and M. Rabaey, *Amer. J. Ophthalmol.* **50**, 793 (1960); W. B. Chodirker and T. B. Tomasi, *Science* **142**, 1080 (1963).
9. T. B. Tomasi, E. M. Tan, A. Solomon, R. A. Prendergast, *J. Exp. Med.* **121**, 101 (1965).
10. G. R. Jago and M. Morrison, *Proc. Soc. Exp. Biol. Med.* **111**, 585 (1962); S. J. Klebanoff and R. G. Luebke, *ibid.* **118**, 483 (1965).
11. P. Jolles and J. Jolles, *Nature* **192**, 1187 (1961); R. C. Chandan, K. M. Shahani, R. G. Holly, *Nature* **204**, 76 (1964).
12. J. F. Petit and P. Jolles, *ibid.* **200**, 168 (1963); H. H. Chauncey, *J. Amer. Dental Assoc.* **43**, 361 (1961).
13. V. Krause, *Acta Ophthalmol. Supp. 53* 1959); J. Allerhand *et al., J. Pediat.* **62**, 85 (1963).
14. A. S. Josephson and D. W. Lockwood, *J. Immunol.* **93**, 532 (1964).
15. M. L. Groves, *J. Amer. Chem. Soc.* **82**, 3345 (1960); B. Johanson, *Acta. Chem. Scand.* **14**, 510 (1960); J. Montreuil, J. Tonnelat, S. Mullet, *Biochim. Biophys. Acta* **45**, 413 (1960); B. Blanc and H. Islike, *Bull. Soc. Chim. Biol.* **43**, 929 (1961); W. G. Gordon, J. Ziegler, J. J. Basch, *Biochim. Biophys. Acta* **60**, 410 (1962).
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17. We thank J. Bright and O. Finley for assistance.

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Electrophoretic Heterogeneity of the Polypeptide Chains of Human G-Myeloma Proteins

Abstract. *The light and heavy polypeptide chains derived from human G-myeloma proteins are electrophoretically heterogeneous as judged by disc electrophoresis of the polypeptide chains in urea-acrylamide gels. Individual myeloma proteins contained as many as eight light-chain and nine heavy-chain components.*

Normal human serum immunoglobulin G (IgG) consists of a group of molecules that are heterogeneous when judged by immunochemical and electrophoretic criteria (*1*). Human G-myeloma proteins, by contrast, have been considered homogeneous (*2*). It has become increasingly apparent, however, that myeloma proteins are only relatively homogeneous by electrophoretic criteria.

Electrophoretic heterogeneity in the starch-gel patterns of intact G-myeloma proteins has been demonstrated by Askonas, Fahey, and others (*3*) using myeloma proteins from both man and mouse. The constituent light and heavy polypeptide chains of human myeloma proteins are also electrophoretically heterogeneous. Cohen and Porter (*4*) demonstrated two bands in alkaline urea starch-gel analyses of light chains from most partially reduced and alkylated G-myeloma proteins. Heavy polypeptide chains (γ-chains) yielded a diffuse electrophoretic zone that did not resolve into bands. Poulik (*5*) reported even greater heterogeneity; he found at least five components in some myeloma light chains analyzed by starch-gel electrophoresis at alkaline *p*H. His studies in acid urea starch gel also suggested the presence of multiple components in myeloma protein γ-chains.

Myeloma proteins are the most homogeneous preparations of immunoglobulin molecules now available. For this reason they have served as models for structural analyses of immunoglobulin molecules. The high resolving power of disc electrophoresis in acrylamide gels prompted us to reexamine the electrophoretic homogeneity of polypeptide chains isolated from myeloma proteins.

A G-myeloma protein molecule is constructed of two light polypeptide chains that are antigenically either of type K (κ-chains) or type L (λ-chains) (*6*) and two heavy polypeptide chains of one of the four antigenic subclasses (γ_{2a}, γ_{2b}, γ_{2c}, γ_{2d}) (*7*). Four human G-myeloma proteins were selected to represent the four heavy chain subclasses and both types of light chains. These proteins were isolated (*8*) and found by immunochemical means to be relatively uncontaminated (*9*).

In previous electrophoretic studies of myeloma polypeptide chains (*4, 5*) the chains were obtained by partial reduction of the myeloma proteins, under conditions that resulted in the breaking predominantly of interpolypeptide-chain disulfide bonds. To minimize the possibility that folding of polypeptide chains (caused by intrachain disulfide bridges) might contribute to electrophoretic heterogeneity, the isolated proteins were extensively reduced in 7*M* guanidine hydrochloride (*10*) with 0.1*M* dithioerythritol, and then alkylated in 5.2*M* guanidine hydrochloride with 0.22*M* iodoacetamide at *p*H 8.2. Light and heavy polypeptide chains were isolated by gel filtration on Sephadex G-200 in 5*M* guanidine hydrochloride (*10*). Since it is not possible to perform electrophoresis in the presence of high concentrations of charged molecules (such as guanidine hydrochloride), the polypeptide chains were dialyzed against 8.5*M* urea and then analyzed by disc electrophoresis on 4 percent polyacrylamide gels at *p*H 9.4 in the presence of 8.5*M* deionized urea (*11*).

The acrylamide-gel patterns of heavy-chain preparations from myeloma proteins representing the four antigenic subclasses of IgG all showed from six to nine discrete bands (Fig. 1, upper). Light polypeptide chains isolated from these same myeloma proteins showed from three to eight discrete components (Fig. 1, lower). Light chains from pooled normal serum IgG showed seven to eight components (Fig. 1, lower). The intensity of staining of light-chain bands of most myeloma preparations differed markedly from the "gaussian distribution" usually seen in patterns of normal light chains (*4*).

These gel patterns indicate considerable degrees of electrophoretic heterogeneity of both the light and heavy chains derived from G-myeloma proteins. The following experiments were performed to examine several possible causes for the multiple electrophoretic components. A single molecular species might produce multiple bands as the result of some unknown process during electrophoresis. In that instance, reelectrophoresis of one isolated band should lead again to the generation of multiple bands. In an experiment performed on the κ-chains from protein Sp, two identical gels were subjected to electrophoresis simultaneously at *p*H 9.4. The multiple bands were

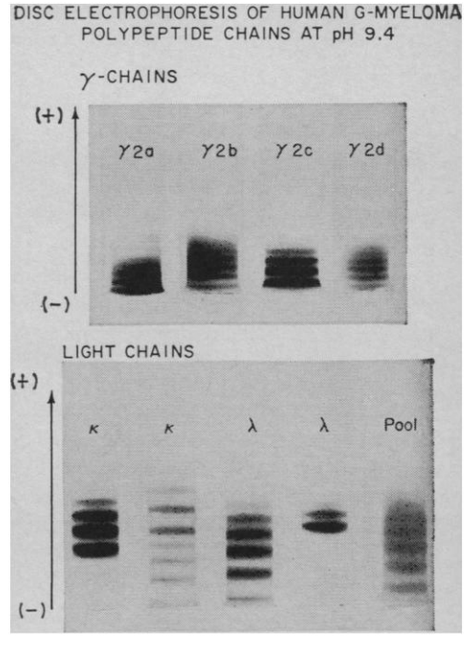

Fig. 1. Electrophoretic patterns of reduced and alkylated G-myeloma polypeptide chains. Upper: heavy chains, type γ_{2a} from Sa; γ_{2b} from Sp; γ_{2c} from Ta; γ_{2d} from Me. Lower: light chains, type κ from Sa; κ from Sp; λ from Ta; λ from Me; and light chains from pooled normal IgG. Disc electrophoresis in direction of arrow in 4 percent acrylamide gel, *p*H 9.4 tris-HCl buffer, 8.5*M* urea.

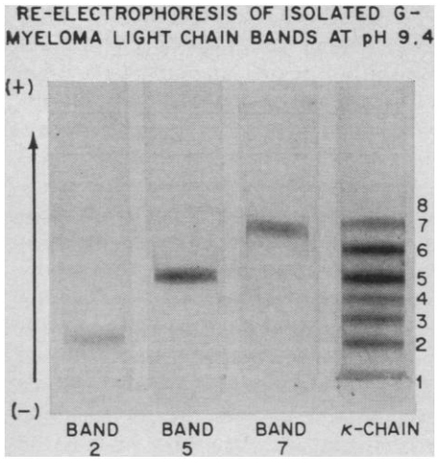

Fig. 2. Electrophoretic patterns of Sp protein light-chain bands after reelectrophoresis. κ-Chain from protein Sp is at the extreme right. Bands 2, 5, and 7 were cut out of an acrylamide gel and subjected to reelectrophoresis at *p*H 9.4, 8.5*M* urea.

quickly detected by immersing one gel in 10 percent trichloroacetic acid to precipitate the protein. Appropriate zones were cut out of the companion gel. Reelectrophoresis of these gel slices yielded single components of the expected mobility (Fig. 2), an indication that the bands are not artifacts of electrophoresis.

Another potential artifact could arise from unequal alkylation of the polypeptide chains. For example, some molecules are alkylated only at cysteine residues, whereas others that are alkylated at cysteine may also be alkylated at histidine, or lysine, or both. These possibilities were investigated by comparing the electrophoretic patterns of a reduced but nonalkylated myeloma protein with the patterns produced by the reduced, alkylated, and separated heavy and light chains from the same protein. This comparison could not be carried out at alkaline *p*H, presumably because of incomplete dissociation of the light and heavy chains in 8.5*M* urea at *p*H 9.4. Therefore, myeloma protein Sp was reduced in 8 to 10*M* urea at *p*H 8.2, and the reduction product was then acidified to *p*H 2 to 3. This reduced protein was compared with its reduced, alkylated, and separated chains by electrophoresis in polyacrylamide gels at *p*H 2.3 in 7*M* urea (*12*). Essentially identical banding patterns of the alkylated and nonalkylated heavy chains were observed (Fig. 3). Some studies of light chains showed comparable banding for the alkylated and nonalkylated proteins, but resolution was relatively poor. In Fig. 3 the alkylated light chains show several bands, while the nonalkylated light chains give a broad zone of the same average mobility, but without discrete banding. Thus, although the data on light chains are not conclusive, the banding of heavy chains could not be shown to be an artifact of alkylation in these experiments.

Deterioration of myeloma proteins during long-term storage of serum seemed to be an unlikely cause of the observed heterogeneity, since chains isolated from myeloma protein obtained from the same patient 1 and 3 years prior to this study gave essentially identical patterns in acrylamide gel electrophoresis (patterns not shown). More rapid deterioration also seems an unlikely cause of heterogeneity, since Askonas (*3*) has shown that the myeloma protein extracted from the microsomal fraction of a mouse plasma cell tumor is as heterogeneous as the serum myeloma protein.

Multiple electrophoretic components might also result from aggregation of the polypeptide chains. To test this possibility, extensively reduced and alkylated light and heavy polypeptide chains were subjected to high-speed sedimentation equilibrium analysis (*13*) in the buffer used for gel electrophoresis (8.5*M* urea, tris, *p*H 9.4). Results calculated for both light and heavy chains from interference fringe measurements obtained at three protein concentrations showed linear curve when the $\log_e$ (ln) of concentration was plotted against the square of the radius (Fig. 4) and gave no evidence of mass heterogeneity (*14*). Mass heterogeneity thus does not account for the observed electrophoretic banding.

Myeloma proteins appear to be more homogeneous than normal serum IgG; however, the polypeptide chains of the myeloma proteins examined in this study are all electrophoretically heterogeneous. The finding of eight electrophoretically different light chains and nine heavy chains in a single myeloma protein would be consistent with the possibility of as many as 72 "different" molecules within a single myeloma protein. The heterogeneity is present in light polypeptide chains of antigenic types K and L and also in heavy chains of antigenic subclasses γ_{2a}, γ_{2b}, γ_{2c}, and γ_{2d}. This heterogeneity has not been shown to result from prolonged serum storage, contamination with other proteins, mass heterogeneity, unequal alkylation, or the effects of electrophoresis per se.

The chemical factors responsible for these multiple electrophoretic components have not yet been elucidated. Our

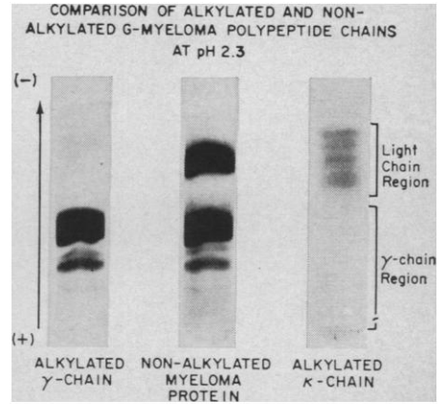

Fig. 3. Electrophoretic patterns comparing reduced myeloma protein Sp with its reduced and alkylated polypeptide chains. From left to right: reduced and alkylated Sp heavy chain; reduced nonalkylated Sp myeloma protein; reduced and alkylated Sp light chain. Electrophoresis in 4 percent acrylamide gel, *p*H 2.3, 7*M* urea.

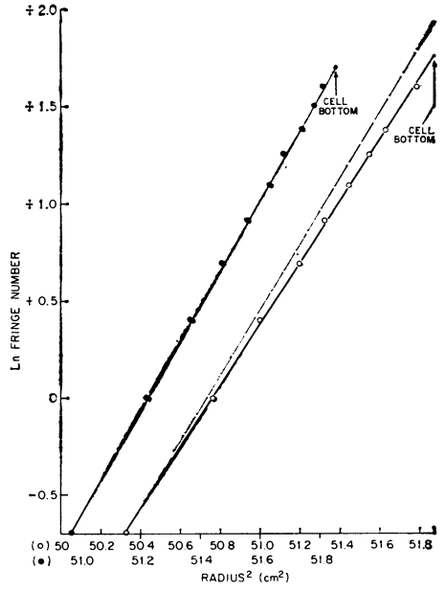

Fig. 4. High-speed sedimentation equilibrium analysis. Open circles are data points for light chain from Sa at an initial concentration of 0.3 mg/ml, centrifuged for 48 hours at 39,460 rev/min at 20°C. Solid circles are data points for the heavy chain from Sa at an initial concentration of 0.3 mg/ml, centrifuged for 48 hours at 35,600 rev/min. Solid lines are the "least squares" fit of the experimental points, while the dashed line represents the line that would have been obtained if the light-chain sample contained 10 percent dimer in addition to the monomeric light chain. Molecular weights were derived from these data (*14*).

data do not bear on the question of whether the observed heterogeneity (whatever its chemical basis) is developed at the time of synthesis, or whether this is a "postsynthesis" phenomenon similar to that described for mouse myeloma protein (*15*).

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References and Notes

1. J. L. Fahey, *Advance. Immunol.* **2** (1962).
2. F. W. Putnam, *Physiol. Rev.* **37**, 512 (1957).
3. J. A. Owen, C. Got, H. J. Silberman, *Clin. Chim. Acta* **3**, 605 (1958); B. A. Askonas, *Biochem. J.* **79**, 33 (1961); J. Fahey, *J. Clin. Invest.* **42**, 111 (1963).
4. S. Cohen and R. R. Porter, *Biochem. J.* **90**, 278 (1964).
5. M. D. Poulik, *Nature* **202**, 1174 (1964).
6. M. Mannik and H. G. Kunkel, *J. Exp. Med.* **116**, 859 (1962); J. L. Fahey and A. Solomon, *J. Clin. Invest.* **42**, 811 (1963).
7. H. M. Grey and H. G. Kunkel, *J. Exp. Med.* **120**, 253 (1964); W. D. Terry and J. L. Fahey, *Science* **146**, 400 (1964).
8. J. L. Fahey and C. J. McLaughlin, *J. Immunol.* **91**, 484 (1963).
9. Sa is a type K γ_{2a}-protein contaminated with less than 5 percent of other immunoglobulins containing λ light chains and heavy chains other than γ_{2a}, when tested by immunodiffusion against appropriate antiserums at 200 mg/ml; Sp is a type K γ_{2b}-protein with less than 1 percent contamination when tested at 110 mg/ml; Ta is a type L γ_{2c}-protein with 5 to 10 percent contamination when tested at 60 mg/ml; Me is a type L γ_{2d}-protein without contamination when tested at 40 mg/ml. No serum proteins other than IgG were detected in any of these preparations. For example, contamination in protein Sp was estimated as follows; Sp is a type K γ_{2b}-protein, and when tested by Ouchterlony analysis at 110 mg/ml showed either no precipitation band or a very faint band with antiserum to λ-chain. This antiserum detects λ-type IgG at concentrations as low as 0.05 to 0.1 mg/ml. On the basis of the known 2:1 ratio of κ:λ, it is assumed that the λ-type contamination represents one-third of the total contamination. Therefore Sp is considered to contain about 0.3 mg of contaminating IgG in 110 mg, which is less than 1 percent. Calculations based on tests with antiserum to heavy chains yield similar estimates of contamination.
10. P. A. Small and M. E. Lamm, *Biochemistry* **5**, 259 (1966).
11. R. A. Reisfeld and P. A. Small, *Science* **152**, 1253 (1966).
12. R. A. Reisfeld, unpublished.
13. D. A. Yphantis, *Biochemistry* **3**, 297 (1964).
14. Size heterogeneity would have been indicated by (i) curvature of the line when the natural logarithm of concentration was plotted against the square of the radius, and (ii) by Z-average molecular weights significantly higher than the weight-average molecular weights. For the light chain of protein Sa, averages of three determinations at 0.1, 0.2, and 0.3 mg/ml were: weight-average molecular weight 23,000 ± 800; Z-average of 20,500 ± 1,100. The averages of three determinations at 0.3, 0.6, and 0.9 mg/ml for the heavy chains were: weight-average molecular weight 49,600 ± 2,400; Z-average of 50,300 ± 1,900. These light- and heavy-chain weight averages do not differ significantly from their Z-averages. Results were based on an assumed partial specific volume of 0.71 and 0.72 respectively in a solvent of density 1.1236.
15. E. Kolsch, *Fed. Proc.* **25**, 490 (1966).
16. We thank Dr. J. L. Fahey for support and encouragement and Miss M. S. Robert and Mr. J. Boone for technical assistance.
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Tritiated Norepinephrine: Release from Brain Slices by Electrical Stimulation

Abstract. *Slices of rat brain and heart that had concentrated H^3-norepinephrine were superfused and electrically stimulated. Stimulation induced a marked release of H^3-norepinephrine with a threshold and a maximum repsonse. Release also occurred with increased concentrations of potassium, presumably due to neuronal depolarization. Inhibition of electrically induced release occurred with low calcium and with chlorpromazine and pentobarbital.*

Norepinephrine has been thought to be a neurotransmitter in the central nervous system, although the evidence is incomplete. Endogenous concentration and regional distribution of norepinephrine have been determined in brain (*1*). The blood-brain barrier prevents entry of administered norepinephrine into the brain from the circulation. Tritiated norepinephrine introduced into the lateral ventricle of rat brain appears to mix with endogenous norepinephrine, since its regional and subcellular distributions parallel those of the endogenous amine (*2*).

In oxygenated physiologic media, slices of tissues rich in adrenergic nerve endings concentrate H^3-norepinephrine (*3*). Norepinephrine taken up by brain slices is concentrated at nerve endings in regions rich in endogenous catecholamine, and the tissue appears to maintain histologic integrity after incubation (*4*). In addition, neurons in such preparations of brain slices maintain their resting membrane potentials and their ability to form action potentials during 2 to 3 hours of incubation (*5, 6*). Brain slices stimulated with alternating current at 1 to 10 volts have a markedly increased respiratory rate (*7, 8*). Such stimuli depolarize neurons in brain slices (*5*) and are known to elicit physiologic responses when applied to the mammalian cortex (*7*).

We used slices of rat heart and rat brain to study the effect of electrical stimulation on release of norepinephrine. Slices of heart and mid-coronal sections of brain (100 to 200 mg) were incubated for 30 minutes at 37°C in Ringer solution supplemented with tricarboxylic cycle intermediates (*3*) containing H^3-norepinephrine (25 ng and 1.25 μc per milliliter). The medium was agitated and saturated with 5 percent carbon dioxide in oxygen. After incubation, concentration of tritium in brain slices was 18 times the concentration in incubation medium; in heart slices, 6 times.

Slices were transferred to a 5-ml organ bath and bathed at 37°C with a continuous flow (2.5 ml/min) of oxygenated Krebs-Ringer medium. Serial fractions of effluent medium were collected during 2-minute intervals and assayed for total radioactivity and for H^3-norepinephrine and its metabolites (*9*). Unaltered H^3-norepinephrine accounted for about 80 percent of total radioactivity in effluent solutions; only traces of methylated catecholamine (normetanephrine) were found, and oxidized metabolites of norepinephrine accounted for most of the remaining radioactivity.

The rate of washout of H^3-norepinephrine from brain slices followed a multiphase course and was greatest during the first 10 to 15 minutes. Electrical stimuli of the order of 5 volts for 60 seconds, when applied about 20 minutes after the start of superfusion, produced striking and rapid increases in efflux of H^3-norepinephrine, with a return to base line within a few minutes (Fig. 1). Heart slices behaved similarly. At the end of these experiments, the slices had lost only a small fraction of their radioactivity.

Stimuli of 0.5 volt applied for 30 seconds did not appear to release tritium; from 1 to 4 volts, there appeared to be a direct relation between release of tritium and applied voltage. Stimuli above 4 volts, applied for 30