morphological, histochemical, and physiological characteristics are very likely connected with the ways in which the different motor unit groups, even within a single muscle, are utilized by the central nervous system in various types of movement [see (*2*) and (*22*)].

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References and Notes

1. H. A. Padykula and G. F. Gauthier, in *Exploratory Concepts in Muscular Dystrophy and Related Disorders*, A. T. Milhorat, Ed. (Excerpta Medica, Amsterdam, 1967), p. 117.
2. E. Henneman and C. B. Olson, *J. Neurophysiol.* **28**, 581 (1965); C. B. Olson and C. P. Swett, Jr., *J. Comp. Neurol.* **128**, 475 (1966).
3. R. J. Barnard, V. R. Edgerton, T. Furukawa, J. B. Peter, *Amer. J. Physiol.* **220**, 410 (1971); D. Denny-Brown, *Proc. Roy. Soc. Ser. B.* **104**, 371 (1929); B. Nyström, *Acta Neurol. Scand.* **44**, 405 (1968).
4. C. S. Sherrington, *Proc. Roy. Soc. Ser. B*, **105**, 332 (1929).
5. L. Edström and E. Kugelberg, *J. Neurol. Neurosurg. Psychiat.* **31**, 424 (1968).
6. R. E. Burke, *J. Physiol. London* **193**, 141 (1967).
7. ———, P. Rudomin, F. E. Zajac, *Science* **169**, 122 (1970).
8. The decline in tension during repetitive stimulus trains over the 2-minute period used to assess the fatigue index did not appear to be due to fatigue of the neuromuscular junction. Muscle fiber action potentials (EMG responses) recorded from the muscle surface over the stimulated units usually changed little over this period. This suggests that the number of muscle fibers participating in the mechanical response was essentially constant and indicates that the diminution of mechanical tension output was due primarily to fatigue in the excitation-contraction coupling mechanism or in the contractile mechanism itself.
9. EMG responses were of constant shape and amplitude throughout unfused tetani showing "sag," which suggests that the tension decline was not due to muscle fibers dropping out of response. Other evidence suggests that the "sag" is not due to true fatigue but rather results from subtle changes in the kinetics of excitation-contraction coupling or in the contractile mechanism.
10. As used here, contraction time denotes the interval between initiation of the EMG response and the peak of the mechanical twitch response. Maximally potentiated twitches, recorded after a series of repeated short tetani (posttetanic potentiation), were used for these measurements in order to have all of the units in the series in an approximately comparable state. This represents a difference in technique from an earlier study (*6*) and appears to account in part for differences from the earlier results.
11. E. Kugelberg and L. Edström, *J. Neurol. Neurosurg. Psychiat.* **31**, 415 (1968).
12. W. K. Engel and M. H. Brooke, in *Neurological Diagnostic Techniques*, W. S. Fields, Ed. (Thomas, Springfield, Ill., 1966), p. 90.
13. J. F. A. McManus and R. W. Mowry, *Staining Methods: Histological and Histochemical* (Harper & Row, New York, 1960), p. 126.
14. E. Farber, W. H. Sternberg, C. D. Dunlop, *J. Histochem. Cytochem.* **4**, 254 (1956).
15. M. M. Nachlas *et al.*, *ibid.* **5**, 420 (1957).
16. H. A. Padykula and E. Herman, *ibid.* **3**, 170 (1955).
17. M. H. Brooke and K. K. Kaiser, *ibid.* **17**, 431 (1969).
18. G. A. Drews and W. K. Engel, *Nature* **212**, 1551 (1966).
19. M. Brandstater and E. H. Lambert, *Bull. Amer. Ass. Electromyogr. Electrodiagn.* **15–16**, 82 (1969); A. M. Doyle and R. F. Mayer, *Bull. Sch. Med. Univ. Md.* **54**, 11 (1969). We have assumed that the glycogen-depleted fibers found in muscles in which a single motor unit had been stimulated actually do belong to the same motor unit. It seems impossible to demonstrate this directly, but because control muscles have not shown glycogen-free fibers of otherwise normal appearance, the above assumption seems justified. Glycogen depletion in muscle fibers of types FF and FR motor units was quite complete, with no residual staining in relatively large numbers of fibers that were readily identified. Completeness of depletion was checked under high magnification. Muscle fibers of type S units were in several cases more difficult to identify, because fewer depleted fibers were found in these units and because fibers histochemically similar to those of S units often have little glycogen to begin with. However, in each of the studied S units, muscle fibers with barely detectable glycogen staining, or no trace of staining at all, were found (see Fig. 2). The discussion of whether or not the techniques used in the present study were adequate to deplete *all* of the muscle fibers belonging to a specific motor unit, and the problem of fiber count per unit is in preparation.
20. The reaction products of the DPHND and SDH stains in fibers of type FF units were distributed in a fine, very interrupted intermyofibrillar network that sometimes showed a tendency to increase in density toward the fiber periphery. In FR unit fibers, the particles were distributed in a coarser, interrupted intermyofibrillar network that usually showed a tendency to increase in density toward the periphery, with subsarcolemmal clumps. In fibers of type S units, the reaction products were distributed uniformly in a dense uninterrupted network without peripheral accumulation, and subsarcolemmal clumps were not prominent. The pattern of distribution of these reaction products has been used in the past as an important criterion in the classification of muscle fiber types [J. M. Stein and H. A. Padykula, *Amer. J. Anat.* **110**, 103 (1962)].
21. F. C. A. Romanul, *Arch. Neurol. Chicago* **12**, 497 (1965).
22. R. E. Burke, *J. Physiol. London* **196**, 631 (1968); ———, E. Jankowska, G. ten-Bruggencate, *ibid.* **207**, 709 (1970).

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Creation of "Amyloid" Fibrils from Bence Jones Proteins in vitro

Abstract. *"Amyloid" fibrils have been created from some human Bence Jones proteins by proteolytic digestion under physiologic conditions. These fibrils with an antiparallel, β-pleated sheet conformation consist of only a portion of the variable region of the immunoglobulin light polypeptide chain and share the physical properties of amyloid fibrils. The relation between amyloidosis and immunoglobulins is thus more firmly established and a pathogenetic mechanism for amyloid fibril formation is suggested.*

Amyloid fibrils, the structures that are generally recognized as the characteristic and lethal component deposited in tissues in the disease amyloidosis (*1*), have certain well-defined properties. These include a green polarization birefringence after being stained with Congo red (*2*), a protein consisting of polypeptide chains in an antiparallel conformation and β-pleated sheet structure, as judged by x-ray crystallography (*3*), a distinguishing (*4*) but occasionally variable appearance (*5*) when viewed by electron microscopy, and a relative resistance to enzymic degradation (*6*). Evidence obtained by amino acid sequence and immunochemical studies of purified amyloid fibril proteins (*7*) suggests that amyloid fibrils consist primarily of the amino-terminal variable segment of the light polypeptide chain of homogeneous immunoglobulins in those cases studied. If indeed this is their source, then it should be possible to produce a variable region fragment having the characteristics of amyloid

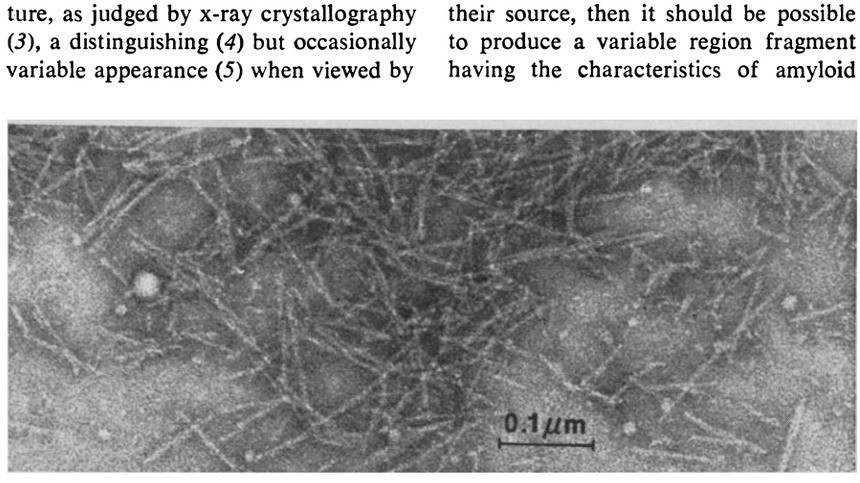

Fig. 1. Electron micrograph of fibrils formed by peptic digestion of a λ Bence Jones protein, Nic, after incubation at *p*H 3.5 for 2 hours at 37°C.

fibrils from homogeneous light chains.

A method for cleaving Bence Jones proteins into variable and constant fragments by proteolysis has been described by Solomon *et al.* (*8*). We subjected three κ and two λ Bence Jones proteins having no polysaccharide constituents from patients not known to have amyloidosis to a 2- to 5-hour incubation in 0.05*M* glycine–HCl buffer, *p*H 3.5, at a concentration of 10 mg/ml in the presence of pepsin (40 μg/ml) at 37°C. During the incubation, precipitates formed with the two λ Bence Jones proteins. These precipitates were centrifuged at 8000*g* for 1 hour in a refrigerated centrifuge (Sorvall), and a portion of the sediments was stained with alkaline Congo red, examined by polarization microscopy, and found to have a green birefringence (*2*). Electron microscopy by the negative staining method (*9*) showed that the precipitates were composed of fibrils measuring from 70 to 80 Å in width and from 1000 to 2000 Å in length, and that they had the appearance of twisted doublet filaments characteristic of amyloid fibrils (Fig. 1). The remaining sediments were throughly washed with 0.1*N* ammonium acetate buffer, *p*H 5.0, and lyophilized. Examination of both sediments by x-ray diffraction methods (*3*) revealed a strong, sharp band at 4.75 Å and a moderately strong, diffuse halo at 9.3 Å (Fig. 2). This picture is typical of the antiparallel β-pleated sheet configuration of amyloid fibrils (*3*). Infrared studies (*10*) confirmed this x-ray diffraction interpretation of the fibril conformation.

One of the λ Bence Jones proteins (Nic BJ) was chosen for further study. The proteolytically derived sediment (Nic fragment) was denatured in 6*M* guanidine hydrochloride buffered to *p*H 8.0 with 0.1*N* tris-HCl containing 1 percent mercaptoethanol, exhaustively dialyzed with distilled water, and lyophilized. The molecular weight of the Nic fragment, as determined by column chromatography on Sephadex G-100 equilibrated with 5*M* guanidine in 1*N* acetic acid (*11*), was 4600. By this method of column chromatography, the Nic fragment was also purified to give a single band when subjected to disc electrophoresis on a sodium dodecyl sulfate–polyacrylamide gel (*12*). Both the Nic BJ and the Nic fragment were aminoethylated (*13*) in 6*M* guanidine hydrochloride buffered to *p*H 8.0 with 0.1*N* tris-HCl. The aminoethylated proteins were digested with trypsin, and

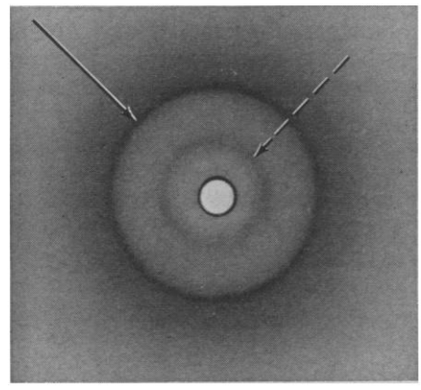

Fig. 2. X-ray diffraction of fibrils (see Fig. 1) formed from the Nic Bence Jones protein after peptic digestion. Solid arrow points to 4.75-Å backbone spacing and dashed arrow indicates 9.3-Å side chain spacing.

peptide mapping was performed (*14*). Common region peptides from the peptide map of the Nic fragment were absent. The partial amino acid sequence of the constant region of Nic BJ has been reported (*15*). The amino acid sequence of the aminoethylated Nic fragment was determined with an automatic amino acid analyzer (Beckman model 890) (*16*). The amino-terminal amino acid sequence of the Nic fragment is compared in Table 1 with that of Ha, a λ Bence Jones protein of variable region subgroup VλI (*17*). They are very similar except that the Nic fragment appears to lack the first three amino acids. The yield of leucine at the first step (position 4) was 25 percent of theoretical, based on a molecular weight of 4600. This may mean that a portion of the Nic fragment may still have a blocked amino terminus. Despite the possible heterogeneity at the amino terminus, the sequence data in conjunction with peptide mapping and molecular weight studies show that the Nic fragment derives exclusively from a portion of the variable region of Nic BJ (*18*).

These studies demonstrate that fibrils having the tinctorial, ultrastructural, and crystallographic properties (*3–5*) of amyloid fibrils can be created from some but not all Bence Jones proteins by peptic digestion at *p*H 3.5 and 37°C. The results of peptide mapping, sequence analysis, and molecular weight determinations prove that these fibrils are derived solely from the variable region of the parent light chain. This ability to form structures with properties similar to those of amyloid fibrils from the variable region of some Bence Jones proteins further corroborates the findings that amyloid fibrils are derived primarily from the variable region of immunoglobulin proteins (*7*).

The ratio of λ to κ light chains of intact myeloma proteins in multiple myeloma is approximately 1 : 2, whereas there is an equal ratio of λ to κ types when Bence Jones proteins only are produced in multiple myeloma without amyloidosis (*19*). However, in amyloidosis associated with Bence Jones proteinuria, the ratio of λ to κ Bence Jones proteins is increased to approximately 3 : 2 (*20*). In the small series of amyloid proteins studied by us the ratio of λ to κ variable region fragments is 2 : 1 (*7*). Our finding of a higher frequency of λ, as compared to κ Bence Jones proteins capable of forming fibril aggregates under the proteolytic conditions employed, parallels the preferential association of amyloidosis with λ light chains. Differences both in primary structure and physical properties are known to distinguish λ from κ light chains (*21*). Such differences may explain the greater "amyloid" fibril-forming capacity of the variable fragments created by proteolysis from the λ as compared to the κ Bence Jones proteins noted here and the relatively higher ratio of λ to κ light chains associated with amyloidosis.

The fact that "amyloid" fibrils can be created from some Bence Jones proteins at a physiologic temperature in the presence of a proteolytic enzyme having an acidic *p*H optimum suggests that one possible pathogenetic mecha-

Table 1. Amino acid sequence of Nic fragment compared to the sequence of the prototype VλI, Ha (*17*). Peptic digestion cleaved the first three amino acids from the Nic Bence Jones protein, therefore, in Nic fragment these deletions are introduced to maximize the homology (*18*).

	1	2	3	4	5	6	7	8	9	10	11
Nic fragment				Leu	Thr	Gln	Pro	Pro	Ser	Val	Ser
Ha	Glp	Ser	Val	Leu	Thr	Gln	Pro	Pro	Ser	Val	Ser
	12	13	14	15	16	17	18	19	20	21	22
Nic fragment	Gly	Ala	Pro	Gly	Gln	Arg	Val	Thr	Ile	Ser	Cys
Ha	Gly	Thr	Pro	Gly	Gln	Arg	Val	Thr	Ile	Ser	Cys

nism for amyloid formation may be by means of intralysosomal catheptic (*22*) digestion of light polypeptide chains of immunoglobulins. This mechanism is supported by the frequent close spatial relationship between amyloid deposits and cells of the macrophage system (*23*) and by the electron microscopic observations of fibrils within plasmalemmal invaginations and membrane-bound vesicles of macrophages (*24*). Since none of the patients whose Bence Jones proteins could be degraded to form "amyloid" fibrils in vitro were known to have amyloidosis, other mechanisms or factors in addition to those noted here may be necessary for the production of amyloid fibrils in vivo. The type (and homogeneity) of variable fragment of immunoglobulin protein deposited in tissues as amyloid fibrils may be dependent on a selection process occurring at the level of either immunoglobulin-synthesizing cells or immunoglobulin-degrading phagocytic cells or both. Furthermore, the possibility must still be considered that some cases of amyloidosis may result from tissue deposition of antiparallel, β-pleated sheet fibrils derived from portions of immunoglobulins other than light chains or of proteins other than immunoglobulins.

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References and Notes

1. A. S. Cohen and E. Calkins, *Nature* **183**, 1202 (1959); W. Barth, G. G. Glenner, T. A. Waldmann, R. F. Zelis, *Ann. Intern. Med.* **59**, 787 (1969).
2. H. P. Missmahl and M. Hartwig, *Virchows Arch. Pathol. Anat.* **324**, 480 (1953); H. Puchtler, F. Sweat, M. Levine, *J. Histochem. Cytochem.* **10**, 355 (1962).
3. E. D. Eanes and G. G. Glenner, *J. Histochem. Cytochem.* **16**, 673 (1968).
4. T. Shirahama and A. S. Cohen, *J. Cell Biol.* **33**, 679 (1967).
5. G. G. Glenner, H. R. Keiser, H. A. Bladen, P. Cuatrecasas, E. D. Eanes, J. S. Ram, J. N. Kanfer, R. A. DeLellis, *J. Histochem. Cytochem.* **16**, 633 (1968); M. Pras, D. Zucker-Franklin, A. Rimon, E. C. Franklin, *J. Exp. Med.* **130**, 777 (1969).
6. L. Arvy and C. Sors, *Acta Histochem.* **6**, 77 (1958); G. D. Sorenson and H. B. Binington, *Fed. Proc.* **23**, 550 (1964).
7. G. G. Glenner, J. Harbaugh, J. I. Ohms, M. Harada, P. Cuatrecasas, *Biochem. Biophys. Res. Commun.* **41**, 1287 (1970); G. Glenner, W. Terry, M. Harada, C. Isersky, D. Page, *Science* **172**, 1150 (1971); C. Isersky, D. Ein, M. Harada, R. A. DeLellis, D. Page, G. G. Glenner, in preparation.
8. A. Solomon and C. L. McLaughlin, *J. Biol. Chem.* **244**, 3993 (1969); ———, C. H. Wei, J. R. Einstein, *ibid.* **245**, 5289 (1970).
9. H. A. Bladen, M. U. Nylen, G. G. Glenner, *J. Ultrastruct. Res.* **14**, 449 (1966).
10. J. Termine, E. D. Eanes, D. Ein, G. G. Glenner, in preparation.
11. M. Harada, C. Isersky, P. Cuatrecasas, D. Page, H. A. Bladen, E. D. Eanes, H. R. Keiser, G. G. Glenner, *J. Histochem. Cytochem.* **19**, 1 (1971); W. W. Fish, K. G. Mann, C. Tanford, *J. Biol. Chem.* **244**, 4980 (1969).
12. K. Weber and M. Osborn, *J. Biol. Chem.* **244**, 4406 (1969).
13. M. A. Raftery and R. D. Cole, *ibid.* **241**, 3457 (1966).
14. A. M. Katz, W. J. Dreyer, C. Anfinsen, *ibid.* **234**, 2897 (1959).
15. L. Hood and D. Ein, *Science* **162**, 679 (1968).
16. P. Edman and G. Begg, *Eur. J. Biochem.* **1**, 80 (1967); J. J. Pisano and T. J. Bronzert, *J. Biol. Chem.* **244**, 5597 (1969).
17. F. W. Putnam, T. Shinoda, K. Titani, M. Wikler, *Science* **157**, 1050 (1967).
18. It would appear that the absence of the amino-terminal tripeptide is a result of proteolytic digestion, since the parent Nic BJ has a blocked amino terminus, pyrrolid-2-one-5-carboxylic acid (Glp). Amino acid sequence analysis of λ Bence Jones proteins by Edman degradation has usually been prevented by the presence of Glp as the unreactive amino-terminal amino acid. The automatic amino acid sequence analysis of the Nic fragment reported here indicates that peptic digestion cleaved the parent, amino-terminal unreactive Nic BJ at position 3, thereby permitting Edman degradation. This suggests the possible applicability of the present method of forming insoluble fibril aggregates by peptic digestion for the automatic amino acid sequencing of other amino-terminal blocked immunoglobulin polypeptide chains.
19. E. F. Osserman and K. Takatsuki, *Medicine* **42**, 357 (1969); E. F. Osserman and D. P. Lawlor, *Amer. J. Med.* **18**, 462 (1955); R. C. Williams, R. D. Brunning, F. A. Wollheim, *Ann. Intern. Med.* **65**, 471 (1966).
20. A. I. Pick and E. F. Osserman, in *Amyloidosis*, E. Mandema, L. Ruinen, J. H. Scholten, A. S. Cohen, Eds. (Excerpta Medica, Amsterdam, 1968), p. 100.
21. G. M. Edelman and W. E. Gall, *Annu. Rev. Biochem.* **38**, 415 (1969); K. Hamaguchi and S. Migita, *J. Biochem.* **56**, 512 (1964).
22. B. A. Ehrenreich and Z. A. Cohn, *J. Exp. Med.* **126**, 941 (1969); *ibid.* **129**, 227 (1969).
23. H. Smetana, *J. Exp. Med.* **45**, 619 (1926); B. Gueft and J. J. Ghidoni, *Amer. J. Pathol.* **43**, 837 (1963); W. A. Heefner and G. D. Sorenson, *Lab. Invest.* **11**, 585 (1962); G. Teilum, *Amer. J. Pathol.* **32**, 945 (1956).
24. G. D. Sorenson and W. A. Bari, and following discussion, in *Amyloidosis*, E. Mandema, L. Ruinen, J. H. Scholten, A. S. Cohen, Eds. (Excerpta Medica, Amsterdam, 1968), p. 58; R. Caesar, *Nova Acta Leopoldina* **31**, 87 (1966).

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DNA Constancy in Heteroploidy and the Stem Line Theory of Tumors

Abstract. *Cellular DNA was measured by high-speed flow microfluorometry in mammalian diploid and heteroploid cell populations stained by the fluorescent-Feulgen procedure. Heteroploid cells with elevated modal chromosome number showed the expected increase in modal DNA content. However, the variability of DNA content was the same in diploid and heteroploid cell populations despite the large variability of chromosome number in the latter populations. This suggests that heteroploidy may include defects in the chromosomal condensation and kinetochore development systems.*

Heteroploidy, abnormal variability of chromosome number and structure (*1*), is a common feature of the cell populations of tumors and of established cell lines in culture (*2*). By contrast, normal animal cells exhibit very little karyotypic variability either in vivo or in vitro (*2*). Many heteroploid cell populations have high modal chromosome numbers as well as high variability of chromosome number; studies of such populations show a positive correlation between modes for chromosome number and cellular DNA content (*3*). It has been assumed that, within each such population, high variability of chromosome number corresponds to high variability of DNA content per cell. It has thus been assumed that such cell populations have abnormal genetic variability, a notion that has encouraged acceptance and extension of Makino's stem line theory (*4*), which has important implications for tumor therapy (*5*).

We present evidence that heteroploid cell populations consist of one or more subpopulations, each with its characteristic DNA content per cell and modal chromosome number. The DNA content per cell is constant within a subpopulation, but chromosome number is variable. Thus, variability of chromosome number for the entire population is the sum of two contributions: one due to the presence of one or more subpopulations, each with the same ratio between modal chromosome number and DNA content per cell; and the other due to variability of chromosome number, despite uniform DNA content, within each subpopulation.

Much of the published data on DNA distribution in cell populations has been obtained by measurement of relatively small numbers of Feulgen-stained cells. Because of poor statistical precision, histograms of these populations have failed to reveal details of shape and fine structure. As a result, it has been difficult to distinguish among components of a population heterogeneous with respect to mitotic cycle phase, stem line, and proportion of cells of each stem line that are in cell cycle traverse. A