Journal of General Microbiology (1974), **80**, 31–36
Printed in Great Britain

Production of Aflatoxins, and Acetate[1-^{14}C] Incorporation, by *Aspergillus parasiticus*

By S. R. GUPTA, H. R. PRASANNA, L. VISWANATHAN* AND T. A. VENKITASUBRAMANIAN

Department of Biochemistry, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi-7, India

(*Received* 5 *January* 1973; *revised* 6 *August* 1973)

SUMMARY

Considerable amounts of aflatoxins were produced when mycelium of *Aspergillus parasiticus* NRRL 3240 grown for 4 days in a synthetic medium (SL medium) or a semi-synthetic medium (YES medium) were resuspended in either medium for 2 days, but not when resuspended in media lacking sucrose, or in buffers. Acetate[1-^{14}C] was incorporated efficiently on addition to cultures grown in YES medium or on addition to mycelium grown and resuspended in SL medium. Changes in pH within the range of 3·5 to 6·5 did not have any pronounced effect on aflatoxin production or on incorporation of labelled acetate by resuspended mycelia.

INTRODUCTION

Several workers have shown that labelled acetate[1-^{14}C] is incorporated into aflatoxins (Adye & Mateles, 1964; Detroy & Hesseltine, 1969; Basappa, Sreenivasamurthy & Parpia, 1970; Biollaz, Büchi & Milne, 1970; Hsieh & Mateles, 1970). However, the media used by them supported only low aflatoxin yields.

A completely synthetic medium giving high yields of aflatoxins has been reported from this laboratory (Reddy, Viswanathan & Venkitasubramanian, 1971): acetate[1-^{14}C] added directly to cultures of *Aspergillus parasiticus* grown on this medium is efficiently incorporated into aflatoxins (Reddy, 1972). As a continuation of these studies, the formation of aflatoxins by *A. parasiticus* grown and resuspended in the synthetic medium has been investigated. The incorporation of acetate[1-^{14}C] into aflatoxins by such resuspended mycelium has also been examined and compared with that by mycelium grown in a semi-synthetic medium.

METHODS

Aspergillus parasiticus NRRL 3240 obtained from the Northern Regional Research Laboratory, Peoria, Illinois, U.S.A., was maintained as a soil culture. A spore suspension in sterile double-distilled water was prepared from 5- to 6-day-old cultures grown on 50 ml of glucose peptone agar and distributed equally among five 500 ml Erlenmeyer flasks, each containing 100 ml of sterile medium.

Two media were used. (i) Yeast extract–sucrose medium (YES medium) (Davis, Diener & Eldridge, 1966): sucrose, 200 g; Difco yeast extract, 20 g; made up to 1 l with double-distilled water. (ii) Synthetic medium (SL medium) (Reddy *et al.* 1971): sucrose, 85 g; asparagine, 10 g; $(NH_4)_2SO_4$, 3·5 g; KH_2PO_4, 750 mg; $MgSO_4.7H_2O$, 350 mg; $CaCl_2.2H_2O$,

* Present address: Department of Biochemistry, National Sugar Institute, Kanpur-17, India.

Table 1. *Aflatoxin production by mycelium grown in YES medium for 4 days and resuspended in different media for 2 days*

Resuspension medium	Aflatoxins in original mycelium (mg)			Increase in aflatoxin content after resuspension (mg)		
	B	G	Total	B	G	Total
YES	2·4	9·6	12·0	2·7	4·1	6·8
SL	2·2	7·8	10·0	2·7	2·2	4·9
0·05 M-phosphate buffer, pH 5·7	1·7	6·2	7·9	0·4	−0·2	+0·2
0·05 M-maleate buffer, pH 5·5	1·7	6·8	8·5	0·5	−0·2	+0·3
0·05 M-succinate buffer, pH 5·5	1·8	7·0	8·8	0·4	−0·6	−0·2

75 mg; $ZnSO_4.7H_2O$, 10 mg; $MnCl_2.4H_2O$, 5 mg; $(NH_4)_6Mo_7O_{24}.4H_2O$ 2 mg; $FeSO_4.7H_2O$, 2 mg; $Na_2B_4O_7$, 2 mg; made up to 1 l with double-distilled water. The pH of the medium was adjusted to 4·5.

The flasks were incubated at 26 ± 1 °C as stationary cultures. After growing for the requisite number of days, mycelium and medium were separated and the mycelium was washed with double-distilled water. In all the resuspension experiments, the washed mycelium was cut into two equal parts and one part transferred to 50 ml of sterile resuspension medium in a 250 ml Erlenmeyer flask and incubated for the requisite period; the other half was not used in the work reported here.

For incorporation studies, acetate[1-^{14}C] (10 μCi) was added directly to the culture or to the resuspension medium. At the end of the experiment, the mycelium and medium were separated. The mycelium was blotted and its wet weight determined. The aflatoxins from the medium and mycelium were extracted with chloroform. The aflatoxins were separated by thin-layer chromatography on silica gel G by using methanol (2%, v/v) in chloroform. All the samples were first run with ether. They were eluted with methanol and estimated spectrophotometrically by measuring extinction at 363 nm (Nabney & Nesbitt, 1965). Aflatoxins B_1 and B_2 were estimated together as B, and aflatoxins G_1 and G_2 were estimated together as G. Radiopurity of aflatoxins was checked by using a number of different solvent systems for aflatoxin separation (Reddy, Viswanathan & Venkitasubramanian, 1970; Ayres, Lee & Sinnhuber, 1971). Counts in methanol eluates were taken with a thin-film gas-flow detector connected to a proportional counting system (Model PCS 14C of Electronics Corp. of India Ltd, Hyderabad, India). The data given are the averages of two separate flasks.

RESULTS AND DISCUSSION

Most reports on the effects of different factors on aflatoxin production have been based on altering the conditions or components of the growth medium. The effects observed could be secondary. A system in which aflatoxins were produced on resuspension of a grown mycelium might be of greater value in studying the effect of different factors on aflatoxin production. The data in this paper show that such a system is experimentally feasible: both YES and SL medium were useful in this respect (Table 1). The presence of sucrose was essential for aflatoxin production by resuspended mycelium (probably because it provided the necessary energy): no aflatoxins were formed when the mycelia were resuspended either in SL medium minus sucrose for 2 days (Table 2), or in different buffers (Table 1).

Table 2. *Aflatoxin production by mycelium grown in SL medium for 4 days and resuspended in different media for 2 days*

Resuspension medium	Aflatoxins in original mycelium (mg)			Increase in aflatoxin content after resuspension (mg)		
	B	G	Total	B	G	Total
YES	1·6	6·4	8·0	4·9	7·6	12·5
SL	2·3	2·9	5·2	3·9	6·4	10·3
SL minus sucrose	3·2	1·8	5·0	+0·1	−0·2	−0·1
SL minus both sucrose and asparagine	3·9	2·0	5·9	−0·2	+0·3	+0·1
SL plus 2% yeast extract	3·2	2·3	5·5	3·5	9·4	12·9
SL plus 2% yeast extract minus sucrose	2·0	1·8	3·8	0·2	0·1	0·3

Table 3. *Radiopurity of [^{14}C] aflatoxins**

Solvent system	Radioactivity incorporated into aflatoxin B (c.p.m.)	Radioactivity incorporated into aflatoxin G (c.p.m.)
1. $CHCl_3$:MeOH, (98:2, v/v)	1436	446
2. Toluene:isoamyl alcohol: methanol (90:32:3, by vol.)	1518	562
3. $CHCl_3$:acetone (9:1, v/v)	1411	458
4. Benzene:EtOH:H_2O (46:35:19, by vol.)	1502	573
5. $CHCl_3$:acetone (95:5, v/v). Dried at 90 °C and again with $CHCl_3$:acetone (9:1, v/v)	1614	997

* Equal samples of the chloroform extract containing aflatoxins were subjected to t.l.c. The chromatoplates were run with ether to eliminate the pigments which were carried with the ether front before using the different solvent systems mentioned above.

In view of the risk of the presence of highly radioactive impurities (Ayres *et al.* 1971), several solvent systems were used to study the pattern of acetate[1-^{14}C] incorporation (Table 3). That observed with 2% methanol in chloroform was lower, indicating that the aflatoxins had no radioimpurity. The absorption characteristics of standard aflatoxin and the sample were studied in the range 240 to 500 nm and only two peaks at 265 and 363 nm were obtained in both instances. The ^{14}C-labelled aflatoxins B and G were recrystallized twice from hexane and their specific activities were determined and found to be constant, further confirming the radiopurity of the aflatoxins.

In the work described below, differences were noted from the results reported by Reddy (1972), who used direct addition of labelled acetate to SL medium. The data obtained on the addition of labelled acetate for 3 h to cultures grown for different periods on YES medium are presented in Table 4. There was a gradual increase in wet weight of mycelium and in aflatoxin content with the age of the culture. Incorporation into aflatoxins was maximal on the fourth day and decreased thereafter. On the other hand, with mycelium grown in SL medium, maximum incorporation was observed on the 5th day (Reddy, 1972). Acetate[1-^{14}C] was incorporated more into aflatoxins in the mycelium than into those in the

Table 4. *Incorporation of acetate*[1-^{14}C] *into aflatoxins in a period of* 3 *h after direct addition of acetate*[1-^{14}C] (10 μ*Ci*) *to cultures grown on YES medium for different times*

Days of growth	Mycelial wet wt (g)	Total aflatoxin content (mg)	Radioactivity incorporated into total aflatoxins (c.p.m.)	Specific activity (c.p.m./mg aflatoxin)					
				Medium		Mycelium		Total	
				B	G	B	G	B	G
2	1·7	0·8	7320	0	0	46000	34222	12600	5106
3	5·7	10·8	55270	103	618	9731	8054	6288	4137
4	8·2	16·2	68765	1100	344	23795	2307	13470	1858
6	12·8	23·3	13870	180	130	3022	884	1319	343
8	13·3	26·7	11655	590	157	1355	406	876	218
11	16·2	34·4	12460	965	192	559	494	546	257

Table 5. *Incorporation into aflatoxins at different periods after direct addition of acetate* [1-^{14}C] (10 μ*Ci*) *to cultures grown on YES medium for* 4 *days*

Period (h)	Total aflatoxin content (mg)	Radioactivity incorporated into total aflatoxins (c.p.m.)	Specific activity (c.p.m./mg aflatoxin)					
			Medium		Mycelium		Total	
			B	G	B	G	B	G
0·25	16·6	13525	259	41	1508	1202	1171	716
0·5	23·9	24090	94	48	1471	1482	1053	855
1	20·3	22280	204	109	1204	1789	852	1277
2	18·1	22170	303	59	2117	2020	1172	1005
3	16·4	17930	436	170	2122	1609	1523	1089
4	16·2	29260	436	271	3187	2753	2028	1691
6	18·9	61145	1286	1448	4188	4332	3045	3003
8	24·8	65770	1372	1445	5452	3469	3901	2356
16	30·3	48880	2140	1733	2113	1278	2098	1452
24	20·5	56640	2279	1877	4186	3256	3482	2442

medium. Specific activities were very high on the second day owing to low aflatoxin content, reached a second maximum on the fourth day, and then decreased with age of the culture.

When 4-day-old cultures on YES medium were incubated with acetate[1-^{14}C] for different periods of time, there was an increase in incorporation from 15 min to 30 min; thereafter the radioactivity in total aflatoxins remained more or less constant up to 4 h, with a further increase between 4 and 6 h (Table 5). Reddy (1972) observed maximum incorporation in 3 h with 5-day-old cultures on SL medium. Total specific activities were maximal at 6 to 8 h. The incorporation and specific activities were again much higher in the mycelium than in the medium.

With mycelium grown in YES medium and resuspended in a synthetic medium, Detroy & Hesseltine (1969) observed an increase in the extent of incorporation even up to 40 h. Similarly, with mycelium grown and resuspended in SL medium the incorporation of acetate[1-^{14}C] into aflatoxins was nearly constant from 2 to 8 h and then increased from 8 to 40 h (Table 6). The increase in counts during the later period was apparently because of synthesis of significant amounts of aflatoxins.

The production of aflatoxins can be markedly affected by changes in the initial pH of the medium (Reddy *et al.* 1971). If the mycelium was grown in SL medium with an initial pH of

Table 6. *Incorporation into aflatoxins by mycelia grown in SL media for 4 days and resuspended in SL media containing acetate[1-^{14}C] (10 μCi) for different periods*

Period (h)	Total aflatoxins after resuspension (mg)	Radioactivity incorporated into total aflatoxins (c.p.m.)	Specific activity (c.p.m./mg aflatoxin)					
			Medium		Mycelium		Total	
			B	G	B	G	B	G
1	1·6	15825	9204	7171	14544	8821	12058	8318
2	2·1	30775	5757	5302	29648	15345	23805	11150
3	2·1	27575	3939	7172	24733	14561	20460	11205
5	2·1	31475	5038	5888	33022	20903	22530	11650
8	2·2	32500	5289	8807	40699	14063	21310	8991
16	3·7	40300	5784	15839	21742	8050	15280	12250
24	3·0	43500	8449	12424	31140	8968	20745	10490
40	5·1	52850	7056	11439	17449	5382	13810	8181

Table 7. *Effect of pH of resuspension medium on the incorporation into aflatoxins by mycelia grown for 4 days in SL media and resuspended in SL media containing actate[1-^{14}C] (10 μCi) for 2 h*

pH	Total aflatoxins after resuspension (mg)	Radioactivity incorporated into total aflatoxins (c.p.m.)	Specific activity (c.p.m./mg aflatoxin)					
			Medium		Mycelium		Total	
			B	G	B	G	B	G
2·5	6·5	14475	866	1422	2986	2381	2346	2049
3·5	8·0	25025	810	2866	3286	4689	2760	3475
4·5	7·5	24575	479	2065	3217	5000	2663	3943
5·5	5·6	28500	1135	5262	4489	8010	3664	7317
6·5	6·0	20375	613	2241	3189	7479	2946	5119

4·5 and then resuspended in SL medium having pH values varying from 2·5 to 6·5, incorporation was not affected by the pH value except for a slight decrease at pH 2·5 (Table 7). A similar pattern was observed for specific activities. With mycelium grown for 4 days and resuspended for 2 days in YES medium, aflatoxin production and incorporation of acetate-[1-^{14}C] were also not affected within the pH range 3·5 to 6·5 (data not presented). This contrasts with the findings of Reddy (1972), who observed that with mycelium grown in SL medium initial pH of the growth medium has a pronounced effect on incorporation.

Appreciable amounts of acetate[1-^{14}C] were incorporated into aflatoxins within a few hours.

This work was supported in part by a PL-480 grant no. FG-In-438.

REFERENCES

ADYE, J. & MATELES, R. I. (1964). Incorporation of labelled compounds into aflatoxins. *Biochimica et biophysica acta* **86**, 418–420.

AYRES, J. L., LEE, D. J. & SINNHUBER, R. O. (1971). Preparation of ^{14}C- and ^{3}H-labelled aflatoxins. *Journal of the Association of Official Analytical Chemists* **54**, 1027–1031.

BASAPPA, S. C., SREENIVASAMURTHY, V. & PARPIA, H. A. B. (1970). Aflatoxin and kojic acid production by resting cells of *Aspergillus flavus* Link. *Journal of General Microbiology* **61**, 81–86.

BIOLLAZ, M., BÜCHI, G. & MILNE, G. (1970). The biosynthesis of the aflatoxins. *Journal of the American Chemical Society* **92**, 1035–1043.

DAVIS, N. D., DIENER, U. L. & ELDRIDGE, D. W. (1966). Production of aflatoxins B_1 and G_1 by *Aspergillus flavus* in a semisynthetic medium. *Applied Microbiology* **14**, 378–380.

DETROY, R. W. & HESSELTINE, C. W. (1969). Net synthesis of ^{14}C-labelled lipids and aflatoxins in resting cells of *Aspergillus parasiticus*. *Developments in Industrial Microbiology* **10**, 127–133.

HSIEH, D. P. H. & MATELES, R. I. (1970). The relative contribution of acetate and glucose to aflatoxin biosynthesis. *Biochimica et biophysica acta* **208**, 482–486.

NABNEY, J. & NESBITT, B. F. (1965). A spectrophotometric method for determining the aflatoxins. *Analyst* **90**, 155–160.

REDDY, T. V. (1972). *Metabolic studies on Aspergillus flavus*. Ph.D. thesis, Delhi University, Delhi, India.

REDDY, T. V., VISWANATHAN, L. & VENKITASUBRAMANIAN, T. A. (1970). Thin layer chromatography of aflatoxins. *Analytical Biochemistry* **38**, 568–571.

REDDY, T. V., VISWANATHAN, L. & VENKITASUBRAMANIAN, T. A. (1971). High aflatoxin production on a chemically defined medium. *Applied Microbiology* **22**, 393–396.