Structural Studies on Normal Horse Immunoglobulin Light Chains. Detection of κ-Type N-Terminal Sequences†

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ABSTRACT: Light chains prepared by partial sulfitolysis of pooled normal (heterogeneous) horse immunoglobulins were analysed by automated Edman degradation in a sequenator. Residues characteristic of κ chains were detected at a low level. By comparison of the yields of κ-like N-terminal sequences in horse and human light chains, it was estimated that horse light chains contain 7–8% κ chain. κ-like N-terminal sequences were also detected in bovine light chains. A κ-enriched fraction was obtained by chromatography of the total horse light chains on SE-Sephadex. Automated Edman degradation was carried out on several independent preparations of the κ-enriched light chain. Residues recovered at each step were converted to free amino acids by the use of hydriodic acid and NaOH–dithionite hydrolysis procedures and the relative levels of different amino acids were determined by amino acid analysis. The major and alternative residues occurring at each position were identified in the region 1–28 of the κ sequence. The major sequence shows a number of unique residues compared with human κ chains and several positions in the sequence were clearly heterogeneous. The results would indicate that most of the horse κ chains derived from a single ancestral sequence which differs from κ subgroups seen to date in other species. The implications of "species-associated" variable-region sequences are discussed with respect to current theories of the genetic origin of these sequences.

Immunoglobulin light chains can be divided into two major types, κ and λ, on the basis of their antigenic properties and chemical structure. Sequence analysis of individual human Bence-Jones proteins or light chains of myeloma proteins has indicated that the two types of chain really represent duplicate light chain systems, as distinct from the subgroups seen within each system (Smith *et al.*, 1971). Each system appears to be coded by a small number of constant region genes (c genes) plus a pool of variable-region genes (v genes)[1] which can associate with any of the c genes of the same type. The number of v genes in each pool is not known, and continues to be a much debated issue (Hood and Talmage, 1970; Milstein and Svasti, 1971).

Although the two types of light chain today differ by 40% of their amino acid sequence, they are both able to combine with the three major classes of heavy chain (Grubb, 1970) and they appear to have overlapping if not equivalent biological function. This became clear when it was found that some species of animal were practically devoid of one or the other type of light chain and yet were perfectly capable of surviving (Hood *et al.*, 1967). One such species was the horse, whose immunoglobulin light-chain pool was found to consist almost entirely of the λ type. The mouse represented the converse situation, having almost exclusively κ light chains.

In the studies reported here, automated Edman degradation (Edman and Begg, 1967) has been used to study the minor κ class of horse immunoglobulin light chains. This approach was feasible since the majority of horse λ chains are blocked at the amino terminus (Hood *et al.*, 1967) and therefore did not interfere with the sequencing of the κ chains, even though the latter were present at low levels in unfractionated horse light chain. In order to extend the sequence of horse κ chain beyond the first 4 positions, the procedure of Franek and Zorina (1967) was used to obtain a fraction of horse light chain enriched in κ chains and this enriched fraction was subjected to more extensive N-terminal sequence analysis using the procedures of Smithies *et al.* (1971). This procedure permits one to identify the major and minor residues at each position and to obtain a quantitative estimate of the relative importance of alternative residues. When this type of analysis is carried out on normal immunoglobulin light chains one obtains a "majority" sequence which would be similar to the sum of the sequences of many individual myeloma protein light-chain sequences. That this is in fact the case was demonstrated by Niall and Edman (1967) who analyzed the N-terminal sequence of normal human light chains and found that the "majority" sequence closely resembled the sequence one would expect to see if one looked for the most frequently occurring amino acids at each position in the Bence-Jones protein sequences.

Previous studies on horse immunoglobulins have indicated the presence of two antigenic types of light chain, and it was suggested that these two types might correspond to κ and λ chains (Klinman *et al.*, 1965; Rockey, 1967). Both antigenic types were found in association with all of the different classes of horse Ig, a situation similar to that found for the distribution of κ and λ chains among human Ig classes. If the two antigenic light-chain types were in fact κ and λ chains, it became clear from the work of Hood *et al.* (1967) that the κ type must be present as a relatively minor constituent.

The present results indicate that normal horse light chain does contain sequences homologous to κ light chains, and the nature of these sequences is the subject of this report.

Materials and Methods

Proteins. Pooled normal equine immunoglobulins were purchased from Miles Research Labs, Kanakee, Ill. (lot no. 5 horse immunoglobulins). Normal human plasma was dialyzed

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[1] Abbreviations used are: PhNCS, phenylthiohydantoin group; v gene, a gene for one variable-region sequence; "variable region," the portion of the light chain which shows sequence variability (it is presumed to represent the N-terminal 110–113 residues of the chain, by analogy with human light chains); V_k gene, a gene coding for one κ-variable region sequence.

against 17.5 mM ammonium phosphate (pH 6.3), clarified by centrifugation, and then passed through a column of DEAE-cellulose equilibrated with the same buffer to obtain the IgG fraction (Levy and Sober, 1960). Immunoglobulin G from pooled normal human sera was purchased from Miles Research Labs.

Light-Chain Preparation. Immunoglobulin was subjected to partial sulfitolysis as described by Franek and Zikan (1964), then desalted on Sephadex G-25 in 0.5% ammonium carbonate and lyophilized. The *S*-sulfoprotein was dissolved in 0.01 M sodium phosphate (pH 6.8), containing 1 mM EDTA and 0.1% sodium dodecyl sulfate along with a slight excess (by weight) of solid sodium dodecyl sulfate (Fisher, cat. no. S-329). The solution was then heated for 10 min at 60° and finally filtered if necessary before loading on a column of Sephadex G-100, equilibrated with the 10 mM sodium phosphate–0.1% sodium dodecyl sulfate buffer. The heavy-chain and light-chain peaks were separately pooled and the light chain was desalted on Sephadex G-25 in 0.5% ammonium carbonate and lyophilized.

Fractionation of Horse Light Chains on SE-Sephadex. The procedure developed by Franek and Zorina (1967) for the separation of normal pig light chains into κ- and λ-rich fractions was used. Horse light chain, in the partial *S*-sulfo form, was dissolved in 8 M urea–0.05 M KCl–5 mM potassium formate (pH 3.0). The column (2.5 × 30 cm) was equilibrated with the same buffer. The separation was effected with a gradient of KCl in the 8 M urea (pH 3.0) buffer, running from 0.08 to 0.28 M in a total volume of 2.5 l. The flow rate was maintained at 0.5 ml/min. Pooled fractions were concentrated using a UM-2 membrane (Amicon Corp.) and desalted on Sephadex G-25 in 0.05 M formic acid.

Reduction and Alkylation. Light chain was dissolved at 10 mg/ml in 8 M urea–0.2 M Tris-HCl (pH 8.3), containing 1 mM EDTA. Dithiothreitol was added to give a concentration of 5 mM and the solution was incubated for 30 min at 37°. [^{3}H]Iodoacetic acid (Amersham, 137 Ci/mol), diluted to 2.68 Ci/mol with cold iodoacetic acid (Matheson), was then added to give a final concentration of 20 mM and the incubation was continued at 37° for a further 30 min. The alkylated protein was then desalted on Sephadex G-25 in 0.5% ammonium carbonate and lyophilized.

Electrophoresis. Sodium dodecyl sulfate acrylamide gel electrophoresis was used to assess the purity of the light chain and to obtain an estimate of its molecular weight. The method employed gels of 10% acrylamide, 8 M urea, and 0.1% sodium dodecyl sulfate, with a cross-linker (*N,N'*-methylenebisacrylamide) to acrylamide ratio of 1:25 (Swank and Munkres, 1971). Alkaline urea acrylamide gels (pH 8.2) contained 10% acrylamide (bis:acrylamide, 1:25), 8 M urea, and 0.02 M Tris-glycine (pH 8.2). Protein bands were visualized by staining with Coomassie Brilliant Blue (Weber and Osborn, 1969).

Gel Isoelectric Focusing. Isoelectric focusing (Awdeh *et al.*, 1968) of various fractions of horse light chain was performed using an LKB Multiphor apparatus. Gels were prepared containing 6 M urea, 5% acrylamide, 0.13% *N,N'*-methylenebisacrylamide, 2% ampholine (LKB-Producter) (pH range 3.5–10), and 0.026 mg/100 ml of riboflavine. After polymerization of the gel (about 2 hr at 8-in. distance from a 20-W fluorescent light), small strips of Whatman No. 3MM filter paper (5 × 15 mm) were wetted with sample solutions (protein samples dissolved at 5 mg/ml in 8 M urea) and placed on the surface of the gel. Electrofocusing was begun using constant current (50 mA) with coolant circulation at 12–15°. After about 1 hr, the power supply was switched to give constant voltage (950 V), after which the current decreased to a plateau of about 15–20 mA. Total electrofocusing time was about 2.5 hr. The pH gradient in the gel was determined by cutting small pieces of gel and suspending them in 1 ml of distilled water. The pH of the solution was then measured. The gels were stained for 15 min at 60° using Coomassie Brilliant Blue, as described by Vesterberg (1972).

Sequenator Procedures. Automated sequence analysis was carried out as described by Edman and Begg (1967) with minor modifications mentioned below. The instrument used in these experiments was an Illitron Model 9001. The protein (6–8 mg) was dissolved in 0.2 ml of 0.1 M formic acid–1 mM ammonium formate for loading. The program was started at the blowoff stage immediately following the normally programmed ethyl acetate extraction (stage 9 of Edman and Begg). The loading cycle was continued through a "dummy" acid cleavage and chlorobutane extraction before the first coupling reaction. The major departure from the Edman–Begg program was the omission of the second acid cleavage and chlorobutane extraction step. The total cycle time was 81.5 min, the belljar temperature (N_2 temperature) was regulated at 60°, the base-plate band heater was set at 33 V and the effluent temperature for the spindle core coolant was regulated at 32–33°.

Analysis of Thiazolinone Derivatives. In experiments where the protein had been alkylated with radioactive iodoacetate, aliquots of 0.2 ml of each chlorobutane fraction (fraction volume was *ca.* 3.5 ml) were taken for scintillation counting in a Packard scintillation counter. Radioactivity in the fraction was used to identify carboxymethylcysteine. The procedure used for the hydrolysis of the thiazolinone derivatives was that described by Smithies *et al.* (1971). Before evaporation of the chlorobutane, exactly 50 nmol of a solution of PhNCS-norleucine (Mann Research Labs), 2.5 mM in ethanol, was added to each fraction. This served as an internal standard for all sample handling thereafter and it also gave an indication of the extent of the hydrolysis. In any one sequenator run the entire sample was used for one hydrolysis procedure (either hydriodic acid or NaOH–sodium dithionite hydrolysis).

Amino Acid Analysis. Each hydrolysed fraction was analyzed for amino acids on a Technicon TSM-1 amino acid analyzer. Peak areas were determined by means of an Infotronics CRS-110 A integrator. A single-column procedure was used (Gibson *et al.*, 1971). For the alkaline hydrolysis samples, a slightly shorter program could be used, since most of the arginine is converted to ornithine, which elutes ahead of lysine.

Results

Light chains were prepared by partial sulfitolysis of horse IgG followed by gel filtration on Sephadex G-100 in the presence of 0.1% sodium dodecyl sulfate. This procedure proved to give virtually quantitative yields of light chain but did necessitate effective removal of the sodium dodecyl sulfate. The latter was possible using Sephadex G-25 columns of large dimensions (at least 7× the volume of the sample to be desalted) equilibrated with 0.5% ammonium carbonate. Purified normal horse immunoglobulin light chain gave a single band on sodium dodecyl sulfate–acrylamide gel electrophoresis, corresponding to the same mobility as human light chain. On alkaline urea acrylamide gel electrophoresis horse light chain migrated as a series of bands, similar to normal human light chains in this respect (Figure 2).

Direct sequential degradation of horse light chain showed the presence of κ-like residues at a low level at position 1

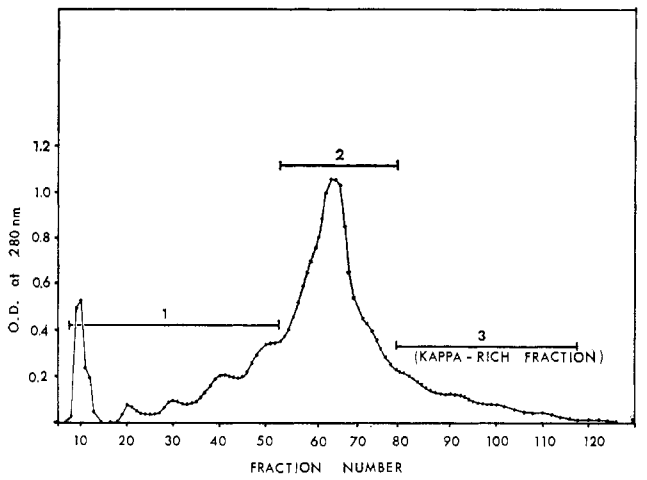

FIGURE 1: Fractionation of horse light chain on SE-Sephadex C-25: 400 mg of normal horse light chain (prepared by partial sulfitolysis of pooled normal horse immunoglobulin) was dissolved in 50 ml of the equilibrating buffer, 8 M urea–0.05 M KCl–5 mM potassium formate (pH 3.0). The column dimensions were 2.5 × 30 cm. Elution was with a linear gradient of KCl in 8 M urea (pH 3.0) from 0.08 to 0.28 M KCl in a total volume of 2.5 l. The flow rate was maintained at 0.5 ml/min.

(Asp), position 2 (Ile), and position 4 (Met). An estimate of the amount of κ chain present in normal horse light chain is made in Table I, where the yields of κ N-terminal sequence are compared for several preparations of horse light chain and several preparations of human light chain. Since the theoretical yield of κ in normal human light chain is 60% it can be estimated that the κ chains of horse represent about 7–8% of the total light-chain population.

Fractionation of Horse Light Chain on SE-Sephadex. In studies on porcine immunoglobulin light chains, Franek developed a chromatographic method by which normal κ and normal λ chains could be grossly separated into two populations (Franek and Zorina, 1967). The method was also applied with success to the fractionation of normal human light chains into κ-rich and λ-rich fractions (Franek *et al.*, 1968). Essentially the same procedure has been applied here in an attempt to obtain a κ-rich fraction. Horse light chain (400 mg) was fractionated using a column of SE-Sephadex in 8 M urea (pH 3.0) with a gradient of KCl for elution. A typical separation is shown in Figure 1. A broad, slightly irregular peak of optical density at 280 nm was obtained and this was pooled into three or four fractions, as indicated. Edman degradation of each of the fractions indicated that only fraction 3 contained detectable amount of κ chain (N-terminal Asp). The yield of protein obtained in this fraction corresponded to 40–45 mg or about 10% of the total applied to the column. This fraction was found to be about sixfold enriched in κ chain as compared with the unfractionated light chain (Table I). Refractionation of this material was attempted but yielded only a 1.5-fold increase in the proportion of N-terminal aspartic acid at a cost of about 75% of the material, so that most of the studies reported here were made with once-fractionated material. Electrophoresis of several of the fractions of horse light chain on alkaline urea gels is shown in Figure 2. By this criterion, all of the fractions tested appeared to be as heterogeneous as was the starting material.

Isoelectric focusing (Awdeh *et al.*, 1968) of different fractions of horse light chain also showed that all of the fractions were extensively heterogeneous. The gel isoelectric focusing patterns of the light-chain fractions as their partial *S*-sulfo

TABLE I: Yields of κ in Normal Horse Light Chain, Normal Human Light Chain and in Fractionated Horse Light Chain as Judged by N-Terminal Sequences Present.

Material	Yield[a] of κ-like N-Terminal Sequence	
Total horse light chain	% of protein applied	
Preparation no. I40	4.1	
I40	3.49	
I40	6.25	
I71	2.10	
I135	5.2	
I168	3.35	$m = 4.08$
Bovine light chain	2.10	
κ-enriched fraction of horse light chain		
Preparation no. FA-99-3	23.7	
FA-99-3	20.9	
FA-102-3	17.6	
FB-24-9	27.4	
FB-24-9	29.1	
FB-28-9	25.2	
Twice-fractionated horse κ	36.5	
Normal human light chain		
Individual 23	22.6	
Individual 23	28.2	
Individual 0494	37.8	
Individual 0494	32.4	
Individual 49006	34.2	
Pooled	36.2	$m = 31.90$[b]

[a] Yield was based on the recoveries of aspartic acid at position 1, isoleucine at position 2, leucine and methionine at position 4. For horse and bovine light chains, aspartic acid at position 1 was taken to represent 86% of the κ chains, isoleucine at position 2 as 30%, methionine and leucine at position 4 both as 50%. For human light chains aspartic acid at position 1 was taken to represent 70% of the κ chains, isoleucine at position 2 as 86%, leucine at position 4 as 33%, and methionine at position 4 as 67%. From the recovery of these amino acids, the number of moles of κ chain was computed and then compared with the weight of protein used in the experiment. A molecular weight of 22,500 was assumed for the light chain. [b] Since human light chains contain approximately 60% κ chain (Hood *et al.*, 1967), it is evident that the overall yield in this procedure is about 50%. This would indicate that horse light chains contain close to 8% κ chains.

derivatives are shown in Figure 3. Complete reduction and alkylation of the light chains with iodoacetamide failed to reduce the heterogeneity observed (Figure 4).

Automated Edman Degradation of the κ Fraction. During the course of the study several sequenator runs were made on the κ-enriched material. Analysis of the thiazolinone derivatives was made by using a combination of two different hydrolysis methods as described by Smithies *et al.* (1971). All of the data were totally consistent as to the major sequence and major alternative residues present.

Quantitation of Sequenator Data. In an attempt to obtain as accurate as possible a picture of the relative amounts of alternative residues at each position, as well as the overall yield of residues at each position, the amino acid analysis

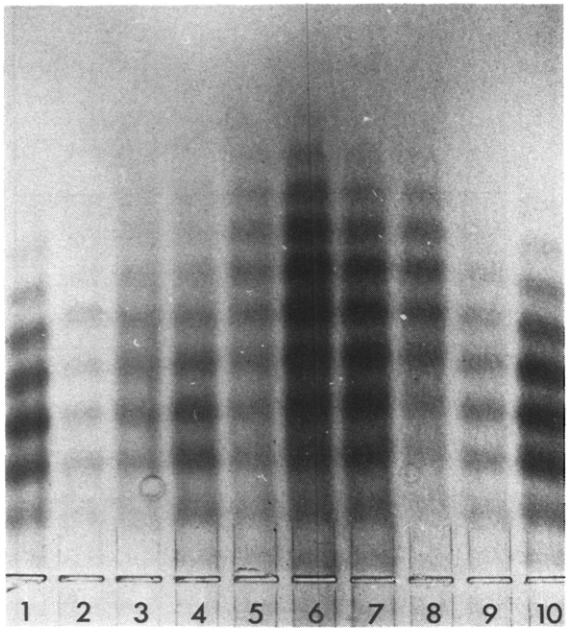

FIGURE 2: Electrophoresis of normal light chains in 8 M urea acrylamide gels (pH 8.2): (1) normal human light chain; (2) unfractionated horse light chain (3); pool 1 (Figure 1); (4) pool 2 (Figure 1); (5, 6, 7) three independent preparations of horse κ fraction, corresponding to pool 3 (Figure 1); (8) twice-fractionated horse κ fraction; (9) unfractionated horse light chain; (10) normal human light chain. It should be noted that the light chains were not completely reduced and alkylated prior to electrophoresis but were run directly as their partial *S*-sulfo derivatives.

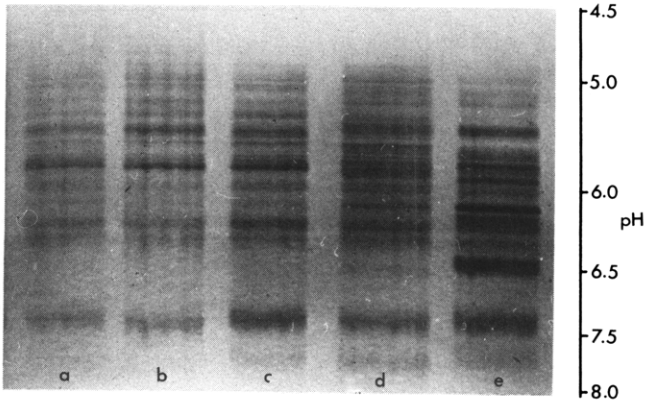

FIGURE 3: Gel isoelectric focusing of various fractions of horse light chain: (a) unfractionated horse light chain; (b) pool 1 (Figure 1); (c) pool 2 (Figure 1); (d) κ-enriched fraction, corresponding to pool 3 (Figure 1); (e) twice-fractionated horse κ chain. The light chains in this case were focused as their partial *S*-sulfo derivatives. Focusing of the chains after complete reduction and alkylation is shown in Figure 4.

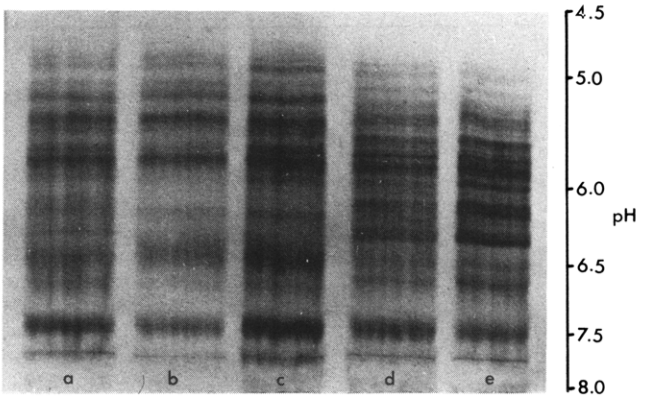

FIGURE 4: Gel isoelectric focusing of horse light-chain fractions after complete reduction and alkylation with iodoacetamide. Light-chain fractions were reduced with 10 mM dithiothreitol in 8 M urea–0.2 M Tris (pH 8.0) then alkylated with 40 mM iodoacetamide. Following dialysis and lyophilization, samples were dissolved in 8 M urea to apply on gel: (a) unfractionated horse light chain; (b) pool 1 (Figure 1); (c) pool 2 (Figure 1); (d) κ-enriched fraction, corresponding to pool 3 (Figure 1); (e) twice-fractionated horse κ chain.

data from several sequenator experiments was normalized[2] and averaged (Figures 5 and 6). The average values for the recovery of each amino acid were then plotted and base-line noise level was estimated as illustrated in Figure 6b for glutamic acid. The values above base line were then taken as the true recovery of that amino acid at each position. A second correction which was then applied was the subtraction of the estimated carry-over from preceding positions. As shown by Smithies *et al.* (1971), the amount of carry-over during a normal sequenator run will increment by a relatively constant value at each cycle, so that it is possible to calculate and correct for this factor. Figure 6d shows the replotted data for the yield of glutamic acid after correction for background and subtraction of a carry-over fraction of 0.025/cycle.

Possibly the most difficult factors to estimate are the relative recoveries of different amino acids, since these depend not only on the yield after hydrolysis but also on the relative efficiency with which different residues are extracted into the chlorobutane. These factors can be estimated by determining the recovery of different amino acids during the degradation of a known protein or a protein of know homogeneity. In such a case a semilogarithmic plot of the yield of major residues versus position should form a straight line. The distance that given amino acids falls below the straight line is then a measure of their relative recovery compared to those residues which fall on the line. In this laboratory, a number of such estimates have been made for most of the amino acids and the relative values have been found to be quite consistent. In the sequenator runs[3] employed for these measurements so far the amino acids leucine and glycine have always fallen close to the 100% relative recovery line, both for the hydriodic acid and NaOH procedures. The values for most of the other residues have been consistent with a standard deviation of in most cases less than 10% of the mean values. The recovery factors are shown in Table II.

The relative recovery factors for each amino acid were applied to the averaged data for the horse κ sequence, and the result is given in Table III. The validity of the corrections can be tested roughly by examining the total yield of all residues at each position. These values should fall close to the exponential decay curve which one normally observes if one plots the yield of major residues during the degradation of a homogeneous protein. The fact that the decay curve agreed reasonably well to the expected exponential fall off (Figure 7) would indicate that no major residue alternatives have either been missed or overestimated, at least up to position 25 in the sequence.

[2] Data from individual sequenator runs were normalized by setting the total of all end groups recovered on step 1 equal to 100%. The totals in all runs were close to 100 nmol.

[3] The "standard" proteins employed for these determinations were three human myeloma light chains (Cal, Edd, and Bet). Sequences of these proteins will be presented elsewhere.

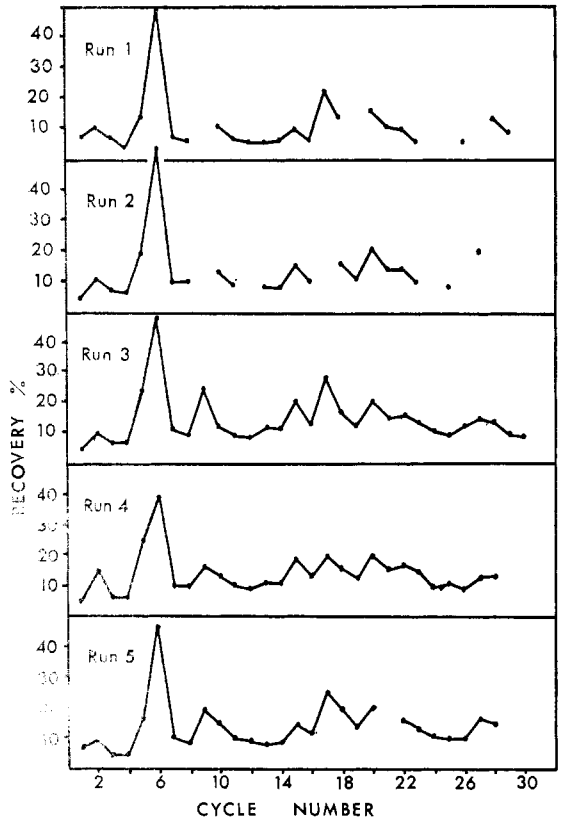

FIGURE 5: The yields of glutamic acid after HI hydrolysis at each position during the automated degradation of the κ-rich fraction of horse light chain. Data from five separate sequenator runs on five independent preparations of the κ fraction. The yields are expressed as a per cent of the total end groups at position 1 in the degradation.

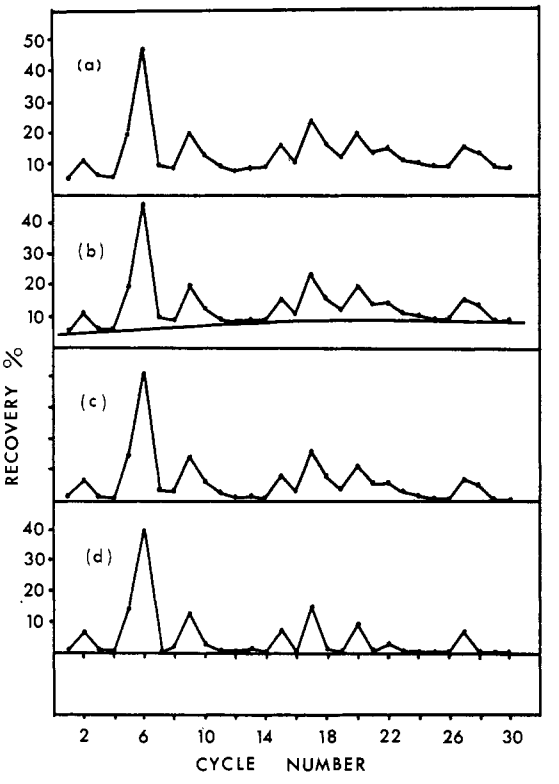

FIGURE 6: Stages in the analysis of the sequenator data. (a) The average yield of glutamic acid at each position for five separate sequenator runs. All of the data in Figure 5 was combined to give the average values. (b) A smooth curve was drawn in as an estimate of the background or base-line value for glutamic acid. (c) The values of glutamic acid after subtraction of the background level. (d) Data corrected for a carry-over factor of 0.025/cycle.

The position of cysteine was determined in a separate sequenator run using ^{3}H-carboxymethylated light chain. When the recovery of radioactivity was plotted over the first 30 positions, it was clear that cysteine occurs at both positions 21 and 23 (Figure 8). The possibility that the methionine residue at position 21 was alkylated can be ruled out since no significant radioactivity was found at position 4, where methionine also occurs. A more likely explanation and one which is consistent with the amino acid analysis data is that cysteine occurs at position 21 but it belongs to a contaminating population of λ light chains which were being cosequenced.

Presence of Unblocked λ Chains. While the majority of horse λ chains are blocked at the amino terminus (Hood *et al.*, 1967), and therefore would not be sequenced, residues corresponding to a λ-like sequence can be seen at a low level if one examines the data of Table III. The most likely λ residues are shown in Table IV, where they are compared with human λ sequences. From the (-Leu-Thr-Glx-Pro-) sequence at positions 3-6, it can be estimated that the contaminating sequence accounts for about 20% of the total at these positions. Although mutual linkage of these residues cannot be established from this type of analysis, the fact that they all occur at the same relative level strengthens the argument that they may be linked together, forming a sequence homologous with human λ chains.

Heterogeneity in the Horse κ Sequence. At least part of the heterogeneity apparent in the sequence data of Table III is probably due to the presence of a λ-like contaminant having a free N terminus, as discussed above. There remain a number of other positions however where the major sequence shows clear heterogeneity. The most heterogeneous position is 9, where 5 amino acids were consistently observed and the major residue (Glx) accounted for only 30% of the total. Other positions showing major alternative residues are position 2 (Ile + Val), position 4 (Met + Leu), and position 13 (Val, Ala).

Discussion

Direct Edman degradation in the sequenator of the fraction of horse light chains enriched in κ permitted the identification of the predominant N-terminal sequence up to position 28. This sequence is given in Table V, which shows the major sequence as well as all alternative residues occurring at a level of 30% or more of the major residue at each position. The first important point is that the major sequence does in fact correspond to a κ sequence. This is clear if one considers the alignment of the sequence with human and mouse κ chains (Table V). The major horse sequence shows the correct alignment of the invariant κ-chain residues (*e.g.*, threonine at position 5, glutamine at position 6, glycine at position 16, and cysteine at position 23). In addition, many positions in the horse κ sequence share identical residues with at least one of the human κ subgroup sequences.

A complicating factor encountered in the study of the horse κ sequence was the presence of a minor λ-like sequence at about the 20% level. The probable λ-chain residues are italicized in Table V. While the majority of λ chains in horse are blocked at the amino terminus (Hood *et al.*, 1967) and therefore would not be sequenced, it is clear that a small subpopulation of horse λ chains is present with free N-terminal resi-

TABLE II: Correction Factors for Recovery of Different Amino Acids.

Amino Acid	HI Hydrolysis			NaOH–Dithionite Hydrolysis		
	Recov Factor[a]	SD	No. of Estimates	Recov Factor[a]	SD	No. of Estimates
Asx	0.64		2	0.51	0.05	3
Gln	0.75	0.01	4	0.44	0.06	3
Glx	0.77		1	nd		
Pro	0.73	0.06	9	0.64	0.18	3
Gly	1.00	0.05	7	0.95	0.09	5
Ala	1.00	0.06	5	1.00	0.01	4
Cys or Ser (as Ala)[b]	0.71	0.07	10	0.16	0.03	5
Thr (as ABA[c])	0.88	0.05	9	0.23	0.02	4
Val	0.75	0.03	6	1.00	0.10	4
Met				0.78	0.06	2
Ile (as *a*Ile + Ile)	0.89	0.02	5	1.00	0.01	3
Leu	1.01		2	1.00	0.13	2
Tyr	0.64	0.06	5	0.91	0.11	4
Lys	0.57	0.10	5	0.52	0.01	2

[a] Recovery factors were determined using data obtained in four separate sequenator runs on three different homogeneous myeloma light chains (Cal, Edd, Bett). The factor includes the relative efficiency of extraction into the chlorobutane as well as the relative recoveries from hydrolysis. [b] The recovery of serine (or cysteine) can be obtained comparing the recovery of alanine from NaOH and HI hydrolysis by the following equations: (1) $A_N = 1.0A + 0.16S$; (2) $A_I = 1.0A + 0.71S$; $A_I = A_N + 0.55S$. $S = (A_I - A_N)/0.55$, where A_N = amount of alanine in the NaOH–dithionite hydrolysis, A_I = amount of alanine in the HI hydrolysis, A = amount of alanine present at that position, and S = amount of serine present at that position. HI hydrolysis: 57% HI, 130°, 20 hr. NaOH–dithionite: 0.2 N NaOH + 0.1 M $Na_2S_2O_4$, 130°, 3.5 hr. [c] ABA = α-aminobutyric acid.

TABLE III: N-Terminal Sequence of κ-Enriched Fraction of Horse Light Chain.

Step	Asx[a]	Glx	Pro	Gly	Ala	Ser[d]	Thr	Val	Met	Ile	Leu	Tyr	Phe	Lys	Arg[b]	Yield[a]
1	61.3	1.3	1.3	3.5	1.0	16.8	–	0.4	–	–	9.8	–	0.4	2.5		98.3
2	3.2	8.2	–	–	1.4	3.1	3.4	41.6	–	20.9	–	–	0.8	3.0		86.1
3	–	1.2	1.2	0.5	–	0.8	4.2	62.2	1.8	0.4	14.8	0.5	–	–		87.6
4	–	–	0.8	–	–	–	20.7	0.7	36.2	0.2	28.3	–	–	–		86.9
5	0.8	18.3	1.8	–	0.5	3.0	56.1	–	–	–	–	–	–	–		80.5
6	–	52.1	12.2	0.75	–	6.1	–	–	0.2	–	0.7	0.4	0.75	–		73.2
7	1.0	–	0.5	1.8	6.25	60.6	1.4	1.1	0.4	0.4	0.4	0.3	0.5	–		74.7
8	–	2.4	40.0	0.6	–	11.2	0.3	1.6	–	0.4	–	–	0.4	–		56.9
9	7.1	16.8	5.8	2.8	5.5	0.1	0.8	12.2	–	0.2	2.6	–	–	2.0		55.9
10	1.25	3.8	1.4	0.9	–	45.6	0.8	1.7	–	–	1.2	–	1.3	–		57.9
11	1.3	0.7	0.4	2.7	3.0	5.8	0.5	9.3	–	–	34.0	0.7	–	–		58.4
12	–	–	1.0	1.2	30.3	4.7	6.4	1.4	0.4	–	7.2	–	–	–		52.6
13	0.8	1.2	1.0	3.5	11.5	–	–	23.3	0.4	–	10.8	–	–	1.0		53.5
14	0.8	0.5	–	11.5	3.4	13.8	–	4.6	0.4	–	1.3	–	–	1.0		37.3
15	0.4	9.5	6.5	–	0.4	0.8	–	0.6	–	0.4	23.3	0.7	–	–		42.6
16	1.3	–	–	22.9	–	1.2	6.6	–	–	–	2.9	–	–	–		34.9
17	1.0	19.1	–	3.7	8.6	–	–	0.4	–	–	–	–	–	–		32.9
18	–	1.6	0.9	–	–	4.3	6.3	–	–	0.4	0.4	–	–	1.2	++	(15.1) + Arg
19	0.4	–	0.4	–	2.9	–	–	17.8	–	4.9	1.7	0.5	–	–		28.6
20	–	11.8	–	0.8	0.9	1.6	5.3	2.1	0.4	0.2	0.4	0.4	–	–		23.9
21	1.2	–	0.8	0.2	–	7.8[e]	–	–	16.6	2.0	0.7	–	–	0.8		30.1
22	0.5	3.4	–	0.1	–	1.9	0.3	–	1.7	–	–	0.4	–	10.1		18.4
23	0.2	–	–	3.8	–	12.7[e]	0.5	–	0.1	–	–	–	–	1.2		18.6
24	–	–	0.9	0.6	–	–	1.4	0.6	–	–	0.5	–	0.6	8.0	+	(13.6) + Arg
25	2.8	–	–	–	10.8	–	–	0.4	–	1.1	0.6	–	3.9	1.6		21.2
26	–	–	–	–	2.0	9.0	0.8	–	–	–	0.4	–	–	–		12.2
27	2.0	8.7	–	–	–	–	–	–	–	–	–	–	–	–		10.7
28	1.7	0.4	1.3	–	–	3.8	1.7	8.1	–	–	–	–	0.4	2.2		19.6

[a] Yields are expressed as a per cent of all end groups recovered on step 1 (see text for details). [b] Arginine was not estimated due to its low recovery in the chlorobutane extraction. [c] Histidine was not detected above background at any position. [d] Serine was determined by differential hydrolysis as described below Table II. [e] Probably cysteine (see Figure 8).

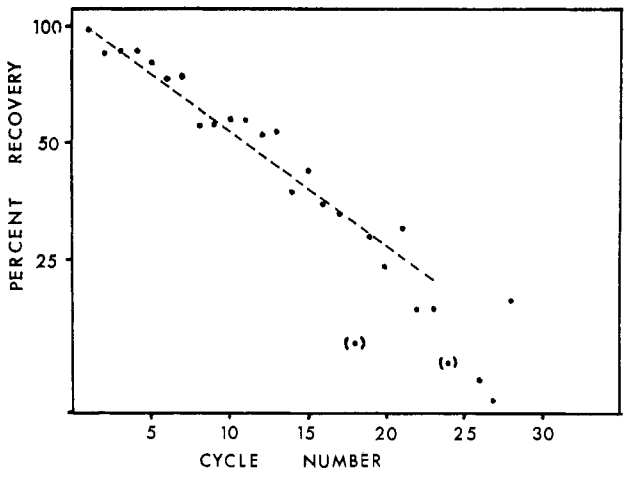

FIGURE 7: Semilogarithmic plot of the total of amino acids recovered at each step in the degradation of the horse κ fraction. Values are taken from the last column of Table III. The low recovery at positions 18 and 24 is due to the fact that arginine at these positions was not included in the total. The values are expressed as a per cent of all end groups recovered on step 1. The dashed line corresponds to a repetitive yield of 92%.

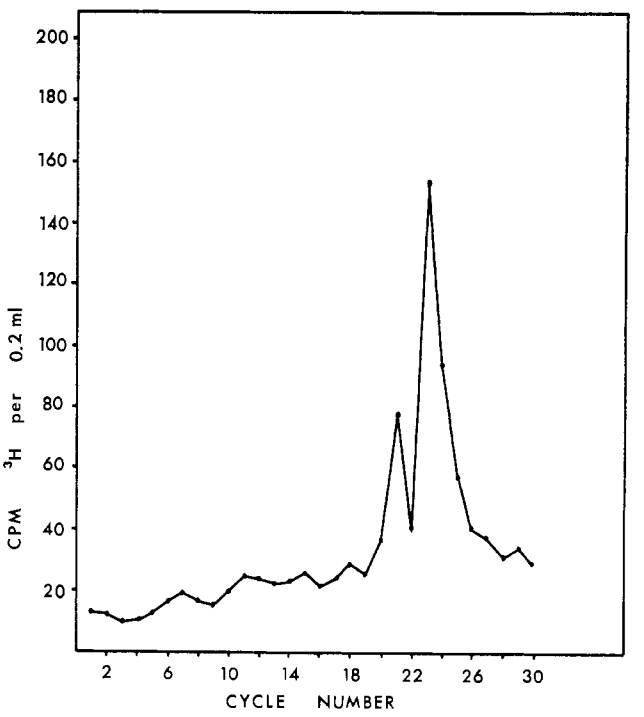

FIGURE 8: The location of carboxymethylcysteine in the first 30 residues of horse light chain. The κ fraction of horse light chain was reduced with 5 mM dithiothreitol in 8 M urea–0.2 M Tris (pH 8.3) and alkylated with 20 mM [³H]iodoacetic acid (2.68 Ci/mol). The protein was then degraded using the sequenator. An aliquot (0.2 ml) of each chlorobutane fraction (total volume, 3.5 ml) was then counted in a liquid scintillation counter

dues. The residues detected which most probably belong to this λ-chain sequence are shown in Table IV, which indicates a strong homology between the minor λ subgroup and the corresponding λ (III) subgroup of human light chains (Smith *et al.*, 1971).

In considering the kappa sequence, it is appropriate to ask whether it is representative of the majority of the κ chains present in the horse light-chain pool. The following considerations would suggest that this is so. Firstly, the amount of κ chain recovered in fraction 3 from the SE-Sephadex column (see Figure 1) accounted for virtually all of the κ-like N-terminal sequence detected in unfractionated horse light chain. Secondly, on one occasion each of the separate pools obtained from the column was analyzed in the sequenator and only pool 3 contained detectable amounts of the κ sequence. Pool 2 (Figure 1) contained a small amount of light chain having N-terminal leucine (also found in the κ fraction, see Table III), but it is not clear whether these chains represent κ or λ light chains. In any case, the level of these chains in pool 2 was lower than in pool 3, so that they are at least represented in the composite data of Table III. The possibility that some horse κ chains went undetected because of blocked N-terminal residues seems unlikely because previous studies on horse light chains have failed to show any κ-like blocked light chains (Hood *et al.*, 1967).

The most significant features of the horse κ sequence are its species-specific nature and its apparent heterogeneity. Comparison of the major horse κ sequence with κ sequences seen to date in other species indicates that the majority of horse κ chains share a distinct N-terminal sequence. Residues characterizing this new linkage group include valine at position 2, glutamic acid or glutamine at position 9, leucine at position 15, and the sequence Glx-Met-Lys-Cys-Lys at positions 20–24. It is clear from the data in Table III that none of these positions is entirely homogeneous with respect to the major residue, so that one cannot be certain that all horse κ chains belong to this linkage group. Indeed, it seems likely that other subgroups of κ sequences are present in horse light chains in addition to the linkage group mentioned above. For example, residues such as isoleucine at position 2, proline at position 15, and isoleucine at position 21 are all characteristic of human and mouse κ subgroups and these residues were clearly de-

TABLE IV: Homology of Minor Sequence With Human λ Sequences.

	1	5	10		15	20		
Probable λ residues:	Ser	Leu Thr Glx Pro	Val	Val	Leu Gly Glx Thr Ala	Ile	Cys	Gly
Level[a]	(17)	(17) (24) (23) (17)	(22)	(16)	(20) (31) (22) (19) (26)	(17)		(20)
Human λ Sequences:								
VIL	His-	Ser- Ala- Leu- Thr- Gln- Pro- Ala- Ser- Val- Ser- Gly- Ser- Leu- Gly- Gln- Ser- Ile-			Thr- Ile- Ser-		Cys- Thr- Gly-	
NEW	Glp-	Ser- Val- Leu- Thr- Gln- Pro- Pro- Ser- Val- Ser- Ala- Ala- Pro- Gly- Gln- Lys- Val- Thr- Ile- Ser-					Cys- Ser- Gly-	
KERN		-Tyr- Ala- Leu- Thr- Gln- Pro- Pro- Ser- Val- Ser- Val- Ser- Pro- Gly- Gln- Thr- Ala- Ser-			Ile- Thr-		Cys- Ser- Gly-	
BO	Glp-	Ser- Ala- Leu- Thr- Gln- Pro- Pro- Ser- Ala- Ser- Gly- Ser- Pro- Gly- Gln- Ser- Val- Thr- Ile- Ser-					Cys- Thr- Gly-	

[a] The recovery of the minor residues is expressed as a percent of all residues recovered at the respective positions. References for human λ-chain sequences are: VIL, Ponstingl and Hilschmann (1969); NEW, Langer *et al.* (1968); KERN, Ponstingl and Hilschmann (1968); BO, Wikler and Putnam (1970).

TABLE V: Major N-Terminal Sequence of Horse κ Chains.

	5							10							15							20							25					
Horse K[a]	ASX-	VAL-	MET-	THR-	GLX-	SER-	PRO-	GLX-	SER-	LEU-	ALA-	VAL-	SER-	LEU-	GLY-	GLX-	ARG-	VAL-	GLX-	MET-	LYS-	CYS-	LYS-	ALA-	SER-	GLX-	VAL...							
	ILE		LEU	*Glx*					Asx				Ala	*Gly*	*Glx*				*Ala*	*Thr*			Thr	*Cys*	Glx	*Gly*	Arg	Phe						Ser
			Thr						*Val*				*Leu*	Val																				
									Pro																									
									Ala																									
	5							10							15							20							25					
Human K_I	Asp-	Ile-	Gln-	Met-	Thr-	Gln-	Ser-	Pro-	Ser-	Ser-	Leu-	Ser-	Ala-	Ser-	Val-	Gly-	Asp-	Arg-	Val-	Thr-	Ile-	Thr-	Cys-	Arg-	Ala-	Ser-	Gln-	Asp ...						
Human K_{II}	Glu-	Ile-	Val-	Leu-	Thr-	Gln-	Ser-	Pro-	Gly-	Thr-	Leu-	Ser-	Leu-	Ser-	Pro-	Gly-	Glu-	Arg-	Ala-	Thr-	Leu-	Ser-	Cys-	Arg-	Ala-	Ser-	Gln-	Ser ...						
Human K_{III}	Asp-	Ile-	Val-	Met-	Thr-	Gln-	Ser-	Pro-	Leu-	Ser-	Leu-	Pro-	Val-	Thr-	Pro-	Gly-	Glu-	Pro-	Ala-	Ser-	Ile-	Ser-	Cys-	Arg-	Ala-	Ser-	Gln-	Ser ...						
Human K_{IV}	Asp-	Ile-	Val-	Met-	Thr-	Gln-	Ser-	Pro-	Asx-	Ser-	Leu-	Ala-	Val-	Ser-	Leu-	Gly-	Glx-	Arg-	Ala-	Thr-	Ile-	Asx...													
Mouse McPC 870	Asp-	Ile-	Val-	Met-	Thr-	Gln-	Ser-	Pro-	Ser-	Ser-	Leu-	Ser-	Val-	Ser-	Ala-	Gly-	Glu-	Lys-	Val-	Thr-	Met-	Ser-	Cys...												
Human λ_{III}	Tyr-	Val-	Leu-	*Thr-*	*Gln-*	Pro-	Pro-	Ser-	*Val-*	Ser-	Val-	Ser-	Pro-	*Gly-*	*Gln-*	Thr-	*Ala-*	Ser-	Ile-	Thr-	*Cys-*	Ser-	*Gly-*	Asp-	Lys-	Leu-	Gly...								

[a] Sequence includes all residues occurring at 30% or more of the major residue. References for comparative sequences are: human K_I–K_{III} and λ_{III}, Smith *et al.* (1971); human K_{IV}, Wang *et al.* (1973); mouse McPC870, Hood *et al.* (1973). Residues in italic type in the horse sequence may correspond to a minor λ sequence present in the κ fraction.

tected in the horse sequence. Comparison of the horse sequences with the human κ linkage groups permits one to say that the human K_I and K_{II} sequences have all but disappeared in the horse, however. This is seen in the virtual absence of glutamine at position 3 (characteristic of human K_I) and the absence of any glutamic acid at position 1 (characteristic of human K_{II}) in the horse κ sequence.

Although it seems unlikely that all of the horse κ chains belong to the linkage group represented by the major sequence, a significant proportion of the chains possess at least one of the "species-specific" residues. For example, at position 21 methionine occurs in 75% or more of the sequences (neglecting the cysteine which is very likely a λ chain residue). The isoleucine which was detected at this position occurs in less than 10% of the sequences. In evolutionary terms, this means that 75% of the v-region sequences of horse κ chains probably originated from a single ancestral gene which coded for a v region having methionine at position 21.

The finding that the majority of horse κ chains share a distinct sequence compared to κ chains in other species is concordant with the emerging picture of the nature of species differences in the v regions of immunoglobulins. Since the original observation of Doolittle (1966) that the N-terminal residues of rabbit and human κ chains differed, a number of studies have shown that the phenomenon of "species-specific" or "phylogenetically associated" residues can be seen in the v-region sequences of many species. Instances of phylogenetically associated residues have now been documented in v-region sequences of rabbit κ chains (Doolittle, 1966; Hood *et al.*, 1970), pig λ chains (Novotny *et al.*, 1972), chicken λ chains (Kubo *et al.*, 1971; Grant *et al.*, 1971), and in the V_{HIII} sequences of several orders of mammals (Capra *et al.*, 1973). The possible origin of these sequences is discussed below.

Many positions in the sequence in addition to the major residue exhibited several alternative residues, some of which were clearly major residues and others of which were minor (Table III). This degree of heterogeneity contrasts with the virtual homogeneity of N-terminal sequence reported in studies on normal immunoglobulin chains from a number of species. For example, Novotny *et al.* (1972) reported that the N-terminal sequence of normal pig λ chains showed little evidence of heterogeneity and little sequence heterogeneity was found in the N-terminal 23 residues of the V_{HIII} subgroup of several mammalian heavy chains (Capra *et al.*, 1973). In order to eliminate the possibility that the heterogeneity observed in the horse sequence was due to artefact, perhaps caused by irregularities in the "background" level of different amino acids during the degradation, the horse κ fraction was subjected to a number of degradations in the sequenator. Virtually all of the heterogeneity observed in the first analysis of the horse kappa fraction was seen in all of the repeat runs. Furthermore, this heterogeneity was not eliminated by averaging the quantitative data from five separate sequenator runs, a procedure which would tend to eliminate background irregularities. In the analysis of a series of homogeneous light chains isolated from human myeloma proteins, using the same methodology employed here (but without averaging repeat runs) the level of minor residues observed has been found to be consistently less than 2% of the major residue over the first 25 positions, in sharp contrast to the results obtained with the horse κ fraction. While these considerations indicate that the observed heterogeneity is real they do not establish whether all of the variation originates in the κ sequence itself. For example, it seems likely that some of the minor residues detected could reflect equally well the presence of additional

variants of the contaminating λ sequence which was identified (Table IV).

A certain amount of the heterogeneity of sequence may be attributable to the presence of a subgroup of λ chains with free N termini, but this cannot be the explanation for the presence of characteristic κ-like alternative residues at a number of positions (*e.g.*, valine + isoleucine at position 2, leucine + methionine at position 4, valine + alanine at position 13, etc). Furthermore, at position 9, the major (κ) sequence was clearly variable, since the major residue recovered accounted for only 30% of the total at that position. Electrophoresis and isolectric focusing of the κ fraction also indicated that it was heterogeneous, and this was equally true of the twice-fractionated horse κ chain, which contained probably more than 60% κ chain (Table I). It is interesting to note that in the isoelectric focusing results the κ fraction showed a larger number of sharply focusing bands than did the unfractionated horse light chain. It also showed the presence of minor bands which were not readily visible in the unfractionated light chain. Surprisingly, however, this observation may actually indicate a *reduced* heterogeneity in the κ fraction.

The evolution of immunoglobulin v-region sequences has been interpreted from two different points of view. One of these is that the sequences within an identifiable v-region subgroup (*e.g.*, V_{HIII}) may be specified by a single germ-line gene which has been passed along through evolution. Amino acid substitutions incorporated in this gene during evolution would immediately appear in the majority of the sequences of that subgroup, thus giving rise to "species-specific" residues. This interpretation has been clearly presented by Capra *et al.* (1973). This of course implies that the bulk of antibody variability is generated by some as yet unknown somatic mechanism (Jerne, 1971). The other view on this subject and the one favored by this author, is that immunoglobulin v-region sequences are specified by a large pool of germ-line genes (Dreyer and Bennett, 1965; Hood and Talmage, 1970), but that the size and composition of this pool are constantly changing in evolution. This would result from the fact that unequal crossing-over would be continually occurring in such a multigene system and it would be continually altering the total numbers and frequencies of different v genes on the chromosome (Smith *et al.*, 1971). Chromosomes carrying altered numbers of certain v genes could become "fixed" in the population by the process of drift, as has been suggested for neutral mutations in general (Kimura, 1968; King and Jukes, 1969). This process, if repeated many times, could lead to the situation where different species of animal might possess quite different sets of v-region genes. It may not be necessary to invoke special gene expansion events as discussed by Hood *et al.* (1970) in order to explain the existence of large families of related sequences (*e.g.*, the human kappa subgroups, or "species-specific" subgroups) since such families of sequences might be a natural consequence of continual unequal crossing-over (Black and Gibson, 1974).

The fact that the levels of κ and λ chains differ markedly in different species (Hood *et al.*, 1967) suggests that each light-chain type is under independent control. It seems possible that the level of expression of each chain type is simply a function of the size of each v-gene pool, or more precisely, of the functional diversity of each v-gene pool. If the relative levels of different types of light chain do in fact reflect the relative numbers of germ-line v genes, consideration of the range of variation in levels of κ and λ chains could give some indication of the actual number of germ-line genes for each type. For example, if there were no more than 20 v-region genes for each type of light chain the minimum κ to λ (or λ to κ) ratio would be 1 to 20. In this case, the minimum level for the expression of a minor type would be about 5%. The lowest level which has so far been observed for the expression of a minor type is in the case of mouse λ chains. These chains make up about 3% of the light chains of the mouse and they display extremely restricted heterogeneity. On this basis it has been suggested that they may represent the limit case where only a single V_λ gene is present (Weigert *et al.*, 1970; Cesari and Weigert, 1973). The present results show that κ chains make up about 8% of horse light chains, a result which is also consistent with a relatively low gene ratio (about 10 to 1). With the methods currently available for fractionation of light chains and for sequence analysis, it should be possible to detect κ chains at considerably less than the 1% level in normal light-chain pools. It should therefore be of considerable interest to examine the light chains of other species having low levels of κ chains. If the numbers of germ-line genes were small, one would expect to find a minimum value for the ratio of κ to λ chains, after which this value would drop to zero.

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Correlation between Guanosine Tetraphosphate Accumulation and Degree of Amino Acid Control of Ribonucleic Acid Accumulation During Nutritionally Slowed Growth in *Escherichia coli*†

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ABSTRACT: Amino acid starvation causes a severe curtailment of RNA accumulation and elicits accumulation of guanosine 3′,5′-bis(diphosphate) in stringent strains of *Escherichia coli* grown in glucose minimal media. However, it is shown here that amino acid starvation only partially inhibits RNA accumulation when the bacterial growth rate is reduced by utilizing poor carbon sources or by limiting the intracellular supply of thiamine. Even under these conditions, the observed rate of RNA accumulation is inversely correlatable to the level of guanosine 3′,5′-bis(diphosphate) accumulated.

When stringent (*rel*$^+$) bacteria are starved for an essential amino acid or fail to aminoacylate a tRNA species, not only does protein accumulation cease but also RNA accumulation is substantially reduced (Sands and Roberts, 1952; Pardee and Prestidge, 1956; Neidhardt, 1966; Gallant and Margason, 1972). This response, called the stringent response, depends on the function of the *rel* gene. Mutations at this locus give rise to relaxed mutants (*rel*$^-$) which continue to accumulate RNA for at least one-third doubling time after amino acid starvation (Borek *et al.*, 1956; Stent and Brenner, 1961; Fiil and Friesen, 1968). Amino acid starvation elicits a second response in *rel*$^+$ cells: rapid accumulation of guanosine 3′,5′-bis(diphosphate) (ppGpp) which does not occur in *rel*$^-$ cells (Cashel and Gallant, 1969; Cashel, 1969; Swanton and Edlin, 1972). The correlation between the kinetics of ppGpp accumulation and the cessation of RNA accumulation suggests that this nucleotide plays a role in the regulation of synthesis of rRNA (Cashel, 1969). However, this presumption has not been directly demonstrated (Haseltine, 1972; Lazzarini and Johnson, 1973; Murooka and Lazzarini, 1973).

The correlation between the high intracellular levels of ppGpp and the cessation of RNA accumulation has been previously established under relatively rich growth conditions such as glucose minimal medium. In the present work, the generality of this relationship is examined. At slow growth rates, created by poor carbon sources or limitation in the intracellular supply of thiamine, stringent (*rel*$^+$) strains of *Escherichia coli* adopt a partially relaxed character: amino acid starvation does not completely halt RNA accumulation. The observed rate of RNA accumulation under these conditions depends on the previous growth rate. Even under these conditions, the inverse correlation between the ppGpp levels and RNA accumulation is maintained.

Experimental Section

Bacteria and Culture Conditions. Three *E. coli* K-12 stringent (*rel*$^+$) strains were used: HY 1, CP 78A, and CP 78B. A prototrophic strain which we have named HY 1 was obtained from Dr. Ira Pastan, National Cancer Institute, Bethesda, Md. CP 78A (leaky for thiamine requirement) and CP 78B (thiamine revertant) were isolated from a single clone of CP 78 (*thi, arg, his, thr, leu, rel*$^+$) (obtained from Dr. Barbara Bachmann, Yale University, New Haven, Conn.) after prolonged cultivation in the absence of thiamine. These strains grow in

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