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Preparation of Labeled Aflatoxins with High Specific Activities

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Resting cells of *Aspergillus parasiticus* ATCC 15517 were used to prepare highly labeled aflatoxins from labeled acetate. High synthetic activity in growing cells was evidenced only during 40 to 70 hr of incubation. Glucose was required for high incorporation efficiency, whereas the concentration of the labeled acetate determined the specific activity of the product. When labeled acetate was continuously added to maintain a concentration near but not exceeding 10 mM, in a culture containing 30 g of glucose per liter, 2% of its labels could be recovered in the purified aflatoxins which have a specific activity more than three times that of the labeled acetate.

Isotope-labeled aflatoxins with high specific activities are useful for investigations on their biosynthesis, structure analysis (3), and metabolism in animals (12). As part of a study on the biosynthesis of aflatoxins, techniques were developed which make possible the preparation of relatively large quantities of highly labeled aflatoxins by procedures similar to those reviewed by Perlman et al. (11).

Chemical degradation of aflatoxin B_1 produced by *Aspergillus parasiticus* containing acetate-*1*-^{14}C or acetate-*2*-^{14}C (3) has shown that the 16 carbon atoms of the ring structure of aflatoxin B_1 are totally derived from acetate, 9 are derived from carboxyl, and 7 are derived from methyl. Therefore, if the aflatoxins were prepared from acetate-*1*-^{14}C, the maximal possible relative isotope content (RIC) of the product, defined as the specific activity of the product relative to that of the precursor, would be 9.

Preliminary studies (1) have shown that about 1% of the labels from acetate-*1*-^{14}C could be incorporated into aflatoxins, but the RIC obtained was only 0.1, indicating that the specific activity of the product is only one-tenth that of the precursor. The present paper describes a search for improved conditions under which both the incorporation percentage of the labels of acetate and the specific activity of the aflatoxins produced could be maximized.

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MATERIALS AND METHODS

Organism. *A. parasiticus* ATCC 15517 was used throughout this study. The suspension of conidia used as the inoculum was prepared by the procedures reported by Donkersloot and Mateles (5).

Media. The basal medium used to grow *A. parasiticus* contained, per liter: glucose, 50 g; $(NH_4)_2SO_4$, 4 g; KH_2PO, 10 g; $MgSO_4 \cdot 7H_2O$, 2 g; and the trace metals as used by Adye and Mateles (1).

The replacement medium used to prepare the resting cell cultures of *A. parasiticus* contained, per liter: KH_2PO_4, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; and the same trace metals as used in the basal medium. The desired precursors were then added to the resting cells. The respiring cells were prevented from growing by the absence of a nitrogen source.

Acetate-1-^{14}C. Sodium acetate-*1*-^{14}C (specific activity, 57 mCi/mmole) was purchased from New England Nuclear Corp., Boston, Mass. For the various experiments, the activity was diluted by unlabeled sodium acetate, and the solution was adjusted to *p*H 6.0 with sulfuric acid.

Culture techniques. Each 100 ml of basal medium contained in a 500-ml baffled flask (Bellco Glass, Inc.) was inoculated with 10^5 conidia. This primary culture was incubated at 30 C on a rotary shaker at 100 rev/min for the first day and 200 rev/min afterwards. In the shaken flask, the mold grew as pellets, which were collected on a cheesecloth after aflatoxin production had begun. The pellets were washed and filtered, and a definite amount (based on wet weight) was resuspended in the replacement medium to form the resting cell culture. After addition of precursors, the culture was incubated at 30 C on a rotary shaker at 200 rev/min.

Purification of aflatoxins. The incubated broth was filtered through a sintered-glass filter and repeatedly shaken with equal volumes of chloroform until all of the aflatoxins were extracted. The chloroform extract was evaporated under reduced pressure to dryness, and the solids were redissolved with a small amount of benzene. The crude aflatoxins were purified by two thin-layer-chromatography (TLC) systems. The concentrated benzene solution was first applied as a band on a Silica Gel G plate which was then developed with chloroform-acetone (9:1). The fluorescent bands corresponding to aflatoxins B_1, B_2, G_1, and G_2 were scraped off and eluted with 10% methanol in chloroform. The eluates were dried and redissolved in benzene for purification with the second TLC system (Silica Gel G plates developed with ethyl acetate-propanol-water, 20:2:1). Further TLC purifications showed that the specific activity of the purified aflatoxin was constant.

For each level of a variable investigated, at least two flasks were run and the mean was reported.

Assays. The dry cell mass was measured by heating the washed pellets at 110 C to a constant weight. The washed pellets collected on a cheesecloth under aspiration are referred to as fresh cells. In kinetic studies, the cell growth was calculated from the consumed ammonium sulfate by using an average yield constant obtained by dividing the total ammonium sulfate consumed by the total dry cell mass. A phenol-sodium hypochlorite method (10) was used to measure ammonium sulfate.

Glucose was determined by micro-Glucostat, and acetate was determined by its radioactivity. The validity of isotopic assay for acetate has been discussed in a previous report (7).

The concentration of aflatoxins in chloroform was calculated from the optical density of the chloroform solutions by using appropriate molar extinction coefficients (B_1, 21,800; B_2, 20,800; G_1, 16,100; G_2, 19,300; references 2, 4). Since about 80% of total aflatoxins was found to be aflatoxin B_1, the concentration of mixed aflatoxins could be very well approximated by using an extinction coefficient of 20,000.

Radioactivity was measured with a Packard Tri-Carb model 2002 liquid scintillation spectrometer; the dioxane-based scintillation fluid (New England Nuclear Corp., Boston, Mass.) used had a counting efficiency of 70% for carbon-14. The amount of precursor incorporated into aflatoxins was calculated by dividing the radioactivity of the aflatoxin by the specific activity of the presursor. The RIC of the product was obtained either by dividing the specific activity of the product by that of the presursor or by dividing the amount of precursor incorporated by the amount of product.

Incorporation efficiency. Two types of incorporation efficiencies were distinguished. One is the percentage of labels of persursor recovered from the product. This percentage of incorporation (PI) shows the material economy of the process. The other efficiency is the RIC of product, which indicates the number of moles of presursor incorporated per mole of product, hence the presursor-product relationship. The relations between the above parameters are summarized as follows:

$$A_2 = RIC \cdot A_1 \quad (1)$$

where A_1 = specific activity of precursor (millicuries per millimole) and A_2 = specific activity of product (millicuries per millimole) and

$$PI = 100 \cdot RIC \cdot X/F \quad (2)$$

where X = amount of product (micromoles) and F = amount of precursor (micromoles).

RESULTS AND DISCUSSION

Initiation and duration of synthetic activity. The growth of *A. parasiticus* and its production of aflatoxins in a shaken flask are shown in Fig. 1. Aflatoxins appeared some 24 hr after the start of incubation when cell mass was increasing rapidly, as indicated by the rate of nitrogen consumption, and the concentrations of glucose and ammonium sulfate were still high. Shortly after their appearance, the aflatoxin concentration increased at a high rate which lasted about 24 hr. A similar fermentation pattern was also observed in a 5-liter agitated fermentor (9).

When the pellets at different culture ages were collected (at 24-hr intervals), washed, and resuspended in the nitrogen-free replacement medium containing 50 g of glucose per liter and incubated for 10 hr, only the 48-hr cultures produced significant amounts of aflatoxins (Table 1). In the 48-hr culture, a linear production of aflatoxins was observed, which lasted for only 20 hr (Fig. 2), indicating a relatively short duration of the synthetic activity of the cells.

Previous studies (7) have shown that the resting cells were constantly taking up glucose and acetate regardless of their synthetic activities, and that, therefore, incorporation of labeled acetate into aflatoxins should be carried out for the duration of synthetic activity to ensure a high incorporation efficiency.

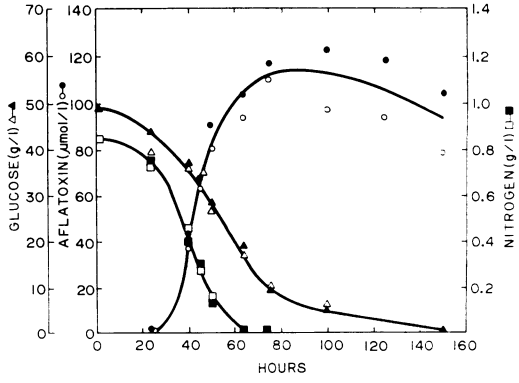

FIG. 1. *Growth of and aflatoxin production by Aspergillus parasiticus in 100 ml of medium contained in a 500-ml shaken flask.*

TABLE 1. *Synthetic activity of the cells at different culture ages*[a]

Time of incubation (hr)	Aflatoxin produced in 10 hr (μmole/flask)		Relative synthetic activity	
	Run 1	Run 2	Run 1	Run 2
24	0.16	0.12	5	6
48	3.21	2.10	100	100
72	0.12	0.15	4	7
96	0.18	0.48	6	23
120	0.07		2	

[a] Each flask contained 0.5 g of glucose and 2 g of fresh cells suspended in 10 ml of the replacement medium.

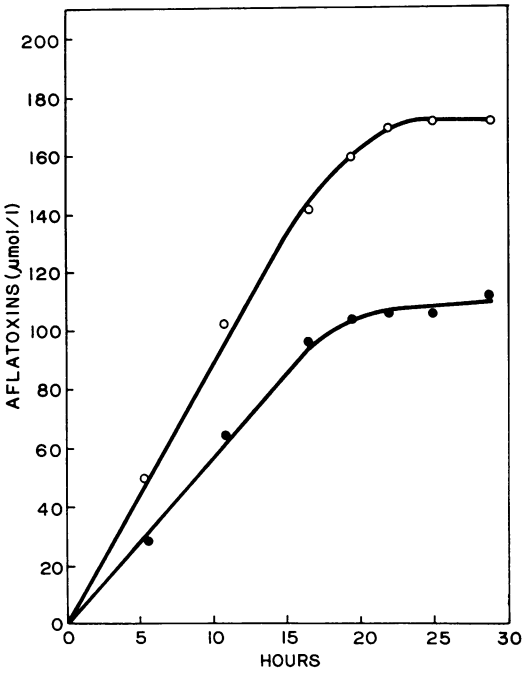

FIG. 2. *Alfatoxin production by the resting cells of Aspergillus parasiticus in 100 ml of medium contained in a 500-ml shaken flask. The cells used were obtained from the growing cultures at 48th hr of incubation.*

Cell concentration. During the 20 hr of active synthesis, the rate of de novo synthesis of aflatoxins would be proportional to the cell concentration if no other factors were limiting. The effect of cell concentration on the relative rates of aflatoxin production in the 10-ml culture in a 50-ml baffled flask is shown in Fig. 3. A linear increase in rate was observed up to a cell concentration of 1.5 g of fresh cells (0.183 g, dry mass) per 10 ml; at higher cell concentrations, the rate dropped drastically. This was possibly due to oxygen limitation, because molecular oxygen has been shown to be a substrate in the biosynthesis of aflatoxins (D. P. H. Hsieh and B. J. Johnson, 160th Nat. ACS Meeting, Chicago, Ill.). In 100-ml cultures in 500-ml baffled flasks, this linear increase was observed only up to a cell concentration of 100 g/liter. Figure 2 shows that the rate of toxin production at a cell concentration of 100 g/liter was slightly lower than twice the rate at a cell concentration of 50 g/liter.

Relative contribution of acetate and glucose. When used as a sole carbon source for the resting cell cultures, acetate is a precursor of aflatoxins; its incorporation into aflatoxins was only 0.5%. Incorporation of acetate in the presence of various levels of glucose concentration (Table 2) shows that a small amount of glucose (e.g., 0.5 mg/ml) increased the incorporation percentage more than fourfold and that both the yield of aflatoxins and the incorporation of acetate were maximal when the initial glucose concentration was 30 g/liter. Therefore, glucose is required for a high de novo synthetic activity. The considerable amount of aflatoxins produced by the culture in the absence of glucose indicates a significant quantity of endogenous precursors in the cells harvested after 48 hr of cultivation. These endogenous precursors are an important diluting factor in the labeling of aflatoxins.

The relative contribution of acetate and glucose to the biosynthesis of aflatoxins has been reported previously (7). It was found that (i) acetate is preferentially incorporated into aflatoxins despite the presence of glucose at a concentration four times higher than that of acetate; (ii) acetate appears to inhibit competitively the incorporation of carbon atoms from glucose into aflatoxins; (iii) when the molar ratio of acetate to

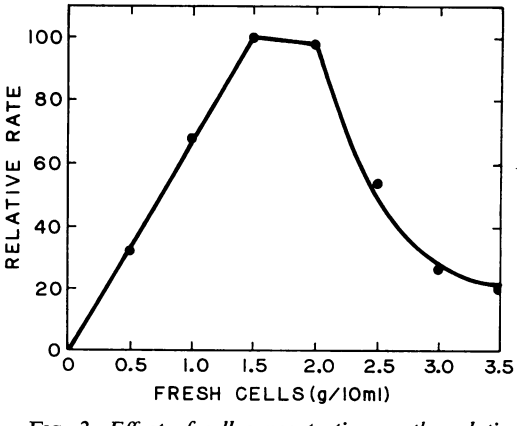

FIG. 3. *Effect of cell concentration on the relative rate of aflatoxin production in 50-ml shaken flasks.*

TABLE 2. *Effect of glucose concentration on the de novo synthesis of aflatoxins*[a]

Glucose concn (g/liter)	Aflatoxins produced (μmole/flask)	Incorporation efficiency of acetate-*1*-^{14}C (%)	RIC[b] of product
0	0.38	0.50	0.13
0.5	0.51	2.01	0.39
5	0.92	2.19	0.24
10	0.95	2.20	0.23
20	1.27	2.18	0.17
30	1.30	2.16	0.17
40	1.30	2.17	0.17
50	1.27	2.17	0.17

[a] Each flask contained 1 g of fresh cells in 10 ml of the replacement medium and was incubated for 10 hr. The concentration of acetate was 1 mM.

[b] Relative isotope content.

TABLE 3. *Effect of acetate concentration on the incorporation efficiency*[a]

Concn of acetate-*1*-^{14}C (mM)	Aflatoxins produced (μmole/flask)	Incorporation efficiency of acetate-*1*-^{14}C (%)	RIC[b] of product
0.5	1.20	0.91	0.04
1.0	1.17	2.22	0.19
5.0	1.19	2.20	0.92
10.0	0.80	1.52	1.90
20.0	0.64	0.91	2.85

[a] Each flask contained 0.5 g (2,780 μmole) of glucose and 1 g of fresh cells suspended in 10 ml of the replacement medium and was incubated for 11 hr.

[b] Relative isotope content.

glucose is 1:4 or higher, the newly formed aflatoxins would be almost exclusively derived from acetate, giving an RIC value close to the theoretical maximum of 9; and (iv) the maximal uptake rates of acetate and glucose are about equal, 1.6 mmolar per hr at a cell concentration of 50 g of fresh mycelia per liter.

Based on the above findings, it is evident that the percentage of acetate incorporation, which is proportional to the de novo synthetic activity, is determined by the glucose concentration of the culture, whereas the specific activity of the product, which is proportional to RIC, is determined by the concentration of the labeled acetate used. The effect of the acetate concentration on the incorporation efficiency is shown in Table 3. High incorporation efficiency was concurrent with initial acetate concentrations lower than 10 mM. In this range, RIC was proportional to the acetate concentration, suggesting that acetate is a tracer rather than a substrate (6). Therefore, in the preparation of labeled aflatoxins from labeled acetate, the acetate concentration should be kept below 10 mM, for otherwise the incorporation percentage would decline and the acetate would become a substrate which may alter the mechanism of the biosynthesis. An RIC value of 2.8 obtained at a glucose-acetate ratio as high as 2,780 to 200 shows clearly the preferential incorporation of acetate and the possibility of production of labeled aflatoxin with a specific activity several times higher than the labeled acetate used. However, due to the dilution effect of the endogenous precursors mentioned above, it is impossible to obtain a product with an RIC equal to 9.

Continuous feeding of labeled acetate. In a 10-ml culture containing 1 g of fresh mycelium, the uptake rate of acetate is calculated to be 32 μmoles/hr (*see above*). If the labeled acetate were added at the same rate, there would be no accumulation of acetate, and any desired starting concentration of acetate can be maintained throughout incubation. In this way, 640 μmoles of acetate can be used in the 20-hr active period. The process was simulated by adding acetate intermittently to the cultures. In one experiment, acetate was added with equal molar amounts of glucose so that at no time was glucose in excess; alternatively, the cultures were started with an initial glucose concentration of 30 g/liter, and acetate was added subsequently. The results (Table 4) show that the incorporation percentage

TABLE 4. *Production of* ^{14}C*-aflatoxins from acetate-1-*^{14}C[a]

Acetate-*1*-^{14}C added		Glucose added		Aflatoxin produced (μmole)	Incorporation efficiency of acetate-*1*-^{14}C (%)	RIC[b] of product
Amt (μmole)	No. of additions	Amt (μmole)	No. of additions			
0	0	1,667	1	3.54		
70	6	1,667	1	3.66	2.16	0.414
350	6	1,667	1	3.74	2.28	2.14
350	6	1,667	1	3.68	2.69	2.56
700	6	1,667	1	3.58	2.00	3.90
0	0	0	0	0.206		
50	1	50	1	0.370	1.89	2.55
100	2	100	2	0.400	1.21	3.03
150	3	150	3	0.400	1.18	4.43
200	4	200	4	0.440	1.05	4.78
250	5	250	5	0.450	0.80	4.45

[a] Each flask contained 1 g of fresh cells suspended in 10 ml of the replacement medium and was incubated for 20 hr. Addition of precursors was made at equal time intervals.

[b] Relative isotope content.

is twice as high when the glucose is in excess, but high RIC of product can be assured by using the equimolar mixture of acetate and glucose.

Preparative runs. A preparative run was made to incorporate acetate-*1*-^{13}C into aflatoxin B_1 for the nuclear magnetic resonance (NMR) analysis of the product to verify its labeling pattern. Five 500-ml baffled flasks, each containing 100 ml of the replacement medium, 2 g of fresh mycelia, and 16.67 mmoles (3 g) of glucose, were used for the incorporation. To each culture, 1,632 μmoles of sodium acetate-*1*-^{13}C labeled with 6 μCi of acetate-*1*-^{14}C (0.1 μmole) was added at six 3-hr intervals (each addition being equal to 272 μmoles). The acetate-*1*-^{14}C was used to determine the incorporation percentage and the RIC of the product. After a total of 21 hr of incubation, 38 μmoles (11.4 mg) of crude aflatoxins was extracted from the combined broths, purified by the two TLC systems described above, and then precipitated out. The purified aflatoxin B_1 was redissolved in deuterated chloroform and subjected to NMR analyses. From the optical density of the solution, it was calculated that 7 mg of purified ^{13}C-aflatoxin B_1 was obtained. (RIC of the product was 2.66.)

Another preparative run was carried out in 50-ml baffled flasks. Each flask contained 1 g of fresh mycelia suspended in 10 ml of the replacement medium. After addition of 5 mg (27.8 μmoles) of glucose and 17.5 μmoles of acetate-*1*-^{14}C with a specific activity of 57 mCi/mmole, the culture was incubated for 10 hr. From 1 mCi of the labeled acetate, 20 μCi of purified aflatoxin B_1 was obtained, which has a specific activity of 84 mCi/mmole corresponding to an RIC of 1.47.

Aflatoxins labeled with tritium have been prepared by several chemical methods (8). In all processes, most of the aflatoxins were decomposed during processing, and the purification of the tritiated aflatoxins was very difficult. The specific activity of the purified product was on the order of 100 mCi/mmole. The present biological method provides an alternative way to prepare labeled aflatoxins and has several advantages. The incorporation and purification processes are comparatively easy at a better material economy. Since *A. parasiticus* produces four major aflatoxins, B_1, B_2, G_1, and G_2, the four labeled compounds can be obtained from a single process. A specific activity of the product as high as 200 mCi/mmole is easily attainable. Since the position and the type of the precursor acetate will dictate the label in the aflatoxins, this method can be employed to prepare aflatoxins labeled with ^3H, ^{13}C, ^{14}C, or ^{18}O. Finally, since nontoxic precursors are employed in the biological method, the potential hazard of exposure to large amounts of aflatoxins can be prevented.

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