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Inhibition of Aminoacyl-Transfer Ribonucleic Acid Synthetases and the Regulation of Amino Acid Biosynthetic Enzymes in *Neurospora crassa*

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Growth conditions that result in the accumulation of the tryptophan intermediate indoleglycerol phosphate or of the histidine intermediate imidazoleglycerol phosphate cause mycelia of *Neurospora crassa* to exhibit an immediate and sustained increase in the differential rate at which the biosynthetic enzymes of the tryptophan, histidine, and arginine pathways are synthesized. These accumulated intermediates are shown to be inhibitors of the activity of aminoacyl-transfer ribonucleic acid (tRNA) synthetases, as judged by an in vitro esterification assay. The tryptophan intermediate is shown to inhibit the charging of tryptophan, and the histidine intermediate is shown to inhibit charging of histidine. The inhibitions noted are consistent with the finding that the level of charged $tRNA^{Trp}$ is decreased significantly in cells that have accumulated indoleglycerol phosphate and that of $tRNA^{His}$ is decreased significantly in cells that have accumulated imidazoleglycerol phosphate. These results are interpreted as support for the involvement of aminoacyl-tRNA species in mediating cross-pathway regulation of the tryptophan, histidine, and arginine biosynthetic pathways as proposed in Lester's polyrepressor hypothesis (G. Lester, 1971). The correlations noted lead to the conclusion that *Neurospora* utilizes regulatory mechanisms that have the ability to react to changes in the level of charging of tRNA species.

The importance of accumulated intermediates of histidine and tryptophan biosynthesis for regulation of the formation of tryptophan synthase in *Neurospora* has been recognized for some time (10, 19, 25, 30). The recent work of Carsiotis and co-workers (9, 11) has shown that deprivation of tryptophan, histidine, or arginine auxotrophs of *Neurospora* with respect to the required amino acid will cause the cells to exhibit derepression of the enzymes of all three biosynthetic pathways. These workers have used the term cross-pathway regulation to denote this phenomenon. The "polyrepressor" model introduced by Lester (19) to account for this phenomenon postulated the existence of a macromolecule that could recognize signals from several biosynthetic pathways simultaneously. The possibility that these signals might be aminoacyl-transfer ribonucleic acids (tRNA's) was also suggested.

The recent work of Nazario and co-workers (27) on an auxotrophic strain of *Neurospora* that required tryptophan for growth but had all the enzymes required to form tryptophan in vitro indicated that the mutant possessed a deficient tryptophan-charging enzyme. This mutant appeared to exhibit higher than ordinary levels of two tryptophan biosynthetic enzymes. These authors suggested, on the basis of their results, that a product of tryptophanyl-tRNA synthetase, probably tryptophanyl-tRNA, was involved in regulation of formation of enzymes of the pathway.

In the work described here, we sought evidence that would relate the accumulation of intermediates, the activity of various aminoacyl-tRNA synthetases, the differential rates of synthesis of biosynthetic enzymes, and the levels of various aminoacyl-tRNA's in vivo. Such correlations, if they could be demonstrated, would provide a framework of circumstantial evidence that might implicate aminoacyl-tRNA's in the regulation of the formation of enzymes of amino acid biosynthetic pathways in *Neurospora*. We show here that the tryptophan intermediate indoleglycerol phosphate and the histidine intermediate imidazoleglycerol phosphate have the ability to inhibit the formation of tryptophanyl- and histidyl-tRNA, respectively, as judged by an in vitro esterifica-

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tion assay. Further, we show that cells that accumulate either of these intermediates exhibit sustained increases in differential rates of synthesis of the biosynthetic enzymes of the tryptophan, histidine, and arginine pathways. Lastly, we demonstrate that in cells that have accumulated indoleglycerol phosphate, the level of tryptophanyl-tRNA is reduced by a factor of 2 and in cells that have accumulated imidazoleglycerol phosphate the level of histidyl-tRNA is reduced by a factor of 4. We suggest that these findings are compatible with the predictions of the polyrepressor model (19) and provide the basis for further experimental approaches to the question of regulation of protein synthesis in eucaryotic organisms.

(This work was the basis of a dissertation submitted by S.L.S. to Washington State University, Pullman, in partial fulfillment of the requirements for the Ph.D. degree [February 1976].)

MATERIALS AND METHODS

Growth of cultures. Stock cultures of wild-type *Neurospora crassa*, strain 74A, were maintained on Vogel's medium N (31), supplemented with 2% sucrose and 1.5% agar. Mycelia for measurement of biosynthetic enzymes and intermediates were grown in shake cultures as described (30). Large amounts of mycelium needed for extraction of aminoacyl-tRNA synthetases and tRNA species were grown in 20-liter carboys fitted with spargers. Mycelia were lyophilized and ground to a find powder in a Wiley mill.

Measurement of biosynthetic enzymes, intermediates, and tryptophan. Extracts for assay of tryptophan biosynthetic enzymes were prepared in 0.1 M potassium phosphate buffer, pH 7.8, containing 0.1 mM ethylenediaminetetraacetic acid and 0.1 mM dithiothreitol. After centrifugation, ammonium sulfate was added to 75% saturation. The pellet obtained after centrifugation was taken up in the same buffer and used in the assays below. Extracts for assay of histidinol phosphate phosphatase were prepared in triethanolamine-HCl buffer, pH 7.5. For assay of histidinol phosphate transaminase, ornithine transcarbamylase, carbamyl phosphate synthetase, and aspartic transcarbamylase extracts were prepared in 0.05 M potassium phosphate buffer, pH 7.0.

The enzymes assayed and references to the methods used are as follows: anthranilate synthase (15); phosphoribosyl-transferase (33); indoleglycerol phosphate synthase (33); tryptophan synthase (14); histidinol phosphate transaminase (3); histidinol phosphate phosphatase (2), as modified by Carsiotis et al. (11); ornithine transcarbamylase, aspartic transcarbamylase, and carbamyl phosphate synthetase (all described by Davis [13]). All assays were incubated at 37°C. One unit of activity is that amount of enzyme required to catalyze the conversion of 1 μmol of substrate to product in 1 min at 37°C. Specific activity is reported in units per milligram of protein. Protein was measured by the method of Lowry et al. (21), using bovine serum albumin as a standard.

Indoleglycerol phosphate was measured using the periodate assay described by Yanofsky (35), and imidazoleglycerol phosphate was measured spectrophotometrically after periodate oxidation and extraction with butanol as described by Ames (1). Indoleglycerol phosphate and imidazoleglycerol phosphate were assayed in crude extracts, which had been boiled for 10 min. Tryptophan was measured with tryptophanase as described by DeMoss (14).

Isolation of aminoacyl-tRNA synthetases. A partially purified preparation of aminoacyl-tRNA synthetases was obtained from log-phase cells of *N. crassa* 74A by a modification of the method of Barnett (4). Lyophilized powdered mycelia were extracted as described. After centrifugation, the extract was treated with protamine sulfate (3 mg/ml), and the precipitated nucleic acids were removed by a second centrifugation. The supernatant thus obtained was back-extracted with ammonium sulfate as described (4). After dialysis of the resulting protein solution, glycerol was added to a final concentration of 25%. This extract was used without further purification. Such extracts have little ribonuclease activity (29).

Isolation of tRNA. tRNA was isolated by a modification of the procedure described by von Ehrenstein (32). Fresh or frozen mycelia were homogenized in a Waring blender with a mixture consisting of equal volumes of buffer [0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, 0.1 M sucrose, 0.002 M magnesium acetate, 0.05 M NaCl, and 1% sodium lauryl sulfate] and 88% phenol. The homogenate was shaken for 2 h at room temperature. After centrifugation, the aqueous layer was removed and nucleic acids were precipitated as described (32). The precipitated nucleic acids were resuspended in 0.2 M NaCl in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, and applied to a column of diethylaminoethyl-cellulose. Elution was as described (32). After recovery of the tRNA, attached amino acids were removed by alkaline hydrolysis (32). The final purification was achieved by isopropanol fractionation. After dialysis, the preparation was lyophilized and stored at −20°C. For assay, the material was dissolved in distilled water. Such preparations had 13 to 14 absorbancy units at 260 nm (A_{260}) per mg and good acceptor activity for a number of amino acids tested.

Assay of aminoacyl-tRNA synthetase activity. Aminoacyl-tRNA synthetase activity was measured as the rate of esterification of ^{14}C-labeled amino acid to tRNA. The reaction mixture used was based on the standard reaction mixture described by Shearn and Horowitz (29). All components except the enzyme were added to the reaction tubes, which were preincubated at 30°C for 5 min. The reaction was started by the addition of enzyme. Samples (0.05 ml) were removed at intervals of 20 s for 2 min, applied to glass-fiber papers (Whatman GF/A, 2.1 cm), and immediately placed in cold 5% trichloroacetic acid to

stop the reaction. Samples were washed by the method of Bollum (5) and placed in vials containing 10 ml of scintillation fluid, prepared by dissolving 0.5% (wt/vol) 2,5-diphenyloxazole and 0.01% (wt/vol) 1,4-bis-2-(5-phenyloxazolyl)benzene in toluene. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

In all experiments, the incorporation of ^{14}C-labeled amino acid was a linear function of time over the period sampled. Rates of reaction were determined by linear regression analysis of the data points obtained. The rates reported are the averages of three determinations.

Preparation of biosynthetic intermediates and derivatives. Indoleglycerol phosphate and indoleglycerol was isolated from culture filtrates of *Escherichia coli* strain $A_2/F'A_2$ as described (24), with the following modification: after concentration of the ethanol washes, the indoleglycerol phosphate and indoleglycerol were further purified by chromatography on Sephadex G-10, which completely resolves these two compounds. Imidazoleglycerol phosphate was chemically synthesized by the method of Ames (1) from barium ribose-5-phosphate and formamidine-HCl. The imidazoleglycerol phosphate was eluted from a Dowex 50 column using 0.5 N HCl (23). Imidazoleglycerol was synthesized in essentially the same manner from ribose and formamidine-HCl in the presence of potassium phosphate buffer (1) and was purified using the same procedure as for imidazoleglycerol phosphate, with an additional chromatography over Sephadex G-10. Identity and purity of the imidazoleglycerol phosphate and imidazoleglycerol were verified by paper chromatography.

Determination of the level of aminoacylated tRNA species in vivo. The percentage of various tRNA species aminoacylated in the cells was measured as the ratio of the acceptor activity of a tRNA species that was periodate resistant to the total acceptor activity for that species present in phenol extracts of cells. The procedure used was based on the method of Folk and Berg (16) as modified by Lewis and Ames (20), with other modifications to adapt these procedures for use with *N. crassa*. Cells treated with trichloroacetic acid, as described (20), were harvested by vacuum filtration and homogenized as above. After recovery of the aqueous layer, nucleic acids were precipitated and subjected to periodate or mock-periodate treatment (16). After removal of esterified amino acids (32), acceptor activity was determined using the tRNA estrification assay described above, except that the reaction was allowed to go to completion. The experimental protocol was as described (20). Acceptor activity is reported in picomoles per A_{260}. The percentage of aminoacylated tRNA in the extracts, for the species of interest, was calculated by division of the acceptor activity of the periodate-treated sample by the acceptor activity of the mock-periodate-treated sample and multiplication of this ratio by 100.

Chemicals. L-Tryptophan (side chain, 3-^{14}C) was purchased from New England Nuclear Corp. Other ^{14}C-labeled amino acids were purchased from Schwarz/Mann and had a specific activity of 50 μCi/μmol. Indoleacrylic acid and 3-amino-1,2,4-triazole (amitrole) were obtained from Nutritional Biochemicals and Sigma, respectively. Reagent-grade phenol (liquefied) was a product of Matheson, Coleman and Bell or Mallinkrodt and contained no preservative. Other biochemicals used were purchased from Sigma or Calbiochem, and all chemicals used were of reagent grade or the highest purity obtainable.

RESULTS

Turner and Matchett (30) showed that wild-type cells of *N. crassa* grown in the presence of indoleacrylic acid would accumulate indoleglycerol phosphate and would subsequently exhibit a 20-fold increase in their differential rate of formation of tryptophan synthase. We have studied this phenomenon in greater detail and have extended the original observation by demonstrating that such conditions of growth cause the cells to exhibit derepression with respect to all the gene products of the tryptophan pathway, two gene products of the histidine pathway, and two gene products of the arginine pathway (Fig. 1). As a control, aspartic transcarbamylase was measured. These conditions had no measurable effect on the formation of this enzyme (Fig. 1I). The data of the individual plots presented in Fig. 1 were subjected to linear regression analysis, and values for the differential rates of synthesis of the various enzymes were obtained. The values determined in this manner are presented in Table 1. Included in this table, for comparative purposes, are values obtained for the differential rates of synthesis of these same enzymes in cells grown in the presence of 3-amino-1,2,4-triazole. This compound had been reported to elicit derepression of enzymes of the tryptophan, histidine, and arginine pathways (11). From the data presented in Fig. 1 and Table 1, it can be seen that the effect of indoleacrylic acid is similar to that of 3-amino-1,2,4-triazole in causing derepression of all three pathways. The differences noted in the magnitude of the effects on various enzymes in cells subjected to each growth condition are quite reproducible. The reason for these differences is unknown at present but could reflect varying rates of breakdown of the several enzymes involved.

Both indoleacrylic acid and 3-amino-1,2,4-triazole act in a similar manner by inhibiting a biosynthetic enzyme, which results in the accumulation of an intermediate of the pathway affected. In the case of indoleacrylic acid, the enzyme blocked is tryptophan synthase, which results in the accumulation of indoleglycerol phosphate (24). 3-Amino-1,2,4-triazole inhibits imidazoleglycerol phosphate dehydrase, an enzyme of the histidine pathway, which results in the accumulation of imidazoleglycerol phos-

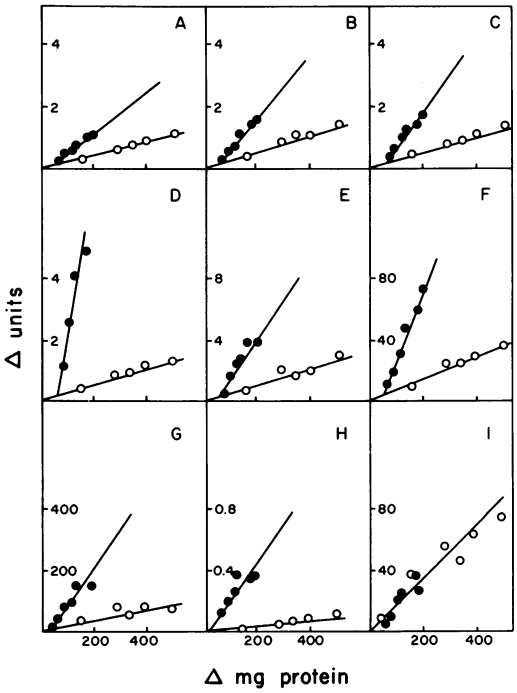

FIG. 1. *Depression of tryptophan, histidine, and arginine enzymes. One-liter shake cultures were inoculated as described (30). These were harvested at 1-h intervals for 6 h. Total enzyme and total protein were determined as indicated in the text. Increases in total enzyme are plotted as a function of increases in total protein. Symbols: ○, minimal medium; ●, minimal medium + 0.5 mM indoleacrylic acid. (A) Anthranilate synthase; (B) phosphoribosyl transferase; (C) indoleglycerol phosphate synthase; (D) tryptophan synthase; (E) histidinol phosphate transaminase; (F) histidinol phosphate phosphatase; (G) ornithine transcarbamylase; (H) carbamyl phosphate synthetase; (I) aspartyl transcarbamylase.*

phate (18). Figure 2 shows the kinetics of accumulation of both these intermediates in cells grown in the presence of indoleacrylic acid and 3-amino-1,2,4-triazole. From these data, it is clear that indoleglycerol phosphate but not imidazoleglycerol phosphate accumulates in cells grown in the presence of indoleacrylic acid. Similarly, imidazoleglycerol phosphate but not indoleglycerol phosphate accumulates in cells grown in the presence of 3-amino-1,2,4-triazole. The pattern observed in the accumulation of the two intermediates is quite different. The concentration of indoleglycerol phosphate increased rapidly during the first hour of growth in medium containing indoleacrylic acid and then remained fairly constant. The concentration of imidazoleglycerol phosphate in cultures grown in the presence of 3-amino-1,2,4-triazole showed a steady increase during the 6-h period in which measurements were made.

The blockage of tryptophan synthase by indoleacrylic acid could be expected to lead to a deprivation of tryptophan in the mycelium. To determine if this was the case, we measured the intracellular level of tryptophan in cells grown in medium containing this compound. The results of these measurements indicated that there was a slight increase in the level of tryptophan under these conditions. After 6 h of growth in medium containing indoleacrylic acid, the tryptophan pool was 0.3 μmol/g (dry weight) compared to a value of 0.1 μmol/g (dry weight) found with growth in minimal medium. The tryptophan pool remained nearly the same as in minimal medium with growth in 3-amino-1,2,4-triazole. The inhibition of imidazoleglycerol phosphate dehydratase by 3-amino-1,2,4-triazole does lead to a decrease in the histidine pool, however, as was shown by Carsiotis and co-workers (personal communication).

To test whether aminoacyl-tRNA's might be involved in the effects noted above, we have used two approaches. First, we examined the effects of indoleglycerol phosphate and imidazoleglycerol phosphate and their respective dephosphorylated derivatives on the formation of tryptophanyl-tRNA and histidyl-tRNA. Second, we measured the levels of these and other aminoacyl-tRNA's in cells that had accumulated the intermediates in question.

Figure 3 shows the results of experiments examining the effect of indoleglycerol phosphate and indoleglycerol on tryptophanyl-tRNA synthetase. Figure 3A shows a double-reciprocal plot of data obtained for the rate of tryptophanyl-tRNA formation as a function of tryptophan concentration. Both indoleglycerol phosphate and indoleglycerol were inhibitory to the reaction. Analysis of these data gave a value of 1.6 μM for the K_m of tryptophanyl-tRNA synthetase with respect to tryptophan. This agrees well with the K_m obtained by others (Suskind, personal communication). The K_i for indoleglycerol phosphate was found to be 4.8 mM, assuming simple competitive inhibition. In the case of indoleglycerol, there was a clear departure from linearity, and so it was not possible to determine a value for the K_i of this compound.

Figure 3B shows the relationship of the inhibitor concentration of the percentage of remaining tryptophanyl-tRNA synthetase activity. These plots show that both compounds inhibit the charging reaction and, of the two, indoleglycerol is more inhibitory.

The structural similarity of indoleglycerol

TABLE 1. *Differential rate of formation of biosynthetic enzymes in cells grown in the presence of indoleacrylic acid and 3-amino-1,2,4-triazole*

Enzyme	Differential rate of enzyme synthesis (mU/mg of protein)[a]			Elevation[b]	
	Minimal medium	0.5 mM indoleacrylic acid	3 mM 3-amino-1,2,4-triazole	0.5 mM indoleacrylic acid	3 mM 3-amino-1,2,4-triazole
Anthranilate synthase	2.17 (0.016)[c]	650 (0.022)	7.50 (0.14)	3.0	3.5
Phosphoribosyl transferase	2.83 (0.068)	10.1 (0.12)	11.5 (0.17)	3.6	4.1
Indoleglycerol phosphate synthase	2.50 (0.029)	9.50 (0.15)	13.0 (0.26)	3.8	5.2
Tryptophan synthase	2.50 (0.047)	46.6 (0.53)	14.0 (0.054)	18.7	5.6
Histidinol phosphate transaminase	5.57 (0.24)	24.6 (0.25)	42.0 (0.84)	4.4	7.5
Histidinol phosphate phosphatase	71.0 (1.6)	472.0 (4.2)	507.0 (7.1)	6.6	7.1
Ornithine transcarbamylase	155.0 (13.0)	842.0 (19.0)	1,302.0 (20.0)	5.4	8.4
Carbamylphosphate synthetase	0.25 (0.012)	1.80 (0.044)	2.52 (0.085)	7.2	10.1
Aspartic transcarbamylase	169.0 (6.7)	169.0 (4.1)	169.0 (3.8)	1.0	1.0

[a] One unit of enzyme activity is equivalent to 1 μmol of product formed or substrate used per min. The differential rates were obtained as the slopes of lines such as those presented in Fig. 1.

[b] The degree of elevation is the ratio of the differential rate found in cells grown in the presence of indoleacrylic acid or 3-amino-1,2,4-triazole to that found in cells grown in minimal medium.

[c] Parenthetic numbers are standard errors of the estimates.

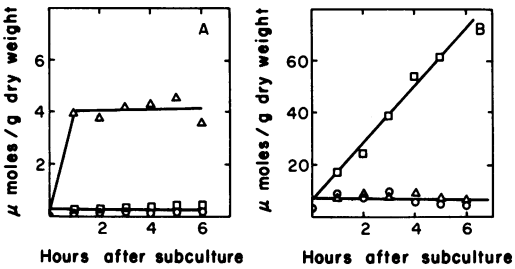

FIG. 2. *Levels of indoleglycerol phosphate and imidazoleglycerol phosphate in cells grown in the presence of indoleacrylic acid (A) and 3-amino-1,2,4-triazole (B). Cells were grown as for measurement of enzyme activities. Cultural conditions: ○, minimal medium; □, 3.0 mM 3-amino-1,2,4-triazole; △, 0.5 mM indoleacrylic acid. (A) Indoleglycerol phosphate; (B) imidazoleglycerol phosphate.*

phosphate to imidazoleglycerol phosphate suggested that both intermediates might have similar effects on charging reactions. That this is not the case is shown in Fig. 4. In Fig. 4A, imidazoleglycerol and imidazoleglycerol phosphate concentrations are plotted versus percentage of remaining activity of tryptophanyl-tRNA synthetase. The effect on this enzyme, if any, is barely detectable at the highest concentrations used. In Fig. 4B, however, histidine charging is shown to be quite sensitive to the same range of concentrations of the two compounds. The effect of indoleglycerol phosphate and indoleglycerol on formation of histidyl-tRNA was examined in similar experiments (Fig. 4C). In this case indoleglycerol had a barely measurable effect, but indoleglycerol phosphate showed easily measured inhibition. In experiments of this general type not reported here, it was shown that α-methylhistidine inhibited specifically the charging of histidine, and that argininosuccinate specifically inhibited the charging of arginine. These results are summarized conveniently in Table 2.

The results in Table 2 and the correlations noted above provided a conceptual framework for proceeding with an experimental analysis of the phenomenon of cross-pathway regulation and specifically suggested a test for one of the predictions of Lester's (19) polyrepressor hypothesis. Even though the concentrations of intermediates used in the experiment in Fig. 4 are high, they are well within the ranges demonstrated to be present in cells challenged with the inhibitors used (cf. Fig. 2). The experiments described above show that the intermediates examined do have the potential for inhibiting the charging reactions of their respective pathways. It was therefore important to examine the percentages of tryptophan, histidine, and arginine tRNA's aminoacylated in cells growing in conditions in which the supposed inhibitors of charging could be shown to have accumulated. To measure the levels of aminoacyl-tRNA's present in the growth conditions of in-

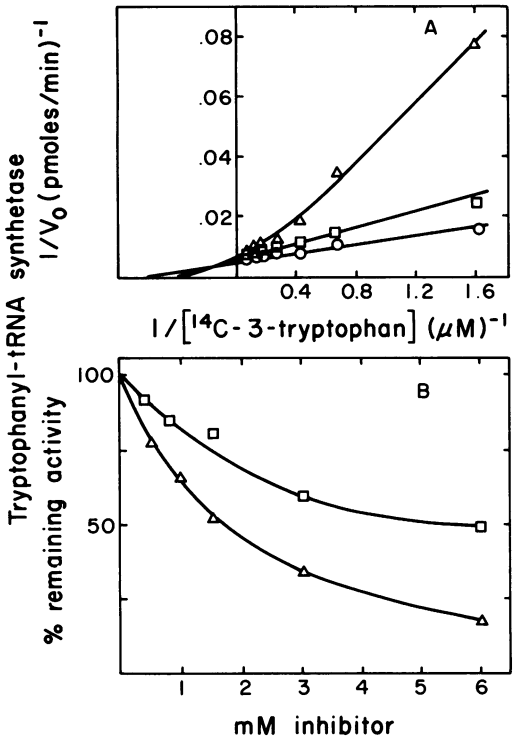

FIG. 3. *Inhibition of tryptophanyl-tRNA synthetase by indoleglycerol phosphate and indoleglycerol. (A) Double-reciprocal plot of the rate of reaction as a function of tryptophan concentration in the presence of 3 mM indoleglycerol phosphate* (□) *or 3 mM indoleglycerol* (△) *or with no inhibitor present* (○). *(B) Effect of varying concentrations of indoleglycerol phosphate and indoleglycerol on the rate of tryptophanyl-tRNA formation. The rate of the uninhibited reaction (140 pmol/min, with a concentration of 2 μM tryptophan) was considered to be 100%. Values at the various concentrations were plotted as the percentage of the rate of the uninhibited reaction. Symbols:* ○, *no addition;* □, *indoleglycerol phosphate;* △, *indoleglycerol.*

terest, log-phase cells were subcultured into one of three conditions. Those transferred to minimal medium continued to exhibit a repressed rate of synthesis of the enzymes in question and did not accumulate intermediates. Those transferred to medium containing indoleacrylic acid promptly began the accumulation of indoleglycerol phosphate and exhibited derepressed synthesis of the enzymes in question. Similarly, those transferred to 3-amino-1,2,4-triazole began to accumulate imidazoleglycerol phosphate and to exhibit derepression. Samples of each subculture were taken at intervals of 3 h, and the levels of tryptophanyl-, histidyl-, and arginyl-tRNA were determined. In addition, and to provide internal controls for the experiment, the levels of isoleucyl- and valyl-tRNA were also determined.

The results of this experiment are presented in Table 3. The data are divided into three sections. The percentage of each tRNA species esterified under the various conditions of growth is presented in the first section. The total acceptor activity relative to the A_{260} for each sample is presented in section 2 of the

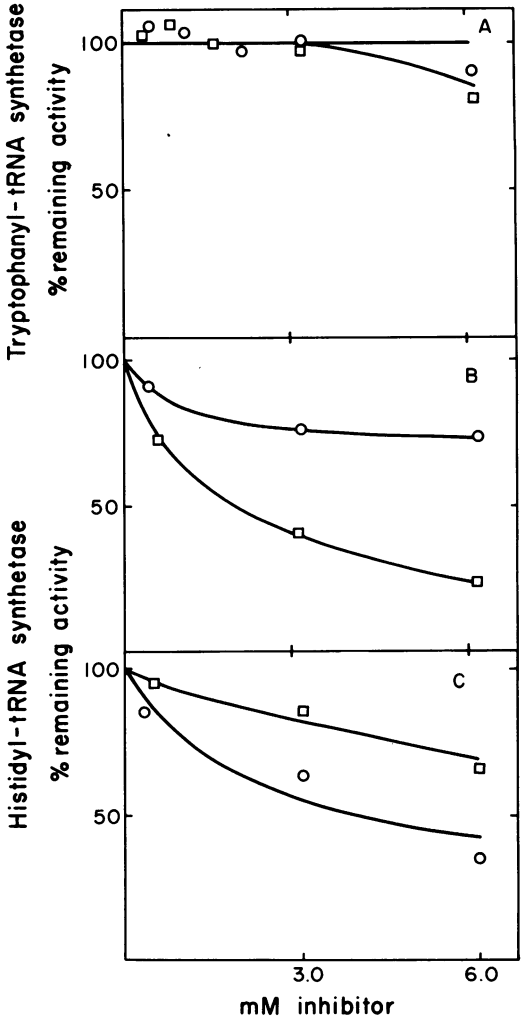

FIG. 4. *Effects of intermediates on tryptophanyl- and histidyl-tRNA synthetase. (A) Tryptophanyl-tRNA synthetase activity in the presence of imidazoleglycerol phosphate* (○) *and imidazoleglycerol* (□). *(B) Inhibition of histidyl-tRNA synthetase by imidazoleglycerol phosphate* (○) *and imidazoleglycerol* (□). *(C) Inhibition of histidyl-tRNA synthetase by indoleglycerol phosphate* (○) *and indoleglycerol* (□).

TABLE 2. *Inhibition of charging*

Compound	Tryptophanyl-tRNA synthetase	Histidyl-tRNA synthetase	Arginyl-tRNA synthetase
Indoleglycerol phosphate	Inhibition	Inhibition	No inhibition
Indoleglycerol	Marked inhibition	Slight inhibition	No inhibition
Imidazoleglycerol phosphate	No inhibition	Inhibition	No inhibition
Imidazoleglycerol	No inhibition	Marked inhibition	No inhibition
α-Methylhistidine	No inhibition	Marked inhibition	No inhibition
Argininosuccinate	No inhibition	No inhibition	Inhibition

table. The total acceptor activity relative to the acceptor activity of $tRNA^{Val}$ is presented in the third section of the table. Others had shown (20) that the acceptor activity relative to an internal standard such as valine was less variable than that relative to the A_{260}. As shown in section 3 of the table, this was the case in the experiment described here.

The cells subcultured into minimal medium that continued to exhibit repressed formation of the enzymes in question showed no obvious change in the level of charging with respect to any of the five species measured. The cells subcultured into medium containing 0.5 mM indoleacrylic acid showed a significant decrease in the level of charged $tRNA^{Trp}$. The other species examined showed no obvious effect. The cells subcultured into medium containing 3.0 mM 3-amino-1,2,4-triazole showed a significant drop only in the level of charged $tRNA^{His}$. None of the other species examined showed any obvious decrease in the percent esterified.

It is doubtful in the case of indoleacrylic acid that the observed decrements were caused by any measurable reduction in the intracellular concentration of tryptophan. Our measurements (see above) indicated slight increases in the intracellular concentration of tryptophan under the conditions of the experiment. With regard to histidine, the situation is less clear. Carsiotis and co-workers (personal communication) have shown 3-amino-1,2,4-triazole-mediated derepression of tryptophan synthase in cells whose rate of growth was not obviously lowered. Whether or not the decrease in level of histidyl-tRNA is caused by accumulation of pathway intermediates or deprivation of histidine, the results of these experiments demonstrate the correlation between the derepressions noted and the reduced levels of charging.

DISCUSSION

The possible involvement of aminoacyl-tRNA's or the aminoacyl-tRNA synthetases in the regulation of amino acid biosynthetic pathways has been the subject of numerous studies in recent years. Particularly in procaryotic organisms, there is now convincing evidence for such involvement in several amino acid biosynthetic pathways (for review, see 6). One of the best-studied examples is the role of histidyl-tRNA in regulation of the histidine operon in *Salmonella typhimurium*. Of six genetic loci that affect regulation of this pathway, four are concerned with $tRNA^{His}$ formation and a fifth is the structural gene for histidyl-tRNA synthetase. Studies of this system indicated that repression was directly correlated with the concentation of histidyl-tRNA (20). Regulation of the *ilv* pathway in *S. typhimurium* and *E. coli*, which is subject to multivalent repression by isoleucine, valine, and leucine, also appears to involve the aminoacyl-tRNA species of their respective pathways (17). A model has recently been proposed to account for the experimental observations related to the regulation of the *ilv* pathway. This model also involves the participation of threonine deaminase in regulation of this pathway (8, 17). Evidence for the involvement of aminoacyl-tRNA's has also been obtained for several other pathways, including arginine, tryptophan, and methionine biosynthesis (6).

There is also evidence for involvement of aminoacyl-tRNA's in regulation of amino acid biosynthetic pathways in eucaryotes, although this question has not been as extensively studied in these organisms. McLaughlin et al. (22) found that the activity of isoleucyl-tRNA synthetase was required for multivalent repression of the isoleucine biosynthetic enzymes in yeast. The possibility that isoleucyl-tRNA was required for repression was suggested. In *N. crassa*, there is evidence that arginyl-tRNA is required for repression of arginine biosynthesis. This was suggested by the work of Nazario on an *arg-10* mutant of *N. crassa* (26). This mutant, when grown on limiting arginine or on nonlimiting arginine plus citrulline, accumulated argininosuccinate and had elevated levels of arginine biosynthetic enzymes. In the latter case this was true even though the pool of argi-

TABLE 3. *In vivo levels of aminoacyl-tRNA's in cells grown in the presence of indoleacrylic acid or 3-amino-1,2,4-triazole and in minimal medium*

Growth conditions	Growth (h)	tRNA charged (%)[a]					Total acceptor activity of tRNA (pmol/A_{260})[b]					Total acceptor activity relative to valine[c]				
		$tRNA^{Trp}$	$tRNA^{His}$	$tRNA^{Arg}$	$tRNA^{Ile}$	$tRNA^{Val}$	$tRNA^{Trp}$	$tRNA^{His}$	$tRNA^{Arg}$	$tRNA^{Ile}$	$tRNA^{Val}$	$tRNA^{Trp}$	$tRNA^{His}$	$tRNA^{Arg}$	$tRNA^{Ile}$
Minimal	16	85	78	94	96	80	1.01	1.02	2.73	1.21	0.79	1.28	1.29	3.48	1.54
Minimal	3	99	94	94	85	74	0.74	0.58	1.41	0.81	0.50	1.48	1.14	2.80	1.60
Minimal	6	85	74	84	79	66	0.57	0.32	0.71	0.34	0.22	2.54	1.44	3.17	1.50
0.5 mM indoleacrylic acid	3	51	80	95	93	88	1.04	0.74	1.49	1.18	0.57	1.82	1.29	2.60	2.06
	6	48	89	100	98	92	1.16	0.72	2.38	1.61	0.67	1.74	1.08	3.58	2.41
3.0 mM 3-amino-1,2,4-triazole	3	100	26	92	85	90	1.19	0.86	2.18	1.51	0.65	1.82	1.32	3.34	2.30
	6	103	22	107	88	103	1.50	0.84	1.74	1.52	0.66	2.28	1.27	2.63	2.30

[a] The percent charged of a particular tRNA species is the ratio of the acceptor activity of that tRNA species resistant to periodate to the total acceptor for that species in the extract, multiplied by 100.

[b] The total acceptor activity was determined on a mock-periodate-treated sample after removal of esterified amino acids by alkaline hydrolysis.

[c] The values reported here are the ratios of the total acceptor activity/A_{260} for the various tRNA species to the total acceptor activity for valine tRNA.

nine was high. The finding that argininosuccinate inhibited charging of $tRNA^{Arg}$ in vitro (26) led Nazario to suggest that arginyl-tRNA formation was required for arginine to be effective in repression.

The possibility that tryptophanyl-tRNA might be involved in repression of tryptophan biosynthetic enzymes in *N. crassa* was first supported experimentally by studies on the *trp-5* mutant, which was characterized as having an altered tryptophanyl-tRNA synthetase (27). Those studies indicated that even when the pool of tryptophan was equal to or higher than that found in wild-type cells, this mutant had elevated levels of tryptophan biosynthetic enzymes.

The work presented here was undertaken to determine if a correlation existed between derepression of tryptophan, histidine, and arginine biosynthetic enzymes, the accumulation of intermediates of these pathways, and the levels of charged tRNA species of these pathways in cells grown in medium containing indoleacrylic acid and 3-amino-1,2,4-triazole.

If such a relationship existed and could be demonstrated unambiguously, then important new information would be available, which might help to explain the phenomenon of cross-pathway regulation. An interrelationship between the regulation of the tryptophan and histidine pathways had been indicated by earlier studies, which showed that starvation for histidine resulted in derepression of tryptophan biosynthetic enzymes as well as those of the histidine pathway (10, 12). Recently, this effect has also been shown to be reciprocal in that starvation for tryptophan resulted in derepression of histidine enzymes (9). In addition, both conditions were found to result in derepression of arginine biosynthetic enzymes, indicating that the regulation of this pathway was also related to the regulation of the tryptophan and histidine pathways (9, 11). Growth in the presence of 3-amino-1,2,4-triazole has also been shown to result in derepression of all three pathways (11). Cross-pathway regulation of the tryptophan, histidine, and arginine pathways is not unique to *Neurospora*. Schürch and coworkers (28) found that 3-amino-1,2,4-triazole would cause derepression of the enzymes of all three pathways in yeast. The relationship between these three pathways in yeast was confirmed by Wolfner and co-workers (34), who showed in addition that regulation of the lysine pathway was also involved. Involvement of the lysine pathway in cross-pathway responses has also been suggested for *Neurospora* (Carsiotis, personal communication). We have examined

the effect of indoleacrylic acid on the levels of histidine and arginine biosynthetic enzymes as well as tryptophan enzymes. The work presented here has shown that growth in the presence of indoleacrylic acid resulted in a similar pattern of derepression of biosynthetic enzymes of these three pathways. Accompanying the derepression of these three pathways was the presence in the cells of high levels of indoleglycerol phosphate with growth in medium containing indoleacrylic acid and imidazoleglycerol phosphate in the case of growth in medium containing 3-amino-1,2,4-triazole.

Earlier work with tryptophan synthase had shown the importance of the presence of the accumulated intermediates in relation to the derepression (30). The experiments described here and elsewhere (9–11) have demonstrated other derepressions in the presence of these intermediates. For this reason we were interested in mechanisms that could account for involvement of these intermediates in such regulation. One such mechanism involved direct participation of the accumulated intermediates in interference with the formation of a required repressor compound (25). The work of Nazario et al. (27) on the *trp-5* mutant suggested the possibility that tryptophanyl-tRNA might be a regulatory effector. The in vitro studies reported here on formation of aminoacyl-tRNA's were designed to determine if these accumulated intermediates could interfere with the formation of these compounds. Our studies with indoleglycerol phosphate and indoleglycerol indicate that both these compounds, at concentrations that may be demonstrated to occur intracellularly, have the potential to inhibit tryptophanyl-tRNA synthetase. These studies have shown further that similarly demonstrated levels of imidazoleglycerol phosphate and imidazoleglycerol have the potential to inhibit histidyl-tRNA synthetase. In other experiments not reported here, it was shown that α-methylhistidine will inhibit the formation of histidyl-tRNA, and others (Carsiotis, personal communication) have shown derepression in response to this histidine analogue.

These results provided evidence for the ability of intermediates and amino acid analogues associated with derepression to inhibit aminoacyl-tRNA synthetases. The pattern of inhibition found was consistent with the suggestion that histidyl-tRNA, tryptophanyl-tRNA, and perhaps also arginyl-tRNA were involved in cross-pathway regulation. This hypothesis was further supported by studies in which the levels of tryptophanyl-tRNA and histidyl-tRNA were measured in cells grown in the presence of indoleacrylic acid and 3-amino-1,2,4-triazole. With growth in the presence of indoleacrylic acid and accumulation of indoleglycerol phosphate, only the level of tryptophanyl-tRNA was decreased, although biosynthetic enzymes of all three pathways were derepressed. With growth in medium containing 3-amino-1,2,4-triazole and accumulation of imidazoleglycerol phosphate, only the level of histidyl-tRNA was lowered, although enzymes of all three pathways were derepressed. A similar pattern is suggested by studies on the *trp-5* mutant, which appears to be defective in tryptophanyl-tRNA synthetase (27), yet also has derepressed levels of all three sets of biosynthetic enzymes (Carsiotis, personal communication). Whether or not the reduced levels of aminoacyl-tRNA's are caused by inhibitions exerted on the synthetases by accumulated intermediates in vivo must remain an open question. Circumstantial evidence supports the notion that the intermediates are indeed responsible, but other explanations are not ruled out by these experiments. Whatever the cause, it is clear that derepression of the pathways noted is correlated with reduction in the levels of one of the cognizant aminoacyl-tRNAs.

The results reported here and the correlations noted are consistant with the polyrepressor model proposed by Lester (19). This model proposed a repressor molecule that required the presence of all three species of aminoacyl-tRNA for activity. According to the model, the absence of any one of these would render the postulated repressor inoperative and derepression with respect to all the pathways under its control would occur. The results reported here confirm the correlations expected between the levels of aminoacyl-tRNA's and derepression of the related pathways. Unfortunately, they offer no hint as to the mechanism by which such a repressor complex might regulate the rate of protein synthesis.

Although we do not have a satisfactory understanding of the regulation of protein biosynthesis in *N. crassa*, it is clear from the studies reported here and elsewhere that it involves a complex mechanism. The studies reported here do provide support for the idea that such regulation involves the participation of aminoacyl-tRNA's. As yet, however, there is no evidence as to the mechanism by which these aminoacyl-tRNA's can effect repression, or how derepression of enzymes for which the structural genes are widely scattered in the genome can be effected by the lowered level of a single species of aminoacyl-tRNA. An answer to this question must await more investigation and will be fully

understood only when our knowledge of retrieval of genetic information in *N. crassa* is more complete.

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LITERATURE CITED

1. **Ames. B. N.** 1957. The biosynthesis of histidine; D-erythroimidazole-glycerol phosphate dehydrase. J. Biol. Chem. **228**:131-143.
2. **Ames, B. N.** 1957. The biosynthesis of histidine, L-histidinol phosphate phosphatase. J. Biol. Chem. **226**:583-593.
3. **Ames, B. N., and B. L. Horecker.** 1956. The biosynthesis of histodine:imidazoleacetol phosphate transaminase. J. Biol. Chem. **220**:113-128.
4. **Barnett, W. E.** 1965. Interspecies aminoacyl-sRNA formation: fractionation of *Neurospora* enzymes involved in anomalous aminoacylation. Proc. Natl. Acad. Sci. U.S.A. **53**:1462-1467.
5. **Bollum, F. J.** 1966. Filter paper disk techniques for assaying radioactive macromolecules, p. 296-300. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research, vol. 1. Harper and Row, New York.
6. **Brenchley, J. E., and L. S. Williams.** 1975. Transfer RNA involvement in the regulation of enzyme synthesis. Annu. Rev. Microbiol. **29**:251-274.
7. **Brenner, M., and B. N. Ames.** 1971. The histidine operon and its regulation, p. 349-387. *In* H. J. Vogel (ed.), Metabolic pathways, vol. 5. Academic Press Inc., New York.
8. **Calhoun, D. H., and G. W. Hatfield.** 1973. Autoregulation: a role for a biosynthetic enzyme in the control of gene expression. Proc. Natl. Acad. Sci. U.S.A. **70**:2757-2761.
9. **Carsiotis, M., and R. F. Jones.** 1974. Cross-pathway regulation: tryptophan-mediated control of histidine and arginine biosynthetic enzymes in *Neurospora crassa*. J. Bacteriol. **119**:889-892.
10. **Carsiotis, M., R. F. Jones, A. M. Lacy, T. J. Cleary, and D. B. Fankhauser.** 1970. Histidine-mediated control of tryptophan biosynthetic enzymes in *Neurospora crassa*. J. Bacteriol. **104**:98-106.
11. **Carsiotis, M., R. F. Jones, and A. C. Wesseling.** 1974. Cross-pathway regulation: histidine-mediated control of histidine, tryptophan, and arginine biosynthetic enzymes in *Neurospora crassa*. J. Bacteriol. **119**:893-898.
12. **Carsiotis, M., and A. M. Lacy.** 1965. Increased activity of tryptophan biosynthetic enzymes in histidine mutants of *Neurospora*. J. Bacteriol. **89**:1472-1477.
13. **Davis, R. H.** 1965. Carbamyl phosphate synthesis in *Neurospora crassa*. II. Genetics, metabolic position, and regulation of arginine-specific carbamyl phosphokinase. Biochim. Biophys. Acta **107**:54-68.
14. **DeMoss, J. A.** 1962. Studies on the mechanism of the tryptophan synthetase reaction. Biochim. Biophys. Acta **62**:279-293.
15. **DeMoss, J. A.** 1965. The conversion of shikimic acid to anthranilic acid by extracts of *Neurospora crassa*. J. Biol. Chem. **240**:1231-1235.
16. **Folk, W. R., and P. Berg.** 1970. Characterization of altered forms of glycyl transfer ribonucleic acid synthetase and the effects of such alterations on aminoacyl transfer ribonucleic acid synthesis in vivo. J. Bacteriol. **102**:204-212.
17. **Hatfield, G. W., and R. O. Burns.** 1970. Specific binding of leucyl transfer RNA to an immature form of L-threonine deaminase: its implications in repression. Proc. Natl. Acad. Sci. U.S.A. **66**:1027-1035.
18. **Hilton, J. L., P. C. Kearney, and B. N. Ames.** 1965. Mode of action of the herbicide, 3-amino-1,2,4-triazole (amitrole): inhibition of an enzyme of histidine biosynthesis. Arch. Biochem. Biophys. **112**:544-547.
19. **Lester, G.** 1971. Regulation of tryptophan biosynthetic enzymes in *Neurospora crassa*. J. Bacteriol. **107**:193-202.
20. **Lewis, J. A., and B. N. Ames.** 1972. Histidine regulation in *Salmonella typhimurium*: XI. The percentage of transfer RNA^{His} charged *in vivo* and its relation to the repression of the histidine operon. J. Mol. Biol. **66**:131-142.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
22. **McLaughlin, C. S., P. T. Magee, and L. H. Hartwell.** 1969. Role of isoleucyl-transfer ribonucleic acid synthetase in ribonucleic acid synthesis and enzyme repression in yeast. J. Bacteriol. **100**:579-584.
23. **Martin, R. G., M. A. Bererich, B. N. Ames, W. W. David, R. F. Goldberger, and J. D. Yourno.** 1971. Enzymes and intermediates of histidine biosynthesis in *Salmonella typhimurium*, p. 3-44. *In* H. Tabor and C. W. Tabor (ed.), Methods in enzymology, vol. 17B. Academic Press Inc., New York.
24. **Matchett, W. H.** 1972. Inhibition of tryptophan synthetase by indoleacrylic acid. J. Bacteriol. **110**:146-154.
25. **Matchett, W. H., J. R. Turner, and W. R. Wiley.** 1968. The role of tryptophan in the physiology of *Neurospora*. Yale J. Biol. Med. **40**:257-283.
26. **Nazario, M.** 1967. The accumulation of argininosuccinate in *Neurospora crassa*. II. Inhibition of arginyl-tRNA synthesis by arginino-succinate. Biochim. Biophys. Acta **145**:146-152.
27. **Nazario, M., J. A. Kinsey, and M. Ahmad.** 1971. *Neurospora* mutant deficient in tryptophanyl-transfer ribonucleic acid synthetase activity. J. Bacteriol. **105**:121-126.
28. **Schürch, A., J. Miozzari, and R. Hütter.** 1974. Regulation of tryptophan biosynthesis in *Saccharomyces cerevisiae*: mode of action of 5-methyl-tryptophan and 5-methyl-tryptophan-sensitive mutants. J. Bacteriol. **117**:1131-1140.
29. **Shearn, A., and N. Horowitz.** 1969. A study of transfer ribonucleic acid in *Neurospora*. I. The attachment of amino acids and amino acid analogs. Biochemistry **8**:295-303.
30. **Turner, J. R., and W. H. Matchett.** 1968. Alteration of tryptophan-mediated regulation in *Neurospora crassa* by indoleglycerol phosphate. J. Bacteriol. **95**:1608-1614.
31. **Vogel, H. J.** 1964. Distribution of lysine pathways among fungi: evolutionary implications. Am. Nat. **98**:435-446.
32. **von Ehrenstein, G.** 1967. Isolation of sRNA from intact *Escherichia coli* cells, p. 588-596. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 12A. Academic Press Inc., New York.
33. **Wegman, J., and J. A. DeMoss.** 1965. The enzymatic conversion of anthranilate to indoleglycerol phosphate in *Neurospora crassa*. J. Biol. Chem. **240**:3781-3788.
34. **Wolfner, M., D. Yep, F. Messenguy, and G. R. Fink.** 1975. Integration of amino acid biosynthesis into the cell cycle of *Saccharomyces cerevisiae*. J. Mol. Biol. **96**:273-290.
35. **Yanofsky, C.** 1956. The enzymatic conversion of anthranilic acid to indole. J. Biol. Chem. **223**:171-184.