Synthesis of the γG Heavy Chain in Rabbit Lymph Node Cells*

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ABSTRACT: γG heavy chains contain an N-terminal variable amino acid sequence and a C-terminal invariant sequence. Labeling studies were performed to determine whether or not these chains, like those of other proteins, grow from the N terminal to the C terminal. In addition an attempt was made to determine whether there are one or two initiation points for the growth of the heavy chain. Suspensions of rabbit lymph node cells were incubated with [^{3}H]leucine for periods of 30 sec, 1 min, and 4 hr. Cell extracts from each experiment were mixed with carrier γG, and with extracts uniformly labeled for 4 hr with [^{14}C]leucine. The γG was then purified by ammonium sulfate precipitation, DEAE-cellulose chromatography, and gel filtration. Fragments of the heavy chain were obtained by cleavage with papain and with cyanogen bromide and subsequent gel filtration. There was a pronounced specific activity gradient between the N- and C-terminal fragments following short incubations with [^{3}H]leucine, demonstrating that the γG heavy chain grows from the N to the C terminal. The labeling pattern was consistent with the growth of the chain from a single initiation point, although a second initiation point could not be definitely excluded.

The primary structure of immunoglobulin polypeptide chains is unusual. The N-terminal halves of light chains vary extensively in amino acid sequence, but the C-terminal halves are relatively invariant (Titani *et al.*, 1965). Heavy chains also have variable and invariant regions (Potter *et al.*, 1965; Frangione and Franklin, 1965; Frangione *et al.*, 1966; Small *et al.*, 1966). The variations in both types of chains probably are the basis of antibody specificity. In heavy chains, complete amino acid sequences are not yet available, and the exact locations of the variable and invariant parts are not known. The variable part of the rabbit γG heavy chain probably begins at the N terminal (Wilkinson *et al.*, 1966) and extends about one-fourth or one-third the length of the chain (Small *et al.*, 1966).

The genetic and biosynthetic origin of two such regions in one type of polypeptide chain is not understood. The variable and invariant regions might be the products of separate genes which could be joined at the DNA level (Dreyer and Bennett, 1965), at the level of mRNA, or by linking separately formed polypeptide chains by a peptide bond (Cioli and Baglioni, 1966). The present experiments attempted to explore the last possibility.

Polypeptide chains grow by sequential addition of amino acids from the N to the C terminal. This has been shown for hemoglobin α and β chains (Dintzis, 1961; Naughton and Dintzis, 1962) and for several other proteins (Canfield and Anfinsen, 1963; Luck and Barry, 1964; Humbel, 1965; Sargent and Campbell, 1965). The unusual primary structure of immunoglobulin polypeptide chains raises two questions about their synthesis. (1) Do these chains also grow from the N to the C terminal? (2) Is there a single initiation point for the growth of the whole chain, or are there two such points corresponding to the variable and invariant parts?

The heavy chain of rabbit γG-immunoglobulin was selected for this study for two reasons. The first is that *in vitro* isotope incorporation methods are available for studying the synthesis of γG in rabbit lymph node cells (Helmreich *et al.*, 1961, 1962; Fleischman, 1963). Secondly, the heavy chain of rabbit γG can be cleaved into identifiable fragments by papain (Porter 1959; Fleischman *et al.*, 1963) and by cyanogen bromide (Press *et al.*, 1966b; Givol and Porter, 1965, 1966; Hill *et al.*, 1966) and the order of some of these fragments in the chain is known. The growth of a polypeptide chain can be demonstrated by incorporation of radioactive amino acids. Chains completed during short incubations with the label have a gradient of increasing specific activity from the N to the C terminal. After longer incubations, the gradient is reduced and the specific activity becomes uniform along the chain (Dintzis, 1961). In the present experiments, rabbit lymph node cells were incubated for short periods with radioactive leucine, and the distribution of the isotope in the heavy chain fragments was analyzed. In response to the first question posed above, the distribution of specific activity showed that these chains, like those of other proteins, are synthesized from the N to the C terminal. The second question could not be answered definitely since the exact location of the variable and invariant parts is not known. However, the

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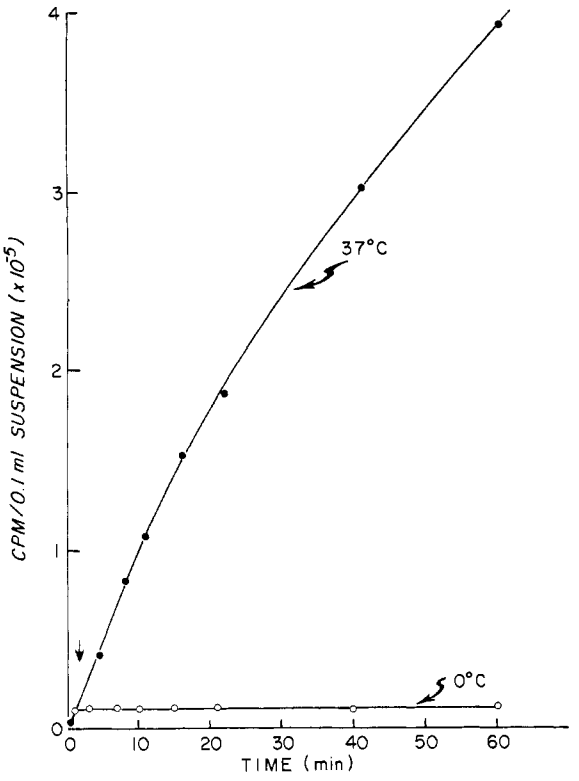

FIGURE 1: Incorporation of [^{3}H]leucine into hot trichloroacetic acid insoluble material by rabbit lymph node cells at 37° (●——●) and at 0° (○——○). Isotope was added to a cell suspension at 37° at time zero. After 1.5 min (arrow), an aliquot was removed and chilled to 0° as described in the text. Samples were then taken at various times from both cultures and counted.

over-all labeling pattern was consistent with a single initiation point for the whole chain.

Materials and Methods

Preparation and Incubation of Cell Suspensions. Cell suspensions were prepared according to Helmreich *et al.* (1961) from the popliteal and axillary lymph nodes of rabbits immunized with DNP-conjugated proteins or ovalbumin. The immunization program was not significant except that the nodes of hyperimmunized animals generally yielded more cells. Cell concentrations were determined by measuring the packed cell volume of a suspension. Media and methods for the incubation of cell suspensions have been described by Helmreich *et al.* (1961) and L. A. Steiner and H. N. Eisen (in preparation). For 4-hr incubations, 20 ml of a suspension containing 2.5×10^7 cells/ml was placed in a 500-ml erlenmeyer flask. For short incubations (5 min or less), 20 ml of a suspension containing $1.2–2.5 \times 10^8$ cells/ml was placed in a 250-ml erlenmeyer flask. Unlabeled L-leucine was omitted from the incubation medium. L-[4,5-^{3}H]Leucine (5000 mc/mmole) and L-[^{14}C]leucine (uniformly labeled) (231 mc/mmole) were obtained from the New England Nuclear Corp.

Freshly prepared cell suspensions were warmed for 20 min in an incubator room, incubated for 5 min in a 37° water bath with occasional gentle stirring, and equilibrated with 95% O_2–5% CO_2 to pH 7.4. Radioactive leucine was added at time zero with rapid mixing. In 4-hr incubations, [^{14}C]leucine was added to a final concentration of 1.5–2.5 μc/ml and [^{3}H]leucine to 30 μc/ml. In short incubations [^{3}H]leucine was added to a final concentration of 100 μc/ml. Incorporation of the label was stopped after the desired time by pouring the suspension onto an equal volume of frozen medium in the bottom of another erlenmeyer flask. This cooled the suspension to 2° within 20 sec, and stopped the incorporation of radioactive leucine (Figure 1). The cells were then centrifuged at 1000 rpm at 0–5°, washed once with 10–20 ml of cold medium to remove excess radioactive leucine, resuspended in 1–2 ml of medium, and frozen.

Purification of γG. Frozen cell suspensions were thawed, pooled, homogenized, and centrifuged at 8000*g* and 100,000*g* (Fleischman, 1963). In doubly labeled experiments, ^{14}C- and ^{3}H-labeled supernatants (each containing $3–9 \times 10^6$ cpm precipitable in 5% trichloracetic acid) were mixed together at this stage; 300 mg of carrier rabbit γG-globulin, prepared from normal rabbit serum by ammonium sulfate precipitation and DEAE-cellulose chromatography as described below, was added.

A saturated solution of ammonium sulfate was added to the mixture dropwise while stirring in the cold to a final concentration of 40% saturation. After standing overnight at 4°, the precipitate was centrifuged and washed three times with cold 40% saturated ammonium sulfate. The precipitate was redissolved in 20 ml of 0.0175 M sodium phosphate buffer (pH 6.3) and dialyzed against two changes of 1 l. of the same buffer. The dialyzed material was clarified by centrifugation and passed through a DEAE-cellulose column (Serva 0.68 mequiv/g, 2.5×31 cm) equilibrated with the same buffer. The effluent fractions were pooled and concentrated. This material was used for papain digestion. An additional purification step was introduced for studies on the cyanogen bromide fragments of the heavy chain. The concentrated effluent from the DEAE-cellulose column was applied to a column of Sephadex G-200 (4.4×44 cm) in 0.15 M NaCl. A substantial portion (10–50%) of the radioactivity separated from the γG carrier. The radioactivity in the pooled and concentrated γG peak was completely precipitable with goat antirabbit γG antiserum, and could not be separated from the carrier γG by filtration on Sephadex G-200 in 6 M guanidine-HCl (Figure 2). The purified radioactive material was, therefore, assumed to be entirely γG-globulin.

Papain Digestion and Separation of Fd Fragment. Labeled γG purified by ammonium sulfate precipitation and DEAE-cellulose chromatography was digested with papain and chromatographed on CM-cellulose as previously described (Fleischman, 1963). Reduced

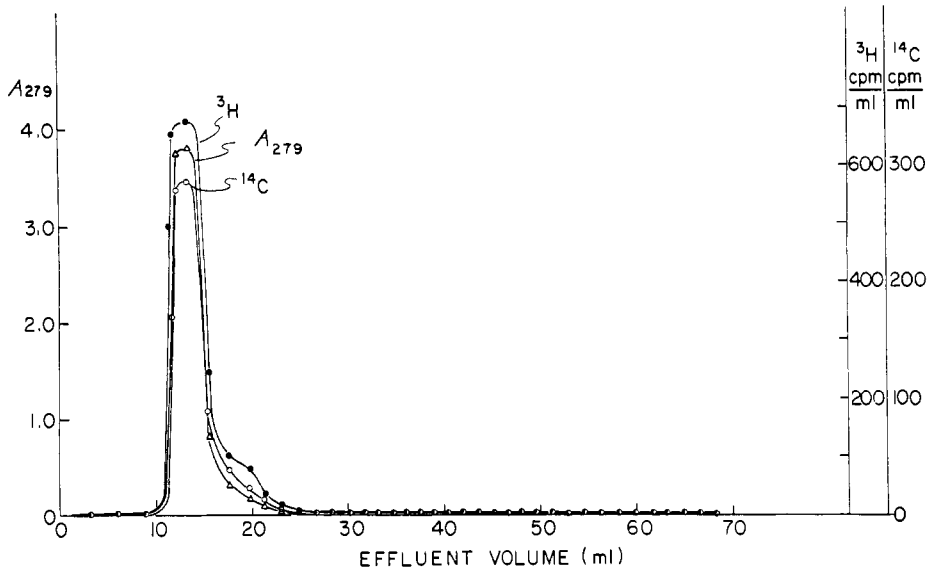

FIGURE 2: Sephadex G-200 filtration of labeled rabbit γG in 6 M guanidine-HCl. (●——●) ^{3}H counts. (○——○) ^{14}C counts. (△——△) absorbancy$_{279}$. Column dimensions are 1.0 × 47 cm.

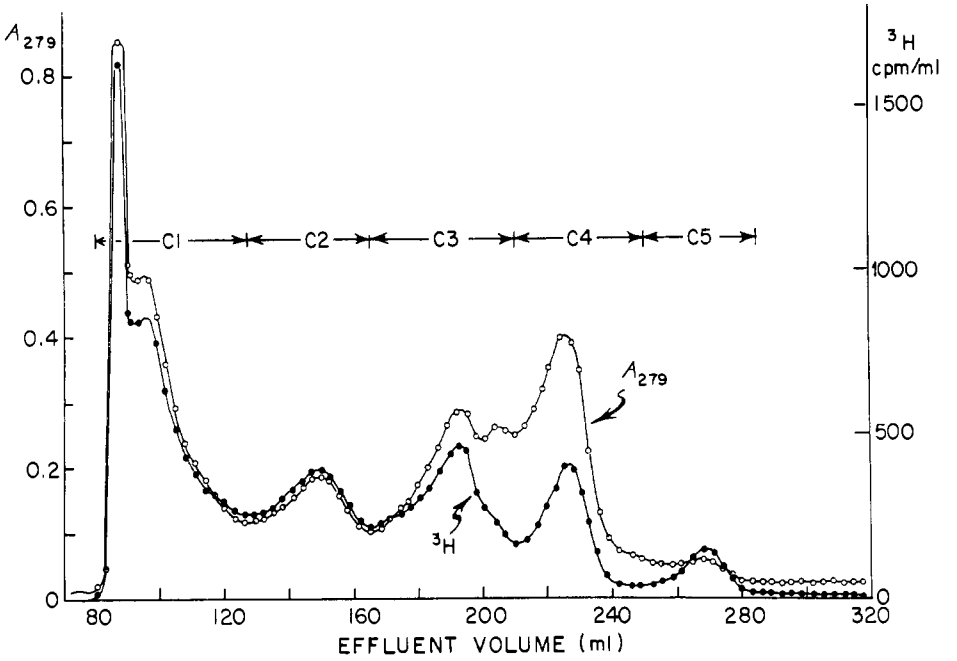

FIGURE 3: Cyanogen bromide fragments of heavy chain labeled for 4 hr with [^{3}H]leucine. (●——●) ^{3}H counts. (○——○) absorbancy$_{279}$. Figures 3–6 are elution diagrams from a Sephadex G-100 column (2 × 103 cm) in 1 N acetic acid.

alkylated Fab(I)[1] and Fab(II) were filtered on Sephadex G-100 (2.0 × 103 cm) in 1 N propionic acid to separate Fd from light chains (Fleischman *et al.*, 1963).

Preparation and Cyanogen Bromide Cleavage of Heavy Chains. Labeled γG was reduced and alkylated as described by Fleischman *et al.* (1963). Heavy chains were separated from light chains on a Sephadex G-100 column (2.5 × 100 cm) in 1 N propionic acid. The yield of heavy chain was consistently 74–75% of the total absorbancy$_{279}$ units recovered from the column. The absorbancy$_{279}$ yield of heavy chain after reduction and gel filtration in guanidine is 72% (Small and Lamm, 1966); hence the maximum contamination of heavy

[1] The nomenclature of immunoglobulins and their subunits is described in *Bull. World Health Organ. 30*, 447 (1964). Fab(I) and Fab(II) refer to papain fragments I and II of Porter (1959).

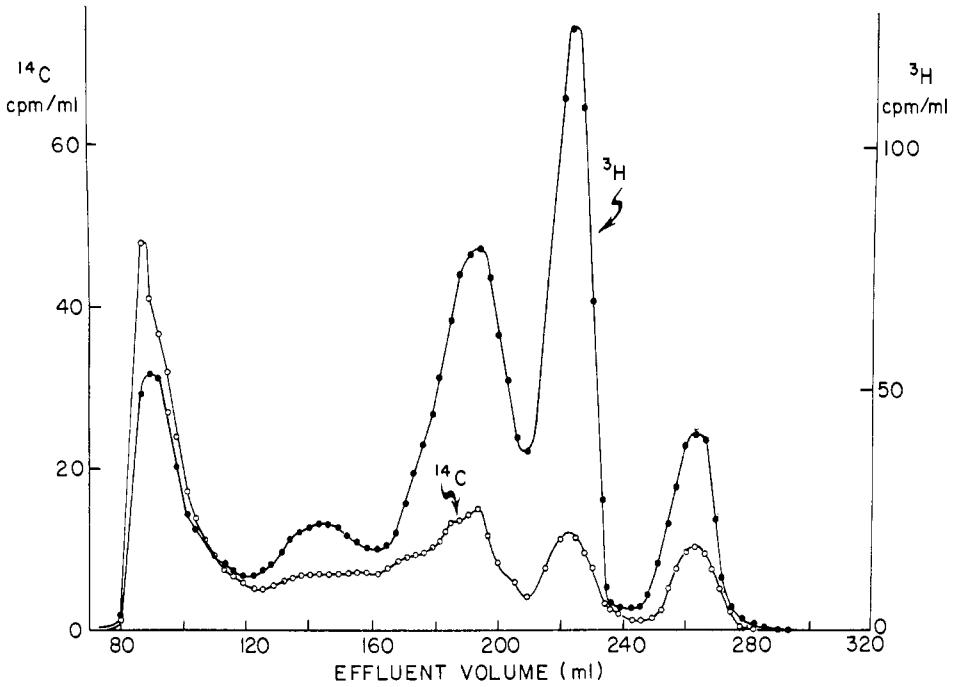

FIGURE 4: Cyanogen bromide fragments of heavy chain labeled for 30 sec with [^{3}H]leucine and for 4 hr with [^{14}C]-leucine. (●——●) ^{3}H counts. (○——○) ^{14}C counts.

chains by light chains in the present experiments was 3–5%. The heavy chain fractions were pooled and concentrated by pressure dialysis. Heavy chain (20–40 mg) in 2–3 ml of 1 N propionic acid was dialyzed against 70% formic acid and cleaved with twice the weight of cyanogen bromide (Eastman) according to Givol and Porter (1965). After 24 hr at room temperature the mixture was diluted tenfold with distilled water and lyophilized. The protein was redissolved in 1.0 ml of 1 N acetic acid and applied to a Sephadex G-100 column (2.0 × 103 cm). A flow rate of 8 ml/hr and collection of 3.0-ml fractions satisfactorily resolved five major components in the digest (Figure 3).

Measurement of Radioactivity. Protein samples up to and including the G-200 filtration step were counted following precipitation in 5% trichloracetic acid, heating to 100°, and filtration on 25-mm Millipore filters. The filters were washed twice with 5 ml of 5% trichloracetic acid and once with 5 ml of 1% acetic acid, dried, and counted in vials using a standard toluene–PPO–dimethyl-POPOP[2] mixture in a Packard Model 3003 Tri-Carb scintillation counter. Counting rates were linear up to 0.3 mg of protein deposited on the filters.

Purified γG, chains, and fragments were counted in Bray's (1960) solution. A sample of 1.0 ml was mixed with 10 ml of Bray's solution. Counting rates were linear at least up to 1.0 mg of protein counted. Standards were prepared for each buffer or solvent used. Samples with low counting rates were counted to at least 1000 counts. ^{14}C and ^{3}H were counted in separate channels. The counts were corrected for background and overlap by an IBM 7072 computer.

Results

Papain Fragments of the Heavy Chain. Papain digestion was performed on γG labeled for various times with [^{3}H]leucine only. The distribution of radioactivity in the three papain fragments of γG after labeling for 4 hr, 5 min, and 1 min is shown in Table I. The results are expressed as the percentage of the total radioactivity recovered from the CM-cellulose column. The sum of Fab(I) and Fab(II) is compared with Fc as discussed previously (Fleischman, 1963). The labeling at 5 min is uniform in that there is no significant difference from the 4-hr pattern. However, at 1 min, a significantly greater proportion of the label is found in the Fc fragment.

TABLE I: Distribution of Counts in Papain Fragments (% of total).

Incubn Time	Fab(I) + Fab(II)	Fc
1 min	55	45
5 min	68	32
4 hr	67	33

[2] Abbreviations used: PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)].

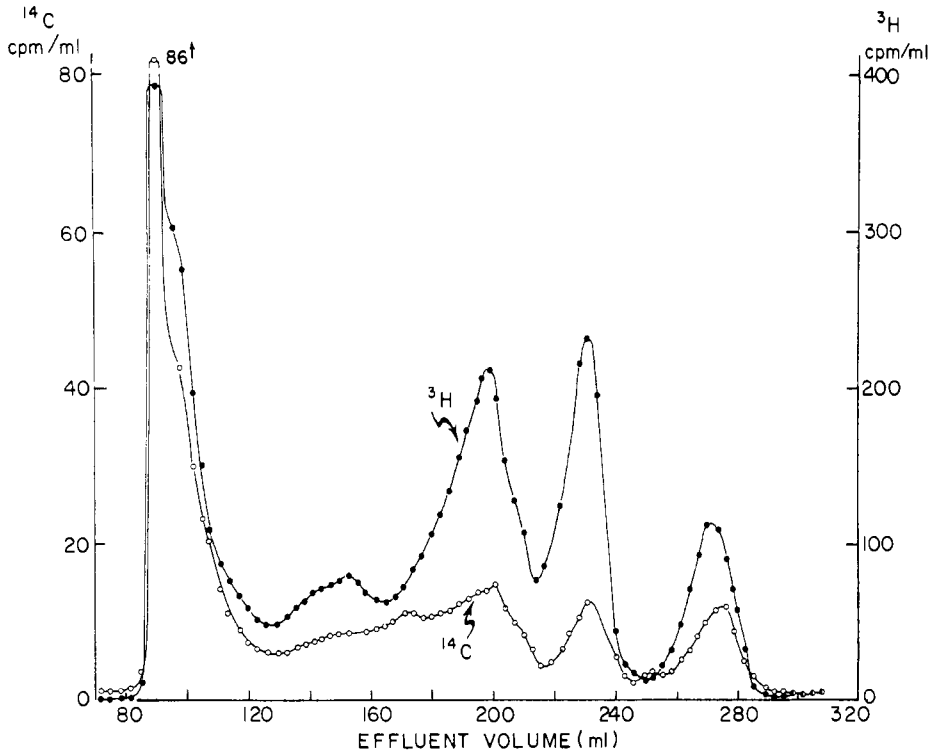

FIGURE 5: Cyanogen bromide fragments of heavy chain labeled for 1 min with [^{3}H]leucine and for 4 hr with [^{14}C]leucine. (●——●) ^{3}H counts (O——O) ^{14}C counts.

Both Fab(I) and Fab(II) from each experiment were reduced and alkylated. Separation of Fd from light chains on Sephadex G-100 in 1 N propionic acid resolved the labeling pattern within the heavy chains. The counts in Fd, Fc, and light chains were normalized to 1.0 for Fd in each experiment and are summarized in Table II. As in Table I, labeling is uniform after 5-min incubation, but at 1 min there are twice as many counts in Fc as in Fd. The results for Fab(I) and Fab(II) are identical.

When reduced alkylated Fab from 5- and 1-min experiments was fractionated on Sephadex G-100 in 1 N propionic acid, 32–38% of the radioactivity appeared in a peak near the void volume of the column. This was not seen in reduced alkylated Fab from 4-hr incubations. The radioactivity in this peak coprecipitated completely with goat antirabbit Fab antiserum plus rabbit γG, but only partially with goat antirabbit Fc antiserum. It may have represented incomplete γG chains carried along in the purification. It was not seen in the cyanogen bromide experiments and presumably was eliminated by purification on Sephadex G-200.

TABLE II: Distribution of [^{3}H]Leucine Counts in Fd, Fc, and Light Chains (normalized to 1.0 for Fd).

Incubn Time	Fab(I)			Fab(II)		
	Heavy Chain		Light Chain	Heavy Chain		Light Chain
	Fd	Fc		Fd	Fc	
1 min	1.0	2.1	1.5	1.0	2.0	1.4
5 min	1.0	0.8	0.7	1.0	0.8	0.6
4 hr	1.0	0.9	0.8	1.0	0.8	0.7

Cyanogen Bromide Fragments of the Heavy Chain. Figure 3 shows the fragments of a cyanogen bromide digest of heavy chain uniformly labeled for 4 hr with [^{3}H]leucine. Variations in the ratio of absorbancy to counts per minute reflect differences in the leucine content of different fragments. The percentage of label in each fragment is listed in Table III. Fragment C1 is the N-terminal half of the heavy chain. It contains the N-terminal peptides of the heavy chain and its amino acid composition resembles that of Fd (Press *et al.*, 1966b). C2 has not yet been identified. The remaining fractions are from the Fc part of the chain. C3 and C4 are probably the 124 and 77 amino acid fragments of Fc obtained after cyanogen bromide cleavage of incompletely reduced heavy chain (Givol and Porter, 1966; Hill *et al.*, 1966). C5 is the C-terminal octadecapeptide and has a single leucine residue (Givol and Porter, 1965; Press *et al.*, 1966b). Givol and Porter (1966) tentatively proposed that the sequence of the fragments from the N to the C terminal was C1, C3, C4, and C5.

Recoveries of the five fragments varied slightly.

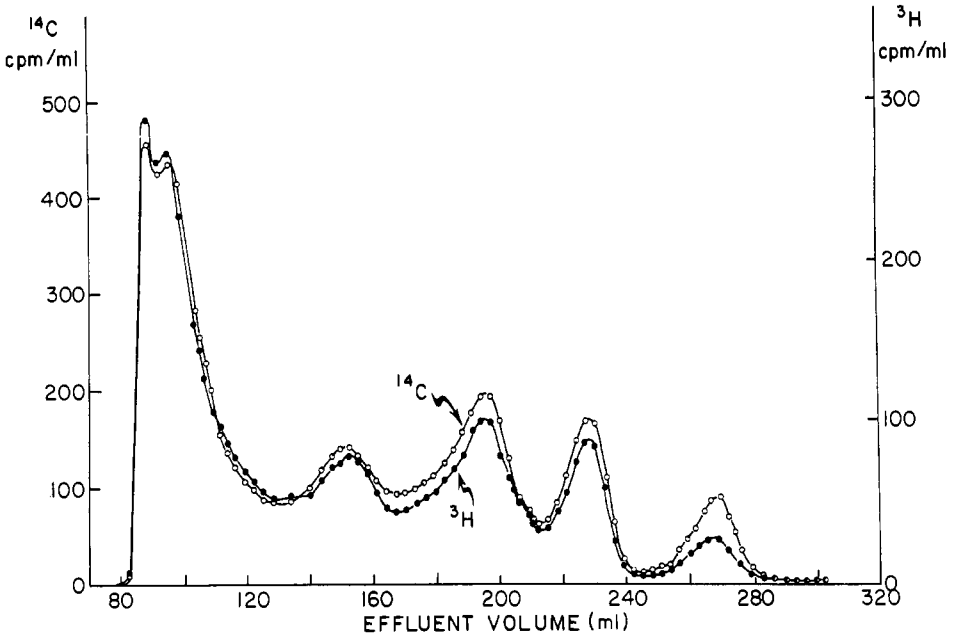

FIGURE 6: Cyanogen bromide fragments of heavy chain labeled for 4 hr with [^{3}H]leucine and for 4 hr with [^{14}C]leucine. (●——●) ^{3}H counts. (○——○) ^{14}C counts.

A cell extract uniformly labeled for 4 hr with [^{14}C]-leucine was, therefore, added to extracts labeled for short times with [^{3}H]leucine before purification of the γG. This provided an internal control for recovery of the fragments in each experiment. The specific activity of each fraction was expressed as the ratio of ^{3}H to ^{14}C counts (Dintzis, 1961).

Figure 4 shows the labeling pattern of the heavy chain fragments after 30-sec incubation with [^{3}H]leucine, and Figure 5 shows the pattern after a 1-min incubation. In both experiments the ratio of ^{3}H counts to ^{14}C counts is considerably higher in C3, C4, and C5 than in C1. This agrees with Table II where after 1 min most of the label is in the Fc part of the heavy chain.

Figure 6 illustrates a control experiment in which both the ^{3}H and ^{14}C labels are from 4 hr incubations. The ^{3}H:^{14}C ratios are similar in all fractions. In all three experiments, the ratios were normalized to 1.0 for that of C1 and are summarized in Table IV.

The specific activities of the fragments from the C-terminal part of the chain, C3, C4, and C5, are much higher after short incubations. The specific activity gradient is steepest at 30 sec, less at 1 min, and absent after 4 hr, when the ^{3}H:^{14}C ratio should be approximately the same in all parts of the chain. Surprisingly, C5, the C-terminal fragment, did not have the highest specific activity. The low specific activity of C5 appears to be an artifact rather than a peculiarity of chain synthesis, since it is also evident in the control experiment where both labels were from 4-hr incubations. Table III shows that 6–10% of the ^{14}C counts from uniformly labeled chains appear in C5, whereas 4–4.5% of the ^{3}H counts from uniformly labeled chains are in C5. C5 has one leucine residue per mole (Givol

TABLE III: Distribution of Counts in the Cyanogen Bromide Fragments of Rabbit γG Heavy Chain (% of total).

Fig.	Isotope	Incubn Times	Fragment C1	C2	C3	C4	C5
3	^{3}H	4 hr	43	19	21	12	4.5
4	^{3}H	30 sec	18	10	34	28	10
	^{14}C	4 hr	45	12	23	11	9
5	^{3}H	1 min	33	10	29	19	9
	^{14}C	4 hr	48	11	22	9	10
6	^{3}H	4 hr	49	17	20	10	4
	^{14}C	4 hr	44	17	22	11	6

TABLE IV: Relative Specific Activities of the Cyanogen Bromide Fragments of Heavy Chain (^{3}H:^{14}C ratios normalized to 1.0 for C1).

Fig.	Incubn Time with [^{3}H]Leucine	Fragment C1	C2	C3	C4	C5
4	30 sec	1.0	2.1	3.8	6.8	3.1
5	1 min	1.0	1.4	1.9	3.0	1.4
6	4 hr	1.0	0.9	0.9	0.9	0.6

and Porter, 1965). Assuming 30 residues of leucine/mole of heavy chain (Fleischman *et al.*, 1963), C5 should contain 3.3% of the counts in a uniformly labeled chain. The recovery of ^{3}H in C5 is, therefore, about 30% too high, but that of ^{14}C is two- to threefold too high.

The small number of counts in C5 prevented an extensive analysis of this material; nevertheless, several attempts were made to account for the excess ^{14}C counts. (1) C5 may contain a ^{14}C-labeled impurity. The C5 fractions from each of the experiments in Figures 3–6 were lyophilized, redissolved in 0.5 ml of 1 N acetic acid, and applied to a Sephadex G-50 column (1.1 × 47 cm) (Givol and Porter, 1965). A typical elution pattern is shown in Figure 7. All the ^{14}C and ^{3}H counts were recovered in a single peak corresponding to the main C5 component, and no labeled contaminant was resolved. Identical results were obtained in all experiments. It is noteworthy, however, that the radioactivity peaks were slightly displaced from the absorbancy$_{279}$ peak of the carrier. This suggests a structural difference between the C5 from the labeled γG which was intracellular, and the C5 from the carrier γG isolated from serum.

(2) Quenching in the C5 counting vials was checked both by an external radium standard and by the channel ratio method (Weltman and Talmage, 1963). No unusual quenching of either isotope was found.

(3) The uniformly labeled L-[^{14}C]leucine used in 4-hr incubations may have been metabolized to other amino acids. An aliquot of doubly labeled heavy chain was hydrolyzed in 6 N HCl for 24 hr at 110°. The hydrolysate was applied to the long column of a Spinco amino acid analyzer and effluent fractions of 1.1 ml were collected. The fractions were monitored with ninhydrin and counted. One hundred per cent of the ^{14}C counts and 91% of the ^{3}H counts applied to the column were recovered in the leucine peak. The remaining ^{3}H counts adhered to the column and were eluted with NaOH. There was no detectable radioactivity in any other amino acid. The excess ^{14}C in C5, therefore, could not be attributed to conversion of [^{14}C]leucine to other amino acids.

(4) The cells incubated for 4 hr with [^{14}C]leucine may have synthesized a structurally different immunoglobulin with additional leucine residues in C5. Such substitutions would significantly affect the labeling pattern of the C5 fragment which contains only one leucine. Trypsin splits C5 into a major fragment containing leucine (T1) and two smaller fragments (T2 and T3) (Givol and Porter, 1965). There may have been additional [^{14}C]leucine residues in the smaller tryptic fragments of C5, as in human heavy chains (Press *et al.*, 1966a).

An aliquot of doubly labeled C5 was hydrolyzed with trypsin and filtered on a Sephadex G-25 (fine) column (1.0 × 69 cm) in 1% formic acid according to Press *et al.* (1966a) and Givol and Porter (1965). Figure 8 shows that all the ^{14}C and ^{3}H counts were eluted in a single peak corresponding to the major tryptic fragment (T1) of C5. The ^{3}H:^{14}C ratio in this peak was the same as that of C5 before digestion. There was thus no evidence of extra [^{14}C]leucine residues in smaller tryptic fragments of C5. However, extra [^{14}C]leucine residues in T1 could not be ruled out.

The excess ^{14}C counts in C5 prevented an accurate estimate of its specific activity. However, the specific activities of the fragments could also be estimated from the distribution of the tritium label only. In this calculation, the uniformly labeled tritium pattern in Figure 3 replaced the ^{14}C pattern in the experiments shown in Figures 4–6. Specific activity was expressed as the ratio of ^{3}H counts in each fragment to the ^{3}H counts in the corresponding fragment of Figure 3. The ratios were again normalized to 1.0 for C1 in each experiment and are listed in Table V. The scatter is greater than in Table IV since the recoveries are not internally controlled in each experiment, but it is evident that the specific activity of C5 is similar to or greater than that of C4 in the short incubations.

Labeling of Heavy vs. Light Chains. The relative specific activities of heavy and light chains eluted from Sephadex G-100 in 1 N propionic acid are shown in Table VI. The specific activities in each experiment were expressed as the ratio of ^{3}H to ^{14}C counts, and the ratios were normalized to 1.0 for the heavy chain. The specific activity of the light chains in newly formed

TABLE V: Relative Specific Activities of the Cyanogen Bromide Fragments of Heavy Chain (^{3}H counts only).[a]

Fig.	Incubn Time with [^{3}H]Leucine	Fragment C1	C2	C3	C4	C5
4	30 sec	1.0	1.3	3.9	5.8	5.5
5	1 min	1.0	0.7	1.8	2.0	2.7
6	4 hr	1.0	0.8	0.8	0.8	0.8

[a] Specific activity is expressed as the ratio of ^{3}H counts in each fragment to ^{3}H counts in the corresponding fragment of the uniformly labeled chain in Figure 3. The ratios have been normalized to 1.0 for C1.

TABLE VI: Relative Specific Activities of Heavy and Light Chains (^{3}H:^{14}C Ratios).

Incubn Time with [^{3}H]Leucine	Heavy Chain	Light Chain
30 sec	1.0	1.5
1 min	1.0	1.0
4 hr	1.0	1.0

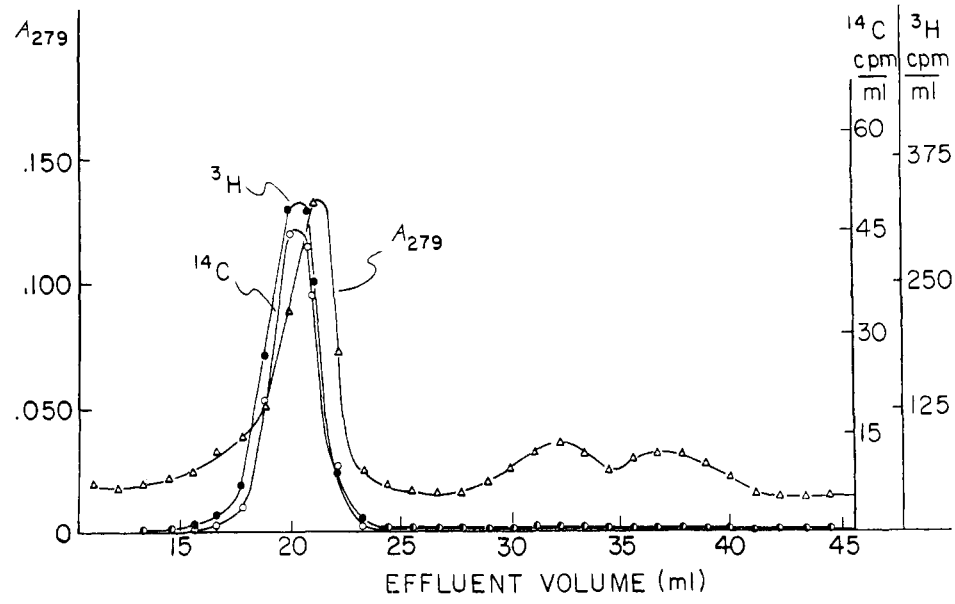

FIGURE 7: Sephadex G-50 filtration of C5 in 1 N acetic acid. (●——●) ^3H counts. (○——○) ^{14}C counts. (△——△) absorbancy$_{279}$. Column dimensions are 1.1 × 47 cm.

γG is higher at 30-sec incubation, but becomes equal to that of the heavy chain after longer incubations.

Discussion

The gradient of specific activity between C1 and C5 (Table V) during short incubations and its disappearance at longer times is consistent with the model proposed by Dintzis (1961) for the growth of a polypeptide chain. Rabbit γG heavy chains, like other polypeptides, appear to grow from the N to the C terminal.

The problem of one or two initiation points for the growth of the heavy chain is more difficult to resolve. The labeling pattern is consistent with the growth of the entire chain from a single initiation point at the N terminal. The sixfold specific activity ratio between C5 and C1 at 30 sec makes it unlikely that there is a separate "hot spot" corresponding to a different initiation point for the variable part of C1. However, this result is not sufficient to rule out such an initiation point since Colombo and Baglioni (1966) found a high specific activity ratio between the β and α chains of hemoglobin after short incubations, and there are obviously two initiation points, one for each chain. In order to demonstrate unequivocally two initiation points in the heavy chain, the specific activity of the last (C-terminal) leucine in the variable region must be shown to be higher than that of the first (N-terminal) leucine in the invariant region. The two regions must first be resolved by sequence studies within the C1 fragment. Low recoveries of the variable peptides of heterogeneous rabbit γG chains make such sequence studies difficult (Small *et al.*, 1966). The problem might be answered definitely only by experiments on more homogeneous mouse or human myeloma proteins.

Continuous translation of the variable and invariant regions of immunoglobulin chains from a single initiation point does not imply that the chain is coded by a single gene or even by two collinear genes. Information for the variable and invariant regions might be joined following transcription of mRNA, or at the DNA level (Dreyer and Bennett, 1965).

The noncoincidence of the radioactivity and the absorbancy$_{279}$ of C5 on Sephadex G-50 (Figure 7) is not understood. Radioactivity was also displaced from absorbancy$_{279}$ in the chromatography of Fc on CM-cellulose in the present and in previous experiments (Fleischman, 1963). Since C5 is part of Fc (Givol and Porter, 1965; Hill *et al.*, 1966), the displacements observed in C5 and in Fc may be related.

The displacement may reflect a structural difference between intracellular γG and the carrier serum γG. Notani *et al.* (1966) have recently demonstrated a structural change in Fc of mouse myeloma protein upon secretion from the cell.

The extra ^{14}C counts in the C5 peak remain unexplained. Most likely there is a ^{14}C-labeled contaminant which cannot be resolved by gel filtration on Sephadex G-50. Alternatively, a structurally different chain may have been formed by the cells incubated for 4 hr with ^{14}C with extra leucine residues in the large tryptic fragment (T1) of C5. Trypsin would not separate them from the leucine residue in T1 found by Givol and Porter (1965).

The higher specific activity of light chains relative to heavy chains in newly formed γG (Table VI) is analogous to the higher specific activity of β chains relative to α chains in newly formed hemoglobin (Colombo and Baglioni, 1966). Colombo and Baglion (1966) proposed that newly formed β chains combin

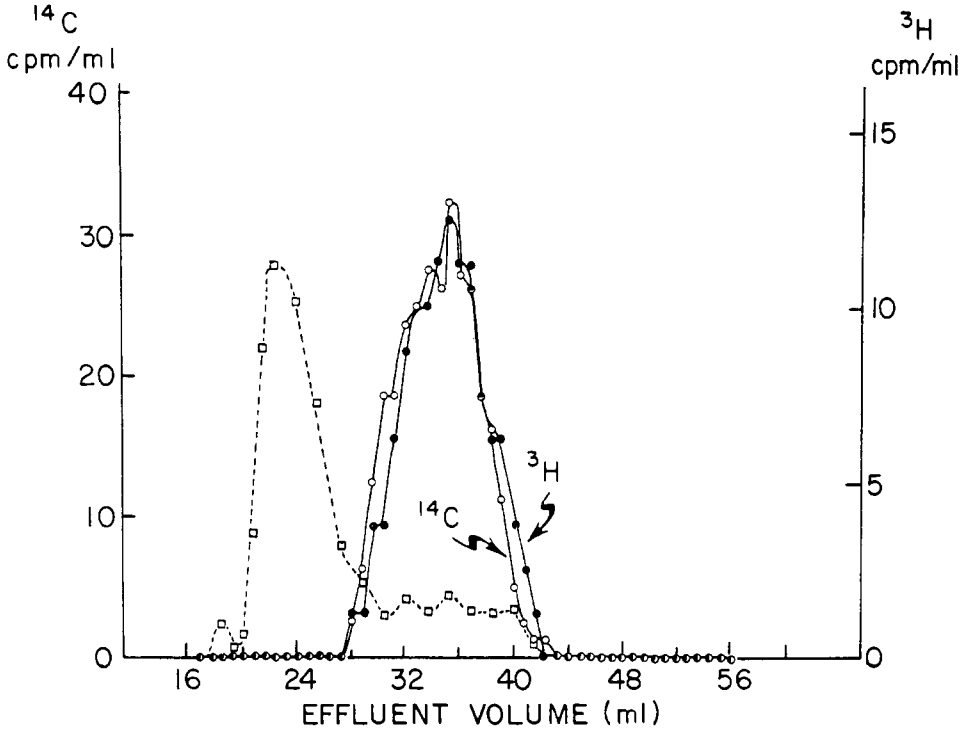

FIGURE 8: Sephadex G-25 filtration of a tryptic digest of C5 in 1% formic acid. (●——●) ^{3}H counts. (○——○) ^{14}C counts. (□——□) shows the position of ^{3}H-labeled undigested C5 on the same column.

with previously completed α chains, releasing them from ribosomes to form new hemoglobin molecules. Shapiro *et al.* (1966a) and Askonas and Williamson (1966) similarly proposed that free light chains release heavy chains from ribosomes to form new immunoglobulin molecules. The higher specific activity of light chains relative to heavy chains after short incubation with labeled leucine is consistent with this proposal. On the other hand, Shapiro *et al.* (1966b) and Askonas and Williamson (1966) found free light chains in immunoglobulin-forming cells. Random sampling of light chains from such a pool for incorporation into γG would reduce rather than increase their specific activity relative to heavy chains at short incubation times. The relative specific activities of the chains in newly formed γG must, therefore, depend on the relative sizes of the pools of both light and heavy chains as well as on the relative rates of synthesis of both types of chains. These parameters must be measured before the higher specific activity of light chains at short times can be satisfactorily explained.

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Kinetics of Iodination. V. Comparison of the Kinetics of Iodination of Isomers and *ortho* Derivatives of 4-Methylphenol*

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ABSTRACT: The kinetics of iodination of a series of methylphenol derivatives has been studied as a model for the iodination of tyrosine. The rates of iodination of the position isomers of methylphenol are in the order 3-methylphenol > 2-methylphenol > 4-methylphenol as a result of inductive and presumably steric effects. The second-order rate constant for the iodination of 4-methylphenol is 1.78×10^3 l. mole^{-1} sec^{-1}. On the basis of this rate being 100, the rates for the *ortho*-substituted 4-methylphenols have the following values: 2-nitro, 0.055; 2-bromo, 2.27; 2-iodo, 4.7; 2-allyl, 341; 2-methyl, 345; 2-*n*-propyl, 546; 2-isopropyl, 620. This order is consistent with a primary inductive effect, and the data fit the Hammett $\sigma\rho$ relationship.

The mean value for ρ for the series is -4.664 ± 0.226, reflecting the sensitivity of the iodination reaction to the inductive effect.

In studies comparing the rates of iodination of *N*-acetyl-L-tyrosine and *N*-acetyl-3-iodo-L-tyrosine, the former became iodinated 20–30 times more rapidly than did the latter over the pH range 5.40–9.80. The reason for this was thought to be the inductive effect of the electrophilic iodine atom (Mayberry *et al.*, 1964, 1965a). Similarly, tyrosine has been shown to become iodinated at a rate 17 times greater than that for 3-iodotyrosine (Mayberry *et al.*, 1965b). Berliner *et al.* (1954) have shown that a general inductive effect is responsible for the differences in the relative rates of iodination of a series of *p*-alkylphenols. Both the *p*-alkylphenol studies and the tyrosine studies involved the effect of a substituent on the rates of iodination *meta* to it. The situations were not completely analogous in that the studies with tyrosine involved only an electrophilic substituent (iodine), while Berliner and co-workers' study involved only electron-donating substituents. In addition, the iodine substituent in the tyrosine studies was *ortho* to the hydroxyl group, while in the study by Berliner and co-workers the alkyl groups were *para* to the hydroxyl group.

The present study investigated the effect of a wider variety of *ortho* substituents (R) on the rates of iodination in the other position *ortho* to the hydroxyl group in a series of phenols in which the *para* position was blocked (structure I). Methylphenol derivatives were

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