fectiveness of dichloromethane is presumably due to its free miscibility with polar and apolar solvents and its limited solubility in water.

The use of dichloromethane opens up new possibilities of treating dry seeds with stimulators or inhibitors of germination. A stimulator would be supplied to dry seeds if it were desired to accelerate germination. A water-soluble inhibitor would be supplied if it were desired to delay seed germination until suitable conditions occur, such as the presence of adequate rainfall. This would be useful if the inhibitor were slowly washed out of the seeds, as seems to occur with endogenous inhibitors in certain cases (*3*).

An additional use of permeating dry seeds with chemicals would be to study the metabolism of exogenously applied compounds. At present no reliable data are available on the metabolism of dry seeds.

HANNA MEYER
A. M. MAYER
Department of Botany, The Hebrew University, Jerusalem, Israel

References and Notes

1. A. M. Mayer and A. Poljakoff-Mayber, *The Germination of Seeds* (Pergamon, New York, 1963).
2. D. Chen, personal communication.
3. F. W. Went, *Experimental Control of Plant Growth* (Chronica Botanica, Waltham, Mass., 1957), pp. 248–251.
4. Supported in part by grant FG-Is-239 from the U.S. Department of Agriculture under P.L. 480 to A.M.M. and A. Poljakoff-Mayber.

22 September 1970; revised 30 November 1970 ■

Aflatoxin P_1: A New Aflatoxin Metabolite in Monkeys

Abstract. *A new phenolic derivative of aflatoxin B_1, appearing mainly in conjugated form, was identified as the principal urinary metabolite of aflatoxin B_1 in rhesus monkeys. Its identification in human urine might facilitate estimation of aflatoxin exposure in human populations.*

Aflatoxin B_1 has many interesting biological and biochemical properties (*1*, *2*), including high potency as a hepatotoxic and hepatocarcinogenic agent in animals (*3*). Different species respond differently to the toxin (*1*), but susceptibility of man is unknown. Human exposure sometimes occurs (*4*, *5*), and evidence presented in case reports implicates aflatoxin in acute poisoning (*6*) and supports the hypothesis that aflatoxin exposure is associated with elevated incidence of primary liver cancer in certain populations (*7*).

Detailed knowledge of metabolism of the toxin is essential for understanding which aspects of the metabolism determine response and also for extrapolation of animal data in estimating human response. We have therefore been investigating the comparative metabolism of aflatoxin B_1 in the rat and monkey, and report here the isolation and chemical identification of a new metabolite from the urine of rhesus monkeys. The metabolite is the phenolic product of *O*-demethylation of aflatoxin B_1 and is present in urine mainly in conjugated form. Because it is a phenol, we propose the trivial name aflatoxin P_1. The major urinary metabolite of aflatoxin B_1, it represents the excretory form of about 20 percent of a single dose within 24 hours after injection.

Male rhesus monkeys (2.5 to 4.0 kg) were provided by and housed at the New England Regional Primate Research Center, Southborough, Massachusetts. Each animal was given a single intraperitoneal injection of a mixture that contained 5 μc [^{14}C]aflatoxin B_1, labeled only in the ring carbons, and nonradioactive aflatoxin B_1 (0.4 mg per kilogram of body weight), dissolved in 0.8 ml of dimethylsulfoxide.

Urine was collected continuously for 96 hours. Samples were processed according to the following scheme, with all procedures being carried out in either darkness or subdued light in order to minimize photolysis of aflatoxin metabolites. All extractions were performed with equal volumes of urine and solvent. Urine was successively extracted seven times, first with a mixture of methanol and chloroform (6 : 4, by volume), then six times with chloroform alone. The seven extracts were pooled, reduced in volume, and finally evaporated to dryness under nitrogen at room temperature (fraction 1). By this procedure we recovered 15 percent of total urine radioactivity.

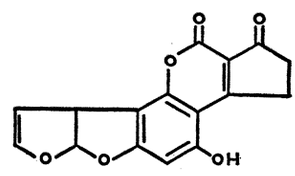

Fig. 1. Structure of aflatoxin P_1.

The remaining aqueous phase contained most (85 percent) of the total radioactivity in the urine, which we presumed to be conjugates of aflatoxin metabolites that were insoluble in chloroform. Major components of this fraction were separated and isolated as follows. The aqueous phase was recycled through an Amberlite XAD-2 (Rohm and Haas) column (4 by 50 cm) for 1.5 hours. This column, originally used for isolating steroid conjugates (*8*), retained 95 percent of the radioactivity contained in the aqueous phase. Radioactivity retained by the column was completely eluted with 1.5 liters of methanol, and the eluate was reduced to dryness (fraction 2). Two further procedures were used to isolate individual components.

In the first method, in view of the activities of the hydrolytic enzymes β-glucuronidase and sulfatase, we redissolved the residue in fraction 2 in 0.2*M* acetate buffer, *p*H 5.0, and incubated it with β-glucuronidase (Ketodase, Warner-Chilcott) for 48 hours at 37°C. The incubation mixture was extracted as described above; the extract, fraction 3, contained 60 percent of the radioactivity of fraction 2.

The aqueous residue remaining after the above treatment was reconstituted to its original volume with acetate buffer. Sulfatase type III (Sigma) was added, and the mixture was again incubated at 37°C for 48 hours. The incubation mixture was extracted and concentrated as described above; the product (fraction 4) contained 12 percent of the initial radioactivity of fraction 2.

The second method of separating components of fraction 2 consisted of column chromatography on diethylaminoethyl (DEAE) Sephadex. The procedures were adapted from those originally devised to separate certain groups of phenolic conjugates (*9*). This method avoided the interference by steroid conjugates that proved troublesome in the enzymatic procedure. A DEAE-Sephadex A-25 column (1.5 by 80 cm) eluted with a linear NaCl gradient (0.0 to 0.8*M*) was used to separate the radioactivity in fraction 2 into four subfractions. The major peak from this column contained 60 percent of the radioactivity of fraction 2; enzymatic hydrolysis with β-glucuronidase and solvent extraction suggested that most (90 percent) of the material in this subfraction was a glucuronide. In-

terfering fluorescent compounds were removed by silica-gel chromatography, by the procedure (*10*) for isolation of aflatoxin M_1. Aflatoxin P_1 was found in this fraction, free of interfering compounds (fraction 5).

The chloroform-soluble fractions (1, 3, 4, and 5) were subjected to thin-layer chromatography (*11*). When viewed under ultraviolet light, all the fractions were found to contain a yellow-green fluorescent compound with an R_F value different from authentic aflatoxins B_1, M_1, or the hemiacetal derivative of aflatoxin B_1. Autoradiography on x-ray film revealed a single radioactive spot that coincided with the fluorescent substance.

On the basis of this evidence, we concluded that all of the fractions contained a common metabolite, present in fraction 2 principally as a glucuronide and to a lesser extent as a sulfate. In order to accumulate sufficient quantities of this substance for chemical identification, three male rhesus monkeys weighing 3.0 to 4.4 kg were dosed once weekly for 10 weeks with aflatoxin B_1 (0.2 mg per kilogram of body weight). Urine was collected for 24 hours after each injection, and pooled samples were subjected to extraction and purification as above.

The isolated compound had ultraviolet absorption maxima (in ethanol) at 267 and 362 nm; in ethanol-sodium hydroxide, the spectrum showed shifts in the maxima to 298, 337, and 420 nm. The mass spectrum resembled that of aflatoxin B_1, offset by 14 mass units, and indicated that the compound had a molecular weight of 298. Methylation of the metabolite with diazomethane produced a product having mass spectrum, ultraviolet spectrum, and thin-layer chromatographic behavior identical with those of an authentic sample of aflatoxin B_1. All of these properties are consistent with the conclusion that the compound is the phenol produced by demethylation of aflatoxin B_1. The structure of aflatoxin P_1 is shown in Fig. 1.

The existence of this metabolic product was predicted by our earlier observations on the metabolism of methoxy-labeled aflatoxin in the rat (*12*), which indicated that *O*-demethylation was a major route of metabolism of the toxin. The reason other investigators have not found aflatoxin P_1 in urine is probably that only a small fraction of the total amount in urine is present in unconjugated, chloroform-soluble form. Previous investigators have concerned themselves almost exclusively with that fraction of urinary extracts containing chloroform-soluble materials.

Our experiments indicate that aflatoxin P_1 represents at least 60 percent of the urinary aflatoxin derivatives; of this, about 50 percent is present as glucuronide, 10 percent as sulfate, and 3 percent as unconjugated phenol. Together, these represent more than 20 percent of an injected dose of aflatoxin B_1. Aflatoxin M_1 (4-hydroxyaflatoxin B_1), the only other known metabolite, accounted for only 2.3 percent of the dose administered to these monkeys.

Aside from their value in interpreting species differences in response, these findings have potential use in estimating aflatoxin exposures of human populations. At present, epidemiologic surveys dealing with relations between liver cancer and aflatoxin in foods rely on dietary surveys that involve systematic collection of food samples, analysis for aflatoxin content, and calculation of ingestion by estimation of food intake. Such surveys yield only semiquantitative information on group (rarely individual) exposure, and are expensive to apply to large populations.

If aflatoxin P_1 is a urinary metabolite of aflatoxin B_1 in man and its excretion is quantitatively related to aflatoxin B_1 intake, then exposure to the toxin could be estimated by analysis of urine samples. Such analysis has been attempted, with amounts of aflatoxin M_1 in urine being used as the index of ingestion (*5*), but the low recovery (1 to 4 percent) prohibited quantitative estimation of toxin intake. If man excretes aflatoxin P_1 in quantities comparable to those excreted by the monkey, this approach may be epidemiologically feasible.

JOHN DALEZIOS
GERALD N. WOGAN
Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge 02139
STEVEN M. WEINREB
Department of Chemistry

References and Notes

1. W. H. Butler, in *Aflatoxin*, L. A. Goldblatt, Ed. (Academic Press, New York, 1969), pp. 223–236.
2. G. N. Wogan, *ibid.*, pp. 151–186.
3. ———, *Bacteriol. Rev.* **30**, 460 (1966).
4. ———, *Fed. Proc.* **27**, 932 (1966).
5. T. C. Campbell, J. P. Caedo, Jr., J. Bulatao-Jayme, L. Salamat, R. W. Engel, *Nature* **227**, 403 (1970).
6. A. Serck-Hanssen, *Arch. Environ. Health* **20**, 729 (1970).
7. H. F. Kraybill and M. B. Shimkin, *Advan. Cancer Res.* **8**, 191 (1964).
8. H. L. Bradlow, *Steroids* **11**, 265 (1968).
9. R. Hahnel and A. Rahman, *Biochem. J.* **105**, 1047 (1967); R. Hobkirk, P. Musey, M. Nilsen, *Steroids* **14**, 191 (1969).
10. M. Masri, *J. Amer. Oil Chem. Soc.* **47**, 61 (1970).
11. R. D. Stubbelfield, G. M. Shannon, O. L. Shotwell, *J. Ass. Offic. Anal. Chem.* **52**, 669 (1969).
12. G. N. Wogan, G. S. Edwards, R. C. Shank, *Cancer Res.* **27**, 1729 (1967).
13. Supported by NIH contract PH 43-62-468. We thank Dr. F. Garcia of New England Regional Primate Research Center for assistance. Contribution No. 1713 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

29 September 1970

Linkage Group–Chromosome Correlation in *Culex tritaeniorhynchus*

Abstract. *Linkage groups of a culicine mosquito,* Culex tritaeniorhynchus, *have been assigned to their respective chromosomes by genetic and cytologic observations of radiation-induced aberrations. Linkage group I is assigned to the smallest chromosome, linkage group II to the submetacentric chromosome, and linkage group III to the metacentric chromosome.*

A few translocations have been reported for two species of mosquitoes belonging to the subfamily Culicinae—*Culex pipiens* and *Aedes aegypti* (*1*). These studies are important because translocations and other chromosomal aberrations are generally associated with a high degree of sterility and thus are potentially useful as a method of genetic control. None of the above translocations have been identified with particular chromosomes of the karyotype. There has not yet been a successful assignment of linkage groups to their respective chromosomes in any culicine mosquito. Even sex has not been identified with a particular chromosome. This is somewhat surprising since these mosquitoes have only three pairs of chromosomes; thus, one would think that the assignment of linkage groups to chromosomes would be comparatively easy. With this thought in mind we exposed 130 males to approximately 3000 rad of gamma radiation from a ^{60}Co source in an attempt to produce a series of radiation-induced aberrations on genetically marked chro-