by Lymph Node Cells, VIII. Order of Synthesis of the Interchain Disulfide Linkages of Immunoglobulins*

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Communicated by Marshall Nirenberg, April 13, 1970

Abstract. Murine myeloma cells (ADJ-PC-5), incubated *in vitro* with ^3H -leucine, secrete ^3H -immunoglobulin G as a single molecular species as judged by the migration characteristics of the labeled product on sodium dodecyl sulfate-acrylamide gel electrophoresis. However, the fact that some of the interchain disulfide linkages of intracellular immunoglobulins had not been acquired permitted the identification of the following intracellular species: LHHL (identical to immunoglobulin G), HHL, HH, and L (H and L refer to heavy and light polypeptide chains, respectively). Although HH and HHL were readily observed, radioactivity was not detected in the region of the gel where HL would be expected. The time course for the appearance of the intermediates indicates that in these cells the first interchain disulfide bond to be formed occurs between heavy chains. In contrast, the interchain disulfide bonds of immunoglobulins derived from rabbit lymph node cells were acquired in a different order. The principal intracellular species observed were LHHL and HL, whereas HHL and HH were not detectable. These findings indicate that in this species the first interchain disulfide bond to be formed is that between the heavy and light chains of immunoglobulin G.

A knowledge of the order of assembly of the heavy and light polypeptide chain subunits of immunoglobulin, besides affording an insight into the mechanism of synthesis of this glycoprotein, has importance for an understanding of the intracellular events involved in secretion. In previous studies in this laboratory concerning the mechanism of secretion,¹⁻³ a difference in the intracellular distribution of at least some kinds of partially assembled molecules was demonstrated when it was shown that rabbit immunoglobulins labeled solely in their protein moiety were segregated from those molecules labeled solely in their carbohydrate component.⁴ The occurrence in myelomas of other types of molecules which were partially assembled with regard to the acquisition of interchain disulfide bridges has also been reported.⁵⁻⁷ Thus, molecules composed of two heavy chains or of two heavy chains and one light chain or of one heavy and one light chain have been observed. To achieve a better understanding of the behavior of such partially assembled molecules in relation to the synthesis and secretion of immunoglobulins, we assessed the order of acquisition of interchain disulfide bonds in rabbit lymph node cells and reexamined this

process in murine myeloma cells. In this report, evidence is presented indicating that the initial disulfide linkage of rabbit immunoglobulin G was between a heavy and a light chain while the initial linkage of murine myeloma (ADJ-PC-5) immunoglobulin G was between heavy chains.

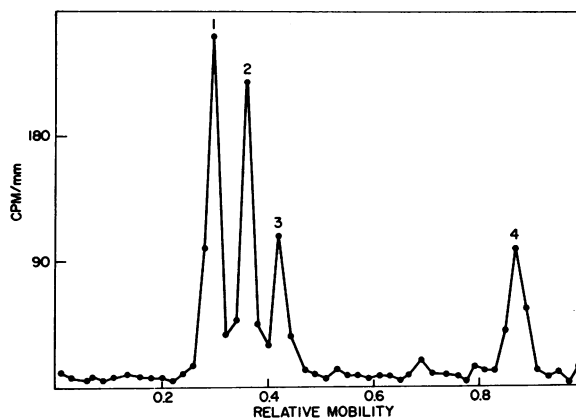
Materials and Methods. The materials used and the procedures followed for the immunization of the rabbits were those previously described.^{8,9} The ADJ-PC-5 myeloma, generation number 137-150, maintained by serial subcutaneous transplantation, was provided by Dr. Michael Potter as transplantation generation number 120.

The isolation and incubation of the cells was as previously described.^{1,8,9} Throughout the study myeloma cells were treated in the same way as those of the rabbit. The specific activity of the L-leucine-4,5-³H (New England Nuclear) was 5000 $\mu\text{Ci}/\mu\text{mole}$, that of the L-leucine-U-¹⁴C (New England Nuclear) was 275 $\mu\text{Ci}/\mu\text{mole}$. Incubation was terminated by rapidly chilling and centrifugally separating the cells from the medium. The cells were lysed by treatment for 10 min at 4°C with 0.5% sodium deoxycholate in 0.15 M NaCl-0.01 M sodium phosphate, pH 7.1, containing 0.05 M iodoacetamide. The viscous mass was diluted 6- to 14-fold with 0.05 M iodoacetamide in 0.15 M NaCl - 0.1 M sodium phosphate, pH 7.1, and warmed at 37°C for 10 min to allow alkylation of thiol groups. Material treated in this way was centrifuged at 100,000 *g* for 1 hr. The clear supernatant fluid was concentrated in a collodion bag (Schleicher and Schuell, no. 100) by negative pressure ultrafiltration and dialyzed against 0.002 M sodium phosphate, pH 7.1. The medium from the incubation was similarly concentrated and dialyzed after centrifugal clarification at 9000 *g* for 10 min.

Samples were prepared for gel electrophoresis by treating a portion for 1 hr with a precipitating mixture of the unrelated immune system, egg albumin, and goat anti-egg albumin to remove nonspecific coprecipitable radioactivity. The labeled immunoglobulin in the supernatant fluid was specifically coprecipitated by the addition of the appropriate combination of either rabbit γ -globulin and goat anti-rabbit γ -globulin or mouse γ -globulin and rabbit anti-mouse γ -globulin. The radioactive coprecipitated material, containing about 0.5 mg of protein, was dissolved in 2% sodium dodecyl sulfate containing 0.5 M urea and 10% acetic acid,¹⁰ then dialyzed in Visking bags against 0.1% sodium dodecyl sulfate, 0.5 M urea, 0.01 M sodium phosphate, pH 7.1, for about 10 hr. The sample was then subjected to electrophoresis on a polyacrylamide gel (5% acrylamide 0.13% *N,N'*-methylenebisacrylamide) with 0.1% sodium dodecyl sulfate, 0.5 M urea, 0.1 M sodium phosphate, pH 7.1, as the buffer,¹¹ and horse heart cytochrome *c* (Mann Research Laboratories) as the reference marker. The gels were sliced with a device resembling an egg slicer,¹² dissolved in 30% hydrogen peroxide at 60°C,¹³ and counted by use of 10% NCS (Nuclear Chicago) in the toluene/Liquifluor (New England Nuclear) scintillant.

Results. The heavy (H) chain polypeptide units of immunoglobulin G are linked to each other and to a light (L) chain subunit by noncovalent forces as well as by covalent disulfide bonds. These covalent and noncovalent linkages can be readily distinguished by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Under such circumstances the detergent nullifies noncovalent interactions permitting the detection of partially assembled molecules linked solely by disulfide bridges. For example, the radioactivity from murine myeloma cells (ADJ-PC-5) that was specifically coprecipitable with carrier mouse γ -globulin and rabbit anti-mouse γ -globulin yielded four peaks on acrylamide gel electrophoresis (Fig. 1). However, when iodoacetamide was not included in the cell lysis medium, thereby permitting further disulfide bond formation, only a single peak (peak 1) was observed. The possible artifactual production of partially assembled molecules from intact immunoglobulin,

FIG. 1.—Electrophoretic pattern of radioactive immunoglobulin components from ADJ-PC-5 cells. ADJ-PC-5 cells were incubated in 30 ml beakers containing 3.0 ml of medium, 2×10^8 cells, and 1.5 μ Ci of 14 C-leucine. After 180 min of incubation the pooled cells were harvested by centrifugation and processed as described in *Materials and Methods*. The specifically coprecipitated immunoglobulin components derived from 5.2×10^7 ADJ-PC-5 cells were applied to the gel for electrophoresis.



as a consequence of disulfide cleavage, appears to be excluded by the fact that the lysis of unlabeled cells in the presence of both iodoacetamide and 3 H-labeled extracellular immunoglobulin yielded a single radioactive peak (peak 1) coincident with the single peak characteristic of the labeled product secreted by these cells.

Several lines of evidence support the view that the material in peak 1 (Fig. 1) is composed of immunoglobulin molecules containing two heavy and two light chains (LHHL). First, the intracellular radioactivity applied to the gel for electrophoresis was most likely immunoglobulin in character because it was initially isolated by coprecipitation in the presence of antisera specific for the heavy and light chains of mouse immunoglobulin. Moreover, ADJ-PC-5 cells are known to secrete immunoglobulin G¹⁴ composed of two heavy and two light chains and, as mentioned previously, the 3 H-labeled extracellular immunoglobulin product yielded a single peak identical with peak 1. Finally, reduction and alkylation of the material extracted from peak 1 yielded two peaks on subsequent electrophoresis which had the migrational characteristics expected for heavy and light chains. By using these criteria, the successively more mobile peaks labeled 2, 3, and 4 (Fig. 1) were identified as HHL, HH, and L, respectively. Evidence relating to some of the characteristics of the first three peaks is presented in Table 1. The molecular weight estimates determined from relative mobilities on electrophoresis are in agreement with molecular weights that can be calculated from the suggested heavy and light chain content. Furthermore, the ratio of radioactive heavy and light chains derived from each of the peaks after reduction and alkylation provides additional support for this identification of the peaks. As would be predicted from theory the heavy chain: light chain ratio of radioactivity in HHL was found to be about twice that of LHHL. Furthermore, at least 93% of the radioactivity of the HH peak was observed as heavy chains subsequent to reduction and alkylation.

Although the observation of molecules such as HH and HHL suggests their role as intermediates in the formation of LHHL, compelling evidence for this relationship would be provided by a kinetic demonstration that HH and HHL are, indeed, precursors of the final product. The time course of appearance of

TABLE 1. Characteristics of the intracellular immunoglobulin of ADJ-PC-5.

| Peak in Fig. 1 | R_{cyto}^* | Molecular Weight† | | H/L‡ | Assigned composition |
|----------------|---------------------|-------------------|-------------|------|----------------------|
| | | Estimated | Theoretical | | |
| 1 | 0.28 | 160,000 | 156,000 | 1.7 | LHHL |
| 2 | 0.34 | 134,000 | 133,000 | 3.1 | HHL |
| 3 | 0.41 | 109,000 | 110,000 | 13.5 | HH |
| 4 | 0.85 | 30,000 | 23,000 | 0 | L |

* Electrophoretic mobility relative to that of cytochrome *c*, in each case the numbers represent the mean of at least 16 observations.

† Estimated graphically (see refs. 15-17) using the present system of sodium dodecyl sulfate-gel electrophoresis and proteins with known, independently estimated, molecular weights. In this system radioactive heavy-chains of ADJ-PC-5 had a relative mobility of 0.63 (est. mol wt 57,000). In deriving the theoretical molecular weight it was assumed that H = 55,000, L = 23,000.

‡ The immunoglobulinlike material present intracellularly after 3 hr of incubation of ADJ-PC-5 cells with ^3H -leucine was prepared as described in *Materials and Methods* and a portion subjected to electrophoresis. The gel was sliced and half of each slice tested for radioactivity to generate a curve such as in Fig. 1. The remaining half-slices for each radioactive peak were then extracted with a small volume of distilled water, reduced with dithiothreitol, alkylated with iodoacetamide, subjected to electrophoresis again, and the radioactivity in the regions of the heavy and light chains compared. The combined results of two such experiments are recorded.

radioactivity in the various intracellular immunoglobulin components is presented in Figure 2. In this experiment, the only distinct peak observed after 2 min of incubation of cells with ^3H -leucine, was in the light chain region (Fig. 2A). Readily discernible peaks having the relative mobility defined for L, HH, and HHL were observed at 10 min (Fig. 2B). In other experiments both HH and HHL were detected as early as 5 min. The small peak in the LHHL region that was marginally detectable at 10 min (Fig. 2B) was clearly evident at 60 min (Fig. 2C). A well-defined heavy chain peak was not discernible at 2, 10, or 60 min despite the relative ease of detection of light chains or the heavy chain dimer (HH). More significantly, a peak that would correspond to a half molecule containing one heavy and one light chain (HL) was never observed on electrophoresis of more than several dozen intracellular contents examined during the course of this study.

A summary of the order of appearance of the various peaks relating to the assembly of the immunoglobulin of ADJ-PC-5 is shown in Table 2. It is note-

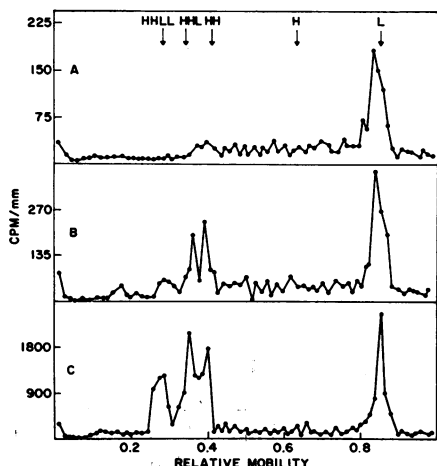


FIG. 2.—Electrophoretic pattern of radioactive immunoglobulin components from ADJ-PC-5 cells as a function of time of labeling. ADJ-PC-5 cells were incubated for 2, 10, or 60 min in 30-ml beakers containing 3.0 ml of medium, 2×10^8 cells, and 30 μCi of ^3H -leucine. The remainder of the procedure is described in *Materials and Methods*. (A) Cells were incubated with ^3H -leucine for 2 min, and the intracellular immunoglobulin of 4.7×10^8 cells was applied to the gel. (B) Cells were incubated with ^3H -leucine for 10 min, and the intracellular immunoglobulin of 2.8×10^8 cells was applied to the gel. (C) Cells were incubated with ^3H -leucine for 60 min, and the intracellular immunoglobulin of 1.4×10^8 cells was applied to the gel.

worthy that the quantitative relationship of these components to one another is also in accord with the view that HH and HHL are precursors of LHHL. In addition, these data establish that the equilibration of radioactivity among the peaks occurs no later than at 120 min. This finding is consistent with the independent observation that the total amount of intracellular radioactivity specifically coprecipitable as immunoglobulin remains constant after about 90–120 min (D'Amico and Kern, unpublished observations).

The specifically precipitable immunoglobulin components derived from rabbit lymph node cells yielded a pattern of peaks that was clearly distinguishable from the over-all pattern we observed for murine myeloma cells (ADJ-PC-5) under identical experimental conditions. The peaks designated 1, 2 and 3 (Fig. 3) were identified as LHHL, HL, and L, respectively, using essentially the same criteria used to identify the intracellular immunoglobulin components of myeloma cells. Additional characterization of LHHL and HL is presented in Table 3. As would be expected from their known heavy and light chain content the heavy chain: light chain ratio of radioactivity of LHHL and HL were found to be equivalent. While the molecular weight estimated for HL, based on acrylamide gel electrophoresis data, was in accord with the known value, the molecular weight estimated for LHHL was lower than is known for this well-characterized protein.¹⁸

TABLE 2. *Distribution of radioactivity in immunoglobulin components of ADJ-PC-5 cells as a function of time.**

| Minutes of incubation | Relative Radioactivity† | | | |
|-----------------------|-------------------------|-----|----|-----|
| | LHHL | HHL | HH | L |
| 2 | ND‡ | ND | § | 100 |
| 5 | ND | 24 | 17 | 59 |
| 10 | 10 | 18 | 18 | 52 |
| 60 | 22 | 30 | 14 | 34 |
| 120 | 35 | 31 | 16 | 18 |
| 180 | 37 | 28 | 18 | 17 |

* Immunoglobulin of ADJ-PC-5, labeled with ³H-leucine for the indicated intervals and isolated as described in *Materials and Methods*, was subjected to electrophoresis and the radioactivity of the sliced gel determined. The results are a composite of several experiments.

† Relative radioactivity = (100) (cpm in peak)/Σ (cpm in peaks).

‡ ND, no detectable peak of radioactivity.

§ In one study no radioactive peak was present. In a second experiment a single peak was present in the region of HH and, although it could have been contaminated with HHL, it contained 29% of the total radioactivity present in peaks.

FIG. 3.—Electrophoretic pattern of radioactive immunoglobulin components from rabbit lymph node cells. Popliteal lymph node cells of the rabbit were incubated for 120 min as described in the captions to Fig. 2. The specifically coprecipitated intracellular immunoglobulin components derived from 6.9×10^7 cells were subjected to electrophoresis.

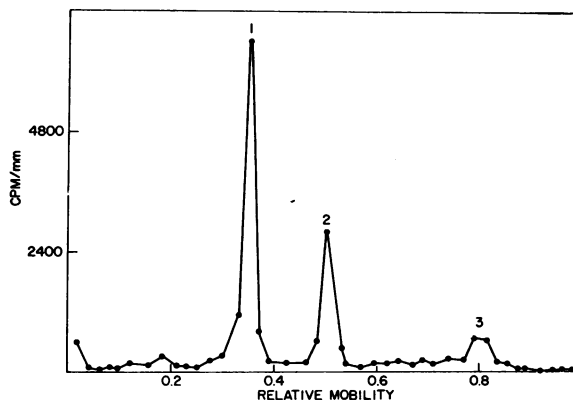


TABLE 3. *Characteristics of the intracellular immunoglobulin components of rabbit lymph node.*

| Peak in fig. 3 | R_{eyto}^* | Molecular Weight† | | H/L‡ | Assigned composition |
|-------------------|---------------------|-------------------|-------------|------|-------------------------|
| | | Estimated | Theoretical | | |
| 1 | 0.37 | 122,000 | 156,000 | 2.20 | LHHL |
| 2 | 0.53 | 76,000 | 78,000 | 2.45 | HL |
| 3 | 0.79 | 36,000 | 23,000 | 0 | L |

* Electrophoretic mobility relative to that of cytochrome c, in each case the numbers represent the mean of at least 23 observations.

† Estimated graphically (see refs. 15-17) using the present system of sodium dodecyl sulfate-gel electrophoresis and proteins with known, independently estimated, molecular weights. In this system radioactive heavy chains of rabbit immunoglobulin G had a relative mobility of 0.64 (est. mol wt., 55,000). In deriving the theoretical molecular weight it was assumed that H = 55,000, L = 23,000.

‡ Same as footnote (†) in legend to Table 1 except that rabbit lymph node cells incubated with ^3H -leucine provided the intracellular immunoglobulins and the combined results of four experiments are presented.

The time course of appearance of the various rabbit immunoglobulin components is shown in Figure 4. At 1 and 2 min after the addition of ^3H -leucine to cells (Fig. 4A and B), a light chain peak was the only peak identifiable with certainty. Incubation of cells for 10 min revealed peaks for LHHL, HL, and H (Fig. 4C). This pattern was equally detectable at 6 min and was marginally present as early as at 3 min.

It was necessary to exclude the possibility that the small heavy chain peak was not an LL peak since the latter would have very nearly the same relative mobility. Therefore, an aliquot of the intracellular materials used to obtain the results shown in Figure 4C was coprecipitated with antisera specific for only the heavy chains of immunoglobulin G and then subjected to electrophoresis. Under these circumstances the light chain peak was no longer detectable while the peaks for LHHL, HL, and H were observed, thereby indicating the presence of heavy chain determinants in each of the peaks.

A summary of the distribution of radioactivity in the various intracellular immunoglobulin components is presented in Table 4. Subsequent to the incubation of cells for 60 min with ^3H -leucine, the peaks for LHHL and HL became

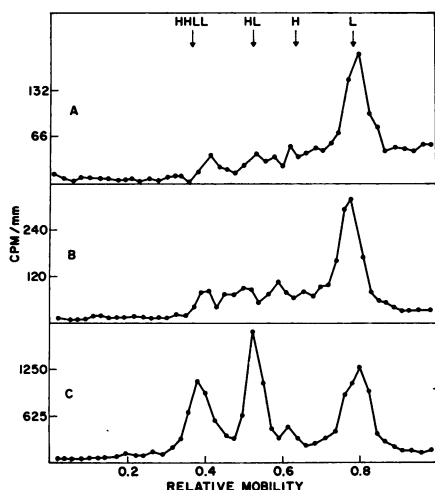


FIG. 4.—Electrophoretic pattern of radioactive immunoglobulin components from rabbit lymph node cells as a function of time. Rabbit popliteal lymph node cells were incubated with ^3H -leucine for 1, 2, or 10 min and the labeled immunoglobulin isolated and subjected to electrophoresis as described in Fig. 2. (A) Cells were incubated with ^3H -leucine for 1 min, and the intracellular immunoglobulin of 9.4×10^8 cells was applied to the gel. (B) Cells were incubated with ^3H -leucine for 2 min, and the intracellular immunoglobulin of 6.6×10^8 cells was applied to the gel. (C) Cells were incubated with ^3H -leucine for 10 min, and the intracellular immunoglobulin of 3.2×10^8 cells was applied to the gel.

equilibrated and the ratio LHHL:HL was about 2.0. For more brief incubations this ratio was closer to 1.0 as would be expected if HL were a precursor of LHHL. It should be emphasized that in contrast to the results obtained with ADJ-PC-5 cells, there was no detectable peak for HHL or HH in the intracellular contents obtained from rabbit lymph node.

Discussion. There are only two alternatives for the nonrandom synthesis of the first interchain disulfide bond of immunoglobulin G. Thus, the initial linkage must occur between heavy chains or between a heavy and a light chain since a light chain to light chain disulfide for immunoglobulin G has not been observed. The observation in murine myeloma cells (ADJ-PC-5) of disulfide linked HH molecules in the absence of HL molecules indicates that the heavy to heavy chain bond is formed first by such cells. In addition, the time-ordered appearance of HH and HHL before the appearance of LHHL indicates that HH and HHL are precursors. Although it was not possible to observe, unequivocally, the presence of HH in the absence of HHL, the finding that the relative amount of radioactivity in HH remained essentially unchanged while the radioactivity in HHL increased with time suggests that HH is a precursor of HHL (see Table 2). Askonas and Williamson⁵ reported the presence of HH and HHL in another strain of murine myeloma (5563). However, the semiquantitative technique used for their time-course study did not provide any evidence for a precursor relationship between the partial immunoglobulin molecules and LHHL.

In contrast to the findings with ADJ-PC-5, rabbit lymph node cells yielded HL as the only disulfide-linked intermediate. Since in time-course studies HL was never observed in the absence of LHHL, the only evidence for a precursor relationship was the observation that the ratio of radioactivity of LHHL:HL changes in the expected direction from about 1 at early times to about 2 at equilibrium (see Table 4).

It is important to ask the question: "Are the interchain disulfide bonds formed before or after the formation of the noncovalent interchain linkages?" While present study does not provide an answer to this question, the nonrandom formation of interchain disulfide bonds suggests the possibility that the affinity characteristics of certain noncovalent linkages may determine the order of disulfide bond acquisition. Indeed, it is necessary to consider the alternative that molecules like HH, HHL, and HL may largely be artifacts of our electrophoresis conditions in which the noncovalent forces are masked. Thus, within the cell such disulfide bond intermediates may exist mainly as four chain molecules (LHHL) as a consequence of noncovalent forces.

TABLE 4. *Distribution of radioactivity in immunoglobulin components of rabbit lymph node cells as a function of time.**

| Minutes of incubation | Relative Radioactivity† | | |
|-----------------------|-------------------------|----|-----|
| | LHHL | HL | L |
| 1 | ND‡ | ND | 100 |
| 2 | ND | ND | 100 |
| 6 | 34 | 32 | 34 |
| 10 | 30 | 32 | 39 |
| 30 | 40 | 38 | 22 |
| 60 | 58 | 32 | 11 |
| 120 | 57 | 31 | 12 |

* Immunoglobulin of rabbit lymph node cells, labeled with ³H-leucine for the indicated intervals and isolated as described in *Materials and Methods*, was subjected to electrophoresis, and the radioactivity of the sliced gel determined. The results are a composite of several experiments.

† Relative radioactivity = (100) (cpm in peak) / Σ (cpm in peaks).

‡ ND, no detectable peak of radioactivity.

The nonrandom order of acquisition of interchain disulfide bonds may be accounted for by other means. For example, if it is assumed that interchain disulfide bonds are formed enzymatically, then the enzyme specificity or affinity for different sites on the molecule could provide for an exclusive sequence of disulfide bond formation. In addition, the relative concentration of heavy and light chains at the site at which such chains first meet could impose restrictions tending to favor one bond type initially.

The findings presented here provide another parameter of immunoglobulin synthesis which will permit study of the intracellular sites for chain assembly. Moreover, the possible relationship of assembly events to the intracellular events involved in secretion and the relationship of interchain disulfide bond acquisition to the biosynthesis of the carbohydrate moiety of these proteins can also be assessed.

* For the previous paper in this series, see ref. 19.

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¹⁸ Despite the anomalous mobility of the radioactive LHHL, we have been unable to assign any reasonable alternative structure to this immunoglobulin peak. Because of its apparent molecular weight, it was possible that the peak consisted of $F_{(ab)2}$ [for abbreviations see *Bull. W. H. O.* **30**, 447 (1964)]. This possibility was ruled out by the finding that, after reduction and alkylation, the fraction yielded two radioactive peaks with mobilities corresponding to those of light- and heavy-polypeptide chains. A more decisive result was obtained by the use of monospecific antiserum to the F_c fragment of rabbit γ -globulin. The LHHL was essentially totally coprecipitable with this reagent, a finding which also excludes the presence of significant amounts of heavy-chains other than of the γ -class.

The mobility for rabbit LHHL is the mean of 26 observations which ranged from 0.32 to 0.40, indicating the ready reproducibility of the value. Indistinguishable values for the mobility were obtained with the extracellular immunoglobulin and the intracellular immunoglobulin, prepared in the presence or absence of iodoacetamide. Rabbit γ -globulin, purified from serum (Sober, H. A., and C. A. Peterson, *Fed. Proc.*, **17**, 1116 (1958)), yielded several bands on stained gels and one major band fell within the observed range of values for radioactive LHHL from lymph node. In addition it should be noted that the frequently reported values for the molecular weight which lie in the 150,000 range may be falsely high estimates resulting from aggregate formation (Mamet-Bratley, M. D., *Biochem. Biophys. Acta*, **207**, 76 (1970)).

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