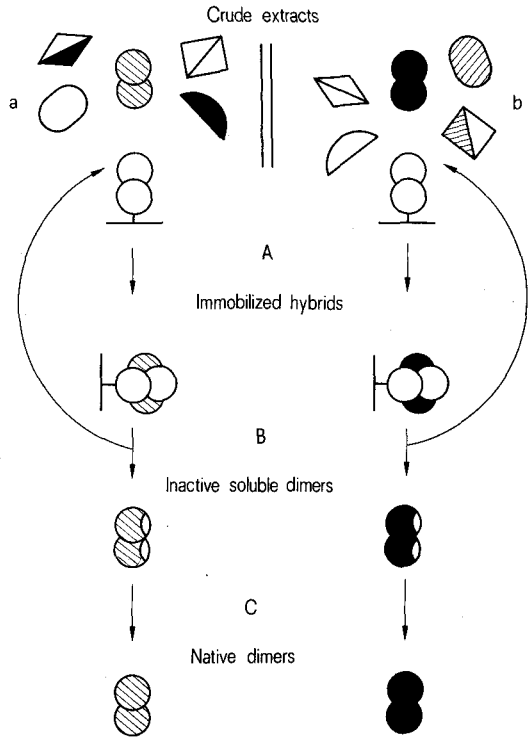
This indicates that a sufficiently pure protein can be obtained by a one-step procedure outlined above (figure, A). It should be pointed out that the reassociation of the immobilized and soluble dimers proceeded under non-denaturating conditions, in the absence of any dissociating agent. This suggests the presence of dimeric forms of the enzyme in the crude tissue extracts. Tetrameric apo-GAPD was shown to dissociate reversibly into dimers on dilution[9]. The incomplete saturation with NAD may account for the partial dissociation of this enzyme in tissue extracts.

To achieve solubilization of the non-covalently bound dimers, immobilized hybrids were treated at 4 °C with 0.15 M NaCl, 5 mM EDTA, 2 mM dithiothreitol (pH 7.6).



Immobilized dimer of GAPD as an affinity sorbent. *A*, adsorption of dimers existing in the crude extracts of different tissues (a,b) with subsequent washing from unbound proteins. *B*, incubation under the dissociating conditions and washing of the sorbent, which may be used repeatedly. *C*, reactivation of the soluble dimers. The figures of different shape refer to various proteins.

A 30-min incubation under these conditions resulted in the splitting of 50% of the matrix-bound protein; the solubilized dimers were inactive (figure, B). Reactivation was accomplished under the conditions previously described to reverse cold inactivation of the soluble rat muscle enzyme[10]; the samples were incubated at 25 °C in the presence of 0.1 M sodium phosphate and 2 mM dithiothreitol for 15–20 min. Enzyme activity then appeared in solution; in the case of the rat muscle dehydrogenase it corresponded to 26 U/mg protein. This value is significantly lower than the sp. act. of the crystalline enzyme preparation (60–100 U/mg protein), which is probably due to the incomplete reactivation of the solubilized protein. The possibility of partial denaturation of the enzyme in the course of dissociation and reactivation (steps B and C) is the main shortcoming of the method. These steps may be omitted when the aim of the investigation is to study some properties of the isolated enzyme which are not altered in the hybrid molecule (for example, kinetic parameters of GAPD). Preliminary experiments of this type were carried out with hybrids of the carboxymethylated yeast dimers and muscle dimers extracted from different mammalian tissues.

The principal advantage of the present method is its applicability in cases where an enzyme is to be isolated from a small portion of material or from a diluted protein solution. Immobilized subunits can be prepared using an accessible oligomeric enzyme capable of forming hybrids with the enzymes to be isolated. This approach may help enzyme purification procedures based on highly specific protein-protein interactions.

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Role of lactone ring of aflatoxin B_1 in toxicity and mutagenicity

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Summary. Cleavage of the lactone ring of aflatoxin B_1 results in a nonfluorescent compound that has greatly reduced biological activity. Mutagenicity, as measured by the Ames test, is reduced 450-fold compared to that of B_1, and toxicity, as measured by the chick embryo test, is reduced 18-fold.

Aflatoxin B_1, a secondary metabolite of *Aspergillus parasiticus* and *A. flavus*, may function as a potent toxin, a carcinogen, a teratogen, and a mutagen[1]. All toxins in this family have a coumarin nucleus fused to a bifurano moiety and contain either a pentenone ring or a 6-membered lactone (figure).

Aflatoxins B_1 and D_1: comparison of mutagenicity and toxicity

Mutagenicity* B_1 (nm/plate)	Net revertants	D_1 (nm/plate)	Net revertants	Toxicity** B_1 (μg/egg)	Deaths (%)	D_1 (μg/egg)	Deaths (%)
0.064	429	35	701	0.5	96	5	64
0.12	823	70	804	0.25	68	2.5	44
0.16	1260	140	1900	0.125	40	0.5	4
0.26	1429	175	2103	0.025	4	0.25	4
0.32	1799	210	2496				

* Duplicate determinations made at each level; the microsomal fraction (protein concentration of 26.6 mg/ml as determined by the method of Lowry) was incorporated into the S-9 mix at a ratio of 0.07 ml/ml. Results shown are with S-9. ** Leghorn, Mt. Hope strain chick embryos; air sack injection of compounds dissolved in vegetable oil; 25 eggs per dose level.

Comparative studies of the mutagenicity of biologically derived aflatoxin analogs to *Salmonella typhimurium* mutants show the following potency order relative to B_1: M_1, 0.032; H_1, 0.020; B_2, 0.002; P_1, 0.001; and B_{2a}, 0.000 (figure)[2]. Hsieh et al.[3] reported that aflatoxin Q_1 was approximately 18 times less toxic to chick embryos than B_1. Buchi et al.[4] showed that aflatoxin P_1 caused a degree of mortality in mice 15 times less than that caused by B_1. Ciegler and Peterson[5] demonstrated that aflatoxin B_{2a} caused fewer deaths in ducklings than did B_1. In studies on rainbow trout, Sinnhuber et al.[6] reported that aflatoxin M_1 is only one-third as carcinogenic as aflatoxin B_1. Wehner et al.[7] and Wong and Hsieh[2] show a positive correlation between mutagenicity and carcinogenicity for most mycotoxins tested. Because mutagenicity and toxicity of biologically-derived compounds are reduced by alteration of the cyclopentenone and the bifuran moieties, these portions of aflatoxin B_1, particularly the bifuran, have been held largely responsible for biological activity.

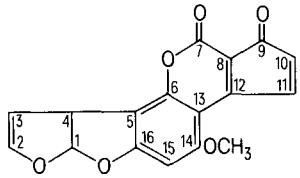
Aflatoxin D_1 (figure) is a nonfluorescent compound derived by the treatment of B_1 with ammonia[8]. Unlike the biologically formed derivatives, D_1 contains the bifuran and the pentenone moieties of B_1 but lacks the lactone ring. We report here the results of the Ames mutagenicity test and the chick embryo toxicity test on this 'derived' aflatoxin.

Aflatoxin D_1 was prepared by the method of Lee et al.[8]. Mutagenic assays were conducted on B_1 and D_1 according to the procedure of Ames et al.[9]. *Salmonella typhimurium* strain TA-100 was obtained from Dr B.N. Ames, University of California, Berkeley. Revertants/nm were calculated from the data shown in the table. Compared to an average of 6511 revertants/nm for B_1, aflatoxin D_1 preparations caused only 14 revertants/nm. The potency ratio of D_1 relative to B_1 is 0.0022, more than a 450-fold decrease.

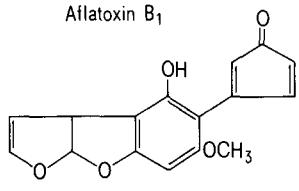
Toxicity of aflatoxins B_1 and D_1 was determined in 4-day-old chick embryos (table)[10]. Embryos were examined every 2nd day for 10 days, and total incubation and deaths were recorded. Toxicity differences were similar to those of other analogs. When deaths were plotted against concentrations of B_1 and D_1, 0.165 μg of B_1/egg caused deaths in 50% of the chick embryos, whereas 3 μg of D_1/egg caused the same degree of mortality, an 18-fold difference.

Our data along with all previous data show a strong reduction of mutagenicity and toxicity of B_1 with alteration of any portion of the molecule. Alterations of the pentenone, the bifuran, or substituted groups in the lactone ring do not alter fluorescence. Cleavage of the lactone ring does result in nonfluorescence.

Even though Lee and Cucullu[11] found that less than 1% of aflatoaxin B_1 was recoverable as D_1 in ammoniated aflatoxin-contaminated cottonseed meal, they detected little fluorescence (as B_1). Subsequent radiolabel studies by Lee et al.[12] showed that only 0.9% of the total radioactivity added as [C-14]-B_1 before ammoniation was detected in fluorescent material that was not B_1. Most of the nonfluorescent radiolabeled material was detected with the non-protein residue[12]. Inability to recover the remaining radiolabel probably resulted from binding of the reactant to macromolecular components of the meal, as found by Beckwith et al.[13] in similar studies involving contaminated corn. In this latter study, the bound nonfluorescent derivatives were also biologically inactive. Hence, there seems to be a direct correlation between loss of the easily measured molecular fluorescence and loss of biological activity. These losses may be used as an indicator in tests of the efficacy of aflatoxin-detoxification procedures.



Aflatoxin B_1



Aflatoxin D_1

Structures of aflatoxins B_1 and D_1. Analogs of B_1 are substituted as follows: M_1, 4-hydroxylated; H_1, 9- and 11-hydroxylated; Q_1, 11-hydroxylated; P_1, 14-hydroxylated; B_2, 2- and 3-hydrogenated; B_{2a}, 2-hydroxylated, 3-hydrogenated.

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