nepetalactone-$^{14}$C showed a large peak with a retention time of 60 minutes; this peak was absent in the chromatogram of a control urine. Methylation with diazomethane (*4*) caused a change in retention time of the predominant peak, an indication that the major metabolite was a carboxylic acid. Further analysis (including chromatography with standard dimethyl nepetalinate) on Carbowax or Apiezon L indicated that the retention time of dimethyl nepetalinate and the methylated unknown were identical. Radio-gas chromatography showed that the major radioactive peak was identical with the peak detected by the hydrogen flame ionization detector and mass spectrometry. Other radioactive peaks corresponded to retention times of unreacted *cis,trans*-nepetalactone peaks, dihydronepetalactone, and an unidentified compound.

Mass spectra obtained with the combination gas chromatograph–mass spectrometer (*9*) after the extract from urine was methylated indicated the presence of $\alpha$-dimethyl nepetalinate, dihydronepetalactone, and unreacted *cis,trans*-nepetalactone.

A sample of the ether extract from urine was subjected to silicic acid chromatography (*10*) and gave the same elution volume as free $\alpha$-nepetalinic acid (9 ml). Direct comparison with authentic $\alpha$-dimethyl nepetalinate after methylation and analysis by gas-liquid chromatography concluded identification.

The specific activity of the recovered $\alpha$-dimethyl nepetalinate was $1.85 \times 10^4$ mc/mmole as compared to $1.87 \times 10^4$ mc/mmole for the administered uniformly labeled *cis,trans*-nepetalactone-$^{14}$C, indicating no significant endogenous dilution. Although the extent of formation varied among cats, the free $\alpha$-nepetalinic acid was always quantitatively the major metabolite (50 to 75 percent).

A probable pathway for the metabolism of nepetalactone by the domestic cat is shown in Fig. 2. Two routes are proposed: (i) the direct delactonization of nepetalactone to yield $\alpha$-nepetalic acid which is subsequently oxidized to $\alpha$-nepetalinic acid, and (ii) the hydrogenation of nepetalactone to yield dihydronepetalactone (*11*) followed by delactonization and oxidation to yield $\alpha$-nepetalinic acid (*12*). Although $\alpha$-nepetalic acid is implicated as a key intermediate in nepetalactone metabolism, the available evidence to support this view is inconclusive.

Our results provide the first evidence for the metabolism of an attractant by a higher animal. It should be pointed out that the metabolism of milligram quantities of attractant may bear no relationship to the few molecules required to stimulate the olfactory receptors and produce the psychopharmacological effects.

G. R. WALLER
G. H. PRICE
E. D. MITCHELL
*Department of Biochemistry, Oklahoma State University, Stillwater*

### References and Notes

1. S. M. McElvain, R. D. Bright, P. R. Johnson, *J. Amer. Chem. Soc.* **63**, 1558 (1941).
2. N. B. Todd, *J. Hered.* **53**, 54 (1962).
3. Some *Nepeta cataria* plants produce *cis-trans*-nepetalactone and *trans,cis*-nepetalactone in the ratio of 99.9 to 0.1, whereas in others a ratio of 70 to 30 may be found. These isomers are difficult to separate; for the preparation of *cis,trans*-nepetalactone-$^{14}$C which had a minimum of *trans,cis*-nepetalactone-$^{14}$C, a single leaf of several plants was analyzed to locate plants producing predominantly *cis,trans*-nepetalactone; these plants were then used in the preparation of *cis,trans*-nepetalactone-$^{14}$C from $^{14}CO_2$.
4. F. E. Regnier, E. J. Eisenbraun, G. R. Waller, *Phytochemistry* **6**, 1271 (1967).
5. To make certain that the radioactivity collected in the NaOH traps was not respired as organic volatile compounds, a trap (—78°C) was inserted between the metabolism chamber and the first NaOH trap in the experiment with the adult female cat. The aqueous solution recovered from the traps was devoid of radioactivity; thus the respired radioactivity was $^{14}CO_2$.
6. All of the final radioactivity was recovered in the ether extract of the steam distillate. Since this represented only a small portion of the administered dose, it was not studied further.
7. Analytical thin-layer chromatography was performed on thin-layer plates (20 by 20 by 0.4 cm or 20 by 6 by 0.6 cm) coated with silica gel G (25 mg/cm$^2$) and developed in a hexane, acetone, ethanol (40 : 10 : 4 by volume) system. The compounds were located by staining with iodine vapor. For chromatography of the free $\alpha$-nepetalinic acid a benzene, methanol, glacial acetic acid (90 : 16 : 8 by volume) system was used. After air-drying until no acetic acid could be detected by smell, the plate was sprayed with a solution of 0.04 percent bromcresol green in ethanol. The acid spots appeared yellow on a blue background. Thin-layer chromatography plates were assayed for radioactivity by (i) external scanning of the plate with a Nuclear-Chicago model 10324$\pi$ Actigraph III and (ii) scraping the plates clean at appropriate areas and counting by liquid scintillation spectrometry (Tri-Carb model 314).
8. J. F. Klebe, J. Finkbeiner, D. M. White, *J. Amer. Chem. Soc.* **88**, 3390 (1966).
9. R. Ryhage, *Arkiv Kemi* **26**, 305 (1967); G. R. Waller, *Proc. Okla. Acad. Sci.* **47**, 295 (1967).
10. C. S. Marvel and R. D. Rands, *J. Amer. Chem. Soc.* **72**, 2642 (1950).
11. Our evidence suggests that this is $\alpha$-dihydronepetalactone based on gas-liquid chromatography retention times.
12. The specificity of the enzyme-catalyzed reactions involved in (i) opening of the lactone and (ii) oxidation to yield $\alpha$-nepetalinic acid is recognized. Chemical opening of the lactone ring followed by oxidation yields approximately equal amounts of the $\alpha$ and $\delta$ isomers of nepetalinic acid [E. J. Eisenbraun and S. M. McElvain, *J. Amer. Chem. Soc.* **77**, 3383 (1955)]. If these reactions were the result of chemical catalysis brought about by the alkaline conditions of the small intestine, approximately equal amounts of the $\alpha$ and $\delta$ isomers of nepetalinic acid would be formed. No $\delta$-nepetalinic acid was found in cat urine; all four isomers, $\alpha$, $\beta$, $\gamma$, and $\delta$, of nepetalinic acids can be distinguished based on their difference in retention times (*4*); consequently, we concluded the stereospecific oxidation of nepetalactone results from the action of mammalian enzymes.
13. Supported in part by NSF grant GB5607. We thank Dr. E. J. Eisenbraun for $\alpha$-nepetalinic acid (2-carboxy-$\alpha$,3-dimethylcyclopentaneacetic acid) and for helpful discussions; Dr. R. Panciera for necropsy examinations and Dr. B. L. Glenn for assistance in maintaining the cats. G.H.P. was an NDEA fellow (1965–67).

6 December 1968

## Collagen Gels: Design for a Vitreous Replacement

Abstract. *Clear, stable gels have been prepared from purified tropocollagen from calf skin; the collagen was solubilized with a proteolytic enzyme (Proctase) and stabilized by ultraviolet irradiation under nitrogen. These gels are clear, possess altered immunologic reactivity, and have properties of an ideal vitreous replacement. Implantations in rabbit and monkey eyes appear to be well tolerated, remain clear, and gradually disappear in about 2 months.*

One of the problems in medicine and surgery today is the procurement of replacements for damaged human organs. Because of its biologic inertness and its structural stability, collagen can be altered in various ways to make it suitable for replacements. Tropocollagen, or molecular collagen, is a triple helical fibrous protein with nonhelical appendages that are critical determinants of its molecular interaction (*1*) and immunologic properties (*2*). Modification of these regions by proteolytic enzymes and by ultraviolet light leads to alterations in collagen which render it suitable for heteroimplantation. Collagen films implanted in rabbit corneas (*3*) have been in place for more than 2 years with no untoward reaction. We now report on the replacement of vitreous with specially prepared collagen gels. The human vitreous humor is a clear gel-like structure located in the posterior part of the eye. In cases of a damaged vitreous, replacement with a clear gel could help restore sight.

Collagen is extracted from calf skin by treating washed collagen fiber with Proctase (*4*), a proteolytic enzyme with

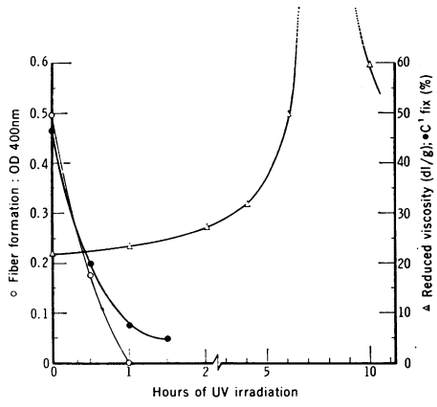


Fig. 1. Effect of ultraviolet irradiation of 0.2 percent collagen solutions treated with Proctase in 0.05 percent acetic acid on the following: ○, Fiber formation as measured by optical density at 400 nm of the collagen after 30 minutes of incubation in phosphate-buffered saline at 37°C; ●, complement fixation (C'fix) as measured with 100 $\mu$g of collagen and a 1/200 dilution of rabbit antiserum to Proctase-treated collagen; and △, reduced viscosity [deciliters per gram (dl/g)]. The dotted lines indicate gel formation.

a *p*H optimum of 3.0. The helical portions of collagen are relatively impervious to proteolytic digestion, whereas the nonhelical appendages are quite susceptible. Thus, enzyme treatment results in solubilization of large amounts of insoluble collagen and in digestion of noncollagenous protein contaminants. Insoluble material is removed from the mixture by centrifugation, and collagen is again precipitated from the supernatant by dialysis against 0.02*M* $Na_2HPO_4$. The reconstituted collagen fiber is again treated with Proctase (1/200), precipitated by dialysis against 0.02*M* $Na_2HPO_4$, and then dialyzed against a borax buffer solution (0.037*M* $Na_2CO_3$, 0.012*M* $Na_2B_4O_7$, *p*H 10) to inactivate the Proctase. The collagen which now has been treated twice with Proctase is further purified by repeated precipitation and solubilization. The final material is dissolved in 0.05 percent acetic acid at a collagen concentration of 0.5 percent. This concentration of collagen was chosen because the refractive index of such a solution approaches that of human vitreous. The material is centrifuged at 108,000*g* for 5 hours, the sediment is removed, and the process is repeated. The clear supernatant may then be diluted to a collagen concentration of 0.2 percent and passed through a Millipore filter (0.4 $\mu$). All further handling is then done aseptically. The collagen is lyophilized, reconstituted to 0.5 percent concentration in 0.01*N* HCl, dialyzed against 10 percent glucose, and irradiated with ultraviolet light from a 19-watt source at a distance of 15 cm. Alternatively, the collagen may first be dialyzed against repeated changes of distilled water and then against 10 percent glucose; it is then subjected to ultraviolet irradiation. The irradiation must be carried out in a nitrogen atmosphere. In air or oxygen, collagen is rapidly depolymerized. In nitrogen, however, this method results in a firm, clear gel which can be passed through a hypodermic needle and still retain its gel structure.

Effects of treating collagen with enzymes are well documented and include effects on intra- and intermolecular bonding (*5*), as well as effects on antigenicity (*2*). Ultraviolet irradiation further alters antigenic and physical properties of collagen solutions (*6*). The treated collagen is weakly antigenic in rabbits, but an occasional animal will respond to repeated injections of collagen treated with Proctase and Freund's adjuvant with a small amount of complement-fixing antibody. When Proctase-treated collagen is subjected to ultraviolet irradiation, reactivity with antibody to the treated collagen is rapidly lost (Fig. 1). This change in antigenicity actually precedes many other measurable physical alterations of the collagen. The viscosity of collagen solutions increases with ultraviolet irradiation until finally a firm gel forms (Fig. 1). If irradiation is continued, the gel breaks down; and if irradition is done in the presence of oxygen, collagen never gels and rapidly depolymerizes. The rate of gel formation depends on the buffer. The refractive index of the gel can be changed by altering the protein concentration, and the irradiation prevents fiber formation when the *p*H or ionic strength of collagen solutions is increased (Fig. 1).

The molecular changes thus result in a firm, stable gel. This crystal-clear gel (Fig. 2) was implanted in 72 rabbit eyes and in 27 monkey eyes after prior removal of some normal vitreous, and the effects in vivo were noted. All the implants were well tolerated. After 0.5 ml of the gel was injected, there was an initial inflammatory response in monkey eyes which was slightly greater than that seen in control eyes (injected with an equal volume of 0.85 percent NaCl). This reaction completely disappeared between day 5 and day 10. In rabbit eyes, the very slight visible reaction did not exceed that observed in control eyes. Sections of eyes removed at intervals beginning at 2 weeks after implantation showed no abnormalities. Fine structure of the retina could be seen through the gels, and the gels gradually decreased in size and were gone in about 2 months. Observations of eyes that had had gel implants were continued for up to 6 months, and there were no abnormalities (*7*). The relatively short life span of collagen in the eye is sufficient for many ophthalmological needs and may be an advantage, since the implant will be replaced.

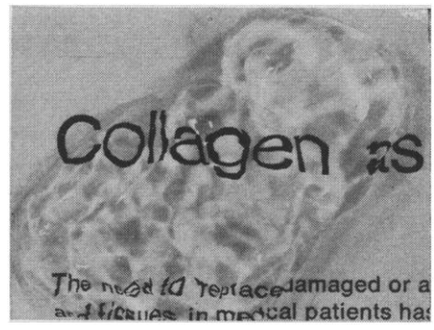


Fig. 2. Clear gel prepared from collagen treated with Proctase, indicating the clarity and rigid structure of the material.

KURT H. STENZEL
MICHAEL W. DUNN
ALBERT L. RUBIN
*Departments of Surgery (Ophthalmology), and Biochemistry, Rogosin Laboratories, New York Hospital–Cornell Medical Center, New York*

TERUO MIYATA
*Collagen Research Division, Japan Leather Company, Ltd., Tokyo*

### References and Notes

1. A. L. Rubin, D. Pfahl, P. T. Speakman, P. F. Davison, F. O. Schmitt, *Science* **139**, 37 (1963).
2. F. O. Schmitt, L. Levine, M. P. Drake, A. L. Rubin, D. Pfahl, P. F. Davison, *Proc. Nat. Acad. Sci. U.S.* **51**, 493 (1964); P. F. Davison, L. Levine, M. P. Drake, A. L. Rubin, S. Bump, *J. Exp. Med.* **126**, 331 (1967).
3. M. W. Dunn, T. Nishihara, A. W. Branwood, A. L. Rubin, *Science* **157**, 1329 (1967).
4. A. L. Rubin, R. R. Riggio, R. L. Nachman, G. H. Schwartz, T. Miyata, K. H. Stenzel, *Trans. Amer. Soc. Artif. Intern. Org.* **14**, 169 (1968).
5. A. L. Rubin, M. P. Drake, P. F. Davison, D. Pfahl, P. T. Speakman, F. O. Schmitt, *Biochemistry* **4**, 181 (1965).
6. T. Miyata, T. Sohde, A. L. Rubin, K. H. Stenzel, in preparation.
7. M. W. Dunn, T. Miyata, K. H. Stenzel, A. L. Rubin, in preparation.
8. Supported in part by PHS grant HE 08736, the John A. Hartford Foundation, and the New York Heart Association.

28 February 1969; revised 21 April 1969