nemia in the F_2 progeny was 22 percent. In the animals backcrossed to NAR, the frequency of the mutant trait was around 50 percent, whereas in the backcross to normal rats, no analbuminemia was found. These data indicate that analbuminemia is inherited as an autosomal recessive trait.

The first case of human analbuminemia was reported by Benhold *et al.* (*3*); since then, others have studied the etiology and metabolic aspects of the human disease (*4*-*7*).

A decrease of albumin accompanied by an increase of globulins occurs in the serum of analbuminemic patients, and high cholesterol level has been reported (*5*). The analbuminemic rats should serve as a model for understanding the human disease, and may also be suitable for studying the function of albumin.

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References and Notes

1. In 1974 and 1976, Nagase first recognized the existence of analbuminemia in dead Sprague-Dawley rats but was unable to find living analbuminemic rats in the colony. Hattori *et al.* (*8*) succeeded in breeding a strain of rat that developed a high degree of hypercholesterolemia after feeding them a high (2 percent) cholesterol diet for 3 days. In these experiments, Sprague-Dawley rats of both sexes were fed a high cholesterol diet; these rats were divided into high and low responders according to their serum cholesterol levels. By repeated matings between siblings that were high responders, progeny of both sexes became progressively more susceptible to dietary hypercholesterolemia; some of them developed spontaneous hypercholesterolemia on a cholesterol diet. These rats were delivered to us in the F_4 generation, and further mating between siblings was performed at our institute.
2. Two lines of NAR and normal Sprague-Dawley rats were bred. The rats were kept on a diet of CE-2 (Clea Japan, Tokyo) and had free access to water. Room temperature was maintained at 23° ± 2°C throughout the experiments.
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Toxaphene, a Complex Mixture of Polychloroterpenes and a Major Insecticide, Is Mutagenic

Abstract. *Toxaphene, the most widely used chlorinated insecticide, is mutagenic in the* Salmonella *test without requiring liver homogenate for activity. This insecticide is a complex mixture (more than 177 polychloroterpenes) with carcinogenic activity in rodents. Some but not all of the mutagenic components are easily separated from the insecticidal ingredients.*

More than 10^9 pounds (0.5×10^9 kg) of the insecticide toxaphene (*1*) have been manufactured since 1947. The yearly production now totals about 40×10^6 pounds (*2*). The insecticide, which is used on cotton (86 percent of the total amount) and food crops (*3*), is a complex mixture of at least 177 polychlorinated compounds (overall composition, $C_{10}H_{10}Cl_8$) (*4*). It is produced by the extensive chlorination of technical-grade camphene obtained by isomerization of α-pinene, a by-product of turpentine distillation. Only ten components have been identified, including the most toxic ingredient, and they account for less than one-fourth of the mass of the mixture (*5*). Eighteen or more components are used in amounts individually exceeding 10^6 pounds per year (*5*, *6*).

The carcinogenic and mutagenic potential of toxaphene is of interest because of its long history of use, continuing importance, and complex composition. Toxaphene is a carcinogen in rodents (*7*). Toxicological problems have also been encountered with two related polychlorocamphene insecticides. Strobane, produced in the United States (*2*), causes liver hepatomas in male mice (*8*). Another polychlorocamphene insecticide, produced in the U.S.S.R. and of unknown composition relative to toxaphene, is associated with chromosomal and other abnormalities in humans at high occupational concentrations (*9*). In pregnant female rats this insecticide is transported to the fetus, and in male rodents it causes testicular degeneration and endocrine changes (*10*).

We used mutagenesis assays as a first step in defining the potential carcinogenic components of toxaphene (*1*). When tested in histidine-requiring strains of *Salmonella typhimurium* (*11*), toxaphene was mutagenic in strains TA98 and TA100, giving 325 revertants per milligram in the latter case (Fig. 1). All further discussion in this report is based on findings with the more sensitive TA100 strain. Mutagenic activity was reduced by 50 percent in the presence of rat or mouse liver homogenate [Fig. 1, toxaphene (+S9)]; this finding suggests possible nonspecific interactions with glutathione or macromolecules (for example, RNA).

Because of the chemical complexity of toxaphene, we fractionated the mixture to determine whether the mutagenic and toxic activities are separable. The direct mutagenic activity observed did not correspond exactly to the major toxic components, as suggested by the following evidence.

1) The most easily isolated major toxic component, heptachlorobornane-I (*5*), did not have mutagenic activity, either with or without liver S9, in any of the standard tester strains (TA1535, TA1537, TA1538, TA98, and TA100) (Fig. 1).

2) The direct mutagenic activity was not lost upon treatment of toxaphene with ethanolic potassium hydroxide under conditions (molar ratios of 1:1 or 1:10, 24 hours, 25°C) that dehydrochlorinate the major identified *gem*-dichloro toxic components.

3) The mutagenic activity of a recrystallized toxaphene fraction (from isopropanol) was less than that of the more polar mother liquor fraction (Fig. 1). Some of the direct mutagenic activity was in the polar fractions from chromatography of toxaphene on a silicic acid column rather than the nonpolar fractions most acutely toxic to animals and usually analyzed by gas chromatography (GC) (*12*) (Fig. 1, methanol and hexane fractions).

4) A series of toxaphene samples produced in different years that were similar in toxicity and retention pattern on open-tubular-column GC had about a fourfold range of direct mutagenic activity (*13*).

We attempted to remove the direct mutagens from toxaphene but were unsuccessful. The mutagenic activity was not reduced when a solution of toxaphene in carbon tetrachloride was washed with water or aqueous methanol (up to 20 percent). A portion of the activity survived passage in hexane solution through a concentrated sulfuric acid column (*14*), as would chlorobornanes and other chloroalkanes. A potent but minor (0.3 percent) mutagenic fraction was retained on a Celite column on chromatography as a hexane solution but was then eluted with methanol; however, most of the direct mutagenic activity remained with the bulk of the material that eluted in the hexane.

Heptachlorobornane-I and possibly other major toxic components of tox-

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aphene were not detected as mutagens in the *Salmonella* test. However, this may be because heavily chlorinated chemicals generally do not appear to be properly evaluated by this assay system. For example, such carcinogens as carbon tetrachloride, dieldrin, and DDE (a metabolite and dehydrochlorination product of DDT) (*15*) are known to cause chromosomal aberrations or to interact with the DNA of mammalian cells, but they are not mutagenic in the *Salmonella* test (*16*). The active metabolic forms may be too short-lived (for example, free radicals) to be detected or the liver homogenate in the in vitro system may not faithfully reproduce in vivo metabolism. Anaerobic systems metabolize carbon tetrachloride and DDT, giving rise in the former case to a reactive free radical (*17*). We are attempting to improve the *Salmonella* assay in various ways, including anaerobic incubations, to determine whether these chemicals and heptachlorobornane-I (*14*, *18*) are then detectable as mutagens.

Little is known about the toxaphene residues in people or in the environment, largely because of the difficulties inherent in analyzing for such a complex mixture and its degradation products. Toxaphene persists for up to several years in soils and lake sediments and, as expected of a fat-soluble chlorinated hydrocarbon, it accumulates in fish (*19*). On the other hand, its residues are dissipated more rapidly than those of DDT and some other chlorinated insecticides in poultry, rats, and cows (*20*). Toxaphene and its heptachlorobornane-I component are also extensively metabolized by mice, hamsters, guinea pigs, rabbits, and monkeys (*21*). The composition of toxaphene undergoes drastic changes in these systems as a result of the more rapid loss of some components than others and the introduction of new compounds formed metabolically. No method is available for the complete analysis of the hundreds or thousands of compounds in toxaphene-derived residues (*4–6*, *21*).

Assessing risks for a substance introduced into the environment is unusually difficult when it is a complex and largely unidentified mixture. The potential for problems is even greater when the material is from a chemical class, such as chlorinated hydrocarbons, containing many mutagens and carcinogens (*22*). In addition, the general use of broad-spectrum pesticides has been questioned (*23*) as has the effectiveness of toxaphene (*24*). Greater emphasis must be given to the development of selective insecticides with acceptable toxicological properties and to integrated pest management programs that minimize pesticide use (*23*, *25*).

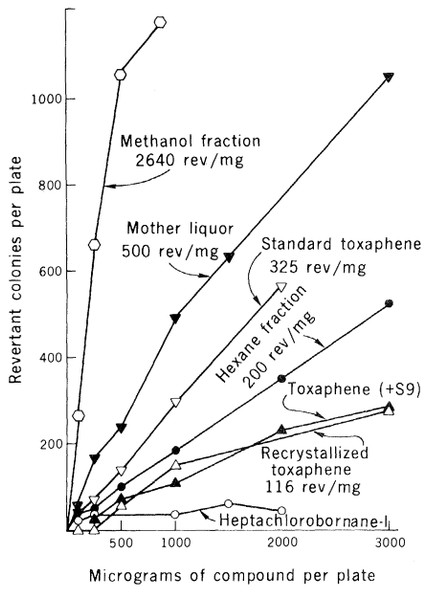

Fig. 1. Mutagenicity of toxaphene and its components or derivatives in *Salmonella* strain TA100. The number of revertants, after subtracting the spontaneous revertants (~150), is plotted against the amount of mutagenic compound or mixture. Where indicated, S9 mix (containing 20 μl of phenobarbital-induced mouse liver S9) was incorporated into the pour plates. (Aroclor 1254-induced rat liver S9 gave similar results.) Standard toxaphene was also mutagenic with *Salmonella* strain TA98 (50 revertants per milligram).

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1. In this study toxaphene refers to a standard sample from Hercules, Inc., Wilmington, Del. (lot X-18825-6, 1974 production).
2. *Synthetic Organic Chemicals: U.S. Production and Sales* (Publication 920, U.S. Trade Commission, Washington, D.C., 1977), p. 321. Toxaphene-like pesticides are used on a large scale in many parts of the world, mainly in cotton-producing countries. About 10^7 pounds of the related insecticide Strobane were manufactured annually until 1975.
3. "Position Document I on Toxaphene, Toxaphene Working Group" (Environmental Protection Agency, Washington, D.C., 19 April 1977). The amount of toxaphene used each year in the United States for insect control on soybeans, wheat, peanuts, corn, and tomatoes ranges from 0.3×10^6 to 2.4×10^6 pounds for each of these food crops.
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7. Publication NIH 79-837 (National Cancer Institute Carcinogenesis Technical Report Series 37, Department of Health, Education, and Welfare, Washington, D.C., 1979). Toxaphene (lot X-16189-49) was carcinogenic to male and female mice, causing liver tumors (hepatocellular carcinomas). Results also "suggest carcinogenicity" for male and female rats, causing thyroid tumors (follicular-cell carcinomas or adenomas). Increased liver neoplasia was also observed in both sexes of rats. Using data from this study, we calculate (N. K. Hooper, C. Sawyer, B. N. Ames, unpublished data) that the daily dose required to produce cancer in half of the mice (TD_{50}) is approximately 5 mg per kilogram of body weight. Toxaphene is about one-tenth as potent in rats. Earlier cancer tests [A. J. Lehman, *Summaries of Pesticide Toxicity* (Association of Food and Drug Officials of the United States, Topeka, Kans., 1965), p. 36] were negative but by current standards seem inadequate.
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12. When toxaphene is chromatographed on a silicic acid column and successively eluted with hexane and methanol, the methanol fraction, which is free of the known major toxic components, is considerably more mutagenic (2640 revertants per milligram) than the hexane fraction (200 revertants per milligram). In another experiment with further fractionation, we obtained an even more mutagenic methanol fraction with more than 17,000 revertants per milligram.
13. Ten samples of toxaphene (commercial preparations of Hercules, 1949 through 1975), quite similar in composition by open-tubular-column GC (*6*), ranged in direct mutagenic activity (TA100, −S9) from 310 revertants per milligram (1954) to 1270 revertants per milligram (1963). Hercules lots X-18825-6 and X-16189-49 gave 325 and 930 revertants per milligram, respectively. The mutagenic activity of 12 commercial samples of related chloroterpene insecticides produced in the United States and abroad, with different compositions but similar toxicities to houseflies and mice (*6*), ranged from 230 revertants per milligram (Procida, Paris, France) to 1530 revertants per milligram (Bison Chemical, Fort Natchez, Tex.).
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24. A major cotton pest, the tobacco budworm (*Heliothis virescens*), has developed resistance to toxaphene in areas of prolonged and extensive use [*Environment* **19**, 23 (1977)].
25. D. Brusick (Litton Bionetics) has independently found that toxaphene is mutagenic without requiring metabolic activation on *Salmonella* strains TA98 and TA100 (unpublished data).
26. We thank D. Maron and J. A. Katzenellenbogen for their considerable help on the project. Supported by Department of Energy contract EY-76-S-03-0034-PA156 to B.N.A. and NIH grant PO1 ES00049 and EPA grant R-803913 to J.E.C.

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Endogenous Inhibitor of Colchicine-Tubulin Binding in Rat Brain

Abstract. *A competitive inhibitor of colchicine binding to tubulin has been found in rat brain. Most of the inhibitor is associated with microsomes but some inhibitor, with an apparent molecular weight of approximately 250,000, is found in the cytosol. Both the microsomal and cytosol inhibitors are heat- and trypsin-sensitive, indicating that a protein moiety is required for activity. The microsomes bind tubulin directly; the microsomal and cytosol fractions both inhibit microtubule assembly in vitro. The inhibitor may function in the living cell to bind and sequester nonpolymerized tubulin. Regulation of tubulin attachment to microsomes could then control the concentration of cytosolic tubulin available for microtubule assembly.*

It is generally believed that microtubules in living cells are in a dynamic equilibrium with their subunit proteins. The number and distribution of assembled microtubules can change rapidly during the cell cycle or in response to external stimuli. Perhaps the most dramatic shift occurs when cells enter mitosis, at which time cytoplasmic microtubules disassemble and their protein constituents reassemble into microtubules of the mitotic spindle. Since Weisenberg's discovery in 1972 (*1*) of conditions for assembly of microtubules from brain supernatants, much has been learned about the factors that participate in or affect polymerization in vitro. Microtubule-associated proteins, Mg^{2+}, and guanosine 5′-triphosphate (GTP) stimulate and calcium inhibits tubulin assembly in vitro (*1*-*7*), but it is not known whether these or other factors regulate microtubule formation and dissolution in cells.

Colchicine, an alkaloid derived from *Colchicum autumnale*, inhibits microtubule assembly both in vitro and in vivo by binding with high affinity to tubulin (*8*-*10*), the major protein constituent of microtubules. Because colchicine is not found in the animal kingdom or in most plants, so far as is known, it has not been considered a candidate for regulation of microtubule assembly. However, it seemed possible that the colchicine-binding site on tubulin might have evolved as an attachment site for an endogenous material, which would be a natural regulator of tubulin assembly in living cells. Previous studies of the colchicine-tubulin binding reaction prompted the suggestion that a factor in brain cytosol lowered the affinity of tubulin for colchicine (*10*). In this report, evidence is presented for the existence of a trypsin-sensitive competitive inhibitor of colchicine binding in the microsomes and cytosol of rat brain.

Microtubule protein was prepared from weanling rat brains by two cycles of assembly-disassembly, using the method of Shelanski *et al.* (*11*). In electrophoresis on sodium dodecyl sulfate polyacrylamide gels (*12*), more than 90 percent of the protein was present in the tubulin band and the remaining 5 to 10 percent was found in the high-molecular-weight region. At saturation, a typical preparation of purified microtubule protein bound 4 pmole of colchicine per microgram. To prepare brain cytosol, rat brains were homogenized at 4°C in 10 m*M* Na_2HPO_4, *p*H 7.0, or 50 m*M* 4-morpholineethanesulfonate (MES), 1 m*M* [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (EGTA), and 1 m*M* $MgCl_2$, *p*H 6.6 (1 ml/g), using a Tekmar tissue disruptor (model SDT), and centrifuged for 90 minutes at 100,000*g*. Unlabeled colchicine was obtained from Sigma and [^{3}H]colchicine from Amersham.

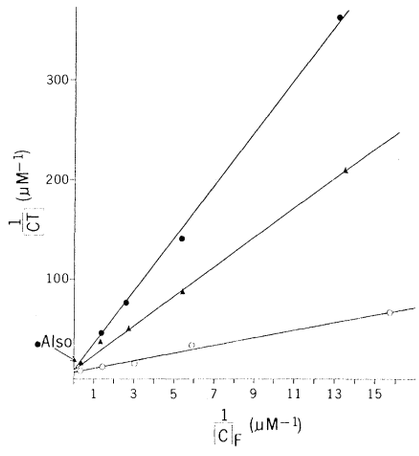

Fig. 1. Effect of brain cytosol inhibitor, partially purified by $(NH_4)_2SO_4$ fractionation (●), and of resuspended microsomal pellet inhibitor (▲) on colchicine-binding affinity of purified tubulin. Open circles represent colchicine binding by tubulin in the absence of inhibitor; $[C]_F$ represents concentration of free colchicine and $[CT]$ the concentration of bound colchicine at the end of the incubation.

Colchicine binding was determined by the charcoal separation method previously described (*13*). In a typical assay, unlabeled colchicine or a putative inhibitor sample was preincubated with tubulin (6 μg per 95 μl) for 2 hours at 37°C before adding 20 μl of labeled colchicine (final concentration, 0.48 μ*M*), followed by incubation for an additional 2 hours. In the kinetic study (Fig. 1), various concentrations of labeled colchicine were incubated with tubulin at 37°C for 4 hours before charcoal separation.

Native tubulin in brain cytosol has a lower affinity for colchicine than does purified tubulin. Addition of purified tubulin to brain cytosol results in a homogeneous class of low-affinity colchicine-binding sites (*10*). These data suggested that the lower colchicine-binding affinity of tubulin in brain cytosol was due to an endogenous factor which inhibits colchicine-tubulin binding. However, in fresh brain cytosol, endogenous tubulin has substantial colchicine-binding activity, which makes it difficult to measure precisely the inhibitory activity of the preparation. We were able to eliminate this problem because the colchicine-binding activity of tubulin is labile, whereas inhibitory activity is stable. Thus, after dialysis of brain cytosol for 48 hours at 4°C (500 volumes of 10 m*M* Na_2HPO_4, *p*H 7.0, three changes) all colchicine-binding activity was lost and inhibitory activity persisted. In multiple experiments, the inhibitor in the dialyzed brain extract decreased the apparent affinity of tubulin for colchicine, but had no effect on the maximum binding, and thus behaved as a competitive inhibitor. Inhibitory activity was precipitated from undialyzed brain extracts between 30 and 60 percent $(NH_4)_2SO_4$. The 60 percent pellet was resuspended in one-fifth the volume of the original supernatant and dialyzed for 48 hours at 4°C. This preparation also behaved as a competitive inhibitor of colchicine binding by tubulin (Fig. 1).

The inhibitor is inactivated by heating

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