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Organization of Polyaromatic Biosynthetic Enzymes in a Variety of Photosynthetic Organisms

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Sucrose density gradient centrifugation was used to estimate the molecular weights and determine possible physical aggregation of the enzymes catalyzing steps 2 to 6 in pre-chorismic acid polyaromatic biosynthesis in *Anabaena variabilis*, *Chlamydomonas reinhardi*, *Euglena gracilis*, *Nicotiana tabacum*, and *Physcomitrella patens*. In *A. variabilis*, the five enzymes are separable. Similar results were obtained for *P. patens*, *N. tabacum*, and *C. reinhardi* extracts, except that dehydroshikimate reductase and dehydroquinase were not separable by this method. Evidence is presented for an enzyme aggregate containing five activities, with a molecular weight of approximately 120,000 in *E. gracilis;* dissociation of this aggregate into components corresponding to molecular weight of ca. 60,000 is also observed. Preliminary evidence concerning the enzymatic composition of the 60,000-molecular-weight components is presented and discussed. Similarities between the *E. gracilis* polyaromatic aggregate and that of *Neurospora crassa* are discussed.

In *Neurospora crassa*, five of the enzymes in the pre-chorismic acid polyaromatic biosynthetic pathway (steps 2 to 6, Fig. 1) are physically associated as a multienzyme aggregate with molecular weight of ca. 230,000 (4); these enzymes are encoded by the five genes of the *arom* gene cluster (9). In *Saccharomyces cerevisiae*, these activities are found primarily in an aggregate of similar molecular weight and are encoded by the 5-cistron *arom-1* region [A. de Leeuw, Genetics **56**:554 (abst.) and Ph.D. Dissertation, Yale University, 1968]. Previous reports have described a similar aggregate of these enzymes in a variety of fungi (1) and low-molecular-weight, for the most part separable, enzymes in bacterial extracts (3). In the present study, the sedimentation patterns of these five enzymes was examined by sucrose density gradient centrifugation in extracts of a variety of photosynthetic organisms, namely, the blue-green alga *Anabaena variabilis*, *Chlamydomonas reinhardi*, *Euglenia gracilis*, *Nicotiana tabacum*, and the moss *Physcomitrella patens*.

MATERIALS AND METHODS

Growth and extraction. The strains and media used are shown in Table 1. The *C. reinhardi* cells were a gift from the laboratory of J. L. Rosenbaum. *N. tabacum* cells were grown at 25 C in 500 ml of medium in 2-liter flasks for 3 weeks on a rotary shaker. *E. gracilis* was grown for 3 to 5 days at 25 C under fluorescent light either in 1,500 ml of medium in Fernbach flasks on a rotary shaker or with forced aeration in a carboy containing 16 liters of medium. *A. variabilis* and *P. patens* were similarly grown in illuminated carboys at 25 C; they were aerated with a mixture of air and CO_2 by the method of Engel (6).

A. variabilis, *C. reinhardi*, and *E. gracilis* were harvested by centrifugation; tobacco and moss cells were harvested by filtration. Cells were washed in cold 0.1 M potassium phosphate buffer (*p*H 7.4), and the pellets were stored at −15 C.

Cell extracts of *A. variabilis*, *C. reinhardi*, *P. patens*, and *E. gracilis* were prepared by passage through a Hughes pressure cell as described previously (1, 3). In some cases, *E. gracilis* cells were disrupted by sonic disintegration in a Branson Sonifier or by lyophilization and grinding in a Wiley-mill. The Gamborg sonic disintegration-lyophilization procedure (8) was used to disrupt tobacco cells. The extraction buffer was either potassium phosphate (0.1 M, *p*H 7.4) or 0.1 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride (*p*H 7.4) and contained 10^{-3} M dithiothreitol. One gram of polyclar AT (Bio-Rad Laboratories, Richmond, Calif.) per 8 g (wet weight) of cells was added to all suspensions in the initial phases of extraction of tobacco. After removal of debris by centrifugation, the supernatant was treated with protamine sulfate (0.1 volume of 1.4% solution) to precipitate nucleic acids. The resulting crude extract either was desalted by dialysis against the original extraction buffer for 4 hr or by filtration through a column (4 by 1 cm; Sephadex G-25,

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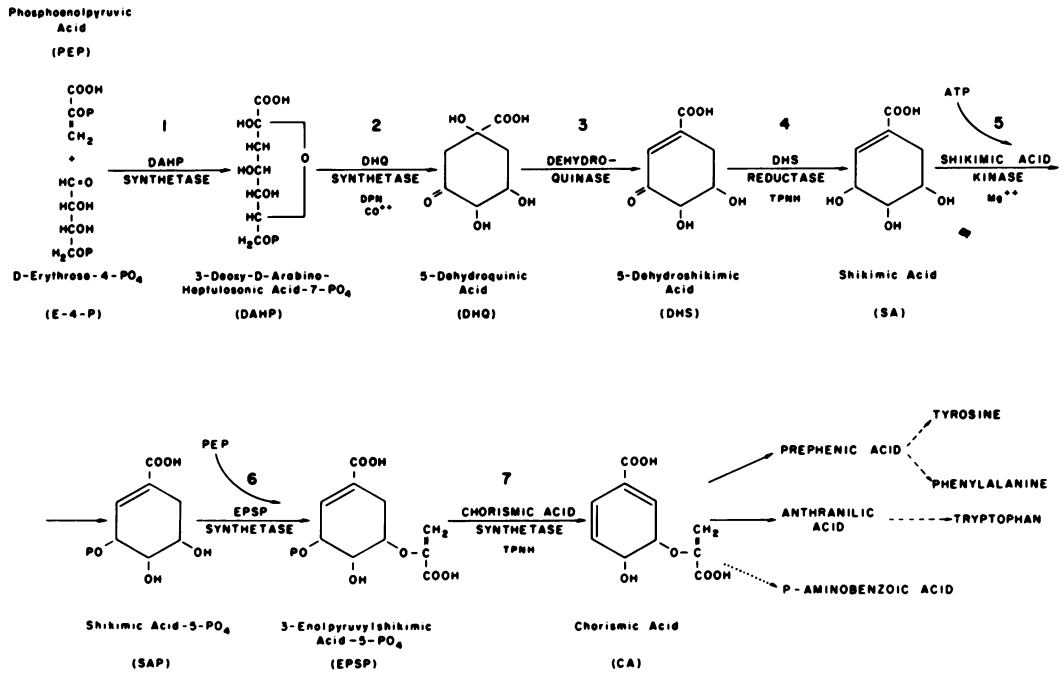

FIG. 1. *Reactions in the biosynthesis of chorismic acid in the polyaromatic pathway.*

Pharmacia, Uppsala; reference 13) or was fractionated by ammonium sulfate and then desalted.

Sucrose density gradient centrifugation. Gradients were prepared and run as described previously (1, 3; *see* 13). Horseradish peroxidase (20 μg; Worthington Biochemical Corp., Freehold, N.J.) and 50 μg of *E. coli* alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) or 20 μg of alcohol dehydrogenase (Worthington), or both, were added to the gradients to serve as internal standards. Calculated recoveries of enzyme activities after density gradient centrifugation were usually in the range of 70 to 100%; however, these data were sometimes incomplete because of apparent masking of some activities in crude extracts. When ammonium sulfate fractionation was used, all fractions containing appreciable activities were analyzed by gradient centrifugation.

Enzyme assays. 5-Dehydroquinic acid (DHQ) synthetase, dehydroquinase, 5-dehydroshikimic acid (DHS) reductase, 3-enolpyruvylshikimic acid-5-PO_4 (EPSP) synthetase, and shikimate kinase were assayed as described previously (9). Shikimate kinase in *N. tabacum* extracts was also assayed by a radioactive procedure (3). Enzyme fractions (10 to 20 μliters) were assayed for peroxidase as described previously (3). Alcohol dehydrogenase was assayed in a reaction mixture containing 20 μliters of enzyme fraction, 0.2 mmole of ethanol, 15 μmoles of Tris (*p*H 8.5), and 5 μmoles of nicotinamide adenine dinucleotide (NAD) in a volume of 0.6 ml. Change in absorbance at 25 C was recorded at 340 nm. Presence of alkaline phosphatase in 10-μliter enzyme fractions was assayed by recording change in absorbance at 410 nm as *p*-nitrophenol was produced from 1 mM *p*-nitrophenylphosphate disodium tetrahydrate (Sigma 104, Sigma Chemical Co.) in a total volume of 0.65 ml containing 0.6 mmole of Tris (*p*H 8.0).

Thermostability tests on dehydroquinase in *E. gracilis* were performed as described previously (1).

RESULTS AND DISCUSSION

Representative gradient profiles for each species are shown in Fig. 2 to 7. Differences in sedimentation patterns of an enzyme found in various ammonium sulfate fractions and crude extracts were observed only for shikimate kinase in *C. reinhardi* and for the enzymes of *E. gracilis*, as discussed below. Estimates of molecular weight by using either alkaline phosphatase or alcohol dehydrogenase as a standard (*see* 13) are shown in Table 2.

In *A. variabilis*, as in other procaryotes studied previously (3), the five enzymes were for the most part separable. Although the positions of DHS reductase and shikimate kinase activity profiles are similar in Fig. 2, there is some indication in other gradients of *A. variabilis* that the apparent molecular weights of the two enzymes are slightly different (Table 2). Separation of the enzymes was also observed for the eucaryotes *C. reinhardi*, *P. patens*, and *N. tabacum* (Fig. 3, 4 and 5), except that dehydroquinase and DHS reductase were not clearly separable. [In Fig. 3,

TABLE 1. *Strains and media*

Species	Origin of strains	Medium
Anabaena variabilis	B381 derivative from C. Peter Wolk, MSU/AEC Plant Research Laboratory, Michigan State University	Allen-Arnon minimal medium (2)
Chlamydomonas reinhardi	Wild-type strain from J. L. Rosenbaum, Biology Department, Yale University (14)	Sager-Granick minimal medium (15)
Euglena gracilis	Strain Z (wild type) from J. Preston, Microbiology Department, Yale University	Hutner's minimal medium (11)
Nicotiana tabacum	XD 120-3 callus culture from P. Filner, MSU/AEC Plant Research Laboratory, Michigan State University	Filner's modified White's medium (7)
Physcomitrella patens	Wild-type strain isolated by HLK Whitehouse and received from P. P. Engel, St. Benedict's College	Half-strength Hutner's minimal medium (6, 12)

EPSP synthetase is not obviously separable from DHS reductase and dehydroquinase; however, this activity in other experiments with *C. reinhardi* consistently sedimented at a slightly slower rate than DHS reductase and dehydroquinase (Table 2).] The molecular weights of DHQ synthetase and dehydroquinase in these organisms are similar to the *Escherichia coli* values, except for dehydroquinase in *A. variabilis* extracts. A progression of molecular weights for dehydroquinase was observed among procaryotes (3), and sedimentation of this enzyme in *A. variabilis* corresponds to an estimated molecular weight of 130,000, which is similar to that for *Streptomyces coelicolor* (3). In *A. variabilis*, both shikimate kinase and EPSP synthetase sedimented as apparently heavier molecules than corresponding enzymes in any of the nonaggregate-containing species previously examined. Shikimate kinase

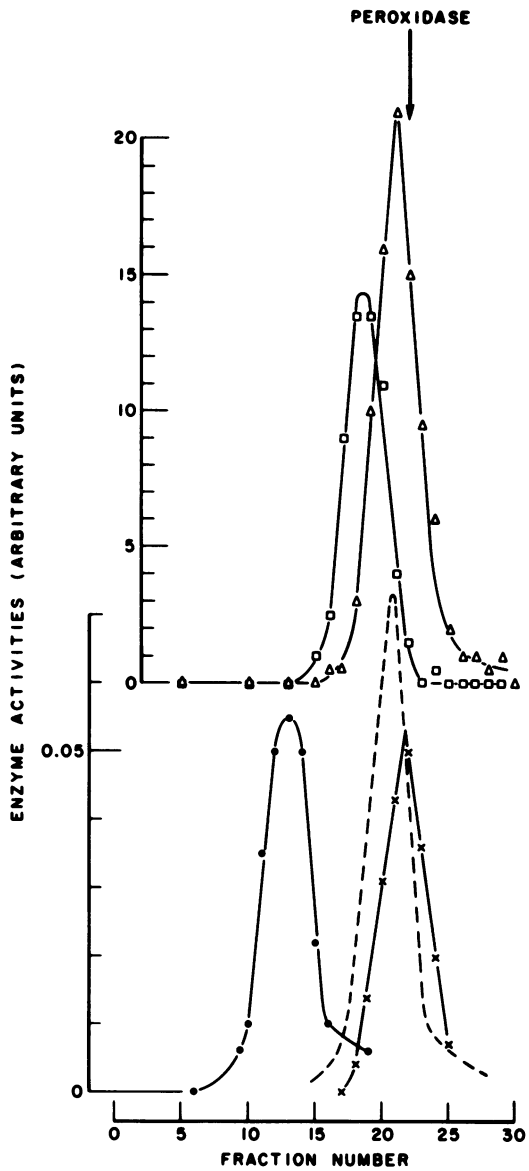

FIG. 2. *Distribution of aromatic enzyme activities after sucrose density gradient centrifugation of Anabaena variabilis crude extract. Arrow indicates peak of activity for the enzyme standard peroxidase (molecular weight, 40,000). The DHQ synthetase profile is taken from a similar gradient of a 50 to 75% ammonium sulfate fraction; this activity was not detected in crude extracts or 0 to 50% ammonium sulfate fractions. Symbols: △, shikimic acid kinase; □, EPSP synthetase; ●, dehydroquinase; ×, DHS reductase; broken line, DHQ synthetase profile (from similar gradient).*

TABLE 2. *Average molecular-weight values for aromatic biosynthetic enzymes in five species*[a]

Species	DHQ synthetase			Dehydroquinase			DHS reductase			Shikimate kinase			EPSP synthetase		
	$\bar{x}$[b]	n	$s_{\bar{x}}$	$\bar{x}$	n	$s_{\bar{x}}$	$\bar{x}$	n	$s_{\bar{x}}$	$\bar{x}$	n	$s_{\bar{x}}$	$\bar{x}$	n	$s_{\bar{x}}$
A. variabilis	58,000	3	1,100	130,000	9	1,900	46,000	8	700	53,000	6	1,700	71,000	5	2,200
C. reinhardi	57,000	2	(400)	42,000	5	1,200	42,000	11	900	Multiple			38,000	11	1,800
P. patens	67,000	4	1,500	46,000	10	700	46,000	13	1,500	23,000	4	800	37,000	3	1,000
N. tabacum	67,000	5	700	41,000	2	(500)	43,000	5	1,400						
E. coli (3)	56,000	4	1,100	40,000	8	800	25,000	6	800	17,000 21,000	6	400	38,000	3	1,800

[a] Abbreviations: DHQ, 5-dehydroquinic acid; DHS, 5-dehydroshikimic acid; EPSP, 3-enolpyruvylshikimic acid-5-PO_4.

[b] Mean molecular-weight value calculated from n gradients, $\bar{x}$. The standard error, $s_{\bar{x}}$ is placed in parentheses when only two values were averaged.

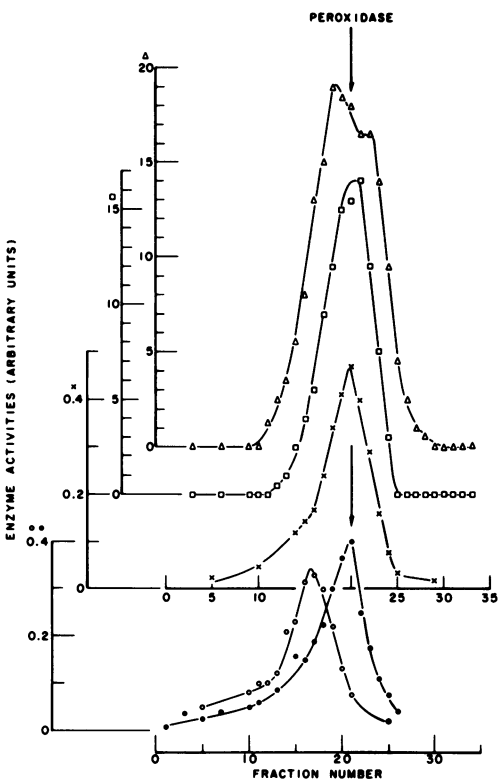

FIG. 3. *Distribution of aromatic enzyme activities after sucrose density gradient centrifugation of Chlamydomonas reinhardi crude extract. The five activity profiles are a composite derived from two gradients of the same extract centrifuged simultaneously. Symbols: arrows, peak of activity for the enzyme standard peroxidase (molecular weight, 40,000) on each gradient; △, shikimic acid kinase; □, EPSP synthetase; ●, dehydroquinase; ×, DHS reductase; and ○, DHQ synthetase.*

and EPSP synthetase were not detected in *N. tabacum* with the assay systems used, and recovery of dehydroquinase was generally low; similar experiments with pea (*Pisum sativum*) and mung bean (*Phaseolus aureus*) also failed to detect these activities and showed the same sedimentation pattern for the other three activities as with *N. tabacum*. In *C. reinhardi*, as in *E. coli*, multiple forms of shikimate kinase were observed; the broad or multiple peaks obtained from gradients of various *Chlamydomonas* fractions did not yield consistent molecular weight values for these forms, but they were quite different from those of *E. coli*.

The most complex results were obtained in analyses of *E. gracilis* extracts. Figure 6 shows four activities sedimenting together, with a molecular weight approximation of ca. 120,000, using typical alkaline phosphatase sedimentation as a standard, or ca. 150,000, comparing with typical alcohol dehydrogenase sedimentation. DHQ synthetase activity was not recovered from this gradient, but in a subsequent experiment, weak DHQ synthetase activity was found to coincide with DHS reductase and dehydroquinase activities. Variation in this pattern was found, however, in different cell batches and with different enzyme extraction procedures; Fig. 7 shows a sedimentation pattern of four activities, suggesting different degrees of aggregation. The profile indicates activities corresponding to a 120,000-molecular-weight component estimated on the basis of alkaline phosphatase sedimentation and, also, active components corresponding to 60,000 molecular weight. Various degrees of heterogeneity and coincidence of activities were observed by hydroxylapatite and diethylaminoethyl (DEAE)-Sephadex column chromatography, as

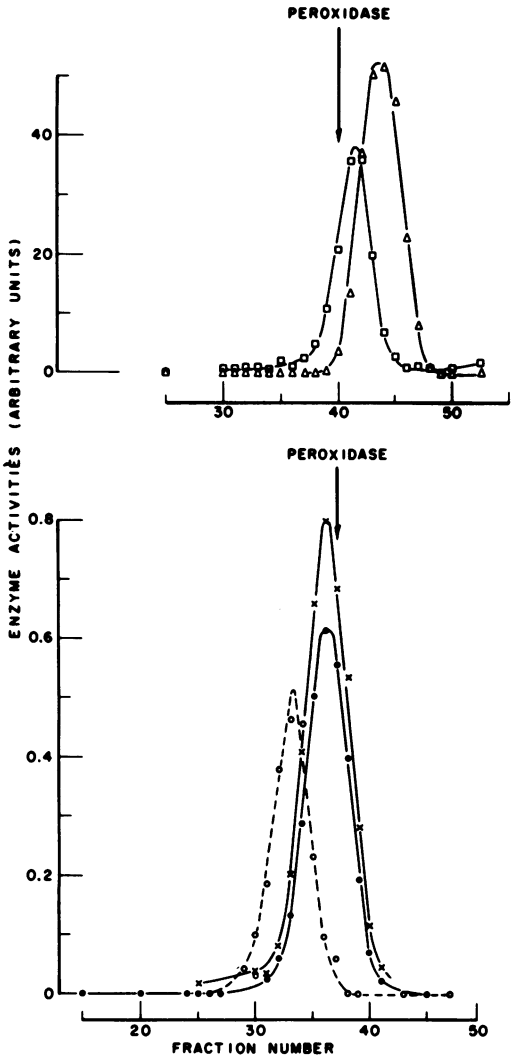

FIG. 4. *Distribution of aromatic enzyme activities after sucrose density gradient centrifugation of Physcomitrella patens 50 to 75% ammonium sulfate fraction. Arrows indicate peak of activity for the enzyme standard peroxidase (molecular weight, 40,000) in each of two gradients centrifuged simultaneously. The DHQ synthetase profile was obtained in a similar gradient of a 0 to 75% ammonium sulfate fraction. Symbols: △, shikimic acid kinase; □, EPSP synthetase; ●, dehydroquinase; ×, DHS reductase; and ○, DHQ synthetase (from similar gradient).*

well as by density gradient centrifugation. These results suggest the presence of a protein aggregate in *Euglena* extracts which has a molecular weight of 120,000 and can dissociate into one or more 60,000-molecular-weight components. These smaller components may be partial aggregates containing more than one activity. Breakdown of the aggregate with some degree of nonspecific reaggregation could account for the further variability observed.

We considered several lines of evidence which lead to a conjecture about the nature of the 60,000 molecular weight subunits. In *Euglena*, EPSP synthetase was found in 45 to 60% ammonium sulfate fractions and in 0 to 45% fractions. In contrast, the other enzyme activities were essentially absent from the 45 to 60% fractions (Table 3). In this higher fraction, the molecular weight of EPSP synthetase was estimated to be around 60,000, whereas in crude extracts, 0 to 60%, or 0 to 45% fractions, indications of a 60,000-molecular-weight species of this enzyme were found along with heavier species, e.g., a 120,000-molecular-weight component and sometimes a component intermediate in size. This appears to indicate that the 60,000-molecular-weight component of EPSP synthetase is not part of a subunit containing dehydroquinase, DHS reductase, or shikimate kinase activity. DHQ synthetase activity has been observed only in the 120,000-molecular weight-aggregate; it is typically low or completely undetectable. Since this activity was not detected

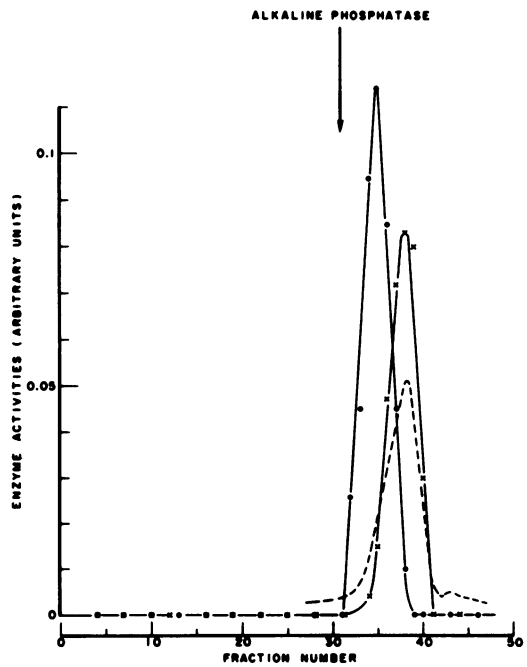

FIG. 5. *Distribution of aromatic enzyme activities after sucrose density gradient centrifugation of Nicotiana tabacum 0 to 50% ammonium sulfate fraction. Symbols: arrow, peak of activity for the enzyme standard alkaline phosphatase (molecular weight, 80,000); ×, DHS reductase; ○, DHQ synthetase; broken line, dehydroquinase profile (from similar gradient).*

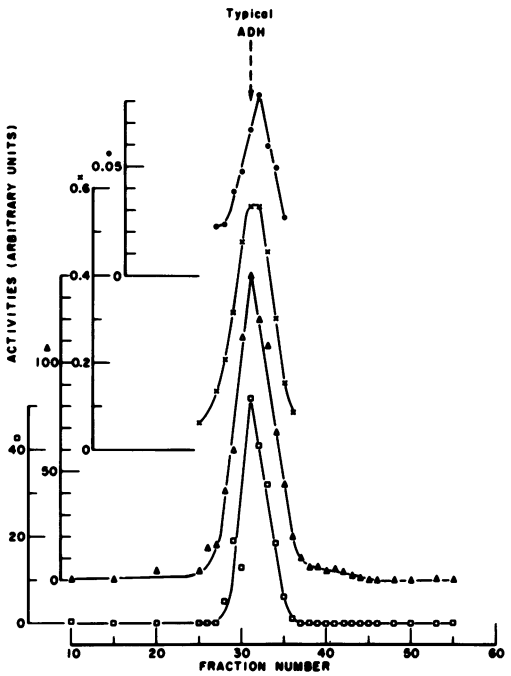

FIG. 6. *Distribution of aromatic enzyme activities after sucrose density gradient centrifugation of Euglena gracilis 0 to 50% ammonium sulfate fraction. Cells were disrupted by passage through a Hughes pressure cell. No enzyme standard was recovered from this gradient. Symbols: arrow, approximate position of the enzyme standard alcohol dehydrogenase (molecular weight, 151,000) from similar gradients; ●, dehydroquinase; ×, DHS reductase; △, shikimate kinase; and □, EPSP synthetase.*

in either the EPSP synthetase-containing 45 to 60% fractions or in corresponding 0 to 45% fractions, it is not possible on the basis of these data to determine whether a DHQ synthetase component is associated with EPSP synthetase or with the other aromatic enzymes.

In the bacterial study (3), the data indicated that in *Bacillus subtilis* several activities had sedimentation values corresponding to apparent molecular weights around 40,000, and it was suggested that some activities could either be associated or by coincidence have similar apparent molecular weights. In *Chlamydomonas*, moss, and tobacco, this again is the case for dehydroquinase and DHS reductase. A preliminary experiment utilizing DEAE-cellulose chromatography on an extract of *P. patens* indicated that these two activities are also not separable by this means. If, in *Euglena*, a 40,000-molecular-weight component contained both reductase and dehydroquinase activities, it might be conjectured that a 60,000-molecular-weight component contains both these activities and shikimate kinase activity. The ammonium sulfate fractionation data in Table 3 are consistent with at least DHS

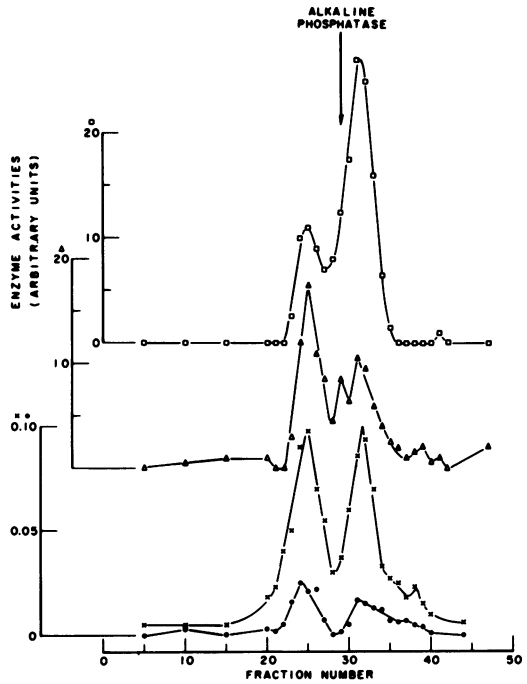

FIG. 7. *Distribution of aromatic enzyme activities after sucrose density gradient centrifugation of Euglena gracilis crude extract. Cells were disrupted by lyophilization and grinding. Symbols: arrow, peak of activity for the enzyme standard alkaline phosphatase (molecular weight, 80,000); □, EPSP synthetase; △, shikimate kinase; ×, DHS reductase; and ●, dehydroquinase.*

TABLE 3. *Enzyme activities in ammonium sulfate fractions of extracts of E. gracilis*[a]

Ammonium sulfate fraction	Specific activity[b]				
	DHS reductase	Dehydroquinase	DHQ synthetase	EPSP synthetase	Shikimate kinase
%					
0–25	1.9	0.17	0	0.47	1.2
25–35	6.1	0.73	0	0	0.35
35–45	7.7	0.95	0	0.05	1.7
45–60	0.13	0.01	0	2.4	0.02

[a] These extracts were prepared by the lyophilization and grinding procedure. Abbreviations as in Table 2.

[b] Expressed as 10^{-3} international units per milligram of protein.

reductase and dehydroquinase aggregation in all fractions. In view of the type of density gradient sedimentation and ammonium sulfate fractionation patterns we have observed for these activities, we are tempted to speculate that these light components consist of an aggregate containing dehydroquinase, DHS reductase, and shikimate kinase, and a second component with only EPSP synthetase activity, although perhaps associated also with inactive DHQ synthetase; these heterologous components could combine to form the 120,000 aggregate. Obviously, additional data are required to substantiate these interpretations.

In previous studies (3), the possible relationship of the sizes of the individual bacterial enzymes to the components of the *Neurospora arom* aggregate was considered. The present results for *Euglena* show considerable similarity to recent results and current interpretations of the structure of this aggregate in *N. crassa*. Burgoyne, Case, and Giles (4, 5) presented evidence that the 230,000-molecular-weight aggregate in *N. crassa* can dissociate into identical subunits of a molecular weight of approximately 115,000, a value strikingly similar to that of the *Euglena* aggregate. Dissociation into enzymatically active components of 60,000 molecular weight as found in *Euglena* has not been clearly demonstrated with wild-type *Neurospora*; however, in density gradient studies of pleiotropic (polarity) mutants in the *arom* gene cluster lacking dehydroquinase, DHS reductase, and shikimate kinase activities, EPSP synthetase activity has been found to be associated with a 60,000-molecular weight-component [Mary Case, Genetics **61**:58 (abst.)].

In *N. crassa*, a quinic acid-induced dehydroquinase can be distinguished from the biosynthetic dehydroquinase (associated with the *arom* aggregate) by heat stability tests and by density gradient centrifugation (10). Our evidence indicates that only a heat-labile biosynthetic enzyme is present in *E. gracilis*.

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