Phytochemistry, 1977, Vol 16, pp. 551–555 Pergamon Press Printed in England

CHEMICAL INDUCTION OF β-CAROTENE BIOSYNTHESIS*

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(*Received* 13 *September* 1976)

Key Word Index—*Citrus paradisi*; Rutaceae; Marsh seedless grapefruit; carotenoid biosynthesis; bioregulators; tertiary amines; lycopene; β-carotene.

Abstract—Marsh white seedless grapefruit were treated with the 2-diethylaminoethanol esters of the following acids: benzoic, phenylacetic, hydrocinnamic, 4-phenylbutyric, 5-phenylvaleric, valeric, hexanoic, heptanoic, octanoic, nonanoic, 5-chlorovaleric, cyclohexanecarboxylic, phenoxyacetic, *p*-chlorophenoxyacetic, 3-phenoxypropionic, cinnamic and *p*-chlorocinnamic. Several of these esters, in particular the hexanoate, 4-phenylbutyrate and cinnamate, caused the accumulation of large amounts of β-carotene. The effects of the hexanoate and of 2-phenoxytriethylamine, which causes only lycopene accumulation, were studied as functions of time. The hexanoate caused the rapid accumulation of lycopene during the first day. The amount of lycopene then began to decrease and that of β-carotene increased until, after 14 days, β-carotene was the major pigment. 2-Phenoxytriethylamine caused rapid lycopene accumulation during the first day and a slow steady increase afterwards. Thus, the mode of action of the β-carotene inducers may be similar to that of the lycopene inducers except that the former are probably rapidly hydrolysed by the esterase(s) in the flavedo, so that they no longer inhibit the cyclase(s), and β-carotene is accumulated at the expanse of lycopene.

INTRODUCTION

Carotenogenesis in citrus is affected by a variety of tertiary amines of the general formula RCH_2NEt_2 [1–7]. The total carotene content is greatly increased and lycopene (ψ,ψ-carotene) becomes a major pigment. Derepression of a gene regulating the synthesis of a specific enzyme(s) and inhibition of the cyclase(s) have been postulated to explain these effects [3]. The biological activity depends upon the lipid solubility of the compound and the electron-withdrawing ability of substituent groups [5–7].

A series of *para*-substituted 2-diethylaminoethyl benzoates caused a much larger accumulation of β-carotene (β,β-carotene) than other lycopene inducers although lycopene remained the major pigment [7]. For the further examination of this effect, the 2-diethylaminoethyl esters of the following acids were synthesized and tested on Marsh white seedless grapefruit: benzoic (**1**), phenylacetic (**2**), hydrocinnamic (**3**), 4-phenylbutyric (**4**), 5-phenylvaleric (**5**), valeric (**6**), hexanoic (**7**), heptanoic (**8**), octanoic (**9**), nonanoic (**10**), 5-chlorovaleric (**11**), cyclohexanecarboxylic (**12**), phenoxyacetic (**13**), *p*-chlorophenoxyacetic (**14**), 3-phenoxypropionic (**15**), cinnamic (**16**) and *p*-chlorocinnamic (**17**). Several of these esters, in particular **4**, **7** and **16**, caused very large increases in β-carotene, so that it was the major pigment, with only a small amount of lycopene. The carotene accumulation was also studied as a function of time.

RESULTS AND DISCUSSION

The esters were applied as free amines in *iso*-PrOH. This method of treatment only stimulates carotenogenesis in the flavedo, the endocarp remains uncoloured. The peel remained healthy on all the fruit except those treated with **5**, **9** and **10**, which damaged about 25, 30 and 60% of the peel area, respectively. The tendency to cause tissue damage with increasing lipid solubility has been noted for other inducers [5]. Only undamaged flavedo was analysed.

Table 1 gives the results of the first group of esters tested. Compounds **7** and **16** caused dramatic changes in the general pattern of carotenes from that previously observed for lycopene inducers. Usually, massive lycopene accumulation is accompanied by only small increases in the cyclic carotenes [5], or by moderate increases when benxoates are the inducers [7]. The two esters, however, caused large increases in β-carotene, smaller increases in α- and γ-carotene (β,ε- and β,ψ-carotenes) and only moderate increases in lycopene. Compound **13** caused only a weak response but of the same pattern. With **6**, the β-carotene content was also high, but the ratio to lycopene was lower. The former pattern was seen with **1** and **2**. Compounds **11** and **12** were ineffective except for inducing the formation

* Part 10 in the series 'Chemical Regulation of Carotenoid Biosynthesis'. For Part 9 see Poling, S. M., Hsu, W. J. and Yokoyama, H. (1976) *Phytochemistry* **15**, 1685.

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Table 1. Effect of compounds **1**, **2**, **6**, **7**, **11**–**13** and **16** at 0.2 M concentration on the carotene content of flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	1	2	6	7	11	12	13	16
Phytofluene	34.8	41.3	44.4	37.1	40.2	33.3	27.6	31.3	43.9
ζ-Carotene	6.51	15.1	13.7	12.1	14.3	5.51	3.58	6.69	19.3
Neurosporene	0.39	1.96	1.72	1.28	0.68	1.01	0.96	1.13	1.52
Lycopene		28.5	32.0	21.1	22.3	0.30	0.71	1.17	15.4
γ-Carotene	0.35	0.64	1.17	1.95	6.65	0.17	0.26	0.42	5.54
α-Carotene	0.45	0.65	1.44	1.76	*	0.59	0.49	0.79	3.46
β-Carotene	2.03	3.38	7.15	32.6	127	2.20	1.77	7.62	99.5
Total carotenes	44.6	91.5	101	108	211	43.0	35.4	49.1	188
Total xanthophylls	22.6	21.5	24.4	19.9	24.9	29.1	22.8	23.0	25.1

* Unresolved from β-carotene.

of a very small amount of lycopene. Compound **12** is the saturated analog of the benzoate (**1**) and lacked activity probably because of steric effects. The ester of 2-ethylhexanoic acid also showed these steric effects (unpublished results). It failed to cause any lycopene formation and decreased the total carotenes.

Table 2 shows the effects of a second group of esters. In this experiment **7**, **13** and **16** were much less effective than in the previous one, although the general pattern was the same. The variability of the effectiveness is discussed later. The activities of **13** and **16** were not lower, but higher, than those of their *p*-Cl analogues, **14** and **17**. Usually, electron-withdrawing groups increase the activity of the inducers [6, 7]. The increased length of its side chain and, therefore, its lipid solubility made **15** markedly more active than **13**. The most active ester tested, **4**, caused a large increase in β-carotene.

Because **7** and **4** were the most active in inducing β-carotene accumulation, the series of aliphatic esters, **6**–**10**, (Table 3) and ω-arylaliphatic esters, **2**–**5**, (Table 4) were tested. Compound **7** was the most effective aliphatic ester and **8** was the next. Both **9** and **10** caused large β-carotene increases, but severely damaged the peel. Again, the ratio of β-carotene to lycopene was low for **6**. The ω-arylaliphatic ester **2** more closely resembled the benzoate (**1**) in its effect than the other members of the series. An increase in the chain length by one methylene group, ie, **3**, reversed the relation between lycopene and β-carotene and the latter became the major pigment. Compound **4** was the most active of the series, whereas **5** was much less active and caused severe peel damage. The most active β-carotene inducers were the hexanoate (**7**), 4-phenylbutyrate (**4**) and cinnamate (**16**).

To see whether β-carotene and lycopene accumulate in the same way with both β-carotene and lycopene inducing compounds, we applied **7** and 2-phenoxytriethylamine (**18**), which is a moderately effective lycopene inducer [5], to grapefruit from the same batch and analysed them after 1, 2, 3, 4, 7 and 14 days. Figure 1

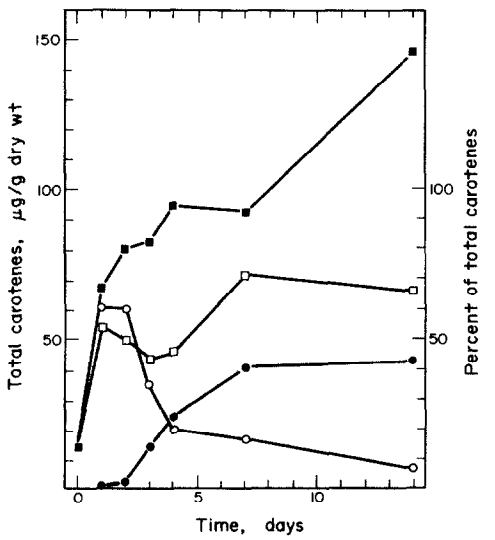

Fig. 1. Changes in the flavedo carotenes of Marsh seedless grapefruit after treatment with carotene inducers. —■— 0.2 M 2-phenoxytriethylamine (**18**), total carotenes; —□— 0.2 M diethylaminoethyl hexanoate (**7**), total carotenes; —○— and —●— lycopene and β-carotene, respectively, as percent of total carotenes for 0.2 M **7**.

Table 2. Effect of compounds **4**, **7**, **13**–**16** and **17** at 0.2 M concentration on the carotene content of flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	4	7	13	14	15	16	17
Phytofluene	27.0	20.3	20.6	26.6	19.2	27.6	26.2	21.3
ζ-Carotene	3.05	3.89	2.89	3.92	1.70	3.69	6.60	2.77
Neurosporene	0.20	0.30	0.36	0.35	0.56	0.45	0.43	0.44
Lycopene		4.12	1.30			1.93	2.83	3.35
γ-Carotene		1.89	0.91			0.82	0.56	0.61
α-Carotene	0.25	3.32	0.98	0.42	0.45	1.24	0.58	1.51
β-Carotene	0.78	40.0	20.2	2.51	1.31	15.6	8.46	8.07
Other carotenes	2.06	1.75	0.79	1.97	2.47	0.75	0.93	0.94
Total carotenes	33.4	72.6	48.1	35.8	25.6	52.1	46.6	38.9
Total xanthophylls	15.8	17.2	16.7	20.8	20.8	16.5	15.6	17.5

Table 3. Effect of compounds **6–10** and **16** at 0.2 M concentration on the carotene content of flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	6	7	8	9	10	16
Phytofluene	36.6	35.9	36.6	32.4	31.2	30.7	39.2
ζ-Carotene	5.11	10.3	19.4	15.7	13.0	8.48	20.5
Neurosporene		0.55		0.61			0.62
Lycopene		9.30	11.4	21.5	8.03	2.79	9.28
γ-Carotene	0.33	1.01	5.57	3.54	2.13	2.01	3.18
α-Carotene	0.33	1.32	3.68	3.05	6.18	3.22	2.22
β-Carotene	1.61	14.2	98.3	58.3	50.5	43.0	50.4
Other carotenes	2.40	1.19	2.52	2.16	2.40	2.04	1.64
Total carotenes	46.4	73.8	177	137	113	92.2	127
Total xanthophylls	24.9	18.2	22.0	24.4	22.1	24.3	21.2

shows the total carotenes plotted for **7** and **18**, and the percentages of the total carotenes that were due to lycopene and β-carotene for **7**. Compound **18** caused a rapid increase in carotenes the first day and then a slow steady increase thereafter. Lycopene comprised 52 and 71 % of the total carotenes after 1 and 14 days, respectively, while there was no significant increase in the cyclic carotenes. Compound **7** caused a much different response. Total carotenes increased rapidly during the first day due to the formation of lycopene, not to β-carotene accumulation. The percentage of lycopene decreased slightly on day 2, dropped rapidly during days 3 and 4 and decreased slowly thereafter. The percentages of β-carotene and of lycopene varied conversely so that β-carotene exceeded lycopene on day 4 and was the predominant pigment by day 14. Compound **7** induced only a relatively small increase in total carotenes between days 1 and 14. The experiment with **7** was repeated with different grapefruit and the responses were the same. The percentage of β-carotene exceeded that of lycopene just after 4 days, while the total carotene content increased slowly between days 1 and 14 from 65 to 100 μg/g dry wt. Analysis of another replicate experiment showed that after 4 days, the β-carotene and lycopene contents were, respectively, 39.9 and 42.5 μg/g dry wt. The β-carotene inducing compounds in Tables 1–4 probably acted the same way. The flavedo was analysed after 14 days, so most of the lycopene should have been converted to β-carotene. The β-carotene inducers thus seemed to cause a very rapid, initial accumulation of lycopene, which was then converted to β-carotene. The conversion was half completed after 4 days and continued afterwards until most of the lycopene was converted.

The question then arose as to whether the increase in cyclic carotenes caused by the benzoates [7] was also due to conversion of lycopene. The ratio of β-carotene to lycopene was much lower when **1** was used at 0.2 M than in earlier work at 0.1 M [7]. Therefore, a low concentration (0.02 M) of the most active of the benzoates, *p*-bromobenzoate (**19**), was applied to grapefruit, and the fruit were examined after 2 and 4 weeks (Table 5). After 2 weeks the increase in β-carotene was moderate and lycopene was the major pigment. But after 4 weeks, lycopene had decreased and the β-carotene increased. Thus, it appears that the benzoates act in the same way as the aliphatic esters but over an extended time scale. These results could be explained if the esters are assumed to hydrolyse after they begin to affect the fruit. The aliphatic ester enters the fruit, stimulates the pathway, but

Table 5. Effect of compound **19** at 0.02 M concentration after 14 and 28 days on the carotene content of the flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	14 days	28 days
Phytofluene	36.6	51.9	81.2
ζ-Carotene	5.11	24.0	37.4
Neurosporene		0.42	0.82
Lycopene		141	80.3
γ-Carotene	0.33	2.00	4.59
α-Carotene	0.33	1.38	5.09
β-Carotene	1.61	14.9	58.5
Other carotenes	2.40	2.20	4.48
Total carotenes	46.4	238	272
Total xanthophylls	24.9	27.4	29.9

Table 4. Effect of compounds **1–5** and **16** at 0.2 M concentration on the carotene content of the flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	1	2	3	4	5	16
Phytofluene	47.8	43.9	47.9	35.9	34.7	29.3	40.8
ζ-Carotene	6.48	11.0	10.7	9.91	9.78	5.06	12.3
Neurosporene	0.41	1.07	0.84	1.20	1.37	0.79	0.84
Lycopene		31.6	22.9	4.72	13.1	2.32	11.0
γ-Carotene	0.34	1.10	0.86	1.04	3.90	0.88	3.05
α-Carotene	0.18	0.38	0.81	0.78	5.48	0.85	2.46
β-Carotene	0.81	1.97	3.85	23.6	93.4	14.1	71.1
Other carotenes	1.68	0.81	0.52	0.34	0.72	0.25	0.62
Total carotenes	57.7	91.9	88.4	77.5	162	52.7	142
Total xanthophylls	29.8	30.4	33.7	26.6	29.0	26.0	33.0

also blocks the cyclase(s) and causes the rapid build up of lycopene. Meanwhile, the ester hydrolyses, and little remains after 2 days. Thus, the pathway is no longer stimulated and the inhibition of the cyclase(s) is removed, and the lycopene is then converted to β-carotene. If benzoates take longer to hydrolyse, they would stimulate the pathway for a longer time and cause both an initial rapid build-up and a steady increase thereafterof the total carotenes. The conversion of lycopene to β-carotene would be delayed and would be completed more slowly with the benzoates than the aliphatic esters. Differences in the rates of hydrolysis and the effects of hydrolysis would explain why **1** and **2** caused a greater increase in total carotenes than **3** (Table 4), even though they are less lipid soluble. The lower β-carotene to lycopene ratios for **1** and **2** as compared to **3** are evidence of the slower hydrolysis of these two compounds, thus, they can act on the pathway for a longer period causing a larger increase in the total carotenes. If this explanation is true, application of an inhibitor of the cyclase(s) with a β-carotene inducer should cause lycopene accumulation. This was done by use of **7** and 1-morpholino-3-phenoxypropane (**20**). Generally, replacing the diethylamino group of active lycopene inducers with the morpholine group greatly reduces their activity but they still act as strong inhibitors of the cyclase(s) (unpublished results). Table 6 shows that **7** caused the usual β-carotene accumulation. Compound **20** caused a slight lycopene increase. But addition of both **7** and **20** stimulated lycopene formation and prevented its conversion to β-carotene.

The esters could undergo passive chemical hydrolysis or active hydrolysis by naturally occurring esterase(s). Esterase activity has been found in the flavedo of citrus [8, 9]. The methyl esters of *p*-aminobenzoic, *p*-bromobenzoic and *p*-nitrobenzoic acids were not hydrolysed, whereas the methyl esters of butyric and propionic acids were hydrolysed faster than methyl benzoate, which was hydrolysed slowly [8]. The activity of the esterase(s) is in general agreement with our qualitative estimates of the hydrolysis rates of the esters we studied. Thus, the aliphatic esters may have been hydrolysed by esterase(s) in the flavedo. The benzoates may either have been hydrolysed by the esterase(s) at a rate that was too small to measure by the techniques used in the earlier studies or they may have undergone passive chemical hydrolysis.

The greater apparent variability of the response caused by the β-carotene inducing esters (compare **16** in Tables 1–4) than by lycopene inducers could be due to one or both of the following reasons. There could be a wide variability of the esterase activity of the flavedo. With very high esterase activity, the inducers could be inactivated before producing much effect. The variability might also be due to variations in the amount of metabolic intermediates needed to synthesize either the enzymes of the biosynthetic pathway or the carotenoids at the time of treatment, or both. The β-carotene inducers only act for a short time, and their action might depend upon the immediate availability of the intermediates. The lycopene inducers, on the other hand, may remain active much longer, as suggested by the action of **18**, and may be able to mobilize the needed intermediates; thus, the lycopene content could increase slowly and steadily, even though the initial burst of activity is missing.

Table 6. Effect of compounds **7**, **20** and **7** + **20** at 0.2 M concentration on the carotene content of the flavedo of Marsh seedless grapefruit (μg/g dry wt)

	Control	**7**	**20**	**7** + **20**
Phytofluene	31.7	42.1	33.5	31.1
ζ-Carotene	4.24	19.6	7.41	11.5
Neurosporene	0.33	1.45	1.56	2.03
Lycopene		7.82	5.08	52.1
γ-Carotene	0.20	2.50	0.80	1.53
α-Carotene	0.32	1.81	1.13	2.09
β-Carotene	1.00	53.3	1.23	2.41
Total carotenes	37.8	129	50.7	103
Total xanthophylls	25.4	24.0	24.7	25.9

Our results can be summarized as follows. The aliphatic esters of 2-diethylaminoethanol, in particular, the hexanoate (**7**) 4-phenylbutyrate (**4**) and cinnamate (**16**) caused β-carotene accumulation by a two stage process. We postulate, that initially, they stimulate the pathway and block the cyclase(s) in the same manner as lycopene inducers, such as **18**, so that lycopene accumulates rapidly during the first day after treatment. Lycopene inducers remain active after this initial burst of biosynthetic activity and cause lycopene to accumulate at a steady but slower rate thereafter. The aliphatic esters, on the other hand, are probably hydrolysed, possibly by the esterase(s) in the flavedo, and lose activity after 2 or 3 days. Inactivation of the aliphatic esters removes both the stimulation of the pathway and the inhibition of the cyclase(s). Most of the accumulated lycopene is then converted to β-carotene, with smaller increases in γ- and α-carotene, during the 3 or 4 days after the inactivation of the inducer. The benzoates are intermediate between the aliphatic esters and the lycopene inducers. Because they are hydrolysed much more slowly than the aliphatic esters, the benzoates cause greater total carotene accumulation; but the conversion of lycopene to β-carotene is greatly delayed.

EXPERIMENTAL

Fruit samples. Marsh white seedless grapefruit were harvested from different fields at different times except those used in Tables 3 and 5. All samples consisted of 6 fruit except those in Table 4, which consisted of 8 fruit.

Post-harvest treatment of fruit. All test compounds were applied at 0.2 M solns in *iso*-PrOH except **19**, which was used at 0.02 M. The control was treated with *iso*-PrOH. The soln was poured over the surface of the fruit to give complete coverage. The fruit were allowed to drain and then moved to a clean surface to air dry for several hours. Then they were placed in polyethylene bags and stored at room temp. (~21°). Flavedo was removed 14 days after treatment from all fruit except those used in the two time studies (Table 5 and Fig. 1).

Isolation and identification of the pigments. Pigments were isolated and identified by published methods [5]. 'Other Carotenes' consisted of the total content of 3 or more bands that were present in amounts too small to identify individually. $E^{1\%}_{1\,cm} = 2700$ was assumed for 'Other Carotenes' and 'Total Xanthophylls'.

Compounds **1**–**17** and **19** were made from the acyl chlorides as previously described for the benzoates [7]. The acyl chlorides for **3**–**5**, **14**, **16** and **17** were not commercially available and were synthesized from the acids by the method of Martin and Fieser [10].

2-Phenoxytriethylamine (**18**) was prepared for an earlier work [5].

1-Morpholino-3-phenoxypropane (**20**) was synthesized from 3-phenoxypropyl bromide and morpholine instead of diethylamine [5].

Acknowledgement—This work was supported in part by the Florida Citrus Commission.

REFERENCES

1. Coggins, Jr., C. W., Henning, G. L. and Yokoyama, H. (1970) *Science* **168**, 1589.
2. Yokoyama, H., Coggins, Hr., C. W. and Henning, G. L. (1971) *Phytochemistry* **10**, 1831.
3. Yokoyama, H., Coggins, Jr., C. W., Henning, G. L. and De Benedict, C. (1972) *Phytochemistry* **11**, 1721.
4. Poling, S. M., Hsu, W. J. and Yokoyama, H. (1973) *Phytochemistry* **12**, 2665.
5. Poling, S. M., Hsu, W. J. and Yokoyama, H. (1975) *Phytochemistry* **14**, 1933.
6. Hsu, W. J., Poling, S. M., De Benedict, C., Rudash, C. and Yokoyama, H. (1975) *J. Agr. Fd Chem.* **23**, 831.
7. Poling, S. M., Hsu, W. J. and Yokoyama, H. (1976) *Phytochemistry* **15**, 1685.
8. MacDonnell, L. R., Jang, R., Jansen, E. F. and Lineweaver, H. (1950) *Arch. Biochem.* **28**, 260.
9. Schwartz, H. M., Biedron, S. I., Von Holdt, M. M. and Rehm, S. (1964) *Phytochemistry* **3**, 189.
10. Martin, E. L. and Fieser, L. F. (1943) in *Organic Syntheses* (Blatt, A. H., ed.), Coll. Vol. II, pp. 569–571. John Wiley, New York.