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The Production of Polyacetylenic Compounds by Basidiomycetes: Glucose Conversion

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Polyacetylenic compounds, i.e. compounds containing two or more triple bonds in conjugation, are produced by a variety of (mainly higher) fungi when grown on synthetic or semi-synthetic media. They occur sufficiently frequently (Anchel, 1954, and unpublished observations) to make it apparent that the production of such compounds, though unusual, is not a rare freak of metabolism. The fungal polyacetylenes belong to a class of compounds which includes the complex antibiotics and an enormous variety of other 'natural products', compounds which play no obvious part in the primary metabolic processes of organisms. Despite the great practical and theoretical interest of such 'secondary metabolites', their relationship to the better-known processes, such as glycolysis or protein synthesis, is very little understood. As relatively simple members of this class of compound, and also as containing chemical units of peculiar thermodynamic instability, the fungal polyacetylenes are of especial biochemical interest. A comparison of the structures of the small number of compounds which have been fully investigated does not yet permit any significant conclusions to be drawn, and it may be noted that chains of both odd and even numbers of carbon atoms are represented.

In order to provide material for chemical studies, we have grown polyacetylene-producing fungi under a variety of conditions. The polyacetylenes have distinctive ultraviolet absorption spectra, and this has made possible the study of the course of polyacetylene production in different cultures in some detail. The present communication records observations made with an unidentified Basidiomycete, B. 841, originally isolated from white cedar by Dr Baxter at the University of Michigan. Cultures of this fungus produce hydrocyanic acid (Robbins, Rolnick & Kavanagh, 1950)

and a mixture of four closely related polyacetylenic antibiotics. The structures of the latter are indicated in Fig. 1, and details of their separation are described elsewhere (Bu'Lock, Jones & Leeming, 1955); it is sufficient to note here that all share a distinctive ultraviolet absorption spectrum (Fig. 2) by measurement of which their combined concentrations may be measured.

Fig. 1. Antibiotics isolated from the fungus B. 841 (Bu'Lock *et al.* 1955).

Fig. 2. Ultraviolet absorption spectrum of nemotinic acid. The spectra of the other polyacetylenes from the fungus B. 841, and of mixtures, differ only in detail.

METHODS

Growth of the fungus B. 841. The fungus was grown as surface cultures supported on glass wool in 2 l. 'penicillin' flasks (Pyrex no. 1420) fitted with sampling tubes. The fungus grows only slowly, so that special care to avoid contamination is needed. The 'full medium' used for growth (Robbins, Kavanagh & Hervey, 1947) contained: NaNO_3 , 3 g.; KH_2PO_4 , 1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; KCl, 0.5 g.; glucose, 40 g.; cornsteep liquor (Woolley and Son Ltd., Manchester), 20 ml.; water, 1 l. Inocula (4 per flask, about 1 cm.² each) were taken from the periphery of 11-day-old malt-agar plate cultures, and the cultures were normally maintained at 25°. For 'reflooding', the medium was poured off and fresh sterile medium added, care being taken not to submerge the mycelium. When the composition of the medium was changed, the mycelial mats were first washed twice with the new medium. About 700 ml. of medium was used in each flask, the surface area of the cultures being about 230 cm.².

Analysis of the medium. Samples of the medium were drawn off as required, usually weekly. For glucose estimation, a 10 ml. sample was passed through a short column of active carbon (Norit; Haller and Phillips Ltd., Basinghall Street, London, E.C. 2), which was then washed with 10 ml. of water, and the combined eluates were made up to 50 ml. and titrated with Soxhlet's modification of Fehling's solution (see Bates *et al.* 1942). For the determination of the polyacetylene concentration, a 10 ml. sample of medium was extracted with 5 ml. of ethyl acetate, and the absorption spectrum of the extract, suitably diluted, was measured. The concentration of polyacetylenes present is proportional to the difference in optical density of the extract at 263 (maximum) and 272 m μ . (minimum); if this difference is ΔD , measured in a cell of 1 cm. thickness at a dilution v , then the concentration in the aqueous medium is $8.0 v \cdot \Delta D$ units/ml. This unit, based on known extinction coefficients and partition ratios, approximates to a concentration of 1 $\mu\text{g.}/\text{ml.}$ of medium, calculated as nemotinic acid, the main polyacetylene present. Polyacetylenes could not be detected in aqueous or ethanolic extracts of the mycelium itself.

Reproducibility. The growth curves (Figs. 3-6) show results obtained from duplicate runs, and are typical of observations which have been made several times. There is in general some variation of the actual level of polyacetylene production from one run to another, and also from one flask to another, but the qualitative progress of the reactions remains the same. Thus, for example, the curves of Fig. 4 record sets of duplicate observations and also illustrate the behaviour found in two other, similar, sets of experiments.

RESULTS

Growth from inocula. The course of polyacetylene formation during growth from inocula is illustrated in Fig. 3, which shows polyacetylene formation and glucose consumption at two different temperatures. Polyacetylene formation and glucose consumption are at first slow, becoming more rapid as the amount of mycelium present increases. The rate of polyacetylene production then begins to fall off, until the polyacetylene concentration reaches a

maximum and then begins to decline. This disappearance of polyacetylenes from the medium is due to an activity of the mycelium and does not occur in mycelium-free medium. In growth from inocula, the mycelial mat reaches a dry weight of 2.0-2.5 g. in 6-8 weeks; the total polyacetylene present at the maximum is of the order of 0.6% of the weight of glucose consumed. At a lower temperature, growth and consumption of glucose are less rapid, and the initial production of polyacetylenes is correspondingly less; however, the stage at which

Fig. 3. Production of polyacetylenes (A) and consumption of glucose (B) in growing cultures of the fungus B. 841, at 25° (full curves) and at 20° (broken curves).

Fig. 4. Production and destruction of polyacetylenes in replacement cultures of the fungus B. 841. Curve 1, on full medium; curve 2, as 1, glucose made up to 4% again after 29 days; curve 3, as 1, extra polyacetylenes (200 units/ml.) added after 29 days.

polyacetylene destruction overtakes production is postponed, so that a somewhat higher maximum yield is obtained (about 1.0% of the weight of glucose consumed). Despite the production of polyacetylenic acids, the pH of the medium rises during growth, from an initial value of 3.9 to about 5.0.

Reflooded cultures. Cultures were normally re-flooded when the stage of maximum polyacetylene yield was reached. Fig. 4, curve 1, shows the polyacetylene production after reflooding with the full

production was investigated (Fig. 4, curve 2). Polyacetylene formation was resumed, but only at a reduced rate. Various observations (cf. Fig. 5) suggested that polyacetylene destruction was most rapid when a high concentration of polyacetylenes had been reached; this was confirmed by observations of the polyacetylene destruction in flasks to which extra polyacetylenes had been added (Fig. 4, curve 3).

Variation of reflooding medium. A remarkable increase in polyacetylene formation was observed when a 4% (w/v) aqueous glucose solution was substituted for the full growth medium in reflooding (Fig. 5). The maximum yield of polyacetylene was increased as much as three-fold (about 1.7% of the weight of glucose consumed) and the actual rate of production was also increased. Reflooding of pre-

Fig. 5. Production of polyacetylene by the fungus *B. 841* in replacement cultures. Curve 1, on full medium; curve 2, on glucose (4%) + cornsteep (20 ml./l.); curve 3, on glucose (4%); curve 4, on glucose (4%) + KNO_3 (3 g./l.) + KH_2PO_4 (1 g./l.); curve 5, on glucose (1%).

growth medium. The course of events is similar to that described above, except that since the amount of mycelium does not increase there is no initial 'lag' in polyacetylene production. Glucose consumption in reflooded cultures is more rapid than in cultures from inocula (compare Figs. 3 and 6). To ascertain whether the destruction of polyacetylenes was conditioned only by a low glucose level, the effect of adding extra glucose, sufficient to restore the concentration to its initial value (4%), to flasks at the stage of maximum polyacetylene

Fig. 6. Glucose consumption in replacement cultures of the fungus *B. 841*. Upper curve, on glucose (4%); lower curve, on full medium.

formed mycelial mats with 4% glucose solution was adopted as the standard method for larger-scale production of the polyacetylenes. The effect was most marked if the mycelial mats were washed before reflooding, as described above; this reduces the carry-over of constituents of the full medium. Reflooding with a medium containing 4% of glucose and cornsteep (20 ml./l.) alone produced the same result as reflooding with the full medium; on the other hand, reflooding with a glucose-nitrate-phosphate solution (the concentrations of

these substances being the same as those used in the full medium) gave the same intensified polyacetylene production as that observed with glucose alone. Reflooding with 1% aqueous glucose gave a greatly reduced yield of polyacetylenes.

DISCUSSION

The production of polyacetylenes by the fungus B. 841 can be discussed in terms of the interaction between three types of reaction route:

- glucose —————→ 'normal' metabolites; (1)
 glucose —————→ polyacetylenes; (2)
 polyacetylenes —→ breakdown products. (3)

The reactions of group (1) include the consumption of glucose for growth, maintenance, respiration etc., and are thus of considerable complexity. It is uncertain, for example, whether the final breakdown to carbon dioxide proceeds by the citric acid

case the glucose available is many times that required for the production of polyacetylenes.

The nature of the dependence of route (2) upon the 'normal' glucose metabolism of routes (1) is clarified by the effect of supplying the cultures with 4% glucose only. The total glucose consumption is reduced, yet the amount converted into polyacetylenes is increased (cf. Table 1).

Since the behaviour of cultures reflooded with the full medium is duplicated by that of cultures reflooded with 4% glucose and cornsteep liquor (Fig. 5), the effect may be ascribed to some factor or factors supplied by the latter, and the cultures reflooded with 4% glucose alone may be considered as deficient cultures. The effect of this deficiency is apparently to suppress a part of the 'normal' glucose metabolism, i.e. to affect some of the reactions of group (1), and also to increase the amount of glucose metabolized along route (2). At the same time, a large proportion of the glucose metabolism remains unaltered; this may be due to

Table 1. *Conversion of glucose into polyacetylenes*

The data apply to cultures at stages of maximum polyacetylene concentration, reached in 6 weeks after inoculation or in 3 weeks after reflooding. Because of their simultaneous destruction, the polyacetylenes present at this stage represent only about 0.7 of the total amount which has been formed. The glucose equivalent of the polyacetylenes (estimated as nemotinic acid) is based on carbon content.

Conditions	II.		
	I. Total glucose used (g./flask)	Glucose equiv. of max. yield of polyacetylenes (g./flask)	III. II expressed as percentage of I
Growth from inocula	18	0.18	1.0
Reflood (full medium)	21	0.13	0.6
Reflood (4% glucose)	14	0.42	3.0

cycle or in part by some other scheme. Equally, the intermediate processes in the overall reactions represented by (2) and (3) are unknown. In all the experiments, group (1) reactions account for most of the glucose consumed. At no time does the amount of polyacetylenes present account for more than 5% of the carbon content of the glucose consumed, though in view of the simultaneous breakdown of polyacetylenes (route 3) it would appear that, under appropriate conditions, up to 7% of the glucose consumed may follow route (2).

The present experiments throw some light on the interaction of the polyacetylene synthesis (2) with the reactions of group (1). It is clear from the results obtained by varying the reflooding medium that routes (1) and (2) are not wholly independent and that the importance of (2) seems to depend upon the amount of glucose that can be 'spared' from route (1). Thus polyacetylene formation is slower during growth, and also—in reflooded cultures, where there is little or no growth—on the 1% glucose medium, even though in the latter

partial retention of the cornsteep factors by the mycelium, or may indicate the presence of a 'cornsteep-independent' group of reactions. The latter explanation seems more likely, since the behaviour on 4% glucose is repeated after several refloodings. Thus it appears possible to represent the metabolism of B. 841 by a scheme such as that in Fig. 7. In this scheme, the effect of a deficiency

Fig. 7. ———, Reactions not requiring cornsteep factors; - - - -, reactions requiring cornsteep factors.

in the cornsteep factors is to divert a greater proportion of the glucose consumed into polyacetylenes; in other words, polyacetylene formation in B. 841 appears to be an alternative metabolic pathway for some intermediate product of 'normal' glucose breakdown.

The competition between formation and breakdown of polyacetylene is shown by the general form of the curves. As the medium becomes depleted of glucose, polyacetylene formation becomes slower and is eventually outstripped by the increasing rate of breakdown. Comparison of the steepness of the descending slopes in Fig. 5 suggests that breakdown is more rapid at higher polyacetylene concentrations, and this is confirmed by the results in Fig. 4. Reduction of temperature has several effects, of which the most marked appears to be a lower rate of breakdown, so that a higher maximum concentration of polyacetylenes is eventually attained (Fig. 3).

SUMMARY

1. The polyacetylenic compounds produced by the Basidiomycete B. 841 during growth and in various replacement cultures are formed from glucose; they are not metabolic end-products but can be broken down by the fungus at rates comparable with their rates of synthesis. They are formed in highest yield in replacement cultures supplied with 4% aqueous glucose only, and the

extent of their synthesis is reduced when cornsteep liquor (a source of essential growth factors for the fungus) is added.

2. It is suggested that the synthesis of polyacetylenes by the fungus B. 841 represents an alternative metabolic pathway for some intermediate in glucose breakdown, the other metabolic routes of which show a partial requirement for factors present in cornsteep liquor.

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The Synthesis of Indole from Anthranilic Acid by *Escherichia coli*

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It is widely accepted that in *Neurospora* anthranilic acid serves as a precursor of indole in the biosynthetic pathway leading to tryptophan (see reviews by Bonner, 1951; Bonner & Yanovsky, 1951; also Partridge, Bonner & Yanovsky, 1952). Evidence is accumulating that tryptophan synthesis in bacteria occurs in a similar fashion to that in *Neurospora*. Thus anthranilate or indole will replace tryptophan for the growth of *Lactobacilli* (Snell, 1943; Green & Black, 1943; Wright & Skeggs, 1945; Schweigert, Sauberlich, Baumann & Elvehjem, 1946; Schweigert, 1947; Rhuland & Bard, 1952) and for some mutant strains of *Escherichia coli* (Davis, 1951). Further, anthranilate has been found to accumulate in the growth medium of a strain of *Bacterium typhosum* blocked, presumably, between anthranilate and indole (Rydon, 1948). The last worker showed also that the inhibition of growth of *Bact. typhosum* by 4- and 5-methylanthranilates was reversed by anthranilate, indole or tryptophan.

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On the other hand, Rhuland & Bard (1952) failed to show indole or tryptophan synthesis by washed cells of *Lactobacillus arabinosus* which metabolized anthranilate. They suggested that the utilization of anthranilate for growth in this organism may be different from that in *Neurospora*.

In the present paper are described some growth characteristics of mutant strains of *Esch. coli* blocked at various points in the biosynthetic chain to tryptophan, and conditions for obtaining indole synthesis from anthranilate in washed suspensions and extracts.

MATERIALS AND METHODS

Strains of Escherichia coli. Strain M 121-35 was kindly supplied by Dr B. Davis, and the other strains were supplied by Dr J. Monod. The bacteria were maintained on heart maceration-glucose agar. For experimental work they were grown for 16-18 hr. in a glucose-mineral broth (Davis & Mingioli, 1950), supplemented with tryptophan unless otherwise stated. Generally the cells were harvested by centrifuging and washed twice with 1% (w/v) KCl.