Three wood preservatives containing chlorinated hydrocarbons (dichlorobenzene, trichlorobenzene, and hexachlorocyclohexane), when used in feeding tests, produced an abnormal flavor distinctly different from that originally found.

The active factor was isolated from wood shavings by extraction with methanol, reextraction from methanol with ethyl ether, and fractionation of the ether extract by thin-layer chromatography on silica gel G (Merck; thickness of layer 1 mm) with ethyl acetate (15 percent) in hexane as the solvent. Feeding experiments with extracts from fractions of the plate material, followed by testing of the eggs, were used to locate fractions containing the active material. Portions of these active fractions were concentrated on cotton wool for distillation directly into the ionization chamber of a mass spectrometer. The resulting spectrum was identified as that of a tetrachloroanisol.

One milligram of pure 2,3,4,6-tetrachloroanisol (prepared by methylation of the parent phenol) per kilogram of weight was fed to hens and broilers. A musty taste, similar to that found when 5 percent active wood shavings was fed to them, developed in their eggs and in broiler meat. The musty taste of eggs was also produced by mixing 20 μg of the active factor into a normal egg.

Related chlorinated anisols and phenols were tested for activity: 2,4,6-trichloroanisol was more active than 2,3,4,6-tetrachloroanisol; but 2,4,5-trichloroanisol, pentachloroanisol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, and pentachlorophenol were inactive.

The presence of tetrachloroanisol has since been demonstrated in eggs from hens fed on active wood shavings, in musty eggs from two commercial houses, in seven lots of active wood shavings, and in built-up litter taken from two commercial houses where musty eggs were produced. The compound has not been detected in normal eggs or in inactive wood shavings. Pentachloroanisol was found in all instances where the presence of 2,3,4,6-tetrachloroanisol had been established. Whether these compounds are present in wood shavings as a result of biotransformation or as a contaminant of wood preservatives is unknown.

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Note

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Purification and Reconstitution of the Periodic Fibril and Unit Structure of Human Amyloid

Abstract. *Extraction with alkaline sodium glycinate of the periodic fibril and unit structure of human amyloid and subsequent acidification of the extract provided a method for the purification as well as for the reconstitution of the periodic rod and of the unit structure. Guanidine completely solubilized and irreversibly dissociated both the rod and the unit structure of the human amyloid fibril.*

Recent electron-microscopic studies on negatively stained extracts of human amyloidotic tissue (*1*) revealed the presence of two distinct types of particles. One was a rod 100 Å wide; the other was a small pentagonal structure (unit structure) 90 Å in diameter (Fig. 1). Both morphologic units were present in all preparations of various tissues obtained from patients with either primary or secondary amyloidosis. Narrow electron-opaque bands divided the rods at intervals into smaller segments, the average distance from the center of one segment to that of the next being approximately 40 Å. A similar periodicity of amyloid fibrils, or segments thereof, has been described by several authors (*2*). Frequently, however, the width of the electron-opaque bands between segments varied irregularly, suggesting that the segments were easily disunited. When preparations containing predominantly long rods were exposed to high-frequency sound, a large increase in the number of unit structures (apparently single or double segments of the rods) was evident. Because the unit structures lay on their flat sides, the ends of the amyloid rod could be seen. The unit structures appeared to be composed of five globular sections surrounding a hollow core. A nonperiodic, 75-Å-wide fibril composed of two laterally aggregated parallel filaments with an apparent 100-Å beading has been described by Shirahama and Cohen (*3*) and solubilized, purified, and precipitated by Newcombe and Cohen (*4*). We attempted to reconstitute the periodic fibril of human amyloid, to purify it, and to characterize it by physical means.

The periodic structural components of amyloid in impure form were isolated, by a previously described method, from the spleen of a patient with secondary amyloidosis (*1*). The supernatant fluids of seven saline washings containing the periodic amyloid fibrils and unit structures were pooled, and the impure fibrils and unit structures were sedimented by centrifugation at 105,000*g* for 20 minutes at 4°C.

We used Newcombe and Cohen's method (*4*) for solubilizing and purifying the periodic fibrils and unit structures. The sediment of the washings centrifuged at 105,000*g* was shaken with Sörenson's glycine buffer (0.1*M*, *p*H 9.5). The glycinate extract was then centrifuged at 105,000*g* for 1 hour at 4°C, and the crystal-clear supernatant fluid was removed. A portion of it was examined with the electron microscope; another portion was used for electrophoresis on polyacrylamide gel. The remaining supernatant of fluid was dialyzed against 2 liters of 0.1*M* sodium acetate buffer (*p*H 4.5), the dialyzing solution being changed every 2 hours for 8 hours. A white, fluffy precipitate was formed, which was further dialyzed overnight against distilled water. The precipitated material was resuspended and centrifuged at 20,000*g* for 1 hour at 4°C. The white pellet obtained was again solubilized and precipitated, and this entire procedure was repeated twice.

Electron-microscopic examination of a portion of the original glycinate extract revealed only the unit structure,

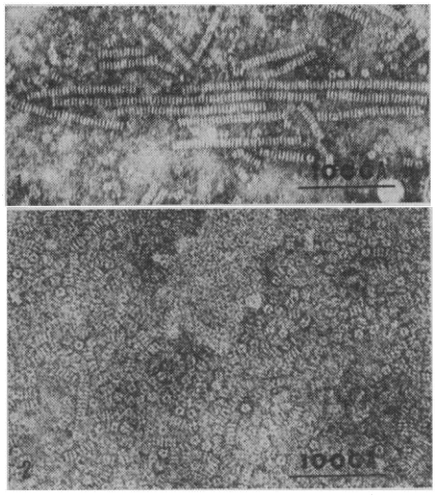

Fig. 1 (top). Periodic fibrils and unit structures ("doughnuts") of human amyloid isolated by differential centrifugation. Fig. 2 (bottom). Periodic rods and unit structures of human amyloid reconstituted from alkaline glycinate extract.

while examination of the purified precipitate revealed not only the unit structure, but numerous, short fibrils (Fig. 2) previously described as characteristic of human amyloid (*1*).

For electrophoretic analyses, the reprecipitated amyloid was lyophilized, extracted with glycinate buffer (*p*H 9.5), and centrifuged for 2 hours at 105,000*g*. The supernatant fluid from this purified sample as well as that from the initial, impure glycinate extract was exhaustively dialyzed against tris (hydroxymethyl) aminomethane (Tris)–glycinate buffer (0.024*M*, *p*H 8.8). Electrophoresis of samples of both solutions (containing 20 to 40 μg of protein as estimated by the Lowry method) on polyacrylamide (7 percent) gel was carried out on the Canalco Model 12, disc-electrophoretic apparatus at room temperature in a current of 1 ma for 20 minutes and in one of 2.5 ma for 60 minutes per 6 cm of gel column. The amidoschwartz stain for protein and standard staining procedure, with only minor modifications, were used on all gels in this study. This electrophoresis revealed the presence of two distinct protein bands and two diffuse bands in the impure extract (Fig. 3a) but only one band in the purified preparation (Fig. 3b). An amyloid extract, purified as above, of myocardial tissue from a patient with primary amyloidosis also yielded a single protein band, which had electrophoretic mobility identical to that of the band obtained from the extract of spleen from a patient with secondary amyloidosis. Electrophoresis on polyacrylamide gel of a glycinate extract of purified nonperiodic fibrils, prepared as described by Newcombe and Cohen (*4*), resulted in, as reported by these authors, several distinct protein bands; the band that stained most intensely and migrated most slowly corresponded in electrophoretic mobility to that obtained from the purified periodic rod and unit structure described herein (Fig. 3c).

In order to determine the effect of mild denaturing agents, we suspended a pellet (1 mg) of the amyloid particles, solubilized in glycinate and precipitated in acid, in 1 ml of distilled water, and added this mixture to a solution containing 0.075 ml of 4.6*M* tris-HCl buffer, *p*H 8.2; 23.2 mg of iodoacetamide; 0.35 ml of distilled water; and 1.5 g of recrystallized guanidine-HCl, in which complete solution occurred (*5*). This guanidine solution was dialyzed against 150 ml of 10 molal urea (recrystallized and deionized) with ten changes over 4 days. No sediment was obtained when this solution was centrifuged at 105,000*g* for 1 hour at 4°C. We examined with the electron microscope the precipitate obtained from a portion of this urea solution dialyzed first against acetate buffer (0.1*M*, *p*H 4.3) and then against distilled water. No periodic fibril or unit structure was observed. A sample (24 to 96 μg) of the amyloid extract (in 10 molal urea) was placed on a 6 percent polyacrylamide gel containing 8.5*M* deionized urea in both the upper and lower gel, and electrophoresis and staining were performed as described above. Five electrophoretically distinguishable bands were observed of which three are readily visible in Fig. 3c. Five distinguishable bands with slightly different electrophoretic mobilities were also obtained upon electrophoresis of the guanidine extract on routine 7 percent polyacrylamide gel. Electrophoresis of the glycinate extract of the purified amyloid on the polyacrylamide-urea gel yielded only one significant, intense band, but it had an electrophoretic mobility different from that of the band produced on the 7 percent polyacrylamide gel. When 6*N* urea alone was substituted as the denaturing agent and the extract was treated as described above, no structurally identifiable material was obtained on precipitation, and disc-electrophoresis of this extract on polyacrylamide-urea gel failed to yield more than two discrete protein bands.

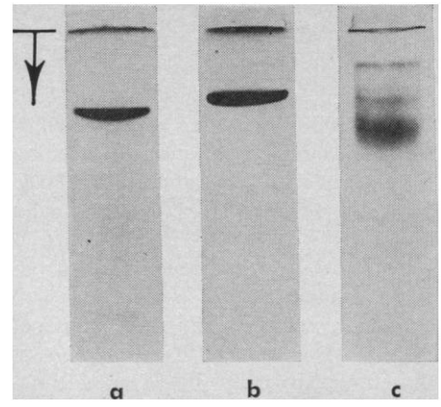

Fig. 3. Polyacrylamide disc-electrophoresis of (a) impure periodic amyloid fraction (7 percent gel); (b) purified periodic amyloid fraction (7 percent gel); (c) purified periodic amyloid fraction identical to that of (b), solubilized and denatured in guanidine (polyacrylamide-urea gel).

The method of Newcombe and Cohen (*4*) for the purification of nonperiodic fibrils of amyloid can be used in the purification and reconstitution of the unit structure and periodic fibrils of both primary and secondary human amyloid. Since electron microscopy provided evidence that the glycinate extract apparently contained in solution only the unit structure, it seems that precipitation in acid of this extract reconstitutes the periodic amyloid fibril. Disc-electrophoresis of the purified glycinate extract results in one slowly moving protein band, which we interpret as containing the intact unit structure or electrophoretically indistinguishable subunits. However, this unit structure is apparently dissociated by guanidine into at least five electrophoretically distinguishable proteins. Our evidence indicates that guanidine and alkaline glycinate are capable of solubilizing completely the unit structure and periodic fibril of amyloid.

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5. We are indebted to Dr. Parker Small for suggesting this method to us. We thank S. Mason and G. Kresovich for technical assistance.

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