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THE NATURE OF FORMATE METABOLISM IN GREENING BARLEY LEAVES

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Key Word Index—*Hordeum vulgare*; *Phaseolus vulgaris*; *Vicia faba*; *Zea mays*; greening; effects of aminopterin and isonicotinyl hydrazide on formate metabolism; glycine; serine and aspartate as products of [^{3}H]formate metabolism.

Abstract—The metabolism of [^{3}H]formate has been examined in etiolated and greening leaves of barley (*Hordeum vulgare*), dwarf bean (*Phaseolus vulgaris*), broad bean (*Vicia faba*) and corn (*Zea mays*). Tritium was extensively incorporated by primary leaves incubated for 20-min periods in light or dark. The organic acids and free amino acids were the principal products of formate metabolism but these and other products were more heavily labelled in green tissues. Time course experiments with barley leaves revealed a rapid labelling of serine, accompanied by increasing amounts of ^{3}H in glycine and aspartate as the feeding period was extended. These amino acid products were formed throughout a 4-day greening period with an approximate doubling in total incorporation being due to large accumulations of tritiated glycine and aspartate. The involvement of tetrahydrofolate-dependent reactions in formate metabolism was indicated by inhibition of [^{14}C] and [^{3}H]formate incorporation by the folate antagonist, aminopterin. Labelling of glycine and serine was also strongly inhibited (up to 90%) when the leaves were incubated with increasing concentrations of isonicotinylhydrazide.

INTRODUCTION

When dark-grown barley seedlings are exposed to light, chlorophyll synthesis is accompanied by increases in the activities of Calvin cycle enzymes [1,2] and by a capacity for photosynthetic CO_2 fixation [3]. In the preceding paper [4] it was shown that this period is also characterized by development of glycollate oxidase, ability to generate formate from glyoxylate and a potential flow of formate into related folate derivatives. In this regard [^{14}C]formate feeding experiments suggested an increasing flow of C–1 units for glycine and serine synthesis during the early stages of chlorophyll production. However, the widespread occurrence of formate dehydrogenase [5,6] and the peroxidatic action of catalase on formate [6,8] could mask these contributions to C_1 metabolism and result in appreciable reincorporation of formate via CO_2 fixation.

In the present work we have assessed the nature of formate metabolism by supplying [^{3}H]formate during the greening period. A flow of ^{3}H to serine, glycine and other products was observed and this increased substantially in barley leaves that were greened in light. The folate antagonist, aminopterin, inhibited the flow of formate hydrogen and carbon into these products.

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RESULTS

[^{3}H]*Formate utilization by leaves of different species*

In an initial survey, [^{3}H]formate was supplied to partially greened and etiolated leaves of four species (Table 1). The primary leaves of barley, corn and dwarf bean were exposed to light (500 μeinsteins/m^2 per sec) until total chlorophyll contents were *ca* 75% of maximum. Broad bean leaves were harvested from greenhouse-grown seedlings as described in the Experimental. Formate utilization by all species resulted in extensive distribution of ^{3}H and more incorporation of the label occurred in the partially greened material. The principal labelled amino acids were serine, glycine and aspartate. In the light-grown bean leaves, the specific activity of glycine was significantly higher than that of serine. In corn, greater amounts of ^{3}H entered serine than glycine and this former product accounted for 50–70% of the ^{3}H that entered the amino acid fraction (Table 1). Small amounts of ^{3}H entered methionine in the bean species. Due to the extremely small pool size of methionine in these leaves, specific radioactivities were not calculated but would be at least as high as those calculated for glycine and serine.

Formate metabolism during greening of barley leaves

The data in Table 1 for barley leaves show that tritated formate, like [^{14}C]formate (ref. [4]) is readily metabolized. For leaves greened for 2 days (Fig. 1a) the rate of incorporation of ^{3}H into the amino acid fraction was a linear function of incubation time for

Table 1. Metabolism of [^{3}H]formate by etiolated and greened leaves

Fraction	Barley		Dwarf bean		Corn		Broad bean
	Etiolated	Green	Etiolated	Green	Etiolated	Green	Green
Ether solubles	12.2 ± 1.0	45.8 ± 2.0	9.1 ± 1.3	75.8 ± 4.5	12.0 ± 0.7	23.4 ± 1.5	19.4 ± 3.0
Sugars	50.0 ± 0.1	153.8 ± 4.1	48.8 ± 2.0	99.0 ± 2.5	40.6 ± 2.0	119.7 ± 15.9	110.9 ± 0.6
Organic acids	746.0 ± 51.0	705.8 ± 18.8	105.8 ± 46.3	609.0 ± 54.2	470.6 ± 53.2	790.9 ± 80.7	932.0 ± 2.8
Free amino acids	223.1 ± 22.4	411.2 ± 55.2	334.1 ± 18.4	777.2 ± 70.7	198.3 ± 36.8	646.9 ± 53.4	470.1 ± 4.0
aspartate sp. act.	31.7 ± 6.5	159.7 ± 18.9	38.9 ± 10.6	103.8 ± 20.3	29.9 ± 4.9	94.2 ± 11.3	49.2 ± 6.8
^{3}H%	10.8	10.8	19.5	14.8	20.6	12.2	5.7
serine sp. act.	101.6 ± 16.7	696.1 ± 29.6	396.1 ± 81.2	238.5 ± 33.8	73.8 ± 25.3	347.5 ± 86.5	311.5 ± 16.6
^{3}H%	45.1	38.0	50.9	26.4	52.6	69.0	28.5
glycine sp. act.	97.1 ± 12.3	569.6 ± 92.9	109.7 ± 33.2	798.2 ± 71.7	22.8 ± 10.3	27.8 ± 4.3	614.2 ± 35.8
^{3}H%	12.5	33.9	14.9	40.5	10.2	6.0	46.4
methionine ^{3}H%	n.d.	n.d.	2.7	5.0	n.d.	n.d.	5.7
Total ^{3}H incorporated	1031.3	1316.6	497.8	1561.0	721.5	1580.9	1532.4

Data are expressed as dpm recovered/three leaves $\times 10^{-1}$ ± s.e.m. and are mean values obtained from two separate experiments run in duplicate. Sp. act.—dpm/μmol $\times 10^{-3}$ ± s.e.m. ^{3}H%—dpm as a percentage of total in free amino acid fraction. The greened leaves of barley, corn and dwarf bean were exposed to light for 1, 2 and 3 days, respectively. For each species, three primary leaves were excised and sectioned for radioisotope feeding (ref. [4]). [^{3}H]Formate, 37.5 μCi (sp. act. 192 μCi/μmol) was then supplied to each tissue sample for 20 min with (greened tissue) or without (etiolated tissue) light.

n.d.—not detected.

up to 20 min. Analyses showed that labelling of serine, glycine and aspartate together accounted for *ca* 85% of the ^{3}H present. Initially the specific radioactivity of serine was more than twice that of glycine or aspartate (Fig. 1b) but by 20 min the serine pool was saturated with ^{3}H. Data for glycine show that the specific activity rose rapidly to the values attained by 20 min.

In other experiments, 6-day-old barley seedlings were exposed to light and, at intervals of up to 4 days, samples of the primary leaves were incubated with [^{3}H]formate for 20 min. Seedlings maintained in darkness provided etiolated, control leaf samples. The data from these studies (Fig. 2) show that greening leaves incorporated much more ^{3}H and this tendency was especially pronounced as the greening period was extended. Most radioactivity was associated with the free amino acids and again, this resided in serine, glycine and aspartate (Fig. 3). A comparison of the data in Figs. 2 and 3 suggests that much of the increase in amino acid labelling during the greening period could be ascribed to increases in ^{3}H of the glycine and aspartate pools.

Effects of aminopterin and isonicotinylhydrazide (INH) on formate metabolism

The heavy labelling of serine in these formate feeding experiments suggests operation of a folate-dependent pathway[9,10]. To examine this possibility we incubated leaf sections in the folate antagonist, aminopterin prior to supplying [^{3}H]formate. By inhibition of dihydrofolate reductase, this analogue reduces generation of tetrahydrofolate[9] and hence reduces the tetrahydrofolate-dependent flow of C–1 units[10]. In these experiments, the leaves were sectioned to facilitate penetration of aminopterin and illuminated to induce greening. The data in Table 2 show that incorporation of [^{14}C]formate was reduced by up to 50% when 10 μM to 1 mM of aminopterin was supplied. This inhibition of formate incorporation affected all labelled fractions and in each case inhibition was related to aminopterin concentration. Similar data were obtained when the treated leaves were incubated with [^{3}H]formate. The data for control and 0.1 mM aminopterin-treated leaves are summarized in Table 3. Clearly the specific and total radioactivities of serine were reduced by the inhibitor. Similar changes were observed for aspartate and for the small levels of ^{3}H that entered glutamate and alanine.

In contrast, incorporation of ^{3}H into glycine was increased by *ca* two-fold and this was accompanied by an enlargement of the glycine pool. The specific activity of glycine was, however, still significantly lower in the aminopterin-treated leaves.

The pyridoxal phosphate antagonist, INH[11] also affected the incorporation of formate (Table 4). In these studies, inhibitory effects were apparent for all labelled fractions except the organic acids. The inhibition of glycine labelling was more than 90% while that for serine labelling approached 80%. The INH-treated leaves contained greater quantities of glycine but less serine than the controls. As a result this inhibitor drastically reduced the specific activity of glycine (Table 4).

DISCUSSION

The substantial flow of formate carbon[4] and of ^{3}H derived from [^{3}H]formate into glycine, serine and aspartate (Fig. 3, Table 1) implies that these compounds are labelled directly or indirectly as a result of C_1 metabolism. It is conceivable that some tritiated formate was oxidized to ^3H_2O and that exchange[12] with hydrogen attached to the α-carbon of amino acids occurred during the feeding period. Such

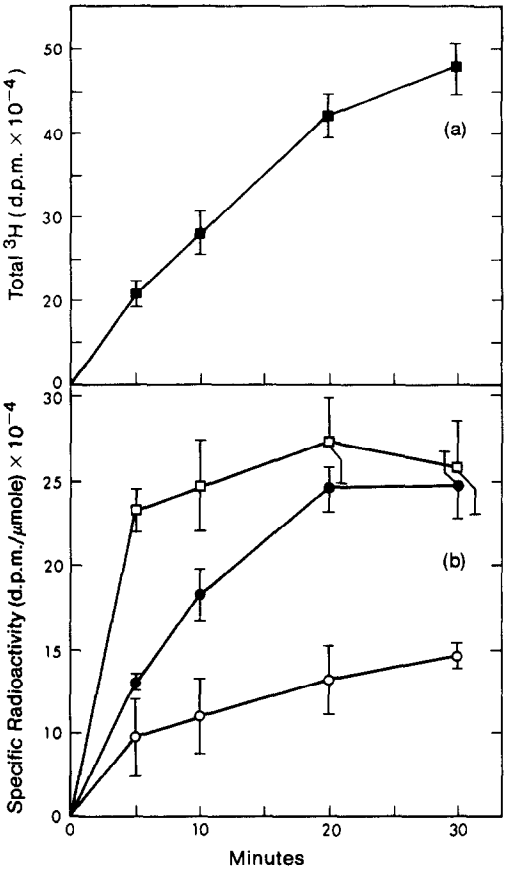

Fig. 1. Time course of [^{3}H]formate incorporation into free amino acids. 6-Day-old, dark-grown barley seedlings were exposed to continuous light (500 μeinsteins/m^2 per sec) for 2 days. The primary leaves were then sectioned and incubated in light for up to 30 min with 37.5 μCi (182 μCi/μmol) of Na [^{3}H]formate. Data are mean values (±s.e.m.) from duplicate determinations performed in three separate experiments. (a) Incorporation into free amino acid fraction; (b) specific radioactivities of serine (□), glycine (●), and aspartate (○).

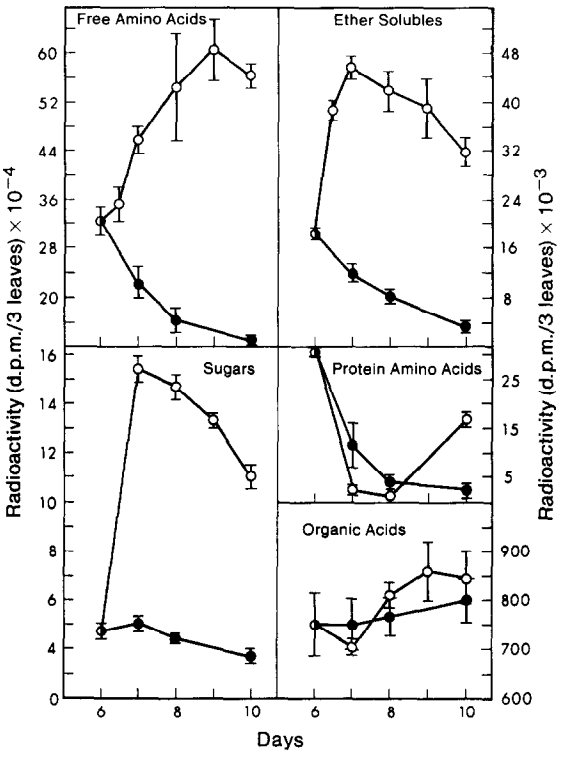

Fig. 2. Incorporation of [^{3}H]formate into major products by greening and etiolated barley leaves. 6-Day-old dark grown seedlings were exposed to light (○) or maintained in the dark (●). Samples of the primary leaves were harvested at the periods indicated and incubated with [^{3}H]formate for 20 min as in Fig. 1. Greening tissues (○) received light during formate feeding; etiolated controls (●) were supplied formate in the dark. Data are mean values (±s.e.m.) from duplicate determinations performed in three separate experiments.

Table 2. Metabolism of [^{14}C]formate by aminopterin-treated barley leaves

Fraction	Aminopterin concentration (mM)			
	0	0.01	0.1	1.0
Ether solubles	22.1 ± 0.6	21.4 ± 0.1	16.5 ± 0.3	11.9 ± 0.3
inhibition(%)		3	25	46
Sugars	260.2 ± 23.0	231.7 ± 8.2	145.8 ± 30.4	128.2 ± 18.5
inhibition(%)		11	44	51
Organic acids	312.3 ± 14.8	283.6 ± 10.6	229.6 ± 27.9	138.1 ± 18.3
inhibition(%)		9	26	56
Soluble amino acids	443.1 ± 9.0	393.3 ± 18.1	283.3 ± 25.7	227.1 ± 26.9
inhibition(%)		11	36	49
Total ^{14}C incorporated	1037.6 ± 47.3	930.1 ± 37.0	675.2 ± 84.4	505.2 ± 64.0

Data are expressed as dpm incorporated/three leaves $\times 10^{-4}$ ± s.e.m. and are mean values obtained from two separate experiments run in duplicate. 6-Day-old etiolated leaves were sectioned and greened in the presence of aminopterin for 24 hr. The tissues (*ca* 0.2 g fr. wt) were then incubated with 12.5 μCi of [^{14}C]formate, sp. act. 60.7 μCi/μmol, for 20 min in light of 500 μeinsteins/m^2 per sec.

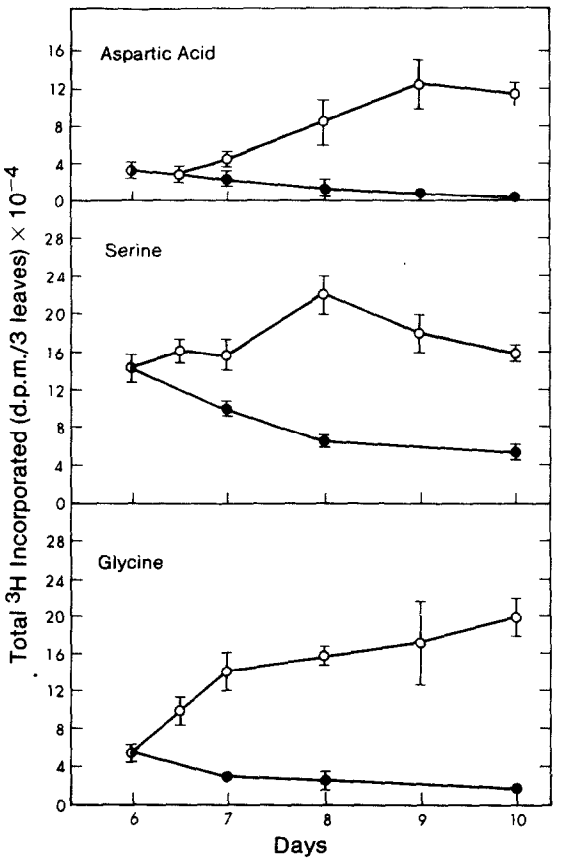

Fig. 3. Labelling of aspartate, serine and glycine during greening. The amino acid fractions (Fig. 2) of greened (○) and etiolated (●) leaves were analysed by ion exchange chromatography[4]. Each data point is a mean value (± s.e.m.) obtained from duplicate determinations of three separate experiments.

Table 3. Incorporation of [^{3}H]formate by aminopterin-treated leaves

Fraction	Aminopterin concentration (mM) 0	0.1
Ether solubles	51.3 ± 0.5	34.6 ± 1.5
Sugars	397.2 ± 47.6	204.3 ± 40.8
Organic acids	648.0 ± 133.1	468.9 ± 27.9
Soluble amino acids	759.6 ± 7.0	518.0 ± 9.1
aspartate	70.6 ± 16.2	32.5 ± 4.3
sp. act.	144.5 ± 3.4	23.9 ± 3.1
μmol	0.49 ± 0.10	1.36 ± 0.13
serine	370.6 ± 16.2	225.4 ± 2.0
sp. act.	339.4 ± 14.9	251.6 ± 2.3
μmol	1.09 ± 0.31	0.90 ± 0.09
glutamate	33.3 ± 2.5	13.5 ± 1.1
sp. act.	33.3 ± 2.5	9.9 ± 0.8
μmol	1.00 ± 0.13	1.36 ± 0.07
glycine	74.4 ± 3.9	148.7 ± 11.4
sp. act.	171.0 ± 4.6	118.0 ± 7.0
μmol	0.44 ± 0.07	1.26 ± 0.20
alanine	17.0 ± 1.8	6.3 ± 0.2
sp. act.	56.0 ± 6.0	13.9 ± 0.5
μmol	0.30 ± 0.10	0.46 ± 0.11
Total [^{3}H] incorporated	1856.0 ± 188.2	1225.8 ± 79.3

Data are expressed as dpm incorporated/three leaves $\times 10^{-3}$ ± s.e.m. and are mean values obtained from two separate experiments run in duplicate. Sp. acts. are expressed as dpm $\times 10^{-3}/\mu$mol ± s.e.m.; μmol data refer to amino acid pool size/three leaves. General procedures for supply of inhibitor are outlined in Table 2. [^{3}H]Formate, 37.5 μCi of ^{3}H; sp. act. 182 μCi/μmol was supplied for 20 min with illumination.

Table 4. Effect of isonicotinylhydrazide on [^{3}H]formate metabolism by greened barley leaves

Fraction	INH concentration (mM) 0	73	360
Ether solubles	43.2 ± 3.3	31.4 ± 1.1	23.2 ± 2.9
Sugars	142.5 ± 5.3	127.0 ± 15.6	80.6 ± 4.2
Organic acids	822.0 ± 49.8	771.8 ± 161.9	973.5 ± 34.3
Soluble amino acids	522.3 ± 49.2	352.9 ± 13.5	232.7 ± 3.5
aspartate	84.0 ± 25.7	35.3 ± 7.3	66.9 ± 2.6
sp. act.	290.8 ± 34.5	178.2 ± 45.9	290.9 ± 70.8
μmol	0.28 ± 0.06	0.20 ± 0.01	0.23 ± 0.06
serine	220.0 ± 22.6	115.5 ± 12.3	45.9 ± 7.8
sp. act.	493.2 ± 51.7	489.3 ± 5.7	209.6 ± 21.0
μmol	0.45 ± 0.03	0.24 ± 0.02	0.22 ± 0.05
glycine	156.8 ± 9.5	87.5 ± 7.2	12.8 ± 0.2
sp. act.	570.2 ± 75.4	25.3 ± 2.0	3.8 ± 0.5
μmol	0.28 ± 0.02	3.46 ± 0.01	3.38 ± 0.39
Total [^{3}H] incorporated	1530.0 ± 107.5	1283.2 ± 192.1	1310.0 ± 44.9

Data are expressed as in Table 3. 6-Day-old seedlings were grown in the dark and then illuminated (500 μeinsteins/m^2 per sec) for 2 days. Primary leaves were then sectioned and incubated with INH for 1 hr. Tritiated formate was supplied as in Table 3.

exchange, catalysed by various amino transferases, has been reported by Humphrey and Davies[12] for *Lemna minor* exposed to ^3H_2O. However, it is perhaps significant to the present work that heavy labelling of serine, glutamate and alanine was accompanied in *Lemna* by much lower levels of radioactivity in glycine. In the [^{3}H]formate feedings (Table 1) glycine and serine were both heavily labelled and ^{3}H was invariably only found in one other amino acid. This apparent selective incorporation of formate and the presumably high dilution of any ^3H_2O arising from oxidation argues against this exchange process as the sole mechanism for labelling of these products. It appears more likely that formate was essentially metabolized as a ^{3}HCO-unit, a view consistent with earlier studies[13] and one that is also supported by the inhibitory effect of aminopterin (Tables 2 and 3). It should also be noted that these tissues have greater levels of folate-dependent enzymes as greening progresses[4].

Serine is a characteristic product of formate metabolism[10] but the pathways for the labelling of aspartate and glycine (Table 1, Fig. 3) are less clear. Kent[13] has presented information on aspartate labelling when [^{3}H]formate was fed to *Vicia faba* leaves. Time course experiments and the intramolecular distribution of ^{3}H in the products indicated a formate pathway with serine, pyruvate and oxaloacetate as intermediates. In the present work (Table 1) the organic acid fraction of all four species was heavily tritiated following formate metabolism in the light. Part of this labelling could result from ^{3}H exchange, especially if serine was converted into glycerate and pyruvate. We have not examined the nature of this fraction but our isolation method implies that the ^{3}H components were non-volatile at acidic pH. Serine diverted to the Kreb's cycle by such a pathway would possibly represent a drain of intermediates from the conventional glycollate pathway and in this regard, it may be significant that less ^{3}H entered the organic acids and aspartate of dark-fed, etiolated leaves (Table 1).

With the exception of corn, all of the species incorporated large amounts of ^{3}H into glycine (Table 1). In barley, this product became increasingly important as the greening period was extended (Fig. 3). The inhibitory effect of INH on this incorporation (Table 3) suggests the involvement of a pyridoxal phosphate dependent reaction. In *Pseudomonas* AMl, glycine can arise from C–1 units by a novel pathway which has glyoxylate as an obligatory intermediate[14]. There is no evidence for a comparable route in higher organisms; in fact the generation of formate from glyoxylate by pyruvic decarboxylase[15] and glycollate oxidase[16] represent reactions that are essentially irreversible. On the other hand, the glycine synthase reaction could be instrumental in the observed glycine labelling. It follows from use of sodium [^{3}H]formate that ^{3}H was incorporated into the methylene group of glycine, a position that can exchange with methylenetetrahydrofolate in animal tissues[17] and conceivably also in plant mitochondria[18]. Reversible exchange reactions involving bicarbonate and the carboxyl group of glycine are also catalysed by glycine synthase[17,19,20]. Furthermore, this enzyme is widely distributed in photosynthetic tissues [10,16,21] and recently[22] we have detected cleavage activity in greening barley leaves. Conceivably, glycine labelling in the [^{3}H]formate experiments represents an exchange reaction rather than a route for synthesis *de novo*. In animal tissues glycine synthesis can occur via this route[17] but normal physiological conditions, as in plant tissues[16], favour the cleavage reaction. In greening barley leaves rates of glycine turnover are high and a precursor role for this amino acid in chlorophyll biosynthesis has been proposed[23,25]. Despite this, it remains to be determined whether formate and the glycine synthase reaction have physiological importance in this biosynthetic pathway during greening.

EXPERIMENTAL

Chemicals. Sodium [^{14}C] and [^{3}H]formate were purchased from Amersham-Searle, Des Plaines, IL. INH was supplied by Nutritional Biochemicals Corp., Cleveland, OH, and aminopterin was purchased from Sigma, St Louis, MO. Other chemicals, of the highest quality available, were purchased from Sigma and from Fisher Scientific, Edmonton, Alberta.

Seedling growth. Seeds of barley (*Hordeum vulgare* L. cv Galt), dwarf bean (*Phaseolus vulgaris* L. cv Bountiful), broad bean (*Vicia faba* L. cv Broad Windsor) and corn (*Zea mays* L. cv Alta Gold) were surface sterilized by soaking in 1% Na hypochlorite containing Tween-80 for 30 min. After rinsing in distilled H_2O, the seeds were germinated as follows. Broad bean seeds were sown in 6-in. pots containing a sterilized loam–peat–sand (3:2:1) mixture and grown for 5 weeks in an environmentally controlled greenhouse (50% r.h., 22°, 14 hr day length). Barley, dwarf bean and corn were germinated in sterilized Vermiculite in darkness at 22° and 30% r.h. The seedlings were watered with half-strength Hoagland–Epstein soln[26]. The growth period in darkness was 6, 14 and 13 days for barley, dwarf bean and corn, respectively. Greening was carried out in growth chambers as previously described[4].

Radioisotope feeding experiments. Procedures for supplying labelled formate and subsequent fraction of radioactive products were as described in the preceding paper[4]. The amounts of formate supplied are summarized in the appropriate figures and tables.

Inhibitor studies. In experiments with INH, 8-day-old leaves that had received light for 2 days were harvested, sectioned and placed in Warburg flasks[4]. The inhibitor soln in 2.8 ml (up to 0.36 M) was added and the tissues were pre-incubated for 1 hr at 25° and 500 μeinsteins/m^2 per sec. [^{3}H]Formate was then added as noted in Table 4. In aminopterin experiments, 6-day-old etiolated barley leaves were sectioned and placed in Warburg flasks containing up to 1 mM aminopterin in 2.8 ml of soln. The flasks were incubated at 25° and illuminated at an intensity of 500 μeinsteins/m^2 per sec for 24 hr. [^{3}H] or [^{14}C]Formate was then added as summarized in Tables 2 and 3.

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