A Comparative Study of the Incorporation of [1-¹⁴C]Acetate into Phospholipids by a Toxigenic and a Non-toxigenic Strain of *Aspergillus flavus*

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SUMMARY

The incorporation of [1-¹⁴C]acetate into phospholipids by a toxigenic and a non-toxigenic strain of *Aspergillus flavus* was investigated, using a glucose salts medium (AM medium) and a sucrose yeast extract medium (YES medium) for growth and for the incorporation of radioactivity. YES medium yielded large amounts of aflatoxins. The toxigenic strain gave a higher incorporation of acetate in AM medium and a lower incorporation in YES medium than did the non-toxigenic strain. The growth and resuspension media strongly influenced the incorporation by the toxigenic strain. The specific activity obtained on AM medium was more than 10 times that on YES medium. The effects of the media on the non-toxigenic strain were less marked. The data indicate a close interrelation between the utilization of acetate for the synthesis of phospholipids and for the formation of aflatoxins.

INTRODUCTION

Aflatoxins, a group of toxic metabolites produced by *Aspergillus flavus*, have been attracting a large number of investigators because of their toxicity and carcinogenicity to a number of experimental animals, and because of the need to prevent their formation in agricultural products (Wogan, 1966; Goldblatt, 1967; Schoental, 1967.) Though a considerable amount of work has been done on their chemistry and biological effects and the factors affecting their formation, no systematic comparison of the metabolism of toxigenic and non-toxigenic strains of *A. flavus* seems to have been carried out. As a part of such a comparative study, the incorporation of [1-¹⁴C]acetate into phospholipids by two strains of *A. flavus* was investigated, using two media which differed markedly in their ability to support the production of aflatoxins. The data obtained suggest a possible relation between lipid biosynthesis and aflatoxin formation.

METHODS*

Aspergillus flavus ATCC 15517, a toxigenic strain, and *A. flavus* ATCC 9643, a non-toxigenic strain were obtained from the Northern Regional Research Laboratory,

* The following abbreviations are used: AM medium, glucose salts medium; YES medium-sucrose yeast extract medium; PI, phosphatidyl inositol; PS, phosphatidyl serine; LPC, lysophosphatidyl choline; LPE, lysophosphatidyl ethanolamine; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PGP, polyglycerophosphatide; NADPH₂, reduced nicotinamide adenine dinucleotide.

Peoria, Illinois, U.S.A., and maintained as soil cultures. Sodium [$1-^{14}\text{C}$]acetate (specific activity 12.66 mCi/mm) was obtained from the Bhabha Atomic Research Centre, Bombay. The liquid media used had the following composition. Glucose salts medium (AM medium) (Adye & Mateles, 1964): Glucose, 50 g.; ammonium sulphate, 3 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg.; ammonium molybdate $4\text{H}_2\text{O}$, 0.5 mg.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.3 mg.; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.11 mg.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 17.6 mg.; $\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O}$, 10 mg.; $\text{Na}_2\text{B}_2\text{O}_7$, 0.7 mg./l.; pH 6.6. Sucrose yeast extract medium (YES medium) (Davis, Diener & Eldridge, 1966): Sucrose, 200 g.; Difco yeast extract, 20 g./l., pH 6.4.

Culture methods. The *Aspergillus* strains were grown first in glucose peptone agar slants and then in 50 ml. of the same medium in bottles. After 1 week, the spores from this growth were used to inoculate sufficient 100 ml. quantities of each of the two liquid media, AM and YES, in 500 ml. Erlenmeyer flasks which were then incubated on a rotary shaker for 3 days at 25° .

Incorporation of [$1-^{14}\text{C}$]acetate. The mycelium from each flask was separated by filtration under suction. Each mycelial mat was then washed well with distilled water, blotted and weighed. They were then resuspended in 50 ml. of sterile AM or YES medium, containing 10 μCi of sodium [$1-^{14}\text{C}$]acetate in 250 ml. Erlenmeyer flasks and incubated for 3 h. on a rotary shaker at 25° . Preliminary experiments indicated that maximum incorporation was obtained at 3 h. The mycelium was separated from the medium, washed well and the lipids extracted by grinding with sand and chloroform-methanol (2:1, v/v). Proteolipids were dissociated by the method of Folch, Lees & Stanley (1957) and the final lipid extract made up to a known volume in chloroform.

Separation and determination of phospholipids. Phospholipids were separated by thin layer chromatography on Silica gel G with chloroform-methanol-7M-ammonium hydroxide (46:18:3, by vol.) (Abramson & Blecher, 1964). The phospholipid spots were detected by exposure to iodine vapours, scraped out and eluted by a mixture of ethanol-chloroform-acetic acid-water (50:15:10:1, by vol.) as described by Biezenski (1967). The identity of the phospholipids was established by comparison with known phospholipid standards and by suitable spray reagents. The eluates were evaporated to a small volume under reduced pressure and a sample was used for the determination of phosphorus by the method of Bartlett (1959) as modified by Marinetti (1962). Another sample was dried in stainless steel planchets and the radioactivity determined in a Tracerlab Windowless gas-flow counter.

RESULTS

The total phospholipid content and the incorporation of radioactivity into total phospholipids are presented in Table 1. The total phospholipid content of the mycelia of the non-toxicogenic strain grown in YES medium was less than that of mycelia grown in AM medium. No clear-cut differences were observed with the toxicogenic strain.

When AM medium was used for both growth and resuspension, the total incorporation and specific activity obtained with the toxicogenic strain were respectively 2.9 times and 2.3 times the values given by the non-toxicogenic strain. On the other hand, with YES medium the toxicogenic strain yielded values that were respectively 0.67 and 0.33 of those given by the non-toxicogenic strain. The growth and resuspension media had a pronounced effect on the incorporation of [$1-^{14}\text{C}$]acetate into phospholipids in the case of the toxicogenic strain, AM medium giving more than 10 times the incorporation

and specific activity obtained with YES medium. The differences were smaller with the non-toxicogenic strain. When the different combinations of media given in Table 1 are considered, it is clear that the medium had a marked effect on the toxigenic strain and that there was a gradual decrease in specific activity in the order given in Table 1. With the non-toxicogenic strain, somewhat higher specific activity was obtained when

Table 1. *Incorporation of [^{14}C]acetate into the total phospholipids by *Aspergillus flavus**

Mycelia grown for 3 days on AM or YES medium were resuspended in one or other of these media containing 10 μCi of [^{14}C]acetate and incubated for 3 h. on a rotary shaker at 25°. All the experiments were performed in duplicate and average values are presented in this table.

Growth medium	Resuspension medium	Total phospholipids as $\mu\text{g. phosphorus/g. wet weight of mycelium}$	Radioactivity incorporated counts/min./g. wet weight of mycelium	Specific activity counts/min./ $\mu\text{g. phosphorus}$
Toxigenic strain ATCC 15517				
AM	AM	189	283,900	1,504
AM	YES	110	64,650	589
YES	AM	147	48,230	329
YES	YES	172	25,320	148
Non-toxicogenic strain ATCC 9643				
AM	AM	151	97,990	649
AM	YES	137	55,670	407
YES	AM	67	32,260	478
YES	YES	85	37,870	447

Table 2. *Incorporation of [^{14}C]acetate into individual phospholipids by the toxigenic strain *Aspergillus flavus* ATCC 15517*

Incorporation of [^{14}C]acetate was carried out as described in Table 1. Phospholipids were separated by thin layer chromatography on Silica gel G with chloroform-methanol-7M-ammonium hydroxide (46:18:3, by vol.). All the experiments were performed in duplicate and average values are presented.

Growth medium	Resuspension medium	PI	PS	LPE	PC	PE	PGP
Radioactivity incorporated counts/min./g. wet weight of mycelium							
AM	AM	8,879	34,650	72,400	72,870	84,360	10,770
AM	YES	4,283	7,173	10,903	25,740	13,530	3,019
YES	AM	2,299	8,881	5,218	18,910	11,830	1,094
YES	YES	1,042	2,188	4,104	9,134	8,274	579
Specific activity counts/min./ $\mu\text{g. phosphorus}$							
AM	AM	796	1,368	1,926	1,108	2,297	885
AM	YES	853	808	1,014	670	329	551
YES	AM	347	541	388	301	312	113
YES	YES	170	117	243	126	168	73

PI = phosphatidyl inositol; PS = phosphatidyl serine; LPE = lysophosphatidyl ethanolamine; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PGP = polyglycerophosphatide.

AM medium was used for growth and resuspension. The specific activities given by the other media combinations did not differ much among themselves.

In general, the total incorporation and the specific activities of the individual phospholipids showed a pattern similar to that obtained for the total phospholipids (Tables 2, 3). For most phospholipids, the toxigenic strain yielded higher total counts and specific activities on AM medium and lower incorporation on YES medium than did the non-toxicogenic strain. AM medium gave higher amounts of radioactivities with both strains, but the differences between the two media were much larger in the case of the toxigenic strain. Again, there was a decrease in the incorporation of [^{14}C]acetate into the individual phospholipids by the toxigenic strain on the different media combinations in the order given in Table 2. Such a regular change in radioactivity was not observed in the case of the non-toxicogenic strain (Table 3).

Table 3. *Incorporation of [^{14}C]acetate into individual phospholipids by the non-toxicogenic strain Aspergillus flavus ATCC 9643.*

Incorporation of [^{14}C]acetate and separation of phospholipids were carried out as described in Tables 1 and 2. All the experiments were performed in duplicate and average values are presented.

Growth medium	Resuspension medium	PI	PS	LPE	PC	PE	PGP
Radioactivity incorporated counts/min./g. wet weight of mycelium							
AM	AM	10,150	13,070	17,010	33,460	22,210	2,093
AM	YES	1,221	12,150	7,065	13,010	19,310	2,914
YES	AM	1,311	2,980	3,663	11,360	12,380	572
YES	YES	1,549	2,920	6,277	12,280	14,150	791
Specific activity counts/min./ μg . phosphorus							
AM	AM	415	727	1,141	795	674	112
AM	YES	131	702	346	246	693	339
YES	AM	222	754	456	432	680	110
YES	YES	398	620	550	332	585	227

PI = phosphatidyl inositol; PS = phosphatidyl serine; LPE = lysophosphatidyl ethanolamine; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PGP = polyglycerophosphatide.

In all the experiments, the highest incorporation was obtained in phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) and the next highest in lysophosphatidyl ethanolamine (LPE).

DISCUSSION

AM and YES media were chosen for this investigation, since the latter gives much higher yields of aflatoxins than AM medium. It was hoped that any relation between aflatoxin formation and phospholipid synthesis would be brought out clearly by a comparison of the two strains of *Aspergillus flavus* on these media.

The toxigenic strain was more efficient in synthesizing phospholipids and gave much higher specific activity than the non-toxicogenic strain on AM medium (Table 1). But, under conditions of high toxin production on YES medium, it yielded one-third the specific activity obtained with the non-toxicogenic strain, suggesting that [^{14}C]acetate was diverted to the formation of aflatoxins and hence the incorporation into phospholipids was reduced.

Both the growth and resuspension media had a very pronounced effect on the incorporation of acetate by the toxigenic strain (Table 1). Since their effect on the non-toxigenic strain was much less, the results obtained on the two media appear to arise from the differences in their ability to support toxin production. The gradual change in the specific activities yielded by the different combinations of media, presented in Table 1, indicate clearly that both the medium on which the fungus was grown and the one in which the incorporation was carried out had a marked effect on the extent of incorporation. AM medium, whether used as growth or resuspension medium, yielded higher specific activity than YES medium. In the case of the non-toxigenic strain, the differences between the different media combinations were not so clear cut, supporting the above reasoning, since this strain does not produce any aflatoxins.

The incorporation of [$1-^{14}\text{C}$]acetate into the individual phospholipids with different media combinations was similar to that into the total phospholipids, showing that the differences observed were not specific for any of the individual phospholipids (Tables 2, 3). PC and PE are the major phospholipids present in *Aspergillus flavus* and in most of the experiments they exhibited the maximum specific activity. LPE, which is probably an intermediate in the synthesis of PE, also incorporated considerable amounts of radioactivity. Lysophosphatidyl choline (LPC) was not detected in the lipid extract of this fungus.

Adye & Mateles (1964) have investigated the incorporation of different labelled substrates into aflatoxins and have shown that acetate is efficiently incorporated. The labelling patterns obtained with [$1-^{14}\text{C}$] and [$2-^{14}\text{C}$]acetate have also been worked out, and it has been suggested that acetate is first converted into a polyacetate compound, which by means of a series of ring closures and rearrangements gives rise to aflatoxins (Biollaz, Büchi & Milne, 1968). Since acetate is a precursor of a number of normal metabolites, competition may be expected between the production of such compounds and secondary metabolites, such as aflatoxins. Evidence that this process is under the control of regulatory mechanisms has accumulated in recent years (Tanenbaum, 1967). For example, an enzyme preparation from *Alternaria tenuis* incorporated radioactive precursors into lipids in media of low ionic strength in the presence of NADPH_2 . In media of high ionic strength or in the absence of NADPH_2 there was practically no incorporation into lipids, but the secondary metabolite alternariol was labelled (Gatenbeck & Hermodsson, 1965). It is possible that a similar control mechanism operates in *Aspergillus flavus* and led to the differences observed in the incorporation of acetate into phospholipids by the toxigenic strain in different media.

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