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THE ROLE OF TRYPTOPHAN IN THE PHYSIOLOGY OF NEUROSPORA‡

The genetics and biochemistry of tryptophan metabolism in the bread mold, *Neurospora crassa,* have received considerable experimental attention during the past 20 years. By now, an impressive body of information has accumulated to the extent that the intimate details of the biosynthesis[1-3] and degradation[4-6] of tryptophan in Neurospora are well known and also to the extent that the genetics of this interesting series of reactions now serves as a textbook model to illustrate the relationship between genes and enzymes.[7] These are subjects that have been reviewed extensively elsewhere.[8,9] In this review we shall concern ourselves with the physiological implications of the metabolism of tryptophan by Neurospora with particular regard to the regulatory mechanisms which impinge on this interesting system.

UPTAKE FROM EXTERNAL MEDIUM

Tryptophan transport across the cell membrane of Neurospora is mediated by a stereospecific transport system. Although the transport system has not been isolated and characterized, the available evidence suggests that it involves a distinct protein with catalytic activity. Tryptophan present in the environment may be concentrated many fold by the transport system. According to the present concept, tryptophan enters the cell and is accumulated in acid soluble pools.

The progress that has been made toward describing the mechanism of tryptophan transport and the way in which the process is functionally integrated into over-all cellular metabolism is summarized here.

Kinetics of pool formation

When labeled tryptophan is added to a logarithmically growing culture of Neurospora, the radioactivity is immediately transferred to a cold

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trichloroacetic acid (TCA) soluble fraction. The transfer of radioactive tryptophan from the TCA soluble to the TCA precipitable fraction (protein)[10] is delayed for approximately 90-120 seconds. Results of a typical experiment are shown in Figure 1. As shown, total uptake and formation of the acid soluble pool are coincident at early times. The initial rate of tryptophan uptake into the TCA-soluble pool is dependent upon the exogenous concentration of tryptophan. The concentration dependency of the process is demonstrated by the Lineweaver-Burke plot[10] shown in Figure 2. The K_m and V_{max} for the process are 5×10^{-5} *M* and 33 μmoles per min. per g cells dry weight, respectively.

Requirements for reactivity with transport system

Table 1 shows the specificity characteristics of the transport system. A number of amino acids inhibit the rate of tryptophan accumulation; for example, methionine, leucine, and phenylalanine are extremely effective

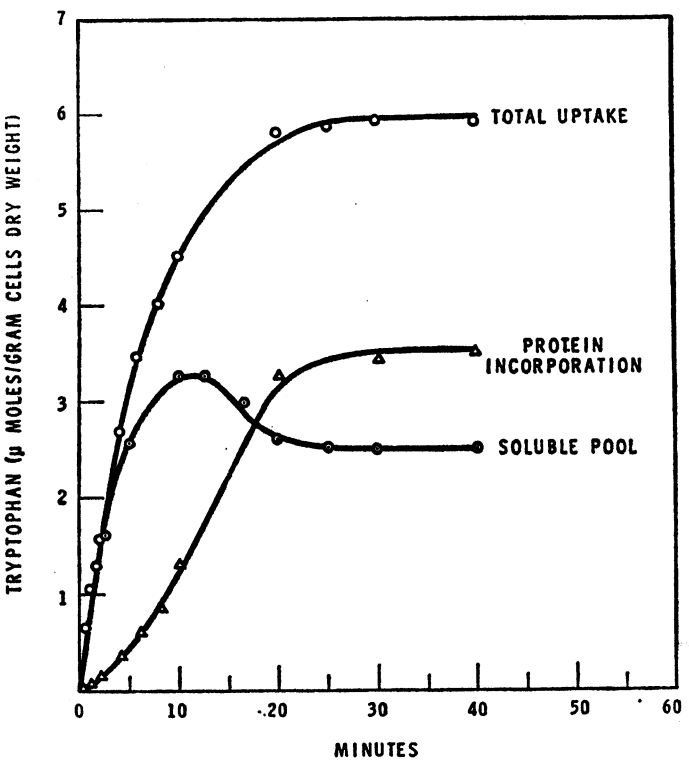

FIG. 1. Time course of tryptophan uptake and incorporation into protein by Neurospora. The uptake medium contained 310 μg/ml cells dry weight. The initial concentration of L-tryptophan-3-^{14}C (specific radioactivity, 2.12 μC/μmole) was 2.1×10^{-6} *M*. (From ref. 10.)

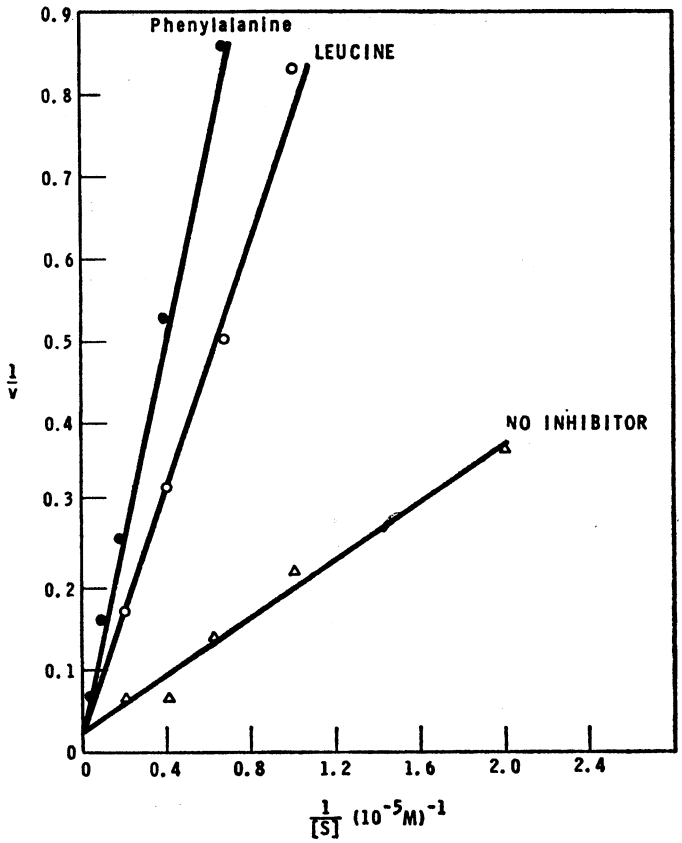

FIG. 2. Kinetics of tryptophan transport. Initial rates of transport were determined from time course studies as illustated in Figure 1. (Unpublished observations.)

inhibitors of tryptophan uptake. The fact that D-tryptophan and D-leucine are noninhibitory shows that the process is stereospecific. Figure 3 shows that the inhibitions exerted by leucine and phenylalanine are competitive. An α-amino group, a carboxyl group, and an uncharged side chain appear to be necessary for reactivity with the transport site(s). In addition, only amino acids in the L configuration show any reactivity. The competition between leucine and tryptophan is restricted entirely to the pool forming system. Leucine has no effect on the flow of tryptophan from the intracellular pool to the sites of metabolism.

The energy requirement for tryptophan uptake has not been clearly established. Since the process involves accumulation against a concentration gradient, it seems clear, however, that energy is required. Further, the temperature characteristic[10] is between 2.0 and 2.5.

TABLE 1. EFFECT OF OTHER AMINO ACIDS ON TRYPTOPHAN UPTAKE

Inhibitor	*Concentration* (M) $\times 10^{-4}$	*Percent inhibition of initial rate of uptake**
L-Leucine	5	90
L-Methionine	5	82
L-Ethionine	5	82
L-Cysteine	5	77
L-Phenylalanine	5	95
L-Tyrosine	5	72
L-Histidine	4	47
L-Serine	4	53
L-Isoleucine	5	11
L-Valine	5	9
L-Aspartic acid	4	0
L-Glutamic acid	4	0
Glycine	5	0
L-Lysine	4	10
L-Alanine	5	20
D-Tryptophan	5	0
D-Leucine	5	0

* External tryptophan 1×10^{-5} M; the inhibitor and tryptophan were added simultaneously to the reaction mixture. The rate of uptake was calculated from time course studies of the type shown in Fig. 1 (from ref. 10).

These observations together support the idea that tryptophan uptake is mediated by a process that is enzymic in nature. A knowledge of the precise mechanism of transport, however, will require isolation and characterization of the catalytic site.

Genetic control of tryptophan transport

Lester[11] has shown that strains of Neurospora that were found to be resistant to 4-methyl tryptophan were unable to transport tryptophan. In inheritance tests, 4-methyl tryptophan resistance behaved as a single gene difference. The 4-methyl tryptophan mutants were shown to be unable to transport many of the amino acids that are transported by the tryptophan transport system.[10] Stadler[12] has made a comprehensive study of the 4-methyl tryptophan resistant (*mtr*) mutants in which 30 resistant mutants were examined. In all of the mutants examined, the *mtr* locus mapped in a restricted region of linkage group IV. The mutation is recessive to the wild-type allele. Tryptophan uptake in six of the *mtr* mutants examined was less than 15 per cent that of wild-type strain 74A.

St. Lawrence, *et al.*[13] described an additional class of tryptophan transport

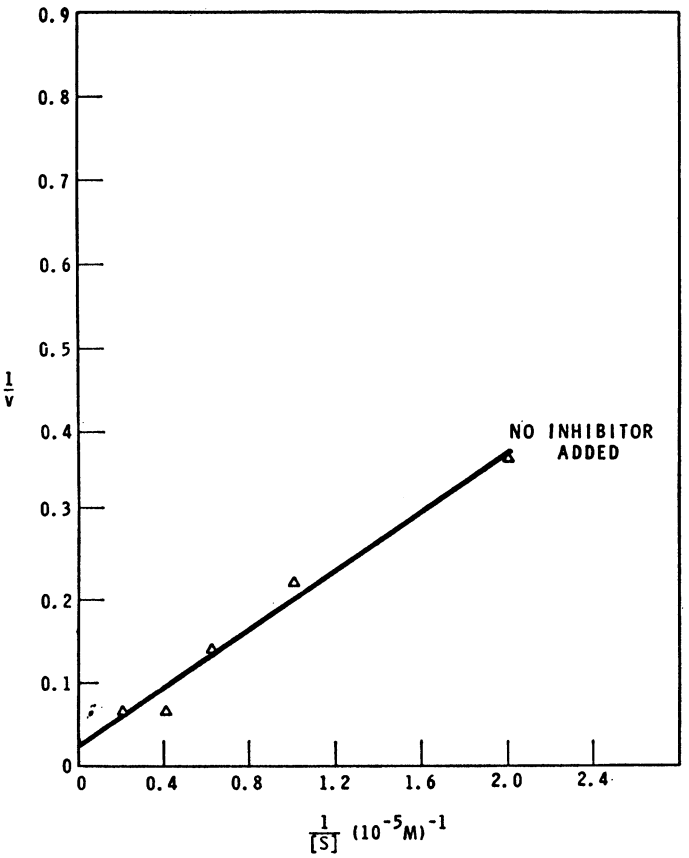

FIG. 3. Competitive inhibition of tryptophan transport by leucine and phenylalanine. Both leucine and phenylalanine were present at an initial concentration of 5×10^{-5} *M*. (Redrawn from data of ref. 10.)

mutants that are genetically and phenotypically distinct from the mutants described by Stadler.[11] This mutation (*mod-5*) is linked to the *tryp-2* (anthranilate synthetase, cf., Fig. 7) locus on linkage group VI and shows a 3 per cent second division segregation. The *mod-5* mutation is recessive to the wild-type allele.[13] Phenotypically, the *mod-5* mutation results in an inability of leucine to inhibit tryptophan uptake. In view of the observation[10] that leucine and tryptophan are transported by the same system (Fig. 3), it would appear that the *mod-5* mutation is a reflection of the alteration of the affinity of the tryptophan transport system for leucine. Alternatively, these mutants might suggest the existence of more than one tryptophan transport system.[14]

Sugar transport systems in microorganisms, except those for glucose and

perhaps galactose, are generally inducible systems.[15,16] Although amino acid transport systems are generally regarded as constitutive,[17] Boezi and DeMoss[18] showed that tryptophan permease of *Escherichia coli* exhibited catabolite repression in response to added glucose. Since the tryptophan transport system in Neurospora is apparently constitutively synthesized,[10] it has until now been difficult to study the influence of the transport system on the regulation of other cellular activities. The isolation of mutants[11,19] unable to transport tryptophan, however, will now permit studies on the role that the transport system in Neurospora plays in the regulation of growth, enzyme induction, repression, and feedback inhibition of enzyme activity. For example, tryptophan is required for the induction of kynureninase in Neurospora.* In low concentrations of inducer, the induction of kynureninase should be dependent on the accumulation of tryptophan by the transport system. Thus, it seems that the rate of induction of kynureninase at low tryptophan concentrations should be markedly decreased in the transport mutants. Future experiments with mutants cryptic for tryptophan should show how the tryptophan transport system contributes to cellular regulation and, as will be discussed below, is itself regulated.

Metabolic regulation of tryptophan transport

When Neurospora strain 74A growing in minimal medium is abruptly shifted to growth in medium supplemented with a high external concentration of tryptophan, a marked reduction is observed in the rate of tryptophan transport.[19] The time course of the reduction is shown in Figure 4. The time required to reduce the rate of uptake 50 per cent was approximately 5-10 minutes. The intracellular pool of tryptophan at zero time was 1.8 μmoles per g cells (dry weight). After 5 minutes the pool size increased to 40 μmoles per g cells. These data suggested that the reduction resulted from the accumulation of tryptophan in the intracellular pool. Pools of tryptophan formed from externally supplied indole are equally as effective in reducing the rate of tryptophan transport. It seems clear from these results that the reduction in the rate reflects a control mechanism which is correlated with the presence of tryptophan in the acid soluble pool. The observed decrease in the rate of transport is not a reflection of increased efflux of tryptophan. Tryptophan transport in Neurospora is unidirectional; in several critical experiments no efflux or exchange diffusion was observed.[10]

Inhibition of tryptophan uptake occurs when any amino acid transported by the tryptophan system is present in the acid soluble pool.[19] Leucine pools, for example, depress the rate of tryptophan uptake. Pools of lysine and

* See references 22 and 31.

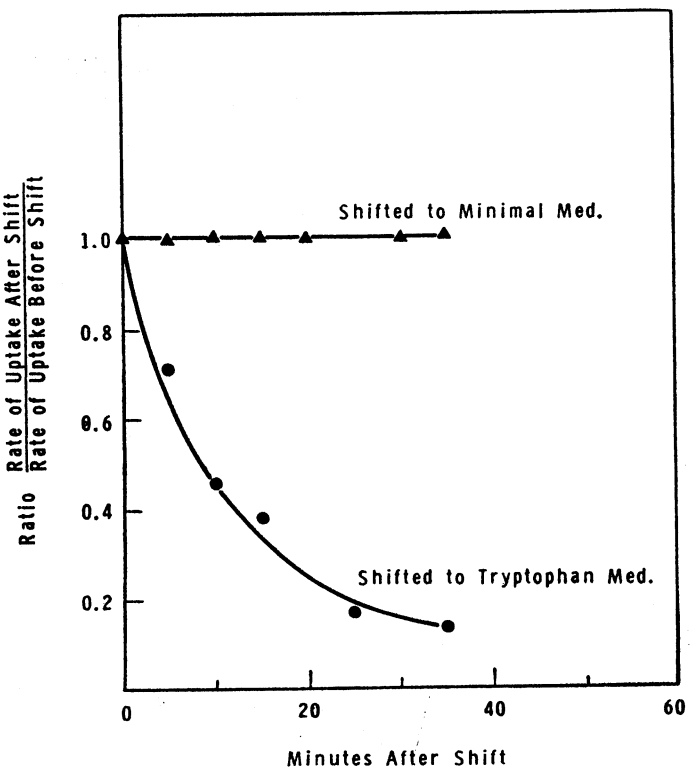

FIG. 4. Reduction in rate of transport following shift to tryptophan medium. Cells grown in minimal medium were transferred to medium containing 3 *mM* L-tryptophan. At the indicated times samples were removed, washed, and uptake measured as indicated in Figure 1. (From ref. 19.)

glutamic acid, amino acids that exhibit no reactivity with the tryptophan system, have no effect on tryptophan uptake. These results suggest that the effect of tryptophan on the transport system is a reflection of a general property of the members of the tryptophan transport group (cf., Table 1). While the partial specificity of the regulatory system can be reconciled on the basis of the concept of amino acid transport families, it is difficult to account for the lack of alternative transport routes for other amino acids of the same family. The selective value of such a control mechanism is difficult to comprehend, although other degenerative control mechanisms have been described (e.g., valine sensitivity[20]). In view of the competition for uptake between the amino acids of the tryptophan family, however, it is possible that when two or more of these amino acids are present in the environment, large pools of neither are formed and therefore this degenerative feedback is not exerted.

Neurospora mutant strains auxotrophic for tryptophan respond to large pools of tryptophan in much the same way as wild-type 74A. Much larger pools, however, are required to produce the same decrease in the rate of uptake in the mutant.[10] The reason for the altered response in mutants is unknown.

When Neurospora mutant strain *td*-201 was starved for an external source of tryptophan, the rate of tryptophan transport was markedly reduced.[21] Figure 5 shows the effect of tryptophan starvation on pool utilization, transport, and protein synthesis. The cessation of protein synthesis marked the beginning of a loss of transport activity.

Although not shown in Figure 5, the loss of transport activity is completely reversed by the addition of high concentrations of tryptophan to the external medium, an addition that permits resumption of protein synthesis.[21]

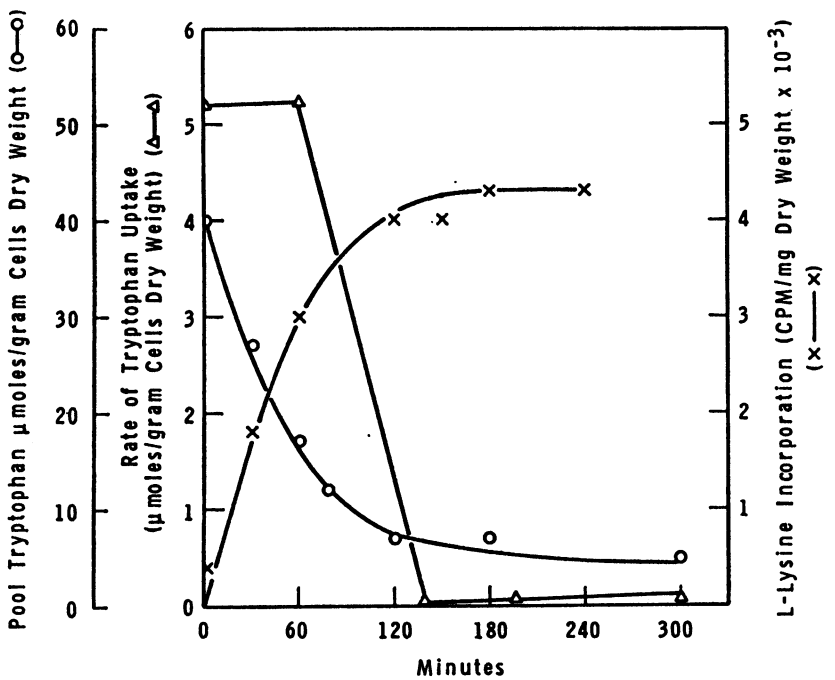

FIG. 5. Effect of tryptophan starvation on pool utilization, protein synthesis, and transport in a *td* mutant. The rate of tryptophan uptake was measured, as previously described,[10] at various times after the onset of tryptophan deprivation. Protein synthesis was assessed by observing the incorporation of labeled lysine (U-^{14}C, specific radioactivity 2.0 μC/μmole). Intracellular tryptophan was assayed with tryptophanase.[8] (From ref. 19.)

These results suggest that continued net protein synthesis is required for the maintenance of a functional transport system. Energy reserves are not depleted by amino acid deprivation since glycogen synthesis and respiration continue unabated during starvation for tryptophan. It should be noted also, that the regeneration of the transport system is inhibited by cycloheximide, an antibiotic that specifically inhibits protein synthesis in Neurospora.

Since continued protein synthesis is required to maintain a functional transport system, it should be possible by specifically inhibiting protein synthesis to measure the decay of the labile transport component. Figure 6 shows the result of such an experiment in which cycloheximide was used to inhibit protein synthesis. Control cells growing in minimal medium at 30° C. maintain a constant transport activity. Control cells treated with

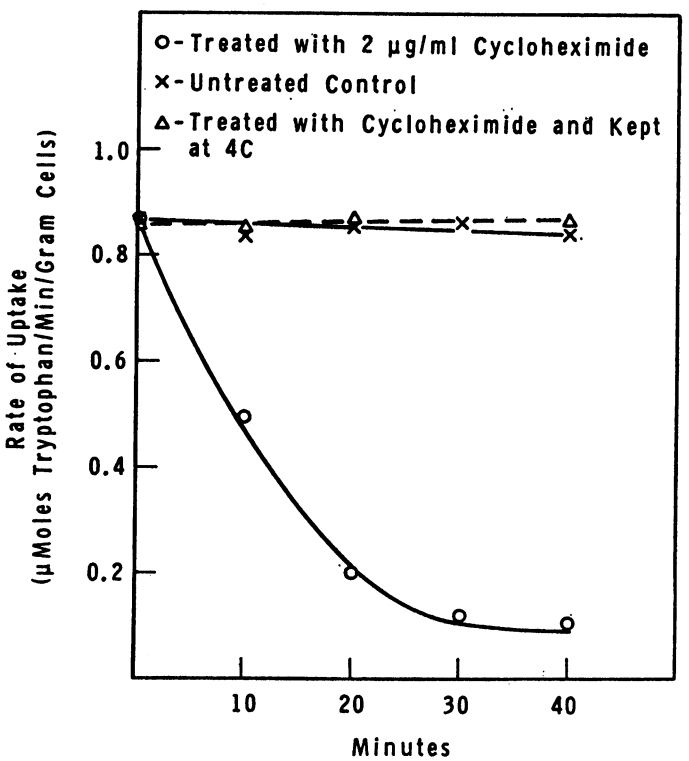

FIG. 6. Turnover of the tryptophan transport system. Wild-type cells were challenged with cycloheximide (2 µg/ml). At various intervals after cessation of protein synthesis, cells were sampled, washed, and their residual transport activity assessed as previously described. Other details are mentioned in the text. (From ref. 19.)

cycloheximide at 4° C. retain an intact transport system. Cells treated with cycloheximide at 30° C. showed a decay in ability to transport tryptophan that began at cessation of protein synthesis. These are the results to be expected if transport is limited by a labile component that requires synthesis of protein for regeneration. The lack of decay in activity at 4° C. suggests that degradation of the labile component is temperature sensitive and probably enzymically mediated. The previously mentioned observation that cycloheximide prevents regeneration of the labile component in the auxotroph[19] suggests that this component is a protein. The genetic evidence cited above is consistent with this view.

The turnover of tryptophan transport activity in Neurospora does not appear to be the result of generalized protein turnover or lability of tryptophan biosynthetic or degradative enzymes. Kynureninase and tryptophan synthetase activities remain essentially constant during experiments like that detailed in Figure 6.

The observation of a rapid turnover of the transport system suggests a mechanism for the regulation of tryptophan uptake. The rate of uptake could be modulated through the maintenance of a delicate balance between the synthesis and degradation of the transport site(s) or other labile constituents. Thus, as the pool size increased, tryptophan or some product would feed back and repress the synthesis of a component that turned over rapidly. In this connection we have observed that regeneration of transport activity subsequent to a period of inhibition of protein synthesis occurs at a considerably reduced rate when either tryptophan or leucine pools are populated.

Summary of the properties of the uptake system

Tryptophan uptake in Neurospora is mediated by a stereospecific transport system with catalytic properties which are very similar to those of enzyme catalyzed reactions. Tryptophan appears to combine with the transport site at the external surface of the membrane and this complex permits the movement of tryptophan to the interior of the cell where it is released in some undefined manner into an expandable pool. Energy is required for the process; however, the mechanism of energy coupling to the process has not been investigated.

The process appears to be genetically controlled. Two unlinked genes have been shown to affect the physiological function of the site(s). These results may indicate the existence of more than one reaction in the transport process.

The rate of tryptophan transport appears to be controlled by some component of the intracellular tryptophan pool. Regulation of the rate of trypto-

phan entry seems to be mediated through the maintenance of a delicate balance between the synthesis and degradation of some labile component of the transport system.

METABOLISM

Biosynthetic reactions

Figure 7 illustrates the salient features of the metabolism of tryptophan. The conversion of chorismic acid to anthranilic acid by the enzyme anthranilate synthetase is generally regarded as the first step specific to the biosynthesis of tryptophan in Neurospora. This activity was first demonstrated in extracts of Neurospora by J. A. DeMoss[1] who also showed that this activity was competitively inhibited by low concentrations of L-tryptophan. The next step in the biosynthesis involves the condensation of anthranilic acid and phosphoribosylpyrophosphate to form a molecule of N-5′-phosphoribosylanthranilic acid. This reaction is catalyzed by the enzyme phosphoribosyl transferase.[2] Phosphoribosylanthranilic acid is isomerized to form the anthranilic deoxyribulotide 1-(0-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CDRP) by the enzyme phosphoribosylanthranilic acid isomerase. CDRP is converted to indole-3-glycerol phosphate by the enzyme indoleglycerol phosphate synthetase.

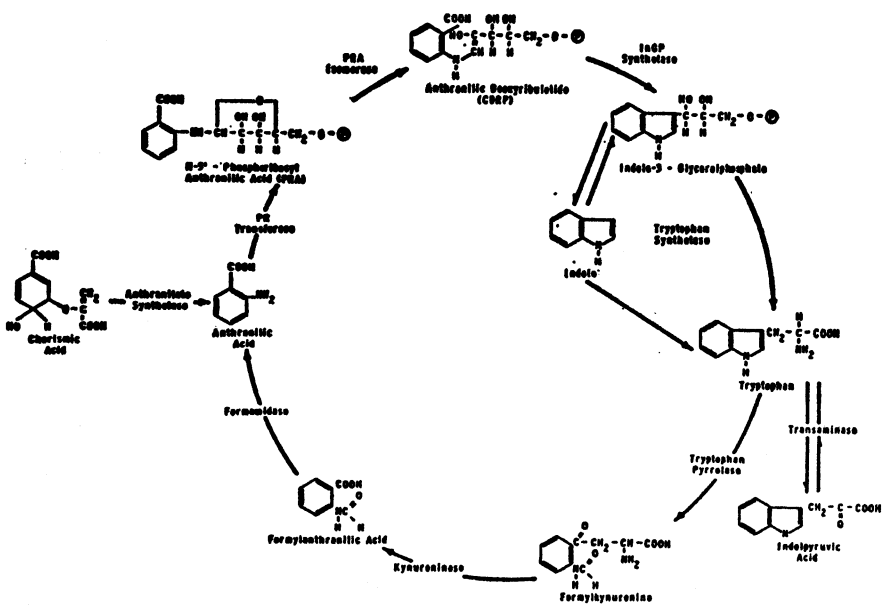

FIG. 7. The biosynthesis and degradation of tryptophan in Neurospora.

Indole-3-glycerol phosphate is the penultimate intermediate in the pathway leading to the biosynthesis of tryptophan. It is the physiological substrate for tryptophan synthetase and is converted by this enzyme to tryptophan. Tryptophan synthetase is the terminal enzyme in the biosynthetic sequence and, in addition to the physiologically important conversion of indoleglycerol phosphate to tryptophan, is known to catalyze the conversion of indole and serine to tryptophan as well as the reversible conversion of indoleglycerol phosphate to indole.[3]

Degradative reactions

Tryptophan formed by these reactions may be activated for subsequent incorporation into protein or alternatively may be oxidatively degraded to either formylkynurenine or indolepyruvic acid. The presence of tryptophan pyrrolase in extracts of *Neurospora crassa* has not been demonstrated. However, its presence is inferred from the patterns of accumulation[5] which are discussed below. The transaminase activity, resulting in the formation of indolepyruvate has been shown to occur in cell-free extracts.[6] The activity is apparently constitutive. If present, tryptophan pyrrolase degrades tryptophan to formylkynurenine, a compound that is further degraded to formylanthranilic acid.[22] Formylanthranilic acid may be excreted into the growth medium or it may be further degraded by the enzyme kynurenine formamidase[23] to form anthranilic acid, a compound that is itself an intermediate in the biosynthetic sequence. Under certain conditions, anthranilic acid will also be excreted into the medium and may subsequently re-enter the biosynthetic sequence for direct conversion to tryptophan.[5]

The tryptophan cycle

Figure 7 shows that the biosynthetic and degradative steps involved in tryptophan metabolism form a cyclic sequence of reactions involving anthranilic acid and tryptophan. The presence of such a cycle was first postulated by Haskins and Mitchell[4] who observed that anthranilic acid appeared to be accumulated by anthranilic mutants growing on tryptophan. Figure 8 illustrates the design of an experiment that tests whether or not this cycle actually turns during the growth of Neurospora. In such an experiment, tryptophan labeled in the two position of the indole nucleus is supplied to a mutant that is blocked prior to anthranilic acid. In this case, tryptophan that enters protein directly will do so without loss of the radioactive atom. However, any tryptophan that is routed through the degradative reactions of the cycle will lose the radioactive atom in the conversion of formylanthranilic to anthranilic acid. Tryptophan derived from the anthranilic acid formed will be nonradioactive. Thus, in such an experiment, the cells have available to them a source of radioactive tryptophan and a source of unlabeled

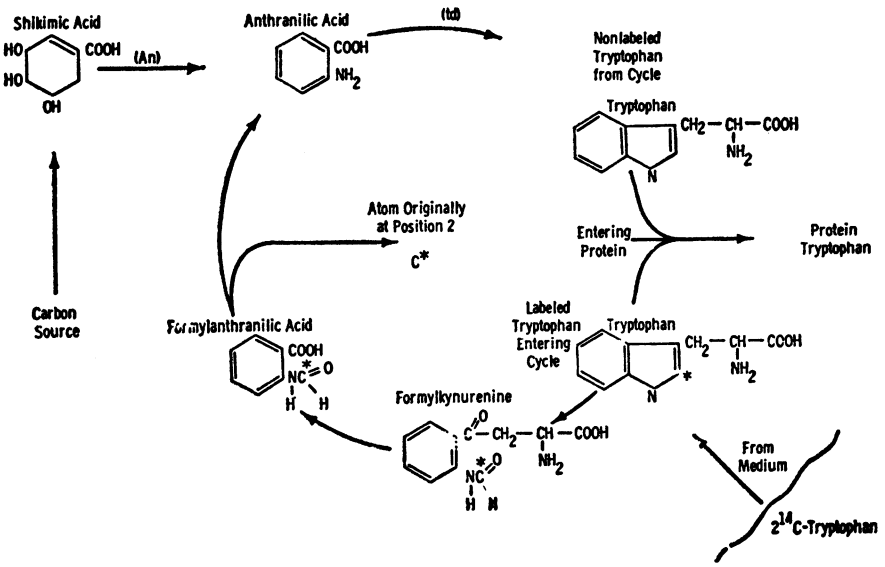

FIG. 8. Replacement of position two of tryptophan via the tryptophan cycle. (For detailed methods, see ref. 6.)

tryptophan arising through the biosynthetic arc of the tryptophan cycle. The amount of tryptophan that is cycled may be assessed by comparing the specific radioactivity of tryptophan isolated from protein with the specific radioactivity of the originally supplied tryptophan. If such measurements are made at various stages during growth under these conditions, one obtains a measure of the relative amount of the total tryptophan that has been incorporated directly into protein as compared with the amount of tryptophan that has been cycled before incorporation into protein. Figure 9 illustrates the results obtained in a typical experiment of the sort shown in Figure 8. The disappearance of externally supplied tryptophan from the filtrate is plotted as a function of the dry weight of the culture. As shown, tryptophan disappears from the filtrate after it has produced approximately 25 mg. dry weight of cells per 20 ml. of filtrate. The specific radioactivity of protein tryptophan at various times during this experiment is illustrated by the curve connecting the open circles. Tryptophan that had been incorporated into protein before the disappearance of externally supplied tryptophan showed no dilution in specific radioactivity. Dilution of the specific radioactivity of protein tryptophan occurred only after the disappearance of externally supplied tryptophan and apparently reflected the release of feedback inhibition exerted by tryptophan on early reactions of the biosynthetic arc of the cycle. This type of experiment is controlled by the use of mutants blocked between anthranilic acid and indoleglycerol

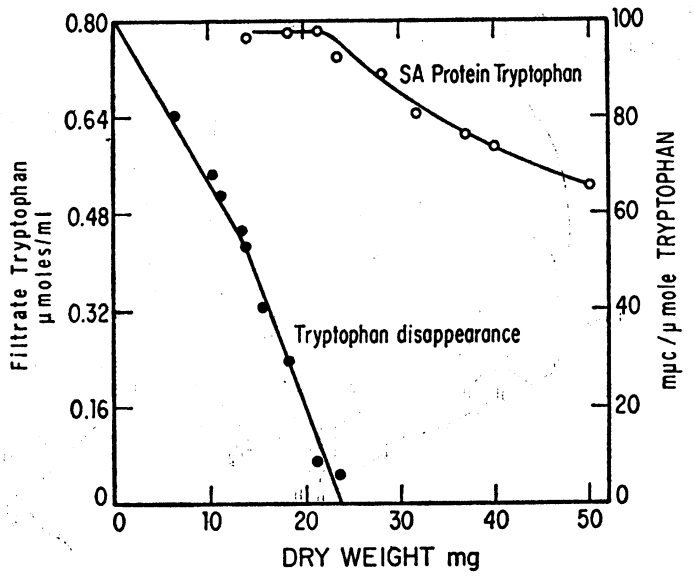

FIG. 9. Dilution of specific radioactivity of protein tryptophan. Experimental design as described in Figure 8 and text. Filtrate tryptophan was measured with tryptophanase.[8] Protein tryptophan was isolated from an alkaline hydrolyzate as indole resulting from digestion with tryptophanase.[5] Original specific radioactivity of filtrate tryptophan was 0.1 μC/μmole. (Unpublished observations.)

phosphate. Such mutants should be able to degrade tryptophan to formylanthranilic acid and anthranilic acid but unable to convert anthranilic acid to indoleglycerol phosphate and hence should show no dilution of specific radioactivity of tryptophan in protein. When these mutants were examined in experiments as described in Figure 9, no dilution was seen. Other controls for this experiment involve the use of externally supplied tryptophan labeled randomly in the benzene ring of the indole nucleus. When tryptophan labeled in these positions was used, no isotopic dilution of tryptophan entering protein was observed. These results are entirely consistent with the existence of the cycle as shown in Figure 7.

The role of indolepyruvic acid

Figure 10 shows that indolepyruvic acid is accumulated in the culture filtrate of mutant strains of Neurospora growing in the presence of an external supply of tryptophan. The importance of this compound in the metabolism of externally supplied tryptophan by Neurospora was discovered in experiments on the effects of temperature on the utilization of externally supplied tryptophan by tryptophan auxotrophs. It had been observed many times that growth yields of *td* mutants grown on limiting quantities of

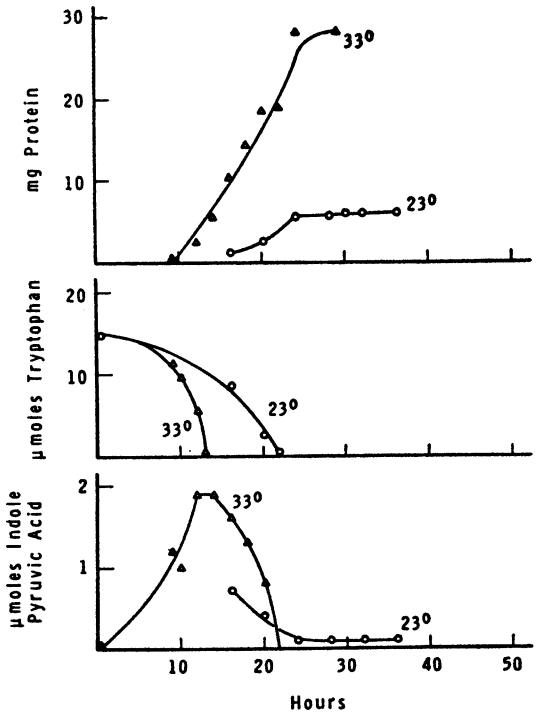

FIG. 10. The relationship of protein synthesis to utilization of supplied and accumulated compounds by a *td* mutant. Alkali soluble protein from cultures (20 ml.) was measured by the method of Lowry.[24] Filtrate tryptophan was measured with tryptophanase.[3] Indolepyruvic acid was identified in filtrates by the methods of Srivastava[25] and measured with the $FeCl_3$ reagent.[26] (From ref. 6.)

tryptophan were considerably reduced when these strains were grown at 20° C. instead of 30° C.[27] These differences in growth yield were originally attributed to differences in the rate of turn of the tryptophan cycle and to differences in the size and prolonged maintenance of the intracellular pool of tryptophan. However, rigorous examination of these points revealed that neither explanation could account for the differences in final yield of mycelia. Figure 10 illustrates an experiment that shows the role of indolepyruvic acid in this temperature response. The upper panel shows the time course of protein synthesis in a *td* mutant grown at 33° C. and at 23° C. on the same limiting quantity of tryptophan. The middle panel shows the time course of utilization of externally supplied tryptophan by these cultures. The final yield of protein at 33° C. is approximately sixfold higher than the yield at 23° C. Protein synthesis at 33° C. continued uninterruptedly for many hours after the disappearance of external tryptophan. At 23° C., however, protein synthesis stopped abruptly when external

tryptophan was exhausted from the filtrate. Filtrates of cultures grown at 33° C. were sampled at early times after tryptophan had disappeared and were found to support continued growth of subcultures. Further investigation of these filtrates revealed the presence of indolepyruvic acid, a compound that was subsequently shown to support the growth of *td* mutants and other tryptophan auxotrophs. The lower panel of Figure 10 shows the time course of appearance and utilization of indolepyruvic acid in filtrates of these cultures. At the time of tryptophan disappearance from 33° C. cultures, approximately 2 μmoles of indolepyruvic acid were present in their filtrate. After this time, approximately 20 mg. of protein were synthesized. During the synthesis of this protein, the accumulated indolepyruvic acid disappeared. The synthesis of 20 mg. of Neurospora protein requires approximately 2 μmoles of tryptophan. This is precisely the amount of indolepyruvic acid that was present in the filtrate and apparently available for conversion to tryptophan and subsequent incorporation into protein. At 23° C., when tryptophan disappeared, net synthesis of protein stopped. Cessation of protein synthesis is consistent with the demonstrated absence of indolepyruvic acid from the 23° C. filtrate. The reason for the accumulation of indolepyruvate at 33° C. but not at 23° C. is presently unknown but may be related to the fact that the size of the intracellular pool of tryptophan is considerably higher at the higher temperature.

Patterns of accumulation

The tryptophan cycle, as it appears in Figure 7, shows that certain compounds might be expected to accumulate in the culture filtrate of mutants growing in an external supply of tryptophan. *Td* mutants growing in an external supply of radioactive tryptophan labeled in the benzene ring exhibit these expected accumulations quite clearly. Formylanthranilic acid accumulates in the filtrate and its specific radioactivity is equal to the specific radioactivity of the originally supplied tryptophan. Anthranilic acid which accumulates before the disappearance of tryptophan has the same specific radioactivity as the originally supplied tryptophan. When externally supplied tryptophan is exhausted, the cells continue to synthesize anthranilic acid from the major carbon source and this compound is excreted into the medium. The specific radioactivity of the accumulated anthranilic acid is therefore diluted by nonradioactive anthranilic acid arising from the carbon source. Figure 11 illustrates a typical experiment in which a *td* mutant growing in the presence of radioactive tryptophan accumulated formylanthranilic and anthranilic acid. The specific radioactivities of these compounds are plotted as a function of the age of the culture. The arrow indicates the time when tryptophan disappeared from the filtrate. The re-

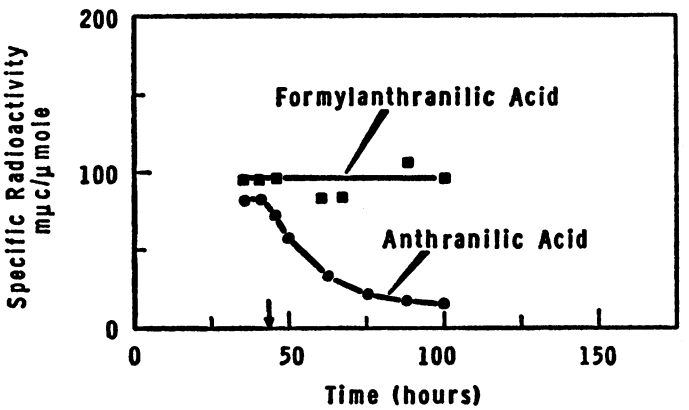

FIG. 11. Specific radioactivities of compounds arising from the degradation of L-[benzene-$^{14}C_6$] tryptophan (0.1 μC/μmole) by Neurospora. Compounds were isolated from filtrates by paper chromatography.[5] Formylanthranilic acid was hydrolyzed to anthranilic acid and this compound was measured spectrofluorometrically. (Redrawn from ref. 5.)

sults show that the specific radioactivity of formylanthranilic acid remained equal to the specific radioactivity of the originally supplied tryptophan for many hours after it disappeared from the filtrate. Anthranilic acid, on the other hand, had the same specific radioactivity as the originally supplied tryptophan at times before externally supplied tryptophan disappeared but showed a reduction in specific radioactivity starting immediately after the disappearance of externally supplied tryptophan. These results are consistent with the cycle turning as written. They offer a further illustration of the ability of tryptophan to inhibit the synthesis of anthranilic acid. Anthranilic acid arising from the carbon source does not become available to dilute the specific radioactivity of the accumulated anthranilic acid until external tryptophan, the apparent inhibitor of anthranilate synthetase, is exhausted. Although not indicated by these data, approximately 70 to 80 per cent of the originally supplied tryptophan is degraded via the tryptophan cycle and eventually appears as formylanthranilic or anthranilic acid in filtrates of *td* mutants.[5]

CHANNELING

Concept of multiple pools

Early work on factors regulating the rate of formation of enzymes involved in the biosynthesis and degradation of tryptophan showed that in prototrophic strains, the biosynthetic enzymes were fully repressed under virtually all conditions of growth. Auxotrophic strains, on the other hand,

where shown to exhibit derepression of the tryptophan biosynthetic enzymes only under conditions of tryptophan deprivation. Briefly, these results may be summarized as follows: Derepression of tryptophan synthetase was observed to occur in auxotrophs under conditions in which the intracellular concentration of tryptophan fell below the level of 1 μmole per gram dry weight of mycelia. In prototrophic strains, on the other hand, even though the intracellular concentration of tryptophan was caused to vary over a 30-fold range from 0.5 to 15 μmoles of tryptophan per gram dry weight of mycelia, no derepression was observed even at the lowest intracellular concentrations of tryptophan. Thus, prototrophic strains appeared to be fully repressed even though the intracellular concentrations of tryptophan were below that required for derepression in auxotrophic strains. An interpretation of these results is that more than one pool of intracellular tryptophan is present in Neurospora and further that tryptophan deprivation of a tryptophan auxotroph is the only means of depopulating the pool concerned with regulation of the formation of biosynthetic enzymes. A corollary of this interpretation is that the products of the biosynthetic sequence populate the regulatory pool preferentially.

Preferential utilization

Certain features of the metabolism of externally supplied tryptophan in Neurospora make it possible to test this interpretation in a fairly direct way. Specifically, it is possible to assess the contribution of the tryptophan biosynthetic sequence to various pools. The operational requirement for such a test is that the observer be able to distinguish between tryptophan from an external source and tryptophan of endogenous origin. In Neurospora this requirement may be satisfied in either of two ways. As shown above, the tryptophan cycle is capable of generating non-radioactive molecules of tryptophan from those previously labeled with radioactivity. Alternatively, it is possible to supply an indole utilizing strain with radioactive indole and with nonradioactive tryptophan. Indole will be taken up by the cells and converted to tryptophan as fast as it enters.[38] Biosynthesized tryptophan in this case would be radioactive and tryptophan of exogenous origin would be nonradioactive. Under conditions in which growth is permitted to occur in the presence of labeled indole and cold tryptophan, both compounds will enter the cells independently and each will enter at a rate sufficient to permit growth of the mycelia in the absence of the other. Figure 12 shows data taken from a culture growing under these conditions. The utilization of radioactive indole and nonradioactive tryptophan are plotted as functions of the dry weight of the culture at various times. At times before the disappearance of external tryptophan, samples of mycelia were withdrawn. The observed specific radioactivities of acid soluble tryptophan

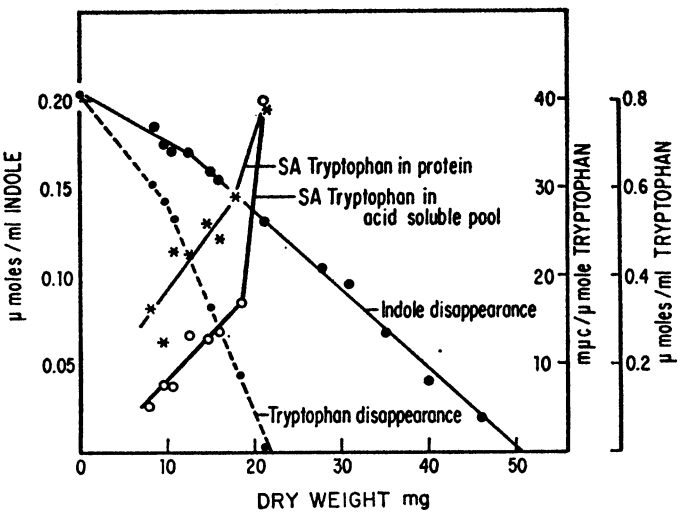

FIG. 12. Specific radioactivities of protein tryptophan and acid soluble tryptophan in cells growing in a medium supplemented with indole-2-^{14}C and unlabeled tryptophan. Methods as described in the legends of Figures 9 and 10. (From ref. 28.)

and of tryptophan isolated from protein of the cells are plotted as functions of the dry weight of the culture. A comparison of these specific radioactivities reveals that at all times before the disappearance of external tryptophan the specific radioactivity of tryptophan isolated from protein was higher than the specific radioactivity of the acid soluble tryptophan. Using the data on uptake of indole and tryptophan and assuming that there is complete mixing of exogenous tryptophan with tryptophan biosynthesized from exogenous indole, it is possible to calculate the theoretical specific radioactivity expected of tryptophan present in the intracellular acid soluble pool. In Fig. 13 these values are plotted for various growth stages of the culture between 10 and 22 mg. (i.e., the generation that immediately preceded the disappearance of tryptophan). These values agree with the values actually observed for tryptophan present in the acid soluble pool. From the known rate of protein synthesis in this experiment[28] and from the fact that Neurospora protein contains approximately 0.1 μmole of tryptophan per milligram of protein,[5] it is possible to calculate by increments the specific radioactivity of tryptophan actually entering protein at these times that would be required to give the increases in specific radioactivity of protein-tryptophan recorded in Figure 12. These values were calculated and are also plotted in Figure 13. There was agreement between observed and expected specific radioactivities of the total acid soluble pool of tryptophan. Tryptophan entering protein at these times had a two to threefold higher

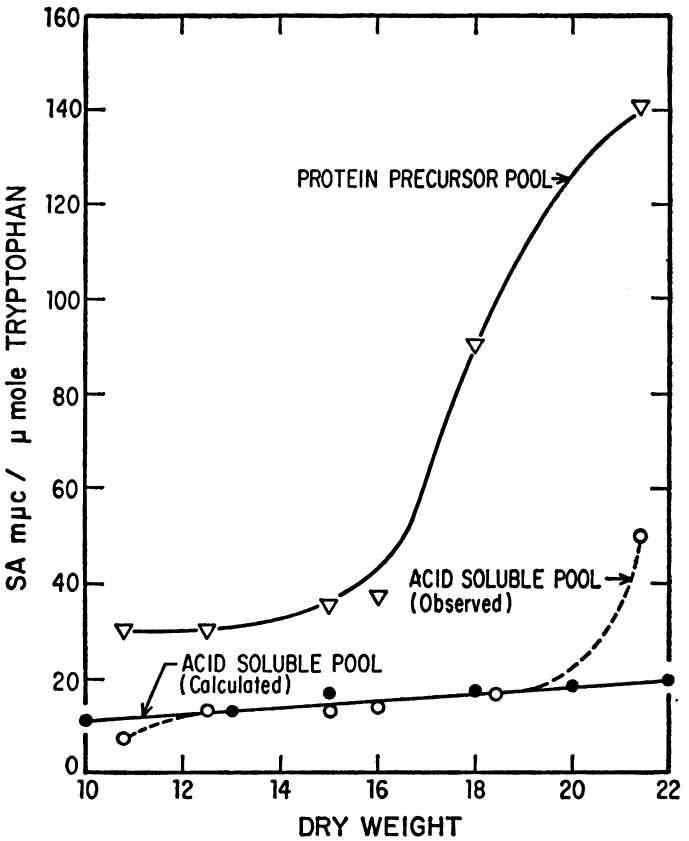

FIG. 13. Comparison of specific radioactivities of tryptophan: precursor pool calculated by increments (see text), acid soluble pool observed, and theoretically derived acid soluble pool based on assumption of complete mixing of biosynthesized and externally supplied tryptophan. (From ref. 28.)

specific radioactivity. Since the only source of radioactive tryptophan under these conditions was that derived from the biosynthetic sequence, these results indicate that tryptophan of biosynthetic origin is incorporated preferentially into protein. They also offer an independent confirmation of the concept of multiple pools of intracellular tryptophan. Other experiments to be described below will show that tryptophan derived endogenously from an external source of indole appears to be a more effective repressor of formation of the biosynthetic enzymes than is tryptophan derived from an exogenous source.

REGULATORY EFFECTS OF INTRACELLULAR TRYPTOPHAN

When Neurospora grows in medium supplemented with tryptophan,

endogenous synthesis of this amino acid is essentially nil. The cells draw upon the external source until it is exhausted and only then do they begin endogenous synthesis. The mechanism that allows the cells to use the external source preferentially almost certainly involves the fact that anthranilate synthetase is feedback inhibited by low internal concentrations of tryptophan.[1] In addition to this fine control on the flow of intermediates through the biosynthetic sequence, intracellular tryptophan has been shown to repress the formation of enzymes involved in its biosynthesis.[27,29,30] Intracellular tryptophan is also known to induce the formation of kynureninase.[29,31] A consideration of these effects leads to the conclusion that intracellular tryptophan has the attributes generally ascribed to a small molecule effector as this term is used in the operon theory.[29]

In the preceding section, experiments were detailed that indicated the existence of more than one intracellular pool of tryptophan. It should be recalled that the multiple pool concept was originally postulated to account for the fact that derepression of tryptophan biosynthetic enzymes was not observed in prototrophs. Experiments have been reported recently[33] that extend the concept of multiple pools and suggest that distinct regulatory functions may be assigned to them.

Repression of biosynthetic enzymes

Certain tryptophan synthetase mutants (cf., Fig. 7) retain the ability to catalyze the condensation of indole and serine to form tryptophan. These

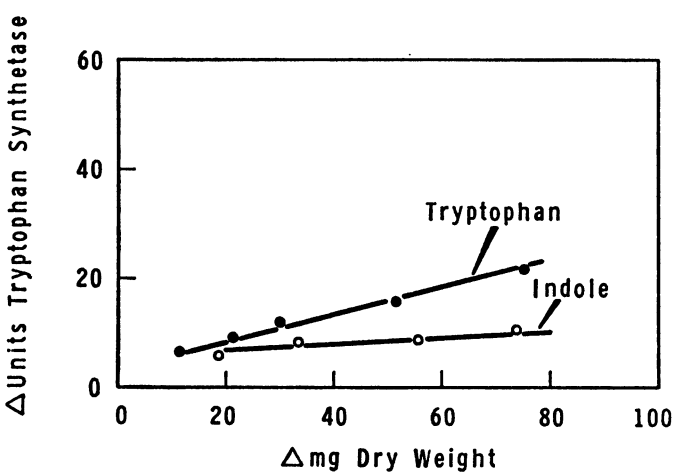

FIG. 14. Relative rate of formation of tryptophan synthetase by *td*-201 (indole utilizer) growing in either tryptophan or indole. Tryptophan synthetase was measured by the method of Yanofsky.[34] One unit of enzyme is the amount required to catalyze the disappearance of 1 μmole of indole per hour at 37° C. Tryptophan was present initially at 0.74 μmoles/ml; indole at 0.18 μmoles/ml.

mutants will grow if supplied with either indole or tryptophan. Growth on indole forces such mutants to generate tryptophan biosynthetically. Figure 14 illustrates the relative rate of formation of tryptophan synthetase in a *td* mutant growing in the presence of either tryptophan or indole. At times before the disappearance of supplement, enzyme is formed at a higher rate in the presence of tryptophan than in the presence of indole. Although not shown by these data, after the disappearance of either supplement, repression is released and both cultures form enzyme at the same increased rate.[37] These results are consistent with the failure to observe release of repression in prototrophs. They suggest that tryptophan of biosynthetic origin is more effective at causing repression than is tryptophan derived from an external source.

The recent work of Carsiotis and co-workers[35-37] with histidine mutants and with 3-amino-1, 2, 4-triazole (3AT) is particularly instructive. They have shown that histidine mutants, when grown on a medium limited in histidine, exhibit a remarkable derepression with respect to tryptophan biosynthetic enzymes. The onset of derepression appears to be coincident with exhaustion of histidine and deceleration of growth. Their work with 3AT has shown that this compound causes wild-type and other strains to exhibit derepression of tryptophan enzymes. These observations are related by the fact that 3AT, a compound which inhibits the activity of imidazoleglycerol phosphate dehydratase,[38] causes wild-type strains to mimic histidine auxotrophs. In both cases the effects were reversed by addition of histidine to the growth medium. In retrospect, and in view of some results we shall describe below, it would appear that in the presence of 3AT, derepression of the tryptophan biosynthetic enzymes is due at least in part to the resultant accumulation of imidazoleglycerol phosphate.

While it seems clear that imidazoleglycerol phosphate has a positive effect on the rate of formation of tryptophan synthetase, the derepression of the enzyme in early histidine mutants that are genetically blocked prior to imidazoleglycerol phosphate, requires further interpretation. Carsiotis[37] has shown that mutants which cannot accumulate imidazoleglycerol phosphate do nevertheless exhibit derepression of tryptophan synthetase when undergoing histidine starvation. Further, he has shown[37] that wild-type cells, when challenged with α-methylhistidine, an inhibitor of the attachment of histidine to sRNA,[39] also exhibit derepression with respect to tryptophan biosynthetic enzymes. These observations would seem to implicate histidinyl-sRNA in a control mechanism impinging on the enzymes of tryptophan biosynthesis.

Crawford and Gunsalus[40] working with *Psuedomonas putida* have reported that indoleglycerol phosphate behaves in this organism as if it were

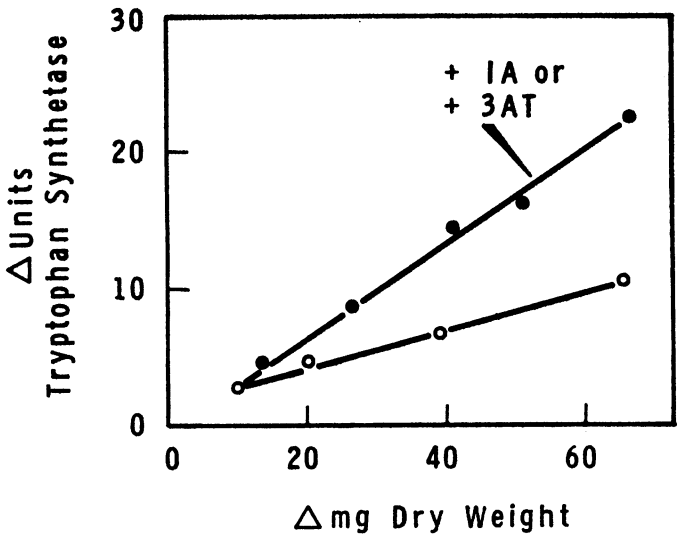

FIG. 15. Derepression of tryptophan synthetase by wild-type (74A) Neurospora in the presence of 3AT or indoleacrylic acid. Indoleacrylic acid was present at an initial concentration of 5.0×10^{-5} *M*; 3AT at 5.0×10^{-3} *M*. Methods as described in Figure 14. (Unpublished data.)

the inducer of tryptophan synthetase. Doolittle[41] has shown that indoleacrylic acid has essentially the same effect on tryptophan synthetase of *E. coli*.

We have examined the effect of indoleacrylic acid and 3AT on the formation of tryptophan synthetase in wild-type Neurospora and have found (Fig. 15) that these compounds cause derepression. Furthermore, we have found that coincident with derepression the cells accumulate imidazoleglycerol phosphate (in response to 3AT) or indoleglycerol phosphate (in response to indoleacrylic acid). Thus, in Neurospora it would appear that indoleglycerol phosphate has essentially the same derepressive effect that imidazoleglycerol phosphate seems to have in the cases described by Carsiotis and co-workers. The structural similarities of these compounds suggest that they have the same mode of action. In our experience the presence of either of these compounds appears to interfere with the ability of intracellular tryptophan to repress tryptophan synthetase.

That 3AT and indoleacrylic acid were without direct effect in these responses was shown by studies with mutants. When a *hist-7* mutant (blocked prior to imidazoleglycerol phosphate) was challenged with 3AT, neither derepression nor accumulation of imidazole compounds was observed. Derepression was observed in this strain in response to indoleacrylic acid,

a treatment that also caused accumulation of indoleglycerol phosphate. Similarly, a *tryp-4* mutant (blocked prior to indoleglycerol phosphate) failed to exhibit derepression in response to indoleacrylic acid but was responsive to 3AT.

Induction of degradative enzymes

Studies on the induction of kynureninase by tryptophan have contributed importantly to our understanding of control mechanisms exerted by tryptophan in Neurospora. Figure 16 shows the induction of kynureninase (cf., Fig. 7) in an indole utilizing *td* mutant of Neurospora under various growth conditions. Externally derived tryptophan is a more effective inducer than is endogenously synthesized tryptophan derived from an external source of

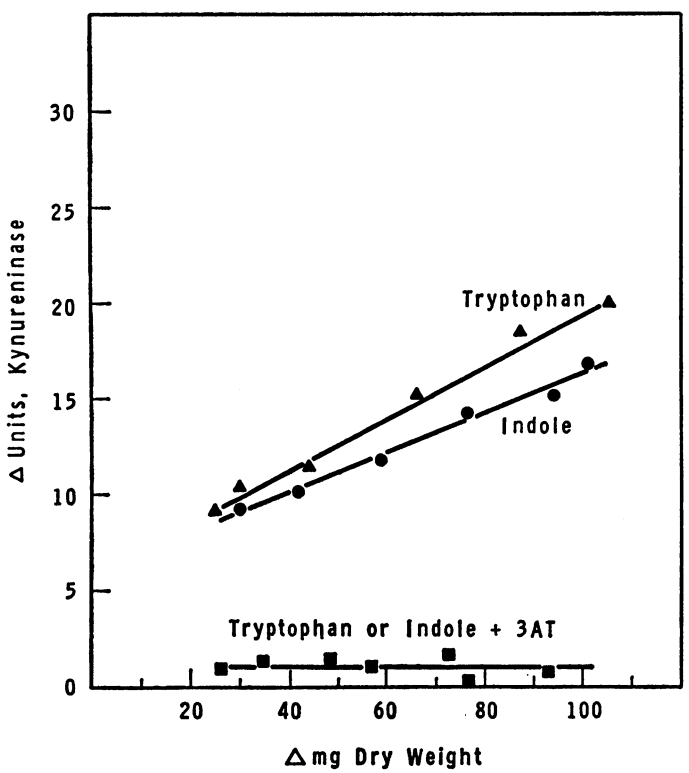

FIG. 16. Induction of kynureninase in the presence of tryptophan or indole and the effect of 3AT. 3AT present at initial concentration of 5.0×10^{-3} *M*. Kynureninase was measured by appearance of anthranilic acid.[22] One unit of kynureninase is that amount of enzyme required to catalyze the appearance of one μmole of anthranilic acid from kynurenine per hour at 37° C. Other methods as described in Figure 14. (Unpublished observations.)

indole. The presence of 3AT completely inhibits the induced synthesis of kynureninase regardless of the source of tryptophan. These results and those presented in Figure 14 point to an interesting conclusion regarding the concept of multiple pools discussed in the preceding section. Under conditions that force the cells to draw upon endogenously synthesized tryptophan, tryptophan synthetase, the biosynthetic enzyme, is maximally repressed and kynureninase, a degradative enzyme, is poorly induced. In contrast, under conditions that force the cells to draw upon an external source of tryptophan and prevent endogenous synthesis, the formation of tryptophan synthetase is poorly repressed and kynureninase is maximally induced. These results suggest that tryptophan in the internal pool mediates repression of biosynthetic enzymes and in the expandable pool mediates induction of the degradative enzymes. Thus, it would appear that these pools are distinguished from each other by three criteria; namely, 1) their primary source of tryptophan, 2) the metabolic fate of their contents, and 3) their apparently distinct regulatory functions.

Involvement of aminoacyl-sRNA in control

Recent reports from several laboratories (for review see reference 42) have introduced the concept that aminoacyl-sRNA derivatives of amino acids are involved in control mechanisms and are perhaps the active effectors controlling the synthesis of protein.

The results reviewed here are consistent with the idea that a derivative of tryptophan is the active effector in Neurospora and further, that indoleglycerol phosphate and the structurally related imidazoleglycerol phosphate interfere with the derivitization and thereby reduce the ability of tryptophan to become active as an effector of protein synthesis. Our work in the near future will focus on testing some predictions of this interesting possibility. Specifically, we shall examine the effects of appropriate indole and imidazole derivatives on the synthesis of tryptophanyl-sRNA in cell free charging systems and on the level of charged tryptophan sRNA *in vivo*. It should be borne in mind, however, that even if tryptophanyl-sRNA is implicated in tryptophan mediated control of protein synthesis in Neurospora, we would still remain far from a satisfactory understanding of regulation in this organism. The operon concept,[39] which seems to apply so well to certain bacterial systems, is of doubtful value in Neurospora. In Neurospora, a eucaryotic organism, not only are the structural genes of tryptophan biosynthesis not linked, they are located on different chromosomes. The identification in Neurospora, of a molecule with the attributes normally ascribed to an effector (in the context of the operon theory) is likely therefore to raise at least as many questions as it answers.

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