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Studies in Carotenogenesis

6. THE EFFECT OF SOME POSSIBLE CAROTENE PRECURSORS ON GROWTH, LIPOGENESIS AND CAROTENOGENESIS IN THE FUNGUS *PHYCOMYCES BLAKESLEEANUS*

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A number of compounds which could theoretically act as primary intermediates (repeating units) in carotenogenesis have been examined using the fungus *Phycomyces blakesleeanus* as the test organism. Apart from the information obtained concerning the formation of β -carotene, interesting observations have also been made on the effect of the compounds tested on lipogenesis and growth in general. Many of the substances tested have proved to be extremely toxic. Some of the results described here have already been briefly reported (Glover, Goodwin & Lijinsky, 1951).

EXPERIMENTAL

Cultural conditions. Two types of experiments were carried out. In the first, well developed mats grown in Petri dishes on filter paper were transferred, when 3-4 days old, to fresh media also in Petri dishes. Details of this technique have already been given (Goodwin & Lijinsky, 1951). In the

second type of experiment, *Phycomyces* was germinated and allowed to grow undisturbed on our standard medium (Garton, Goodwin & Lijinsky, 1951) modified only by the substitution of part of the glucose by an equivalent amount (calculated on C content) of the substance under examination.

Materials used. The materials used for the standard medium were as described previously (Garton *et al.* 1951). The following intermediates were synthesized: 3-methylbut-2-enoic acid (β -methylcrotonic acid; $\text{Me}_2\text{C}:\text{CH}.\text{CO}_2\text{H}$, m.p. 66.5-67.5°) from mesityl oxide (Vogel, 1948); DL-2-hydroxy-4-methylpentanoic acid (DL- α -hydroxyisocaproic acid; $\text{Me}_2\text{CH}.\text{CH}_2.\text{CHOH}.\text{CO}_2\text{H}$, m.p. 79-81°) and DL-2-hydroxy-3-methylbutanoic acid (DL- α -hydroxyisovaleric acid; $\text{Me}_2.\text{CH}.\text{CHOH}.\text{CO}_2\text{H}$, m.p. 84°) by the conventional method via the corresponding α -bromo acids (Vogel, 1948); 4-methylpentamide (isocaproamide, m.p. 118°) and 3-methylbutanamide (isovaleramide, m.p. 137°) by the usual method from the corresponding acid (Vogel, 1948) and 2-keto-4-methylpentanoic acid (α -ketoisocaproic acid; $\text{Me}_2\text{CH}.\text{CH}_2.\text{CO}.\text{CO}_2\text{H}$, b.p. 83°/17 mm.) (2:4-dinitrophenylhydrazones, m.p. 146-148°) from isovaleryl chloride (Mauthner, 1909); and 3-methylbut-2-enal (β -methylcrotonaldehyde; $\text{Me}_2\text{C}:\text{CH}.\text{CHO}$, b.p. 68-72°/95 mm.) (2:4-dinitrophenylhydrazone, m.p. 182°) by the method of

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Young & Linden (1947) from dimethylallylbromide (b.p. 28–33°/12 mm.) prepared according to Staudinger, Kreis & Schilt (1922). Other possible intermediates were obtained commercially and purified in the appropriate manner when it was found necessary.

RESULTS

Transfer experiments

A preliminary series of experiments was carried out to determine the effect on carotenogenesis of the variation in the glucose concentration of the standard medium to which the 3- to 4-day-old mats were transferred. The results (Fig. 1) show that a

Fig. 1. The production of carotene (and dry weight) by well developed mats of *Phycomyces* transferred for 4 days to the standard medium (Garton *et al.* 1951) containing N (0.2%, w/v, asparagine) and varying amounts of glucose. ○—○, dry wt. production; ●—● carotene production.

glucose concentration of between 0.5 and 1.0% (w/v) is required to maintain the carotene at its transfer level, but rather less to maintain dry weight. Very similar results were obtained for carotene when the transfer medium contained no nitrogen, and are not recorded here; production of dry weight was, of course, much less on the nitrogen-free medium (see also Goodwin & Willmer, 1952). It was therefore decided to test the intermediates by transferring mats to the standard medium (either with or without nitrogen) containing just sufficient glucose to maintain the pre-transfer carotene level, together with small amounts of intermediates in concentrations so arranged that in all media the final C concentration was the same. Under these conditions the controls should synthesize only small

amounts of carotene, whilst the presence of small amounts of a direct intermediate should considerably stimulate synthesis. Any energy necessary for the utilization of these intermediates was available in the glucose.

Goodwin & Lijinsky (1951) found that L-leucine greatly stimulated carotenogenesis in *Phycomyces*; they suggested that β -methylcrotonaldehyde was the repeating unit in carotene synthesis and that it was formed via the following steps: leucine $\rightarrow$ α -ketoisocaproic acid $\rightarrow$ isovaleraldehyde $\rightarrow$ β -methylcrotonaldehyde. These and related substances were chosen for test. The other main group tested were mixtures of C₂ and C₃ units, which might be expected to be formed during glycolysis (or fermentation or both), and which could conceivably condense to give an effective repeating unit. Two other compounds were examined for reasons which will be discussed later.

The results of many experiments are summarized in Tables 1 and 2. In all these experiments, mycelia were examined 3, 5 and 7 days after transfer; only the 3-day data are given here, because those for the 5- and 7-day cultures always confirmed the 3-day results. Under these conditions all the test substances are inactive in stimulating carotenogenesis; furthermore, the branched-chain acids, β -methylcrotonaldehyde and crotonaldehyde are extremely toxic. Mycelia transferred to media containing these compounds soon became moribund and quickly began to lose their pigment; within about 6 hr. almost all had disappeared. Other mycelial constituents are not lost rapidly; the total dry weight disappears less quickly, whilst the lipid fraction is almost completely unaffected.

The potassium salts of these branched-chain acids were as toxic as the free acids; introduction of an α -keto group also left the toxicity unaltered (Table 2), but the introduction of an α -hydroxyl group completely eliminated the toxic effects (Table 1). The amides of the branched-chain acids were examined because it was thought that they would be hydrolysed slowly, thus liberating the toxic free acid in amounts with which the organism could deal. Table 1 shows that the amides were almost completely non-toxic and had no positive effect on carotenogenesis. *iso*Valeraldehyde (saturated) is much less toxic than β -methylcrotonaldehyde (unsaturated), and the corresponding saturated alcohol (*iso*amyl alcohol) is not toxic.

Because of the toxicity of these possible precursors, they were tested at a lower concentration. One-twentieth of the original concentrations was used, for even at these levels sufficient precursor is available for synthesis of much more than the normal amounts of carotene found. At these lower concentrations, none of the intermediates was toxic and none of them stimulated carotenogenesis.

Table 1. *Dry weight, carotene and lipid produced by well developed mycelia of Phycomyces transferred for 3 days to the standard medium (Garton et al. 1951) (+ N) containing 0.84 % (w/v) glucose and sufficient of the possible precursor to make the final C content equivalent to 1 % (w/v) glucose*

Substance tested	Concn. (% w/v)	Amount produced after transference		
		Dry wt. (mg.)	Carotene (μ g.)	Lipid (mg.)
Acetate (potassium salt)	0.264	65	35	0
Acetate (potassium salt) + Acetone	0.132 } 0.065 }	124	31	9
DL- α -Hydroxyisocaproic* acid (DL-2-hydroxy-4-methylbutanoic acid)	0.234	124	18	-9
DL- α -Hydroxyisovaleric acid* (DL-2-hydroxy-3-methylbutanoic acid)	0.252	116	53	-6
isoAmyl alcohol (3-methylbutan-1-ol)	0.113	160	15	0
isoValeramide (3-methylbutanamide)	0.107	70	67	-11
isoCaproamide (4-methylpentamide)	0.102	93	30	-12
α -Ketoisocaproamide (2-keto-4-methylpentanoic acid)	0.116	92	9	-2
isoValeraldehyde (3-methylbutanal)	0.115	17	28	-20
isoValeric acid (3-methylbutanoic acid)	0.117	-3	-5	-15
isoCaproic acid (4-methylpentanoic acid)	0.112	-60	-49	8
isoValerate (potassium salt)	0.117	-54	-51	1
isoCaproate (potassium salt)	0.112	-69	-40	1
β -Methylcrotonic acid (3-methylbut-2-enoic acid)	0.106	-51	-44	13
Crotonaldehyde (but-2-enal)	0.091	-92	-44	-20
isoPropylacrylic acid (3-methylpent-2-enoic acid)	0.102	-63	-43	
Glucose 1 % (control)		119	52	8
Content of mats at transfer (mean values)		210	52	45

* Twice the normal concentration of these acids was used on the assumption that only one isomer would be utilized.

Table 2. *Dry weight, carotene and lipid production by well developed mycelia of Phycomyces, transferred for 3 days to the standard medium (Garton et al. 1951) without nitrogen and containing 1.0 % (w/v) and sufficient of the possible precursor to make the final C content equivalent to 1.16 % (w/v) glucose*

Substance tested	Concn. (% w/v)	Amount produced after transference		
		Dry wt. (mg.)	Carotene (μ g.)	Lipid (mg.)
Acetone + Acetaldehyde	0.065 } 0.074 }	16	40	2
Acetone + Pyruvate	0.065 } 0.062 }	16	18	-1
Pyruvate + isoPropanol	0.062 } 0.071 }	17	31	-1
isoPropanol + Acetaldehyde	0.071 } 0.074 }	18	29	0
β -Methylcrotonaldehyde	0.09	-70	-34	-10
α -Ketoisocaproic acid	0.115	-40	-35	-6
Glucose (control)	1.16	22	47	-2
Content of mats at transfer (mean values)		103	37	20

Experiments on growing cultures

When the transfer experiments failed to yield positive results it was decided to repeat the experiments of Goodwin & Lijinsky (1951) in which it was found that L-leucine and L-valine stimulated carotenogenesis in media low in glucose, but to substitute for the amino-acids the probable intermediates. Because of their known toxicity, the

intermediates were used in the same concentrations as in the experiments just described in the previous paragraph. The results obtained in a number of experiments on 5- to 9-day-old mycelia are recorded in Table 3. β -Methylcrotonaldehyde shows quite marked stimulation of β -carotene synthesis, whilst α -hydroxyisocaproic and α -ketoisocaproic acids show a slight stimulation; these stimulations are somewhat more marked when the concentrations

Table 3. *Dry weight, carotene and lipid produced in 5 to 9 day-old mycelia*

(Mycelia grown in 8 oz. medicine bottles, 15 ml. standard medium; glucose (1 % w/v); lipid and β -carotene expressed as % of dry wt.)

Substance tested	Concn. (% w/v)	No. of exps.	Dry wt. (mg.)	Lipid (%)	Amounts produced		
					β -Carotene		
					(μ g.)	Limits of scatter	Concn. (% $\times 10^3$)
<i>iso</i> Valeraldehyde	0.00465	7	53	23.4	17	14-21	32.1
β -Methylcrotonaldehyde	0.00405	16	54	27.4	27	19-37	50.0
DL- α -Hydroxyisocaproic acid	0.0053	7	57	22.0	23	15-26	40.4
β -Methylcrotonic acid	0.00477	6	52	30.2	18	16-20	34.6
α -Ketoisocaproic acid	0.00531	7	56	25.2	22	16-28	39.3
Glucose (control)	1.0	16	60	27.8	17	15-20	28.4

Table 4. *Dry weight, carotene and lipid produced in 4-day-old mycelia grown in 8 oz. medicine bottles containing 15 ml. of standard medium (Garton et al. 1951) containing 1 % (w/v) glucose and sufficient precursors to make the final concn. equivalent in C concn. to 1.16 % (w/v) glucose*

(The concn. of the intermediates tested are so arranged that both contribute the same amount of C.)

Precursors	Concn. (w/v)	Dry wt. (mg.)	Amounts produced			
			Lipid		Carotene	
			Wt. (mg.)	(%)	Wt. (μ g.)	(% $\times 10^3$)
Acetone	0.065	70	14.1	20.1	10	14.3
+ Acetaldehyde	0.074					
Acetone	0.065	67	11.7	17.4	12	17.9
+ Acetate (potassium salt)	0.132					
Acetone	0.065	70	15.0	21.4	11	15.7
+ Pyruvate	0.062					
Pyruvate	0.062	71	16.9	23.8	11	15.5
+ <i>iso</i> Propanol	0.071					
<i>iso</i> Propanol	0.071	67	14.4	21.5	9	14.0
+ Acetaldehyde	0.074					
Glucose (control)	1 %	60	13.2	21.9	10	16.7

of β -carotene produced are considered. *iso*Valeraldehyde and β -methylcrotonic acid were without significant effect.

Extension of these experiments using mixtures of C₂ and C₃ compounds likely to combine to form β -methylcrotonaldehyde have never yielded clear-cut results; although it is possible to use much higher concentrations without any signs of toxicity. Values obtained for 4-day cultures in a series of experiments are given in Table 4 which show that a possible slight stimulation appears to have occurred with a mixture of acetone and acetate. In other experiments this has not been reproduced, whilst a similar slight stimulation has occasionally been revealed with other mixtures.

DISCUSSION

In a medium containing insufficient glucose for maximal carotene synthesis, L-leucine stimulates production of this pigment (Goodwin & Lijinsky,

1951). It was suggested that β -methylcrotonaldehyde was produced (via α -ketoisocaproic acid and *isovaleraldehyde*) and acted as the repeating unit for the synthesis of carotene. Experiments carried out under the same conditions, but using these possible precursors (Table 3), have shown that β -methylcrotonaldehyde is the most effective stimulant, whilst α -keto- and α -hydroxy-*isocaproic* acids are slightly active; *isovaleraldehyde* is, however, non-stimulatory. As β -methylcrotonic acid also shows no definite stimulation, it seems possible that β -methylcrotonaldehyde is the active repeating unit, but that it is not produced from the α -keto (or α -hydroxy) acid via *isovaleraldehyde*. Preliminary experiments using ¹⁴CH₃COOH indicate that *isovaleraldehyde* is not in the pathway from acetate to carotene (Glover, Goodwin & Willmer, 1952), but this does not definitely rule out *isovaleraldehyde* as an intermediate when leucine is the precursor.

The demonstration that β -methylcrotonaldehyde is possibly the immediate precursor of carotene when leucine is the starting material, does not prove that it is the repeating unit when glucose is the substrate. If it were so, β -methylcrotonaldehyde might be formed from more normal products of glucose dissimilation, one possibility being the condensation of acetone and acetaldehyde, thus:

This and other possible combinations have been examined (Table 4) in growing cultures. When tested under the same conditions as β -methylcrotonaldehyde, none has stimulated carotenogenesis, although, because of their non-toxicity, they were tested at higher levels. Even acetate, thought by Schopfer & Grob (1950, 1952) to be the primary carotene precursor, was inactive. Such negative experiments obviously do not prove that none of these combinations can form β -methylcrotonaldehyde, but the question whether β -methylcrotonaldehyde is the immediate precursor of carotene when glucose is the basic substrate, must be left open.

A very large number of transfer experiments, a typical selection of which is given in Tables 1 and 2, have failed in any instance to stimulate carotenogenesis above that produced by glucose. It must be concluded that experiments of this type are not suitable for this kind of investigation.

Some interesting observations, unconnected with carotenogenesis, have been made concerning the toxicity of some compounds tested as carotene precursors. The branched-chain acids, *isovaleric*, *isocaproic* and β -methylcrotonic (either free or as potassium salts) are extremely toxic and at a concentration of about 0.02% (w/v) quickly kill the fungus *Phycomyces*. There are a few other instances where it has been found that fatty acids are fungicidal. Keeney, Ajello, Lankford & Mary (1945) found that octanoate (caprylate) and propionate were effective against the pathogenic *Fonsecaea*

pedrosoi; Rothman, Smiljanic & Shapiro (1945) discovered that the fatty acids from adult hair inhibited growth of the ringworm fungus *Microsporon audouinii*, the fraction containing the acids with 7 to 11 C atoms being most effective; Levine & Novak (1950) found that straight-chain acids above octanoic almost completely inhibited respiration of *Blastomyces dermatitis*, that the lower members stimulated respiration, and that the branched-chain (*isovaleric*) or unsaturated (*crotonic*) acids had no effect on respiration.

SUMMARY

1. When mats of *Phycomyces* are transferred to media containing various concentrations of glucose, carotene synthesis does not begin until the concentration is between 0.75 and 1.0% (w/v).

2. A large number of substances tested for ability to stimulate carotenogenesis in transferred cultures all gave negative results.

3. In growing cultures containing 1.0% (w/v) glucose, carotenogenesis was stimulated slightly by α -ketoisocaproic and α -hydroxyisocaproic acids and appreciably by β -methylcrotonaldehyde (all at concentrations of about 0.001%); *isovaleraldehyde* and β -methylcrotonic acid were without obvious effect, when tested at the same level. Tested at higher levels a number of binary mixtures, thought likely to give rise to β -methylcrotonaldehyde, were inactive.

4. The following were extremely toxic to *Phycomyces* at a concentration of about 0.02% (w/v): *isovaleric*, *isocaproic*, α -ketoisocaproic and α -methylcrotonic acids, and β -methylcrotonaldehyde and crotonaldehyde. Both α -hydroxylation and amidation of *isocaproic* and *isovaleric* acids rendered them non-toxic. *isovaleraldehyde* was much less toxic than β -methylcrotonaldehyde, whilst *isoamyl alcohol* was non-toxic.

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