excess of 200 μg were applied. These distinct electrophoretic bands very nicely define a normal distribution curve, as shown in the scan of the gel at 750 nm (Fig. 2, top). The scan hints at an eighth and ninth band which cannot be seen visually. Figure 2 (bottom) shows the results from a caseinolytic assay of an unstained acrylamide gel in which consecutive slices were cut from the gel and assayed after activation with urokinase. The optical density at 280 nm is directly proportional to the activity and indicates that the different electrophoretic bands from the affinity chromatography plasminogen preparation are active. Since, as we have shown in parallel experiments, no activity develops unless the bands are treated with urokinase, the different forms are apparently all proenzyme. Other studies show that these plasminogen preparations form (i) a single band on polyacrylamide gel in the presence of ε-ACA at *p*H 8.3 and (ii) a single band without inhibitor (ε-ACA) present on starch gel at *p*H 2.5 (*11*). When a sample of the proenzyme is activated for 6 minutes at 25°C with the bacterial exotoxin streptokinase, 0.73 mole of active enzyme (plasmin) is formed per mole of plasminogen. The titration of plasmin is performed with *p*-nitrophenyl-*p'*-guanidino benzoate hydrochloride according to the procedure of Chase and Shaw (*12*). The titrable plasmin (73 percent active enzyme) is at the same level as that found by Chase and Shaw in a preparation of highly purified plasmin supplied by K. C. Robbins, which had a proteolytic activity of 31.7 casein units per milligram of protein (*12*).

While distinct electrophoretic forms of the proenzyme on polyacrylamide gel contradict results of some workers who have reported plasminogen preparations which give one band on disc-gel electrophoresis (*13*), others have demonstrated more than one band on starch and acrylamide electrophoresis (*14*). Summaria and Robbins have reported that polyacrylamide gel electrophoresis of human plasminogen in 8*M* urea or 0.3*M* ε-ACA (but not untreated) showed three to five bands and that the proenzyme could be separated into distinct fractions by isoelectric focusing in 7*M* urea. From other experiments, they suggest that the determinants for the multimolecular forms of the proenzyme reside in one region of the plasminogen molecule having a molecular weight of 25,700, called the light chain (*15*).

We think that the few manipulations of the proenzyme during purification by affinity chromatography and the maintenance of neutral *p*H throughout the procedure account for the unusual stability and free solubility at neutral *p*H. Because of the simplicity and speed of the method and apparent integrity of the product, this procedure will make large amounts of plasminogen readily available for clinical and structural studies.

DALE G. DEUTSCH
EDWIN T. MERTZ
Department of Biochemistry, Purdue University, Lafayette, Indiana 47907

References and Notes

1. For example: L. F. Remmert and P. P. Cohen, *J. Biol. Chem.* **181**, 431 (1949); D. L. Kline and J. B. Fishman, *ibid.* **236**, 3232 (1961); N. Alkjaersig, *Biochem. J.* **93**, 171 (1964); K. H. Slotta and J. D. Gonzalez, *Biochemistry* **3**, 285 (1964); K. C. Robbins, L. Summaria, D. Elwyn, G. H. Barlow, *J. Biol. Chem.* **240**, 541 (1965); J. Y. S. Chan and E. T. Mertz, *Can. J. Biochem.* **44**, 475 (1966); A. J. Johnson, K. Bergstrom, A. O. Tse, *Fed. Proc.* **26**, 647 (1967); T. Onishi, *Shikoku Igaku Zasshi* **23**, 58 (1967).
2. D. G. Deutsch and E. T. Mertz, *Fed. Proc.* **29**, 647 (1970).
3. *British Patent Specifications, 770693* (Her Majesty's Stationary Office, London, 1957); N. Alkjaersig, A. P. Fletcher, S. Sherry, *J. Biol. Chem.* **234**, 832 (1959).
4. P. Cuatrecasas, M. Wilchek, C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.* **61**, 636 (1968).
5. H. G. Barlow, L. Summaria, K. C. Robbins, *J. Biol. Chem.* **244**, 1138 (1969).
6. Whole human blood, preserved in a citrate phosphate dextrose anticoagulant solution (Fenwal, GF-15E Blood Pack), was centrifuged for 15 minutes at 2000*g*. The resulting plasma was adjusted to *p*H 7.4 and plasma pooled from two donors was subjected to affinity chromatography.
7. The effect of tris in facilitating the removal of the bound enzyme from the inhibitor-substituted agarose has been noted by G. Kohlhaw, personal communication, and J. C. Schmit, S. Artz, H. Zalkin, *J. Biol. Chem.*, in press.
8. A. J. Johnson, D. L. Kline, N. Alkjaersig, *Thromb. Diath. Haemorrh.* **21**, 259 (1969).
9. Obtained from Leo Pharmaceutical Products (Denmark) with 85,000 units per ampoule.
10. L. Ornstein, *Ann. N.Y. Acad. Sci.* **121**, 321 (1964); B. J. Davis, *ibid.*, p. 404.
11. D. G. Deutsch, T. H. Liu, E. T. Mertz, unpublished data.
12. T. Chase, Jr., and E. Shaw, *Biochemistry* **8**, 2212 (1969).
13. For example: Y. Abiko, N. Iwamoto, M. Shimizu, *J. Biochem. Tokyo* **64**, 743 (1968); M. Muramatu, Y. Hayakumo, T. Onishi, T. Sato, S. Fujii, *ibid.* **65**, 329 (1969).
14. For example: N. Alkjaersig, *Biochem. J.* **93**, 171 (1964); P. K. Chang, W. R. McCoy, G. Pincus, *J. Biol. Chem.* **240**, 93 (1965); W. F. Barg, Jr., E. Beggiano, E. C. DeRenzo, *Biochim. Biophys. Acta* **104**, 470 (1965); J. R. S. Chan and E. T. Mertz, *Can. J. Biochem.* **44**, 469 (1966).
15. L. Summaria and K. C. Robbins, *Fed. Proc.* **29**, 316 (1970).
16. Supported by NIH grant HE-08401; Journal Paper No. 4084 from the Agricultural Experiment Station, Purdue University, Lafayette, Indiana 47907. We thank Dr. P. Elliott and Dr. H. Russell for the human blood samples, also S. Artz, Dr. V. Rodwell, G. Doellgast, Dr. F. Regnier, Dr. M. Laskowski, Jr., and R. Schneeman for their help.

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Collagen Molecules: Distribution of Alpha Chains

Abstract. *To ascertain the distribution of alpha chains within the collagen molecule, intramolecular cross-links were introduced into tropocollagen by reacting formaldehyde with dilute homogeneous molecular dispersions of collagen extracted from lathyritic guinea pig skin. Denaturation, chromatography on carboxymethyl cellulose, measurement of molecular weight, and analyses of the amino acids of the cross-linked product indicate that most, if not all, of the collagen molecules consist of two α1 chains and one α2 chain* $[(\alpha 1)_2(\alpha 2)_1]$.

Collagen from a number of vertebrate species is composed of twice as many α1 as α2 chains, α2 having the same molecular weight as, but different amino acid composition from, α1 (*1*). Since each tropocollagen molecule is composed of three α chains, Piez and his colleagues (*1*) suggested that the molecule be defined by the formula $(\alpha 1)_2(\alpha 2)_1$. However, the 2 : 1 ratio is only a statistical value for the bulk collagen, and it has not been demonstrated that each molecule has two α1 and one α2 chains. For example, if there were twice as many molecules of the type $(\alpha 1)_3$ as $(\alpha 2)_3$, denaturation which results in chain separation would yield the 2 : 1 ratio of α1 to α2. This report provides direct evidence for the great predominance of $(\alpha 1)_2(\alpha 2)_1$ type of collagen molecules that can be extracted from lathyritic guinea pig skin and describes a new method for revealing and quantitating molecular heterogeneity. This demonstration is based on the production of intramolecular cross-links by reaction with formaldehyde (*2*) solutions of collagen dilute enough to avoid intermolecular reactions; the cross-linked product was then isolated and

Table 1. Molecular weight distribution of cross-linked collagen at sedimentation equilibrium from concentration 0.250 to 1.021 fringes.

Type	Range
M_n	222,970 to 282,690
M_w	274,730 to 329,477
M_z	317,035 to 354,860

the denatured triple-stranded molecules were characterized.

Neutral salt-extracted collagen was prepared from the skin of lathyritic guinea pigs (300 g) and purified by repeated precipitation with salt (*3*). Collagen from lathyritic animals was used here in an effort to minimize the amounts of aggregation and of naturally occurring intramolecular cross-links (β and γ components). The lyophilized protein was dissolved in 0.1*M* sodium acetate buffer, *p*H 3.3, dialyzed against the buffer for 24 hours at 4°C, and clarified by centrifugation at 44,000*g* for 60 minutes. Molecular homogeneity and the absence of aggregates in the starting solution was established by equilibrium sedimentation (*4*). Formaldehyde (37 percent, analytical reagent), *p*H 2.8, was added dropwise to the solution containing 0.67 mg of collagen per milliliter, with stirring, to a final concentration of 6.2 percent, as described by Veis and Drake (*2*). The mixture was incubated at 4°C for 3 days, after which it was dialyzed for 3 days against several changes of the starting buffer. This was followed by dialysis sequentially against 0.2 percent Dimedon (5,5-dimethyl-1,2-cyclohexanedione) in the starting buffer for 2 days with changes, and then against 0.02*M* Na_2HPO_4 to precipitate the collagen. The samples were then dissolved in 0.1*M* acetic acid and lyophilized. The recovery of protein as judged by hydroxyproline assay (*5*) was complete. Control collagen was treated in the same manner, except that exposure to formaldehyde was omitted.

The samples were dissolved in 0.06*M* sodium acetate buffer, *p*H 4.8, denatured by warming to 40°C for 10 minutes, and chromatographed on columns (2.5 by 8 cm) of carboxymethyl cellulose equilibrated with 0.06*M* sodium acetate, *p*H 4.8, at 40°C. A linear gradient of NaCl, 0 to 0.1*M* over a total volume of 800 ml was superimposed at a flow rate of 312 ml/hour (*1*). Figure 1A shows the chromatogram of denatured control collagen, and Fig. 1B shows that of the denatured cross-linked collagen. Of the formaldehyde-treated material 78 percent was eluted at a region between the two β positions. This elution position has tentatively been assigned to naturally cross-linked $(\alpha 1)_2(\alpha 2)_1$ in dogfish skin collagen by Lewis and Piez (*6*) and in rat tail tendon collagen by Veis and Anesey (*7*). The small peak and elevated base line may represent minor heterogeneity, but not enough material could be recovered for further analysis. Recovery of hydroxyproline from the columns was comparable for the formaldehyde-treated collagen (37 percent) and the control (46 percent). These values have not been corrected for mechanical and other losses that occur during the frequent transfer of material.

Table 2. Amino acid composition of guinea pig skin collagen, α chains, and formaldehyde-treated collagen. The results are expressed as the number of residues per 1000 residues.

Amino acid	$\alpha 1$	$\alpha 2$	Calculated $(\alpha 1)_2(\alpha 2)_1$	Collagen	
				Original	HCHO-treated*
Hydroxyproline	101	113	105	100	94
Aspartic acid	44	49	46	49	46
Threonine	17	20	18	20	19
Serine	38	35	37	39	37
Glutamic acid	76	63	72	69	68
Proline	128	121	126	123	123
Glycine	325	321	324	331	341
Alanine	121	99	114	103	110
Valine	17	30	21	23	25
Methionine	4.2	12	5.4	5.8	6.2
Isoleucine	7.6	12	9.1	9.7	9.2
Leucine	19	26	22	23	23
Tyrosine	2.8	2.0	2.5	2.7	1.3
Phenylalanine	11	11	11	10	11
Hydroxylysine	5.7	8.9	6.1	5.5	6.0
Lysine	30	24	28	33	28
Histidine	1.9	6.9	3.6	4.8	4.1
Arginine	49	49	49	48	50

* Analyses were performed on the fraction isolated from carboxymethyl cellulose chromatography.

The molecular weight of the major fraction (0.13 mg/ml) was determined by the equilibrium sedimentation method of Roark and Yphantis (*4*). The solvent was 0.5*M* NaCl, 0.05*M* tris, *p*H 7.6, and the centrifugation was conducted at 8.5°C (12,000 rev/min, 60 hours, double-sector cell, Spinco model E ultracentrifuge). The slope of ln *c* plotted against $r^2/2$ gives an apparent M_w of 290,000. Partial specific volume used was 0.705. Table 1 presents number- (M_n), weight- (M_w), and *z*-average (M_z) molecular weights throughout the cell at equilibrium. The fact that $M_z > M_w > M_n$ reflects some heterogeneity with a low-molecular-weight species. This contamination must be less than

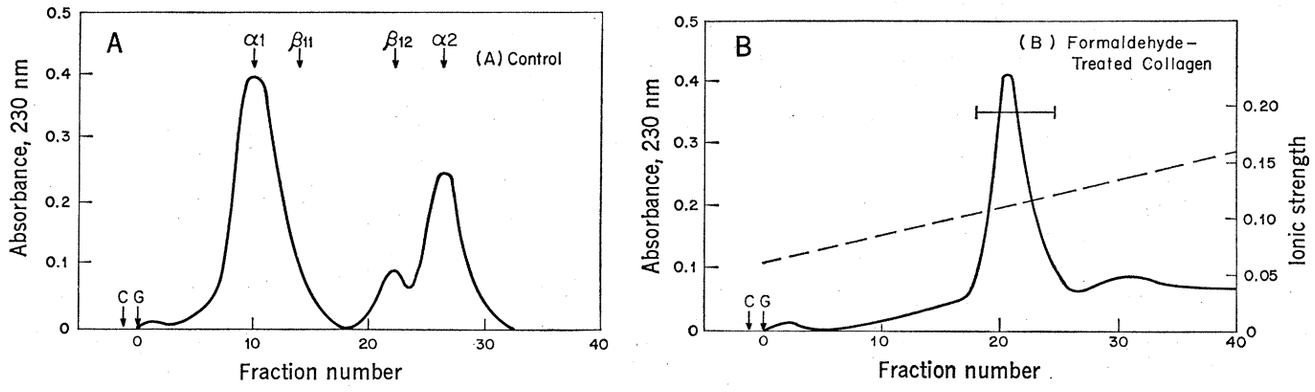

Fig. 1. Carboxymethyl cellulose elution patterns. Collagen (20 mg) was applied at *C*. The gradient began at *G*; 15.5 ml per fraction. (A) Control collagen; arrows indicate elution positions of components. (B) Formaldehyde-treated collagen; bar indicates which fractions were used for further study.

5 percent because, on acrylamide-gel electrophoresis, the cross-linked material was found not to travel in a 7.5 percent gel, an indication that the molecular weight was 300,000 or greater. Under the same sedimentation conditions α chains isolated by ion exchange and molecular sieve chromatography had a molecular weight 99,500.

Amino acid compositions were determined on an automatic instrument by the single column method (*8*) for the cross-linked component isolated from the carboxymethyl cellulose column and for the original guinea pig skin collagen (Table 2). The cross-linked fraction has a composition most closely resembling the sum of two $\alpha 1$ chains and one $\alpha 2$ chain, $(\alpha 1)_2(\alpha 2)_1$.

The technique of introducing cross-links into collagen dispersions was used effectively by Veis and Drake (*2*) to demonstrate the formation of both inter- and intramolecular cross-links in solutions containing free molecules and aggregates. This procedure, modified to remove aggregates, has been used here to prove the mixed chain composition and predominance of $(\alpha 1)_2(\alpha 2)_1$ distribution within collagen molecules of mammalian skin, proposed by Piez *et al.* (*1*).

Recently, however, Miller and Matukas (*9*) have obtained evidence for the existence of a native collagen molecule in chick xiphoid cartilage, which contains three $\alpha 1$ chains, thus renewing interest in the possible heterogeneity of collagen in terms of chain distribution in individual molecules. The formaldehyde cross-linking method has now been used in our laboratory (*10*) to separate and quantitate the two different collagen species in cartilage. The technique of formaldehyde cross-linking in molecular dispersions of collagen can be used to explore aspects of collagen heterogeneity in different tissues, during embryonic development, and in disease states.

JULIANNE G. NOLD
ANDREW H. KANG
JEROME GROSS

Developmental Biology Laboratory, Massachusetts General Hospital, Boston 02114

References and Notes

1. K. A. Piez, M. S. Lewis, G. R. Martin, J. Gross, *Biochim. Biophys. Acta* **53**, 596 (1961); K. A. Piez, E. A. Eigner, M. S. Lewis, *Biochemistry* **2**, 58 (1963).
2. A. Veis and M. P. Drake, *J. Biol. Chem.* **238**, 2003 (1963).
3. A. H. Kang, K. A. Piez, J. Gross, *Biochemistry* **8**, 1506 (1969).
4. D. E. Roark and D. A. Yphantis, *Proc. N. Y. Acad. Sci.* **164**, 245 (1969).
5. I. Bergman and R. Loxley, *Anal. Chem.* **35**, 1961 (1963).
6. S. Lewis and K. A. Piez, *J. Biol. Chem.* **239**, 3336 (1964).
7. A. Veis and J. Anesey, *ibid.* **240**, 3899 (1965).
8. E. J. Miller and K. A. Piez, *Anal. Biochem.* **16**, 320 (1966).
9. E. J. Miller and V. J. Matukas, *Proc. Nat. Acad. Sci. U.S.* **64**, 1246 (1969).
10. R. E. Trelstand, A. H. Kang, J. Gross, *Biochemistry*, in press.
11. This is publication No. 517 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Department of Medicine, Massachusetts General Hospital, Boston 02114. These investigations were supported by PHS grant AM 3564 and a grant from the RGK Foundation. J.G.N. was supported by PHS predoctoral training grant GM 00451.

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Distal Lobe of the Pilifer: An Ultrasonic Receptor in Choerocampine Hawkmoths

Abstract. *Evidence is presented for a novel mechanism mediating the auditory sense in the Choerocampinae (Lepidoptera: Sphingidae). Vibrations in the scale-free medial wall of the enlarged labial palp segment are transmitted by contact to the distal lobe of the pilifer, a derivative of the labrum, and are there transduced into afferent impulses in the labral nerve.*

Hawkmoths of the subfamily Choerocampinae react behaviorally to ultrasound. That the enlarged labial palps characteristic of this group are concerned in ultrasonic reception (*1*) was shown by recording second-order spike responses in neural units within the cervical connectives. Lateral deflection of both palps from their resting position, or amputation of their bulbous distal portions, reduces sensitivity 50- to 100-fold, but neither this treatment nor total removal of both palps completely abolishes the acoustic response. A number of experiments exploring the possibility that sound energy throws the labial palps into vibration about their cranial articulations confirmed the amplifying property of the palps but yielded no definite information about the receptor site (*2*). No structures that might serve as the receptor mechanism could be discovered either within the palps or near

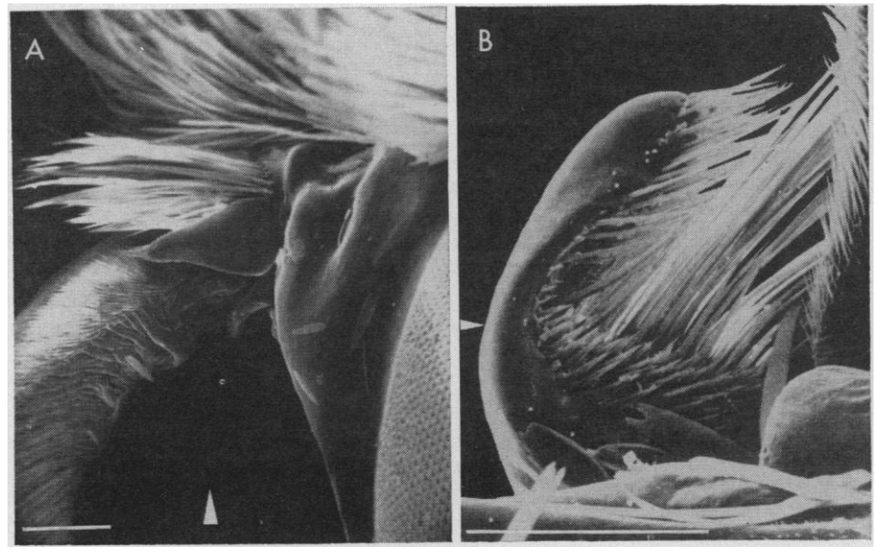

Fig. 1. The left pilifer of *Celerio lineata* exposed by removal of the labial palp (*5*). The arrow in each picture indicates the approximate angle from which the other picture was taken. The scales represent 0.2 mm. (A) Lateral view of anterior region of the head. The proboscis extends downward to the left; a part of the left compound eye is at lower right. Long sensilla chaetica from the basal part of the pilifer are visible near the apex of the distal lobe. (B) Ventral view of the same region. The background has been retouched. Lateral is to the left of the frame; the lower surface of the proboscis is visible at upper right. Several detached scales lie near the base of the distal lobe. When the labial palp is fully adducted some part of its medial surface appears to make contact with the smooth, convex, lateral surface of the distal lobe (at the left in B). The concave medial surface of this lobe bears short sensilla chaetica that do not touch the proboscis base (right). Long sensilla on the basal part of the pilifer (visible also in A) make contact with the proboscis.