ture relationships, but only between 14 and 23°C. At temperatures between 23 and 35°C the heart rates of the winter toads did not increase when their body temperatures were increased. Seasonal differences in heart rate-temperature relationships had been reported for excised frog hearts and shown to be linked to seasonal changes in thyroid and anterior pituitary activities. The similar seasonal differences in heart rate-temperature relationships shown here in intact toads may also reflect endocrine changes. This working hypothesis remains to be tested directly.

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Purification and Toxicity of Aflatoxin G1.* (31428)

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There has been increasing recognition of the important role played by fungal metabolites as acute and chronic toxins in animals with possible implications in man. Attention has been focused particularly on the aflatoxins and much work has been done on their effects in animals(1,2) and on their chemistry. The structures of 4 of these coumarin derivatives have been established(3,4). In the course of our preparation of substantial quantities of some of these compounds from a mixture of aflatoxins derived from *Aspergillus parasiticus* grown on groundnuts, several interesting observations have been made and a reliable method for analyzing the crude mixtures has been developed.

Materials and methods. The aflatoxin mixtures (prepared by Dr. K. Sargeant, M.R.E., Porton) were resolved on thin layer (TLC) plates of silica gel H (Kensington Scientific Co., Oakland, Calif.) 20 × 20 cm, 1 mm thick for the preparative work, or of silica gel G, 5 × 20 cm, 0.5 mm thick for the analytical studies. The material was applied in chloroform solution as a thin streak 2 cm from the bottom of the plate. The developing solvent was chloroform-diethyl ether-acetic acid 2:2:1, which took a little more than 1 hour to ascend the plate; if separation of the components was not adequate, the plate was removed, allowed to dry for 30 minutes and redeveloped in the same solvent. At this stage the several fluorescent bands were separated by non-fluorescent zones. Each fluorescent band was scraped from the plate and placed in either a centrifuge tube or a flask (depending on the quantity of silica gel). For direct spectrometric estimation of the aflatoxins using standard absorptivity values (4) adsorbed fluorescent material was eluted with ethanol-chloroform 3:1. For preparation of aflatoxins the adsorbed material was eluted thrice with acetone-chloroform 1:1, followed by evaporation of the solvent. After repeated chromatography the separated components were sufficiently pure to be crystallized from chloroform by addition of methanol. The purity of each chystalline product was determined by analysis of 0.5 to 1 mg on a 5 × 20 cm TLC plate.

Acute toxicity determinations were made using white Pekin ducklings (average weight 57 g) collected from the breeders. These were given graded doses of the aflatoxins by mouth within 24 hours of hatching. The aflatoxins were dissolved in dimethylformamide (DMF), maximum volume 0.15 ml, and given by mouth before the ducklings were offered food. No deaths were recorded in control animals; the only effect was a diminished growth rate if the ducklings were given 0.20 ml or more DMF. At the doses used

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the growth curves of the control animals given DMF were similar to those of untreated animals.

As a preliminary to the biological testing in rats of purified aflatoxins B1 and G1 by feeding in drinking water it was necessary to examine the stability of the two compounds in aqueous solution. A saturated solution of the aflatoxin in water was divided into two portions. One was kept in the dark, the other in light (on a window ledge). After 1 and 9 days both solutions of each aflatoxin were examined, both by direct measurement of U.V. absorbance of the aqueous solution and by extraction of 50 ml of aqueous solution with 10 ml of chloroform, followed by taking of the absorption spectrum of the chloroform solution (it was observed that the aqueous portion remained fluorescent even after removal of the measurable aflatoxin by several extractions with chloroform). There was little difference in absorbance between the aqueous solutions of either B1 or G1 kept in the dark or in the light. To ascertain whether decomposition had occurred, nevertheless, the chloroform extracts were evaporated to dryness and chromatographed on thin layer plates of silica gel G (as described in the assay procedure). Each fluorescent band observed was scraped from the plate, the adsorbed material eluted and the absorption spectrum taken.

Results and discussion. After purification by TLC and crystallization, aflatoxins B1 and G1 with a purity exceeding 99.0% (as determined by thin layer chromatography) were obtained. Three other compounds, B2, G2 and another with R_f lower than that of aflatoxin G2 were separated and crystallized; the quantity of these isolated was small. Several minor fluorescent zones of lower R_f could not be identified and were not isolated.

It was observed that, while zones B1, B2 and G2 were blue fluorescent in ultraviolet light, zone G1 presented the picture of green fluorescence at the leading edge, but blue fluorescence in the region of lower R_f. The green fluorescent portion of the zone coincided with a yellow color in visible light. This indicated strongly that aflatoxin G1 is not a green fluorescent compound, but that the green color is due to the presence of a yellow impurity. This was confirmed when it was found that the yellow color tended to be retained on the silica gel when the aflatoxin was eluted with chloroform-acetone. Repeated chromatography of the aflatoxin G1 zone resulted in a diminution of the green fluorescence until the chromatogram showed only a light blue fluorescence; at this stage the zone was quite colorless in visible light. Examination of the NMR spectra of the blue fluorescent G1 and the green fluorescent G1 (using a Varian HA-100 Spectrometer) showed that, in the latter, in addition to the peaks corresponding to aflatoxin itself, at least 3 absorptions were present in the alkyl region. These included apparent singlets at 2.06 and 1.25 ppm and a broad unresolved multiplet at 1.71 ppm. In the spectrum of the blue fluorescent compound the small singlet at 2.06 ppm appeared to be absent. The difference between the two spectra is consistent with the presence of a small proportion of an impurity in the green fluorescent G1 sample.

It was possible that some of the material removed in the chromatographic procedure was partly responsible for the toxic effect of aflatoxin G1. Therefore, the toxicity of this 'blue' G1 was compared with that of another sample crystallized from the green fluorescent portion of the chromatogram.

In the acute toxicity experiments the ducklings dying within 6 days were recorded and the LD_{50} estimated by the method of Weil (5). The results obtained from these estimations were: aflatoxin B1, 17.5 μg (14.3-21.57) or 0.335 mg/kg; green aflatoxin G1, 54.08 μg (44.25-66.36) or 0.95 mg/kg and blue aflatoxin G1, 45.7 μg (28.3-74.0) or 0.785 mg/kg. As can be seen the LD_{50} of both the blue and green G1 fell within each fiducial limit and are distinct from the limits of the aflatoxin B1. The liver lesions produced by the blue and green G1 are similar.

The results of similar tests by Asao *et al*(3) were: LD_{50} for aflatoxin B1 of 28.2 μg/duckling (50 g) and for aflatoxin G1 of 90 μg/duckling. These are 2-day estimates using propylene glycol as the solvent. Also Carnaghan *et al*(6) reported figures for B1

TABLE I. Quantitative Analysis of Aflatoxin Mixtures.

Crude aflatoxin sample No.	36		39		40		41		41	
Applied to T.L.C. plate	1010 μg		960 μg		1020 μg		1140 μg		1120 μg	
Total recovered	1060 μg		975 μg		1000 μg		1130 μg		1090 μg	
	μg	%	μg	%	μg	%	μg	%	μg	%
B1	432	41	342	35	348	34	504	44	490	44
B2	118	11	53	5.5	49	4.8	61	5.3	67	6
G1	460	43	550	56	570	56	525	46	503	45
G2	44	4.1	31	3.1	36	3.5	40	3.5	27	2.5
Band 5	4	.3	—	—	—	—	4	.3	8	.7
Band 6	8	.7	—	—	—	—	—	—	—	—
Band 7	—	—	—	—	—	—	—	—	—	—
		100.1		99.6		98.3		99.1		98.2

and G1 of 18.2 μg and 39.2 μg for 50 g Khaki Campbell ducklings respectively using DMF in a solvent. These figures were calculated from deaths occurring within 6 days. As can be seen the results reported here compare favorably with those previously reported.

Since there is no observable difference in acute toxicity, it appears that aflatoxin G1 is a blue fluorescent compound, not green fluorescent and that the green fluorescence often observed is due to the presence of a small proportion of yellow impurity. (In our studies portions of the aflatoxin B1 zone occasionally appeared green fluorescent due to the presence of a yellow compound.) Reliance on the green fluorescence as a means of indentification of aflatoxin G1 can, therefore, be misleading, since it is possible to have a G1 zone on a chromatogram that is not green fluorescent.

As a result of our experience with fluorescence, this was not considered reliable as a means of identification and estimation of aflatoxins. Because of its susceptibility to interferences(7) fluorescence should be used only with great caution for quantification. In our studies with aflatoxins there was no relationship between the size and intensity of a fluorescent spot and the quantity of material in it. Consequently, in our analysis of aflatoxin mixtures absorption spectrometry was used for the estimation of concentration of the components.

The results of analysis of approximately 1 mg aliquots of each aflatoxin mixture are given in Table I. On the TLC plate 7 bands were usually discerned. The 4 with highest R_f were, in order, B1, B2, G1 and G2. Band 5 was usually a prominent one, but of unknown structure. The concentrations of the compounds in bands 5, 6 and 7 were calculated using the absorptivity value for aflatoxin G1. In the few cases where the separation of B2 and G2 from the contiguous bands was not complete, they were rechromatographed alone and the procedure repeated.

In the studies of the stability of aflatoxins B1 and G1 in water it appeared that while little decomposition had occurred in the solutions kept in the dark, even after 9 days, considerable decomposition had taken place in the solutions kept in the light even after 24 hours. After 9 days in the light many bands were observed (9 in the B1 solution, 5 in the G1 solution) and there remained little, if any, of either aflatoxin B1 or G1. The results of the experiments are shown in Table II (all of the decomposition products were estimated at 363 mμ as the respective aflatoxins).

Therefore, it appears that aflatoxins are quite labile in light in aqueous solution, as well as when refluxed in methanol(8) or autoclaved in steam(9), whereas it is fairly stable in aqueous solution in the dark. This led us to carry out our feeding experiments with aflatoxins in drinking water in brown bottles, the animals being given the solutions only overnight.

Summary. A method for quantitative analysis of crude aflatoxin mixtures is described, using thin layer chromatography and absorption spectrometry. Highly purified aflatoxin G1 has been prepared by thin layer

TABLE II. Decomposition of Aflatoxins B1 and G1 in Aqueous Solution.

	B1		G1	
	Initial conc—1220 μg/100 ml		Initial conc—816 μg/100 ml	
1-day exp	Dark	Light	Dark	Light
Concentration from absorbance of aqueous solution	*1220 μg/100 ml	*1130 μg/100 ml	†816 μg/100 ml	†760 μg/100 ml
Proportion of original aflatoxin remaining as estimated by T.L.C.	97%	62%	98%	60%
Proportion of decomposition products	1 Band—3%	2 Bands—38%	1 Band—2%	2 Bands—40%
	Initial conc—1260 μg/100 ml		Initial conc—1000 μg/100 ml	
9-day exp	Dark	Light	Dark	Light
Concentration from absorbance of aqueous solution	*1215 μg/100 ml	*815 μg/100 ml	†960 μg/100 ml	†670 μg/100 ml
Proportion of original aflatoxin remaining as estimated by T.L.C.	95%	3%	96.5%	8%
Proportion of decomposition products	2 Bands—5%	8 Bands—97%	3 Bands—3.5%	4 Bands—92%

* Estimated as aflatoxin B1 from absorption at 363 mμ.
† Estimated as aflatoxin G1 from absorption at 363 mμ.

chromatography and is a blue fluorescent compound, not, as previously reported, green fluorescent. The toxicity of the purified compound does not differ from that of the less pure compound containing a yellow impurity. Both aflatoxins B1 and G1 are fairly stable in aqueous solution in the dark, but undergo rapid decomposition in light (40% in 1 day; almost 100% after 9 days).

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