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Studies in Carotenogenesis

16. THE ACTION OF SOME ANTIBIOTICS, ESPECIALLY STREPTOMYCIN, ON CAROTENOGENESIS IN *PHYCOMYCES BLAKESLEEANUS**

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Bracco & von Euler (1947, 1948) observed that when barley seedlings were germinated on a medium containing streptomycin they produced white coleoptiles and first leaves which contained neither chlorophyll nor carotenoids but normal amounts of anthocyanins. Green seedlings transferred to a streptomycin medium did not lose their plastid pigments, although etiolated seedlings failed to become green or to develop chloroplasts on exposure to light in the presence of streptomycin. These findings have been largely confirmed by using *Pinus jeffreyi* (Bogorad, 1950) and *Pisum* spp. (Schopfer, Bein & Besson, 1952). The green crown gall tumours of carrots also turn white in the presence of streptomycin (Ropp, 1948).

Similar colour changes were first observed in micro-organisms by Provasoli, Hutner & Schatz (1948), using *Euglena gracilis* v. *bacillaris*; in this case there was a permanent loss of chloroplasts, the algae remaining colourless on serial transfer to streptomycin-free media in the light. This has been confirmed by Lwoff & Schaeffer (1949a, b) and Jirovec (1949); Goodwin & Jamikorn (1954) showed that although considerably reduced by streptomycin, carotenoid synthesis is less affected than chlorophyll synthesis. The whole streptomycin molecule is necessary for inhibition of pigment formation in *E. gracilis* (Provasoli, Hutner & Pinter, 1951) although dihydrostreptomycins A and B are also active (Hutner & Provasoli, 1951). Provasoli *et al.* (1951) also reported no obvious colour change in cultures of five strains of photosynthetic Athiorhodaceae, in which growth had been partly inhibited by streptomycin. Goodwin &

Osman (1953) found, however, in quantitative experiments with *Rhodospirillum rubrum*, that there was a narrow range of concentration over which streptomycin inhibited carotenoid (spirilloxanthin) synthesis to a greater extent than growth; the effect was very much less marked than with *E. gracilis*.

Because of these reports the effect of streptomycin on carotenogenesis in a non-photosynthetic organism, *Phycomyces blakesleeanus*, has been examined in detail. A preliminary report of the inhibition of carotenogenesis in this organism by streptomycin has already been made (Goodwin & Griffiths, 1952) and this has been confirmed by Schopfer, Grob, Besson & Keller (1952).

Although no other antibiotic was found which had the same effect as streptomycin on *E. gracilis* (Hutner & Provasoli, 1951), it has been briefly reported that chloromycetin inhibits carotenogenesis in both pea seedlings and *P. blakesleeanus*, that tetroneic acid is active only on pea seedlings and that penicillin is inactive on both organisms (Schopfer, Grob, Besson & Keller, 1952; Schopfer, Grob & Besson, 1952a, b). In the present investigation these and other antibiotics have also been examined, but in less detail than streptomycin.

EXPERIMENTAL

Cultures. *Phycomyces blakesleeanus* (- strain) obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland, was used throughout the investigation.

The standard medium was that described by Garton, Goodwin & Lijinsky (1951) modified to contain 2.5% (w/v) glucose. The basal acetate medium used was medium C of Friend, Goodwin & Griffiths (1955), based on that of Schopfer & Grob (1950, 1952).

* Part 15: Friend, Goodwin & Griffiths (1955).

The antibiotics were added to the autoclaved media (15 lb./sq.in./15 min.) either from sterile ampoules or after sterilization by filtration.

Inoculation was carried out with spore suspensions prepared and dispensed as described by Garton *et al.* (1951).

Mycelia were normally grown either in 8 oz. medicine bottles containing 15 ml. of medium or in penicillin flasks containing 100 ml. of medium, and incubated at 25° in a glass incubator (Garton *et al.* 1951). In order to obtain mats for transfer to fresh medium, mycelia were grown in Petri dishes and the method of transference used was that described by Goodwin & Willmer (1952).

Dry-weight lipid and carotene determinations. In all cases, except where otherwise stated, three cultures were combined for each analysis; this saves a considerable amount of time and is justifiable, because Lijinsky (1951) has shown that when groups of 10 cultures are examined separately the mean values of highest and lowest groups of three never differed from the mean of the 10 analyses by more than 6% in dry weight and 10% in carotene.

Dry-weight determinations of mycelia grown on glucose media were carried out by the method of Garton *et al.* (1951) and of mycelia grown on acetate by the method of Friend *et al.* (1955), which involves a slight modification of the original method owing to the precipitation in the medium of inorganic phosphates during growth. These are removed by washing the mycelium with 5 ml. of 2N-HCl. Lipid and carotene determinations were carried out in all cases using the method of Garton *et al.* (1951).

Chromatographic separation of the carotenoids was carried out with alumina as adsorbent (Goodwin, 1952).

Formation of 2:4-dinitrophenylhydrazones. Two methods were used, one for routine identification and one for isolation. In the first procedure, Brady's reagent was added to the medium which was then kept at 37° overnight; on cooling to room temperature, the orange-yellow precipitate was collected and dissolved in ethyl ether freshly distilled over reduced iron [if undistilled ether is used the hydrazones eventually give anomalous colours in alkaline solution (Whittam & Davies, 1954)]. The ethereal solution was then shaken in a separating funnel with an equal vol. of 5% (w/v) Na₂CO₃, which extracts acid hydrazones (Goodwin & Williams, 1952). After separation from the ether layer (A) the aqueous solution is immediately acidified with 2N-HCl and the acid hydrazones are extracted back into ethyl ether; considerable destruction of the hydrazones occurs if they are allowed to stand in alkaline solution for any length of time. After the ether solution has been washed free from acid with successive small volumes of water, it is ready for paper chromatography. The original ether extract (A) is similarly washed free from alkali preparatory to chromatography.

In the second method the pH of the medium was adjusted to 10 with 2N-NaOH and extracted with freshly distilled ethyl ether in a continuous extractor for 6 hr. The solvent was then removed from the extract *in vacuo* and the 2:4-dinitrophenylhydrazones (DNPH's) prepared from the residue in the usual manner. The medium was then taken to pH 1 with conc. HCl and again extracted with ethyl ether; the ether extract was taken to dryness *in vacuo* and the DNPH's prepared. Finally the residual medium was treated with Brady's reagent, and any precipitate formed was treated as described in the first method.

Chromatography of the 2:4-dinitrophenylhydrazones. The neutral DNPH's were chromatographed on paper by the

ascending method, using the two-phase solvent system of Meigh (1952) in which heptane was the mobile and methanol the stationary phase. The DNPH's were applied in ether. The acid hydrazones were separated using the method of Altmann, Crook & Datta (1951), which employs a *tert.*-amyl alcohol-ethanol-water solvent system. The hydrazones were applied in a glycine/NaOH buffer (0.1M) of pH 8.2-8.4.

The yellow-orange spots could easily be located visually on the paper, and further characterized by spraying with 2N-NaOH, which produced characteristic colours varying from brown to blue. In order to measure the absorption spectrum of the separated spots they were eluted with warm glacial acetic acid, transferred to ethyl ether and washed free from acid, and the spectrum was measured. The ethyl ether extracts were then evaporated to dryness *in vacuo*, dissolved in 3 ml. of ethanol, diluted with an equal vol. of 1.5N-NaOH and the absorption spectra measured as quickly as possible.

The method of Kulonen (1953) in which the DNPH's of keto acids are reduced with Al-amalgam to the corresponding amino acids, which are then chromatographed in the conventional manner, has also been employed.

Spectral measurements. These were carried out with the Unicam SP. 600, SP. 500 or Beckman DU instruments. Measurements were made at 5 mμ. intervals, except near maxima, where the interval was reduced to 1.0 mμ. or 0.5 mμ.

RESULTS

Effect of streptomycin on carotene synthesis

Even very high concentrations of streptomycin have no marked effect on growth of 7-day cultures of *Phycomyces blakesleeana* (Fig. 1). With β-carotene, however, there was a gradual but marked decrease in synthesis with increasing concentration of streptomycin up to a level of 0.075-0.10% (w/v), when the carotene level was 30-40% of its normal value. Further increases in streptomycin had no added effect on carotene production.

When mycelia are examined at various stages of development, it will be seen (Table 1) that streptomycin slows down growth but, confirming the results shown in Fig. 1, does not alter the amount of growth finally achieved. Inhibition of carotenogenesis does not take place in young growing mycelia. The results in Table 1 confirm the earlier observations (Garton *et al.* 1951) that carotenogenesis in the dark is only about 50% of that in the light, although growth is normal, and show that the 'dark' synthesis of β-carotene is also inhibited by streptomycin.

When the glucose concentration of the standard medium is reduced to 1% (w/v), growth is reduced to a lesser extent than is carotenogenesis (Goodwin & Willmer, 1952). The results given in Table 2 show that this reduced level of synthesis is further lowered by streptomycin. On the other hand, carotenogenesis by vegetative mats placed on a glucose/salts medium (i.e. in the absence of N) is almost completely inhibited by the antibiotic (Table 3).

It is justifiable to assume in each experiment that all mats at transfer have the same weight and contain the same amount of β -carotene (Lijinsky, 1951). Although variations between flasks in a single experiment are small, mats from experiments

carried out at different times can vary considerably, especially during the early stages of growth (2-4 days). This accounts for the different basal values in the experiments quoted in Table 3.

When asparagine in the standard medium is replaced by leucine or valine, a considerable stimulation of β -carotene synthesis results (Goodwin & Lijinsky, 1952). Table 4 shows that this stimulation is also streptomycin-sensitive. When asparagine is replaced by NH_4NO_3 or $(\text{NH}_4)_2\text{SO}_4$ there is a considerable reduction of growth and of carotenogenesis (Garton *et al.* 1951). This change in the nitrogen source, however, results in almost complete elimination of the streptomycin effect on carotene synthesis (Table 5). Even the presence of NH_4NO_3 , together with asparagine, reduces the sensitivity of the carotenogenic system to streptomycin. The final pH of the media are recorded for these experiments because it was possible that the slightly lower values on the NH_4NO_3 media might have affected the action of the antibiotic (see Discussion).

In 1940, Leonian & Lilly showed that the addition of a number of carboxylic acids (0.02M) to the glucose/ NH_4NO_3 medium considerably

Fig. 1. Effect of streptomycin on growth and carotenogenesis by *P. blakesleeanus*. Standard glucose medium with varying amounts of streptomycin; amounts produced in 15 ml. of medium in 8 oz. medicine bottles in the light; 7-day cultures, temp. 25°. Each point represents the mean value for three bottles. ●, Total dry wt.; ○, β -carotene.

Table 1. *Effect of streptomycin on growth and β -carotene synthesis by Phycomyces blakesleeanus at various times after inoculation*

Standard medium; amounts produced in 15 ml. of medium in 8 oz. medicine bottles; temp. 25°; two bottles bulked for each analysis.

Age of culture	...	3 days			5 days			7 days		
		β -Carotene			β -Carotene			β -Carotene		
		Dry wt. (mg.)	Amount (μ g.)	Concn. (p.p.m.)	Dry wt. (mg.)	Amount (μ g.)	Concn. (p.p.m.)	Dry wt. (mg.)	Amount (μ g.)	Concn. (p.p.m.)
Light cultures										
1.	Standard medium	46.3	12.3	266	105.2	88	837	106.3	115	1083
2.	1 + 0.4% (w/v) streptomycin	21.4	7.5	351	121.8	56	460	110.4	60	544
Dark cultures										
1.	Standard medium	52.1	6.3	120	115.1	45	400	120.0	61	510
2.	1 + 0.4% (w/v) streptomycin	30.2	7.6	250	128.1	31	240	129.0	41	360

Table 2. *Effect of streptomycin on β -carotene synthesis by Phycomyces blakesleeanus*

Standard medium with glucose concn. reduced from 2.5 to 1.0% (w/v); amounts produced in 15 ml. of medium in 8 oz. medicine bottles; temp. 25°; 7-day cultures in the light; three mycelia bulked for each analysis.

Expt. no.	Streptomycin absent			Streptomycin present (0.2%, w/v)			Inhibition of β -carotene production by streptomycin (%)
	Dry wt. (mg.)	β -Carotene		Dry wt. (mg.)	β -Carotene		
		Amount (μ g.)	Concn. (p.p.m.)		Amount (μ g.)	Concn. (p.p.m.)	
1	76.5	19.4	253	95.6	11.0	115	43.3
2	57.2	10.7	187	59.2	7.7	130	28.0
3	49.4	21.5	435	62.3	10.1	160	53.0
4	32.9	14.3	435	41.6	6.7	161	53.2

stimulated growth of *P. blakesleeanus*. This has been confirmed by Friend *et al.* (1954, 1955), who also showed that the accompanying stimulation of carotenogenesis varied with the acid used; this is due to variations in the final pH of the different media, the greatest stimulation at a level of

0.02M being obtained with acetate and succinate. A large number of experiments have been carried out on the effect of streptomycin on growth and carotenogenesis by *P. blakesleeanus* on the glucose/ NH_4NO_3 medium in the presence of different carboxylic acids. In no case was there an effect on

Table 3. *Effect of streptomycin on β -carotene synthesis by vegetative mats of Phycomyces blakesleeanus*

3-day mats grown in Petri dishes containing 15 ml. of standard medium with glucose concn. reduced to 1% (w/v), washed with sterile water and transferred to 15 ml. standard medium without asparagine in the presence or absence of 0.2% (w/v) streptomycin. 2 days incubation at 25° in the light; three mats bulked for controls and 'normal' transfers, six mats for streptomycin transfers.

	β -Carotene ($\mu\text{g.}$)			Inhibition (%)
	Amount at transfer	Amount after transfer	Increment	
Expt. A				
Without streptomycin	135*	264	129	—
With streptomycin		156	21	83.7
Expt. B				
Without streptomycin	41*	88	47	—
With streptomycin		42.4	1.4	97.1

* The different amounts of carotene present in the two cases is due to the different weights of the mats when harvested, the mean values being 108 and 47 mg. respectively.

Table 4. *Effect of streptomycin on leucine-stimulated carotenogenesis in Phycomyces blakesleeanus*

Amounts produced on 15 ml. of standard medium containing 1% (w/v) glucose and with either L-asparagine or DL-leucine (0.2%, w/v) as N source; concn. of streptomycin 0.2%, (w/v); temp. 25°; three mycelia bulked for each determination.

Nitrogen source	7-day cultures			11-day cultures		
	Dry wt. (mg.)	β -Carotene		Dry wt. (mg.)	β -Carotene	
		Amount ($\mu\text{g.}$)	Concn. (p.p.m.)		Amount ($\mu\text{g.}$)	Concn. (p.p.m.)
Without streptomycin						
Asparagine	32.9	14.3	435	43.2	9.5	220
Leucine	19.9	16.3	820	37.2	49.9	1341
With streptomycin						
Asparagine	41.6	6.7	161	58.8	3.0	51
Leucine	21.5	10.0	465	38.3	33.5	875

Table 5. *Effect of streptomycin on carotenogenesis in Phycomyces blakesleeanus cultured on various nitrogen sources*

Amounts produced in 8 oz. medicine bottles containing 15 ml. of standard medium with asparagine replaced by different N sources; streptomycin 0.2% (w/v); 7-day cultures; temp. 25°. Three mats combined for each analysis.

Nitrogen source	Without streptomycin					With streptomycin			
	Concn. (% w/v)	Dry wt. (mg.)	β -Carotene		pH of medium	Dry wt. (mg.)	β -Carotene		Final pH of medium*
			Amount ($\mu\text{g.}$)	Concn. (p.p.m.)			Amount ($\mu\text{g.}$)	Concn. (p.p.m.)	
Asparagine	0.20	94.5	128	1356	4.5	118.2	33.3	282	4.4
NH_4NO_3	0.25	17.7	26.2	1482	3.3	24.7	36.9	1498	2.8
$(\text{NH}_4)_2\text{SO}_4$	0.25	13.7	34.2	2500	3.7	20.1	40.7	2025	3.1
NH_4NO_3 and asparagine	0.175 } 0.075 }	37.7	105	2785	2.6	52.1	106	2035	2.6
NH_4NO_3 and asparagine	0.25 } 0.20 }	79.8	143	1792	3.7	86.9	114	1312	3.4

* All media were originally adjusted to pH 6.0.

growth. The effect on carotenogenesis is illustrated in Table 6, where the mean carotene production in the presence of streptomycin in each separate experiment is represented as a percentage of that produced at the same time in the absence of streptomycin. Marked and consistent inhibition only occurs when succinate is the stimulatory carboxylic acid. Even when carotene production is increased in the presence of malate, fumarate, etc. by reducing the concentration of these acids to 0.01M (Friend *et al.* 1955) no marked streptomycin inhibition was observed.

containing medium. Fig. 2 shows that growth and carotenogenesis are both affected equally, so that (except with very high concentrations of streptomycin which reduces growth so considerably that accurate analyses are impossible) the concentration of carotene does not alter; the lipid levels followed the same pattern and are not recorded. The same results are obtained when yeast extract (0.1%), which stimulated growth and carotenogenesis on an acetate medium (Friend *et al.* 1954, 1955), is added to the acetate medium; they are, therefore, not reproduced here.

Table 6. *Effect of streptomycin on carotenogenesis by Phycomyces blakesleeana on a glucose/NH₄NO₃ medium, in the presence of various carboxylic acids*

Results recorded as percentage of control value in same experiment; three bottles bulked for each determination; 7-day cultures; streptomycin 0.2% (w/v); temp. 25°.

Carboxylic acid added	Concn. (M)	Amount of β -carotene (% of control)	Final pH of medium (mean)	
			Without streptomycin	With streptomycin
None	—	98, 113	3.3	2.8
Acetate	0.02	113, 121, 106, 105	2.4	2.4
Succinate	0.02	53, 51, 64, 51	2.7	2.8
Citrate	0.02	77, 82, 133	4.0	4.0
	0.01	96, 80	3.3	3.1
α -Oxoglutarate	0.02	88, 92	2.8	3.3
	0.01	86	3.2	4.4
Fumarate	0.02	109, 91	3.5	2.6
	0.01	115, 72	3.2	4.4
Malate	0.02	97, 90, 116	2.5	3.0
	0.01	82	2.9	2.8

Table 7. *Effect of streptomycin on β -ionone-stimulated carotenogenesis in Phycomyces blakesleeana*

Amounts produced in 100 ml. of standard medium in penicillin flasks: β -ionone 10 μ l./flask; streptomycin concn. 0.2%, (w/v); 7-day cultures; temp. 25°; two flasks bulked for each analysis.

	Normal cultures	β -Ionone cultures	β -Ionone + streptomycin cultures
Total dry wt. (mg.)	477	467	581
β -Carotene (μ g.)	672	1300	360
β -Carotene concentration (p.p.m.)	1409	2780	620

Effect of streptomycin on β -ionone stimulation

Mackinney, Nakayama, Buss & Chichester (1952) found that addition of small amounts of β -ionone to glucose/asparagine cultures of *P. blakesleeana* considerably stimulates carotenogenesis. The results given in Table 7 confirm this finding, and also demonstrate that this stimulation is streptomycin-sensitive.

Effect of streptomycin on carotenogenesis by Phycomyces blakesleeana on acetate media

The effect of streptomycin on *P. blakesleeana* grown on a medium containing acetate as the sole carbon source is different from that on a glucose-

Effects of various degradation products of streptomycin on carotenogenesis

The results of a small number of experiments carried out with the limited quantities available to us of some fragments of the streptomycin molecule (Table 8) show that methyl streptobios-aminidine dimethyl acetal inhibits carotenogenesis in *P. blakesleeana*. All the other fragments examined were inactive.

Attempts to reverse the inhibition of carotenogenesis by streptomycin

Loo, Carter, Kehm & Anderlik (1950) reported that fructose 1:6-diphosphate reversed the streptomycin inhibition of growth in *Lactobacillus*

arabinosus, *Staphylococcus aureus* and the yeast *Torula utilis*. At the concentration recommended by Loo *et al.* (1950) (0.512%, w/v), this compound had no effect on the inhibition of carotenogenesis by streptomycin. Similarly, pantothenic acid, reported to reverse the growth inhibition of streptomycin in *Saccharomyces fragilis* (Lichstein & Gilfillian, 1951), was without effect at levels of up to 200 mg./100 ml. Further, neither of these two compounds had any effect on growth or carotenogenesis by *P. blakesleeanus* in the absence of streptomycin, although, in agreement with the observations of Hawker (1948), it was found that production of sporangiophores occurred sooner in the presence than in the absence of hexosediphosphate.

Streptomycin and the production of carbonyl compounds by Phycomyces blakesleeanus

The possibility that the building unit for carotenogenesis may be an aldehyde such as β -methylcrotonaldehyde or isovaleraldehyde (Goodwin, 1953), and that these might accumulate in the presence of streptomycin, prompted an investigation into the carbonyl compounds present in the normal and streptomycin media.

Neutral carbonyl compounds. The 2:4-dinitrophenylhydrazones (DNPH's) which are not extracted from ethyl ether by Na_2CO_3 were chromatographed with the solvent system of Meigh (1952); only one spot was obtained which remained at the origin. It gave a blue coloration with 3N-NaOH indicating a dicarbonyl compound; glucose,

Fig. 2. Effect of streptomycin on growth and carotenogenesis by *P. blakesleeanus*. The all-acetate medium with varying amounts of streptomycin. Amounts produced in 100 ml. of medium in penicillin pots in the light; temp. 25°; 7-day cultures. Each point represents the mean value of two pots bulked for analysis. ●, Total dry wt.; ○, β -carotene.

Table 8. *Effect of various fragments of the streptomycin molecule on carotenogenesis by Phycomyces blakesleeanus*

Amounts produced in 8 oz. medicine bottles containing 15 ml. of medium; 7-day cultures; temp. 25°. Concn. of inhibitor 0.1% (w/v); three experiments carried out at different times; three bottles bulked for each analysis.

Fragment	Expt.	Dry wt. (mg.)	β -Carotene	
			Amount (μ g.)	Concn. (p.p.m.)
Control	A	98.8	87	881
	B	100.5	91	903
	C	103.2	90	872
Streptobiosaminidine dimethyl acetal	A	91.3	39	428
	B	92.4	41	454
	C	90.0	36	402
L-N-Methylglucosamine	A	99.9	77.5	837
	B	95.5	80	768
	C	103.2	79	883
Streptosamine	A	91.2	80.5	883
Streptidine sulphate	A	88.3	87	981
	B	86.6	88	1016
	C	87.0	86	988
Streptamine hydrochloride	B	92.5	72	778
	C*	89.0	90	998

* Only two determinations.

dihydroxyacetone and glyceraldehyde were ruled out because they form DNPH's only on boiling, also all their DNPH's except that of dihydroxyacetone, exhibited different absorption spectra from that of the unknown. The remaining possibilities were methylglyoxal and glyoxal; the latter was ruled out because of the different absorption spectrum of its DNPH (Fig. 3), while the spectrum

Fig. 3. Absorption spectra in ether of the 2:4-dinitrophenylhydrazones of: methylglyoxal, ---; glyoxal, —; neutral metabolite from culture medium of *P. blakesleeanus*, —●—. The $E_{\max}$ values have been adjusted to the same level for the three compounds.

of the unknown DNPH was almost identical with that of methylglyoxal. All the compounds gave the same absorption spectrum with a broad maximum at 560 mμ. when treated with NaOH. As the unknown was present in the medium only in minute traces, it was not possible to obtain it crystalline, but the above evidence makes it almost certain that it is methylglyoxal.

The acid carbonyl fraction. Chromatography of the acid DNPH's isolated by methods A (Experimental) and by using the solvent system of Altmann *et al.* (1951) revealed three spots *A*, *B* and *C*, with R_f values of 0.36, 0.75 and 0.80 respectively. *A* and *C* occurred only in traces. Compound *A* was shown to be the DNPH of pyruvic acid by the identity of its absorption spectrum in ethanol and 1.5N-NaOH with that of an authentic specimen (Goodwin & Williams, 1952) and by its failure to

separate from an authentic specimen of pyruvic acid DNPH on paper chromatography of the mixture. Further, in media deficient in thiamine, the intensity of this spot increased very considerably, and Haag (1940) has found that the extracellular pyruvate produced by a number of yeasts and moulds was very much increased when these organisms were grown in the presence of sub-optimal amounts of thiamine.

Spot *C* was yellowish brown and gave a blue coloration with NaOH; because of the small amounts produced, it was not investigated further. The major spot, *B*, was khaki-coloured and gave a bright purple-blue colour with alkali. When the keto acids were extracted as such from the medium (with ethyl ether at pH 1), the DNPH's produced did not contain fraction *B*. Subsequent treatment of the residual medium with DNPH produced compound *B*. In streptomycin media, *B* could easily be separated from the DNPH of streptomycin by extracting the treated medium with ethyl ether, in which the latter is insoluble. From 2 l. of medium (20 penicillin flasks) about 6 mg. of crystalline *B* was obtained which decomposed above 350° without melting and contained 20.3% of N ($\equiv$ mol.wt. of 178 assuming a bis-derivative). The absorption maxima of its spectrum in ethyl ether and 0.75N-NaOH were at higher wavelengths than any of those recorded for the DNPH's of the keto acids derived from the naturally occurring amino acids (Cavallini, 1950). Treatment of the DNPH with aluminium amalgam, with subsequent paper separation of the products (Kulonen, 1953), produced three spots which fluoresced in ultraviolet light with colours varying from bright orange to bright green; none of the spots, however, was ninhydrin-positive, thus confirming the supposition that *B* was not a DNPH of a keto acid.

The possibility that *B* was the DNPH of an enediol was examined by comparing its absorption spectrum in ether and NaOH with those of a number of such or related compounds. Its spectral properties correspond very closely in position (in both ethyl ether and 0.75N-NaOH) and shape with that of hydroxytetronic acid but are quite different from those of the other related compounds examined (Table 9). A mixed paper chromatogram of the DNPH's of the metabolite and hydroxytetronic acid showed that the two compounds are easily separable, indicating their non-identity. It would seem, however, that the metabolite is probably a derivative of tetronic acid but that it is not ascorbic acid.

Neither of these two metabolites could be detected when vegetative mats were dissimilating glucose or when the fungus was grown on an acetate medium.

Table 9. *Absorption spectrum maxima (m μ .) of 2:4-dinitrophenylhydrazones of a number of ene-diols*

	Solvent	
	Ethyl ether (m μ .)	0.75N-NaOH (m μ .)
Metabolic product of <i>P. blakesleeanus</i>	395, 430	535
Hydroxytetronic acid	390, 435	535
Terrestrial acid	340	449
Penicillic acid	357	410
Carlosic acid	315	412
Carlic acid	343	417
Carolic acid	341	413
Carolinic acid	343	415
isoAscorbic acid	348	420
Ascorbic acid	343	520, 544
Reductone	348, 345	Destroyed in alkali
Reductic acid	370	Destroyed in alkali

Table 10. *Effect of various antibiotics on growth and carotenogenesis in Phycomyces blakesleeanus*

Amounts produced in 8 oz. medicine bottles containing 15 ml. of standard medium; three mycelia bulked for each analysis; 7-day cultures; temp. 25°.

Antibiotic added	Concn. (%, w/v)	% of control values	
		Dry wt.	β -Carotene
Chloromycetin	0.02	103.1, 968	90.9, 95.1
	0.10	77.7, 843	44.6, 46.7
	0.16	84.2	27.9
	0.20	46.3	12.4
Aureomycin	0.001	106.0	105.9
	0.01	Complete inhibition	
Penicillin	0.02	95.7	129.2
	0.16	Complete inhibition	
Heximide	0.000015	114.2	49.7
	0.00015	82.0	69.1
	0.0010	88.9	49.7
	0.0015	80.1	52.6
	0.020	79.8	68.7
	0.050	Complete inhibition	
Terrestrial acid	0.010	90.7, 100.5	53.9, 85.7
	0.015	35.4	28.6
	0.020	57.4	32.7
	0.025	Complete inhibition	
Penicillic acid	0.010	95.9	86.6
	0.025	100.6, 86.8	58.2, 26.6
	0.05	47.0	9.9
	0.10	Complete inhibition	
Carlosic acid	0.025	112.6	86.7
	0.25	Complete inhibition	
Carlic acid	0.025	111.9	134.0
	0.25	Complete inhibition	
Carolic acid	0.025	78.9	86.0
	0.25	Complete inhibition	
Carolinic acid	0.01	99.1	99.4
	0.025	87.3	72.0
	0.05	83.5	62.1
	0.10	Complete inhibition	

Effect of streptomycin on the production of methylglyoxal and the ene-diol

It was always obvious from visual inspection of chromatograms of the DNPH's of 'streptomycin media' that *B* was almost completely absent and that the amount of methylglyoxal-like compound was reduced. In a number of experiments the ene-diol was determined quantitatively as for pyruvate (Goodwin & Williams, 1952), using 560 m μ . instead of 412 m μ . for determining the *E* value; the methylglyoxal was similarly determined in the ethyl ether extract after removing the ene-diol with 5% (w/v) Na₂CO₃. Comparison of the normal and streptomycin media showed that in the latter the synthesis of *B* was reduced on the average by 93% and that of methylglyoxal by 58%.

Effect of antibiotics other than streptomycin

A number of other antibiotics have been reviewed for their effect on growth and carotenogenesis in *P. blakesleeanus*. Table 10 shows that chloramphenicol, penicillic acid and terrestrial acid inhibit carotenogenesis to a much greater extent than they do growth, while aureomycin, penicillin, and carlosic, carlic, carolic and carolinic acids did not have a marked effect on either growth or carotenogenesis at concentrations below those at which they inhibit growth completely. It has not been possible to examine some of the acids in any further detail because of lack of material.

The results with chloramphenicol and with penicillin confirm those of Schopfer and his group.

DISCUSSION

Mode of action of streptomycin

Carotenogenesis in *P. blakesleeanus* cultured on the standard medium can be divided into two phases: (a) about 30% of the total, produced during the growing period, and (b) about 70% of the total, produced after growth is completed (Goodwin & Willmer, 1952). The results obtained with streptomycin would justify the working hypothesis that the antibiotic inhibits the second phase only, for the following reasons: (i) the maximal inhibition of carotenogenesis on the standard medium is about 60% (Fig. 1), (ii) carotene synthesis by vegetative cells (phase b) is almost completely inhibited, (iii) no inhibition occurs in young cultures (Table 1) and (iv) in the absence of excess of glucose (i.e. when the concentration is reduced from 2.5 to 1.0%) the degree of inhibition is perhaps slightly reduced. (In this case the reduction is not as clear-cut as might be expected, but it is likely that phase b is proceeding to some degree even during growth.) The main difference between the two phases might be that during growth, asparagine is also utilized

for carotene synthesis whilst after growth is completed glucose only is used, a conclusion compatible with the observations of Chichester, Mackinney & Wong (1954) on the incorporation of labelled fructose into β -carotene by *P. blakesleeanus*. When the effects of streptomycin on the acetate and the glucose/asparagine media are compared, at least three possible interpretations exist: (A) That as growth and carotenogenesis on the acetate medium are equally inhibited, the primary effect is on growth. This would lead to the conclusion that neither growth nor the streptomycin-sensitive synthesis of β -carotene on the glucose/asparagine medium proceeds via acetate. For general anabolic processes (growth) this conclusion would appear unlikely and thus it may be that streptomycin acts primarily by inhibiting the activation of acetate. (B) If streptomycin were not directly affecting carotenogenesis on the acetate medium, then the acetate not being utilized for growth could theoretically be 'shunted' into carotene synthesis so that the concentration would increase. As this is not the case, it could be concluded that acetate incorporation into β -carotene is directly affected by streptomycin and that the streptomycin-sensitive carotene synthesis on the glucose/asparagine medium proceeds via acetate. (C) As the final pH of the acetate medium is about 7.8, which is near the optimum for streptomycin activity against bacteria (Henry, Henry, Berkman & Housewright, 1947; quoted by Florey *et al.* 1949), an effect is apparent which cannot be produced at a lower pH (glucose medium); this implies that carotenogenesis is more sensitive than is growth to streptomycin at the lower pH. A decision as to which is correct cannot be made at present and, indeed, the situations envisaged may be oversimplified. For example, when asparagine in the standard medium is replaced by an ammonium salt, streptomycin inhibition of carotenogenesis disappears. This could be ascribed to the lower final pH obtained on the ammonium salt medium (Table 5), for there are many reports of the reduced antibacterial activity of streptomycin at low pH values; for example, it is about sixty times more active against *Staph. aureus* at pH 7.7 than at pH 5.2 (Wolinsky & Steenken, 1946). It is unlikely that streptomycin is destroyed at the lower pH prevailing in the ammonium salt media, for Regna, Wasselle & Solomons (1946) showed that no appreciable hydrolysis of streptomycin occurred over 900 hr. at pH 1.7.

The failure of streptomycin also to inhibit the increased carotene production on the glucose/ NH_4NO_3 medium in the presence of traces of carboxylic acids would probably be similarly explicable except for one outstanding fact: stimulation of carotenogenesis in the presence of

succinate is inhibited by streptomycin. Until more is known of the mechanism of this stimulation on the glucose/ NH_4NO_3 medium by the carboxylic acids, it is possible to do no more than emphasize this important anomaly.

It is important in conclusion to point out that the concentration of streptomycin required to produce maximal inhibition of carotenogenesis in *P. blakesleeanus* is much higher than that used in studying bacterial inhibition (0.1–0.2%, (w/v), compared with 0.5–50 $\mu\text{g./ml.}$) and that required to produce permanent apochlorosis in *E. gracilis* (40–500 $\mu\text{g./ml.}$ according to strain) (Hutner & Provasoli, 1951). Inhibition of carotenogenesis in the photosynthetic bacterium *Rsp. rubrum* is also achieved at a much lower concentration (10–100 $\mu\text{g./ml.}$) (Goodwin & Osman, 1953), although the effect is very much less marked than in *P. blakesleeanus*.

Methylglyoxal and the ene-diol

Although the synthesis of these two compounds is reduced in the presence of streptomycin, it is unlikely that they are connected with carotenogenesis, for neither is produced in detectable amounts when β -carotene is being synthesized either on acetate media or by vegetative cells of *P. blakesleeanus*.

Other antibiotics

Perhaps the most interesting observation emerging from the brief survey of the action of other antibiotics on carotenogenesis in *P. blakesleeanus* is that heximide inhibits carotenogenesis, although it has been reported to increase the number of yellow (carotene-containing) colonies in cultures of *Allomyces arbuscula* (Whiffen, 1952) and *Blastocladiella* spp. (Cantino & Hyatt, 1953).

SUMMARY

1. On a glucose/asparagine medium streptomycin inhibits carotenogenesis but not growth. Maximal inhibition is obtained with 0.05–0.1% (w/v) streptomycin; increased concentrations of streptomycin up to 1.6% (w/v) do not further affect carotenogenesis. Carotenogenesis in the dark and the carotenogenic action of leucine and β -ionone are also streptomycin-sensitive.

2. Replacement of asparagine in the glucose/asparagine medium wholly or partly by an ammonium salt results in failure of streptomycin to inhibit carotenogenesis. Stimulation of growth and carotenogenesis on the glucose/ NH_4NO_3 medium by (0.02M) carboxylic acids is unaffected by streptomycin, with the exception of carotene stimulation in the presence of succinate.

3. Streptobiosamidine dimethyl acetal, but no other component of the streptomycin molecule, inhibits carotenogenesis.

4. On a medium in which acetate replaces glucose, streptomycin inhibits growth and carotenogenesis equally.

5. Two carbonyl compounds occur in the *P. blakesleeanus* medium; one is almost certainly methylglyoxal and the other is an ene-diol related to hydroxytetronic acid. The synthesis of the ene-diol is almost completely suppressed in the presence of streptomycin and that of methylglyoxal reduced.

6. A brief survey of the effect on carotenogenesis of a number of other antibiotics is recorded.

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