somewhat wider, that of *Vanadis* II a little narrower. The departures are not large enough to rule out a possible chemical relationship, yet neither do they support it.

The most striking departure from the Dartnall function is the large inflection at about 550 nm displayed by *Vanadis* I. It is tempting to suppose that this arises from the accessory retina, which in *Torrea* peaks in sensitivity near this wavelength. But why then is it not, as in *Torrea*, reversed in polarity?

In the little work we were able to do with *Vanadis*, we never observed a cornea-positive response. Yet is should be noted that even in *Torrea*, with its paired accessory retinas, it is not easy to place the active electrode over one of them so as to record the ERG through its base, hence with inverted polarity. In *Vanadis* this would be still more difficult, and in the short time at our disposal, such achievement eluded us. It seems reasonable to suppose that if the active electrode happened to be at some distance from the accessory retina, more or less across from it (see Fig. 9), it could pick up responses from the tips of the accessory retinal receptors as well as from those of the main retina, hence with the same polarity. That and its enormously greater sensitivity at long wavelengths might well permit the accessory retina to account for the inflection near 550 nm in the spectral sensitivity of *Vanadis* I.

We had also direct evidence that more than one photosensitive system functions in *Vanadis* I, in that the response on occasion changed its character markedly at different wavelengths. Thus in one experiment, whereas the response at 480 nm was a simple monophasic wave, that at 380 nm had a distinct inflection on the rising limb, as though a second, shorter-wavelength mechanism intruded. At another time a preparation that we had worked with for several hours and had treated with MS 222 suddenly began to display a very complex response to single flashes at both 380 and 580 nm; at the latter wavelength, however, this response alternated regularly with a single, very small cornea-negative blip. We have no idea what this meant, apart from its important implication that different mechanisms were at work at 380 and 580 nm. The curves for *Vanadis* I in Fig. 11A and Fig. 12A show that these wavelengths hit respectively the short- and long-wavelength inflections. It may well be that *Vanadis* I possesses three distinct photosystems, represented by the main peak at 460 nm, and the subsidiary inflections near 380 and 550 nm. We saw no comparable differences in the responses of *Vanadis* II at 380 nm and 560 nm.

We have already emphasized the extraordinary evolutionary convergence represented by the possession of accessory retinas in alciopid worms, and in deep-sea fishes and cephalopods. Our observations on *Vanadis* provide another remarkable instance of such convergence, in that our specimens taken at 300 m share with a wide range of other deep-sea organisms, including deep-sea fishes, visual sensitivity peaks in the blue, at 460 to 480 nm. These are the wavelengths of sunlight that penetrate most deeply into clear ocean water (Fig. 6). At a depth of 200 to 250 m the entire spectrum of sunlight is reduced to a band of blue light stretching from about 450 to 505 nm, and peaking in intensity at 460 to 480 nm (*13*). At still greater depths, and at night, organisms with eyes must depend for vision upon bioluminescence, particularly that of luminescent bacteria, and this, in turn, is concentrated primarily at the same wavelengths (*14*). A visual sensitivity peaking at 460 to 480 nm is therefore optimal for deep-sea life; and our deep-sea alciopids have achieved this aspect of fitness along with a wide array of deep-sea organisms belonging to other phyla that possess eyes.

Note added in proof: Since writing this report we learned of ERG measurements of spectral sensitivity in the relatively primitive cup-eye of another marine annelid worm, *Nereis* (*15*).

GEORGE WALD
STEPHEN RAYPORT
Biological Laboratories,
Harvard University,
Cambridge, Massachusetts 02138

References and Notes

1. A. Krohn, *Arch. Naturgesch.* **11**, 171 (1845); R. Greeff, *Nova Acta Kais. Leopold. Carol. Akad. Naturforsch.* **39** (No. 2), 35 (1876); V. Graber, *Arch. Mikrosk. Anat. Entwicklungsmech.* **17**, 243 (1880); N. Kleinenberg, *Z. Wiss. Zool.* **44**, 1 (1886); R. Hesse, *ibid.* **65**, 446 (1898–1899).
2. For present classification see P. V. Ushakov, *Fauna of the U.S.S.R.*, vol. 1, *Polychaetes* (Academy of Sciences, U.S.S.R., Leningrad, 1972), translated by E. D. Gordon with O. Theodor, Ed., Jerusalem, 1974 (available from National Technical Information Service, Springfield, Va.).
3. E. Béraneck, *Rev. Suisse Zool.* **1**, 65 (1893).
4. C. O. Hermans and R. M. Eakin, *Z. Morphol. Tiere* **79**, 245 (1974).
5. G. Wald, H. Griesinger, T. Cronin, M. Adamian, in preparation.
6. H. J. A. Dartnall, *Br. Med. Bull.* **9**, 24 (1953).
7. P. Ruck and T. L. Jahn, *J. Gen. Physiol.* **37**, 825 (1954).
8. M. Hanani and P. Hillman, *ibid.* **67**, 235 (1976).
9. R. D. DeVoe, *ibid.* **59**, 247 (1972).
10. (a) G. D. Lange and P. H. Hartline, *J. Comp. Physiol.* **93**, 19 (1974); (b) P. H. Hartline and G. D. Lange, *ibid.*, p. 37.
11. A. Brauer, *Verh. Dtsch Zool. Ges.* **12**, 42 (1902); V. Franz, *Biol. Zentralbl.* **27**, 271 (1907); O. Munk, *Dana Rep. Carlsberg Found. No. 70* (1966).
12. C. Chun, *Verh. Dtsch. Zool. Ges.* **13**, 67 (1903); *Deutsche Tiefsee-Expedition "Valdivia", vol. 18* (Gustav Fischer, Jena, 1910–1915; English translation by Israel Program for Scientific Translations, Jerusalem, 1975) (available from National Technical Information Service, Springfield, Va.).
13. B. Lundgren and J. Hojerslev, *Copenhagen Univ. Rep. Inst. Phys. Oceanogr.* **14**, 1 (1971); cited by T. H. Waterman in N. G. Jerlov and E. S. Nielsen, Eds., *Optical Aspects of Oceanography* (Academic Press, New York, 1974), p. 420; N. G. Jerlov, *Marine Optics* (Elsevier, Amsterdam, 1976), chap. 10.
14. See T. H. Waterman [in N. G. Jerlov and E. S. Nielsen, Eds., *Optical Aspects of Oceanography* (Academic Press, New York, 1974), chap. 18] on underwater light and animal vision; and B. P. Boden and E. M. Kampa (in *ibid.*, chap. 19) on marine bioluminescence. For a discussion of the evolutionary implications of these arrangements see G. Wald, *Wistar Inst. Symp. Monogr. No. 5* (1967), p. 59.
15. D. R. Yingst, H. R. Fernandez, L. G. Bishop, *J. Comp. Physiol.* **77**, 225 (1972).
16. This investigation was supported in part with funds from the NSF and NIH. We are grateful to the staff of the Stazione Zoologica for many kindnesses and much help, particularly to E. Muzii, and to its director, A. Monroy. We also thank H. Griesinger of our Harvard laboratory for Figs. 2, 8, and 9B; and T. Cronin for species identifications.

17 January 1977

Laser Fluorimetry: Subpicogram Detection of Aflatoxins Using High-Pressure Liquid Chromatography

Abstract. *The use of high-pressure liquid chromatographic separation in conjunction with laser-induced fluorescence detection permits the analysis of trace fluorescent species at new limits of sensitivity. This technique was applied to the carcinogens aflatoxins B_1, B_2, G_1, and G_2, which were linearly quantitated to 7.5×10^{-13} gram. The procedure consists of forming more fluorescent aflatoxin derivatives, eluting the aflatoxins from a reverse-phase column, focusing the 325-nanometer output of a helium-cadmium ion laser into a suspended droplet of the eluent, and measuring the resulting fluorescence using phase-sensitive detection.*

The well-documented potency of aflatoxins, carcinogenic mold metabolites, as causative agents for tumor formation in laboratory animals gives rise to a need for extremely sensitive and selective detection methods in food products at the level of a few parts per billion (ppb) (*1–3*). We report here the development of an analytical method for detecting ultratrace amounts of fluorescent species, based on the use of high-pressure liquid chromatographic separation followed by

laser-induced fluorescence detection. The technique described here has a linear response of more than three orders of magnitude and a lower detection limit of 750 fg (750 × 10^{-15} g) for the four commonly occurring aflatoxins (B_1, B_2, G_1, and G_2). This limit of detection might be compared to about 1 ng (1 × 10^{-9} g) for high-pressure liquid chromatography (HPLC) with ultraviolet (UV) absorption detection (*4*) and about 200 pg (200 × 10^{-12} g) for thin-layer chromatography (TLC) with time-resolved laser-induced fluorescence (*5*). A 4-μl suspended droplet of solvent eluting from the column (eluent) serves as a "windowless" fluorescence cell. The peak signal at the detection limit corresponds to 7 fg of aflatoxin in the eluent droplet, illustrating the capability of this technique for microanalysis. We also report the application of this method for determination of aflatoxin B_1 in yellow corn extracts to 2 ppb.

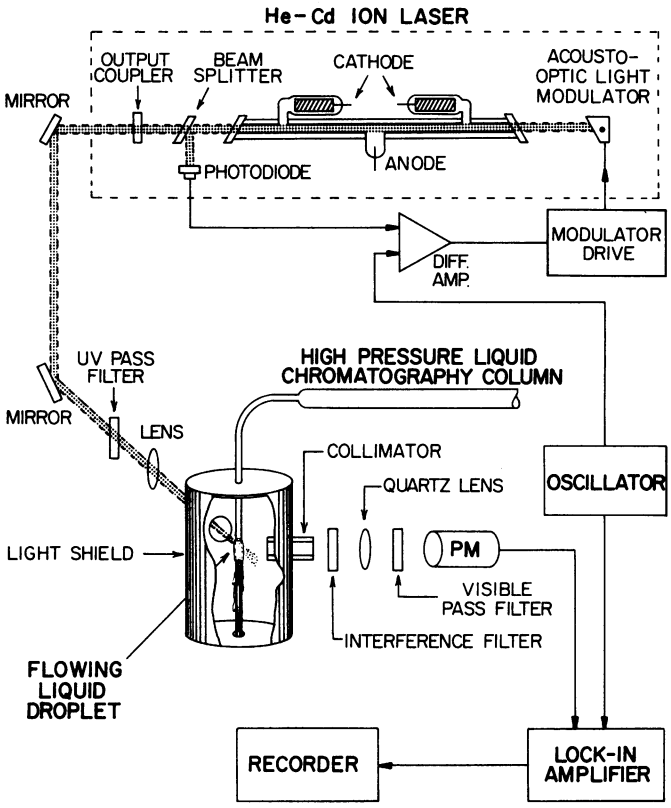


Fig. 1. Diagram of the HPLC laser fluorescence detector. A droplet of eluent from the chromatographic column flows under gravity in the gap (2 mm) between a stainless steel tube and a solid rod to form a windowless fluorescence cell. The 325-nm output of a He-Cd ion laser (Liconix model 405UV) passes through a filter to remove visible background emission from the plasma tube and is then focused to a small spot inside the droplet. The fluorescence is detected by a photomultiplier (Centronics 4249BA), which views the droplet through several filters. The laser output is amplitude-modulated, and an internal feedback loop, consisting of a photodiode, difference amplifier, and acousto-optic light modulator, reduces the root-mean-square noise of the laser and ensures that the laser power is directly proportional to the applied oscillator voltage. The signal from the photomultiplier which is in phase with the oscillator is detected by a lock-in amplifier, whose output drives a stripchart recorder.

The laser fluorescence detector is shown schematically in Fig. 1. The 325-nm line of a He-Cd ion laser (8 mw) excites fluorescence in a flowing droplet of eluent suspended between a piece of stainless steel tubing 0.159 cm in diameter and a solid rod of the same diameter. Focusing the beam to a small spot inside the droplet avoids direct scattering of the laser light into the detection optics. The liquid droplet forms a windowless fluorescence cell (4 μl), which avoids the problem of fluorescence from cell walls irradiated with intense UV light. The eluent, after passing through the laser beam, flows down the outside of the solid rod and is discarded. The laser beam is 100 percent modulated at 50 khz and the photomultiplier output is detected in phase with the modulation frequency.

By far the most frequently used method for aflatoxin detection is TLC. The higher separation efficiency and rapidity of analysis characteristic of HPLC suggest that its use would be advantageous. Several reports of normal-phase HPLC in conjunction with UV absorption de-

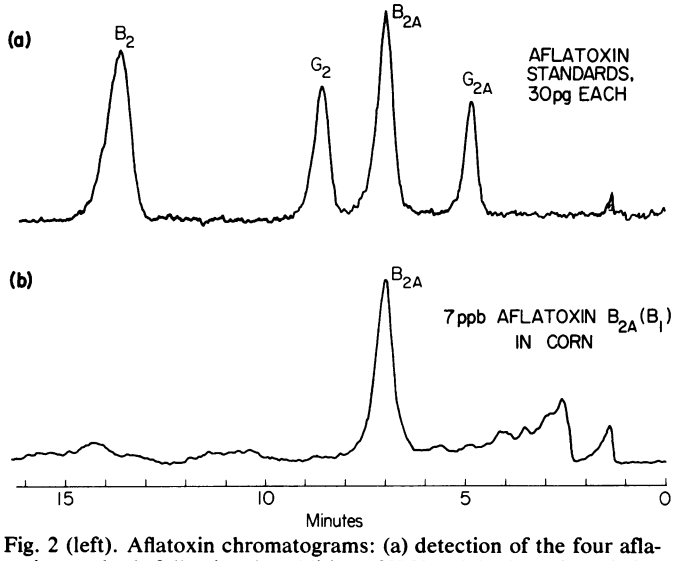
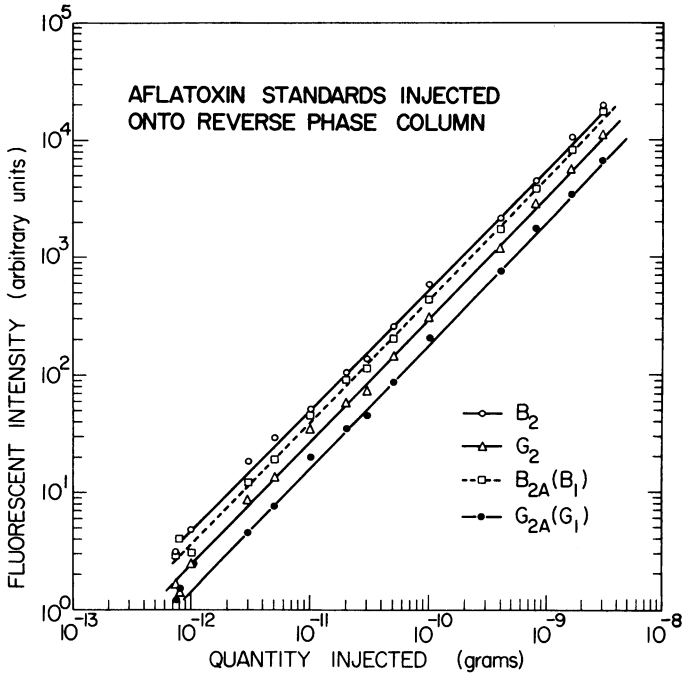


Fig. 2 (left). Aflatoxin chromatograms: (a) detection of the four aflatoxin standards following the addition of HCl and (b) detection of aflatoxin B_1 in corn extract by the same procedure, using a 10-μl sample injection. The chromatogram represents 45 pg of B_1 in the injected sample. Fig. 3 (right). Linear quantitation of the four aflatoxin standards and their derivatives, using laser fluorimetry. Each data point represents a separate injection onto the column.



tection have appeared in the literature (*4*, *6–8*). While adequate resolution of the aflatoxins is readily achieved, the detection sensitivity has been in the low nanogram range. The more sensitive technique of fluorescence detection, while successful for aflatoxins in the solid state, has met with difficulty in HPLC (*9*), since aflatoxin B_1, the most important from a toxic and carcinogenic viewpoint, has a poor fluorescence quantum yield in solution (*10*, *11*). A novel technique developed by Thorpe and Stoloff (*12*) obviates this problem. By the addition of trifluoroacetic acid (TFA), aflatoxin B_1 is converted to aflatoxin B_{2a} (*13*), which is known to be highly fluorescent in hydrogen-bonded solvents (*14*). Aflatoxin G_1 is also transformed into G_{2a}, but B_2 and G_2 are unaltered (*15*). All four aflatoxins can then be separated on a reverse-phase HPLC column and quantitated fluorimetrically.

We have adopted this procedure but have substituted hydrochloric acid for TFA to make the derivatives of B_1 and G_1, since TFA causes additional unidentified peaks to appear in the chromatogram. Our method consists of drying the aflatoxin-containing extract or standard under a stream of nitrogen, adding a few drops of 1*N* HCl, allowing 15 minutes for reaction, evaporating under nitrogen on a steam bath, and finally, redissolving the sample in elution solvent. This procedure is reported (*16*) to have a high yield (> 90 percent) of aflatoxin B_{2a} and G_{2a}, which is consistent with our observation of a single peak per aflatoxin eluting from the column. Mixtures of water and ethanol (75:25 by volume) are used to elute the aflatoxins on a μBondapak C_{18} column (*17*). The resolution as well as the retention time of the aflatoxins can be varied by changing the proportions of water and ethanol. Both solvents were of high purity (*18*).

Figure 2 shows some typical aflatoxin chromatograms obtained by using the above procedure and the laser fluorescence detection scheme illustrated in Fig. 1. Figure 2a represents 30 pg each of the derivative aflatoxins G_{2a} and B_{2a} and the aflatoxins G_2 and B_2. The absence of secondary peaks and the lack of baseline fluctuations are noteworthy. Figure 2b is a chromatogram of an extract prepared (*19*) from 10 g of yellow corn known to contain 7 ppb of aflatoxin B_1. At present, the limit of detection in the corn sample with this method is approximately 2 ppb of aflatoxin B_1, the sensitivity being limited by interferences from other compounds in the corn extract. However, with better cleanup procedures it is expected that this detection limit can be further reduced.

In quantitating aflatoxin contamination it is important to establish the linear dynamic range of the detector. By injecting known quantities of appropriately diluted standards and their derivatives onto the column we have established a linear response of more than three orders of magnitude, as shown in Fig. 3. Scatter in these data, especially at higher signal levels, is largely a result of inaccurate graphical integration of the peaks. The signal-to-noise ratio at the lower limit of detection, 750 fg, was roughly 2 to 1 with a 3-second time constant on the lock-in amplifier.

Since the aflatoxins are considerably diluted as they elute from the column, it is of interest to determine what quantity of aflatoxin is in the detection volume itself. We assume that the peak has a Gaussian profile, $f(x) = (2\pi)^{-1/2}\ \sigma^{-1} \exp(-x^2/2\sigma^2)$, where 3.56 σ represents the full width of the peak at half-maximum intensity (FWHM). At the detection limit, the FWHM is 0.4 minute at a flow rate of 1.5 ml/min, giving a value of $\sigma = 0.17$ ml. The normalization of a Gaussian profile is such that the integral of $f(x)$ over all x is unity. Hence, the concentration in femtograms per milliliter at the detection limit is $C(x) = 750 f(x)$, and the aflatoxin concentration at the maximum of the peak is $C(0) = 1800$ fg/ml ($6 \times 10^{-12} M$). Thus the amount of aflatoxin in the 4-μl detection volume is only 7 fg or about 1×10^7 molecules at the signal maximum, and the amount of aflatoxin in the laser beam is even less. Although still higher detection sensitivities have been obtained in gas-phase laser-induced fluorescence (*20*), the present study illustrates that laser fluorimetry of condensed media in conjunction with chromatographic separation can detect and quantitate extremely low levels of fluorescent species.

GERALD J. DIEBOLD
RICHARD N. ZARE
Department of Chemistry, Columbia University, New York 10027

References and Notes

1. L. A. Goldblatt, *Aflatoxin: Scientific Background, Control, and Implications* (Academic Press, New York, 1969).
2. W. H. Butler, in *Mycotoxins*, I. F. H. Purchase, Ed. (Elsevier, Amsterdam, 1974), pp. 1–28.
3. R. C. Shank, in *Mycotoxins and Other Fungal Related Food Problems*, J. Rodricks, Ed. (American Chemical Society, Washington, D.C., 1976).
4. W. A. Pons, *J. Assoc. Offic. Anal. Chem.* **59**, 101 (1976).
5. M. R. Berman and R. N. Zare, *Anal. Chem.* **47**, 1200 (1975).
6. G. H. R. Rao and M. W. Anders, *J. Chromatogr.* **84**, 402 (1973).
7. J. Sieber and D. Hsieh, *J. Assoc. Offic. Anal. Chem.* **56**, 827 (1973).
8. R. C. Garner, *J. Chromatogr.* **103**, 186 (1975).
9. L. Seitz, *ibid.* **104**, 81 (1975).
10. J. A. Robertson, W. A. Pons, Jr., L. A. Goldblatt, *J. Agric. Food Chem.* **15**, 798 (1967).
11. J. Chelkowski, *Photochem. Photobiol.* **20**, 279 (1974).
12. W. Thorpe and L. Stoloff, in *89th Annual Meeting of the Association of Official Analytical Chemists* (Association of Official Analytical Chemists, Washington, D.C., 1975), abstr. 58.
13. G. Büchi, D. M. Foulkes, M. Kurono, G. F. Mitchell, *J. Am. Chem. Soc.* **88**, 4534 (1966).
14. K. K. Maggon, S. Gopal, L. Viswanathan, T. A. Venkitasubramanian, S. Rathi, *Ind. J. Biochem. Biophys.* **9**, 195 (1972).
15. W. Przybylski, *J. Assoc. Off. Anal. Chem.* **58**, 163 (1975).
16. A. E. Pohland, M. E. Cushmac, P. J. Andrellos, *ibid.* **51**, 907 (1968).
17. All chromatographic equipment is from Waters Associates, Inc., Milford, Mass.
18. A. B. Bradley and R. N. Zare, *J. Am. Chem. Soc.* **98**, 620 (1976).
19. L. M. Seitz and H. E. Mohr, *Cereal Chem.*, in press.
20. R. N. Zare and P. J. Dagdigian, *Science* **185**, 739 (1974).
21. We thank L. M. Seitz (U.S. Grain Marketing Research Center, Manhattan, Kan.), who supplied us with corn extracts and aflatoxin standards, and J. B. Kinney (summer undergraduate research assistant), who helped us in the development of the detection apparatus. This investigation was supported by National Cancer Institute grant 1-R01-CA-18271-01.

20 December 1976; revised 26 January 1977

Monomeric Forms of the Acid Ionophore Lasalocid A (X-537A) from Polar Solvents

Abstract. *X-ray structural analyses have been carried out on the free acid of lasalocid A (X-537A) and on the sodium salt, both crystallized from methanol solution. In each case the structure is monomeric with one molecule of methanol complexing to the free acid and to the salt.*

Among acid ionophores, lasalocid A (X-537A) (1) has several unique features and has been the subject of many chemical and biochemical studies. Since the initial establishment (*1*) of the detailed structure and stereochemistry of lasalocid, x-ray studies have been carried out on the barium salt (*1*, *2*) and the silver salt (*3*) of lasalocid, the silver salt of 5-nitrolasalocid (*4*), two forms of the sodium salt of 5-bromolasalocid obtained from acetone and from carbon tetrachloride solutions (*5*), and the free acid of 5-bromolasalocid hemihydrate that had been crystallized from a hexane–methylene chloride solution (*6*). In these crystals, the conformation of the backbone of the ionophore has been essentially in-