JOURNAL OF BACTERIOLOGY, Dec. 1967, p. 1896-1907

Vol. 94, No. 6
Printed in U.S.A.

Organization of the Tryptophan Pathway: a Phylogenetic Study of the Fungi

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Received for publication 17 July 1967

The enzymes involved in tryptophan biosynthesis have been analyzed in a variety of fungal strains and a few other microorganisms. The same five biosynthetic reactions occur in all organisms tested, but differences have been found in the stability requirements for the enzymes, in their differential precipitation with ammonium sulfate, and in their sedimentation pattern after zone centrifugation. Based on the sedimentation behavior of anthranilate synthetase, phosphoribosyl-transferase, *N*-(5'-phosphoribosyl)-anthranilate isomerase, and indole-3-glycerophosphate synthetase, five different patterns of enzyme association could be recognized. The distribution of these patterns was used to evaluate several specific features of proposed phylogenetic relationships in the fungi. A closer relationship between *Chytridiales* and *Aspergillales* is postulated, eliminating the *Zygomycetes* and the *Endomycetales* as probable intermediates; the latter groups are considered to be sidelines. The data support the idea of a polyphyletic origin of the phycomycetes and suggest that anascosporogenous yeasts tested are related to the heterobasidiomycetes rather than to the *Endomycetales*. A possible sequence of changes leading to the various patterns of organization of the tryptophan pathway during the course of evolution is also proposed.

The presently held schemes of phylogenetic relationships among fungi are based mainly on morphological criteria and on life cycle studies (7, 19, 30). Some understanding of evolutionary trends, such as the development of the dikaryophase and the elaboration of fruiting bodies, has been attained, but many aspects of phylogenetic relationships remain uncertain. Apart from the composition of the cell wall (2, 38), little use had been made in studies on fungi of physiological or biochemical approaches as a basis for phylogenetic interpretations. However, with plants and animals, as well as with bacteria, extensive studies of macromolecules, nucleic acids, and proteins, and comparative physiological investigations, have provided additional bases for evolutionary considerations (10, 25).

The diversity found in the biochemical organization of the tryptophan pathway (9, 16) provides a possible approach to phylogenetic studies. Tryptophan biosynthesis involves the same five enzymatic steps in all organisms which have been studied. However, in the species previously analyzed in detail, differences have been found both in the gene-enzyme relationships and in the organization of the reactions in separable protein components (Fig. 1). The genetic control of the enzymes is illustrated on the left half of Fig. 1; the arrows indicate the enzymes which are influenced by the various loci. In *Escherichia coli* and *Salmonella typhimurium*, the genes are closely linked and arranged in operons (3, 5, 8, 22, 27, 32, 41); in the fungi, the tryptophan genes are each located on a different linkage group (1, 4, 28, 31). The genetic pleiotropism observed is due to differences in the molecular organization of the enzymes as illustrated on the right half of Fig. 1. In *E. coli* and *S. typhimurium*, tryptophan synthetase is reversibly dissociable into two active subunits (9, 12, 40; E. Balbinder, *personal communication*), the active components which catalyze anthranilate synthetase and phosphoribosyl (PR)-transferase form a complex (6, 23), and activities 3 and 4 are found in a single component (8, 13). Components which catalyze multiple reactions are also present in *Neurospora crassa*, *Aspergillus nidulans*, and *Saccharomyces cerevisiae* (9, 16, 18, 21) but they differ in the enzymes involved.

We have used the differences illustrated in

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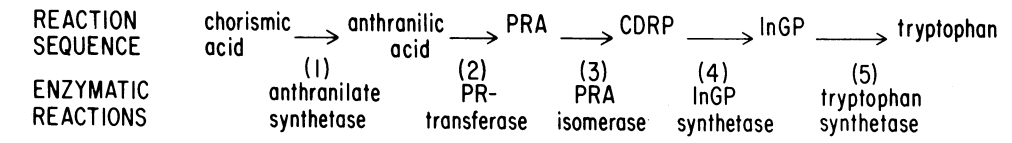

ORGANISMS	GENETIC LOCI AND THEIR CONTROL OF ENZYME ACTIVITIES	ENZYME REACTION COMPONENTS SEPARABLE ON SUCROSE GRADIENTS
Escherichia coli	E D C B A (1) (2) (3) (4) (5)	[1+2] [3+4] [5] ⇌ [5A] + [5B]
Salmonella typhimurium	A B E D C (1) (2) (3)? (4) (5)	[1+2] [3+4] [5] ⇌ [5A] + [5B]
Saccharomyces cerevisiae	trp-2 trp-4 trp-1 trp-3 trp-5 (1) (2) (3) (4) (5)	[1+4] [2] [3] [5]
Neurospora crassa	trp-2 trp-4 trp-1 trp-3 (1) (2) (3) (4) (5)	[1+3+4] [2] [5]
Aspergillus nidulans	A D C B (1) (2) (3) (4) (5)	[1+3+4] [2] [5]

FIG. 1. *Gene-enzyme relationships and association of enzyme components in the tryptophan pathway. Abbreviations: PRA, N-(5'-phosphoribosyl)-anthranilate; CDRP, 1-(O-carboxyphenylamino)-1-deoxyribulose-5-phosphate; InGP, indole-3-glycerophosphate; PR, phosphoribosyl.*

Fig. 1 to examine possible relationships among a variety of fungi and other microorganisms. Since a genetic analysis of most fungal strains is impossible, our investigations have been restricted to the study of the protein components. Isolates of the blue-green alga *Anabaena variabilis*, the myxomycete *Physarum polycephalum*, and two strains of *E. coli* have been included in our study in order to consider possible connections between the fungal strains tested and other groups of organisms.

MATERIALS AND METHODS

Organisms and culture conditions. A list of the strains tested is given in Table 1. The strains were maintained on slants of yeast extract-malt extract-agar (29) or nutrient agar (15). Fermentation flasks containing from 1 to 10 liters of medium I or II were inoculated and then incubated either on a rotary shaker (1-liter flasks) or by forced aeration (2- to 10-liter flasks) at 21, 30, or 37 C (Table 1). The unicellular organisms (*Anabaena variabilis*, *E. coli*, yeasts) were harvested by centrifugation; the mycelial organisms were harvested by filtration. The cell material was lyophilized, except that with *A. variabilis* and *E. coli* the fresh cell material was used. The lyophilized material was ground to a fine powder with alumina and quartz sand in a mortar.

Preparation and fractionation of extracts. For investigation of the enzymes, the powders from lyophilized materials were extracted and fractionated with protamine sulfate and solid ammonium sulfate as described (17). *A. variabilis* and *E. coli* were broken in a Branson sonic oscillator (17) and fractionated by the same procedure as the extracts from lyophilized powders. In most cases, extracts and fractionations were made in 0.05 M potassium phosphate buffer (*p*H 7.0) containing 10^{-4} M ethylenediaminetetraacetic acid (EDTA). Some exceptions had to be made, however. In the case of *Giberella fujikuroi*, the buffer had to be used without EDTA but with a supplement of 1 M potassium chloride to prevent destruction of the enzymes. For *Cryptococcus laurentii* var. *flavescens*, *Mucor hiemalis*, *Phycomyces blakesleeanus*, *Rhizopus arrhizinus*, *Rhodotorula glutinis*, *Sporobolomyces salmonicolor*, *Tremella mesenterica*, and *Ustilago maydis*, the buffer had to be supplemented by the addition of 0.01 M L-glutamine to prevent the loss of anthranilate synthetase activity. The most active fractions have been used for further investigations.

Zone centrifugation. Zone centrifugation was carried out as previously described (16). The sucrose solutions were usually made up in 0.05 M potassium

phosphate buffer (*p*H 7.0) containing 10^{-4} M EDTA. To guarantee satisfactory stability of the enzymatic activities, the same modifications of the buffers as mentioned above were necessary in the extraction and fractionation procedures. Addition of 20 μg of pyridoxal phosphate per ml to buffer was necessary with *G. fujikuroi* and *Rhizophlyctis rosea* in order to preserve the *N*-(5′-phosphoribosyl)-anthranilate (PRA) isomerase activity.

Assays. Enzymes were assayed as described previ-

TABLE 1. *Strains and culture conditions*

Organism	Strain[a]	Growth medium[b]	Temp of growth
			C
Fungi			
Allomyces macrogynus (Emerson) Emerson et Wilson	UCB 3-35	I	21
Aspergillus nidulans (Eidam) Wint. in Rabh.	A 160	I	30
Byssochlamys nivea Westl.	ETH M 4626	I	21
Coprinus lagopus Fr.	CAES H 9	I	21
Cryptococcus laurentii var. *flavescens* (Saito) Lodder et Kreger-van Rij	NRRL Y-1401	I	21
Dipodascus uninucleatus Biggs	NRRL Y-1268	I	30
Endomyces bisporus Verrall	ATCC 14628	I	24
Gibberella fujikuroi (Sawada) Wollenweber	NRRL 2278	I	21
Morchella esculenta (Linnaeus) Persoon	NRRL 2603	I	21
Mucor hiemalis Wehmer	NRRL 2461	I	21
Neurospora crassa Shear et Dodge	74-OR23-1A	I	30
	trp-3(td48R)	I	30
Phycomyces blakesleeanus Burgeff	NRRL 1554	I	21
Polyporus circinatus Fr.	NRRL 2903	I	21
Pythium sp.	UCB 47-11	I	21
Rhizophlyctis rosea (DeBary et Woronin) Fischer	UCB 57-5	I	30
Rhizopus arrhizinus Fischer	NRRL 2582	I	21
Rhodotorula glutinis (Fresenius) Harrison	NRRL Y-2503	I	30
Saccharomyces cerevisiae Hansen	XT-300-4A	I	30
Saprolegnia sp.	UCB 47-15a	I	30
Sporobolomyces salmonicolor (Fischer et Brebeck) Kluyver et van Niel	NRRL Y-850	I	30
Tremella mesenterica Retz.	NRRL Y-6151	I	21
Ustilago maydis (DeCandolle) Corda	CAES U 4	I	21
Myxomycete			
Physarum polycephalum Schw.	M3C (CREC)	—[c]	—[c]
Bacterium			
Escherichia coli (Migula) Castellani et Chalmers	K-12	II	30
	9830	II	30
Alga			
Anabaena variabilis Kütz.	F. Haxo, SIO	—[d]	—[d]

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CAES, Department of Genetics, Connecticut Agricultural Experiment Station, New Haven, Conn.; CREC, Centre de Recherches Exp. sur le Cancer, Lausanne VD, Switzerland; ETH, Institut für spezielle Botanik, Eidg. Technische Hochschule, Zürich, Switzerland; NRRL, Northern Regional Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.; SIO, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, Calif.; UCB, Department of Botany, University of California, Berkeley, Calif.

[b] Medium I: minimal medium (34) supplemented with (per liter) 10 g of dextrose, 10 g of peptone (Difco), and 4 g of yeast extract (Difco); to reduce growth of contaminating bacteria, 50 mg (per liter) of chloramphenicol was added to the medium. Medium II: minimal medium (36) supplemented with 2% sucrose.

[c] The lyophilized powder of *P. polycephalum* was furnished by R. Braun, Lausanne VD, Switzerland. For culture conditions, *see* Daniel and Rusch (14).

[d] Cells of *A. variabilis* were obtained from D. O. Holm-Hansen, SIO, La Jolla, Calif. For culture conditions, *see* Holm-Hansen (20).

ously: anthranilate synthetase (16), indole-3-glycerophosphate (InGP) synthetase (37), PR-transferase and PRA isomerase (21), and tryptophan synthetase (39). In each assay, 1 unit is defined as that amount of enzyme which catalyzes the conversion of 1 μmole of substrate or the formation of 1 μmole of product per hr. Protein was assayed according to the procedure of Lowry et al. (26).

RESULTS

A preliminary study of the enzymes of the tryptophan pathway in various fungal extracts indicated that the conditions required for stabilizing the enzymes varied considerably. Because of this variability, modifications of the buffer composition, by the addition of EDTA, potassium chloride, pyridoxal phosphate, and L-glutamine in various combinations, were necessary. But even under optimal stability conditions, the enzymatic activities in the crude extracts of most strains tested were too low for an analysis on sucrose gradients. Therefore, it was necessary to concentrate and partially purify the activities by means of ammonium sulfate fractionation (Table 2). The crude extracts which were frac-

TABLE 2. *Fractionation of the tryptophan enzymes with ammonium sulfate*

Organism	Fractions[a] containing enzyme activities				
	Anthranilate synthetase	PR-transferase	PRA isomerase	InGP synthetase	Tryptophan synthetase
Allomyces macrogynus	0–40	40–50	0–40	0–40	—
Aspergillus nidulans	40–50	50–60	40–50	40–50	0–40
Byssochlamys nivea	40–50	50–60	40–50	40–50	0–40
Coprinus lagopus	0–40	40–50	0–40	0–40	—
Giberella fujikuroi	40–50	40–50	40–50	40–50	0–40
Morchella esculenta	40–50	40–50	40–50	40–50	0–40
Neurospora crassa	40–50	40–50	40–50	40–50	0–40
Polyporus circinatus	40–50/50–60	50–60	40–50/50–60	40–50/50–60	—
Pythium sp.	50–60	50–60	50–60	50–60	—
Rhizophlyctis rosea	50–60	50–60	50–60	50–60	—
Saprolegnia sp.	40–50	40–50	40–50	40–50	—
Dipodascus uninucleatus	0–40	40–50	40–50	0–40	0–40
Endomyces bisporus	50–60	50–60/60–80	50–60/60–80	50–60	—
Saccharomyces cerevisiae	40–50	50–60	50–60	40–50	50–60
Cryptococcus laurentii var.	40–50[c]	—	40–50	40–50	—
flavescens	40–50[c]	—	0–40	0–40	—
Mucor hiemalis	40–50	50–60	40–50	40–50	0–40
	40–50	50–60	0–40	0–40	0–40
Phycomyces blakesleeanus	40–50	—	40–50	40–50	0–40
	40–50	—	0–40	0–40	0–40
Rhizopus arrhizinus	40–50	50–60	40–50	40–50	0–40
	40–50	50–60	0–40	0–40	0–40
Rhodotorula glutinis	50–60	50–60	50–60	50–60	—
	50–60	50–60	40–50	40–50	—
Sporobolomyces salmonicolor	40–50	50–60	40–50	40–50	—
	40–50	50–60	0–40	0–40	—
Tremella mesenterica	40–50	50–60	40–50	40–50	—
	40–50	50–60	0–40	0–40	—
Ustilago maydis	40–50	50–60	40–50	40–50	0–40
	40–50	50–60	0–40	0–40	0–40

[a] Solid ammonium sulfate was added (24.2 g per 100 ml of protamine sulfate supernatant solution) to obtain 40% saturation. After centrifugation, 6.3 g of solid ammonium sulfate was added per 100 ml of supernatant fluid to reach 50% saturation. After another centrifugation, 6.5 g of solid ammonium sulfate was added per 100 ml of supernatant fluid to bring the solution up to 60% saturation. After an additional centrifugation, 14.0 g of solid ammonium sulfate was added per 100 ml of the supernatant fluid to bring the solution to 80% saturation.

[b] Not available because of instability or low activity.

[c] Extraction and fractionation were carried out either without the addition of L-glutamine to the buffer (second row of data) or in the presence of 10^{-3} M L-glutamine (first row of data).

tionated by the procedure outlined in Table 2 contained from 6 to 12 mg of protein per ml, except in the cases of *Dipodascus uninucleatus*, *Physarum polycephalum*, and *Rhizophlyctis rosea* for which the protein concentration of the crude extracts was from 1 to 2 mg per ml. Variation within this range did not alter the pattern of precipitation shown. With the exceptions noted below, more than 60% of activity present in the crude extracts was recovered in the ammonium sulfate fractions. Although no particular significance should be placed in the fractionation pattern of a given enzyme activity in the extracts of various organisms, the patterns of differential precipitation of the various activities are of significance and reveal some interesting similarities and differences. PR-transferase frequently did not precipitate at the same ammonium sulfate concentration as did the other enzymes but was recovered only after higher levels of saturation were reached. Tryptophan synthetase frequently was very unstable and could not be tested reliably. When this activity was present in sufficient amounts, it generally precipitated at a lower level of ammonium sulfate concentration than did the other activities.

On the basis of the fractional precipitation of the remaining three enzymes (anthranilate synthetase, PRA isomerase, and InGP synthetase), three categories could be distinguished. The organisms listed in Table 2 are grouped into the three categories found. In the 11 organisms of the first group, anthranilate synthetase, PRA isomerase, and InGP synthetase precipitated at the same ammonium sulfate concentration. In the three organisms of the second group, anthranilate synthetase and InGP synthetase were recovered in the same precipitate, whereas PRA isomerase fractionated differently. In the third group, the fractionation behavior of the three enzymes was dependent on the presence of L-glutamine which, as mentioned, is necessary for maintaining anthranilate synthetase activity. In the presence of glutamine, all three activities precipitated in the same fraction; in the absence of glutamine, PRA isomerase and InGP synthetase were consistently precipitated at lower ammonium sulfate concentrations than in the presence of glutamine but the solubility of the remaining anthranilate synthetase was unchanged. In the absence of glutamine, however, only 10 to 20% of the anthranilate synthetase was recovered in these extracts.

The differential fractionation patterns suggested that the various strains fall into different categories with respect to the biochemical organization of the reactions of the tryptophan pathway. To determine the organizational pattern, ammonium sulfate fractions containing anthranilate synthetase, PR-transferase, PRA isomerase, and InGP synthetase were analyzed on sucrose gradients. When these activities did not appear in the same fractions, a range of ammonium sulfate fractionation was chosen which guaranteed the presence of all four activities in the same precipitate. Some differences were noted in relative ratios of the four activities in various strains. However, since the optimal reaction conditions were not determined for each strain, we have placed no special significance on these differences but rather have focused our attention on the distribution of each activity on sucrose gradients to detect possible differences in biochemical organization. In all but two instances, the recovery of activities on sucrose gradients ranged from 50 to 70%. In the two exceptions, the extracts from *Cryptococcus* and *Travella*, about 25% of each of the activities was recovered.

Based on the sedimentation behavior of anthranilate synthetase, PR-transferase, PRA isomerase, and InGP synthetase, five different enzyme types could be distinguished (Fig. 2). Three of these types have previously been described (16): from *Neurospora crassa* (type I), *Saccharomyces cerevisiae* (type II), and *Escherichia coli* (type V). PR-transferase, which was not included in the previous study, sedimented as a separate component in *N. crassa* and *S. cerevisiae* but was present in a complex with anthranilate synthetase in *E. coli* (23). Another sedimentation pattern was found in *Saporlegnia* species (type IV): anthranilate synthetase sedimented as a separate component at approximately 7.5*S*, PR-transferase was observed also as a separate component at approximately 5.5*S*, and PRA isomerase and InGP synthetase occurred together in a peak corresponding to 3.5*S*. Still a different sedimentation pattern was observed in *Mucor hiemalis* (type III). Anthranilate synthetase, PRA isomerase, and InGP synthetase were found in a common band in the 10.5*S* region in the presence of L-glutamine and EDTA. In the absence of glutamine and EDTA, anthranilate synthetase was unstable and only a small amount of anthranilate synthetase was detectable after sucrose gradient centrifugation in the 10.5*S* region, while PRA isomerase and InGP synthetase sedimented together at approximately 7.5*S*. PR-transferase sedimented at approximately 6*S* under both conditions. The addition or omission of glutamine or EDTA (or both) had no influence on the sedimentation behavior of the enzymes in the other types (types I, II, IV, V).

All of the fungi analyzed fell into categories I to IV, with the following minor aberrations in certain type I strains: the pattern shown for type

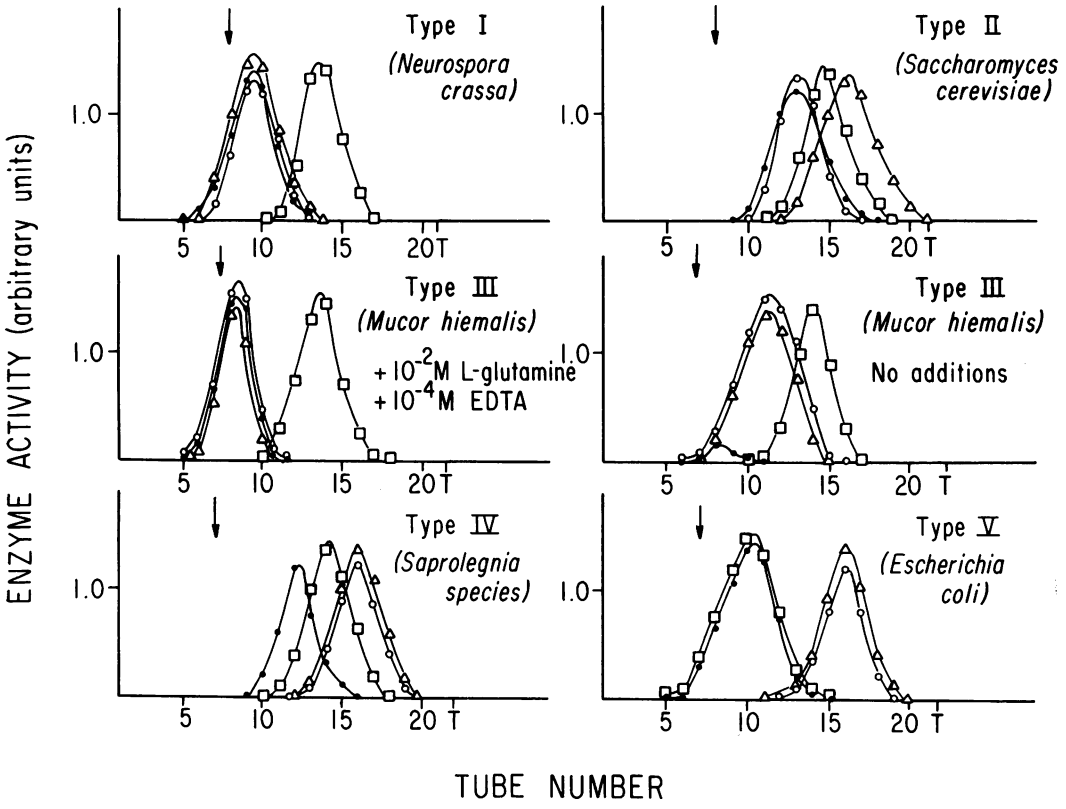

FIG. 2. *Distribution of enzyme activities after zone centrifugation. The activities of anthranilate synthetase (●), PR-transferase (□), PRA isomerase (△), and InGP synthetase (○) are presented in normalized units to allow presentation of the data on the same graph. Concentrated extracts were layered on 4.2-ml sucrose gradients (5 to 20%) and centrifuged for 11 hr at 139,000 rev/min in the SW 39 head of a model L Spinco ultracentrifuge. Ten-drop fractions were collected and analyzed for the activities.*

I (Fig. 2) was obtained with a derepressed *trp-3* mutant of *N. crassa*. The wild-type 74-OR23-1A, however, exhibited a 7.5*S* shoulder of PRA isomerase and InGP synthetase, in addition to the main 10.5*S* component (Fig. 3). The significance of this shoulder is not understood since it occurred in some purified preparations of the aggregate as well (18). Another modification was observed in the zone centrifugation pattern of *Coprinus lagopus*: a band with all three activities occurred at approximately 10.5*S*, accompanied by a shoulder with only anthranilate synthetase activity in the 6.0*S* region (Fig. 3). No particular significance has been attached to these shoulders of activity in assigning these organisms to the type I category.

The distribution of sedimentation patterns in the various species is shown in Table 3. Most widely distributed are the type I and III patterns and only a few representatives of types II and IV have been found. Type I was found in the chytridial fungi, in all higher ascomycetes and holobasidiomycetes tested, and in the myxomycete *Physarum polycephalum*. All strains of ascosporogenous yeasts (*Endomycetales*) had the type II pattern; no other organisms exhibited this pattern. Type III was found in all *Zygomycetes*, heterobasidiomycetes, and anascosporogenous yeasts tested. Both *Oomycetes* tested, as well as the blue-green alga *Anabaena variabilis*, fell within type IV. Although the presence of the type V pattern has been demonstrated by us only in *E. coli*, the same pattern may be expected for *Salmonella typhimurium*.

DISCUSSION

We shall consider these results from two distinct but related points of view. First, we will discuss the phylogeny of fungi from a consideration of the distribution of the enzyme patterns and then speculate on the possible evolution of the tryptophan enzymes.

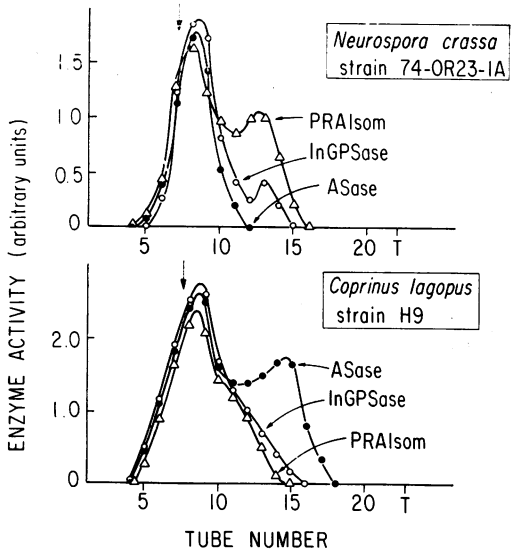

FIG. 3. *Anomalous zone centrifugation patterns of wild-type Neurospora crassa and Coprinus lagopus. The activities are normalized to allow presentation of the data on the same graph. See Fig. 2 for conditions.*

For the purposes of this discussion we have made the following assumptions. (i) Similar patterns of enzyme association (as demonstrated on sucrose gradients) reflect similar genetic backgrounds. The similarity in the gene-enzyme relationships and the aggregation pattern in each of two pairs of organisms (*E. coli* and *S. typhimurium*; *Neurospora crassa* and *Aspergillus nidulans*) would seem to support this assumption (Fig. 1). (ii) Changes in the enzyme organization are not likely to have been caused by simple, reversible mutational events. This assumption relies on the observation that, within any given group of clearly related organisms, only one type of pattern is observed (Fig. 4). (iii) The change from type I (four genes) to type II (five genes) or vice versa (Table 1) must have involved a virtually irreversible event such as genetic translocation. (iv) No evident and severe selective force has been exerted on any changes in the organization of the tryptophan enzymes. Based on these assumptions, we suggest that organisms which have evolved from one another will exhibit the same pattern of organization of their tryptophan enzymes. Furthermore, we suggest that, once a change in the gene-enzyme relationships occurred, it was irreversible and all descendants after this point will possess the same new pattern.

The distribution of the various enzyme patterns can best be considered with respect to existing schemes of phylogeny and can be used to clarify doubtful relationships or to verify or reject suspected relationships. The occurrence of different enzyme types in the phylogenetic system of fungi presented by Gäumann (19) is shown in Fig. 4.

TABLE 3. *Distribution of sedimentation patterns of anthranilate synthetase, PR-transferase, PRA isomerase, and InGP synthetase in zone centrifugation*

Sedimentation type	Organism
Type I	*Allomyces macrogynus*[a]
	Aspergillus nidulans
	Byssochlamys nivea
	Coprinus lagopus[a]
	Gibberella fujikuroi
	Morchella esculenta
	Neurospora crassa
	Physarum polycephalum
	Polyporus circinatus
	Rhizophlyctis rosea
Type II	*Dipodascus uninucleatus*
	Endomyces bisporus
	Saccharomyces cerevisiae
Type III	*Cryptococcus laurentii* var. *flavescens*
	Mucor hiemalis
	Phycomyces blakesleeanus
	Rhizopus arrhizinus[a]
	Rhodotorula glutinis
	Sporobolomyces salmonicolor
	Tremella mesenterica[a]
	Ustilago maydis[a]
Type IV	*Pythium* sp.
	Saprolegnia sp.
	Anabaena variabilis
Type V	*Escherichia coli*

[a] Levels of PR-transferase activity were too low to be analyzed by zone centrifugation in *Allomyces*, *Coprinus*, *Rhizopus*, *Tremella*, and *Ustilago*.

A consideration of this distribution reveals several significant features. The phycomycetes are separated into four groups, based on their flagellation (33): the anteriorly biflagellated *Oomycetes*, the anteriorly uniflagellated *Hyphochytriales*, the posteriorly uniflagellated chytridial fungi (*Chytridiales*, *Blastocladiales*, and *Monoblepharidales*), and the nonflagellated *Zygomycetes*. Differences in cell wall composition (2, 38) and the lysine pathway agree with this subdivision (Table 4). Vogel (35) has shown that the diaminopimelic acid pathway of lysine biosynthesis is found in bacteria, blue-green algae, green algae, vascular plants, and *Oomycetes*, as well as in *Hypochytriales*, whereas all ascomycetes and basidiomycetes, the chytridial fungi, the *Zygomycetes*, and the euglenids show the α-

amino adipic acid pathway. The results of the analyses of the tryptophan enzymes are in good agreement with this grouping and with a possible polyphyletic origin of the phycomycetes. The data give further emphasis to the considerable biochemical differences between "cellulose-fungi" and "chitin-fungi," and the studies on both the lysine and tryptophan pathways point to a closer

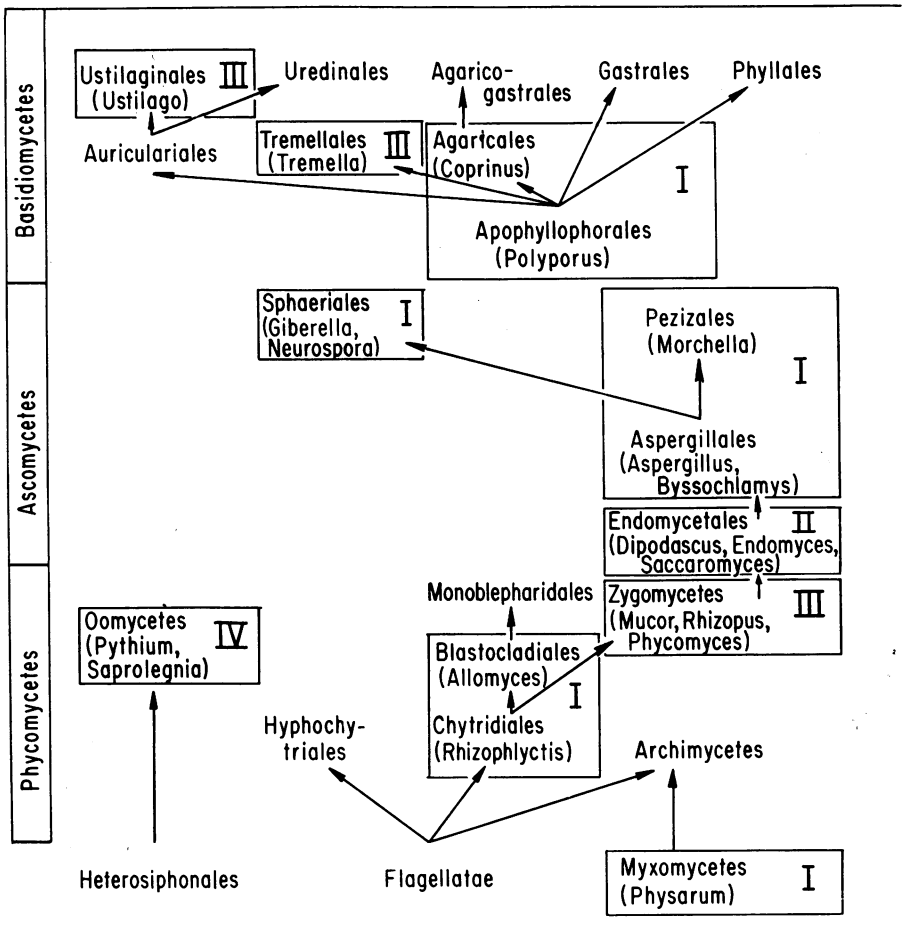

FIG. 4. *Distribution of the various patterns of enzyme association in the phylogenetic system of Gäumann (19). The roman numerals indicate the distribution of the various types shown in Fig. 2. The genera shown in parentheses indicate those organisms which have been tested in that particular order. All the organisms within a single box exhibit the pattern indicated for that box.*

TABLE 4. *Grouping within the phycomycetes*

Organisms	Flagellation	Main cell wall components	Lysine pathway[a]	Organizational type of tryptophan enzymes
Oomycetes	Anteriorly biflagellated	Cellulose	DAP	Type IV
Hyphochytriales	Anteriorly uniflagellated	Cellulose and chitin	DAP	Not tested
Chytridial fungi (*Chytridiales, Blastocladiales, Monoblepharidales*)	Posteriorly uniflagellated	Chitin	AAA	Type I
Zygomycetes	Nonflagellated	Chitin	AAA	Type III

[a] Abbreviations: AAA, α-aminoadipic acid; DAP, α,ε-diaminopimelic acid.

relationship of the *Oomycetes* to other groups of organisms (e.g., blue-green and green algae) than to the "chitin-fungi."

Further investigations are necessary to establish the possible algal or protozoal ancestors of all the groups of phycomycetes and especially the "cellulose-fungi" in order to determine whether the *Oomycetes* and *Hypochytriales* form a single phylogenetic group apart from the chytrids and *Zygomycetes* or whether two (or more) additional ancestral lines must be considered.

The relationship between the *Chytridiales* and the *Zygomycetes* will be discussed in connection with the possible origin of the ascomycetes.

The origin of the ascomycetes still remains in doubt; Gäumann (19) tentatively suggested the following relationships: *Chytridiales* → *Zygomycetes* → *Endomycetales* → *Aspergillales*. On the other hand, other investigators, basing their phylogeny of the fungi on a floridean ancestry, regard both intermediary groups as reduction lines [see Bessey (7) for a discussion of the yeasts, and Chadefaud (11) for a discussion of the *Zygomycetes*]. The enzymatic analysis demonstrated the occurrence of the type I pattern in chytrids and aspergilli, type II in *Endomycetales*, and type III in *Zygomycetes*. According to Gäumann's scheme, a change of type I → type III → type II → type I would have occurred for the tryptophan pathway. *Dipodascus uninucleatus*, considered to be one of the key species in the scheme deriving the *Aspergillales* from the *Endomycetales*, possesses the type II pattern, as opposed to the type I pattern of *A. nidulans*. These results argue against *D. uninucleatus* being an immediate ancestor of forms related to the aspergilli. Rather, the results favor the elimination of the *Zygomycetes* and *Endomycetales* as possible intermediates between the chytrids and *Aspergillales*, and suggest a more direct *Chytridiales* → *Aspergillales* relationship, with the *Zygomycetes* and *Endomycetales* being sidelines of evolution. There are at present, however, no species which can be considered as intermediary forms between the sporangial chytrids and the ascus-forming aspergilli.

Another point of interest arises from the distribution of aggregate type III in *Zygomycetes*, heterobasidiomycetes, and the anascosporogenous yeasts (*Sporobolomyces*, *Rhodotorula*, and *Cryptococcus*). The presence of type III in heterobasidiomycetes as well as in anascosporogenous yeasts may indicate a close relationship between these two groups of organisms. Such a relationship, at least for some cases, has been suggested earlier by Kluyver and van Niel (24) and other investigators. The finding of type III in heterobasidiomycetes and *Zygomycetes* must, at present, be regarded as a coincidence, since no indication of intermediary forms is available. The presence of enzyme type III in the heterobasidiomycetes of the *Auricularia* type as well as of the *Tremella* type (19), and the presence of type I in the holobasidiomycetes, might be a clue to the polyphyletic origin of the basidiomycetes. However, again no ascomycetal forms have been found to possess type III of enzyme-aggregation which might represent possible precursors to the heterobasidiomycetes.

In addition to deriving these implications concerning the phylogeny of fungi, we can, with the original assumptions in mind, discuss the results with respect to evolution of enzyme organization. Bonner et al. (9) suggested that, in the case of tryptophan synthetase, a system of completely independent components under the control of distinct genes evolved to a system of interdependent components and finally gave rise to a single component system under the control of a single gene. Since the fungi are a group of organisms in which some general trends of evolution have been established, it is possible to consider the possible sequence of changes which has occurred in the organization of the tryptophan enzymes during the course of evolution of the various groups of fungi.

In nearly all examples studied, the enzymes PRA isomerase and InGP synthetase are catalyzed by a single component; organisms as distinct as the procaryotic *E. coli*, the ascomycetes *N. crassa* and *A. nidulans*, the chytridial fungi, and the myxomycete *Physarum polycephalum* exhibit this pattern. Exceptions are found only in the ascosporogenous yeasts (*Endomycetales*). The fact that the separate genetic control of the two enzymes is apparently present in one restricted group favors the interpretation that the joint control of PRA isomerase and InGP synthetase by a single gene was established at a very early stage of evolutionary history.

A second striking fact is that anthranilate synthetase is usually associated with another gene product, when in its active form, and loses its activity when dissociated. Exceptions are only rarely found, namely, in the *Oomycetes* and the blue-green alga *Anabaena variabilis*, summarized in type IV, and possibly also in *Coprinus*. Two forms of anthranilate synthetase complexes have been established, i.e., with PR-transferase or with the gene product containing InGP synthetase activity. The complex with PR-transferase has been found only in *E. coli* and *S. typhimurium*. The association with the InGP synthetase-containing gene product, however, is very widespread; it has been observed in the myxomycete *P. polycephalum* and in all chitin-fungi tested

(chytridial phycomycetes, *Zygomycetes*, ascomycetes, basidiomycetes). The aggregation can be a stable one (types I and II) or an unstable one (type III), although the basis for this difference is not yet understood. The presence of a stable aggregate in the myxomycete *Physarum*, the chytridial fungi, and the large body of higher ascomycetes and the holobasidiomycetes favors the interpretation that the stable aggregate binding is more fundamental than the unstable association. Whether either type has arisen only once in a remote ancestor or has developed in parallel several times cannot yet be decided.

Based on the two aspects discussed, joint genetic control of PRA isomerase and InGP synthetase and aggregation of anthranilate synthetase, the evolutionary sequence presented in Fig. 5 may be suggested. Joint genetic control (of PRA isomerase and InGP synthetase) with no aggregation is considered to be the most primitive organization found in any of the organisms studied here, and is postulated to be the first change from separate genetic control with no aggregation. A sideline leads to the situation present in *E. coli* and *S. typhimurium*, i.e., the association of anthranilate synthetase activity with PR-transferase activity. During the course of evolution, the aggregation of anthranilate

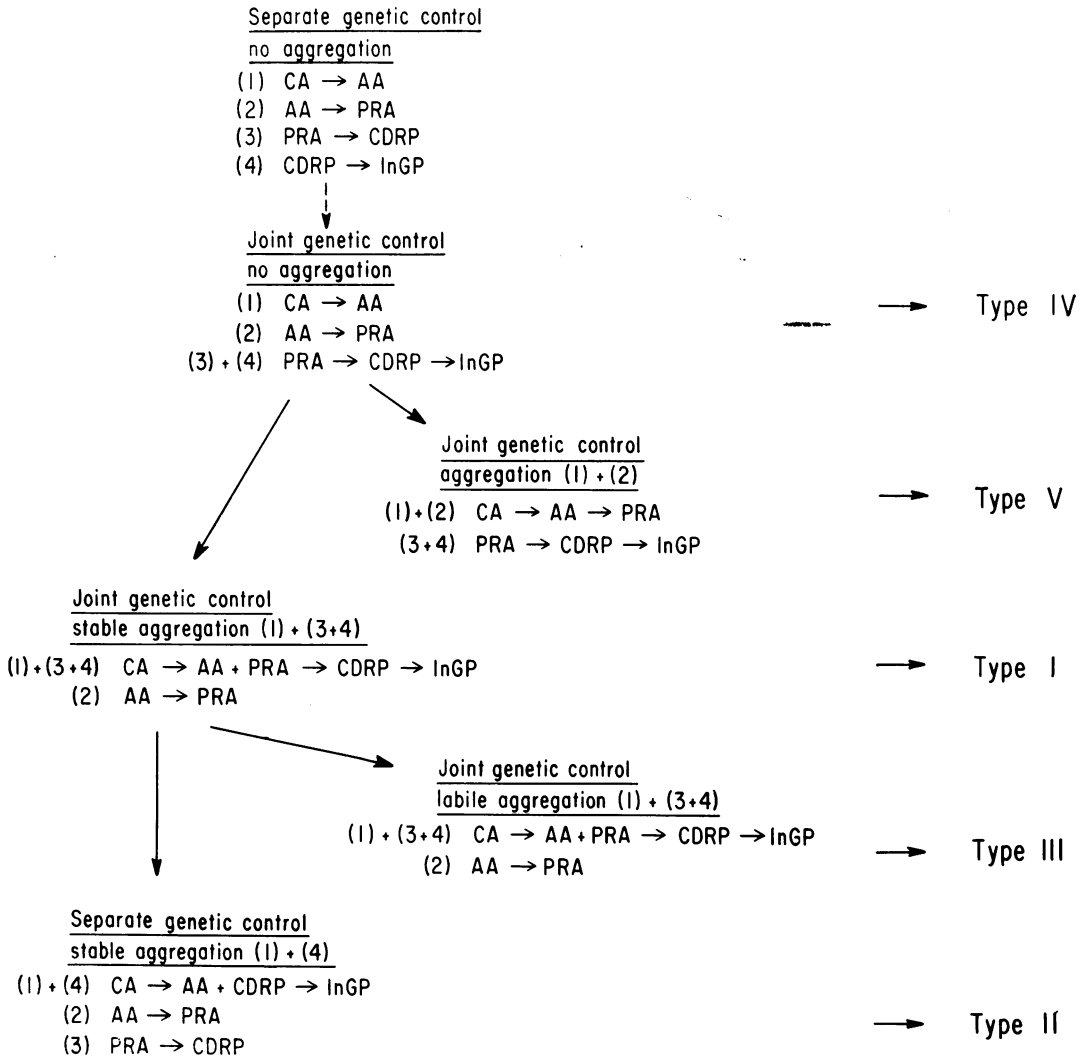

FIG. 5. *Possible sequence of changes which may have led, during the course of evolution, to the various patterns of enzyme association. CA, chorismic acid; AA, anthranilic acid; other abbreviations defined in Fig. 1.*

synthetase with the jointly controlled PRA isomerase and InGP synthetase is presumed to have developed. Secondary modifications can be visualized as having occurred from this stage. Either the stability properties of the aggregate were changed, leading to the type III pattern, or the joint genetic control was broken, resulting in a reversal of the original trend of increasing interdependence of the components to an increased independence of components as observed in the ascosporogenous yeasts.

Although it is not possible to follow the course of evolution directly, the results and discussion presented here show that studies at the molecular level can provide useful parameters for considering possible phylogenetic relationships. Hopefully, the extension of these types of studies to other biochemical pathways and other organisms will eventually provide a clearer picture of the evolutionary history of existing organisms.

ACKNOWLEDGMENT

This investigation was supported by grant GB-4687 from the National Science Foundation.

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