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TRENDS IN METABOLIC SPECIALIZATION AMONG THE FUNGI

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Summary

The basis for, and advantages of, the use of sequence analysis of proteins are presented. Cytochrome *c* is used as an example and it is suggested that not enough work has been done upon the amino acid sequences of proteins of fungi, especially cytochrome *c*. Other means of studying proteins and enzymes in order to acquire data useful to evolutionists who deal with fungi are discussed, including analysis of mechanisms of control of enzyme activity, electrophoretic migration and properties of enzymes such as response to activators and inhibitors, pH and temperature optima etc. Finally, micromolecules are discussed in the context of their function in the fungi, and suggestions are made as to their present and potential use in phylogenetic studies of these organisms.

My look at metabolism in the fungi will be retrospective, progressive and idiosyncratic. In no way could I expect, or be expected, to review completely all of metabolism so I intend instead to stress those areas that I believe will yield major results in the future and mention only *en passant* those that have been important, but in which the intensity of effort will diminish. In any case, my approach must be through the use of examples, rather than exhaustive, and I hope it will be exemplary in a qualitative sense as well.

The organization I will use is to proceed from a discussion of enzymes, as elements of metabolism, to pathways, networks and controls, following which micromolecules and some approaches to them that I find significant for the future will be discussed. So on to enzymes.

Enzymes as proteins

It is now axiomatic that evolution at the molecular level stems from events that occur in DNA molecules. These include duplication, as distinguished from replication, shortening or deletion of portions of DNA strands and point-mutations. The translation of these various changes into proteins by the genetic code is a metabolic event from which stem the morphological, ecological and other complexities with which the evolutionary process is concerned. Therefore, it is crucial that the properties of proteins and enzymes be understood before the entire process of evolution can be known. Furthermore, proteins are the most variable and versatile of all types of biological molecules so that mutations will allow many to continue to function, although that of others will be destroyed.

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Use of amino acid sequences

Because they are direct translations from the code of the corresponding structural genes, analysis of the amino acid sequences of proteins could be an independent test of the concepts of evolution because this process at the molecular level need not proceed by the same rules as gross morphology or even micromolecules (micro-metabolites). Thus, the multiplicity of genes involved in the formation of the latter, including one or more structural and regulatory ones, relegates micro-molecules to the status of most other characters used for evolutionary studies.

How are homologies detected from the primary structure of proteins? First, of course, the amino acid sequences must be known. This may sound trivial but it is not enough to infer the sequence from peptide maps and comparisons of these from other proteins, for the argument assumes homology and leads to circularity.

The rules that must be followed in assessing the relatedness of proteins include a determination of whether the similarities in sequence observed are the results of random occurrences (mutations) and whether they are significantly more prevalent than is required by the biological requirements of the protein. If homology is not immediately obvious by direct comparison of sequences then the calculation of "minimal mutation distances" is carried out, that is "the minimal number of single nucleotide changes required to transform the gene or the gene segment coding for one protein segment to that coding for the other amino acid sequence" (Nolan and Margoliash, 1968). Often an "average mutation value" is computed by dividing the minimal mutation distance by the number of amino acid residues compared. Fitch (1966) has provided a statistical approach by assessing the probability of random changes occurring in sequences by calculating minimal mutation distances for peptide segments of fixed length from the sequences under comparison, using all such pairs that are possible. When the frequency of occurrence of individual mutation distances in *all* these comparisons is plotted against the mutation distances themselves, a Gaussian distribution is indicative of random occurences (Figure 1). Reciprocally, the very low numbers beyond the Gaussian part of the curve denote possible homology because the mutation distances are smaller than would be expected for random events. Another way of dealing with this problem is by use of the "composition divergence" index, which has just been described (Horris and Teller, 1973), That there are ambiguities in the use of such techniques has been noted by Holmquist and Jukes (1972) but they suggest means to overcome these.

More serious difficulties arise from the need to distinguish between ancestral homology and convergence. In order to overcome this difficulty it is necessary to understand the critical biological requirements of a protein and then to estimate the minimal number of residues that must be retained unchanged over the course of evolution because of these requirements. Means to deal with this problem have been suggested (Fitch and Margoliash, 1967; Moore, Barnabas and Goodman, 1973) whereby phylogenetic trees have been derived statistically by defining the minimal number of single nucleotide changes needed to derive current sets of genes from an ancestral gene. This number for eukaryotic cytochrome *c* has been given as twenty-seven to twenty-nine (Fitch and Margoliash, 1967), although it may be subject to revision as a result of work on the *Candida krusei* enzyme (Lederer, 1972).

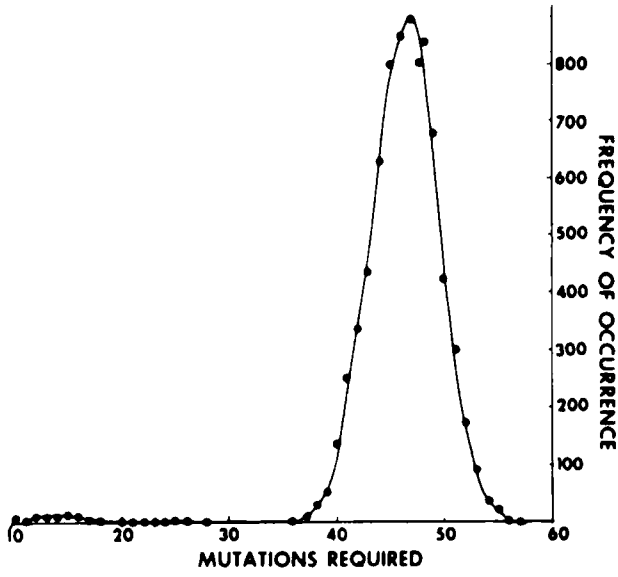

Fig. 1. Comparison of minimal mutation distances (abcissa) for all possible 30-residue segments of human cytochrome *c* and baker's yeast iso-1-cytochrome *c* (7.056 comparisons). The numbers of times various values of minimal mutation distances occur in the comparisons are given on the ordinate. The Gaussian portion of the curve on the right represents the random part of the distribution. The extended line on the left indicates those comparisons for which the minimal mutation distances are less than expected for a random distribution and hence suggest ancestral homology. The probability that such a distribution occurred by chance is $< 10^{-80}$.

(Taken from Nolan and Margoliash, 1968)

It is important to observe that the total number of variant amino acid residues or the minimal mutation distances are very narrowly distributed in the comparison of cytochromes c from higher taxa (Nolan and Margoliash, 1968) in contrast to comparisons within a single class. A possible explanation of this phenomenon is that the number of variant residues acquired during evolution is related closely to elapsed time so that a relatively constant number of mutations will be found in homologous genes in different ancestral lines, given enough time for their divergence from a common ancestor. Thus, during the two hundred million years of evolution of cytochrome *c* the numerous factors affecting mutations in all groups having this enzyme have been averaged out, producing the observed relation to time. Consequently, it is possible to calculate the average time needed for the establishment of a single amino acid change in the sequence, or a single nucleotide change in the corresponding structural gene, for two evolutionary lines. A "unit evolutionary period" has been calculated using the divergence of mammals and birds as a reference point based upon an elapsed time of 280 million years (Margoliash and Smith, 1965) in terms of amino acid substitutions ($\frac{280}{10.6}$ = 26 million years) and minimal mutation distances ($\frac{280}{13.6}$ = 21 million years) (Nolan and Margoliash,

1968). Now simple proportions can be used to estimate the duration of other major evolutionary changes. To bring these numbers closer to the fungi, their divergence from the animals occurred some 1.1 billion years ago, according to the characteristics of cytochrome *c* from the few species that have been examined in detail.

I have given considerable attention to cytochrome *c* not only because it is a valuable metabolic indicator in its own right but because it serves as an example which may be used in the future for other fungal proteins which are, in the words of Vogel (1965), "suitably poised between the extremes of biochemical unity and diversity." As for the potential of cytochrome *c* itself, it is felt by some systematists (e.g., R. A. Crowson, 1972) that as a result of a broad survey of the amino acid sequences of this protein over "the next couple of decades, our systematic and phylogenetic understanding of the living world will probably have experienced its biggest advance of the whole century." How well have the fungi fared as objects of study through this means?

Relatively few fungal proteins have been sequenced, even if we consider recent additions to the literature (Bitar *et al.*, 1972; Morgan *et al.*, 1972). An evidence of the rudimentary state of our knowledge of the primary structure of cytochrome *c* in the fungi is the fact that none of the Phycomycetes have been investigated from this point of view and only the sequence from *Ustilago sphaerogena* is known from among the Basidiomycetes (Bitar *et al.*); in fact, fewer than ten species have been studied in all. Among those that have been studied some interesting differences have been found such as the presence of methylated lysine at both positions seventy-two and eighty-six in *Humicola lanuginosa* (Morgan *et al.*, 1972) so that further exploration of fungi will undoubtedly be very rewarding. For example, the well-known polyphyletic nature of the Phycomycetes would bear examination in this way although it is possible that the Ascomycetes will be less rewarding as is suggested by the similarity of the *Humicola* and *Neurospora* cytochromes *c* (Mayor *et al.*, 1972). In this connection, a real challenge to the use of this technique is to determine whether differences in mutable sequences can be used to assess relationships between taxa at lower levels.

This has been a rewarding area for other proteins as well for there has been the development of rich detail on the diversification of hemoglobins, fibrinopeptides, proteolytic enzymes and histones. Evidence of the success of this approach, based upon the techniques discussed above, is provided in Table 1, in which the rate of mutation of some of the proteins mentioned above is listed. These data reveal great differences in the rate of change of amino acid residues, with the histones being the most conservative in this respect. This is not surprising, given what is known about the similarity of these proteins from a wide variety of organisms. Nevertheless, it might be very rewarding from several standpoints to study fungal histones. Thus, they have only been isolated thus far from *Neurospora* (Hsiang and Cole, 1973) and differ in interesting ways from those of other organisms. To illustrate this point I need only note that whereas one of them is similar to histone F2b of calf thymus in amino acid composition and other properties, the other resembles only slightly the other slightly lysine-rich histone, $F2a_2$. Moreover, no other of the five general types of histones were found and the total amount of this material that could be isolated is much lower than that found in other organisms. Given the essential role of this group

TABLE 1. The rates of mutation of selected proteins

Proteins	Mutations per 100 million years
Fibrinopeptides	90
Growth hormones	60
Immunoglobulins	34
Kappa C region	40
Kappa V region	34
Heavy C region	28
Ribonucleases	30
Hemoglobins	12
Beta	13
Alpha	11
Myoglobins	9
Gastrins	9
Adenohypophyseal hormones	9
Encephalitogenic proteins	7
Insulins	4
Cytochromes *c*	3
Glyceraldehyde-3-PO_4 dehydrogenases	2
Histones	0.06

The figures are given in PAMs, or Accepted Point Mutations per 100 residues, which we have estimated would occur in 100 million years of evolution. For most of the proteins, the rate was based on the time of divergence of the mammalian orders at 75 million years ago. (Taken from Dayhoff, 1971)

of proteins in condensation of chromosomes, and possibly in differentiation, such analyses are crucial to our understanding of ontogeny and probably for that of evolution as well.

There is reason to believe (Pfleiderer and Zwilling, 1972) that the analyses of amino acid sequences of proteolytic enzymes will be very revealing of a focus of metabolic diversity. Yet, among the ten species he used, none were fungi. Many other opportunities of this kind exist, as, for example, in the case of the NADP-dependent glutamate dehydrogenase of *Neurospora*, which may be restricted to the higher fungi (LéJohn, 1971), and for which there is evidence from amino acid sequences for evolution in common with vertebrates (Wootton, Chambers, Taylor and Fincham, 1973).

Use of other properties of proteins

A wide variety of other properties of enzyme proteins have been used to distinguish between them. For example, Haneishi and Shirasaka (1968) compared the cytochromes *c* of eighteen different yeasts with respect to their mobility on a resin column, response to inhibition, precipitability with antisera against two yeasts and reactivity with cow cytochrome oxidase. These results are summarized in Table 2 wherein the four groups into which the eighteen species could be placed are given. In general, this arrangement supports those derived by other techniques.

TABLE 2. Summary of relationships of eighteen different yeasts, based upon the properties of their cytochromes *c*

Group of yeasts	Mobility on CG-50 (moving distance)	Inhibition of activity with		Precipitability with Can-as [1]	Reactivity with cytochrome a [3]
		Can-as [1]	Sacc-as [2]		
I	0.05 cm	20–30%	40–60%	—	++
II	0.7–1.2	20–30	40–60	—	++
III	1.3–4.0	40–45	20–30	+	++
IV	20–40	(70–90)	(70–90)	—	+

[1] Antiserum against *Candida krusei* cytochrome *c*
[2] Antiserum against *Saccharomyces oviformis* Mz cytochrome *c*
[3] cytochrome a from *S. oviformis* Mz
(Taken from Haneishi and Shirasaka, 1965)

Other means of studying enzymatic diversity are outlined in Table 3 along with the source of the enzyme studied. Such properties as the K_M (Michaelis constant), pH optimum, substrate specificity, number of subunits, molecular weight, requirement for cofactors, inhibitors and activators and others have been used with a number of enzymes from many species. These properties have been used to assay the diversity of fungal proteins in only a few cases. One of these is the effect of D(+)-tartaric acid upon acid phosphatase, which was shown by Kilsheimer and Axelrod (1958) to distinguish between the enzymes from separate groups of the Phycomycetes. However, it should be noted that the use of impure extracts can be misleading, given the presence of specific protein inhibitors (Cabib and Ulane, 1973; Garrett *et al.*, 1972) and activators (Money, 1968; Katsunama *et al.*, 1972), and other interfering factors which make such experiments difficult to control. Furthermore, the meaning of differences of these kinds evolutionarily has not been studied in much detail for the fungi and instances where the function of enzymes are very similar, even from very diverse groups, are known (Mishra and Fridovich, 1972; Rapp *et al.*, 1973).

Proteins which may or may not have enzymatic activity may become useful as indices of diversity when more is learned about them. Thus, the inhibitors and activators mentioned above are worth examining in the way the incompatibility factors of ascomycetes (Caten, 1972) and basidiomycetes (Raper, 1966; Koltin *et al.*, 1972) and structural proteins of cells and organelles have been. Finally, the "killer" factors that have recently been discovered in yeast (Bussey, 1972; Bussey *et al.*, 1973) surely lend themselves to this kind of analysis as well.

I have deliberately excluded immunological means of identifying and comparing proteins because they have been reviewed frequently and are an established technique. Another technique of perhaps even wider application because of its simplicity and sensitivity is electrophoresis which will be dealt with next.

Electrophoretic properties.

The high resolving power, convenience and easy availability of gel electrophoresis as a means of assessing the diversity of proteins has led to the explosive development of the technique for use with fungi and other organisms. Electrophoresis vies with immunology as the technique of choice in determining relatedness, based upon similarities and differences in proteins.

TABLE 3. Methods through which the diversity of fungal enzymes have been studied. Analysis of amino acid sequences has been omitted.

Protein	Organism	Property determined	Reference
malic dehydrogenase	*Polysphondylium pallidum*, *Neurospora crassa*	molecular weight	Kaplan, 1965
phosphoglucomutase	"yeast"	stimulation by imidazole, pH optimum	Joshi *et al.*, 1965
alcohol dehydrogenase	"yeast"	rates of reaction with analogs of pyridine nucleotides	Kaplan, 1965
triosephosphate dehydrogenase	"yeast"	inhibition by pyridine-3-aldehyde diphosphopyridine nucleotide	Kaplan, 1965
NAD-specific isocitric dehydrogenase	*Saccharomyces cerevisiae*, *Blastocladiella emersonii*	reversibility of reaction	Barnes *et al.*, 1972; LéJohn *et al.*, 1969
fructose diphosphate aldolase	*Saccharomyces cerevisiae*, *Aspergillus niger*	molecular weight, sedimentation constant, number of subunits, metal requirement, activability of K^+, pH profile for exchange, -SH groups	Rutter, 1965
glycogen phosphorylase	*Saccharomyces cerevisiae*	response to AMP	Sagardía *et al.*, 1971
carboxypeptidase	*Aspergillus saitoi*	substrate specificity, pH optimum, K_m	Ichishima, 1972
aminopeptidase	*Hapalopilus nidulans*, and *Pleurotus ostreatus*	substrate specificity	Blaich, 1973
acid protease	*Cladosporium* sp.	resistance to pepsin inhibitor	Murao *et al.*, 1973
"proteases"	39 common fungi	milk-clotting ability	Knight, 1966
acid phosphatase	*Fusarium moniliforme*	substrate specificity, inhibition by L(+) tartrate, requirement for cations	Yoshida and Hanamitsu, 1958
acid phosphatase	*Aspergillus niger*	substrate specificity	Ikawa *et al.*, 1964
cycloaldolase	*Neurospora crassa*	inhibition by EDTA, activation by NH^+_4, pH range of activity, response to inhibitors	Mogyoros *et al.*, 1972
β-D-galactosidase	*Corticium rolfsii*	substrate specificity pH minimum	Kaji *et al.*, 1972
α-glucosidase	*Mucor javanicus*	substrate specificity and ability to transglucosylate	Yamasaki *et al.*, 1973
glucose oxidase	*Aspergillus niger*, *Penicillium amagasakiense*	number of subunits	O'Malley and Weaver, 1972; Yoshimura and Isemura, 1971
alcohol oxidase	*unident. basidiomycete*	substrate specificity,	Janssen *et al.*, 1965

RNA polymerase	*Neurospora crassa, Saccharomyces cerevisiae*	resistance to rifampicin	Wintersberger, 1972
tRNA nucleotidyl transferase	*Neurospora crassa*	identity of catalytic and inhibitory sites	Hill and Nazario, 1973
tRNA ligases	*Saccharomyces cerevisiae*	subunit structure	Rymo *et al.*, 1972
ribonuclease	*Ustilago sphaerogena*	mechanism of hydrolysis	Blank and Dekker, 1972
cytochrome *c*	18 yeast species	mobility on columns, inhibition by antisera, activity with cow cytochrome oxidase etc.	Haneishi and Shirasaka, 1968
cytochrome *c*	*Candida krusei, Saccharomyces oviformis, Kloeckera* sp.	affinity for Amberlite, reactivity with cytochrome oxidases	Yamanaka *et al.*, 1964
cytochrome *c*	*Hemispora stellata*	absorption peaks, oxidation-reduction potential	Sala and Burgos, 1973

Although the former has its detractors in this comparison (c.f. Heger *et al.*, 1968), the rapid pace of improvement in the technique and in the understanding thereof, as illustrated in the large number of papers reviewed in a symposium at the 1973 meetings of the American Institute of Biological Sciences (to be published in the Bulletin of the Torrey Botanical Club), suggests that it is here to stay as a viable complement to immunology. In fact, it has been asserted that "differentiation of enzyme proteins by polyacrylamide gel electrophoresis is much better than immunological analysis" (Ohara and Nasuno, 1972) because the alkaline proteases of *Aspergillus oryzae* and *A. sojae* were distinguishable by the former method and not by the latter. How good is this technique for the fungi?

The electrophoretic era for the fungi was ushered in by Chang, Srb and Steward in 1962, who separated proteins in extracts of *Neurospora* using paper, starch and acrylamide as supporting media. The superiority of using acrylamide for fungal protein separations was established in this study in which it also was shown that *N. crassa, N. sitophila, N. intermedia* and an unidentified strain from the Philippines had distinctive protein complements. Nevertheless, despite these initial and later successes, failures also have been recorded as in the case of races of *Phytophthora fragariae* (Gill and Powell, 1968) and strains of *Verticillium* from hops (Webb *et al.*, 1972). What are some of the considerations in using gel electrophoresis for the separation of fungal proteins?

The properties of the organism and the way it is grown and treated otherwise, affect the electrophoretic patterns obtained. Thus, whereas the electrophoretic profiles of mutant strains of *Neurospora* were easily distinguishable by Barber *et al.* (1969), races of *Phytophthora fragariae* and of *Verticillium*, as discussed above, were not. Table 4 reveals other ways in which electrophoretic patterns may be affected by various characteristics of the organism. For example, Zink (1969) found that the alcohol dehydrogenases formed on sucrose and ethanol differed. Moreover, the widespread effect of glucose in repressing the formation of certain enzymes must be considered as well (Hanks and Sussman, 1969). Mutations probably ac-

count for the extensive changes in protein bands found in extracts of *Verticillium albo-atrum* from two-year-old cultures, as compared with those from the original isolates (Whitney *et al.*, 1968). Extensive differences in proteins are found as a result of development as, for example, in the enzymes studied by Solomon *et al.* (1964) in *Dictyostelium discoideum*, and many similar cases exist. On the other hand, Wilson and Huisingh (1972) report that the isozymes of peroxidase and O-diphenol oxidase are identical during the development of *Glomerella magna*. That extensive differences in the protein complement of a single organism can be introduced by a few genes such as, perhaps, incompatibility factors, was suggested by Wang and Raper (1970) in their work with the tetrapolar *Schizophyllum commune*. Using isogenic strains of different mating types they discovered that the isozymic profile of fourteen of the fifteen enzymes analyzed differed after dikaryotization, so that profound changes were effected during the introduction of the diploid condition in this organism. These results were extended to a dipolar species of *Coprinus* by Ross *et al.* (1973) who concluded that, in this organism as well, the shift from the monokaryon to the dikaryon was accompanied by a major shift in the types of proteins synthesized as well. On the other hand, the work of Dave *et al.* (1973) with yeast indicates that the levels of ploidy did not affect isozymes of enolase at all.

Another consideration is the localization of the proteins used for electrophoresis. Extracellular enzymes often are used but may have no intracellular equivalents, as in the case of the invertase (Trevithick and Metzenberg, 1964), and β-galactosidase (Johnson and DeBusk, 1970) of *Neuro-*

TABLE 4. Conditions relating to the growth, treatment and properties of fungi which affect the electrophoretic separation of their proteins

Organism	Condition	Reference
Fusarium spp.	size of inoculum	Glynn and Reid, 1968
Fusarium spp.	composition of medium	Glynn and Reid, 1968
Neurospora crassa	composition of medium	Zink, 1969
Candida lipolytica	composition of medium	Lloyd *et al.*, 1971
9 species of polypores	composition of medium	Shannon *et al.*, 1973
Fusarium spp.	temperature of growth	Meyer *et al.*, 1964
Aspergillus nidulans	temperature of growth	Dorn, 1964
Verticillium albo-atrum	length of storage of culture	Whitney *et al.*, 1968
Sclerotium rolfsii	developmental age	Chet *et al.*, 1972
Coccidioides immitis	developmental age	Shechter, 1967
Dictyostelium discoideum	developmental age	Solomon *et al.*, 1964; Riepel and Gerisch, 1969
Neurospora crassa	developmental age	Williams and Tatum, 1966; Zink, 1972
Drechslera spp.	developmental age	Shipton and McDonald, 1970
Schizophyllum commune	whether mono- or dikaryon	Wang and Raper, 1970
Coprinus lagopus	whether mono- or dikaryon	Ross *et al.*, 1973
Neurospora crassa	location of enzyme	Trevithick and Metzenberg, 1964; Johnson and DeBusk, 1970
Trametes versicolor	location of enzyme	Schanol *et al.*, 1971

TABLE 5. Those electrophoretic techniques and enzymatic properties that have been shown to affect patterns of fungal proteins delineated by electrophoresis

Organism	Observation	Reference
Neurospora crassa	preparation of extract	Yu, Kula and Tsai, 1973; Glatzer, Eakin and Wagner, 1972
Aspergillus spp.	volume of extract	Garber, 1973; Mishra and Fridovich, 1972
Neurospora crassa	storage of extract	Reddy and Threlkeld, 1972
N. crassa, Fusarium oxysporum	storage of extract	Rapp, Adams and Miller, 1973
Phycomyces	heat-activation of enzyme	
Penicillium spp.	pH of electrophoresis	Yokoyama and Ichishima
Neurospora crassa	pH of electrophoresis	Benveniste and Munkres, 1973
N. crassa	ionic concentration of extract	Benveniste and Munkres, 1973; Hecker and Sussman, 1973
Saccharomyces cerevisiae	coenzymes in extract	Grisolia, Quijada and Fernandez, 1964
S. cerevisiae	substrates in extract	Derechin *et al.*, 1972
N. crassa	preparation of gel	Reddy and Threlkeld, 1973
Drechslera spp.	preparation of gel	Shipton and McDonald, 1970
Drechslera spp.	bands difficult to quantify	Shipton and MacDonald, 1970

spora crassa. Furthermore, the well-known difference between enzymes of mitochondrial and soluble cytoplasmic origin (c.f. Collins and Wagner, 1973) is another illustration of the need to screen proteins from all parts of cells and organisms.

Genetic studies of the control of isozymes in the fungi include those of Wang and Raper (1970) and Ross *et al.* (1973) discussed above. Earlier, Dorn (1965) had shown differences between mutant and wild-type forms of phosphatase in *Aspergillus nidulans*. In addition, Mishra and Tatum (1970) found that two isozymes of phosphoglucomutase are controlled by the *ragged*-1 and *ragged*-2 genes of *Neurospora sitophila* and Scott and Tatum (1970) proposed that those of glucose-6-phosphate dehydrogenase in *N. crassa* are determined by *balloon*, *frost* and *colonial*-2, which are considered the structural genes for these loci. Quantitative differences in the three laccases of *Podospora anserina* were found to occur in the pleiotropic mutant "zonata", when compared with wild-type strains (Esser and Minuth, 1970). Then, Yu *et al.* (1971) showed that a single gene controlled the slow and fast forms of trehalase in *N. crassa* and that an unlinked one modified the electrophoretic properties of these. On the other hand, Reddy and Threlkeld (1972) have established that two unlinked, codominant, duplicate genes control the two esterase isozymes in *N. crassa* and *N. sitophila*.

As Table 5 indicates, the electrophoretic techniques used have a strong influence upon the number and types of protein bands and/or enzymatic profiles obtained. Such variables as the supporting medium, degree of cross-linkage in the gel, pH and whether a continuous or discontinuous system is used are of importance. Thus, most of the work done until now has been with acid proteins but the basic ones, like histones, are of consider-

able interest but have not been studied very ofen. Moreover, the preparation and subsequent treatment of the extract often is crucial to the reproducibility of the system. A case in point is that of superoxide dismutase from *Fusarium oxysporum* and *Neurospora crassa* for which it has been shown (Mishra and Fridovich, 1972, and Rapp *et al.*, 1972) that storage in the cold yields several different isozymes than are found otherwise. The stability of some proteins is influenced by the presence of coenzymes (Grisolia *et al.*, 1964) and substrates (Derechin *et al.*, 1972), and by the ionic strength of buffers (Hecker and Sussman, 1973), degree of association or dissociation of the enzyme (Hecker and Sussman, 1973; Yokoyama and Ichisima, 1972). Thus, the conformation of the protein may be subject to control by small molecules like nicotinamide-adenine dinucleotide (NAD), as in the case of the glyceraldehyde 3-phosphate dehydrogenase (Deal, 1969), and dissociation and sensitivity to proteolysis may also be, according to Yang and Deal (1969). Enzymatic interconversion has been described with increasing frequency (Segal, 1973) in several organisms so that incubation of extracts would lead to changes in profiles. Finally, much remains to be learned about the "microheterogeneity" of proteins, which most often appears to be due to quantitative differences in their carbohydrate moiety. Such microheterogeneity has been described for the phenoloxidases of *Podospora anserina* (Esser and Minuth, 1971) and should be expected elsewhere.

Enzymatic controls

Estimates vary but it is clear that a substantial part of the genome is composed of punctuation and regulatory sequences, in addition to structural ones. Consequently, it is at least possible that metabolic diversity at this level of organization will show trends which are unique to the fungi. What is known of these matters at present?

Control of enzyme biosynthesis

First it should be observed that there is no reason to believe that protein synthesis in the fungi differs in significant ways from that found in other organisms. On the other hand, the relatively frequent occurrence of self-inhibitors (Sussman and Douthit, 1973), and the finding that one of these inhibits translation (Bacon, 1973), may be a characteristic means of control in these organisms.

Several excellent reviews of controls have been published, including those of Calvo and Fink (1971) on bacteria and fungi, Gross (1969) on fungi, DeRobichon-Szulmajster and Surdin-Kerjan (1971) on yeasts and that of Metzenberg (1972) on *Neurospora.*

Although several well-known cases exist where enzymes for specific pathways are coded for by genes on several different chromosomes – for example, on seven and nine different ones in the case of histidine biosynthesis in *Neurospora* and *Aspergillus,* respectively – there are other cases where two or more genes are clustered and contiguous and control steps in a single metabolic sequence. Several such are listed in Table 6 which also indicates that such "clusters" have been found in several different fungi. A remarkable case of this kind is the linkage of the determinants of incompatibility factors in *Schizophyllum* (Raper, 1966). But are they "operons," à *la* bacteria?

TABLE 6: Mechanisms utilized by fungi to control enzyme biosynthesis

Mechanism	Organism	Reference
"gene clusters"	*Neurospora crassa*	Metzenberg, 1972
	Schizophyllum commune	Raper, 1966
Non-clustered but coordinated cistrons:		
nitrate reductase	*N. crassa, Aspergillus nidulans*	Gross, 1969
galactose metabolism	*Saccharomyces cerevisiae*	Gross, 1969
leucine biosynthesis, sulfate metabolism and tryptophan synthetase	*Neurospora crassa*	Gross, 1969
catabolite repression:		
carbon source	*N. crassa* and others	Hanks and Sussman, 1969
nitrogen source	*Saccharomyces cerevisiae*	Dubois *et al.*, 1973
sulfur source	*N. crassa*	Metzenberg, 1972
phosphate source	*N. crassa, S. cerevisiae*	Schmidt *et al.*, 1963; Nyc, 1967
induction	several	Metzenberg, 1972

There are at least two reasons for concluding that it is premature to analogize the gene clusters in fungi with operons. First the gene cluster is really an enzyme cluster, or a multi-enzymatic aggregate of all of the enzymes coded for by the cluster and none not coded for. By contrast, the usual operon consists of separate enzymes, sometimes localized in the membrane as well as soluble phase. Metzenberg (1972) speculates that the selective advantage of the gene cluster is to facilitate "channeling" by means of the enzyme cluster. Channeling is postulated to result in the ordered transfer of a linked series of metabolites, as in the arginine pathway described by Davis (1967). On the other hand, as Metzenberg (1972) observes, not all enzyme aggregates are the products of clustered genes, nor do all such aggregates that are the products of clustered genes participate in channeled reactions.

Second, operons are controlled by operators and the latter are defined by the existence of mutants that are operator-constitutive (O^c), that is those that have lost their responsiveness to repressors. However, no operator-constitutive mutants have been found in the fungi despite diligent searches, and only one case that can be formally interpreted in this way has been found in yeast (Douglas and Hawthorne, 1966). However, even in this case, the enzymes of galactose metabolism, there are differences from prokaryotic operons because the yeast "operator" controls the production of an activator, not the structural genes themselves.

"Non-clustered but coordinated" cistrons (Table 6) appear to be more common in the fungi than clustered one (Gross, 1961). For example, three structural genes and one regulatory gene for sulfur metabolism in *Neurospora* have been found on *four* different chromosomes (Metzenberg, 1972). It appears that the *cys-3* gene forms a product that is essential for the expression of several unlinked structural genes. In addition, the *scon* gene may code for a control element that diffuses within the nucleus and may be confined to that region. The exact mechanism of control in these cases is not known, that is, whether the control element is protein, nucleic acid or

something else. However, in the case of clustered and non-clustered cistrons alike, the control probably is positive, rather than negative, as in the case of some prokaryotic systems.

"Catabolite repression" is the phenomenon whereby the presence of certain metabolites in the medium prevents the synthesis of enzymes involved in catabolism. The best-known instance of this kind is glucose repression, which has been found in the case of trehalase and invertase (Hanks and Sussman, 1969) and the soluble malate dehydrogenase (Benevistes and Munkres, 1970) of *Neurospora crassa.* Furthermore, Flavell and Woodward (1971) suggest that a glycolytic intermediate functions as a repressor of the glyoxylate shunt enzymes in this organism.

Nitrogen starvation derepresses nitrate and nitrite reductases in *Neurospora* (Cook and Sorger, 1969), although induction may be involved as well. That NADP-glutamate dehydrogenase (anabolic) is repressible by NH_4+, along with arginase, urea amidolyase and allantoinase has been shown by Dubois *et al.* (1973) in *Saccharomyces cerevisiae.* Furthermore, other substrates, such as sulfur-containing compounds (Metzenberg, 1972) and inorganic phosphate (Nyc, 1967) repress enzyme biosynthesis as well, so a wide range of enzymes must be controllable in this way.

The selective value of catabolite repression probably lies in permitting the exhaustion of substrates which are available in plenty before enzymes needed for the degradation of less plentiful ones are synthesized. That catabolite repression probably is tied to differentiation in the fungi is suggested by Klebs' experiments which related exhaustion of the medium to fruiting. In fact, Hanks and Sussman (1969) showed that conidiation in *Neurospora* and derepression of carbohydrases occur almost simultaneously and that aconidial mutants derepress at a much lower concentration of sugar. Furthermore, the ubiquitous need for the proper "carbon: nitrogen ratio" in media for the growth of fungi (Cochrane, 1958) could relate to the derepression or repression of coordinated sets of enzymes needed for growth and differentiation.

Although there are some known cases of "enzyme induction" in fungi, that is, synthesis induced by substrate or related molecules, care must be taken in developing the proof. Thus, it must be shown that the presence of an inducer and *not* the exhaustion of substrate results in enzyme biosynthesis. For example, Kobr *et al.* (1965) suggested that acetate induces the glyoxylate shunt enzymes in *Neurospora* but Flavell and Woodward (1971) conclude otherwise, based upon some complex features of acetate metabolism. Another necessity is to distinguish between "inducer exclusion" at the level of uptake from true catabolite repression at the level of gene expression. And, finally, endogenous inducer concentrations may vary, leading to more or less induction, even in the absence of external inducer.

Nevertheless, there is good evidence that kynureninase and the enzymes of quinic acid synthesis are inducible in *Neurospora* and others have been found to be so in other organisms as well (Gross, 1969).

Although "quantal control" (M. Sussman and Newell, 1972), or the controlled transcription of definite amounts of enzymes only, has been postulated for *Dictyostelium discoideum,* and perhaps baker's yeast, it is a potential control that may be characteristic of fungi at large so it will be worthy of further study.

Control of enzyme activity.

In addition to controls at the level of synthesis there are others which

TABLE 7. Mechanisms utilized by fungi to control enzyme activity

Mechanism	Organism	Reference
Inhibition or inactivation:		
feed-back	many	Gross, 1969; Metzenberg, 1972
"interdependence"	*Neurospora crassa*	Gross, 1969
formation of inhibitors	*N. crassa*	Metzenberg, 1972; Garrett and Sussman, 1972
proteolytic inactivation	*Saccharomyces cerevisiae*	Yang and Deal, 1969
dissociation	*S. cerevisiae*	Deal, 1969
Activation:		
allosteric	*Phycomyces blakesleeanus*	Van Assche *et al.*, 1972
proteolytic	*S. cerevisiae*	Cabib and Ulane, 1973
Localization:		
mitochondria and other organelles	all fungi	many references
"channeling"	*N. crassa*	Davis, 1967
wall/membrane	*N. crassa*	Hecker and Sussman, 1973

affect enzymes already formed. The general types of these controls are listed in Table 7. By now "feed-back inhibition," especially in the regulation of amino acid synthesis, is so well established that not much more need be said about it. On the other hand, Gross (1969) has suggested that "interdependence" among enzymes, whereby tandem inhibitions occur, is less well known but is found in several fungi. As for enzyme inhibitors, nucleases (Hasunuma and Ishikawa, 1972) and trehalase (Garrett and Sussman, 1972) in *Neurospora* and tyrosinase in *Aspergillus nidulans* (Bull and Carter, 1972) appear to be regulated in this way. Proteolytic decomposition of the NADP-glutamic dehydrogenase of *Saccharomyces cerevisiae* has been invoked as a control mechanism by Yang and Deal (1969), as has the dissociation and reaggregation of the same enzyme (Deal, 1969).

As for activation of enzymes, Van Assche et al. (1972) have shown that the activity of trehalase in spores of *Phycomyces blakesleeanus* is immediately increased many-fold by the application of 50°C, the very temperature that is required to break the dormancy of these cells. An "activator" of glycogen phosphorylase in baker's yeast turns out to be a proteolytic enzyme, thereby mimicking such transformations as the pepsinogen — pepsin one in mammals.

Sequestering of certain pathways in mitochondria and other organelles has been shown to be a means whereby pathways are regulable, through permeability barriers, surface effects etc. Such a mechanism has been proposed as the means whereby trehalose is not degraded in ascospores of *Neurospora,* even though both the enzyme and the substrate are present simultaneously (Hecker and Sussman, 1973). In this case, immunofluorescent studies have revealed that trehalase is localized on the outer membrane of the cell whereas its substrate is in the interior. However, it is not necessary for organelles to be present in order that sequestering and sequencing of reactions occur. Thus, channeling (Davis, 1967) may occur, as described before, without formed structures which position the enzymes involved.

Before this section is closed it should be noted that enzymes are subject

to control in other ways as well. For example, small molecules, through allosteric and other effects can strongly affect such activity. In fact, Barbara Wright has reminded us, through her detailed examination of the development of *Dictyostelium discoideum* that the flux of substrates over a given metabolic pathway may be a determinant of the rate of reactions, as well as a result of these. The work of Chance and his co-workers, and of others, upon mitochondrial oxidations, leads to similar conclusions. These effects of small molecules now introduce a review of "micromolecules" of fungi as they reflect trends in the metabolic specialization of these organisms.

Micromolecules as indicators of metabolic diversity

To set the stage for this section I quote some remarks of J. D. Bu'lock (1961) who, along with Turner (1971), has published an extensive review detailing many of the micromolecules produced by fungi: "...the organic chemist's view of nature is unbalanced, even lunatic, but still in some ways more exciting than that of the biochemist. While the enzymologist's garden is a dream of uniformity, a green meadow where the cycles of Calvin and Krebs tick round in disciplined order, the organic chemist walks in an untidy jungle of uncouthly named extractive, rainbow display of pigments, where in every bush there lurks the mangled shape of some alkaloid, the exotic perfume of some new terpene, or some shocking and explosive polyacetylene." Here is a realm in which the metabolic diversity of the fungi is manifest but I will not say much about these secondary products because they have been the subject of the reviews noted above, as well as of shorter summaries; in addition, for the most part, the role of these materials in the organisms which produce them is largely unknown so that their evolutionary position cannot be optimally approached at this time. Consequently, I

TABLE 8. Substances produced by fungi that affect their development or growth positively

Developmental Role	Compound	Organism	Reference
sex hormone	trisporic acid antheridiol sirenin sterols (?)	Mucorales *Achlya* spp. *Allomyces* spp. *Pythium, Phytophthora, Saccharomyces cerevisiae*	Gooday, 1973 Arsenault *et al.*, 1968 Nutting *et al.*, 1968 Takao *et al.*, 1970
sexual agglutinin	protein	*Hansenula wingei*	Taylor and Orton, 1970
chemotaxis and differentiation	cyclic AMP	cellular slime molds	Bonner *et al.*, 1972
phototaxis (light receptor)	unidentified	*Dictyostelium discoideum*	Poff *et al.*, 1973
fruiting inducers	AMP, cyclic AMP and a protein	*Coprinus macrorhizus*	Uno and Ishikawa, 1973
morphogens	cytochalasins, griseofulvin	*Metarrhizium anisopliae, Helminthosporium* spp., etc.	Betina *et al.*, 1972; Aldridge *et al.*, 1967

TABLE 9. Substances produced by fungi that inhibit growth and development

Effect	Compound	Organism	Reference
"killer" strain determinant	double-stranded RNA	*Saccharomyces cerevisiae*	Vodkin and Fink, 1973
self-inhibitor of spore germination	cis-methyl ferulic acid, methyl 3, 4-dimethoxy cinnamic acid, etc.	rusts	Allen, 1972; Macko *et al.*, 1971
	5-isobutyroxy-β-ionine	*Peronospora tabacina*	
	N, N'-dimethyl-guanosine	*Dictyostelium discoideum*	Bacon *et al.*, 1973
self-inhibitor of growth	γ-L-glutamyl hydroxybenzene	*Agaricus bisporus*	Vogel and Weaver, 1972
	3-β-hydroxyindole, 2-phenylethanol	*Candida albicans*	Lingappa *et al.*, 1969
inhibitor of perithecium formation	"small molecule"	*Sordaria fimicola*	Pollock and Johnson, 1972
inhibitor of growth	acetylenes and many others	many fungi	Fries, 1973; Hutchinson, 1971
sporostatic factor	acetone, acetaldehyde and others	many fungi	Robinson and Garrett, 1969
vacuolation factor, "aging" hormone	sterol (?)	many fungi	Park and Robinson, 1967
necrobiosis inducers	gibberellins, gibberellin synergists,	*Gibberella* spp. *Pestalotia* spp.	Lewis, 1973 Kimura and Tamura, 1972
	indole acetic acid etc.	*Rhizopus* spp.	Gruen, 1965

will deal mainly with substances whose role is established. The power of this approach is revealed in the work of Vogel (1965) and others with lysine, data which have been used in phylogenetic considerations of the fungi. The way I have chosen to do this is through the assembly of tables arbitrarily distinguishing between substances that affect the growth and development of fungi positively or negatively. Let us consider those that are positive affectors first, as in Table 8.

There are exciting times ahead in the search for sex hormones, some of which already have been identified as listed in the table. Sterols have been implicated in this way and undoubtedly will be a fruitful group of substances to explore, for their diversity and characteristic structures have led Bloch (1965) and others to use them as evolutionary indicators. Also this group of compounds has been implicated as vacuolation factors (Table 9) so their role is diverse. But, in addition, there are hormones of other types, such as trisporic acid and sirenin and their investigation will lead to interesting findings as well. Not as much is known about sexual agglutinins except that they are probably glycoproteins and can hardly be considered micromolecules, but they are included because of their effect, rather than their chemical nature.

Taxes of various types probably are controlled by chemical signals like

cyclic AMP and other small molecules, as has been described for *Dictyostelium* (Table 8), and it will be on interest to explore such triggers in other motile fungi as well in order to compare the substances involved. Adenylates also appear to be involved as inducers of fruiting in basidiomycetes (Table 8) so the role of these substances is very broad indeed.

Morphogens of great potential interest to those interested in the mechanism of fungal growth and development include the cytochalasins and griseofulvin. These substances influence the branching patterns of fungi, as Betina and coworkers (1972) have shown.

Metabolic diversity is to be found among the substances fungi produce which affect the growth of other organisms, including higher plants, nematodes etc. Among these compounds are the gibberellins, indole acetic acid, nemin and others produced by a wide variety of fungi (Table 9).

Negative affectors of fungal growth and development are listed in Table 9. As in the case of the sexual agglutinins listed among positive affectors, doublestranded RNA has been included in this table. It is representative of a class of macromolecules described as viruses in fungi, almost one hundred of which have been found. These may be very useful indicators of fungal relationships if their specificity is like that of prokaryotic viruses.

Another very interesting recent development is the characterization of at least three groups of self-inhibitors of fungal spore germination, those of rusts, *Peronospora tabacina* and *Dictyostelium discoideum* (Table 9). All three of these classes of substances differ from one another so the analysis of the many other such compounds that probably exist (Sussman and Douthit, 1973) will undoubtedly show further metabolic diversity.

It is very arbitrary to separate morphogens like cytochalasins etc., from the self-inhibitors of growth, perithecium formation etc., for they may work by related mechanisms. Nevertheless, these inhibitory materials, however classified, are only now being investigated in detail so little can be said about their role and, sometimes, chemical compositon.

Closing remarks

This brings me to the end of this review. I have ignored many aspects of metabolism, for example, the nucleic acids, whose diversity is the subject of intensive study and which may show unique attributes in the fungi, as Cantino and co-workers (Cantino and Myers, 1972) have shown for the γ-particles of *Blastocladiella*. On the other hand, base ratios have not proven to be very useful indicators of diversity. Nor have I talked about nutrition for it is an approach that has been used successfully so, like immunology, its usefulness has been established. Because the exigencies have forced me to be selective I hope the materials I have chosen to discuss will be among the significant bridges to future work in fungal metabolism.

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