mate; General Diagnostics). Bovine Factor X and Factor VIIa were provided by Dr. Yale Nemerson and were used at 6.48 μg/ml and 0.158 μg/ml, respectively. Bovine plasma and cephalin were prepared as described (*4*). A typical standard curve generated with bovine brain TF had clotting times of 15.7 seconds for 100 TF units per milliliter and 48.5 seconds for 6.25 TF units per milliliter. All coagulation times were determined twice and averaged.

6. Tissue factor (10 μl, 38 μg/ml), mixed bovine brain lipids [2 mg/ml in 0.01*M* tris with 0.25 percent sodium deoxycholate (*4*)], 50 μl of a mixture of 0.5*M* tris-HCl, 1*M* NaCl (*p*H 7.6) with bovine serum albumin (10 mg/ml), test cations, and water to 0.5 ml were combined in 1.5-ml conical polypropylene tubes. The tubes were covered with dialysis membrane and floated inverted on tris-NaCl at 4°C (*4*, *8*). The lipid concentration (milligrams per milliliter) was calculated on the basis of a phosphorus content of 4 percent (*4*).
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Hepatocarcinogenicity of Glandless Cottonseeds and Cottonseed Oil to Rainbow Trout (*Salmo gairdnerii*)

Abstract. *Glandless cottonseed kernels are available for purchase and consumption by the general public. These kernels contain no gossypol but still have a full complement of naturally occurring cyclopropenoid fatty acids, which in rainbow trout are active as synergists with aflatoxins and primary liver carcinogens. Diets containing glandless cottonseed kernels or a lightly processed cottonseed oil produced significant numbers of hepatocellular carcinomas in rainbow trout after 1 year. The much greater incidence of cancer induced by the kernel than by the oil indicates that synergists or other carcinogens may be present in the kernel in addition to the cyclopropenoid fatty acids.*

By-products of the seeds of the cotton plant *Gossypium hirsutum* are constituents of both animal and human foodstuffs. Cottonseed meal is used extensively as a protein supplement in cattle, swine, poultry, and fish rations, and cottonseed oil is an important ingredient of margarines and cooking oils for human use. Cottonseed, however, contains two toxic compounds that limit its use. Pigment glands of cotton plants contain the polyphenolic compound gossypol, which is toxic to livestock (*1*), swine (*2*), poultry (*3*), dogs (*4*), rodents (*5*), and trout (*6*). The presence of gossypol in cottonseed meal has been the primary limitation to its use in human food.

Cottonseed also contains malvalic acid (MA) and sterculic acid (SA) as glycerides in the oil. These cyclopropenoid fatty acids (CPFA) produce several undesirable effects in birds and mammals, including pink discoloration of stored egg whites (*7*); inhibition of the fatty acyl desaturase system (*8*); increased saturated fatty acids in body lipids (*9*); delayed sexual maturity in female rats (*10*); lung, liver, and kidney abnormalities and high prenatal and postnatal mortalities in rat pups (*11*, *12*); increased cholesterol levels, aortic atherosclerosis, and liver damage in rabbits (*13*); and mitogenic activity in rat liver and pancreas (*14*, *15*).

In rainbow trout, SA and, to a lesser degree, MA are powerful synergists in combination with aflatoxin B_1 and its metabolites, greatly increasing the incidence and severity of liver cancer (*16*–*18*). Both SA and MA are also primary hepatocarcinogens in rainbow trout (*18*–*20*). However, neither synergistic nor carcinogenic properties of SA have been unequivocally demonstrated in mammals (*21*, *22*).

Selective breeding of the cotton plant has resulted in varieties that produce glandless seeds that are free of gossypol but still contain a full complement of CPFA. The CPFA content is approximately 0.5 to 1.0 percent of the total lipid content of the seed. Of this, about 65 percent is MA and 35 percent SA (*23*). Nevertheless, glandless cottonseeds have been approved by the Food and Drug Administration as a nut substitute and snack item for human consumption.

We designed experiments to test the effects of glandless cottonseeds on rainbow trout. A mildly processed cottonseed oil was also fed to rainbow trout for comparison. (Heavy processing of cottonseed oil for use in margarine or salad oils destroys much of the CPFA content.)

Roasted glandless cottonseed kernels (GCK) were ground and added to semipurified trout feed (the control diet) (*24*) so that they constituted 25 percent of its dry weight. Protein, carbohydrate, lipid, and mineral components of the control diet were reduced to keep the two diets isonitrogenous and isocaloric. Cottonseed oil containing 0.35 percent CPFA (*22*) was added to the control feed (*24*) at 7.5 percent of its dry weight, and carbohydrate and lipid levels were again reduced to accommodate the increased level of lipid. Duplicate lots of 80 rainbow trout fingerlings (3 months old) were given free access to the control diet or one of the two experimental diets for 12 months and were sampled at 6, 9, and 12 months to determine histopathology—in particular, liver cancer incidence. Food consumption, weight gain, and ratios of liver weight to body weight were also recorded.

The CPFA content of the GCK was determined with the nuclear magnetic resonance (NMR) method of Pawlowski *et al.* (*25*). The GCK contained 39.8 percent lipid, as determined by extraction with ethyl ether. Electronic integration of the terminal methyl and cyclopropene region of the GCK lipid on a Varian FT-80 NMR spectrograph demonstrated that 0.73 percent of the fatty acids contained an isolated 1,2-disubstituted cyclopropene ring (MA and SA). These values permit estimation of the total CPFA content of the diets. The GCK diet contained 9.95 percent cottonseed lipid, of which 0.73 percent, or 726 mg/kg, was CPFA. Therefore, 35 percent, or approximately 250 parts per million (ppm), was SA. In the cottonseed oil diet the cottonseed oil (7.5 percent of the diet) contained 0.35 percent CPFA, which means there were 262 mg of CPFA per kilogram of the diet, or about 90 ppm SA.

Duplicate 25-g samples of GCK were analyzed for aflatoxins with the method described in (*26*): none were detectable. The level of any aflatoxin B_1 present would have been < 0.1 part per billion (ppb) in the GCK or < 0.025 ppb in the diet.

Another GCK sample was prepared with the method of Goodhead and Gough (*27*) and was analyzed by combined gas chromatography and thermal energy analysis for volatile *N*-nitroso compounds (*28*); the sensitivity of this method is 0.1 ppb. No *N*-nitroso compounds were present.

Growth of the trout was significantly ($P < .001$, Student's *t*-test) reduced by the diet containing GCK. At 12 months of age, the fish fed the GCK diet had an average body weight of 424 ± 110 g compared with 565 ± 164 g for the control fish. The fish fed the cottonseed oil diet weighed slightly less than the control fish (533 ± 140 g), but the difference was not significant. Ratios of liver weight to body weight indicated the presence of CPFA toxins. The ratio was significantly higher ($P < .001$) in the cottonseed oil–fed fish (1.28 ± 0.26) than in the controls

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(0.94 ± 0.16) and in the GCK-fed fish (1.60 ± 0.52) than in the cotton seed oil-fed fish (1.28 ± 0.26).

Both experimental diets—especially the GCK diet (Fig. 1A)—produced liver carcinomas in the fish (Table 1). The tumors were histologically similar to those induced in rainbow trout by aflatoxins or other liver carcinogens (*24*). They were characterized by broad trabeculae of deeply basophilic cells, numerous mitotic figures, and various degrees of hyperplastic bile duct involvement (Fig. 1B). Liver pathology due to CPFA was similar to that described previously (*29, 30*).

The cottonseed oil diet contained SA (≈ 90 ppm) as the glyceride, but yielded results similar to those for diets containing 50 ppm of methyl sterculate (MS) (*18*). In several feeding trials, a 30 to 40 percent incidence of liver cancer was produced in rainbow trout with MS at the latter concentration (*18*). The low number of tumors and the long latency period (no tumors were detected before 12 months) in the present study were also consistent with the previous results.

Because of the results of a previous MS dose-response study (*19*), we did not expect a greater incidence of tumors to result from the higher CPFA content of the GCK diet. The previous study showed a maximum tumor incidence at about 50 ppm of CPFA and slightly decreased incidences at higher concentrations. The large number of tumors that developed in the fish in the present study is also not consistent with carcinogenesis induced by CPFA alone. These inconsistencies may be due to the presence of synergists or other carcinogens in the diet. For example, there may be a very low level of aflatoxin in the GCK—< 0.1 ppb in the GCK or < 0.025 ppb in the GCK diet. Previous studies in our laboratory indicate that this would probably be adequate to produce the synergistic effect we believe occurred in this experiment. (There were no detectable nitrosamines in the GCK, so they can be eliminated as a contributing factor.)

These results verify the hepatocarcinogenicity of CPFA in cottonseed products to rainbow trout, and call attention to the added danger of possible microcontamination by aflatoxins or other carcinogens. Although CPFA have not as yet been shown to be carcinogenic or synergistic in mammals or humans, there is a need for additional research on the safety of glandless cottonseeds for human consumption.

Table 1. Liver cancer incidence in rainbow trout that were fed diets containing cottonseed products.

Diet	After 6 months	After 9 months	After 12 months	Percentage	Total number of tumors	Cumulative mortality
Control*						
Tank 1	0/10	0/10	0/26	0.0	0	4
Tank 2	0/10	0/10	0/30	0.0	0	0
Cottonseed oil						
Tank 1	1/10	0/10	17/59	28.8	20	1
Tank 2	0/10	0/10	21/58	36.2	27	2
GCK						
Tank 1	0/10	1/10	41/58	70.7	110	2
Tank 2	0/10	3/10	46/58	75.9	106	2

*The number of controls sampled was smaller than the number of experimental animals because one-half of the controls also served as controls for another experiment that lasted an additional 3 months. Liver cancer was not detected in those controls at 15 months either.

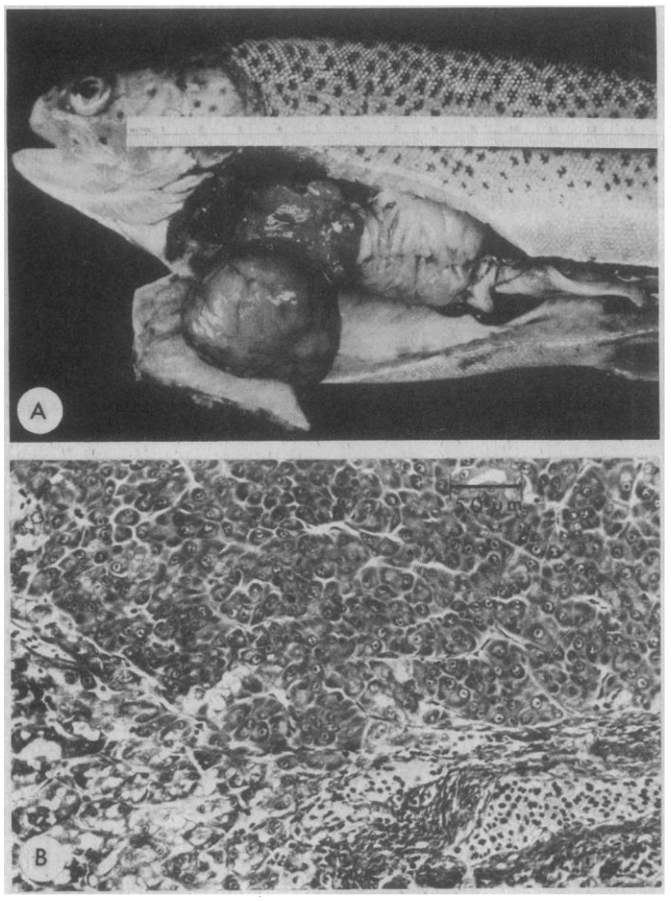

Fig. 1. (A) Rainbow trout with a large hepatocellular carcinoma. It had been fed GCK at 25 percent of the diet for 1 year. (B) Photomicrograph of a section of a hepatocellular carcinoma. Note the broad cords of deeply basophilic cells, the mitotic figures, and the compression of normal liver tissue. Stain: hematoxylin and eosin (×450).

J. D. HENDRICKS
R. O. SINNHUBER
P. M. LOVELAND
N. E. PAWLOWSKI
J. E. NIXON

Department of Food Science and Technology, Oregon State University, Corvallis 97331

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Order in the Optic Nerve of Goldfish

Abstract. *A small amount of horseradish peroxidase, injected into the goldfish optic nerve and transported into the retina, filled an annulus of ganglion cells. Since the retina grew by annular addition of cells, this result shows that axons from cells of similar age clustered together in the nerve.*

Most nervous systems are composed of collections of nerve cell bodies connected by groups of axons. The connections are often highly ordered, and proper function depends on this order. Much of our knowledge of the formation, maintenance, and modification of such orderly connections has been derived from studies of the retinotectal system of lower vertebrates, especially fish and amphibians. Ganglion cells of the retina send axons to the tectum via the optic nerve. The axons from ganglion cells at different locations on the retina terminate on the tectal surface in a retinotopic map; that is, neighboring retinal sites project to neighboring tectal sites. Although the spatial relations between the retina and tectum have been extensively studied, relatively little information exists concerning order in the optic nerve. Recent anatomical studies have shown that axons of neighboring retinal ganglion cells are often near each other in the nerve (*1*-*3*). This result has been interpreted as evidence that the nerve is retinotopically ordered, and, in addition, that the order was instrumental in the original establishment of connections (*4*). There are, however, reasons to doubt that the nerve is so simply ordered. Electrophysiological attempts to reveal retinotopic order have failed (*5*). Also, no evidence exists for the necessary consequence of the retinotopic hypothesis: that axons from widely separated retinal sites are separated in the nerve. Scholes has suggested, from the appearance of the retinas of cichlid fish, that axons near each other in the nerve originate from ganglion cells located in annuli centered on the optic disk (*2*). We have confirmed Scholes's predictions in the goldfish and conclude that the nerve is ordered, but chronologically rather than retinotopically.

We have used the histochemical marker horseradish peroxidase (HRP) to trace clusters of optic nerve axons to their retinal origins. A small amount of HRP, injected into the optic nerve just behind the eye, was taken up by a discrete group of axons that transported it both anterogradely to the tectum and retrogradely into the retina (*6*). A longitudinal section of an optic nerve which received such an injection is shown in Fig. 1a. Axons filled with HRP reaction product form a dense cluster surrounded by relatively clear areas. Figure 1b shows the whole retina, prepared as a flat mount, which was attached to this optic nerve. The HRP reaction product filled axons radiating from the optic disk and an annulus (arrow) of ganglion cell bodies. Filled axons were not found peripheral to the annulus, nor were filled ganglion cell bodies found either central or peripheral to it. (The other dark areas in Fig. 1 are blood vessels, most prominent peripheral to the annulus, and clumps of pigmented epithelium which stuck to the retina near the optic disk and margin.) Figure 1c is a view of the central edge of the annulus; individual ganglion cell bodies, some dendrites (single arrow) and axons leaving the annulus (double arrow) are filled with HRP reaction product.

In six cases in which HRP injection was confined to part of the optic nerve, one retina had a complete annulus of stained ganglion cells and five had partial annuli ($< 360°$ in circumference). The annuli varied in width and in distance from the optic disk. In one retina, two partial annuli at different distances from the disk were filled. Since the goldfish retina grows by adding new cells at the margin (*7*), the cells in each annulus are of similar age. Hence the annular labeling pattern indicates that axons of similar age cluster together in the optic nerve (*8*). The HRP injections tended to be confined by glial boundaries in the nerve. Since glial compartmentation of the optic nerve appears to be random (*9*, *10*), we suggest that each partial annulus resulted when the HRP was confined to a region which contained only a fraction of an age-related cluster of axons. The filled ganglion cells were always tightly grouped rather than scattered around the annulus, implying that within an age-related cluster of axons, those from neighboring ganglion cells were relatively near one another. This inference is consistent with previous reports that axons from neighboring places on the retina tend to stay together in the optic nerve of goldfish (*1*).

The results of our HRP injections do not allow us to determine if neighboring clusters of axons in the optic nerve arise from adjacent retinal annuli, although the ultrastructural appearance of the nerve itself suggests this. The optic nerves of small (young) goldfish contain a cluster of nonmyelinated axons. This cluster always lies adjacent to clusters containing slightly larger axons surrounded by thin myelin sheaths. Axons in more distant clusters are larger still and surrounded by even heavier myelin sheaths (*10*). We suggest (i) that this gradient of axon size and myelination results from the addition of new (nonmyelinated) axons near others recently added and (ii) that myelination of the axons has proceeded further in the older clusters. These suggestions are supported by several pieces of evidence. (i) Only a few scattered nonmyelinated axons are found in the optic nerves of large (old) goldfish (*10*); the absence of a cluster of nonmyelinated axons rules out their existence as a stable population and suggests that they are not efferent axons since efferents exist in both large and small goldfish. [In addition, Schmidt has recently shown that efferent axons in the goldfish optic nerve are large, myelinated axons (*11*).] (ii) Lesions of the peripheral retina disrupt only axons in the nonmyelinated cluster and in nearby clusters of thinly myelinated axons (*12*). This evidence strongly suggests that nonmyelinated axons are from ganglion cells at the peripheral margin of the retina. Lesions that damage axons from both peripheral and more central (older) retina disrupt axons in the nonmyelinated and thinly myelinated clusters

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