BIOSYNTHETIC PRODUCTION OF [^{14}C]AFLATOXINS

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Received July 28, 1977
Revised September 7, 1977

SUMMARY

A method for producing large amounts of ring-labeled [^{14}C] aflatoxins using Aspergillus parasiticus cultures is reported. Conditions were optimized as to mold incubation medium, time of incubation, and ^{14}C-acetate: glucose ratio. The yield of labeled aflatoxins was 1.2% of the [1-^{14}C] acetate precursor; 60% of this was aflatoxin B_1 having a specific radioactivity of 158.5 μCi/μmole.

Key Words: ^{14}C-aflatoxin, Mycotoxin, Aspergillus

INTRODUCTION

Aflatoxins are a group of metabolites produced mainly by several members of the genus Aspergillus. Interest in the carcinogenic properties of these compounds has created a need for a method of preparing [^{14}C]aflatoxins for use as tracers in biochemical and metabolic studies. A number of previous studies have concerned themselves with this problem (1-6), but most had low yields and/or low specific activity products. By employing 3 day old yeast-like cultures of Aspergillus parasiticus, we have devised a method of producing millicurie amounts of high specific activity ring-labeled [^{14}C]aflatoxins from [1-^{14}C]acetate in reasonable yield.

MATERIALS AND METHODS

Spores of Aspergillus parasiticus NRRL 2999 (the generous gift of D. Fennell, R. Detroy and E. Lillehoj, Northern Regional Research Lab., U.S.D.A., Peoria, Ill., U.S.A.) were cultured at room termperature on potato-dextrose-agar

0362-4803/78/0015-0041$01.00
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slants (10 g dextrose, 200 g old white diced potatoes and 18 g agar/liter of distilled water) (7) to induce sporulation. Spores were harvested from 5 to 7 day slant cultures by covering them with sterile water and agitating with an inoculation loop. The spore suspension was either used directly to inoculate the basal culture media or placed in sterile vials which were lyophilized and stored at -20°C for later use. The basal culture medium used to grow A. parasiticus from spores contained (per liter of distilled water): glucose, 50 g; $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 10 g; $MgSO_4 \cdot 7H_2O$, 2 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5 mg; $Fe_2(SO_4)_3 \cdot 6H_2O$, 0.3 mg; $CuSO_4 \cdot 5H_2O$, 0.3 mg; $ZnSO_4 \cdot 7H_2O$, 15.0 mg; boric acid, 1.0 mg; and $MnCl_2 \cdot 4H_2O$, 0.4 mg (8). This medium was inoculated with 10^6 spores/ml and incubated for 72 hours in a cotton stoppered Erlenmeyer flask. The incubation was done in the dark on an enclosed rotary shaker set at 100 r.p.m. and 28 ± 1°. The mycelia grew in white yeast-like balls which were collected by filtration through cheesecloth and washed twice with distilled water.

For [^{14}C]aflatoxin synthesis the mycelia were suspended in fresh culture medium (adjusted to pH 6.0 with H_2SO_4) supplemented with 10 mM $ZnSO_4$ and [1-^{14}C] acetate at the concentration indicated, and incubated as described above in open Erlenmeyer flasks (9). Preliminary tests showed that aflatoxin synthesis was greatest at a mycelial concentration of 0.1 g wet weight/ml (test range 0.025 to 0.2 g/ml). The incubation was stopped at 24 hr (unless otherwise indicated) and the mycelia separated from the broth by filtration through cheesecloth. Both fractions were extracted 4 times with an equal volume of chloroform: methanol (9:1, v/v), using a blender in the case of the mycelial fraction.

The organic extracts were pooled, evaporated to dryness, and spotted on thin layer chromatography (TLC) plates (Silica GHR-25, Brinkman Instruments, Westbury, N.Y., U.S.A.). The TLC plates were developed twice in the same direction, first with diethylether and then with chloroform: acetone (9:1, v/v). This provides a relatively pure aflatoxin preparation, as most of the contaminating pigments are carried close to the solvent front by the diethylether, while the aflatoxins migrate only during the second development. The aflatoxins were identified on

the basis of co-migration with aflatoxin B_1, B_2, G_1, and G_2 standards and by their characteristic fluorescent color under long wave uv illumination. The aflatoxins were quantitated by scraping the fluorescent spots into vials and counting by liquid scintillation using a xylene-detergent based scintillation fluid (Scintiverse, Fisher Scientific Co., Pittsburg, Pa., U.S.A.).

For larger scale aflatoxin preparation, the original organic extract was streaked on the TLC plates and, following development, the aflatoxin bands were scraped off and extracted with chloroform: acetone: methanol: (8:1:1, v/v/v). The dried extract was dissolved in chloroform and quantitated both by liquid scintillation counting and by absorption at 360 nm, using ε = 20,000 for mixtures of all four aflatoxins (2) and ε = 21,800 for purified aflatoxin B_1 (10).

RESULTS AND DISCUSSION

Label from [1-^{14}C]acetate was incorporated into the aflatoxins in a nearly linear fashion for 24 hr following resuspension of 3-day mycelia in fresh medium (Table 1). The [^{14}C] from this precursor has previously been shown not to be

Table 1. Biosynthetic activity of the mycelia[a] at different culture ages

Incubation Time (hr)	Aflatoxin B_1		Total Aflatoxins[b]		Specific Activity (µCi/µmole)	RI[c]	Final pH
	10^6 DPM	% yield	10^6 DPM	% yield			
6	0.55	0.2	0.85	0.3	3.9	1.2	5.0
12	1.10	0.4	1.80	0.7	6.4	1.9	4.5
24	1.80	0.7	3.05	1.1	7.2	2.1	3.8
48	1.85	0.7	3.15	1.1	4.7	1.3	3.0

[a]Each flask contained 20 ml of basal media, 125 µCi of [1-^{14}C]acetate, (3.34 µCi/µmole) and 2 g of freshly resuspended mycelia. Each number is the average of two determinations.

[b]Total aflatoxins: $B_1 + B_2 + G_1 + G_2$.

[c]RI: Relative incorporation (specific activity of [^{14}C]aflatoxin/specific activity of [^{14}C]acetate).

incorporated into the metabolically labile methoxy group of aflatoxins (12). Incubation for longer periods did not improve the yield of aflatoxins and resulted in

substantially lower specific activity, suggesting that the [^{14}C]acetate was depleted by 24 hr. These results are qualitatively similar to those of other investigators employing both the same and another strain of A. parasiticus (2, 13). Therefore all subsequent incorporation experiments were carried out for 24 hr using freshly resuspended mycelia from 3-day cultures.

Addition of 10 mM zinc sulfate to the incubation medium increased aflatoxin B_1 production by 33% (Table 2). The $ZnSO_4$ was tested because previous investigators (1, 5) had reported it had a beneficial effect on aflatoxin production in

Table 2. Effect of zinc and other factors on the incorporation [1-^{14}C]acetate into aflatoxins

Medium	% Conversion of Acetate to Aflatoxin B_1	% Conversion of Acetate to Total Aflatoxins
Control[a]	0.6	1.1
" at 1/5 normal glucose concentration	0.3	0.4
" - glucose + maltose (50 g/l)	0.6	1.1
" with split addition of ^{14}C acetate[b]	0.6	1.1
" + 10 mM $ZnSO_4$	0.8	1.2
" + 10 mM $ZnSO_4$ with 1/4 normal mycelia inoculum	0.5	0.7
" + 10 mM $ZnSO_4$ with 1/2 normal mycelia inoculum	0.7	1.0
" + 10 mM $ZnSO_4$ with 2 x normal mycelia inoculum	0.8	1.1

[a]Each control flask contained 20 ml of basal incubation medium inoculated with 2 g of mycelia and 125 μCi of [1-^{14}C]acetate, specific activity 3.4 μCi/μmole. The flasks were incubated with shaking at 28 ± 1° for 24 hrs. Each number is the average of two flasks.

[b]Half of the 125 μCi of [^{14}C]acetate was added at the beginning of incubation and the other half was added 12 hrs. later.

two other A. parasiticus strains. In an attempt to increase the yield and specific activity of the aflatoxin the [^{14}C]acetate: glucose ratio was raised by lowering the glucose concentration or replacing it with maltose. Neither of these measures nor administration of the [^{14}C]acetate on a divided dose schedule

improved yields, and, in fact, these procedures resulted in a product with lower specific activity.

To test whether this procedure could be successfully scaled up to produce larger amounts of [^{14}C]aflatoxin, two preparative experiments were carried out, each using five 500 ml Erlenmeyer flasks. Each flask contained 10 g of 3 day-old mycelia freshly suspended in 100 ml of basal medium supplemented with [1-^{14}C] acetate (55.7 μCi/μmole) and 10 mM $ZnSO_4$. The flasks were incubated in the dark at 28 ± 1°C on a rotary shaker (100 r.p.m.) for 24 hours. The overall yield of the two experiments (details are reported in Table 3) showed that an average of 1.2% of the acetate was converted into aflatoxins with the majority of this (0.71%)

Table 3. Distribution and yield of [^{14}C]aflatoxins from mycelia and broth extracts

No.	Fractions	Aflatoxins (yield, μCi)							
		B_1		B_2		G_1		G_2	
		μCi	% yield	μCi	% yield	μCi	% yield	μCi	% yield
I[a]	Broth	824.0	0.41	103.4	0.05	268.3	0.13	142.0	0.07
	Mycelia	495.8	0.25	99.8	0.05	159.4	0.08	94.6	0.05
	Total	1319.8	0.66	203.2	0.10	427.7	0.21	236.6	0.12
II[b]	Broth	705.5	0.43	101.5	0.05	183.0	0.10	128.0	0.06
	Mycelia	564.1	0.34	148.6	0.09	328.1	0.20	203.8	0.12
	Total	1269.6	0.77	250.1	0.14	511.1	0.30	331.8	0.18

[a]Starting material: 200.9 mCi of [1-^{14}C]acetate (7.2 mM).

[b]Starting material: 164.5 mCi of [1-^{14}C]acetate (5.9 mM).

being aflatoxin B_1. This yield is higher than that reported by most previous investigators, in part because they frequently had not extracted the mycelial fraction. The organic extract was streaked on TLC plates and purified by the dual-development method described above (diethylether followed by chloroform: acetone, 9:1,v/v). The radiochemical purity of the purified aflatoxin B_1 was greater than 97% and its specific activity was 158.5 μCi/μmole, a value which

remained constant (± 1.5%) through two further TLC purification steps employing other solvent systems. This 2.85 fold increase in specific activity of the [^{14}C]aflatoxin B_1 over its acetate precursor is 32% of the theoretically achievable value, as [1-^{14}C]acetate has been shown to label nine of the aflatoxin ring carbons (11).

ACKNOWLEDGEMENT

This project was partially supported by NCI grant CA14784 and NIH-GRS grant S01-RR-5359, DHEW.

REFERENCES

1. Adye J. and Mateles R.I. - Biochim. Biophys. Acta 86:418 (1964)
2. Hsieh D.P.H. and Mateles R.I. - Appl. Microbiol. 22:79 (1971)
3. Mabee M.S., Chipley J.R. and Applegate M. - J. Labelled Compd. 9:277 (1973)
4. Schoenhard G.L., Sinnhuber R.O. and Lee D.J. - J. Assoc. Off. Analyt. Chem. 56: 643 (1973)
5. Gupta S.K. and Venkitasubramanian T.A. - Z. Lebensmitt. Untersch. Forsch. 159: 107 (1975)
6. Jackson L.K. and Ciegler A. - J. Env. Sci. Health B11 (4):317 (1976)
7. Manual of Clinical Microbiology, 2nd Ed. American Society for Microbiology (1974)
8. Detroy R.W. and Ciegler A. - J. Gen. Microbiol. 65:259 (1971)
9. Joffe A.Z. and Lisker N. - Appl. Microbiol. 18:517 (1969)
10. Asao T., Buchi G., Abdel Kadar M.M., Chang S.B., Wick E.L. and Wogan G.N.- J. Am. Chem. Soc. 87:882 (1965)
11. Biollaz M., Buchi G. and Milne G. - J. Am. Chem. Soc. 92:1035 (1970)
12. Wogan G.N., Edwards G.S. and Shank R.C. - Cancer Res. 27:1729 (1967)
13. Detroy R.W. and Hesseltine C.W. - Can. J. Microbiol. 16:959 (1970)