

Polyacrylamide-gel Electrophoresis of Enzymes during Morphogenesis of Sclerotia of *Sclerotinia sclerotiorum*

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SUMMARY

Succinate dehydrogenase (SDH) and glucose-6-phosphate dehydrogenase (Glu-6-PDH) from *Sclerotinia sclerotiorum* (Lib.) de Bary were moderately active in submerged mycelium while in non-sclerotial aerial mycelium arylesterase and acid phosphatase were very active. In sclerotial initials, glyceraldehyde-3-phosphate dehydrogenase (Gly-3-PDH) and SDH were at their highest level of activity, Glu-6-PDH and phosphogluconate dehydrogenase (PGDH) were moderately active, laccase activity increased markedly, and tyrosinase was detected for the first time, its activity being moderate. In young compacting sclerotia, the activities of Glu-6-PDH and PGDH increased, Gly-3-PDH and SDH showed lowered activities, and laccase activity decreased. Suppression of the glycolytic Krebs-cycle pathway and the stimulation of the pentose phosphate pathway seem important during the compaction and maturation of sclerotia. Tyrosinase may be involved in sclerotial initiation.

INTRODUCTION

Many aspects of the development and structure of sclerotia have been reviewed by Willetts (1972). However, there have been few investigations of the biochemical mechanisms involved in initiation and growth of sclerotia (Trevethick & Cooke, 1971; Chet, Retig & Henis, 1972) or of other fungal vegetative structures and sporocarps. The ease with which the sclerotia of most fungi can be grown in culture enables their morphogenesis to be studied conveniently; information obtained from such investigations could have direct relevance to other multi-hyphal structures.

This paper describes the results of an electrophoretic study of selected enzymes from different developmental stages of the sclerotium of *Sclerotinia sclerotiorum* (Lib.) de Bary (= *Whetzelinia sclerotiorum* (Lib.); Korf & Dumont, 1972). The relative activities of the enzymes have been estimated semi-quantitatively according to the relative widths and the staining intensities of the bands on the zymograms (Solomon, Johnson & Gregg, 1964; Hart, 1970; El-Sharkawy & Huisingsh, 1971; Reddy & Stahmann, 1972). A major advantage of this approach is that an extensive survey of several enzymes can be made from which a general understanding can be obtained of the biochemical processes involved in morphogenesis. However, there are obvious quantitative restrictions on the interpretation of data obtained by using electrophoretic methods and further confirmatory work is in progress in our laboratories using quantitative enzyme assays and radiorespirometric (C₁/C₆ ratio) techniques.

METHODS

Sclerotinia sclerotiorum, isolated from French beans, was cultured at 20 °C under continuous fluorescent light of about 100 lux in large glass dishes (19 cm diam and 3 cm high) on modified Czapek Dox Liquid (Oxoid; 100 ml) supplemented with L-asparagine (2 g/l) and thiamine hydrochloride (2 mg/l). The depth of the liquid medium was about 0.7 cm. The fungus was also grown under the same light and temperature conditions on a gyratory shaker (speed: 95 rev./min; throw: 1.5 cm) in 250 ml conical flasks each containing 50 ml medium. When stationary the depth of the liquid in the flasks was about 1.5 cm. The flasks were plugged with cotton wool.

The mycelial and sclerotial stages used in our study included (i) submerged mycelial pellets from 4-day-old shake cultures; (ii) aerial mycelia harvested from regions showing no differentiation into sclerotial initials after 1 to 2 weeks growth in static liquid culture; (iii) fluffy, loose aggregates of hyphae representing an early stage of sclerotial development; (iv) sclerotia which were firm and white but had not attained maximum size; (v) sclerotia fully developed in size but lightly pigmented to give a yellow to light-grey colour; (vi) recently matured, darkly pigmented sclerotia; (vii) sclerotia removed from cultures 2 weeks after maturation.

The contents of five shake cultures were sieved through a 1.5 × 1.5 mm mesh and the mycelial pellets collected in this way were rinsed with distilled water. Aerial mycelia, growing on ten stationary cultures in large dishes, were removed from the vessels with forceps; sclerotia at different stages of development were also harvested from the cultures in this manner. The early stages of sclerotial development (stage iii) usually adhered to the colony but could be readily detached with a pair of fine forceps. No difficulties were experienced with the other sclerotial stages. Exudation droplets were removed by rinsing the sclerotia with distilled water and then blotting dry with filter paper. Normally during a single harvesting several sclerotial stages were collected from each dish. Immediately after harvesting all the samples were frozen rapidly with crushed dry-ice (solid CO₂).

Proteins were then extracted from the samples by using pestles and mortars chilled to 0 to 4 °C, each mortar containing a small amount of acid-washed sand; 3 ml of buffer (0.05 M-tris adjusted to pH 8.3 with glycine, at room temperature) were added to each gram of fungal material. After thorough grinding, the mixture was centrifuged at 20000 g for 1 h at 0 to 4 °C, then 0.5 ml samples of the supernatant were pipetted into glass ampoules. The extracts in the samples were frozen rapidly with crushed dry-ice, freeze-dried and sealed under vacuum by means of a Dynavac freeze-drier. They were then stored at 4 °C until required.

The method of Lowry, Rosebrough, Farr & Randall (1951) was used to determine the amount of protein in the freeze-dried extracts with bovine serum albumin as the standard.

Acrylamide-gel electrophoresis was carried out on a modified 18-slot, horizontal flat-bed system (Lund, 1965). A 7.5 % gel was used and this was made up according to the method of Dietz & Lubrano (1967). The gel was buffered using 0.025 M-tris adjusted to pH 8.9 with glycine at room temperature. The freeze-dried materials were dissolved in distilled water to give a concentration of 10 µg protein/µl; samples containing 300 µg protein were dispensed into slots in the gels. Electrophoresis was carried out at 4 °C with a tank buffer of 0.005 M-tris adjusted to pH 8.3 with glycine and at a constant current of 35 mA which gave an initial potential of about 15 to 20 V/cm across the gel slab (20 × 12 × 0.6 cm). Electrophoresis was stopped when the tracker dye, bromophenol blue, had moved 6 to 7 cm (usually after 3 to 4 h). Each gel was sliced horizontally into four pieces of equal thickness. The

upper piece was discarded and the remaining three slices were stained to detect proteins and selected enzymes. The gel slabs were stained for soluble proteins with Coomassie blue (Chrambach, Reisfeld, Wyckoff & Zaccari, 1967).

The enzymes selected for study and the techniques used were: tyrosinase (EC. 1.10.3.1) – either 0.006 M-L-dihydroxyphenylalanine or a saturated solution of L-tyrosine in 0.1 M-phosphate buffer at pH 7.4; laccase (EC. 1.10.3.2) – either 0.02 M-*o*-dianisidine or benzidine in 0.2 M-acetate buffer at pH 4.5; peroxidase (EC. 1.11.1.7) – 50 mg *o*-dianisidine, 35 ml 95 % ethanol, 1 ml 3 % H₂O₂ and 14 ml 0.2 M-acetate buffer at pH 4.5; glyceraldehyde-3-phosphate dehydrogenase (NAD) (EC. 1.2.1.12) and phosphogluconate dehydrogenase (EC. 1.1.1.43) – methods of Brewer & Sing (1970); succinate dehydrogenase (EC. 1.3.99.1), glucose-6-phosphate dehydrogenase (NADP) (EC. 1.1.1.49), acid phosphatase (EC. 3.1.3.2) and arylesterase (EC. 3.1.1.2) – methods of Cole, Blondin & Temple (1968). The pH values were measured at room temperature (approx. 20 °C).

All gels were incubated at 37 °C in the dark during the staining process. After staining they were photographed and the electrophoretic patterns were recorded as interpretative drawings. The analyses were repeated at least three times for each enzyme.

RESULTS AND DISCUSSION

The electrophoretic patterns of selected enzymes and soluble proteins of submerged mycelia, aerial hyphae and five stages of sclerotium development are shown in Fig. 1 and 2; these Figures are tracings of representative gels. The data have been summarized in Table 1. The results were consistently reproduced and the information given is from at least three different experiments.

Submerged mycelium

When *Sclerotinia sclerotiorum* was inoculated on to a suitable substrate, aerial and submerged hyphae developed. Presumably the submerged hyphae absorb nutrient from the medium and this is used for further mycelial development. Succinate dehydrogenase, a Krebs-cycle enzyme, and glucose-6-phosphate dehydrogenase (NADP), a pentose phosphate shunt enzyme associated with synthetic processes, were detected in the extracts from the submerged mycelium. Four isoenzyme bands of the former were moderately intense while five bands of the latter were observed and, of these, two were very intense.

Aerial mycelium

After a colony of *Sclerotinia sclerotiorum* had become established on a substrate, sclerotial initials were produced and some of these developed and usually matured within about 5 days. The energy and nutrient requirements for sclerotial development must have been considerably greater than those for mycelial growth and our previous observations suggest that the nutrient status of the substrate is of great importance in determining how many sclerotia mature (Willettts & Wong, 1971). However, it seems unlikely that the large amount of energy and nutrient needed for the rapid growth of the sclerotia could be obtained from the substrate at the time of sclerotium formation. A possible source of reserve nutrients may be the vegetative aerial mycelium, and its degradation could supply the sudden demands of the developing sclerotia. This would explain the considerable esterase activity detected in the aerial hyphae – 16 different isoenzyme bands and eight of these very intense. Also, high activity of acid phosphatase, another degrading enzyme, was detected.

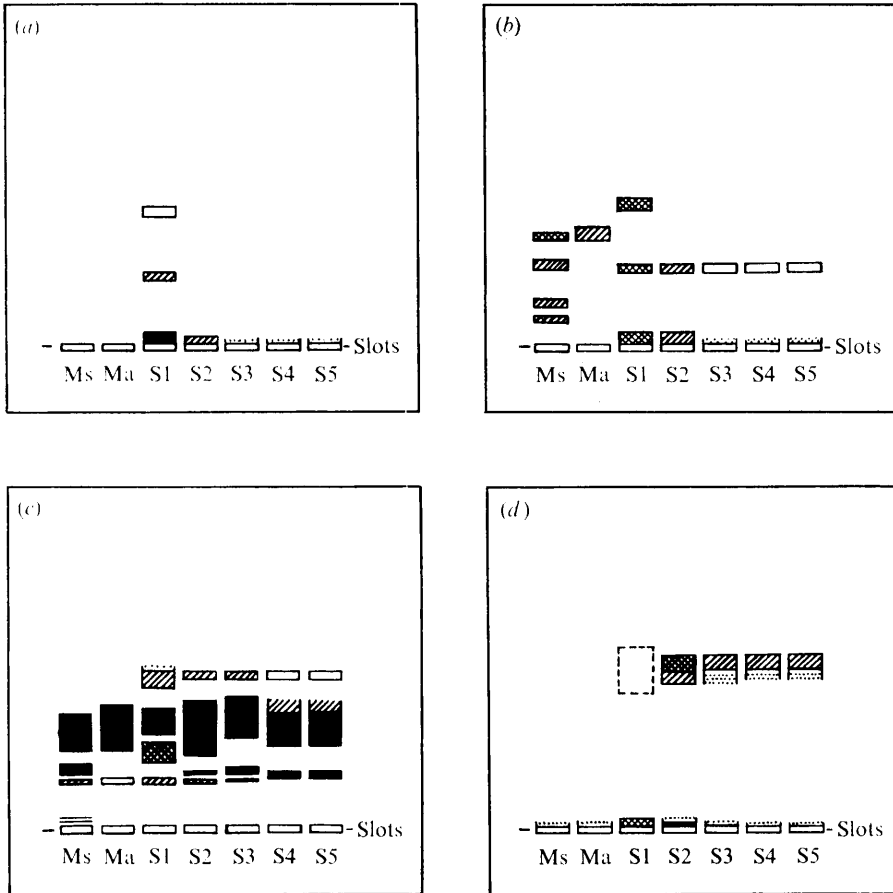


Fig. 1. Diagrammatic interpretation of electrophoretic patterns of enzymes from different stages of sclerotial development. Submerged mycelium, Ms; aerial mycelium, Ma; sclerotium initials, S1; young sclerotia, S2; maturing sclerotia, S3; mature sclerotia, S4; and old sclerotia, S5. (a) Glyceraldehyde-3-phosphate dehydrogenase (NAD). (b) Succinate dehydrogenase. (c) Glucose-6-phosphate dehydrogenase (NADP). (d) Phosphogluconate dehydrogenase. The intensities of the bands are shown by the degree of shading. Stippled areas are sites of enzyme activity not resolved into discrete bands.

Sclerotial initials

Active metabolism to provide the energy and the substances needed for hyphal growth would be expected at sites of sclerotium initiation. Almost all of the enzyme systems included in our studies were detected in young sclerotial initials. Glyceraldehyde-3-phosphate dehydrogenase and succinate dehydrogenase, which are glycolytic and Krebs-cycle respiratory enzymes respectively, showed their highest activities at this stage, suggesting a high level of normal aerobic respiration. Glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase, which are associated with biosynthetic processes, were moderately active. Tyrosinase, which was not detected in submerged or aerial mycelia, was also moderately active, and there was a marked increase in laccase activity.

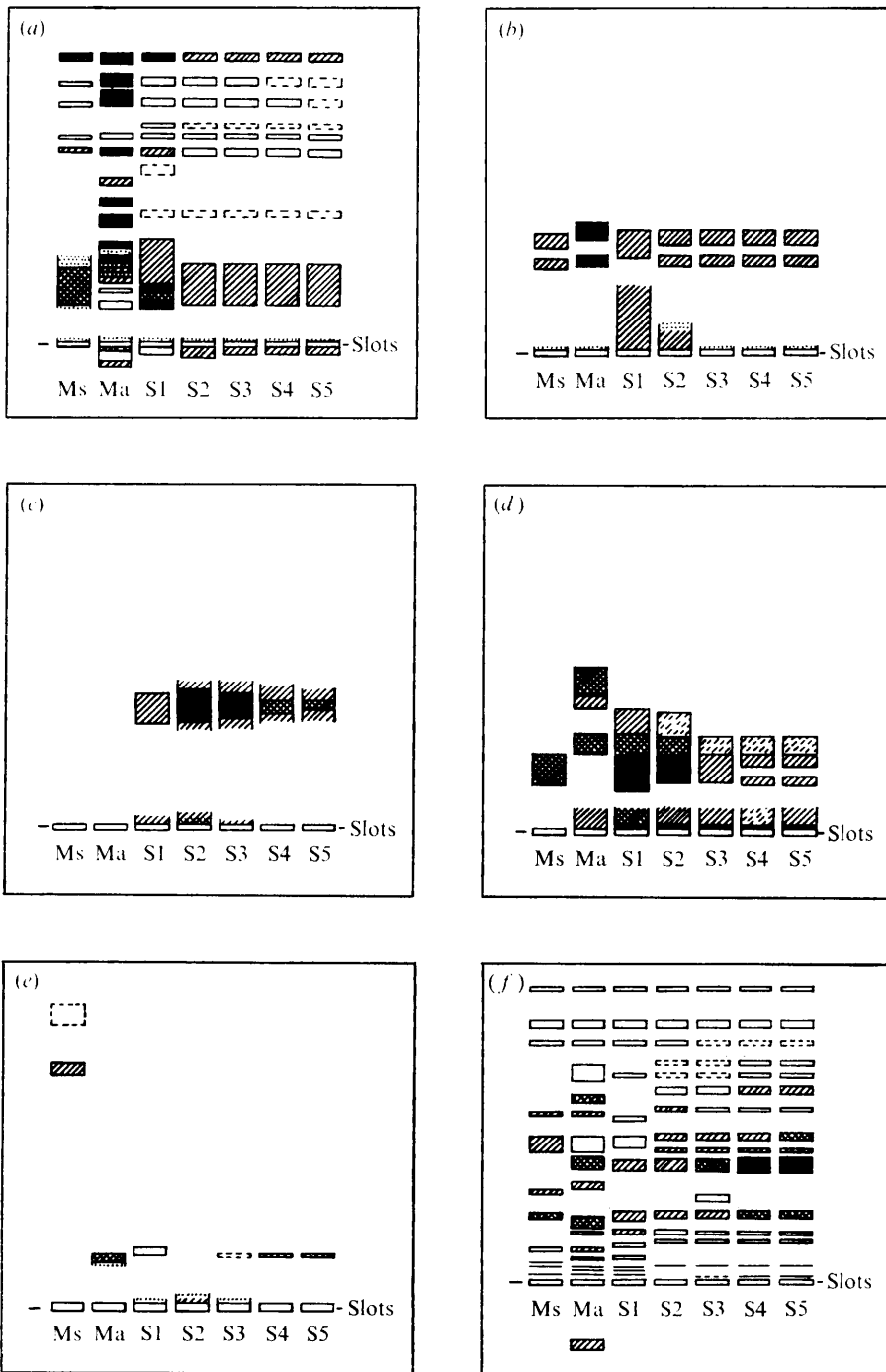


Fig. 2. Diagrammatic interpretation of electrophoretic patterns of enzymes and general proteins from different stages of sclerotial development. Abbreviations and shading as in Fig. 1. (a) Aryl-esterase. (b) Acid phosphatase. (c) Tyrosinase. (d) Laccase. (e) Peroxidase. (f) Soluble proteins.

Table 1. *Enzyme activities during different stages of sclerotial morphogenesis in Sclerotinia sclerotiorum*

	Degree of enzyme activity							
	Submerged mycelium	Aerial mycelium	Sclerotial initials	Young sclerotia	Maturing sclerotia	Mature sclerotia	Old sclerotia	
Metabolic pathways and associated enzymes								
Glycolytic pathway								
Glyceraldehyde-3-phosphate dehydrogenase	Not detected	Not detected	Moderate (3 ¹ , 1 ¹)	Low (1 ¹)	Trace	Trace	Trace	
Krebs cycle								
Succinate dehydrogenase	Moderate (4 ¹)	Low (1 ¹)	Moderate (3 ¹)	Low (2 ¹)	Trace	Trace	Trace	
Pentose phosphate pathway								
Glucose-6-phosphate dehydrogenase	Moderate (5 ¹ , 2 ¹)	Moderate (2 ¹ , 1 ¹)	Moderate (4 ¹ , 1 ¹)	High (5 ¹ , 2 ¹)	High (4 ¹ , 2 ¹)	Moderate (3 ¹ , 2 ¹)	Moderate (3 ¹ , 2 ¹)	
Phosphogluconate dehydrogenase	Trace	Trace	Low to moderate (2 ¹)	Moderate (2 ¹)	Low to moderate (2 ¹)	Low (2 ¹)	Low (2 ¹)	
Degradative metabolism								
Arylesterase	Moderate (7 ¹ , 1 ¹)	Very high (16 ¹ , 8 ¹)	Moderate (13 ¹ , 3 ¹)	Low (9 ¹)	Low (9 ¹)	Low (9 ¹)	Low (9 ¹)	
Acid phosphatase	Moderate (3 ¹)	High (3 ¹ , 2 ¹)	Moderate (2 ¹)	Moderate (3 ¹)	Moderate (3 ¹)	Moderate (3 ¹)	Low to moderate (3 ¹)	
Polyphenoloxidases and peroxidase								
Tyrosinase	Not detected	Not detected	Moderate (2 ¹)	High (2 ¹ , 1 ¹)	High (2 ¹ , 1 ¹)	Moderate (1 ¹)	Moderate (1 ¹)	
Laccase	Moderate (1 ¹)	Moderate (3 ¹ or 4 ¹)	Very high (3 ¹ or 4 ¹)	High (3 ¹ or 4 ¹)	Moderate (2 ¹ or 3 ¹)	Moderate (3 ¹ or 4 ¹)	Moderate (3 ¹ or 4 ¹)	
Peroxidase	Low (2 ¹)	Moderate (1 ¹)	Very low (1 ¹ or 2 ¹)	Very low (1 ¹)	Very low (1 ¹)	Low (1 ¹)	Low (1 ¹)	

3¹: Total no. of bands = 3; 1¹: no. of intense bands = 1; a similar notation is used for the rest of the Table.

Young unpigmented sclerotia

The highest activities of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase were observed in young compacting sclerotia. This coincided with maximum hyphal production. However, glyceraldehyde-3-phosphate dehydrogenase and succinate dehydrogenase were less active. Tyrosinase became very active but there was a decrease in laccase activity.

Maturing and matured sclerotia

After sclerotia had grown to their maximum size and matured, the activities of all the enzymes were reduced, apart from tyrosinase which remained active. This generally low metabolism would be expected of a resting structure. Presumably the tyrosinase was involved in the pigmentation that took place at this stage.

When several hyphae form close together they have the potential of developing into a sclerotium. If sufficient energy for rapid growth is obtained by these hyphae, a site of active metabolism will be created and a sink develop to which nutrient flows. At first, the hyphae will be able to obtain sufficient energy from the glycolytic Krebs-cycle pathway, but as branching and interweaving lead to a greater density of hyphae, oxygen levels are probably reduced within the compacting sclerotium. The pentose phosphate pathway, although an aerobic route, requires less oxygen than the glycolytic Krebs-cycle pathway and consequently the former will probably assume greater importance in the metabolism of the medullary hyphae. These hyphae form the main part of the structure and thus the bulk of the material sampled.

We found that the pentose phosphate shunt enzymes were most active in the young compacting sclerotia and the increase in their activities coincided with a reduction in the activities of the enzymes of the glycolytic Krebs-cycle pathway. Thus as one pathway became less involved in the development of the sclerotium, its role was taken over by the other. Arimura & Kihara (1968) showed that the mitochondria of the sclerotial cells of *Sclerotinia sclerotiorum* have fewer cristae than those of vegetative hyphae and they suggested that aerobic respiration in the sclerotial cells is low. This agrees with our electrophoretic data for succinate dehydrogenase.

Turian & Bianchi (1972) and Ng, Smith & Anderson (1972) suggested that the pentose phosphate shunt may be important in the conidiation of *Neurospora crassa* and conidiophore differentiation of *Aspergillus niger* respectively. Activation of the pentose phosphate pathway in *Neurospora* may be brought about by the blockage of the glycolytic pathway by iodoacetate and other antiglycolytic agents, the consequence of which is conidiation. *Sclerotinia sclerotiorum* lacks a conidial stage but Willetts (1972) suggested that the sclerotium of this fungus might have evolved from undifferentiated conidial chains and conidiophores. The induction of sclerotial initials may be associated with a suppression of glycolysis and/or the Krebs cycle, and a corresponding enhancement of the pentose phosphate shunt at some sites in the mycelium. Metabolism of hexoses through the pentose phosphate pathway would generate the NADPH₂ essential for the biosynthetic reactions associated with differentiation.

Tyrosinase has been implicated in the initiation of the perithecia of *Neurospora crassa* (Hirsch, 1954) and *Hypomyces solani* (Wilson, 1968) and the basidiocarp of *Schizophyllum commune* (Leonard & Phillips, 1973). We failed to detect tyrosinase in aerial and submerged vegetative mycelium of *Sclerotinia sclerotiorum*, but the enzyme was moderately active in sclerotial initials and highly active during the rapid growth of the sclerotium. No pigmentation was apparent during these two stages and it appears that tyrosinase might be involved

in more than just melanization of the rind. The work of Solti & Telegdi (1972) on the effect of tyrosinase on glyceraldehyde-3-phosphate dehydrogenase appears to be of relevance here. Tyrosinase may act on the tyrosyl residues of proteins, including those of glyceraldehyde-3-phosphate dehydrogenase. The -SH groups of the enzymes may become oxidized by the oxidation products of tyrosyl side chains, and the ensuing inactivation of the glyceraldehyde-3-phosphate dehydrogenase could suppress glycolysis and possibly stimulate the pentose phosphate pathway.

Laccase was detected in vegetative mycelium and at all stages of sclerotial development, but its activity was greatest in initials and young sclerotia. This corresponded with the greatest activity of tyrosinase. Laccase has also been reported as having morphogenetic functions (Esser, 1968). No obvious role can be suggested for peroxidase in sclerotial morphogenesis.

It appears that some of our results parallel those obtained by Cantino (1961) and Cantino & Lovett (1964) from their studies on the development of sporangia of *Blastocladiella*. They found that bicarbonate ions suppressed the Krebs cycle and stimulated the pentose phosphate pathway. Increased tyrosinase activity and melanin formation were also observed. Often substances that are regularly associated with metabolism are overlooked as morphogens and this may be so for carbon dioxide and for bicarbonate ions, which are natural staling substances of metabolism. A biochemical investigation of the relationship between bicarbonate ions and the morphogenesis of fungal sclerotia might provide useful data on the initiation and subsequent development of these structures.

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