Chemical and Immunochemical Characterization of Electrophoretic Subfractions of Rabbit γG-Immunoglobulin Light Chains with b4 Allotypic Specificity*

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ABSTRACT: Rabbit γG-immunoglobulin light polypeptide chains with b4 allotypic specificity have been fractionated into at least seven major components by preparative multiphase zone electrophoresis on columns of polyacrylamide gel. Amino acid analyses showed significant differences among these components. Marked differences found in the number of amide residues could account for the electrophoretic resolution of the seven components since an increase in amide content was associated with a decrease in electrophoretic mobility. End-group analyses performed by several methods showed essentially only alanine at the amino-terminal end of the unfractionated light chain and some of its seven electrophoretic components. Each of these seven fractions, when isolated from a partially reduced, carboxamidomethylated light-chain preparation, behaved upon reelectrophoresis as a single moiety. However, four to five components were resolved from components 1, 3, and 6 upon reelectrophoresis at pH 9.4 in acrylamide gel containing 8 M urea when each of these single bands was extensively reduced and alkylated in 7 M guanidine hydrochloride.

This same resolution was also obtained when partially reduced, carboxamidomethylated components 1, 3, and 6 were simply exposed to a freshly deionized 10 M urea solution. Both quantitative precipitin studies and allotype determinations indicated that the slowest and fastest migrating of the seven major electrophoretic components differed most in their behavior as precipitating antigens when compared in this regard to the unfractionated light chain. Chemical heterogeneity was also indicated by peptide maps of tryptic digests of unfractionated light chains and its electrophoretic components which showed three times and two times the number of peptides expected from their respective lysine and arginine content.

There is considerable evidence for the heterogeneity of immunoglobulins, both as antigens and antibodies, which has been described and discussed in detail in a number of recent reviews (Cohen and Porter, 1964a,b; Franklin, 1964; Nisonoff and Thorbecke, 1964; Fleischman, 1966). The pronounced electrophoretic heterogeneity of light polypeptide chains of several species has been described (Poulik and Edelman, 1961; Poulik, 1964; Cohen and Porter, 1964a,b; Feinstein, 1966). An example of a genetic control of heterogeneity of such molecules was provided by the discovery of allotypes (Oudin, 1956), the genetically controlled antigenic forms of serum proteins which differ among individuals within a species. In our laboratory we have carried out studies in order to understand the relationship between the genetic control of allotypic specificities and the structure and biosynthesis of rabbit γG-immunoglobulins. To elucidate some of the chemical differences associated with allotypic specificities we investigated amino acid composition (Reisfeld, *et al.*, 1965) and peptide maps of both light (Small *et al.*, 1965) and heavy chains (Small *et al.*, 1966). We found distinct differences in amino acid composition among light chains with different allotypic specificities. Peptide maps of either heavy or light chains with different allelic allotypes varied distinctly. Evidence for considerable immunochemical heterogeneity of antigens and antibodies of the allotype–antiallotype system has been obtained by a number of investigators and has been recently reviewed (Oudin, 1966). Some of the complexities of the b4–anti-b4 system were documented in a recent report from our laboratory evaluating the minimum number of b4 allotypic determinants on rabbit γG-immunoglobulins (Mage *et al.*, 1966).

The objective of this study was to investigate chemical and immunochemical differences among the major electrophoretic components of rabbit light polypeptide chain with b4 allotypic specificity and to look for evidence of heterogeneity within the individual components.

Materials and Methods

Rabbits. The rabbits which were used to obtain serum pools were $a^1a^1b^4b^4$ progeny of parents with known genotype. They were from colonies which were bred over a number of years at the National Institutes of Health.

Preparation of Light Polypeptide Chains of γG-Immunoglobulins. The γG-immunoglobulin was pre-

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pared from the rabbit serum pool by sodium sulfate precipitation followed by chromatography on diethylaminoethylcellulose as described previously (Reisfeld *et al.*, 1965). Partial reduction followed by carboxamidomethylation was performed prior to isolation of the light polypeptide chains by gel filtration on Sephadex G-100 as previously described (Reisfeld *et al.*, 1965). Protein concentrations were determined spectrophotometrically using a value of $E_{1\,cm}^{1\%}$ 15.0 at 280 mμ or by a Kjeldahl nitrogen method followed by colorimetry with Nessler's solution (Lanni *et al.*, 1950; Vannier and Campbell, 1961).

Reagents. All the solvents used in chromatography were reagent grade. Acrylamide and methylenebisacrylamide were recrystallized from acetone. Phenyl isothiocyanate (Eastman Organic Chemicals) was purified by distillation *in vacuo*. Dioxane was distilled over sodium metal and stored frozen. *N*-Ethylmorpholine (Eastman Organic Chemicals), practical grade, was redistilled (bp 137–139°) and stored at 5°. Iodoacetamide and iodoacetic acid were recrystallized from petroleum ether (bp 30–60°) and chloroform, respectively. Urea was either recrystallized from ethanol or deionized just prior to use. Guanidine hydrochloride was filtered and recrystallized from water until the optical density of a 7 M solution measured at 280 mμ was less than 0.025. 1-Dimethylaminonaphthalene-5-sulfonyl chloride was recrystallized from hexane. Trypsin (Worthington Biochemical Corp., lot 6241) was recrystallized after treatment with L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone to eliminate residual chymotryptic activity (Kostka and Carpenter, 1964).

ANTISERA. Rabbit anti-b4 antiserum used for quantitative precipitin studies and for precipitations of ^{125}I-labeled light chain fractions was a pool of antisera from two rabbits which had been immunized with γG-immunoglobulin prepared by DEAE chromatography. Rabbit anti-b5 antiserum used as "Coombs" reagent in precipitations of labeled light chains was from a single rabbit which had received immunizations of b5 immunoglobulin in the form of an ovalbumin–antiovalbumin specific precipitate. These antisera have been described previously (Mage and Dray, 1965). A goat was immunized with unfractionated b4 light chains. Protein (1 mg) in complete Freund's adjuvant was injected intramuscularly in multiple sites. One month later additional intramuscular injections of 1 mg in Freund's incomplete adjuvant were given, and the animal was bled for serum 8 days later. Another goat was similarly immunized with isolated component 3 using a 0.5-mg dose each month.

Quantitative Precipitin Studies. For establishing precipitin curves with rabbit antisera, we used a micro method (Mage and Dray, 1965) measuring precipitate nitrogen (Lowry *et al.*, 1951). With the goat antisera we used a larger scale, modified Folin method (Heidelberger and MacPherson, 1943a,b).

Multiphase Zone Electrophoresis on Polyacrylamide Gels. Preparative gel electrophoresis was performed using essentially the apparatus and method described by Jovin *et al.* (1964). The Polyprep apparatus manufactured by Buchler Instruments, Fort Lee, N. J., was used for all preparative electrophoreses. The buffer system described by Jovin *et al.* (1964) was slightly modified as described previously (Reisfeld and Small, 1966) and both analytical and preparative electrophoreses were generally performed at pH 9.4 and 25° in the absence of urea in gels with a concentration of acrylamide ranging from 7.5 to 15%. Extensively reduced and carboxymethylated preparations were analyzed in gels containing 8 M urea and having an acrylamide content from 4 to 6%. The relative mobilities of protein bands obtained by analytical acrylamide gel electrophoresis were expressed as R_F values which were computed from the position of each band with reference to the bromophenol blue tracking dye front. In a typical experiment 25 mg of light polypeptide chain was dissolved in 2 ml of upper gel buffer (0.046 M Tris–0.032 M H_3PO_4, pH 6.9) containing 5% (w/v) sucrose and thoroughly dialyzed against this same solution. The sample was carefully layered on top of a preparative acrylamide column, 4.5 cm in height with a 15-cm² cross-sectional area (50-ml lower gel, 20-ml upper gel), and a trace of bromophenol blue dye was added. A constant current of 15 ma and 100 v was applied until the sample had completely entered the upper gel. Then the current was increased to 60 ma and 280 v. The acrylamide concentration of the upper gel was 2.5% and that of the lower gel 8%. The bromophenol blue dye front emerged from the bottom of the gel 2.5 hr after the dye band had entered the lower gel. The elution rate was 0.8 ml/min. Fractions were collected at 5-min intervals. It was found advantageous to decrease the elution rate gradually from 0.8 to 0.2 ml per min after 4 hr of running time had elapsed. This aided in obtaining less diffuse elution patterns. Recovery of protein from the columns ranged from 70 to 80%.

Extensive Reduction and Alkylation. Light polypeptide chains were extensively reduced with dithiothreitol (0.01 M) and alkylated with either iodoacetamide or iodoacetic acid (0.022 M) at pH 8 in the presence of 7 M guanidine hydrochloride. Reduced and alkylated proteins were thoroughly dialyzed against several changes of deionized, 10 M urea solutions prior to electrophoresis.

Quantitation of Allotype. Samples of light polypeptide chain preparations and their electrophoretic components isolated by preparative electrophoresis on polyacrylamide gels were labeled with ^{125}I (McFarlane, 1958). The fraction precipitable with anti-b4-allotype antiserum was determined. This was followed by indirect precipitation of b4–anti-b4 complexes, using anti-b5 antiserum as an antiglobulin reagent. The method has been previously described in detail (Dray and Nisonoff, 1963).

Amino Acid Analyses. Three samples (0.2–0.5 mg each) from each electrophoretic component and from the unfractionated light chain were hydrolyzed *in vacuo* in 6 *N* hydrochloric acid for 24, 48, and 72 hr, respectively. Hydrolysates were analyzed with a Beckman–Spinco Model 120 C amino acid analyzer (Spackman *et al.*, 1958) using an accelerated buffer system (Hubbard, 1965; Hubbard and Kremen, 1965).

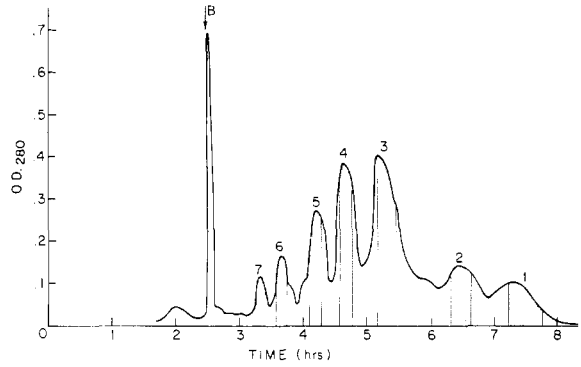

FIGURE 1: Elution pattern resulting from preparative multiphase zone electrophoresis of 25 mg of light polypeptide chain. The shaded areas denote the volume fractions of each respective peak which were pooled and further analyzed. B indicates the position of the bromophenol blue dye front. Conditions are described in the text.

Normalized mole recoveries for all three hydrolysis times for a given component were averaged arithmetically with the following exceptions. Only 72-hr values of valine and isoleucine were considered. Serine and threonine values were linearly extrapolated to zero hydrolysis time by the method of least squares. The averaged and extrapolated values were renormalized to a total of 204 residues exclusive of tryptophan, half-cystine, and carboxymethylcysteine. The total number of residues was chosen arbitrarily to correspond to a molecular weight of about 22,000.

Amide Determinations. The method of Chrambach (1960) was used with some minor modifications for determination of total amide content. Light polypeptide chains and their electrophoretic components as well as a sample of ribonuclease (Worthington two times crystallized) were carefully dried to constant weight at 37° and analyzed for nitrogen content by the Kjeldahl method followed by colorimetry with Nessler's reagent. Samples were hydrolyzed with 2 N HCl *in vacuo*, for 3 hr at 110°. Hydrolysates were placed in Plexiglass Conway diffusion dishes of identical size, and the liberated ammonia was titrated with a standardized solution of HCl. All determinations were made in triplicate. Each sample was also carefully analyzed for free ammonia content prior to hydrolysis.

Amino-Terminal End-Group Analyses. Samples of 0.02 μmole of light polypeptide chains were dinitrophenylated with [^{14}C]FDNB[1] by the method of Sanger as cited by Fraenkel-Conrat *et al.* (1955). The phenylthiohydantoin derivative of the N-terminal amino acid was prepared by the paper strip modification of the method of Fraenkel-Conrat *et al.* (1955).

Amino-terminal end groups were also determined by the cyanate method of Stark and Smyth (1963). Samples of 0.4 μmole of light polypeptide chain were carbamylated in a total volume of 5 ml of *N*-ethylmorpholine buffer (pH 8) containing 8 M urea and 0.6 M potassium cyanate. Carbamylation was carried out for 18 hr at 50°.

N-Terminal analyses were also carried out by the "Dansyl" method of Gray and Hartley (1963a,b). Aliquots of polypeptide chains ranging from 1 to 5 × 10^{-3} μmole were evaporated to dryness and dissolved in 15–20 μl of 0.1 M sodium bicarbonate in ammonia-free water. An equal volume of freshly prepared 1-dimethylaminonaphthalene-5-sulfonyl chloride in acetone (2 mg/ml) was added and the mixture was incubated for 3 hr at 37°. The reaction mixture was dried and the residue was placed in 20 μl of constant-boiling HCl and hydrolyzed in a sealed tube at 110° for 18 hr. The product was evaporated *in vacuo*, spotted on Whatman No. 3MM paper, and subjected to electrophoresis on a cooled flat plate in pyridine–acetate buffer (pH 4.55).

Trypsin Digestion. For tryptic digestion 5 mg of light polypeptide chain was oxidized with performic acid according to the method of Moore (1963), but without the use of hydrogen bromide as a reducing agent. After lyophilization, the sample was placed in 0.5% ammonium bicarbonate, trypsin was added, and digestion was carried out at 37° for 12 hr. A light chain to trypsin ratio of 100:1 was routinely used. After digestion, samples were lyophilized. The mapping of the tryptic hydrolysate was carried out by a standard technique (Katz *et al.*, 1959). Between 2 and 3 mg of lyophilized digest was applied to Whatman No. 3MM filter paper. The chromatographic separation was carried out with butanol–acetic acid–water (4:1:5). After chromatography the papers were dried and subjected to electrophoresis at 3000 v for 1 hr under Varsol with a pyridine–acetic acid–water buffer (1:10:289) at pH 3.6. Peptides were detected with a ninhydrin spray reagent (Levy and Chung, 1963).

Results

Preparative multiphase zone electrophoresis on polyacrylamide gel columns separated partially reduced and

TABLE I: R_F Values and Relative Amounts of the Seven Major Electrophoretic Components of Rabbit Light Polypeptide Chain.

Component	R_F Values	% of Total Area	% Area of Peak Analyzed (shaded)
1	0.35	6.2	63
2	0.40	7.0	51
3	0.50	12.2	41
4	0.55	26.4	61
5	0.60	29.0	58
6	0.69	10.7	31
7	0.75	8.3	44

[1] Abbreviations used: FDNB, fluorodinitrobenzene; PTH, phenylthiohydantoin derivative.

TABLE II: Amino Acid Composition of Unfractionated Light Chain and Its Electrophoretic Subfractions Expressed as Residues.[a]

Amino Acid	Component 1	2	3	4	5	6	7	Unfractionated L Chain	Calcd Unfractionated Light Chain	Std Error of Mean for 3 Runs[b]
Lys	9.6	8.4	8.7	8.6	8.9	9.2	8.9	8.7	8.8	±0.09
His	1.64	1.31	1.18	1.33	1.35	2.16	1.97	1.24	1.47	0.046
Arg	4.4	3.0	3.2	3.1	3.6	4.6	3.9	3.1	3.6	0.08
Asp	19.2	19.4	18.7	18.8	18.6	18.8	20.0	18.7	18.9	0.07
Thr	25.2	28.6	29.9	28.8	28.8	24.3	25.8	28.1	28.0	0.16
Ser	20.3	20.5	21.5	21.0	21.7	19.0	19.3	20.5	20.8	0.25
Glu	20.0	20.2	19.9	20.1	19.5	20.6	20.2	20.1	20.0	0.09
Pro	10.7	11.4	10.9	11.8	11.1	10.6	12.0	11.2	11.3	0.21
Gly	2.06	18.9	18.5	18.8	19.2	21.9	20.4	19.5	19.5	0.12
Ala	17.9	16.0	15.9	16.0	16.2	18.3	17.8	16.0	16.5	0.09
Val	19.6	20.7	20.7	20.5	20.1	18.7	19.2	21.2	20.1	0.13
Met	1.16	0.95	0.83	0.84	0.99	1.51	1.42	0.90	1.03	0.047
Ile	7.3	7.3	7.2	7.1	7.1	7.8	7.4	7.4	7.2	0.12
Leu	11.6	10.8	10.5	10.5	10.9	12.1	11.2	10.5	10.9	0.06
Tyr	8.2	10.0	9.9	10.1	9.5	8.0	8.3	10.2	9.4	0.08
Phe	6.8	6.5	6.6	6.6	6.5	6.4	6.3	6.5	6.5	0.07
CM-Cys	0.99	1.00	1.01	0.93	0.94	1.04	0.90	0.95	0.96	0.053
Amide	23.5	18.6	17.2	15.7	14.5	13.3	10.1	16.4	15.4	

[a] Calculated on the basis of 204 residues for all amino acids except tryptophan, half-cystine, and carboxymethylcysteine. [b] Standard error of the mean for sets of three determinations (single bands) averaged at the level of sums of squares. Standard errors for band 7 (based on a single determination) should be increased by the factor $\sqrt{3}$. Standard errors of Val and Ile are estimates of weighted averages from data on seven bands. Standard errors for Thr and Ser were calculated with deviations from the least-squares line of regression rather than from the arithmetic mean.

carboxamidomethylated light polypeptide chain with b4 allotypic specificity into at least seven major electrophoretic components as shown in Figure 1. Each component isolated from the central area of each peak (shaded) moved as a single band upon reelectrophoresis at pH 9.4, with essentially the same R_F value as in the original light-chain preparation. A representative pattern of the seven major components is shown in Figure 2. Table I shows the R_F values of each of the major components calculated from both preparative and analytical acrylamide electrophoreses. The same table also lists the relative amounts of each of the seven major effluent peaks shown in Figure 1 as calculated from measurement of the total area of each peak. The relative percentage of the shaded area within each peak is also represented. Comparison of the total areas of each of the major peaks shows a general distribution which somewhat resembles a Gaussian curve. As depicted in Figure 3, the areas between the shaded portion of some effluent peaks show additional electrophoretic components as well as considerable overlap due to incomplete resolution of the electrophoretic light-chain populations. This observation, as well as the appearance of new bands whenever analytical acrylamide gels were "overloaded" with light polypeptide chains, indicated that we see only part of the total population, namely the major electrophoretic populations of light polypeptide chains. The amino acid compositions of the seven major components and of the unfractionated light chain are given in Table II. The average value obtained for carboxymethylcysteine is 0.98 residue. Carboxymethylcysteine values for all components fall within ±1.1 times the standard error of the mean for a single component. The result suggests that the *S*-carboxamidomethylation was uniform and restricted to a single half-cystine residue, most likely the one participating in the interchain disulfide bridge. Also, it appears likely that variations in average peptide chain length (exceeding about 6%) do not occur among the components, and that the value assigned to the total number of residues in a chain is a fair estimate.

Significantly nonintegral values are obtained for methionine in components 6 and 7 and for histidine in components 1, 2, 4, and 5 if one allows a combined experimental error of up to four times the standard error plus a 10% error in the assigned value for total

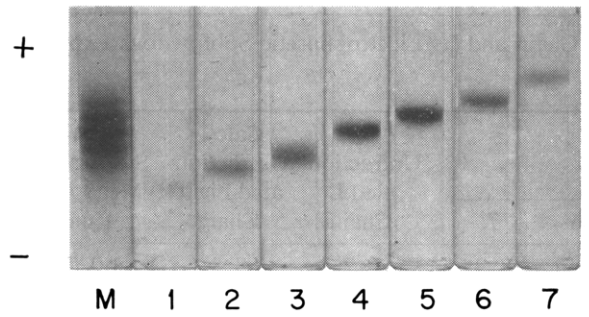

FIGURE 2: Analytical acrylamide electrophoresis patterns of unfractionated light chain (M) and its seven major electrophoretic components (shaded areas in Figure 1). Volume fractions comprising each shaded area were pooled and concentrated to 1-ml volumes. An aliquot (50 μl) of each concentrated fraction was applied to each gel column. The protein concentrations of these aliquots comprising components 1–7 were 20, 30, 45, 150, 160, 40, and 30 μg, respectively; 300 μg of unfractionated light chain (M) was applied.

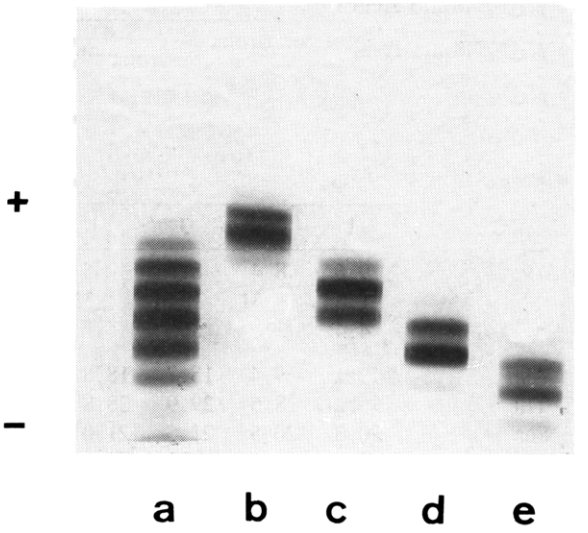

FIGURE 3: Electrophoretic patterns of components isolated from areas between major peaks of an "overloaded" (50 mg) preparative acrylamide column. Unfractionated light chain (a); area between peaks 6 and 7 (b); area between peaks 3 and 4 (c); area between peaks 2 and 3 (d); and area between peaks 1 and 2 (e).

residues. These components are thus demonstrated to be heterogeneous.

In regard to amino acid composition the seven components fall into two separate groups which may be designated group I, comprising components 2, 3, 4, and 5, and group II, comprising components 1, 6, and 7. A significant difference in content of an amino acid between two band fractions is interpreted as a difference equal to or exceeding four times the corresponding average standard error of the mean for a component. Members of group II vary significantly and characteristically from members of group I (when all pairs are compared) in having more histidine (0.3–1.0 residue), arginine (0.3–1.6 residues), glycine (1.3–3.4 residues), alanine (1.5–2.4 residues), methionine (0.2–0.7 residue), and leucine (0.3–1.6 residues), and less threonine (2.8–5.6 residues), valine (0.5–2.0 residues), and tyrosine (1.2–2.0 residues). When comparing pairs of components which fall within a group, one finds no amino acid which varies by more than 1.5 residues. Within group I no significant differences appear in regard to either phenylalanine or isoleucine content. Differences exceeding three or four times the standard error of the mean occur for all other amino acids between components 2 and 5. Members of group II vary significantly among themselves for all amino acids measured except tyrosine. Component 6 varies more widely from components 1 and 7 than the latter two vary from one another in spite of their extreme separation in electrophoretic mobility. Component 6 has more glutamic acid, glycine, alanine, and leucine and less aspartic acid and threonine than components 1 and 7. Similarly, component 5 varies somewhat more from other members of group I than components 2–4 do from one another. Throughout the seven bands the ratio of histidine to methionine is constant (1.43 $\pm$ 0.15) while each of these amino acids varies nearly twofold.

The data shown in Table II also show a marked difference in amide residues among the components which in itself could account for their electrophoretic resolution since an increase in amide groups was associated with a decrease in electrophoretic mobility. The reliability of the amino acid analyses data for the seven major electrophoretic components is pointed out by a comparison of the number of amino acid residues obtained by analysis of unfractionated light chain and by calculation from the weighted averages of the composition of the seven components. Almost all amino acids varied less than 0.5 residue with only valine and tyrosine varying from 0.8 to 1.0 residue.

The data presented thus far imply that differences in the number of charged carboxyl groups are mainly responsible for the electrophoretic resolution of the light-chain populations. This was further evaluated by electrophoresis of light-chain preparations at pH 2.8 where, in all probability, only a minor portion of the carboxyl groups is charged. At this pH one can still observe essentially seven components (not shown); however, we do not know whether these components migrated in the same order as those observed at pH 9.4. Furthermore, amino groups apparently do not greatly contribute to electrophoretic resolution since the electrophoretic patterns of light chain were virtually identical at pH 9.4 and 7.8 (not shown).

In an attempt to determine the extent of electrophoretic heterogeneity we subjected partially reduced, carboxamidomethylated whole light-chain preparations as well as component 3 to extensive reduction in 7 M guanidine hydrochloride and either carboxamidomethylation or carboxymethylation. Amino acid anal-

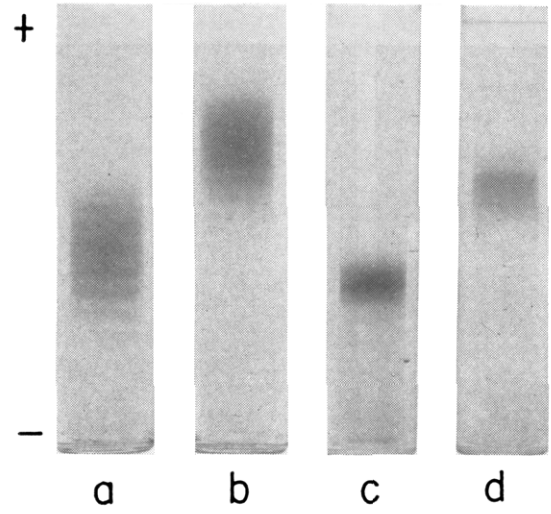

FIGURE 4: Electrophoretic patterns of extensively reduced and alkylated preparations. Unfractionated light chain, carboxamidomethylated (a); unfractionated light chain, carboxymethylated (b); component 3, carboxamidomethylated (c); and component 3, carboxymethylated (d).

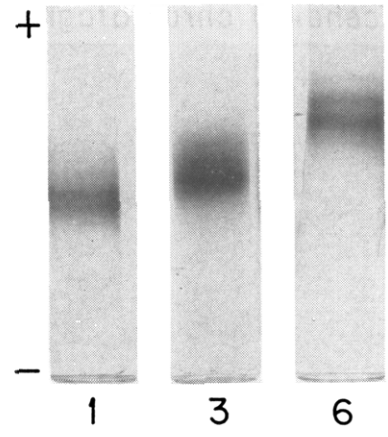

FIGURE 5: Electrophoretic patterns of extensively reduced, carboxamidomethylated preparations. Component 1 (a), component 3 (b), and component 6 (c).

yses revealed that at least five of the seven expected half-cystine residues reacted in each case and that no amino acids other than half-cystine were alkylated. Figure 4 depicts the electrophoretic patterns of those preparations in the presence of 8 M urea. It is evident that the addition of at least five carboxyl groups in the case of carboxymethylation (Figure 4b) caused a definite shift in electrophoretic mobility of whole light polypeptide chain, without, however, essentially altering the electrophoretic pattern, *i.e.*, no additional components could readily be detected. It should be pointed out that unfractionated L-chain preparations (Figure 4a,b) are clearly poorly resolved showing much unresolved material between major components. In contrast, when the heretofore seemingly single electrophoretic component 3 (Figure 4c,d) was treated in this manner it showed additional bands and exhibited the expected increase in electrophoretic mobility. To further test this apparent increase in electrophoretic resolution, we compared the electrophoretic patterns in 8 M urea gels of components 1, 3, and 6 after their extensive reduction and carboxamidomethylation in 7 M guanidine hydrochloride. The result (Figure 5) showed each of the originally single components to consist of at least four to five electrophoretic subfractions. No charged groups were added. Similar electrophoretic patterns were observed when partially reduced and carboxamidomethylated components 1, 3, and 6 were simply exposed to three changes of freshly deionized 10 M urea solution over a period of 8 hr.

To further determine the degree of heterogeneity of light polypeptide chains, we investigated the amino-terminal residues of unfractionated light chains and its electrophoretic components 1, 3, 4, 6, and 7. We did not observe with any of the methods used significant qualitative differences in N-terminal amino acids between unfractionated light chains and its electrophoretic components. Under the usual dinitrophenylation conditions, with the addition of 8 M urea, spots on chromatograms were observed with R_F values of alanine and aspartic acid, the former being present in considerably larger amounts. We also applied the Edman procedure and found PTH-alanine as the principal derivative detected chromatographically, with traces of aspartic acid, glutamine, and glycine. Estimation of PTH-alanine by spectral measurements gave the value of 0.86 mole/mole of light chain. Attempts to identify the adjacent residue showed in a preliminary experiment a single derivative, PTH-valine. Amino-terminal analyses were also made by the Dansyl method and the 1-dimethylaminonaphthalene-5-sulfonylamino acids were resolved and identified by high-voltage electrophoresis on Whatman No. 3MM paper. Results clearly showed alanine, smaller amounts of glycine, and traces of aspartic acid. However, when the light-chain preparations were repeatedly precipitated with trichloroacetic acid (10%, w/v), and the resultant supernatant was extracted with ether and analyzed directly on the Beckman analyzer, the following amino acids (residues per mole of light chain) were found: glycine (0.2), serine (0.11), aspartic acid (0.05), alanine (0.04), threonine (0.03), glutamic acid (0.01), and valine (0.01). Reagent and glassware blanks did not show any detectable amino acids. Thus it would seem that at least in our light-chain preparations obtained from γG-immunoglobulin of b4 homozygous rabbits there are these noncovalently bound amino acids present and that all the detectable light chain populations have essentially only alanine present at the amino-terminal end. Amino-terminal end-group analyses were also carried out by the cyanate method (Stark and Smyth, 1963). Control experiments were performed in which noncarbamylated light-chain preparations were carried through the analyses. The extent of carbamylation as estimated by amino acid analysis of

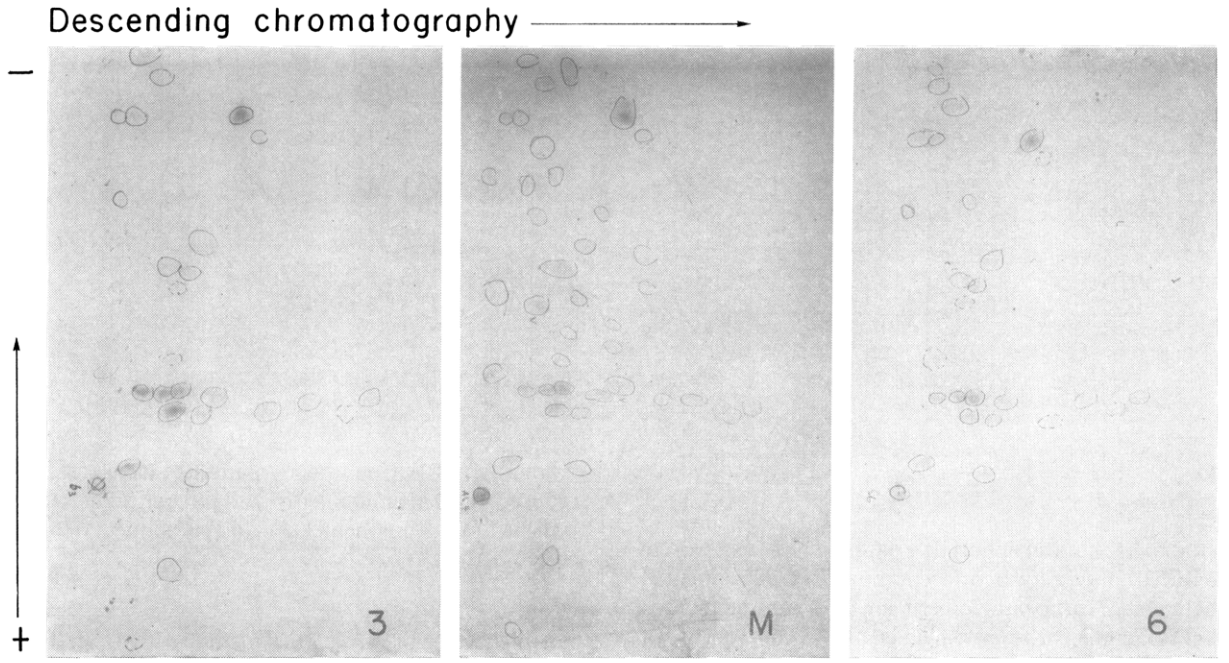

FIGURE 6: Tryptic peptide maps of unfractionated light chain (M), component 3 (3), and component 6 (6).

acid hydrolysates for lysine and homocitrulline showed that the reaction had gone 60% to completion, *i.e.*, lysine was still present to the extent of 40%. After the fractionation of the hydrolysate on Dowex 50 and alkaline hydrolysis of the resulting hydantoins, we recovered essentially only alanine with traces of aspartic acid, glutamic acid, and glycine. In the control experiments, traces of these same amino acids were detected in the amount of approximately 0.005 μmole which represents approximately $^1/_{30}$ of the expected molar residue recovery.

To further study the heterogeneity of light polypeptide chain and its electrophoretic components, we have compared the peptide maps of tryptic digests of unfractionated light chain and of components 3 and 6, respectively, as shown in Figure 6. The maps of these components are strikingly similar and show only very minor differences. There is a decrease in the number of spots by a factor of 1.5 when the fingerprints of components 3 and 6 are compared with the peptide map of unfractionated light chain. However, heterogeneity in both components 3 and 6 is clearly indicated since the maps show essentially twice the number of peptides expected on the basis of the arginine and lysine content. Unfractionated light chain shows three times the number of expected peptides.

We examined the question of whether the electrophoretically separated light-chain fractions differed in their behavior as precipitating antigens using both rabbit anti-b4 allotype and goat anti-b4 light-chain antisera. In Table III we show that all the components contained molecular species which were directly precipitable with rabbit anti-b4 (from immunized b5 rabbits). The ^{125}I-labeled, unfractionated light chain (60%) was precipitable directly by anti-b4 and all but 8% of the remaining labled light-chain population was precipitated upon addition of anti-b5 as a "Coombs" antiglobulin reagent to precipitate light chains specifically complexed with the b5-anti-b4 antibodies. Some light chains in the mixture (about 8% of the total) did not combine with anti-b4 antiserum; these light chains have been designated *b negative* (Oudin, 1966). The data in Table III indicate that components 1 and 7 contained fewer molecules with detectable b4 determinants and conversely had slightly higher per cents of *b-negative* molecules. Component 7 especially was

TABLE III: Quantitative Determination of Allotype Content of ^{125}I-Labeled Unfractionated Light Chain and Its Electrophoretic Components.

Component	^{125}I Precipitable Directly by Anti-b4 (%)	Total ^{125}I Precipitable (direct and indirect ppt) (%)
1	39	86
2	66	94
3	45	91
4	35	89
5	56	90
6	45	86
7	13	80
M	60	92

poorly precipitated by direct addition of anti-b4 antiserum, forming more soluble complexes which required the addition of anti-b5 for "indirect" precipitation.

Precipitin studies using the antiserum of a goat (Figure 7A,B) which had been immunized with the unfractionated b4 light-chain mixture (M) showed that the antiserum could not distinguish between M and components 2–4. These four preparations gave identical precipitin curves, precipitating a maximum of 310 μg of antibody N/ml of antiserum. Whereas all antibodies precipitable by the immunizing mixture were also precipitable by the light chains in components 2–4, the different slopes and lower total antibody precipitated indicated that a small per cent (6–10%) of antibodies precipitable by the mixture may not have been precipitable by components 1, 5, and 6. We did not have enough material to extend the curve for component 7 and determine the maximal precipitable antibody; it is probable that this component was also unable to precipitate all the antibody precipitable by the immunizing mixture. A goat immunized with purified component 3 made antibody with similar properties to that shown suggesting that at least this particular component has most of the immunogenic determinants present in the unfractionated light chain.

The goat antisera are highly specific for the allotype of the immunizing antigen; b5 light chains (Figure 7A, closed squares) precipitated less than 10% of the antibody precipitable by the b4 light chains. The homologous b4 light chains used for immunization were also better precipitating antigens than the b4 γG- mmunoglobulin from which they were separated; the latter precipitated only about one-third of the total precipitable antibody (Figure 7B, closed squares). With rabbit anti-b4 antiserum the situation was reversed; whole b4 γG-immunoglobulin used for immunization was a better precipitating antigen than separated light chains (Figure 7C). The antibody in rabbit sera not precipitated with light chains was precipitable from supernatants by whole b4 γG-immunoglobulin. We compared isolated b4 light chains before further electrophoretic steps (Figure 7C, open circles) with a control mixture taken through the electrophoretic steps and relyophilized and it was this latter control mixture which was used in subsequent comparison (Figure 7C–E, closed circles).

The curves shown in Figure 7C–E were obtained with a microquantitative precipitin method because of the limited amounts of available reagents. The same general picture emerged as was seen with the goat antisera. The mixture and component 4 produced identical curves; components 2 and 3 were similar, rising with identical slopes. The curves of components 1, 5, 6, and 7 were different. The study was repeated using an independently separated set of electrophoretic subfractions as precipitating antigens and strikingly similar relationships of the components to the mixture were found with the exception of components 6 and 7. In the new set, they were both very poor precipitating antigens for the rabbit antiserum although they were similar to the previous preparations of components 6 and 7 when tested with the

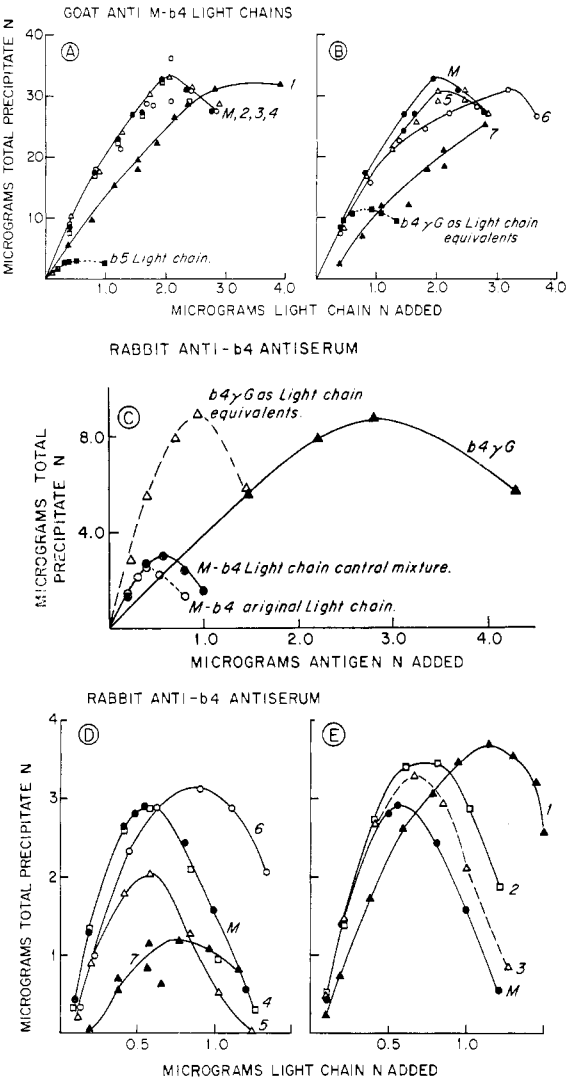

FIGURE 7: Quantitative precipitin curves of unfractionated light chain and its electrophoretic subfractions. M denotes unfractionated light chain. We used 0.1 ml of goat antiserum or 0.15 ml of a 1 : 5 dilution of rabbit anti-b4 antiserum per precipitin point.

goat antiserum. Since direct precipitation with anti-b4 antiserum does not precipitate all light chains capable of binding antibody even with larger amounts of reactants (Table III), ^{125}I-labeled light chains were used in precipitin tests on the microscale used to establish the curves shown in Figure 7C–E in order to estimate the amount of ^{125}I-labeled antigen in precipitates. Per cent precipitated label ranged from a low of 5% for component 7 to a high of 47% (component 2) and depended upon the degree of antibody excess; a greater percentage of labeled antigen was found in the precipitates formed at a point in moderate antibody excess than was found in the precipitates formed at a point in antibody excess but nearer to the equivalence region of the curve. Using estimates of antigen in the precipitate based on ^{125}I label we calculated that the total antibody pre-

cipitable by components 1–6 was equal to or slightly more than that precipitable by M; component 7, however, precipitated only 43% of the antibody precipitable by M. The state of aggregation of the L-chain mixture (M) and its electrophoretic subfractions could affect their behavior as direct precipitating antigens. In order to evaluate aggregation, we subjected several subfractions (1, 3, 6, and 7) to gel filtration on Sephadex G-100 in saline–borate buffer (pH 8.1) (0.16 M H_3BO_3–0.02 M NaOH–0.13 M NaCl). These subfractions emerged with essentially the same elution volume as did unfractionated L chain on Sephadex G-100 in 1 M propionic acid.

Discussion

The analyses of light-chain preparations both by preparative and analytical electrophoresis on polyacrylamide gels have clearly indicated that in partially reduced, carboxamidomethylated preparations at least seven major components can be distinguished. Although these components have genetically determined b4 antigenic determinants in common, they are chemically heterogeneous and each contains a number of electrophoretic subpopulations. The main electrophoretic differences which distinguish these components can be ascribed to changes in the number of carboxyl groups as demonstrated by amide analyses. It was evident from these determinations that an increase in electrophoretic mobility was associated with a decrease in amide content. The effect of carboxyl groups on the electrophoretic mobility of light chains in acrylamide gels was further demonstrated by the marked increase in the mobility of all electrophoretic subfractions upon carboxymethylation of five half-cystine residues (Figure 4). Similar results have been reported previously with light chains isolated from normal human IgG and from myeloma proteins (Feinstein, 1966). Estimation of total net negative charges at pH 9.4 of components 1–7 obtained from amino acid and amide compositions gave the following values: 3.5 (1), 11.4 (2), 11.3 (3), 13.4 (4), 13.0 (5), 14.0 (6), and 19.0 (7). The unfractionated light chain by the same estimation had a total negative charge of 12.5. It is evident that in regard to net charge, components 1 and 7 differ considerably from each other and from the rest of the components which, in turn, do not differ markedly from each other. This is probably another reflection of heterogeneity since there may be more electrophoretically unresolved subpopulations in components 2–6 than in component 1 and 7.

Another phenomenon which can, at least in part, account for electrophoretic resolution is the state of the tertiary structure, *i.e.*, folding and unfolding and resultant available charged groups in a given charge environment. For example, it has been shown that beef heart lactate dehydrogenase with a net negative charge of 69, estimated from amino acid and amide analyses, moved much more rapidly toward the anode in starch gel electrophoresis than did chicken heart enzyme with an estimated charge of 90 at pH 7 (Pesce *et al.*, 1964). Preparations of β-lactoglobulin isolated from two different species showed differences in electrophoretic mobility despite identity of amide content and only minor differences in four neutral amino acids (Phillips and Jenness, 1965). The finding of additional bands among components 1, 3, and 6 (Figure 5) upon extensive reduction and carboxamidomethylation in 7 M guanidine hydrochloride could be due to reorientation of charged groups in a new environment. One has to be aware, however, of the possibility of unequal or incomplete reduction and alkylation and that this may actually cause the appearance of additional electrophoretic components. However, this could not explain the additional bands which were also seen upon electrophoresis of partially reduced and alkylated components which had simply been exposed to 10 M urea solutions. It is unlikely that these newly resolved bands can be ascribed entirely to carbamylation of the ε-amino group of lysine residues since cyanate effects were minimized by the use of several changes of freshly deionized urea solutions the conductance of which was constantly monitored at 3–5 μmho. The additional finding that lysine content did not change after this treatment and that no homocitrulline whatsoever could be detected makes it unlikely that any significant carbamylation did occur.

The data from amino acid analyses suggest heterogeneity of the light-chain subfractions as indicated from significantly nonintegral values for amino acid residues, especially histidine and methionine. In regard to amino acid composition, the seven subfractions fall into two groups which differ from each other and may be designated group I comprising components 2–5 and group II consisting of components 1, 6, and 7. Group II varies from group I and from unfractionated light chains by a total of approximately 16 residues, involving 15 different amino acids. This is a similar variation involving, however, more different amino acids than was observed in a comparison of unfractionated light chains with b4 and b5 allotypic specificity. It is possible that some of these amino acid differences between groups I and II are related to the differences in their behavior as precipitating antigens (Reisfeld, *et al*, 1965) and that the differences in amino acid composition between groups I and II actually may reflect the existence of two major antigenic populations similar to κ and λ types observed in man and mouse. In this regard, it is of interest that extreme anodal and cathodal electrophoretic subfractions of normal human IgG light chains have a greater proportion of λ chains than do the more central electrophoretic components (Cohen and Gordon, 1965). Thus it is possible that rabbit light chains with amino acid composition characteristics of group II components 1, 6, and 7 may have larger proportions of populations with different antigenic types than group I components 2–5. This hypothesis is strengthened by data from quantitative allotype determinations given in Table III which show fractions 1 and 7 to contain fewer molecules with detectable b4 determinants. These two components conversely contain slightly larger amounts of *b-negative* molecules which do not combine with anti-b4 antiserum. These *b-negative* molecules represent another

antigenic population and could possibly be analogous to the population(s) responsible for the differences in amino acid composition between groups I and II. There are some interesting correlations between amino acid composition and quantitative precipitin data of subfractions of light chain. Thus components 1, 6, and 7 differ from components 2–5 in their behavior as precipitating antigens (Figure 7A–D) as well as in their respective amino acid compositions (Table II). Component 5 differs somewhat more from other group I components 2–4 in both amino acid composition and in its behavior as a precipitating antigen.

Amino-terminal analyses of both unfractionated light chain and some of its electrophoretic components have shown essentially one major terminal residue (alanine) and small traces of a number of other amino acids. The latter consist mainly of noncovalently bound amino acids which can be removed by trichloroacetic acid precipitation of unfractionated light-chain preparations. From these data and the finding of only valine at the second position after the N terminal, we cannot distinguish the electrophoretic subfractions of light chain in groups I and II as we can from their respective amino acid compositions.

The peptide maps indicate that components 3 and 6 are quite heterogeneous since they show approximately two times the number of peptides expected from the arginine and lysine content of a single polypeptide chain with a molecular weight of 22,000. It is, of course, possible that some of the spots located in the position of neutral amino acids could be due to free amino acids. However, we doubt that this is the case for all the additional spots since the quantities of the seven free amino acids were such that only two of them (glycine and serine) would be detectable at all on the basis of the sensitivity of the spray reagent used (Rydon and Smith, 1952). Furthermore, all the detectable peptides are strikingly similar in both components. The great similarity in peptide maps of these two components which differ in amino acid composition and immunochemical properties may at least in part be explained by a relatively large common peptide pattern shared by these components and a consequent dilution of variant spots which are thus quite difficult to detect. However, we cannot, at present, differentiate whether some of the peptides common to both components 3 and 6 are part of the same antigenic group.

The heterogeneity of related light polypeptide chains in a homozygous rabbit poses an interesting question. What is the mechanism which maintains an area of antigenic determinants under genetic control in a relatively large proportion of chemically heterogeneous light polypeptide chains?

From the work on human Bence-Jones proteins it is known that the variable portion of the κ- and λ-type light chains is between residues 1 and 107 while antigenic determinants controlled by alleles Inv (a) and Inv (b) are associated with the substitution of a single amino acid in the common portion at position 191 (Hilschman and Craig, 1965; Milstein, 1966; Baglioni *et al.*, 1966). It is evident from our data that we have chemical variations as well as common antigenic determinants. The common *b4* determinants may indicate that there is also an invariant region present in rabbit light chains. However, our data do not distinguish whether this is so or whether common *b4* antigenic determinants are expressed by homologous peptides scattered throughout the light polypeptide chain.

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In Vitro Metabolism of Testosterone-4-^{14}C and Δ^4-Androstene-3,17-dione-4-^{14}C in Human Skin*

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ABSTRACT: Testosterone-4-^{14}C was incubated with specimens of human skin. Metabolites were separated by paper chromatography and thin layer chromatography. Androstenedione, 5α-dihydrotestosterone, 5α-androstanedione, androsterone, and epiandrosterone were identified as metabolites. The metabolites were identified by reverse isotopic dilution and the preparation of derivatives (formation of acetates or reduction with $NaBH_4$). Incubation of androstenedione-4-^{4}C with human skin produced metabolites with similar chromatographic mobilities, and the formation of testosterone was established by reverse isotopic dilution. No radioactive etiocholanolone or etiocholanedione could be detected among the metabolites.

Human skin has been shown to metabolize testosterone at a rate considerably greater than liver (Wotiz *et al.*, 1956). This finding is of particular interest because the skin is one of the largest organs of the body. Wotiz *et al.* (1956) detected several metabolites by autoradiography after testosterone-4-^{14}C was incubated with human skin *in vitro*, and one of these was identified as androstenedione[1] by mixed chromatography and infrared spectrophotometry. The identification of several additional metabolites by mixed chromatography and reverse isotopic dilution was reported in a preliminary communication from our laboratory (Gomez and Hsia, 1966), but no evidence was found which supported the formation of etiocholanolone, a major urinary metabolite of testosterone. Independently Rongone (1966a,b), using gas chromatography, identified etiocholanolone as one of several metabolites of testosterone in the skin of a patient with Klinefelter's syndrome. In an effort to ascertain if normal skin transforms testosterone to etiocholanolone or any other 5β metabolites, we have studied skin specimens from several subjects of both sexes and varying ages. This paper reports our findings on the metabolism of testosterone-4-^{14}C and the conversion of androstenedione to testosterone in human skin.

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[1] The following abbreviations and trivial names are used: NAD, nicotinamide–adenine dinucleotide; NADH, reduced nicotinamide–adenine dinucleotide; NADP, nicotinamide–adenine dinucleotide phosphate; 5α-androstanedione, 5α-androstane-3,17-dione; androstenedione, Δ^4-androstene-3,17-dione; androsterone, 5α-androstan-3α-ol-17-one; 5α-dihydrotestosterone, 5α-androstan-17β-ol-3-one; epiandrosterone, 5α-androstan-3β-ol-17-one; etiocholanedione, 5β-androstane-3,17-dione; etiocholanolone, 5β-androstan-3α-ol-17-one.

Experimental Section

Chemicals. Testosterone-4-^{14}C and androstenedione-4-^{14}C with specific activities of 45.2 mc/mmole were purchased from New England Nuclear Corp. and purified by chromatography in the ligroin–propylene glycol system described by Savard (1953). Stock solutions