Proc. Nat. Acad. Sci. USA
Vol. 70, No. 7, pp. 1943–1946, July 1973

Collagen and Procollagen Production by a Clonal Line of Schwann Cells

(peripheral neurinoma/acrylamide gel electrophoresis/S100 protein/myelin basic protein)

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Communicated by Nathan O. Kaplan, April 18, 1973

ABSTRACT **A well-characterized line of rat Schwann cells has been examined for its ability to produce collagen. About 14% of the [^{3}H]proline in the proteins that were secreted into the culture medium by the cells was hydroxylated, while only 1% of the labeled proteins in the cell layer contained [^{3}H]hydroxyproline. Three sizes of procollagen polypeptides, of molecular weights about 105,000, 120,000, and 155,000, were present in the medium, as well as tropocollagen molecules that contained the usual α1 and α2 chains. Subsequently, the Schwann cells ceased producing the smaller collagenous polypeptides, although the total [^{3}H]hydroxyproline content of the medium was unchanged. The [^{3}H]hydroxyproline was almost entirely accounted for by the polypeptide of 155,000 daltons; this peptide was rapidly digested by collagenase or pepsin or chymotrypsin. The destruction by pepsin and chymotrypsin indicates that the large polypeptide, in contrast to procollagen and tropocollagen, is not in the collagenous (helical) conformation. Possibly, this substance is a very early form of procollagen that does not fold into the collagen conformation. The data show that cells of neuroectodermal origin can synthesize collagen, and also suggest that Schwann cells may be responsible for a large proportion of the collagen seen in peripheral neurinomas *in vivo*.**

Production of collagen fibrils and other collagen-related products by cells of neuroectodermal origin has been a controversial subject (1–4). Recently, the synthesis and secretion of collagen by chick embryonic neuroepithelium has been suggested by electron microscopic observations (5). The presence of considerable amounts of collagen in peripheral neurinomas, thought by some on the basis of morphology (6–9) and biochemistry (10) to represent Schwann-cell tumors, has led some investigators to postulate that Schwann cells can produce collagen. In contrast, others have suggested that the presence of collagen means that the principal cell type present in these peripheral neurinomas must be fibroblastic in origin rather than neuroectodermal (1, 11–13).

A key solution to this problem would be to examine a pure, well-characterized population of peripheral neurinoma cells in culture for their ability to produce collagen. In this report, we describe that a clonal line of rat Schwann cells in culture (14) elaborate collagen-related molecules in amounts comparable to fibroblasts in cell culture (15).

MATERIALS AND METHODS

Tissue Culture. Line RN-2, a clonal line of rat Schwann cells (14, 16), was maintained in Ham's Medium F-12 (17) supplemented with 10% horse serum (Grand Island Biological Co.) and 2.5% fetal-calf serum (Reheis Chemical Co.). Line 3T6, a clonal line of mouse fibroblasts kindly provided by Drs. Richard Wang and Howard Green, was maintained in Dulbecco's Modified Eagle Medium (DME; ref. 18) supplemented with 10% calf serum (Grand Island Biological Co.) and 50 μg/ml of ascorbic acid. Both cell lines were grown as monolayer cultures on plastic tissue culture dishes (Falcon) in a humidified atmosphere of either 5% CO_2–95% air (RN-2) or 10% CO_2–90% air (3T6) at 37.5°. Cells were subcultured by standard procedures, with 0.25% Viokase (Viobin Corp., Monticello, Ill.) in phosphate-buffered physiological saline lacking calcium and magnesium.

Proline Hydroxylation Assay. The dialyzed medium and cell-layer fractions were assayed for [^{3}H]proline and [^{3}H]-hydroxyproline by conventional means (19).

Enzymatic Treatment. Bacterial collagenase (Worthington) was purified and used as described by Peterkofsky and Diegelmann (20). α-Chymotrypsin (Worthington) was incubated with samples for 1 hr at pH 7.6 and room temperature at a concentration of about 10 μg of enzyme to 100 μg of sample protein. Pepsin treatment was done by adjusting the pH of the sample to 3.0 with concentrated acetic acid and incubating with pepsin for 18 hr at 4° at a final enzyme concentration of 10 μg/ml. After incubation, the pH was returned to 7.6 with NaOH to inactivate the pepsin.

Sodium Dodecyl Sulfate–Acrylamide Gel Electrophoresis. Large volumes of serum-supplemented Medium F-12 were harvested from confluent RN-2 cultures after at least 3 days of incubation. The medium was centrifuged at 2000 $\times$ *g* for 15 min to remove cells and debris, made 20% (w/v) with respect to ammonium sulfate, and stored at 4° for 25 hr after which the precipitate was collected by centrifugation at 10,000 $\times$ *g* for 30 min. The 20% ammonium sulfate precipitate (P-20) was dissolved in a small volume of 0.05 M Tris·HCl buffer (pH 7.4) and dialyzed against this buffer to remove ammonium sulfate. Portions of this P-20 solution were denatured with Na dodecyl sulfate (1%), reduced with 10 mM dithiothreitol, and heated at 100° for at least 3 min to disaggregate proteins. Alternatively, portions of the P-20 solution were treated with purified collagenase or α-chymotrypsin before denaturation. After denaturation, the P-20 samples were layered onto 5.7% Na dodecyl sulfate–acrylamide gels and subjected to electrophoresis and staining by the method of Fairbanks *et al.* (21). The destained gels were scanned at 660 nm.

The protein standards and molecular weight markers used were purified rat-tail collagen and purified dermatosparactic calf-skin collagen (kindly donated by Dr. C. Lapiere) (22).

To radioactively label polypeptides related to collagen, we incubated cultures of RN-2 cells for 24 hr in DME medium (minus serum) containing 50 μg/ml each of ascorbic acid

TABLE 1. *Distribution of nondialyzable [^{3}H]proline and [^{3}H]hydroxyproline incorporated in cultures of RN-2 cells and 3T6 fibroblast cells**

Exp.	^{3}H radioactivity (cpm × 10^{-3}) Hydroxyproline	Total	H/T†
	RN-2 medium		
1	7.4	41.7	17.9
2	28.7	283.6	10.1
3	23.3	163.9	14.2
		Average =	14.1
	3T6 medium		
1	27.5	138.1	19.9
2	26.7	127.9	20.9
3	107.5	538.9	19.9
		Average =	20.2
	RN-2 cell layer		
1	9.5	634.2	1.5
2	327.0	27176.0	1.2
3	14.8	1134.2	1.3
		Average =	1.3
	3T6 cell layer		
1	191.5	1367.7	14.0
2	145.9	1195.9	12.2
3	295.2	3517.0	8.0
		Average =	11.4

* Virtually all of the [^{3}H]hydroxyproline in the medium had been precipitated by 20% ammonium sulfate. Proline hydroxylation in cell cultures was assayed by addition of supplemented (ascorbic acid) DME medium containing L-[5-^{3}H]proline (33.8 Ci/mmol; 1 μCi/ml) to confluent cell cultures. The labeled medium remained on the cells for 2–3 days, after which it was removed and dialyzed at 4° against several changes of distilled water. The cell layer was washed twice with phosphate-buffered saline and scraped into a screw-capped tube for hydrolysis. Samples were hydrolyzed at 6 N HCl for 18 hr at 105°, and [^{3}H]-hydroxyproline was separated from [^{3}H]proline on a small Dowex 50 column.

† H/T = the ratio of [^{3}H]hydroxyproline to total ^{3}H label in the experimental sample, expressed as a percentage.

and β-aminopropionitrile fumarate (BAPN) plus [5-^{3}H]-proline (33.8 Ci/mmol; 10 μCi/ml). The medium was removed and centrifuged at 5000 × *g* for 10 min. The supernatant fraction was divided into three portions, which were either not treated (control), treated with collagenase, or treated with pepsin or chymotrypsin. Each sample was then made 1% with respect to Na dodecyl sulfate in Tris·HCl buffer (pH 8.0) containing 40 mM dithiothreitol and 1 mM EDTA, heated at 100° for 1–3 min to completely denature all the proteins, and then extensively dialyzed. The samples were concentrated to about 0.1 ml with Aquacide (Calbiochem) and were then analyzed on a 1 × 50 cm Agarose (4%) column in the Na dodecyl sulfate-Tris·HCl buffer or were subjected to electrophoresis on 5.7% Na dodecyl sulfate–polyacrylamide gels. The polyacrylamide gels were sliced on a Gilson gel fractionator and 0.5 ml of 10% Na dodecyl sulfate was added to each fraction. The radioactivity was then measured in 10 ml of Triton-toluene scintillation fluid.

TABLE 2. *Determination of molecular weight of RN-2 collagen-related proteins by Na dodecyl sulfate–acrylamide gel electrophoresis.*

Designation	Collagen fraction Source	Ref.	Reference molecular weight
α1	Rat tail	(23)	98,000
α2	Rat tail	(23)	98,000
β12	Rat tail	(23)	196,000
pα1	Cattle	(22)	105,000
			Estimated molecular weight
"α1"	RN-2		98,000
"α2"	RN-2		98,000
"pα1"	RN-2		105,000
1.3-cm peak	RN-2		120,000
1.9-cm peak	RN-2		155,000

RESULTS

Cell Line RN-2 was derived from a chemically-induced tumor of the rat peripheral nervous system and classified as a Schwann cell line on the basis of the primary tumor location and histology and by its ability to perform several biochemical processes that are characteristic of Schwann cells. These include the syntheses of S-100 protein, 2′,3′-cyclic nucleotide-3′-phosphohydrolase, and a protein similar to the myelin basic protein (14, 16).

Proline Hydroxylation and Collagenase Sensitivity. Initial experiments were done to determine if RN-2 cells were capable of hydroxylating proline to hydroxyproline, a specific assay for collagen production. Several separate experiments demonstrated a ratio of [^{3}H]hydroxyproline/total ^{3}H label (H/T) in the RN-2 medium of 14%, while the cell layer had a ratio of only 1.3% (Table 1). In comparison, the H/T ratio in 3T6 fibroblasts was 20% in the medium and 12% in the cell layer.

About 35% of the [^{3}H]proline-labeled protein in the medium was sensitive to collagenase, as judged by the product's solubility in 5% trichloroacetic acid–0.5% tannic acid (20). The collagen-related proteins in the medium were partially purified by ammonium sulfate precipitation (P-20) and examined by gel electrophoresis. The P-20 fraction had a H/T ratio of 31%, and 63% of it was digested by collagenase.

Na Dodecyl Sulfate–Acrylamide Gel Electrophoresis. To further examine the collagenase-sensitive proteins produced by RN-2 cells in culture, the P-20 fraction was treated with collagenase or with chymotrypsin, and then subjected to electrophoresis in 5.7% Na dodecyl sulfate–acrylamide gels. The results are shown in Fig. 1. The control sample contains peaks corresponding to standard tropocollagen α1 and α2 chains, and to procollagen pα1 (22) chains. The *hatched areas* denote proteins that were specifically digested by collagenase, as shown in the middle panel (the four new bands seen in the right hand part of the middle panel are due to the collagenase added to the reaction mixture). The lower panel shows the results after treatment with chymotrypsin, namely the apparent conversion of all procollagen peaks into α1 and α2

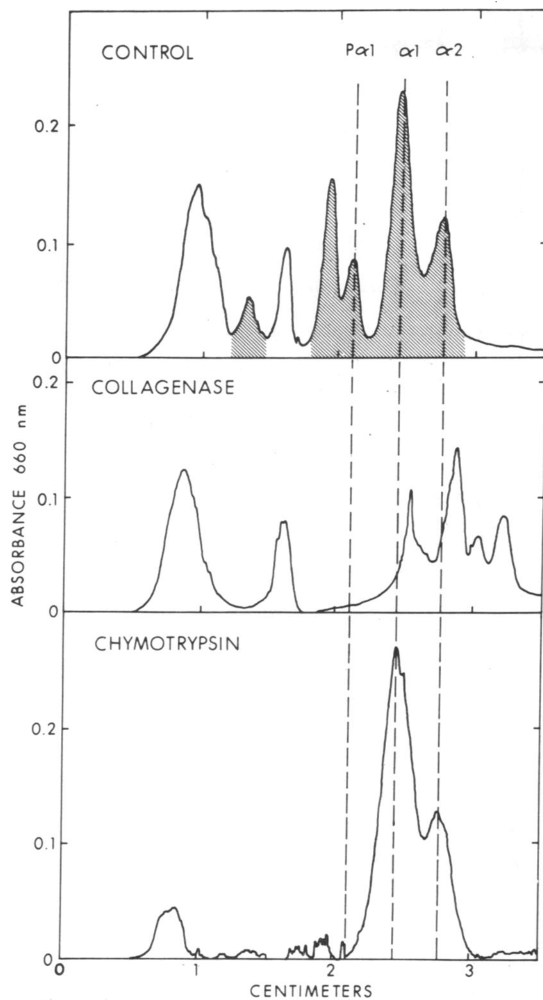

FIG. 1. Densitometric scan of stained acrylamide gels obtained from Na dodecyl sulfate electrophoresis of the P-20 proteins. Untreated (*top*), collagenase-treated (*middle*), and chymotrypsin-treated (*bottom*). The *origin* is to left of the figure (0 cm).

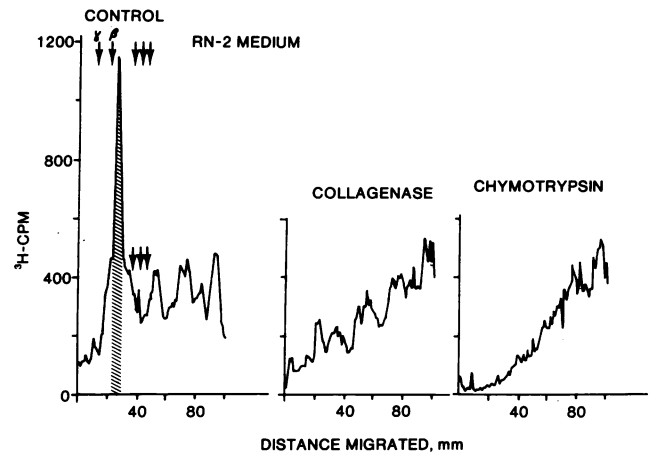

FIG. 2. Radioactive analysis of sliced acrylamide gels obtained from Na dodecyl sulfate electrophoresis of the proteins in the medium. Untreated (*left*), collagenase-treated (*middle*), and chymotrypsin-treated (*right*). Similar results were obtained when the procedures of Fig. 1 were used. The *unmarked arrows* in the control panel represent the location, from left to right, of pα1, α1, and α2, respectively.

peaks as well as the maintenance of the original α1 and α2 chains. Stark *et al.* (24) have reported that chymotrypsin treatment at neutral pH converts procollagen pα1 and pα2 chains into tropocollagen α1 and α2 chains by cleaving noncollagenous peptides from the molecule.

Comparison of the five collagen-related peptide chains in Fig. 1 with authentic markers run in parallel (Table 2) indicates that the five components are α2, α1, and pα1 plus two larger chains of about 120,000 and 155,000 daltons.

About 3 months after these observations, consisting of six consecutive experiments, we found that the Schwann cells ceased producing the smaller collagenous polypeptides and now produce only the largest. Samples analyzed on 5.7% Na dodecyl sulfate–polyacrylamide gels (Fig. 2) demonstrate one collagen-related polypeptide of about 155,000 daltons. This collagen-related polypeptide apparently is not in collagen triple-helical molecular form because both chymotrypsin (Fig. 2) and pepsin (not shown) destroy it. When the culture medium was analyzed on a 4% agarose–Na dodecyl sulfate column, only one collagen-related peak was observed, which eluted sooner than collagen α-chains (not shown). This collagen-related polypeptide contains about 14% hydroxyproline and it is identical with the peak in Fig. 2 (control).

DISCUSSION

Collagenous elements have been observed in close proximity to cells morphologically identified as Schwann cells in peripheral neurinomas (6–8, 10, 25–27) as well as in regenerating nerve tissue (4, 28). From these observations, some investigators have concluded that Schwann cells produce collagen and basement membrane (4, 6–8, 28), while other workers have concluded that peripheral neurinomas are fibroblast-derived tumors (1, 11, 12). However, only inferential data were used to form these conclusions. Since all of the tissues studied, even in tissue culture, were composed of mixtures of cells including fibroblasts, there was no definite way to prove the origin of the collagenous elements. We are able to state conclusively, by using a clonal line of Schwann cells in tissue culture, that neuroectodermal cells, Schwann cells in particular, are capable of elaborating copious amounts of collagen. Thus, the majority of the collagen-like material observed in peripheral neurinomas may be produced by Schwann cells.

Most of the studies cited above remarked on the scarcity of mature collagen fibers in peripheral neurinomas despite an abundance of reticulin and of a type of collagen deposition termed "Luse bodies" (25, 29), which have a repeating periodicity of from 1000–1500 Å (in contrast to the usual periodicity of 640 Å for native collagen fibers).

A similar form of fibril, called fibrous long spacing (FLS) collagen, can be formed *in vitro* by mixing collagen solutions and specific precipitants, usually glycoproteins (30). Therefore, FLS-like structures found in the tumors may be formed by an abnormal production of glycoproteins by the tumor cells. Alternatively, they may be formed as a result of polymerization and precipitation of procollagen molecules that have not been converted into normal collagen molecules.

Stark *et al.* (24) and Bellamy *et al.* (31) ascribe to the additional peptide sequences in procollagen molecules the role of controlling extracellular fibril formation, the extra peptide sequences serving to inhibit fibril formation until an extracellular proteolytic enzyme (32, 33) cleaves off these peptides,

initiating fibrogenesis. Table 1 shows that very little collagen seems to be present in RN-2 cell layers, compared with that in 3T6 fibroblast cell layers. To suggest that the enzyme(s) responsible for converting procollagen to collagen (thus initiating fibrogenesis) in RN-2 cells are missing or inactive is not tenable since Fig. 1 shows that considerable amounts of the tropocollagen components, $\alpha 1$ and $\alpha 2$, are present. Moreover, the limited effect of chymotrypsin confirms that they exist in tropocollagen molecules. It is not clear why this tropocollagen does not precipitate into typical collagen fibrils. Perhaps fibril formation requires cofactors that are missing in the RN-2 cell cultures, possibly as a result of its original neoplastic state.

Several laboratories have now reported the existence of procollagen molecules in various tissues (31, 34–39), such procollagens having weights ranging from 105,000 to 500,000. In particular, Grant *et al.* (39) have studied basement-membrane collagen production by embryonic chick lens cells and have described the conversion of pα-chains of 140,000 daltons to α-chains of 115,000 daltons, which they suggest is the final size of basement-membrane α-chains. By comparison, the largest single chain of collagen-related material secreted by RN-2 cells is about 155,000 daltons. This chain could represent α-chains from basement-membrane collagen since Schwann cells are thought to make basement membranes (40). However, in contrast to Grant *et al.* (39), we observed $\alpha 1$ and $\alpha 2$-sized chains as well. Perhaps Schwann cells produce two types of procollagen molecule, one destined for basement-membrane collagen and the other destined for striated collagen fibers. Alternatively, a single primordial procollagen molecule may be initially synthesized, followed by its cleavage into specific basement-membrane components or ultimately into tropocollagen. Finally, the 155,000-dalton polypeptide may represent a collagen molecule that is unique to Schwann cells.

The apparent phenotypic change in expression with the loss of all collagen-related polypeptides except for the largest suggests that the ability of the cell to convert procollagen to collagen has been lost, much like the dermatosparoctic condition (22). However, the large collagen-related polypeptide is also not in the correct collagen molecular form since it is destroyed by chymotrypsin and pepsin. Therefore, a defect in processing the collagen into molecular form also exists unless the 155,000-dalton polypeptide by its very nature cannot assume the collagen conformation.

We thank Mrs. Barbara Glastris and Mrs. Ding Young Lyn for excellent technical assistance. This work was partially supported by NIH Grants AM-12683-04, and NSCA 10861-01, NSF Grant GB-31077, Life Insurance Medical Research Fund Grant G-69024, and American Cancer Society Grant VC 124-A. R.L.C. was a postdoctoral fellow of NIH (CA-52091-01) during this work.

1. Raimondi, A. J. & Beckman, F. (1967) *Acta Neuropathol.* **8,** 1–23.
2. Barton, A. A. (1962) *Brain* **85,** 799–808.
3. Causey, G. & Barton, A. A. (1959) *Brain* **82,** 594–598.
4. Nathaniel, E. J. H. & Pease, D. C. (1963) *J. Ultrastruct. Res.* **9,** 550–560.
5. Cohen, A. M. & Hay, E. D. (1971) *Develop. Biol.* **26,** 578–605.
6. Murray, M. & Stout, A. P. (1942) *Amer. J. Pathol.* **18,** 585–589.
7. Pineda, A. (1965) *Neurology* **15,** 536–547.
8. Murray, M. R. & Stout, A. P. (1940) *Amer. J. Pathol.* **16,** 41–60.
9. Cravioto, H. & Lockwood, R. (1968) *J. Ultrastruct. Res.* **24,** 70–85.
10. Pfeiffer, S. E., Kornblith, P. L., Levine, L., Cares, H. L. & Seals, J. (1972) *Brain Res.* **41,** 187–193.
11. Penfield, W. (1932) in *Cytology and Cellular Pathology of the Nervous System*, ed., Penfield, W. (Hoeber, New York), pp. 953–990.
12. Tarlov, I. M. (1940) *Amer. J. Pathol.* **16,** 33–40.
13. Bailey, P. & Hermann, J. D. (1938) *Amer. J. Pathol.* **14,** 1–38.
14. Pfeiffer, S. E. & Wechsler, W. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 2885–2889.
15. Goldberg, B. & Green, H. (1964) *J. Cell Biol.* **22,** 227–258.
16. Pfeiffer, S. E. and Wechsler, W. (1972) *Naturwissenschaften* **59,** 518–519.
17. Ham, R. G. (1965) *Proc. Nat. Acad. Sci. USA* **53,** 288–293.
18. Smith, J. D., Freeman, G., Vogt, M. & Delbecco, R. (1960) *Virology* **12,** 185–196.
19. Peterkofsky, B. & Udenfriend, S. (1961) *Biochem. Biophys. Res. Commun.* **6,** 184–190.
20. Peterkofsky, B. & Diegelmann, R. (1971) *Biochemistry* **10,** 988–994.
21. Fairbanks, G., Steck, T. L. & Wallach, F. H. (1971) *Biochemistry* **10,** 2606–2624.
22. Lenaers, A., Ansay, M., Nusgens, B. V. & Lapiere, C. M. (1971) *Eur. J. Biochem.* **23,** 533–543.
23. Lewis, M. S. & Piez, K. A. (1964) *Biochemistry* **3,** 1126–1131.
24. Stark, M., Lenaers, A., Lapiere, C. & Kuhn, K. (1971) *FEBS Lett.* **18,** 225–227.
25. Luse, S. A. (1960) *Neurology* **10,** 881–905.
26. Ramsey, H. J. (1965) *J. Neuropathol. Exp. Neurol.* **24,** 40–48.
27. Cravioto, H. (1969) *Acta Neuropathol. (Berlin)* **12,** 116–157.
28. Thomas, P. K. (1964) *J. Cell Biol.* **23,** 375–382.
29. Luse, S. A., Zopf, D. & Cox, J. W. (1963) *Anat. Rec.* **145,** 254.
30. Gross, J., Highberger, H. J. & Schmitt, F. O. (1954) *Proc. Nat. Acad. Sci. USA* **40,** 679–688.
31. Bellamy, G. & Bornstein, P. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 1138–1142.
32. Lapiere, C. M., Lenaers, A. & Kohn, L. D. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 3054–3058.
33. Bornstein, P., Ehrlich, H. P. & Wyke, A. W. (1971) *Science* **175,** 544–546.
34. Lenaers, A. B., Nusgens, B. V., Ansay, M. & Lapiere, C. M. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352,** 14.
35. Layman, D. L., McGoodwin, E. B. & Martin, G. R. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 454–458.
36. Ramaley, P. B. & Rosenbloom, J. (1971) *FEBS Lett.* **15,** 59–64.
37. Jimenez, S. A., Dehm, P. & Prockop, D. J. (1971) *FEBS Lett.* **17,** 245–248.
38. Church, R. L., Pfeiffer, S. E. & Tanzer, M. L. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 2638–2642.
39. Grant, M. E., Kefalides, N. A. & Prockop, D. J. (1972) *Fed. Proc.* **31,** 480.
40. Thomas, P. K. (1963) *J. Anat.* **97,** 35–48.