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INHIBITION BY ISONICOTINYL HYDRAZIDE OF PIGMENT FORMATION IN HIGHER PLANTS

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Key Word Index—*Zea mays*, maize; *Hordeum vulgare*, barley; Gramineae; *iso*nicotinyl hydrazide; glycollate pathway; L-serine; pyruvate; β-carotene; chlorophyll.

Abstract—The inhibition of greening of illuminated etiolated maize seedlings by *iso*nicotinyl hydrazide can be alleviated by serine or pyruvate. The similar inhibition in barley can be reversed only by pyruvate. In both plants earlier intermediates in the glycollate pathway and other related compounds were ineffective in overcoming the inhibition of greening produced by *iso*nicotinyl hydrazide. In maize seedlings radioactivity from L-serine-[3-^{14}C] is poorly incorporated into β-carotene, a typical chloroplast terpenoid, unless glycine and formate or, more effectively, glycine together with *iso*nicotinyl hydrazide are supplied. These supplementations may minimize interconversion of serine and glycine, and hence dilution of radioactivity at C-3 of L-serine by unlabelled C-1 units, before incorporation into terpenoids. The results support the view that in young greening tissue the C2-3 fragment of L-serine can give rise to acetyl-CoA, an obligatory precursor of chloroplast terpenoids.

INTRODUCTION

*Iso*NICOTINYL hydrazide (isoniazid, INH) probably acts as an inhibitor of pyridoxal phosphate-requiring enzymes by combining with the coenzyme, the reaction *in vitro* being slow and progressive[1,2] and leading to formation of the pyridoxal phosphate *iso*nicotinyl hydrazone (Fig. 1). In particular in photosynthetic organisms INH has been reported to inhibit the conversion of glycine to serine.[3-5] Thus it has been shown that in photosynthesizing *Chlorella* INH blocks the metabolism of glycollate via glycine and serine to hexoses[3,6,7] and this results in an accumulation of glycollate and glycine. In *Pisum sativum*, after vacuum infiltration with INH, the levels of glycollate and glycine increased whilst that of serine decreased.[8] However, being a general inhibitor of pyridoxal phosphate-requiring enzymes we might expect INH to have a variety of effects on amino acid metabolism and

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biosynthesis, including formation of δ-amino laevulinic acid, in addition to the glycine–serine interconversion. Ultimately protein synthesis will be affected, and it is known that chloroplast pigment formation is inhibited if protein synthesis is blocked.[9,10]

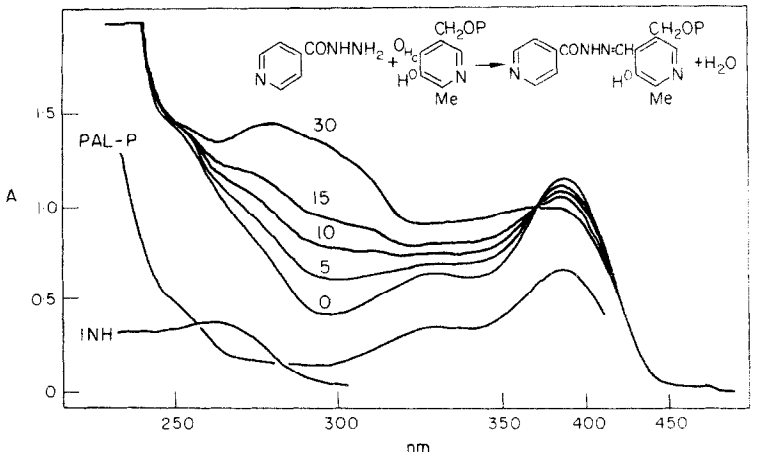

FIG. 1. THE REACTION BETWEEN *iso*NICOTINYL HYDRAZIDE AND PYRIDOXAL PHOSPHATE.
Absorption spectra were obtained immediately and 5, 10, 15 and 30 min after addition of *iso*nicotinyl hydrazide to pyridoxal phosphate. The final conen of both in the cuvette was *ca* 16×10^{-5} M. The lower traces are those for *iso*nicotinyl hydrazide and pyridoxal phosphate, respectively, at *ca* 8×10^{-5} M. The reagents were in 10^{-2} M phosphate buffer, pH 7·2, at 23°. Also shown, in the inset, is the reaction thought to occur between *iso*nicotinyl hydrazide and pyridoxal phosphate.

We have previously used INH as an inhibitor of the glycollate to serine pathway in an attempt to define a possible role for this metabolic route in chloroplast pigment synthesis in higher plants.[11,12] The route proposed involved the sequence, CO_2 ⟶ glycollate → glyoxylate → glycine → serine ⟶ pyruvate → acetyl-CoA. Although in the simplest case all these conversions would take place in the chloroplast it would only be necessary, to explain our earlier isotope incorporation and isotope dilution studies.[11,12] that the initial formation of glycollate and a step(s) in the conversion of serine to pyruvate be chloroplastidic. These earlier studies showed that INH inhibited the formation of chlorophyll and carotenoids in illuminated etiolated maize seedlings and that the incorporation of radioactivity into β-carotene from $^{14}CO_2$, glyoxylate-[2-^{14}C], glycine-[2-^{14}C] and formate-[^{14}C] was inhibited, whereas radioactive label from L-serine-[U-^{14}C] was readily incorporated into the pigments formed upon illumination. In contrast there was no marked effect of INH on sterol formation, which takes place outside the chloroplasts, in any of these experiments. The studies supported an hypothesis of compartmentation of terpenoid biosynthesis in higher plants[13] and were thought to be a possible route for the synthesis of the large amounts of acetyl-CoA which are formed from fixed CO_2 during greening. The evidence in support of this and alternative proposals has been reviewed elsewhere.[14]

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We now report further studies which demonstrate that under some conditions it is possible to overcome the observed inhibition of pigment synthesis by INH by supplementation with metabolites from the biosynthetic sequence postulated for acetyl-CoA formation.

RESULTS AND DISCUSSION

The mechanism of action of INH appears to be through combination with pyridoxal phosphate to form the *iso*nicotinyl hydrazone,[1] a reaction that can be readily followed in a spectrophotometer (Fig. 1). We found that the rate of formation of the hydrazone was maximal at pH 6·1, rather less at pH 7·2, and fell off markedly at higher pHs; pH 7 was selected for incubation of plant material with the inhibitor. As shown by changes in absorption spectra INH did not react with pyruvate, the other metabolites used in the present studies, or with pyridoxol phosphate. Nicotinamide, which bears considerable structural resemblance to INH, did not affect greening of etiolated plants upon illumination, nor did it appear, in spectrophotometric studies, to react with pyridoxal phosphate.

Two methods of exposing tissue to INH were employed. In initial studies inhibitor was supplied to excised etiolated seedlings;[1,2] however, for most studies it was found both more convenient and effective to incubate lengths of etiolated shoot firstly in the dark for 60 min in the presence of inhibitor, and subsequently under illumination for 23 hr in Petri dishes containing inhibitor and the metabolites under test. In the latter case INH penetrates the tissue by diffusion processes and in many cases a narrow central portion of the shoot segment remained free of inhibitor, as evidenced by greening of this region of tissue on illumination.

Preliminary studies were aimed at determining the optimal age of seedlings for use in greening experiments. Samples of etiolated maize and barley shoots were taken at daily intervals after unrolling of the leaves in maize seedlings; and after barley seedlings, where leaf unrolling was less clearly defined, had reached a height of some 7·5 cm. The results of this study are presented in Fig. 2. In the case of barley leaf tissue the greening process, as shown by chlorophyll synthesis over a 24 hr period, was maximal when seedlings were 10·0–12·5 cm tall. Carotenoid formation appeared to be maximal with the youngest tissue tested but only slowly diminished over a period of several days during which time seedlings doubled in height. In experiments with barley *ca* 12·5 cm tall seedlings were therefore routinely used. With maize the formation of both chlorophylls and carotenoids decreased with age of tissue after leaf unrolling, and in this case experiments were carried out as soon as possible after this was observed.

The effect of INH on the greening of etiolated leaves selected in this way was studied by exposure of leaf segments to various concentrations of inhibitor (Fig. 3) and shows that chlorophyll formation in maize was inhibited some 60% by 10 mM INH, and in barley was almost entirely inhibited by this concentration. This concentration of INH, selected for routine use, abolished carotenoid formation in the case of both maize and barley. Indeed, final carotenoid levels following illumination in the presence of inhibitor were often lower than originally present in the etiolated tissue. In these circumstances, the tissue developed a whitish pallor in place of the original yellow colouration. This situation is reflected in a negative change in $A_{480}^{\text{carotenoid}}$ compared to the 85% acetone extract from etiolated shoots. No estimate could be made of the amount of INH which penetrated the tissue but access was restricted, because a narrow central region of leaf commonly turned green upon illumination even in the presence of inhibitor.

The effect of glycine, serine and related metabolites on the INH-produced inhibition of greening of etiolated maize is shown in Table 1. The controls in each series of experiments included estimation of chlorophylls and carotenoids in etiolated tissue, and in tissue after the period of illumination in presence or absence of INH. The difference in chlorophyll level in the first and last of these incubations was taken as the reference for expression of greening as a percentage for incubations under other conditions. The values of these controls in Table 1 are the mean values of several experiments; however, the per cent greening of tissue in the presence of metabolites in each case is given by comparison with the chlorophyll levels in etiolated and green tissue in the appropriate experiment.

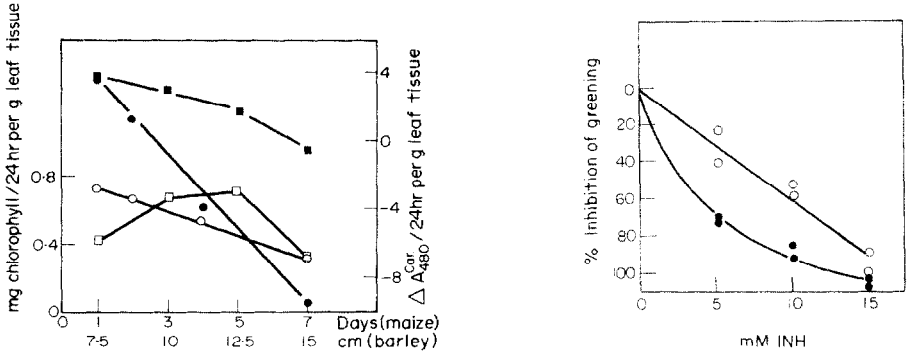

FIG. 2. PIGMENT SYNTHESIS IN SEGMENTS OF ETIOLATED MAIZE AND BARLEY AS A FUNCTION OF AGE OF TISSUE.

Etiolated plants were sampled at daily intervals after maize leaves had unrolled and barley had reached a growth height of *ca* 7·5 cm. Segments of shoot (0·5 cm length) were incubated in H_2O in open Petri dishes and the chlorophyll and carotenoid levels after 24 hr illumination (*ca* 30000 lx) were determined. Maize, —○—○—, chlorophyll; —●—●—, carotenoid: barley, —□—□—, chlorophyll: —■—■—, carotenoid.

FIG. 3. INHIBITION OF PIGMENT SYNTHESIS BY *iso*NICOTINYL HYDRAZIDE.

Segments of etiolated maize and barley shoots were incubated in 40 ml H_2O containing *iso*nicotinyl hydrazide over the range 0–15 mM. Following 1 hr in the dark the samples were illuminated for 23 hr and then analysed for chlorophyll and carotenoid. Samples of leaf (*ca* 1 g) were selected 3 days after leaf unrolling in the case of maize, and when seedlings were 12·5 cm high in the case of barley. Maize, —○—○—; barley, —●—●—.

In maize, supplementation of the incubation medium with glycollate or glycine did not restore chlorophyll or carotenoid formation in tissue treated with inhibitor. Indeed, in these incubations, as in that of tissue incubated with inhibitor alone, carotenoid levels were substantially less than those of etiolated tissue. In contrast, supplementation of the

TABLE 1. EFFECT OF METABOLITE SUPPLEMENTATION ON THE *iso*NICOTINYL

	E.T.*	G.T.*	G.T.I.*	Glycollate		
Metabolite concentration (mM)	–	–	–	6·5	3·25	6·5
*Iso*nicotinyl hydrazide (mM)	–	–	10	–	10	10
Chlorophyll (a + b) (μg/g tissue)	35(6)‡	263(7)	65(6)	280	121	93
% Greening	0§	100§	13(6)	138	22	23
ΔA_{480}^{car}	0¶	4·9(7)	−0·5(6)	6·8	3·0	−2·1

* E.T., etiolated tissue; G.T., greened tissue; G.T.I., tissue greened in the presence of inhibitor.
† Formate (11·0 mM) also present.
‡ Figures in brackets indicate the values quoted are the means of the indicated number of experiments.

medium with L-serine markedly restored greening; in the best experiment carotenoid level was the same as, and chlorophyll level 80% that of, tissue illuminated for the same period in the absence of inhibitor. At comparable concentration pyruvate was almost as effective in restoring greening. In the absence of inhibitor both glycollate and glycine enhanced greening as reflected in both formation of chlorophyll and carotenoid.

The results of similar studies with barley differ in one important respect (Table 2). As with maize, glycine did not restore greening in INH-inhibited tissues. However, in barley, unlike maize, supplementation with L-serine was relatively ineffective. Although it restored carotenoid levels to some 60% those of tissues allowed to green in the absence of inhibitor, it did not restore chlorophyll formation. Indeed, at 9·5 mM L-serine proved less effective in restoring chlorophyll formation than at lower (2·5–5 mM) concentrations. The inhibition of greening by INH was overcome, however, by supplementation of incubations with pyruvate. Both chlorophyll and carotenoid formation was restored by some 80% by 8·5 mM pyruvate.

The observation that serine restored greening in maize tissue treated with INH is in agreement with an earlier study[12] which showed that INH inhibited the incorporation of radioactivity from glyoxylate-[2-^{14}C], glycine-[2-^{14}C] and formate-[^{14}C] into β-carotene of illuminated excised etiolated maize seedlings, but did not affect incorporation from L-serine-[U-^{14}C].

In an unrelated investigation it was shown that when L-serine-[3-^{14}C] was supplied to illuminated etiolated maize seedlings little radioactivity appeared in the β-carotene subsequently isolated. Serine supplied to plants may be subject to considerable interconversion into glycine, resulting in dilution of the radioactivity at C-3 of serine, before incorporation of the serine into pigments. An attempt was therefore made to enhance the incorporation of radioactivity from L-serine-[3-^{14}C] into β-carotene by supplying seedlings at the same time with glycine and formate, or with glycine together with INH, to inhibit conversion of L-serine to glycine. In the latter case glycine was also supplied to meet demands of cell metabolism since its formation *in vivo* from glyoxylate, a pyridoxal phosphate-dependent transamination, might be inhibited by INH. The results of these studies are summarized in Table 3 where for convenience some previous data[12] for L-serine-[U-^{14}C] is also included.

Incorporation of radioactivity from L-serine-[3-^{14}C] into β-carotene of illuminated etiolated maize seedlings was extremely low, but could be enhanced by supplying the plants at the same time with glycine and formate, or more effectively with glycine in the presence of INH. As suggested earlier, the interconversion of serine and glycine may result in the radioactivity at C-3 of serine being diluted by unlabelled C-1 units present *in situ*. Inhibi-

HYDRAZIDE INHIBITION OF GREENING OF ETIOLATED MAIZE

Glycine				L-Serine					Pyruvate	
6·5†	3·25	6·5	6·5	3·25	2·5	2·5	3·0	3·5	5·5	5·5
—	10	10	10	—	10	10	10	10	—	10
260	82	48	57	250	134	161	193	224	277	151
127	10	3	8	121	26	52	63	79	136	46
7·4	−0·3	−0·2	−4·2	7·3	4·3	3·4	5·8	4·5	6·0	3·3

§ These values form the basis for calculation of greening in each experiment. Greening in cases of metabolite addition are based on comparison with the actual values obtained in the appropriate experiment.

¶ The A_{480} for etiolated tissue is taken as the reference from which ΔA_{480} is calculated.

TABLE 2. EFFECT OF METABOLITE SUPPLEMENTATION ON THE *iso*NICOTINYL HYDRAZIDE INHIBITION OF GREENING OF ETIOLATED BARLEY

	E.T.*	G.T.*	G.T.I.*	Glycine		L-Serine					Pyruvate	
Metabolite concentration (mM)				6·5	6·5	5·0	0·62	1·25	2·5	5·0	9·5	8·5	8·5
*Iso*nicotinyl hydrazide (mM)			10		10		10	10	10	10	10		10
Chlorophyll (a + b) (μg g tissue)	81	480	78	501	82	493	99	123	162	162	124	450	371
% Greening	0†	100†	0	107	0	105	5	10	20	20	11	95	73
ΔA_{480}	0‡	9·7	−1·0	4·1	1·7	4·9	−0·1	2·6	5·3	4·7	4·5	7·0	8·3

* E.T., etiolated tissue; G.T., greened tissue; G.T.I., tissue greened in the presence of inhibitor.
† These values form the basis for calculation of greening.
‡ The A_{480} for etiolated tissue is taken as the reference from which ΔA_{480} is calculated.

tion of this conversion by the presence of INH results in a 40-fold enhancement of incorporation of C-3 of added serine into carotenoids. In the metabolic route for conversion of glycollate or glyoxylate to acetyl-CoA, INH would be expected to inhibit the conversions of glyoxylate to glycine (glyoxylate aminotransferase), glycine to L-serine (serine hydroxymethyltransferase), and any direct conversion of L-serine to pyruvate (L-serine dehydratase). Under the conditions used all the metabolites would be able to penetrate the chloroplast membranes freely.[15-20]

TABLE 3. EFFECT OF *iso*NICOTINYL HYDRAZIDE IN INCREASING INCORPORATION FROM L-SERINE-[3-^{14}C] INTO β-CAROTENE

Additions	L-Serine-[U-^{14}C]	L-Serine-[U-^{14}C] 15 mM inhibitor	L-Serine-[3-^{14}C]	L-Serine-[3-^{14}C] glycine HCOOH	L-Serine-[3-^{14}C] glycine 15 mM inhibitor
Radioactivity supplied (μC)	10	10	6·7	6·7	6·7
Weight of seedlings (g)	36	20	20	20	20
Unsaponifiable lipid (mg)	76	66	48	47	44
β-Carotene (dis/min/mg)	81700	88000	960	17500	36400

Specific activity of β-carotene given is after a single recrystallization.
Excised seedlings were exposed during 24 hr illumination to [U-^{14}C]- or [3-^{14}C]-serine with supplementation with approx. 10-fold excess of unlabelled compounds or 15 mM *iso*nicotinyl hydrazide as indicated.

The intracellular location of the enzymes catalysing the formation of L-serine is a matter of controversy. Tolbert and his coworkers[21] believe that though glycollate formation occurs in the chloroplast its subsequent conversion to glyoxylate, and hence glycine, is located in the peroxisomes. Whilst the further formation of L-serine from glycine is primarily located in the mitochondria some L-serine hydroxymethyltransferase activity may be associated with chloroplasts.[22] Other workers[23] claim that glycine and serine can be synthesized in the chloroplast from CO_2, and isolated chloroplasts have been shown to pos-

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[23] ROBERTS, G. R., KEYS, A. J. and WHITTINGHAM, C. P. (1970) *J. Exp. Botany* **21,** 683.

sess the enzymic capacity to carry out individual steps in the sequence from glycollate to L-serine.[24–26] It is likely, therefore, that synthesis of serine can occur in more than one cellular compartment and that different sites of synthesis may assume different degrees of importance as the leaf ages. Evidence has been presented elsewhere that this may be so in the postulated sequence for the formation of acetyl-CoA from glycollate.[27]

However, the proposals regarding the synthesis of the serine used in pigment synthesis *in vivo* are compatible with a collaborative cellular function involving chloroplast enzyme systems together with other cellular membranes or soluble enzyme systems. Although the previous radioisotope incorporation and radioisotope dilution studies[12] are most easily explained on the basis that the route occurs in chloroplasts the data are also consistent with the exclusive occurrence in the chloroplast of the formation of glycollate from CO_2 and also of later stage(s) of the conversion of serine to pyruvate. This latter view would be in accord with another report[28] which suggests that chloroplasts may not possess the entire capacity to form acetyl-CoA from CO_2.

Although in terms of cell economics a direct conversion of L-serine to pyruvate is appealing we do not yet have conclusive evidence that chloroplasts possess this capacity. An alternative sequence is the route L-serine → hydroxypyruvate → glycerate → 2-phosphoglycerate → phosphoenol pyruvate → pyruvate; a route we indicated earlier,[12] and which has also been proposed[29] for acetyl-CoA formation from glycollate in *Chlorella*. The first conversion in this sequence, catalysed by L-serine–pyruvate aminotransferase, is pyridoxal phosphate dependent, and therefore liable to inhibition by INH. However, in isotope dilution studies (Evans and Rogers, unpublished data) with illuminated etiolated maize, glycerate did not suppress incorporation of radioactivity from $^{14}CO_2$ into β-carotene. Moreover, previous radioisotope experiments[12] indicate that the formation of pyruvate from its immediate precursor must be located in the chloroplast, whereas pyruvate kinase, which catalyses the terminal step in the above sequence, does not appear to be a chloroplast enzyme.[30]

This reservation would also apply to a suggestion[14] that pigment formation may originate from 2-phosphoglycerate, formed as a result of photosynthetic CO_2 fixation. This may be the case for mature green plants, in particular monocotyledonous plants where there is only limited turnover of chloroplast terpenoids, but the proposal would appear to be in conflict with our data obtained with young etiolated monocotyledonous plants.

Reservations concerning involvement of a glycollate pathway in synthesis of acetyl-CoA have been detailed elsewhere.[14] They include the suggestion that incorporation into β-carotene of radioactivity from glyoxylate-[2-^{14}C], and presumably of glycine-[2-^{14}C] and L-serine-[U-^{14}C] via appropriately labelled glyoxylate, arises not from a true incorporation into acetyl-CoA but by an exchange of radioactivity between C-2 labelled glyoxylate and acetyl-CoA. This explanation is not in accord with the present work since a net synthesis of acetyl-CoA from added serine, but not from glyoxylate or glycine, is clearly demonstrated in maize by restored chlorophyll and carotenoid formation in INH-inhibited tissues.

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The present experiments show that in maize the inhibition of greening by INH can be reversed by L-serine. Apart from glycollate and glycine, other metabolites ineffective in restoring pigment formation under the same conditions were acetate, acetylphosphate, malonate, lactate and succinate, all intermediates in alternative routes of acetyl-CoA synthesis.[12,14] Supplying phosphoenolpyruvate only partly restored greening, though glucose was almost as effective as serine. Glucose was less effective in the case of barley. None of the compounds inhibited greening in the absence of INH. The inability of acetate, acetylphosphate or malonate to restore greening may be evidence against the possible role of acetate as the immediate precursor of acetyl-CoA in preference to pyruvate.[12,14]

The simplest conclusion to be drawn from our observations is that L-serine may be used in the formation of chloroplast pigments as a precursor of acetyl-CoA. Possible effects of INH through inhibition of δ-amino laevulinic acid biosynthesis or protein synthesis are unlikely since in this event L-serine would be unlikely to restore chlorophyll and carotenoid formation.

In barley L-serine was comparatively ineffective and only pyruvate would restore greening in the presence of INH. This difference may reflect the degree of inhibition of L-serine hydroxymethyltransferase and possibly L-serine dehydratase, or the alternative L-serine–pyruvate aminotransferase, by INH in the two plants. Thus, in maize INH certainly affects the glycine to serine conversion, though previous pyridoxal phosphate dependent enzymes involved in glycine formation may also be inhibited. In *Chlorella*, however, INH does not inhibit incorporation of $^{14}CO_2$ into glycine,[3] while in some higher plants glycine accumulates as well as glycollate.[8] In barley, the conversion of serine to pyruvate is inhibited and only the latter metabolite will restore pigment formation. This distinction in maize and barley may be explained by differences in binding of pyridoxal phosphate to the respective enzymes in the two plants.

The potential quantitative importance of the serine route for pigment formation is indicated by the almost complete restoration of pigment formation in INH-treated maize and barley seedlings by serine and pyruvate respectively. However, the route may be of primary importance in young greening tissue where the chloroplasts are not fully functional, and its significance in mature green plants was not determined by the present studies.

EXPERIMENTAL

Treatment with isonicotinyl hydrazide. Etiolated maize (*Zea mays*, cv. Rhodesian White Tooth Double Hybrid) and etiolated barley (*Hordeum vulgare*, cv. Proctor) were grown from seed in vermiculite on trays in the dark at 25°. For exposure to inhibitor and/or metabolites, etiolated shoots were taken at appropriate maturity (see Results and Discussion), and cut into segments *ca* 0·5 cm in length. A known weight of leaf was incubated with INH in the dark for 60 min in a boiling tube containing an appropriate concn of inhibitor at pH 7. The leaf segments were then transferred to Petri dishes containing 40 ml soln of the same inhibitor concn supplemented as necessary with the metabolites under test, and the lidded Petri dishes were illuminated (*ca* 20000 lx) for the succeeding 23 hr. Appropriate controls lacking INH and/or illumination period were also included. The pH of the suspending soln (originally pH 7) was measured after illumination and incubations in which the pH had changed markedly were discarded. In experiments with radioisotopes etiolated seedlings were excised at the node and the excised ends immersed in H_2O, containing the compounds to be supplied, in a shallow beaker to a depth of 2 cm. The shoots were allowed to take up unlabelled compounds, and, when appropriate, inhibitor, for 60 min before presentation of radioactive L-serine. The shoots, after excision and feeding, were illuminated for 24 hr. To encourage transpiration and increase uptake, the shoots were placed in an air stream.[12]

Pigment extraction and assay. An extraction procedure based on Appelqvist *et al.*[31] was used. Leaf tissue was homogenised in 85% Me_2CO until the tissue was colourless. The cell debris was then removed by filtration

[31] APPELQVIST, L.-Å., BOYNTON, J. E., STUMPF, P. K. and VON WETTSTEIN, D. (1968) *J. Lipid. Res.* **9**, 425.

through a sintered glass funnel and the A of the soln measured at 480, 645 and 663 nm. The values of total chlorophyll, and of chlorophyll *a* and chlorophyll *b* separately, were taken from a nomogram.[32] To obtain a measure of carotenoid synthesis, that part of the absorbance of the Me_2CO extract at 480 nm due to carotenoids (A_{480}^{car}) was determined by correcting the observed A 480 for the contribution of the chlorophylls at this wavelength, using the equation derived by Kirk and Allen.[9] The increase in A 480 which is due to carotenoid synthesis is referred to as ΔA_{480}^{car}.

Extraction of lipid and separation of terpenoids. The extraction of lipid, saponification, extraction of unsaponifiable lipid, and separation and crystallization of β-carotene were as described previously. The β-carotene was recrystallized once before assay of radioactivity.[12]

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[32] KIRK, J. T. O. (1968) *Planta* **78**, 200.