Inhibitions of Growth and Metabolism by 3-Amino-1,2,4-triazole (Amitrole)

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The herbicide, 3-amino-1,2,4-triazole (amitrole), selectively controls growth of both microorganisms and higher plants. Inhibition of the dehydratase enzyme in the histidine biosynthetic pathway is the major growth-controlling action in heterotropic microorganisms. At least two additional sites of action in purine and one-carbon metabolism, plus numerous indirect effects, are implicated by interferences in the metabolism of adenine, riboflavin, methionine, and serine. Inhibition of histidine biosynthesis occurs also in photosynthetic organisms but does not satisfactorily explain phytotoxicity. Toxicity of amitrole can be circumvented by riboflavin acting through a photochemical mechanism in the light or through a biological interaction in the dark. The riboflavin-amitrole interaction in bacteria is normally masked by the inhibition of histidine biosynthesis. In bacteria, riboflavin appears to protect growth by sparing of adenine. The protective mechanism does not involve either the destruction of amitrole or the use of riboflavin as a precursor for purines.

A general hypothesis has developed that many herbicides control weeds through the cumulative effects of interferences in a number of different physiological and biochemical processes. For some herbicides, we have reason to believe that a single process may be sufficiently more sensitive than others, and of sufficient importance to survival of the plant that its inhibition constitutes a satisfactory explanation of major herbicidal actions. However, for many compounds numerous processes are affected before the plant accumulates the chemical to herbicidal concentrations. Efforts have been made to identify individual sites of action at cellular and molecular levels and to evaluate their physiological significance. These efforts are frustrated frequently by interferences from direct and indirect effects at other action sites.

Greatest progress in establishing principles of the mechanism of action for the multiple-site herbicides has been accomplished with 3-amino-1,2,4-triazole (amitrole). This herbicide is one of the few for which inhibition of growth of microorganisms is comparable to that found with green plants. The variety of organisms available for toxicity studies permits investigators to select biological materials in which one of the multiple sites is unusually sensitive. Therefore, interferences caused by additional actions are experimentally minimized. The multiple-organism approach with amitrole is beginning to expose a number of general principles relating to the mechanism of toxic actions which probably apply equally well to other multiple-action pesticides. For example, the action site primarily responsible for toxicity to one organism or plant species may be of minor significance to growth control of another species. There is some evidence to suggest that the principal mechanism of action is not necessarily the same at two different stages in the life cycle of a single species or possibly even in two different sensitive organs of a single plant. In some organisms, the control of growth appears to result first from the secondary (indirect) effects of the primary inhibition rather than from the direct effects of the inhibition. And finally, the most sensitive site is not necessarily the one primarily involved in growth control. Thus, sublethal actions may produce visible symptoms which are unrelated to herbicidal action.

The biological activity of amitrole was discovered in 1952 and the chemical was released as an experimental herbicide in 1953. In the literature of the subsequent five years, numerous hypotheses concerning the mechanism of amitrole action were proposed, examined extensively, and then rejected. Most of the research designed to elucidate the toxic action of amitrole centered around three major metabolic processes: chlorophyll and porphyrin biosynthesis, cation functions, and metabolism of purines and their derivatives. The absence of chlorophyll in newly expanded leaves is the most striking symptom of amitrole toxicity. Biochemical and anatomical data indicate that amitrole interferes with development of leaf plastids rather than inhibiting the porphyrin biosynthetic pathway directly (Hilton *et al.*, 1963; Naylor, 1964; Wolf, 1960). Amitrole is a weak chelating agent. However, biochemical data do not support the hypothesis that chelation is of any major significance in most growth inhibitions (Guérin-Dumartrait, 1966; Hilton *et al.*, 1963; Naylor, 1964). Amitrole interrupts numerous aspects of purine metabolism. Consequently, the working hypothesis was proposed that amitrole might act as a general inhibitor of purine metabolism (Hilton *et al.*, 1963), or that the phosphorylated glycoside of amitrole was competitive with AMP (Frederick and Gentile, 1967). Subsequent research data, presented here, do not substantiate such a broad concept. The total data favor the hypothesis that multiple and highly specific metabolic sites are involved in amitrole actions. As yet an adequate number of action sites have not been characterized sufficiently to determine possible similarities between them in terms of molecular structure.

THEORY AND FINDINGS

First evidence for the physiologically significant inhibitions came in 1960 when a number of different investigators simultaneously and independently reported pro-

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tection by histidine, purines, or riboflavin against amitrole inhibitions of growth of a variety of organisms. Literature citations of the reported antagonisms are summarized in Table I. The early literature on growth antagonisms strongly suggested a phylogenetic relation for amitrole inhibitions. Inhibition in histidine metabolism appeared responsible for amitrole toxicity to microorganisms but not to green plants. An interference in purine metabolism seemed involved in toxicity to green algae. Riboflavin-amitrole interaction seemed to hold the clue to amitrole toxicity to higher plants. As understanding of the individual antagonisms increased, investigators obtained evidence for each of the actions in additional classes of organisms. For example, initial efforts to detect the riboflavin-amitrole antagonisms in bacteria failed because the interaction was completely masked by the histidine inhibition. When this inhibition is negated with exogenous histidine, riboflavin can be shown to protect growth of bacteria against amitrole inhibitions (Figure 1). Furthermore, through use of techniques other than growth antagonisms, evidence is accumulating for histidine and purine inhibitions in higher plants. Additional research on metabolic effects will probably provide evidence for all the interactions in all of the organisms listed.

The metabolite-amitrole interactions for different organisms (Table I) strongly suggest multiple sites of action for amitrole. However, the hypothesis that all of the interactions result from a single inhibition is not eliminated since both histidine and riboflavin are derived from purine precursors. Even the more recently reported methionine and serine protections in bacteria could fit a single-action hypothesis since these metabolites serve

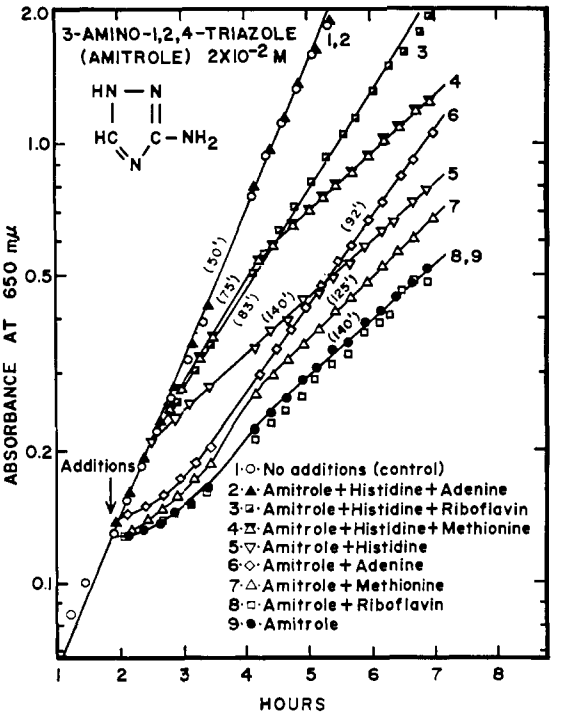

Figure 1. Interaction of metabolites, $2 \times 10^{-4}M$, and amitrole, $2 \times 10^{-2}M$, on growth of *S. typhimurium* (Hilton and Kaufman, 1968)

Doubling times in minutes shown in parenthesis

Table I. Metabolites which Protect Growth against Amitrole Inhibition[a]

Class of Organism	Metabolite: Histidine	Purines	Riboflavin	Methionine or serine	References
Yeast	++++	+	0[b]		Hilton, 1960; Klopotowski and Bagdasarian, 1966; Weyter and Broquist, 1960
Bacteria	+++	++	++	++	Bond and Akers, 1961; Hilton and Kaufman, 1968; Hilton *et al.*, 1965; Weyter and Broquist, 1960
Fungi	+++	0	0		Hilton, 1963
Colorless algae	+++	0	0		Casselton, 1964, 1966
Red algae		+++			Boney, 1963
Green algae	0	+++	0		Castelfranco and Bisalputra, 1967; Wolf, 1962
Higher plants	0	+,0	++++	0[b]	Castelfranco *et al.*, 1963; Hilton, 1962; Naylor, 1964; Sund *et al.*, 1960
Parts of plants	+++,0	++,0			Burt, 1967; Jackson, 1961

[a] Subjective rating scale:
++++ Protection observed at amitrole concentrations more than 100 times minimum toxic concentration.
+++ Major protection observed.
++ Protections in addition to the major protection.
+ Minor protective actions.
0 Antagonisms sought but not found.
Blank spaces indicate no reports.

[b] Hilton, 1963

as one-carbon donors, and therefore as precursors in purine biosynthesis. The sequence in which the amitrole-metabolite interactions are observed for bacteria (Table II) suggests that additional sites of action become involved as amitrole concentrations increase but do not eliminate the single-action hypothesis. The multiple-site hypothesis is demonstrated most conclusively by an evaluation of inhibitor structure *vs.* metabolic activity (Table II). Structural changes should not affect all sites equally. Thus, when more than one site of action is involved, some inhibitions can be enhanced and others eliminated by structural changes.

Alterations in structure of the amitrole molecule did not affect equally the antagonisms of histidine, adenine, riboflavin, or methionine-serine (Table II). The results seem to indicate four separate processes and no fewer than three sites of amitrole action in *Salmonella typhimurium* (Loeffler) Cast. and Chalm. The inability to separate antagonism to methionine and serine by changes in inhibitor structure indicates that these two metabolites are involved in circumvention of a single metabolic inhibition. Currently, the data in Table II are interpreted as evidence for direct inhibitions in the separate processes of histidine metabolism, purine metabolism, and one-carbon metabolism, plus an independent interaction with riboflavin which nullifies the effect of amitrole on purine metabolism (Hilton and Kaufman, 1968).

Histidine Metabolism. Amitrole is a competitive inhibitor of imidazoleglycerol phosphate (IGP) dehydratase, an enzyme of the histidine biosynthetic pathway. This inhibition is the primary mechanism through which the herbicide produces all physiological and biochemical responses attributable to interrupted histidine metabolism. Secondary effects, induced by a shortage of histidine, appear to produce many of the additional results frequently cited as evidence for alternate hypotheses on the mechanism of amitrole action.

Concurrent with the discoveries of amitrole interferences with histidine metabolism in yeast and bacteria, the histidine biosynthetic pathway (Figure 2) was being elucidated from studies with fungi and bacteria (Ames and Hartman, 1962; Smith and Ames, 1964) and then shown to exist also in yeast (Fink, 1964). Subsequently, interest in the herbicidal action of amitrole led to studies which provided partial evidence that the identical pathway exists also in algae (Casselton, 1966; Siegel and Gentile, 1966), and in higher plants (Davies, 1968; Klopotowski *et al.*, 1968). Available evidence seems to indicate that the pathway is basically the same in all histidine producing organisms.

Histidine biosynthesis in *S. typhimurium* (Ames and Hartman, 1962) is regulated by two metabolic control mechanisms: feedback inhibition, in which the end product, histidine, inhibits the first enzyme of the pathway; and enzyme repression, in which excess free histidine in the cell inhibits formation of all 10 histidine biosynthetic enzymes. There is evidence that histidine does, indeed, shut off the biosynthesis of histidine in vivo in bacteria (Magasanik and Karibian, 1960; Moyed, 1959), yeast (Fink, 1964; Hilton and Kearney, 1965), algae (Siegel and Gentile, 1966), and higher plants (Davies, 1968; Dougall, 1965). The first enzyme of the pathway isolated from *S. typhimurium* (Ames *et al.*, 1961b), *Neurospora crassa*, and *Saccharomyces cerevisiae* (Fink, 1964), and several species of higher plants (Klopotowski *et al.*, 1968) is inhibited in vitro by low concentrations of histidine. Feedback inhibition, therefore, is probably involved as a control mechanism in metabolic regulation of histidine biosynthesis in essentially all histidine producing organisms. Histidine regulation of formation of the histidine biosynthetic enzymes, however, seems to be more variable among organisms. Histidine starvation of *S. typhimurium* results in a 20-fold increase in biosynthetic enzymes (derepression) (Ames and Garry, 1959; Ames and

Table II. Major Interactions of Triazole or Tetrazole Inhibitors and Selected Metabolites Observed in Growth Responses of *S. typhimurium*[a]

Inhibitor	Inhibitor Concentration, *M*	Metabolites Added to Basic Medium	Metabolites Circumventing Growth Inhibitions[b]				
			Histidine	Adenine	Riboflavin	Methionine	Serine
Amitrole	2×10^{-2}	—	++	+	0	0	0
Amitrole	2×10^{-2}	Histidine	0	++	++	+	0
Amitrole	10^{-1}	Histidine and adenine	0	0	0	++	++
3-Hydroxytriazole	10^{-1}	—	0	++	0	0	0
3-Chlorotriazole	2 to 200×10^{-4}	—	0	0	0	++	++
5-Amino-1,2,4-tetrazole	2×10^{-2}	—	0	0	0	++	++
1,2,4-Triazole	2×10^{-2}	—	0	0	0	++	++

[a] From Hilton and Kaufman (1968). Does not include minor interactions presumed to result from indirect rather than direct actions.
[b] Symbols represent relative importance of the interactions to growth: 0, little or no protection; + the smaller of two major protective interactions; ++ the major interaction, representing maximum protection obtained.

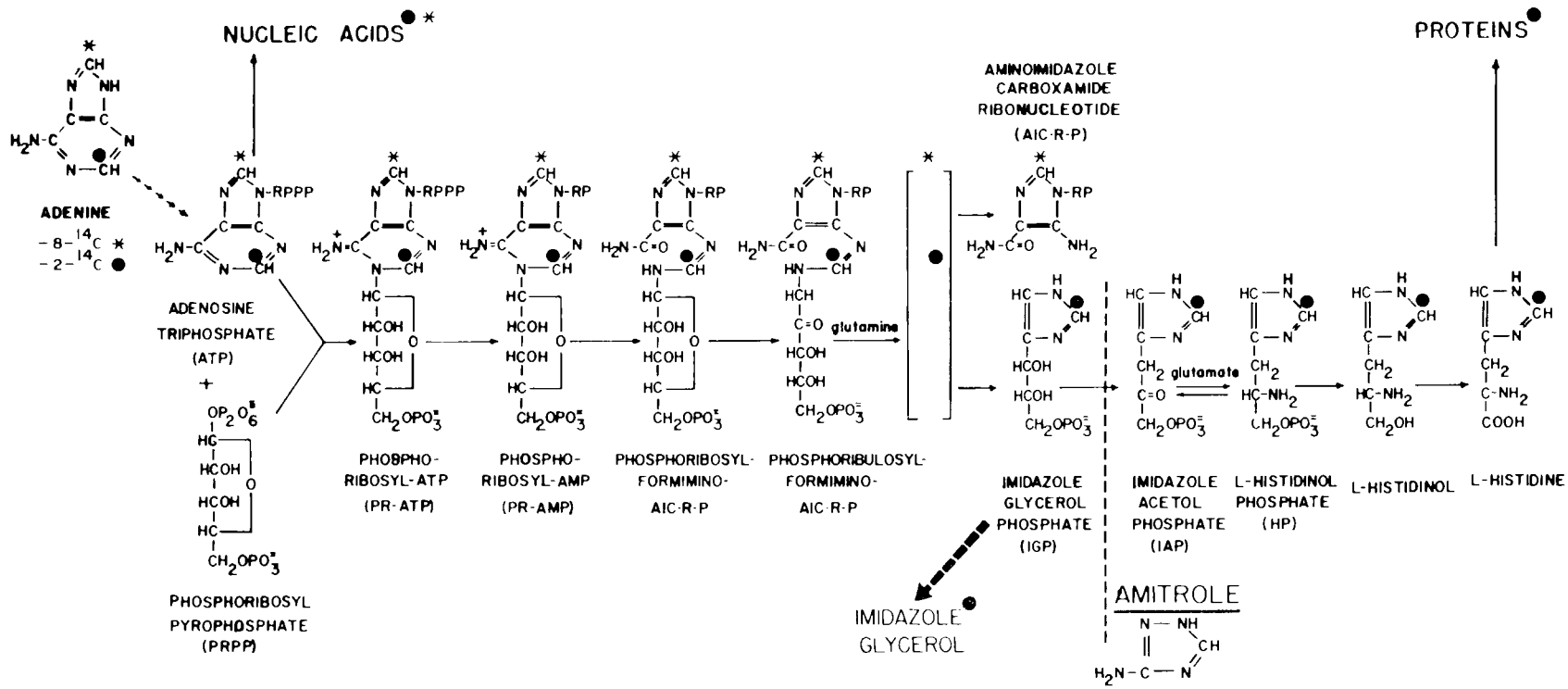

Figure 2. Schematic presentation of metabolism of adenine-2-^{14}C (dot) or adenine-8-^{14}C (asterisk) through the pathway of histidine biosynthesis showing metabolic site (dashed line) of amitrole action

Pathway modeled after that of Smith and Ames (1964)

Hartman, 1963). *S. cerevisiae* does not appear to derepress so readily (Klopotowski, 1963; Klopotowski and Hulanicka, 1963; Klopotowski and Wiater, 1965). Both feedback inhibition and enzyme repression become involved in interpretations of amitrole-induced responses of the various organisms.

Still another aspect of histidine metabolism that has influenced amitrole research is the inability of the phosphorylated histidine precursors to pass through cell membranes. Since the dephosphorylated products are not utilized as enzyme substrates, it has been impossible to utilize histidine precursors to support growth of histidine-requiring mutants or to overcome amitrole toxicities.

Following the initial implication that amitrole inhibited histidine biosynthesis rather than histidine utilization (Hilton, 1960), a precursor of histidine, imidazoleglycerol phosphate (IGP), was found to accumulate in yeast cells inhibited by the herbicide (Hilton and Kearney, 1965; Klopotowski and Hulanicka, 1963). The precursor was dephosphorylated and excreted into the external nutrient medium. The obvious implication of IGP dehydratase as the site of amitrole action was subsequently demonstrated in vitro with the enzyme isolated from yeast (Klopotowski and Wiater, 1965) and bacteria (Hilton *et al.*, 1965). More recently Wiater (Klopotowski *et al.*, 1968) has obtained and characterized the enzyme from higher plants. The IGP dehydratase enzymes from all organisms exhibit an enzyme-amitrole dissociation constant (K_i) of about $3 \times 10^{-5}M$ compared to an enzyme-IGP dissociation constant (K_m) at least 10 times larger. Thus, the affinity of amitrole for its site of action is considerably greater than that of the enzyme's natural substrate.

Additional enzymic sites for amitrole inhibition within the histidine biosynthetic pathway have been sought by the various investigators, but none have been found. At least eight of the 10 enzymes have been tested in vitro. The earlier hypothesis that amitrole or its phosphorylated glycoside is a general inhibitor of purine utilization is now untenable as an explanation of amitrole inhibition of histidine metabolism. Inhibition of histidine formation necessarily releases the biosynthetic pathway from the normal suppression due to feedback inhibition. Consequently, the histidine pathway becomes even more competitive for available purine precursors—as evidenced by excess production of IGP. Thus, amitrole actually increases adenine utilization by the pathway. When adenine-2-^{14}C is introduced into a yeast (Hilton and Kearney, 1965) or bacterial growth system (Table III), one finds that ^{14}C normally incorporated into nucleic acids and into histidine is diverted largely into IGP. The journey for the labeled carbon terminates in the external medium as IG-2-^{14}C. Additional consequences of the release from feedback inhibition as an explanation of other expressions of amitrole toxicity will be discussed with the effects of amitrole on purine metabolism.

The yeast *S. cerevisiae* Meyen ex Hansen (bakers' yeast) is the most amitrole-sensitive microorganism yet discovered. Cell multiplication is inhibited 50% by $3 \times 10^{-5}M$ amitrole whereas 3000 times more herbicide is required when histidine is added to the nutrient medium (Hilton, 1960). The toxicity of amitrole to growth is apparently enhanced by the dephosphorylation and elimination of IGP from cells, thereby limiting the extent to which the competitive substrate can accumulate and displace the inhibitor from its site of action.

In contrast to yeast, most bacteria are only slightly and temporarily affected by concentrations as high as $2 \times 10^{-3}M$. These differences in sensitivity do not involve differences in sensitivity of the two IGP dehy-

Table III. Distribution of ^{14}C from Adenine-2-^{14}C and Adenine-8-^{14}C in Growth Medium and Cellular Fractions of *S. typhimurium* as Affected by Amitrole and Histidine[a]

Isotope and Sample Fraction	Treatment			
	None	Amitrole, $2 \times 10^{-2}M$	Histidine, $2 \times 10^{-4}M$	Amitrole + histidine
		10^3 Counts/Min.		
Adenine-2-^{14}C				
External medium[b]	30.4	214.0	31.6	34.0
Cell fractions[c]				
Metabolites	5.1	10.2	7.1	7.0
Nucleic acids	108.6	24.0	152.8	135.6
Proteins	33.1	3.2	0.6	0.7
Adenine-8-^{14}C				
External medium[b]	8.5	210.5	8.3	8.5
Cell fractions[c]				
Metabolites	8.2	28.7	10.2	10.0
Nucleic acids	193.8	53.4	203.6	218.1
Proteins	1.6	0.3	0.7	1.0

[a] Average of two experiments containing two replications each. Bacterial cells cultured in glucose-minimal medium (Ames *et al.*, 1961a) at 37° C. with aeration until a logarithmic growth rate was obtained. To 1 ml. of prewarmed treatment solution containing 1 μc. of adenine-^{14}C was added 9 ml. of culture containing 4.3 to 5.3 mg. dry wt. of bacteria. Cells harvested after 30-minute incubation and fractionated as reported for similar experiments with yeast (Hilton and Kearney, 1965).
[b] Counts per 10 ml.
[c] Counts per mg. dry wt. of bacteria.

dratase enzymes (Hilton *et al.*, 1965; Klopotowski and Wiater, 1965); and do not appear to involve differences in permeability of organisms to amitrole or to inactivation of the inhibitor (Hilton and Kaufman, 1967). Growth curves for the bacteria (Figure 1) show that $2 \times 10^{-2} M$ amitrole immediately inhibits growth, but that inhibition is followed by a slow recovery. The recovery phenomenon results from a greatly increased formation of histidine biosynthetic enzymes (derepression) triggered by the shortage of histidine that resulted from amitrole inhibition of histidine biosynthesis (Hilton *et al.*, 1965). The resulting increase in IGP dehydratase content therefore increases the rate of histidine biosynthesis. Yeast does not derepress (Klopotowski, 1963; Klopotowski and Hulanicka, 1963; Klopotowski and Wiater, 1965) as readily and is, therefore, much more sensitive to amitrole than *Salmonella*. Thus, differences in the recovery mechanism appear to explain selective toxicity of amitrole in some microorganisms.

The significance of derepression as a protective mechanism against amitrole toxicity is illustrated by the much enhanced sensitivity (Hilton *et al.*, 1965) of the bacteria to amitrole in the presence of 1,2,4-triazole-3-alanine (triazolealanine), a histidine analog that prevents derepression (Levine and Hartman, 1963). Individually, amitrole and triazolealanine are relatively ineffective as bacteriocides; but with triazolealanine present to prevent the recovery mechanism, toxicity of amitrole to bacteria is comparable to that observed with yeast. If the repression-derepression principle should be found eventually to be involved in sensitivity or resistance of higher plants to some herbicides, the prospect of adding nontoxic chemicals to prevent recovery mechanisms has obvious practical implications for altering patterns of selectivity. [There is little hope that the amitrole-triazolealanine synergism can be demonstrated in plants under field conditions. Even if it could, the practice could not compete economically with the present and less expensive commercial practice utilizing synergism of thiocyanate and amitrole. This synergism appears to reside in the thiocyanate inhibition (Carter, 1965) of amitrole detoxication brought about by enzymic combination of the herbicide with serine to form β-(3-amino-1,2,4-triazolyl-1-)α-alanine (Massini, 1963). Thiocyanate is not synergistic with amitrole against *Salmonella* growth (Hilton, 1967) because this bacterium does not form the amitrole metabolite (Hilton and Kaufman, 1967).]

Inhibition of histidine biosynthesis is the major mechanism by which amitrole controls growth of most heterotrophic or achlorophyllous organisms. The inhibition can be demonstrated also in autotrophic, chlorophyllous organisms but some other inhibition of greater physiological significance appears to control growth in them. The suggested phylogenetic division of organisms (with respect to inhibition of histidine biosynthesis as the primary mechanism of amitrole growth-controlling action) is based on reported protections of growth by histidine. Statements that histidine protects growth of chlorophyllous algae can be found in the literature, but these appear to involve erroneous interpretation of the work of others. If growth protection by histidine in green autotropic algae has been observed by anyone, the data have not been reported. In fact, Castelfranco and Bisalputra (1967) found just the opposite with *Scenedesmus quadricauda*, a colonial green alga related to *Chlorella*. In this culture, adenine—but not histidine—protected growth. Wolf (1962) found that *Chlorella pyrenoidosa* responded to purines but not to riboflavin for growth protection. He did not evaluate histidine. In contrast, *Prototheca zopfii* (Casselton, 1964, 1966), an achlorophyllous alga believed to be a colorless *Chlorella*, was protected by histidine but not by adenine or riboflavin. However, as evidenced by IGP accumulation, amitrole inhibited histidine biosynthesis in both chlorophyllous *Chlorella vulgaris* and in achlorophyllous *Prototheca zopfii*. Siegel and Gentile (1966) found that growth of *Chlorella* was more sensitive to amitrole than that of *Prototheca*, whereas *Prototheca* accumulated three times as much IGP as *Chlorella*—a difference which conforms to the hypothesis (Guérin-Dumartrait, 1966) that inhibition of purine biosynthesis is the most sensitive mechanism for amitrole action in green algae.

Feedback inhibition facilitates evaluation of the physiological significance of inhibited histidine biosynthesis as the mechanism of amitrole growth-controlling action. Thus, when exogenous histidine eliminates both IGP accumulation and growth inhibition, then inhibited histidine biosynthesis seems established as "the" mechanism of action. However, when exogenous histidine eliminates IGP accumulation without affecting amitrole inhibition of growth, then the growth-controlling action must be sought at some site other than in histidine biosynthesis. Davies (1968), working with cells of higher plants in suspension culture, found that IG and trace amounts of IGP accumulated in amitrole-treated cells. Histidine prevented this excess IGP production without circumventing amitrole-induced inhibition of growth. A similar combination of interactions appears likely for most green algae.

The inhibition of histidine biosynthesis alone cannot be considered a satisfactory explanation of herbicidal actions of amitrole in higher plants. Attempts to negate amitrole inhibition of growth of intact seedlings or mature plants by treatments with histidine, its salts, or its esters applied to seeds, roots, shoots, or severed veins have invariably produced negative results. The failure cannot be attributed to poor penetration or translocation, because histidine is readily absorbed and translocated in barley (Crafts and Yamaguchi, 1964).

The most convincing evidence against IGP dehydratase as the major site of growth-controlling action, however, was obtained from studies (McWhorter and Hilton, 1967) with maize seedlings. These studies suggest that adequate levels of free histidine are available to shoots from reserves stored in the seed and that the high levels of free histidine persist longer in tops of the amitrole-treated seedlings than in the untreated. Consequently, the histidine biosynthetic pathway is possibly nonexistent (repressed) but more probably nonfunctional (feedback inhibited) during a period in which amitrole exhibits an herbicidal action. Since

amitrole inhibits growth of seedlings while the plant has a stored supply of free histidine which is not being utilized for protein synthesis, the major mechanism of amitrole action in germinating seedlings can hardly be the same as that demonstrated for yeast, bacteria, and colorless algae. However, in vitro, IGP dehydratase from plants is inhibited by amitrole (Klopotowski *et al.*, 1968). Furthermore, histidine biosynthesis in cells from a rose is inhibited (Davies, 1968) and in the absence of seed reserves the total histidine content of maize is reduced (McWhorter and Hilton, 1967). Therefore, inhibited histidine biosynthesis may be a contributing factor in herbicidal action in mature plants. Even so, Davies' evidence that exogenous histidine prevented IGP accumulation, without protecting growth of rose cells in suspension culture, argues that still another site of action, at least equal in sensitivity to that of IGP dehydratase, must be involved in growth-controlling action.

Two exceptions have been reported to the generalization that histidine does not negate amitrole inhibition of higher plant growth. The earliest, by Jackson (1961), reported that histidine partially reversed toxicity of amitrole to growth of root hairs on intact seedlings of *Agrostis alba* L. The study was complicated by the fact that histidine was considerably more toxic than amitrole to root hair growth. Nevertheless, growth of the root hairs in $0.01M$ amitrole was increased almost 50% by $10^{-4}M$ histidine. More recently, Burt (1967) found that histidine partially protected growth of cultured tissue of *Cirsium arvense* (L.) Scop. (Canada thistle) against amitrole inhibition. Adenine also nullified amitrole inhibition but did so only in combination with histidine. The relation is comparable to that found with bacteria (Figure 1).

The various investigations of amitrole-histidine interactions in higher plants provide the basis for questioning whether the mechanism primarily responsible for herbicidal action under field conditions is necessarily the same in all plants; whether the primary site of herbicide action may change during the life cycle of a plant; and even whether the primary mechanism of growth-controlling action might differ among the different parts of a single plant—e.g., root hairs may be affected by a site and mechanism which is not the principal controlling action throughout the plant.

Purine and One-Carbon Metabolism. The purine-amitrole interactions, unlike the histidine interactions, cannot be explained on the basis of a single inhibition. Different explanations seem to be demanded for purine reversals of the various physiological expressions of amitrole action in the different organisms. Even within a single organism the metabolic interactions between purines and amitrole become so numerous that assignment of physiological significance to individual amitrole actions becomes exceedingly difficult. In bacteria, for example, inhibition of histidine biosynthesis affects purine metabolism indirectly (Table III); purines prevent amitrole accumulation by cells (Hilton and Kaufman, 1967); amitrole inhibits metabolism of one-carbon units (Boguslawski *et al.*, 1967; Hilton and Kaufman, 1968); adenine nucleotides also inhibit formation of one-carbon units (Dalal and Gots, 1965; Neuman and Magasanik, 1963); amitrole inhibits a site in the purine biosynthetic pathway (Klopotowski *et al.*, 1968); and amitrole inhibits certain enzymes of the purine degradation pathway (Castelfranco and Brown, 1963; Rabinowitz and Pricer, 1956).

The first report of purine nullification of amitrole phytotoxicity was made in an oral presentation in 1958 (Aldrich, 1958). Two years later, purine circumventions of amitrole inhibition were reported also for apically treated tomatoes (Sund *et al.*, 1960); in nutrient culture of bacteria and yeasts (Bond and Akers, 1961; Hilton, 1960; Weyter and Broquist, 1960) and still later for green and red algae (Boney, 1963; Wolf, 1962). Failures to detect purine-amitrole antagonisms were reported for higher plant seedlings, colorless algae, and fungi (Table I). With some green plants (Aldrich, 1958) and with yeast (Hilton, 1960; Klopotowski and Bagdasarian, 1966), pyrimidines also partially circumvented amitrole actions. However, the problems concerning amitrole actions seem to lie in purine metabolism and little attention has been given to the pyrimidine relations. As a tentative hypothesis, pyrimidine protection might be attributed to increased effectiveness of limited purine supplies. [A comparable situation has been documented with the herbicide 2,2-dichloropropionic acid, an antimetabolite competitive with pantoic acid, which is antagonized by β-alanine in organisms with impaired capacity for β-alanine production (Van Oorschot and Hilton, 1963).]

In some organisms, the initial biochemical aberration responsible for control of growth and of other responses appears to be a purine deficiency resulting from the inhibition of histidine biosynthesis. In addition to inhibiting histidine biosynthesis, amitrole has been shown to inhibit both growth and riboflavin biosynthesis in *Eremothecium ashbyii* (Hilton, 1966; Sund and Little, 1960) and both growth (Hilton, 1960) and nucleic acid formation in *S. cerevisiae* (Hilton and Kearney, 1965). All of these inhibitions are circumvented or nullified by exogenous histidine. Since purines serve as precursors for metabolic processes (including biosynthesis or nucleic acids, riboflavin, and histidine), the reasonable interpretation is that the inhibition of histidine biosynthesis by amitrole releases the histidine pathway from metabolic control (feedback inhibition) and makes it more competitive for limited supplies of purine precursors. As a result, precursors are diverted into IGP accumulation at the expense of growth or excess riboflavin production. This type of indirect effect on purine metabolism is further supported by the facts that purines can replace histidine for protection of yeast growth against the lowest toxic concentrations of amitrole (Hilton, 1960; Klopotowski and Bagdasarian, 1966) and that the purine protection can be duplicated (Hilton, 1966) by *N*-hydroxy-*N*-formyl glycine (hadacidin), an inhibitor of the first of two steps involved in the conversion of inosine monophosphate to adenosine monophosphate (Shigeura and Gordon, 1962a, 1962b). Thus, within certain limits, hadacidin action appears to sub-

stitute for the missing feedback inhibition. Presumably it slows the flow of purines into the amitrole-induced, excess IGP production. As predicted, hadacidin is no more effective than are purines for circumvention of amitrole toxicity; and neither purines nor hadacidin can approach the effectiveness of histidine.

Not all of the observed indirect effects of amitrole on purine metabolism can be explained by a purine shortage caused by release of the histidine biosynthetic pathway from feedback inhibition. The utilization of carbon-labeled adenine for nucleic acid biosynthesis in yeast and bacteria is inhibited also under circumstances in which histidine, but not purines, could protect growth.

Table III contains data from an experiment on the distribution of ^{14}C from adenine-2-^{14}C and adenine-8-^{14}C among various cellular components of bacterial cells during a 30-minute experimental period following amitrole treatment. The results are comparable to those previously reported for adenine-2-^{14}C applied to yeast cells (Hilton and Kearney, 1965) and are predictable from a knowledge of histidine metabolism (Figure 2). Thus, in untreated cultures, (Table III, column 1) adenine-2-^{14}C was utilized mostly for nucleic acid biosynthesis; but approximately a third as much ^{14}C was utilized in histidine-^{14}C biosynthesis, thereby yielding radioactive protein. Amitrole (column 2) inhibited protein labeling by inhibiting histidine biosynthesis; and the isotope diverted by amitrole from protein (and from nucleic acids) accumulated in the cell as IGP, and was excreted to the external medium as IG. Exogenous histidine (column 3) prevented protein labeling by feedback inhibition of the histidine biosynthetic pathway and the diverted isotope showed up in nucleic acids. The effect of the amitrole-histidine combination treatments (column 4) was identical to that of histidine alone. The adenine-8-^{14}C data in Table III appears identical to those for adenine-2-^{14}C except for two predictable differences: adenine-8-^{14}C did not label protein appreciably since it cannot yield radioactive histidine; and the ^{14}C accumulated in the external medium of amitrole-inhibited cultures was not IG, but rather was predominantly one unidentified compound presumed to be a derivative of amino-imidazole carboxamide ribonucleotide (Figure 2).

In the presence of either adenine-2- or adenine-8-^{14}C, amitrole inhibited labeling of nucleic acids (Table III). Since this inhibition was prevented by histidine, the effect was an indirect result of IGP dehydratase inhibition. Since adenine did not protect growth during these 30-minute tests (Figure 1), the effects on nucleic acid cannot be attributed to a shortage of purines. An alternative hypothesis is needed and is available from the literature on amino acid starvation. In bacteria, RNA formation is limited by nutritional deficiencies of individual amino acids (Ezekiel, 1964; Kurland and Maaloe, 1962; Stent and Brenner, 1961). Amino acid starvation is believed to produce cells containing transfer RNA (t-RNA) free of amino acids. It has been postulated that t-RNA, when unesterified with amino acids, inhibits RNA synthesis whereas t-RNA esterified with amino acids does not. Tissieres *et al.* (1963) observed that soluble RNA, not bound to amino acids, inhibits the enzyme RNA polymerase in vitro. The inhibition was considerably less when the RNA was bound to amino acids. Amitrole-induced histidine starvation in bacteria and in yeast undoubtedly produces a similar effect on formation of microbial ribonucleic acid. Although some of the early literature on amitrole effects on nucleic acid metabolism in green plants could be interpreted in light of this form of indirect action, more detailed and recent studies seem to require a more specific direct action on purine metabolism (Bartels and Wolf, 1965; Guérin-Dumartrait, 1966; Siegel and Gentile, 1966).

Still different problems in purine metabolism are detected in bacteria (Hilton and Kaufman, 1968; Hilton *et al.*, 1965) and also in yeast (Klopotowski and Bagdasarian, 1966), once the indirect effects of histidine deficiencies are eliminated by addition of exogenous histidine. Even in the presence of biologically saturating quantities of histidine, amitrole at external concentrations above $10^{-2}M$ still cause a delayed inhibition of growth of *Salmonella* (Figure 1). The delay is proportional to amitrole concentration and the growth inhibition is totally offset by adenine. Protection is complete as long as the added adenine supply lasts (Figure 3). These data suggest that either purine biosynthesis is inhibited or that purines inhibit amitrole uptake. Both inhibitions have been found, but only the former seems significant to growth control.

One of the direct interactions between purines and amitrole in *S. typhimurium* is the inhibition of amitrole accumulation by purine (Hilton and Kaufman, 1967). Studies on the fate of amitrole in the bacteria revealed that less than 2% of the applied amitrole was absorbed during a 3-hour period, and that over 90% of the amitrole absorbed by growing cultures was bound tightly within the cell (Figures 4 and 5). The reduction in amitrole absorbed and bound, without corresponding reduction in toxicity to growth, is illustrated for hypoxanthine in Figure 4. Adenine was the most effective purine for elimination of bound amitrole. When histidine was omitted from the growth medium and bound amitrole determined at a time at which adenine exerted no growth protection, adenine inhibited amitrole uptake (or binding) as much as 95%. Therefore, much of the bound amitrole appears to be inactive as a growth inhibitor, and apparently only a very small percentage of the amitrole absorbed by bacteria is involved in growth toxicities. One further concludes that purine inhibition of uptake or internal binding does not explain adenine circumvention of growth toxicities.

That portion of the inhibition of bacterial growth which is not counteractable by histidine but is overcome by adenine is presumably an effect on purine biosynthesis, since inhibited uptake and binding do not account for growth protection. Recent developments suggest that at least two sites of action must be involved in the inhibition of purine biosynthesis. One of these sites apparently lies directly in the purine biosynthetic pathway while the other is associated with one-carbon metabolism. Klopotowski *et al.* (1968), working with

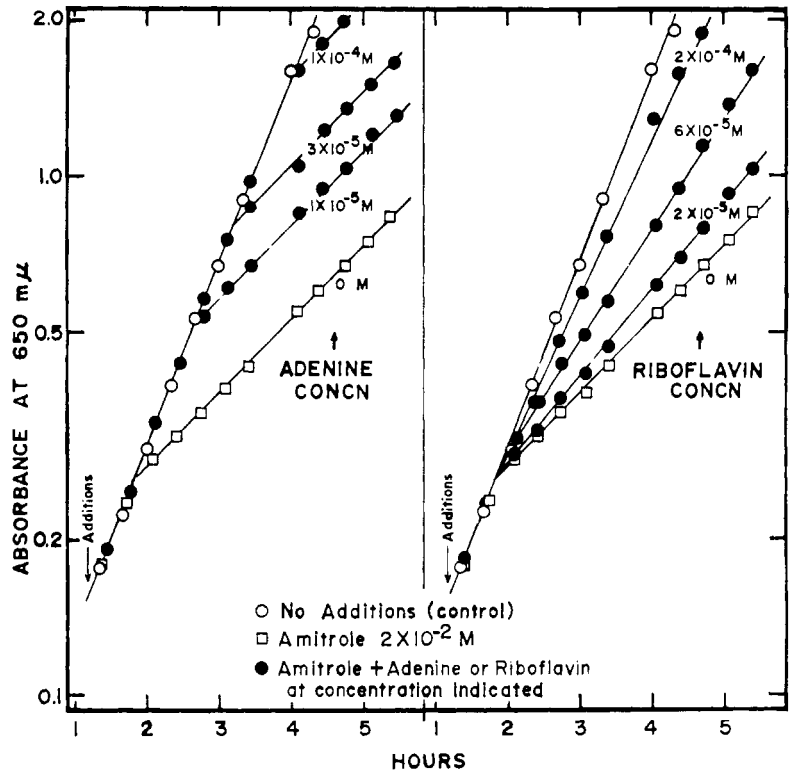

Figure 3. Relation of adenine and riboflavin concentrations to growth response of *S. typhimurium* LT 2 inhibited by amitrole

All cultures were grown with aeration at 37°C. in glucose-minimal medium (Ames *et al.*, 1961a) containing 2 × $10^{-4}M$ histidine

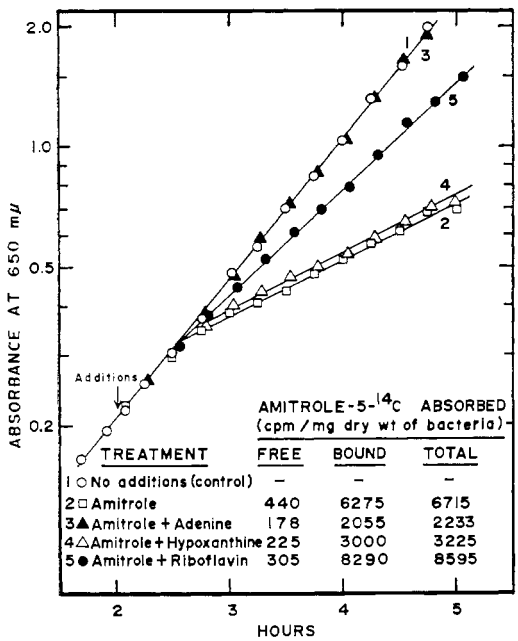

Figure 4. Effect of adenine, hypoxanthine, and riboflavin on amitrole inhibition of growth and on accumulation of amitrole-5-^{14}C by *S. typhimurium* in medium supplemented with histidine

Final concentration of additions were 2 × $10^{-4}M$ metabolites and 2 × $10^{-2}M$ amitrole plus 1.0 μc. amitrole-5-^{14}C (specific activity, 1.2 mc./mmole)

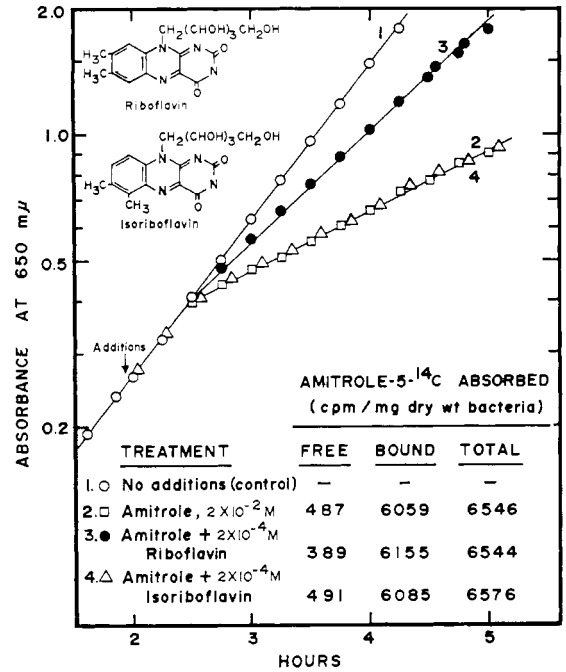

Figure 5. Effect of riboflavin and isoriboflavin on amitrole inhibition of growth and on accumulation of amitrole-5-^{14}C by *S. typhimurium* in medium supplemented with histidine

Amitrole-5-^{14}C (specific activity, 1.2 mc./mmole) added with unlabeled amitrole

purine-requiring mutants of *Salmonella,* have obtained evidence of an inhibition in the purine biosynthetic pathway that prevents formation of the imidazole ring. Our own attempts to use glycine-^{14}C to demonstrate accumulation of a labeled intermediate in the purine pathway of the wild type organism were unsuccessful. But our failures possibly resulted from an additional inhibition which prevented the formation of one-carbon units required for build up of the intermediate.

The inhibition of one-carbon metabolism in *Salmonella* by triazoles was first discovered by Boguslawski *et al.* (1967), who were attempting to find a structure capable of affecting purine biosynthesis without affecting histidine biosynthesis. Instead, they found that unsubstituted triazole inhibition of growth was potentiated by adenine and glycine, was unaffected by histidine, and was reversed by methionine, serine, or cysteine. From work with glycineless and serineless mutants they concluded that triazole prevented the production of one-carbon units derived from glycine. Subsequently, Hilton and Kaufman (1968) showed similar metabolite-inhibitor interactions for amitrole and other substituted triazoles (Table II). The possibility that amitrole inhibited a site in the pathway for methionine biosynthesis was a serious consideration, since rhizobitoxine (a partially identified low molecular weight phytotoxin synthesized by *Rhizobium japonicium*) produces amitrole-like symptoms in higher plants. In *Salmonella,* this toxin is a specific inhibitor of β-cystathionase (Owens *et al.*, 1968), an enzyme of methionine biosynthesis. Toxicity of rhizobitoxine was circumvented by methionine but not by serine. Thus, even though methionine was more effective than serine against the triazoles in *Salmonella* growth, the serine circumvention of inhibition argues against a site of action for triazoles in the methionine biosynthetic pathway. Consequently, the triazoles as a group appear to be inhibitors of production of one-carbon units.

Amitrole, however, differs from the other triazole inhibitors of one-carbon production in that adenine reverses amitrole toxicity whereas it potentiates toxicity of the others. Inhibition of *Salmonella* growth by adenine alone has been observed also, and attributed to interference with one-carbon metabolism through inhibition by adenine nucleotides of folic acid biosynthesis from guanine nucleotides (Dalal and Gots, 1965). In addition, adenine and other purines are believed to repress the enzyme system in *Escherichia coli* required for metabolism of single-carbon units derived from glycine (Neuman and Magasanik, 1963). The inhibition of production of one-carbon units by adenine apparently outweighs the reduced requirement for one-carbon units (sparing action) that adenine would be expected from feedback inhibition of the purine biosynthetic pathway. Serine, the main source of one-carbon units, does not satisfy the cell's total requirements. Neuman and Magasanik (1963) indicated that carbon 2 of glycine must supply the rest. As pointed out by Boguslawski *et al.* (1967), this is the apparent process inhibited by triazole; thus, adenine synergism with triazoles is to be expected. The fact that adenine nullifies amitrole toxicity rather than enhancing it, requires that amitrole have an additional action on purine metabolism not achieved with the other triazoles. This presumably is the inhibition of the purine biosynthetic pathway being currently elucidated by the investigators in Warsaw. The fact that 3-hydroxytriazole shares with amitrole an inhibition of purine metabolism, but not the inhibition of one-carbon metabolism or the interaction with riboflavin (Table II), would seem to add circumstantial evidence to the hypothesis that two sites of action for amitrole are involved in purine metabolism.

From experiments such as those in Figure 1, one finds that the maximum protection afforded by methionine is that pictured. In contrast, adenine affords complete circumvention of toxicity of $2 \times 10^{-2}M$ amitrole. However, as amitrole concentration is increased to $2 \times 10^{-1}M$, the maximum protection afforded by adenine (or by riboflavin) is progressively reduced. At the higher concentrations of amitrole, with histidine and adenine added to the growth medium, either methionine or serine is capable of complete protection of growth in contrast to incomplete protection obtained at lower amitrole concentrations with histidine present but adenine missing (Hilton and Kaufman, 1968). Apparently, the amitrole inhibition of growth does involve both an inhibition of one-carbon production and an inhibition of purine biosynthesis. Adenine initially can circumvent both inhibitions by supplying the end-products of the purine pathway and by eliminating the major requirement for one-carbon units. Methionine, on the other hand, can serve as a one-carbon donor, thus circumventing that inhibition, but is incapable of offsetting the inhibition in the purine biosynthetic pathway. At higher concentrations of amitrole, the inhibition of one-carbon production seems so severe that the sparing effect of adenine on one-carbon units is not sufficient to permit the required production of other one-carbon products, such as methionine and serine.

In green algae, a direct inhibition of purine metabolism by amitrole is the inhibited process most directly related to growth control. Purines negate amitrole toxicity whereas riboflavin or histidine apparently do not (Table I). Guérin-Dumartrait (1966) demonstrated with synchronous cultures of *C. pyrenoidosa* Chick that inhibition of DNA and RNA biosynthesis preceded inhibition of cell division. She concluded that the primary mode of action of amitrole on *Chlorella* was by a competitive inhibition which prevented nucleic acid biosynthesis. Similarly, Siegel and Gentile (1966), in studies which demonstrated amitrole inhibition of histidine biosynthesis in *C. vulgaris,* concluded that indirect effects resulting from histidine deficiencies could not explain amitrole action on nucleic acid metabolism. From their studies one can predict that a combination of histidine and adenine should be more effective than adenine alone in circumventing amitrole toxicity to green algae.

Current research on purine metabolism has concentrated on bacteria as research organisms for identifying sites of action. The frequent inability to extend such studies to higher organisms often results in criticism of

the microbial approach. With higher plant materials, Davies (1968) and Burt (1967) have obtained results that lead us to believe the principles developed for *Salmonella* will aid in understanding phytotoxicity of amitrole. Burt, for example, working with higher plant tissue cultures, found one tissue in which adenine circumvents amitrole toxicity but only in the presence of histidine—exactly the situation observed with bacteria. Davies (Table IV) found that growth of cells of *Rosa* hybrid (Paul's Scarlet Rose) in suspension culture is inhibited by amitrole and this inhibition is enhanced by adenine—comparable to the effect between triazoles and adenine in bacteria. Furthermore, the combination of histidine, adenine, and serine reduced amitrole inhibition from 89 to 58%, whereas the three metabolites had little effect individually. Inhibition of histidine biosynthesis by amitrole was documented in these plant cells by demonstration of IG accumulation. Although any attempt to explain the adenine and serine protections of plant cell growth are probably premature, one must be impressed by the similarities of Davies' results to those obtained with bacteria. A combination of histidine, adenine, and serine almost totally reverses the toxicity of 0.1*M* amitrole to *Salmonella* whereas any one metabolite alone is without effect (Hilton and Kaufman, 1968).

Still another mechanism exists in higher plants for interference with glycine-serine metabolism. Carter and Naylor (1960, 1961) observed that a variety of higher plant species utilized glycine-^{14}C or serine-^{14}C for formation of a nontoxic metabolite of amitrole, that is now generally accepted to be β-(3-amino-1,2,4-triazoly-1-)-α-alanine (Carter, 1965; Massini, 1963). The depletion of glycine-serine reserves resulting from amitrole metabolism represents yet another hypothesis for the mechanism of amitrole phytotoxic action that would limit purine biosynthesis. Reductions in glycine and serine levels in amitrole-treated plants have indeed been reported (McWhorter and Hilton, 1967; Naylor, 1964) but are not consistently observed (Bartels and Wolf, 1965; Burt, 1967). A physiological significance for growth control by this mechanism of glycine-serine depletion cannot be documented. Repeated attempts to reduce amitrole damage by applications of serine or glycine to germinating seeds, established seedlings, or mature plants have invariably ended in failure. The only data yet available to suggest that amitrole controls growth of plant material by tying up serine and glycine are those shown in Table IV. Even here, the small effect observed could possibly result from a mechanism causing a shortage of one-carbon units similar to that postulated for bacteria. The protective action of glycine, however, seems to favor the detoxication hypothesis.

Direct evidence of amitrole inhibition of purine biosynthesis in higher plants was obtained with wheat seedlings grown in light. Bartels and Wolf (1965) found that the RNA content and the incorporation of glycine-^{14}C and formate-^{14}C into RNA was reduced in amitrole-treated tissue. The incorporation of formate into adenine and guanine was inhibited equally, thereby suggesting inhibition of an early step in purine biosynthesis. However, dark-grown tissue showed no effect of amitrole on nucleic acid or acid-soluble nucleotide content. As pointed out by Bartels and Wolf (1965), etiolated tissue of dark-grown plants receives its purines only from the grain endosperm, whereas light-grown tissue receives nucleic acid precursors both from endosperm and by de novo synthesis. In this respect, it is of interest that the partial nullifications of amitrole toxicity by purines applied to intact higher plants have been reported only for established plants (independent of seed reserves). Inhibited purine biosynthesis possibly contributes to growth control in older plants. However, since nucleic acid formation is unaffected by amitrole in dark grown seedlings, the herbicidal action of amitrole must involve yet another site of action. Early growth of wheat or barley seedlings in the dark represents one of the two most amitrole-sensitive biological processes on record. (Growth of these seedlings and growth of bakers' yeast are both inhibited 50% by $3 \times 10^{-5}M$ amitrole.) The failure of adenine, serine, or methionine to circumvent amitrole toxicity to wheat seedlings suggests that none of several inhibitions—inhibition of the purine biosynthetic pathway, the hypothetical inhibition of formation of one-carbon units, and depletion of glycine and serine by amitrole detoxication—comprises a satisfactory explanation of phytotoxicity to young seedlings. However, these inhibitions, like inhibition of histidine biosynthesis, may be able to control growth of plant tissues under specialized conditions.

Riboflavin Metabolism. At least three independent processes are involved in amitrole-riboflavin interactions. Amitrole inhibition of riboflavin formation is an indirect effect of its action on histidine biosynthesis. Exogenous riboflavin negates amitrole toxicity by either of two mechanisms. A nonbiological mechanism takes precedence in light; but a very definite biological mechanism is observed in the total absence of light. Most

Table IV. Interactions of Several Metabolites and Amitrole on Cells of *Rosa* hyb. (Paul's Scarlet Rose) in Suspension Culture[a]

Supplemental Amino Acid Treatments	Relative Growth[b] of Amitrole-Treated Cultures			
	Water	Adenine, 0.5m*M*	Histidine, 0.25m*M*	Adenine + histidine
None	11.5	1.5	14.3	20.0
Glycine, 1m*M*	15.0	1.0	34.0	38.0
Serine, 1m*M*	16.0	3.0	29.2	42.0

[a] Data of Davies (1968), published by permission.
[b] Growth of cultures treated with amitrole (1m*M*) expressed as percentage of corresponding control cultures containing no amitrole.

of the recent literature has dealt with the nonbiological interaction. Consequently, the biological mechanism for nullification of amitrole toxicity has been dismissed frequently without adequate consideration.

The purpose here is not only to review the amitrole-riboflavin interactions which are apparently understood, but also to present the additional information demonstrating a biological mechanism for riboflavin nullification of amitrole toxicity; put related studies in proper perspective; and record efforts made to resolve the biological interactions.

That riboflavin overcomes the toxicity of amitrole was first shown by Sund *et al.* (1960), who used mature tomato plants grown in the greenhouse. The early proposal that amitrole inhibits an enzyme of the riboflavin biosynthetic pathway (Sund, 1961) seemed justified for three reasons: amitrole inhibited an enzyme catalyzing a comparable reaction in the purine degradative pathway (Rabinowitz and Pricer, 1956); amitrole inhibited production of riboflavin by *Eremothecium ashbyii* (Hilton, 1966; Sund and Little, 1960); and riboflavin content was reduced in maize and pea plants treated with amitrole. The circumstances of these experiments, however, permitted alternate or additional interpretations.

E. ashbyii is a yeast-like organism which produces excessive quantities of riboflavin and is the organism used most commonly for studies on riboflavin biosynthesis. Riboflavin production by the organism is much more sensitive to amitrole than is its growth. However, histidine, which stimulates both growth and riboflavin production by the untreated organism, completely negates amitrole inhibition of both processes (Hilton, 1966). Since both histidine and riboflavin are derived from common precursors, *viz.*, purines, one suspects that the inhibition of IGP dehydratase releases metabolic controls on the histidine pathway, allowing it to become more competitive for purine precursors. As a result, there are inadequate precursors for the usual amount of excess riboflavin production. In the presence of amitrole, the organism manufactures IGP instead of riboflavin (Hilton, 1967).

Following the initial discovery of a riboflavin-amitrole interaction, the suspected chemical destruction of amitrole by photoactivated flavins was reported (Castelfranco *et al.*, 1963; Hilton, 1962), and partially elucidated (Plimmer *et al.*, 1967). Castelfranco and Brown (1963) observed destruction of amitrole by other free-radical generating systems in addition to photoactivated flavins and proposed that amitrole is converted to a free radical, that they demonstrated can either undergo ring cleavage or be adsorbed to protein. Cleavage of the triazole ring of amitrole-5-^{14}C by photoactivated riboflavin or by other free-radical induced decomposition yields unlabeled urea, unlabeled cyanamide (Plimmer *et al.*, 1967), and labeled CO_2 (Table V). In addition to products arising from ring cleavage, other products are formed which possibly arise by polymerization of amitrole radicals.

The chemical interaction of riboflavin and amitrole is visualized in Figure 6. Solutions of light- and dark-exposed riboflavin-2-^{14}C, with and without amitrole-^{12}C, and of amitrole-5-^{14}C, with and without riboflavin-^{12}C, were compared by thin-layer chromatography. In light, riboflavin photodecomposed to three products. (Unknown F in Figure 6 is the major breakdown product and may be used as an indicator of light exposure in biological experiments.) Amitrole protected riboflavin from photodecomposition (Figure 6, second column) but was itself degraded in the process (Figure 6, column 4 and Table V). In darkness, no decomposition of either riboflavin or amitrole could be detected. Isoriboflavin, a nonphysiological flavin which is also capable of photodestruction of amitrole (Hilton, 1962), behaved like riboflavin in similar nonradioactive experiments, except that only two photodecomposition products of isoriboflavin were observed. The facts that amitrole is degraded by photoactivated riboflavin, that riboflavin-amitrole interactions on growth of any organism have been reported only in tests in which the two chemicals are applied together, and that both plants (Mer, 1957) and bacteria (Wilson and Pardee, 1962) are considered relatively impermeable to riboflavin would seem to represent a strong case for a nonbiological mechanism to explain riboflavin nullification of amitrole toxicity.

However, the evidence now seems overwhelming that riboflavin nullification of amitrole inhibitions can involve

Table V. Ability of Riboflavin to Degrade Amitrole-5-^{14}C to $^{14}CO_2$ in Photochemical and Bacterial Systems

System and Treatment	Light Exposure	Duration, Hours	Distribution of ^{14}C,[a] % of Total			
			CO_2	Medium	Bacteria	Total recovered
Photochemical system						
Control	Light[b]	24	6.4	94.9		101.3
Riboflavin 5 × $10^{-4}M$	Light	1	3.9			
	Light	4	55.4			
	Light	24	74.5	25.6		100.1
Riboflavin 5 × $10^{-4}M$	Dark	24	4.0	91.9		95.9
Bacterial system[c]						
Control	Dark	24	1.3	83.7	10.1	95.1
Riboflavin 5 × $10^{-4}M$	Dark	24	1.2	86.6	10.7	98.5

[a] Initial amitrole concentration: 1.3 × $10^{-5}M$ (0.5 μc.).
[b] 250 foot-candles total intensity from incandescent and fluorescent light.
[c] *S. typhimurium* cultured as described in Figure 3 except that aeration was by bubbling air through a closed system rather than by rotary shaking. Comparable medium used for nonbacterial tubes. CO_2 traps consisted of 25 ml. of 2-methoxyethanol–monoethanolamine (1 to 7). One-milliliter samples of CO_2 trapping solutions were removed for analysis by liquid scintillation counting at times indicated.

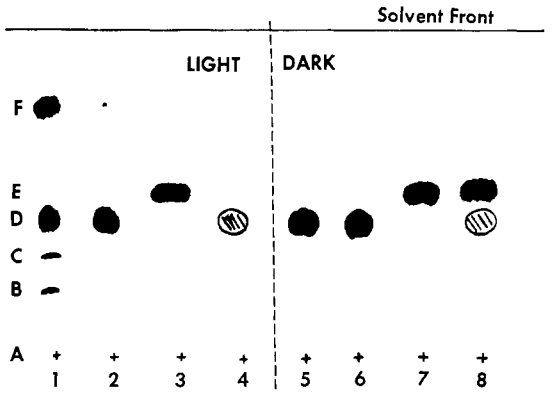

Figure 6. Thin-layer radiochromatograms showing riboflavin-amitrole interactions after 3-hour exposure to light (250 foot-candles, columns 1 to 4) or darkness (columns 5 to 8) in growth medium (Ames *et al.*, 1961a) supplemented with $2 \times 10^{-4}M$ histidine

Solutions 1 and 5. Riboflavin-2-^{14}C $3 \times 10^{-6}M$
2 and 6. Riboflavin-2-^{14}C plus amitrole $2 \times 10^{-2}M$
3 and 7. Amitrole-5-^{14}C $1.3 \times 10^{-5}M$
4 and 8. Amitrole-5-^{14}C plus riboflavin $2 \times 10^{-4}M$

Vertical designations, A. Origin
B, C, F. Unidentified photodegradation products of riboflavin
D. Riboflavin (hatched spots show non-radioactive riboflavin)
E. Amitrole

Solvent system; 1-butanol–propionic acid–water (15:7:10)

physiological mechanisms as well as photochemical mechanisms. Riboflavin nullifies amitrole toxicity to plant seedlings and to bacteria (Figure 1) in darkness. This fact should be sufficient to establish a biological mechanism for growth antagonisms; but the criticism that stray light has penetrated so-called dark experiments can cast doubt on the results unless additional evidence is presented.

The first direct evidence for a physiological mechanism of riboflavin nullification of amitrole toxicity was obtained with early growth of seedlings. Nullification in the absence of stray light was demonstrated by three experimental approaches (Hilton, 1962). First, comparative tests were made by using riboflavin and isoriboflavin, on the assumption that the nonphysiological isoriboflavin would behave like riboflavin in any chemical or photochemical reaction, but that it could not substitute for the physiological flavin in biological reactions. Chemical destruction of amitrole was mediated by both flavins in the light but not in darkness. Amitrole toxicity was nullified by both flavins in the light, but only by riboflavin in darkness. Second, toxicity of amitrole and of 2,4-dichlorophenoxyacetic acid (2,4-D) to seedling growth was nullified by riboflavin in the light but only toxicity of amitrole was nullified when the experiment was conducted in darkness. Hundreds of biologically active chemicals are known to be inactivated by riboflavin, but nullification of biological activity in the absence of light is not commonly reported. Third, nine species of plants, which varied in their sensitivity to amitrole from I_{50} values (50% inhibition of growth) of $3 \times 10^{-5}M$ to values of $10^{-2}M$, were compared for the riboflavin-dark nullification. In the most amitrole-sensitive plants, wheat and barley, over 100 times more amitrole was required to inhibit growth of riboflavin-treated plants. In contrast, amitrole sensitivity of buckwheat, the most amitrole-resistant plant, was unaffected by riboflavin treatment. All nine tested species became equally insensitive to amitrole in the presence of riboflavin, regardless of their initial sensitivity. If the riboflavin protection in the dark depended on nonphysiological inactivation of amitrole external to the plants, one would expect tolerant species to be protected as effectively as sensitive ones.

The discovery (Hilton and Kaufman, 1968) that the nullification of amitrole toxicity in darkness could be demonstrated with bacteria as well as with plant seedlings provided additional evidence for a biological antagonism. Earlier efforts to detect riboflavin nullification of amitrole toxicity to microorganisms had been unsuccessful [one of the criticisms against the physiological hypothesis (Naylor, 1964)] because the interaction was masked by the inhibition of histidine biosynthesis. Once this inhibition was relieved by exogenous histidine, the riboflavin antagonism was demonstrated (Figure 1). These data again indicated the biological nature of the riboflavin-amitrole interaction; chemical or photochemical inactivation of amitrole by riboflavin should have provided protection in the absence, as well as in the presence, of histidine.

Nevertheless, more convincing evidence was sought to exclude photochemical or chemical destruction of amitrole by riboflavin in the external nutrient medium as an explanation of results on growth shown in Figure 1. In the first series of tests, degradation of amitrole was detected by measuring $^{14}CO_2$ released from amitrole-5-^{14}C. Dark-grown bacteria with and without riboflavin treatment did not degrade amitrole-5-^{14}C to $^{14}CO_2$ (Table V). In another series of tests, bacteria were grown in the dark and treated with riboflavin-2-^{14}C or amitrole-5-^{14}C with and without unlabeled amitrole or riboflavin, respectively. External media from these bacterial cultures chromatographed like the uninoculated control media (similar to the dark series shown in Figure 6). Yet, in the same cultures, growth protection was demonstrated in the absence of photochemical destruction of compounds in the media. Since carelessness in exposing such experiments to stray light is detected by appearance of unknown F (Figure 6) on chromatograms, the absence of compound F-^{14}C indicated the validity of the biological data.

Additional evidence to exclude photochemical processes as an explanation of riboflavin nullification of amitrole toxicity to bacteria is seen in comparative evaluations of effectiveness of riboflavin and isoriboflavin (Figure 5). Since photoactivated isoriboflavin destroys amitrole in the same manner as photoactivated riboflavin, the failure of isoriboflavin to protect growth in the dark indicates that stray light was not involved in the dark-protection by riboflavin. Occurrence of any other hypothetical chemical interaction between amitrole

and flavins also seems unlikely, unless the interaction is in some way mediated by the organism.

The process by which riboflavin nullifies amitrole toxicity to bacteria involves a mechanism which prevents, or circumvents, the amitrole-induced deficiency of adenine. Riboflavin nullifies the same amitrole inhibitions that are circumvented by adenine, but not those circumvented by histidine, methionine, or serine (Hilton and Kaufman, 1968). However, two major differences in the adenine and riboflavin protections are illustrated in Figures 1 and 3. First, adenine—but not riboflavin—shows a delayed protection of growth in the absence of exogenous histidine. The delayed adenine protection is observed only after the histidine deficiency is satisfied by histidine produced as a result of the recovery mechanism (derepression of histidine biosynthetic enzymes). Riboflavin protection, on the other hand, is totally dependent on exogenous histidine (Figure 1). Second, the shape of the growth curves for the adenine-treated series of amitrole-inhibited cultures indicates that adenine is a growth limiting nutrient—i.e., protection is complete for as long as the adenine supply lasts. In contrast, the shape of the growth curves for the riboflavin-treated series indicates that the flavin is affecting a rate process (Figure 3).

Several hypotheses were considered in determining whether riboflavin might affect rate of purine metabolism by preventing amitrole from reaching its site of action. Negative results were obtained in a direct test of the hypothesis that metabolic energy mimics light energy and is used to activate riboflavin in the external medium for formation of free radicals of amitrole which then decompose. No measurable amounts of $^{14}CO_2$ were released from amitrole-5-^{14}C by riboflavin-treated bacteria (Table V). Degradation or metabolism of amitrole mediated by riboflavin within bacterial cells also seems unlikely, since ^{14}C from amitrole-5-^{14}C is neither released as CO_2 nor detected in natural products formed by *Salmonella* cells. The possibility of biologically mediated formation of an amitrole-riboflavin complex in the medium was considered but rejected because the absorption spectrum for exogenous riboflavin was unaltered by the presence of amitrole plus a culture of *S. typhimurium*. Furthermore, the protective concentrations of riboflavin are only 1/100 the concentration of amitrole used to control growth. Riboflavin protection through an extracellular mechanism, therefore, would seem to require a catalytic process.

Negative results were also obtained for the hypothesis that riboflavin prevented amitrole uptake and for the hypothesis that riboflavin inactivated amitrole mediating its binding to inert sites within cells. Riboflavin did not mimic the purine inhibition of amitrole-^{14}C uptake (Figures 4 and 5). In fact, riboflavin frequently seemed to increase the amount of amitrole extractable from bacterial cells. The riboflavin-induced increase reported in Figure 4 represents the maximum increase ever observed. Since methionine-treated cells showed similar increases, the effect was considered tentatively of no interpretive value. Another hypothesis was suggested by the facts that in vitro free radical-activated amitrole is tightly adsorbed by protein (Castelfranco and Brown, 1963), over 90% of the amitrole sorbed by bacteria is tightly bound (Figures 4 and 5), and that amitrole bound by bacteria is considered largely nontoxic (Hilton and Kaufman, 1967). Assuming that unbound, intracellular amitrole represents the toxic moiety, riboflavin-mediated adsorption of free (toxic) amitrole to inert sites on macromolecules might constitute an inactivation mechanism. Three independent experiments, for which data are averaged in Figure 5, offered some initial support for this hypothesis. Riboflavin, but not isoriboflavin, increased the amount of bound amitrole at the expense of free amitrole in the cell. Since these differences correlated with growth responses, the additional experiment shown in Figure 4 was undertaken. In this test and in others similar to it, hypoxanthine, which does not protect growth against amitrole toxicity, was more effective than riboflavin in reducing the amount of free amitrole present in cells. Therefore, the hypothesis that riboflavin protections result from conversion of free, toxic amitrole to bound, nontoxic forms seems untenable.

The investigations with amitrole-5-^{14}C conducted in darkness, therefore, led to the conclusion that riboflavin did not significantly influence the absorption, accumulation, metabolism, or degradation of amitrole by dark-grown *Salmonella* through either biological or nonbiological mechanisms. A similar conclusion was reached for higher plants from preliminary experiments with wheat seedlings.

Studies were conducted also to determine the fate of riboflavin-2-^{14}C in *S. typhimurium*. Utilization of isotope during a 30-minute or 3-hour growth period (Table VI) accounted for about 0.2% of the total applied riboflavin-2-^{14}C ($3 \times 10^{-6}M$; specific activity 34.1 mc. per mmole). Most of that taken up was extractable with 80% ethanol and was usually present only as the unmetabolized molecule. Conversion of trace amounts of the ethanol-extractable isotope to flavin mononucleotide (FMN) was observed in some experiments. Conversion to flavin adenine dinucleotide (FAD) was never detected. Amitrole partially inhibited riboflavin-^{14}C uptake in 30-minute and 3-hour experiments (Table VI) but tripled uptake in 24 hours. Conversion to physiological flavins was not enhanced by amitrole during the short-term experiments with exogenous histidine either present or absent. In 24-hour experiments, inconsistent results were obtained for amitrole enhancement of riboflavin conversion to physiological forms. No evidence of metabolic degradation of riboflavin was observed on paper chromatograms. Addition of unlabeled riboflavin, $2 \times 10^{-4}M$, to the cultures produced the surprising response of a 3- to 10-fold increase in isotope uptake during a 30-minute, 3-hour, or 24-hour incubation. The unexpected ability of riboflavin to increase its own uptake was not further investigated since it did not influence the general results or conclusions for amitrole actions.

Studies with riboflavin-2-^{14}C were initiated principally to examine the hypothesis that riboflavin serves as a precursor of purines. Thimann and Radner (1958,

Table VI. Comparative Distribution of ^{14}C in Various Fractions from Cultures of *S. typhimurium* Treated with Adenine-2-^{14}C, Riboflavin-2-^{14}C, and Amitrole plus Riboflavin-2-^{14}C

Fractional Distribution	Extraction Method	^{14}C Distribution (10^3 Counts/Min.)[a] after Application as: Adenine-2-^{14}C[b]	Riboflavin-2-^{14}C[c]	Amitrole plus riboflavin-2-^{14}C[d]
External medium		183.0	1522.0	1436.0
Bacterial fractions				
Metabolites	80% Ethanol, 55°C., 30 min.	80.0	2.5	1.7
Nucleic acids	5% TCA, 100°C., 1 hr.	910.0	0.7	0.8
Proteins	6*M* HCl, 100°C., 1 hr.	67.7	0.4	0.3

[a] Total counts extracted from a volume of 10 ml. of glucose-minimal medium containing $2 \times 10^{-4}M$ histidine.
[b] Logarithmically growing cultures harvested 30 minutes after treatment with 1 μc. of adenine-^{14}C (specific activity 15.6 mc./mmole).
[c] Logarithmically growing cultures harvested 3 hours after treatment with 1 μc. of riboflavin-^{14}C (specific activity 34.1 mc./mmole). (Recovered isotope was predominantly unmetabolized riboflavin. Qualitatively similar results were obtained for 30-minute and 24-hour incubations.)
[d] Same as footnote [c] but with $2 \times 10^{-2}M$ amitrole.

1962) found that certain purine analogs inhibited anthocyanin biosynthesis in the duckweed *Spirodela oligorrhiza*. The inhibition was circumvented by riboflavin, purines, or pyrimidines and, therefore, resembles the interactions observed between amitrole and metabolites. They suggested that riboflavin acts by serving as a readily available source of a purine moiety for continual resynthesis of a specific and unstable nucleic acid. If a similar hypothesis can be invoked to explain riboflavin and purine reversal of amitrole toxicity to *S. typhimurium*, then it must involve a process operating at minute levels (the ethanol-insoluble materials in Table VI). Comparisons of riboflavin-^{14}C and adenine-^{14}C utilization (Table VI) indicate that riboflavin metabolism is totally unlike that of adenine, that riboflavin does not serve appreciably as a precursor of nucleic acids, and that amitrole treatment does not induce it to do so.

The failure of amitrole to enhance bacterial conversion of exogenous riboflavin to its physiological forms with subsequent binding to enzyme protein demonstrates that amitrole does not induce a severe riboflavin deficiency in *Salmonella*. Since histidine is required before riboflavin protection of growth is observed, the metabolism of histidine-2-^{14}C was examined also. Neither riboflavin nor amitrole affected metabolism of histidine-^{14}C in a total histidine concentration of $2 \times 10^{-4}M$.

Although the evidence for a biological mechanism for riboflavin nullification of amitrole toxicity seems convincing, no progress has been made in elucidating the basic mechanism responsible for the interaction. The most logical hypotheses have been tested by methods expected to reveal their validity, but the results have been uninformative.

CONCLUSIONS

The most productive approach to understanding the mode of action of amitrole to date has been the identification of sites and elucidation of mechanisms in microorganisms. Qualitative principles established by this approach invariably have been extended—with minor alterations—to more complex organisms. While research with microorganisms offers no assurance of detection of the most significant action underlying herbicidal actions, it has provided information that facilitates proper interpretation of a number of responses in higher plants which appear to be related to each other but are actually caused by separate inhibitions.

The obvious interpretation of documented amitrole actions is that the herbicide has multiple sites of action. Three primary inhibitions appear to be involved in growth control for heterotropic microorganisms although one predominates in the more sensitive organisms. The three inhibitions lead to numerous secondary (indirect) effects, that have appeared to support incorrect hypotheses. (Much of the amitrole mechanism research has involved tentative acceptance and subsequent rejection of such hypotheses.) The indirect effects are made all the more complicated because the three primary inhibitions for microbes occur in metabolic pathways sufficiently interrelated that an inhibition of one produces secondary effects on the others. For most heterotropic cultures, an enzyme of histidine biosynthesis is the inhibited site of greatest significance for growth controlling action. Second to it, amitrole inhibitions of a specific site in the purine biosynthetic pathway and of some early aspect of methionine-serine metabolism (apparently the formation of one-carbon units) seem to be of more or less equal sensitivity. In amitrole inhibitions of green algae, purine biosynthesis is likely of greater significance than is histidine biosynthesis. Of the three sites of inhibition, only the inhibition of IGP dehydratase in histidine biosynthesis is satisfactorily established. The effect of amitrole on an enzyme of purine biosynthesis probably will be equally well-documented and elucidated in the near future; but action at the enzyme level in one-carbon metabolism will not be established so readily.

Although inhibition of histidine and purine biosynthesis occurs also in higher plant tissue, it does not seem to explain total herbicidal action. The basic objection to its having a significant role in growth control under field conditions is that the herbicide is highly phytotoxic at a time when adequate supplies of histidine and purines are available from reserves stored in the seed. The total data accumulated seem sufficient to substantiate this as a valid objection. This conclusion does not imply that the herbicide cannot control growth of a weed at

some point in its life cycle by either of the two known mechanisms, or that one of them is not the most sensitive in growth control of certain specialized cells or tissues of an individual plant. It does suggest that still another very sensitive site or mechanism of action remains to be discovered before the physiological significance for individual inhibitions can be properly assigned. The relative physiological significance of the three established amitrole actions are probably placed in proper perspective by the work of Davies (Table IV) with plant cells in solution culture. No individual metabolite or combination of metabolites was completely effective in overcoming amitrole inhibitions.

The riboflavin interaction with amitrole is the outstanding antagonism observed with growth of higher plants. The tentative conclusion, based on studies of a similar interaction expressed by bacterial growth, is that the physiological action of riboflavin represents a process not directly a part of mode of action of the inhibitor. The possibility that amitrole-induced deficiencies in riboflavin production explains riboflavin protections is apparently excluded for most microorganisms. It also seems an unlikely explanation for higher plants. In the bacteria at least, riboflavin appears to act by preventing amitrole-induced deficiencies of purines. In green plants, the question is whether riboflavin specifically affects purine metabolism or whether it would prevent inhibition at any site of action. There are reasons to believe that riboflavin protections are directed specifically toward purine metabolism. First, riboflavin fails to nullify the inhibition of histidine biosynthesis and does not duplicate the actions accomplished with methionine in bacteria. Second, the riboflavin-amitrole interaction is similar to the riboflavin and purine-analog interactions demonstrated by Thimann and Radner (1958, 1962) in duckweed. If the protections are specific for purine metabolism, clarification of riboflavin protections may offer the best hope for elucidating the mode of herbicidal action of amitrole.

Most attempts to evaluate physiological significance of known amitrole inhibitions have dealt with the growth response. There are, however, additional symptoms of amitrole toxicity and indications that different syndromes result from different metabolic inhibitions. The most obvious symptom of amitrole in green plants is albinism in new shoot tissue produced after treatment. The effect is not the result of a direct inhibition in the pathways for formation of chloroplastic pigments, but is rather a consequence of failure of the chloroplasts to develop (Bartels, 1965; Castelfranco and Bisalputra, 1967, Pyfrom *et al.*, 1957; Wolf, 1960). The underdeveloped chloroplasts of wheat seedlings have been found lacking in 70S ribosomes, fret membranes, and grana (Bartels *et al.*, 1967). In some species another symptom of amitrole treatment is the accumulation of nonchloroplastic anthocyanins (Bartels and Wolf, 1967). In other species, brown necrotic spots occur in foliage sprayed with amitrole solutions. It is of interest that growth inhibition, albinism, and development of brown spots on the leaves can be differently correlated with separate environmental conditions. Growth inhibition is greatest under conditions in which chlorosis and browning are minimized (Jansen, 1960). Thiocyanate (which enhances amitrole translocation and prevents detoxication) eliminates local injury (the discolored leaf spots) but enhances systematic action of amitrole. Purines partially nullify growth inhibitions but not albinism in tomato (Sund *et al.*, 1960). On the other hand, histidine retarded albinism in *Euglena* (Naylor, 1964) but does not do so in higher plants. Observations such as these suggest multiple mechanisms of amitrole action with some of the different symptoms resulting from different metabolic inhibitions. An effort to correlate specific symptoms with specific metabolic inhibitions seems profitable for future attempts to evaluate physiological implications of amitrole actions.

Any final explanation of herbicidal action of amitrole must take into account the fact that plants are more sensitive to amitrole in light than in darkness. In many instances this difference can be correlated with purine biosynthesis. Siegel and Gentile (1966) reported that light-grown green algae were more sensitive to amitrole but accumulated less IGP than dark-grown achlorophyllous algae. If purine biosynthesis is the most amitrole-sensitive process in the green algae as is suspected, then limited purine supplies would automatically and necessarily limit IGP production. Bartels and Wolf (1965) observed amitrole interferences with purine metabolism in wheat seedlings which were not observed in darkness. Anthocyanin accumulated in amitrole-treated, light-grown seedlings but not in dark-treated seedlings (Bartels and Wolf, 1967). In addition, an interaction of light plus amitrole is apparently necessary for inhibition of chlorophyll development (Naylor, 1964). The suggestion by Castelfranco and Brown (1963) that free-radical formation is an early step in amitrole action represents a possible interpretation for the effect of light. Photoactivated processes are among the systems capable of producing the hypothetical free radical of amitrole which binds to protein. Tenaciously bound amitrole is detected in all organisms.

The current status of research on mode of action is that the primary site of action of amitrole and a basis for its selective actions are rather adequately explained for heterotropic microorganisms. Additional but less sensitive sites of action are being defined and elucidated. The background being established with these additional sites in microorganisms offers reasonable hope that the primary site and mechanism of action in autotropic algae can be defined in the near future and, very likely, implicated as a partial explanation of phytotoxicity in higher plants. In contrast, the most important site and mechanism of action in higher plants seems yet to be detected.

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