The Preparation of Aflatoxins Labeled with Tritium*

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SUMMARY

Aflatoxins B_1 and G_1 were labeled with tritium by reaction with tritiated water in the presence of a catalyst. Although considerable decomposition occurred, it was possible to purify the products by thin layer chromatography and crystallization following addition of unlabeled compound. The products were chemically homogeneous and the radiochemical purity was above 82 %. When the exchange was carried out with a natural aflatoxin mixture, the specific activity of the purified G_1 was only one-tenth that of the B_1. When the aflatoxin G_1 was tritiated alone, the specific activity after dilution was 194 μCi/mg. Similarly, the specific activity of purified tritium labeled aflatoxin B_1 was 300 μCi/mg. The problems encountered in labeling with tritium such unstable molecules as the aflatoxins are discussed.

Aflatoxin B_1 is a naturally occurring carcinogen of a very high order of potency (as little as 1 mg has given rise to liver tumors in rats), produced, along with other aflatoxins of similar structure, by several strains of *Aspergillus*. Because of their unique chemical structure, studies of the metabolism of aflatoxins and of their interaction with the constituents of cells might provide some new insight into the mechanism of carcinogenesis. To enable such studies to be carried out, radioactively labeled compounds were needed and it was decided to attempt to label these compounds with tritium by an exchange procedure (the chemical synthesis being long and complex [1]). Some of the problems encountered in the attempt to prepare these compounds with fairly

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high specific activity illustrate the precautions necessary in dealing with these unstable compounds.

Because the aflatoxins are unsaturated compounds, the standard Wilzbach labeling procedure [2] could not be used, since it would lead to reduction products. An alternative procedure [3] involving short term exposure to tritium gas during passage of an electric arc was used (carried out by Tracerlab, Inc., Waltham, Mass.). The crude aflatoxin mixture (containing approximately 45 % each of aflatoxins B_1 and G_1, together with 5 % and 4 %, respectively, of aflatoxins B_2 and G_2) was 400 mg, with a specific activity of 160 μCi/mg *. Analysis of this product by chromatography on thin layer plates of silica gel G as previously described [5], followed by spectrometric and radiometric examination of the fractions, revealed that much of the activity was not associated with aflatoxin. Furthermore, the specific activities of the aflatoxins B_2 and G_2 were about 100 μCi/mg, much higher than those of aflatoxin B_1 (12 μCi/mg) or G_1 (10 μCi/mg), showing that much of the tritium activity was contained in the reduction products of aflatoxins B_1 and G_1. After purification of the aflatoxins B_1 and G_1 by repeated chromatography on T.L.C. plates of silica gel, followed by crystallization from chloroform/methanol, 43 mg of B_1 and 32 mg of G_1 were obtained (from 250 mg of crude mixture) with specific activities of 9.5 and 0.7 μCi/mg, respectively. Although the products had a high degree of radiochemical and chemical purity, the specific activities were inadequate for biochemical studies.

An alternative method of tritium labeling was by exchange with tritiated water in trifluoroacetic acid in the presence of a catalyst. This exchange (and subsequent ones) were carried out by New England Nuclear Corporation, Boston, Mass. The product, 48 mg, 228 mCi, was resolved by thin layer chromatography and it was found that considerable decomposition of the aflatoxins had taken place during exposure [6] (presumably due to the strong acid at an elevated temperature) and only 1.6 mg of aflatoxin B_1 and 2.2 mg of G_1 remained, although these did contain much of the radioactivity (38 mCi and 30 mCi, respectively). Only 6 mg of the product was soluble in chloroform, the remainder being a black solid. Each of the bands corresponding to aflatoxins B_1 and G_1 was eluted with chloroform/methanol. Each of the two labeled compounds was diluted with unlabeled aflatoxin (70 mg B_1 and 60 mg G_1) and crystallized twice, assayed for radiochemical purity, crystallized again (Yield : 36 mg B_1, 42 mg G_1), and re-assayed. The specific activity (300 μCi/mg B_1, 30 μCi/mg G_1) changed insignificantly following the third crystallization and the radioassay (by T.L.C. chromatography on a small plate and radioassay of all the fractions, fluorescent and non-fluorescent, on

* The assays of radioactivity were carried out as in [4]. Weighed aliquots of solutions, or accurately measured volumes, were added to 2 ml ethanol, 13 ml of scintillation "cocktail" was added and the solutions counted in a liquid scintillation counter. Correction for chemical quenching was made by using internal standards.

the plate) showed that in the aflatoxin B_1 preparation, 91 % of the radioactivity was in the fluorescent zone (a second crop recovered from the mother liquors had a slightly lower purity—86 %). The aflatoxin G_1 preparation showed a similar purity (90 %), although the specific activity was only one-tenth that of the B_1, and really inadequate for biochemical studies. Because of the extensive decomposition that had occurred during the exchange procedure, giving rise to compounds of unknown nature, it was felt that demonstration that the fluorescent zone contained 90 % of the total activity of the crystalline material was insufficient evidence of purity. If the compound is radiochemically pure, then the radioactivity should be distributed uniformly throughout the compound and each part of the fluorescent zone should have the same specific activity (within normal statistical limits). With this in mind, the fluorescent zone was divided into two portions (leading and following) and both assayed separately for radioactivity and aflatoxin content. In the case of the aflatoxin B_1 preparation the leading portion (higher R_f) containing most of the aflatoxin had a specific activity of 54 μCi/mg, while the following portion (lower R_f) had a specific activity of 1.2 mCi/mg. This showed that the aflatoxin B_1 contained a radiochemical impurity of similar R_f and very high specific activity. It was obviously hazardous to use such material for further experiments. A similiar examination of the aflatoxin G_1 showed that there was little radiochemical impurity of the kind present in the B_1 sample.

Since the previous preparations were obviously unsatisfactory, it was decided to attempt an exchange procedure less likely to lead to decomposition of the aflatoxin. By substitution of dimethylformamide for trifluoroacetic acid and by carrying out the exchange in the dark, it was hoped to minimise or to eliminate chemical decomposition. The procedure (again carried out by New England Nuclear Corporation) used 25 mg of aflatoxin mixture, dissolved in 0.5 ml of dimethylformamide containing 10 curies of tritiated water and 25 mg of pre-reduced platinum catalyst. The mixture was stirred magnetically for 2 days at 70° C, after which labile tritium was removed by evaporation with methanol under reduced pressure. The product contained a total of 620 mCi, and was chromatographed on a T.L.C. plate of silica gel G, using a mixture of chloroform/ether/acetic acid/methanol (70 : 20 : 10 : 2) as developing solvent.

Some yellow and brown impurities were present near the solvent front. Two major blue fluorescent bands had R_f's corresponding to those of aflatoxins B_1 and G_1 (there were also several smaller bands near to the origin). Each of these two bands was divided into a leading portion and a trailing portion (bearing in mind the previous findings), which were eluted separately. The total aflatoxins measured spectrometrically were 4 mg of B_1 and 2.6 mg of G_1, showing that again almost three quarters of the aflatoxin mixture had been destroyed during the tritiation procedure. In each case, the leading portion of aflatoxin B_1 and G_1 zones contained more activity than the trailing portion.

The leading portions of both zones were diluted with 72 mg of, respectively, purified aflatoxin B_1 and purified aflatoxin G_1 and crystallized twice from chloroform/methanol yielding, in each case, 50 mg of crystalline material. The specific activities of 360 μCi/mg for aflatoxin B_1 and 57 μCi/mg for aflatoxin G_1 were not significantly changed by further crystallization. Analysis of the two crystalline materials by T.L.C. chromatography of a small sample, including division of the main fluorescent zone, in each case, into a leading and trailing portion, revealed a difference between the two samples.

The two portions of the B_1 fluorescent zone had specific activities of 290 and 330 μCi/mg, showing that the activity was distributed fairly uniformly between the aflatoxin B_1 molecules; the activity in the fluorescent zone was more than 82 % of the total activity, indicating a high degree of radiochemical purity. On the other hand, the two portions of the G_1 fluorescent zone had specific activities of 24 and 73 μCi/mg, a difference too large to be accounted for by errors in the assay *. Also the activity in the fluorescent zone due to aflatoxin G_1 was only 44 % of the total activity.

It appeared that the exchange of tritium for hydrogen in aflatoxin G_1 was less favorable than in aflatoxin B_1, by an order of magnitude, when the two compounds were exposed together. After four attempts, no progress was made towards preparing aflatoxin G_1 of high specific activity, and it was decided to attempt this by exposure of aflatoxin G_1 alone to tritium. A pure sample of aflatoxin G_1 (prepared as described [5])—25 mg—was labeled by exchange with tritiated water in dimethylformamide precisely as was the mixture of aflatoxins. In this case, again, much of the aflatoxin appeared to have been destroyed, only about 4 mg remaining. The product was chromatographed on a T.L.C. place of silica gel G, developed with chloroform/methanol (95 : 5). The major band was green fluorescent in UV light, and just below was a blue fluorescent band; the remainder of the chromatogram consisted of several lesser fluorescent bands. Approximately 34 % of the total activity was in the green fluorescent band (79 mCi) that corresponded with aflatoxin G_1. The material in this band was extracted from silica gel with a mixture of chloroform, ethanol and acetone, the silica gel separated by centrifugation and the supernatant liquid evaporated to dryness. Unlabeled aflatoxin (79 mg) was added and the mixture of labeled and unlabeled material was crystallized from chloroform/methanol three times. The crystalline product was examined as previously described for radiochemical purity after both the second and third crystallization. There was only a slight decrease in specific activity of the compound after the third crystallization as compared with the specific activity after the second crystallization : the radiochemical purity also was almost constant with more than 86 % of the total activity in the aflatoxin G_1 fluorescent band. The yield of crystalline material was 58 mg and the specific

* The error in the assay of specific activity was estimated as ± 4 %, comprising errors arising from incomplete elution, spectrophotometric inaccuracies and counting variations.

activity 194 μCi/mg. A second crop of crystals from the mother liquors was much less pure radiochemically.

Although these two preparations did not represent the ultimate in radiochemical and chemical purity, they did appear to be adequate for biochemical studies, and had sufficiently high specific activities for the measurement of quite low levels of interaction with cell constituents. However, it must be borne in mind that in this case (as in every instance in which radioactive nuclides are only a small proportion of the atoms of an element in a molecule), the possible presence of a trace of chemical impurity of high specific activity can make the interpretation of the results of such experiments quite difficult. Some experiments with the tritium-labeled aflatoxins described above will be published elsewhere.

Because of the destruction which aflatoxins undergo during exposure to tritium oxide, even in solution in dimethylformamide, (with production of hydroxylated compounds, amongst others [6], a further method of labeling was suggested. This involved exposure to 100 curies of tritium gas, but at liquid nitrogen temperature (73° K) to minimise reduction of the aflatoxins. Such an exposure was carried out on 10 to 20 mg of aflatoxins B_1 and G_1, at the Radiochemical Centre, Amersham, England.

The products were 20 mg of aflatoxin G_1 containing 24.7 mCi and 6.3 mg of aflatoxin B_1 containing 1.7 mCi. The incorporated activity was much less than was expected (high specific activities in steroids have been achieved by this method), but the products were, nevertheless, purified, each on a single 20 × 20 cm plate of silica gel G, developed with 5 % methanol in chloroform. The main fluorescent zone and the remainder of the plates were separated, eluted and radioassayed.

The (fluorescent) aflatoxin G_1 band contained 840 μCi or 8 % of the total activity on the chromatogram. The aflatoxin B_1 fluorescent band contained 135 μCi or 17 % of the total activity on the plate. Dilution of the G_1 band with unlabeled aflatoxin G_1 and crystallizing gave a product which was still not pure (the fluorescent band contained only 20 % of the radioactivity and was itself inhomogeneous—the specific activity was 2 μCi/mg). These two products were so unsatisfactory that they were not proceeded with. It was obvious that this method of labeling was no more satisfactory than liquid exchange.

It is hoped that these observations of the difficulties encountered in labeling with tritium such unstable compounds as the aflatoxins will be helpful and that they might provoke thought leading to a more satisfactory way of preparing labeled aflatoxins.

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