This is to certify that the

thesis entitled

THE BIOSYNTHESIS OF Δ-AMINOLEVULINIC ACID AND TETRAPYRROLES AND THEIR REGULATION IN GREENING BARLEY: INVESTIGATIONS WITH METABOLIC INHIBITORS

presented by

PETER WILLIAM BERGUM

has been accepted towards fulfillment
of the requirements for

Master of Science degree in Botany and Plant ~~Pathology~~

Kenneth D. Nadler
Major professor

Date Sept. 5, 1979

THE BIOSYNTHESIS OF Δ-AMINOLEVULINIC ACID AND TETRAPYRROLES AND THEIR REGULATION IN GREENING BARLEY: INVESTIGATIONS WITH METABOLIC INHIBITORS

By

Peter William Bergum

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1979

ABSTRACT

THE BIOSYNTHESIS OF Δ-AMINOLEVULINIC ACID AND TETRAPYRROLES AND THEIR REGULATION IN GREENING BARLEY: INVESTIGATIONS WITH METABOLIC INHIBITORS

By

Peter William Bergum

The effect of isonicotinic acid hydrazide on the biosynthesis of chlorophyll in greening barley was investigated. Isonicotinic acid hydrazide is an antagonist of pyridoxal phosphate and as such can be used to detect such pyridoxal phosphate-dependent enzymes as δ-aminolevulinatic acid synthase. The hydrazide inhibited δ-aminolevulinate formation but appeared to have an additional inhibitory site in the chorophyll biosynthetic pathway. It caused an increased accumulation of protoporphyrin with a concomitmant decrease in protochlorophyllide in aminolevulinate-treated seedlings. Labelling experiments with ^{14}C-aminolevulinate not only indicated a site of action for the hydrazide between protoporphyrin and protochlorophyllide but also suggested an increased protoporphyrin turnover. Pre-treatment with light or nicotin amide attenuated these effects of isonicotinic acid hydrazide. These observations are consistent with a dual inhibitory action of isonicotinic acid hydrazide on chlorophyll synthesis: it appears to act on δ-aminolevulinate synthesis as a pyridoxal

phosphate antagonist and on tetrapyrrole synthesis via an effect on the pyridine nucleotide pool. Aminolevulinate synthase does not appear to play a major role in δ-amino-levulinate synthesis in greening barley.

". . . Chlorophyll . . . is the real
Prometheus, stealing fire from the heavens."

Timiryazev

This thesis cannot be dedicated to just one person. I would therefore like to dedicate it with the sincerest gratitude and deepest heartfelt feelings to -

My parents, Dr. Frank A. Loewus, Doug, Yael, Mona, and the memory of Ernest Erickson -

who, each in their own very special and unique way, have given so much not only to the realization of this thesis but also to me through their support, encouragement, and understanding.

ACKNOWLEDGMENTS

It is very difficult, due to the limitation of space, to include all these people who have helped me in so many ways during my graduate studies here at Michigan State. For these people, I would like to express a special thanks.

For my interest in plant science, I am deeply indebted to Dr. Frank A. Loewus, my undergraduate advisor. His enthusiasm for plant biochemistry, his support and encouragement were inspirational for me in pursuing a graduate career in plant physiology.

I would also like to thank two of the best lab colleagues one could ever possibly have - Drs. Yael Avissar and Ronald Myers for providing a delightful atmosphere for scientific work and intellectual discussion. I am also grateful for their undying support when it was needed and their friendship.

I thank Dr. R. S. Bandurski for the use of his spectrophotometers and Dr. C. P. Wolk for generously providing me "refuge" in his laboratory during the writing of my thesis. To the women of Dr. Wolk's lab, I owe a warm thanks for providing such a pleasant atmosphere for writing my thesis - even if all the distractions didn't help me finish faster writing my thesis.

To Dennis, Nien-tai, and Adiva, I thank them for their "sympatheic ears," continuous moral support and friendship.

I'm eternally grateful to Yael for all her very useful suggestions for the revision of my thesis, for the many stimulating intellectual exchanges not only about science but about literature, art and life, and for our very special friendship.

I would like to thank the members of my committee, Drs. Philip Filner and Norman Good for their critical review of my thesis work. I am grateful to my advisor, Dr. Kenneth Nadler, for providing me with the opportunity to grow as a scientist. I believe that we both have learned a great deal from one another.

I will be forever grateful to my best friend, Doug, who was able to "put up" with me during this entire time. His constant support, limitless understanding and caring have shown me that there can be no better friend.

I am thankful to Barb for her excellent expertise in graphics, her listening ears and heart, and her warm friendly smile.

And lastly I thank Mona, who bore with patience, understanding and love our separation and my frequent "silence" over so many miles.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALA	δ-aminolevulinic acid
ALAS	δ-aminolevulinic acid synthase (EC 2.3.1.37)
CHLD(D)	chlorophyll(ide)
CHL	chlorophyll
DOVA	γ, δ-dioxovaleric acid
α-HPMS	α-hydroxy-2-pyridine-methane sulfonic acid
INA	isonicotinic acid
INH	isonicotinic acid hydrazide
LEV	potassium levulinate
MgPROTO	magnesium protoporphyrin
MgPROTO ME	magnesium protoporphyrin monomethyl ester
NAmD	nicotinamide
PALP	pyridoxal phosphate
PROTO	protoporphyrin
PCHLD	protocholorophyllide
PXN	pyridoxine
*SA	radiospecific activity
SAM	S-adenosyl methionine

INTRODUCTION

The Biosynthesis of δ-Aminolevulinic Acid (ALA)

δ-Aminolevulinic acid (ALA) is the first intermediate committed to the biosynthetic pathway leading to such tetrapyrroles as heme, chlorophyll, bilins, and vitamin B_{12} (37). Like most other tetrapyrroles found in various organisms, chlorophyll is synthesized by the condensation of eight molecules of ALA (75). In bacteria, including photosynthetic bacteria, and in animal tissue, ALA formation is catalyzed by the pyridoxal phosphate-dependent enzyme, ALA synthase (ALAS) [succinyl CoA: glycine C-succinyl transferase (decarboxylating), EC 2.3.1.37] (37). In animals, ALAS is found within the mitochondrion, existing close to a source of its substrate succinyl CoA and its end-product, heme, This close proximity of ALAS and heme may play an important role in the feedback regulation of the pathway. In Rhodopseudomonas spheroides, ALAS is feedback inhibited by heme (20); in addition, there is some evidence to suggest that heme may also repress ALAS formation (68). In cultured liver cells, ALAS is subject to induction (96a), repression (96b), and product inhibition by protoporphyrin and heme (18, 37).

In contrast to the well-elucidated model for ALA synthesis in bacteria and animals, a clear understanding of the mechanism of ALA formation in higher plants remains elusive. It has been long argued that chlorophyll is formed by a pathway identical to that of heme synthesis in bacteria and animals - the only difference being at the metal chelation step. The existence of ALAS in plants - in contrast to its proven existence in animals - is a subject of much controversy (cf 5, 85). There have been scattered demonstrations, appearing in the literature, of a "presumed" ALAS in such tissue as callus culture of soybeans (119) and greening potatoes (89, 90), even though the validity of such reports has been called into question (for a review: Beale, 5). The evidence supporting the presence of ALAS in plants is usually based on one of two criteria: 1) a glycine- and succinyl CoA-dependent formation of ALA in extracts of plants, or 2) the incorporation of label from ^{14}C-glycine or -succinate into chlorophyll or ALA. Most investigators have not shown that the ALAS activity which they were reporting, was actually glycine- and succinyl CoA-dependent (cf 119). When the presence of ALAS was concluded from the incorporation of ^{14}C-glycine or -succinate into chlorophyll or ALA, careful controls were not reported - such as to whether C-2 of glycine was preferentially incorporated into C-5 of ALA or whether glycine and succinate were incorporated significantly better than any other intermediate common to general metabolism (see 5, cf 48, 118). In opposition to this criticism, however, there are several reports

which suggest the presence of ALAS in certain greening tissues. Ramaswamy and Nair (89, 90) demonstrated an ALAS activity requiring ATP, $MgCl_2$, pyridoxal phosphate, succinyl CoA thiokinase, glycine and succinate in greening potatoe skins. In addition, ^{14}C-labelled glycine and succinate were equally incorporated into ALA, whereas α-ketoglutarate and glutamate were not. Furthermore the incorporation of glycine was dependent on the presence of succinate and vice versa. Labelling experiments with two green algae, Chlorella fusca (86) and Scenedesmus obliquus (62), suggest that the ALAS pathway for ALA formation, indeed, appears to be in operation in these organisms (see Figure 1A).

Alternative Pathways for ALA Synthesis

Since the existence of ALAS in higher plants has appeared equivocal, and many investigators have been unable to find ALAS in a vast number of greening plants, which under normal conditions make enormous amounts of chlorophyll, the possibility of alternative pathways for ALA formation has been considered.

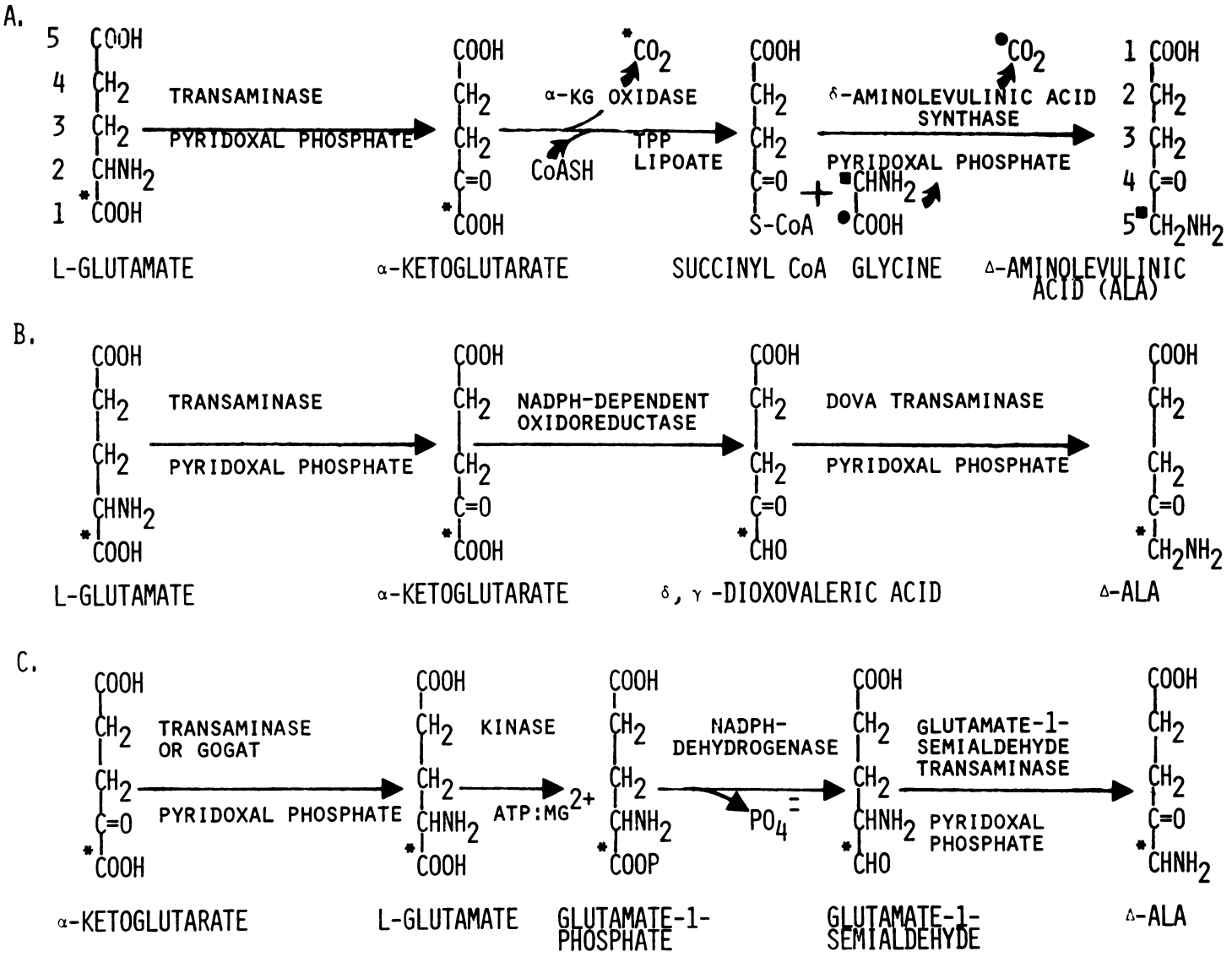
The work of Beale and Castelfranco (8, 9) showed that greening tissues of higher plants, such as cucumber cotyledons, barley and bean leaves, will preferentially incorporate ^{14}C-labelled α-KG, glutamate, and glutamine into ALA compared to ^{14}C-glycine or ^{14}C-succinate. Furthermore, they were able to demonstrate that the carbon skeleton of these five carbon precursors was incorporated intact into ALA. When the labelling

Figure 1. The proposed pathways of δ-aminolevulinic acid biosynthesis.

Pathway A - the classical or ALA synthase pathway involves the pyridoxal phosphate-dependent enzyme, ALA synthase, which condenses succinyl CoA and glycine. In the conversion of 1-^{14}C-glutamate to ALA via the ALAS pathway, the label is evolved as $^{14}CO_2$.

Pathway B - the DOVA transaminase pathway involves a transamination of γ, δ -dioxovaleric acid (DOVA) to ALA. Ketoglutarate as opposed to glutamate is the more immediate precursor of ALA in this pathway. In the conversion of 1-^{14}C-glutamate to ALA via the DOVA transaminase pathway, the label appears in C-5 of ALA.

Pathway C - the glutamate-1-semialdehyde aminotransferase pathway involves the transamination of glutamate-1-semialdehyde to ALA. Glutamate as opposed to α-ketoglutarate is the more immediate precursor of ALA. In the conversion of 1-^{14}C-glutamate to ALA via the glutamate-1-semi-aldehyde pathway, the label appears in C-5 of ALA.


A.
TRANSAMINASE
PYRIDOXAL PHOSPHATE
α-KG OXIDASE
CoASH
TPP
LIPOATE
δ-AMINOLEVULINIC ACID SYNTHASE
PYRIDOXAL PHOSPHATE
L-GLUTAMATE
α-KETOGLUTARATE
SUCCINYL CoA
GLYCINE
Δ-AMINOLEVULINIC ACID (ALA)
B.
TRANSAMINASE
PYRIDOXAL PHOSPHATE
NADPH-DEPENDENT OXIDOREDUCTASE
DOVA TRANSAMINASE
PYRIDOXAL PHOSPHATE
L-GLUTAMATE
α-KETOGLUTARATE
δ, γ -DIOXOVALERIC ACID
Δ-ALA
C.
TRANSAMINASE OR GOGAT
PYRIDOXAL PHOSPHATE
KINASE
ATP:MG2+
NADPH-DEHYDROGENASE
GLUTAMATE-1-SEMIALDEHYDE TRANSAMINASE
PYRIDOXAL PHOSPHATE
α-KETOGLUTARATE
L-GLUTAMATE
GLUTAMATE-1-PHOSPHATE
GLUTAMATE-1-SEMIALDEHYDE
Δ-ALA

pattern of ALA derived from glutamate was examined, it was concluded that this pattern was incompatible with that which could be derived from the ALAS pathway (6). These experiments gave rise to the conception of a new pathway for ALA formation in plants, referred to generally as the C-5 pathway. This is probably the primary biosynthetic route for tetrapyrrole synthesis in plants (5, 7, 53, 43), due to its wide demonstration in such diverse tissues and organisms as greening leaves of maize and pea, immature spinach leaves, the green algae Scenedesmus, Euglena, the red algae Cyandium caldarium, and the blue-green algae Anabaena variabilis. The mechanism for ALA synthesis via the C-5 pathway in higher plants is as controversial as the presence of ALAS. There are several hypothetical pathways proposed for ALA formation, all of which stem from either α-ketoglutarate, glutamate, or glutamine (5, 7, 53, see Figure 1). In the cell-free system from maize, α-KG is the better precursor of ALA (43, 78) whereas in isolated chloroplasts from cucumber cotyledons, glutamate appears to be the better precursor (8); this inconsistency has certainly fueled the controversy.

One of the proposed C-5 pathways involves the intermediate, γ, δ-dioxovaleric acid (DOVA) as the direct precursor of ALA. An enzyme catalying the transamintion of DOVA to ALA was detected in extracts of Chlorella, bean leaves, and Euglena (32, 33, 96). The activity of this DOVA transaminase in Chlorella could not, however, be linked to the light-induced

synthesis of ALA. Mechanisms for ALA synthesis via DOVA require an initial conversion of glutamate or α-KG to DOVA (see Figure 1B). Lohr and Friedmann (73) partially purified two protein fractions from extracts of maize leaves, one of which reportedly catalyzed the dehydrogenation of α-KG to DOVA in the presence of NADH and the other transamination of DOVA to ALA. Unfortunately, their communication lacked so much information concerning their methods and experimental data that their results cannot be considered unequivocal (5, 43, 54). Although the DOVA transaminase pathway has been viewed as a viable mechanism for ALA formation in greening organisms, there exists substantial evidence that it may be nothing more than a "fortuitous association of enzymes encountered under experimental conditions" (Porra and Grimme, 85, 5). The reasons are as follows: 1) the actual presence of DOVA has not been shown in any of the systems where DOVA transaminase was reported (cf 5, 4) nor has any enzymatic synthesis of DOVA been shown; 2) the enzyme ALA transaminase, which catalyzes the conversion of ALA to DOVA has been reported (66, cf 100) and it appears that in the reversible interconversion of ALA and DOVA (60) the amination of DOVA is less significant than the deamination of ALA (83, 66); 3) DOVA transaminase could serve to degrade AlA in vivo, since DOVA is readily metabolized (82, 4, 5), and an ALA oxidase activity has been demonstrated in extracts from barley shoots (31) (although a relationship with DOVA transaminase has not been shown); and finally 4) DOVA undergoes a nonenzymatic transamination to ALA (4),

since the aldehyde group of DOVA possesses a high degree of reactivity with primary amines such as glycine, TRIS, and ALA. All of this suggests that the aminotransferase activities involving DOVA reported up to this time may simply be without physiological significance, even though some amino acid-specificity has been shown for the enzyme activity (32).

There have been other mechanisms suggested for ALA synthesis. Since glutathione could not be replaced by any other sulfhydryl reagent, Weinstein and Castelfranco (116) suggest a mechanism for ALA formation involving glutathione in a reaction similr to that catalyzed by glyoxylase. In this system, DOVA is converted via a thiol-ester adduct to α-hydroxyglutarate (50). They propose an ATP-dependent formation of a thiol-ester adduct between glutamate and glutathione, which undergoes reduction to the 1-semialdehyde and transamination to ALA. Unfortunately, the only report of this glyoxylase system has been in beef liver.

The most convincing mechanism for ALA synthesis up to this time involves a L-glutamate-1-semialdehyde aminotransferase in chloroplasts from greening barley (54, 53). In the pathway catalyzed by this enzyme, the biosynthesis of ALA from glutamate proceeds according to the three following proposed steps: 1) glutamate undergoes phosphorylation from ATP:Mg^{2+} by a kinase, 2) the glutamate-1-phosphate is reduced by a NADH-linked dehydrogenase to glutamate-1-semialdehyde, and 3) glutamate-1-semialdehyde is transaminated to ALA (see Figure 1C). The reaction is analogous to the biosynthesis of ornithine

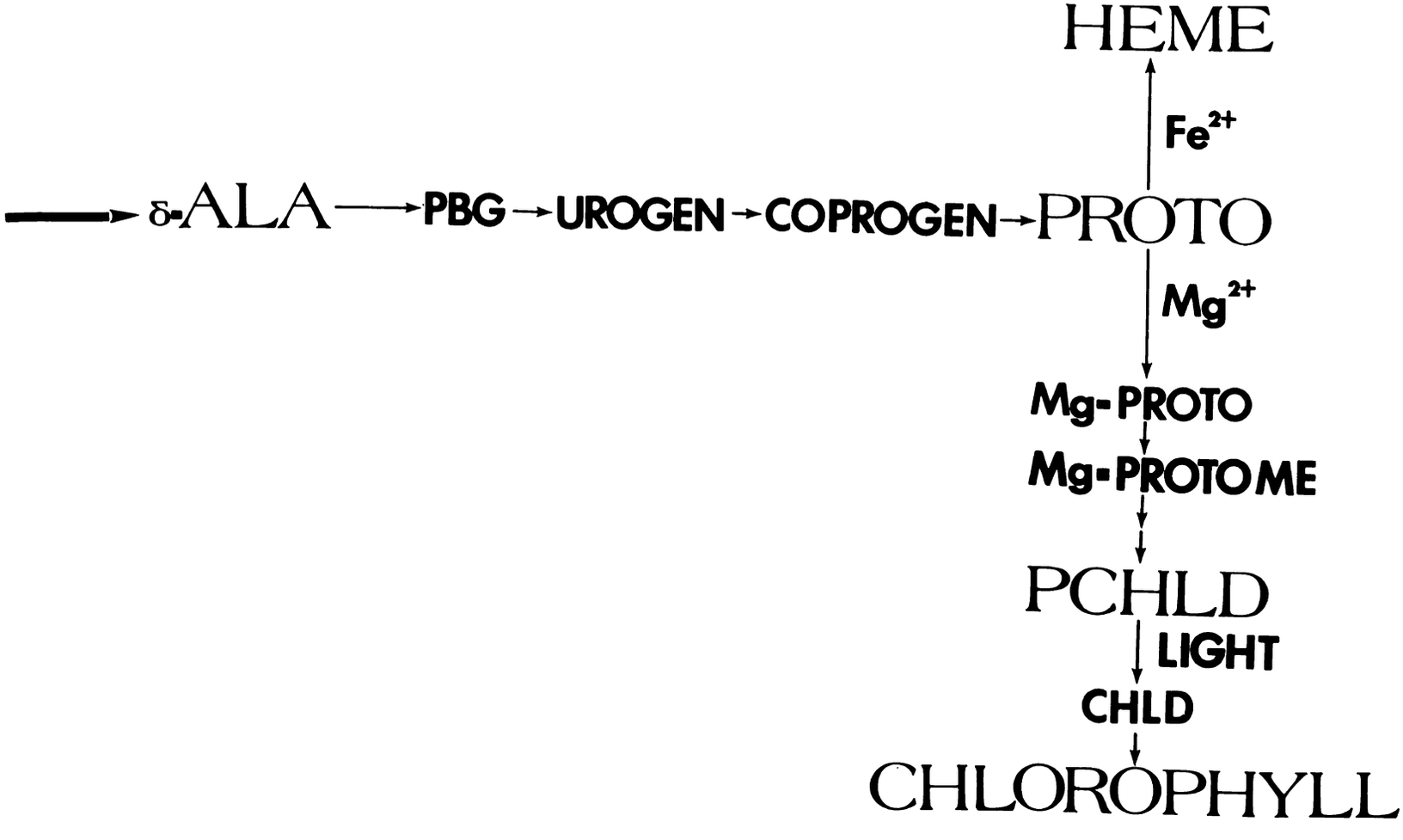
Figure 2. The biosynthetic pathway of chlorophyll and heme.

The biosynthesis of ALA has been proposed to be feedback regulated by three tetrapyrroles: heme, protoporphyrin, and protochlorophyllide.

Abbreviations:

δ ALA -	δ-aminolevulinic acid
PBG -	porphobilinogen
Coprogen -	coprobilinogen
Proto -	protoporphyrin
Mg-Proto -	magnesium protoporphyrin
Mg-ProtoME -	magnesium protoporphyrin monomethyl ester
PCHLD -	protochlorophyllide
CHLD -	chlorophyllide

CHOROPHYLL AND HEME BIOSYNTHESIS



and initial stages of proline biosynthesis. In these cases, however, the glutamate-5-semialdehyde is formed instead of glutamate-1-semialdehyde. The evidence for the glutamate-1-semialdehyde aminotransferase pathway is as follows: 1) soluble protein preparations from plastids which support ALA synthesis from ^{14}C (U) - glutamate in the presence of ATP, NADPH, and Mg^{2+} were also capable of synthesizing ALA from glutamate-1-semialdehyde in the absence of added cofactors, 2) the transaminase inhibitors aminooxyacetate and cycloserine inhibited ALA formation from glutamate-1-semialdehyde, 3) β-hydroxy-glutamate, an analog of glutamate and potent inhibitor of the conversion of ^{14}C-glutamate to ALA, had no effect on the conversion of glutamate-1-semialdehyde to ALA, and 4) the column fractions which possessed the ability to synthesize ALA from labelled-glutamate corresponded to the fractions containing the aminotransferase activity. These results suggest that an aminotransferase catalyzing the formation of ALA from glutamate-1-semialdehyde is likely involved in the synthesis of ALA in barley.

The Existence of Multiple Pathways in Barley For the Formation of ALA

The finding that both the ALAS and C-5 pathway exist together in a greening organism, Scenedesmus obliquus, raises the possibility that both pathways may exist in higher plants (54, cf 85). In Scenedesmus, it appears that ALA is formed predominantly via the ALAS pathway, but both pathways "co-exist"

simultaneously and lead to chlorophyll formation. On the other hand, Stobart and Hendry (104) have suggested that that the ALAS pathway is in operation only in the dark in barley, while the glutamate pathway operates in greening leaves. Since they obtained incorporation of labelled-glycine into ALA in the dark, it was suggested that this might be mitochondrial heme synthesis as there was little chloroplastic formation of tetrapyrroles in the dark (cf 5). The fact remains however that heme synthesis appears to occur via the C-5 pathway in barley leaves, and regardless of how poor of a radioactive precursor a compound was, heme and chlorophyll were labelled equally, suggesting that only one pathway, the C-5 pathway, gave rise to both metallo-tetrapyrroles (22). The presence of two pathways in higher plants, both of which synthesize ALA, remains controversial.

The Biosynthetic Pathway Chlorophyll and Other Magnesium Tetrapyrroles

The first step in the biosynthesis of chlorophyll (CHL) (Figure 2) is the rate-limiting formation of ALA - discussed in detail in the previous section. Following ALA synthesis, two molecules of ALA are condensed to form the monopyrrole porphobilogen (PBG) by the enzyme ALA dehydrase. PBG is the monomeric pyrrole which serves as precursor of the subsequent porphyrin compounds. Four molecules of PBG are combined by the coordinated action of two enzymes, PBG-deaminase and uroporphyrinogen III oxidase to protoporphyrin IX (PROTO), the

last intermediate common to both heme and CHL biosynthesis. After the synthesis of PROTO - at the point of metal insertion - the pathway bifurcates into the branches of heme and chlorophyll. If Fe(II) is inserted into PROTO by ferrochelatase, heme is formed. If Mg(II) is incorporated into PROTO by Mg chelatase, Mg protoporphyrin (MgPROTO) is formed. In its conversion to chlorophyll, the newly synthesized MgPROTO undergoes a series of structural modifications which include the closure of a fifth ring and the addition of a phytol side chain as well as a photoreduction of one of the pyrrole rings. The first modification of MgPROTO occurring is on the carboxyl group of the propriate residue of ring C. This residue is esterified with a methyl group derived from S-adenosyl methionine. The transfer is catalyzed by MgPROTO IX methyl transferase, yielding MgPROTO monomethyl ester (MgPROTO ME). In the events prior to ring closure, the activated proprionate residue undergoes a biosynthetic β-oxidation; the sequence for which is as follows: the β-carbon is desaturated, and hydroxylated yielding the acrylic methyl ester. The acrylic derivative is then oxidized to a ketone at the β-carbon, activating the α-carbon. This α-carbon condenses with the γ-methane bridge carbon of the Mg-tetrapyrrole, yielding the cyclopentanone ring (for review: 16, 23, 27). The resulting tetrapyrrole is called Mg-2, 4- divinyl pheoporhyrin a_5 ME and belongs to a group of Mg-tetrapyrroles which contain this fifth ring called phorbins. The reduction of the vinyl side chain on ring B of the phorbin macrocycle to an ethyl group

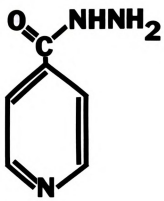
is required to form Mg 2-vinyl pheoporphyrin a_5 ME, or protochlorophyllide (PCHLD) - the naturally accumulating pigment of dark-grown angiosperms. PCHLD, in subsequent reactions, will be photoreduced and phytylated to give chlorophyll. Treatment of dark-grown leaves of angiosperms with exogenous ALA gives rise to the accumulation of a PCHLD species which is not transformable in light, that is, is not photoreduced to CHLD. This $PCHLD_{628}$ absorbs maximally between 628nm and 636nm (38, 106). The native, phototransformable species, $PCHLD_{650}$ - with an absorption maximum at 650nm - is intercontertible with $PCHLD_{628}$ species (52, 19, 38). The interconversion of $PCHLD_{628}$ to $PCHLD_{650}$ is dependent on the free sites available on the photoconverting enzyme, holochrome (38, 107). The attachment of $PCHLD_{628}$ to the holochrome results in a $PCHLD_{650}$ -holochrome complex (PCHLD-H). The actual binding of the $PCHLD_{628}$ to the holochrome is apparently responsible for the spectral shift from 628 to 650 ($PCHLD_{650}$) and appears to require ATP and NADPH (49, 42, 19, 38). Normally, native $PCHLD_{650}$ fully saturates all available apoenzyme. Under conditions where $PCHLD_{628}$ accumulates, its accretion is a consequence of the lack of available sites on the holochrome to which the $PCHLD_{628}$ can bind (38). If brief flashes are given to convert the $PCHLD_{650}$ to CHLD, $PCHLD_{628}$ will be rapidly converted to $PCHLD_{650}$ depending on how quickly the $PCHLD_{628}$ saturates the holochrome binding sites (106, 108, 38). In the presence of light, the $\Delta^{7,8}$-double bond of ring D of the $PCHLD_{650}$ is reduced, generating $CHLD_{678}$ (holochrome-bound). The actual

source of the reductant responsible for the photoreducing event is still a matter to be resolved. The holochrome has been proposed to act as a reductase, undergoing oxidation and reduction itself with NADPH as reductant (16). It has also been suggested that the light absorbed by the PCHLD chromophore is responsible for the photoreduction (see 91 for discussion). We can summarize the overall process as follows to correspond to the in vivo spectral shifts [as proposed by Brodersen (19) and modified by Horton (49)]: 1) $PCHLD_{628}$ + holochrome + NADPH + ATP → $PCHLD_{650}$ [enzyme-substrate-NADPH-ATP] complex. The ATP is not only necessary for photoconversion of $PCHLD_{628}$ but also appears to stabilize the $PCHLD_{650}$-holochrome (49); 2) $PCHLD_{650}$ + light energy → $CHLD_{678}$ [enzyme-product-NADP + ADP complex]; 3) $CHLD_{678}$ + dark → $CHLD_{684}$, and $CHLD_{684}$ + dark → $CHL(D)_{672}$ [product + holochrome + NADP + ADP]. The last spectral shift is presumably the phytylation of CHLD to CHL, the last step in CHL synthesis (16).

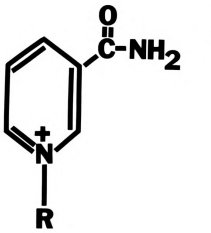
Contrary to what used to be believed about the phytylation process, chlorophyllase does not appear to esterify free phytol to the proprionate residue of ring D of CHLD (cf reviews 16, 91). Results from kinetic and inhibitor experiments suggest that the phytylation of CHLD is a multistep pathway involving the reduction of a terpenoid alcohol adduct to a phytol side chain (93). The esterifying enzyme isolated from maize shoots were shown to affix geranylgeranyl pyrophosphate to CHLD giving CHL a- geranylgeraniol. The CHL-geranylgeraniol

Figure 3. Structure of isonicotinic acid hydrazide and two physiologically important analogs.

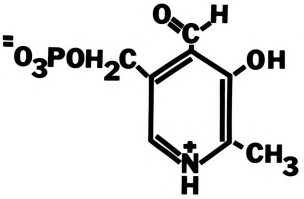
INH is a structural analog of both pyridoxal phosphate and the nicotinamide moiety of the pyridine nucleotide, NAD^+.



Isonicotinic Acid Hydrazide



The Nicotinamide Moiety of NAD^+



Pyridoxal Phosphate

ester if formed first; subsequent stepwise hydrogenation of this intermediate gives rise to CHL-dihydrogeranylgeraniol, CHL-tetrahydrogeranylgeraniol, and CHL phytyl ester (93, 94).

Regulation of Chlorophyll Biosynthesis

The pathway of chlorophyll biosynthesis appears to be regulated at three points (see Figure 2): 1) at the level of ALA formation, where control over the flux of the ALA into the pathway is exerted; 2) at the point of metal chelation of PROTO IX, where the flow into the branch pathways of heme and Mg-tetrapyrroles is regulated; and 3) at the site of photoreduction of PCHLD to CHLD, where light regulates the conversion in angiosperms. (In gymnosperms and most algae, this conversion has no light requirements.) All of the enzymes of the CHL pathway are contained in the etioplast, although studies with inhibitor of protein synthesis show that they are synthesized by cytoplasmic ribosomes. With the exception of the ALA synthesizing enzyme(s) - which is light inducible - all of the biosynthetic enzymes needed for CHL formation are present in non-limiting quantities in the etiochloroplast (97).

The availability of ALA is generally accepted as being the rate-controlling factor in CHL biosynthesis (37, 10). In addition, the enzyme responsible for ALA formation is believed to be under feedback regulation (110). For example, if a block occurs in the pathway, whether naturally as in the case of etiolated plants with the physiological accumulation of PCHLD, or genetically, as in mutants defective in CHL

biosynthesis, little of the tetrapyrrole intermediates prior to the blockage will accumulate (36). When ALA synthesis is examined under these "obstructing" conditions, it is found to be reduced and the accumulation of intermediates before the block occurs only when the plant is fed ALA (110). From these observations as well as from extensive analysis of Mendelian mutants blocked in porphyrin synthesis, it has been suggested that PROTO and PCHLD have a feedback regulatory function on ALA synthesis (111, 112, 76, 36). It has also been proposed that another feedback loop exists in the Mg-tetrapyrrole pathway from PCHLD to the step converting PROTO to MgPROTO in Chlamydomonas (112). Etiolated bean leaves treated with iron chelators, such as α,α'-dipyridyl, produce large amounts of MgPROTO ME and smaller amounts of MgPROTO, PROTO, and PCHLD as well as increased amounts of ALA (25). These results appear to indicate that the iron chelators, by making Fe(II) unavailable, antagonize the feedback inhibition of ALA synthesis by some iron-containing compound probably heme. A regulatory role for protoheme has also been implied in the case of greening barley, since protoheme appears to turn over rapidly in the absence of net synthesis during rapid CHL accumulation (22).

Isonicotinic Acid Hydrazide (INH)

Isonicotinic acid hydrazide (INH) is an antagonist of pyridoxal phosphate-dependent enzymes such as transaminases

and decarboxylases (114). It is widely used as a chemotherapeutic agent in the treatment of tuberculosis in man.

It is known, for example, that INH inhibits the mitochondrial conversion of glycine to serine by glycine decarboxylase in the photorespiratory glycolate pathway of plants (59, 87). INH also inhibits heme synthesis in both rat liver mitochondria (65) and rabbit reticulocytes (17); this effect of INH is due to its inhibition of pyridoxal phosphate-dependent ALA synthase (14). The inhibition of pyridoxal phosphate-dependent enzymes by INH is a consequence of hydrazone formation between the hydrazide of INH and the aldehyde of pyridoxal phosphate (14, 114) (Figure 3).

INH is known to be oxidized to pyridine-4-carboxyaldehyde by the horseradish peroxidase/$Mn^{2+}/0_2$ system (124). In addition, INH appears to be metabolized to isonicotinic acid in wild type and INH-resistant cell lines of tobacco callus (13).

The Use of Isonicotinic Acid Hydrazide in an Attempt to Determine the Presence of δ-Aminolevulinic Acid Synthase in Greening Barley

In consideration of the question: whether or not the ALAS pathway exists in higher plants, we attempted to resolve the issue by using the metabolic inhibitor, INH. As an antagonist of PALP-dependent enzymes, INH should inhibit ALA formation, if ALAS plays an active role in the formation of ALA in greening barley (cf 14). We have, in fact, demonstrated that INH inhibits ALA formation; our experiments indicate however

that the inhibition does not appear to be due to the presence of ALAS. It is more likely due to the presence of a transaminase activity involved in ALA synthesis. When it was discovered that INH acted on CHL synthesis beyond the point of ALA formation, it became important to redirect our study in clarifying the precise site and nature of this effect. Isonicotinic acid hydrazide was shown to inhibit the formation of PCHLD, at a site between PROTO and PCHLD. In light of the significant regulatory function that these two tetrapyrroles play in CHL biosynthesis - as feedback inhibitors of ALA synthesis - it was imperative to elucidate this particular effect by INH. If INH acted indeed as pyridoxal phosphate antagonist, then how could we explain the effect on tetrapyrrole synthesis, where no transamination reactions are known to be required? We attempted to compare the effects of INH to other inhibitors, whose modes of action were better understood. Comparison of INH action with the actions of known chemical blockers of the porphyrin pathway, showed that only ethionine operated like INH. Both apparently block tetrapyrrole synthesis between PROTO and PCHLD.

We then directed our attention towards clarifying the mechanism of this action of INH on tetrapyrrole synthesis. From our experimental results and reports in the literature, it seemed likely that INH may exert its inhibitory effect on tetrapyrrole synthesis by affecting the pyridine nucleotide pool, and consequently the redox balance of the plant cell. Our observations of the antagonistic interplay of light and

INH inhibition support such a hypothesis. This lead us to re-investigate the nature of the INH effect on ALA synthesis; it was discovered that the effect on ALA formation was not primarily via pyridine nucleotides. Attempts were then made to examine the effect of INH on the actual synthesis of ALA from labelled glutamate. Although glutamate has been shown to be incorporated into ALA in barley (6), preliminary attempts proved unsuccessful. We then turned our attention to an organism similar in metabolism to a higher plant chloroplast, that is, the blue-green alga Anabaena variabilis, which synthesizes ALA exclusively via the C-5 pathway. In Anabaena, INH appeared to inhibit the transamination of α-ketoglutarate to glutamate, thereby inhibiting the synthesis of ALA from α-KG but not from glutamate. We concluded then that ALA synthesis in Anabaena involved glutamate as the immediate precursor of ALA, supporting the proposed glutamate-1-semialdehyde pathway. These results coupled with additional inhibitor studies of ALA synthesis in barley suggest that INH inhibits ALA synthesis in a manner consistent with an antagonism of pyridoxal phosphate.

The mode of action of INH offers us some insight into the biosynthetic requirements and regulation of the chlorophyll pathway - in spite of its apparent lack of specificity.

MATERIALS AND METHODS

Growth of Seedlings

Barley seeds, Hordeum vulgare var. Larker, purchased from Donald Keinath, Caro, Michigan, were surfaced-disinfected for 1 minute in 1% NaOCl, and rinsed thoroughly in running tap water. The seeds were then soaked in a Mg^{2+}-supplemented Hutner's nutrient medium (Appendix 1) for 3 hours, and were sown in a flat (56cm X 26cm X 6.5cm) containing vermiculite moistened with 2.5 liters of Hutner's medium. The seeds germinated within 3 days in a dark, humidistated (80%) room at 28°C. Six- to seven-day old etiolated seedlings were used, unless noted otherwise.

Manipulations of Plant Tissue

The seedling tops were harvested under a dim, green safelight by cutting off the shoots just above the level of the bed. Seedlings (~8 g) were trimmed to a uniform length of 8 cm by excision of the bottom section of the shoots, and placed into 15 ml beakers containing 6 ml of solution. The transpiration stream served as the means of uptake. For experiments conducted in the dark, a small fan was placed 30 cm in front of the seedlings. For the greening experiments conducted in light, the beakers - containing shoots and

and solutions - were placed in an illumination chamber (95cm X 65cm X 62cm). In this case, air was drawn across the shoots by a small fan positioned in the back wall of the chamber. Incandescent lights provided the illumination necessary for greening. The light intensities were determined by a Kettering Radiant Power Meter (LDC). For low light experiments, 2 bulbs (Sylvania) @ 7½ watts were used, generating a light intensity of 3.0×10^3 ergs/cm^2/sec. For high light experiments, 4 soft white bulbs (General Electric) @ 100 watts were employed, producing 9.0×10^4 ergs/cm^2/sec of light. Constant ambient temperature was maintained in the chamber. The shoots were preincubated for 1 hour - unless indicated otherwise - to ensure adequate uptake of experimental solutions into the leaves.

Extraction and Pigment Determinations

At the end of the treatment period, the seedlings were removed from their solution, blotted to remove any excess moisture, and 2.75-3.50 g portions were weighed out. The tissue was extracted into 50 ml of ice-cold (initially -20°C) alkaline acetone (9 volumes of acetone to 1 volume of .1 M Na_2CO_3) in a Waring Blender for 1 minute at the highest speed setting; the acetone extract was suction filtered through Whatman No. 1 paper, and the trapped residual plant material was re-extracted with an additional 50 ml of alkaline acetone. The filtrates were combined and an aliquot of the filtrate was transferred to a test tube and clarified by centrifugation at a speed setting of 6 for 5 minutes in a table top centifuge.

The absorbances of the clear filtrate were determined with a Gilford 240 Spectrophotometer at the following wavelengths (in nm): 480, 539, 628, 645, and 663. The pigment concentrations of PCHLD and CHL were determined according to the equations given by Klein and Schiff (64) in mg/l: CHL = 12.2 A_{663} - 0.07 A_{628}, and PCHLD = 28.7 A_{628} - 5.21 A_{663}; for carotenoids according to (A_{480} + .114 A_{663} - 0.638 A_{645})/ 250,000 and PROTO according to the derived equation 46.1 A_{539} - 9.2 A_{628} (Gough, 36). In addition, the absorption maxima of the pigments were confirmed using a Cary 15 recording spectrophotometer.

Pigment synthesis was expressed as net synthesis corrected for the pigment content of shoots grown in the dark.

Identification, Separation, and Fluorimetric Quantitation of Tetrapyrroles

The porphyrins were identified according to the methods of Duggan and Gassman (25) as follows: the crude acetone filtrate was extracted into anhydrous diethyl ether, the volume of which was reduced by bubbling gaseous N_2 through the ether; the porphrins contained in the ether extract were then separated by chromatography in the dark on thin-layer plates of silica gel (EM Laboratories, Inc.) in benezene-ethyl acetate-ethanol (100:25:25, v/v) (25). The individual bands of pigments were located by their red fluorescence under UV light and their R_f values recorded. Before the chromatogram dried out, the pigmented bands were scraped from the plates and

eluted with acetone-methanol (4:1, v/v). The particles of silica gel were separated from the solvent by centrifugation, and an absorption spectrum was taken of each pigmented supernatant with a Cary 15 recording spectrophotometer. Pigments were identified by a comparison of their absorption maxima and their mobilities (R_f) in this solvent system to those previously reported in the literature (25).

A quantitated separation of the porphyrins was accomplished by thin-layer chromatography on silica gel plates in lutidine: H_2O (100:30. v/v) and NH_3 vapor (from NH_4OH) [a modification of the method as described by Gough (36)] and the separated porphyrins were measured by fluorimetry. This solvent system permitted an easy elution of PROTO from the silica gel - much better than from the benzene-ethyl acetate-ethanol system (data not shown). A 200 µl aliquot of the acetone filtrate was applied in a narrow band to a thin-layer plate; the tetrapyrroles were then chromatographed. The separated pigment bands were scraped from the plates into an eluting solvent. Acetone: methanol (4:1) served as the eluent for metalloporphyrins and methanol: 2 N NH_4OH (4:1, v/v) for protoporphyrin. Small test tubes containing the pigmented silica gel and eluent were placed in a covered, ultra-sonicator both for 30 minutes to facilitate the elution process. After centrifugation, the concentration of tetrapyrrole in the supernatants was determined fluorimetrically with a Turner filter fluorimeter, model No. 111. For the quantitation of PROTO, Wratten filter No. 405 was used for excitation and No. 23A for emissions;

for the quantitation of PCHLD, filter No. 47B was used for excitation and No. 23A for emissions. The concentrations were calculated from standard curves prepared for PROTO and PCHLD from pure samples. The pure sample of PCHLD was obtained chromatographically from an extract of ALA-fed, dark-grown seedlings; the PCHLD was estimated spectrophotometrically. The disodium salt of protoporphyrin IX (Sigma Chemical) was used as the standard.

Extraction and Determination of ALA

ALA was estimated by the method of Mauzerall and Granick (79) as modified by Urata (109). Seedlings were incubated in 30 mM potassium levulinate, a competitive inhibitor of ALA dehydrase, permitting the accumulation of ALA as described by Beale (7). Shoot tissue (2.75 to 3.5 g) was extracted as before into 100 ml of cold, 5% trichloroacetic acid (v/v) followed by suction filtration. The crude filtrate was allowed to stand overnight at 4°C; the extract was clarified by centrifugation at 10,000 x g for 15 minutes. The resulting supernatant was then decanted and a 1 ml aliquot of the TCA supernatant was adjusted to pH 4.6 with 1 ml of 2 M Na-acetate buffer and combined with .1 ml of 2,4-pentanedione in a test tube. The test tube, containing the mixture, was stoppered with a glass marble and immersed in a boiling water bath for 15 minutes to form the ALA-pyrrole. One ml of modified Ehrlich's reagent was then added to 1 ml of the cooled solution, containing the ALA-pyrrole, and the absorbance at 555 nm was

determined 15 minutes later. The concentration of ALA-pyrrole was calculated using E_{mM}^{1cm} = 15,600. The identity of the pyrrole was confirmed from its absorption spectrum and the ratio of the absorbances at 525 nm and 555 nm (A_{525}/A_{555} = 0.69).

Radiochemical Procedures

Measurement of Respired $^{14}CO_2$

The effect of various inhibitors on respired $^{14}CO_2$ was measured according to the method of Beale (6). Two grams of seedlings were exposed to 4 hours of illumination in beakers containing water. The seedlings were then removed, blotted, and 3 cm segments excised from the greenest, middle sections of the shoots. The cut sections were placed tops up, maintaining the original orientation of the seedlings, into 2 ml solutions containing a labelled amino acid and inhibitor, where indicated. The employed radiochemicals, purchased from New England Nuclear: [2-^{14}C] glycine (14.1 mCi/mmol); [1-^{14}C] glycine (11.58 mCi/mmol); [3,4-^{14}C] glutamate (14.2 mCi/mmol); and [1-^{14}C] glutamate (48.75 mCi/mmol) were individually added to the incubation solution giving a label concentration of 0.5 µCi/ml. The beakers containing the seedlings and label were placed in glass jars (14.5cm x 7cm (diameter)) fitted with lids from which 2.1 cm glass fiber filter discs wetted with 0.1 ml of 1 N NaOH were suspended. At specified intervals, the filter discs were replaced, and the radioactivity of the ^{14}C-carbonate, formed from the $^{14}CO_2$ trapped on the

discs, was measured by liquid scintillation with a Packard Tri-carb scintillation spectrometer Model 3003; counting efficiency for ^{14}C was 82.0%. The filter discs were placed in vials each containing 10 ml of Aqueous Counting Scintillant (Amersham) and allowed to remain in the scintillation cocktail at least 1 hour prior to counting.

Incorporation of [^{14}C]-ALA into Tetrapyrroles

Four grams of etiolated barley shoots were placed in beakers containing 5 ml of 10 mM ALA and 1.51 µCi [4-^{14}C] ALA (51.5 mCi/mmol, New England Nuclear), and allowed to green for 8 hours under low light. The pigments were extracted, and two 200 µl aliquots of each extract were chromatographied in the lutidine: H_2O solvent system.

One chromatogram served in the pigment quantitation, as described before. The other was used in the determination of the specific activities of [^{14}C]- PROTO, -PCHLD, -CHL. The individual pigmented bands were located under UV light, and the silica gel, containing the pigmented bands, was scraped from the chromatogram into vials to which was added liquid scintillant. The radioactivity was measured by liquid scintillation as before.

Colorimetric Assay for Isonicotinic Acid Hydrazide

Since INH has a strong affinity for the Cu(II) ion (3), it will react with the Lowry reagents used for protein determination

(74) to give a blue color. The INH contained in 5% TAC extracts, was estimated colorimetrically by the following procedure: .2 ml of TCA extract was added to 1 ml of Lowry reagent C [50 volumes solution A (2% Na_2CO_3 in 0.1 N NaOH) + 1 volume solution B (0.5% $CuSO_4$, 1% potassium citrate in H_2O)] for 10 minutes. Then 2 ml of Folin-Ciocolteau reagent (diluted 3x) was added. After 30 minutes, the absorbance at 500 nm was read. The concentrations of INH in the experimental samples were determined from a standard curve.

Experimental Procedures for the Synthesis of [^{14}C]-ALA in Blue-Green Algae

The blue-green alga, Anabaena variabilis Kütz (A.T.C.C. 29413) was grown autotrophically in an 8-fold dilution of the nitrogen-free medium of Allen and Arnon (2) at 30°C in a Microferm Fermentor (New Brunswick Scientific) in the laboratory of Dr. C. P. Wolk (Plant Research Lab, Michigan State University). The growing cultures were continuously illuminated by cool-white fluorescent lamps (General Electric) and aerated until a density of 0.5-1.5 µg of CHL/ml was obtained. Filaments of blue-green algae were separated from the medium by suction filtration through Whatman No. 4 filter paper. The cells were then thoroughly washed with distilled water and resuspended in 40 mM HEPES buffer (pH 8.0) to give a final concentration of 200-300 µg CHL/ml. Warburg flasks were used as the reaction vessels in these experiments. A folded piece of paper wetted with .1 ml of 1 N NaOH was placed in the center

well to trap the respired $^{14}CO_2$. The following solutions - where indicated - were placed in the outer well in ml: 1 M levulinate, 0.1; 200 mM INH, 0.4; labelled precursor, 0.1; 1 mM cold precursor, 0.03; .2 M ATP, 0.1; cell suspension, 1; and water to give a total volume of 2 ml. The radiolabelled precursors used were: [^{14}C(U)] glutamate (260 mCi/mol), [1-^{14}C] glycine (11.58 mCi/mmol, [2-^{14}C] glycine (14.1 mCi/mmol), [1,4-^{14}C] succinate (51.3 mCi/mmol), and [^{14}C(U)]α-ketoglutarate (287 mCi/mmol), purchased from New England Nuclear. The stock concentration of all radioactive precursors was 0.1 mCi/ml. The reaction vessels were stoppered and placed under illumination for 3 hours with continuous agitation provided by a shaker. The reaction was stopped upon the addition of .4 ml 3 N perchloric acid. The reaction mix was then transferred to a test tube, and 15 ml 1 N KOH was added to raise the pH to ~2. The filter papers, containing the ^{14}C-carbonate, were also removed, placed into vials to which liquid scintillant was added, and the radioactivity counted. The acid-killed cells were permitted to stand overnight at 4°C. The cell mix was then centrifuged at 10,000 x g for 15 minutes to pellet the cell debris; the supernatant was then decanted. The ALA contained in the supernatant was then chromatographed on Dowex 50, and purified as the ALA-pyrrole according to the procedures of Beale (6). The radioactivity contained in the ALA-pyrrole was measured by liquid scintillation, as described previously. The amount of ALA was measured by chemical means as described earlier.

RESULTS

A. Effect of Isonicotinic Acid Hydrazide on δ-Aminolevulinic Acid and Chlorophyll Formation

1. Variations in Greening Capacity

Barley seedling of different ages showed substantial variation in their capacity to synthesize δ-aminolevulinate (ALA) and chlorophyll (CHL). Preliminary examination of INH effects on chlorophyll synthesis confirmed the need for uniformity in the age of the plant tissue as well as the ratio of the tissue mass to the volume of extracting solution (3 g of tissue per 100 ml of extracting solution proved to be ideal). The accumulation of ALA and chlorophyll was assayed during a 20 hr illumination period (Figure 4). A significant difference between 6½- and 7½-day old leaves could be seen in total levels of both ALA and CHL produced (cf Hendry & Stobart, 47). After 8 hr, the older tissue contained only 55% of the ALA and 63% of the CHL that the tissue a day younger did. Six-day old shoots were found to possess the highest synthetic capacity (both ALA and CHL) of all ages tested, and consequently were the seedlings of choice.

2. The Effect of INH

Isonicotinic acid hydrazide effectively inhibited CHL accumulation by 85% during a 19 hr greening period (Figure 5).

Figure 4. Time course of δ-aminolevulinic acid and chlorophyll formation during the greening period. Effect of varying seedling age.

The ALA and chlorophyll content of $6\frac{1}{2}$- and $7\frac{1}{2}$-day old seedling were measured after various lengths of illumination. Seedlings used for ALA determinations were incubated in 30 mM levulinate, those used for chlorophyll determinations were incubated in water. The light intensity was 5600 ergs/cm^2/sec.

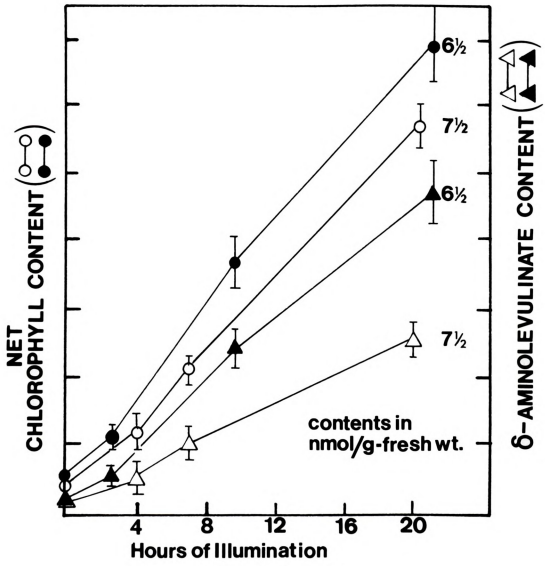
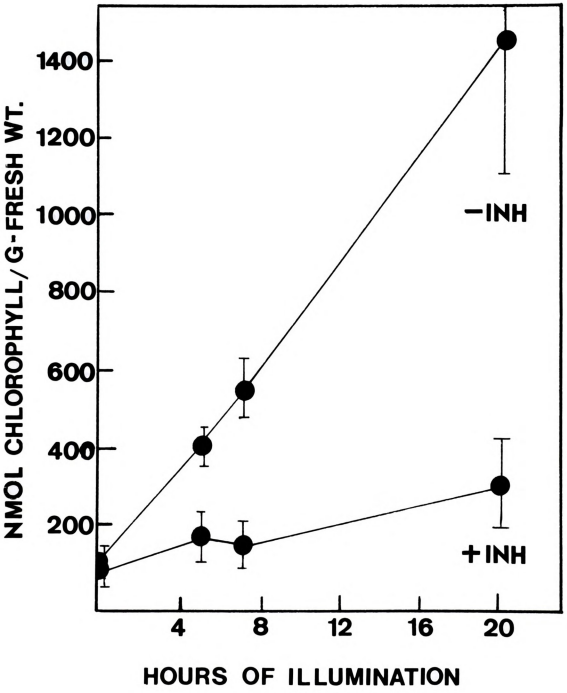

NET
CHLOROPHYLL CONTENT
δ-AMINOLEVULINATE CONTENT
6½
7½
6½
7½
contents in
nmol/g-fresh wt.
4
8
12
16
20
Hours of Illumination

Figure 5. Effect of isonicotinic acid hydrazide (INH) on chlorophyll formation.

Seven-day old barley shoots were incubated in either water or a 0.1 M solution of INH for 20 hours in a light intensity of 5600 ergs/cm^2/sec. The chlorophyll content of the tissue was assayed after various times of illumination.


NMOL CHLOROPHYLL/G-FRESH WT.
1400
1200
1000
800
600
400
200
−INH
+INH
4
8
12
16
20
HOURS OF ILLUMINATION

The effect of INH was rather rapid: more than one half of the total inhibition was attained within the first 4 hr of greening. The potency of INH was clearly demonstrated through its inhibition of ALA and CHL synthesis in low light (Figure 6). Increasing the concentration of INH causes a dramatic rise in the inhibition of both ALA and CHL synthesis. At a concentration of 20 mM, INH maximally inhibited CHL formation by 78% in low light, whereas ALA was inhibited only 52%. Since CHL synthesis was significantly more sensitive to the effects of INH than ALA synthesis, it suggested - as one possibility - a second site of inhibition for INH. The apparent K_i's for these inhibitions differed, being ~ 3 mM for CHL synthesis and ~ 10 mM for ALA synthesis. INH appeared, therefore, to be a more effective inhibitor of CHL than of ALA synthesis. The fact that both CHL and ALA synthesis were significantly inhibited, however, indicates that INH may act in part on ALA synthesis. The inhibitiory effects of INH appear to be dependent on light intensity. The inhibitions by INH (20 mM) of both ALA and CHL synthesis were reduced by 20-25% by increasing the light intensity from 3,000 to 90,000 ergs/cm^2/sec. From the curve (Figure 6), it was evident that the inhibitions never reach a plateau at any concentration of INH in high light - quite contrary to the effect observed in low light where the inhibition curves plateau sharply. This light effect was examined more closely: the efficacy of 10 mM INH was monitored at several light intensities from

Figure 6. The effect of various concentrations of INH on the inhibitions of chlorophyll and ALA synthesis in low and high light.

Barley shoots were incubated in various concentrations of INH for an 8 hr illumination period under either low (L) light (3000 ergs/cm^2/sec) or high (H) light (90,000 ergs/cm^2/sec). Seedlings destined for the chlorophyll assay were incubated in water and/or INH; seedlings used for the ALA assay were incubated in 30 mM LEV and/or INH. Inhibitions of synthesis represent the percent of CHL or ALA synthesis which was inhibited by INH as compared to the control. The controls for ALA, L and H in nmol/g-fresh wt. : 468 ± 15, 781 ± 37 and CHL, L and H in nmol/g-fresh wt.: 44.6 ± 8, 101.1 ± 20.

% Inhibition of:
Chlorophyll Synthesis(•—•)
δ-Aminolevulinic Acid (ALA)
Synthesis(□—□)
100
80
60
40
20
5 10 20 30 40 50 60 70 80 90 100
INH concentration (mM)
L-low light intensity: 3,000 ergs/cm²/s
H-high " : 90,000 "
* STANDARD DEVIATIONS LESS THAN 2%
ARE NOT SHOWN
•H
•L
□L
□H

3,000 to 90,000 ergs/cm^2/sec^1 (Figure 7). Increasing the light intensity attenuated the inhibition by INH of ALA synthesis. By increasing the intensity 4-fold, the inhibition was diminished by a factor of 2 (from 44% to 23%). A 30-fold increase in light intensity essentially eliminated the inhibitory effect of INH on ALA synthesis (from 44% to 6%). These results demonstrated an inverse relationship between the inhibition of ALA formation and light intensity. Conceivably, the attenuation of INH potency by light could be due to a photodestruction of the inhibitor. Direct analysis of the INH levels did not bear out this hypothesis: TCA-extracts of shoots treated with INH under high and low light were assayed for INH. Extracted INH was plotted against the INH concentration of the incubation solution (Figure 8). The INH content of tissue exposed to high light was slightly greater than that of tissue exposed to low light. Shoots incubated in 20 mM INH contained 15.9 ± .2 μmoles of INH/g-fresh wt. in high light - a level 32% greater than that found in low light. The higher levels of assayable INH in tissues treated in high light suggest that INH was not undergoing photodestruction. It should be noted that the estimated levels of INH represent the total INH content of the leaf, however, and not the intracellular concentration which is the physiologically active one. The reduced potency of INH under high light could not be shown by assay to be a light-induced breakdown of the inhibitor. In addition, both pyridine-4-carboxyaldehyde (12) and isonicotinic acid (13) - reported

Figure 7. The effect of increasing light intensity on the inhibition by INH of ALA synthesis.

The inhibition of ALA synthesis in barley shoots was determined after 8 hr incubations in 30 mM LEV + 10 mM INH at each of the following light intensities: 3,000, 12,000, and 90,000 ergs/cm^2/sec.

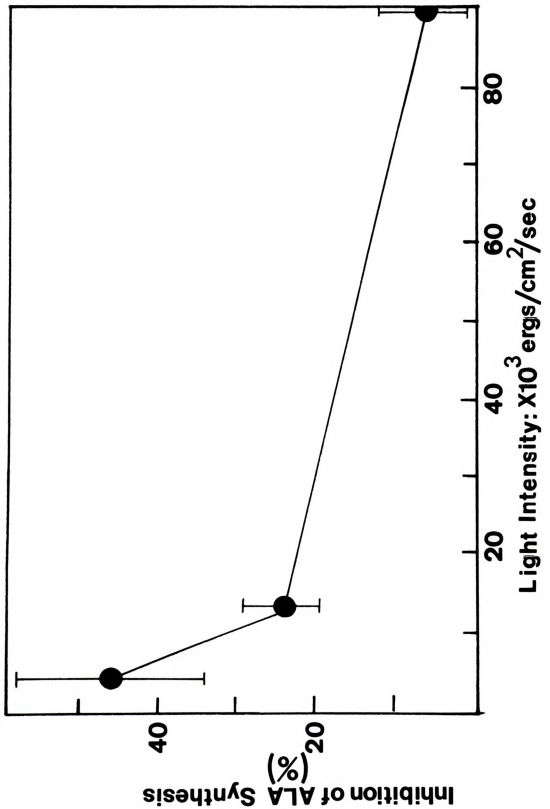
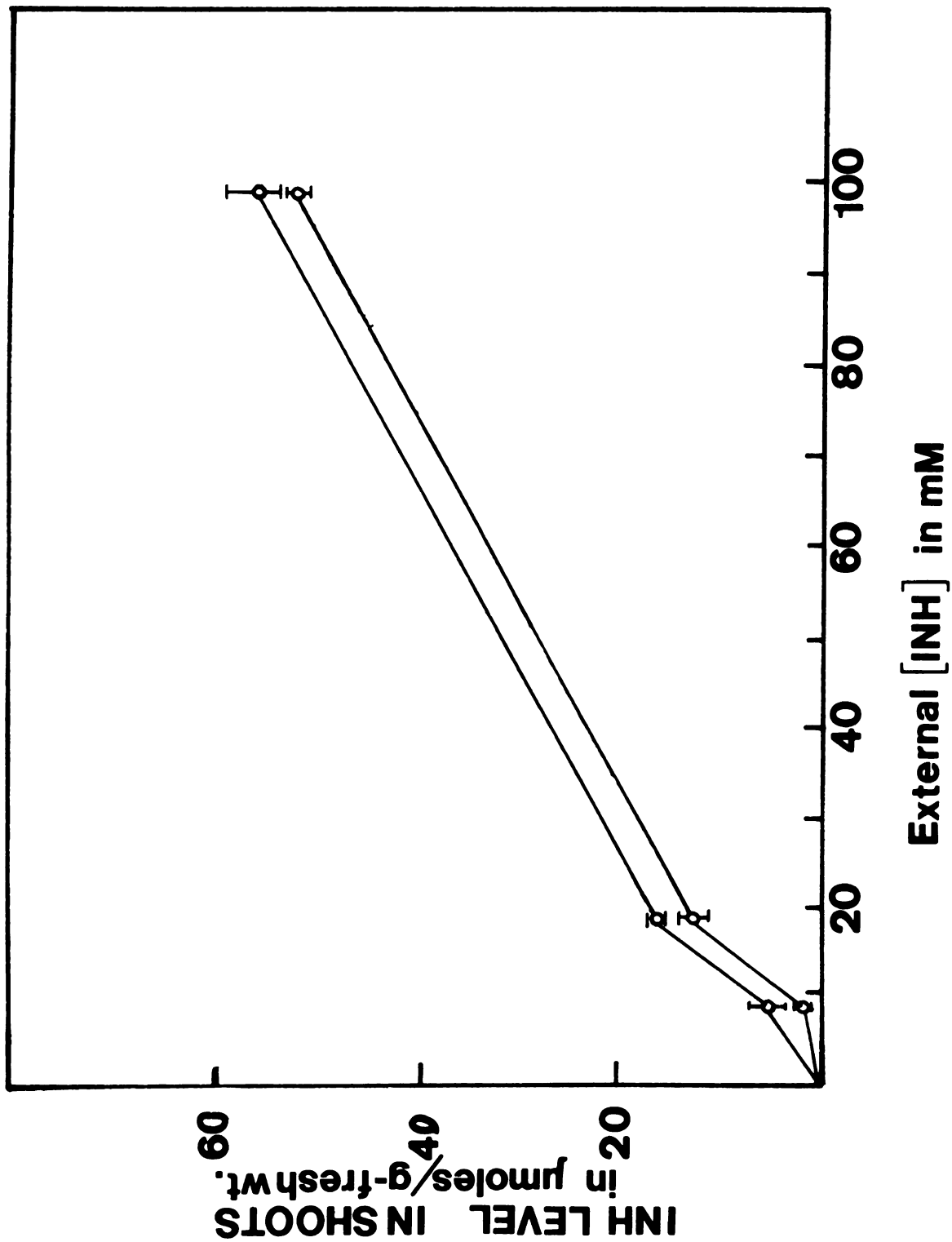

Inhibition of ALA Synthesis (%)
20
40
20
40
60
80
Light Intensity: X10^3 ergs/cm^2/sec

Figure 8. Accumulation of INH in barley tissue under low and high light.

The levels of INH in barley shoots, incubated in various concentrations of INH, were determined after 8 hr of illumination under low (L) = 3,000 ergs/cm^2/sec or high (H) = 90,000 ergs/cm^2/sec light. Isonicotinic acid hydrazide reacts with Lowry reagents to form a blue-colored complex. The INH contained in a 5% TCA extract of the barley seedlings was assayed colorimetrically at 500 nm.


INH LEVEL IN SHOOTS
in μmoles/g-fresh wt.
60
40
20
20
40
60
80
100
External [INH] in mM

degradation products of INH - did not give any Lowry-positive color. If a breakdown of INH to pyridine-4-carboxyaldehyde or INA had occurred in high light, than a discernible decrease in the amount of Lowry-positive material should have been seen. The concentration of INH in tissue incubated in 20 mM INH was calculated to be 17.7 and 13.3 mM for high and low light, respectively. (This calculation was based on the assumption that barley is 90% water by mass.) The hydrazide was freely taken up by the shoots, since the internal concentration of INH approached that of the incubation medium. The uptake of INH followed a curve very similar to the curve for chlorophyll inhibition in high light.

3. Attempts to Reverse the INH-inhibition of Chlorophyll Synthesis

Several compounds were examined for their ability to reverse the effects of INH on the synthesis of ALA and CHL. Precursors of ALA as well as ALA itself were added to the incubation solution containing INH to determine whether or not INH directly inhibited the formation of ALA. The only added compound which significantly reversed the INH-inhibition of CHL synthesis was ALA (30 mM) (Table 1). The tissue treated with both ALA and INH produced 60% of the control level of chlorophyll instead of 8% for INH alone. Precursors of ALA via the C-5 pathway - glutamate, glutamine, α-ketoglutarate - as well as of the classical pathway - glycine

Table 1.--Effects of various amino and organic acids on the inhibition of chlorophyll synthesis by INH: an attempt to reverse the inhibition.

Added Amino/Organic Acids	20 mM INH	(% of control) Chlorophyll Synthesis
none	-	100
none	+	7.6 ± 2
50 mM glutamate	+	11.0 ± .5
50 mM glutamine	+	9.9 ± 11
50 mM α-ketoglutarate	+	9.0 ± 11
50 mM glycine	+	8.2 ± 8
50 mM succinate	+	5.4 ± 11
50 mM glycine + 50 mM succinate	+	14.4 ± 6
1% casamino acids	+	11.3 ± 6
30 mM δ-aminolevulinic acid	+	59.5 ± 11
30 mM pyridoxine·HCl	+	18.6 ± 1

Etiolated seedlings were incubated in either water or 20 mM INH plus none or one amino acid or organic acid solution, as indicated, for 8 hr in a light intensity of 5600 ergs/ cm^2/sec. The control level of chlorophyll accumulation was 39.4 ± 7 nmol/g-fresh wt.

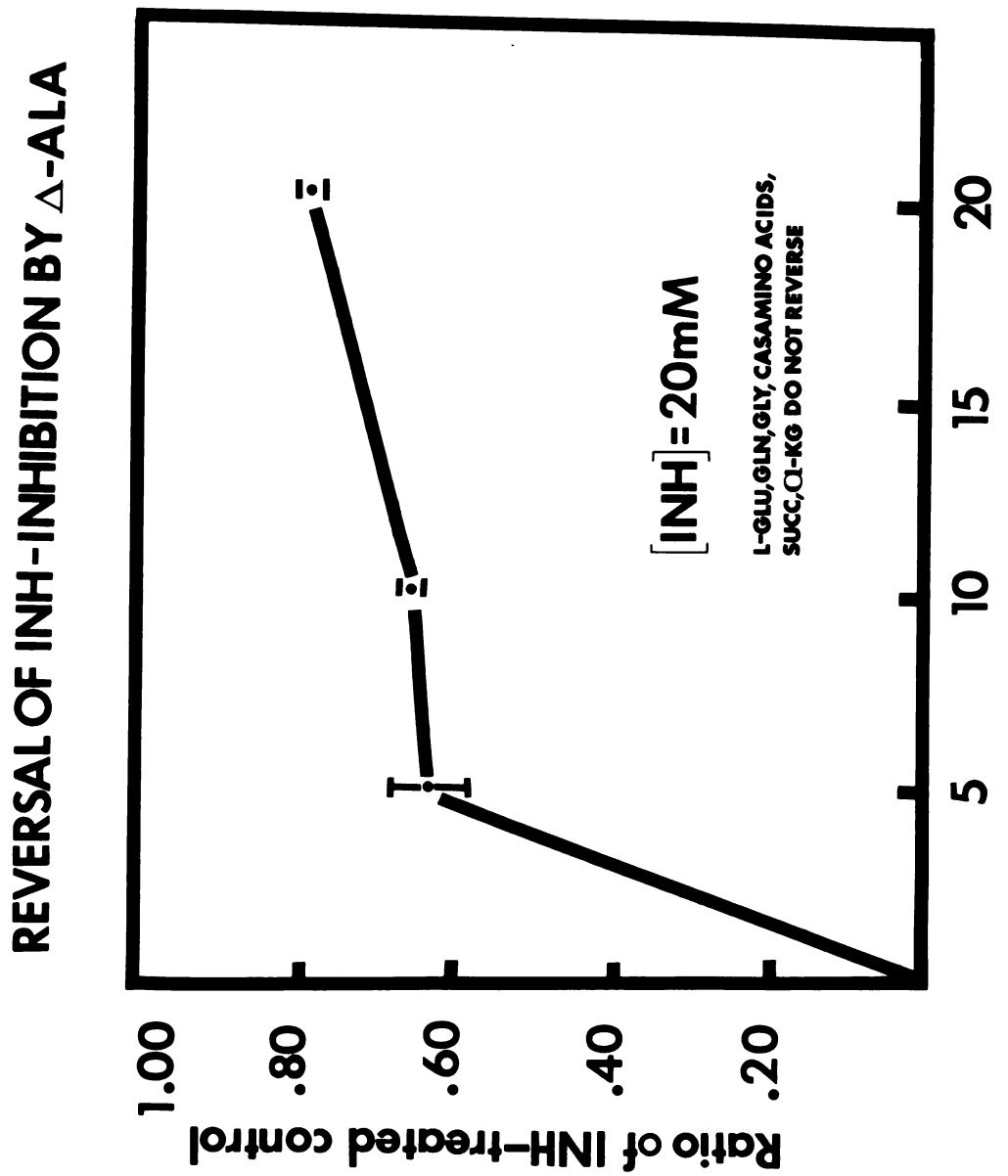
and succinate - did not show any capacity for reversing the inhibition by INH of CHL synthesis.

Glycine and succinate in combination - the substrates for ALA synthase - did not significantly reverse, nor did 1% casamino acids. Pyridoxine, a precursor of pyridoxal phosphate, also did not reverse. In addition, the possibility that INH might have acted by interferring with the pyridine nucleotide pool was examined by using added nicotinamide. Various concentrations of nicotinamide were added to the 20 mM INH incubation solutions, and CHL formation was determined. In both high and low light, nicotinamide did not reverse the inhibition of CHL accumulation by INH in concentrations from 10 to 100 mM (data not shown). Since nicotinamide did not counteract the effects of INH-inhibition, this initially suggested that INH's mode of action on CHL synthesis was most likely not via the pyridine nucleotide pool (in disagreement with subsequently determined results, cf. Table 5).

Exogenous ALA supplied to seedlings simultaneously with INH, sharply reversed the inhibition of CHL synthesis seen with INH alone (Figure 9). The ability to reverse the INH-inhibition was dependent on the concentration of added ALA. It was also shown that this reversal was not due to a chemical "inactivation" of INH by ALA as measured by the Ehrlich's and Lowry assays. At 10 mM ALA, 65% of the inhibition was reversed, whereas 20 mM (80%) of the inhibition could be reversed. Higher concentrations of ALA did not give total

Figure 9. Reversal by ALA of the INH-inhibition of chlorophyll synthesis.

Etiolated shoots were incubated in water or 20 mM INH plus varying concentrations of ALA (from 0-20 mM). Reversal of the INH-inhibition of chlorophyll synthesis is defined so that a ratio of 1.0 indicates that there is no inhibition at that particular concentration of ALA. The light intensity was 3,000 ergs/cm^2/sec. The ordinate is the ratio of net chlorophyll formation in INH treated seedlings over chlorophyll formation in the controls (91.8 ± 6 nmol/g-fresh wt.). (The stimulation of chlorophyll formation by ALA without INH has been substracted from each point.)


REVERSAL OF INH-INHIBITION BY Δ-ALA
Ratio of INH-treated control
1.00
.80
.60
.40
.20
5
10
15
20
[δ-ALA] in mM
[INH] = 20mM
L-GLU, GLN, GLY, CASAMINO ACIDS, SUCC, α-KG DO NOT REVERSE

reversal of the inhibition (data not shown). The apparent concentration of ALA reversing one half of the total inhibition was ~5 mM, suggesting that INH does indeed inhibit the synthesis of ALA.

A noteworthy observation was made concerning the appearance of shoots supplied with 10 mM ALA and 20 mM INH in low light; they showed a symptomatic wilt caused by a loss of turgidity that shoots fed ALA in high light also exhibited. The seedlings exposed more directly to the light - positioned closest to the sides of the beaker - appeared blanched compared to the greener, shielded tissue - closer to the inside. This was not observed in tissue treated with either water or ALA alone. This blanching effect is often characteristic of photosensitization (cf. 11, 113) and might be attributed to the accumulation of pigmented precursors of CHL. The INH-treated tissue also bore a faint brownish color, suggesting the accumulation of some brown pigment, presumably derived from ALA and a site of INH action beyond ALA synthesis.

4. The Effect of INH on ALA-stimulated Tetrapyrrole Synthesis

To pinpoint additional, possible sites of action for this inhibitor, we examined the effects of INH on the synthesis of other intermediates of the biosynthetic pathway leading to CHL. We conducted these experiments in the dark - where little ALA and no CHL are formed - in the presence of added ALA. This experimental design enabled us to investigate that portion of the pathway between the formation of

ALA and PCHLD, the Mg-tetrapyrrole which accumulates in darkness. Spectrophotometric and chromatographic analyses of pigmented extracts from tissue incubated in ALA in the dark revealed the presence of only two tetrapyrrolic compounds: PROTO and PCHLD - even with added INH (Figure 10).

The effect of 20 mM INH on PCHLD and PROTO formation was examined over the course of a 24 hr dark incubation period (Figure 11). The shoots were incubated in 10 mM ALA solutions to facilitate tetrapyrrole synthesis, which is quite low without light activation. During the first 9 hr, there appeared to be no significant differences in either PCHLD or PROTO synthesis between INH-treated and untreated seedlings [cf the light experiment, where CHL synthesis was inhibited 78% (Figure 5) and 40% in the presence of 30 mM ALA within 8 hrs (Table 1)]. Between 9 and 24 hr, INH inhibited PCHLD synthesis about 20% and seemed to slightly stimulate PROTO synthesis. These results implied a possible site of action between PROTO and PCHLD. In order to investigate this possibility further, various concentrations of INH were tested for their effects on ALA-stimulated tetrapyrrole synthesis in the dark (Figure 12). All concentrations of INH less than 20 mM stimulated PROTO synthesis between 17 and 31%, whereas PCHLD was inhibited between 0 and 20% over these same concentrations. At the higher INH concentrations (50 and 100 mM), synthesis of both tetrapyrroles from exogenous ALA was inhibited but not to the same extent; PROTO accumulation

Figure 10. An absorption spectrum of an ether extract of barley seedlings treated with both INH and ALA.

Seedlings were fed 20 mM ALA in the presence of 20 mM INH for 21 hr in the dark. At the end of this time, seedlings were extracted into alkaline-acetone and filtered. The acetone filtrate was then extracted with diethyl ether to transfer the pigments into the ether phase. An absorption spectrum was recorded. The absorbance between 350-450 nm is given on the 0-1 absorbance scale; the absorbance between 450-700 nm is expressed on an 0.1 absorbance scale.

Key to absorbance maxima (in nm):

Photochlorophyllide: 432, 534, 571, 624

Protoporphyrin: 403, 503, 534, 575

Chlorophyll a: 663

Carotenoids: 469

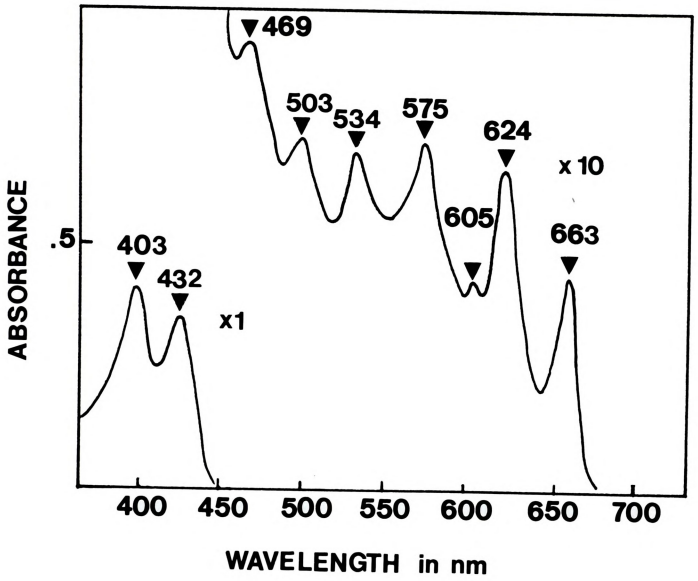

ABSORBANCE
.5
403
432
x1
469
503
534
575
605
624
x 10
663
400
450
500
550
600
650
700
WAVELENGTH in nm

Figure 11. The effect of INH on the increase in ALA-induced protoporphyrin (PROTO) and protochlorophyllide (PCHLD) accumulation in the dark.

Seedlings were incubated in 10 mM ALA ± 20 mM INH for 24 hr in the dark. Uptake of the solutions was facilitated by air blown across the shoots from a small fan. At various times, seedlings were removed and the PROTO and PCHLD contained therein was extracted and quantified fluorimetrically. (See materials and methods for further details.)

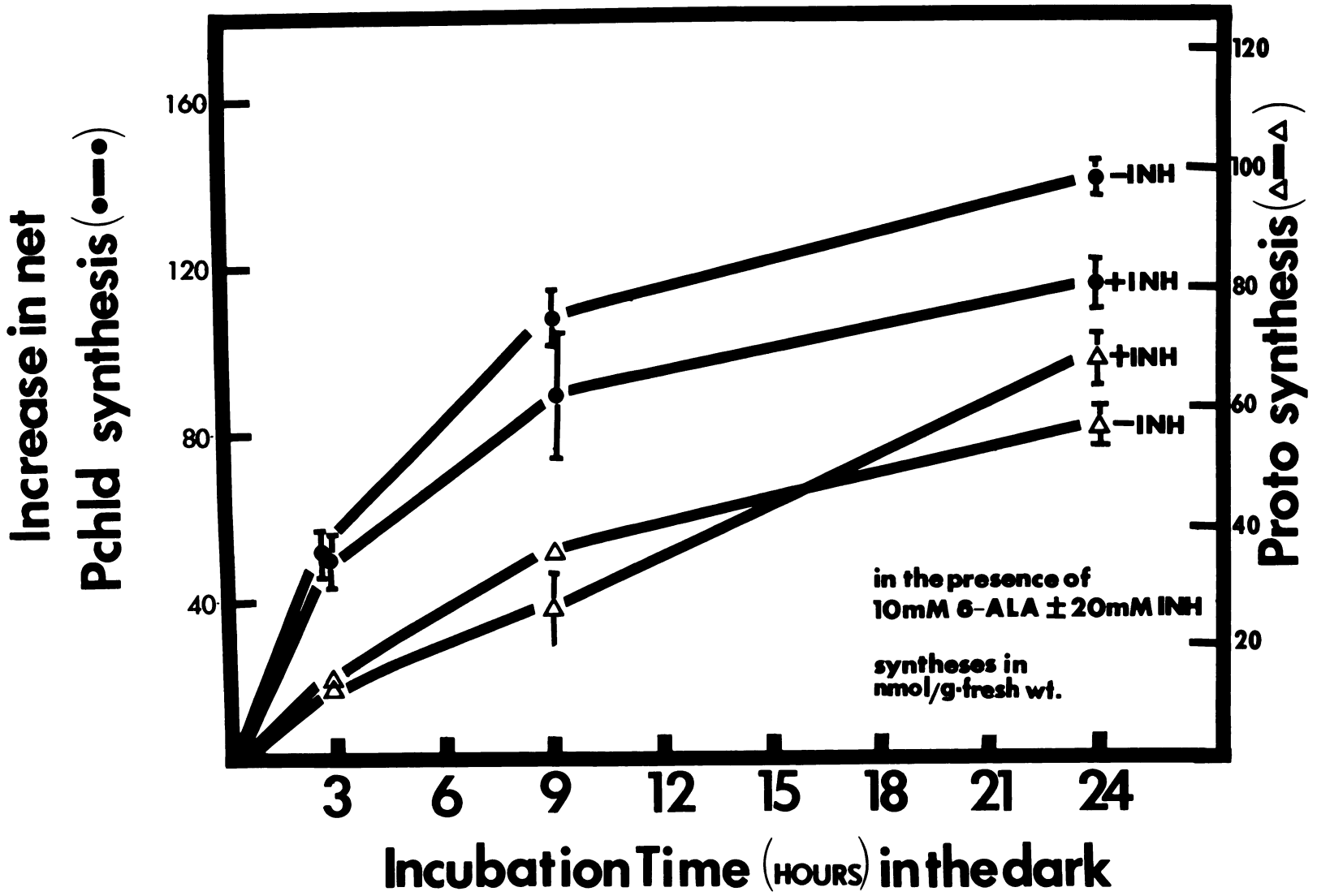
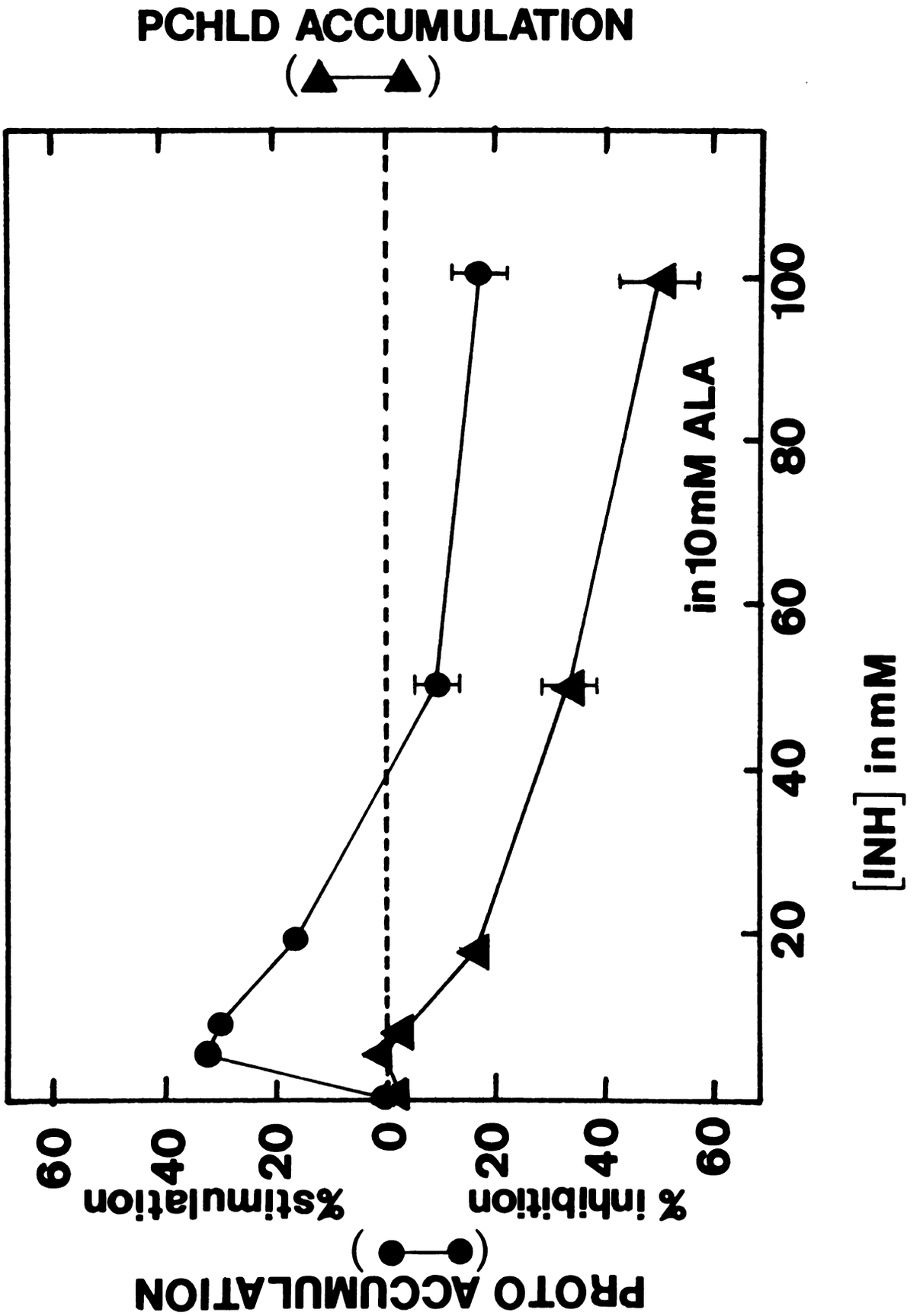

Increase in net
Pchld synthesis (•—•)
160
120
80
40
120
100
80
60
40
20
Proto synthesis (Δ—Δ)
–INH
+INH
+INH
–INH
in the presence of
10mM δ-ALA ± 20mM INH
syntheses in
nmol/g-fresh wt.
3
6
9
12
15
18
21
24
Incubation Time (HOURS) in the dark

Figure 12. The stimulation of ALA-dependent PROTO accumulation by low concentrations of INH in the dark.

Seedlings were incubated in 10 mM ALA with/without various concentrations of INH for 24 hr in the dark. PROTO and PCHLD accumulation were assayed at the end of these time periods. The figure shows the effects of INH on tetrapyrrole accumulation after 24 hr of incubation. Control levels of PROTO and PCHLD were in nmol/g-fresh wt.: 66.9 ± 6 and 140.9 ± 16, respectively.


PCHLD ACCUMULATION
(▲—▲)
PROTO ACCUMULATION
(●—●)
% inhibition
%stimulation
0
20
40
60
20
40
60
80
100
[INH] in mM
in 10mM ALA

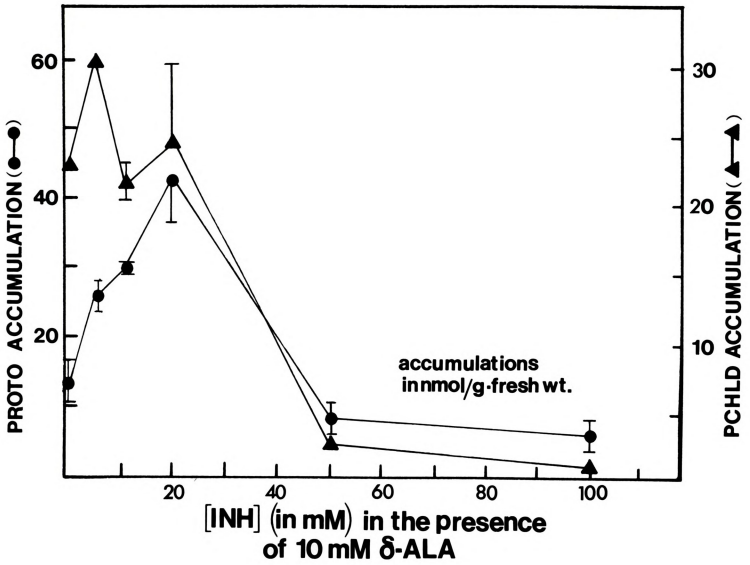
was inhibited only 10-17% compared to inhibitions of 38-50% for PCHLD. The differential inhibitory effect of INH on the synthesis of these tetrapyrroles may suggest a site of action somewhere between PROTO and PCHLD. Although this higher concentration of inhibitor may be acting in a different manner.

The fact that 5 mM INH stimulated PROTO accumulation by 31% and had no effect on PCHLD seemed rather curious. If INH were blocking the conversion of PROTO to PCHLD, then we would have expected to see an inhibition of PCHLD (we will address this inconsistency in greater detail in the discussion). At a higher INH concentration (20 mM), however, PROTO was stimulated by 20% and PCHLD was inhibited by 20% - which is what we would have expected.

The effect of INH on tetrapyrrole formation in low light was examined. When PROTO levels of ALA-fed barley shoots were estimated after exposure to low light in increasing concentrations of INH, we noted a dramatic rise of 244% in the content of PROTO in the INH-treated tissues as compared to controls (Figure 13). PCHLD synthesis remained refractory to INH over these same concentrations of inhibitor - the same effect observed in the dark. At higher concentrations, the inhibitory effects of INH on the synthesis of both tetrapyrroles were displayed; PCHLD was inhibited maximally by 71% whereas PROTO was inhibited by only 32%. The formation of PCHLD appeared, therefore, to have a 2-fold greater sensitivity to INH than did PROTO, suggesting perhaps an

Figure 13. The stimulation of ALA-enhanced PROTO accumulation by low concentrations of INH in low light.

Seedlings were incubated in 10 mM ALA with/without various concentrations of INH for 8 hr in low light (3,000 ers/cm^2/sec). PROTO and PCHLD were assayed at the end of the 8 hr illumination period.


PROTO ACCUMULATION (●—●)
PCHLD ACCUMULATION (▲—▲)
60
40
20
30
20
10
accumulations
in nmol/g·fresh wt.
20
40
60
80
100
[INH] (in mM) in the presence
of 10 mM δ-ALA

inhibitory site of action somewhere between these two tetrapyrroles. The inhibition of both PROTO and PCHLD at higher concentrations of INH may be due, however, to other general toxic effects.

Further investigation into the effects of INH on pigment synthesis in shoots exposed to low light revealed that INH has no demonstrable effect on the synthesis of carotenoids (Table 2) - a process intimately associated with the formation of the chloroplast membrane (92). This in addition to other evidence argues against an explanation of INH's differential mode of action on PROTO and PCHLD involving the inhibition of membrane formation. In the presence of added ALA, CHL synthesis was less sensitive to INH (50 mM) than PCHLD, an inhibition of 40% compared to 67% for PCHLD. This mitigates an inhibitory role for INH on membrane synthesis, since both PCHLD and CHL are presumably synthesized on membranes (102) and would have displayed similar extents of inhibition. We also demonstrated that the syntheses of PROTO, PCHLD, and CHL were differentially sensitive to INH (Table 2). The inhibitor strongly stimulated PROTO synthesis - as demonstrated earlier - between 28 and 66% for 5-20 mM INH. PCHLD remained unaffected and CHL slightly inhibited by 15% over these same concentrations. Increasing the INH concentration to 50 and 100 mM, resulted in differential inhibitions. The levels of PROTO and CHL synthesis were reduced by similar percentages: 32 and 44% for PROTO and

Table 2.--Effects of INH on the synthesis of tetrapyrroles and carotenoids in low light in the presence of ALA.

Treatment	% of Control			
INH concentration	PROTO	PCHLD	CHL	CAROTENOIDS
0	100 ± 10	100 ± 12	100 ± 12	100 ± 16
5 mM	128 ± 7	124 ± 2	89 ± 8	98 ± 5
10 mM	131 ± 4	96 ± 3	87 ± 8	102 ± 8
20 mM	166 ± 30	105 ± 21	85 ± 14	100 ± 5
50 mM	68 ± 1	33 ± 16	60 ± 1	98 ± 16
100 mM	56 ± 7	15 ± 2	55 ± 2	81 ± 3

Etiolated shoots were incubated in 10 mM ALA + various concentrations of INH (0-100 m) for 8 hr in low light (3,000 ergs/cm^2/sec). The levels of pigments were assayed at the end of this illumination period. Control levels of net synthesis for PROTO, PCHLD, CHL, and carotenoids in nmol/g-fresh wt. were: 12.5 ± 3, 23.0 ± 3, 94.5 ± 11, and 64.0 ± 4, respectively.

40 to 45% for CHL. On the other hand, the 2-fold greater sensitivity of PCHLD synthesis to the hydrazide was again shown by its inhibition, ranging from 67 to 85%. These results raise the possibility that at higher concentrations (50-100 mM) INH may inhibit a step between PCHLD and CHL.

To clarify whether there is indeed site of action of INH on the steps between the formation of PROTO and PCHLD, shoots were incubated in 5 mM ALA containing [4-^{14}C] ALA in the dark with and without INH. The radiospecific activity (*SA) of the ALA in the incubation solution was .17 μCi/μmol (300 cpm/nmol). After 3 hr incubation, the *SA's of the PROTO and PCHLD in the control tissue were 722 and 600 cpm/nmol respectively, or 30% and 25% of a theoretical *SA for tetrapyrroles, formed solely from the exogenous ^{14}C-ALA, of 2400 cpm/nmol (Table 4). [We are making the assumptions that all synthesis is derived from the unadulterated exogenous ALA (*SA= 300 cpm/nmol), and 8 molecules of ALA are used in forming one molecule of tetrapyrrole (75).] The *SA's of both PROTO and PCHLD were approximately one-quarter of the theoretical estimation, suggesting the presence of an endogenous pool of cold ALA or other intermediates leading to tetrapyrroles, which diluted the labelling by 75%. A differential effect of INH on the amount of label incorporated into PROTO and PCHLD was clearly seen; the *SA of PROTO rose 52% over the control whereas the *SA of PCHLD remained constant - evidence supporting, the idea of a site of action

Table 3.--Effects of INH on the radiospecific activity (*SA) of accumulating PROTO and PCHLD in barley shoots fed ALA in the dark.

hours of incubation in 5 mM ^{14}C-ALA	Radiospecific Activity in cpm/nmol of:					
	PROTO			PCHLD		
	-INH	+INH	ratio(*SA's)	-INH	+INH	ratio(*SA's)
3	722 ± 32	1099 ± 66	1.52 ± .09	600 ± 20	653 ± 49	1.09 ± .08
11½+	1656 ± 18	2048 ± 46	1.24 ± .03	892 ± 85	714 ± 22	.80 ± .02
23	835 ± 47	662 ± 41	.79 ± .06	821 ± 105	746 ± 39	.91 ± .05

+ *SA of ALA was halved.

Shoots were fed 5 mM ALA with a *SA of 300 cpm/nmol for 11½ hr in the dark with and without 20 mM INH. At 11½ hr the *SA of the ALA in all incubation solutions was halved to 150 cpm/nmol. At incubation times 3, 11½, and 24 hr, shoots were harvested, extracted, and extracts chromatographed. The individual pigment bands of the chromatograms were eluted and assayed fluorimetrically for PROTO and PCHLD or the radioactivity quantified by liquid scintillation counting. The columns designated ratio (*SA's) in the table above represent the ratio of the *SA of the INH-treated (+INH) to the (-INH) control. After 23 hr, the net synthesis of PROTO and PCHLD with and without INH in nmol/g-fresh wt: 37 ± 6, 34 ± 3; 83.5 ± 6, 51.2 ± 5, respectively.

for INH presumably after the step of PROTO synthesis. By 11½ hr in the incubation period, the *SA of the PROTO pool had reached a level equivalent to 70% of the theoretical estimated value of 2400 cpm/nmol whereas the *SA of the PCHLD pool reached a level of only 37% of the theoretical value. The equilibration of label into all the pools of intermediates in the pathway appeared to require more than 11½ hours, but clearly the PROTO pool appeared to be more rapidly equilibrated than the PCHLD pool. The *SA of PROTO for INH-treated shoots was 124% of the untreated, suggesting INH might act to facilitate the labelling of the PROTO pool, or essentially stimulate its turnover. At the 11½ hr point, the *SA of the exogenous ALA was diluted by a factor of 2 giving a new *SA for ALA of 150 cpm/nmol. This was done to test the effect of INH on the turnover of both tetrapyrroles in a modified pulse-chase experiment. After 23 hr of incubation, we observed that the *SA of the PROTO dropped to 835 cpm/nmol, a value less than the calculated theoretical value of 1200 cpm/nmol but approximately one-half of the *SA of PROTO seen before we diluted the labelled ALA. This suggests that the pool of PROTO undergoes a rather rapid turnover. INH appeared to enhance the loss of label from PROTO by 72% (from 2048 to 662 cpm/nmol), essentially enhancing its turnover rate. On the other hand, the *SA of the PCHLD remained relatively unaffected during this period of 11½ hr; INH had just a slight effect on the *SA of PCHLD -

at most an inhibition of 10%. The pool of PCHLD did not appear to be turned over significantly; when the *SA of the ALA was halved, a change in the *SA of PCHLD was observed which was entirely consistent with the dilution of label by new accumulation of PCHLD. We concluded therefore that INH acted in a manner which appeared to stimulate the turnover of PROTO.

A similar experiment - analogous to the proceeding one - was performed in low light in order to see if these same effects of INH on *SA's could be produced in the light. Shoots were incubated in 5 mM, ^{14}C-ALA with or without 20 mM INH (Table 4). The 8 hr illumination period was divided into two 4 hr periods so that treatments could be varied for any two sequential 4 hr periods. This was done to investigate the role of light (or darkness) in bringing about the inhibitory effects of INH. When the tissues were exposed to 4 hr of light prior to 4 hr of INH, the inhibitor had no effect on the synthesis or *SA of the tetrapyrroles (cf treatments 4 and 8), whereas INH treatment in light followed by just light and no INH resulted in inhibitions of tetrapyrrole formation and changes in *SA's. Light given before INH appeared therefore to spare the effects of the inhibitor. It was clear that INH exerted its maximum inhibitory effects with only 4 hr of treatment (cf treatments 9 and 5, 7, 8).

Table 4. Effect of INH on the *SA and synthesis of tetrapyrroles derived from ^{14}C-ALA in the light.

	Treatment in 5 mM ALA				Inhibition of Synthesis (% Inh) or Ratio of *SA					
	|←1st 4hr→|		|←2nd 4hr→|		PROTO		PCHLD		CHL	
	INH	LIGHT	INH	LIGHT	% Inh	Ratio(*SA)	% Inh	Ratio(*SA)	% Inh	Ratio(*SA)
1.	-	+	-	+	0 ± 2	1.0 ± .12	0 ± 10	1.00 ± .07	0 ± 7	1.00 ± .11
2.	-	+	-	-	-9 ± 1	0.96 ± 0	-125 ± 3	0.99 ± .01	54 ± 2	1.01 ± .02
3.	-	+	+	-	-8 ± 2	0.82 ± .03	-106 ± 1	1.05 ± .06	54 ± 2	0.91 ± .02
4.	-	+	+	+	-6 ± 3	1.02 ± .07	6 ± 5	1.06 ± .06	5 ± 8	1.10 ± .06
5.	-	-	+	+	22 ± 8	1.77 ± .06	75 ± 11	1.39 ± .04	35 ± 1	0.99 ± .14
6.	-	-	-	+	19 ± 4	1.82 ± .4	9 ± 7	1.43 ± .08	47 ± 4	1.16 ± .12
7.	+	-	-	+	27 ± 13	2.07 ± .11	77 ± 5	1.72 ± .07	46 ± 0	1.10 1 .15
8.	+	+	-	+	30 ± 12	2.20 ± .40	64 ± 1	1.41 ± .03	35 ± 5	1.00 ± .13
9.	+	+	+	+	27 ± 6	1.89 ± .30	77 ± 11	1.57 ± .08	39 ± 2	1.36 ± .04

Seedlings were fed a solution of 5 mM ^{14}C-labelled ALA (110 cpm/nmol) ± 20 mM INH during the course of an 8 hr treatment period. This 8 hr period was divided into two sequential 4 hr treatments: the 1st 4 hr and the 2nd 4 hr. We could alter treatments (INH and light) during the 8 hr period. The light treatment could be varied so that the seedlings could be exposed to illumination at 3000 ergs/cm^2/sec (light: +) or darkness (light: -) or both during the 8 hr period. INH treatment (INH: +) or no treatment (INH: -) could also be varied. The *SA's and the syntheses of PROTO, PCHLD, and CHL were determined after 8 hr of treatment. % Inhibition represents the % of synthesis inhibited by each treatment as compared to the control (treatment 1.) [(+) %Inh= an inhibition of synthesis, (-)%Inh= a stimulation of synthesis.] Ratio(*SA) represents the ratio of *SA of treated to control. The control values (treatment 1.) are for the synthesis of PROTO, PCHLD, CHL in nmol/g-fresh wt.: 19 ± 1, 58.7 ± 6, 130.1 ± 9 and *SA in cpm/nmol: 354 ± 43, 249 ± 18, 426 ± 48, respectively.

Treatment with INH also caused a significant rise in the *SA of PROTO (treatments 5, 7, 8, and 9). This stimulation was ~100%, and was not observed when the tissue was first exposed to light followed by INH (treatment 4). In addition, INH inhibited PCHLD synthesis by 64-77% as opposed to PROTO and CHL inhibitions of only 22-30% and 35-46%, respectively (treatments 5, 7-9). [Note again the 2-fold greater sensitivity of PCHLD to INH.] It was disturbing to observe an inhibition of PROTO synthesis under these conditions, since we had previously seen INH-induced stimulations in PROTO accumulation. Evidently, by lowering the concentration of exogenous ALA to 5 mM (compared to 10 mM previously), we have made tetrapyrrole synthesis more sensitive to INH effects. Whether the effect of the hydrazide at lower ALA concentrations is the same as at higher concentrations is a factor to be considered.

Overall, these data may imply more than one site of action for INH: one between PROTO and PCHLD, and another between PCHLD and CHL; however, the latter site appeared to be much less sensitive to inhibition by INH than the former. The increased *SA's of both PROTO and PCHLD with INH-treatment may indicate an enhanced turnover of both these tetrapyrroles.

In conclusion, light given prior to INH appeared to attenuate the inhibitory effects of the hydrazide. The inhibitor also appeared to have specific although differential effects on the biosyntheses of tetrapyrroles, suggesting, perhaps, sites of INH action.

5. Nicotinamide - An Antagonist of the INH-inhibition of Tetrapyrrole Synthesis

The inhibitory effects of INH on tetrapyrroles could be greatly diminished, if barley shoots [prior to their exposure to INH] were pre-treated with 50 mM nicotinamide - a reputed antagonist of INH in mammalian tissues (Table 5). Seedlings, pre-treated in nicotinamide or water for 4 hr in the dark, were then treated with 5 mM ALA + 50 nM nicotinamide, or 5 mM ALA + 50 nM nicotinamide + 20 mM INH in low light. The 8 hr illumination period was divided, as before, into two 4 hr periods, where treatement could be varied from one 4 hr period to another. The nicotinamide pre-treatment mitigated the inhibitory effects of INH not only on the synthesis of CHL but also on the synthesis of the tetrapyrroles, PROTO and PCHLD. This result was seen, if the shoots were treated with INH in either light or dark immediately after the nicotinamide pre-treatment in the dark (cf treatments 3 and 4; 5 and 6). The inhibitions of tetrapyrrole synthesis were effectively attenuated by nicotinamide pre-treatment (cf treatments 5 and 6) from 66% to -3% for PCHLD and from 31% to 6% for PROTO. The elimination of the INH-inhibition of PCHLD (treatment 5) was not due just to an increased accumulation of PCHLD due to the dark (cf treatment 6). Of special interest, was the observation that the *SA ratio of PROTO decreased from 1.81 with INH treatment alone to .67 with nicotinamide pre-treatment - a stark and

Table 5.--The reversal by nicotinamide of the inhibitory effects of INH on the *SA and synthesis of tetrapyrroles.[a]

Pre-treatment	Treatment in 5mM ALA						PROTO		PCHLD		CHL	
| + 4 hr + |	|+1st 4hr+|			|+2nd 4hr +|							
NAmD(in dark)	NAmD	INH	Light	NAmD	INH	Light	% Inh	Ratio(*SA)	% Inh	Ratio(*SA)	% Inh	Ratio(*SA)
1. -	-	-	+	-	-	+	0 ± 10	1.0 ± .11	0 ± 17	1.0 ± .19	0 ± 7	1.0 ± .12
2. +	+	-	+	+	-	+	1 ± 8	1.05± .09	11 ± 8	1.1 ± .35	3 ± 3	1.09± .05
3. -	-	+	+	-	-	+	22 ± 1	1.81± .02	82 ± 4	1.53± .15	35 ± 5	1.01± .06
4. +	+	+	+	+	-	+	3 ± 2	0.67± .11	49 ± 6	1.14± .04	11 ± 4	1.09± .30
5. +	+	-	-	+	+	+	6 ± 4	0.69± .11	-3 ± 7	1.00± .04	21 ± 3	1.12± .07
6. -	-	-	-	-	+	+	31 ± 10	2.07± 0	66 ± 9	1.48± .08	37 ± 2	1.29± .18
7. +	+	-	+	+	+	+	-4 ± 4	1.16± .12	15 ± 2	1.25± .05	10 ± 1	1.03± .07
8. -	-	-	+	-	+	+	-2 ± 17	1.13± .39	3 = 13	1.21± .23	9 ± 5	1.38± .38

[a]Average of two independent experiments

Etiolated seedlings were pre-treated in either H_2O or 50 mM NAmD for 4 hr in the dark. The seedlings were then transferred to solutions containing 5 mM ^{14}C-labelled ALA ± 50 mM NAmD ± 20 mM INH. As before the 8 hr treatment was divided into two sequential periods, during which time treatments, both chemical and light, could be varied. Inhibition of synthesis (% Inh) represents the percent of tetrapyrrole synthesis inhibited by each treatment as compared to the control (treatment 1.). Ratio(*SA) represents the ratio of the *SA of each tetrapyrrole after treatment to the control (treatment 1.). The control values (treatment 1.) are for synthesis of PROTO, PCHLD, CHL in nmol/g-fresh wt.: 11 ± 1, 32.6 ± 15, 125.9 ± 13 and for *SA in cpm/nmol, 395 ± 44, 304 ± 33, and 276 ± 34, respectively.

opposite effect. Nicotinamide also mitigated the inhibition by INH of CHL synthesis (cf treatment 3 and 4); pre-treatment with nicotinamide reduced the inhibition of CHL synthesis from 35 to 11%. As a control, nicotinamide treatment had no effect on either the synthesis or *SA of any of the tetrapyrroles (treatment 2). We observed once again that light given before INH eliminates the inhibitory effects (treatment 8). These results suggest that nicotinamide acts in a manner dramatically opposite to that observed with INH - in a manner which antagonizes the effects of INH as does light.

In an attempt to determine whether or not nicotinamide might possibly mitigate the inhibitory effect on ALA formation as well, we pre-treated shoots, as before, with 50 mM nicotinamide. The tissue was then subjected to solutions of varying contents - 30 mM levulinate with and without 50 mM nicotinamide with and without 20 mM INH. In addition, we varied the treatments during each 4 hr of the 8 hr illumination period (Table 6A). INH treatment for 4 hr gave the full inhibition of ALA formation (treatment 2). Nicotinamide did not reverse the effects of INH on ALA synthesis. Pre-treatment with nicotinamide was no different than pre-treatment with water in the extent of INH-inhibition (cf treatments 3 and 4); the reduction in inhibition appeared to be due to the light treatment. Exposing the tissue to 4 hr of light prior to INH-treatment reduced the inhibition of ALA synthesis from 53% to 13-14% (treatments 3, 4) - an effect observed in previous

Table 6.--Effect of pre-treatment and treatment with nicotinamide or pyridoxine on the inhibition by INH of ALA formation in low light.

A.	Pre-treatment ← 4hr →	Treatment in 30 mM LEV ← 1st 4hr →			← 2nd 4hr →			Inhibition of ALA Formation
	NAmD	NAmD	INH	Light	NAmd	INH	Light	
1.	-	-	-	+	-	-	+	0 + 5
2.	-	-	+	+	-	-	+	52.8 ± 8
3.	+	+	-	+	+	+	+	14.1 ± 11
4.	-	-	-	+	+	+	+	13.3 ± 2
5.	+	+	+	+	+	-	+	45.9 ± 6
6.	+	+	-	-	+	+	+	42.9 ± 10
7.	-	-	-	-	+	+	+	35.2 ± 19
B.	PXN	PXN	INH	Light	PXN	INH	Light	
1.	-	-	-	+	-	-	+	0 ± 12
2.	-	-	+	+	+	-	+	48.6 ± 7
3.	+	+	-	+	+	+	+	15.7 ± 3
4.	+	+	+	+	+	-	+	25.4 ± 5
5.	+	+	-	-	+	+	+	32.9 ± 2

Seedlings were pre-incubated with either water or 50 mM NAmD (6A) or 50 mM pyridoxine (PXN) (6B) in the dark. The seedlings were then transferred to solutions containing 30 mM LEV ± 50 mM NAmD (6A) or 50 mM PXN (6B) ± 20 mM INH. As in earlier experiment, the 8 hr treatment period was divided into two 4 hr periods, so that treatments could be varied. The control levels of ALA were in nmol/g-fresh wt.: 6A, 610 ± 4, and 6B, 762 ± 7, respectively.

experiments with tetrapyrrole synthesis. If, however, the shoots were first treated with nicotinamide in the dark followed then by INH-treatment in the light (treatment 6), the full inhibitory effects were expressed. No difference between pre-treatment with nicotinamide and with water was observed (cf treatments 5 and 2); in these cases the inhibition of ALA synthesis was 46 and 53%, respectively.

This suggests that INH does not appear to act on ALA synthesis in the same way it acts on tetrapyrrole synthesis. These results also infer that INH may have an additional mode of action in its interference with ALA synthesis - other than, for example, on pyridine nucleotides.

Since INH is an antagonist of pyridoxal phosphate, we attempted to see if pre-treatments with precursors of pyridoxal phosphate such as pyridoxine and pyridoxal might reverse the inhibition of ALA synthesis by INH. Preliminary results suggest that 50 mM pyridoxine might reverse as much as one-half of the total inhibition caused by INH, from 49% to 25% (cf treatments 2 and 4, Table 6B). Again, exposure to light prior to INH treatment "protected" the synthesis of ALA from the inhibitory effects of INH (treatment 3). Pyridoxal proved to be quite toxic to the seedlings, causing severe wilt. In conclusion, it appears that the inhibitory effects of INH on ALA synthesis are different from those on tetrapyrrole synthesis. Light and perhaps pyridoxine have a sparing effect on the inhibition by INH of ALA formation whereas nicotinamide does not.

B. Comparative Effects of Other Metabolic Inhibitors on δ-Aminolevulinic Acid and Tetrapyrrole Synthesis

1. Analogs of INH

In an attempt to understand the mode of action of INH, several structural analogs of INH as well as other substituted pyridines were examined for their relative efficacy as inhibitors of ALA and CHL synthesis. From the results, we could divide the compounds into four categories based on the degree to which they inhibited ALA synthesis in low light (Table 7). The most efficacious inhibitors were shown to be picolinic acid, quinolinic acid, isonicotinic acid (INA), and nicotinic acid with inhibitions ranging between 85-96%. The more moderately efficacious compounds included pyridine-2-aldoximine, INH, and 3-acetyl pyridine, exhibiting inhibitions of 48-58%. Iproniazide and pyridine-4-carboxyaldehyde gave weak inhibitions (24-25%), whereas nicotinamide and pyridoxine·HCl were not inhibitory. A carboxylic acid substitution of the pyridine ring seemed to bestow the highest inhibitory activity to a compound. Substitution of the carboxyl groups on the pyridine ring drastically reduced the efficacy of the compound (cf INA:INH, nicotinic acid:nicotinamide). The nicotinic acid series also displayed a strong inhibition of CHL synthesis; in fact INA appeared to be more efficacious than INH, at least at 100 mM. On closer examination of INA's effect on CHL synthesis over several concentrations, we saw that INA was not as potent on inhibitor as INH (Figure 14).

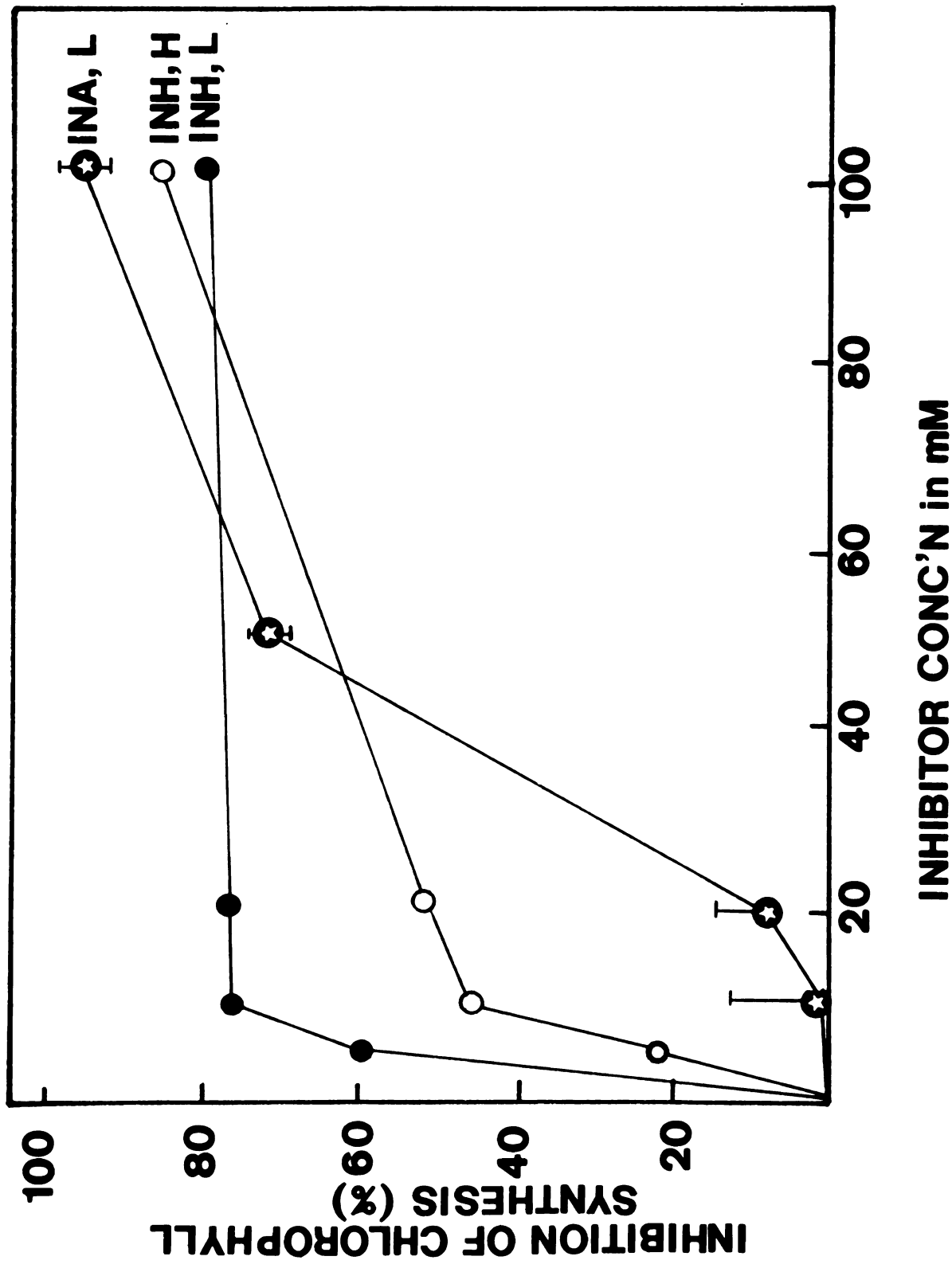
Table 7.--Effect of analogs of INH on ALA and chlorophyll biosynthesis.

Inhibitor (100 mM)	Pyridine ring substitution	% Inhibition of Synthesis	
		ALA	CHL
Picolinic acid	2= COOH	96.4 ± 0	-
Quinolinic acid	2,3= COOH	87.7 ± 5	-
Isonicotinic acid	4= COOH	86.9 ± 2	95.1 ± 2
Nicotinic acid	3= COOH	84.7 ± 1	59.6 ± 10
Pyridine-2-aldoximine	2= CH=NOH	57.7 ± 4	-
Isonicotinic acid hydrazide	4= $CONHNH_2$	49.7 ± 2	78.2 ± 2
3-Acetylpyridine	3= $COCH_3$	48.4 ± 5	-
Iproniazid phosphate (Isonicotinic acid 2-isopropyl hydrazide PO_4)	4= $CONHNH(CHCH_3)CH_2OPO_3^-$	24.7 ± 4	-
Pyridine-4-aldehyde	4= CHO	24.3 ± 3	-
Nicotinamide	3= $CONH_2$	-1.8 ± 7	16.8 ± 7
Pyridoxine	3,4= CH_2OH; 5,6= CH_3	-3.2 ± 0	-

For ALA determinations, seedlings were incubated in 30 mM LEV, for chlorophyll determinations in water, plus an inhibitor for 8 hr in light, 3,000 ergs/cm^2/sec. The control levels of synthesis for ALA and CHL are in nmol/g-fresh wt.: 850 ± 10 and 64.9 ± 9, respectively.

Figure 14. Effect of isonicotinic acid (INA) on chlorophyll synthesis.

Seedlings were incubated for 8 hr in varying concentrations of INA (0-100 mM) under 3,000 ergs/cm^2/sec of light. The inhibitions of chlorophyll synthesis in INH (low and high) light were replotted from figure 6 for comparison.


INA, L
INH, H
INH, L
100
80
60
40
20
INHIBITION OF CHLOROPHYLL SYNTHESIS (%)
20
40
60
80
100
INHIBITOR CONC'N in mM

In fact, at a concentration of 20 mM - where INH inhibited 78% - INA inhibition was only 6%. The nature of the dose response of INH resembled more closely that of INH in high light. The attenuated potency of INA may be due to its relative impermeability as compared to INH, since it is a more polar molecule than INH.

2. Additional Inhibitors

In order to determine if a certain degree of similarity exists between the modes of action of various inhibitors, we must first examine the extent of their inhibitions on a wide range of biosyntheses, in this case, on the biosyntheses of intermediates of the chlorophyll pathway.

Several inhibitors used in previous studies were screened to determine their potential to inhibit ALA-stimulated tetrapyrrole synthesis in a manner most similar to INH (Table 8). Ethonine - an antagonist of methionine and presumed inhibitor of SAM-dependent formation of Mg-PROTO ME (24) - had an inhibitory profile most nearly akin to that of INH. Protochlorophyllide synthesis proved to be ~2-times more sensitive to ethionine than were either PROTO or CHL. (In addition, ethionine inhibited ALA synthesis in a manner similar to INH, $K_{i,\ app}$ = 20 mM). Structural analogs of INH showed little similarity to INH in their manner of action. Isonicotinic acid inhibited all tetrapyrroles examined uniformly by 54-67%, whereas pyridine-4-carboxyaldehyde displayed little effect on the synthesis of tetrapyrroles. Cobalt

Table 8.--Effect of various inhibitors on ALA-stimulated tetrapyrrole synthesis: possible modes of action similar to INH.

Inhibitor (in 20 mM ALA)	Tetrapyrrole Synthesis as % of Control		
	PROTO	PCHLD	CHL(D)*
50 mM isonicotinic acid	58.2 ± 1	54.5 ± 1	66.7 ± 18
20 mM cobalt chloride	46.4 ± 4	21.0 ± 1	7.4 ± 18
50 mM ethionine	70.5 ± 2	39.5 ± 2	100
50 mM pyridine-4-aldehyde	84.1 ± 4	92.9 ± 0	100

* CHL(D) the photoconverted $PCHLD_{650}$ measured as chlorophyll Seedlings were incubated in a solution of 20 mM ALA plus an inhibitor for 21.5 hr in the dark. The accumulating PROTO, PCHLD, and CHL(D) (formed from the photoconversion of $PCHLD_{650}$ to CHL(D) under the low levels of illumination encountered during the assay procedure) were measured. The control values for PROTO, PCHLD, and CHL(D) were in nmol/g-fresh wt.: 116.9 ± 5, 170 ± 6, and 14.0 ± 1, respectively.

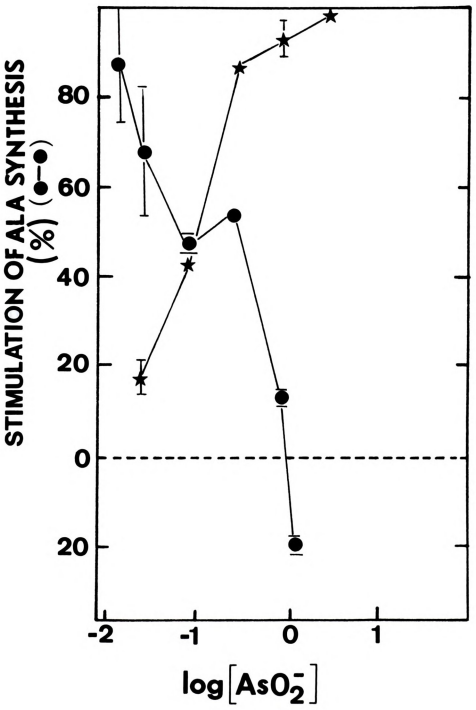
exhibited a differential inhibition of PROTO and the Mg-tetrapyrroles. Chlorophyll, was, by far, the most affected (92%), followed by PCHLD (79%), whereas PROTO was only moderately inhibited (54%). These results were consistent with a site of action for Co^{2+} at the conversion of PROTO to Mg-PROTO In conclusion, ethionine appears to act similarly to INH on the synthesis of tetrapyrroles.

3. Arsenite: Effect on ALA and Chlorophyll Formation

To clarify the site of inhibition of INH on ALA formation, whether directly on the synthesis of ALA or else on the synthesis of a precursor of ALA, we examined the effects of arsenite (AsO_2^-) on the accumulation of ALA. Arsenite is known to inhibit lipoate-requiring enzymes, and more specifically the TCA cycle by blocking the oxidation of α-ketoglutarate (39). By using AsO_2^-, we hoped to regulate the flow of precursors to ALA. When ALA synthesis was estimated in the presence of AsO_2^- at concentrations between 25 μM and 1 mM, a sharp stimulation in ALA formation was observed with decreasing concentrations of AsO_2^-; a maximal stimulation of 87% was obtained at 25 μM AsO_2^- (Figure 15). As the concentration of AsO_2^- was increased, the enhancement of ALA formation decreased; at 1.4 mM no stimulation was achieved and at 2 mM a 19% inhibition of ALA synthesis was observed. Arsenite effectively inhibited, on the other hand, CHL synthesis; at 500 μM AsO_2^-, CHL was inhibited by 85% whereas ALA was stimulated by 52%. The apparent K_i for the CHL inhibition was 100 μM. INH

Figure 15. Effect of arsenite on ALA and chlorophyll synthesis.

Seedlings were incubated for 8 hr under 3,000 ergs/cm^2/sec of light in 30 mM LEV plus various concentrations of $NaAsO_2$ (25 μM- 2 mM) for ALA determinations or water plus various concentrations of $NaAsO_2$ (50 μM- 5 mM) for CHL determinations. The control values of the synthesis of ALA and CHL in nmol/g-fresh wt.: 423 ± 14 and 81.2 ± 2, respectively.


STIMULATION OF ALA SYNTHESIS (%) (●—●)
INHIBITION OF CHLOROPHYLL SYNTHESIS(%) (★—★)
80
60
40
20
0
20
-2
-1
0
1
log[AsO2-]

completely attenuated the AsO_2^--induced stimulation of ALA synthesis (Table 9). This enhancement of 82% by AsO_2^- was eliminated with the addition of INH; in fact total ALA synthesis was inhibited by 25% in the presence of both INH and AsO_2^- compared to 50% in the presence of just INH. The fact, however, that AsO_2^--treatment was able to overcome one-half of the inhibition caused by INH suggested that the site of action on ALA by AsO_2^- and INH may be closely related.

Arsenite was also used to see whether or not it would inhibit ALA-stimulated tetrapyrrole synthesis in the dark. After a 20 hr incubation period, 25 μM AsO_2^- caused only a slight inhibition of PROTO and PCHLD, to the extent of 20-21% (Table 10). Arsenite also inhibited respiration by 25-30% as measured by the evolution of $^{14}CO_2$ from [1-^{14}C] glycine and [3,4-^{14}C] glutamate. These results suggested that AsO_2^-'s effect on tetrapyrrole synthesis might simply be due to the inhibition of respiration.

C. Photorespiratory Inhibitors: Their Effects on ALA and Chlorophyll Formation

Since INH is a known inhibitor of photorespiration (59), other inhibitors of photorespiration were tested for their ability to act on ALA and CHL synthesis in a manner similar to that of INH. If ALA synthesis was measured in tissue treated with the photorespiratory inhibitors α-hydroxy-2-pyridine-methanesulfonic acid (α-HPMS) and glycine hydoxamate,

Table 9.--Effect of INH on arsenite-stimulated ALA formation.

Treatment in 30 mM LEV	Percent of Control (ALA Synthesis)
none	100.0 ± 8
25 μM AsO_2^-	182.3 ± 17
25 μM AsO_2^- + 20 mM INH	74.9 ± 11
20 mM INH	49.7 ± 2

Seedlings were incubated in 30 mM LEV ± 25 μM AsO_2^- ± 20 mM INH for 8 hr in 3,000 ergs/cm^2/sec of light. The control levels of ALA were 348 nmol/g-fresh wt. ± 29.

Table 10.--Effect of arsensite on ALA-stimulated tetrapyrrole formation in the dark.

Treatment	Percent of Control (Tetrapyrrole Accumulation)	
	PROTO	PCHLD
10 mM ALA	100 ± 4	100 ± 7
10 mM ALA + 25 uM AsO_2^-	79.6± 1	79.0± 7

Seedlings were incubated in 10 mM ALA ± 25 μM AsO_2^- for 20 hr in the dark. Control levels of PROTO and PCHLD were in nmol/g-fresh wt.: 29.0 ± 1 and 123.4 ± 5, respectively.

a marked stimulation in ALA formation was noted at concentrations of 10 mM, both α-HPMS and glycine hydroxamate gave enhancements of ALA formation of 110 and 52%, respectively (Table 11). Additions of intermediates of the photorespiratory pathway generated slight stimulations in ALA accumulation; glycine, serine, or glycolate (20 mM) augmented the synthesis of ALA by a mere 10-26%. This enhancement was most likely due to a generalized "carbon-seeding" of metabolic pathways. In addition, none of the photorespiratory inhibitors affected CHL synthesis at concentrations where ALA was stimulated. Evidently, α-HPMS and glycine hydroxamate did not block the flow of ALA-equivalents to CHL and consequently stimulate the formation of ALA by blocking chlorophyll synthesis. It was clear that the photorespiratory inhibitors tested possessed little similarity to INH in mode of action. To determine how INH, as a photorespiratory inhibitor, might affect ALA synthesis, we examined the effects of INH on the respiratory $^{14}CO_2$ released from labelled precursors of ALA: [1-^{14}C] glycine and [3,4-^{14}C] glutamate (Table 12). It was apparent that the C-1 of glycine was respired to a much greater extent than C-3 and C-4 of glutamate. These results were consistent with what we might expect from the photorespiratory glycolate pathway, since the C-1 of glycine is lost in its conversion to serine (cf 59). INH dramatically inhibited the evolution of $^{14}CO_2$ from glycine by 83% and hardly inhibited the respiration of glutamate by only 18%. These results confirm INH as

Table 11.--Effect of various inhibitors and intermediates of the photorespiratory glycolate pathway on the formation of ALA and chlorophyll.

Treatments	Percent of Control	
	ALA	CHL
none	100 ± 14	100 ± 4
10 mM glycine hydroxamate	210 ± 7	101.3 ± 18
20 mM glycine hydroxamate	191 ± 2	104.1 ± 8
10 mM glycine	113 ± 11	-
20 mM glycine	126 ± 1	-
10 mM α-HPMS	152 ± 2	102.4 ± 5
20 mM α-HPMS	135 ± 4	-
10 mM α-HPMS + 20 mM glycine	-	108.0 ± 2
20 mM glycolate	120 ± 9	-
20 mM serine	110 ± 2	-

Various photorespiratory inhibitors and intermediates were added to 30 mM levulinate for experiments involving ALA determinations or to H_2O for experiments involving chlorophyll determinations. The incubation time was 8 hr under 3,000 ergs/cm^2/sec of light. (α-HPMS = α-hydroxy-2-pyridine-methanesulfonic acid)

Table 12.--Effect of INH on $^{14}CO_2$-evolution from barley seedlings fed labelled amino acids.

Labelled amino acid	20 mM INH	Respired $^{14}CO_2$(cpm/gm-FW)	% Inhibition
1-^{14}C-glycine	-	14,080 ± 1753	0 ± 12
1-^{14}C-glycine	+	2,435 ± 190	82.7 ± 1
3,4-^{14}C-glutamate	-	3,202 ± 638	0 ± 20
3,4-^{14}C-glutamate	+	2,628 ± 271	17.9 ± 8

an inhibitor of photorespiration. The photorespiratory inhibitors which had stimulated ALA synthesis were also examined for their effect on the respiration of [1-^{14}C] glycine (Table 13). At 20 mM, α-HPMS inhibited this respiration by 34%, whereas 10 mM glycine hydroxamate effectively inhibited the respiration by 64%. Since both INH and glycine hydroxamate inhibited the evolution of $^{14}CO_2$ to similar extents, it suggested that they might act on the same site of the photorespiratory pathway (59).

D. The Effect of INH on the Synthesis of δ-Aminolevulinic in Anabaena variabilis

The blue-green alga, Anabaena variabilis Kütz, appears to synthesize ALA via the C-5 pathway (unpublished results, Avissar). Anabaena contains, therefore, a biosynthetic system for ALA formation similar to that of higher plant chloroplasts (103). Since INH has been shown to inhibit the in vivo synthesis of ALA in barley, we attempted to determine whether or not INH would inhibit the synthesis of ALA from ^{14}C-labelled precursors in Anabaena. Isonicotinic acid hydrazide was not only ineffectual in inhibiting the synthesis of ALA from [^{14}C(U)] glutamate, but appeared on the other hand to stimulate the incorporation of the labelled amino acid into ALA as determined by the method of Beale et al. (6) (Table 14). In the presence of 40 mM INH, the incorporation of labelled glutamate into ALA was stimulated by 30%. It was also apparent

Table 13.--Effect of AsO_2^-, α-HPMS, and glycine hydroxamate on $^{14}CO_2$-evolution.

Treatment	Respired $^{14}CO_2$(cpm/gm-FW)	% Inhibition
1-^{14}C-glycine	26,073	0
1-^{14}C-glycine		
+ 25 μM AsO_2^-	18,090	30.6
+ 20 mM α-HPMS	17,078	34.5
+ 10 mM glycine hydroxamate	9,300	64.3
3,4-^{14}C-glutamate	10,226	0
+ 25 μM AsO_2^-	7,632	25.4

Two g of seedlings were illuminated at a light intensity of 3,000 ergs/cm^2/sec for 4 hr in beakers containing water. At the end of 4 hr, the seedlings were removed and the greenest 3 cm section of the seedling was excised and placed into 2 ml solutions containing .5 μCi/ml of ^{14}C-glycine or 3,4-^{14}C-glutamate ± inhibitor. The beakers and seedlings were placed into jars fitted with lids from which discs wetted with .1 M NaOH were suspended. The jars were returned to the light for 4 additional hours of illumination. The $^{14}CO_2$ trapped on the discs were then measured by liquid scintillation counting.

Table 14.--Effect of INH on the biosynthesis of ALA from ^{14}C-precursors in Anabaena variabilis.

Labelled precursor added	50 mM LEV	40 mM INH	$^{14}CO_2$ respired (cpm)	Total cpm in ALA-pyrrole	*SA of ALA-pyrrole in cpm/nmol
$^{14}C(U)$-glutamate	-	-	5552	779	$\sim 0^+$
+	+	-	7565 ± 87	109,433 ± 8733	15,390 ± 1237
+	-	+	3824	17,982	$\sim 0^+$
+	+	+	4766 ± 39	142,154 ± 1988	21,437 ± 217
1-^{14}C-glycine	-	-	17,421	978	$\sim 0^+$
+	+	-	25,990	1494	71
2-^{14}C-glycine	-	-	2987	1173	$\sim 0^+$
+	+	-	3893	1283	22
+	-	+	1036	1277	$\sim 0^+$
+	+	+	3308 ± 1182	1314 ± 47	22 ± 7
1,4-^{14}C-succinate	-	-	4971	122	$\sim 0^+$
+	+	-	4992	296	34
$^{14}C(U)\alpha$-ketoglutarate	-	-	3665	398	$\sim 0^+$
+	+	-	2529 ± 71	17,354 ± 958	2174 ± 123
+	+	+	3159 ± 25	2324	326 ± 20

$\sim 0^+$: since the level of chemically detectable ALA was below the sensitivity of the assay, the *SA is expressed as ~ 0.

A 1 ml cell suspension of Anabaena was incubated in a Warburg flask in a solution (total volume=2 ml) containing 50 mM LEV, 10 mM ATP, 15 µM cold precursor, 5 µCi/ml of labelled precursor ± 40 mM INH for 3 hr under illumination. After 3 hr, the reaction was stopped with 3N PCA. The folded filter paper wetted with NaOH was removed from the center well of the Warburg and placed in a liquid scintillation vial. The respired $^{14}CO_2$ was determined radiospectrometrically. The ^{14}C-ALA was purified as the ALA-pyrrole, quantified, and the radioactivity measured as before.

that, of all precursors tested, glutamate was preferentially converted to ALA - at least 6-times better than [^{14}C(U)] α-ketoglutarate- the second best precursor, confirming the presence of the C-5 pathway for ALA synthesis in Anabaena. Neither [1- or 2-^{14}C] glycine nor [1,4-^{14}C] succinate were incorporated to any significant extents into ALA. Anabaena, therefore, did not appear to possess ALA synthase activity, suggesting that ALA was formed exclusively via the C-5 pathway from C-5 precursors. Although INH did not inhibit label incorporation from glutamate into ALA, it did have a dramatic effect on the incorporation of label from α-ketoglutarate into ALA. The hydrazide inhibited the conversion of α-KG to ALA by 89%. In addition, INH stimulated the respiratory release of $^{14}CO_2$ from [^{14}C(U)] α-ketoglutarate by 25% whereas it had the opposite effect on the release of $^{14}CO_2$ from [^{14}C(U)] glutamate, inhibiting the evolution by 37%. These results are not only consistent with a site of inhibitory action for INH between α-KG and glutamate, but also suggest that INH may act on ALA synthesis as an antagonist of the pyridoxal phosphate-dependent transamination of α-KG to glutamate.

DISCUSSION

The effects of INH on the biosynthetic pathway of chlorophyll in greening barley shoots were examined. This study was undertaken to investigate the biosynthesis of ALA in higher plants. Isonicotinic acid hydrazide, a metabolic inhibitor reportedly an antagonist of pyridoxal phosphate (114), was intended to serve as a chemical probe for the presence of the pyridoxal phosphate requiring ALAS pathway for ALA formation in barley (cf 14). The existence of the C-5 pathway for ALA synthesis is well documented in a variety of greening organisms (5). The presence of the ALAS pathway in higher plants has also been reported, although less well documented than the C-5 pathway (85). The hydrazide of isonicotinic acid was chosen as a potential inhibitor of ALA formation primarily due to its antagonism of pyridoxal phosphate-dependent enzymes and, therefore, of ALAS (65, 14). However, during the course of these investigations, it became clear that INH had several sites of action in the biosynthetic pathway leading to chlorophyll, some of which could not be related to an antagonism of pyridoxal phosphate-dependent enzymes.

In order to eliminate any possible confusion concerning these multiple effects of INH, I will organize this discussion along the following lines: I. the effects of INH on tetrapyrrole

biosynthesis, II. the reversal of the INH-inhibition of tetrapyrrole biosynthesis by light, ALA, and nicotinamide, III. INH: mechanism of action against the biosynthesis of tetrapyrroles and ALA, and IV. additional modes of INH action.

I. The Effects of INH on Tetrapyrrole Biosynthesis in Greening Barley Shoots

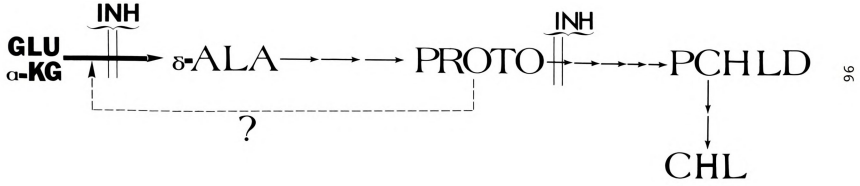
INH appeared to exert at least two inhibitory effects on the greening process in barley: one on the synthesis of ALA, and another on the synthesis of chlorophyll. This was demonstrated by the different extents to which INH inhibited the syntheses of ALA and chlorophyll. The apparent inhibition constants for these inhibitions were 10 and 3 mM, respectively. The greater sensitivity of CHL to the inhibitor suggests that INH may act not only on the synthesis of ALA but also on a step in CHL biosynthesis subsequent to the formation of ALA. Had CHL synthesis been inhibited to the same extent as that of ALA, we could have ascribed both inhibitions to the action of INH on ALA synthesis. INH also appears to photosensitize tissue which has been fed ALA under low light - an effect not observed in tissue incubated in water or ALA alone. These observations suggested to us a site of action for INH on tetrapyrrole synthesis rather than on ALA formation alone. The accumulation of pigmented intermediates of the biosynthetic pathway leading to CHL are known to photosensitize plant tissue (11, 113). When barley was incubated in the dark in

ALA, to by-pass the effect of INH on ALA synthesis and, to examine the effect of INH on just that portion of the pathway from ALA to PCHLD, we saw a stimulation in the accumulation of PROTO and an inhibition of the accumulation of PCHLD (Figure 12). The stimulation of PROTO accumulation by INH was shown also to occur in the light and was consistent with the visual observation that the tissue appeared to have a slightly brownish color. The accumulated PROTO could account for the photosensitization of the INH-treated seedlings in low light (cf 113). It appears therefore that INH may act at a site between PROTO and PCHLD (Figure 16). If an inhibition is to occur between two points in the pathway of chlorophyll biosynthesis, then we should expect to see an accumulation of that tetrapyrrole just before the blockage by INH. This is what we see when PROTO accumulates in the presence of INH. However, we should also see a decrease in the accumulation of the tetrapyrrole subsequent to the blockage - which we do not see. The formation of protochlorophyllide does not appear to inhibited at those concentrations of INH which stimulate the accumulation of PROTO. This inconsistency is puzzling. If, however, the formation of PCHLD is saturated due to the excess availability of ALA, then an INH block subsequent to PROTO synthesis may not necessarily result in an inhibition of PCHLD accumulation. This implies that the kinetic limitations on the synthesis of PROTO and PCHLD may not be the same. The synthesis of PROTO, for example, is a stromal process whereas

Figure 16. Proposed sites of action of INH on the biosynthetic pathway leading to chlorophyll.

Site 1. ALA synthesis

Site 2. Between protoporphyrin and protochlorophyllide


INH
GLU
α-KG
δ-ALA
PROTO
INH
PCHLD
?
CHL

the synthesis of PCHLD is a membrane-associated process. Since there is a physical separation in the two syntheses, PCHLD formation may not necessarily be inhibitied, when INH, at low concentrations, causes a stimulation in PROTO accumulation. There may be ample PROTO passing along the pathway to PCHLD so that INH does not inhibit the accumulation of PCHLD. Unfortunately, very little is known about the actual pool sizes of PROTO and PCHLD and the kinetic limitations of their syntheses. A t higher concentrations of INH, PCHLD is inhibited 2-times more than PROTO, inferring that PCHLD synthesis is more susceptible to the inhibition by INH. Apparently, at higher INH concentrations, the hydrazide is most effective in inhibiting PCHLD accumulation. The question which then arises - is the mode of action of INH on tetrapyrrole synthesis the same for low and high concentrations of the hydrazide? First, we observe a stimulation in PROTO accmulation with low concentrations of INH but no inhibition of PCHLD formation. Second, with high hydrazide concentrations, both PCHLD and PROTO are inhibited. The inhibition of PCHLD is two times larger, however, than that of PROTO. Are these effects different in terms of site of action?

Ethionine, for example, is known to inhibit the SAM-dependent methylation of Mg PROTO (69), a step between the biosyntheses of PROTO and PCHLD. PCHLD formation proved to be 2-times more sensitive to ethionine than PROTO or CHL(D) but PROTO was indeed inhibited. This suggests a strong similarity in the

mode of action of ethionine and INH at high concentrations on tetrapyrrole formation (cf Demain, 24). We might also conclude that both effects of the hydrazide, whether at high or low concentration, are due to INH's inhibitory action at a site subsequent to the synthesis of PROTO.

We have also noted that both CHL and PROTO are equally sensitive to the inhibitory effects of INH at higher concentrations. At first this seems puzzling, since PCHLD is a precursor of CHL, and is inhibited to a 2-fold greater extent by INH than is CHL. If we consider the kinetic limitations of the conversion of PCHLD to CHL, however, this does not appear too unreasonable. Under low light, CHL synthesis is limited only by the formation of transformable PCHLD which in turn is dependent on the available binding sites on the holochrome. In the presence of exogenous ALA, free PCHLD accumulates in excess of the level needed for saturating the holochrome (38). In these PCHLD-saturating conditions, the rate of CHL synthesis is dependent of the rate of photoconversion of PCHLD 650 - bound to the holochrome - to CHL. An inhibition of the synthesis of this large, unbound pool of PCHLD 628 by INH will not necessarily mean that the accumulation of CHL will be inhibited to the same extent. Our data shows that only about one-half of the inhibition of PCHLD is manifested in an inhibition of CHL synthesis.

A site of INH action between PROTO and PCHLD was partly supported by using ^{14}C-ALA as a precursor of tetrapyrrole

formation. The hydrazide effected both an increase in the *SA of PROTO and a slight decrease in the *SA of PCHLD, suggesting that INH inhibits the formation of PCHLD from PROTO.

Somewhat unsettling, however, was the observation, that PROTO accumulation was not stimulated significantly under the conditions of this experiment. One explanation for these results is that we used a lower concentration of exogenous ALA (5 mM cf 10 mM) to increase the *SA of the external ALA solution. Lowering the amount of ALA available for tetrapyrrole synthesis, resulted in an enhanced sensitivity of PROTO and PCHLD to INH. We therefore saw no stimulation in PROTO accumulation. This may be essentially the same effect which we see at higher INH concentrations when PROTO formation is slightly inhibited. As explained before, it appears that these two apparently different effects may be on the same site in the pathway, however.

INH causes a large rise in the *SA of PROTO in the light. On closer examination of this facilitated labelling of PROTO using a pulse-chase experiment in the dark, we discovered that INH appears to stimulate the turnover of PROTO with little effect on PCHLD. This rapid turnover of PROTO may be indicative of its presumptive role in the regulation of the biosynthetic pathway of chlorophyll. There is evidence to suggest that PROTO acts as a feedback regulator of ALA formation (36, 113, 76, see Figure 2). In turning over a feedback inhibitor, such as PROTO, we can imagine a mechanism - present

in plants - for controlling the levels of PROTO which might accumulate (cf 36). In fact, PCHLD has been suggested to feedback regulate the conversion of PROTO to MgPROTO (112); a consequence thereof would be an accumulation of PROTO. If the levels of PROTO rise significantly, then this porphyrin may act to feedback inhibit the entire pathway by inhibiting ALA formation. This would reduce the overall flux through the pathway and thus prevent the wasteful and even detrimental accumulation of labile intermediates, such as PROTO, which cannot be converted to CHL in the dark and which can photosensitize tissue in the light. In fact, in the dark very low levels of ALA and PCHLD as well as barely detectable levels of PROTO are found (36).

Additional evidence for a feedback mechanism is obtained from the genetic analysis of mutants. The xan-f mutants of barley - blocked at the conversion of PROTO to MgPROTO - do not accumulate PROTO unless supplied with ALA in the light (36). This suggests that there is a mechanism which limits the levels of PROTO which may normally accumulate. Furthermore, the enzymes responsible for the rate-limiting step(s) of ALA formation are believed to undergo rapid turnover (30, 81). This implies that those steps essential in regulating the rate and flux of ALA-equivalents through the pathway to CHL are, themselves, subject to control. For example, PCHLD regulates its own synthesis and consequently the flux into the Mg-tetrapyrrole branch of the pathway (112). The effects of

INH on tetrapyrrole synthesis bear out a regulatory mechanism on the levels of tetrapyrroles which are allowed to accumulate. In the presence of INH, the conversion of PROTO to PCHLD maybe partially blocked, resulting in the accumulation of PROTO (Figure 13). In addition, the *SA of the PROTO doubles, suggesting an increased turnover of the PROTO pool or perhaps a decreased dilution of labelled ALA from endogenous synthesis. If the block were to result in only an expansion in the size of the PROTO pool, then the *SA of the PROTO should not be altered significantly since the *SA of the labelled precursors of PROTO should remain unaltered; this is not what we observe. If, on the other hand, the PROTO pool undergoes turnover, then an increase in the *SA should indicate an increased labelling of the pool by precursors with a higher *SA than PROTO as we have observed. It is not clear whether this INH-induced turnover, is a consequence of INH-stimulated metabolism of PROTO or whether the INH-induced accumulation of PROTO itself effects an enhanced turnover in much the same way PCHLD accumulation shuts itself off (cf 112). It is clear, however, that INH stimulates the normal rate at which PROTO is metabolized.

In the Chlamydomonas mutant br_s-1, which accumulates PROTO in the dark without added ALA, there appears to be a rapid photodestruction of the porphyrin upon being transferred to the light. Within 8 hr after illumination, 50% of the dark accumulated PROTO is lost. This suggests that in light there is a mechanism for reducing the levels of PROTO which could

accumulate. In experiments with ALA-fed tissue, kept in the dark for 4 hr followed by 4 hr of INH-treatment in the light (Table 4), we observe a doubling in the *SA of PROTO. With regards to our observations on PROTO turnover in the dark and the reported lability of accumulating PROTO in light, it seems certain that the change in *SA of the PROTO, observed in the light, is associated with an enhanced turnover of that porphyrin. It is interesting to note that Castelfranco and Jones (22) found a stimulated turnover of protoheme in rapidly greening barley. Since heme is formed from PROTO, it is conceivable that there is a connection between the rapid turnover of both these regulatory tetrapyrroles.

PROTO appears to play a crucial role in the regulation of the chlorophyll pathway, whether directly (113, 110, 35), or indirectly through its conversion to heme (25, 22). The br_c mutant of Chlamydomonas - bearing a mutation in one of the four structural genes involved in the conversion of PROTO to MgPROTO - accumulates PROTO which appears to inhibit the overall rate of CHL synthesis (113). This feedback inhibition is born out by barley mutants with blocked PCHLD synthesis; these structural gene mutants do not accumulate porphyrins in the absence of exogenous ALA. They do pile up large amounts of porphyrins and trace acmounts of PCHLD when their leaves are supplied with ALA. In one such mutant, xan-f^{10}, which appears to lack a protein necessary for the insertion of Mg^{2+} into PROTO IX, the induction of ALA synthesis does not occur upon

illumination, suggesting a feedback role for PROTO on the rate-limiting step as effective as that for PCHLD.

Since ALA formation is inhibited by INH in greening barley (Figure 6) (12) - although less severely than tetrapyrrole synthesis - it is conceivable that part of this observed inhibition may be attributable to feedback inhibition by PROTO. Several mutant strains of Chlamydomonas and varieties of barley also support such a regulatory mechanism. For example, the br_s-1 mutant of Chlamydomonas, which produces 75% of its tetrapyrroles as PROTO, possesses only 1.9% of the ALA synthetic capacity of wild type cells. In the double mutant br_s-1, r-1, where the r-1 allele makes ALA synthesis insensitive to PROTO, the levels of ALA synthesis are 112% of the r-1 control (41% of wild type). This strongly suggests that PROTO feedback regulates ALA synthesis in wild type cells. In addition, an analogous mutant of barley, xan-f^{10}, tig-o^{34}, which is blocked in the formation of MgPROTO and possesses deregulated ALA synthesis, constitutively accumulates porphyrins in the absence of ALA (110). Gough and Granick reported that the ALA-synthesizing enzyme is derepressed in these tigrina mutants of barley (unpublished results, cited in 110). This evidence clearly indicates a direct regulatory role for PROTO on the synthesis of ALA. If INH chemically blocks tetrapyrrole synthesis in a manner analogous to the genetic mutations which block MgPROTO synthesis, then why don't we observe PROTO accumulation in the presence of INH without exogenous

ALA? Gough showed that barley mutants blocked at various steps in the pathway prior to PCHLD will not accumulate detectable amounts of porphyrin intermediates unless fed ALA or are combined with regulatory mutants (36). It seems likely that INH, which appears to block between PROTO and PCHLD, will not cause a PROTO accretion without added ALA due to the strict regulation of ALA synthesis by PROTO. If an intermediate such as PROTO accumulates beyond a certain threshold, it inhibits its own synthesis simply by inhibiting the synthesis of ALA. In INH-treated barley leaves under conditions of PROTO accumulation, ALA synthesis is inhibited by only 50% at most. This limited - as opposed to complete - reduction in ALA synthesis may imply that: 1) INH causes an incomplete block between PROTO and PCHLD, 2) INH acts on ALA synthesis other than or in addition to PROTO feedback inhibition (see section IIB), 3) the levulinate technique (used in measuring ALA) deregulates ALA synthesis, masking the true extent of any possible INH-induced feedback of ALA synthesis. All three alternatives are valid possibilities; however, since the interactions of 1) and 2) are pertinent to another discussion, we will limit ourselves to alternative 3). We have observed that the levulinate (LEV) method (7) for ALA measurement results in an apparent deregulation of ALA formation in barley seedlings; this effect was also noted in maize leaves (78). The assay is based on ALA formation after treatment with LEV, a competitive inhibitor of ALA dehydrase. LEV blocks the conversion of ALA to

porphobilinogen (see Figure 2), resulting in the accumulation of ALA which we can chemically assay. Tissue treated with 30 mM levulinate, a concentration maximizing ALA accumulation, show an inhibition of CHL synthesis of 25-30%. If the ALA, detected by assay, is compared to the ALA-equivalents calculated from the amount of CHL inhibited by LEV-treatment, we find an overproduction of ALA of 30-40%. This suggests that LEV-treatment brings about a partial deregulation of ALA synthesis, perhaps by reducing the levels of PROTO or PCHLD which act to "restrain" the flow of ALA down the pathway to CHL. With increasing dose, INH inhibits linearly this overproduction phenomenon (data not shown). INH (20 mM) effectively eliminates this excess ALA accumulation - the same concentration which gives maximal inhibition of both ALA and CHL formation, and causes maximal stimulation of PROTO accumulation. It is relevant to note that the apparent K_i's for both PROTO accumulation and ALA inhibition are 10 mM. In overview, it is not certain, however, whether this effect can be attributed to a direct inhibition of the synthesis of ALA by PROTO accumulation, but it remains a distinct possibility.

It was rather surprising to discover that PCHLD does not appear to undergo any significant turnover, considering how rapidly PROTO is turned over. Th pulse-labelling experiment (Table 3) showed that the *SA of PCHLD decreased to a level which was predictable by calculation. This relatively stable $PCHLD_{628}$ pool contrasts to the ca. 4 hr half-life calculated for $PCHLD_{650}$ reported in 8-day old barley (46). PCHLD is also

suggested to be a regulator of CHL biosynthesis (110) (see Figure 2). Unexpectedly, even at PCHLD levels well above those which occur naturally (cf 136 nmol/gm-FW and 7nmol/gm-FW), no appreciable turnover could be detected (cf 30). Mattheis and Rebeiz (77) report in their cucumber system that no detectable photodestruction of PCHLD occurs. It seems that the feedback mechanisms controlling ALA and MgPROTO formation are sufficient to control the level of PCHLD which would normally accumulate in a plant. Conceivably, turnover of PCHLD is less likely to occur due to its prominent association with the newly developing membranes of the etiochloroplast (cf 102, 42). In contrast, PROTO appears to be synthesized in the stroma (102). In addition, light plays an important role in controlling the levels of PCHLD by photoconversion to CHLD. This photocontrol as well as the feedback mechanisms stemming from PCHLD may be sufficient to regulate PCHLD accumulation. INH has a slight effect on the *SA of PCHLD in the dark, producing a decrease in *SA of 20% after 11½ hr. This drop in *SA is probably attributable to INH blocking the formation of PCHLD and can be explained by a decrease in flow from the more radioactivity labelled precursors of PCHLD. We have observed previously that INH has little effect on PROTO or PCHLD accumulation in the dark until after 8 hr (Figure 11). The decrease in *SA of PCHLD which we noted at 11½ hr (Table 3) - may then correspond to the "onset" of the inhibition by INH of PCHLD. The decrease is directionally opposite to the stimulation, we

observe, in the *SA of PROTO (+24%). Even INH does not stimulate the loss of label from PCHLD during the pulse-chase experiment. This is a clear indication that INH does not have any significant effect on the turnover of PCHLD or even on bringing about a detectable turnover of this unbound pool of $PCHLD_{628}$.

It was clear during this investigation that INH was not acting on tetrapyrrole synthesis through an inhibition of plastidic lamellae formation, although INH does have the potential to antagonize amino acid transaminases. An inhibition of membranes could have been expressed in a general inhibition of pigment synthesis and given the results we observed. There are several lines of evidence, however, opposing such an argument: 1) the inhibition of CHL synthesis by INH was reduced under high light. If INH had affected membrane formation, then membrane-associated CHL should have been rapidly photodestroyed with higher light intensities. 2) We should not have seen differential inhibitions of tetrapyrrole synthesis, if membranes were being affected. 3) The *SA of CHL remained constant regardless of whether or not INH was present. If membrane synthesis was inhibited, then the incorporation of CHL into the membranes should have also been affected, resulting in some fluctuation in the *SA of CHL. If CHL was undergoing turnover, then a notable decrease in the *SA of CHL should have been observed as a consequence of the significantly lower *SA of its precursor, PCHLD. 4) Addition

of casamino acids had no effect on reversing CHL inhibition. If INH was acting on transaminases and consequently on protein and membrane formation, then an ample supply of amino acids should have alleviated a portion of the inhibition and 5) carotenoid synthesis is unaffected by INH (Table 2), suggesting the etiochloroplast remains intact (cf 92).

II. The Reversal of the INH-inhibition of Tetrapyrrole Biosynthesis by Light, ALA, and Nicotinamide

A. Reversal by Light

Light appears to antagonize the inhibitory effects of INH. It was clear that under high light conditions the inhibitions of both ALA and CHL formation were significantly reduced compared to inhibitions observed under low light (Figure 6). The extent of INH-inhibition of ALA synthesis appeared to decrease logarithmically with increasing light intensity (Figure 7). This decrease in inhibition could not be explained by a photodestruction of the INH by higher light intensities (Figure 8). In addition, when ALA-fed shoots were illuminated prior to INH treatment there was a complete mitigation of the inhibitions and changes in *SA of the tetrapyrroles which we ascribe to the effect of INH (Table 5). If light and INH were supplied simultaneously, then the full inhibitory effects were expressed. If the ALA-fed tissue were placed in darkness, followed by INH in light or were in INH in dark, followed by light, the effects were the same: INH inhibited tetrapyrrole

synthesis and caused a significant rise in the *SA of both PROTO and PCHLD. In summary: exposure of seedlings to light prior to INH treatment prevented the manifestation of INH's effects (the mechanism for this action will be discussed in section IIIA).

The doubling of the PROTO *SA could indicate a rapid turnover, as was observed before in the dark, and correlates well with the protoheme turnover observed in greening barley (22). The change in the *SA of PCHLD could indicate an effect of INH between PCHLD and CHL. In addition, we should probably address the fact that a significant portion of the ALA available for tetrapyrrole synthesis - in these ALA-fed shoots - is derived from ALA synthesis. The decrease in PROTO *SA, seen when ALA-fed shoots exposed to 4 hr of light were placed into darkness for 4 hr, could conceivably indicate an increase in the proportion of PROTO derived from cold ALA. We have noted that the total levels of tetrapyrroles following dark incubation are significantly lower than following the corresponding light incubation (cf 81 nmol/gm and 259 nmol/gm), suggesting that ALA uptake may be lower in the dark. This is consistent with Sundqvist's findings that there is a transient permeability of etiochloroplasts to exogenous ALA depending on whether in the light or dark (105). A decreased uptake of labelled ALA, after the seedlings have been transferred to darkness, could be responsible for the observed dilution. However, in a light-dark transition we should observe a decrease in endogenous ALA, since the ALA synthesizing enzymes turnover

rather rapidly in the dark (30). The contradiction between this fact and our observation remains unexplained.

B. Reversal by ALA

The INH-inhibition of chlorophyll was chemically reversible only by ALA. Feeding potential precursors of ALA to seedlings did not reverse the INH-inhibition, neither did pyridoxine, a precursor of pyridoxal phosphate, nor nicotinamide, an antagonist of INH in mammalian tissues; nor did amino acid mixtures, products of transaminase action (Table 1). The reversal by ALA of the INH-inhibition of CHL synthesis was demonstrated to be concentration dependent (Figure 9). This ALA concentration effect saturates at ~5 mM ALA, with only 80% of the CHL inhibition being reversible. This suggests that a portion of the total inhibition of CHL formation - which we observe - is caused by an inhibition of ALA synthesis. This inhibition of ALA might be the consequence of: 1) either an inhibition of the synthesis of ALA itself or one of its precursors and/or 2) the feedback regulation of ALA formation via INH-induced PROTO accumulation (see Figure 16). The remaining 20% of the total inhibition of CHL synthesis may perhaps be due to an INH-block between PROTO and PCHLD. Consistent with this explanation is the observation that none of the precursors of ALA or other chemicals, which we used, would reverse the INH-inhibition of CHL. These compounds were simply unable to overcome the multiple inhibitory effects of INH, resulting from its several sites of action.

C. Reversal by Nicotinamide

If barley shoots were fed ALA to by-pass the effects of INH on ALA formation, we could attempt a chemical reversal of those particular effects of INH directed against sites in tetrapyrrole formation. The reversal by light of the INH-inhibition as well as various reports of INH acting antagonistically against the NAD pool (121, 122, 120, 88) prompted us to explore the possibility that INH acts on tetrapyrrole formation via the pyridine nucleotides.

In mammalian cells, INH is known to enzymatically exchange for the nicotinamide moiety of NAD (121, see Figure 3). Nicotinamide also antagonizes this exchange by NADase in certain mammalian tissues (122). In addition, NADase has been found in plants and is competitively inhibited by nicotinamide [K_i= 28 mM] (44). We believed that if INH was acting via the pyridine nucleotides on tetrapyrrole synthesis, and if this action was manifested through NADase, then pre-treatment of shoots with NAmD should, presumably, inhibit the NADase and prevent the INH effects. When barley shoots were pre-incubated in 50 mM nicotinamide and then fed ALA - with and without INH - we observed a significant decrease in the inhibitions by INH of all tetrapyrrole accumulation as well as a directional change, that is rise or drop, in their *SA's when compared to the INH controls (Table 5). Not only did the nicotinamide appear to reduce the INH-inhibition of tetrapyrrole formation, particularly PCHLD (from 82% to -3%), but also eliminated the large rise in the *SA attributed to an

INH-stimulated turnover of PROTO (from a ratio of 1.81 to .63). Moreover, a change in *SA, opposite in direction to that observed in INH controls, implies that nicotinamide mitigates the effects of INH. These results suggest that INH may act on tetrapyrrole synthesis via the pyridine nucleotide pool.

III. The Mechanisms of INH Action on the Biosynthesis of Tetrapyrroles and ALA: A Hypothesis

Our results, suggesting an INH involvement with pyridine nucleotides, is consistent with a mechanism of INH action described in other organisms (120). INH is clinically used as a tuberculostatic agent against Mycobacterium tuberculosis the bacterium responsible for tuberculosis. Since there is a direct correlation between INH's lethality and NAD content, the primary mode of action of INH against Mycobacterium appears to be on the pyridine nucleotide pool. INH not only inhibits the synthesis of NAD, but also stimulates the degradation of NAD, lowering the NAD content of the cell and affecting numerous metabolic processes of the organism. In the brain tissue of pigs, INH is readily exchanged with the nicotinamide moiety of NAD by NADase (55, 122). The NADase from lupines (44) and from certain mammalian tissue (51) is inhibited by nicotinamide; the lupine NADase has an apparent K_i = 28 mM. The inhibition of mammalian NADases by nicotinamide is uncompetitive whereas that of the lupine NADase is competitive (51). The NADase from pig brain promotes the formation of

the corresponding pyridine analog of NAD from both INH and 3 -acetylpyridine; these analogs in turn stimulate the activity of NADase. Although there is an apparent difference in the kinetic properties of the NADases from the tissues of lupine and pig brain, we can attempt a correlation of the effects of various pyridine analogs on the activity of NADase in pig brain with their effects on chlorophyll synthesis in barley. For example, nicotinamide, under specified experimental conditions, inhibits pig brain NADase by 50%. Under these same conditions, nicotinic acid inhibits the NADase by only 2%, whereas INH stimulates the activity of the enzyme by 3% (55). In barley, nicotinamide has little effect on CHL synthesis over a range of concentrations, inhibiting at best 17% (Table 7). Nicotinic acid, on the other hand, is a potent inhibitor of CHL synthesis, inhibiting by 60% compared to 78% for INH. It seems, therefore, that pyridine analogs, which inhibit NADase, have little effect on the synthesis of CHL, whereas those analogs which do not inhibit NADase (e.g., NA), or stimulate NADase (e.g., INH) are respectively more effective inhibitors of CHL synthesis. This lends support to the notion that INH may act through its effect on NADase. A stimulation in NADase could result in not only a net reduction in the pool of NAD present but also an increase in the amount of the pyridine nucleotide analog, INH-NAD. It also seems possible that the INH-NAD analog might be the "active" compound responsible for the inhibitory effects of INH on tetrapyrrole synthesis, which

we observe (cf 121). Pyridine analogs are known to inhibit dehydrogenases such as lactate dehydrogenase (114), for example. A direct consequence of decreased dehydrogenase activity as well as a decreased pool of physiologically active NAD may be an alteration of the redox potential of the cell (114). 3-Acetylpyridine, for example, which like INH, stimulates NADase in pig brain, very effectively inhibits glucose-6-phosphate dehydrogenase (K_i= 30 μM). The inhibitory effects of the analogs of NAD on dehydrogenases are most probably due to the stereospecific nature of these enzymes and their required coenzyme. The NAD-linked dehydrogenases require C-4 hydrogens of NAD to be in A or B configuration. An analog of NAD, substituted in C-4 of the pyridine moiety as in the case of INH, could be expected to interfere with the normal function of a NAD-linked dehydrogenase (114). We might therefore speculate on a mode of action for INH involving anabolic reactions requiring pyridine nucleotides.

A. Mechanism of INH Action on the Biosynthesis of Tetrapyrroles

INH action on tetrapyrrole biosynthesis via the pyridine nucleotide pool is a reasonable possibility. It is known, for example, in Rhodopseudomonas spheroides that bacteriochlorophyll synthesis is regulated by the ratio of reduced/oxidized pyridine nucleotide (101). Redox also appears to be important for ALA synthase activity. In addition, there may be several sites in the Mg-branch of the porphyrin pathway from PROTO to CHL in higher plants which appear to require pyridine

nucleotide (see Figure 17): 1) the conversion of MgPROTO ME to PCHLD appears to follow a series of β-type oxidation reactions (27, 28, 29) which by analogy to fatty acid oxidation involves NAD-linked dehydrogenase and therefore contains a potential site for INH(-NAD) action. If INH inhibits at a point between MgPROTO ME and PCHLD then in the presence of ALA and INH, we should expect however an accumulation of MgPROTO, MgPROTO ME, or some other intermediate, which has not yet been observed in any of our experiments. 2) The attachment of PCHLD to the holochrome requires NADPH (19) to form a PCHLD-holochrome-NADPH complex. Apparently, NADPH is necessary for the conversion of the photoinactive $PCHLD_{628}$ species - which accumulates in the presence of exogenous ALA - to the phototransformable $PCHLD_{650}$ species. This could be a major site of INH action, preventing the conversion of $PCHLD_{628}$ to $PCHLD_{650}$. This also tenders a possible explanation for PCHLD being more sensitive to INH than other tetrapyrroles. In fact, it has been suggested that a possible site for the regulation of CHL synthesis may be the $PCHLD_{628}/PCHLD_{650}$ conversion which is controlled by the NADPH:NADP ratio (42). 3) Pyridine nucleotide may also be involved in the phytylation of CHLD to CHL. The geranylgeraniol adduct of CHLD is reduced through a dihydro- and tetrahydro-intermediate to phytol, presumably by pyridine nucleotide (98). A site of action for INH beyond PCHLD formation is also suggested by the effects of INH on PCHLD and CHL in our [^{14}C]-ALA labelling experiments. Inhibitors of phytol synthesis are good inhibitors of CHL

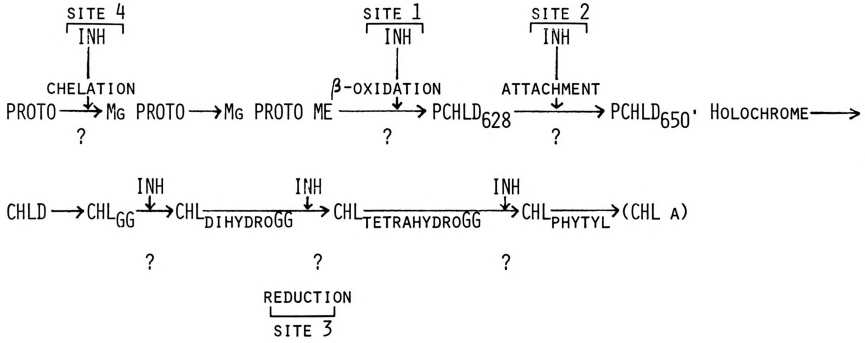
Figure 17. Potential sites for INH action on the biosynthetic pathway between protoporhyrin and chlorophyll.

Site 1. The β-oxidation of Mg PROTO ME to PCHLD

Site 2. The attachment of $PCHLD_{628}$ to the holochrome

Site 3. The stepwise reduction of CHL-geranylgeraniol to CHL-phytyl

Site 4. The Mg-chelation of PROTO


SITE 4
INH
CHELATION
PROTO → MG PROTO → MG PROTO ME → PCHLD628 → PCHLD650, HOLOCHROME →
?
SITE 1
INH
β-OXIDATION
?
SITE 2
INH
ATTACHMENT
?
CHLD → CHLGG → CHLDIHYDROGG → CHLTETRAHYDROGG → CHLPHYTYL (CHL A)
INH
INH
INH
? ? ?
REDUCTION
SITE 3

synthesis. SAN 6706, an inhibitor of carotenoid synthesis (92) and amitrol, which causes the accumulation of intermediates between CHLD and CHL, inhibit both phytol accretion and CHL synthesis (94). It is therefore, possible that INH could inhibit CHL synthesis by interfering in the stepwise reduction of geranylgeraniol to phytol. 4) Another possibility for INH action via the pyridine nucleotide pool, explaining the fact that we never see any tetrapyrrolic intermediates accumulate other than PROTO and PCHLD, is that INH inhibits MgPROTO formation from PROTO. Indeed, the addition of pyridine nucleotide to isolated chloroplasts stimulates the formation of MgPROTO from L-glutamate (116). Intermediates of the tricarboxylic acid cycle stimulate the formation of MgPROTO in Rhodopseudomonas (34) and in cucumber chloroplasts (Castelfranco, personal communication). This stimulation of MgPROTO synthesis by TCA intermediates appears to be an effect on pyridine nucleotides. If, indeed, pyridine nucleotide is required for the insertion of the Mg^{2+} ion into the tetrapyrrolic ring of PROTO, then an INH site of action at this step in the CHL pathway is conceivable.

In summary: there appear to be at least four sites in the Mg-branch of the tetrapyrrole pathway between PROTO and CHL formation, requiring pyridine nucleotide. These sites may be susceptible to INH, if the action of the hydrazide is via an antagonism of the pyridine nucleotide pool.

The INH analog of NAD may have an additional mode of action on metabolism other than on pyridine nucleotides. At an optimal

concentration of 500 μM, the INH-NAD analog catalyzes photophosphorylation in illuminated chloroplasts from spinach (15). At higher concentrations, the INH-NAD analog has been suggested to act an an uncoupler (15). Conceivably, the effects of INH an tetrapyrrole synthesis are manifested in an inhibition of ATP synthesis. There is an essential requirement for ATP not only in the synthesis of MgPROTO (21, 34) in the insertion of the Mg^{2+} but also in the photoconversion of PCHLD to CHLD (49). It is possible to envision a mechanism for INH action via an uncoupling of ATP synthesis which, in turn, affects the synthesis of these tetrapyrroles. In this regard, we may also see differential inhibitions of synthesis of ALA, PROTO, PCHLD, and CHL, if the K_m's for ATP of each of these synthetic steps are different. If the primary mechanism for INH action is through its effect on ATP synthesis, then the inhibitions by INH of the intermediates of CHL biosynthesis should be expressed as a general inhibition of all anabolic processes through a reduction in the available energy supply. We would, therefore, expect an inhibition of ATP synthesis to be much more detrimental than an interference of the pyridine nucleotide, since ATP is more crucial for metabolism.

A Possible Mechanism of Action for INH: The Interaction of the INH-NAD Analog and the Pyridine Nucleotide Pool and Its Relation to Light.--The antagonism of the INH-inhibition of tetrapyrrole synthesis by light might be explained in terms of an alteration of the concentration of the INH-NAD analog in

barley tissue. It is known, for example, that the 3-acetyl pyridine and INH analogs of INH can be photoreduced by chloroplasts (15). Only the oxidized, as opposed to reduced, form of the INH-NAD appears to be active in chloroplasts, catalyzing photophosphylation; the reduced form is inactive (15). If we could speculate that by altering the ratio of reduced to oxidized pyridine nucleotide in a shoot, then we might be able to reduce the inhibitory effects of INH, which we observe. NADase will only attack the oxidized forms of NAD(P), that is, catalyze the exchange between INH and oxidized NAD (120, 122). In the dark, only 25% of the total chloroplastic NADP(H_2) and 2-5% of the NAD(H_2) are in the reduced form (45). This is the ideal redox state for the full expression of INH's inhibitory effects. INH inhibits maximally under dark conditions which is precisely the effect we observe in our experiments with light/dark transitions (cf Table 4). In the dark, NADase may readily promote the formation of INH-NAD from the highly oxidized pyridine nucleotide pool. An increased concentration of INH-NAD may then in turn inhibit tetrapyrrole synthesis upon illumination. Admittedly, in light the INH-NAD analog should undergo reduction. It is not known, however, whether the reduced form of INH-NAD is as efficacious an inhibitor against dehydrogenases as its oxidized counterpart - this would seem likely - or whether the rate of analog reduction is slower than the rate of native NAD reduction. Both alternatives offer plausible explanations for our observations that there is no reduction in INH efficacy when INH and light

are given to seedlings simultaneously. If seedlings are illuminated prior to INH treatment, we see an attenuation of the inhibitory effects of INH. Upon exposure to light, up to 60% of total chloroplastic NAD(P)H_2 is reduced, this represents, however, a transient state. Within minutes after light exposure, the reduced state becomes oxidized (45). A reducing environment would represent a redox condition unfavorable for the expression of INH's inhibitory effects. Since NADase catalyzes the formation of INH-NAD only from NAD - not from NADH - there would be a significant drop in available substrate for the NADase. Presumably, a reduction in the concentration of INH-NAD bespeaks a reduction in the expressed inhibitory effects of INH on tetrapyrrole synthesis. However, this presumed reducing environment in a chloroplast lasts for only a few minutes and could not adequately explain why exposure to light prior to INH treatment will attenuate the effects of INH. We have also not determined the minimum duration of light exposure necessary for mitigating the inhibitory effects of the hydrazide. It would seem that a requirement for just a brief exposure (minutes) would support a hypothesis involving pyridine nucleotides; longer exposures (hours) might suggest another mechanism. Unfortunately, we do not know enough about the redox status of an illuminated chloroplast for long term (4 hrs in our experiments) to justify or disqualify a hypothesis involving light and a mechanism of INH action involving the pyridine nucleotide pool.

As noted earlier, nicotinamide elicits a strong inhibition of NADases (51, 122) and at 50 mM (Table 5) appears to reverse the inhibitory effects of INH on tetrapyrrole synthesis in barley shoots. When seedlings are pre-incubated in nicotinamide in the dark, to inactivate the NADase, and followed then by simultaneous exposure to light and INH treatment, we see a dramatic reduction in the effects of INH compared to those observed without nicotinamide treatment (Table 5). Although the redox status of the cells in the dark is oxidized, the presence of the nicotinamide presumably causes an inactivation of NADase (cf 114) and thereby prevents the conversion of NAD to its potent analog, INH-NAD upon the addition of INH in the light.

The seedlings require a period of nicotinamide feeding prior to INH treatment, before reduced inhibitory effects are seen. Nicotinamide has little effect in reversing the inhibition by INH if both chemicals are supplied at the same time. This suggested a slow uptake of NAmD and/or a lag in the inactivation of the NADase. In conclusion, we can speculate that INH may have its effect on tetrapyrrole synthesis via a perturbation of the pyridine nucleotide pool.

B. Mechanism of INH Action on the Biosynthesis of δ-Aminolevulinic Acid

ALA formation is inhibited by INH to a lesser degree (50%) than is CHL formation (78%) (Figure 6). Previously, we could attribute only a portion of this total inhibition of ALA

formation to a feedback inhibition of its synthesis (section I). It, therefore, seems likely that INH is inhibiting either the synthesis of one of ALA's precursors or the actual biosynthetic reaction(s) of ALA itself.

By using various structural derivatives of pyridine, we could demonstrate that the inhibitors can be divided into several classes based on the extent to which they inhibit ALA formation (Table 7). In order to determine whether or not the inhibition of ALA synthesis is via an effect on pyridine nucleotides - as that of chlorophyll may be - we attempted to show a relationship between the effects of these analogs on NADase (from pig brain) and the extent of their inhibition of ALA formation. 3-Acetylpyridine, pyridine-4-carboxyaldehyde, and INH stimulate NADase by 75, 24, 3% respectively, whereas nicotinic acid and nicotinamide inhibit by 2 and 50% (55); the corresponding percent-inhibitions of ALA formation for these analogs are: 48, 24, 50, 85, -3% (Table 7). There is consequently no obvious relationship between the effect of the analogs on NADase and their inhibition of ALA synthesis unlike the relationship we could draw in the case of chlorophyll synthesis. This suggests that the mode of action of INH on ALA synthesis may be different from the mode of action which we have proposed for INH on the formation of tetrapyrroles derived from ALA. In agreement is the observation that nicotinamide pre-treatment had little effect on the inhibition of ALA formation by INH (Table 6A). Since nicotinamide would

not reverse the INH effect, we may assume that the direct action of INH on ALA synthesis is not primarily due to an interference with the pyridine nucleotides. If it is not a NAD effect, then what is the mechanism of action of INH on ALA formation?

A pre-treatment of light will mitigate more than half of the total INH-inhibition of ALA synthesis (Table 6A). This light reversible portion of the total inhibition of ALA may be attributable to one of two possibilities: 1) the effect is the direct manifestation of INH-induced PROTO feedback inhibition, and light will reverse this effect, or 2) NAD is, indeed, involved in the INH action on ALA synthesis, consistent with light reversibility but less probable in view of our earlier arguments. In consideration of the second possibility, NADPH is required in the synthesis of ALA, regardless of which "variation" of the C-5 pathway we are discussing. In the glutamate-1-semialdehyde aminotransferase pathway (Figure 1), NADPH serves as the reductant for the dehydrogenase responsible for the synthesis of glutamate-1-semialdehyde (54). In the DOVA transaminase pathway, a NADPH-dependent dehydrogenase converts α-KG to DOVA (43, 73). Since pyridine nucleotide is involved in all presently proposed hypotheses for ALA formation, it seems possible that a portion of the INH-inhibiiton of ALA synthesis may be a reflection of some perturbation of the pyridine nucleotide pool. If, indeed, this is the case then the contradiction that nicotinamide does not reverse a portion of the observed inhibition is not fully

understood - unless there is an additional site for INH on pathway for ALA synthesis, which is not a NAD effect. INH may therefore have two sites of action on ALA synthesis and one of which is not nicotinamide reversible.

That portion of the inhibition of ALA which is not light-reversible may be due to an inhibition of pyridoxal phosphate. Preliminary results suggest that pre-treatment with pyridoxine - a presumptive precursor of pyridoxal phosphate - may facilitate a partial reversal of the INH-inhibition of ALA synthesis (Table 6B); pyridoxine appears to reverse ca. one-half of the total inhibition. By itself, this piece of data is merely suggestive, however, it is consistent with: 1) an inferred role for pyridoxal phosphate in the transamination of either DOVA or glutamate-1-semialdehyde to ALA, and 2) a clear requirements of pyridoxal phosphate for the synthesis of ALA in Euglena chloroplasts (96) and in cell-free preparations from maize (43). Additionally, Kanangara et al. (53) have shown that the biosynthesis of ALA from L-glutamate-U-^{14}C involves a transamination step. The transamination inhibitors aminooxyacetate and cycloserine effectively inhibit glutamate-1-semialdehyde aminotransferase in a soluble protein fraction from greening barley plastids (54). These results suggest that INH may act on ALA synthesis through an antagonism of not only pyridoxal phosphate but also of NAD.

We had hoped to show the effect of INH on the incorporation of [1-^{14}C] glutamate into ALA in order to clarify the

site of action of INH on ALA formation in barley. Although this procedure has been previously done in barley shoots (6), we had much difficulty in incorporating significant amounts of label into the ALA and unfortunately, the experiment could not be done.

It has recently been shown, however, that ALA is synthesized in the blue-green alga, Anabaena variabilis exclusively via the C-5 pathway (Avissar, unpublished results). In addition, it is a well accepted fact that blue-green algae are the progenitors of higher plant chloroplasts and have an identical carbon metabolism to that of chloroplasts (67); for example, both possess the Calvin cycle and pentose phosphate shunt (103). Therefore, due to these apparent metabolic similarities between chloroplasts and blue-green algae, and the algae's "propensity" for making ALA, we examined the effects of INH on the incorporation of various precursors of ALA into ALA in Anabaena. Much to our surprise, we found that INH (40 mM) stimulated by 30% the incorporation of the best precursor, [^{14}C(U)] glutamate, into ALA (Table 14). However, when we examined the effects of INH on the incorporation of [^{14}C(U)]α-ketoglutarate into ALA, we found that it inhibited the incorporation by 89%. These results infer a site of action for INH between α-KG and glutamate. The $^{14}CO_2$ evolution data support this hypothesis. The hydrazide stimulated the respiration of labelled α-KG and inhibited the respiration of labelled glutamate by 25 and 37%, respectively. It has been suggested that the synthesis of ALA involves the

transamination of α-KG to glutamate (54). The presence of such transaminases is known in both blue-green algae and higher plant chloroplasts (58): these enzymes require pyridoxal phosphate. An equally plausible alternative for the synthesis of glutamate from α-KG is glutamate synthase (GOGAT), which reductively aminates α-KG from glutamine. Glutamate synthase is found exclusively in the cloroplasts of higher plants and vegetative cells of blue-green algae; it too is inhibited by the transaminase inhibitor aminooxyacetate (72, 114, cf 54). Glutamine, a substate for GOGAT, is synthesized by the enzyme glutamine synthetase (GS) in the cytoplasm (cf 57), and appears to be the major product of ^{14}C-glutamate metabolism in leaves of Vicia faba (80). In barley, we have noted that methionine sulfoximine - a specific inhibitor of GS - maximally inhibits CHL synthesis by 50% (data not shown), the same extent to which we can inhibit ALA synthesis with INH. This suggests that glutamate for ALA synthesis might be derived from GS-GOGAT.

Since INH also inhibits the conversion of α-KG to glutamate in blue-green algae, it appears likely that this mode of action is an antagonism of pyridoxal phosphate as is aminooxyacetate's mode of action on GOGAT. Our data on the effect of arsenite on ALA formation support such a claim for a site of action for INH between α-KG and glutamate in barley. Arsenite (AsO_2^-), known to inhibit lipoate acid-requiring enzymes, blocks the oxidation of α-KG to succinyl CoA in the TCA cycle, resulting in the accumulation of α-KG (cf 115).

When barley shoots are treated with 25 μM AsO_2^- (Table 9), we observe a stimulation of ALA formation by 87%, presumably due to an increased conversion of accumulating α-KG to ALA. When AsO_2^- and INH are supplied together, INH eliminates the stimulation, we observed earlier. However, 50% of the inhibition of ALA synthesis - seen with INH alone - was reversed. This suggests that INH and AsO_2^- may have closely related sites of action, as shown in the scheme (Figure 18). Arsenite treatment causes an increased flux of α-KG into the biosynthetic pathway for ALA. The hydrazide, as a transaminase inhibitor, inhibits the conversion of a significant portion of the accumulating α-KG, induced by AsO_2^-, to glutamate in tissue treated with both INH and AsO_2^-. Since AsO_2^- causes a partial reversal of the INH-inhibition of ALA synthesis, it may infer that the primary site of INH-action on ALA synthesis is the transamination of α-KG to glutamate (cf 53).

Our data may also suggest that the pathway using glutamate-1-semialdehyde is the main pathway for ALA synthesis in Anabaena and barley (cf 54, 53). If DOVA is the immediate precursor of ALA, then we should see an inhibition of ALA synthesis from [^{14}C(U)]-glutamate, since the transaminase converting glutamate to α-KG would be inhibited, such is not the case (Table 14).

We may speculate that the effects of INH on ALA formation may be caused by two very different mechanisms of actions: 1) a pyridoxal phosphate antagonism inhibiting the transaminase

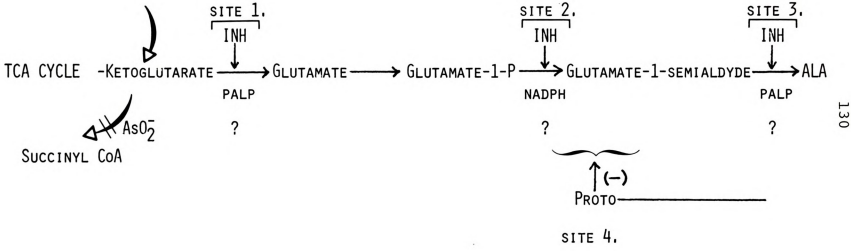
Figure 18. Potential sites for INH action on δ-aminolevulinic acid biosynthesis in greening barley.

Site 1. The pyridoxal phosphate-dependent transamintion of α-KG to glutamate

Site 2. The NADPH-dependent dehydrogenation of glutamate-1-phosphate to glutamate-1-semialdehyde

Site 3. The pyridoxal phosphate-dependent transamination of glutamate-1-semialdehyde to ALA

Site 4. The feedback inhibition by PROTO on ALA synthesis


SITE 1.
INH
TCA CYCLE
-KETOGLUTARATE
GLUTAMATE
GLUTAMATE-1-P
SITE 2.
INH
GLUTAMATE-1-SEMIALDYDE
SITE 3.
INH
ALA
PALP
NADPH
PALP
AsO_2^-
?
?
?
SUCCINYL CoA
(–)
PROTO
SITE 4.

responsible for glutamate synthesis and/or the glutamate-1-semialdehyde transaminase, and 2) a light-reversible antagonism of pyridine nucleotides inhibiting, perhaps, the dehydrogenase responsible for glutamate-1-semialdehyde formation.

δ-Aminolevulinic Acid Synthase (ALSA): Does It Exist In Greening Barley Shoots?--Could any of the INH effects on ALA synthesis be attributed to the presence of ALAS? In consideration of the evidence in the barley system that ALA is formed via the glutamate-1-semialdehyde pathway, it seems somewhat remote. The apparent pyridoxal phosphate-effect of INH involved in ALA synthesis seems to be due to either a direct interference of INH with the semi-aldehyde transaminase or the glutamate-forming transaminase and not with ALAS. It is clear from our labelling data in Anabaena that the *SA of the ALA formed from ^{14}C-2-glycine was .14% that from ^{14}C(U)-glutamate (cf 22 cpm/nmol and 15,390 cpm/nmol). There is essentially no contribution of ALAS to ALA synthesis in blue-green algae. Whether this is equally true for barley shoots is not certain. Our experiments with AsO_2^- and INH (Table 9) may suggest that the C-5 pathway is the main pathway for ALA formation. We would expect the AsO_2^-, an inhibitor of succinate formation, would severely inhibit ALA synthase were it responsible for ALA formation in barley since succinate is a substrate for this enzyme (cf 70). We see, on the other hand, a stimulation in ALA synthesis with arsenite.

Stobart and Hendry (104) suggest that the small amount of label incorporated into ALA from [^{14}C]-glycine is due to the levulinate method for ALA determination. At 30 mM levulinate, for example, the levels of glycine are elevated by 66% compared to controls, this suggests that any labelling by glycine of ALA could be diluted due to an increased glycine pool and therefore explain the low levels of label incorporation into ALA. The intracellular pools of glutamate and glycine in barley have been reported to be 670 and 140 nmol/g-fresh wt. (22). If the glycine pool is increased by 66%, there should relatively little effect on the *SA of glycine as compared to glutamate - since the amount of labelled precursor supplied in these comparative labelling experiments is the same for both amino acids. (In addition, the glycine level which they claim is 14-fold larger than that reported by Castelfranco and Jones, 22). Furthermore, levulinate treatment has no effect on the label incorporated into ALA from [^{14}C]-glycine in Anabaena; controls without LEV incorporate the same amount of radioactivity as the LEV-treated (Table 14).

Stobart and Hendry (47) suggest, moreover, that the ALAS pathway is operative in dark-grown leaves while the C-5 pathway is of quantitative importance in the light. They claim the incorporation of [^{14}C]-glycine into ALA in the dark; however, they show neither their data nor an analysis of the labelling pattern determining the position of the radioactivity in the ALA carbon skeleton. In order to eliminate the possiblity that ALA is labelled non-specifically, it is

necessary to demonstrate that the label from [2-^{14}C] glycine appears in C-5 of ALA, consistent with a labelling pattern that ALAS would give.

If however the ALAS pathway does exist in dark-grown barley, it seems that it would have little significance for the plastids, since the levels of ALA are barely detectable in the dark. The light-inducible system for ALA formation and therefore chlorophyll synthesis appears to be the glutamate pathway. The presence of ALAS in barley remains equivocal, although the possibility exists that the incorporation of ^{14}C-glycine into the minute quantity of ALA observed in the dark is attributable to the synthesis of ALA used in hemoprotein formation in the mitochondria (cf 5). [It should be noted however that heme synthesis in rapidly greening barley has been suggested to be via the C-5 pathway (22).]

IV. Additional Modes of Isonicotinic Acid Hydrazide Action

A. INH as a Metal Chelator

INH demonstrates a very strong affinity for heavy metals (1). INH, for example, chelates Cu^{2+} as in the Lowry assay (unpublished observation) and forms, when mixed with an equal molar solution of Cu^{2+}, an INH-Cu^{2+} complex (3). In light of the fact, that chelators of iron - such as α,α'-dipyridyl and 8-hydroxyquinoline - induce the synthesis of Mg-porphyrins in etiolated beans and barley (95, 25, 40), it seems rather

important to consider the effects of INH on tetrapyrroles as a consequence of metal chelation. The most notable effect on tissue treated with iron chelators is the large accumulation of MgPROTO ME (25, 95). INH-treated barley seedlings do not appear to accumulate MgPROTO ME. Moreover, dipyridyl stimulates both the biosynthesis of Mg-porphyrins, in the absence of ALA, and the biosynthesis of ALA itself in beans; we observe neither effect in barley. These chelators act to inhibit heme synthesis, thereby removing a reputed feedback inhibition by heme on the rate-limiting step(s) of tetrapyrrole formation (25), thus deregulating the synthesis of ALA and Mg-tetrapyrroles. We observe, however, the opposite effects with INH: inhibitions of both ALA and PCHLD. If INH were acting as an iron chelator, then we should not observe the reversals of INH effects with light or nicotinamide treatments. Other porphyrinogenic compounds like pyridine-2-aldoximine, pyridine-2-aldehyde, and picolinic acid do not display the inhibitory profile of INH. This suggests that the modes of action of INH, which we see, are not due to any intrinsic potential to chelate metals.

B. INH as a Photorespiratory Inhibitor of the Glycolate Pathway

INH inhibits the photorespiratory conversion of glycine to serine in Chlorella and in leaves of higher plants (87, 59, 99). This conversion is catalyzed by the combined action of glycine decarboxylase and serine hydroxymethyltransferase

in the mitochondrion (59). Apparently, INH affects the pyridoxal phosphate-dependent decarboxylation reaction responsible for the photorespiratory evolution of CO_2 in higher plants (59). In order to determine whether the effects of INH which we see were due to an inhibitory effect on photorespiration, we screened several chemical inhibitors - known to block the glycolate pathway - for their ability to inhibit ALA and chlorophyll synthesis. The two inhibitors (10 m): glycine hydoxamate, a glycine analog which presumably blocks the conversion of glycine to serine, and α-HPMS, an inhibitor of glycolate oxidase, stimulate ALA synthesis by 110 and 52%, respectively (Table 11). This is quite inconsistent with the effect we obtain with INH. Additional pieces of data do not support a role of INH's action on ALA and CHL as a consequence of its effect on the photorespiratory pathway: 1) glycine, which accumulates in the presence of INH (87, 99), stimulates ALA formation by 26% at 20 mM (Table 11), 2) INH is slightly inhibitory of the glutamate-glyoxylate aminotransferase (87), which, according to Zelitch (123, 71), causes an accumulation of glutamate. Glutamate should, at least, stimulate ALA formation, and 3) unlike INH, both glycine hydroxamate and α-HPMS* do not inhibit CHL formation (Table 11). From these

---

*Hendry and Stobart (48) have found, to the contrary, that 10 mM α-HPMS inhibits CHL synthesis by 39%. Their illumination period was 22 hr compared to 8 hr for our experiments. α-HPMS is known to be unstable after exposures of light longer than several minutes (Tolbert, personal communication). It is possible that their effects are a consequence of the relase of the sulfonic acid group upon light-stimulated degradation of α-HPMS).

observations, it seems apparent that INH does not act on ALA and CHL formation in the same manner as any of the inhibitors of the glycolate pathway. The apparent stimulations of ALA synthesis by glycine hydroxamate and α-HPMS may be due to an inhibition of carbon flow through the glycolate pathway, which may compete indirectly with the biosynthetic pathway of CHL. Blocking glycolate oxidation with α-HPMS, for example, inhibits photorespiration in leaf discs from tobacco and increases net photosynthesis by 50% (84). Perhaps, this accounts for the increased ALA formation. Glycine hydroxamide may stimulate glutamate accumulation, since an inhibition of the glycine to serine conversion results in the accumulation of glyoxylate and glutamate (cf 84). In addition, we have shown that INH is fully capable of acting as an inhibitor of the mitochondrial conversion of [1-^{14}C]-glycine to serine, inhibiting by 83% (Table 12). Although INH clearly inhibits the photorespiratory glycolate pathway, its effects on ALA and tetrapyrrole synthesis do not appear to be a manifestation of this particular site of action.

V. Summary

In overview, we have seen that INH does not act with one specific mode of action on the pathway of chlorophyll biosynthesis. Isonicotinic acid hydrazide proved therefore to be a non-specific inhibitor, and not just the pyridoxal-phosphate antagonist for which it was originally intended.

The hydrazide appears to have several inhibitory sites in the tetrapyrole pathway. There seems to be a site of action on ALA synthesis and a site on tetrapyrrole synthesis, presumably between the synthesis of PROTO and PCHLD. The partial reversal of chlorophyll synthesis by ALA supports a direct effect of INH on ALA synthesis. The accumulation of PROTO and large increase in the *SA of this porphyrin with INH treatment in [^{14}C]ALA-fed tissue support a site for INH action after PROTO formation. Both light and nicotinamide pretreatment mitigate the inhibitory effects of INH on tetrapyrroles. We have offered a hypothesis for the INH effects on tetrapyrroles formation involving a perturtation of the pyridine nucleotide pool. In contrast, the hydrazide appears to act differently on ALA synthesis. In this case, INH may act like a pyridoxal phosphate antagonist.

In conclusion, INH has offered a means to examine the sensitivity of the chlorophyll biosynthetic pathway to an inhibitor with several modes of action. Its apparent involvement in antagonizing both pyridoxal phosphate - requiring reactions and the pyridine nucleotide pool has given us some insight on the regulation of the biosynthesis of tetrapyrroles in greening barley. Since INH appears to be rather nonspecific due its various modes of action, the hydrazide is not an ideal inhibitor for in vivo experimentation.

LITERATURE CITED

LITERATURE CITED

1. Albert A. 1953. Quantitative studies of the avidity of naturally occurring substrances for trace metals. Biochem. J. 54:646-654.

2. Allen, M. B. and D. I. Arnon. 1955. Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen-fixation by Anabaena cylindrica Lemm. Plant Physiol. 30:366-372.

3. Antony, A., Ramakrishnan, J., Mikelens, P., Jackson, J. and W. Levinson. 1978. Effect of INH-Cu^{2+} complex on Rous Sarcoma virus and its genome RNA. Bioinorg. Chem. 9:23-34.

4. Beale, S. and S. Granick. 1979. Chemical synthesis of 4,5-dioxovaleric acid and its nonenzymatic transamination to 5-aminolevulinic acid. Pytochem. 18:441-444.

5. Beale, S. 1978. δ-ALA in plants: Its biosynthesis, regulation and role in plastid development. Ann. Rev. Plant Physiol. 29:95-120.

6. Beale, S. I., S. P. Gough, and S. Granick. 1975. Biosynthesis of δ-ALA from the intact C-skeleton of glutamate in greening barley. Proc. Natl. Acad. Sci. U. S. 72:2719-2723.

7. Beale, S. I. and P. A. Castelfranco. 1974. The biosynthesis of δ-ALA in higher plants. I. Accumulation of δ-ALA in greening plant tissues. Plant Physio. 53:291-296.

8. Beale, S. I. and P. A. Castelfranco. 1974. The biosynthesis of δ-ALA in higher plants. II. Formation of ^{14}C-ALA from labelled precursors in greening plant tissues. Plant Physiol. 53:297-303.

9. Beale, S. I. and P. A. Castelfranco. 1973. ^{14}C-Incorporation from exogenous compounds into ALA by greening cucumber cotyledons. Biochem. Biophys. Res. Commun. 52:143-149.

10. Beale, S. I. 1970. The biosynthesis of δ-ALA in Chlorella. Plant Physiol. 45:504-506.

11. Bendix, S. and M. B. Allen. 1962. UV-induced mutants of Chlorella pyrenoidosa. Arch. Mikrobiol. 41:115.

12. Bergum, P. W. and K. D. Nadler. 1978. Effects of INH on the synthesis of δ-ALA and chlorophyll in greening barley shoots. Plant Physiol. 61S:82.

13. Berlyn, M. B. and J. Zelitch. 1978. Growth and metabolism of [^{3}H]-isonicotinic acid hydrazide by INH-resistant tobacco callus. Plant Physiol. 61S:101.

14. Biehl, J. P. and R. W. Vilter. 1954. Effects of isoniazid on pyridoxine metabolism. J. Amer. Med. Assoc. 156:1549-1552.

15. Boger, P., C. C. Black, and A. San Pietro. 1967. Photosynthetic reactions with pyridine analogs: I. INH-NAD. In Biochemistry of Chloroplasts, Proceedings of a NATO Study Institute Held at Aberystwyth, August, 1965, vol. II, ed. T. W. Goodwin, Academic Press.

16. Bogorad, L. 1976. Chlorophyll biosynthesis. In Chemistry and Biochemistry of Plant Pigments. Ed. T. W. Goodwin. New York: Academic Press, pp. 64-148.

17. Borova, J., P. Ponka, and J. Neuwirt. 1973. Study of intracellular iron distribution with normal and inhibited heme synthesis. Biochim. Biophys. Acta. 320:143-156.

18. Bottomley, S. S. and G. A. Smithee. 1969. Purification and characterization of bone marrow δ-ALAS. Blood 34:857.

19. Brodersen, P. 1976. Factors affecting the photoconversion of PCHLD to CHLD in etioplast membranes from barley. Photosynthetica 10:33-39.

20. Burham, B. F. and J. Lascelles. 1963. Control of porphyrin biosynthesis through negative feedback mechanism. Studies in preparations of δ-ALAS and δ-ALAD from Rhodopseudomonas spheroides. Biochem. J. 87:462-472.

21. Castelfranco, P. A., J. D. Wienstein, S. Schwarz, A. D. Pardo, and B. E. Wezelman. 1979. The Mg^{2+}-insertion step in chlorophyll biosynthesis. Arch. Biochem. Biophys. 192:592-598.

22. Castelfranco, P. A. and O. T. G. Jones. 1975. Protohaem turnover and chlorophyll synthesis in greening barley tissue. Plant Physiol. 55:485-490.

23. Cox, M. T., T. T. Howarth, A. H. Jackson, and G. W. Kenner. 1972. The formation of the isocyclic ring in chlorophyll. J. Amer. Chem. Soc. 91:1232-1233.

24. Demain, A. L. and R. Y. White. 1971. Porphyrin overproduction by Pseudomonas denitrificans: Essentiality of betaine and stimulation by ethionine. J. Bacteriol. 107:456-460.

25. Duggan, J. and M. Gassman. 1974. Induction of porphyrin synthesis in etiolated bean leaves by chelators of iron. Plant Physiol. 53:206-215.

26. Ellsworth, R. K., R. M. Tusk, and L. A. St. Pieree. 1976. Studies on chlorophyllase. IV. Attribution of hydrolytic and esterifying "chlorophyllase" activities observed in vitro to two enzymes. Photosynthetica 10:312-323.

27. Ellsworth, R. K. and S. Arnoff. 1969. Investigations of the biogenesis of chlorophyll a. IV. Isolation of and partial characterization of some biosynthetic intermediates between MgPROTO IX ME and Mg-vinylpheophorphyrin a_5 obtained from Chlorella mutants. Arch. Biochem. Biophys. 130:374-387.

28. Ellsworth, R. K. and S. Arnoff. 1968. Investigations on the biogenesis of chlorophyll a III. Biosynthesis of Mg-vinylpheophorphrine a_5 ME from Mg-protopheoporphine IX ME as observed in Chlorella mutants. Arch. Biochem. Biophys. 125:269-277.

29. Ellsworth, R. K. and S. Arnoff. 1968. Investigations on the biogenesis of chlorophyll a II. Chlorophyllide accumulation by a Chlorella mutant. Arch. Biochem. Biophys. 125:35-39.

30. Fluhr, R., E. Harel, S. Klein, and E. Miller. 1975. Control of δ-ALA and chlorophyll accumulation in greening maize upon light-dark transitions. Plant Physiol. 56:497-501.

31. Gassman, M. and P. Castelfranco. 1977. Oxidation of 5-ALA to CO_2 by extracts of barley seedlings. Plant Physiol. 59S:103.

32. Gassman, M., J. Plusec, and J. Bogorad. 1968. δ-ALA transaminase in Chlorella vulgaris. Plant Physiol. 43:1411-1414.

33. Gassman, M., J. Plusec, and L. Bogorad. 1966. δ-ALA transaminase from Chlorella and Phaseolus. Plant Physiol. 41:xiv.

34. Gorchein, A. Control of Mg-protoporphyrin chelatase activity in Rhodopseudomonas spheroides: Role of light, 02, and electron and energy transfer. Biochem. J. 134:833-845.

35. Gough, S. P. and C. G. Kannangara. 1977. Synthesis of ALA by a chloroplast stroma preparation from greening barley leaves. Carlsberg Res. Commun. 42: 459-464.

36. Gough, S. Defective synthesis of porphyrins in barley plastids by mutations in nuclear genes. Biochim. Biophys. Acta. 286:36-54.

37. Granick, S. and S. Sassa. 1971. δ-ALA and the control of heme and chlorophyll synthesis. In: Metabolic Regulation. Ed. H. J. Vogel, Vol. V, Academic Press, pp. 77-139.

38. Granick, S. and M. Gassman. 1970. Rapid regeneration of protocholorophyllide$_{650}$. Plant Physiol. 45: 201-205.

39. Granick, S. and S. Urata. 1963. Increase in activity of δ-ALA synthetase in liver mitochondria induced by feeding of 3,5-Dicarbethoxy-1,4-dihydrocollidine. J. Biol. Chem. 238:821-827.

40. Granick, S. 1959. Mg-porphyrins formed by barley seedlings treated with δ-ALA. Plant Physiol. 34S: xviii.

41. Griffiths, W. T. and O. T. G. Jones. 1975. Mg 2,4,-divinylphaeoporphyrin a_5 as a substrate for chlorophyll biosynthesis in vitro. FEBS letters 50: 355-358.

42. Griffiths, W. T. 1974. Source of reducing equivalents for the in vitro synthesis of chlorophyll from protochlorophyllide. FEBS letters 46:301-304.

43. Harel, E., E. Meller, and M. Rosenburg. 1978. Synthesis of 5-ALA(^{14}C) by cell-free preparations from greening maize leaves. Phytochem. 17:1277-1280.

44. Hasse, K. and H. Schleyer. 1961. Eine DPN-Nucleosidase der höheren Pflanze. Biochemische Zeitschrift 334:360-368.

45. Heber, U. W. and K. A. Santarius. 1965. Comparmentation and reduction of pyridine nucleotides in relationship to photosynthesis. Biochim. Biophys. Acta 109:390-408.

46. Hendry, G. A. F. and A. K. Stobart. 1977. Protochlorophyllide(P_{650}) turnover in dark-green barley leaves. Phytochem. 16:1663-1664.

47. _____. 1977. Glycine metabolism and chlorophyll synthesis in barley leaves. Phytochem. 16:1567-1570.

48. _____. 1977. Haem and chlorophyll formation in etiolated and greening leaves of barley. Phytochem. 16:1545-1548.

49. Horton, P. and R. M. Leech. 1975. The effect of ATP on the photoconvrsion of protochlorophyllide in isolated etioplasts of Zea mays. Plant Physiol. 56:113-120.

50. Jerzykowski, T., R. Winter, and W. Matuszewski. 1973. γ,δ-dioxovaleric acid as a substrate for the glyoxylase enzyme system. Biochem. J. 135:713-719.

51. Johnson, W. J. 1963. Dinucleotide analogues and related substances. In: Metabolic Inhibitors. Ed. R. M. Hochster and J. H. Quastel, vol. II. New York: Academic Press, pp. 1-22.

52. Kahn, A. and O. F. Nielsen. 1974. Photoconvertible protocholorophyll(ide) 635/650 in vivo: A single species or two species in dynamic equilibrium? Biochim. Biophys. Acta 333:409-414.

53. Kannangara, C. G. and S. P. Gough. 1977. Synthesis of ALA and chlorophyll by isolated chloroplasts. Carlsberg Res. Commun. 42:441-457.

54. Kannangara, C. G. and S. P. Gough. 1978. Biosynthesis of δ-ALA in greening barley leaves: Glutamate-1-semialdehyde aminotransferase. Carlsberg Res. Commun. 43:185-194.

55. Kaplan, N. O., M. M. Ciotti, J. van Eys, and R. M. Burton. 1959. Effect of pyridine derivatives on animal tissue diphosphopyridine nucleotidases. J. Biol. Chem. 234:134-138.

56. Kenner, G. W., S. W. McCombie, and K. M. Smith. 1972. Chem. Commun. 844-845.

57. Key, A. J., I. F. Bird, M. J. Cornelius, P. J. Lea, R. M. Wallsgrove, and B. J. Miflin. 1978. Photorespiratory nitogen cycle. Nature 275:741-743.

58. Kirk, P. R. and R. M. Leech. 1972. Amino acid synthesis by isolated chloroplasts during photosynthesis. Plant Physiol. 50:228-234.

59. Kisaki, I., N. Yoshida, and A. Imai. 1971. Glycine decarboxylase and serine formation in spinach leaf mitocondrial preparations with reference to photorespiration. Plant Cell Physiol. 12:275-288.

60. Kissel, H. J. and L. Heilmeyer. 1969. Nachweis und Bestimmung von γ,δ-Dioxovaleriansäure: Reversibele Umwandlung von γ, δ-Dioxovalerinsäure und δ-Aminolävulinsaure in Ratten. Biochim. Biophys. Acta 177:78-87.

61. Klein, O. and H. Senger. 1978. Biosynthetic pathways to ALA induced by blue light in the pigment mutant C-2A' of Scenedesmus obliquus. Photochem. Photobiol. 27:203-208.

62. _____. 1978. Two biosynthetic pathways to ALA in a pigment mutant of the green alga, Scenedesmus obliquus. Plant Physiol. 62:10-13.

63. Klein, S., E. Harel, E. Ne'eman, E. Katz, and E. Meller. 1975. Accumulation of ALA and its relation to chlorophyll synthesis and development of plastid structure in greening leaves. Plant Physiol. 56: 486-496.

64. Klein, S. and J. A. Schiff. 1972. The correlated appearance of prolamellar bodies, protochlorophyll-(ide) species, and the shibata shift during development of bean etioplasts in the dark. Plant Physiol. 49:619-626.

65. Koller, M. E., P. H. Prante, R. Ulvik, and I. Romslo. 1976. Effect of haemin and isonicotinic acid hydrazide on the uptake of Fe^{3+} from transferin by isolated rat liver mitochondria. Biochem. Biophys. Res. Commun. 71:339-345.

66. Kowalski, E., A. Dancewicz, and Z. Szot. 1957. Aminolaevulinic acid transaminase in tissue. Bull. Acad. Pol. Sci. Cl.2 5:223-226.

67. Kung, S-d. 1977. Expression of chloroplast genomes in higher plnats. Ann. Rev. Plant Physiol. 28:401-437.

68. Lascelles, J. and T. P. Hatcher. 1969. Bacteriochlorophyll and heme synthesis in Rhodopseudomonas spheroides: Possible role of heme in regulation of the branches biosynthetic pathway. J. Bacteriol. 98:712-720.

69. Lascelles, J. 1966. The regulation of synthesis of iron and mg-tetrapyrroles: Observations with mutant strains of Rhodopseudomonas spheroides. Biochem. J. 100:184-189.

70. Lascelles, J. 1956. The synthesis of porphyrins and bacteriochlorophyll in cell suspensions of Rhodopseudomonas spheroides. Biochem. J. 62:78-93.

71. Lawyer, A. L. and I. Zelitch. 1978. Inhibition of glutamate: Glyoxylate amino transferase activity in tobacco leaves and callus by glycidate, an inhibitor of photorespiration. Plant Physiol. 61:242-247.

72. Lea, P. J. and B. J. Miflin. 1975. The occurrence of glutamate synthase in algae. Biochem. Biophys. Res. Commun. 64:856-862.

73. Lohr, J. B. and H. C. Friedman. 1976. New pathway for δ-ALA biosynthesis: Formation from α-KG by two partially purified plant enzymes. Biochem. Biophys. Res. Commun. 69:908-913.

74. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

75. Manetas, Y. and G. Akoyunogiou. 1975. The stoichiometric relationship between δ-ALA and protochlorophyllide. Plant Sci Letters 5:375-378.

76. Mascia, P. 1978. Analysis of precursors accumulated by several chlorophyll biosynthetic mutants of maize. Molec. gen. Genetics 161:237.

77. Mattheis, J. R. and C. A. Rebeiz. 1978. Chloroplast biogenesis XXIII. The conversion of exogenous protochlorophyllide into transformable protochlorophyllide in vitro. Photochem. Photobiol. 28:55-60.

78. Meller, E., S. Belkin, and E. Harel. 1975. The biosynthesis of δ-ALA in greening maize leaves. Photochem. 14:2399-2402.

79. Mauzerall, D. and S. Granick. 1956. The occurrence and determination of ALA and porphobilinogen in urine. J. Biol. Chem. 219:435-446.

80. Miflin, B. J. and P. J. Lea. 1977. The pathway of nitrogen assimilation in plants. IN: Progress in Phytochemistry 4. Ed. L. Rheinhold, J. B. Harborne, T. Swain. Oxford: Pergamon Press, pp. 1-26.

81. Nadler, K. D. and S. Granick. 1970. Controls on chlorophyll synthesis in barley. Plant Physiol. 46: 240-246.

82. Nemeth, A. R., C. S. Russel, and D. Shemin. 1957. The succinate-cycle II. metabolism of δ-aminolevulinc acid. J. Biol. Chem. 229:415-422.

83. Neuberger, A. and J. M. Turner. 1963, γ,δ-Dioxovaleric acid aminotransferase activity in Rhodopseudomonas spheroides. Biochim. Biophys. Acta 67:342-345.

84. Oliver, D. J. and I. Zelitch. 1977. Metabolic regulation of glycolate synthesis, photorespiration and net photosynthesis in tobacco by L-glutamate. Plant Physiol. 59:688-694.

85. Porra, R. J. and L. H. Grimme. 1978. Tetrapyrrole biosynthesis in algae and higher plants: A discussion of the importance of the ALAS and DOVA transaminase pathways in the biosynthesis of chlorophyll. Internat. J. Biochem. 9:883-886.

86. Porra, R. J. and L. H. Grimme. 1974. Chlorophyll synthesis and intracellular fluctuations of ALA formation during regreening of nitrogen-deficient Chlorella fusca. Arch. Biochem. Biophys. 164:312-321.

87. Pritchard, G. G., C. P. Whittingham, and W. J. Griffin. 1963. The effect of INH on the photosynthetic incorporation of radioactive CO_2 into ethanol-soluble compounds of Chlorella. J. Exper. Bot. 14:281-289.

88. Ramakrishnan, T., P. S. Murphy, and K. P. Gopimathan. 1972. Intermediary metabolism of Mycobacteria. Bacteriol. Rev. 36:65-108.

89. Ramaswamy, N. K. and P. M. Nair. 1976. Pathway for the biosynthesis of δ-ALA in greening potatoes. Ind. J. Biochem. 13:394-397.

90. Ramaswamy, N. K. and P. M. Nair. 1973. ALA synthetase from cold-stored potatoes. Biochim. Biophys. Acta 293:269-277.

91. Rebeiz, C. A. and P. A. Castelfranco. 1973. Protochlorophyll and chlorophyll biosynthesis in cell-free systems from higher plants. Ann. Rev. Plant Physiol. 24:129-172.

92. Ridley, S. M. and J. Ridley. 1979. Interaction of chloroplasts with inhibitors: Location of carotenoid synthesis and inhibition during chloroplast development. Plant Physiol. 63:392-398.

93. Rüdiger, W., P. Hedden, H.-P. Köst, and D. J. Chapman. 1977. Esterification of chlorophyllide by geranylgerianyl pyrophosphate in a cell-free system from maize shoots. Biochem. Biophys. Res. Commun. 74: 1268-1272.

94. Rüdiger, W., J. Benz, U. Lempert, S. Schochs, and D. Seffens. 1976. Inhibition of phytol accumulation with herbicides. Geranylgeraniol and dihydrogeranylgeraniol-containing chlorophyll from wheat seedlings. Zeitschrift für Pflanzenphysiologie 80:131-143.

95. Ryberg, M. and C. Sundqvist. 1976. The influence of 8-hydroxyquinoline on the accumulation of porphyrins in dark-grown wheat leaves treated with δ-ALA. Plant Physiol. 36:356-361.

96A. Salvador, G. J. 1978. δ-ALA synthesis from γ,δ-dioxovaleric acid by cellular preparations of Euglena gracilis. Plant Sci. Letters 13:351-355.

96B. Sassa, S. and S. Granick. 1970. Induction of δ-aminolevulinic acid synthetase in chick embryo liver cells in culture. Proc. Nat. Acad. Sci. (USA) 67:517-522.

97. Schneider, H. A. W. 1976. Enzymic capacties for chlorophyll biosynthetic activation and de novo synthesis of enzymes. Zeitschrift für Naturforschung 31:55-63.

98. Schoch, S., U. Lempert, and W. Rüdiger. 1977. On the last steps of chlorophyll biosynthesis : intermediates between chlorophyllide and phytol-containing chlorophyll. Zeitschrift für Pflanzenphysiologie. 83:427-436.

99. Servaites, J. C. and W. L. Ogren. 1977. Chemical inhibition of the glycolate pathway in soybean leaf cells. Plant Physiol. 60:461-466.

100. Shemin, D., C. S. Russel, and T. Abramsky. 1955. The succinate-glycine cycle. I. the mechanism of pyrrole synthesis. J. Biol. Chem. 215:613-626.

101. Sistrom, W. R. 1963. A note on the effect of inhibitors of electron transport and phosphorylation of photopigment synthesis in Rhodopseudomonas spheroides. In: Bacterial Photosynthesis. Ed. H. Gest. Antoich Press.

102. Smith, B. B. and C. A. Rebeiz. 1979. Chloroplast Biogenesis XIV. intrachloroplastic localization of the biosynthesis and accumulation of protoporphyrin IX, MgPROTO ME, and longer wavelength metalloporphyrins during greening. Plant Physiol. 63: 227-231.

103. Smith, A. J. 1973. Synthesis of metabolic intermediates. In: The Biology of Blue-green algae. Botanical Monographs. vol. 9. ed. N. G. Carr, B. A. Whitton. Berkeley.

104. Stobart, A. K. and G. A. F. Hendry. 1978. Chlorophyll formation and glycine metabolism in levulinate treated barley leaves. Phytochem. 17:993-994.

105. Sundqvist, C. 1978. Red light-stimulated accumulation of protochlorophyllide in dark grown leaves treated with δ-ALA. Plant Sci. Letter 12:69-76.

106. Sundqvist, C. 1973. The relationship between chlorophyllide accumulation, the amount of protochlorophyllide$_{636}$ and protochlorophyllide$_{650}$ in dark grown wheat leaves treated with δ-ALA. Physiol. Plant. 28:464-470.

107. Sundqvist, C. 1969. Transformation of protochlorophyllide formed from exogenous δ-ALA in continuous light and in flash light. Physiol. Plant. 22: 147-156.

108. Süzer, S. and K. Sauer. 1971. The sites of photoconversion of protochlorophyllide to chlorophyllide in barley seedlings. Plant Physiol. 48:60-63.

109. Urata, G. and S. Granick. 1963. Biosynthesis of δ-aminoketones and the metabolism of aminoketone. J. Biol. Chem. 238:811-820.

110. von Wettstein, D., A. Kahn, O. F. Nielsen, and S. Gough. 1974. Genetic regulation of chlorophyll synthesis analyzed with mutants in barley. Science 184: 800-802.

111. Wang, W.-Y., J. E. Boyton, and N. W. Gillham. 1977. Genetic control of chlorophyll biosynthesis: Effect of increased δ-ALA synthesis on the phenotype of the y-1 mutant of Chlamydomonas. Mol. Gen. Genetics 152:7-12.

112. Wang, W.-Y., J. E. Boynton, N. W. Gillman, and S. Gough. 1975. Genetic control of chlorophyll biosynthesis in Chlamydomonas: Analysis of a mutant affecting synthesis of δ-ALA. Cell 6:75-84.

113. Wang, W.-Y., J. E. Boynston, and N. W. Gillman. 1974. Genetic control of chlorophyll biosynthesis in Chlamydomonas. Analysis of mutants at two loci mediating the conversion of PROTO IX to Mg PROTO. J. Cell Biol. 63:806-823.

114. Webb, J. L. 1966. Analogs of pyridoxal. In: Enzyme and Metabolic Inhibitors, vol. II. Ed. J. L. Webb, pp. 561-579.

115. Webb, J. L. 1966. Arsenicals. In: Enzymes and Metabolic Inhibitors, vol III. Ed. J. L. Webb, pp. 595-794.

116. Weinstein, J. D. and P. A. Castelfranco. 1978. Mg protoporphyrin IX and δ-ALA synthesis from glutamate in isolated greening chloroplasts: δ-ALA synthesis. Arch. Biochem. Biophys. 186:376-382.

117. Wellburn, A. R. 1975. δ-ALA formation in greening Avena laminae. Phytochem. 14:699-701.

118. Wellburn, F. A. M. and A. R. Wellburn. 1971. Chorophyll synthesis by isolated intact etioplasts. Biochem. Biophys. Res. Commun. 45:747-750.

119. Wider de Xifra, E. A., A. M. D. C. Batlle, and H. A. Tigier. 1971. δ-ALA synthetase in extracts of cultured soybean cells. Biochim. Biophys. Acta 235:511-517.

120. Youatt, J. 1969. A review of the action of isoniazid. Amer. Rev. Respir. Disease 99:729-749.

121. Zatman, L. J., N. O. Kaplan, and S. P. Colowick. 1954. Effect of isonicotinic acid on diphosphopyridine nucleotidases. J. Biol. Chem. 209:453-466.

122. Zatman, L. J., N. O. Kaplan, and S. P. Colowick. 1953. Inhibition of spleen diphosphopyridine nucleotidase by nicotinamide, an exchange reaction. J. Biol. Chem. 200:197-213.

123. Zelitch, I. 1978. Effect of glycidate, an inhibitor of glycolate synthesis in leaves, on the activity of some enzymes of the glycolate pathway. Plant Physiol. 61:236-241.

124. Zinner, K., C. C. C. Vidigal, N. Duran, and G. Cilento. 1977. Oxidation of isonicotinic acid hydrazide by the proxidase system. The formation of an excited product. Arch. Biochem. Biophys. 180: 452-458.

APPENDIX

APPENDIX

Hutner's Nutrient Medium

Hutner's stock ($-Mg^{2+}$): 59 X normal strength

Solution A: in ~400 ml glass distilled water, chemical in grams

$Ca(NO_3)_2$ - 35.4

EDTA(free acid)* - 50.0

K_2HPO_4 - 40.0

KOH (85% pellets) 24-26

NH_4NO_3 - 20.0

* EDTA not very soluble unless under alkaline conditions

Solution B: in ~300 ml glass distilled water

$ZnSO_4 \cdot 7H_2O$ - 6.59

H_3BO_3 - 1.42

$Na_2MO_4 \cdot 2H_2O$- 2.52

$CuSO_2 \cdot 5H_2O$ - 0.394

+ 1 N HCl until cloudiness disappears

Solution C: ~100 ml of water

$FeSO_4 \cdot 7H_2O$ - 2.49

Add solution A and B, adjust pH with 1 N HCl, then add solution C and bring up to correct volume.

Mg^{2+} Stock: 25 gm $MgSO_4 \cdot 7H_2O$/liter of glass distilled water

Hutner's Nutrient Solution: 25 ml of Hutner's stock + 25 ml Mg^{2+} stock and bring volume up to 2½ liter with water.