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Studies of aromatic biosynthetic and catabolic enzymes in *Ustilago maydis* and in mutants of *U. violacea*

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SUMMARY

A multienzyme complex for five of the enzymes in the prechorismate portion of the aromatic biosynthetic pathway has been demonstrated in *Ustilago violacea*, and has previously been reported in *U. maydis* (Ahmed & Giles, 1969). This complex is similar to that found in *Neurospora crassa* and other fungi. In *U. violacea* polyaromatic-requiring mutants show pleiotropic deficiencies for all five of these enzymes, similar to the extreme pleiotropic polar mutants of the *arom* gene cluster in Neurospora (Giles, Case, Partridge & Ahmed, 1967*a*; Case & Giles, 1971). This result is interpreted as mutational evidence for an *arom* gene cluster in *U. violacea* comparable to that in *N. crassa*. A second low molecular weight, heat-stable isozyme of dehydroquinase is shown to be present at high (constitutive) levels in *U. maydis*, as previously indicated by Ahmed & Giles (1969), but this activity is increased to extraordinarily high levels in cells grown in the presence of quinate. In contrast, *U. violacea* strains do not grow on quinate, have a single, heat-labile dehydroquinase species, and lack activities for other enzymes in the quinate catabolic pathway.

1. INTRODUCTION

In extracts of *Ustilago maydis* five enzymes of the early, common pathway for the biosynthesis of aromatic amino acids (Reactions 2–6 in Fig. 1) have been shown to be physically aggregated (Ahmed & Giles, 1969). This *arom* multienzyme complex is similar in size to that reported first for *Neurospora crassa* (Giles, Case, Partridge & Ahmed, 1967*a*) and later for a variety of other fungi (deLeeuw, 1968; Ahmed & Giles, 1969). In Neurospora and yeast the aggregate is encoded in a cluster of five genes (the *arom* cluster), in which mutants with pleiotropic as well as single enzyme defects can be demonstrated. The hypothesis has been made that the presence of the multienzyme complex is correlated with and provides evidence for the occurrence of a gene cluster (Ahmed & Giles, 1969; Berlyn & Giles, 1969). In bacterial species, in which the homologous genes are not clustered, the enzymes are not aggregated *in vitro* (Berlyn & Giles, 1969). This report presents genetic and biochemical evidence which suggests the presence of a gene cluster and demonstrates aggregation of the gene products in a second species of *Ustilago*, *U. violacea*.

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Ustilago violacea, like *U. maydis*, is a parasitic fungus which can be cultured on artificial media as unicellular, uninucleate, haploid sporidia, or as diploid sporidia which can be induced to undergo mitotic recombination. In *U. violacea* mitotic haploidization of the diploid sporidia can also be induced (Day & Jones, 1969). This species was examined for the presence of an *arom* aggregate and for the occurrence of pleiotropic mutants indicative of a gene cluster.

In *U. maydis* grown in media lacking quinic acid the presence of a second species of dehydroquinase, heat-stable and presumably analogous to the heat-stable, inducible dehydroquinase of the quinic acid degradative pathway of *Neurospora crassa* (Giles, Partridge, Ahmed & Case, 1967*b*), was previously reported (Ahmed & Giles, 1969). This result has been further examined in *Ustilago maydis* and the enzymic and growth responses of *U. violacea* in the presence of quinic acid are also reported.

2. MATERIALS AND METHODS

(i) *Strains and media*

The media used for *U. violacea* were those described by Day & Jones (1968): complete medium (CM) and minimal medium (MM) with supplementation of 10 μg/ml tryptophan, 50 μg/ml tyrosine, 50 μg/ml phenylalanine, 0·25 μg/ml p-amino benzoic acid, and 10 μg/ml inositol, where required. For testing induction by quinic acid, the dextrose concentration was reduced to 0·2% and 0·3% quinic acid was added, or where indicated, 0·3% quinic acid was used in the absence of dextrose. *U. violacea* strains were also tested on 1% quinic acid. The multiply requiring *arom* mutants were grown on supplemented MM only; they did not grow on CM and growth on liquid supplemented MM was slow. Other strains grew more slowly on MM than on CM. *U. maydis* was grown on MM (Holliday, 1961) with adjustments of quinate and dextrose concentrations as indicated above. For enzyme assays strains were grown from 5 to 10% inoculum in 500 ml of medium in 2 l. flasks on a rotary shaker or in 13 l. of medium in aerated carboys at 22 °C in the case of *U. violacea* and at 30 °C for *U. maydis*.

The *U. maydis* wild-type strain U4 was obtained from Dr Peter Day, Conn. Agric. Expt. Station, New Haven, Conn. The *arom* mutants of *U. violacea* were isolated by Dr Mary Case, Yale Univ., after UV treatment and inositolless death selection (Holliday, 1962) of the inositol-requiring strain 1·729, obtained from Dr A. W. Day, Univ. of Western Ontario, London, Ontario.

(ii) *Biochemical methods*

Cells were disrupted in a Hughes pressure cell or by lyophilization and grinding in a Wiley mill and extracted as described in previous studies (Berlyn, Ahmed & Giles, 1970). The buffer used throughout the extraction was either 0·1 M tris (hydroxymethyl) aminomethane-HCl, pH 7·4, or 0·1 M potassium phosphate, pH 7·4, with addition of 2×10^{-4} M α-thioglycerol or dithiothreitol for extracts assayed for biosynthetic enzymes and with addition of 10^{-3} M dithiothreitol and 10^{-3} M ethylenediaminetetraacetic acid for extracts assayed for degradative

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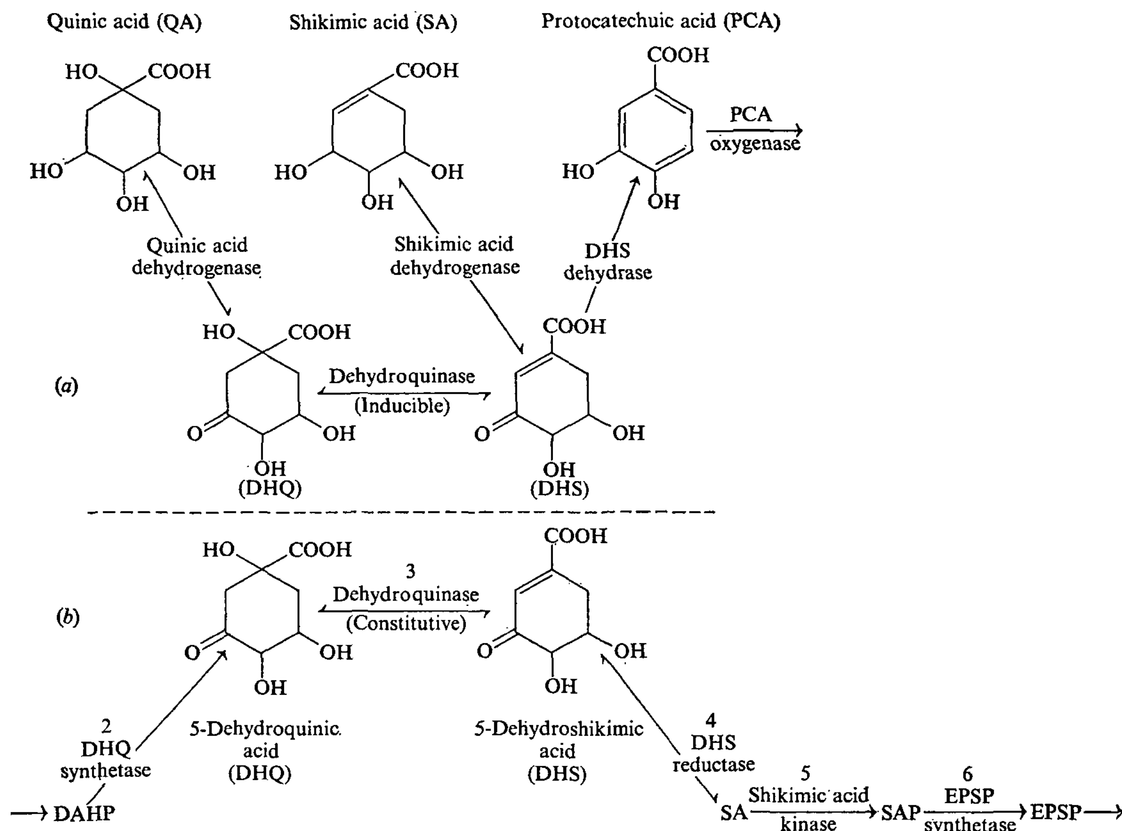

Fig. 1. Reactions in the quinic acid catabolic (*a*) and aromatic synthetic (*b*) pathways, indicating the relationships of the two dehydroquinase isozymes in *Neurospora crassa*.

enzymes. Enzyme assay and sucrose density gradient procedures have also been described previously (Berlyn & Giles, 1969; Ahmed & Giles, 1969), except for the following modifications: A heat-treated quinate grown Neurospora extract containing heat-stable dehydroquinase was used to provide the dehydroquinase activity necessary for the DHQ synthetase assay; the quinate dehydrogenase and shikimate dehydrogenase reaction mixtures contained a final concentration of 10 mM quinate and 13 mM shikimate, respectively. EPSP synthetase and shikimate kinase were detected only after sucrose density gradient centrifugation of extracts.

3. RESULTS

Sucrose density gradient profiles of the five enzyme activities of *U. violacea* and *U. maydis* are shown in Figs. 2 and 3. In both species the enzymes are coincident; the position indicates a molecular weight of approximately 180000, using alkaline phosphatase or malate dehydrogenase as a standard, or approximately 220000 when catalase is used as a standard (cf. Case & Giles, 1971).

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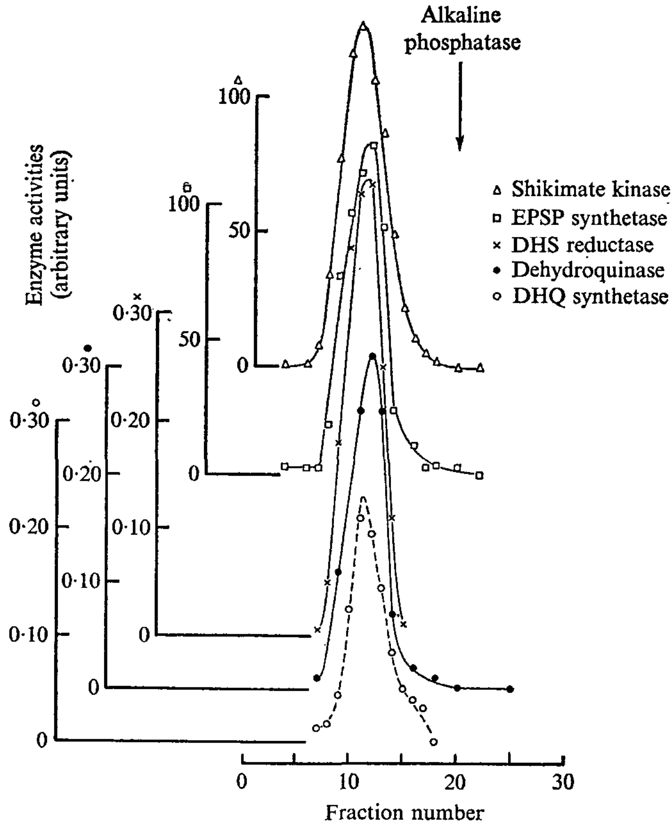

Fig. 2. Distribution of aromatic enzyme activities after sucrose density gradient centrifugation of a 30–50% ammonium sulphate fraction of *U. violacea* 1·729. The DHQ synthetase profile was obtained from an identical extraction and centrifugation procedure performed subsequently. Arrow indicates the peak of the activity profile for the enzyme standard, *Escherichia coli* alkaline phosphatase.

Specific growth requirements of twenty mutants of *U. violacea* deficient in aromatic amino acid biosynthesis were determined. Of these, ten required tryptophan supplementation, four required phenylalanine, and four required the combination of all aromatic supplements. Enzyme activities of the four multiply requiring pleiotropic mutants and two of the tryptophan mutants are given in Table 1. Mutant 15 had a low level of chorismate synthetase activity and wild-type levels of the other enzymes. The tryptophan-requiring mutants were not deficient for any of these activities. Two mutants (12 and 32) have normal chorismate synthetase activity and lack significant activity for the other five enzymes. Mutant 24 has reduced but significant levels of DHS reductase and dehydroquinase, but lacked DHQ synthetase and probably shikimate kinase and EPSP synthetase.

Both growth and enzymic studies indicate that the quinic acid degradative pathway, as it exists in Neurospora and *U. maydis* (Fig. 1) is lacking in *U. violacea*. Fig. 4 shows growth curves of *U. violacea* and *U. maydis* on minimal medium

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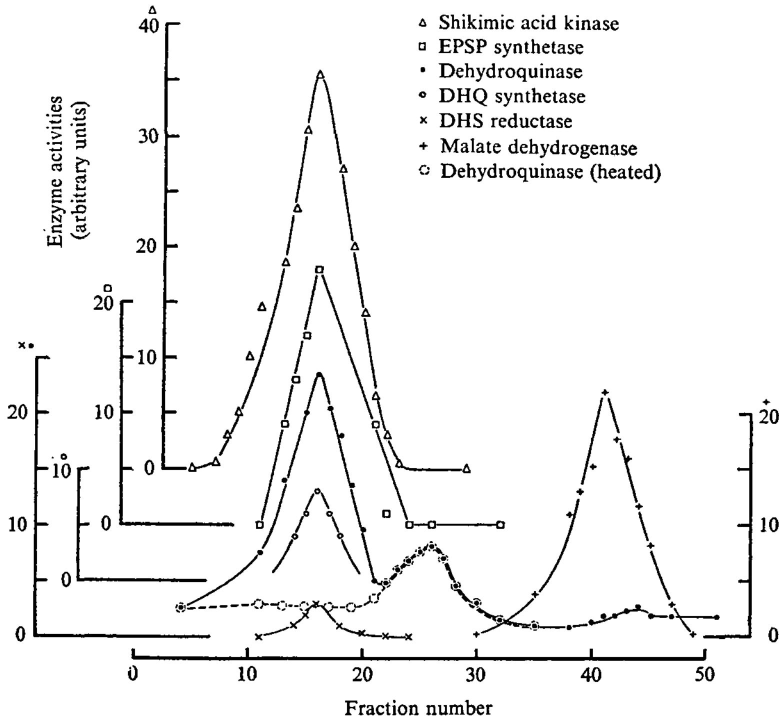

Fig. 3. Distribution of aromatic enzyme activities after sucrose density gradient centrifugation of a 0–50% ammonium sulphate fraction of *U. maydis* grown in the absence of quinate. Dehydroquinase activity was assayed before and after heating fractions at 58 °C for 10 min. This figure has appeared in *J. Bacteriol.* **99** (Ahmed & Giles, 1969) and is republished with permission of the American Society for Microbiology.

containing either dextrose or quinic acid as carbon source. Results of assays for degradative pathway enzymes in *U. maydis* and in mutant and wild-type strains of *U. violacea* grown for 5 days on dextrose or on dextrose plus quinic acid are shown in Table 2. In all cases these activities are absent from *U. violacea* extracts except for an occasional low level of QDH or SDH activity, which is presumably a non-specific reaction of a dehydrogenase present in the extract. A shift of a 5-day culture of *U. violacea* from dextrose medium to quinic acid medium also failed to induce the enzymes of this pathway in *U. violacea*; growth on succinate prior to a switch to quinate-containing medium did not alter this negative response.

4. DISCUSSION

The density gradient sedimentation rates indicate the presence in *U. violacea* of a multienzyme complex similar to that found in *N. crassa*. The presence of pleiotropic mutants (32 and 12) deficient for all five activities also parallels the situation in Neurospora. Although other explanations of pleiotropy are possible, it seems most likely that these mutations are analogous to the pleiotropic

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Table 1. *Aromatic biosynthetic enzyme activities for wild-type and mutant strains of* Ustilago violacea

Strain no.	Growth requirement	Ammonium sulphate fraction	DHQ synthetase	Specific activities (International units × 10^{-3}) Dehydroquinase	DHS reductase	Shikimate kinase	EPSP synthetase	Chorismate synthetase
1·729	Inos	0–33	0	4·4	6·7			
		33–50	12	10	12	6·2	7·3	1·2
		50–75	0	0·7	2·8			
32	Arom, inos	0–33	0·4	0	0			
		33–50	0	0	0	0	0	1·4
12	Arom, inos	0–33	0	0·01	0			
		33–50	0·2	0·01	0·02	0	0	1·8
		50–75	0·02	0·002	0·02			
24	Arom, inos	0–33	0	5·0	5·5	*	*	
		33–50	0	1·0	2·2	0	0	2·3
		50–75	0	0	0			
15	Arom, inos	0–33	1·1	0	0			
		33–50	18	22	28	3·3	18	0·2
		50–75	0·7	1·3	2·4			
25	Tryp, inos	0–33	0·8	3·8	1·8			
		33–50	4·1	11	14	3·2	3·0	1·3
29	Tryp, inos	0–33	0·6	1·5	0·9			
		33–50	14	13	19	6·8	4·0	6·3
		50–75	0·25	0	0			

* Fluorescence was sometimes observed in a few very heavy fractions of gradients of 0–33% ammonium sulphate fractionated extracts, but its limited and nonsymmetrical distribution seemed not to represent evidence for true shikimate kinase or EPSP synthetase activity.

mutants of the Neurospora *arom* gene cluster, and indicate a similar type of genetic organization for the *arom* genes of *Ustilago violacea*. If this is the case, further mutational and genetic analyses in *U. violacea* should provide conclusive evidence for such a cluster. The 'partial pleiotropy' of mutant 24 bears a general although not specific similarity to mutants of Neurospora (Giles *et al.* 1967*a*) and yeast (deLeeuw, 1968); whether this mutation represents a polar mutation or a missense mutation resulting in loss of three activities due to disruption of the aggregate (cf. Case & Giles, 1968, 1971) cannot be determined in the absence of complementation data. Attempts to isolate these alleles in a suitable background with respect to mating type and forcing markers for use in complementation studies have been hindered by a predominance of aneuploids rather than haploids after para-fluorophenylalanine treatment. Although the reductase and dehydroquinase activities are significant they were too low to allow suitable recovery from sucrose density gradients of the ammonium sulphate fractionated extracts which would have provided evidence about the state of aggregation of the enzymes.

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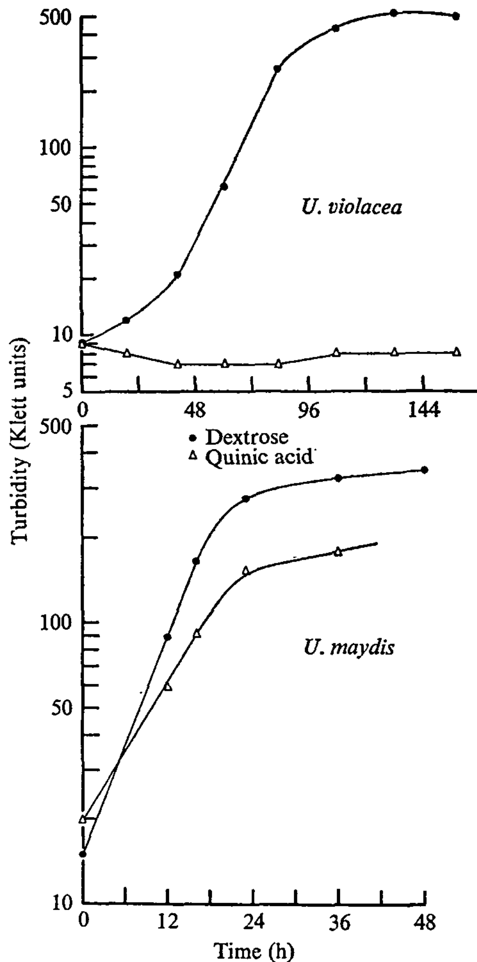

Fig. 4. Growth curves for *U. violacea* and *U. maydis* grown on dextrose or quinic acid as carbon source. Two percent inocula of cells grown on complete media were used. A heavier inoculum of cells grown on minimal medium alleviated the lag in growth of *U. violacea*.

Increased activity in the 0–33 % as compared to the 33–50 % ammonium sulphate fraction for this mutant is indirectly suggestive of a possible change in the physical state of the aggregate.

Current evidence indicates a clearly delineated distribution of this multienzyme complex along major phylogenetic lines. All fungi thus far examined (three basidiomycetes, three ascomycetes, and three phycomycetes) possess the high molecular weight aggregate, whereas procaryotes (five bacterial species and one blue-green alga), mosses, higher plants (tobacco, mung beans, and peas) and *Chlamydomonas* do not (Ahmed & Giles, 1969; Berlyn & Giles, 1969; Berlyn, Ahmed & Giles 1970). In *Euglena gracilis* a smaller, less stable aggregate has been demonstrated (Berlyn, Ahmed & Giles, 1970). Examination of other 'borderline' organisms would be interesting in this respect.

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Table 2. *Quinic acid degradative enzyme activities in* Ustilago maydis *and strains of* Ustilago violacea *grown for 5 days on dextrose or quinic acid*

	Ammonium sulphate fraction	Specific activities (International units × 10^{-3}) Quinate dehydrogenase	Shikimate dehydrogenase	Dehydroquinase	Heat stable dehydroquinase	DHS reductase
U. maydis (U4) on dextrose	0–30	33	32	21	2·6	14
	30–50	300	220	32	29	0·67
	50–75	110	96	0·7	1·6	0·10
U. maydis (U4) on dextrose + quinic acid	0–30	21	18	80	110	3·4
	30–50	220	200	1400	2400	3·4
	50–75	120	100	73	90	0
U. violacea (1·729) on dextrose	0–30	0	0	0	0	0
	30–50	0	5·4	37	0	40
	50–75	0	0	0	0	4·1
U. violacea (1·729) on dextrose + quinic acid	0–30	0	0	0	0	0
	30–50	0	2·4	36	0	31
	50–75	0	0	0	0	0
U. violacea (*arom*-32) on dextrose	0–30	0	0	0	0	0
	30–50	0	0	0	0	0
	50–75	0	0	0	0	0
U. violacea (*arom*-32) on dextrose + quinic acid	0–30	0	0	0	0	0
	30–50	0	0	0	0	0
	50–75	0	0	0	0	0
U. violacea (*arom*-12) on dextrose	0–30	0·1	0·2	0·2	0	0
	30–50	0·2	0·05	0	0	0
	50–75	0·4	0·4	0	0	0·7
U. violacea (*arom*-12) on dextrose + quinic acid	0–30	0·2	0·2	0·4	0	0
	30–50	0	0·3	0	0	0
	50–75	0·4	0·3	0	0	0·6
U. violacea (*arom*-24) on dextrose	0–30	0	0	2·7	0	5·4
	30–50	0	0	2·1	0	3·0
	50–75	0	0	0·6	0	2·1
U. violacea (*arom*-24) on quinic acid	0–30	0	0	1·4	0	2·5
	30–50	0	0	1·0	0	2·4
	50–75	0	0	0·2	0	1·8

Previous studies indicated that *U. maydis* can grow well with quinic acid as sole carbon source (Ahmed & Giles, 1969; Kent Keeton, unpublished). Ahmed & Giles (1969) reported a thermostable dehydroquinase in extracts of cells grown in the presence or absence of quinic acid. This activity is distinct from the heat-labile peak on sucrose gradient profiles. The presence of quinate dehydrogenase and DHS dehydrase in *U. maydis* was also reported. A constitutive rather than an inducible degradative pathway in this organism was proposed. The current study indicates that relatively high levels of degradative pathway enzymes were present in cells grown in the absence of quinate; however, presence of quinate leads to greatly increased levels of dehydroquinase (Table 2). This high level of

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induction was not observed for quinate dehydrogenase and shikimate dehydrogenase. Levels of these activities varied several fold in different extractions and under different growth conditions, but did not vary significantly in parallel quinate-grown and dextrose-grown cultures. In a given extract quinate dehydrogenase and shikimate dehydrogenase activities were quite similar. In Neurospora the two activities are apparently associated with a single enzyme (Rines, 1969; Chaleff, 1971).

In contrast to *U. maydis*, *U. violacea* cannot grow at the expense of quinic acid, and extracts lack significant amounts of the degradative enzymes; a single species of dehydroquinase activity is recovered from gradients and only the heat-labile form of the enzyme is observed. It is striking that these two closely related species should differ in their ability to utilize this carbon source. Two of the ascomycetes examined, *Aspergillus nidulans* and *Neurospora crassa*, possess the quinate catabolic enzymes whereas a third, *Saccharomyces cerevisiae*, does not. The three phycomycetes examined as well as the basidiomycete *Coprinus lagopus* and *Euglena gracilis* also lacked this pathway. Thus, the presence of the aggregate is not always correlated with the existence of the degradative pathway. Perhaps the retention of this pathway is determined by the availability of quinic acid in the normal substrata of a particular organism. High levels of quinic acid have been found in various organs of *Zea mays*, the host plant for *U. maydis* (Carles, 1957); quinate levels of the *U. violacea* host *Silene alba* remain to be determined.

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