Double-Stranded Ribonucleic Acid Formation in vitro by MS 2 Phage-Induced RNA Synthetase

Abstract. *Part of the RNA synthesized from nucleoside triphosphate precursors by partially purified RNA synthetase, an enzyme induced in* Escherichia coli *by the RNA-containing phage MS 2, is resistant to hydrolysis by ribonuclease. Upon heating in 0.15*M *sodium chloride, 0.015*M *sodium citrate followed by fast cooling the material becomes ribonuclease-sensitive with a sharp transition at 102° to 104°C. The suggestion that the ribonuclease-resistant product is double-stranded RNA is reinforced by restoration of the ribonuclease resistance of the heat-denatured material by reannealing at temperatures just below the transition point and by its buoyant density in cesium sulfate. It is suggested that double-stranded RNA is the replicative form of MS 2 phage RNA.*

Infection of *Escherichia coli* with the RNA (*1*) phage MS 2 induces the formation of an RNA-synthesizing enzyme (RNA synthetase) not found in extracts of normal bacteria (*2, 3*). Analogous results have been reported for animal cells infected with RNA viruses (*4*) and RNA phage-infected bacteria (*5*).

With the four ribonucleoside triphosphates—ATP, GTP, UTP, and CTP—as substrates, partially purified *E. coli* RNA synthetase forms a product which has been characterized as RNA (*2*). Its base composition, as determined analytically, is quite similar to that of MS 2 RNA (*6*). The preparations of synthetase obtained so far have always been quite rich in endogenous RNA. Significant stimulation of enzymatic activity by further addition of RNA has not been observed. We believe that the enzyme, as isolated, is already firmly associated with its primer (*3*). Addition of ribonuclease to reaction mixtures containing the synthetase and the four triphosphates *before* incubation inhibits the formation of acid-insoluble product almost completely. Inhibition is also observed when synthetase preparations are incubated with ribonuclease, followed by ammonium sulfate fractionation to remove this enzyme (*3*) or incubated with the Ca^{++} dependent micrococcal nuclease (*7*), followed by removal of Ca^{++} as calcium oxalate (*6*) before assay of the synthetase. In contrast, we have observed that addition of ribonuclease to the synthetase system after incubation causes only a partial degradation of the reaction product, thus suggesting the possibility that a double-stranded RNA, tentatively proposed (*3*) as the replicative form of MS 2 phage, was actually being detected in vitro after incubation of RNA synthetase with radioactive substrates.

The synthetase was prepared from infected whole cells, rather than from infected protoplasts (*2*). Partial purification by magnesium ion and ammonium sulfate fractionation led to 20- to 30-fold purification in about 25 percent yield (*6*).

The effect of ribonuclease on the synthetase product isolated by phenol extraction (*8*), was determined both before heating the mixture and after heating it for 10 minutes at 120°C, followed by rapid cooling. As shown in Fig. 1, nearly 70 percent of the unheated material (curve *A*) was resistant to 1.0 μg of ribonuclease per milliliter and about 50 percent resistant to concentrations of the enzyme ranging from 30 to 100 μg/ml. In marked contrast to this behavior, over 90 percent of the product was rendered sensitive to 1.0 μg of ribonuclease per milliliter by heating (curve *B*). In this experiment the ribonuclease digestion was carried out in 0.15*M* sodium chloride containing 0.015*M* sodium citrate. The experiment of Fig. 2, in which the susceptibility to ribonuclease of both the native (curve *A*) and heated and fast-cooled synthetase product (curve *B*) were measured in sodium chloride–sodium citrate solutions of different concentrations, shows that resistance to ribonuclease was negligible at low salt concentrations but increased with increasing ionic strength, very rapidly in the case of the native and slowly in the case of the heated and fast-cooled product. Magnesium chloride was found to be more effective than sodium chloride–sodium citrate in enhancing ribonuclease resistance (see *9*). Since the low susceptibility to ribonuclease could conceivably be due to the presence of a heat-labile ribonuclease inhibitor in the crude product of the synthetase reaction, a large excess of cold MS 2 RNA was added to the radioactive synthetase product. Figure 3 indicates that the added viral RNA (curve *C*) was degraded to the same extent, and almost as rapidly as the heated and fast-cooled synthetase product (curve *B*) whereas the native product (curve *A*), as expected, was degraded to a much lesser extent.

Samples of the radioactive, deproteinized synthetase product (0.08 mμmole/ml as mononucleotide) were heated to different temperatures in either 0.15*M* sodium chloride, 0.015*M* sodium citrate or 0.0015*M* sodium chloride, 0.00015*M* sodium citrate, fast cooled, and examined for ribonuclease resistance in 0.15*M* sodium chloride–0.015*M* sodium citrate solution. Its susceptibility to ribonuclease increased abruptly at around 100°C (Fig. 4)

Table 1. Reannealing of heat-denatured synthetase product. Radioactive synthetase product (legend to Fig. 1) (0.92 mμmole/ml as mononucleotide) was denatured by heating to 120°C for 10 minutes in a sealed glass tube followed by fast cooling at −10°C. For renaturation, 0.3 ml of the heat-denatured material was taken to dryness in a stream of nitrogen, dissolved in 0.015 ml of 0.375*M* sodium chloride, 0.0375*M* sodium citrate, sealed into a capillary tube, heated to 96°C for 3 hours, and allowed to cool to 40°C for 1 hour. The material was rinsed out of the tube with 0.5 ml of distilled water. Part of this product was denatured once more. Ribonuclease-susceptibility was assayed, in portions with a total radioactivity of 200 count/min or more, as described for Fig. 2. As a control, a sample of the synthetase product was heated for 3 hours at 96°C. This did not appreciably reduce the amount of acid-precipitable material.

Synthetase product	Ribonuclease-resistant radioactivity (percent of control)
Native	51
Heat-denatured	5
Renatured by annealing	37 (35)*
Heat-denatured after annealing	7

* Incubation with 100 μg of ribonuclease per milliliter.

Table 2. Summary of buoyant densities of nucleic acids and polynucleotides as determined by banding in cesium sulfate density gradients.

Material	Density
This paper	
RNAase-resistant synthetase product	1.60
E. coli ribosomal RNA	1.65
Montagnier and Sanders (11)	
Replicative form of EMC* virus	1.57
Krebs II ascites cells ribosomal RNA	1.63
Doi and Spiegelman (15)	
MS 2 phage RNA	1.63
E. coli ribosomal RNA	1.66
Stanley (20)	
Tobacco mosaic virus RNA	1.604
E. coli ribosomal RNA	1.646
PolyA	1.675
PolyU	1.721
PolyA + U	1.688
PolyA + 2U	1.663
Warner et al. (9)	
DNA-RNA hybrid	1.490
Single-stranded DNA (calf thymus)	1.451
Double-stranded DNA (calf thymus)	1.425

* Encephalomyocarditis.

when heated in the solution of higher ionic strength (curve *A*), and above 80°C when heated in the solution of lower ionic strength (curve *B*), with a sharp transition (T_m) at 104°C and 87°C, respectively. The same experiment (not shown in the figure), conducted in 0.15*M* sodium chloride, 0.015*M* sodium citrate with synthetase product prepared with a different batch of enzyme, yielded a T_m value of 102°C. A decrease of T_m with decreasing ionic strength has been observed for the thermal denaturation transition of double-stranded DNA (*10*) and the replicative form of encephalomyocarditis virus (*11*).

The curves of Fig. 4 are similar to those obtained by Geiduschek *et al.* (*12*) for annealed complementary RNA (T_m, about 61°C, measured by susceptibility to ribonuclease in 0.005*M* sodium chloride, 0.001*M* phosphate, *p*H 7.1, 10^{-4}*M* EDTA) and by Warner *et al.* (*9*) for a DNA-RNA hybrid (T_m, about 88°C measured by susceptibility to ribonuclease in tris-HCl buffer, *p*H 7.6, ionic strength, 0.15). Sharp thermal denaturation transitions have recently been reported by Gomatos and Tamm (*13*) for the double-stranded (*14*) reovirus RNA (T_m, 90° to 95°C in 0.15*M* sodium chloride, 0.015*M* sodium citrate), and by Montagnier and Sanders (*11*) for the apparently double-stranded replicative form of encephalomyocarditis virus RNA (T_m, 96°C, in 0.15*M* sodium chloride, 0.015*M* sodium citrate).

If the ribonuclease resistance of the synthetase product were indeed due to its double-stranded nature, and its conversion into a ribonuclease-sensitive form by heating and fast cooling represented a melting out process with liberation of complementary RNA strands, it should be possible to renature the synthetase product by heating and slow cooling under appropriate conditions, with recovery of ribonuclease-resistant material. As shown in Table 1, this experiment was carried out successfully with relatively high concentrations of heat-denatured synthetase product (18 mμmole/ml as mononucleotide) at high ionic strength, and an annealing temperature close to the T_m. As would be expected, the reannealed product could again be rendered sensitive to ribonuclease by heating and fast cooling.

The ribonuclease-resistant synthetase product obtained by ribonuclease digestion of the reaction mixture after incubation with cold ATP, GTP^{32}, UTP-C^{14} and CTP-C^{14}, phenol treatment (*8*), subsequent dialysis against 0.1*M*, and finally against 0.001*M* sodium chloride, was centrifuged to equilibrium in cesium sulfate (mean density, 1.64) as described by Doi and Spiegelman (*15*). A single peak of radioactivity was found at a density of 1.60; *E. coli* ribosomal RNA was found at a density of 1.65 under the same conditions. These

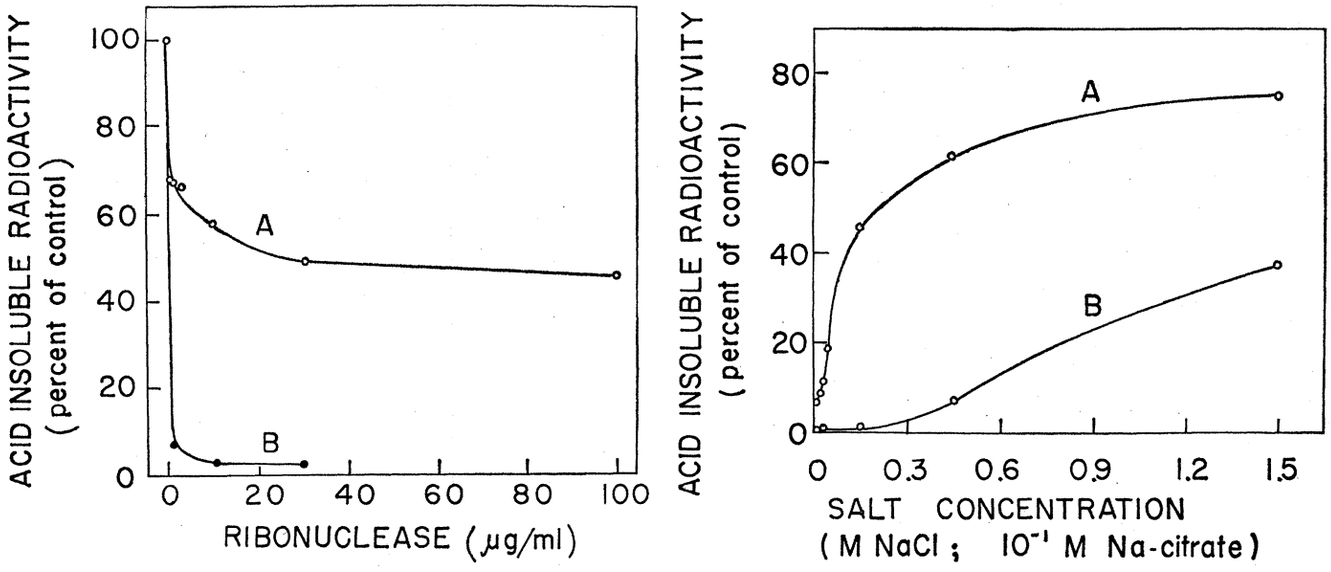
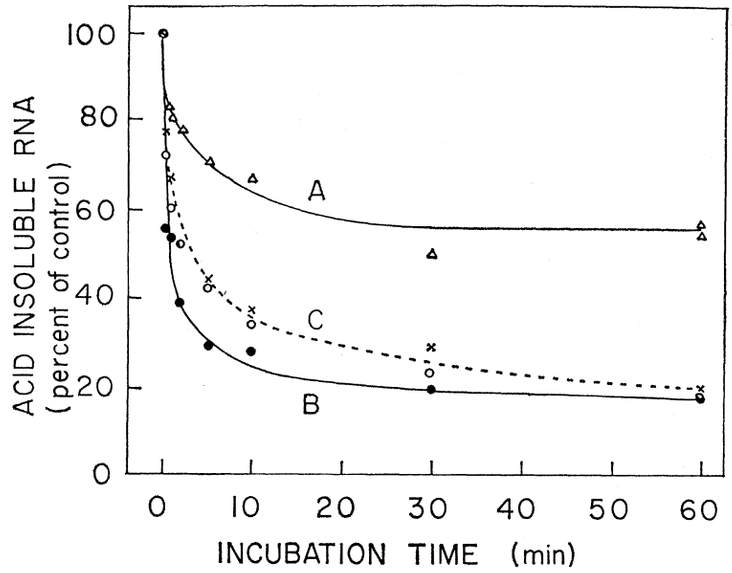


Fig. 1 (left). Resistance of native and heat-denatured synthetase product to incubation with different concentrations of ribonuclease. Radioactive synthetase product was obtained by incubating RNA synthetase [Step II fraction (*6*)] under standard assay conditions (*2*) for 10 min at 37°C with cold ATP, GTP^{32}, UTP-C^{14} and CTP-C^{14}. After cooling to 0°C, neutralized sodium pyrophosphate was added to a final concentration of 3 percent. The solution was deproteinized with phenol (*8*) and the nucleic acids in the aqueous layer were precipitated with ethanol. The precipitate was dried and dissolved in 0.005*M* tris-HCl buffer, *p*H 7.2, to give a total RNA concentration of about 0.3 mg/ml and about 3 mμmole/ml (as mononucleotide) of newly synthesized, radioactive RNA. Of the radioactivity incorporated into acid-insoluble material 85 percent was recovered. Portions (0.05 ml) containing about 300 count/min were incubated with different amounts of ribonuclease for 30 min at 25°C in 0.15*M* sodium chloride containing 0.015*M* sodium citrate, at *p*H 7.0, in a final volume of 1.0 ml. After cooling to 0°C, 0.1 ml of bovine serum albumin (10 mg/ml) and 10 ml of 0.4*N* perchloric acid were added. The precipitate was collected by centrifugation, washed twice with 0.4*N* perchloric acid, dissolved in 1.5*N* NH_4OH, plated, dried, and its radioactivity measured in a windowless gas flow counter. The results are expressed as percentage of the radioactivity in the acid-insoluble fraction after ribonuclease treatment as compared to a control kept at 0°C without ribonuclease. Curve *A*, native product; curve *B*, heat-denatured product which was obtained by heating the native material in 0.15*M* sodium chloride, 0.015*M* sodium citrate, *p*H 7.0, for 10 min at 120°C in a sealed tube, followed by rapid cooling at −10°C. Fig. 2 (right). The effect of ionic strength on the ribonuclease resistance of native and denatured synthetase product. These were obtained as described in the legend to Fig. 1, except that GTP^{32} (65,000 count/min per mμmole) was the only radioactive nucleoside triphosphate. Portions (about 700 count/min) were incubated for 30 min at 25°C with RNAase (5 μg/ml) in 0.5 ml of sodium chloride-sodium citrate solutions, *p*H 7.0, of different concentration. After cooling to 0°C, 1.0 ml of 0.8*N* perchloric acid was added and, after standing for 5 min, the acid-insoluble material was collected on Millipore HA filters (25 mm diameter) and washed with 0.4*N* perchloric acid. The filters were placed on planchets and after drying the radioactivity was measured in an end-window gas-flow counter. Curve *A*, native product; curve *B*, heat-denatured product.



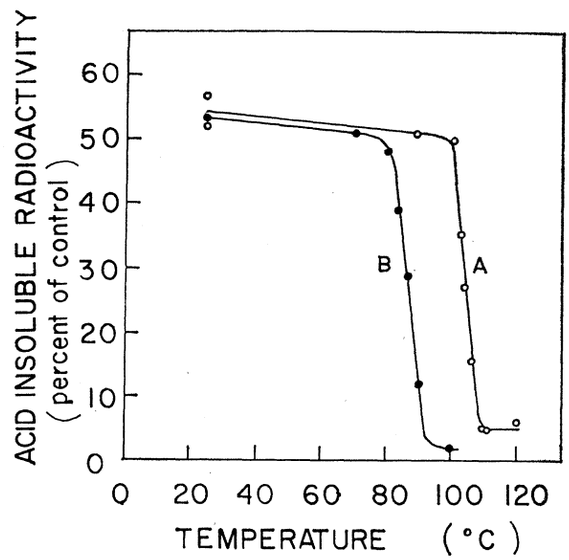


Fig. 3 (left). Time course of ribonuclease digestion of native and denatured synthetase product. Mixtures were prepared containing, in a final volume of 3 ml, either native or heat-denatured synthetase product (prepared as described in legend of Fig. 2, with a total of 10,000 count/min), 1.2 mg of MS 2 RNA, and 0.15*M* sodium chloride, 0.015*M* sodium citrate. Four portions (each 0.2 ml), to be used as controls, were removed, the solution was brought to 25°C and, at time zero, 0.01 ml of ribonuclease (200 μg/ml) was added to the main reaction mixture with stirring. At various times, portions (0.2 ml) were withdrawn and pipetted into 3 ml of 0.4*N* perchloric acid at 0°C. After 5 min the samples were filtered through Millipore HA filters and the absorbancy of the filtrates at 260 mμ was determined. The filters were further washed with 0.4*N* perchloric acid and their radioactivity was measured as in Fig. 2. The total absorbancy of the incubation mixture was determined in a portion (0.2 ml) after adding 0.2 ml of 1.0*N* NaOH, heating to 100°C for 5 min, and adding 6 ml of 0.4*N* perchloric acid. Curve *A*, native product; curve *B*, denatured product. For these two curves the results are expressed as percentage of radioactivity in the acid-insoluble fraction after incubation with ribonuclease as compared to controls kept at 0°C without ribonuclease. Curve *C*, percentage of ultraviolet-absorbing material in the acid-insoluble fraction after incubation with ribonuclease as compared to controls kept at 0°C without ribonuclease. Curve *C* is a composite curve from the same experiments from which curves *A* and *B* were derived. Fig. 4 (right). Thermal transition curves of ribonuclease susceptibility of synthetase product. Radioactive synthetase, prepared as described for Fig. 2, was precipitated twice with ethanol. Samples (0.12 ml) containing 0.024 mμmole of radioactive product (400 count/min), either in 0.15*M* sodium chloride, 0.015*M* sodium citrate (curve *A*) or in 0.0015*M* sodium chloride, 0.00015*M* sodium citrate (curve *B*), *p*H 7.0, final volume, 0.3 ml, were heated to different temperatures for 10 min in sealed glass tubes and rapidly cooled to 0°C. The contents of each tube were then incubated with ribonuclease (5 μg/ml) for 30 min at 25°C in 0.15*M* sodium chloride, 0.015*M* sodium citrate, in a final volume of 0.6 ml. After cooling, the samples were processed and the results expressed as described in the legend to Fig. 2. In a separate control experiment heating for 10 min at 110°C without subsequent ribonuclease treatment did not decrease the amount of radioactivity in the acid-precipitable fraction.

values may be compared with the buoyant densities of other nucleic acids and polynucleotides (Table 2). The density of the ribonuclease-resistant synthetase product is substantially lower than that of ribosomal or MS 2 RNA.

These results show that a substantial fraction of the radioactivity recovered as acid-insoluble material, after incubation of labeled ribonucleoside triphosphates with the MS 2 phage-induced RNA synthetase of *E. coli*, is present as double-stranded RNA. The sharp thermal transition observed for the susceptibility of this acid-insoluble material to ribonuclease is characteristic of highly ordered secondary structure and the reannealing experiments provide further support that complementary strands are involved. The occurrence of DNA-RNA hybrids is excluded by the fact that synthesis of the product was carried out in the presence of added deoxyribonuclease and by the high buoyant density in cesium sulfate of the ribonuclease-resistant material.

Formation of double-stranded RNA through the action of RNA synthetase in vitro would lend support to our previous suggestion (*3*) that MS 2 RNA replication in vivo may involve a double-stranded RNA as an intermediate in replication. It is of interest in this connection that double-stranded RNA is strongly implicated as the replicative form of encephalomyocarditis virus RNA (*11*). The existence of a replicative form of RNA in MS 2 distinct from the viral RNA finds some support in the preliminary observation (*6*) that the RNA of P^{32}-labeled MS 2 virus was partially converted to a ribonuclease-resistant form 15 minutes after entering the host cells.

The sequence of events leading to RNA replication in MS 2 might be pictured as follows. The incoming parental strand ("plus" strand) serves first as messenger and programs the synthesis of RNA synthetase. The newly formed enzyme binds the parental "plus" strand and, using it as template, synthesizes a complementary RNA strand ("minus" strand). The duplex thus formed would be the replicative form. It could conceivably serve as template for production of copies of the "plus" strand without separation of the individual strands (conservative mechanism).

Alternatively, the newly formed "plus" strands might each time displace their counterparts from the duplex (semi-conservative mechanism). In either case the replicative form is here assumed to produce only "plus" strands in analogy with the in vivo formation of messenger RNA from only one of the two DNA strands (*16*). An analogy in vitro may be provided by the observation (*17*) that, with the double-stranded complex composed of polyA + polyU as template, *Azotobacter* RNA nucleotidyl transferase (RNA polymerase) forms polyU but little or no polyA. The same is true of a 1:1 complex of PolyA and polyriboT (*18*). Single-stranded polyA or polyU (or

polyriboT), on the other hand, primes the synthesis of polyU or polyA, respectively.

If the replicative form of poliovirus RNA were double-stranded, the observation (*19*) that in HeLa cells infected with this virus, the base ratios of the RNA newly synthesized in the presence of actinomycin D are similar to those of poliovirus RNA, and therefore do not reflect an equal synthesis of complementary RNA, might be explained by the assumption that only "plus" strands are produced on the double-stranded template. However, these results as well as those on the detection in vivo of only one kind of messenger RNA strand might also be interpreted as meaning that, while both strands are produced, one of them is subsequently eliminated. There is no evidence so far, although it is not unlikely, that newly formed viral RNA is exclusively of the "plus" type.

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References and Notes

1. Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, GTP, UTP, CTP, 5′-triphosphates of adenosine, guanosine, uridine, cytidine; polyA, polyadenylic acid; polyU, polyuridylic acid; polyriboT, polyribothymidylic acid; tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine-tetraacetate.
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Aflatoxin B_2: Chemical Identity and Biological Activity

Abstract. *Aflatoxin B_2, a blue-fluorescent metabolite of* Aspergillus flavus, *was isolated from cultures grown on crushed wheat. Chemical structure of the compound was elucidated as dihydroaflatoxin B_1. Biological activity was determined in day-old male white Pekin ducklings. The criteria of activity were reduction in growth and liver size and the extent of bile-duct hyperplasia.*

Aflatoxins B and G, two metabolites of the fungus *Aspergillus flavus*, which were associated with the toxicity of animal feeds (*1*), have recently been isolated and their structures elucidated (*2*). When cultured under laboratory conditions, this fungus produces a complex mixture of blue and yellow-green fluorescent compounds which are separable by chromatography. A system of nomenclature has been suggested in which the two major components (B and G above) would become B_1 and G_1 (*3*).

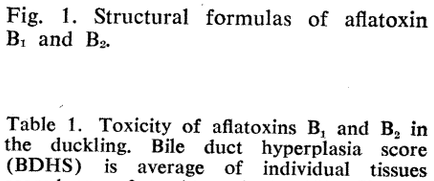
We have identified and evaluated the toxicity of a second blue-fluorescent compound, aflatoxin B_2, produced in relatively small quantities by the fungus. The compound was isolated from cultures of *A. flavus* grown for 7 days at 30°C on sterilized crushed wheat. Cultures were thoroughly extracted with chloroform and the crude mixture of toxic fluorescent substances was precipitated by addition to 20 volumes of petroleum ether. Crude extracts were thus obtained in yields of approximately 750 mg per kilogram of wheat.

Fractionation of the crude mixtures was accomplished by thin-layer chromatography on silica gel G (Merck) with a mixture of chloroform and methanol (97:3) as developing solvent. The blue-fluorescent substance migrating with a slightly smaller R_F value than aflatoxin B_1 was isolated by extraction of the adsorbant with methanol. This compound, aflatoxin B_2, was purified by recrystallization from chloroform-pentane.

Its molecular weight (m.w.) was 314 (mass spectrograph); m.p. 286° to 289°C (decomposed); $\lambda_{max}^{ethanol}$ 222, 265, 362 mμ (ε19,600, 9,200, 14,700); $\nu_{max}^{CHCl_3}$ 1760, 1685, 1625, 1600 cm^{-1}; $[\alpha]_D^{CHCl_3}$ −429°. These data are very similar to those of aflatoxin B_1 (*2*): $C_{17}H_{12}O_6$, m.w. 312; m.p. 268° to 269°C (dec.); $\lambda_{max}^{CHCl_3}$ 223, 265, 362 mμ (ε 25,600, 13,400, 21,800); $\nu_{max}^{CHCl_3}$ 1760, 1665, 1630, 1600 cm^{-1}; $[\alpha]_D^{CHCl_3}$ −558°. The data strongly suggest that aflatoxin B_2 is dihydroaflatoxin B_1 (Fig. 1).

In order to test this hypothesis, dihydroaflatoxin B_1 was prepared by catalytic hydrogenation of aflatoxin B_1 in ethanol over 1 percent palladized calcium carbonate. The reduction was interrupted after one mole of hydrogen had been absorbed. The resulting product had m.w. 314 (mass spectrograph); m.p. 287 to 289°C (dec.); $\lambda_{max}^{ethanol}$ 222, 265, 362 mμ (ε17,600, 11,000, 20,800); $\nu_{max}^{CHCl_3}$ 1760, 1685, 1625, 1600 cm^{-1}; $[\alpha]_D^{CHCl_3}$ −430°. On thin-layer chromatograms, its R_F value was identical with that of aflatoxin B_2. These physical data agree well with those reported by other investigators (*4*, *5*). Thus aflatoxin B_2 is dihydroaflatoxin B_1 and has the structure shown in Fig. 1.

The toxic properties of aflatoxin B_2 isolated from culture extracts were compared with those of aflatoxin B_1



$C_{17}H_{12}O_6$ MOL. WT. 312 $C_{17}H_{14}O_6$ MOL. WT. 314

Fig. 1. Structural formulas of aflatoxin B_1 and B_2.

Table 1. Toxicity of aflatoxins B_1 and B_2 in the duckling. Bile duct hyperplasia score (BDHS) is average of individual tissues scored on a 0 to 4+ scale (× 10). Six animals at each dose.

Dose (μg)	Body wt. 8-day (g)	Liver weight		BDHS
		Actual (g)	Relative to body wt. (%)	
		Aflatoxin B_1		
0.0	133	7.1	5.3	0
2.0	129	6.0	5.0	10.0
3.9	125	6.6	5.3	16.0
7.8	101	4.6	4.6	30.0
15.7	112	4.8	4.3	30.0
		Aflatoxin B_2		
0.0	142	8.2	5.9	0
50.0	147	7.1	4.9	16.0
80.0	127	6.2	4.9	20.0
125.0	116	6.3	4.7	20.0
200.0	100	4.5	4.5	30.0